

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

ABSTRACT

Diabetes mellitus (DM) is a serious metabolic disorder. Pomegranate has various biological effects. The present study evaluated pomegranate juice (PJ) therapeutic effects on the liver of Streptozotocin (STZ) induced DM mice model. Fifty male mice were 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), the diabetic received pomegranate juice (STZ+PJ) group, the diabetic received metformin (250 mg/kg) (STZ+MET) group. The experiment duration was 6 weeks. Initial and final body weights and liver weights were measured. At the experimental end, blood glucose levels and liver enzymes serum levels as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured. A liver histological examination was made under a light microscope. STZ led to a significant decreased in final body weight, and an increase 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 the STZ group but AST and ALP levels were still elevated versus control. Histopathological liver examination of PJ-and MET-treated groups showed amelioration of histological changes of DM. Metformin repairs 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 the metabolism of foreign substances entering the body [1]. “The enzymes alanine aminotransferase (ALT), aspartate amino transferase (AST), and alkaline phosphatase (ALP) are often used in assessing liver integrity. AST is a hepatic enzyme that catalyzes the 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 the 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** the structure and function of many organs such as the liver, kidney, heart, and blood vessels” [3]. “World Health Organization reported that Saudi Arabia positions seventh in the world for diabetes rates. About seven million of the community in Saudi Arabia are diabetic and 3 million are pre-diabetic. Diabetes elevated ten times among the Saudi population in the previous three decades” [4]. DM is associated with increased free radicals formation leading to oxidative stress. High levels of free radicals led to damage to 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** the glucose transporter and **leads to** DNA damage that induces poly ADP-ribosylation activation. Poly ADP-ribosylation depletes cellular NAD^+ and ATP which led to a defect in insulin secretion [5].

“Pomegranate (*Punica granatum* L.) is a member of the Punicaceae family, one of the most ancient edible fruits that widely grown in Mediterranean regions such 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 the fruit. These parts are divided into seeds, peel, and arils. An important product gets from pomegranate fruit is the 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 such as Ca, Fe, Mg, Se, and Zn that exert antioxidant actions and prevent oxidative stress [7]. Pomegranate juice has the highest amount of phenolic compounds compared with commonly consumed fruit juices, like grape, grapefruit, cranberry, or orange juice [8]. Pomegranate has different biological activities, such as potent antioxidant, antiproliferative, and antiatherogenic apoptotic [7]. The potential properties of pomegranate are useful in the 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) improves insulin resistance, suppresses 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 is attributed to its ellagitannins [16].

“Metformin (dimethyl biguanide) is a drug that decreased blood glucose level and improves 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 the liver of diabetic adult male mice.

2. MATERIAL AND METHODS

2.1 Drugs

Pomegranate fresh fruits were obtained from the Taif region (Al Bustan farm), Saudi Arabia. Streptozotocin was purchased from Sigma Aldrich Chemical Co., Ltd. (Egypt Cairo). Metformin 500 mg was obtained from AL-Nhdi pharmacy, Jeddah, Saudi Arabia. The 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 was obtained using a commercial blender (Moulinex) and filtrated with a Buchner funnel to remove water-insoluble materials and immediately diluted with distilled water to a 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 were obtained from the 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 were housed under similar standard environmental conditions in suitable cages (20 x 32 x 20 cm for every 5 mice). Animals were 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% vitaminized 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 were collected on 3rd day to ensure the production of diabetes. Mice with fasting blood glucose (FBG) higher than 175 mg/dl are considered diabetic” [21].

2.5 Experimental protocol

Mice were divided randomly and equally into 5 groups as follows: Negative control group: Mice fed on a normal standard chow diet with free water supply. Pomegranate juice group (PJ): Mice received PJ orally in a 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 a 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 a dose of 250 mg/kg/day [22] for 6 weeks by nasogastric tube.

2.6 Determination of weights

The body weights were monitored at the beginning and end of the experiment before sacrificing using a digital scale. The biological values of diets are assessed by estimation of body weight gain percent (BWG %) as follows 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, the abdomen of mice was opened after reaching the stage of surgical anesthesia, as evidenced by the absence of withdrawal reflex. The liver was excised, cleaned, and washed and liver weight was determined. The liver index was calculated by dividing liver weight by final body weight and multiplying by 100.

2.7 Collection of blood samples

At the experimental end, 4 ml of blood was gathered from the retro-orbital plexus using a capillary tube inserted in the medial canthus. To obtain serum, the blood was 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 -20°C till used. Fasting blood glucose (FBG) and liver function tests such as ALT, AST, and ALP in sera were determined with suitable kits.

2.8 Histopathological studies

At the experimental end, the 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 expressed as mean +/- standard error of the mean. Analysis was made by IBM SPSS Statistics for Windows, version 23 (IBM SPSS, IBM Corp., Armonk, N.Y., USA). Shapiro – Wilk test is 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 in 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 the STZ group versus control, PJ, STZ+PJ, and STZ+MET (*P* <0.0001 for all). Meanwhile, FBG level was significantly decreased in the PJ group versus the control (*P* <0.050). Serum levels of ALT were significantly increased in the STZ group versus control, PJ, STZ+PJ, and STZ+MET (*P* <0.0001 for all) and in the 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 the 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 the central vein to the 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 a portal artery, portal vein, and bile duct lined with cuboidal cells with a circular nucleus (Fig. 1b).

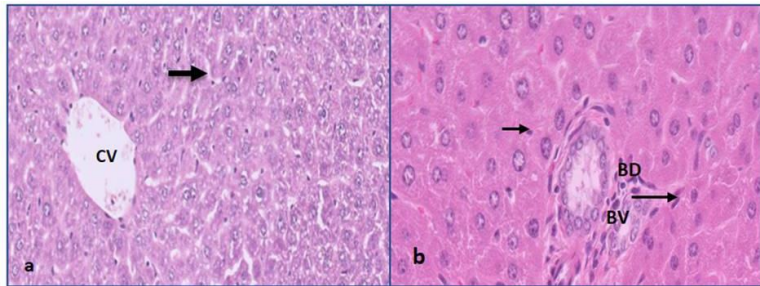


Fig.1. Light micrograph of mice liver section in the control group (a) showing the normal structure of central vein (CV) surrounded by the well-distributed hepatocytes (HC) and hepatic sinusoids (arrow) (H&E, X200), (b) showing the portal area which contains portal artery, portal vein (BV) and bile duct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

The examination of liver tissue in the 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).

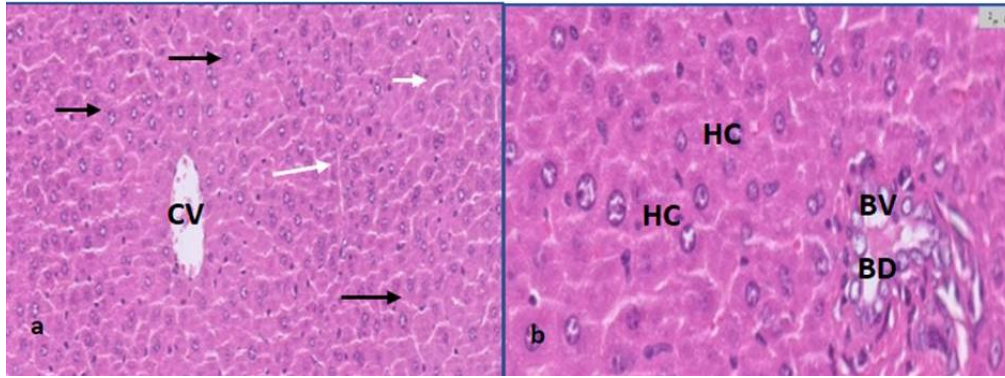


Fig.2. Light microphotographs of liver section from mice treated for 30 days with PJ (a) showing the normal structure of the liver with the central vein (CV), blood sinusoids (white arrow), and normal hepatocytes cell cords (black arrow) (H&E, X 200), (b) showing the portal area with the normal structure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

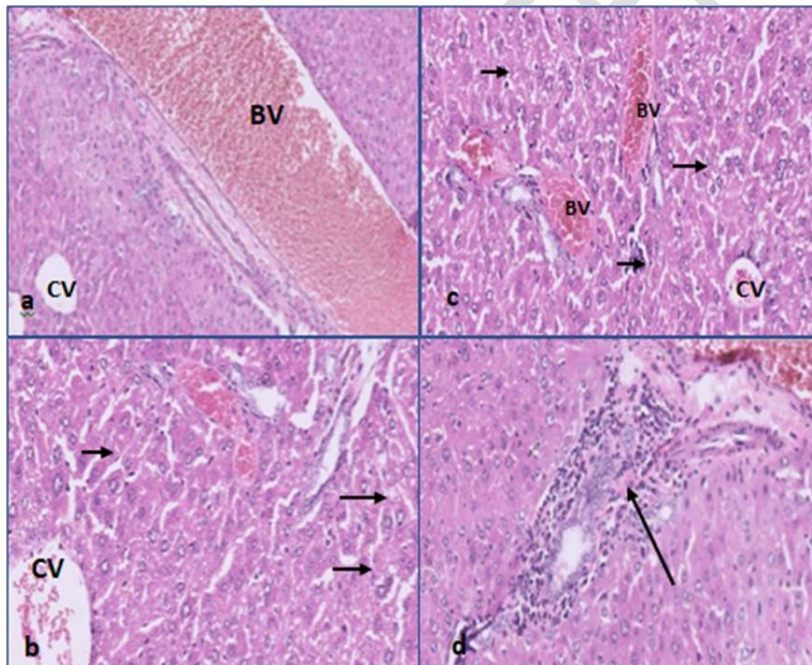


Fig. 3. Light microphotographs of liver sections from male mice treated for 30 days with STZ (a) showing high dilatation and congestion of blood vessels (BV), destruction of the lining epithelial cells in a central vein (CV) (H&E, X 200), (b) hepatocytes showed highly vacuolated cytoplasm (arrow), congestion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

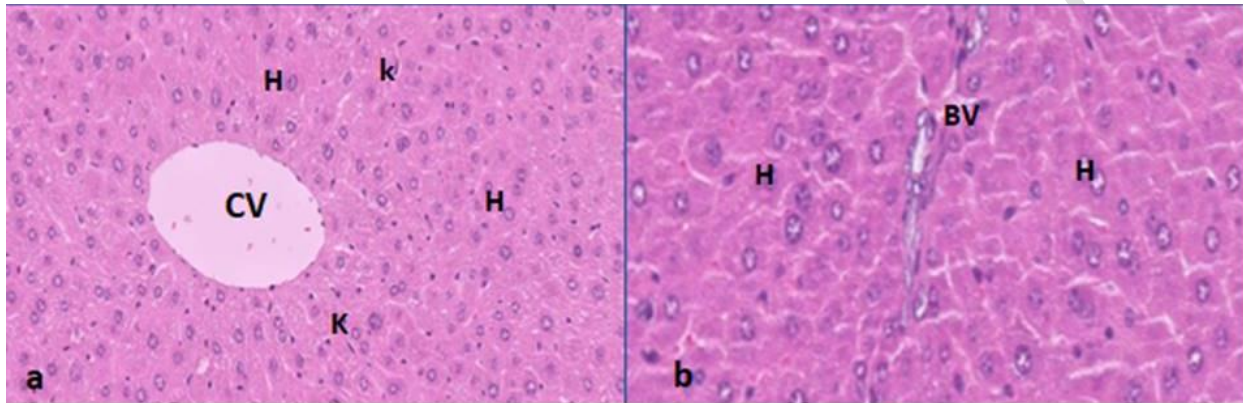


Fig. 4. Light microphotographs of liver sections from male mice treated for 30 days with STZ combination with PJ (a) showing the normal structure of the liver, central vein (CV), hepatocytes (H), and Kupffer cell (K), (b) showing the histological structure of the portal area with normal hepatocytes (H),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

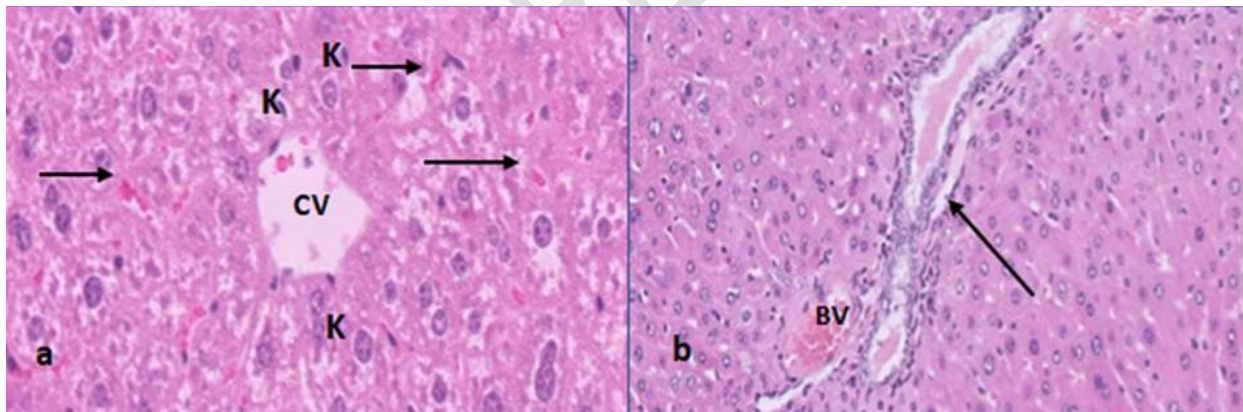


Fig. 5. Light microphotographs of liver sections from male mice treated for 30 days with STZ combination with MET (a) showing bleeding between hepatocytes (arrow), and proliferation in Kupffer cells (K), (H&E, X400), (b) showing the cellular infiltration in the portal area (arrow) and showing dilatation 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 the largest important organ that deals with metabolic processing of many nutrient substances as a 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 hyperglycemia” [23]. “Elevated free radicals in oxidative stress destruct several organs including the liver” [24]. In spite of the presence of known antidiabetic medicines in the pharmaceutical market, remedies from medicinal plants are used with success to treat diabetes mellitus and its complications. In the present study, the therapeutic effect of PJ evaluated regards its effectiveness in decreasing the level of blood glucose in STZ-diabetic mice and its effect on liver functions and structure.

The result of this research revealed that the 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 an 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 PJ could be the underlying factor for improving appetite and increasing weight gain, and decreasing possible diabetic complications [13]. Also, it was reported that PJ supplementation to mice reduces long-term weight in animals that were fed a high-fat diet [28, 29] which could explain the decrease in the percentage of weight gain to 4.32% in the 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 an increase in 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 a significant increase in FBG level in diabetic groups (STZ, STZ+PJ, and STZ+MET) versus the negative control group. The mechanisms by which STZ brought about its diabetic state included selective destruction of pancreatic insulin-secreting β -cells, which make cells less active and lead to poor glucose utilization by tissues” [33]. Meanwhile, FBG in this study was significantly decreased in the PJ group versus the negative control. Also, the result of this study revealed that treatment of STZ-induced diabetic mice with PJ and with MET significantly reduced FBG levels versus the STZ group. Most pomegranate products were proven to have a hypoglycemic effect both in experimental animals and human research [34]. The anti-hyperglycemic activity of pomegranate may be through a stimulatory effect on insulin secretion or through the improvement of insulin action [33]. Amri et al. [7] attributed the anti-diabetic effect of pomegranate contents to phenolic compounds, ellagic acids, tannic acids, gallic acids, and flavonoids that have hypoglycemic actions. “Metformin is a very well-known insulin sensitizer; its effects on insulin resistance are very much familiar. This drug is recommended by the American diabetic association for the 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 the STZ group versus negative control, PJ, STZ+PJ, and STZ+MET. Administration of metformin in diabetic mice for 6 weeks did not decrease ALT levels in to control group. Serum levels of AST and ALP were significantly increased in the STZ group versus the 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 the negative control. In diabetic groups, the elevated serum levels of liver enzymes (ALT

and AST) indicate cellular leakages and loss of functional integrity of the hepatic cell membrane. While elevated serum levels of ALP and bilirubin are related to hepatic cell functions [36]. In this study, the administration of PJ showed an insignificant difference in liver enzyme activities versus negative control, thus PJ could be considered safe causing no harmful effects on liver parenchyma structure and function. Also, PJ oral administration for 6 weeks in diabetic mice significantly decreased serum ALT toward a 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] who 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 in ALT, AST, and ALP in rats that received PPE (200 mg/kg) for 11 weeks and were then injected with STZ (55 mg/kg). “Oxidative stress induced by STZ and increased blood glucose were most probably linked to increased liver enzymes” [38] and explained “their decline by the antioxidant action of PJ. Pomegranates decreased liver toxicity by enhancing enzymatic and non-enzymatic antioxidant defense systems” [39]. “The presence of the polyphenolic compound in PJ proved to play a role in a therapeutic activity that is associated with its effective antioxidant action both *in vitro* and *in vivo*” [40].

Light microscopic examination of the 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, and destructed central veins lining. Similar results were 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]. Hepatocyte necrosis results in the release of enzymes and elevated 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 the case of diabetes versus negative control. Diabetic changes are due to oxidative stress, which explained the improvement that occurred via the 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 levels and protecting against hyperglycemic-induced hepatic damage. The therapeutic action of PJ was most probably associated with the antioxidant effects of polyphenol content. More studies on the same samples must be made to confirm this mechanism. Furthermore, more work is required to define the exact active ingredients of PJ responsible for its antioxidant therapeutic action against diabetic complications including hepatic affection.

Ethical Approval:

Animal Ethic committee approval has been collected and preserved by the author(s)

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