Kailash Sharma* and Milind Parle

PhD Research Scholar, Department of Pharmaceutical Sciences, Guru Jambheshwar University of Science & Technology, Hisar-125001 (Haryana), India.

ABSTRACT

Anxiety is one of the most common psychiatric disorders that we experienced in our everyday life. Benzodiazepine and serotonergic agents are used to treat anxiety. Long term intake of these agents may cause side effects such as sleep disturbances, weight gain, sexual dysfunction, paradoxical effects etc. Therefore, the demands of new potential anxiolytic agents are increases with negligible side effect. High importance is given to herbal medicines and phytoconstituents due to their wide therapeutic properties with minimal side effects. Annona squamosa is an excellent combination of healthy nutrients and essential ingredients. Therefore, we aimed to investigate the anxiolytic potential of Annona squamosa fruit juice in unstressed and stressed mice using anxiety models viz. Light-dark model, Elevated plus maze model and Hole-board model. Annona squamosa fruit juice (3%, 6% and 9% v/v, p.o) was administered for 14 days in different groups of unstressed and stressed mice. Annona squamosa fruit juice significantly enhanced the time spent in the lit compartment in light-dark model, number of entries and time spent in the open arms in elevated plus maze model and head dip counts in hole-board model in unstressed and stressed mice. Blood plasma nitrite levels were reduced effectively by Annona squamosa, thereby suggesting enhancement in scavenging of free radicals. Interestingly, brain GABA and Serotonin levels were markedly increased indicate anxiolytic effect of Annona squamosa. These findings, when taken together showed that Annona squamosa possesses promising antianxiety like effect.

KEYWORDS: Anxiety, Anxiolytics, Annona squamosa, Sugar Apple.
INTRODUCTION
Anxiety is a condition of human emotion, which is characterized by cognitive, emotional, and behavioral components and an uncomfortable feeling associated with nervousness, apprehension or worry.\(^1\) It has two components like physical component (Nervousness, fear, worry, insecurity) and emotional component (trembling, ulcers, nausea, vomiting, sweating, diarrhea, increased blood pressure and heart rate, etc.), which affect the cognitive functions (thinking, decision ability, learning, memory and concentration) of a person.\(^2,3,4,5\) The GABA, serotonin and dopamine neurotransmitter system have been entailed in the development of anxiety. Chronic use of antianxiety agents can cause many side effects such as blurred vision weight gain, sexual dysfunctions and gastrointestinal disorders. Therefore, the demands of such kind of medicines are increases which have negligible side effects. Herbal drugs have great importance in this context because of their potential ingredients, safe and therapeutic effects. Annona squamosa (Annonaceae) commonly known as Sugar apple, is famous for its excellent nutritional and medicinal importance throughout the world. This plant is reported for their diverse ranges of pharmacological and therapeutic properties including neuroprotective, antioxidants, antiinflammatory, analgesic, antipyretic, antiulcer, antiseptic, abortifacient etc.\(^6,7\) Therefore, we undertook the present study to evaluate the anxiolytic potential of Annona squamosa, by using different animal models and studied their effect on exploratory behaviors as well as biochemical levels.

MATERIALS AND METHODS

Plant Material
Fresh Annona squamosa (Sugar apple) fruits were purchased from the local market Hisar-Haryana (India) and authenticated from National Herbarium of Cultivated Plants (NHCP), Division of Plant Exploration and Germplasm Collection, National Bureau of Plant Genetic Resources, New Delhi (vide. No. NHCP/NBPGR/2015-37).

Preparation of Annona Squamosa Fruit Juice
Annona squamosa (Sugar apple) fresh fruit juice was prepared in a juicer (Crompton, India) eliminating seeds. Pure fruit juice of Annona squamosa was diluted with distilled water to make 3%, 6% and 9% v/v juice concentrations.

Experimental Animals
Swiss male albino mice, weighing 25-30g were procured from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar,
Haryana (India). The animals were acclimatized for at least seven days before the start of experiments. The animals had free access to feed and water except the duration of experiment and they were housed in a natural (12h each) light-dark cycle. Food administered to animals comprised of wheat porridge. Experiments were carried out between 09:00 AM – 5:00 PM. The experimental protocol was approved by Institutional Animals Ethics Committee (IAEC) and the care of animals were taken as per the guidelines of CPCSEA, Ministry of Environment and Forests, Government of India, New Delhi, India (Registration No. 0436).

**Drug protocol**

Diazepam (2 mg/kg, i.p.) obtained in the form of ampoule from Ranbaxy Laboratories Ltd. was diluted with normal saline and administered for 14 successive days in the present study.

**Experimental Design**

In the present study a total of 180 mice divided into 30 groups (15 stressed and 15 unstressed groups) were employed (Picture 1 and 2). Each group was consisting of six mice each. Unstressed mice were exposed to Light-Dark Model, Elevated-Plus Maze and Hole-Board Model for a normal duration (5 minutes), sufficient to evaluate anxiety levels in rodents.[8] Stress was formed in mice by immobilizing them for 6 hours by taping all their four limbs and trunk on a wooden board. In unstressed mice, behavioral testing was done 30 minutes after administration of the drug. In the case of stressed mice, behavioral testing was started 10 minutes after setting the animals free from immobilization.

![Picture 1: Experimental design for unstressed mice](image-url)
Light-Dark Model (LDM)
Light and dark model is commonly employed for evaluation of anxiolytic activity. The apparatus consisted of two boxes (25 cm x 25 cm x 25 cm) joined together. One box was made dark by covering its top with plywood whereas a 40 W lamp illuminated the other box. The light source was placed 25 cm above the open box. The mice were treated with the test drug or vehicle for 30 min before being placed in the lit box. The latency time for the first passage from the light compartment to the dark one, the number of transitions between the two compartments, the movement in each compartment and the time spent in each compartment were recorded for 10 min.\(^9\)

Elevated-Plus Maze (EPM)
The Elevated Plus-Maze served as the exteroceptive behavioral model.\(^{10}\) The Elevated plus Maze consisted of two open arms (16 cm x 5 cm) and two enclosed arms, (16 cm x 5 cm x 15 cm), arranged opposite to each other, extended from a central platform (5 cm x 5 cm) and the maze was elevated to a height of 25 cm from the floor. Each mouse was placed individually at the central platform of maze with its head facing towards an open arm and observed for 5 min to record the number of entries into open arm. Entry into an arm was considered valid only when all four paws of the mouse were inside that arm. The plus maze
was carefully wiped with hydrogen peroxide and dried with sponge after each trial. Test was conducted in quite room to avoid disturbances to animals.

**Hole-Board Model (HBM)**

Hole-board model is commonly employed for evaluation of anxiolytic activity. The Hole-board apparatus consisted of a wooden box (40 x 40 x 25 cm$^3$) with 16 holes (each of diameter 3 cm) evenly distributed on the floor. For a period of 5 min. the number of head dippings was counted by placing the animal in the centre of the apparatus. Head dipping in hole-board test reflected exploratory behavior of mice.[11]

**Biochemical estimations**

**Collection of blood samples**

Blood (about 1 ml) was collected from retro-orbital plexus of separate fresh groups of mice. Plasma was separated from the blood by centrifuging it at 2500 rpm at 4°C for 10 min using a refrigerated centrifuge (Remi Instruments, Mumbai, India). The nitrite levels were estimated in plasma.

**Estimation of Plasma Nitrite Levels**

Plasma nitrite was measured by using the method of 1982.[12] A mixture of 1% w/v sulphanilamide in 5% aqueous solution of m-phosphoric acid (1 part) and 0.1% w/v N-(1-Naphthyl) ethylene diaminedihydrochloride (1 part) was prepared and kept at 0°C for 60 min. 0.5 ml plasma was mixed with 0.5 ml of the above mixture and kept in dark for 10 min at room temperature. The absorbance was read at 546 nm using UV-visible spectrophotometer (Varian Cary 5000 UV-VIS-NIR Spectrophotometer, Netherland CHRIST).

**Isolation of brain**

**Estimation of Brain Serotonin Levels**

After behavioural experiments on 14th day, mice were sacrificed by decapitation and the brain was isolated, which was rinsed with isotonic saline. Weighed the brain tissue and homogenized in 3 ml HCl-Butanol (0.1 M HCl in butanol) in ice cool environment. The homogenate was used for estimation of serotonin levels The HCl-Butanol brain homogenate was then centrifuged for 10 min at 2000 rpm. 0.8 ml of supernatant phase was removed and added to an Eppendorf reagent tube containing 2 ml of heptane and 0.25 ml 0.1 M HCl. After 10 min of vigorous shaking the tube and centrifuged under same conditions to separate two phases. Upper organic phase was discarded and the aqueous phase was used for serotonin assay. 1.25 ml of o-phthalaldialdehyde reagent was added to 1 ml of the aqueous phase.
fluorophore was developed by heating to 100°C for 10 min. after the samples reached to equilibrium with the ambient temperature, fluorescence at 360-470 nm were taken using systronic photofluorometer (Model 152, Ahmedabad, Gujrat). Compared the tissue values (fluorescence of tissue extract minus fluorescence of tissue blank) with an internal reagent standard (fluorescence of internal reagent standard minus fluorescence of internal reagent blank). For serotonin tissue blank, 0.025 conc. HCl without o-phthaldialdehyde was added. Internal standard was obtained by adding 500ng of serotonin creatinine sulfate monohydrate in 0.125 ml distilled water and 2.5 ml HCl-Butanol, which was then carried through the entire extraction procedure. For the internal reagent blank, 0.125 ml distilled water was added to 2.5 ml HCl-Butanol.\textsuperscript{13}

**Estimation of Brain GABA Levels**

Brain GABA content was estimated using the established method of Brains were rapidly removed from mice after completing behavioral testing, and isolated brains were weighed and transferred to 5 ml of ice-cold trichloroacetic acid (10% w/v), homogenized and centrifuged at 10,000 × g for 10 min at 0°C. Then, 0.1 ml of tissue extract was added to 0.2 ml of 0.15 M ninhydrin solution in a 0.5 M carbonatebicarbonate buffer (pH 9.95), which was incubated in a water bath at 60°C for 30 min and then cooled and treated with 5 ml of copper tartrate reagent (0.16% disodium carbonate, 0.03% copper sulfate and 0.03% tartaric acid). After 10 min, a fluorescence reading was taken at excitation/emission wavelengths of 377/451 nm in a spectrofluorimeter (Shimadzu RF-1501).\textsuperscript{14}

**Statistical analysis**

Results were expressed as mean ± SEM. Significant differences between groups were determined using one-way ANOVA followed by Dunnett’s test. Differences between data sets were considered as significant when \( p < 0.05 \) and \( p < 0.01 \).

**RESULTS**

**Effect of Annona squamosa fruit juice using light – dark model**

Administration of *Annona squamosa* (ASJ) fruit juice at the concentration of 6%, 9% v/v for 14 days remarkably (\( p<0.01 \)) increased the time spend in the lit box in unstressed and stressed mice, in the light - dark model, while at 3% v/v dose, significantly (\( p < 0.05 \)) increased the time spend in the lit box in unstressed and stressed mice (Fig. 1).
Fig. 1: Effect of ASJ & Diazepam on time spent in lit box of light-dark model in unstressed and stressed mice
Values are Mean ± SEM (n = 6).
* denotes (p < 0.05) & ** denotes (p < 0.01) as compared to respective control group treated unstressed mice.
# denotes (p < 0.05) & ## denotes (p < 0.01) as compared to respective control group treated stressed mice.

Effect of Annona squamosa fruit juice on time spent in open arms using Elevated Plus Maze
Administration of Annona squamosa (ASJ) fruit juice at the concentration of 6%, 9% v/v for 14 days remarkably (p < 0.01) enhanced the time spent in open arm in unstressed mice, in the elevated plus maze test, while at 3% v/v dose, showed significantly (p < 0.05). But in stressed mice ASJ at the concentration of 3%, 6% and 9% v/v remarkably increased the time spent in open arm (Fig. 2).

Fig. 2: Effect of ASJ & Diazepam on time spent in open arms of EPM in unstressed and stressed mice
Values are Mean ± SEM (n = 6). One way ANOVA followed by Dunnett’s t-test.

ASJ denotes *Annona squamosa* fruit juice

**Effect of *Annona squamosa* fruit juice on number of entries using Elevated Plus Maze**

ASJ (p.o.) at the concentration of 9% v/v remarkably (p<0.01) and 6% v/v significantly (p<0.05) increased the number of entries in open arm in unstressed mice while at 3% v/v dose, showed significantly (p < 0.05), in the elevated plus maze test, while at 3% v/v dose, no significant (p < 0.05) effect was observed. But in stressed mice ASJ at the concentration of 3%, 6% and 9% v/v remarkably (p<0.01) increased the number of entries in open arm (Fig. 3).

![Graph showing number of entries in open arms of EPM in unstressed and stressed mice](image)

**Fig. 3:** Effect of ASJ & Diazepam on number of entries in open arms of EPM in unstressed and stressed mice

Values are Mean ± SEM (n = 6).

* denotes (p< 0.05) & ** denotes (p<0.01) as compared to respective control group treated unstressed mice.

# denotes (p< 0.05) & ## denotes (p<0.01) as compared to respective control group treated stressed mice.

**Effect of *Annona squamosa* fruit juice using Hole-board model**

ASJ (p.o.) at the concentration of 3%, 6% and 9% v/v remarkably (p<0.01) increased the number of head dips in unstressed mice, while in stressed mice the concentration of 3% v/v dose, showed significantly (p < 0.05) and 6% and 9% v/v remarkably (p<0.01) increased the number of head dips in hole-board model (Fig. 4).
Fig. 4: Effect of ASJ & Diazepam on number of head dips of Hole - board model in unstressed and stressed mice. Values are Mean ± SEM (n = 6).

* denotes (p < 0.05) & ** denotes (p<0.01) as compared to respective control group treated unstressed mice.

# denotes (p< 0.05) & ## denotes (p<0.01) as compared to respective control group treated stressed mice.

Effect of Annona squamosa fruit juice on brain GABA levels

Brain GABA levels is an important marker in anxiety disease. The administration of ASJ (p.o.) for 14 days at the concentration of 3%, 6% and 9% v/v remarkably (p<0.01) enhanced Brain GABA levels in unstressed mice, while in stressed mice the concentration of 3% v/v dose, showed no effect but 6% and 9% v/v remarkably (p<0.01) increased the Brain GABA levels (Fig. 5).

Fig. 5: Effect of ASJ & Diazepam on Brain GABA levels in unstressed and stressed mice
Values are Mean ± SEM (n = 6). One way ANOVA followed by Dunnett’s t-test.

ASJ denotes *Annona squamosa* fruit juice

**Effect of *Annona squamosa* fruit juice on brain Serotonin levels**

The administration of ASJ (p.o.) for 14 days at the concentration of 6% and 9% v/v remarkably (p<0.01) enhanced Brain Serotonin levels in unstressed and stressed mice, while at the concentration of 3% v/v dose, showed no effect in unstressed and stressed mice (Fig. 6).

![Graph showing effect of ASJ on brain serotonin levels](image)

**Fig. 6: Effect of ASJ on Brain Serotonin levels in unstressed and stressed mice**

Values are Mean ± SEM (n = 6). One way ANOVA followed by Dunnett’s t-test.

ASJ denotes *Annona squamosa* fruit juice

**Effect of *Annona squamosa* fruit juice on blood Plasma Nitrite levels**

The administration of ASJ (6%, 9%) showed highly significant and 3% showed significant in unstressed mice while at the concentration of 6% and 9% v/v remarkably (p<0.01) reduced plasma nitrite levels in stressed mice (Fig. 7).

![Graph showing effect of ASJ on plasma nitrite levels](image)

**Fig. 7: Effect of ASJ on Plasma nitrite levels in unstressed and stressed mice**
VALUES are Mean ± SEM (n = 6). One way ANOVA followed by Dunnett’s t-test.

ASJ denotes *Annona squamosa* fruit juice

**DISCUSSION**

In the present study, we have focused upon the effects of *Annona squamosa* fruit juice in stressed and unstressed mice. The common targets for the treatment of anxiety are GABA and serotonin. Benzodiazepines are the most commonly prescribed medicines, which act through GABAergic system. Benzodiazepines are associated with abuse, dependence, and withdrawal symptoms. Moreover, anxiety patients also face various difficulties associated with these antianxiety drugs. Therefore, the demand of herbal medicines is increasing due to their wide application and therapeutic efficacy with least side effects. *Annona squamosa* fruit is the powerhouse of excellent ingredients including p-coumaric acid, caffeic acid, protocatecuic acid, gallic acid and vitamin C. Protocatechuic acid and vitamin-C has neuroprotective effect due to by promoting endogenous antioxidant enzymatic activities and inhibiting free radical generation. It is reported that p-coumaric acid to have activates the GABA-A receptor and produce anxiolytic activity. Gallic acid produced anti-anxiety like activity possibly involvement of Nitriergic System through inhibition of nNOS and iNOS. The inhibitory effect of caffeic acid on the production and release of nitric oxide have the ability to inhibit emotional abnormality such as anxiety. Based on this literature, we decided to investigate the effect of *Annona squamosa* fruit juice (ASJ) against anxiety employing various anxiety models. We observed that the administration of *Annona squamosa* fruit juice to mice for 14 successive days showed antianxiety potential with increased time spent by mice in open arm in Elevated plus maze test and enhanced time spent in lit box using light-dark model and number of head-dips increased in Hole-board model. In our study, GABA and serotonin levels were increased but plasma nitrate levels were decreased after the administration of ASJ for 14 successive days. An abundance of evidence suggests that increased levels of serotonin and GABA but decreased levels of plasma nitrate are helpful in the recovery of the patient suffering from anxiety disorder. These results might be due to presence of p-coumaric acid, caffeic acid, protocatecuic acid, gallic acid and vitamin-c and their properties (Picture 3). Furthermore, it can act synergistically or other minor bioactive constituents may also contribute to the observed effect. These findings suggest that *Annona squamosa* fruit juice may be looked as a promising antianxiety agent.
CONCLUSION

*Annona squamosa* (Sugar apple) are the rich source of bioactive compound with excellent nutrients. In the present study, we observed that *Annona squamosa* fruit juice reduced anxiety of mice in anxiety models viz. Light-Dark Model, Elevated-Plus Maze and Hole-Board Model and enhanced brain GABA, brain serotonin levels and reduced blood plasma nitrate levels. Thus, we conclude from our study that *Annona squamosa* fruit juice possess antianxiety effect which is mediated through GABA and serotonin receptors. Therefore, it is worthwhile to explore *Annona squamosa* fruit clinically in managing anxiety.

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