Original Article

Anxiolytic and Antidepressant Effects of Aqueous Extract of *Stachys lavandulifolia* Vahl. in Mice

Abstract

Background: *Stachys lavandulifolia* Vahl. is a widely used plant in Iranian traditional medicine. It has long been used as an anxiolytic herb. The aim of this work was to investigate the anxiolytic and antidepressant properties of aqueous extract of aerial parts of *Stachys lavandulifolia* Vahl. in mice. **Materials and Methods:** For evaluation of anxiolytic and antidepressant effects of the plant, different doses (500, 1000, 1200, and 1400 mg/kg) of the extract were used in the mouse models of elevated plus maze (EPM) and forced swimming test (FST), respectively. The possible anxiolytic mechanism of the extract was determined by using pentylenetetrazol (PTZ) (10 mg/kg), propranolol (0.2 mg/kg), and atropine (0.5 mg/kg). Sedative effect of the extract was evaluated by ketamine sleeping time test, and rotarod was used to determine the effects of the extract on motor function. Statistical analysis was performed using one-way analysis of variance (ANOVA). **Results:** The results showed that the percentage of time spent and number of entries is significantly increased ($P < 0.05$) with doses of 1000 and 1200 mg/kg of the extract. PTZ but not propranolol or atropine reversed the effects of the extract (1000 mg/kg) on EPM. Neither doses of the extract could decrease the immobility of the mice in FST ($P > 0.05$). Motor coordination was impaired (all doses) by the plant. **Conclusion:** This study confirms the anxiolytic properties of aqueous extract of *S. lavandulifolia* and suggests that its effects are mediated through $GABA_A$ receptors. The extract does not have sedative properties but the results are indicative of a coordination impairing potential of the extract.

Keywords: *Antidepressant, anxiolytic, mechanism, Stachys lavandulifolia Vahl.*

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Received: 01 Oct 2019
Accepted: 19 Feb 2020
Published: 26 Jun 2020

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Access this article online

Website: www.jrpsjournal.com

DOI:10.4103/jrptps.JRPTPS_108_19

Quick Response Code:

Introduction

Anxiety disorders are a group of mental disorders that as a group make the most prevalent mental health problems in the world (anxiety). The common features of all anxiety disorders are that the response of the affected individual is not proportional to the actual or potential risk or threat posed.[1] The treatments for anxiety disorders can be grouped as psychological, pharmacological, or the practice of complementary and alternative medicine (CAM).[2] Natural products such as herbal remedies are a category of CAM therapies. Many people prefer herbal products to conventional medications because the latter has more side effects and limited efficacy.[3] Among pharmacological options, antidepressants are the first-line treatment today. They have a better safety profile and absence of misuse potential in comparison to the benzodiazepines, another widely used treatment option in anxiety disorders. But antidepressants also have numerous unwanted effects on major organs of the body, including cardiovascular, central nervous system, genitourinary, and gastrointestinal tract.[4]

The genus *Stachys* belongs to the Lamiaceae family, which includes approximately 300 species worldwide from which 13 are endemic to Iran.[5] *Stachys lavandulifolia* Vahl. is distributed wildly in Iran, and traditionally has been used as a herbal tea in a variety of conditions, including inflammatory diseases, anxiety, cough, gastrointestinal disorders, ulcers, fevers, and diarrhea.[6] Previous studies showed antimicrobial,[7,8] analgesic,[9] anxiolytic,[10,11] and antioxidant[12,13] properties of different extracts of *S. lavandulifolia*.

In two separate studies, Rabbani *et al.*[10,11] have investigated the anxiolytic effects of the hydroalcoholic (HA) extract and fractions of the plant in mice. They have shown that the HA (50 mg/kg), petroleum ether (25 and 50 mg/kg), ethyl acetate (25 and 50 mg/kg), and water (50 mg/kg) fractions of *S. lavandulifolia* significantly increased the percentage of time spent and number of entries in the open arms. As the aqueous extract of the plant is used in traditional practice of the herb in this study,

How to cite this article: Modarresi M, Mohammadi-Farani A, Amirshahrokhi K, Beheshtizadeh R. Anxiolytic and antidepressant effects of aqueous extract of *Stachys lavandulifolia* Vahl. in mice. J Rep Pharm Sci 2020;9:31-40.

we have decided to study the aqueous formulation of the plant extract. In previous studies, the mechanism of action of the extract was not investigated; hence, here we have also evaluated the mechanism of action of the drug. Other possible properties such as sedative, antidepressant, and effects of the drug on general motor behavior were also investigated.

Materials and Methods

Preparation of aqueous solution

Arial parts of *S. lavandulifolia* were collected from Bostanabad area in East Azarbaijan province in Iran (May 2018) and dried in sunlight. The botanical identity of the plant was confirmed by a botanist. A voucher specimen number (5594) was obtained from the Herbarium of Research Center of Agriculture and Natural Resources of Kermanshah Province (Kermanshah, Iran). For preparation of aqueous extract, 30 g of coarsely powdered plant material was boiled in 1500 mL of distilled water. The mixture was heated on water bath (90°C) and occasionally stirred for 1 h. Aqueous extract was filtered and dried under vacuum at 40°C.

Determination of total flavonoid content

Briefly, 0.5 mL of extract solution was mixed with 4.5 mL of distilled water. Next, 0.5 mL of sodium nitrite solution (5%) was added to the mixture. After 5 min, 0.5 mL of aluminum chloride (10%) was added and left for six more minutes. Then 4 mL of sodium hydroxide (1 M) was added to the mixture. Fifteen minutes later, the absorbance of the mixture was read at 510 nm (Apel PD-303 UV; Saitama, Japan). Calibration curve was created using quercetin as a standard flavonoid.[14]

Determination of total iridoid content

For the determination of iridoid content of the plant, 3 mL of extract solution (2 mg/mL, in distilled water) was added to 1 mL of glycine solution (10%) and 1 mL of sulfuric acid (0.1 M). The mixture was maintained in a boiling water bath for 20 min. The volume of the extract was increased to the final volume of 10 mL by distilled water. Blank samples were simultaneously prepared, and the absorbance was measured at 554 nm.[15]

Chemicals

Atropine sulfate, pentylenetetrazol (PTZ), and propranolol hydrochloride were purchased from Merck (Darmstadt, Germany). Imipramine hydrochloride and diazepam were obtained from Ciba-Geigy (Basle, Switzerland). All drugs were dissolved in normal saline in desired concentrations in a way that the final injection volume was 10 mL/kg.

Animal experiments

Male NMRI mice (25–30 g) were used in the experiments. They were kept in a room with controlled environmental conditions (temperature, 22°C ± 2°C; humidity, 65% ± 2%; and light/dark time, 12/12 h). The mice had free access to water and standard diet. Experiments were performed between 9 am and 1 pm. Drugs were administered intraperitoneally (i.p.). Seventy two mice were randomly divided into 12 groups (6 mice in each group). Different doses of plant extract (500, 1000, 1200, and 1400 mg/kg), diazepam (1.5 mg/kg), normal saline (10 mL/kg), PTZ (10 mg/kg), PTZ + plant extract (1200 mg/kg), propranolol (0.2 mg/kg), propranolol + plant extract (1200 mg/kg), atropine (0.5 mg/kg), and atropine + plant extract (1200 mg/kg) were administered to the mice (i.p.). The minimum dose of the extract (500 mg/kg) was determined by pilot studies, and the last dose (1400 mg/kg) was chosen according to the ineffective anxiolytic action of the extract. All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at Kermanshah University of Medical Sciences (ethical code, 1396.356).

Elevated plus maze

Elevated plus maze (EPM) is a common model for the evaluation of anxiety in rodents. This model is based on the natural aversion of rodents of open spaces placed higher than the ground surrounding. The apparatus is a plus-shaped maze, elevated to a height of 65 cm. There are two opposite open (35 × 5 × 15 cm) and two opposite closed arms (35 × 5 cm), and the junction of the four arms is measured 5 × 5 cm.[16]

Thirty minutes after the administration of plant extract and drugs, mice were placed on the closed arm of EPM with their faces toward the end of the arm. Total number of entries into open and closed arms and the time spent in the open and closed arms were recorded during the 5-min test period. The percentage of open and close arm entries and the percentage of open and close arm time were calculated for each mouse. Between each trial, the maze was cleaned with a piece of wet cotton.

Locomotor activity

This test is based on photobeam recording where locomotion of the mice is calculated as the number of beams crossed in a certain amount of time.[17] Thirty minutes after administration of plant extract and drugs, the mice were placed on the locomotor apparatus. Locomotor activity was measured at a 5-min period.

Ketamine-induced sleeping time

Ketamine-induced onset of sleep time was measured to evaluate the hypnotic activity of the extract. Thirty minutes after administration of plant extract and drugs, the mice received a single injection of ketamine (3 mg/kg). Induction time was recorded as the time needed for the mice to lose their righting reflex and to show the onset of sleep. The time before regaining the righting reflex was recorded as duration of sleep.[18]

Rotarod

This test is used to evaluate the motor coordination of the mice. The rotarod apparatus consists of a metal rod attached to a motor, which rotates at the speed of 20 rpm. Each mouse was injected with the plant extract (500, 1000, 1200, and 1400 mg/kg), diazepam (3 mg/kg), and normal saline

(10 ml/kg). After 30 min, the mice were placed on the rod, and the time before falling of each mouse was recorded in a 180-s interval.[19]

Forced swimming test

This is a test in which the mice are forced to swim for 6 min in a cylindrical container (height 25 cm, diameter 10 cm, 15 cm of water at 21°C–23°C). Each mouse was injected with the plant extract (500, 1000, 1200, and 1400 mg/kg), imipramine (30 mg/kg), and normal saline (10 mL/kg). After 30 min, the mice were put into the water, and the immobility time was recorded in the last 4 min.[20]

Statistics

Statistical analysis was performed with one-way analysis of variance (ANOVA) using *post hoc* Duncan test. Probability values $P < 0.05$ were considered statistically significant. Data were expressed as mean ± standard error.

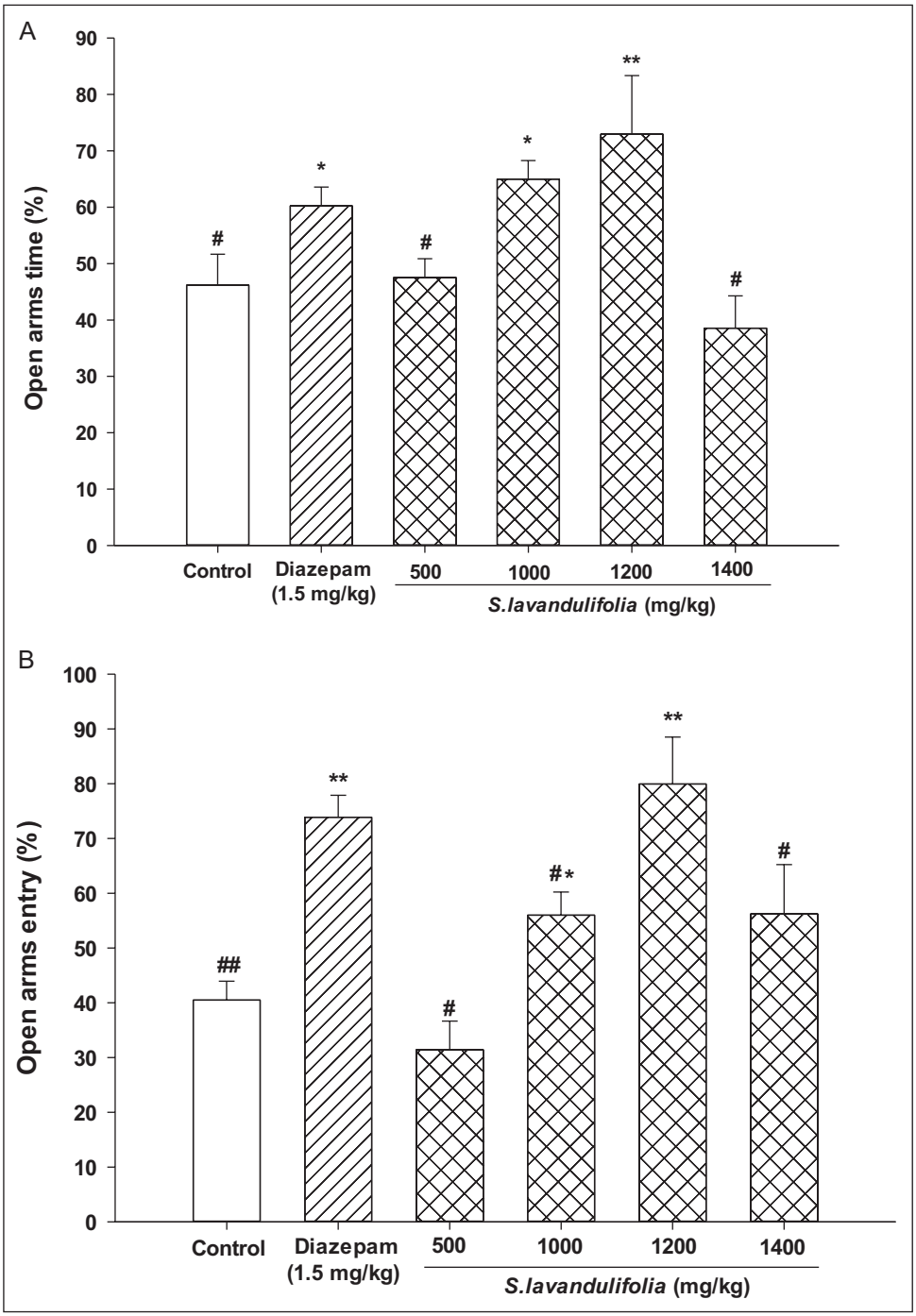

Figure 1: Effect of diazepam and various doses of plant extracts on (A) the percentage of time spent in open arms and (B) the percentage of open arm entries in the elevated plus maze for 5 min in mice. *$P < 0.05$, **$P < 0.01$ compared with vehicle-treated control. #$P < 0.05$ compared with diazepam. Data are reported as mean (± standard error of the mean [SEM]) values. The number of mice is 6–8 per group. All injections were administered 30min before the test

Results

Total flavonoid and total iridoid contents

Total flavonoid and total iridoid contents of aqueous extract of the aerial parts of *S. lavandulifolia* were estimated by quercetin and aucubin standards, respectively. The results showed that the linear relationship was good in the detection range. The total flavonoid and total iridoid contents were determined as 103.76 ± 1.55 mg quercetin equivalent/g of dried extract and 69.20 ± 2.51 mg aucubin equivalent/g of dried extract, respectively.

Elevated plus maze

As evident in Figure 1A and B, diazepam increased the percentage of time spent and number of entries in open arms ($P < 0.05$), and it decreased the percentage of time spent

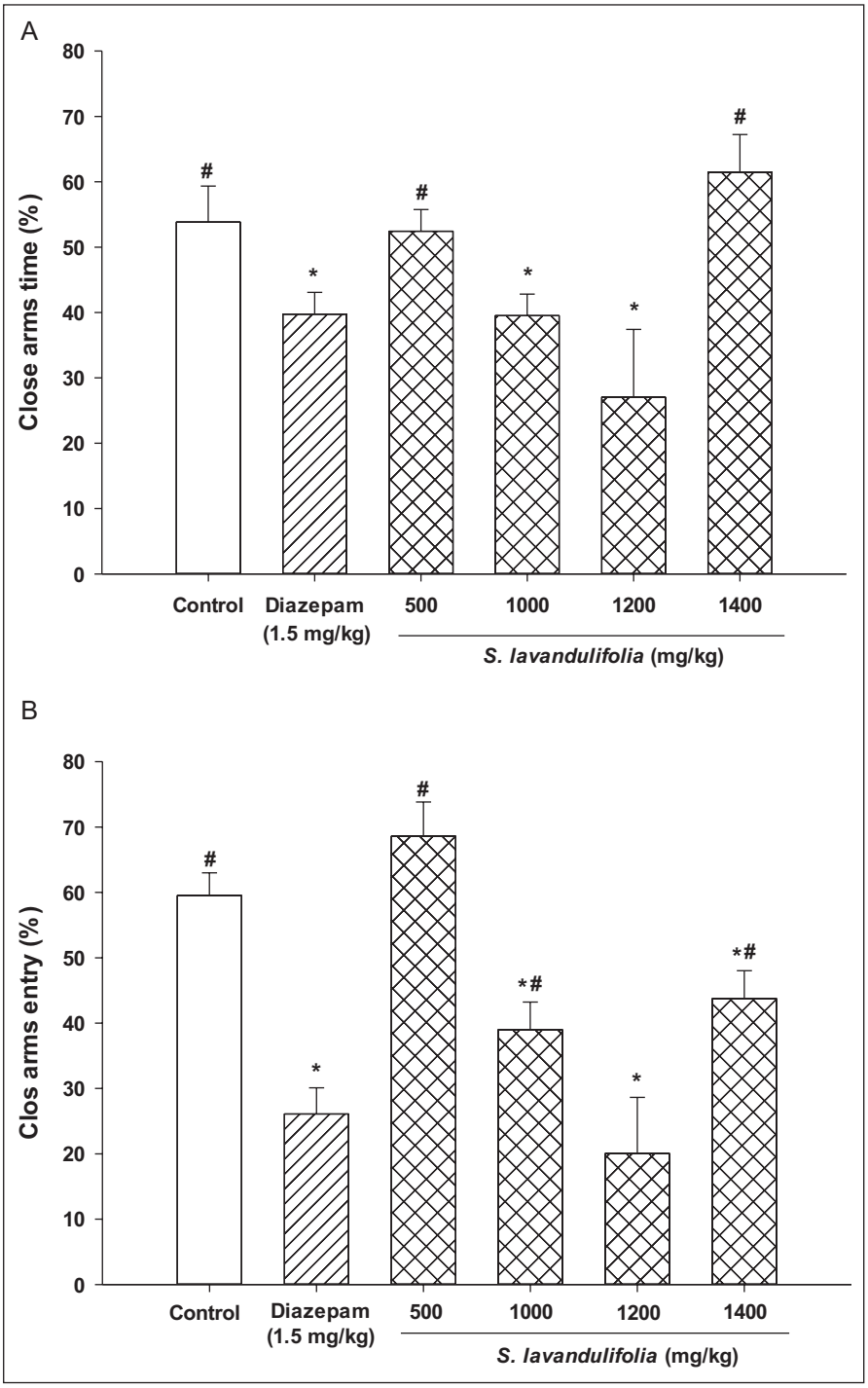

Figure 2: Effect of diazepam and various doses of plant extracts on (A) the percentage of time spent in close arms and (B) the percentage of close arm entries in the elevated plus maze for 5min in mice. *$P < 0.05$ compared with vehicle-treated control. #$P < 0.05$ compared with diazepam. Data are reported as mean (±standard error of the mean [SEM]) values. The number of mice is 6–8 per group. All injections were administered 30min before the tests

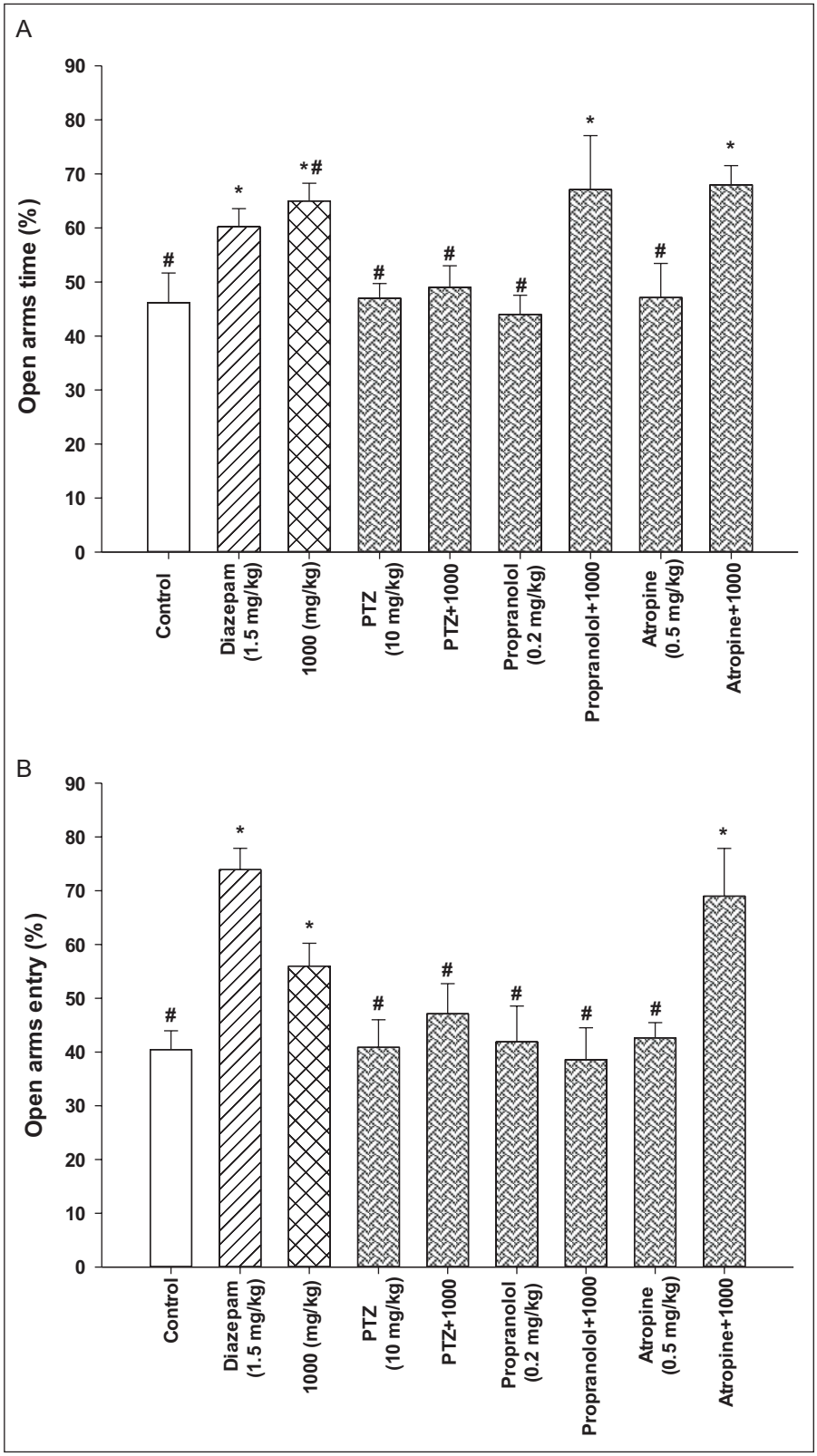

Figure 3: The effects of $GABA_A$ receptor antagonist (pentylenetetrazol; PTZ), β-receptor antagonist (propranolol), and muscarinic receptor antagonist (atropine) on (A) the effects of the plant extract on percentage of time spent in open arms and (B) the percentage of close arm entries in elevated plus maze in mice (*n* = 6–8). **P* < 0.05 compared with vehicle-treated control. #*P* < 0.05 compared with diazepam. Data are reported as mean (±standard error of the mean [SEM]) values. The number of mice is 6–8 per group

and number of entries in closed arms [Figure 2B] of EPM ($P < 0.05$). Doses of 1000 and 1200 mg/kg of *S. lavandulifolia* increased the amount of time spent ($P < 0.05$ and $P < 0.01$, respectively) and the number of entries ($P < 0.05$ and $P < 0.01$, respectively) in open arms of the EPM [Figure 1A and B]. Meanwhile, these doses decreased the amount of time spent ($P < 0.01$) and the number of entries ($P < 0.01$) in closed arms of the EPM [Figure 2A and B].

Administration of PTZ (10 mg/kg) decreased the amount of time [Figure 3A] and the number of entries [Figure 3B] in the plant extract (1000 mg/kg), such that it was not statistically significant with the control group ($P = 0.14$). On the contrary, propranolol (0.2 mg/kg) and atropine (0.5 mg/kg) did not change the effects of *S. lavandulifolia* (1000 mg/kg), and the amount of time and the number of entries were similar to the extract (1000 mg/kg) group ($P > 0.05$).

Locomotor activity

S. lavandulifolia decreased the spontaneous motor activity of the extract at the dose of 1200 mg/kg ($P < 0.05$). All doses of the extract had greater locomotor activities compared to diazepam ($P < 0.05$) [Figure 4].

Ketamine-induced sleeping time

As shown in Figure 5A, the latency to fall asleep was decreased ($P < 0.001$) by diazepam (1.5 mg/kg), and it was unchanged ($P = 0.02$) by the aqueous extract (1200 mg/kg) compared to the control group. Diazepam increased the duration of sleep ($P < 0.05$) as compared to the control but our extract did not have a statistically significant effect on sleep duration compared to the control group [Figure 5B].

Rotarod test

Results of the rotarod test showed that diazepam impaired ($P < 0.05$) the performance of the mice as compared to the control. Different doses of the extract also decreased ($P < 0.05$ for 500 mg/kg, $P < 0.01$ for 1000 mg/kg, and $P < 0.001$ for 1200, 1400 mg/kg) the riding time of the mice on the apparatus. The level of performance was significantly ($P < 0.001$) less than diazepam for doses of 1200 and 1400 mg/kg of the extract [Figure 6].

Forced swimming test

Immobility time of the mice receiving various doses of plant extract (500, 1000, 1200, and 1400 mg/kg) was compared to imipramine (30 mg/kg) and vehicle as respective positive and negative controls [Figure 7]. Imipramine as expected decreased ($P < 0.001$) the immobility time of the mice in water as compared to the vehicle control group. Plant extract increased the immobility of the mice at doses of 1000 ($P < 0.05$), 1200 ($P < 0.01$), and 1400 mg/kg ($P < 0.001$).

Discussion

This study showed that the aqueous extract of *S. lavandulifolia* (1000 and 1200 mg/kg, i.p.) increased both the percentage of entries and the percentage of time spent in open arms and decreased the percentage of entries and time in closed arms in the EPM model of anxiety in mice [Figures 1 and 2], thus indicating its anxiolytic properties. These results are in accordance with previous reports about the anxiolytic properties of diazepam.[21-23] Several neurotransmitters (such as norepinephrine, dopamine, serotonin, gamma-aminobutyric acid (GABA), glutamate, and acetylcholine)[24,25] and some neuropeptides (such as substance

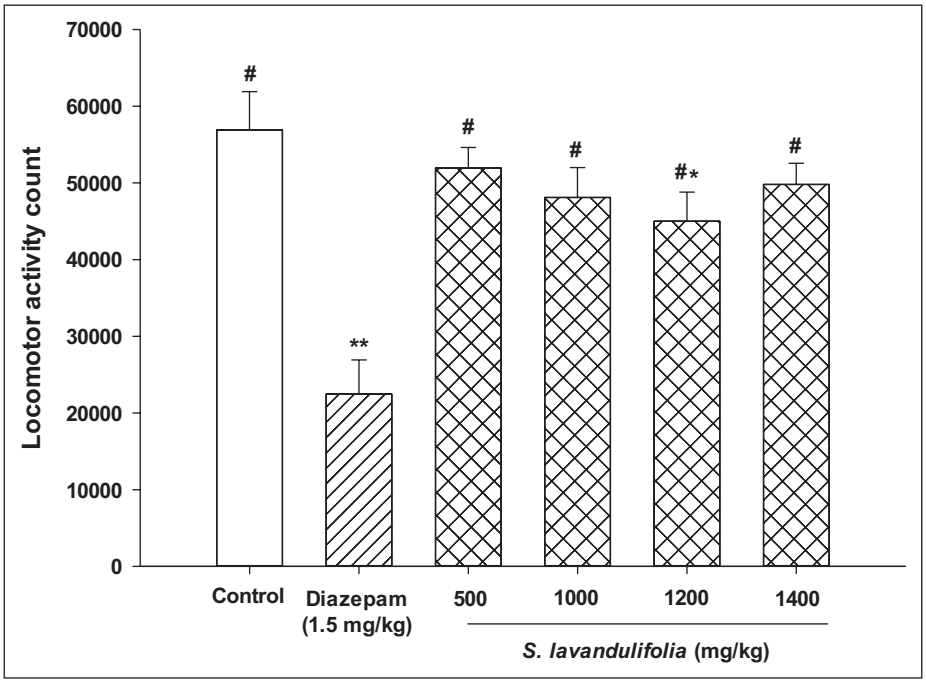

Figure 4: Effects of diazepam and various doses of aqueous extract of *Stachys lavandulifolia* on locomotor activity in mice (n = 6–8). *$P < 0.05$ compared with vehicle-treated control. **$P < 0.001$ compared with vehicle-treated control. #$P < 0.05$ compared with diazepam. Locomotor activity counts are calculated over 5min, which began 30min after the drugs were used

P, neuropeptide Y, and corticotropin-releasing factor)[26] are important in the neurobiology of anxiety disorders. It has been known for a long time that benzodiazepines as an old class of anxiolytics exert their action through facilitation of GABAergic neurotransmission.[27] The anxiolytic action of the extract is prevented by the pretreatment of the mice with subeffective doses of a $GABA_A$ receptor antagonist (i.e., PTZ) [Figure 3]. This indicates that the anxiolytic action of the extract depends on the ionotropic GABA receptors. Beta blockers and antimuscarinic agents are two other important classes of drugs that are used in anxiety-related conditions.[28] Subeffective doses of atropine or propranolol could not add to the effects of the least effective dose of extract (1000 mg/kg) in our study. This means that its effects are not mediated through blocking beta or muscarinic receptors.

Currently used anxiolytic drugs have major drawbacks such as having debilitating side effects (e.g., on motor function) or not being effective in some forms of anxiety disorders.[29] For further evaluation of the effects of the extract on motor behavior,

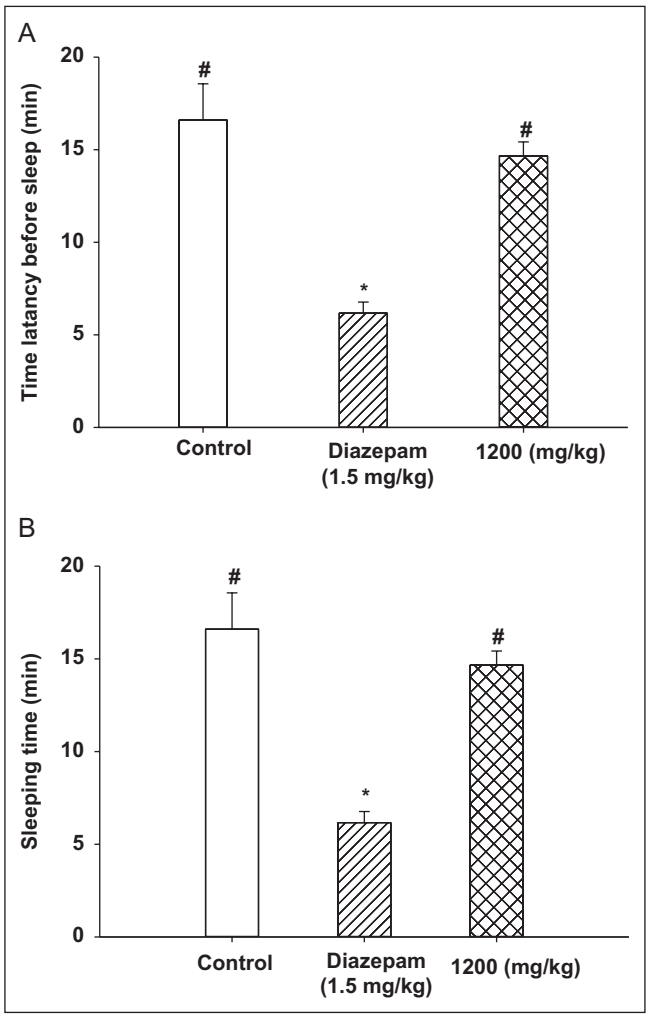

Figure 5: Effects of diazepam and *Stachys lavandulifolia* aqueous extract on (A) the latency to fall asleep and (B) total sleeping time in mice (n = 6–8). *P < 0.05 compared with vehicle-treated control. #P < 0.05 compared with diazepam group. Results are shown as mean ± standard error of the mean (SEM)

spontaneous motor activity and rotarod performance were also assessed in the mice using the most effective anxiolytic dose of the extract (1200 mg/kg). The results showed that this dose of the extract significantly decreased the locomotor activity of the mice [Figure 5A] measured in locomotometer. Locomotor activity can represent the psychostimulant or sedative properties of a compound.[30] Therefore, this extract must have sedative properties compared to the control group. Locomotor activity in the extraction group is also significantly higher compared to the diazepam group [Figure 5A]. This indicates that it has less sedative effects compared to diazepam. The rotarod test is a screening method for the evaluation of the neuromuscular impairment of the mice.[31] The results showed that our aqueous extract significantly decreased the latency to fall from the rotarod in a dose-dependent manner [Figure 6]. This implies that the extract has a deleterious effect on neuromuscular coordination.[31] The results of the rotarod test also suggest that the inhibitory effects of the extract on locomotor activity might be exerted through peripheral neuromuscular mechanisms rather than having a central sedative effect.[32] This assumption is reinforced by the results from the ketamine test because these results showed that the extract (1200 mg/kg) is devoid of sedative or hypnotic effects as it does not change time latency before sleep or sleep duration of the mice compared to the control group [Figure 5]. Having no effects on these sleep parameters can be considered as a positive feature for the extract, as sedation is a major problem with many traditional anxiolytic drugs, especially benzodiazepines.[33]

The effects of the extract on motor function also changes the interpretation of the results obtained from forced swimming test (FST). This test is sensitive to all major classes of antidepressant drugs such as selective serotonin reuptake inhibitors, tricyclics, and monoamine oxidase inhibitors.[34] In our experiments, the time of immobility is increased in a dose-dependent way [Figure 7]. In FST, it is assumed that immobility is correlated with behavioral despair.[35] Considering the results of locomotor and rotarod tests, we can say that the elevated time of immobility is attributed to the decreased basal activity level rather than a psychomotor effect.[36] So a straight and simple conclusion would be that the extract does not show antidepressant activity, and the increased time of immobility is the result of the effects of the extract on motor function of the mice instead of having a pro-depressive effect.

Flavonoids, diterpenoids, quinines, iridoids, and phenolic acids are found as secondary metabolites in different species of the genus *Stachys*.[37,38] There are a lot of reports on the anxiolytic effects of plant flavonoids.[39-44] Many natural ligands for $GABA_A$ receptors have flavonoid structures.[45] Iridoids are also found to have anxiolytic effects;[46-48] however, to the best of our knowledge, there is no known iridoid with direct $GABA_A$ agonistic action. Consequently, the anxiolytic properties of the extract might be related to its flavonoid and iridoid compounds.

Rabbani *et al.*[11] have shown that the HA extract of *S. lavandulifolia* has anxiolytic effects at 100 mg/kg, and the

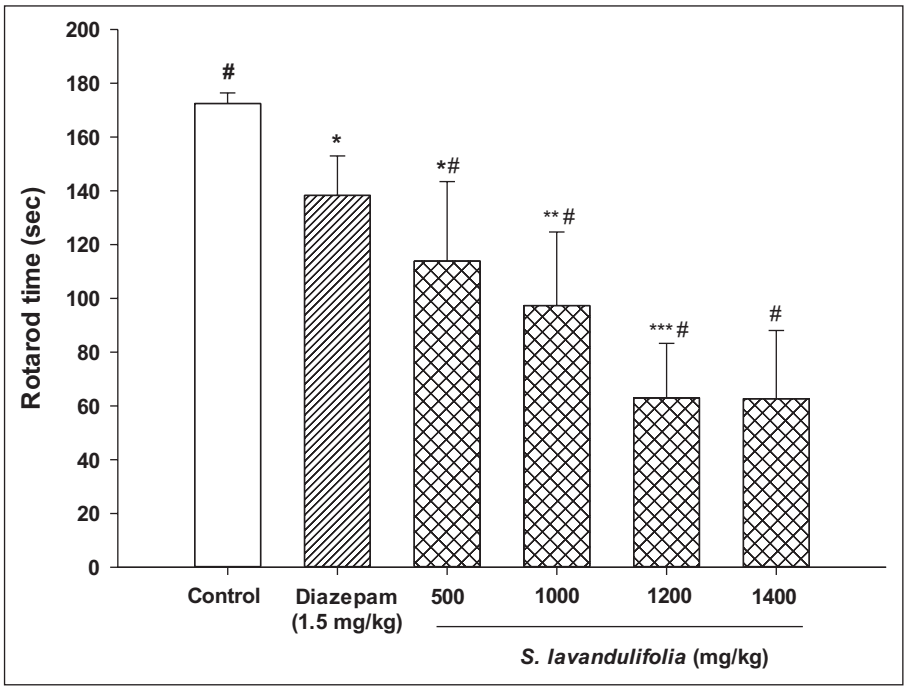

Figure 6: Effects of diazepam and various doses of plant extract on latency to drop off from rotating rotarod (20rpm) in mice (*n* = 6–8). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with control group. #*P* < 0.05 compared with diazepam. Results are shown as mean ± standard error of the mean (SEM)

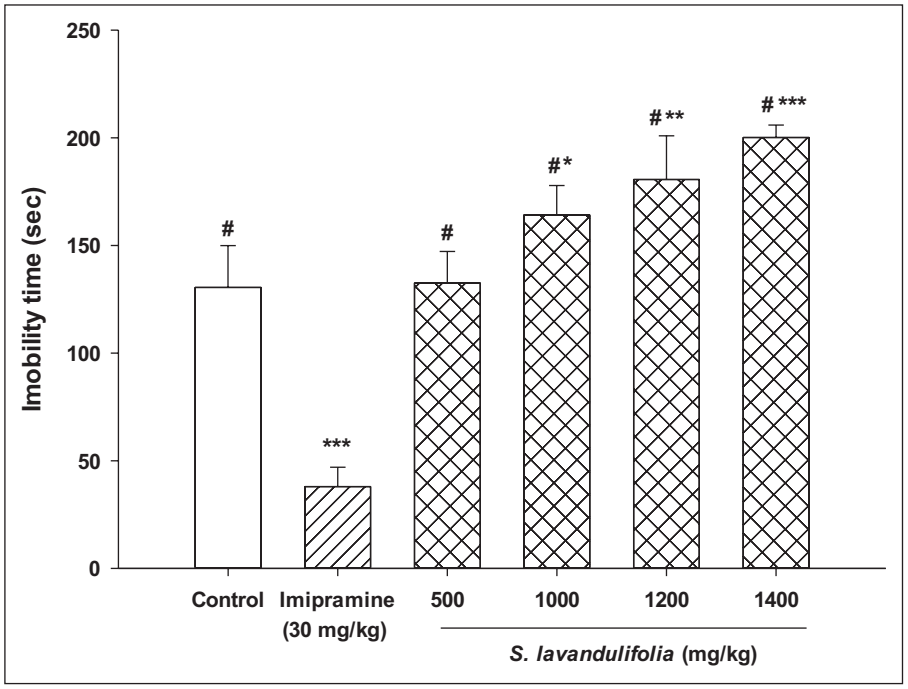

Figure 7: Effects of the treatment of mice with various doses of aqueous extract of the plant on the immobility time in the forced swimming test in mice (*n* = 6-8). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with control group. #*P* < 0.05 compared with diazepam. Results are shown as mean ± standard error of the mean (SEM)

essential oil of the plant has no effects on anxiety level at doses up to 100 mg/kg. They have not reported any defect in the motor activity of the mice.[11]

In another study on HA extract and fractions of the plant, it was shown that the HA (50 mg/kg), petroleum ether (25 and 50 mg/kg), ethyl acetate (25 and 50 mg/kg), and aqueous (50 mg/kg) fractions of *S. lavandulifolia* significantly increased the percentage of time spent and the percentage of arm entries in EPM test in mice.[10] The reason behind getting anxiolytic effects at high doses of the extract might be the fact that aqueous extracts are less enriched in lipid-soluble compounds and hence less prone to cross blood–brain barrier. As we have

used high doses of an aqueous extract, the effects on motor activity may be attributed to the group of compounds that are more water soluble, and hence may have peripheral effects rather than central effects.[49] It is also probable that the effect of active compounds of the extract is masked by some other chemicals in the mixture. This antagonistic relation between different compounds in plant extracts is well-documented in other natural products.[50]

Conclusion

Our study showed that aqueous extract of *S. lavandulifolia* has anxiolytic properties (1000 and 1200 mg/kg), which is not different from diazepam (1.5 mg/kg), and it mediates its action probably through $GABA_A$ receptors. It has less sedative and less effects on spontaneous locomotor activity compared to diazepam. The results also showed that the extract disturbs motor coordination of the mice, which is presumably mediated through peripheral mechanisms.

Acknowledgement

The content of this paper was extracted from the Pharm. D thesis (No. 96442) submitted by Rezvan Beheshtizadeh, which was financially supported by research council of Kermanshah University of Medical Sciences, Kermanshah, Iran.

Financial support and sponsorship

This study was supported by the Kermanshah University of Medical Sciences.

Conflicts of interest

There are no conflicts of interest.

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