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Antidepressant Effects of the Aqueous and Hydroalcoholic Extracts of *Salvia mirzayanii* and *Salvia macrosiphon* in Male Mice

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Abstract

Objectives: As some species of the *Salvia* family have been shown to exert antidepressant-like activity, the aim of this study was to investigate the antidepressant effects of the aqueous and hydroalcoholic extracts of *Salvia mirzayanii* and *Salvia macrosiphon*, two endemic species of salvia in Iran.

Methods: In total, 148 eight-week-old male albino mice (25 - 35 g) were used to carry out the forced swimming test. The effects of different doses of the aqueous (100 - 1800 mg/kg) and hydroalcoholic extracts (75 - 900 mg/kg) of *Salvia mirzayanii* and *Salvia macrosiphon* on immobility, climbing, and swimming behaviors were examined. Fluoxetine and imipramine were used as control drugs. The effects of extracts on locomotor activity were also evaluated.

Results: High doses of the aqueous extracts of both plants and hydroalcoholic extract of *Salvia mirzayanii* produced a significant reduction in immobility and increase in swimming compared to the control group. The hydroalcoholic extract of *Salvia macrosiphon* did not show any significant effect on immobility and swimming. Only some doses of hydroalcoholic extract of *Salvia mirzayanii* and aqueous extract of *Salvia macrosiphon* showed a significant increase in climbing behavior. The aqueous and hydroalcoholic extract of both plants caused a decrease in spontaneous activity.

Conclusions: The findings of this study indicated that the aqueous and hydroalcoholic extracts of *Salvia mirzayanii* and the aqueous extract of *Salvia macrosiphon* had antidepressant-like activity. In addition, the hydroalcoholic extract of *Salvia mirzayanii* and the aqueous extracts of *Salvia macrosiphon* were more effective fractions of these plants. These endemic Iranian *Salvia* species may have potential therapeutic effects for depression.

Keywords: Forced Swimming Test, *Salvia mirzayanii*, *Salvia macrosiphon*, Mice

1. Background

Depression, the most common mood disorder, affects 350 million people of all ages around the world (1) and has been raised as a "Global Crisis" by WHO (2). Depression is associated with a group of psychosocial and physical problems with a high rate of suicide (3). Nonetheless, many approved pharmacological agents for treating depression have low rates of efficacy and high unwanted side effects (4). Then, the investigation for novel, more-tolerated, and efficacious antidepressant agents without the disadvantages remains necessary.

It is believed that natural products are relatively safe for human as compared with synthetic compounds. As a result, there has been an increasing interest for the use of botanical compounds for the treatment of CNS disturbances including depression. An increasing number of studies have addressed that bioactive compounds of medicinal plants are good candidates for the management of mild to moderate depression. In this regard, there are some reports about the CNS effects of *Salvia* in literatures (5).

Salvia is the largest genus of the Lamiaceae family, with over 900 species worldwide and almost 60 species in Iran. *Salvia* has been traditionally used for treating many health problems (6); and its reported therapeutic effects in neuropsychological, cardiovascular, metabolic, and endocrine diseases (7), has led to suggestions that different parts of

Salvia species can be used for drug development.

Salvia officinalis, commonly known as sage, has been indicated for different medical purposes in Iranian folk medicine including a sedative, analgesic, and tonic (8); and to treat convulsion, unilateral headache, tremor, fatigue, and dizziness (8, 9). Sage tea has been traditionally used for the treatment of depression (7). Recent studies have been shown that *S. officinalis* has many pharmacological properties such as anti-anxiety, antidepressant and improving effects on mood and cognition (10, 11). In addition, various *Salvia* species including *lavandulaefolia, leriifolia, guaranitica, elegans, sclarea*, and *divinorum* have been reported to have potential activities for treating a wide range of CNS disturbances (11-15). Several species of *Salvia* genus such as *officinalis, elegans* (15), *sclarea* (16), and *divinorum* (17) have also demonstrated an antidepressant-like activity in rodents.

S. mirzayanii (Marv-e-Talkh in Persian) and *S. macrosiphon* (Marvak in Persian) are two well-known endemic species of the Salvia genus in Iran. *S. mirzayanii* has been used in Iranian folk medicine especially in Hormozgan and Kerman provinces for the treatment of diabetes, spasms, gastrointestinal disorders, infections, and inflammations (18). Local healers in Iran still use *S. mirzayanii* as a tonic and astringent. Pharmacological studies have shown antimicrobial, anti-hyperglycemic, antioxidant, neuroprotective and anti-inflammatory properties for different preparations of *S. mirzayanii* (19, 20). In regards to *S. macrosiphon*, folk practitioners have reported its effectiveness in treating a sore throat and cough (21). However, there is no study reporting the antidepressant effects of these two endemic species of *Salvia*.

2. Objectives

Based on previously reported antidepressant-like activity of several species of Salvia as well as the biological properties of Iranian endemic *Salvia* species, the aim of this study was to evaluate the possible antidepressant effects of aqueous and hydroalcoholic extracts of *S. mirzayanii* and *S. macrosiphon* using the forced swimming test (FST).

3. Methods

3.1. Plant Collection and Authentication

Aerial parts of *S. mirzayanii* and *S. macrosiphon* were collected from Hormozgan and Fars Provinces, Iran, respectively. *S. mirzayanii* was authenticated by M. Kamali Nejad (Department of Traditional Medicines, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences). The taxonomic identification of *S. macrosiphon* was confirmed by A. Khosravi (Department of Biology, Faculty of Science, Shiraz University). The voucher specimens of *S. mirzayanii* (#1120) and *S. macrosiphon* (#1110) were deposited at the herbarium of Shahid Beheshti University of Medical Sciences.

3.2. Preparation of the Aqueous Extracts of Plants

The aerial parts of plants were air dried in the shade and grounded. For aqueous extraction, 50 grams of powdered *S. mirzayanii* or *S. macrosiphon* were macerated in distilled water (400 mL) at an ambient temperature for 24 hours. Thereafter, the mixtures were filtered by Watman paper; and the filtrates were concentrated over a water bath and dried under a vacuum. The yields were 18.4% and 14.5% (w/w) for *S. mirzayanii* and *S. macrosiphon*, respectively.

3.3. Preparation of the Hydroalcoholic Extracts of Plants

For preparing hydroalcoholic extracts, the powdered *S. mirzayanii* (50 g) or *S. macrosiphon* (25 g) was percolated with ethanol 70% (500 mL) for 72 hours. The percolates were then extracted with petroleum ether three times in order to remove the fat. The extracts were concentrated over a water bath and dried under a vacuum. The yields were 22.0% and 16.8 % (w/w) for *S. mirzayanii* and *S. macrosiphon*, respectively.

3.4. Phytochemical Screening Test

The aqueous and hydroalcoholic extracts were preliminarily screened for the presence of alkaloids with Mayer reagent, anthrax quinones by the Borntraeger reaction, tannins by ferric chloride solution, and flavonoids by Zn and HCl (22).

3.5. Experimental Animals

Eight-week-old male albino mice (N = 148), 25 - 35 g were obtained from the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. Mice were kept in cages with a standard condition. All experiments were approved by the Ethics Committee of Shiraz University of Medical Sciences (#ec-p-91-5317). Mice were randomly assigned into different groups (N = 6 - 8/groups) for various behavioral tests.

3.6. Drugs

Imipramine (15 mg/kg) and fluoxetine (20 mg/kg) (Pars Daru, Tehran, Iran) were used as reference drugs and dissolved in distilled water. Doses of imipramine and fluoxetine were chosen based on the previous studies (23). Different concentrations of the *S. mirzayanii* and *S. macrosiphon* extracts were prepared by serial dilution from

a stock solution of hydroalcoholic extract (90 mg/mL in 10% ethanol) and aqueous extract (180 mg/mL in distilled water). Ethanol 10% or distilled water was used as negative controls for the hydroalcoholic and aqueous extracts, respectively. All freshly prepared solutions were intraperitoneally injected (i.p., 0.1 mL/10 g body weight).

3.7. Assessment of Acute Toxicity of Extracts

Mice (N = 6/group) were i.p. injected different doses of plant extracts (100 - 5400 mg/kg) and observed for 48 hours for any possible mortality. The maximum dose that did not provoke death was considered as a maximal non-fatal dose (MNFD). The doses of aqueous and hydroalcoholic extracts of *S. mirzayanii* and *S. macrosiphon* used for antidepressant study were determined based on their respective MNFD (75 - 900 mg/kg for hydroalcoholic extracts and 100 - 1800 mg/kg for aqueous extracts).

3.8. Measurement of Locomotors Activity

Spontaneous locomotors activity was measured using the Animex Activity Meter (AB FARAD model, Sweden) (24). The animals were allowed to become adapted with the apparatus before the experiment. Mice (N = 6/group) were received the i.p. injection of ethanol 10%, distilled water, different doses of the hydroalcoholic extracts (100 - 5400 mg/kg), or different doses of the aqueous extracts (100 - 2700 mg/kg), and 30 minutes later their locomotors activities were recorded at 5-minute intervals for 20 minutes.

3.9. Assessment of Antidepressant-Like Activity by Forced Swimming Test (FST)

Depression-like behavior was assessed using FST (25). Briefly, mice were individually placed in a glass cylinder (18 cm in diameter, 25 cm high) filled with 25°C tap water to a height of 15 cm for 15 minutes for adaptation to the test environment. Twenty-four hours later, mice were again placed in the water and numbers of the climbing, swimming, and immobility behaviors of mice were recorded at 5 second intervals during the 5-minute test period. Mice (N = 8/group) received the i.p. injection of different doses of the hydroalcoholic extracts (75 - 900 mg/kg), different doses of the aqueous extracts (100 - 1800 mg/kg), fluoxetine (20 mg/kg), imipramine (15 mg/kg), ethanol 10%, or distilled water three times at: 23.5, 5, and 0.5 hours prior to the FST. Decreases in number of immobility and increases in numbers of climbing or swimming behaviors were considered as an antidepressant-like action.

3.10. Statistical Analysis

Data were presented as mean ± SEM. One-way analyses of variance (ANOVA) was used to compare the mean differences between groups. Dunnett *t* was used as a post hoc test for multiple comparisons. All analyses were performed using SPSS software (version 18); and $P < 0.05$ was considered as a statistically significant level.

4. Results

4.1. Phytochemical Screening Test

The phytochemical screening revealed that the aqueous and hydroalcoholic extracts of *S. mirzayanii* and the hydro alcoholic extract of *S. macrosiphon* contained saponins, tannins, and flavonoids. However, the aqueous extract of *S. macrosiphon* contained tannin and few amounts of saponin.

4.2. Acute Toxicity of Plant Extracts

Hydroalcoholic extract of *S. mirzayanii* caused 83.3% mortality at a dose of 5400 mg/kg, while the aqueous extract showed no mortality at any of the studied doses. The hydroalcoholic extract of *S. macrosiphon* caused 63.3% mortality at doses of 1800 and 2700 mg/kg. The aqueous extract of *S. macrosiphon* caused death in animals at doses of 1200 (16.6%), 1500 (50%), 1800 (50%), and 2700 mg/kg (63.3%).

4.3. Effects of the Plant Extracts on Locomotors Activity

4.3.1. Effects of S. mirzayanii Extracts on Locomotor Activity

One-way ANOVA revealed a significant effect of the *S. mirzayanii* extracts on locomotor activity (Figure 1A, $P < 0.001$). The aqueous extract at doses of 1800 (35.5%) and 2700 mg/kg (80.1%) significantly decreased locomotor activity as compared with the control group ($P < 0.001$) (Figure 1A). Significant decreases in locomotor activity were also observed by the hydroalcoholic extract at doses of 1800 (52.2%), 2700 (49.9%) and 5400 mg/kg (94.1%) in comparison to the control group ($P < 0.01$) (Figure 1A).

4.3.2. Effects of the S. macrosiphon Extracts on Locomotor Activity

One-way ANOVA showed a significant effect of the *S. macrosiphon* extracts on locomotor activity (Figure 1B, $P < 0.001$). The aqueous extract of *S. macrosiphon* significantly decreased locomotor activity at doses of 300 (32.8%, $P < 0.001$), 900 (31.1%, $P < 0.001$), and 1800 mg/kg (25.0%, $P < 0.05$), in comparison to the control group (Figure 1B). The hydroalcoholic extract of *S. macrosiphon* at doses of 100 (30.9%, $P < 0.001$), 300 (29.3%, $P < 0.001$), 900 (51.4%, $P < 0.001$), 1800 (19.9%, $P < 0.05$), and 2700 mg/kg (19.7%, $P < 0.05$) significantly decreased locomotor activity, as compared with the control group (Figure 1B).

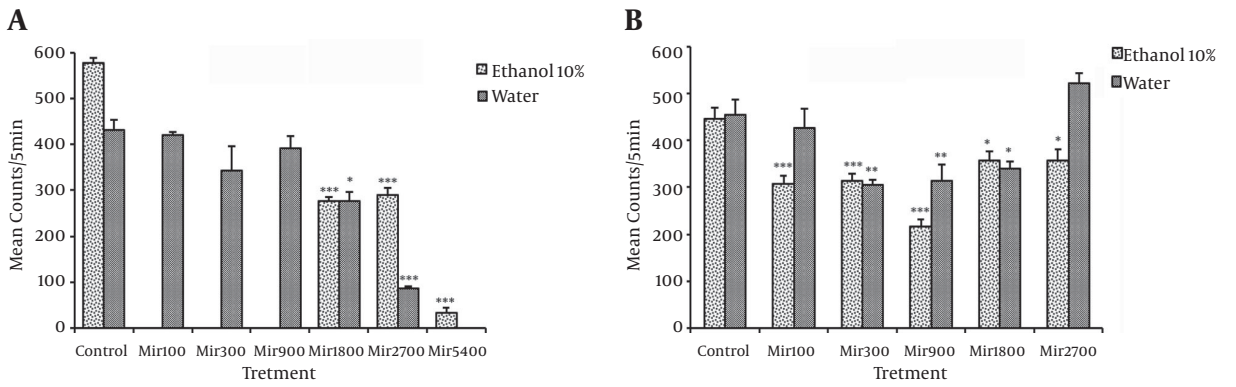

Figure 1. Bars represent mean ± SEM of the mean activity counts per 5 min during a 20-min test period for the hydroalcoholic extracts (100 - 5400 mg/kg) and the aqueous extracts (100 - 2700 mg/kg) of *S. mirzayanii* (A) and *S. macrosiphon* (B) on spontaneous motor activity in mice (N = 6/group). *P < 0.05 compared with control group (ethanol 10% or water) ***P < 0.001 compared with control group (ethanol 10% or water)

4.4. Effects of Plant Extracts on FST

4.4.1. Effects of S. mirzayanii Extracts on FST

Figure 2A shows the effects of different doses of hydroalcoholic extract of *S. mirzayanii* on immobility, swimming, and climbing behaviors. Doses of 150, 300, 600, and 900 mg/kg of the hydroalcoholic extract caused a significant decrease in immobility (11.3%, 19.6%, 13.2%, and 11.3%, respectively) in comparison to control group (P < 0.001). In addition, the hydroalcoholic extract caused significant increases in swimming at doses of 150 and 300 mg/kg (97.6% and 156.0%, respectively) (P < 0.001); and in climbing at doses of 600 and 900 mg/kg (79.8% and 42.1%, respectively) (P < 0.001) in comparison to the control group (Figure 2A).

The aqueous extract of *S. mirzayanii* significantly decreased immobility at doses of 900 and 1800 mg/kg (11.1% and 10.8%, respectively, P < 0.01); and increased swimming at a dose of 1800 mg/kg (68.6%, P < 0.01) as compared with the control group (Figure 2B). The aqueous extract at a dose of 300 mg/kg caused a decrease in immobility (7.6%, P = 0.06) and an increase in swimming (48.5%, P = 0.08) as compared with control, however, it did not statistically reach a significant level.

Both fluoxetine and imipramine significantly decreased immobility, while fluoxetine increased swimming and imipramine increased climbing behaviors in comparison to the control group (P < 0.001).

4.4.2. Effects of the S. macrosiphon Extracts on FST

One-way ANOVA did not show any significant effect of hydroalcoholic extract of the *S. macrosiphon* on immobility, swimming, and climbing behaviors. However, Dunnet *t* test indicated that the dose of 100 mg/kg hydroalcoholic extract of the *S. macrosiphon* significantly increased climbing behavior (17.8%, P < 0.05) in comparison to the control group (Figure 3A).

The aqueous extract of *S. macrosiphon* decreased immobility at doses of 300, 600, and 900 mg/kg (11.1%, 13.3%, and 14.2%, respectively, P < 0.05); and increased swimming at doses of 600 and 900 mg/kg (53.3% and 61.8%, P < 0.05) as compared with the control group (Figure 3B).

Fluoxetine increased swimming and imipramine increased climbing behaviors; while both fluoxetine and imipramine significantly decreased immobility in comparison to the control group (Figure 3B).

5. Discussion

The present study showed that the aqueous and hydroalcoholic extracts of *Salvia mirzayanii* and the aqueous extract of *Salvia macrosiphon* had antidepressant-like activity in the forced swimming test.

FST is the most valuable model that is used for assessment of antidepressant activity in rodents (26, 27). Although all antidepressant drugs reduce immobility as an index of their antidepressant activity in the FST, different drugs with different mechanism of action produce two distinct behavioral patterns including climbing and swimming. It has been shown that climbing is increased by drugs acting at noradrenergic system such as imipramine; and swimming is sensitive to increased serotoninergic transmission by drugs like fluoxetine (25, 28).

In the present study, reduced immobility was observed by both the hydroalcoholic and aqueous extracts of *S. mirzayanii*, which suggests that this plant has an antidepressant-like activity. The reduced immobility and antidepressant-like activity of *S. mirzayanii* are not related

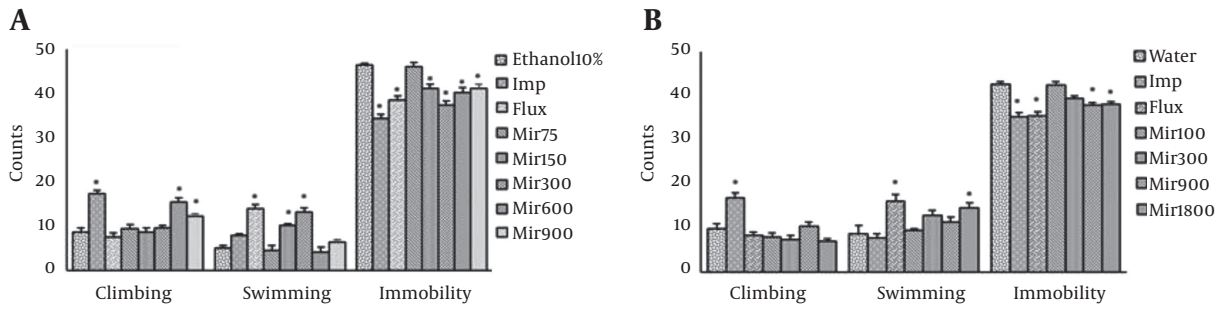

Figure 2. Bars represent means ± SEM of the numbers of climbing, swimming and immobility behaviors for imipramine (imp, 15 mg/kg), fluoxetine (flux, 20 mg/kg), the hydroalcoholic extract (75 - 900 mg/kg) (A) and the aqueous extract (100 - 1800 mg/kg) (B) of *S. mirzayanii* in the forced swimming test (N = 8/group). *P < 0.05 compared with control group (ethanol 10% or water)

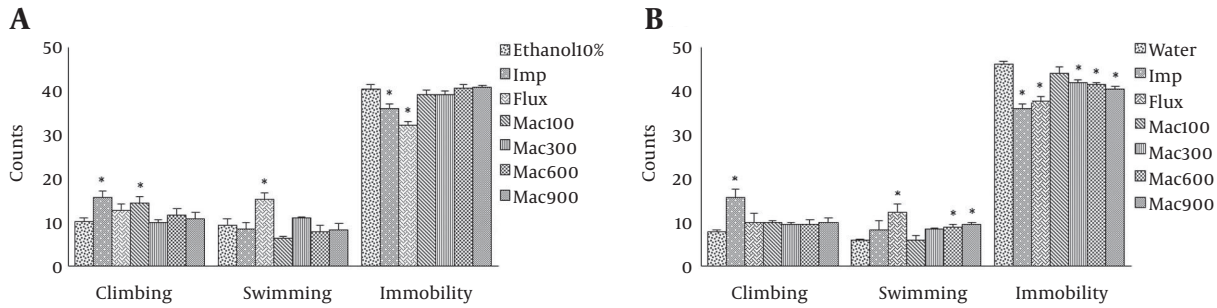

Figure 3. Bars represent means ± SEM of the numbers of climbing, swimming and immobility behaviors for imipramine (imp, 15 mg/kg), fluoxetine (flux, 20 mg/kg), the hydroalcoholic extract (100 - 900 mg/kg) (A) and the aqueous extract (100 - 900 mg/kg) (B) of *S. macrosiphon* s in the forced swimming test (N = 8 mice/group). *P < 0.05 compared with control group (ethanol 10% or water)

to the stimulatory effects of the extracts on motor activity, since the extracts did not increase locomotor activities.

The increased climbing behavior at higher doses of the hydroalcoholic extract of *S. mirzayanii* resembles to that of imipramine; while increased swimming behavior at lower doses of the hydroalcoholic extract is similar to what is seen by fluoxetine. These findings suggest that the hydroalcoholic extract contains compounds that act at both noradrenergic and serotonergic systems. However, the aqueous extract of *S. mirzayanii* increased swimming only at its highest studied dose (i.e. 1800 mg/kg) with no effects on climbing behavior. This suggests that the phytoconstituents of the aqueous extract act through serotonergic system similar to that of fluoxetine.

Comparing the findings of the hydroalcoholic and aqueous extracts of *S. mirzayanii*, it was observed that the antidepressant activity of the hydroalcoholic extract started at lower doses (150 mg/kg) than that of the aqueous extract (900 mg/kg). In addition, the lower dose of the hydroalcoholic extract was more effective in increasing swimming behavior than that seen with the high dose of the aqueous extract. Therefore, it can be implied that compounds responsible for antidepressant-like activity of *S. mirzayanii* are more soluble in ethanol than in water; and further studies for determining and isolating the constituent (s) with antidepressant activity should focus on the hydroalcoholic extract of *S. mirzayanii*.

The phytoconstituent is responsible for the antidepressant-like activity of *S. mirzayanii* and the mechanism by which it acts is currently unclear. However, in this study, phytochemical screening revealed that the extracts of *S. mirzayanii* like many other species of salvia genus contain tannins and flavonoids (29, 30). The current finding is also in agreement with a previous report indicating that the methanolic extract of *S. mirzayanii* contained flavonoids including rutin and luteolin (31). In addition, phenolic compounds such as rosmarinic acid and catechin were found in methanolic extract of *S. mirzayanii* (31). Noteworthy, the antidepressant-like activity has been demonstrated for rosmarinic acid (32), luteolin (33), rutin (34, 35) and catechin (36). Moreover, previous studies have shown that tannins and flavonoids can possess antide-

pressant effects (37, 38). In this regard, it is notable that the antidepressant activity of *S. elegans* has been attributed to a flavon, named isosakuranetin-5-O-rutinoside (39). Therefore, it is possible that these phytochemicals present in *S. mirzayanii* have contributed to the antidepressant effects of *S. mirzayanii*. Certainly, further investigations are required to clarify the key component of *S. mirzayanii* accountable for its antidepressant action. The mechanisms by which the extracts of *S. mirzayanii* exert antidepressant activity have to be elucidated in future studies as well. However, the patterns of behaviors showed by *S. mirzayanii* are suggestive of its action on both noradrenergic and serotonergic systems. In agreement with this notion, it has been suggested that rutin and catechin, phytochemicals present in the extracts of *S. mirzayanii*, produce their antidepressant activities possibly by increasing serotonin and norepinephrine availabilities in the synaptic cleft (34). In addition, rosmarinic acid, present in the extracts of *S. mirzayanii*, has been shown to act as an antidepressant through down regulation of mitogen-activated protein kinase phosphatase-1 and upregulation of brain-derived neurotrophic factor (32). Rosmarinic acid has also modulated dopamine and corticosterone synthesis (32). Furthermore, the antidepressant-like activity of luteolin, another phytochemical of *S. mirzayanii*, has been partly related to the suppression of endoplasmic reticulum stress (33). Any of these mechanisms may be contributed to the antidepressant effects of *S. mirzayanii* extracts. Nevertheless, further studies should address its precise mechanism of action.

In the current study, the aqueous, but not the hydroalcoholic, extract of *S. macrosiphon* showed antidepressant-like activity as indicated by decreasing immobility in the FST. The antidepressant action of the aqueous extract of *S. macrosiphon* could not be due to an enhancement effect on locomotor activity, since the extract decreased spontaneous motor activity.

Comparing the aqueous and hydroalcoholic extracts of *S. macrosiphon*, it can be suggested that the aqueous extract is the effective fraction of *S. macrosiphon* in regards to antidepressant activity. The phytoconstituent(s) responsible for the antidepressant effect has to be identified and isolated in further studies. However, the phytochemical tests in the present study identified tannins in the aqueous extract of *S. macrosiphon*. Tannins are water-soluble compounds of the polyphenolic group. Tannins have exerted antidepressant action in previous studies (38, 40), and might be responsible for antidepressant effects of the aqueous extract of *S. macrosiphon*. There is no other previous report regarding the phytochemicals present in the aqueous extract of *S. macrosiphon* to be justified for its antidepressant activity. Certainly, future phytochemical studies are warranted for detection and isolation of antidepressant active ingredients of the aqueous extract of *S. macrosiphon*. In addition, the mechanism by which the extract exerts its antidepressant effect is currently unknown. However, since the extract increased swimming, but not climbing, behavior, it can be proposed that the *S. macrosiphon* extract mainly acts through serotonergic system. This is in agreement with previous reports regarding the involvement of serotonergic brain systems on antidepressant-like effect of other *Salvia* species extracts i.e. *S. elegans* and *S. verticillata* (15, 39, 41, 42). In addition, salvinorin A, a major active compound of *S. divinorum* extract, has also exhibited antidepressant-like effect by reduced immobility and enhanced swimming behaviors in the FST (17), which is suggestive of serotonergic involvement similar to the *S. macrosiphon* extract. Surely, future studies should clarify this notion.

Comparing the two endemic Iranian *Salvia* species, the findings of this study indicated that the hydroalcoholic extract of *S. mirzayanii* is more effective than the hydroalcoholic extract of *S. macrosiphon*; while the aqueous of *S. macrosiphon* was more potent than the aqueous extract of *S. mirzayanii* in antidepressant-like activity. This might be due to quantitatively and/or qualitatively different phytoconstituents of these species.

The findings of this study are in agreement with previously reported antidepressant-like activity for other species of salvia genus. Previous reports are mainly used as methanolic, but not aqueous, extract of salvia genus. Thus, studies on rodent model and a single case report have indicated that *S. divinorum* had antidepressant effect (17, 43, 44). In addition, the antidepressive effects have been reported for the hydroalcoholic extracts of *S. elegans* (125 - 2000 mg/kg) and *S. verticillata* (250 - 2000 mg/kg) and the ethanolic extract of *S. lachnostachys* Benth leaves (100 mg/kg) (15, 41, 42, 45). Furthermore, essential oil of *S. sclarea*, another *Salvia* species, had antidepressant-like effect by modulation of dopamine activities in rats (16). The range of antidepressant doses and patterns of behaviors observed in FST for other species of salvia are close to those of two endemic Iranian *Salvia* species used in the current study. This suggests that possibly similar compounds are responsible for antidepressant properties of *Salvia* species.

5.1. Conclusions

The results of this study indicated that both *S. mirzayanii* and *S. macrosiphon* had antidepressant effects. In addition, the hydroalcoholic extract of *S. mirzayanii* and the aqueous extract of *S. microsiphon* were more efficacious fractions of these plants; therefore, further studies to isolate the phytoconstituents responsible for antidepressant activity of *S. mirzayanii* and *S. macrosiphon* should focus on

these fractions. The findings of this study support the use of these plants to treat depression as suggested in traditional medicine.

Footnotes

Authors' Contribution: Masoumeh Emamghoreishi and Katayoun Javidnia conceived and designed research. Fatemeh Ghasemi conducted experiments. Mohammad Fathalipour and Parisa Sarkoohi analyzed data. Mohammad Fathalipour, Masoumeh Emamghoreishi, and Parisa Sarkoohi wrote the manuscript. All authors read and approved the manuscript.

Conflict of Interests: The authors declare that they have no conflict of interest.

Ethical Approval: All experiments were approved by the Ethics Committee of Shiraz University of Medical Sciences (#ec-p-91-5317).

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