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Investigate the Immuno-Histological Changes of Renal Tissue in Alloxan Induced Diabetic in Albino Male Rats Treated with Ethanolic Herbal Extract (*Moringa oleifera* seeds)

Fateh Kadhim [a*]

[a] *Department of Microbiology and Parasitology, College of Veterinary Medicine, University of Kerbala, Iraq.*

Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

Aim: This study aimed to assess the nephron-protective effect of *Moringa oleifera* seeds extract against alloxan induced diabetic male rats. The seeds of *Moringa oleifera*, have long been utilized in traditional medicine and as a source of nutritional supplements in various parts of the world. Numerous studies have been published highlighting the pharmacological properties of *M. oleifera* seeds (MOS), which encompass the ability to lower blood glucose levels and reduce inflammation. Nevertheless, the mechanisms that underlie its therapeutic effects is not well understood. Therefor this study was designed to define the alterations that may occur in response to MOS use.

**Corresponding author: Email: Fatih.o@uokerbala.edu.iq;*

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Methods: The present study comprised a total of eighteen adult albino male rats, which were split into three groups (**A**, **B**, and **C**), each consisting of six rats. Diabetes was induced in two groups (**B** and **C**) using alloxan at a dosage rate of 150 mg/kg body weight intraperitoneally following a pre-induction fast period of 24 hours. While, group (**A**) served as (Normal Control) was administered normal saline. After confirming the incidence of diabetes, the treatment was started as follows: Group A and B (negative and positive control) received no treatment. Whereas. Group C received 100 mg/kg/day of *Moringa oleifera* seeds extract orally for 21 consecutive days. The levels of serum IL-6 were analysed. Pathological tissue damage was assessed using Periodic Acid-Schiff staining. The presence of CD3T cells was detected through IHC.

Results: in comparison to the DN group, the experimental rats treated with MOS exhibited a remarkable reduction in serum IL-6 levels and kidney tissue damage. Furthermore, the MOS extract led to a decrease in CD3 expression.

Conclusion: Overall, this study suggests that ethanolic extract of MOS could inhibit IL-6 synthesis and the infiltration of T cells, which leads to delay the development of DN by improving the outcomes of the kidney.

Keywords: Diabetic; Moringa oleifera seed extract; alloxan; interleukine 6; diabetic nephropathy; CD3 (T cells).

1. INTRODUCTION

Diabetes mellitus (DM) is described as a chronic metabolic disease that is characterized by hyperglycaemia (an increase in blood glucose) and dyslipidaemia, which can lead to various complications overtime such as retinopathy, nephropathy and neuropathy [1]. It arises from either an insufficient production of insulin in the body or defects in the body's ability to use insulin [2]. The prevalence figure of the disease is rabidly changed with an annual increases in cases, recently in 2021 the estimation of the International Diabetes Federation (IDF) has published that globally around 537 million people had diabetes and it is expected that this number to reach 643 million by 2030, and 783 million by 2045 [3]. Diabetic nephropathy has a complex pathophysiology that involves changes in hormones and hemodynamics, growth factor expansion, advanced glycation end products synthesis and accumulation. Perhaps these could lead to increase the oxidative stress and the inflammatory response, which subsequently impaired renal functions, with glomerular hypertension, renal hypertrophy, and changes in glomerular composition [4]. A research has been established that diabetes induce nephron function abnormalities, which result in decreased renal excretion of potassium and hydrogen and cause hyperkalemia [5]. It is becoming more and more crucial to investigate T cell function in DKD. T cells were known to play either protective or pathogenic roles, they can behave in a variety of ways, alteration of insulin resistance, inducing podocyte cell damage and renal fibrosis, and controlling proteinuria, among other protective or pathogenic functions [6]. *Moringa oleifera* (horse radish tree) is a crucial traditional plant that is used to cure a variety of illnesses, including hypoimmunity, diabetes, hypertension, anemia, and inflammation. *M. oleifera* may have anti-inflammatory properties due to a variety of antioxidant components, including ascorbic acid, flavonoids, and phenols, as well as certain flavonoid pigments like kaempferol and isoquercitrin [7]. Furthermore, it has been documented that some of the active ingredients in *M. oleifera* reduce certain types of chronic inflammation [8]. It has been found that some of the bioactive components of M. O extract can lower hyperglycemia [9]. Furthermore, both in vitro and in vivo studies have shown the antioxidant properties of extracts derived from all parts of *M. oleifera* [10]. Administration of the extract of M.O seeds had a nephron-protective effect by lowering blood creatinine levels, enhancing overall structure, and upregulating the production of SOD, which considered as a key antioxidant [11]. The aim of the current study is to investigate the potential of using MOS in mitigating the adverse effects of alloxan induced diabetics on renal tissue in male rats.

2. MATERIALS AND METHODS

2.1 Animals and Experimental Design

This study was curried at the college of veterinary medicine, University of Kerbala, Kerbala, Iraq. A total number of eighteen adult albino male rats were divided into three groups (A, B, and C) with 6 rats in each group, weighing between (180-200 gm). The animals were kept in separate cages in the animal house for the duration of the experiment. They were given 10

days' period prior to intervention for adaptation with a standard environment (food, care, and consistent temperature, and light/dark cycle conditions).

2.2 Preparation of MOS Extract

The plant seeds were properly cleaned with distilled water and then allowed to dry at room temperature. Next, the seeds of *Moringa oleifera* were grinded into a fine powder. The powder was then subjected to the ethanol soxhlet extraction. The resulting extract was evaporated with a rotary evaporator set at 50°C. Then, it was weighed and stored at -4°C in a refrigerator until used.

2.3 Diabetes Induction and Dose Schedule

In order to induce diabetes in rats, the experimental groups (B and C) were treated with freshly prepared alloxan dissolved in 0.9% saline at the dose rate of 150 mg/kg body weight intra-peritoneally following a pre-induction fast period of 24 hours according to [12] protocol. While, group A (negative Control) was administered normal saline. Then after 72 hours, fasting blood glucose was measured. Rats with ≥250 mg/dl glucose levels were included and considered as diabetic-induced rats. Group A and B (negative and positive control) were received no treatment. Whereas. Group C received 100 mg/kg/day of *Moringa oleifera* seeds extract orally for 21 consecutive days. Rats were fed normal diet for the duration of the study (21 days).

2.4 Interleukin-6 Measurements

At the end of the experiment, rats were anaesthetized with 30% chloroform. Blood sample were collected from heart and centrifuged for serum isolation for cytokines analysis, using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam, US) and following the manufacturer's instructions, the IL-6 levels in the blood of the experimental groups were determined.

2.5 Tissues Collection and Staining

Following scarification, the kidneys' tissues were extracted and fixed by using 10% neutral buffered formaldehyde preserved in 10% neutral buffered formaldehyde before being prepared for Formalin fixing and paraffin embedding (FEPE). For histological evaluations, kidney sections (4µm) were stained with Periodic Acid-Schiff (PAS) in order to track the changes in the histology. Using the markers CD3 (a T cell marker), immunohistochemistry (IHC) labeling to assess the infiltration of the immunological T cells [13,14]. To stain FFPE kidney sections for CD3 analysis, the first step involved deparaffinizing the sections in xylene and then gradually rehydrating them using a series of ethanol exposures (100%, 90%, and 70%). The sections were then treated with 3% hydrogen peroxide for a duration of 10 minutes. To further prevent non-specific binding, Vector Laboratories' 2.5% goat serum was used for blocking. The primary antibody, anti-rabbit CD3 (Dako), were diluted to 1:200 in PBS and incubated on the sections for a period of 2 hours at room temperature. Following thorough washes with PBS, a peroxidase conjugated secondary antibody was applied to the sections. Once again, the sections were washed and then counterstained using haematoxylin (Sigma) before being washed with distilled water. To complete the process, the sections were dehydrated using graded ethanol concentrations for 3 min and then in xylene for 5 min. Finally, the sections were mounted using DPX mounting media (Sigma) and visualized using an Olympus light microscope.

2.6 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism software (10.1.2). One-way ANOVA was used with selection of Tukey's multiple comparisons test to compare between groups. The data were shown as mean ± SD, and findings were deemed significant if * $P < 0.05$, **** $P < 0.0001$.

3. RESULTS AND DISCUSSION

3.1 Effect of MOS Extract on Serum IL-6 Level in Diabetic Male Rats

The levels of IL-6 in the serum of male rats treated with alloxan and alloxan+MOS, as well as saline-treated controls, were measured using ELISA. Fig. 1 displays the IL-6 concentrations in each of the experimental groups. As shown that the administering of alloxan resulted in a substantial elevation of protein levels of IL-6 compared with saline-injected control group*** $P < 0.0001$. Whereas, MOS treatment (group C) was significantly reduced the of IL-6 serum levels

compared to alloxan alone (group B) * P < 0.05. Altogether, these results indicate that treating diabetic rats with MOS extract may help to reduce renal inflammation by lowering the production of IL-6.

3.2 Effects of MOS Treatment on the Morphological Changes of Kidneys in Diabetic Male Rats

On the other hand, slices from rats treated with alloxan showed distinct histological alterations. Acute tubular necrosis, fibrosis, interstitial inflammation, tubular dilatation, tubular cell brush boundary loss, and intratubular inflammatory cast were among the alterations seen (Fig. 2.B). Interestingly, MOS treatment (Fig. 2.C) effectively reduced glomerular and tubular damage in alloxan treated rats and restore the normal structure.

3.3 Infiltration of CD3 T Cells in the Renal Interstitium of Diabetic Male Rats

To demonstrate the expression of CD3 protein of infiltrating T cells in the renal sections of diabetic rats, immunohistochemistry was done on FFPE renal tissue sections. Interestingly Fig. 3 C shows CD3 protein in the renal interstitium (tubules and glomeruli) of diabetic rats less non-treated diabetic group (C) Fig. 3 C, whereas non-diabetic group shows no expression (Fig. 1 A).

Diabetes alters the basement membrane's composition both qualitatively and quantitatively, which promotes protein production, reduces the membrane's capacity to degrade further, increases its permeability, and damages endothelium [3]. It is characterised by diffuse or nodular glomerulosclerosis [15]. The destruction of insulin-secreting cells in the pancreas, leading to hypoinsulinemia and hyperglycemia, is a direct result of alloxan's induction of diabetes. Alloxan specifically targets and damages the pancreatic beta cells, causing hyperglycemia. This cytotoxic effect is attributed to the production of free radicals, which has been observed both in laboratory settings and within living organisms [16,17].

Hyperglycemia is an important factor that known to drive the development and progression of DN. In response to tissue damage, the body produces IL-6, a potent protein that triggers inflammation and serves a crucial function in immune regulation. Additionally, IL-6 plays a prominent role in the progression of inflammatory processes. It has been determined that interleukin 6 (IL-6) is a crucial modulator of immunological responses, inflammation, and glucose metabolism. Serum IL-6 level is closely related to renal injury and poor prognosis in patients with diabetic nephropathy [18]. Serum IL-6 was positively correlated with urinary albumin/creatinine ratio (ACR) and negatively correlated with eGFR [19].

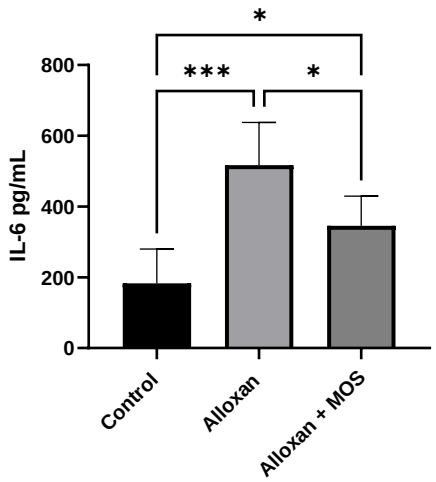

Fig. 1. Effect of MOS treatment on IL-6 levels in serum of diabetic male rats

*IL-6 protein levels were measured in saline-treated control rats or diabetic rats with or without MOS by ELISA. Results are presented as mean ± SD of 6 rats per group. Alloxan group (**B**) *** P < 0.0001compared with normal control group (**A**). Alloxan group (**B**) * P < 0.05 compared with MOS treated Alloxan group (**C**) (ANOVA)*

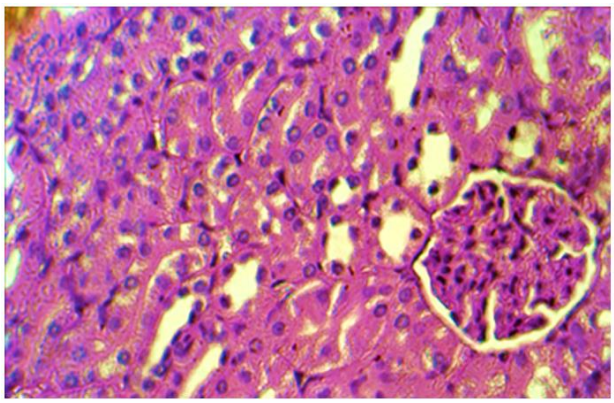

Fig. 2. A. Photomicrograph of renal section of normal control male rats (group A)
Showing normal PAS staining with normal morphology of renal glomeruli, Bowman's capsule, the capsular spaces, tubular basement membrane, and brush border of proximal tubules. (x40)

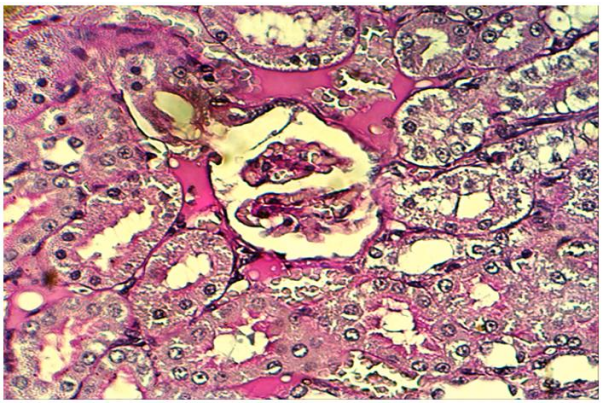

Fig. 2. B. Photomicrograph of kidney sections of diabetic rats (group B)
Showing abnormal PAS reaction revealed the destruction in brush borders of some of convoluted tubules, also a thickning in the basement membrane of Bowman's capsule and glomerulosclerosis, the capsular spaces wider than those of the other groups and some renal tubules were vacuolated. Inflammatory cast are seen in renal interstitium (x40)

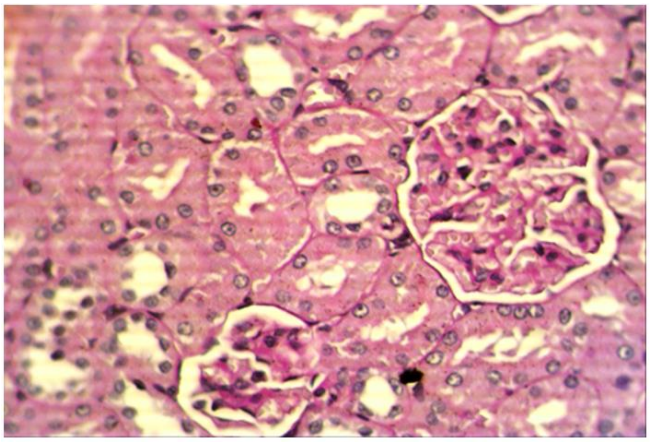

Fig. 2.C. Photomicrograph of sections of the kidney of MOS treated diabetic rats (group C)
Interestingly showing signs of improvement with a weak PAS reaction in glomeruli and brush borders of renal tubules. The capsular spaces look normal and there is no thickening in the basement membrane of Bowman's capsule or vacoulation in the tubules (x40)

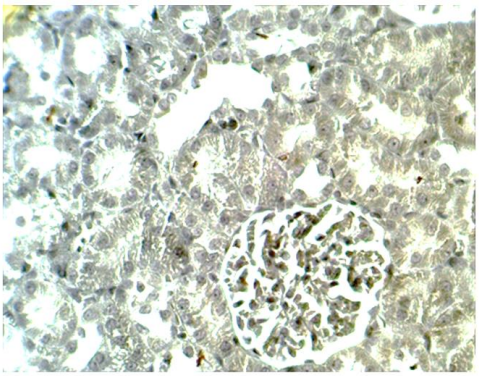

Fig. 3.A. Immunohistochemistry analysis of CD3 in rat kidney sections of normal control rats (group A)

FFPE sections from renal tissues of non-diabetic rats were used to demonstrate the presence of CD3. Sections were incubated with a rabbit anti rat CD3 and an anti-rabbit secondary antibody. The results showing a negative CD3 immunoreactivity for T cells infiltration in the glomerular and tubulointerstitium, assessed at day 24 of experiment. (CD3, x40)

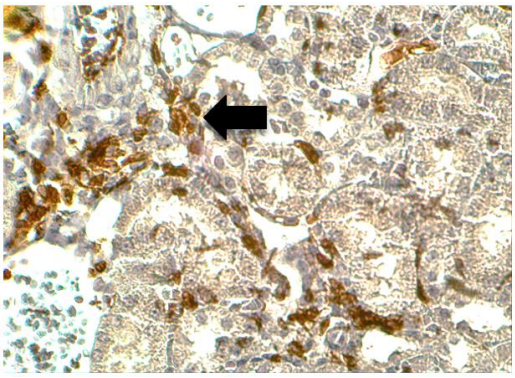

Fig. 3.B. Immunohistochemistry analysis of CD3 in rat kidney sections of diabetic rats (group B)

FFPE sections from renal tissues of diabetic rats were used to demonstrate the presence of CD3. Sections were incubated with a rabbit anti rat CD3 and an anti-rabbit secondary antibody. The results showing an intense (sever) CD3-positive immunoreaction for T cells infiltration in the tubulointerstitium (black arrow), assessed at day 24 of experiment. (CD3, x40)

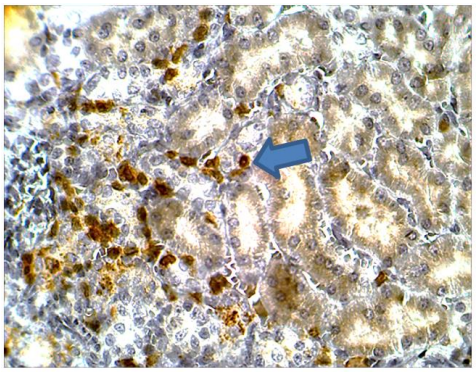

Fig. 3.C. Immunohistochemistry analysis of CD3 in rat kidney sections of MOS treated diabetic rats (group C)

FFPE sections from renal tissues diabetic rats treated with MOS extract were used to demonstrate the presence of CD3 in kidney tissue. Sections were incubated with a rabbit anti rat CD3 and an anti-rabbit secondary antibody. The results show that CD3 (of infiltrating T cells) positively expressed in renal interstitium with less intensity (black arrow) than non-treated diabetic group. Assessed at day 24 of experiment. (CD3, x40)

The results of current study interestingly show a significant decrease in IL-6 level in the serum of MOS extract treated diabetic rats compared with non-treated diabetic group, which possibly lead to decrease the pro-inflammatory environment and promoting the anti-inflammatory properties. According to [20,21] *Moringa oleifera* contains phytochemicals such as flavonoids, including quercetin and kaempferol, These compounds are thought to have potential in promoting the anti-inflammatory effects. Although some of the published studies used leaves extract of the plant, which revealed that ethanolic extract of *Moringa oleifera* (EEMO) administration had a significant improvement in renal functions in diabetic rats along with improved kidney morphology. Additionally, EEMO therapy reduced the levels of renal reactive oxygen species in diabetic animals [22]. Another study in line with the current findings is done by [23] revealed that serum levels of RF, TNF-alpha, IL-1, and IL-6 of MOEE treated significantly decreased compared to those in the diseased control group with less infiltration of lymphocytes suggesting that *Moringa oleifera* may possess a promising anti-arthritic property. The mechanism of how the extract of MOS inhibit the proinflammatory mediators (IL-6) is yet to be study, here it is a speculate that this plant contains several elements with medical important that might influence the inflammatory environment at the molecular level (signalling pathway): either by affecting the inflammatory cells itself which probably changing its behaviour form pro-inflammatory toward anti-inflammatory (phenotyping shift), or by targeting the particular renal cells of the kidney through the activation-proliferation or inhibition –apoptosis. This explanation could be supported by [24] study which has demonstrated that quercetin can regulate pro-inflammatory IL-6, inflammation signaling pathways, and transcription pathways, leading to the inhibition of inflammatory mediators such as histamine and tryptase, as well as the production of cytokines like IL-6. A recent published study [25] concluding that Based on the outcomes of molecular docking, the interaction between quercetin and IL-6 has the potential to reduce the expression of IL-6 protein, thereby inhibiting its functions, therefor these in silico results, the selected polyphenol compounds derived from ethanolic *Moringa* leaf extract exhibit more promising therapeutic potential compared to synthetic compounds.

Histological examination of renal sections of this study illustrate more changes in the renal glomeruli and tubules in response to alloxan induced diabetes in male rats, these like glomerular sclerosis, mesangial matrix expansions and enlargement of bowman capsule, also the epithelial straightening vacuolization and dilatation were seen in renal tubules. Most notably that changes were mitigated by using the extract of MOS to treat alloxan induced diabetic rats. Clearly, the recovery signs can be seen in the histology sections that include the arrangement of bowman's capsule, the glomeruli, and renal tubules with no signs of cast deposition or vacculations. The results of this study agree with [26] findings that treating diabetic rats with 50 or 100 mg Moringa seed powder/kg body weight were restored normal histology of the kidney and pancreas compared to the diabetic positive control group. Furthermore, the expression pattern of CD3 of infiltrating T cells which were assessed by HI, revealed that CD3 highly expressed in renal section from alloxan induced diabetic group compared with other groups, surprisingly the MOS extract treated group showing markedly less CD3 staining for infiltrating T cells, which in turn may associate with the improvement of the histological damage of kidney tissue. This pattern of expression could link to the serum pro-inflammatory cytokine. These observations suggest that the extract of MOS is likely to have a nephro-protective effects against alloxan induced renal tissue damage in male rats. This is in line with the previous study that reported *M. oleifera* seeds extract exert anti-renal fibrosis activities through glycogen synthase kinase-3 beta (GSK-3β) pathway [27]. Importantly, in flow cytometry study the intrarenal CD3+ T cells were found to be a significantly increased in proteinuric mice with diabetic nephropathy [28]. Further study that may support the current finding regarding the effect of MOS extract on CD3 T cells, well documented that *M. oleifera* polyphenol extract (MOPE) significantly alleviated the symptoms of DSS-induced colitis by reducing the infiltration of CD3+ T cells, CD177+ neutrophils, and F4/80+ macrophages, and significantly reduced the secretion of IL-6 and TNF-α [8].

4. CONCLUSION

Moringa oleifera seed extracts potentially protect the renal tissue from the adverse effect of alloxan induced diabetic in male rats. MOS extract reducing the pro-inflammatory cytokine IL-6 and mitigating the histological damage (reducing the infalammtory changes and T cells infiltration) of

rat's kidneys. Generally, this study revealed that MOS were capable to improve the inflammatory changing of renal tissues that associated with induced diabetic in rats.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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