ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Protective effects of pomegranate seed extract on streptozotocin-induced β-cell damage in Rats : Inhibition of pancreatic nuclear factor kappa beta, transforming growth factor beta and matrix metalloproteinase-2 genes expression

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Manuscript Info

Manuscript History:

Received: 11 November 2013
Final Accepted: 28 November 2013
Published Online: December 2013

Key words:
pomegranate seed extract; diabetes; NF-κβ; TGF-β ; MMP-2

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Abstract

Aim of the Work: This study evaluated the possible protective effect of pomegranate seed extract (PSE) on streptozotocin (STZ)-induced ß-cell dysfunction in rats and its probable mechanism of action by analyzing nuclear factor kappa beta (NF-κβ), transforming growth factor beta (TGF-β) and matrix metalloproteinase (MMP)-2 genes expression in the pancreas.

Materials and Methods: Diabetes was induced in rats by single intraperitoneal injection of STZ (50 mg/kg body weight). Rats were divided into three groups (10 each): group I: control; group II: diabetic rats and group III: diabetic rats treated with PSE (300 mg/kg/day) administered orally for 4 weeks. Pancreatic NF-κβ, TGF-β and MMP-2 expressions were determined by RT-PCR. Reduced glutathione (GSH), was also measured in the pancreas. Immunohistochemical (IHC) staining of insulin was done on pancreatic sections.

Results: Pancreatic expression of NF-κβ, TGF-β and MMP-2 genes were significantly decreased with significant increase in pancreatic GSH content in pomegranate treated diabetic rats compared to non-treated diabetic rats. A significant increase in the mean area percent of insulin in pomegranate treated diabetic rats compared to non-treated diabetic rats by IHC.

Conclusion: PSE treatment prevented STZ-induced pancreatic ß-cell damage and protects β-cells from apoptosis and destruction in diabetes mellitus induced in rats which may be related to its antioxidant effect and to the significant decrease of TGF-β and MMP-2 genes expression in the pancreas via suppression of pancreatic NF-κB gene expression. Thus PSE could be used as a dietary supplement in patients with diabetes mellitus.

Introduction

Medicinal plants are a good source of compounds with hypoglycemic effects and are used as adjuncts to existing therapies for the treatment of diabetes mellitus (Sharma et al., 2008). Pomegranate (Punica granatum L.; Punicaceae) is a plant consumed either as an edible fruit or in the form of a beverage such as fruit juice and has been used in folkloric medicine for the treatment of various diseases. Edible parts of pomegranate fruit comprise 78% juice and 22% seed (El-Nemr et al., 2006).

Pomegranate seeds are a rich source of total lipids; of which comprised to 12% to 20% of total seed weight and characterized by a high content of polyunsaturated (n-3) fatty acids such as linolenic, linoleic, and other lipids such as punicic acid, oleic acid, stearic acid, and palmitic acid (Ozgul-Yucel, 2005). The seeds also contain protein, crude fibers, vitamins, minerals, pectin, sugars, polyphenols, isoflavones (mainly genistein), the phytoestrogen coumestrol, and the sex steroid, estrone (El-Nemr et al., 2006; Syed et al., 2007). The hypoglycemic activity of

seeds of pomegranate has been reported previously by Katz et \al. (2007). Meanwhile, the protective effect of pomegranate seed extract (PSE) on pancreatic β-cell and its probable mechanism of action in diabetes is not well studied.

Nuclear factor kappa beta (NF-κβ) is a major transcription factor and has been shown to regulate the expression of numerous genes that play important roles in cellular stress responses, cell growth, survival, and apoptosis (Karin and Ben-Neriah, 2000; Karin and Lin, 2002). In diabetes, NF-κβ is an important cellular signal in initiating the cascade of events culminating in ß-cell death and islet destruction (Cnop et al., 2005).

Transforming growth factor beta (TGF-β) is a member of a family of dimeric polypeptide growth factors and is involved in a myriad of cellular processes including cell proliferation, differentiation, and apoptosis (Attisano and Wrana , 2002; Feng and Derynck, 2005). There is a role of TGF-β pathway in regulating expression of genes that control β-cell function. Reduced TGF-β/Smad3 signaling markedly enhances insulin content and insulin secretion (Lin et al., 2009). TGF-β can also directly and indirectly (via its effects on tissue inhibitor matrix metalloproteinases) modulates matrix metalloproteinase (MMP)-2 activities (Overall et al., 1991; Martin et al., 1994). Matrix metalloproteinases are a family of secreted zinc proteases capable of degrading collagen and other matrix components. Matrix metalloproteinase-2, -12, and -14 were greatly increased in the Zucker diabetic fatty islets, and these increases are contemporaneous with the onset of β-cell dysfunction (Zhou et al., 2005).

Streptozotocin (STZ), a glucosamine-nitrosourea compound, can be selectively toxic to the pancreatic β-cells and has been used to induce β-cell injury in vivo, resulting in an experimental model of diabetes mellitus and β-cell dysfunction (Watanabe et al., 2010).

Thus the aim of the present study was to evaluate the possible protective effect of PSE on STZ-induced ß-cell dysfunction in rats and its probable mechanism of action by analyzing NF-κβ, TGF-β and MMP-2 genes expression in the pancreas by RT- PCR.

Materials and Methods

Animals

A total of 30 (20 diabetic surviving rats, 10 normal rats) adult male Wistar albino rats weighing 250 ± 10 g were used for this study. They were obtained from the animal facility of Faculty of Medicine, Cairo University. They were kept under controlled condition of illumination (12 h light/dark), and temperature 20-25° C. They were housed under ideal laboratory conditions, maintained on standard pellet diet and water *ad libitum* throughout the experimental period. All animal procedures were performed after approval from the ethics committee of the National Research Centre Cairo, Egypt and in accordance with the international regulations for the use and care of experimental animals (Canadian Council on Animal Care Guidelines, 1993).

Induction of diabetes in rats

Streptozotocin was obtained from Sigma Aldrich (St Louis, Missouri, USA). After overnight fasting, rats were made diabetic by a single intraperitoneal injection of STZ (50 mg/kg body weight) freshly prepared in 0.1 mol/l citrate buffer, pH 4.5 (Naskar et al., 2011). After 72 h of STZ administration, fasting plasma glucose was measured by obtaining blood samples from the tail in all animals. The rats showing plasma glucose level of 250 mg/dl or more were considered diabetic (Kanter et al., 2004).

Experimental design

The rats were randomly divided into 3 groups of ten rats each as follows:

Group I (Control group): Rats were injected with the same volume of citrate buffer as that used as a vehicle in the diabetic group (0.1 mol/l citrate buffer, pH 4.5) alone.

Group II (Diabetic group): Overnight-fasted rats received a single intraperitoneal injection of STZ (50 mg/kg body weight).

Group III (Diabetic+pomegranate treated group): Rats received a single intraperitoneal injection of STZ (50 mg/kg body weight). On the third day (72 h) after STZ injection, rats were treated with PSE (300 mg/kg/day) given orally by gavages for 4 weeks. Pomegranate Seed Extract (Neocell, USA), commonly used as dietary supplements, was supplied as powder, dissolved in distilled water to be given in a single dose of 300mg/kg/day (Das et al., 2001).

At the end of the experiment, blood samples were taken from the rat tail vein in collecting heparinized capillary tubes in overnight fasting rats. The samples were centrifuged at 1000g and plasma was obtained and frozen in polypropylene tubes at −20°C until assayed. Animals from all groups were then sacrificed by decapitation and the pancreas was isolated, washed with saline, and then part of the pancreas was blotted with a piece of filter paper, kept in foil paper, and freezed at −70°C till used for gene expression. Another part of the pancreas was fixed in 10% formol saline for histological studies.

Biochemical analysis

I-Determination of plasma glucose and insulin

Fasting plasma glucose was determined using an autoanalyzer (Hitachi 747 auto-analyzer, Hitachi, Tokyo, Japan). Insulin level was measured with a chemiluminescence immuno-metric assay using a UniCell DXI 800 analyzer (Beckman Coulter Inc., Fullerton, CA, USA).

II- Determination of pancreatic reduced glutathione (GSH)

GSH content was determined in pancreatic sections by using a commercially available assay (Glutathione Assay Kit II, Merck Chemicals, Hull, UK) according to the protocol provided by the manufacturer. Briefly, pancreatic tissue (1 mg) was homogenized in 150 µl ice-cold lysis buffer and centrifuged at 10,000 *g* for 15 min at 4°C. After protein determination, supernatants were deproteinized with 5% metaphosphoric acid (Sigma-Aldrich Chemie) and 4 M triethanolamine (Sigma-Aldrich Chemie). Samples were then mixed with the Assay Cocktail reagents. After incubation at 25°C for 25 min, absorbance was measured at 405 nm. GSH levels were calculated by using a standard curve generated by standards provided by the manufacturer.

III- Molecular Biology Methods:

1- RNA extraction:

Total RNA from pancreas was extracted by the acid guanidinum thiocyanate-phenol- chloroform method (Chomczynski and Sacchi, 1987). The extracted RNA was quantitated by spectrophotometer (Tokyo, Japan) at 260 nm.

2- Primers synthesis:

Different primer sets were used. The sequence of the primers was enlisted in Table (1).

3- Reverse transcription-polymerase chain reaction (RT-PCR) experiments:

RT- PCR was done using the extracted RNA for detection of the expression of NF-κ ß, TGF- β and MMP-2 genes.

a) RT-PCR of NF-*k*β:

The cDNA was synthesized under the following conditions: 1µg total RNA was incubated with 5x buffer, 1mmol/L each dNTP, $MgCl_2$ (5mmol), 50 U of RNAse inhibitor and reverse primer. After denaturation at 65^oC for 15 minutes, 20 U of AMV was added. Then, the followings were added: $MgCl_2$ (1.25µmol/L), dNTP (200 µmol/L each), primers (10 pmol/L each) and 1U Taq polymerase. The reaction was incubated for 1 hour at 42^oC. The PCR condition was 90 sec at 95^oC, followed by 30 cycles of 40 sec at 95^o C, 40 sec at 60^oC and 60 sec at 72^oC.

b) RT-PCR of TGF-β:

Total RNA was reverse transcribed using the RT-PCR Stratagene Kit (Foster city, California, USA) according to the manufacturer's instructions. Then, the reaction tube was placed at 42°C for 1 h followed by heating to 95°C for 5 min. For PCR, 3 µl cDNA was mixed with reaction buffer (500 mmol/l KCl, 100 mmol/l Tris-HCl pH 8.3, 1.5 mmol/l $MgCl_2$, and 0.001% gelatin), dNTPs mix (2.5 mmol/L), Taq polymerase(2U), primers (0.4 µmol/L each). The reaction mixture was then subjected to 30 cycles of PCR amplification as follows: denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 2 min.

c) RT-PCR of MMP-2

Reverse transcription was performed using an annealing temperature of 70°C in a final volume of 25µL containing 250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$,50 mM dithiothreitol, 0.5 mM dNTPs, reverse primer, 26U ribonuclease inhibitor (Promega , Madison, WI, USA) and MMLV- RT (200U). PCR was performed in a total volume of 50 µL of the reaction mix. The thermocycling conditions were: initial denaturation for 5 min at 94, 40 cycles of amplification of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min, followed by a final elongation of 10 min at 72°C.

4. Quantitation of the PCR product

The PCR products were then quantitated by using a quantitation kit (from Promega Corporation, Madison, WI, USA). This method depends on purification of the PCR using Promega Wizard PCR preps DNA purification kit (Promega Corporation, Madison, WI, USA). The mixture for quantitation consisted of DNA quantitation buffer, sodium pyrophosphate, NDPK enzyme solution, T4 DNA polymerase and DNA. All these contents were incubated at 37°C for 10 min. Then, 100 µL of Enliten L/L reagent was added. Immediately, the reaction was read using a luminometer. The same steps were done on DNAs of known concentrations provided by the kit, and a standard curve was performed by plotting the readings of the luminometer against the concentrations. Then, the readings of the amplified PCR product of NF- *k*β, TGF-β and MMP-2 after using the luminometer were read from the standard curve. The results were expressed as pg/gm tissue (Shaker et al., 2006).

Light microscopic study

Specimens of the tail of the pancreas were fixed in 10% formol saline. Paraffin-embedded sections were cut at 7 µm thickness and were subjected to:

a - Hematoxylin and eosin (H&E) stain (Kiernan, 2001).

b -Immunohistochemical stains for insulin: Using streptavidine-biotin peroxidase technique (Bancroft and Cook, 1994) the endogenous peroxidase activity was eliminated using 10% H_2O_2 for 15 min. Sections were then incubated for one hour with specific antibody against:

- Insulin (a mouse monoclonal antibody, Dako). Immunoreactivity of insulin appeared as brown cytoplasmic staining in β-cells of islets of Langerhans.

Quantitative morphometric measurements

The mean area percent of insulin immunostaining-positive cells in pancreatic stained sections was measured. This was accomplished using Leica Qwin 500 C software image analyzer computer system Ltd. (Cambridge, England). Measurements were carried out within 10 nonoverlapping fields for each animal at a magnification of × 400.

Statistical analysis

The data were expressed as means ± standard deviation of the means (SD). Analysis of variance (ANOVA) was performed on the means to determine whether there were significant ($P < 0.05$) differences among the groups. When ANOVA indicated statistical significance, Tukey-Kramer's multiple comparison test follows up, for intergroup comparisons. SPSS version 12 (Chicago, Illinois, USA) was used for all statistical analyses. The results were considered significant when P value <0.05.

Results

I. Biochemical results:

Effects of PSE on fasting plasma glucose and insulin levels in STZ-induced diabetic rats

As shown in table 2, the fasting plasma glucose levels were significantly elevated (P<0.001) in STZ treated diabetic rats (group II) as compared to the control rats (291.6 ± 8.16 mg/dl vs 86.05 ± 4.98 mg/dl respectively). This was accompanied with significant decrease (P<0.05) in plasma insulin levels in STZ-treated rats compared to control rats (16.2±1.16 vs 22.38± 3.46 respectively, table, 2). Treatment of STZ diabetic rats with PSE for 4 weeks (group III) significantly decreased (P<0.001) fasting plasma glucose level by 42% compared to non-treated diabetic rats (170.16±12.09 vs 291.6±8.16 respectively, table, 2). Meanwhile, PSE treatment in diabetic rats (group III) did not normalize fasting plasma glucose as there was significant difference (P<0.001) in group III as compared to control group (table, 2). There was also significant increase (P<0.01) in plasma insulin level in pomegranate treated diabetic rats (group III) by 61% as compared to non-treated diabetic rats (26.04±5.9 vs 16.2±1.16 respectively) with non-significant difference (P>0.05) between group III and control group (table, 2).

Effects of PSE on pancreatic expressions of NF-κß, TGF-β and MMP-2 mRNA in STZ-induced diabetic rats

The present study showed that NF-κß, TGF-β and MMP-2 mRNA expressions determined by RT-PCR were significantly increased (P<0.001) in rat pancreas in STZ diabetic rats (group II) as compared to control rats (table, 2). STZ induced diabetic rats treated with PSE for 4 weeks (group III) showed significant decrease (P<0.001) in expressions of mRNA levels of NF-κß by 23%, TGF-β by 46% and MMP-2 by 20% as compared to non-treated STZ diabetic rats. Meanwhile, pomegranate seed treatment did not return mRNA levels of NF-κß, TGF-β and MMP-2 to control values as there was significant difference (P<0.001) in these parameters in group III as compared to control group (table, 2).

Effects of PSE on pancreatic GSH content in STZ-induced diabetic rats

The present study showed significant decrease (P<0.001) in pancreatic antioxidant parameter, GSH, in STZ diabetic rats as compared to control rats (table, 2). STZ-induced diabetic rats treated with PSE (group III) showed significant increase (P<0.001) in pancreatic GSH by 29% as compared to non-treated diabetic rats but there was significant difference (P<0.001) in group III as compared to control group (table, 2).

II. Histological results:

A. H&E results

Pancreatic sections from control rats (group I) showed islets of Langerhans that appear as lightly stained area in-between the darkly stained exocrine acini. Cells of the islets are crowded and separated by blood capillaries. Most of the central cells which are mostly beta cells are polyhedral with rounded nuclei. The islet is closely related to the pancreatic acini with no detectable space in-between (Fig. 1).

Pancreatic sections from diabetic rats (group II) showed distorted islet of Langerhans with decreased cellular density, increased intercellular space as well as wide separation between islets of Langerhans and the surrounding pancreatic acini with the presence of many apoptotic cells (Fig. 2a). Congestion as well as destruction in the wall of blood capillaries with blood extravasation is also seen (Fig. 2b). There are two small islets which are formed of few cells where the islets are surrounded with pancreatic acini (Fig. 2c).

Pancreatic sections from diabetic rats treated with PSE (group III) showed islets with the surrounding acini. Islets are formed of crowded cells with no wide spaces between them. Most of islets' cells appear normal. Apparently normal blood capillaries are also noted (Fig. 3).

B. Insulin immunohistochemical stained results:

Islets of Langerhans in the pancreatic sections from control rats (group I) showed positive immunostaining for insulin in almost all central islets' β-cells. Insulin immunostaining appeared as diffuse brown cytoplasmic deposits. Cells at the periphery of the islets showed negative reaction (Fig. 4). The diabetic rats (group II) showed reduced insulin expression in the islets (Fig. 5). While diabetic rats treated with PSE (group III) revealed diffuse positive insulin immunoreactivity within the cells of the islets of Langerhans (Fig. 6).

Morphometric results

As shown in table (3), the mean area percent of insulin immunoreactivity was significantly decreased ($p<0.001$) in diabetic group (group II) as compared to control group. Diabetic rats treated with PSE (group III) showed significant increase ($p<0.001$) in the mean area percent of insulin immunoreactivity as compared to diabetic non-treated rats with non-significant difference ($p>0.05$) between group III and the control group (table, 3).

Table (1): Sequence of all primers used in the experiment

Parameter	Primer sequence	PCR product (bp)
NF- *k*β	F: 5' TACCATGCTGTTTTGGTTAC 3' R: 5' TCAAGCTACCAATGACTTTC 3'	208
TGF-β	F: 5' AGCCTGTGGGGCTATATGAC 3' R: 5' CTTGGCAGCTCAACTCTCTG 3'	760
MMP-2	F: 5-CTTTGCAGGAGACAAGTTCTGG-3 R: 5-TTAAGGTGGTGCAGGTATCTGG-3	701
β-actin	F:5'TCACCCTGAAGTACCCCATGGAG3' R:5'TTGGCCTTGGGGTTCAGGGGG3	150

Table (2): Effects of PSE treatment on different biochemical parameters in diabetic rats

Parameters	**Control Group I**	**Diabetic Group II**	**Diabetic+PSE Group III**
Fasting plasma glucose (mg/dl)	86.05±4.98	291.6±8.16*	170.16±12.09$^{\#}$*
Plasma Insulin (µ U/ml)	22.38±3.46	16.2±1.16**	26.04±5.9$^{\$}$
Pancreatic NF-κ ß gene (pg/gm tissue)	583±9.67	992.5±8.46*	765.6±9.9$^{\#}$*
Pancreatic TGF-β gene (pg/gm tissue)	347.16±5.49	879.16±6.91*	477.5±4.24$^{\#}$*
Pancreatic MMP-2 gene (pg/gm tissue)	323.83±8.13	722.66±9.62*	580.1±6.96$^{\#}$*
Pancreatic GSH (pg/gm tissue)	52.4±1.38	36.16±1.72*	46.78±1.5$^{\#}$*

Values are expressed as means ± SD (n = 10 each group). **PSE**: pomegranate seed extract**, NF-κß:** nuclear factor kappa beta**, TGF-β:** transforming growth factor-beta**, MMP-2:** matrix metalloproteinase-2, **GSH:** reduced glutathione. Analysis of variance (ANOVA) followed by Tukey-Kramer analysis was used for the comparison between the groups.

*$p<0.001$, **$p<0.05$ vs control group

$^{\#}p<0.001$, $^{\$}$ $p<0.01$ *vs* diabetic group

Table (3): The mean area percent of insulin immunostaining in the studied groups

Groups	Mean area percent of insulin
Control (Group I)	23.79±2.77
Diabetic (Group II)	6.84±1.57*
Diabetic +Pomegranate treated (Group III)	20.92±1.90**

Values are expressed as means ± SD (n = 10 each group). Analysis of variance (ANOVA) followed by Tukey-Kramer analysis was used for the comparison between the groups.

*p<0.001 vs control group

**p<0.001 *vs* diabetic group

Fig. 1: Photomicrographs of a pancreatic section from a control rat (group I) showing an islet of Langerhans that appears as lightly stained area in-between the darkly stained exocrine acini (A). Cells of the islet are crowded and separated by blood capillaries (arrowheads). Most of the central cells which are mostly beta cells are polyhedral with rounded nuclei (arrows). Note that the islet is closely related to the pancreatic acini with no detectable space in-between. H&E X400.

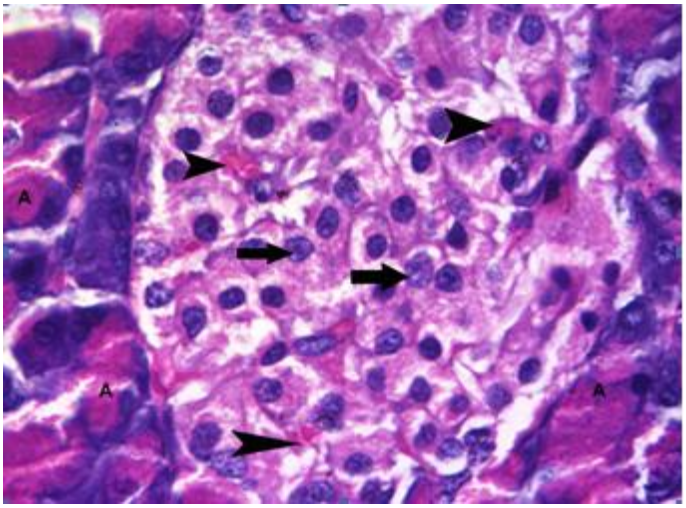


Fig. 2a: Photomicrographs of a pancreatic section from a diabetic rat (group II) showing distorted islet of Langerhans with decreased cellular density, increased intercellular space( )as well as wide separation ( ) between islet of Langerhans and the surrounding pancreatic acini (A) with the presence of many apoptotic cells (arrows). H&E X200.

Fig. 2b: Photomicrographs of a pancreatic section from a diabetic rat (group II) showing distorted islet of Langerhans (I) that is surrounded with pancreatic acini (A). Congestion (arrowheads) as well as destruction in the wall of blood capillaries with blood extravasation (spiral arrow) is also seen. H&E X400

Fig. 2c: Photomicrographs of a pancreatic section from a diabetic rat (group II) showing two small islets (I) which are formed of few cells. The islets are surrounded with pancreatic acini (A). H&E X200.

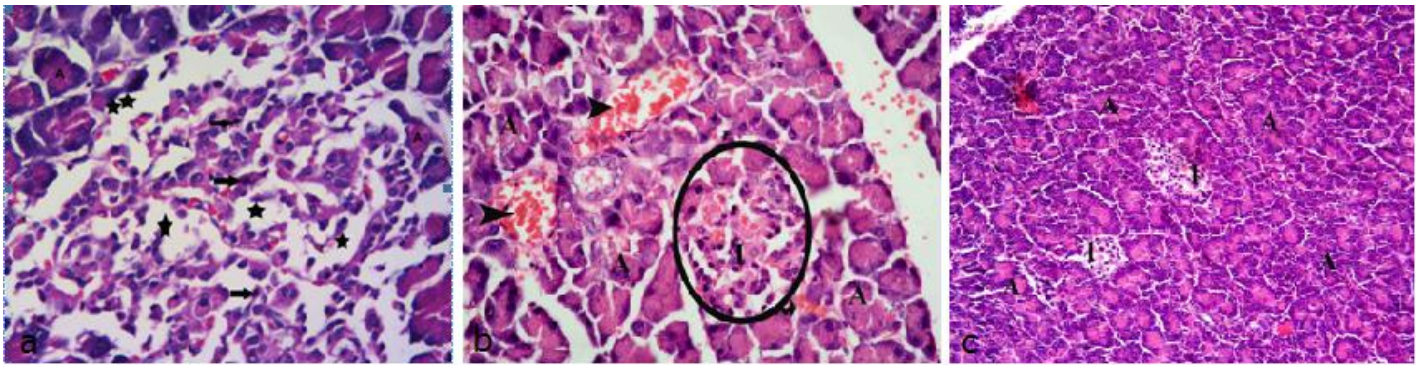


Fig. 3: Photomicrographs of a pancreatic section from a diabetic rat treated with pomegranate seed extract (group III) showing an islet with the surrounding acini (A). Islet is formed of crowded cells with no wide spaces between them. Most of islets' cells appear normal. Apparently normal blood capillaries are also noted (arrowheads). H&E X400

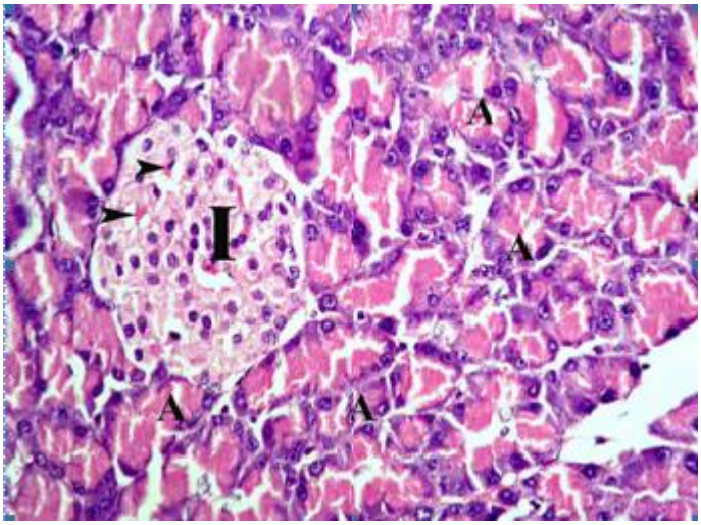


Fig. 4: A photomicrograph of a pancreatic section from a control rat (group I) showing an islet surrounded with pancreatic acini (A). Almost all central islets' β-cells show positive immunostaining for insulin. Insulin immunostaining appears as diffuse brown cytoplasmic deposits (arrows). Cells at the periphery of the islets show negative immunoreaction (arrowheads). Insulin immunostaining X400

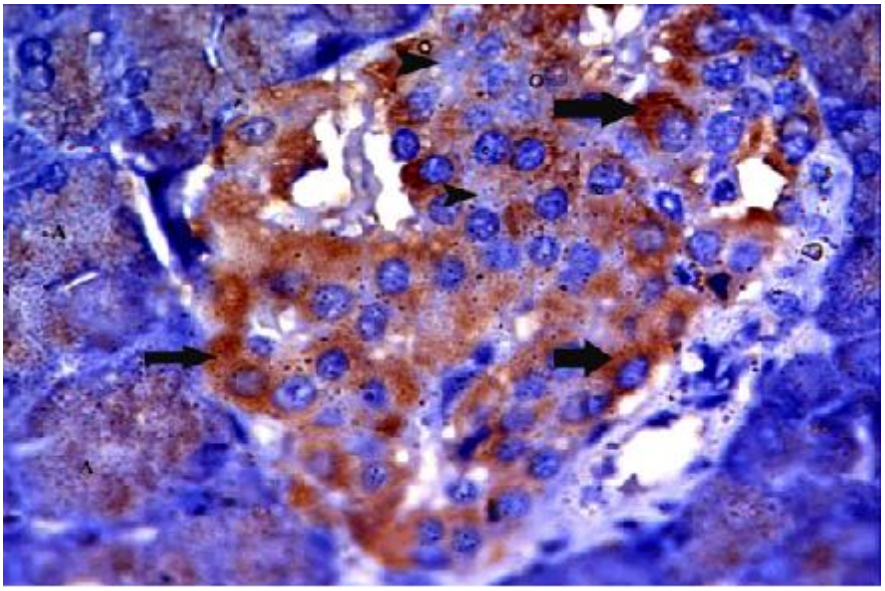


Fig. 5: A photomicrograph of a pancreatic section from a diabetic rat (group II) showing an islet surrounded with pancreatic acini (A). Reduced insulin expression (arrows) within the islet with increased area of negative immunoreactivity (arrowheads) is seen. Insulin immunostaining X400

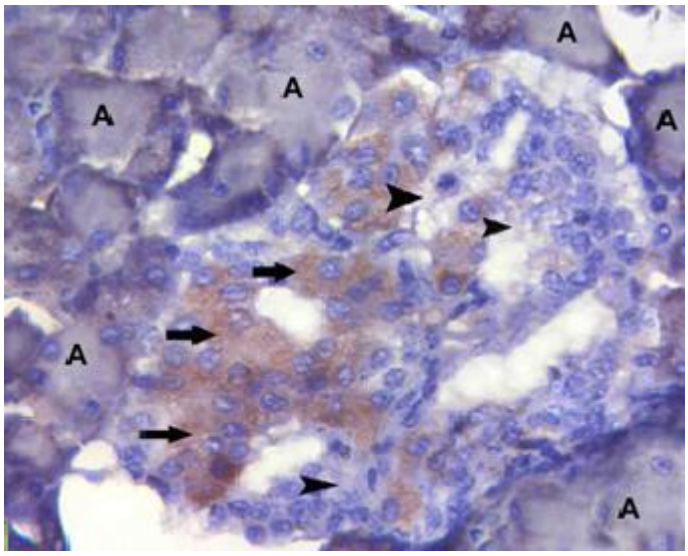
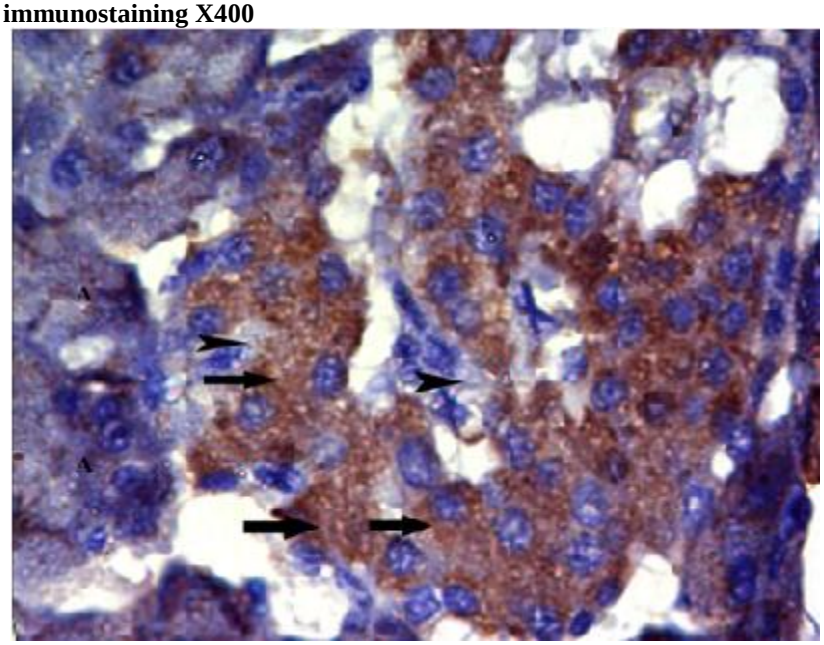


Fig. 6: A photomicrograph of a pancreatic section from a diabetic rat treated with pomegranate seed extract (group III) showing an islet of Langerhan surrounded with pancreatic acini (A). Diffuse positive insulin immunoreactivity (arrows) within the islet is seen, while its periphery shows negative immunoreaction (arrowheads). Insulin immunostaining X400



Discussion

Streptozotocin is the most usual applicable substance for induction of experimental diabetes mellitus (Szkudelski, 2001). In the present study, a single intraperitoneal injection of STZ (50 mg/kg body weight) leads to development of diabetes in rats manifested by hyperglycemia and hypoinsulinemia 4 weeks after STZ injection in STZ treated diabetic rats compared to control rats. STZ is a nitrosourea compound which specifically induces DNA

strand breakage in β-cells causing diabetes mellitus (Lenzen, 2008). This leads to insulin deficiency which in turn increases the blood sugar level. The changes in blood glucose and insulin concentrations reflect abnormalities in β-cell function as stated by Bedoya et al. (1996).

In our study, PSE treatment in diabetic rats for 4 weeks significantly reduced STZ-induced high blood glucose levels, and significantly elevated plasma insulin levels as compared to non-treated STZ diabetic rats. These findings suggest a significant antihyperglycemic effect of PSE in diabetic rats which are in agreement with the findings of Das et al. (2001) where they found that PSE led to significant reduction of blood glucose in STZ induced diabetes in rats. On the contrary, pomegranate seed did not exert significant hypoglycemic effect in alloxan induced diabetic rats as stated by Jelodar et al. (2007). They explained this apparent discrepancy between pomegranate seeds and their extract as possibly due to a compound in pomegranate seeds with hypoglycemic effects that, in extract form, was more concentrated and therefore more effective in lowering blood sugar than the seed form.

The antidiabetic effect of the seed of pomegranate may be, partly, due to their positive effect on glycogen synthesis in liver, skeletal muscle and heart muscle, and partly, due to the insulin-like or insulin releasing action of the ingredients present in the seed of the plant as stated by Das and Sarma (2009). Known compounds in pomegranate seed, such as ellagic, gallic and ursolic acid, have been identified as having anti-diabetic actions (Banihani et al., 2013). Also, the presence of one or more bioactive antihyperglycemic principles, such as flavonoids, isoflavones, and their synergistic effects (Manoharan et al., 2009) are known to be natural antioxidants and thus protecting the existing β-cells from dying by their free radical scavenzing action (Kaneto et al., 1999). The increased plasma insulin levels in pomegranate treated diabetic rats in the present study suggested that the PSE might have stimulated insulin secretion from surviving pancreatic β-cells and thus blood glucose is controlled.

In the current study, STZ treated diabetic rats showed significant increase in NF-κβ mRNA expression in the pancreas determined by RT-PCR compared to control rats. The previous findings are in accordance with those of previous studies where Ho et al. (2000) demonstrated that in mice, STZ treatment results in NF-κβ activation in the pancreas. Also, Kwon et al. (2006) revealed increased NF-κβ binding activity in pancreatic nuclear extracts derived from STZ-treated hyperglycemic diabetic mice as assayed by using gel mobility shift assays.

Streptozotocin enters the pancreatic β-cell via glucose transporter-GLUT2 and causes diabetes by inducing DNA alkylation (Szkudelski, 2001). In addition, STZ destroys islet cells through several other mechanisms, including the production of reactive oxygen species (ROS) and nitric oxide (Kwon et al., 2006), activation of pancreatic NF-κβ (Eldor et al., 2006), and induction of pronounced immune and inflammatory responses (Iwakiri et al., 1990). As a result of the STZ action, β cells undergo destruction by necrosis (Szkudelski, 2001).

The transcription factor, NF-κβ participates in the transcriptional regulation of pro-inflammatory genes, such as inducible nitric oxide synthases, cyclooxygenase-2 and tumor necrosis factor (TNF)-.α, that contribute to ß-cell destruction and their activation results in the production of pro-inflammatory mediators (Blackwell and Christman, 1997). NF-κβ can be activated by STZ, ROS (Adcock et al., 1994) and cytokines, such as interleukin- 1 β, TNF-.α and interferon gamma. In β cells NF-κβ activation can also be triggered by glucose (Maedler et al., 2002). NF-κβ is initially located in the cytoplasm as an inactive form complexed with Iκβ, an NF-κβ inhibitory factor. However, various inducers cause this complex to dissociate, presumably via the phosphorylation of Iκβ, thereby allowing NF-κβ to be released from the complex. NF-κβ then translocates to the nucleus, where it interacts with its DNA recognition sites to mediate gene transcription (Melloul, 2008) and eventually leads to islet cell death (Hayden and Ghosh, 2004).

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The current study showed that PSE treatment in diabetic rats significantly reduced pancreatic NF-κβ mRNA expression compared to non-treated diabetic rats which are in agreement with the following studies. Aggarwal and Shishodia (2004) reported that the pathway that activates the transcription factor NF-κβ can be interrupted by phytochemicals as pomegranate (ellagic acid). It has also been found that dietary punicic acid, which is a conjugated linolenic acid isomer and a component of pomegranate seed, decreases fasting plasma glucose concentrations, improves the glucose-normalizing ability and suppresses NF-κβ activation (Hontecillas et al., 2009).

In the current study, STZ treated diabetic rats showed significant increase in pancreatic TGF-β gene expression compared to control rats. TGF-β, in addition to stimulating the deposition of increased amounts of extracellular matrix, is also known to regulate cell growth, differentiation, and function in the pancreas (Yoshikawa

et al., 2002). TGF-β regulates these cellular processes by binding to cell-surface receptors types which are linked to serine-threonine protein kinases and after ligand stimulation; intracellular signaling is initiated via transcription factors known as SMADs (Attisano et al., 1993; Heldin et al., 1997). TGF-β/Smad3 signaling regulates genes involved in β-cell function whereas loss of TGF-β/Smad3 signaling was accompanied by activated insulin receptor signaling and increased gene expression of factors that promote β-cell function. In contrast, amplified TGF-β signals repressed expression of genes that promote insulin biosynthesis, pro-insulin processing, glucose sensing, glucose metabolism, incretin signaling, insulin exocytosis, and glucose stimulated insulin secretion (Lin et al., 2009).

In the present study, diabetic rats treated with PSE for 4 weeks showed significant decrease in pancreatic TGF-β gene expression compared with non-treated diabetic rats. Previous studies have demonstrated a close relationship between NF-κB and TGF-β signaling pathways where NF-κβ can elicit TGF-β gene transcription (Strauch et al., 2003). Thus in the present study PSE inhibition of pancreatic TGF-β gene expression might be due to inhibition of pancreatic NF-κβ gene expression.

The current study showed that STZ treated diabetic rats led to significant increase in pancreatic MMP-2 gene expression as compared to control rats. Matrix metalloproteinases constitute a family of secreted or transmembrane proteins that degrade extracellular matrix proteins (Vu and, Werb, 2000) and are normally expressed in islets (Barro et al., 1998).) The mRNAs for MMP-2, -12, and -14 were greatly increased in the Zucker diabetic fatty rat's islets, and these increases are contemporaneous with the onset of β-cell dysfunction and diabetes (Zhou et al., 2005). They stated that MMPs limit expansion of β-cell mass since MMPs avidly cleave matrix proteins, and this might interfere with critical cell-matrix interactions. MMPs may also degrade growth factors or receptors that are required for β-cell survival and expansion.

The results of the present work revealed that PSE treatment significantly decreased pancreatic MMP-2 gene expression in STZ treated rats compared to non- treated diabetic rats which might be due to significant decrease in pancreatic TGF-β gene expression since Ishihara et al. (1998) stated that TGF beta 1 induced in pancreatic duct cells also induced MMP-2 in an autocrine or paracrine manner. Pomegranate seed has inhibitory effect on MMP-2 as demonstrated by Afaq et al. (2009) who stated that pomegranate seed was able to inhibit UVB-induced expressions of MMP-2 and MMP-9 activity in the EpiDerm.

This study showed significant decrease in the antioxidant parameter; GSH content, in the pancreas of STZ treated rats compared to control rats. Both enzymatic and non-enzymatic antioxidant defense systems were impaired in STZ-induced diabetic rats suggesting that these antioxidants are exhausted to combat the deleterious effects of increased oxidative stress generated in STZ treated beta cells (Pavana et al., 2007). Pancreatic β-cells are susceptible to oxidative stress damage, which is generated by chronic exposure to high levels of glucose and toxic agents because the islets express very low levels of antioxidant enzymes and activity causing pancreatic β-cell dysfunction and apoptosis (Sakai et al., 2003).

In the current work, PSE treatment in STZ induced diabetes in rats produced significant increase in the antioxidant GSH content in the pancreas compared to non-treated diabetic rats. These results suggest an antioxidant effect of PSE in diabetes which is not well studied as compared to literature. Meanwhile, previous authors reported that extracts from pomegranate seed exhibit strong antioxidant activity (Singh et al., 2002; Wang et al., 2004 and Gill et al., 2012). Pomegranate juice and seed extracts have 2-3 times the in vitro antioxidant capacity of either red wine or green tea (Schubert et al., 1999; Gil et al., 2000). Pomegranate seed extracts are found to be a rich source of polyphenolic compounds (flavonoids and tannins) which account for 92% of their antioxidant activities (Gil et al., 2000), and has scavenging activity against oxygen free radicals.

In the present study, histological examination of pancreatic tissue from STZ diabetic rats revealed distorted islet of Langerhans which was associated by the significant decrease in the mean area percent of insulin immunoreactivity detected by immunohistochemical morphometric analysis. Meanwhile, PSE treatment in diabetic rats prevented islet destruction and preserved islet architecture where most of islets' cells appear normal in H&E stained sections. This was accompanied with significant increase in the mean area percent of insulin immunoreactivity in PSE treated diabetic rats compared to non-treated diabetic rats. These results suggested that PSE has a protective effect against pancreatic islets damage and potentiate insulin secretion from surviving ß cells of the islets of Langerhans in an experimental model of diabetes which might be provided by repair/regeneration of the

endocrine pancreas as stated by Shanmugasundaram et al. (1990). They reported that one of the mechanisms of the hypoglycemic actions of plants is by increasing beta cells in the pancreas by activating regeneration of these cells. On the contrary to these results, pancreas of diabetic rats treated with pomegranate seed showed close similarity to diabetic untreated group in section stained with H&E as stated by Jelodar et al. (2007).

In conclusion, the results of the present study showed that PSE treatment has hypoglycemic effects, prevented STZ-induced pancreatic ß-cell damage and protects the β-cells from apoptosis and destruction in an experimental model of diabetes in rats. These effects may be mediated through its antioxidant action and suppression of TGF-β and MMP-2 genes expression in the pancreas via suppression of pancreatic NF-κβ gene expression. Thus PSE has could serve as an effective adjunct in the management of diabetes mellitus.

Acknowledgements

The authors wish to thank Dr. Dalia El Marakby, Assistant Professor of Histology, Faculty of Medicine, Cairo University for carrying out the histological examination done in the present study.

References

Adcock IM, Brown CR, Kwon O, Barnes PJ. (1994): Oxidative stress induces NF kappa B DNA binding and inducible NOS mRNA in human epithelial cells. Biochem Biophys Res Commun 199:1518–1524.

Afaq F, Zaid MA, Khan N, Dreher M, Mukhtar H. (2009): Protective effect of pomegranate-derived products on UVB-mediated damage in human reconstituted skin. Exp Dermatol 18(6):553-61.

Aggarwal BB, Shishodia S. (2004): Suppression of the nuclear factor-kappaB activation pathway by spice-derived phytochemicals: reasoning for seasoning. Ann N Y Acad Sci 1030:434-41.

Attisano L, Carcamo J, Ventura F, Weis FM, Massague J, Wrana JL. (1993): Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. Cell 75:671-680.

Attisano L, Wrana JL. (2002): Signal transduction by the TGF-beta superfamily. Science 296(5573):1646-7.

Bancroft JD and Cook HC. (1994): Immunocytochemistry. In: Bancroft JD, Cook, HC, editors. Manual of histological techniques and their diagnostic application. Edinburgh, Churchill Livingstone, p. 263-287.

Banihani S, Swedan S, Alguraan Z. (2013): Pomegranate and type 2 diabetes. Nutr Res.33(5):341-8.

Barro C, Zaoui P, Morel F, Benhamou PY. (1998): Matrix metalloproteinase expression in rat pancreatic islets. Pancreas17:378 –382.

Bedoya, FJ, Solano F, Lucas M. (1996): N-monomethyl-arginine and nicotinamide prevent streptozotocin-induced double strand DNA break formation in pancreatic rat islets. Experientia, 52:344-347.

Blackwell TS, Christman JW. (1997): The role of nuclear factor-kappa B in cytokine gene regulation. Am J Respir Cell Mol Biol 17:3–9.

Canadian Council on Animal Care (CCAC), 1993. Guide to the Care and Use of Experimental Animals, Volume 1 (2nd Edition), Canada.

Chomczynski P, Sacchi N. (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem 162(1), 156–159.

Cnop M, Welsh N, Jonas JC, Jörns A, Lenzen S, Eizirik DL. (2005): Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 54 Suppl 2:S97-107.

Das AK, Mandal SC, Banerjee SK, Sinha S, Saha BP, Pal M. (2001): Studies on the hypoglycaemic activity of Punica granatum seed in streptozotocin induced diabetic rats. Phytother Res 15(7): 628-629.

Das S, Sarma G (2009): Antidiabetic action of ethanolic extracts of Punica granatum Linn. In alloxan induced diabetic albino rats. S J PharmSci 2(1): 14-21.

Eldor R, Yeffet A, Baum K, Doviner V, Amar D, Ben-Neriah Y, Christofori G, Peled A, Carel JC, Boitard C,Klein T, Serup P, Eizirik DL, Melloul D. (2006): Conditional and specific NF-kappaB blockade protects pancreatic beta cells from diabetogenic agents. Proc Natl Acad Sci U S A. 103(13):5072-7.

El-Nemr SE, Ismail IA, Ragab M. (2006): Chemical Composition of Juice and Seeds of Pomegranate Fruit. Die Nahrung 34 (7):601-606.

Feng XH, Derynck R. (2005): Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol 21:659-93.

Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA. (2000): Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agric Food Chem 48(10):4581-9.

Gill NS, Dhawan S, Jain A, Arora R, Bali M. (2012): Antioxidant and anti-ulcerogenic activity of wild Punica granatum ethanolic seed extract", Research Journal of Medicinal plants 6(1); 47-55.

Hayden MS, Ghosh S. (2004): Signaling to NF-kappaB. Genes Dev 18(18):2195-224.

Heldin CH, Miyazono K, ten Dijke P. (1997): TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 390:465-471.

Ho E, Chen G, Bray TM (2000):Alpha-phenyl-tert-butylnitrone (PBN) inhibits NFkappaBactivation offering protection against chemically induced diabetes. Free Radic Biol Med.28(4):604-14.

Hontecillas R, O'Shea M, Einerhand A, Diguardo M, Bassaganya-Riera J.(2009): Activation of PPAR gamma and alpha by punicic acid ameliorates glucose tolerance and suppresses obesity-related inflammation. J Am Coll Nutr 28(2):184-95.

Ishihara T, Hayasaka A, Yamaguchi T, Kondo F, Saisho H.(1998): Immunohistochemical study of transforming growth factor-beta 1, matrix metalloproteinase-2,9, tissue inhibitors of metalloproteinase-1,2, and basement membrane components at pancreatic ducts in chronic pancreatitis. Pancreas 17(4):412-8.

Iwakiri R, Nagafuchi S, Kounoue E, Nakamura M, Kikuchi M, Nakano S, Niho Y(1990): Immunohistochemical study of insulitis induced by multiple low doses of streptozocin in CD-1 mice. Diabetes Res Clin Pract 9:75–82.

Jelodar G, Mohsen M, Shahram S. (2007): Effect of walnut leaf, coriander and pomegranate on blood glucose andhistopathology of pancreas of alloxan induce d diabetic rats. Afr J Tradit Complement Altern Med 4(3):299-305.

Kaneto H, Kajimoto Y, Miyagawa J, Matsuoka T, Fujitani Y, Umayahara Y, Hanafusa T, Matsuzawa Y, Yamasaki Y, Hori M. (1999): Beneficial effects of antioxidants in diabetes: possible protection of pancreatic β–cells against glucose toxicity. Diabetes. 48: 2398–2406.

Kanter M, Coskun O, Korkmaz A, Oter S. (2004): Effects of Nigella sativa on oxidative stress and beta-cell damage instreptozotocin-induced diabetic rats. Anat Rec A Discov Mol Cell Evol Biol 279(1):685-91.

Karin M, Ben-Neriah Y. (2000): Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol. 18:621-63.

Karin M, Lin A. (2002): NF-kappaB at the crossroads of life and death. Nat Immunol. 3(3):221-7.

Katz SR, Newman RA, Lansky EP. (2007): Punica granatum: heuristic treatment for diabetes mellitus. J Med Food **10**(2):213–7.

Kiernan JA (2001): Histological and histochemical methods: Theory and practice. 3rd ed. Arnold Publisher, London, pp:111-162.

Kwon KB, Kim EK, Jeong ES, Lee YH, Lee YR, Park JW, Ryu DG, Park BH (2006):Cortex cinnamomi extract prevents streptozotocin- and cytokine-induced beta-cell damage by inhibiting NF-kappaB. World J Gastroenterol.12(27):4331-7.

Lenzen S (2008): The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia 51(2):216-26.

Lin HM, Lee JH, Yadav H, Kamaraju AK, Liu E, Zhigang D, Vieira A, Kim SJ, Collins H, Matschinsky F,Harlan DM, Roberts AB, Rane SG.(2009): Transforming growth factor beta/Smad3 signaling regulates insulin gene transcription and pancreatic islet beta-cell function. J Biol Chem 284(18):12246-57.

Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY. (2002): Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. J Clin Invest 110(6):851-60.

Manoharan, S.; Kumar, R. Anish; Mary, A. Linsa; Singh, R. B.; Balakrishnan, S.; Silvan, S. (2009): Effects of Punica granatum Flowers on Carbohydrate Metabolizing Enzymes, Lipid Peroxidation and Antioxidants Status in Streptozotocin Induced Diabetic Rats. The Open Nutraceuticals Journal 2:113-117.

Martin J, Knowlden J, Davies M, Williams JD (1994): Identification and independent regulation of human mesangial cell metalloproteinases. Kidney Int 46 :877 –885.

Melloul D. (2008): Role of NF-kappaB in beta-cell death. Biochem Soc Trans 36(Pt 3):334-9.

Naskar S, Mazumder UK, Pramanik G, Gupta M, Kumar RB, Bala A, Islam A (2011): Evaluation of antihyperglycemic activity of Cocos nucifera Linn. on streptozotocin induced type 2 diabetic rats. J Ethnopharmacol.138(3):769-73

Overall C, Wrana JL, Sodek J (1991): Transcriptional and post transcriptional regulation of 72kDa gelatinase/typeIV collagenase by transforming growth factor β1 in human fibroblasts. *J Biol Chem*266 :14064–14071.

Ozgul-Yucel S (2005): Determination of Conjugated Linolenic Acid Content of Selected Oil Seeds Grown in Turkey. Journal of the American Oil Chemists' Society, 82 (12):893-897.

Pavana P, Sethupathy S, Manoharan S. (2007): Antihyperglycemic and antilipidperoxidative effects of Tephrosia purpurea seed extract in streptozotocin induced diabetic rats. Indian J Clin Biochem 22: 77-83.

Sakai K, Matsumoto K, Nishikawa T, Suefuji M, Nakamaru K, Hirashima Y, Kawashima J, Shirotani T,Ichinose K, Brownlee M, Araki E. (2003): Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells. Biochem Biophys Res Commun ;300(1):216-22.

Schubert, S.Y., Lansky, E.P. & Necman, I. (1999): Antioxidant and Eicosanoid Enzyme Inhibition Properties of Pomegranate Seed Oil and Fermented Juice Flavonoids. Journal of Ethnopharmacology 66(1):11-17.

Shaker OG, Moustafa W, Essmat S, Abdel-Halim M, El-Komy M. (2006): The role of interleukin-12 in the pathogenesis of psoriasis. Clin Biochem 39(2):119-25.

Shanmugasundaram ER, Gopinath KL, Radha Shanmugasundaram K, Rajendran VM. (1990): Possible regeneration of the islets of Langerhans in streptozotocin-diabetic rats given Gymnema sylvestre leaf extracts. J Ethnopharmacol.30(3):265-79.

Sharma B, Balomajumder C, Roy P. (2008): Hypoglycemic and hypolipidemic effects of flavonoid rich extract fromEugenia jambolana seeds on streptozotocin i nduced diabetic rats. Food Chem Toxicol 46(7):2376-83.

Singh, R.P., Chidambara Murthy, K.N., Jayaprakasha, G.K. (2002): Studies on the antioxidant activity of pomegranate (Punica granatum) peel and seed extracts using in vitro models. Journal of Agricultural and Food Chemistry 50 (1):81–86.

Strauch ED, Yamaguchi J, Bass BL, Wang JY. (2003): Bile salts regulate intestinal epithelial cell migration by nuclear factor-kappa B-induced expression of transforming growth factor-beta. J Am Coll Surg 197:974–984.

Syed, DN, Afaq F, Mukhtar, H (2007): Pomegranate Derived Products for Cancer Chemoprevention. Seminars in Cancer Biology 17(5):377-385.

Szkudelski T. (2001): The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res 50(6):537-46.

Vu TH, Werb Z (2000): Matrix metalloproteinases: effectors of development and normal physiology. Genes Dev14 :2123 –2133.

Wang RF, Xie WD, Zhang Z, Xing DM, Ding Y, Wang W, Ma C, Du LJ (2004): Bioactive compounds from the seeds of Punica granatum (pomegranate) J Nat Prod.;67:2096–2098.

Watanabe K, Thandavarayan RA, Harima M, Sari FR, Gurusamy N, Veeraveedu PT, Mito S, Arozal W, Sukumaran V, Laksmanan AP, Soetikno V, Kodama M, Aizawa Y. (2010): Role of differential signaling pathways and oxidative stress indiabetic cardiomyopathy. Curr Cardiol Rev 6(4):280-90.

Yoshikawa H, Kihara Y, Taguchi M, Yamaguchi T, Nakamura H, Otsuki M (2002): Role of TGF-β1 in the development of pancreatic fibrosis in Otsuka Long-Evans Tokushima Fatty rats. Am J Physiol Gastrointest Liver Physiol 282 :G549 –G58.

Zhou YP, Madjidi A, Wilson ME, Nothhelfer DA, Johnson JH, Palma JF, Schweitzer A, Burant C, Blume JE,Johnson JD. (2005): Matrix metalloproteinases contribute to insulin insufficiency in Zucker diabetic fatty rats. Diabetes 54(9):2612-9.