Original Research Article

Therapeutic Effects of Pomegranate (Punica Granatum L.) Juice on Liver of Diabetic Mice

ABSTRACT

Diabetes mellitus (DM) is serious metabolic disorder. Pomegranate has various biological effects. Present study evaluated pomegranate juice (PJ) therapeutic effects on liver of Streptozotocin (STZ) induced DM mice model. Fifty male mice distributed into 5 equal groups (10 each). Groups were negative control, PJ group received orally PJ (180 mg/kg), diabetic group received STZ (60 mg/kg), diabetic received pomegranate juice (STZ+PJ) group, diabetic received metformin (250 mg/kg) (STZ+MET) group. Experiment duration was 6 weeks. Initial and final body weights and liver weights measured. At experimental end, blood glucose levels and liver enzymes serum levels as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) measured. Liver histological examination made under light microscope. STZ led to significant decreased in final body weight, increased in blood glucose and serum levels of AST, ALT and ALP versus control. PJ administration to diabetic mice significantly decreased blood glucose and serum levels of liver enzymes versus STZ group but AST and ALP levels still elevated versus control. Histopathological liver examination of PJ- and MET- treated groups showed amelioration of histological changes of DM. Metformin repair damaged hepatic cells, but was not as effective as PJ. In conclusion, pomegranate juice improves liver health in diabetic mice.

Keywords: *Diabetes mellitus; Liver; Metformin; Mice; Pomegranate juice.*

1. INTRODUCTION

The liver is the main organ responsible for many important functions and has an essential role in metabolism of foreign substances entering the body [1]. The enzymes alanine aminotransferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP) are often used in assessing liver integrity. AST is a hepatic enzyme that catalyzes transamination reaction of alanine amino acid. AST is found in highest concentration chiefly in heart muscles versus other tissues like skeletal muscles, kidney, and liver [2]. ALT and ALP are more specific compared with AST in assessment of liver functions [2].

Diabetes mellitus (DM) is a chronic, complicated metabolic disorder manifested by hyperglycemia, which often results from defects in insulin action, insulin secretion, or both. DM is associated with severe abnormalities of carbohydrate, fat, and protein metabolism. Hyperglycemia affected structure and function of many organs as liver, kidney, heart and blood vessels [3]. World Health Organization reported that Saudi Arabia positions seventh in world for diabetes rates. About seven million of community in Saudi Arabia is diabetic and 3 million are pre-diabetic. Diabetes elevated ten times between Saudi population in previous three decades [4]. DM associated with increased free radicals formation leading to oxidative stress. High levels of free radicals led to damage of cellular organelles and increased lipid peroxidation in cellular membranes [3]. Streptozotocin (STZ), an antibiotic with diabetogenic actions, acutely and excessively destructed selective pancreatic β cells. STZ-induced diabetic animals provide a model of β-cell deterioration through glucose toxicity. STZ enters β cell through glucose transporter and leads to DNA damage that induces poly ADP-ribosylation activation. Poly ADP-ribosylation deplete cellular NAD^+ and ATP that led to defect in insulin secretion [5].

Pomegranate (Punica granatum L.) is a member of Punicaceae family, one of the most ancient edible fruits that widely grown in Mediterranean regions as Iraq, Iran, Egypt, and India, but sparsely cultivated in Japan, China, USA, and Russia [6]. Pomegranate fruit has valuable compounds in different parts of fruit. These parts divided into seeds, peel, and arils. An important product get from pomegranate fruit is juice that can be obtained from arils or whole fruit [7]. Pomegranate juice (PJ) is rich in bioactive substances like polyphenols, tannic acid, gallic acid, anthocyanidins, ellagic acid, ascorbic acid, and minerals as Ca, Fe, Mg, Se and Zn that exert antioxidant actions and prevent oxidative stress [7]. Pomegranate juice has highest amount of phenolic compounds compared with commonly consumed fruit juices, like grape, grapefruit, cranberry, or orange juice [8]. Pomegranate has different biological activities, as potent antioxidant, antiproliferative and antiartherogenic apoptotic [7]. The potential properties of pomegranate is useful in treatment and prevention of cancer [9], cardiovascular disorders [10], treatment of acquired immune deficiency syndrome [11] and antidiabetic activity [12]. Pomegranate peel extract (PPE) provided a prophylactic action versus diabetes-induced alteration in rat liver [13]. Pomegranate flower (PGF) improve insulin resistance, suppress postprandial hyperglycemia [14] and decreased cardiac fibrosis [15] in Zucker diabetic fatty (ZDF) rats, a genetic model of type 2 DM and obesity. The antidiabetic activity of pomegranate attributed to its ellagitannins [16].

Metformin (dimethyl biguanide) is a drug that decreased blood glucose level and improve glucose tolerance without changing plasma insulin profile [17]. Direct effects of metformin include suppressed activity of respiratory electron transport chain in mitochondria and activation of cytoplasmic protein kinase referred to as AMP activated protein kinase (AMPK). AMPK is an important sensor of cellular energy homeostasis and is sensitive to AMP: ATP ratio [18].

The aim of the present research was to evaluate the therapeutic effects of Pomegranate juice administration for six weeks on liver of diabetic adult male mice.

2. MATERIAL AND METHODS

2.1 Drugs

Pomegranate fresh fruits were obtained from Taif region (Al Bustan farm), Saudi Arabia. Streptozotocin was purchased from Sigma Aldrich Chemical Co., Ltd. (Egypt Cairo). Metformin 500 mg obtained from AL-Nhdi pharmacy, Jeddah, Saudi Arabia. Mouse alanine transaminase ELISA kit was purchased from Geno Technology, Inc. (USA). Mouse total alkaline phosphatases and mouse aspartate aminotransferase ELISA kits purchased from My Bio- Source, Inc., California, San Diego (USA).

2.2 Pomegranate juice preparation

The fruits of fresh pomegranate were washed and manually peeled, without separating the seeds. Pomegranate juice obtained using a commercial blender (Moulinex) and filtrated with a Buchner funnel to remove water insoluble materials and immediately diluted with distilled water to volume of (1:10 water/volume) [19].

2.3 Animals

Adult healthy 50 Swiss Webster mice (SWM) approximately 6-8 weeks old and weighing (25 - 30) gm obtained from Experimental Animal Laboratory of King Abdul-Aziz University at King Fahd Center for Medical Research, Jeddah, Saudi Arabia. The Biomedical Ethics Committee at Umm Al-Qura University, Makkah, Saudi Arabia confirmed the research. All animals were treated in accordance with the Principles of Laboratory Animals Care. The animals housed under similar standard environmental conditions in suitable cages (20 x 32 x 20 cm for every 5 mice). Animals kept at room temperature (25°C) with constant humidity (40–70%) and 12h/12h light/ dark cycle prior to use in the experimental protocols for 7 days for adaptation. A commercial pellet diet and fresh drinking water were given *ad libitum*. This diet contains 55% cornstarch, 20% casein, 15% corn oil, 5% vitaminzed starch and 5% salt mixture.

2.4 Induction of diabetes mellitus

Mice were fasted 12 h before Streptozotocin injection. Diabetes was induced by a single i.p. injection of Streptozotocin in 0.1 M citrate buffer (pH 4.0) at a dose of 60 mg/kg [20]. Glucose (5%) was given in drinking water to overcome STZ induced hypoglycemia. Blood samples collected on 3rd day to ensure production of diabetes. Mice with fasting blood glucose (FBG) higher than 175 mg/dl considered as diabetic [21].

2.5 Experimental protocol

Mice were divided randomly and equally into 5 groups as follows: Negative control group: Mice fed on normal standard chow diet with free water supply. Pomegranate juice group (PJ): Mice received PJ orally in dose of 180 mg /kg [21] by nasogastric tube for 6 weeks. Diabetic control group (STZ): Mice subjected to induction of diabetic mellitus by a single i.p. of STZ (60 mg/kg) [20] and fed on normal standard rat chow diet. Pomegranate juice-treated diabetic group (STZ+PJ): Mice subjected to induction of diabetes and received orally pomegranate juice for 6 weeks by nasogastric tube. Metformin -treated diabetic group (STZ+MET): Mice subjected to induction of diabetes and received orally metformin in dose of 250 mg/kg/day [22] for 6 weeks by nasogastric tube.

2.6 Determination of weights

The body weights were monitored at beginning and end of experiment before sacrificing using a digital scale. The biological values of diets assessed by estimation of body weight gain percent (BWG %) as following final body weight minus initial body weight divided by initial body

weight and multiply by 100. At end of 6th week and under ether anesthesia, abdomen of mice was opened after reaching the stage of surgical anesthesia, as evident by absence of withdrawal reflex. Liver was excised, cleaned and washed and liver weight was determined. Liver index was calculated by dividing liver weight by final body weight and multiply by 100.

2.7 Collection of blood samples

At experimental end, 4 ml of blood was gathered from retro-orbital plexus using capillary tube inserted in medial canthus. To obtain serum, the blood gathered into a dry clean graduated glass centrifuge tube. Blood was rapidly set to centrifuge at 3000 r.p.m. for 15 minutes. Supernatant serum was sucked into Eppendorf tubes and stored frozen at -20ºC till used**.** Fasting blood glucose (FBG) and liver function tests as ALT, AST and ALP in sera were determined with the suitable kits.

2.8 Histopathological studies

At experimental end, liver was cut into small pieces and fixed in % formaldehyde solution and processed for light microscopic examination to get paraffin sections of 5 µm thickness. Sections stained with Hematoxylin and Eosin (H&E). Slides were mounted using entellan and covered with coverslips prior to viewing and photography by light microscope.

2.9 Statistical analysis

Data were expresses as mean +/- standard error of mean. Analyzed was made by IBM SPSS Statistics for Windows, version 23 (IBM SPSS, IBM Corp., Armonk, N.Y., USA). Shapiro – Wilk test used to evaluate normal data distribution. One-way ANOVA test followed by least significant difference (Tukey's), assuming groups' equal variance, utilized to calculate significance. *P*-values of <0.05 were considered statistically significant.

3. RESULTS

3.1 Biological results

There were insignificant changes of the initial body weight, liver weight and liver index between different studied groups. Final body weights significantly decreased in STZ, STZ+PJ and STZ+MET groups versus control (*P* <0.010, *P* <0.0001 and *P* <0.050, respectively. The percentage weight gains in control, PJ, STZ, STZ+PJ and STZ+MET were 33.40%, 22.62%, 11.45%, 4.32% and 16.61%, respectively (Table 1).

Table 1: Weights of different studied groups.

Variables	Control	PJ	STZ	STZ+PJ	STZ+MET
Initial body weight (grams)	28.74±0.17	29.44±0.37	29.88±0.33	29.20±0.48	29.50±0.58
Final body weight (grams)	38.34±1.29	36.10±0.97	33.30±0.91**	30.46±0.94***	34.40±0.70*
Body weight gain (%)	33.40%	22.62%	11.45%	4.32%	16.61%
Liver weight (gram)	1.98±0.09	1.64±0.08	1.66±0.11	1.49±0.07	1.56±0.24
Liver index (%)	5.17±0.15	4.55±0.23	5.01±0.40	4.87±0.10	4.54±0.67

*: Significance versus control. *: *P* <0.050, **: *P* <0.010, ***: *P* <0.0001.

3.2 Biochemical results

The FBG levels were significantly increased in STZ group versus control, PJ, STZ+PJ and STZ+MET (*P* <0.0001 for all). Meanwhile, FBG level was significantly decreased in PJ group versus control (*P* <0.050). Serum levels of ALT were significantly increased in STZ group versus control, PJ, STZ+PJ and STZ+MET (*P* <0.0001 for all) and in STZ+MET group versus control (*P* <0.0001) that indicated that MET was not efficient to return ALT level to normal. Serum levels of AST and ALP were significantly increased in STZ group versus control, PJ,

STZ+PJ and STZ+MET (P <0.0001 for all) and in STZ+PJ and STZ+MET groups versus control (P <0.0001 for all) (Table 2).

Table 2: Fasting blood glucose and liver function tests in different studied groups.

Variables	Control	PJ	STZ	STZ+PJ	STZ+MET
FBG (mg/dl)	141.40±3.45	117.60±5.35*,###	297.80±7.44***	131.80±4.03###	132.00±4.42 ###
ALT (U/L)	99.80±4.57	115.50±1.89###	241.40±8.76***	123.80±5.77###	159.90±8.41***, ###
AST (U/L)	121.80±5.52	126.20±1.10###	337.60±2.85***	220.50±4.34***, ###	222.20±4.64***, ###
ALP (U/L)	63.90±2.09	65.44±2.00###	148.77±2.30***	110.77±2.48***, ###	114.49±2.81***, ###

FBG: fasting blood glucose; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase. *: Significance versus control, #: Significance versus STZ. *: P <0.050, ***: P <0.0001.

3.3 Histological results

The histological examination of the liver sections collected from mice in the control group appeared normal in structure with classic hepatic lobules. The lobule was formed of cords of hepatocytes radiating from central vein to periphery of the lobule. The hepatocytes were polyhedral in shape with normal cytoplasm and nuclei. Normal hepatic sinusoids were observed between the hepatic cords (Fig. 1a). The portal area appeared with a normal structure containing portal artery, portal vein and bile duct lined with cuboidal cells with a circular nucleus (Fig. 1b).

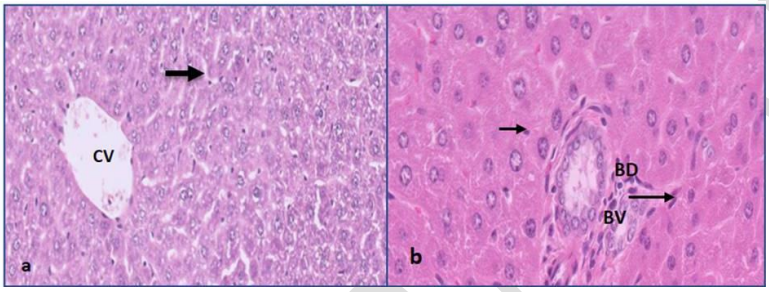

Fig.1. Light micrograph of mice liver section in control group (a) showing the normal structure of central vein (CV) surrounded by the well-distributed hepatocytes (HC) and hepatic sinusoids (arrow) (H&E, X200), (b) showing portal area which contains portal artery, portal vein (BV) and bile duct (BD) lined with cuboidal cells with circular nucleus and Kupffer cell (arrows) (H&E, X200).

The examination of liver tissue in PJ group showed a normal structure of the hepatic tissues, cords of hepatocytes, and central vein. Also, the hepatocytes, hepatic sinusoids, and portal area appeared normal (Figs. 2a and 2b).

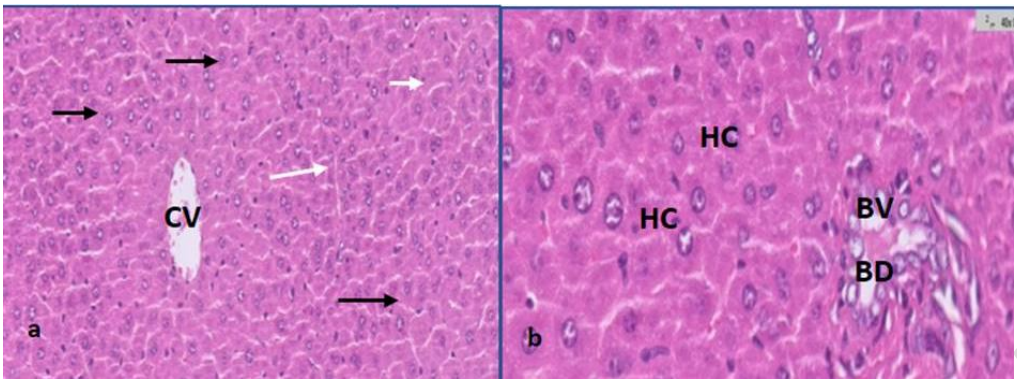

Fig.2. Light microphotographs of liver section from mice treated for 30 days with PJ (a) showing the normal structure of the liver with central vein (CV), blood sinusoids (white arrow) and normal hepatocytes cell cords (black arrow) (H&E, X 200), (b) showing the portal area with normal structure of portal vein (BV), bile duct (BD) and hepatocytes (HC) (H&E, X 400).

Light microscopic examination of the liver sections of rats treated with STZ for 30 days showed obvious histopathological alterations in the liver structure, which included the presence of highly dilatation and congestion of blood vessel, destruction of the lining epithelial cells in a central vein (Fig. 3a). The hepatocytes, highly vacuolated cytoplasm, congestion and destruction of the lining epithelial cells in a central vein (Fig. 3b), dilatation and congestion of blood vein, proliferation in Kupffer cells and abnormal central vein (Fig. 3c) in addition to cellular infiltration in the portal area (Fig. 3d).

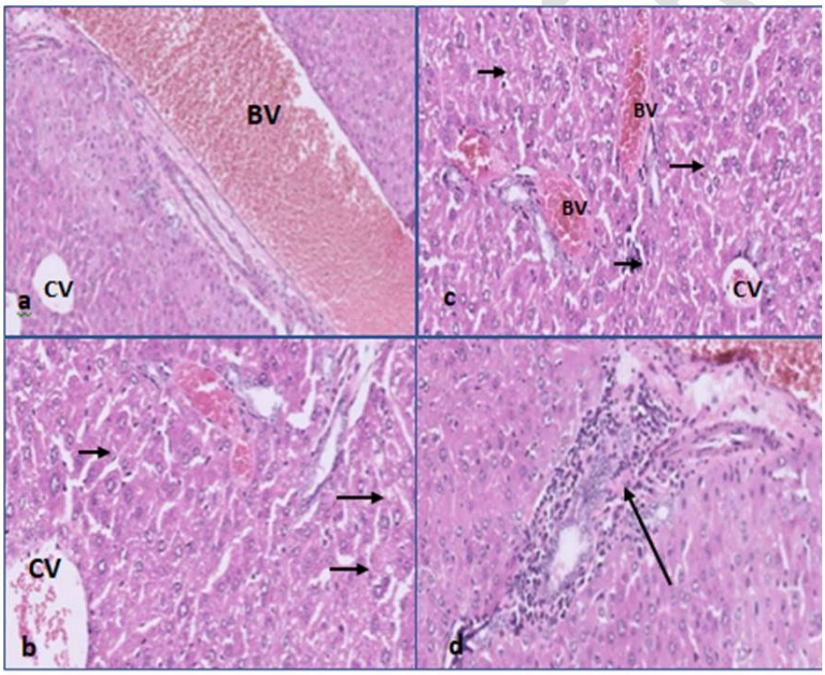

Fig. 3. Light microphotographs of liver sections from male mice treated for 30 days with STZ (a) showing highly dilatation and congestion of blood vessel (BV), destruction of the lining epithelial cells in a central vein (CV) (H&E, X 200), (b) hepatocytes showed highly vacuolated cytoplasm (arrow), congestion and destruction of the lining epithelial cells in a central vein (CV) (H&E, X 400), (c) dilatation and congestion of blood vein (BV), proliferation in Kupffer cells

(K) (arrow) and abnormal central vein (CV) (H&E, X 200), (d) showing cellular infiltration in the portal area (arrow) (H&E, X400).

Combined treatment of STZ with PJ showed that the histological structure of the liver was nearly similar to the control group. The hepatocytes, central vein, and hepatic sinusoids appeared normal in structure (Fig.4a). The portal area appeared with a normal bile duct, portal vein, and hepatic artery (Fig. 4b).

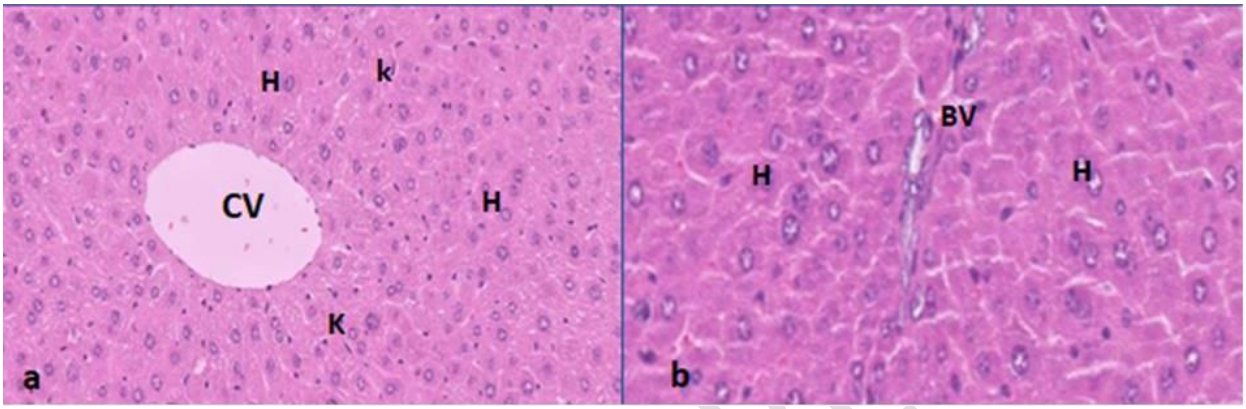

Fig. 4. Light microphotographs of liver sections from male mice treated for 30 days with STZ combination with PJ (a) showing the normal structure of the liver, central vein (CV), hepatocytes (H), and Kupffer cell (K), (b) showing the histological structure of the portal area with normal hepatocytes (H), bile duct and blood vessel (BV) (H&E, X400).

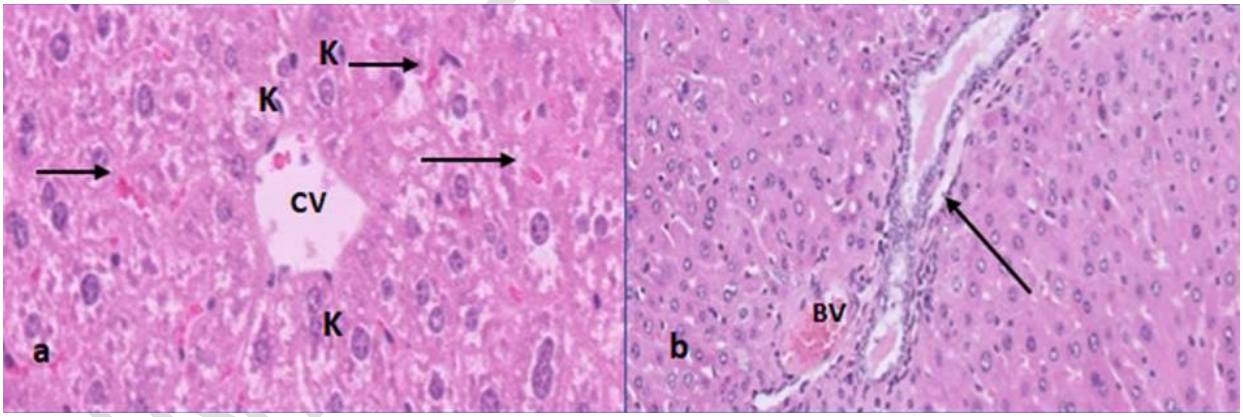

Fig. 5. Light microphotographs of liver sections from male mice treated for 30 days with STZ combination with MET (a) showing bleeding between hepatocytes (arrow), and proliferation in Kupffer cells (K), (H&E, X400), (b) showing the cellular infiltration in the portal area (arrow) and showing dilatation and congestion of blood vein (BV) (H&E, X200).

Combined treatment of STZ + MET showed bleeding between hepatocytes, and proliferation in Kupffer cells (Fig.5a) as well as cellular infiltration in the portal area dilatation and congestion of blood vein (Fig. 5b).

4. DISCUSSION

The liver is considered largest important organ that deals with metabolic processing of many nutrient substances as carbohydrate as well as drug and toxin detoxification in both normal and diseased status [1]. Diabetes mellitus is associated with increased oxidative stress caused by hyperglycemias [23]. Elevated free radicles in oxidative stress destruct several organs including the liver [24]. In spite of the presence of known antidiabetic medicines in pharmaceutical market, remedies from medicinal plants are used with success to treat diabetes mellitus and its complications. In the present study, therapeutic effect of PJ evaluated regards its effectiveness in decreasing level of blood glucose in STZ-diabetic mice and effect on liver functions and structure.

The result of this research revealed that final body weights of diabetic mice (STZ, STZ+PJ and MET+STZ) groups were significantly reduced versus negative control. These findings were similar to other researchers [13, 25, 26]. Decrease in body weights in DM is usually due to abnormalities of glucose metabolism and decreased of glucose uptake by body cells with subsequent shifting to adipose tissue and muscles as sources of energy that leads to weight loss [27]. Meanwhile, the results of this study revealed that mice administered PJ for 6 weeks after induction of diabetes revealed increase in body weight gains. The percentage weight gains in control, PJ, STZ, STZ+PJ and MET+PJ were 33.40%, 22.62%, 11.45%, 4.32% and 16.61%, respectively. These results hypothesized that utilizing of PJ could be underlying factor for improving appetite and increased weight gain, and decreasing possible diabetic complications [13]. Also, it was reported that PJ supplementation to mice reduces long-term weight in animals that fed high-fat diet [28, 29] that could explained decrease in percentage of weight gain to 4.32% in STZ+PJ group. In contrast, other studies by Amin et al. [30], Nwozo et al. [31] and Gabr [32] revealed significant increases in BWG% of diabetic rats versus control rats. Those authors explained the increase in body weight of diabetic rats might be due to increase of feed and caloric intake by rats. Meanwhile, the result of this study revealed insignificant changes in liver weight and liver index between different studied groups.

The results of the present research revealed significant increase in FBG level in diabetic groups (STZ, STZ+PJ and STZ+MET) versus negative control group. The mechanisms by which STZ brought about its diabetic state included selective destruction of pancreatic insulin secreting ß-cells, which make cells less active and lead to poor glucose utilization by tissues [33]. Meanwhile, FBG in this study was significantly decreased in PJ group versus negative control. Also, the result of this study revealed that treatment of STZ-induced diabetic mice with PJ and with MET significantly reduced FBG level versus STZ group. Most pomegranate products were proved to have hypoglycemic effect both in experimental animals and human researches [34]. The anti-hyperglycemic activity of pomegranate may be through a stimulatory effect on insulin secretion or through improvement of insulin action [33]. Amri et al. [7] attributed the anti-diabetic effect of pomegranate contents as phenolic compounds, ellagic acids, tannic acids, gallic acid, and flavonoids that have hypoglycemic actions. Metformin is very well known insulin sensitizer; its effects on insulin resistance are very much familiar. This drug is recommended by American diabetic association for prevention of type 2 DM and as first line therapy for Type 2 DM therapy in obese patients [35].

The present study revealed that serum levels of ALT were significantly increased in STZ group versus negative control, PJ, STZ+PJ and STZ+MET. Administration of metformin in diabetic mice for 6 weeks did not decreased ALT level to control group. Serum levels of AST and ALP were significantly increased in STZ group versus negative control, PJ, STZ+PJ and STZ+MET. Meanwhile, serum levels of AST and ALP in STZ+PJ and STZ+MET groups were still significantly higher than negative control. In diabetic groups, the elevated serum levels of liver enzymes (ALT and AST) indicate cellular

leakages and loss of functional integrity of hepatic cell membrane. While, elevated serum levels of ALP and bilirubin related to hepatic cells functions [36]. In this study, administration of PJ showed insignificant difference in liver enzymes activities versus negative control, thus PJ could be considered safe causing no harmful effects to liver parenchyma structure and function. Also, PJ oral administration for 6 weeks in diabetic mice significantly decreased serum ALT towards normal level. These indicate that PJ preserved the structural integrity of the hepatocellular membrane and liver cell architecture which is confirmed by histopathological studies. The results of this study were in line with Toklu and coworkers [37] whom assess the effect of chronic PPE administration on liver fibrosis made by bile duct ligation in rats and found that serum AST and ALT levels were significantly decreased by PPE treatment. Toklu and coworkers concluded that pomegranate flowers possess potent antioxidants that have hepato-protective properties. Also, Faddladdeen and Ojaimi [13] reported improvement of ALT, AST and ALP in rats received PPE (200 mg/kg) for 11 weeks and then injected with STZ (55 mg/kg). Oxidative stress induced by STZ and increased blood glucose were most probably linked to increase liver enzymes [38] and explained their decline by the antioxidant action of PJ. Pomegranates decreased the liver toxicity by enhancing enzymatic and non-enzymatic antioxidant defense systems [39]. The presence of polyphenolic compound in PJ proved to play a role in therapeutic activity that associated to its effective antioxidant action both *in vitro* and *in vivo* [40].

Light microscopic examination of liver in the present study showed alteration in diabetic mice liver in the form of disordered hepatic cords, appearance of scattered apoptotic cells, dilation in sinusoids and central veins, destructed central veins lining. Similar results obtained by Aboonabi et al. [41], Al-Attar et al. [42], Faddladdeen [43] and Rodríguez et al. [44]. STZ-cytotoxicity on pancreatic islet β-cells resulting in hyperglycaemic status that interferes with cellular metabolic oxidative mechanisms and promotes de novo generation of free radicals [45]. Hepatocytes necrosis is result in release of enzymes and elevated their serum levels [38]. In animals receiving PJ and MET, hepatocytes showed the absence of changes induced by diabetes where hepatocytes, sinusoids, and portal areas looked similar to those of negative control especially in PJ treated group [13].

5. CONCLUSIONS

From this study, it could be concluded that morphological features of liver tissue using light microscopic studies provided an idea concerning cellular changes in case of diabetes versus negative control. Diabetic changes are due to oxidative stress, which explained the improvement occurred via administration of PJ and MET; the natural supplement that proved to possess high antioxidant activity. Administration of PJ had potential therapeutic action on reducing blood glucose level and protect against hyperglycemic-induced hepatic damage. Therapeutic action of PJ was most probably associated with antioxidant effects of polyphenol content. More studies on the same samples must be made to confirm this mechanism. Furthermore, more work required to define the exact active ingredients of PJ responsible for its antioxidant therapeutic action against diabetic complications including hepatic affection.

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