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Original article

Evaluation of Anti-depressant and Anti-anxiety Activity of Methanol Extract of *Stachys annua* L. in Mice

Azadeh Motavallian[1,2*], Fatemeh Yousefbeyk[3], Bahram Soltani Tehrani[1], Zahra Zeinoddini[1], Forough Aghajani Torshkooh[1], Sasan Andalib[4,5,6,7]

[1]Department of Pharmacology and Toxicology, School of Pharmacy, Guilan University of Medical Sciences, Rasht, Iran.
[2]Rhino-sinus, Ear, and Skull Base Diseases Research Center, Department of Otolaryngology and Head and Neck Surgery, Amiralmomenin Hospital, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran.
[3]Department of Pharmacognosy, School of Pharmacy, Guilan University of Medical Sciences, Rasht, Iran.
[4]Research Unit of Clinical Physiology and Nuclear Medicine, Department of Clinical Research, Department of Nuclear Medicine, Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark.
[5]BRIDGE-Brain Research-Inter-Disciplinary Guided Excellence, Department of Clinical Research, University of Southern Denmark, Odense, Denmark.
[6]Research Unit of Psychiatry, Department of Psychiatry, Psychiatry in the Region of Southern Denmark, University of Southern Denmark, Odense, Denmark.
[7]Neuroscience Research Center, Department of Neurosurgery, Poursina Hospital, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran.

Abstract

Background and objectives: Development of new medicines with fewer side effects and more efficacy is needed for treatment of depressive and anxiety disorders. The present study was designed to investigate the antidepressant and anti-anxiety effects of *Stachys annua* L. methanol extract in mice. **Methods:** The extract was prepared by maceration method and the total phenolic and flavonoid contents were determined by Folin-Ciocalteu and aluminum chloride methods, respectively. Elevated plus-maze (EPM) and open field tests (OFT) were applied to evaluate the anti-anxiety and locomotor activity of animals treated with the intraperitoneal (i.p.) extract (12.5, 25, 50, and100 mg/kg), respectively. Antidepressant activity was evaluated by forced swim test (FST) and tail suspension test (TST). **Results:** The total phenolic content of the extract was 54.13±0.01 mg of gallic acid equivalents per gram of dry extract and total flavonoid content was 67.89±0.005 mg of quercetin as equivalents/ g of extract. Intraperitoneal administration of the extract (50 and 100 mg/kg) significantly increased the percentage of time spent and the percentage of arm entries into the open arms of EPM and decreased locomotor activity, compared with the vehicle control group. In addition, the immobility time of animals significantly decreased in both FST and TST with doses of 25, 50, and 100 mg/kg of the extract, compared with the vehicle control group. **Conclusion:** The extract of *Stachys annua* L. might be used as an adjunct therapy in clinical studies for the treatment of depressive and anxiety disorders. However, determination of active ingredients needs further evaluation.

Keywords: anti-anxiety agent; anti-depressive agent; methanol extract; mice; *Stachys annua*

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*Corresponding author: motavallian@gums.ac.ir

Introduction

Depression is a major contributor to the overall global burden of diseases [1]. Depressed patients are at a higher risk of serious physical health problems. The characterizations of depression are loss of interest in activities, guilt and hopelessness, sleep and appetite changes, fatigue, concentration problems, restlessness, and suicidal ideation [2]. Depression is likely to coincide with anxiety. Comorbid anxiety and depression exert a reciprocal negative impact; however, there is evidence to suggest that pre-existing anxiety is a risk factor for later depression [3]. Most commonly used types of classical antidepressants are tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and selective serotonin reuptake inhibitors (SSRIs) [4,5]. Despite the efficacy of approved antidepressants, some patients fail to respond efficiently to these drugs [6]. Moreover, they have a wide range of adverse effects [7]. Thus, there is a need for research in order to adopt new therapeutic approaches for anxiety and depression. There is a growing interest in research in the anti-depressive effect of herbal medicines. Having lower side effects, herbal medicine have shown comparable efficacy to classical antidepressant medications [8,9]. *Stachys annua* L. is a plant belonging to the Lamiaceae family and native to Mediterranean, south-western Asia, and North America [10]. This plant is widely distributed in north, north west, western and middle regions of Iran [11]. The leaves and aerial parts of the plant have been traditionally used as antidiabetic, antipyretic, anti-catarhal, tonic, and wound-healing agents [10,12]. Antibacterial, anti-inflammatory, anti-toxic, anti-nephritic, antihepatitis, antioxidant, and cytotoxic effects of different species of *Stachys* have been reported by experimental studies [13]. Despite all of these reports, to date, no pharmacological studies have evaluated the effects of *S. annua* L. on psychiatric disorders. The present study aimed to examine the antidepressant and anxiolytic effects of *Stachys annua* L. methanol extract in mice.

Materials and Methods

Ethical considerations

The present study was approved by Ethics Committee of Guilan University of Medical Sciences in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals (Approval No: IR.GUMS.REC.1398.25).

Chemicals and treatment

All drugs and methanol extract of *Stachys annua* L. (12.5, 25, 50, and 100 mg/kg) were freshly prepared in the distilled water and the Tween® 80 (1%), and administered intraperitoneally (i.p.) 30 min before each experiment.. Fluoxetine (10 mg/kg) and imipramine (10 mg/kg) as positive control drugs in depression-related behavioral tests were obtained from Dr. Abidi Pharm. Co. (Iran) and Sobhan Daru Co. (Iran), respectively. Diazepam (2 mg/kg), as a positive control in the elevated plus maze test, was obtained from Caspian Tamin Pharm. Co (Iran). Distilled water and Tween® 80 were applied as vehicle control. All solutions were freshly prepared in the distilled water and the Tween® 80 (1%) and administered intraperitoneally (i.p.) 30 min before each experiment. Dosage selection was specified based on previous studies [14,15].

Plant material and preparation of the methanol extract

The aerial parts of *Stachys annua* L. were collected from Rostamabad, Guilan province in the North-West of Iran, during May 2018. The voucher specimen (183 HGUM) was deposited at the Herbarium of School of Pharmacy, Guilan University of Medical Sciences, Rasht, Iran. The aerial parts (500 g) were shade dried, powdered, and extracted with methanol by maceration method for percolation method for 72 h (the solvent was refreshed every 24) [16].The plant: solvent ratio was 1:10. The solvent was evaporated by rotary evaporator to obtain the methanol extract (g). The extracts were stored in a refrigerator until required.

Total phenolics content

Total phenolic content was determined by the Folin-Ciocalteu method [17]. One mL of methanol extract was mixed with 5 mL of Folin-Ciocalteu reagent (previously diluted tenfold with distilled water). After 10 min, sodium bicarbonate solution (4 ml, 75 g/L) was added. The mixture was allowed to stand for a further 30 min in the dark at room temperature. The absorbance was measured at 765 nm using a UV/VIS spectrophotometer (Lambda 25 PerkinElmer, USA). Different concentrations of

gallic acid standard (25, 50, 70, 100, 200 µg/L) were used to plot calibration the curve. The concentration was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract [18].

Total flavonoids content

Total flavonoid content was determined by aluminum chloride method using quercetin as the standard [19]. Five mL of aluminum trichloride ($AlCl_3$) (2% in methanol) was added to 5 mL of extract (1 mg/mL). The absorbance of the mixture was measured at 415 nm after 10 min. Blank sample contained 5 mL of the extract and 5 mL of methanol without $AlCl_3$. The absorbance of different concentrations of quercetin (10, 25, 50, 70, 100, 200 µg/L) was measured to plot the standard curve. Total flavonoid content was expressed as mg of quercetin equivalents (QE)/g of the extract.

Animals

This study was carried out on Male albino mice (25±5 g) bred in the Animal House of Guilan University of Medical Sciences. One hundred seventy-three animals were housed in groups of 6 per cage with access to food and water ad libitum, under standard housing conditions with a 12:12 h light-dark cycle at 22±2 °C. Each animal was used for only one experimental condition after its acclimatization to room conditions. At the end of each experiment, animals were sacrificed by inhalation of carbon dioxide (CO2) [20].

Acute toxicity study

Acute toxicity study was performed based on OECD 425 guidelines [21]. Five animals were treated with a single oral dose of the extract (2000 mg/kg body weight) and tap water was given to the other group of five mice as a control. The mice were observed continuously for 2 h and thereafter once daily over 14 days for determination of possible mortality and behavioral neurological toxicity. Furthermore, bodyweight changes were recorded for each animal during the period of the experiment.

Elevated plus-maze (EPM)

Thirty-six animals were randomly divided into the following groups of 6 mice each: Group I: Vehicle control group (Tween 80+normal saline); Group II-V: plant extract (12.5, 25, 50 and, 100 mg/kg); Group VI: Diazepam 2 mg/kg. Animals received a single i.p. dose of the vehicle, standard drug, or plant extract 30 min prior to commencement of the test and were individually put at the center of a wooden box cross located 40 cm above the floor. As described previously by Pellow et al. [22], the EPM has two open (30 × 5 × 0.25 cm) and two closed (30× 5× 40 cm) arms. Each animal was put in the center of the apparatus which faced one of the open arms. Any entries into an arm were defined as all four paws of the animal placing in the lines limiting a squared area. To evaluate anxiety, the entries to open arms/total entries and the time spent in the open arms/time spent in all arms of the apparatus were recorded by a camera and analyzed using a video tracking software (EthoVision, The Netherlands) during a 5-min period.

Open field test (OFT)

Locomotor activity of animals was assessed by a plexiglass chamber (40 × 40 × 40 cm) in a quiet and lighted room. Forty-eight animals were randomly divided into the following groups of 6 mice in each: Group I: Vehicle control group (Tween 80+normal saline); Group II-V: plant extract (12.5, 25, 50 and, 100 mg/kg); Group VI: Diazepam 2 mg/kg; Group VII: fluoxetine 10 mg/kg; Group VIII: imipramine 10 mg/kg. Thirty minutes after a single i.p. dose of the test compounds, they were placed individually in the center of the chamber and during the 5 min, the number of squares crossed with four paws of the animal as their locomotor activity was recorded by using a digital camera. All the videos were assessed by Ethovision XT (The Netherlands) software [23].

Forced swimming test (FST)

As described by Porsolt et al., the FST test was conducted to assess antidepressant properties of methanol extract of *S. annua* [24]. Forty-two animals were randomly divided into the following groups of 6 mice in each: Group I: Vehicle control group (Tween 80+normal saline); Group II-V: plant extract (12.5, 25, 50 and, 100 mg/kg); Group VI: fluoxetine 10 mg/kg; Group VII: imipramine 10 mg/kg. Thirty min after i.p. injection of the test compounds, were individually placed in a plexiglass cylindrical container (15 cm in diameter), filled with a 35-cm height of water (temperature 22-25 °C). The animals were monitored for 6 min, and the time of immobility was measured in the last 4 min.

The immobility time was defined as the period of time in which the animals stopped swimming and floated floated motionless (regardless of little movements to just maintain balance) on the surface of the water.

Tail suspension test (TST)

TST is a screening test to assess depression and antidepressant-like properties of new drugs. Forty-two animals were randomly divided into the following groups of 6 mice in each: Group I: Vehicle control group (Tween 80+normal saline); Group II-IV: plant extract (12.5, 25, 50 and, 100 mg/kg); Group VI: fluoxetine 10 mg/kg; Group VII: imipramine 10 mg/kg. Thirty min after i.p. treatment with test compounds, each mouse was suspended from the tail using adhesive tape placed approximately 1-2 cm from the tip of the tail. The mice were monitored for 6 min and immobility time was evaluated as absence of movements of animals over the last 4 min [25].

Statistical analysis

Graph Pad Prism 8 software was used for statistical analysis. All data were expressed as the mean ± standard error of the mean (SEM). Comparison between groups was made using one-way analysis of variance (ANOVA) followed by Tukey post hoc test and p value < 0.05 was considered as statistically significant.

Results and Discussion

The total phenolic content of *S. annua* methanol extract was 54.13±0.01 mg of gallic acid equivalents (GAE) per gram of dry extract in reference to the standard curve for gallic acid ($y = 0.00088x - 0.0388$, $R^2 = 0.9975$). The total flavonoid content was measured regarding to the standard curve of quercetin ($y = 0.0178x - 0.0119$, $R^2 = 0.995$) and was reported 67.89± 0.005 mg of quercetin as equivalents (QE)/ g of extract.

Following the oral administration of the extract (2000 mg/kg), all animals were alive during the experimental period (14 days) of determination of toxicity. Moreover, mice treated with oral extract of *S. annua* did not show any signs of toxicity and changes in the bodyweight compared to the control group. In EPM test, *S. annua* methanol extract at the doses of 50 and 100 mg/kg showed a significantly high percentage of time spent in the open arms/time spent in all arms and the percent of entries to open arm/total entries in mice placed at the center of EPM, compared with those of the vehicle ($p<0.05$) (Figures 1A and 1B). Moreover, these parameters significantly increased in the positive control group treated with diazepam (2 mg/kg, i.p.), in comparison with the vehicle-treated control group ($p<0.05$).

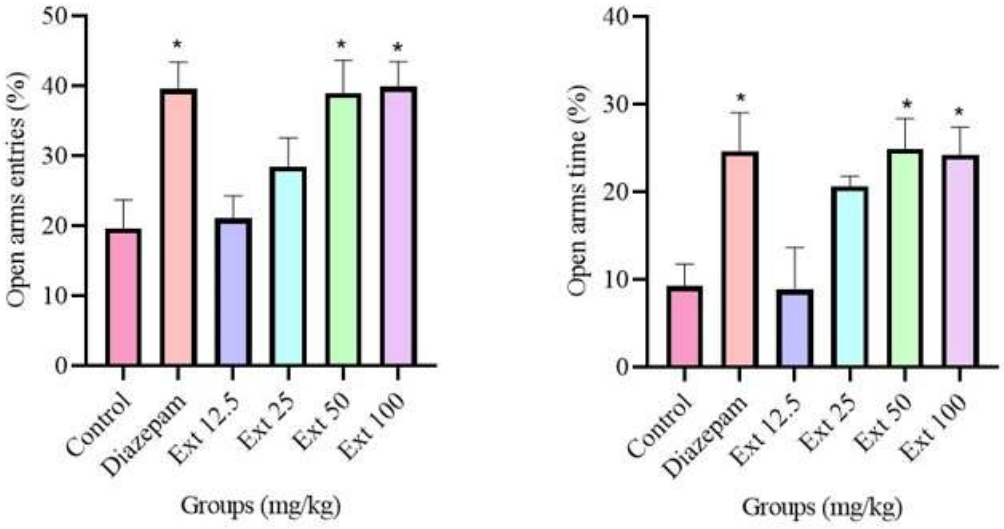

Figure 1. Effects of *Stachys annua* methanol extract and diazepam on (A) the percentage of open arm entries to total entries; (B) the percentage of time spent in the open arms to time spent in all arms at EPM test during a 5 min in mice. The animals received injections of the vehicle, plant extract (12.5, 25, 50 and 100 mg/kg) and diazepam 30 min prior to test; Data are expressed as mean±(SEM); n =6; *p <0 .05: Significant difference compared to vehicle-control group.

As shown in Table 1, the plant extract with doses of 50 and 100 mg/kg, showed a significant decrease in the number of lines crossed by the four paws of the mice ($p<0.05$) and a significant increase in the time spent in the central zone ($p<0.05$) compared with those of the vehicle-treated control group, during the 5-min observation period. Of the positive control groups, only the diazepam group experienced a significant decrease in the total squares crossed by animals ($p<0.05$) and the number of rearing ($p<0.05$), as well as a significant increase in time spent in the central zone ($p<0.01$) in comparison with the vehicle-treated control group.

As shown in Figure 2, in FST, time spent immobile with reference drugs (imipramine and fluoxetine) was significantly less than that of the vehicle-treated control group ($p< 0.001$). The mice treated with all doses of extract showed significantly less immobility time, as compared with those receiving vehicle. In addition, doses of 50 and 100 mg/kg of *S. annua* L. methanol extract showed antidepressant-like activity comparable to the positive control groups.

In TST, all doses of the extract significantly reduced immobility time in comparison with the vehicle-treated control group ($p<0.05$ for 12.5, 25, and 50 mg/kg; $p< 0.01$ for 100 mg/kg). The time spent immobile significantly decreased in animals treated with fluoxetine and imipramine as the positive control groups ($p<0.01$) (Figure 3).

In the present study, we evaluated the antidepressant and anxiolytic effects of *Stachys annua* methanol extract in mice. The results of the present study demonstrated the antidepressant-like activity of the extract in both FST and TST. The immobility time in animals treated with *S. annua* methanol extract (12.5, 25, 50, and 100 mg/kg, i.p.) significantly decreased, compared to the vehicle-treated control group in a dose-dependent manner.

Table 1. Effects of *Stachys annua* methanol extract in the open field apparatus during 5-min

	Control	Imipramine	Fluoxetine	Diazepam	Extract 12.5	Extract 25	Extract 50	Extract 100
Lines crossed by animals	92.7 ± 11.2	79.7± 5.8	72.8± 9.3	51.3± 2.2 *	79.3± 7.3	57.8 ± 4.8	53 ± 10.4 *	57.5 ± 6.5 *
Time spent in central zone (s)	13.5 ± 1.6	19.5 ± 2.3	18 ± 1.8	26 ± 2.5 **	14.1 ± 2.8	21.7 ± 1.7	22.8 ± 1 *	23.7 ± 1.3 *
Number of rearing	33.7±2.3	31.3± 1.3	32.8 ± 1.5	23.3 ± 1.6 *	28.7 ± 2.3	29.5 ± 1.9	27 ± 3.5	29.1 ± 1.4

Mice received injections of vehicle, plant extract (12.5, 25, 50, and 100 mg/kg, i.p.), diazepam (2 mg/kg, i.p.), fluoxetine (10 mg, i.p.), imipramine (10 mg/kg, i.p.), 30 min prior to test; values are means±SEM; n =6; *p< 0.05 and **p<0.01: significant difference compared to vehicle-control group

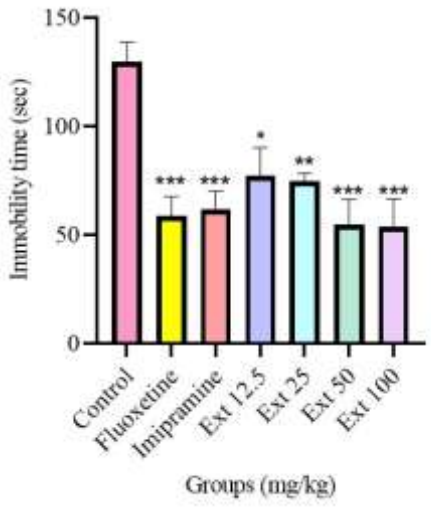

Figure 2. Effects of *Stachys annua* L. methanol extract on the period of immobility time (sec) in the forced swimming test. The mice were injected with vehicle, plant extract (12.5, 25, 50, and 100 mg/kg, i.p.), fluoxetine (10 mg/kg, i.p.), and imipramine (10 mg/kg, i.p.), 30 min prior to test; Values are in means ± SEM; n =6; *p < 0.05, **p < 0.01, ***p < 0.001 indicate significant differences, compared with the vehicle-control group.

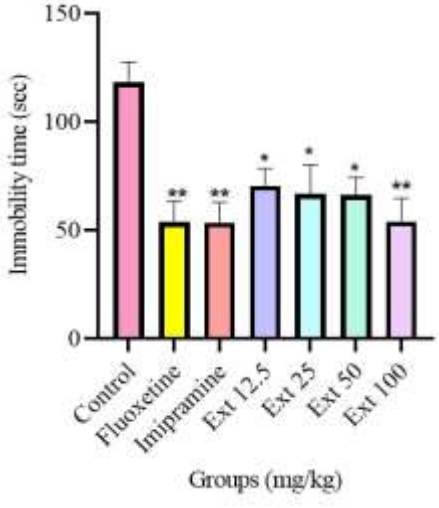

Figure 3. Effects of of *Stachys annua* methanol extract on time (sec) spent immobile in the TST. Mice were injected with vehicle control, plant extract (12.5, 25, 50, and 100 mg/kg, i.p.), fluoxetine (10 mg/kg, i.p.), and imipramine (10 mg/kg, i.p.), 30 min prior to test; Values are means ± SEM; n =6; *p<0.05 and **p<0.01 indicate significant differences compared with the vehicle-control group.

In the OFT, locomotor activity significantly decreased by the extract at doses of 50 and 100 mg/kg, compared to the vehicle. Decreasing locomotor activity of animals treated with the extract rules out the hypothesis that the reduction in immobility time is a result of psycho-stimulant effects of the extract. This reveals that the antidepressant-like effects in the TST and FST were unlikely to be attributed to the false positive results [26]. Furthermore, the locomotor activity and the number of rearing significantly decreased in animals treated with diazepam among the positive control groups, which could be related to the sedation effect of diazepam [27]. In the EPM test, the animals spend more time in the closed area due to natural avoidance of mice for open area. However, animals receiving the extract at doses of 50 and 100 mg/kg spent more time and had more entries to the open arm of EPM, indicating an anti-anxiety-like effect. In addition, diazepam as a reference drug exhibited anxiolytic activity, which was in agreement with findings of a previous study [28]. Protocatechuic acid, p-hydroxybenzoic, caffeic acid, (−)-epicatechin, (+)-catechin, ferulic acid, benzoic acid, rutin, rosmarinic acid, and apigenin have been demonstrated as the main phenolic and flavonoid compounds of *S. annua* [12]. In depressive disorder, monoamine function is decreased in the brain, which is known as monoamine hypothesis of the disease [29]. There is evidence that chlorogenic acid can prevent astrocytes monoamine oxidase (MAO)-B activation, following reactive oxygen species (ROS) production, and has antidepressant-like effects in mice [30]. In a study, *Eucommia ulmoides* extract, which is rich in chlorogenic acid, exhibited antidepressant activity by promoting serotonin release and enhancement of synapsin I expression in the cultured cells of fetal rat raphe neurons [31]. Involvement of the serotonergic and noradrenergic and/or dopaminergic systems has been shown in the mechanism of antidepressant-like action of rutin in *Schinus molle* L. [32]. Several studies have so far demonstrated the antidepressant effect of rosmarinic acid. Sasaki et al. observed that rosmarinic acid-enriched extract from *Rosmarinus officinalis* produced significant antidepressant-like effects through modulation of monoaminergic and cholinergic functions in turn via upregulation of tyrosine hydroxylase and pyruvate carboxylase involved in dopaminergic, serotonergic, and GABAergic pathway regulations [33]. Another study elucidated the antidepressant-like effect of rosmarinic acid via its up-regulatory action-induced cell proliferation in mice hippocampus [34]. Reduction of brain-derived neurotrophic factor (BDNF) [35] and overexpression of mitogen-activated protein kinase phosphatase-1 (MKP-1) [36,37] were shown to be key factors in the pathophysiology of depression. Rosmarinic acid has antidepressant activity mediated by downregulation of Mkp-1, upregulation of BDNF, and modulation of dopamine and corticosterone synthesis and therefore might be a potential target for treatment of depressive disorders [38]. Furthermore, protocatechuic acid represented antidepressant activity in two animal models of depression, which was possibly influenced by monoaminergic system and upregulation of BDNF level [39]. Antidepressant-like activity of ferulic acid has been shown to be regulated via various pathways. Zeni et al. found that ferulic acid as a glutamate antagonist and an antioxidant compound diminishes the depressive-like behavior and oxidative stress in mice [40]. Furthermore, antidepressant-like effect of ferulic acid has been demonstrated via inhibition of MAO-A in mouse brain [41]. A recent study revealed that ferulic acid enhanced cell survival and proliferation, energy metabolism, and dopamine synthesis in the depressed mouse brain [42]. Epicatechin alleviates anxiety in association with elevated monoamine and BDNF levels, in mouse hippocampus [43]. In Catechin decreases depressive symptoms in rats undergoing chronic unpredictable mild stress through declining oxidative stress [44]. Monterio et al. reported sedative, anxiolytic, and antidepressant-like effect of *Annona coriacea*, likely mediated by the modulation of the $GABA_A$ receptors and monoaminergic systems, probably depending in turn on the caffeic acid presence in the extract [45]. A recent study elucidated involvement of autophagy in the pathology and treatment of depression. Apigenin induces antidepressant effects in chronic restraint stress mice potentially by improving autophagy through the adenosine monophosphate-activated protein kinase (AMPK) /mammalian target of rapamycin (mTOR) pathway [46]. As mentioned above, the reduction of BDNF was confirmed to have role in the pathophysiology of depression. Up-regulation of BDNF levels in the mouse hippocampus by apigenin was demonstrated in FST [47].

Pathophysiological relationships between oxidative stress/inflammation and psychiatric disorders such as anxiety and depressive disorders have been shown [48]. Amongst the phenolic compounds, chlorogenic acid and rosmarinic acid have been extensively investigated for their antioxidant and anti-inflammatory effects [49]. Chlorogenic acid represented the inhibitory effects on expression of NO, pro-inflammatory cytokines, and Ninjurin1 regulated by the NF-jB pathway in LPS-stimulated RAW 264.7 cells [50]. Another study reported the anti-inflammatory and anti-oxidant effects of RA by inhibition of neutrophil activity, inhibition of MMP-9 activity, and modulation of the NF-κB pathway [51]. Kocak et al. reported that the phenolic and flavonoid compounds are responsible for the main biological activities of *S. annua* collected in Turkey [12]. By measuring total phenolic and flavonoid contents of different of different solvent extracts, methanol extract of this plant was found to contain the highest amount of phenolic (35.36 mg GAEs/g extract) and flavonoid (51.38 mg REs/g extract) compounds [12]. Interestingly, the findings of the present study showed a higher content of phenolic (54.13 mg GAEs/g extract) and flavonoids (67.89 mg REs/g extract) in *S. annua* gathered from North of Iran.

In DPPH and ABTS radical scavenging assays, methanol extract of *S. annua* has shown a high antioxidant and strong radical scavenging activity potential, which might be due to the high amounts of chlorogenic, benzoic, and rosmarinic acids in the extract [12]. Therefore, one of the pathways involved in antidepressant and antianxiety activity of the extract is probably its antioxidant and anti-inflammatory properties.

Conclusion

To the best of our knowledge, the present study is the first to demonstrate the antidepressant-like activity and anxiolytic effect of *Stachys annua* in animal models of psychiatric disorders. It seems that the phenolic and flavonoid compounds in the extract are responsible for the beneficial above-mentioned effects. However, further studies on the main isolated compounds with the anti-anxiety and antidepressant-like effect and their potential mechanisms are required. Using *S. annua* in conventional medications would be an additional value in future experimental investigations for single or add-on therapy of depression. Moreover, due to the fact that the route and duration of the administration may affect pharmacokinetics of bioactive compounds, assessment of chronic and oral administration of *S. annua* extract on depression and anxiety, as well as toxicological studies are suggested.

Acknowledgments

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Author contributions

Azadeh Motavallian contributed to data collection, data analyses, data interpretation, and drafting the manuscript; Zahra Zeinoddini and Forough Aghajani Torshkooh contributed to data collection and drafting the manuscript; Bahram Soltani Tehrani contributed to statistical analysis and drafting the manuscript; Fatemeh Yousefbeyk contributed to data collection and drafting the manuscript; Sasan Andalib contributed to data collection and drafting the manuscript. All authors approved and read the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

AMPK: monophosphate-activated protein kinase; ANOVA: one-way analysis of variance; BDNF: brain-derived neurotrophic factor; EPM: elevated plus-maze; FST: forced swimming test; GABA-A: gamma-aminobutyric acid-A; GAE: gallic acid equivalents; IP: intraperitoneal; MAO-B: monoamine oxidase-B; MAOIs: monoamine oxidase inhibitors; MKP-1: mitogen-activated protein kinase phosphatase-1; mTOR: mammalian target of rapamycin; OECD: Organization for Economic Co-operation and Development; OFT: open field tests; QE: quercetin equivalents; ROS: reactive oxygen species; SEM: standard error of the mean; SSRIs: selective serotonin reuptake inhibitors; TCAs: tricyclic antidepressants; TST: tail suspension test.