Neuroprotective effect of *Annona squamosa* & (-) Anonaine in decreased GABA receptor of epileptic rats

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ABSTRACT

Annona squamosa and its active alkaloid content (-) Anonaine are known for antiepileptic activity on the basis of literature review. The objective of this study was to establish neuroprotection by *Annona squamosa* and its active alkaloid content (-) Anonaine as an antiepileptic agent by acting on GABA receptor. For neuroprotective assessment, variations of GABA, $GABA_A$, $GABA_B$ receptors in the cortex region of the brain in epileptic rats and potential applications of *Annona squamosa* and its isolated chemical constituent (Anonaine) were investigated by using confocal microscopy. Nootropic activity in epileptic rats was also studied by radial and Y-maze model. GABA receptor binding analysis in the cortex region of epileptic rats showed significant decrease in B_{max} ($p<0.001$) compared to control. Microscopic (confocal) study reveals confirmed decreased GABA receptors in epileptic rats. The extract of *Annona squamosa* leaves and its isolated constituent- anonaine showed memory boosting and memory regaining effects in radial arm and y-maze model. From the above data and findings, we may conclude that extract of *Annona squamosa* leaves and its alkaloidal constituent- anonaine improves significant changes in epileptic rat's behavior and their decreased GABA receptors. Both extract and its isolated component in future may be evaluated in further studies.

INTRODUCTION

Epilepsy is a major neurological disorder and up to 5% of the world population develops epilepsy in their Lifetime (Binder, 2004). Epileptogenesisis associated with the loss of a subset of GABAergic neurons as well as altered expression of GABA receptor subunits. Altered physiology and pharmacology of both GABAA and GABAB receptors may contribute to hyper excitability in hippocampal and cortical networks, and specific knowledge of receptor subtype pharmacology may suggest novel therapeutic targets (Porwal and Sharma, 2011). Herbal medicine has long been used to treat neural symptoms. Although the precise mechanisms of action of herbal drugs have yet to be determined, some of them have been shown to exert anti-inflammatory and/or antioxidant effects in a variety of peripheral systems. Now, as increasing evidence indicates that neuroglia-derived chronic inflammatory responses play a pathological role in the central nervous system, anti-inflammatory herbal medicine

and its constituents are being proved to be a potent neuroprotector against various brain pathologies. Structural diversity of medicinal herbs makes them a valuable source of novel lead compounds against therapeutic targets that are newly discovered by genomics, proteomics, and high-throughput screening (Raza *et al.*, 2001).

Ethanopharmacological 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 the Indian traditional medicinal System (Saluja and Santani, 1994). *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 leaf extract of this plant has been used as antidiabetics, hypolipidemic, anticancer, expectorant and insecticidal agents. The leaves contain several alkaloids (annonaine), flavanoids and acetogenins (Wu *et al.*, 2013).

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(-) Anonaine an isoquinoline alkaloid isolated from *Annona squamosa* has variety of biological activities like-vasorelaxant, antibacterial, antifungal, antioxidant and antidepressant effects (Porwal *et al.*, 2013). *Annona squamosa* has been found to treat epilepsy (Kurioka, 1981). But so far there are very few studies reporting their role in the functional regulation of GABA receptors. In this study, we investigated neuroprotective study (taking epilepsy as a parameter) of *Annona squamosa* on the GABAergic receptor binding in the cerebral cortex and spatial learning and memory in epileptic rats.

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 the specimen was deposited at the Herbarium, Department of Botany, Hindu college, Moradabad, for reference (voucher no. H/0139). The leaves were a shade dried, grounded. The powdered material was then extracted twice using hydro alcoholic (30:70) solvent system in a soxhlet apparatus for 24 hrs. The extract was concentrated under reduced pressure using rotary evaporator and stored at $100^{o}C$ (yield: 10%, w/w).

Animals

Adult male wistar rats (50) weighing 180-250 g, bred in Animal House facility of B.N College of Pharmacy Udaipur were used. The animals were acclimatized for ten days under laboratory conditions. They were housed in polypropylene cages and maintained at $27^{o}C \pm 2^{o}C$, relative humidity $65 \pm 10\%$ under 12 hours light/dark cycle. The animals were fed with commercial diet and water ad libitum. Ethical clearance for performing the experiments on animals was obtained from the Institutional Animal Ethics Committee (IAEC) with CPCSEA no.870/ac/05/CPCSEA.

Drug administration

The chemicals (diazepam, pilocarpine, atropine etc.) used in the present study were purchased from Sigma, MO, USA. 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. [3H] GABA, [3H] baclofen, [3H] bicuculline were purchased from NEN life sciences products, Inc., BOSTON, MA, USA.

Introduction of Epilepsy in adult male rats

Male wistar rats were used to develop status epilepticus (SE) by injecting rats with pilocarpine at a dose of 350 mg/kg b.w., i.p., preceded by 30 min with atropine 1 mg/kg, b.w., i.p. Within 20 to 40 min after the pilocarpine injection, all the animals developed status epilepticus. Status epilepticus was allowed to continue for 1 hr and then all the animals were treated with diazepam (4 mg/kg, i.p.). Rats which showed recurrent seizures

were used for further experiments. The behavioral and seizure activities in rats were closely observed. Experimental rats were divided into five groups-

Group 1- Control treated with normal saline [C]
Group 2- Epileptic rats [E]
Group 3- Epileptic rats (E) treated with extract of *Annona squamosa* (AS) [E+AS]
Group 4- Epileptic rats (E) treated with Anonaine (A) [E+A]
Group 5- Epileptic rats (E) treated with Carbamazepine [E+C]

Dose of 250 mg/kg, p.o. of *Annona squamosa* leaves extract and anonaine were given in rats for 15 days (Saluja and Santani, 1994). A standard drug used for the treatment of epilepsy was Carbamazepine (150 mg/kg, p.o.). After the treatment, the rats were sacrificed and the tissues were stored in $-80^{o}C$.

GABA receptor binding assay

All receptor binding studies were assayed in triton x-100 treated synaptic membranes. The crude synaptic membrane was prepared using sodium free 10 Mm Tris buffer (pH 7.4). Each assay tube contained a protein concentration of 100 mg. In saturation binding experiments, 5-40 nM of [3H] GABA incubated with and without excess of unlabeled GABA (100 µM). The incubation was continued for 20 min at $40^{o}C$ followed by centrifugation at 35000 xg for 20 min. [3H] GABA in the pellet was determined by scintillation spectrometry. Specific binding was determined by subtracting nonspecific binding from the total binding (Murthy and Friesen, 1985).

Scatchard analysis

The data were analyzed according to scatchard (Vogel, 2002). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on x-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. K_d is inversely proportional to receptor affinity.

Behavioral testing

Epileptic and drug treated rats were tested for behavioral study-Radial maze and Y-maze.

Radial maze test (Ma *et al.*, 2007)

Radial arm maze has been extensively used in learning and memory studies. . The rat uses spatial information provided by the distal cues in the room to efficiently locate the baited arms. The radial arm-maze allows the study of spatial reference and working memory processes in the rat. In reference memory procedures, information is useful for many sessions/days and may usually be needed during the entire experiment. On the contrary,

working memory procedures have a major temporal component as the information presented in the maze (arms baited) is useful for one session but not for subsequent ones; the rat has to remember the information during a delay interval (min to h). Correct choices in the radial arm-maze are rewarded with food. Animals can be trained extensively and then receive specific brain lesions (hippocampus, septum or fimbria-fornix); after recovery from the surgery the rats are re-trained to determine the cognitive ability or the speed of recovery as these lesions severely disrupt the processing of spatial information.

The apparatus is a wooden elevated eight-arm radial maze with the arms extending from a central platform 26 cm in diameter. Each arm is 56 cm long and 5 cm wide by 2 cm high rails along the length of the arm. The maze is well illuminated and numerous cues are present. Food pellets (reward) are placed at the end of the arms. During the test, rats are fed once a day and their body weights maintained at 85% of their free feeding weight to motivate the rat to run the maze. Animals are trained on a daily basis in the maze to collect the food pellets.

Y-maze test

The Y-maze was made of grey wood, covered with black paper, and consisted of three arms with an angle of 120° between each of the arms. Each arm was 8 cm wide ×30 cm length ×15 cm height.

The three identical arms were randomly designated: Start arm, in which the mouse started to explore (always open); Novel arm, which was blocked at the 1st trial, but open at the 2nd trial; and the other arm (always open). The maze was placed in a separate room with enough light. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed on the walls of the maze.

The Y-maze test consisted of two trials separated by an inter-trial interval (ITI). The first trial (training) was 10 min duration and allowed the mouse to explore only two arms (start arm and the other arm) of the maze, with the third arm (novel arm) blocked. After a 1 h ITI (Akwa *et al*., 2001), the second trial (retention) was conducted, during which all three arms were accessible and novelty vs. familiarity was analyzed through comparing behavior in all three arms.

For the second trial, the mouse was placed back in the maze in the same starting arm, with free access to all three arms for 5 min. The time spent in each arm were analyzed data were expressed as percentage of performance in all three arms during the 5-minutes of test (Chen *et al*., 1996).

Confocal photography system

Control and experimental rats were deeply anesthetized with ether. The rat was transcardially perfused with PBS (pH- 7.4) followed by 4% paraformaldehyde in PBS (Pawley, 2006). After perfusion the brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hour and then equilibrated with 30%

sucrose solution in 0.1 M PBS. 40 μm sections were cut using Cryostat (thermo scientific). The sections were treated with PBST (PBS in 0.05% Triton X-100) for 20 min. Brain slices were incubated overnight at 4°C with rat primary antibody for $GABA_{A\alpha}1$. The brain slices were then rinsed with PBST and then incubated with Rhodamine coated secondary antibody (Rang, 2007). The sections were observed and photographed using confocal imaging system (Cell Viogar CV1000).

RESULTS

Scatchard analysis of [³H] GABA binding against GABA in the cerebral cortex of treated groups

Scatchard analysis of [³H] GABA against GABA in the cerebral cortex showed a significant decrease ($P < 0.001$) in the B_{max} of epileptic rats compared to controls. K_d showed a significant decrease ($P < 0.05$) in the epileptic group compared to control. Treatment using *Annona squamosa*, Anonaine and Carbamazepine reversed the B_{max} to near control (Table 1).

Scatchard analysis of [³H]bicuculline against Bicuculline and [³H]baclofen against baclofen in the cerebral cortex of treated groups

Scatchard analysis of [³H]bicuculline against bicuculline and [³H]baclofen against baclofen in the cerebral cortex showed a significant decrease ($P < 0.001$) in the B_{max} of epileptic rats compared to controls. K_d showed significant decrease ($P < 0.05$) in epileptic group compared to control. Treatment using *Annona squamosa*, Anonaine and Carbamazepine reversed the B_{max} to near control (Table 2 and 3).

Radial Maze Assessment

There was significant increase ($p < 0.001$) in the number of trials required to achieve five consecutive criterion performances in the epileptic rats compared to control. Treatment using *Annona squamosa*, Anonaine (250 mg/kg, p.o.) reversed this change to near control (Figure 1).

Y-Maze Assessment

Time spent in the novel arm was decreased significantly ($p < 0.001$) in the epileptic group compared to control. - *Annona squamosa*, Anonaine treated epileptic rats were shown improved performance (Figure 2).

GABA receptor staining analysis

$GABA_{A\alpha1}$ receptor subunit antibody staining in the hippocampus showed a significant decrease ($p < 0.01$) in the $GABA_{A\alpha1}$ receptor subunit in epileptic rat compared to control. *Annona squamosa*, Anonaine and Carbamazepine treated epileptic rats showed a significant reversal ($p < 0.05$) of the decrease in $GABA_{A\alpha1}$ receptor subunit staining in the hippocampus compared to epileptic rats (Figure 3).
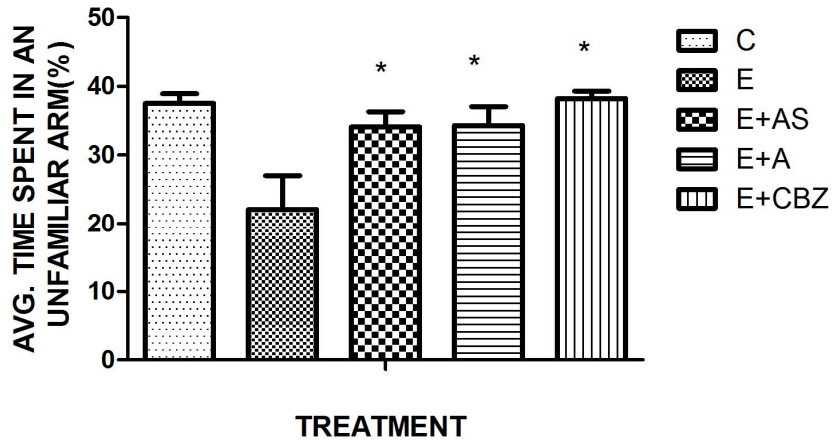
Representative graph showing radial arm maze performance of control and experimental rats.C-control E-epileptic, E+AS-epileptic +annona aquamosa E+A-epileptic +anoniane, E+CBZ-epileptic+carbamazepine

Fig . 1: Graph Showing Radial Arm Maze Performance. Data are in mean ± sem (n=5)
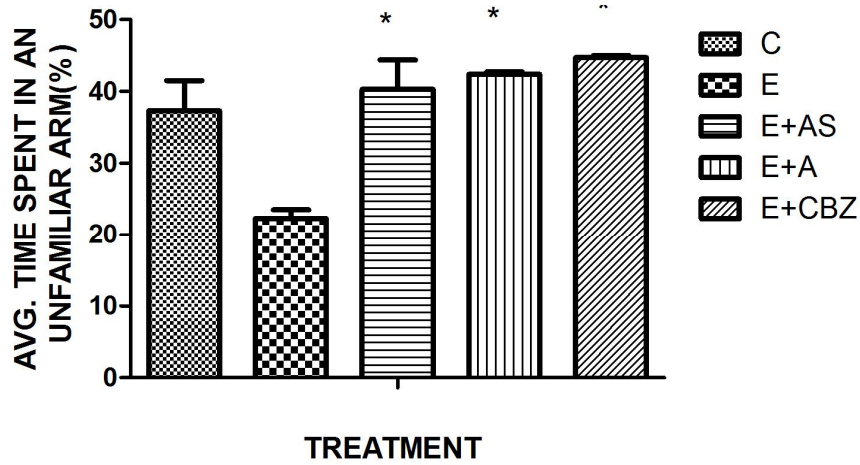
Representative graph showing Y maze performance of control and experimental rats. Epileptic, rats showed less exploratory behavior compared to control C-Control, E-Epileptic, E+AS-Epileptic+*Annona squamosa*, E+A-Epileptic+anonaine, E+CBZ-Epileptic+carbamazepine

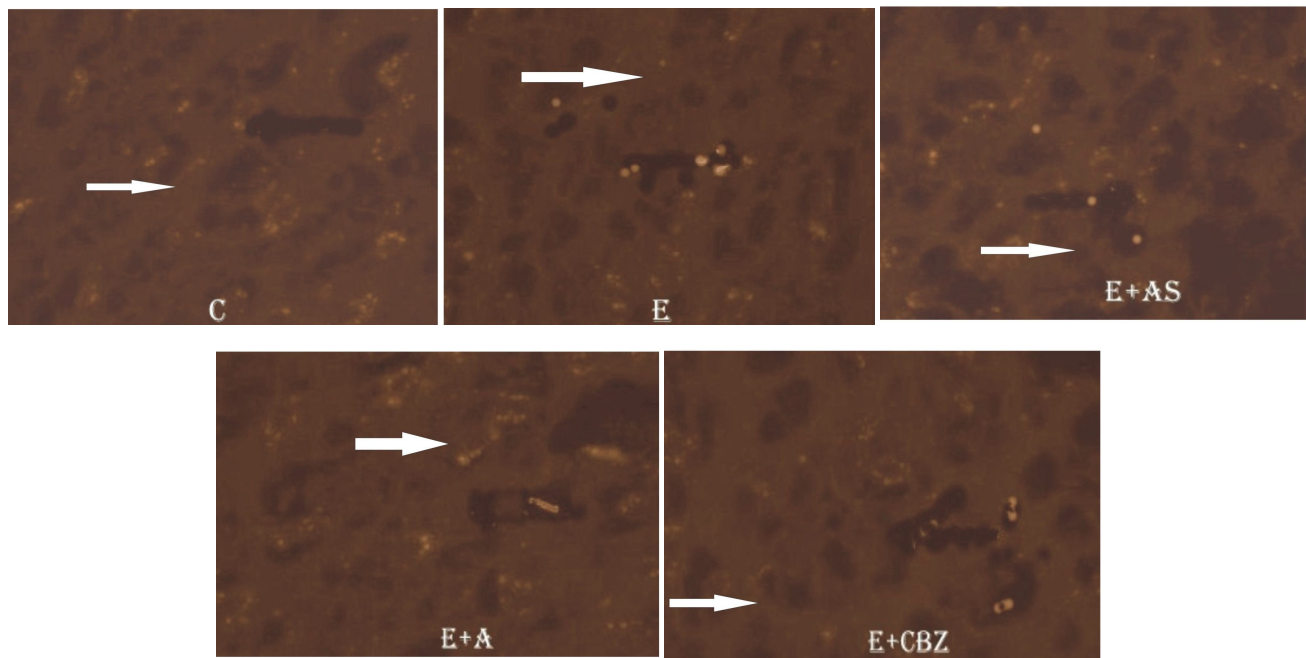**Fig. 2:** GRAPH SHOWING Y-MAZE PERFORMANCE. Data are in mean ± sem (n=5))

Fig. 3: GABAAα1 receptor subunit antibody staining in the cerebral cortex of Control and experimental rats. C- Control, E- Epileptic, E+AS- Epileptic + *Annona squamosa*, E+A- Epileptic + Annonaine, E+CBZ- Epileptic + Carbamazepine treated rats)

DISCUSSION

In the present investigation, it is evident that the *Annona squamosa* and Anonaine, was able to ameliorate epileptic seizures when induced externally in rats. It is understood from the literature that GABAergic neurotransmission is closely related to induction of epilepsy in the animals (Katzung, 2009) (Jin *et al*., 2003). GABA is the major inhibitory neurotransmitter in the central nervous system (Joseph, 2002) (Smart, 1997).

It exerts an inhibitory action in all forebrain structures and plays a role in the physiopathogenesis of epilepsy (Labrakakis, 1997). $GABA_A$ receptor binding influences the early portion of the GABA mediated inhibitory postsynaptic potential, whereas $GABA_B$ binding influences the late portion. $GABA_A$ receptor activation in neurons induced a complex physiological response, namely the activation of a Cl^- conductance in concert with a blockade of the resting K^+ outward conductance results in hyperpolarisation. Both responses were mediated by the activation of $GABA_A$ receptors, since they were both mimicked by the $GABA_A$ receptor agonist muscimol and antagonized by picrotoxin and bicuculline (Kanner and Soto, 1998)

There are various reports of the anticonvulsant and neuroprotective properties of *Annona squamosa* and its active alkaloid component Anonaine, an effort has been extended to understand their pharmacological action on the GABA receptors in the cerebral cortex of the epileptic rats. We observed a significant decrease in the B_{max} of GABA receptors in the cerebral cortex of epileptic rats compared to control GABA receptors.

There are many evidences which highlight the higher incidence of psychiatric diseases in epileptic patients when compared to the normal patients (Mendez *et al*., 1993) (Honig *et al*., 1993). Anxiety disorders have long been associated with disturbances of GABA function because of the ability of the benzodiazepine anxiolytics to facilitate brain GABA neurotransmission (Goddard *et al*., 2001). Interestingly, as with plasma studies MRS also reveals lowered concentrations of GABA in the occipital cortex in panic disorder (Butler and Zeman, 2008). Also cerebral cortex plays a key role in memory, attention, perceptual awareness, thought, language, mood and consciousness. Epileptic patients are often suffering from memory and cognitive problems.

In this study, we used two parameters for behavioral study-radial and y-maze to test anxiety and depression like behavior induced by the decreased GABA receptors in the cerebral cortex of the epileptic rats. Our study showed an improved performance of *Annona squamosa* and its active alkaloid component Anonaine (250 mg/kg, p.o.) in both radial and y-maze analysis compared to epileptic rats. This can be correlated with the decreased GABA receptors in the cerebral cortex of the epileptic rat interaction through 5-HT pathways. Cerebral cortex plays a key role in memory, attention, perceptual awareness, thought, language, mood and consciousness. The results also confirmed the memory enhancing property of above extract and its active component in epileptic rats.

CONCLUSION

We conclude from our studies that *Annona squamosa* and its active alkaloid component Anonaine treatment potentiates a therapeutic effect by reversing the alterations in general GABA, $GABA_A$, $GABA_B$ receptor binding, $GABA_A$ receptor subunits, that occur during epilepsy, resulting an increased GABA mediated inhibition in the over stimulated cerebral cortex neurons. These results also confirmed the memory enhancing property of *Annona squamosa* in epileptic rats. Overall, our study concludes that an extract of leaves of *Annona squamosa* and its isolated component-Anonaine, helps in the management of epilepsy along with associated mood disorders and memory related problems. The study needs to be further extended for best results.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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