

Effect of Xanthenone Versus Irisin in Alleviating Renal Ischemic Reperfusion Injury through Modifying the PI3K/AKT/eNOS and TLR-4/NFκB Pathways

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Abstract

Ischemic reperfusion injury (IRI) in the kidney is a common cause of acute-kidney-injury. Our aim is to compare the effects of Xanthenone /Irisin on alleviating inflammation, cell apoptosis and oxidative stress injury in kidney ischemic reperfusion injury (IRI) via modulating the PI3K/Akt/eNOS and TLR4-NFκB pathways. Thirty two male rats were equally divided into groups: Control, Ischemic reperfusion (IR), IR treated with (i.v) Xanthenone (10mg/kg), IR treated with (i.p) Irisin (100 μg/kg). Renal functions, blood pressure, renal tissue Angiotensin converting enzyme-2 (ACE2), Angiotensin 1-7 (Ang 1-7), inflammatory, oxidative stress and apoptotic markers were evaluated. Also, PI3K/AKT/eNOS and TLR-4 relative mRNA levels in renal tissue were assessed. Also, the protein concentration of pAKT, eNOS, histomorphological, and immunohistochemical analysis were done. In IR group, renal function tests, blood pressure, and apoptotic parameters were elevated. ACE2 and Ang (1-7) tissue levels, relative gene expression of PI3K/AKT/eNOS and TLR-4, the protein concentration of pAKT and eNOS, histomorphological and immunohistochemical analysis of NFκB in renal tissue were all distorted in IR group. However, Xanthenone and Irisin treatments improved renal function, inflammation, and oxidative stress through PI3K/AKT/eNOS and TLR-4/ NFκB pathway. Moreover, Xanthenone acts through ACE2/Ang (1-7) pathway, while Irisin acts mainly through PI3K/AKT/eNOS and TLR4/NFκB pathway. This may increase the potential for therapeutic interventions for IRI in the kidney, with a focus on investigating their clinical efficacy.

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1. Introduction

Ischemic/reperfusion injury (IRI) is a condition where an organ experiences restriction of blood supply followed by re-oxygenation and restoration of blood flow. This whole process not only results in the production of reactive oxygen species (ROS) and cytokines but also amplifies tissue damage by triggering an inflammatory chain reaction [1]. The medical condition known as acute kidney injury (AKI) is characterized by rapid renal failure and a high death rate. One of the pathological conditions linked with AKI in the kidney is IRI [2].

The renin-angiotensin system (RAS) is a vascular homeostatic controller-signaling pathway. Angiotensin-converting enzyme (ACE) and angiotensin-converting enzyme 2 (ACE2) are opposing arms of RAS, with ACE/Ang II/Angiotensin type-1 (AT1) receptor promoting inflammation and ACE2/Ang (1-7)/Mas receptor minimizing it in renal tissue [3]. Angiotensin I (Ang I) is converted to Angiotensin II (Ang II) by carboxypeptidase and ACE. Ang II is further broken down into the vasodilator Ang (1-7) by carboxypeptidase and ACE2. The ACE2 enzyme may be more significant than ACE in regulating local levels of Ang II and Ang 1-7. The balance of the RAS axis is disrupted in cases of renal ischemia. The negative effects of Ang II on renal tissue can be countered by Ang (1-7). Thus, in cases of renal IRI, stimulating the ACE2/Ang (1-7)/Mas axis provides new therapeutic opportunities [4].

Abnormalities in the expression of the ACE-2 enzyme have been linked to the heart, blood vessels and kidneys injuries. It has been observed that the cardiac ACE2 expression can be

increased by Ang-converting enzyme inhibitors or Ang II receptor blockers. The ACE2 enzyme has been found to be effective in lowering structural remodeling and fibrosis [5]. Based on these findings, it is speculated that increasing pharmaceutical ACE2 activity could have positive effects and protect against the pathophysiology of renal IR damage.

Xanthenone is a newly discovered ACE2 activator, and previous research has shown that activating this enzyme could be a potential therapeutic approach for cardiovascular disorders [6]. AKI caused by renal IRI can be treated by preventing the production of ROS. Irisin, originally discovered as a metabolic regulator, has been found to increase mitochondrial function and reduce ROS production. Administering Irisin after renal IRI can reduce kidney damage by decreasing oxidative stress, upregulating mitochondrial activity, and downregulating the inflammatory response. This suggests that Irisin can be a promising new therapy option for AKI resulting from renal IRI [7].

IRI multiple pathways have been identified. One of these pathways is the toll-like receptor 4-nuclear factor kappa B (TLR-4-NFkB) pathway. This pathway triggers several kinases which in turn activate downstream kinases, leading to the release of inflammatory cytokines and the initiation of inflammatory responses [8].

Numerous studies have linked TLRs, especially TLR-2 and TLR-4, to the harmful inflammatory outcomes observed in IRI associated with conditions such as trauma, stroke, myocardial infarction, and organ transplants. The TLR-4 is expressed in both the proximal and distal tubules as well as the thin limb of the loop of Henle and

the collecting ducts. Following IRI, expression of TLR-4 is increased in these regions [9].

In addition, the phosphatidylinositol 3-kinase / protein kinase B (PI3K/AKT) signaling pathway plays a crucial role in the pathophysiology of renal IRI damage, which is also necessary for cell survival and death [10]. According to **Nicholson and Anderson**, AKT is a phosphorylation-activated downstream kinase of PI3K [11]. It has been shown that the activation of PI3K/AKT reduces IRI and enhances the survival of renal tubular epithelial cells [10].

Nitric oxide (NO) is a vasoactive derivative of L-arginine via the nitric oxide synthase (NOS) enzyme that has three isoforms: neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS) [12]. The eNOS plays many important roles as it relaxes the smooth muscle, regulates blood flow and blood pressure, and prevents thrombocyte adhesion and aggregation. The deficiency of NO has been linked to IRI damage, and it has been observed that IRI was alleviated after NO administration [13]. Based on the above facts, we studied the effects of both Irisin and Xanthenone on an IRI rat model to evaluate their impact on alleviating inflammation, ROS injury and cellular apoptosis through PI3K/AKT/eNOS and TLR4/NF-kB.

1. Material and Methods

1.1. Chemicals and drug preparation

Xanthenone was derived in the form of powder form (25 g) (Xanthone (9-Xanthenone 97%, Cat NO X 600)) from Sigma Aldrich Company, Beijing, China. The pouch of 10 mg of Xanthenone was dissolved in 1 ml saline solution to be given in a dose of 10 mg/kg intravenous

(i.v) according to the dose described by **Hernández Prada et al.** [14]. Irisin was derived in the form of white powder (100µg) purchased as (recombinant Irisin, Phoenix Biotech, Burlingame, CA, Cat NO 067-29A). According to manufacture instructions, it was reconstructed in 1 ml distilled water (100 µg/ml) and given in a dose of 100µg/kg by intraperitoneal (i.p) injection according to dose reported by **Zhang et al.** [15].

1.2. Experimental animals

Thirty-two Sprague-Dawley adult male rats, weighing 180–200 g, aged (30–35 weeks), were used in this study. The rats were obtained from the animal breeding house of rats in the Faculty of Medicine at Tanta University, Egypt. Standard diets were given in a pathogen-free environment under 12h light–dark cycles at a temperature of 23–25°C. Experimental protocol was started after two weeks' acclimatization. The ethical standards for using and handling laboratory animals in research were followed during all experimental procedures.

1.3. Ethics statement

All experimental procedures on animals used for this study were performed in accordance with the Research Ethics Committee ethical guidelines of the Faculty of Medicine, Tanta University (Approval code: 35027/11/21). Animals were handled gently, housed in suitable environmental and nutritional conditions, assessed for general health and body weight (BW), and anesthetized before injury and scarification. The experimental procedures were also done in accordance with the recommendations of the National Institutes of Health for experimental

animals' care and use (NHI Publication; No. 8023, revised 1996).

1.4. Rat model of renal IRI

As previously described, all rats were surgically prepared for renal IR [16]. Rats were anesthetized with sodium pentobarbital (40mg/kg) and xylazine (10 mg/kg, i.p). After that, the kidneys of the animals were exposed via incisions (about 1.5 cm) along both sides of the spine at the lower border of the costal margin. Both kidneys were carefully separated, and the fat around the kidney pedicle was removed. Then, for 45 min, noninvasive arterial clamps were inserted into the bilateral kidney pedicles. The darkening of the kidneys from bright red to dark red confirmed ischemia. Removing the clamps and restoring the blood supply after 45 minutes enabled effective reperfusion, with the color of the kidneys changing from dark red to bright red. The surgical incisions were repaired, and sterile gauze was used to cover them. The rats were returned to their clean cages with free access to food and water after recovering from anesthesia. To compensate for the number of deaths in rats, 25% were added in each group as dropout, $n=8$ rat/ group.

1.5. Study design

1.5.1. Protocol of the Experiment

Rats were divided randomly into four groups ($n=8$ /group). **Control group:** Rats in this group underwent all the procedures except the renal ischemia step. Then, 45 minutes after reperfusion initiation they received a 1ml/kg saline i.v via the tail vein and 1ml/kg distilled water by i.p. injection **Ischemic reperfusion group (IR);** Rats in this group underwent IRI as mentioned before [16] and then, 45 minutes after reperfusion initiation, they received a 1ml/kg

saline by i.v injection via the tail vein and 1ml/kg distilled water i.p. **IR treated with Xanthenone group (IR + Xanthenone);** Rats in this group underwent IRI as mentioned before [16], as in the IR group; then, 45 minutes after reperfusion initiation, they were treated with Xanthenone at a dose of (10 mg/kg) by i.v injection via the tail vein [14]. **IR treated with Irisin group (IR + Irisin);** Rats in this group underwent IRI, as mentioned before [16], as in the IR group. 45 minutes after reperfusion initiation, they were treated with Irisin solution by i.p. injection at a dose of (100 µg/kg) [15].

All rats were placed in metabolic cages (Tecniplast, Hohenpeissenberg, Germany) for 24 hours before scarification to collect 24 hours urine volume which were used to measure urinary creatinine and urinary albumin concentration.

After 24h postoperative and before scarification, BW was measured using the electronic animal weight scale and the arterial blood pressure of rats was measured in non-anesthetized rats using BIOPAC Systems software noninvasive rat tail blood pressure method.

Then, rats were anesthetized by i.p. injection of sodium pentobarbital (40mg/kg). Blood samples were collected from the abdominal aorta, left to coagulate for about 15 min and were centrifuged at 3000 rpm for 20 min to obtain separated serum and kept frozen at -20°C for biochemical tests. The kidney tissue weight (KW) was obtained immediately after removal of the body by using sensitive digital scale. The right kidney tissue was frozen rapidly in liquid nitrogen and stored at -80°C , and the left kidney tissue was fixed in 4% paraformaldehyde for further analysis. Finally, the sacrificed animals were

packed in special packages according to safety precautions and infection control measures, as

shown in **Figure 1**.

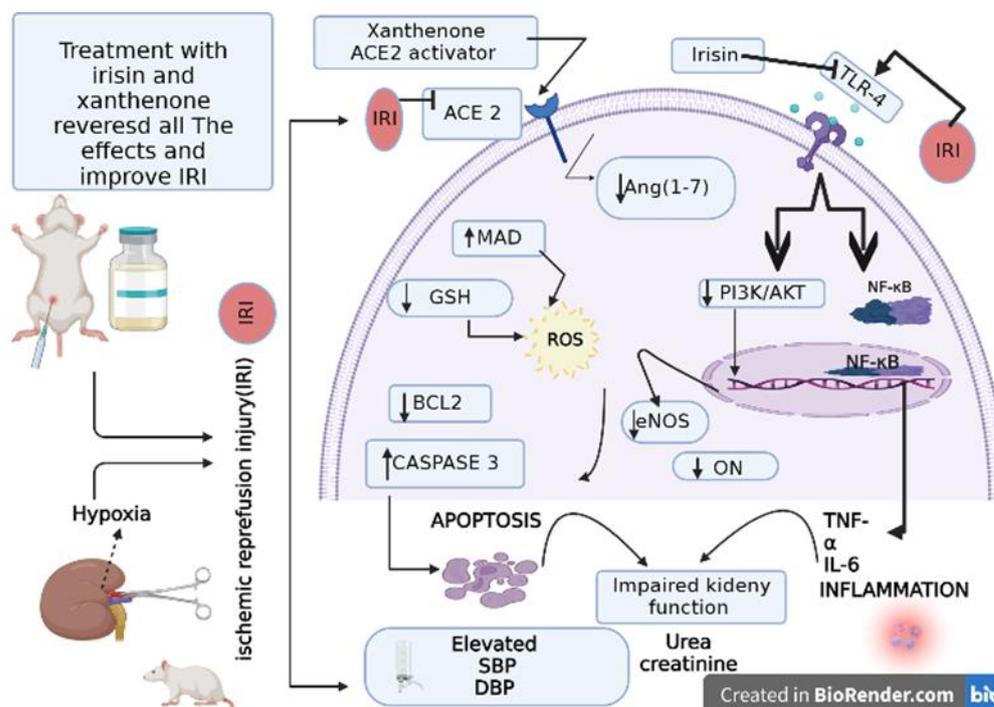


Figure 1 Summary of the experimental design and protocol. IRI resulted in activation of TLR4 and subsequently of NF-kB that resulted from increased expression of proinflammatory cytokines IL-6 and TNF- α . Decreased PI3K /AKT resulted in decreased expression eNOS and decreased NO production with elevated systolic and diastolic blood pressure, decreased ACE 2, Ang1-7, increased ROS and activation of apoptosis; all this resulted in impaired kidney function treatment, but Irisin and Xanthenone treatment reversed all the above-mentioned changes and improve kidney function. Created with BioRender.com

1.5.2. Urinary albumin and creatinine level were measured to calculate albumin / creatinine ratio (ACR)

Urinary concentrations of albumin and creatinine were determined by using Albumin-to-Creatinine Ratio (ACR) Assay Kit (BioVision Incompany, Milpitas, California, USA, Catalog # K551-100) and ACR was calculated.

1.5.3. Systolic (SBP) and diastolic blood pressure (DBP)

SBP and DBP were measured before scarification at the end of the study by blinded researcher in awake un-anaesthetized rats using the non-invasive rat-tail blood pressure module in research Biopac Data Acquisition MP150 device (BIOPAC Systems Inc., Santa Barbara, CA) connected to PC professional software. For

accurate non-invasive blood pressure measurement, the rat was warmed to 32°C, so the animal heating chamber was turned on for 5 minutes. The rat was placed inside the “animal holder” with holes provided for ventilation with the tail left outside. The tail cuff with a sensor was connected to the tail of the rat inside the restrainer. The recording had stopped automatically after 24 seconds, three successive cycles were recorded for each animal and the average was calculated.

2.5.4. Serum biochemical analysis of renal function: serum creatinine and Blood urea nitrogen (BUN),

The colorimetric method was used for the estimation of concentrations of serum creatinine and BUN using (Egyptian Company for Biotechnology, Cairo, Egypt, Cod No 235 003)

kits for creatinine and (Egyptian Company for Biotechnology, Cairo, Egypt, *Cod No 321 004*) kit for BUN according to the described protocols.

2.5.5 Measurement of renal enzymes ACE 2 and Ang (1-7)

Enzyme-linked immunoassay technique (ELISA) was used to measure concentrations of ACE2 levels using ELISA kits for rat ACE2 (*Cat No. CSB-E14308r*, CUSABIO, Houston, USA) and Ang (1-7) levels using ELISA kits for rat Ang (1-7) (*Cat No. MBS2604372*, MyBioSource Inc., San Diego, California, USA) in renal tissue according to described protocols.

2.5.6. Measurement of renal oxidative stress markers: Malondialdehyde (MDA), and reduced glutathione (GSH)

A Spectrophotometric method was used to measure the levels of MDA by using the Biodiagnostic Kits (*No MD 25 29* Biodiagnostic Co., Egypt). In addition, according to described protocols, GSH was detected using a Biodiagnostic Kits (*No GR 25 11* Biodiagnostic Co., Egypt) in renal tissue.

2.5.7. Measurement of renal apoptotic markers level: B-cell leukemia/lymphoma 2 protein (BCL-2), caspase-3

The ELISA technique was used to measure concentrations of BCL-2 and Caspase-3 levels by using BCL-2 kits (Rat BCL-2 ELISA Kit; MyBioSource, Inc. San Diego, USA, *NO MBS2515143*), Caspase-3 kits (Rat Caspase-3 ELISA Kit MyBioSource, Inc. San Diego, USA, *NO MBS018987*) in renal tissue according to described protocols.

2.5.8. Measurement of renal inflammatory markers: Tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6)

The Levels of TNF- α and IL-6 in renal tissue were measured using the ELISA technique with the Rat TNF- α ELISA kit (Shanghai Sunred Biological Technology Co., Ltd., China. *CatNo. 201-11-0765*) and the Rat IL-6 ELISA kit (*Cat No. CSB-E04640r*, CUSABIO, Houston, USA).

2.5.9. Measurement of renal nitric oxide (NO)

The colorimetric method was used for the estimation of concentrations of levels of NO by using the Biodiagnostic Kit (*No. NO 25 33* Biodiagnostic Co., Egypt) in renal tissue according to the described protocols.

2.5.10. RNA Extraction and Quantitative RT-PCR detection of renal TLR-4, PI3K, AKT, and eNOS Genes

The RNeasy Mini Kit (*Cat No 74104*, Qiagen, Germany) was used for extraction of RNA from renal tissue. The concentration and purity of RNA were determined using the Nanodrop 8000 (Thermo Scientific, Waltham, MA, USA) at the absorbance of 260 and 280. A ratio between 1.8 and 2.2 was considered acceptable for further genetic analysis.

The (Quanti Tect two-step Reverse Transcription Kits (*Cat. No. 205311*, Qiagen, Germany) was used for reverse transcription. The SYBR Green PCR master mix (Quantinova SYBR Green PCR Kit (*Cat. No. 208052*, Qiagen, Germany) were used for detecting mRNA expression level and the used primers for PCR reaction are mentioned in **Table 1**.

The primer was designed using primer3web version 4.1.0 and NCBI and tested in silico using Sequence manipulation suit (SMS) version 2, PCR Primer Stat tool & UCSC In silico PCR and in silico PCR amplification tools. GAPDH was used as a house keeping gene and

was included in the primer sequence list. The $2^{-\Delta\Delta Ct}$ method of analysis was used to analyze the results according to Livak, (2001), based on the (threshold) cycle (Cq or CT) quantification value as follows [17]

Relative expression = $2^{-\Delta\Delta Ct}$, where ΔCt = (CT of target gene – CT GAPDH) and this was calculated for target group and for control $\Delta\Delta Ct$ = (ΔCt of target group- ΔCt of control).

2.5.11. Measurement of renal pAKT and eNOS

The ELISA technique was used to measure concentrations of pAKT by using Rat Phosphorylated AKT ELISA (MyBioSource Inc. Cat. No MBS1600201, San Diego, California, USA) and eNOS by using Rat NOS3/eNOS (Nitric Oxide Synthase 3, Endothelial), (Biotech Inc. Cat. No ER0148, Wuhan, Hubei, China) in renal tissue according to the manufacturer's instructions

2.5.12. Histomorphological analysis of kidney

The renal tissues that were collected in 4% paraformaldehyde were embedded in paraffin, sliced into 5 μ m thick sections and subsequently stained with hematoxylin and eosin (H&E) stain. Kidney morphological changes were observed using an Olympus light microscope model

BX50F4 ($\times 400$). Ten serial sections were examined by each animal in each study group. An independent, blinded observer determined all the histological changes in each study group.

2.5.13. Immunohistochemical analysis of nuclear factor kappa B (NF κ B)

(Cat. No: MBS9701121) was used to assess NF κ B in renal tissue immunohistochemically. The positive expression of NF κ B was detected using a LEICA light microscope model DM500 ($\times 400$) and was assessed. The changes in the histological and immune-histochemical characteristics of renal tissue were quantitatively evaluated using an image J analysis program and represented as a percentage area.

Statistical analysis

Data were expressed as Mean \pm standard deviation (SD) and all statistical comparisons were made by means of a One-Way ANOVA test, followed by Tukey's post hoc analysis, and *p* values less than 0.05 were considered statistically significant. The analysis was performed by the statistical package for the social science software (SPSS version 22.0).

Table (1): Primers for PCR reaction

Primer	Primer	Accession number/NCBI Reference sequence	Exon/intron position	Product length	Location of primer within the gene	Location of primer within the gene
PI3K	FOR: 5'-GATCGCTTGCTGTGAGCTGGAT-3' REV: 5'-GGATGTTTCCCAATGCCCTGGA-3'	<u>NM_0011085</u> <u>00.1</u>	exon 18	104	FOR: Template 4041..... 4062 REV: Template 4187..... 4166	1 170600741 to 170600762 (-)
AKT	FOR: 5'-GGACTACTTGCACTCCGAGAAG-3' REV: 5'-CATAGTGGCACCGTCCTTGATC-3'	<u>NM_033230.3</u>	exon 8	136	FOR: Template 1325 1346 REV: Template 1460..... 1439	6 131717295 to 131717316 (-)
eNOS	FOR: 5'-TATTTGATGCTCGGGACTGC-3' REV: 5'-AAGATTGCCTCGGTTTGTTG-5'	<u>NM_021838.2</u>	exon 5	89	FOR: Template 587 606 REV: Template 675 656	4 10809878 to 10809897 (-)

TLR4	FOR: 5'-GCACTCTCACTTCCTCTTGC-3' REV: 5'-GCAGGAAAGGAACAATGCCA-3'	<u>NM_019178.2</u>	<u>exon 1</u>	225	FOR: Template 7089 REV: Template 294 275	5 80145936 to 80145955 (+)
GAPDH	FOR: 5'-CCCCATTTGATGTTAGCGGG-3' REV: 5'-AACGACCCCTTCATTGACCT-3'	<u>NM_017008.4</u>	<u>exon 2</u>	164	FOR: Template 332 313 REV: Template 169 188	3 43213115to 43213134(+)

FOR: Forward

REV: Reverse

3. Results

3.1. Results for markers of renal injury (ACR, KW/100gmBW, serum creatinine, and BUN)

Figure 2 (A) showed that in IR group there was a significant increase in ACR level when compared with all other groups ($P < 0.05$). However, it was significantly ($P < 0.05$) reduced in both the Xanthene-treated group and the Irisin-treated group when compared to the IR group, but there was insignificant ($P > 0.05$) change when compared to the control group and when compared with each other.

For KW / 100gm BW changes, KW was normalized to BW and reported as tissue kW/100 g of BW. **Figure 2** (B) showed that there was significant ($P < 0.05$) increase in kidney weight/100gm BW in the IR group when compared to all other groups. While, in both, the Xanthene-treated group and the Irisin-treated group it was significantly ($P < 0.05$) reduced when compared to the IR group, but this decrease was insignificant ($p > 0.05$) in comparison to control group and in comparison, to each other.

For renal function test: serum creatinine and BUN was accessed, and **Figure 2** (C&D) showed that there was a significant ($P < 0.05$) increase in creatinine and BUN in the IR group when compared with all other groups. However, they showed a significant ($P < 0.05$) decrease in both the Xanthene-treated group and the Irisin-

treated group when compared to the IR group, with no significant change ($P > 0.05$) when compared to the control group and when compared with each other.

3.2. Blood pressure changes

Figure 3 (A, B&C) showed that SBP and DBP in the IR was significantly ($P < 0.05$) increased compared to all other groups. While in the Xanthene-treated group, they showed a significant decrease ($P < 0.05$) compared to the IR group and Irisin-treated group, but there was insignificant ($P > 0.05$) change when compared to the control group. Moreover, in the Irisin-treated group, SBP and DBP were significantly ($P < 0.05$) lower than the IR group, but they were still significantly ($P < 0.05$) higher when compared to the control and Xanthene-treated groups as shown in **Figure 3** (D&E).

3.3 Renal levels of ACE 2 and Ang (1-7)

As shown in **Figure 4** (A&B), renal ACE2 and Ang (1–7) were significantly ($P < 0.05$) lower in the IR group in comparison to the control and Xanthene-treated groups, but it was insignificantly ($P > 0.05$) changed when compared to the Irisin-treated group. In the Xanthene-treated group, they were significantly ($P < 0.05$) higher when compared to the IR and Irisin-treated groups as well, with insignificant ($P > 0.05$) change when compared to the control group. Finally, in Irisin treated group, they were significantly ($P <$

0.05) decreased when compared to control and Xanthenone-treated groups, with insignificant ($P > 0.05$) change when compared to the IR group.

3.4 Renal Tissue parameters (renal oxidative stress markers, Apoptotic markers, inflammatory markers, and Renal NO)

As shown in **Figure 5** (A-F), in the IR group, there was a significant ($P < 0.05$) increase in renal tissue MDA, caspase-3, TNF α and IL-6. While there was a significant ($P < 0.05$) decrease in renal tissue GSH and BCL2 when compared with other groups. On other hand, in the Xanthenone-treated and Irisin-treated groups, there was a significant ($P < 0.05$) decrease in renal tissue MDA, Caspase-3, TNF α and IL-6 with significant ($P < 0.05$) increase in renal tissue GSH and BCL2 compared to the IR group, but those were insignificant ($P > 0.05$) when compared with the control group. Moreover, in the Irisin-treated group when compared with the Xanthenone-treated group, renal tissue MDA, Caspase-3, TNF α , and IL-6 were significantly ($P < 0.05$) lower. In addition, renal tissue GSH and BCL2 were significantly ($P < 0.05$) higher than in the Xanthenone-treated group.

As regard to renal NO, it was shown in **Figure 5** (G) that in the IR group, renal NO showed insignificant ($P > 0.05$) change when compared to the control group, but it showed a significant ($P < 0.05$) decrease when compared to both Xanthenone and Irisin-treated groups. While in the Xanthenone and Irisin treated groups, it was significantly ($P < 0.05$) increased when compared with the control and IR groups. While there was insignificant ($P > 0.05$) change when comparing the Xanthenone-treated group to the Irisin-treated group.

3.5 Relative mRNA expression level of renal PI3K, AKT, TLR-4, eNOS Genes by RT-PCR, and Protein concentration of pAKT and eNOS proteins in Renal tissue

Figure 6 (A, B&C) showed that the relative mRNA expression levels of PI3K, AKT and eNOS in renal tissue were significantly ($P < 0.05$) reduced in the IR group when compared to all other groups. While in the Xanthenone-treated group they significantly ($P < 0.05$) increased when compared with control and IR groups, with a significant ($P < 0.05$) decrease when compared to the Irisin-treated group. Finally, in the Irisin-treated group, their relative mRNA expression levels were significantly ($P < 0.05$) elevated compared with all other groups.

As regards the relative mRNA expression level of TLR4 in renal tissue, **Figure 6** (D) showed significant ($P < 0.05$) increase in the IR group when compared to all other groups. While, in the Xanthenone treated group, it showed a significant ($P < 0.05$) increase when compared with control group and Irisin- treated group with a significant ($P < 0.05$) decrease when compared to the IR group. However, in the Irisin-treated group, it was significantly ($P < 0.05$) reduced when compared with the IR group and Xanthenone treated group, with no significant ($P > 0.05$) change when compared to the control group.

Figure 6 (E&F) showed the pAKT and eNOS protein concentration in renal tissue, and there was a significant ($P < 0.05$) reduction of these protein concentrations in the IR group when compared to all other groups. While in the Xanthenone-treated group, they were significantly ($P < 0.05$) increased when compared with the IR group and significantly ($P < 0.05$) decreased when

compared to the Irisin-treated group, but it was insignificantly ($P > 0.05$) changed when compared to the control group. On the other hand, in the Irisin-treated group, they were significantly ($P < 0.05$) increased compared to all other groups.

3.6 Kidney histomorphological examination

The control group showed the normal structure of the renal glomeruli, Bowman's space, vascular pole, proximal convoluted tubules, and distal convoluted tubules, shown in **Figures 7** (A1&A2). While the IR group showed glomerular atrophy and glomerular hypercellularity with multiple areas of hemorrhage in the interstitial tissue and sloughing of tubular epithelial cells with intraluminal casts and desquamated cells, which are shown in **Figure 7** (B1-B4). Some glomeruli appeared near normal in the Xanthenone-treated

group, while others were still atrophic. Some proximal convoluted tubules appear normal, and others show tubular sloughing. Multiple areas of interstitial hemorrhage are still seen, as shown in **Figure 7** (C1&C2). However, in the Irisin-treated group, the glomeruli returned to a nearly normal structure. The proximal convoluted tubules are apparently normal with little tubular sloughing, while the distal convoluted tubules show slight dilatation, as shown in **Figure 7** (D1&D2).

3.7 Immunohistochemical staining of NFκB

According to immunohistochemistry, NFκB expression in renal tissues in the IR group was significantly ($P < 0.05$) increased compared to all other groups. While Xanthenone and Irisin treatment resulted in a significant ($P < 0.05$) decrease in renal NFκB expression, renal NFκB expression was nearly normalized by the treatment effect with Irisin, are shown in **Figure 8** (A-E).

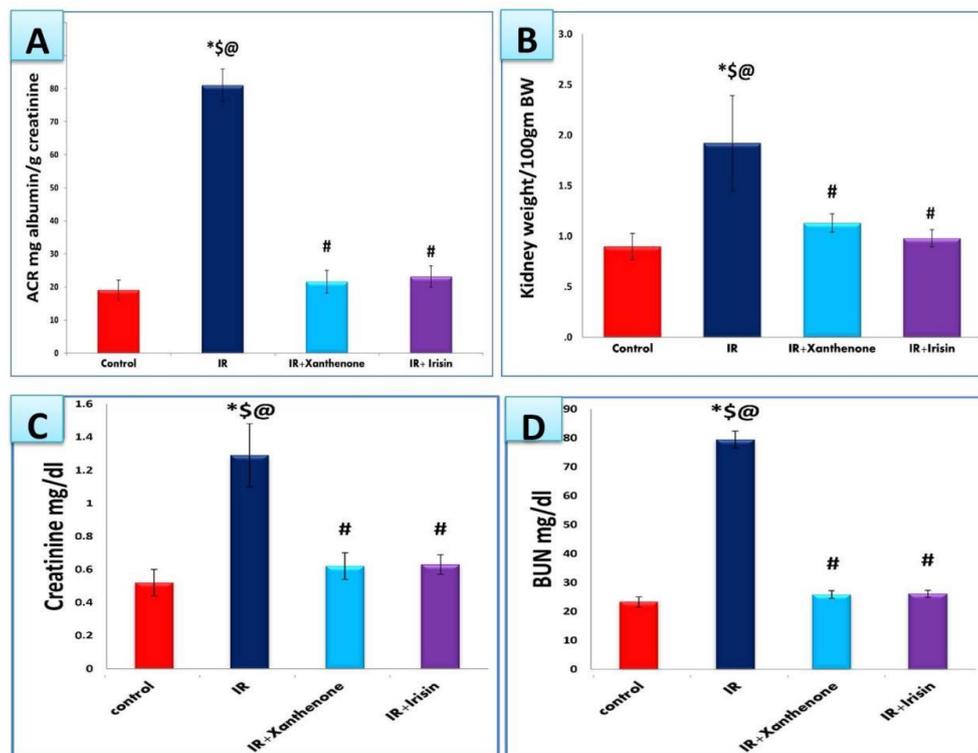


Figure 2 (A-D) Parameters of renal injury in all studied groups. (A) ACR (mg Albumin / g creatinine). (B) kidney weight/ 100gm BW. (C) Serum creatinine (mg/dl). (D) Serum BUN (mg/dl). Mean \pm SD. n=8. All data were analyzed using one way ANOVA, post hoc tukey's test at $p < 0.05$. * $P < 0.05$ versus control group, # $P < 0.05$ versus IR, $^{\$}P < 0.05$ versus IR+ Xanthenone, $^{\textcircled{P}}P < 0.05$ versus IR+ Irisin. IR: Ischemia reperfusion.

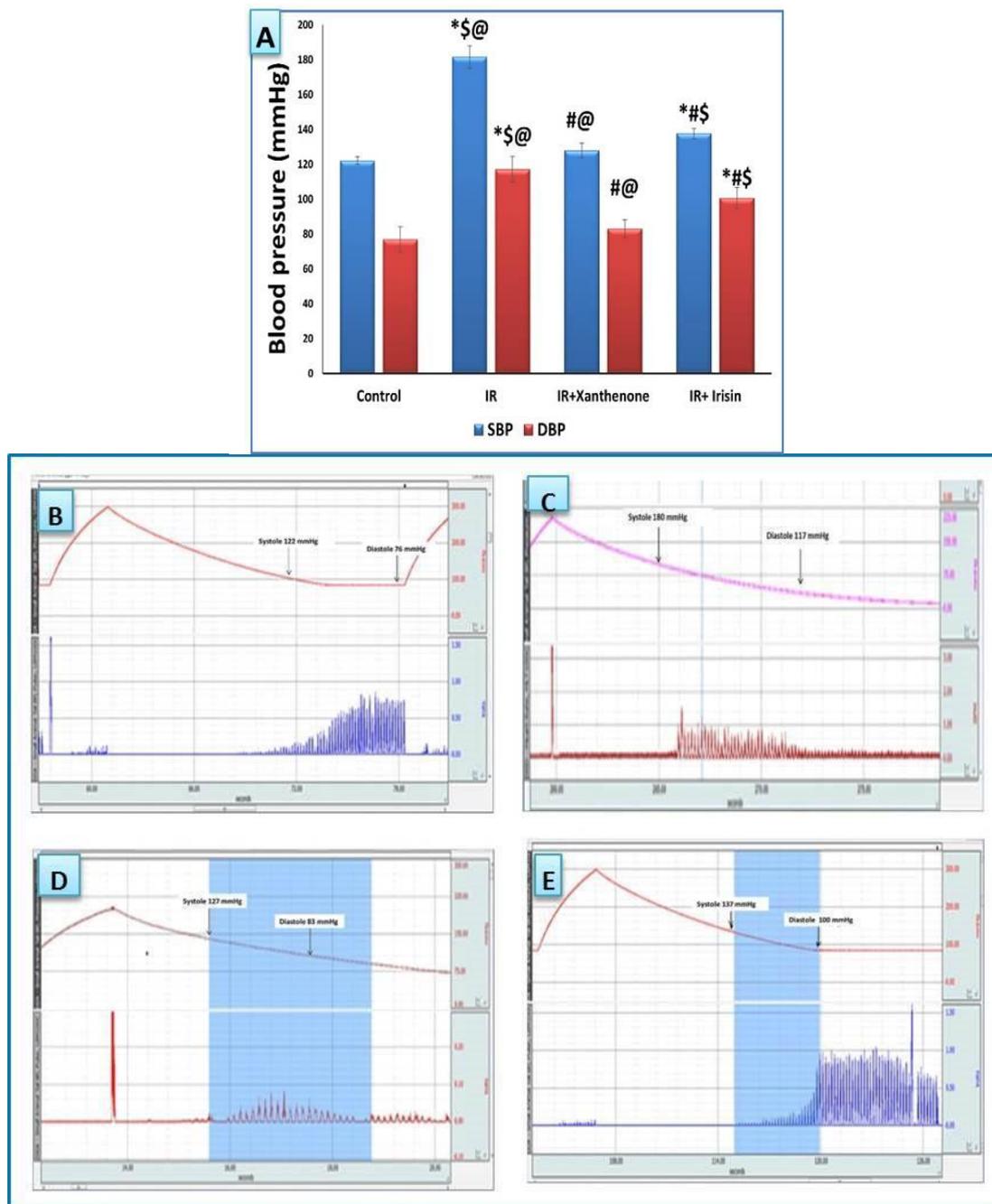


Figure 3 (A-E) Blood pressure changes (mmHg) among all studied groups. **(A)** histogram for SBP and DBP in all studied groups. Mean \pm SD. n=8. All data were analyzed using one way ANOVA, post hoc tukey's test at $p < 0.05$. * $P < 0.05$ versus control group, # $P < 0.05$ versus IR, $^{\$}P < 0.05$ versus IR+ Xanthenone, @ $P < 0.05$ versus IR+ Irisin. IR: Ischemia reperfusion. **(B-E)** Curves of SBP and DBP (mmHg) in all the studied groups using Biopac MP 150 data Acquisition system. **(B)** BP in control, **(C)** BP in IR, **(D)** BP in IR+ Xanthenone, **(E)** BP in IR+ Irisin IR: Ischemia reperfusion.

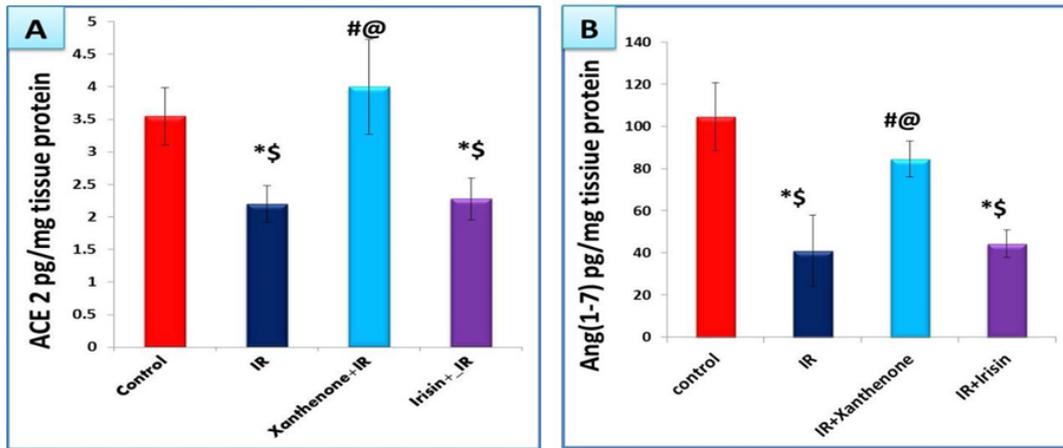
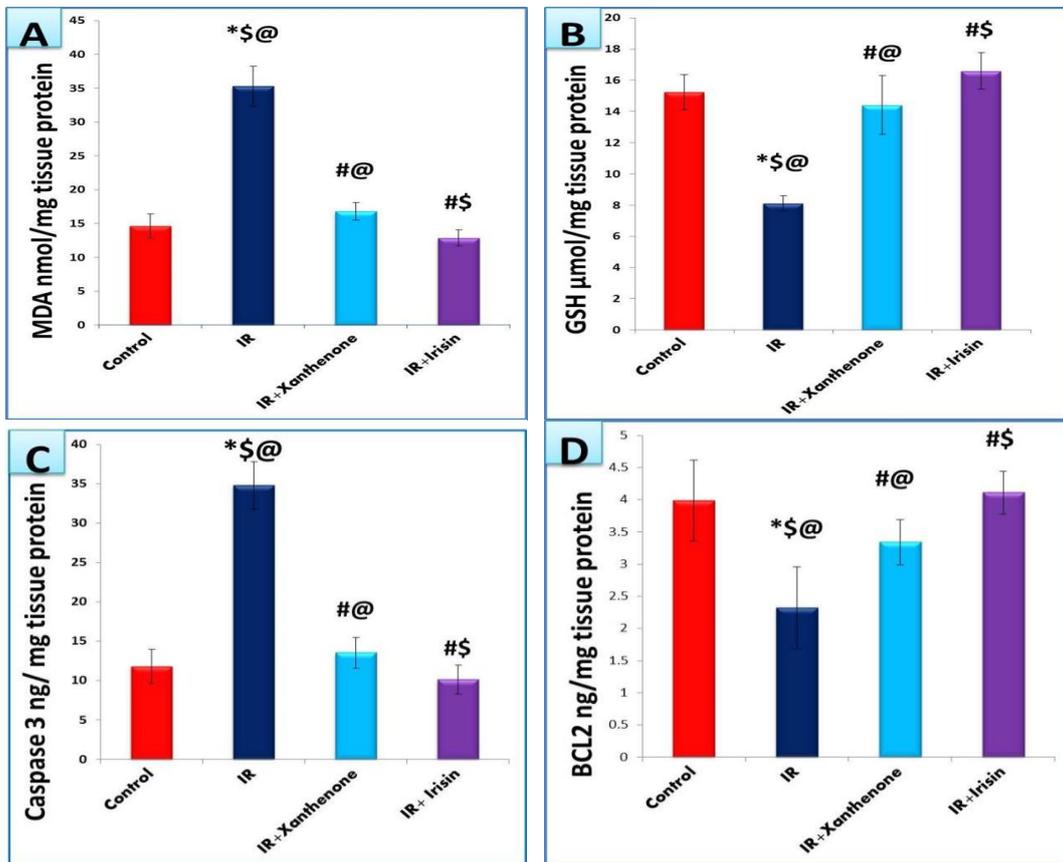


Figure 4 (A&B) Renal levels of ACE 2 and Ang (1-7) in all studied groups. **(A)** Renal ACE2 Pg/mg tissue protein. **(B)** Renal Ang (1-7) Pg/mgtissue protein. Mean ± SD. n=8. All data were analyzed using one way ANOVA, post hoc tukey's test at $p < 0.05$. * $P < 0.05$ versus control group, # $P < 0.05$ versus IR, \$ $P < 0.05$ versus IR+ Xanthene, @ $P < 0.05$ versus IR+ Irisin. IR: Ischemia reperfusion.



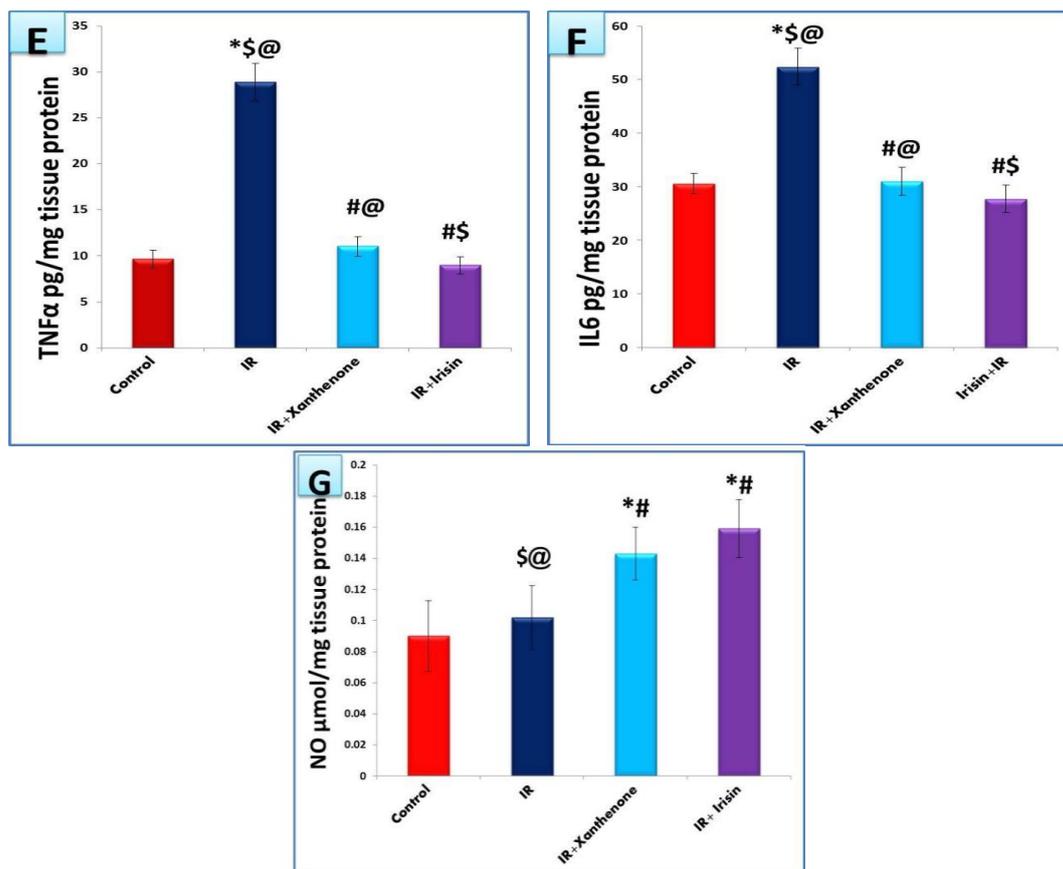
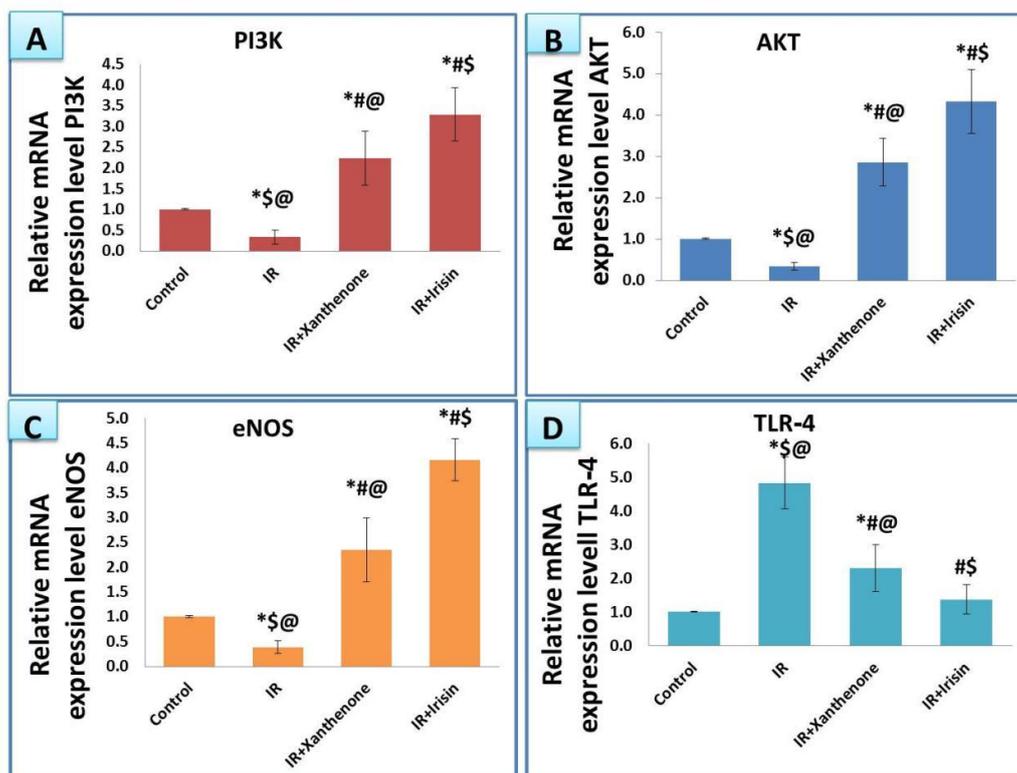


Figure 5 (A-G) Renal tissue oxidative stress, apoptotic, inflammatory markers, and NO in all studied groups (A) Renal MDA nmol/mg tissue protein, (B) Renal GSH μmol/mg tissue protein, (C) Renal Caspase-3 ng/mg tissue protein, (D) Renal BCL2 ng/mg tissue protein, (E) Renal TNFα pg/mg tissue protein, (F) Renal IL6 pg/mg tissue protein, (G) Renal NO (μmol/mg tissue protein). Mean ± SD. n=8. All data were analyzed using one way ANOVA, post hoc tukey's test at p < 0.05. *P < 0.05 versus control group, #P < 0.05 versus IR, \$P < 0.05 versus IR+ Xanthene, @P < 0.05 versus IR+ Irisin. IR: Ischemia reperfusion.



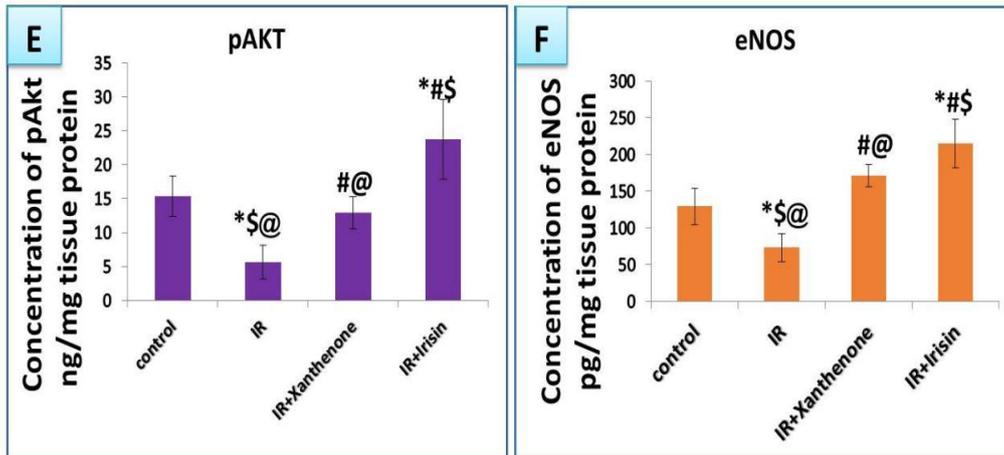
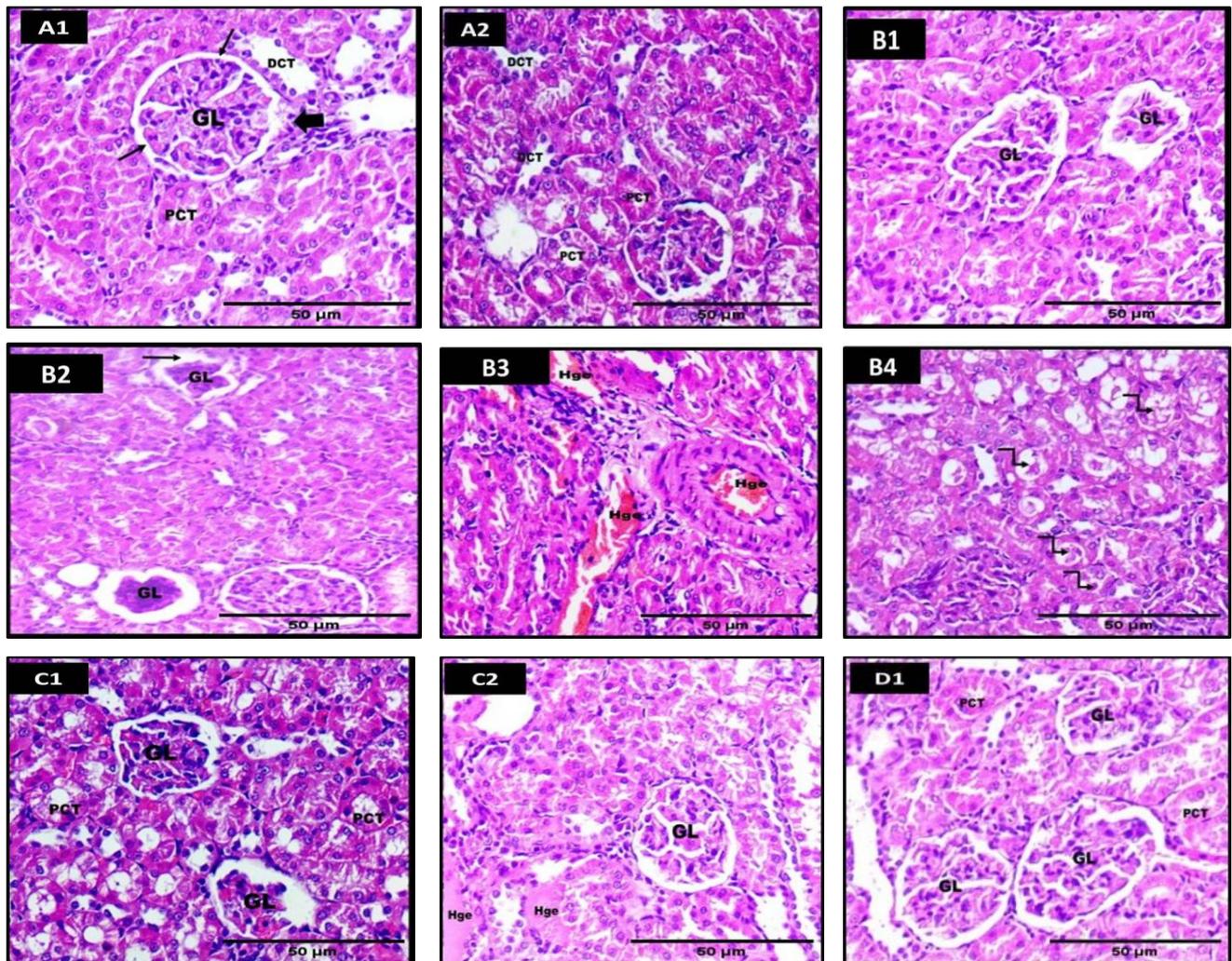


Figure 6 (A-F) Relative gene mRNA expression, & protein concentration in renal tissue of all studied groups (A) PI3k mRNA expression, (B) AKT mRNA expression, (C)eNOS mRNA expression, (D) TLR4 mRNA expression, (E): pAKT concentration (ng/mg tissue protein) (F)eNOS (pg/mg tissue protein). Mean±SD. n=8. All data were analyzed using one way ANOVA, post hoc tukey's test at $p < 0.05$. * $P < 0.05$ versus control group, # $P < 0.05$ versus IR, \$ $P < 0.05$ versus IR+ Xanthenone, @ $P < 0.05$ versus IR+ Irisin. IR: Ischemia reperfusion.



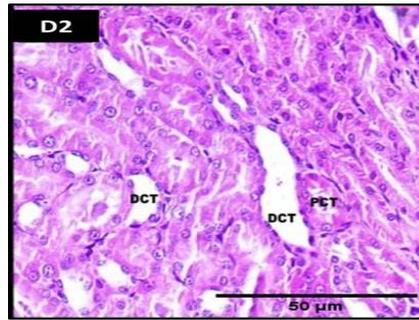
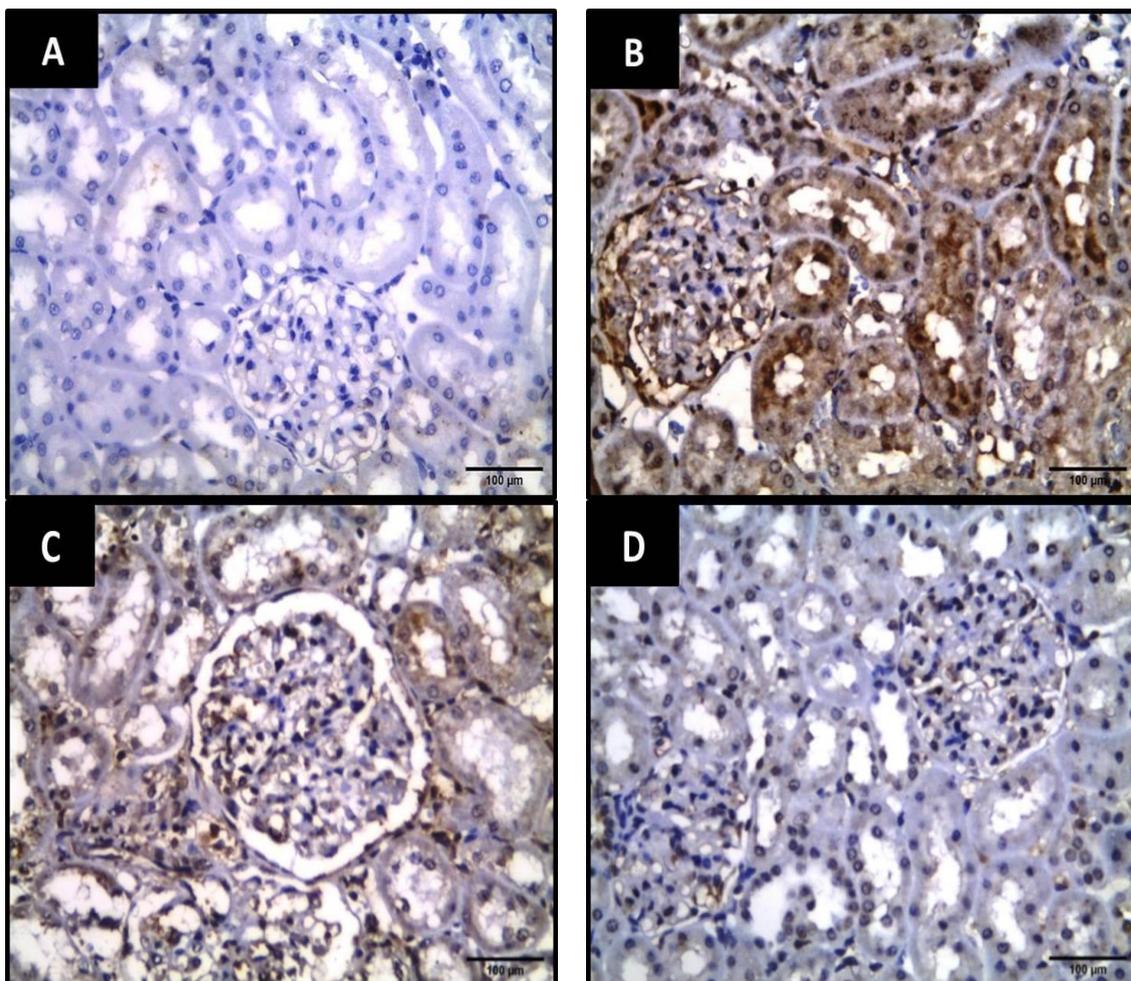


Figure 7 (A-D) A photomicrograph of a renal section of an adult albino rat from (A) **Control group**, (B) **IR group**, (C) **IR + Xanthenone**, & (D) **IR + Irisin**. Hematoxylin& Eosin staining x400. **Figure (6 A1 &A2)** from the control group showed normal structure of the renal glomeruli (GL), the Bowman's space (thin arrow), the vascular pole (thick arrow), and the proximal convoluted tubules (PCT) and distal convoluted tubules (DCT). **Figures (6B1-B4)** from the IR group showed (B1) Glomerular atrophy (GL), (B2) glomerular hypercellularity (GL), (B3) multiple areas of hemorrhage in the interstitial tissue (Hge), (B4) sloughing of tubular epithelial cells with intraluminal casts and desquamated cells (curved arrows). **Figures (6C1&C2)** from the IR + Xanthenone showed some glomeruli (GL) appear near normal, and others are still atrophic. Some proximal convoluted tubules (PCT) appear normal, and others show tubular sloughing. Multiple areas of interstitial hemorrhage (Hge) are still seen in (C2). **Figures (6D1&D2)** from the **IR + Irisin groups** showed a return of the glomeruli (GL) near normal. The proximal convoluted tubules (PCT) are normal with few tubular sloughing in (D1), and the distal convoluted tubules (DCT) showed slight dilatation in (D2).



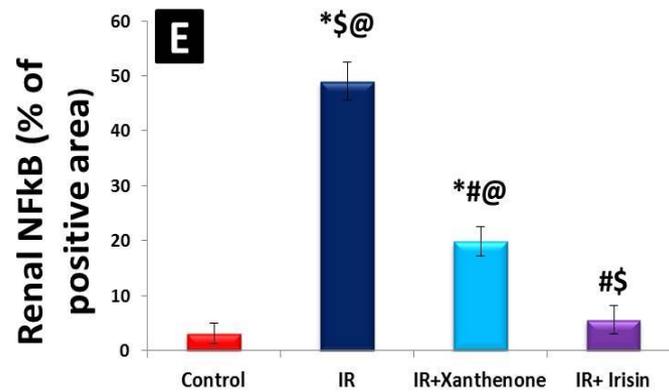


Figure 8 (A-D) Immunohistochemical analysis of NFκB expression in renal tissue (Magnification x400). **(A) Control group showing a negative NFκB reaction. (B) IR group showing strong positive NFκB reaction in the form of brown stain within the cytoplasm (C) IR+Xanthene group showing weak positive reaction compared to IR group (D) IR+ Irisin group showing negative NFκB reaction. (E)** Area percentage of immuno-positive reaction, data are presented as mean ± SD. n=8. All data were analyzed using one way ANOVA, post hoc Bonferroni test at $p < 0.05$. * $P < 0.05$ versus control group # $P < 0.05$ versus IR \$ $P < 0.05$ versus IR+ Xanthene < 0.05 versus IR+ Irisin. IRI: Ischemic renal injury.

4 Discussion

Renal IRI refers to tissue or organ damage after ischemia when the tissue or organs restore blood flow. The kidney, which has a high blood perfusion rate, is particularly susceptible to ischemia and reperfusion damage [18]. Renal ischemia was generated in the current study by clamping the renal pedicles bilaterally for 45 min, followed by a 24-hour reperfusion period, resulting in functional and structural renal injury. Reperfusion is associated with an increase in KW/100gm BW. This aligns with the findings of Azarkish et al. [19]. The study also found that kidney injury induced by IRI was demonstrated biochemically by a significant increase in the urine A/C ratio and an increase in serum nephrotoxicity indicators, including creatinine and BUN, which aligns with Fang et al. [20].

Tissue MDA, a marker for lipid peroxidation, shows an increase during Ischemia-Reperfusion IRI. This indicates that the damaged tissue generates excessive ROS, which leads to oxidative stress and membrane lipid peroxidation [21]. Furthermore, oxidative stress and the production of

inflammatory mediators are linked to the reperfusion phase and may be the reason for the increase in KW. Blood flow during reperfusion triggers oxidative DNA damage, which leads to apoptosis and cell death, as seen by an increase in renal caspase-3 (apoptotic marker) and a decrease in renal BCL2 (anti-apoptotic marker) [22]. Activation of the PI3K/AKT pathway is an endogenous regulatory mechanism that helps cells' survival when they are exposed to damage by external stimuli. The pathway regulates the transcription of genes that encode antioxidant proteins such as GSH when the body is under stress, as reported by Wang et al. [23].

In the current study, there was a reduction in relative PI3K/AKT mRNA expression and a reduction in Protein concentration of pAKT in IRI, which is in agreement with Liu et al. [10].

The TLRs act as receptors for the cellular response of the innate immune system, and their molecular pathways generate pro-inflammatory cytokines and chemokines, such as TNF-α and IL-6. Several studies have reported that ischemia upregulates TLR2 and TLR4 expression [24].

Ahmadvand and Mahdavifard [25] found that IRI leads to increased levels of TNF- α and IL-6. This is the result of overexpression of TLR-4 mRNA, which activates TLR4 receptors through various ligands. The activation and recruitment of TLR4 receptors in IRI causes the activation of several kinases, including inhibitor of NF κ B (I) kinases (IKKs), leading to the release of NF κ B from IKKB and its translocation to the nucleus. This, in turn, increases the gene expression of the transcription factors NF κ B and upregulates the production of proinflammatory substances like IL-6. Activation of NF κ B was proved in this study by immunohistochemical analysis. Also, ischemia-induced endothelial dysfunction was recorded by a reduction of relative eNOS mRNA expression and a reduction of protein concentration of eNOS. This was also reported by **Smith et al. [26]**.

Different types of NOS isoforms generate NO in different ways. iNOS generates NO in high concentrations while eNOS and nNOS create NO in brief bursts at low concentrations for physiological reasons. It is believed that eNOS is a protective enzyme, whereas NO generated by iNOS can be harmful [14]. The balance between the activation of iNOS and the inhibition of eNOS may be the reason why there is no significant change in NO levels in IRI group. RAS activation has been reported in AKI, and it was proposed that RAS plays an important role in the transition from AKI to chronic kidney damage (CKD). Patients recovering from AKI may benefit from therapy with a RAS inhibitor to prevent the AKI/CKD transition [27].

During IRI, an inflammatory cascade occurs that leads to the recruitment of

inflammatory cells to the intima. These recruited cells can produce Ang II, a component of the intracellular angiotensin system, which can then initiate a positive feedback loop that perpetuates the inflammatory response. This vicious cycle can cause severe damage to the body's tissues [28]. In a study by **Lakshmanan et al. [29]**, it was found that Ang II can cause oxidative stress by activating NADPH oxidase through the AT1 receptor, resulting in lipid and lipoprotein oxidation, DNA damage, and compromised endothelial function. Ang II enhances the renal inflammatory response by activating the AT1 receptor and the NF κ B pathway, which releases chemokines (IL-6, TNF- α). Moreover, the binding of Ang II to AT1 activates a G protein, phospholipase C, and inositol trisphosphate synthesis, resulting in a rise in intracellular calcium and vascular remodeling. This was evident in the present study by the elevation of ABP in IRI.

In the process of IRI, NO and oxygen free radicals combine to produce peroxynitrite, which nitrates AKT, thereby limiting its catalytic activity. This explains the decrease in pAKT protein concentration observed during IRI, as well as the relative expression of PI3K/AKT mRNA. On mesangial cells, Ang II triggers the upregulation of TLR4, which has functional consequences such as increased NF κ B activation, TNF- α generation, and IL-6 expression. TLR4 expression can also be regulated by the RAS, Ang II-forming pathway, providing new insights into the role of Ang II in renal inflammation [30].

Histopathological changes were observed in the IR group, including glomerular atrophy and hypercellularity, interstitial tissue haemorrhage, and sloughing of tubular epithelial cells with

intraluminal casts and desquamated cells. These changes confirm the biochemical finding of the present study and are also consistent with **Villa et al. [31]**.

This study investigated the potential of Xanthenone, an inhibitor of Ang II and activator of Ang (1-7), to protect rats from renal IRI. Furthermore, this work examined the antioxidant capacity of Xanthenone by analyzing its impact on TLR4 expression, the PI3K/AKT cell survival pathway, and the resulting inflammatory and apoptotic responses.

Xanthenone injection has been found to enhance renal function, including urine ACR, BUN, and creatinine, while also reducing histopathological abnormalities. Additionally, it has been observed to decrease KW/100gm BW. The increased activity of GSH and a decrease in MDA levels indicate that Xanthenone is rebalancing the oxidant/antioxidant state affected by IRI. This leads to an improvement in renal function. Xanthenone treatment has not only decreased the IRI inflammatory response, as shown by lower levels of TNF- α and IL-6 in kidney tissue but also has an anti-apoptotic effect. This is demonstrated by an increase in renal tissue BCL2 and a decrease in renal tissue caspase 3. This information highlights the powerful anti-inflammatory qualities of Xanthenone. Additionally, Xanthenone has been shown to lower ABP. These findings are in agreement with the research conducted by **Abdel-Fattah et al. [32]**.

Xanthenone has been observed to raise ACE2 and enhance the ACE2/Ang (1-7)/Mas receptor axis, which opposes the traditional ACE/Ang II/AT1 receptor axis. This action may help reduce Ang II, activate ACE2 enzyme, and

convert it to Ang (1-7), leading to anti-inflammatory, anti-apoptotic, and antioxidant properties. The study also found that Xanthenone therapy increased tissue ACE2 and Ang (1-7) levels, in line with **Abdel-Fattah et al. [33]**.

Xanthenone was found to increase the eNOS protein content and relative mRNA expression. These effects may be attributed to an increase in tissue NO, caused by an increase in the protein concentration of AKT and a rise in the relative mRNA expression of PI3K/AKT. These findings are consistent with the study conducted by **Zhao et al. [34]**, who stated that the Mas/PI3K/AKT/NO axis is the primary Ang (1-7) signalling pathway, and that Ang (1-7) activates NO synthase and NO generation in adult ventricular cardiomyocytes via the PI3K/AKT axis. Xanthenone was also observed to lower both systolic and diastolic blood pressure, which may be due to Ang II's direct vasoconstrictor action. This decrease resulted from an increase in ACE2 activity that facilitates the breakdown of Ang II into Ang (1-7), which activates blood vessel Mas receptors and has a vasodilator effect. This is in accordance with **Gupta et al. [35]**.

In addition to its known effects on AKT and eNOS through Gq protein and Mas receptors, Ang (1-7) also has a direct vasodilator impact [36]. In addition, **Biancardi et al. [37]** reported that AT1 receptors and TLR4 work together to mediate Ang II-dependent microglial activation and oxidative damage. Thus, Xanthenone causes downregulation of relative TLR4 mRNA expression with subsequent decreases in proinflammatory mediators such as NF κ B, TNF, and IL-6 by enhancing Ang (1-7) against the

classic axis ACE/AngII/AT1, as verified in this study by immunohistochemical staining of NFkB.

It has been found that during the reperfusion phase, the production of ROS plays a crucial role in the development of AKI caused by IRI [4]. One of the methods to treat AKI caused by IRI involves blocking the production of ROS. In this regard, the impact of irisin, a metabolic regulator, has been studied which enhances mitochondrial performance and reduces the production of ROS [38]. Furthermore, numerous studies have shown that irisin protects the liver and lungs against damage [39].

However, its role in kidney injury was unknown. In the present study, we showed that Irisin plays a protective role against kidney injury in renal IRI. It was clear that Irisin decreased KW/100gm BW and improved renal function by lowering urinary ACR, BUN and serum creatinine and having an antioxidant effect, as evidenced by reducing MDA and increasing GSH levels. It also has an anti-inflammatory effect, as evidenced by decreasing levels of NFkB as evidenced by immunohistochemical changes and lowering tissue TNF- α and IL-6. It also has an anti-apoptotic effect, as evidenced by its ability to reduce caspases 3. Irisin improves an already established inflammatory condition and exerts a protective effect on the vascular endothelium, stimulating the capability for the synthesis of NO by increasing relative mRNA expression of eNOS and increasing protein level of eNOS, resulting in elevation of tissue NO that has a VD effect. This may explain the reduction of ABP with Irisin treatment. Nevertheless, the underlying mechanisms of Irisin are not fully understood. This study found that Irisin might improve the PI3K/AKT signaling

pathway by increasing the relative mRNA expression of PI3K/AKT in addition to increasing the protein concentration of AKT. Moreover, Irisin inhibits the TLR4 inflammatory signaling pathway. Activation of the PI3K/AKT pathway and suppression of TL4 are in concordance with **Waseem et al. [40]**. However, Irisin has no effect on tissue ACE2 and Ang (1-7).

Upon comparing the groups treated with Xanthene and Irisin, it was evident that the Xanthene group exhibited a significantly lesser change in ABP as compared to the Irisin group. This observation can possibly be attributed to the dual action of Xanthene, which raises Ang (1-7) and NO. However, the significant increase in the relative mRNA expression of PI3K/AKT/eNOS and the protein concentration of AKT and eNOS in the Irisin group, as compared to the Xanthene group, demonstrated the greater antioxidant, anti-apoptotic, and anti-inflammatory effects of Irisin. Moreover, the relative mRNA expression of TLR4 in the Irisin group was significantly lower than that of the Xanthene group.

5 Conclusion

This is the first study that sheds light on the potential of Irisin and Xanthene in the context of mitigation of ischemic insults to the kidney. Both Irisin and Xanthene were found to improve renal IRI. Irisin acts on PI3K/AKT/eNOS and TLR4/NFkB pathways. Xanthene operates through PI3K/AKT/eNOS, TLR4/NFkB, and the RAS pathways. Although Xanthene may not possess the same antioxidant, anti-apoptotic, and anti-inflammatory properties as Irisin, it still has an impact on the PI3K/AKT/eNOS and TLR4/NFkB pathways. This suggests the

possibility of using Xanthenone and Irisin for treating AKI.

6 Recommendation

This study represented a future step on the way of modulating the ischemic insults in the kidney and maybe other organs, and to examine the therapeutic effect of Xanthenone and Irisin. However, further research is required to better understand the long-term effects of these compounds. Xanthenone and Irisin may have a longer-lasting therapeutic impact, but extensive clinical trials are needed to confirm this. Moreover, it is important to investigate whether the effects of these compounds are reversible when therapy ends, which could improve the quality of life of patients in the future.

Ethical Approval

All experimental procedures on animals used for this study were performed in accordance with the Research Ethics Committee ethical guidelines of the Faculty of Medicine, Tanta University (Approval code: 35027/11/21). The experimental procedures were also done in accordance with the recommendations of the National Institutes of Health for experimental animals' care and use (NHI Publication; No. 8023, revised 1996).

Authors contributions:

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Rehab A. A. El-Shaer: Conceptualization, Methodology, investigation, Writing – review & editing, Formal analysis, corresponding author.

Shymaa A. Maher: Conceptualization, Methodology, investigation, Writing – review & editing, Formal analysis.

Dina A Ali: Conceptualization, Methodology, investigation, Writing – review & editing, Formal analysis.

Amal M. A. Hammada: Imaging, reading and interpretation of histopathological and immunohistochemistry slides, Writing – review & editing.

Marwa M. Awad: Conceptualization, Methodology, investigation, Writing – review & editing, Formal analysis.

The authors declare that all data were generated in-house and that no paper mill was used.

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