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RESEARCH ARTICLE

Effect of Olive Leaf Extract on the Kidney of Pregnant Diabetic Rats and Their Fetuses

By

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Abstract

Aim of the work: Diabetes during pregnancy leads to fetal congenital malformations and long-term postnatal diseases. Antidiabetic plants are used as supportive therapy in the treatment of diabetes during pregnancy, so the present study aims to investigate the protective effect of olive leaf extract on the kidney of the pregnant rats and their fetuses. **Material and methods:** forty pregnant albino rats were used and categorized into four groups after mating; group 1: control group, group 2: olive leaf extract (1 ml/100gm. b. wt), group 3, streptozotocin induced diabetic rats (STZ 35 mg/kg b.wt.) , group 4, diabetic rats treated with olive leaf extract (as in groups 2&3) . The pregnant females were dissected on the 19th day of pregnancy. Blood sample were collected to estimate the biochemical parameters of the kidney functions. Also, kidney samples were taken for the histological and histochemical studies. **Results:** administration of olive leaves extract didn't change the external characters of the fetuses while, fetuses of the diabetic mothers showed some developmental retardation such as, apalpebralia, anotia and small in size.. On the other hand, fetuses of the diabetic mothers which were treated with olive leaves extract showed somewhat normal morphological development. According to the biochemical, histopathological and histochemical observations, the olive leaf extract succeeded to minimize the drastic changes which were observed in the diabetic rats and their fetuses.. **Conclusion:** it is recommended that the use of the olive leaf extract has the ability to minimize the damage of hyperglycemia.

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Introduction

Historically, infants born to mothers with diabetes have been at significantly greater risk for spontaneous abortion, stillbirth, congenital malformations, and perinatal morbidity and mortality (Kicklighter, 2001). Mogensen et al. (1985) found that elevated blood glucose and related microvascular disease is associated with slow, but progressive damage to the kidneys. This damage becomes detectable when protein (primarily albumin) is excreted in the urine in higher concentrations than normal. As the severity of damage increases, the quantity of protein in the urine also increases, coupled with increasing blood pressure. Eventually, the condition can lead to renal failure (Berch-Johnsen et al., 1985).

Rossing et al. (1995) established that the kidney disease is a known complication of diabetes. Diabetic kidney disease occurs in 20 - 40 % of patients with diabetes and is the leading cause of End Stage Renal Disease (ESRD). Sakata (1999) had proved that long term diabetes was associated with a number of other physical disorders; affecting the kidney (nephropathy). Diabetic nephropathy accounts for about 20-30% of patients (Defronzo, 1988).

It is characterized by proteinuria and progressive kidney failure occurred more frequently when uncontrolled hyperglycemia and hypertension are present (Haffner et al., 1998). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys and nerves and arteries. Diabetic nephropathy raised arterial blood pressure, increased relative mortality for cardiovascular disease and can progress to renal failure (Lovell, 2000; Morrish et al., 2001).

The earliest clinical evidence of nephropathy is the increase in urine volume and creatinine clearance, low abnormal levels of albumin in the urine (30 mg/day), high blood pressure, swollen feet and ankles, leg cramps especially at night, weakness, paleness, anemia, dry and itchy skin especially at night. This referred to microalbuminuria and patients have incipient nephropathy (Defronzo and Goodman, 1995 ; Gunes et al., 1999; Robert, 2002 and Murali et al., 2003). Mogensen et al. (1995) found that 80% of subjects with type 1 diabetes who develop microalbuminuria have increased urinary albumin excretion at a rate of 10-20% per year. Diabetic nephropathy is characterized by nephrotic syndrome and diffuse glomerulosclerosis (Ragavan and Krishnakumari, 2006; Maeda and Shiigai, 2007). Also, it is characterized by excessive massing of extracellular matrix with thickening of glomerular and tubular basement membranes and increased amount of mesangial matrix, which ultimately progress to glomerulosclerosis and tubulointerstitial fibrosis (Yashpal et al., 2008). There is a gradual increase in the kidney damage which is indicated by the increase in blood pressure, abnormal blood tests (creatinine tests) and need to urinate more often, hypertension and of fluid retention in the body (oedema) (Hostertter et al., 1982; Kinchen et al., 2002 ; Wahren et al., 2007). The end-stages of kidney disease are nephropathy, renal failure; peripheral neuropathy with risk of foot ulcers, amputations and damaging the nerves of the bladder (Genuth et al., 2003; Umesh et al., 2013). According to Chen et al. (2007) and Prakash et al. (2007) kidney failure often leads to death in diabetes.

The pathology of diabetic nephropathy manifests histologically as , damaged distal tubular epithelium, cytoplasmic changes, medial thickening of the small arteries and glomerulosclerosis. Glomerulosclerosis is characterized by glomerular basement membrane thickening with increased extracellular matrix deposition (Ruggenti et al., 2003; Maeda and shiigai, 2007 ; Kurt et al., 2012). The hyperglycemic maternal environment has also been associated with neonates that are at greater risk for future development of negative health outcomes such as future obesity, insulin resistance, type 2 diabetes mellitus and metabolic syndrome (Calkins and Devaskar, 2011).

Olive leaf extract: olive leaf is the leaf of the olive tree (*Olea europaea*).Olive leaf extract was used by the ancient Egyptian and Mediterranean cultures to treat a variety of health conditions, including infections, fever and pain (Omar, 2010).The active medical constituents find in unprocessed olive leaf are oleuropein, oleuropeoside and hydroxytyrosol, as well as several other polyphenols and flavenoids including oleocanthal

Some benefits of oleuropein : Antioxidant activity (Visioli et al., 2002;Andreadou et al., 2006), Anti-inflammatory effect(Visioli et al. ,1998) , Anti-atherogenic effect (Visioli and Galli , 2001; Carluccio et al. ,2003) , Anti-cancer effect (Hamdi and Castellon , 2005; Menendez et al. ,2007) , Antimicrobial effect (Bisignano et al., 1999 ;Furneri et al., 2002; Caturla et al., 2005), Antiviral effect (Fredrickson, 2000;Ma et al. , 2001) ,Skin protectant (Ancora et al. , 2004) , Anti-aging (Katsiki et al. ,2007) , Neuroprotective activity(Moosmann and Behl ,1999; German and Walzem, 2000; Petkov and Manolov, 1978), anti-platelet aggregation (Petroni et al., 1995), antipyretic effects (Visioli et al., 1995) and hypotensive (Khayyal et al., 2002). Prevention of free radical formation (Andrikopoulos et al., 2002). Oleuropein was reported to have an anti-hyperglycemic effect in the diabetic rats (Gonzalez et al., 1992 ;Al-Azzawie and Alhamdani, 2006; Mohamed, 2014).

Material and methods:-

The present work was carried out on forty mature pregnant albino rats, weight 200 ± 20 gm. They were obtained from El Rammed Medical Hospital, Cairo. The experimental animals were randomly divided into four groups, control (C), olive (O), diabetic (D) and diabetic+olive (D+O). They fed on rodent diet and some vegetables. The rats were stayed for 2 weeks for adaptation then the experiment was started. Streptozotocin (STZ) was purchased from Sigma, St .Louis, MO, USA. Diabetes mellitus was induced in fasted animals of **D** and **D+O** groups (12 hours) by a single intraperitoneal injection of Streptozotocin (35 mg/kg b.wt.). It was dissolved in 0.01 mole/l citrate buffer (pH 4.5) then animals were orally injected with 2 ml of glucose solution. After 48 hours of STZ injection, blood glucose levels were measured by glucometer. Rats with fasting blood glucose level more than 250 mg /dl are considered diabetic (Waer and Helmy, 2012).After that olive leaf extract (5.5 grams of the olive leaf powder were soaked in 100 ml boiled dist. water and covered for ten minutes, then cooled to room temperature and filtered).It was given orally with a dose of 1ml/100gm of b. wt. (using the stomach tube) every day till the 19th day of pregnancy **O**, **D+O** groups. This dose is equivalent to the therapeutic human dose (500mg) (Wainstein et al., 2012).

Rat's estrus cycle usually begins at 6 – 7 weeks of age; the estrus cycle repeats itself every 4 - 5 days. The stage of estrus cycle was determined by the vaginal smear technique as determined by **Taylor (1986)**. In the absence of vaginal plug, a drop from vaginal contents was prepared and examined under the microscope for the presence of spermatozoa. The presence of spermatozoa in smears confirmed that mating had taken place and this is considered as zero day of pregnancy (**Eda et al., 2009**).

Directly, after the animal was anesthetized by ether, blood was collected from the heart puncture by plastic syringes and left to coagulate and serum was separated by centrifugation at 3000rpm for 15 min. for the biochemical analysis serum glucose , insulin level, serum albumin concentration, serum glutamic pyruvate transaminase (GPT) activity, serum creatinine and urea level were determined.

The pregnant females were dissected and the uterine horns were removed freshly and then photographed. Fetuses were dissected out for gross examination. Abnormalities or any morphological changes such as, uterine form and resorptions, fetus's malformations, uterine shape, number of implantation sites, number of living fetuses and dead ones were realized.

The specimens of the kidney were taken from the pregnant rats of all groups. The specimens were fixed in 10% neutral buffer formol and Carnoy's fluid for the histological and histochemical studies. Specimens were washed and dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax.

Sections were then cut at 5µm thickness and stained by haematoxylin and eosin according to the method of **Drury and Wallington (1980)**; periodic acid Schiff's reaction for demonstrating polysaccharides (**Pearse ,1977**) ; mercuric bromophenol blue method for detecting total proteins (**Mazia et al., 1953**); Mallory's trichrome stain for demonstrating collagen fibers (**Pearse , 1977**) and modified aldehyde fuchsin method for detecting different cells of islets of Langerhans (**Halami, 1952**).

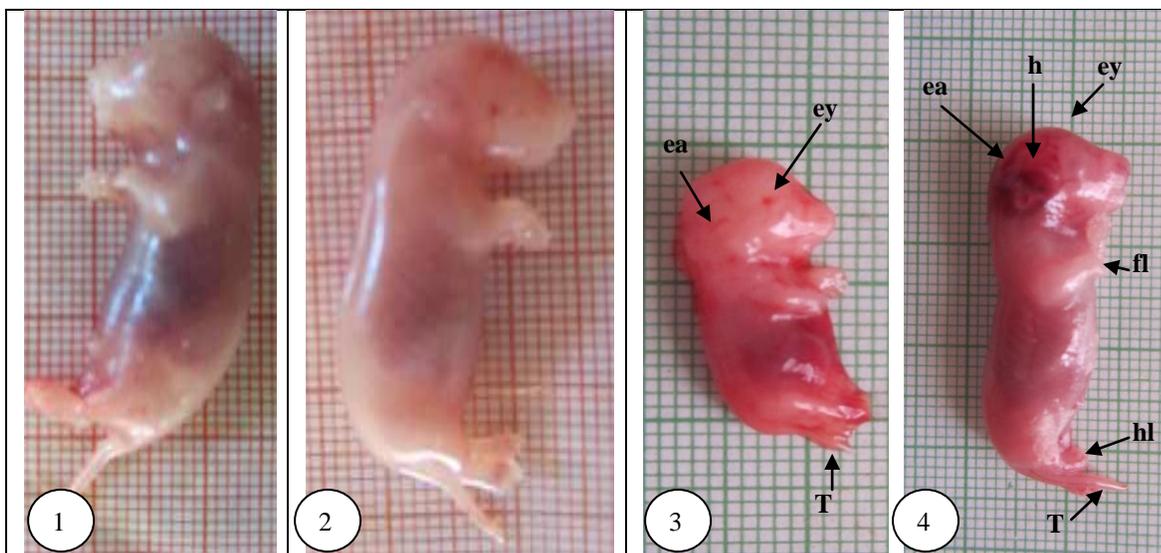
The optical density of PAS +ve materials and mercuric bromophenol blue stained sections of the kidney of the control and treated groups were recorded using software image analysis Pro Plus ipwin 32. The mean optical density was used to compare the PAS positive materials and total protein content in the different groups. The comparison was established as the percentage of change for the treated and the control groups.

Statistical analyses were performed using analyses of significant differences between treatment means of the physiological data (glucose, insuline, albumine, GPT, créatinine and urea), anatomical data (number of implantation sites, number of living fetuses and resorbed ones) and histochemical data (PAS+ve materials and total protein content for all groups) were determined by using T-test Microsoft Excel 2007. Data were presented as mean \pm SE and $P \leq 0.05$ was considered statistically significant.

Results

Fetus's malformations:-

Normal morphological characters of fetuses appeared clearly in the control group which isolated from the uteri of pregnant rats on the 19th day of gestation (**Fig. 1**). Administration of olive leaves extract (Group **O**) didn't change the external characters of fetuses (**Fig. 2**),while fetuses of the diabetic mothers showed a decrease in size, very thin skin, very thin muscle layer under the skin, microcudate (short tails), apalpebralia (absence of eyelids) and anotia (absence of ear pinna) (**Fig. 3**). On the other hand, fetuses of the diabetic mothers which treated with olive leaves extract showed somewhat normal appearance in their size, fore and hind limbs, eyelids and tails , but some fetuses showed elongated and narrowed head and subcutaneous hemorrhage (**Fig. 4**).



Figs. (1- 4) Showing isolated fetuses on day 19 of gestation. **Figs.(1, x1&2, x 0,9)** showing normal morphological characters of fetuses of the control and olive groups respectively. **Fig. (3, x1)** showing a retarded fetuse with very thin skin, very thin muscle layer under the skin, microcaudate (T), absence of eyelid (ey) and ear pinna (ea) in group **D** and **fig.(4 , x0.8)** showing improvement in size, normal fore and hind limbs (fl+hl), normal eyelid (ey), normal tail (T), but some fetuses showed elongated narrow head and subcutaneous hemorrhage (h) in group **D+O**.

Serum creatinine and urea level:-

The results tabulated in **table (1)** and illustrated in **figure(5)** showed no significant change ($P \geq 0.05$) in the mean values of the serum creatinine level in **O** and **D+O** groups (0.87 ± 0.21 mg/dl and 0.83 ± 0.22 mg/dl) respectively. But there was highly significant increase ($P \leq 0.01$) in the diabetic group (1.77 ± 0.27 mg/dl), compared to the control group. The measurements recorded an increase in the percent of change to 117.56% in the diabetic mothers in comparison with the control group. While, in **O** and **D+O** groups they reached 7.12% and 2.21% respectively.

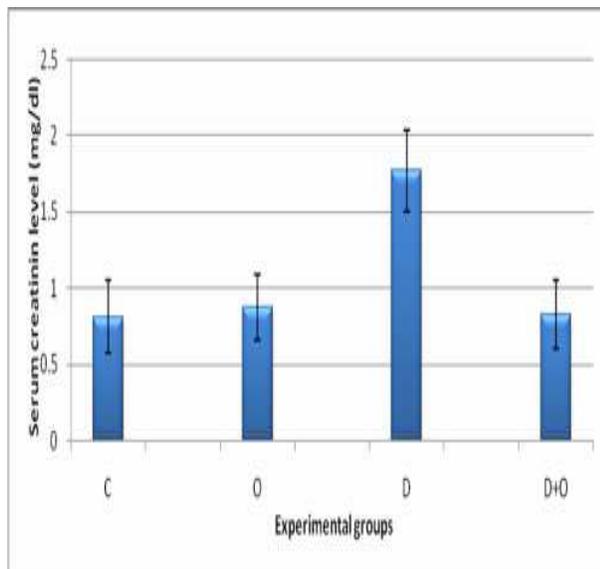
As shown in **table (1)** and **figure (6)** there was no significant change ($P \geq 0.05$) in the mean values of the serum urea level in groups **O** and **D+O**, where the values reached 4.10 ± 0.77 mg/dl and 4.48 ± 0.95 mg/dl respectively. But it showed highly significant increase ($P \leq 0.01$) in the diabetic group (6.49 ± 0.41 mg/dl) compared to the control group (3.97 ± 1.34 mg/dl). In comparison with the control group, administration of olive leaves extract decreased the percent of change in group **D** from 63.28% to 12.77% in **D+O** group respectively. While it was 3.21% only in the group of animals received olive leaves extract alone.

Table (1) Showing the statistical analysis of the serum creatinin level (mg/dl) and serum urea level (mg/dl) values in the different experimental groups.

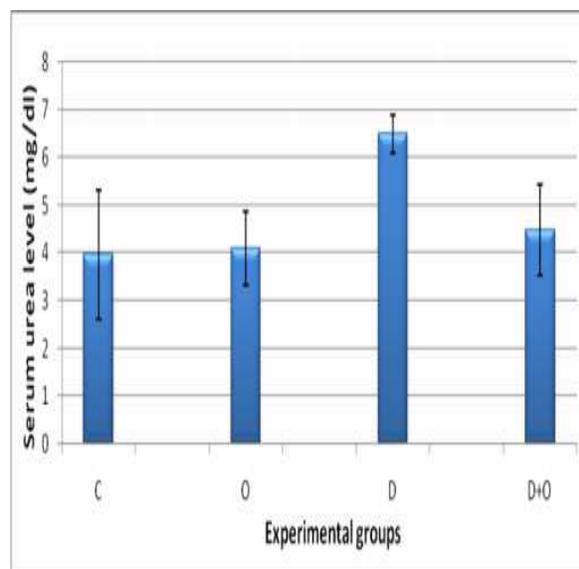
Groups \ Parameters	C	O	D	D+O
serum creatinin level (mg/dl)	0.81 ± 0.24	0.87 ± 0.21 7.12%	1.77** ± 0.27 117.56%	0.83 ± 0.22 2.21%
serum urea level (mg/dl)	3.97 ± 1.34	4.10 ± 0.77 3.21%	6.49** ± 0.41 63.28%	4.48 ± 0.95 12.77%

- Each value represented the mean and \pm standard deviation (SD).
- The values are considered significant at $*P \leq 0.05$ and highly significant at $**P \leq 0.01$ compared to the control group.
- C, control, O, olive leaves extract, D, diabetic and D+O, diabetic and olive leaves extract .

5



6



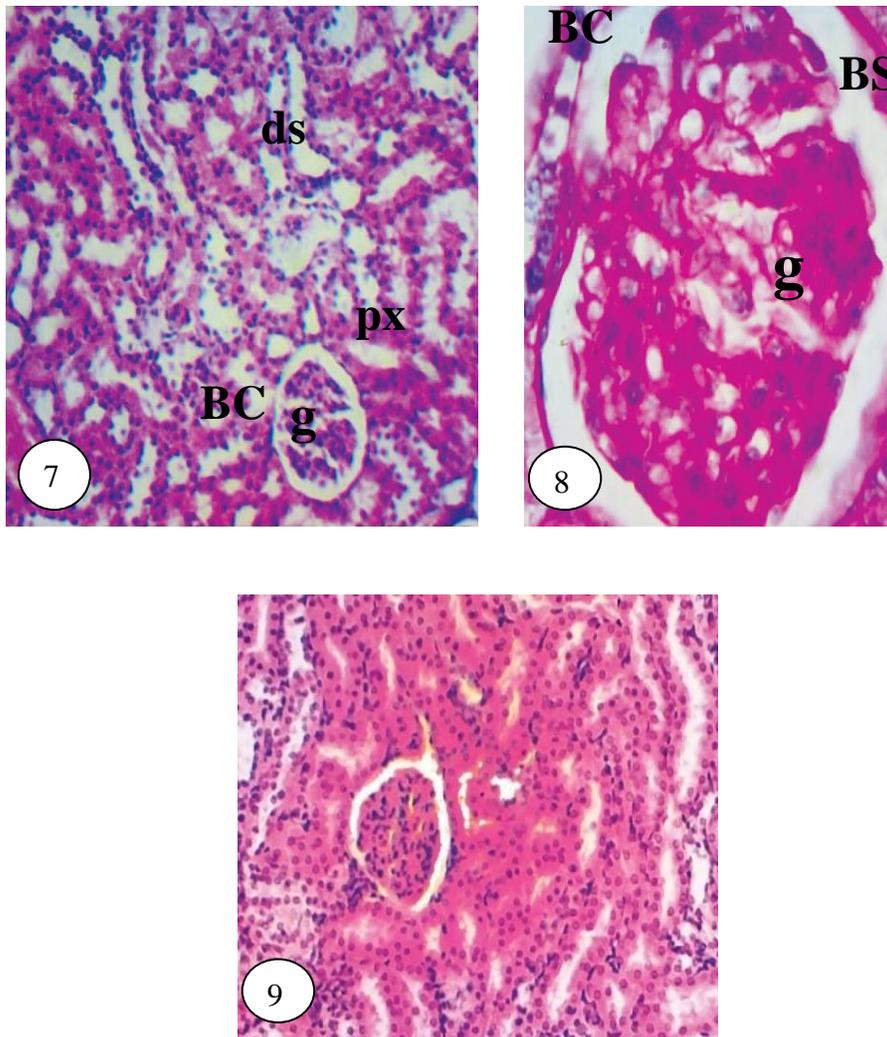
Figs. (5, 6) Showing the mean values in serum creatinine (mg/dl) and urea level (mg/dl) of the different experimental groups.

The histopathological study of Kidney cortex of the pregnant rats:-

Normal kidney cortex of the control pregnant rats was detected (Figs. 7,8). Kidney cortex of the pregnant rats of group **O** showed normal appearance of Bowman's corpuscle and the convoluted tubules (Fig. 9) with little increased collagen fibers in the Bowman's capsules, glomeruli and brush borders of the proximal convoluted tubules (Fig. 15).

Severe changes were observed in kidney cortex of group **D** (Figs. 10, 11). These changes include: atrophied and shrunk glomeruli with wide empty spaces and wide Bowman's spaces. Some of the convoluted tubules showed hydropic degeneration and cloudy swelling with faint staining affinity, poorly detected brush borders of the proximal convoluted tubules, highly decreased collagen fibers in the kidney cortex especially in the brush borders and in the basement membranes of the convoluted tubules and small hemorrhagic areas were also realized (Fig. 16).

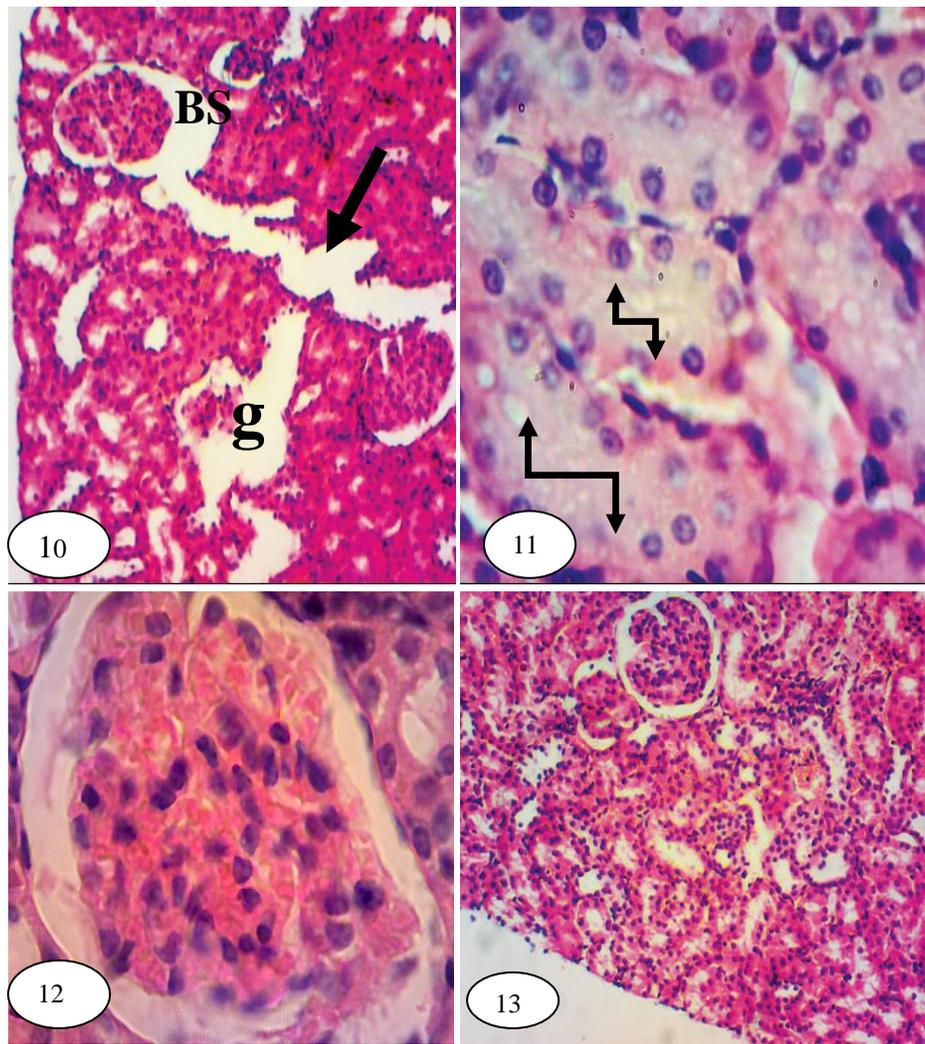
Somewhat normal appearance of the kidney cortex of group **D+O** was observed, but some glomeruli were still lobulated (Figs.12, 13) with somewhat normal distribution of collagen fibers (Fig. 17).



Figs. (7,8) Photomicrographs of kidney cortex of the control pregnant rat showing normal Bowman's capsules (BC), Bowman's space (BS), glomeruli (g), proximal (px) and distal (ds) convoluted tubules. (H&E X200 & H&E x800)

Fig. (9) A photomicrograph showing normal appearance of the kidney cortex of group **O**.

(H&E X200)



Figs. (10, 11) Photomicrographs showing many changes in the kidney cortex of group **D** .Notice: atrophied glomerulus (g), Bowman's capsule surrounded by hydronephrosis (→) and many pyknotic nuclei (P). The convoluted tubules show hydropic degeneration and cloudy swelling (↘) with pale staining affinity in some convoluted tubules , poorly detected brush borders of the proximal convoluted tubules and wide Bowman's spaces (BS).
(H&E X200 & H&E x800)

Figs. (12,13) Photomicrographs of sections of kidney cortex of group **D+O** showing somewhat normal appearance of the kidney cortex, but some glomeruli are corrugated.

(H&E X200 & H&E x800)

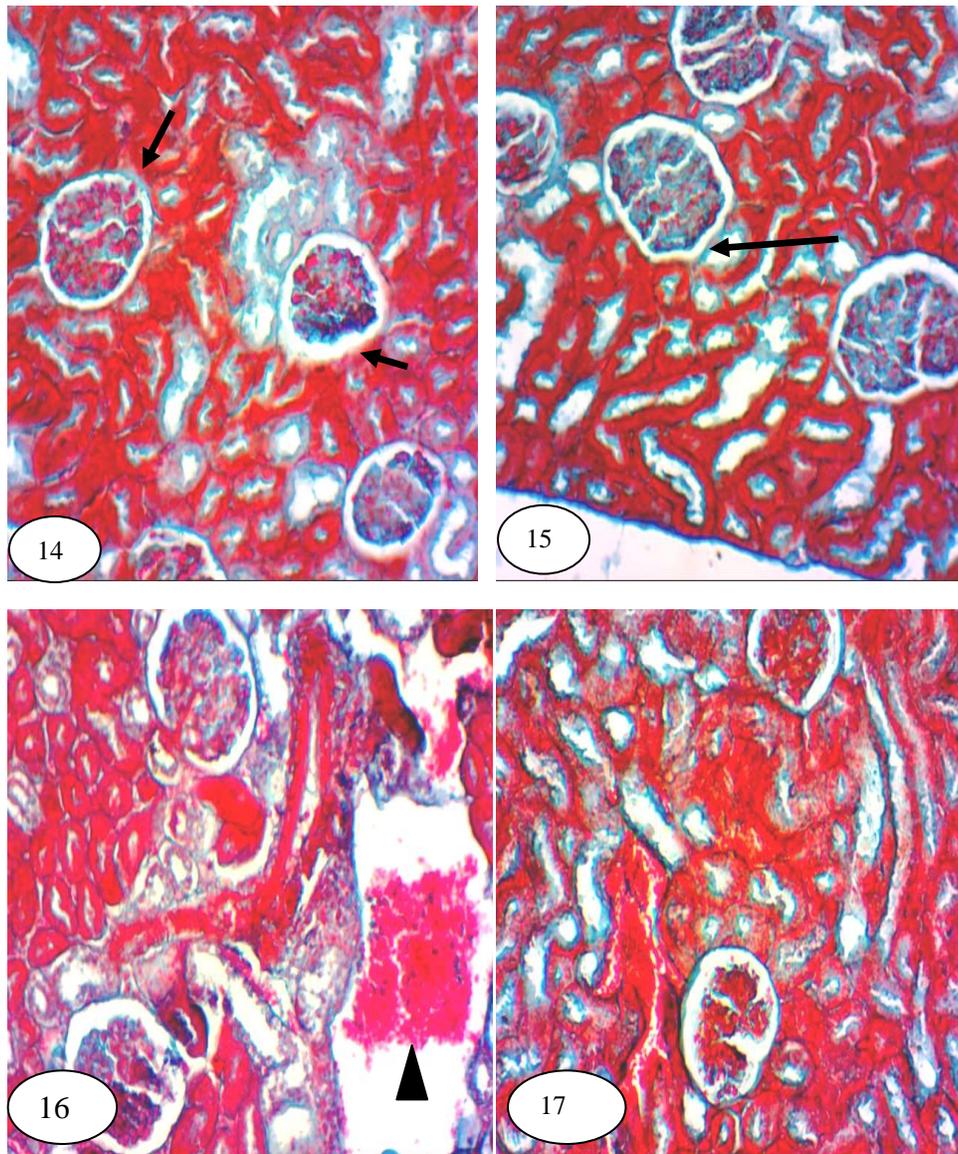


Fig. (14) A photomicrograph of a section of kidney of the control pregnant rat showing normal distribution of collagen fibers. Notice: collagen fibers in the Bowman's capsules (\rightarrow), brush borders of the proximal convoluted tubules, glomeruli and the basement membranes of the convoluted tubules.

Fig. (15) A photomicrograph of a section of kidney cortex of group **O** showing increased collagen fibers in Bowman's capsules, glomeruli and brush borders of the proximal convoluted tubules (\rightarrow).

Fig. (16) A photomicrograph showing highly decreased collagen fibers in the kidney cortex of group **D** especially in the brush borders and in the basement membranes of the convoluted tubules, but they increased in Bowman's capsules. Hemorrhagic areas (\blacktriangle).

Fig. (17) A photomicrograph showing somewhat normal distribution of collagen fibers in the kidney cortex of group **D+O**.
(Mallory's trichrome stain X200)

The quantitative study:-

Table (2) and **figure (26)** showed the measurements of optical density values of total protein in the kidney of mothers in the different experimental groups.

The mean value of total proteins in the cortex region of the kidney of the control group recorded 102.24 ± 8.61 pixel in the glomeruli and 94.06 ± 6.80 pixel in the convoluted tubules (**Fig.18**). Total protein in the glomeruli of kidney cortex of group **O** reached 111.51 ± 12.65 pixel, with less staining affinity of total protein in the convoluted tubules (89.31 ± 8.88 pixel) when compared to the control group (**Fig.19**). Faintly stained protein was demonstrated in the glomeruli (82.58 ± 8.50 pixel) and convoluted tubules of group **D** (86.62 ± 7.17 pixel), but some glomeruli were densely stained than the control group (**Fig.20**). Total protein content of group **D+O** reached 102.13 ± 8.93 pixel in the glomeruli, but they reached 100 ± 10.85 pixel in the convoluted tubules (**Fig.21**).

The percentage of change of protein content recorded -19.22% in the glomeruli and -7.90% in the convoluted tubules of group **D**. The percentage of change of protein content in the glomeruli reduced - 0.10% and increased to 6.31% in the convoluted tubules of group **D+O**.

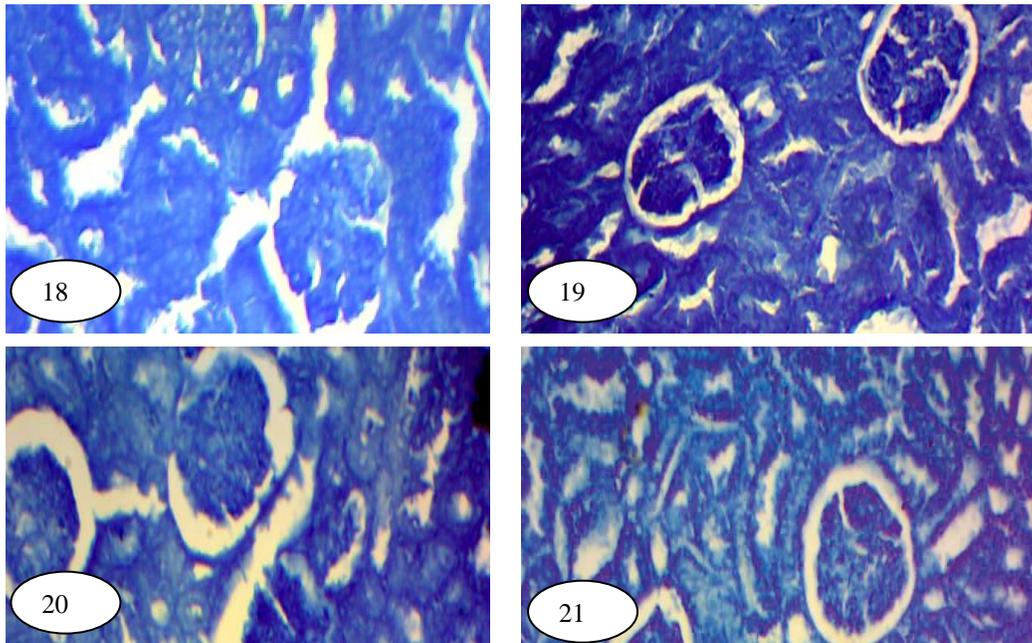


Fig. (18) A photomicrograph of a section of kidney cortex of the control group showing normal distribution of total protein.

Fig. (19) A photomicrograph of a section of kidney cortex of group **O** showing dense staining affinity of total protein.

Fig. (20) A photomicrograph of a section of kidney cortex of group **D** showing faintly stained total protein in the convoluted tubules. Some glomeruli are densely stained (←).

Fig. (21) A photomicrograph of a section of kidney cortex of group **D+O** showing faintly stained total protein.

(Mercuric bromophenol blue X 200)

Table (3) and **figure (27)** showed the measurements of optical density values of PAS positive materials in the kidney cortex of mothers in the different experimental groups.

Normal polysaccharides content recorded 157.72 ± 9.86 pixel in the glomeruli and 137.68 ± 9.45 pixel in the convoluted tubules (**Fig.22**). Decreased content of polysaccharides in group **O** was detected in the glomeruli (136.72 ± 11.63 pixel) and the convoluted tubules (120.27 ± 9.59 pixel) when compared to the control group (**Fig.23**). Faintly stained polysaccharides were detected in the convoluted tubules of group **D** compared to the control group (87.75 ± 9.12 pixel), while they reached 81.54 ± 9.97 pixel in Bowman's corpuscles (**Fig.24**). Polysaccharides restored their normal appearance in the glomeruli and reached 117.84 ± 11.13 pixel and convoluted tubules showed 114.42 ± 10.07 pixel in group **D+O**, but these levels are still less than those observed in the control group (**Fig.25**).

The percentage of change of polysaccharides content in the glomeruli reduced to -48.30% and -36.26% in the convoluted tubules of group **D**. The percentage of change of carbohydrates increased to -25.28% in the glomeruli and -16.89% in the convoluted tubules of group **D+O** when compared to group **D**.

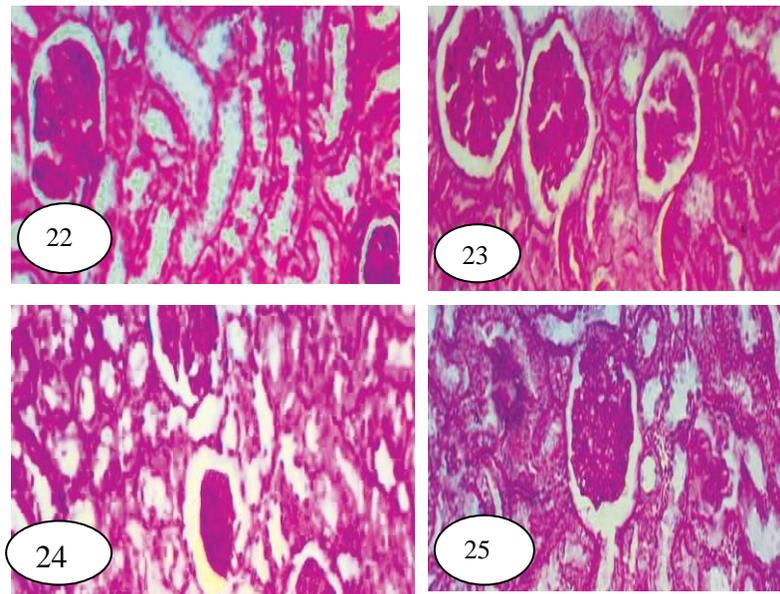


Fig. (22) A photomicrograph of a section of kidney cortex of the control pregnant rat showing normal distribution of polysaccharides.
Fig. (23) A photomicrograph of a section of kidney cortex of group **O** showing normal distribution of polysaccharides.
Fig. (24) A photomicrograph of a section of kidney cortex of group **D** showing faintly stained polysaccharides in the convoluted tubules and in Bowman's capsules ,but some glomeruli are deeply stained.
Fig. (25) A photomicrograph of a section of kidney cortex of group **D+O** showing decreased polysaccharides compared to the control group.

(PAS X200)

Table (2) Showing the optical density values of total protein in the kidney cortex of mothers in the different experimental groups.

Groups Parameters	C		O		D		D+O	
	G	T	G	T	G	T	G	T
Mean	102.24	94.06	111.51	89.31	82.58	86.62	102.13	100
±SD	±8.61	±6.80	±12.65	±8.88	±8.50	±7.17	±8.93	±10.85
% of change			9.06	-5.04	-19.22	-7.90	-0.10	6.31

- Each value represented the mean and ± standard deviation (SD).
- The values are considered significant at *P ≤ 0.05 and highly significant at **P ≤ 0.01. compared with the control group.
- C, control, O, olive leaf extract, D, diabetic and D+O, diabetic and olive leaf extract, G glomeruli and T, convoluted tubules .

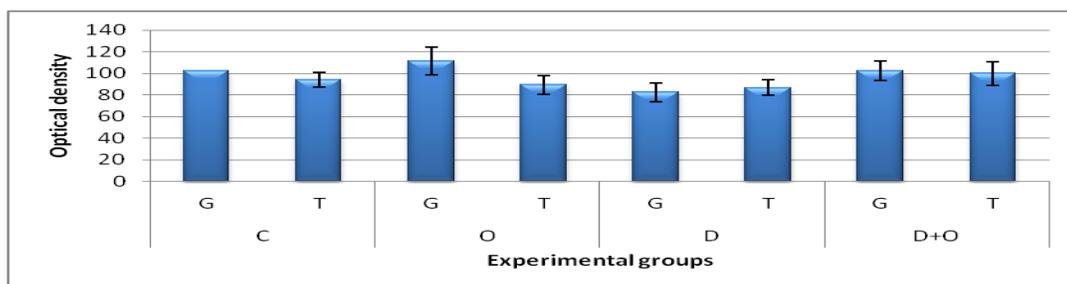


Fig. (26) Showing the optical density values of total protein in kidney cortex of mothers in the different experimental groups.

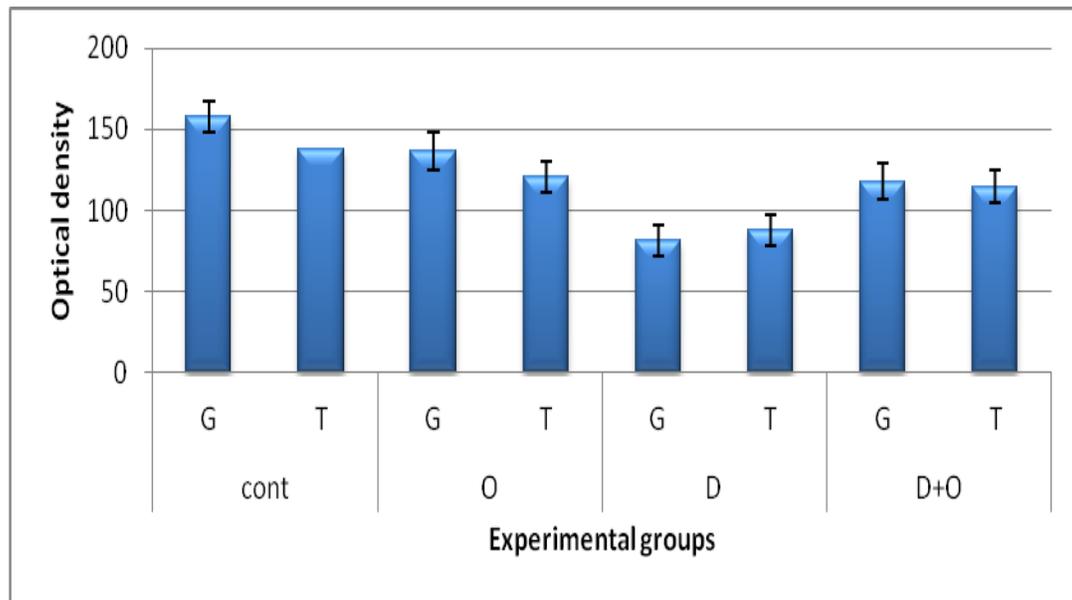
Table (3) Showing the optical density values of PAS +ve materials in the kidney cortex of mothers in the different experimental groups.

Groups	C		O		D		D+O	
	G	T	G	T	G	T	G	T
Parameters								
Mean	157.72	137.68	136.72	120.27	81.54	87.75	117.84	114.42
±SD	±9.86	±9.45	±11.63	±9.59	±9.97	±9.12	±11.13	±10.07
% of change			-13.31	-12.64	-48.30	-36.26	-25.28	-16.89

- Each value represented the mean and \pm standard deviation (SD).

- The values are considered significant at $*P \leq 0.05$ and highly significant at $**P \leq 0.01$ compared with the control group.

- C, control, O, olive leaf extract, D, diabetic and D+O, diabetic and olive leaf extract, G, glomeruli and T, convoluted tubules.

**Fig. (27) Showing the optical density values of PAS +ve materials in the kidney cortex of mothers in the different experimental groups.**

The histopathological study of the fetal kidney:-

Histological pattern of the fetal kidney cortex of the control and O group showed well developed architecture (Figs.28, 29) with normal distribution of collagen fibers in both groups (Figs. 34, 35).

Fetal kidney tissue of group D showed disappearance of the medulla, the medullary area was surrounded by thick fibrotic area, glomeruli showed many severe changes such as atrophy and congestion, distortion, elongation or lost their normal architecture, ruptured Bowman's capsules and completely degenerated glomeruli. Walls of the distal convoluted tubules appeared highly stratified due to increased proliferation with degenerated cuboidal cells and increased fibrotic stroma. Cells of the proximal convoluted tubules contained pyknotic nuclei (Figs. 30-32). Increased collagen fibers were observed in the stroma and around the medullary region, but they were decreased in Bowman's capsules and in the basement membranes of the convoluted tubules (Fig. 36).

Well-developed fetal kidney cortex of group D+O was detected with normal glomeruli, Bowman's capsules and convoluted tubules (Fig. 33) with somewhat normal appearance of collagen fibers (Fig. 37).

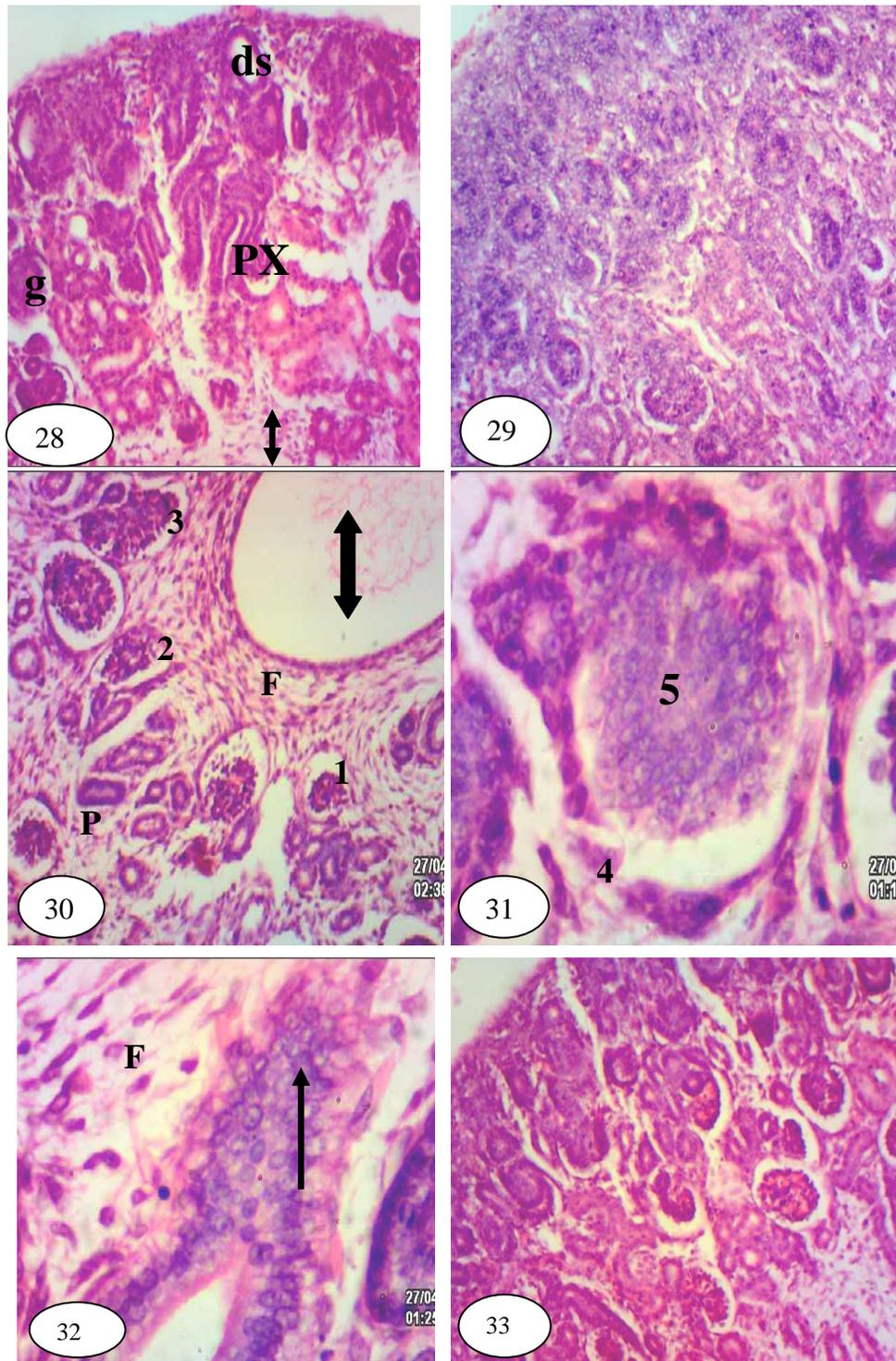


Fig. (28) A photomicrograph showing the fetal kidney cortex of the control group. Notice : the glomeruli (g), proximal (px) and distal (ds) convoluted tubules with the stroma in between them (←) .

(H&E X 200)

Fig. (29) A photomicrograph showing well developed architecture of the fetal kidney cortex of group O .

(H&E X 200)

Figs. (30-32) Photomicrographs of the fetal kidney cortex of group D showing disappearance of the medulla (←), the medullary area is surrounded by thick fibrotic area, the glomeruli showed many changes such as (1) atrophy and congestion, (2) distortion, (3) elongation and lost its normal architecture, (4) ruptured Bowman's capsule and (5) completely degenerated glomeruli. The wall of the distal convoluted tubule appears highly stratified with degenerated cuboidal cells (↑), widened and fibrotic stroma (F) and the cells of the proximal convoluted tubules contain pyknotic nuclei (P).

(a-H&E x200& b, c- H&E x800)

Fig. (33) A photomicrograph showing well developed fetal kidney cortex of group **D+O**.

(HX&E X200)

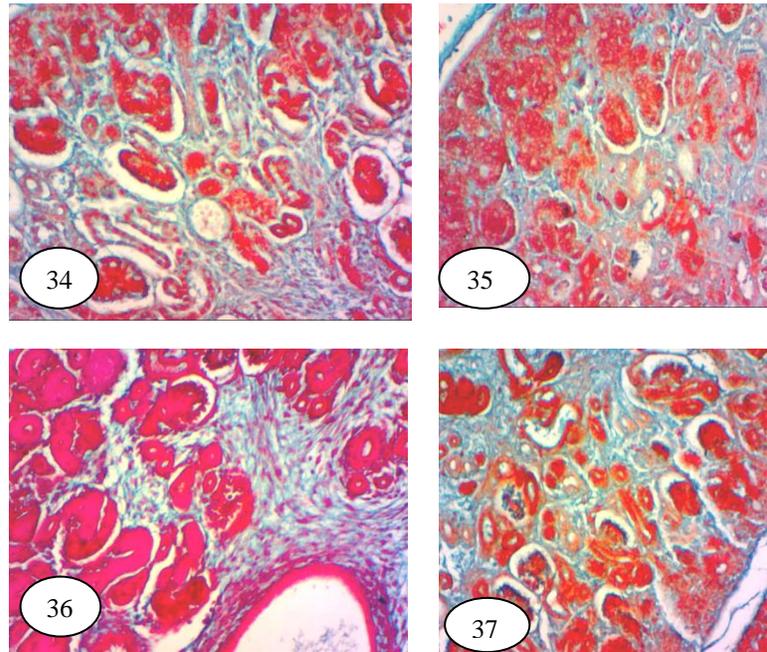


Fig. (34) A photomicrograph of fetal kidney cortex of the control group showing numerous collagen fibers supporting the stroma, the capsule, walls of the convoluted tubules, brush borders of the proximal convoluted tubules and Bowman's capsules.

Fig. (35) A photomicrograph of fetal kidney cortex of group **O** showing normal distribution of collagen fibers.

Fig. (36) A photomicrograph of fetal kidney cortex of group **D** showing increased collagen fibers in the stroma and around the medullary region, but they are decreased in Bowman's capsules and in the basement membranes of the convoluted tubules.

Fig. (37) A photomicrograph of the fetal kidney cortex of group **D+O** showing somewhat normal distribution of collagen fibers.

(Mallory's trichrome stain X200)

The histochemical study:-

Table (4) and **figure (46)** showed the measurements of optical density values of total protein in the fetal kidney cortex in the different experimental groups.

The mean value of normal total protein in the fetal kidney cortex of the control group recorded 129.57 ± 7.13 pixel in the glomeruli and 116.88 ± 10.15 pixel in the tubules. The distal convoluted tubules were less stained than the proximal ones (**Fig.38**). Somewhat normal appearance of total protein content was noticed in the fetal kidney glomeruli of the fetal kidney cortex of group **O** (114.03 ± 6.56 pixel) and convoluted tubules (120.96 ± 11.80 pixel) when compared to control group (**Fig.39**). Reduced staining affinity of total protein was demonstrated in glomeruli of the fetal kidney cortex (98.76 ± 5.50 pixel) and in the convoluted tubules (106.88 ± 9.83 pixel) of group **D** (**Fig.40**). Decreased staining affinity of total protein was observed in the glomeruli (111.03 ± 7.04 pixel) and convoluted tubules (99.03 ± 7.24 pixel) of the fetal kidney cortex of group **D+O** (**Fig.41**).

The percent of change of total protein content recorded -23.77% in the glomeruli and - 8.55% in the convoluted tubules of group **D**. Total protein percent of change recorded -14.30% in the glomeruli and -15.27% in the convoluted tubules of group **D+O**.

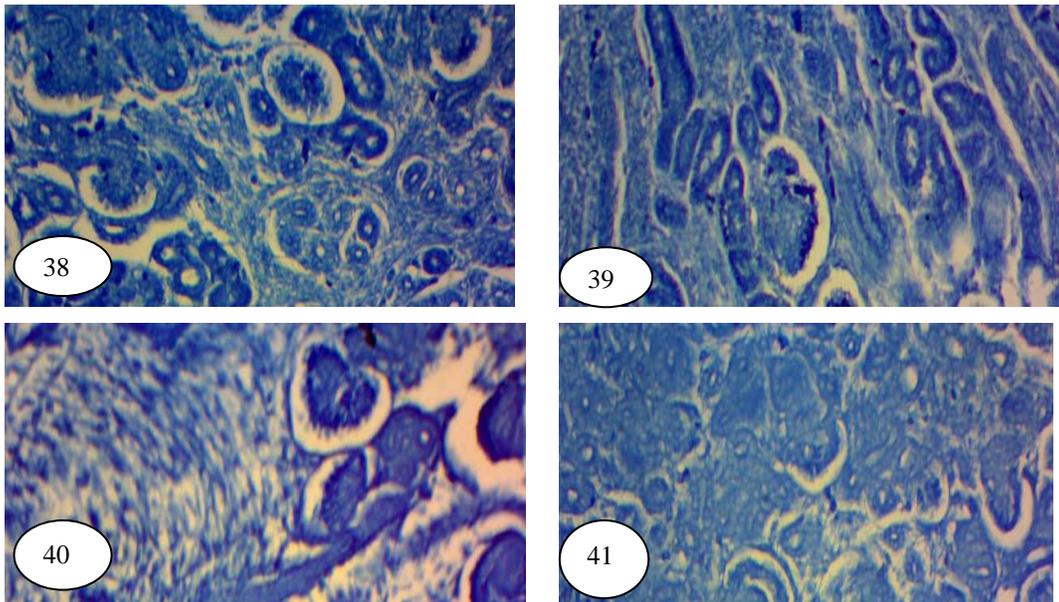


Fig. (38) A photomicrograph showing normal distribution of total protein in the fetal kidney cortex of the control group. Notice: the distal convoluted tubules are less stained than the proximal ones

Fig. (39) A photomicrograph showing somewhat normal appearance of total protein content in the fetal kidney cortex of the group **O**.

Fig. (40) A photomicrograph showing reduced staining affinity of total protein in the necrotic and fibrotic areas in the fetal kidney cortex of the group **D**.

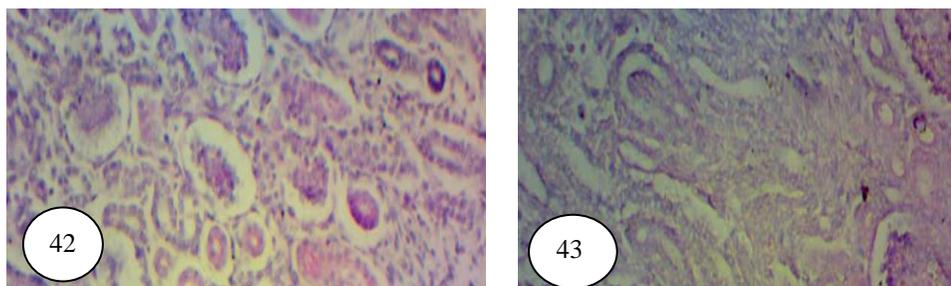
Fig. (41) A photomicrograph showing decreased staining affinity of total protein in the glomeruli and convoluted tubules in the fetal kidney cortex of the group **D+O**.

(Mercuric bromophenol blue X200)

Table (5) and **figure (47)** showed the measurements of optical density values of PAS positive materials in the fetal kidney cortex in the different experimental groups.

Polysaccharides recorded 121 ± 19.06 pixel in the glomeruli and 102.6 ± 9.19 pixel in the convoluted tubules in the kidney cortex of group **C** (**Fig.42**). Increased staining affinity of polysaccharides were detected in the glomeruli (131.26 ± 16.01 pixel) and convoluted tubules (123.5 ± 8.51 pixel) of group **O** (**Fig.43**). In group **D**, deeply stained polysaccharides in the glomeruli were noticed, but few of them were faintly stained (116.93 ± 15.27 pixel). Convoluted tubules were moderately stained, fibrotic areas were less stained and degenerated areas were negatively stained (101 ± 13.57 pixel) (**Fig.44**). Somewhat normal appearance of polysaccharides was observed in the glomeruli of group **D+O** (123.73 ± 11.56 pixel), but increased in the convoluted tubules (109.93 ± 9.77 pixel) (**Fig.45**).

Polysaccharides percent of change recorded -3.36% in the glomeruli and -1.55% in the convoluted tubules of group **D**. The percentage of change recorded 2.25% in the glomeruli and 7.14% in the convoluted tubules in group **D+O**.



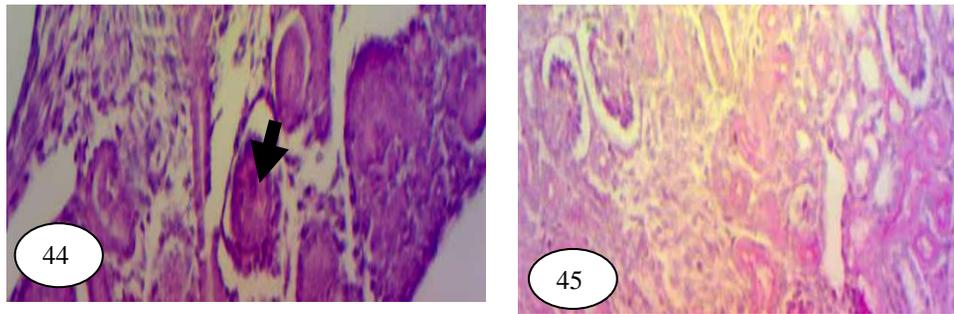


Fig.(42) A photomicrograph showing normal distribution of PAS +ve materials in the fetal kidney cortex of the control group.

Fig. (43) A photomicrograph showing normal distribution of PAS +ve materials in the convoluted tubules, but some glomeruli showed increased staining affinity in the fetal kidney cortex of the group **O**.

Fig. (44) A photomicrograph showing deeply stained glomeruli, but few of them are faintly stained (←), convoluted tubules are moderately stained, fibrotic areas are less stained and degenerated areas are negatively stained in the fetal kidney cortex of group **D**.

Fig. (45) A photomicrograph showing somewhat normal appearance of PAS +ve materials in the fetal kidney cortex of group **D+O**.

(PAS X200)

Table (4) Showing the optical density values of total protein of the fetal kidney cortex in the different experimental groups.

groups	C		O		D		D+O	
	G	T	G	T	G	T	G	T
Mean	129.57	116.88	114.03	120.96	98.76	106.88	111.03	99.03
±SD	±7.13	±10.15	±6.56	±11.80	±5.50	±9.83	±7.04	±7.24
% of change			-11.99	3.49	-23.77	-8.55	-14.30	-15.27

- Each value represented the mean and ± standard deviation (SD).
- The values are considered significant at *P ≤ 0.05 and highly significant at **P ≤ 0.01. compared with the control group.
- C, control; O, olive leaf extract, D, diabetic, D+O, diabetic and olive leaf extract, G, glomeruli and T, tubules .

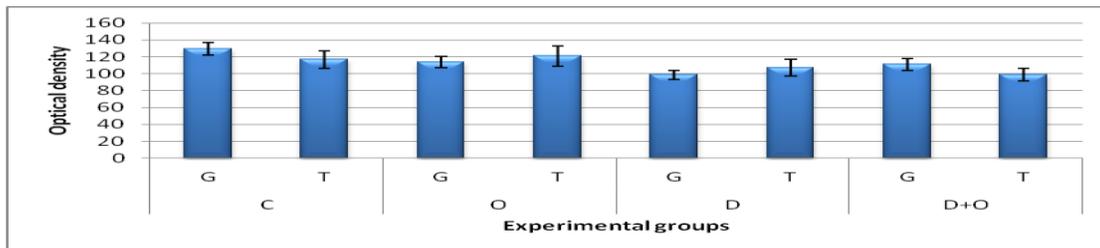


Fig. (46) Histogram showing the optical density values of total protein of the fetal kidney cortex in the different experimental groups.

Table (5) Showing the optical density values of PAS +ve materials of the fetal kidney cortex in the different experimental groups.

Groups	C		O		D		D+O	
	G	T	G	T	G	T	G	T
Mean	121	102.6	131.26	123.53	116.93	101	123.73	109.93
±SD	±19.06	±9.19	±16.01	±8.51	±15.27	±13.57	±11.56	±9.77
% of change			8.47	20.4	-3.36	-1.55	2.25	7.14

- Each value represented the mean and ± standard deviation (SD).
- The values are considered significant at *P ≤ 0.05 and highly significant at **P ≤ 0.01. compared with the control group.
- C, control, O, olive leaf extract, D, diabetic, D+O, diabetic and olive leaf extract, G, glomeruli and T, tubules .

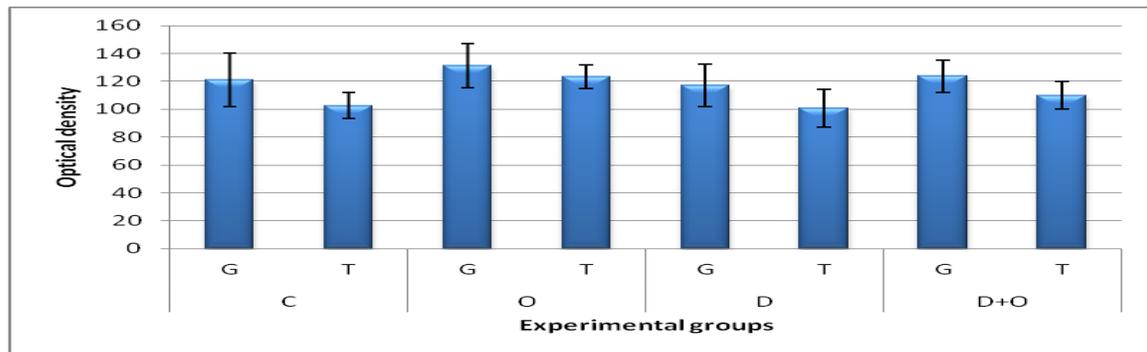


Fig. (47) Histogram showing the optical density values of PAS +ve materials of the fetal kidney cortex in the different experimental groups.

Discussion

The effects of excess glucose level in the serum resulted in the fetal growth retardation. In the first half of pregnancy, the fetus is exposed primarily to hyperglycemia, which, without secondary hyperinsulinemia, results in slowing the fetal growth. During the second half of pregnancy, hypertrophic islet cells respond to hyperglycemia with an increase in insulin production. This potent combination of hyperinsulinemia, a major anabolic hormone and hyperglycemia, a major anabolic fuel, results in a cascade of the third-trimester events that culminate in a striking increase in fat stores and increase in protein stores (Nold and Georgieff, 2004). The diabetic patients need alternative therapies to control all the pathological aspects of the diabetes (Meral et al., 2001).

Herbal medicines are better and safer than conventional medicines (Marles and Farnsworth, 1995). Some medical plants are rich sources of antidiabetic, antihyperlipidemic and antioxidant agents such as flavonoids, gallotannins, amino acids and other related polyphenols (Muruganandan et al., 2005; Ashok-Kumar et al., 2012).

Some of these plants have a greater consumption during pregnancy as they are considered safe and are also reported to have beneficial effects in the treatment of intrauterine growth retardation (Mostafa et al., 2013). In this respect, the antioxidant properties of oleuropein and hydroxytyrosol in olive leaves extract allow them to be efficient in the protection against diabetes (Jemai et al., 2009). So, this study is a step to evaluate the effects of water extract of olive leaves as an antidiabetic agent during gestation period.

In the present study, the fetuses isolated on the 19th day of gestation showed normal morphological characters in the control and olive groups. These results proved the safety of olive leaves extract in the pregnant rats and their fetuses. Also, the histological and histochemical results confirmed this opinion.

The fetuses of the diabetic mothers in the present study, showed decreased body size, very thin skin with a thin layer of muscle fibers, microcudate, apalpebralia, anotia and microsomia. This may be due to the delay of the fetus's development in the diabetic animals than the normal ones. Ishihara et al. (2000) observed that the fetal or newborn body weight of fetuses of pregnant rats was highly variable, ranging from developing microsomia to no change (Gerber et al., 2000) or to macrosomia (Merzouk et al., 2000). Whereas a positive correlation between maternal glycemia and fetal weight was found in mildly diabetic rats (Aerts et al., 1990). At birth, fetuses from severe diabetic dams were small at birth and had decreased pancreatic weight (Portha et al., 2011).

Pettepher et al. (1991) and Damasceno et al. (2011) demonstrated that severely diabetic rats presented higher DNA damage confirming the interaction between hyperglycemia-induced genotoxicity and teratogenesis. Damasceno et al. (2014) found that oxygen and nitrogen species, the products of free radicals, which are dependent on fatty acid oxidation, can induce chromosome breaks in streptozotocin-induced diabetes models. Zabihi and Loeken (2010) used a similar diabetes model, recovered embryos from the diabetic rats at day 10 or 11 of pregnancy. These embryos were cultured within their intact visceral yolk sac for 24 or 48 h and presented decreased Bcl-2 levels and increased Bax levels and increased activation of caspase 3. Thus, exposure to diabetes during organogenesis increased cellular apoptosis and embryonic dysmorphogenesis.

Diabetes during pregnancy causes an abnormal intrauterine metabolic and hormonal milieu that result in congenital malformations and neonatal hyperglycemia (Fuhrmann et al., 1983; Martin et al., 1987). It also enhances the risk of short and long-term postnatal disease, including macrosomia (Cowett and Shwartz, 1982; Small et al., 1987), glucose intolerance, insulin resistant (Pettitt et al., 1988; Martin et al., 1995), type 2 diabetes later in life (Vohr et al., 1980; Pettitt et al., 1993) and obesity (Boloker et al., 2002; Portha et al., 2011; ADA, 2014). Diabetes during pregnancy is associated with increased morbidity (hypoglycemia, hypocalcemia, polycythemia, hyperbilirubinemia) and fetal mortality (Griz et al., 2003). Maternal diabetes constitutes an unfavorable environment for fetal-placental and embryonic development (Rudgy et al., 2013; Damasceno, 2014).

In the present study administration of olive leaf extract showed improvement in the external characters of fetuses. This improvement was observed in their size, fore and hind limbs, eyelids and tails. This may be due to chemical structure of olive leaf extract which like steroid hormones which have the ability to improve numerous physiological activities and repair the damaged internal tissues in the kidney, liver, brain and others (**Khalid et al., 2009**). **Omar (2010)** reported that olive leaf extract has important role in controlling blood glucose level and reduce the high blood pressure in the diabetic animals and that may improve the development processes of fetuses inside the uteri.

In 2014, **El- Nabarawy** reported that the percentage of tail DNA and tail moment values were also higher in both embryo and placenta of the diabetic -induced rats. But, DNA damage was partly ameliorated after *O. europaea* leaves water extract treatment.

In the present study the fetuses of group **D+O** has many patches of subcutaneous hemorrhage and this may be due to the slow aggregation of platelets. This finding is in agreement with those of **Singh et al. (2008)** who reported that polyphenols found in olive leaf extract are capable of inhibiting in vitro platelet activation in health. Pharmacological activity of oleuropein includes diverse healing properties due to its vasodilatory role (**Petkov and Manolov, 1978**) and anti-platelet aggregation (**Petroni et al., 1995**).

The physiological results:-

The results of present study showed no significant change in the mean values of creatinin in the treated groups **O** and **D+O**. But there was highly significant increase in the diabetic group compared to the control group.

Serum creatinin often rises in type 2 diabetes due to the renal arterial disease and/or cardiac failure rather than to diabetic nephropathy. Where, hyperglycemia causes kidney damage through glycosylation, activation of protein kinase C, release of several cytokines (**Stanfield, 2011**). In addition, this may be result from failure of the body to excrete the metabolic products of proteins (**Guyton and Hall, 2006**). Where, proteins metabolic rate increased in diabetes because of gluconeogenesis increasing rate. Moreover, this result can be caused by the hyperglycemia, hypertension, or hyperlipidemia that occurs with diabetes (**Stanfield, 2011**).

The present study recorded an improvement in serum creatinin in the treated group **D+O** when compared to the diabetic animals, which may be due to improvement of the kidney functions and decreased the excessive loss of albumin in urine in the diabetic rats. Therefore, based on these findings, antioxidants have a protective effect against the diabetic nephropathy (**Eidi et al., 2009; Stanfield, 2011; Helal et al., 2013; Mohamed, 2014**).

Results of the present study showed highly significant increase in the serum urea level recorded in the diabetic group. This elevation of urea in the blood always indicated that there is a defect in kidney function and may be result from failure of the body to excrete the metabolic products of proteins (**Guyton and Hall, 2006; Stanfield, 2011**).

On the other side, no significant change in the mean values of the serum urea level in groups **O** and **D+O** was detected in the present study. The improvement of serum urea levels in rats treated with olive leaf extract may be due to regeneration of kidney glomeruli and that improved the kidney filtration process. This opinion is agreement with those of **Eidi et al. (2009); Abd El-Rahman and Al-ahmari (2013) and Mohammed (2014)**. Multiple animal studies have shown the effectiveness of olive leaf extract is normalizing a variety of cardiovascular, hepatic and metabolic signs, most likely through reversing related chronic inflammation and oxidative stress (**Zari and Al-Attar, 2011; El- Nabarawy, 2014**).

Oleuropein is an ester of 2-(3,4-dihydroxyphenyl) ethanol (hydroxytyrosol) and has the oleosidic skeleton that is common to the secoiridoid glucosides of oleaceae, mainly in its aglycone form, which makes the sugar moiety insoluble in oil (**Soler-Rivas et al., 2000**). Therefore, it may prevent glucose perfusion during the cell membrane. **Caturla et al. (2005) and Visioli et al. (2002)** have used biophysical assays to study the interaction between oleuropein and membrane lipids. A scavenging effect of oleuropein was demonstrated with respect to hypochlorous acid.

Also, oleuropein inhibits low-density lipoproteins (LDL) oxidation in vitro and lipid peroxidation *in vivo* and scavenges free radicals (**Kanner et al., 2012**). **Ahmadvand et al. (2014)** showed that oleuropein has beneficial effects in decreasing the elevated glycated hemoglobin (HbA1c) and serum glucose, lipid profile, atherogenic index in alloxan-induced-diabetic rats.

The histological results:-

Kidney of the pregnant rats

Histological results of this study showed normal structure of kidney cortex of the control rats and those of group **O** with normal glomeruli which are surrounded by the Bowman's capsules beside proximal and distal convoluted tubules. The microscopic appearance of kidney cortex of the diabetic rats showed severe changes. These changes include: atrophied glomeruli with wide empty spaces, wide Bowman's spaces. Some of the convoluted tubules showed hydropic degeneration and cloudy swelling with faint staining affinity, poorly detected brush borders of the proximal convoluted tubules, highly decreased collagen fibers in the kidney cortex especially in the brush borders and in the basement membranes of the convoluted tubules and small hemorrhagic areas. **Yassin et al. (2004)** noticed that in the diabetic rats the glomerular tufts were obviously contracted, lobulated, degenerated and infiltrated

by chronic inflammatory cells and RBCs. The glomeruli were more or less shrunken. The urinary space became wide. The nuclei of some deteriorated cells displayed obvious signs of karyorrhexis, while few of these nuclei showed marked karyolysis and change of architecture in the mother tissues.

Also, **Selvant et al. (2008)** reported that in the diabetic rats the kidney showed degenerative changes in the cortex, medulla and necrosis of tubules. In addition **Zeeuw et al. (2006)** observed that in diabetics, the kidney sections showed damaged glomeruli, proximal tubules and interstitial inflammation.

Thakran et al. (2004) and **Teoh et al. (2010)** noticed early nephropathic changes in the kidney of the diabetic rats and attributed the swelling of endoplasmic reticulum and mitochondria of the convoluted tubules cytoplasm to the cloudy swelling. The cell becomes water logged due to swelling of the organelles and true vacuoles appear in the cytoplasm. At this stage, cells are said to exhibit hydropic degeneration and these results agreed with the present study.

In the present work diabetic rats treated with olive leaf extract showed no inflammatory infiltration in the kidney sections. The anti-inflammatory role of medicinal plants was also noticed by several authors (**Ruberto et al., 2000** ; **Ozbek et al., 2003**). **Ozbek et al. (2003)** proposed a biological mechanism that may explain these anti-inflammatory and anticancer effects. This mechanism involves the shutting down of an intercellular signaling system called tumor necrosis factor (or TNF)-mediated signaling. Normal distribution of collagen fibers was demonstrated in kidney cortex of group **D+O**.

In the present work, the diabetic rats showed a decrease in protein content in the renal tubules of the kidney cortex. This decrease may be due to the decrease in ribosomal granules of rough endoplasmic reticulum or due to the decrease in DNA content. The decrease of DNA content was associated with a decrease in protein content in kidney cells of the diabetic rats. These results go in agreement with those of **Blasiak et al. (2003)**, they reported that alloxan can damage DNA in normal cells, operating therefore as a genotoxic compound. The observed DNA damage might be due to the induction of DNA strand breaks and/or the formation of alkali labile sites, which can be transformed into strand breaks in the alkaline comet assay. Also, **El-Nabarawy (2014)** reported that diabetes can generate oxidative stress and DNA damage to embryo and placenta and this can be ameliorated by oral doses of olive leaves extract by using alkaline comet assay. The author added that the ability of streptozotocin to generate free radicals in the presence of suitable reducing agents, like reduced glutathione and oxygen is well known. Streptozotocin exerts its DNA-damaging action, at least in part, by the production of free radicals and this action can be modulated by common antioxidants.

Increased protein content indicating that olive leaf extract is more effective in improving kidney cell dysfunction induced by streptozotocin. It may also cause increased amount of ribosomes in the rough endoplasmic reticulum in cells, reflecting their ability to stimulate protein synthesis (**Tuenz et al., 2003**).

In the present study, results of the diabetic rats showed marked diminution in PAS +ve materials in the brush borders and basement membranes of some tubules in kidney sections. These results go in agreement with those of **El-missiry and El-Gindy (2000)**. They observed a decrease in glycogen content in sections of kidney tissue of mother and fetuses. Diminution of carbohydrates content that was observed in the present work was most probably consequent to signs of degeneration and inflammation manifested in this work, or due to damaging effect of streptozotocin on the cytoplasmic organelles especially Golgi apparatus and the associated enzymes. Decrease in mucopolysaccharides content in the kidney of diabetic rats has been explained by **Tunez et al. (2003)**, they postulated that the decrease of glycogen content of rats treated with streptozotocin might be due to express of glycogenolysis.

In the present work, the treatment of the diabetic rats with olive leaf extract (Group **D+O**) showed an improvement in polysaccharides content when compared to the diabetic group, but still less than the normal content. These effects may be due to antioxidant nature of this plant. According to **Poop and Cattley (1991)**, it seems clear that the increase of polysaccharides deposition in basement membranes and brush borders of the renal tubules is a sign of glycogenesis.

-Fetal kidney

In this experiment fetal kidney cortex of the control and **O** groups showed normal glomeruli, proximal and distal convoluted tubules with the stroma in between them. Numerous collagen fibers are supporting the stroma, the capsule, walls of the convoluted tubules, brush borders of the proximal convoluted tubules and Bowman's capsules. **Daković-Bjelaković et al. (2005)** and **Tank et al. (2012)** examined the normal fetal kidney and they observed lobulation which was well marked in early gestation, as days of gestation increased, lobulations were less marked.

The lobes were separated only in the superficial part of the cortex. In the deeper part of cortex they were fused with each other. The kidney is covered by a thin capsule made up of fibrous tissue. Beneath the capsule the medullary differentiation was not well marked in early days of gestation, as late of gestation cortex and medulla were very well differentiated. The thickness of cortex and medulla increased at the late of gestation, in which the nephrogenic zone beneath the capsule was not seen. The cortex contained mature glomeruli with lobulated capillaries present just beneath the capsule. The vascularity of the cortex was also increased. The medulla showed well differentiated collecting tubules with thick and thin segments of loop of Henle. Close to the renal pelvis, the ducts of Bellini with columnar epithelium were identified.

Histological examination of fetal kidney tissue of group **D** in this experiment showed disappearance of the medulla, the medullary area was surrounded by thick fibrotic area, glomeruli showed many dystrophic changes such as atrophy and congestion, distortion and lost their normal architecture with ruptured Bowman's capsules and completely degenerated glomeruli. Walls of the distal convoluted tubules appeared highly stratified due to proliferated cells with degenerated cuboidal cells and expanded fibrotic stroma. Cells of the proximal convoluted tubules contained pyknotic nuclei. Increased collagen fibers were observed in the stroma and around the medullary region, but they were decreased in Bowman's capsules and in the basement membranes of the convoluted tubules. **Horn et al. (1985)** declared that the presence of collagen in the presinusoidal spaces might affect the blood supply to liver cells and would reduce the exchange of metabolites, perhaps causing hepatocellular dysfunction and necrosis.

Tran et al. (2008) observed small kidneys in the fetuses of the diabetic rats. They also reported that glomeruli were smaller and there was a relatively low number of nephrons and there was some evidences of nephron collapse.

In 1988, Brenner et al. hypothesized that low glomerular endowment or fewer numbers of nephrons are a risk factor for hypertension and ESRD in adulthood. In principle, decreased nephron number leads to renal hyperfiltration (higher filtration pressure and an increased GFR per glomerulus). Although outcomes such as low birth weight (LBW), small kidneys and fewer nephron numbers resulting from an adverse intrauterine environment that might predispose to future hypertension are known.

Zhang et al. (2007) suggested that a high-glucose milieu in uteri retards renal morphogenesis by inducing a significantly higher number of apoptotic podocytes in the developing glomeruli.

The usage of olive leaf extract in the present study improved the histological architecture of fetal kidney. Well-developed fetal kidney cortex was demonstrated in group **D+O** with normal glomeruli, Bowman's capsules and convoluted tubules and somewhat normal appearance of collagen fibers. Olive leaf extract protects gentamicin-induced nephrotoxicity possibly by inhibition of lipid peroxidation, enhancing renal glutathione content, and antioxidant enzymes activity. The findings suggested the potential therapeutic use of OLE as a new nephroprotective agent against acute kidney failure (**Tavafi et al., 2012**).

Histochemical results in this work showed reduced staining affinity of total protein in the necrotic and fibrotic areas in the fetal kidney cortex of group **D** and these results agreed with those of **Shaffie et al. (2010)** who found marked diminution of protein content of renal tubular cells in alloxan diabetic rats. They added that this decrease may be due to the ability of alloxan to generate free radicals in the presence of suitable reducing agents, like reduced glutathione and oxygen. Alloxan exerts its DNA-damaging action, at least in part, by the production of free radicals and that this action can be modulated by common antioxidants, which can easily supplement the diet.

The results of this study showed that diabetic rats treated with olive leaf extract showed an increase in protein content in the renal tubules of the kidney. Olive leaf extract is an effective antioxidant which can protect proteins against oxidation. **Sierens et al. (2001)** stated that the antioxidant species may act in vivo to decrease damage of protein content in tissues. Increased protein content in group **D+O** indicating that olive leaf is more effective in improving kidney cell dysfunction induced by streptozotocin.

In the present work, deep staining affinity of PAS+ve materials was detected in glomeruli of the kidney cortex of group **D**, but few of them were faintly stained, convoluted tubules were moderately stained, fibrotic areas were less stained and degenerated areas were negatively stained. Increased PAS+ve materials in some glomeruli may be due to congestion observed in them, since RBCs contain 10% of their weight carbohydrates (**Abd Rabu, 2011**). These results are in agreement with those of **Shaffie et al. (2010)** who noticed diminution in PAS +ve materials in some tubules in kidney sections of the diabetic rats. However, **Poop and Cattley (1991)** and **Al Dossary (2007)** reported that the decrease in mucopolysaccharides content in tissue made by several factors may be due to the disturbed role of Golgi apparatus, which is responsible for synthesis of polysaccharides.

In the present work, the treatment of the diabetic rats with olive leaf extract showed normal content of PAS+ve materials in the kidney cortex of their fetuses. **Tavafi et al. (2012)** found that OLE is a new nephroprotective agent against acute kidney failure.

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