EVALUATION OF ANTICONVULSANT ACTIVITY OF ANNONA SQUAMOSA L. LEAVES IN MICE

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Abstract: The aim of the present study was to evaluate the effects of Annona squamosa (AS) leaves on seizures induced by pentylenetetrazol (PTZ) and picrotoxin (PTX). Pretreatment with hydroalcoholic extract of Annona squamosa leaves (125-500mg/kg) induced a dose-dependent decrease in the incidence of both clonic and seizures generalized tonic–clonic seizures (Pd<0.05) following PTZ and PTX administration. Co-administration of a sub-effective dose of AS (125 mg/kg, po) with a sub-protective dose of diazepam (0.5 mg/kg, ip) increased the latency to seizure. The combination significantly enhanced percent protection against PTZ and PTX induced convulsions. The results suggested that the anticonvulsant effect of Annona squamosa leaves against PTZ and PTX induced convulsions may be mediated, at least partly, through GABA<sub>A</sub>-benzodiazepine receptor complex.

Key words: Anticonvulsant, Annona squamosa;

INTRODUCTION

Epilepsy is a major neurological disorder and up to 5% of the world population develops epilepsy in their lifetime. Seizure is the characterstic feature in epilepsy and is associated with disordered and rhythmic high frequency discharge of impulses by a group of neurons in the brain. Abnormal cellular discharge may be associated with a variety of causative factors such as- trauma, oxygen deprivation, tumors, infection and metabolic derangements. However, no specific factors are found in about half of patients suffering from epilepsy [1,2].

The current therapy of epilepsy with modern antiepileptic drugs (AEDs) is associated with side effects, dose-related and chronic toxicity, teratogenic effects and approximately 30% of the patients continue to have seizures with current AED therapy [3-6]. Ethnopharmacological research on natural products can contribute to the discovery of new, safe active compounds with novel structure that may serve as leads to the development of new antiepileptic drugs. Several plants of the families Euphorbiaceae, Leguminaceae, Labiatae, Liliaceae, Gentianaceae, Solanaceae, and Umbelliferae are used for the treatment of epilepsy in Indian traditional medicinal system [7]. An example is Brahmi Ghrita a phanchagavya formulation of Indian traditional medicinal system containing 4 medicinal plants [8]. It displayed antiepileptic activity in different types of models [9]. Another polyherbal formulation, Ummadnashak Ghrita containing 4 medicinal plants also shows anticonvulsant activity in many animal models [10].

Annona squamosa Linn., Annonaceae, commonly known as sitaphal and custard-apple or sugar-apple, is a native of West Indies and is now cultivated throughout India, mainly for its edible fruit. The alcoholic extract of defatted seeds of Annona squamosa has been reported to possess anticonvulsant activities in rats [11]. However, there
is no such study on the leaves of *Annona squamosa*. Leaves extract of this plant has been used as antidiabetics, hypolipidemic, anticancer, expectorant and insecticidal agents. The leaves contains several alkaloids (annonaine, roemerine), flavanoids and acetogenins [12]. Traditional healers ask epilepsy patients to eat one leaf of *Annona squamosa* for relief from the epileptic attacks, but this claim is not yet scientifically validated. With this background the present study was designed to investigate anticonvulsant potential of *Annona squamosa* leaves against various models of convulsions. The study was further extended to explore the involvement of benzodiazepine receptors in its action.

**MATERIALS AND METHODS**

**Plant material and preparation of extract:** The leaves of *Annona squamosa* were collected during the month of April-May from Moradabad. The botanical identity of the plant material was verified and specimen was deposited at the Herbarium, Department of Botany, Hindu college, Moradabad, for reference (voucher no. H/0139). The leaves were shade dried, grounded. The powdered material was then extracted twice using hydro alcoholic (30:70) solvent system in Soxhlet apparatus for 24 hrs. The extract was concentrated under reduced pressure using rotary evaporator and stored at 100°C (yield: 10%, w/w).

**Animals:** Male albino mice weighing 20-25 g and Wistar rats (180-250 g), bred in Animal House facility of BN College of Pharmacy Udaipur were used. The animals were housed under standard laboratory conditions and maintained on natural light and dark cycle and had free access to food and water. Animals were acclimatized to laboratory conditions before the experiment. Each animal was used only once. Each experimental group consisted of 6-8 animals. All the experiments were carried out between 0900 and 1500 hrs. The experimental protocols were approved by Institutional Animal Ethics Committee (IAEC) (CPCSEA no. 870/ac/05/CPCSEA) and conducted according to Indian National Science Academy Guidelines (icmr.nic.in/ bioethics/ INSA Guidelines.pdf) for the use and care of experiments.

**Acute toxicity test of the extract:** The acute toxicity test of extract in mice was estimated by p.o. route [13]. The extracts were administered at 10, 100, 1000 mg/kg in the first stage. When no mortality was induced the doses were increased. Mice were kept under observation for the following 14 days and their weights registered.

**Drug administration:** The following drugs were used in the present study. pentylentetrazol (Sigma, MO, USA), diazepam (Sigma, MO, USA), picrotoxin (Sigma, MO, USA), diazepam from Ranbaxy Laboratories, Tween 80 from Rankem labs, ethanol from S.D. Fine Chemical Ltd. Pentylentetrazol and picrotoxin were dissolved in normal saline and diazepam was prepared using Tween 80 (one drop) and solutions were made with sterile water. The *Annona squamosa* leaf extract was reconstituted by dissolving it in 0.9% NaCl solution and then suspending the resultant solution in 0.5% tween 80 suspension freshly before use. The extract was administered in a fixed dose of 10ml/kg of body weight 60 minutes before animals were challenged with the convulsive drug.

**Pentylentetrazol induced convulsions:** Pentylentetrazol (PTZ) (80 mg/kg, ip) was administered to induce clonic convulsions [14]. Animals were observed for a period of 30 min post-PTZ administration. The parameters noted were mean onset time of convulsions, duration of clonus and recovery/death (% recovery or % survival) due to PTZ. The drug extracts were administered 60 min before the PTZ challenge. In mechanistic study, various agonists/antagonists were administered 15 min before drug extracts and after 60 min were challenged with convulsive dose of PTZ. The experimental protocol comprised of the following groups, each consisting of 6–10 animals:

- **Group 1- control group treated with vehicle (distilled water) followed by PTZ.**
- **Group 2, 3, and 4:** given graded doses of diazepam (0.5, 1.0, 2.0 and 4.0mg/kg; i.p.).
- **Group 5, 6, and 7:** recieved *Annona squamosa* (125, 250 and 500 mg/kg), followed by PTZ. Diazepam was administered 15 min. before *Annona squamosa* extract.
- **Group 8:** given a combination of sub-protective dose of diazepam and sub-protective dose of *Annona squamosa* (125 mg/kg, p.o.) followed by PTZ. Diazepam was administered 15 min. before *Annona squamosa* extract.

**Picrotoxin-induced convolution:** Picrotoxin (PTX) 8mg/kg bw, was administered subcutaneously to induce convulsions. Immediately after PTX administration mice were individually placed in plastic observation chambers for a period of 30 minutes.
The parameters noted were onset of clonic seizures, duration of clonic seizures and mortality. The animals were observed for 48 hrs for the mortality [15]. The treatment groups were made as above, instead of PTZ all the animals were challenged with picrotoxin.

**Locomotor activity:** The locomotor activity was monitored by using actophotometer (IMCORP, India). An array of 16 infrared emitter detector pairs measured activity of animals along a single axis of motion, the digital data being displayed on the frontal panel meters as ambulatory movements. Mice were allowed to acclimatize to the observation chamber for a period of 2 min. Locomotion was expressed in terms of total number of ambulations (total photobeam counts) per 5 min/per animal [16].

Group 1 (n = 6) served as control group. Distilled water was administered p.o. to this group 60 min. later, the locomotor count was measured for five minutes. Group 2, 3 and 4 - *A. squamosa* leaf extract (125,250 and 500 mg/kg; p.o. respectively) and after 60 min., animals were placed for 5 min on actophotometer for observation. Group 5 - Diazepam (2 mg/kg, i.p.) was administered to this group of animals and after 30 minutes animals were noted for any change in locomotor activity by placing them in actophotometer as above.

**Statistical analysis:** One specific group of mice was assigned to one specific drug treatment condition and each group comprised 5–8 mice. All the values were expressed as mean ± S.E.M. The data were analyzed by using analysis of variance followed by Dunnett’s test. In all tests, the criterion for statistical significance was p<0.05.

**RESULTS**

**Acute toxicity study:** The LD50 value of *Annona squamosa* leaf extract was found to be 2.5g/kg.

**Effect of *Annona squamosa* leaf extract on spontaneous locomotor activity:** The hydro alcoholic extract of leaves of *Annona squamosa* L. produced a significant decrease in the activity of mice as shown in Fig. 1.

**Effect of *Annona squamosa* leaf extract on pentylentetrazol induced convulsions:** Pentylentetrazol (PTZ, 80 mg/kg i.p.) produced generalized clonic and hind-limb tonic seizures in all the animals. AS leaf extract (AS, 125, 250 and 500 mg/kg, p.o.) offered a significant protection (p<0.05) against PTZ-induced seizures. The plant extract (AS, 125, 250 and 500 mg/kg, p.o.) significantly delayed (p < 0.05) the onset of myoclonic jerks, clonus and extensor phase of PTZ-induced seizures. The
reference anticonvulsant diazepam also profoundly delayed the onset of convulsion parameters, and significantly protected the animals (p<0.05) against PTZ-induced seizures (Table 1).

**Effect of Annona squamosa leaf extract on picrotoxin induced convulsions:** Picrotoxin (PTX, 8 mg/kg i.p.) produced hind-limb tonic seizures in all the animals used. AS leaf extract (AS, 125, 250 and

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment groups (mg/kg)</th>
<th>OJ (Mean±SEM)</th>
<th>OS (Mean±SEM)</th>
<th>OC (Mean±SEM)</th>
<th>DC (Mean±SEM)</th>
<th>OE (Mean±SEM)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (Vehicle)</td>
<td>125.768 ± 17.405</td>
<td>197.876 ± 16.353</td>
<td>283.83 ± 12.830</td>
<td>200.532 ± 3.143</td>
<td>479.438 ± 45.161</td>
<td>0</td>
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<tr>
<td>2</td>
<td>DZ (0.5)</td>
<td>263.408 ± 14.522</td>
<td>383.392 ± 15.45</td>
<td>387.280 ± 19.506*</td>
<td>130.236 ± 12.464*</td>
<td>605.846 ± 23.586*</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>DZ(1.0)</td>
<td>369.508 ± 21.826*</td>
<td>649.156 ± 16.325*</td>
<td>700.884 ± 9.793*</td>
<td>85.208 ± 6.517*</td>
<td>897.562 ± 23.586*</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>DZ(2.0)</td>
<td>378.252 ± 21.126*</td>
<td>770.606 ± 15.45</td>
<td>819.638 ± 1.906*</td>
<td>819.638 ± 1.906*</td>
<td>1374.300 ± 23.586*</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>DZ(4.0)</td>
<td>838.096 ± 14.000*</td>
<td>1056.178 ± 130.236*</td>
<td>1343.186 ± 4.781*</td>
<td>5475.0 ± 1.906*</td>
<td>1684.736 ± 130.236*</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>AS (125)</td>
<td>229.386 ± 15.295</td>
<td>313.312 ± 15.010</td>
<td>355.546 ± 14.843</td>
<td>161.974 ± 5.258*</td>
<td>548.172 ± 19.042</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>AS (250)</td>
<td>310.538 ± 17.048*</td>
<td>421.080 ± 17.272</td>
<td>606.224 ± 33.337*</td>
<td>146.474 ± 7.388*</td>
<td>893.812 ± 36.959*</td>
<td>0</td>
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500 mg/kg; p.o.) produced significant protection ($p < 0.05$) against PTX-induced seizures (as in the PTZ-induced tonic seizures) as evidenced by decreased hind limb tonic extension. Moreover, the plant’s extract significantly delayed ($p < 0.05$) the onset of PTX-induced seizures (as in the PTZ-induced tonic seizures). The reference anticonvulsant drug diazepam significantly delayed the onset of convulsions and (DZ; 4 mg/kg i.p.), completely abolished clonic and tonic ($p < 0.05$) convulsions (Table 2).

**Effect of combination of sub-effective dose of Annona squamosa leaf extract and sub-effective dose of diazepam against PTZ and PTX induced convulsions:** 125 mg/kg was selected as sub-effective dose of *Annona squamosa* against PTZ and PTX induced convulsions. This dose was combined with sub-protective dose of diazepam (0.5 mg/kg). Combination of these two at sub-protective doses resulted in enhanced anticonvulsant effect. The combination also significantly delayed onset of clonus ($p < 0.05$) (Fig. 2A) and onset of extensor ($p < 0.05$) (Fig. 2B). Similarly, there was enhanced recovery ($p < 0.05$) in animals when these two drugs were combined in their sub protective doses as all the animals recovered. The combination of sub protective doses of diazepam (0.5 mg/kg, i.p.) and hydroalcoholic extract of leaves of *Annona squamosa* L. (125 mg/kg, p.o.) significantly delayed onset of clonus and extensor ($p < 0.05$) against PTX induced convulsions as well (Fig. 3 A and B).

**DISCUSSION**

The present study revealed that the hydroalcoholic extract of leaves of *Annona squamosa* L. blocked both clonic-tonic seizures induced by PTZ, PTX. Results suggest that *Annona squamosa* L. leaves may be effective in blocking generalized tonic-clonic partial and generalized clonic seizures.

Molecular targets of currently used antiepileptic drugs comprise voltage-activated sodium and calcium channels, GABA $\lambda$ receptors, ionotropic glutamate receptors, GABA transporters, GABA transaminase and others [17]. However, despite theoretically optimal regimen of drug treatment, about one out of three epileptic patients remains refractory to the pharmacological therapy [18]. Therefore, there is need for more efficacious and safer antiepileptic drugs. Most of conventional antiepileptic drugs are associated with many side effects such as neurotoxic effects, cognitive deficits and teratogenic effects, which decrease their clinical utility [19]. Recently, the search for novel pharmacotherapy from medicinal plants for neurological and psychiatric diseases has progressed significantly owing to their less side effects and better tolerability [20]. The observation emanated in the present study provided the evidence in the favour of anticonvulsant activity of hydroalcoholic extract of leaves of *Annona squamosa* L. in PTZ and PTX induced convulsions in mice. The dose of 500 mg/kg, p.o. increased the latency to the onset of seizures and significantly suppressed the tonic convulsions induced by PTZ and PTX.

Experimental evidence obtained in the present laboratory animal study shows that *Annona squamosa* leaf extract significantly delayed the onset of seizures induced by pentylenetetrazole (PTZ), and also significantly antagonized picrotoxin induced seizures. The hydroalcoholic extract of leaves of *Annona squamosa* L. produced a dose dependently delayed the latency to clonic convulsions in picrotoxin induced convulsion model. Since PTZ- and PTX-induced seizures have been shown to be due to inhibition and/or attenuation of GABAergic neurotransmission [21-23], it is not unlikely that extract probably produces its anticonvulsant effect directly like GABA, or indirectly by enhancing GABAergic neurotransmission and/or action in the brain. The PTZ test identifies drugs with efficacy against non-convulsive absence or myoclonic seizures, acting due to increase of the seizure threshold. The antagonism of PTZ induced seizures suggests the interaction of hydroalcoholic extract of leaves of *Annona squamosa* L. with GABAergic neurotransmission.

The experimental animal models of epilepsy induced by the systemic administration of chemicals have been used to study the mechanism of drug action. Picrotoxin (PTX), a potent, selective GABA $\lambda$ receptor antagonist, produces seizures by blocking the effect of GABA at central GABA $\lambda$ receptors which would reduce the inhibitory synaptic transmission to promote excitatory neurotransmission [24], while, PTZ has been reported to diminish the GABAergic tone [25] by the inhibition of BZ site of the GABA receptors [25,26]. In order to determine the role of benzodiazepine (BZ) receptors participation in the AS induced anticonvulsant effects, sub protective dose of diazepam, a specific agonist of...
the benzodiazepine site in the GABA_A-BZ receptor complex was combined with the sub protective dose of AS. The results obtained after from pretreatment with the combination of these two drugs in their sub protective concentrations against PTZ and PTX-induced seizure models in mice, suggest that AS could facilitate the inhibitory activity of the GABAergic system probably through the same site of action as that of diazepam. Diazepam is known to produce anticonvulsant effect by modulating BZ site of the GABA receptors.

Thus, it could be concluded that the hydroalcoholic extract of *Annona squamosa* possesses anticonvulsant activity particularly against PTZ induced convulsions. Therefore, it is likely that the extract might possibly be producing anticonvulsant action by increasing level of GABA, an inhibitory neurotransmitter in the central nervous system. The potentiation of anticonvulsant activity diazepam by the hydroalcoholic extract of leaves of *Annona squamosa* L. indicates that it may be useful as an adjuvant therapy and can lower the potency and side effects of diazepam.

REFERENCES