



Anxiolytic Effect of Ethanolic Extract of Citrus Lemon Leaves on Rats in Comparison with Diazepam on Elevated Plus Maze Model

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ABSTRACT

Introduction: Anxiety is an unpleasant state of tension, apprehension, or uneasiness. Disorders involving anxiety are among the most common mental disorders. In India, it is reported to be the highest among all psychiatry disorders. Benzodiazepines are the most commonly prescribed drugs for treatment but their clinical uses are limited due to side effects. Citrus lemon has been widely exploited in complementary and alternative medicine but no significant work has been carried out on the anxiolytic effects of this plant extract. Our study was designed to experimentally evaluate the anxiolytic effects of ethanolic extract of leaves of Citrus Lemon on Wistar rats using three different doses by oral route i.e. 50,100,200 mg/kg on the elevated plus-maze model. **Materials and method:** A total of 30 Wistar rats of either sex were divided into 5 groups with 6 rats each. Group I (control): received distilled water. Group II (standard): Diazepam 2 mg/kg is given. Group III 50 mg/kg of the extract is given. Group IV-100 mg/kg is given. Group V 200 mg/kg is given. Data analyzed by one-way ANOVA and Post hoc comparisons were performed by Tukey Kramer multiple comparison test. $P < 0.05$ was considered significant. **Results:** Ethanolic extract of citrus lemon in a dose of 100 mg/kg showed effective anxiolytic activity compared to a 50,200 mg/kg dose and the effect is comparable to Diazepam. **Conclusion:** The present study suggests that citrus lemon may be developed as a potential anti-anxiety agent.

Keywords: Citrus lemon, Diazepam, Elevated plus maze, Anxiety

INTRODUCTION

Anxiety is an unpleasant state of tension, apprehension, or uneasiness. Disorders involving anxiety are among the most common mental disorders [1]. When it becomes disproportionate and excessive to the situation, it interferes with performance and constitutes anxiety disorder which usually presents in a chronic state [2,3].

Anxiety disorders are increasingly recognized as highly prevalent with onset during the teenage years with lifetime prevalence ranging between 13.6% and 28.8% in western countries. In India the estimated prevalence rate of anxiety disorders is 20.7% which is reported to be the highest among all psychiatry disorders, thus highlighting the importance of treating anxiety disorders [4]. Benzodiazepines have been the most widely used anxiolytic agents for many years. Even though they are considered to be safe during short-term therapy, the long-term use of benzodiazepines has been associated with different adverse effects such as impaired motor coordination, drowsiness, development of tolerance, cognitive and memory changes, physical dependence, and withdrawal reaction on discontinuation [5]. Thus a search for a drug with the same therapeutic efficacy as conventional drugs but with fewer side effects would be a valuable addition to the medical practice [6].

Various plants are being used in complementary and alternative medicines for the management of anxiety. Citrus is a highly reputed plant and has been widely employed in herbal medicine and aromatherapy but no significant work

has been carried out on the anxiolytic effects of this plant extracts, So the present study has been designed to evaluate the anxiolytic activity of ethanolic extract of leaves of citrus lemon in Wistar rats using elevated plus-maze model [7].

AIMS AND OBJECTIVES

- To evaluate the anxiolytic activity and effective dose of ethanolic extract of leaves of Citrus lemon on rats.
- To compare the anxiolytic activity with the standard drug Diazepam

MATERIALS AND METHOD

A total of 30 Wistar rats of either sex weighing between 150-200 grams were obtained from Central Animal House, Gandhi Medical College, and Hyderabad. The animals were kept under standard laboratory conditions with free access to food and water and a 12 h light-dark cycle. All efforts were made to minimize animal suffering and used only the number of animals necessary to produce reliable scientific data. The alcoholic extract of leaves of citrus lemon was prepared by using homogenized powder of dried leaves of Citrus lemon in soxhlet apparatus at Women's College Koti, Hyderabad.

Study Design

Animals were randomly divided into five groups namely group I (control), group II (The standard), group III (50 mg/kg oral), group IV (100 mg/kg oral), and group V (200 mg/kg oral) with 6 animals in each group (Table 1).

The drugs and the doses used were as follows:

Table 1 Drugs and their dosages used in different groups of rats

Group	Drugs/Dose	Number of Rats
I-Control	Distilled water (1 ml/100 gms)	6
II-Standard	Diazepam (2.0 mg/kg)	6
III-test	Alcoholic extract-50 mg/kg orally	6
IV-test	Alcoholic extract -100 mg/kg orally	6
V-test	Alcoholic extract -200 mg/kg orally	6

All the test solutions, the standard drug, and control were administered orally 60 min before the procedure. Animals were housed separately for the entire duration of the study. Anxiolytic activity of different doses of test compound has been compared with standard drug diazepam by using the elevated plus-maze anti-anxiety model [8-10].

The parameters observed on an elevated plus-maze:

- The first preference of rats to open or closed arm
- Several entries in open and closed arms. (an arm entry is defined as the entry of four paws into the arm)
- Percentage number of open arm entries
- Time spent in open and closed arms
- Percentage time spent in open arms

RESULTS

Observations

The observations of the behavior of rats in an elevated plus-maze are as follows

Table 2 Behaviour of rats on elevated plus-maze: Group-I (Control)

Parameter Observed	Observations of Individual rats						Mean \pm SD
	1	2	3	4	5	6	
First arm preference	Open	Closed	Closed	Open	Closed	Closed	-
No. of open arm entries	1	3	2	2	3	2	2.16 \pm 0.70
No. of total arm entries	7	7	6	7	9	6	7.0 \pm 1.41
% open/total arm entries	14.28	42.85	33.3	28.57	33.33	33.33	30.92 \pm 13.44
Time spent in open arm (sec)	20	15	35	20	20	10	20 \pm 7.07
Time spent in closed arm (sec)	270	260	270	280	270	250	266 \pm 14.14
% Time spent in open arms	6.6	5	11.6	6.6	6.6	3.3	6.61 \pm 2.33

From the above Table 2, it is observed that out of 6 rats, 4 rats preferred to enter the closed arm first and only 2 rats preferred to enter the open arm. The mean number of open arm entries was 2.16 ± 0.70 and mean the number of total arm entries was 7.0 ± 1.41 . The mean percentage of open arm entries was 30.92 ± 13.44 .

Out of the total duration of 300 seconds, the mean time spent in the open arms was 20 ± 7.07 sec and in the closed arms was 266 ± 14.14 sec with the remaining time being in the center of the maze. The mean percentage of time spent in open arms was calculated using the time spent in open arms divided by total time i.e. 300 sec, which came to 6.61 ± 2.33 .

Table 3 Behaviour of rats on elevated plus-maze: Group-II (Diazepam 2 mg/kg)

Parameter	Observations of Individual rats						Mean \pm SD
	1	2	3	4	5	6	
First arm preference	Open	Open	Open	Open	Closed	Open	-
No. of open arm entries	6	5	6	4	8	8	6.16 \pm 1.41
No. of total arm entries	10	9	10	6	11	12	9.66 \pm 1.41
% open / total arm entries	60	55.5	60	66.6	72.7	66.6	63.5 \pm 14.0
Time spent in open arms (sec)	140	110	120	140	120	165	132.5 \pm 17.6
Time spent in closed arms (sec)	120	130	140	120	95	100	117.5 \pm 14.14
% Time spent in open arms	46.66	36.66	40	46.66	40	55	44.16 \pm 5.89

From the above Table 3, it is observed that out of 6 rats, 5 rats preferred to enter the open arm first and only 1 rat preferred to enter the closed arm. The mean number of open arm entries was 6.16 ± 1.41 and mean the number of total arm entries was 9.66 ± 1.41 . The mean percentage of open arm entries was 63.5 ± 14.0 . To assess factors affecting bladder preservation therapy.

Out of the total duration of 300 seconds, the mean time spent in the open arms was 132.5 ± 17.67 sec and in the closed arms was 117.5 ± 14.14 sec with the remaining time being in the center of the maze. The mean percentage of time spent in open arms was 44.16 ± 5.89 .

Table 4 Behaviour of rats on elevated plus maze: Group-III (50 mg/kg)

Parameter Observed	Observations of Individual rats						Mean \pm SD
	1	2	3	4	5	6	
First arm preference	Closed	Open	Closed	Closed	Open	Closed	-
No. of open arm entries	2	3	2	2	3	1	2.16 \pm 0.70
No. of total arm entries	5	7	6	6	6	7	6.16 \pm 1.41
% open/total arm entries	40	42.85	33.3	33.3	50	14.28	35.6 \pm 18.18
Time spent in open arms (sec)	12	10	10	8	15	20	12.5 \pm 5.65
Time spent in closed arms (sec)	270	280	280	290	280	260	276.6 \pm 7.07
%Time spent in open arms	4	3.33	3.33	2.66	5	6.6	4.15 \pm 1.83

The following were the observations of rats after administration of alcoholic extract of citrus Limon (50 mg/kg) orally. From the above Table 4, it is observed that out of 6 rats, 4 rats preferred to enter the closed arm first and only 2 rats preferred to enter the open arm. The mean number of open arm entries was 2.16 \pm 0.70 and mean the number of total arm entries was 6.16 \pm 1.41. The mean percentage of open arm entries was 35.6 \pm 18.18.

Out of the total duration of 300 seconds, the mean time spent in the open arms was 12.5 \pm 5.65 sec and in the closed arms was 276.6 \pm 7.07 sec with the remaining time being in the center of the maze. The mean percentage of time spent in open arms was 4.15 \pm 1.83.

Table 5 Behaviour of rats on elevated plus maze: Group-IV (100 mg/kg)

Parameter Observed	Observations of Individual rats						Mean \pm SD
	1	2	3	4	5	6	
First arm preference	Open	Open	Open	Open	Open	Closed	-
No. of open arm entries	5	5	4	5	6	4	4.83 \pm 1.41
No. of total arm entries	10	12	8	10	11	5	9.33 \pm 3.53
% open/total arm entries	50	16.6	50	50	54.5	80	50.18 \pm 21.2
Time spent in open arms (sec)	100	85	70	90	110	120	95.83 \pm 14.14
Time spent in closed arms (sec)	180	160	220	200	160	170	181.6 \pm 7.07
%Time spent in open arms	33.33	28.33	23.3	30	36.6	40	31.91 \pm 4.73

The following were the observations of rats after administration of alcoholic extract of citrus Limon (100 mg/kg) orally.

From the above Table 5, it is observed that out of 6 rats, 5 rats preferred to enter the open arm first and only 1 rate preferred to enter the closed arm. This was similar to that observed in Group II. The mean number of open arm entries was 4.83 \pm 1.41 and the mean several total arm entries was 9.33 \pm 3.53. The mean percentage of open arm entries was 50.18 \pm 21.2.

Out of the total duration of 300 seconds, the mean time spent in the open arms was 95.83 \pm 14.1 sec and in the closed arms was 181.6 \pm 7.07 sec with the remaining time being in the center of the maze. The mean percentage of time spent in open arms was 31.91 \pm 4.73.

Table 6 Behaviour of rats on elevated plus-maze: Group V (200 mg/kg)

Parameter Observed	Observations of Individual rats						Mean ± SD
	1	2	3	4	5	6	
First arm preference	Closed	Closed	Closed	Closed	Closed	Open	-
No. of open arm entries	1	2	1	1	1	2	1.33 ± 0.70
No. of total arm entries	4	3	2	2	2	3	2.66 ± 0.70
% open/total arm entries	25	66.6	50	50	50	66.6	51.3 ± 29.41
Time spent in open arms (sec)	20	35	20	20	10	15	20 ± 3.53
Time spent in closed arms (sec)	260	280	270	250	260	270	265 ± 7.07
%Time spent in open arms	6.6	11.6	6.6	6.6	3.3	5	6.61 ± 1.13

The following were the observations of rats after administration of alcoholic extract of citrus Limon (200 mg/kg) orally.

From the above Table 6, it is observed that out of 6 rats, 5 rats preferred to enter the closed arm first and only 1 rat preferred to enter the open arm. The mean number of open arm entries was 1.33 ± 0.70 and the mean several total arm entries was 2.66 ± 0.70. The mean percentage of open arm entries was 51.3 ± 29.41.

Out of the total duration of 300 seconds, the mean time spent in the open arms was 20 ± 3.53 sec and in the closed arms was 265 ± 7.07 sec with the remaining time being in the center of the maze. The mean percentage of time spent in open arms was 6.61 ± 1.13

Statistical Analysis

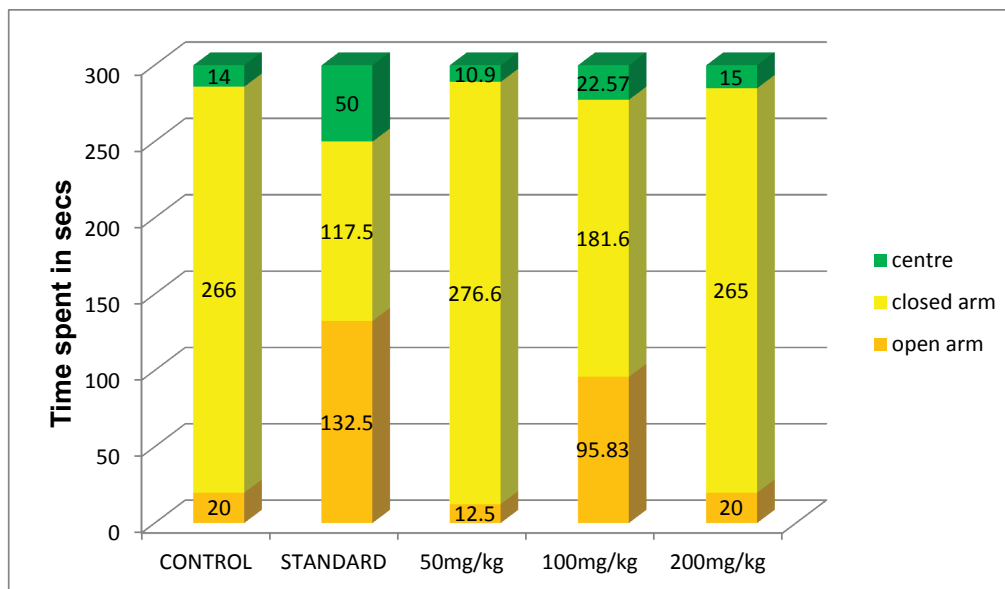


Figure 1 Comparison of time spent in open arms, closed arms and in the center of elevated plus-maze among Group I (control), Group II (Standard), Group III (50 mg/kg), Group IV (100 mg/kg) and Group V (200 mg/kg)

From the above Figure 1 it is observed that in groups I, III, and V, rats spent most of the time in closed arms whereas in groups II and IV a significant increase in time spent in open arms was observed. The proportion of time spent in open arms was highest in group II when compared to group IV. It was also observed that time spent in the center of the maze is increased in groups II and IV when compared to groups I, III, and V.

DISCUSSION

We have selected Citrus lemon in our study because its anxiolytic activity was found to be almost close to the Diazepam. However, the majority of the studies conducted previously had evaluated the anxiolytic activity of citrus on mice and very few studies were there on rats. In our study, the anxiolytic activity of citrus was evaluated using different species *viz* rats, and also the anxiolytic property was compared in three doses 50 mg/kg, 100 mg/kg, and 200 mg/kg. The oral route was chosen because, if the anxiolytic activity of citrus lemon was proved then it will be the most convenient route to implement the findings to the general population as it can be used as an add-on to their diets.

Khan, Riaz *et al.* evaluated the behavioral effects of citrus lemon in rats in 2014 at three different doses *i.e.* 0.2 ml/kg, 0.4 ml/kg, 0.6 ml/kg considered as low, moderate, and high doses [11]. They observed an increase in several open arm entries, time spent in open arms, no. of entries decreases in several rearings by 0.4 ml/kg, and that this anxiolytic activity is similar to standard drug Diazepam. They suggested that citrus lemon might serve as a promising alternative therapeutic target, particularly for treatment-resistant anxiety and depression. The advantage of this study is chronic administration of the extract to the rats and evaluation of its anxiolytic activity which is similar to disease patterns as most of the time anxiety manifests as a chronic disorder.

L. M. Lopes Campelo *et al.* examined sedative, anxiolytic and antidepressant effects of essential oil of Citrus lemon on Swiss albino mice. The effects of essential oil were demonstrated by an open field, elevated plus maze, rota rod, pentobarbital-induced sleeping time, and forced swimming tests [12].

They concluded that these sedative, anxiolytic effects of essential oils might involve action on benzodiazepine type receptors and also suggested that noradrenergic and serotonergic mechanisms involve in antidepressant effect. In the present study, the equivalent dose in rats produced similar results in elevated plus-maze. This may be because of the alcoholic extract used in the present study and also may be because of interspecies or inter-lab variability.

In the present study, the anxiolytic activity of the citrus leaves extract was observed at a dose of 100 mg/kg in rats. Anxiolytic activity of citrus lemon is likely to be associated with its essential oil content and flavonoids. The components of the essential oil include Limonene (65%), Linalool (1.73%), Citronella (2.77), Neral (6.85), Geranial (5.9), *etc.* Lima and Collaborators observed that limonene inhalation exerts an anxiolytic-like effect in male mice, by using an elevated plus-maze model at a dose that did not interfere with motor activity [13]. A similar anxiolytic-like effect of limonene was also found by Satou and collaborators who observed an increase in open arm exploration (increase in time spent and percentage of entries) in the elevated plus-maze, and an increase in the time spent on the light side of the light/dark apparatus [14,15].

According to some studies, it has been found that Linalool produced different effects on CNS, such as hypnotic, anticonvulsant, anxiolytic, and sedative in human subjects [16]. In a recent study, in the light/dark box test, inhalation of linalool oxide led to an increase in the time spent by the mice in the brightly-lit chamber and the number of times the animal crossed from one compartment to another, without affecting the performance on the rotarod [17].

Mechanism of action by which citrus lemon shows anxiolytic activity may also be because of the presence of Flavonoids. They have structural similarities to Diazepam acts via the Gamma-Aminobutyric Acid (GABAA) receptor complex to produce its anxiolytic activity [18]. Furthermore, a sedative effect on the central nervous system has been shown for quercetin and Isoquercetin glycosides in mice [19].

Many studies have demonstrated that the anxiolytic activity of essential oils and flavonoids in leaves of citrus lemon is because of its interaction with the Gamma-Aminobutyric Acid (GABAA) receptor complex. Active constituents of citrus lemon are primarily monoterpene compounds and thus chemically similar to essential oils. These monoterpenes exhibit anti-anxiety activity which is thought to be due to GABAergic mechanisms.

Recommendations for further work

To date, only a very limited number of studies have been conducted to evaluate the anxiolytic activity of Citrus lemon. To extrapolate the results obtained to humans, more studies must be conducted.

Further studies are required to identify the phytochemical constituent responsible for the observed anxiolytic effect of ethanolic extract at a dose of 100 mg/kg and to explain the anxiolytic mechanism.

CONCLUSION

The test compound Ethanolic extract of Citrus Lemon leaves, 100 mg/kg dose has Anxiolytic activity similar to that of Diazepam. These results support the previous studies where the anxiolytic activity of citrus lemon in a dose of 100 mg/kg showed significant activity similar to Diazepam. If further preclinical and clinical studies show positive results then, these compounds could prove to be an effective alternative to the existing anti-anxiety drugs with better safety profiles.

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Conflicting Interests

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