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Protective Effects of *Artemisia persica* Essential Oil Against Pentylenetetrazol-Induced Seizure in Male Mice with Emphasizing Its Mechanism of Action

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Abstract

Background: A new approach in the treatment of epilepsy is to use new drugs with neuroprotective, antioxidant, and anti-inflammatory effects.

Objectives: The study aimed to investigate the protective effects of *Artemisia persica* essential oil (EO) against pentylenetetrazol (PTZ)-induced seizure in mice.

Methods: This experimental study was conducted at the Izeh Islamic Azad University, Iran. 70 male BALB/c mice were divided into seven groups of 10 using simple random allocation, including control (normal saline), PTZ (35 mg/kg i.p. with 48 hours intervals and then 60 mg/kg on the 10th day), interventions (PTZ plus daily i.p. injection of EO at doses of 50, 75, and 100 mg/kg), diazepam (PTZ plus EO at a dose of 100 mg/kg + diazepam), and flumazenil (PTZ plus EO at a dose of 100 mg/kg + flumazenil) groups.

Results: The treatment of PTZ-kindled mice with 50 mg/kg of EO significantly reduced the seizure onset latency ($P < 0.05$). EO at a dose of 100 mg/kg significantly decreased tonic seizures, head tics, and repeated spinning and jumping ($P < 0.05$). Diazepam improved, and flumazenil weakened the anticonvulsant effects of the EO. The treatment of PTZ-kindled mice with EO (100 mg/kg) significantly decreased nitric oxide and malondialdehyde, and increased the total antioxidant capacity in both serum and brain ($P < 0.05$). EO at a dose of 100 mg/kg could significantly decrease *IL-1*β and *TNF-*α expression in the brain of epileptic mice ($P < 0.05$).

Conclusions: *A. persica* EO shows anticonvulsant effects through benzodiazepine receptor binding activity and modulation of oxidative stress and the inflammatory process.

Keywords: *Artemisia persica*, Diazepam, Flumazenil, Pentylenetetrazol, Seizures

1. Background

Epilepsy is a chronic neurological disorder, in which the altered neural activity in the brain leads to seizure. During an epileptic seizure, abnormal behavior, symptoms, and feelings, including loss of consciousness, may occur (1). Epilepsy is the most common neurological disorder second to stroke and affects about 1% of the world's population (2). Despite the unprecedented advances in the development of antiepileptic drugs in recent years, the number of patients who do not respond to drug therapy has not changed significantly (3). In addition, most of the available drugs only alleviate the symptoms without having any significant effects on the progression of the disease. Moreover, the continuous consumption of these drugs also causes drug resistance and certain side effects (3).

Kindling is a well-known technique to study epilepsy. In this method, the repeated administration of a subconvulsive chemical or electrical stimulus leads to the development of gradual seizures, which ultimately generalize into a tonic-clonic seizure (4).

Epileptic seizures disrupt the levels of excitatory and inhibitory neurotransmitters. The seizure-induced excitotoxicity is associated with excessive production of free radicals and reactive oxygen species (ROS), which leads to neurodegeneration and dramatically affects the neuronal function (5). In vitro studies have also shown that a rapid inflammatory response occurs in the glial cells immediately after the induction of epilepsy by electrical or chemical stimuli. High expression of inflammatory cytokines, such as tumor necrosis factor alpha (*TNF-*α) and interleukin 6 (IL-6), in astrocytes, can reduce the seizure threshold and the frequency of spontaneous seizures (6). Hence, in recent years, the main strategy of epilepsy treat-

ment has been seeking out new drugs with neuroprotective activities that can prevent or delay disease progression and development. Researchers have found that natural compounds with anti-inflammatory and antioxidant activity hold great potential to prevent neuronal loss and neurological disorders (5).

Artemisia persica is one of the most valuable plants of the *Artemisia* genus (7). In Iranian traditional medicine, *A. persica* has been used as an antiseptic, carminative, appetizing, antiparasitic, antipyretic, and analgesic agent (8). The phytochemical analysis of the *A. persica* essential oil revealed the presence of α-pinene, 1, 8-cineole, sabinene hydrate, pinocarveol, pinocarvone, Artedouglasia oxide C, and Artedouglasia oxide D (8). Studies have demonstrated antioxidant (7), anticancer (9), antimicrobial (10), and antinociceptive (11) effects of *A. persica* essential.

2. Objectives

The present study was conducted to investigate the protective effects of *A. persica* essential oil against pentylenetetrazol (PTZ)-induced seizure.

3. Methods

3.1. Preparation of Essential Oil

First, the dried *A. persica* whole-plant from was bought from a local market in Izeh, Khuzestan Province, and identified by an herbalist. Then, a reference sample was kept in the Herbarium of the Islamic Azad University, Izeh Branch, with voucher herbarium specimen No., 34565. The essential oil was prepared by water distillation using a Clevenger apparatus. For this purpose, 50 g of the pulverized plant was weighed and transferred into a 500-mL round-bottomed flask, mixed with 200 mL of distilled water and then heated to reach the distillation rate of 2 - 3 mL per min. After four hours, the essential oil was collected, dried with anhydrous sodium sulfate for 24 h, and stored in dark-colored glass bottles at -20°C until use (7).

3.2. Laboratory Animals and Grouping

This experimental study was conducted at the Izeh Islamic Azad University, Khuzestan Province, Iran, during summer and autumn 2018. The study protocol was approved by the Ethics Committee of Izeh Islamic Azad University (Code number: 15330513962002) in 2018. 70 male BALB/c mice weighing 25 - 30 g were purchased from the Animal Breeding Facility Centre of Pasteur Institute, Karaj, Iran. They were kept in four standard cages in groups of four in the Breeding Centre and Animal House of Izeh Islamic Azad University, under controlled conditions (12:12-hour light/dark cycle at 22 $\pm$ 2°C) with *ad libitum* constant access to standard water and food. All animal procedures were based on the Guide for the Care and Use of Laboratory Animals (12). After two weeks of acclimatization, the mice were divided into seven groups of 10 using simple random allocation. The intraperitoneal (i.p.) injection of PTZ at a dose of 35 mg/kg every 48 hours for nine days and then at a dose of 60 mg/kg on the 10th day was used to develop the kindling model as described previously (13). The control group received i.p. injection of normal saline (1 mL/kg body weight) for 10 days. The PTZ-kindled mice received the i.p. injection of PTZ as previously mentioned. The intervention groups received daily i.p. injection of *A. persica* essential oil at doses of 50, 75, and 100 mg/kg body weight, 30 minutes before PTZ injection for 10 days. The positive control group received the daily i.p. injection of *A. persica* essential oil at a dose of 100 mg/kg for 10 days and the i.p. injection of diazepam (2 mg/kg) on the 10th day, 30 minutes before PTZ injection. The flumazenil-treated group received the daily i.p. injection of *A. persica* essential oil at a dose of 100 mg/kg for 10 days, and the i.p. injection of flumazenil (2 mg/kg body weight) on the 10th day, 30 minutes before PTZ injection.

The seizure threshold (ST)/seizure onset latency of each animal was recorded for 30 minutes after the final injection of PTZ on the 10th day. The seizure severity parameters including whole body seizures, head and upper limb seizures, head tics, and spinning and jumping were recorded. The seizure threshold (ST) and severity were recorded for all animals by a blind person how was unaware of treatment in each group. Then, the animals underwent general anesthesia by chloral hydrate and blood samples were collected from the heart of the animal. The collected blood samples were then centrifuged (15 minutes at 3000 rpm) to separate sera that were immediately stored at -70°C for biochemical analysis. The mice were then sacrificed and brain tissues were removed. The 10% brain tissue homogenate (W/V) was prepared in 0.1 M sodium phosphate buffer (pH 7.4) and stored at -70°C until biochemical analysis.

Measurement of lipid peroxide level: Briefly, 200 μL of serum/brain homogenate was added to a test tube and mixed with 1.5 mL of acetic acid (20%, pH 3.5), 1.5 mL of thiobarbituric acid (TBA, 0.8%), and 200 μL of sodium dodecyl sulfate (SDS, 8.1%). Then 700 μL of distilled water was added to each tube and heated in a boiling water bath for 60 minutes. After cooling under tap water, 1 mL of distilled water and 5 mL of n-butanol/pyridine were added to each tube and shaken vigorously. The resulting solutions were then centrifuged (4000 rpm for 10 minutes), and the optical ab-

sorption of the supernatant was recorded at 532 nm. Lipid peroxide levels were determined using a standard calibration curve and expressed as μmol of malondialdehyde (14).

Measurement of total antioxidant capacity: The antioxidant capacity of serum and tissue homogenate was measured using the ferric reducing antioxidant power (FRAP) assay. The working FRAP reagent was prepared by mixing 10 mL of 0.25 M acetate buffer (pH 3.6), 2.5 mL of 10 mM TPTZ in 40 mM HCl, and 2.5 mL of 20 mM $FeCl_3.6H_2O$. 25 μL of tissue homogenate/serum was added to 1.5 mL of freshly prepared FRAP solution and left at 37°C for 10 minutes. After incubation, the optical absorption at 593 nm was recorded (14).

3.3. Measurement of Nitric Oxide ($NO^{\bullet}$) Level

$NO^{\bullet}$ is a diatomic free radical with extremely short physiological half-life. In biological systems, it is rapidly oxidized to stable end products such as nitrates (NO^{3-}) and nitrite (NO^{2-}). Therefore, the total nitrite content in serum and tissue homogenate was assessed as the index of $NO^{\bullet}$ production. Briefly, 300 μL of serum/tissue homogenate was added to 600 μL of 75 mM $ZnSO_4$ solution and centrifuged at 1000 g for five minutes at room temperature. The resulting supernatant was incubated with copper cadmium granules in a glycine-NaOH buffer in order to convert nitrate to nitrite. The total nitrite level was measured by the Griess reaction. 1 mL of the sample was added to a Griess solution (1 mL of 0.5% sulfanilamide and 0.05% n-naphthalene diamine hydrochloride), and after 30 minutes of incubation in the dark, the absorbance at 545 nm was recorded (13).

3.4. Measurement of IL-1β and TNF-α Gene Expression

The total cellular RNA of mice brain samples was extracted using the method of acid guanidium thiocyanate phenol/chloroform extraction. Mice brain samples had been frozen previously in an adequate volume of acid guanidium thiocyanate solution and kept at -80°C until use for RNA extraction. The extracted RNA was monitored by spectrophotometric absorbance (260 nm) and agarose gel electrophoresis with ethidium bromide staining. For reverse transcription, 1 μg of purified total RNA was used for incubation with 1 μM oligo (dT) and 200 units of MMLV reverse transcriptase from a Clontech first strand cDNA synthesis kit. The RT-PCR was performed using an aliquot (5 μL of a 1/10 dilution) of the cDNA of each sample. The PCR primers used are listed in Table 1. DNA amplification was carried out in 1x Taq polymerase buffer, 1.5 mM $MgCl_2$ (supplemented with 50 μM dNTP), 0.25 μM of 5' and 3'-specific primers, 1 μCi of [α-32p], and 2 units of Taq polymerase (Promega C) in a final volume of 50 μL. The mixture was overlaid with mineral oil and amplified for 30 cycles (denaturation: 1 minute at 94°C, annealing: 1 minute at 60°C, extension: 1 minute at 72°C), followed by a final extension for 7 minutes at 72°C and storage at 4°C in a Triothermoblock. 10 μL of cDNA products were size-separated by electrophoresis on a 10% acryl/bisacrylamide gel and stained with ethidium bromide (15 μg/mL). Each band was excised from the gel and the quantity of 32p incorporated was measured in a scintillation counter (15).

3.5. Statistical Analysis

Data were analyzed using SPSS20 software. The Kolmogorov-Smirnov test was used to determine the normal distribution of data. All data were normally distributed and therefore were subjected to a parametric test. In addition, the Levene's test showed that all groups had an equal variance. Thus, the one-way analysis of variance (ANOVA) followed by Turkey's test was used to identify statistical differences between the mean values of the groups. All data were presented as mean $\pm$ SD and a P value of less than 0.05 was considered statistically significant.

4. Results

Seizure latency and the frequencies of tonic seizures, whole body seizures, head and upper limb seizures, head tics, and spinning and jumping in the study groups are illustrated in Figure 1. According to the results, treatment with *A. persica* essential oil at a dose of 50 mg/kg significantly increased the seizure latency compared to the PTZ-kindled group ($P < 0.05$). *A. persica* essential oil at a dose of 100 mg/kg significantly reduced tonic seizures, and at all three doses significantly decreased the frequency of head tics and spinning and jumping ($P < 0.05$ and < 0.001, respectively). In mice given 100 mg/kg of essential oil and flumazenil, head tics and spinning and jumping were not significantly different from those in the PTZ kindling group. Treatment with 100 mg/kg of essential oil and diazepam significantly increased seizure latency and decreased the frequencies of tonic seizures, head and upper limb seizures, body seizures, head tics, and repeated spinning and jumping compared to the PTZ-treated group ($P < 0.01, < 0.05$, and < 0.001, respectively).

Table 2 indicates that the successive injection of PTZ into mice led to a significant increase in the malondialdehyde (MDA) level and a significant reduction in the antioxidant capacity of the brain and serum ($P < 0.001$). The treatment of PTZ-kindled mice with *A. persica* essential oil at doses of 50, 75, and 100 mg/kg significantly decreased the brain and serum levels of MDA ($P < 0.001$). The total antioxidant capacity significantly improved in the sera of

Table 1. Forward and Reverse Primers for Each Gene

Genes	Forward	Reverse
GAPDH (control gen)	GTA TTG GGC GCC TGG TCA CC	CGC TCC TGG AAG ATG GTG ATG G
IL-1β	5 ' - TTGACGGACCCCAAAAGATG	AGAAGGTGCTCATGTCCTCA- 3 '
TNF-α	ACT GAA CTT CGG GGT GAT TG	GCT TGG TGG TTT GCT ACG AC

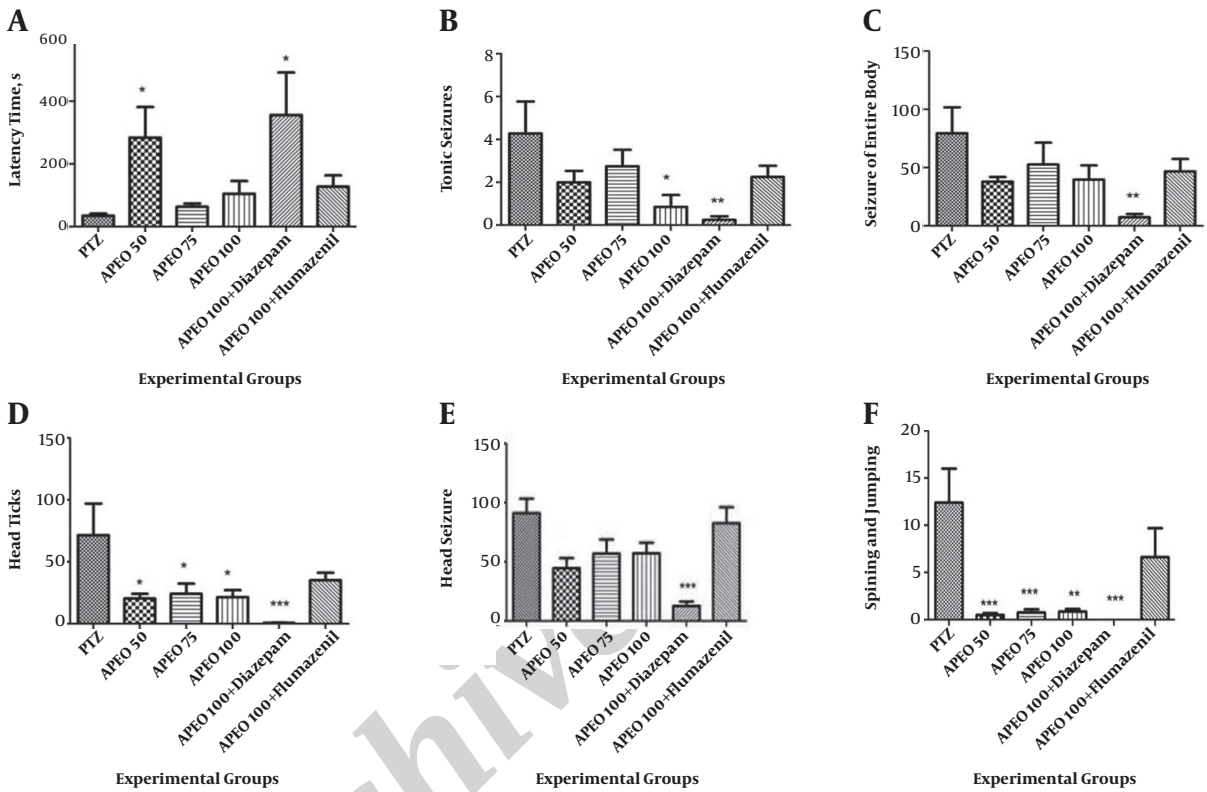

Figure 1. Comparison of seizure latency (A) and the frequencies of tonic seizures (B), whole body seizures (C), head tics (D), upper and lower limb seizure (E), and repeated spinning and jumping (F) between study groups. Data are given as means ± SD (n = 9). Significant difference compared to the PTZ-treated group. * P < 0.05; ** P < 0.01; *** P < 0.001. APEO, *Artemisia persica* essential oil; PTZ, pentylenetetrazol; APEO50, 50 mg/kg; APEO75, 75mg/kg; APEO100, 100 mg/kg.

PTZ-kindled mice receiving 50, 75, and 100 mg/kg of *A. persica* essential oil and the brains of PTZ-kindled mice receiving 100 mg/kg of *A. persica* essential oil (P < 0.001). There were no significant differences in serum MDA and brain antioxidant capacity between the PTZ-kindled group and the group treated with flumazenil plus 100 mg/kg of essential oil.

As shown in Figure 2, PTZ injections caused a partial and insignificant increase in the serum nitric oxide levels. The treatment of PTZ-kindled mice with *A. persica* essential oil at doses of 50, 75, and 100 mg/kg significantly decreased the serum level of nitric oxide (P < 0.001). There was no significant difference in the serum nitric oxide level between the PTZ-kindled group and the group treated with flumazenil plus 100 mg/kg of essential oil.

The comparison of *IL-1β* and *TNF-α* expression between the study groups is shown in Table 3. As shown, the treatment of PTZ-kindled mice with different doses of *A. persica* essential oil significantly reduced *IL-1β* expression compared to PTZ-kindled mice. *IL-1β* expression also showed a significant reduction in the groups treated with *A. persica* essential (100 mg/kg) plus diazepam or Flumazenil (Table 3). *A. persica* essential oil at a dose of 100 mg/kg significantly reduced *TNF-α* expression compared to PTZ-kindled mice but increased its expression at doses of 50 and 75 mg/kg.

Table 2. Comparison of Total Antioxidant Capacity and Malondialdehyde Between the Study Groups[a]

	Total Antioxidant Capacity	
	Serum, μM/mL[b]	Brain, μM/g[b]
Experimental Group		
Control	146.78 ± 58.36***	312.85 ± 75.93***
PTZ	456.57 ± 66.57	117.85 ± 53.72
APEO50	274.12 ± 37.84***	203.75 ± 49.58
APEO75	301.75 ± 130.38***	225.37 ± 28.11
APEO100	320.00 ± 24.61***	240.47 ± 98.81**
APEO100+Diazepam	355.50 ± 26.09***	235.00 ± 124.63*
APEO100+Flumazenil	243.06 ± 117.51***	180.71 ± 40.88
Lipid peroxidation		
Control	147.42 ± 37.46***	129.00 ± 18.12***
PTZ	353.14 ± 51.86	476.64 ± 50.36
APEO50	217.62 ± 112.80***	265.00 ± 76.63***
APEO75	217.62 ± 50.73***	227.50 ± 56.23***
APEO100	235.87 ± 51.10**	146.12 ± 25.73***
APEO100+Diazepam	127.00 ± 70.93***	134.50 ± 36.96***
APEO100+Flumazenil	264.57 ± 23.52	336.51 ± 28.59***

Abbreviations: APEO, *Artemisia persica* essential oil; PTZ, pentylenetetrazol; APEO50, 50 mg/kg; APEO75, 75 mg/kg; APEO100, 100 mg/kg.
[a] Values are expressed as means ± SD (n = 10).
[b] Significant difference compared to PTZ-kindled group (* P < 0.05; ** P < 0.01; *** P < 0.001).

Table 3. Comparison of *IL-1*β and *TNF-*α Expression Between the Study Groups[a]

Groups	*IL-1*β Expression[b]	*TNF-*α Expression[b]
PTZ	1.00 ± 0.27	1.00 ± 0.45
APEO50	0.74 ± 0.28*	4.12 ± 1.00*
APEO75	0.18 ± 0.02*	3.55 ± 1.1*
APEO100	0.06 ± 0.08*	0.64 ± 0.12*
APEO100+Diazepam	0.14 ± 0.01*	2.86 ± 0.43*
APEO100+Flumazenil	0.08 ± 0.002*	0.41 ± 0.12*

Abbreviations: APEO, *Artemisia persica* essential oil; PTZ, pentylenetetrazol; APEO50, 50 mg/kg; APEO75, 75 mg/kg; APEO100, 100 mg/kg.
[a] Values are expressed as mean ± SD (n = 9) and show the fold increase of genes expression compared to PTZ-kindled mice.
[b] Significant difference compared to the PTZ-kindled group (* P < 0.05).

5. Discussion

This study investigated the protective effects of *A. persica* essential oil against PTZ-induced seizures in mice. The results showed that treatment with *A. persica* essential oil at a dose of 50 mg/kg significantly increased seizure onset latency. *A. persica* essential oil at a dose of 100 mg/kg significantly decreased the frequencies of tonic seizures, head tics, and repeated spinning and jumping. Treatment with 100 mg/kg of the essential oil plus diazepam (GABA agonist) produced the greatest anticonvulsant effects in epileptic mice (significant increase of seizure onset latency and significant reduction of whole body seizures, tonic seizures, head tics, and repeated spinning and jumping compared to the PTZ group). Flumazenil (GABA antagonist) reduced the anticonvulsant effects of *A. persica* essential oil; thus, the frequency of tonic seizures, head tics, and spinning and jumping did not significantly differ between the PTZ-treated group and the group given essential oil (100 mg/kg) plus flumazenil.

Researchers argue that *GABAergic* and *glutamatergic* systems play important roles in the process of epilepsy. It was shown that drugs that enhance the synaptic lev-

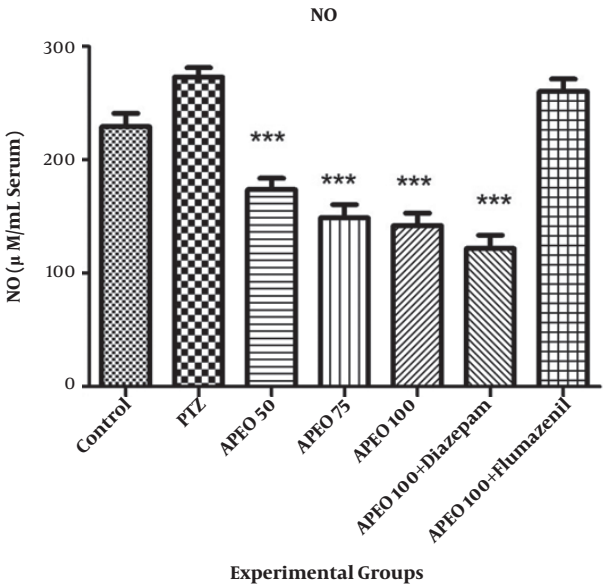

Figure 2. Comparison of serum nitric oxide levels between the study groups. Data are given as means ± SD (n = 9). Significant difference compared to the PTZ-kindled group. *** P < 0.001. APEO, *Artemisia persica* essential oil; PTZ, pentylenetetrazol; APEO50, 50 mg/kg; APEO75, 75 mg/kg; APEO100, 100 mg/kg.

els of GABA by inhibiting its catabolism or increasing its reabsorption are effective antiepileptic drugs. Benzodiazepines are one of such drugs that increase the binding of GABA to its receptors, thereby increasing the frequency of chloride channels opening. In addition, it has been argued that a number of GABA inhibitors such as deoxypyridoxine, isoniazid, thiosemicarbazide, and L-allylglycine can cause seizures (16). In the present study, diazepam boosted the anticonvulsant effects of *A. persica* essential oil and flumazenil alleviated its anticonvulsant effects, indicating that the activity of the essential oil is exerted via the GABAergic system.

In the present study, a significant increase in the lipid peroxides and nitric oxide levels and a significant reduction in the total antioxidant capacity of both serum and brain were observed in PTZ-kindled mice. Oxidative stress of the central nervous system has been reported in several animal models of epilepsy, such as the amygdala-kindling model of temporal lobe epilepsy (17), the kainic acid model of temporal lobe epilepsy (18), pentylenetetrazol (PTZ)-kindling model of focal epilepsy (19), and acute PTZ-induced seizures (20). Increased lipid peroxidation and hemolysis of erythrocytes have also been reported in patients with epilepsy compared to healthy people (21).

It has been stated that PTZ triggers a variety of biochemical processes including the activation of membrane phospholipases, proteases, and nucleases (22). Changes in the metabolism of membrane phospholipids lead to the over-production of lipid peroxides and free radicals. Free radicals can exacerbate epileptic seizures directly by inactivating glutamine synthetase and glutamate decarboxylase, which disturbs the process of excitatory (glutamate) and inhibitory (GABA) neurotransmitters synthesis (23). In the present study, the treatment of epileptic mice with *A. persica* essential oil was observed to increase the antioxidant capacity and decrease the nitric oxide and MDA levels of both serum and brain. Therefore, it can be argued that *A. persica* essential oil exert protective effects against PTZ-induced epilepsy by fighting against oxidative neuronal injury and boosting the antioxidant defense.

So far, no study has been conducted to investigate the in vivo antioxidant effects of *A. persica* essential oil but its in vitro antioxidant effects have been demonstrated in some studies (8, 24). As mentioned above, the phytochemicals identified in *A. persica* essential oil include α-pinene, 1,8-cineole, sabinene hydrate, pinocarveol, pinocarvone, Artedouglasia oxide C, and Artedouglasia oxide D (8). In a study, α-pinene and 1, 8-cineole were observed to produce protective effects against hydrogen peroxide-induced oxidative damage of PC12 cells (25).

In the present study, the treatment of epileptic mice with 100 mg/kg of *A. persica* essential oil resulted in a significant reduction in *IL-1*β and *TNF-*α expression. In recent years, accumulating evidence has suggested that numerous immune and inflammatory processes such as the over-production of pro-inflammatory cytokines occur in the brain after the induction of seizure in experimental animal models or in clinical cases of epilepsy (26). Some inflammatory cytokines such as IL-1 and beta-HMGB1 have been reported to modify the activity of NMDA receptors through increasing their phosphorylation. Inflammatory cytokines have also been reported to overstimulate seizure by inhibiting glutamate reuptake in astrocytes (27). In addition, *IL-1*β and *TNF-*α injections were shown to decrease the expression of GABAA receptors and GABA-induced chloride ion flow, which ultimately exacerbated seizure severity and duration in mice. In this regard, it was shown that anti-inflammatory drugs could reduce the severity of some types of epilepsy in animal models (28). In the present study, the efficacy of *A. persica* essential oil in reducing brain inflammation was demonstrated for the first time, as 100 mg/kg of the essential oil significantly reduced the expression of *IL-1*β and *TNF-*α in PTZ-kindled mice. In previous studies, the anti-inflammatory effects of α-pinene and 1, 8-cineole have been demonstrated (15, 29). Given that these compounds are abundantly found in the essential oil of *Artemisia*, it seems that the neuroprotective effects of *A. persica* essential oil are due to the presence of the compounds with anti-inflammatory effects.

Our results demonstrated that the essential oil extract of *A. persica* possesses anticonvulsants activity via the suppression of oxidative stress and pro-inflammatory cytokines. One of the strengths of this study was the assessment of several mechanisms involved in the PTZ-induced seizure process to determine the exact mechanism of the essential oil effects. This study has intrinsic limitations common to the pharmacological assessment of herbal remedies. One of the most important steps in the production of herbal remedy is to produce a standard herbal drug with special and constant components that can be reproduced. In general, the type and amount of chemical compounds found in the medicinal plants are affected by several factors such as the genetic differences, geographical location, harvest time, soil quality, plant part used, and method of plant processing. Therefore, it is necessary to determine the active components of the plants and evaluate their activities (30).

5.1. Conclusions

A. persica essential oil at a dose of 100 mg/kg showed protective effects against PTZ-induced seizure, which may be due to the modulation of GABA receptors and the inhibition of oxidative stress markers and brain inflammation.

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Footnotes

Authors' Contribution: Mojgan Daneshkhah did all the testes. Mahbubeh Setorki designed test-analyse and prepared the article.

Conflict of Interests: The authors declare that there is no conflict of interest.

Ethical Considerations: The study protocol was approved by the Ethics Committee of Izeh Islamic Azad University (Code number: 15330513962002) in 2018.

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