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Research article

Proconvulsant effects of *Nepeta menthoides* hydro alcoholic extract in different seizure tests: behavioral and biochemical studies

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ABSTRACT

In Iran, both *Nepeta menthoides* - the endemic species of Nepeta genus - and *Lavandula officinalis* are known as Ustukhuddoos and used widely as medicinal herbs. In Iranian traditional medicine, Ustukhuddoos has been recommended for several neuronal diseases including depression and epilepsy. While the antiepileptic effects of *Lavandula officinalis* have been investigated in a number of studies, no reports are available taking into account the effect of *Nepeta menthoides* on epilepsy. Since convulsion is an important side effect of some medicinal plants, a thorough study of the effects of *Nepeta menthoides* on epilepsy seems necessary.

This study was designed to investigate the potential anti- or pro-convulsant activity of *Nepeta menthoides* and its effects on oxidative stress markers. Since an herbal medicine showed opposite effects in two animal models of epilepsy in our laboratory, authers decided to study Nepeta effects through several seizure tests including the intravenous pentylenetetrazol (i.v. PTZ) infusion, the maximal electroshock (MES), acute PTZ and PTZ-kindling tests. These seizure models are generally used for screening pro- or anti-epileptic drugs. *Nepeta menthoides* (400 mg/kg) significantly reduced the dose of PTZ necessary for clonus seizure induction. Combining either phenytoin (Phen) or Valproate (Val) with Nepeta decreased their antiepileptic effects. Therefore, *Nepeta menthoides* not only failed to prevent the seizures but also increased sensitivity to them. Nepeta raised brain NO levels in different seizure tests. It seems there is a relation between NO elevation by Nepeta and increased sensitivity to seizures that should be investigated later.

1. Introduction

Epilepsy is among the diseases for which the use of complementary and alternative medicine (CAM) is relatively common [1]. While many consumers consider Herbal medicine both healthy and effective, some widely used herbs may in fact have epileptogenic properties and raise the risk of seizures [2]. More extensive studies are needed to determine the exact effects of many commonly used herbs on epilepsy. Epilepsy as a neurological disease affects 50 million people worldwide [3]. In many cases it may be treated with common antiepileptic drugs (AED), but nearly a third of patients have proved to be resistant to routine treatments. No antiepileptic drug can abolish the interictal activity [4]. Many studies support the efficacy of treatments using agents with antioxidant properties [5]. Hence, considering the antioxidant properties of some compounds used in herbal medicine, many researchers have focused on the study of the antiepileptic effects of these herbs.

Nepeta menthoides, also known as "Ustukhuddoos" in Iranian traditional medicine [6, 7, 8, 9, 10], is one of the species of Nepeta genus commonly found in herbal markets. Ustukhuddoos has long been recommended for a number of nervous diseases including epilepsy and depression [11]. The Nepeta genus (Lamiaceae) is a diverse genus with about 300 herbaceous species. These species are widely diffused in southwestern Asia and the western Himalayas, as well as Iran, Turkey, and the Hindu Kush. Nepeta species are used for their sedative, diuretic, spasmolytic, expectorant, antiseptic, antitussive, antiasthmatic, and febrifuge effects in traditional medicine [7, 8, 12, 13].

Nepeta menthoides (Boiss & Buhse) is a domestic species of Nepeta in Iran spread in various parts of the country, especially around Mount Sabalan and Marand region in north western Iran [6, 8, 9, 14, 15, 16]. It is a permanent, upright and powerful plant with violet flowers, normally growing from 15 to 40 cm high [6, 7].

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It is worth noting that *Nepeta menthoides* exhibits similar medicinal properties to other Nepeta species because of shared chemical compounds. However, some studies suggest that *Nepeta menthoides* has therapeutic effects on the improvement of memory and depression [17, 18, 19]. Moreover, there are studies reporting antilarvicidal, antibacterial, anti-inflammatory, and analgesic effects of this species [20, 21, 22].

The *Nepeta bractaeta* flower extract has been reported to show anti-seizure properties through the increased current electroshock seizures test (ICES) and the PTZ test [23]. On the other hand, it has been shown that *Nepeta cataria* (Catnip) leads to increased stereotype behaviors and sensitivity to seizures in mice [24]. Nonetheless, there is no available report about the effects of *Nepeta menthoides* on epilepsy.

There are two species referred to as Ustukhuddoos in Iran; *Nepeta menthoides* and *Lavandula officinalis* [6, 7, 8, 25]. While there are a number of studies examining the antiepileptic effects of the latter, there are no reports available on the effects of the former (*Nepeta menthoides*) on epilepsy.

Nepeta menthoides is used for depression disorder [19] and has been reported to have beneficial effects on memory impairment [17, 18], as well as morphine dependence and tolerance [26]. Given these facts and considering that convulsion is one of the dangerous side effects of some medicinal plants, it seems necessary to perform a study focused on investigating the effects of *Nepeta menthoides* on epilepsy.

Several seizure tests such as PTZ infusion, acute PTZ, PTZ-induced kindling, and maximal electroshock (MES) tests have been used to measure potential anti- or pro-convulsant effects of *Nepeta menthoides*. Some of these studies attribute neuronal loss in PTZ kindling to free radical production [27]. Therefore, the role of oxidative stress markers such as nitric oxide (NO) and malondialdehyde (MDA) in each of the seizure tests and the effects of *Nepeta menthoides* on them were also examined.

2. Materials and methods

2.1. Animals

A total of 278 adult male NMRI albino mice ranging from 25 to 30 g in weight (Baghiyyatallah University, Tehran, Iran) were housed 10 mice per cage at most. A 12-h light/dark cycle was maintained with lights switched on at 6 a.m. Food pellet and tap water were freely accessible. For the duration of the one-week period leading to the experiments, the animals were allowed to adapt to the colony room. The protocol was approved by the Research Ethics Committee of Shahed University (IR. KAUmS. MEDNT. REC. 1396. C).

2.1.1. Classification of the animals

We randomly divided 268 male mice into 4 main groups corresponding to the 4 animal models of epilepsy. Each group was subdivided into four to eight subgroups (i.e. 7-10 mice per subgroup), each corresponding to one kind of pretreatment.

2.1.1.1. PTZ kindling groups pretreatment. The mice were divided into five groups of 10 each. The control PTZ group received isotonic saline, the Valproate (Val) group was given 150 mg/kg of Val., and the other groups were administered three different doses of *Nepeta menthoides* extract (100, 200 and 400 mg/kg) [28] every 48 h, 30 min before the PTZ were injected. Drug administration was intraperitoneal (i.p.).

2.1.1.2. Timed intravenous PTZ infusion groups pretreatment. Again, the animals were divided into five groups of 10 each. The control PTZ group received isotonic saline, the Diazepam (Diaz) group was given 1 mg/kg of Diaz, and the rest of the groups received *Nepeta menthoides* extract (in 100, 200 and 400 mg/kg doses). This was done repeatedly for seven days, in addition to once 30 min before PTZ infusion on the test day.

2.1.1.3. Acute PTZ group pretreatment. The mice were divided into two main classes; the first class composed of four groups and the second class composed of eight groups based on receiving single or repeated pretreatments. There were seven mice in each group. For the first class, pretreatments were carried out 30 min before PTZ (100 mg/kg i.p) injection, with a single dose of Nepeta (400 mg/kg), Val (300 mg/kg) and their combination. For the second class, on the other hand, pretreatments were performed for a week, once a day, with Nepeta (100, 200, 400 mg/kg), Val (300 mg/kg) and their combinations. Normal saline was used as solvent for all of the drugs and the administrations were i.p. The PTZ group received normal saline as pretreatment.

2.1.1.4. MES groups pretreatment. The same classification and groups as the ones described in 2.1.1.3 were repeated with a different animal model of epilepsy. For the first class, pretreatments were performed with a single dose of Nepeta (400 mg/kg), Phen (25 mg/kg) and their combination 30 min before the MES test. For the second class, pretreatments were carried out for a week once a day, with Nepeta (100, 200, 400 mg/kg), Phen (25 mg/kg) and their combinations. The control MES group received normal saline as pretreatment.

2.2. Drugs

PTZ (Sigma, USA), Diaz (Roozdaroo, Iran), Phen (Roozdaroo, Iran), Val (Darootebb, Iran), and *Nepeta menthoides* extract were dissolved and diluted by adding normal saline.

2.2.1. Plant extraction

We purchased *Nepeta menthoides* dried aerial parts (1000 g) from the herbal medicine market. The plant was identified and authenticated by a botanist and the voucher specimen number was deposited under reference number PMP-315. The plant is listed in www.theplantlist.org as *Nepeta menthoides* Boiss. & Buhse. We ground the plant and allowed it to soak for 3 days in ethanol 70% (1:4) double distilled water at room temperature (25 °C). Afterwards, we filtered it three times. The filtrates were dried in a water bath at 50 °C. The extraction yield was about 10%. We kept it at 4 °C until the test day. On the test day, we dissolved and diluted the extract in normal saline. As is investigated in another study in our laboratory on *Nepeta menthoides* toxicity, the oral administration of 4000 mg/kg of the extract caused the death of one out of eight mice after 48 h.

2.2.2. Isolation of essential oil from the hydroalchoholic extract

In order to analyze the contents of the extract, the essential oil was isolated from the extract by hydrodistillation. In hydrodistillation, the hydroalcoholic extract is placed in a distillation apparatus, sufficient water is added, and brought to a boil. Alternatively, one can inject live steam into the extract. The mixed vapor of water and oil flows through a coil and gets condensed back to liquid by indirect cooling. The distillate flows from the condenser into a separator removing oil from water. Table 1 lists the main ingredients as revealed by gas chromatography-mass spectrometry (GC/MS).

2.2.3. Extract analysis

2.2.3.1. Phytochemical screening tests. We performed standard screening tests for alkaloids, flavonoids, saponins, tannins, and cardiac glycosides [26, 29, 30, 31]. The results showed that flavonoids, pseudo tannins, saponins, and cardiac glycosides are present in the extract.

2.2.3.2. Total phenolic content assay. In order to measure the total amount of phenols, Folin-Ciocalteu reagent was used as described in [32] with minor adjustments. The absorbance of the distinct concentrations of standard solutions of gallic acid (25–150 μg/mL in 80% methanol) was measured. The resulting calibration curve was used to quantify total phenolic ingredients [32, 33, 34]. The data showed that the total amount

Table 1. The compositions of *Nepeta menthoides* essential oil isolated from hydroalchoholic extract and % of abundance.

Compound	(%) of the measure units.
α-terpineol	15.93
Silphiperfolan-7-β-ol	7.27
Linalool	7.14
Terpinene-4-ol	6.24
d-terpineol	5.7
4α-α, 7-α, 7α-β-Nepetalactone	4.96
Carvacrol	4.48
Hotrienol	3.72
2-methoxy-para-Cresol	3.6
1,8-cineole	3.29
Geraniol	2.92
Thymol	2.79

of phenols in the *Nepeta menthoides* extract was 35.57 ± 3.7 μg, based on corresponding gallic acid equivalent (GAE)/250 μg of dry extract.

2.2.3.3. Total flavonoids content assay. In order to measure the total amount of flavonoids in the extract, aluminum chloride colorimetric assay was used [32, 33, 34]. The results showed that the total amount of flavonoids in the *Nepeta menthoides* extract was 73.4 ± 5.5 μg as catechin equivalent (CE) per 500 μg dry extract.

2.2.3.4. Phenolic compounds HPLC analysis. The HPLC method was employed to measure phenolic ingredients including Rutin, rosmarinic acid (RA), quercetin, and caffeic acid (CA) (Herbal Processing Center of Jihad Daneshgahi, Karaj-Ghazvin) as described in our previous work [26].

An HPLC apparatus (Smartline, Kenuer, Germany) consisting of a quaternary pump, a C18 Eurospher-100 reversed phase column, and a D-14163 UV-VIS detector was used. The software tool ChromGate 3.1 was used for data processing. A concentration gradient mobile phase (water plus 0.2% glacial acetic acid as solvent A and acetonitrile as solvent B, flow rate of 1 mL/min) performed the separation. The solvents were linearly changed from A-B (90:10, v/v) to A-B (75:25, v/v) over a 15-minute period. Mobile-phase A had an 80% reduction after 40 min, reached 0% after 45 min, remained constant for 50 min, and linearly increased to 90% during the next 5 min. A volume of 20 μl was injected causing the common UV absorbance to peak at 280 nm. A hydrophilic PTFE membrane (0.45 μm pore size) was used to filter the sample prior to injection. The retention times were compared to those of the standards to identify the peaks. The corresponding calibration curves were used to calculate the contents of each phenolic compound. The whole evaluation process was repeated three times. The details can be found in Table 2 and the appendices (All analysis reports is given in the supplementary file).

2.3. Behavioral study

2.3.1. PTZ kindling

Repetitive administration (11 injection periods) of a subconvulsant PTZ dose of 35 mg/kg i.p. every other day could induce kindling. The mice were pretreated every second day thirty minutes before each PTZ injection. The animals were under observation for half an hour after the last drug injection. The following scale was used for seizure intensity measuring [35]: 0 no response; 1 facial and ear twitching; 2 seizure waves moving axially throughout the body; 3 myoclonic seizures; 4 generalized clonic seizures and falling to one side; 5 generalized seizures with tonic extension and status epilepticus; and 6 mortalities. A challenge dose of PTZ (75 mg/kg) on day 24 generated tonic and clonic convulsions and lethality. Seizure latency and duration of phases 2 and 5 were measured.

Table 2. Content of the phenolic compounds.

Phenolic compounds name	Phenolic compounds content mg/g DW
Rosmarinic acid	19.74
Rutin	14.02
Quercetin	0,12
Caffeic acid	2.19

2.3.2. Timed intravenous PTZ infusion

After one week of extract and Diaz (1 mg/kg) pretreatment, on the test day, thirty minutes after the last injection, intravenous PTZ infusion was performed. In order to measure the threshold dose of PTZ for the appearance of generalized clonic convulsions, a 30 gauge dental needle was inserted into the tail vein of the restrained mouse [36]. The needle was fixed to the tail by a special tape, and was attached to a plastic syringe with a pump inside it. The PTZ solution (5 mg/ml) was infused at a constant rate of 0.5 mL/min using a pump to unrestrained mice. The infusion was stopped as soon as generalized clonic seizures appeared in each mouse. The lowest dose of PTZ (mg/Kg) for induction of total body clonic seizures was calculated by the following formula [36]:

PTZ (mg/kg) = duration of PTZ infusion (s) × infusion rate (ml/s) × PTZ concentration (mg/ml) / body weight (kg)

2.3.3. Acute PTZ induced seizures test

Thirty minutes after single or repeated pretreatments, on the test day, the mice were injected with PTZ (100 mg/kg) and observed for at least 30 min to detect tonic and clonic seizure latencies, mortality latency, % of mortality, the occurrence of tonic, clonic, generalized seizures, and HLTE [37, 38].

2.3.4. MES test

Thirty minutes after single or repeated pretreatments, on the test day, MES (50 mA, 60 Hz, 0.7 s duration) was applied through ear-clip electrodes using a stimulator apparatus. Durations of hind limb tonic extension (HLTE), % of mortality, and % of protection of HLTE were recorded. Phen (25 mg/kg) was used as positive control [38, 39].

2.3.5. Chimney test

Motor coordination was assessed in mice by the chimney test in order to rule out any motor activity perturbation induced by the drugs. The test was performed according to the method described by Luszczki et al. [40]. Before seizure induction, after administration of the tested drugs such as different doses of *Nepeta menthoides,* Val, Diaz, and Phen, the animals (5 mice in each group were randomly tested) were placed individually in tubes that were 25 cm long and 2.5 cm in diameter. We then moved the tubes to a vertical position in order to observe whether the mouse can climb up to exit the pipe in 60s. The number of mice unable to perform the test in 60s was recorded as Motor deficit [40].

2.4. Sample preparation and biochemical assay

At the end of the behavioral experiments on the test day, in order to evaluate the biochemical factors the animals were decapitated and their brains were quickly removed. The brains were washed in cold normal saline two times and were kept in glass bottles in a freezer (-20 °C) before they went through other procedures. Then, the brains were split and homogenized for 2 min at 5000 rpm using four volumes of ice-cold Tris-HCl (50 mM, pH 7.4). MDA, NO, and protein contents were measured. The homogenized solution was then centrifuged for 60 min at 5000 × g to remove debris. The supernatant solution was then extracted with a mixture of ethanol/chloroform (a volume with a ratio of 5:3). After centrifugation at 5000 × g for 30 min, the clear upper layer (the ethanol phase) was taken and used for measuring the protein levels. All experiments were performed at +4 °C.

2.4.1. MDA evaluation

Brain MDA content was measured by a method according to the reaction with thiobarbituric acid (TBA) (at pH 2–3 at 90 °C for 15 min) for the generation of a pink pigment with maximum absorption at 532 nm [41]. The sample was mixed with 2 times of cold trichloroacetic acid 10% (w/v) to precipitate protein. The precipitate was pelleted by centrifugation and the supernatant was left to react with a volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm.

2.4.2. NO evaluation

Since NO detection is considerably difficult in biological preparations, the total nitrite levels of the sample were measured using the Griess method as an index of nitric oxide [42]. In this method, the sample's nitrate is converted to nitrite using cadmium, followed by color development by Griess reagents (sulfanilamide and N-naphthyl ethylenediamine) in an acidic medium. The absorbance was read at 540 nm with a spectrophotometer [43].

2.5. Statistical analysis

The results were expressed as mean ± S.E.M. Statistical analysis was performed using one way ANOVA followed by Tukey post-hoc test for parametric data and Kruskal–Wallis one way analysis of variance by ranks followed by Wilcoxon-Mann-Whitney test.

Regarding non-parametric data, repeated measure ANOVA was used for PTZ kindling. P values less than 0.05 were considered significant. The software tool used for statistical analysis was Sigma State 3.5.

3. Results

3.1. Behavioral studies

3.1.1. PTZ kindling

3.1.1.1. Effects of different doses of Nepeta menthoides on the intensity of PTZ induced seizure. Mortality rates for the PTZ challenge dose (75 mg/kg, i.p.) in control PTZ and *Nepeta menthoides* pretreatment groups (100, 200 and 400 mg/kg) were 20, 10, 30, and 10 % respectively (Table 4). In the Val group, there was no mortality. In the PTZ group, PTZ kindling caused seizure activity enhancement leading to the generalized clonic-tonic seizure. The results in Figure 1 show that pretreatment with Val as an antiepileptic drug reduced seizure intensity ($p < 0.05$). Moreover, as Tables 3 and 4 show, Val pretreatment generally enhanced latency but reduced duration of seizures ($p < 0.05$). While 100 and 200 mg/kg doses of Nepeta decreased seizure scores only in the 10th and 5th injection times respectively ($P < 0.05$), the 400 mg/kg dose of the extract was not able to reduce the seizure intensity in any of the injection times (Figure 1).

Our data also show that different doses of *Nepeta menthoides* pretreatment failed to significantly change latency and duration of the seizure phases relative to the PTZ group (Tables 3 and 4). It is worth noting that only the 400 mg/kg dose of *Nepeta menthoides* reduced the duration and increased the latency of seizures in the PTZ test dose (Table 4). Experiments with the chimney test showed that administration of Val (150 mg/kg), Diaz (1 mg/kg), Phen (25 mg/kg), and *Nepeta menthoides* (100, 200 and 400 mg/kg) did not induce any motor disturbance (a quality test) because all of the tested mice managed to climb up and escape the tube in 60s.

3.1.2. Timed intravenous PTZ infusion

3.1.2.1. The effect of Nepeta menthoides at different doses and Diaz on the threshold dose of PTZ induced convulsions. The threshold dose of PTZ for seizure induction with progressive intravenous (i.v.) infusion was calculated for 7-day pretreatment with Diaz and different doses of *Nepeta menthoides*. Figure 2 shows that Diaz (1 mg/kg) - an antiepileptic drug - significantly increased the threshold dose of PTZ for clonic seizures in mice (from 62.3 ± 4.6 mg/kg in the PTZ group to 128.9 ± 8.08; $p < 0.05$). In other words, Diaz reduced the possibility of seizure induction by PTZ. Figure 2 also shows that the *Nepeta menthoides* extract (100 and 200 mg/kg) did not have a significant effect on the threshold dose of PTZ for clonus seizure production, as compared with the PTZ group. Furthermore, the 400 mg/kg dose of *Nepeta menthoides* significantly reduced the dose of PTZ necessary for clonus seizure induction as compared with the PTZ group (62.3 ± 4.6 mg/kg in PTZ group to 48.64 ± 1.87; $p < 0.05$). In fact, *Nepeta menthoides* pretreatment not only failed to reduce the probability of seizure induction by PTZ but also increased susceptibility to seizure.

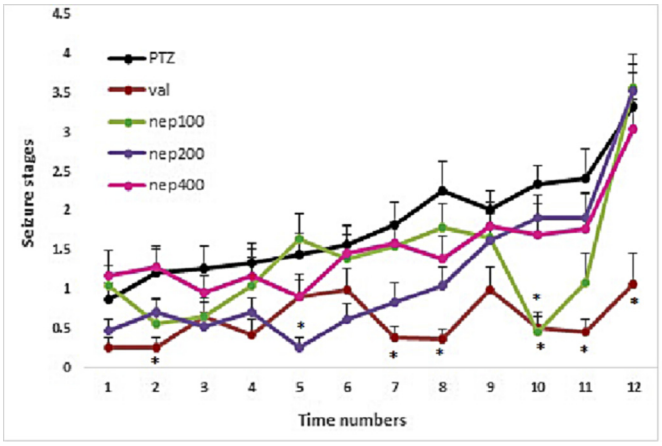

Figure 1. The effect of *Nepeta menthoides* at different doses on PTZ-kindled seizures. Val, an antiepileptic drug, reduced the intensity of seizures generated by PTZ kindling significantly. Pretreatment with *Nepeta menthoides* (100 and 200 mg/kg) reduced seizure intensity only in the 10th and 5th injections respectively ($p < 0.05$), and the 400 mg/kg dose of the extract was unable to reduce seizure intensity. * Significant difference with the PTZ group ($P < 0.05$). Tukey test was done. Val, Nep, and PTZ indicate Valproate, *Nepeta menthoides*, and pentylenetetrazol respectively.

3.1.3. Convulsions induced by acute PTZ

3.1.3.1. The effect of the single dose of Val, Nepeta menthoides and their combination on acute PTZ induced convulsions. PTZ (100 mg/kg) was administered 30 min after the pretreatment of the mice in different groups with Val (300 mg/kg), *Nepeta menthoides* (400 mg/kg) and their combination (Val + *Nepeta menthoides)*. Table 5 shows the effects of Val - an antiepileptic drug - as well as *Nepeta menthoides* extract and a combination of Val and the extract on tonic and clonic seizure latency, % of mortality, and the number of clonic, tonic, generalized, and HLTE seizures. Unlike Val, pretreatment with *Nepeta menthoides* extract did not show antiepileptic effects as it did not enhance tonic and clonic seizure latency (it increased the number of clonic seizures in comparison with the control PTZ group). The addition of Nepeta to Val reduced the antiepileptic effect of Val such that clonic and tonic seizure latencies affected by Val (308.33 ± 41.49 and 462.5 ± 128.08) were reduced to (165.71 ± 16.16 and 174.28 ± 28.79) respectively. Similarly, the addition of Nepeta to Val increased the number of clonic seizures from (0.8 ± 0.37) to (7.28 ± 1.73). Therefore, *Nepeta menthoides* not only failed to prevent convulsions induced by PTZ but also increased sensitivity to seizures in comparison to the Val and control PTZ groups ($P < 0/05$).

3.1.3.2. The effect of repeated administration of Val and Nepeta menthoides at different doses and their combinations on acute PTZ induced convulsions. PTZ (100 mg/kg) was injected on the test day 30 min after the last pretreatments with Val (300 mg/kg), *Nepeta menthoides* (100, 200, 400 mg/kg) or their combinations (Val + *Nepeta menthoides*). Table 6

Table 3. The effect of *Nepeta menthoides* at different doses on seizure factors induced by PTZ.

Animal groups	2nd phase Latency (min)	2nd phase Duration (sec)	5th phase Latency (min)	5th phase Duration (sec)
PTZ	2.95 ± 0.19	30.52 ± 2.75	3.4 ± 0.47	15.5 ± 1.76
Val + PTZ	*10.62 ± 2.03	* 2.85 ± 0.56	*15.5 ± 2.5	12.5 ± 2.5
Nep 100 + PTZ	2.42 ± 0.15	25.56 ± 3.83	5.31 ± 0.45	19.09 ± 3.77
Nep 200 + PTZ	2.96 ± 0.2	31.2 ± 4.34	3.7 ± 0.63	20.9 ± 2.95
Nep 400 + PTZ	4.2 ± 1.03	30.6 ± 3.34	6.08 ± 0.5	19.22 ± 1.88

Data expressed as mean ± SEM, n = 10 in each group. Val, Nep, and PTZ indicate Valproate, *Nepeta menthoides,* and pentylenetetrazol respectively. * Significant difference with the PTZ group, $p < 0.05$. Tukey test was done.

Table 4. The effect of *Nepeta menthoides* at different doses on duration and latency of the 5th phase of seizure in PTZ challenge dose.

Animal groups	mortality Incidence (%)	5th phase Duration (sec)	5th phase Latency (min)
PTZ	20	14.11 ± 2.25	2.88 ± 0.67
Val + PTZ	0	*10	*18
Nep 100 + PTZ	10	30 ± 8	6.55 ± 0.8
Nep 200 + PTZ	30	20 ± 3.3	2 ± 0.47
Nep 400 + PTZ	10	*9.8 ± 0.95	*8 ± 1.62

Data expressed as mean ± SEM for duration and latency of the 5th phase, n = 10 in each group, Val, Nep, and PTZ indicate Valproate, *Nepeta menthoides*, and pentylenetetrazol respectively. * Significant difference with the PTZ group, $p < 0.05$. Kruscal- Wallis oneway analysis of variance by ranks followed by post-hoc Mann-Whitney test.

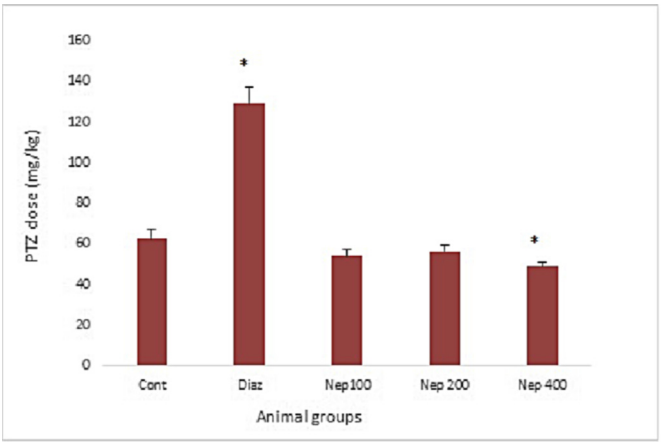

Figure 2. The effects of *Nepeta menthoides* at different doses and Diaz on the threshold dose of PTZ induced convulsions. * Significant difference with the PTZ group ($p < 0.05$). Tukey test was done. Diaz, PTZ, and Nep indicate Diazepam, Pentylenetetrazol, and *Nepeta menthoides* respectively.

shows the effects of repeated administration of Val - an antiepileptic drug - as well as *Nepeta menthoides* at different doses and combinations of Val and Nepeta on the tonic and clonic seizure latency, mortality latency, % of mortality, and the number of clonic, tonic, generalized, and HLTE seizures. Different doses of Nepeta did not have a significant effect on seizure and mortality latencies in comparison to the control PTZ group. We can therefore conclude that Nepeta did not show any antiepileptic effects. Furthermore, a 400 mg/kg dose of *Nepeta menthoides* showed epileptogenic effects; it increased the number of clonic seizures in comparison to the control PTZ group. With regard to the reduction of antiepileptic properties of repeated administration of Val (300 mg/kg), the addition of Nepeta did not change the effect of Val.

3.1.4. MES

3.1.4.1. The effect of a single dose of Phen, Nepeta menthoides, and their combination on MES induced convulsions. Tonic seizures were generated by MES (50 mA, 60 Hz, 0.7 s duration) 30 min after pretreatment of the mice with Phen (25 mg/kg), *Nepeta menthoides* (400 mg/kg) or their combination (Phen + *Nepeta menthoides)*. Table7 shows the effects of Phen - an antiepileptic drug - as well as *Nepeta menthoides* and the combination of Phen and Nepeta on the duration of HLTE, the percentage of mortality and the percentage of protection. Unlike Phen, pretreatment with the *Nepeta menthoides* extract did not reveal antiseizure properties, as it did not decrease the duration of the HLTE phase and did not provide any protection against MES induced seizures. Adding Nepeta to Phen reduced the antiepileptic effect of Phen such that the 100% protection provided by Phen decreased to 28.57 ± 18.44 and unlike the Phen group, HLTE did occur in the combination group. Therefore, *Nepeta menthoides*

Table 5. The effect of Val, *Nepeta menthoides*, and their combination on acute PTZ induced convulsion factors.

Animal groups	Clonic seizure latency	Tonic seizure latency	% of Mortality	Number of clonic seizuers	Number of tonic seizures	Number of generalized seizures	Number of HLTE seizures
Cont PTZ	74.25 ± 7.38	165.4 ± 45.006	100 ± 0	2.8 ± 0.58	5.4 ± 1.36	2 ± 0.55	0.8 ± 0.2
Val	308.33 ± 41.49 *,**,***	462.5 ± 128.08 *,**,***	0 ± 0	0.8 ± 0.37	1.25 ± 0.56	0 ± 0	0 ± 0
Nep 400	83.57 ± 13.92	148.33 ± 45.73	71.42 ± 18.44	9 ± 1.40	3 ± 0.69	2 ± 0.44	0.85 ± 0.14
Nep + Val	165.71 ± 16.16 *,**	174.28 ± 28.79	0 ± 0	7.28 ± 1.73	2.71 ± 0.52	1 ± 0.58	0 ± 0

Data expressed as mean ± SEM, n = 7 in each group, Val, Nep, and PTZ indicate Valproate, *Nepeta menthoides,* and pentylenetetrazol respectively. *, **, *** Significant difference with the control PTZ, Nep and Nep + Val groups respectively, $p < 0.05$. Tukey test was done.

Table 6. The effect of repeated administration of Val, different doses of *Nepeta menthoides* and their combinations on acute PTZ induced convulsion factors.

Animal groups	Clonic seizure latency (S)	Tonic seizure latency (S)	Mortality latency (S)	% of Mortality	Number of clonic seizuers	Number of tonic seizures	Number of generalized seizures	Number of HLTE seizures
Cont	74.25 ± 7.38	165.4 ± 45.006	485 ± 95.89	100 ± 0	2.8 ± 0.58	5.4 ± 1.36	2 ± 0.55	0.8 ± 0.2
Nep 100	108 ± 18.20	119.14 ± 18.89	348 ± 52.99	57.14 ± 20.20	4.4 ± 0.78	3 ± 0.48	1.86 ± 0.59	0.66 ± 0.19
Nep 200	91.66 ± 14.42	97 ± 19.58	381.5 ± 14.83	85.71 ± 14.28	4.16 ± 0.81	3.83 ± 0.69	2.57 ± 0.72	0.85 ± 0.14
Nep 400	80.83 ± 9.21	77.5 ± 6.38	373 ± 12.36	100 ± 0	7 ± 0.57	4.66 ± 0.62	2.28 ± 0.42	1 ± 0
Val	102.14 ± 10.74	91.14 ± 8.79	401.25 ± 41.24	71.42 ± 18.44	5 ± 0.71	4.16 ± 0.87	3.43 ± 0.61	0.71 ± 0.18
Nep100 + Val	86.66 ± 14.82	65 ± 12.75	396.25 ± 63.76	85.71 ± 14.28	6.83 ± 1.25	2.83 ± 0.37	2.28 ± 0.64	0.57 ± 0.20
Nep200 + Val	115 ± 20	143.75 ± 33.31	528.33 ± 39.39	66.66 ± 21.08	4.8 ± 0.67	2.83 ± 0.83	2 ± 0.36	0.83 ± 0.16
Nep400 + Val	96.66 ± 19.37	98.83 ± 17.53	550 ± 75.21	85.71 ± 14.28	7.2 ± 0.94	5.57 ± 1.09	2.14 ± 0.55	0.86 ± 0.26

Data expressed as mean ± SEM, n = 7 in each group, Val, Nep, and PTZ indicate Valproate, *Nepeta menthoides,* and pentylenetetrazol respectively. Tukey test was done for the first, second and third columns.

Table 7. The effect of a single dose of Phen, *Nepeta menthoides*, and their combination on MES induced convulsion, mortality, and protection.

Animal groups	Time of HLTE (S)	% of Mortality	% of protection
Cont	18.57 ± 2.61	85.71 ± 14.28	0
Nep4oo	21 ± 1.31	42.85 ± 20.20	0
Phen	0 *,**,***	0	100
Nep400 + Phen	9 ± 2.97 *,**	14.28 ± 14.28	28.57 ± 18.44

Data exhibited as mean ± SEM, n = 7 in each group, MES: maximal electroshock, Phen: Phenytoin, Nep: *Nepeta menthoides*. *, **, *** Significant difference with the control, *Nepeta menthoides,* and Nepeta + Phen groups respectively, $p < 0.05$. Tukey test was done for the first column.

not only failed to prevent convulsions induced by MES but also enhanced susceptibility to seizures in comparison with the Phen group ($P < 0/05$).

3.1.4.2. The effect of repeated administration of Phen and Nepeta menthoides at different doses and their combinations on MES induced convulsions, mortality, and protection. Animal pretreatment was performed with Phen (25 mg/kg), *Nepeta menthoides* at different doses (100, 200 and 400 mg/kg), and their combinations for seven days. Tonic convulsions were induced by MES, 30 min after the last injections on the test day. Table 8 shows the effects of repeated administration of Phen as well as different doses of *Nepeta menthoides* and their combinations on the duration time of HLTE, the percentage of mortality, and the percentage of protection. Unlike Phenytoin, one-week pretreatment with different doses of the *Nepeta menthoides* extract did not reveal antiseizure properties, as the duration of HLTE phase in MES induced seizures and the mortality percentage did not decrease in comparison with the control group ($P < 0/05$). Furthermore, the extract did not provide any protection against MES. Repeated pretreatment with the combination of a 400 mg/kg dose of *Nepeta menthoides* and Phen resulted in a decrease of antiepileptic effects in comparison with Phen alone; the duration of the HLTE phase increased from 3.57 ± 2.51 to 12.71 ± 1.41($P < 0/05$) and the protection percentage decreased from 71.43 ± 18.44 to zero. The results show that the extract not only failed to prevent convulsions induced by MES but also enhanced sensitivity to seizures.

3.2. Biochemical studies

3.2.1. PTZ kindling

3.2.1.1. The effect of Nepeta menthoides at different doses on oxidative stress markers in the PTZ kindling model. Table 9 shows the brain levels of oxidative stress markers in the control and PTZ kindled mice. PTZ kindling enhanced the brain MDA levels in comparison to the control group. However, pretreatment with *Nepeta menthoides* at different doses

Table 8. The effect of repeated administration of Phen, different doses of *Nepeta menthoides,* and their combinations on MES induced tonic convulsions, % of mortality and % of protection.

Animal groups	Time of HLTE (S)	% of Mortality	% of Protection
Cont	18.57 ± 2.61	85.71 ± 14.28	0
Nep100	14.57 ± 0.72	85.71 ± 14.28	0
Nep200	16.29 ± 0.75	100	0
Nep4oo	18.43 ± 1.23	71.43 ± 18.44	0
Phen	3.57 ± 2.51 *, **	14.28 ± 14.28	71.43 ± 18.44
Nep100 + Phen	3.57 ± 2.37 *, **	0	71.43 ± 18.44
Nep200 + Phen	5.28 ± 2.53 *	0	57.14 ± 20.20
Nep400 + Phen	12.71 ± 1.41	28.57 ± 18.44	0

Data showed as mean ± SEM, n = 7 in each group, MES: maximal electroshock, Phen: Phenytoin, Nep: *Nepeta menthoides*. * Significant difference with the control and Nepeta groups (100,200,400 mg/kg), $p < 0.05$. ** Significant difference with Nepeta 400 + Phen, $p < 0.05$. Tukey test was don for the first column.

Table 9. The effect of *Nepeta menthoides* at different doses on oxidative stress markers in the PTZ kindling model.

Test groups	NO mg/g protein)	MDA (μg/g protein)
Control normal saline	0.01 ± 0.003	0. 633 ± 0.15
PTZ	0.021 ± 0.003	1.8 ± 0.4*
Val + PTZ	0.0173 ± 0.006	1.218 ± 0.1
Nep100 + PTZ	0.115 ± 0.017 *,**	1.08 ± 0.14
Nep200 + PTZ	0.0408 ± 0.014	0.94 ± 0.14
Nep400 + PTZ	0.1 ± 0.027 *,**	0.67 ± 0.094

Data expressed as mean ± SEM. PTZ: Pentylenetetrazol, Val: Valproate, Nep: *Nepeta menthoides.*
*, ** Significant difference with the control and Val groups respectively, $p < 0.05$. Tukey test was done.

Table 10. The effect of *Nepeta menthoides* at different doses on oxidative stress markers in the PTZ infusion model.

Test groups	NO (mg/g protein)	MDA (μg/g protein)
Control normal saline	0.01 ± 0.003	0.633 ± 0.15
PTZ	0.0165 ± 0.003	0.685 ± 0.166
Diaz + PTZ	0.0067 ± 0.002	0.331 ± 0.136
Nep100 + PTZ	0.0214 ± 0.001 $	0.452 ± 0.042
Nep200 + PTZ	0.0253 ± 0.005 $	0.457 ± 0.11
Nep400 + PTZ	0.015 ± 0.002	0.48 ± 0.09

Data expressed as mean ± SEM. PTZ: Pentylenetetrazol, Diaz: Diazepam, Nep: *Nepeta menthoides*. $ Significant difference in comparison to the Diaz group, $p < 0.05$. Tukey test was done.

Table 11. The effect of *Nepeta menthoides* at different doses on oxidative stress factors in the MES model.

Test groups	NO (mg/g protein)	MDA (μg/g protein)
Control normal saline	0.01 ± 0.003	0.633 ± 0.15
MES	0.0097 ± 0.002	0.58 ± 0.15
Phen + MES	0.003 ± 0.0004	0.303 ± 0.063
Nep100 + MES	0.003 ± 0.0006	0.98 ± 0.26
Nep200 + MES	0.0236 ± 0.002 #	0.41 ± 0.03
Nep400 + MES	0.013 ± 0.002	0.317 ± 0.058

Data expressed as mean ± SEM. MES: maximal electroshock, Phen: Phenytoin, Nep: *Nepeta menthoides*. # Significant difference versus control, MES, Phen and Nep 100 mg/kg groups, $p < 0.05$. Tukey test was done.

and Val did not significantly reduce brain MDA content in comparison to the PTZ group. While brain NO levels did not change significantly in the PTZ and Val groups, pretreatment with 100 and 400 mg/kg doses of *Nepeta menthoides* significantly increased brain NO levels in comparison to control and Val groups ($P < 0.05$).

3.2.2. Timed intravenous PTZ infusion

3.2.2.1. The effect of seven-day pretreatment with Nepeta menthoides at different doses and Diaz on oxidative stress markers in the PTZ infusion model. Table 10 shows the amounts of brain oxidative stress markers in the PTZ infused and control groups. Brain MDA levels did not change significantly in any of the experimental groups. It is worth noting that repeated administration of PTZ in PTZ kindling enhanced brain MDA levels, while a single infusion of PTZ did not cause any change. On the other hand, while brain NO levels did not change significantly in the PTZ and Diaz groups, *Nepeta menthoides* pretreatment (100 and 200 mg/kg) caused elevation of brain NO levels in comparison to the Diaz group ($P < 0.05$).

3.2.3. MES

3.2.3.1. The effect of pretreatment with Nepeta menthoides at different doses and Phen on oxidative stress markers in the MES model. Table 11 shows the amounts of brain oxidative stress markers (NO and MDA) in the MES subgroups and the control mice. Brain tissue MDA levels did not change significantly in any of the experimental groups in comparison to the control group. Therefore, our results demonstrate that brain MDA levels did not change in the MES test, in contrast with PTZ kindling. Moreover, the results show that pretreatment with *Nepeta menthoides* (200 mg/kg) significantly increased brain NO levels in contrast with the control and MES groups ($p < 0.05$).

4. Discussion

Nepeta menthoides is one of the domestic species of the Nepeta genus in Iran, locally referred to as Ustukhuddoos along with *Lavandula officinalis*. Despite some reports about the antiepileptic effects of Ustukhuddoos [44], there are no reports regarding the effects of *Nepeta menthoides* in particular on epilepsy.

During drug development, some acute seizure tests including the PTZ infusion threshold test, PTZ, and MES are widely used to screen drugs with anti- or pro-convulsant properties [45]. Moreover, the combination of herbal extracts with standard antiepileptic drugs such as Phen and Val may produce anti- or pro-epileptic effects.

The results of the present study show that pretreatment with different doses of *Nepeta menthoides* not only failed to prevent seizures, but also increased susceptibility to them in different animal models of epilepsy. As opposed to Val, repeated pretreatment with different doses of *Nepeta menthoides* was unable to prevent mortality in PTZ kindling. Moreover, *Nepeta menthoides* at none of its doses could reduce seizure intensity or duration. Val as an antiepileptic drug increased latency and reduced the duration of seizure phases. *Nepeta menthoides*, however, did not change

any of these variables in PTZ kindling. Furthermore, in contrast to Diaz, *Nepeta menthoides* (400 mg/kg) not only failed to increase the threshold dose of PTZ but also decreased it in the PTZ infusion model.

Similarly, Phen reduced the duration of the HLTE phase in the MES test, whereas *Nepeta menthoides* (400 mg/kg) not only was unable to decrease the duration of the HLTE phase, but also reduced the antiepileptic effect of Phen when combined with it. Moreover, in the acute PTZ induced seizure model, unlike Val, pretreatment with *Nepeta menthoides* (400 mg/kg) extract did not show antiepileptic activity, as it did not enhance tonic and clonic seizure latency and in fact increased the number of clonic seizures in comparison with the control PTZ group. Combining Val with Nepeta reduced the antiepileptic effect of Val. Therefore, *Nepeta menthoides* not only failed to prevent convulsions induced by PTZ but also enhanced susceptibility to seizures in comparison with Val and control PTZ groups ($P < 0/05$).

Additionally, our results showed that only PTZ kindling caused elevation of brain MDA levels and that *Nepeta menthoides* did not change it. On the other hand, *Nepeta menthoides* increased brain NO levels in PTZ kindling, PTZ infusion, and MES tests.

Phytochemical screening and HPLC analysis of the *Nepeta menthoides* extract showed that it contains phenolic compounds such as rosmarinic acid (RA) and caffeic acid (CA) and flavonoids such as rutin and quercetin.

Studies on the neuroprotective, antioxidant and antiepileptic effects of RA and CA are available in different animal models, but certain points still remain unclear. For example, it has been reported that some doses of RA and CA display neuroprotective effects against the oxidative stress generated in epilepsy even though they do not show any anti-epileptogenic effects in the kindling epilepsy model [46]. Another study showed that RA dose-dependently increased latency in PTZ induced clonic and generalized seizures as well as latency in pilocarpine induced myoclonic jerks. However, repeated administration of RA in the chronic epilepsy model did not prevent the generation of spontaneous recurrent seizure [47]. RA has been shown to decrease seizure intensity, mitigate oxidative stress and hippocampal neuronal loss, and potentiate the defensive system's activity in the temporal lobe epilepsy induced by kainic acid [48]. Therefore, it seems that RA can reduce seizures dose dependently only in some animal models of epilepsy.

Flavonoids such as quercetin are present in many plants in glycosidic form (rutin). Moreover, many vegetable diets, diet supplements, and pharmaceutical preparations used by epileptic patients contain quercetin. The results obtained from studies on some animal models of epilepsy such as intravenous infusion pentylenetetrazol tests, maximal electroshock, and seizures induced by 6Hz stimulation showed that quercetin and rutin in some doses attenuate seizure factors only in the 6Hz induced seizure test and do not change seizure parameters in other models [49]. Proconvulsant effects have also been reported for quercetin on N-methyl D-aspartate (NMDA) and pentylenetetrazol induced seizures [50]. Therefore, it seems that free radical scavengers and antioxidants do not necessarily attenuate seizure symptoms.

In order to test the relationship between the antioxidant properties and the antiepileptic effects of some herbal compounds, the effects of Trolox (a vitamin E analog), melatonin, vitamin C, and alpha-lipoic acid on the latency of seizures induced by kainic acid, PTZ, and pilocarpine are reported. The results show anticonvulsant activity for alpha-lipoic acid, vitamin C, and Trolox only against pilocarpine. No effect against kainic acid or PTZ induced seizures was detected, except mortality reduction by vitamin C in the PTZ model [51]. The absence of anticonvulsant effects in some animal models of epilepsy suggests that the antioxidant properties of the herbal compounds do not necessarily account for their antiepileptic activity. We studied the effect of one herbal medicine extract in two animal models of epilepsy recently (not published). The results showed that the extract decreased seizure intensity in one model but intensified seizure-related factors in another one.

Chemical analysis of the essential oil isolated from the hydroalcoholic extract of Nepeta showed that nepetalactone is the main component of the essential oil of *Nepeta menthoides*. This is in agreement with previous studies [52]. Other studies have also reported that nepetalactone is the main ingredient of *Nepeta cataria* [53], and long-term exposure to this compound increases sensitivity to picrotoxin and strychnine induced seizures in rodents [24]. On the other hand, nepetalactone was not detected in the oil of *Nepeta bracteata* [54]. The flower extract of this plant showed antiseizure effects in ICES and PTZ tests [23].

Given the above facts, one can argue that nepetalactone has a role in the proconvulsant effects of some Nepeta species such as *Nepeta menthoides* and *Nepeta cataria*.

It is demonstrated that 4α-α, 7-α, 7α-β-Nepetalactone is a new opioid analgesic and exhibits agonistic properties for specific opioid receptors [55]. Some studies show that single and repeated administrations of tramadol and morphine intensify PTZ-induced seizures. Proconvulsant effects of tramadol and morphine on seizures induced by PTZ have been attributed, to some extent, to their effect on GABAergic pathways [56]. Therefore, it seems that the stimulatory or proconvulsant effects of *Nepeta menthoides* in the present study are partly due to the presence of nepetalactone and its anti-GABAergic effects.

In our previous study, we reported that *Nepeta menthoides* extract (400 mg/kg) prevented morphine dependence and tolerance [26]. This study also indicated that there are some substances in *Nepeta menthoides* extract that interact with morphine.

Chemical analysis showed that 1, 8 cineol (eucalyptol) is another main ingredient in the essential oil isolated from the *Nepeta menthoides* extract. There is some evidence on the occurrence of seizures following eucalyptus and camphor oil overdose/poisoning [57]. It has also been reported that essential oils that have monoterpene compounds, such as camphor and 1, 8 cineole, may induce epileptic seizures in humans and animals [58]. Therefore, it seems that 1, 8 cineol may be partly responsible for epileptogenic effects of *Nepeta menthoides* extract.

It is necessary to remember that the results of the present study showed that brain MDA content increased only in PTZ kindling, and Val as an antiepileptic drug failed to reduce brain MDA levels. These results support previous studies [28]. However, in PTZ infusion and MES induced seizures; brain MDA levels did not change significantly. These data are similar to previous data [59, 60, 61, 62]. It appears that PTZ kindling triggers different processes such as lipid peroxidation [63]. Therefore, it seems that oxidative stress may be involved in the generation of epilepsy in the PTZ kindled animal. *Nepeta menthoides* did not change brain MDA levels significantly. No studies are available about the role of *Nepeta menthoides* on brain MDA contents in animal models of epilepsy.

Our finding also showed that *Nepeta menthoides* increases brain NO levels in PTZ kindling, PTZ infusion, and MES tests. There is evidence regarding the role of free radicals in the pathogenesis of many diseases such as epilepsy [64]. Normally, antioxidants neutralize the destructive effects of free radicals in body tissues [65, 66]. Free radicals inhibit glutamine synthesis which results in the elevation of brain L-glutamate levels [64, 67]. However, many studies demonstrate that only activation of the NMDA receptor and NO production are involved in the seizure without glutamine synthesizes inhibition [68]. NO is a membrane permeable molecule that can affect different biological processes [69]. NO acts as a neurotransmitter and is involved in epileptic activity, regulation of neuronal excitability, and synaptic plasticity [70]. Three different nitric oxide synthase (NOS) isozymes catalyze the production of NO from arginine [69]. Experiments involving the administration of NOS inhibitors demonstrate involvement of NO in epilepsy [71]. It is shown that large amounts of nitric oxide induce limbic seizures. There is also evidence attributing hippocampal neuron damage to rises in the release of glutamate and nitric oxide [72]. However, the role of NO is unknown in the pathophysiology of epilepsy. It is reported that NO is involved in the pathogenesis of epilepsy via glia proliferation and neuronal death [73].

It is demonstrated that PTZ stimulates No production through enhancement of neuronal nitric oxide synthase (nNOS) expression, and

brain NO levels may be a risk factor for PTZ induced convulsions [69]. However, in the present study – similar to a previous study [28] - PTZ kindling did not have a significant effect on brain NO levels in comparison to the control group. Moreover, PTZ infusion and MES did not change brain NO levels. With regard to the role of the hippocampus in epilepsy, it seems that hippocampal NO levels need to be measured specifically.

It seems there is a relation between NO elevation by Nepeta and increased sensitivity to seizures that should be investigated later.

In the previous study, the antiepileptic effects of *Lavandula officinalis* were associated with brain NO suppression [28]. Therefore, it may be suggested that nitric oxide is a promoter of epilepsy.

Declarations

Author contribution statement

Batool Rahmati: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Fatemeh Zaeri: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Azhdar Heydari: Performed the experiments; Contributed reagents, materials, analysis tools or data;

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The authors declare no conflict of interest.

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