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ORIGINAL ARTICLE

The Impact of *Artemisia absinthium* Hydro-ethanolic Extract on Oxidative Stress in the Brain in a Seizure Model Induced by Pentylenetetrazole in Mice

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KEYWORDS

Artemisia absinthium;
Epilepsy;
Pentylenetetrazole;
Oxidative stress

ABSTRACT: Oxidative stress has a crucial role in epileptic seizures. Several studies have shown the protective effect of *Artemisia absinthium (A. absinthium*) against neuronal damage and oxidative stress. In the current research, the effect of *A. absinthium* on oxidative stress indicators in an animal model of seizure provoked by injecting pentylenetetrazole (PTZ) was estimated in mice. The mice were allocated into the following groups: a control group in which vehicle was administered; PTZ group (a single dose of 100 mg kg^{-1}, ip); and other groups, which daily received 6.25-200 mg kg^{-1} of the *A. absinthium* extract during 3 consecutive days before PTZ. The first recorded MCS and minimal clonic seizure and the first generalized tonic-clonic seizure (GTCS) latencies were analyzed. The brain segments, including the cortex and hippocampus of the animals, were then removed and harvested for oxidative stress evaluation. The extract significantly postponed the onsets of the MCS and GTCS when injected before PTZ. The seizure attacks provoked by PTZ also increased MDA in the cortex and hippocampus to levels greater than the control ($P<0.001$). In addition, the extract had an ameliorative effect on MDA concentration in the cortex and hippocampus ($P<0.05$-$P<0.001$). A lower concentration of total thiol was observed in the brain of the PTZ injected mice than the control ones ($P<0.01$- $P<0.001$). Pretreatment with the extract corrected the thiol level in the brain tissue ($P<0.05$-$P<0.001$). The current research shows that *A. absinthium* hydro-ethanolic extract has considerable anti-oxidant properties in a PTZ-induced seizure model in mice.

INTRODUCTION

Seizure attacks are due to episodes of asynchronous neural activities in the brain and manifest with physical features and behavioral changes. They occur in epileptic patients and other pathological conditions such as fever, hypocalcemia, and hypoglycemia [1]. Epilepsy, as a primary neurological disease, has been known to occur in approximately 1% of people [2]. It has been reported that a significant impairment of cognition, memory, and

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learning occurs in epileptic patients [3]. Also, a high level of free radicals has been reported to be produced after seizure attacks seizures, which can damage proteins, lipids, and DNA [4]. Since brain tissue contains high levels of lipids, it is exposed to a high level of oxidative stress. Neurological diseases are caused mainly by brain tissue oxidative damage. Numerous studies have reported that oxidative damage to brain tissue is responsible for some of the seizure complications [5]. Also, pentylenetetrazol (PTZ) induces neurotoxicity and convulsion partly due to overproducing free radicals and reactive oxygen species (ROS) [6]. Several studies have reported that oxidative stress is the primary mechanism of epilepsy [4, 5, 7]. Therefore, a substance with anti-oxidant properties can be useful in treating seizures [8]. In a previous study, we showed that some plant extracts with anti-oxidant properties could be useful in treating of seizures [6].

Secondary metabolites and essential oils are important therapeutic compounds in medicinal plants. Medicinal plants play important roles in treating disorders because of their low cost, high availability, and fewer side effects [9]. Numerous studies have reported anxiolytic, analgesic, antidepressant, and anti-epileptic properties of medicinal plants. *Artemisia absinthium (A. absinthium*) Linn. as a plant belonging to the Asteraceae family [1, 10, 11], is known as Wormwood and Afsantin. Its origin is Persia and Pakistan [12]. Using *A. absinthium* has been common in traditional medicine as an antispasmodic, stomachic, anthelmintic, febrifuge, and cardiac stimulant for declining liver inflammation and improving memory [13, 14]. This plant is also used to treat central nervous system (CNS) disorders, including ischemia and depression, in various parts of the world [15, 16]. In a recent study, *A. absinthium* hydro-ethanolic extract improved pentobarbital induced hypnosis in mice [11] regarding the effect of its extract or other ingredients on gamma-Aminobutyric acid (GABA) receptors.

Some studies have reported anti-oxidant and neuroprotective effects of *A. absinthium*. In an experimental study, an acute oil administration lowered the H_2O_2 toxicity [17]. Another research showed that the fraction of *A. absinthium* produced by ethyl acetate decreased oxidative stress caused by ischemia/reperfusion in the brain tissue [16]. Some ingredients of essential oil of *A. absinthium* include β-pinene (23.8%) and β-thujone (18.6%) [18]. Here, β-pinene is volatile monoterpenes that possess antidepressant-like activity [19]. The anti-oxidant effects of *A. absinthium* essential oil and extracts have been attributed to the sesquiterpene and dimer of sesquiterpene lactones, such as artabsin and absinthin [20].

Considering the probable effects of *A. absinthium* extract or its ingredient on GABA receptors and the anti-convulsant effect of the natural products with anti-oxidant effects [21-23], the current research was done to estimate the effects of *A. absinthium* extract on PTZ-induced seizures and oxidative damage in brain tissue in mice. It has been previously reported that the extraction method affects the anti-oxidant effects of the plants. In this respect, two types of extract, namley Soxhalet and macerated, were examined [24, 25].

MATERIALS AND METHODS

The chemicals and the plant extract

PTZ was bought from Sigma-Aldrich Company (St. Louis, USA). Other chemicals used for biochemical measurements, including ethylenediaminetetraacetic acid (EDTA), thiobarbituric acid (TBA), hydrochloric acid (HCL), trichloroacetic acid (TCA), and DTNB (2, 2'-dinitro-5, 5'-dithiodibenzoic acid), were purchased from Merck Company.

The aerial parts (leaves, stems, and twigs) of *A. absinthium* were obtained from the mountains around Dargaz City (North Khorasan Province, Iran). The plant was confirmed in the Herbarium of Research Center of Khorasan Razavi Agricultural and Natural Resources and was allocated with a voucher number (No. 11856). The plant was dried and then grounded by a mortar. The Soxhlet form of the extract was prepared by extracting 100 g of the dried materials of the plant with 1500 mL of ethanol-water (70/30 v/v) using the Soxhlet equipment [10]. Also, the macerated form of the extract was prepared by mixing 100 g of the powders of the plant with 875 mL of ethanol-water (70/30, v /v). The mixture was placed on a shaker at room temperature. After 72 h, the mixture was filtered using a filter paper. A reduced pressure method was used to remove the solvent from

both Soxhlet and macerated forms of the extract. The extracts were then dried, and saline was used to prepare the appropriate extract doses [11, 26].

The animals and treatments

The experiments were performed on 110 BALB/ C mice, 28 ± 3g in weight and age of 8-10 weeks. The mice were kept at the laboratory animal center. The standard conditions were provided, and the temperature was set at 22-23℃. A 12-h periodic dark/light was provided, and appropriate humidity with laboratory chow and water were provided. The animals were randomly allocated into 11 groups. They received the following treatments (n = 10 in each group): 1) Control (saline), 2) PTZ (100 mg kg^{-1}), 3-8) 6.25, 12.5, 25, 50, 100, and 200 mg kg^{-1} of the macerated extract before PTZ injection, and 9-11) 50, 100, and 200 mg kg^{-1} of the Soxhlet extract before PTZ. The doses were selected from the previous studies [16, 27]. The number of mice in each group was also selected considering the other experiments. The animals of Groups 2-8 were intraperitoneally (i.p.) treated with different doses of the extract during three consecutive days [1, 23, 28]. Saline was injected in the mice of Groups 1 and 2 in these days. The mice also received saline or the *A. absinthium* extract 30 min prior to i.p. injection of PTZ (100 mg kg^{-1}). Our previous studies revealed that PTZ in this dose provokes generalized tonic-clonic seizures (GTCS) in mice and rats [6, 29-31]. Then, the cortical and hippocampal regions were separated for the biochemical measurements. Also, the brains were collected without inducing seizures in the control group.

Induction of seizures by PTZ

The seizure behaviors were induced by locating PTZ injection. Next, the mice were placed in a Plexiglas container (35 cm × 35 cm × 35 cm), and their behaviors were recorded during 60 min [6, 27, 29, 31, 32]. The behavioral responses of the mice to PTZ administration were observed, and their latency to the first minimal clonic seizure (MCS) and the first GTCS was recorded [27, 30, 31].

Biochemical assessment

After performing the behavioral experiments, a deep anesthesia was induced, the brains were collected, and the cortex and hippocampus were separated and harvested for biochemical measurements. A competent person made the animal killing with minimum pain-suffering and distress. The experiments were conducted considering the Annex IV of the guidelines from Directive EU/2010/63 of the European Parliament. Before doing the biochemical measurements, the cortex and hippocampus were dissected and homogenized on an ice-cold surface.

Estimation of thiol concentration

For total thiol (SH) content measurement, DTNB was used as a reagent. From the reaction of DTNB with thiol, a yellow solution is produced. To this end, 1 mL tris-EDTA buffer (pH = 8.6) was added after the brain homogenates, and the sample absorbance was observed at 412 nm (A1). Then, 20 µL of DTNB was added to the mixture, and after 15 min of storing at laboratory temperature, the absorbance was observed again (A2). The absorbance of DTNB also was considered a blank (B). Total thiol concentration was estimated from the following equation [6, 32-35]. Total thiol concentration (mM) = (A2-A1-B) × 1.07/0.05 × 13.6.

Lipid peroxidation assay

Malondialdehyde (MDA) level was measured as an index of lipid peroxidation. MDA has a peak absorbance at 535 nm. In addition, it reacts with TBA as a TBA reactive substance to produce a red color solution. The TBA/TCA/HCL reagent was dispensed to the homogenate samples, and the samples were boiled for 40 min using a boiling water bath. After reaching room temperature, the samples were centrifuged within 1000 g for 10 min. The absorbance of the samples was observed at 535 nm [6, 33-35]. The following formula was used to calculate MDA: C (M) = Absorbance/ (1.56×10^5).

Statistics analysis

One-way analysis of variance (ANOVA) and Tukey post hoc test were performed to show the statistical differences between the groups. The results were expressed as mean ± SEM, and those with a P-value lower than 0.05 were considered significant.

RESULTS

The effect of Soxhlet and macerated extracts on seizure behaviors

The behavioral results showed that both 50 and 100 mg kg^{-1} of Soxhlet extract prolonged MCS latency (P<0.05 for both), but 200 mg kg^{-1} of the extract had no effect. In addition, 100 mg kg^{-1} of Soxhlet extract prolonged GTCS latency, but 50 and 200 mg kg^{-1} of the extract did not show a significant effect (Figures 1a and 1b).

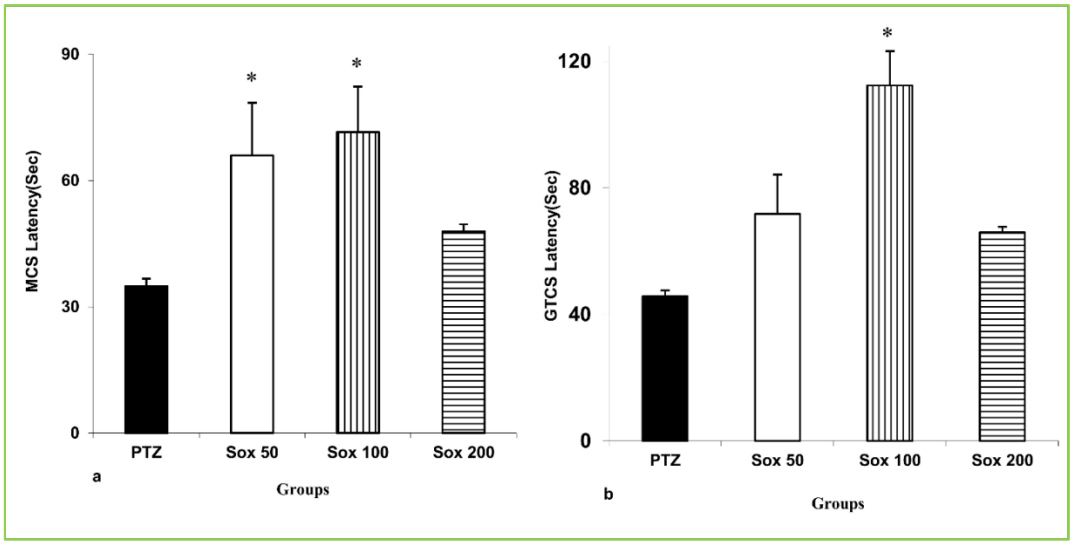

Figure 1. The effects of *A. absinthium* Soxhlet extract on (A) MCS and (B) GTCS seizures latencies (*P<0.05 compared to the control mice).

The behavioral results also revealed that both 6.25 mg kg^{-1} and 200 mg kg^{-1} of the macerated extract prolonged MCS latency (P<0.05 and P<0.01, respectively). However, 12.5, 25, 50, and 100 mg kg^{-1} of this extract were not effective. Among different doses of the macerated extract, only 200 mg kg^{-1} of the macerated extract could increase the GTCS latency, but 6.25, 12.5, 25, 50, and 100 mg kg^{-1} were not effective (Figures 2a and 2b).

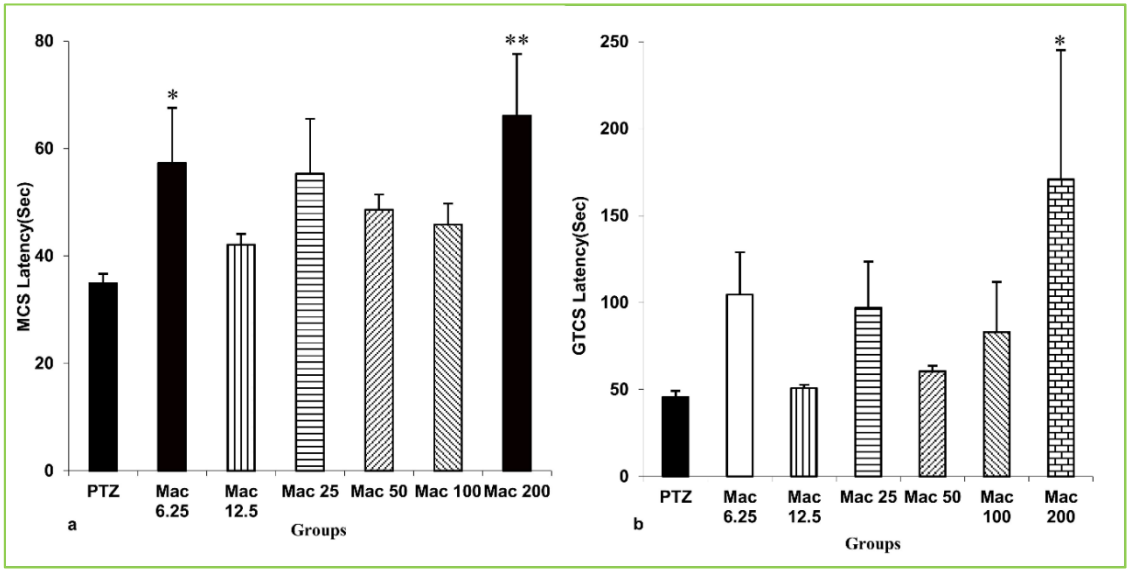

Figure 2. The effects of *A. absinthium* macerated extract on (A) MCS and (B) GTCS seizures latencies (*P<0.05, **P<0.01 compared to the control mice).

The effect of Soxhlet extract on the concentrations of

MDA and thiol

The results showed that PTZ- induced seizure was followed by a decrease in the thiol content and an increase in the MDA in the cortex of PTZ-injected mice compared to the brain of the control animals (P<0.001 and P<0.01, respectively). All doses (i.e., 50, 100, and 200 mg kg^{-1}) of the Soxhlet extract improved the thiol concentration (P<0.001 for all cases) and decreased MDA level (P<0.001, P<0.01, and P<0.05, respectively) in the cortex of Sox 50, Sox 100, and Sox 200 groups compared to the PTZ injected mice (Figures 3a and 3b).

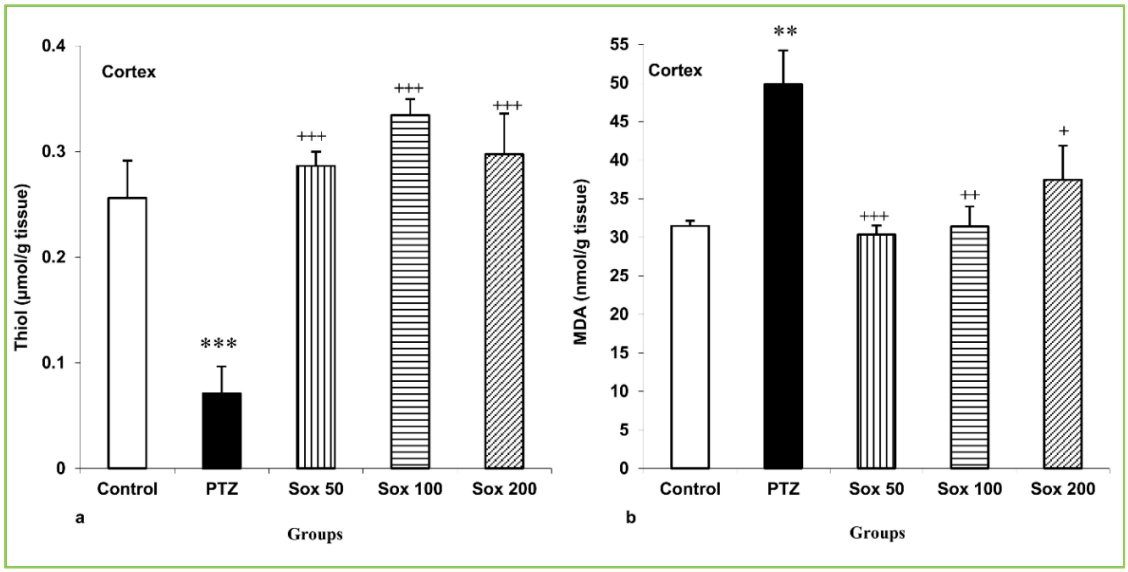

Figure 3. The effects of *A. absinthium* Soxhlet extract on (A) thiol and (B) MDA concentration in the cortex (**P<0.01 and ***P<0.001 compared with the control mice; $^{+}$P<0.05, $^{++}$P<0.01, and $^{+++}$P<0.001 compared to the PTZ-injected group).

The results also revealed that thiol content was lower while MDA level was higher in the hippocampus of PTZ injected mice compared to the control mice (P<0.01 for thiol and P<0.001 for MDA). All doses of the Soxhlet extract corrected thiol concentration in the hippocampus of Sox 50, Sox 100, and Sox 200 groups compared to the PTZ injected mice (P<0.01, P<0.001, and P<0.05, respectively). The hippocampal MDA levels in all Sox 50, Sox 100, and Sox 200 groups were lower than that in the PTZ injected group (P<0.05, P<0.01, and P<0.05, respectively) (Figures. 4a and 4b).

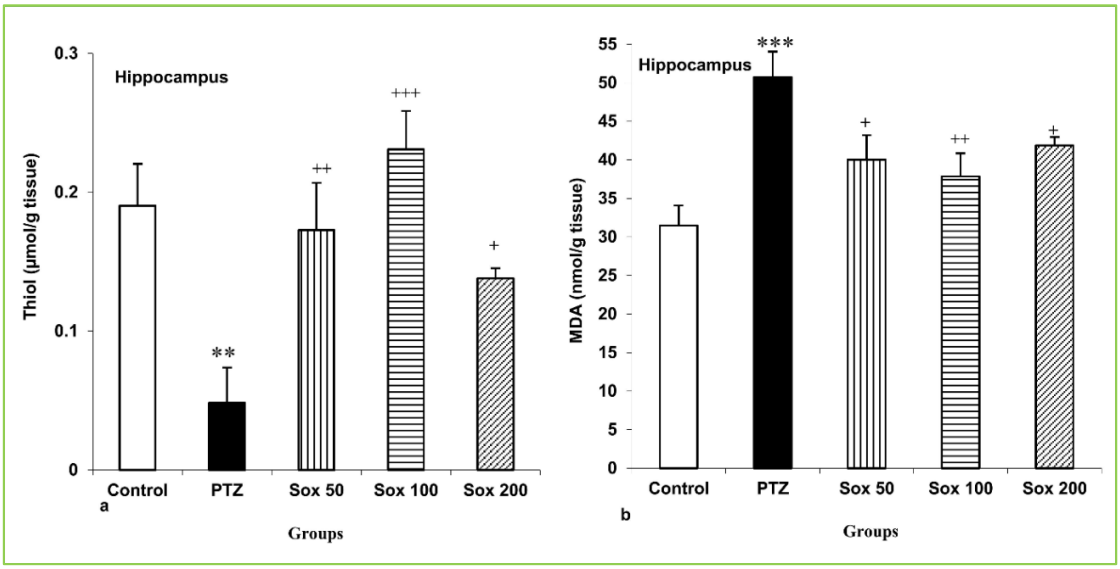

Figure 4. The effects of *A. absinthium* Soxhlet extract on the (A) thiol and (B) MDA concentration in the hippocampus. **P<0.01, ***P<0.001 compared to the control mice, $^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$P<0.001 compared to the PTZ injected group.

The effect of the macerated extract on MDA and thiol

Among different doses of macerated extract, 6.25, 25, 50, and 200 mg kg^{-1} of the extract improved thiol content in the cortex compared to the PTZ group (P<0.01, P<0.05, P<0.05, and P<0.05, respectively). The results also showed that MDA level in the cortex of the rats pretreated by 6.25, 12.5, 50, 100, and 200 mg kg^{-1} of the extract was lower than the PTZ injected mice (P<0.001, P<0.01, P<0.01, P<0.05, and P<0.01, respectively) (Figures 5a and 5b).

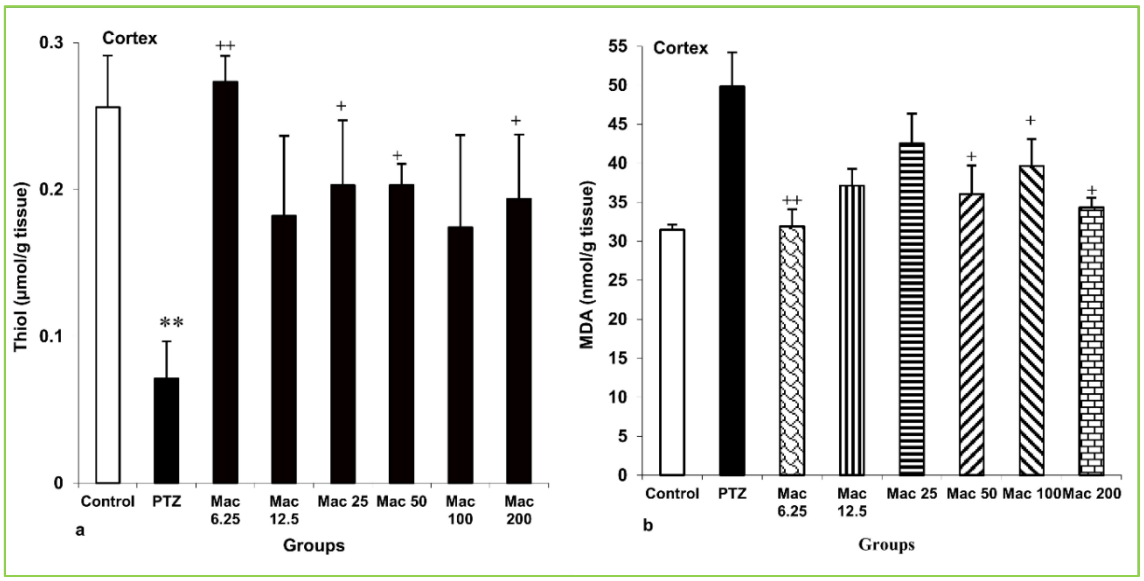

Figure 5. The effects of *A. absinthium* macerated extract on (A) thiol and (B) MDA concentration in the cortex (**P<0.01, ***P<0.001 compared to the control group; $^{+}$P<0.05, $^{++}$P<0.01, and $^{+++}$P<0.001 compared to the PTZ group).

The results of the biochemical analysis showed that thiol concentration in the hippocampus of the rats pretreated by all doses (i.e., 6.25, 12.5, 25, 50, 100, and 200 mg kg^{-1}) of the macerated extract was higher than the PTZ group (P<0.05 to P<0.001). Among different doses of macerated extract, only 6.25 mg kg^{-1} dose was effective in decreasing MDA level in the hippocampus compared to the PTZ injected mice (P<0.01) (Figures 6a and 6b).

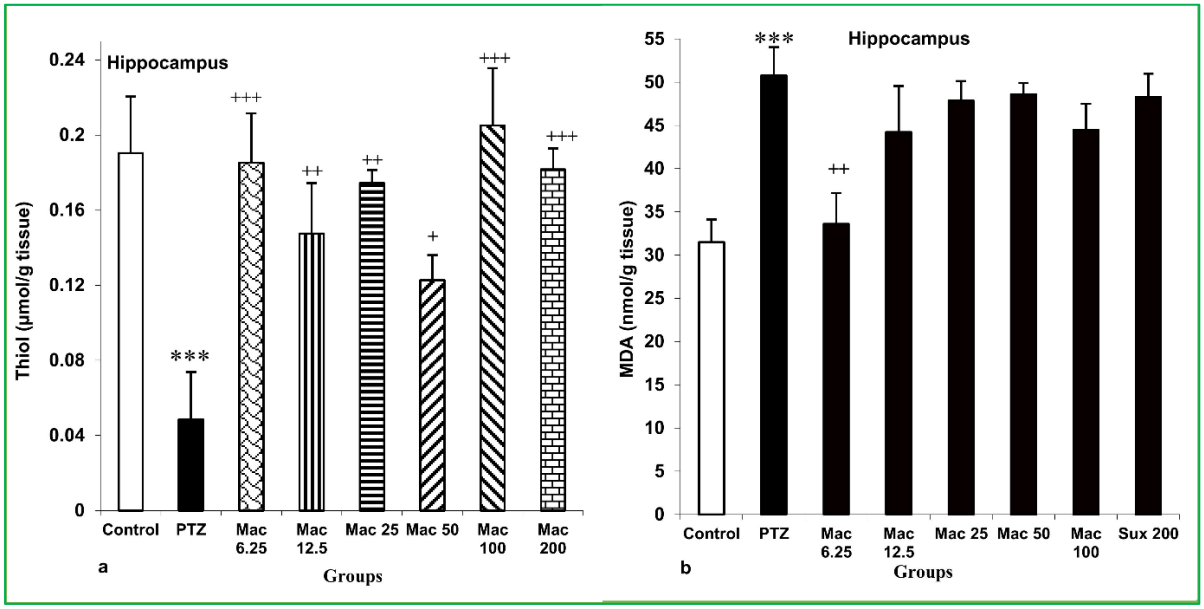

Figure 6. The effects of *A. absinthium* macerated extract on (A) thiol and (B) MDA concentration in the hippocampus (***P<0.001 compared to the control mice; $^{+}$P<0.05, $^{++}$P<0.01, and $^{+++}$P<0.001 compared to the PTZ injected mice).

Comparison of the effects soxhlet and macerated extract of the plant

The results revealed that the mice treated with 100 mg kg^{-1} of macerated extract had a shorter MCS latency than the mice treated with the same dose of Soxhlet extract (P<0.05). No significant difference was observed between the effects of 50 and 200 mg kg^{-1} of macerated extract and the same doses of Soxhlet extract on MCS latency. In addition, no significant difference was observed between the effects of 50, 100, and 200 mg kg^{-1} of macerated extract and the same doses of Soxhlet extract on GTCS latency. The biochemical data showed that thiol content in the cortex of the mice that received 50 and 100 mg kg^{-1} macerated extract was lower than those that received the same doses of Soxhlet (P<0.001 and P<0.05, respectively). However, no significant difference was seen between the effect of 200 mg kg^{-1} of macerated extract and the same dose of Soxhlet extract. In addition, there was no significant difference between the effects of 50, 100, and 200 mg kg^{-1} of macerated extract and the same doses of Soxhlet extract on thiol content in the hippocampus. Finally, the MDA level in the hippocampus of the mice which received 50 and 200 mg kg^{-1} of the macerated extract was higher than those treated by the same dose of Soxhlet extract (P<0.05 for both), but no significant difference was observed between the effect of 100 mg kg^{-1} of macerated extract and the same dose of Soxhlet extract (Table 1).

Table 1. Comparison of the effects Soxhlet and macerated extract of *A. absinthium.**P<0.05 and ***P<0.001compared to the same dose of Soxhlet extract.

	MCS(Sec)	GTCS(Sec)	Thiol-Cortex	MDA-Cortex	Thiol-Hippocampus	MDA-Hippocampus
Sox 50	66±12.45	71.80±8.91	0.28±0.01	30.35±1.16	0.17±0.03	40.03±3.15
Sox 100	71.53±10.80	112.46±23.86	0.33±0.01	31.39±2.58	0.23±0.02	37.86±2.98
Sox 200	47.90±1.72	66±3.50	0.29±0.03	37.43±4.42	0.13±0.00	41.87±1.08
Mac 50	48.6±2.87	60.5±3.10	0.20±0.01***	36.05±3.66	0.12±0.01	48.58±1.32*
Mac 100	45.83±3.92*	83±28.86	0.17±0.06*	39.66±3.45	0.20±0.03	44.48±3.02
Mac 200	66.11±11.48	170.88±74.28	0.19±0.04	34.32±1.23	0.18±0.01	48.26±2.71*

DISCUSSION

The current study showed that *A. absinthium* had an anti-oxidant effect in the brain tissue in a seizure model induced by PTZ. Structural and functional damages have been frequently reported to occur as complications of epilepsy and seizure [36, 37]. It has also been reported that cognitive dysfunctions are common in epileptic patients [3, 36, 38]. The consequences of epilepsy have been repeatedly attributed to apoptosis and neuronal death in the brain areas, including the hippocampus and cortex [1, 39, 40]. Brian tissue oxidative damage also plays a major role in epilepsy and seizure attacks [41]. Besides, oxidative stress is suggested to have a role in epilepsy and seizure [42]. In the present study, PTZ-induced seizure was accompanied by an oxidative stress status in both the hippocampus and the cortex, which was presented by a decrease in thiol content and an increased level of MDA. Several studies have reported that PTZ-induced seizures are associated with an oxidative stress status in the brain [6, 43, 44]. The anti-oxidant natural products have been frequently shown to act as anti-epileptic and anti-seizure agents [23, 45, 46]. The anti-oxidants were also able to attenuate epilepsy and seizure-related cognitive impairments [23, 47]. Various studies have shown that the plants with anti-oxidant effects also exhibit a protective effect in epilepsy by scavenging the free radicals [48, 49].

A. absinthium is a medicinal plant with anti-inflammatory and anti-oxidant activities [50]. In the current study, both Soxhalet and macerated extracts of *A. absinthium* showed anti-oxidant effects. In support of these results, *A. absinthium*

has been reported to have beneficial effects on neurodegenerative disorders [16, 51, 52]. It was previously shown that a methanol extract of *A. absinthium* decreased infarct size and showed a neuroprotective effect in a rat model of cerebral ischemia/reperfusion[16]**.** Elsewhere, it was reported that the aqueous extract of *A. absinthium* decreased the peroxidation of lipids in liver tissue and increased the SOD activity in lipopolysaccharides (LPS) intoxicated mice [53]. Moreover, 100-200 mg kg^{-1} doses of *A. absinthium* methanol extract protected the brain in a rat model of cerebral ischemia/reperfusion and increased the SOD and GSH levels in the rat brains [54]. The anti-oxidant effects of essential oil of the leaves of *A. absinthium* were also confirmed in different studies [55]. It was also shown that *A. absinthium* extract reduced mercuric chloride-induced oxidative stress in the brain, which was presented by a decrease in MDA level and increased catalase and SOD activities [56].

In the present research, the low (50mg kg^{-1}) and the medium (100mg kg^{-1}) doses of the Soxhlet extract prolonged MCS latency, but the highest dose (200mg kg^{-1}) was not effective. In addition, only 100mg kg^{-1} of Soxhlet extract increased GTCS latency, but none of 50 or 200mg kg^{-1} of the plant extract was effective. Based on these results, it is assumed that the anti-convulsant ingredients of the plant might be heat-sensitive. Therefore, a wide range of macerated extract was examined for this purpose. The results showed that the lowest dose (6.25mg kg^{-1}) and the highest dose (200mg kg^{-1}) of the macerated extract prolonged the MCS latency, but other doses (i.e., 12.5, 25, 50, and 100mg kg^{-1}) of the extract had no effect. In addition, the highest dose (200mg kg^{-1}) of the macerated prolonged the GTCS latency, but 6.25, 12.5, 25, 50, and 100mg kg^{-1} of the plant extract had no effect. Considering these results, it is difficult to conclude about the anti-seizure effects of the *A. absinthium*, and some more studies have to be done in the future. The protective effect of *A. absinthium* on seizure and epilepsy has not been previously reported. In this respect, even some neurotoxic and convulsive effects have been reported for it. However, it needs to be more investigated in future studies.

Since mechanisms of the beneficial properties of the extracts were not evaluated in the current work, further studies are suggested to be done in the future. A recent study showed that the hydro-ethnolic extract of the plant had hypnotic properties attributed to the effects of the plant or its ingredients on the GABA or its receptors [11]. Therefore, the anti-convulsant effects of the plant extract observed in the present study might be due to its effect on GABA release or GABA receptors.

The results showed that all doses (i.e., 50, 100, and 200mg kg^{-1}) of the Soxhlet extract declined the MDA concentration and improved the thiol level in both the hippocampus and cortex. Considering these results, it seems that some of the anti-convulsant effect of the Soxhalet extract observed in the present research might be partly related to its anti-oxidant effects. In addition, the plant extract is suggested to be protective against neuronal damage due to epilepsy and seizure. Based on these results and the results of oxidative stress, it seems that some of the attenuating effects of the Soxhlet extract on MDA and its improving effect of thiol content are due to its anti-convulsant effects; however, further studies are suggested in this regard.

The results also showed 6.25-200mg kg^{-1} of the macerated extract decreased MDA and increased thiol levels in the cortex. In addition, 6.25-200 mg kg^{-1} of the macerated extract corrected thiol content, but only the lowest dose effectively decreased MDA in the hippocampus. In addition, the anti-convulsant effect of the macerated extract was negligible. In this respect, among different doses of the extract, one dose postponed the GTCS onset, and the two doses increased the MCS latency. Therefore, it seems that the anti-oxidant properties of the macerated extract are more considerable than the anti-convulsant effects. However, further investigations are suggested to be done.

In the present study, the effects of the same doses of macerated and Soxhlet extract were also

compared. The results revealed that MCS latency in the mice treated with 100mg kg^{-1} of the macerated extract was shorter than those treated with the same dose of Soxhlet extract. Nevertheless, no significant difference was observed between the effects of 50 and 200mg kg^{-1}. In addition, no significant difference was also observed between the effects of macerated and Soxhlet extract on GTCS. Considering these results, it seems that the Soxhlet extract was a little more effective on seizures; however, again, it needs more investigations. In addition, thiol content in the brain of the mice treated by two doses of the macerated extract was lower, but MDA was higher than those treated by the same doses of Soxhlet extract. Therefore, it seems that Soxhlet extract had higher anti-oxidant effects than the macerated extract. Similar to our results, it was previously reported that the Soxhlet extract of the plants has a higher anti-oxidant activity than the macerated extract [24, 25].

Finally, the plant extracts' anti-apoptotic and protective effects against neuronal death were not evaluated in the current study. However, considering the protective effects of the plant revealed in the current research, it seems that the *A. absinthium* extract can decline the side effects of seizure and epilepsy on the brain.

The components responsible for the protective effects of *A. absinthium* seen in the current research were not determined in the current research. Nevertheless, Shikimic Acid as a component of the leaves of *A. absinthium* was able to reduce MDA level and increase the GSH and SOD in diabetic rats [57]. It was also shown that caruifolin D inhibited neuroinflammation via lowering the generation of intracellular reactive oxygen species in LPS-stimulated BV-2cells [51]. The anti-oxidant effects of *A. absinthium* essential oil and extracts have been attributed to the sesquiterpene and dimer of sesquiterpene lactones such as artabsin and absinthin [20]. Another component of essential oil and extract of *A. absinthium* is reported to be β- Pinene. In this respect, the anti-convulsant effects of the plant extract may at least partly be due to this component [18, 19]. Further studies, however, have to be done to clear these issues.

In conclusion, the present study shows the beneficial effects of *A. absinthium* extract on seizures and its subsequent outcomes, including brain damage. However, more studies using other animal models and more precise experiments need to be done in the future. For instance, electrophysiological studies seem to be elucidating this issue.

ACKNOWLEDGEMENTS

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ETHICAL CONSIDERATION

All efforts were made to maintain the animals in good general health according to the European Communities Council Directive (2010/63/UE). The Mashhad University of Medical Sciences, Iran, Ethical Committee (IR.MUMS.MEDICAL.REC.1398.581) confirmed animal handling and all related procedures.

Conflict of interest

We declare that we have no conflict of interest.

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