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Preliminary Phytochemical, Acute Oral Toxicity and Anticonvulsant Activity of the Seed Extract of *Brassica juncea*

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Authors' contributions

This work was carried out in the collaboration between both authors. Author NLBD designed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Author DTDT performed experiments, managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this research is to investigate the preliminary phytochemical properties, acute oral toxicity and anticonvulsant activity of the seed extract of *Brassica juncea* (*B. juncea*).
Study Design: Qualitative analysis of for phytochemicals was performed. Experimental mouse model was contributed for toxicity test and anticonvulsant activity of pentylenetetrazole (PTZ) - induced seizure.
Place and Duration of Study: Applied Biochemistry Laboratory, Department of Applied Biochemistry, School of Biotechnology, International University, Vietnam National University, Ho Chi Minh, between June 2014 and December 21014.
Methodology: Phytochemicals from the methanol seed extract were screened by standard methods. Acute oral toxicity study was conducted as per Organization for Economic Co-operation and Development (OECD) 425 guidelines while anticonvulsant activity was assessed against PTZ-induced seizure in mice. The effect of the extract at dose levels of 200, 300, 400 and 500 mg/kg body weight was evaluated in an experimental mice model, using diazepam as positive control (5

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mg/kg, *p.o*). At the end of the observation period, the animals were sacrificed and their brains were removed for histopathological examination.
Results: The phytochemical study showed the presence of alkaloids, flavonoids, saponins, tannins, terpenoids, and phenolic compounds in the seeds of *B. juncea*. The Acute oral toxicity study indicated that the extract was safe and non-toxic to mice up to 5000 mg/kg body weight. The extract significantly delayed the latency of convulsion ($p < 0.05$) induced by PTZ at the dose of 500 mg/kg *p.o.* The extract also reduced the frequency of convulsion and provided up to 100% protection (500 mg/kg *p.o*) against death.
Conclusion: The data suggest that the methanol seed extract of *B. juncea* is safe and possesses anticonvulsant activity in PTZ-induced seizure in mice.

Keywords: Brassica juncea; methanol extract; phytochemical; anticonvulsant, pentylenetetrazole, lethal dose; acute toxicity; histopathological analysis.

1. INTRODUCTION

Brassica juncea (*B. juncea*) is grown worldwide, especially central, and South Asia, belonging to the family of Brassicaceae [1]. It is an oilseed crop, mainly grown as a food crop and also used for its medicinal purposes. *B. juncea* has studied for treatment of diabetic cataract [2], anti-inflammatory, reduction of the frequency of migraine attacks [3], antioxidant activity [4], anti-nociceptive, and anti-hyperglycemic activity [5].

The brain is made up of millions of nerve cells called neurons. They generate electrical impulses and messages to control the human thoughts, emotions and movement. Epilepsy is a disorder of the brain that occurs when sudden bursts of electrical activity in the brain disrupt normal pattern of these impulses. This can cause changes in sensation, awareness and behavior, with or without body convulsion, muscle spasms or loss of consciousness, depending on where the seizure starts and spreads in the brain [6, 7]. There are three common mechanisms by which seizures can develop in either normal or pathologic brains: 1) Diminution of inhibitory mechanism (especially synaptic inhibition due to GABA); 2) Enhancement of the excitatory synaptic mechanism (especially those mediated by NMDA); and 3) Enhancement of endogenous neuronal burst firing (usually by enhancing voltage dependent calcium currents). Different forms of human epilepsy may be caused by any one or combination of the above mechanisms [8].

Epilepsy is among the most prevalent of the serious neurological disorders, The World Health Organization (WHO) estimates that eight people per 1000 worldwide have this disease. Interestingly, the prevalence of epilepsy in developing countries is generally higher than in developed countries [9]. Modern Antiepileptic Drugs (AEDs) are available as anti-epileptic agents but to have control over the seizures is very difficult because of their side effects, dose-related and chronic toxicity, teratogenic effects and large number of interactions [10,11]. Thus, it is necessary to investigate for an antiepileptic agent that is highly efficacious as well as safe in items of drug related toxicity. Medicinal plants used for the therapy of epilepsy in traditional medicine have been shown to possess promising anticonvulsant activities in animal models [12-16] with better safety and efficacy profiles. Our study was carried out to investigate a non-toxic plant which has a potential in treatment of epilepsy.

2. MATERIALS AND METHODS

2.1 Plant Materials

The seeds of *B. juncea* were provided and authenticated by agricultural Hi-tech park of Ho Chi Minh City. A voucher specimen was deposited in the herbarum of Applied Biochemistry laboratory, Department of Applied Chemistry, School of Biotechnology, International University, Viet Nam National University-Ho Chi Minh City, Viet Nam with voucher No HB-BIO-14-04-30.

2.2 Chemicals and Drugs

Pentylenetetrazole (PTZ) was purchased from Sigma-Aldrich (St. Spruce, Saint Louis, MO 63103, USA), Methanol was procured from Shanghai Demand Chemical Co., Ltd. Diazepam (5 mg, Vidipha Central Pharmaceutical Joint Stock Company, Viet Nam). All drugs/chemicals were prepared fresh in distilled water to the desired concentration.

2.3 Experimental Animals

Healthy Swiss mice *Mus musculus* var. *Albino*, weighing 25 - 30 g, were procured from Pasteur Institute of Ho Chi Minh City. They were housed in clean cages and had free access to standard pallet diet and water *ad libitum*. During the experiment, the mice were kept in a controlled environment of 12 h light/dark cycle. All the animals were acclimated to laboratory conditions for a week prior to commencement of the experiments. All the animal studies followed the guidelines enunciated in the "Guide for the Care and Use of Laboratory Animals", as well as specific national laws where applicable [17].

2.4 Preparation of Methanol Seed Extract of *B. juncea*

One hundred gram (100 g) of the powder from the grounded dried seeds of *B. juncea* was soaked in 250 mL of methanol for 48 hours with constant vigorous shaking. The extract was then filtered and evaporated in vacuum to get the concentrated crude extract. The crude extract was stored in a refrigerator until required for further use [18,19].

2.5 Phytochemical Screening

Phytochemical analysis of the extract was carried out for the detection of various constituents using standard methods [20-24]

2.6 Oral acute Toxicity Test

Acute toxicity of the plant extract was carried out as per the OECD guideline 425 (Up and Down method). A limit test was performed using healthy female albino rats weighing 175-200 g. Prior to dosing, animals were fasted overnight and the dose for each animal was determined based on body weight. The crude extract was suspended in distilled water [25].

Initially the extract was administered to one animal in a single dose of 5000 mg/kg by oral gavage using a feeding tube. After the administration, food was withheld for a further 3-4 hours. The animal was observed once during the first 30 minutes after dosing, then periodically, during the first 24 hours. As the animal was not died, 2 additional animals were given the same dose and observed similarly.

All the survived animals were then kept for a 14-day observation period. The LD_{50} was calculated using the software program-AOT425statpgm.

2.7 Determination of Anticonvulsant Activity

Mice were randomly divided in to six groups of five animals each. Group I served as control received an equivalent amount of distilled water, group II served as reference standard received diazepam 5 mg/kg body weight (*p.o.*), groups III, IV, V, and VI were treated with methanol extract as 200, 300, 400, and 500 mg/kg body weight (*p.o.*) respectively, 30 min after oral administration of diazepam and 60 min after oral administration of extracts, 85 mg/kg PTZ was injected intraperitoneally [14-16]. The animals were observed for 1 hour by placing in a separate cage. The onset of clonic convulsion was noted. The frequency of convulsions and number of death were also recorded. Manifestations of seizures were rated on a 6-point scale according to Racine's scale, which is widely used in studies on animal models of epilepsy (Table 1) [26].

2.8 Statistical Analysis

The Data were expressed as mean ± standard error mean (S.E.M). Analysis of the data was made using SPSS, version 16.0. One-way ANOVA and Paired sample T-test was used as tools for data analysis. p values less than 0.05 ($p < 0.05$) were considered as significance.

Table 1. Six-point scale for anticonvulsant activity

Light seizures	Intermediate seizures	Heavy seizures
0.5: Immobility, piloerection, salivation, narrowing of eyes, face and vibrissae twitching, ear rubbing with forepaws	**1.5**: Clonic movements of forelimbs and mild whole body convulsions, exophthalmia, aggressive behavior	**2.5**: Rearing and falling, eye congestion
1.0: Head nodding and chewing movements	**2.0**: Rearing and running with stronger tonic-clonic motions including hind limbs, tail hypertension, lock jaw	**3.0**: Loss of postural tone with general body rigidity

2.9 Histopathological Study

At the end of the observation period, the animals were sacrificed and the brain was immediately removed. The brain tissues were dehydrated with 4% sucrose for 24 hours and kept in buffered 10% formalin. Paraffin sections of 4-5 µm were then made and stained with hematoxylin and eosin (H&E) for microscopic examination [27,28]. Stained slides were observed for the situation of cerebral cortex, and the distribution of neurons. These observations were compared with the normal brain tissues.

3. RESULTS

3.1 Phytochemical Profile

Phytochemical analysis of the methanol seed extract of *B. juncea* revealed the presence of alkaloids, flavonoids, saponins, tannins, terpenoids, and phenolic compounds (Table 2).

3.2 Acute Oral Toxicity Study

Data collected from Acute Oral Toxicity test by Up – And - Down procedure was described in Table 3. After 14 days of observation, long-term outcomes were recorded and used to estimate LD_{50} value in Table 4**.**

Statistical estimate based on the long-term outcomes; estimated LD_{50} is greater than 5000 mg/kg (based on an assumed sigma of 0.5).

There was no mortality among the graded dose groups of animals and they did not show any toxicity or behavioral changes at a dose level of 5000 mg/kg of body weight. This finding suggests that methanol seed extract of *B. juncea* was safe and non-toxic to mice up to 5000 mg/kg.

3.3 Anticonvulsant Activity of Methanol Seed Extract of *B. juncea*

The results demonstrate that the methanol seed extract of *B. juncea* have anticonvulsant activity in PTZ-induced seizure models. Table 5 & Fig. 1 indicate that the methanol seed extract of *B. juncea* significantly delayed the latency of convulsions ($p < 0.05$) but it was not follow the dose-dependent manner. The latency in control group was 49.89±5.33 seconds. In group V and VI, 400 and 500 mg/kg of the extract markedly prolonged the latency of seizures to nearly double as compared to that of control group.

Notably, the results shown in Table 5 & Fig. 2 indicate a rapid decline in the frequency of convulsions by times. In control group, convulsion in tested mice was lasted for 7.2 ± 1.74 times. This number decreased slightly to 6.6±2.13 in group III (200 mg/kg), continued to decrease to 3.2±1.02 in group IV (300 mg/kg), and steepest to 2.6±0.51 in group VI (500 mg/kg).

As shown in Table 5, all the animals in control group died, while in group VI the survival was 100%. This is comparable to the values obtained for Diazepam group where non mortality was recorded. In group V, the percentage of protection was 60%, indicating the potential anticonvulsant activity of the extract at low concentration.

Table 2. Phytochemical constituents of the methanol seed extract of *B. juncea*

Phytochemical constituents	Phytochemical test	Inference
Alkaloids	*Wagner's test, Dragendroff's test, Hager's test*	**+**
Flavonoids	*Ammonium test, Aluminum Chloride test, Lead acetate test, Ferric Chloride test*	**+**
Saponins	*Frothing test*	**+**
Steroids	*Lieberman-Burchard's test*	**-**
Tannins	*Ferric Chloride test, Lead acetate test*	**+**
Terpenoids	*Salkowski test*	**+**
Glycosides	*Borntrager's test*	**-**
Carbohydrates	*Fehling's test*	**-**
Phenolic compounds	*Ferric Chloride test*	**+**
Oil and fat	*Sudan(III) test*	**-**

**+ indicates positive test result*; *- indicates negative test result*

Table 3. Dose progression and results

Test Seq.	Animal ID	Dose (mg/kg)	Short-term result	Long-term result
1	1	5000	O	O
2	2	5000	O	O
3	3	5000	O	O

**X: Died.O: Survived*

Table 4. Summary of long-term results

Dose	O	X	Total
5000	3	O	3
All Doses	3	O	3

Table 5. Effect of methanol seed extract of *B. juncea* on PTZ-induced seizure mice

Group (n=5)	Latency of convulsion (second)	Frequency of convulsion (time)	No. of convulsion	No. of deaths	Protection (%)
Control	49.89±5.33	7.2±1.74	5	5	0
III	57.61±8.45	6.6±2.13	5	4	20
IV	78.26±6.33	3.2±1.02	5	3	40
V	81.83±8.66	5.6±1.17	5	2	60
VI	98.07*±15.19	2.6*±0.51	5	0	100
Diazepam	80.40±20.20	1.60±0.40	5	0	100

**Each value represents mean ± SEM; * p < 0.05 compared with the control*

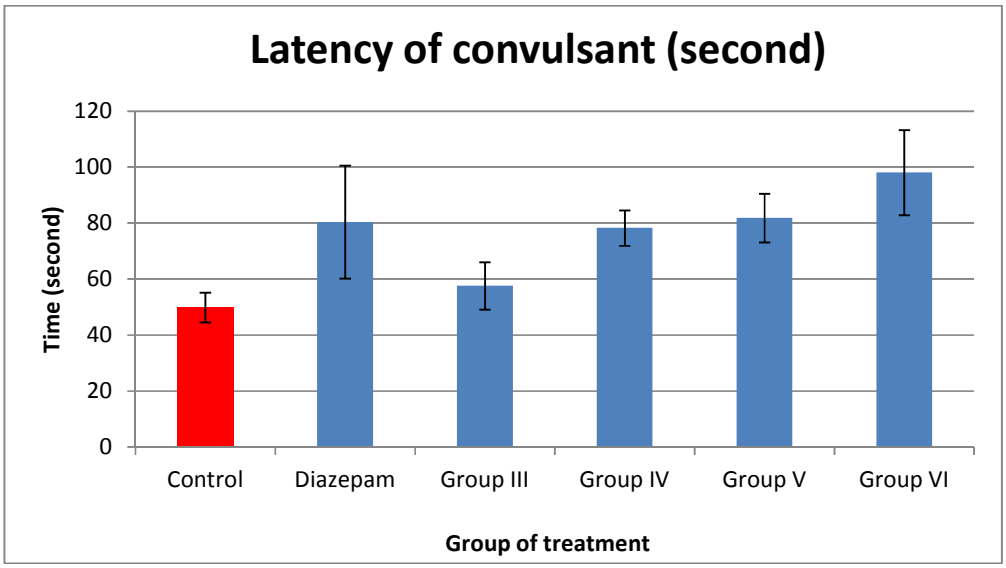

Fig. 1. Effect of different doses of methanol seed extract of *B. juncea* (200, 300, 400, 500 mg/kg) on the latency of convulsions in mice

3.4 Histopathological Study

Histopathological studies provide supportive evidence for the biochemical analysis. PTZ is often used in experimental models of epilepsy, and it is known that PTZ causes neuronal damage on the cerebral cortex (C). The neurons in the cerebral cortex of brain section of normal mouse revealed no abnormal histological appearance (Fig. 3A1). In contrast, most of the cortical neurons in the negative control group showed severe degenerative changes, including: decrease in size and number of neurons, shrinkage of nuclei, degeneration of meninges,

and a number of neurons' nuclei had been completely karyorrhexis, pyknosis, and karyolysis in comparison with brain section of normal mouse (Fig. 3B1). Compared to negative control group, brain section of male treated with methanol seed extract of *B. juncea* (200 mg/kg and 300 mg/kg) showed significant changes, number of injured cells are slightly decreased, just remained pyknosis, and karyorrhexis nuclei. Simultaneously, the meninges are moderately impacted as compared to that of negative control group (Figs. 3 C1, D1). The above phenomena have really plunged in group V (treated with methanol seed extract of *B. juncea* 400 mg/kg). There were no karyorrhexis or karyolysis and just existed a small number of pyknosis (Fig. 3 E1). Electron microscopy revealed that neuronal architecture was nearly intact in the *B. juncea* treated group (500 mg/kg) and positive control group (Figs. 3F1, G1). The histopathological analysis indicated the instrumental efficiency of the methanol seed extract of *B. juncea* in reducing seizures not only phenomena of convulsant but also the impact on nerve cells.

4. DISCUSSION

The results of the present study indicate that methanol seed extract of B. *juncea* possesses anticonvulsant activity on PTZ-induced seizures in mice. The mechanism by which PTZ is believed to exert its anticonvulsant effect is by acting as an antagonist at the $GABA_A$ receptor complex [29,30]. GABA is the major inhibitory neurotransmitter in the brain which is implicated in epilepsy. The enhancement and inhibition of the neurotransmission of GABA attenuates and enhances convulsion. Standard antiepileptic drugs like diazepam can prevent seizures induced by PTZ by enhancing GABA mediated inhibition in the brain [31,32]. Since the extract delayed latency and reduced the reoccurrence of PTZ convulsions, it is probable that it may be by interfering with GABA mechanism to exert its anticonvulsant effect.

Preliminary phytochemical analysis performed showed that the tannins, saponins and flavonoids are the major components of the extract. There are some evidences about anticonvulsant effect of some flavonoid compounds [33]. Flavonoids are ligands for benzodiazepine binding sites on $GABA_A$ receptors. It shows that anticonvulsant effects of some natural and synthetic flavonoids exerted their action through the central benzodiazepine receptors in mice [34]. Therefore, it seems that the anticonvulsant effect of *B. juncea* may be related in part to flavonoid compounds displayed in the extract.

Histopathological examination has showed reasonable results, providing an enormous potentiality for further research on the effects of *B. juncea* seed extract of anticonvulsant activity, especially in field of histopathological analysis. The further studies should focus on the impact of the extract in others part of brain and should conduct chronic test.

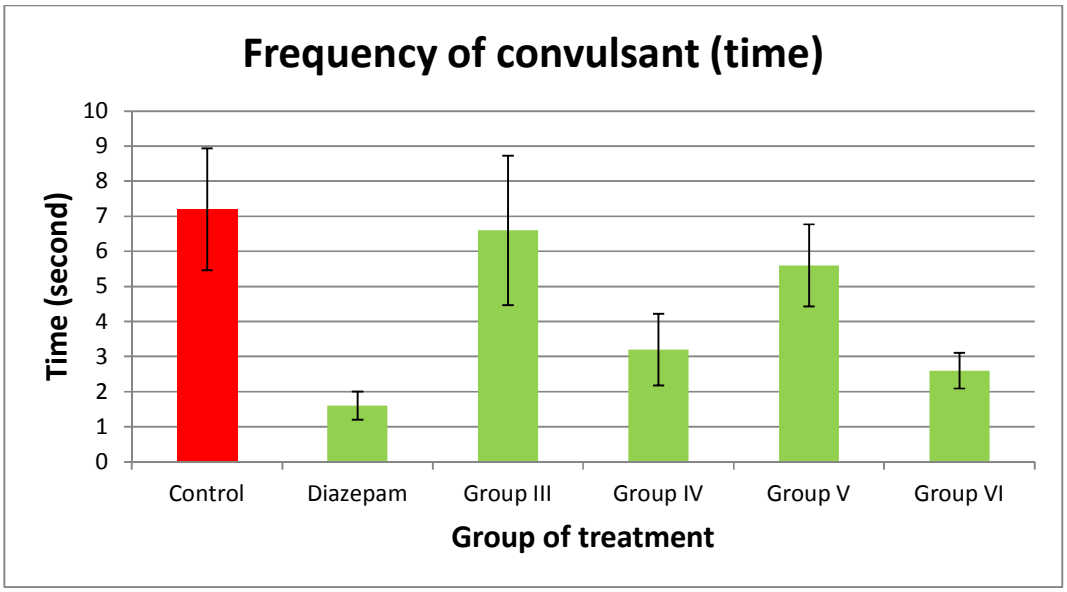

Fig. 2. Effect of different doses of methanol seed extract of *B. juncea* (200, 300, 400, 500 mg/kg) on the frequency of convulsions in mice

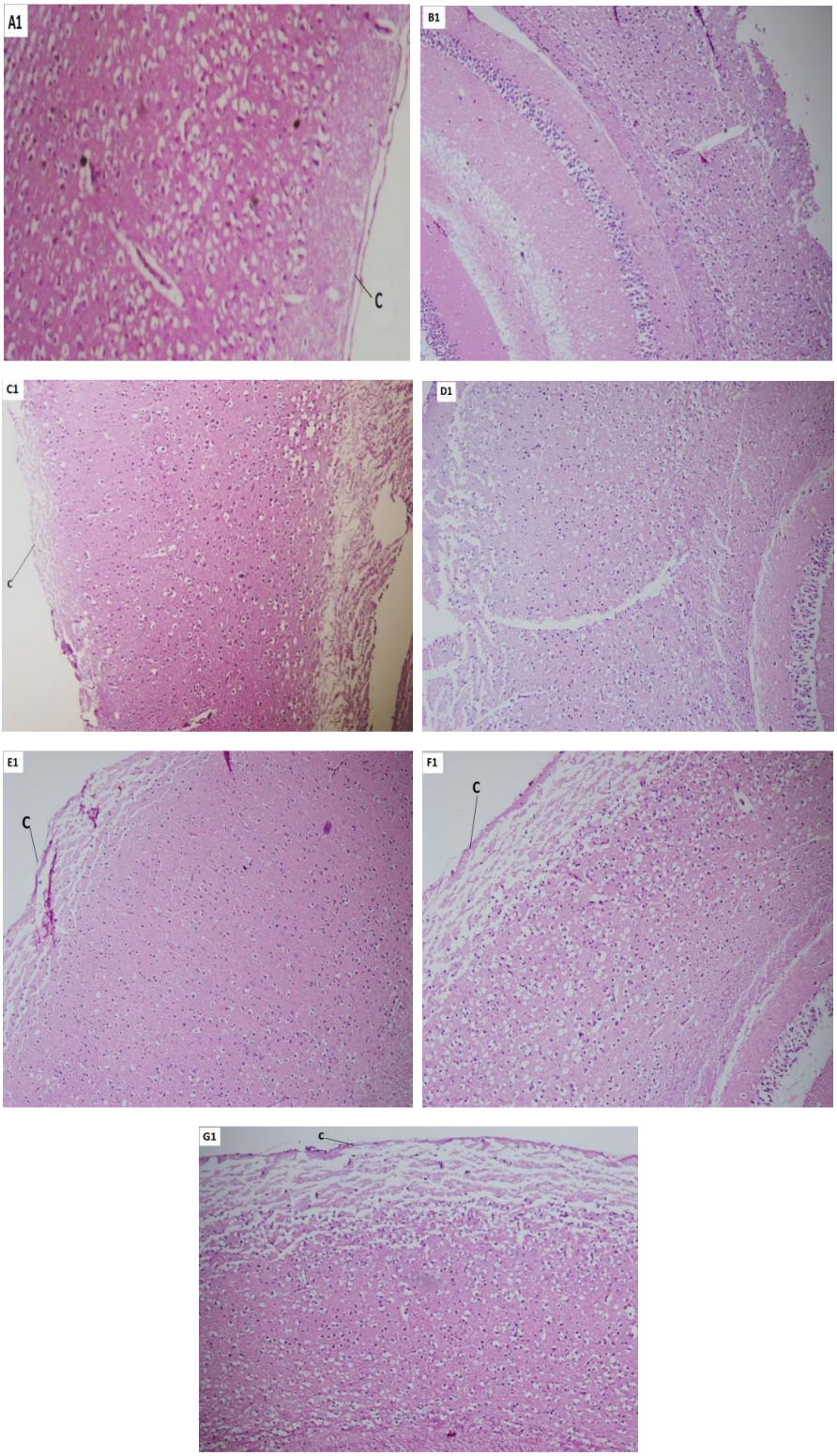

Fig. 3. Histological features of mouse brain treated with the extract

(A1) Normal brain; (B1) Negative control; (C1) 200 mg/kg; (D1) 300 mg/kg; (E1) 400 mg/kg; (F1) 500 mg/kg; and (G1) positive control (section stained with H&E, x100)

5. CONCLUSION

From the above study, it was concluded that the methanol seed extract of *B. juncea* possess a significant anticonvulsant activity against PTZ-induced seizure in mice. These findings provide a basis for further pharmacological investigations; it also suggests the application of *B. juncea* in the treatment of convulsive disorders as a need of modern health science.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments were conducted in accordance with animal use ethics as accepted internationally.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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