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Antidepressant activity of *Citrus limetta* leaves in mice using battery of behavior models modulating via serotonergic systems

Antidepressant activity of *Citrus limetta* leaves in mice using battery of behavior models modulating via serotonergic systems

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Abstract

The methanolic extract of *Citrus limetta* leaves was estimated in the present study for antidepressant activity. This activity was evaluated by using the rat behavioral model i.e. forced swimming and tail suspension model for 14 days, with estimation of neurotransmitters in animals brain. The sedative effect was evaluated by actophotometer. The extract (200 mg/kg) administered orally showed significant ($p < 0.05$) decrease in immobility time against the standard drug fluoxetine (20 mg/kg, i.p), and imipramine (15 mg/kg, i.p.) respectively but no significant reduction was found in the locomotor activity against diazepam (2 mg/kg, i.p.). In depression, the brain reflected low monoamines level like release of norepinephrine, dopamine and serotonin but 14 days after successive administration of the extract, their levels were significantly increased. In conclusion, *C. limetta* showed significant anti-depressant activity.

Introduction

Depression is considered as a mood disorder in which mixed feelings of sadness, loss of appetite, frustration, fatigue and anger interfere with day to day life. The causes are complex and involve a combination of miscellaneous factors such as biologic, genetic, and environmental. People suffering from depression may have abnormal levels of certain monoamine transmitter in brain, including serotonin, acetylcholine, and catecholamine (such as dopamine) (Gold et al., 1988). Numerous synthetic drugs are being used as the standard treatment for this heterogeneous mood disorder but they cause severe adverse effects (Hadizadeh et al., 2009).

In recent years, the search for novel pharmacotherapy agents obtained from medicinal plants like *Curcuma longa* (Yu et al., 2002), *Emblica officinalis* (Sharma and Nain., 2011), *Ginkgo biloba* (Rojas et al., 2011), grape (Rabiei et al., 2017), *Withania somnifera* (Bhattacharya et al., 2000), etc for psychiatric illnesses has progressed, which has been reflected by experimentation on large

number of herbal medicines in a battery of animal models.

Citrus limetta is a species of genus *Citrus* belonging to family Rutaceae. Citrus plants are rich in naturally-occurring flavonoids (Cao et al., 1997). A variety of vital bioflavonoids like hesperidin, narirutin, naringin, neohesperidin, eriocitrin, neoeriocitrin, rutin, diosmin, neoponcirin, and nobiletin are abundant in these citrus plants and have proved their efficacy as most prominent cancer preventing agents (Albach et al., 1969; Castillo et al., 1992; Jourdan et al., 1985; Kawaii et al., 1999). Amongst the citrus bioactive flavonoids, the flavanone and polymethoxy flavones glycosides attract significant attention for their constituents and biological activities (Tripoli et al., 2007). They possess a wide variety of biological activities through inhibition of key enzymes in mitochondrial respiration like in treatment of coronary heart disease, anti-spasmodic, anti-inflammatory, free radical scavengers, estrogenic, cytotoxic, antitumor, antihyperglycemic, larvicidal, antifungal and antibacterial activities (Harborne and Williams,



2000). It has been revealed by traditional and ethno medicinal literature that fruit and leaves of this important species is very effective in treating different pathologies and ailments such as common cold, fever, control of cholesterol, regulation of inflammatory and digestive disorders, prevention and treatment of skin problems and a remarkable modulator of blood pressure (KunduSen et al., 2010; Perez et al., 2010). Phytochemical profile and traditional use of *C. limetta* as a multipurpose medicinal agent appealed us to scientifically evaluate its use as an anti-depressant.

Materials and Methods

Plant material

The leaves of *C. limetta* were collected from CDL National herbal park, Yamunanagar, Haryana in April-May, 2009 and authenticated by NISCAIR (NISCAIR/RHMD/CONSULT/2009-10/1347/150). All chemicals were obtained from the Sigma Aldrich.

Preparation of extracts

The leaves of *C. limetta* were shed dried, powdered and subjected to successive solvent extraction with four different solvents namely petroleum ether (40-60°C), chloroform (61°C), methanol (65°C) and water in soxhlet extractor.

Phytochemical screening

The extracts were screened for different classes of phytoconstituents such as alkaloids, triterpenoid, carbohydrates, flavonoids and tannins using standard procedures (Trease and Evans, 2002; Edeoga et al., 2005).

Animals

Swiss albino mice of either sex (approximately 3 months old and weighing about 20-25 g) were collected from the institutional animal house and kept under standard laboratory conditions (maintained at ambient temperature of 23°C with changes of light and dark every 12 hours and constant access to food and water). The animals were housed at least one week in the laboratory animal room before testing.

Drugs

Fluoxetine (20 mg/kg), imipramine (15 mg/kg) and diazepam (2 mg/kg) were used as standard drugs for forced swimming test, tail suspension test and locomotor activity respectively. These drugs were dissolved in 2% aqueous Tween 80 suspension and administered orally.

Acute toxicity study

Acute oral toxicity study of the methanolic extract of *C. limetta* was carried out in Swiss albino mice (20-25 g) of

either sex. Mice comprising 6 animals in each group, fasted for 18 hours before experiment, were given the methanol extract (50, 100, 200, 400, 500, 1000, and 2000 mg/kg) p.o. The control group received distilled water 10 mL/kg p.o. Mice were closely observed for 2 hours post-treatment for any signs of toxicity as well as behavioural changes. The rate of mortality shown within 24 hours was recorded. The surviving mice were further observed for another 7 days for any significant signs of delayed toxicity. The lethal dose 50 (LD₅₀) value was estimated by log-probit analysis (Akindele and Adeyemi, 2006).

Preparation of test doses

The methanolic extract of *C. limetta* did not cause death and was found to be completely nontoxic up to a dose of 2 g/kg. Thus, it was considered to be safe for use. One-tenth of this dose, i.e., 200 mg/kg body weight and half of this one-tenth dose, i.e., 100 mg/kg, were used as minimum dose for the elucidation of different pharmacological activities.

Based on acute toxicity study, 100, 200, 400 mg/kg of the extracts were prepared by suspending the dried extracts in vehicle (2% aqueous Tween 80 suspension). The doses of methanol extracts were so adjusted as to administer 0.5 mL of the suspension of extracts. Diazepam 2 mg/kg suspended in the vehicle was used as standard anxiolytic. The suspending vehicle (0.5 mL) was used as control.

Experimental protocol

All mice were randomly divided into 18 groups. Each group contained 6 mice.

Tail suspension test

The tail suspension test was the second effective method for evaluating the antidepressant effect of the methanol extract. Thirty min after the standard drug or vehicle administered, mice were subjected to the test. Each animal was individually suspended in the wooden box with the help of hanging clip 50 cm above the floor and 1 cm part of the tail was clipped. During the test each animal taken was both acoustically and visually isolated from other animals. The total period (six minutes) of immobility/mobility shown by the animals was recorded manually. It was observed that each animal showed vigorous movement during the initial 2 min period out of the total 6 min testing period. The immobility of animals was manually recorded during the next 4 min of total experimental period. After the initial period of vigorous motor activity, the mice became still and the time of immobility was measured with a stopwatch, for a total duration of 6 min. The testing animals were considered immobile when they hung passively and completely motionless (Steru et al., 1985).

Box 1: Forced Swimming Test**Principle**

Measurement of immobility time is carried out by observing the motoric activity of the mouse, which is placed in a pool of water.

Requirements

Glass cylinder (diameter 25 cm, height 25 cm); Methanol extract; Mouse; Stop watch; Thermometer

Procedure

Step 1: A glass cylinder was filled with water up to a height of 15 cm.

Step 2: The temperature of water was maintained at $25 \pm 1^\circ\text{C}$ using hot water and ice. Check the temperature using a thermometer.

Step 3: Each mouse was administered either a) none (control), b) 2% aqueous Tween 80 suspension (vehicle control), or c) extract (different doses: 100, 200, or 400 mg/kg orally once daily for 14 days) or d) fluoxetine. On the day of forced swimming test, vehicle, extract or fluoxetine was administered 30 min before the test.

Step 4: Thirty minutes later, the mouse was subjected to the behavioral test.

Step 5: During the test session, the immobility time was recorded by blind observer who has been trained for the observation.

Step 6: The total duration of immobility was measured during the 6 min test; for the first 2 min the animal was allowed to adjust to the new conditions; after these 2 min, the immobility time that alternated with conditions of enhanced motor activity was measured.

Note

1. The height of the cylinder should be high enough to prevent the mouse from escaping from the cylinder.
2. The test was conducted in a dim lighted room and each mice was used only once in the test.
3. Shorter immobility time is an indicator of the stronger antidepressant effect of the tested substance.
4. The mouse was considered immobile when neither hind leg was moving nor the mice were slightly hunched forward.
5. The test is a relatively short and low cost behavioral test. It does not require any training of the mouse and can be conducted with minimal equipment.

References

Porsolt et al., 1978; Aslam, 2016

One group was control naive intact mice while another group was treated with 2% aqueous Tween 80 suspension which were used as vehicle for tail suspension test model. Another three groups were treated with the prepared extract at doses of 100, 200, 400 mg/kg under the same conditions 30 min before antidepressant testing/activity via oral route once daily for 14 days. The last group was treated with imipramine (standard drug) 30 min before behavioral test.

Locomotor activity

Animals were placed in the digital actophotometer (Inco, India), which consists of a cage which was 30 cm long and 30 cm deep with a wire mesh at the bottom. A continuous beam of light from about six lights was made to fall on corresponding photoelectric cell. The photoelectric cell got activated when an animal crossed the beam of light and thereby cuts off the rays of light falling on it. These cutoffs were counted for a period of 5 min.

Actophotometer: One group was control naive intact mice and another group was treated with 2% aqueous tween 80 suspension which was used as vehicle for actophotometer. Another three groups were treated with the prepared extract at doses of 100, 200, 400 mg/kg under the same conditions 30 min before antidepressant testing via oral route for 14 days once daily. The last group was treated with standard drug diazepam (2 mg/kg) 30 min before test.

Behaviors evaluation

The behavioral profiles were assessed daily after single dose and after repetitive administration of the extract for continuously 14 days (once daily).

Neurotransmitter measurements

On day 14, experimental animals were sacrificed by cervical dislocation (within 5 min) after being exposed to antidepressant models. The samples of brain were collected immediately on an ice plate. The brain tissue was weighed, homogenized by using cold *n*-butyl alcohol at a 1:10 volume, shaken well for 5 min and centrifuged at $3,000 \times g$ for 5 min. Both 5 mL of *n*-heptane and 0.1 mol/L HCl were added to the supernatant. After this the mixture was vortexed for 5 min and then re-centrifuged at $3,000 \times g$ for another 5 min. Serotonin, norepinephrine, and dopamine was found in the water phase and 5-HIAA, a 5-HT metabolite were present in the organic phase. The amines were estimated by fluorimetric method (Welch and Welch, 1969; Jacobowitz and Richardson, 1978).

Estimation of norepinephrine and dopamine

The water phase (1 mL) was added to 1/15 mol/L of phosphate saline buffer (1.7 mL, pH 7.2). To this, 0.1 mL of iodine reagent was added and allowed to stand for another 2 min, followed by addition of 0.5 mL of alkaline sodium sulfite solution and 0.6 mL of 6 mol/L glacial acetic acid was added after 2 min. Now the mixture was boiled for 20 min, followed by cooling and finally fluorescence of norepinephrine was read at 385/475 nm, the dopamine fluorescence at 322/370 nm

(Welch and Welch, 1969; Jacobowitz and Richardson, 1978).

Estimating of serotonin

The aqueous phase (0.2 mL) was added to *O*-phthalaldehyde reagent (0.25 mL). The fluorophore was developed by heating to 100°C for 10 min. After the samples reached equilibrium with the ambient temperature, readings were taken at wavelengths of 360-470 nm in the spectrofluorometer (Balamurugan et al., 2009).

Statistical analysis

The study data are represented as mean \pm SEM. The comparison between before and after treatment was made with Student's *t*-test. Data were analyzed by using a ANOVA (One-way analysis of variance) followed by Dunnett's test.

Results

Phytochemistry

The results of phytochemical screening indicated the presence of flavonoids in the extract (Table I).

Behaviors evaluation

It was observed that both single and even repetitive administrations of vehicle did not produce any significant change in the immobility time in forced swimming and tail suspension model. But single and repetitive administration of fluoxetine and imipramine

significantly decreased the immobility time in both models ($p < 0.05$) (Table II). The single administration of all extracts of *C. limetta* viz. petroleum ether, chloroform and aqueous at all dosage range used failed to show any significant changes on the percentage of changes of immobility time but the methanolic extract at dose of 200 mg/kg and 400 mg/kg markedly decreased the immobility time in both the models after repeated once daily treatment for 14 days (Table II). The behaviors assessments were performed within a time span of 30 min after the single and repetitive treatment on 1st and 14th day.

The effects of extract on spontaneous locomotor activity (alertness) in mice are shown in Table III. The methanolic extract administered at the doses of 100, 200 and 400 mg/kg did not modify any considerable effect on locomotor activity in mice *i.e.* no sedation was induced by the methanolic extract of *C. limetta*.

Brain serotonin, norepinephrine and dopamine levels

The methanolic extract (200 mg/kg) of *C. limetta* to mice significantly increased the brain serotonin (35 ± 4.5 $\mu\text{g/g}$ protein), norepinephrine (29 ± 3.8 $\mu\text{g/g}$ protein) and dopamine (23 ± 3.7 $\mu\text{g/g}$ protein) levels when compared with control group (19 ± 2.3 , 17 ± 2.7 and 11 ± 2.6 $\mu\text{g/g}$ protein) respectively (Figure 1).

Discussion

The results of our experimental study depicted that the

Table I

Phytochemical screening of leaves extract of *C. limetta*

Index	Inference			
	Petroleum ether	Chloroform	Methanol	Aqueous
<i>Test for alkaloids</i>				
Dragendorff's reagent	-	-	-	-
Mayer's test	-	-	-	-
Hanger's reagent	-	-	-	-
<i>Test for flavonoids</i>				
Lead acetate test	-	-	++	+
Sodium hydroxide test	-	-	++	++
<i>Test for tannins</i>				
Bromine water test	-	-	-	-
Dilute iodine solution test	-	-	-	-
<i>Test for triterpenoid</i>				
Liebermann-Burchard's test	++	+	-	-
<i>Test for carbohydrate</i>				
Molisch's reagent	-	-	-	+
Fehling solution	-	-	-	+
Benedict's reagent	-	-	-	+

Table II

Effect of methanolic extract of *C. limetta* on immobility time in mice

Treatment	Immobility period			
	Forced swimming test		Tail suspension test	
	Day 1	Day 14	Day 1	Day 14
Naive	190.3 ± 6.4	191.3 ± 5.7	186.5 ± 7.2	187.4 ± 4.7
Control (2% Tween 80)	186.2 ± 5.7	192.4 ± 3.5	180.2 ± 8.9	173.8 ± 8.2
Extract (100 mg/kg)	143.7 ± 8.3	97.2 ± 5.2	157.8 ± 8.2	101.2 ± 1.4
Extract (200 mg/kg)	112.5 ± 7.9 ^a	55.6 ± 4.7 ^a	119.6 ± 5.1 ^a	69.2 ± 2.3 ^a
Extract (400 mg/kg)	110.4 ± 3.1 ^a	63.1 ± 7.9 ^a	121.0 ± 3.4 ^a	77.2 ± 2.4 ^a
Imipramine (15 mg/kg) or fluoxetine (20 mg/kg)	87.6 ± 8.6 ^a	42.3 ± 2.4 ^a	79.6 ± 3.7 ^a	37.0 ± 4.9 ^a

Data are mean ± SEM; n=6; ^ap<0.05 compared to control

Table III

Effect of methanolic leaves extract of *C. limetta* on spontaneous locomotor activity in mice

Treatment	Activity counts (Cutoff time 5 min)	
	Before treatment	After 30 min of treatment
Naive	173 ± 13	180 ± 15
Control (2% Tween 80)	176 ± 16	189 ± 09
Extract (100 mg/kg)	245 ± 20	231 ± 11
Extract (200 mg/kg)	206 ± 15	233 ± 19
Extract (400 mg/kg)	190 ± 18	201 ± 23
Diazepam (2 mg/kg)	215 ± 13	72 ± 27 ^a

Data are mean ± SEM; n=6; ^ap<0.05 when after treatment compared to before treatment

extract can significantly decrease immobility time in both the behavioral test without showing sedative effect (decrease in alertness). It was observed that after 14 days of treatment the extract can produce anti-depressant activity at the dose of 200 mg/kg in the tested models whereas less significant changes were observed at low and high doses. The decrease in immobility time observed in this study depicts the real antidepressant like activity of the extract. Previous studies demonstrated that many neurotransmitters are involved to play a role in the pathophysiology of depression. It has been proved by numerous studies that antidepressant drugs, such as fluoxetine, aids in facilitating the action of serotonin. Because of this it is widely used as an antidepressant and also agreed with animal studies, such as forced swimming test (Lucki, 1997). Further it was demonstrated that swimming behaviour shown by animals was sensitive to certain serotonergic compounds like SSRI (e.g. fluoxetine) (Detke et al., 1995).

Based on these observations, it can be supposed that the extract which can decrease the immobility time and simultaneously increases swimming behavior in the animals exposed to these paradigms can always exert its effect through a mechanism similar to that of the fluoxetine via the serotonin system. It was also found that the decrease in immobility was under the influence of motor behavior and sedative effect. We also determined the effect of plant extract on these motor behaviors and sedation. The results of the present study clearly showed that the extract did not produce any significant changes on alertness.

Analysis of the neurohormonal data supports the behavioral models results as well. There is now considerable evidence to implicate the serotonergic system with depression. The serotonergic system has been implicated in the changes in energy, cognitive functioning, sleep, mood, appetite and libido which is commonly seen in depression and affective disorders. Thus, decreased serotonin activity is always associated with depression and the most effective antidepressants have been shown to increase the functioning of serotonin in the brain. In addition, low activity of serotonin may also permit the dysregulation of other neurotransmitters, including norepinephrine (Ninan, 1999). In this study, the extract showed similar effects like imipramine which is a tricyclic antidepressant by causing inhibition of serotonin, norepinephrine and dopamine reuptake. It might be possible that the extract exert any one of these mechanism for antidepressant like activity. Preliminary phytochemical screening of crude methanol extract showed the presence of flavonoids. Flavonoids possess multiple neuroprotective actions in treating central nervous pathophysiological conditions including depression and it was reported that they possess potent antidepressant property via central serotonergic and noradrenergic system (Yi et al., 2010).

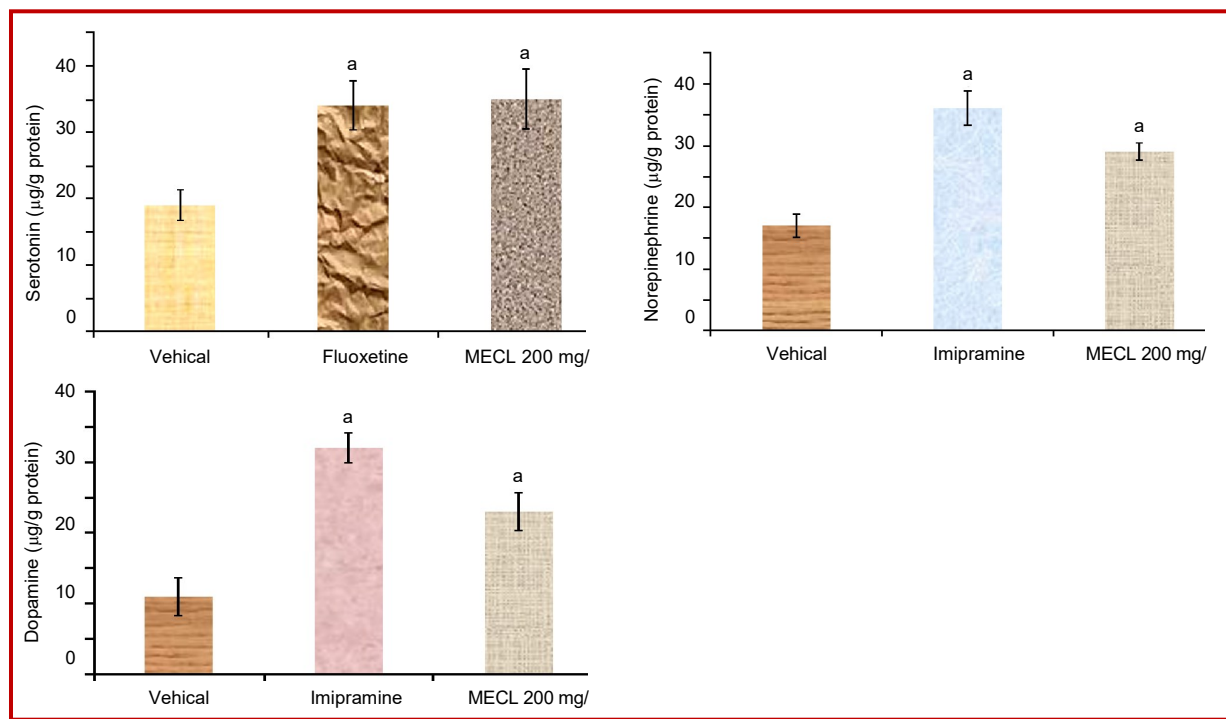


Figure 1: Effect of methanolic leaves extract *C. limetta* (200 mg/kg) on serotonin (A), norepinephrine (B) and dopamine (C) after the trial of antidepressant model

All values are mean \pm SEM; n=6; *p<0.05 compared to control

Conclusion

Flavonoids present in the methanol extract of *C. limetta* produces antidepressant effect in mice mediating an interaction through serotonergic, dopaminergic and noradrenergic system.

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Ethical Issue

All experiment procedures and protocol used in the study were reviewed and approved by University Ethical Committee reference No. 828/AC/04/CPCSEA. Acute oral toxicity study of *C. limetta* was carried out in Swiss albino mice of either sex according to OECD guidelines No. 423.

Conflict of Interest

Authors declare no conflict of interest

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