Research Article

Assessment of anti-oxidant, anti-epileptic and anti-anxiety activities of pomegranate leaf (*Punica grantum*) in mice

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Abstract

Objective: The aim of this research was to assess the anti-oxidant, anti-epileptic and anti-anxiety properties of the flavonoids obtained from the Pomegranate (*Punica granatum* L.). **Materials and Methods:** Methanol and ethyl acetate solvent was used for Soxhlet extraction and water was used for cold maceration and the extracts were screened for the presence of different phytochemicals. Anti-oxidant activity was assessed using DPPH method by using ascorbic acid as standard and IC50 value was calculated. **Results and Conclusion:** Flavonoid obtained from the methanolic extract showed highest free radical scavenging activity with IC50 value of 31.48µg/ml. Anti- epileptic activity was assessed using the MES model using Phenytoin (25 mg/kg) as standard and MEP and EAP were given at the doses of 100mg/kg and 200 mg/kg in which 200mg/kg methanolic extract showed the least duration of tonic clonic convulsion (26.67 $\pm$ 0.33 sec). Actophotometer test was done to evaluate the anti-anxiety activity in which Diazepam (3 mg/kg) standard drug was used and MEP and EAF (100mg/kg and 200mg/kg) was administered to the mice. The result showed that the MEF 200mg/kg showed the least movement during the 3 minutes time.

Keywords: *Punica grantum,* phytochemical screening, anti-epileptic activity, anti-anxiety, antioxidant activity

Introduction

Traditional medical practices have been the base for development of modern allopathic medicines. Primary metabolites are directly involved in growth and development while secondary metabolites present in the plant directly work as biocatalysts.it includes the use derivatives of plants, minerals, bacteria, and animals. In case of plants, they contain secondary metabolites like alkaloids, glycosides, flavonoids, tannins and many more. More than 35000 species of plants are being used for treatment, prevention and cure of many clinical conditions (Seeram et al., 2006).

Pomegranate is the edible fruit known from the ancient times, and its juice is also highly consumed specially during illness. Research findings support the traditional use of pomegranate as a medicinal plant as almost all parts of plant have several bioactive metabolite (Sodipo et al., 1991). Polyphenols are the major phytochemicals that is present in pomegranate which includes flavonoids, condensed tannins and hydrolysable tannins. Other phytochemicals are steroids, triterpenoids, fatty acids, triglycerides and alkaloids (Seeram et al., 2006).

Studies that have been conducted till now showed that pomegranate and its secondary metabolites possess anti-oxidant (McMahon et al., 1995) anti-microbial, antifungal properties (Gil et al., 2000), anti-inflammatory (Les et al., 2015), anti-hypertensive (Nainwani et al., 2014), anti-diabetic and hypolipidemic, vasoprotective, wound healing and anti-proliferative properties (Radhika et al., 2011).

So as to emphasize on the safety concerns relating to the drugs, the present review comprises of seeking the safer and convenient way to relief disease with less side effects and easy availability of the raw materials. The polyphenols present in the pomegranate extract have been reported to show different pharmacologically important actions like;

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anti-diabetic, anti-cancer, anti-inflammatory etc. Thus recent study was an attempt to carry out the antioxidant, anti-epileptic and anti-anxiety properties of leaf extract (from 3 solvents of different polarity) of *Punica granatum* in Swiss *Albino mice* using electroconvulsiometer for anti convulsant action, Actophotometer for anti-anxiety and DPPH scavenging method for anti-oxidant activity.

Materials and Methods

Plant materials

The plant leaf for investigation was collected from Sano Thimi, Bhaktapur, Nepal and was identified from the help of taxonomist from department of Herbarium and plant resources, Godavari, government of Nepal.

Experimental animals

Healthy Albino mice (25-35 gm) were used from our animal house facility. Animals were housed separately in polycarbonate cage (29 cm × 22 cm ×14 cm) under standard laboratory conditions with Food and water. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Nepal [Permit: Asian college (Bagmati) 2019-20], and animal welfare and experimental procedures were strictly in accordance with the guide for the care and use of laboratory animals.

Macroscopic and powder microscopy

Different microscopic parameters such as color, order, size and appearance were observed. A pinch of sample powder was soaked in chloral hydrate for 1 hour and was stained with phlorogluconic acid followed by the addition of 1-2 drops of concentrated HCl. Slide was prepared and was observed under the microscope at powers 10X, 40X and 100X.

Physical Parameters

Determination of loss on drying

2gm of sample was put in Petridish and was weighed. It was then put in hot air oven at 105°C until constant weight was obtained. The sample along with Petridish was weighed in gap of minutes and after obtaining the constant weight, the final weight of sample was noted to calculate loss on drying.

Determination of ash value

Firstly, total crucible was weigh and 2.00gm of sample was added to it. The sample was heated for 4 hours in a muffle furnace at a temperature of 450° C. further down it was cooled in dessicator at a room temperature and final weight was noted to calculate the total ash value.

Extraction by cold maceration

Forty gram of sample powder was taken in 1000 ml beaker and 500 ml of distilled water was added to it. It was stirred and was left covered with aluminum foil. After 24 hours the mixture was again stirred and covered. This process was continued for 7 days and after the 7th day, it was filtered and filtrate was subjected for evaporation and was stored in the porcelain basin for further use.

Soxhlet extraction

Accurately 40.05 gram of sample powder for methanolic extraction and 40.04 gram for ethyl acetate extraction was taken and put in 1000 ml round bottom flask of Soxhlet Apparatus. Porcelain chips were added to the flask and apparatus was fitted. 3 cycle of respective solvent were added to the apparatus which was approximately 250 ml. then, the content was heated in heating mantle at 30°C until the clear solvent ran through the siphon tube.

Removal of solvent

The content of R.B. flask was filtered after cooling and the filtrate was transferred to 500 ml beaker and evaporated in water bath until only small amount of content was left. At last, the content was transferred to porcelain basin, was evaporated to dryness and was preserved by covering with aluminum foil in desiccator until further use.

Phytochemical Screening

Phytochemical screening was conducted for detection of flavonoids, detection of alkaloids, detection of glycosides, detection of protein and amino acid, detection of saponin and detection of tannins per the standard procedures. A Chromatographic (thin layer chromatography) study was also performed (Dongdong et al., 2018).

Anti-epileptic assessment

Electroconvulsiometer test

Albino mice were randomly divided into six groups each consisting of 3 animals and were treated according to the protocol: Group I: Control group (3% tween p.o); Group II: Standard drug (Phenytoin 25mg/kg i.p); Group III and IV: flavonoid from methanolic extract (100 and 200 mg/kg p.o); Group V and VI: flavonoid from ethyl acetate extract (100 and 200mg/kg p.o).

After 1 hr seizure was induced with a single 12 mA intensity of 50 Hz stimulus for 0.2 s using Electroconvulsiometer. Time duration (in seconds) for tonic – clonic convulsions were recorded (Chauhan et al., 1988).

Anti-anxiety evaluation

Actohotometer Test

Mice were randomly divided into six groups consisting of 3 animals each (n=3). One group was treated with standard drug (Diazepam 3mg/kg i.p) or control (3% tween i.p) or

flavonoids from methanolic extract and ethyl acetate extract (100 and 200 mg/kg p.o).

The locomotor behavior of mice was monitored using Actophotometer. Animals were placed in Actophotometer individually 1 hour after the administration of the drugs, and basal activity was recorded over the period of 5 min (Kumar et al., 2012).

Rota rod test

The effect on motor coordination was assessed using a rota-rod apparatus (LE 8500). Rota rod consisted of a base plant form and aniron rod of 3 cm diameter and 30 cm length, with a non-slippery surface. The rod was divided into four equal sections by three disks. The animals were pre-selected in a training session 24 h before the test, based on their ability to remain on the bar (at 12 rpm) for 2 min, and then allowing four mice to walk on the rod at the speed of 12 rpm at the same time observed over a period of 30, 60, and 90 min. Intervals between the mounting of the animal on the rotating bar and falling off of it were registered automatically as the performance time. Time spent in the apparatus was observed for 5 min duration (300 s).

Results and discussion

In this project, we studied anti-oxidant, anti-epileptic and anti-anxiety properties of flavonoid extracts from leaves of *Punica granatum* collected from Sano Thimi Bhaktapur, Nepal. Epidermis, fibers, starch grains, vessels, endodermis and oxalate crystals were observed in powder microscopy. Loss on drying on leaf of *Punica granatum* was found to be 0.33 gram. Total ash value of leaf of *Punica granatum* was found to be 1.42 gram.

Extraction was done by maceration for aqueous extraction using distilled water and Soxhlet extraction was conducted using methanol and ethyl acetate.

Phytochemical tests for each extract was conducted which showed the presence of alkaloid, glycoside and tannin in all three solvents however flavonoid was present only in methanolic and ethyl acetate extract (Table 1). All three extracts showed positive response for glycoside test (Molisch's test and Fehling's test) but showed negative for Killer-Kiliani test which suggested that there is no presence of cardiac glycoside. Positive test for Biuret test and negative for Ninhydrin test suggested that protein is present but free amino acid is absent. Column chromatography was conducted for the separation of flavonoids from the methanolic and ethyl acetate extract but the aqueous extract was discarded since it showed no response of flavonoids. It was done using Chloroform: Methanol (1:1) as solvent. For TLC, Toluene: Acetic acid (4.6:0.4) was induced as mobile phase to detect the flavonoid in elute (Table 2).

Antioxidant activity of the methanolic flavonoid extract (MEF) and ethyl acetate flavonoid (EAF) was performed by calculating the IC50 value.

Table 1. Phytochemical screening of different extracts of *Punica granatum*

S. No.	Tests	Methanol extract	Ethyl acetate extract	Distilled water extract
1	**Flavonoids**			
	Shinoda test	+	+	-
	Alkaline reagent test	+	+	-
2	**Alkaloid**			
	Mayer's test	+	+	+
	Wagner's test	+	+	+
	Hager's test	+	+	+
3	**Glycosides**			
	Killer-kiliani	-	-	-
	Molisch's test	+	+	+
	Fehling's test	+	+	+
4	**Protein and amino acid**			
	Ninhydrin test	-	-	-
	Biuret test	+	+	-
5	**Detection of saponin**			
	Foam test	+	+	+
6	**Detection of tannins**			
	Ferric chloride	+	+	+
	Gelatin test	+	+	+
	Lead acetate test	+	+	+
	Wagner's test	+	+	+
	Hager's test	+	+	+
7	**Glycosides**			
	Killer-kiliani	-	-	-
	Molisch's test	+	+	+
	Fehling's test	+	+	+
8	**Protein and amino acid**			
	Ninhydrin test	-	-	-
	Biuret test	+	+	-
9	**Detection of saponin**			
	Foam test	+	+	+
10	**Detection of tannins**			
	Ferric chloride	+	+	+
	Gelatin test	+	+	+
	Lead acetate test	+	+	+

Table 2. TLC values of different flavonoid detection

Extracts	Distance moved by mobile phase	Distance moved by solute	Rf value
MEF	5.1	1.3	0.25
		2.1	0.41
EAF	5.1	1.3	0.25
		2.1	0.41

MEF: methanolic flavonoid extract; EAF: ethyl acetate flavonoid

Antiepileptic activity

Electroconvulsiometer test

For the study of anti-epileptic activity, Phenytoin was used as standard drug where the activity was carried out using MES model. The MEF and EAF (100 and 200 mg/kg) was showed

Table 3. Anti-oxidant evaluation

Extracts	Concentration (µg/ml)	% inhibition	IC_{50}
Ascorbic acid	25	39.05	26.89
	50	52.89	
	100	64.51	
	200	79.98	
	400	86.26	
MEF	25	46.5	31.48
	50	60.45	
	100	68.05	
	200	84.18	
	400	90.53	
EAF	25	36.59	33.62
	50	48.17	
	100	72.76	
	200	79.01	
	400	87.2	

MEF: methanolic flavonoid extract; EAF: ethyl acetate flavonoid

Table 4. Results of anti-epileptic activity (Electroconvulsiometer test)

Groups	Treatment	Dose	Weight of mice (gm)	Duration of HLTE (sec)
I	Phenytoin	25mg/kg i.p.	26.47± 0.45	21 ± 1.15
II	Tween 3%	0.3ml p.o.	27.71 ± 1.19	54.33 ± 2.03
III	MEF	100mg/kg i.p.	24.21 ± 1.21	36.33 ± 2.85
IV	MEF	200mg/kg i.p.	27.13± 0.13	26.67 ± 0.33
V	EAF	100mg/kg i.p.	27.16 ± 0.73	47.33 ± 0.88
V	EAF	200mg/kg i.p.	27.15± 1.04	42 ± 1.16

P value <0.05; MEF: methanolic flavonoid extract; EAF: ethyl acetate flavonoid

Table 5. Results of anti-anxiety activity (Actophotometer test)

Groups	Treatment	Dose	Weight of mice (gm)	No. of movement before treatment	No. of movement after treatment
I	Diazepam	3mg/kg i.p.	23.82 ± 0.37	177 ± 1.15	75 ± 2.08
II	Tween	3%	23.04 ± 1.00	171.33± 3.75	165.33 ± 3.52
III	MEF	100mg/kg p.o.	23.71 ± 0.62	174 ± 3.51	133.67 ± 2.73
IV	MEF	200mg/kg p.o.	22.56 ± 0.92	172.33± 2.18	122.33 ± 0.67
V	EAF	100mg/kg p.o.	23.85 ± 0.16	173.67± 3.92	131.67 ± 1.45
VI	EAF	200mg/kg p.o.	23.05 ± 0.82	170.67± 1.85	125.67 ± 1.20

P value <0.05; MEF: methanolic flavonoid extract; EAF: ethyl acetate flavonoid

significant dose dependent decrease in duration of tonic-clonic convulsion induced by MES (P<0.05) (Table 3).

Anti-anxiety activity

Actophotometer test

Actophotometer test was used for the evaluation of the anti-anxiety properties of the flavonoids obtained from *Punica granatum* in which number of movement was recorded before and after treatment. MEF and EAF at the dose of 100mg/kg and 200 mg/kg showed significant changes (p<0.05) compared to standard drug Diazepam (3mg/kg i.p) which concludes that flavonoids obtained from the plant has anxiolytic effects.

Table 6. Results of anti-anxiety activity (Rota-rod apparatus)

Groups	Treatment	Dose	Time (sec) of animals remained without falling from rod		
			30min	60min	90min
I	Diazepam	3mg/kg i.p.	92.33± 22.45	199.8± 35.34	217.5 ± 32.58
II	Tween 3%	0.3ml p.o.	300	300	300
III	MEF	100mg/kg i.p.	227 ± 33.87	260.7 ± 18.39	265.2 ± 31.19
IV	MEF	200mg/kg i.p.	262.5 ± 22.48	264.8 ± 22.99	277.2 ± 22.83
V	EAF	100mg/kg i.p.	240.2 ± 34.51	270.1 ± 17.25	280.1 ± 19.90
VI	EAF	200mg/kg i.p.	246.1 ± 17.29	257.12 ± 19.22	271.11 ± 21.11

P value <0.05; MEF: methanolic flavonoid extract; EAF: ethyl acetate flavonoid

Rota rod test

The data shows that on average the mice treated with 100, 200 and 400 mg/kg p.o. of the methanolic extract of *Punica granatum* were able to maintain equilibrium on the rotating rod and stayed on longer without falling, whereas diazepam (at 1 mg/kg only) showed a significant decrease in the locomotor score when compared to other groups.

Conclusion

The conducted study ascertains that the flavonoids contained in *Punica granatum* possesses significant anti-epileptic, anti-oxidant and antianxiety activity in mice which is supported by the phytochemical screening. The methanolic flavonoid (MEP) has shown better result in all assessed activities than ethyl acetate flavonoid (EAF) which suggests that the flavonoid content is more in methanolic extract.

Conflict of interest

The authors whose names are listed certify that they have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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