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RESEARCH ARTICLE

Neuroprotective effect of alcoholic extract of *Selaginella bryopteris* leaves in experimental models of epilepsy

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Abstract

Epilepsy, a neurological disorder, is characterized by recurrent, uncontrolled seizures due to an imbalance between inhibitory and excitatory neuronal interactions in the central nervous system (CNS). This study explores the neuroprotective effects of an alcoholic extract from *Selaginella bryopteris* leaves in experimental epilepsy models. Swiss albino mice (25–30 g) were used, and epilepsy was induced *via* pentylenetetrazol (PTZ, 60 mg/kg) and maximal electric shock (MES). The extract was administered orally at varying doses and compared with conventional antiepileptic drugs, phenytoin and diazepam. LC-MS analysis identified amentoflavone as a key bioactive compound with antiepileptic properties. The extract demonstrated significant dose-dependent protection in both PTZ and MES models, delaying convulsions in the PTZ model at 500 mg/kg, comparable to diazepam, and providing convulsion protection in the MES model similar to phenytoin. Additionally, the extract increased gamma-aminobutyric acid (GABA) and glutathione (GSH) levels while reducing lipid peroxidation (LPO) levels, indicating its neuroprotective properties. These findings suggest that *S. bryopteris* leaves possess significant antiepileptic properties and may serve as a promising treatment for epilepsy.

Keywords: Antiepileptic, *Selaginella bryopteris* leaves extract, Seizure model, Neuroprotection, LC-MS.

Introduction

Epilepsy is a neurological condition characterized by spontaneous seizures resulting from an imbalance between the excitatory and inhibitory elements of the central nervous system (CNS) (Hugte *et al.,* 2023). This imbalance often arises from decreased gamma-aminobutyric acid (GABA) activity and increased glutamatergic activity (Alshabi *et al.,* 2022).

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Although the exact pathophysiology of seizures remains unclear, it is widely accepted that excessive glutamate release, the primary excitatory neurotransmitter, plays a significant role. Glutamate activates receptors such as N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, which can contribute to seizures onset (Chen *et al*., 2023). Conversely, inhibitory interneurons release GABA, which helps suppresses this excitatory activity by providing a counterbalance. Consequently, medications that block glutamate receptors or enhance GABA function are effective in preventing seizures (Da Guedes *et al.,* 2022).

Epilepsy can be caused by factors such as head injuries, infections, tumours, and idiopathic origins (Doerrfuss *et al.,* 2024). Although there is no evidence to supporting a hereditary component of epilepsy, the condition nevertheless poses a serious health risk by impairing normal brain cell function (Chowdhury *et al.,* 2022).

In India, epilepsy prevalence ranges from 2.2 to 10.4 cases per 1,000 persons (Wal *et al.,* 2024). Despite being lower than in some other developing nations, this prevalence raises public health concerns (More *et al.,* 2023).

Therapeutic drugs like carbamazepine, sodium valproate, phenytoin, and diazepam are commonly used to treat epilepsy by regulating abnormal electrical activity

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in brain neurons (Talevi *et al.,* 2024). These drugs work by blocking calcium and sodium channels and balancing excitatory and inhibitory neurotransmitters in the CNS. Researchers continue to identify new targets and develop innovative therapeutic compounds (Hu *et al.,* 2023).

The demand for herbal drugs is growing, as they are perceived as natural, safe, and associated with fewer side effects compared to prescription medications (Choudhury *et al.,* 2023). Plants may offer a novel source for synthesizing antiepileptic drugs due to their biological activity (Ohanme *et al.,* 2024). Numerous plants that have historically been used to treat epilepsy in different medical systems have shown antiepileptic efficacy in animal models, indicating potential for managing the condition (Chaachouay *et al.,* 2024).

Medicinal plants are being explored for epilepsy treatment because synthetic drugs often have unwanted side effects, high costs, and limited effectiveness. Plants are more affordable and relied upon by many developing countries for treatment (Waris *et al.,* 2024).

Due to traditional beliefs and limited access to modern medical treatments, many people use medicinal plants for neurological diseases. One such plant that has long been used to treat epilepsy is *S. bryopteris*, traditionally used to treat epilepsy (Shankar *et al.,* 2012).

Chemical studies of this plant have identified numerous secondary metabolites, including flavonoids, tannins, phenols, alkaloids, saponins, and terpenoids. Flavonoid-rich *S. bryopteris* is a desiccation-resistant Indian resurrection plant and therapeutic herb (Gautam *et al.,* 2023). *S. bryopteris* methanolic extract (SBME) contains amentoflavone, a naturally occurring bioflavonoid with a broad spectrum of biological functions (Jain *et al.,* 2020).

Reports suggest flavonoids like amentoflavone have neuroprotective or antiepileptic properties (Rong *et al.,* 2019). Given the high concentrations of these compounds in the plant, they may contribute to its potential antiepileptic effects. However, the literature reveals a lack of research on the antiepileptic activity of *S. bryopteris*. This study explores the antiepileptic effects of *S. bryopteris* using maximal electroshock and PTZ-induced seizure models.

Materials and Methods

Plant Material Collection and Certification

S. bryopteris was collected from Yuvika herbs, DKC Agrotech Pvt. Ltd, Delhi. The Central Ayurveda Research Institute confirmed the species and archived the voucher specimen under reference number SMPU/CARI/BNG/2023-24/2640.

Plant Material Extraction

About 400 grams of dried *S. bryopteris* leaves were finely powdered and extracted using a soxhlet apparatus with methanol as the solvent (Paswan *et al.*, 2020). The extraction was performed at approximately 65°C for 8 to 10 hours until the solvent in the siphon tube appeared clear. After extraction, methanol was removed by rotary evaporation at 40 to 50°C. The crude extract was then desiccated in a vacuum chamber to eliminate residual solvent. Soxhlet extraction with methanol remains a popular and effective method for isolating bioactive compounds from plant materials.

LC-MS Analysis of Plant Extract

For the LC-MS test, a 4.6 mm ID × 100 mm, 3 µm Inertsil ODS-3 column was used, with a flow rate of 1-mL/min. Mobile Phases A and B included 0.1% formic acid in acetonitrile and 0.1% formic acid in water, respectively. The gradual elution commenced with 90% A and 10% B, shifting to 2% A and 98% B by 3.00 minutes and maintained until 6.00 minutes. The slope was changed back to 90% A and 10% B at 6.50 minutes, maintaining this composition until the total run time of eight minutes was reached. The Electrospray Ionization (ESI) mode of the mass spectrometer was adjusted to 140°C of heat, 400°C of desolvation heat, and 800L/h of gas circulation rate. The mass range scanned was 100 to 1000 m/z, with data acquisition managed by MassLynx software. Before the injection, the *S. bryopteris* extract underwent purification using a 0.22 µm filter. The LC-MS results were analyzed for the identification and quantification of phytoconstituents.

Drugs and Chemicals

Pentylenetetrazol (Sigma Aldrich), phenytoin, diazepam, DTNB, TBA (Central drug house Pvt Ltd.)

Test Animals and Ethical Considerations

The Institutional Animal Ethics Committee of Krupanidhi College of Pharmacy approved the study bearing approval no. 2018/PCOL/24/KCP/IAEC. Male Swiss strain mice (albino) weighing 25 to 30 gm was kept in a ventilated facility with a 12-hour light/dark cycle at an ambient temperature of 25 ± 5°C. Mice were given a pellet diet and perpetual access to water.

Experimental Design

MES induced seizures

The study involved five groups: Group 1 (Normal), Group 2 (Control with MES), group 3 (MES + SBME 250 mg/kg, *p.o.*), group 4 (MES + SBME 500 mg/kg, *p.o.*), and group 5 (MES + phenytoin, 25 mg/kg, *p.o.*). Electroshock was delivered to each mouse using ear-clip electrodes for 0.2 seconds with a current of 150 volts (resistances of 25, 50, and 1 ohm per second). Six mice of either sex, weighing 25 ± 5 g, received extract treatments for seven days, and on the experimental day, the test was conducted 60 minutes after extract administration and 30 minutes after the standardized drug (Phenytoin 5 mg/kg *i.p.*). The time duration of stupor

and tonic hind limb extension (THLE) was recorded, and observations for mortality and THLE were made for 15 minutes, respectively.

Pentylenetetrazole (PTZ) induced convulsion

There were five groups of animals. Group 1 received a vehicle, groups 3 and 4 were given SBME at different doses, group 2 served as the PTZ control, and group 5 received diazepam (5 mg/kg) as the standard treatment. Vehicles and extracts were administered orally, while diazepam was given intraperitoneally. After 7 days of treatment, PTZ (65 mg/kg) was injected intraperitoneally 45 minutes after the vehicle or extract and 30 minutes after the standard drug. Mice were observed for the onset of convulsions, seizure duration, and mortality over 30 minutes (Alshabi *et al*., 2022).

Estimation of brain GABA content

Upon completion of the trial, the animals were euthanized, and the brains were collected. A 0.5 g sample of brain tissue was mixed with 5 mL of ice-cold 10% w/v trichloroacetic acid for homogenization and centrifugation at 10,000 rpm for 5 minutes at 0°C. The supernatant was utilized for GABA quantification. After a 30-minute incubation at 60°C with 0.1 mL of the homogenized tissue and 0.2 mL of 0.14M ninhydrin in 0.5M carbonate-bicarbonate buffer (pH 9.95), 5 mL of copper tartrate reagent was added. The fluorescence was subsequently assessed spectrophotometrically at an excitation wavelength of attraction of 377 nm and an emission wavelength of 455 nm (Feng *et al*., 2022).

In-vitro Antioxidant Assay

Determination of reduced glutathione (GSH)

Reduced glutathione (GSH) levels were evaluated using Ellman's technique. 250 µL of tissue homogenate was mixed with 1-mL of 5% trichloroacetic acid (TCA) in a 2 mL Eppendorf tube. The tube was then centrifuged at 3000 rpm for 10 minutes. The supernatant was combined with 1.5 cc of 0.2 M phosphate buffer. Following a 10-minute incubation, 250 µL of 0.6 mM of DTNB was added. The concentration of GSH was estimated by comparing the absorbance value to a standard curve generated using a glutathione solution (1-mg/mL) and quantified as µg of GSH per mg of protein (Zong *et al*., 2022).

Determination of lipid peroxidation (LPO)

Lipid peroxidation was measured by detecting malondialdehyde (MDA) levels. A 1-mL sample of brain homogenate was combined with 2 mL of 4% sodium dodecyl sulfate, 15 mL of 0.8% thiobarbituric acid (TBA, pH 7.4), and 1-mL of 20% acetic acid in 0.27 mL of hydrochloric acid (pH 3.5). The mixture was heated up to 85°C for one hour, followed by centrifugation at 1200 rpm for ten minutes. The pink hue was detected spectrophotometrically at 532 nm. MDA concentrations were estimated using 1,1,3,3-tetraethoxypropane as a reference and represented as nanomoles of MDA per mg of protein (Łukawski & Czuczwar, 2023).

Microscopical examination

The brain tissue sample was dissected and promptly fixed in a 10% formalin solution for up to 24 hours. The histological sections of the brain were then dehydrated, embedded in paraffin, sliced into 5 µm thick segments, and stained with eosin and hematoxylin. The resulting tissue slices were investigated under an optical microscope (Mathon *et al*., 2023).

Statistical Analysis

Specify the statistical tests applied (e.g., ANOVA, post hoc tests), referencing GraphPad Prism for analysis. Report how significance was determined (e.g. $*p < 0.05$).

Results

Extraction of Plant

Dried plant material (400 g) of *S. bryopteris* was procured and shipped to Green Chem Pvt. Ltd., Bengaluru, Karnataka, India, for extraction. The extraction was performed using a soxhlet apparatus with methanol as the solvent, resulting in a dry powdered extract with a percentage yield of 7.5%.

LC-MS Evaluation of SBME

The LC-MS evaluation of SBME identified several key phytoconstituents. The LC data revealed multiple peaks, with the most prominent at 3.82 minutes, suggesting a major compound, likely a bioflavonoid such as amentoflavone. Other notable peaks appeared at 3.57, 4.18, and 5.07 minutes, indicating smaller quantities of phenolic acids and sterols. The MS analysis confirmed the presence of amentoflavone, shown by a strong signal at mass by charge 537.57, along with additional signals at mass by charge 265.4026 and 135.0474, suggesting fragments and smaller phenolic compounds (Figure 1 and Table 1). These results align with the known phytochemistry of SBME and support the presence of bioactive compounds like bioflavonoids and phenolic acids, indicating potential therapeutic applications, including the management of epilepsy.

Impact of SBME on duration ofdistinct periods of convulsion in Experimental Animals using MES model

All phases of seizures (flexion, extensor, clonus, and stupor) were significantly less severe in the SBME extract 250 and 500 mg/kg groups compared to the MES control group (Figure 2a, b, c and d). Both doses demonstrated dose-dependent efficacy, with the 500 mg/kg dose showing a stronger impact. The standard drug provided the most significant reduction in all seizure phases.

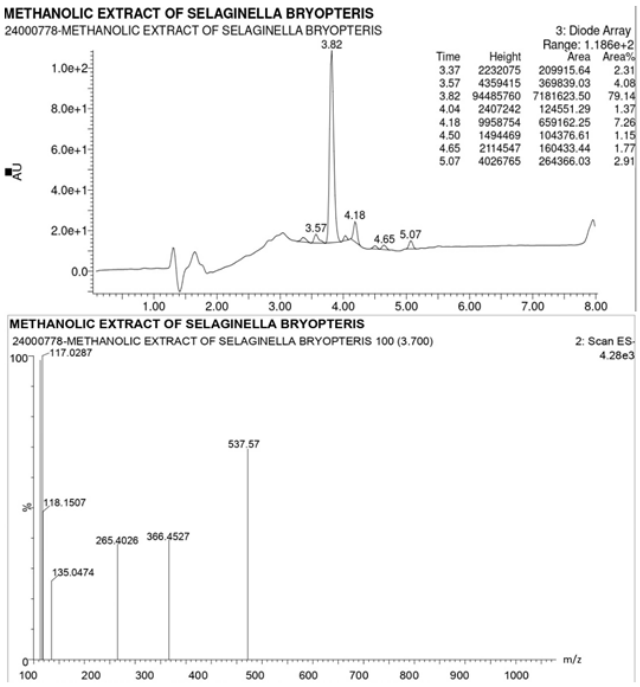

Figure 1: LC-MS analysis of SBME

Impact of SBME in Experimental Animals Using the PTZ-induced Convulsion Model

Figures 3a and b show that compared to the PTZ control group, SBME at 250 and 500 mg/kg effectively delayed the start of seizures and shortened their duration. SBME 500 mg/kg provided a stronger effect than the 250 mg/kg dose, with results approaching those of the standard antiepileptic drug. Both the onset delay and convulsion duration reduction were dose-dependent, with the standard drug showing the greatest effect, though SBME 500 mg/kg demonstrated comparable efficacy.

Impact of SBME on Brain GABA Levels and Oxidative Stress Markers (GSH and LPO)

GABA levels

In the PTZ group, GABA levels were significantly reduced ($^{###}p$ <0.0001). Both SBME doses (250 and 500 mg/kg) significantly increased GABA levels ($^{**}p < 0.001$), with the 500 mg/kg dose showing a stronger impact (Figure 4a). The standard treatment (STD) had the most significant increase ($^{***}p <$ 0.0001).

GSH levels

GSH levels were effectively lower in the PTZ group ($^{###}p<$ 0.0001), indicating oxidative stress (Figure 4b). SBME 250 mg/kg significantly raised GSH ($^{**}p$ <0.001), and 500 mg/kg depicts an even greater increase ($^{***}p$ <0.0001). The STD group had the largest increase ($^{***}p$ <0.0001).

LPO levels

LPO was significantly elevated in the PTZ group ($^{###}p$ <0.0001), indicating oxidative damage in Figure 4c. SBME 250 mg/kg significantly reduced LPO ($^{**}p$ <0.001), while the 500 mg/kg dose produced a stronger reduction ($^{***}p$ <0.0001). The STD group showed the greatest reduction ($^{***}p$ <0.0001).

Histopathology

Mice brain sections photomicrographed are shown in Figures 5 & 6. The normal control group's animals' brain tissue showed intact neuronal cells and no evidence of cerebral edema or congestion. The positive control group's brain sections, on the other hand, showed notable gliosis, cerebral congestion, and neuronal eosinophilia. Standard medication and 500 mg/kg of SBME did not cause any symptoms of neuronal eosinophilia, meningeal inflammation, cerebral congestion, or edema in the animals. Meningeal congestion, inflammation, cerebral congestion, and edema were all reduced in brain tissue from mice given 250 mg/kg of SBME.

Discussion

An imbalance between inhibitory and excitatory signals in the CNS can lead to uncontrollable seizures (Boleti *et al*., 2024). Long-term use of conventional antiepileptic drugs can result in adverse effects, drug resistance, and high treatment costs, particularly in resource-limited areas (Al-Worafi, 2024). This has led to a growing interest in plant-based treatments, which are often considered safer and more accessible.

This study evaluated the antiepileptic impact of SBME using two established epilepsy models: MES and PTZ-induced seizures. These models simulate different types of seizures, allowing us to assess the broad potential of SBME as an anticonvulsant.

The MES model, used to study generalized tonic-clonic seizures, revealed that SBME significantly reduced the time duration of THLE in a dose-dependent manner. This

Table 1: LC-MS analysis of SBME

Retention time	*LC peak area*	*Possible phytoconstituent*	*MS m/z*	*Identification*
3.37	209,915.64	Gallic acid	135.0474	Phenolic compound
3.57	369,839.03	Flavonoid	117.0287	Organic acid fragment
3.82	7,181,623.50	Amentoflavone	537.57	Amentoflavone
4.18	659,162.25	ß- sitosterol	265.4026	Amentoflavone fragment
5.07	264,366.03	Phenolic or sterol	366.4527	Possible flavonoid or sterol

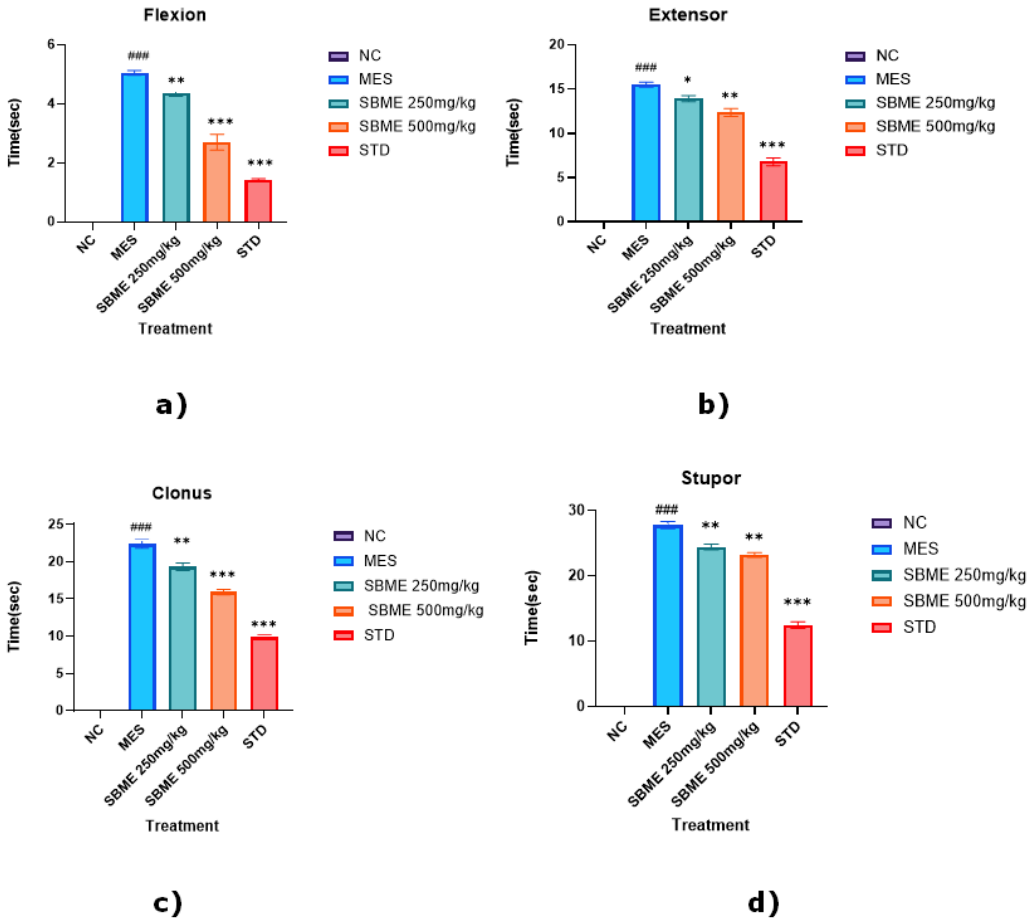

Figure 2: Impact of SBME on convulsions caused by MES for all phases of seizure (a) Flexion, (b) Extensor, (c) Clonus and (d) Stupor. For six animals each group, values are given as MEAN ± SEM. As compared with positive control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and $^{###}p < 0.001$ were found

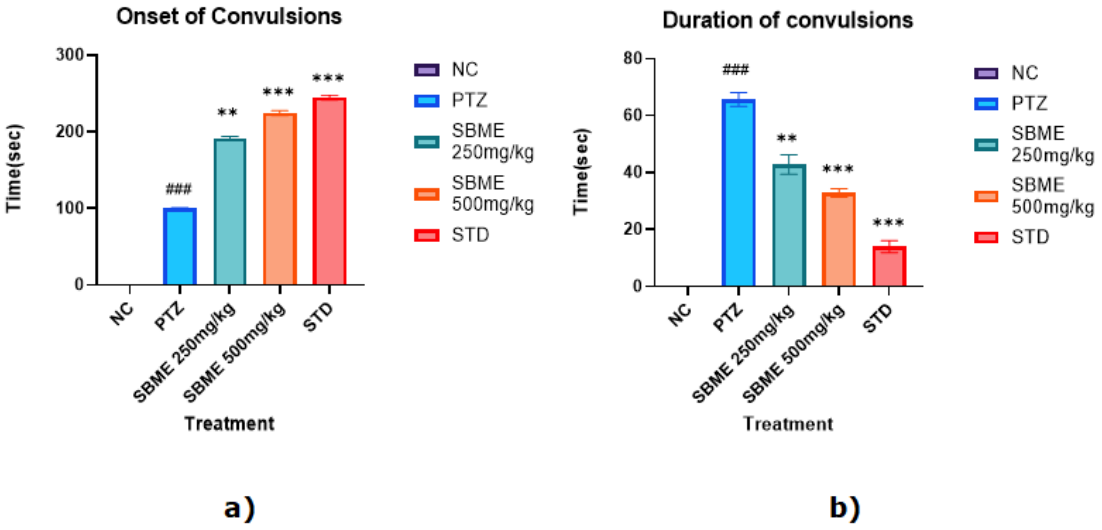

Figure 3: Impact of SBME on convulsions caused by PTZ (a) Onset, (b) Duration. For six animals each group, values are given as MEAN ± SEM. In compared with positive control, *p <0.05, **$p < 0.01$, ***$p < 0.001$, and $^{###}p < 0.001$ were found.

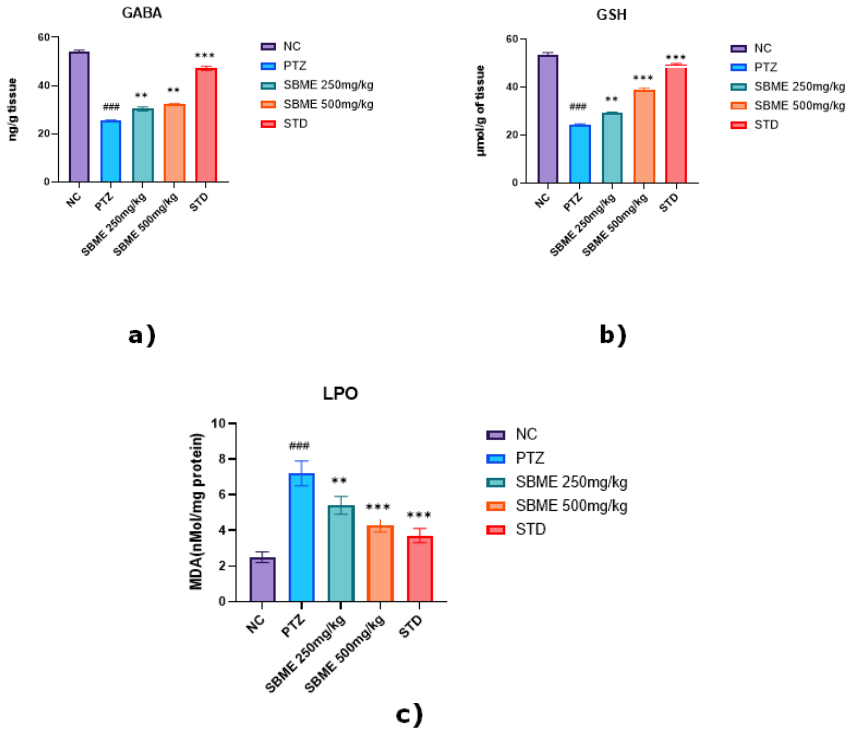

Figure 4: Impact of SBME on Brain (a) GABA Levels, oxidative stress markers (b), (c) (GSH and LPO). For six animals in each group, values are given as MEAN ± SEM. In comparison with positive control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and $^{###}p < 0.001$ were found

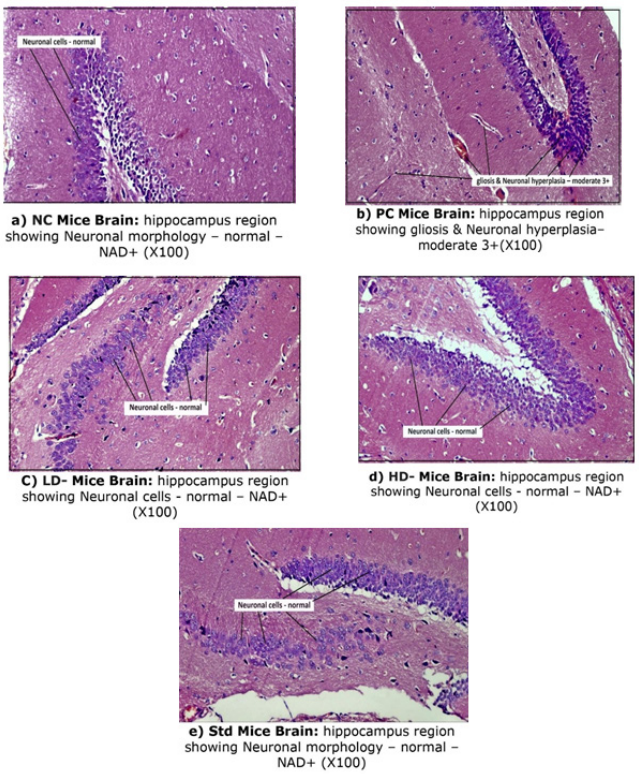

Figure 5: Mice brain tissue photomicrographs (100X) in the MES model. a) NC- Normal control; b) PC-Positive control (MES); c) LD-Low dose of SBME 250 mg/Kg; d) HD-High dose of SBME 500 mg/Kg; e) STD-Standard (Phenytoin 25 mg/kg)

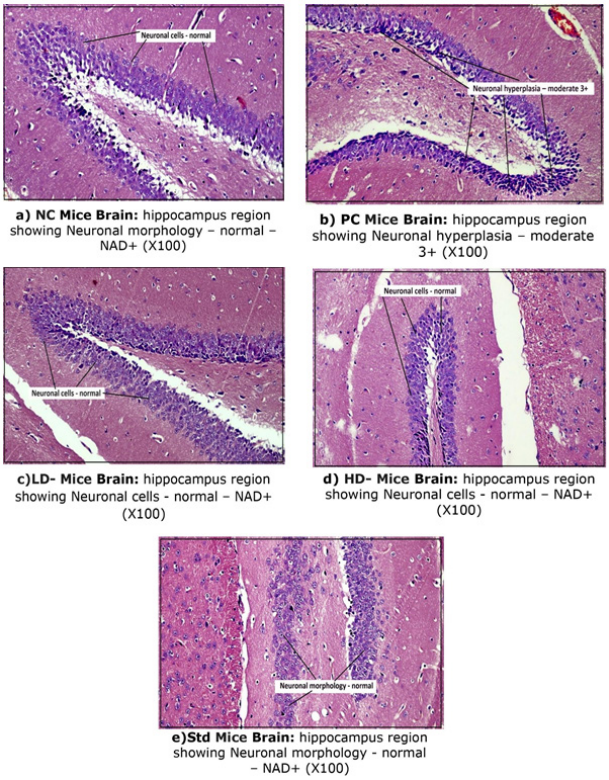

Figure 6: Mice brain tissue photomicrographs (100x) in the PTZ model. a) NC- Normal control; b) PC-Positive control (PTZ); c) LD- Low dose of SBME 250 mg/Kg; d) HD-High dose of SBME 500 mg/Kg; e) STD-Standard (Diazepam 10 mg/kg)

suggests SBME may modulate ion channels, similar to how phenytoin blocks voltage-gated sodium channels, reducing abnormal neuronal firing (Guerrini *et al*., 2023; Agbo *et al*., 2023). Amentoflavone, a bioactive compound in SBME, is likely responsible for this effect by interacting with sodium channels (Rong *et al*., 2019).

The PTZ-induced seizure model examines the influence of drugs on GABAergic neurotransmission. SBME delayed seizure onset and reduced seizure duration, with the higher dose (500 mg/kg) yielding effects comparable to diazepam. Biochemical analysis showed increased brain GABA levels, suggesting SBME enhances GABAergic transmission. Amentoflavone likely plays a role by modulating GABA-A receptors boosting inhibitory neurotransmission (Tariq *et al*., 2024; Monteiro *et al*., 2024).

Oxidative stress is a key contributor to epilepsy pathophysiology, as excessive reactive oxygen species cause neuronal damage and increase seizure susceptibility (Pant *et al*., 2024). SBME demonstrated strong antioxidant activity by reducing lipid peroxidation (LPO) levels and increasing glutathione (GSH) levels. These effects, along with reduced neuronal damage observed in histopathology, may be attributed to amentoflavone and other phenolic compounds (Ijaz *et al*., 2023).

The combined actions of SBME modulating sodium channels, enhancing GABAergic transmission, and reducing oxidative stress create a comprehensive neuroprotective effect, reducing seizure severity and protecting neurons from damage.

In summary, the antiepileptic effects of SBME in the MES and PTZ models suggest it works through multiple mechanisms, including sodium channel inhibition, GABAergic modulation, and antioxidant activity. Amentoflavone appears central to these effects, making SBME a promising candidate for natural epilepsy treatments. Future studies should isolate the specific active compounds and further investigate SBME's potential in clinical settings.

Conclusion

In conclusion, the SBME has shown considerable potential in protecting against seizures, indicating its possible application in managing both generalized tonic-clonic and absence seizures. However, these results are preliminary, and additional research is essential to validate its benefits and stability in preclinical animals and humans. Future work will focus on isolating specific bioactive compounds, like amentoflavone, to explore their role in the development of natural anticonvulsant treatments.

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