

Anticonvulsant Effects of *Lippia citriodora* (Verbenaceae) Leaves Ethanolic Extract in Mice: Role of GABAergic System

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ABSTRACT

Background: *Lippia citriodora* Kunth is one of the Iranian traditional medicines for the treatment of convulsive disorders. The goal of this study is to investigate the anticonvulsant activity of the plant's leave ethanolic extract against electro- and chemoconvulsant-induced seizures in mice.

Methods: The anticonvulsant activity of the extract (200, 400, 800 mg/kg, *per os*, p.o.) was investigated in pentylenetetrazole (PTZ) and maximal electroshock (MES)-induced seizures in mice. Diazepam (1 mg/kg) and phenytoin (25 mg/kg) intraperitoneally (i.p.) were used as reference drugs. In addition, for investigating the role of GABAergic system, flumazenil (2 mg/kg, i.p.) was also injected before *L. citriodora*.

Results: The extract had not any toxicity and significantly decreased the duration and increased the latency of the seizures induced by PTZ (90 mg/kg). In the MES test, *L. citriodora* displayed statistically significant reduction in hind limb tonic extension duration in a nondose-dependent manner. Flumazenil reversed the anticonvulsant activity of the plant's extract in the PTZ model.

Conclusions: The results propose that *L. citriodora* leave ethanolic extract has anticonvulsant activity against convulsive disorders. It seems that this plant's extract generates its antiseizure effect through GABAergic system potentiation. Further studies will be needed in order to investigate the exact mechanisms of it. Moreover, one may conclude that the present results are in accordance with the positive effect of *L. citriodora* extract to treat convulsion mentioned in old Iranian literature.

Keywords: Anticonvulsant, *Lippia citriodora*, maximal electroshock, mice, pentylenetetrazole

INTRODUCTION

Epilepsy is a common neurological disorder described by episodic seizures that affects 50 million people

worldwide.^[1] About 70–80% of epileptic patients are able to be commonly treated with modern anticonvulsant drugs that prevent from/lessen the number of seizure attacks. However, 30% of epileptic patients suffer from uncontrolled seizures even when they are using available modern drugs.^[2] Moreover, adverse effects associated with anticonvulsants, limit their use by patients and hence,

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there is an increasing need for searching new drugs with more effective and safer profile.

During the ancient times, Iranian people used medicinal plants to treat this debilitating neurological disorder. Moreover, nowadays, traditional practitioners persuade Iranian people to consume medicinal plants for the treatment of epilepsy.^[3]

Lippia citriodora (lemon verbena) is a perennial shrub belongs to *Verbenaceae* family which is being utilized as a spice and medicinal plant. It is native to Western South America and is cultivated in North Africa, Southern Europe, and Middle East. It has been reported that this species' leaves have digestive, antipyretic, antispasmodic, sedative, and stomachic properties.^[4,5] In addition, one review study has reported the traditional use of *L. citriodora* for the management of diabetes, digestive system disorders, dermatological problems, allergy, and respiratory problems in Oriental Morocco.^[6] Infusions of *L. citriodora* leaves have been commonly used in Iranian traditional medicines for the treatment of anxiety and epilepsy because of its tranquilizing effect.^[7] Previous studies demonstrated antihyperalgesic, antispasmodic, antiviruses, antioxidant, and sedative-hypnotic activities after the administration of essential oils and extracts of *L. citriodora*.^[8-12] GABAergic is the major system used to study the anticonvulsant mechanism of medicinal plants and natural products in animal models.^[13] Ragone *et al.* reported the sedative activity of *L. citriodora* aqueous extract in mice on the open-field test that was potentiated by diazepam and inhibited by flumazenil.^[14] The traditional usage of the plant *L. citriodora* in the treatment of convulsion has not been examined in a scientific manner yet; therefore, the aim of this study is to examine the anticonvulsant features of the ethanolic extract of *L. citriodora* in experimental animals.

METHODS

Plant material

The leaves of *L. citriodora* were collected from the Medicinal Plants Research Farm, Jihad-e Daneshgahi Research Complex, Alborz, Iran, during May 2013. The aerial parts were identified and authenticated by Miss. Farahmand, a plant taxonomist at the Iranian Biological Resource Center, where a voucher specimen with the number - 531 was deposited.

Preparation of *Lippia citriodora* ethanolic extract

Dried leaves were milled into powder using a commercial grinder. The coarse powder (100 g) was extracted with 70% ethanol for 72 h through refluxing extraction. The extract was then concentrated by a rotary evaporator apparatus at a temperature not exceeding 40°C. For animal experiments, *L. citriodora* ethanolic extract (LCEE) was suspended in carboxymethylcellulose (CMC) 0.5%.

Animals

In this study, male NMRI mice weighing 26 ± 3 g (Razi Institute, Karaj, Iran) were used. These mice were kept in standard polycarbonate cages in groups containing 4–5 members and housed in a temperature-controlled room ($24 \pm 2^\circ\text{C}$) with a 12-h dark/12-h light cycle. Animals got acclimated at least 2 days before experiments with free access to food and water. The protocol of experiments was approved by the Institutional Ethical Committee for the use of Human or Animal Subjects, the Declaration of Helsinki for human subjects, and NIH Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985), the UK Animals Scientific Procedures Act 1986, and European Communities Council Directive of 24 November 1986 (86/609/EEC). In addition, we made sure to reduce animal suffering and also to operate on the just enough the number of animals needed to produce reliable scientific data.

Drugs and chemicals

Drugs used in this study are as follows: Pentylenetetrazole (PTZ) (Sigma, USA), flumazenil (2 mg/kg) (Roche, Switzerland), diazepam (Chemidaru, Iran), phenytoin (Loghman, Iran). Dissolvent of PTZ, diazepam, and phenytoin was normal saline. All compounds got prepared freshly each time and were administered intraperitoneally. Maximal electroshock (MES) apparatus (SATADIS-8810, Iran) was used in the present study.

Phytochemical screening

Phytochemical screening of the ethanolic extract of *L. citriodora* leaves was done using the reagents and chemicals mentioned below: Alkaloids with Dragendorff's reagent, flavonoids with the use of Mg and HCl, tannins with 1% gelatin, and 10% NaCl solutions, and saponins, which is able to produce suds and therefore induce hemolysis reaction.^[15]

Studying on acute toxicity

According to OECD guideline no. 425, toxicity study of the extract was performed. A limit test was done primarily with NMRI mice weighing 26 ± 3 g. A 2000 mg/kg dose of extract prepared in CMC 1%, as recommended in guideline, was serially administered to six mice. After a single dosage administration, each mouse was closely observed hour by hour for the symptoms of toxicity and/or abnormality in behavior up to 48 h. Afterward, they were observed every day for toxicity and mortality up to 14 days.

Anticonvulsant activity

Maximal electroshock seizure test

The mice were randomly divided into five groups of six animals each: (1) Negative control group with normal saline (10 mL/kg), (2) positive control group with

phenytoin (25 mg/kg, intraperitoneally [i.p.]), (3, 4, 5) ethanolic extract-treated groups (200, 400, and 800 mg/kg, p.o., respectively). The mice were given ethanolic extract and controls 30 min prior to the induction of MES. Tonic convulsions were induced in 99.99% of the untreated animals by alternative electrical current (sinusoidal pulses, 120 mA and 60 Hz, for 0.2 s) through ear-clip electrodes by a stimulator apparatus. Before placing the electrode, a drop of 0.9% saline solution was applied on each ear of the mice. The duration of hind limb tonic extension (HLTE) was recorded.^[16,17]

Pentylentetrazole-induced seizure

The animals got divided into groups with 6 members. The mice of three groups were given LCEE at the doses of (200, 400, 800 mg/kg, p.o.) 30 min before injecting PTZ (90 mg/kg, i.p.). Two groups were given diazepam (1 mg/kg, i.p.) and normal saline 30 min before administrating PTZ (90 mg/kg, i.p.).^[16] Each mouse was placed into an individual plastic cage for observation lasting for 30 min. The general clonus was characterized by forelimb clonus followed by full body clonus. The recorded data was the latency of clonic convulsion, the duration of clonic convulsion, and the percentage of seizure and mortality protection.^[18,19]

The flumazenil effect on the anticonvulsant activity of Lippia citriodora ethanolic extract

In addition, we studied the effects of flumazenil, a GABA/benzodiazepine receptor complex antagonist, on the anticonvulsant activity of LCEE in order to examine the probable involvement of GABAergic system.^[20] We selected 6 groups each containing 6 mice. Flumazenil (2 mg/kg, i.p.) was administrated to mice of the first group, 5 min before administrating LCEE (800 mg/kg, p.o.), and 35 min before the injection of PTZ. The mice of the second group received flumazenil (2 mg/kg) 5 min before the injection of diazepam (1 mg/kg). Moreover, four groups received injection of diazepam (1 mg/kg), flumazenil (2 mg/kg), LCEE (800 mg/kg, p.o.), and normal saline (10 mL/kg) 30 min before the injection of PTZ (90 mg/kg), respectively.^[20-23] Moreover, the anticonvulsant activity of LCEE and diazepam in animals pretreated with flumazenil was assessed and compared with normal saline (10 mL/kg), flumazenil (2 mg/kg), diazepam (1 mg/kg), and LCEE (800 mg/kg)-treated mice.

Statistical analysis

The results of the study are reported as mean \pm standard error of mean. We performed the statistical analyses by One-way Analysis of Variance using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) software. Calculation of the group differences was by *post hoc* analysis using Dunnett's multiple comparison test. For all tests, statistically significant differences were considered the ones with values of $P < 0.05$.

RESULTS

Acute toxicity study

In the limit test, neither death nor any observable neurobehavioral effects were observed in the acute toxicity study. Because of the lack of observable toxicities, there was no LD50 determination carried out. This suggests that the plant extract at the doses used in this study is rather safe or nontoxic in mice.

Phytochemical screening

Initial phytochemical screening of the extract showed the presence of flavonoids and alkaloids. However, tannins and saponins were absent.

Anticonvulsant activity

Maximal electroshock test

In MES test, the administration of ethanolic extract at doses (200, 400, and 800 mg/kg) reduced the duration of HLTE compared to saline control group ($P < 0.001$) in a nondose-dependent manner. These doses were as effective as phenytoin (25 mg/kg) [Figure 1].

Pentylentetrazole-induced seizure

LCEE at the dose (800 mg/kg) made the latency of seizure longer, compared to saline group ($P < 0.01$); but two doses (200 and 400 mg/kg) were not effective ($P > 0.05$). Moreover, LCEE at doses (200 mg/kg) ($P < 0.05$), (400 and 800 mg/kg) ($P < 0.001$) decreased the duration of seizure compared to saline control group dose-dependently [Table 1]. LCEE displayed its seizure protection in a dose-dependent manner [Table 1]. In addition, compared to saline group, diazepam prolonged the onset time of seizure, ($P < 0.01$) and made the duration of seizure shorter ($P < 0.001$). Seizure protection (%) in diazepam and LCEE at doses (200, 400, and 800 mg/kg) were 100%, 16%, 33%, and 89%, respectively. Mortality protection (%) in diazepam and LCEE at doses (200, 400, and 800 mg/kg) were 100%, 33%, 67%, and 100%, respectively [Table 1].

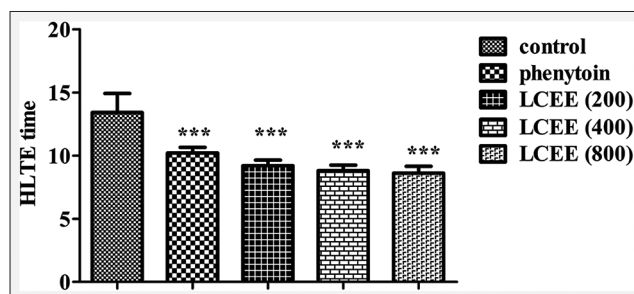


Figure 1: The effect of *Lippia citriodora* ethanolic extract on maximal electroshock-induced seizure in mice. The ethanolic extract (200, 400, 800 mg/kg, p.o.) and phenytoin (25 mg/kg, intraperitoneally) administered 30 min prior to induction of maximal electroshock seizure. *** $P < 0.001$ compared to saline group, Dunnett's multiple comparison test. Data represent mean \pm standard error of mean of three independent experiments ($n = 3$).

Table 1: Effects of *Lippia citriodora* ethanolic extract on pentylenetetrazole-induced convulsion in mice

| Treatment (dose) | Onset (s) | Duration (s) | Seizure protection (%) | Mortality protection (%) |
|--------------------------|--------------|--------------|------------------------|--------------------------|
| Normal saline (10 mL/kg) | 57.2±9.45 | 19.20±0.8 | 0 | 0 |
| Diazepam (1 mg/kg) | 405.6±35.9** | 6±0.45*** | 100 | 100 |
| LCEE (200 mg/kg) | 78.40±4.27 | 16±0.71* | 16 | 33 |
| LCEE (400 mg/kg) | 109.6±23.08 | 14±0.63*** | 33 | 67 |
| LCEE (800 mg/kg) | 388.2±78** | 7.6±0.24*** | 83 | 100 |

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to saline group, Dunnett's multiple comparison test. Data represent mean±SEM of three independent experiments (n=3). SEM=Standard error of mean, LCEE=*Lippia citriodora* ethanolic extract

Flumazenil effect on the *Lippia citriodora*'s anticonvulsant activity

In the PTZ-induced seizure model, flumazenil administration (2 mg/kg) 5 min before LCEE (800 mg/kg) saved the LCEE effect of prolonging seizure latency and dropping the duration of clonic seizure. There was no statistically significant difference between the latency and the duration of seizure in mice that had received LCEE (800 mg/kg) pretreated with flumazenil and the saline group ($P > 0.05$). Therefore, flumazenil reversed the anticonvulsant activity of LCEE and diazepam [Table 2]. Flumazenil reduced mortality protection (%) in LCEE (800 mg/kg) from 100% to 50%.

DISCUSSION

Convulsive disorder is a common phenomenon worldwide. Most of the pharmaceutical anticonvulsant drugs being used for the control and/or treatment of individuals with convulsion or epilepsy presently, have lots of toxic adverse effects. Therefore, there is an obligation to explore effective and safe newer anticonvulsant agents with less toxicities from natural sources such as plants.^[24] MES-induced tonic extension is an experimental model of seizure similar to grand mal epilepsy. Any drug inhibiting use-dependent Na^+ -channel such as valproate phenytoin and lamotrigine or drugs such as felbamate that block glutamatergic excitation mediated by N-methyl-D-aspartate receptor, can be used for the prevention of this type of seizure.^[25,26] The results of our study indicated that LCEE extract possesses anticonvulsant activity in mice in a nondose-dependent manner. PTZ-induced seizure model is among the standard tests suggested to screen the anticonvulsant agents. PTZ causes the spread of seizure similar to petit mal epilepsy by inhibiting and/or attenuation of GABAergic neurotransmission. Therefore, the type of seizure mentioned is able to be prevented by drugs which enhance Cl^- ion influx in response to GABA and promoting GABA release such as benzodiazepines, phenobarbital, and valproate.^[27] However, the results of this laboratory animal study showed that the ethanolic extract of *L. citriodora* has dose-dependent anticonvulsant effect in PTZ-induced seizures. Thus, the finding of the present study displayed that LCEE

Table 2: Effect of flumazenil on the anticonvulsant activity of *Lippia citriodora* ethanolic extract and diazepam in pentylenetetrazole-induced convulsion in mice

| Treatment (dose) | Onset (s) | Duration (s) | Mortality protection (%) |
|--------------------------|---------------|--------------|--------------------------|
| Normal saline (10 mL/kg) | 57.2±9.45 | 19.20±0.8 | 0 |
| Flumazenil (2 mg/kg) | 48±1.9 | 17±0.55 | 0 |
| Diazepam (1 mg/kg) | 405.6±35.96** | 6±0.45*** | 100 |
| Diazepam + flumazenil | 169.2±1.16 | 17.8±1.47 | 67 |
| LCEE (800 mg/kg) | 388.2±78** | 7.6±0.24*** | 100 |
| LCEE + flumazenil | 169.2±1.16 | 17.8±1.47 | 50 |

** $P < 0.01$, *** $P < 0.001$ compared to saline group, Dunnett's multiple comparison test. Data represent mean±SEM of three independent experiments (n=3). SEM=Standard error of mean, LCEE=*Lippia citriodora* ethanolic extract

has protective activity against both grand mal and petit mal epilepsy. As we know, this study is the first one about the anticonvulsant effect of this plant's extract in biomedical literature. Acute toxicity test obtained in this study for *Lippia citriodora* leaves ethanolic extract proposed the extract of this plant is safe in, and/or nontoxic to, mice. Preliminary phytochemical screening of this plant's extract in this study showed that the crude extract contained flavonoids and alkaloids. Bhutada *et al.* indicated anticonvulsant effects of berberine, an isoquinoline alkaloid, on pentylenetetrazole (PTZ) and MES-induced convulsion.^[28] Another study showed that piperine alkaloid delayed the onset of PTZ-induced seizure in mice.^[29] In addition, a recent study reports that quercetin, a famous flavonoid, prolonged the latency of seizures, reduced the duration of seizures, and seizure severity score compared to control group in PTZ-induced seizure.^[30] To investigate the possible interaction between GABAergic system and anticonvulsant activity of *L. citriodora*, flumazenil, a GABA/benzodiazepine receptor complex antagonist, was used.^[20] As it was shown in Table 2, flumazenil reversed the effects of *L. citriodora* on seizure latency and the duration of clonic seizure in PTZ model. It is considerable that the anticonvulsant effect of LCEE is antagonized by an antagonist of GABA/benzodiazepine receptor complex. Therefore, this effect may be related to benzodiazepine receptor activation. Fernández *et al.* indicated that in the central nervous system, many flavonoids are capable of acting as benzodiazepine-like molecules and modulate chloride

ion influx generated by GABA in experimental model of convulsion.^[31] Anyhow, our present state of knowledge about the chemical constituent of *L. citriodora* leaves is limited. Therefore, we suggest that the flavonoids and alkaloids present in the plant's leaves might be responsible for the anticonvulsant activity of *L. citriodora*. However, additional experiments are needed to make clear this speculation.

CONCLUSIONS

Experimental evidences provided in this study suggest that LCEE possesses anticonvulsant activity. This finding confirmed its folkloric uses as a natural supplementary remedy in the control and/or management of convulsive disorders mentioned in old Iranian literatures. It further shows that the observed effects are most likely because of the presence of flavonoids and alkaloids in the ethanolic extract. Moreover, the important role of GABA/benzodiazepine receptor complex in the effects of *L. citriodora* leaves extract should be considered.

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Conflicts of interest

There are no conflicts of interest.

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