

The Hepatoprotective Effect of Olive Leaf Extract on Diabetic Pregnant Mice and Their Fetuses

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Abstract

Gestational diabetes mellitus (GDM) defined as is a disease with hyperglycemia, insulin resistance and fetal abnormality development. This study was designed to evaluate the

hypoglycemic effect and hepatoprotective properties of olive leaves extract. The experimental mice divided into, the control group GI (not diabetic group), and the diabetic pregnant mice groups were divided into: the diabetic pregnant group (GII) single intraperitoneal injected by streptozotocine (STZ, 240mg/Kg b.wt.). The pregnant mice were given a daily oral dose of olive leaf extract (OLE) only (100 mg/kg) from day 1 to 18 of gestation group (GIII). The diabetic pregnant mice were given daily oral dose of olive leaf extract from day 1 to day 18 of gestation, group (GIV). The STZ-induced diabetic group(GII) exhibited a significant ($p<0.05$) hyperglycemia, accompanied with a significant increase in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), enzyme activities when compared with control group. This result is confirmed with extreme histopathological changes in liver tissues and decreased in glutathione peroxidase (GPx) expression. A significant improvement in glucose level, serum AST and ALT enzyme activities showed in (GIV). Also, OLE succeeded to minimize the severe changes in liver tissues of diabetic pregnant mice and their fetuses. Furthermore, level of (GPx) was returned to near normal level. The findings suggest that OLE have a hepatoprotective effect on pregnant diabetic mice and their fetus.

Keywords: gestational diabetes, olive leaf extract, liver, liver enzyme, fetus

1. Introduction

Diabetes is the most common endocrine disorder characterized by hyperglycemia and resulting from the defects in insulin secretion, insulin action or both (Ghosh *et al.*, 2011). Hyperglycemia contributes to the progression and maintenance of overall oxidative environment. The evidence from both the experimental and clinical studies indicates that oxidative stress plays a major role in the diabetic pathophysiology (Das *et al.*, 2012). Maternal diabetes is a result of either pre-existing diabetes in pregnant women, or due to insulin resistance and high blood glucose during pregnancy, Gestational Diabetes Mellitus, (GDM). Persaud (2007) reported that the fetuses of diabetic mothers are in risk of fetal and neonatal anomalies which results in increased infant mortality and morbidity rates. Streptozotocin (STZ) is used to induce diabetes in experimental animals via its toxic effects on pancreatic β -cells. STZ produce a reactive oxygen species (ROS) which, causing oxidative damage (Szkudelski 2001).

Natural antioxidant products in medicinal plants are used to treat various pathological injuries in the liver considering the role of oxidative stress in their pathogenesis. Olive leaves are traditionally used in many medical conditions for its potent antioxidant activity (Hedeab *et al.* 2015). Recently, Liu *et al.* (2014) reported that olive leaf has been considered as an anti-inflammatory, antioxidant, and anti diabetic agent. The main component of the olive leaves is oleuropein, which is thought to be responsible for pharmacological effects. Furthermore, the olive leaves contain triterpenes (oleanolic and maslinic acid), flavonoides (e.g., luteolin, apigenine, rutin), and chalcones such as olivin, olivindigluconide (Meirinhos *et al.* 2005, Pereira *et al.* 2007). The OLE can promote the insulin resistance and inflammation response in rats with type 2 diabetes induced by high-fat diet and streptozotocin (Liu *et al.* 2014).

The liver was frequently damaged during diabetes, as a consequence of increased levels of oxidative stress and dysregulation of immune function, this damage was confirmed by histological analysis of the liver, which also suggested that olive leaf powders may decrease tissue damage by inhibiting autoimmune reactions and modulating oxidative stress. Park et al.(2013) reported that the effect of olive leaf powder treatment on liver and kidney tissue indicated a mild reduction in tissue damage. The treatment of Cyclosporine in rats with olive leaf extract showed an ameliorative effect in levels of glutathione peroxidase compared to cyclosporine treated group (Hedeab et al.2015). The OLE treatment led to decrease ALT and AST activities in serum levels, increase in GSH levels in organs and ameliorate histopathological findings in liver tissues. The amelioration in histopathological findings supported the hepatoprotection effect by olive leaf extract due to its potent antioxidant activity (Topalović et al. 2015).

In this study, we determine the protective effect of olive leaf extract on pregnant diabetic mothers and their fetuses histologically and biochemically.

2. Materials and Methods

2.1 Preparation of Olive Leaves Extract

Olive leaves were purchased from a commercial market, Hail, Saudi Arabia. The leaves were scientifically defined by the botany staff of biology department, and voucher specimens were deposited at the Herbarium of Biological Sciences Department, Faculty of Sciences, Hail University, Hail, Saudi Arabia. For the preparation of aqueous olive leaves extract, the leaves were washed and dried at room temperature. The dried olive leaves (50 g) were powdered and added to 2 L of hot water in a flask. After 6 h, the mixture was slowly boiled for 1 h. then, cooled at room temperature. The mixture was gently subjected to an electric mixer for 10 min. After that, the solution of olive leaves was filtered. Finally, the filtrates were evaporated in an oven at 40 °C to produce dried residues (active principles). Furthermore, these extracts were prepared every 2 weeks and stored in a refrigerator for experimentation (Al-Attar & Abu Zeid., 2013, Sakr & Lamfon., 2012).

2.2 Experimental Animals

The present experimental study was carried out on CD-1 mice, with an average body weight of 20 – 30g obtained from the breeding unit of King Saud University, Rhyaid, KSA. All animal experimental protocols were approved by Committee of Scientific Ethics at University of Hail (UOH), and were carried out in accordance with its guidelines for animal use. The females and males were kept separately in wire cages under regular conditions of 12/12 hours light/dark cycles. They were fed on cubes consisting of crude protein, minerals and fibers. Vitamins were added as fresh vegetables and the animals were provided with milk and tap water *ad libitum*. The hyperglycemic were induced by single intraperitoneal injection of streptozotocine (STZ, 240mg/Kg body weight, from Sigma chemical Co., St. Louis, Mo) diluted in phosphate buffered saline pH 7.2. After 7 days of injection, the plasma glucose concentration was measured from tail vein by using glycosmeter (Accu Chek (Bayer Diagnostics)). The mice defined as diabetic if the blood glucose value exceeding 250mg/dl.

The diabetic female were mated with non diabetic male over night. If the vaginal plug appeared in the morning, this day defined as 0 day of gestation. The pregnant mice divided into four groups 10mice/each. The control group (GI). The diabetic group (GII) single intraperitoneal injected by streptozotocine (STZ, 240mg/Kg b.wt.). The third group (GIII) the pregnant mice were given a daily oral dose of olive leaf extract only (100 mg/kg) from day 1 to 18 of gestation. Group 4 (GIV) the diabetic pregnant mice were given daily oral dose of olive leaf extract from day 1 to day 18 of gestation.

2.3 Blood Sample Collection

At the end of the experimental period, the animals were anesthetized with chloroform and blood was collected in vacuum tube clot activator from the heart. Then the blood samples were centrifuged at 3500 rpm for 10 min in a centrifuge to separate the serum. The Alanine amino transferase (ALT) and Aspartate aminotransferase (AST) were measured using kits from Reflotron and Liquicolor Analyticals. All assessment assays and kits were performed in accordance with the manufacturers' instructions and protocols.

2.4 Histological Examination

The fetuses and mother's liver tissues of both control and all experimental groups were fixed in 10% formalin for 48 hr, after which they were kept in 70% alcohol. The specimens were dehydrated in ascending series of alcohol, one hour each, cleared in terpineol for 3 days and embedded in 3 changes of pure paraffin wax, one hour each. Serial longitudinal and transverse sections, 5 microns thick, were cut and mounted on clean glass slides. The paraffin sections were stained with Harris's hematoxylin and eosin, cleared in xylol and mounted in neutral canada balsam. Sections of liver of the different groups were carefully examined and photomicrographs were made as requested.

2.5 Immunohistochemistry Examination

Liver tissues of mothers fixed with phosphate-buffered formalin, and embedded in paraffin. Immunohistochemical staining was performed using the standard labeled streptavidin–biotin–peroxidase complex method (LSAB kit; Dako Japan, Kyoto, Japan). Briefly, after routine deparaffinization, 5micron thick sections were rehydrated and antigen retrieved with the Dako 3 in 1 AR buffer EDTA pH 9.0 in a DAKO PT link. Slides were peroxidase blocked in 3% H₂O₂ in methanol for 10 mins. They were then blocked with Dako Protein block for 10 minutes (containing casein 0.25% in PBS) then incubated with primary antibody for 20 min and detected with Dako Envision Flex amplification kit for 30 minutes. Colorimetric detection was completed with Diaminobenzidine for 5 minutes. Slides were counterstained with Haematoxylin and covers lipped.

2.6 Statistical Analysis

All data are presented as mean \pm SD. Results were tested for variance using t- student test. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1 Biochemical Results

a) Mother glucose levels

The plasma glucose concentration was significantly increased in STZ group (GII) compared with the control group (450.2±49.95). The blood glucose levels in diabetic group treated with olive leaf extract (GIV) tended to be lower than those of the diabetic group (GII) (145.2±70.37). In addition, there was no significant difference in plasma glucose level in a group of pregnant mice treated with olive leaf extract only (GIII) comparing with the control group. (Table 1&Fig.1).

Table 1. Effects of olive leaf extract and/or STZ administration on the glucose level of pregnant mice.

Groups	GI	GII	GIII	GIV
Glucose level(mg/dL)	127.8±14.41	450.2±49.95*	132±8.91	145.2±70.37
Mean ±SD		252.2%	3.2%	13.6%
% Change to con				

Data are presented as mean (± SD). *Significantly different from the control group (P<0.05). % change to con: % change to control level

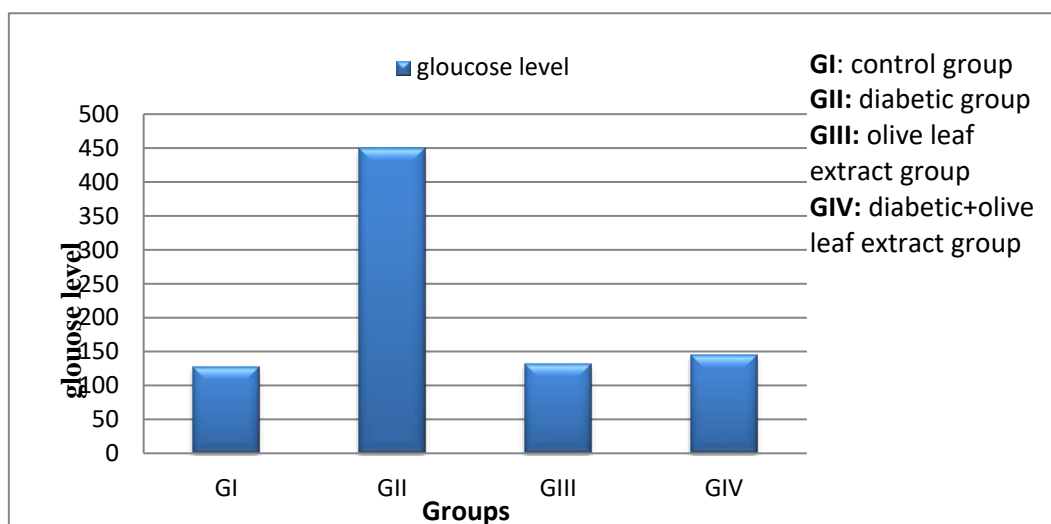


Fig.1: Effects of olive leaf extract and/or STZ administration on the glucose level of pregnant mice

b) Liver enzymes

Liver function was detected by measuring the AST and ALT activities in serum pregnant mice. Significant increases (P<0.05) were recorded in all measuring parameters due to the injection of STZ (240mg/Kg body weight) in pregnant mice (GII), the percentage increase of serum AST and ALT activities was calculated 122.7%, 251.76% respectively when compared to the

normal control level (GI) (Table 2). Administered pregnant mice with olive leaf extract (100mg/kg) (GIII) caused no significant change in the concentration of serum AST activity compared to the normal control level (Table 2). On the other hand, a significant ($P<0.05$) decrease was shown in the level of serum ALT activity recording -37.6% comparing to the normal control group (GI).

Table (2) showed a full restoration in serum AST and ALT activities in diabetic group treated with olive leaf extract (100mg/kg) (GIV) as regarded to the normal control value since all parameters recorded non significant change ($p>0.05$) comparing to the normal control level (GI). On the other hand, serum AST and ALT activities showed a significant reduction ($p<0.05$) compared to diabetic group (GII), with percentages change (-50.67% and -75.25 %.respectively).

Table 2. Effects of olive leaf extract and/or STZ administration on serum ALT and AST levels of non-diabetic and diabetic pregnant mice.

Parameters	GI	GII	GII	GIV
AST (U/L) Mean ± SD % Change to con.	198.5± 14.8	442± 45.25* 122.7%	199.5± 14.8 0.5%	218± 8.48 10.1%
ALT (U/L) Mean ± SD % Change to con.	42.5± 3.5	149.5± 33.2* 251.76%	26.5± 2.12* -37.6%	37± 7.07 -12.9%

Data are presented as mean (± SD). *Significantly different from the control group ($P<0.05$). % change to control level

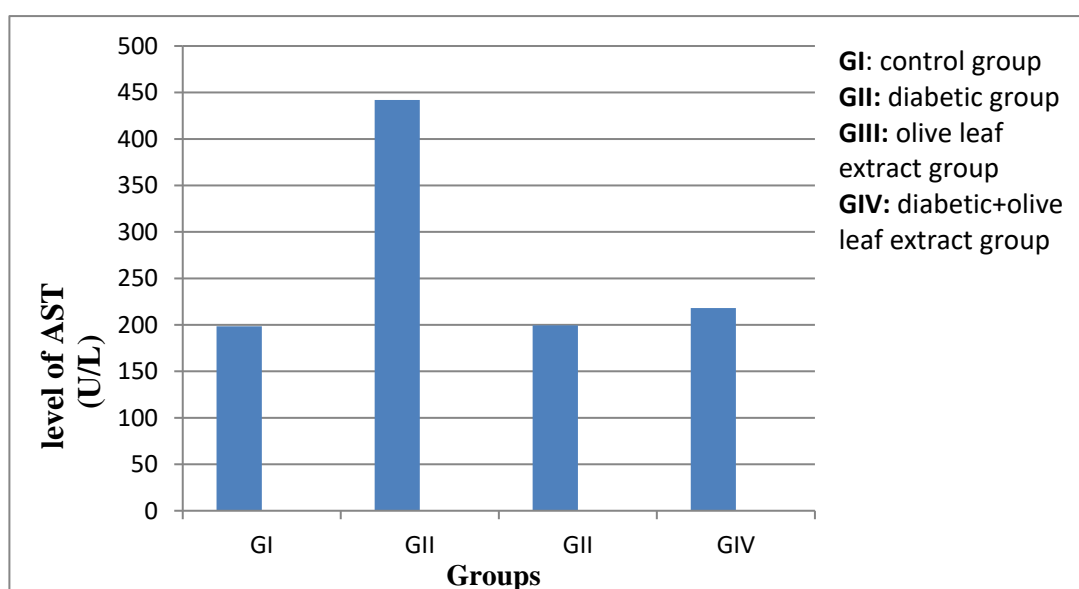


Fig. 2a. Effects of olive leaf extract and/or STZ administration on serum AST levels of non-diabetic and diabetic pregnant mice.

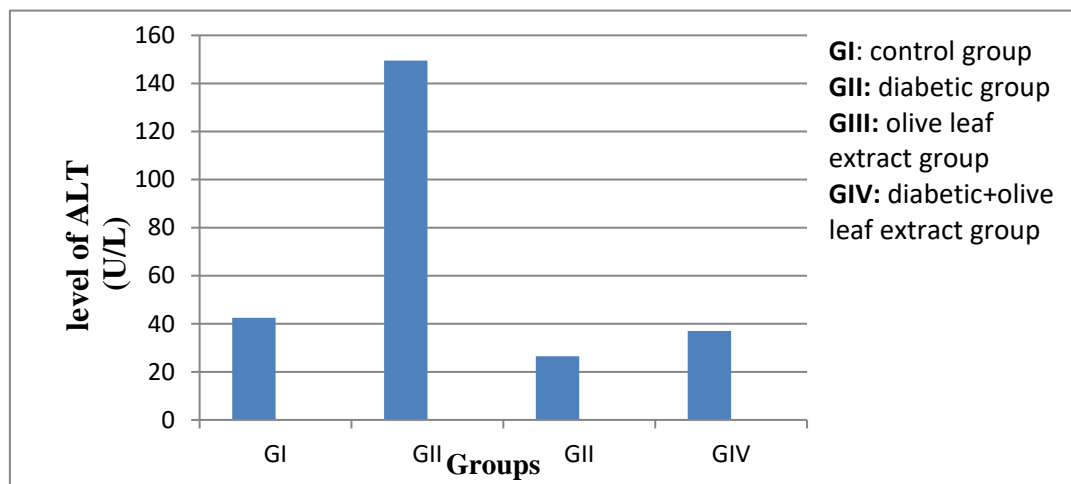


Fig. 2b. Effects of olive leaf extract and/or STZ administration on serum AST levels of non-diabetic and diabetic pregnant mice.

3.2 Histological Results

a) Liver of mothers

The liver section of control mice (GI) showed the classical polygonal hepatic lobules separated by portal tracts. Each lobule consists of a number of polygonal hepatocytes, with rounded nuclei. Blood sinusoids lie between the hepatic cords were lined by sinusoidal and Kupffer cells (Figure. 3a). Hepatic tissues obtained from diabetic pregnant mice (GII) showed extreme extend of histopathological changes which, include severe cellular damage, cellular swelling, necrosis and nuclear pyknosis (Figures. 3b&3c). Oral administrations of the olive leaf extract (GIII) showed the common architecture of the hepatic lobules and the characteristic features of the hepatocytes with mostly normal architecture (Figure. 3d). The treatment of diabetic mice with the olive leaf extract (GIV) caused somewhat variable degrees of amelioration of the histopathological changes induced by the diabetes as shown in (Figures 3e& 3f).

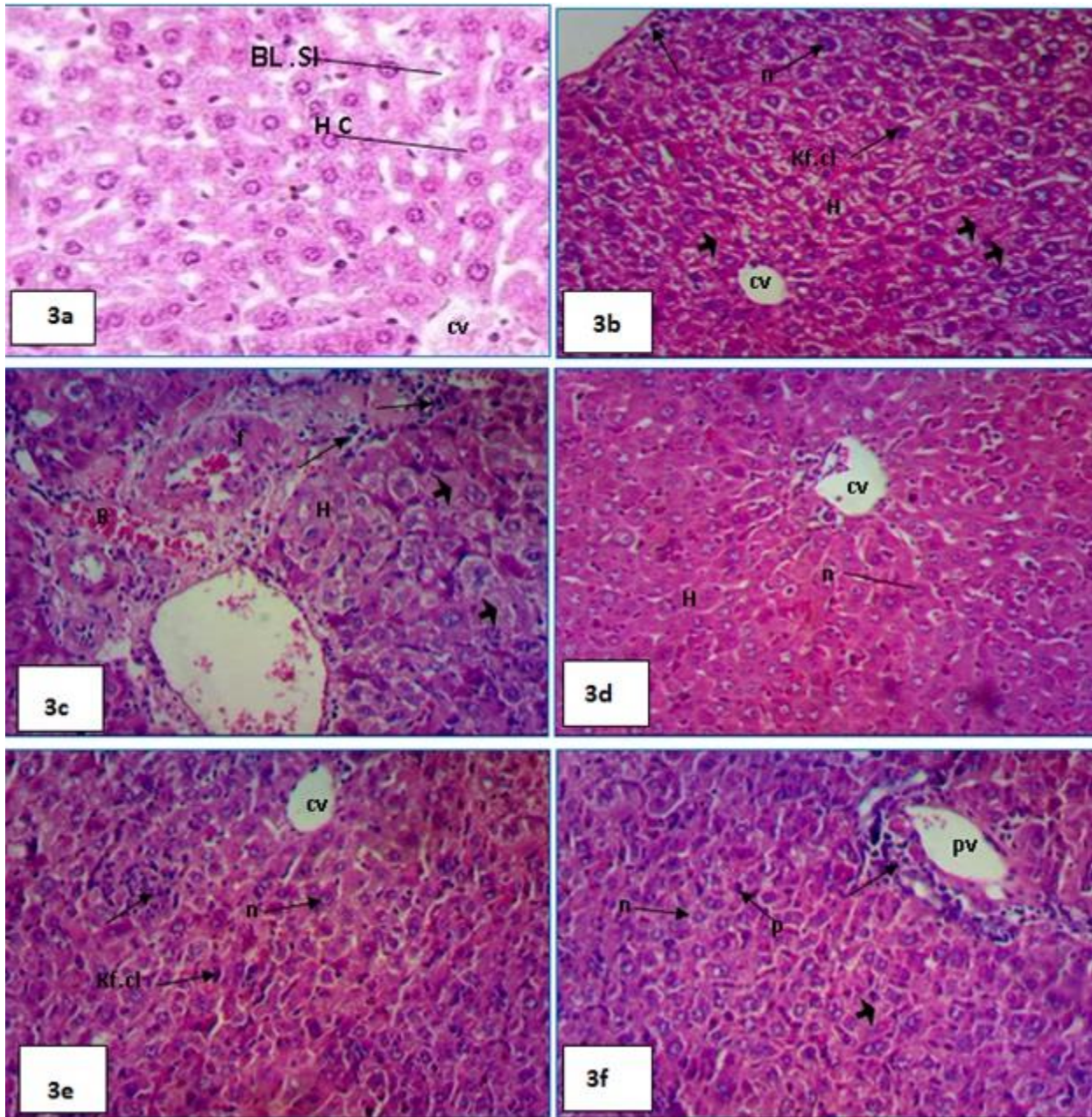


Fig.3. Photomicrograph of H&E stain of liver section (40X). Liver of (GI) showing normal hepatocytes (HC), blood sinusoids (BL.SI) and a central vein (CV) (3a). Diabetic liver (GII) (3b) with swelling hepatic cell (H), pyknotic nuclei (n) and activated Van- Kupffer cells (kf.cl). Some of damaged hepatocytes lost their nuclei (head arrows). Appearance of numerous inflammatory cells between the hepatocytes (long arrows) . Liver section of a diabetic mouse (3c) showing nucleated hepatocytes (head arrows) .The portal areas were invaded with numerous lymphocytes (long arrows) and fibrocytes (f). Section of liver of (GIII) showing normal architecture (3d). Liver of (GIV) showing somewhat normal structure with inflammatory cells (arrow)(3e) and cytoplasmic vacuolation (head arrow) (3f).

b) Liver of fetus

The liver of 18-days-old control fetuses (GI) is surrounded by a delicate connective tissue capsule. It is composed of numerous hepatic lobules with indistinct outlines, since the interlobular connective tissue is poorly developed. The portal spaces in between the hepatic lobules are hardly distinguished. Each lobule possesses a central vein and contains numerous strands of hepatic cells. The hepatic strands anastomose to form a network enclosing a system of tortuous blood sinusoids (Figures.4a&4b). The liver of 18-days-old fetuses (GIII) showed to somewhat no difference with the control group (Figures.4e&4f). However, the liver of 18-days-old fetuses of (GII) showed congestion of the central vein and blood sinusoids. The liver lost its normal architecture and the hepatocytes showed marked vacuolar degeneration with necrosis and contained pyknotic nuclei (Figures.4c&4d). The treated fetus liver tissue of group 4 (GIV) showed mild amelioration in its architecture (Figures.4g&4h).

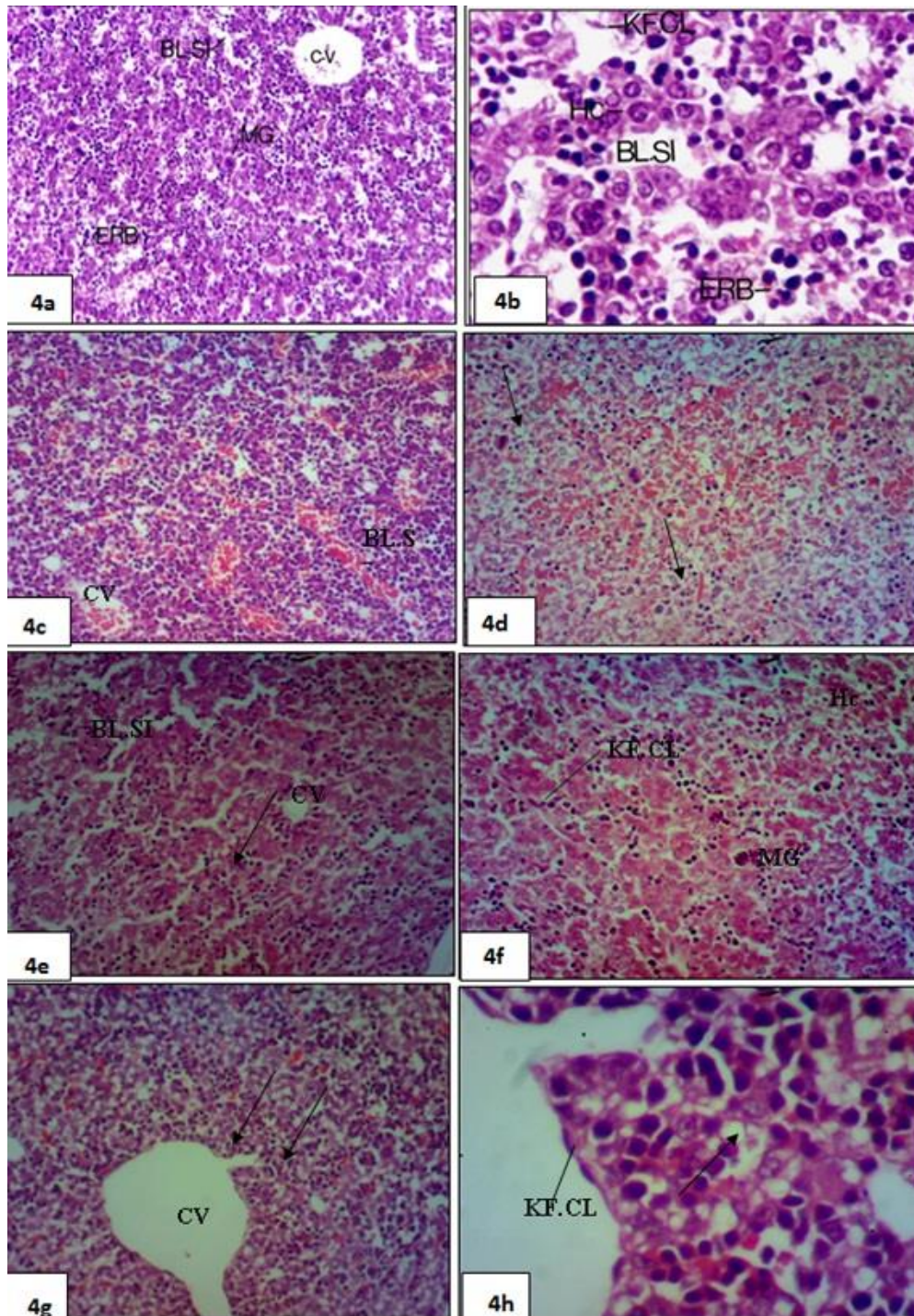


Fig. 4(a- h). H&E stain photomicrograph of liver section from 18-days-old fetus. (4a&b) for control fetus (GI) (20-40X respectively). (Fig.4c) for (GII) showing congestion in central vein (CV), blood sinusoid (BL.SI) (20X) and vacuolated cytoplasm of hepatic cells (arrows) (20X) (4d). Liver of (GIII) (4e&f,20X) showing normal liver tissue. (Fig.4g) showing liver of fetus of (GIV) with central vein (CV) which surrounding by mild inflammatory cells (arrows) (20X) and some vacuolated cytoplasm (arrows) (4h) (40X).

3.3 Immunohistochemical Results

Diabetic pregnant mice (GII) showed decrease in the amount of glutathione peroxidase (GPx) indicated by the faint brown stain (Figure.5b) as compared to normal control group (GI) (Figure. 5a). The administration of OLE only (GIII) showed nearly normal appearance of GPx (Figure. 5c). The treatment pregnant diabetic mice with olive leaf extract (GIV) increased the GPx amount to nearly normal appearance (Figure. 5d).

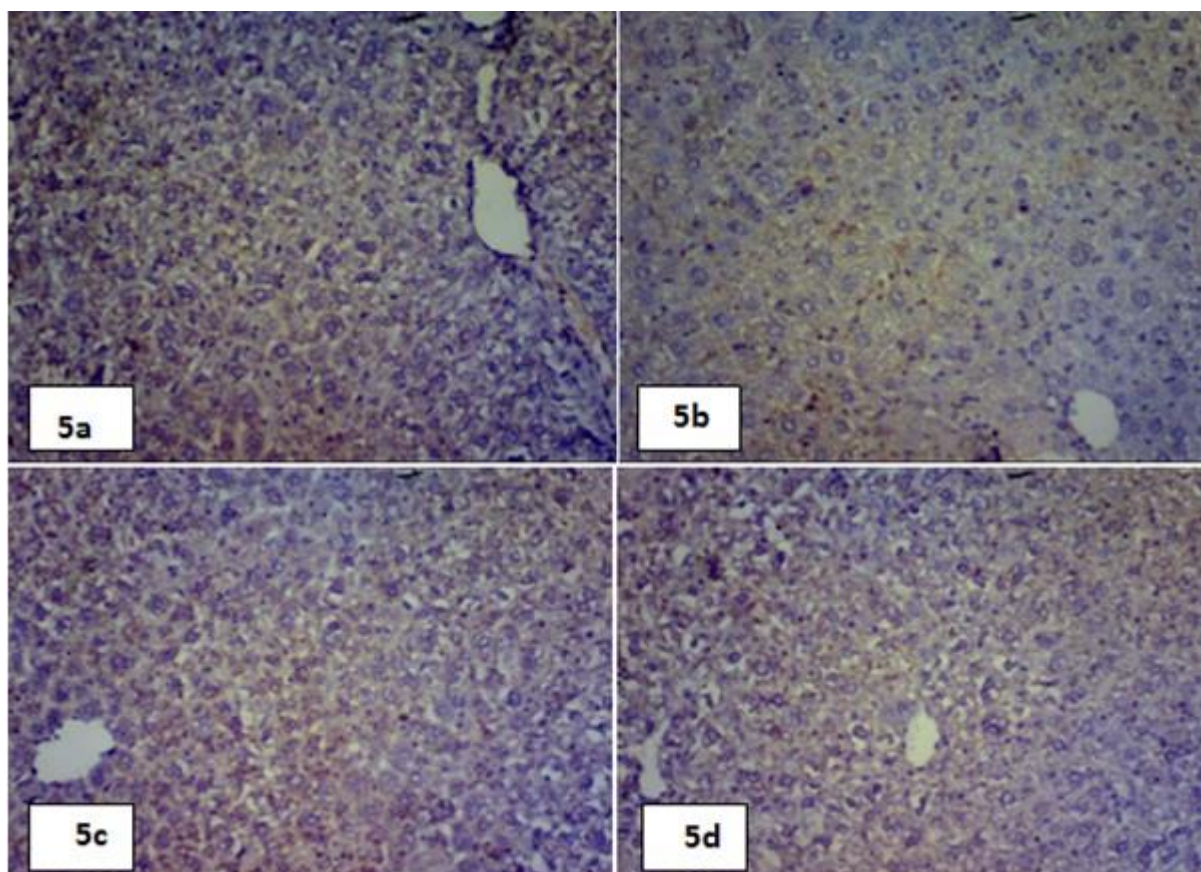


Fig.5. **GPx- immunostaining (40X)** photomicrograph of section of Liver of mice showing positive immunoreactivity for glutathione peroxidase (GPx) (5a) of (GI) and (5c) for (GIII). Liver of (GII) showing faint brown staining in the cells and around the central vein (5b). Liver of (GIV) showing marked increase in the GPx amount (5d).

4. Discussion

The present study demonstrates the ameliorated role of olive leaf extract against hyperglycemic hepatic complications. In the present study, STZ-induced diabetic pregnant mice (GII) showed a significant increase in glucose level as compared to the control group. These results are in agreement with those of many authors using STZ- diabetic animals (El-Naggar et al. 2005; Hassan et al. 2008; Helmy et al. 2007). Gojo et al. (2007) concluded that diabetes can be produced in animals by i.p. injection of STZ which is toxic to β -cells and it is widely used to induce experimental diabetes in laboratory animals.

The results of the present study indicated that oral administration of olive leaves extract to

diabetic group (GIV) showed a full return of blood glucose level comparing to control group. These results are in agreement with other finding, Sanarya et al.(2011) found a significant decrease in blood glucose level in the STZ-diabetic male mice treated with *Olea europaea* aqueous extract, and the hypoglycemic effect recorded with aqueous extract of olive leaves in diabetic rats (Comeyli & Miri Moghadam ,2008). The hypoglycemic effect of olive leaf extract is due to antioxidant properties of olive leaves and also, oleuropein and tannins in olive leaves act as a-glucosidase inhibitors, reducing the absorption of carbohydrates in the gut (Jemai et al., 2009). Moreover, the oleuropein in olive leaf accelerated the cellular uptake of glucose, leading to reduced plasma glucose (Gonzalez et al., 1992).

The damage of the liver caused by diabetes induced by STZ was determined by the alteration in serum marker enzymes beside the histopathological changes in liver tissue. Serum AST, ALT and ALP are the enzyme biomarkers to monitor the liver status and aids in the liver toxicity conditions (Amin & Hamza, 2005). The obtained results in this study revealed that the diabetes induced by STZ induced significant increases in the serum levels of AST and ALT which are evidences of liver cells injury and leakage of enzymes from cells (Bashandy & Wasel, 2011). In addition, Ravikumar et al. (2005) found that rise in ALT activity is almost due to hepatocellular injury and is usually associated with rise in AST. Our results are in agreement with other findings showing that hyperglycemia is associated with an increase in the levels of serum levels of AST and ALT in experimental diabetes studies (Ahmed, 2005; Deros et al.,2007; Rawi et al. 2001). The elevation in AST and ALT levels may be due to the destructive changes in the hepatic cells (Kim et al.,2006; Rawi, 1995). Furthermore, diabetic complications such as increased gluconeogenesis and ketogenesis referred to the increased in the levels of these enzymes (Monday & Uzoma ,2013). This suggestion is supported by histopathological alternation in liver observed during the present study.

The histological studies of liver in diabetic pregnant mice (GII) showed severe changes which include disorganization of the hepatic cords, cellular swelling, few lymphocytic infiltration, necrosis, vacuolization of the cytoplasm together with pyknotic changes in the nuclei of the hepatocytes. These findings are in agreement with those of many authors using STZ- diabetic animals (Arya et al., 2014; Motshakeri et al., 2014; Oryan et al., 2014; Zhou et al., 2008). Hyperglycemia has been found to play a key role in reactive oxygen species (ROS) generated damage (Maritim et al., 2003). Palanduz et al. (2001) indicated that overproduction of ROS and accumulation of lipid peroxidation is by-products in diabetic complications

Till now there is a lack of much work on the effect of maternal diabetes on fetal hepatotoxicity. We found in our present investigation, that maternal diabetes interfered with hepatocyte proliferation and differentiation, causing the liver tissue lost its normal architecture and the hepatocytes showed marked vacuolar degeneration, necrosis and contained pyknotic nuclei associated with congestion of the central vein and blood sinusoids. The maternal diabetes led to severe alterations in livers of developing fetuses by increased apoptic cell death (El-Sayyad et al., 2014). These effects are likely to be as a result of elevated oxidative stress and liberation of free radicals (Brownlee, 2005; Goh & Cooper, 2008).

The treatment of pregnant mice with olive leaf extract (GIV) caused a detectable decrease of the transaminases activity thus improving hepatic function. Similar results were reported by many authors who suggested that oleuropein had hepatoprotective effects (Domitrovic et al., 2012; Eidi et al., 2009; Kim et al., 2014; Mousa et al., 2014). Also, the histopathological findings support the hepatoprotection of olive leaf extract where, the treatment of diabetic group with OLE (GIV) improved the histological architecture with existents of the cytoplasmic vacuoles in some hepatocytes this could be due to antioxidative stress or anticitotoxic effect of the olive leaf extracts. The present findings come in agreement with the results which, indicate that, olive leaf extract has potent antioxidant effects, ameliorated the oxidative liver injury and led to decreases in serum ALT and AST activities (Topalović et al., 2015). The beneficial effect of olive leaf extract appears to be linked to its antioxidant activity which was found to be helpful in the prevention of diabetic complications associated with oxidative stress. These results are in agreement with the findings concluded by Khalil (2004) that an aqueous extract of olive leaf has antioxidant property and hepatoprotective activity.

Antioxidant enzymes, including SOD, CAT and GPx, have a role in scavenging the toxic intermediate of incomplete oxidation. In this study, Glutathione peroxidase (GPx) showed significantly decreased in diabetic liver tissue (GII), which indicates decline scavenging of H₂O₂ and lipid hydroperoxides. This result is coordinate with other study (Friesen et al., 2004). Depletion of tissue glutathione (GSH) levels increased cellular damage caused by oxidative stress in diabetic rats (Tachi et al.2001). The decreased GPx activity illustrate degradation of H₂O₂ (Manju & Nalini 2005; Young et al.,2005). A deficiency in the antioxidant enzymes activities can produce an excess formation of the superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) in cells, which in turn generate more hydroxyl radicals as well as the regeneration of propagation of lipid peroxidation. The decreased of hepatic SOD and GPx activities as a result of increasing ROS generation (Maritim et al., 2003).

In our study treatment of the diabetic pregnant mice with olive leaf extract (GIV) improve GPx activities. The antioxidant enzyme activities (superoxide dismutase and glutathione peroxidase) were improved to nearly normal levels in the olive leaf extract treated rats (Olmez et al., 2015). Also, the antioxidative and antigenotoxic properties of the oleuropein-rich dry olive leaf extract against hydrogen peroxide induced DNA damage in human peripheral blood leukocytes due to its capacity to act as potent free radical scavenger (Topalović et al., 2015). The polyphenolic compounds increased enzyme expression of SOD and GPx in transcriptional level (Vina et al., 2006).

5. Conclusion

In conclusion, the maternal diabetes led to sever alterations in livers of pregnant mice and their developing fetuses. Nevertheless, the results of this study shows that oral administration of the olive leaf extract improve the histological architecture of liver tissue of diabetic mothers and their fetuses, reduces serum ALT and AST levels and increases the GPx activities which may be attributed to the antioxidant effects of the leaves extract.

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