Journal of Traditional and Complementary Medicine 11 (2021) 318–327

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Original Article

Therapeutic effects of eugenol in a rat model of traumatic brain injury: A behavioral, biochemical, and histological study

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ARTICLE INFO

Article history:
Received 18 August 2020
Received in revised form
5 January 2021
Accepted 5 January 2021
Available online 8 January 2021

Keywords:
Traumatic brain injury
Eugenol
Blood-brain barrier permeability
Brain edema
Neuroprotection

ABSTRACT

Background and aim: Traumatic brain injury (TBI) results in death or long term functional disabilities. Eugenol is demonstrated to be beneficial in a range of experimental models of neurological disorders via its anti-inflammatory and antioxidant properties. Thus, the present study was designed to investigate the neuroprotective effects of eugenol in a weight-drop induced rat model of TBI.

Experimental procedure: Rats were assigned into five groups; control and TBI groups pretreated with vehicle, and three TBI groups pretreated with different doses of eugenol (25, 50, and 100 mg/kg/day, *p.o.*, seven consecutive days). Except for the control, all other groups were subjected to TBI using Marmarou's weight-drop method. 24 h after TBI, locomotor functions and short term memory were evaluated. Lastly animals were scarified and the estimation of lipid peroxidation in brain tissue, blood-brain barrier (BBB) integrity, brain water content (brain edema) and histopathology of the brain tissue were performed.

Results: Weight-drop induced TBI caused functional disabilities in the rats as indicated by impairment in locomotor activities and short term memory. The TBI also resulted in augmented neuronal cell death designated by chromatolysis. The results also showed disruption in the BBB integrity, increased edema, and lipid peroxidation in the brain of the rats exposed to trauma. Pretreatment with eugenol (100 mg/kg) ameliorated histopathological, neurochemical, and behavioral consequences of trauma.

Conclusion: For the first time this study revealed that eugenol can be considered as a potential candidate for managing the functional disabilities associated with TBI because of its antioxidant activities.

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1. Introduction

Worldwide, traumatic brain injuries (TBI) are the foremost cause of death and disabilities in young individuals. The incidences of TBI are increasing yearly. TBI affects the global healthcare system as well as socioeconomic status and is considered to be a silent epidemic. TBI is defined as an impairment of brain physiology resulted from an external mechanical impact.[1] Brain function impairment includes unconsciousness, memory loss, neurologic deficits, sensory loss, and any impairment in the psychological state at the time as well as after the injury.

External mechanical forces are produced from any events that include head striking at an object or being struck by an object, blast or explosion, acceleration/deceleration movement of the brain without undergoing direct blow to the head, road traffic accidents, recreational accidents *i.e.* parachute jumping, sports injuries (boxing, football, etc.), the use of firearms, child abuse and several rare reasons. These have been described as the causes of TBI.[2,3]

Considerable success has been achieved in managing short-term outcomes in victims of severe trauma; however, the functional recovery of patients after severe TBI is still limited.[4] This may be due to an incomplete understanding of the mechanisms involved in the secondary injury. Those patients of TBI who survive commonly suffered from chronic impairment in personality traits, sensori-motor functions, and cognition.[5] For the past several years, several agents have been studied and failed in clinical trials to demonstrate any considerable improvement in functional outcomes of trauma patients.[6] Thus the major challenge to reduce the burden of TBI is the rehabilitation of patients.

The pharmacological and therapeutic interventions of the

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2021.01.003

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List of abbreviations

BBB	Blood-brain barrier
DC	Decompressive craniectomy
HBSS	Hank's balanced salt solution
ICP	Intracranial pressure
MDA	Malondialdehyde
PG	Propylene glycol
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
TBA	Thiobarbituric acid
TBI	Traumatic brain injury
TEP	1, 1, 3, 3–tetra ethoxy propane
TTR	Time to right

chemicals isolated from medicinal plants were reported to have neuroprotective effects and therefore, can be potential candidates for neurological disorders. Eugenol is chemically 4-allyl-2-methoxyphenol. It is found in various kinds of spices and herbs like clove, cinnamon, and nutmeg. It is listed as substances "generally recognized as safe" and approved as a natural food additive by the U.S. Food and drug administration.[7] Eugenol despite its relatively simple molecular structure, has very broad biological protective actions against nephrotoxicity,[8] chronic inflammation,[9] as well as cancer.[10] Eugenol has hydrophobic properties so for that reason when it is taken orally it can pass through the blood-brain barrier (BBB) and can reach the brain. Therefore, it is found to be effective in various neurological disorders. Eugenol is reported to be an anti-stress agent[11] and dampened the activity of neurons and attenuated the epileptic-like activity in cortical slices.[12] Experimental data on rodents suggest that eugenol is protective against neurotoxicity induced by aluminum,[13,14] scopolamine,[15] acrylamide,[16] chlorpyrifos[17] as well as 6-hydroxydopamine.[18] It is also proved to be protective against ischemia-induced brain toxicity in gerbils.[19] Furthermore, eugenol mitigated oxidative stress, inflammation, and neural death and also endorsed functional recovery after spinal cord injury in rats.[20] The neuroprotective effect of eugenol may at least in part be attributed to its anti-oxidative property.

Considering the beneficial effects of eugenol in several studies, the present work aims to study the neuroprotective potential of eugenol in a weight-drop induced rat model of TBI and to understand the underlying mechanisms involved in the probable neuroprotective effect of eugenol.

2. Material and methods

2.1. Drugs, reagents, and solvents

Eugenol, thiobarbituric acid (TBA) and Evans blue were procured from Sigma Aldrich. Hank's balanced salt solution (HBSS) was procured by Thermo Fisher Scientific. TEP (1, 1, 3, 3–tetra ethoxy propane), sodium dodecyl sulphate (SDS), glacial acetic acid, n-butanol, pyridine, phosphate buffer, hydrogen peroxide (H_2O_2) and all the other reagents and solvents were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai. Eugenol is lipophilic and therefore it is insoluble in water. Hence it was suspended in 40% propylene glycol (PG) in phosphate buffer (pH 6.8).

2.2. Animals

Sprague Dawley rats (350–450 g of both sexes) (8–12 weeks of age) were used in the present study. Animals were housed 3 per cage in polypropylene cages lined with husk. Male and female animals were housed separately. The standard environmental conditions (relative humidity of 60 ± 5% and a temperature of 25 ± 3°C) were maintained in the animal room. Additionally, a 10% air exhaust in the air conditioning unit along with a 12 h light/dark cycle was maintained for the experimental animals. Animals were given food and water freely during the experimental period. Animal handling and housing were performed as per good laboratory practice mentioned in the guidelines by committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Department of Animal Husbandry and Dairying, Government of India. All experimental protocols were reviewed and accepted by the Institutional Animal Ethics Committee before the initiation of the experiment (IP/PCOL/MPH/25/2019/006).

2.3. Experimental design

A schematic diagram of the study design and the schedule of treatment are shown in Fig. 1. Sprague Dawley rats of both sexes were taken. Each group consisted of equal number of male and female rats. Animals were randomly assigned into five groups with twelve animals each. The groups were as follows:

Group I: Control (vehicle + no TBI injury).

Group II: TBI (vehicle + TBI injury).

Group III: Eugenol (25 mg/kg) + TBI (eugenol (25 mg/kg/day, *p.o.*, seven days) + TBI injury).

Group IV: Eugenol (50 mg/kg) + TBI (eugenol (50 mg/kg/day, *p.o.*, seven days) + TBI injury).

Group V: Eugenol (100 mg/kg) + TBI (eugenol (100 mg/kg/day, *p.o.* seven days) + TBI injury).

Rats of control and TBI groups received the 40% PG (1 ml/kg, *p.o.*) as a vehicle and the other three groups of TBI received three different doses of eugenol (25, 50, and 100 mg/kg/day, *p.o.*) for seven consecutive days. On the sixth day of vehicle/eugenol treatment, all the groups except control were subjected to weight-drop induced TBI. There were two subgroups for each group (n = 6). Animals of subgroup 1 were utilized for behavioral, biochemical (lipid peroxidation) and histopathological studies while animals of subgroup 2 were utilized for measuring cerebral edema and permeability of the blood-brain barrier.

In subgroup 1, 24 h after TBI, clinical endpoint of time to right (TTR) were measured in all the animals. Finally, on the seventh seventh day after the last dose of the vehicle or drug, alterations in motor coordination and locomotor activity of all the animals were assessed using beam-walking test, actophotometer, rearing test, and rotarod apparatus while the memory was assessed using the Y-maze test. All animals were sacrificed by cervical dislocation and brains were taken out for the estimation of malondialdehyde (MDA) levels which is a marker of lipid peroxidation. Lastly, brain samples were fixed in 10% formalin for histopathological studies.

In subgroup 2, 30 min before exposure to TBI, animals of all the groups were injected with Evans blue (2 % W/V) (2 ml/kg, *i.p*) as per Manaenko et al.[21] Finally, on the seventh day after the last dose of the vehicle or drug, all the animals were anesthetized and transcardially perfused with ice-cold heparinized saline and then all the animals were killed by cervical dislocation and their brains were excised out. The right lob was used for BBB permeability while the left lobe was used to measure brain water content (brain edema).

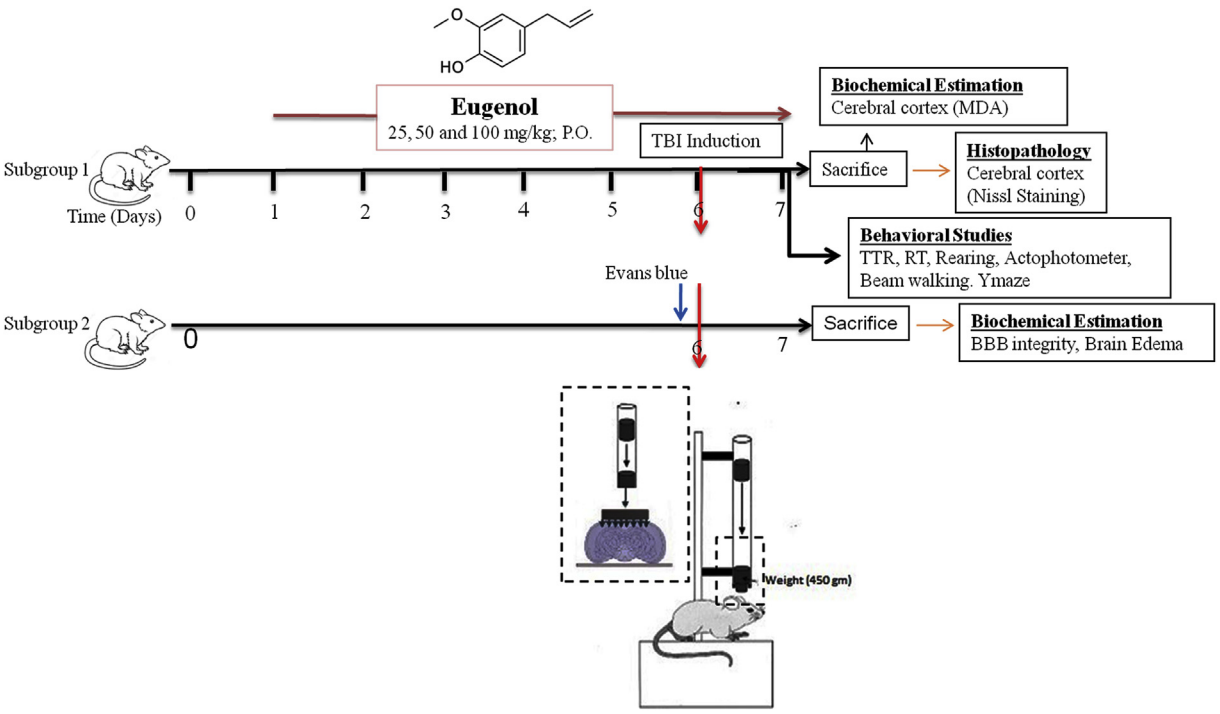

Fig. 1. A schematic diagram of the study design and the schedule of treatment. TBI: Traumatic brain injury; P.O.: per oral; MDA: malondialdehyde; TTR: time to right; RT: rotarod test; BBB: blood-brain barrier.

2.4. Weight-drop induced TBI model

Diffuse close-head TBI was induced in rats by weight-drop model as described previously by Marmarou et al.[22,23] Animals were anesthetized with a thiopentone sodium at a dose of 40 mg/kg (*i.p.*) and maintained with 2% in 98% oxygen. Once anesthetized, the animals were placed in a prone position on a sponge based bed of 6 cm thickness. A midline incision was made on the head, and the coronal and lambdoid sutures were identified. A metallic disc cap of 5-mm diameter and 2-mm thickness was fixed to the cranium between the two cranial sutures and the midline using bone wax. A weight mass of 450 g was securely attached to a string and allowed to fall freely from a height of 100 cm through a tube on to the metallic cap placed over the skull of the rat. The metallic cap avoided the direct impact of weight on the rat's brain. Immediately after the impact on the rat's head, the rat was taken out and metal cap was removed. The surgical area was cleaned, the skin was sutured and topical lidocaine was applied on to the rat's head with a cotton plug. The rat was then placed in a supine position in a cage. Time taken by the rat to wake from the anesthesia and flip from the supine position to prone i.e., TTR was noted down. The animal was then returned to the home cage on achieving normal behavior like grooming, exploring, and walking.

2.5. Assessment of neurobehavioral functions

2.5.1. Actophotometer

The animal locomotor behavior was monitored using an actophotometer as described in the previous report.[24] An actophotometer is an equipment which consists of a photoelectric cell, a digital counter, and a light source. The horizontal locomotor activity of animals can be recorded by employing an actophotometer which principally functions on photoelectric cells that are connected in the circuit with a counter. The circuit is complete when the beam of light falls on the photoelectric cell. When the beam of light falling on the photoelectric cell is cut off by the movement of animals, a count is recorded. Animals were weighed, marked, and then each animal was placed in the instrument for 2–3 min to get acclimatized with the instrument environment. In the main test, animals were kept for 5 min and the readings were noted as activity score.

2.5.2. Rotarod test

The rotarod test is commonly used for the estimation of rat's ability to balance on a rotating rod this gives the idea about the proper motor coordination of rats.[25] The acceleration of the rotation of rotarod leads to increased difficulty level for the rat to keep balance. The rotarod was set to start at an initial speed of 5 rpm and accelerate to 5 rpm over every 40 s or accelerate to 20 rpm over 180 s. Here we measure the time taken by the rat to fall at a range of different speeds. Firstly, training was given to the rat for a maximum of 3 min. Rats were given four trials per day for three days consecutively. On the test day, the rat was placed on the rotarod instrument and allowed to move for 3 min with accelerated speed. The average latency to the first fall off the rod was recorded in sec.

2.5.3. Spontaneous forelimb elevation (cylinder) test

The cylinder test evaluates the spontaneous forelimb use (rearing behavior) of rodents, which can be used to evaluate the sensory-motor function in many injury models that cause altered forelimb use.[26] The cylinder test is a frequently used behavioral assessment to measure the impairment of motor function in experimental models of TBI. The animal moves within a clear glass cylinder with an open and clear top. The forelimb activity of the rat while rearing against the wall of the cylinder is recorded. In the procedure for 5 min, the numbers of rearing activity shown by rats were calculated.

2.5.4. Beam-walking test

Motor function was accessed by using a modified beam-walking

test, as per the earlier reports.[27] The beam-walking task was done to analyze the motor coordination by estimating the locomotor function and loss of balance in rats. The wooden beam has a surface of 2.5 cm in width and 112 cm in length. It was elevated 60 cm above the ground level. A 20 × 25 × 24 cm box with a 10 cm opening was placed at one end of the beam. Before TBI induction, two trials at the interval of 10 min were given to train the rats to transverse on a narrow beam and enter a darkened box placed at the rear end of the beam using stimulation with a loud noise. Once the rat entered the darkened box, the loud noise stimulation was immediately stopped. The beam walking task was performed on the first day of post-TBI and time to transverse the beam is recorded. Longer times to transverse the beam indicates impaired motor function.

2.5.5. Y-maze test

Spatial memory was measured by Y-maze, as previously described by Dellu and colleagues.[28] The Y-maze is used for the estimation of short-term memory of the rats, for the assessment of spatial working memory and spontaneous alternation. In brief, the three arms of Y maze were randomly assigned to the starting arm, the novel arm, and another arm. The Y maze task consists of two phases of 5 min with an interval of 1 h. During the first phase, the novel arm was blocked, and the rats entering the starting arm were allowed to move between the other two arms of the apparatus. During the second phase, the novel arm was opened and the rats entering starting arm could freely move throughout the three arms. The curiosity of rodents to explore blocked or previously unvisited arm or area is estimated. A rat with intact spatial reference memory should enter and spend the majority of time in the blocked arm or the novel arm in comparison to other arms of the maze. Rats with impaired spatial memory or spatial reference memory will not be able to identify the blocked arm or the novel arm as they have lost the memory of which arm was blocked in the first phase of the study. The impairment of memory is signified by lacking in differentiation between the novel and the blocked arms by the rat. The number of entries into the novel arm as well as the percentage of time spent in the novel arm was measured.

2.6. Estimation of lipid peroxidation

MDA is formed as the product of the peroxidation of polyunsaturated fatty acids. Thus MDA level is the marker of the lipid peroxidation. MDA levels in the brain tissues of control and traumatized animals were estimated as described earlier.[29,30] Brain tissue was isolated and the cortex region (100 mg) was transferred to tubes containing 5 ml HBSS buffer and homogenized in 3 cycles of 30 s each at 3000 rpm with a 30 s gap. Tissue homogenate was centrifuged at 3000 rpm for 10 min at 25 °C. The cell pellet was collected while the supernatant was discarded. The cell pellet was transferred into tubes containing 0.2 ml of 8.1 % SDS, 1.5 ml of 20 % acetic acid, 1.5 ml of 0.8 % aqueous solution of TBA, and 0.7 ml of MilliQ water. 0.1 ml HBSS was added in the control tubes instead of homogenate. The tubes were kept in a boiling water bath for 1 h. After boiling, 1 ml Milli Q added to each tube. 5 ml butanol: pyridine (15:1) was added to each tube & vortexed for 5 min. The organic layer was centrifuged at 3000 rpm for 10 min at 25 °C and the amount of MDA formed was measured by absorbance of the upper organic layer at 532 nm. The concentration of MDA was calculated in μM/mg tissue using a standard curve prepared with TEP.

2.7. Histopathological study

The brain tissues were taken out, washed with ice-cold 0.9% saline solution, and fixed in 10% buffered formalin for histopathological studies. Fixative was cleaned from the brain tissues by washing with running tap water. The tissues were again washed and cleaned with methyl benzoate and dehydrated through a graded series of alcohol and finally embedded in paraffin wax. Tissue sections of 5 μm thickness were cut, deparaffinized, and hydrated with water followed by staining with cresyl violet (0.5% W/V) for 3–5 min. After staining tissue sections were dehydrated and fixed by DPX mountant. Sections were studied under a light microscope.

2.8. Evaluation of blood-brain barrier permeability and brain edema

Evans blue dye was extracted from brain samples and measured according to the previously reported method.[31,32] Briefly, the collected brain samples were kept in 0.5 N KOH overnight at 37°C. On the next day, 2.5 ml of a mixed solution of 4 N H_3PO_4 and acetone were mixed in each tube in the ratio of 3:15. The tube was vortexed for 1 min and then centrifuged at the 3000 rpm for 15 min at 25 °C. The absorbance of the Evans blue layer was taken at 620 nm. Evans blue concentration in the brain was calculated using the standard curve prepared from Evans blue. The amount of the dye extracted from the brain was expressed as μM per 100 mg of tissue.

The brain water content method is used to measure the edema that took place in the brain. This edema occurs in the brain due to the breakdown of the BBB leading to the accumulation of the fluid in the brain. The brain water content estimation was done as per the previously reported method.[33] After the rats were sacrificed their brains were excised out and weighted (wet weight) and stored by dipping in absolute alcohol for about 30 min and were kept for 24 h for drying at a temperature of 55±5°C. After 24 h, the brains were reweighted (dry weight) and the brain water content was measured using this equation.

$$\%\,\text{Brain water content} = [(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100$$

The brain water content is expressed as % brain water content per 100 gm body weight of the animal.

2.9. Statistical analysis

Results were expressed as the mean ± standard error mean (SEM) (n = 6). Means were normally distributed. The data were analyzed with the trial version of GraphPad Prism 8 (SanDiego, CA). Data were statistically analyzed by one-way ANOVA, followed by Tukey's test; $p < 0.05$ was considered statistically significant.

3. Results

3.1. Eugenol alleviated the TBI induced increased time to right (TTR) in rats

To test the clinical significance of this TBI model we measured TTR (Fig. 2). Statistical analysis revealed that there were significant differences in TTR value among various groups [$F(4, 29) = 76.83$, $p < 0.0001$]. The TTR was significantly ($p < 0.05$) increased in animals subjected to trauma in comparison to the control group. Eugenol in the doses of 50 and 100 mg/kg significantly ($p < 0.05$) attenuated the TBI induced increase in TTR, while the 25 mg/kg dose of eugenol was found ineffective.

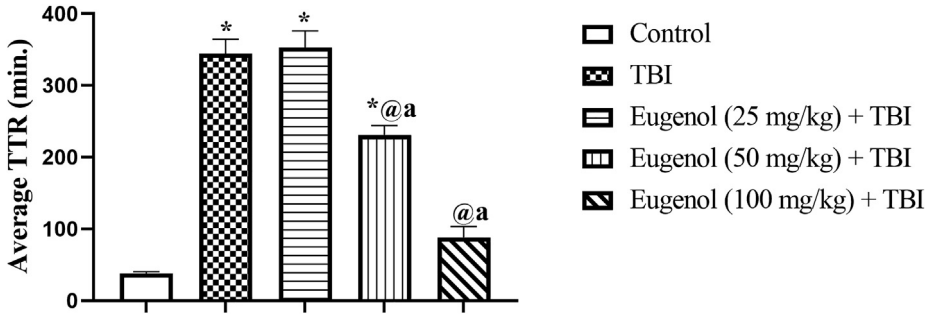

Fig. 2. Effect of eugenol on average time to right (TTR) in a weight-drop induced rat model of TBI. Each bar represents mean ± SEM of six animals per group. *$p < 0.05$ compared to control, @$p < 0.05$ compared to TBI, a$p < 0.05$ compared to eugenol 25 mg/kg using one way ANOVA (Tukey's multiple comparisons test).

3.2. Eugenol enhanced the TBI induced decreased activity score of the rat in actophotometer

Fig. 3(a) illustrates the effect of pretreatment with eugenol on the mean activity score per 5 min as assessed in actophotometer in the weight-drop induced rat model of TBI. Statistical analysis of the results revealed that there were significant differences in mean activity scores among different groups [$F(4, 29) = 12.99$, $p < 0.0001$]. In trauma exposed rats, the mean activity score decreased significantly ($p < 0.05$) compared to the control group. Pretreatment with eugenol in the doses of 50 and 100 mg/kg showed significant ($p < 0.05$) improvement in performance of animals exposed to TBI in actophotometer as compared to the animals of TBI group without eugenol pretreatment.

3.3. Eugenol improved motor coordination of rats exposed to TBI on rotarod apparatus

Fig. 3(b) depicts the effect of pretreatment with eugenol on the motor coordination of rats on rotarod apparatus in the weight-drop induced rat model of TBI. Statistical analysis revealed that there were significant differences in the performance of rats in the rotarod apparatus [$F(4, 29) = 43.22$, $p < 0.0001$] among different groups. Post-hoc test showed a significant ($p < 0.05$) difference in the performance of rats in rotarod between control and animals exposed to trauma. Eugenol pretreatment in a dose of 100 mg/kg had shown significant ($p < 0.05$) improvement in performance of rats exposed to trauma on rotarod apparatus when compared with the vehicle treated trauma group.

3.4. Eugenol improved rearing behavior of rats exposed to TBI

The results of pretreatment with eugenol on the rearing behavior of the rats in the cylinder test exposed to trauma are shown in Fig. 3(c). Statistical analysis revealed that there were significant differences in the rearing behavior of rats in various groups [$F(4,29) = 17.06$, $p < 0.0001$]. In the present study, a significant ($p < 0.05$) drop in the rearing behavior of the rats was observed in TBI in contrast to the control group. Repeated pretreatment with eugenol significantly ($p < 0.05$) reversed the TBI induced changes in rearing behavior only at a dose of 100 mg/kg while other doses were found ineffective.

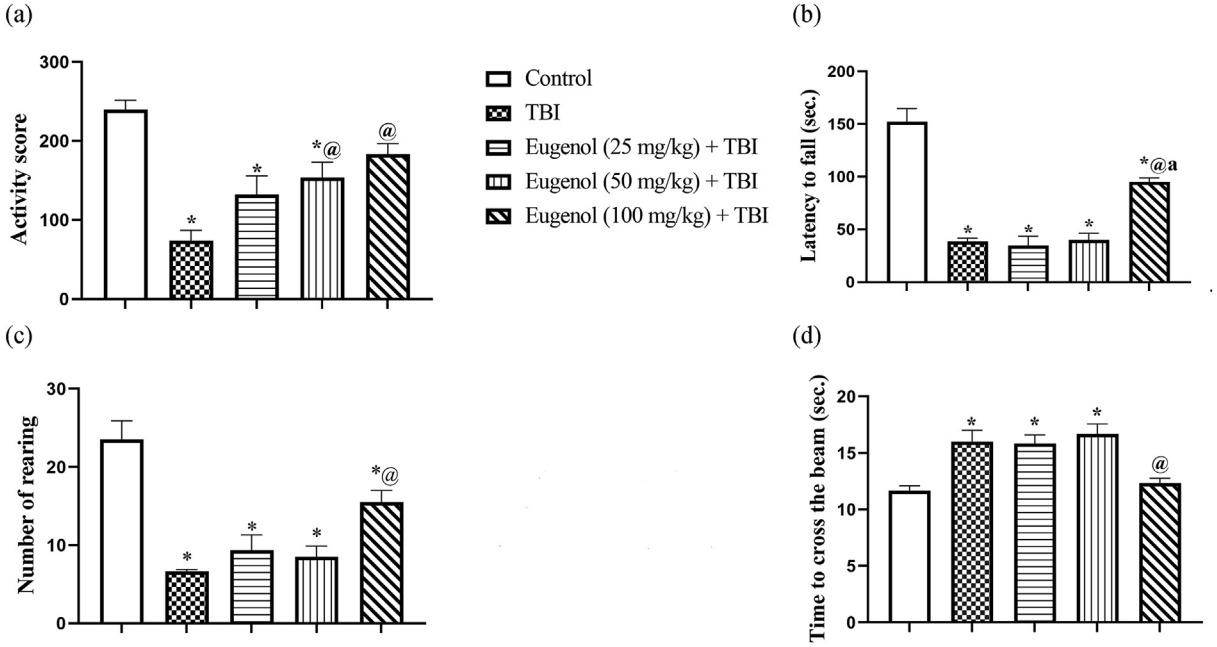

Fig. 3. Effect of eugenol on activity score in actophotometer (a), motor coordination (latency to fall) in rotarod test (b), number of rearing in cylinder test (c) and time to cross the beam in beam-walking test (d) in a weight-drop induced rat model of TBI. Each bar represents mean ± SEM of six animals per group. *$p < 0.05$ compared to control, @$p < 0.05$ compared to TBI, a$p < 0.05$ compared to eugenol 25 mg/kg using one way ANOVA (Tukey's multiple comparisons test).

3.5. Eugenol improved performance of rats exposed to TBI in beam-walking test

Fig. 3(d) represents the effect of repeated eugenol pretreatment on the performance of rats in beam-walking test in the weight-drop induced rat model of TBI. Statistical analysis revealed significant differences in the performance of rats in beam-walking test among various groups [$F(4,29) = 17.22$, $p < 0.0001$]. The time to cross the beam (sec) was found to be significantly ($p < 0.05$) higher in the rats exposed to trauma than obtained from the control. Thus, TBI deteriorated the performance of rats in a beam-walking test. This TBI induced deteriorated performance of rats in beam-walking test was significantly ($p < 0.05$) improved by pretreatment with 100 mg/kg eugenol while other doses were found ineffective.

3.6. Eugenol exhibited anti-amnesic activity in Y-maze task

The results of pretreatment with eugenol on the percentage of time spent in the novel arm in a weight-drop induced rat model of TBI are shown in Fig. 4(a). Statistical analysis of results showed significant differences among various groups [$F(4,29) = 37.82$, $p < 0.0001$]. It was observed from the results that the percentage of time spent in the novel arm significantly decreased in rats of the TBI group pretreated with vehicle and TBI groups pretreated with eugenol at the doses of 25 and 50 mg/kg in comparion to rats of control group. Eugenol pretreatment in a dose of 100 mg/kg significantly ($p<0.05$) improved the TBI induced decrease in the percentage of time spent by animals in the novel arm.

Fig. 4(b) aptly demonstrates the effect of pretreatment with eugenol on the number of entries in novel arm in Y-maze in a weight-drop induced rat model of TBI. Statistical analysis revealed a significant difference in the number of entries in the novel arm of Y-maze among various groups [$F(4,29) = 15.39$, $p < 0.0001$]. Results showed that the number of entries in the novel arm of Y-maze was significantly ($p < 0.05$) decreased in trauma-exposed rats as compared to the control group. Repeated eugenol (100 mg/kg) pretreatment mitigated the TBI-induced decrease in number of entries in the novel arm of Y-maze while other two doses were found ineffective.

3.7. Eugenol mitigated the TBI induced increased lipid peroxidation in rat brain

Fig. 5 aptly demonstrates the effect of eugenol pretreatment on the levels of MDA, a marker of lipid peroxidation. Statistical analysis revealed significant differences in the MDA levels among various groups [$F(4,29) = 32.72$; $p < 0.0001$]. Trauma increased the MDA levels in the brain significantly ($p < 0.05$) while eugenol pretreatment with the doses of 50 and 100 mg/kg effectively mitigated the TBI-induced increase in MDA levels while the dose of 25 mg/kg was found ineffective.

3.8. Effect of eugenol on TBI induced neuronal damage

Nissl staining was performed on brain sections from a control, TBI, and TBI groups pretreated with eugenol (25, 50, and 100 mg/

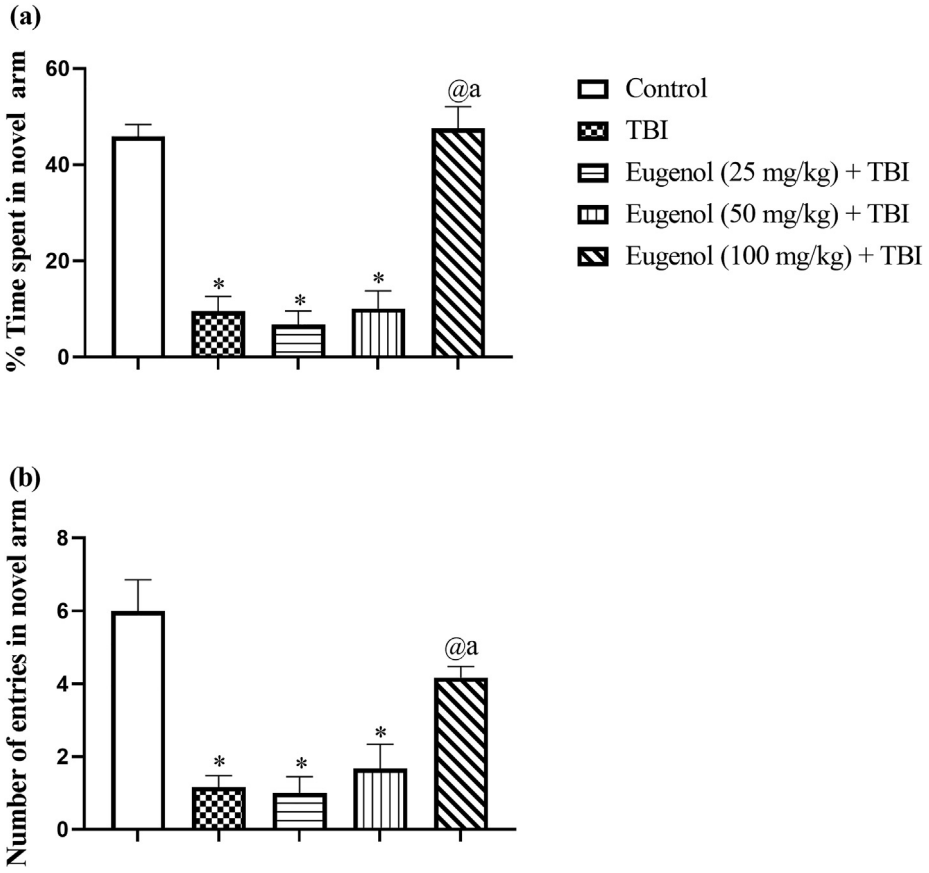

Fig. 4. Effect of eugenol on % time spent in novel arm (a) and number of entries in novel arm (b) in Y-Maze test in a weight-drop induced rat model of TBI. Each bar represents mean ± SEM of six animals per group. *$p < 0.05$ compared to control, $^{@}p < 0.05$ compared to TBI, $^{a}p < 0.05$ compared to eugenol 25 mg/kg using one way ANOVA (Tukey's multiple comparisons test).

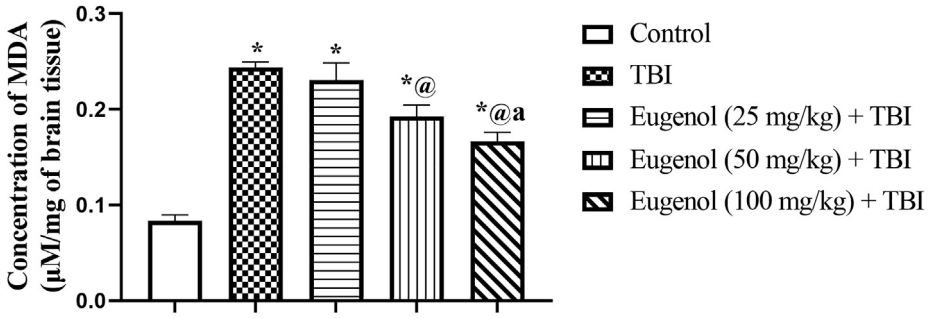

Fig. 5. Effect of eugenol on the concentration of malondialdehyde (MDA) in a weight-drop induced rat model of TBI. Each bar represents mean ± SEM of six animals per group. $^{*}p < 0.05$ compared to control, $^{@}p < 0.05$ compared to TBI, $^{a}p < 0.05$ compared to eugenol 25 mg/kg using one way ANOVA (Tukey's multiple comparisons test).

kg). Sections were stained with cresyl violet. Nissl staining using cresyl violet was done in brain sections for the detection of neuronal death and damage. Cresyl violet specifically stains the Nissl bodies which are large granular bodies in neurons. Chromatolysis (indicated by arrow), *i.e.*, dissolution of Nissl bodies was found in the brain sections of rats of TBI (Fig. 6(b)), eugenol 25 mg/kg + TBI (Fig. 6(c)), and eugenol 50 mg/kg + TBI (Fig. 6(d)) groups. Chromatolysis is an induced response of cells triggered by axotomy, neurotoxicity, cell exhaustion. Chromatolysis is a precursor to apoptosis. However, chromatolysis was absent in the eugenol 100 mg/kg + TBI group (Fig. 6(e)). Thus, eugenol in the dose of 100 mg/kg decreased the neuronal death.

3.9. Effect of eugenol on blood-brain barrier integrity in the TBI model

The effect of eugenol pretreatment on the Evans blue concentration in the right hemisphere of the brain was studied and the results were shown in Fig. 7. Significant differences were observed in the Evans blue concentration among the groups [$F(4, 29 = 293.4$, $p < 0.0001$]. A significant ($p < 0.05$) increase in Evans blue concentration was observed in the brain of animals exposed to TBI in comparison to the animals of control group. Eugenol in the doses of 50 and 100 mg/kg had shown a significant ($p < 0.05$) decrease in Evans blue concentration in the brain of animals exposed to TBI in comparison to vehicle treated TBI exposed animals.

3.10. Eugenol attenuated the TBI induced increased brain water content in rats

Fig. 8 illustrates the effect of eugenol pretreatment on the water content of the brain (brain edema) in animals exposed to TBI. Statistical analysis of results showed significant difference among the groups [$F(4,29) = 10.85$, $p < <0.0001$]. Post-hoc analysis showed that the brain water content increased significantly ($p < 0.05$) in the rats exposed to trauma compared with control rats. Brain edema induced by TBI was attenuated with the eugenol pretreatment at the dose of 100 mg/kg while the other two doses of the eugenol were found ineffective.

4. Discussion

In the present study, we have evaluated the neuroprotective effects of eugenol in the weight-drop induced rat model of TBI. The important findings of the present investigation are as follows:

(1) Weight-drop induced rat model of TBI resulted in increased brain water content (edema), altered BBB permeability, augmented lipid peroxidation, and increased neuronal cell death indicated by

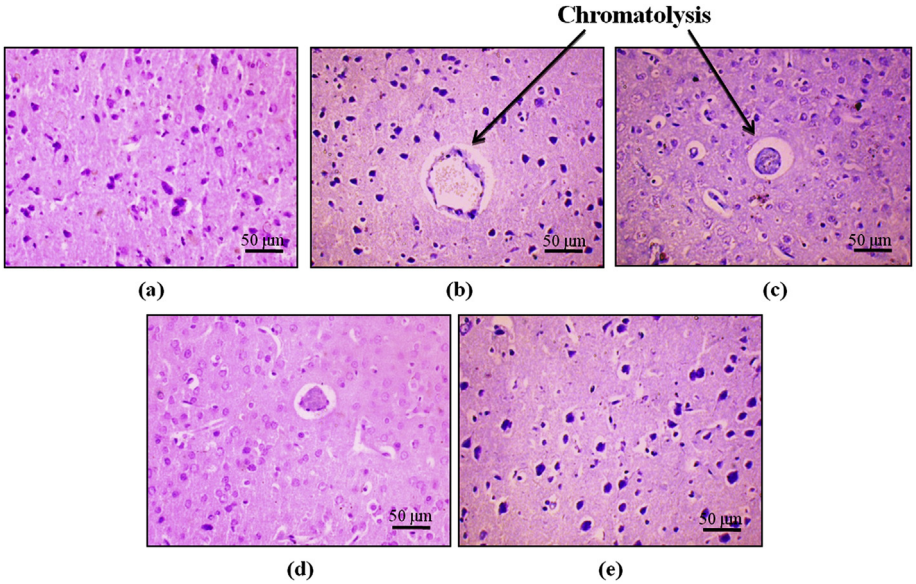

Fig. 6. Nissl staining of brain sections of different groups (400 X magnification). Control (a) TBI (b) Eugenol (25 mg/kg) + TBI (c) Eugenol (50 mg/kg) + TBI (d) Eugenol (100 mg/kg) + TBI (e).

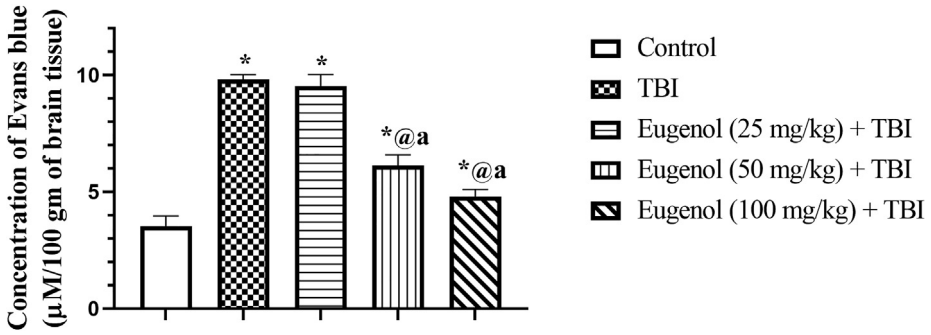

Fig. 7. Effect of eugenol on blood-brain barrier permeability (concentration of Evans blue) in a weight-drop induced rat model of TBI. Each bar represents mean ± SEM of six animals per group. *$p < 0.05$ compared to control, $^{@}p < 0.05$ compared to TBI, $^{a}p < 0.05$ compared to eugenol 25 mg/kg using one way ANOVA (Tukey's multiple comparisons test).

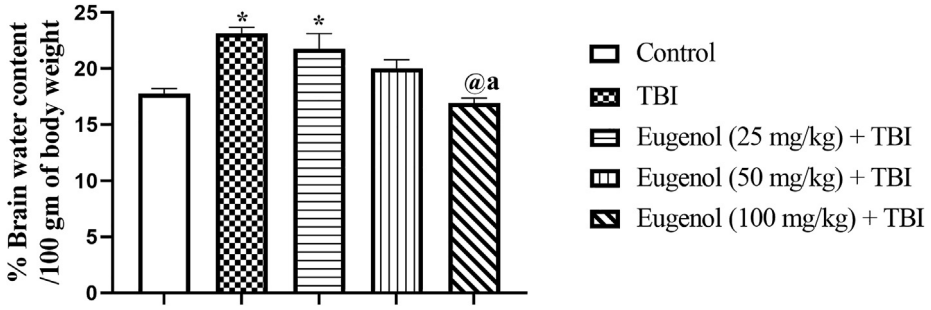

Fig. 8. Effect of eugenol on brain edema (% brain water content/100 g of body weight) in a weight-drop induced rat model of TBI. Each bar represents mean ± SEM of six animals per group. *$p < 0.05$ compared to control, $^{@}p < 0.05$ compared to TBI, $^{a}p < 0.05$ compared to eugenol 25 mg/kg using one way ANOVA (Tukey's multiple comparisons test).

augmented chromatolysis

(2) TBI also induced impairment in locomotor activity, motor coordination and short term memory (functional disabilities)

(3) Pretreatment with eugenol in rats ameliorated histopathological, neurochemical and behavioral consequences of trauma.

Pathogenesis of TBI has comprised of two phases of pathogenesis, primary and secondary injury. The primary injury is caused by the immediate focal and diffuse mechanical damage of brain parenchyma at the time of the initial impact while secondary injury takes place hours or days after the inciting traumatic event. The impact on the brain in TBI causes fracture of the cranial vault which sometimes depressed into the brain. This leads to the shearing of axons and blood vessels and thereby intracerebral bleeding and hemorrhage within brain parenchyma.[34] TBI also results in brain edema and accumulation of cerebrospinal fluid due to blockage of outflow. This cumulatively results in the increase in the intracranial pressure (ICP) and ultimately herniation which is a major cause of mortality. The decompressive craniectomy (DC) is a widely used neurosurgical treatment for increased ICP following TBI.[35] In DC, a part of the skull is removed to allow the swollen brain to expand without being squeezed. However, reports showed that DC itself caused brain injury.[32,36] Thus DC reduces the mortality; however, it converts fatality into survival with severe disability.[37]

The weight-drop induced rat model of TBI is a clinically appropriate mechanical model of inducing diffuse close-head TBI in rodents. It imitates cerebral contusion, which results in a shearing injury to the brain.[38] The weight-drop method results in cognitive impairments, neuroinflammation, apoptosis, and diffuse neuronal loss.[39] The present study also shows that weight-drop induced TBI resulted in behavioral alteration (motor incoordination and memory loss), lipid peroxidation, BBB disruption, brain edema, and neuronal loss.

TBI results in neuromotor impairment that causes disabilities affecting mobility, and motor coordination.[40] To elucidate the effect of eugenol on impairment in mobility, balance, and motor coordination induced by TBI, we performed four functional tests such as actophotometer, rotarod, rearing, and beam-walking test. Our results showed that weight-drop induced TBI resulted in sustained neurological motor dysfunction, as indicated by the increased time taken to transverse the beam in the beam-walking test and decreased activity score in the actophotometer. Rearing phenomenon and Motor coordination was also deteriorated in the rats exposed to TBI as observed in cylinder and rotarod tests respectively. Earlier it was reported that the cortical area is associated with coordinated walking in the rat and cortical lesion results in a motor abnormality when the rats transverse a narrow elevated beam.[41] Beam-walking test is an excellent tool for studying the functional recovery of locomotor ability after sensorimotor cortex injury in the rats.[42] Similarly, actophotometer, rearing test, and rotarod are useful method for evaluating the movement disorder. Hence weight-drop method induced trauma causes the cortical lesions and thus abnormality in mobility and motor coordination. The present study showed that eugenol in the doses of 100 mg/kg attenuated weight-drop induced motor defects in beam-walking, rotarod, rearing, and actophotometer tests. Thus eugenol resulted in increased functional recovery after trauma.

In addition to motor disorders, the TBI victim also suffered from cognitive impairment.[43] Post-traumatic amnesia is the state of perplexity that occur immediately after TBI in which the patient is unable to recall events that occur after the injury. This period of memory is one of the most common indexes used to determine the severity of injury and prognosis for recovery.[44] Memory and learning deficits in the traumatized rats were studied using the Y-maze test. The results from the Y-maze showed that TBI resulted in impaired memory, which was depicted as a reduced entry and time spent in the novel arm. Eugenol pretreatment at the dose of

100 mg/kg in rats followed by trauma improved memory performances in the Y-maze test. Thus, eugenol improved memory in the traumatic rats.

Oxidative stress plays a crucial role in the pathogenesis of TBI and ultimately development and progression of neurobehavioral deficits.[45] The results of the present study showed that TBI results in the augmented MDA levels in the brain of rats. MDA is a marker of lipid peroxidation and reflects oxidative stress. Eugenol has already been reported to exert direct free radical scavenging activity.[46] Therefore; it seems that the improving effect of eugenol against TBI induced cognitive and motor deficit in rats was mediated, at least partially, by its antioxidant and free radical scavenging capability.

Results of both preclinical and clinical studies suggest that head trauma results in frequent BBB disruption. Focal mechanical impact on the head causes concomitant shear injury to the endothelium wall of small blood vessels. Disruption of the endothelial cell wall of blood vessels results in the loss of BBB integrity and increases its permeability. BBB breakdown leads to exposure of the brain tissue to serum-derived molecules and in uncontrolled transport of the ion and protein from the intravascular to extracellular brain compartments with the definite accumulation of water. This pathology anatomically increases the volume of extracellular space in the brain and thus results in vasogenic edema.[47] Cytotoxic brain edema occurred due to intracellular water accumulation in neurons, astrocytes, and microglia. It is observed that in patients cytotoxic edema appears more frequent than vasogenic edema after TBI. However, both of these entities are related to increased ICP and ischemic events in the secondary phase of brain injury.[48] Results of the present study also show that weight-drop induced TBI results in brain edema as well as disruption in the BBB. Pretreatment with 100 mg/kg of dose eugenol found to attenuate the TBI induced increased in brain edema and disruption of BBB.

The Evans blue is a high molecular weight dye and bound to albumin in the plasma. In normal conditions, Evans blue is not able to cross the BBB. BBB disruption results in increased permeability of Evans blue to the brain. Hence, Evans blue concentration in the brain is indicative of the BBB disruption. Traumatized animals have shown a marked increase in Evans blue level in the brain as well as % brain water content/100 g animal body weight (brain edema). Eugenol at the dose of 100 mg/kg, has shown a significant decrease of Evans blue concentration as well as % brain water content/100 g animal body weight (brain edema) in the brain of rats exposed to trauma. Thus eugenol improves the disrupted BBB integrity and decreased brain edema caused by trauma.

Neural membranes consist of phospholipids in large amounts, which are essential for normal vascular permeability. Phospholipid peroxidation within the membrane results in the alteration of membrane fluidity and permeability, thereby allowing ions such as Ca^{2+} to leak into the cell.[49] Thus, oxygen-free radicals generated in TBI are most likely to play a crucial role in increased cerebral vascular permeability and thus in vasogenic brain edema.[50] Eugenol has been reported to exert direct free radical scavenging activity.[46] This beneficial effect of eugenol on disturbed BBB integrity and increased brain edema in weight-drop induced trauma might be due to its antioxidant activity.

Free radical generations, disrupts the BBB and increased brain edema results in the neuronal loss. Nissl-staining is a widely used method to study morphology and pathology of neuronal tissue. Histopathological examination of Nissl stained brain sections reveals the chromatolysis in animals exposed to trauma, which indicates neuronal cell death. The absence of chromatolysis was observed in the Nissl stained brain sections of animals treated with eugenol (100 mg/kg doses) followed by trauma. Thus eugenol attenuated the neuronal cell death.

5. Conclusion

Our animal studies have shown that eugenol possesses promising neuroprotective effects in a weight-drop induced TBI model as it mitigates the histopathological, neurochemical, and behavioral consequences of trauma. The observed neuroprotective activity of eugenol may be because of its antioxidant activity. Several preclinical studies as well as clinical trials were conducted earlier on the repurposing of antioxidants in the prevention and treatment of trauma. Hence, eugenol can be a potential candidate for the management of functional disabilities associated with trauma patients. These findings may attract food and pharmaceutical industry to come up with safe and effective therapeutic products. However, further preclinical and clinical studies are required to validate the use of eugenol in the treatment of TBI.

Declaration of competing interest

There is no conflict of interest to disclose.

Acknowledgments

Authors acknowledge the Institute of Pharmacy, Nirma University, Ahmedabad, India for financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2021.01.003.

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