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Contributed Paper

Insights Into the Causes and Anti-hyperglycemic Effects Of *Punica granatum* Rind in Alloxan-induced Diabetic Rats

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ABSTRACT

In the present investigation, the anti-hyperglycemic potential and scientific validation of *Punica granatum* rind/peel, a waste material and herbal drug, from Kumauni region was assessed in alloxan-induced diabetes model. The effects of oral administration of different doses *i.e* low (LP) and high (HP) (LP; 75 mg/kg and HP; 150 mg/kg body weight) of methanolic fragment of *P. granatum* peel (PGPE) on blood glucose, lipid peroxidation (MDA; malondialdehyde), antioxidant enzymes (SOD; superoxide dismutase and GPx; glutathione peroxidase) were estimated and compared with glibenclamide and insulin for 45 days. Treatment with PGPE, glibenclamide and insulin resulted in a significant reduction of blood glucose levels as compared to solvent control ($p < 0.05$). PGPE enhanced the plasma insulin level by five fold. PGPE also resulted in a significant decrease in MDA values ($p > 0.001$) in kidney (LP; 16.80 and HP; 52.08%) and an increase in antioxidants level ($p > 0.05$) such as SOD 39.68% and 75.03%, GPx 20.07% and 67.60% in plasma and SOD 44% and 66%, GPx 50% and 80% in kidney, though MDA level was not significantly decreased ($p < 0.05$) in plasma, when compared to diabetic controls. Histopathological studies also validated our studies of interest. To conclude, PGPE supplementation leads to an increase in insulin level in experimental animals, confirming restoration of pancreatic β -cells and other parameters clearly indicating the anti-hyperglycemic and antioxidant effect of PGPE.

Keywords: *Punica granatum* rind, diabetes, alloxan, oxidative stress, antioxidant enzymes, malondialdehyde

1. INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder characterized by hyperglycemia associated with microvascular and macrovascular complications including retinopathy, nephropathy, neuropathy and cardiovascular disorder [1]. Oxidative damage

has been suggested to be a contributory factor in the development and complication of diabetes [2]. Traditional antidiabetic plants might provide an useful source of new oral hypoglycemic compounds for the development as pharmaceutical entities or as sample dietary adjuncts to existing therapies[3].

The *Punica granatum* tree, which is believed to be flourished in the Garden of Eden, has been used extensively in the folk medicine of many cultures. *Punicagranatum* Linn, a fruit bearing tree belonging to the family *Punicaceae* typically grows 12-16 feet, has many spiny branches and can be extremely long lived[4]. The potential therapeutic properties of *P. granatum* are wide ranging and include treatment and prevention of cardiovascular disease, diabetes, dental problems, arthritis dysfunction and protection from ultraviolet radiation [5]. Other potential applications include infant brain ischemia, Alzheimer's disease, male infertility, arthritis, cancer prevention and obesity[4,6]. The major antioxidant potential constituents of peel are phenolic punicalagins, gallic acid, fatty acids, catechin, epigallocatechingallate, quercetin, rutin, flavanoids, flavones, flavanones, and anthocyanidins[4-7].

It is known that formation of free radicals is elevated in diabetes and its complications[8,9]. In accordance, the antioxidant potential of *P. granatum* fruit, juice and rind (peel) has been previously reported [4-6]. Methanolic fragment of *P. granatum* peel extract (PGPE) showed more potential as a health supplement natural antioxidant than the pulp extract [10]. *Punica* peel is used by traditional healers (vaidya) (49%) in India; accordingly our previous study also supported the anti-hyperglycemic effect using *in vitro* glucose oxidase method [11]. The phenolic

profile in the methanolic extracts was investigated and flavonoids, phenolic acids, their derivatives including quercetin, rutin, gallic acid, ellagic acid and punicalagin as a major ellagitannin, were reported in our previous report [9]. In view of the above consideration, the present study was designed to investigate the comparative antidiabetic efficacy of methanolic extract of *Punicagranatum* rind in alloxan-induced diabetic rats; levels of antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), insulin level and lipid per-oxidation were measured in plasma and kidney of the experimental rats along with the histopathological evaluation.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of Plant Material

P. granatum was collected from the regional market of Bhowali, Bhimtal (Uttarakhand, India) during the month of July-August and were authenticated, deposited (KU/D007) to Botany department herbarium, Kumaun University, Nainital, Uttarakhand. Plant material was dried under shade at temperature less than 40°C.

2.2 Preparation of PGPE Extract

PGPE extract was prepared as per our previous studies [12]. The dried plant material was grounded into a moderately coarse powder using domestic electric grinder. Dried powder (5kg) was extracted with methanol (1:10 *w/v*) for 6 days three-times, by hot extraction method. The filtrate was evaporated under reduced pressure, dried and used for further experiment.

2.3 Preparation of Animals

Male *wistar* albino rats (140-160gBW; body weight) were housed under standard

laboratory conditions of light and dark cycles of 7:00 am to 7:00 pm, temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $60\% \pm 1\%$ relative humidity. The animals were given standard rat pellet (Lipton India Ltd, Bangalore) and tap water *ad libitum*. The study protocols were approved by Maharani Lakshmi Ammanni College Ethical Committee, clearance from Ethical Committee (1368/ac/10/CPCSEA/BT-SKM/07), Bangalore.

2.4 Alloxan-induced Wistar Albino Rat Model for Diabetes Mellitus

Diabetes mellitus was induced by administering alloxan (180 mg/kg BW *i.p.*) in 500mM Phosphate buffer (pH 7.0) in a volume of 1ml/kg. After two days of alloxan (2, 4, 5, 6-tetraoxypyrimidine, 5, 6-dioxyuracil) injection, animals showing the fasting blood glucose (FBS) level of more than 220 mg/dl were considered as diabetes positive [8].

2.5 Experimental Groups

Male rats were randomly allotted into six groups of six animals ($n=6$) each. Group I served as normal control and received distilled water (NL). Group II served as DM control and received distilled water (DC). Group III DM animals were treated orally with methanolic fraction of PGPE at a dose of 75mg/kg/1ml/day (LP). Group IV DM animals treated orally with methanolic extract of PGPE at a dose of 150mg/kg/1ml/day (HP). Group V DM animals treated with glibenclamide at a dose of 600 $\mu\text{g/kg/day}$ (DG). Group VI DM animals treated with insulin (Insugen-N, Biocon, Bangalore, India) at a dose of 2 U/kg/day by *i.p.* route (DI). The drug treatment was carried out every day morning with the help of 16 gauge ball-tipped feeding needle for a period of six weeks.

FBS was determined weekly by blood sweeping by Accu-Chek Glucometer (Apollo Pharmacy, Bangalore, India). BW was determined everyday. After six weeks of drug treatment, antioxidant contents in plasma and kidney were evaluated.

2.6 Separation of Blood Plasma

Blood plasma was separated by collecting the blood in a test tube adding anticoagulant at room temperature for 15-30 min followed by centrifugation (RV/FM, super spin, Plasto Craft, India) at 300g for 5 min. The upper layer of plasma was carefully aspirated and employed for biochemical analyses.

2.7 Preparation of Tissue

Tissue preparation was done as per our previous studies [8]. Rats were euthanized by an overdose of anesthetic ether. The kidneys were immediately excised and chilled in ice cold 0.9% sodium chloride. The tissues were kept at -80°C until processed. The tissue was homogenized in 50 mM phosphate buffer pH (7.0). The homogenate was centrifuged at 600g for 15 min at 4°C (RV/FM, super spin, Plasto Craft, India). The supernatant was collected for analytical procedures.

2.8 Enzyme Assays

Glutathione peroxidase (GPx; *E.C.1.11.1.9*) was measured at 37°C by the method of Flohe and Gunzler (1984) [13]. Superoxide dismutase (SOD; *E.C.1.5.1.1*) was determined at room temperature according to the method of Mishra and Fredovich (1972) [14]. The total protein concentration was estimated by Lowry et al., (1951) [15] using BSA as standard.

2.9 Lipid Peroxidation

This assay was used to analyze malondialdehyde (MDA) by the method of Ohkawa et al., (1979)[16] using 1, 1, 3, 3-tetra methoxypropane (TMP) as standard.

2.10 Estimation of Insulin

Insulin level was measured by Insulin kit (Mercodia Insulin ELISA) using spectrophotometer (Elico, India)[17].

2.11 Statistical Analysis

Data were expressed as mean \pm SE. Comparison between different groups were done using one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism, version 4.0 (Graph Pad Software, San Diego, CA, USA). A significant difference was judged at a level of $p < 0.05$.

3. RESULTS

3.1 Plant Yield

The yield of the methanolic extract was 4.76% (w/w).

3.2 Effect of PGPE on Body Weight

Figure 1 illustrates the effect of various agents tested on the relative body weight of experimental animals (in grams) for a period of six weeks. Animals in alloxan-treated group (DC) showed reduced body weight as compared to solvent (water)-treated control group (NL). There was a two-to-three-fold increase

in body weight of rats treated with both the doses of PGPE, glibenclamide and insulin in comparison with diabetic control rats (DC).

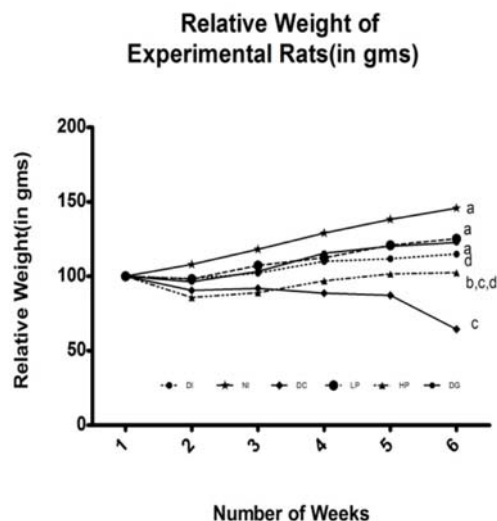


Figure 1. Effect of PGPE, glibenclamide or insulin treatment on body weight of diabetic experimental rats.

NL, Normal; DC, Diabetic Control; DG, Diabetic Glibenclamide; DI, Diabetic Insulin; LP, Low Punica peel (75 mg/kg B.W.); HP, High Punica peel (150 mg/kg B.W.). Values are mean \pm SE of six rats per group ($n=6$). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p < 0.05$. Those which are not sharing the same letters are significantly different.

3.3 Effect of PGPE on Hyperglycemia

Figure 2 summarizes the levels of glucose in normal and diabetic animals.

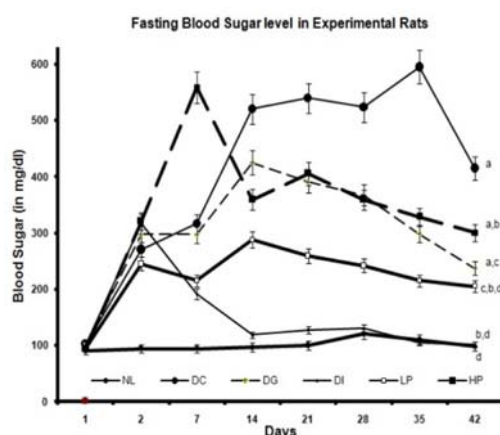


Figure 2. Effect of PGPE on blood glucose level in diabetic experimental rats.

NL, Normal; DC, Diabetic Control; DG, Diabetic Glibenclamide; DI, Diabetic Insulin; LP, Low Punica peel (75 mg/kg B.W.); HP, High Punica peel (150 mg/kg B.W.). Values are mean \pm SE of six rats per group ($n=6$). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p<0.05$. Those which are not sharing the same letters are significantly different.

In all the groups prior to alloxan administration, the basal blood glucose levels of the rats were not significantly different. However, there was a significant elevation in glucose after 72 hours of alloxan administration. After alloxan administration, significant ($p<0.05$) increase in blood glucose was observed in DC when compared to NL rats. Although an anti-hyperglycemic effect was evident from the first week onwards, the significant decrease in blood glucose was going through up to six weeks in PGPE group, receiving high dose. The administration of PGPE (Groups III and IV) significantly ($p<0.001$) decreased glucose level in diabetic rats as compared with diabetic control rats. Thus, the levels of blood glucose returned to near normal range in diabetic rats treated with PGPE. Moreover, the effect of high

PGPE was more significant than low PGPE dose.

3.4 Changes in Plasma Insulin Level

Plasma insulin level was significantly declined in diabetic control group treated with alloxan (figure 3). But, there were approximately three-to-five-fold significant increase in the insulin levels of experimental animals treated with lower and higher dosages of PGPE ($p<0.05$) respectively, as compared to solvent control rats.

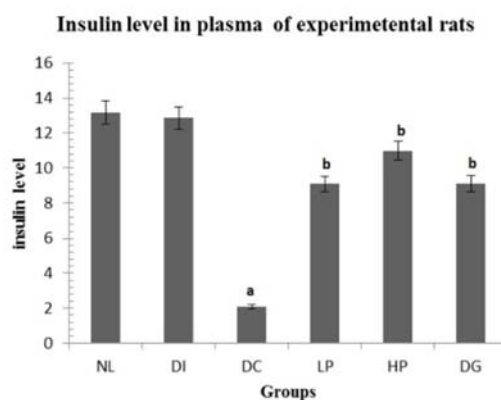


Figure 3. Effect of supplementation of PGPE on insulin level in plasma of diabetic experimental rats.

NL, Normal; DC, Diabetic Control; DG, Diabetic Glibenclamide; DI, Diabetic Insulin; LP, Low Punica peel (75 mg/kg B.W.); HP, High Punica peel (150 mg/kg B.W.). Values are mean \pm SE of six rats per group ($n=6$). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p<0.05$. Those which are not sharing the same letters are significantly different.

3.5 Antioxidant Enzymes

The concentrations of antioxidant enzymes (SOD and GPx) in normal and experimental rats were presented in table 1 for kidney and figure 4 and 5 for plasma, respectively. There were significant decrease

of these enzymes in plasma and kidney in diabetic control group. Lower and higher dosage recovered the antioxidant level such as SOD 39.68% and 75.03%, GPx 20.07% and 67.60% in plasma and SOD 44% and 66%, GPx 50% and 80% in kidney

respectively. Oral administration of higher dose (150mg/kg BW) of PGPE showed a significant effect and brought back all the above parameters to near normal values, comparable to that of insulin.

Table 1. Effect of supplementation of PGPE on SOD, GPx and MDA levels in kidney of diabetic rats.

	SOD (U/mg protein)	GPx(μ g GSH consume/ mg protein)	MDA (nM/mg protein)
NL	18.34 \pm 2.02	6.57 \pm 2.02	1.01 \pm 0.12
DI	13.56 \pm 1.15 ^a	5.03 \pm 1.15 ^a	1.640 \pm 0.15 ^a
DC	7.61 \pm 1.72 ^b	2.84 \pm 1.72 ^b	3.09 \pm 0.32 ^b
LP(75 mg/kg)	10.63 \pm 0.67 ^c	3.41 \pm 0.67 ^{ab}	1.79 \pm 0.47 ^b
HP(150 mg/kg)	13.32 \pm 1.93 ^a	4.76 \pm 1.93 ^a	1.59 \pm 0.13 ^a
DG(600 μ g/kg)	12.30 \pm 1.74 ^a	3.67 \pm 1.74 ^{ab}	1.65 \pm 0.17 ^a

NL, Normal; DC, Diabetic Control; DG, Diabetic Glibenclamide; DI, Diabetic Insulin; LP, Low Punica peel (75 mg/kg B.W.); HP, High Punica peel (150 mg/kg B.W.). Values are mean \pm SE of six rats per group (n=6). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p < 0.05$. Those which are not sharing the same letters are significantly different.

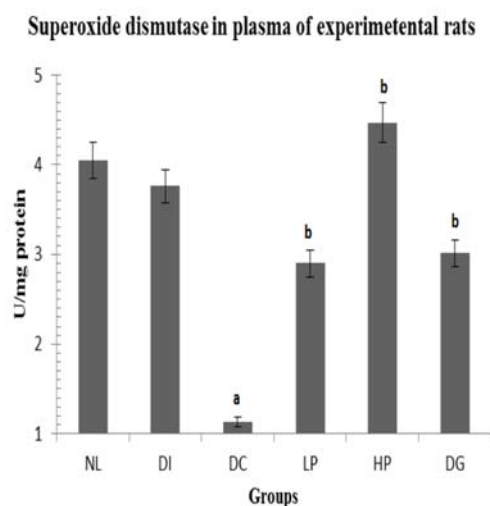


Figure 4. Effect of supplementation of PGPE on superoxide dismutase (SOD) in plasma of diabetic experimental rats.

NL, Normal; DC, Diabetic Control; DG, Diabetic Glibenclamide; DI, Diabetic Insulin; LP, Low Punica peel (75 mg/kg B.W.); HP, High Punica peel (150 mg/kg B.W.). Values are mean \pm SE of six rats per group (n=6). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p < 0.05$. Those which are not sharing the same letters are significantly different.

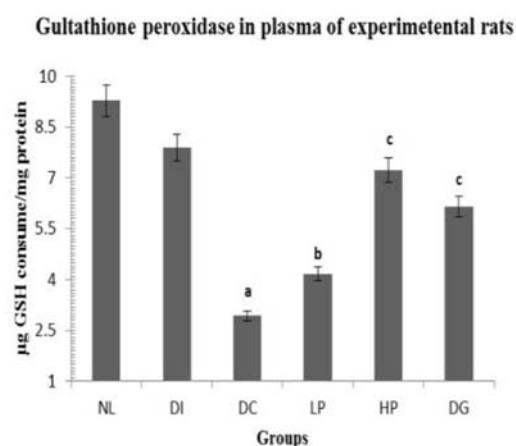


Figure 5. Effect of supplementation of PGPE on superoxide dismutase (SOD) in plasma of diabetic experimental rats.

NL, Normal; DC, Diabetic Control; DG, Diabetic Glibenclamide; DI, Diabetic Insulin; LP, Low Punica peel (75 mg/kg B.W.); HP, High Punica peel (150 mg/kg B.W.). Values are mean \pm SE of six rats per group (n=6). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p < 0.05$. Those which are not sharing the same letters are significantly different.

3.6 Lipid Proxidation

Table 1 and figure 6 provide the concentration of MDA level in different test sample of normal and experimental animals.

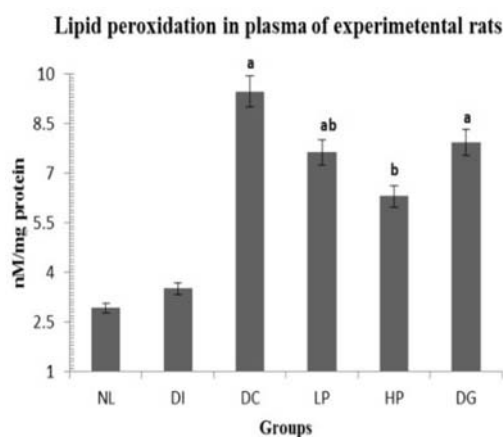


Figure 6. Effect of supplementation of PGPE on lipid peroxidation (MDA) in plasma of diabetic experimental rats.

NL, Normal; DC, Diabetic Control; DG, Diabetic Glibenclamide; DI, Diabetic Insulin; LP, Low Punica peel (75 mg/kg B.W.); HP, High Punica peel (150 mg/kg B.W.). Values are mean \pm SE of six rats per group (n=6). Statistical analysis was done by one-way ANOVA between groups and values were considered significant at $p < 0.05$. Those which are not sharing the same letters are significantly different.

There was a significant decrease in MDA level in kidney (LP; 16.8 and HP; 52.08%) and no significant decrease found in plasma (LP; 42.07% and HP; 48.54%) ($p < 0.001$) when compared with the corresponding control group. Administration of PGPE at 150mg/ml/Kg BW, insulin and glibenclamide tends to bring the value to near normal. PGPE at the dose of 75 mg/ml/Kg BW did not show any significant effect. PGPE at 150 mg/Kg BW was more effective than glibenclamide.

3.7 Histopathological Studies

As shown in Figure 7, in the diabetic group, degeneration with reduction in the number and reduced dimensions of the islets were observed in the pancreas. Experimental rats treated with LP (C) refurbished less number of β cell region, however, HP (D) showed a significant increase in the cellular population, size of the islets and in the number of islets also illustrated as compared to DC (B). The rejuvenation of β cells were comparable to DG group (E).

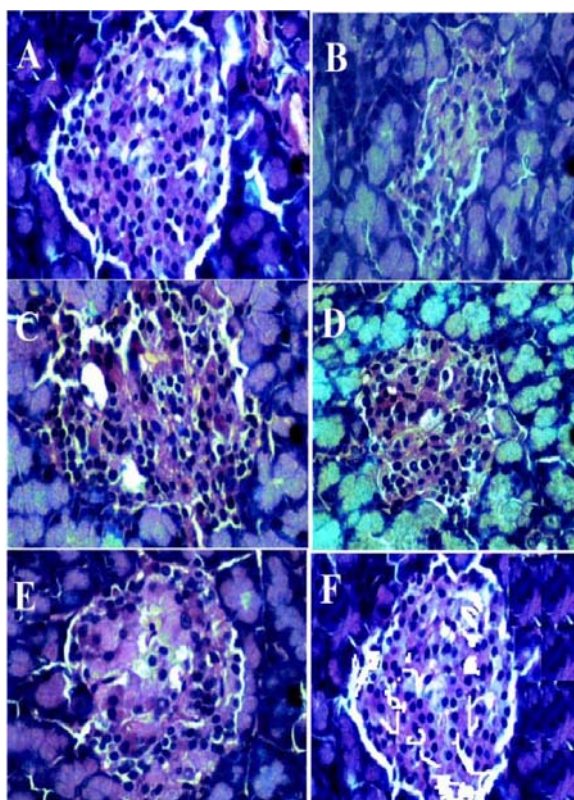


Figure 7. Histopathological evaluation of pancreas stained by H and E stain of (A) normal, (B) control diabetic, effects of ethanolic extract of *Punica granatum* (C) 75 mg/kg ethanolic extract, (D) 150 mg/kg, and (E) glibenclamide, and (F) Insulin experimental rats, Microscope magnification (40x).

4. DISCUSSION

Diabetes mellitus is possibly the world's largest growing metabolic disorder and as the knowledge on the heterogeneity of

this disorder is advanced, the need for more appropriate therapy increases [3,18]. Recent data commended that 80% of the drug molecules are either used traditionally or

derived from the natural sources[19,20]. The study of such medicines might offer a natural key to unlock a diabetologist's pharmacy for the future[3,21]. Hence, this *in-vivo* study was designed to assess the antioxidant effect of PGPE on diabetes induced in rats. For this study, a widely used diabetic model of alloxan-induced hyperglycemia in rodents was considered [8]. The cytotoxic action of alloxan, a diabetogenic agent is mediated by reactive oxygen species. Dialuric acid, a byproduct of alloxan reduction, establishes a redox cycle forming superoxide radicals that undergo dismutation to hydrogen peroxide which generates highly reactive hydroxyl radicals by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of β -cells[22].

Like the glibenclamide, PGPE also produced significant reduction in blood glucose levels of alloxan-treated diabetic rats. Augmented insulin level explicates the effect of PGPE on pancreas. The possible mechanism by which PGPE brings about its hypoglycemic action may be by potentiation of the insulin effect of plasma by increasing either the pancreatic secretion of insulin from β -cells of islets of Langerhans or its release from the bound form. Our study is in accordance to previous studies by Parmar and Kar (2007)[23]. Their study also revealed the histopathological studies of the pancreas, indicated improved volume density of islets and improved percentage of β -cells, of the rats that supplemented with peel, which may be a sign of regeneration potential of the extract. A higher dose of the extract has a greater impending recuperative effect on the islet cells of the animals than a lower dose of PGPE. The body weight was decreased in alloxan-induced diabetic rats [24,8]. Administration of PGPE improved

the body weight in alloxan diabetes. The ability of PGPE to protect body weight loss seems to be a result of its ability to reduce hyperglycemia.

The PGPE had markedly higher antioxidant capacity than citrus peel [24]. Oxidative stress in diabetes may result from overproduction of precursors for reactive oxygen species and/or lowered efficiency of scavenging and inhibitory systems. SOD and GPx are the two major antioxidant enzymes that remove the toxic free radicals *in vivo*. Increased production of free radicals is associated with depletion of antioxidants.

In our study, we have also observed decrease activities of GPx and SOD in diabetic rats as compared to solvent-control rats. A decreased level of these antioxidants can lead to excessive accumulation of superoxide and peroxy radicals, which in turn generate hydroxyl radical resulting in the initiation and propagation of lipid peroxidation[8, 23]. This is in agreement with other reports which showed that the antioxidant system was suppressed by alloxan administration [22, 25]. In the present study, the observed increased concentration of MDA in plasma and kidney of alloxan-treated rats may be due to oxidative damage induced by alloxan. This observation is consistent with our earlier reports [8, 9, 25].

Our previous studies showed the *Punica* peel is rich in several antioxidants like pelargonidin-3-glucoside, rutin and quercetin [9, 26]. Reduction of blood glucose and MDA level would be because of these antioxidants. Supplementation of PGPE to diabetic rats daily for a period of 45 days resulted in a significant decrease in MDA and a significant increase in GPx and SOD levels. Previous phytochemical analysis indicated a high content of total polyphenol in the methanolic extract of PGPE [9, 28], which might be related to the

antidiabetic and antiperoxidative effects of the test peels [25-29].

5. CONCLUSION

Given the potential effects of the PGPE extracts in comparison with the modulated effects seen with glibenclamide-treated groups, further isolation of the major effective phyto-constituents from this extract might provide us with a valuable natural derivative for the effective treatment of diabetes.

Finally to conclude, PGPE exhibited significant hypoglycemic activities in diabetic rats. The methanolic fraction of PGPE also showed an improvement in insulin level which would be an indication of regeneration or mimicking of β -cells of pancreas and so might be of value in treatment of diabetes. Mechanism centered studies are needed to understand the mode of action of PGPE.

6. ACKNOWLEDGMENTS

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AUTHORS DISCLOSURE STATEMENT

Authors declare no conflict of interest.

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