WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 5, 1429-1440. Research Article ISSN 2277– 7105

CNS ACTIVITIES OF AQUEOUS EXTRACTS OF CITRUS LIMON AND CITRUS SINENSIS AND ITS COMBINATION STUDIED IN MICE

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Article Received on
24 March 2016,
Revised on 12 April 2016,
Accepted on 02 May 2016
DOI: 10.20959/wjpr20165-6149

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ABSTRACT

The *Citrus limon* and *Citrus Sinensis* exhibits many important natural components, including citric acid, ascorbic acid, minerals and phenolic compounds, such flavonoids. Although their biological properties have always been associated with their content of vitamin C, it has recently been shown those flavonoids and other nutrients and non-nutrients (vitamins, minerals, dietary fiber, essential oils and carotenoids). Therefore, their health-promoting effects, such as obesity, diabetes, blood lipid lowering, cardiovascular diseases, brain disorders and certain types of cancer, have been associated with their contents, especially vitamin C and flavonoids, due to their natural antioxidant characteristics. Therefore, this work was designed based on phytochemical constitution such as flavonoids in Citrus limon Citrus Sinensis peel extracts because flavonoids are rich in the treatment for evaluation of CNS activities in mice. Fresh and crushed peel of Citrus limon and Citrus Sinensis peel were collected and then extracted with solvents such as water, and the doses of 20 mg/kg body weight is given for an mice. CNS activities were performed by using a Forced swim test, Tail Suspension test, Actophotometer test, Rota rod test and Muscle Grip strength in mice. Diazepam 5mg/kg was used as standard drug for these studies. In the present study, the aqueous peel extracts of Citrus limon, Citrus Sinensis and its combination was demonstrated significant CNS activities in the tested models.

KEYWORDS: *Citrus limon* and *Citrus Sinensis*, Forced swim test, Tail Suspension test, Actophotometer test, Rota rod test, Muscle Grip strength and Diazepam.

1. INTRODUCTION

According to the world health report approximately 450 million people suffer from a mental or behavioural disorder, but only a small number them receive even the foremost basic treatment, this accounts for 12.3% of the global burden of disease and will increase to 15% by 2020.[1] Drugs acting on the central nervous system were the first to be used by the primitive human and are still the most broadly utilized group of pharmacological agents. Mental ailments are heterogeneous diseases and will probably require a selected arsenal of drugs with different modes of action for successful treatment of their various manifestations.[2]

In today's lifestyle of stress and strain, there is a general need for agents having neuroprotective and neuropharmacological activity by enhancing learning and memory function of the brain.[3] Stress involves complicated biochemical, neural and immunological mechanisms and plays a crucial role in the progression of a variety of disease states ranging from psychiatric disorders like depression, anxiety, immunosuppression, endocrine disorders including diabetes mellitus, male impotency and cognitive dysfunctions to cardiovascular disease, high blood pressure, peptic ulcers, migraine, allergies, asthma, carcinoma, premature aging, rheumatic diseases and ulcerative colitis.[4] Severe stressful conditions are responsible for the etiopathogenesis of various psychosomatic disorders. Homeostasis is controlled by various physiological mediators working in concert by interacting with receptors placed at various physiological levels and the functional identity of neurotransmitters is challenged during stressful conditions.

Out of various neurotransmitters, nor adrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) are the necessary which are widely distributed in brain and their practical role is well established during stressful conditions.[5] Cognitive dysfunction, a major health problem in twenty first century and the foremost functionally debilitative side of many neuropsychiatric disorders and neurodegenerative disorders, such as schizophrenic disorder, depression, AD dementia, seizure disorders, head injury and parkinsonism. Memory function is vulnerable to a variety of pathologic processes including neurodegenerative diseases, depression, anxiety and the adverse effects of medication and normal ageing.[6] Learning and memory are generated by an experience dependent and long-lasting modification of the central nervous system.

Herbal drugs are widely used for the treatment of various diseases. Although herbal drugs often contain highly active pharmacological compounds but much importance is not given to their safety evaluation, may be due to a popular notion "anything herbal is safe." Lately, with recent increasing interest in traditional or herbal drugs for the prevention and treatment of various disorders, there is also increasing concern about the safety of traditional, herbal product based medicines.[7]

The current research also focuses on the extraction and CNS activities of peels of citrus fruits which are easily available at zero cost thus decreasing the cost for production. The following fruits have been targeted in the present study.

A) The lemon is both a small evergreen tree (*Citrus limon*) native to Asia, and the tree's ellipsoidal yellow fruit. The fruit is used for culinary and non-culinary purposes throughout the world – primarily for its juice, though the pulp and rind (zest) are also used, mainly in cooking and baking. Lemon juice is about 5% to 6% (approximately 0.3 M) citric acid, which gives lemons a sour taste, and a pH of 2–3.[8]

B) Orange (*Citrus sinensis*) trees are widely cultivated in tropical and subtropical climates for the sweet fruit, which is peeled or cut (to avoid the bitter rind) and eaten whole, or processed to extract orange juice, and also for the fragrant peel. In 2008, 68.5 million tons of oranges were grown worldwide, primarily in Brazil and the US states California and Florida. Oranges probably originated in Southeast Asia and were cultivated in China by 2500 BC.[9]

Numerous bioactive compounds such as flavonoids, Saponins, Phenolic and tannins have been isolated from peels of *Citrus Limon and Citrus Sinensis*. Some of these bioactive compounds have been worked out for one or the other medicinal attributes.[10] But till date, the CNS activities of *Citrus Limon and Citrus Sinensis* have not been scientifically evaluated. Hence, in the present study, the effect with *Citrus Limon and Citrus Sinensis* peels extracts at a dose of 20 mg/kg body wt on CNS activities has been studied.

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

Drugs and Chemicals used in this study were of analytical grade and of highest purity procured from standard commercial sources in India.

2.2 Experimental animals

Healthy adult albino mice weighing 40-50 grams of either sex were selected for the study. Animals were housed in appropriate cages in uniform hygienic conditions and fed with standard pellet diet (Amrul Laboratory Animal Diet) and water ad libitum. They were fasted overnight before the day of experiment. Animals were housed within the departmental animal house and the room temperature was maintained at 27°C. Animal studies had approval of IAEC.

2.3 Plant Material Collection

The fruits of *Citrus Limon and Citrus Sinensis* were collected from local market in the month of January. The peels were separated from the fruits and it was cleaned, reduced to small fragments, air dried under shade at room temperature and coarsely powdered in a mixer. The powdered material was stored or taken up for extraction process.

2.4 Preparation of plant extracts

The powdered peels of *Citrus Limon and Citrus Sinensis* were successively extracted in 100-150ml each of distilled water by using Soxhlet extractor. The plant material was suspended in the main chamber of Soxhlet extractor which was then placed onto a flask containing the extraction solvent. The Soxhlet was then equipped with a condenser. The flask was heated; the solvent evaporated and moved up into the condenser where it was converted into a liquid that trickled into the extraction chamber containing the sample. This extraction process kept for 8hrs at 20-40^{0}C. At the end of the hot extraction process each extract was filtered. The filtered extract was dried in oven to remove remaining moisture, if present, and finally weighed and sealed up for further use.

2.5 Phytochemical screening

The peels of *Citrus Limon and Citrus Sinensis* was screened for various chemical constituents (tannins, alkaloids, cardiac glycosides, flavonoids, steroidal compounds, saponins) using established methods.[10]

2.6 CNS Activities

Despair Swim Test[11]

For the determination of antidepressant activity, forced swim test (FST) protocol was employed. During the test, animals were individually placed in a glass cylinder (20 cm in height, 14 cm in diameter) filled with water up to a height of 10cm, at 25 ± 2°C. All animals

were forced to swim for 5 min and the duration of immobility was observed and measured during the 5 min interval of the test. Immobility period was regarded as the time spent by the rats to float in water with no struggle and making only those movements necessary to keep its head above the water. In order to check the fitness level of each test animal, a pre-test was carried out 24 h before the FST by subjecting each test animal to a session of 15 min swimming.

Tail suspension test[12]

Tail suspension test was performed based on the method prescribed[18]. The mice were suspended 58cm above the floor by means of an adhesive tape, placed approximately 1cm from the tip of the tail. The total duration of immobility was quantified during a test period of 5min. Mice were considered immobile when they were completely remain motionless.

Actophotometer test[13]

The locomotor activity can be easily studied with the help of actophotometer, the rats were grouped and treated with drugs.Turn on the equipment (check & make sure that all the photocells are working for accurate recording) and placed individually each rat in the activity cage for 10 minutes. Note the basal activity score of all the animals. Inject the drug diazepam (Dose: 5 mg/kg, ip; make a stock solution containing 0.5 mg/ml of the drug & inject 1 ml/100 g body wt of mouse), and after 30 mins re-test each mouse for activity scores for 10 mins. Note the difference in the activity, before & after chlorpromazine. Calculate percent decrease in motor activity.

Rota rod test[14]

The skeletal muscle relaxations together with taming or calming effect these agents reduce anxiety and tension. The loss of muscle grip is an indication of musclerelaxation. This effect can be easily studied in animals using inclined plane or rotating rods. The difference in the fall off time from the rotating rod between the control and drug treated animal is taken on an index of muscle relaxations. The angle of the slope of the inclined plane or the rate of rotation of the rod should be adjusted such (20-25 rpm is ideal) that a normal mouse can stay on the plane or on the rod for an appreciable period (3-5 min) of time. Inject the diazepam and test drugs. After 30min the animals is placed once again on rotating rod and note the fall-off time. Compare the fall-off time of animals before and after treatment of test and standard drug.

Muscle grip strength test[20]

This test is used to assess muscular strength in rodents which can be influenced by muscle relaxants and sedative drugs. In a preliminary experiment the animals are tested for their normal grip strength by exposing them to horizontal thin metallic wire suspended about 30cm in the air, which they immediately grasp with their forceps. The mouse is then released to hang on with its forelimbs. Normal animals are able to catch the wire with the hind limbs and climb on it with 5seconds. Only animals which fulfil this criterion are included in the test. Then now test groups should tested for every 15min for 2 hours. The percentage of animals loosing the grip strength is recorded using different test and standard drugs.

3. RESULTS

The preliminary phytochemical screening of the dry residue showed the presence of saponins, flavonoids, glycosides, tannins and phenolic compounds.

➢ FORCED SWIM TEST

The anti-depressant activity of AQCL, AQCS and AQCO was assessed using Forced Swimming Test in Swiss albino rats were illustrated in Table No:1. It was observed that AQCS and AQCO at a dose of 20mg/kg exhibited significant reduction in immobility time when compared to control and Standard in dose dependent manner. Similarly the animals treated with diazepam (5mg/kg) as expected showed significant decrease in immobility time.

Table No:1 Data obtained from Forced Swim Test

S.No	Treatment	Dose(mg/kg)	Drug Administration (sec)		% change in activity
			Before	After 60 min	
1.	Control	--	26	25	4%
2.	Diazepam	5	58	9	84.48%
3.	AQCL	20	39	24	38.46%
4.	AQCS	20	40	21	47.50%
5.	AQCO	20	47	21	55.31%

The results are expressed as means ± S.E.M Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA). Statistical significance was assessed as $p < 0.05$.

➢ TAIL SUSPENSION TEST

In tail suspension test, the aqueous extracts of peel of *C.Siniuses and* Its combination at a dose of 20 mg/kg i.p. significantly decreased the immobility time. The magnitude of the

antidepressant effects of 20 mg/kg i.p. of aqueous peel of *C.limon* and of *C.Siniuses* was comparable to that of Diazepam 5 mg/kg i.p. (Table 2)

Table: 2- Data obtained from Tail Suspension Test

S.No	Treatment	Dose(mg/kg)	Drug Administration (sec)		% change in activity
			Before	After 60 min	
1.	Control	--	45	63	-0.4%
2.	Diazepam	5	66	12	81.81%
3.	AQCL	20	54	33	38.88%
4.	AQCS	20	60	32	46.66%
5.	AQCO	20	46	24	47.82%

The results are expressed as means ± S.E.M Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA). Statistical significance was assessed as $p < 0.05$.

- **ACTOPHOTOMETER TEST**

The percentage of reduction in spontaneous motor activity with diazepam (5 mg/kg i.p) after 1 hour is 79.61 % i.e. there is highly significant (P<0.05) when compare to control, where as dose of AQCS, AQCO (20mg/kg i.p) showed dose and time dependent decrease in locomotor activity that is 47.66% and 56.33% respectively when compared to standard. The values are highly significant (P<0.05) (Table No:3)

Table: 3 - Data obtained from Actophotometer Test

S.No	Treatment	Dose (mg/kg)	Drug Administration (sec)		% change in activity
			Before	After 60 min	
1.	Control	--	305	294	3.60%
2.	Diazepam	5	368	75	79.61%
3.	AQCL	20	319	181	43.26%
4.	AQCS	20	321	168	47.66%
5.	AQCO	20	398	173	56.53%

The results are expressed as means ± S.E.M Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA). Statistical significance was assessed as $p < 0.05$.

- **ROTAROD TEST**

Treatment with extracts at a dose of 20mg/kg and Diazepam at dose of 5 mg/kg decreased fall off and sliding time and increase climbing time (motor coordination). The result obtained

from both standard and extract treated groups were compared with the control group. A highly significant P<0.05 reduction in the motor coordination was observed in the AQCS and AQCO drug after 60 minutes of duration. (Table 4)

Table: 4 - Data obtained from Rotarod Test

S.No	Treatment	Dose (mg/kg)	Drug Administration (sec)		% change in activity
			Before	After 60 min	
1.	Control	--	22	24	-9.09%
2.	Diazepam	10	49	12	75.5%
3.	AQCL	20	34	20	41.17%
4.	AQCS	20	48	23	52.08%
5.	AQCO	20	36	15	58.33%

The results are expressed as means ± S.E.M Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA). Statistical significance was assessed as $p < 0.05$.

- **MUSCLE GRIP STRENGTH**

Treatment with extracts at a dose of 20mg/kg and Diazepam at dose of 5 mg/kg decreased grip strength (motor coordination). The result obtained from both standard and extract treated groups were compared with the control group. A highly significant P<0.05 reduction in the motor coordination was observed in the AQCS and AQCO drug after 60 minutes of duration.(Table 5)

Table: 5 - Data obtained from Rotarod Test

S.No	Treatment	Dose(mg/kg)	Drug Administration (sec)		% change in activity
			Before	After 60 min	
1.	Control	--	124	120	3.22%
2.	Diazepam	10	142	28	80.02%
3.	AQCL	20	101	60	40.05%
4.	AQCS	20	113	52	53.9%
5.	AQCO	20	300	190	60.33%

The results are expressed as means ± S.E.M Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA). Statistical significance was assessed as $p < 0.05$.

4. DISCUSSION

In this study we have evaluated the antidepressant activity of *Citrus limon and Citrus sinensis and* combination of aqueous extracts in FST, TST, Actophotometer, Rotarod and Muscle Grip Strength tests were performed.

In this study AQCL, AQCS and AQCO (20 mg/kg) produced significant antidepressant effect in FST and TST. These models of depression are widely used to screen new antidepressant drugs. This test is quite sensitive and relatively specific to all major classes of antidepressant drugs including TCAs, SSRIs, MAOI, Atypical antidepressants. The Forced swim test and Tail suspension test is the most widely used tool for assessing antidepressant activity pre-clinically.

The widespread use of this simple model is mainly due to its ability to detect a broad spectrum of antidepressant agents. It has been argued that FST (Forced swim test) and TST (Tail Suspension Test) is less stressful and has greater pharmacological sensitivity. Environmental factors and hereditary factors play a major role in producing deficient monoaminergic transmission in central nervous system thereby producing symptoms of depression.

Locomotor activity indicates alertness and the decrease indicates sedative action. The $GABA_A$ receptor complex is involved in sedation, muscle relaxant and anxiety in CNS. Various neurological and psychological disorders such as epilepsy, depression, Parkinson syndrome, Alzheimer's disease are involved with this receptor. Benzodiazepines like diazepam in the actophotometer test act by potentiation of the $GABA_A$ receptors, causing membrane hyperpolarization, ultimately leading to decrease in the firing rate of neurons in the brain or by directly acting on the GABA receptor where increased GABA neurotransmission has a damping effect on the stimulatory pathways causing a psychologically calming effect.

Diazepam taken as a standard at a dose of 5 mg/kg orally showed significant ($p<0.05$) CNS depressant activity. Aqueous extracts of *C.limon and C.Sinensis* peel in this study could also act by the same mechanism that is either GABA facilitatory or GABA mimetic action by reducing the locomotor activity in laboratory animals. The objective of this study was to investigate in-depth the skeletal muscle relaxant activity by Rota Rod and Muscle Grip Strength test of the AQCL, AQCS and AQCO (20 mg/kg). The present results showed that

the Aqueous extract of AQCS and AQCO possess a significant skeletal muscle relaxant activity in experimental rats. At dose of 20 mg/kg it showed highly significant skeletal muscle relaxant activity at 60 min of duration. Since the pharmacological profile of the present investigation of the aqueous extract of *C.limon and C.Sinensis* peel was significant to that of benzodiazepines, it is also possible that they might interact with benzodiazepine receptor located adjacent to the GABA receptor. This activity again could be due to the inhibition of excitatory mechanisms in the CNS. Treatment with AQCS and AQCO causes significant increase in the level of norepinephrine, dopamine and acetylcholine activity in the brain.

5. CONCLUSION

The peel extracts named the *C.limon, C.Sinensis* and its combinations has been used as a test drug and Pharmacological activities have been done on the experimental animals using Mice. The forced swim test and Tail Suspension test was performed for anti depressant activity. In this methods the immobility period of the mice before and after injecting the drug were noted. Actophotometer is performed for measuring the locomotor activity.

Rota rod and Muscle Grip Strength were performed for Skeletal Muscle Relaxant Activity. In this experiment the mice were placed on an apparatus and the time of fall is noted before and after injecting the drug. Here diazepam has been used as standard drug. There was a significant dose dependent decrease in duration of fall of time and decrease in immobility time in animals treated with 20mg/kg doses.

Therefore, It is concluded that *C.limon, C.Sinensis* and its combinations of aqueous extracts has good anti depressant, locomotar and skeletal muscle relaxant activity on mice and hence it can also be used as an anti depressant or skeletal muscle relaxant drug in the society. So, further detailed investigations are needed to isolate and identify the active compounds present in the plant extract and its various fractions and their efficacy need to be done. It will help in the development of novel and safe drugs for the treatment of different types of CNS disorders.

6. ACKNOWLEDGEMENTS

The authors are very much thankful to Geethanjali College of pharmacy, Hyderabad for their support in carrying out the research work.

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