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Protective and Therapeutic Effects of Licorice in Rats With Acute Tubular Necrosis

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Objectives: Various protective and therapeutic effects such as antioxidant, anti-inflammatory, anticancer, antihistaminic, and antibacterial effects have been depicted for licorice. However, its biological effects in the kidney are still not clear. Therefore, we aimed to investigate the efficiency of licorice in rats with gentamicin (GM)-induced acute tubular necrosis.

Design and Methods: Rats were randomized into the control group (only saline for 12 days), licorice group (licorice for 12 days), GM group (GM for 12 days), GM + licorice group, and licorice-treated GM group (licorice for 12 days after taking GM for 12 days). Blood urea, creatinine, and uric acid levels were measured and histopathological analyses of the kidneys were performed. The oxidant-antioxidant balance was evaluated by detecting lipid peroxidation (LPO) and total peroxide levels, and antioxidative side was determined by measuring total antioxidant capacity (TAC) and reduced glutathione (GSH) levels in plasma and kidney tissues.

Results: The oxidant-antioxidant balance seemed to be shifted to the oxidative side in the GM group when compared with the control and GM + licorice groups. In GM group, biochemical profiles showed a remarkable increase in blood uric acid, urea, and creatinine levels, and depletion of renal tissue and plasma TAC and GSH levels. In addition, histopathologic studies revealed severe acute tubular necrosis, congestion, and hyaline casts, verifying GM-induced nephrotoxicity. Licorice was effective in reduction of blood urea, creatinine, and uric acid levels, and also effective in decreasing the tubular necrosis score. Licorice treatment also significantly reduced LPO and total peroxide levels, and increased TAC and GSH levels in both renal tissue and blood. Moreover, these changes in rats subjected to the combined therapy (GM + licorice) were significantly less than those of GM group.

Conclusions: Licorice ameliorates GM-induced nephrotoxicity and oxidative damage by scavenging oxygen free radicals, decreasing LPO, and improving antioxidant defense.

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ACUTE TUBULAR NECROSIS (ATN) is a medical condition involving the death of tubular cells that form the tubule that transports urine to the ureters while reabsorbing 99% of the water (and highly concentrating the salts and metabolic byproducts). Tubular cells replace

themselves continuously, and the recovery is likely if the cause of ATN is removed. ATN presents with acute renal failure (ARF) and is one of the most common causes of ARF which is a rapid loss of kidney function.¹ ARF is diagnosed on the basis of clinical history, such as decreased urine

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production, and characteristic laboratory findings, such as elevated blood urea nitrogen and creatinine levels. Depending on its severity, ARF may lead to several complications, including metabolic acidosis, high potassium levels, changes in body fluid balance, and effects to other organ systems. ATN may be classified as either toxic or ischemic. Toxic ATN occurs when the tubular cells are exposed to a toxic substance (nephrotoxic ATN), such as some antibiotics (e.g., gentamicin [GM]). Ischemic ATN occurs when the tubular cells do not get enough oxygen, a condition to which they are highly sensitive and susceptible, because of their very high metabolism.¹⁻³

It is well known that there are many diseases that can be treated by good nutrition, including specific vitamins and herbs.^{4,5} Licorice is commonly used to purify the liver, the body's detoxification center, helping its defenses against liver diseases, such as cirrhosis and hepatitis by modern herbalists. The herb contains a natural hormone that induces the adrenal cortex to produce larger amounts of cortisone and aldosterone and assists the body to handle stress and relieve exhaustion. It also produces mild estrogenic effects and is said to normalize ovulation in women experiencing infrequent menstruation and ease menopausal symptoms.⁶⁻⁹ Besides these known properties, licorice is widely used in Southeastern Anatolia as a traditional medicine, to treat renal diseases particularly to protect kidneys from the injury caused by renal stones. However, there seems to have been no investigation of the licorice on the renal injuries. Therefore, the present study has been planned to investigate its protective and therapeutic effects on renal function, antioxidant-oxidant status, and histopathological changes in ATN induced by a nephrotoxic agent, GM, and to focus on the public health issue.

Subjects and Methods

Animals and Experimental Design

Experimental study was performed in a total of 25 Wistar-albino rats aged 3 months and weighing 180 to 220 g. All animals were maintained under standard conditions and treated in compliance with the National Institutes of Health guidelines. They were housed on a 12-hour dark/light cycles schedule, with lights on at 06:00 hours. Food and water was available ad libitum. Experiments were approved by the local ethics committee of Harran

University and performed in Harran University Experimental Research Center. Rats were randomized into 5 groups (5 rats in each); the control group (only saline for 12 days) (group 1), licorice group (licorice for 12 days) (group 2), GM group (GM at a dose of 100 mg/kg/day intraperitoneally for 12 days) (group 3), GM + licorice group (GM + licorice together for 12 days) (group 4), and licorice treatment group (licorice for 12 days after taking GM for 12 days) (group 5).

Preparation of Licorice Extract

Licorice roots (1,000 g) were incubated in distilled water for 24 hours at room temperature and filtrated using a thick filtration paper. The obtained extract was stored in a refrigerator (4°C) until it had to be given to the rats. It was diluted 60 times, and its optic density was detected by a spectrophotometer before using.

Preparation of Blood and Renal Tissue Samples

The rats were killed at the end of the procedures, and blood samples and renal tissues were obtained from all the rats. A part of kidney and blood samples were stored at -80°C for biochemical studies until analyzing. A tissue homogenate was prepared by using a polytron tissue homogenizer (Sartorius Stedim Biotech S.A., Germany).

Biochemical Analyses

The plasma uric acid, urea, and creatinine concentrations were estimated by using commercially available kits in an autoanalyzer (Aeroset Abbott Laboratories, Chicago, IL).

Determination of Lipid Peroxidation

Plasma lipid peroxidation (LPO) was evaluated by the fluorimetric method based on the reaction between malondialdehyde (MDA) and thiobarbituric acid.¹⁰ Briefly, 50 mL of plasma was added to 1 mL of 10 mmol/L diethylthiobarbituric acid reagent in phosphate buffer (0.1 mol/L, pH 3). The mixture was mixed for 5 seconds and incubated for 60 minutes at 95°C. Samples were placed on ice for 5 minutes and then 5 mL of butanol was added. The mixture was shaken for 1 minute to extract the diethylthiobarbituric acid-MDA adduct, followed by centrifugation at 1,500g for 10 minutes at 4°C. Fluorescence of the butanol

extract was measured at excitation wavelength of 539 nm and emission wavelength of 553 nm. 1,1,3,3-Tetraethoxypropane was used as a standard solution and the values were presented as mmol/L.

Measurement of Total Antioxidant Capacity

Total antioxidant capacity (TAC) levels of plasma and supernatants of the homogenized and centrifuged kidney tissues were determined by using an automated measurement method developed by Erel.¹¹ Hydroxyl radicals, the most potent biological radicals, are produced by this method. In the assay, ferrous ion solution present in Reagent 1 is mixed with hydrogen peroxide, which is present in Reagent 2. The subsequently produced radicals such as brown-colored dianisidine radical cations produced by the hydroxyl radicals are also potent radicals. Using this method, the antioxidative effect of the sample is measured against the potent-free radical reactions initiated by the produced hydroxyl radicals. The assay has excellent precision values lower than 3%. The results are expressed as mmol Trolox Equiv./L for plasma, mmol Trolox Eqv./g protein for tissue samples.

Measurement of Total Peroxide Concentration

Total peroxide (TPO) concentrations were determined by an enzymatic assay, as described previously.¹² The ferrous ion oxidation xylenol orange (FOX)-2 test system is based on oxidation of ferrous ion to ferric ion by various types of peroxides contained within the samples, to produce a colored ferric-xylenol orange complex whose absorbance can be measured. Briefly, the FOX2 reagent was prepared by dissolving ammonium ferrous sulfate (9.8 mg) in 250 mM sulfuric acid (H_2SO_4) (10 mL), to give a final concentration of 250 μ M of ferrous ion in acid. This solution was then added to 90 mL of high-performance liquid chromatography (HPLC)-grade methanol containing 79.2 mg butylated hydroxytoluene. Finally, 7.6-mg xylenol orange was added with stirring to make the final working reagent (250 μ M ammonium ferrous sulfate, 100- μ M xylenol orange, 25-mM sulfuric acid, and 4-mM butylated hydroxytoluene in 90% v/v methanol in a final volume of 100 mL). The blank reagent contained all the components of the solution except ferrous sulfate.

Aliquots (200 μ L) of the samples were mixed with 1,800 mL FOX2 reagent. After incubation at room temperature for 30 minutes, the vials were centrifuged at 12,000g for 10 minutes. Absorbance of the supernatant was then determined at 560 nm. TPO content of the samples was determined as a function of the absorbance difference between sample and blank tubes using a solution of hydrogen peroxide as standard. The coefficient of variation for individual serum samples was <5%.

Determination of Glutathione

The glutathione (GSH) content of the samples was estimated by the Beutler method based on the development of a stable yellow-colored complex, with 5,50-dithiobis-2-nitrobenzoic acid.¹³ The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration.

Histopathologic Evaluation

Kidney of each animal was taken for histological evaluation. Samples of kidney were placed in formalin and embedded in wax according to standard protocols. They were subsequently sectioned at 7 μ m and stained with hematoxylin and eosin. Samples were then graded histologically from 1 to 4 according to the severity of injury by using a predetermined scoring system.¹⁴ The histological parameters evaluated were tubular necrosis and interstitial edema. A minimum of 10 fields for each kidney slide were examined and assigned for severity of changes. Histological analysis was performed by an expert who was blinded to the experimental data.

Statistical Analysis

For statistical analyses, nonparametric independent group comparisons were made. For multiple comparisons, the Kruskal-Wallis test was used, and for comparisons between groups, the Mann-Whitney test was used if any statistical significance was found. $P < .05$ was considered statistically significant. Data were expressed as mean \pm SD.

Results

All animals survived the experimental protocol. In GM group, biochemical profiles showed a remarkable increase in blood uric acid, urea, and

creatinine levels. In addition, histopathologic studies revealed severe ATN, congestion, and hyaline casts, verifying GM-induced nephrotoxicity. Renal functions such as blood uric acid, urea, and creatinine concentrations of rats subjected to licorice treatment after GM showed a marked recovery as compared with the GM-treated rats ($P < .001$ for all). Blood uric acid, urea, and creatinine concentrations were also significantly decreased in licorice + GM treatment group as compared with the GM-treated rats ($P < .001$ for all). Renal functional parameters are shown in Table 1.

TAC and GSH levels in renal tissue and plasma were lower in GM treatment group than those in control- and licorice-treated GM groups, and these were statistically significant ($P < .001$ for all). TAC and GSH levels in plasma were significantly higher in the licorice-treated GM group ($P < .001$ for both) and also licorice + GM treatment group than those in the GM-treated group ($P < .001$ and $P < .01$, respectively). TPO and LPO levels of plasma and renal tissue were significantly higher in the GM-treated group than those in the licorice-treated GM group ($P < .001$ and $P < .01$, respectively). TPO and LPO levels of plasma and renal tissue were also significantly higher in the GM-treated group than those in the licorice + GM group ($P < .05$ and $P < .01$; $P < .05$ and $P < .001$, respectively). There were no statistically significant differences between the licorice treatment group and control group with regard to the TPO and LPO levels ($P > .05$). The results are summarized in Tables 2 and 3 and shown in Figs. 1 and 2.

In groups 1 and 2, the results of the histopathological examinations of the renal tissues were normal (Fig. 3). Histopathological examination of the tissues in group 3 revealed severe lesions (fourth degree of necrosis) such as tubular necrosis, proximal tubular epithelium necrosis (no nuclei, intense

eosinophilic homogeneous cytoplasm, but preserved shape), and damage characterized by a loss of brush border, no nuclei, intense eosinophilic homogeneous cytoplasm, lumen dilatation or collapse, and cellular detachment from tubular basement membranes observed in the kidney of the rats (Fig. 4). Co-treatment of licorice with GM (group 4) considerably decreased these signs of ATN compared with only GM-treated group (group 3) (Fig. 5). Also, treatment of the GM-treated group with licorice (group 5) reduced the morphological alterations associated with nephrotoxicity of GM significantly to the normal (Fig. 3).

Discussion

ATN causes severe damage to the kidney epithelial tubular cells and is often associated with severe renal dysfunction and ARF which is the rapid breakdown of renal function. ARF occurs when high levels of uremic toxins (waste products of the body's metabolism) accumulate in the blood and when the kidneys are unable to excrete (discharge) the daily load of toxins in the urine.^{1-3,15} It is known that ATN can be induced by GM, whose clinical uses have so far been restricted.¹⁶⁻¹⁸ The exact mechanism of nephrotoxicity is still unknown; however, it appears that free radicals may be involved.^{19,20}

Licorice has previously been shown to alleviate oxidative stress involved in cancer, bronchitis, colitis, diverticulosis, gastritis, stress, colds, nausea, and inflammation because of its antioxidant properties. Moreover, various pharmacological activities of licorice, including anti-inflammatory, immunomodulatory, antiulcer, and anti-allergy activities have been reported.^{6-9,21} Licorice also has antiviral activity against various DNA and RNA viruses including HIV and severe acute respiratory syndrome-associated coronavirus.²²⁻²⁴

Table 1. Clinical Parameters in Rats of the Study Groups

Parameters	Group 1 (n = 5)	Group 2 (n = 5)	Group 3 (n = 5)	Group 4 (n = 5)	Group 5 (n = 5)
Urea (mg/L)	318.0 ± 172.0	338.0 ± 102.3 ^a	2938.0 ± 733 ^{b,c,†‡}	452.0 ± 69.1 ^{d*,e*,f,†‡}	402.0 ± 72.2 ^{g*,h*,i,j,†‡}
Creatinine (mg/L)	6.4 ± 0.5	7.0 ± 0.7 ^a	52.8 ± 23.6 ^{b,c,†‡}	9.2 ± 1.8 ^{d*,e*,f,†‡}	6.6 ± 0.5 ^{g,i,j,*}
Uric acid (mg/L)	14.4 ± 3.6	24.0 ± 6.0 ^{a*}	151.8 ± 24.9 ^{b,c,e,†‡}	38.0 ± 2.8 ^{d*,e*,f,†‡}	53.4 ± 5.02 ^{g,h,i,j,†‡}

Values are mean ± SD.

Differences between groups 1 and 2^a, 3^b, 4^d, 5^g; groups 2 and 3^c, 4^e, 5^h; Groups 3 and 4^f, 5ⁱ; Groups 4 and 5^j.

* $P < .05$.

† $P < .01$.

‡ $P < .001$.

Table 2. Plasma Oxidative and Antioxidative Parameters in Rats of the Study Groups

Parameters	Group 1 (n = 5)	Group 2 (n = 5)	Group 3 (n = 5)	Group 4 (n = 5)	Group 5 (n = 5)
TAC (mmol Trolox Eqv./L)	0.39 ± 0.11	0.56 ± 0.18 ^{a*}	0.13 ± 0.03 ^{b†,c‡}	0.53 ± 0.02 ^{d*,e,f‡}	0.77 ± 0.25 ^{g*,h*,i‡,j*}
TPO (mmol H ₂ O ₂ Eqv./L)	0.10 ± 0.00	0.10 ± 0.00 ^a	0.32 ± 0.04 ^{b†,c‡}	0.20 ± 0.00 ^{d*,e*,f*}	0.12 ± 0.04 ^{g,i‡,j*}
LPO (nmol/L)	1.37 ± 0.10	1.24 ± 0.03 ^{a*}	1.74 ± 0.23 ^{b*,c*}	1.34 ± 0.16 ^{d,e*,f†}	1.41 ± 0.19 ^{g,h*,i†,j}
GSH (μmol/L)	0.18 ± 0.08	0.22 ± 0.01 ^a	0.12 ± 0.01 ^{b†,c‡}	0.28 ± 0.12 ^{d*,e,f†}	0.43 ± 0.09 ^{g†,h*,i‡,j*}

TAC, total antioxidant capacity; TPO, total peroxide; H₂O₂, hydrogen peroxide; LPO, lipid peroxidation; GSH, glutathione. Values are mean ± SD.

Differences between groups 1 and 2^a, 3^b, 4^d, 5^g; groups 2 and 3^c, 4^e, 5^h; groups 3 and 4^f, 5ⁱ; Groups 4 and 5^j.

*P < .05.

†P < .01.

‡P < .001.

Glycyrrhizin, a major bioactive compound derived from the underground parts of licorice plants, has been used in Japan for more than 20 years as a hepatoprotective agent for chronic hepatitis.²⁵ Therefore, a large amount of licorice and its extracts are available in the world market as sweetening agents and medicinal materials. In our region, it is traditionally used for the treatment of kidney diseases by people without prescription. Although the various pharmacological properties of licorice have been extensively studied, its beneficial effect on renal diseases remains poorly understood. Thus, the effect of licorice supplementation on the GM-induced nephrotoxicity was examined in this study.

In our experimental study, GM caused significant renal dysfunction as assessed by a markedly

increase in the plasma concentrations of urea, uric acid, and creatinine in the GM-treated group, which were decreased to the normal values with the licorice treatment. We also evaluated oxidative-antioxidative status in plasma and renal tissue of the rats and showed the presence of severe oxidative stress in the GM-treated group. In this study, we measured LPO and TPO to determine oxidative status, and TAS and GSH to determine antioxidative status. Our results implicated that oxidative-antioxidative balance shifted toward the oxidative side in the GM-treated group. However, when GM was used together with licorice, the imbalance between oxidants and antioxidants disappeared. Moreover, the treatment with licorice after using GM provided a certain recovery in the renal tissue histopathologically and also

Table 3. Oxidative and Antioxidative Parameters in Renal Tissue of the Rats

Parameters	Group 1 (n = 5)	Group 2 (n = 5)	Group 3 (n = 5)	Group 4 (n = 5)	Group 5 (n = 5)
TAC (mmol Trolox Eqv./g protein)	0.37 ± 0.04	0.39 ± 0.04 ^a	0.16 ± 0.02 ^{b†,c‡}	0.40 ± 0.07 ^{d,f†}	0.36 ± 0.07 ^{g,i†,j}
TPO (mmol H ₂ O ₂ Eqv./g protein)	0.07 ± 0.00	0.06 ± 0.00 ^a	0.39 ± 0.11 ^{b†,c‡}	0.21 ± 0.00 ^{d†,e†,f*}	0.07 ± 0.01 ^{g,i†,j‡}
LPO (nmol/g protein)	1.40 ± 0.31	1.47 ± 0.39 ^a	3.33 ± 0.44 ^{b†,c‡}	1.22 ± 0.15 ^{d,f†}	2.16 ± 0.50 ^{g*,h*,i†,j*}
GSH (μmol/g protein)	0.19 ± 0.15	0.22 ± 0.01 ^a	0.13 ± 0.01 ^{b,c*}	0.17 ± 0.01 ^d	0.21 ± 0.00 ^{g,h,i*,j}

Values are mean ± SD.

Differences between groups 1 and 2^a, 3^b, 4^d, 5^g; groups 2 and 3^c, 4^e, 5^h; groups 3 and 4^f, 5ⁱ; groups 4 and 5^j.

*P < .05.

†P < .01.

‡P < .001.

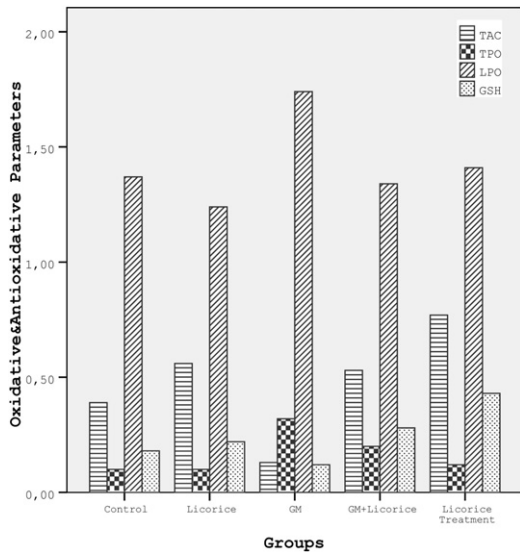


Figure 1. Plasma oxidative and antioxidative parameters in rats of the study groups.

balanced the oxidant and antioxidant biochemical parameters.

Oxygen free radical generation can be assessed by measuring the values of LPO products. The measurement of TPO and LPO provides a sensitive index of LPO and oxidative stress.²⁶⁻²⁸ The results of this study confirm that GM causes oxidative stress, by either modulating production of free radicals and reactive oxygen species, and also of toxic cytokines leading to inflammation and

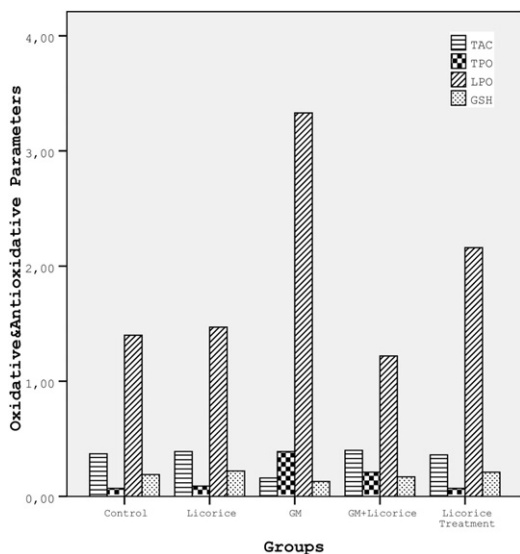


Figure 2. Oxidative and antioxidative parameters in renal tissue of the rats.

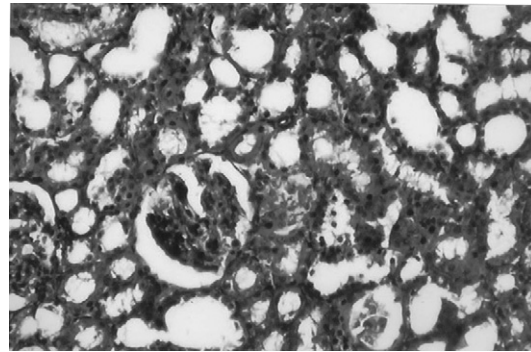


Figure 3. (Groups 1, 2, and 5): Histological features of normal renal tissue were observed (grade 0) (hematoxylin and eosin stain, original magnification ×200).

leukocyte infiltration, or direct tissue damage, which is shown in previous studies.²⁹⁻³¹ We also evaluated the effects of licorice on the parameters implicating oxidative and antioxidative status. We observed significantly decreased LPO and TPO levels and increased TAC and GSH levels with the administration of licorice, which may, probably, be related to its antioxidant and free-radical scavenging effects. Similar results were reported for several herbs having antioxidative effects such as sesame oil, eugenol, nigella sativa, and ginseng.^{16-18,20,32,33}

In this study, we defined a significant decrease in plasma urea, uric acid, and creatinine levels, and plasma and renal tissue oxidative parameter levels in the licorice treatment group after inducing

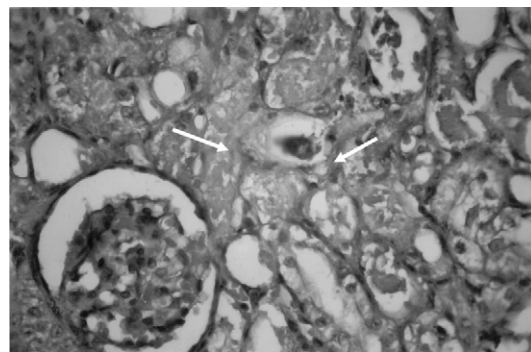


Figure 4. (Group 3): Severe lesions (grade 4) such as tubular necrosis, proximal tubular epithelium necrosis (no nuclei, intense eosinophilic homogeneous cytoplasm, but preserved shape), and cellular detachment from tubular basement membranes observed in the renal tissue of the rats (arrows), (hematoxylin and eosin stain, original magnification ×400).

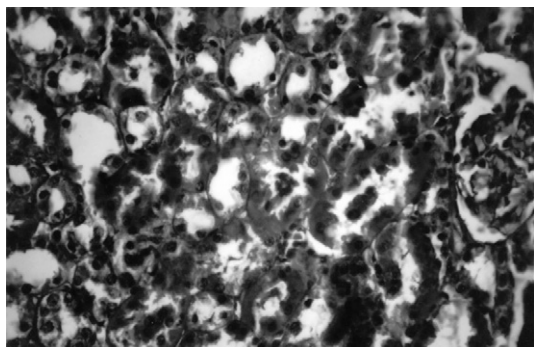


Figure 5. (Group 4): Co-treatment of licorice with GM considerably decreased these signs of ATN compared with only GM-treated group (grade 1) (Figure 2) (hematoxylin and eosin stain, original magnification $\times 400$).

ATN with GM and also in the GM + licorice group compared with GM-treated group. The protective and therapeutic effects of licorice against GM-induced nephrotoxicity appear to be associated with its antioxidative effects. In the light of these findings, it may be possible to conclude that licorice seems to be beneficial as having protective and therapeutic effects because of its antioxidative properties and may be used as a supportive treatment. However, further investigations are required to evaluate the beneficial effects of licorice on kidney tissue damage in clinical and experimental models.

Practical Application

This study calls attention to the beneficial effects of licorice on renal pathologies caused and/or resulted in oxidative damage by scavenging oxygen free radicals, decreasing LPO and improving antioxidant defense. The result implicates a greater emphasis on nutritional interventions to support the usage of licorice by people for protection and treatment of renal diseases.

Acknowledgments

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