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Original Article

Anxiolytic effects of *Lippia citriodora* in a mouce model of anxiety

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Abstract

Lippia citriodora is commonly used in Iranian folk medicine for treatment of many disorders. Since there scientific data to prove the anxiolytic properties of this plant in Iran are scarce, we aimed to evaluate the sedative and anxiolytic activity of the leaf extract and essence of *L. citriodora* in an animal model of anxiety. The extract and the essence used were obtained after maceration and hydro-distillation of the leaves of *L. citriodora*, respectively. We evaluated the anti-anxiety profile and sedative activity of diazepam (1 mg/kg i.p. as the standard), hydroethanolic extract (200 and 400 mg/kg i.p.) and the essence (10, 15, and 50 mg/kg i.p.) of leaves of *L. citriodora* using elevated plus-maze and locomotor activity. We also used flumazenil, to find out if the possible effects are mediated through gamma-aminobutyric acid (GABA)/benzodiazepine receptor complex. The results showed that the essence of *L. citriodora* at a dose of 15 mg/kg is the most effective anxiolytic dose. Interestingly, flumazenil reversed this action of the essence as well as that of diazepam. The extract even at a dose of 400 mg/kg did not show significant anxiolytic effect. In locomotor activity studies, the essence caused sedation to a lesser extent than diazepam. The results suggest that the essence of this plant could be a better candidate for further analysis and fractionation. As the anxiolytic effect of the essence is reversed by flumazenil, it is possible that the GABA receptor could be involved in mediating these effects.

Keywords: *Lippia citriodora*; Anxiety; Sedative activity; Plus-maze; Locomotor activity

INTRODUCTION

Temporary anxiety is a part of life that happens for everyone. Anxiety disorders, however, do not go away and may get worse over time. The feelings of discomfort can interfere with daily activities such as job performance, schoolwork, and relationships (1). Around one eighth of the total world populations are affected by one form of anxiety disorders (2). Patients with anxiety disorders are more likely to experience other comorbidities such as depression, drug abuse, asthma, and cardiovascular disease (3).

Several groups of drugs, synthetic and natural, have been used throughout the history of anxiety treatment. Amongst the synthetic drugs, benzodiazepines and antidepressants have been mostly prescribed with a wide range of side effects and drug interactions (4). The high cost of synthetic drugs and their side effects has forced scientists to search the traditional medicine as an option for concerted search for new chemical entities (5). Various plants (*Fumeria indica, Azadirachta indica, Piper methysticum, Hypericum perforatum, Stachys lavandulifolia, Valeriana officinalis, Melissa officinalis, Ocimum basilicum L.*) from different parts of the world have been studied for the possible sedative and anxiolytic effects (6-12). *Lippia citriodora* from Verbenaceae family is indigenous to Western South America and is cultivated in North Africa, Southern Europe and Middle East (13). In Iran *L. citriodora* (known as Behlimoo) is cultivated in northern parts of the country. The leaves are traditionally used for treatment of fever, colds, flatulence, spasms, asthma, diarrhea, colic, indigestion, insomnia, and anxiety (14).

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The extract and essential oil of *L. citriodora* mainly contains citral (geranial, neral), geraniol, limonene, and cineol. These components have been shown to have anti-oxidative, anti-microbial, anti-inflammatory, anti-fungal, and analgesic effects (15,16).

No data are available on the anxiolytic action of *L. citriodora.* Since the plant has various traditional therapeutic uses such as anxiolytic, and the presence of terpenes in *L. citriodora,* we aimed to examine the anxiolytic effects of the extract and essential oil of this plant.

MATERIALS AND METHODS

Plant extraction and fractionation

The leaves of *L. citriodora* cultivated in northern part of Iran were purchased from local market in August 2016. A voucher sample (No. 3538) of the plant was deposited in the herbarium of the School of Pharmacy and Pharmaceutical Science, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

Extract preparation

Air-dried and powdered leaves of *L. citriodora* (200 g) were extracted at room temperature with 2 L ethanol:water (7:3) using maceration method for 24 h. After filtration of the extract with Buchner funnel, the solvent was evaporated in a rotary evaporator at 70 °C under reduced pressure to produce concentrated extract that was then freeze-dried to produce a dry powder. Appropriate amount of the extract was diluted with normal saline (containing 2 drops of Tween 80 per 10 mL) to obtain the final concentration.

Isolation of essential oil

About 100 g of the air-dried plant materials was subjected to hydrodistillation for 3 h using Clevenger-type apparatus according to the method described in British Pharmacopoeia (17). The volatile oil was stored in a sealed vial at 4 °C until use. Afterward the appropriate volume of essential oil of the plant was diluted with normal saline (containing 2 drops of Tween 80 per 10 mL) to obtain the final concentration.

Animals

Male Syrian mice (Pasture institute, Iran) weighing about 25 g were kept in a room with controlled temperature and light. Animals had free access to food and water. Two hours before experiments, mice were acclimated to the main environment. In order to avoid diurnal cycle, all tests carried out between 9:00 AM to 13:00 PM. Each mice received a single intraperitoneal (i.p.) injection and used just for one test. Four experimental groups with at least 6 mice in each group were used for locomotor activity test and ten experimental groups with at least 6 mice in each group were used for elevated plus-maze (EPM) test.

All animal experiments were approved by the Animal Research Ethics Committee of Isfahan University of Medical Science (ethical approval ID: 394702) and performed in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals.

Drugs

Diazepam hydrochloride injectable solution in glass ampoule (10 mg/2 mL) was provided by Caspian Tamin Drug Company (Iran). Each ampoule was diluted with saline solution (containing 0.5% Tween 80) to reach desired concentration. Flumazenil injectable solution was obtained from Sandoz Company (0.5 mg/5 mL).

The *L. citriodora* extract and essential oil were diluted with normal saline containing 0.5% Tween 80. All injectable solutions were prepared on the day of experiment and administered i.p. in a volume of 0.1 mL/10 g mice body weight.

Elevated plus-maze

Elevated plus-maze is used to measure the anxiolytic activity. The maze has two open (30 × 5× 0.2 cm) and two closed (30 × 5× 15 cm) arms extended from a central platform (5 × 5 cm) and is located 45 cm above the floor. The whole maze is made of wood and painted in black. The maze totally cleaned by ethanol and allowed to dry between subjects in order to eliminate any odor cues.

A single i.p. dose of diazepam (1 mg/kg), extract at 200 or 400 mg/kg, essential oil at 10, 15, or 50 mg/kg, or the vehicle was administered to a mouse 30 min before the onset of the test. Each mouse was used just once and for one test. Experiments were carried out in a sound-proof room with a dim light. At the beginning of the test the mouse was placed at the center of the maze facing a close arm and during a 5 min observation the total number of open and closed arms entries and the time spent in open and closed arms were recorded.

Open arm entries were defined as existence of two paws in an open arm. For each mouse the percentage of open/closed arm entries is calculated as follows (12,18):

$$\% \frac{Open \text{ arm entries}}{Closed \text{ arm entries}} = \frac{Entries\ to\ open\ arm}{Total\ entries} \times 100 \quad (1)$$

The percentage of time spent in open/closed arm for each animal was determined as follows:

$$\% \frac{Time\ spent\ open\ arm}{Time\ spent\ closed\ arm} = \frac{Time\ spent\ in\ open\ arm}{Total\ time\ spent\ in\ both\ arms\ (5\ \min observastion)} \times 100 \quad (2)$$

In addition, we studied the effects of flumazenil, which is a GABA/benzodiazepine receptor complex antagonist, on the anxiolytic effect of the essential oil of *L. citriodora* in order to examine the probable involvement of GABAergic system. We selected 3 groups each containing 6 mice. Flumazenil (2 mg/kg, i.p.) administrated to mice of the first group, 5 min before administration of the essential oil (15 mg/kg, i.p.), and 35 min before the test. The mice of the second group received flumazenil (2 mg/kg) 5 min before the injection of diazepam (1 mg/kg). The third group was served as the control which received flumazenil (2 mg/kg) 5 min prior to the normal saline. The anxiolytic activity of essential oil (15 mg/kg), diazepam (1 mg/kg) and normal saline in animals pretreated with flumazenil was assessed and compared with essential oil (15 mg/kg), diazepam (1 mg/kg) and normal saline-treated mice (19,20).

Locomotor activity

In order to record spontaneous locomotor activity, a rectangular dark cage with a computerized infrared tracking device which automatically records two parameters of activity (time and count) during a 3 sessions of 5 min observation (total 15 min) were used. The total locomotor activity count was calculated by multiplication of time and the number of movements. The movement count unit is measured by the number of breaking beams and the movement time unit is based on the total time of beam breaks by the mouse. Each mouse was injected with a single dose of diazepam (1 mg/kg), control component, essential oil (15 mg/kg) or extract (400 mg/kg) 30 min before the onset of the test. After each test, the cage was cleaned with ethanol and allowed to dry in order to avoid any odor cues (12).

Statistical analysis

All the results were expressed as mean ± SEM, and were analyzed using a one-way analysis of variance (ANOVA) or Student's t-test where appropriate. All statistical analyses were carried out using Sigma Stat 3.1. (Systat Software, Inc). The results were considered statistically significant if $P < 0.05$.

RESULTS

Plant extract

In this study, the extract and essential oil of *L. citriodora* were prepared. The amount of extract and the essential oil was 29.66% w/w and 0.6% v/w, respectively.

Elevated plus maze

In the elevated plus maze test, diazepam (1 mg/kg) significantly increased the number of entries to the open arm (Fig. 1A) and the percentage of time spent (Fig. 1B) ($P < 0.001$). Two different doses of the extract including 200 and 400 mg/kg were tested that results in no significant effect on the number of entries to the open arm (Fig. 1A) and the time spent (Fig. 1B). Three different doses of essential oil including 10, 15, and 50 mg/kg were administered. Although significant effect for all three doses was observed in the open arm entry ($P < 0.05$), only 15 mg/kg of essential oil caused significant increase in the percentage of time spent in the open arm ($P < 0.05$) (Figs. 1A and 1B).

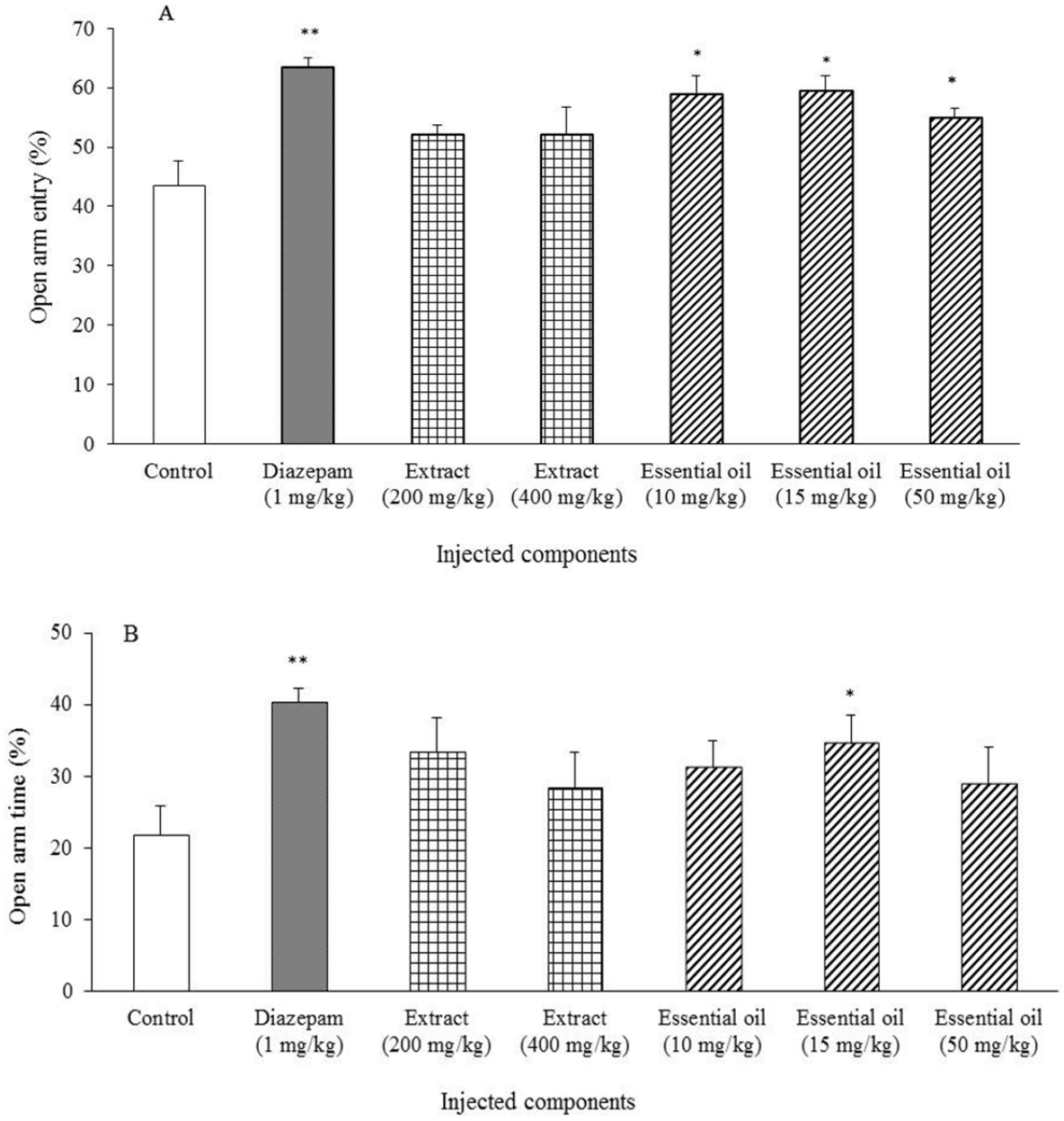

Fig. 1. Effects of diazepam, vehicle, and different doses of *L. citriodora* and hydroalcoholic extract on (A), the percentage of entries into the open arms and (B), the percentage of time mice spent in the open arms during a 5 min test. 200 and 400 mg/kg of the plant's hydroalcoholic extract, 10, 15, and 50 mg/kg of the plant's essential oil, 1 mg/kg diazepam, or control were injected 30 min prior to testing. Data are presented as mean ± SEM for each group of at least 6 mice. *$P < 0.05$ compared to control group. **$P < 0.001$ compared to control group.

Flumazenil effect on the L. citriodora's anxiolytic activity

In the elevated plus-maze model, administration of flumazenil (2 mg/kg) 5 min before the administration of the most effective dose of *L. citriodora* essential oil (15 mg/kg) and 1 mg/kg of diazepam significantly decreased the the number of entries to the open arm (Fig 2A) and the time spent. (Fig 2B) ($P < 0.05$ and $P < 0.001$, respectively). Therefore, flumazenil reversed the anxiolytic activity of *L. citriodora* essential oil and diazepam by decreasing the number of entries and the prolongation of time spent in open arms induced by diazepam ($P < 0.001$) or the essential oil ($P < 0.05$) of *L. citriodora*. However, no significant effect was observed in the control group. (Fig. 2).

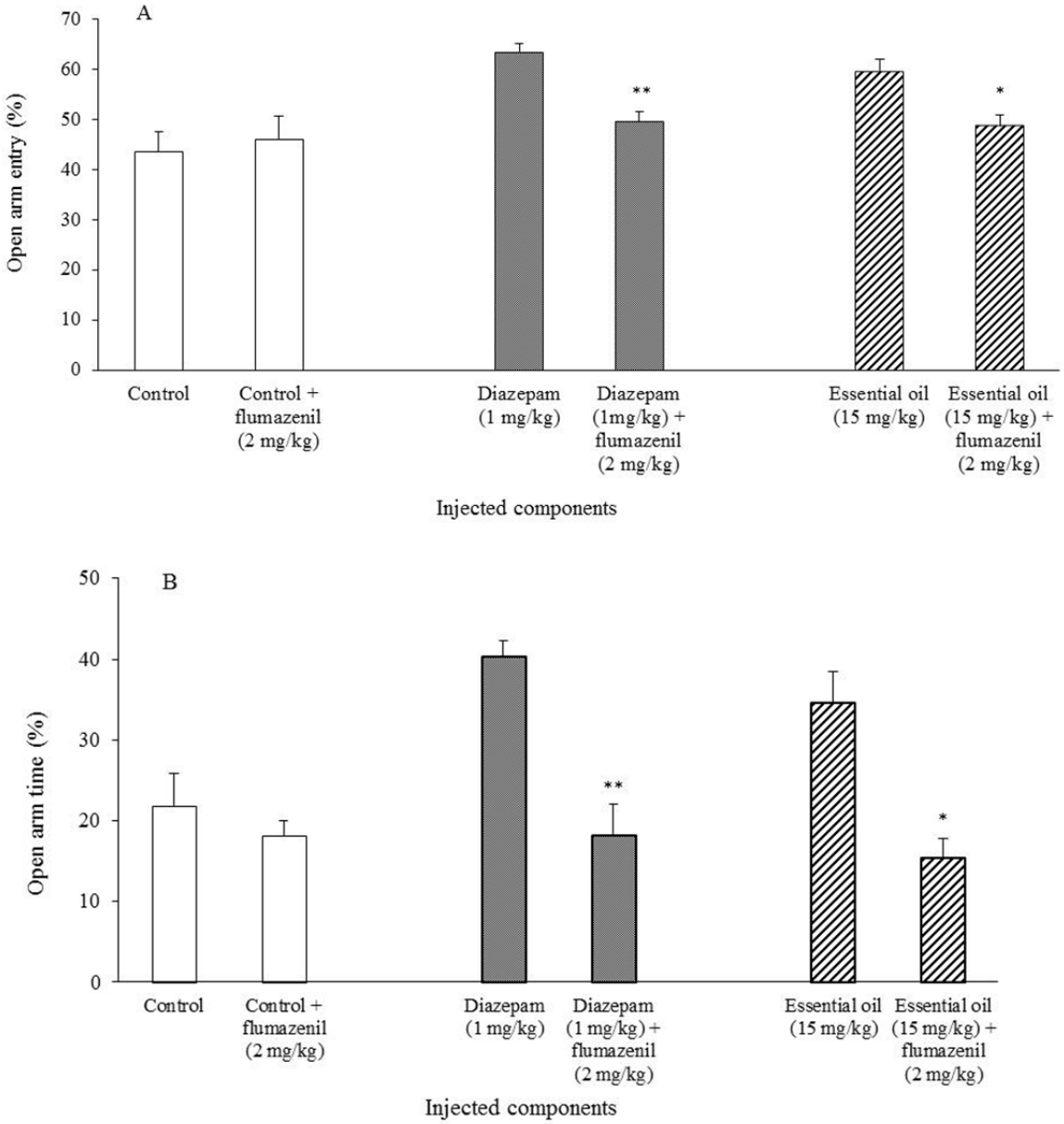

Fig. 2. Effects of flumazenil on the anxiolytic activity of *Lippia citriodora* ethanolic essential oil and diazepam on (A), the percentage of entries into the open arms and (B), the percentage of time mice spent in the open arm. 2 mg/kg flumazenil administered 5 min prior to the injection of 1 mg/kg diazepam or 15 mg/kg essential oil and 35 min before the test. Data are presented as mean ± SEM for each group of at least 6 mice. **P* < 0.05 compared to control group. ***P* < 0.001 compared to control group.

Locomotor activity

In this experiment, in general, diazepam (1 mg/kg) decreased the locomotor activity significantly as compared to the control group ($P < 0.001$). The most anxiolytic dose of the extract, which was found to be 400 mg/kg, resulted in a decline in motor activity of the animal in comparison to the control group ($P < 0.05$).

The essential oil at dose of 15 mg/kg considerably decreased the motor activity of the animals compared to control group ($P < 0.001$) (Fig. 3B). However, only diazepam and the essence caused significant decreased in motor activity in all three 5 minutes and the extract showed significant decreased just in first 5 minutes (Fig. 3A).

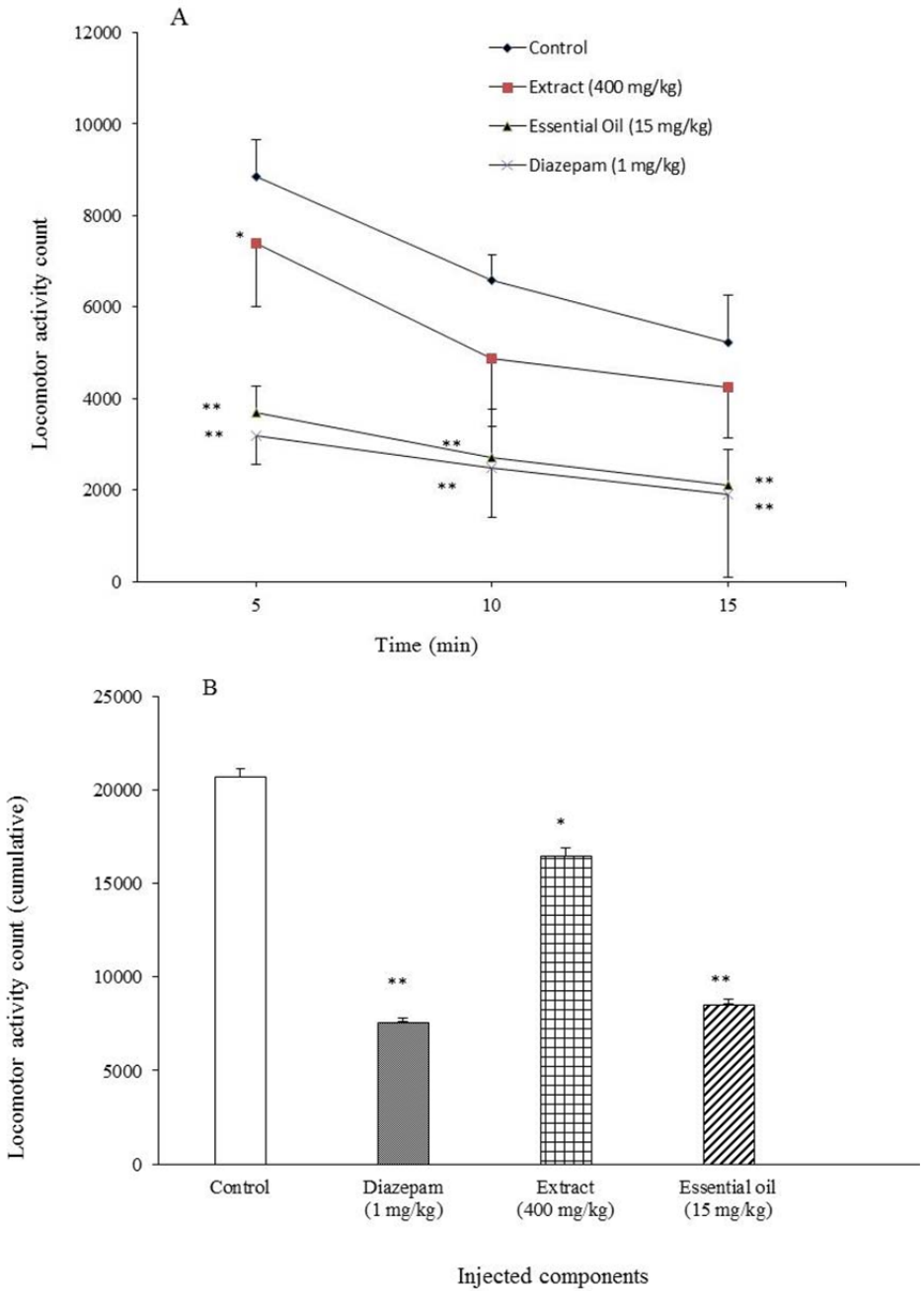

Fig. 3. The effects of diazepam, vehicle, and *Lippia citriodora* ethanolic extract and essence on (A), spontaneous locomotor activity during three 5-min intervals and (B), hydroalcoholic extract and essential oil on spontaneous locomotor activity during the total 15 min of testing. The locomotor activity counts (mean ± SEM) were measured over a 15 min period which starts 30 min after the administration of control, diazepam, plant essential oil, or hydroalcoholic extract. Data are presented as mean ± SEM for each group of at least 6 mice. *$P < 0.05$ compared to control group. **$P < 0.001$ compared to control group.

DISCUSSION

In EPM model of anxiety, in agreement with previous studies, diazepam as an anxiolytic substance increased the time spent and the numbers of entries to the open arms. The increase in time and entries in the open arms directly reflects a reduction of anxiety and stress as were shown by Lister (1990). Although the anxiolytic dose of diazepam varies from study to study, in most cases including this one, diazepam at 1 mg/kg significantly increased the time spent in the open arms ($P < 0.001$) as well as the number of entries to the open arms ($P < 0.001$) (12,20,21).

Doses of 10, 15, and 50 mg/kg of the essential oil showed a significant increase in the number of entries to the open arms ($P < 0.05$). As far as the time in the open arms was important, the essential oil only at a dose of 15 mg/kg caused an increase in this parameter. As a result, the optimum dose of essential oil was determined to be 15 mg/kg. Essential oil at doses above 50 mg/kg produced sedation and lack of movement in different arms of the apparatus. The lack of normal activity by essential oil at doses of 50 mg/kg or higher was further confirmed by locomotor activity meter data.

No data are available on the active ingredient of the essential oil of this plant. Therefore, one cannot relate the anxiolytic action of the essential oil to any of the plant ingredients. However, phytochemical work done on a similar genus of this plant have shown that major content of the essential oil are terpenes such as geranial, neral, and limonene. It is possible that terpenes content of the plant is causing the anxiolytic action. Studies on the plants that have terpenes in their essential oils, have shown to increase both parameters (entries and time in open arms) of EPM (23,24).

In contrast to the essential oil, the hydroalcoholic extract of *L. citriodora* did not produce a significant anxiolytic action. These findings are consistent with previous experiments in which *Lippia alba*'s extract have failed to produce anxiolytic effects (22,23). Major content of the plant extract are now known to be flavonoids like verbascoside, salvigenin, eupatorin, eupafolin, luteolin, hispidulin, diosmetin, cismaritin, cirsiliol, pectolin-arigenin, and 6-hydroxyluteolin. These components are believed to be responsible for antiviral and antimalarial activities (24). In our study, we did not observe any significant changes in EPM model. This could be due to low percentage of terpenes content (22,23).

Flumazenil, a GABA/benzodiazepine receptor complex antagonist, reversed the diazepam action on both parameters of the EPM. When tested against the action of essential oil, flumazenil also reversed the time and the number of entries into the open arms. Since flumazenil acts by inhibiting the GABA/benzodiazepine receptor complex, it is possible that the essential oil which mainly contains terpenes work by activating the GABA receptor. There are some studies in which the role of GABA receptor in the action of *L. alba* have been proven. For example in a work done by Neto (2009), volatile terpenes were shown to produce anticonvulsant action in rats by inhibiting GABA uptake or by binding to GABA receptor (25).

The anxiolytic action of *L. citriodora* cannot be limited to their action on the GABA receptors. There are a number of other receptors that are involved in the action of anti-anxiety of drugs. For example, 5-HT1 receptor agonists or partial agonists produce their anxiolytic effects by activating these receptors. Terpenes also have been shown to bind to the 5-HT1 receptor in order to show anxiolytic action. Thus, terepenes could indeed act through several receptors that have a role in anxiolytic function (26). Verification of the exact mechanism of action of this ingredient requires further assays and experiment. The plant essential oil at optimal dose of 15 mg/kg significantly decreased the spontaneous locomotor activity while no significant effect on locomotor activity was observed by the extract (400 mg/kg). This reduction in locomotor activity could be either due to the plant's essential oil effect on the central nervous system or a direct effect on the periphery. Diazepam showed greater impact on spontaneous locomotor activity than plant's extract or essential oil and that difference show that diazepam is more sedative than hydroalcoholic extract and essential oil of *O. basilicum*. Further studies are required to determine the exact mechanism of the sedative effect of this plant (24).

CONCLUSIONS

In conclusion, the essence of the plant *L. citriodora*, exhibits anxiolytic activity similar to diazepam without a significant reduction in locomotor activity. This anxiolytic-like action may involve the GABAergic system as flumazenil reverses this effect. The major active constituents responsible for the anxiolytic action of the plant need to be identified.

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