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Research Article

Effects of ethanolic leaf extract of *Psidium guajava* Linn. on L-arginine induced obsessive compulsive disorder in mice

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Abstract: Obsessive compulsive disorder (OCD) is a high-prevalence psychiatric disorder affecting 2–5% of the population. It is a type of anxiety disorder in which people suffer from recurrent, unwanted thoughts or ideas (obsessions), engage in repetitive, irrational behaviors or mental acts (compulsions) or both. Although its pathophysiology remains unclear, current evidence implicates contributions of the serotonergic and dopaminergic neurotransmitter systems and a neural circuitry that includes the orbitofrontal cortex, the thalamus and the striatum. In this present research work the pharmacological effect of ethanolic extract of *Psidium guajava* Linn.(EEPG) against L-arginine induced obsessions were evaluated along with *in-vitro* studies like estimation of serotonin and dopamine. From the results we found that the EEPG shown significant ($p<0.05$ - $p<0.01$) improvement in behavioral parameters *viz.* marble burying behavior, locomotion and memory retention. It is also identified that the EEPG treated groups had shown the significant ($p<0.01$ and $p<0.05$) increase in levels of dopamine and serotonin which may be responsible for behavioral functions.
Keywords: OCD, Marble burying behavior**,** *Psidium guajava* and Anxiety.

INTRODUCTION

Anxiety is implicated in a number of psychiatric disorders, such as depression, panic attacks, phobias, generalized anxiety disorder, obsessive-compulsive disorder and post-traumatic stress disorder. Anxiety disorders affect women twice as frequently as they do men. Many studies shown that people with depression often experience symptoms of an anxiety disorder. Anxiety classified into various types such as Generalized anxiety disorder (GAD), Panic disorder, Phobias, Obsessive compulsive disorder (OCD), Post traumatic stress disorder (PTSD) [1].

Generalized anxiety disorder (GAD) often is hard to distinguish from panic disorder, common anxiety disorders, or social phobia because the symptoms are often similar and they often cross over between the disorders. Panic disorder is another type of generalized anxiety disorder that often co-exists with depression. Panic disorder affects 6 million Americans every year, most often young adults. Panic disorder involves the sudden onset of overwhelming fear and terror. Phobic disorder is an unreasonable or irrational fear of something that poses little or no real danger. If people with phobias can't avoid what they fear, then it immediately results in a marked anxiety response. Social anxiety disorder, also called social phobia, is a psychological condition that causes an overwhelming fear of situations that require interacting with another person or performing in front of others. Post-traumatic stress disorder (PTSD) happens after exposure to a traumatic event. It may be an event the person witnessed, or a situation in which the person was confronted with a threat of death or serious injury to himself or others. Obsessive-compulsive disorder (OCD) is a type of anxiety disorder in which people suffer from recurrent, unwanted thoughts or ideas (obsessions); engage in repetitive, irrational behaviors or mental acts (compulsions); or both [2].

Obsessions are unwanted, recurrent, and disturbing thoughts which the person cannot suppress and which can cause overwhelming anxiety [3]. A minority are regarded as over-valued ideas and rarely, delusions [4].

Common obsessive thoughts in obsessive-compulsive disorder (OCD) [5]

Obsession	Percent
Contamination from dirt, germs, viruses (e.g. HIV), bodily fluids or faeces, chemicals, sticky substances, dangerous materia(e.g. asbestos)	37.8%
Excessive concern with order or symmetry	10.0%
Fear of harm (e.g. door locks are not safe)	23.6%
Obsessions with the body or physical symptoms	7.2%
Religious, sacrilegious or blasphemous thoughts	5.9%
Sexual thoughts (e.g. being a paedophile or a homosexual)	5.5%
Urge to hoard useless or worn out possessions	4.8%
Thoughts of violence or aggression (e.g. stabbing one's baby)	4.3%

Compulsions are repetitive, ritualized behaviors that the person feels driven to perform to alleviate the anxiety of the obsessions. Compulsions are repetitive behaviors or mental acts that the person feels driven to perform. A compulsion can either be overt and observed by others (e.g. checking that a door is locked) or a covert mental act that cannot be observed (e.g. repeating a certain phrase in one's mind). Covert compulsions are generally more difficult to resist or monitor than overt ones as they are 'portable' and easier to perform [3].

Common compulsive behaviors in obsessive-compulsive disorder (OCD) [5]

Compulsion	Percent
Checking (e.g. gas taps)	28.8%
Cleaning, washing	26.5%
Repeating acts	11.1%
Mental compulsions (e.g.special words or prayers repeated in a set manner)	10.9%
Ordering, symmetry or exactness	5.9%
Hoarding/collecting	3.5%
Counting	2.1%

Guava (*Psidium guajava* Linn.), belonging to the Family Myrtaceae with about 133 genera and more than 3,800 species [6]. Guava is rich in antioxidant compounds and contains a high level of ascorbic acid ranging from 174.2 to 396.7 mg/100 g fresh fruit [7]. Myricetin and apigenin were reported to be 549.5 and 579.0 mg/kg dry weight, respectively [8]. It plays a vital role in fulfilling the vitamin C deficiency among the people of the country since 100 g of fruit contains about 260 mg of vitamin C [9], which is 2–5 times higher than the fresh orange [10]. The leaf extracts *Psidium guajava* Linn. Posses various folk medicinal properties and used to treat gastrointestinal disturbances such as vomiting, diarrhea, inhibition of the peristaltic reflex, gastroenteritis, spasmolytic activity, dysentery, abdominal distention, flatulence and gastric pain [11]. Many scientific works were also carried out on various extracts of leaves, fruit, and seeds of *Psidium guajava* proved the importance of it in modern medicine as Antioxidant, Anti-diabetic, Hepatoprotective, Spermatoprotective, Anti-cancer, Immunomodulatory etc [10].

In this present research work the pharmacological effect of *Psidium guajava* Linn against L-arginine induced obsessions were evaluated along with *in-vitro* studies like estimation of neurotransmitters serotonin and dopamine.

MATERIALS AND METHODS

Collection and authentification of plant material:

Fresh leaves of the *Psidium guajava* were collected from local areas of Warangal and authenticated by Prof. Vatsavaya.S.Raju**,** Department of Botany, Kakatiya University, Warangal, Telangana, India and voucher specimen was submitted in our institution (SAM/03/2012) for future reference.

Preparation of leaf extract [12]

The leaves were air-dried at room temperature (25°C) for 2 weeks, after which it was grinded to a uniform powder. The ethanolic extract (EEPG) was prepared by soaking the dry powdered plant material in sufficient amount of ethanol at room temperature for 48 h. The extract was filtered through a Whatmann filter paper and concentrated using a rotary evaporator with the water bath set at 40°C. The obtained crude extract was stored in desiccators at 4 ^{0}C [13]. Phytochemical screening of ethanolic leaf extract of *Psidium guajava* was performed using standard procedures [14].

Experimental animals:

Adult Swiss albino mice (18-22g) were procured from Sanzyme labs, Hyderabad (Reg no.93/1999/CPCSEA). Animals were housed at CPCSEA approved animal house of St. John College of Pharmacy (Reg no.1278/ac/09/CPCSEA), Warangal

under a standard 12 hour light/dark cycle and controlled conditions of temperature and humidity (25 ± 2°C, 55–65%). Mice received standard rodent pellet diet and water *ad libitum*. The study was approved by the Institutional animal ethics committee of St. John College of Pharmacy (IAEC no.018/IAEC/StJCOP/2012).

For *in vivo* pharmacological studies the animals were acclimatized to the laboratory conditions for a week prior to the experimentation and randomly divided into four and five groups of each six animals respectively. Principles of animal handling were strictly adhered to CPCSEA regulations and the handling of animals was made under the supervision of animal ethics committee of the institute.

Acute oral toxicity study:

The acute oral toxicity procedure was followed according to OECD 423 guidelines. The acute toxic class method is a stepwise procedure with 3 animals of a single sex per step by using EEPG. It was observed that the test extract was not mortal even at 2000mg/kg dose.

Experimental design

Mice were divided into 5 different groups (n=6 per group)

GROUP 1: Control group received normal saline.

GROUP 2: Animals treated with L-arginine of 400mg/kg (i.p)

GROUP 3: Animals treated with standard drug Fluoxetine of 30mg/kg (i.p)

GROUP 4: Animals treated with EEPG of 200 mg/Kg (p.o)

GROUP 5: Animals treated with EEPG of 400 mg/Kg (p.o)

Study Plan

In the present study the animals were pretreated with EEPG for a period of 21days (200 and 400 mg/kg/p.o). OCD was induced by L-arginine weekly twice for 10doses. During drug treatment the behavioral studies were conducted using water maze, elevated plus maze, actophoto meter. Marble burying behavior and open field method were also observed. After the drug treatment the animals were sacrificed and the brains were removed and homogenized. The homogenized content was used for the estimation of neurotransmitter levels.

***In-Vivo* Pharmacological Evaluation**

Locomotor Activity Test:

Locomotor activity was assessed using Actophotometer, which had a circular arena of 40 cm covered with the lid, equipped with three infrared beams and photo-cells connected to digital count. Locomotor activity was measured for a 15-min period [15, 16]

Morris Water maze Test

A spatial test was performed by the method of Morris with minor modification. The water maze is a circular pool (120cm in diameter and 50cm in height) with a featureless inner surface. The pool was filled to a depth of 35cm with water containing 500mL of milk (20±1 ◦C). The pool was divided into four quadrants of equal area. A white platform (6cm in diameter and 29cm in height) was then placed in one of the pool quadrants.

The first experimental day was dedicated to swimming training for 60 s without the submerged platform. During the five subsequent days, the mice were given two daily trials with an inter-trial interval of 30 min in the presence of the platform in place. When a mouse located the platform, it was permitted to remain on it for 10 s. If the mouse did not locate the platform within 120 s, it was placed on the platform for 10 s. The animal was taken to its home cage and was allowed to dry up under an infrared lamp after each trial after several trials; the test was conducted on the 21st day injection of L-arginine. In each trial, the time required to escape on to the platform was recorded [17].

Open Field Activity Test

The open field was made of plywood and consisted of a floor (96 x 96 cm) with high walls. Entire apparatus was painted black except for 6 mm thick white lines that divided the floor into 16 squares. Each animal was placed at one corner of the apparatus and for the next 5 min, it was observed for the ambulations (number of squares crossed), total period of immobility (in sec), number of rearings, groomings and fecal pellets [18, 19].

Marble burying behavior

The anti-compulsive effect was assessed by the widely used model of studying the marble-burying behavior of mice [19, 20]. Marble-burying is the most sensitive to the selective serotonin reuptake inhibitors (SSRI) class of psychotherapeutics, which is currently used as a drug of choice for OCD, hence this test is considered as model for compulsive behavior.

Mice were placed individually in a small mouse-size Plexiglas cage containing bedding that was 5 cm in depth [21], along with 25 small marbles arranged in six evenly spaced rows of four, with one marble in the middle. Testing was conducted for a 30-min period. Mice were initially pretested as described above, and only those that buried at least 12 marbles were used for further testing. After a 30-min exposure to the marbles, mice were removed and the unburied marbles were counted [24]. Marbles were considered buried if they were at least one-half covered with bedding [16, 25].

Elevated plus maze

An elevated plus-maze test was performed on all nest-builders previously tested in the open field. BIG and SMALL male mice (n = 10) used in the open field test were taken to another testing room 3–7 days before the elevated plus maze test. The plus-maze was made of grey Perspex and elevated to a height of 75 cm. It consisted of two open arms (30cm×5 cm, with 4mm high ledges) and two enclosed arms (30cm×5 cm, with a closed roof). All tests took place between 13:00 and 15:00 under dim red light. Each mouse was placed in the central square (5cm×5 cm) facing an open arm, and allowed to explore the maze for 5 min. The times spent on the open arms, closed arms and in the center were measured as well as the number of entries into a closed arm, into an open arm, and the total number of entries. An entry was defined as at least three of the four paws being on the arm. The maze was cleaned before each test. The mice were not used for any additional experiments [18].

***In Vitro* Methods**

Dopamine Assay

The assay represents a miniaturization of the trihydroxide method. To 0.02ml of HCl phase, 0.05ml 0.4M and 0.01ml EDTA/Sodium acetate buffer (pH 6.9) were added, followed by 0.01ml iodine solution (0.1M in ethanol) for oxidation. The reaction was stored after two minutes by addition of 0.01ml Na2SO3 in 5m NaOH. Acetic acid was added 1.5 minutes later. The solution was then heated to 100 for 6 minutes. When the sample again reached room temperature, excitation and emission spectra were read in the microcuvette at 330-375nm[22, 23].

Serotonin Assay

As mentioned earlier some modifications in reagent concentration became necessary together with changes in the proportions of the solvent, in order to obtain in a good fluorescence yield with reduced volume for serotonin determination, the O-pthaldialdehyde (OPT) method was employed. From the OPT reagent 0.025ml were added to 0.02ml of the HCl extract. The fluorophore was developed by heating at 100°C for 10 min. After the samples reached equilibrium with the ambient temperature, intensity reading at 360-470 nm were taken using the micro cuvette [21, 22].

RESULTS:

Effect of EEPG on marble burying behavior

Fluoxetine treated mice shows 100% protection against L-arginine induced compulsions where as EEPG 200 mg/kg, 400 mg/kg have protection against L-arginine. The EEPG treated group of 200 and 400 mg/kg shows a significant decrease in marble burying ($p<0.05$ and $p<0.01$) when compared to the standard group as shown in Table 1.

Effect of EEPG on locomotion

There was a decrease in the number of beam interactions in the group II animals ($p<0.01$) where as in the standard group there is no significant decrease in the beam interactions which indicates the locomotion of the animal is not affected when compared to that of the control group. The EEPG treated groups 200 and 400 mg/kg produced a significant difference ($p<0.05$ and $p<0.01$) in locomotion (Table 2).

Effect of EEPG on morris water maze

There is an increase in 'escape latency' in group II when compared with the control group. Both groups III and IV showed significant ($p<0.05$ and $p<0.05$) decrease in 'escape latency'(Table 3).

Effect of EEPG on open field test

The group II animals have shown decrease in the ambulation, rearing and grooming when compared with the control group. The EEPG treated group of 200 and 400 mg/kg shows a significant decrease in ambulation, rearing and grooming ($p<0.05$ and $p<0.01$) when compared to the standard group (Table 4).

Effect of EEPG on elevated plus maze

The group II animal shown decrease in the time spending in open arm and increase in the time spending in closed arm when compared with the control group. The EEPG treated group of 200 and 400 mg/kg shows a significant increase in the time spent in open arm and decrease in the time spent in closed arm ($p<0.05$ and $p<0.01$) when compared to the control group (Table 5).

Effect of EEPG on dopamine level

Significant increase in the dopamine levels was observed in mice brain of EEPG and fluoxetine treated when compared with the group II and group II shows the decreased levels dopamine when compared with that of control group (Table 6).

Effect of EEPG on serotonin level

Significant decrease in the serotonin levels was observed in mice brain in L-arginine treated animals i.e. group II when compared with the control group animals. Whereas the treatment with EEPG 200mg/kg and 400 mg/kg and fluoxetine increased the serotonin levels (Table 6).

Table-1: Effect of EEPG on marble burying behavior

	Observation	Group-I	Group- II	Group-III	Group- IV	Group-V
5	No. of marbles buried	5.50±0.61	4.50±0.61***	0.16±0.16***	6.83±0.41**	4.12±0***
10		7.2±1.01	16.3±0.76***	0.83±0.54***	9.5±0.56***	4.24±0***
15		6.8±0.96	20.6±0.80***	2.83±0.94***	12.3± 0.42***	1.33±0.33**
20		8.6±.95	19.5±0.42**	2.66±0.98***	14.3±0.66***	3.8±0.4***
25		5.3±1.05	21±0.44***	1.5±0.61***	12.3±0.71**	5.16±0.7***
30		6.6±0.61	23.3±0.21***	1.2±0.51***	11.1±0.6***	5.15±0.5**

Values are expressed as mean ± SEM of 6 animals Comparisons were made between: Group I and group II, III, IV, V. Statistical significance done by ANOVA, by using GraphPad Software (version-5.0.0.288), followed by Dunnet's "t" test, ***p<0.001, **p<0.01 and *p<0.05.

Table-2: Effect of EEPG on locomotion

GROUPS	TREATEMENT	READINGS(15 mins)
I	Control	107 ± 2.46
II	L-arginine of 400mg/kg (i.p)	78.67±2.27***
III	fluoxetine (30 mg/kg, i.p)	100±1.12*
IV	EEPG-200mg/kg, p.o	90.33±1.99***
V	EEPG-400mg/kg, p.o	99.83±0.79*

Values are expressed as mean ± SEM of 6 animals Comparisons were made between: Group I and group II, III, IV, V. Statistical significance done by ANOVA, by using GraphPad Software (version-5.0.0.288), followed by Dunnet's "t" test.***P<0.001, **P<0.01 and *P<0.05.

Table-3: Effect of EEPG on Morris Water Maze

GROUPS	TREATEMENT	Time identification of stand in secs
I	CONTROL	11±0.46
II	L-arginine of 400mg/kg (i.p)	14.6±1.54*
III	fluoxetine (30 mg/kg, i.p)	4.66±0.49***
IV	EEPG-200mg/kg, p.o	7.33±1.02**
V	EEPG-400mg/kg, p.o	5.66±0.9***

Values are expressed as mean ± SEM of 6 animals Comparisons were made between: Group I and group II, III, IV, V. Statistical significance done by ANOVA, by using GraphPad Software (version-5.0.0.288), followed by Dunnet's "t" test.***P<0.001, **P<0.01 and *P<0.05.

Table 4: Effect of EEPG on open field test

GROUP	I	II	III	IV	V
Ambulation	41.8±0.54	8.8±0.74***	28.83±2.05***	23.83±1.8***	30±1.88***
Rearing	11.22±0.6	5.2±0.20***	8.02±0.71*	7.5±0.90**	8.4±0.77*
Grooming	11.76±1.01	2.33±0.29***	7.87±0.62***	3.05±0.4***	6.31±0.25***

Values are expressed as mean ± SEM of 6 animals Comparisons were made between: Group I and group II, III, IV, V. Statistical significance done by ANOVA, by using GraphPad Software (version-5.0.0.288), followed by Dunnet's "t" test.***P<0.001, **P<0.01 and *P<0.05.

Table-5: Effect of EEPG on elevated plus maze

GROUPS	OPEN ARM	CLOSED ARM
I	3.22±0.05	3.65±0.02
II	1.52±0.27***	3.41±0.26***
III	4.48±0.20**	0.76±0.18***
IV	2.45±0.10***	3.39±0.03**
V	2.85±0.07***	2.89±0.07**

Values are expressed as mean ± SEM of 6 animals Comparisons were made between: Group I and group II, III, IV, V. Statistical significance done by ANOVA, by using GraphPad Software (version-5.0.0.288), followed by Dunnet's "t" test.***P<0.001, **P<0.01 and *P<0.05.

Table-6: Effect of EEPG on dopamine and serotonin level

GROUPS	Dopamine (Pictogram/mgtissue)	Serotonin (Pictogram/mg tissue)
CONTROL	650.2±3.41	832.3±4.61
L-arginine of 400mg/kg (i.p)	503.2±4.34***	708.5±1.1***
fluoxetine (30 mg/kg, i.p)	670.7±2.56**	861.3±4.5***
EEPG-200mg/kg, p.o	578.7±3.57***	784.5±5.25***
EEPG-400mg/kg, p.o	638.2±4.07*	816.2±2.1*

Values are expressed as mean ± SEM of 6 animals Comparisons were made between: Group I vs group II and group II vs III, IV, V. Statistical significance done by ANOVA, by using GraphPad Software (version-5.0.0.288), followed by Dunnet's "t" test.***P<0.001, **P<0.01 and *P<0.05.

DISCUSSION:

Obsessive–compulsive disorder (OCD) is a high-prevalence psychiatric disorder affecting 2–5% of the population. Although its pathophysiology remains unclear, current evidence implicates contributions of the serotonergic and dopaminergic neurotransmitter systems and a neural circuitry that includes the orbitofrontal cortex, the thalamus and the striatum.

The neurohumoral studies indicated that OCD is associated with serotonin dysfunction and/or hyperactivity of dopamine & glutamate. Incidentally, nitric oxide (NO) donor and endogenous NO suppress serotonin levels, whereas nitric oxide synthase inhibitors are reported to elevate the same in various brain areas. NO donors are also reported to increase the release of dopamine, and facilitate glutamate release in several brain structures. The effect of employed nitrergic agents (L-arginine) in present investigation on marble-burying behavior may be related to their influence on nitric oxide, serotonin, dopamine and/or glutamate, which are implicated in pathophysiology of OCD [23].

The marble-burying task has been characterized as a model of compulsive behavior (obsessive compulsive disorder) [20]. Furthermore, it is most sensitive to the selective serotonin reuptake inhibitor (SSRI) class of psychotherapeutics, which is currently the medication of choice for obsessive compulsive disorder. Acute administration of L-arginine, a nitric oxide precursor significantly influenced the marble-burying behavior without affecting locomotor activity. L-arginine treated mice exhibited significantly higher number of marbles buried when compared with control group. EEPG treated mice exhibited the decrease in the number of marbles buried. In water maze test the impaired spatial learning by L-Arginine and the improvement by EEPG shows the significant property of memory retention. The extract also decreased the escape latency to the platform. In the elevated plus maze test the EEPG shows the decrease in the time spent in open arm and increase in closed arm. The EEPG contains flavonoids, terpenoids and saponins, a number of scientific reports indicated that terpenoids produced CNS depressant action. In recent years, it has become accepted that neuronal pathways in the brain whose transmitter functions are sub served by dopamine and 5HT have great behavioral significance in animals and man. Here the EEPG extract has elevated the levels of dopamine and 5-HT, this clearly reveals the prominent behavioral actions of the extract.

CONCLUSION

Psidium guajava (Linn.) is popularly known as 'poor man's apple of the tropics, has a long history of traditional use for a wide range of diseases. The fruit as well as its juice is consumed for its great taste and nutritional benefits. Much of the traditional uses have been validated by scientific research as Antioxidant, Anti-diabetic, Hepatoprotective, Spermatoprotective, Anti-cancer, Immunomodulatory etc. In this present research work we have studied for its activity on obsessive compulsive disorder and found to be effective.

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