

**Antidiabetic Action of Ethanolic Extracts of
Punica granatum Linn. in Alloxan-induced
Diabetic Albino Rats**

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ABSTRACT

Antidiabetic action of ethanolic extracts of seed and rind of *Punica granatum* L. was evaluated on alloxan-induced diabetic albino rats. Both the extracts, each at a dose of 200mg/kg/d, were administered orally for two weeks to alloxan-induced diabetic rats. Blood glucose was estimated every week for two consecutive weeks. For evaluation of probable mechanism of action of the extracts, glycogen estimation was carried out in liver, heart and skeletal muscle and effect on adrenaline-induced hyperglycemia was observed. Both the extracts significantly ($p < 0.05$) reduced the rise in blood glucose induced by alloxan, with the rind extract exhibiting significantly ($p < 0.05$) better activity than seed extract. Both the extracts also produced significant ($p < 0.05$) increase in liver glycogen and significantly ($p < 0.05$) reduced adrenaline-induced hyperglycemia. These results support strong antidiabetic action in favor of *P. granatum* seed and rind extracts

Key words: *Punica granatum* L., Ethanolic extracts, Antidiabetic, Alloxan.

INTRODUCTION

Punica granatum L. (Family-Punicaceae), commonly known in India as 'Anar' or 'Dalim', is a highly ornamental large deciduous shrub or small tree widely distributed and cultivated in many parts of India. The rind of the fruit is anthelmintic and is useful in diarrhoea and dysentery. The seeds are used in scabies, liver and kidney disorders (Kirtikar and Basu, 1988). Phytochemical analysis of the seeds have revealed the presence of ursolic acid, β -sitosterol, estrogens and phenolic glycosides (Ahmed *et al.*, 1995) while the rind has been reported to contain tannins (ellagitannins- punicalagin), flavonoids and colouring matters (Evans, 2002). Biological testing of the various extracts of the leaves, seeds roots and stem barks revealed their hypotensive, antispasmodic and anthelmintic effects (Sharaf *et al.*, 1967). Antibacterial effect of *P. granatum* leaves (Nair and Chanda, 2005) and hypoglycemic action of flowers (Huang *et al.*, 2005), seeds (Das *et al.*, 2001) and rind (Khalil *et al.*, 2004) have been reported. Literature reviews indicated that no studies showing comparison on the antidiabetic potential of the rind and seed of this plant on alloxan-induced diabetes have so far been undertaken. In this backdrop, the present study was aimed to compare the antidiabetic activity of ethanolic extracts of seed and rind of *P. granatum* in alloxan-induced diabetic albino rats and also to examine the probable mechanism underlying the antidiabetic action of the extracts.

EXPERIMENTAL

Plant material

The fruits of *P. granatum* were collected from the local market in Dibrugarh in the months of June to August. The plant material was authenticated by Dr. L.R. Saikia, Reader, Department of Life Sciences, Dibrugarh University, Dibrugarh. A voucher specimen (No. DU/LS/211) was deposited at the Department of Life Sciences, Dibrugarh University, Dibrugarh.

Extraction

The seeds and rinds of *P. granatum* were manually separated from the whole fruits, air dried, and powdered (1200 g each of seed and rind powder). Ethanolic extracts were prepared using 90%

ethanol by percolation method (Nairn JG, 2000). The extracts were evaporated to dryness under vacuum and dried in vacuum desiccators to obtain a final yield of 257.5 g of the seed extract (21.5% w/w) and 188 g of the rind extract (15.7% w/w).

Animals

The study was carried out in healthy adult albino rats (*Rattus norvegicus*) of either sex (100-200 g each). Rats were housed in clean polypropylene cages with food pellets and tap water provided *ad libitum*. Permission from the Institutional Animal Ethical Committee for laboratory use of animals (Registration no: 634/02/a/CPCSEA; dated 19/05/2002) was duly obtained and the animals were taken care of as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Acute Toxicity Study

Acute oral toxicity test for the ethanolic extract of seed of *P. granatum* (EESP) and ethanolic extract of rind of *P. granatum* (EERP) was carried out as per OECD Guidelines 425 (OECD, 2006).

Chemicals Used

Crude powder of glibenclamide was obtained from Aventis Pharma Ltd., Goa while alloxan monohydrate was purchased from Sigma Aldrich India, Bangalore. The glucose kit for blood glucose estimation was obtained from Sigma Diagnostic (India) Pvt. Ltd., Baroda.

Experimental Design for Antidiabetic Study:

The study was done as described by Akhtar *et al.*, (2007). A total of thirty-six animals were equally divided into five groups with six animals in each group:

- Group–A : Normal Control. Received normal saline, 10 ml/kg/d.
- Group–B : Diabetic Control. Received normal saline, 10 ml/kg/d.
- Group–C : Diabetic Test. Received EESP, 200 mg/kg/d.
- Group–D : Diabetic Test. Received EERP, 200 mg/kg/d.
- Group–E : Diabetic Standard. Received glibenclamide, 0.5 mg/kg/d (Ghosh, 2005a).

The above drugs were administered orally, once daily, for two weeks.

Induction of Diabetes

Leaving aside six rats for Normal Control Group, 30 rats were induced diabetes by a single intraperitoneal injection of alloxan monohydrate in the dose of 150 mg/kg body weight. The fasting blood glucose was determined after 72 hours. Only 24 rats showing blood glucose level greater than 200 mg/100 ml were taken for the study (Prajapati *et al.*, 2008). Blood glucose was estimated every week for two consecutive weeks. Blood samples were collected from the orbital sinus of rats (Ghosh, 2005b) and glucose estimation was done by glucose oxidase method (McLauchlan, 1988).

Probable Mechanism of Antidiabetic Action

1. Glycogen Estimation of Liver, Skeletal Muscle and Cardiac Muscle:

The study was carried out as per the method of Carroll *et al.*, (1956). Out of 36 rats, 30 rats were induced diabetes by alloxan monohydrate (150 mg/kg body weight) intraperitoneally and 24 rats with blood glucose level greater than 200 mg/100ml were taken after 72 hours of diabetes induction. All the rats were kept fasting for 18 hours before the experiment. The rats were divided into five groups with six animals in each, as before.

- Group–A : Normal Control. Received normal saline, 10 ml/kg/d.
- Group–B : DiabeticControl. Received normal saline, 10 ml/kg/d and alloxan.
- Group–C : Diabetic Test. Received EESP, 200 mg/kg/d and alloxan.
- Group–D : Diabetic Test. Received EERP, 200 mg/kg/d and alloxan
- Group–E : Diabetic Standard. Received glibenclamide, 0.5 mg/kg/d and alloxan.

After two hours of administration of above drugs. The animals were killed by decapitation. The liver, leg muscle and heart tissues were taken out with care and their glycogen content was estimated by use of Anthrone reagent.

2. Effect on Adrenaline-induced Hyperglycemia :

The method described by Anturlikar *et al.*, (1995) was adopted to study the effect on adrenaline-induced hyperglycemia. The rats were divided into four groups with six animals in each as before.

Group–A : Normal Control. Received normal saline, 10 ml/kg/d

Group–B : Test Drug. Received EESP, 200 mg/kg/d

Group–C : Test Drug. Received EERP, 200 mg/kg/d

Group–D : Standard Drug. Received glibenclamide, 0.5 mg/kg/d

The above drugs were administered orally after drawing fasting blood samples. Adrenaline hydrochloride 100 µg was administered intraperitoneally to all the rats one hour after drug administration. Blood samples were again collected half an hour later.

Statistical Analysis

The data was statistically analysed using One-way ANOVA followed by Dunnett's multiple comparison test and Bonferroni (Chiplonkar, 1996). The statistical analysis was done using computerised GraphPad Prism software version 5.00. Values of $p < 0.05$ were considered significant.

RESULTS

Acute toxicity tests

There was no mortality recorded among the rats up to the maximum dose of 2000 mg/kg. Hence, the LD₅₀ of both the extracts can be said to be above 2000mg/kg. One-tenth of the maximum dose tested was selected for the experiments (Koneri and Balaraman, 2008).

Table–1: Effects of EESP and EERP on blood glucose level of alloxan-induced diabetic rats

Groups	Mean Blood Glucose Level in mg/100ml				
	'0 Day' (Base line)	'1 st day' (After 72 Hours)	'8 th Day'	'15 th Day'	
Normal Control (Normal Saline; 10ml/kg/d))	89 ± 1.00	112 ± 0.93	109 ± 1.34	111 ± 0.86	
Diabetic Control (Alloxan + Normal saline; 10ml/kg/d)	90 ± 1.34	296 ± 2.32 ^a	330 ± 1.00 ^a	372 ± 1.34 ^a	
Diabetic Test (Alloxan + EESP; 200mg/kg/d)	88 ± 1.53	310 ± 0.68 ^a	235 ± 2.27 ^{b,c,d}	151 ± 1.77 ^{b,c,d}	
Diabetic Test (Alloxan +EERP; 200mg/kg/d)	89 ± 2.28	300 ± 1.98 ^a	196 ± 1.55 ^{b,e}	139 ± 1.16 ^{b,e}	
Diabetic Standard (Alloxan + Glibenclamide; 0.5mg/kg/d)	92 ± 1.21	279 ± 1.07 ^a	184 ± 0.77 ^b	127 ± 1.48 ^b	
	<i>f</i>	0.9746	2930	2983	6380
ANOVA	<i>df</i>	4, 25	4, 25	4, 25	4, 25
	<i>p</i>	>0.05	<0.05	<0.05	<0.05

Values are expressed as Mean ± SEM; n=6 rats in each group. One-way ANOVA followed by Dunnett's multiple comparison test and Bonferroni was done. ^ap<0.05 when compared to Normal Control Group. ^bp<0.05 when compared to Diabetic Control Group. ^cp<0.05 when compared to Diabetic Test (EERP) Group. ^dp<0.05 and ^ep<0.05 when compared to Diabetic Standard Group.

Effect on fasting blood glucose level of diabetic rats

On repeated administration of the extracts and glibenclamide for two weeks, a significant ($p < 0.05$) decrease in blood glucose was found in EESP administered Group, EERP administered Group and Diabetic Standard Group respectively as compared to Diabetic Control Group which showed a significant ($p < 0.05$) rise in blood glucose as compared to Normal Control Group. The blood glucose lowering activity of EERP was significantly ($p < 0.05$) more as compared to that of EESP. However, among the three administered drugs i.e EESP, EERP and glibenclamide, the greatest blood glucose lowering action was exhibited by glibenclamide (Table-1).

Effect on Glycogen Estimation

There was a significant ($p < 0.05$) increase in the glycogen content of liver, skeletal muscle and cardiac muscle in EESP administered Group, EERP administered Group and Diabetic Standard Group respectively as compared to Diabetic Control Group which showed a significant ($p < 0.05$) reduction in glycogen content in the above tissues as compared to Normal Control Group. However, the increase in glycogen content in these tissues caused by EERP was significantly ($p < 0.05$) more than that by EESP (Table-2).

Table-2: Effect on glycogen concentration in liver, skeletal muscle and cardiac muscle

Groups	Glycogen Concentration (mg/100 g)			
	Liver	Skeletal Muscle	Cardiac Muscle	
Normal Control (Normal saline; 10ml/kg)	45 ± 1.00	38 ± 0.68	32 ± 0.89	
Diabetic Control (Alloxan + Normal saline; 10ml/kg)	6 ± 1.07 ^a	5 ± 0.93 ^a	3 ± 0.58 ^a	
Diabetic Test (Alloxan + EESP; 200mg/kg)	31 ± 0.93 ^{b,c}	20 ± 1.67 ^{b,c,d}	12 ± 1.69 ^{b,c,d}	
Diabetic Test (Alloxan + EERP; 200mg/kg)	39 ± 1.61 ^b	28 ± 1.95 ^{b,e}	21 ± 1.71 ^{b,e}	
Diabetic Standard (Alloxan + Glibenclamide; 0.5mg/kg)	48 ± 1.86 ^b	37 ± 2.21 ^b	30 ± 1.39 ^b	
	<i>f</i>	156.5	72.77	84.19
ANOVA	<i>df</i>	4, 25	4, 25	4, 25
	<i>p</i>	< 0.05	< 0.05	< 0.05

Values are expressed as Mean ± SEM; n=6 rats in each group. One-way ANOVA followed by Dunnett's multiple comparison test and Bonferroni was done. ^a $p < 0.05$ when compared to Normal Control Group. ^b $p < 0.05$ when compared to Diabetic Control Group. ^c $p < 0.05$ when compared to Diabetic Test (EERP) Group. ^d $p < 0.05$ and ^e $p < 0.05$ when compared to Diabetic Standard Group.

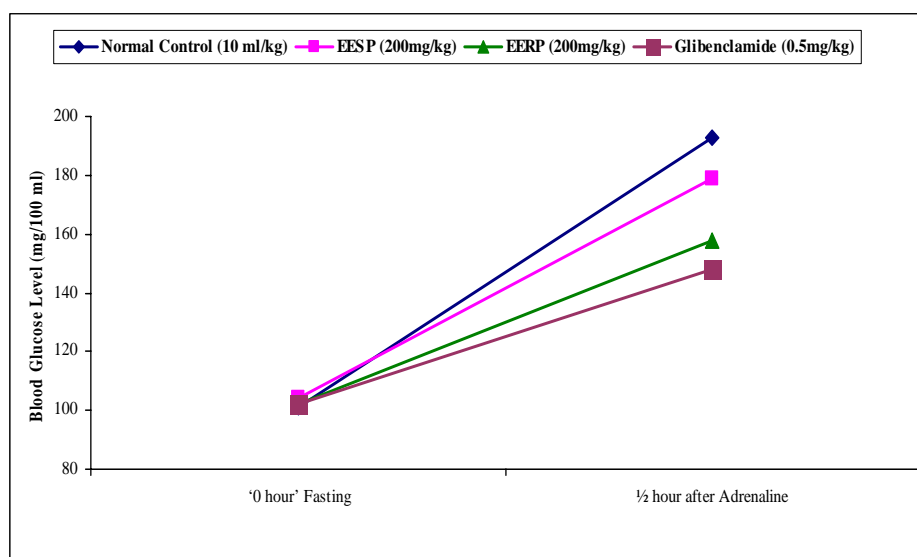
Effect on Adrenaline-induced Hyperglycemia

Both test drugs and the standard drug significantly ($p < 0.05$) reduced hyperglycemia induced by adrenaline, with the reduction caused by EERP being significantly ($p < 0.05$) more than that by EESP. However, the maximum reduction of adrenaline-induced hyperglycemia was brought about by glibenclamide (Table-3 and Figure 1)

Table–3: Effect on adrenaline-induced hyperglycemia in albino rats

Groups	Blood Glucose Level (mg/100 ml)				
	'0 hour' Fasting	½ hour after Adrenaline	Change	% of Increase	% of Decrease
Normal Control (Normal saline; 10ml/kg)	101 ± 1.77	193 ± 2.11	92 ± 0.73	91.09	
Test Drug (EESP; 200mg/kg)	104 ± 1.86	179 ± 1.73 ^{a,b,c}	75 ± 1.07 ^{a,b,c}	72.12	20.83
Test Drug (EERP; 200mg/kg)	102 ± 3.03	158 ± 2.32 ^{a,d}	56 ± 0.93 ^{a,d}	54.90	39.73
Standard Drug (Glibenclamide; 0.5mg/kg)	102 ± 2.50	148 ± 1.16 ^a	46 ± 1.21 ^a	45.10	50.49
	<i>f</i>	0.2870	116.2	416.9	
ANOVA	<i>df</i>	3, 20	3, 20	3, 20	
	<i>p</i>	> 0.05	< 0.05	< 0.05	

Values are expressed as Mean ± SEM; n=6 rats in each group. One-way ANOVA followed by Dunnett's multiple comparison test and Bonferroni was done. ^ap<0.05 when compared to the Normal Control Group. ^bp<0.05 when compared to the Test Drug (EERP) Group. ^cp<0.05 and ^dp<0.05 when compared to Diabetic Standard Group.

**Figure 1: Effect on adrenaline-induced hyperglycemia in albino rats**

DISCUSSION

The results found in the present study reflect that the ethanolic extracts of seed and rind of *P. granatum* possess significant ($p < 0.05$) antidiabetic action. The antidiabetic action of the seed and rind of *P. granatum* may be due to the insulin-like or insulin releasing action of the ingredients present in the seed and rind of the plant. The insulin-like and insulin releasing action of ingredients of many herbal plants have previously been reported (Bates *et al.*, 2000; Gray *et al.*, 1999).

Alloxan, a β -cytotoxic agent, rapidly and selectively accumulates in pancreatic β -cells (Gorus *et al.*, 1982) and causes β -cell death and apoptosis by generation of reactive oxygen species (ROS), superoxide radicals and hydrogen peroxide (Szkudelski, 2001). β cell death causes hyperglycemia due to insulin deficiency which further aggravates the oxidative stress induced by alloxan (Kaneto *et al.*, 1996). The antidiabetic activity of the seed and rind of *P. granatum* might be attributed to the presence in them of tannins, flavonoids and phenolic glycosides, known to be natural antioxidants (Evans, 2002), which protect the existing β -cells (which escaped alloxanization) from dying by their free radical scavenging action (Kaneto *et al.*, 1999). The rind of *P. granatum* has been reported to possess the highest antioxidant activity among the peel, pulp and seed fractions of 28 kinds of fruits commonly consumed in China (Mohamed, 2007). Insulin is a potent activator of the enzyme glycogen synthase while inhibiting the enzyme glycogen phosphorylase responsible for glycogenolysis in liver and muscle (Bollen *et al.*, 1998). Insulin deficiency in diabetes, as such, results in reduced concentrations of glycogen in liver and muscle. EESP and EERP caused an increase in glycogen concentration of the liver probably by stimulating the enzymes glycogen synthase and hexokinase, both of which contribute to increased glycogen synthesis. Kumar *et al.*, (2006) have reported the glycogen synthase stimulating action of fruits of *Terminalia chebula*. The increase in liver glycogen may also have been brought about by inhibition of the enzyme glucose-6-phosphatase leading to accumulation of glucose-6-phosphate, which allosterically inhibited the enzyme glycogen phosphorylase (Schaffingen *et al.*, 2002). Diminished phosphatidylinositol 3-kinase (PI-3K) activation in diabetes as a result of insulin deficiency has been reported to be associated with impaired skeletal muscle glycogen synthase enzyme (Nikoulina *et al.*, 2001). The EESP and EERP, due to the insulin-like action of their ingredients, probably increased PI-3K activation leading to stimulation of muscle glycogen synthase. The increased concentration of glycogen in skeletal and cardiac muscle also might be due to increased expression and translocation of GLUT-4 glucose transporters as a result of increased PI-3K activation, leading to increased peripheral uptake of glucose (Davis, 2006). Adrenaline produces hyperglycemia by inhibiting insulin release; stimulating glycogenolysis in muscle and thus providing substrate in the form of lactate for hepatic gluconeogenesis; stimulating glucagon secretion; and stimulating ACTH secretion which, in turn, stimulates glucocorticoid secretion from the adrenal cortex (Kraus-Friedmann, 1984). It has also been reported that adrenaline produces hyperglycemia by increasing glucose uptake from both the large and small intestine (Alada *et al.*, 2001). The test drugs significantly ($p < 0.05$) reduced the adrenaline-induced hyperglycemia probably by inhibiting adrenaline-induced stimulation of α_2 receptors in β -cells of pancreas and thus promoting further insulin release (Tripathi, 2008). On comparing the antidiabetic action of the ethanolic extracts of seed and rind of *P. granatum*, it was found that the rind exhibited better activity than the seed extract. Among the three drugs, glibenclamide was found to possess the best antidiabetic action.

CONCLUSION

Thus, it can be concluded that both the seed and rind extracts of *P. granatum* possess significant antidiabetic action, with the rind extract exhibiting better activity than the seed extract. The antidiabetic effect of these extracts may be, partly, due to their positive effect on glycogen synthesis in liver, skeletal muscle and heart muscle, and partly, due to their stimulatory action on insulin release. Further studies on molecular level are required to determine the exact mechanism of antidiabetic action after isolation of active principles from the seed and rind extracts of *P. granatum*.

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