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RESEARCH ARTICLE

Anti-inflammatory Effect of *Juniperus procera* Extract in Rats Exposed to Streptozotocin Toxicity

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ARTICLE HISTORY

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Abstract: ***Background*:** Chronic inflammation is a critical health issue and implicated in several chronic health problems such as tumors, auto-immune disorder, hypertension or diabetes. However, *Juniperus procera* is one of the famous ancient plants that has been traditionally used to treat several diseases such as hyperglycemia, hepatitis, jaundice, bronchitis, and pneumonia.

***Objective*:** Current study is an attempt to investigate the anti-inflammatory effect of *Juniperus procera* extract on rats exposed to cytotoxicity caused experimentally by streptozotocin injections.

***Methods*:** Five groups of adult Wistar rats (10 rats each) were examined as (Normal control, Normal rats treated with *Juniperus procera* extract, rats administrated with streptozotocin, rats administrated with streptozotocin and treated with insulin and, rats administrated with streptozotocin and *Juniperus procera* extract). At the end of the experiment, blood was collected from experimented rats. Animals then were killed and small parts of both pancreas and liver were collected for gene expression and histopathological examination.

***Results*:** Serum analysis showed a significant increase in glucose, IL-6, IL-2 and TNF-α levels in rats exposed to streptozotocin. That change was reduced in rats cotreated with insulin or *Juniperus procera* extract. Moreover, streptozotocin showed a significant upregulation of IL-6, TNF-α and A2M genes, while, either insulin or *Juniperus procera* treatment was restored to normal status. Streptozotocin induced inflammation within hepatic tissues which clearly reduced in hepatic tissues of both insulin and junipers cotreated groups.

***Conclusion*:** Streptozotocin toxicity induces acute inflammation and increases serum glucose, IL-6, IL-2 and TNF-α levels. However, *Juniperus procera* extract was found to significantly prevent that reaction within four weeks experimented frame time.

Keywords: Anti-inflammatory, IL-2, IL-6, *Juniperus procera*, streptozotocin, TNF-α, α2 microglobulin.

1. INTRODUCTION

Inflammation is a complicated biological process that usually starts as a part of body defense mechanism but when lasts longer than required causes several medical problems [1]. Inflammation is classified into two types depending on the duration of the inflammatory reaction [1]. These types are acute inflammation that lasts for a few days and chronic inflammation which lasts weeks to months [2]. Acute inflammation starts with increasing blood vessels permeability that increase the localized migration of macrophages, proteins, and fluids in the injured tissue [2]. This classical mechanism causes redness, swelling, warmth and pain or discomfort [2]. Although inflammation is beneficial for healing and infection elimination, the prolonged inflammatory response (chronic inflammation) can cause very complicated health

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problems such as tumors, auto-immune disorder, hypertension or diabetes [2].

Initiation of the inflammatory response is controlled by several secreted proteins called proinflammatory mediators. Proinflammatory mediators are secreted from different cell types such as lymphocytes, endothelial cells, macrophages and injured cells [3]. Interleukin-6 (IL-6) is one of the proinflammatory mediators called the master proinflammatory cytokines in some references. It is a glycoprotein that plays different roles in immune system such as hematopoietic stimulation, T and B lymphocytes differentiation. IL-6 induces the production of acute phase proteins such as C-reactive protein and one of the chronic inflammatory causes [3, 4]. Tumor Necrosis Factor-alpha (TNF-α) is a proinflammatory cytokine produced mainly by activated macrophages, mast cells, neutrophils, and lymphocytes. It is a glycoprotein involved in different immune functions inducing fever, acute phase reaction and inflammation [3, 4].

Several reports have linked proinflammatory mediators either directly or indirectly with diabetes and insulin resistance incidence [5, 6]. Recently, scholars have found that the increase of serum levels of TNF-α and IL-6 were associated with insulin resistance in type2 diabetic patients [5-8]. In addition, several cross-sectional studies on the onset of type 2 diabetes confirmed that acute phase proteins such as C-reactive protein and proinflammatory cytokines such as TNF-α are strongly linked with type 2 diabetes development and the risk of diabetic complications [5-8].

In fact, plants are the main source of many modern medicines. *Juniperus procera* is one of the famous ancient plants that has been traditionally used to treat several diseases such as hyperglycemia, hepatitis, jaundice, bronchitis, and pneumonia [9-13]. Therefore, the current study is an attempt to investigate the anti-inflammatory effect of ethanolic extract of *Juniperus procera* leaves on rats exposed to streptozotocin, an alkylating chemical that is broadly used to induce hyperglycemia in experimental animals due to its cytotoxicity effect on beta cells of the pancreas *via* DNA damage induction and its inflammatory response stimulation.

2. MATERIALS AND METHODS

2.1. Materials

Three months old adult male Wistar rats were purchased from King Fahad Research Institute, Jeddah, Saudi Arabia. Streptozotocin (STZ) was purchased from Sigma Aldrich St. Louis, MO, USA. Insulin was purchased from Novo Nordisk, Københavnsvej 216, 4600 Køge, Denmark. Oligo dT, chloroform, ethanol were purchased from Wako Pure Chemicals, Osaka, Japan and primers were from Macrogen Company, Seoul, Korea. TriZol reagent was from Invitrogen, Carlsbad, CA. Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (Sib Enzyme, Ltd., Ak, Novosibirsk, Russia). PCR master mix was from Promega Corporation, Madison, WI. Rat serum/plasma IL-6, IL-2 and TNF-α estimation kits were purchased from Abcam plc, 330 Cambridge Science Park Cambridge, CB4 0FL UK. Serum glucose levels were estimated using a fully automated analyzer Cobas 6000 Roche.

2.2. Methods

2.2.1. Juniperus procera Ethanolic Extraction

Fresh *Juniperus procera* leaves were dried and powdered in dark. 0.2 kg of powder were dissolved in an equal amount of ethanol: water (50:50) to yield 30 grams of extract.

2.2.2. Experimental Design

Five groups of adult Wistar rats weighing 250-300 grams (75-90 days old) 10 rats each were examined as (Normal control, Normal rats treated with 200mg/kg of *Juniperus procera* extract, rats administrated with streptozotocin (STZ), rats administrated with (STZ) and treated with 1U/ kg of insulin and rats administrated with (STZ) and treated with 200mg/kg of *Juniperus procera* extract [10]. All procedures were approved by the Animal Care Committee of Taif University, Iran for the project #4825/1437/1.

2.2.3. Streptozotocin Administration

STZ was prepared using fresh powder dissolved in 0.05 M Citrate buffer, pH 4.5. After 12 hours fasting, rats were injected by a single intraperitoneally dose (60 mg/kg of body weight). In dorsal

position, designated rats were injected in the caudal abdominal cavity using a sterile 25g needle. Streptozotocin induces inflammation and diabetes in 2- 3 days by destroying beta cells. STZ animals and control groups were kept in metabolic cages individually and separately and underfeeding and metabolic control. Rats with fasting blood glucose between 200- 250 mg/dl were considered diabetic and included in the experiments for further treatment [14, 15]. Random blood glucose levels were measured daily for 4 successive weeks to assure diabetic incidence. At the end of the experiment, rats were fasted overnight and anesthetized using diethyl ether inhalation; blood was collected from the inner canthus of the eyes to obtain serum. Animals then were killed and small parts of both pancreas and liver were collected. Some of these parts were immediately immersed in TriZol reagent and kept at -70°C until RNA extraction. Other parts of both organs were preserved in 10% buffered neutral formalin for histopathological examination.

2.2.4. Serum Analysis

Serum concentrations of glucose were measured spectrophotometrically using Cobas 6000 Roche. For serum IL-6, IL-2 and TNF-α estimation, samples were diluted by 1:2 in diluent provided with the kit. Serum levels of IL-6, IL-2 and TNF-α were measured *via* Enzyme-Linked Immune Sorbent Assay (ELISA) using commercial kits purchased from Abcam plc catalog number (ab 100772), (ab221834) and (ab46070) respectively.

2.2.5. RNA Extraction and cDNA Synthesis

Approximately 50 mg per tissue was used for total RNA extraction from either pancreas or liver samples. Samples were flash frozen in liquid nitrogen and subsequently stored at -70°C in 1 mL Trizol. Frozen samples were homogenized using a Polytron 300 D homogenizer (Brinkman Instruments, Westbury, NY). Then, 0.3 mL chloroform was added to the homogenate. The mixtures were vortexed for 30 seconds and centrifuged at 4°C and 13000 rpm for 20 min. Supernatants were collected into new Eppendorf tubes. Equal volumes of isopropanol were added to the samples and vortexed for 15 seconds, and centrifuged at 4°C for 15 min and 13000 rpm. Pellets were washed with 70% ethanol, briefly dried up, and then dissolved in Diethyl Pyrocarbonate (DEPC) water. Purity, concentration and total RNA yielded were estimated spectrophotometrically at 260nm. The optical density of the 260/280 ratio of all RNA samples was 1.7-1.9. For cDNA synthesis, 3 μg total RNA was mixed with 0.5 ng oligo dT primer (Wako Pure Chemicals, Osaka, Japan) in a total volume of 11 μL sterilized DEPC water and incubated in the Bio-Rad T100TM Thermal Cycler at 65°C for 10 min for denaturation. A mixture of 20μL total volume of (2 μL of 10-RT-buffer, 2 μL of 10 mM dNTPs, and 100 U Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (Sib Enzyme, Ltd., Ak, Novosibirsk, Russia) and completed with DEPC water) was reincubated in the Thermal Cycler at 37°C for 1 hour at 90°C for 10 min and stored at -20°C until used.

2.2.6. Semi-Quantitative RT-PCR Analysis

For semi-quantitative RT-PCR analysis, specific primers for examined genes listed in Table **1** were designed using the Oligo-4 computer program and synthesized by Macrogen (Macrogen Company, Seoul, Korea). PCR was conducted in a final volume of 25 μL consisting of 1 μL cDNA, 1 μL of 10 pM of each primer (forward and reverse), and 12.5 μL PCR master mix (Promega Corporation, Madison, WI); the volume was completed up to 25 μL using sterilized and deionized water. PCR was carried out using Bio-RadT100TM Thermal Cycler machine with the cycle sequence at 94°C for 5 min of one cycle, followed by variable cycles stated in Table **1** each of which consisted of denaturation at 94°C for 1 min, annealing at a specific temperature corresponding to each primer (Table **1**), and extended at 72°C for 1 min to 7 min of final extension at 72°C. As a technical control, expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was examined (Table **1**). PCR products were electrophorized on 1.5% agarose gel stained with ethidium bromide in Tris-borate-EDTA buffer. Products were visualized under UV light and photographed using a gel documentation system. The density of Bands were analyzed densitometrically using Image J software version 1.47.

2.2.7. Histopathological Investigation

Pancreatic tissues were obtained from sacrificed rats then fixed in 10% buffered neutral formalin

Table 1. Polymerase chain reaction conditions for the genes analyzed.

Gene	Product Size	Annealing Temperature	Sense	Antisense
A2M	325	56	gtctctgtctgtttccttagtt	attggcctttcgtggtttag
TNF- α	357	59	tttcataccaggagaaagtcagc	gagccacaattccctttctaagt
IL-6	415	59	gttctcagggagatcttggaaat	ctttcaagatgagttggatggtc
G3PDH	269	59	tgttcctacccccaatgtgt	tgtgagggagatgctcagtge

A2M: alpha-2-Macroglobulin; TNF-α: Tumor Necrosis factor-alpha; IL-6: Interleukine-6; G3PDH: Glyceraldehyde 3-phosphate dehydrogenase.

Table 2. Effect of *Juniperus procera* extract and insulin administration on serum glucose and proinflammatory cytokines levels.

	Normal Control	JPE	(STZ)	(STZ) + Insulin	(STZ) + JPE
Glucose (mg/dl)	107 ± 9.4	127 ± 5.2	422 ± 11.7*	161± 5.6**	277± 23.2
IL-6 (µg/ml)	13.58 ± 0.46	13.3 ± 1.2	18.9 ± 0.4*	12.8 ± 0.5**	14.6 ± 0.3**
IL-2 (µg/ml)	0.83 ± 0.04	0.88 ± 0.1	1.15 ± 0.01*	0.79 ± 0.05**	0.96 ± 0.06**
TNF-α (U/ml)	64 ± 0.6	65 ± 1.8	85.3 ± 3.1*	67.7 ± 2.4**	70.3 ± 2.7**

Values are means ± SEM for five different rats per each treatment. Values are statistically significant at *p ≥ 0.05 corresponding to control; **p ≥ 0.05 corresponding to STZ exposed rats.

for 48 h. The samples were washed then dehydrated in ascending grades of alcohol. The clearing was performed in xylene followed by embedding then casting. Sectioning into 5 µ thickness was done followed by staining with normal routine stain (H&E).

2.2.8. Statistical Analysis

Data were collected and analyzed using Analysis of Variance (ANOVA) and post hoc descriptive tests by SPSS software version 11.5 for Windows with the p-value < 0.05 considered as statistically significant. Results are presented in means ± Standard Error of Means (SEM).

3. RESULTS

3.1. Positive Restoration Effect of *Juniperus procera* Extract of Serum Levels of Glucose and Pre Inflammatory Markers that Influenced by STZ Injection

Table **2** clearly shows a significant increase in serum glucose levels in streptozotocin-injected rats. That increase was reduced by 50% in both rats co-treated with JPE and rats co-treated with insulin. While there is no clear change in serum glucose levels of both normal fed rats and rats treated with JPE alone (Table **2**). In addition, STZ toxicity significantly increases serum levels of IL-6, TNF-α, and IL-2 up to about 50% while, either treatment of JPE or, insulin significantly restores serum levels of these cytokines as shown in Table **2**. However, JPE treatment alone does not affect the serum levels of measured cytokines comparing with normal control rats.

3.2. Histopathological Pictures of Pancreatic Tissues of Experimented Animals

Fig. (**1**) clearly shows that normal tissue architecture with normal cellular details of beta cells in the pancreatic tissue of control rats (Fig. **1A**). Similarly, the pancreatic tissue of Juniperus administered rats showed a normal histological picture of islets of Langerhans (Fig. **1B**). While pancreas of streptozotocin-administered rats showed severe regression and atrophy of islets of Langerhans (Fig. **1C**). However, the pancreas of streptozotocin-administered rats co-treated with JPE showed regeneration of beta cells of islets of Langerhans (Fig. **1D**) and a similar picture of pancreatic tissue were found in rats co-treated with insulin (Fig. **1E**).

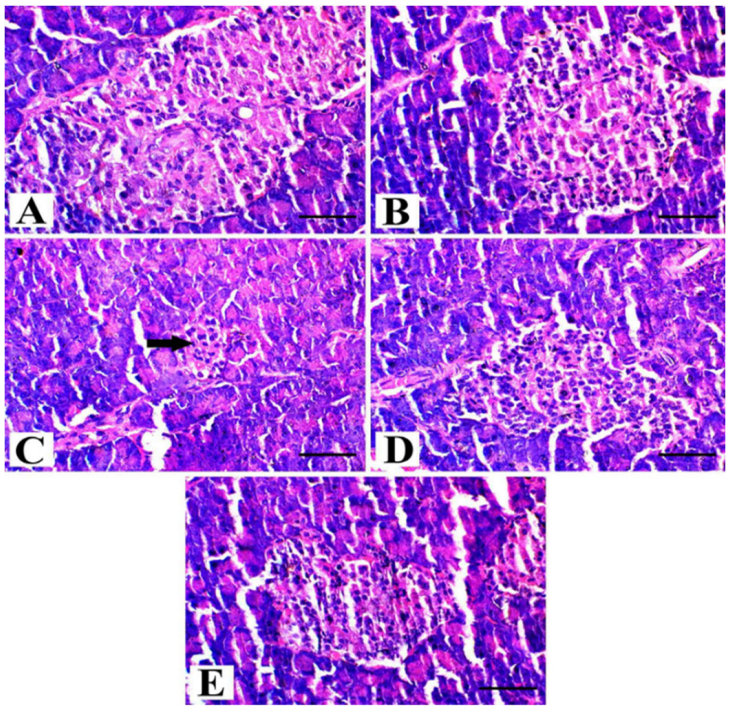

Fig. (1). Histopathological picture of pancreatic tissues of experimental rats. A). The pancreatic tissue of control rats showed normal tissue architecture with normal cellular details of beta cells. **B**). The pancreatic tissue of Juniperus administered rats showed a normal histological picture of islets of Langerhans. **C**). The pancreas of streptozotocin-administered rats showed severe regression and atrophy of islets of Langerhans (arrow). **D**). The pancreas of streptozotocin-administered rats co-treated with *Juniperus procera* extract showed regeneration of beta cells of islets of Langerhans. **E**). The pancreatic tissue of streptozotocin-administered rats treated with insulin showed restoration of a normal picture of beta cells of islets of Langerhans. (Scale bar=100 μm).

3.3. Protective Effect of *Juniperus Procera* Extract on mRNA Expression of IL-6, TNF-α, and A2M genes

Streptozotocin toxicity causes a striking up-regulation of mRNA expression of some pre inflammatory genes illustrated in Figs. (**2-4**). Fig. (**2**) shows a clear up-regulation of IL-6 gene under SZN toxicity and similar effect have been shown in the expression of TNF-α and A2M genes as represented in Figs. (**3** and **4**) respectively. These changes have been restored to almost normal levels in rats that are co-treated with JPE as shown in Figs. (**2-4**). Moreover, there was no clear difference in mRNA expression of IL-6, TNF-α and A2M genes between rats treated by insulin or rats treated by JPE as well as there was no clear difference between normal control rats and rats treated by JPE alone as shown in Figs. (**2-4**).

All clinical investigations must be conducted according to the declaration of Helsinki principles. Authors must comply with the guidelines of the international committee of medical journal editors (www.icmje.org).

4. DISCUSSION

Inflammation is an essential physiological mechanism for pathogens removal and to restore damaged tissue. The inflammatory mechanism involves many complemented and cooperated cytokines, chemokines, and metabolites. On the other hand, prolonged inflammatory response (chronic inflammation) may cause very complicated health problems such as tumors, auto-immune disorder, hypertension or diabetes [2].

Interleukin-6 (IL-6) is one of the inflammatory mediators that stimulates hematopoiesis, T and B lymphocytes differentiation and C-reactive protein production [3, 4]. In addition to IL-6, tumor necrosis factor-alpha (TNF-α) is a cytokine produced mainly by activated macrophages, mast cells, neutrophils, and lymphocytes. TNF-α is involved in different immune functions such as fever reactions,

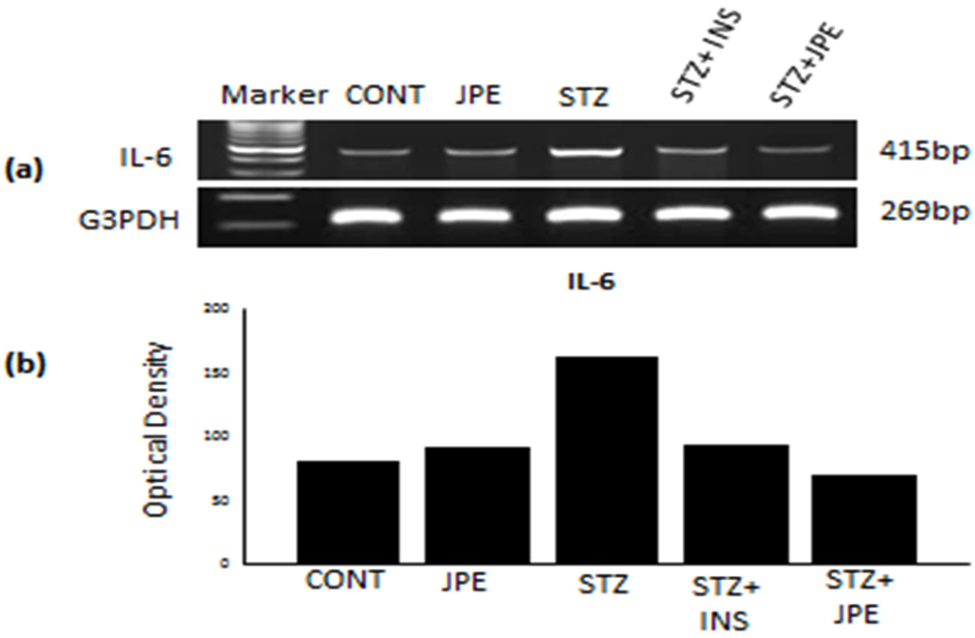

Fig. (2). Effect of *Juniperus procera* on changes in mRNA expression of IL-6 gene in hepatic tissues of experimental rats. (**a**) panels are the mRNA expression of examined genes. (**b**) columns are the densitometric analysis of gene expression. Data shown represent three technical replicates.

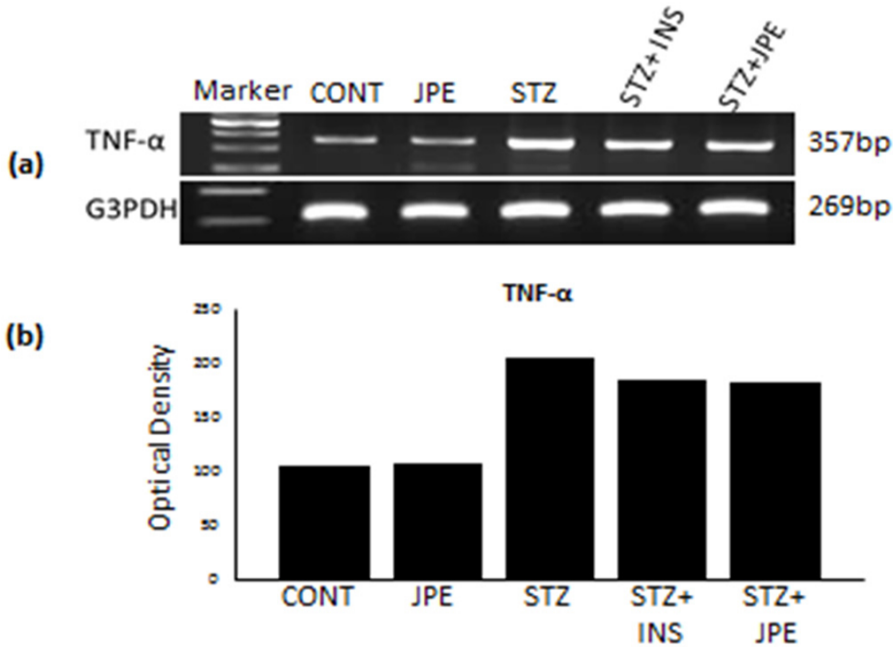

Fig. (3). Effect of *Juniperus procera* on changes in mRNA expression of TNF-α gene in hepatic tissues of experimental rats. (**a**) panels are the mRNA expression of examined genes. (**b**) columns are the densitometric analysis of gene expression. The data shown represent three technical replicates.

acute phase reaction and inflammation [3, 4]. Several studies have reported that IL-6 and TNF-α are associated either directly or indirectly with diabetes and insulin resistance incidence [5, 6]. Recently, scholars have found that the increase of serum levels of TNF-α and IL-6 were associated with insulin resistance in type 2 diabetic patients [5-8]. Therefore, the current study was concentrated on these two cytokines as inflammatory condition indicators in our way to investigate the anti-inflammatory effect of using JPE as alternative and preventive treatment. This kind of native

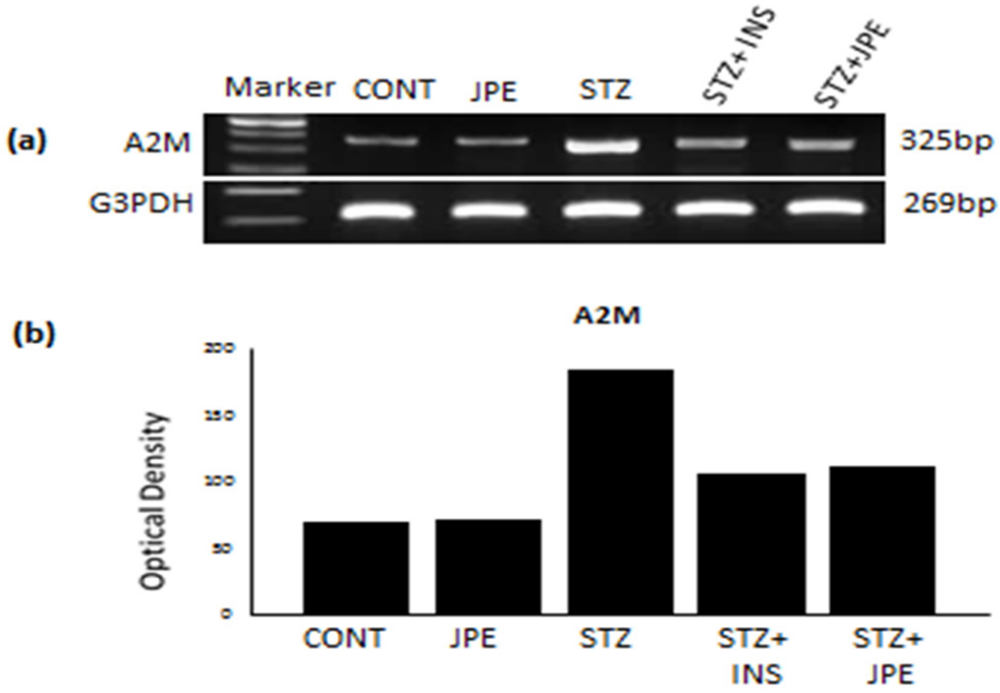

Fig. (4). Effect of *Juniperus procera* on changes in mRNA expression of α-2-microglobulin gene in hepatic tissues of experimental rats. (**a**) panels are the mRNA expression of examined genes. (**b**) columns are the densitometric analysis of gene expression. Data shown represent three technical replicates.

ancient plant is traditionally used for treating several medical conditions such as liver injury, cholestasis, nephrotoxicity and hyperglycemia [10-13]. According to Burits, *et al*. and others the extract of the leaves of this kind of plants contains different types of terpenes that have a remarkable anti-inflammatory action [11, 12, 16]. As shown in Table **2** STZ toxicity caused an acute and severe inflammation as observed *via* 50% increase in serum levels of IL-6, TNF-α as well as IL-2 that were statistically significant in which P values were < 0.05 for each cytokine. However, these changes were strikingly recovered with treating animals by either insulin injections or oral administration of JPE (Table **2**). Visually, these data were confirmed *via* histopathological investigations of pancreatic tissues as presented in Fig. (**1**). Fig. (**1A**) clarifies that slides of pancreatic tissue of control rats show a normal tissue architecture with normal cellular details of beta cells. Similarly, the pancreatic tissue of Juniperus administered rats showed a normal histological picture of islets of Langerhans (Fig. **1B**). While, pancreas of streptozotocin-administered rats showed severe regression and atrophy of islets of Langerhans (Fig. **1C**). However, the pancreas of streptozotocin-administered rats co-treated with JPE showed regeneration of beta cells of islets of Langerhans (Fig. **1D**) and a similar picture of pancreatic tissue were found in rats co-treated with insulin (Fig. **1E**). Gene expression study clearly showed that STZ toxicity upregulated both IL-6 and TNF-α genes which may indicate a positive feedback and confirming inflammation caused by cytotoxicity of STZ Figs. (**2** and **3**). However, treatment with either insulin injections or oral administration of JPE restored that genetic activity to normal regulation particularly IL-6 as shown in Figs. (**2** and **3**). A2M gene is a protein-coding gene located on the P arm of chromosome 12 [17]. A2M gene codes alpha-2-Macroglobulin is a homotetrameric glycoprotein plays several physiological roles. Alpha-2-Macroglobulin inhibits several proteases such as collagenase, thrombin, and trypsin [18-20]. During inflammation α2M protein acts as a carrier for pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α [21-24]. Therefore, herein current study A2M gene expression has been investigated as an indicator for the proinflammatory condition caused by STZ toxicity. As presented in Fig. (**4**) STZ toxicity upregulated the mRNA expression of A2M gene which may indicate that hepatocytes tried to

resist the stress caused by STZ and that may be more striking with simultaneous upregulation of gene expression of both IL-6 and TNF-α. However, this reaction was declined in both experimental groups; insulin injected group and JPE oral co-treated group.

CONCLUSION

In conclusion, STZ toxicity induces acute inflammation as visually observed in pancreatic histology slides and indicated *via* both increased serum levels of IL-2, IL-6 and TNF-α and, *via* the upregulation of the gene expression of IL-6, TNF-α as well as A2M. The anti-inflammatory effect of JPE was significantly observed *via* the limited techniques used in this study which of course require further studies to identify what were the active components in JPE extract that exerted that sort of action and how?

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures were approved by the Animal Care Committee of Taif University, Iran, for the project #4825/1437/1.

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are the basis of this research. All the reported experiments on rats were carried out due to Implementing Regulations of the Law of Ethics of Research on Living Creatures Published *via* National Committee of BioEthics (NCBE), 2nd edition, 2016.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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Declared none.

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