

Evaluation of *Punica granatum* fruit juice for anti-anxiety activity

Shivaraj Kulkarni^{*1}, Ritesh Bathe² and Akshay Javalgikar²

¹Department of Pharmacology, N.E.T. Pharmacy College, Raichur, India

²Department of Pharmacology, Sahyadri College of Pharmacy, Methwade, India

*Correspondence Info:

Mr. Shivaraj Kulkarni

Department of Pharmacology,

Sahyadri College of Pharmacy, Methwade, India

E-mail: shivaraj3286@gmail.com

Abstract

Background and Objectives: In traditional system of Indian medicine, *Punica granatum L.* fruits are widely used for treatment of brain diseases, fever, heart diseases, diarrhea, dysentery, piles, inflammation and bronchitis. However, there is no authentic scientific data reported regarding anxiolytic activity of *Punica granatum* (Linn) fruit juice. To evaluate the anxiolytic activities of *Punica granatum L.* fruit juice in various validated animal models of anxiety in mice.

Methods: The preliminary phytochemical investigation was carried out with the fruit juice of *Punica granatum* for qualitative identification of phytoconstituents. Animals were administered with orally 0.1, 0.2, 0.4 and 0.8 ml of *Punica granatum* fruit juice and observed for its mortality upto 48 hours study period (short term toxicity) to determine LD₅₀. From the LD₅₀ dose, 1/20, 1/10 & 1/5 doses were selected and considered as low, medium and high dose respectively. For assessing the anxiolytic activity, models like Elevated Plus Maze, Hole-Board were used Diazepam was used as a standard reference for anxiolytic.

Results: Preliminary phytochemical investigation of the PGFJ (*Punica granatum L.* fruit juice) revealed the presence of flavonoids, saponins, tannins, sterols, polyphenols, alkaloids, carbohydrates and proteins. LD₅₀ (acute oral toxicity) of *Punica granatum* fruit juice was determined in mice with a dose limit of 2000 mg/kg as per OECD guidelines no.425 and even upto 2000 mg/kg dose no mortality was recorded. Hence the experimental doses selected were 1/20, 1/10 and 1/5 of the LD₅₀ value and considered as low, medium and high doses of *Punica granatum* fruit juice 100, 200 and 400 mg/kg respectively. In Elevated Plus Maze model, low, medium and high doses PGFJ and Diazepam (2 mg/kg) had significantly increased number of entries, time spent in open arms and decreased the number of entries and time spent in closed arms. In Hole-board model, Diazepam (2 mg/kg), medium and high doses (200 and 400mg/kg) but not the low dose (100mg/kg) of PGFJ had significantly increased the number of head dips, latency of first head dip and number of rearings.

Conclusion: The present study ascertains that the plant *Punica granatum L.* fruit juice possesses significant antianxiety activity in mice and study concludes the beneficial effects of *Punica granatum* fruit juice in treatment of anxiety signifies the rational basis for its traditional use.

Keywords: *Punica granatum*, anxiety, Diazepam, Elevated Plus Maze, Hole-board model.

1. Introduction

In recent years, anxiolytic drugs have been among the front-runners in terms of the number of prescription written in medical practice. This may be due to the tense life style imposed on man by the competitive atmosphere. Some degree of anxiety is a part of normal life, but treatment needed when it is disproportionate to the situation and excessive.[1] Anxiety disorders may be due to life experiences, heredity and brain chemistry. Chemical messenger's (neurotransmitters) in the brain regulate thought and feeling. Sometimes has a problem with brain messages sent out because of a chemical imbalance. Approximately 4-6% of the population suffers from anxiety so severely, that it disrupts routine life functions.[2] In today's world of stressful life style with the individuals, anxiety is one of the common symptoms associated, affecting almost 1/8 th of the total

population worldwide and has become a very important area of research interest in psychopharmacology during this decade and is also obvious component of many psychiatric and medical conditions.[3] Currently, the most widely prescribed medications for anxiety disorders are the Benzodiazepines. However, the clinical uses of Benzodiazepines are limited by their side effects such as psychomotor impairment, potentiation of other central depressant drugs and dependence liability. Therefore, the development of new herbal medications possessing anxiolytic effect without the complication of BZD would be of great importance in the treatment of anxiety-related disorders. In recent years, interest is growing in alternative medicines that include plant derived medications, polyherbal formulations that affect the mind. During last few years there has been increase in usage of alternative medicines by the patients for

such ailments, Therefore many herbal medicines have been accepted in our country for treating anxiety disorders as *Bacopa monniera* (Brahmi)[4] and *Withania somnifera* (Ashwagandha).[5] *Punica granatum* is one such medicinal plant that has been extensively used in the traditional system of medicine. In ayurveda, this plant is used to treat brain disorders, heart disease, anthelmintic, diarrhea, dysentery, piles, colic, inflammation, aphrodisiac diuretic and bronchitis.[6] Ingesting healthy *Punica granatum* fresh fruit juice has also been known to alter anxiety, insomnia and improves circulation of the heart.[7] No major investigation reports were found pertaining to its CNS activity. Hence, the present study is proposed to evaluate the anxiolytic activity of *Punica granatum* fruit juice by using different validated animal models for anxiety based on exploratory behaviour in mice.

2. Materials and methods

2.1 Plant Material

Fresh fruits of the plant *Punica granatum* were collected around the fields of Agricultural University, Raichur and authenticated by Prof. Vedavyas, Department of Botany, L.V.D College Raichur.

2.2 Preparation of fruit juice of *Punica granatum*:

The freshly collected fruit was taken and peel was removed then, arils/seeds were taken from the fruit then squeezed in muslin cloth. The obtained juice was collected and filtered in whatmann filter paper. The juice was kept in refrigerator at 0-8°C until used.

2.3 Preliminary phytochemical screening

The preliminary phytochemical investigation was carried out with the fruit juice of *Punica granatum* for qualitative identification of phytoconstituents and to detect carbohydrates, proteins, amino acids, flavonoids, saponins, alkaloids, glycosides, phytosterols, tannins and phenolics.

2.4 Experimental animals:

Swiss albino mice of either sex weighing between 20-25g were procured from Shri Venkateshwara Enterprises, Bangalore for experimental purpose. The animals were fasted overnight and during the experiment. All experiments were carried out during the light period. The approval of the Institutional Animal Ethical Committee (IAEC) of N.E.T Pharmacy College, Raichur (Karnataka) was taken prior to the experimentation. All the protocols and the experiments were conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), with registration number 576/02/bc/CPCSEA.

2.5 Acute toxicity studies:

The oral acute toxicity of *Punica granatum* was determined by using albino mice of either sex (20-25 g), maintained under standard conditions. The animals were fasted for 3h prior to the experiment. Animals were

administered with single dose of *Punica granatum* fruit juice (PGFJ) and observed for its mortality up to 48 h study period (short term toxicity). Based on the short-term toxicity profile, the next dose was determined as per OECD guidelines No 425. From the LD₅₀ dose 1/20, 1/10 and 1/5th doses were selected and considered as low, medium and high dose respectively. The PGFJ was administered orally at the doses of 50, 100, 200, 400, 800, 1000 and 2000 mg/kg to different groups of mice. No mortality was observed for 48hr study period. PGFJ was found to be safe at the given doses.

2.6 Experimental design:

In the investigation, Albino mice of either sex weighing between 20-25g were used for the study. The animals were divided into five groups, each group containing six mice.

Group A - Normal control (distilled water 0.2ml p.o)

Group B - Standard (Diazepam 2 mg/kg p.o)

Group C - Fruit juice of *Punica granatum* (100mg/kg p.o)

Group D - Fruit juice of *Punica granatum*(200 mg/kg p.o)

Group E - Fruit juice of *Punica granatum* (400 mg/kg p.o)

2.7 Determination of antianxiety activity: [1-3,8]

Elevated plus-maze model:

The Elevated plus-maze apparatus comprises of two open arms (16×5cm) and two closed arms (16×5×12cm) that extend from a common central platform (5×5cm). The entire maze is elevated to a height of 25cms above the floor level. During a five minutes test period, the following parameters were recorded using Sony handy camera:

- Number of entries into open arm,
- Number of entries into closed arm,
- Time spent in the open arm
- Time spent in the closed arm
- Total number of entries in open and closed arm

Hole-board apparatus:

The apparatus used in this model consists of wooden chamber (40x40x25 cm) with 16 holes (diameter 3 cm) on the floor, elevated from the ground so that the rats could peep through the holes. During a five minutes test period the following parameters were recorded using Sony handy camera.

- Latency to the first head dips
- The number of head dips through the holes
- Number of rearings

2.8 Statistical Analysis

The data were expressed as mean ± S.E.M from 6 animals [n=6]. The results were subjected to statistical analysis by using Unpaired t-test to calculate the significance difference if any among the groups. P<0.05 was considered as statistical significance using Graph Pad Prism Software.

3. Results

The preliminary phytochemical studies with the *Punica granatum* fruit juice revealed the presence of flavonoids, saponins, tannins, sterols, polyphenols, alkaloids, carbohydrates and proteins. LD₅₀ (acute oral toxicity) of

Punica granatum fruit juice was determined in mice with a dose limit of 2000 mg/kg as per OECD guidelines no.425 and even upto 2000 mg/kg dose no mortality was recorded. Hence the experimental doses selected were 1/20, 1/10 and 1/5 of the LD₅₀ value and considered as low, medium and high doses respectively.

Elevated plus Maze model (EPM):

Punica granatum fruit juice was screened for anxiolytic activity using EPM model in mice. The vehicle treated mice spent 21.75±10.781 sec in the open arm, 269.25±9.98 sec in the closed arm, with 2.0±0.40 number of entries in the open arm and 14.75±1.49 number of entries into the closed arm.

The different doses of PGFJ i.e 100, 200 and 400 mg/kg were administered orally daily once for 8 consecutive

days. On the eighth day, 1h after treatment, it was observed that low, medium and high doses of PGFJ had significantly increased time spent in the open arm and number of entries into open arm dose dependently and significantly decreased time spent in the closed arm and number of entries in the closed arm dose dependently as compared to control group.

Standard drug Diazepam (2 mg/kg) has long been reported for its anxiolytic activity in mice with EPM model. Animals treated with Diazepam showed significantly ($P<0.01$ and $P<0.001$) increased time spent in the open arm, number of entries in the open arm and significantly ($P<0.001$ and $P<0.01$) decreased time spent in the closed arm and number of entries in the closed arm respectively as compared to control group.

Table 1: Anxiolytic effect of PGFJ with Elevated Plus Maze model in mice

S. No	Treatment	Time spent in open arm (Sec)	Time spent in closed arm (Sec)	Number of entries into open arm (Count/ 5 min)	Number of entries into closed arm (Count/ 5 min)
1	Control	21.75±10.781	269.25±9.98	2.0±0.40	14.75±1.49
2	Diazepam (2mg/kg)	110.00±31.893**	174.00±27.19***	8.5±1.74***	7.5±2.05**
3	PGFJ (100 mg/kg)	53.25±5.121**	247.75±4.4**	3.25±0.85**	6.5±0.64***
4	PGFJ (200 mg/kg)	84.5±20.089**	202.5±16.52***	6.0±0.85**	6.5±0.64***
5	PGFJ (400 mg/kg)	107.75±30.051**	178.25±24.77***	7.5±1.7***	6.75±1.37***

n = 6 in each group. Values are expressed as mean±SEM. Statistical analysis was carried out by using Unpaired 't' test. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ statistically significant as compared to control group.

Hole Board Model:

Each mice was placed individually in the Hole board apparatus and the parameters like the number of head dips, duration of head dips, latency to first head dips and no of rearings were recorded.

PGFJ at the dose of 100 mg/kg p.o. produced no significant increase in number of head dips and no of rearings but, significant increase ($P<0.05$) in the latency to first head dips were noted when compared with control group. PGFJ at the dose of 200 mg/kg p.o had significantly ($P<0.01$ and $P<0.001$) increased the number of head dips, number of

rearings and latency to first head dips when compared with control group. PGFJ at the doses of 400 mg/kg p.o had significantly ($P<0.001$ and $P<0.01$) increased latency to first head dips, number of rearings and the number of head dips when compared with control group.

The reference standard Diazepam (2mg/kg) p.o treated group showed highly significant ($P<0.001$) increase in the number of head dips, duration of head dips, latency to first head dips and no of rearings as compared with control group.

Table 2: Anxiolytic effect of PGFJ with Hole Board Model in mice

S. No	Treatment	Number of head dips	Latency to first head dips	Number of rearing
1	Control	16.75±9.56	8.75±2.56	4.5±1.25
2	Diazepam (2mg/kg)	53.00±4.95***	24.00±4.37***	11.75±1.79***
3	PGFJ (100 mg/kg)	24.25±4.48	13.5±1.70*	5.75±1.19
4	PGFJ (200 mg/kg)	41.25±2.68**	22.5±3.22***	9.2±2.17**
5	PGFJ (400 mg/kg)	47.75±5.79**	24.25±1.93***	11.25±1.31***

n = 6 in each group. Values are expressed as mean±SEM. Statistical analysis was carried out by using Unpaired 't' test. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ statistically significant as compared to control group.

4. Discussion

The significance of anxiety in health and disease is well recognised, its underlying neurobiological mechanisms are not well understood.[2] Anxiety disorders may be due to life experiences, heredity and brain chemistry.

Benzodiazepines, the widely prescribed and clinically effective anxiolytics, are thought to produce their pharmacological actions via specific high affinity binding sites on a supramolecular complex composed of GABA-A and BZD receptor coupled with a chloride ion channel. At the cellular level, stimulation of GABA-A receptor results in an

increased chloride conductance and usually hyperpolarisation. In intact animals activation of this receptor is known to be associated with antianxiety and anticonvulsant actions.[2]

Animal behavioural models of anxiety have played an important role in the assessment of anxiolytics. Choice of the animal models for anxiety studies and to search for new classes of anxiolytic compounds with potential therapeutic applications is an important parameter in drug development research.[2] *Punica granatum* is a medicinal plant that has been extensively used in the traditional system of medicine. In ayurveda, this plant is used to treat brain disorders, heart disease, diarrhea, dysentery, piles, colic, inflammation and bronchitis.[6] There are few reports available for its scientific evaluation of the pharmacological effects on CNS activity.[9] The present work was aimed to demonstrate the anxiolytic effects of *Punica granatum L.* fruit juice in mice in several behavioural models like EPM, Hole-board, Open field and Light-Dark paradigms.

EPM is one of the most popular animal test for research on behavioral pharmacology of anxiety. In EPM, naive mice will normally prefer to spend much of their allotted time in the closed arm. This preference appears to reflect an aversion towards open arms that is generated by fear of open spaces. Drugs that increase open arm exploration are considered as anxiolytics.[10] In our study, we observed that PGFJ (100, 200 and 400 mg/kg) and Diazepam (2 mg/kg) induced significant increase in both the number of entries and time spent in the open arms. The number of entries and time spent in the closed arms were reduced in the PGFJ treated groups as compared to the control group. These observations clearly show statistically significant anxiolytic activity in EPM model in mice.

Hole-board model indicates that head-dipping behaviour is sensitive to changes in the emotional state of the animal and suggests, the expression of an anxiolytic state in animals may be reflected by significant increase in head-dipping behaviour. The PGFJ medium and high doses (200 & 400mg/kg) shows increase in number, latency of head dipping and the number of rearing in the hole board test. But the effect of low dose (100mg/kg) was found to be non-significant when compared with control. Diazepam (2 mg/kg) had significantly increased the number, latency of head dipping and the number of rearing. Therefore the increased number of head dippings and rearings are representation of anxiolytic effect.

The present work demonstrated that the fruit juice of *Punica granatum L.* has anxiolytic effects in mice. This action of PGFJ represents the functional similarity to BZD's which are widely used as anxiolytic agents. The benzodiazepines are known to act through the BZD-GABA receptors. The role of GABA in anxiety is well established. The mechanism of anxiolytic action of PGFJ may involve an action on

GABAergic transmission;[11] however, further studies are needed to ascertain this.

Earlier reports on the chemical constituents of plants and their pharmacology suggest that plant containing flavonoids, saponins and tannins possesses activity against many CNS disorders.[12] Phytochemical tests of PGFJ revealed the presence of flavonoids, saponins and tannins. It is possible that the mechanism of anxiolytic action of PGFJ could be due to the binding of any of these Phytochemicals to the GABA-A – BZD complex.

Acknowledgement

The authors are thankful to Dr Shivakumar, Associate Professor, Dept of pharmacology, N.E.T College of Pharmacy Raichur and Dr. Satish s, Associate Professor, Srinivas College of Pharmacy, Mangalore for providing necessary facilities to carry out this work.

References

- [1] Agarwal MN, Itankar PR, Patil AT, Vyas JC, Kelkar AJ. Anxiolytic activity of *Annona squamosa* leaf extracts in mice. *Ind J Pharm Educ Res*, 2009; 43(1):99-103.
- [2] Kulkarni SK. Handbook of experimental pharmacology. 3rd edition. Vallabh Prakashan, New Delhi, 1999: 28-39.
- [3] Yadav AV, Kawale LA, Nade VS. Effect of *Morus alba L.* (mulberry) leaves on anxiety in mice. *Ind J Pharmacol*, 2008; 40(1): 32-36.
- [4] Singh HK, Dhawan BN. Drugs affecting learning and memory. Lectures in neurology. New Delhi: Wiley Eastern, 1992: 189-202.
- [5] Battacharya SK, Kumar A, Gohsal S. Effect of glycol withanolides from *Withania somnifera* on an animal model of Alzheimer's disease and perturbate cholinergic markers of cognition in rats. *Phytother Res* 1995; 9:110-13.
- [6] Kirtikar KR, Basu BD. Indian Medicinal Plants. 2nd ed. Periodical Experts Book Agency, New Delhi, 1935; volume-II: 1084-87.
- [7] <http://www.pogainfo.com/Pomegranate.htm> assessed on: 10/13/2008.
- [8] Vogel H. Gerhard, Vogel Wolf gang H. (Eds). Drug discovery and evaluation pharmacological assays (Springer), 2000; 2: (E) 230-244.
- [9] Sokindra Kumar, Kamal Kishore, Vijender singh. Central nervous system activity of acute administration of ethanol extract of *Punica granatum L.* seeds in mice. *Ind J Exp Biol*, 2008; 46:811-816.
- [10] Hellion-Ibarrola MC, Ibarrola DA, Montalbetti Y, Kennedy ML, Heinichen O, Campuzano M, et al. The anxiolytic-like effects of *Aloisia polystachya* in mice. *J Ethnopharmacol*, 2006; 105:400-8.
- [11] Bhatacharya SK, Satyan KS. Experimental methods for evaluation of psychotropic agents in rodents: Antianxiety agents. *Indian J Exp Biol*, 1997; 46:565-75.
- [12] Adeyemi O, Yetmitan OK, Taiwo AE. Neurosedative and musclerelaxant activities of ethyl acetate extract of *Baphia nitida* AFZEL. *J Ethnopharmacol*, 2006; 106: 312.