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The Protective Effect of Hydroalcoholic Extract of the Southern Maidenhair Fern (*Adiantum capillus-veneris*) on the Depression and Anxiety Caused by Chronic Stress in Adult Male Mice: An Experimental Randomized Study

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Abstract

Background: Antioxidant compounds are novel approaches in the treatment of neurological disorders.

Objectives: The purpose of this study was to examine the antidepressant and anxiolytic effects of the hydroalcoholic extract of *Adiantum capillus-veneris* (rich in flavonoids with antioxidant properties) in mice under chronic restraint stress (CRS).

Methods: This experimental study was conducted in a university-affiliated Experimental Animal Unit, Khuzestan, Iran, from April to June 2018. Forty male Balb/C mice were randomly divided into five groups (n = 8), including under chronic restraint stress (CRS) receiving normal saline, hydroalcoholic extract of *A. capillus-veneris* (100, 200, and 400 mg/kg/day, i.p), or diazepam (10 mg/kg/day, i.p). After 21 days of the consecutive treatment, anxiolytic and antidepressant activities were evaluated using elevated plus-maze (EPM) and forced swim test (FST). Moreover, serum and brain levels of total antioxidant capacity (TAC) and malondialdehyde (MDA), as well as serum corticosterone, were measured.

Results: Immobility time in the FST was significantly decreased ($P = 0.002$, $P = 0.001$, $P < 0.0001$) after treating CRS mice with all doses of the extract. CRS-exposed mice treated with all doses of the extract showed a significantly increased percentage of entries into the open arm ($P < 0.0001$, $P = 0.001$) and reduced closed arm entries in the EPM ($P = 0.012$, $P = 0.024$). Extract at all doses significantly increased serum ($P < 0.0001$) and brain ($P = 0.011$, $P = 0.004$, $P = 0.001$) TAC in CRS-exposed mice. The extract (200 and 400 mg/kg) also reduced CRS-induced serum and brain MDA ($P < 0.0001$, $P = 0.001$). Serum corticosterone did not significantly change following the extract treatment.

Conclusions: *A. capillus-veneris* extract showed antidepressant and anxiolytic effects by reducing oxidative stress markers.

Keywords: *Adiantum capillus-veneris*, Adjustment Disorders, Antioxidants, Anxiety, Corticosterone, Depression, Flavonoids, Mice, Oxidative Stress

1. Background

Depression and anxiety are chronic and recurring disorders associated with cognitive, biochemical, psychological, and behavioral changes (1). Generalized Anxiety Disorder (GAD) is one of the prevalent anxiety disorders, the main characteristics of which are excessive and unreasonable worry about every day's life events and activities (2). The GAD is a strong predictor of subsequent secondary disorders, including depression and other anxiety disorders. The comorbidity rates of GAD and depression was reported to be as high as 70%; however, the basis for this relationship is still unknown (3). The occurrence of stressful events has been reported as a common risk factor associated with both depression and GAD (4).

Chronic restraint (CRS) is used widely to make rodents depressed or anxious (5). The impaired activity of the hypothalamus-pituitary-adrenal axis (HPA) (6), altered brain monoamines levels (5), oxidative and nitrosative damage of the neural cell (7, 8), and cerebral inflammation (8)are the hallmarks of depression and anxiety due to the exposure to chronic stress. Today, the novel approaches in the treatment of neurological disorders such as anxiety and depression are to find new compounds with several modes of action, including antioxidant, inflammatory, and neuroprotective effects (6).

Adiantum capillus-veneris (Pteridaceae) is a cosmopolitan species widely distributed in the areas of the world with tropical climates and high humidity. It also grows wieldy in the northern parts of Iran (9). This plant has been traditionally used in Iran as an antitussive, anti-fever, expectorant, and diuretic drug, and also is utilized in the treating respiratory diseases and digestive disorders (10). Laboratory studies have demonstrated its antimicrobial (11), analgesic (9), anti-inflammatory (9), and antioxidant (12, 13) effects. Phytochemical analyses have shown the presence of flavonoids, alkaloids, tannins, saponins, terpenoids, glycosides, steroids, and reducing sugars in the plant extract (11). Its flavonoids consist of rutin, quercetin, quercetin-3-o-glucoside, querciturone, nicotiflorin, naringin, astragalin, populnin, procyanidin, prodelphinidin, and kaempferol-3-sulfate (14).

2. Objectives

This plant has not been previously explored for its protective effect against neurological disorders; Therefore, the present study was carried out to examine the antidepressant and anxiolytic effects of the plant extract and also some of its modes of action.

3. Methods

3.1. Drugs and Chemicals

Diazepam was obtained from the Sobhan Pharmaceutical Co. Iran. Acetic acid, thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), $FeCl_3.6H_2O$, 2,4,6-Tri (2-pyridyl)-s-triazine (TPTZ)and other reagents were purchased from Sigma-Aldrich Chemical Co (USA) and Merck Co. (Germany).

3.2. Preparation of A. capillus-veneris Extract

A. capillus-veneris was purchased from a local market of Izeh, Iran and confirmed by an herbalist, and then a reference sample was kept in the Herbarium of Islamic Izeh University with voucher herbarium specimen No. 7543. Extraction was conducted by a maceration method. The dried plant sample was pulverized by an electric mill and then mixed with 70% ethanol at 1:5 solid-to-liquid ratio. The resulting mixture was kept at room temperature for 72 hours. Then the solution was filtered using a Whatman No 1 filter paper and the filtrated solution was concentrated by a rotary evaporator under 40°C. Finally, the yielded mixture was incubated at 37°C until complete dryness (9).

3.3. Animals and Ethical Statement

This experimental study was conducted in the Experimental Animal Unit of Islamic Azad University of Izeh, Iran, Khuzestan, in the spring of 2018. A total number of 40 male BALB/c mice weighing 25 - 30g were obtained from the Animal Breeding Facility Centre of Pasteur Institute, Karaj, Iran. The mice were kept in the temperature of 23 $\pm$ 2°C while it was 12h light and 12h dark and they had the same water and food. The Guideline for the Care and Use of Laboratory Animals was the basis for treating the animals (15). The study was reviewed on 3 March 2018 by the Research Committee of Islamic Azad University of Izeh and approved by the code of 15330557962002.

The sample size was calculated using the following formula:

$$n = \frac{\Psi^2 \left(\frac{\sum s_i{}^2}{k}\right)}{\sum \frac{\left(x_i - \frac{x_1 + x_2 + x_3}{k}\right)^2}{k-1}} \quad (1)$$

$\alpha = 0.05$, $\beta = 0.10$, k: number of groups, $\Psi\alpha$, β, k-1, ∞ = 2.52, x_i, s_i: mean (x_1 =, x_2 = ...) and standard deviation (s_1 =, s_2 = ...).

3.4. Animals Grouping and Treatment

In this experimental study, which was conducted from April to June 2018, 40 male BALB/c mice were randomly (simple randomization) divided into five groups (n = 8): Group 1 (chronic restraint stress (CRS)): underwent restraint stress for six hours a day and received i.p injection of normal saline for 21 consecutive days; groups 2, 3, and 4 (intervention groups): underwent chronic restraint stress and received i.p injection of *A. capillus-veneris* extract at doses of 100, 200, and 400mg/kg, respectively; and group 5 (positive control group): underwent chronic restraint stress and for 21 consecutive days they received diazepam at a dose of 10 mg/kg. The extract and diazepam injections were given 30 minutes prior to the stress induction (16). The elevated plus maze and forced swim tests were used to evaluate depression and anxiety after treating the mice for 21 days. Once the behavioral examination was done, the animals went under deep anesthesia to take heart blood samples and the samples were centrifuged to separate sera. Then for biochemical analysis, the sera and the removed brain tissues were stored at -80°C.

3.5. Forced Swim Test

The forced swim test (FST) is a reliable and common test to study the antidepressant-like effects of drugs. In this test, a glass cylinder (25 $\times$ 12 $\times$ 15 cm) is filled with 25°C water and the mouse from a 20-cm distance to the water surface is gently released into the water. The animal is allowed to acclimatize for the first two minutes. The time of immobility (seconds) during the last 4 minutes of the 6 minutes tests was recorded. The period when there is no activity except that needed to hold the animal's head above the surface is called immobility and the time when the animal has active movement of extremities and circling in the container is referred to as swimming. All measurements of variables were conducted by one individual (17).

3.6. Elevated Plus Maze Test

To measure anxiety a device called elevated plus maze was used. This device has a central sheath, which is 50 cm above the floor, two opposite closed arms, and two opposite open arms. This test was done in a rather dark, silent chamber, and each mouse was put mildly in the device center in front of the open arms and was let to explore it for 5 minutes. The time spent in each arm and the number of entries were recorded. One day after the last day of feeding, animals' behavior in the experimental sessions (10 minutes) was recorded by a video camera located above the maze, interfaced with a monitor and a computer in an adjacent room. The recorded behavior in the computer was subsequently scored for conventional indices of anxiety (17).

3.7. Measurement of Malondialdehyde (MDA)

First, 200 μL of tissue homogenate/serum was combined with 1.5 mL of 20% acetic acid, 1.5 mL of 0.8% thiobarbituric acid, and 200 μL of 8.1% sodium dodecyl sulfate. Then 700 μL of distilled water was added to the mixture and it was heated in a boiling water bath for 60 minutes. After the mixture was cooled under tap water, distilled water (1 mL) and n-butanol/pyridine solution (5 mL) were added to the reaction mixtures and shaken vigorously. Eventually, the acquired solutions were centrifuged at 4000 rpm for 10 minutes and the spectrophotometry was used to record the optical absorbance of the supernatant at 532 nm (Shimadzu, Japan) (we used blank for calibration) (18).

3.8. Measurement of Total Antioxidant Capacity (TAC)

Using ferric reducing antioxidant power (FRAP) assay, the total antioxidant capacity of serum and tissue homogenate were evaluated. The working FRAP reagent was prepared by mixing acetate buffer (10 mL, 0.25M, pH = 3.6), 2 TPTZ (5 mL, 10 mM, prepared in 40 mM HCl), and $FeCl_3.6H_2O$ (2.5 mL, 20mM). Then 25 μL of tissue homogenate/serum was added to 1.5 mL of working FRAP solution and put aside at 37°C for 10 minutes. After incubation, the spectrophotometry at 593 nm (Shimadzu, Japan) was used to read the optical absorbance (we used blank for calibration) (18).

3.9. Measurement of Serum Corticosterone Level

The corticosterone ELISA Kit (ab108821) was used to measure serum corticosterone level based on the manufacturer's instructions by ELISA reader (Shimadzu, Japan).

3.10. Statistical Analysis

Data were analyzed using the IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, N.Y., USA). The data were quantitative so the Kolmogorov-Smirnov (K-S) test was used to confirm the assumption of the normal distribution of data frequency ($P > 0.05$) (Table 1). To identify statistical differences between the means, analysis of Variance (ANOVA) followed by LSD test was used. The whole data were presented as mean $\pm$ SD (Table 2), as well as mean $\pm$ SEM (In figures), and P value less than 0.05 was considered statistically significant.

Table 1. Kolmogorov-Smirnov Test (Test Distribution Is Normal)

	K-S
Immobility Time	0.076
Number of entries open arm in 5 minutes	0.402
Percentage of entries into the open arm	0.479
Time of attendance in the open arm, second	0.641
Number of entries closed arm in 5 minutes	0.327
Time of attendance in the closed arm, second	0.641
Serum frap level	0.724
Brain frap level	1.864
Serum MDA level	0.593
Brain MDA level	1.296
Serum corticosterone level	0.472

4. Results

As shown in Figure 1, the treatment of CRS exposed mice with *A. capillus-veneris* extract at doses of 100, 200, and 400 mg/kg significantly decreased the immobility time compared with the CRS group receiving normal saline ($P = 0.002$, $P = 0.001$, and $P < 0.0001$). Diazepam showed no significant effect on the duration of animal immobility time in the forced swim test (Figure 1).

Based on the results of Figure 2, the time elapsed in the open arm of EPM and the number of entries were not significantly different between the experimental groups (A and C). However, the percentage of open arms entries

Table 2. Mean ± SD of Depression, Anxiety, and Biochemical Tests

	Groups[a]							
	Saline	Extract 100	P Value	Extract 200	P Value	Extract 400	P Value	Diazepam	P Value
Immobility time, S	72.375 ± 45.93	27.25 ± 16.22	0.002**	26.5 ± 15.66	0.001**	16.125 ± 8.8	< 0.0001***	48.625 ± 27.35	0.079
Number of entries open arm in 5 minutes	3.57 ± 2.22	5.625 ± 2.33	0.060	4.25 ± 1.28	0.525	3.75 ± 2.25	0.867	4.125 ± 1.96	0.603
Percentage of entries into the open arm	28.58 ± 13.39	49.24 ± 6.74	< 0.0001***	55.11 ± 7.28	< 0.0001***	46.45 ± 9.75	0.001**	47.07 ± 9.1	0.001**
Time of attendance in the open arm, S	51.43 ± 32.59	102.875 ± 67.93	0.077	100.5 ± 47.05	0.091	91.625 ± 58.77	0.163	101.00 ± 56.87	0.088
Number of entries closed arm in 5 minutes	7.14 ± 3.71	5.875 ± 2.9	0.346	3.625 ± 1.77	0.012*	4.00 ± 1.41	0.024*	5.00 ± 2.56	0.116
Time of attendance in the closed arm, S	248.57 ± 32.59	197.125 ± 67.93	0.077	199.5 ± 47.05	0.091	208.375 ± 58.77	0.163	199.00 ± 56.87	0.088
Serum Frap level, μmol	195.375 ± 32.43	449.43 ± 74.38	< 0.0001***	431.875 ± 136.23	< 0.0001***	488.57 ± 69.13	< 0.0001***	327.83 ± 222.9	0.050*
Brain Frap level, μmol	161.25 ± 42.85	196.71 ± 5.47	0.011*	201.25 ± 6.88	0.004**	208.00 ± 10.88	0.001**	140.33 ± 34.64	0.138
Serum MDA level, μmol/L	464.00 ± 63.76	440.29 ± 25.22	0.282	379.00 ± 45.49	< 0.0001***	283.86 ± 24.4	< 0.0001***	344.00 ± 28.46	< 0.0001***
Brain MDA level, μmol/L	356.5 ± 40.49	307.14 ± 79.08	0.165	233.00 ± 105.12	0.001**	136.71 ± 6.24	< 0.0001***	271.5 ± 50.27	0.025*
Serum Corticosterone level, ng/mL	96.71 ± 58.1	94.62 ± 63.2	0.938	76.41 ± 34.39	0.397	85.32 ± 40.86	0.657	93.00 ± 30.18	0.881

Abbreviations: FRAP, ferric reducing antioxidant power; MDA, malondialdehyde.
[a] Underwent chronic restraint stress for six hours a day and received i.p injection of normal saline, *A. capillus-veneris* extract at doses of 100, 200, and 400mg/kg/day, or diazepam at a dose of 10 mg/kg/day.

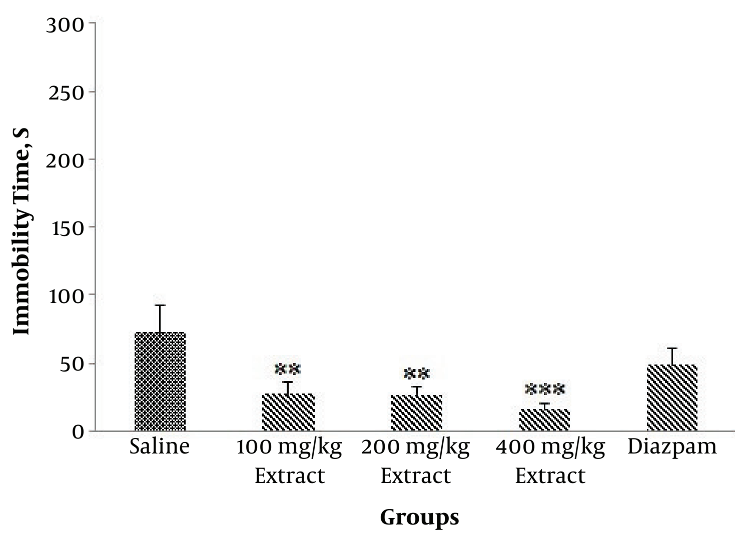

Figure 1. Comparison of the immobility time in the forced swim test in mice under chronic restraint stress (CRS) receiving normal saline, different doses of *A. capillus-veneris* extract, and diazepam. **P < 0.01 and ***P < 0.001 represent significant differences between intervention groups and CRS mice receiving normal saline.

showed a significant increase in CRS mice receiving the extract at doses of 100, 200, and 400 mg/kg and diazepam compared with CRS group receiving normal saline (P < 0.0001, P = 0.001).

As shown in Figure 3, the treatment of CRS-exposed mice with the extract at doses of 200 and 400 mg significantly reduced the number of entries into the closed arms of EPM compared with the CRS group receiving normal saline (P = 0.012, P = 0.024). The time spent in the closed arms of EPM showed no differences between the groups (Figure 3).

The mean of serum and brain TAC and MDA levels in the experimental groups are shown in Figure 4. As shown, serum and brain TAC levels were significantly increased in CRS mice receiving all doses of the extract when compared with the CRS mice receiving normal saline (serum TCA level; P < 0.0001, brain TCA level; P = 0.011, P = 0.004, and P = 0.001). Also, treating CRS mice with diazepam significantly increased serum TAC level (P = 0.05). A significant decrease was also found in the MDA levels of the brain and serum in CRS mice receiving 200 and 400 mg/kg of the extract as well as diazepam (serum MDA; P < 0.0001 and brain MDA; P = 0.025, P = 0.001, P < 0.0001).

According to the results of Figure 5, a non-significant and partial reduction was seen in the serum level of corticosterone in mice receiving 200 and 400 mg of the extract compared with CRS mice receiving normal saline.

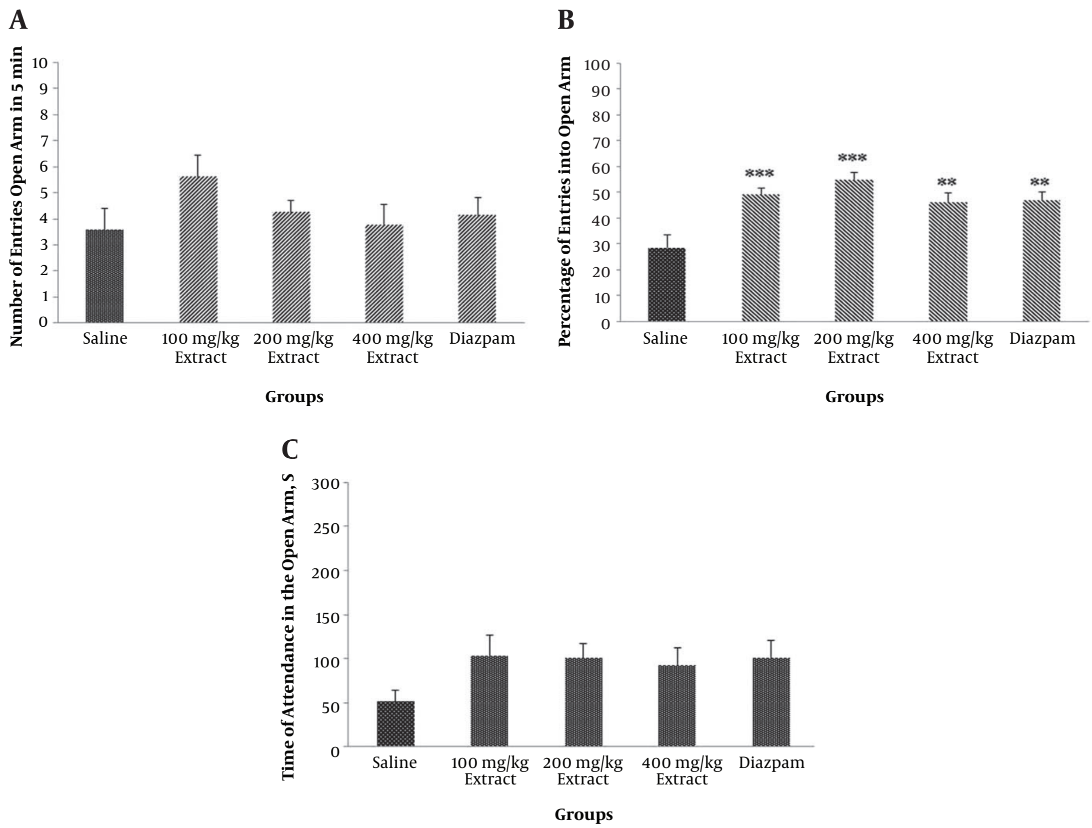

Figure 2. Comparison of the number of entries (A), percentage of entries (B), and time spent (C) in the open arms of Elevated Plus Maze (EPM) in mice under chronic restraint stress (CRS) receiving normal saline, various doses of *A. capillus-veneris* extract and diazepam. $^{**}P < 0.01$ and $^{***}P < 0.001$ represent significant differences between intervention groups and CRS mice receiving normal saline.

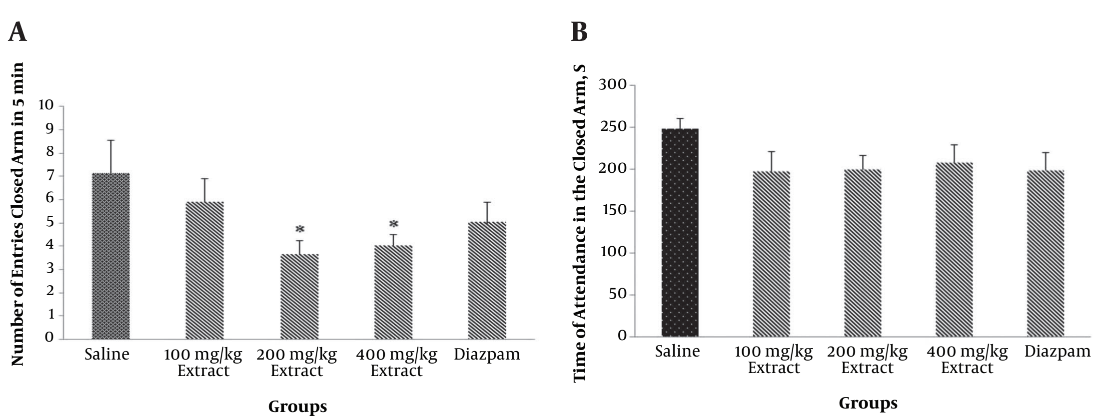

Figure 3. Comparison of the number of entries (A) and percentage of entry (B) in the closed arm of Elevated Plus Maze (EPM) in mice under chronic restraint stress (CRS) receiving normal saline, various doses of *A. capillus-veneris* extract, and diazepam. $^{*}P < 0.05$ indicates a significant difference between intervention groups and CRS mice receiving normal saline.

5. Discussion

In this study, the protective effects of *A. capillus-veneris* extract were evaluated against anxiety- and depression-like behaviors caused by chronic restraint stress in male mice. Based on the results, the treatment of CRS mice with

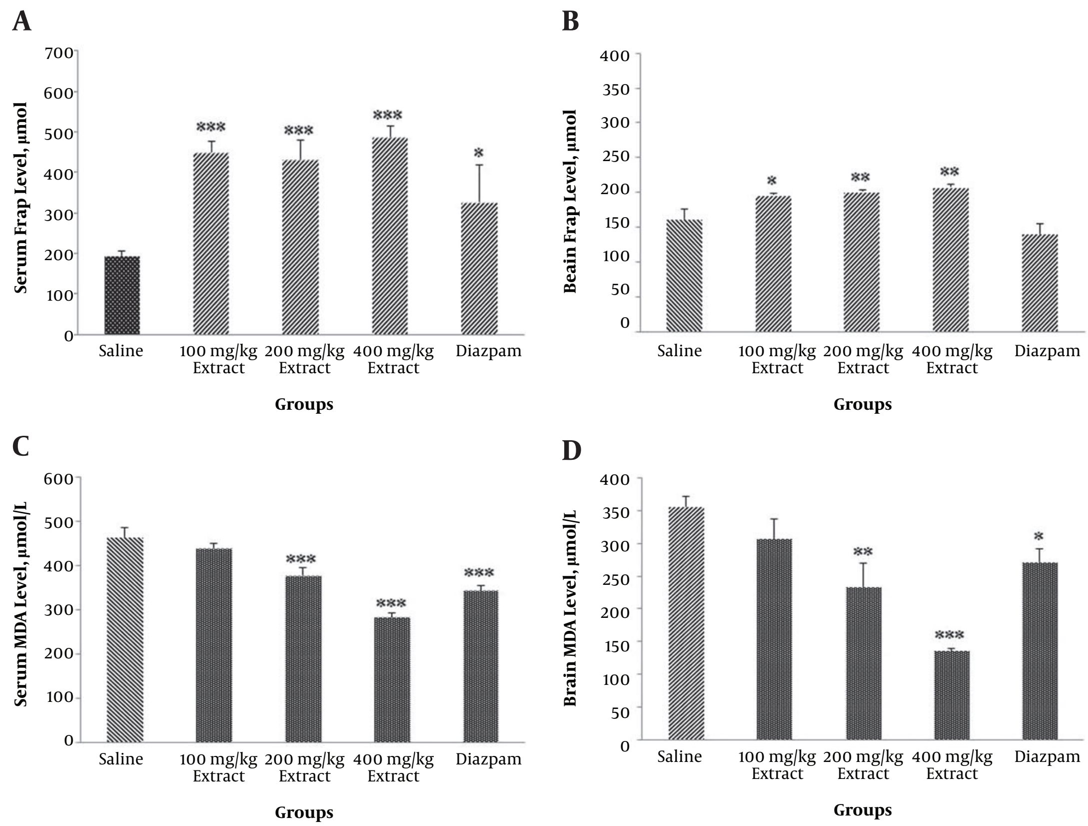

Figure 4. Comparison of serum (A) and brain (B) total antioxidant capacity and serum (C) and brain (D) MDA in mice under chronic restraint stress (CRS) receiving normal saline, various doses of *A. capillus-veneris* extract, and diazepam. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ Indicate significant differences between intervention groups and CRS mice receiving normal saline.

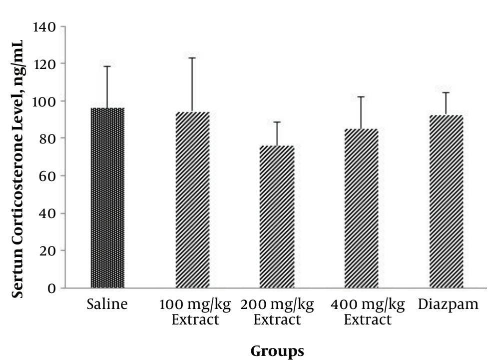

Figure 5. Comparison of serum corticosterone levels in the mice under CRS receiving normal saline, various doses of *Adiantum capillus-veneris* extract, and diazepam.

all doses of the extract significantly decreased immobility time in the forced swim test. *A. capillus-veneris* extract (200 and 400 mg/kg) also increased open arm entries and reduced closed arm entries in the EPM test. These findings suggest the antidepressant and anxiolytic effects of *A. capillus-veneris* extract, which may be due to its high amount of flavonoids with neuroprotective actions. Phytochemical studies have shown the presence of flavonoids such as rutin, quercetin, quercetin-3-o-glucoside, querciturone, nicotiflorin, naringin, astragalin, populnin, procyanidin, prodelphinidin, and kaempferol-3-sulfate in the plant extract (14). Some of these compounds, including quercetin and rutin, have been reported to exert antidepressant and anti-anxiety effects in chronic stress models (19-22). These compounds exhibit their activities through multiple mechanisms, including the normalization of HAP activity and reducing oxidative stress and neuroinflammation (19-22).

In general, HPA axis dysfunction plays an essential role in the development and progression of anxiety- and depression-like behaviors (20). The main characteristic feature of a stress response is the increased secretion of stress hormone (corticosterone in rodents and cortisol in humans) due to the activation of the HPA axis. Stress hormone secretion tends to reduce gradually after a stressor occurs (23). However, chronic hyperactivation of the HPA axis during a stressful situation can lead to the development of depression and anxiety (16, 24). In this regard, it has been found that chronic injections of corticosterone cause depression-like behaviors in animal models (25, 26). The increased serum levels of glucocorticoids have also been reported in patients with major depression, which were decreased after treatment with antidepressants (27). Furthermore, the higher prevalence of depression was reported in patients with autoimmune diseases or Cushing's syndrome receiving cortisol analogs such as dexamethasone and prednisolone (28). In the present study, administration of *A. capillus-veneris* extract into stressed mice caused an insignificant and partial reduction of serum corticosterone, which may somehow play a role in the observed antidepressant and anxiolytic activities.

Recently, the roles of oxidative stress in the pathogenesis of depression and anxiety in humans as well as in animal models were discovered (23). Chronic restraint stress (CRS) has been shown to result in elevated brain malondialdehyde as an indicator of lipid peroxidation (16). It also increases the protein and DNA oxidation in different regions of the brain (29). In a meta-analysis conducted by Jimenez-Fernandez et al (30), higher MDA, lower uric acid and zinc, and altered activity of superoxide dismutase (SOD) were reported in the serum of depressed patients compared with healthy subjects. Forlenza and Miller also reported an increased serum 8-hydroxy-2'-deoxyguanosine (8-OHdG) as an index of oxidative DNA damage in patients with depression (31).

The exact mechanism of increasing the oxidative stress parameters during chronic stress exposure has not been yet fully understood (19). It seems that HPA axis activation and elevated levels of circulating glucocorticoids plays a role in this process (22). It has been observed that repeated cellular exposure to glucocorticoids increases the production of ROS. Glucocorticoids exposure also inhibits the detoxification capacity of enzymatic (SOD, GPX, and CAT) and non-enzymatic (glutathione) endogenous antioxidants (32). In this study, treatment of CRS-exposed mice with *A. capillus-veneris* extract resulted in a significant increase of TCA and significant reduction of MDA in both brain and serum. Consistent with these results, the antioxidant effects of *A. capillus-veneris* extract have been shown in mice exposed to carbon tetrachloride (33) and cisplatin (13).

5.1. Conclusions

According to the results of this study, *A. capillus-veneris* extract significantly ameliorated anxiety and depression in chronically stressed mice, which may be due to the decreased lipid peroxidation and improved antioxidant capacity of the brain and serum. Considering the role of *A. capillus-veneris* extract, we suggest to characterize the active chemical constituents of the extract, evaluate their effects on animal models, and then develop an appropriate clinical trial.

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Footnotes

Authors' Contribution: Jafar Ahmadpoor conceived and extracted the data, Saeid Valipour Chahardahcheric 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 takes responsibility for the integrity of the data and the accuracy of the data analysis and is guarantor.

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

Ethical Approval: The study was reviewed on 3 March 2018 by the Research Committee of Islamic Azad University of Izeh and approved by the code of 15330557962002.

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