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Original Paper

Protective Effects of Betulinic Acid on Pentylenetetrazol-Induced Seizures in Rats

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Introduction: Seizure is one of the most important diseases of the Central Nervous System (CNS) that affects about 1% to 2% of humans. Seizure is caused due to a symmetrical electrical discharge in a group of neurons in the CNS. Betulinic Acid (BA) is known as a potent antioxidant with different effects that have been mentioned in many research studies. In this study, the impact of BA was evaluated on pentylenetetrazol-induced seizure.

Materials and Methods: In this study, 48 male Wister rats were used weighing between 215 and 250 g. The animals were randomized into six groups, each consisting of eight rats. Treatment groups were treated with different doses of BA (25, 50, and 100 mg/kg); positive and negative control groups received phenobarbital (80 mg/kg) and normal saline (10 mg/kg), respectively, 30 minutes before pentylenetetrazol injection (50 mg/kg, intraperitoneally). Then, parameters such as convulsion time, latency to convulsion, pain, passive avoidance memory, and antioxidant status were studied.

Results: The results revealed that BA possesses dose-dependent influence and the dose of 100 mg/kg has the maximum effect for increasing the latency to convulsion and reducing the convulsion time. Moreover, BA significantly reduced oxidative damage in the rats' brain tissue compared with the PTZ-kindled group.

Conclusion: Based on the results obtained in this study, BA can effectively control pentylenetetrazol-induced seizures.

Keywords: Pentylenetetrazol, Seizure, Betulinic acid, Rats

Introduction

Epilepsy is the term for a set of conditions described by intermittent impulsive seizures. Up to 5% of the world's population suffers from epilepsy (Hosseinzadeh and Sadeghnia, 2007). Numerous theories have been put forth to elucidate the reason for primary or idiopathic epilepsy, such as changes in some typical neurotransmitter systems, such as glycine, glutamatergic, and GABAergic neurotransmitter systems (Engelborghs et al., 2000, Ure and Perassolo, 2000). Several molecules such as opioids and nitric oxide have been proposed as possible neurotransmitters or backward messengers, but none of these explanations is entirely satisfactory (Rakhade et al., 2012, Rowley et al., 2012). Pentylenetetrazol (PTZ), a selective blocker of the chloride channel attached to the $GABA_A$ receptor complex, is the most common chemoconvulsant utilized for the assessment of Antiepileptic Drugs (AEDs) (Dhir, 2012). An adequate high dose of PTZ can yield a range of seizure activities that vary from slight myoclonic jerks to face and forelimbs clonus without loss of righting reflex which is identified as Minimal Clonic Seizure (MCS) to clonic seizures of limbs with loss of righting reflex) to a full tonic extension of both forelimbs and hind limbs (generalized as tonic- Generalized Tonic-Clonic Seizures (GTCS) (Koutroumanidou et al., 2013). Oxidative stress in the central nervous system has been observed in several rodent models of experimental epilepsy, like the amygdala-kindling model, the kainic acid model, the PTZ-kindling model, and acute PTZ-induced seizures (Obay et al., 2008). Oxidative stress is a toxicological condition that happens in the body and stimulates various sorts of reactive species, including Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) (Ermak and Davies, 2002). Oxidative stress is considered as one of the pathological processes that result in the onset and development of central nervous system injury by forcing a permanent change

in lipid membranes, proteins, DNA, and more significantly, controlling paths that control biological tasks (Scannevin et al., 2012). The present treatment of epilepsy by recent Antiepileptic Drugs (AEDs) is accompanied by adverse effects and chronic toxicity, and teratogenic effects. In other words, with the existing AEDs treatment, about 30% of the patients still suffer from seizures (Perucca and Gilliam, 2012). Betulinic Acid (BA) is a pentacyclic triterpenoid commonly found in fruit shells, leaves, and stem barks. BA is primarily recognized for its anti-tumor and anti-inflammatory properties (Lu et al., 2011). Based on the theory that oxidative stress is implicated in epilepsy, some methods designed for decreasing such stress can help stop or reduce the progression of seizures. In recent years, a growing body of evidence has pointed to a possible antioxidant role for BA (Lu et al., 2011, Fu et al., 2011). Therefore, the present study was conducted to evaluate the BA's effect on seizures elicited by PTZ in rats. The potential antioxidant effects of BA were considered as well.

Materials and Methods

Chemicals

Pentylenetetrazol (PTZ) was obtained from Sigma, and phenobarbital was obtained from Tolidaru Pharmaceuticals (Tehran, Iran). Thiobarbituric Acid (TBA), Bovine Serum Albumin (BSA), and Bradford reagent were bought from Sigma Company (Sigma-Aldrich Company, St. Louis, MO, USA). All substances and reagents were of analytical grade. Betulinic acid was acquired from Roche Company (Germany).

Animals

Adult male Wistar rats weighing 215-250 g were obtained from the animal house of Ahvaz Jundishapur University of Medical Sciences (AJUMS). The rats were held in polypropylene cages and had ad libitum access to standard diet and tap water. The animals were kept under a controlled temperature (20±2°C) in a 12 h light, 12 h dark cycle. The animals had a minimum adaptation period of one week before the experiments. The experiments were done in conformity with the Animal Ethics Committee's guidelines for using experimental animals approved by AJUMS (IR.AJUMS.REC.1395.138).

Study Design

To conduct the tests, the animals were divided into 6 groups and then weighed, numbered, and treatment administered. The treatment groups were intraperitoneally injected with 25, 50, and 100 mg/kg (in 10 mL/kg normal saline) of BA. The negative and positive controls were treated with saline (10 mL/kg) and phenobarbital (80 mg/kg), respectively, through intraperitoneal injections. The PTZ group only received PTZ (50 mg/kg) without any treatment. Thirty minutes after BA or saline injection, PTZ was injected intraperitoneally to all groups. This process was followed by measuring the convulsion time, latency to convulsion, pain, and passive avoidance memory.

Dose-Response Study (Seizure Onset and Duration of Seizure)

PTZ was solved in 0.9% saline and injected Intraperitoneally (IP). After PTZ injection, each animal was individually caged, and the intervals between PTZ injections and the first observation of convulsion symptoms were measured to determine the time of onset. The time of seizure was calculated as the interval between the beginning and the end of convulsions.

Tail-Flick Test

An automatic tail-flick algesiometer (U. Basile, Comerio, Italy) was utilized to measure the reaction latencies, according to the original method of D'Amour and Smith. The light beam was focused on the animal's tail almost 4 cm from the tip, and the power was fine-tuned, so baseline digits were between 2 and 3 s (D'AMOUR and SMITH, 1941).

Step-Through Passive Avoidance Task

Passive avoidance performance was carried out in two identical light and dark square boxes (20×20×20 cm) separated by a sliding door (5×5 cm), as described by Kim et al. The illuminated compartment contained a 50 W bulb, and the floors of both compartments were composed of 2-mm thick stainless steel rods spaced 1 cm apart. The rats were initially placed in the illuminated compartment for the acquisition trial, and the door between the two compartments was opened 10 s later. Immediately after rats entering the dark compartment, the door was automatically closed, and an electrical foot shock (0.5 mA for 3 s) was delivered through the stainless steel rods. To test memory, after 24 h, each rat was placed in the light chamber again, and after 10 s, the sliding door was opened, and the delay before entering the dark compartment was recorded as memory latency (step-through latency). The maximum time considered in the passive avoidance test was 300 s (Kim et al., 2007).

Sample Collection

After measuring the convulsion parameters, the rats were put under deep anesthesia by ketamine/xylazine (90/10 mg/kg). The animals were decapitated, and their brains were removed and rapidly rinsed with cold saline. Then the brains were weighed and homogenized for tissue antioxidant evaluations.

Estimation of Oxidative Stress Parameters of Tissues

The brain tissues were sliced into tiny pieces with scissors and homogenized in cold phosphate buffer (pH=8) at 10% (w/v) concentration (weight by volume) by a homogenizer (Heidolph, Germany). The uninterrupted cells and their fragments were moved using a refrigerated centrifuge (1000 rpm, 10 min, 4°C) (Hettich Zentrifugen, Germany). Protein content in the homogenates was estimated by Bradford's method (Bradford, 1976), and the crystalline BSA was used as the standard. The supernatant was used to assess the Malondialdehyde (MDA), Superoxide Dismutase (SOD), and Glutathione Peroxidase (GPx) by standard methods.

Lipid Peroxidation Assays

The peroxidation of lipid was assessed by determining the MDA amount by the Thiobarbituric Acid (TBA) color reaction utilizing the method depicted by Buege and Aust. In short, 0.5 mL of brain supernatant was mixed with 1.5 mL of Trichloroacetic acid (TCA, 10%, w/v); the samples were centrifuged (4000 rpm, 10 min), and 1.5 mL of every supernatant sample were moved to a test tube that included 2 mL of TBA solution (0.67%, w/v). It was kept in boiling water for 30 min, creating a pink solution. Then the combination was cooled instantly, and the absorbance was measured at 532 nm by a spectrophotometer. The MDA concentration was computed upon the absorbance coefficient of the TBA–MDA compound ($\varepsilon=1.56\times10^5$ cm^{-1} M^{-1}) and stated as

nmol/mg protein [17].

Superoxide Dismutase Activity (SOD) Assay

The activity of SOD was measured by a calorimetry ELISA assay kit (Zell Bio, GmbH, Ulm, Germany). In this test, the SOD activity was stated as units per microgram of brain protein.

Glutathione Peroxidase (GPx)

GPx activity was measured by Fecondo and Augusteyn's method (Ma et al., 2010). The method was based on the indirect measurement of GPx activity. Glutathione disulfide (GSSG) produced by GPx is reduced at a constant rate by GR with NADPH (Sigma-Aldrich, USA) as a cofactor. The oxidation rate of Nicotinamide adenine dinucleotide phosphate (NADPH) was observed to be 340 nm. The results were reported as U/g protein.

Statistical Analysis

The obtained data were expressed as Mean±SEM, and all statistical assessments were conducted by a one-way ANOVA test followed by Tukey's post hoc analysis. P values less than 0.05 were considered significant.

Results

Convulsion Parameters

The average delay in the onset of seizures was significantly increased with 100 mg/kg of BA compared to the PTZ group (P<0.05). Besides, the average interval in the onset of seizures on the doses of 25 and 50 mg/kg did not show a significant increase compared to the PTZ group (Figure 1A). The convulsion times in groups injected with BA in doses of 50 and 100 mg/kg were significantly decreased compared with the positive control group (P < 0.05, Figure 1B).

Tail-Flick Test

PTZ was administered intraperitoneally (50 mg/kg) to produce an increment in tail-flick reaction latency compared with saline control animals (Figure 2). However, this increase was not significant. The tail-flick response decreased in the group that received BA. Moreover, the tail-flick response in the group injected with 50 mg/kg BA significantly reduced compared with the PTZ group (P<0.05).

Effect of Betulinic Acid on Step-Through Passive Avoidance Task

PTZ was administered intraperitoneally (50 mg/kg) to produce a significant decrease in step-through latency task compared to the saline control animals (P<0.05, Figure 3). Administration of BA and phenobarbital increased step-through latency. Moreover, this latency was significantly increased in groups injected with 100 mg/kg BA and phenobarbital compared to the PTZ group (P<0.001, P<0.01, respectively).

Effect of Betulinic Acid on Oxidative Stress

For additional evaluation of the probable mechanism engaged with the protecting influence of BA on PTZ-induced seizures, MDA formation and antioxidant enzymes (SOD and GPx) were evaluated. Antioxidant enzymes play a prominent role in resistance against the toxicity induced by free radicals. Figure 4A presents the brain rates of oxidant/antioxidant stress indicators in the non-kindled and kindled rats. Compared with other groups, PTZ kindling (PTZk) created a substantial rise in the MDA content of brain tissue, which is a sign of lipid peroxidation. The PTZk-induced increase in MDA content of the tissue was significantly restricted by BA action. Moreover, the groups that received 25 and 100 mg of BA exhibited a significant decrease in MDA compared with the PTZk group (P<0.05).

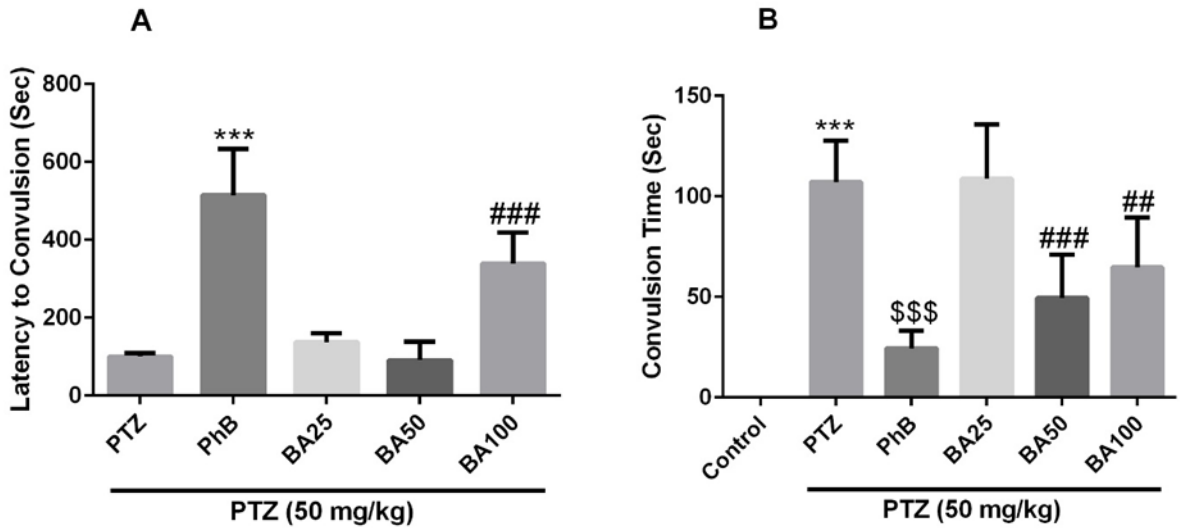

Figure 1: Comparison of latency to convulsion (A) and average convulsion time (B) in various groups
Abbreviations: PTZ: Pentylenetetrazol; PhB: Phenobarbital; BA: Betulinic Acid (25, 50, and 100 mg/kg).
In Figure 1A: ***Significant difference (P<0.001) between the PhB group and the PTZ group (P<0.001).
###Significant difference (P<0.001) between the BA100 group and the PTZ group (P<0.001).
In Figure 1B: ***Significant difference between the PTZ group and the control group (P<0.001).
$$$Significant difference between the PhB group and the PTZ group (P<0.001).
& ### There are significant differences between the BA50, BA 100, and PTZ groups (P<0.01 and P<0.001, respectively).
Statistical analyses were performed by one-way ANOVA followed by Tukey's post hoc test. Values are presented as the mean±SEM (n-8).

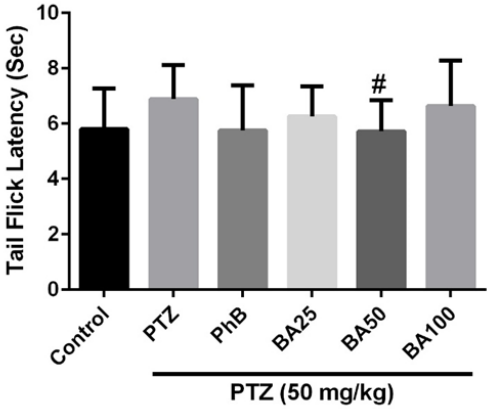

Figure 2: Comparison of average tail-flick latency in various groups

Abbreviations: PTZ: Pentylenetetrazol; PhB: Phenobarbital; BA: Betulinic Acid (25, 50, and 100 mg/kg). There were no significant differences between various groups, except between BA50 and PTZ groups (#P<0.05). Statistical analyses were performed by one-way ANOVA followed by Tukey's post hoc test. Values are presented as the mean±SEM (n-8).

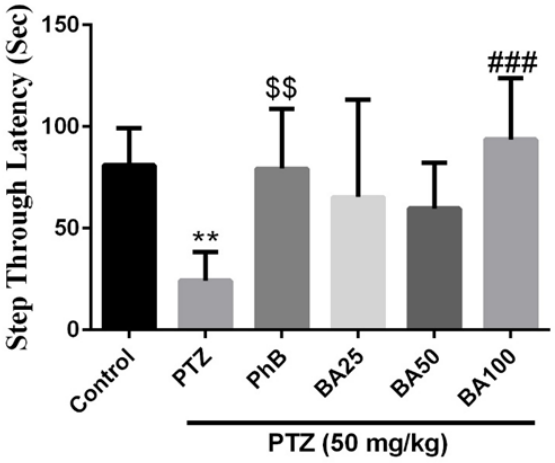

Figure 3: Comparison of average step-through latency in various groups

Abbreviations: PTZ: Pentylenetetrazol; PhB: Phenobarbital; BA: Betulinic Acid (25, 50, and 100 mg/kg).

****Significant difference (P<0.01) between the control group and the PTZ group. $$Significant difference between the PhB group and the PTZ group (P<0.01). ###Significant difference between the BA100 group and the PTZ group (P<0.001). Statistical analyses were performed by one-way ANOVA followed by Tukey's post hoc test. Values are presented as the mean±SEM (n-8).**

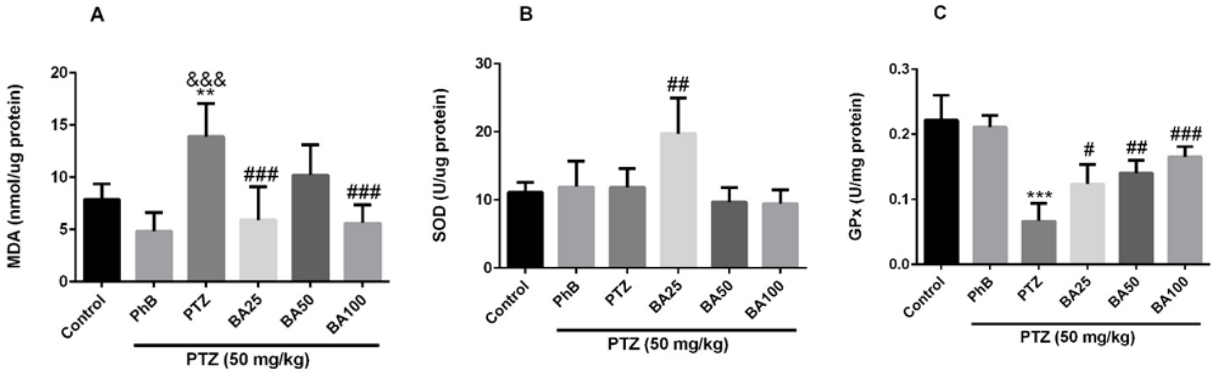

Figure 4: Comparison of average MDA (A), SOD (B), and GPx (C) between various groups.

Abbreviations: PTZ: Pentylenetetrazol; PhB: Phenobarbital; BA: Betulinic Acid (25, 50, and 100 mg/kg).

In Figure 4A: **Significant difference (P<0.001) between the control group and the PTZ group (P<0.001).

&&&Significant difference between PhB group and PTZ group (P<0.001). ###Significant difference (P<0.001) between BA25 and BA100 groups with the PTZ group (P<0.001).

In Figure 4B: ##Significant difference between the BA25 group and the PTZ group (P<0.01).

In Figure 4C: ***Significant difference between the PTZ group and the control group (P<0.001).

#, ## & ### Significant differences between BA25, BA50, and BA100 groups and the PTZ group (P<0.05, P<0.01, and P<0.001, respectively).

Statistical analyses were carried out by one-way ANOVA followed by Tukey's post hoc test. Values are presented as the mean±SEM (n-8).

The MDA rates of brain tissues in the treated groups stayed close to the control group.

Superoxide Dismutase (SOD) activity was nearly similar in all the groups except group 4, which was pretreated with 25 mg of BA. This group showed a significant increase ($P<0.05$) in the SOD level (Figure 4B). There was a fundamentally lower amount of GPx in the PTZk group compared with the others (Figure 4C, $P<0.05$). BA-treated groups demonstrated significantly greater levels of GPx compared with the PTZk group ($P<0.05$). Additionally, this increase was dose dependent.

Discussion

In the present study, PTZ deceased latency to convulsion, produced seizures, decreased memory, and increased oxidative stress in all rats. In animals that received BA, the convulsion time decreased, and the latency to convulsion increased. In the treated groups, resistance to pain increased, and memory improved in the passive avoidance task (Figures 1-3). These findings (decreased convulsion time and increased latency to convulsion) indicated the anticonvulsant properties of BA. Also, PTZ increased oxidative stress in the whole brain; and treatment with BA increased the antioxidant capacity of the brain.

With regard to the amelioration of antioxidant parameters (Figure 4), it was concluded that this effect might be related to the antioxidant property of BA. This result is consistent with the study of Rajaei et al., which showed that antioxidant and anti-inflammatory agents could improve aversive memory in the oxidative damage of the brain (Rajaei et al., 2016). In another study, Manigandan and Ramanathan studied mangrove-derived ligands such as avicenol A, BA, and lupeol against Alzheimer receptor proteins. Their findings showed that caspase inhibitors, antioxidants, and anti-inflammatory agents, such as BA and triterpenoid, could effectively be used to treat Alzheimer disease (Manigandan and Ramanathan, 2014).

Growing evidence has shown that cellular injury aided by oxidative stress contributes to the beginning and development of several brain diseases. Numerous research studies have been completed to improve supplementary and alternate drugs for decreasing oxidative stress and refining brain tasks. Beneficial agents from widely-accepted sources, for example, BA, are mainly effective for the treatment of oxidative stress states since they have the potential and capacity to decrease the noxiousness of drugs (Lu et al., 2011).

Oxidative stress in the central nervous system has been found in some rodent models of experimental epilepsy, for instance, the PTZ-kindling model (Rauca et al., 1999). The PTZ-kindling model is considered an improved vulnerability to seizures after injection of primarily sub-convulsive dosages of PTZ terminating in general tonic-clonic seizures. PTZ is a particular blocker of the chloride ionophore complex of the $GABA_A$ receptor, and later repetitive or single-dose injection results in a reduction in the GABAergic role (Ilhan et al., 2005) and to the motivation and adjustment of the compactness or sensibility of diverse glutamate receptor subtypes in various brain areas (Schroeder et al., 1998). PTZ may also activate different biochemical procedures, including the activation of membrane phospholipases, proteases, and nucleases (Dhir, 2012). Noticeable changes in membrane phospholipid metabolism lead to the release of Free Fatty Acids (FFAs), diacylglycerols, eicosanoids, lipid peroxides, and free radicals (Ilhan et al., 2005).

Both enzymatic and non-enzymatic antioxidant systems are crucial for cellular reaction to manage oxidative stress in the physiological state. Thus, an increment in different oxidative stress markers such as MDA and a reduction in another antioxidant such as Glutathione (GSH) and antioxidant enzymes comprising CAT and SOD intensely support the presence of oxidative stress impairment (Zhu et al., Dey and Lakshmanan, 2013, Karabulut et al., 2010). Antioxidant enzymes (SOD, GPx, and CAT) are the main line of defense against oxidative tissue injury (Rodriguez et al., 2004). SOD has an essential role in purifying toxic intermediates and changing O_2 into H_2O_2. Furthermore, CAT breaks down H_2O_2 into non-poisonous products (Wu et al., 2016). MDA has been utilized as a marker of lipid peroxidation for years and has remained one of the lipid peroxidation items. It has been used as an easy tool for evaluating lipid peroxidation in biological resources (Draper and Hadley, 1990). Some investigations have established that the deactivation of free radicals by antioxidant agents may decrease the quantities of PTZ oxidative injury and, along these lines, defend the brain (Rahmati et al., 2013, Kiasalari et al., 2012).

This study backs up this theory that PTZ-induced seizure action concurs with higher oxidative stress in brain tissue. The raised amount of MDA, a biomarker of lipid peroxidation, reveals augmented free radical production in the Pentylenetetrazol-kindled (PTZk) rats. The PTZk-induced increase of the MDA amount in the brain tissue was considerably averted by BA action. Thus, the considerably lower amounts of MDA in the brain of BA+PTZk rats compared with PTZk rats specifies the reduction of lipid peroxidation. There was a concurrent diminution in the GPx action in PTZk rats. GPx is an intrinsic antioxidant enzyme, and it acts in response to the free radicals and inhibits the generation of hydroxyl radicals, the greatest toxic form of free radicals. Throughout this protective procedure, Glutathione peroxidase (GSH-Px) changes reduced glutathione to its oxidized form. The reduced GPx action in PTZk rats observed in this study specifies that there was an expanded generation of free radicals, and the reduced GPx was exhausted through the way of opposing oxidative stress. The reduction in MDA content and increment in the SOD and GPx actions in the BA+PTZk group might result from the antioxidant quality of BA.

Many researchers have studied the effects of natural products on chemically-induced seizures. In a similar study, Souza et al. studied the antioxidant activity of caffeine in PTZ-induced convulsions and oxidative injury in rats. Their results demonstrated that a chronic rather than acute (single dose) caffeine regime reduced the length of PTZ-induced convulsions in mature male Wistar rats as documented via cortical Electroencephalographic (EEG) and behavioral studies (Souza et al., 2013).

Conclusion

The present study's findings showed that BA, with its antioxidant activity, is an anticonvulsant compound. It is further concluded that the possible protective effect of BA might be the result of its free radical-scavenging, antioxidant activity, and inhibition of lipid peroxidation.

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Conflict of interest: The authors declared no conflict of interest.

Ethical Permission: The experiments were done in conformity with the Animal Ethics Committee's guidelines for using experimental animals approved by AJUMS (IR.AJUMS.REC.1395.138).

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Analysis and interpretation of data: Yaghoob Farbood, Seyyed Ali Mard
Drafting of the manuscript: Rasool Baitsayyah, Yaghoob Farbood
Statistical analysis: Seyyed Ali Mard, Seyyed Ali Mard
Study supervision: Alireza Sarkaki

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