Methanolic extract of *Biarum carduchrum* ameliorates seizures, oxidative stress, and cognitive impairment in experimental models of epilepsy in rats

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Abstract

Introduction: Given the high prevalence of epilepsy and low availability of usual therapies, finding more effective drugs is essential for the treatment of epileptic patients. In the present study, the anti-epileptic property of the methanol extract of *Biarum carduchrum* (*B. carduchrum*) leaves was investigated on pentylenetetrazole (PTZ) kindled rats.

Materials and methods: In this experimental study, fifty Wistar rats were randomly grouped into five groups including 1: Control, 2: PTZ, 3: PTZ + methanolic extract (100 and 200 mg/kg), and 4: PTZ + methanolic extract + flumazenil. The scores of epilepsy were investigated 30 min after PTZ injection. Behavioral tests including Shuttle box, Morris Water Maze, and tail suspension tests were done in the experimental groups. Finally, the rats under deep anesthesia; serum samples were given, and their brain tissue removed for biochemical tests, including malondialdehyde level, anti-oxidant capacity, and nitrite and nitrate levels.

Results: *B. carduchrum* methanolic extract reduced the number of tunic seizures and jumps in treated animals. The extract also induced an improvement in passive avoidance memory and spatial memory in the Morris Water Maze test and reduced the immobilization time in the tail suspension test. Treatment of PTZ kindled rats with *B. carduchrum* methanolic extract resulted in a decrease in the levels of nitric oxide and malondialdehyde as well as a significant increase in the antioxidant capacity of the brain tissue and serum.

Conclusion: *B. carduchrum* methanolic extract can be used as an anti-epileptic agent for depression control, improvement of learning, and memory after complementary testing.

Keywords: *Biarum carduchrum*, PTZ-kindled rats, Behavioral tests, Antioxidant activity

Introduction

Epilepsy which characterized by frequent seizures, is one of the most common global neurological disorders. Available anti-epilepsy drugs cause a broad spectrum of systemic side effects that are related to the dose, and the presence of cognitive disorders (1). Therefore, it is necessary to find new drugs that inhibit the destructive effects of epilepsy without affecting cognitive performance.

The neurons in the hippocampus are closely related to seizure activity and the loss of neurons among pyramidal cells of the hippocampus has been shown in patients with epilepsy and also in the experimental model of epilepsy (1). Oxidative stress is involved in the pathogenesis and progress of epilepsy, and cognitive impairment in epileptic

patients. The production of free radicals can induce epilepsy through direct inactivation of glutamine synthetase, leading to abnormal production of glutamic acid as a stimulating neurotransmitter (2).

Kindling is one of the most common types of animal epilepsy model that has been used for initial evaluation of anti-epileptic drugs. Pentylenetetrazole (PTZ) is a selective blocking factor for chloride channel, which is paired with the $GABA_A$ receptor complex, and induces chemical seizure. It is well known that PTZ has destructive effects on the neurons membranes and affect potassium and calcium ions transport leading to releasing of calcium reservoirs in the cell, and also reduces the neurotransmitter induced chloride ion conductance (3).

The medicinal plants that had been used in traditional medicine for treating epilepsy have shown significant anti-seizure activity in animal models, and has also been reported to have desirable effects on cognitive function (4). *B. carduchrum* is one of the most valuable medicinal plants that belongs to Areaceae family is found in Zagros Mountains (Fars and Kohgiluyeh-Boyer-Ahmad provinces, Iran) and in some areas of Turkey, Syria, and Iraq (4). The existence of flavonoids and anthocyanins in *B. carduchrum* was first reported by Williams et al. (1981). Also the presence of alkaloids, amines, saponins, cyanotic acids, and flavonoids have been shown in Areaceae family (5, 6). The present study was aimed to evaluate the protective effects of *B. carduchrum* methanolic extract in PTZ-induced seizure in rats.

Materials and Methods

Extract Preparation

B. carduchrum was provided from the local market in Izeh; was identified by Dr Setorki, herbalist, and then was kept in the Herbarium of Islamic Azad University of Izeh as a reference sample (voucher No. 80145). The leaves were washed and dried in an oven at 40ºC under vacuum condition. Then the dried leaves were grinded and sifted. For extract preparation, methanol was added to the powdered leaves and stored for 72 h at room temperature. The extract was then filtered and dried by vacuum rotation at 40 ºC (7).

Measurement of Total Phenol Compounds

The measurement was done by Folin Ciocalteu method. In summary, 0.5 mL of the extract solution prior to drying was well mixed with 2.5 ml of diluted (1:10) Folin Ciocalteu reagent and 2 ml sodium carbonate solution (7.5%). The test tube contained the mixture was placed in a 45°C water bath for 15 min. Then the absorbance of the mixture at the wavelength of 760 nm was measured by a spectrophotometer in contrast to the blank solution containing methanol (80%) and the diluted reagent (8).

Measurement of the Flavonoids Compounds

The volume of 0.5 ml of solution prepared from methanolic extract (0.01 g of dried extract in 10 ml methanol 60%) was mixed with 0.5 ml aluminum chloride (2%) and 3 ml potassium acetate (5%). After standing for 40 min at room temperature, the absorption of the mixture was measured at the wavelength of 415 nm in contrast to distilled water. The amounts of flavonoids (mg per g of dried extract) were determined by comparison to the standard curve that was prepared based on the light absorbance of different concentrations of quercetin (8).

Free Radical Scavenging Activity

Different concentrations of the extract in distilled water were mixed with the same volume of 0.1 mM DPPH solution (1, 1diphenyl-2-picryl hydrazyl prepared in 95% ethanol) and allowed to stand for 15 min at room temperature. Then, the light absorbance of the mixtures was recorded at 517 nm in contrast to blank containing distilled water instead of the extract. The percentage of

DPPH radicals scavenging activity was calculated by the following formula:
DPPH radical scavenging activity (%) = [(Acontrol - Asample) / Acontrol] × 100
The IC50 values were determined by plotting a graph of concentration versus the percentage of inhibition (8).

High Performance Liquid Chromatography (HPLC) Analysis

A water liquid chromatography apparatus consist of Waters 2695 separations module (USA) and Waters PDA detector 996 (USA) was used for HPLC analysis. Injection was equipped with auto sampler injector, and the chromatographic assay was performed on a 150×4.6 mm column (Eurospher 100-5 C18 analytical column). Elution was carried out in a gradient system by using methanol as the organic phase (solvent A) and distilled water (solvent B) with the flow-rate of 1 mL/min. Injection volume was 20 µL and the temperature was maintained at 25°C. The obtained peaks were monitored at the wavelength of 195-400 nm. Data acquisition and integration was performed with Millennium32 software and was compared to the data obtained by quercetin as the standard.

Laboratory Animals Grouping

Male Wistar rats weighing 150-200 g were used. The animals were kept in appropriate conditions including the temperature of 21 ± 2°C with a time period consist of exposure to light (12 hrs) followed by 12 hrs dark exposure. The animals had similar free access to water and food in all keeping times. The experimental animals were randomly divided into five groups each containing 10 animals including the control group (receiving normal saline via intraperitoneal injection every 48 hrs for 10 days), the negative control group (receiving PTZ through intraperitoneal injection every 48 hrs for 10 days), the treatment groups (receiving *B. carduchrum* methanolic extract daily at the doses of 100 and 200 mg/kg + PTZ every 48 h for 10 days, 30 min after receiving the extract), the flumazenil-treated group received the daily i.p. injection of *B. carduchrum* at the dose of 200 mg/kg + flumazenil (2 mg/kg), 30 min before PTZ injection. The epilepsy model received PTZ (35 mg/kg) for 9 days, every 48 h intraperitoneally and on the tenth day received PTZ (60 mg/kg). The scores of seizures were recorded during 30 min after recived PTZ (60 mg/kg). Finally, blood samples were collected and the brain tissue of rats were removed under deep anesthesia and kept at -80°C for biochemical analysis.

Tail Suspension Test

for this purpose, a 50 cm string was extended horizontally between the two bases, and the tail of the rat was stabilized. The test began with a sharp rat movement. The rat, which was suspended from the tail, was completely immobile, passive and without reaction. The inert period was recorded with a chronometer (9).

Passive Avoidance Memory Test

The test was done for analysis of learning and memory in the experimented animals. Plexiglass Shuttle box with dimensions of 20×80×20 cm consisted of a bright and darkened chamber. An electrical shocker was placed in the floor of the darkened chamber and the two chambers were connected via a guillotine door. The avoidance test was performed in three stages. The 1st stage was adaptation step. In this stage the rat was left in the bright chamber for 5 min during 2 days before the next stage in order to train in the apparatus. The 2nd stage was acquisition step. In this stage, in the third day the animal was placed in bright chamber for 2 min and then the sliding door between the bright and darkened chambers was opened. The latency time in which the rat was entered to the darkened chamber was recorded and

considered as initial latency (t1). When the rat entered the darkened chamber, an electrical shock (1 mA/s) was exerted to it and after 1 min the rat was went out of the box. The 3rd stage was retention step. This stage was similar to the previous stage with the difference that there was no shock when the animal entered the darkened chamber. The latency time that lasted for the rat entrance into the darkened chamber was recorded and considered as step-through latency (T2).

Spatial Memory Test Using Morris Water Maze

The test was done to evaluate learning, memory, and motor function of the rats. The device included a water pond (136 cm diameter and 60 cm height), which was filled up to 25 cm of the height by water (temperature of 22 ± 1°C). A 10-cm diameter Plexiglass platform was placed in the center of the southwest quadrant and about 1 cm below the water surface. Each rat was trained four times a day during 4^{th} and 5^{th} (probe) days. The training was repeated one time without a platform (10).

Biochemical Testing

Measurement of Serum and Brain Tissue Anti-oxidant Capacity

The method of Beniz and Strain was used. Fresh FRAP (ferric reducing antioxidant power) reagent consist of 2.5 ml TPTZ2,4,6-tripyridyl-s-triazine (10 mM in HCl 40 mM), 2.5 ml $FeCl_3$ and 2.5 ml acetate buffer (0.3 M, pH=3.6) was prepared and its temperature was adjusted to 37 ºC. Ferric reducing ability based on the ability of serum to restore ferric ions in the presence of tripyridyl-s-triazine was measured by colorimetric method at the wave length of 593 nm (8).

Measurement of Malondialdehyde Levels in Serum and Brain Tissue

The amount of 2.5 ml thiobarbituric acid acid (TBA) dissolved in sodium sulfate was added to the mixture of 100 µl serum or homogenized tissue and 100 µl SDS (8.1%); then heated in a boiling water bath for 60 min. The mixture was centrifuged at 4000 rpm for 10 min after cooling in tap water and the light absorbance of the supernatant was recorded at the wave length of 523 nm (9).

Measurement of Serum Nitrite and Nitrate

Nitrite and nitrate measurements were performed based on the rate of reduction of nitrate to nitrite by cadmium and the Griess1 and Griess2 reagents (8).

Statistical Analysis

Data were analyzed using SPSS16 software. One way analysis of variance (ANOVA) was used to identify the differences between the mean results. All data were presented as mean ± standard deviation and 95% confidence was considered for detection of significant differences.

Results

Phytochemical Analysis

The results showed that each 1 mg of *B. carduchrum* dried methanolic extract contained 42.63±0.7494 µg phenol compounds and 22.5±3.299 µg flavonoids. The result of HPLC analysis revealed that quercetin with the amount of 1 µg was present in each g of *B. carduchrum* methanolic extract (Figure 1). Also, the result of the DPPH scavenging activity showed that the IC50 level of the extract was 350 µg/mL.

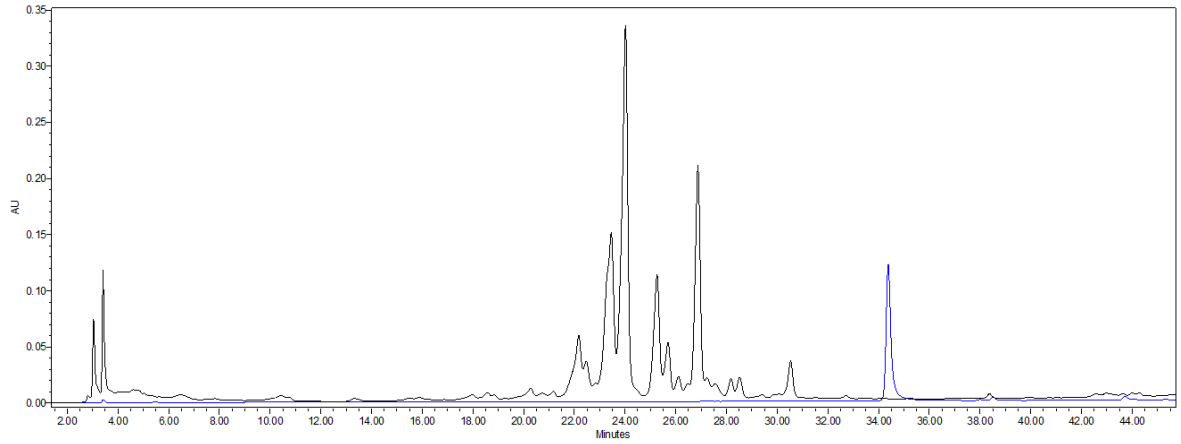

Figure 1. The results obtained from High performance liquid chromatography (HPLC) analysis of *Biarum carduchrum* methanolic extract(quercetin used as standard).

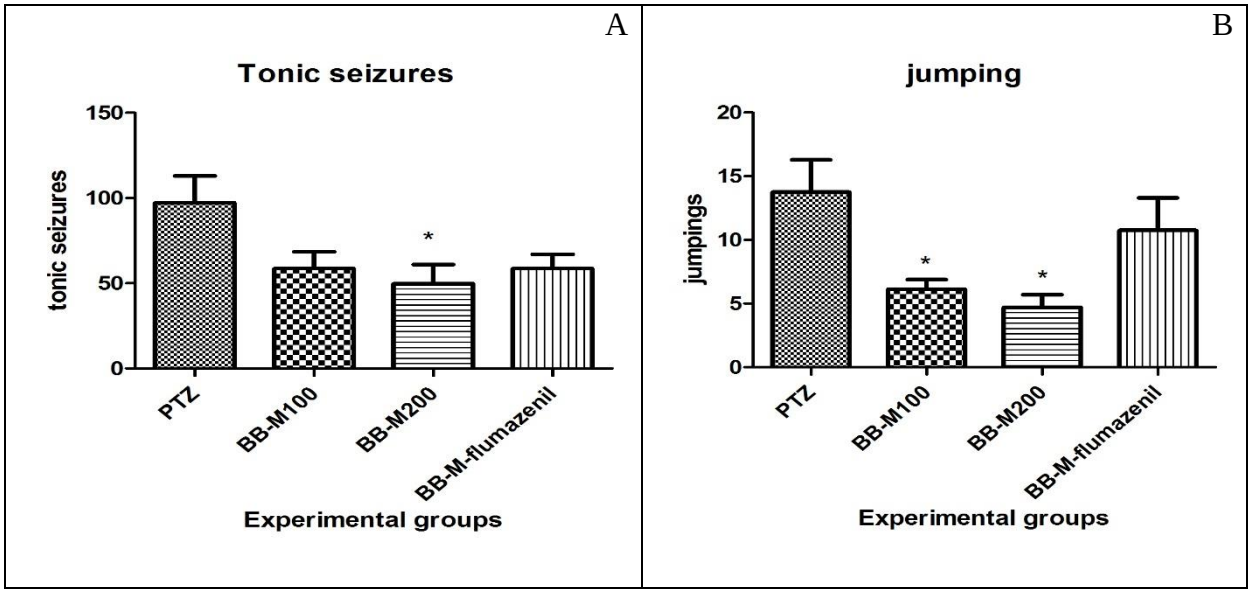

Figure 2. Comparison of the frequency of tonic seizures (A) and jump numbers (B) between the experimental groups. * indicates the significant difference between the experimental group and PTZ receiving group (P<0.05), and BB-M indicates *Biarum carduchrum* methanol extract.

Behavioral Tests Results

The frequencies of the tonic seizures and the numbers of jumps in the experimental groups are shown in figure 2. Treatment of the rats with 200 mg/kg *B. carduchrum* methanolic extract resulted in a significant decrease in the number of tonic seizures compared to PTZ receiving group. Also the number of jumps was significantly decreased by the treatment with *B. carduchrum* methanolic extract (100 and 200 mg/kg) in the recipient groups compared to PTZ receiving group. No significant difference was shown in the group receiving flumazenil combined with *B. carduchrum* methanol extract (200 mg/kg body weight) in comparing PTZ receiving group.

The results of the tail suspension test are shown in figure 3. According to the results, the immobilization period in the PTZ receiving group is significantly higher than

the control group. Treatment with *B. carduchrum* methanolic extract (100 and 200 mg/kg) resulted in significant reduction of immobility duration comparing PTZ receiving group.

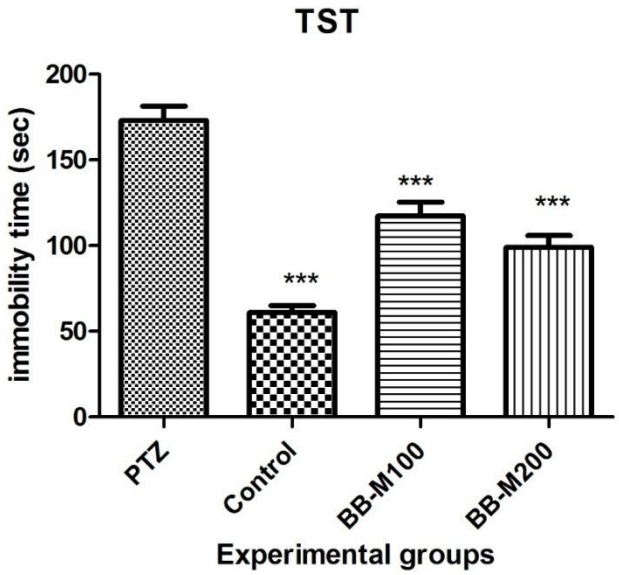

Figure 3. Comparison of the immobility duration between the experimental groups in tail suspension test. *** indicates the significant difference between the experimental group and PTZ receiving group (P<0.001), and BB-M indicates *Biarum carduchrum* methanol extract.

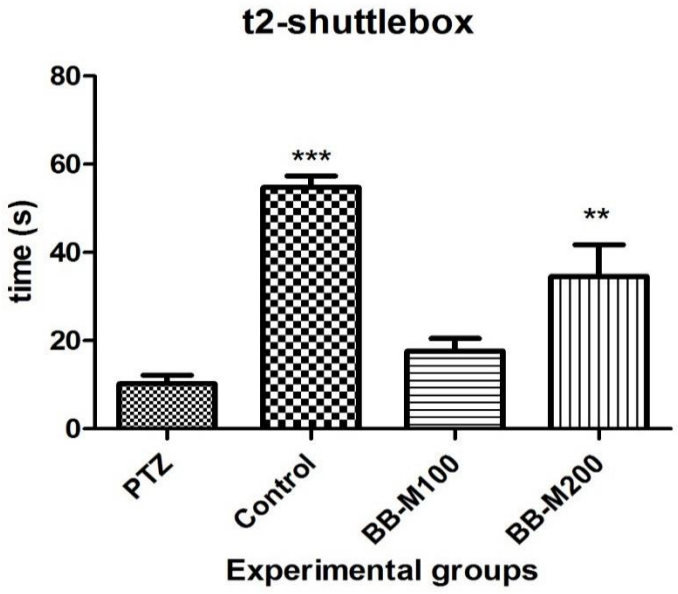

Figure 4. Comparison of the secondary delay time (t2) between the experimental groups in Shuttle box test. ** and *** indicate the significant difference between the experimental group and PTZ receiving group at P<0.01 and P<0.001 respectively. BB-M indicates *Biarum carduchrum* methanol extract.

Sequential injections of PTZ resulted in a significant reduction in secondary delay time (t2) comparing control group in the Shuttle box test (figure 4). The group receiving *B. carduchrum* methanolic extract (200 mg/kg body weight) showed a significant increase in t2 when entering the darkened chamber compared to PTZ receiving group.

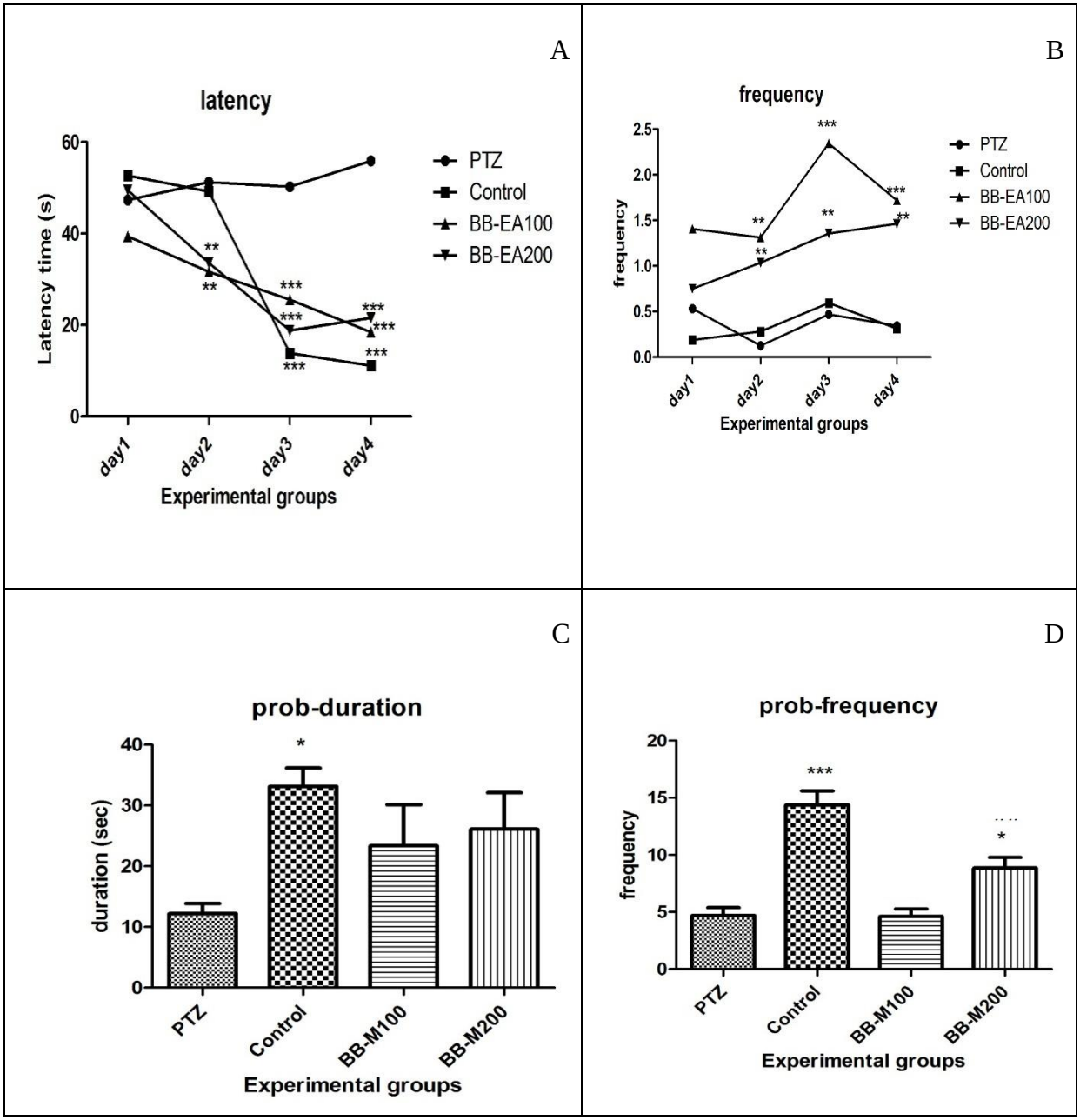

Figure 5. Comparison of the delay in reaching the hidden platform (A), the swimming frequencies within the target quadrant during the training days (B), the swimming time durations in the target quadrant during the probe day (C), and the swimming frequencies the target quadrant during the probe day (D) between the experimental groups in Morriss Water Maze test. *, ** and *** indicate the significant difference between the experimental group and PTZ receiving group at P<0.05, P<0.01 and P<0.001 respectively. BB-M indicates *Biarum carduchrum* methanol extract.

The results of the spatial memory in Morriss Water Maze test are shown in figure 5. During the second, third and fourth trials, the groups receiving *B. carduchrum* methanolic

extract (100 and 200 mg/kg) showed less delay time in reaching the hidden platform comparing to PTZ receiving group. Also, the swimming frequencies by these extract treated groups were significantly higher than PTZ receiving group. The results of the test in probe day showed that the control group has been able to swim in a longer period of time than PTZ receiving group. Also the group receiving *B. carduchrum* methanolic extract (200 mg/kg) had a higher swimming frequency in target quadrant on the probe day than PTZ receiving group.

According to the figure 6 sequential injections of PTZ have resulted in a significant reduction in the antioxidant capacity of the serum and brain tissue as well as a significant increase in the level of malondialdehyde, compared to the control group.

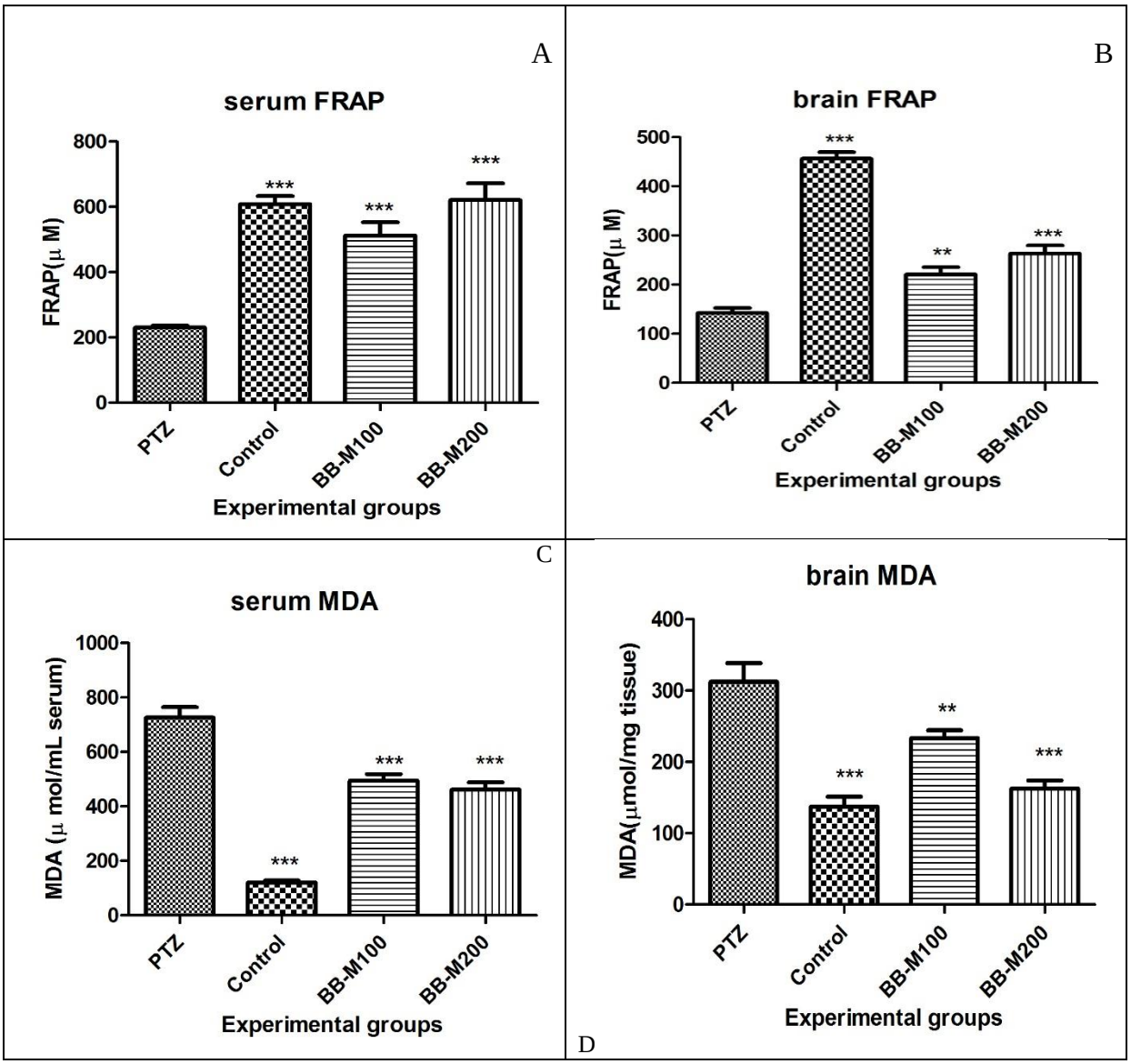

Figure 6. Comparison of the antioxidant capacity of the serum (A) and brain (B) as well as the malondialdehyde level of the serum (C) and brain (D) between the experimental groups. ** and *** indicate the significant difference between the experimental group and PTZ receiving group at P<0.01 and P<0.001 respectively. BB-M indicates *Biarum carduchrum* methanol extract.

Although *B. carduchrum* methanolic extract (100 and 200 mg/kg) resulted in a significant increase in the antioxidant capacity of the serum and brain tissue as well as a significant decrease in the level of the serum and brain tissue malondialdehyde level compared to PTZ receiving group.

According to the results shown in figure 7 the level of nitric oxide (NO) of the brain tissue in the control group was significantly lower than in PTZ receiving group. *B. carduchrum* methanolic extract with the doses of 200 mg/kg resulted in significant reduction in NO level of serum and brain tissue compared to PTZ receiving group.

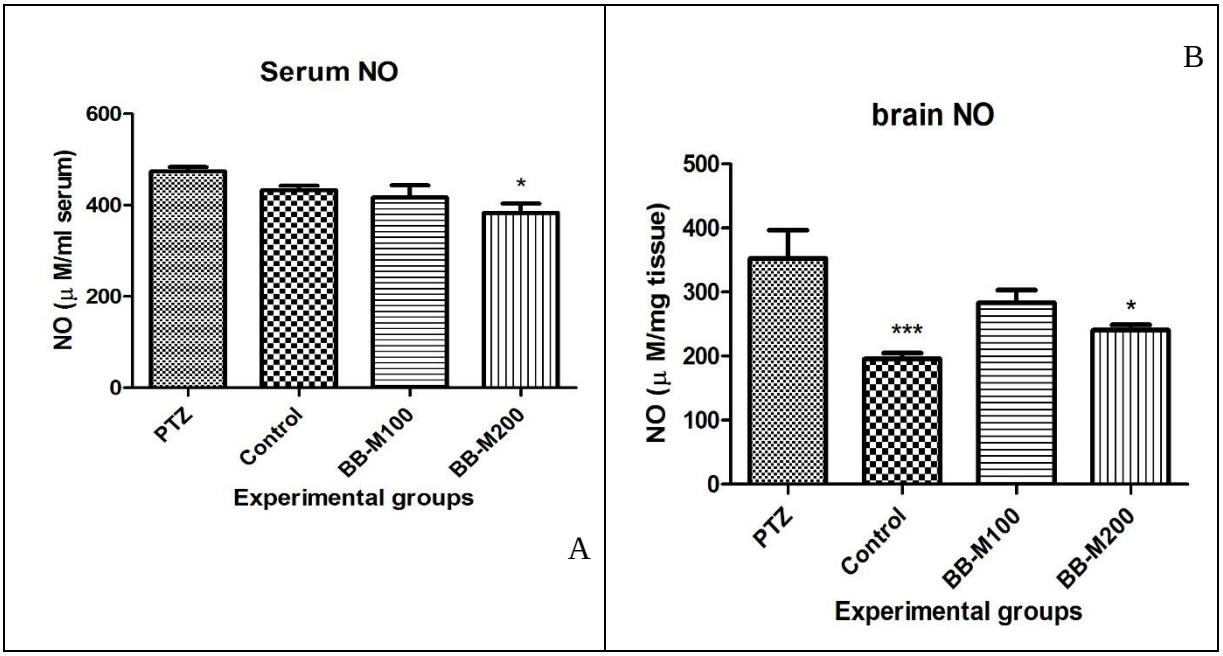

Figure 7. Comparison of the nitric oxide level of the serum (A) and brain (B) between the experimental groups. * and *** indicate the significant difference between the experimental group and PTZ receiving group at P<0.05 and P<0.001 respectively. BB-M indicates *Biarum carduchrum* methanol extract.

Discussion

This study aimed the investigating the effect of *B. carduchrum* extract on the severity of seizure and behavioral impairments in PTZ kindled rats. It was observed that the treatment with the extract caused a significant decrease in the frequency of jumping, rotation and tonic seizure although flumazenil was significantly inhibited the effectiveness of *B. carduchrum* extract with the dose of 200 mg/kg. Oxidative stress that causes oxidation of lipids, proteins and DNA causes severe damage of neurons. A significant decrease in the amount of GSH, GSSG, cysteine and protein thiol as well as increasing the carbonyl protein and protein disulfide have been observed after the PTZ induced seizure in rat brain cortex (11). It has shown that the seizure due to PTZ injection causes a significant increase in cerebral cortex nitric oxide (12). The results of the present study showed that PTZ injection causes a significant increase in the amount of lipids peroxidation in the brain tissue. Our results conform to previous studies that reported elevation of lipid peroxidation in brain, erythrocytes and liver during epileptic seizures (13, 14).

In this study, the treatment of rats by *B. carduchrum* methanolic extract resulted in the induction of antioxidant capacity of the

brain tissue along with a decrease in nitric oxide and malondialdehyde levels in the brain tissue. Also, high strength in scavenging DPPH radicals indicated the presence of antioxidant capacity in the extract. The high content of phenol and flavonoids that was evaluated in *B. carduchrum* methanolic extract might be the reason for its protective effects against oxidative damage of neurons and the strengthening of antioxidant defense. Hosseini et al. (2014) showed that *B. carduchrum* hydromethanol extract was more powerful than butylated hydroxytoluene (BHT) and alpha-Tocopherol in scavenging of DPPH radicals. They also showed that by increasing the concentration of the extract, the antioxidant activity has also increased because of more entrance of phenol compounds to the reaction environment. With increasing the concentration of phenol compounds, hydrogen is more feasible binds to free radicals leading the elevation of antioxidant activity (7). Glutamate and gamma-aminobutyric acid (GABA) are the main neurotransmitter that has been broadly studied in relation to chemical kindling induced seizure. Both glutamatergic and GABAergic systems are important in epilepsy phenomenon. It is proposed that seizure is the high stimulation of neurons caused by imbalance between glutamate induction and GABA inhibition (15).

HPLC analyzes of *B. carduchrum* methanolic extract showed that the extract contains high levels of quercetin. Nassiri-Asl et al. (2013) reported that quercetin with the dose of 50 mg/kg reduced the seizure severity and increased the avoidance memory (16). Also it has been shown that treatment with quercetin (50 and 100 mg/kg) inhibited the increasing expression of β1 and β3 subunits of $GABA_A$ receptors 2 h after injection of kainic acid (16). Flumazenil is also a strong receptor antagonist whose competitive effect prevents from GABA activities on benzodiazepine receptors (17). In the present study inhibitory effects of flumazenil that observed on anti-seizure activity of *B. carduchrum* methanol extract was proposed the function of the extract on benzodiazepine receptors.

The hippocampus plays an important role in learning and memory in mammals. Accordingly, any damage in the hippocampus causes cognitive impairment. Previous studies have shown that the seizure caused by PTZ leads to the damage of neurons in the hippocampus, thus resulting in impaired memory and learning (18, 19). In this study, *B. carduchrum* extracts caused significant improvement in spatial and avoidance memories in the PTZ receiving rats. It can be proposed that the extract prevents memory loss by preventing damages in tissues involved in memory and learning.

It has been shown that quercetin which was the major flavonoid in *B. carduchrum* extract in this study, improves learning and spatial memory through reducing oxidative stress and increasing GSH in mice (20). Also injection of a dose of quercetin 1 h before receiving scopolamine has been resulted in memory and learning improvement in zebrafish (21).

Depression has a significant impact on the quality of life in patients with epilepsy and is also the main cause of increasing suicide cases in these patients. Recent epidemiological studies showed that there is a reciprocal relationship between depression and epilepsy (22, 23). Injection of *B. carduchrum* methanolic extract caused significant reduction of the inactivation time in tail suspension test. It has been shown that treatment with quercetin would increase the time of social interaction and reduce the immobility time in rat, which shows the anti-anxiety and anti-depressant effects of this bioflavonoid (24, 25). Similarly, quercetin has been contributed to the reduction of inactivation duration in compulsory swim

test in diabetic mice (26). Regarding these studies, it is possible to say that the antidepressant effects of *B. carduchrum* extract may be due to the quercetin content of it.

Conclusion

According to the findings of the present study, anti-seizure effects was shown in *B. carduchrum* methanolic extract on epileptic rats which was associated with antioxidant properties and reduction of oxidative stress. However, more studies on the molecular level are required to obtain the exact mechanism of the protective effect of this extract and usage as an anti-epileptic drug for depression control.

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Conflict of Interests

The authors declare that there is no conflict of interest.

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Authors' Contribution

Youness Teymorivand conceived and extracted the data, Zahra Hooshmandi designed the study, analyzed the data and wrote the manuscript. Mahbubeh Setorki revised the paper and had full access to all of the data in the study and Sabrieh Amini takes responsibility for the integrity of the data and the accuracy of the data analysis and is guarantor.

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