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Effect of *Stachys lavandulifolia* on Pentylenetetrazole-Induced Seizures, Passive Avoidance Learning, and Oxidative Stress in Male Wistar Rats

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Abstract

Objective: Mountain tea with the scientific moniker of *Stachys lavandulifolia* has been used in traditional medicine for osteoarthritis and rheumatic disorders. Its leaf extract has also been adopted to treat epilepsy and other central nervous system disorders. To evaluate the effect of *S. lavandulifolia* extract, the experimental model of memory impairment caused by seizures was performed in rats.

Methods: Seizures in male Wistar rats (200–250 g) were induced with a challenge dose (60 mg/kg) of pentylenetetrazole. Animals were treated with either *S. lavandulifolia* extract (50 mg/kg) or sodium valproate (100 mg/kg) alone or in combination intraperitoneally 30 minutes before pentylenetetrazole administration. The rate of memory was assessed using the passive avoidance test. At the end of the experiments, the rats were put down painlessly via deep anesthesia, and their blood sera were isolated to assess antioxidative factors such as nitric oxide, catalase, and superoxide dismutase.

Results: Pentylenetetrazole-treated group revealed a memory deficit compared with the control group. *S. lavandulifolia* extract exhibited protection at the dose of 50 mg/kg in pentylenetetrazole-induced seizures. *S. lavandulifolia* extract also reduced seizure-induced memory impairment, which resulted in a significant improvement in memory retrieval in passive avoidance compared with the pentylenetetrazole-treated group. In addition, *S. lavandulifolia* extract treatment protects the seizure-induced memory deficit by lowering nitric oxide levels and restoring the antioxidant enzyme catalase and superoxide dismutase level.

Conclusion: The findings revealed that *S. lavandulifolia* extract exhibits significant inhibitory activity and impedes memory impairment by inhibiting oxidative stress damage.

Keywords: Learning and memory; oxidative stress; passive avoidance; seizure; *Stachys lavandulifolia*

INTRODUCTION

Seizure is a condition in which nerve cells make sudden and simultaneous nerve discharges and is often accompanied by changes in the network and neural function. The term epilepsy is also defined as the presence of two or more seizures, which is one of the most common diseases in the world.[1] Along with seizures, epilepsy is also associated with several other comorbidities, including cognitive deficits, which are widespread in patients with epilepsy.[2] At present, most cases of epilepsy are treated or controlled with anti-epileptic drugs (AEDs), which, as have been shown, have limitations in performance, safety, and efficacy.[3] Sodium valproate (VAL) is one of the most common drugs used to treat epilepsy. It is also used in the treatment of bipolar disease.[3] As mentioned, AEDs have side effects, of which VAL is no exception and can cause memory loss and behavioral ramifications.[4,5]

Although there are many studies on how comorbidities with epilepsy develop, there is little information on how epileptic seizure causes memory impairment associated with learning.[6] In addition to memory impairment, studies have indicated that epileptic seizures are associated with increased oxidative stress (OS) and the production of reactive oxygen species (ROS). There is ample evidence that OS plays a pivotal role in promoting seizures and epilepsy, causing membrane lipid peroxidation and depletion of antioxidant enzyme levels.[7]

With 7000 species worldwide, the Laminase family is one of the largest species of flowering plants. Mountain tea with the scientific name *Stachys lavandulifolia* grows in different parts of Iran.[8] Due to its therapeutic properties in Iranian traditional medicine, it is widely used in the treatment of diseases such as gastrointestinal disorders, inflammatory maladies, and anxiety, and also as a sedative.[9-11] Furthermore, in recent decades, researchers have paid special attention to this plant, documenting that the essential oil and the main ingredient of (-)-α-bisabolol from *S. lavandulifolia* have anti-inflammatory and analgesic properties.[12] Another study on the antioxidant properties showed that *S. lavandulifolia* contained flavonoids and tannins.[13,14] Other studies have also shown antidepressant and antianxiety properties in mice.[15]

To the best of our knowledge, so far, there has been no report on the effect of *S. lavandulifolia* on the seizure process, as well as memory impairment associated with seizures in humans or animal models; likewise, how it affects OS in the epileptic condition is little known. Thus, this study was designed and tested to identify the neuroprotective properties of *S. lavandulifolia* alone or in combination with VAL in epilepsy-induced or pentylenetetrazole (PTZ) rats following memory deficits, and to investigate the possible antioxidant mechanisms that *S. lavandulifolia* may propose.

METHODS

Plant Collection and Extraction

Fresh leaves of *S. lavandulifolia* were collected from Medicinal Plant Garden at the Shahid Beheshti University. Leaves of *S. lavandulifolia* were authenticated by a botanist at the School of Life Sciences Herbarium, Shahid Beheshti University, and the voucher number SBU. SLSH.98.032 was assigned. The leaves were air-dried for 14 days and powdered. An amount of 200 g of the powdered leaves was soaked with 2 L of 70% ethanol for 72 hours, filtered, and concentrated using a rotary evaporator (EYEL A, Japan) under reduced pressure and temperature (50°C). The *S. lavandulifolia* extract (SLE) was further dried and preserved in a desiccator containing activated silica until it was ready for use. The yield obtained was 5.7% w/w. The extract was reconstituted for use in the experiments by gently triturating to prepare a solution of it with normal saline as the vehicle.

Animals

Locally bred male Wistar rats (n = 25, 200–250 g) were used in the present study. These rodents were kept in standard cages in the animal room under controlled conditions (room temperature 22 ± 2°C and 12 h light/dark cycle). Standard food for rats (Pars Animal Feed Co., Iran) and water were made available to the animals in an unlimited manner. All experiments were performed between 9 and 12 AM to reduce the effect of the light cycle on the susceptibility to seizures. Working with animals and the implementation of the experiments were completely done in accordance with the international ethical principles. The research protocol was also approved by the University's Animal Research Ethics Committee (Number: IR.SBU.REC.1399.084, Date: 04.05.2020).

Drugs and Chemicals

PTZ was prepared from Sigma Company as a crystalline white powder. VAL (ampoule, 400 mg/4 mL, catalog No. 86) was prepared by RAHA Pharmaceutical Company, Tehran, Iran.

Experimental Design and Treatment Protocol

Twenty-five rats were randomly divided into five groups (n = 5/group). Seizures were induced by intraperitoneal (i.p.) injection of PTZ (60 mg/kg) dissolved in normal saline.[16] SLE was injected i.p. at the dose of 50 mg/kg.[9,17,18] VAL was dissolved in normal saline and injected into rat i.p. at a dose of 100 mg/kg.[19] The volume of injection in all animals was considered constant at 0.5 mL. The test protocol used to evaluate the effect of SLE and VAL alone or in combination on behavioral activities was as follows:

- Group I (control group): Rats that received only normal saline.
- Group II (PTZ group): Rats that received normal saline half an hour before the PTZ injection.
- Group III (VAL group): Rats that received sodium valproate (100 mg/kg, i.p.) half an hour before the PTZ injection.
- Group IV (SLE group): Rats that received *S. lavandulifolia* extract (50 mg/kg, i.p.) half an hour before the PTZ injection.
- Group V (SLE+VAL group): Rats receiving sodium valproate (100 mg/kg, i.p.) and *S. lavandulifolia* extract (50 mg/kg, i.p.) half an hour before the PTZ injection.

Behavioral Evaluation Based on Racine Average Score

The motor behavior of the animals in each group was recorded and stored by a computer-connected camera for half an hour after the PTZ injection and was examined by a researcher in a blind manner. Based on the Racine scale in animals, the six-stage stereotypical behavioral manifestations that were displayed after PTZ injection and the latency and number of myoclonic jerks and generalized tonic-clonic seizure (GTCS) latency and duration were evaluated (Table 1).[20]

Evaluation of Passive Avoidance Memory

The rate of memory recovery in animals was assessed by a shuttle box (Borj-e Sanaat Co., Tehran, Iran) one hour after PZT injection.[21] The device consists of two dark and light compartments separated by a guillotine door. The floor of this chamber has steel rods that can transmit electric shock to the limbs of living entities. Briefly, on the first day of the acquisition phase, each rat was placed separately in a clear compartment. After 30 seconds of habituation, the guillotine door was opened and the initial latency (IL) was measured to enter the dark chamber. The rats that showed IL for more than 60 seconds were excluded from further analysis. When the rats entered the dark area, the guillotine door would quickly be closed and an electric foot shock (75 V, 0.2 mA, 50 Hz) was applied to them for 3 seconds. The animal would be transferred to its cage 30 seconds after the electric shock, and this operation was repeated 5 minutes later. The rats were shocked every time they put all four limbs in the darkness. The training would end when the animal stayed in a bright room for 120 consecutive seconds. The number of shocks (SN) was measured until acquisition. Twenty-four hours later, like before, retention latency (RL) and the total time spent in the light compartment (TLC) were measured after seizure induction, but no electric shock was applied. Recovery time was measured in 300 seconds.

Animal Euthanasia and Serum Extraction

After behavioral tests, the animals underwent deep anesthesia with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and their blood was collected after cardiac puncture by a sterile syringe. The blood was allowed to clot for half an hour at room temperature and then the serums were separated by a centrifuge at 3000 rpm for 15 minutes and stored at −20°C.

Measurement of Antioxidative Markers

In order to measure antioxidative markers from serum samples, conventional kits (Novin Navand Salamat Co., Iran) available in the market and microplate reader (BioteTek ELx808, USA) were used. In short, for catalase (CAT) assay, 50 mL of phosphate buffer is removed and

Table 1. Modified Racine's Scale for Pentylenetetrazole-Induced Seizure in Rats

Score	Behavioral Manifestation
0	No behavioral sign
1	Ear and facial twitching
2	Head nodding and myoclonic jerks
3	Unilateral forelimb clonus with lordotic posture
4	Bilateral forelimb clonus with rearing and falling
5	Generalized tonic–clonic seizure (GTCS) with loss of postural tone

0.05 mL of H_2O_2 is added. To study the changes in the optical density of the CAT enzyme in the samples of different groups, after combining them, the optical density of the CAT enzyme activity was measured at 550 nm wavelength by a microplate reader device. In order to measure superoxide dismutase (SOD) activity, briefly, for every ten samples, mix 200 µL of R1 with 1.8 mL of distilled water and bring the pH to 8.2. Subsequently, mix five µL of R2a with 495 µL of R2b reagent and vortex thoroughly. After 5 minutes of incubation at room temperature and away from light, the absorption of the samples was read at 405 nm by a microplate reader. The measurement of nitric oxide (NO) level in the serum samples was assessed according to the Griess method. The activity of CAT, SOD, and NO was measured according to the kit instructions at a wavelength of 550, 405, and 570 nm and was reported in mU/mL, mU/mL, and µmoL/L, respectively.

Statistical Analysis of Data

The results of the present study were shown as median (min, max). The normality of the data was tested by the Shapiro-Wilk test. If the data were normal, then one-way ANOVA and Tukey's post hoc test were used to examine the differences between the groups. If the hypothesis of normality of the data was rejected, then nonparametric Kruskal–Wallis test and Dunn's test post hoc test were used to examine the differences between the groups. All statistical analyzes were performed by GraphPad Prism (GraphPad Software Inc., San Diego, California). Moreover, in all analyses, the value of *P* was set at less than 0.05.

RESULTS

The Effect of Different Treatments on the Activity of PTZ-Induced Seizures

The effect of different treatments on the manifestations of PTZ-induced convulsive behavior is displayed in Figure 1A–D. Regarding the myoclonic jerk latency, statistical analysis revealed a significant increase in the SLE and VAL groups versus PTZ group. Further statistical analysis also showed a significant difference in the latency of myoclonic jerk between the SLE group and the SLE + VAL group (Figure 1A). As exhibited in Figure 1B, the number of myoclonic jerks in different groups was affected; the effect had a significant decrease in the SLE and VAL groups compared to the PTZ group. Regarding the GTCS latency factor, a significant increase was witnessed in the SLE + VAL group compared to the PTZ group. However, this increase need not be significant in SLE or VAL group alone compared with the PTZ group (Figure 1C). In the scrutiny of factors, GTCS duration revealed a significant decrease in the SLE, VAL, and SLE + VAL groups in comparison to the PTZ group (Figure 1D).

The Effect of Different Treatments on Passive Avoidance Memory

There was no statistically significant difference between IL and SN in the different treatment groups (Figure 2A and B). However, RL in the PTZ group showed a significant decrease compared to the control group, indicating memory impairment. Further analysis between the groups showed that the VAL, SLE, and SLE + VAL groups increased

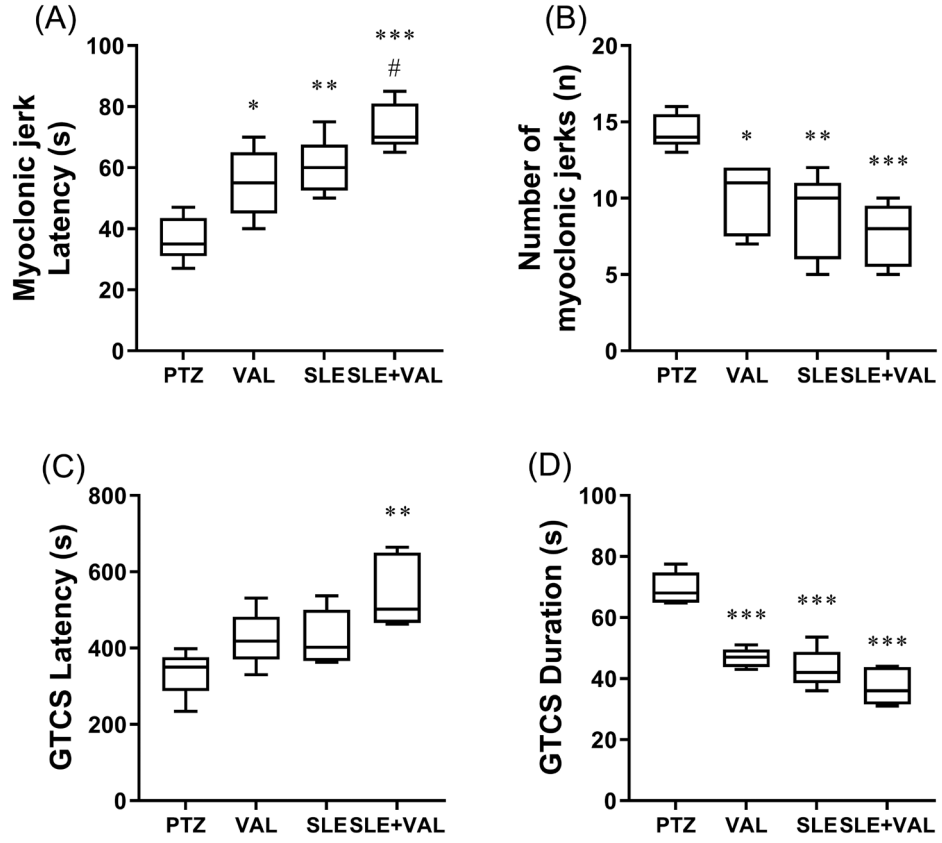

Figure 1. The effect of different treatments after PTZ challenge (60 mg/kg, i.p.) on latency of myoclonic jerk (A), number of myoclonic jerks (B), GTCS latency (C), and GTCS duration (D) in male Wistar rats. In a box plot, the distance between two upper and lower box line represents the interquartile range. The central line represents the median value (n = 5 rats per group). **P* < .05; ***P* < .01; ****P* < .001 significant difference between VAL, SLE, or SLE + VAL group with PTZ group. #*P* < .05 significant difference between SLE + VAL group with VAL group. *SLE*, *Stachys lavandulifolia* extract; *PTZ*, pentylenetetrazole; *GTCS*, generalized tonic-clonic seizure; *VAL*, sodium valproate.

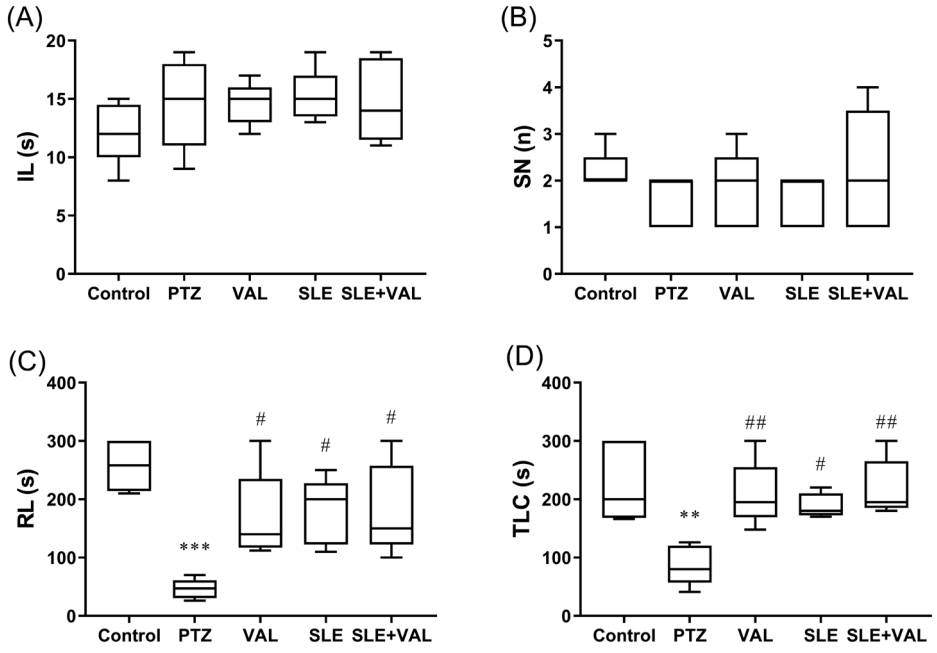

Figure 2. The effect of different treatments after PTZ challenge (60 mg/kg, i.p.) on IL (A), SN (B), RL (C), and TLC (D) in male Wistar rats. In a box plot, the distance between two upper and lower box line represents the interquartile range. The central line represents the median value (n = 5 rats per group). ***P* < .01; *** *P* < .001 significant difference between PTZ group with control group. #*P* < .05; ##*P* < .01 significant difference between VAL, SLE, or SLE+VAL group with PTZ group. *SLE*, *Stachys lavandulifolia* extract; *PTZ*, pentylenetetrazole; *IL*, initial latency; *SN*, shock number; *RL*, retention latency; *TLC*, total light compartment; *VAL*, sodium valproate.

the RL level significantly compared to the PTZ group (Figure 2C). Regarding the TLC factor, the PTZ group exhibited a significant decrease compared to the control group. SLE, VAL, and SLE+VAL treatments significantly increased TLC compared to the PTZ group (Figure 2D).

The Effect of Different Treatments on Antioxidative Markers

As shown in Figure 3A, there was a significant decrease in serum CAT levels in the PTZ group compared with the control group. Moreover, VAL and SLE+VAL treatment caused a significant increase in the serum level of CAT compared to the PTZ group. Statistical analysis of SOD serum levels in the PTZ group displayed a significant decrease compared to the control group. Conversely, in the SLE+VAL group, there was no significant difference observed vs. the control group. Further statistical analysis signaled that there was a significant difference in terms of increasing serum SOD levels in the SLE, VAL, and SLE+VAL groups versus the PTZ group (Figure 3B). There was a significant increase in serum NO levels in the PTZ group compared to the control group. Moreover, VAL, SLE, and SLE+VAL treatments caused a significant decrease in the serum level of NO compared to the PTZ group (Figure 3C).

DISCUSSION

In the present study, the effect of SLE alone or in combination with VAL on seizures and PTZ-induced PA memory deficits in rats was investigated. The results revealed that SLE alone or in combination with VAL had a statistically significant effect on improving the behavioral seizure manifestation compared to the PTZ group.

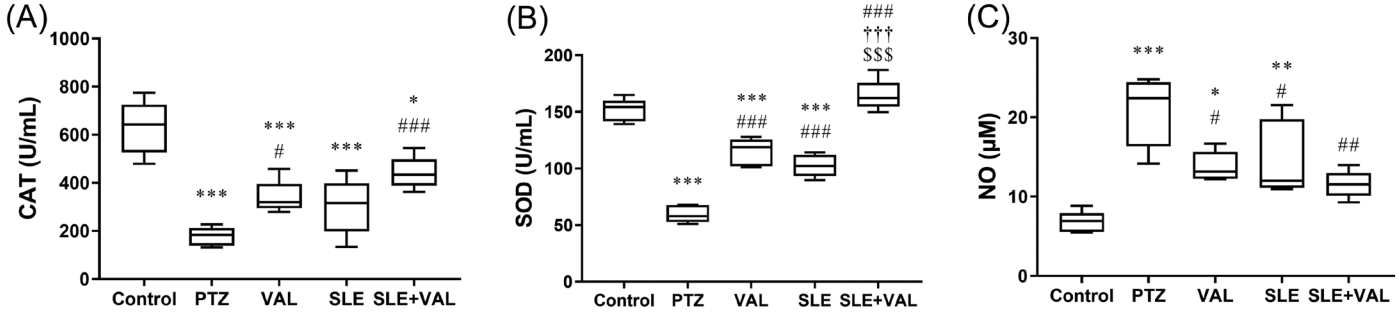

Figure 3. The effect of different treatments after PTZ challenge (60 mg/kg, i.p.) on serum level of CAT (A), SOD (B), and NO (C) in male Wistar rats. In a box plot, the distance between two upper and lower box line represents the interquartile range. The central line represents the median value (n = 5 rats per group). **P* < .05; ***P* < .01; ****P* < .001 significant difference between PTZ, VAL, SLE, or SLE+VAL group with control group. #*P* < .05; ##*P* < .01; ###*P* < .001 significant difference between VAL, SLE, or SLE+VAL group with PTZ group. †††*P* < .001 significant difference between SLE+VAL group with VAL group. $$$*P* < .001 significant difference between SLE+VAL group with SLE group. SLE, Stachys lavandulifolia extract; CAT, catalase; SOD, superoxide dismutase; NO, nitric oxide; PTZ, pentylenetetrazole; VAL, sodium valproate.

The PA test also showed that PTZ-reduced RL and TLC, resulted in memory impairment in animals receiving PTZ. The results of this study are in line with reports that have been documented on memory impairment due to PTZ-induced seizures.[21,22] With its effect on RL and TLC and its significant increase compared to the PTZ group, SLE reverses the effect of PTZ-induced memory impairment in rats, indicating its protective role against seizures and seizure-induced memory impairment. While no studies have been reported on the anticonvulsant effect or the effect of SLE on memory so far, there are some reports on the inhibitory effects of SLE on depression and anxiety in mouse models.[9,15]

Various mechanisms have been proposed for how PTZ injection causes seizures and the associated memory impairment. One of the most important factors in the development of seizures and the resulting behavioral changes is the OS and the deviation of ROS from its normal level.[7] ROS, which comprise highly active oxygen-containing molecules, are common oxidizing compounds that attack and oxidize various molecules to produce secondary oxidized products. A number of enzymes play a quintessential antioxidant role against the attack of ROS. One of the most important of these enzymes is CAT. CAT is a common enzyme in mammalian and nonmammalian cells which catalyzes the breakdown of hydrogen peroxide into water and oxygen and can convert millions of hydrogen peroxide (H_2O_2) molecules into water and oxygen every second. H_2O_2 is one of the productive compounds of ROS and is the product of the body's natural aerobic metabolism. However, this by-product is toxic to eukaryotic cells and can cause DNA damage, oxidation of lipids and proteins, and lead to mutation or even cell death. To prevent damage to cells and tissues, hydrogen peroxide is converted to oxygen and water by the CAT enzyme.[23,24] One of the most important antioxidant mechanisms of the body against the attack of ROS is the presence and activity of the SOD enzyme. SOD enzymes are metalloproteins. These enzymes catalyze the dismutation reaction of superoxide anion (O_2^-) to oxygen and hydrogen peroxide. The presence of a sufficient amount of SOD in cells and tissues keeps the concentration of O_2^- at a very low level. SOD activity in cells and extracellular environments is critical to prevent OS-related diseases.[24,25] NO is a molecular mediator of many physiological processes such as vasodilation, inflammation, thrombosis, immunity, and neurotransmission. NO is made by the NO synthase (NOS) enzyme from L-arginine, oxygen, and NADPH and participates in vascular homeostasis by inhibiting vascular smooth muscle contraction, platelet aggregation, and leukocyte adhesion to the endothelium. People with atherosclerosis, diabetes, and high blood pressure often manifest NO pathway disorders.[26] In the present study, the results showed a decrease in the CAT and SOD serum levels and an increase in NO following the PTZ injection. The present study, similar to various prior studies, showed that the injection of PTZ in animal models increases OS and decreases antioxidant levels.[16,22,27] Furthermore, data from the present study showed that SLE affected the serum level of CAT and SOD and counteracted the effects of PTZ by increasing it and decreasing the serum level of NO. The direct effect of SLE on CAT, SOD, and NO has not been reported, but there are some reports that it has antioxidant activities, and it can affect ROS formation.[10,11] In a previously reported study, SLE decreased lipid peroxidation and increased total antioxidant power in human.[10] Therefore, the positive effects of SLE on seizures and the improvement of memory impairment caused by seizures which were observed in the present study can be partially ascribed to the antioxidant properties of SLE.

The present study for the first time examined the antioxidant effects of SLE, as well as its possible mechanism. The elicited results could pave the way for future studies. Despite this strength, the present study had a limitation: this study did not investigate the pathway and inflammatory cytokines due to the small number of samples and time constraints. Also, another limitation of the current study is that it uses the plant extract instead of using the effective chemical, the formulation of which is known due to small amount of funding. This issue can be addressed in future research designs.

CONCLUSION

Overall, the present study provided evidence for the potential neuroprotective effects of SLE alone or in combination with VAL. In addition, it was shown that SLE can be protective against induced OS during seizures. Therefore, a treatment strategy that could address the potential therapeutic effect of SLE with VAL in the treatment of seizures and memory impairment associated with seizures calls for further research. Additional study designs are also needed to fully elucidate the mechanisms of anticonvulsant function and safety in their chronic use.

Ethics Committee Approval: The research protocol was approved by the Animal Ethics Committee of Shahid Beheshti University (Date: May 4, 2020, Decision No: IR.SBU.REC.1399.084).

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Author Contributions: Concept - A.H., V.A.; Design - A.H., V.A.; Supervision - A.H., V.A.; Resources - A.H., V.A.; Materials - F.A., V.A.; Data Collection and/or Processing - A.H., F.A.; Analysis and/or Interpretation - A.H., F.A.; Literature Search - A.H., F.A., V.A.; Writing Manuscript - A.H., F.A., V.A.; Critical Review - A.H., V.A.; Other - A.H., F.A., V.A.

Declaration of Interests: The authors state that there was no conflict of interest in this study.

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