MONASH University

Pharmacological evaluation of embelin in epilepsy induced cognitive impairment in zebrafish

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M. Pharmacy (Pharmacology)

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(*Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia*)

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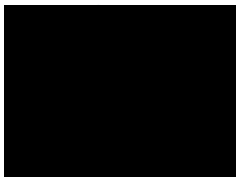
Notice 1

Abstract

Epilepsy is a serious neurological disorder characterized by abnormal neuronal electrical firing that result in epileptic seizures. Despite having more than 20 anti-epileptic drugs (AEDs), all of them only provide symptomatic treatment. As epilepsy is an unpredictable disease and a social stigma for patients, it can lead to a loss of autonomy in daily life. The people affected with epilepsy are increasing and it is estimated that about 70 million people are suffering worldwide. Moreover, findings are emerging that currently available AEDs also display cognitive alterations in addition to retarding seizures. This leads to a burning need to explore new molecules that can retard seizures as well as improve cognitive impairment. Embelin (EMB) is a natural product-based benzoquinone which has already demonstrated its pharmacological potentials against an array of neurological conditions. As the overall costs of studies which involve large animals are expensive and laborious, small rodent or zebrafish models are preferred to study the safety profile of a drug and for hypothesis testing. In recent years, the primary focus has turned towards non-mammalian epilepsy models in zebrafish due to numerous factors. Therefore, the first part of the study involved a complete systematic review on the use of EMB in various CNS disorders and zebrafish model development for acute seizure induced cognitive dysfunction. A model system was developed using acute pentylenetetrazole (PTZ) seizures model and testing the memory function by T-maze analysis. Different AEDs were tested against PTZ seizures and assessed for T-maze memory function. After confirmation of an animal model, the next study performed by using different doses of EMB and confirming its activity against acute epileptic seizure induced cognitive impairment. The second part of the study includes the development of a chronic epilepsy induced cognitive dysfunction model by PTZ kindling and confirming the effect of EMB. The results showed that daily dose of PTZ 80 mg/kg for 10 days successfully induces a kindling effect in zebrafish, whereas the single dose of KA did not. As compared to control, PTZ and KA administered group demonstrates impairment in memory as demonstrated by three-axis maze. PTZ group treated with a series of EMB doses ranging from (0.156 mg/kg to 0.625) retards seizure as well as significantly reduces epilepsy-induced memory alteration. In addition, EMB treatment reduces the expression of inflammatory markers implicating its anti-inflammatory potential. Overall, findings demonstrate that EMB might be a potential candidate against chronic epilepsy related cognitive dysfunction as EMB prevents the seizures, so we expect it to prevent the associated neuroinflammation and learning deficit. Thus, in the present thesis, we tried to describe the overall activity of EMB by considering pharmacology, docking, immunochemistry, and bio-molecular studies for epilepsy induced cognitive dysfunction.

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.

Signature: 

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❖ Achievements

- **Publications during enrolment**

1. Tan QY, **Kundap UP**, Kumari Y, Shaikh MF (2015) IA. Review on Natural Therapy for Seizure Disorders. Pharm Pharmacol Int J 3(2): 00051. DOI: 10.15406/ppij.2015.02.00051
2. **Kundap, U**., Jaiswal, Y., Sarawade, R., Williams, L., & Shaikh, M. F. (2016). Effect of Pelargonidin isolated from Ficus benghalensis L. on phenotypic changes in zebrafish (Danio rerio) embryos. Saudi Pharmaceutical Journal. http://dx.doi.org/10.1016/j.jsps.2016.06.010
3. **Kundap UP**, Bhuvanendran S, Kumari Y, Othman I and Shaikh M (2017). Plant derived phytocompound, Embelin in CNS disorders: A systematic review. Front. Pharmacol. 8:76. doi: 10.3389/fphar.2017.00076
4. **Kundap, U. P**., Kumari, Y., Othman, I., & Shaikh, M. (2017). Zebrafish as a Model for Epilepsy-Induced Cognitive Dysfunction: A Pharmacological, Biochemical and Behavioral Approach. Frontiers in Pharmacology, 8, 515.
5. Choo BKM, **Kundap UP**, Kumari Y, Hue S-M, Othman I and Shaikh MF (2018) Orthosiphon stamineus Leaf Extract Affects TNF-ὰ and Seizures in a Zebrafish Model. Front. Pharmacol. 9:139. doi: 10.3389/fphar.2018.00139.

- **Poster presentation during enrolment**

1. **U. P. Kundap**, Y. Kumari, I. Othman, N. Ahmed, M. F. Shaikh (2016) Embelin's affinity towards GABAA receptor and the ability to modulate neurotransmitters makes it an anticonvulsant compound. Conference Abstract: IBRO-APRC 2016 Thailand & Thai Neuroscience Society International conference, July 4th -8th, 2016
2. **Kundap UP**, Bhuvanendran S, Kumari Y and Shaikh M (2016). Development of a zebrafish model for epilepsy and antiepileptic drugs induced cognitive dysfunction. Front. Cell. Neurosci. Conference Abstract: 14th Meeting of the Asian-Pacific Society for Neurochemistry, 27 Aug - 30 Aug 2016. doi: 10.3389/conf.fncel.2016.36.00169.
3. **Kundap, U.,** Tiang, N., Kumari, Y., Othman, I., & Shaikh, M. F. (2016). Embelin, a potential molecule to ameliorate seizure induced cognitive decline in T-maze zebrafish model. European Neuropsychopharmacology, 26, S273-S274. Conference Abstract: 29th ECNP Congress, Austria Centre Vienna (ACV), Vienna, Austria, 17th to 20th Sep.2016. doi: 10.1016/S0924-977X(16)31159-2.
4. **Kundap, U. P**., Kumari, Y., Othman, I., & Shaikh, M. F. (2017, December). EFFECT OF EMBELIN IN CHRONIC EPILEPSY INDUCED COGNITIVE DYSFUNCTION IN THREE-

AXIS MAZE MODEL OF ADULT ZEBRAFISH. In EPILEPSIA (Vol. 58, pp. S48-S48). 111 RIVER ST, HOBOKEN 07030-5774, NJ USA: WILEY. 14. 32nd International Epilepsy Congress, took place in Barcelona, Spain between 2nd– 6th September 2017.

- ## Oral presentation during enrolment

1. Oral presentation session entitled "Cognitive Dysfunctioning by Epilepsy & AEDs in Zebrafish Model", at Malaysia Zebrafish Disease Model Workshop 2015 (MZDM 2015), CARIF - Cancer Research Malaysia, USJ Laboratory, Selangor, Malaysia on 11th & 12th Nov 2015.
2. Platform presentation session entitled "To study the effect of Embelin on neurogenesis and cognition in PTZ induced epilepsy model of adult zebrafish" at Basic science session of 13th European Congress on Epileptology, Vienna, Austria on 26th to 30th of August 2018.

- ## Awards during enrolment

1. Best Poster Presentation Award – "Assoc. Prof. Naiphinich Kotchabhakdi Award" for title "Embelin's affinity towards GABAA receptor and the ability to modulate neurotransmitters makes it an anticonvulsant compound" at IBRO-APRC Associated School of Neuroscience, Thailand & 20th in Neuroscience Society International Conference July 4th-8th, 2016.
2. MSN Education Grant (ID:00380) - 29th European College of Neuropsychopharmacology (ECNP) Congress, Austria, Vienna, honor date: Sep 2016 honor issuer: Malaysian Society of Neurosciences. (RM 3600)
3. 3MT - Three Minute Thesis Competition (1st Place Winner), honor date: May 2017, honor issuer: Jeffery Cheah School of Medicine and Health Sciences, Monash University Malaysia.
4. IBRO International Travel Grant, honor date: Sep 2017 honor issuer: International Brain Research Organization (IBRO). 32nd International Epilepsy Congress, Barcelona, Spain between 2nd– 6th September 2017. (EUR 1800€)
5. Visiting Research Scholar Grant – University Hospital Leuven, Department of Pediatric Neurology, KU Leuven, Belgium (Nov-2017 to Dec-2017). (EUR 2000€)
6. International League Against Epilepsy (ILAE) - sponsored travel bursary to attend Young Epilepsy Section's (YES) Kick-off workshop in University College London (UCL), London, 12th – 13th May – 2018. (US 750$)
7. Malaysian Society of Neuroscience (MSN) Best Publication Awards for 2018 – for research publication – "Zebrafish as a Model for Epilepsy-Induced Cognitive Dysfunction: A Pharmacological, Biochemical and Behavioral Approach" Frontiers in Pharmacology, 2017, 8:515, 1-13. (RM 2400)

8. Jeffrey Cheah School of Medicine and Health Sciences Travel Award, honor date: Aug. 2018 honor issuer: Jeffery Cheah School of Medicine and Health Sciences, Monash University Malaysia for Platform presentation at Basic science session of 13th European Congress on Epileptology, Vienna, Austria on 26th to 30th of August 2018. (RM 2200)

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 research paper published in peer reviewed journals, 1 accepted and 1 submitted publications. The core theme of the thesis is pharmacological evaluation of embelin in epilepsy induced cognitive impairment in zebrafish. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia under the supervision of Dr. Mohd. Farooq Shaikh.

In the case of 4 research publication, my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
1	*Zebrafish as a Model for Epilepsy-Induced Cognitive Dysfunction: A Pharmacological, Biochemical and Behavioral Approach.*	*Published*	*60%. Performing experiment, data analysis, drafting manuscript and respond to reviewer's comments.*	*1) 20%, Mohd. Farooq Shaikh, conceptualised the idea, designed the study, manuscript editing, proofreading and responded to reviewer's comments.* *2) 15%, Yatinesh Kumari input into designing gene expression study and result analysis.* *3) 5%, Iekhsan Othman input into LCMS/MS methods and result analysis*	*No* *No* *No*

2	*Embelin protects against pentylenetetrazole-induced seizures and associated memory decline in a zebrafish model.*	*Submitted*	*60%. Involved in design of the study, performed experiment, data analysis, manuscript writing and responded to reviewers' comments.*	*1) 15%, Mohd. Farooq Shaikh, conceptualised the idea, designed the study, proofreading responded to reviewer's comments.* *2) 5%, Nafees Ahmed input into Docking study and manuscript writing.* *3) 10%, Yatinesh Kumari input into designing gene expression study and ICC study and result analysis.* *4) 5%, Brandon Choo input into result analysis and manuscript writing.* *5) 5%, Iekhsan Othman input into LCMS/MS methods and result analysis.*	*No* *No* *No* *Yes* *No*
3	*Embelin prevents seizure and associated cognitive impairments in a pentylenetetrazole -induced kindling zebrafish model*	*Accepted*	*60%. Involved in designing, performed experiment, data analysis, manuscript writing and responded to reviewers' comments.*	*1) 20%, Mohd. Farooq Shaikh, conceptualised the idea and designed the study, manuscript editing, proofreading, responded to reviewer's comments.* *2) 10%, Yatinesh Kumari input into designing gene expression study and result analysis.* *3) 5%, YamNath Paudel input into result analysis and manuscript writing.*	*No* *No* *Yes* *No*

				4) 5%, Iekhsan Othman input into LCMS/MS methods and result analysis.	

**If no co-authors, leave fields blank*

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

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Name: Uday P Kundap

Signature: [redacted]

Main Supervisor: Dr. Mohd. Farooq Shaikh

Date: 09/12/2018

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.

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Dedicated to my parents, my guru, and my supervisor

Table of Contents

List of abbreviations and acronyms

α: Alfa

β: Beta

γ: Gamma

µl: Microliter

°C: Degree Celsius

i.e.: That is

e.g: Example

mg: Milligram

min: Minute

ml: Milliliter

Ach: Acetylcholine

AEDs: Anti-epileptic drugs

AD: Alzheimer's disease

BDNF: Brain-derived neurotrophic factor

BBB: Blood-brain barrier

BrdU: Bromodeoxyuridine (5-bromo-2'-deoxyuridine)

CCL2: C-C motif ligand – 2

CSEs: cognitive side effects

CNS: Central nervous system

CREB: cAMP response element-binding protein

DCs: Dendritic cells

DMSO: Dimethyl sulfoxide

DZP: Diazepam

EAE: Experimental allergic encephalomyelitis

EMB: Embelin

EPM: Elevated plus maze

FST: Forced swimming test

GABA: γ-aminobutyric acid

$GABA_A$: γ-aminobutyric acid receptor – α unit

GBP: Gabapentin

GI: Gonadally intact

Glu: Glutamate

HMGB1: High mobility group box protein-1

HLTE: Hind leg tonic extension

HD: Huntington’s disease

HI: Hypoxia-ischemia

ICA: Internal carotid artery

ICC: Immunocytochemistry

IFN-γ: Interferon-γ

IL-1: Interleukin-1

ICH: International Council for Harmonization

KA: Kainic acid

LC-MS/MS: Liquid chromatography/tandem mass spectrometry

LPS: lipopolysaccharide

MARP: Monash Animal Research Platform

MCAO: Middle cerebral artery occlusion

MES: Maximal electroshock

MOSTI: eScience Fund of Ministry of Science, Technology and Innovation

MS: Multiple Sclerosis

NPY: Neuropeptide – Y

NA: Noradrenaline

OFT: Open field test

OGD: Oxygen-glucose deprivation

ORTK: Omniscript Reverse-transcription Kit

OXC: Oxcarbazepin

PFA: Paraformaldehyde

PFT: Pifithrin-a

PHY: Phenytoin

PTZ: Pentelentetrazole

PIC: Pro-inflammatory cytokines

RSV: Rivastigmine

TBI: Traumatic brain injury

TLR4: Toll like receptor – 4

TL: Transfer latencie

TNF-α: Tumor necrosis factor - α

TST: Tail suspension test

STAT3: Signal transducer and activator of transcription - 3

SD: Standard deviation

SOD: Superoxide dismutase

WHO: World Health Organization

XIAP: X-chromosomal linked inhibitor of apoptosis

3-NP: Nitropropionic acid

5-HT: 5-hydroxytryptamine receptors

Chapter-1

INTRODUCTION

Epilepsy associated cognitive decline with available therapeutic approach: An overview

Epilepsy is a serious neurological disorder characterized by abnormal neuronal electrical firing that result in epileptic seizures (Paudel et al., 2018). It is estimated that about 70 million people are suffering from epilepsy worldwide (Copmans et al., 2018). Due to its complex pathology, the actual cause epilepsy is still unknown, but efforts are ongoing to understand the real cause of the disease. A new practical clinical definition of epilepsy was proposed by the International League Against Epilepsy (ILAE) in 2014 to define epilepsy as a fulfilment of any one criterion: (1) at least two unprovoked or reflex seizures occurring more than 24 h apart; (2) one unprovoked or reflex seizure and a probability of further seizures that is similar to the general recurrence risk after two unprovoked seizures, occurring over the next 10 yrs. (3) diagnosis of an epilepsy syndrome (Fisher et al., 2014). Epilepsy patients are still considered to be socially stigmatized and can lose their autonomy in daily life (Espinosa Jovel et al., 2016). Moreover, epilepsy is also associated with various comorbidities including neuropsychiatric and neurobehavioral comorbidities (Roopra et al., 2012;Paudel et al., 2018). Cognitive ability such as in learning, memory, attention and problem solving, is important for day to day functioning (Oostrom et al., 2003). Cognitive function is one of the most common and severe comorbidities associated with epilepsy. These comorbidities have a serious impact on the quality of life of individuals suffering from epilepsy (Kleen et al., 2010).

The fundamental aim in treating epilepsy patients is the reduction of seizures, due to its poor etiological understanding it is difficult to find out the exact way to treat epilepsy (Goldenberg, 2010). Anti-epileptic drugs (AEDs) are successful in controlling epilepsy on a long-term basis rather than allowing patients to be epilepsy free for a life time (Ahmed, 2017). The first use of bromide as an AED was reported by Dr. Charles Locock in his research comment at ‘The Lancet’ journal, 1857 (Löscher et al., 2013). The next drug used as an AED was the result of a serendipitous finding of phenobarbital in 1912 and then followed by others such as phenytoin, diazepam, valporic acid, etc., which are used as a first line drug in most of the developing countries (Perucca et al., 2007). Chronic use of AEDs in people with epilepsy is reported to be associated with a number of neuropsychiatric comorbidities such as memory

decline, depression, anxiety and others (Asconapé, 2002). Pharmacoresistance is another major problem associated with the use of AEDs and hence there is a pressing need for the development of safer and more effective AEDs (Dalic and Cook, 2016).

unpredictability of epileptic seizures and treatment related side effects affects the quality of life of patients. Moreover, no significant effect has been observed by available antiepileptic drugs (AED) in prevent epileptogenesis (Perucca and Gilliam, 2012). Thus, novel drug molecules and therapies that are capable in seizure control in epileptic patients are needed (Amini et al., 2015). Introduction of new plant based medicines with unique mechanisms of action is one option, along with other sources of drugs that are marine or mineral in nature. (Witt and Helmstaedter, 2017). The utero exposure of AEDs medication can start the neurocognitive burden in patients with epilepsy. Epilepsy surgery can also induce certain cognitive deficits, Clinicians should consider cognitive side effect profiles of antiepileptic medications, particularly in extreme age groups. While no effective treatments are available for cognitive and behavioral impairments in epilepsy, comprehensive pretreatment evaluation and meticulous selection of antiepileptic drugs or surgical approach may minimize such untoward effects.

Nevertheless, despite considerable progress in drug and epilepsy research and carefully optimized AED treatment, approximately 35% of all epilepsy patients experience recurrent non provoked spontaneous seizures (Taylor et al., 2011). Drug resistance is a specific problem in patients with epilepsy in temporal lobe, which is the most epileptogenic region of the brain. Unbearable toxic effect for AEDs, impaired memory function, and depressive symptoms are commonly recorded with epilepsy related side effects and comorbidities. Advance in drug discoveries have provided novel AEDs with remarkable advantages in terms of better tolerance, fewer interactions, less side effects and simpler pharmacokinetics (Sørensen and Kokaia, 2013). But still they fail to have the superior efficacy compared to older AEDs. Different approach in controlling epilepsy is by giving the ketogenic diet at least for certain type of epilepsy with metabolic syndromes (Tyagi and Delanty, 2003). Surgical intervention is also one the possibilities, in patients with focal epilepsy that have excellent outcomes and leads to post-surgical freedom and drug resistance problems in 80% patients. It is being well understood that, current scenario of refractor epilepsy represents a major problem in front of doctors and scientist in finding better approach to control or treat epilepsy(Stafstrom and Carmant, 2015).

The consumption and use of herbal medications and dietary supplements has increased dramatically in most of the developed countries in recent times (Colalto, 2018). A sufficient number of these remedies are used for treating patients with neurological or psychiatric disorders (Tyagi and Delanty, 2003). There are enormous benefits from the use of such plant based medications for central nervous system (CNS) disorders (e.g., St. John's wort for treating depression, the *Lavandula officinalis* herb for epilepsy in Iranian traditional medicine) (Liu et al., 2017). However, the mechanism of action (MOA) and explicit evidence of benefits from most of the herbal and complementary medications have not been shown in controlled clinical trials (M Manchishi, 2018). One of the reasons is lack of scientific evidences and basis which hinder the translation of these traditional medicines into patient with great safety (Wachtel-Galor and Benzie, 2011). This shortcoming could be addressed by using the reverse pharmacological approach to determine the specific MOA of those drugs in pre-clinical studies (Helmstädter and Staiger, 2014).

The newly developed AEDs has provided substantial advantages for those affected with epilepsy of different kinds. In patients with newly treated AEDs as many as 60-70% result outcome had been observed in successful control of seizures (Canevini et al., 2010). The newer AEDs introduced during the past decade offer advantages in this respect over older agents (Löscher et al., 2013). Phenytoin is the most widely used AED in the United States, but its hepatic metabolism and associated enzyme induction, as well as its nonlinear pharmacokinetics, are disadvantages for elderly patients. Because of their potential effects on cognitive function, sedating AEDs such as phenobarbital and primidone have little place in the treatment of new-onset seizures in elderly patients. Carbamazepine also is an enzyme-inducing agent with significant potential for drug interactions (Asconapé, 2002). Among the newer AEDs, gabapentin and levetiracetam have good safety and cognitive effect profiles and do not interact with other drugs, and lamotrigine offers many of the same benefits. Oxcarbazepine has better tolerability than carbamazepine, and topiramate and zonisamide (Kanda et al., 2017), although they have more cognitive side effects than the other new AEDs, herbal medicines with anti-epileptic effect can be considered for some elderly patients (M Manchishi, 2018).

In the present study, a similar approach has been adopted. Embelin, a plant based benzoquinone obtained from the fresh berries of the plant "*Embelia ribes*" Burm (Family:

Myrsinaceae) (Durg et al., 2017) was used to evaluate effectiveness of embelin in epilepsy-induced cognitive dysfunction. Embelin is known for its pharmacological activity and non-hazardous nature. Embelin was used traditionally to treat convulsions, as an anti-oxidant and also against worm infection and in sickness behavior (Shaikh et al., 2016). Various review articles on Embelin described it as a promising drug for CNS disorders and it can exhibit strong neuroprotective activity.

The development of new AEDs depends upon pre-clinical research using animal models to establish efficacy and safety prior to the introduction to humans (Löscher and Schmidt, 2006). Various pre-clinical models have been used to investigate the role of different brain functions and to understand disease development (Samarut, 2016). As a small vertebrate of aquatic origin, zebrafish (*Danio rerio*) have become increasingly popular organism for biomedical research (Howe et al., 2013). The usefulness of both adult and larval zebrafish in neurology and neuroscience has grown tremendously in the past decades (Farrar et al., 2018). Zebrafish gained its popularity due to close physiological & genetic homology to humans, and ease in manipulating genetic materials and similarity in CNS morphology (Lakstygal et al., 2018). Other factors for its popularity includes cost-effectiveness, ease of maintainance and rapid breeding.

So, the overall goal of the thesis was to evaluate the effectiveness of embelin in epilepsy-induced memory impairment using zebrafish as an *in-vivo* animal model. The primary objective was to develop animal models of acute and chronic seizure induced–cognitive dysfunction using pharmacological, behavioral and biochemical approach. Secondarily, embelin was screened using developed models and mechanism of action of embelin was studied. The first part of this thesis involved a systematic review on the use of embelin in various CNS disorders. This review gathered all the CNS related activities reported with embelin. This helped in understanding the various features of embelin like safety, dose, pharmacokinetics and BBB permeability.

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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

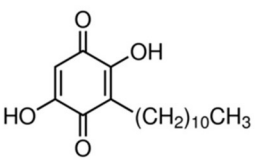
primary health care requirements. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs (World Health Organization, 1999; Saraf, 2012).

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.

Plant of *Embelia ribes*



Chemical structure of embelin

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

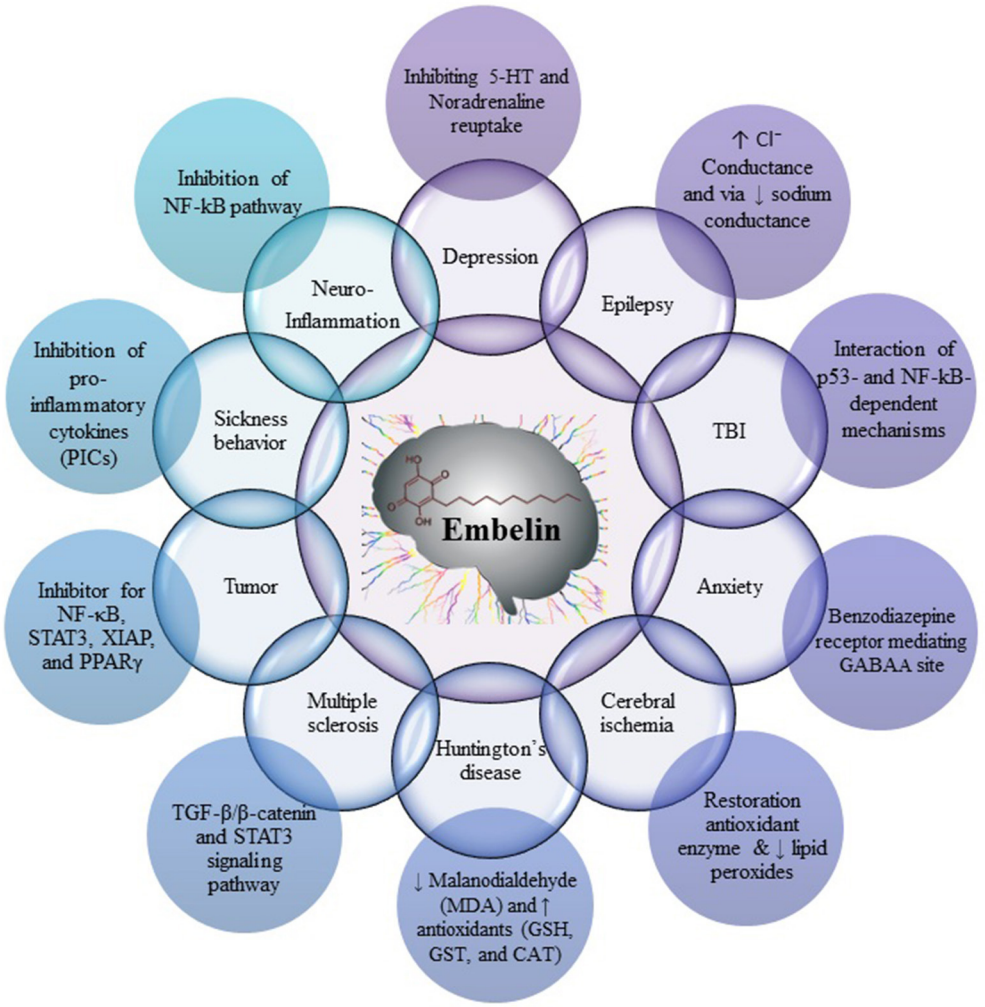


FIGURE 2 | Mechanism of action of embelin on various CNS disorders.

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

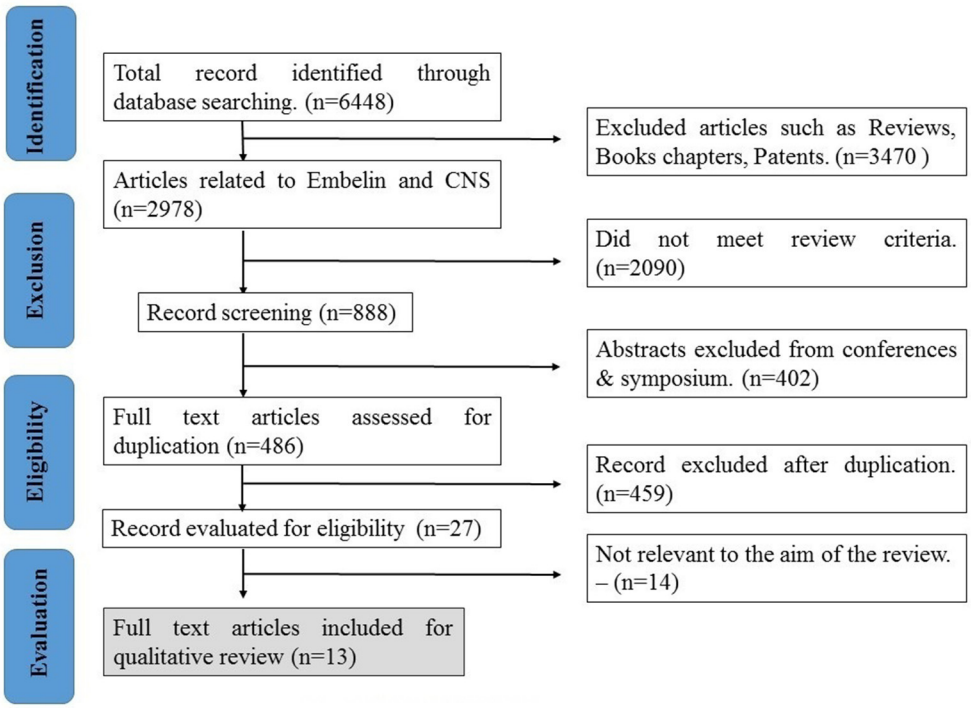


FIGURE 3 | Flow chart of study selection procedure.

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

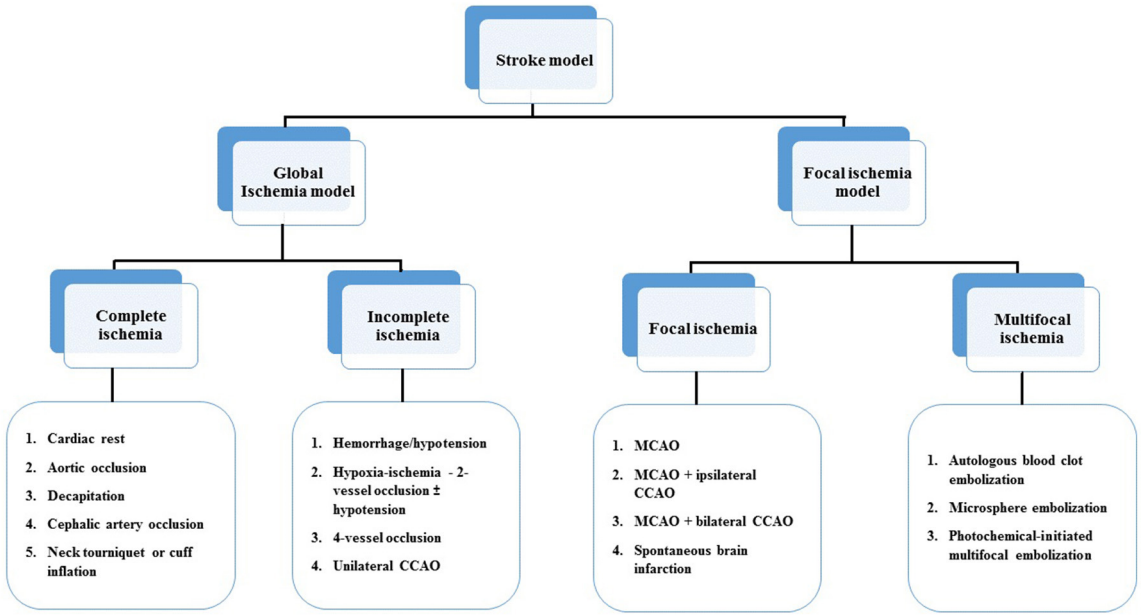


FIGURE 4 | Animal models of ischemic stroke model.

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-2

INTRODUCTION:

In this section, the vertebrate model organism, zebrafish (*Danio rerio*) is discussed in the context of its ability to be developed into a biological screening model to test a large number of potential AEDs in order to select the best acting molecule for further studies in higher animals. Zebrafish are ideal for major neurodevelopment and behavior studies as they have comparable seizure score stages when induced with pro-convulsants like PTZ at a particular dose range (Lakstygal et al., 2018). Behavior and neurotransmitter studies along with molecular components of the CNS in the lower vertebrates can be compared with some higher vertebrates like humans, primates, and rodents, thus giving cost-effective models for further research (Bailey et al., 2013). An important feature of the zebrafish as an organism for neurodevelopmental research is that it has complex, well-defined behaviors (Bugel et al., 2014). Chemical screening in the adult brain can be performed using automated techniques and thus provide neurodegeneration data for a large number of drug molecules lacking this information (Hill, 2013). As advances continue to increase the throughput of gene expression, immunochemistry, and pharmacological analysis, zebrafish are poised to further expansion of knowledge for more CNS related disorders like epilepsy and neurodegeneration (Mitchell et al., 2013).

In the quest to combat human brain disorders, the search for new potential therapeutics is becoming a major hurdle. Therefore, screening for unique CNS molecules is significantly important for translational biomedical research (de Abreu et al., 2018). A very important approach in this field includes developing newer, more valid neurobehavior animal models and paradigms as well as creating high output screening platforms (Denayer et al., 2014). Using zebrafish for seizure locomotor behavior and T-maze analysis is a suitable combination to study epilepsy induced cognitive dysfunction (Kundap et al., 2017). Cognitive problem is an important impairment in one of the six cognitive domains of complex attention, executive function, learning, and memory, language, perceptual motor and social cognition (Snyder et al., 2015). The animal (zebrafish) models of memory problems are specifically divided into three major categories, the pharmacological, developmental and genetic models (Choo and Shaikh, 2018). In this study, we decided to use PTZ to produce acute seizure induced cognitive problems in zebrafish, in which memory was evaluate using a T-maze test (Zhang et al., 2017)

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Zebrafish as a Model for Epilepsy-Induced Cognitive Dysfunction: A Pharmacological, Biochemical and Behavioral Approach

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Epilepsy is a neuronal disorder allied with distinct neurological and behavioral alterations characterized by recurrent spontaneous epileptic seizures. Impairment of the cognitive performances such as learning and memory is frequently observed in epileptic patients. Anti-epileptic drugs (AEDs) are efficient to the majority of patients. However, 30% of this population seems to be refractory to the drug treatment. These patients are not seizure-free and frequently they show impaired cognitive functions. Unfortunately, as a side effect, some AEDs could contribute to such impairment. The major problem associated with conducting studies on epilepsy-related cognitive function is the lack of easy, rapid, specific and sensitive *in vivo* testing models. However, by using a number of different techniques and parameters in the zebrafish, we can incorporate the unique feature of specific disorder to study the molecular and behavior basis of this disease. In the view of current literature, the goal of the study was to develop a zebrafish model of epilepsy induced cognitive dysfunction. In this study, the effect of AEDs on locomotor activity and seizure-like behavior was tested against the pentylenetetrazole (PTZ) induced seizures in zebrafish and epilepsy associated cognitive dysfunction was determined using T-maze test followed by neurotransmitter estimation and gene expression analysis. It was observed that all the AEDs significantly reversed PTZ induced seizure in zebrafish, but had a negative impact on cognitive functions of zebrafish. AEDs were found to modulate neurotransmitter levels, especially GABA, glutamate, and acetylcholine and gene expression in the drug treated zebrafish brains. Therefore, combination of behavioral, neurochemical and genenetic information, makes this model a useful tool for future research and discovery of newer and safer AEDs.

Keywords: zebrafish model development, epilepsy, anti-epileptic drugs, cognitive dysfunction, T-maze

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INTRODUCTION

Epilepsy is a chronic neurological disorder characterized by unpredictable seizures, which may differ from a brief lapse of attention and muscle cramps to severe and long-lasting convulsions (Zashikhina, 2014). It has a poorly understood pathologic mechanism and is an intricate brain disorder with numerous fundamental causes (Galanopoulou et al., 2012). The multifactorial nature

of epilepsy needs to be taken into consideration when developing therapeutic strategies overcoming selected mechanisms. Its clinical management is predominantly based on the administration of anti-epileptic drugs (AEDs) aiming to suppress the seizure activity. Although more than 20 AEDs are available, nearly 30% of the epileptic patients are refractory to drug-treatment. The AEDs are used to modify the processes involved in epileptogenesis and promote inhibition over excitation and thus prevent epileptic seizure (White et al., 2007). The AEDs act by several mechanism but the major mechanisms of action, include γ-aminobutyric acid (GABA) enhancers, glutamate blockers, calcium current inhibitors and sodium channel blockers (Czapinski et al., 2005). AEDs enhance inhibitory neurotransmission or suppress neuronal excitability. (Aldenkamp et al., 2003). Owing to the advantages of wide availability, lower cost and long-term experience older AEDs are still been prescribed, but greater effects are often exhibited by older AEDs (Eddy et al., 2011). Newer agents which are currently established have differences in their pharmacokinetic properties, mode of pharmacological action (Bootsma et al., 2009).

Cognitive impairment is a common comorbidity in multiple brain disorders like epilepsy, Alzheimer's disease (AD), schizophrenia, Huntington's disease (HD) and autism (Vingerhoets, 2006). Brain areas affected due to electric discharge in epilepsy are temporal lobe, hippocampus, medial frontal brain regions, bilateral superior temporal and subthalamus brain regions in epileptic patients (Laufs et al., 2007). It is better agrued that seizure-induced neuronal modeling during epilepsy and recurrent seizures can cause continuous neuronal reorganization (Pitkänen and Sutula, 2002). As temperol lobes and hippocampus is associated with memory formation, it is not shocking that epilepsy in such area can cause memory dysfunctioning (Helmstaedter and Kockelmann, 2006). Neurotransmitters are important in maintaining the normal brain functions and also known to be modulated during a brain insult. Alteration of neurotransmitters has been found to be closely associated with epilepsy. Some important neurotransmitters known to paly a significant role in epilepsy and cognition are GABA, glutamate and acetylcholine (Sancheti et al., 2013). GABA is an inhibitory transmitter and known for its role in suppressing epilepsy (Rico et al., 2011). Glutamate is an excitary chemical which causes neuronal death and is associated with glutamate neuro toxicity in epilepsy (Ozawa et al., 1998). Acetylcholine (ACh) plays the key role in modulating glutamate release and memory formation. It is reported that AEDs reduce neuronal irritability but also may impair neuronal excitability, neurotransmitter release, enzymes, genes and factors critical for information processing and memory (Hamed, 2009). As per a clinical study done at Columbia University, New York, NY, United States over all AEDs related cognitive side effect (CSEs) was found to be 12.8%. Drug specific CSEs for gabapentin (7.3%), oxcarbazepine (11.6%), phenytoin (12.9%). From the study, it was concluded that patient-reported CSEs are most common with topiramate (TPM), followed by zonisamide, phenytoin, and oxcarbazepine (Arif et al., 2009).

Zebrafish has a complex nervous system capable of sophisticated behaviors and susceptible to seizures. A full range of mature behavior can be studied in adult zebrafish which makes them particularly desirable for model development. In last few years, the use of zebrafish has gained popularity as an alternative to rodents and other experimental animals for the study of the molecular mechanisms underlying cognition deficit and for the screening of potential therapeutic compounds (Kalueff et al., 2014). There is much scientific evidence illustrating the benefits of animals, such as zebrafish, as a replacement and better animal model for drug discovery (**Figure 1**). Genetic structure of zebrafish is similar to human. Around 70 percent of genes are shared with humans and about 84 percent of genes known to human disease are also expressed in zebrafish (Norton and Bally-Cuif, 2010). The overall cost of the studies which involves large animals are expensive and laborious. The small animal or rodent models are preferred to study safety profile of a drug and for testing of the hypothesis (Jucker, 2010). The non-human primate and rodents models are similar to humans in terms of their anatomy, physiology and behavior, but they are not so common due to ethical and economical concerns (Jucker, 2010). The cost and time required to carry out the study in zebrafish model is less as compared to rodents (Mussulini et al., 2013). For studying various brain disorders, zebrafish (*Danio rerio*) is quickly mounting as a promising model creature (Kundap et al., 2016). It is important to minimize animal distress by using the least sensitive organism possible to answer the question at hand. One of the biggest challenges in conducting research on epilepsy and cognitive function is the absence of a precise, sensitive, and reproducible disease model (Vining, 1987).

Pentylenetetrazole (PTZ) is used in zebrafish to induce epileptic seizure-like condition and to study the mechanisms of seizures (Desmond et al., 2012). γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain, PTZ exerts its convulsive effect by inhibiting the activity of GABA at $GABA_A$ receptors (Shaikh et al., 2013). There are several reports which provide strong evidence on the usefulness of zebrafish model for studying cognitive functions. Investigators used active avoidance paradigm to discover a high impact about learning and memory in zebrafish. In this method zebrafish are trained to associate light with shock stimulus in a fish shuttle-box (Xu and Goetz, 2012). Zebrafish has natural color preference choice and it has received little attention in past few research. Natural color preference concerning a precise color may lead to changes in learning, decision making, memory and visual discrimination (Avdesh et al., 2010). The zebrafish is becoming an popular model increasingly for investigating treatment method and understanding the process behind memory problems in AD (Newman et al., 2014). The T-maze is a forthright method to test the learning skill, long- and short-term memory, and memory plasticity in zebrafish (Vignet et al., 2013). The T-maze has been most comprehensively used to examine specific features of spatial working and learning memory (Wenk, 2001).

In the view of current literature, the prime goal of the study was to develop a zebrafish model of epilepsy induced cognitive dysfunction and simulate the clinical condition which shows that both epilepsy and AEDs negatively affect the cognitive functions. This zebrafish model will serve as an important tool

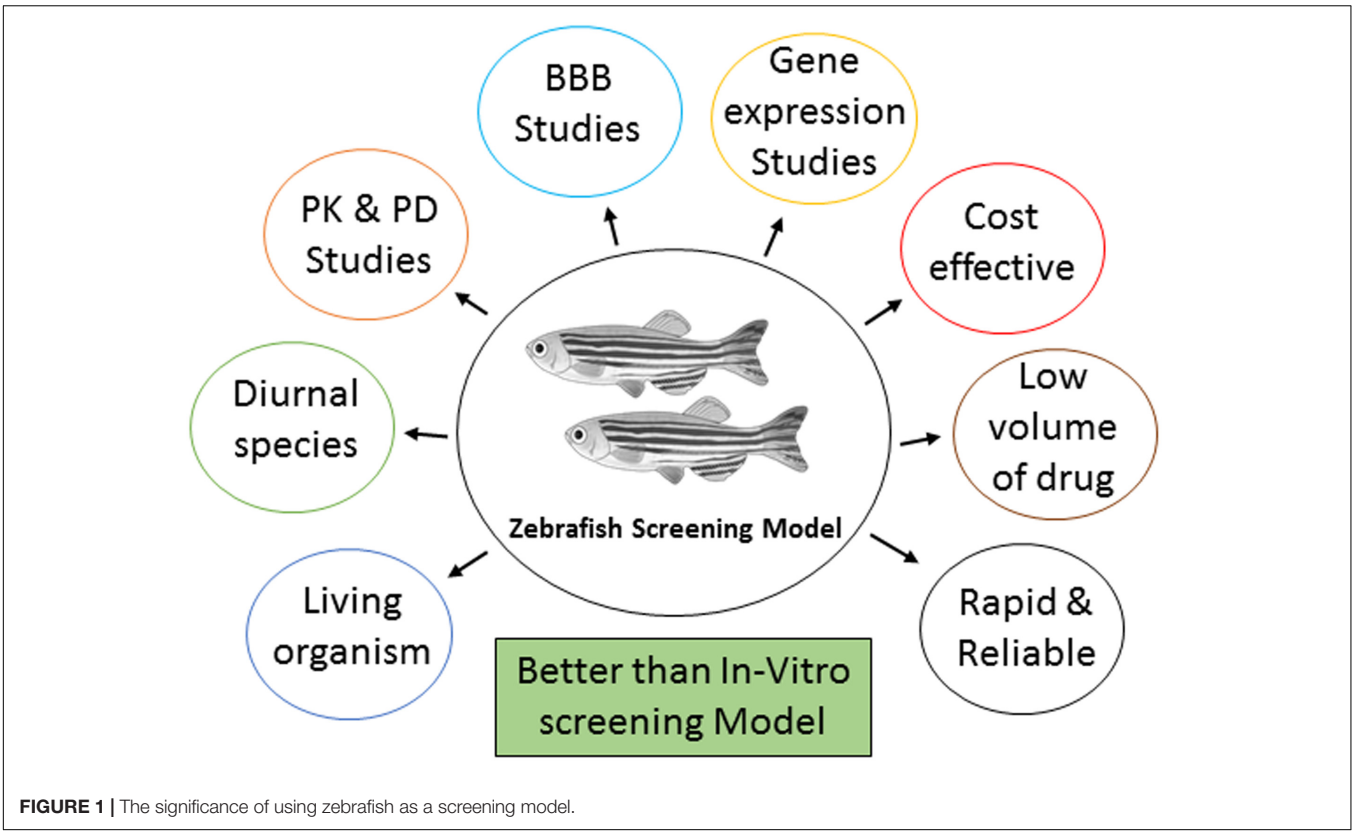

FIGURE 1 | The significance of using zebrafish as a screening model.

for the development and screening of newer and safer anti-epileptic drugs with intact memory functions. As epilepsy involve important biological and physiological mechanisms, studying zebrafish behavior, neurotransmitters level and gene expression are of great significance and key parameters in the development of an impressive animal model.

MATERIALS AND METHODS

Chemicals and Equipment

Glutamic acid (Glu), γ-aminobutyric acid (GABA), Acetylcholine (ACh) and Pentylenetetrazole (PTZ) were from Sigma–Aldrich (United States). All the standard AEDs such as Phenytoin (PHY), Oxcarbazepin (OXC), Gabapentin (GBP), Diazepam (DZP), Rivastigmine (RSV) were from Sigma–Aldrich (United States). Dimethyl sulfoxide (DMSO) was purchased from Vivantis Inc (United States). Sony video recorder, Smart V3.0.05 tracking software (Pan Lab, Harvard apparatus), Agilent 1290 Infinity UHPLC, coupled with Agilent 6410 Triple, Quad LC/MS, Milli-Q system from Millipore (Bedford, MA, United States), Applied Biosystems StepOnePlus^{TM} Real-Time PCR Systems.

Animal Care

Adult zebrafish (*Danio rerio*; 3–4 months-old) of heterogeneous strain wild-type stock (standard short-fin phenotype) were obtained from a Akarium Batukarang, Subang Jaya, Malaysia. All fish were housed in the animal facility of Monash University Malaysia under standard husbandry conditions. Fish were maintained under temperature 28°C ± 2°C, pH between 6.8 and 7.1 and light intensity 250 lux with 14 h light: 10 h dark regime (light onset: 8am; light offset: 10pm). Fish were fed with TetraMin® Tropical Flake and live Artemia from Bio-Marine Brand (Aquafauna, Inc. United States) three times a day to ensure a constant source of nourishment with *ad libitum* feeding. Standard zebrafish tank which are equipped with circulating water system with constant aeration having tank dimensions of (36 cm × 26 cm × 22 cm) were used as shown in **Figure 2**. Group housing (10–12 fishes/tank), males and females having seperate housing arrangement. All the animal experimentations were approved by Monash Animal Research Platform (MARP), Australia.

Drug Treatment and Groups

Adult male zebrafish (*Danio rerio*) were used. PHY, OXC, GBP, DZP, RSV and PTZ were dissolved in 10% DMSO. Three months old fish were selected with a weight range of 0.5–0.6 g. Animal were divided into following groups, Group I: Vehicle control (10% DMSO); Group II: Pentylenetetrazole (PTZ-Negative control group); Group III: Phenytoin 80 mg/kg (PHY) + PTZ (170 mg/kg); Group IV: Oxcarbazepin 80 mg/kg (OXC) + PTZ (170 mg/kg); Group V: Gabapentin 800 mg/kg (GBP) + PTZ (170 mg/kg); Group VI: Diazepam 1.25 mg/kg (DZP) + PTZ (170 mg/kg); and Group VII: Rivastigmine 1.5 mg/kg (RSV) + PTZ (170 mg/kg). In the experiment 10–12 fisher group were used (**Figure 2**).

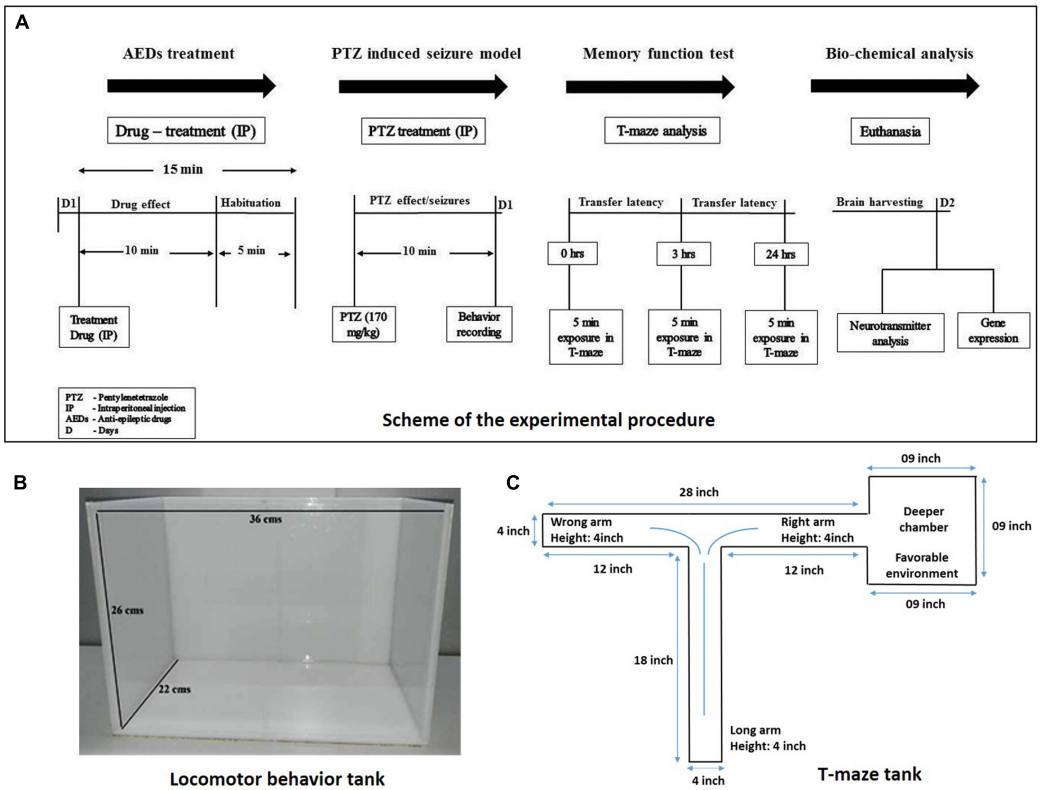

FIGURE 2 | Experimental procedure and experiment tanks used. **(A)** Represents scheme for the experiment procedure used to carry out the study. **(B)** Represent the tank used for the locomotor behavior study. **(C)** Illustrates the typical size and specification of T-maze apparatus.

Intraperitoneal Injection in Zebrafish

The vehicle, PTZ and AED treated groups were injected intraperitoneally (via posterior to the pelvic girdle into the abdominal cavity), using Hamilton syringe 10 μl (700 series, Hamilton 80400) (Stewart et al., 2011). The experiment was performed in a separate behavior room with constant room temperature of 28°C ± 2°C and humidity 50–60%. All the fish were acclimatized in the behavior room 2 h prior to experiment to avoid novel tank response. Precautions include using a small injection volume of 10 μl per gram of fish and a 35 gauge needle. Fish were restrained in water saturated sponge under benzocaine anesthesia to reduce the distress (Júnior et al., 2012). This technique of IP injection in zebrafish was found to be effective and did not cause any mortality throughout the experiment.

Fish was captured individually by fish holding net, then transfer into anesthesia solution (30 mg/L Benzocaine), Fish was taken out once anaesthesized and weighed to calculate the dose and the injection volume. A soft sponge of approximately 20 mm in height was saturated with water and set into 60 mm Petri dish. A cut of 10–15 mm deep was made on the sponge to restrain and hold the fish for injection. Intraperitoneal injection was made using a dissecting microscope by inserting the needle into the midline between the pelvic fins. Appropriate volume was injected according to the body weight. After injection, fish was immediately transferred to the tank.

PTZ-Induced Seizure Model

The fish were treated with vehicle/AEDs via intraperitoneal injection and then habituated for 15 min in the observation tank before administration of PTZ. The vehicle control group only received 10% DMSO and suspended for behavior recording. Fifteen minutes after vehicle/AEDs administration, the animals were exposed to PTZ (170 mg/kg, IP) which presented different seizure profiles, intensities and latency to reach the scores. The seizures last for 10 min post-PTZ administration, which gradually decreases with time.. Adult zebrafish were tested in an observation tank, where the seizure score were measured using a special scoring system as mentioned in **Table 1**. Under the directives of Monash Animal Research Platform (MARP)-Australia, the dose of PTZ was adjusted to 170 mg/kg in order

TABLE 1 | Seizure score analysis modified from Mussulini et al. (2013).

Seizure scores	Event of Behavior
Score 1	Short swim mainly at the bottom of the tank
Score 2	Increased swimming activity and high frequency of opercula movement.
Score 3	Burst swimming, left and right movements and erratic movements
Score 4	Circular movements

to get the highest seizure score of 4. Seizure score, seizure onset, total distance traveled, time spent in upper and the lower half of the tank were the parameters noted (Baraban et al., 2005).

T-maze Test

The T-maze is composed of one long (18′) and two short (12′) arms. One of the short arms is connected to a deeper square chamber (9 × 9′) which serve as a favorable environment for the fish (see **Figure 2**). Favorable environment is the chamber which is deeper and wider compare to other arms in T-maze and once fish finds it, they spend the majority of their time in it. The T-maze behavior test was performed in the behavior room of constant room temperature of 25°C – 26°C and humidity 50– 60%. Each fish was placed at the beginning of the long arm and the time required to reach deeper chamber was recorded in the 5 min exploration period. The time taken by the fish to travel into the deeper chamber was determined as transfer latency (TL). Transfer latencies were recorded at 0, 3, and 24 h post-PTZ exposure. The TL was expressed as inflection ratio (Kasture et al., 2007). Inflexion ratio (IR) = (L0-L1)/(L1), (IR) = (L0-L2)/(L2), where L0 is the initial latency (s) at 0 h and L1 and L2 is the latency (s) at 3 and 24 h trial. Behavior recording from seizure activity and T-maze test were analyzed for locomotor patterns. Tracking of the locomotor pattern was done by using computer software SMART v3.0-Panlab Harvard Apparatus®.

Brain Harvesting

At the end of the behavioral studies, fish brains were harvested. The brain from each group was further divided into two sets and transferred into trizol for gene expression studies and methanol for LC-MS/MS respectively. The Brain was harvested by removing the skull of the fish and extracting the brain directly into the respective solvent. Brains were stored immediately at −80°C until further use.

Neurotransmitter Analysis Using LC-MS/MS

Important neurotransmitters like GABA, glutamate, and acetylcholine (ACh) were analyzed using LC-MS/MS. Stock solutions of 1 mg/ml were prepared for all the standard neurotransmitters in methanol (0.1% formic acid). The solution was kept at 4°C until use.

Serial dilution from 100 to 2000 ppb was used for calibration. The brain was homogenized in 200 µl ice-cold methanol (1% formic acid). The homogenate was vortex-mixed for 1 min and then centrifuged at 18,000 × *g* for 10 min at 4°C. Finally, the supernatant was pipetted and placed into vials for LC-MS/MS analysis.

LC–MS/MS was run on an Agilent 1290 Infinity UHPLC, coupled with Agilent 6410 Triple Quad LC/MS, ZORBAX Eclipse plus C18 RRHD 2.1 × 150 mm, 1.8-micron (P/N 959759-902) column, the auto-sampler system (Agilent Technologies, Santa Clara, CA, United States). The samples were separated on a SMol-RRHD-Eclipse-C18-8 (15) UHPLC-160129-00011-Pos-DMRM used at 30°C. The mobile phase consisting of 0.1% formic acid in water (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B) was used with a gradient elution: 0–3 min, 50% B; 3–6 min, 95% B; 06–07 min, 95% B at a flow rate of 0.1 ml/min. ESI-MS/MS Conditions were set as follows: ESI ion source, positive ion polarity, gas temperature 325°C, drying gas flow 9.0 L/min, nebulizer pressure 45 psi, Vcap 4000V. MS acquisition of GABA, Glu, ACh as performed in electrospray positive ionization multiple reaction monitoring (MRM) mode.

Gene Expression

All brain samples were collected in ice-cold 200 µl TRIzol® reagent (Invitrogen, Carlsbad, CA, United States) and stored at −80°C until use. Gene expression study was carried out for Neuropeptide Y (NPY), Brain-derived neurotrophic factor (BDNF) and cAMP-responsive element-binding protein1 (CREB1) genes.

Isolation of RNA and First Strand cDNA Synthesis

Total mRNA were isolated by following the manufacturer's protocol. In brief, brain tissues were appropriately homogenized in TRIzol® reagent followed by mixing with chloroform and centrifuged at 13,500 rpm (revolutions per minute) for 15 min at 4°C. The upper aqueous supernatant was transferred into new tubes and isopropanol was added, mixed and were incubated for 10 min at room temperature and later centrifuged for 10 min at 13,500 rpm at 4°C. The supernatant was discarded and the pellets were subjected for rinsing with 75% ethanol. The pellets were then left for air drying between 5 and 8 min. Finally, nuclease-free water was added to each tube to dissolve the mRNA pellet. The concentration and purity of the isolated mRNA were measured by using NanoDrop Spectrophotometer. The mRNA samples were converted to cDNA with the help of Omniscript Reverse-transcription Kit (QIAGEN).

StepOne® Real-Time PCR

Gene expression for NPY, BDNF and CREB1 were computed using real-time quantitative RT-PCR (Applied Biosystems) using QuantiTect SYRB Green dye (Qiagen, Valencia, CA, United States). All the primer sets were purchased by Qiagen (npy: Dr_npy_1_SG QuantiTect Primer Assay (QT02205763), bdnf: Dr_bdnf_1_SG QuantiTect Primer Assay (QT02125326), creb:Dr_crebbpa_1_SG QuantiTect Primer Assay (QT02197503). Samples were incubated at 95°C for 2 min prior to thermal cycling (40 cycles of 95°C for 5 s and 60°C for 15 s). Relative expression of these three genes were attained by normalizing threshold cycle (Ct) values against Ct value of eef1a1b (housekeeping gene) (2 ^ [Ct eef1a1b – Ct Gene of interest]).

Statistical Analysis

All the data in the results are expressed as Mean ± Standard Errors of the Mean (SEM). Data is analyzed by Analysis of Variance (ANOVA) followed by Dunnett's tests. The *P*-value $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ is considered as

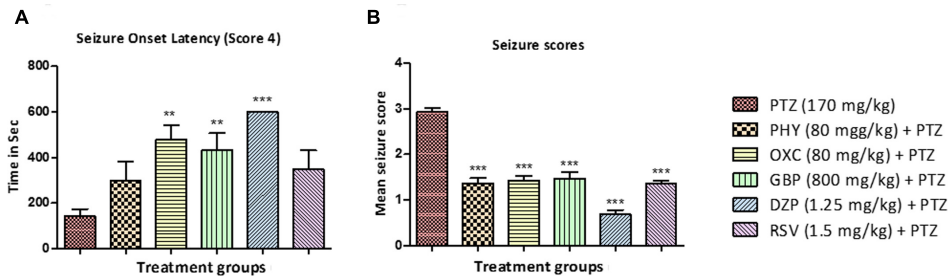

FIGURE 3 | Seizure onset latency and behavior seizure score. **(A)** Represent seizure onset latency (score 4) of AEDs treated fish when compared with PTZ treated group. **(B)** Represent the effect of AEDs on PTZ induced seizure in zebrafish. Data are expressed as Mean ± SEM, $n = 10$ and statistical analysis by one-way ANOVA followed by Dunnett test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

statistically significant. All the groups were compared with the PTZ negative control group.

RESULTS

Seizure Onset Latency and Seizure Score Analysis

All the animals in the PTZ treated (Group II) reached seizure score 4 within 150 – 180 s after PTZ administration. In contrast, the onset was delayed in animals treated with standard AEDs as shown in **Figure 3A**. Drugs like, OXC, GBP, and DZP significantly delayed the onset of seizure whereas delay in onset with PHY and RSV was statistically insignificant. Seizures are measured using a special scoring system (**Table 1**). All the animals treated AEDs displays seizure not more than score 2 and exhibited seizure suppression activity. Thus a significant anti-epileptic activity was observed in all the animals treated with AEDs as shown in **Figures 3A,B** ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Locomotor Pattern

The locomotor pattern in the vehicle control group was demonstrated by normal swimming all over the tank. The PTZ group has spontaneously provoked seizures which is represented by abnormal and circular tracking pattern. The locomotor tracking pattern of PHY, RSV, OXC, GBP and DZP treated groups showed attenuation of PTZ seizure effect and a swimming pattern nearly similar to control group as shown in **Figure 4A**. The total distance traveled was significantly higher in all AED treated groups as compare to PTZ group. The total distance traveled was higher in control group as compare to PTZ group but it was found to be statistically insignificant as shown in **Figure 4B**. The control fish have spent the equal duration of time in both the halves of the tank, whereas PTZ group was found to be inconsistent in their swimming and spent more time in the lower half compared to the upper half of the tank. As fish were protected from seizure by treatment of AEDs, drug treatment reversed the PTZ seizure effect and time spent in lower half of the tank. So, AED treated group spent more time in upper half than compare to PTZ group as depicted in **Figures 4C,D** ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Zebrafish T-maze Test for Anti-epileptic Drugs

The control group showed less to no repeated entry into the wrong arm whereas PTZ group exhibited an opposite effect, so the time spent and total distance traveled was found to be significantly less as compared to PTZ treated group as shown in **Figures 5A,B**. T-maze tracking pattern of GBP and DZP shows single wrong entry in the T-maze. Whereas PHY, RSV and OXC treated group showed repeated entry to the wrong arm. Time spent in wrong arm by DZP, OXC, GBP and RSV was found to be significantly less as compared to PTZ group. PHY showed no significant reduction in time spend in wrong arm as shown in **Figure 5B**. AEDs treated group took the high/long time to reach the deepest chamber, and time spent in the wrong arm was also increased exhibiting impaired memory functions similar to PTZ group as shown in **Figure 5C**. Fish from vehicle control group exhibited an improved IR (memory function) at 3 and 24 h in the absence of seizures. PTZ treated group exhibited decreased IR both at 3 and 24 h. All the AEDs do not significantly increase IR (memory function) at 3 and 24 h when compared with PTZ group as shown in **Figures 5D,E**. In AED-treated groups, PTZ challenge had affected their IR, none of the AEDs significantly improved memory function when compared with PTZ group ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Estimation of Neurotransmitters by LC/MS-MS

Neurotransmitter analysis showed that, as PTZ is a GABA-$_{A}$ receptor blocker the levels GABA in PTZ treated group were lower compared to control. No significant increase in GABA was found in PHY, OXC and DZP treated groups when compared to the PTZ group. GBP and RSV exhibited a significant increase in GABA as compared to the PTZ group as shown in **Figure 6A**. The level of glutamate in PTZ treated group was higher as compared to control group. Fish treated with AEDs significantly protected against PTZ induced glutamate surge and maintained glutamate levels similar to control group as shown in **Figure 6B**. Brain acetylcholine levels were significantly decreased by the PTZ group as compared with control. All the AEDs except DZP showed a similar ACh level as of PTZ group. DZP was the only

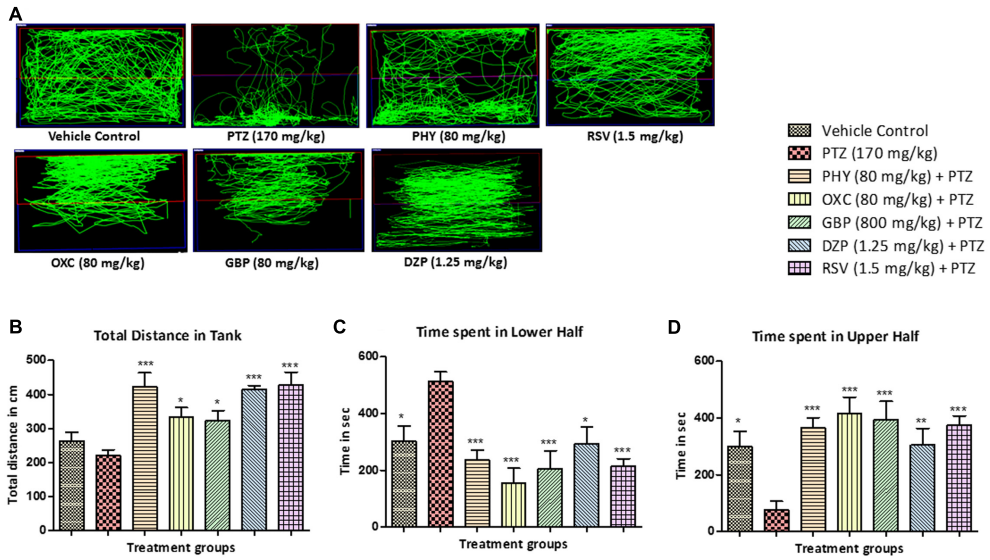

FIGURE 4 | Pentylenetetrazole (PTZ) induced locomotor pattern and behavior. **(A)** Representative locomotion tracking patterns of vehicle control, PTZ treated and all the AEDs treated groups. **(B)** Represent the total distance traveled by the fish in experiment tank in different groups. **(C,D)** Represent the total time spent by each fish in the lower and upper half of the experimental tank. Data are expressed as Mean ± SEM, $n = 10$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

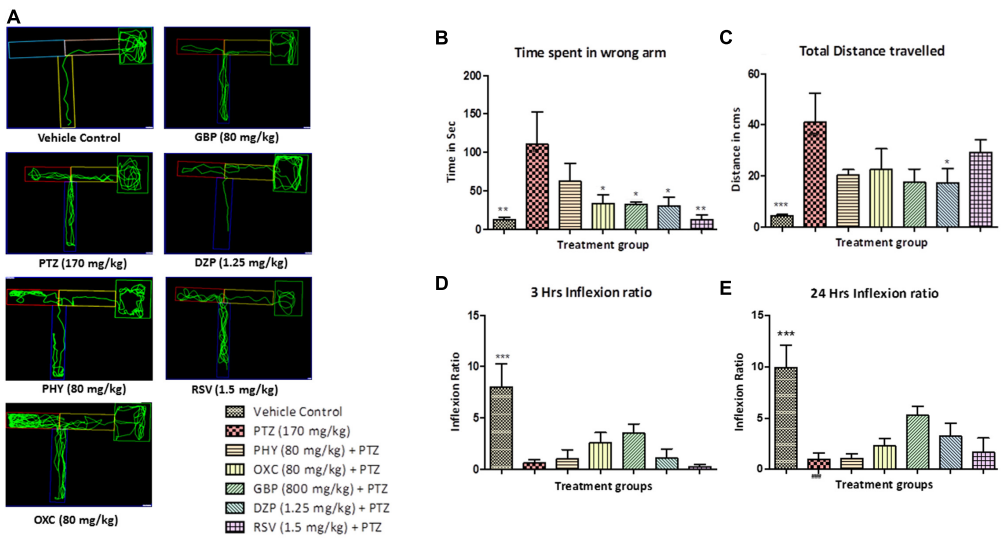

FIGURE 5 | T-maze analysis and tracking pattern. In **(A)** each plot represents locomotor pattern of vehicle control, PTZ treated and all the AEDs treated groups. **(B,C)** represent total time spent in the wrong arm of T-maze by each fish and total distance traveled to reach the deeper chamber. **(D,E)** represents graph plot of inflecion ratio at 24 and 3 h T-maze trial. Data are expressed as Mean ± SEM, $n = 10$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

drug that exhibited increased ACh levels as shown in **Figure 6C** ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Estimation of Gene Expression by RT-PCR

Brain-derived neurotrophic factor mRNA levels were downregulated in the PTZ treated group when compared with control. BDNF mRNA expression in PHY and DZP was found to be statistically insignificant. However, OXC, GBP, and RSV significantly upregulated mRNA expression of BDNF, as compared to PTZ treated group as shown in **Figure 7A**. CREB1 mRNA expression was up-regulated in the PTZ treated group as compared to the vehicle control group. In all AEDs treated fish except DZP and RSV, the mRNA expression of CREB_1 was similar as that of PTZ group, however, PHY, OXC, and GBP

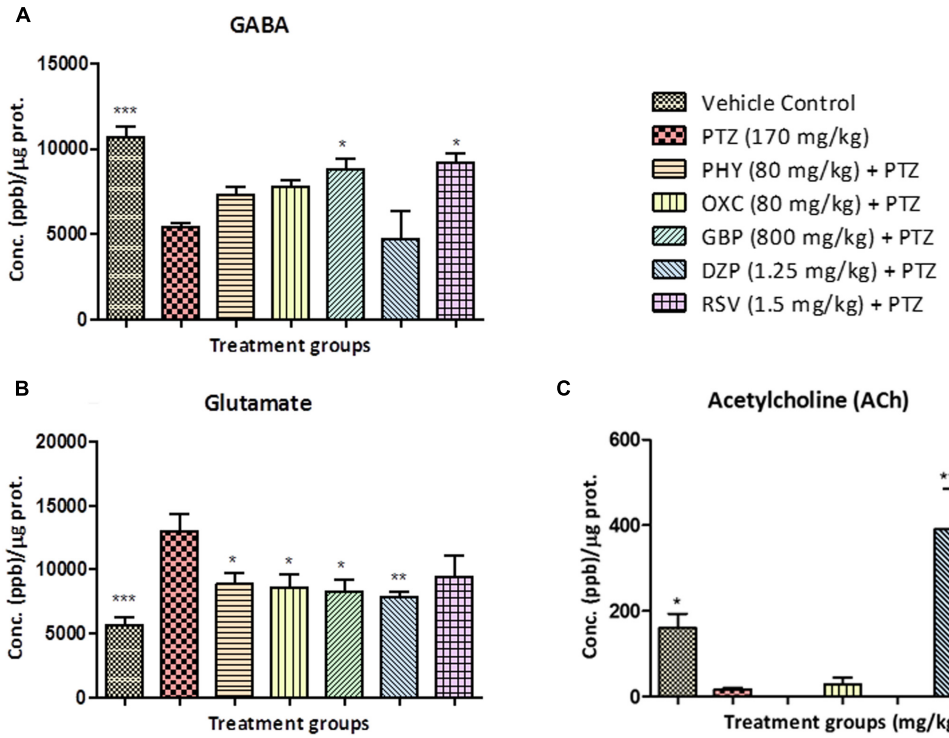

FIGURE 6 | Concentration of neurotransmitters in zebrafish brain after T-maze test. Figure represents zebrafish neurotransmitter levels. **(A)** GABA, **(B)** Glutamate **(C)** Acetylcholine. Data are expressed as Mean ± SEM, $n = 6$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

significantly upregulated the expression as shown in **Figure 7B**. NPY mRNA expression was downregulated in the PTZ treated group. However, this down-regulation was ameliorated by DZP pre-treatment as compared with PTZ group. This effect was same as observed in the control group. There was no significant difference observed in PHY, OXC and RSV pre-treatment when compared with PTZ treated group as shown in **Figure 7C** ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

DISCUSSION

This work present development of a new model for epilepsy-induced cognitive dysfunction in zebrafish. This model has shown the effect of epilepsy on behavior, locomotion, important neurotransmitters and related genes expression. A considerable modulation in the parameters related to cognition such as learning and memory, proteins and genes were observed after epilepsy seizures. Effect of different AEDs' was evaluated using the developed model. Findings of the this study suggests that AEDs significantly reverse the epileptic conditions but have some negative impact on the cognitive functions of the zebrafish.

For studying various brain disorders, zebrafish (*Danio rerio*) are gaining importance and emerging as a promising model organism (Guo et al., 2015). Seizure-like behavioral responses can be induced in adult zebrafish by several pharmacological approaches and there is an increasing trend of utilizing zebrafish model in epilepsy research (Kalueff et al., 2014). They are smaller in size and easy to maintain. Furthermore, it is a diurnal species with several fundamental similarities to humans (Stewart et al., 2014). As there is a limited spectrum of antiepileptic drugs (AEDs) available. A lot of patients are resistant to the existing therapy and many suffer from drug related comorbidities. In order to develop safe and efficient AEDs, there is a lack of model systems that fully recapitulate the condition of epilepsy induced cognitive dysfunction (Phillips and Westerfield, 2014). Zebrafish as an alternative to existing animal models is gaining popularity in the field of cognitive research. There are several reports which provide strong evidence on the usefulness of zebrafish model for studying cognitive functions (Stewart and Kalueff, 2012).

Pentylenetetrazole is a chemoconvulsant act via $GABA_A$ receptor and act on an allosteric site (Desmond et al., 2012). A dose of 220 mg/kg of PTZ produce clonic tonic seizure in adult zebrafish (Banote et al., 2013). In the present study, a dose of 170 mg/kg was used as ethics committee suggested to use a lower dose to prevent further distress. Mussulini et al., 2013, utilized PTZ exposure to fish by dissolving pro-convulsant in the tank water (Mussulini et al., 2013). Whereas, IP injection in adult zebrafish was introduced by Kinkel et al. (2010). One of the method for administration of test substances to zebrafish is by dissolving the drug or chemical into the tank water, and it expected that test substance will be taken up by the fish. Nonetheless, tank water method do not give any idea about how much of the drug is actually absorbed or taken up

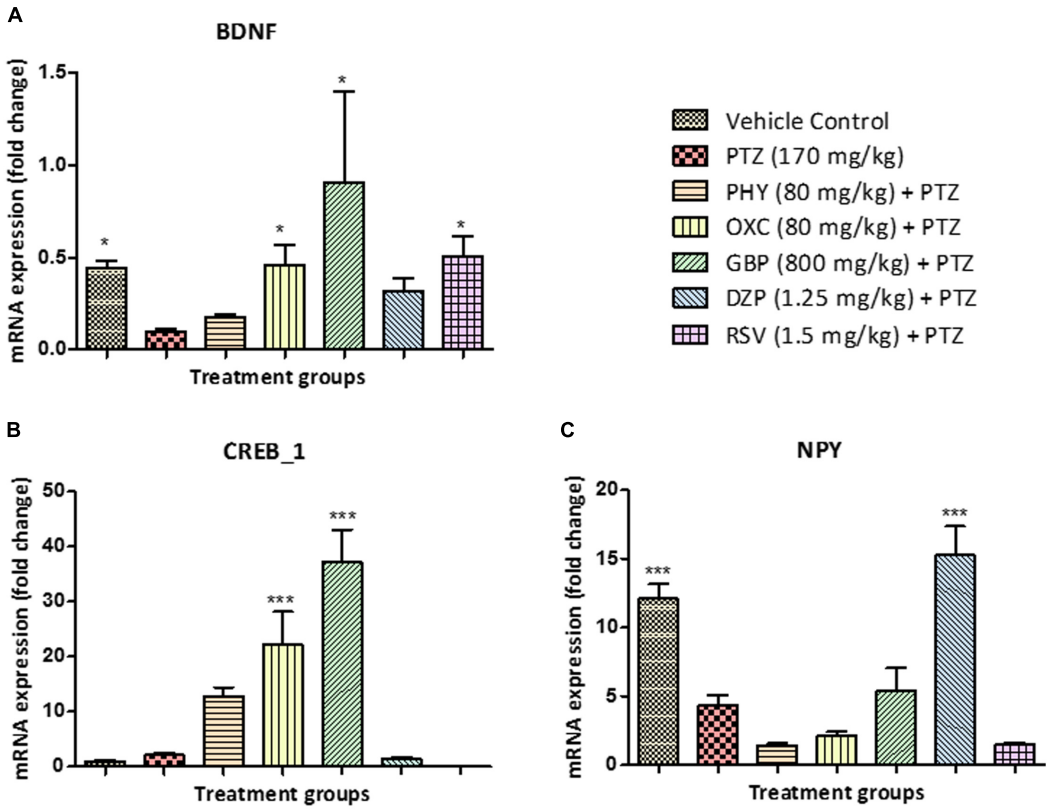

FIGURE 7 | Gene expression in zebrafish brain determined by real time-PCR. **(A)** represents graph plot for BDNF mRNA expression in zebrafish brain, the level of BDNF fold change was found to be high in AEDs treated and control group as compared to PTZ treated group. **(B)** represents graph plot of CREB_1 mRNA expression levels in zebrafish brain, the level of CREB_1 fold change were found to be high in AEDs treated as compared to PTZ treated group. **(C)** represents graph plot of NPY mRNA expression in zebrafish brain, the level of NPY fold change were found to be high in DZP treated and control group as compared to PTZ treated group. This result shows that AEDs alters the gene expression fold change in the brain and affect the cognitive functioning of the brain. Data are expressed as Mean ± SEM, $n = 6$ and $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

by the fish (Kinkel et al., 2010). Intraperitoneal injection is considered as a better option to deliver a definite amount of drug to each fish, based on body weight calculations (10 μl for 1 gm fish). This route is of greater significance in order to correlate drug concentration to the efficacy and also important in metabolic studies (Traver et al., 2003). Intraperitoneal injection is relatively safe with zero mortality when tested in our laboratory. Hence, IP route of drug administration was preferred in our research.

In the present study, we have used seizsure score as described by Mussulini et al. (2013) with minor modifications. All the AEDs tested, delayed the onset of seizure in zebrafish after PTZ insult. This indicates the positive role of AEDs in preventing the seizure episode in patients (Patsalos et al., 2008). Latency to seizure score 4 is the time required by the fish to reach seizure score 4 in a given time frame of 600 s. Drugs like PHY, OXC, GBP, DZP and RSV significantly reduced seizure score when compared to PTZ treated group. The animals in the PTZ treated (Group II, negative control) exhibited seizure-like activity and full blown seizures of score 4 as shown in **Figures 3A,B**. Seizure scores mimic the clinical condition in the patients (Huang et al., 2001). Higher the score, more intense is the seizure episode. All the AEDs significantly helped in reducing the severity of the seizures as indicated by seizure score less than 2. It is also depicted by locomotor pattern as described earlier. Locomotor pattern clearly explains the abnormal behavior in PTZ group. The behavioral analysis and locomotor tracking patterns explain that the AEDs helped fish to overcome the PTZ effect and restored usual swimming movements nearly all over the tank. As seen in PTZ treated group the locomotor moment of the fish was observed to be at the bottom most part of the tank and time spent by fish was also more, which is similar to clinical stupor like behavior and anxiety in epileptic condition.

Seizures are the uncontrolled firing of neurons which produces involuntary moment of the body, which could be a partial or generalized type of seizures (Berg et al., 2010). Since greater the seizure frequency-duration and severity, it is likely to increase cognitive impairment in patients (Inoyama and Meador, 2015). Epidemiological studies reveal that dementia or memory problems is largely a unseen problem and the number is increasing (Farooq et al., 2007). Cognition in epilepsy can adversely be affected by multiple factors, including the seizure etiology, hereditary factors, psychosocial factors, and sequelae of epilepsy treatment, including AEDs (Motamedi and Meador,

2003). AEDs, primarily affects psychomotor speed, vigilance, and attention. Secondarily, it disturbs cognitive functions like memory and learning abilities (Chung et al., 2007). Children with age below 12 have a developing nervous system which is more susceptible to the long-standing side effects of AEDs-induced cognitive impairment. It is a serious problem and it is imperative to recognize and strategies to subside the negative effect of AEDs on cognition (Lagae, 2006). In a similar way, pharmacodynamic and pharmacokinetic factors both in combination are responsible for the cognitive effects of AEDs in individuals. For example, in the clinical setting, levetiracetam (LEV) and carbamazepine (CBZ) has reported adverse pharmacodynamic interactions (Sisodiya et al., 2002). LEV, TPM, CZB and other AEDs exhibit toxicity during combination therapy having a pharmacodynamic interaction (Perucca, 2006). Switching to another drug may help in preventing the damage and in improving cognitive function, memory, alertness, or ability to concentrate. Reports also shown that shifting from multi-drug therapy to monotherapy has been advantageous in reducing cognitive adverse effects (Vining et al., 1987; Wenk, 2001).

T-maze is used as an assessment tool which provides the brief overview of the cognitive status in a zebrafish model. T-maze is one of the most widely used behavioral paradigm to study detailed features of spatial working memory (Wenk, 2001). In this repetitive learning task, individual zebrafish were trained to explore and travel to the deeper end of the T-maze. Correct choices were rewarded with big space and favorable environment and incorrect choices result in getting small congested environment confinement (Lamb et al., 2012). In the present study, AEDs have significantly ameliorated seizure provoked by PTZ exposure but they have a negative impact on cognitive functions. Results from the T-maze test clearly demonstrated that AED-treated fish gets repeatedly lost in the T-maze, which shows epilepsy-related impaired spatial memory function in zebrafish. It is also observed that zebrafish have the capabilities to successfully learn, navigate and discriminate between wrong and right arm to reach the deeper chamber. Drugs like OXC, GBP and DZP have less negative impact than PHY and RSV on memory functions. The T-maze locomotor pattern seen in PTZ group was found to be significantly different as compared to control group. Most of the AEDs exhibit a similar locomotor pattern as of PTZ treated group in T-maze. The telencephalon connected to the olfactory organ is responsible for governing spatial memory, reproductive behavior, feeding behavior, and color vision in zebrafish. The spatial memory is accountable for recording information of individuals's environment. A fish with poor working spatial memory may result in, getting repeatedly lost in the maze (Yu et al., 2006). The alteration in the neurotransmitter by AEDs and by epilepsy contributes to cognitive dysfunction in epilepsy patients (Greengard, 2001). There is a greater evidence available which explains the negative impact of AEDs on different aspects of cognitive functions.

Neurotransmitters are chemical agents in the body that modulate, initiate, and amplify signals across the brain (Thippeswamy et al., 2011). Abnormal alteration of neurotransmitters levels has been found to be closely associated with many neurological diseases including epilepsy (Huguenard, 2003). One of the reasons for the epileptic seizure and cognition in clinical conditions is neurotransmitter modulations by both epilepsy and AEDs (Park and Kwon, 2008; Kleen et al., 2010). LC–MS/MS method was used to quantify the neurotransmitters simultaneously in zebrafish brain (Santos-Fandila et al., 2015). It is well studied in rodents that neurotransmitters plays a significant role in modulating memory and learning function (Levin and Cerutti, 2009). A similar modulation was found in zebrafish brain when tested after T-maze test for learning and memory. GABA is an inhibitory transmitter in the central nervous system and known for its role in epilepsy (Shaikh et al., 2013). In the current research, all the fish treated with PTZ shows decrease level of GABA that contribute to epileptogenesis. The level of GABA was found to be high in the control group and some of the AEDs like DZP and GBP which acts via a GABAergic pathway. Drugs such as PHY and OXC which do not act via this pathway showed reduced GABA levels. A study performed by Silva (2003) in rodent, proves the fundamental role of GABA in controlling epilepsy (Silva, 2003; Werner and Covenas, 2011). The Increase in glutamate (Glu), an excitatory amino acid, is closely related to the etiology of epilepsy. This glutamate release causes a cascade of changes resulting in increased intracellular calcium that ultimately results in cell death (Holmes, 2002). It was found that level of glutamic acid in zebrafish brain of the control group was low as compared to PTZ treated group. In most of the AEDs treated group the level of glutamate was found to be high, which might have contributed to the neuronal loss, and impaired the memory function. By modulation of glutamate signaling, many biological events are related to brain functioning are affected (Ozawa et al., 1998), such as learning and memory (Izquierdo and Medina, 1997). Acetylcholine (ACh), a signaling molecule elicit several actions at neuromuscular junctions and in the CNS and also plays a key role in modulating glutamate release and maintaining memory formation. (Atzori et al., 2003). It was found that level of ACh in PTZ and all the AEDs treated group was low as compared to control group and this is in correlation with high levels of glutamate. Memory function was not improved due to the low level of ACh release in most of the AEDs treated group. But ACh was found to be high in DZP group and which could be due to acetylcholinesterase (AChe) inhibition property of DZP (Lundgren et al., 1987). Although the level of ACh in DZP treated group was high, there was not much improvement in memory functions in zebrafish when tested in T-maze.

Brain-derived neurotrophic factor, a member of the "neurotrophin" family, enhances the survival and differentiation of several classes of neurons (Mercer et al., 1997). In our result, we found that level of BDNF mRNA expression (fold change) in PTZ treated group was decreased. Similarly, we found that there was no significant difference in BDNF mRNA expression (fold change) in PHY and DZP treated groups, which showed that AEDs contributes toward memory impairment although correcting epilepsy. Fish treated with OXC, GBP, and RSV showed a slight increase in mRNA expression of BDNF gene. A study conducted by Binder (2004), have shown that

upregulation of BDNF is involved in epileptogenesis, and modulation of BDNF signal transduction helps in preventing an epileptic condition (Binder, 2004). The transcription factor, CREB is crucial for important functions of cognition like memory and synaptic plasticity. Earlier studies reported an increase in CREB phosphorylation in rodent models of epilepsy (Zhu et al., 2012). In all the fish treated with AEDs, the level of CREB mRNA expression (fold change) was higher than PTZ treated group. This states that AEDs along with epilepsy contributes to loss of memory function in zebrafish after 24 h T-maze trial. The level of CREB mRNA expression was high in PTZ treated fish which shows that CREB too plays the important role in the process of epileptogenesis. Theories around cAMP-response element binding protein (CREB) and memory are still developing. Formation of long lasting memory is completely dependent on activation of (CREB)-dependent gene expression which is a crucial phase in the molecular pathway. Lower the expression of CREB gene higher will be the memory function (Benito and Barco, 2010). NPY play a significant role in regulating various physiological events in the brain, including energy balance, learning and memory, and epilepsy. NPY gene is generally responsible for regulation of neurotransmitters in the brain. It is reported that NPY gene therapy decreases chronic spontaneous seizures in a rat model of temporal lobe epilepsy (Noe et al., 2008). It selectively reduced synaptic excitation mediated by glutamate release (Hollopeter et al., 1998). In the zebrafish brain, neurons containing NPY mRNA are widely distributed in particular to regions like telencephalon, optic tectum, and rhombencephalon (Soderberg et al., 2000). In our result we found that level of NPY mRNA expression (fold change) in PTZ treated group was decreased. The mRNA expression of NPY gene in control group was found to be high. Similarly, fish treated with DZP and GBP, showed an increase in the mRNA expression of NPY gene. We found that there was no significant difference in NPY mRNA expression (fold change) in PHY, OXC, and RSV-treated group as compared with PTZ group. A study performed by Stroud et al., demonstrated that NPY suppresses absence seizures in Genetic Absence Epilepsy Rats of Strasbourg (GAERS) (Stroud et al., 2005). NPY also play a significant role in human epilepsy and it is supported by an increased NPY expression in biopsy samples from temporal lobe epilepsy patients (Furtinger et al., 2001).

This study suggest that the negative impact of standard anti-epileptic drugs on learning and memory, which is very common among epileptic patients. The successful development of zebrafish model was confirmed by the effect of epilepsy and AEDs impairing the learning and memory abilities in zebrafish. It was found that AEDs suppresses seizure-like behavior but cannot reverse seizure associated learning and memory dysfunction in a zebrafish model. This model, therefore, potentially extend the significant use of zebrafish and technique for screening or developing newer drugs for epilepsy-related cognition dysfunction in humans.

CONCLUSION

For investigating the cause and pathology of human disease animal models are considered as a useful tool. It is better known that such models can never represent the complete pathology that is observed in human diseases. To develop a model in the animal for brain disorder particularly epilepsy is very difficult because of its disease complexity and variation among the species. However, by using a number of different techniques and parameters in the zebrafish, we can incorporate the unique feature of specific disorder to study the molecular and behavior basis of this disease. The behavioral study, neurotransmitter analysis and gene expression studies in the aforementioned work above provide a first proof-of-principle model for screening basic drugs in epilepsy-related cognitive research using zebrafish as a choice of animal.

ETHICS STATEMENT

The experimental protocol was approved by the Monash Animal Research Platform (MARP) Animal Ethics Committee, Monash University, Australia (MARP/2016/009).

AUTHOR CONTRIBUTIONS

UK performed all the experimental procedure along with result analysis, manuscript writing and figures designing. YK contributed in designing gene expression study, result analysis and figures in the manuscript. IO contributed in LC-MS/MS method. MS contributed in designing the study, result analysis and manuscript writing.

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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-3

INTRODUCTION:

With a huge number of plant species on earth, we are endowed with a colossal wealth of therapeutic remedies. Natural products and their subordinates represents to 50% of the considerable number of medications in current therapeutics. As a result of the low achievement rate and enormous capital speculation need, the innovative work of customary medications are expensive and troublesome (Pan et al., 2013). But still, almost half of the pharma industries and drug development companies derive pharmaceutical medications from natural plants. Thus, natural products have served a major source of drug molecules for centuries (Karim et al., 2018). One major cognitive problems associated with many CNS and neurodegenerative disorders, are parkinsonism, Alzheimer's, seizures, traumatic head injury, disease, impairment in the cerebrovascular system (Kumar and Veeranjaneyulu, 2018). However, in the Indian medicinal system of Ayurveda, which is 5000 years old, selected plants have been long classified as "medhya rasayanas", from the Sanskrit words "medhya", meaning intellect or cognition, and rasayana", meaning "rejuvenation" (Ray S, 2015). Medhya Rasayanas are gathering of medicinal plants depicted in Ayurveda (Indian system of medicine) with multi-overlap benefits, explicitly to enhance memory and judgement and intellect by Prabhava. Embelin is one of the plants from the Indian medicinal system which is mainly used to treat worm infections, but also has a strong positive neuroprotective effect on the nervous system and is used against epilepsy and paralysis (Souravi and Rajasekharan, 2014). The aim of the following study was to investigate the complete pharmacological, behavior and molecular activity of Embelin (EMB) on PTZ induced acute seizures and post-seizure cognitive effects in healthy and epileptic zebrafish.

Phytochemical and pharmacological investigations discovered that the presence of EMB is one of the vital elements in treating CNS disorders (Kundap et al., 2017). A herbal-based benzoquinone known as Embelin (2,5dihydroxy-3-undecyl-1,4 benzoquinone) is the main component in the red berry fruits produced by *Embelia ribes* (Dubéarnès et al., 2015). In view of the above, the first objective was to evaluate the effectiveness of EMB against PTZ induced seizures and associated cognitive dysfunction. We also tried to explore the possible role on EMB in healthy adult zebrafish, which would help to shed light on the memory enhancing property of EMB in the zebrafish brain. The second goal was to assess the molecular system of EMB through the investigation of synaptic release of neurotransmitters which assume to play a critical role in epilepsy and memory absconds. In addition being

supplemented by gene expression study and immunohisto chemistry analysis of neuronal migration and differentiation.

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Title: Embelin protects against pentylenetetrazole-induced seizures and associated memory decline in a zebrafish model.

ABSTRACT:

Purpose of the research: Epilepsy is a neurological disorder associated with uncontrol electrical firing in the brain, with a majority of the approximately 70 million cases having unknown etiologies. Cognitive impairment and behavioral disturbances are among the more alarming co-morbidities of epilepsy. Anti-epileptic drugs (AEDs) are reported to worsen cognitive status in 1/3rd of patients with epilepsy. Embelin (EMB) is a benzoquinone derived from the plant *Embelia Ribes* and is reported to have CNS activity. This study aims to evaluate the effectiveness of EMB against pentylenetetrazole (PTZ) induced acute seizures associated cognitive dysfunction. The zebrafish animal model was used study the effect of EMB against PTZ induced seizures. A T-maze trial was incorporated to test zebrafish memory function after epileptic seizures.

The principal results: The behavioral observations were in line with molecular docking studies and found that EMB has a higher affinity for the gamma-aminobutyric acid ($GABA_A$) receptor and also modulates the behavior, genes and neurotransmitters which improve epilepsy and cognitive function after seizures. Healthy zebrafish treated with EMB alone had no behavioral and biochemical interference or side effects. EMB was also found to promote neuronal protection and migration in zebrafish brains.

Major Conclusions: EMB could be a promising candidate against epilepsy induced learning and memory dysfunction in zebrafish. However, there is possibility that these results can be generalized to mammals, particularly humans, due to genetic and functional similarities. Further investigations utilizing different seizure models are warranted to strengthen the potential for clinical translation.

KEYWORDS: Embelin; Epilepsy; Cognitive Disorder; T-maze Behavior; Zebrafish

RUNNING TITLE: Embelin against epilepsy and related cognitive dysfunction.

BULLET POINT SUMMARY:

1) What is already known:

- Epilepsy related comorbidities like cognitive dysfunctions are common in many people with epilepsy.
- Most of the anti-epileptic drugs help in preventing seizures but have a negative impact on cognitive functions.

- A crude extract of the plant *Embelia ribes* is used to treat epilepsy in alternative medicine.

2) What this study adds:

- Embelin isolated from *Embelia ribes* was found to be effective against seizures and prevented memory decline in a zebrafish.

- Embelin modulates neurotransmitters, gene expression and exhibits a neuroprotective effect.

- Embelin could be a promising molecule against seizures and related cognitive dysfunction.

1. INTRODUCTION:

Epilepsy is the fourth most common neurological disorder (Newton and Garcia, 2012), affecting over 70 million people of all ages around the world (Copmans et al., 2017). The significant feature of epilepsy are the seizures, but it also affect cognition, leading to a poor quality of life (Van Rijckevorsel, 2006;Kim and Ko, 2016). The prevalence of memory problems in patients with epilepsy is 40-44% and they experience difficulties in problem-solving, learning and have psychomotor retardation (Mula, 2015).

In recent years, the primary focus has turned towards non-mammalian epilepsy models for behavior testing due to numerous factors. These factors including greater cost-effectiveness, high genetic correlation with humans and rapid breeding (Arief et al., 2018). The cost and time required to carry out gene expression and brain cell genesis studies in a zebrafish model are less as compared to rodents (Mussulini et al., 2013). The main limitation in executing research in epilepsy and cognitive abnormalities is the lack of precise and reproducible animal models for the testing of new drug molecules (Yam Nath Paudel et al., 2018). To overcome this limitation, our laboratory has developed a zebrafish model of epilepsy induced cognitive dysfunction and confirmed the hypothesis that both epilepsy and anti-epileptic drugs (AEDs) affect the cognitive functions in zebrafish (Kundap et al., 2017c). Although a study is conducted using zebrafish, the findings can still be translated to mammals, particularly humans as over 70% of zebrafish genes are substantially similar to their mammalian orthologues (MacRae and Peterson, 2015). Another reason is that 70% of zebrafish genes also have one or more human orthologues and 47% of human genes have a one to one relationship with a corresponding zebrafish gene (Howe et al., 2013). There is also evidence that several important zebrafish brain regions show homologous functions to their mammalian counterparts, despite the substantial neuroanatomical differences (Fontana et al., 2018).

Numerous natural products including derivatives of quinone, which are thought to have better efficacy and safety profiles, are known for their central nervous system (CNS) related activity (Durg et al., 2017). Embelin (EMB) exhibits favourable chemical and physical properties and its capacity for penetrating the blood-brain barrier (BBB) (Bhuvanendran et al., 2018) makes it a suitable candidate for the treatment of CNS related complications (Pathan et al., 2009). EMB is a highly water-insoluble compound with a LogP value of 4.71 (octanol-water) (Xu et al., 2005). Phytochemical and pharmacological investigations discovered that the presence of EMB is a vital element in treating CNS disorders (Kundap et al., 2017b).

The primary goal was to evaluate the effectiveness of the plant-based EMB against pentylenetetrazole (PTZ)-induced seizures and associated cognitive dysfunction using adult zebrafish as an animal model. In spite of the proven role of EMB in several neurological disorders (Kundap et al., 2017a), few attempts have been made to explore the effectiveness of EMB against PTZ-induced seizures and related cognitive impairment. Thus, in the current study, we attempted to elucidate the overall activity of EMB by using molecular docking, immunohistochemistry, pharmacological, biochemical and behavioral experimentation.

2. TECHNIQUES AND MATERIALS:

2.1. Experimental equipment and chemicals list

All reagents were analytical grade used unless specified otherwise. Water was purified and filtered with a specific LC-MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). Glutamate (Glu), gamma-amino butyric acid (GABA), acetylcholine (Ach), diazepam (DZP), pentylenetetrazole (PTZ), paraformaldehyde (PFA), phosphate buffered saline (PBS), Benzocaine (BZ), Bromodeoxyuridine (BrdU) were purchased from Sigma Aldrich (USA). The pure form of embelin (EMB) was purchased from YUCCA Enterprises, Mumbai, India. Ethanol 95% (EtOH) was purchased from Kolin Chemicals Co. Ltd, Korea, methanol (MeOH), chloroform (CHCl3), isopropanol (IPA), and formic acid (FA) were purchased from Friedemann Schmidt Chemicals, Parkwood 6147, Western Australia. The pure form of plant extract of EMB was purchased from YUCCA Enterprises, Mumbai, India. Fish tanks (10 liters capacity) were purchased from PETCO-Pet keeper, Malaysia. The Sony Handycam (AVCHD 5x) recorder, Sony Camcorder stand, Smart 3.0.05 tracking software (Pan Lab, Harvard apparatus), Hamilton syringe 700-702 series 25µl, BD disposable needle (30G), FSC 22 Frozen Section Media (Leica Biosystems, Nussloch, Germany), Leica CM1860 Cryostat (Leica Biosystems, Nussloch, Germany), Agilent Infinity 1290 UHPLC, coupled with Agilent 6410 Triple Quad LC/MS and the Applied Biosystems StepOnePlus™ Real-Time PCR Systems were also used in this work.

2.2. Zebrafish (*Danio rerio*) care and maintenance

Adult zebrafish (*Danio rerio*) of heterozygous wild-type-AB stock (standard short-fin phenotype) were obtained from the Institute of Molecular and Cell Biology (IMCB), 61 Bioplis Drive Proteos, Singapore 138673. All fish were kept in the Monash University Malaysia fish facility at 28° C, with a 10/14 h dark/light cycle (white incident light off at 10 pm, white incident light on at 8 am) under aquarium conditions. Care was taken to maintain the system water pH between pH 6.8 - 7.1 by using an electronic pH pen (Classic PH Pen Tester, Yi Hu Fish Farm Trading, Singapore 698950) and the intensity of light was maintained uniformly all over the housing area at 250 Lux. Fish were nourished with TetraMin® Tropical Flake twice a day and livestock of Artemia from Bio-Marine (Aquafauna, Inc. USA) once a day to ensure a constant source of the food supply with ad libitum feeding. Zebrafish tanks with dimensions of 36 cm x 26 cm x 22 cm were used to house the fish and the tanks were equipped with a water circulation system for constant aeration (Kundap et al., 2017c). Group housing was used were 10-12 fishes/tank, males and females were housed separately. The Monash Animal Research Platform (MARP), Australia, approved all the zebrafish experimental procedures (MARP-2015-084).

2.3.Drug Treatment and groups

The treatment group of animals was initially injected with the vehicle/test drug, and then injected with PTZ to check their seizure behavior. Later their cognitive status was examined using a T-maze. EMB and standard DZP were dissolved in 10% Dimethyl sulfoxide (DMSO). The animals

were divided into the following groups, each group were divided individually with n=10 or else as mentioned.

Set-1 (only EMB treatment)

Group I: Vehicle control (VHC - 10 % DMSO) + Distilled water; Group II: Diazepam (DZP) 1.25 mg/kg + Distilled water: Group III: EMB-0.156 mg/kg + Distilled water; Group IV: EMB-0.312 mg/kg + Distilled water; Group V: EMB-0.625 mg/kg + Distilled water.

Set-2 (Epilepsy behavior test)

Group I: Vehicle control (VHC - 10 % DMSO) + Distilled water; Group II: VHC + Pentylenetetrazole (PTZ) 170 mg/kg; Group III: Diazepam (DZP) 1.25 mg/kg + PTZ (170 mg/kg); Group IV: EMB-0.078 mg/kg + PTZ (170 mg/kg); Group V: EMB-0.156 mg/kg + PTZ (170 mg/kg); Group VI: EMB-0.312 mg/kg + PTZ (170 mg/kg); Group VII: EMB-0.625 mg/kg + PTZ (170 mg/kg); Group VIII: EMB-1.25 mg/kg) + PTZ (170 mg/kg); Group IX: EMB-2.5 mg/kg + PTZ (170 mg/kg); Group X: EMB-5 mg/kg + PTZ (170 mg/kg); Group XI: EMB-10 mg/kg + PTZ (170 mg/kg).

Set-3 (BrdU immunochemistry)

Group I: Vehicle control (10% DMSO); Group II: VHC + Pentylenetetrazole (PTZ-Negative control group); Group III: EMB 0.156 mg/kg + PTZ (170 mg/kg); Group IV: EMB 0.625 mg/kg + PTZ (170 mg/kg).

All the drugs were administered via intraperitoneal injection as per the procedure described previously by Kundap et al. (2017c).

2.3.1. Procedure for a zebrafish anesthesia and intraperitoneal injection

PTZ and the EMB were intraperitoneally infused into the zebrafish as indicated by the convention given by Kundap et al. (2017d), and is given beneath. At the point when different intraperitoneal infusions were required, the infusions were given at alternating lateral ends, instead of the midline between the pelvic fins.

Each zebrafish was caught individually utilizing a fish holding net, and after that moved into an anesthesia arrangement (30 mg/L Benzocaine). The fish were kept into the anesthesia water for 30 secs until they stop moving. The zebrafish was taken out once anesthetized and weighed afterward to calculate the dose and subsequently the infusion volume. A delicate sponge roughly 20 mm in stature was soaked with water and set inside a 60 mm Petri dish. A cut between 10– 15 mm inside and out was made in the sponge to control and hold the fish for the intraperitoneal infusion. The intraperitoneal infusion was given while utilizing a dismembering magnifying lens by embeddings the needle into the midline between the pelvic fins. An appropriate volume was then injected into the zebrafish, after considering the body weight of the zebrafish.

All intraperitoneal infusions were administered into the stomach pit at an area back to the pelvic support, utilizing a 10 µl Hamilton syringe (700 arrangement, Hamilton 80400) (Stewart et al., 2011). The experiment was performed in a separate behaviour room with the room temperature kept between 26°C - 30°C and humidity between 50% - 60%. All zebrafish were acclimatized in the said behaviour room for two hours prior to experiment for minimising any novel tank response. Other precautions taken include using a small injection volume of 10 µl per

gram of fish and using a 35-gauge needle. The zebrafish were restrained in a water saturated sponge under benzocaine anaesthesia to reduce the distress inflicted on the zebrafish (Júnior et al., 2012). This intraperitoneal injection technique was found to be effective in zebrafish (Kundap et al., 2017d) and did not cause any mortality throughout the experiment. After the intraperitoneal injection, the zebrafish was immediately transferred to an observation tank.

2.4. Zebrafish behavior study for epilepsy and cognition:

2.4.1. Dose determination study for embelin:

A wide range of EMB doses was tested, which were 0.078 mg/kg to 10 mg/kg. The zebrafish were divided into 11 groups containing 12 fish in each group. DZP was used as a positive control and the fish received a 1.25 mg/kg dose. The vehicle-treated group received 10% DMSO, followed by a distilled water injection. To induce epileptic seizures, all the animals were first treated individually with the vehicle/DZP/EMB and then injected with 170 mg/kg of PTZ. The resulting seizure behavior were observed for seizure scoring for a period of 10 min post-PTZ injection. the seizure score was used as a parameter to determine the EC_{50} value of the EMB (Vincent, 2010). A graph was plotted as the log of drug concentration against the percentage (%) effect of the drug, giving a familiar sigmoidal shaped graph, to select the effective dose of EMB (Mensor et al., 2001).

2.4.2. Epilepsy behavior, and seizure score recording

Adult zebrafish were tested in an observation tank, with the seizure intensity being measured using a special scoring system as per a previously developed protocol (Kundap et al., 2017c). Seizure score, seizure onset time (mins), total distance traveled (cm) as well as time spent (s) in upper & the lower half of the tank were noted. After seizure score analysis. the fish were tested for their cognitive ability using a T-maze test (Banote et al., 2013). Animals administered with a specific PTZ concentration demonstrate different seizure scores, seizure frequencies, seizure profiles, and latencies to reach the seizure scores. Seizures normally last for 10 mins after administration of 170mg/kg of PTZ and progressively decreases with time (Desmond et al., 2012).

2.4.3. T-maze test

The T-maze is an apparatus which consists of a "T" shaped box containing one straight long arm and two short arms on the left and right-hand side (Wenk, 2001). The right short arm has a bigger opening known as the "deeper chamber", which acts as a favorable environment for the fish and thus the fish tend to spend a maximal amount of time there. The detailed specifications regarding the maze was as per the previous study. Transfer latencies (TL) were recorded at 0, 3 and 24 hrs. post-PTZ administration. An inflexion ratio (IR) = (L0-L1)/L1), (IR) = (L0-L2)/L2) was calculated, where L0 is the initial latency(s) at 0hrs & L1 and L2 is the latency(s) at the 3 hrs. and 24 hrs. trial respectively. The behavior recordings during seizure activity and the T-maze test were analyzed to track the locomotor patterns. Tracking of the locomotor pattern was done by using the computer software SMART v3.0-Panlab Harvard Apparatus® (Kundap et al., 2017c).

2.5. Biochemical estimation

2.5.1. Brain harvesting

The zebrafish brains were harvested at the end of behavior study in order to determine the molecular changes in the brain. The animal groups were divided into two halves and each brain was then transferred into TRIzol® for gene expression studies and another half into methanol for LC-MS/MS studies. The whole process of brain harvesting was done under ice-cold conditions and the brains were immediately transferred into dry ice. All the brains were stored at -80°C until further use.

2.5.2. Neurotransmitter analysis.

Glutamate, GABA, and Ach are the significant neurotransmitters in investigating epilepsy and cognition. These neurotransmitters were analyzed using the Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) technique. All the standard neurotransmitters were prepared in methanol (0.1% formic acid) as a stock solution of 1 mg/ml and were kept at 4°C until use. Standards for calibration were prepared from the original stock solution. Serial dilutions from 100-2000 ppb were used for calibration. The brain was homogenized in 200µl of ice-cold methanol (0.1 % formic acid). The homogenate was vortex-mixed for 1 min and then centrifuged at 18, 000 × g for 10 min at 4^0C. Finally, for LC-MS/MS analysis, the supernatant was pipetted and placed into vials.

LC-MS/MS was run on an Agilent 1290 Infinity UHPLC, coupled with Agilent 6410 Triple, Quad LC/MS, ZORBAX Eclipse plus C18 RRHD 2.1 x 150mm, 1.8-micron (P/N 959759-902) auto-sampler system (Agilent Technologies, Santa Clara, CA, USA). The samples were separated on a SMol-RRHD-Eclipse-C18-8 (15) UHPLC-160129-00011-Pos-DMRM used at 30°C. The mobile phase consisting of 0.1% formic acid in water (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B) was used with a gradient elution: 0–3 min, 50% B; 3-6 min, 95% B; 06–07 min, 95% B at a flow rate of 0.1 ml/min. ESI-MS/MS Conditions were set as follows: ESI ion source, positive ion polarity, gas temperature 325°C, drying gas flow 9.0 L/min, nebulizer pressure 45psi, Vcap 4000V. MS acquisition of GABA, Glu, ACh was performed in electrospray positive ionization multiple reaction monitoring (MRM) mode.

2.5.3. Gene expression:

Gene expression studies were carried out for the Neuropeptide Y (NPY), Brain-derived neurotrophic factor (BDNF) and cAMP-responsive element-binding protein 1 (CREB_1) genes. All the brain samples were placed in ice-cold 200 µl TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and immediately stored at -80°C until further usage. The study was divided into three steps, namely isolation of mRNA, synthesis of cDNA strand and then real-time PCR to estimate the level of the gene expressed.

Isolation of RNA and first strand cDNA synthesis

The mRNA was isolated by following the protocol provided by the kit's manufacturer. In brief, brain tissue was properly homogenized in TRIzol® reagent, mixed with chloroform and centrifuged at 13,500 rpm (revolutions per minute) for 15 minutes at 4 °C. The upper aqueous supernatant was transferred into new tubes and isopropanol was added, mixed and was incubated for 10 minutes at room temperature and later centrifuged for 10 minutes at 13,500 rpm at 4°C. The supernatant was discarded, and the pellets were subjected to rinsing with 75% ethanol. Then, the pellets were left for air drying for between 5 to 8 minutes. Finally, nuclease-free water was added to each tube to dissolve the mRNA pellet. The concentration and purity of the isolated mRNA were measured by using a NanoDrop Spectrophotometer. The mRNA samples were converted into cDNA using the QuantiTect Reverse-Transcription Kit (QIAGEN) according to the manufacturer's protocol.

StepOne® Real-time PCR

The gene expression of NPY, BDNF and CREB_1 were measured by real-time quantitative RT-PCR (StepOne Applied Biosystems) using QuantiTect SYRB Green dye (Qiagen, Valencia, CA). All the primer sets were provided by Qiagen (npy: Dr_npy_1_SG QuantiTect Primer Assay (QT02205763), bdnf: Dr_bdnf_1_SG QuantiTect Primer Assay (QT02125326), CREB_1: Dr_CREB_1 bpa_1_SG QuantiTect Primer Assay (QT02197503). The PCR mixture contained 1X SYBR Green PCR Master Mix (Qiagen), 0.7 µM of both forward and reverse primers and 1 µl of sample cDNA. The samples were incubated at 95 °C for 2 min prior to thermal cycling (40 cycles of 95°C for 5 Sec and 60°C for 15 Sec). Relative expression values of the above genes were obtained by normalizing the threshold cycle (Ct) values of genes of interest against Ct value of eef1a1b (housekeeping gene) (2 ^ [Ct eef1a1b – Ct Gene of interest]).

2.6.Molecular modeling studies:

All molecular docking studies were performed in Biovia Discovery Studio 4.5 (www.accelrys.com). Since the 3D structure of the gamma-aminobutyric acid receptor ($GABA_A$ subunit β3) of zebrafish is not available, we performed molecular docking studies of EMB on the human GABA subunit β3 to predict and correlate *in-vivo* results. The x-ray crystal structure of the human $GABA_A$ subunit β3 complexed with the agonist, benzamidine was retrieved from the Protein Databank (PDB code: 4COF) (Miller and Aricescu, 2014). The water molecules were deleted, and hydrogen atoms were added. Finally, the protein was refined with a CHARMm force field at a physiological pH. To validate the docking reliability, co-crystallized ligand (benzamidine) was first re-docked to the binding site of GABA. Consequently, DZP and EMB were docked into the same active site and 30 conformations of each compound were obtained through CDOCKER. The conformations with the lowest energy were selected as the most probable binding conformation for each ligand.

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 (Brooks et al., 1983). The CDOCKER algorithm adopts a strategy involving the generation of several initial ligand orientations in the active site

of the target protein, followed by molecular dynamics based simulated annealing and final refinement by energy (Mo et al., 2012). The CDOCKER was used for the docking of all compounds. The molecular docking study was carried out to understand the binding mode of EMB within the active site of the $GABA_A$ β3 subunit using the Discovery Studio suit 4.5 software.

2.7. Bromodeoxyuridine (BrdU) immunohistochemistry

2.7.1. Mode of administration

Adult fish were anesthetized in system water tank (1 ltr) containing 0.016% benzocaine (pH 7.0; Classic PH Pen Tester, Yi Hu Fish Farm Trading, Singapore 698950). All the fish from each group was individually injected with BrdU (100 mg/kg) intraperitoneally (Mao et al., 2009), with a time interval of 4 h two times. The vehicle/EMB/PTZ was injected intraperitoneally as per the dose described. The animal in the treatment group was injected with vehicle / EMB doses and habituated for 15 min in an observation tank. To induce epileptic seizures, fish from EMB and PTZ group were individually exposed to 170 mg/kg dose of PTZ. The post injection survival period ranged between 2 h to 15 days, before the brain was extracted for fixation. Later on, after 2 hours of PTZ treatment, animals from each group (n=6) were euthanatized and brain samples were harvested for Immunochemistry studies to check the neuronal loss at day-0. The remaining n=6 animals were housed separately in the individual tanks for next 15 days to check the survival and the proliferating ability of neurons.

2.7.2. Zebrafish Brain fixation

Immunohistochemistry was performed on the whole brain of adult zebrafish. The animals were deeply anesthetized by immersing them into benzocaine dissolved in system water. The brain was extracted completely by opening the skull and, was fixed overnight at 4°C in 4% PAF in saline phosphate buffer (pH 7.4). The specimen was then soaked into 10 % Sucrose solution at room temperature until brain sinks to the bottom of the tube (up to 5-6 hours). Later, 10 % sucrose solution was replaced with 20 % sucrose solution and the specimen was soaked overnight at 4°C. Later, 20 % sucrose solution was replaced with 30 % sucrose solution at 4 °C and left for up to a week, until used for cryostat sectioning (Ekström et al., 2001).

2.7.3. Preparation of cryostat brain section:

To prepare cryo-sections, the cryostat machine was set at -20 °C (Ekström et al., 2001). Specimens were molded into a cryomold block by using FSC 22 frozen section media inside the cryostat machine at -20 °C. The cryostat was set at a 20 microns section and each section thickness and each section was divided into two equally sized wells glass slide. The sections were allowed to adhere to the slide at room temperature for at least 1 hours and later stored at -20 °C until the immunochemical procedure.

2.7.4. Immunohistochemistry procedure in the brain of adult zebrafish

Day-1 Procedure: The protocol followed was described earlier by (Malberg et al., 2000). The sections were thoroughly washed three times in PBS with an interval of 5min. The sections were then immersed in a 50% formamide solution (Vivantis, Inc. USA) for 2 h at 65°C. After 2 h of incubation, the section slides were washed once with 2X-SSC (Sodium Citrate; 0804-4L, Ameresco) for 5 min. Then, sections were incubated in 2N HCl for 30 min at 37°C and washed in a solution of 0.1 M boric acid for 10 min. Blocking solution was added to block the section with 10% normal horse serum (Gibco, Thermo Fisher Scientific, USA) dissolved in PBS containing 0.1% Triton-X-100 (Amresco, Ohio) for 1.5 h. Subsequently, the sections were incubated with mouse anti-BrdU antibody (1:500; Roche Diagnostics, IN, USA) in 10% horse serum with 0.5% bovine serum albumin (BSA) (Sigma Life Science, USA) in PBS containing 0.1% Triton-X-100 for 18 hrs at 4°C.

Day-2 Procedure: Sections were washed three times with PBS at the interval of 5min and incubated for 2 h with the biotinylated horse anti-mouse secondary antibody (1:250; BA-2001, Vector Laboratories, Burlingame, U.S.A). After incubation, the sections were followed by three times wash with PBS and incubated with avidin-biotin complex (1:55; Vectastain ABC Kit, Vector Laboratories, CA, U.S.A.) for 2 h at the room temperature. After three washes with PBS, the sections were visualized with diaminobenzidine solution (DAB, D4293; Sigma), prepared in 0.1M phosphate buffer (pH 7.4) for 4-5 min. The DAB reaction was stopped by adding PBS when a minimum background color appeared. The sections were then re-rinsed first in PBS and then in distilled water, ordered, mounted onto poly-lysine-coated slides, and dried overnight. Finally, they were dehydrated through ascending grades of alcohol, and cleared in xylene before being covered in DPX (Sigma Life Science, USA) and a glass cover slip. The slides were observed for analysis later.

2.7.5. Cell quantifications/cell counting:

To quantify BrdU-positive cells, 10-µm-thick serial coronal plastic sections through the whole tectal region were prepared as described above (n=6). BrdU-positive cells were counted on a BX50 microscope (Olympus) with a UPlanFLN 20×/10×/4× (NA0.90) objective lens. Since the size of the teleost (zebrafish) brain is slightly different among samples of the same age, we divided all sections into 10 groups along the rostro-caudal axis and calculated the mean cell number of each group in each region in the brain. This mean cell number was compared with the corresponding group of samples.

2.8.Statistical analysis:

For statistical analyses, GraphPad Prism 5 software (GraphPad Software, Inc.) was used. Data are represented as mean and standard errors of the mean (SEM). The results acquired were analyzed by One-way ANOVA and subsequent Dunnett's multiple comparison tests to assess the differences in seizure, latency, inflexion ratio, neurotransmitter levels and gene expression levels between treatments and also to assess the difference in cell count at day 0 and the number of cells migrated to different regions of the brain at 15 days post-PTZ injection. For all analyses,

differences between a treatment group and the equivalent negative-control group were considered statistically significant if the p-value was below 0.05 ($p < 0.05$).

3. RESULTS:

3.1. Seizure analysis and onset latency:

All the animals in the PTZ only treatment group showed full blown seizures up to score 4 during the 10 mins recording. All the animals pretreated with EMB displayed a seizure score of not more than 1.5, in a dose-dependent manner. It was also observed that animals treated with EMB-2.5 mg/kg to EMB-10 mg/kg (+PTZ) display seizure scores that are similar to the PTZ treated group. DZP was used as a standard drug for the epilepsy study, it was found to suppress seizures in the DZP + PTZ treated group as shown in figure 2.

Latency to seizure score 4 is the time required by the fish to reach seizure score 4 in a given time frame of 10 mins. The time taken by all the animals to reach seizure score 4 from the negative control group (PTZ treated group) was 160 - 180 Sec after PTZ injection. In animals treated with DZP + PTZ, we found that the latency to reach seizure score 4 was more than 500 Sec. On the other hand, the onset latency was also delayed in animals treated with EMB in a dose-dependent manner. As seen in figure 2B, EMB-0.078 mg/kg to EMB-0.312 mg/kg (+PTZ) groups show a significant increase in seizure onset latency compared to the PTZ treated group. As the dose of the EMB increases, the activity of the drug was found to decrease and could not delay the seizure onset latency in the EMB-0.625 mg/kg to EMB-10 mg/kg (+PTZ).

3.2. Locomotor Pattern

A normal swimming pattern was seen in the vehicle control group, in which the tracking pattern shows swimming all over the tank. These control fish were found to spend an equal amount of time all over the tank as shown in figure 3A. An involuntary, rapid movement of the body that includes a corkscrew (spiral) swimming pattern and hyperactivity was seen in the negative control group (PTZ treated) fish. PTZ provoked seizures are a spontaneous behavior which produced the tremor and jittery locomotion tracking pattern seen mainly at the bottom of the tank. The locomotor tracking pattern for most of the EMB treated groups challenged with PTZ exhibited improved tracking patterns which were almost similar to the control group. It was found from the tracking pattern of the EMB-0.156 mg/kg to EMB-0.625 groups that fish from these groups swam all over the tank, in all directions and in both the halves of the tank, without any unwanted seizure and circular movements. However, the EMB-0.078 mg/kg group tracking pattern was similar to the PTZ treated group, as it was observed that the fish spent more time in the lower half of the tank, with many visible circular movements. DZP was used as a standard drug and zebrafish treated with it showed a swimming pattern from the left to right side of the tank as seen in figure 3A.

The total distance traveled by the fish in the DZP, EMB-0.156 mg/kg and EMB-0.625 mg/kg groups was significantly higher as compared to the PTZ group. The total distance traveled in the EMB-0.312 mg/kg and the control group was higher as compared to the PTZ treated group, but it

was not statistically significant. But in the EMB-0.078 mg/kg treated group, the distance traveled was significantly less than the PTZ treated group, as shown in figure 3B. It was found that the control fish spent nearly equal amount of time in both the halves of the tank, on the other hand, fish from the PTZ group were found to spend significantly more time in the lower half of the tank. The EMB treatment (EMB-0.312 mg/kg to EMB-0.625 mg/kg) reversed the seizure behavior and all the fish were found to spend more time in the upper part of the tank as shown in figure 3. The total distance traveled in the upper half of the tank was also found to be significantly more in all the EMB treated groups as compared with PTZ treated group, as shown in figure 3E.

3.3. Zebrafish T-maze test

In the T-maze test, fish from the vehicle control group exhibited an improved memory function in the absence of seizures. The PTZ treated group exhibited a negative effect, i.e. worsening memory function, which is depicted by a decreased inflexion ratio at both 3 hrs. and 24 hrs. On the other hand, in contrast to the PTZ treated group, EMB exhibited a better improvement of memory and showed an increased inflexion ratio in a dose-dependent manner. An increase in inflexion ratio was seen in the EMB treated group, but the results were not significant after the 3hrs trial. EMB-0.312 mg/kg to EMB-0.625 mg/kg showed a significant increase in the inflexion ratio as compared to the PTZ treated group at the 24 hrs. trail but not at 3 hrs. The inflexion ratio was found to be low in the DZP and EMB-0.078 mg/kg treated groups and was not found to be significant when compared to the PTZ treated-group as shown in figure 4B & C.

As the inflexion ratio was low, the time required for the fish to reach the deeper chamber and the time spent in the wrong arm (left turn) was found to be higher in the PTZ group as compared to control. The control group showed little to no repeated entry into the wrong arm and thus time spent, and distance traveled to reach the deeper chamber was found to be less. Fish from the PTZ treated group frequently failed to navigate its way to the deepest chamber and had more wrong entries into the left arm (wrong arm) before entering the deepest chamber. As the inflexion ratio of fish treated with EMB (+PTZ) was high, the time taken, and distance travelled to reach the deeper chamber were less and found to be significant as compared to the PTZ treated group, which ultimately decreased the total distance traveled and time spent in the wrong arm, as shown in figure 4C & D.

The locomotor pattern of the healthy adult zebrafish in the vehicle control group was found to be normal with a single right turn toward the deepest chamber. The locomotor pattern of adult zebrafish treated with DZP-1.25 mg/kg showed repeated back turns into the long arm and thus spent more time in the T-maze before reaching the deepest chamber. The fish treated with EMB-0.156 mg/kg and EMB-0.312 mg/kg showed similar locomotor activity as that of the control group and was found to travel towards deeper chamber with fewer entries into the wrong arm. However, the fish treated with the EMB-0.625 mg/kg dose showed some weird behavior by spending more time in the T-maze before entering the deepest chamber as shown in figure 5A-E. On the other hand, the EMB-0.156 mg/kg and EMB-0.312 mg/kg treated groups showed a

positive effect on the 3hrs and 24 hrs. inflexion ratio when compared with the control group. EMB-0.625 mg/kg failed to show increased inflexion when compared to the control group. DZP-1.25 mg/kg showed a slight increase in the inflexion ratio at 3hrs but was found to be decrease at 24hrs when compared to the control group. The time spent in the wrong arm was found to be high in the DZP treated group. The EMB-0.156 mg/kg and EMB-0.312 mg/kg group showed an inflexion ratio similar to that of the vehicle-treated fish as shown in figure 5B&C. There was no significant difference found in total distance traveled to reach the deeper chamber in all the groups when compared with the vehicle control group, as shown in figure 5E.

3.4. Estimation of neurotransmitters by LC/MS-MS:

Neurotransmitter analysis by LC/MS-MS demonstrated elevated levels of GABA in the control group when compared to the PTZ treated groups. A significant increase in the level of GABA was found in the EMB-0.078 mg/kg – EMB-0.156 mg/kg treated groups when compared to the PTZ group. Low levels of GABA were found in the DZP, EMB-0.312 mg/kg and EMB-0.625 mg/kg treated groups as shown in figure 6A1. The level of glutamate was found to be high in the PTZ treated group in comparison to the vehicle-treated group. Fish treated with EMB were protected against PTZ induced seizures due to the decreased level of glutamate. It was found that all the fish treated with EMB-0.078 mg/kg to EMB-0.625 mg/kg had a significantly lowered glutamate levels as shown in figure 6A2. The level of glutamate was also found to be lower in the control group and the DZP treated group. It was found that brain Ach levels were significantly decreased by the PTZ group as compared with the control. DZP and the EMB-0.625 mg/kg treated groups showed an increased level of Ach as compared to the PTZ group. There was a slight increase in the level of Ach in EMB-0.312 mg/kg group, but this was not found to be significant, as shown in figure 6A3.

The level of GABA in healthy zebrafish treated with EMB-0.312 mg/kg and EMB-0.625 mg/kg was found to be significantly lower when compared with the vehicle control group. However, the level of GABA was not significantly lower in EMB-0.156 mg/kg and DZP-1.25 mg/kg treated group when compared to the vehicle control group, as shown in figure 6B1. The level of glutamate in the EMB-0.625 mg/kg only treated group was found to be significantly lower when compared with the vehicle control group. The level of glutamate was not significantly low in the EMB-0.156 mg/kg, EMB-0.312 mg/kg and DZP-1.25 mg/kg treated groups when compared to the vehicle control group, as shown in figure 6B2. Similarly, there was no significant difference found in the level of Ach in all the EMB treated groups and the DZP treated group when compared with the vehicle control group, as shown in figure 6B3.

3.5. Estimation of gene expression by RT-PCR:

Brain-derived neurotrophic factor (BDNF) mRNA expression was upregulated in the control treated group as compared to the PTZ treated group. A significant elevation in the expression level of BDNF mRNA was observed in the EMB-0.625 mg/kg group. However, the DZP, EMB-0.156 mg/kg, and EMB-0.312 mg/kg treated groups also demonstrated elevated levels of BDNF

mRNA expression but was not significantly upregulated as compared to PTZ treated group. There was no increase in BDNF mRNA expression in the EMB-0.078 mg/kg treated group as compared to the PTZ treated group as shown in figure 7A1. CREB_1 mRNA expression was down-regulated in the control group as compared to the PTZ treated group. In all the EMB treated groups, the level of CREB_1 mRNA expression was found to be significantly down-regulated as compared to the PTZ treated group in epileptic fish. CREB_1 mRNA expression in the DZP treated group was found to be statistically insignificant when compared to the PTZ group as shown in figure 7A1. NPY mRNA expression was up-regulated in the control group when compared with PTZ treated group. However, the up-regulation of NPY mRNA was improved by DZP, EMB-0.156 mg/kg and EMB-0.625 mg/kg pre-treatment as compared with the PTZ group. There was no significant difference observed with EMB-0.078 mg/kg and EMB-0.312 mg/kg pre-treatment when compared with the PTZ treated-group, as shown in figure 7A3. On the other hand, there was no significant BDNF mRNA fold change observed in the EMB treated group when compared to the vehicle control group. The level of BDNF was high in the DZP treated group but was not significant when compared with the control-treated group as shown in figure 7B1. The level of CREB_1 mRNA expression was found to be high in the DZP and EMB-0.625 mg/kg treated groups but was not significantly higher when compared to the control group. The level of CREB_1 mRNA expression in the EMB-0.156 mg/kg and EMB 0.625 mg/kg treated groups had no significant difference when compared with the vehicle control group as shown in figure 7B2. Similarly, the EMB and DZP treated fish did not show any significant change in mRNA expression fold change of the NPY gene when compared to the vehicle control group, as shown in figure 7B3.

3.6. Molecular docking predictions for embelin

The docked conformation of benzamidine is shown in Figure 8 (I). As described by (Miller and Aricescu, 2014), our result also showed that the phenyl ring of benzamidine forms π-stacking with Phe200 while the amidinium group interacts with Glu155, Ser 156 and Tyr 157 via hydrogen bonds. Furthermore, the electrostatic cation-π interaction was also observed with Tyr 205. The RMSD and CDOCKER interaction energy (CDIE) were found to be 0.85Å and -25.06 kcal/mol respectively. Since DZP is used as a standard drug for the treatment of epilepsy, we have used it as a positive control. Therefore, we have docked DZP into the same active site of the $GABA_A$ receptor. DZP also fits well in the active site, showing a CDIE of -20.28 kcal/mol. As illustrated in figure 8 (II), it formed hydrogen bonds with Tyr97, Tyr157, and Glu155 residues, in a similar manner to benzamidine and showed hydrophobic interaction with Ala201. When EMB was docked in the active site of the receptor, a highly favorable lower CDIE of -42.09 kcal/mol was obtained. As depicted in Figure 8 (III), it formed a hydrogen bond and an electrostatic salt bridge with Arg207. Similarly, to the agonist benzamidine, the phenyl ring of EMB stacked with Phe200 and Tyr157. Thus, molecular docking studies indicated a good relationship between IC_{50} values and CDIE, thus supporting the biological results seen later.

3.7. Zebrafish brain cell genesis study - BrdU labeling

3.7.1. BrdU labeled cells and Proliferation zone at day-1:

To identify proliferation zones in the brain of adult zebrafish, the distribution of BrdU-labeled cells, as obtained after intraperitoneal injection of labeling reagent and by employing a post-administration survival time of 1 hour on day-1, was analyzed. To categorize proliferation zones in the encephalon of adult zebrafish, the scattering of BrdU-labeled cells, as obtained after intraperitoneal injection of BrdU reagent and by employing a post administration survival time of 1 hour on day-1, was analyzed. At day – 0 it was found that the number of BrdU positive labeled cells were significantly higher in the control group as compared to the PTZ treated group. Embelin in a dose-dependent manner significantly protect neurons from PTZ seizures, so the significant increase in BrdU positive cells was found in the EMB treated group when compared to the PTZ treated group. It was also found that at day-0 newborn cells tagged with the BrdU label were found to be originating from the Tectum Opticum TeO), Torus Longitudinal (TL), Tectal ventricle (TeV) and cerebellum region of the zebrafish brain as shown in figure 9 A.

3.7.2. Distribution of positive BrdU-labelled cells after 15 days of survival:

To find out a possible survival and migration pattern of the new cells after EMB and PTZ administration, the distribution of BrdU-positive cells we analyzed in five brain regions olfactory bulb, cerebellum (rhombencephalon), tectum opticum (mesencephalon), telencephalic area (telencephalon) and hypothalamus (diencephalon). As seen, most of the cells from molecular layers migrated towards the granular layer and were found to be high in control and EMB treated brain when compared to PTZ treated fish brain. The migration of cells from the molecular layers (mesencephalon) towards the granular layer in each area of the encephalon. The number of positive BrdU cells found in the olfactory bulb, cerebellum, tectum opticum, telencephalic area, and hypothalamus were significantly higher in the control and EMB treated group as compared to PTZ treated the group as shown in figure 10.

4. DISCUSSION:

A common method for inducing animal epilepsy are chemoconvulsants, which are chemical agents that can produce seizures (Choo et al., 2018). In a dose deciding study, we found that three doses of EMB (0.625 mg/kg, 0.312 mg/kg, and 0.156 mg/kg) had significant seizure protection activity. Further increasing the EMB dose reduced its seizure protection activity. This suggests that EMB has a small therapeutic window, which corroborates findings in a rodent study (Bhuvanendran et al., 2018).

PTZ treated fish showed anxiety behavior as they mainly resided at the bottom half of their tank, in contrast to control fish which spent equal amounts of time in both halves. EMB treatment of up to 0.625 mg/kg reduced PTZ-induced seizures and treated fish exhibited normal swimming movements consisting of repeated short swims.

The T-maze is a standardized learning and memory paradigm (Hamilton et al., 2016) whereby zebrafish attempt to reach a deeper chamber using their spatial memory for a spacious and favorable environment (Lamb et al., 2012). The current study suggests that epileptic zebrafish treated with EMB had the ability to successfully learn, navigate and discriminate between the wrong and right arms to reach the deeper chamber. A similar effect of EMB was also observed in healthy adult zebrafish. The 3 hrs. and 24 hrs. inflexion ratios showed a significant increase in memory function in the EMB-0.312 mg/kg and EMB-0.625 mg/kg groups as compared to the PTZ treated group. In the zebrafish brain, the olfactory bulb in the telencephalon is responsible for governing egocentric navigating myopia that helps the fish reach the deeper chamber (Lindsey et al., 2014). During an epileptic episode, the connection between the olfactory bulb and the telencephalon is likely impaired and this may affect the learning and memory ability of the fish. But when the fish is pre-treated with EMB, it prevents the fish from becoming epileptic and also increasing its learning ability, which leads to the increased 24 hrs. inflexion ratio.

Abnormal neurotransmitter levels are related to many neural diseases including epilepsy (Levin and Cerutti, 2009). GABA is the primary neuroinhibitor in the CNS (Kaila et al., 2014) and plays a well-studied and important role in epilepsy as well as learning and memory (Luo et al., 2011). In the current study, we reported increased GABA levels in all EMB groups, proving the role of GABA in learning and memory. EMB may have some ability to suppress Ca^{2+} influx and increase chloride conductance as epileptogenesis is aided by increased Ca^{2+} influx (Zamponi et al., 2010), reduced chloride conductance and decreased GABAergic presynaptic inhibition (Werner and Covenas, 2011). Extremely augmented excitatory glutamate release is one of the leading causes of epileptic seizures (Rowley et al., 2012). Glutamate toxicity is associated with neuronal death in neurodegenerative disorder cases (Guerriero et al., 2015), and glutamate also affects memory and learning (Izquierdo and Medina, 1997). In the present study, a lower level of glutamate was found in all groups as compared to the PTZ treated group. PTZ acts at the $GABA_A$ receptor and reduces chloride conductance (Zhang et al., 2017), leading to glutamate excitation (Banote et al., 2013). Fish getting repeatedly lost in the wrong arm of the T-maze in the PTZ treated group shows a relation between memory loss and glutamate toxicity, which was

reversed by EMB treatment. Modulating memory formation is a key role of Ach, which also alters neuron excitability, prompts synaptic plasticity and controls the firing of groups of neurons (Picciotto et al., 2012). An increase in Ach levels was also observed in the EMB treated groups but was not significant when compared to the PTZ treated group. DZP is reported to be an acetylcholinesterase (AChE) inhibitor and thus the level of Ach was high in the DZP treated group as the level and duration of Ach in the brain becomes elevated (Lundgren et al., 1987).

BDNF has a remarkable role in synaptic plasticity, survival and differentiation of neurons in the brain (Roopra et al., 2012) as well as increasing the persistence of short and long-term memory storage (Bekinschtein et al., 2008). It has been discovered that BDNF is linked to the inception of epileptogenesis, and the epileptic condition can be avoided by BDNF upregulation (Binder, 2004). In the present study, we found that BDNF mRNA expression was down-regulated in the PTZ treated group when compared with the control, but increased in the EMB treated groups, indicating memory improvement, neuronal survival and differentiation of new growing neurons (Choi et al., 2018). cAMP response elements (CREs) are promoters that are phosphorylated to generate epilepsy (Zhu et al., 2012). Studies in rodents show that a decrease in CREB levels has a 50% chance to decrease seizure episodes (Zhu et al., 2012) and that CREB_1 modulates synaptic plasticity for the intrinsic excitability of the neurons (Benito and Barco, 2010). CREB_1 mRNA expression was high in PTZ treated fish, confirming its role in epileptogenesis. In all the EMB treated groups, CREB_1 mRNA expression was found to be down-regulated, which indicates that EMB helps in reducing epilepsy and enhances memory. Neuropeptide Y (NPY) has an important role in regulating physiological processes such as various brain events, memory, and learning (Kas et al., 2005). Earlier rodent studies reported that chronic spontaneous seizures are reduced by NPY in a temporal lobe epilepsy model of rats (Noe et al., 2010) and that high levels of brain NPY is crucial for memory and learning (Gøtzsche and Woldbye, 2016). The level of NPY mRNA expression in the PTZ treated group was significantly downregulated as compared to the control group. However, EMB treated fish showed increases in NPY expression, indicating that it plays a crucial role in modulating neurotransmitters, increasing neuronal growth and preventing epilepsy in zebrafish brains (Vezzani and Sperk, 2004).

Docking studies are mainly performed to determine the affinity of a particular compound for a receptor or a similar molecule (Meng et al., 2011). We found that EMB has a high affinity for the benzodiazepine active binding site of the $GABA_A$ receptor at the β3 subunit. During epileptic seizures, the extra release of glutamate and increased breakdown of GABA causes neuronal firing and seizure episodes (Engel, 2013). By decreasing GABA release, there is much less binding of GABA to the $GABA_A$ receptor, decreasing the influx of Cl^- ions and hence causing excitability of the neuron cell. EMB's high affinity towards the GABA receptor, may help in GABA release and reducing cellular excitability by prolonged inactivation of voltage-gated neuronal Na^+ channels, preventing intracellular Na^+ accumulation and inhibiting high-frequency discharge. A reduction in Ca^{2+} influx and inhibition of glutamate may also take place after EMB treatment (Eraković et al., 2000). As EMB binds to the benzodiazepine G-protein

coupled receptor at the α or γ subunit, it facilitates GABA mediated Cl^- channel opening with cellular hyperpolarization and a reduction in seizure frequency (Benarroch, 2007). This could serve as a clue towards unraveling the precise mechanism of EMB against epilepsy and related cognitive dysfunction.

Generation of neurons from neuronal stem cells is known as the process of neurogenesis (Fuchs and Gould, 2000). During neurodegeneration, the sub granular zone (SGZ) which is the part of the hippocampus and the dentate gyrus and the subventricular zone (SVZ) are active continuously (Ming and Song, 2011). Proliferation and neurogenesis are not clearly understood in adult zebrafish. There is only one study that describes the proliferative zones and the migration of cells in the telencephalon, preoptic region, thalamus, hypothalamus, midbrain and cerebellum (Kaslin et al., 2008). A similar result was found in the present study, where newborn cells at day-0 were found to be tagged in abundant with BrdU at TeO, TL, TeV and the cerebellum region of the brain. The number of BrdU tagged cells was found to be high in control and EMB treated group as compared to the PTZ treated group. Epileptic seizures induced by PTZ caused increases in oxidative stress and inflammation which leads to neuronal death in the brain (Naseer et al., 2014).

Labeling of dividing cells with the BrdU thymidine analog provides insight into EMB's effect on cell proliferation and migration after a 15 days survival period (Grandel et al., 2006). Mitotic activity was specifically pronounced in the olfactory bulb, hypothalamus, telencephalic area (dorsal telencephalon), preoptic area of the diencephalon, optic tectum of the mesencephalon, torus longitudinal (TL) and in all three cerebellum subdivisions (Zupanc et al., 2005). In the zebrafish optic tectum, there was a lack of evidence for a long-distance migration of the new cells from their proliferation zones. Among all brain regions, the different subdivisions of the cerebellum demonstrated a maximal number of mitotic cells in control and EMB treated zebrafish. A similar result has been reported in other teleosts including guppies and the brown ghost (Rubenstein and Rakic, 2013). As the administration of PTZ damages newly born cells at the phase s4 of mitosis, the number of cells which migrated to different parts of the brain was significantly lower. Evidence of PTZ causing neurodegeneration was also previously observed (Park et al., 2006).

5. CONCLUSION:

EMB is reported to have various activities such as being an anti-oxidant, anti-inflammatory and can cross the BBB. Findings from the current study demonstrate that EMB can also suppress seizure-like behavior and improve cognitive function in zebrafish. The docking study showed that EMB has a higher affinity towards the $GABA_A$ receptor. In addition, the current study also explored the basic mechanism of embelin, its possible site of receptor binding and its ability to promote cell migration and differentiation. The study conducted on healthy zebrafish with only EMB treatment showed similar memory behavior and no alteration in gene expression level affecting the memory status of the fish. This implicates that EMB does not interfere with the normal functioning of body processes and has no adverse effect. T-maze data, behavioral study,

immunohistochemistry staining, and biochemical analysis supported the observed effect of EMB. Herein, we suggest that EMB could be a promising candidate against epilepsy induced learning and memory dysfunction. Further investigations utilizing different seizure models are warranted that will strengthen the potential of EMB towards clinical translation. Current findings shed light on the utilization of plant-based natural compounds against epilepsy and related cognitive impairment. These will overcome the limitations of mainstream AEDs and will be an economical option as well.

6. ETHICS STATEMENT:

The experimental protocol was approved by the Monash Animal Research Platform (MARP) Animal Ethics Committee, Monash University, Australia (MARP-2015-084).

7. AUTHOR CONTRIBUTIONS:

UPK performed most of the experimental procedure along with analysis of the results, writing of the manuscript. BC helped in performing behavior recording, data analysis, writing of the manuscript and proofreading. NA contributed to docking study of EMB. YK contributed to designing the gene expression study, result analysis. IO contributed to LC-MS/MS study. MFS contributed in designing of the study, result analysis, manuscript writing, and proofreading.

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10. COMPETING INTERESTS:

All the authors display no conflict of interest.

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FIGURE LEGENDS:

Figure 1: Experimental setup and design procedure. The flowchart represents the scheme for drug treatment, PTZ administration and behavior recording for epilepsy and T-maze used in the study.

Figure 2: Seizure score and Onset latency for epileptic seizures: (A) Represent the effect of Embelin on PTZ induced seizures in adult zebrafish. (B) Represents onset of seizure latency score 4 for EMB and DZP treated fish when compared with PTZ treated group. Data are represented as Mean ± SEM, n=10 and Statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.

Figure 3: Locomotor pattern and behavior analysis of embelin treatment against Pentylenetetrazole (PTZ) induced seizures: (A) Represent the tracking pattern of locomotor behavior for the Control, PTZ treated and EMB treated groups. (B) Represents the total distance traveled by fish in each group during locomotor behavior tracking. (C, D) represents the time spent in total by each fish in the lower and upper half of the behavior tank. (E) Represents the total distance traveled in the upper half of the tank. The data are represented as Mean ± SEM, n=10 and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.

Figure 4: T-maze tracking pattern and behavior analysis of embelin treatment against Pentylenetetrazole (PTZ) induced seizures and cognitive dysfunction. (A) Represents the T-maze tracking pattern of locomotor behavior for the Control, PTZ treated and EMB treated groups. (B, C) Represents the graph plot of the inflection ratio at the 3h and 24 h T-maze trial. (D, E) represents the time spent in the wrong arm and the total distance traveled by each fish to reach the deepest chamber of T-maze behavior tank. Data are represented as Mean ± SEM, n=8 and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.

Figure 5: T-maze tracking pattern and behavior analysis of embelin treatment in adult healthy zebrafish: (A) Represents the T-maze tracking pattern of locomotor behavior for the Control, DZP treated and embelin (EMB) treated groups. (B, C) Represents the graph plot of the inflection ratio at 3hrs and 24 hrs. T-maze trail in adult healthy zebrafish. (D, E) represents the time spent in the wrong arm and total distance traveled by each fish to reach the deeper chamber of the T-maze in adult healthy zebrafish. Data are represented as Mean ± SEM, n=8 and

statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.

Figure 6: (A) Neurotransmitters analysis in epileptic zebrafish brains after embelin treatment and 24 hrs. T-maze behavior: (A1) Represents concentration of GABA in the zebrafish brain. **(A2)** Represents concentration of glutamate in the zebrafish brain. **(A3)** Represents concentration of acetylcholine (Ach) in the zebrafish brain. Data are represented as Mean ± SEM, n=6 and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.
(B) Neurotransmitters analysis in healthy zebrafish brains after embelin treatment and 24 hrs T-maze behavior: (B1) Represents concentration of GABA in the zebrafish brain. **(B2)** Represents concentration of glutamate in the zebrafish brain. **(B3)** Represents concentration of acetylcholine (Ach) in the zebrafish brain. Data are represented as Mean ± SEM, n=6 and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.

Figure 7: (A) Gene expression analysis in epileptic zebrafish brains after embelin treatment and 24 h T-maze behavior: (A1) Represents graph plot for BDNF mRNA expression in the zebrafish brain. **(A2)** Represents graph plot of CREB_1 mRNA expression level in the zebrafish brain. **(A3)** Represents graph plot of NPY mRNA expression in the zebrafish brain. Data are represented as Mean ± SEM, n=6 and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.
(B) Gene expression analysis in adult healthy zebrafish brains after embelin treatment and 24 h T-maze behavior: (B1) Represents graph plot for BDNF mRNA expression in the zebrafish brain. **(B2)** Represents graph plot of CREB_1 mRNA expression level in the zebrafish brain. **(B3)** Represents graph plot of NPY mRNA expression in the zebrafish brain. Data are represented as Mean ± SEM, n=6 and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤0.05, **P ≤ 0.01, and ***P ≤ 0.001.

Figure 8: (A) Docking study of embelin with respect to diazepam and the $GABA_A$ receptor**. (B)** Proposed mechanism of action of Embelin at the $GABA_A$ receptor to reverse PTZ induced seizures**.** 8B1 - During a seizure, a prolonged opening of the voltage-gated Na^+ channel causes an influx of sodium ions which leads to depolarization and repetitive neuronal firing. The opening of $Ca2^+$ channels increases the influx of positive calcium ions causing, prolonged spikes and T current waves. As there is no affinity for the GABAA receptor, the decreased Cl^- influx causes epileptic excitation inside the neuron cell. 8B2 - EMB shows higher affinity for $GABA_A$ receptor binding, it facilitates GABA mediated Cl^- channel opening with cell hyperpolarization and reduced seizure frequency, prolonged inactivation of the voltage-gated neuronal Na+ channel, prevented intracellular Na+ accumulation, reduced $Ca2^+$ influx and inhibited glutamate.

Figure 9: (A) BrdU immunohistochemistry analysis of the protective effects of embelin at the proliferation (day-0) and survival (day-15) stage against pentylenetetrazole-induced epilepsy the zebrafish brain. **(A I) BrdU-positive staining** revealed labeling of mitotic cells of immature neurons in the subgranular zone of the periventricular gray zone of tectum opticum with BrdU. Photomicrographs of the sagittal section of treatment groups were (A) Control (B) PTZ 170 mg/kg (C) EMB 0.156 mg/kg + PTZ (D) EMB 0.625 mg/kg + PTZ. Representative photomicrographs were taken at magnifications of 40X and 200X. **(II) Quantification of BrdU positive cells.** Data are expressed as means Mean ± SEM, n = 6 and statistical analysis by one-way ANOVA followed by Dunnett test $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. **(A II)** BrdU immunohistochemistry analysis of the effects of embelin in improving neurogenesis and migration of cells generated during 15 days from the molecular layer to the granular layer within the dorsal zone of the periventricular hypothalamus against pentylenetetrazole-induced epilepsy the zebrafish brain. **(I) BrdU-positive staining** revealed labeling and migration of cells at day 15 of cell maturation and differentiation stage within the valvula cerebelli zone of cerebellum with BrdU labeling. Photomicrographs of the sagittal section of treatment groups were (A) Control (B) PTZ 170 mg/kg alone (C) EMB 0.156 mg/kg + PTZ (D) EMB 0.625 mg/kg + PTZ. Representative photomicrographs were taken at magnifications of 40X and 100X. **(II) Quantification of BrdU population:** Data are expressed as Mean ± SEM, n = 6 and statistical analysis by one-way ANOVA followed by Dunnett test $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. **(B) Graphical representation of cell migration in zebrafish brain** (I) Represent location of positive BrdU cells at day-0 (II) Represent migration and location of positive BrdU cells at day-15

Figure 10: BrdU immunohistochemistry analysis of the effects of embelin in improving neurogenesis and migration of cells generated during adulthood from the molecular layer to the granular layer within the molecular zone of the hypothalamus, the internal cellular layer of the olfactory bulb, of the periventricular gray zone of tectum opticum and the medial zone of the dorsal telencephalic area against pentylenetetrazole-induced epilepsy the zebrafish brain. **(I) BrdU-positive staining** revealed labeling and number of migration of cells at day 15 of maturation and differentiation stage at the molecular zone of each section of zebrafish brain with BrdU labeling. Photomicrographs of the sagittal section of treatment groups were (A) Control (B) PTZ 170 mg/kg alone (C) EMB 0.156 mg/kg + PTZ (D) EMB 0.625 mg/kg + PTZ. Representative photomicrographs were taken at magnifications of 40X and 100X. **(II) Quantification of BrdU population:** Data are expressed as Mean ± SEM, n = 6 and statistical analysis by one-way ANOVA followed by Dunnett test $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

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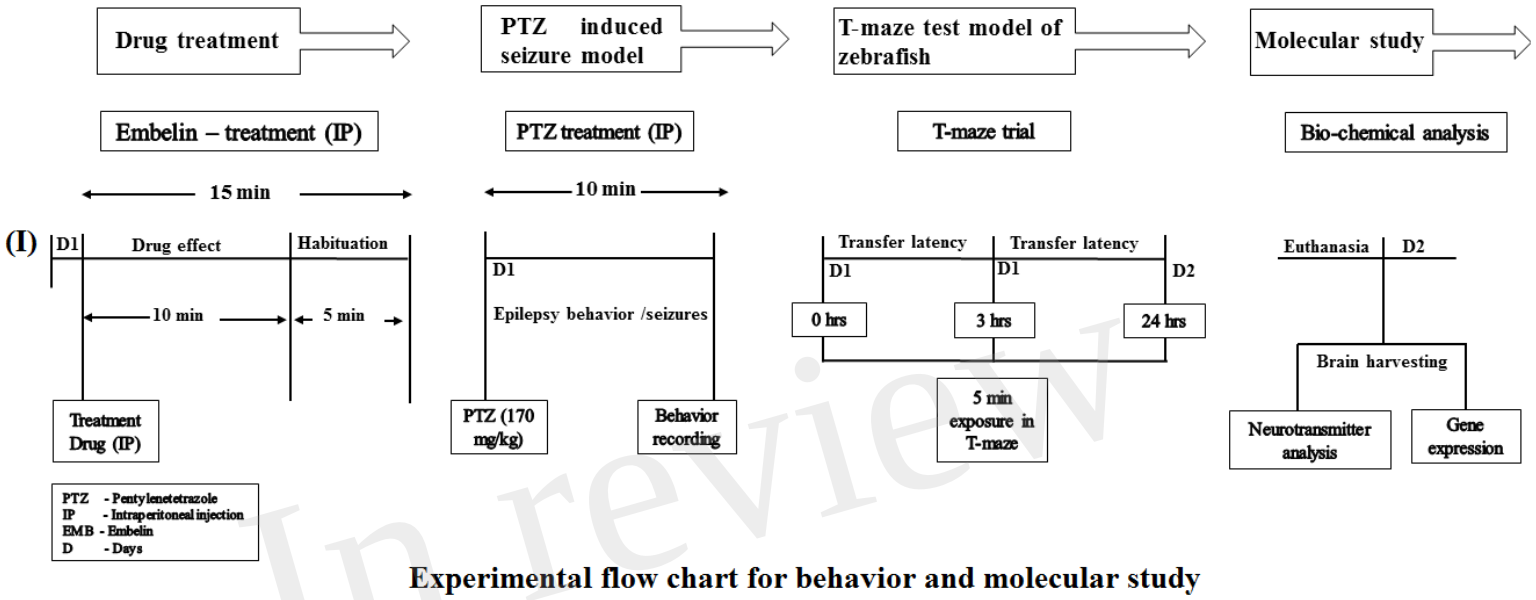
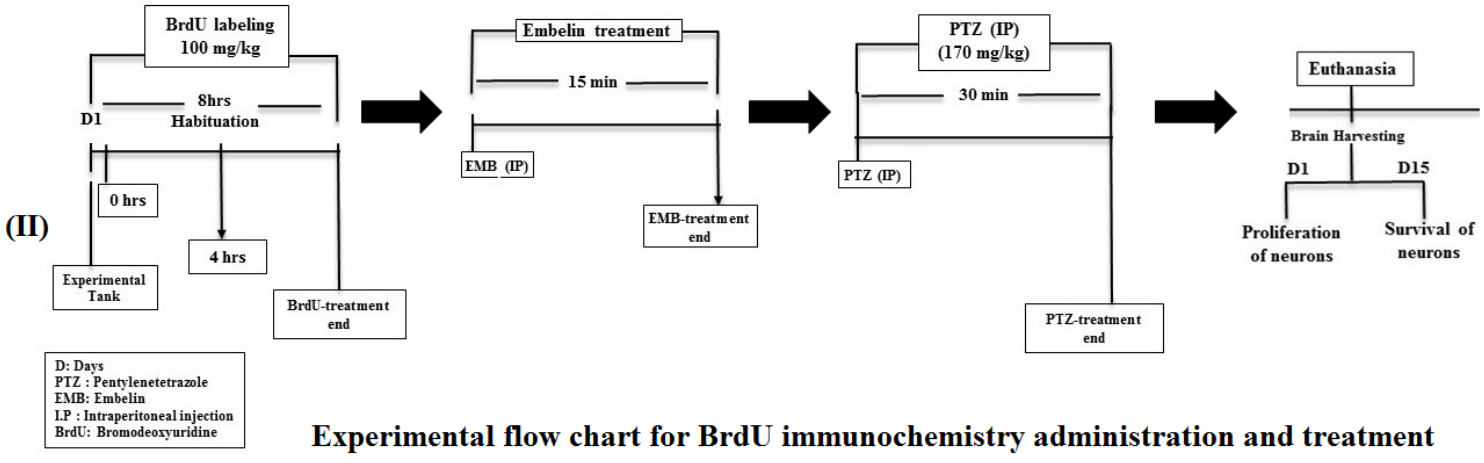


Experimental flow chart for behavior and molecular study



Experimental flow chart for BrdU immunochemistry administration and treatment

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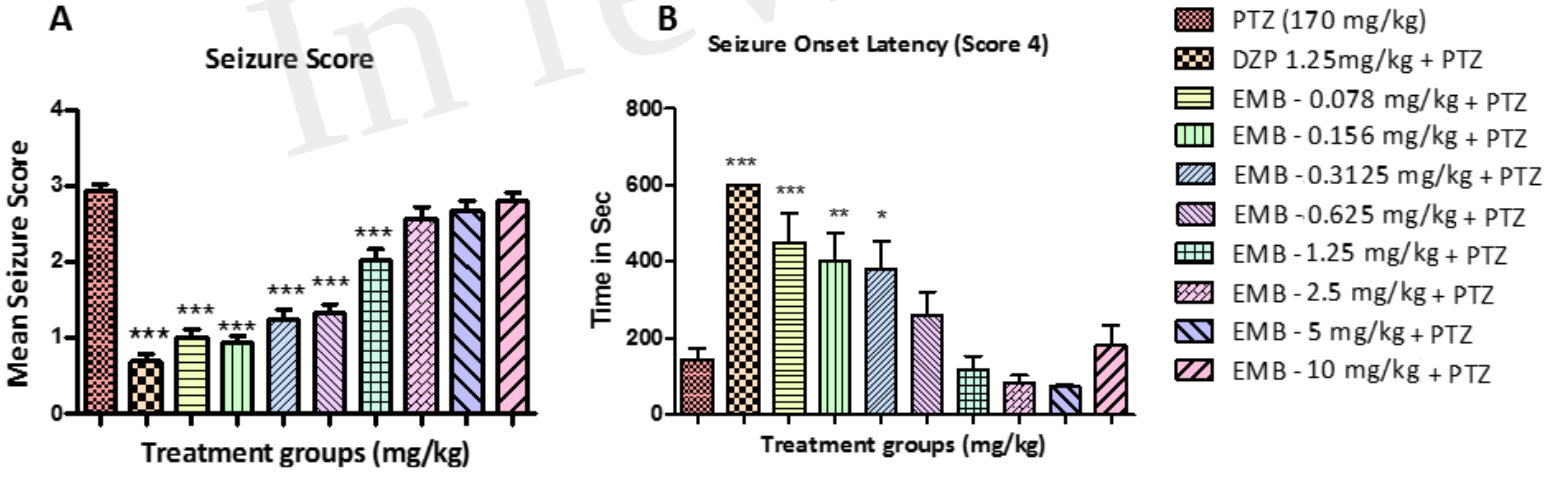

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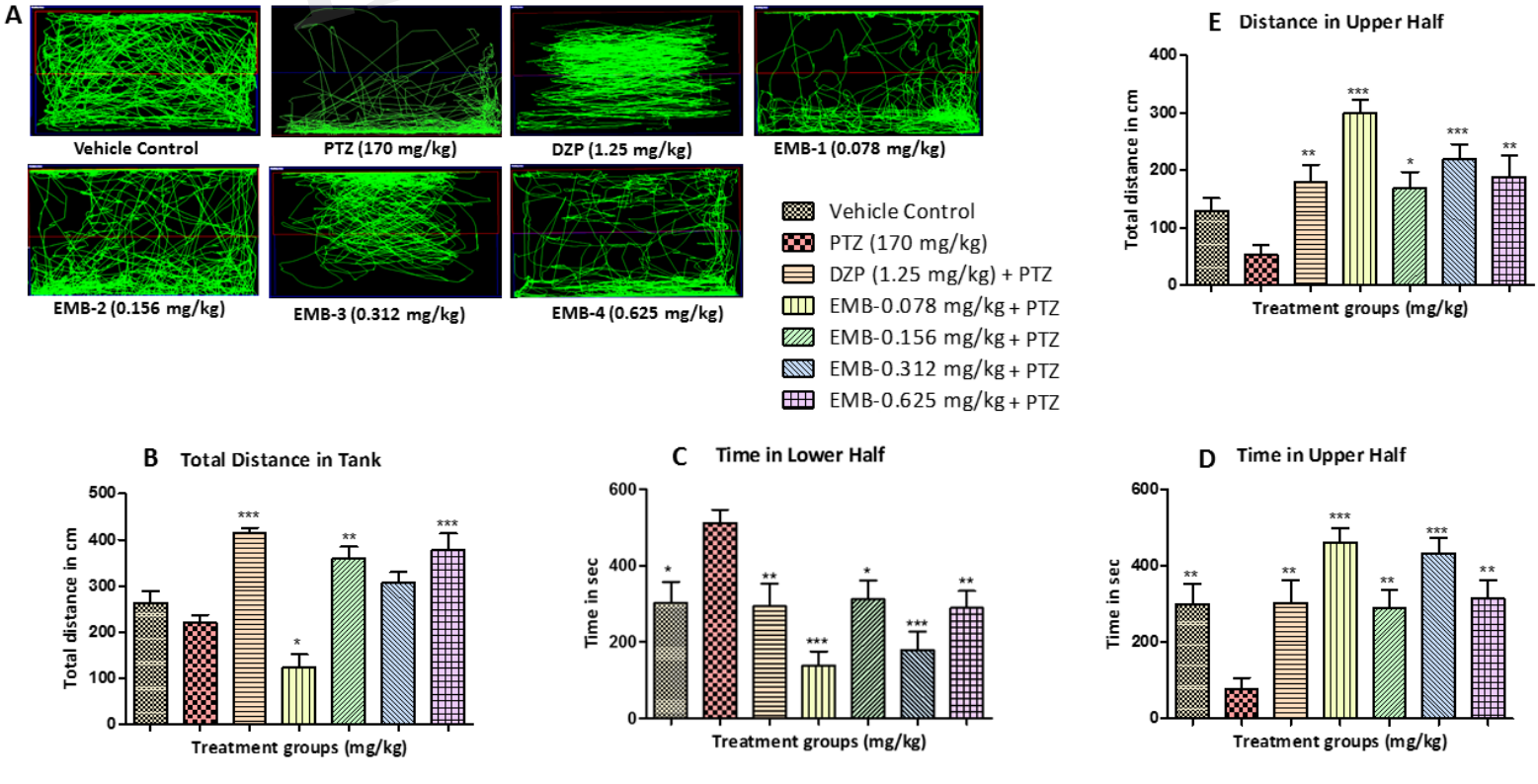

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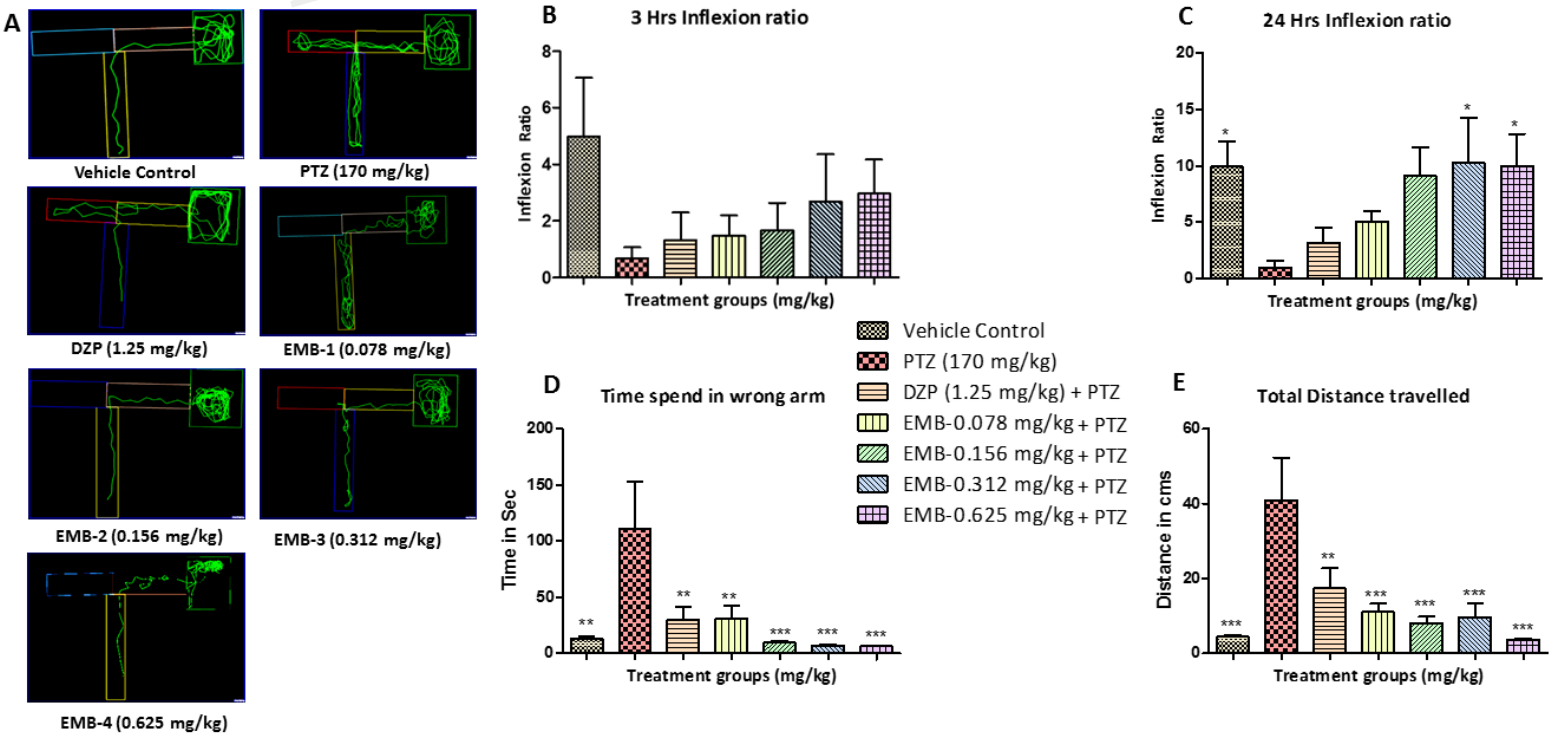

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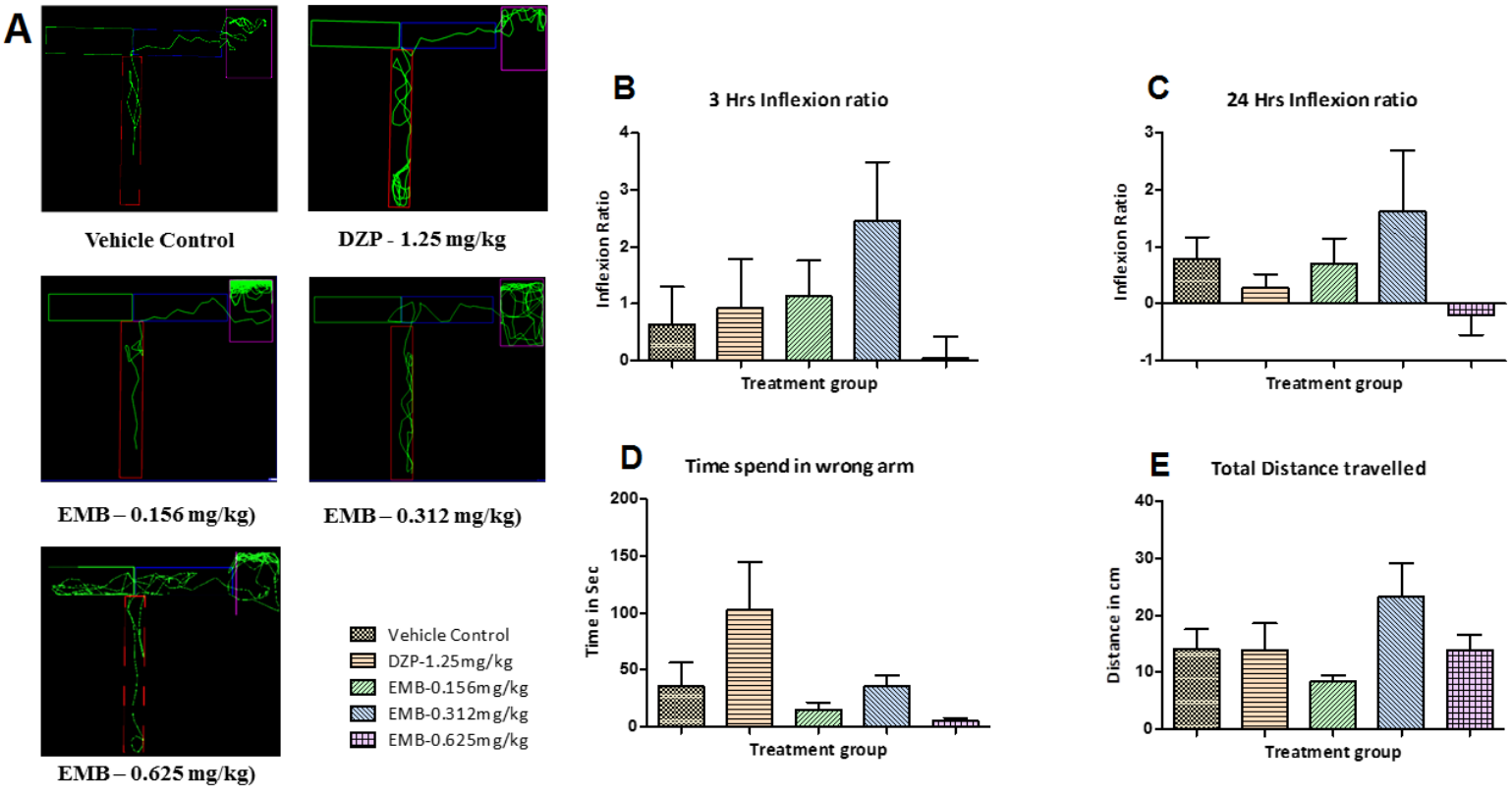

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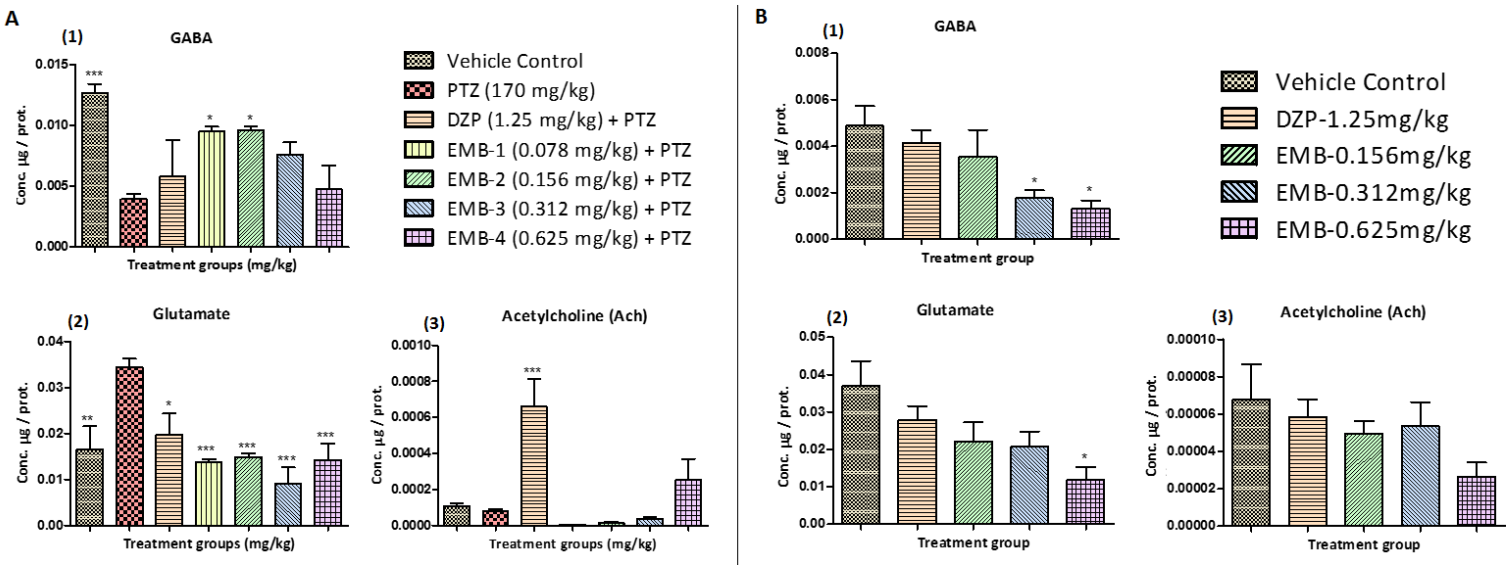

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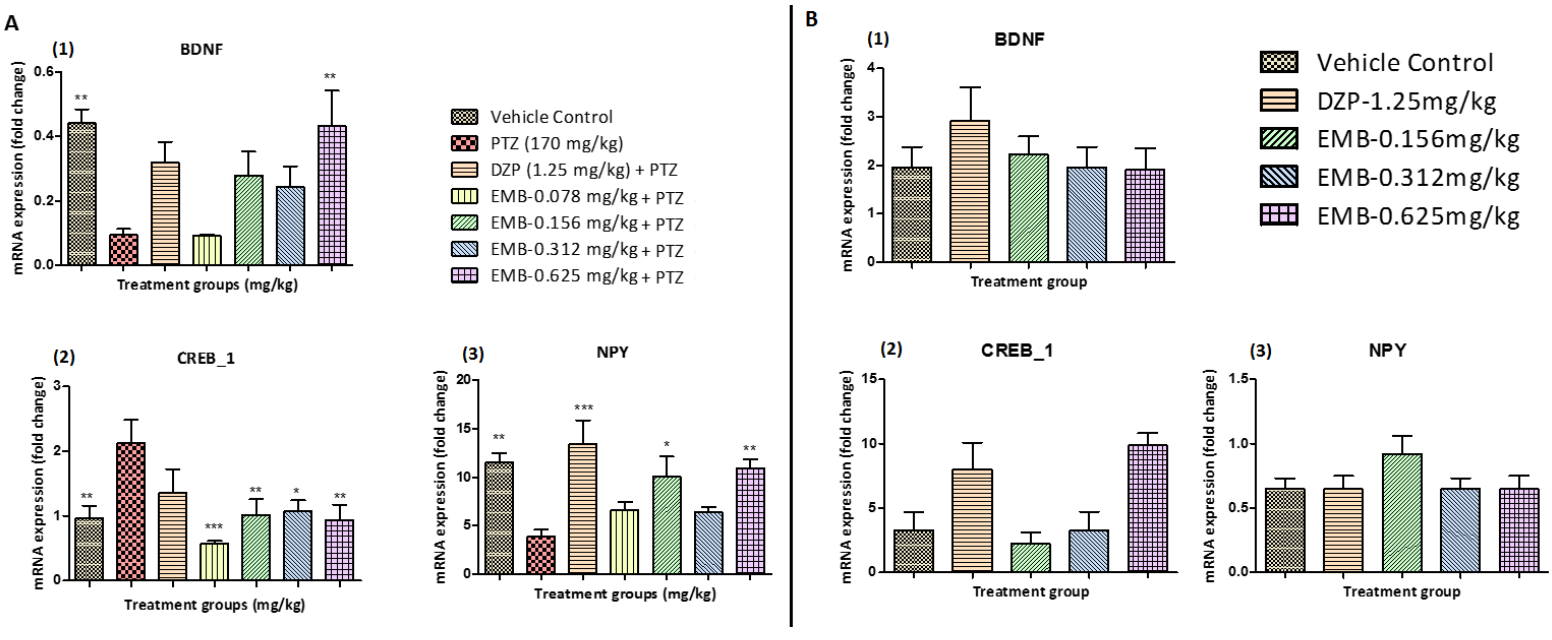

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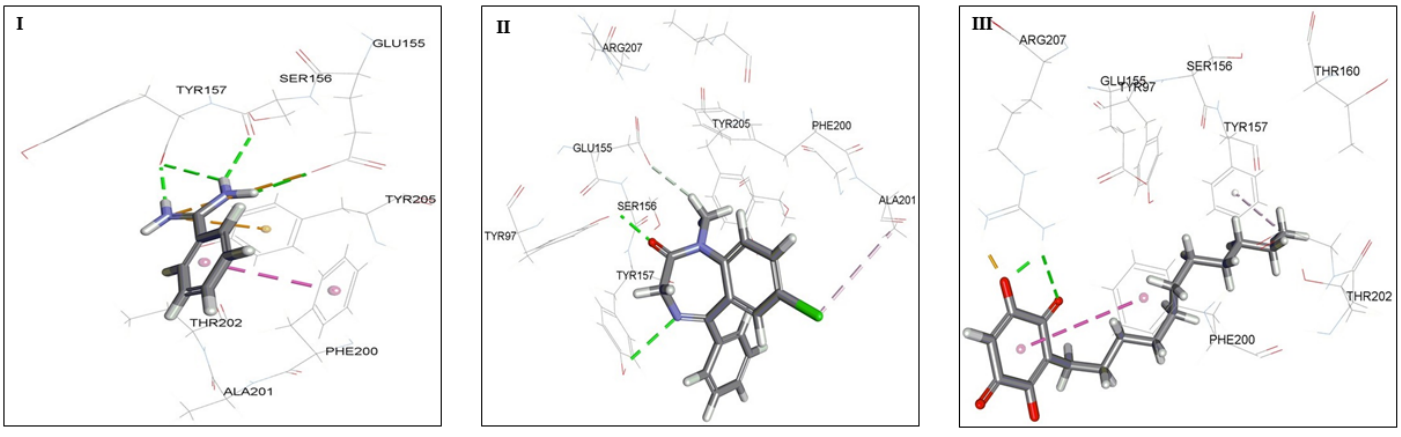


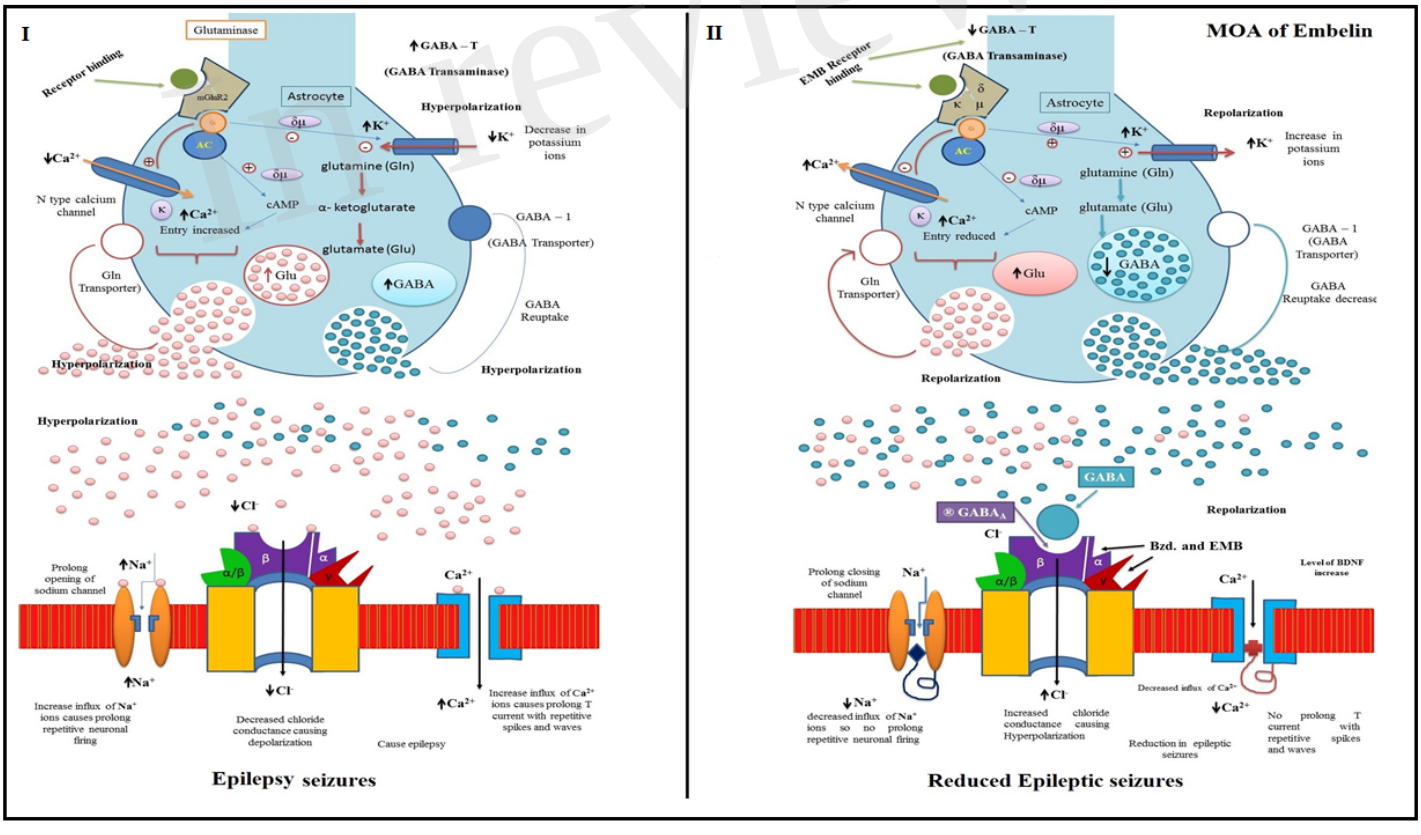
Interaction of agonist benzamidine with active site of GABAA receptor

Binding of Diazepam in the active site of $GABA_A$ receptor

Binding of Embelin in the active site of $GABA_A$ receptor

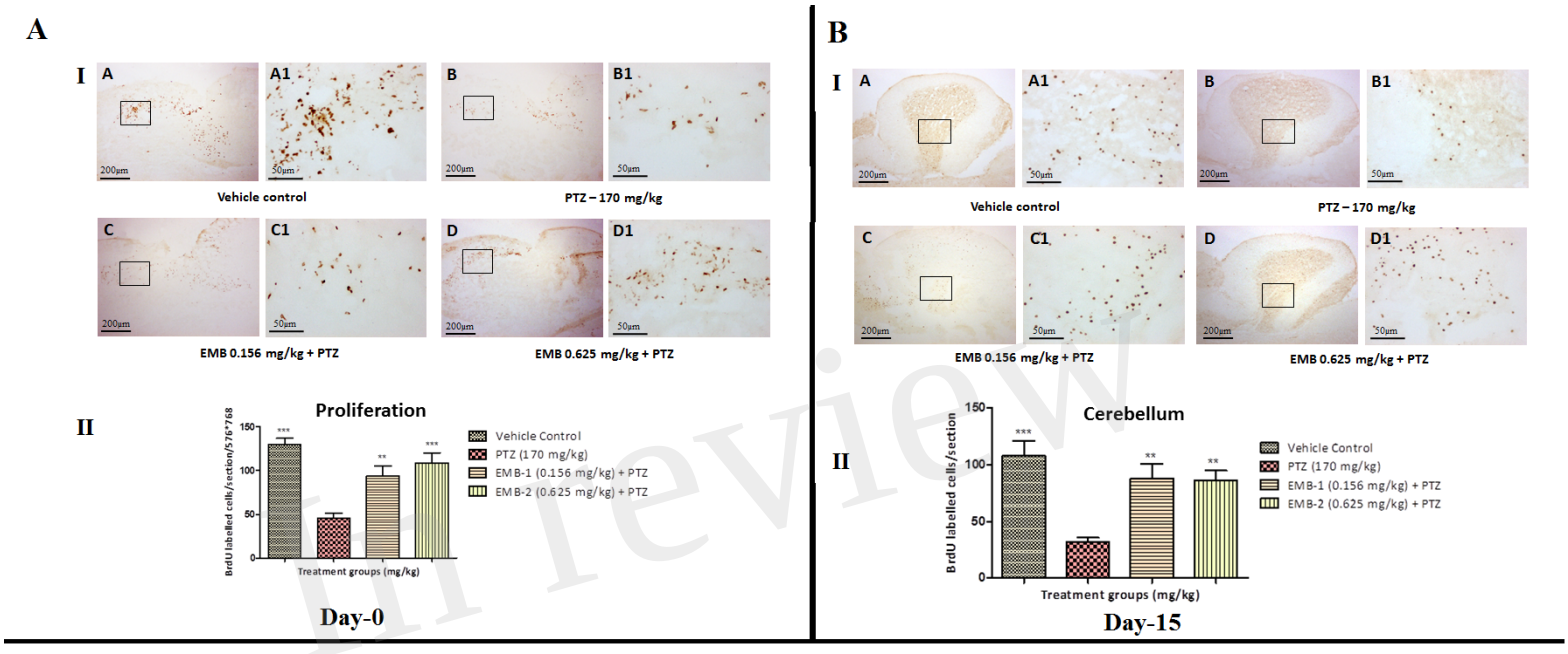
Docking study of embelin with respect to diazepam and the $GABA_A$ receptor

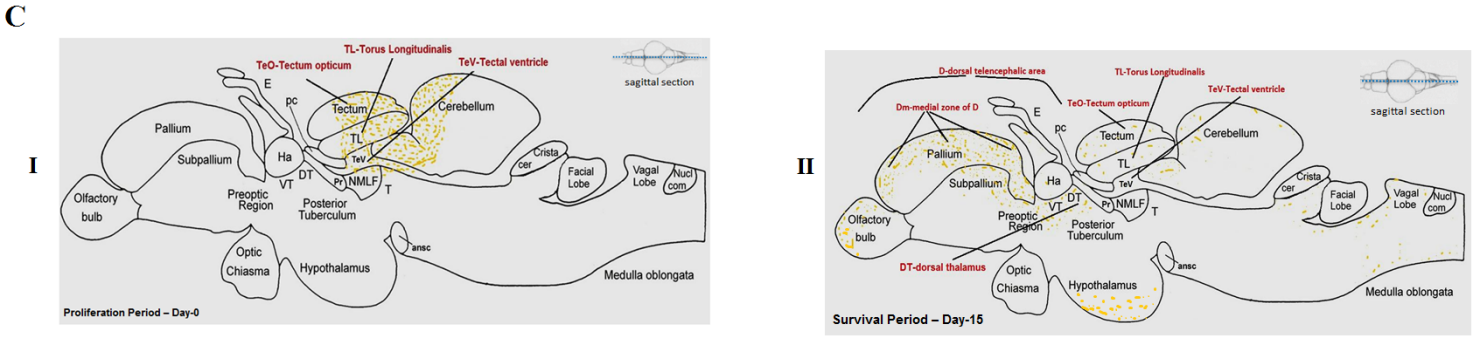
B



Mechanism of action of Embelin at the $GABA_A$ receptor to reverse PTZ induced seizures

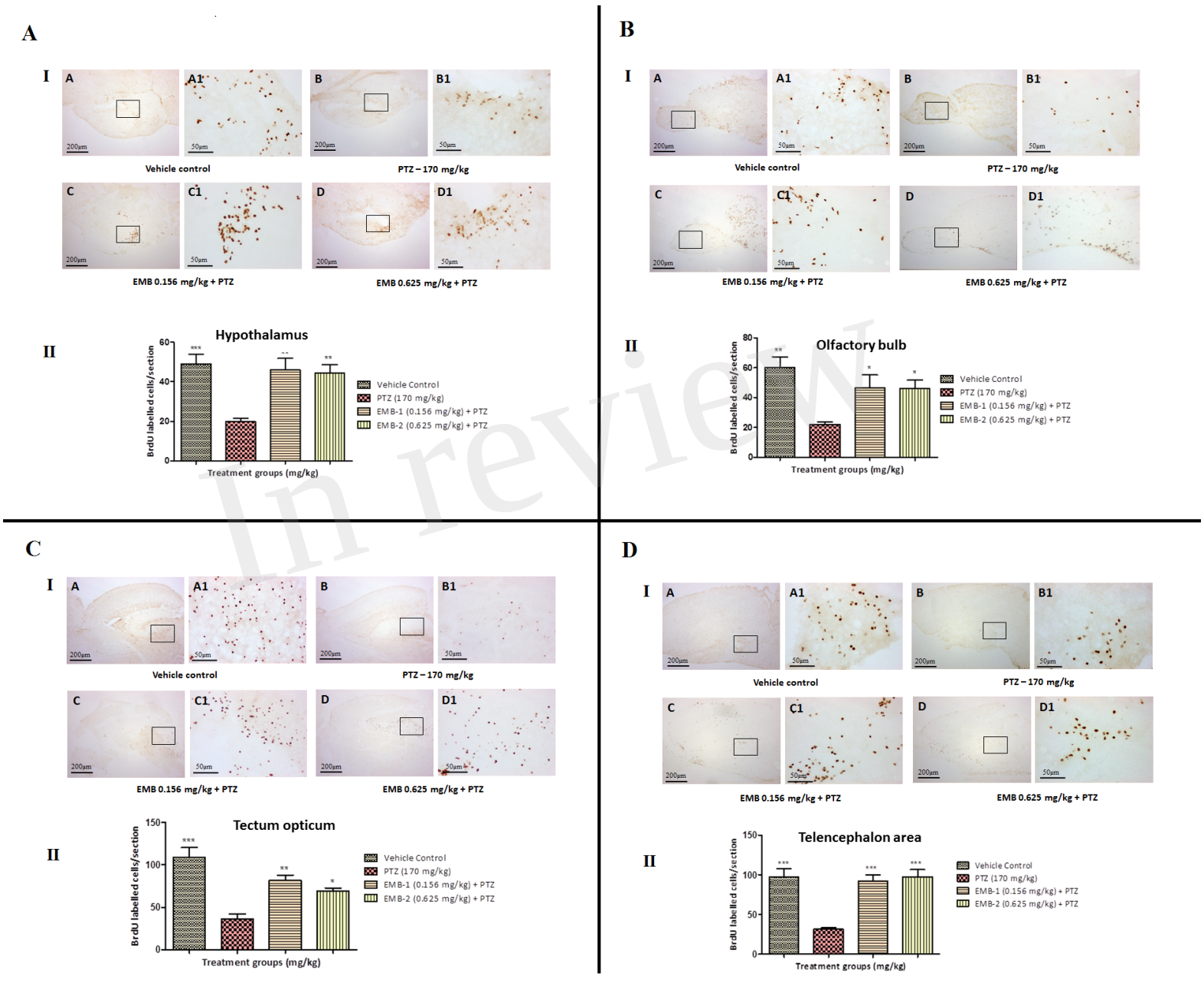
Figure 9.TIF





Graphical representation of cell migration in zebrafish brain

Figure 10.TIF



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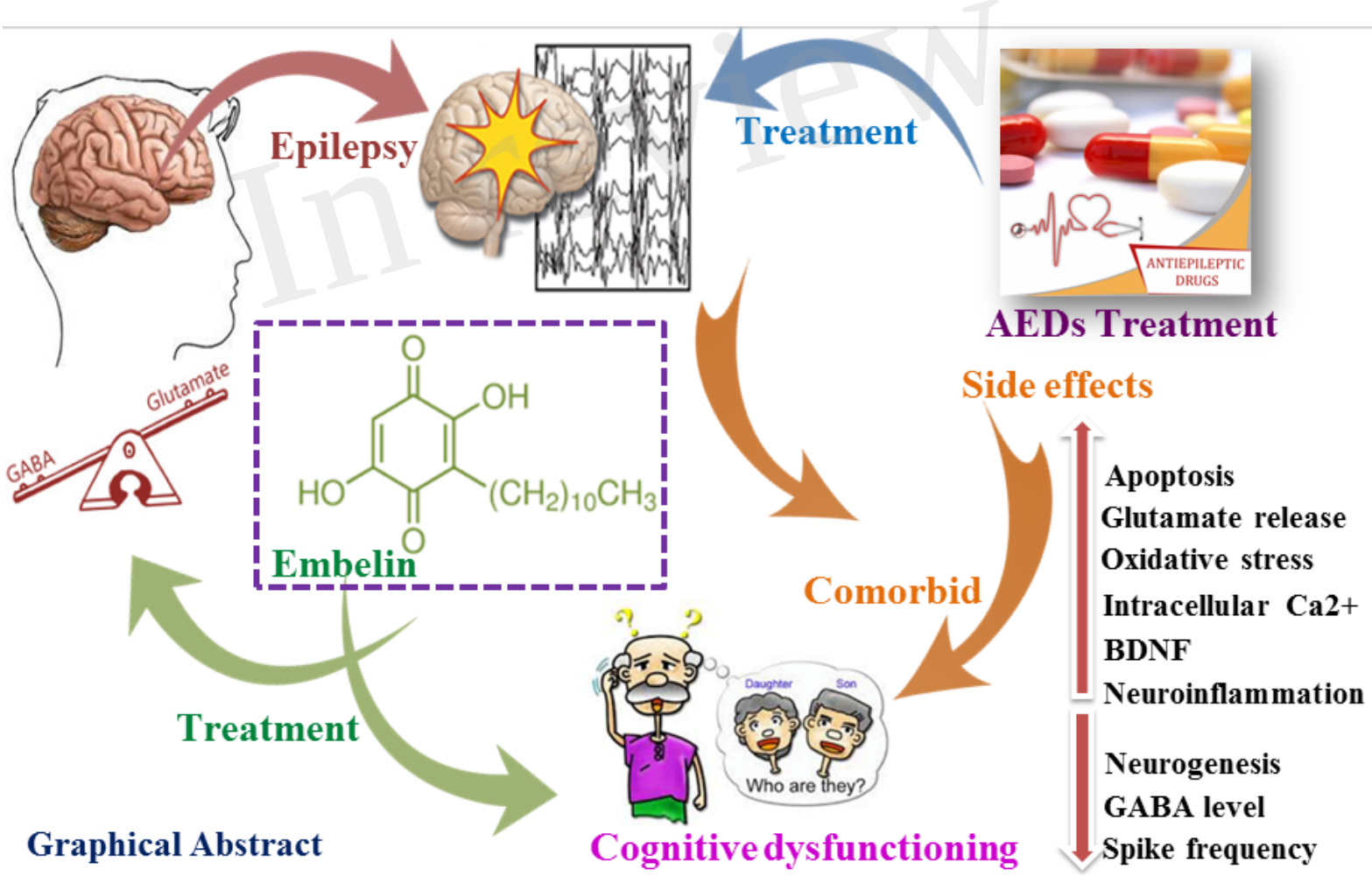

Chapter-4

INTRODUCTION:

Although there is a high interest in utilizing zebrafish as an animal model for neurodegenerative disorders, little is known about the regenerative and spatial cognitive ability of the teleosts compared to largely used mammalian models (Nasir et al., 2012). Despite the increasing fame of zebrafish as an animal model, the use of such models should be more explored in specific neurological studies such as epilepsy, Huntington's disease, Alzheimer's disease etc (Bugel et al., 2014). After developing an acute seizure model of induced cognitive dysfunction in zebrafish and proving the positive effect of embelin (EMB) against it, there was a further need to test the activity of EMB on a chronic epilepsy model of zebrafish. As there was no availability of chronic epilepsy induced cognitive dysfunction models in zebrafish, we decided to develop a model and then test the effect of EMB against it. Chronic epilepsy was induced into the fish by PTZ kindling and then the cognitive ability of the fish was tested by repeatedly daily testing of the fish in a three-axis maze trial for 10 days.

This unit explains a protocol to use PTZ as a pro-convulsive agent to perform chemical kindling in adult zebrafish. Kindling is a chronic animal model of epilepsy that has been extensively used and studied in rodents to understand the process of epileptogenesis and to test new anti-epileptic drugs (AEDs) (Dhir, 2012). Kindling is a process in which small amounts of pro-convulsant drug is administered for a period of 10-15 days in order to produce chronic epilepsy like state. The seizure score was calculated daily and three-axis maze trials were performed to check the memory status. As per rodent models, we tried to induce chronic epilepsy by administrating single dose of KA - 3mg/kg to the fish, the day before the start of the experiment. In a zebrafish three-axis maze model, fish need to navigate along the horizontal, vertical and forward/backward axes and reach the feeding ring where food is given as a reward. The trial is conducted daily after administration of a sub-convulsive dose of PTZ for 10 days. The same procedure was employed after treatment with EMB and the effect of EMB was analysed. Seizure score, inflexion ratio, gene expression fold change and neurotransmitters levels were quantified in order to confirm the positive effect of EMB against chronic epilepsy induced cognitive dysfunction in an adult zebrafish model.

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Nasir, R., Choezom, T., Cunningham, J., Bajaj, B., Rubi, C., Butler, J., Arroyo, K., and Schroeder, J.A. (2012). "Measuring Effects of Psychostimulants on Egocentric Spatial Learning and Memory in Adult Zebrafish," in *Zebrafish Protocols for Neurobehavioral Research*. Springer), 247-256.

Embelin prevents seizure and associated cognitive impairments in a pentylenetetrazole-induced kindling zebrafish model

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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

Author contribution statement

UPK performed most of the experimental procedure along with analysis of the results and, writing of the manuscript. YNP contributed to manuscript writing, result analysis and proofreading. YK contributed to the gene expression study and result analysis. IO contributed to LC-MS/MS study and result analysis. MFS contributed in designing of the study, result analysis, writing and proofreading of the manuscript.

Keywords

chronic epilepsy, Zebrafish, three-axis maze, Cognition, Behavior - Genetics - Molecular

Abstract

Word count: 347

Epilepsy is a neuronal disorder associated with several neurological and behavioral alterations characterized by recurrent spontaneous epileptic seizures. Despite having more than 20 anti-epileptic drugs (AEDs), they only provide a symptomatic treatment. As well as, currently available AEDs also displayed cognitive alterations in addition to retarding seizure. This leads to the need for exploring new molecules that can not only retard seizure but also improve cognitive impairment. Embelin (EMB) is a benzoquinone derivative which has already demonstrated its pharmacological potentials against arrays of neurological conditions. Current study developed a chronic kindling model in adult zebrafish by using repeated administration of small doses of pentylenetetrazole (PTZ) and by a single dose of Kainic acid (KA) to investigate the associated memory impairment. This has been done by using the three-axis maze which is a conventional method to test the learning ability and egocentric memory in zebrafish. As well as, the ameliorative potential of EMB has been evaluated against chronic epilepsy-related memory alterations. As well as the expression level of pro-inflammatory genes such as C-C motif ligand 2 (CCL2), toll-like receptor-4 (TLR4), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interferon-γ (IFN-γ) were evaluated. Level of several neurotransmitters such as γ-aminobutyric acid (GABA), acetylcholine (Ach) and glutamate (Glu) was evaluated by liquid chromatography-mass spectrometry (LC-MS). The results showed that daily dose of PTZ 80 mg/kg for 10 days successfully induces a kindling effect in zebrafish, whereas the single dose of KA did not. As compared to control, PTZ and KA group demonstrates impairment in memory as demonstrated by three-axis maze. PTZ group treated with a series of EMB doses ranging from (0.156 mg/kg to 0.625) retards seizure as well as significantly reduces epilepsy-induced memory alteration. In addition, EMB treatment reduces the expression of inflammatory markers implicating its anti-inflammatory potential. Moreover, levels of GABA, Ach, and glutamate are increased in EMB administered group as compared to the PTZ administered group. Overall, findings demonstrate that EMB might be a potential candidate against chronic epilepsy-related cognitive dysfunction as EMB prevents the seizures, so we expect it to prevent the associated neuroinflammation and learning deficit.

Funding statement

NA

Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: Yes

Please provide the complete ethics statement for your manuscript. Note that the statement will be directly added to the manuscript file for peer-review, and should include the following information:

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- Consent procedure used for human participants or for animal owners
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Ensure that your statement is phrased in a complete way, with clear and concise sentences.
The experimental protocol was approved by the MARP Animal Ethics Committee, Monash University, Australia (MUM/2017/03 and MARP/2017/003).

Data availability statement

Generated Statement: The datasets generated for this study are available on request to the corresponding author.

Title: Embelin prevents seizure and associated cognitive impairments in a pentylenetetrazole-induced kindling zebrafish model

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ABSTRACT:

Epilepsy is a neuronal disorder associated with several neurological and behavioral alterations characterized by recurrent spontaneous epileptic seizures. Despite having more than 20 anti-epileptic drugs (AEDs), they only provide a symptomatic treatment. As well as, currently available AEDs also displayed cognitive alterations in addition to retarding seizure. This leads to the need for exploring new molecules that can not only retard seizure but also improve cognitive impairment. Embelin (EMB) is a benzoquinone derivative which has already demonstrated its pharmacological potentials against arrays of neurological conditions. Current study developed a chronic kindling model in adult zebrafish by using repeated administration of small doses of pentylenetetrazole (PTZ) and by a single dose of Kainic acid (KA) to investigate the associated memory impairment. This has been done by using the three-axis maze which is a conventional method to test the learning ability and egocentric memory in zebrafish. As well as, the ameliorative potential of EMB has been evaluated against chronic epilepsy-related memory alterations. As well as the expression level of pro-inflammatory genes such as C-C motif ligand 2 (CCL2), toll-like receptor-4 (TLR4), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interferon-γ (IFN-γ) were evaluated. Level of several neurotransmitters such as γ-aminobutyric acid (GABA), acetylcholine (Ach) and glutamate (Glu) was evaluated by liquid chromatography-mass spectrometry (LC-MS). The results showed that daily dose of PTZ 80 mg/kg for 10 days successfully induces a kindling effect in zebrafish, whereas the single dose of KA did not. As compared to control, PTZ and KA group demonstrates impairment in memory as demonstrated by three-axis maze. PTZ group treated with a series of EMB doses ranging from (0.156 mg/kg to 0.625) retards seizure as well as significantly reduces epilepsy-induced memory alteration. In addition, EMB treatment reduces the expression of inflammatory markers implicating its anti-inflammatory potential. Moreover, levels of GABA, Ach, and glutamate are increased in EMB

administered group as compared to the PTZ administered group. Overall, findings demonstrate that EMB might be a potential candidate against chronic epilepsy-related cognitive dysfunction as EMB prevents the seizures, so we expect it to prevent the associated neuroinflammation and learning deficit.

Keywords: Chronic epilepsy; Zebrafish; Three-axis maze; Cognition; Behavior

Running title: Embelin ameliorates chronic PTZ kindling and associated memory dysfunction.

Highlights:

1) A repeated small dose of PTZ (80mg/kg) (kindling) for 10 days produces chronic epilepsy like condition in adult zebrafish.

2) EMB was reported to reduce epileptic seizures and improve long term memory as evidenced by three-axis maze test.

3) EMB demonstrated anti-inflammatory effect via downregulating several inflammatory markers (CCL2, TLR4, IL-1, and IFN-γ) in the epileptic brain

4) EMB might be a potential candidate against chronic epilepsy and related cognitive dysfunction as well as can overcome the limitations of mainstream AEDs.

1. INTRODUCTION:

Epilepsy is a neurologic disorder affecting more than 70 million people worldwide (Singh and Trevick, 2016). Epilepsy is mainly characterized by the occurrence of spontaneous recurrent seizures (SRS) and high prevalence of comorbidities, such as cognitive impairments, depression, and anxiety, affecting the lives of individuals (Keezer et al., 2016). Chronic epilepsy is characterized by repeated unpredictable seizures, impairing memory in 30-40 % of patients under long-term anti-epileptic drugs (AEDs) treatment, so epilepsy and AEDs both are responsible for memory problems (Helmstaedter, 2002; Canevini et al., 2010; Zhang et al., 2017). In spite of more than 20 mainstream AEDs, they only provide symptomatic treatment rather than interfering with diseases mechanism as well as 30% patients does not respond to current AEDs (Remy and Beck, 2005; Chen et al., 2018). These data indicate the pressing needs of developing novel and innovative therapy that can reduce seizures and maintain good memory status. Despite recent advances in research, the underlying pathomechanism of epilepsy is still less known. However, findings are emerging out implicating the role of inflammation in epilepsy (Vezzani, 2005; Paudel et al., 2018b; van Vliet et al., 2018). High mobility group box 1 (HMGB1) protein is an initiator and amplifier of neuroinflammation and has been implicated in the seizure via activating toll-like receptor 4 (TLR4) (Ravizza et al., 2017; Paudel et al., 2018a). Moreover, C-C motif ligand 2 (CCL2), tumor necrosis factor-α (TNF-α), interleukin-1(IL-1) and interferon-γ (IFN-γ) has been implicated in seizure generation as well as found to be up-regulated during a seizure (Marchi et al., 2014; Dey et al., 2016).

Local or systemic administration of kainate in rodents leads to repetitive limbic seizures and status epilepticus (SE), lasting for several hours. It is a useful model of temporal lobe epilepsy (TLE) in rodents (Dal-Pizzol et al., 2000). Kainic acid (KA) is an epileptogenic and the

neuroexcitotoxic agent acting on specific kainate receptors (KARs) in the central nervous system (CNS) (Zheng et al., 2011). The complete seizure characterization of KA-induced epilepsy is studied in zebrafish and it produces continues seizures with neuronal toxicity (Alfaro et al., 2011; Menezes et al., 2014; Mussulini et al., 2018). Although traditional models like maximal electroshock seizure (MES) and pentylenetetrazole (PTZ) seizure model have been instrumental for pre-clinical screening since a long time, however, these models have limited themselves in identifying novel compounds with improved efficacy against different chronic disorders (Löscher, 2011). PTZ induced acute seizure model has been already developed in adult zebrafish and recapitulates all the clinical phenotypes of seizure (Mussulini et al., 2013; Kundap et al., 2017a). Kindling is a chronic animal model of epilepsy that has been extensively studied to understand the underlying mechanism of epileptogenesis (Kandratavicius et al., 2014). In addition, kindling is a phenomenon where a sub-convulsive stimulus (either chemical or electrical), if applied repetitively and intermittently, will ultimately lead to the generation of full-blown convulsions (Dhir, 2012b). The study conducted by using PTZ kindling discusses detail methodology to execute a chemical induced kindling in mice (Dhir, 2012b). A number of different promising models which fulfill this criterion have been developed over recent years, but has not modeled in zebrafish (Wilcox et al., 2013). Moreover, the understanding of the molecular involvement underlying such diseases is still under study and it is preventing adequate therapeutic outcomes (Samarut et al., 2016). Various numbers of pre-clinical models have been studied to investigate the role of different brain functions and understand the structure of disease development (Samarut, 2016).

In recent days, zebrafish has received increased attention due to its genetic similarity with human, economic value, a suitable alternative for human disease model and large-scale drug screenings (Shams et al., 2018). Zebrafish has rapid embryonic development as compared to rodents, easy

122 cellular observation, and phenotypic analysis, which makes it a better model to study the
123 neurological aspect of brain disorder (ref).
124 The three-axis maze is a method to evaluate the learning ability and egocentric memory in
125 zebrafish. In three-axis maze, fish requires to navigate a route based on x (forward/backward), y
126 (horizontal) and z (vertical) axes (Nasir et al., 2012a). Egocentric navigation is an evidence-based
127 process, where fish navigates to the feeding ring independent of environmental cues and deals
128 directly with the spatial relationship between subject and reward goal (Nasir et al., 2012a).
129 Embelin (EMB) (2,5dihydroxy-3-undecyl-1,4 benzoquinone) is the main component in the red
130 berry fruits produced by *Embelia ribes* (Dubéarnès et al., 2015). EMB has been studied extensively
131 using various in-vitro and in-vivo animal models and have exhibited strong anti-convulsant,
132 anxiolytic and anti-depressant properties as well as improves conditions such as sickness behavior,
133 Huntington's disease (HD), multiple sclerosis (MS), cerebral ischemia and traumatic brain injury
134 (TBI) (Mahendran et al., 2011; Poojari, 2014). Moreover, earlier findings reported that EMB
135 significantly inhibited seizures induced by electroshock and PTZ in a dose-dependent manner
136 (Shankar et al., 2012). In spite of the huge pharmacological significance of EMB, no study has
137 earlier reported the ameliorative potential of EMB against PTZ kindling induced chronic epilepsy
138 and related cognitive alterations using zebrafish as an animal model.

139 The current study explained a protocol to use PTZ as a pro-convulsive agent to perform chemical
140 kindling in adult zebrafish. The small amount of proconvulsant drug (PTZ) was administered for
141 a period of 10 days in order to produce progressive chronic epilepsy like behavior. To KA-treated
142 fish, as per rodent model, we tried to induce chronic epilepsy by administrating single dose of KA-
143 3mg/kg the day before the start of the experiment. The seizure scores were calculated, and three-
144 axis maze trial was performed in order to check the memory status and seizure progression on a

daily basis. The same procedure was employed in the fish pretreated with EMB and the effect EMB was analyzed. In addition, fold change of neuroinflammatory genes and neurotransmitter levels were quantified in order to confirm the positive effect of EMB against chronic epilepsy induced cognitive dysfunction model in adult zebrafish.

2. MATERIAL AND METHODS:

2.1. Experimental equipment and chemicals list

All analytical grade reagents were used unless specified otherwise. Water was purified and filtered with a specific LC-MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). Glutamate GABA, KA, and PTZ were purchased from Sigma Aldrich (USA). The pure form of plant extract of EMB was purchased from YUCCA Enterprises, Mumbai, India. Ethanol 95% (EtOH) was purchased from Kolin Chemicals Co. Ltd, Korea, methanol (MeOH), chloroform (CHCl3), isopropanol (IPA), and formic acid (FA) was purchased from Friedemann Schmidt Chemicals, Parkwood 6147, Western Australia.

2.2. Zebrafish maintenance and housing conditions:

Adult zebrafish (Danio rerio) of heterozygous wild-type-AB stock (standard short-fin phenotype) were obtained from the Institute of Molecular and Cell Biology (IMCB), 61 Bioplis Drive Proteos, Singapore 138673. All fish were kept in the Monash University Malaysia fish facility at 28° C, with a 10/14 h dark/light cycle (white incident light off at 10 pm, white incident light on at 8 am) under standard aquarium conditions. Care was taken to maintain system water pH between 6.8 - 7.1 by using an electronic pH pen (Classic PH Pen Tester, Yi Hu Fish Farm Trading, Singapore 698950) and intensity of light was maintained at 250 Lux to get the uniform light all over the housing area. Fish were fed, thrice a day to ensure a constant source of nourishment. Nutrition for fish was maintained by Tropical TetraMin® Flakes and live brine shrimps Artemia from Bio-Marine (Aquafauna, Inc. United States). Circulating water system with zebrafish tank which is equipped with constant aeration having (36 cm x 26 cm x 22 cm) tank dimensions (Kundap et al.,

2017a). Monash Animal Research Platform (MARP), Australia, approved all the zebrafish experimental procedures (MUM-2017-03) (MARP-2017-003).

During the experimental procedure the animals were housed individually in each tank of 3 L in size. The animals were not feed in the home tank as they received the food a reward during each trial daily. The fish those do not reach the feeding ring (goal chamber) were fed once in the home tank. To avoid the encounter of aggressive behavior with each other and to individually track the behavior of each fish individually, the fish were housed individually in each tank (Parker et al., 2012). The water temperature of 28° C was maintained same as the system water, with 7.2pH, the light-dark cycle of 14/10 hours and intensity of light was maintained at 250 Lux to get the uniform light all over the housing area.

2.3. Animal grouping/randomization and treatment

Groups of animals exposed to drug treatment were divided as follows. Animals selected for testing were randomized as per the groups with n=12. The experiment was not blinded as videos were recorded and then manually analyzed and checked by 2 investigators individually for confirmation. The fish were pre-treated with embelin and then exposed to PTZ before the start of the Three-axis maze trial. The behavior recording for an epilepsy seizure score and transfer latency was recorded daily for the period of 10 days for each fish. Animal treated with embelin were observed for its epilepsy behavior, cognitive performance in three-axis maze and biochemical changes. Later on, the zebrafish brains were isolated for LCMS/MS studies and gene expression analysis. For experimental procedure and drug administration follow figure 1.

Set 1: Development of chronic epilepsy induced cognitive problems in zebrafish.

Group I: Vehicle control; **Group II:** PTZ 80 mg/kg; **Group III:** KA 3 mg/kg.

Set 2: Elucidating the therapeutic potential of EMB against chronic epilepsy induced cognitive dysfunction

EMB and PTZ were dissolved in 10% DMSO.

Group I: Vehicle control (10% DMSO); **Group II:** Pentylenetetrazole (PTZ) – 80 mg/kg; **Group III:** Embelin (EMB) - 0.156 mg/kg + PTZ; **Group IV:** Embelin (EMB) - 0.312 mg/kg + PTZ; Group V: **Embelin (EMB)** - 0.625 mg/kg + PTZ.

2.4. Software and equipment

The Smart V3.0.05 tracking software (Pan Lab, Harvard Apparatus), the Sony (Handycam HDR-PJ340E) video camera and Sony Camcorder stands was used for the automated tracking of zebrafish swimming patterns and locomotion parameters. The Applied Biosystems StepOnePlusTM Real-Time PCR System was used for the gene expression study. The MilliQ system from Millipore (Bedford, MA, United States) was used to produce pure water for the experiment purpose. The equipment used for quantifying brain neurotransmitters was the Agilent 1290 Infinity UHPLC, coupled with the Agilent 6410 Triple, Quad LC/MS. Fish tank -10 liters capacity (PETCO-Pet keeper, Malaysia), The syringe used for intraperitoneal injection was Hamilton syringe 700-702 series 25µl along with BD disposable needle - 30G (Becton Dickinson, USA).

2.5. Procedure for zebrafish anesthesia and intraperitoneal injection

PTZ and the EMB were intraperitoneally infused into the zebrafish as indicated by the convention given by Kundap et al. (2017b) and is given beneath. At the point when different intraperitoneal infusions were required, the infusions were given at alternating lateral ends, instead of the midline between the pelvic fins. Each zebrafish was caught individually utilizing a fish holding net, and after that moved into an anesthesia arrangement (30 mg/L Benzocaine). The fish were kept into

the anesthesia water for 30 secs until they stop moving. The zebrafish was taken out once anesthetized and weighed afterward to calculate the dose and subsequently the infusion volume. A delicate sponge roughly 20 mm in stature was soaked with water and set inside a 60 mm Petri dish. A cut between 10– 15 mm inside and out was made in the sponge to control and hold the fish for the intraperitoneal infusion. The intraperitoneal infusion was given while utilizing a dismembering magnifying lens by embeddings the needle into the midline between the pelvic fins. An appropriate volume was then injected into the zebrafish, after considering the body weight of the zebrafish.

All intraperitoneal infusions were administered into the stomach pit at an area back to the pelvic support, utilizing a 10 µl Hamilton syringe (700 arrangement, Hamilton 80400) (Stewart et al., 2011). The experiment was performed in a separate behavior room with the room temperature kept between 26°C - 30°C and humidity between 50% - 60%. All zebrafish were acclimatized in the said behavior room for two hours prior to the experiment for minimizing any novel tank response. Other precautions taken include using a small injection volume of 10 µl per gram of fish and using a 30-gauge needle. The zebrafish were restrained in water saturated sponge under benzocaine anesthesia to reduce the distress inflicted on the zebrafish (Júnior et al., 2012). This intraperitoneal injection technique was found to be effective in zebrafish (Kundap et al., 2017b) and did not cause any mortality throughout the experiment. After the intraperitoneal injection, the zebrafish was immediately transferred to an observation tank.

2.6. Seizure score analysis:

2.6.1. PTZ-induced seizure score:

PTZ is a proconvulsant drug. As per earlier reported studies, PTZ at 220-250 mg/kg dose produces full-blown seizures in zebrafish (Banote et al., 2013a). In order to produce kindling effect, PTZ at lower doses (80 mg/kg) was administered daily for 10 days which was almost 1/3 of the actual dose reported earlier. Kindling is a process by which a seizure or other brain event is initiated and its recurrence is made more likely (Dhir, 2012a). PTZ Kindling model is well established in rodents and in other animals so in accordance to those animal model the dose of PTZ administration is calculated for zebrafish (80 mg/kg dose/10 days) (Ohno et al., 2010). After fish was prone to seizures for at least 10 days it was observed for its locomotor behavior, three-axis maze memory analysis, and other biochemical alterations. For EMB treated group fish was pre-treated with a single dose of EMB dissolved in 10% DMSO prior to daily PTZ administration. However, in control group fish was pre-treated with 10% DMSO and the distilled water was administered to maintain the same number of injection in all the groups.

PTZ injected zebrafish displays unique seizure characters, intensities and latency in generating the different seizure scores. PTZ induced seizures behavior will remain around 10 minutes after the PTZ administration and gradually decrease with time. The PTZ injected adult zebrafish were then transferred to the observation tank water for behavior and three-axis maze test. The behavior of the zebrafish was then recorded for 10 minutes after recovery from anesthesia and the video was later viewed using a computer to determine the highest seizure score during the 10 minutes. The zebrafish seizure score was recorded as per the earlier scoring protocol (Banote et al., 2013b; Mussolini et al., 2013; Kundap et al., 2017a); Kundap et al. (2017b) and is given below.

Score 1- Short swim mainly at the bottom of the tank

Score 2- Increased swimming activity and high frequency of opercula movement

Score 3- Burst swimming, left and right movements as well as the erratic movements

Score 4- Circular movements

Score 5- Clonic-tonic full body system rhythmic contractions

Score 6- Fall at the bottom of the tank

Score 7- Death

2.6.2. KA-induced seizure score

Throughout the time of pre-exposure to KA, the behavior of the zebrafish was monitored for 10 min after the fish was fully recovered from anesthesia. The seizure behavior for KA-induced seizure was modified from the earlier findings (Menezes et al., 2014) and described below.

Score 1- Rigidity and hyperventilation of the animal

Score 2- Whirlpool-like swimming behavior

Score 3- Rapid muscular uncontrol movements from right to left

Score 4- Abnormal and spasmodic muscular contractions

Score 5- Rapid whole-body clonus-like convulsions

Score 6- Sinking to the bottom of the tank and spasms for several minutes

Score 7- Death

Under the directives of the Monash Animal Research Platform (MARP)-Australia, the PTZ dose was standardized at 80 mg/kg of zebrafish body weight in order to produce the kindling seizure progression. The dose of KA-3mg/kg (single dose) (Alfaro et al., 2011) was titrated in order to

produce the maximum survival of zebrafish in a group for more than 48 hrs until day-10. The highest observed seizure score was the highest seizure score noted within the entire 10 minutes duration of the recording. The zebrafish swimming pattern and locomotion parameters were determined via analysis using the Smart tracking software.

2.7. Three-axis maze test and Behavior analysis:

In three-axis maze (Figure 1) fish requires to navigate a route based on x (forward/backward), y (lateral) and z (depth) axes and is designed as a measure of spatial memory. The maze is constructed from white 0.25″ acrylic held together with acrylic epoxy and sealed with aquarium sealant. The maze consists of a 20 × 20 × 60-cm tank divided into five 12 × 20 × 20-cm chambers with a 7 × 7-cm window cut into the corner or center of each insert as shown in figure 1. A floating NutraFin Max feeding ring (PetCo, Inc.) was attached to the end of the maze in the goal chamber. The walls and inserts of the maze are constructed of white acrylic to minimize any external visual cues that could be used by the fish as markers. In addition, the maze was uniformly illuminated from above to minimize shadows and visual cues external to the maze. With the inserts in place, fish swim from one chamber to another through the windows in the inserts to reach a food reward in the goal chamber. The order of inserts in the maze was constant throughout the experiment, to allow the route to remain constant until the feeding ring as shown in figure 1.

The detailed specifications regarding the maze were as per the given standard protocol (Nasir et al., 2012b). Transfer latencies (TL) were recorded from day-1 to day-10 post-PTZ administration. An inflexion ratio (IR – day-1) = (TL0-TL1)/TL1), (IR-day-2) = (TL0-TL2)/TL2) rest as follows was calculated, where TL0 is the initial latency(s) at day-0 & TL1 and TL2 is the latency(s) at the Day-1 and day-2 trial respectively. The IR was calculated as compared to day-0 to measure the amount of memory increase each day with the progression of days/treatment. The behavior

recordings during seizure activity and the three-maze test were analyzed to track the locomotor patterns. Tracking of the locomotor pattern was done by using the computer software SMART v3.4-Panlab Harvard Apparatus®

2.7.1. Training:

The Fish was food-deprived for 1 day prior to the start of both the training and testing periods and are not fed outside of the maze throughout the duration of the experiment. Training consisted of two back-to-back trials on the day before testing. During training, the inserts were removed from the maze; the fish was netted from their home tank and placed at the end of the maze opposite the feeding ring. A small amount of Tetramin flakes was placed in the feeding ring and fish are trained to swim the length of the maze to receive food. The Fish was permitted to feed for more than 30 s up to maximum 1 min before being netted and returned to their home tank. Training allows the fish to acclimate to the testing apparatus and learn the location of the feeding ring at the opposite end of the tank. Food-deprived fish learn to associate the testing apparatus with food and actively search for the feeding ring when the inserts will be present. Training is necessary in order to avoid novel tank anxiety effect in fish during the actual testing period. Food deprivation is part of the experimental procedure. The reason behind food-deprivation is that fish learn to associate the testing apparatus with food and actively search for the feeding ring when the inserts are present. After repeated trials fish learns the task to reach the feeding ring and eat the food (Nasir et al., 2012a).

2.7.2. Testing:

Testing consists of one trial per day for 10 consecutive days. Vehicle control animals will be administered I.P with vehicle agent first and then administered with distilled water before placing it in the maze. For PTZ treated group, each fish were administered with 80 mg/kg dose I.P daily

prior to the testing period. The fish were habituated for 10 min after PTZ administration to check the memory test in Three-axis maze. For the KA-treated group, each fish was administered with 3 mg/kg dose at the start of the experiment on day-1. For EMB treated group, the fish was pre-treated with EMB (0.156 mg/kg / 0.312 mg/kg / 0.625 mg/kg) daily prior to administration of PTZ. After specific treatment, the fish are placed in the start chamber and the response latency to reach the feeding ring in the last chamber is recorded. Fish will be allowed to feed for more than 30 s maximum up to 1 min before being returned to their home tanks. If fish failed to complete the maze within 10 min, they are fed and returned back to the home tank.

2.7.3. Gene expression:

Gene expression studies were carried out to determine the expression level of several inflammatory genes such as CCL2, HMGB1, TLR4, IFN- γ, TNF-α and IL-1. All the brain samples were collected in ice-cold 200 µl TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and immediately stored at -80°C until further usage. The study was divided into three steps such as isolation of mRNA, synthesis of cDNA strand and then real-time PCR to estimate the level of genes expressed.

Isolation of RNA and first strand cDNA synthesis: The mRNA was isolated by following the manufacturer's protocol. In brief, brain tissue was properly homogenized in TRIzol® reagent, mixed with chloroform and centrifuged at 13,500 rpm (revolutions per minute) for 15 minutes at 4 °C. The upper aqueous supernatant was transferred into new tubes and isopropanol was added, mixed and was incubated for 10 minutes at room temperature and later centrifuged for 10 minutes at 13,500 rpm at 4°C. The supernatant was discarded, and the pellets were subjected to rinsing with 75% ethanol. Then, the pellets were left for air drying between 5 to 8 minutes of air drying. Finally, nuclease-free water was added to each tube to dissolve the mRNA pellet. The concentration and purity of the isolated mRNA were measured by using a NanoDrop

Spectrophotometer. The mRNA samples were converted into cDNA using Omniscript Reverse-transcription Kit (QIAGEN) according to the manufacturer's protocol.

All the primer sets were provided by Qiagen (NL)

CCL2: Dr_ccl2_1_SG QuantiTect Primer Assay (Cat no: QT02205763) TLR4: Dr_tlr4ba_va. 1_SG QuantiTect Primer Assay (Cat no: QT02198539)

IFN-G: Dr_ifng1-2_1_SG QuantiTect Primer Assay (Cat no: QT02064328)

TNF-α: Dr_tnf_1_SG QuantiTect Primer Assay (Cat no.QT02097655)

IL-1: Dr_il1rapl1a_1_SG QuantiTect Primer Assay (Cat no.QT02131850)

eef1a1b: Dr_eef1a1b_2_SG QuantiTect Primer Assay (Cat no.QT02042684)

2.7.4. Estimation of neurotransmitters by LC/MS-MS:

Glutamate, GABA, and Ach are significant neurotransmitters to study epilepsy and cognition. These neurotransmitters were analyzed using the LC-MS/MS technique. All the standard neurotransmitters were prepared in methanol (0.1% formic acid) as a stock solution of 1 mg/ml and were kept at 4°C until use. Standards for calibration were prepared from the original stock solution. Serial dilution from 100-2000 ppb was used for calibration. The brain was homogenized in 200µl of ice-cold methanol (0.1 % formic acid). The homogenate was vortex-mixed for 1 min and then centrifuged at 18, 000 × g for 10 min at 4°C. Finally, the supernatant was pipetted and placed into vials for LC-MS/MS analysis.

LC-MS/MS was run on an Agilent 1290 Infinity UHPLC, coupled with Agilent 6410 Triple, Quad LC/MS, ZORBAX Eclipse plus C18 RRHD 2.1 x 150mm, 1.8-micron (P/N 959759-902) auto-sampler system (Agilent Technologies, Santa Clara, CA, USA). The samples were separated on a SMol-RRHD-Eclipse-C18-8 (15) UHPLC-160129-00011-Pos-DMRM used at 30°C. The mobile phase consisting of 0.1% formic acid in water (Solvent A) and acetonitrile with 0.1% formic acid

(Solvent B) was used with a gradient elution: 0–3 min, 50% B; 3-6 min, 95% B; 06–07 min, 95% B at a flow rate of 0.1 ml/min. ESI-MS/MS Conditions were set as follows: ESI ion source, positive ion polarity, gas temperature 325°C, drying gas flow 9.0 L/min, nebulizer pressure 45psi, Vcap 4000V. MS acquisition of GABA, Glu, ACh was performed in electrospray positive ionization multiple reaction monitoring (MRM) mode.

2.8. Statistical analysis:

The data were analyzed by GraphPad Prism v.7.02 (San Diego, CA) with repeated measure (mixed model) Two-way ANOVA followed by Bonferroni post-test to compare replicated means by row. Data are presented as means and standard errors of the mean (SEM) to assess the differences in seizure, latency and inflexion ratio. The results acquired for neurotransmitter levels and gene expression between treatments and control were analyzed by One-way ANOVA and subsequent Dunnett's multiple comparison tests. A P-value of < 0.05 indicates statistical significance.

3. RESULTS:

3.1. Daily administration of PTZ induces chronic epilepsy in adult zebrafish:

Daily administration of small doses of PTZ for 10 days induced full-blown seizures in zebrafish. It was observed that 80 mg/kg dose of PTZ cause a gradual increase in seizure scores from day-4 until day-10. Seizure score increase from score 1.5 to score 5 from day-1 to day-10. In addition, a single dose of KA-induced epileptic seizures, however, it was not able to maintain a high seizure score on daily observation and the score was eventually reduced from score 5 at day-1 to no seizure to score 1 at day-10 (Figure 2 (I)).

Moreover, in three-axis maze, it was observed that all the fish from the control group showed a significant decrease in time taken to reach the feeding ring (goal chamber) from day-1 to day 10 and it was less than 100 Sec (figure 2 (II)). However, all the fish from PTZ and KA-treated group found to worsen the cognitive function in zebrafish on observation of three-axis maze study. Indeed, the latency to reach the feeding chamber was high in PTZ and KA-treated fish when compared to the control group (Figure 2 (II)). It was observed that PTZ treated fish took more than 450 Sec to reach the feeding ring until day-10. However, the time was increased to 250 Sec in KA-treated fish when compared to the control group as shown in Figure 2 (II).

3.2. Daily administration of EMB ameliorates PTZ seizures and memory decline:

PTZ at 80 mg/kg, when administrated daily for 10 days, produce kindling like behavior with a continuous unprovoked seizure. Fish, when treated with EMB, ameliorates the gradual increase in seizure generated due to PTZ kindling (Figure 3 (I)). It was found that EMB-0.156 mg/kg to EMB-0.625 reversed chronic epilepsy induced due to PTZ kindling and maintained a low seizure score from day-1 to day-10. All the animals from PTZ treated group demonstrated disruption in cognitive

functions when analyzed on the three-axis maze model (Figure 3 (II)). Due to the alteration in cognitive functions induced due to PTZ kindling, fish get lost in maze compartment of three-axis maze. The tracking pattern of PTZ administered fish demonstrated complex behavior pattern, as well as the fish, cannot analyze the path towards the feeding ring and hence spend more time in each compartment. As seen in figure 2, the time is taken, and distance traveled by PTZ kindled fish to reach the feeding ring was higher as compared to the control group. Simple and straight tracking pattern was observed in the control group which might be due to the continuous daily trial of control fish in three axis maze for 10 days. As the fish was daily treated with a single dose of EMB prior to PTZ administration, EMB ameliorated the epileptic seizure generated from PTZ kindling and improved the locomotor tracking pattern. EMB pre-treated fish with a dose ranging from 0.156 to 0.625 mg/kg demonstrated simple and improved tracking pattern similar to the control group at 10^{th} day of three-maze trial shown in figure 3. Due to PTZ kindling effects, epileptic fish exhibited loss in memory function which is detrimental for egocentric navigation. EMB (0.156 mg/kg - 0.625mg/kg) significantly improved maze navigation times and reduced performance errors in three-axis maze with P-value significant *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.

3.3. Estimation of neurotransmitters by LC/MS-MS:

Neurotransmitters analysis was performed to check the amount of chemical change in the brain after chronic epilepsy treatment. Neurotransmitter analysis by LC/MS-MS demonstrated a significant reduction ($^{*}P < 0.05$) in the level of GABA in the PTZ administered group when compared to the control groups. Significant elevation in the level of GABA was observed on the EMB (0.156 mg/kg) ($^{**}P < 0.01$) and the EMB (0.625 mg/kg) ($^{*}P < 0.05$) administered groups when compared to the PTZ group (Figure 4). The level of glutamate was non-significantly different

between the control group as compared to the PTZ treated group. However, there was a significant elevation in EMB (0.156 mg/kg) ($^{**}P < 0.01$), EMB (0.312 mg/kg) ($^{*}P < 0.05$) and the EMB (0.625 mg/kg) ($^{**}P < 0.01$), administered group when compared to PTZ injected group. However, the brain Ach level was significantly decreased on the PTZ administered group as compared to the control group. In addition, there was significant upregulation in the level of Ach in EMB (0.156 mg/kg) ($^{***}P<0.001$) and the EMB (0.625 mg/kg) ($^{***}P<0.001$) treated group when compared to the PTZ treated group shown in figure 4.

3.4. EMB pre-treatment, reverse epilepsy that affects expression levels of several inflammatory genes

CCL2 mRNA expression level was non-significantly upregulated in the PTZ treated group when compared with the control group. However, CCL2 mRNA expression level was significantly down-regulated in EMB (0.156 mg/kg) ($^{*}P < 0.05$) and the EMB (0.312 mg/kg) ($^{*}P < 0.05$) treated group as compared to PTZ administered group. In addition, there was non-significant downregulation in the CCL2 mRNA expression level on EMB (0.625 mg/kg) treated group when compared to the PTZ treated the group as shown in figure 5A.

TLR4 mRNA expression was significantly ($^{***}P<0.001$) decreased in the control group when compared to the PTZ treated group. When compared to PTZ administered group, TLR4 mRNA expression level was decreased in EMB (0.312 mg/kg) ($^{*}P < 0.05$) and the EMB (0.625 mg/kg) ($^{**}P < 0.01$). However, non-significant downregulation of TLR4 mRNA expression level was observed in EMB (0.156 mg/kg) treated group when compared to the PTZ treated the group as shown in figure 5B.

TNF-α mRNA expression was non-significantly upregulated in the control group as compared to the PTZ treated group. However, no significant difference was observed in the TNF-α expression

levels in all the EMB (0.156, 0.312 and 0.625 mg/kg) treated group when compared to PTZ treated group as shown in figure 5C.

IL-1 mRNA expression was found to be significant ($^{***}P<0.001$) down-regulated in the control group when compared with the PTZ administered group. An IL-1 mRNA expression level was significantly ($^{***}P<0.001$) down-regulated in EMB (0.156 and 0.312 mg/kg) treated the group as compared to the PTZ treated group. However, non-significant downregulation of the IL-1 mRNA expression level was observed in EMB (0.625 mg/kg) treated group when compared to the PTZ treated the group as shown in figure 5D.

Significant ($^{*}P < 0.05$) downregulation in the level of INF-γ mRNA expression was observed in the control group as compared to the PTZ treated group. However, INF-γ mRNA expression was found to be significantly downregulated in EMB (0.156mg/kg) ($^{*}P < 0.05$) and the EMB (0.312mg/kg) ($^{**}P < 0.01$). (the treated group as compared to the PTZ treated group. In comparison with PTZ administered group, non-significant downregulation in INF-γ mRNA expression level was observed in EMB (0.635 mg/kg) treated the group as shown in figure 5E.

4. DISCUSSION:

The development of new drug treatment for chronic epilepsy induced cognitive dysfunction has largely stalled with very minor advances over the past few decades (Witt and Helmstaedter, 2017). In fact, with the concern in epilepsy treatment with standard AEDs, more than one-third of patients with epilepsy develop a cognitive problem (Witt and Helmstaedter, 2017). As per the current study, our focus on a multifaceted approach of developing chronic epilepsy induced memory impairment model in zebrafish using PTZ kindling was found to be successful in comparison with KA administration in three-axis maze. Our results also describe the evaluation of the ameliorative potential of EMB against PTZ kindled chronic epilepsy and related cognitive alterations using adult zebrafish as an experimental model. In addition to that, modulation of several neurotransmitters and inflammatory genes by EMB treatment of varying doses (0.156, 0.312 and 0.612 mg/kg) has opened new therapeutic approach to deal with epilepsy and related memory problems (van Vliet et al., 2018).

Chronic epilepsy is a complex brain disorder exhibiting multiple underlying knowns and unknown causes with poorly understood mechanisms (Staley, 2015). Impairment of learning and memory is frequently observed in long-term epileptic patients and chronic epilepsy (Paudel et al., 2018b). To the best of our knowledge, current options to treat epilepsy only help with seizures and however they did not help to improve cognitive impairment, or standard AEDs contribute to impaired memory in patients with epilepsy (Santulli et al., 2016). In relation to that, current investigation shed light on the development of natural product-based novel therapy of using pro-inflammatory targets that can ameliorate chronic seizure as well as associated cognitive alterations (Barker-Haliski et al., 2017).

In spite of growing interest of utilizing zebrafish as an experimental model, much more remained to learned about the spatial cognitive abilities of zebrafish in epileptic condition as compared to more widely used mammalian species (Meshalkina et al., 2017a). Zebrafish is one of the most emerging model systems to study neurologically related disorders and memory function (Fontana et al., 2018). Use of zebrafish as a model system is considered to be one of the most highly productive animal models with low cost, ease in doing an in-vivo pharmacodynamic study and easy tracking analysis of behavior study (Khan et al., 2017). Recently the large number of studies related to PTZ-kindling are conducted on rodents which have high cost and is a time-consuming procedure (Kumar et al., 2016; Shimada and Yamagata, 2018). It is also known that once the rodents and large animals are epileptic they become difficult to handle, which is exactly opposite to zebrafish model (Meshalkina et al., 2017b). Zebrafish are easy to handle and injection, they are cost-effective, easy to maintain and feed, can adapt to new condition quickly and produce a robust accurate result (Kearney, 2018). Moreover, zebrafish as an experimental model has been significantly important in the investigation elucidating epilepsy-related cognitive alterations (Stewart and Kalueff, 2012; Kundap et al., 2017a).

Seizure-like behavioral and neurophysiological responses can be evoked in adult zebrafish by various genetic modification, pro-convulsive chemical and that collectively strengthen the growing utility of this model for studying epilepsy (Kalueff et al., 2014). Moreover, learning and memory function can be tested in the zebrafish using various types of mazes like T or Y axis maze, light/color preference test and three-axis maze (Nasir et al., 2012a). In the neurological translational research, many advances have been made in understanding CNS and epilepsy-related problems, but there is still a lack of an animal model that can fully recapitulate the clinical phenotypes of human epilepsy-related cognitive dysfunction. In this regard, there is an increased

understanding about the usability of zebrafish as an animal model in epilepsy research and has been demonstrated the features of human epilepsy (Mussulini et al., 2013; Mussulini et al., 2018).

PTZ (80 mg/kg) kindling act via $GABA_A$ receptor induce epileptic seizures and disrupts the cognitive function for 10 days in adult zebrafish as observed on three-axis maze test. Repeated administration of PTZ produces chronic epilepsy like condition in zebrafish along with the significant loss of cognitive performance in one of the three-axis maze which is considered to be one of a complex maze. Repeated administration small doses of PTZ (80 mg/kg) successfully induce chronic epilepsy like condition at least for 10 days as evidenced by increased seizure score. These findings were in agreement with an earlier reported study conducted using PTZ in rat model reporting impaired memory and epileptic seizures (Golechha et al., 2010). Similar findings were reported earlier regarding PTZ kindling in rodents, where administration of PTZ resulted in a progressive increase in sensitivity of epileptic seizures (Löscher, 2017). Similarly, in the present study, a single dose of KA (3 mg/kg) failed to maintain the progressive increase in seizure sensitivity throughout the experiment. This finding was different from the findings from rodents where a single high dose of KA produces full-blown seizure (Demars et al., 2018). This might be due to the virtue of zebrafish and its regenerative property which regenerates the damage neurons and makes the fish less sensitive (Ceci et al., 2018). These findings implicate that a single dose of KA (3 mg/kg) lack produce long term sensitivity in zebrafish for epileptic seizures.

The cognitive behavior of PTZ treated fish showed that latency to reach the feeding ring was high in PTZ treated fish as compared to the control group. It might be since PTZ treated fish could not find its way to the feeding ring and gets repeatedly lost and backtrack to the previous compartment instead of moving forward to the feeding ring. This implicates that PTZ kindling impairs memory in adult zebrafish. Interestingly, latency to reach the feeding ring in KA-treated

fish was less than PTZ treated group but was higher than the control group. This implicates that though a single dose of KA (3 mg/kg) does not maintain chronic epilepsy like a stage for 10 days, and it still manages to disrupt the memory function for 10 days. All these findings are in agreement with the earlier findings reporting cognitive alteration in epileptic conditions (Black et al., 2010; Leeman-Markowski and Schachter, 2016). We observed that pre-treatment with different EMB doses rescued the fish from the epileptic stage as well as ameliorate its cognitive function. In addition, pre-treatment also reduces seizure scores and seizure intensity until day 10 as compared to PTZ treated fish. This finding strengthens the usability of EMB against chronic seizure induced cognitive disruptions. However, evaluating the ameliorative potential of EMB against ranges of seizure model and related cognitive impairments would further verify this statement.

Neurotransmitters play a crucial role in regulating neuronal excitation and maintaining normal behavior of the cognitive function (Moavero et al., 2017). Hypoactivity GABA leads to the increase in higher dopamine release, causing dopaminergic neurons to influence GABAergic system to shut down through $GABA_A$ receptor (Werner and Coveñas, 2011). Moreover, glutamate hyperactivity influence by n-methyl-D-aspartate (NMDA) receptor, inhibit serotonin release via postsynaptic glutaminergic receptors, which can induce epileptic seizures (Lewerenz and Maher, 2015). In the current investigation, in EMB treated group the level of GABA was found to be significantly rescued back to control level as compared to PTZ treated group implicating the release of dopamine causing epileptic seizures. As well as, an increase in the level of GABA in EMB treated group helps in controlling epileptic seizures. On the other hand, the level of glutamate was found to be elevated in EMB treated group in comparison to PTZ treated group, however, it does not affect epileptic behavior because the inhibitory GABA was controlling the hyperexcitation of neurons from glutamate (Medrihan et al., 2014). Ach has a crucial role in

589 regulating attention, learning and short-term memory (Klinkenberg et al., 2011). Moreover, Ach
590 has been implicated in controlling the release of glutamate in the brain during epileptic activity
591 (Belousov et al., 2001), Current study documented the increased level of Ach in EMB treated
592 group compared to PTZ treated group. Current study reported a similar line of neurotransmitters
593 level as observed on our earlier studies after PTZ administration (Kundap et al., 2017b).

594 The alteration of neurotransmitter is closely associated during the epileptic event. Many
595 studies have shown the change in inhibitory neurotransmitter levels like GABA goes high in the
596 epileptic brain (Swaminathan et al., 2018). The study suggests that proinflammatory biomarkers
597 contribute to regulation of epilepsy-associated biochemical changes CNS. The neuroinflammatory
598 markers like IL-1 TNF-α and INF-ϒ play important role in regulating GABA expression and
599 transportation of extracellular GABA in the brain (Su et al., 2015). TLR-4 and RAGE
600 inflammatory pathway is stimulated by one of the most important pro-inflammatory cytokines
601 knows as HMGB1, which is responsible for the release of glutamate and causing hyperexcitability
602 of the brain during epilepsy (Paudel et al., 2019).

603 There is an increased understanding of the role of inflammation in epileptic seizure
604 (Vezzani and Granata, 2005; Vezzani et al., 2013). Inflammatory mediators are be produced by
605 glia, neurons, endothelial cells of the blood-brain barrier (BBB), and peripheral immune cells and
606 might contribute to the onset and perpetuation of seizures in a various type of epilepsies (LIU and
607 HUANG, 2011). Induction of recurrent seizures or single prolonged seizures by chemoconvulsants
608 or electrical stimulation triggers rapid induction of inflammatory mediators in brain regions and
609 upregulates CCL2 expression (Vezzani et al., 2011). Moreover, findings are emerging implicating
610 the same inflammatory pathways in epilepsy and related neurobehavioral comorbidities including
611 cognitive dysfunctions (Mazarati et al., 2017; Paudel et al., 2018b). Current study reported an

increased level of CCL2 in PTZ kindled group. These findings are in corroboration with earlier findings reporting upregulation of CCL2 in the epileptic brain (Bozzi and Caleo, 2016). Interestingly, EMB treatment reduces the expression level of CCL2 as compared to PTZ treated group. These findings are in agreement with the earlier findings reporting anti-inflammatory activities of EMB (Lee et al., 2018). These results are in line with the recent finding of anti-inflammatory activity of EMB in A549 cells and epithelial cell line (Ahmed, 2017).

TLR4 is the principal receptor for HMGB1 and has been implicated in seizure generation (Iori et al., 2017). The contribution of TLR4 in seizure generation is more than that of RAGE (Iori et al., 2013). This makes TLR4 worth exploring against several seizures models in order to elucidate the contribution of TLR4 in seizure generation. Similar to earlier findings (Maroso et al., 2010), we observed an increased level of TLR4 in PTZ administered group suggesting its role in a seizure. EMB pre-treatment reduces the expression level of TLR4 which might be due to the prevention of epileptic seizure by EMB pre-treatment. The increased level of HMGB1 and TLR4 after pro-convulsant administration in current investigation supports the notion that HMGB1-TLR4 signaling may contribute to generating and perpetuating seizures (Maroso et al., 2010).

The effect of TNF-α on seizures depends mainly on its endogenous brain levels and the receptor subtypes predominantly stimulated by this cytokine (Vezzani and Granata, 2005). Few studies have reported the seizure inhibiting the activity of TNF-α mainly via p75 receptors. Generally, the levels of TNF-α found to be elevated in seizure conditions, either in rodents (Ashhab et al., 2013) or in adult zebrafish (Choo et al., 2018). Surprisingly, currently observed a non-significant decrease in the level of TNF-α after PTZ administration. However, this finding is in corroboration with the earlier reported study, reporting a decrease in TNF-α levels even after the pro-convulsant (pilocarpine) administration in rats (Marchi et al., 2007).

635 It was reported that IL-1 mediates upregulation of NMDA receptor in PTZ induced seizures
636 causing epileptogenesis in the rat model (Yi et al., 2017). In an experimental model of status
637 epilepticus (SE) triggered by electrical stimulation, brain mRNA expression level of IL-1 has been
638 elevated (De Simoni et al., 2000). In a similar line, brain mRNA expression level of IL-1 was
639 upregulated after PTZ administration. On the other side, EMB pre-treatment have reduced the
640 brain mRNA expression level of IL-1 suggesting its plausible anti-inflammatory potential.

641 IFN-γ plays an important role in the development of the brain's excitatory seizure pathways,
642 and it has been associated with the development of limbic seizures (Borham et al., 2016). IFN-γ
643 has been implicated in the seizure generation; this makes IFN-γ worth exploring in the current
644 investigation. We observed an increased level of IFN-γ after PTZ administration, which is in
645 support of earlier study reporting elevated IFN-γ level in epileptic condition (Mao et al., 2013).
646 Interestingly, EMB pre-treatment decreases the level of IFN-γ which might be since EMB pre-
647 treatment prevents the epileptic seizure which in turn helps in preventing neuroinflammation.
648 These overall findings from the current study suggest a possible anti-inflammatory potential of
649 EMB in addition to seizure retarding and related memory improving potential. More studies
650 describing the fact that embelin in the absence of PTZ or seizures can reduce neuroinflammation
651 can be the point of interest for further researchers.

652 **5. CONCLUSION:**

653 Herein, we conclude that embelin suppresses seizure-like behavior & improved cognitive function
654 altered due to chronic epilepsy condition. Embelin significantly modulates inflammatory genes
655 affected by seizure and also affect neurotransmitter levels which eventually improves the cognitive
656 status of the animals. Moreover, behavioral observation, and neurotransmitter analysis and gene
657 expression results suggest that embelin can reverse learning and memory dysfunction associated

with chronic epilepsy in a zebrafish model. Though we developed a KA-induced seizure model, we did not assess the ameliorative effect of embelin against KA-induced seizure and related cognitive alterations as single KA dose did not produce chronic epilepsy like behavior similar to PTZ kindling. We acknowledge this part as the limitation of the current study. However, overall findings suggest that embelin has a potential therapeutic value for the management of epilepsy and related cognitive alterations in experimental studies. However, further work is highly recommended to evaluate the therapeutic effects of embelin against genetic models and to compare its efficacy with current AEDs.

6. ETHICS STATEMENT:

The experimental protocol was approved by the MARP Animal Ethics Committee, Monash University, Australia (MUM/2017/03 and MARP/2017/003).

7. AUTHOR CONTRIBUTIONS:

UPK performed most of the experimental procedure along with an analysis of the results and, writing of the manuscript. YNP contributed to manuscript writing, result in analysis and proofreading. YK contributed to the gene expression study and result analysis. IO contributed to LC-MS/MS study and result analysis. MFS contributed in designing of the study, result analysis, writing and proofreading of the manuscript.

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9. CONFLICT OF INTEREST:

The authors would like to declare no conflict of interest.

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925

926 **Figure legends**

927 **Figure 1: Experimental setup and design procedure.** The flowchart represents the scheme for
928 EMB treatment, PTZ administration and behavior recording for epilepsy and three-axis maze used
929 in the study.

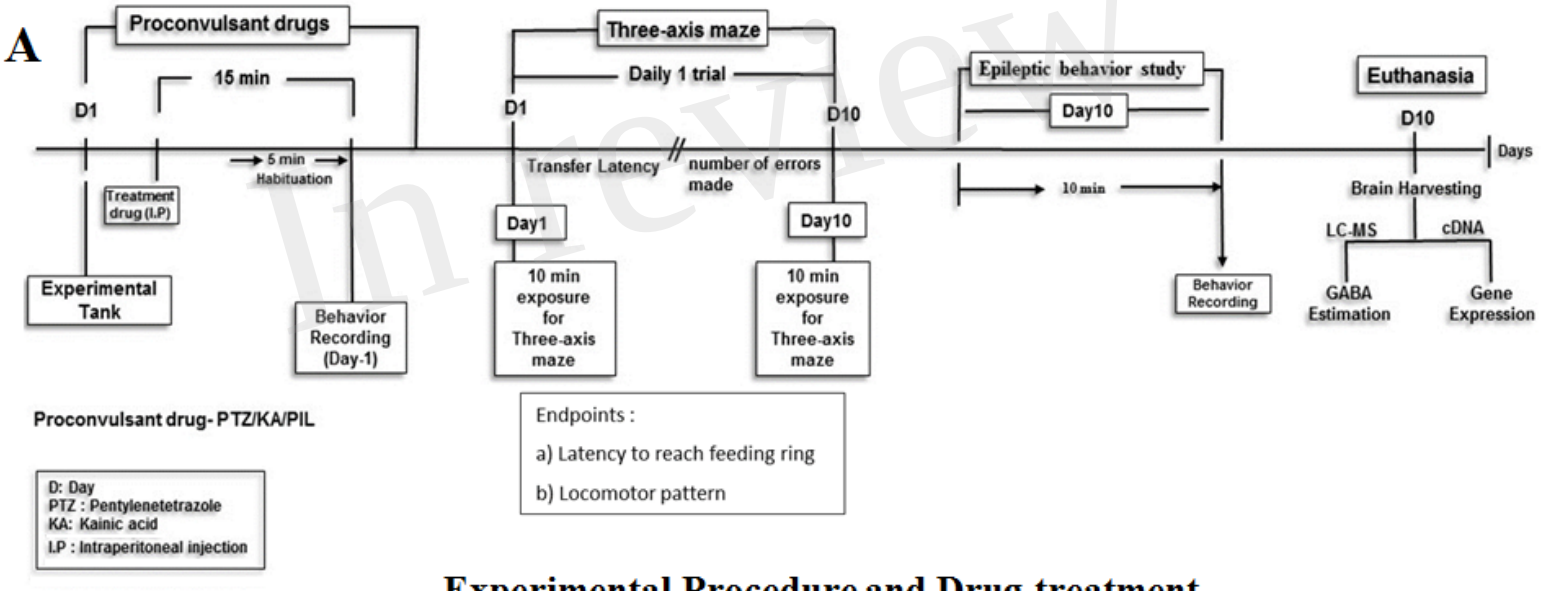
930 **"Figure 2: Daily administration of PTZ induces chronic epilepsy in adult zebrafish.** (AI)
931 Represents the seizure progression pattern of PTZ and KA from day-1 to day-10 with F value of
932 413.7, DF-18 and mean square - 19.41. (AII) represents transfer latency, the time taken by PTZ
933 and KA-treated fish against a control group. PTZ (80 mg/kg) and KA – 3 mg/kg significantly
934 increase the time taken to reach the feeding ring. The data were analyzed by GraphPad Prism
935 v.7.02 (San Diego, CA) with repeated measure (mixed model) Two-way ANOVA followed by
936 Bonferroni post-test to compare replicated means by row. Data are presented as means and
937 standard errors of the mean (SEM) to assess the differences in seizure, latency, and inflexion ratio.:
938 with F value of 22.24, DF-18 and mean square – 70622 *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. (B)

939 Locomotor tracking pattern of a single representative fish in a treated group with the proconvulsant
940 drug at day-10 in three-axis maze.
941
942 **Figure 3: Daily administration of EMB ameliorates PTZ seizures and memory decline.** (AI)
943 Represents the seizure progression pattern of EMB against PTZ kindling from day-1 to day-10
944 with F value of 106.4, DF-36 and mean square – 5.489. (AII) Represents transfer latency, the time
945 taken by EMB treated fish against PTZ treated fish. (AIII) Represents the inflexion ratio of EMB
946 treated group compared to the PTZ treated group. The data were analyzed by GraphPad Prism
947 v.7.02 (San Diego, CA) with repeated measure (mixed model) Two-way ANOVA followed by
948 Bonferroni post-test to compare replicated means by row. Data are presented as means and
949 standard errors of the mean (SEM) to assess the differences in seizure, latency and inflexion ratio
950 with F value of 9.850, DF-36 and mean square- 41046, *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. (B)
951 Locomotor tracking pattern a single representative fish in a treated group with EMB against PTZ
952 treated, control and PTZ negative control at day-10 in a three-axis maze.
953
954 **Figure 4: Neurotransmitters analysis in zebrafish brain after 10-days of three-axis maze**
955 **trial.** (A) Represents the concentration of GABA in the zebrafish brain. (B) Represents
956 concentration of glutamate in the zebrafish brain with F-value = 3.976, R-square = 0.4429, P-value
957 = 0.0156. (C) Represents the ratio of glutamate over GABA showing a significant increase in
958 glutamate levels against PTZ treated group with F-value = 5.5.4, R-square = 0.5240, P-value =
959 0.0037. (D) Represents concentration of acetylcholine (Ach) in the zebrafish brain with F-value =
960 41.86, R-square = 0.9331, P-value = 0.0001. In each neurotransmitter analysis, all the control and
961 EMB treated groups are compared with negative control PTZ treated group. Data are represented

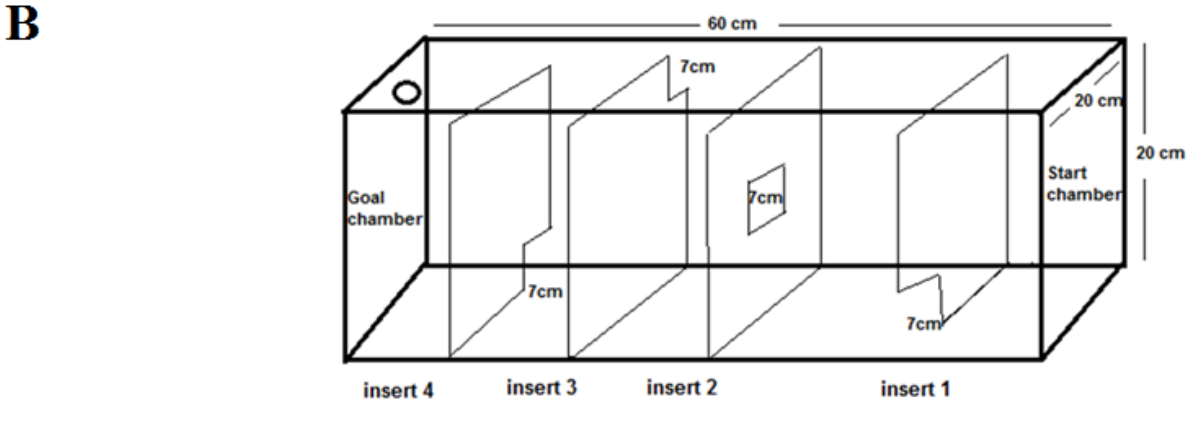
as Mean ± SEM, n=5 and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.

Figure 5: EMB pre-treatment, reverse epilepsy that affects expression levels of several inflammatory genes. (A) Represents graph plot for CCL2 mRNA expression in the zebrafish brain with F-value = 3.564, R-square = 0.4712, P-value = 0.0292. (B) Represents graph plot of TLR-4 mRNA expression in the zebrafish brain with F-value = 12.98, R-square = 0.7644, P-value = 0.0001. (C) Represents graph plot for INF-γ mRNA expression in the zebrafish brain with F-value = 5.414, R-square = 0.5751, P-value = 0.0059. (D) Represents graph plot of IL-1 mRNA expression levels in the zebrafish brain with F-value = 12.4, R-square = 0.7121, P-value = 0.0001. (E) Represents graph plot of TNF-α mRNA expression in the zebrafish brain with F-value = 1.127, R-square = 0.2198, P-value = 0.3788. In each gene, all the control and EMB treated groups are compared with negative control PTZ treated group. Data are represented as Mean ± SEM, n=5 and statistically analyzed by one-way ANOVA followed by Dunnett's test *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001."

Figure 1.TIF

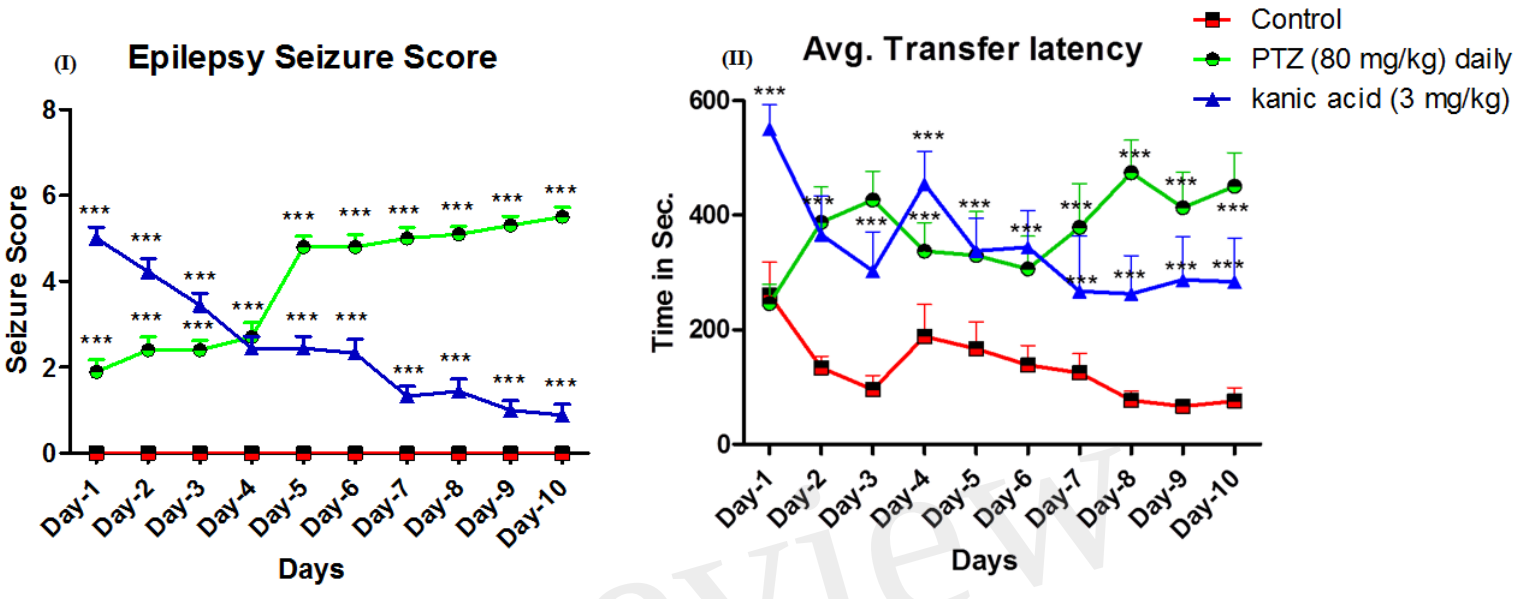


Experimental Procedure and Drug treatment



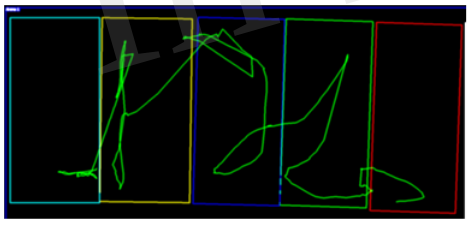
Three-axis maze

A

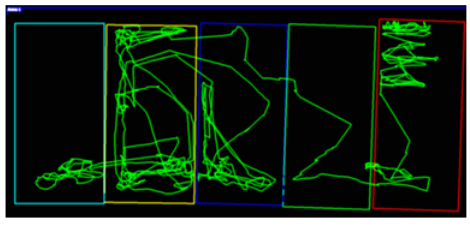


Chronic epileptic seizure score and Transfer latency of proconvulsant drugs

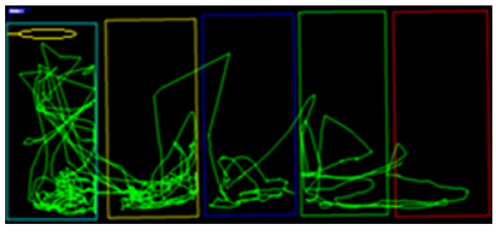
B



Vehicle Control



PTZ (80mg/kg) - 10days

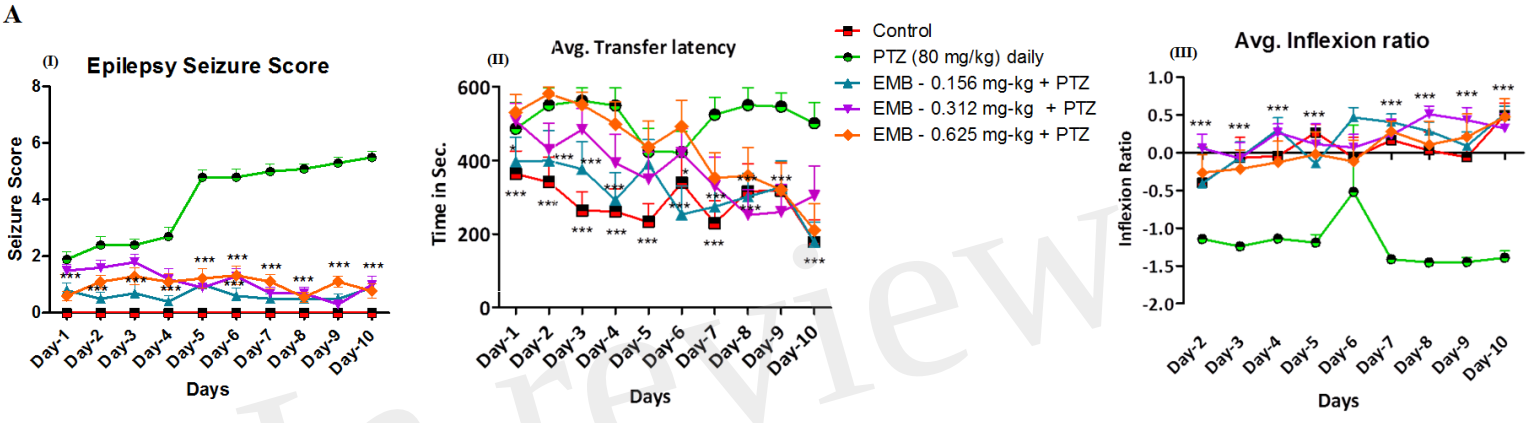


KA (3 mg/kg) - single

Locomotor tracking pattern in three-axis maze

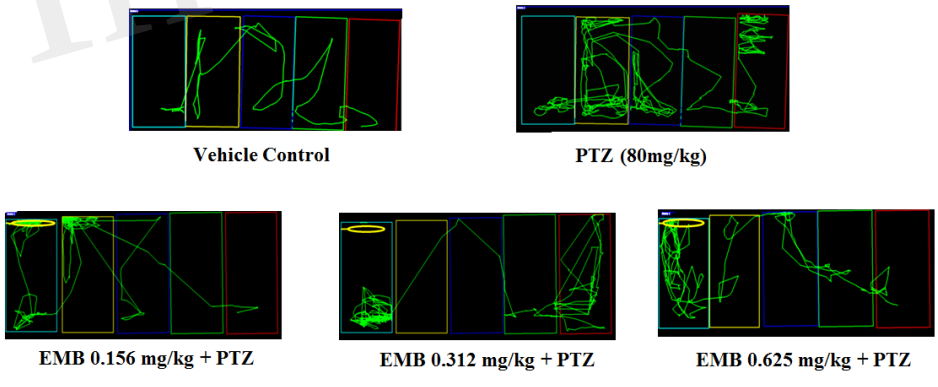
Figure 3.TIF

A



Seizure Score analysis and Transfer Latency

B



Locomotor tracking pattern in three-axis maze

Figure 4.TIF

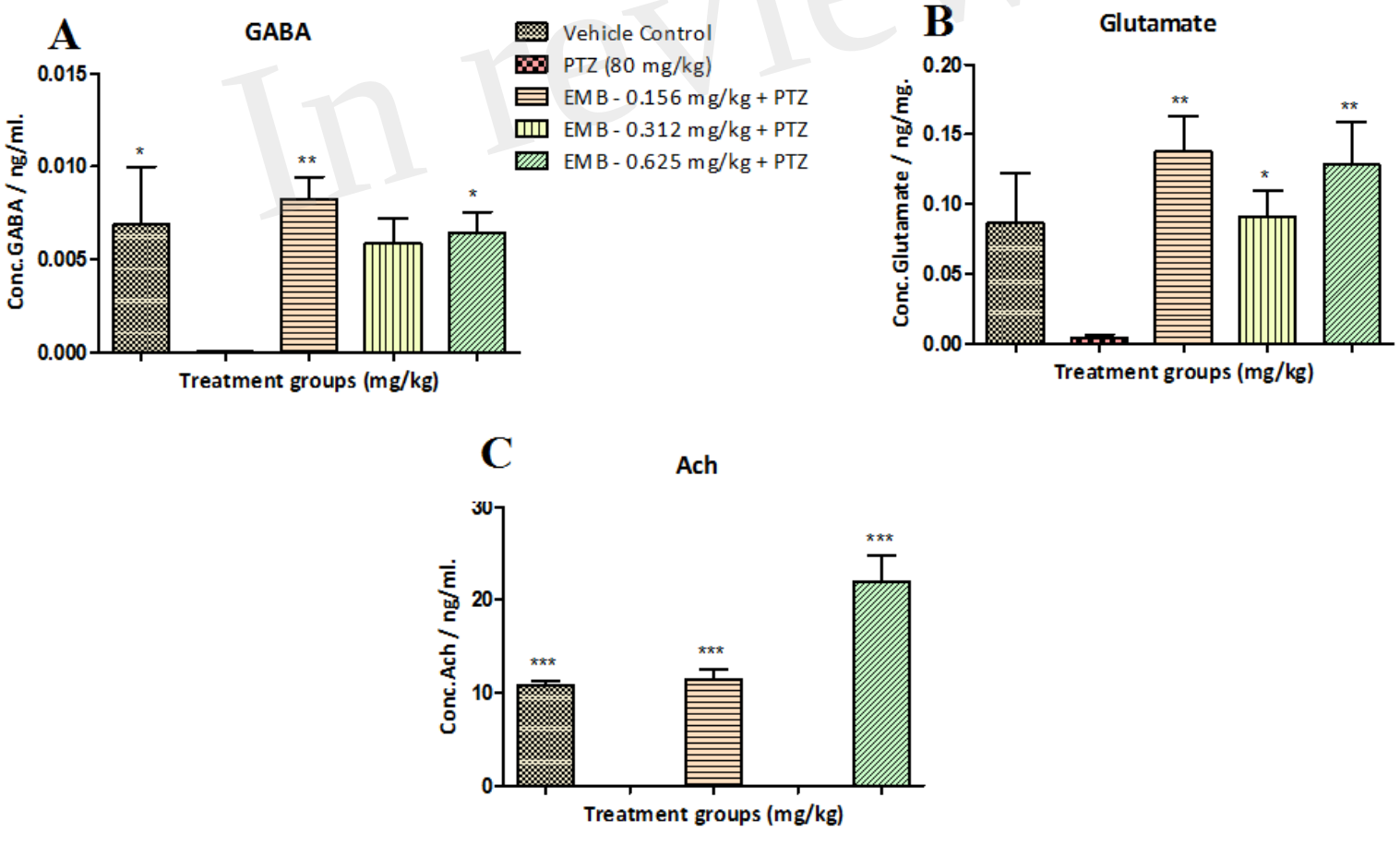
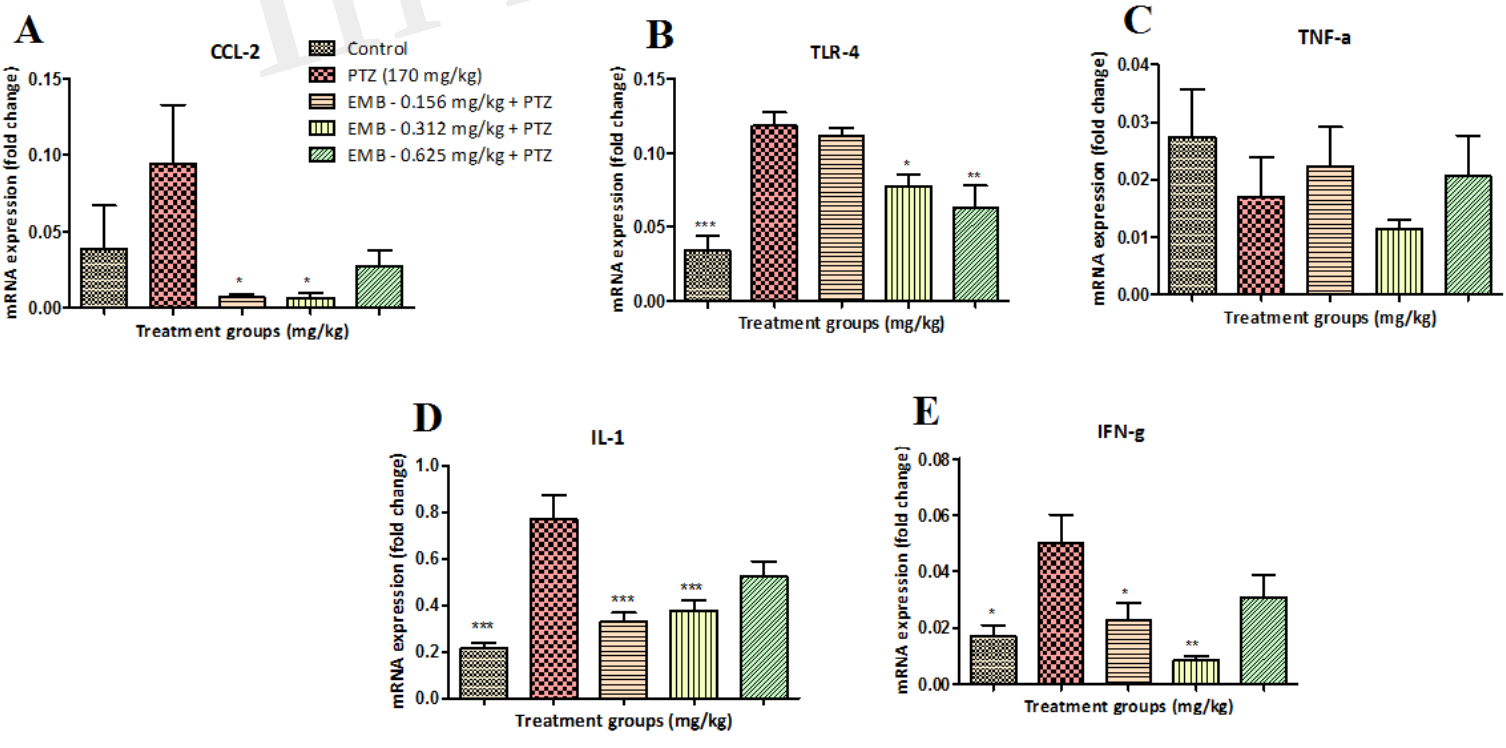


Figure 5.TIF



Discussion

Integrated discussion and future work:

Epilepsy is a neurological condition with complications associated with diverse neurobiological and behavioral alterations characterized by recurrent spontaneous epileptic seizures (Sourbron et al., 2017). The uncontrolled firing of neurons produces involuntary movements all over the body, which are considered as seizures (Berg et al., 2010). According to the revised definition of epilepsy by the International League Against Epilepsy (ILAE) in 2014, epilepsy can also be considered to be present after one or more unprovoked seizures occurring more than 24 hours apart in a person who has other factors that are associated with a high risk of seizure recurrence (Fisher et al., 2014).

Cognition refers to a range of high-level brain functions, together with the ability to calculate, remember and learn information (Kleen et al., 2010). Impairment of cognitive functions such as memory and learning has been repeatedly observed in epileptic patients (Lagae, 2006). It can be better argued that both epilepsy and AEDs in combination affect the neurotransmitter functions necessary for cognitive processes (Kleen et al., 2010). Adverse effects on cognition is a major issue associated with AEDs (Gayatri and Livingston, 2006). An abnormal alteration of neurotransmitters has also been found to be closely associated with many neurological diseases including epilepsy (Park and Kwon, 2008).

For investigating the cause and pathology of human disease, animal models are considered as a useful tool (Löscher, 2017). It is better known that such models can never represent the complete pathology that is observed in human diseases. To develop an animal model for brain disorders, particularly epilepsy, is very difficult because of the disease complexity and variation among species (Vandamme, 2014). However, by using a number of different techniques and parameters in zebrafish, we can incorporate the unique feature of specific disorders to study the molecular and behavior basis of the disease (Stewart et al., 2014).

Herein, we suggest that EMB could be a promising candidate against epilepsy induced learning and memory dysfunction against acute and chronic seizure induced memory problems. EMB modulates the neurotransmitters and genes which are responsible for reversing epileptogenesis and memory loss in zebrafish. It was found to exhibit neuro-protection as well as induced neuronal migration and differentiation in the zebrafish brain. Repeated small dose of PTZ (80mg/kg) (kindling) for 10 days produced chronic epilepsy like condition in adult zebrafish. EMB was reported to reduce epileptic seizures and improve long term memory as evident by three-axis maze test. EMB demonstrated an anti-inflammatory

effect by downregulating several inflammatory markers (CCL2, TLR4, IL-1 and IFN-γ) in the epileptic brain. Zebrafish were successfully used to model acute and chronic seizure related cognitive dysfunction. Zebrafish was found to be a rapid, cost effective and excellent in vivo animal model to study CNS related disorders.

Although a vast number of activities have been reported with embelin in experimental settings, there has not been a single human study found on embelin that is related to CNS activity. None of the animal experimental outcomes were translated into human clinical research. One of the potential reasons for the nontranslation of research could be a lack of detailed safety and toxicity profiles. Further investigations utilizing different seizure models are warranted that will strengthen the potential of EMB towards clinical translation. Therefore, pre-clinical and clinical trials are required to support the idea that this active compound is safe and efficacious. Once a safety profile has been established, embelin should be taken into clinical trials. As embelin is being studied for a myriad of CNS activities, a controlled human clinical trial will open up new horizons for this promising molecule. The current findings thus shed light on the utilization of plant-based natural compounds against epilepsy and related cognitive impairment. These will help overcome the limitations of mainstream AEDs and will also be an economical option as well.

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Certificate of Analysis

EMBELIN

Batch : Yucca/EM/2017/10/01 **Date :** 05.10.2017

Synonym: EMBELIN; Emberine; Embelic acid; 2,5-dihydroxy-3-undecylcyclohexa-2,5-diene-1,4-dione; 2,5-Dihydroxy-3-undecyl-1,4-benzoquinone

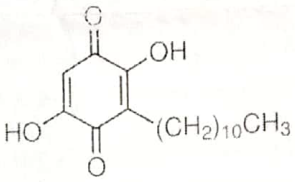
Molecular Formula: $C_{17}H_{26}O_4$

Molecular Weight: 294.39

CAS No.: 550-24-3

Stability: 2 years at -20°C

Molecular structure :

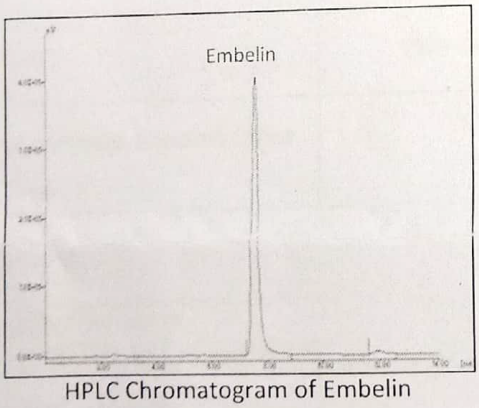


Description : Orange colored powder

Solubility: Soluble in hot organic solvents and in alkali hydroxide solutions; very slightly soluble in petroleum ether; practically insoluble in water.

Moisture content : 1.9 %

Purity by HPLC on dry weight basis : 97.90 %



HPLC Chromatogram of Embelin

Analyzed by [redacted]

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