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ORIGINAL PAPER

Neuro-pharmacological-based in vivo and behavioral exploration of antiepileptic activity of leaves extract of *Terminalia bellirica* Roxb. in chronic pentylenetetrazole-induced kindling model in mice

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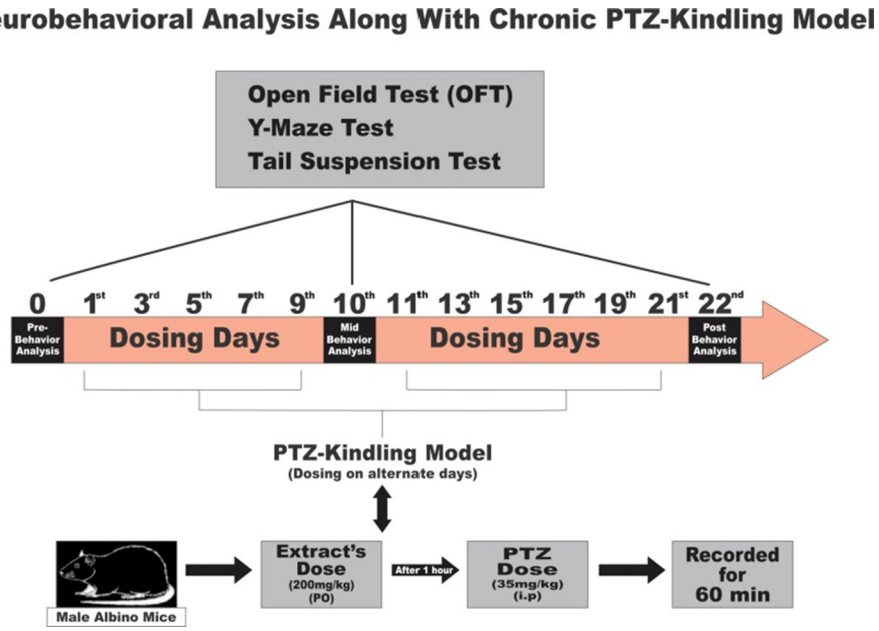
Abstract

Epilepsy affects 70 million people worldwide. An abnormal brain electrical rhythm causes frequent seizures in this neurologic disease. *Terminalia bellirica* Roxb., called as "Behada" has been used in Ayurvedic medicine for millennia. Unani, Tibb, Ayurveda, and Siddha employ its hemostatic, carminative, dysenteric, liver tonic, digestive, antidiarrheal, analgesic, anthelmintic, antibacterial, and skin condition benefits. Its neuroprotective potential in leaves has not been studied as much as in fruit. Leaf extracts of *T. bellirica* were evaluated for their antiepileptic properties in a chronic PTZ-kindling epilepsy model, accompanied by behavioral assessments of learning, memory, and motor function. The methanolic (MeOH) extract from the leaf of *T. bellirica* demonstrated reduced seizure frequency (MeOH; 15 ± 3, DZP; 10 ± 2), mean seizure score (MeOH; 1.5 ± 0.5, DZP; 1.19 ± 0.4, secs) and cumulative duration (MeOH; 285 ± 31, DZP; 220 ± 45, secs), comparable to diazepam (DZP). Significant behavioral improvements were observed in mice treated with MeOH extract in post-test levels: open field (MeOH; 20.7 ± 3.6, DZP; 11.4 ± 2.1, secs), Y-maze (MeOH; 33.1 ± 5.6, DZP; 24.7 ± 4.1, secs) and tail suspension (MeOH; 87.1 ± 13.4, DZP; 48.3 ± 17.5, secs). The MeOH extract of the leaf of *T. bellirica* demonstrated comparable activity in controlling PTZ-induced seizures with behavioral improvements in mice. The methanolic extract of *T. bellirica* leaves shows promise as a novel neuroprotective herbal treatment for epilepsy and its associated behavioral and cognitive impairments.

Extended author information available on the last page of the article

Graphical abstract
Timeline for kindling and behavioral experiments



Keywords Epilepsy · Behavioral analysis · Neuroprotection · Pentylenetetrazole

Introduction

Epilepsy is a neurological disorder characterized by the recurrence of seizures for no apparent reason. Millions of people worldwide suffer from this debilitating neurological condition, and it is responsible for a great deal of illness and death. When a convulsion occurs without a clear cause, it is referred to as "epileptic." Epilepsy is occurrence of signs or symptoms related to abnormal excessive or synchronous neuronal activity in the brain (Fisher et al. 2017).

Using a worldwide active epilepsy occurrence rate of 8%, Sadr et al. (2018) showed that epilepsy is one of the most common chronic neurological illnesses. Seizures can be categorized into four broad categories: generalized, focal, combination, and epilepsy of unclear etiology (either generalized or focal) (Falco-Walter et al. 2018). The lifetime prevalence of epilepsy is 7.6 per 1000 people, making it one of the more prevalent and severe neurological illnesses. With the existing antiepileptic medicines (AEDs), 30% of patients can usually not obtain a long-term remission. Novel drugs with antiepileptic efficacy or supportive effects on seizure management are thus urgently needed. Nearly 70% of people with epilepsy might have a seizure-free life if they were identified at the right time (Lum et al. 2020). Because of the hazardous side effects and prohibitively high prices of synthetic drugs, the pharmaceutical industry has switched to using natural products as the primary source of treatment (Bernardini et al. 2018). Traditional herbal medicines and well-tolerated seizure medications are commonly utilized, but further research is required to reach more definitive, precise, and evidence-based results (Losher et al. 2011). Researchers around the corner are now targeting the plants with therapeutic potential for the search and discovery of such antiepileptic agents.

Terminalia bellirica Roxb., a sizable deciduous tree belonging to the Combretaceae family, has a cluster of broadly elliptic leaves at the termination of its branches (Meena et al. 2010). Numerous phytochemicals are separated from various parts of the plant, including alkaloids, coumarin, flavones, steroids, lignans, tannins, glycosides, terpenoids, saponin, and flavones (Abraham et al. 2014). Based on a cursory review of the *T. bellirica's* scientific literature, it is evident that this plant endures medicinally relevant constituents and is used to treat a variety of ailments; however, the leaves have not yet been explored thoroughly in terms of antiepileptic probe. This is why leaves of *T. bellirica* have been selected to execute the present study with the aim to gauge their antiepileptic prospective.

Materials and methods

Animals

Male Albino mice (25–30 g) were sourced from and managed at the Punjab University, Lahore. They were housed in polypropylene cages under standard laboratory conditions at 22 ± 2 °C with a natural light–dark cycle. The mice had ad libitum access to a pellet diet and water. Ethical approval for the experimentation was obtained prior to the commencement of the research (Certificate No: 154/FIMS), in compliance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA). Proper care was administered in the conventional laboratory setup.

Plant material and preparation of extracts

Leaves of *T. bellirica* were collected from Punjab University, New Campus Lahore. Plant was authenticated under voucher no. GC. Herb. Bot. 3761 from the Department of Botany, Government College University, Lahore, Pakistan. The leaves were cleaned and allowed to dry in the sun for about two days. The dried leaves were gathered and put through a ball mill grinder. For extraction, a variety of solvents have been employed including petroleum ether, chloroform, methanol, ethanol, and water. Cold extraction was carried out by maceration using ethanol and water, whereas hot extraction was performed using the Soxhlet apparatus using petroleum ether, chloroform, and methanol (Saleem et al. 2021).

Chemicals

Chemicals were purchased from authentic sources: Pentylenetetrazole (PTZ) (Sigma chemicals, Germany), Diazepam (Mediate Pharmaceutical, Pakistan), Normal saline (Medipak limited Pakistan limited), Tween 20 (Sigma chemicals, Germany), Distilled water, Petroleum ether (Sigma-Aldrich), Chloroform (MAY & BAKER LTD), Methanol (Merck), Ethanol (Merck).

Chronic epileptic model

Chemical kindling, characterized by the development of seizures after repeated administration of a sub-convulsive dose of a convulsant medication like PTZ, mimics human partial complex seizures (Gahataraj et al. 2020). Effects of *T. bellirica* leaf extract on kindling development were assessed in eight treatment groups, seven mice in each group. These groups were as follows: Group 1 ($n = 7$): Normal control, supplemented with normal saline only, Group II ($n = 7$): Positive control or Standard control receiving Diazepam (5 mg/kg), Group III ($n = 7$): Negative control, receiving PTZ (35 mg/kg); PTZ, known for CNS stimulation through GABAergic inhibition, administered intraperitoneally on alternate days for 21 days, one hour after extract administrations (200 mg/kg p.o) (Bharal et al. 2006), Group IV ($n = 7$): Petroleum ether + PTZ, Group V ($n = 7$): Chloroform + PTZ, Group VI ($n = 7$): Methanol + PTZ, Group VII ($n = 7$): Ethanol + PTZ and Group VIII ($n = 7$): Aqueous + PTZ (ERKEÇ and Arihan. 2015).

Seizure activity and behavioral changes were observed and graded using the Racine scale, a standard paradigm for evaluating medication efficacy in chronic epilepsy models (Goldenberg. 2010). Animals were deemed fully kindled upon the occurrence of three consecutive stages of 4 and/or 5 seizures. Each mouse's brain was dissected after post-behavioral analysis, and the midbrain region was isolated and homogenized in normal saline solution to create a solution of 10% (w/v).

Assessment of behavioral alterations in chronic PTZ-kindling mice model

Neurobehavioral analysis is a common approach for assessing the impact of long-term drug therapy on anxiety, locomotion, memory, and depression in individuals with epilepsy, considering the frequent coexistence of neuropsychiatric disorders (Mazarati 2016; Bagheri et al. 2019). Our experimental protocol involved behavioral tests at three critical stages of the PTZ-kindling model:

- Before initiating the PTZ-kindling model on "Day 0" (Pre-behavior analysis)
- In the middle of the PTZ-kindling model on the "11th day" (Mid-behavior analysis)
- After completing the PTZ-kindling model on the "22nd day" (Post-behavior analysis)

Three non-dosing days were dedicated to conducting behavioral tests, including the Open Field (OFT), Y-maze, and Tail Suspension tests.

Open field test (OFT)

The open field test (OFT) is a widely recognized sensorimotor and behavioral test to assess spontaneous locomotor activity and aspects of anxiety-like behavior in rodents, often employed to measure the impact of various drug treatments (Gould et al. 2009; Kraeuter et al. 2019). This test capitalizes on mice's natural inclination to explore new environments and their avoidance of exposed and illuminated areas. Our study utilized a 40 × 40 × 40 cm open-field labyrinth to assess treatment efficacy. Each mouse underwent

a single 3-min trial in the maze's center, ensuring cleanliness between trials with 70% isopropanol to eliminate any potential influence from previous experiment. Experimental videos were recorded. ToxTrac software analyzed the following parameters:

- Time spent in the central zone,
- Time spent in the periphery

Y-maze test

The Y-maze spontaneous alternation test was employed to examine the impact of chronic drug treatment on mice's acute/short-term spatial memory (Kraeuter et al. 2019). Built on rodents' inherent inclination to explore novel environments, this test measures the likelihood of rodents choosing a new arm over revisiting a previously explored one. In our study, a Y-maze apparatus (15 × 3.5 × 3 inches) arranged in a Y shape at a 120-degree angle was utilized. Each mouse, placed individually in the center of the maze, freely explored its three arms for three minutes. Entries in each arm were recorded by a camera, and the percentage of spontaneous alternation was calculated using the formula:

$$\%\ \text{Spontaneous alternation (SAP)} = \left[\frac{(\text{No. of alterations})}{(\text{Total arm entries}) - 2}\right] \times 100$$

Tail suspension test

The tail suspension test is a widely used behavioral test useful in screening potential anti-depressant drugs. Animals, particularly rodents, when subjected to the inescapable stress of being suspended by their tail will display an immobile posture (Cryan et al. 2005; Can et al. 2012). In our experiment, mice were suspended 35 cm above the surface using a paper clip secured around the tail with adhesive tape. The total duration of immobility was recorded on camera and assessed blindly during a 6-min testing period.

Histological investigations

Brains were fixed in 10% saline/paraformaldehyde (pH 7.6) for 24 h. The sections were examined by light microscopy for histopathological changes, and photomicrographs were taken.

Results

Chronic PTZ- kindling model

Effect of plant extracts on seizure frequency

Terminalia bellirica leaf extracts at a dose of 200 mg/kg/day (p.o) in conjunction with PTZ at a dose of 35 mg/kg (i.p) on alternate days demonstrated a robust protective effect against the onset of kindling (Fig. 1A). The mice in PTZ-induced negative control group experienced significantly (Two-way ANOVA, $p < 0.0001$) more seizures compared to the mice in conventional positive control group treated with diazepam, which had the lowest seizure frequency over 21 days period (Fig. 1B). Compared to diazepam-treated mice (5 mg/kg, i.p), mice treated with methanol extract and petroleum ether (PE) demonstrated similar patterns in seizure frequency (Fig. 1B–D). Comparing seizure scores among groups treated with Diazepam, methanol, and petroleum ether extracts showed almost similar trend (Fig. 1B–D). While, the mice treated with ethanol, water and chloroform extracts demonstrated insignificant effects of the extractives on seizure frequency, comparable to the negative control group (Fig. 1E–G).

Effect of plant extracts on mean seizure score

Next, we examined the effect of plant extracts, leaves of *T. bellirica,* on mean seizure score as shown in Fig. 2H. Data demonstrated only methanol extract exhibited reduced mean seizure score compared to all other extracts during 21 days of treatment–comparable to standard control (Diazepam) (Fig. 2H). The treatment groups of extracts showed significantly reduced mean seizure scores as compared to the only PTZ (35 mg/kg, i.p.) group (Fig. 2H). The normal control group showed approximately no seizures. The positive control group showed fewer seizures, and the negative control showed a high seizure score. Chloroform and water extract groups represented a high seizure score.

Effect of plant extracts on cumulative seizure duration

Next, the impact of various extracts of leaf of *T. bellirica* was examined on cumulative seizure duration during 21 days of treatment (Fig. 2I). Cumulative seizure duration is calculated by adding seizures over 21 days of the experiment. Data suggested that the majority of the plant extracts of *T. bellirica* decreased the duration of PTZ-induced seizures; however, only methanol extract demonstrated cumulative seizure duration comparable to Diazepam (Fig. 2I). Besides,

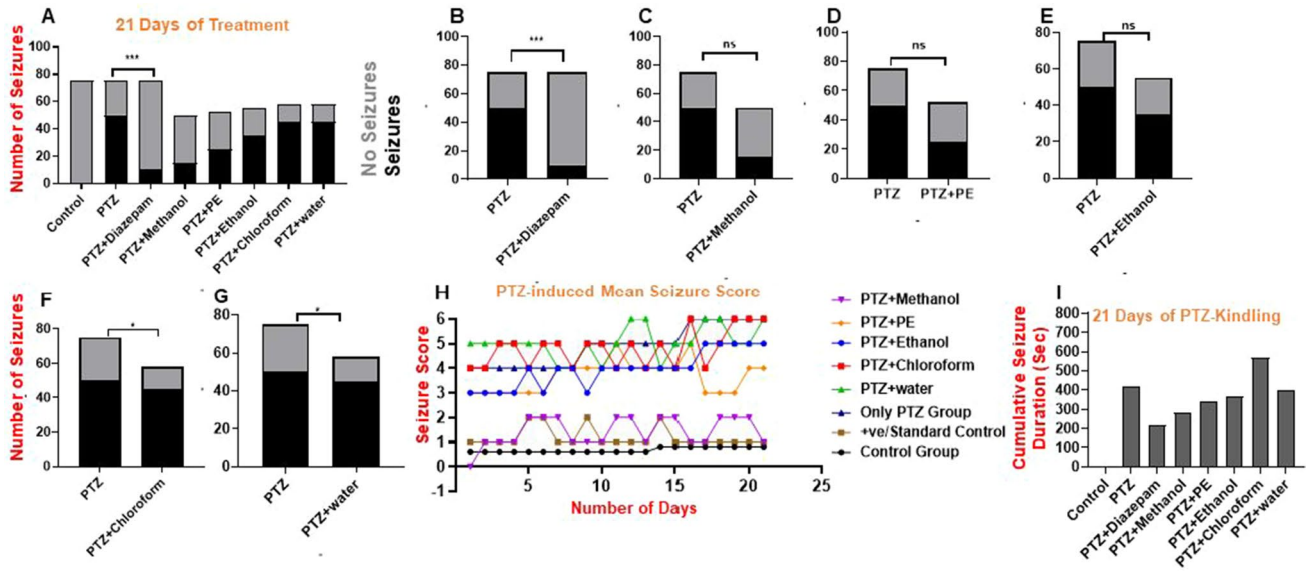


Fig. 1 The effects of extracts of leaf of *T. bellirica*, on the number of seizure, mean seizure score and cumulative seizure duration in PTZ-kindled mice. **A** Number of seizure after 21 days of treatment, **B** Number of seizure; PTZ vs PTZ-diazepam, **C** Number of seizure; PTZ vs PTZ-methanol (MeOH), **D** Number of seizure; PTZ vs PTZ-PE (petroleum ether), **E** Number of seizure; PTZ vs PTZ-ethanol, **F** Number of seizure; PTZ vs PTZ-chloroform, **G** Number of seizure; PTZ vs PTZ-water, **H** Mean seizure score; 5–25 days, **I** Cumulative seizure score of 21 days of treatment

higher cumulative seizure scores were obtained for chloroform and water extracts (Fig. 2I).

Assessment of behavioral alterations in chronic PTZ-induced kindling model

Behavioral assessments were performed via open field test and Y-maze test reported previously.

Open field test (OFT)

The mice were allowed to explore in an open field arena for the evaluation of anxiety-related parameters at 3 test levels, i-e., pre, mid and post. Data suggested that mice tend to spend most of their time in the central zone during and after PTZ-kindling days in negative control (Fig. 2A–C). Compared to PTZ kindled mice, in OFT–mid-level, diazepam-treated mice exhibited a significant reduction in time spent in the central zone followed by *Terminalia's* ethanol and methanol extract-treated mice (Fig. 2B)—indicating a noteworthy reduction in stress levels with the administration of the methanolic extract, demonstrating anxiogenic activity compared to the PTZ group. Notably, this reduction in time spent in the central zone became almost comparable between diazepam and methanol-treated *Terminalia's* leaf extract in OFT post-level (Fig. 2C).

The behavior of mice in the treatment groups was also analyzed using ToxTrac software. The pathway trajectory of mice showing their time of stay in central or peripheral zones of the provided arena is shown in Fig. 2D–F. Mice treated with PTZ alone displayed prolonged periods of stay in the central zone in contrast to normal healthy mice treated only with normal saline. However, mice treated with diazepam and *Terminalia's* methanol extract demonstrated the group treated with methanolic extract significantly mitigated this behavior ($P < 0.0001$), suggesting a pronounced impact on anxiety.

Y-maze test

Y-maze test was performed to assess the impact of plant extract therapy on the acute/short-term recognition abilities at 3 stumps (pre, mid, post). The one-way ANOVA revealed a statistically significant inter-group difference in the percentage of spontaneous alternations (Fig. 3A–C).

In a pre-test level, in all groups, mice tend to spend more time in the opposite arm (Fig. 3A), while in the mid-test and post-test levels, mice in PTZ-kindled, PTZ-chloroform and PTZ-aqueous groups tend to spend more time in same arm (Fig. 3B) with a reduced tendency to explore novel arm. However, in both mid-test and post-test levels, same as the healthy mice, mice in diazepam and PTZ-methanol groups spent more time in the opposite arm with higher tendency to explore novel arm (Fig. 3B&C).

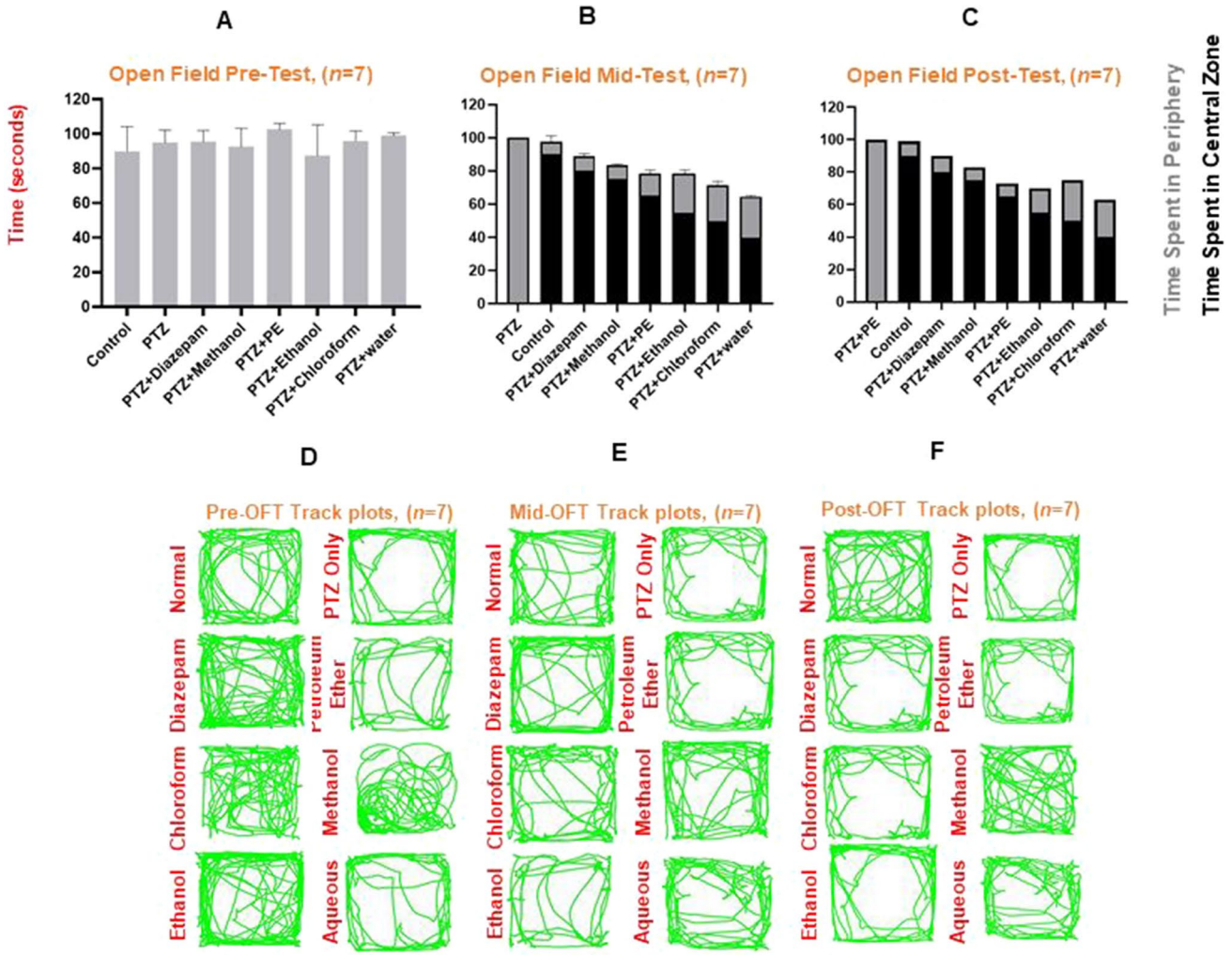


Fig. 2 Effects of extracts of leaf of *T. bellirica* on behavioral alterations in PTZ-induced kindled mice ($n=7$). **A** Pre-level Open Field Test (pre-OFT, day 0)—time spent in the periphery depicted as gray color bars**, B** Mid-level OFT (day 11)—time spent in the periphery depicted as gray color bars and time spent in central zone depicted as black color bars. **C** Post-level-OFT (day 22)—time spent in the periphery depicted as gray color bars and time spent in central zone depicted as black color bars. **D** Pre-OFT (day 0) track plots depicting mice movements in open field arena, **E** Mid-OFT (day 11) track plots depicting mice movements in open field arena, **F** Post-OFT (day22) track plots depicting mice movements in open field arena—green lines represent mice movements colour figure online

Tail suspension test

In tail suspension test, a significant change in the duration of immobility was observed at 3 different levels of experiments. In pre-test level, the mice in all groups were struggling and showed reduced immobility times (Fig. 3D). In mid- and post-test levels, PTZ-kindled mice demonstrated increased immobility time, while PTZ-chloroform and PTZ-aqueous extracts exhibited no reduction in immobility time compared to PTZ-kindled mice (Fig. 3E–F). However, significant reduction in immobility time was observed in mice treated with diazepam and PTZ-methanol extracts, PTZ-petroleum ether and PTZ-ethanol extracts (Fig. 3E–F).

Histopathological investigation

Next, we examined histological changes in the brains of the mice in control, PTZ-kindled, diazepam and PTZ-methanol groups. As shown in Fig. 4A, mice in normal group demonstrated characteristic neuronal architecture (Fig. 4A), while in PTZ-kindled mice signs of brain edema was notable (red arrows) (Fig. 4B). Whereas, treatment with Diazepam showed the presence of normal neurons (Fig. 4C), yet, at a specific focal point, there was evidence of some sort of inflammation. However, mice treated with the methanolic extract exhibited normal neural tissue, including intact neurons (red arrows) and axons (Fig. 4D) (Cavalheiro et al. 1996).

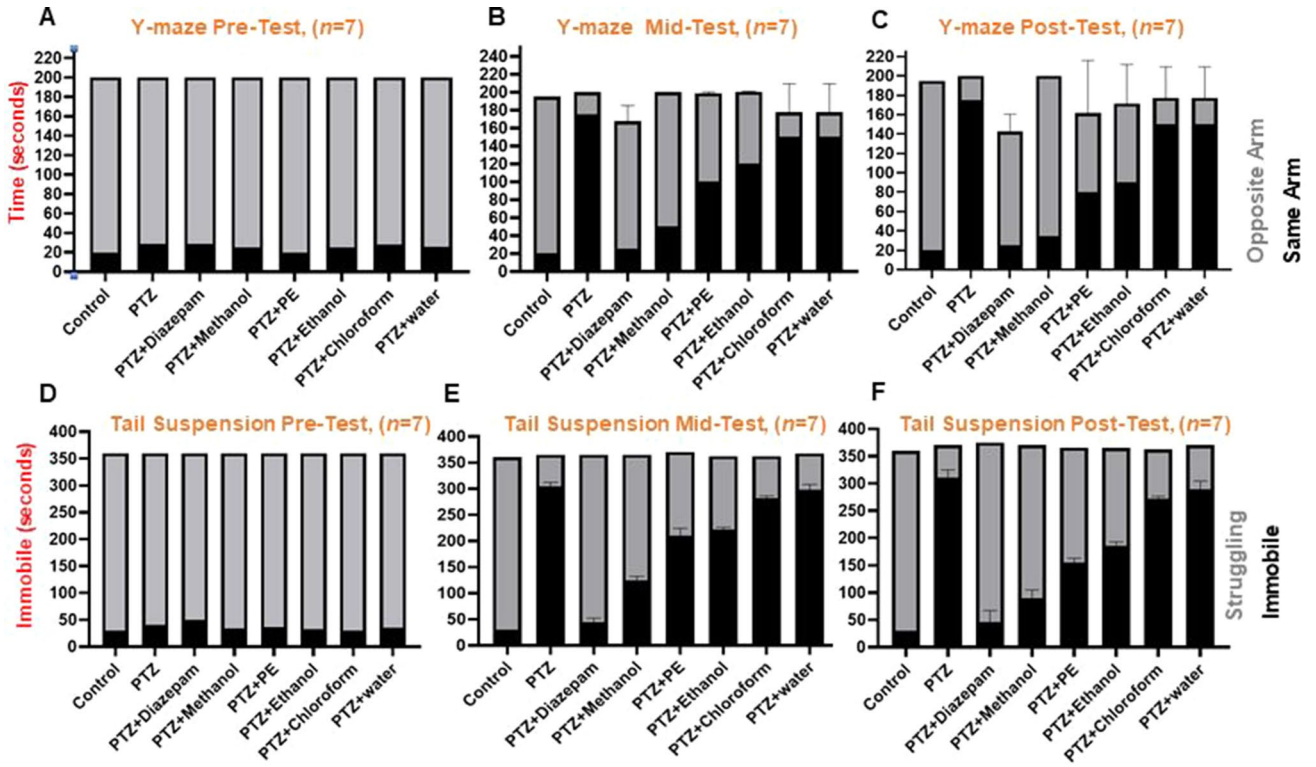


Fig. 3 Assessment of acute/short-term recognition and duration of immobility in PTZ-kindled mice treated with extracts of leaf of *T bellirica*. **A** Pre-level Y-maze test (day 0), gray color bars represent opposite arm and black color bars represent same arm explorations, **B** Mid-level Y-maze test (day 11), gray color bars represent opposite arm and black color bars represent same arm explorations, **C** Post-level Y-maze test (day 22), gray color bars represent opposite arm and black color bars represent same arm explorations, **D** Pre-level Tail Suspension Test (TST, day 0)**,** gray color bars represent struggling time and black color bars represent immobility time, **E** Mid-level TST (day 11), gray color bars represent struggling time and black color bars represent immobility time. **F** Post-level TST (day 22), gray color bars represent struggling time and black color bars represent immobility time colour figure online

Discussion

Widely employed as an Ayurvedic medicinal plant, *T. bellirica* has garnered attention for its antidepressant attributes, notably within the renowned formulation "Triphala" (Kumar and Khurana 2018). The antiepileptic activity has been established in the fruit of *T. bellirica*; nonetheless, the potential of its leaves in this regard remains largely unexplored. In particular, the current study explores the antiepileptic activity of *T. bellirica* leaves, resulting in behavioral manifestations that show promise as antiepileptic agents.

Pentylenetetrazole (PTZ) was administered as a subconvulsive dose to induce PTZ-induced kindling with behavioral manifestations of stage 4–5 seizures. The study compared the neuroprotective effects of the methanolic extract of *T. bellirica* leaves with the standard antiepileptic drug diazepam (ERKEÇ and Arihan 2015). The results demonstrated that the methanolic extract significantly reduced the number, mean seizure score, and duration of seizures, comparable to diazepam, in PTZ-kindled mice. These effects could be attributable to potential modulation of GABA synapses, similar to diazepam, and by significantly decreasing the brain dopamine levels (Nuss 2015). The positive impact of methanolic extract of *T. bellirica* was further strengthened by the histological findings, where brain sections treated with methanolic extract showed nearly normal neurons in comparison with signs of edema and inflammation in the brains of PTZ-kindled mice. As per the literature evidence, PTZ-induced anxiogenic effects have been attributed to GABA-antagonistic effects on the brain (Reddy 2020). Another likely reason could be the oxidative stress that directly regulates pathways inducing neuronal damage and cognitive deficit with reports of a strong association of PTZ-kindled neuronal discharge and oxidative stress (Dehkordi et al. 2023; Zhu et al. 2017). Oxidative stress plays an important role in brain tissue damage during seizure in the PTZ kindling model of epilepsy (Obey et al. 2008; Geronzi, et al. 2018). Moreover, *T. bellirica's* potential an antioxidant , which have been previously documented from fruit extracts, may act as a contributing factor to the reduction of oxidative stress linked to seizures (Gupta et al. 2021) and may also

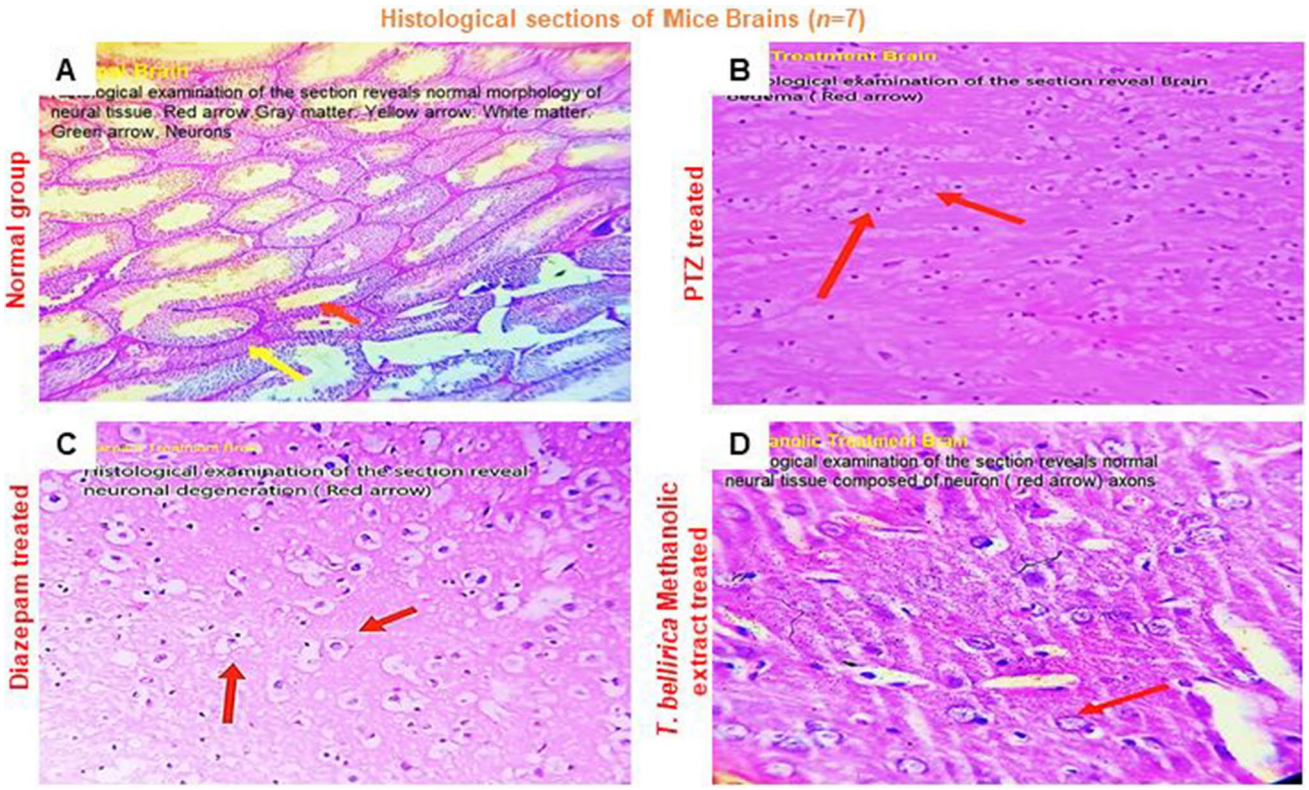


Fig. 4 Histological Examination of PTZ-kindled mice brains treated with extracts of leaf of *T bellirica*. **A** Transverse section of normal mice brain showing normal morphology, **B** Transverse section of PTZ-kindled mice showing edema (red arrows), **C** Transverse section of mice brain treated with diazepam showing some neuronal degeneration, **D** Transverse section of mice brain treated with MeOH extract of *T. bellirica* with signs of normal tissue

be a contributing factor in reducing oxidative stress linked to seizures.

Furthermore, mice were tested in an actimeter to assess the effects of various extracts of leaf of *T. bellirica* on mice locomotor activity. Data indicated that PTZ-kindled mice tend to spend most of their time in the central zone, while, in mid- and post-test levels, after the administration of leaf extractives of *T. bellirica,* mice in the methanolic extract group showed a significant reduction in time spent in the central zone which is comparable to diazepam. These findings are in complete agreement with a study documented that the administration of *T. bellirica* fruit extract significantly reduced the locomotor activity of rats in a dose-dependent manner (Kadian and Parle 2015).

When assessed for long-term memory and tendency toward novelty, again mice treated with methanolic extracts of *T. bellirica* tend to spend more time in the opposite arm, which was comparable to diazepam control. Further behavioral assessment indicated that mice treated with methanolic extracts of *T. bellirica* demonstrated a reduction in the immobile time in the tail suspension test, again comparable to the control at the post-test level. These findings indicated that methanolic extract of *T. bellirica* significantly improved stress, anxiety and depressive symptoms in PTZ-kindled mice–corroborating previous findings, that ethanolic extract of *T. bellirica* fruit reversed reserpine induced extension in immobility time similar to imipramine and fluoxetine (Dhingra and Valecha 2007). These follow-up studies demonstrated the neuroprotective role of methanolic extracts from the leaves of *T. bellirica* in experimental models. This may suggest that plant's leaves could complement epilepsy treatment by targeting biochemical mechanisms and behavioral manifestations. To isolate the bioactive components responsible for these effects, further research is needed. These findings support the plant's traditional use in managing seizures and treating epilepsy.

Conclusion

In conclusion, the data provided here show that *T. bellirica's* methanolic extract greatly reduced the length and averted the severity of PTZ-induced seizures, showing its antiepileptic action. Given their significant antiepileptic role in chronic and behavioral models, it is obvious that *T. bellirica* leaf extracts could be used as an alternative to antiepileptic

medication in patients with epilepsy and might prevent neuronal damage in such patients, which frequently results in behavioral manifestations. More research is needed to identify the extract's active components and assess its pharmacodynamic characteristics.

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Author contributions Conceptualization was done by A.S.; methodology was done by A.S, A.A, M.I, H.S; software and resources were done by A.A.; validation was done by A.A, A.S, and H.S. M.A.; formal analysis was done by A.A., A,S and M.A.; investigation was done by A.A., H.S and A.S; data curation was done by A.A., A.S, H.S; writing—original draft preparation was done by A.S and AA; writing—review and editing was done by A.A., AS.; supervision was done by A.A.,AS, H.S; and final editing was done by AA. All authors have read and agreed to the published version of the manuscript.

Data availability The data generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval and consent to participate A voucher specimen was deposited under the reference number GC. Herb. Bot.15823 The plant specimen was identified by Prof. Dr. Zaheer-ud-din Khan (taxonomist). All the procedures were followed in accordance with relevant guidelines.

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