




Anchusa italica Retz. Hydro-Alcoholic Extract through Attenuation of Oxidative Stress Exerts an Anticonvulsant Effect on the Pentylentetrazole-Induced Seizure in Mice

Mohammad Rahimi-Madiseh^{1,*} Shakiba Nasiri-Boroujeni^{1,*} Zahra Lorigooini¹ Meysam Nazari¹ Reihaneh Sadeghian¹ Mahmoud Rafieian-Kopaei¹ Hossein Amini-Khoei¹ 

¹Medical Plants Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

Address for correspondence Hossein Amini-Khoei, PhD, Medical Plants Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord 12345782, Iran (e-mail: aminikhoyi@gmail.com; aminikhoyi.h@skums.ac.ir).

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Abstract

Introduction It has been shown that oxidative stress is involved in the pathophysiology of seizure. Current anticonvulsants have side effects, thus further studies are needed to find new agents with low side effects. *Anchusa italica* Retz. has been suggested to have antioxidant and neuroprotective effects. The present study aimed to determine the anticonvulsant effects of *A. italica* hydro-alcoholic extract on pentylentetrazole (PTZ)-induced seizures in mice focusing on its possible antioxidative stress properties.

Materials and Methods Sixty mice were randomly divided into six groups. The intervention groups received the *A. italica* extract at the doses of 50, 100, and 200 mg/kg, 30 minutes before the injection of PTZ, whereas the positive control group received phenobarbital. The seizure threshold was then recorded.

Results *A. italica* extract significantly increased the seizure threshold. The extract significantly increased serum and prefrontal cortex total antioxidant capacity compared with the control group. The *A. italica* extract significantly reduced serum and prefrontal cortex malondialdehyde and nitrite levels compared with the control group.

Conclusions The results showed the anticonvulsant effects of *A. italica* extracts on the PTZ-induced seizure in mice is partially due to the attenuation of oxidative stress.

Keywords

- ▶ seizure
- ▶ *Anchusa italica* Retz. extract
- ▶ oxidative stress
- ▶ mice

Introduction

Epilepsy is one of the most common chronic neurological disorders that affect ~1% of the world population.^{1,2} Varied range of etiologies such as central nervous system infections,

brain injuries, brain tumors, and cerebrovascular disorders is involved in the development of seizure.³ While the causes of seizures are widely classified, the full mechanisms involved in the disease are not fully understood. Commonly prescribed drugs are associated with various side effects, and, in addition, some patients are resistant to these drugs.^{4,5}

* These are the co-first authors.

Therefore, the introduction of new drugs with fewer side effects and more effectiveness is strongly felt.

Nitric oxide (NO) plays a key role in various pathophysiological and physiological processes in the nervous system and its related disorders.^{6,7} Recently, NO has been shown to be involved in stimulating and exacerbating seizures in rodents.⁸ Researchers have shown that the increased levels of NO is associated with decrease in seizure threshold in the pentylenetetrazole (PTZ) model of seizure in rodents.⁹

Besides, several experimental models of seizures have demonstrated an increase in the oxidative stress markers, such as malondialdehyde (MDA), as the end-product of lipid peroxidation, in the brain.¹⁰ High oxidative stress state and free radical generation are possible causes of seizure-induced neuronal apoptosis and death.¹¹ Previously, it has been determined that seizures are associated with decrease in total antioxidant capacity (TAC) in the brain and serum samples.^{12,13} Therefore, agents that can reduce the level of oxidative stress markers and increase antioxidant capacity may potentially have anticonvulsant effects.

Using plant- or herbal-derived compounds for treatment of diseases has currently been specially considered. Hence, researchers are trying to introduce herbal medicines for management of diseases.¹⁴ Medicinal plants have long attracted much attention and have been used extensively in traditional medicine. Today, the effects of a large number of these plants in seizures have been studied and their anticonvulsant effects have been confirmed.^{14–16} Recent studies showed that consumption of medicinal plant or natural products as antioxidants is an approach for management of neurological diseases. *Anchusa italica* Retz. belongs to the Boraginaceae family, mostly found in temperate regions, especially in the Mediterranean and tropical regions. *A. italica* Retz. has many pharmacological properties, including anticancer, antiviral, cardioprotective, and antioxidant effects.^{17,18}

Considering all the information mentioned above, in the current study, using PTZ, a seizure-provoking agent in rodents, anticonvulsant effects of hydro-alcoholic extract of the *A. italica* was investigated, focusing on its possible antioxidative stress properties.

Materials and Methods

Plant Material and Preparation of Hydro-Alcoholic Extract of *A. italica*

The plant was collected from the margins of Saman city in Chaharmahal va Bakhtiari province. *A. italica* flowers were gathered and botanically confirmed by a botanist (Shirmardi, Hamzeh Ali, PhD, Research Center of Agriculture and Natural Resources, P.O. Box 415, Shahrekord, Iran) and deposited as specimens in Herbarium of Medical Plants Research Center in Shahrekord University of Medical Sciences, Shahrekord, Iran (SKUMS-504). In the next step, by maceration method, 1,000 g of it was macerated in 3,000 mL of 70% ethanol and kept on a shaker at room

temperature for 48 hours. The resulting mixture was filtered and the solvent was evaporated by a rotary evaporator at 38°C. The extract was completely dried in the incubator under 40°C.

Standardization of Extract

The total phenolic content of *A. italica* extract was calculated using Folin–Ciocalteu method. To this end, 1 mg/mL extract achieved a volume of 3 mL by adding up distilled water and then mixed with 0.5 mL Folin–Ciocalteu reagent for 10 minutes. After that, 4 mL of sodium carbonate was added to the consequential mixture. The obtained mixture was kept in the dark for half an hour and the optical absorbance was read at 765 nm wavelength. The experiments were conducted in triplicate. The total phenolic content was measured by using a calibration curve, and the results were expressed as mg of Gallic acid equivalent/g dried extract.¹⁹

Ethical Approval

The present study was conducted following the guidelines for the care and use of laboratory animals as adopted by the committee on the care and use of laboratory animals of the Shahrekord University of Medical Science (IR.SKUMS.REC.1396.103).

Experimental Animals

The male NMRI mice, weighing 25 to 30 g, were used in the present study. Animals were kept under standard laboratory conditions, including 24°C temperature and a 12-hour darkness cycle with free access to water and food. They were randomly divided into six groups ($n = 10$). Animals in group 1 (positive control) received phenobarbital at a dose of 4 mg/kg (i.p.) as a standard antiseizure drug, whereas those in group 2 (negative control group) received normal saline at a dose of 90 mg/kg (i.p.). Animals in groups 3, 4, and 5 (therapeutic groups) received *A. italica* extract at the doses of 50, 100, and 200 mg/kg (i.p.). All agents in groups 1 to 5 were administrated 30 minutes before PTZ (i.v.). Animals in group 6 were healthy controls who received normal saline (i.p.) without PTZ. The dose and time of administrations were chosen based on our pilot study.

Evaluation of Seizure Threshold

To induce a seizure, PTZ (10 mg/mL) was infused into the tail vein of freely moving mice at a constant rate (0.3 mL/min) using a 30-gauge needle. After fixing the mice's tail, the PTZ was injected by a seizure pump. The injection was stopped as soon as the clonus of the anterior limb was seen. The minimum dose of PTZ for the seizure was considered as the dose of seizure threshold. In this method, the seizure threshold was dependent on the PTZ dose and time.²⁰

Measuring Serum and Prefrontal Cortex Antioxidant Capacity

In the next step, the animals were sacrificed, and their prefrontal cortex and serum were isolated and transferred for biochemical measurements of the tissue or serum. Three solutions were used to measure the antioxidant capacity of samples

including solution 1 (1.55 mL of sodium acetate, 8 mL of acetic acid, and 500 mL of distilled water), solution 2 (270 mg iron chloride, which dissolved in 250 mL of distilled water), and solution 3 (prepared by dissolving of 47 mg treeazin in 40 mL of hydrochloric acid [40 mM]). The work-up solution was prepared from 10 mL of solution 1, 1 mL of solution 2, and 1 mL of solution 3. Also, 25 μ L of the serum or homogeneous prefrontal cortex samples was added to 1.5 mL of the work-up solution and placed at 37°C for 10 minutes, and then the optical absorption at the wavelength of 593 nm was recorded.²¹

Measurement of Serum Malondialdehyde

In summary, 0.5 g of thiobarbituric acid was mixed with 80 mL of acetic acid (20%), adjusted to pH 5/3 by sodium hydroxide, and the volume was diluted with 20% acetic acid per 100 mL. Next, 100 μ L of the serum sample was mixed with 100 μ L of sodium dodecyl sulfate solution (1.8%) and 2.5 mL of the work-up solution. The samples were placed in boiling water for 1 hour, then cooled and centrifuged at 4,000 rpm. The optical absorbance of the supernatant was recorded at 523 nm.²²

Measurement of Prefrontal Cortex Malondialdehyde

One gram of the prefrontal cortex tissue was homogenized in 2.5% potassium chloride (10%) and incubated at 37°C \pm 1 in a metabolic shaker for 60 minutes. After 1 hour of incubation, 1 mL of trichloroacetic acid (5%) plus 1 mL of 67% thiobarbituric acid was added and mixed well after each stage. The combination of each vial was transferred to a centrifuge tube and centrifuged at 2,000 g for 15 minutes. Afterwards, the supernatant was transferred to another tube and placed in a boiling water bath. Ten minutes later, the test tubes were cooled and the absorbance of each portion was measured at 535 nm.²²

The Nitrite Assay

The Griess reaction was adapted to measure nitrite as depicted. Briefly, the standard curves for nitrite were prepared, and the samples (50 μ L of serum and 100 μ L of tissue suspensions) were added to the Griess reagent. Proteins were precipitated by adding 50 μ L of 10% trichloroacetic acid (Sigma-Aldrich). The contents were centrifuged, and the supernatants were transferred to a 96-well flat-bottomed microplate. Absorbance was read at 520 nm using a microplate reader, and the final values were calculated from the standard calibration plots.²³

Statistical Analysis

Statistical analysis was performed using SPSS18 software. One-way analysis of variance followed by Tukey's post-hoc test was used for data analysis. Values are expressed as mean \pm standard error, and $p < 0.05$ was considered statistically significant.

Results

Standardization of Extract

Based on Folin-Ciocalteu method, the total phenol content of *A. italica* extract was determined: 72.2 \pm 0.6 mg of Gallic acid equivalent/g of the dried extract.

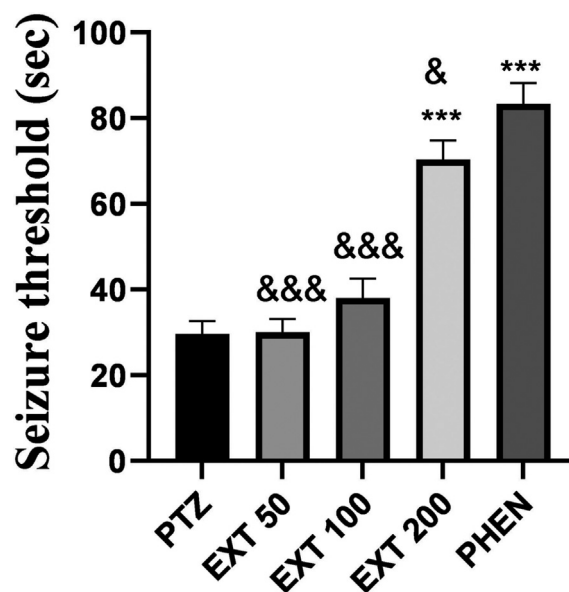


Fig. 1 The seizure threshold (seconds) in the experimental groups. Data are presented as mean \pm standard error from 10 mice and were analyzed using one-way analysis of variance followed by Tukey's post-hoc test. *** $p < 0.001$ compared with the PTZ group, & $p < 0.05$, and &&& $p < 0.001$ compared with the PHEN group. EXT, *Anchusa italica* Retz. hydro-alcoholic extract; PHEN, phenobarbital; PTZ, pentylenetetrazole.

Seizure Threshold

The results of the mean delay in the onset of seizure (seizure threshold) are presented in **Fig. 1**. Findings showed that the mean delay significantly increased in the extract-treated group (200 mg/kg; $p = 0.0007$) as well as phenobarbital group ($p = 0.0003$), compared with the PTZ group. Furthermore, results showed that seizure threshold in the group that received phenobarbital was significantly higher than groups treated with extract at doses of 50 ($p = 0.0002$), 100 ($p = 0.0005$), and 200 mg /kg ($p = 0.0397$).

Serum Total Antioxidant Capacity

Fig. 2 indicates that the TAC in the PTZ group significantly decreased, compared with the control group ($p = 0.0452$). We observed that all three doses of extract significantly increased the TAC in comparison with the PTZ group ($p = 0.0004$). Furthermore, results showed that serum TAC in groups treated with extract at doses of 50 ($p = 0.0003$), 100 ($p = 0.004$), and 200 mg/kg ($p = 0.0006$) was significantly higher than the group receiving phenobarbital.

Serum Malondialdehyde (MDA) Level

According to **Fig. 3**, serum MDA level in the PTZ group is significantly higher than in the control group ($p = 0.008$). Also, *A. italica* extract at doses of 50, 100, and 200 mg/kg as well as phenobarbital significantly reduced MDA in the serum samples in comparison with the PTZ group ($p = 0.0007$). In addition, finding showed that serum MDA level in group treated with extract at dose of 50 mg/kg was significantly higher than group receiving phenobarbital ($p = 0.0216$).

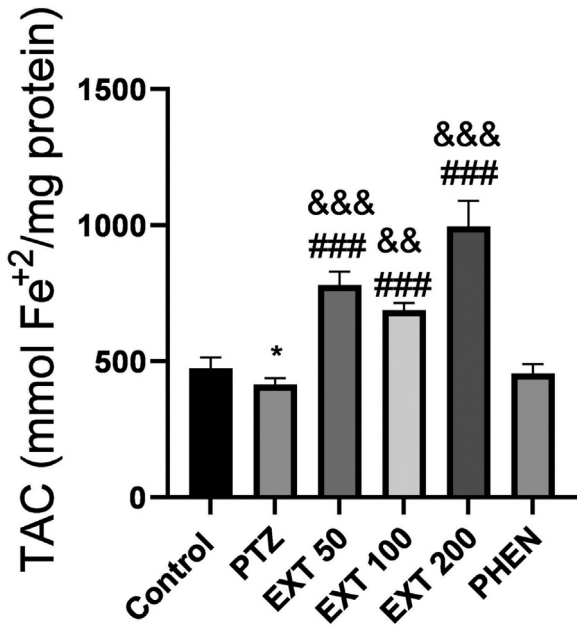


Fig. 2 Serum antioxidant capacity (micro mol Fe⁺²/mg protein) in the experimental groups. Data are presented as mean ± standard error from 10 mice and were analyzed using one-way analysis of variance followed by Tukey’s post-hoc test. **p* < 0.05 compared with the control group, ###*p* < 0.001 compared with the PTZ group, and &&&*p* < 0.01 and &&&*p* < 0.001 compared with the PHEN group. EXT, *Anchusa italica* Retz. hydro-alcoholic extract; PHEN, phenobarbital; PTZ, pentylenetetrazole; TAC, total antioxidant capacity.

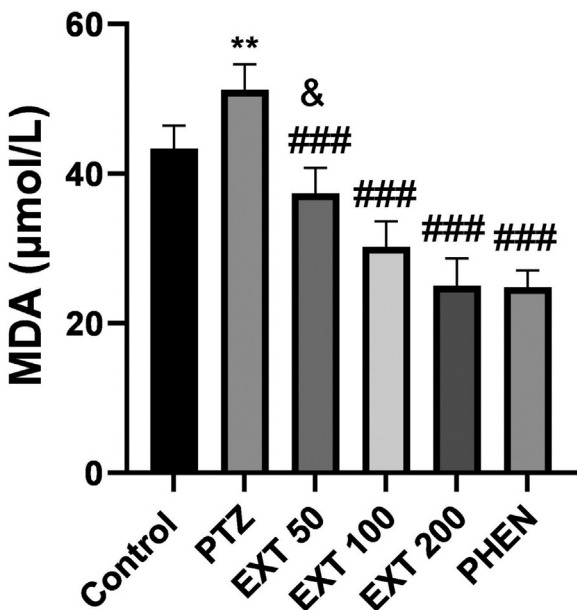


Fig. 3 Serum malondialdehyde levels (micro mol/L) in the experimental groups. Data are presented as mean ± standard error from 10 mice and were analyzed using one-way analysis of variance followed by Tukey’s post-hoc test. ***p* < 0.01 compared with the control group, ###*p* < 0.001 compared with the PTZ group, and &*p* < 0.05 compared with the PHEN group. EXT, *Anchusa italica* Retz. hydro-alcoholic extract; MDA, malondialdehyde; PHEN, phenobarbital; PTZ, pentylenetetrazole.

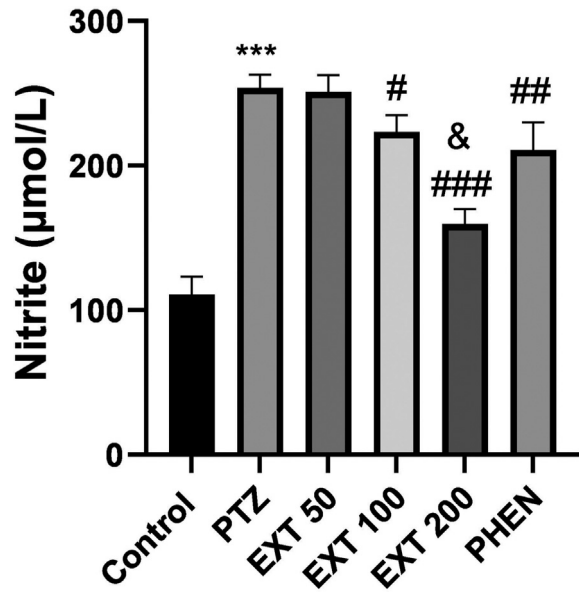


Fig. 4 Serum nitrite levels (micro mol/L) in the experimental groups. Data are presented as mean ± standard error from 10 mice and were analyzed using one-way analysis of variance followed by Tukey’s post-hoc test. ****p* < 0.001 compared with the control group, #*p* < 0.05, ##*p* < 0.01, and ###*p* < 0.001 compared with the PTZ group, and &*p* < 0.05 compared with the PHEN group. EXT, *Anchusa italica* Retz. hydro-alcoholic extract; PHEN, phenobarbital; PTZ, pentylenetetrazole.

Serum Nitrite Level

Results showed that nitrite level in serum samples in the PTZ groups is significantly higher than in the control group (*p* = 0.0002; ▶Fig. 4). We found that *A. italica* extract at doses of 100 (*p* = 0.035) and 200 mg/kg (*p* = 0.0008) as well as phenobarbital (*p* = 0.003) significantly reduced the nitrite level in comparison with the PTZ group. In addition, finding showed that serum nitrite level in group treated with extract at dose of 200 mg/kg was significantly lower than group receiving phenobarbital (*p* = 0.0251).

Prefrontal Cortex TAC

As ▶Fig. 5 presents, the TAC in the prefrontal cortex in the PTZ group is significantly lower than in the control group (*p* = 0.0004). Results showed that *A. italica* extract at doses of 50, 100, and 200 mg/kg significantly increased the TAC in the prefrontal cortex in comparison with the PTZ group (*p* = 0.0002). Moreover, results showed that prefrontal cortex TAC in groups treated with extract at doses of 50, 100, and 200 mg/kg was significantly higher than group receiving phenobarbital (*p* = 0.0007).

Prefrontal Cortex MDA Level

Based on ▶Fig. 6, the MDA in the prefrontal cortex in the PTZ group is significantly higher than in the control group (*p* = 0.0005). We showed that *A. italica* extract at doses of 50, 100, and 200 mg/kg as well as phenobarbital significantly decreased the MDA in the prefrontal cortex in comparison with the PTZ group (*p* = 0.0003). In addition, finding showed

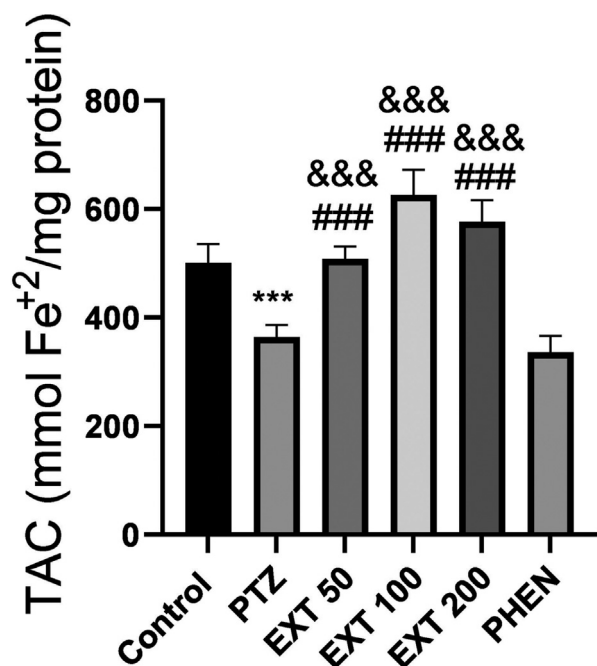


Fig. 5 Antioxidant capacity of prefrontal cortex (micro mol Fe²⁺/mg protein) in the experimental groups. Data are presented as mean \pm standard error from 10 mice and were analyzed using one-way analysis of variance followed by Tukey's post-hoc test. *** $p < 0.001$ compared with the control group, ### $p < 0.001$ compared with the PTZ group, and &&& $p < 0.001$ compared with the PHEN group. EXT, *Anchusa italica* Retz. hydro-alcoholic extract; PHEN, phenobarbital; PTZ, pentylenetetrazole; TAC, total antioxidant capacity.

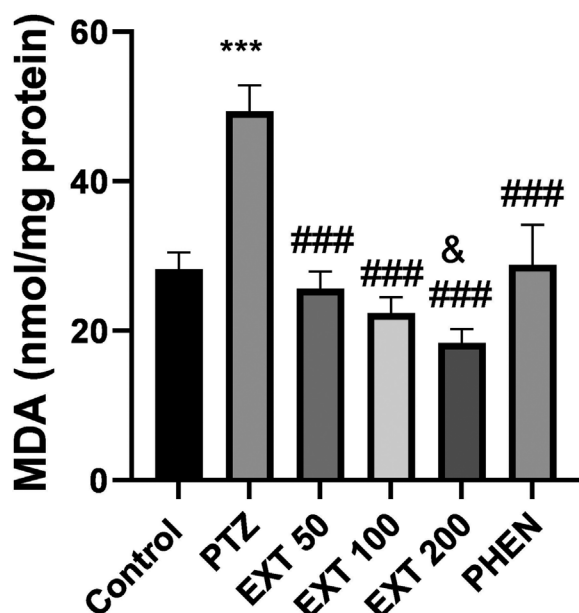


Fig. 6 Level of malondialdehyde (nano mol/mg protein) of prefrontal cortex in the experimental groups. Data are presented as mean \pm standard error from 10 mice and were analyzed using one-way analysis of variance followed by Tukey's post-hoc test. *** $p < 0.001$ compared with the control group, ### $p < 0.001$ compared with the PTZ group, and & $p < 0.05$ compared with the PHEN group. EXT, *Anchusa italica* Retz. hydro-alcoholic extract; MDA, malondialdehyde; PHEN, phenobarbital; PTZ, pentylenetetrazole.

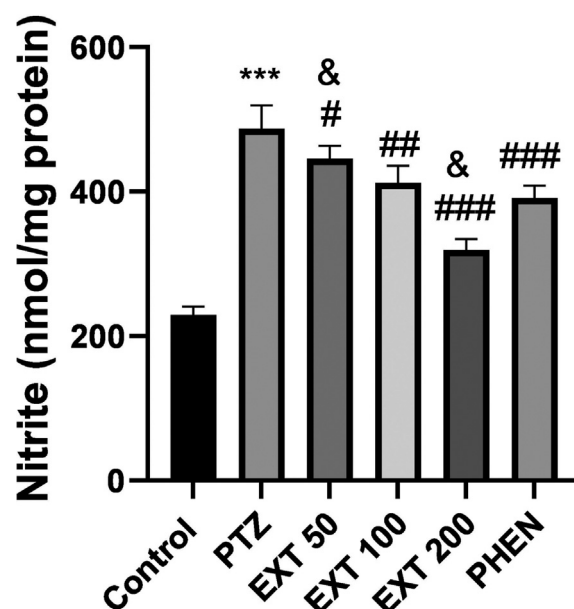


Fig. 7 Level of nitrite (nano mol/mg protein) of prefrontal cortex in the experimental groups. Data are presented as mean \pm standard error from 10 mice and were analyzed using one-way analysis of variance followed by Tukey's post-hoc test. *** $p < 0.001$ compared with the control group, # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ compared with the PTZ group, and & $p < 0.05$ compared with the PHEN group. EXT, *Anchusa italica* Retz. hydro-alcoholic extract; PHEN, phenobarbital; PTZ, pentylenetetrazole.

that prefrontal cortex MDA level in group treated with extract at dose of 200 mg/kg was significantly lower than group receiving phenobarbital ($p = 0.0274$).

Prefrontal Cortex Nitrite Level

As **Fig. 7** shows, the level of nitrite in the prefrontal cortex of the PTZ group is significantly higher than in the control group ($p = 0.0004$). Results showed that *A. italica* extract at doses of 50 ($p = 0.0475$), 100 ($p = 0.0042$), and 200 mg/kg ($p = 0.0006$) as well as phenobarbital ($p = 0.0002$) significantly reduced the nitrite level in the prefrontal cortex in comparison with the PTZ group. In addition, finding showed that prefrontal cortex nitrite level in group treated with extract at dose of 200 mg/kg was significantly lower than group receiving phenobarbital ($p = 0.0482$) while prefrontal cortex nitrite level in group treated with extract at dose of 50 mg/kg was significantly higher than group receiving phenobarbital ($p = 0.0214$).

Discussion

The findings of the present study showed that hydro-alcoholic extract of *A. italica* Retz. increased the seizure threshold in PTZ-induced seizure in mice. Results showed that hydro-alcoholic extract of *A. italica* Retz. increased the antioxidant capacity as well as decreased the MDA and nitrite levels in the prefrontal cortex and serum samples (**Fig. 8**).

Given the increasing rate of epilepsy worldwide and the low efficacy and various side effects of many synthetic drugs,

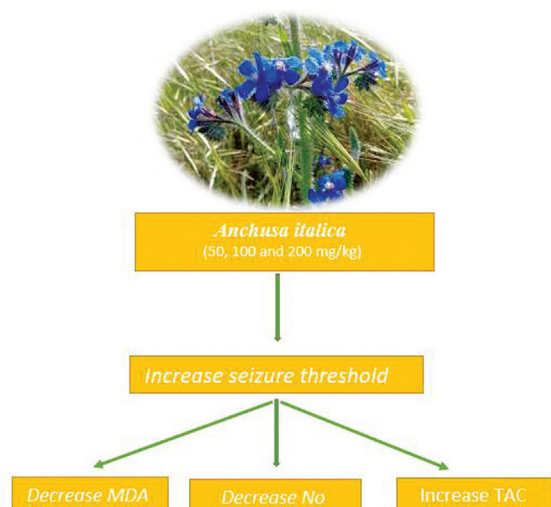


Fig. 8 Graphical abstract of the study.

finding new effective agents with low side effects seem to be necessary.²⁴ Researchers are considering herbal medicines as potential therapies in treatment of diseases.^{25,26} Previous studies have demonstrated that the hydro-alcoholic extract of some medicinal plants like *Viola tricolor* significantly enhanced the seizure threshold.²⁷ However, some herbal medicine has not been effective in epilepsy.²⁸ Previous studies have demonstrated that *A. italica* extract possessed various pharmacological properties in animal model of diseases.^{19,29} It has been determined that *A. italica* has antioxidant properties.³⁰ Also, Toriki et al (2018) revealed that *A. italica* through attenuation of oxidative stress state exerted neuroprotective effects in brain ischemia model in rats.¹⁹ The results of present study showed that *A. italica* Retz. extract, partially at least, through attenuation of oxidative stress state increased the seizure threshold. We observed that *A. italica* Retz. extract at dose of 200 mg/kg had high anticonvulsant efficacy compared with doses of 50 and 100 mg/kg in PTZ-induced seizure in mice.

The role of oxidative stress in the pathophysiology of seizure has been determined in several studies.^{3,31} In this regard, it has been demonstrated that biomarkers of oxidative stress in patients with epilepsy are significantly higher than healthy subjects.³² Animal studies indicated that the induction of seizures by PTZ increased production of free radicals and led to oxidative damage to cells.³³ Imbalance in oxidative/antioxidative systems may play a role in oxidative damage to neurons following seizure attacks.³¹ In the current study, hydro-alcoholic extract of *A. italica* Retz. displayed acceptable antioxidant activity in inhibition of 2,2-diphenyl-1-picrylhydrazyl radicals. Therefore, this ability appears to be related to the antioxidant property of phenolic compounds present in *A. italica* Retz. extract. In this study, we found that hydro-alcoholic extract of *A. italica* Retz. contains high amounts of phenolic contents. The anticonvulsant effects of *A. italica* Retz. extract seem to be because of the presence of these compounds. Baradaran et al (2013)

revealed that antioxidant activities of medicinal plants are due to secondary metabolites such as polyphenols.³⁴

Prefrontal cortex is a part of the brain that is involved in the pathophysiology of seizures.³⁵ In the present study, the antioxidant capacity of prefrontal cortex and serum samples in the PTZ group was lower than the control group. Besides, MDA levels in the prefrontal cortex and serum samples in the PTZ group were significantly higher than in the control group. In line with previous studies, we found that decrease in antioxidant capacity as well as increase in lipid peroxidation are associated with seizures.¹² Our results showed that *A. italica* Retz. extract significantly increased the antioxidant capacity in serum and prefrontal cortex samples. Furthermore, *A. italica* extract significantly decreased the MDA levels in serum and prefrontal cortex samples. In general, it can be concluded that *A. italica* Retz. extract possessed antioxidant effects in PTZ model of seizure in mice.

Previous studies indicated that NO has an excitatory role in the pathogenesis of seizures.³⁶ In this regard, it has been shown that inhibition of NO synthase led to anticonvulsant effects in different known models of seizure and attenuated seizure development.³⁷ Moreover, it has also been reported that the increase in NO production led to mitochondrial dysfunction of neuronal cells leading to oxidative stress state and finally increased the incidence of seizures.³⁸ In this study, nitrite levels in the serum and prefrontal cortex samples increased in the PTZ groups. We showed that *A. italica* Retz. extract significantly decreased nitrite levels in the serum and prefrontal cortex samples.

Overall, our finding demonstrated that *A. italica* Retz. extract, partially at least, via reducing nitrite and MDA levels as well as increasing antioxidant capacity in the prefrontal cortex possessed anticonvulsant effects in PTZ-induced seizure in mice.

Conclusion

According to the present study results, administration of the hydro-alcoholic extract of *A. italica* Retz. increased the seizure threshold in PTZ-induced seizure in mice. We found that anticonvulsant effects of hydro-alcoholic extract of *A. italica* Retz., partially at least, mediated through its antioxidant properties.

Conflict of Interest

None declared.

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