Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6 Issue 2, 2014

Research Article

A STUDY ON THE ANTICONVULSANT AND ANTIANXIETY ACTIVITY OF ETHANOLIC EXTRACT OF *PUNICA GRANATUM* LINN.

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Received: 26 Jan 2014, Revised and Accepted: 07 Mar 2014

ABSTRACT

Objective: To evaluate the anticonvulsant and anxiolytic effect of ethanolic extract of Leaves of *Punica granatum* Linn.

Methods: The anticonvulsant effect of the Ethanolic extract of *Punica granatum* Linn (EEPG 100 and 200 mg/kg) were evaluated in rat using the Maximal electroshock (MES) and Pentylenetetrazole (PTZ) induced seizure models. Standard drug taken for MES model was Phenytoin 25mg/kg. In PTZ model standard drug taken was diazepam 4mg/kg. Anxiolytic activity was evaluated in mice using the elevated plus maze (EPM) and the mirror chamber methods. Diazepam 2mg/kg was taken as standard anxiolytic drug in both the models. The acute toxicity studies and phytochemical analysis of the extract were also carried out.

Results: in MES model Ethanolic extract of *Punica granatum* Linn (EEPG) completely abolished hind limb extensor phase and decreased the duration of tonic flexor phase. In PTZ model it significantly delayed onset of jerks, number and average duration of convulsion in a dose dependent manner. In EPM number of entry to open arm and average time spent per entry in open arm significantly increased. In mirrored chamber latency decreased but number of entry, total time spent per entry and average time per entry all of these are significantly increased.

Conclusion: It is concluded that EEPG possess significant anticonvulsant and anti-anxiety activity.

Keywords: Anti anxiety, Anti-convulsant, *Punica Granatum,* Maximal electroshock, Pentylenetetrazole, Elevated plus maze, Mirror chamber.

INTRODUCTION

Epilepsy describes a condition in which a person has recurrent seizures due to chronic underlying process. [1] Although a number of antiepileptic drugs (AEDs) are available, seizures remain uncontrolled in more than 20% of the patients. [2].

Anxiety is a feeling of apprehension, uncertainty or tension streaming from the anticipation of an imagined or unreal threat. [3]. The primary treatments for anxiety related disorders include selective serotonin reuptake inhibitors (SSRI), Serotonin-norepinephrine reuptake inhibitors (SNRI), benzodiazepines, the azipirone buspirone and beta adrenergic antagonists. [4]. But all these drugs have lots of side effects and potential for dependence and abuse. The pomegranate, *Punica granatum* Linn, belongs to Punicaceae family, grows 12-16 feet and has many spiny branches. Leaves are glossy and lance-shaped. Found in entire region from Himalayas to Iran. [5]

Traditionally, the plant and rind of fruit is used for treatment of various diseases, such as ulcer, hepatic damage, snakebite, dysentery and ulcer [6]. Other potential applications include brain ischemia, male infertility, Alzheimer's disease, arthritis, obesity [5, 7] breast carcinoma [8] and cerebral malaria [9].

Recently anti-anxiety and anti-epileptic activity of fruit and peel of *Punica Granatum* Linn was observed [10, 11,12]. The Leaves of *Punica granatum* Linn. are used in traditional medicine in epileptic patients and in patients with anxiety. But till now no study is available on the antiepileptic and anti-anxiety action of *Punica Granatum* Linn. leaves.

Hence the present study was undertaken to evaluate the effect of Ethanolic extract of *Punica granatum* Linn. leaves in experimental models of seizure and anxiety.

MATERIALS AND METHODS

Collection and authentication of Plant Material

The leaves of *Punica granatum* Linn. were collected from Assam Medical College and Hospital campus (AMCH), Dibrugarh and identified by Dr L. R. Saikia, Professor, Department of Life Science, Dibrugarh University (Voucher specimen No DUL.Sc.460/2013). A voucher specimen was deposited in the Herbarium of the institute.

Preparation of plant extract

Fresh leaves of Punica granatum Linn were cleaned, air dried and powdered. The powder was then packed into Soxhlet apparatus and extraction was done by hot continuous percolation using solvent ethanol (95% v/v). The extract was concentrated using vacuum evaporator (Rotary evaporator). They were further concentrated and dried in desiccators. The yield of Ethanolic extract was found to be 9.63% (W/W).

Phytochemical analysis

EEPG was subjected to qualitative phytochemical analysis as per standard methods. [13]

Drugs and chemicals

Phenytoin sodium was obtained from Abott Healthcare Pvt. Ltd (Mumbai) and Diazepam was obtained from Ranbaxy Laboratories Limited (Solan). Ethanol was procured from Merck (Mumbai, India).

Experimental animals

Healthy Wister rat (150-200gm) and Swiss albino mice (25-35 grams) of either sex were taken from the Central Animal House, Assam Medical College (registration no. 634/02/a/CPCSEA dated 19/05/02).

The animals were housed in standard cages under standard conditions of light and dark cycle and maintained under normal room temperature. The animals were fed with normal diet and water *ad libitum*. Before commencing the work permission from the Institutional Animal Ethics Committee was taken and conducted according to CPCSEA guidelines.

Acute oral Toxicity Test

EEPG was subjected to acute oral toxicity test following OECD guidelines 425 (up and down Method) and was found safe at 2000mg/kg dose. Two arbitrary doses 100mg/kg and 200 mg/kg were selected for the study. [14]

Treatment schedule and assessment of anticonvulsive activity

Healthy Wister Albino rats weighing 150-200 grams were used for assessment of anticonvulsant activity.

Maximal electroshock induced convulsion

In the maximal electroshock induced convulsion, electrical stimulus (150mA, 50Hz, 0.2 sec duration) was applied through ear lobe electrodes using an electroconvulsiometer [15]. Prior to delivery, current output was checked by multimeter. [16] Animals were grouped into four (n=5). Group A was taken as control. Groups B & C treated with test drug (100mg/kg and 200mg/kg EEPG i.p.) & group D was treated with Standard drug Phenytoin (25mg/kg i.p.). Electroshock was given by ear electrodes 30 minutes after the administration of standard drug & plant extract. The decreased duration of Hind Limb Tonic Extension (HLTE) was considered as a protective measure against MES induced seizures. [15, 16, 17]

Pentylenetetrazole induced clonic convulsion

In this test, animals were grouped into four (n=5). Group A was taken as control. Group B & C were treated with test drug (EEPG 100mg/kg and 200mg/kg i.p.) & group D was treated with Diazepam (4mg/kg i.p.). After 30 minutes, PTZ was administered at a dose of 85 mg/kg i.p. & animals were observed for 1 hour. The parameters like latency of convulsion, number of convulsion in 1 hour, average duration of convulsion, seizure score, recovery/ death are observed as measures of anticonvulsive property. [15]

Treatment schedule and assessment of anxiolytic activity

Healthy Albino mice weighing 25-35 grams were used for assessment of anxiolytic activity.

Elevated Plus Maze Test

Elevated plus maze consists of two open arms (16×5 cm) and two enclosed arms (16×5×12 cm) with an open roof, both connected to a central platform (5×5 cm) and the whole apparatus is elevated to a height of 25 cm. The animals are placed individually at the centre of the elevated plus maze with their head facing an open arm [3]. The walls and floor of the apparatus were painted with black paint. The apparatus was kept in a sound proof room. The room was illuminated with a 200 lux lamp [19].The percentage of time spent (duration) in open arms and percentage of the number (frequency) of open arm entries were counted during 5 min. Subsequently, the percentage of time spent in the open arms [100 × open / (open + enclosed)] and percentage of the number of open arm entries (100 × open /total entries) were calculated for each animal.

The apparatus was thoroughly cleaned after each trial. Arm entry is defined as all 4 paws having crossed the dividing line between an arm and the central area. [18]

Animals were grouped in four (n=5). Group A was taken as control. Groups B & C treated with test drug (100mg/kg & 200mg/kg EEPG i.p.) & group D was treated with Diazepam (2mg/kg i.p.).

Mirror Chamber Test

The mirror chamber consisted of a wooden chamber (40×40×30.5 cm) having a mirror chamber (30×30×30 cm) enclosed within it. Placement of the mirrored cube into the centre of the container forms a 5 cm corridor that completely surrounding the mirrored chamber.

The animals were placed individually in the chamber of mirrors at a fixed corner. During the 5 min test session, following parameters were noted: (a) latency to enter the mirror chamber, (b) number of entry, (c) total time spent in mirror chamber, (d) time spent per entry in mirror chamber [3].

Animals were grouped in four (n=5). Group A was taken as control. Groups B & C treated with test drug (100mg/kg & 200mg/kg EEPG i.p.) & group D was treated with standard dug Diazepam 2mg/kg (i.p.).

Statistical analysis

All the values were expressed as arithmetic mean ± SEM & were analysed by one-way analysis of variance (ANOVA), followed by Dennett's multiple comparison test. $p< 0.05$ was the criterion for statistical significance.

RESULTS

Phytochemical analysis

Qualitative phytochemical analysis of EEPG showed presence of flavonoids, glycosides, tannins and carbohydrates.

Acute toxicity study

Oral administration of EEPG at a dose of 2000mg/kg did not produce any toxic effect in female Rat. No mortality was observed and the extract was found to be safe at the given dose. (LD50>2000mg/kg).

Maximal Electroshock Test

There was significant ($p<0.05$) decrease in the duration of Hind limb tonic Extension (HLTE) after administration of EEPG (100 & 200 mg/kg) compared to control group. Phenytoin completely protected the mice against Maximal Electroshock induced convulsion.

Table 1: Effect of Ethanolic extract of Punica Granatum (EEPG) on MES induced convulsions

Groups	Treatment	HLTE (sec)	Recovery/Death
A	3% gum acacia	12.17±0.030	R
B	EEPG 100mg/kg	5.3333±0.6146*	R
C	EEPG 200mg/kg	4.000±0.3651*	R
D	Phenytoin 25 mg/kg	0*	R

Values expressed as mean ± SEM, n=5, ANOVA followed by Dunnett's test. * $p<0.05$ vs gum acacia treated group.

Pentylenetetrazole (PTZ) induced seizures: The Ethanolic extract of *Punica granatum* Linn significantly ($p<0.05$) increased the latency, but decreased the number & average duration of convulsion in the treated group (EEPG 100 & 200mg/kg) compared to control group. Diazepam completely protected the animals against PTZ induced seizures.

Table 2: Effect of Ethanolic extract of Punica Granatum leaves on Pentylenetetrazole (PTZ) induced convulsions

Group	Treatment	Latency (sec)	No of convulsions	Av. duration of convulsions (sec)	Seizure score	Recovery/ death
A	3% gum acacia	714.3±4.66	93.83±1.97	14.67±0.84	4.8±0.2	R
B	EEPG 100mg/kg	1799±3.98*	56.33±1.62*	3.0000±0.36*	4±0.31	R
C	EEPG 200mg/kg	1980±5.57*	3.167±0.6*	1.0000±0.36*	2.1±0.32*	R
D	Diazepam 4mg/kg	----------	0*	0*	0.3±0.2*	R

Values expressed as mean ± SEM, n=5, ANOVA followed by Dunnett's test. * $p<0.05$ vs gum acacia treated group.

Elevated Plus Maze: There was significant increase the number of entry to open arm and also total time spent in open arm in both the test groups (EEPG 100 & 200 mg/kg) when compared to control. But the effects were little less than compared to standard drug Diazepam.

Table 3: Effect of EEPG on behaviour of mice in Elevated Plus Maze test

Group	Treatment	No of entries		% OAE	Time spent (sec)		% TSOA
		Open arm	Closed arm		Open arm	Closed arm	(Sec)
A	3% gum acacia	3.2±0.2	24.2±0.8	11.8±1.1	17.20±1.5	207.2±1.3	7.5±0.8
B	EEPG 100mg/kg	6.6±0.2*	14.4±0.2*	32.6±0.3*	34.20±3.1*	164.4±2.1*	20.1±1.2*
C	EEPG 200mg/kg	9.8±0.4*	11.6±1.1*	44.3±2.1*	52.4±1.5*	152.8±1.8*	26.1±0.8*
D	Diazepam 2mg/kg	10.4±0.5*	11.8±0.5*	50.2±0.4*	72.40±4.2*	139±2.4*	35.3±1.2*

Values are expressed as MEAN ± SEM. n=5, ANOVA followed by Dunnett's test. *p<0.05 vs gum- acacia treated group. % OAE= percentage of open arm entries. % TSOA= percentage of total time spent in open arm in seconds.

Mirror Chamber

In mirror chamber, there was significant decrease in latency to enter mirror chamber, but significant dose dependent increase was seen in number of entry, total time spent, time/entry to mirror chamber in the Test groups (EEPG 100 & 200 mg/kg) when compared to control.

Table 4: Effect of EEPG on behaviour of mice in Mirror Chamber test

Groups	Treatment	Latency (sec)	No of entry	Total time spent (sec)	Time/entry
A	3% gum acacia	237.8±16.04	0.8±0.2	1.8±0.6	1.4±0.4
B	EEPG 100mg/kg	188.2±5.47*	1.6±0.24*	6.2±0.7*	3.5±0.27*
C	EEPG 200mg/kg	151.8±4.49*	2.2±0.37*	8±0.94*	5.56±0.94*
D	Diazepam 2mg/kg	112.8±4.24*	3.6±0.50*	18.2±1.1*	5.56±0.952*

Values are expressed as MEAN ± SEM. n=5. ANOVA followed by Dunnett's test. *p<0.05 vs gum acacia treated group.

DISCUSSION

Ethanolic extract of punica granatum leaves inhibited both MES and PTZ induced seizures. These two tests are most widely used for testing of anticonvulsant activity.

In the present study EEPG attenuated the duration of HLTE phase in MES induced seizures indicating anticonvulsant activity. The MES test identifies agents with activity against generalized tonic clonic seizures. It is also proposed that maximal electroshock test predicts anticonvulsant effects against partial seizure [2].

In PTZ model EEPG increased the latency period but decreased the number of convulsions, average duration of convulsion and seizure score in a dose dependent manner. Chemo-convulsions due to pentylenetetrazol which produce clonic type of convulsions resemble petit mal type of convulsions in man [3].

EEPG was an effective anxiolytic in both the behavioural models for anxiolytic testing. The elevated plus maze (EPM) test is a sensitive behavioural test that reveals animal's neophobia or anxiety [3]. The EPM test is based on a premise where the exposure to an open arm evokes an approach- avoidance conflict [19]. Anxiolytics are expected to increase the proportions of entries into and time spent in open arm. [3]. In case of EEPG an increase in number of entries to open arm and time spent in open arm was observed compared to control.

Many animal species exhibit approach-avoidance conflict upon placement of a mirror in their environment. Furthermore response to an apparent animal reflected in the mirror might be also a source of anxiety. The extended latency to enter the chamber of mirrors is used as a parameter of anxiety analogy [3]. In mirror chamber EEPG significantly decreased the latency to enter mirror chamber, increased number of entry, total time spent and time per entry into mirror chamber compared to control group.

MES- induced convulsion model causes facilitation of Ca^{2+} [20] and other positive ion like Na^{+} into the cells, blockade of which can prevent MES induced tonic extension [21, 22]. Potentiation of GABA receptor may offer protection against MES induced seizures [22].

Antagonism of PTZ induced seizures may be through the interaction with GABAergic receptor. Flavonoids and Diazepam are structurally similar [23] Flavonoids are ligands for Benzodiazepine binding sites on $GABA_A$ receptors [24]. At cellular level stimulation of $GABA_A$ receptor results in an increased chloride ion conductance and usually a concomitant hyperpolarization. In intact animals activation of this receptor is known to be associated with antianxiety and anticonvulsant action [3]. Flavonoids present in Punica Granatum extract may be responsible for its anti-anxiety and anti-epileptic action.

CONCLUSION

The present study concludes that the Ethanolic Extract of Punica granatum leaves possess significant anticonvulsant and anxiolytic activity. Further study is required for isolation & identification of active constituents & to confirm exact mechanism.

Conflict of interest: Nil.

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