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Antidepressant and Anxiolytic Effects of Geraniol in Mice: The Possible Role of Oxidative Stress and Apoptosis

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Abstract

Background: Depression is a severe mental disorder. Current antidepressants are effective in only one-half to one-third of the patients. Besides, these medications might bring about adverse effects. Therefore, the need for newer anti-depressant medications or complementary compounds is utterly felt.

Objectives: We tested the hypothesis that geraniol (GE) attenuates anxiety and depression via the amelioration of oxidative stress and apoptosis in mice.

Methods: In an experimental study, thirty-six BALB/c mice were randomly divided into three control, chronic restraint stress (CRS), and GE groups. CRS and GE groups underwent CRS for two weeks. Accordingly, the CRS group received normal saline (2 mL/kg, i.p.) whereas the GE group received GE (50 mg/kg, i.p.). The behavioral outcomes were assessed using the open-field test (OFT), elevated plus maze (EPM), and tail suspension test (TST). Moreover, superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-px) activity, total antioxidant capacity (TAC), and reactive oxidative species (ROS) levels in the brain were assessed using the spectrophotometric method. The brain's BAX, Bcl-2, and caspase-3 levels were measured using Western blotting.

Results: CRS increased anxiety in stressed mice compared to the control group as indicated by OFT and EPM ($P < 0.01$ for both comparisons). Furthermore, CRS increased the immobility time in TST compared to control animals ($P < 0.001$). Biochemically, CRS decreased SOD activity ($P < 0.01$), GSH-px activity ($P < 0.01$), TAC level ($P < 0.001$), and ROS level ($P < 0.001$). It also increased the BAX/Bcl-2 ratio ($P < 0.001$) and caspase-3 level ($P < 0.001$) compared to the control group. GE reversed all the behavioral and biochemical changes in stressed mice compared to the CRS group.

Conclusions: GE renders potent anxiolytic and antidepressant effects possibly through the modulation of oxidative stress and apoptosis in the mouse brain.

Keywords: Geraniol, Depression, Anxiety, Oxidative Stress, Apoptosis

1. Background

Depression is a severe and occasionally fatal mental disorder that occurs in 4.4% to 20% of the general population. It can also occur at any time peaking in older adulthood, and it is more prevalent in women than in men. According to the world health organization report, depression will be the second most burdensome disease in terms of treatment and care costs by 2020 (1). It has been shown that chronic stress plays an important role in the initiation and progression of depression (2). Evidence suggests that current antidepressants are effective in only one-half to one-third of patients. Besides, these medications might bring about adverse side-effects that limit their use (3). Therefore, the need for newer anti-depressant medications or complementary compounds is utterly felt.

On the one hand, studies have shown that oxidative stress plays an important role in the pathogenesis of depression. This might result from the sensitivity of the central nervous system to free radicals (4). On the other hand, the role of enzymes such as glutathione peroxidase (GSH-px) and superoxide dismutase (SOD) is undeniable in defense against oxidative stress (5). Accumulating data also show that neuronal apoptosis plays a key role in depression via decreasing the regional brain volumes and reducing neuronal and glial cell body numbers (6). Apoptosis is also linked to the onset of depression. This might be accompanied by decreased Bcl-2 and increased caspase-3 levels (7).

Geraniol (GE), as a monoterpene, is found in essential oils of various herbs such as ginger and coriander (8). Recent evidence suggests the antioxidant and anti-apoptotic properties of GE (9, 10). Neuroprotective effects of GE have also been proven in various studies (8, 11), simultaneously rendering minimal liver toxicity (10). GE increases the activities of SOD and neuronal GSH-px and escalates the glutathione content. It also decreases lipid peroxidation and reactive oxygen species (ROS) (11, 12).

2. Objectives

We investigated the effect of chronic administration of GE on anxiety and depressive-like behaviors in the chronic restraint stress (CRS) model of depression in mice. The downstream pathways, i.e., oxidative stress and apoptosis, were also evaluated.

3. Methods

3.1. Animals

A group of 36 male BALB/c mice, approximately eight weeks of age weighing 25 - 30 g, was purchased from the laboratory animal care center of the Tabriz University of Medical Sciences (TUOMS). Mice were socially kept in standard polypropylene cages (five in each cage) under the controlled conditions of relative humidity (55 $\pm$ 5%) and ambient temperature (25 $\pm$ 2°C) on a standard light/dark cycle (lights on from 07:00 to 19:00) before and over the study period along with access to tap water and standard pellet food ad libitum. The animals were adapted for one week before the initiation of the experiments. All efforts were made to minimize the suffering of animals and the number of animals used. The experimental procedures performed in this study were in full conformity with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH; Publication No. 85-23, revised 1985), and were confirmed by the Ethics Committee of Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1397.393).

3.2. Chemicals and Kits

GE (trans-3,7-Dimethyl-2,6-octadien-1-ol) was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, http://www. sigmaaldrich.com). The Western blotting detection reagent was purchased from Amersham (United Kingdom) and the resulting signal was visualized using Kodak autoradiography film (Kodak, Rochester, NY, USA). Polyvinylidene difluoride was obtained from Roche (PVDF; Roche, United Kingdom). The RANSOD Laboratory Kit was purchased from Randox (Randox Laboratories Ltd, Crumlin) for the assessment of TAC and SOD. The RANSEL laboratory kit was obtained from Randox (Randox Laboratories Ltd, Crumlin) for the evaluation of GSH-px. All equipment used in this study was calibrated according to the standard protocols. The reliability and validity were the two features used to assess any measurement instrument applied in this study.

3.3. Study Design and Experimental Procedures

This experimental study was performed at the Neurosciences Research Center (NSRC), TUOMS, Tabriz, Iran, during the summer and fall of 2018. Animals were randomly divided into three groups (n = 12 each) using simple randomization. The first group (control group) did not receive any injection or treatment, the second group (CRS group) was subjected to chronic restraint stress (CRS) for two weeks and treated with normal saline, and the third group (CRS + GE group) was subjected to chronic stress and treated with GE at 50 mg/kg via the intraperitoneal (i.p.) route for two weeks (13). Behavioral tests were performed at the end of the treatment period. All behavioral tests were conducted by a single experimenter.

3.4. Chronic Restraint Stress Protocol

The animals were placed in a cylindrical tube (3 cm in diameter and 10 cm in length) and remained in the tube where they could not move forward or backward. One end of the tube possessed holes to facilitate animals' ventilation. Each animal was kept in a single tube for six hours a day, and the process was repeated for two weeks (14, 15).

3.5. Behavioral Tests

3.5.1. Open Field Test

EthoVisionTM video tracking system was used for the measurement and analysis of all behavioral tests. Open field test (OFT) was used to measure animals' locomotor activity and anxiety level. The open-field area (60 $\times$ 60 $\times$ 40 cm) was divided into two peripheral and central areas. Locomotor activity was considered as the total distance moved while locomotion in the peripheral arena indicated the anxiety level.

3.5.2. Elevated Plus Maze

Elevated plus maze (EPM) was used to measure the animals' anxiety level. The mice were placed within the center of the maze so that they were facing an open arm. The

light was provided by a lamp located above the maze center. Within five minutes, the animal freely moved in different parts of the maze and the time (in seconds) spent in the open arms (%OAT) and open arm entries (%OAE) and the distance moved in the arena center (cm) were recorded. The device was entirely cleansed with ethanol 70% to remove olfactory cues after each trial (16).

3.5.3. Tail Suspension Test

Tail suspension test (TST) was used to evaluate animals depressive-like behavior. For this purpose, the tip of the animals tail was fastened to a metal bar using an adhesive tape. The metal bar was attached to a box with black walls to remove any visual distractions for the mice. The test began with a sharp movement of the mouse. When the animal was completely immobilized (defined as the lack of limb movement), the time was recorded over a six-minute period by a blind observer to the nature of the study and considered as the immobility time (in seconds) (14).

3.6. Biochemical Assays

3.6.1. Sampling

The animals were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) after the last behavioral test. Mice were then decapitated and their brains were excised, frozen at -70°C, and maintained for further use.

3.6.2. Total Reactive Oxygen Species Levels

Fluorescent vital dye dichloro-dihydro-fluorescein diacetate (DCFDA) was applied to measure the total ROS level. The fluorescence intensity caused by dichloro-dihydro-fluorescein (DCF) production from DCFDA was determined. The ROS level was expressed as fluorescence intensity and normalized to the sample proteins (16).

3.6.3. Superoxide Dismutase Activity

The SOD activity was measured using the RANSOD Laboratory Kit based on the method applied in Pourmemar et al. study (17). The brain tissue was homogenized to prepare tissue supernatant and required solutions. The absorbance of the prepared solution was measured by a spectrophotometer at a wavelength of 505 nm.

3.6.4. Glutathione Peroxidase Activity

The GSH-px activity was measured using the RANSEL Laboratory Kit based on the method applied by Pourmemar et al. (17). Glutathione reductase ($\geq$ 0.5 units/L) and NADPH (0.28 mmol/L) solutions were prepared. The decrease in absorbance was measured using a spectrophotometer at a wavelength of 340 nm (37°C) and then the concentration of GSH-px was calculated.

3.6.5. Total Antioxidant Capacity

TAC was calculated using the Randox total antioxidant status kit based on the method applied in Pourmemar et al. study (17). In this method, ABTS+ was decolorized by antioxidant substances, depending on their concentration and antioxidant capacity. The change in sample color was measured using a spectrophotometer at the 600-nm wavelength.

3.6.6. Apoptosis Markers

Western blotting was conducted via the previously explained method (16). Briefly, the radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors was homogenized with brain tissues. Bradford's method was used to determine the protein concentrations of the samples (18). The primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) including anti-caspase-3 (1:500, sc-7148), anti-Bcl-2 (1:500, sc-492), and anti-Bax (1:500, sc-493) antibodies and then the horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (1:5000, sc-2004) were used to incubate the membranes. Signals emitted from detection reagent was visualized via the Kodak autoradiography film, quantified using ImageJ 1.62 software (National Institutes of Health, Bethesda, MD, USA), and normalized to the corresponding internal control. The internal control of the procedure was achieved using anti-β-actin (1:500, sc-130656) antibody.

3.7. Statistical Analysis

The IBM SPSS Statistics Software for Windows, version 22.0 (IBM Corp., Armonk, N.Y., USA) was used to perform all statistical analyses. The data were expressed as means $\pm$ standard deviation (SD), frequencies, and percentages. The normal distribution of the data was evaluated using the Kolmogorov-Smirnov test. Comparison of different groups was made using the one-way ANOVA followed by a post hoc Tukey test. The sample size was calculated according to a previous study (13) and using the following formula:

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

The parameters of α and power were considered 0.05 and 90%, respectively. This yielded a total of 14 animals for each group (C = 10.51, SD = 20, and S = 25). However, due to the ethical issues, only were 12 animals allocated to each group. The P values of < 0.05 were considered statistically significant.

4. Results

Mean ± SD of each response variable according to each independent variable, its respective statistical tests, and p values are presented in Table 1.

4.1. Behavioral Tests

4.1.1. Open Filed Test

We found no significant difference between the groups in locomotor activity in OFT ($P > 0.05$) (Figure 1A). Nevertheless, the distance moved in the arena center was significantly lower in the CRS group than in controls ($P < 0.01$). Treatment with GE significantly increased the distance moved in the arena center compared to the CRS group ($P < 0.001$) (Figure 1B).

4.1.2. Elevated Plus Maze

%OAE and %OAT were significantly lower in the CRS group than in the control group ($P < 0.01$ for both comparisons). Treatment with GE, however, significantly increased %OAE and %OAT compared to the CRS group ($P < 0.001$ for both comparisons) (Figure 2A and B).

4.1.3. Tail Suspension Test

CRS significantly increased the immobility time in the CRS group compared to the control group ($P < 0.001$). However, chronic GE treatment remarkably decreased the immobility time compared to the CRS group ($P < 0.001$) (Figure 3).

4.2. Oxidative Stress Indices

4.2.1. ROS generation

CRS increased the ROS content in depressed mice compared to the control group ($P < 0.001$). Accordingly, GE significantly reduced the ROS generation in CRS mice compared to the model group ($P < 0.001$) (Figure 4A).

4.2.2. Superoxide Dismutase Activity

The SOD activity was meaningfully lower in the CRS group than in the control group ($P < 0.01$). However, treatment with GE significantly increased the SOD activity compared to the CRS group ($P < 0.001$) (Figure 4B).

4.2.3. Glutathione Peroxidase Activity

CRS significantly reduced the GSH-px activity compared to the control group ($P < 0.01$). On the other hand, chronic GE treatment increased the GSH-px activity compared to the CRS group ($P < 0.05$) (Figure 4C).

4.2.4. Total Antioxidant Capacity

The CRS group had significantly lower TAC than the control group ($P < 0.001$). On the other hand, the chronic administration of GE increased TAC compared to the CRS group ($P < 0.001$) (Figure 4D).

4.3. Apoptosis Parameters

4.3.1. Bax/Bcl-2 Ratio

CRS significantly increased the Bax/Bcl-2 ratio compared to the control group ($P < 0.001$). The GE treatment, however, decreased the ratio compared to the CRS group ($P < 0.001$) (Figure 5A and C).

4.3.2. Caspase-3 Level

CRS significantly increased the caspase-3 level compared to the control group ($P < 0.001$); however, chronic GE treatment reduced the caspase-3 level compared to the CRS group ($P < 0.001$) (Figure 5B and C)

5. Discussion

We showed that chronic GE treatment alleviated anxiety and depressive-like behaviors through the amelioration of oxidative stress and apoptosis in CRS mice. In this study, CRS was used to mimic depression and produce depressive-like behavior in mice. This model has been used in several studies to evaluate the impacts of chronic stress on behavior and the brain structure of rodents (14, 19). We also used OFT and EPM tasks to test the animals' anxiety level. EPM is a standard task to test anxiety in rodents (19). Moreover, OFT is a quite popular test to measure anxiety-like behavior in animals (20).

We found that CRS decreased the distance moved in the arena center, %OAT, and %OAE in OFT and EPM, respectively. This indicated that CRS increased the animals' anxiety level without changing the animals' locomotor activity. In line with this finding, Chiba et al. found that CRS decreased the distance traveled in the arena center and the number of rearings in mice. This study also showed that CRS decreased OAE in the EPM task, indicating the increased anxiety level in the animals (21).

We used TST to measure depressive-like behavior in mice and found that CRS increased the immobility time compared to the control group. Similarly, Christiansen et al. showed that CRS increased the immobility time by 38% in TST, suggesting the state of depression in stressed mice (14). GE, on the other hand, was found to alleviate both anxiety and depressive-like behaviors. In a direct relevance to this finding, Deng et al. found that chronic GE treatment

Table 1. Mean ± SD of Each Response Variable According to Each Independent Variable, Their Respective Statistical Tests, and P Value[a]

Independent Variable	Behavioral or Biochemical Test	Dependent Variable	Mean ± SD		P Value[b]
Study groups	OFT	Locomotor activity, cm	Control	1425 ± 233.3	P > 0.05 for both comparisons
			CRS + NS	1296 ± 157.2	
			CRS + GE	1487 ± 134.4	
		Distance moved in arena center, cm	Control	29.64 ± 6.37	P < 0.01 and P < 0.001
			CRS + NS	18.91 ± 5.92	
			CRS + GE	36.07 ± 8.00	
	EPM	Time spent in open arms, %	Control	34.79 ± 5.57	P < 0.01 and P < 0.001
			CRS + NS	23.10 ± 6.39	
			CRS + GE	37.90 ± 9.28	
		Open arm entries, %	Control	39.17 ± 6.67	P < 0.01 and P < 0.001
			CRS + NS	32.83 ± 3.41	
			CRS + GE	40.80 ± 6.05	
	TST	Immobility time, s	Control	55.32 ± 28.21	P < 0.001 for both comparisons
			CRS + NS	176.3 ± 45.59	
			CRS + GE	94.06 ± 33.53	
	Oxidative stress markers	ROS level, DCFDA intensity	Control	5.09 ± 1.22	P < 0.001 for both comparisons
			CRS + NS	13.22 ± 2.51	
			CRS + GE	7.9 ± 2.05	
		Superoxide dismutase activity, U/mg protein	Control	0.57 ± 0.07	P < 0.01 and P < 0.001
			CRS + NS	0.43 ± 0.05	
			CRS + GE	0.68 ± 0.09	
		Glutathione peroxidase activity, U/mg protein	Control	0.88 ± 0.11	P < 0.01 and P < 0.05
			CRS + NS	0.71 ± 0.11	
			CRS + GE	0.84 ± 0.08	
		Total antioxidant capacity, Mmol/L	Control	063 ± 0.12	P < 0.001 for both comparisons
			CRS + NS	0.36 ± 0.07	
			CRS + GE	0.60 ± 0.05	
	Apoptosis markers	Bax/Bcl-2 ratio, fold of change	Control	1.00 ± 0.00	P < 0.001 for both comparisons
			CRS + NS	2.13 ± 0.19	
			CRS + GE	1.06 ± 0.09	
		Caspase-3 level, fold of change	Control	1.00 ± 0.00	P < 0.001 for both comparisons
			CRS + NS	1.70 ± 0.13	
			CRS + GE	1.08 ± 0.08	

Abbreviations: CRS, chronic restraint stress; DCFDA, 2',7' -dichlorofluorescin diacetate; EPM, elevated plus-maze; GE, geraniol; OFT, open field test; TST, tail suspension test.
[a] Statistical test: One-way ANOVA test followed by a post-hoc Tukey test.
[b] The first and second p values are for CRS vs. control and CRS+GE vs. CRS group comparisons, respectively.

for three weeks decreased the immobility time both in TST and in forced swimming test in a chronic unpredictable mild stress mice model (13). However, to the best of our knowledge, the anxiolytic effects of GE have not been assessed until now.

It has been found that the markers of oxidative stress and lipid peroxidation, including malondialdehyde and 8-hydroxydeoxyguanosine may increase during the depres-

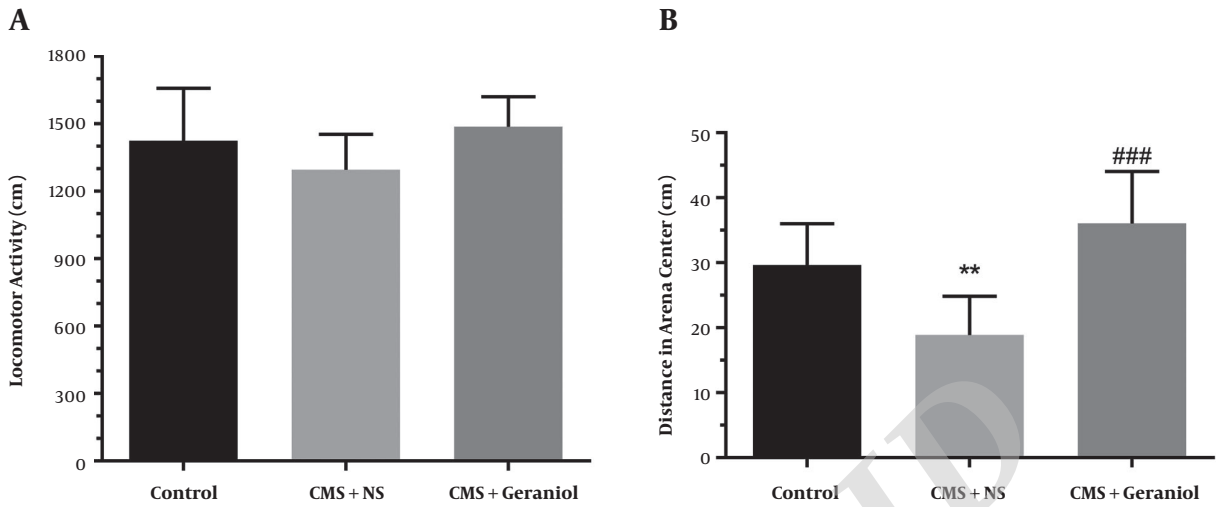

Figure 1. Anxiety-like behavior in the study groups in OFT. A, locomotor activity and B, distance moved in the center of the arena (cm). Each bar represents the mean ± SD, (n = 12). Significant differences were tested by the one-way ANOVA followed by Tukey's post hoc test; **P < 0.01 compared to the control group. ###P < 0.001 compared to the CRS group.

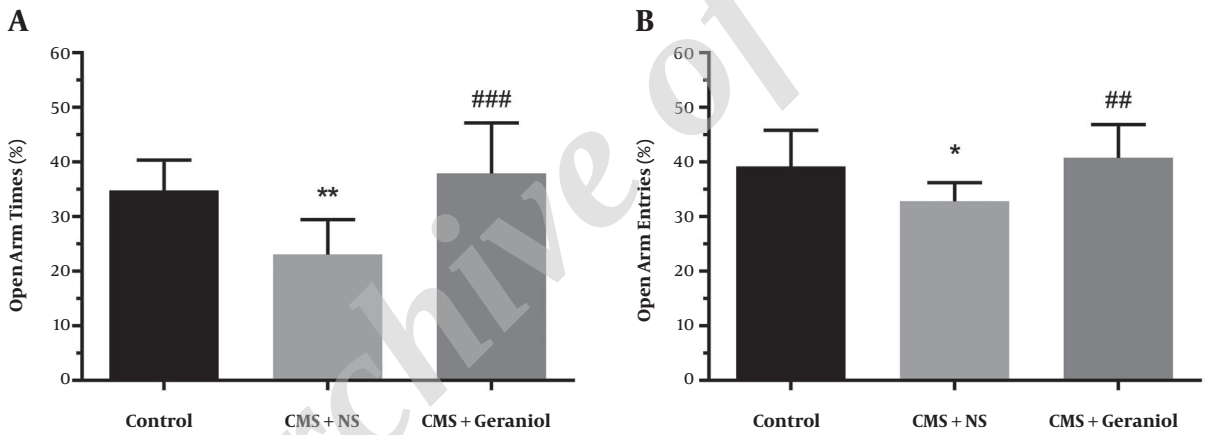

Figure 2. Anxiety-like behavior in the study groups in EPM. A, percentage of the time spent in the open arms of EPM, and B, percentage of entries to the open arms of EPM. Each bar represents the mean ± SD, (n = 12). Significant differences tested by the one-way ANOVA followed by Tukey's post hoc test; *P < 0.05 and **P < 0.01 compared to the control group. ##P < 0.01 and ###P < 0.001 compared to the CRS group.

sion. Moreover, the decreased activity of SOD, GSH-px, catalase, and antioxidant capacity of the brain have been reported in depressed patients (22, 23). Higher levels of oxidative stress can disturb mitochondrial function, which, in turn, deteriorates oxidative stress and neuronal damage during the depression (24).

We found that CRS increased oxidative stress by increasing the ROS level and decreasing the activity of antioxidant enzymes such as SOD and GSH-px and thus, TAC. On the other hand, GE reversed the mentioned changes. Similarly, Che et al. demonstrated that chronic stress impaired the antioxidant defense system, escalated protein and lipidic peroxidation, increased catalase activity, and decreased SOD activity in the rat brain (25). Recently, the antioxidant activity of GE has been demonstrated in various experimental models (11, 26). Wang et al. showed that chronic treatment with GE reversed oxidative changes by a decrease in the MLA level and an increase in the SOD and GSH-px activities via the regulation of NF-κB and p38 MAPK pathways in the traumatic injury of the spinal cord (SCI). This indicates the potent antioxidant activity of GE in the nervous system (11).

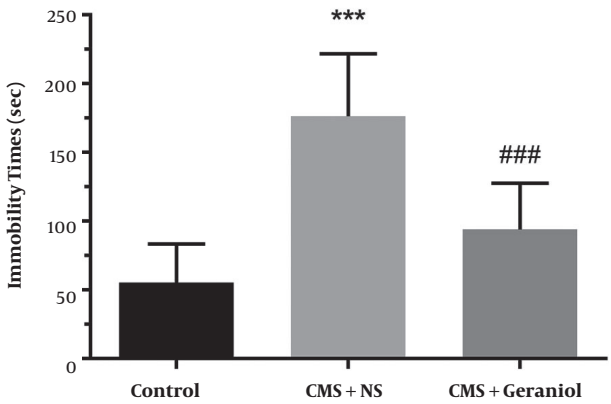

Figure 3. Depressive-like behavior as manifested by immobility time (seconds) in the study groups in TST. Each bar represents the mean ± SD, (n = 12). Significant differences were tested by the one-way ANOVA followed by Tukey's post hoc test; ***P < 0.001 compared to the control group. ###P < 0.001 compared to the CRS group.

Evidence also suggests that chronic stress, as a precipitator of depression, increases apoptosis and neuronal death, and on the other hand, antidepressant medications prevent cellular death (27). It has been found that repeated chronic stress increases the levels of proapoptotic Bax and decreases antiapoptotic Bcl-2 and Bcl-xL in the brain (28). Our results showed that CRS increased the Bax/Bcl-2 ratio and caspase-3 level in the mouse brain. Accordingly, chronic GE treatment reversed apoptotic changes in stressed mice. Similarly, Huang et al. found that CRS aggravated the expression of Bax in the hippocampus of mice. However, it reduced Bcl-2 expression in the CRS mice's hippocampus (29). On the other hand, Wang et al. demonstrated that high-dose chronic GE treatment (250 mg/kg/day for four weeks) suppressed caspase-3 and 9 activities and thus apoptosis in the mice model of SCI (11).

5.1. Strengths and Weaknesses

Our study has several limitations. First, a limited number of animals was used in this study due to the ethical issues involving the use of laboratory animals. Second, our study assessed the effects of GE on two parameters, i.e., oxidative stress and apoptosis, which both increased during the depression. Nevertheless, we were not able to assess the impact of GE on other downstream pathways such as mitochondrial damage, neuroinflammation, excitotoxicity, and synaptic plasticity, which also plays a role in the pathophysiology of depression. Third, our results were only hypothesis-generating, and thus studies with a larger number of animals involving other mentioned mechanisms should be performed. We regard this as a strength that, to the best of our knowledge, this study was among the leading studies to assess the effects of GE on the anxiety and depressive-like behaviors in mice.

5.2. Conclusions

Depression and the resulting/concomitant anxiety are of the most common psychological disorders worldwide. However, current antidepressants/anxiolytics are not effective for all patients. Thus, the need for newer agents is utterly felt. Through the findings of this study, it appears that GE is a potent anti-depressant and anxiolytic in mice. These effects, at least in partial, are mediated through its antioxidant and anti-apoptotic properties. However, this was a preliminary study and its results should be validated in future studies.

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Footnotes

Authors' Contribution: Study concept and design: Alireza Mohammadi. Acquisition of data: Seyed Hojjat Hosseini and Alireza Majdi. Analysis and interpretation of data: Seyed Hojjat Hosseini and Alireza Majdi. Drafting of the manuscript: Seyed Hojjat Hosseini and Alireza Majdi. Critical revision of the manuscript for important intellectual content: Alireza Mohammadi. Statistical analysis: Mehrdad Roozbeh. Administrative, technical, and material support: Alireza Mohammadi. Study supervision: Alireza Mohammadi.

Conflict of Interests: The authors declare that they have no conflicts of interest to disclose.

Ethical Considerations: The experimental procedures performed in this study were in full conformity with the Guide for the Care, and Use of Laboratory Animals of the National Institutes of Health (NIH; Publication No. 85-23, revised 1985), and were confirmed by the Ethics Committee of Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1397.393).

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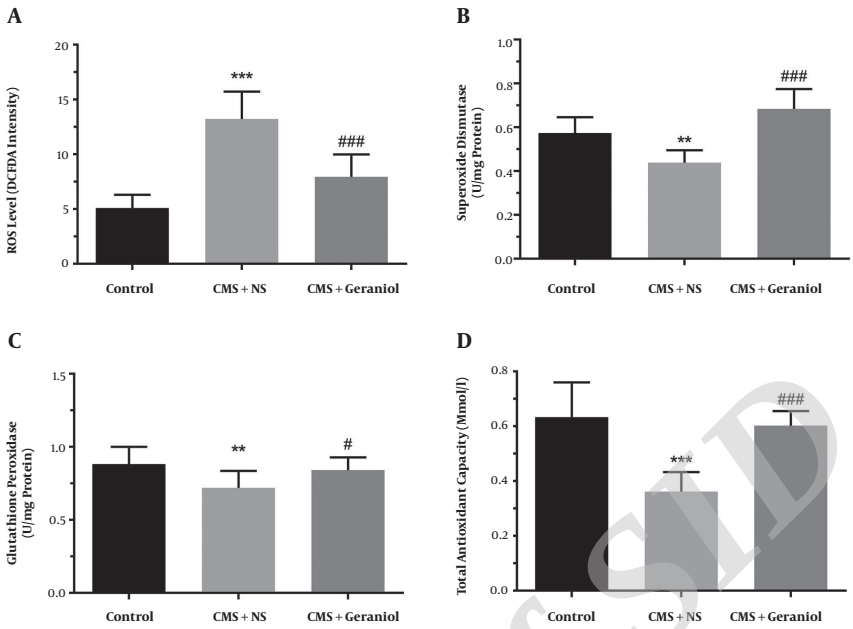

Figure 4. Oxidative stress markers in the study groups. A, ROS level (DCFDA intensity); B, SOD activity (U/mg protein); C, GSH-px activity (U/mg protein); and D, TAC (mMol/L). Each bar represents the mean ± SD, (n = 12). Significant differences were tested by the one-way ANOVA followed by Tukey's post hoc test; **P < 0.01 and ***P < 0.001 compared to the control group. ##P < 0.01 and ###P < 0.001 compared to the CRS group.

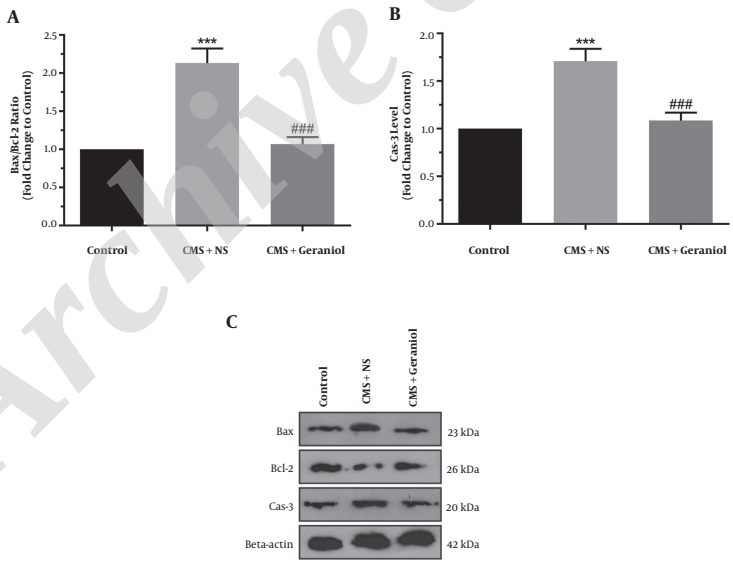

Figure 5. Apoptotic markers in the study groups. A, Bax/Bcl-2 ratio; B, caspase-3 level; and C, representative image of Bax to Bcl-2 protein and caspase-3. Each bar represents the mean ± SD, (n = 12). Significant differences were tested by the one-way ANOVA followed by Tukey's post hoc test; ***P < 0.001 compared to the control group. ###P < 0.001 compared to the CRS group.

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