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Evaluation of Antidepressant, Antianxiolytic, and Antioxidant Effects of *Echium amoenum L.* Extract on Social Isolation Stress of Male Mice

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Abstract

Background: One of the most important herbal remedies in Iran is *Echium amoenum L.* It has been used in traditional Iranian pharmaceutical formulations and has provided an interesting area of research for various drug activities.
Objectives: The present study aimed to investigate the antidepressant and antianxiolytic effects of hydroalcoholic extract of *Echium amoenum L.* in a socially isolated model of male mice.
Methods: In this experimental study, 50 male Balb/c mice weighing 25 to 30 g were divided into five groups of 10 mice. The control group received normal saline. The treatment groups received the *Echium amoenum L.* hydroalcoholic extract for five days at doses of 50, 75, and 100 mg/kg via i.p injection and the negative control group received social isolation and normal saline.
Results: *Echium amoenum L.* hydroalcoholic extract at doses of 50, 75, and 100 mg/kg significantly reduced immobility in the forced swimming test in mice exposed to social isolation stress. *Echium amoenum L.* extract at doses of 75 and 100 mg/kg significantly increased the number of crossings in the center of the open-field box. The time of staying in the open arms increased significantly in groups receiving the extract at 50, 75, and 100 mg/kg. Treatment with *Echium amoenum L.* extract reduced the serum and brain tissue levels of nitric oxide and malondialdehyde in mice exposed to social isolation stress and enhanced the total antioxidant capacity of serum and brain tissue.
Conclusions: Modulating the nitric oxide system and reducing oxidative stress may be essential mechanisms of *Echium amoenum L.* extract in reducing depression and anxiety in mice.

Keywords: Anxiety, Medicinal Plant, Depression, Mice

1. Background

Depression is one of the most important psychological disorders associated with symptoms such as negative mood, physical activity reduction, helplessness, and cognitive function disorder (1). It has been shown that various factors, including social, genetic, biological, and psychological factors, contribute to depression. However, one of the main reasons for depression is the decrease in neurotransmitter levels, such as serotonin, dopamine (DA), and noradrenaline (NA) (2).

Recently, the stress pattern of social isolation (SIS isolation stress, SIS) is proposed as an animal model to investigate the underlying mechanisms involved in the incidence of anxiety and depression (3). Social isolation is a model for investigating the behavioral consequences of rodents deprived of social interaction. Many of the symptoms of social isolation are similar to the symptoms of depression and anxiety. Moreover, the long separation of male mice can cause aggressive behaviors such as attacks (4).

Iranian *Echium amoenum* is an annual grass plant that belongs to the *Boraginaceae* family and the Echium genus (5). Iranian *Echium amoenum* is one of the most important herbal remedies in traditional medicine in Iran. All parts of the plant including stems, leaves, and flowers, except for the root, have medicinal applications (6). Persian *Echium amoenum* is used for relaxation and mood improvement, as well as the treatment of sore throat, pneumonia, and cough. The neuroprotective effects of *Echium amoenum* flowers including anti-ischemic (5), antinociceptive (7) and anti-anxiolytic (8) effects have been shown in animal models. New research suggests that the aqueous extract of *Echium amoenum* is an effective drug for the treatment of patients with mild to moderate depression (6) and patients with obsessive-compulsive disorder (1) and general anxiety disorder (9). Purple-blue petals of *Echium amoenum* are known to be one of the most important sources of phenolic compounds such as rosmarinic acid, cyanidin, and delphinidin (10). Cyanidin-3-glucoside, the most impor-

tant plant anthocyanin, has protective effects against cerebral ischemia-induced brain injury and apoptosis (11). Several studies have been done on the antidepressant effects of *Echium amoenum*; however, no study has assessed the effects of *Echium amoenum* on anxiety and depression induced by social isolation in mice.

2. Objectives

The purpose of the present study was to investigate the antidepressant and anxiolytic effects of the hydroalcoholic extract of *Echium amoenum L.* in a socially isolated model of male mice.

3. Methods

3.1. Extract Preparation

After flowering and confirming the scientific name of the plant, the plant specimen number 90821 was kept in the herbarium of Azad University of Izeh. Extraction was carried out by the maceration method. The dried sample of the plant was powdered by electric grinding and 70% ethanol powder was added to it. The resulting liquid was placed in a magnetic carrier with a magnet and kept at room temperature for 72 h. Then, the contents of the container were filtered and the filtered solution was placed in a rotary apparatus to evaporate water and alcohol. It was finally placed in a 37°C incubator to dry (10).

3.2. DPPH Radical Scavenging Activity

Different amounts of *Echium amoenum* extract were mixed in dilute distilled water and 1 mL of it was mixed with 0.1 mM DPPH solution. It was allowed to stay at room temperature for 15 min. Then, the absorption was measured at 517 nm with a spectrophotometer. For the control sample, 1 mL of distilled water was used. The percentage of inhibitors of DPPH radicals was calculated by the following formula:

$DPPH = [(A_{control} - A_{sample})/A_{control}] 100$

The IC_{50} was obtained by plotting the concentration graph (X-axis) against the inhibition percentage (Y-axis) (12).

3.3. ABTS Radical Scavenging Activity

ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was prepared by the reaction of ABTS (4 mM, 10 mL) with potassium sulfate (2.6 mM, 10 mL) for 12 h at room temperature in the ABTS solution. Before testing, the solution was diluted with methanol to reach an absorbance of 1.1 $\pm$ 0.02 at 734 nm. Thereafter, 150 μL of the hydrolyzed protein was added to 2,850 μL of ABTS solution at various times. After incubation for 12 h, optical absorption was recorded. For the control sample, 150 μL of distilled water was used. The ABTS inhibition activity was determined by the following formula:

Scavenging activity (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$

The IC_{50} was determined from the plot of the scavenging activity against the sample concentration (13).

3.4. Hydroxyl Radical Scavenging Activity

In a test tube, the 1,10-phenanthroline solution (1.865 mM, 1 mL) was mixed with the extract (2 mL) and then, 1 mL of the $FeSO_4$ solution (1.865 mM) was added. The reaction started by adding 1 mL and placed in a water bath at 37°C. The optical absorption was measured after 60 min at 536 nm. The solution containing the extract in the absence of hydrogen peroxide was considered as the blank and the solution without the extracts as the negative control. The hydroxyl radical scavenging activity was determined using the following formula:

Hydroxyl radical scavenging activity (%) = [(As-An)/(Ab-An)] $\times$ 100

The absorbance of the blank IC_{50} was determined from the plot of the scavenging activity against the sample concentration (14).

3.5. Laboratory Animals

The test animals were male mice weighing 25 to 30 g. The animals were kept at an appropriate temperature (25 $\pm$ 3°C) and 55 $\pm$ 5% humidity, 12 h light/12 h dark with free access to equal food and water. The mice were purchased from the Tehran Pasteur Institute.

Ethical considerations: The Ethics Committee of the Azad University of Izeh reviewed all procedures and experiments as the local referral Biomedical Committee for Research Ethics (Code: 15330525972002). Protocols and guidelines were carried on following the National Institutes of Health (NIH) for the care and use of experimental animals. The supervision of animal protocols was carried out according to the research design (15).

In this study, 50 male mice were divided into five groups of 10 mice, four of which were subjected to stress and anxiety by social isolation. In the control group, animals received normal saline intraperitoneally for five days at 10 mg/kg. In the treatment groups, animals received intraperitoneal *Echium amoenum* extract for five days at 50, 75, 100 mg/kg. The negative control group was subjected to social isolation and received normal saline intraperitoneally at a dose of 10 mg/kg for five days.

Animals were isolated for one week and then the extract and normal saline were given for five days after animals were separated administration continued during behavioral tasks. At the end of the behavioral tests, animals were killed in deep anesthesia and their blood and brain tissue were collected. Only one observer was used in this study.

3.6. Social Isolation Method

Animals were randomly kept in both social conditions and social isolation for one week. Mice were housed in plexiglass boxes (six mice per cage) in social condition and isolated mice were housed individually in plexiglass boxes. The isolated animal cage was weekly cleaned by a tester (1).

3.7. Tail Suspension Test

The total duration of Tail Suspension Test (TST) was 6 min, in which animals were allowed to adapt to the apparatus for the first two minutes. The immobility time (s) in the next four minutes was measured using a chronometer. All the measurements of variables were conducted by the same individual (16).

3.8. Forced Swimming Test

It is considered to be immobilized. The whole mandatory swimming test lasted 7 min. The first two minutes devoted to the adaptation of the animal to the current conditions and no immobilization time was recorded. However, the immobilization time was measured in the next 5 min (17).

3.9. Elevated Plus-Maze Test

An apparatus called elevated plus maze was used to measure anxiety. This apparatus had two opposite open arms, two opposite closed arms, and a central sheath elevated 50 cm above the floor. This test was performed in a relatively dark, silent chamber. Each animal was placed gently in the center of the device facing the open arm and allowed to explore for 5 min. The number of entrances and the time spent in each arm were recorded (18).

3.10. Open-Field Test

We set the animal at the center of the tool and recorded the animal behavior within 5 min (19).

3.11. Determination of Total Antioxidant Capacity of Serum and Brain Tissue by FRAP Method

The basis of this method is the ability of serum and brain homogenate tissue to recover ferric Fe^{3+} (Fe^{2+}) ions in the presence of TPTZ. In this method, the reaction (Fe^{2+}) with TPTZ reagent gave the blue complex of Fe^{+2}-TPTZ with a maximum absorbance at 593 nm as measured by spectrophotometry (20).

3.12. Malondialdehyde in Plasma and Brain Tissue

In this test, 1 mL of homogenized brain tissue or serum was inserted into a 20 mL glass tube and incubated at a temperature of 37°C in a metabolic shaker for 60 min. After one hour of incubation, 1 mL of 5% tetrachloroacetic acid was added with 1 mL of 67% thiobarbituric acid. The mixture was transferred from each vial to a centrifuge tube and centrifuged at 2,000 g for 15 min. The supernatant was, then, transferred to another tube, placed in a boiling water bath, cooled for 10 min, and the absorbance of each fraction was measured at 535 nm (21).

3.13. Nitric Oxide in Serum and Brain Tissue

The amount of nitric oxide was determined by measuring its nitrate, serum nitrite, and homogenized brain tissue using a colorimetric kit.

3.14. Statistical Analysis

Comparisons between groups were made using one-way ANOVA, followed by Tukey's post hoc test. A P value of < 0.05 was considered statistically significant. The sample size was calculated by power calculations using G power software (Ver. 3.1.7, Franz Faul, Universitat Kiel, Germany). We set the α error at 0.05 and power (1-β) at 0.8; thus, the required total sample size per group was calculated as 6 to 8 mice in behavioral tests. We also calculated the power value in each experimental group and analyses showed that the power values were larger than 0.8 in all ANOVA tests. The effect size for ANOVA tests was calculated by Cohen's formula.

4. Results

In this study, the antioxidant activity of *Echium amoenum L.* extract was evaluated in vitro by using five antioxidant tests. The results of this study showed that *Echium amoenum L.* extract had strong DPPH free radicals (IC_{50} = 50.85 μg/mL) and moderate ABTS (IC_{50} = 256.79 μg/mL) and hydroxyl radical (IC_{50} = 164.79 μg/mL) scavenging activity.

The results of the immobility time in the forced swimming test (A) and the tail suspension test (B) are shown in Figure 1. The results of this study showed that social isolation stress in mice induced a significant increase in immobility time in forced swimming test compared to control group animals. *Echium amoenum L.* extract at doses of 50, 75, and 100 mg/kg significantly reduced immobility in the forced swim test compared to the SIS group in rats exposed to social isolation stress. In the tail suspension test,

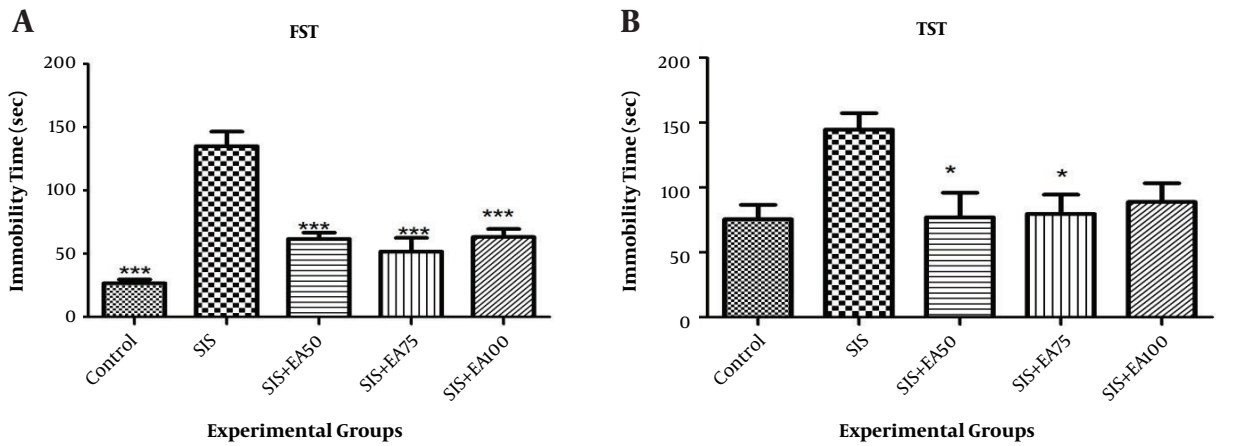

Figure 1. Comparison of immobility duration in forced swimming test (A) and tail suspension test (B) in experimental groups. All groups were compared with the SIS group. EA, *Echium amoenum L.*; SIS, social isolation stress; *P < 0.05, ***P < 0.001

Echium amoenum L. extract at doses of 50 and 75 mg/kg significantly reduced immobilization time compared to the SIS group.

According to the results of the open-field test, the number of entrances into the center of the open-field box was significantly lower in the group under social isolation stress than in the control group. *Echium amoenum L.* extract at doses of 75 and 100 mg/kg significantly increased the number of crossings in the center of the box compared to the SIS group (Figure 2).

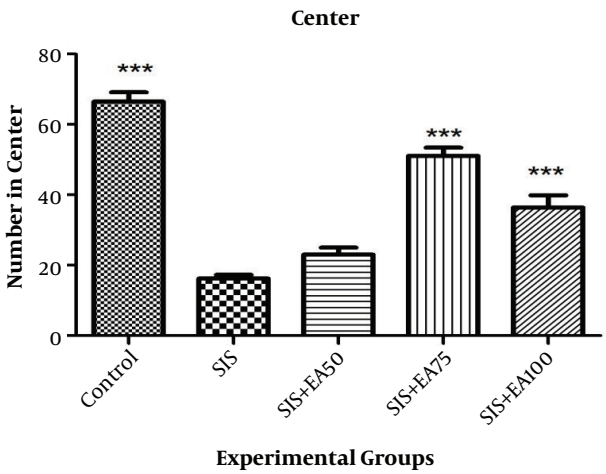

Figure 2. Comparison of the number of crossings in the center of the open-field box in the experimental groups. All groups were compared with the SIS group. EA, *Echium amoenum L.* and SIS, Social Isolation Stress. ***P < 0.001

The results of the plus-maze test are shown in Figure 3. According to the results of this study, the time spent and the number of animals entering the open arm (A, B) were significantly lower in the social isolation group (SIS) than in the control group. The time spent in the open arms in the groups receiving the extract of *Echium amoenum L.* at 50, 75, and 100 mg/kg significantly increased compared to the SIS group. In the group receiving *Echium amoenum L.* extract at 75 mg/kg, the number of entrances into open arms was significantly higher than in the SIS group. The results showed that the SIS group spent significantly more time in the closed arms than the control group. The time spent in the closed arms was significantly lower in the groups receiving the extract at 50, 75, and 100 mg/kg than in the SIS group.

The results of the antioxidant capacity of serum (A) and brain (B) are presented in Figure 4. The antioxidant capacity of serum and brain tissue was significantly lower in animals under social isolation stress than in the control group. Treating with *Echium amoenum L.* extract at doses of 50, 75, and 100 mg/kg significantly increased the antioxidant capacity of serum and brain tissue compared to the SIS group.

According to Figure 5, mice with social isolation stress had significantly higher levels of serum and brain tissue malondialdehyde than the control group. *Echium amoenum L.* extract at three doses of 50, 75, and 100 mg/kg used in this study significantly reduced serum and brain tissue malondialdehyde levels compared to the SIS group.

The results for serum and brain tissue Nitric Oxide (NO) levels are shown in Figure 6. Serum and brain tissue NO levels were significantly higher in the group under social isolation than in the control group. The *Echium amoenum L.* extract at a dose of 75 mg/kg could significantly decrease serum NO levels compared to the SIS group. Nitric oxide

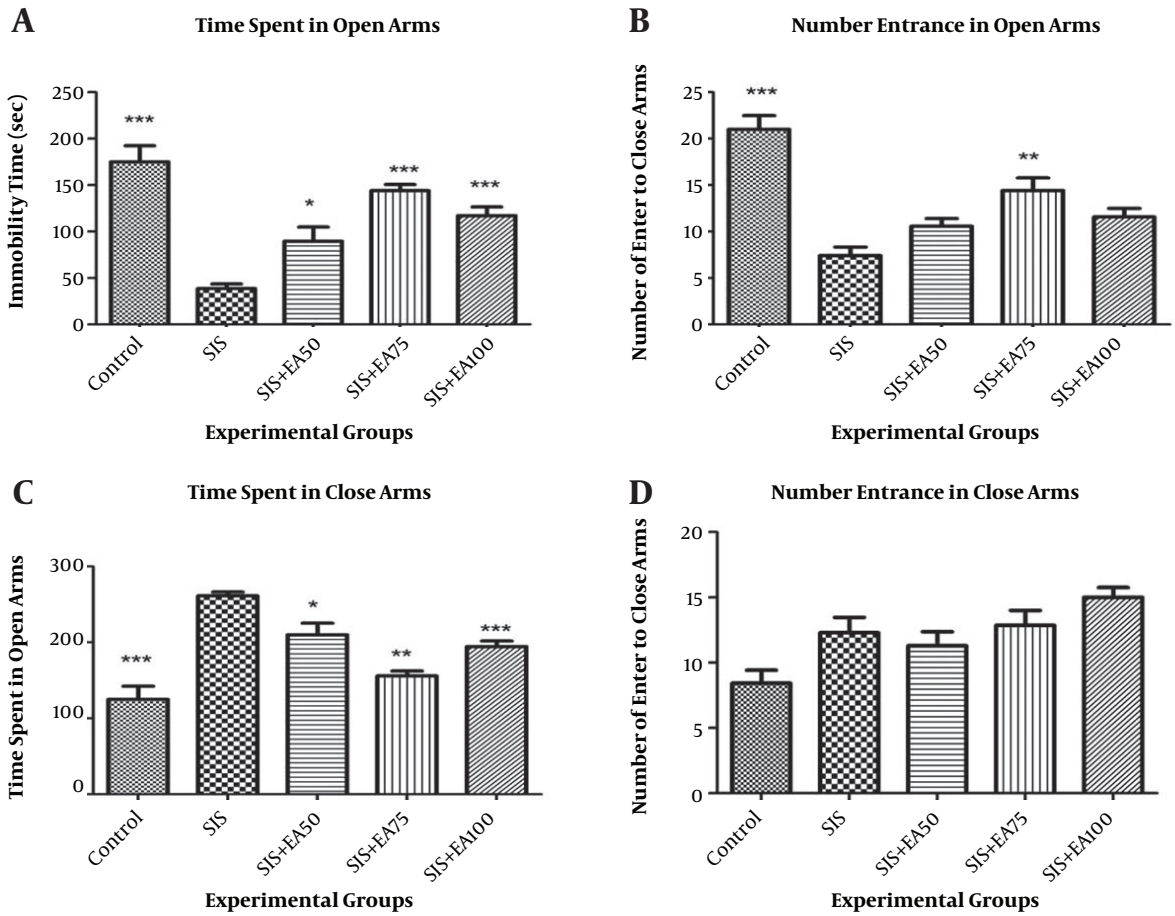

Figure 3. Comparison of time spent (A) and the number of entrances into open arms (B) and time spent in closed arms (C) and the number of entrances into closed arms (D) in experimental groups. All groups were compared with the SIS group. EA, *Echium amoenum L.* and SIS, Social Isolation Stress; *P < 0.05, ***P < 0.001, **P < 0.01

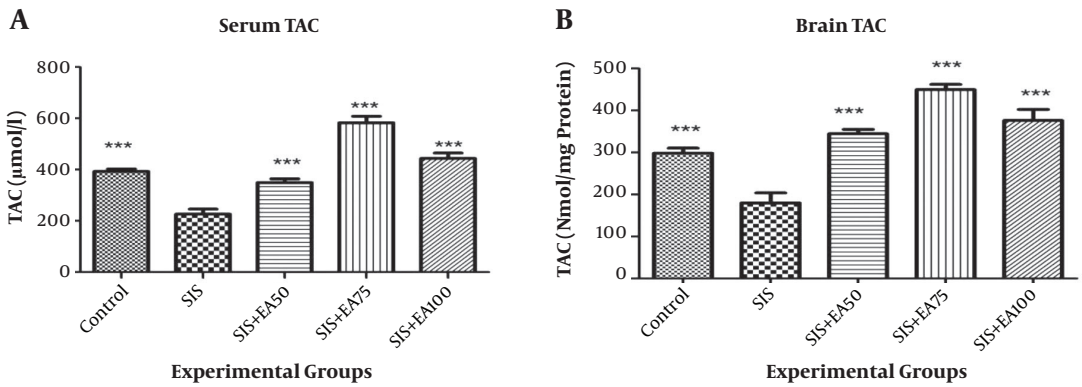

Figure 4. Comparison of antioxidant capacity of serum (A) and brain tissue (B) in experimental groups. All groups were compared with the SIS group. EA, *Echium amoenum L.* and SIS, social isolation stress; ***P < 0.001

levels in brain tissue were significantly lower in the groups receiving the extract at doses of 75 and 100 mg/kg than in the SIS group.

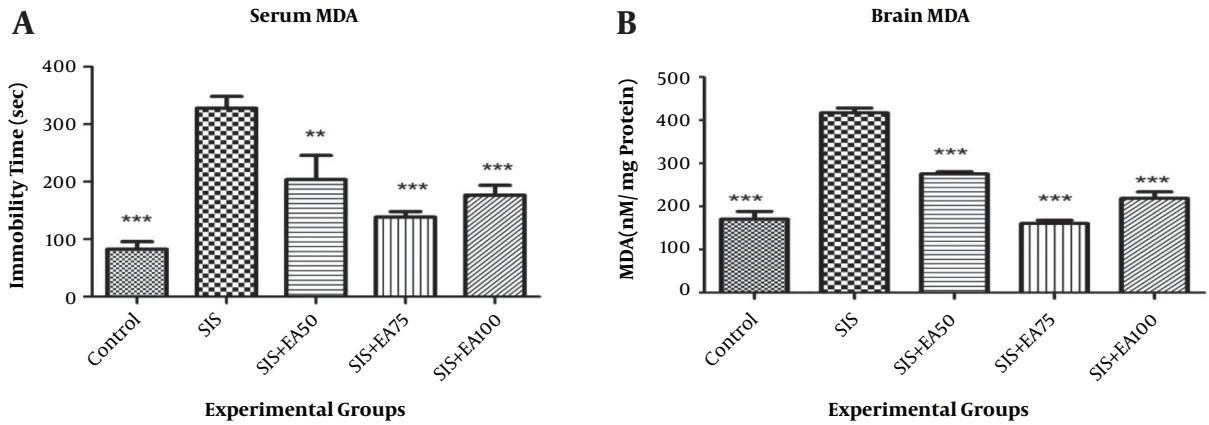

Figure 5. Comparison of serum (A) and brain tissue (B) malondialdehyde levels in the experimental groups. All groups were compared with the SIS group. EA, *Echium amoenum L.* and SIS, social isolation stress; ***P < 0.001, **P < 0.01

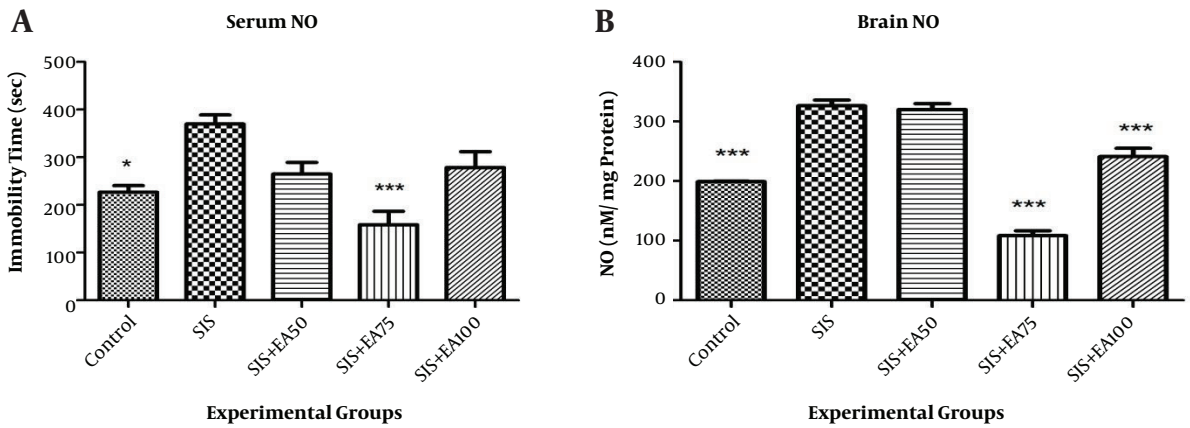

Figure 6. Comparison of serum (A) and brain tissue (B) NO levels in the experimental groups. All groups were compared with the SIS group. EA, *Echium amoenum L.* and SIS, social isolation stress, ***P < 0.001, *P < 0.05

5. Discussion

In modern societies, anxiety disorders have become a common disease and are associated with many psychiatric disorders. Stress plays an important role in the pathophysiology of psychiatric disorders such as anxiety and depression. Stress alters various neural functions at the central and peripheral levels by activating the hypothalamic-pituitary-adrenal axis. Any kind of stress can affect brain function by making long-term changes in the nervous system. There is evidence that SIS experience in early life is associated with depression and anxiety behaviors (22).

Stress-induced behavioral changes in rodents can be closely examined. The plus-maze test and open-field test are the most common experiments to investigate the anxiolytic effects on animal behavioral parameters. In the present study, social isolation stress caused anxiety-like behaviors such as a significant decrease in elevated plus maze parameters including the number of entrances into open arms and the time spent in open arms. Similarly, social isolation stress significantly reduced the animal entrance into the center of the open-field apparatus, indicating fear and anxiety. *Echium amoenum L.* dramatically increased animal entrance into the center of the open-field apparatus. The results are consistent with previous studies (23, 24). The anxiolytic effects of *Echium amoenum L.* may be due to its chemical composition. The antioxidant effects of *Echium amoenum L.* are attributed to the presence of phenolic, vitamin C, flavonoid, anthocyanins beta-carotene, and tannin components (7). Rosmarinic acid has been recognized as one of the main phenolic compounds of the

plant with protective effects against the oxidative damage of dopaminergic neurons by hydrogen peroxide (7). In addition, cyanidin and dolphinidin, which are abundant in *Echium amoenum L.* extract, can decrease hydrogen peroxide production and increase glutathione reductase activity and glutathione content in the cell culture medium (25). According to the findings, it can be stated that the extract of *Echium amoenum L.* can reduce the oxidative stress parameters by oxidative damage of neurons and consequently reduce neuronal damage and decrease the level of anxiety and depression. Oxidative stress can produce ROS, which has deleterious effects on cellular components such as proteins, lipids, and DNA, leading to cellular damage and neurodegeneration (26). Social isolation stress is a method of inducing oxidative damage by disrupting the antioxidant defense mechanism. In the present study, social isolation stress could induce oxidative damage, which was associated with increased lipid peroxidation and decreased antioxidant capacity of serum and brain tissue. In this study, the treatment of mice with *Echium amoenum L.* extract decreased malondialdehyde levels and increased serum antioxidant capacity in brain tissue.

Stress can affect different areas of the brain including the cortex and the hippocampus, especially due to its important role in regulating emotion. In stress conditions, these regions of the brain are affected by various chemical changes including excessive production of NO. Nitric oxide is a signaling molecule, which regulates the key functions in the Central Nervous System (CNS) as a neurotransmitter, as well as a nervous system. Previous studies have shown that the nitric oxide system plays a role in mood pathogenesis disorders. Nitric oxide has also been implicated in the mechanism of action of some antidepressants and anxiolytics (27). Chronic stress increases oxidative and nitrosative stress, stimulates neurotransmission, and disrupts mitochondrial function in areas of the brain that are related to the pathophysiology of depression (28).

Previous studies have shown that the nitric oxide system plays a role in mood disorders (29). Nitric oxide inhibitors seem to have anti-depressant and anti-anxiolytic properties. Nitric oxide also plays a role in the performance mechanism of some antidepressant and anti-anxiolytic drugs (27). The results of the study showed that the treatment of mice with *Echium amoenum L.* extract decreased the serum and brain tissue NO levels as a result of decreased nervous damage and decreased anxiety and depression.

In this study, we showed that immobility time in FST and TST was reported as a characteristic behavior in SIS mice compared to non-stressed control mice, indicating a depressive or mood disorder. Treatment of mice with *Echium amoenum L.* reduced the immobilization time in FST and TST. Consistent with the present results, the anxiolytic and antidepressant effects of *Echium amoenum L.* flowers were demonstrated in previous studies (8, 30-32) and double-blind clinical trials (9).

5.1. Conclusion

In this study, we showed that *Echium amoenum L.* could play an essential role in reducing depression and anxiety in mice by modulating nitric oxide system and reducing oxidative stress. The strength of our work was that it is first to study the effect of *Echium amoenum L.* extract on social isolation. The weakness of our work was that the plant components were not isolated and studied separately.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

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Footnotes

Authors' Contribution: Nasrin Abdol conducted all the testes. Mahbubeh Setorki designed the test, analyzed the data, and prepared the article.

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

Ethical Approval: The study protocol was approved by the Ethics Committee of Izeh Islamic Azad University in 2019 (cod number: 1533052972002).

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