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# EFFECT OF IRAQI ETHANOLIC PROPOLIS EXTRACTS ON THE REACTIVE NITROGEN SPECIES AND GENOTYPE ALTERATION IN DIABETIC NEPHROPATHY RATS

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## ABSTRACT

The purpose of this research is to identify the effect of the Iraqi extract ethanolic propolis(EEP) on the nitrogen radicals and most important of which Peroxynitrite and nitric oxide, in rat laboratory infected kidney failure as a result of diabetes induce by streptozotocin (60 mg / kg), as well as its effect on the candidate genes in diabetic nephropathy are glucose transporter1(GLUT1) and nephrin promoter were found local EEP cannot reduced genotoxicity for STZ, but an effective influence on the free radicals where found a decrease in the concentration of Peroxynitrite radical was detected by detection of Nitrotyrosine (3- NT) by (ELISA), where the concentration increasing in diabetic groups while groups that treated by local EEP showed a decrease in the concentration of (3-NT) compared with control group, which indicates the low concentration of Peroxynitrite. As well as the results show the effect of local EEP on the low concentration of nitric oxide, and reduce the level of glucose in the blood, kidney function (creatinine, uric acid) and the presence of an improvement in the pathological changes of kidney tissues in the treated groups compared to the diabetic, this indicates that propolis is a strong immunological factors and antioxidant. The results we have obtained through this study to conclude that propolis cannot reduce the genotoxicity induced STZ but has immunological properties through its effect on reducing the concentration of 3-NT and $NO^{\bullet}$ in Pre-treated and Post-treated groups and this is due to contain rich in flavonoids, phenolic acid, and others. It also has anti-oxidant properties through its effect anti-hyperglycemia and his effect moderate on kidney function by reducing the concentration of creatinine and uric acid in the blood through the improvement of histological changes in the kidney of rats with diabetes induced by STZ.

**KEYWORD:** ***GLUT1, Nephrin promoter, EEP, 3-NT, Peroxynitrite and Mutation.***

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# INTRODUCTION

Diabetes is characterized by the absence or relative lack of insulin and this has the effect in increasing the level of glucose in the blood, it is classified to insulin-dependent diabetes mellitus (IDDM); called Type I, non-insulin-dependent diabetes mellitus (NIDDM); called Type II and other specific types associated with genetic defects in beta cell function[1]. Diabetic nephropathy is a clinical syndrome characterized by the decline in glomerular filtration rate and excessive deposition of extracellular matrix proteins [2,3]. Oxidizing free radicals consist of two classes are reactive oxygen species (ROS) and reactive nitrogen species (RNS). Radicals of nitrogen common is nitric oxide ($NO^{\cdot}$) and Peroxynitrite ($^{\cdot}ONOO^{-}$)[4]. In vivo, peroxynitrite consist in macrophages, and endothelial cells, platelets, white blood cells, nerve cells, and the other by the reaction between superoxide ($^{\cdot}O_2^{-}$) and the radical of the nitric oxide ($NO^{\cdot}$). Peroxynitrite is a species of short life (about 10 ms in vivo) [5]. Attributed of cytotoxicity to the nitric oxide $NO^{\bullet}$ and peroxynitrite $^{\cdot}ONOO^{-}$ radicals. Peroxynitrite reacts with the lipids, DNA and proteins cause oxidative damage and other series of reactions caused by free radicals. Peroxynitrite can cause DNA damage through oxidative modifications in each of the nucleobases and the backbone sugar-phosphate [6,7]. Nitrogen free radicals play a role in diabetic nephropathy where there is an accumulation of evidence supporting the key role of each of NO, superoxide ($^{\cdot}O_2^{-}$) and peroxynitrite, in the pathogenesis of diabetes and diabetic complications[8,9,10]. Several studies have been proposed that increased oxidative stress, and the paths influential formation of peroxynitrite such as poly-ADP ribosyl polymerase (PARP) is involved in causing the disease diabetes microvascular nephropathy[11,12]. For the diabetic nephropathy effect on the genes have been proposed, where many of the metabolic pathways and related categories of genes as candidates to play a role in genetic susceptibility to diabetic nephropathy. Genes that give susceptibility to diabetic nephropathy can be soliciting in different ways. It is candidate genes in diabetic nephropathy: Glucose transporter 1 gene (GLUT1): mainly facilitates the transfer of glucose in mesangial cells[13]. The experiments indicate that GLUT1 may be associated with impairment of renal glomeruli (glomerulopathy)[14]. Increase GLUT1 occur relatively quickly after the onset of diabetes, in addition, high levels of sugar in stimulating insulin growth factor1 (IGF-1) glucose uptake in cells mediated mesangial[15], indicating that glucose can stimulate activity glucose transporters individual, as well as gene expression of GLUT1 in these cells. Where the expression of GLUT1 gene leads to the morphology features of diabetic nephropathy and the emergence of this gene GLUT1 be related to the development of diabetic nephropathy. Nephrin promoter gene: Nephrin is a protein a recently identified, which is a key element in the slit diaphragm, which has a major role in the nomination of the plasma. Mutations in the genes of the human nephrin are the result in the absence of slite diaphragm, massive proteinuria, and killer disorder known as congenital nephrotic syndrome of the Finnish type[16]. Decreases in nephrin gene expression in diabetic nephropathy refer to the organization on the transcriptional level will weaken. The antioxidants have an important role against free radical damage, and extremely important to maintain optimum health. Where free radicals cause damage to cells and therefore have a key role in many diseases[17]. Fortunately, free radicals are controlled naturally by configuring the useful compounds known as antioxidants different. Propolis is a strong antioxidant, scavenges free radicals directly[18], which contains the components of a typical phenolic flavonoids, phenolic acids and esters[19]. EEP led to lower levels of blood glucose, fructose amin, Malondialdehyde (MDA), and nitric oxide (NO), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol, low density lipoprotein cholesterol in serum of fasting mice. And increase the level of serum cholesterol, high-density lipoprotein cholesterol (HDL-C)[20]. Streptozotocin (STZ) 2-deoxy -2 - ([ methyl (nitrous oxide) amino] carbonyl amino) -â-D- glucopyranose. (STZ; -

N nitro derivative of glucosamine) induce experimental diabetes in rats and mice [21]. Which lead to the degeneration of the islets of Langerhans in the beta cells[22].

## MATERIALS AND METHODS

Was used 60 rats Sprague-Dawley weighing between 150 ± 10 g and housing in the house of the animal in the College of Veterinary Medicine at the University of Al-Qadisiyah were divided animals into four groups (control), (diabetes), (pre-treated) and (post-treated) groups. The induction of diabetes in rats after fasting overnight before intraperitoneal injection by a single dose by (STZ, Sigma-Aldrich) at a dose of (60 mg / kg of body weight). Followed by high blood sugar in the rats over a period of up to 72 hours, using test strips glucose[23], where the level of glucose in the blood of more than 250 mg /dl guide on diabetes [24]. The experiment continued for 60 days. In the last day were sacrificed animals and collecting serum samples and kidney tissue samples for the purpose of testing them.

### *Preparation of local ethanolic extract propolis*

Local EEP Was prepared extract ethanolic by way Krell[25]. Determination of mutations of GLUT1 and nephrin promoter genes in kidney tissue by polymerase chain reaction -single strand confirmation polymorphism PCR-SSCP technique [26,27]. Primers have been designed by National Center for Biotechnology Information(NCBI) online: primers of GLUT1 gene were: ′5GACTTGGAGGAAGCAGCAAAG3′ (forward) and ′5ATCTTCGGGTTTGGACCCAG 3′ (Reverse) the product length size is 691 bp. Primers of nephrin promoter gene were: 5'GGGCCTAGACAAGTGTTGCT3'(forward) and GTGTTCCCGGTAGAGCGAAA(Reverse) the length size is 387bp. Determination of the concentration serum 3-nitro tyrosine (3- NT) in the rats by using technology immunohistochemistry (ELISA) by method quantitative sandwich enzyme[28]. Determination of serum(NO),uric acid and creatinine in rats by spectrophotometric method[29,30, 31].

### *Histopathological examination*

kidney tissues preserved in 10% neutral formalin buffer solution, after fixation, the tissue was trimmed and the specimen were washed with saline for (1-2hrs) and transferred to following Steps:1. Dehydration: Specimen were passed through ascending grades of ethanol alcohol (70%, 80%, 90%, 100%). For 1 hour in each concentration, 2. Clearing: Two solutions of xylol commonly used for clearing. The specimens rested 1 hour in each step, 3. Impregnation with Paraffin wax, 4. Blocking. 5. Sectioning, 6. Staining with hematoxilin &eiosin. [32,33,34].All groups read the data statistically by the program SPSS, version 17 software (2010). Test methods include a way ANOVA one-way and two-way to make comparisons between groups below the level of probability ($p < 0.05$). Mean ± standard error is used to express all data.

## RESULTS AND DISCUSSION

The figures (3) and (4) show the results that we have obtained by PCR-SSCP technique a genetic mutation in certain locations of genes GLUT1 and Nephrin promoter respectively.

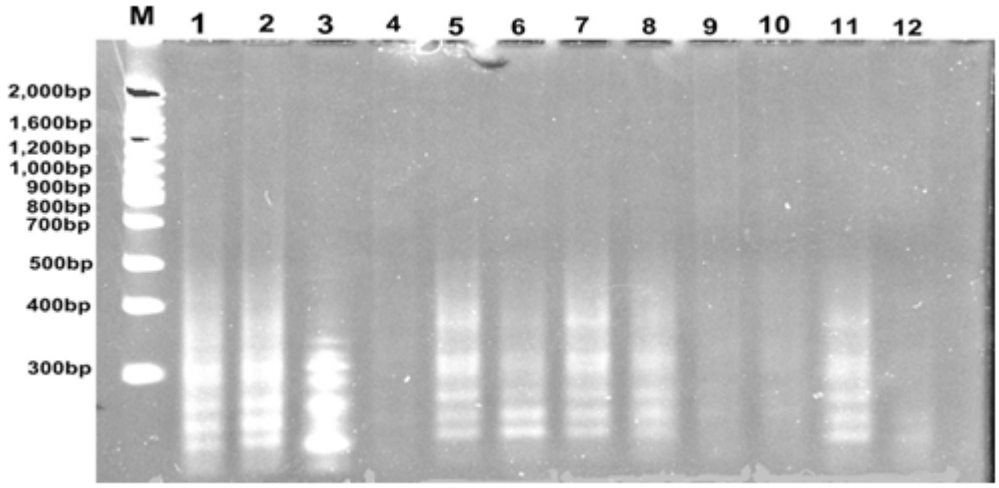


**Figure 1**
***Shows genetic mutation in the GLUT1 gene in diabetes group induced by STZ***

where M refers to the parameter 100bp DNA Ladder, either numbers (1-3) samples G1(control), (4-6) G2 samples (Diabetic), (7-9) G3 samples (Pre-treated), (10-12) G4 samples (Post-treated).

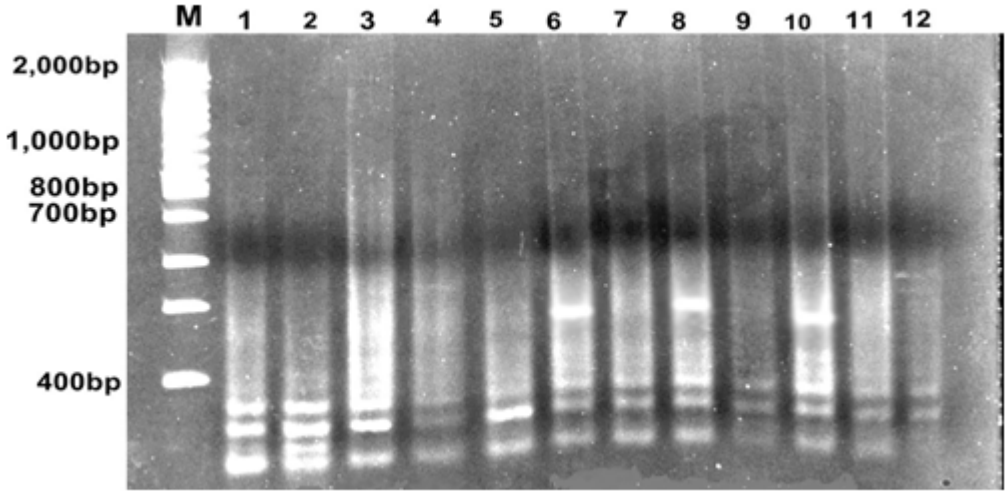


**Figure 2**
***Shoe genetic mutation of the Nephrin promoter gene shows in diabetes groups induced by STZ***

where M refers to the parameter 100bp DNA Ladder, either numbers (1-3) samples G1 (control group), (4-6) samples G2 (Diabetic), (7-9) G3 samples (Pre-treated), (10-12) G4 samples (Post-treated).

**Table1**
***Number of lanes according to genotype of GLUT1 and Nephrin promoter genes.***

| Genes name | Samples | Genotype | No. lanes |
|---|---|---|---|
| **GLUT1** | (1,2,8) | AA | 4 |
| | (5,6,7,11) | AB | 5 |
| | (4,9,10,12) | AC | 0 |
| **Nephrin promoter** | (1,3,4,5,9,11,7) | AA | 3 |
| | (2) | AB | 4 |
| | (6,8,10) | AC | 4 |
| | (12) | AD | 2 |

The results indicated of a genetic mutation in certain locations of genes GLUT1 and Nephrin promoter in infected groups with diabetes induced by STZ in each of G2, and G3 and G4. The table(2) shows the difference in the four groups in the number of lanes this difference indicates that for genetic mutation, suggesting a lack of effect of a substance on the EEP to reduce material STZ genotoxicity.

Cytotoxic effects of STZ rely on DNA alkylation by the work of a particular site with DNA bases[35] and by free radicals generated during metabolism STZ[36]. Therefore local EEP not effect on the mutation that induced STZ but local EEP decreases from peroxynitrite and nitric oxide radicals.the table (2) and figure (3) show the changes in the body weight after diabetes stimulation.

**Table2**
***The effect of local EEP on the body weight in diabetic nephropathy male rats.***

| Day | Group1 (control) Means ± SE | Group2 (Diabetes) Means ± SE | Group3 (pre-treatment) Means ± SE | Group3 (post-treatment) Means ± SE |
|---|---|---|---|---|
| 1 | 156.9±1.44 | 157.5±1.24 | 156.45±1.7 | 156.3±1.53 |
| 6 | 168±1.87 | 169.88±2.09 | 167.8±1.83 | 168±2.97 |
| 12 | 186.7±2.39 | 184.22±1.82 | 184.22±1.82 | 180.9±1.9 |
| 18 | 201.3±1.3 | 202.6±1.36 | 203.3±1.96 | 195.4±3.94 |
| 24 | 218.8±3.5 | 218.3±3.33 | 219.5±4.4 | 215.8±2.9 |
| 30 | 239.1±1.4 | 220.8±4.02 | 225.5±4.24 | 230±3.4 |
| 36 | 252.8±2.37 | 229.5±1.84 | 234.2±1.89 | 239.3±2.06 |
| 42 | 276±1.24 | 235±1.94 | 242±2.05 | 252±2.57 |
| 48 | 307.3±3.96 | 243.15±1.57 | 256.1±5.15 | 288.5±3.08 |
| 54 | 339.7±1.71 | 258.1±1.3 | 270.5±2.27 | 319.5±3.33 |
| 60 | 355.5±2.66 | 255.8±2.1 | 295±2.2 | 329.8±4.04 |

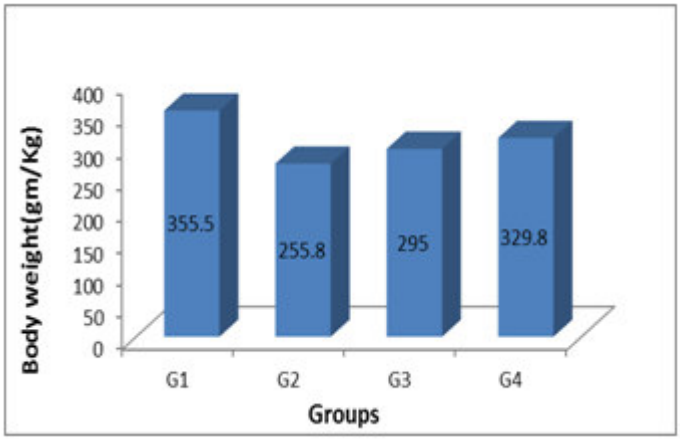


**Figure 3**
***The effect of EEP on the increase body weight in diabetic nephropathy induced by STZ in male rats.***

After diabetes stimulation began to show changes in body weight, where the G1in the case of a continuous increase gradually, while the G2 be in the case of a decrease in body weight significantly compared to the G2. The G3 and G4 show a decrease in body weight compared to the G1but after treatment by local EEP showed a significant improvement in body weight as in the table (2), propolis has a strong anti-oxidant and the scavenging effect against free radicals[37], this result suggests that propolis may improve metabolism troubled associated with diabetes. Either the table (3) and figure (4) show the effect of local EEP on the level of blood glucose in diabetic nephropathy rats.

**Table 3**
***The effect of local EEP on the level of glucose in diabetic nephropathy male rats.***

| Day | Group1 (control) | Group2 (Diabetes) | Group3 (pre-treatment) | Group3 (post-treatment) |
|---|---|---|---|---|
| 24 | 89±1.13 | 87.7±2.13 | 87.8±2.23 | 92.2±2.3 |
| 30 | 90.7±1.45 | 534±16.34 | 288.4±15.17 | 371±28.08 |
| 36 | 89.1±1.88 | 511.7±18 | 348.7±25.91 | 305.6±22.93 |
| 42 | 92.2±2.3 | 541.7±13.24 | 311.6±19.97 | 288.5±16.03 |
| 48 | 87.7±2.13 | 539.6±15.37 | 488.4±8.25 | 282.4±10.61 |
| 54 | 91±1.11 | 520.1±15.9 | 296.8±8.87 | 255.4±11.97 |
| 60 | 92.1±1.92 | 528.4±11.33 | 315.81±13.94 | 267.9±15.66 |

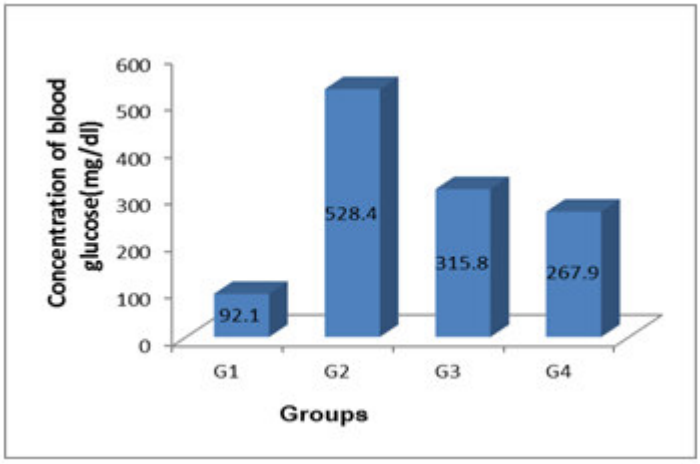


**Figure 4**
***Effect of local EEP on the level of blood glucose in the diabetic nephropathy male rats.***

Where there was a significant increase in blood glucose levels (P< 0.05). It is estimated that the effect of EEP where the reduction in glucose in the blood clearly in G3 and G4 comparison with the (G2). On the other hand, the table (3), explains the low level of blood glucose in the G4 rats more than G3 rats, but were significantly higher than the control group (P< 0.05). These results are consistent with[38],which showed that the local EEP in rats had an impact hypoglycemic effectively. The presence of flavonoids and polyphenolic components of active ingredients effective anti-oxidant, strengthen systems antioxidant defense in the tissues of the pancreas[39].The figure(5) show effect of local EEP on the concentration of (3- NT).

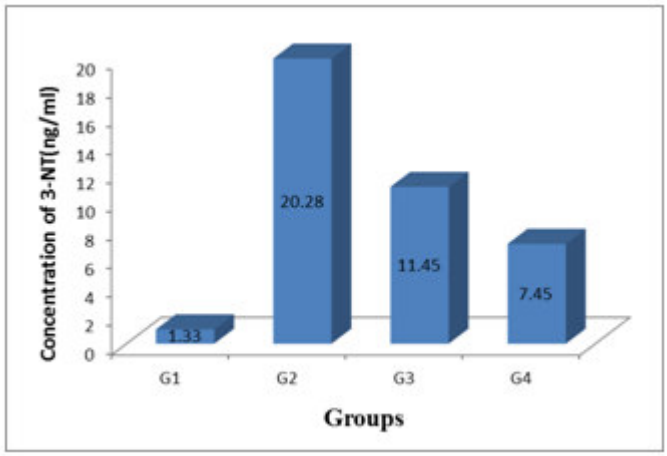


**Figure 5**
***The effect local EEP on the concentration of (3-NT) in diabetic nephropathy male rats.***

where 3-NT concentration increased in diabetic nephropathy rats (G2) (P< 0.05) compared with G1, so indicates increased in oxidative stress and peroxynitrite generation. As the effect of local EEP it has been shown that it leads to a reduction in the concentration of 3-NT in G4, while G3 a low concentration of 3-NT, but more concentration than the G4, but the decline in these groups are not up to the G1 as shown in table (4). These results are consistent with[40,41]. Either figure(6) explain the effect of local EEP on the concentration of serum nitric oxide (NO).

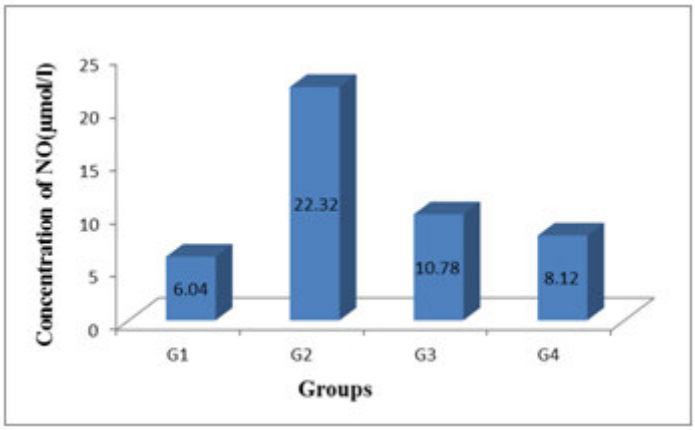


**Figure 6**
***The effect of local EEP on the concentration of NO in diabetic nephropathy male rats.***

It has been found that there is a significant increase in the concentration of NO in a diabetic rats (G2), compared with the G1 (P<0.05), while the G3 and G4 observed a decrease in the level of $NO^{\cdot}$ compared with a diabetic nephropathy rats, but still significantly higher than the control group (P<0.05), as shown table (4).These findings are consistent with[38]. The effect of EEP on the level of uric acid in the rats serum show in figure (7).

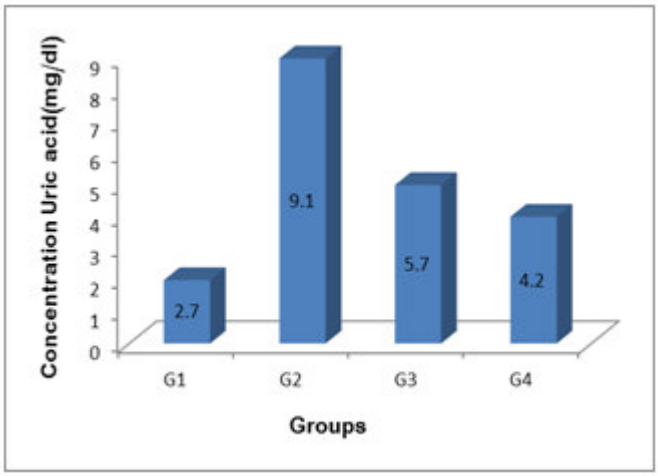


**Figure 7**
***The effect of Iraqi EEP on the level of uric acid in the diabetic nephropathy male rats.***

During this study found a significant increase in the level of uric acid in the serum of diabetic rats (G2) compared with control rats (G1), (P<0.05) but G3 and G4, there is an improvement in the level of uric acid in the blood, where it is significantly reduced compared to G2 (P<0.05), but much more than the G1 (P<0.05), as shown in table (4). These findings are consistent with [36]. And the figure (8) explains the effect of local EEP on the level of creatinine in the serum rats.

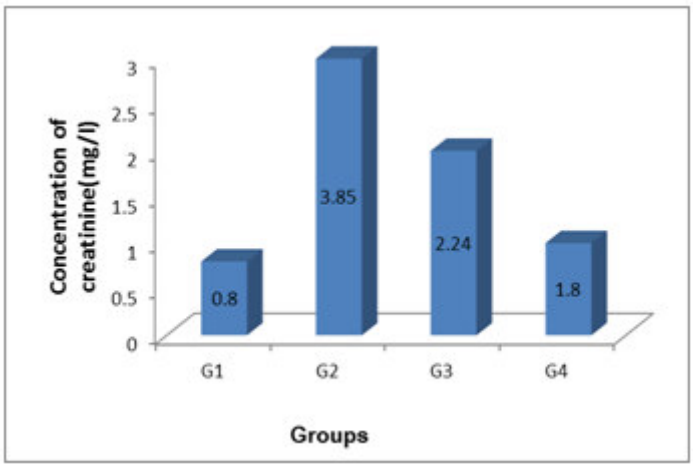


**Figure 8**
***The effect of EEP on the level of creatinine in the serum of diabetic nephropathy male rats.***

there was a significant increase in the levels of serum creatinine in diabetic rats G2 compared to G1 (P<0.05), but the groups treated by local EEP G3 and G4 respectively, it was observed that the local EEP works on a decrease in the level of creatinine (P< 0.05), this result is consistent with the research[38,42], it was noted that an increase in the level of creatinine in diabetic nephropathy rats (G2) refers to the development of diabetic nephropathy as show in table (3).

**Table 3**
***The effect of EEP on the biochemical measurements in the serum of male rats.***

| Groups | Conc. (3-NT) ng/ml | Conc. NO µmole/l | Conc. Uric acid mg/dl | Conc. Creatinine mg/l |
|---|---|---|---|---|
| **Group1 control** | 1.33±0.499 | 6.04±0.31 | 2.7±0.44 | 0.8±0.62 |
| **Group2 diabetic** | 20.28±0.436 | 22.32±0.66 | 9.1±0.25 | 3.85±0.76 |
| **Group3 pre-treated** | 11.45±1.46 | 10.78±0.15 | 5.7±0.39 | 2.24±0.29 |
| **Group4 post-treated** | 7.45±0.74 | 8.12±0.21 | 4.2±1.22 | 1.8±0.38 |

***Histopathological changes***

The figures (9), (10), (11) and (12) showed the histopathological changes in four groups under microscopic examination

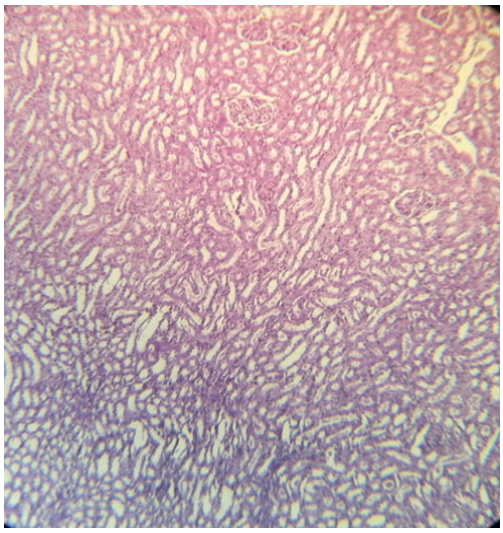

**Figure 9**
***the kidney of normal histoarchitecture induced by STZ after 24-day show normal kidney tissues and showed tubes and glomeruli intact. 400 X H & E.***

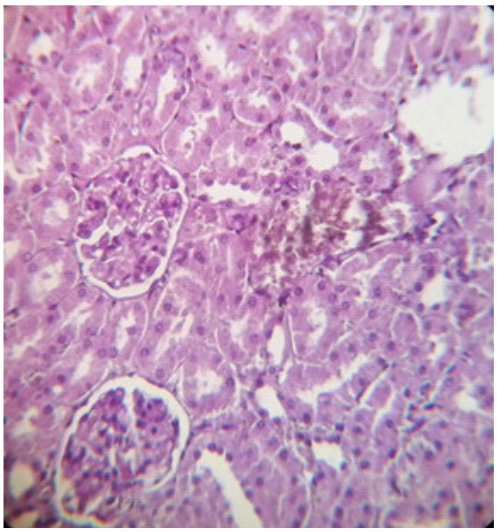

**Figure 10**
***diabetic kidney(G2) of rats the control group, where congestion and severe atrophy of the blood vessels with the destruction of the vascular wall 400 X H & E.***

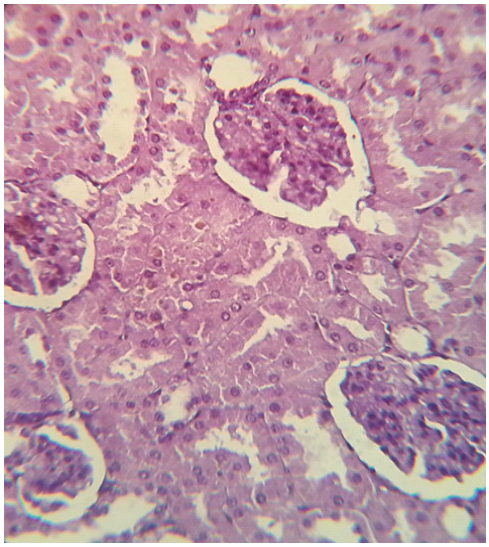

**Figure 11**
***kidney rats in G3 by local EEP that pre-injected with STZ diabetes which stimulates the presence congestion in the shows the breadth light of pipes blood vessels with small areas of red blood cells in the interstitial leak, and also there is a partial improvement in***

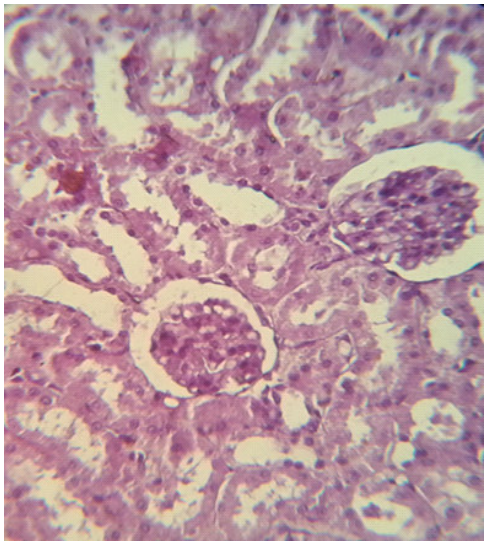

**Figure 12**
***kidney of rats in G4 by local EEP that induced by STZ show congestion and vascular moderate and of mild to moderate capsules Bowman well-preserved, and shown the structure close to the tissue of normal kidney. 400 XH & E.***

***histoarchitecture kidney Bowman capsule is retained. 400X H & E.***

Microscopic examination showed in the G1 no changes in the microscopic structure of the kidney where it was normal kidneys in the control group. The G2 (diabetic) showed microscopic examination of kidney damage in several inflation and the deterioration of the epithelium of the renal tubules, there vascular congestion, and severe atrophy, with the destruction of the vascular wall, these findings are consistent with[38,43,44]. The G3 pre-treated, which is caused by STZ in rats, where the microscopic examination, the presence of mild to moderate congestion in the blood vessels, with small areas of red blood cells in the interstitial leak. Bowman capsule is retained. The effect of local EEP, where the microscopic examination showed reduced pathological changes were partially successful in terms of reduction of kidney damage consistent with these results[38,45]. The G4 of rats post-treated, were showed a significant improvement in pathological changes. Microscopic examination showed, the presence of vascular congestion mild, light

and shows the breadth of the pipe and maintain good Bowman capsules, and showed the structure close to normal kidney tissue, this is consistent with[42,46]. In this study, propolis wizard to diabetic nephropathy rats reversed the effective protection and improvement of histopathological changes.

## CONCLUTION

Iraqi propolis cannot reduce the genotoxicity induced STZ but has immunological properties through its effect on reducing the concentration of 3-NT and $NO^{\cdot}$. It also has anti-oxidant properties through its effect as anti-hyperglycemic reagent.

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