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EVALUATION OF EFFECT ETHANOLIC EXTRACT OF *ANNONA SQUAMOSA* LEAF ON GABA MEDIATED ANXIETY

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ABSTRACT
The objective of the present investigation was to evaluate the evaluate the anxiolytic action of ethanolic extract of Annona squamosa leaf using light/dark box model and elevated plus maze model and to establish the possible involvement of GABA in the action of the extract. Extraction was carried out using ethanol after defatting the dried leaf powder with n-hexane. The total phenolic content in the leaf extract would be determined quantitatively using Folin-Ciocalteu reagent method, using gallic acid as the reference standard. Light/dark box model as well as elevated plus maze model were used to determine the anxiolytic action of the extract at doses of 200 and 400 mg/Kg administered intraperitoneally in mice. Co administration of flumazenil was used to study the involvement of GABA in the anxiolytic activity. The ethanolic extract was dark green in color and sticky and obtained in 16.1% yield. The total phenolic content of the ethanolic extract of Annona squamosa leaf was found to be 25.16 ± 0.175 GAE/mg. The extract at a dose of 400 mg/kg was able to significantly increase the time spent by the mouse in the lit box. The extract at 200 mg/kg dose did not exhibit a significant improvement in open arm entries by the mouse whereas the extract at 400 mg/kg dose exhibited a significant result compared to control ($p<0.001$) in Two way ANOVA. The number of movements in open arm on administration of ethanolic extract of Annona squamosa leaf (400 mg/kg) was found to be 5.16 ± 0.048 as compared to a negligible movement of 1.5 ± 0.047 on the normal saline treated mice.

KEYWORDS: GABA, anxiety, elevated plus maze, light/dark box test, *Annona squamosal.*

INTRODUCTION
Nature remained as an only source for providing all human needs ranging from foods, cosmetics to medicines. Plants, in particular, have formed the basis of sophisticated traditional medicine.[1] Approximately, 6% of all plants have been studied for their biological activities and about 15% for their phytochemistry.[2] Medicinal plants in the defined mixture were classified as botanical drugs approved by the FDA for decades.[3]

Anxiety is a normal emotion when it is appropriate to the environmental situation. Inappropriate or pathological anxiety is a well-recognized and common condition, which causes considerable distress to individuals, families and society in general.

Gamma-aminobutyric acid (GABA) is the primary inhibitory transmitter in the central nervous system. One third of all CNS neurons are thought to be GABAergic. GABA is present in relatively high concentrations in the spinal cord and in all regions of the brain but does not exist in neurons outside the CNS. Interactions between multiple neurobiological systems, including the GABAergic system, are important to the adaptation to stress and protection against the development of pathology of anxiety disorders. Normal anxiety is a natural evolutionary response to stressful stimuli; pathologic anxiety, however, is situationally debilitating and harmful. lipophilic extracts from *Piper nigrum* fruits, *Angelica pubescens* roots, *Acorus calamus* roots, *Biota orientalis* leaves and twigs, *Kadsura longipendunculata* fruits, *Bupleurum chinense* roots, *Pholidota chinensis* stems and roots, *Adenocarpus cincinnatus* roots and tubers, and *Boswellia thurifera* resin possessed positive GABAA receptor modulating activities.[4,5]

Annona squamosa is a small, semi-deciduous tree with edible fruits, belonging to the family Annonaceae. The leaves of Annona squamosa are a rich source of several phytoconstituents and essential oils. The phytochemical profile of Annona squamous leaves can be broadly classified as acetogenins, alkaloids, flavonoids, phenols, saponins, tannins, glycosides, sesquiterpenes, anthocyanins, steroids, diterpenes, terpenoids, quinones, amino acids, and fatty acids.[6] Several pharmacological actions like neuroprotective[7], antioxidant[8,9],

antimicrobial[8] and antitumor[10] have been reported in the plant.

The objective of the present investigation is to prepare ethanolic leaf extract of *Annona squamosa* and to assess the CNS mediated neurobehavioral actions of *Annona squamosa* and evaluating its effect in presence of GABA antagonist.

MATERIAL AND METHODS

All the reagents and chemicals used for the present investigation have been procured from various suppliers and were of analytical or reagent grade. The instruments used were available in the laboratory of the institution and were used without calibration. Ethanol was procured from Sigma Aldrich whereas diazepam injection (Valium 5mg/mL) and flumazenil injection (Fludot 0.5mg/5mL) were procured from local pharmacy.

Collection and Identification of the Plant

The leaves of *Annona squamosa* (AS) was collected from the surrounding regions of Bhopal, Madhya Pradesh in the month of March and authenticated at Saifia Science College; Bhopal**.** The authenticated bark was dried in shade and coarsely powdered using a low speed blender. The powdered material was stored in air tight container till use.

Extraction of soluble phytochemicals

Powdered leaf of AS (120 g) was defatted with hexane at room temperature for 24 h. The marc was dried and packed in the extractor of the soxhlet apparatus and extracted with ethanol by hot continuous extraction process for about 72 h. The extract was filtered and the solvent was evaporated on water bath. The extracts obtained were collected and placed in desiccator to get rid of the excess moisture content**.** The dried extracts were stored in desiccator for phytochemical screening and pharmacological evaluation.

Qualitative Phytochemical Screening[11]

The extract was evaluated by phytochemical qualitative reactions for identifying the presence or absence of usual plant secondary metabolites. The screening was performed for triterpenes/steroids, alkaloids, glycosides, flavonoids, saponins, tannins, and phenolic acids. The color intensity or the precipitate formation was used as analytical responses to these tests.

Quantitative estimation of total phenolics in the extract[12]

The total phenolic content in the leaf extract would be determined quantitatively using Folin-Ciocalteu reagent method, using gallic acid as the reference standard. For total phenolic content determination, 200 µL of sample was mixed with 1.4 mL purified water and 100 µL of Folin-Ciocalteu reagent. After incubating at room temperature for 15 min, 300 µL of 20% Na_2CO_3 aqueous solution was added and the mixture was allowed to incubate at room temperature for 2 h. The absorbance of the solution was measured at 760 nm with a UV-Vis spectrophotometer. Standard solutions of gallic acid (10-100 ppm) were similarly treated to plot the analytical curve. The control solution contained 200 µL of methanol and suitable reagents, and it was prepared and incubated under the same conditions as the rest of the samples. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of the dry sample.

Pharmacological Evaluation

Mice of either sex weighing 20-25 g were used for the study. The animals were housed in cages during the course of experimental duration and maintained at 12 day/night cycle at a 17-26°C. The animals were fed with standard rodent pellet feed and water *ad libitum*. The animals were acclimatized for at least seven days before the start of experiments. The animals were fasted 12 hours before the experiment with free access to only water. The animal were grouped into five groups of six animal each.

Group I – Control (Administered normal saline 0.5 mL i.p)
Group II – Test group (Administered extract solution, 200 mg/kg, i.p)
Group III – Test group (Administered extract solution, 400 mg/kg, i.p)
Group IV – Reference (Administered diazepam 2 mL/kg, i.p)
Group V – Antagonist group (Administered flumazenil 2 mL/kg, i.p 30 minutes prior to administration of extract solution 400 mg/kg)

Preparation of test samples and standard drugs

The test samples were prepared by formulating the dried ethanolic extract as a solution by dispersing the extract in normal saline solution.

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 cardboard 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.[13]

Elevated-Plus Maze (EPM)

The Elevated Plus-Maze served as the exteroceptive behavioral model.[14] The Elevated plus Maze consisted of two open arms (16 cm × 5 cm) and two enclosed arms, (16 cm × 5 cm × 15 cm), arranged opposite to each other, extended from a central platform (5 cm × 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.

Evaluation of role of GABA in activity of the extract

To evaluate the role of GABA in the action elicited by the extract, Group V animal were administered a GABA antagonist drug (Flumazenil) 30 minutes prior to the administration of the extract solution. The effect of extract was now evaluated using both the described animals.

RESULTS AND DISCUSSION

The defatted extract was subjected to extraction with ethanol. The ethanolic extract was dark green in color and sticky and was obtained in 16.1% yield by dry weight of the leaf powder. The findings of phytochemical analysis of the ethanolic extract suggest the presence of alkaloids, glycosides, phenolics, terpenoids, sterols, and flavonoids. The presence of alkaloids anonaine, diterpenoid Annosquamosin has previously been reported.[15,16]

Total Phenolic Content Determination

The ethanolic extract was evaluated for quantification of the total phenolic content concentration in extract. Standard curve of gallic acid was plotted in distilled water for determining absorption data. The linear equation of gallic acid was found to be $y = 0.0048x + 0.005$ with a R^2 value of 0.9993. The total phenolic content in extracts, expressed as gallic acid equivalents. The total phenolic content of the ethanolic extract of *Annona squamosa* leaf was found to be 25.16 ± 0.175 GAE/mg.

Evaluation of anxiolytic effect

Light/Dark Test

The light/dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stressors, that is, novel environment and light. A natural conflict situation occurs when an animal is exposed to an unfamiliar environment or novel objects. The conflict is between the tendency to explore and the initial tendency to avoid the unfamiliar (neophobia). The exploratory activity reflects the combined result of these tendencies in novel situations. Thus, in the light/dark test, drug-induced increase in behaviours in the white part of a two-compartment box, in which a large white compartment is illuminated and a small black compartment is darkened, is suggested as an index of anxiolytic activity. An increase in transitions without an increase in spontaneous locomotion is considered to reflect anxiolytic activity.

The time spent by the mouse in light or the latency to move to dark was a measure of the ability of the extract to avert anxiety.

Table 1: Results of light/dark box test.

Group	Latency to first movement (sec)	Total Time Spent in Lit Box	Total Time spent in Dar Box
I (Control)	27	297	303
II (Extract 200)	36	345	255
II (Extract 400)	44	421	179
IV (Diazepam)	61	510	90

From the results it was clear the after administration of diazepam, the light spent in the lit box was increased remarkably. Similarly, the extract at a dose of 400 mg/kg was able to significantly increase the time spent by the mouse in the lit box (Table 1, Figure 1).

According to the model, any drug that increases both transitions and locomotion is considered a general motor stimulant and is regarded as a putative anxiolytic. Hence the results suggest that the ethanolic extract of *Annona squamosa* leaf have anxiolytic action.

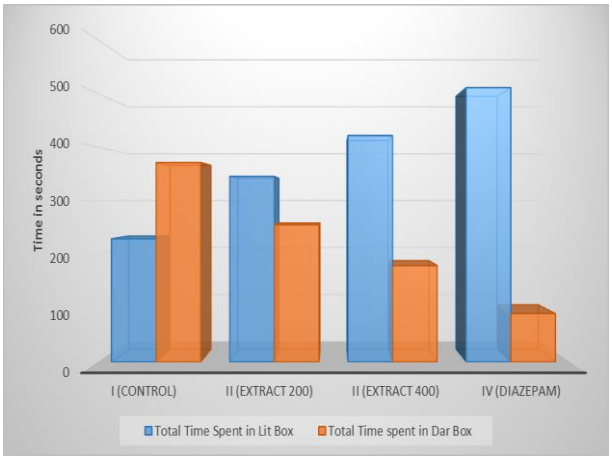

Figure 1: Effect of test solutions on mice in light/dark box test.

Elevated Plus-Maze test

The elevated plus-maze exploration was originally validated as a predictive test of rodent, anxiety-like behavior wherein rodent prefers to remain in the closed arms than open arm.

The extract solution was found to be significantly improving the open arms activity (both the parameters) compared to control group (Table 2). The extract at 200 mg/kg dose did not exhibit a significant improvement in open arm entries by the mouse whereas the extract at 400 mg/kg dose exhibited a significant result compared to control ($p<0.001$) in Two way ANOVA. The number of movements in open arm on administration of ethanolic extract of *Annona squamosa* leaf (400 mg/kg) was found to be 5.16 ± 0.048 as compared to a negligible movement of 1.5 ± 0.047 on the normal saline treated mice (Figure 2).

Table 2: Results of elevated plus-maze test.

Group	Number of open arm entries	Number of close arm entries	Total arm Entries
I (Control)	1.5 ± 0.547	6.16 ± 0.752	7.66 ± 0.816
II (Extract 200)	2.16 ± 0.408	4.66 ± 0.516	6.82 ± 0.752
II (Extract 400)	5.16 ± 0.408	2.5 ± 0.0547	7.66 ± 0.516
IV (Diazepam)	6.33 ± 0.516	1.66 ± 0.816	8.0 ± 0.632

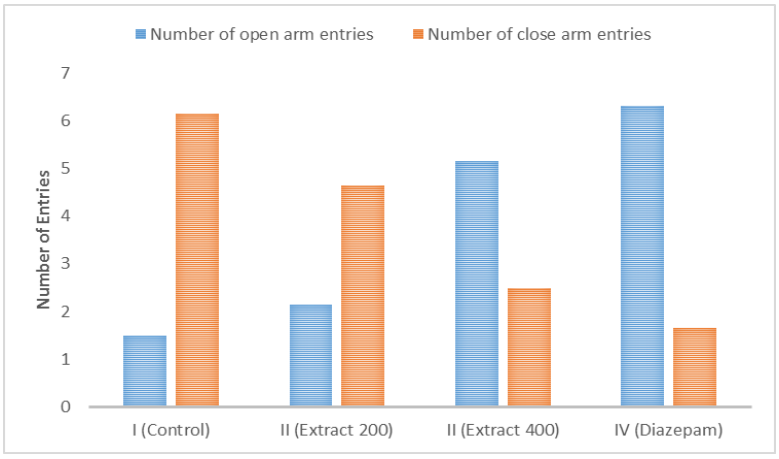

Figure 2: Effect of test solutions on mice in elevated plus-maze test.

Effect of test solutions on GABA

To establish the mediation of the anxiolytic effects by facilitation of GABA levels, the light/dark box test and the elevated plus-maze test were carried out in presence of GABA antagonist drug (Table 3).

Table 3: Results of test solution + GABA antagonist in light/dark model.

Treatment	Latency to first movement (sec)	Total Time Spent in Lit Box	Total Time spent in Dar Box
(Extract 400)	44	421	179
Flumazenil + Extract 400	11	136	464

Table 4: Results of test solution + GABA antagonist in elevated plus-maze model.

Treatment	Number of open arm entries	Number of close arm entries	Total arm Entries
(Extract 400)	5.16 ± 0.408	2.5 ± 0.0547	7.66 ± 0.516
Flumazenil + Extract 400	0.33 ± 0.516	1.33 ± 0.516	1.66 ± 0.717

The findings presented in table 3 & 4 indicate that the ethanolic extract of *Annona squamosa* leaf exert their anxiolytic action in mice by facilitating the effects of GABA.

CONCLUSION

Annona squamosa is a rich source of bioactive compounds with numerous pharmacological and nutritional applications. In the present work, the anxiolytic action of ethanolic extract of its leaves was studied using dark/light model and elevated plus maze model in mice. The involvement of GABA in the anxiolytic effect of the plant extract was also investigated. The results of the study led to the conclusion that the ethanolic extract of *Annona squamosa* leaf exerts its anxiolytic effect by facilitating the action of GABAergic neurotransmission in the central nervous system.

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