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Antidepressant and Anxiolytic Effect of *Echium amoenum* in Restraint Stress Model: The Role of Neuroinflammation in the Prefrontal Cortex and Hippocampus

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Abstract

Background: *Echium amoenum* (*E. amoenum*) is an Iranian medicinal plant with mood-enhancing effects.
Objectives: This study was designed to investigate the effect of standardized *E. amoenum* hydroalcoholic extract on restraint stress (RS)-evoked anxiety- and- depressive-like behaviors in mice.
Methods: This experimental study was conducted at the Tabriz University of Medical Sciences, Tabriz, Iran, in 2018. Doses of the hydroalcoholic extract of *E. amoenum* were optimized for rosmarinic acid (> %2 w/w) concentration of the extract. Other phytochemical indices, including total phenolic and flavonoid contents and radical scavenging activity, were also measured. For behavioral studies, 65 mice were randomly assigned into five groups (n = 13) of control, RS, RS + E75, RS + E150, and RS + E300. Animals in the RS group were subjected to the RS (3 h/day for 14 days) and treated with normal saline, while treatment groups received *E. amoenum* extract (75, 150, and 300 mg/kg, p.o.) concomitantly with RS exposure. Anxiety-like behaviors were assessed by Elevated Plus Maze (EPM) and Open Field Test (OFT). Depression was assessed by the forced swim test (FST) and Tail Suspension Test (TST). Western blotting was performed to determine the protein levels of IL-1β, NF-κB, TNF-α, and IL-6 in the prefrontal cortex (PFC) and hippocampus (HIP). The concentrations of corticosterone, alanine aminotransferase, aspartate aminotransferase, and alanine phosphatase were also measured in serum.
Results: Moderate and high doses of the extract ameliorated RS-induced anxiety- ($P < 0.05$ in OFT and EPM) and depressive-like ($P < 0.05$ and $P < 0.01$ in FST; $P < 0.01$ and $P < 0.001$ in TST) behaviors. These results were approved by decreased serum corticosterone levels ($P < 0.05$ and $P < 0.001$). Furthermore, *E. amoenum* reduced the protein expression of neuroinflammatory markers in the HIP and PFC subregions (significant at least at $P < 0.05$ for IL-1β, NF-κB, and TNF-α). Although RS slightly increased the serum levels of liver enzymes, no histopathological changes were seen in the liver of the RS or *E. amoenum*-treated groups.
Conclusions: *E. amoenum* can be an effective and safe complementary strategy for the treatment of stress-associated inflammation and behavioral changes.

Keywords: Anxiety, Depression, Hippocampus, Inflammation, *Echium amoenum*, Corticosterone, Prefrontal Cortex, Rosmarinic Acid, Stress

1. Background

Stressful life events can disrupt the body's homeostasis, resulting in a cascade of physiological, biochemical, and behavioral changes that ultimately provoke anxiety and depressive disorders. The detrimental effect of stress is strongly linked to the type and duration of the stressor. Although acute stress evolves adaptive mechanisms for survival, sustained exposure to stress can cause the hypothalamic-pituitary-adrenal (HPA) axis dysregulation, resulting in immoderate secretion of cortisol which, in turn, negatively impacts mood and behavior and increases vulnerability to mental disorders such as anxiety and de-

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pression (1).

As a cause of disability, depression is associated with a persistent feeling of despair and sadness, loss of interest and appetite, sleep disturbance, and lack of energy (2). Researchers have proposed stress-induced HPA axis dysfunction and high cortisol levels as the major causes of depressive mood (1). Chronic exposure to stress has profound impacts on the structure and function of the brain. The hippocampus (HIP) and prefrontal cortex (PFC), important brain structures for the control of mood and cognition, are the main areas of the brain most affected by stress, possibly due to abundant glucocorticoid receptors expression (3).

Moreover, stress can induce neuroinflammatory responses in the brain, which can affect neurotransmitters and neurocircuits, potentially leading to the development of depression (4). Accumulating evidence shows increased levels of pro-inflammatory markers in the circulation and cerebrospinal fluid (CSF) of depressive patients (5, 6). Moreover, preclinical findings have shown that the experience of psychological or physical stressors can elevate pro-inflammatory cytokines, such as nuclear factor-kappa B (NF-κB), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and IL-6, in the brain and periphery associated with the development of sickness behavior symptoms such as anhedonia and low appetite (7, 8).

Treating psychotic disorders has become difficult possibly due to their multi-factorial pathophysiology. Although effective treatments are available, many patients are dissatisfied with their conventional antidepressant therapies and may frequently experience relapse (9). On the other hand, many patients suffering from depression and anxiety disorders trust herbal medicines and prefer them to chemical drugs (10). However, the potential side effects and drug interactions must be considered (11).

Echium amoenum Fisch. and Mey (Boraginaceae) is a biennial Iranian medicinal plant with russet-red and funnel-shaped flowers, which is naturally grown in the North of Iran, as well as in Europe and Caucasus regions (12). In traditional medicine, its dried violet-blue flowers have been extensively used as a sedative and mood enhancer, as well as for treatment of anxiety, sore throat, cough, dyspnea, and pneumonia (13).

Accumulating in vitro and in vivo evidence has proven anti-inflammatory, antioxidant, antidiabetic, anxiolytic, antidepressant, and antibacterial properties of *Echium amoenum* (*E. amoenum*), which are mainly attributed to its bioactive phenolic compounds such as rosmarinic acid (RA), anthocyanidins, and flavonoids (14-17). Recently, Naseri et al. demonstrated that *E. amoenum* decreases gene expression of pro-inflammatory cytokines, namely cyclooxygenase-2 (COX2), TNF-α, IL-1β, and IL-6 in the macrophage cell line (18). Furthermore, preclinical and clinical studies have reported the effectiveness of this plant against depressive and anxiety disorders (19-25).

2. Objectives

In spite of the extensive use of *E. amoenum* as an anxiolytic and antidepressant agent, data are limited on the exact mechanisms underlying its therapeutic effects, as well as phytochemical properties and hepatotoxicity. The current study aimed to explore the effect of standardized hydroalcoholic extract of *E. amoenum* on anxiety and depressive-like behaviors in mice exposed to restraint stress.

3. Methods

This experimental study was conducted at Tabriz University of Medical Sciences, Tabriz, Iran, in 2018.

3.1. Plant Material and Preparation of Standardized Extract

Fresh flowers of *E. amoenum* were collected from East Azerbaijan, Iran. The species were identified and the voucher specimens were authenticated by the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran (No., Tbz-Fph 4033). The flowers were dried (22°C - 24°C), then powdered and carefully extracted with distilled water and methanol (30:70) via the maceration method under reduced pressure. A rotatory evaporator was used to evaporate the solvent under reduced pressure at 40°C. For chemical standardization of the extract, the lowest dose of the extract was optimized for 2% w/w of RA level. Other phytochemical parameters of the extract including total phenolic content (TPC), flavonoid content, and radical scavenging activity were also evaluated. Then, three doses of the extract were chosen based on the RA index in the extract and continued with an in vivo model using BALB/c mice.

3.2. In Vitro Study

3.2.1. Determination of RA Contents

The RA content was analyzed using a reversed-phase high-pressure liquid chromatography (HPLC) apparatus (Shimadzu-Japan) equipped with an analytical column, C18 Knauer column (250 mm L. × 4.6 mm I.D.). Then, a preparative column, Shim-pack PRC-ODS Shimadzu (250 mm L. × 50 mm I.D.), was applied for further analysis of the extract. The mobile phase consisting of acetonitrile (eluent A) and 5% trifluoroacetic acid in water (eluent B) was used for separation at a flow rate of 15 mL.min^{-1}. The gradient elution program was as follows: 0 - 15 min of a linear gradient of 0% - 10% acetonitrile (A) and 5% trifluoroacetic

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acid in water (B), 15 - 25 min of isocratic 10% (A), and 25 - 50 min of linear gradient of 10% - 55% (A). The detection wavelength was set at 280 nm. Working standard solutions of RA were prepared by diluting the stock standard solution in methanol and injected (0.5 mL) into the HPLC apparatus and peak areas of RA were identified by comparison with the retention times of samples using the corresponding standard curve (26).

3.2.2. Determination of Total Phenolic Content (TPC)

The Folin Ciocalteu (FC) assay was used for the assessment of TPC in the *E. amoenum* extract. In brief, 10 mg/mL of the extract solution was added to 1 mL of FC reagent and 5 mL of distilled water. After 1 min, 2 mL of 20% Na_2CO_3 was added to the mixture vortexed and incubated for two hours at 25°C. A UV-visible Shimadzu spectrophotometer was used for the measurement of absorbance of the solution at 765 nm. The standard curve was plotted with gallic acid, and TPC was represented as mg gallic acid equivalents (GAE) per g of the extract (27).

3.2.3. Assessment of Radical Scavenging Activity

Radical scavenging activity of the *E. amoenum* extract was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method for determining DPPH free radical scavenging ability. For this purpose, 3 mL of the sample solution was thoroughly mixed with 3 mL of DPPH solution in methanol and incubated at 37°C in the dark for 30 min, and the absorbance was measured at 517 nm. Quercetin was used as the positive control, and the ability of the extract to scavenge DPPH was expressed as RC50 value that was calculated using the following equation: Radical scavenging activity (%) = absorbance of the control (quercetin) - absorbance of the sample/absorbance of the control ×100 (28).

3.2.4. Determination of Total Flavonoids Contents (TFC)

The TFC of the *E. amoenum* extract was estimated using the aluminum chloride method. Briefly, 10 mg/mL of extract solution was mixed with 1.5 mL of solvent, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 0.35 mL of distilled water and maintained at room temperature for 30 min. The spectrophotometer was set at 415 nm to read the absorbance of the mixture. The TFC was determined using quercetin as a standard for a calibration curve and the results were represented as mg of quercetin equivalents (QE) per g of the extract (27).

3.3. In Vivo Study

3.3.1. Animals

Sixty-five adult male BALB/c mice, weighing 28 - 32 g, were purchased from the animal house of Tabriz University of Medical Sciences, Iran. Animals were housed five per cage and kept under a standard laboratory condition of 25 ± 1°C temperature, 50 ± 10% humidity, and 12/12 h light/dark cycle provided with food and water ad libitum. All experimental procedures were accomplished following the guidelines of the Tabriz University of Medical Sciences for care and use of laboratory animals and approved by the Ethics Committee of Tabriz University of Medical Sciences (Approval number: IR.TBZMED.VCR.REC.1397.357).

The sample size was calculated according to previous studies and using the following formula:

$$n = 1 + 2C\left(\frac{s}{d}\right)^2 \quad (1)$$

where s is the standard deviation, d is the difference to be detected, and C is a constant related to the values of α and β selected, in which α and β were considered 0.05 and 90%, respectively.

3.3.2. Study Groups Design

Following a week for adjustment to the new condition, mice were randomly (simple randomization) assigned into five groups (n = 13 in each group), as follows: control group receiving 0.9% saline (NS) gavage, restraint stress (RS) group receiving daily NS gavage, RS + E75 group receiving *E. amoenum* 75 mg/kg, RS + E150 group receiving *E. amoenum* 150 mg/kg, and RS + E300 group receiving *E. amoenum* 300 mg/kg. The plant standardized extract was dissolved in NS for gavage administration. Animals in the stress groups were subjected to RS for 3 h/day for 14 consecutive days, by placing the animals inside a well-ventilated 50 mL falcon tube (with 12 holes), while control animals were maintained in their home cages and they only received saline administration.

3.4. Behavioral Tests

Animals were subjected to the Open Field Test (OFT) and Elevated Plus Maze (EPM) for the assessment of anxiety-like behaviors, as well as to the forced swimming test (FST) and Tail Suspension Test (TST) for the evaluation of depressive-like behaviors. All behavioral tests were analyzed using a video tracking program (EthoVision™; Noldus, The Netherlands).

3.4.1. Open Field Test (OFT)

A square Plexiglass open field arena (33 × 33 × 33 cm) was used for the test. Mice were separately positioned in the center of the arena, and the activity of the animal was recorded for 10 min (29). Behavioral parameters, such as total distance travelled and the time spent in the central area were measured.

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3.4.2. Elevated Plus Maze (EPM)

The EPM apparatus consisted of two open arms (30 × 5 × 0.5 cm) and two enclosed arms (30 × 5 ×15 cm) elevated 50 cm from the floor. Mice were gently placed at the center part of the apparatus facing toward one of the closed arms and allowed to search the maze for 5 min. The factors, including the percentage of entries into open arms (%OAE) and the percentage of time spent in the open arms (%OAT) were calculated (30).

3.4.3. Forced Swimming Test (FST)

The FST device was a clear cylinder (14 cm in diameter, 20 cm in height) filled with tap water (25 ± 2°C). The individual mice were subjected to the swimming session for 6 min, and the total immobility time was digitally recorded in the last 4 min (31). The immobility was defined as remaining motionless in the water with no struggling and making just slight movements to hold the head above water.

3.4.4. Tail Suspension Test (TST)

The TST apparatus was a panel (60 × 30 × 40 cm) made from wood with a metal hook positioned in the middle top of the panel. To suspend the mouse, the tip of the tail (1 cm) was fixed 50 cm above the floor by an adhesive tape. The total immobility time during the last 4 min of the 6-min test session was recorded (32).

3.5. Sampling

One day following the last behavioral test, a mixture of ketamine and xylazine (80 and 8 mg/kg, respectively) was intraperitoneally injected to anesthetize the animals. After serum sampling, brain tissues were immediately removed from the skull, and PFC and HIP were carefully isolated on ice and stored at -70°C. Moreover, liver tissue samples were collected and stored in 10% formalin.

3.6. Western Blotting

The protein expressions of pro-inflammatory cytokines, including NF-κB, TNF-α, IL-1β, and IL-6, were evaluated in the PFC and HIP tissue samples based on the method previously described (29). Briefly, PFC and HIP samples were homogenized in RIPA lysis buffer containing a protease inhibitor cocktail. Then, the lysed tissue was centrifuged at 12000 × g for 15 min at 4°C and the supernatant was collected. Following the assessment of the total protein concentration in the supernatant by the Bradford method, SDS-polyacrylamide gel electrophoresis was used for protein separation. Then, the protein content was transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, UK) and incubated with 5% nonfat milk in Tris-buffered saline (TBST) pH 7.5 for 2 h with shaking at room temperature. Next, the membranes were probed with primary antibodies (all purchased from Santa Cruz, Biotechnology, USA) including NF-κB (sc-8008), anti-TNFα (sc-130349), IL-1β (sc-32294), IL-6 (sc-28343), and anti-GAPDH (sc-32233), as a loading control, in 1:500 concentrations, overnight. After three times of washing with PBS for 10 min, the membranes were incubated with horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG secondary antibody (1:10000, sc-2004) for 2 h at room temperature. Subsequently, the blots were washed with PBS and detected by enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL) and exposed to an X-ray film (Kodak, Rochester, NY, USA). Images of protein bands were obtained and quantified using Image J 1.62 software.

3.7. Serum Corticosterone and Liver Enzyme Activity Assessments

The serum concentration of corticosterone was assessed using an enzyme-linked immunosorbent assay (ELISA) method, based on the manufacturers' protocols (Elabscience, China). Furthermore, serum levels of liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were determined using an auto-analyzer (Technicon, RA1000, USA) (33) and commercial kits (Pars Azmoon, Tehran, Iran).

3.8. Histopathological Studies

In order to examine the liver's histopathological changes, liver tissues were fixed in 10% formalin solution and then dehydrated in graded alcohol and embedded in paraffin wax. The paraffin-embedded tissues were cut at 5 μm thicknesses using a microtome and mounted on slides, then stained with Hematoxylin and Eosin (H & E) according to standard protocols. Images were acquired by light microscopy (Nikon, Japan).

3.9. Statistical Analysis

The data were represented as mean ± standard error of the mean (SEM) in graphs and mean ± standard deviation in tables. The normality of data was checked by the Kolmogorov-Smirnov test. All data were analyzed using Graph Pad Prism 6.01 (Graph Pad Software Inc., La Jolla, CA, USA) using one-way ANOVA, followed by the Tukey post hoc test. A P value of < 0.05 was considered statistically significant.

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4. Results

4.1. RA Contents

HPLC analysis of the *E. amoenum* hydroalcoholic extract afforded RA in the retention time of 36 min consisting of 2.8% (w/w) of the dried extract (Figure 1).

4.2. TPC and TFC Contents and DPPH Radical Scavenging Activity

The results of the in vitro experiment showed that 100 g dried petals of *E. amoenum* yielded 26.63 g powdered extract. Moreover, TPC and TFC were found to be 274.35 $\pm$ 9.5 (mg GAE/g extract) and 23.94 $\pm$ 1.17 (mg QE/g extract), respectively. Additionally, the DPPH free radical scavenging activities of the extract were found to be RC50 = 56.18 μg/mL.

4.3. Effect of Extract on Anxiety- and Depressive-Like Behaviors

4.3.1. OFT

The results of the OFT demonstrated a significant difference in the center time ($F(4, 60) = 8.44$, $P < 0.001$; Figure 2A) between the groups. However, there was no significant difference in locomotor activity ($F(4, 60) = 1.24$, $P > 0.05$, Figure 2B) between the groups. Post hoc analysis revealed that RS markedly ($P < 0.001$) decreased the percentage of the time spent in the central zone. Moreover, animals in the E150 and E300 groups ($P < 0.05$ for both) spent prolonged time in the central zone of the arena compared to the RS-subjected animals (Table 1).

4.3.2. EPM Test

There were significant differences in %OAT ($F(4, 60) = 4.13$, $P < 0.01$) and %OAE ($F(4, 60) = 8.96$, $P < 0.001$) in the EPM test between different groups. As shown in Figure 2C and D, RS significantly decreased %OAT ($P < 0.05$, left panel) and %OAE ($P < 0.01$, right panel) in the EPM test, suggesting an anxiogenic effect. However, the administration of *E. amoenum* at doses 150 and 300 mg/kg significantly increased %OAT and %OAE in RS-subjected mice (Table 1).

4.3.3. FST and TST Behavioral Tests

The results of one-way ANOVA showed a significant difference between groups in immobility time in the FST ($F(4, 60) = 6.18$, $P < 0.001$) and TST ($F(4, 60) = 6.71$, $P < 0.001$). Post hoc comparisons revealed that RS increased immobility time in both FST ($P < 0.01$, left panel) and TST ($P < 0.05$, right panel) behavioral tests compared to the control group (Figure 3). Nevertheless, *E. amoenum* at doses 150 mg/kg ($P < 0.05$ in FST and $P < 0.01$ in TST) and 300 mg/kg ($P < 0.01$ in FST and $P < 0.001$ in TST) could significantly decrease immobility time in the FST and TST as compared to the RS-exposed mice (Table 1).

4.4. Effect of Extract on Serum Concentration of Corticosterone

Our results also showed that serum corticosterone levels were significantly different between the study groups ($F(4, 45) = 18.02$, $P < 0.001$). Multiple comparisons revealed higher serum corticosterone levels in RS-exposed mice (Figure 4, $P < 0.001$) than in the control group. Conversely, E150 ($P < 0.05$) and E300 ($P < 0.001$) groups displayed lower serum corticosterone levels than normal saline-treated mice (Table 1).

4.5. Effect of Extract on Inflammatory Mediator Proteins in the PFC and HIP

4.5.1. NF-κB

The results of Western blotting showed significant differences in the protein expression of NF-κB in the PFC ($F(4, 10) = 11.69$, $P < 0.001$) and HIP ($F(4, 10) = 44.44$, $P < 0.001$) regions between different groups. As shown in Figure 5A, NF-κB significantly increased in the PFC ($P < 0.01$) and HIP ($P < 0.001$) of RS animals. However, *E. amoenum* extracts significantly decreased the protein levels in the PFC ($P < 0.01$ for 75 and 150 mg/kg doses, $P < 0.001$ for 300 mg/kg) and in the HIP (for all doses $P < 0.001$) compared to the normal saline-treated group (Table 2).

4.5.2. TNF-α

The result of one-way ANOVA demonstrated a significant difference in the protein expression of TNF-α in the PFC ($F(4, 10) = 25.71$, $P < 0.001$) and HIP ($F(4, 10) = 4.98$, $P < 0.05$) between different groups. Post hoc analysis showed that exposure to RS markedly up-regulated TNF-α protein expression (Figure 5B) in the PFC ($P < 0.001$) and HIP ($P < 0.05$). Whereas, *E. amoenum* treatments significantly down-regulated TNF-α in the PFC ($P < 0.001$ for all doses) and HIP at doses of 150 and 300 mg/kg ($P < 0.05$) (Table 2).

4.5.3. IL-1β and IL-6

There were significant differences between the groups in the protein levels of IL-1β (Figure 5C) and IL-6 (Figure 5D) in the PFC [(IL-1β: $F(4, 10) = 10.28$, $P < 0.001$); (IL-6: $F(4, 10) = 2597$, $P < 0.001$)] and HIP [(IL-1β ($F(4, 10) = 12.96$, $P < 0.001$); (IL-6: $F(4, 10) = 2123$, $P < 0.001$)]. Multiple comparisons indicated that the protein expressions of IL-1β and IL-6 significantly increased in the PFC ($P < 0.05$ for IL-1β and $P < 0.001$for IL-6) and HIP ($P < 0.01$ for IL-1β and $P < 0.001$ for IL-6) of RS-subjected mice compared to the control group. However, E150 and E300 groups showed lower protein expressions of IL-1β in the PFC ($P < 0.01$) and HIP ($P < 0.05$ for dose 150 mg/kg and $P < 0.001$ for dose 300 mg/kg) than RS-exposed mice. Moreover, *E. amoenum* at dose 300 mg/kg significantly ($P < 0.01$) decreased protein levels of IL-6 in the PFC area (Table 2).

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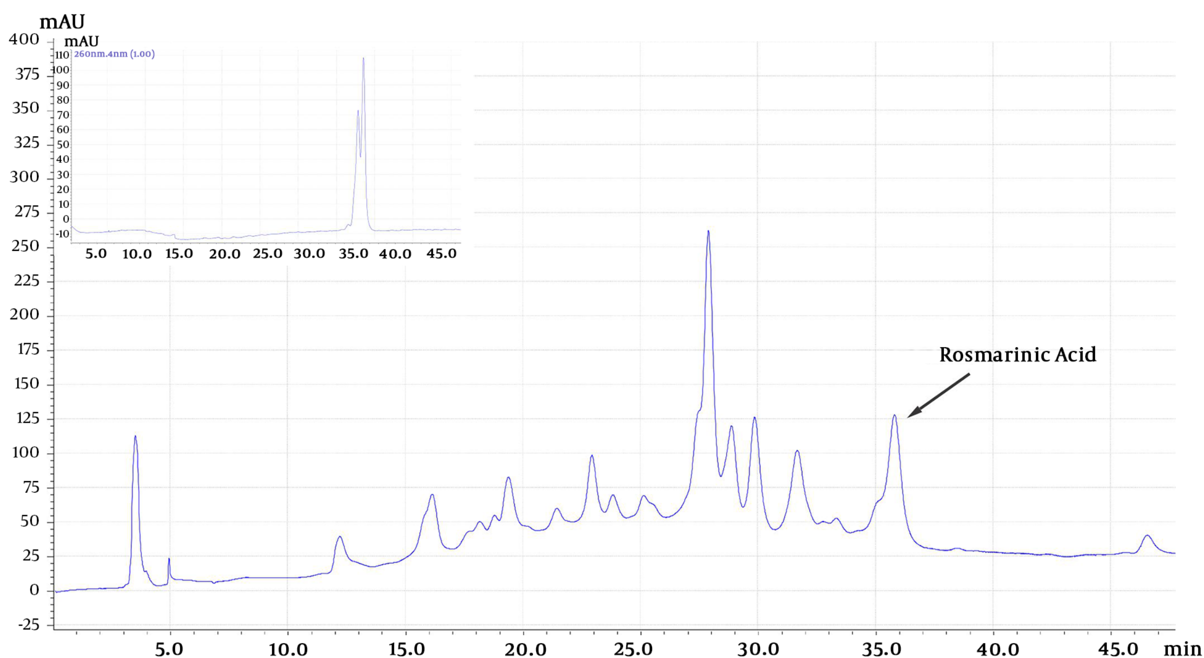

Figure 1. HPLC chromatograms of standard rosmarinic acid (left up) and *Echium amoenum* hydroalcoholic extract demonstrating rosmarinic acid at a retention time of 36 min depicted at a wavelength of 280 nm.

Table 1. Behavioral Tests and Serum Biochemical Parameters Results[a, b]

	Control	Normal Saline	P Value*	E75	P Value#	E150	P Value#	E300	P Value#
OFT, Time in arena center, %	17.38 ± 4.51	10.32 ± 3.79	< 0.001	11.07 ± 4.53	> 0.05	14.95 ± 3.58	< 0.05	15.72 ± 2.75	< 0.05
OFT, Locomotor activity, cm	1240 ± 334	1303 ± 258	> 0.05	1376 ± 357	> 0.05	1447 ± 280	> 0.05	1444 ± 295	> 0.05
EPM, Open arms time, %	40.37 ± 9.79	27.32 ± 9.33	< 0.05	34.63 ± 15.4	> 0.05	41.47 ± 14.45	< 0.05	42.35 ± 9.88	< 0.05
EPM, Open arms entries, %	54.23 ± 7.67	34.73 ± 9.68	< 0.01	46.41 ± 14.54	> 0.05	54.53 ± 12.75	< 0.01	60.42 ± 12.74	< 0.001
FST, Immobility time, s	91.24 ± 30.95	142.1 ± 32.39	< 0.01	127.6 ± 28.79	> 0.05	104.8 ± 34.52	< 0.05	97.98 ± 29.55	< 0.01
TST, Immobility time, s	111.6 ± 29.30	171.9 ± 54.03	< 0.05	138.8 ± 59.04	> 0.05	109.7 ± 31.67	< 0.01	95.38 ± 34.82	< 0.001
Corticosterone, ng/mL	137.8 ± 7.74	224 ± 44.06	< 0.001	210.1 ± 13.76	> 0.05	185.5 ± 20.20	< 0.05	160.7 ± 25.01	< 0.001
Alanine aminotransferase, U/L	50.65 ± 10.43	64.65 ± 10.39	< 0.001	68.02 ± 4.15	> 0.05	53.90 ± 8.75	> 0.05	69.61 ± 5.89	> 0.05
Aspartate aminotransferase, U/L	73.72 ± 8.35	88.38 ± 10.19	< 0.05	87.96 ± 7.01	> 0.05	76.97 ± 8.89	> 0.05	82.21 ± 7.53	> 0.05
Alkaline phosphatase, U/L	132.7 ± 17.31	154.1 ± 15.82	< 0.05	162.3 ± 22.09	> 0.05	139.7 ± 12.46	> 0.05	146.9 ± 10.96	> 0.05

Abbreviations: EPM, elevated plus maze; FST, forced swimming test; OFT, open field test; TST, tail suspension test.
[a] Values are expressed as mean ± SD.
[b] Data were analyzed using one-way ANOVA followed by the Tukey post hoc test. * vs. control group; # vs. normal saline-treated stress group.

4.6. Effect of Extract on Liver Injury Markers

In order to investigate the possible hepatotoxicity effect of different doses of *E. amoenum*, the serum levels of three major markers for hepatotoxicity, including ALT (Figure 6A), AST (Figure 6B), and ALP (Figure 6C) were measured in different groups. The results of enzymes assay showed a significant difference in the serum concentrations of ALT ($F(4, 45) = 5.35$, $P < 0.01$), AST ($F(4, 45) = 5.48$, $P < 0.01$), and ALP ($F(4, 45) = 5.55$, $P < 0.001$) between the groups. Post hoc analysis revealed that the RS significantly increased the serum levels of ALT ($P < 0.05$), AST ($P < 0.05$), and ALP ($P < 0.05$) compared to control animals. Although the serum levels of these liver enzymes in *E. amoenum* receiving groups slightly decreased, there were no significant differences between these groups and normal saline-treated mice (Table 1).

4.7. Histopathological Changes

Although the biochemical assays showed that the RS increased liver enzyme levels, the results of the histologi-

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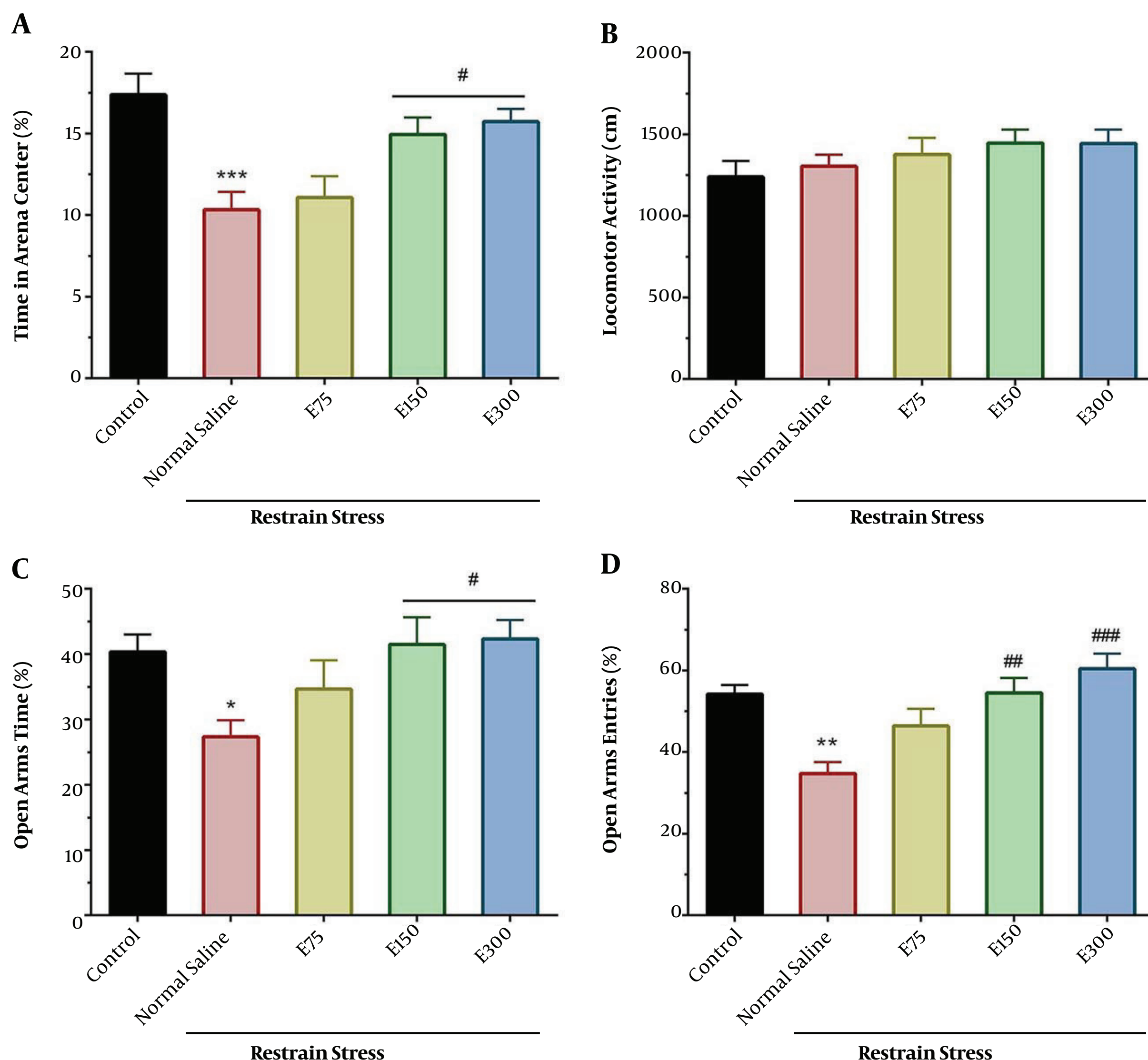

Figure 2. A) The time spent in the central zone and B) locomotor activity in the OFT. C) Effects of *E. amoenum* treatment on the percentage of open arms time (%OAT, left panel) and D) the percentage of open arms entries (%OAE, right panel) in the EPM; values are expressed as mean ± S.E.M (n = 13). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. normal saline (NS)-treated group. (OFT: open field test; EPM: elevated plus maze; E75: *Echium amoenum* 75 mg/kg; E150: *Echium amoenum* 150 mg/kg; E300: *Echium amoenum* 300 mg/kg).

cal examination showed no pathohistological changes in different groups. As Figure 7 shows, the histological structure of liver sections of all groups exhibited a normal lobular pattern with a clear cytoplasm without nuclear displacement.

5. Discussion

The main results of this research showed that *E. amoenum* treatment attenuated RS-evoked anxiety- and depressive-like behaviors. In particular, *E. amoenum* showed a protective effect against neuroinflammation induced by RS in the PFC and HIP regions.

RA is one of the major phenolic compounds of *E. amoenum* (34), which has been reported to have several properties including neuroprotection, anxiolytic, antioxidant, anti-inflammatory, and antidepressive-like effects (35-37). An early study showed that RA at 2% concentration had promising antidepressant effects in mice (35). Hence, in the present study, we optimized the lowest dose of the

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Table 2. Protein Levels of Neuroinflammatory Parameters in the Prefrontal Cortex and Hippocampus in the Western Blot Test[a, b, c]

	Control	Normal Saline	P Value*	E75	P Value#	E150	P Value#	E300	P Value#
NF-κB									
Prefrontal	1	2.67 ± 0.82	< 0.01	0.94 ± 0.21	< 0.01	0.99 ± 0.25	< 0.001	0.52 ± 0.31	< 0.001
Hippocampus	1	2.82 ± 0.38	< 0.001	1.16 ± 0.04	< 0.001	1.09 ± 0.19	< 0.001	0.56 ± 0.25	< 0.001
TNF-α									
Prefrontal	1	2.63 ± 0.62	< 0.001	0.94 ± 0.07	< 0.001	0.81 ± 0.14	< 0.001	0.41 ± 0.11	< 0.001
Hippocampus	1	2.18 ± 0.83	< 0.05	1.24 ± 0.38	> 0.05	0.95 ± 0.13	< 0.05	0.83 ± 0.18	< 0.05
IL-1β									
Prefrontal	1	1.77 ± 0.29	< 0.05	1.33 ± 0.19	> 0.05	1.06 ± 0.21	< 0.01	0.89 ± 0.10	< 0.01
Hippocampus	1	2.27 ± 0.31	< 0.01	1.83 ± 0.33	> 0.05	1.48 ± 0.36	< 0.05	0.83 ± 0.24	< 0.001
IL-6									
Prefrontal	1	2.75 ± 0.29	< 0.001	2.68 ± 0.32	> 0.05	2.13 ± 0.34	> 0.05	1.49 ± 0.15	< 0.01
Hippocampus	1	2.14 ± 0.18	< 0.001	2 ± 0.17	> 0.05	1.98 ± 0.21	> 0.05	1.70 ± 0.19	> 0.05

Abbreviations: IL-1β, interlukin-1 beta; IL-6, interlukin-6; NF-κB, nuclear factor-kappa B; TNF-α, tumor necrosis factor-alpha.
[a] The expression ratio of detected signals for each protein was calculated for the control group.
[b] Values are expressed as mean ± SD.
[c] Data were analyzed using one-way ANOVA followed by the Tukey post hoc test. * vs. control group; # vs. normal saline-treated stress group.

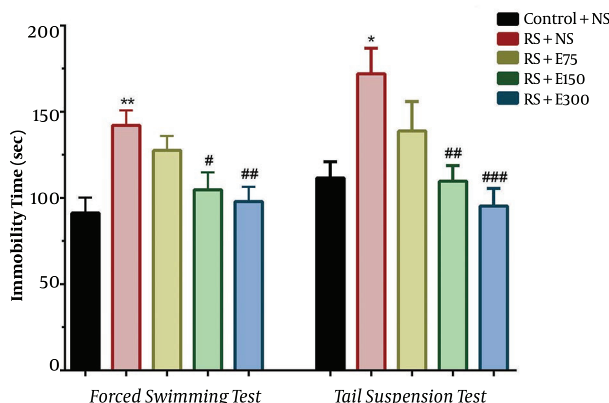

Figure 3. Immobility time in the FST (left) and TST (right) following restraint stress exposure. Values are expressed as mean ± S.E.M (n=13). *P < 0.05, *P < 0.01 vs. control group, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. normal saline (NS)-treated group. (FST: forced swimming test; TST: tail suspension test; RS: restraint stress; NS: normal saline; E75: *Echium amoenum* 75 mg/kg; E150: *Echium amoenum* 150 mg/kg; E300: *Echium amoenum* 300 mg/kg).

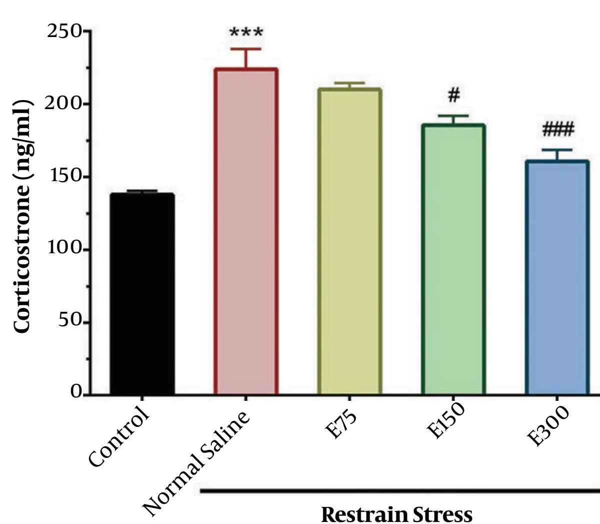

Figure 4. Effects of *E. amoenum* administration on serum corticosterone concentration in the study groups. Values are expressed as mean ± S.E.M (n = 10). ***P < 0.001 vs. control group. #P < 0.05, ###P < 0.001 vs. normal saline-treated mice. (E75: *Echium amoenum* 75 mg/kg; E150: *Echium amoenum* 150 mg/kg; E300: *Echium amoenum* 300 mg/kg).

extract for 2% w/w of RA content and increased higher doses exponentially.

Some studies have shown that RS as a psychophysical stressor induces anxiety and depression in rodents (38, 39). Similarly, the result of this study demonstrated that RS-subjected mice displayed the development of anxiety-like behavior. Moreover, exposure to RS increased depressive symptoms. However, *E. amoenum* treatments, particularly at doses 150 and 300 mg/kg, reversed these behavioral changes. Several animal studies have reported the anxiolytic effect of *E. amoenum* in acute and chronic administrations (19, 20, 40). Furthermore, Sayyah et al. showed that chronic administration (for six weeks) of aqueous extract of *E. amoenum* decreased obsessive and compulsive disorders in the human (41).

The antidepressant properties of *E. amoenum* have also been reported in preclinical and clinical studies. Sayyah et al. first reported the antidepressant effect of *E. amoenum* in a preliminary randomized, double-blind clinical trial (24). Sadeghi et al. recently showed that chronic administration of *E. amoenum* improved depression symptoms in rats, which was attributed to the reduction of oxidative stress and elevation of catecholamine levels in the HIP (42). A recent comparative human study reported that *E. amoenum*

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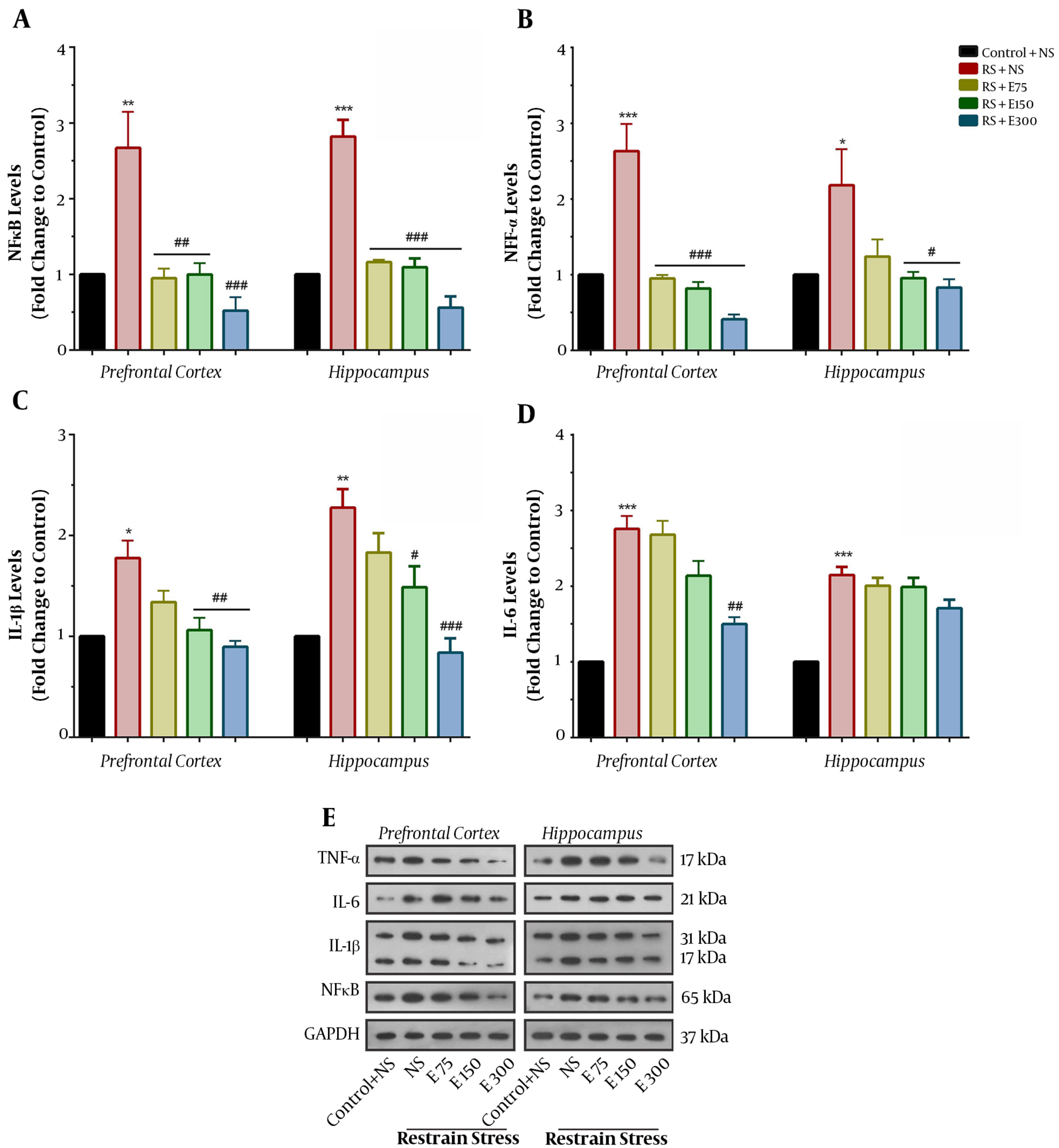

Figure 5. Effects of different doses of *E. amoenum* treatments on the protein levels of (A) NF-κB, (B) TNF-α, (C) IL-1β, and (D) IL-6 in the PFC and HIP subregions in the study groups. E) Representative images of the corresponding protein levels assessed by Western blot. Values are expressed as mean ± S.E.M (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control group. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. NS-treated group. (RS: restraint stress; NS; normal saline; E75: *Echium amoenum* 75 mg/kg; E150: *Echium amoenum* 150 mg/kg; E300: *Echium amoenum* 300 mg/kg; HIP: hippocampus; PFC: prefrontal cortex; IL-1β: interlukin-1 beta; IL-6: interlukin-6; TNF-α: tumor necrosis factor-alpha; NF-κB: nuclear factor-kappa B).

was more effective than citalopram in decreasing depressive symptoms associated with fewer complications than citalopram (23).

Despite extensive evidence reporting the anxiolytic and antidepressant effect of *E. amoenum*, limited data exist addressing the molecular mechanisms underlying these effects. Previous studies demonstrated that flavonoids exerted mild sedative and anxiolytic effects possibly due to

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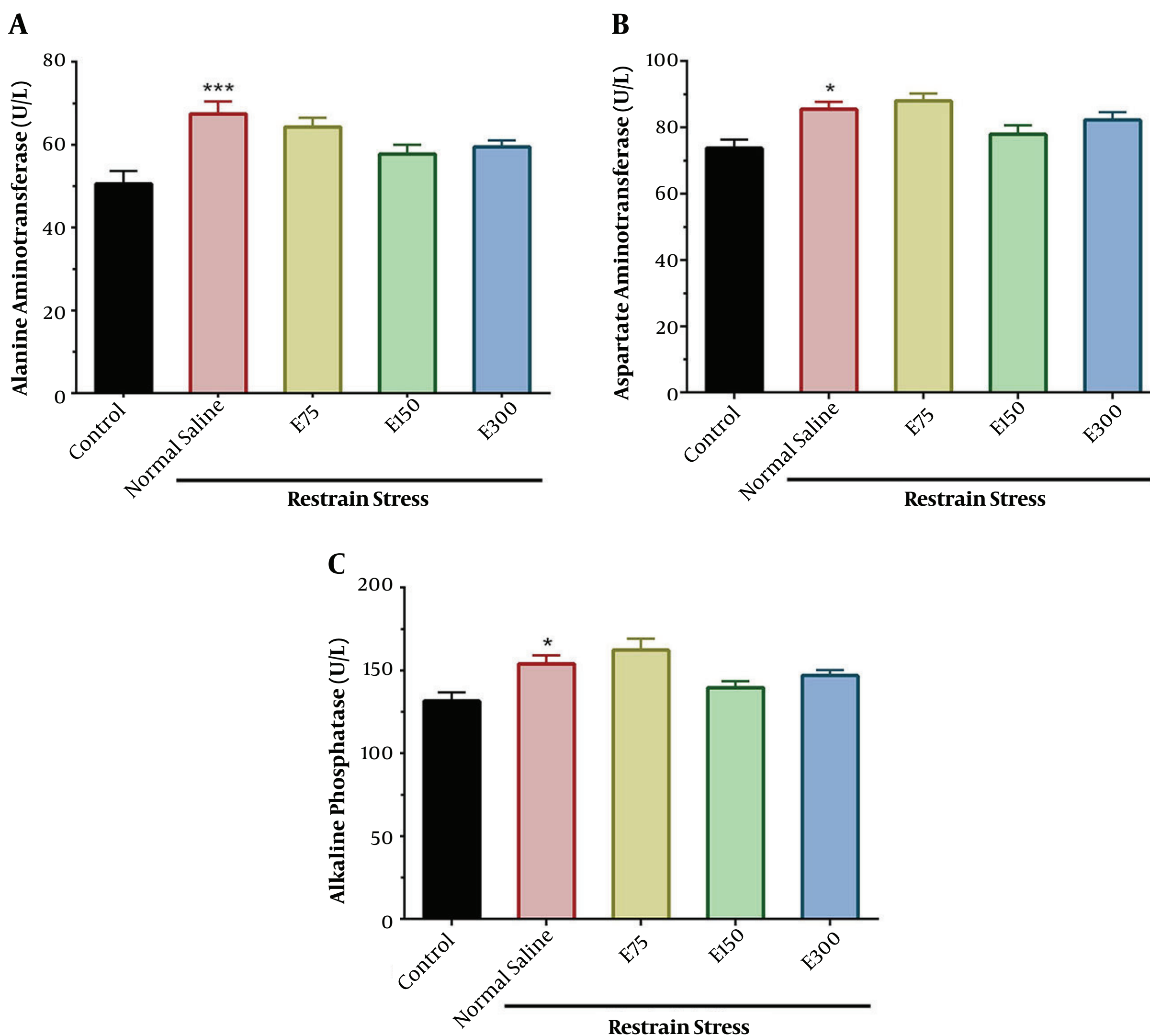

Figure 6. Effects of *E. amoenum* treatments on serum levels of (A) ALT, (B) AST, and (C) ALP in the study groups. Values are expressed as mean ± S.E.M (n = 10). *P < 0.05 vs. control group. (ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: Alkaline phosphatase; E75: *Echium amoenum* 75 mg/kg; E150: *Echium amoenum* 150 mg/kg; E300: *Echium amoenum* 300 mg/kg).

their affinity to bind the central benzodiazepine receptors (43). In this study, the result of the phytochemical analysis revealed that *E. amoenum* had high levels of flavonoid and RA. Therefore, the anxiolytic effect of *E. amoenum* can be presumably explained by the presence of flavonoids and RA constituent in the extract.

Regarding its antidepressant effects, Faryadian et al. demonstrated that the administration of *E. amoenum* increased serotonin and dopamine levels in the cerebrospinal fluid of reserpine-induced depression in rats (25). Moreover, Sadeghi et al. reported that *E. amoenum* improved depression-like behaviors by attenuating oxidative stress and apoptosis in the HIP (42). Additionally, oxidative damage is involved in brain structural and functional impairments, leading to psychiatric disorders, namely anxiety and depression (44, 45). On the other hand, agents with antioxidant properties and the ability to enhance antioxidant defense systems have been suggested as a new strategy for the prevention or treatment of these disorders (46, 47). Previous studies also showed antidepressive-like effects of RA in animal models of depression, which is mainly via the modulation of neurotrophic factor levels and syn-

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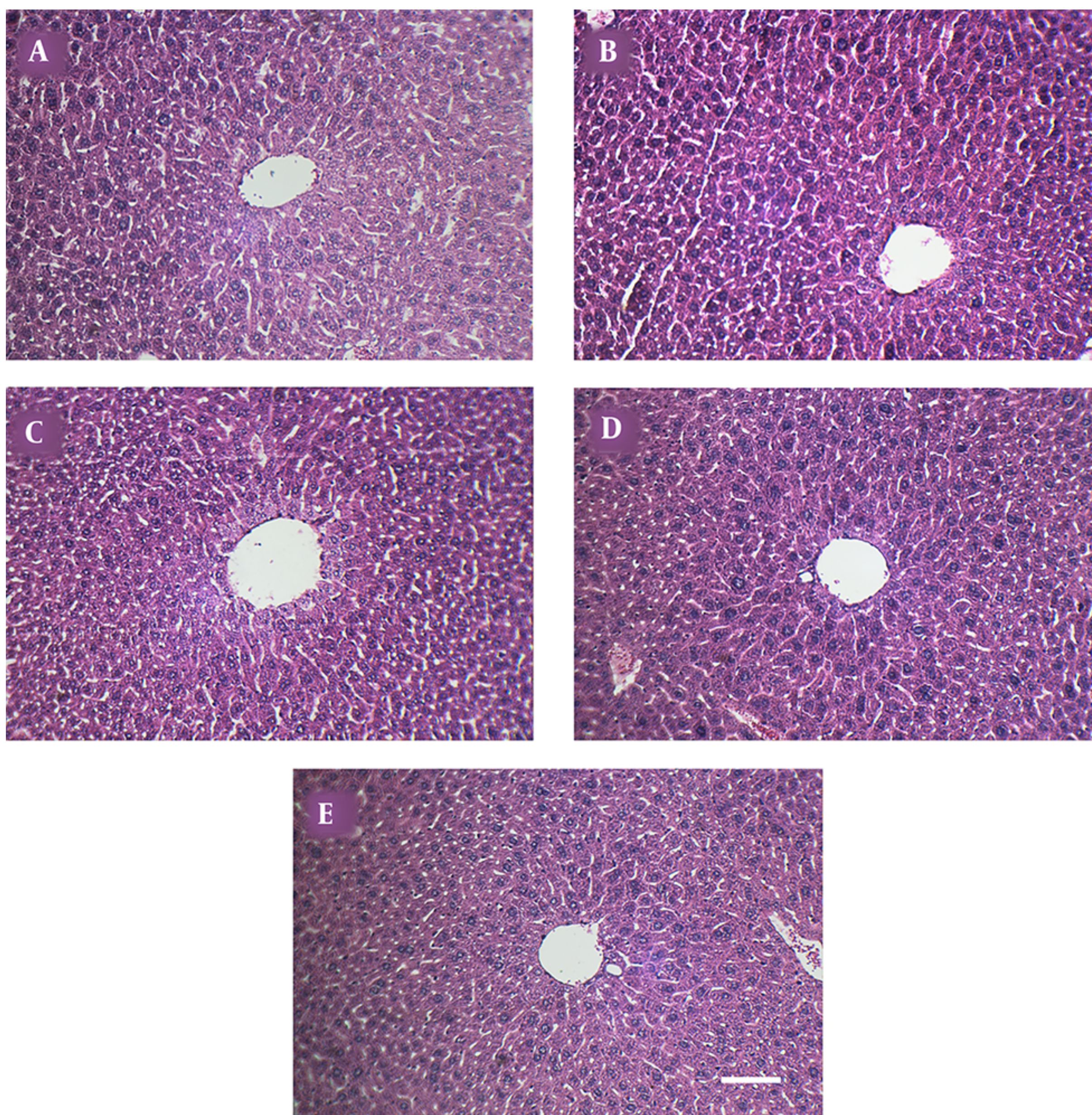

Figure 7. Effect of *E. amoenum* administrations on the histological structure of the liver tissue. Photomicrographs of Hematoxylin and Eosin (H & E)-stained liver sections in (A) control, (B) restraint stress, (C) E75, (D) E150, and (E) E300 (Scale bar: 50 μm). (E75: *Echium amoenum* 75 mg/kg; E150: *Echium amoenum* 150 mg/kg; E300: *Echium amoenum* 300 mg/kg).

thesis of dopamine and corticosterone (35, 36, 48). In this study, *E. amoenum* extract exhibited acceptable levels of RA, polyphenols, natural antioxidants, and DPPH radical scavenging activity, which can be proposed as possible mechanisms underlying the anxiolytic and antidepressant effects of *E. amoenum* extract.

Several experimental studies reported that RS induces the dysregulation of the HPA axis resulting in increasing serum corticosterone levels in rodents (49, 50). The aberration of the HPA axis may worsen brain damage, particularly in the PFC and HIP, due to the extent of expression of glucocorticoid receptors, contributing to the development of depressive symptoms (1, 51). In this study, in agreement with previous reports, we found that RS exposure el-

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evated corticosterone concentration (38, 52, 53), which was markedly declined by *E. amoenum* treatment at doses of 150 and 300 mg/kg. Of interest, these results were accompanied by a noticeable attenuation of anxiety and depressive-like behaviors. Previous studies reported that RA decreased serum corticosterone levels accompanied by attenuation of depressive-like behaviors in rodents (36, 48). Therefore, the attenuation of serum corticosterone levels by *E. amoenum* could be attributed to its RA content.

Neuroinflammation is involved in the onset of mental disorders and depressive mood (8, 54). Accumulating studies in individuals with depression and animal models showed that depression was associated with high levels of pro-inflammatory cytokines and low levels of anti-inflammatory cytokines in the blood and brain (39, 55). Since neuroinflammation plays a causative role in the pathophysiology of depression, targeting this pathway may hold promise for targeted therapy. Furthermore, several studies have proven that acute or chronic RS up-regulates pro-inflammatory cytokines associated with the development of depressive-like behaviors (56, 57). Similarly, we found that RS led to the up-regulation of NF-κB, TNF-α, and IL-6 protein expressions in the PFC and HIP subregions. Nevertheless, *E. amoenum* dose-dependently could decrease protein levels of these pro-inflammatory proteins, indicating an anti-inflammatory effect. A recent in vitro study showed that the methanolic extract of *E. amoenum* down-regulated the gene expression of cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS), TNF-α, IL-1β, and IL-6, as well as the secretion of IL-1β, IL-6, and nitric oxide (NO), in lipopolysaccharide-treated macrophage cells (18). Phytochemical studies confirmed the presence of several bioactive compounds in *E. amoenum* such as flavonoids, anthocyanidins, RA, and gamma-linolenic acid (14, 15), which their ability to inhibit inflammatory responses have been shown in several studies (58, 59). Therefore, we suggest that the anti-inflammatory effect of *E. amoenum*, in this study, stems from these components.

Besides the effectiveness of a given compound, safety is a fundamental feature. In this study, we also evaluated the hepatotoxicity of different doses of *E. amoenum*. The results revealed no changes in the serum levels of major liver markers for hepatotoxicity, namely AST, ALT, and ALP, accompanied by no histopathological changes in liver tissue sections. In agreement with our results, Mehrabani et al. showed that the chronic administration of *E. amoenum* had no hepatotoxicity effect on rats (60). Likewise, Zamansoltani et al. reported that *E. amoenum* could decrease the serum levels of ALT and ALP in rats (61). On the other hand, Zahedi et al. reported that the administration of *E. amoenum* increases ALT and AST levels (62). Although the toxicity of *E. amoenum* was attributed to the presence of pyrrolizidine alkaloids (63, 64), the total alkaloid content of *E. amoenum* was 0.01% (63).

We had limitations in the evaluation of phytochemical parameters. Also, our study assessed the effects of *E. amoenum* on limited neuroinflammatory and hepatic parameters. Nevertheless, we could not assess the impact of treatment on other downstream pathways. However, to the best of our knowledge, this study has a comprehensive view in both of neuromodulatory and hepatotoxic effects of *E. amoenum* in mice.

Overall, the results showed the supportive effects of standardized *E. amoenum* extract against RS-induced anxiety and depression in mice through the inhibition of neuroinflammatory responses in the PFC and HIP. The results suggest that chemical constituents of *E. amoenum* are responsible for its pharmacological activities. It can be concluded that *E. amoenum* may have beneficial effects in the treatment of stress-induced psychiatric disorders.

Footnotes

Authors' Contribution: Mohammad Nouri, Fereshteh Farajdokht, Mohammadali Torbati, Fatemeh Ranjbar Kuchaksaray, Sanaz Hamedyazdan, Saeed Sadigh-Eteghad, and Mostafa Araj-Khodaei performed the experiments, interpreted the results, and wrote the first draft of the manuscript. Saeed Sadigh-Eteghad and Mostafa Araj-Khodaei designed the experiments. SS-E critically interpreted data and critically revised and approved the manuscript.

Conflict of Interests: The authors declared no conflict of interest.

Ethical Approval: All experimental procedures were accomplished following the guidelines of the Tabriz University of Medical Sciences for care and use of laboratory animals and approved by the Ethics Committee of the Tabriz University of Medical Sciences (Approval number: IR.TBZMED.VCR.REC.1397.357).

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