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Histopathological Changes in Diabetic Rats Induced by Alloxan Treated With Olive Leaf Extract and Silymarin Extract with Silver Nanoparticles

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ABSTRACT— The aim of this study was to investigate the antidiabetic effect of olive leaf extract, silymarin extract with silver nanoparticle on diabetic rat at histopathological study. Silver nanoparticle were prepared via green chemistry methods using olive leaf and silymarin extract as capping and reducing agent which reduce silver nitrate (AgNo3) into silver nanoparticles. The present study included 50 rat weight range from 120 – 200 grams. Animal divided randomly into 5 group (each group=10) as follow: the first group (G1) as healthy non diabetic control (control negative) don't received any type of treatment. The other groups were treated intraperitoneally once with 120 mg/kg b.w of alloxan, then when these rats became hyperglycemic according to)Accu-check Roche Diagnostics GmbH, Mannheim, Germany), this group consider (G2) as (control positive), (G3):Diabetic rats were treated orally daily dose(50 mg) of silver nanoparticle of olive leaf extract, (G4):Diabetic rats treated orally daily dose(16 mg) of silver nanoparticle of silymarin extract, while (G5) Diabetic rats treated orally daily dose (33 mg)of nanoparticle for both olive leaf extract and silymarin extract. At the end of the experiment after 3 months, tissue samples were taken from the pancreas, liver and kidney of all groups for histopathological study. Histopathological investigation of pancreas, liver, kidney and heart tissues of diabetic rats represented the presence of different changes in rats with induced diabetes by alloxan. Meanwhile treatment with olive leaf extract and silymarin extract for AgNPs overcome those changes.

KEYWORDS: diabetes, alloxan, olive leaf, silymarin, silver nanoparticles, histopathology.

1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic and life-threatening condition affecting people worldwide [23]. It is a chronic condition characterized by abnormalities in glucose, protein, and lipid metabolism resulting from absolute or inadequate insulin insufficiency [9], [3]. DM is characterized by physiological and cellular abnormalities that lead to the death of beta (β) cells as the illness progresses [26], [36]. *β*-cell failure is caused by glucose and lipid toxicity, and increased glucose absorption by the islet beta (β)-cells induces glucose toxicity [26]. The oxidative stress that results from the process causes a reduction in insulin synthesis and secretion, which sets off a chain of cellular events that eventually leads to death [26]. Anomalies in serum lipids have also been linked to diabetes [24], [37]. Alloxan has been administered in single or multiple doses, through different routes (intraperitoneal, intravenous and subcutaneous); with single intraperitoneal administration apparently the most employed mode. Animal species, the route of administration and nutritional status have been considered to play a role in determining the dose of alloxan appropriate for induction of diabetes. However, single intraperitoneal administration of the drug at 120 mg/kg b.w appears to be most effective [18]. Alloxan causes diabetes via a mechanism which basically

includes partial degradation of the beta (β) cells of pancreatic islets and subsequent compromise in the quantity and quality of insulin produced via these cells. The model employs two distinct pathological effects which include selective inhibition of glucose-stimulated insulin secretion& induced formation of (ROS) reactive oxygen species which promotes selective necrosis of beta cells of the pancreas. Both effects collectively result in a pathophysiological state of insulin-dependent diabetes or type 1-like diabetes mellitus in cells [15]. The chemical features of alloxan and their contribution to its diabetogenicity. The diabetogenicity of alloxan is underlined by its selective cellular uptake by beta cells of the pancreas and consequent accumulation in these cells [30]. The olive tree (Olea europaea) is one of the more famous plants that have commonly been used as a traditional treatment for combating diseases, due to the fact that they possess abundant polyphenolic compounds (i.e., more than 40 g/kg of dry tissue), [11]. Silymarin is a flavonoid containing mixture extracted from the fruits and seeds of the milk thistle plant—*Silybum marianum* (L.) Gaertn. It is available as dietary supplements and can be registered as a drug [6]. Silymarin used in diabetes mellitus and its complications [1], [38]. Nanotechnology refers to the terms manufacturing, illustration, control and uses of structures to control nanoscale size and shape at the nanoscale [32]. Presently, the plant-mediated green synthesis of silver nanoparticles has grown into a new and important branch of nanotechnology. It has gained prominence due to its ecofriendly and cost effective, lesser toxicity when associated with chemical hazards [29]. The aims of research determine the effect of the silver nanoparticles of the olive leaves and silymarin in diabetic rats.

2. Materials and methods

2.1 Experimental animals

A total number of 50 rats used in this experiment at age 2 weeks , the weight range 120_200gm supplied from animal house of veterinary laboratories and cancer Iraq of Iraqi center/ al -Mustansiyria University were used for period of the study ,they are housed and preserver in traditional animals a tact, with role of the condition of temperature (25°c) and animals that feed specific formula fed pellet and given water in *ad libtium* during (90 days) put rats in plastic gage contain hard wood bedding and change it contentiously and ensure its clean surface that experimental in Baghdad at veterinary laboratories. The experimental was achieved under the supervision and approval of the animal's ethical committee at unit of.

2.2 Preparation of experimental plants extract

Olive leaves and silymarin leaves were collected and washed several times with water, then sterilized by 50% alcohol for 5 minutes to remove foreign materials such as dust, particles, and microorganism, then washed with distilled water more than 5 times, and then the leaves were air_ dried for 10 days. The leaves were washed with distilled water three times to remove of dust and adhering impurities, and the dried in a drying oven at 40 °C for 12 hours. The leaves were weighed at 10 g each and ground by an electric grinder. There it was placed in a glass beaker and 100 ml of distilled water added to, then the solution was heated to 80 °C with continuous stirring for 30 minutes. The Whatman 4 tube was filtered to obtain a clear extract and then filtered through a microfiltration paper (0.45 um). The plant extraction was prepared according to the procedure of [17]. The olive and silymarin extract were obtained and a mixture of them was made, following the same steps, except that the two plants were weighed and placed in the same glass beaker, and then extraction was done for them according to the above.

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Figure (1): olive leaves & chopped olive leaves

Figure (2): silymarin leaves &chopped silymarin

2.3 Preparation of silver nanoparticles (AGNO3)

1. Preparation of silver nanoparticles (AGNO3)

AgNO3 from Sigma (169.873 g/mol), 0.169 gm of AgNO3 was dissolved in 1 litter from distilled water at room temperature, then the solution was stirred for 15 minutes to make the solution homogenous. The solution is kept in a dark container to prevent oxidation. At Al-Fadil Foundation, Hilla branch according to the procedure of [8].

2. Synthesis of Silver Nanoparticles

Silver nanoparticles were prepared according to the procedure of [8]. 5 ml of Silver nitrate solution at a concentration of 0.02 M was added to 100 ml of olive leaf extract and silymarin extract and their mixture was mixed separately, and the final concentration of silver ions was 1 mm. The mixtures were heated separately with continuous stirring at a temperature of 70 °c for a period of 20 minutes. It was observed that the color of the extract turned into a dark reddish _ brown color as a result of the reduction of ions by the active substances in the extract. The three solutions of silver nanoparticles were filtered with microfiltration paper (0.22um) to remove large particles and ensure sterilization, and preserved in a dark environment.

2.4 Experimental design

The number of animals of the study about (50) rats were used, at age (2-3 weeks), were divided in to (5) groups and treated as following:

G1(NO: 10)	**Healthy normal rat (negative groups)**
G2(NO:10)	**Diabetic non treated rat (positive groups)**
G3 (NO:10)	**Diabetic rat treated with silver nanoparticle with olive leaf extract (orally given)(50mg)**
G4(NO:10)	**Diabetic rat treated with silver nanoparticle with silymarin extract (orally given)(16 mg)**
G5(NO:10)	**Diabetic rat treated with silver nanoparticle with olive leaf and silymarin extract (orally given)(33 mg)**

The period of the experiment was 90 days. At the end of the experiment, the rats sacrificed by general anesthesia (chloroform). Samples from the liver, kidney, and pancreas tissues were taken and washed with normal saline to eliminate bloody cells. The samples from all rats were fixed in buffered neutral 10% formalin solution and then embedded in paraffin. The tissues were sectioned at 5 μm thicknesses for Hematoxylin and Eosin dye staining. The tissues were further examined using an optical microscope Olympus. Treatment with plant extracts started 48 hours after alloxan injection.

2.5 Induction of diabetes

The animals were fasted overnight (12-24 hours) [25], they were only access to water and their weighed and fasting blood glucose level was recorded. The diabetes was induced by intraperitoneal injection of alloxan monohydrate (120mg/ kg body weight). Alloxan was first weighed individually for each animal, Alloxan prepared by weighted and dissolved in distilled water. Then the diabetes was induced by intraperitoneal injection of 0.5 of freshly prepare solution of alloxan, after alloxan injection food and water were presented after 30 minutes of injection (Carvalho et al., 2003; kanan and Prince, 2003; Nagappa et al., 2003), and given 5% glucose solution for 48 hours instated of water to reduce the severity after hypocalcemia in the body of rats, as the alloxan caused damaged the pancreatic beta cells and caused releasing insulin in large amount. Two days after alloxan injection fasting blood glucose levels of each animals was determined above 250 mg / dl were used as diabetes (Kumar et al., 2006; Gidado et al., 2005; Pari and Venkateswaran, 2003).

2.6 Histopathological study

After scarified the animals of experimental, samples of pancreases, liver and kidney from each animals of all groups were fixed with neutral buffered formalin after at least 48- 72 hours the samples treated with the following :- The tissues (pancreas, liver and kidney)was removed immediately from each animal and then washed within a physiological saline solution (0.9% NaCl) for the removal of the debris or any microbes, which might obstructive the process of fixation. Samples were allowed to remain in fixative (10% Neutral buffered formalin) for 24 hours. The dehydration & clearing of the tissues were processed routinely and embedded in paraffin wax. About 5 microns thickness sections were prepared with microtome & mounted on clean glass slides and then they were deparaffinized in xylene twice for 5 minutes and then rehydrated with graded alcohol & stained with hematoxylin & eosin (H&E) dye. The stained sections were examined by using microscope. Tissues (pancreases, liver and kidney) of diabetic group were compared with tissues of the different treatment groups for histopathological changes. According to (Luna and lee, 1968).

3. Result & Discussion

The result of histopathological changes varying according to the type of treatment for each group that diabetic group give sever changes and these changes noted at different degree in severity for both treated with silver nanoparticles for each plant extract (olive leaves & salymarin) and ameliorate with period that sever changes reduced at 3 month.

3.1 Histopathological study of pancreatic tissue of study group:

In the control groups normal histopathological appearance of pancreas was seen that normal islet cells and other structure fig. (1a), In the diabetic group more sever lesion noted as compared with the control group the most important change in DM group characterized by degeneration and necrosis of beta pancreatic cells of islets as well as the cellular with disrupted, atrophy, and congestion of pancreatic blood vessels also showed fig. (1b). In DM with silymarin ANPS treated groups showing moderate expansion of pancreatic islet with prominent hyperplastic islet seen in figure(1c), in DM with olive leaves AgNPS treated groups present mild expansion and absence of dilation figure(1d), in DM treated with combination groups showing

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the tissue nearly to normal figure(1e).

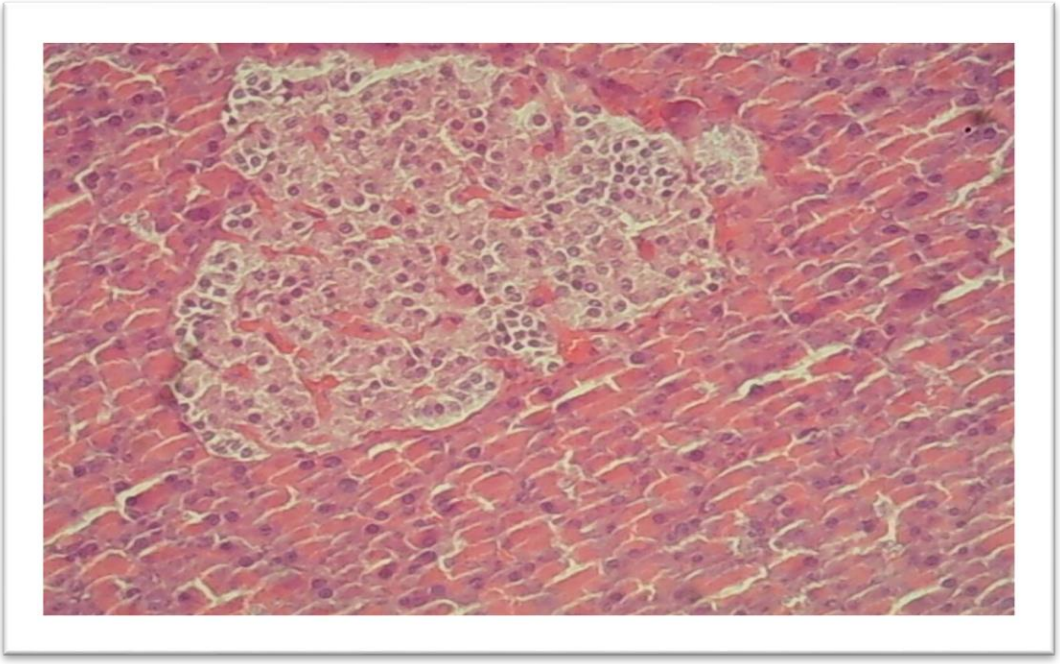

Figure (1 a): normal histological appearance of pancreas was seen that normal islet cells and other structure.

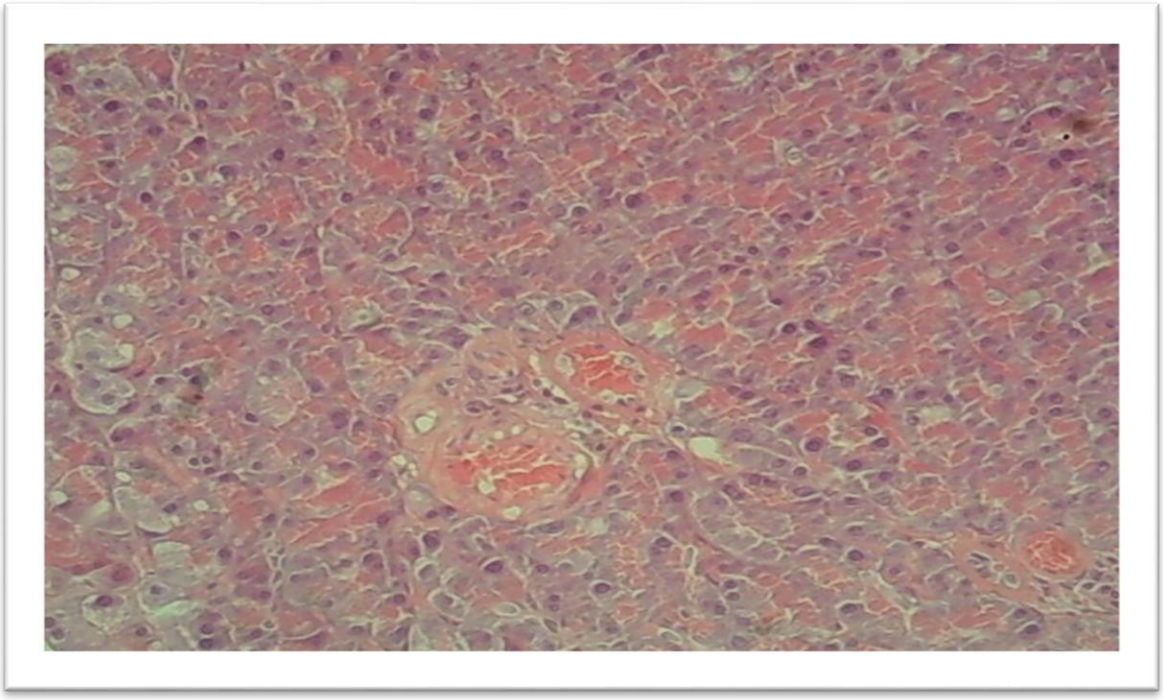

Figure (1b): in DM group presence of degeneration and necrosis of beta pancreatic cells of islets as well as the cellular with disrupted, atrophy, and hydropic degeneration, congestion of pancreatic blood vessels also showed fig.. (H&E X 200)

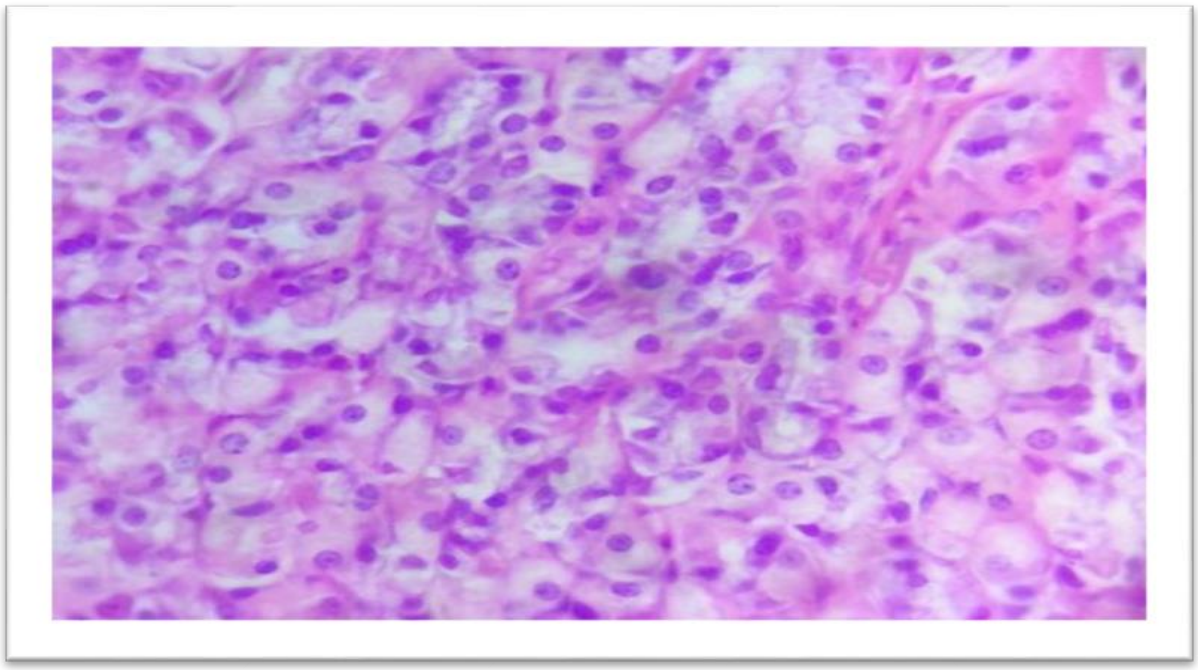

Figure (1c) in DM with silymarin AgNPS treated groups showing moderate expansion of pancreatic islet with prominent hyperplastic islet seen in figure, (H&E X200).

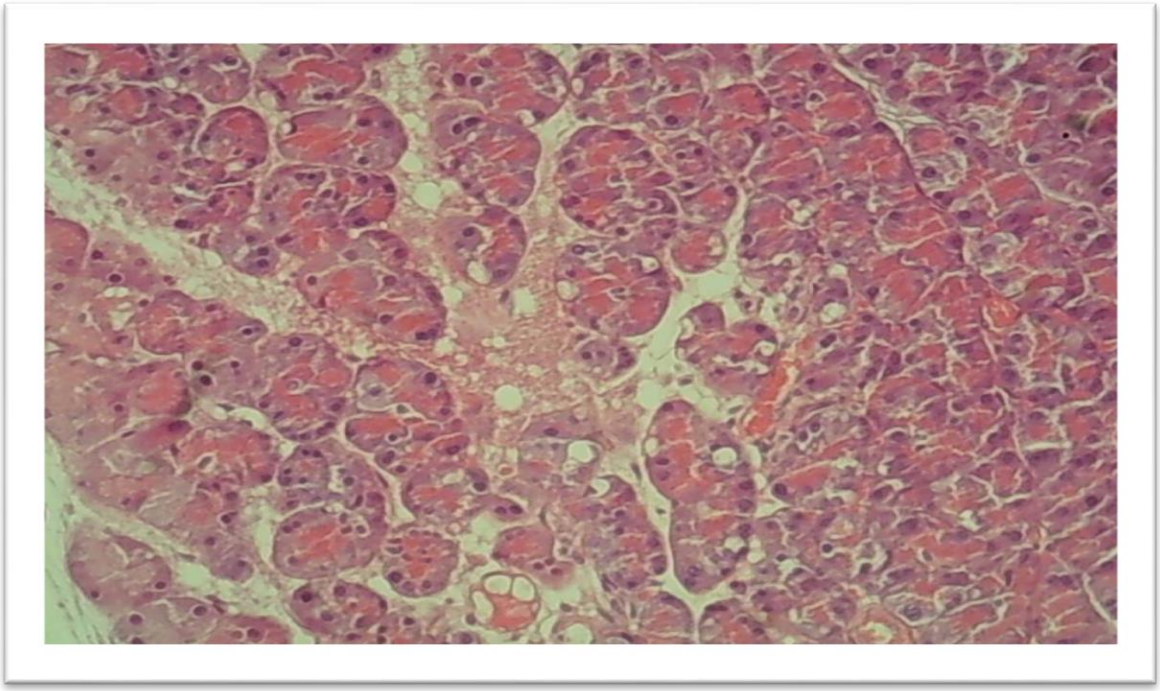

Figure (1 d): in DM with olive leaves AgNPS treated groups present mild expansion and absence of dilation

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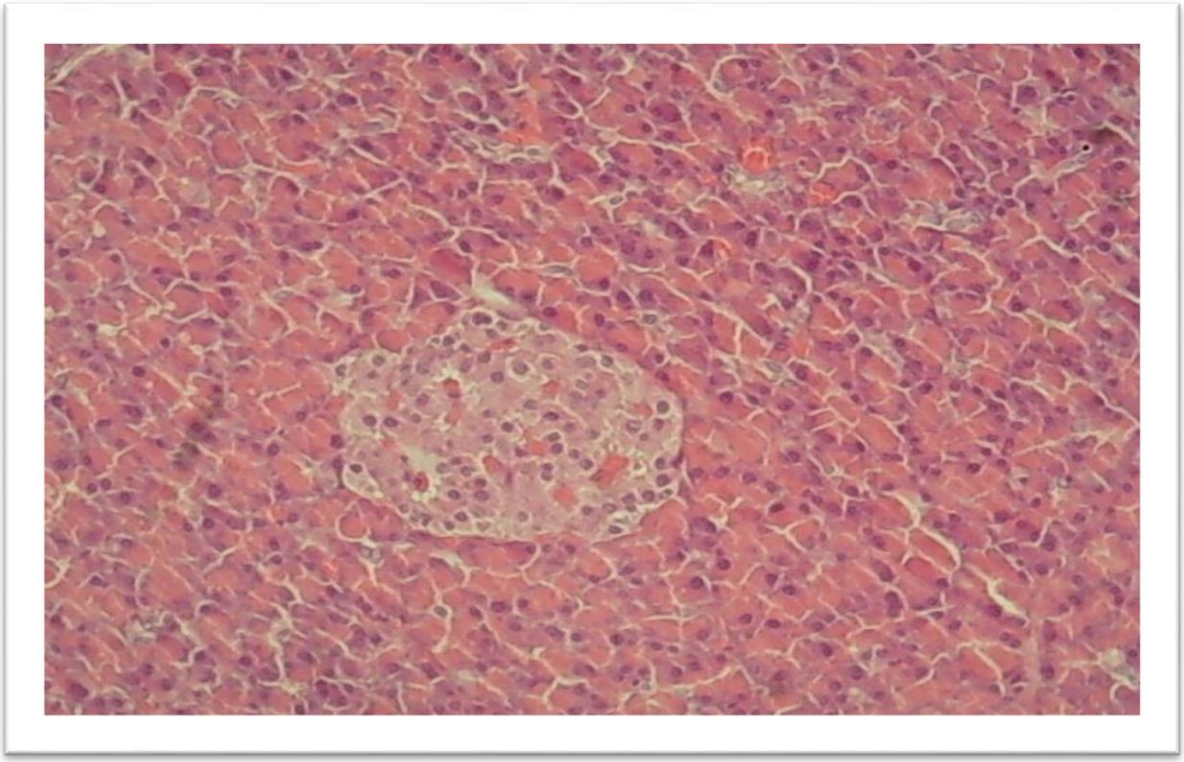

Figure (1e): in DM treated with combination groups showing the tissue nearly to normal

3.2 Histopathological study of hepatic tissue of study group:

Control negative group observed normal structure of liver (hepatocyte, portal area) seen in figure(2a), compared with in control group (DM) showing of infiltrations of inflammatory cells around the central vein with presence of shrinkage of hepatocytes, some of hepatocyte appears with fatty change like, extensive hydropic degeneration and necrosis of DM, dilation and congestion of blood vessels, normal vacuoles were observed in the cytoplasm and mild fibrosis of blood vessels wall, bile duct proliferation and presence of inflammatory cells in portal area, seen in figure(2b), in DM treated with olive leaves of silver nanoparticles showing improvement of the degenerative of hepatocytes, seen in the figure(2 c), in DM treated with silymarin of silver nanoparticles showing improvement of liver tissue and mild change seen in figure(2d), in DM treated with combination showing improvement of liver tissue and nearly return to normal tissue seen in the figure (2e).

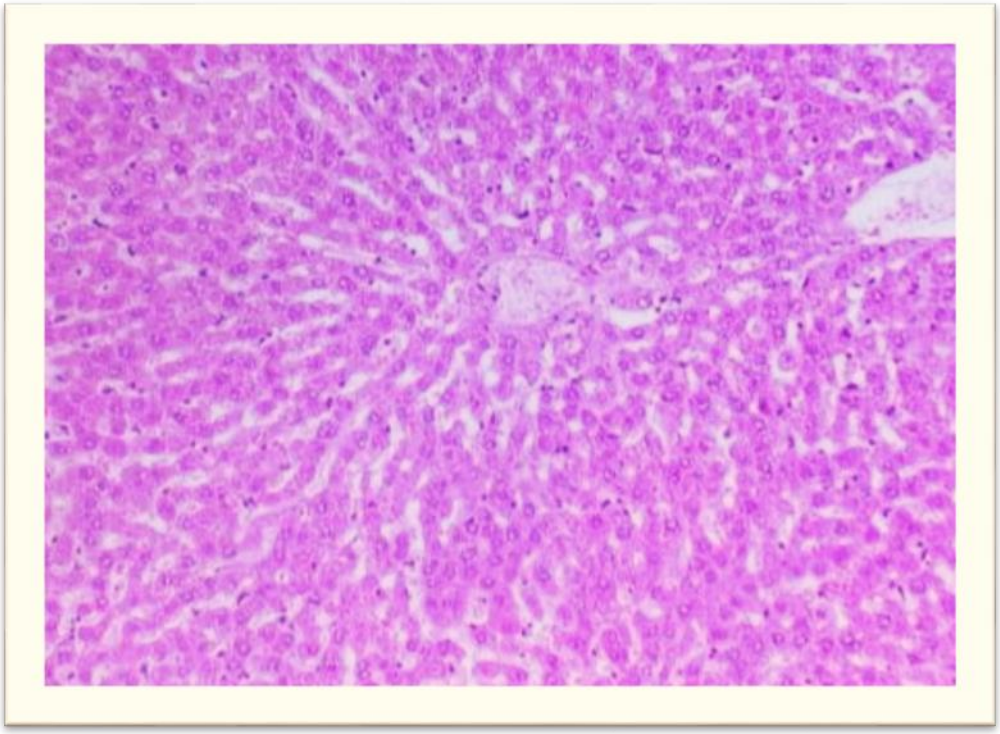

Figure (2 a): Control negative group observed normal structure of liver (hepatocyte, portal area).

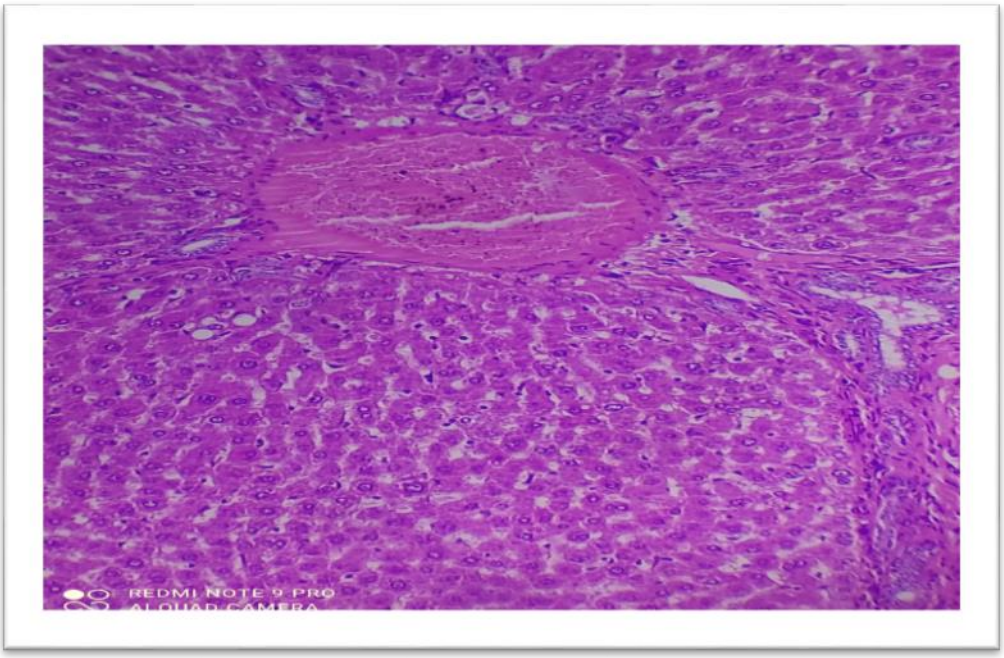

Figure (2b): (DM) showing of infiltrations of inflammatory cells around the central vein with presence of shrinkage of hepatocytes, some of hepatocyte appears with fatty change like, extensive hydropic degeneration and necrosis of DM, dilation and congestion of blood vessels, normal vacuoles were observed in the cytoplasm and mild fibrosis of blood vessels wall, bile duct proliferation and presence of inflammatory cells in portal area.

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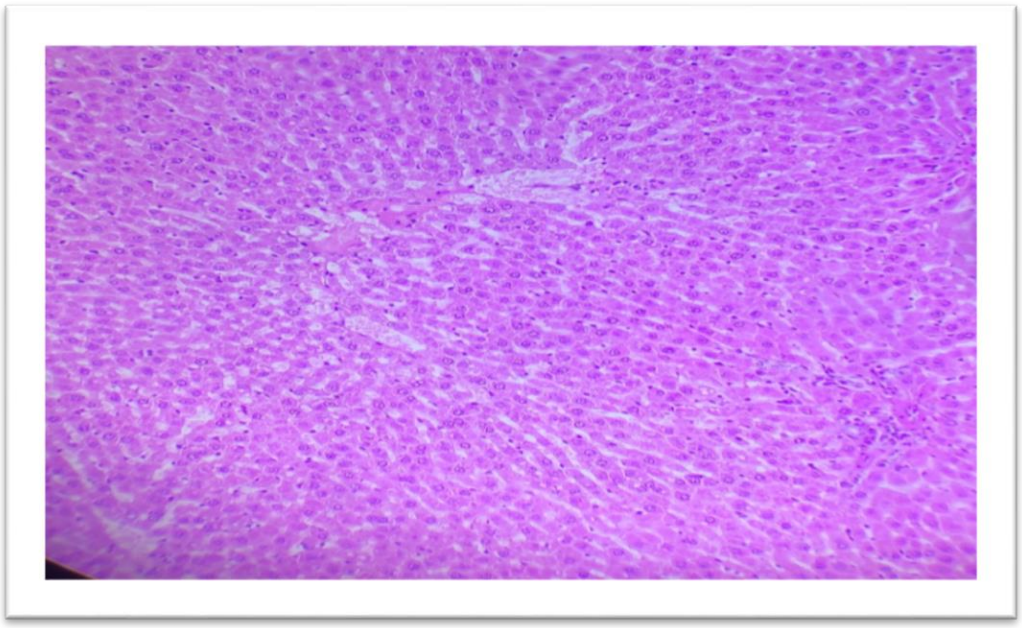

Figure (2 c): in DM treated with olive leaves of silver nanoparticles showing improvement of the degenerative of hepatocytes

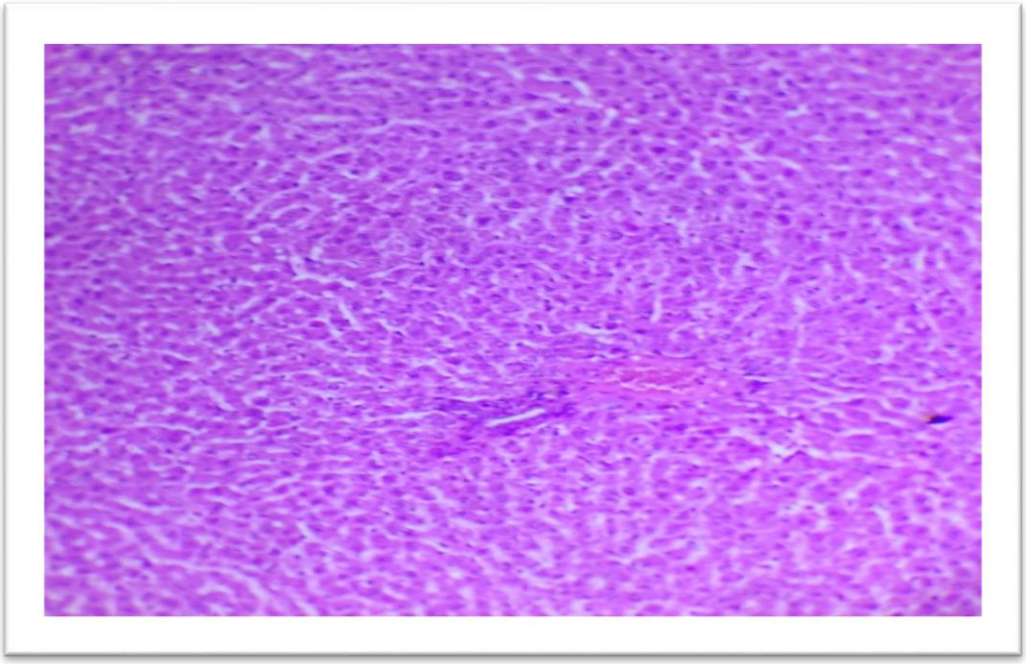

Figure (2d): in DM treated with silymarin of silver nanoparticles showing improvement of liver tissue and mild change.

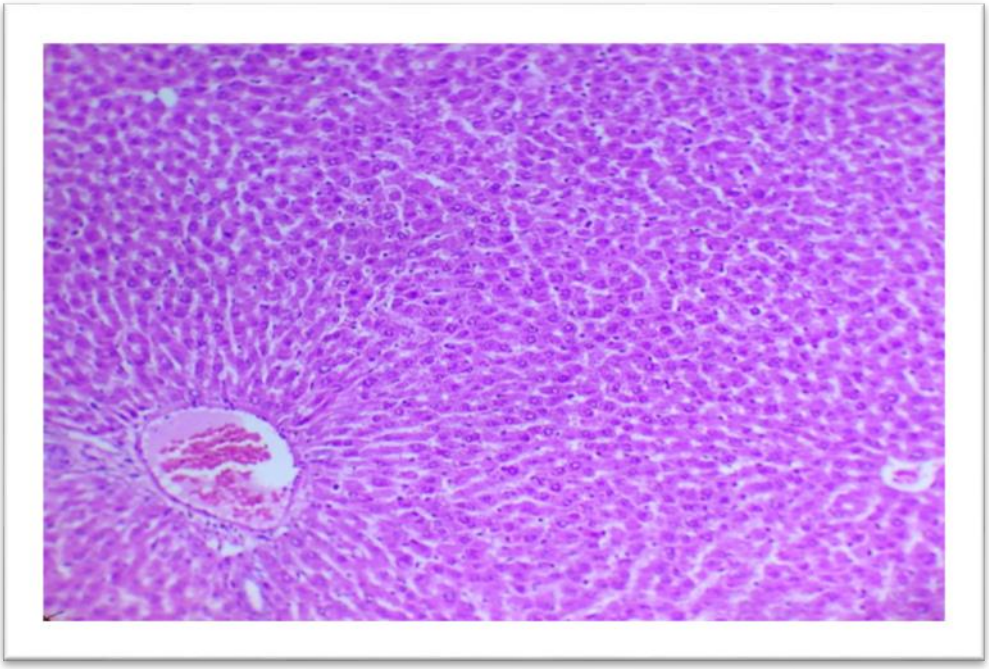

Figure (2 e): in DM treated with combination showing improvement of liver tissue and nearly return to normal tissue.

3.3 Histopathological study of renal tissue of study group:

The normal histological view of the kidney was observed in control negative group seen in figure(3 a), in DM groups the kidney showing hydropic degeneration, necrosis were present and in the tubules epithelial cells. renal tissue from diabetic rats showing glomerular tuft damage with cell disruption seen in figure (3 b), in DM group treated with silymarin of silver nanoparticles represented by ameliorate these changes as (thickening of the capillary for bowman capsule, enlargement of glomerular tuft, lumen of tubules narrowing) showing in the figure(3c), in olive leaves of silver nanoparticles treated group showed a slight improvement and signs of tissue repair in renal glomeruli just dilatation capsule in bowman were seen and represented the via the number of renal glomeruli and the return the bowman capsule to nearly normal structure seen in figure (3d), in combination treated groups showing mild hydropic degeneration and necrosis and the tissue return to nearly normal seen in figure (3 e).

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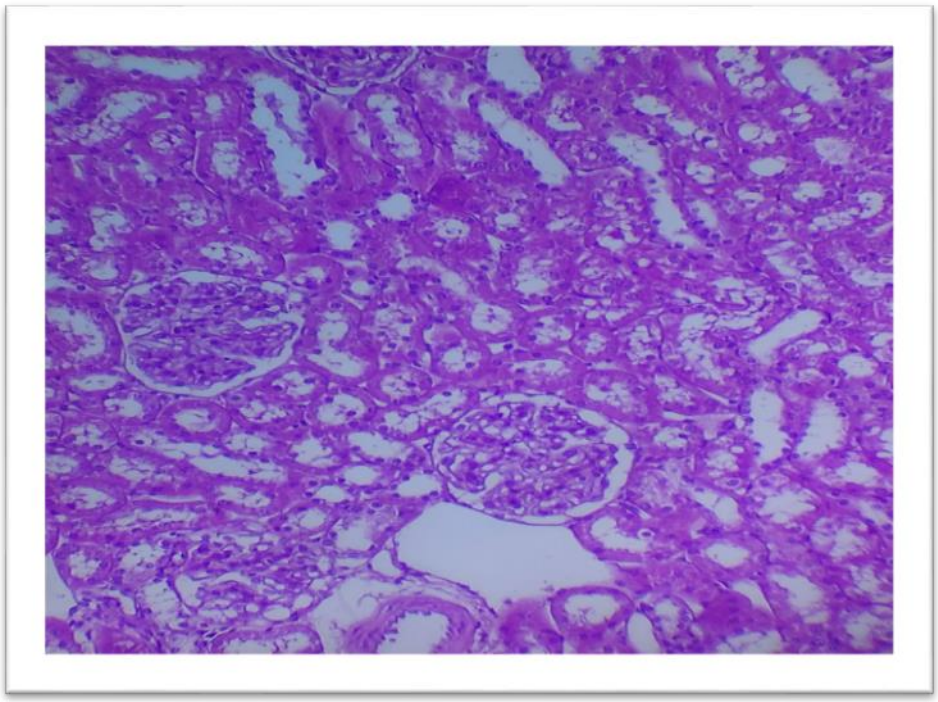

Figure (3 a): The normal histological view of the kidney was observed in control negative group

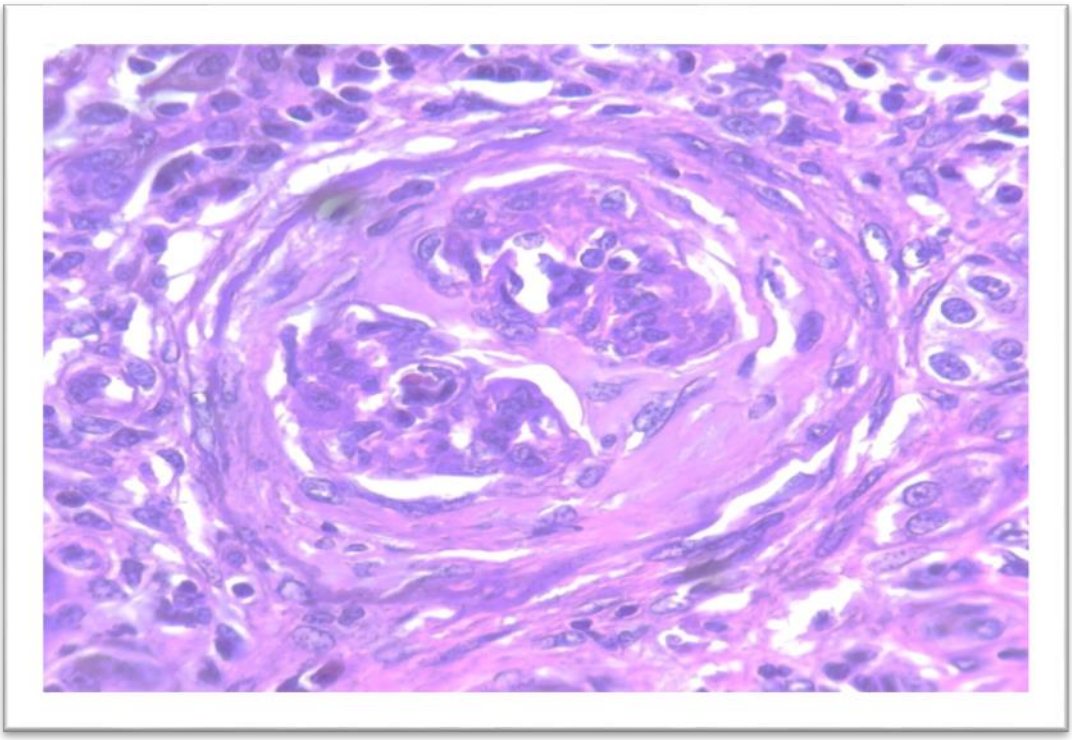

Figure (3 b): in DM groups the kidney showing hydropic degeneration, necrosis were present and in the tubules epithelial cells. Renal tissue from diabetic rats showing glomerular tuft damage with cell disruption

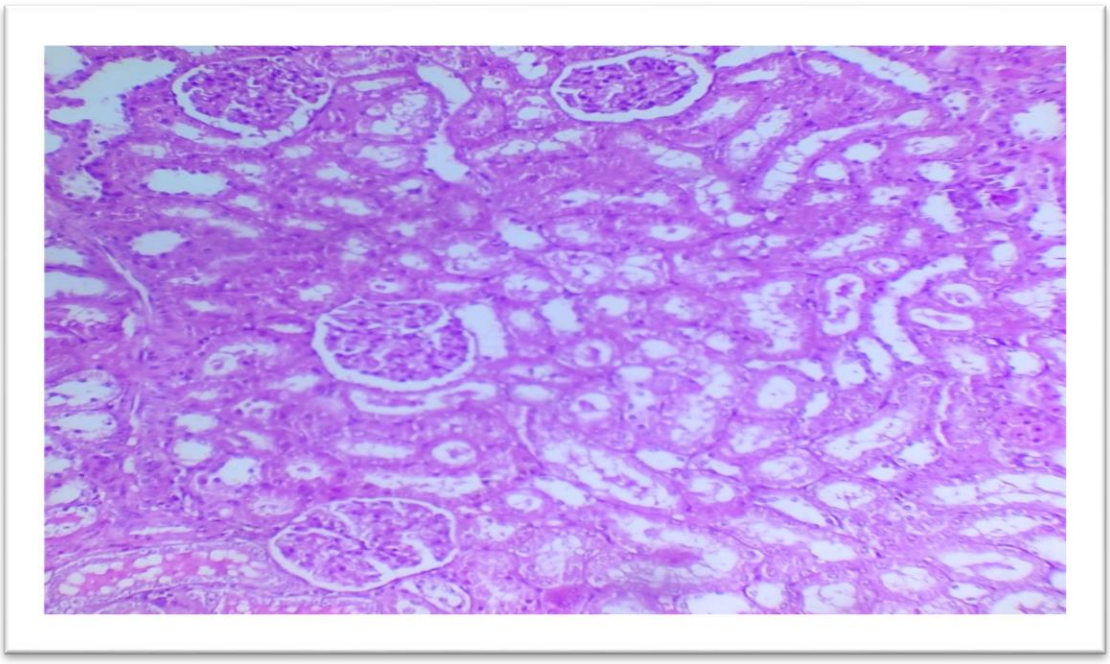

Figure (3 c): in DM group treated with silymarin of silver nanoparticles represented by ameliorate these changes as (thickening of the capillary for bowman capsule, enlargement of glomerular tuft, lumen of tubules narrowing)

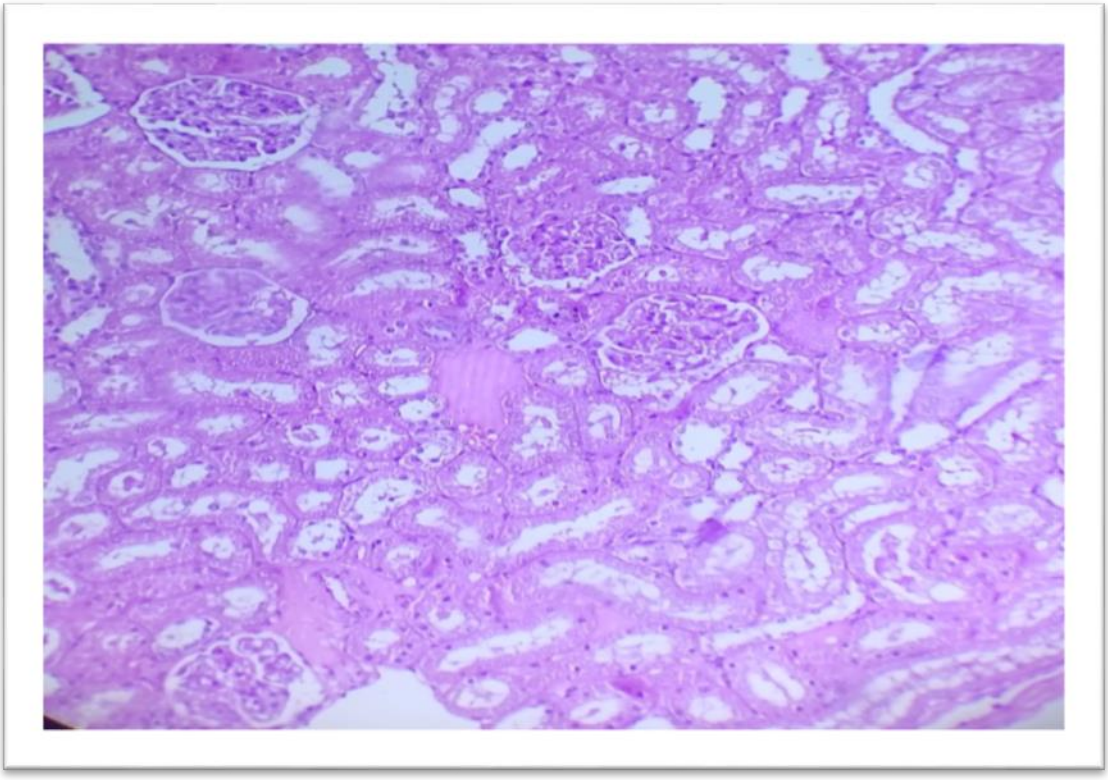

Figure (3 d): in olive leaves of silver nanoparticles treated group showed a slight improvement and signs of tissue repair in renal glomeruli just dilatation capsule in bowman were seen and represented the via the number of renal glomeruli and the return the bowman capsule to nearly normal structure.

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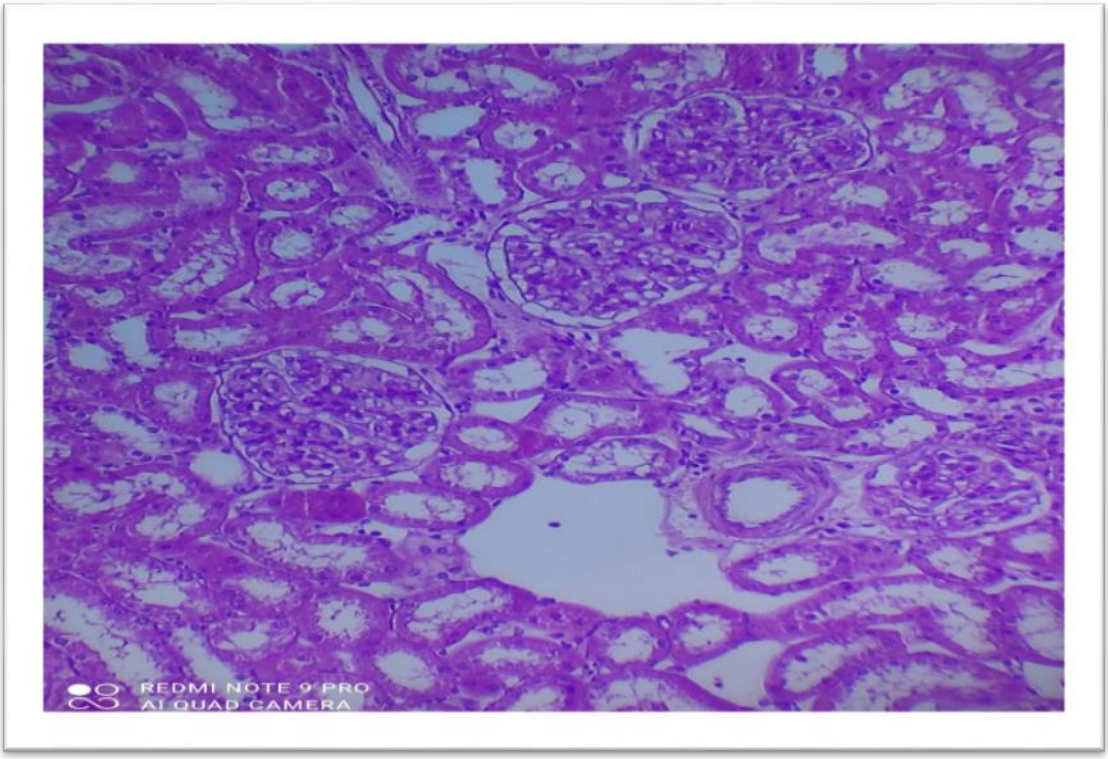

Figure (3 e): combination treated groups showing mild hydropic degeneration and necrosis and the tissue return to nearly normal

4. DISCUSSION

The present study was conducted to investigate the histopathological effects of olive leaves extract, silymarin and silver nanoparticles in many organs in alloxan induced diabetic rats. Present results exhibited that rats treated with alloxan caused various histopathological changes in pancreas, liver, kidney. Alloxan was first isolated via Brugnatelli in 1818 and initially described by [13]. Alloxan causes diabetes by a mechanism which basically involves partial degradation of the beta (β) cells of pancreatic islets and subsequent compromise in the quality and quantity of insulin produced by these cells. Excess nitric oxide and oxygen free radicals in response to alloxan treatment trigger cell death during early stages [14], [35]. Several studies have reported that oxidative stress plays a major role in acute inflammation, especially in acute pancreatitis [5], [20]. Pancreatic islets are more vulnerable to oxidative stress than any other tissues because of the low expression level of antioxidant enzymes, as previously described [19]. Oxidative stress exhibits crosstalk with inflammation [2]. Alloxan enhanced the recruitment of macrophages in the islets of the pancreas, also alloxan enter to βcell of islets of Langerhans of pancreas through GLUT-2 (glucose transporter) which leads to DNA alkylation, and necrosis consequently declined insulin, and progress hyperglycemia [31], [42]. All these reports agree with our study in which the histopathological changes of pancreas of diabetic rats show necrosis and degeneration of beta cells and infiltration of inflammatory cells. It has been reported that high blood sugar levels as a result of the long-term failure of blood glucose control, as a marker of DM, can cause damage to organs and may potentially result in life-threatening complications, such as cardiovascular, neurogenic, eye and kidney diseases [33]. In alloxan -induced diabetic control group, hepatic tissues indicate congestion in a central vein and infiltration of inflammatory cells which agree with [4]. Cellular swelling might be accompanied by leakage of lysosomes hydrolytic enzymes that lead to cytoplasmic degeneration and macromolecular crowding [10]. Hydropic degeneration is a result of ion and fluid homeostasis that lead to an increase of intracellular water [28].

Histopathological section of renal tissue of diabetic group show symptoms of renal injury, there are many factors lead to renal injury, such as oxidative stress injury, immune inflammatory responses, mitochondria-related apoptosis, and other factors were demonstrated to interact with each other and play crucial roles in the pathogenesis of renal injury [12]. Prior studies illustrated that TLR4, a pattern recognition receptor can recognize endogenous ligands and then induce signal transduction in response to high blood sugar levels, playing an important role in the progression of renal injury [21].

The histopathological changes of pancreas, liver, kidney in diabetic rats treated with olive leaf extract& silymarin extract AgNPs restore the normal structure, these results in agreement with [7], which reported olive leaf extract& silymarin AgNPs supplement can partially reduce the imbalance between the generation of reactive oxygen species and the scavenging enzyme activity. According to this study, the olive leaf & silymarin AgNPs supplement, as an antioxidant therapy, may be beneficial for correcting the hyperglycemia and preventing diabetic complications due to lipid peroxidation and free radicals in diabetic rats.

The histopathological section of pancreas of diabetic rats treated with green synthesized silver nanoparticles, show the most of beta cells appear normal, which may be due to the antioxidant ability of AgNPs which counteracting the generation of free radicals via alloxan in the pancreas according to [4], [40], [39]. Also, it can diminish the alloxan persuaded toxicity and potentiates β-cells to proliferate more insulin secretion [4], [41].

Micro-sized silver nanoparticles were the most effective antioxidant at smaller doses and prevent toxicity via decrease accumulations as reported by [27]. Hepatic tissue of diabetic group treated with green synthesized silver nanoparticles was brought back to near normal architecture, which indicated substantial protection of the liver against diabetic induced hepatotoxicity. The capability of a drug to diminish injuries/damages or to reserve the normal physiological function of the liver after induction of toxicity is the index of its hepatocurative effect, which agree with [22]. Plant extracts used to produce AgNPs can lead to synergistic effects, including an antioxidant effect [16].

The histopathological changes in the renal tissue in diabetic rats treated with AgNPs restore the normal structure. Therefore, these nanoparticles capable of decrease oxidative stress and restore the complications of diabetes mellitus, restore tissue damage and increase activities of endogenous [34].

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