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Original Article:

Protective Role of α-Pinene in Cuprizone-Induced Multiple Sclerosis in Mice

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Abstract

Introduction: It is clamied that α-pinene has properties against Multiple Sclerosis (MS) which is known as demyelination of the neurons. Given that, the aim of this study was to investigate the effect of α-pinene on Cuprizone-induced (CPZ) MS.

Materials and Methods: A total of forty C57BL/6 mice were allocated to 4 groups. Mice in group 1 (control) were treated with a normal diet. In group 2, CPZ-induced demyelination was done by chew palate containing .2% (w/w) CPZ for 5 weeks. In group 3, a normal diet was provided and mice were injected with *α*-pinene (1 mg/kg; i.p.) 3 times a week for 5 weeks. In group 4, mice were fed with the CPZ containing diet and injected with *α*-pinene (1 mg/kg; i.p.) three times a week for 5 weeks. At the end of the study, reflexive motor behavior and depressive- like behavior tests were performed. Additionally, serum anti-oxidant activity was determined.

Results: Results show that the CPZ had an adverse effect on reflexive motor behavior tests (P<.05) and co-administration of the CPZ+*α*-pinene diminished the adverse effect of the CPZ on the reflexive motor behavior tests (P<.05). Moreover, CPZ significantly amplified immobility time (P<.05) and co-administration of the CPZ+*α*-pinene reduced the adverse effect of the CPZ on depressive-like behavior tests (P<.05). CPZ significantly increased malondialdehyde (MDA) and decreased glutathione peroxidase (GPx), superoxide dismutase (SOD) and total antioxidant status (TAS) and also these effects were reversed by α-pinene (P<.05). The data indicate that co-administration of the CPZ+*α*-pinene significantly improved the adverse effect of the CPZ on serum antioxidants (P<.05).

Conclusion: α-pinene demonstrated protective outcome against CPZ-induced MS in mice.

Keywords: α-pinene, Cuprizone, Multiple Sclerosis, Mice

1. Introduction

Multiple sclerosis (MS) is known as demyelination of the neurons in the central nervous system (CNS) which can be developed by attachment of the immune system to myelin sheath of the neurons [1, 2]. Based on the literature, the demyelination in MS mostly affects white matter lesions related to motor symptoms. Yet, the recent studies suggest that cognitive impairment in MS patients happens because of the fluctuations in grey matter [3]. CPZ leads to imbalance between demyelination and remyelination processes. In the CPZ-induced experimental demyelination model, various white and grey matter regions are observed in various areas of the nervous system such as cortex, hippocampus, cerebellum and peripheral nervous system (PNS) [4]. Most available MS therapies act via

the immune system by decreasing immunomodulatory or immunosuppressive functions [5]. These medications are highly effective in preventing autoimmune disease, but they are less effective in controlling the neurodegenerative disease [6].

Pinene ($C_{10}H_{16}$) is a nature terpenoid and two isomers as α and β are found in. They have anti-fungal, bactericidal and pain relief activities [7]. *α*-pinene has positive effects on the brain. *α*-pinene supplementation would reduce sleep latency in rats [8]. Several neurodegenerative diseases are prompted by brain-derived neurotrophic factor (BDNF) and oxidative imbalance [9]. Also, *α*-pinene administration raises hippocampus BDNF levels [10]. *α*-pinene has antioxidant properties which can lower serum MDA level and improve catalase, SOD, GPx and glutathione reductase levels [11]. Porres-Martínez et al. [12] reported that *α*-pinene prevented reactive oxygen species (ROS) in H_2O_2-sensitized oxidative stress.

MS is a progressive demyelinating disorder which unfortunately affects millions of people and is growing year by year [13]. There are drugs that can help lessen the symptoms, but there is no cure [14]. Oxidative stress and inflammation play a key role in the pathobiology of the MS, and antioxidants and anti-inflammatory agents are routinely used for the treatment of this disease [13]. Given the therapeutic activity of the *α*-pinene in the nervous system, and lack of information regarding its possible protective activity in MS, the purpose of this study was to examine the effect of α-pinene on CPZ-induced MS in mice.

2. Materials and Methods

Animals and study protocol

Male C57BL/6 mice (aged 4–6 weeks; weighing 19 ± 2 g) were kept in laboratory conditions with *ad libitum* access to food and tap water. One week after acclimatization, the mice were randomly allocated to four experimental groups (n=10). The control mice were provided with standard chew pellet. In group 2, the mice were provided with standard chew pellet mixed with .2% (w/w) CPZ for 5 weeks [1].

In group 3, a normal diet was provided and the mice were i.p. injected with *α*-pinene (1 mg/kg) 3 times a week for 5 weeks. In group 4, the mice received diet containing CPZ (.2% w/w) for 5 weeks and also during this time they were i.p. injected with *α*-pinene (1 mg/kg) [12] three times a week. At the end of the study, reflexive motor behavior and depressive- like behavior tests were administered (Figure 1).

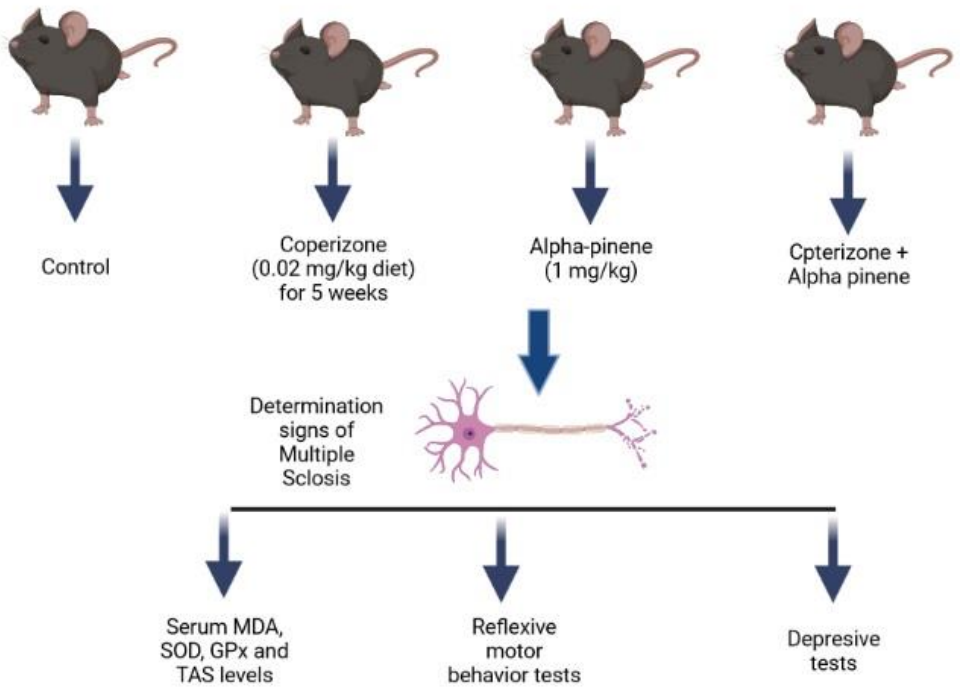

Figure 1. Flowchart of the study procedure. The time line was reflexive motor behavior, depressive - like behavior tests and finally serum antioxidant activity

Clinical score of demyelination

Throughout disease progression, the CPZ-treated mice were scored to indicate neurological damage. Grade 0 – no clinical signs; Grade 1 – paralyzed tail; Grade 2 – loss of coordinated movements, hindlimb paresis; Grade 3 – both hindlimbs paralyzed; Grade 4 – forelimbs paralyzed; Grade 5 – moribund [16].

Ambulation

Crawling behavior is used for ability to walk following MS signs [15]. Animals were located in a transparent enclosure. Gentle tail prod was used to stimulate mice to walk. The ambulation score was given as zero for no movement, score 1 for ability to crawling with asymmetric limb movement, score 2 as the mice with slow crawling but symmetric limb movement and score 3 as fast crawling/ walking [17].

Hind-limb Foot Angle

Following the signs of the MS, hind limb posture changes wherein walking the hind limbs are positioned under the body [17]. A plain open field box equipped with camera was used to record the mice movements. The foot angle was determined by recorded videos. In the recorded videos, a line was drawn from the end of the heel/shin to the tip of the toe [17].

Front- limb Suspension

This test was done on mice to hang onto the wire with both forepaws. Afterward grasping the wire, animal was released and the time was recorded until the mouse fell[17].

Hind-limb Suspension

For this test, the mice were placed face down into the conical laboratory tube. The mice's hind legs hung over the wire and hind-limb posture was given based on score as zero for constant clasping of the hind-limb onto the tube, score one: faintness was apparent and the hind-limb in clasped position, score two: hind-limb were close to each other; score three: hind-limb was closer together and rarely touched each other and finally score four as normal hind limb separation with tail raised [17].

Surface Righting

In the surface righting test, the mice were kept for 5 seconds in supine position, then time needed to return to their prone position was recorded [17, 18].

Grip Strength

Fiberglass screen (16 × 18) in horizontal position is slowly rotated in a vertical position in order to determine the grasping of all four limbs [19]. The hanging impulse against gravity was determined as [weight (g) × latency to fall (s)] [20].

Negative Geotaxis

The mice were placed downward on a 45° wooden surface and time needed by them to face the slope upward was recorded [17].

Forced Swimming Test (FST)

The mouse was placed in a glass cylindrical contain with 25 °C water for 6 min. When it ceased struggling and remained floating motionless immobility was recorded at last 4 min of this duration [21].

Tail Suspension Test (TST)

The mouse was suspended 50 cm above the floor by adhesive tape placed for 6 min and immobility were recorded at last 4 min of this duration [22-24].

Open Field Test (OFT)

The mouse was allocated to the floor of the 45×45×30 cm^3 wooden box which divided into nine square. Number of squares crossed was recorded during 6 min [25].

Rotarod Test

The mouse was placed on a rotarod device at 0–20 rpm for 8 min until its fall which was recorded [26].

Antioxidant activity

After determining the behavioral tests, blood samples were taken from all the mice. Cardiac, serum MDA, SOD, GPx and TAS levels were determined using Zell Bio GmbH (Germany) assay kits.

Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA), shown in the mean ± standard error (SE). Also, the mean differences were determined by Tukey HSD test ($P < .05$).

3. Results

Ambulation score

As seen in Figure 2, the administration of the diet containing CPZ (.2% w/w) significantly decreased ambulation score compared to the controls (P<.05). α-pinene significantly increased ambulation score compared to the controls (P<.05). CPZ+α-pinene significantly decreased adverse effect of the CPZ on ambulation score (P<.05).

Hind-limb foot angle

As shown in Figure 3, the administration of the CPZ significantly increased hind-limb foot angle compared to the controls (P<.05). α-pinene significantly decreased hind-limb foot angle compared to the control group (P<.05). CPZ+α-pinene significantly reduced the adverse effect of the CPZ on hind-limb foot angle (P<.05).

Hind-limb suspension

According to the results, hind-limb suspension was significantly decreased in CPZ-treated mice (P<.05). α-pinene significantly amplified hind-limb suspension compared to the controls (P<.05).

CPZ+α-pinene minimized the adverse effect of the

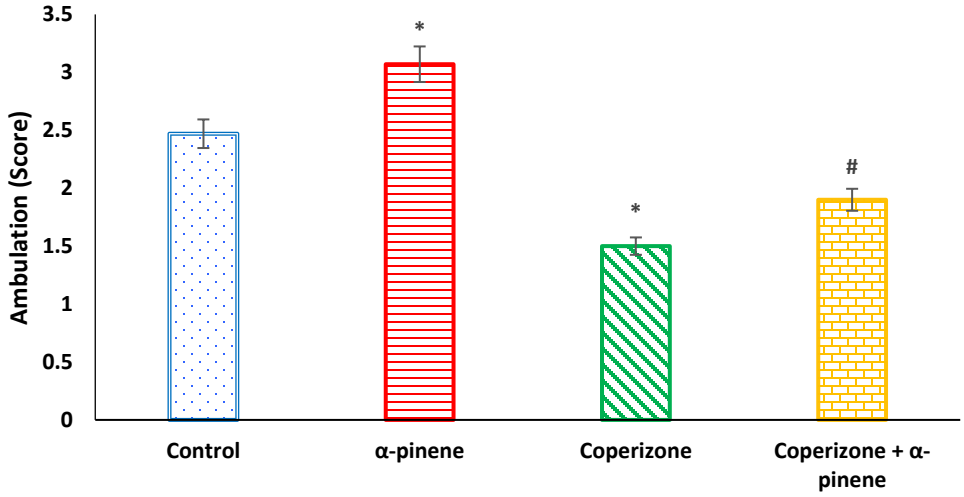

Figure 2. Effects of cuprizone and *a*-pinene and their combination on ambulation score for Cuprizone-induced model of multiple sclerosis in mice(n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

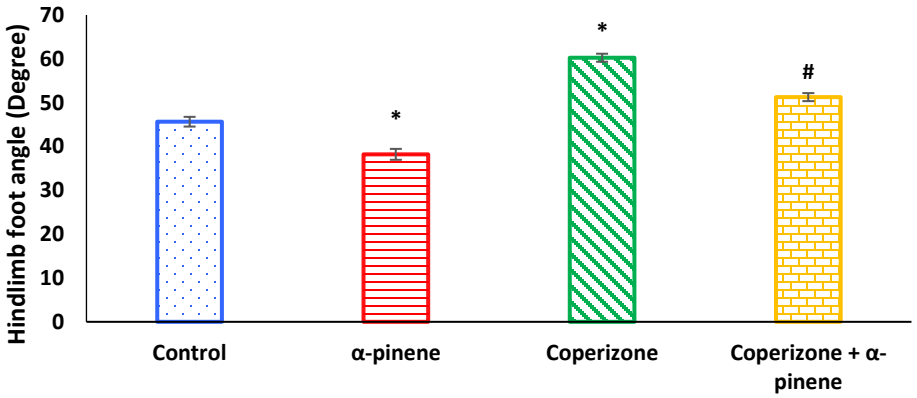

Figure 3. Effects of cuprizone and *a*-pinene and their combination on hind-limb foot angle for of Cuprizone-induced model of multiple sclerosis in mice(n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

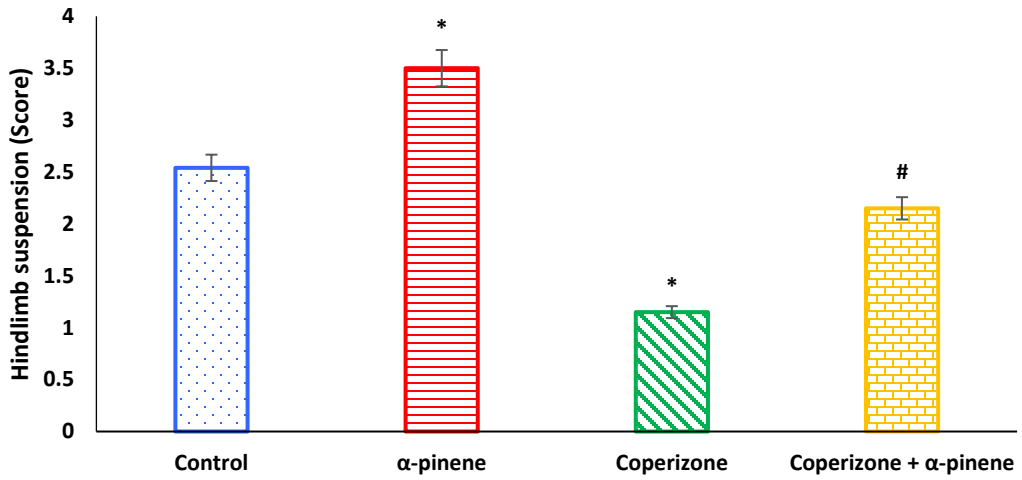

Figure 4. Effects of cuprizone and *a*-pinene and their combination on hind-limb suspension for Cuprizone-induced model of multiple sclerosis in mice(n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

CPZ on hind-limb suspension (P<.05; Figure 4).

Surface righting

The administration of the CPZ significantly increased surface righting compared to the controls (P<.05). Surface righting was significantly reduced following *α*-pinene administration when compared with the control group (P<.05). CPZ + *α*-pinene suppressed CPZ-induced elevation in surface righting (P<.05; Figure 5).

Grip strength

As depicted in Figure 6, the administration of the CPZ (.2% w/w) significantly decreased grip strength compared to the controls (P<.05). Grip strength was significantly elevated following *α*-pinene administration as compared with the control group (P<.05). CPZ + *α*-pinene significantly decreased the adverse effect of the CPZ on grip strength (P<.05).

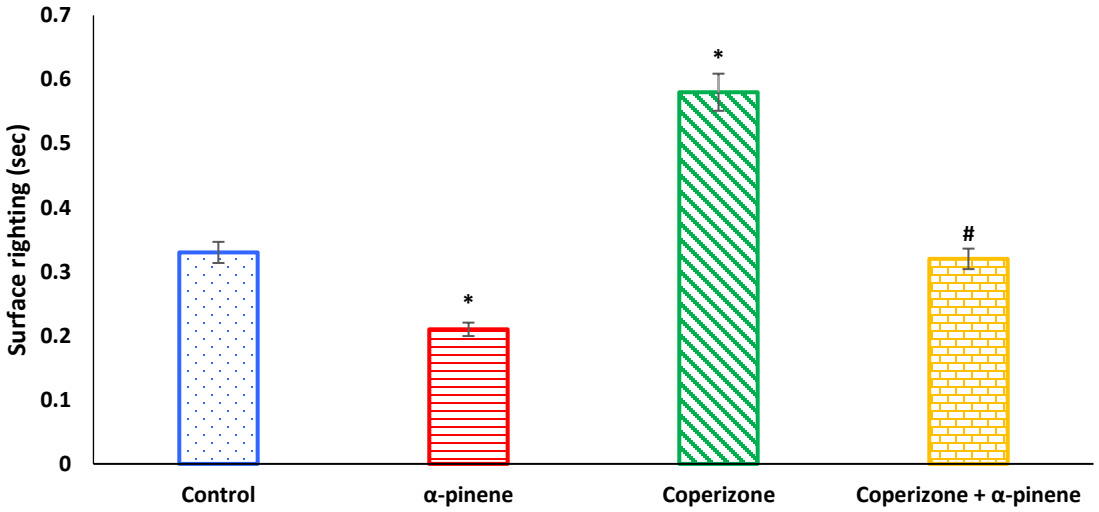

Figure 5. Effects of cuprizone and *a*-pinene and their combination on surface righting for Cuprizone-induced model of multiple sclerosis in mice(n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

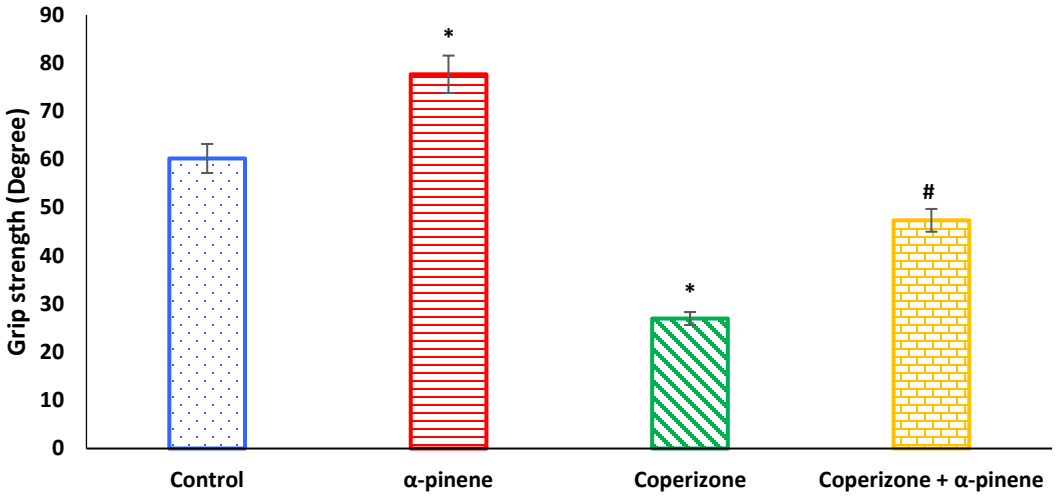

Figure 6. Effects of cuprizone and *a*-pinene and their combination on grip strength for Cuprizone-induced model of multiple sclerosis in mice(n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

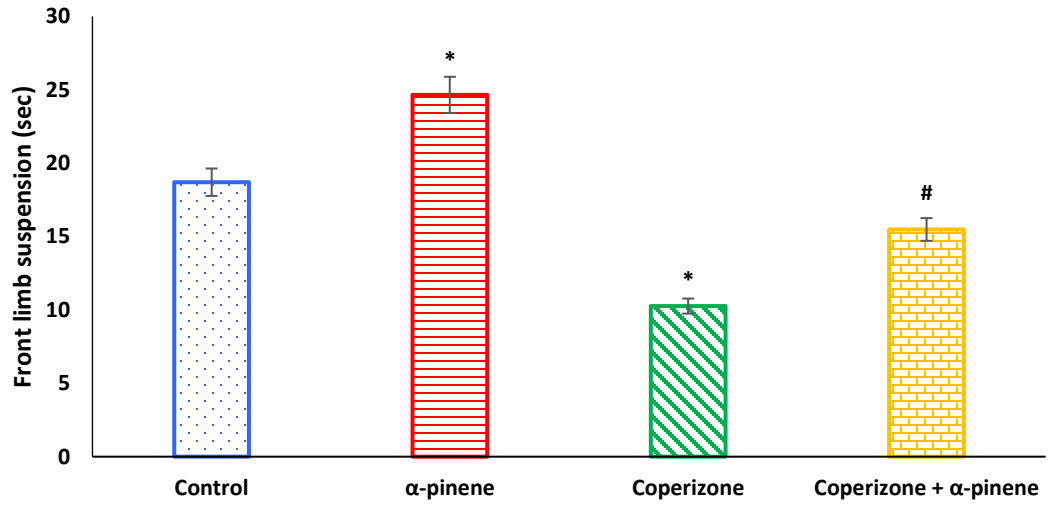

Figure 7. Effects of cuprizone and *a*-pinene and their combination on front limb suspension for Cuprizone-induced model of multiple sclerosis in mice(n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

Front limb suspension

As shown in Figure 7, the administration of the CPZ significantly diminished front limb suspension compared to the controls (P<.05). α-pinene significantly improved front limb suspension compared to the controls (P<.05). CPZ + α-pinene significantly increased front limb suspension (P<.05).

Negative geotaxis

According to the results, negative geotaxis was significantly augmented in the CPZ-treated mice (P<.05). α-pinene significantly decreased hind-limb suspension compared to the controls (P<.05). CPZ + α-pinene decreased the adverse effect of the CPZ on hind-limb suspension (P<.05; Figure 8).

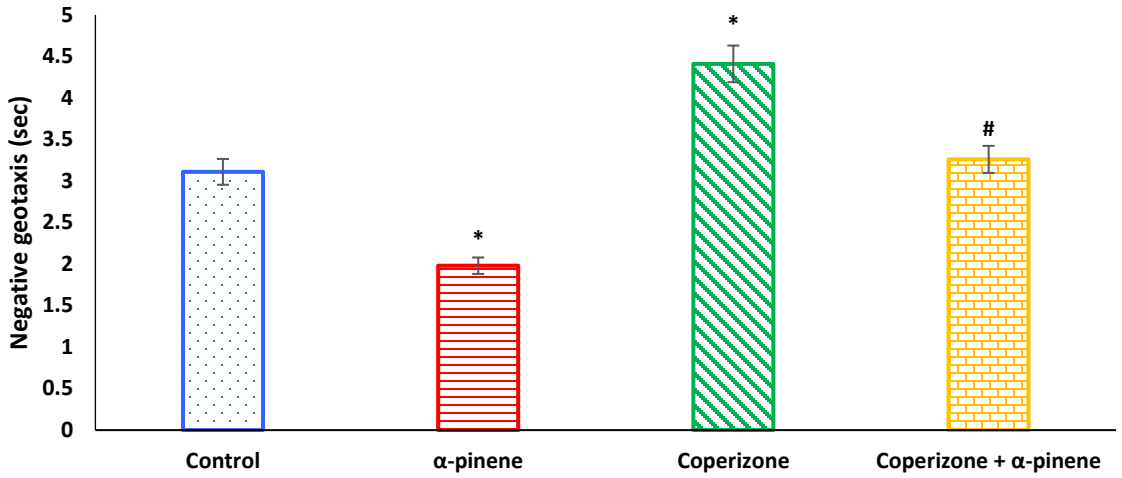

Figure 8. Effects of cuprizone and *α*-pinene and their combination on negative geotaxis forCuprizone-induced model of multiple sclerosis in mice(n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

FST

As depicted in Figure 9, CPZ significantly amplified immobility time in FST as compared with controls (P<.05). Administration of the α-pinene (1 mg/kg) significantly decreased immobility time in FST compared to control group (P<.05). CPZ+*α*-pinene decreased elevation in FST caused by CPZ compared to the controls (P<.05).

TST

As seen in Figure 10 , CPZ significantly amplified immobility time in TST compared with the controls (P<.05). Administration of the α-pinene (1 mg/kg) significantly reduced immobility time in TST (P<.05). CPZ +*α* -pinene increased immobility time suppressed by CPZ compared to the controls (P<.05).

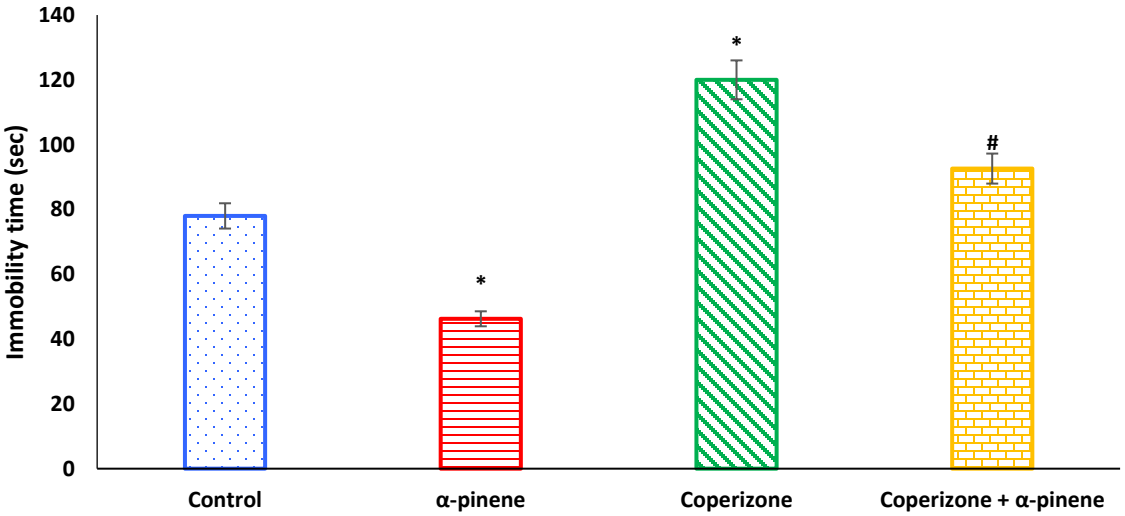

Figure 9. Effects of cuprizone and *α*-pinene and their combination on immobility time (sec) in forced swimming test (FST) for cuprizone-induced model of multiple sclerosis in mice (n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

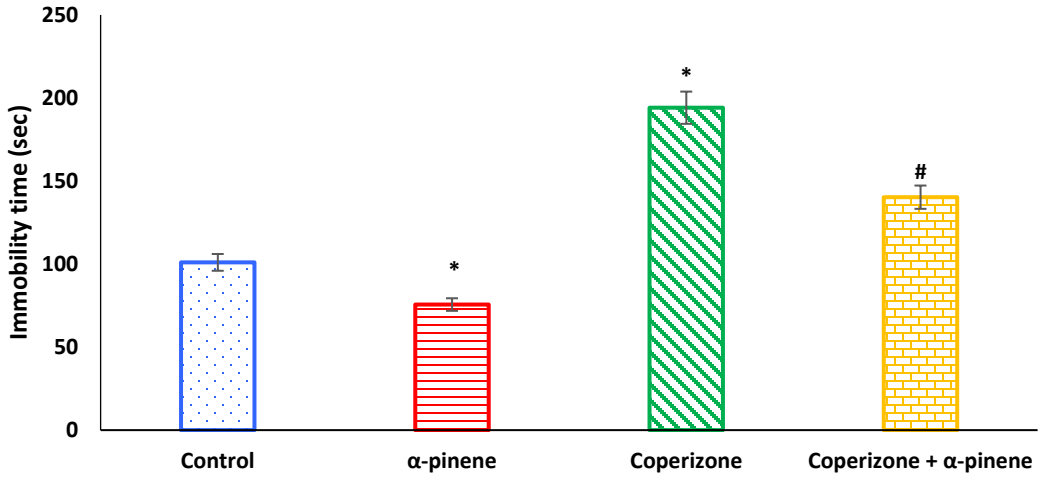

Figure 10. Effects of cuprizone and *a*-pinene and their combination on immobility time (sec) in tail suspension test (TST) for cuprizone-induced model of multiple sclerosis in mice (n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

OFT

In this study, CPZ significantly lowered the number of crosses in the OFT (P<.05). α-pinene (1 mg/kg) significantly raised the number of crosses in the OFT compared to control group (P<.05). CPZ + *α*-pinene significantly diminished the suppressive effect of the CPZ on number of crosses compared with the controls (P<.05; Figure 11).

Staying on rotarod

As shown in Figure 12, the administration of the CPZ significantly diminished the time of staying on the rotarod compared to the controls (P<.05). *α*-pinene significantly increased the time of staying on the rotarod compared to the control mice (P<.05). CPZ + *α*-pinene significantly diminished the adverse effect of the CPZ limb (P<.05).

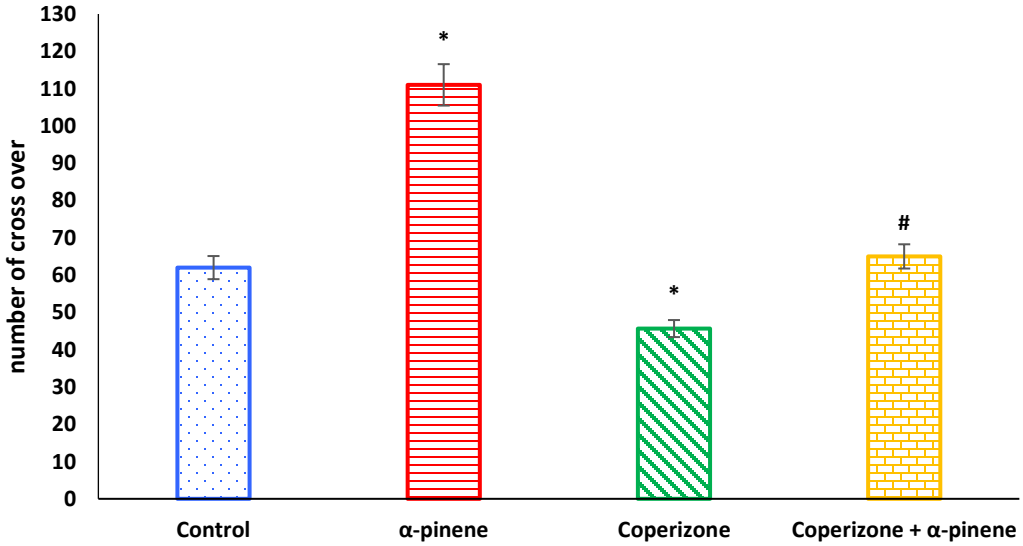

Figure 11. Effects of cuprizone and *a*-pinene and their combination on the number of cross in open field test (OFT) for cuprizone-induced model of multiple sclerosis in mice (n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

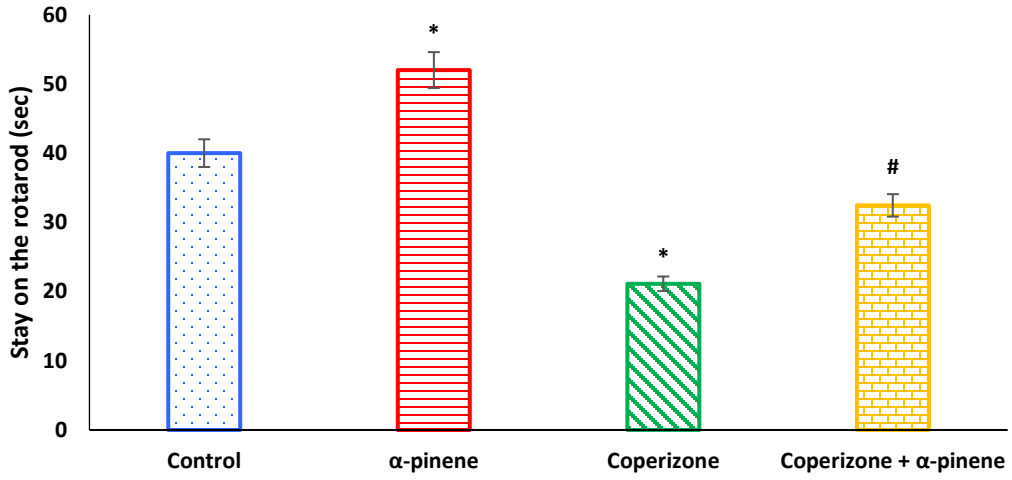

Figure 12. Effects of cuprizone and *a*-pinene and their combination on stay on the rotarod for cuprizone-induced model of multiple sclerosis in mice (n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

Antioxidant assay

The effects of cuprizone, α-pinene and their combination on staying in serum MDA levels are presented in Figure 13. As it is shown in Figure 13, serum MDA levels were significantly elevated by CPZ (P<.05). *α*-pinene significantly diminished serum MDA levels (P<.05). CPZ + *α*-pinene significantly decreased CPZ-induced elevation in serum MDA levels compared to control group (P<.05).

According to the results, CPZ significantly decreased serum SOD levels compared to control group (P<.05). *α*-pinene significantly increased serum SOD levels in comparison to controls (P<.05). CPZ + *α*-pinene significantly improved serum SOD levels suppressed by CPZ compared to the controls (P<.05) (Figure 14).

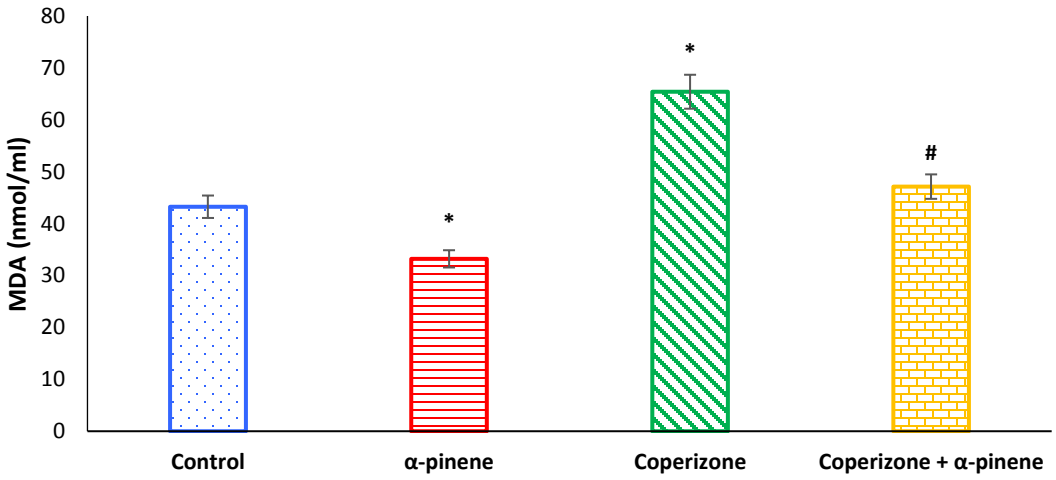

Figure 13. Effects of cuprizone and *a*-pinene and their combination on stay on serum Malondialdehyde (MDA) levels for cuprizone-induced model of multiple sclerosis in mice (n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

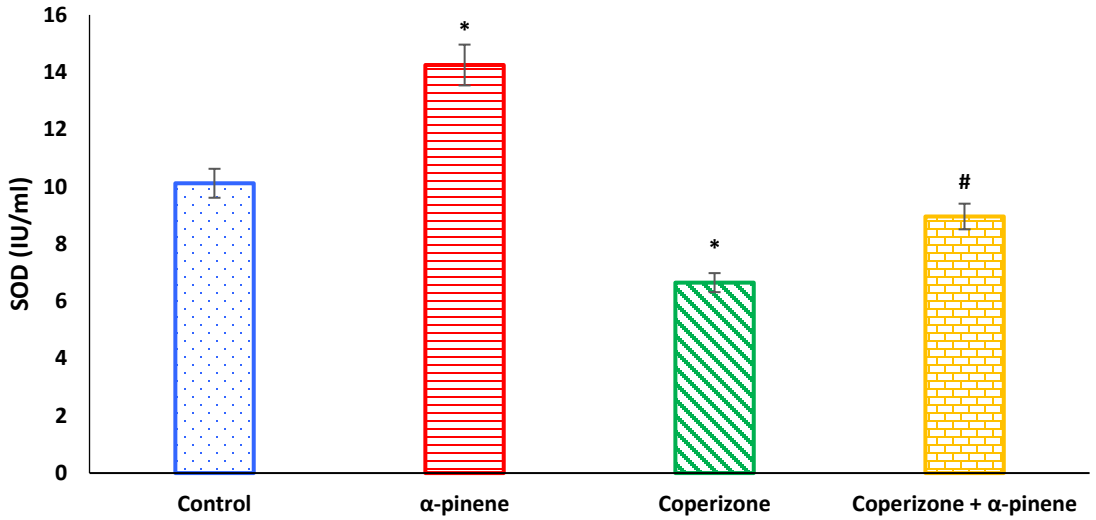

Figure 14. Effects of cuprizone and *α*-pinene and their combination on stay on serum superoxide dismutase (SOD) levels for cuprizone-induced model of multiple sclerosis in mice (n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

As shown in Figure 14, CPZ significantly decreased serum GPx levels compared to control group (P<.05). *α*-pinene significantly increased serum GPx levels in comparison to controls (P<.05). Co-administration of the CPZ+*α*-pinene significantly improved serum GPx levels suppressed by CPZ compared to the controls (P<.05) (Figure 15).

As shown in Figure 16, CPZ significantly suppressed serum TAS levels compared with the controls (P<.05). Administration of the α-pinene (1 serum TAS mg/kg) significantly improved serum TAS levels (P<.05). CPZ+*α*-pinene increased serum TAS levels suppressed by CPZ compared to the controls (P<.05).

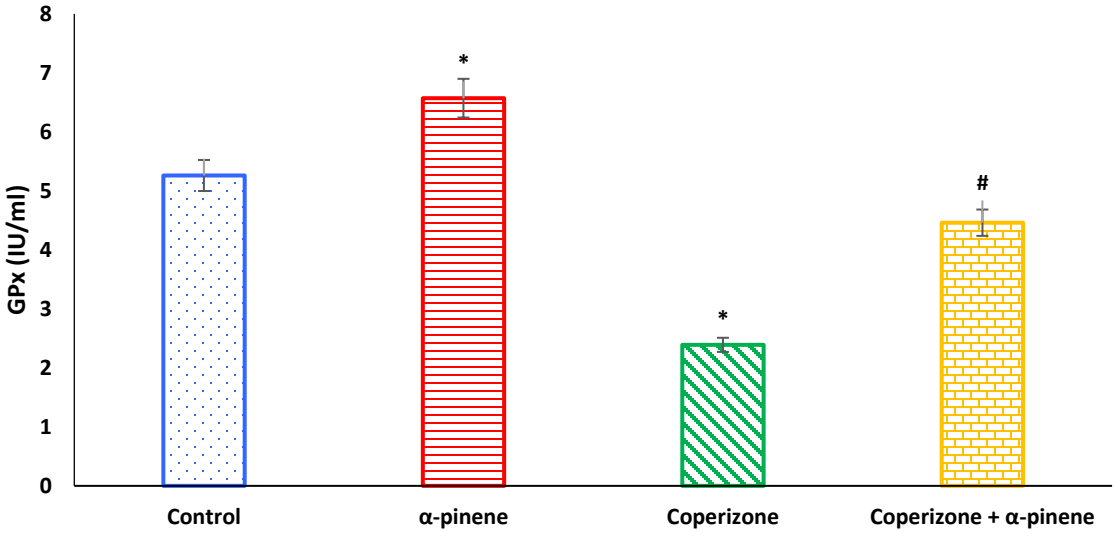

Figure 15. Effects of cuprizone and *α*-pinene and their combination on stay on serum glutathione peroxidase (GPx) levels for cuprizone-induced model of multiple sclerosis in mice (n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

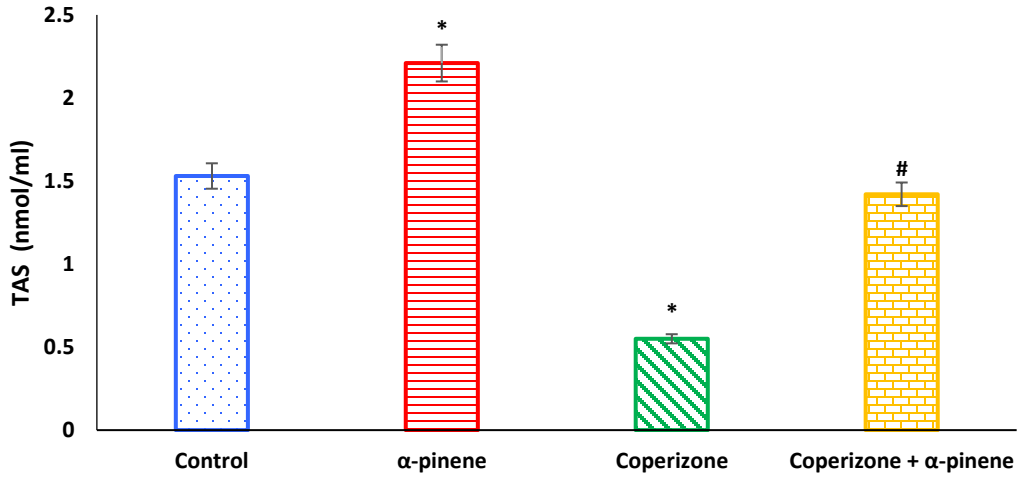

Figure 16. Effects of cuprizone and *α*-pinene and their combination on stay on serum total antioxidant status (TAS) levels for cuprizone-induced model of multiple sclerosis in mice (n=10). * P ≤ 0.05 compared to control group and # P ≤ 0.05 compared to cuprizone group.

4. Discussion

To our knowledge, this was the first study on effects of *α*-pinene protects against CPZ-induced MS in mice. MS is an autoimmune disease with targeted myelin attack that causes demyelination. C57BL/6 mice are one of the most popular strains for experimental MS. In this study, we used C57BL/6 mice [27]. Cuprizone is a toxin, and the model was used to induce demyelination by killing oligodendrocytes in white and gray matter of CNS [5]. Feeding CPZ for 4-6 weeks leads to Oligodendrocytes damage, followed by microglia and astroglia activation. CPZ-induced Oligodendroglia death acts via disrupting the energy metabolism in the mitochondria. These models were very useful for the pathophysiology of the MS [28]. It was observed that CPZ significantly impaired reflexive motor behavior while *α*-pinene per se enhanced reflexive motor behavior in mice. In animals treated by CPZ and *α*-pinene, the *α*-pinene significantly decreased the adverse effect of the CPZ on the reflexive motor behavior. Ojha et al. [5] reported that solid lipid nanoparticles loaded with dimethyl fumarate significantly improved deficit score, grasping ability, forelimb strength, and motor function in CPZ model of rats, which was consistent with our study.

Additionally, CPZ significantly increased immobility time in FST, and *α*-pinene diminished immobility time. CPZ decreased the cross number in the OFT as well as time spent on the rotarod while α-pinene exerted adverse effects. Co-administration of the CPZ+*α*-pinene significantly decreased the adverse effect of the CPZ on depressive tests. Ojha et al. [5] reported that solid lipid nanoparticles loaded with dimethyl fumarate significantly improved locomotor function in OFT and decreased in number falls on rotarod in CPZ model of rats, which was in line with our results. Demyelination is widely seen in the white matter of these patients; however, there are reports for its relation to the grey matter. Seemingly its pathophysiology is not clear [1]. In this regard, Ueno et al. [28] reported that being pre-exposed to α-pinene had no effect on the activity, but the inhalation of α-pinene decreased it in the locomotor activity test. Nevertheless, in the OFT, the amount of activity did not decrease in the mice which were pre exposed to *α*-pinene [29]. Immobility time in TST and FST resembles a state of despair and mental depression which is similar to depression in human. Khalilian et al. [13] reported that CoQ10 (150 mg/kg/day) significantly improved FST and TST in CP-induced MA in mice, which was consistent with our study .

The observed discrepancy between reports might be related to animal species, concentration and duration of administration [30]. Kong et al. [31] found that the inhalation of α-pinene attenuated depressive-like behavior using FST in rats. It was noted that α-pinene reduced the β-amyloid-induced depressive behavior and neuron death in rats [32]. However, due to the limitations of the present study, we were not able to determine pathological effects of the CPZ as well as

protective activity of the **α**-pinene on the hippocampus in model mice of MS.

Based on the results, CPZ significantly increased serum MDA and decreased GPx, SOD and TAS levels and these effects were reversed by α-pinene. Co-administration of the CPZ+α-pinene significantly improved the adverse effect of the CPZ on serum MDA, GPx, SOD and TAS levels. It is reported that α-pinene (2 and 4 mg/kg) decreased MDA and increased thiol and GPx activity [33]**.** The oxidative stress plays an important role in the pathogenesis of MS. The lowest antioxidant activity and the highest neuro-inflammation are the main factors in MS patients [34]**.** Thus, antioxidants and anti-inflammatory agents may be beneficial for both the prevention and treatment of MS [35]**.** α-pinene is able to across the blood –brain barrier [36]**.** α-pinene improved CAT, SOD and GPx inhibiting apoptosis [12]. α-pinene lowers the peroxidation of lipid in rat with Parkinson disease [37]. α-pinene inhibits ROS generation and lipid peroxidation and prevents cell damages. α-pinene can impress antioxidant activity by diminishing ROS generation and lipid peroxidation [1]. α-pinene at 25 and 50 mg/L increased in TAS but at 200 mg/L decreased cell viability in human lymphocytes without mutagenic effects [11].

The positive effect of the antioxidants in the treatment of MS is well documented [13]. They have reported that CoQ10 (150 mg/kg/day) significantly increased total antioxidant capacity and SOD activities in CP-induced MA in mice [13], which was in agreement with our study. Oligodendrocytes are more sensitive to elevated levels of the ROS. Microglia and astrocytes are vulnerable to the ROS. So, amplified ROS leads to demyelination. SOD is an indicator for myelin loss and MS disease [38]. The first line of the oxidative stress defense system against ROS is SOD. By inhibiting SOD activity, CPZ leads to mega mitochondria formation, perikaryon and myelin sheath degeneration. Also, decrease in SOD leads to apoptosis [13]. Moreover, GPx acts as a donor to eliminate ROS and prevent oxidative damage to neurons and oligodendrocytes [38].

5. Conclusion

Based on our findings, it seems that α-pinene has protective effect against MS via improving antioxidant defense system. However, there is no report to check these findings against it. Also, given the limitations of this study, we were not able to investigate histological or immunological effects of α-pinene on CPZ-induced MS in mice. The results of our study suggest that α-pinene has protective effect against CPZ-induced MS in mice.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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This study received no specific grant from any funding agency.

Author's contributions

The authors equally contributed to this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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