INHIBITION OF DPP-IV ACTIVITY AND ENHANCEMENT OF GLP-1 EXPRESSION BY AQUEOUS PEEL EXTRACT OF *PUNICAGRANATUM*IN ALBINO WISTARRATS

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ABSTRACT

The aim of the present study was to determine theeffect of aqueouspeelextract of *Punicagranatum* (*P. granatum*) onantihyperglycemia, *in vitro*and *in vivo*antioxidant, intestinal DPP-IV and GLP-1RmRNA expression levels in alloxan induced diabeticrats.The *P. granatum* extract (50 and100 mg/kg body weight) was administered orally once a day for 2 weeks after the animals were confirmed diabetic.On end of the experimental period,biochemical parameters and histology of the pancreas were investigated.A significant increase in blood glucose, glycosylated hemoglobin (HbA1c) with decrease serum insulin and total protein levels were observed in diabetic rats. Treatment with *P. granatum* extract reduced the elevated levels of blood glucose, HbA1cwith significant increase in insulin and total proteins levels in diabetic rats. Significant decrease in malondialdehyde with increase in the levels of superoxide dismutase, catalase, glutathione peroxidase in the pancreas of diabetic rats, they were reversed in *P. granatum* extract treated diabetic rats. *P. granatum* treatment showed the pancreatic β-cells regeneration. Intestinal DPP-IV mRNA expression levels were elevated in diabetic ratswith decreased GLP-1RmRNAexpression levels. However, in *P. granatum* treated diabetic rats, DPP-IV levels were decreased with corresponding increase in GLP-1R levels. This indicates *P. granatum* has inhibitory effects on DPP-IV enzyme which is further responsible for elevated levels of GLP-1R activity. It concludes that *P. granatum*has significant antioxidant, reduction in blood glucose levels and DPP-IV inhibitory activities, which may be due the presence of high bioactive compounds in *P. granatum* extract.

Key words: Diabetes; Blood glucose; Dipeptidyl peptidase IV; Glucagon-like peptide 1; *Punicagranatum*.

INTRODUCTION

Dipeptidyl peptidase-IV (DPP-IV, E.C.3.4.14.5) is a plasma membrane glycoprotein exopeptidase with very broad substrate specificity that cleaves of N-terminal dipeptides from polypeptides with L-proline or L-alanine residues situated at the penultimate position of the substrates (De Meester et al., 2003). In addition, DPP-IV has been shown to cleave many hormones such as glucagon like peptide 1 (GLP-1) (Mentle in, 2004).GLP- 1 is an incretin hormone that contributed to the control of blood glucose levels via potential to induce insulin secretion from the islet β-cells in a glucose-dependent manner (MacDonald et al., 2002). GLP-1 may also mediate its effects on glucose-induced stimulation of insulin biosynthesis and secretion, inhibition of glucagon secretion, β-cell proliferation and survival (Deacon, 2005). Further important effects of GLP-1 include inhibition of gastrointestinal secretion and motility and food intake (Naslund et al., 1999). However, the half life of GLP-1 is very short (1-2 min) due to the cleavage and inactivation of these proteins by dipeptidyl peptidase IV (DPP IV). Hence, the use of DPP-IV inhibitors increases the GLP-1

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time of action (Stephan et al., 2011). There are several synthetic DPP-IV inhibitors available in the market such as sitagliptin, vildagliptin, saxagliptin and alogliptin, but these upon long term usage reported to cause adverse effects like pancreatitis, angiodema and infective disorders (Matteucci & Giampietro, 2011). In order to prevent the risk associated with these synthetic drugs, natural substances came into existence for promoting better health with minimal (or) no side effects (Siró et al., 2008).

Punicagranatum Linn.(*P. granatum*), commonly known as pomegranate, is a shrub or a small tree, native to the Mediterranean region. The various parts of the plants are used in folklore medicine forthe treatment of various diseases, such as ulcer, hepatic damage, snakebite, etc. The peel of the fruit is antihelminthic, useful indysentery and ulcer (Lansky and Newman, 2007).The plant also possess a number of biological activities such as antitumor (Khan.,2009), antibacterial (Fawoleet al., 2012), antidiarrhoeal (Das et al., 1999), antifungal (Abdollahzadeh et al., 2011), antiulcer (Elwakil, 2011).Furthermore, antioxidant activity accompanied with anti-inflammatory, anti-cholinesterase and cytotoxic activities properties of *P. granatum* has been demonstrated recently (Bekir et al., 2013).There is established claim that the *P. granatum*peel reduces the blood glucose levels, but the exact mechanism is not yet known (Parmar and Kar, 2007). So, in the present study, the DPP-IV inhibitory activity of *P. granatum* was evaluated*,* which might be the reason for the reduction of blood glucose through increasing the GLP-1 levels.

MATERIALS AND METHOD
Chemicals and drugs

Metformin, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St.Louis, MO, USA).Glucose, total protein andHbA1cestimated kits were procured from Span Diagnostics, Surat, India. Plasma insulin was estimated by using an enzyme linked immunosorbent assay (ELISA) kit (Roche diagnostics, Germany). TRIzol reagent was purchased from GeNei, Bangalore, India. Taq DNA polymerase was acquired from Invitrogen (Carlsbad, CA, USA). All other chemicals and reagents used were of analytical grade.

Preparation of aqueous extract

To obtain the aqueous extract of *P. granatum*peels, 200 g of the air-dried peel was boiled in 5 L of distilled water for 30 min with continuous stirring. The resultant solution was filtered through a filter paper. The filtrate was completely evaporated under reduced pressure at 60ºC (yield 3 g) and stored in refrigerator.

Preliminary phytochemical screening of the *P. granatum*peel extract

The preliminary phytochemical analysis was carried out the *P. granatum*peelextracts using standard phytochemical methods described by Trease and Evans, (2001).

IN VITRO ANTIOXIDANT ACTIVITIES

The DPPH scavenging activity of aqueous *P. granatum*peel extractwas measured according to the method of Liu and Zhao, (2006). The ability of the *P. granatum*to scavenge hydrogen peroxide was determined according to the method of Ruch*et al.*, (1989). According to Winterbourn and Sutton, (1984) hydroxyl radical scavenging activity was calculated. Scavenging activity of superoxide anion radical was determined by the method of Stewart and Bewley (1980). The nitric oxide scavenging activity was calculated according to the method of Marcocci et al., (1994).The reducing power of the extracts was determined by the method of Oyaizu (1986).

Animals

Adult albino Wistar rats (180 ± 10 g), Swiss albino mice (18-20 g bw) were obtained from the Mahaveer Enterprizes, Hyderabad, India. They were kept under temperature of (23 ± 2) ºC, humidity of 50 % and 12 h: 12 h of light and dark cycles, respectively. They were fed with a Commercial pellet diet (Rayon's Biotechnology Pvt Ltd, India) and water was provided *ad libitum*. The prior approval for conducting the experiments in rats was obtained from our Institutional Animal Ethics Committee and our lab is approved by CPCSEA, Government of India (Regd. No. 516/01/A/CPCSEA).

Acute toxicity studies:

Healthy adult albino mice of either sex, starved overnight were divided into five groups (n=6). They were orally fed with the *P. granatum* aqueous peel extract in increasing dose levels of 100, 500, 1000, 1500, 2000 mg/kg b.wt.(Patel et al., 2008). The animals were observed continuously for 72 h for any signs of behavioural, neurological and autonomic profile, toxicity and mortality.

Induction of diabetes

Diabetes was induced by the administration of alloxan, at a dose of 150 mg/kg body weight through intraperitoneal route in rats after being deprived of food for 18 hours to induce hyperglycemia (Das and Barman, 2012). The blood glucose levels in the animals were measured at 72 hours after the drug administration and those rats with fasting blood glucose levels greater than 250 mg/dl were considered to be diabetic and were used in the experiment (Kim and Lim, 2013).

Experimental design

The rats were divided into five groups; each group contains 6 animals as follows
Group 1: Normal control rats
Group 2: Diabetic control rats
Group 3: Diabetic rats treated with 50 mg/kg bw of *P. granatum*
Group 4: Diabetic rats treated with 100 mg/kg bw of *P. granatum*
Group 5: Diabetic rats treated with 100 mg/kg bw of metformin

P. granatum was administered once daily for a period of 2 weeks. During the experimental period body weight, food and water intake was estimated. While, the fasting blood glucose levels were measured at 0, 7, 14[th]days by glucose oxidase (GOD) and peroxidase (POD) endpoint method using commercially available kits (Span diagnostics LTD, Surat, India). By the end of 2nd week blood samples were collected from the retro-orbital plexus and used for biochemical analysis.

Biochemical estimation

Serum total protein levels were estimated by using kits (Span Diagnostics, Surat, India).Plasma insulin levels were estimated by using an enzyme linked immunosorbent assay (ELISA) kits (Roche diagnostics, Germany). 2 ml of blood was collected in the tubes containing potassium oxalate and sodium fluoride as anticoagulants for the estimation of glycosylated hemoglobin (HbA1c) in Semi-auto analyzer (Screen master-3000).

Histopathological studies

A portion of pancreatic tissue was dissected out and fixed at 10% buffered neutral formal saline and processed. After fixation, tissues were embedded in paraffin. Fixed tissues were cut at 5 μm and stained with hematoxylin and eosin. The sections were estimated under phase-contrast microscope (Nikon phase contrast 0.9 dry, Japan, NIS- Elements D) and photographs were taken.

Determination of lipid peroxidation and antioxidant enzymes in the pancreas

The pancreas was isolated and cut into small pieces, place in chilled 0.25 M sucrose solution and blotted on a filter paper. The tissues were then homogenized in 10 % chilled Tris hydrochloride buffer (10 mM, pH 7.4) by tissue Homogenizer (Remi Motors, Mumbai, India) and centrifuged at 12000rpm for 15 min at 0ºC using cooling centrifuge (R-247, Refrigerated Centrifuge, Mumbai, India) Lipid peroxidation (LPx) was estimated in terms of malondialdehyde (MDA) content and determined by using the thiobarbituric acid (TBA) by the method of Hiroshi et al. (1979). The activity was expressed as μmol of malondialdehyde formed/g wet weight of tissue. Superoxide dismutase (SOD) (EC 1.15.1.1) was assayed for its ability to inhibit the auto-oxidation of epinephrine in alkaline medium (Misra and Fridovich, 1972). The SOD activity levels were expressed in units per mg protein per min. Catalase (EC 1.11.1.6) was assayed by the method of Maehly and Chance (1954) by determining the decrease in the concentration of hydrogen peroxide (H_2O_2).

The activity of the enzyme was expressed in μmol of hydrogen peroxide (H_2O_2) metabolized/mg protein/min. Glutathione peroxidase (GPx) was assayed by the method of Rotruck et al. (1973). The activity was expressed as μmol of GSH consumed/mg protein/min. The protein concentration was determined by using bovine serum albumin as the standard (Lowry et al., 1951).

Total RNA extraction and reverse transcription and polymerase chain reaction

Total RNA was isolated from the intestinal samples using TRIzol reagent (GeNei, Bangalore, India) according to manufactures instructions. The quality of RNA was confirmed by formaldehyde gel in comparison with 28S and 18S RNA. The RNA concentration was measured at 260 nm using a UV spectrophotometer (UV-1800 UV-Vis Spectrophotometer, Japan). The RNA pellet was dissolved in diethylpyrocarbonate (DPEC) -treated water. cDNA was synthesized using 9μL of total RNA and reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Polymerase chain reaction (PCR) was performed ona Thermal Cycler PCR Machine (Model No.LT- 240)using Taq DNA polymerase with the following thermal cycle profiling: initial denaturation at 95°c for 5 min followed by 30 cycles (Denaturation at 95°c for 5 min, annealing at 60°c for 30 Sec, renaturation at 72°c for 30 Sec, and extension at 72°c for 10 min).

The following primer sets were used to amplify DPP-IV, GLP-1 (Liu et al., 2004; Jian Yang et al., 2007). GAPDH gene was used as a housekeeping gene. All primers were ordered from GeNei, Bangalore, India.
DPP-IV
Forward: 5′-CTCCAGAGGACAACCTTGAC-3′
Reverse: 5′-GGACAAGTGTGCTCTTGAGT-3′
GLP-1R
Forward: 5'-GGCTGTCTTGTACTGCTTTGTC-3′
Reverse: 5'-ATGCCTGTTTGATAGGTTTGAG-3′
GAPDH
Forward: 5′-CTGAGAATGGGAAGCTGGTCAT-3′
Reverse: 5′-TGGTGCAGGATGCATTGCT-3′

The PCR products were detected by electrophoresis on 2%agarose gels containing ethidium bromide. The relative intensities of the DPP-IV, GLP-1 bands were normalized to the corresponding GAPDH band intensities.

Statistical analysis

Values are expressed in mean ± SD. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 13.5 (SPSS Inc, Chicago, IL, USA) and the individual comparisons were obtained by Duncan's multiple range test. Differences were considered to be significant at $p < 0.01$.

RESULTS

Phytochemical analysis of *P. granatum* peelaqueous extract

The preliminary phytochemical screening of aqueous peel extract of *P. granatum* showed the presence of glycosides, carbohydrates, tannins, alkaloids, and flavonoids were identified.

Effect of *P. granatum* on *in vitro* anti oxidant activity

The *in vitro* antioxidant activity of *P. granatum* was shown in table 1. The various concentrations of *P. granatum* and standard ascorbic acid showed antioxidant activity in a dose dependent manner. The IC_{50} values of *P. granatum* for scavenging of DPPH, hydroxyl, superoxide radical, nitric oxide, hydrogen peroxide and reducing power scavenging activity of *P. granatum* extract were 33.26 ± 0.8, 6.65 ± 0.5, 85.4 ± 1.2, 100.41 ± 0.4, 194.85 ± 0.9 and 14.57 ± 1.6 μg/ml respectively.

Effect of *P. granatum* on body weights, food and water intake and organ weight in diabetic rats

The changes of body weight, food and water intake and organ weight in normal and alloxan induced diabetic rats were shown in Table 2. A significant ($p < 0.05$) decrease in body weight was observed in alloxan induced diabetic rats when compared to the normal control rats. *P. granatum* peel extract and metformin administered rats showed progressive increase in body weight.

The diabetic group showed a significant (p<0.05) increase in food and water intake as compared to normal rats, whereas in *P. granatum* extract and metformin treated diabetic rat food and water intake was marked fluctuations was observed when compared with diabetic rats. A statistically significant (p<0.05) reduction in relative pancreatic weight was observed in diabetic rats when compared with normal rats, but those treated with *P. granatum* peel extract had higher pancreatic weight than control rats.

Effect of *P. granatum* on blood glucose, insulin, HbA1cand total protein levels

Alloxan induced diabetic rats showed significant increase in blood glucose and HbA1c levels when compared with controls (Tables 3 and 4). Whereas *P. granatum* and metformin treated diabetic ratsblood glucose and HbA1c levels were significantly (p<0.05) decreased. In diabetic rats insulin and total proteins were significantly (p<0.05) decreased when compared to normal rats, whereas in *P. granatum* peel extract treated diabetic rat insulin and total protein levels were significantly (p<0.05) increased when compared to diabetic rats.

Effect of *P. granatum* on lipid perioxidatin and antioxidant enzyme levels in pancreas

The MDA contents were significantly (p<0.05) increased in the pancreas of diabetic rats when compared to that of normal control rats. However, *P. granatum* peel extract and metformin treated diabetic rats showed significant (p<0.05) declinein MDA contents when compared to diabetic rats.

The antioxidant enzymes like SOD, catalase, GPx levels were significantly (p<0.05) decrease in pancreas of diabetic rats. Furthermore, *P. granatum* peel extract and metformin treated diabetic rats showed significant (p<0.05) increase in SOD, catalase, GPxactivity levels when compared to diabetic rats (Table 5).

Effect of *P. granatum* on intestinalDPP-IV and GLP-1R mRNA expression levels

Alloxan induced diabetic rats significantly (p<0.05) increasedin intestinal DPP-IVmRNA levels as compared to control rats.Whereas, in *P. granatum* and metformin treated diabetic rats, DPP-IV levels were significantly decreased (Fig. 1) when compared with diabetic rats. The intestinalGLP-1RmRNA levels were significantly (p< 0.05) decreased in diabetic rats when compared to control rats. However, in *P. granatum* and metformin treated diabetic rats GLP-1R levels were significantly (p< 0.05) increased when compared with diabetic rats (Fig. 2).

Effect of *P. granatum* on histopathology of the pancreas in normal and diabetic rats

Histology of the islets of Langerhans of normalcontrol rats showed no pathological changes. They showed several rounds to elongated, normal islets of Langerhans in the histology of the pancreas (Fig.3A). In the alloxan induced diabetic rats, the islet morphology was altered with vacuoles and only a few islets were present in tissue sections (Fig.3B).

The *P. granatum* (50 mg/kg) treated diabetic group rats showed mild to moderate degree of necrosis of the islets of Langerhans*P. granatum* (Fig.3C).

Histopathological finding of pancreas ofdiabetic animals treated with *P. granatum*at a dose of 100 mg/kg B.W revealed improvement in pancreatic damage, it shows slight vacuolar degeneration and there was marke dimprovement in islets structure (Fig.3D). Histopathological study of pancreatic tissues indiabetic rats treated with metforminrevealed slight improvement after 14 days (Fig.3E).

Table 1: Effect of *Punica granatum* peel extract on *in vitro* antioxidant activities

Activity	IC_{50} Values (µg/ml)	
	Punica granatum	Ascorbic acid
DPPH radical scavenging activity	33.26 ± 0.8	59.2 ± 0.5
Superoxide radical scavenging activity	85.4 ± 1.2	35.75 ± 1.5
Hydroxyl radical scavenging activity	6.65 ± 0.5	33.05 ± 0.9
Hydrogen peroxide radical scavenging activity	194.85 ± 0.9	491.0 ± 0.9
Nitric oxide radical scavenging activity	100.41 ± 0.4	67.61 ± 0.6
Reducing power	14.57 ± 1.6	6.33 ± 1.3

Values are mean ± S.D three replicates

Table 2: Effect of *Punica granatum* peel extract on body weight, pancreas weight, food and Water intake in normal and alloxan-induced diabetic animals.

Groups	Changes in body weight (g)		Water intake (ml/rat per day)	Food intake (g/rat per day)	Pancreas weight (g/100 g. B.W.)
	Initial	Final			
Normal control rats	185.35[a] ± 6.87	197.64[a] ± 8.63	42.43[a] ± 4.26	0.41[a] ± 0.05	0.75[a] ± 0.03
Diabetic control rats	188.21[a] ± 7.54 (1.54)	166.37[b] ± 9.36 (-15.82)	83.28[b] ± 9.44 (96.27)	0.28[b] ± 0.08 (-31.71)	0.43[b] ± 0.05 (-42.66)
Diabetic + *P. granatum* (50 mg/kg)	193.56[a] ± 4.73 (4.42)	210.79[a,c] ± 8.46 (6.65)	67.43[c] ± 7.64 (58.92)	0.45[a] ± 0.03 (9.75)	0.61[c] ± 0.06 (-18.66)
Diabetic + *P. granatum* (100 mg/kg)	190.46[a] ± 6.27 (2.75)	214.67[c] ± 7.56 (8.61)	55.67[d] ± 6.22 (31.20)	0.58[c] ± 0.04 (41.46)	0.69[a,c] ± 0.05 (-8)
Diabetic + Metformin (100 mg/kg)	183.57[a] ± 5.25 (-0.96)	202.44[a] ± 5.46 (2.42)	52.23[a,d] ± 5.74 (23.09)	0.44[a] ± 0.06 (7.31)	0.73[a] ± 0.08 (-2.67)

Values are mean ± S.D of six rats in each group.
Values in parentheses are percent change from that of control.
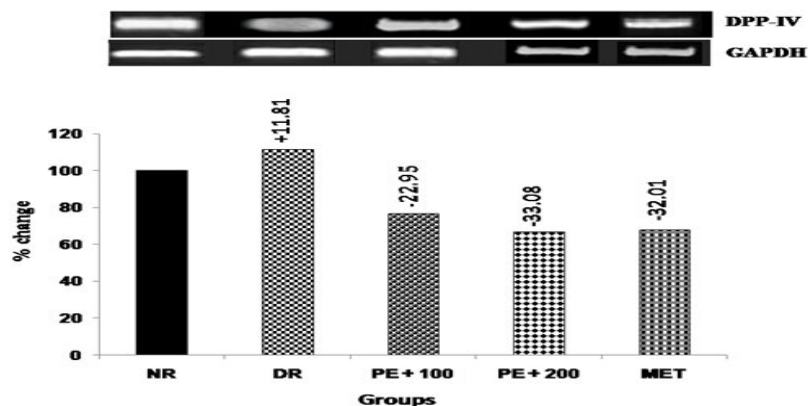Values not sharing a common superscript differ significantly with each other (p<0.05).

Figure 1: Effect of *P. granatum* peel aqueous extract on DPP-IV mRNA expression levels in normal and diabetic rats.

Table 3: Effect of *Punica granatum* peel extract on blood glucose levels in normal and alloxan induced diabetic rats

Groups	Average glucose values (mg/dl)		
	0th day	7th day	14th day
Normal control rats	83.75[a] ± 2.57	85.64[a] ± 3.73	90.53[a] ± 3.64
Diabetic control rats	332.25[b] ± 10.48 (296.71)	328.75[b] ± 11.92 (283.87)	337.25[b] ± 12.52 (272.52)
Diabetic + *P. granatum* (50 mg/kg)	338.25[b] ± 12.11 (303.88)	290.13[c] ± 9.71 (238.77)	181.75[c] ± 7.04 (100.76)
Diabetic + *P. granatum* (100 mg/kg)	342.75[b] ± 11.02 (309.25)	224.67[d] ± 13.83 (162.34)	132.48[d] ± 8.32 (46.33)
Diabetic + Metformin (100 mg/kg)	329.75[b] ± 10.96 (293.73)	203.75[e] ± 11.37 (137.91)	146.25[d] ± 8.26 (61.54)

Values are mean ± S.D of six rats in each group.
Values in parentheses are percent change from that of control.
Values not sharing a common superscript differ significantly with each other ($p < 0.05$).

Table 4: Effect of *P. granatum* peel extract on intestine insulin, total hemoglobin, Glycosylated hemoglobin and total proteins in normal and diabetic rats.

Groups	Plasma insulin (uIU/mL)	Glycosylated hemoglobin (%)	Total Protein (g/dL)
Normal control rats	5.18[a] ± 0.21	3.42[a] ± 0.14	14.31[a] ± 0.86
Diabetic control rats	3.22[b] ± 0.54 (-37.83)	9.65[b] ± 0.56 (182.16)	53.24[b] ± 2.57 (272.04)
Diabetic + *P. granatum* (50 mg/kg)	4.26[c] ± 0.53 (-17.76)	6.84[c] ± 0.68 (100)	34.5[c] ± 2.16 (141.16)
Diabetic + *P. granatum* (100 mg/kg)	4.73[a,c] ± 0.34 (-8.68)	5.08[d] ± 0.25 (48.53)	29.67[d] ± 2.44 (107.33)
Diabetic +Metformin (100 mg/kg)	5.04[a] ± 0.27 (-2.7)	3.36[a] ± 0.18 (-1.75)	26.47[d] ± 1.81 (84.97)

Values are mean ± S.D of six rats in each group.
Values in parentheses are percent change from that of control.
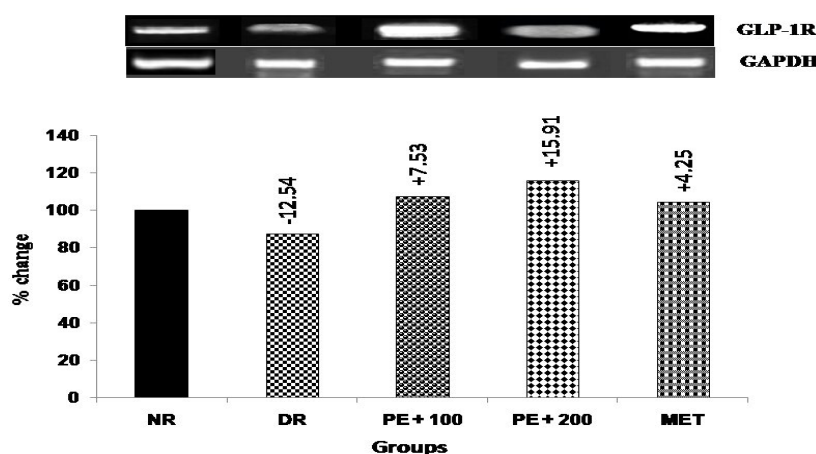Values not sharing a common superscript differ significantly with each other ($p < 0.05$).

Figure 2: Effect of *P. granatum* peelaqueous extract on GLP-1R mRNA expression levels in normal and diabetic rats

Table 5: Effect of *P. granatum* on lipid peroxidation, superoxide dismutase, catalase and Glutathione peroxidase in STZ induced diabetic rat pancreas.

Groups	Lipid peroxidation (μ moles of malondialdehyde formed/g wet wt)	Superoxide dismutase (Units/mg protein/min)	Catalase (μ moles of H_2O_2 metabolised/mg protein/min)	Glutathione peroxidase (μ mole NADPH oxidized per min/g net wt tissue)
Normal control rats	$6.53^a \pm 1.65$	$1.68^a \pm 0.06$	$0.64^a \pm 0.06$	$1.26^a \pm 0.08$
Diabetic control rats	$18.54^b \pm 2.08$ (183.92)	$0.98^b \pm 0.11$ (-41.66)	$0.31^b \pm 0.07$ (-51.56)	$0.92^b \pm 0.06$ (-26.98)
Diabetic + *P. granatum* (50 mg/kg)	$15.65^{b,c} \pm 2.36$ (139.66)	$1.38^c \pm 0.15$ (-17.85)	$0.46^c \pm 0.07$ (-28.12)	$1.13^c \pm 0.03$ (-10.31)
Diabetic + *P. granatum* (100 mg/kg)	$11.57^c \pm 3.45$ (77.18)	$1.52^{a,c} \pm 0.13$ (-9.52)	$0.55^{a,c} \pm 0.09$ (-14.06)	$1.19^{a,c} \pm 0.06$ (-5.55)
Diabetic + Metformin (100 mg/kg)	$9.49^a \pm 2.83$ (45.32)	$1.48^{a,c} \pm 0.12$ (-11.9)	$0.56^{a,c} \pm 0.08$ (-12.5)	$1.24^a \pm 0.05$ (-1.58)

Values are mean ± S.D of six rats in each group.
Values in parentheses are percent change from that of control.
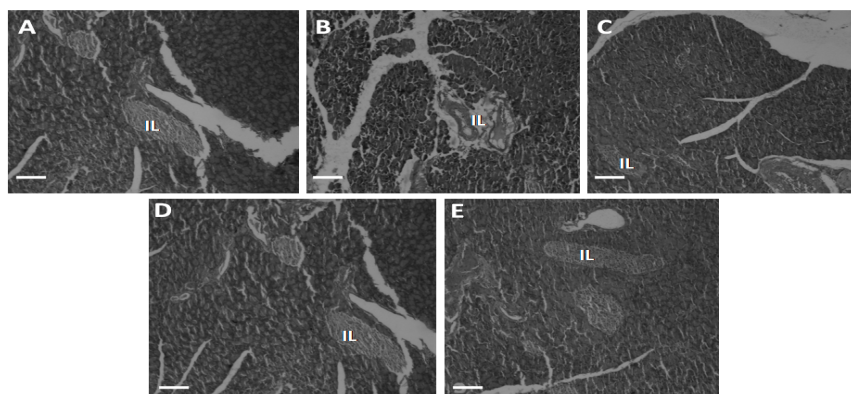Values not sharing a common superscript differ significantly with each other ($p<0.05$).

A: Normal control ; B: Diabetic control; C: 50 mg/kg *P. granatum* ; D: 100 mg/kg *P. granatum*; E: 100 mg/kg Metformin
IL: Islet of Langerhans

Figure 3: Effect of *P. granatum* peel aqueous extract on histopathology of pancreas in normal and diabetic rats. Histopathological section of pancreas of control and experimental rats (20×).

3A. Pancreatic section from normal control rats the islets of langerhans and well definied granulated dark beta cells.

3B. pancreatic section from diabetic induced rat shows the islets of langerhans with imperfected atrophied beta cells.

3C. Pancreatic section from diabetic rats after 50 mg/kg b. wt. of *P. granatum* peel aqueous extract treatment shows the islets of langerhans with well organized beta cells.

3D. Pancreatic section from diabetic rats after 100 mg/kg b. wt. of *P. granatum* peel aqueous extract treatment shows revealed improvement in pancreatic damage, it shows light vacuolar degeneration and there was marked improvement in islets structure.

3E. Represents the pancreatic section from diabetic rat treated with metformin shows that there was a regeneration of beta cells and acinar looked normal with increased number of beta cells.

DISCUSSION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia and often associated with oxidative stress; it causes significant morbidity and mortality (Wild et al., 2004). Treatment with allopathic drugs affects basic metabolic disturbances with serious physiological effects. DPP-IV inhibitors are one of the recent therapies used in the treatment of type 2 diabetes (Lotfy et al., 2011). It causes the degradation of GLP-1 hormone, whose function is to maintain the glucose homeostasis by stimulating the insulin secretion from the pancreas β-cells. In the present study, it was planned to investigate the possible role of the aqueous peel extract *P. granatum* is increasing the GLP-1 levels in intestinal through DPP-IV inhibition by a consequent reduction in the blood glucose levels. Metformin was used as a standard in the present study since, it reported to increase intestinal GLP-1 levels by DPP-IV inhibition in normal rats (Yasuda et al., 2004; Green et al., 2006).

Alloxan-induced diabetes is characterized by severe loss in body weights and similar results were observed in the present study. It may be caused by an increased protein wasting due to unavailability of carbohydrate for utilization as an energy source (Jin et al., 2012). However, oral administration of *P. granatum* peel extract significantly prevented the loss in body weight in diabetic rats (Enasand Khalil, 2004).This could be due to emaciation of skeletal muscle, dehydration and catabolism of fats and proteins (Umrani and Goyal, 2002). Our results indicated that the significant decrease in serum total protein levels in diabetic rats suggest that disruption of protein synthesis or its metabolism in diabetic rats, it was reversedwith *P. granatum* peel extract treatment in diabetic rats. In addition, marked fluctuations in food and water intake were observed in diabetic rats. *P. granatum* peel extract treatment significantly reduced the elevated food and water intake, it may be due to elevated GLP-1 levels as reported in earlier studies that sGLP-1 potently reduces food intake when administrated either into the brain or peripheral tissue in rats (Turton et al., 1996).

Alloxan, a β-cytotoxin, destroys β-cells of islets of Langerhans of the pancreas, resulting in a decrease in endogenous insulin secretion and paves the way for the decreased utilization of glucose by the tissues; it leads to increased glucose levels (Szkudelski, 2001). Our results also indicated that, a raise in the serum glucose level in alloxan induced diabetic rats. Further *P. granatum* peel extract treatment significantly decreasedthe blood glucose levels, it may be due to an improvement in insulin secretion from remnant pancreatic β-cell in diabetic rats. Our resultssupported the studies of Das and Barman, (2012) who reported significant decrease in serum glucose levels in diabetes rats, when treated with *P. granatum* extract.

Administration of alloxan destroys β-cells of the islets of Langerhans in the pancreas. Destruction of β-cells in the pancreas cause marked decrease in serum insulin levels (Szkudelski, 2001). In present investigation alloxan-induced diabetic rats showed significant decrease in serum insulin level when compared with normal control rats. However, treatment with *P. granatum* peel extract shows increased insulin levels. These results indicate that *P. granatum* peel extract might induce regeneration of pancreas β-cells. This results further supported by the pancreatic histopathology of pancreas, which showed the proliferation of the islet cells, this indicates that the production of insulin might be increased in *P. granatum* peel extract treated diabetic rats.

Glycosylated hemoglobin was an indicator of irreversible condensation of glucose with the N-terminal residue of the β-chain of hemoglobin (O'Hea et al., 2009). In our study, the diabetic rats had higher levels of glycosylated hemoglobin, the significant decrease in glycosylated hemoglobin was observed in diabetic rats after treatment with *P. granatum*peel extract indicates that the overall blood glucose levels were controlled which might be due to an improvement in insulin secretion. This is in concurrence with previous studies on Anusha Bhaskar and Anish Kumar (2012).

DPP-IV has been shown to have a significant role in the treatment of diabetes, it is a highly glycosylated serine protease broadly distributed throughout the tissue of the body.

DPP-IV causes the degeneration of gastrointestinal hormones like GLP-1, whose function is to maintain the glucose homeostasis by stimulating the insulin secretion from the pancreas β-cells (Siddiqui, 2009). In the present study, DPP-IV mRNA levels were significantly increased in diabetic rats. Increased in DPP-IV levels may contribute to elevated glucose levels in diabetic rats, which in turn results in increased DPP-IV activity (Bharti et al., 2012). In Palaet al., (2003) study indicated that increased mRNA for DPP-IV in human glomerular endothelial cells dose dependently with increased glucose concentration, it indicates that expression of DPP-IV mRNA levels were positive correlation with glucose levels. Yang et al., (2007) study reported that an increase in DPP-IV in the intestine, liver and kidney of the rat was associated with an insulin deficiency. In the present study, decreased DPP-IV mRNA expression levels in *P. granatum* extract treated diabetic rats might be due to *P. granatum* inhibitory effects on DPP-IV enzyme. Earlier Bharti et al., (2012) studies demonstrated that alkaloidspresent in *Castanospermumaustralia*hass how potential DPP-IV inhibitor activity.

While, natural DPP-IV inhibitors like berberine (an alkaloid) isolated from plants like *Berberisaristata*, *Berberisaquifolium* and *Hydrastiscanadensis* showed effective inhibition against the DPP-IV enzyme and enhancing the GLP-I release (Demuth et al., 2005; Lubbers et al., 2007; Al-Masri et al., 2009; Zhang et al., 2010). GLP-1 stimulates insulin secretion from islets of β-cells and lowers blood glucose, stimulates β-cell proliferation, reduction in food ingestion and glucagon secretion (Holst, 2007). In the present study intestinal GLP-1 mRNA levels were significantly decreased in diabetic rats due to decrease in GLP-1 action which is highly glucose dependent, whereas *P. granatum*extract treated diabetic rats intestinal GLP-1 mRNA expression levels were increased. Our current findings suggest that *P. granatum* peel extracts given orally to diabetic rats exhibited an anti-diabetic effect by lowering of blood glucose levels through the inhibitory activity on DPP-IV enzyme. Hence, GLP-1R mRNA levels were increased in intestine. Although in earlier studies resveratrol treated diabetic mice increase

the GLP-1 levels via enhanced DPP-IV inhibitor activity (Dao et al., 2011). In Liu et al. (2012) study, the DPP IV inhibitor LAF237 shows the renoprotection in diabetic rats was also associated with down-regulation of DPP-IV activity and the enhancement of active GLP-1 levels. Another study indicated that the L-glutamine treated diabetic rats showed a significant increase in plasma active GLP-1 concentration compared to diabetic control group (Badole et al., 2013).

Diabetes mellitus is associated with increased formation of free radicals and decreased antioxidant potential. An imbalance of oxidant/antioxidant defense systems results in alteration in the antioxidant enzyme activity (Maritim et al., 2003). Hyperglycemia causes generation of reactive oxygen species which in turn causes lipid peroxidatin.*P. granatum* peel extract possesses significant antioxidant activity in various *in vitro* models, it may be due to *P. granatum*peel extract contains compounds that are electron donors, which can react with free radicals to convert them to more stable products and terminate radical chain reaction. In the present study *P. granatum* peel extract shows significant *in virto*antioxidant activity when compared to ascorbic acid. In earlier studies free radical scavenging activity was also been reported for *P. granatum* juice (Gil et al., 2000), peel and seed extract (Singh et al., 2002).

In the present study in alloxaninduced diabetic rats, MDA content was increased. Treatment with *P. granatum* peel extract for 14 days significantly reducedthe pancreatic MDA content indicate a protective role of extract, this may be attributed the presence of phytochemicals such as glycosides, carbohydrates, tannins, alkaloids, and flavonoids. This is furthersupported by *P. granatum* extract treated diabetic rats showed a significant abrogates MDA level, suggested that *P. granatum* extract might have antioxidant principles to produce such response (Bagri et al., 2009). The SOD plays a pivotalrole in oxygen defense metabolism by reducing superoxide to hydrogen peroxide (Blokhina et al., 2003). The decrease in SOD activity in diabetic rats could result from inactivation of H_2O_2or by glycosylation of the enzyme which have been reported to occur in diabetes (Ravi et al., 2004).

CAT and GPx are involved in the elimination of H_2O_2. CAT activity decreased due to inactivation by superoxide radical and glycation of the enzyme by the treatment with *P. granatum* peel extract. Also, CAT is also involved in detoxification of high H_2O_2 concentration, whereas GPx is sensitive to lower concentration of H_2O_2. Reduction of GPx activity levels in diabetic rats may result from radical-induced inactivation (Bagri et al., 2009). *P. granatum* peel extract treated diabetic rats pancreatic antioxidant enzymes were significantly increased which could be attributable to strong antioxidative properties (Althunibat et al., 2010).

In conclusion, this study demonstrated that DPP-IV is a key regulator of insulin-stimulating hormones, inhibition of intestinal DPP IV enzyme leads to enhanced intestinal GLP-1 activity, which ultimately results in the potentiation of insulin secretion by pancreatic β-cells and subsequent lowering of blood glucose levels, HbA1c.Furthermore, *P. granatum*peel extract inhibited lipid peroxidation in the diabetic rat, as well as the extract significantly enhanced the activities ofantioxidant enzymes (CAT, SOD and GPx) in the pancreas of diabetic rats. Thus, *P. granatum* peel extract could be beneficial in ameliorating GLP-1 levels through DPP-IV inhibition in intestine by a consequent reduction in the blood glucose levels.

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