



EXPERIMENTAL EVALUATION OF ANXIOLYTIC ACTIVITY OF ALCOHOLIC EXTRACT OF *CITRUS LIMON* LEAVES ON RATS.

Pharmacology

Dr Vandana Godella

Assistant Manager, Pharmacovigilance, Aurobindo Pharma Limited, Hyderabad-500090.

Dr Avula Naveen*

Assistant Professor, Dept of Pharmacology, Government Medical College, Nizamabad-503002. *Corresponding Author

Dr.T.S.Usha Shree

Professor and Head of the Department, Dept of Pharmacology, ESIC Medical College, Hyderabad-500038.

Dr P Vijayakrishna

Pharmacovigilance physician, Vigi medsafe pvt Limited, Hyderabad-.500033.

Dr MR Sravani

Assistant Professor, Dept of Pharmacology, Maheshwara Medical College, Hyderabad-502307.

ABSTRACT

INTRODUCTION : Anxiety as a disorder is increasing worldwide due to complexity of daily life in modern society. In India it is reported to be highest among all psychiatry disorders, thus highlighting the importance of treating anxiety disorders. Benzodiazepines are widely prescribed drugs for treatment but their clinical uses are limited due to side effects. *Citrus Limon* is a highly reputed plant and has been widely employed in complementary and alternative medicine but no significant work has been carried out on the anxiolytic effects of this plant extract. The present study was designed to experimentally evaluate the anxiolytic effects of ethanolic extract of leaves of *Citrus Limon* on wistar rats using three different doses orally i.e 50,100,200 mg/kg on open field test.

MATERIAL & METHODS: 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 2mg/kg is given. Group III -50mg/kg of extract is given. Group IV-100mg/kg is given. Group V- 200mg/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 Limon* in dose of 100mg/kg showed effective anxiolytic activity compared to 50,200 mg/kg dose and effect is compared to Diazepam.

CONCLUSION: The present study suggest that *Citrus Limon* may be developed as a potential anti anxiety agent.

KEYWORDS

Anxiety, *Citrus Limon*, Diazepam, Elevated Plus Maze, Open Field Test.

INTRODUCTION:

Anxiety is a commonly encountered emotion that usually presents as a response to a negative situation⁽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).

In India the estimated prevalence rate is 20.7% which is reported to be highest among all psychiatry disorders, thus highlighting the importance of treating anxiety disorders⁽⁴⁾.

Benzodiazepines have been the most widely used anxiolytic drugs 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 adverse effects such as impaired motor coordination, drowsiness, development of tolerance, cognitive and memory changes, physical dependence and withdrawal reaction on discontinuation⁽⁵⁾. Thus remedies possessing the same efficacy as conventional drugs, but with fewer side effects, would be a valuable addition⁽⁶⁾.

Various plants are being used in complementary and alternative medicines for 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⁽⁷⁾. So, the present study has been designed to evaluate the anxiolytic activity of ethanolic extract of leaves of *Citrus Limon* in wistar rats using open field test.

AIMS&OBJECTIVES :

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

MATERIAL AND METHODS:

A total of 30 wistar rats of either sex weighing between 150-200 grams were obtained from Central Animal House, Gandhi Medical College Hyderabad. The animals were kept under standard laboratory conditions with free access to food & water and a 12hr light dark cycle.

All efforts were made to minimize animal suffering & used only the number of animals necessary to produce reliable scientific data. Alcoholic extract of leaves of *Citrus limon* was prepared using homogenized powder of dried leaves of *Citrus limon* in Soxhlet apparatus at Womens college, koti, Hyderabad. The study was initiated after approval from Institutional Animal Ethical Committee of Gandhi Medical College, Hyderabad and the CPCSEA registration number is 428/01C/CPCSEA/15-GMC-IAEC.

Study design: Animals were randomly divided into five groups namely group I (control), group II (Standard), group III (50mg/kg oral), group IV (100mg/kg oral.) and group V (200mg/kg oral) with 6 rats in each group.

The drugs and their doses used were as follows:

Table – 1: Drugs And Their Dosages Used In Different Groups Of Rats

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

All the test solutions, standard drug and control were administered orally 60 min prior to procedure. Animals were housed separately for the entire duration of study. Anxiolytic activity of different doses of test compound have been compared with standard drug diazepam by using different anti-anxiety models like Open Field Test.

Open Field Test (OFT) is a circular arena with a diameter of 100 cm with walls 30cm high. The floor is divided up into quarters with a central circle of 33cm diameter in the middle of the arena. (Choleris E et al,2001, Carola V, et al;2002)

Animals were placed in the centre of open field. Video recording and

stop watch will be started . Each trial will lasts for 10 mins after which rats were returned in their home cages and arena was cleaned with 70% ethanol solution.The following parameters will be assessed.

1. Number of squares crossed
2. Number of rearings

RESULTS:

OBSERVATIONS: We observed the following results with regard to the different parameters (behaviour of rats) on Open Field Test.

Table –2: Behaviour Of Rats On Open Field Test – Group I (control)

PARAMETER OBSERVED	OBSERVATIONS OF INDIVIDUAL RATS						MEAN ± SD
	1	2	3	4	5	6	
Number of squares passed	10	16	12	10	12	12	12 ± 1.41
Number of rearings	6	4	8	4	5	8	5.83± 1.41

The following were the observations of control group which received distilled water.From the above table it is observed that the mean number of squares passed was 12 ± 1.41 and mean number of rearings was 5.83 ± 1.41.

Table – 3: Behaviour Of Rats On Open Field Test – Group II(standard/Diazepam)

PARAMETER OBSERVED	OBSERVATIONS OF INDIVIDUAL RATS						MEAN ± SD
	1	2	3	4	5	6	
Number of squares passed	16	16	20	24	16	20	18.66± 2.82
Number of rearings	14	16	18	16	15	18	16.16± 2.82

The following were the observations of rats after administration of diazepam(2mg/kg).From the above table it is observed that the mean number of squares passed was 18.66 ± 2.82 and mean number of rearings was 16.16 ± 2.82.

Table – 4: Behaviour Of Rats On Open Field Test – Group III (50mg/kg)

PARAMETER OBSERVED	OBSERVATIONS OF INDIVIDUAL RATS						MEAN ± SD
	1	2	3	4	5	6	
Number of squares passed	8	10	14	12	8	10	10.33± 1.41
Number of rearings	10	5	8	12	15	14	10.66 ± 2.82

The following were the observations of rats after administration of ethanolic extract of citrus limon (50mg/kg).From the above table it is observed that the mean number of squares passed was 10.33± 1.41 and mean number of rearings was 10.66 ± 2.82.

Table – 5: Behaviour Of Rats On Open Field Test – Group IV (100mg/kg)

PARAMETER OBSERVED	OBSERVATIONS OF INDIVIDUAL RATS						MEAN ± SD
	1	2	3	4	5	6	
Number of squares passed	16	16	20	16	16	20	17.33± 2.82
Number of rearings	12	10	15	16	12	16	13.5 ± 2.82

The following were the observations of rats after administration of ethanolic extract of citrus limon (100mg/kg).From the above table it is observed that the mean number of squares passed was 17.33± 2.82 and mean number of rearings was 13.5 ± 2.82.

Table – 6: Behaviour Of Rats On Open Field Test – Group V (200mg/kg)

PARAMETER OBSERVED	OBSERVATIONS OF INDIVIDUAL RATS						MEAN ± SD
	1	2	3	4	5	6	
Number of squares passed	4	2	2	2	4	3	2.83± 0.70
Number of rearings	4	3	3	5	2	6	3.83± 1.41

The following were the observations of rats after administration of ethanolic extract of citrus limon (200mg/kg).From the above table it is observed that the mean number of squares passed was 2.83± 0.70 and mean number of rearings was 3.83 ± 1.41.

STATISTICALANALYSIS:

All data calculated were expressed as Mean ± SD for each group. The

data were analyzed by one-way ANOVA and Post-hoc comparisons were performed by applying Tukey - Kramers multiple comparison test. P<0.05 was considered statistically significant.

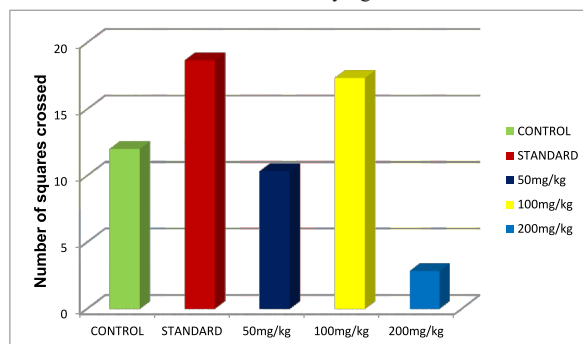


Figure – 9: Comparison of Number of squares crossed in Open Field Test Among Group I (control), Group II (Standard), Group III (50mg/kg), Group IV (100mg/kg) and Group V(200mg/kg)

The mean (±SD) number of squares crossed in group I (control) was 12 ± 1.41, in group II (standard) it was 18.66 ± 2.82 , group III (50 mg/kg) 10.33 ± 1.41 , group IV (100 mg/kg) 17.33 ± 2.82 and group V (200 mg/kg) it was 2.83 ± 0.70

The mean number of squares crossed in group II ,group IV and group V showed P value <0.001 which is highly significant when compared with group I.The mean difference between group II, group IV showed P value >0.05 which is not significant whereas the mean difference between group II and group V showed P value <0.001 which is highly significant.When compared with group I, group III showed P > 0.05 which is not significant.

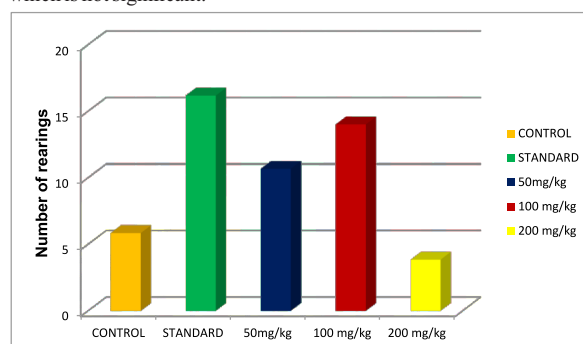


Figure – 10: Comparison of Number of rearings in Open Field Test Among Group I (control), Group II (Standard), Group III (50mg/kg), Group IV (100mg/kg) and Group V(200mg/kg)

The mean (±SD) number of rearings in group I (control) was 5.83 ± 1.41, in group II (standard) it was 16.16 ± 2.82 , group III (50 mg/kg) 10.66 ± 2.82, group IV (100 mg/kg) 13.5± 2.82 and group V (200 mg/kg) it was 3.83 ± 1.41.

The mean number of rearings in group II ,group IV and group V showed P value <0.001 which is highly significant when compared with group I.The mean difference between group II, group V showed P value >0.05 which is not significant whereas the mean difference between group II and group IV showed P value <0.05 which is significant. When compared with group I, group III showed P > 0.05 which is not significant.

All the above observations indicate that ethanolic extract of *Citrus limon* leaves significantly increased number of squares crossed and in rearings compared to control group .This is more significant with 100 mg/kg dose of extract compared to its lower and higher doses and this anxiolytic activity at this dose is found to be similar to Diazepam.

DISCUSSION :

In recent years, many studies using herbal remedies and supplements to treat mild to moderate anxiety disorders have emerged. We have selected *Citrus limon* in our study because it is widely available and also very economical. In the previous studies, its anxiolytic activity was found to be almost close to the Diazepam. However, all the studies

conducted previously have evaluated the anxiolytic activity of citrus on mice, no study have since far been conducted on rats.

Rafeeq Alam Khan ,Azra Riaz evaluated behavioral effects of *Citrus limon* in rats in 2014 at three different doses i.e,0.2,0.4,0.6ml/kg considered as low, moderate and high doses. In this study they observed increase in number of open arm entries, time spent in open arms, no. of entries decrease in number of rearings by 0.4ml/kg and that this anxiolytic activity is similar to standard drug Diazepam⁽⁸⁾ They suggested that *Citrus limon* might serve as an appealing alternative therapeutic target, particularly for the high occurrence of treatment resistant anxiety and depression.

L.M.Lopes Campelo et al examined sedative, anxiolytic and antidepressant effects of essential oil of leaves from *Citrus limon* on Swiss albino mice. They concluded that these sedative, anxiolytic effects of essential oils might involve an action on benzodiazepine type receptors and they suggested that noradrenergic and serotonergic mechanisms involvement in antidepressant effect⁽⁹⁾.

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 *C. limon* is likely to be associated with its essential oil content and flavonoids. It is reported that essential oil of leaves of citrus contains limonene(60–70%), linalool(1.73%), citronella(2.77%), nerol(6.85%), geraniol(5.9). Lima and collaborators observed that limonene inhalation exerts an anxiolytic-like effect in male mice tested in the elevated plus-maze (increases in time spent, and the number of open arm entries), at a dose which did not interfere with motor activity (number of entries in the closed arms). Flumazenil pre-treatment did not block the anxiolytic-like effect of limonene, indicating that the benzodiazepine receptor site did not mediate this effect⁽¹⁰⁾. Similar anxiolytic-like effect of limonene was also found by Satou and collaborators⁽¹¹⁾.

Male mice treated orally (1 and 14 days) with (+)-limonene epoxide reduced the number of marbles buried in the marble-burying test⁽¹²⁾. No difference between acute and repeated treatments was seen; suggesting that tolerance did not occur for the anxiolytic-like effect of limonene epoxide. However, no measurement of motor activity was done.

Linalool has marked effects at the CNS, including hypnotic and anticonvulsant properties, and anxiolytic and sedative effects are also shown by linalool in human subjects⁽¹³⁾.

Mechanism of action by which *C. Limon* shows anxiolytic activity may also be because of the presence of Flavonoids. They have structural similarity to Diazepam acts via GABA_A receptor complex in order to produce its anxiolytic activity⁽¹⁴⁾.

Even though many studies have been conducted to demonstrate the anxiolytic effect of essential oils and flavonoids, there is still a need to isolate, characterize and screen other active principles from the different parts of *Citrus limon* that may be responsible for its anxiolytic activity. Also there is need to find out the exact mechanism by which *Citrus limon* exerts above effects.

CONCLUSION:

Anxiolytic activity of Ethanolic extract of Citrus Limon leaves, 100mg/kg dose is similar to that of Diazepam which is used as standard and thus can be used in the management of anxiety. These results support the previous studies where anxiolytic activity of citrus limon in dose of 100mg/kg showed significant activity similar to Diazepam⁽¹⁵⁾.

If further preclinical and clinical studies show positive results then these compounds could prove to be an effective alternative to the presently available drugs for anxiety and the significant adverse effects associated with the present medication can be avoided.

ACKNOWLEDGEMENTS: we would like to thank our professors Dr. Ushasree and Dr. Indira for their constant support and scientific inputs. We also would like to appreciate the efforts of non-teaching staff of Gandhi Medical College Mr. Hasan, Mrs. Kanakamma and others.

FUNDING: self

DECLARATION OF CONFLICTING INTERESTS: all authors have none to declare.

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