

Neuropharmacological Exploration of Standardized Extract of *Annona squamosa* (L.) Fruit Pulp in Experimental Animals

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ABSTRACT

Aims: The present study aimed to investigate the neuroprotective potential of standardized *Annona squamosa* Linn fruit pulp extract using various in-vitro and in-vivo models.

Methodology: Neuroprotective potential of standardized extract was screened against dopamine-induced contraction of isolated rat vas deferens, serotonin-induced contractions of isolated rat fundus, acetylcholine-induced contractions of isolated goat tracheal chain. *In-vivo* models such as elevated plus maze, light and dark model, force swim test, tail suspension test, lithium-induced head twitches, haloperidol-induced catalepsy, PTZ induced seizure and foot shock-induced aggression were implemented to screen various doses intervals (50-200mg/kg) of extracts in experimental animals.

Results: Standardization of extract showed content of polyphenols 65.37 mg/g of GAE, total flavonoid 5.33 mg/g of RE and HPLC fingerprinting of ASP-ME showed identical retention time as that of standard gallic acid, quercetin and rutin, viz 3.830, 5.765 and 3.830 respectively. Inhibition of DPPH radical reflected as 91.32±0.19 % while percent inhibition of RRI of DPPH was observed as 95.99±0.47 at 150 min. ASP-ME significantly inhibited dopamine and serotonin induced contraction on isolated rat vas deferens and rat fundus respectively at log dose (1.3, 2.5) for dopamine and log dose (2.2, 2.5) for serotonin. ASP-ME potentiated ach-induced contractions on goat tracheal chain preparation. Ach alone produces 106.90±4.6 % response, while ASP-ME in presence of Ach potentiates response and produces 141.80±10 % response. The extract demonstrated anxiolytic activity by increasing the time spent in open arms and light zone in elevated plus maze and light dark test respectively. The duration of immobility was significantly decreased in force swim & tail suspension test respectively demonstrating antidepressant activity. Administration of ASP-ME shown antipsychotic effect in dose dependent manner by minimising aggression induced by foot shock (reduced number of flights), while potentiation of catalepsy induced by haloperidol. The extract also exhibited serotonergic system inhibitory effect by significantly reducing head twitches imparted by lithium.

The ASP-ME significantly delayed the onset of first myoclonic and clonic spasms induced by PTZ indicating anticonvulsant effects. Extract also shown ability to decrease the behavior facilitated by the serotonergic and dopaminergic coordination, while potentiated the actions produced by GABA.

Conclusion: Finding of the study suggests anxiolytic, antidepressant, antipsychotic effects of ASP-ME probably mediated through dopamine D2 and 5-HT receptors, with neuroprotective activity.

Key Words: *Annona squamosa*, Anxiolytic, Antidepressant, Antipsychotic, Anticonvulsant, Antiaggression

INTRODUCTION

Brain ischemia induces the release of excitatory amino acids, with subsequent receptor activation leading to metabolic and electrophysiological dysfunction, along with oxidative stress (including lipid peroxidation).¹ Subsequent reperfusion worsens this oxidative stress, potentiating ischemic injury.² Many plants have been reported to be effective against

CNS disorders and thus providing opportunity to evaluate ethno pharmacologically potential medicinal plants against neurological disorders.^{3, 4} *Annona squamosa* Linn (*Annonaceae*), which is popularly known as Custard Apple, has been cultivated all over India. It is traditionally used as an abortifacient, for the treatment of cardiac problems, constipation, dysentery, dysuria, fever, fainting, hemorrhage,

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malignant tumors, thirst, ulcers and worm infections.^{5, 6} The unripe fruit, when dried and powdered, has been used to treat ulcers, diarrhea, dysentery, and atonic dyspepsia. It is also mixed with gram flour and used to destroy vermin. Ripe fruit made into paste with betel leaves accelerates suppression in tumors.⁷ Aqueous extract of fruit peel has exhibited not only larvicidal action but also exhibited mortalities against adult as well as larval parasitic.^{8, 9}

The present investigation has been undertaken to study the *in-vitro* and *in-vivo* neuroprotective effect of methanol extract of *Annona squamosa*, Linn as perusal of literature revealed lack of systematic studies for neuropharmacological potential along with its qualitative standardiation.

MATERIALS AND METHODS

Plant material

Fresh *Annona squamosa*, Linn fruits were collected in the month of November from a local market of Nanded, Maharashtra, India. Identified and authenticated by J. Jayanthi Scientist 'C' and HOD, Botanical Survey of India, Pune, Maharashtra, India. Voucher specimen was stored at this site for future reference (BSI/WRC/Tech./2012/ANSKAR1).

Instrumentation

Agilent-1200 HPLC with UV-Vis detector, manual sample injector system (20 µl fixed loop), Agilent-TC C₁₈ reverse-phase column (250 X 4.6 mm, particle size 5 µm) proceeded by an ODS guard column (10 µm, 10 mm X 5 mm ID) at an ambient temperature was used. HPLC Isocratic mode, column: C₁₈ ODS, 4.6 x 250 mm, 5 µ particle size of packing material.

Experimental animals

All experimental animal procedures were conducted by the approval of the committee for the purpose of control and supervision on experiments on animals (CPCSEA) (Reg. No. 1613/PO/a/12/CPCSEA) of CRPS Nanded Pharmacy College, Nanded, Maharashtra, India. Animals were housed under standard animal husbandry conditions and acclimatized to the laboratory environment for at least one week before commencement of experiment.

Extraction procedure

According to nature of phytochemicals present in the drug and literature review pulp of *Annona squamosa* fruit was weighed uniformly (1kg), prepared paste was soaked in 3000 ml of the methanol and kept in a dark for 3 days. After 3 days, extract was filtered and the kept on a water bath at about 40°C in order to concentrate them. The concentrated filtrate obtained was used for further studies.

Phytochemical evaluation and standardization of extract

Preliminary phytochemical screening

The methanolic extract of *Annona squamosa* used to test the phytochemical compounds, including alkaloids, saponins, carbohydrate, phenols, triterpenoids, and steroids, in accordance with the previous methods.^{10, 11}

Determination of total phenol and flavonoid content

Total phenol content of the extract was determined by using the method of Folin-Ciocalteu, polyphenolics contents was expressed as gallic acid equivalent in mg/gm of the extract. Total flavonoid content was determined using on the basis of aluminum trichloride colorimetric method and expressed as rutin equivalent in mg/gm of the extract.^{12, 13}

Marker based HPLC fingerprint analysis

The extract was dissolved in 0.1% ortho phosphoric acid to get stock solution of 100 µg/ml. The phenolic markers such as caffeic acid gallic acid, quercetin, rutin and *p*-hydroxy cinnamic acid were dissolved in methanol to get stock of 10 µg/ml. Mobile phase implemented was 0.1% orthophosphoric acid: acetonitrile, with flow rate 1ml/ min.

In vitro anti-oxidant activity:

Determination of DPPH radical scavenging activity and Percent RRI of DPPH (% RRI)

DPPH (2, 2-diphenyl 1, 1-picrylhydrazyl) assay was performed according to previously mentioned protocol to find percent inhibition of various concentration of extract (50-250 µg/ml). Ascorbic acid was used as a positive control. Furthermore by finding effective concentration of ASP-ME (100 µg/ml) percent residual rate of inhibition (% RRI) of DPPH was found at different time intervals.¹⁴

In-vitro neuropharmacological studies

Effect of dopamine induced contraction of isolated rat vas deferens, serotonin-induced contractions of isolated rat fundus and acetylcholine-induced contractions of isolated goat tracheal chain

Adult male wistar rats were sacrificed by cervical dislocation and the vas deferens was removed and kept in Krebs's solution. The dose-responses to dopamine (10, 20, 40, 80 and 160 µg/ml) were recorded on the vas deferens and the same were repeated in presence of ASP-ME (0.5 ml of 25 mg/ml). The contact time between dopamine and tissue was maintained 60 sec. whereas the above mentioned dose-responses was repeated for serotonin-induced contractions on isolated rat fundus in which contact time with tissue was maintained for 60 sec while in case of acetylcholine-induced contractions on isolated goat tracheal chain tissue contact time was maintained for 90 sec.^{15,16}

IN-VIVO NEUROPHARMACOLOGICAL STUDIES

Anxiolytic Activity

Elevated plus maze

Rats were divided in five groups such as control, standard and three test groups, test ASP-ME group (50, 100 and 200 mg/kg p.o.) and standard drug group diazepam (1mg/kg i.p.) were treated 1 hr before evaluation. After 1 hr rats were placed in centre of maze, facing one of enclosed arms, entire maze was elevated to a height of 50 cm above the ground level. During 5 min study period, animals were observed using video tracking system (master maze 3 software) for number of entries in open and enclosed arms, time spent in open, enclosed arms and in centre.¹⁷

Light and Dark model

The testing apparatus consists of two compartment chamber (47X27X27 cm) comprising of two-third brightly illuminated area and one-third dark area separated by a wall with a round hole (7.5 cm long X 7.5 cm high). It also consist a partition containing opening which separate the dark one third from the bright two third of the cage. Rats were divided in groups as described in above method and treated with tests ASP-ME (p.o.) and standard group diazepam (1mg/kg i.p.) 1 hr prior testing, rats were placed individually in the illuminated part of the cage and the electronic video tracking system was used to count movements through the partitions up to 5 min study duration.¹⁸

Antidepressant activity

Force swim test

Experimental rats were preliminarily tested for the fitness level by performing pre-test, which was conducted 24 hr before the forced swim test by subjecting each test animal to a session of 15 min swimming. Rats were individually forced to swim inside a vertical plexiglass cylinder (40 cm height X 18 cm diameter) containing 15 cm of water maintained at (22±2-25±2 °C). ASP-ME (50, 100 and 200 mg/kg) and standard drug (imipramine 15 mg/kg) was administered 1hr prior to testing, all the rats were forced to swim for 5 min, during this latency and duration of immobility was observed.¹⁹

Tail suspension test

Swiss albino mice weighing 25-35 gm were divided into five groups as described above. They were treated with test ASP-ME (50, 100 and 200 mg/kg) or standard drug imipramine 15 mg/kg. 30 min later, animals were put on hold on the step 58 cm above the table top by sticky tape and positioned at 1 cm from the end of the tail. Latency as well as period of immobility were noted for duration of 6 min.²⁰

Antipsychotic activity

Lithium-induced head twitches

Experimental rats were divided into five groups as described above and ASP-ME (50, 100 and 200 mg/kg, i.p.) or Ondansetron (5 mg/kg, i.p.) or vehicle were administered 30 min before the assessment. After 30 min of treatments, lithium sulfate (i. p., 200 mg/kg) was administered and number of head twitches was counted for 60 min.²¹

Haloperidol-induced catalepsy

Experimental rats groups were pretreated with vehicle, or ASP-ME (50, 100 and 200 mg/kg, i.p.) 30 min prior to administration of haloperidol (1 mg/kg, i.p.). The duration of catalepsy was measured at 0, 30, 60, 90, 120, 150 and 180 min using the Bar test. Both the forepaws of rat were positioned on a horizontal bar elevated 3 cm above the table and time taken to withdrawn the forepaws from the bar was noted as the catalepsy period.^{09, 22,}

Anticonvulsant

Pentylenetetrazol (PTZ)-induced seizures

Experimental mice groups were treated with ASP-ME (50, 100 and 200 mg/kg, i.p.) or diazepam (1 mg/kg, i.p.) or vehicle 30 min before injection of PTZ (80 mg/kg, s.c.). The latency to seizures and number of animals surviving after 24 hr were noted. Either delay or abolition of seizures was taken as the criteria of anticonvulsant activity.²³

Antiaggression

Foot shock- induced aggression (FSIA)

Aggression in experimental rats was achieved by means of a series of impulses induced by electronic stimulator. Animals were divided into 5 groups of 10 rats (5 pairs of male rats) per group. The vehicle, haloperidol (1 mg/kg, i.p.) as a standard and ASP-ME (50, 100 and 200 mg/kg; i.p.) were administered 30 min before initiation of experiment. Aggression in animals was recorded by means of latency as well as number of fights.²⁴

Statistical analysis

The statistical analysis was determined by one-way ANOVA followed by Dunnett's test. The analyses were performed with Graphpad prism 6 Software and values of $p < 0.05$, $p < 0.001$ were considered significant. All results were presented as the mean ± SEM.

RESULTS AND DISCUSSION

Phytochemical screening of extract revealed the presence of alkaloids, flavonoids, saponins, carbohydrates, phenols and glycosides. Standardization & HPLC fingerprinting

of ASP-ME showed content of polyphenols 65.37 mg/g of GAE, total flavonoid 5.33 mg/g of RE. HPLC fingerprinting which revealed same retention time as that of standard gallic acid (3.830), quercetin (5.760) and rutin (3.820), ASP-ME shown 3.830, 5.765 and 3.830 respectively (Fig. 1). While as *p*-hydroxy cinnamic and caffeic acid were found absent (Table 1).

ASP-ME exhibited dose dependent inhibition of DPPH radical reflected as 91.32 ± 0.19 % as compared to ascorbic acid 99.12 ± 0.22 % at $150 \mu\text{g/ml}$. When observed up to 150 min, ASP-ME and standard ascorbic acid exhibited percent inhibition of RRI of DPPH 70.82 ± 1.18 and 95.99 ± 0.47 respectively. Both studies of DPPH indicates ability of extract to inhibit residual concentration of DPPH up to 150 min. Results of antioxidant evaluation hints that ASP-ME not only shows concentration dependent inhibition of DPPH but also exhibit time dependent, long term inhibition ability (Table 2).

Dopamine and serotonin at ($100 \mu\text{g/ml}$) log dose of 2.5 exhibited 104.60 ± 3.8 and 134.80 ± 3.6 of percent response while ASP-ME at (25 mg/ml ; 0.5 ml) in presence of dopamine and serotonin showed 78.60 ± 3.2 and 86.20 ± 1.6 of percent response indicating ASP-ME is having ability to significantly inhibit dopamine and serotonin induced contraction on isolated rat vas deferens and rat fundus respectively. The significance was observed mainly at log dose (1.3, 2.5) for dopamine and log dose (2.2, 2.5) for serotonin. This outcome helps to contemplate the anti-dopaminergic as well as anti-serotonergic activity mediated through dopamine D_2 and 5-HT receptors. Cholinergic M_3 receptors are predominantly present in isolated goat tracheal chain preparation. Ach produces dose dependent contractions of isolated goat tracheal chain preparation. The result of the *in-vitro* test indicated that ASP-ME potentiated ach-induced contractions on goat tracheal chain preparation. Ach alone produces 106.90 ± 4.6 percent response, while ASP-ME in presence of Ach potentiates response and produces 141.80 ± 10 percent response. The major difference between dopamine, serotonin and Acetylcholine in previous two effects has been inhibited by ASP-ME while the later is potentiating. Experimental results are in accordance to previous theories supporting neuroprotective effect of ASP-ME against dopamine and serotonin, where as potentiating for Acetylcholine (Table 3). ASP-ME has been screened for its anxiolytic effect by implementing elevated plus maze by evaluating time spent (sec) in open arms, enclosed arms, central zone and entries in open arms and enclosed arms (Table 4).

The vehicle treated group rats spent 43.67 ± 3.30 sec in open arms, 317.5 ± 57.89 sec in enclosed arms and 17.67 ± 1.54 sec in central zone while ASP-ME (100 and 200 mg/kg) and diazepam (1 mg/kg) showed significant ($P < 0.05$, $P < 0.001$) increase in the occupancy in the open arms (199.3 ± 19.35 , 99.17 ± 3.99 and 182.8 ± 14.00) as well as in central zone

(30.17 ± 4.06 , 40.83 ± 5.36 and 56.67 ± 4.66), while decrease in enclosed arm 160.0 ± 9.65 , 108.0 ± 14.02 and 60.17 ± 7.19 sec respectively. ASP-ME (50 mg/kg) showed insignificant decrease in time spent in enclosed arms (244.8 ± 24.93 sec) and increase in open arms (52.33 ± 7.81 sec) as well as central zone (19.00 ± 1.82 sec). The animals treated with diazepam (1 mg/kg) and ASP-ME (100 , 200 mg/kg) exhibited significant increased count of entries to the open arms (30.33 ± 4.89 , 14.83 ± 2.42 and 27.83 ± 4.57) and decreased preference of entries to the enclosed arms (8.83 ± 0.70 , 15.17 ± 1.99 and 8.50 ± 2.07) with insignificant change at ASP-ME 50 mg/kg entries in open arms (9.66 ± 1.83) and entries in enclosed arms (22.33 ± 2.30) as compared to control (9.16 ± 1.01 and 27.67 ± 4.47) respectively. Combining results of all the anxiolytic evaluation assist to contemplate the anxiolytic action of ASP-ME is comparable to standard drug diazepam.

The etiology of the most anxiety disorders, although not fully understood, has come into sharper focus in the recent past. The benzodiazepines (BZDs) are relatively safe and widely used anxiolytic agent and recognized to exhibit effect on BZD-GABA receptors. One of commonly implemented animal model to study behavioral pharmacology of anxiety is EPM as it encompasses natural or spontaneous stimuli viz. height, novelty and unprotected opening.²⁵

Drugs that increase open arm exploration are considered as anxiolytics and the reverse holds true for anxiogenics.²⁶ In EPM test, ASP-ME at both doses 100 and 200 mg/kg enhanced the duration spent in open arms, while the number of entries and time spent in the enclosed arms were decreased as compared to control values. The anxiolytic effect of the ASP-ME was more prominent at 200 mg/kg . In this study, diazepam treated animals increased the number of open arm entries, reducing the natural animal's aversion to the open arms and promoting the exploration thereof, indicating an anxiolytic effect.

Anxiolytic activity of ASP-ME has also been evaluated using light dark model (Table 4). In this study test extract at both doses (100 and 200 mg/kg) and standard drug diazepam (1 mg/kg) exhibited significant ($P < 0.05$ and < 0.001) increase in time spent in light zone (63.83 ± 9.80 , 207.2 ± 10.57 and 234 ± 16.34), number of crossing (18.17 ± 1.92 , 47.50 ± 3.99 and 60.83 ± 5.03), transfer latency (27.33 ± 3.45 , 51.33 ± 4.87 and 57.50 ± 3.31) respectively as compared to control (17.17 ± 3.57 , 2.50 ± 0.56 , 12.17 ± 1.42), while significant decrease in time spent in dark zone (170.7 ± 8.60 , 95.67 ± 7.40 and 79 ± 6.66) was observed as compared to control (217.5 ± 17.51). The dose 50 mg/kg of ASP-ME did not produce any significant change in any of parameters. The light and dark paradigm is based on the natural aversion of rats to bright lit places. Since anxiolytics reduce the natural aversion to light and increase the time spent in the lit compartment. It has been suggested that 5-HT₃ receptors may be

involved in the fear provoked by L/D paradigm and that the nucleus accumbens or the amygdala may be involved in mediating the disinhibitory effect of 5-HT₃ receptor antagonists.²⁷ In this model, ASP-ME produced significant increase in time spent in lit box as compared to vehicle, contemplating an anxiolytic potential.

ASP-ME showed potentiating effect on serotonin induced contractions of rat fundus. In view of this effect, ASP-ME was studied for its anti-depressant property. The anti-depressant effect of ASP-ME was evaluated by using forced swim test (FST) and tail suspension test (TST). Depression is a mood disorder that causes a disturbance in an individual's emotions and feelings. In depressive state there is diminished level of central catecholamine level and also the lower level of 5-hydroxyindolacetic acid which is a principle serotonin metabolite. This suggests that depression is associated with a reduction in central 5-HT function.

Animals treated with ASP-ME (100 mg/kg, 200 mg/kg) and imipramine (15 mg/kg) significantly ($P<0.05$, $P<0.001$) decreased duration of immobility in tail suspension test (105.3 ± 9.38 , 54.83 ± 16.22 and 43.17 ± 12.62) and in forced swim test (95.83 ± 7.53 , 38.00 ± 6.68 and 18.83 ± 2.44) respectively as compared to control (143.70 ± 27.26 and 128.83 ± 9.05). Whereas increased latency of immobility in tail suspension test (104.0 ± 14.18 , 114.5 ± 22.07 and 113.2 ± 9.38) and in forced swim test (63.50 ± 10.05 , 114.2 ± 8.66 and 57.67 ± 7.84) respectively as compared to control (31 ± 8.29 and 7.83 ± 1.07). The administration of ASP-ME (50 mg/kg) showed no statistical significant in duration of immobility as well as latency of immobility (Table 5).

In vehicle treated rats, lithium sulphate produced 49.80 ± 1.39 head twitches whereas ASP-ME at 100 and 200 mg/kg significantly ($P<0.001$) decreased number of head twitches to 17.80 ± 1.15 , 8.20 ± 1.06 respectively. Ondansetron (5-HT₃ antagonist) also reduced the number of head twitches (8.00 ± 0.70) showing its effect on serotonergic system (Fig. 2). Lithium sulphate administered to rats releases serotonin from serotonergic neurons that stimulates serotonin receptors and produces head twitches. These head twitches are antagonized by drugs that blocks 5-HT receptors.²⁷ The results showed that ASP-ME at all dose level significantly decreased lithium-induced head twitches. The extract also inhibited contractions induced by serotonin on rat fundus. Thus, suggesting possible inhibitory effect of ASP-ME on serotonergic system.

In vehicle treated animals, haloperidol (1 mg/kg) produced maximum catalepsy of 253.20 ± 3.97 sec. The ASP-ME (50, 100, 200 mg/kg) significantly potentiated haloperidol induced catalepsy at each time interval (0-180 min at 30 min interval) in dose dependent manner 181.4 ± 3.47 , 287.00 ± 3.89 and 331.6 ± 18.17 respectively ($P<0.001$) in presence of haloperidol at 90 min. However, only ASP-ME at 200 mg/kg did

not exhibit any catalepsy and appeared same as the normal animals (Fig. 3).

Haloperidol, a typical neuroleptic agent produces catalepsy in rodents but exert extrapyramidal side effects in humans. Haloperidol induced catalepsy is one of the animal models to test the extrapyramidal side effects of antipsychotic drugs.⁰⁹ The haloperidol, (a non-selective D₂ antagonist) induced catalepsy is primarily due to blockade of dopamine receptors in the striatum. The agent, increasing dopamine transmission inhibits neuroleptic induced catalepsy.^{25,30} The striatum as well as nucleus accumbens have been recognized as the crucial brain structural components associated with antipsychotic induced catalepsy, which might be due to the inhibition of dopamine neurotransmission.^{28,29} In the present study, ASP-ME (50, 100 and 200 mg/kg) significantly potentiated haloperidol induced catalepsy. The potentiation of catalepsy is indicative of the ability of the test extract drug to antagonize dopamine D₂ receptor in striatum.

PTZ (80 mg/kg) induced myoclonic (50.20 ± 6.07) and clonic spasm (48.40 ± 6.24) in all of control group mice (100%), pre-treatment with ASP-ME (100 and 200 mg/kg) and diazepam (1 mg/kg) significantly ($P<0.001$) delayed the onset initially in myoclonic (163.2 ± 3.86 , 238.6 ± 24.69 and 286.6 ± 11.28) and then in clonic spasms (185.8 ± 5.97 , 318.0 ± 5.85 and 466.2 ± 10.73) respectively (Table 6). PTZ induced convulsion is the frequently used experimental model for preliminary screening to test potential of anticonvulsant drugs.²³ The indications perceived in the absence seizures are similar to seizures induced by PTZ and hence treatment of absence seizures found to suppress PTZ-induced seizures.²⁵ ASP-ME delayed occurrence of seizures and reduced mortality indicating its anticonvulsant potential. Diazepam, GABA_A agonist is highly effective to prevent convulsions induced by PTZ. The anticonvulsant effects of ASP-ME observed here might be correlated to its possible interaction with GABA-benzodiazepine receptor complex. The test extract might be useful for the treatment of absence seizures and anxiety.

In foot shock induced aggression study animal treated with ASP-ME (100 and 200 mg/kg) and haloperidol (1mg/kg) showed significant ($P<0.05$, $P<0.001$) as well as dose dependent decrease in number of fights, 26.60 ± 0.92 , 12.20 ± 0.86 and 6.00 ± 0.70 respectively as compared to control 52.60 ± 0.67 . Whereas increased in latency to fight was observed 80.00 ± 4.48 , 133.2 ± 3.80 and 253.4 ± 9.92 respectively as compared to control 17.60 ± 4.65 . While the dose 50 mg/kg of ASP-ME produces insignificant change in terms of above parameters (Fig. 4).

CONCLUSION

From the above results, it can be concluded that the methanolic extract of the *A. squamosa* fruit pulp showed potent

in-vitro antioxidant activity which might be due to presence of phenolic and flavonoid compounds in the extract. Extract have been found responsible neuroprotective activity in terms of anxiolytic, anti-depressants, antipsychotic and anti-convulsant potential in addition to anti-aggressive, protection of experimentally induced catalepsy. However, assertion of these preliminary pharmacological effects need mechanism based studies to be performed which are currently in process.

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Individual author's contribution

Kawade Rajendra - Carried out plant collection, processing, extraction and pharmacological activities. Actively involved in planning, implementation

Ghante Mahavir - Carried out phytochemical screening, Chromatographic and spectroscopic studies, Standardization of extracts, data compilation and statistical studies

Warokar Amol - Standardization of extracts and preparation of manuscript.

Abbreviation

% RRI	-	% Residual Rate of Inhibition
5-HT	-	5-Hydroxy triptamine (Serotonin)
Ach	-	Acetylcholine
ASP	-	Methanolic extract of <i>Annona squamosa</i> , Linn Pulp
ASP-ME	-	<i>Annona squamosa</i> pulp methanolic extract
CNS	-	Central nervous system
CPCSEA	-	Committee for the Purpose of Control and Supervision on Experiments on Animals
DA	-	Dopamine
DPPH	-	Diphenyl-picryl-hydrazyl
EPM	-	Elevated plus maze
FSIA	-	Foot shock-induced aggression
FST	-	Forced swim test
GABA	-	Gamma amino butyric acid
hr	-	Hour

i.p.	-	Intraperitoneal
IAEC	-	Institutional Animal Ethical Committee
min	-	Minute
OECD	-	Organization of Economic Co-operation development
p.o	-	Per Oral
PTZ	-	Pentylenetetrazol
sec	-	Second(s)
SSRIs	-	Selective serotonin reuptake inhibitors
TST	-	Tail suspension test
UDP	-	Up and Down procedure

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Table 1: Phytochemical screening and standardization of *Annona squamosa*; Linn Pulp methanolic extract

Phytochemical Evaluation		Standardization		Phytoconstituents	Retention Time (min)	
Test	ASP-ME	of ASP-ME		markers used for standardization	Standard	ASP-ME
Alkaloids	+	TFC mg/g of RE	15.33	Caffeic acid	3.875	ND
Flavonoids	+			Gallic acid	3.830	3.830
Saponins	+			Quercetine	5.760	5.765
Carbohydrates	+	TPC mg/g of GAE	46.37	Rutin	3.820	3.830
Phenols	+			<i>P</i> -hydroxy cinnamic acid	5.893	ND
Triterpenoids	-					
Steroids	-					
Glycosides	+					

TFC- Total flavonoid content, RE-Rutin, TPC- Total Polyphenolic content, GAE-Gallic acid, ND-Not Detected, ASP-ME: *Annona squamosa*; Linn. Pulp methanolic extract, + Present, - Absent

Table 2: DPPH Scavenging activity and % Residual Rate of Inhibition of DPPH by *Annona squamosa* Linn. Pulp extract

Extract/ Std	Concentration (µg/ml)	% Inhibition	Time (min)	% RRI at 100 µg/ml
ASP-ME	50	78.96 ± 0.27	50	75.39 ± 0.60
	100	84.66 ± 0.60	90	73.45 ± 0.81
	150	91.32 ± 0.19	150	70.82 ± 1.18
Ascorbic acid	50	97.43 ± 0.33	50	98.07 ± 0.09
	100	98.26 ± 0.20	90	96.88 ± 0.51
	150	99.12 ± 0.22	150	95.99 ± 0.47

Values are expressed as mean ± SEM (n = 3). ASP-ME: *Annona squamosa*; Linn Pulp extract. In % Residual rate of inhibition activity, ASP-ME extract shows % inhibition decreases i.e. antioxidant activity decreases with the time. At 50 min ASP-ME showed maximum activity 75.39 ± 0.60.

Table 3: Effect of ASP-ME (25 mg/ml; 0.5ml) on % Response in dopamine induced contractions of isolated rat vas deferens, serotonin induced contractions on isolated rat fundus and acetylcholine induced contractions on isolated goat tracheal chain preparation

Log Dose	Effect of ASP-ME (25 mg/ml; 0.5ml) on % Responses					
	Dopamine-induced contractions of isolated rat vas deferens		Serotonin-induced contractions on isolated rat fundus		Acetylcholine-induced contractions on isolated goat tracheal chain	
	Dopamine (100 µg/ml)	ASP-ME +Dopamine	Serotonin (100 µg/ml)	ASP-ME + Serotonin	Acetylcholine (100 µg/ml)	ASP-ME +Acetylcholine
1.0	25.40±1.91	12.80±3.07**	60.60±2.89	24.80±2.63	37.60±4.41	73.40±5.89
1.3	48.40±1.98	20.00±2.42	71.60±4.47	34.80±3.94	53.20±4.91	80.80±5.48
1.6	56.00±4.14	34.60±2.61	89.20±3.38	53.40±3.15	65.60±7.13	94.80±5.95
1.9	71.00±2.60	51.20±2.45	98.20±5.07	68.20±2.85	82.60±4.69	105.40±5.19
2.2	85.80±3.67	69.40±2.50	116.20±4.53	79.80±2.80*	93.80±5.91	130.40±8.39**
2.5	104.60±3.89	78.60±3.23**	134.80±3.65	86.20±1.65**	106.90±4.64	141.80±10.19**

Each point represents the mean ± SEM (n = 5). * $P < 0.05$, ** $P < 0.001$ (Student t-test).

Table 4: Effects of methanolic extract of *Annona squamosa* Linn pulp in Elevated plus Maze and Light and Dark box models

Model evaluated	Parameters observed	Control	Diazepam 1 mg/kg	ASP-ME (mg/kg)		
				50	100	200
Effect in elevated plus maze	Time spent in Open arm (sec)	43.67±3.30	199.3±19.35**	52.33±7.81	99.17±3.99*	182.8±14.00**
	Time spent in Enclosed arm (sec)	317.5±57.89	60.17±7.19**	244.8±24.93	160.0±9.65*	108.0±14.02**
	Entries in Open arm	9.16±1.01	30.33±4.89**	9.66±1.83	14.83±2.42	27.83±4.57**
	Entries in Enclosed arm	27.67±4.47	8.83±0.70**	22.33±2.30	15.17±1.99*	8.50±2.07**
	Time spent in Central zone (sec)	17.67±1.54	56.67±4.66**	19.00±1.82	30.17±4.06	40.83±5.36**
Effect in light/dark exploration	Time spent in Light zone (sec)	17.17±3.57	234±16.34**	52.33±11.06	63.83±9.80*	207.2±10.57**
	Time spent in Dark zone (sec)	217.5±17.51	79±6.66**	199.3±11.80	170.7±8.60*	95.67±7.40**
	No. of Crossings	2.50±0.56	60.83±5.03**	8.16±1.24	18.17±1.92*	47.50±3.99**
	Transfer Latency	12.17±1.42	57.50±3.31**	17.00±2.89	27.33±3.45*	51.33±4.87**

Values are expressed as mean ± SEM (n = 6). ** $P < 0.001$, * $P < 0.05$ vs. Vehicle (One-way ANOVA followed by Dunnett's test). ASP-ME: *Annona squamosa*; Linn. Methanolic extract

Table 5: Effects of methanolic extract of *Annona squamosa* Linn Pulps on Duration of Immobility & Latency of immobility in Tail Suspension Test & Forced Swim Test

Treatment (mg/kg)	Duration of Immobility (sec)		Latency of Immobility (sec)	
	Tail Suspension Test	Forced Swim Test	Tail Suspension Test	Forced Swim Test
Control	143.70 ± 27.26	128.83 ± 9.05	31 ± 8.29	7.83 ± 1.07
Imipramine 15	43.17 ± 12.62** ^a	18.83 ± 2.44**	113.2 ± 9.38 ^a	57.67 ± 7.84** ^a
ASP-ME 50	135.5 ± 17.56	114.5 ± 8.25	49.83 ± 9.76	16.83 ± 2.67
100	105.3 ± 9.38 ^a	95.83 ± 7.53*	104.0 ± 14.18*	63.50 ± 10.05**
200	54.83 ± 16.22** ^a	38.00 ± 6.68**	114.5 ± 22.07** ^a	114.2 ± 8.66** ^a

Values are expressed as mean ± SEM (n = 6). ** $P < 0.001$, * $P < 0.05$ vs. Vehicle (One-way ANOVA followed by Dunnett's test). ^a Compared with standard treated group. ASP-ME: *Annona squamosa*; Linn. Methanolic extract

Table 6: Effects of ASP-ME on Pentylentetrazole-induced (PTZ) Anticonvulsant activity

Treatment (mg/kg)	No. of animal convulsed/ No. of animal used	Animal protected (%)	Latency to Clonic Seizures	Latency to Myo- clonic Seizure
Control Veh. + PTZ 80 s.c.	5/5	0	48.40±6.24	50.20±6.07
Diazepam 1 i.p. + PTZ 80 s.c	0/5	100	466.2±10.73**	286.6±11.28**
ASP-ME 50 i.p + PTZ 80 s.c.	4/5	20	71.80±4.88	74.80±4.64
ASP-ME 100 i.p + PTZ 80 s.c.	3/5	40	185.8±5.97**	163.2±3.86**
ASP-ME 200 i.p + PTZ 80 s.c.	1/5	80	318.0±5.85**	238.6±24.69**

Values are expressed as mean ± SEM (n = 5). **P < 0.001 vs. Vehicle (One-way ANOVA followed by Dunnett's test). ASP-ME: *Annona squamosa*; Linn. Methanolic extract

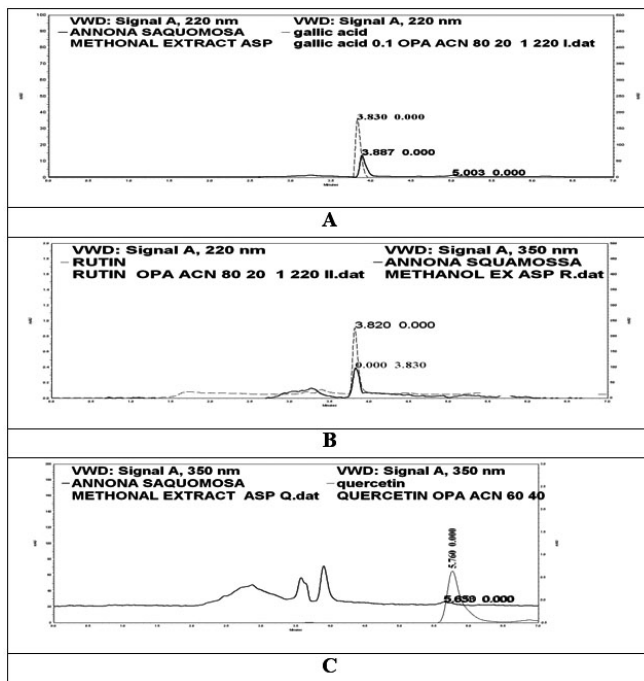
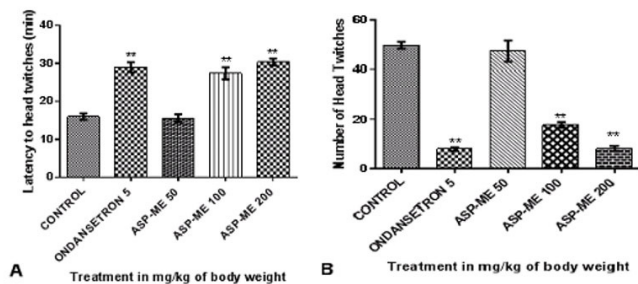

Figure 1: Overlay Chromatograms of A. Gallic acid, B. Rutin and C. Quercetin in ASP-ME.


Figure 2: Effect of ASP-ME on Lithium-induced (A) Latency to Head Twiches (B) Number of Head Twiches. Each column represents as mean ± SEM (n = 5). **P < 0.001 vs. Vehicle (One-way ANOVA followed by Dunnett's test). ASP-ME: *Annona squamosa*; Linn Methanolic extract.

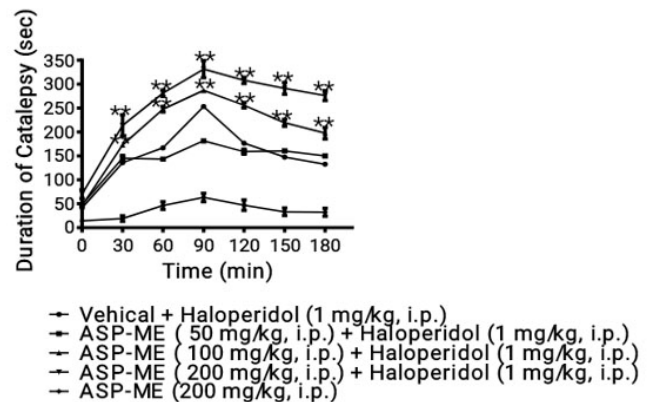


Figure 3: Effect of ASP-ME on haloperidol-induced catalepsy in Rats. Each point indicates mean ± SEM (n = 5). **P<0.001 vs. Vehicle (One-way ANOVA followed by Dunnett's test).

In vehicle treated animals, haloperidol (1 mg/kg) produced maximum catalepsy after 90 min (253.20±3.97). The ASP-ME (50, 100, 200 mg/kg) significantly potentiated haloperidol induced catalepsy at each time interval in dose dependent manner. ASP-ME at dose 50, 100 and 200 mg/kg showed maximum cataleptic score 181.4±3.47, 287.00±3.89 and 331.6±18.17 respectively at 90 min (P<0.001) in haloperidol treated animals. The ASP-ME (200 mg/kg) treated Rats did not exhibit any catalepsy and appeared same as the normal animals.

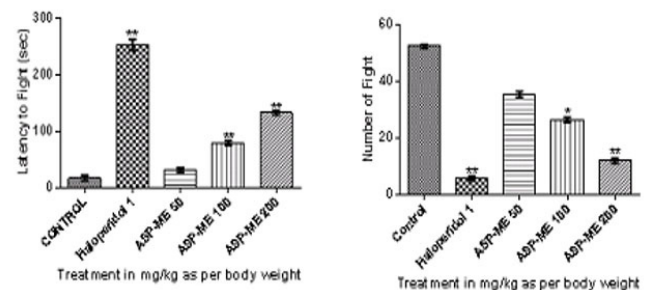


Figure 4: Effect of ASP-ME on Foot Shock Induced Aggression (A) Latency to Fight (B) Number of Fights. Each column represents as mean ± SEM (n = 5). *P < 0.05, **P < 0.001 vs. Vehicle (One-way ANOVA followed by Dunnett's test). ASP-ME: *Annona squamosa*; Linn Methanolic extract.