INTRODUCTION

The bioactivated xenobiotic, naphthalene (NA), is a pervasive environmental contaminant found in ambient air and ground water [1]. Humans are exposed to NA from different sources, including industrial applications, such as the production of phthalic anhydride, which is used in the synthesis of resins, plastics, pharmaceuticals, and insect repellents [2]. Non-occupational exposures also arise from diesel and fuel emissions [3]. It has become obvious that tobacco smoke poses a health risk to nonsmokers and NA has been found to be the most abundant polycyclic aromatic hydrocarbon in sidestream cigarette smoke [4]. Exposure to NA at home has classically occurred from its use in mothballs as a moth repellent, although this use is becoming less common due to the toxicity and flammability of NA [5]. There is some evidence that NA may cause developmental toxicity as it may cross the placenta giving rise to neonatal hemolytic anemia [6]. NA is toxic by all routes of exposure, whether it is ingested, inhaled or comes into contact with skin and eyes [7]. NA exposure is associated with several toxic manifestations in humans and laboratory animals, with the lens of the eye and the lungs being most sensitive. Most human toxicities involve low dose, chronic exposure to NA [8]. NA caused an increase in incidence and severity of olfactory epithelia metaplasia, chronic inflammation and hyperplasia of respiratory epithelium in the nose and chronic inflammation in the lungs [9]. Histological examination of rat lungs following intraperitoneal administration of 200 mg/kg NA resulted in damage to the non-ciliated, bronchiolar epithelial (Clara) cells [10]. Preuss et al. [2] reported the development of cataract.
following exposure to NA. Subsequently, NA has become widely used to induce cataracts in experimental animal models [11]. In tests with hamster ovary cells, NA induced sister chromatid exchanges with and without exogenous metabolic activation [12]. The above studies indicate a wide range of effects that can be produced by NA. NA has been classified by the International Agency for Research on Cancer as being possibly carcinogenic in humans [13]. Rosemary (Rosmarinus officinalis) is an herb commonly used as spice and flavoring agents in food processing [14]. Rosemary composed of dried leaves and flowers constitutes a particularly interesting source of biologically active phytochemicals as it contains a variety of phenolic compounds with substantial antioxidant activity [15]. Leaves of rosemary possess a variety of bioactivities including anti-tumor, antioxidant [16] and anti-inflammatory actions [17]. It is also useful in treatment or prevention of bronchial asthma, spasmodic disorders, peptic ulcer, inflammatory diseases, hepatotoxicity, atherosclerosis biliary upsets, as well as for tension headache, renal colic, heart disease, and poor sperm motility [18]. Up to the best of our knowledge, very few studies were performed to demonstrate the renal injury associated with NA exposure. Hence, the aim of this study was to demonstrate the effect of NA on the kidney of adult male albino rat and evaluate the possible protective role of rosemary.

**MATERIALS AND METHODS**

**Animals**

A total of 25 Sprague-Dawley adult male albino rats weighting 150-200 g were used in this experiment. Food and water were provided *ad libitum* for 5 days before use in the Anatomy Department, Faculty of Medicine, Menoufiya University. All aspects of animal care and treatment were carried out according to the local guidelines of the ethical committee for animal research.

**Chemicals**

(1) NA (molecular weight 128.19 g/mol) was obtained as a powder from adwic Co., Cairo, Egypt.

(2) Rosemary leaves were purchased from the chemistry department, Agricultural Research Center, Cairo, Egypt.

**Experimental Design**

The experimental period lasted for 30 days. Animals were randomly divided into three groups:

**Group I (control group)** included 15 rats and were further divided into 3 equal subgroups:

- Subgroup Ia was kept without any treatment throughout the experimental period.
- Subgroup Ib received corn oil (5 ml/kg/day) orally by gastric tube.
- Subgroup Ic received rosemary extract 10 ml/kg/day orally by gastric tube [19].

**Group II (NA treated group)** included 5 rats that received NA at a dose of 200 mg/kg/day dissolved in 5 ml/kg corn oil orally by gastric tube [19].

**Group III (protected group)** included 5 rats received rosemary extract at the same previous dose followed after 60 min by NA at the same previous dose orally by gastric tube.

**Preparation of Rosemary Extract**

Rosemary leaves were washed thoroughly with distilled water. The air dried leaves were coarsely powdered. 8 g of the powder dissolved in 100 ml of distilled water was boiled for 2 min and infused for 10 min. After cooling and passing through filter paper, a clear solution was obtained. During 24 h, 10 ml/kg/day of the extract was given to the rats by gastric tube [15].

**Biochemical Assessment of Renal Function**

24 h following the last dose, renal function was assessed in all experimental groups by measuring serum urea and creatinine levels. Blood samples were collected from the retroorbital venous plexus, under mild anesthesia, using a fine heparinized capillary tube introduced into the medial epicanthus of the rat’s eye. 2 mL of blood was collected in a clean graduated centrifuge tube, allowed to clot at room temperature for 10 min and then centrifuged at 3000 rpm for 20 min. The supernatant serum was collected in a dry clean tube to estimate serum urea and creatinine using an autoanalyzer (Hitachi 912 auto-analyzer; Germany) in the Clinical Pathology Department of the Faculty of Medicine, Menoufiya University.

**Histological and Immunohistochemical Studies**

At the end of the experiment, rats were killed under general anesthesia. Both kidneys from each animal were carefully dissected out and removed. The kidney was fixed in a 10% neutral buffered formalin solution and then processed to prepare 3 mm thick paraffin sections suitable for use in the following histological and immunohistochemical techniques: Hematoxylin and eosin (H and E), periodic acid Schiff’s reaction (PAS) for identification of changes in the mesangial matrix and the basement membranes, which appeared magenta red in color and with Masson’s trichrome to demonstrate collagen fibers [20,21]. Sections were also used in immunohistochemical staining for detection of alkaline phosphatase and inducible nitric oxide synthase (iNOS) immunoreactivity. Briefly, sections were deparaffinized, rehydrated, and, after antigen retrieval with 10 mmol/l citrate acid solution (pH 6), specimens were preincubated with goat serum for 5 min and were then incubated overnight at 4°C with polyclonal anti-alkaline phosphatase and anti-iNOS (Working dilution 1:500). Binding was detected using biotinylated secondary antibody (goat anti-mouse IgG; Sigma Aldrich) for 10 min. The specimens were then incubated with streptavidin-peroxidase complex for 5 min, followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich) for 3 min. Slides were counterstained with hematoxylin and mounted.

**Morphometric Study**

Data were obtained using a Leica Qwin 500 image analyzer computer system (Leica, Hessen, Germany).
(1) Glomerular changes:

- Glomerular matrix index: The glomerular matrix index represents the ratio of the mesangial matrix area to the glomerular tuft area in PAS-stained sections. It is used to determine the degree of increased glomerular matrix. It was measured as the percentage of the area positive for PAS reaction within the glomerular tuft in 10 randomly selected glomeruli per animal.

- Glomerular fibrosis: To evaluate the fibrosis in the glomeruli, Masson’s trichrome-stained sections were used. The percentage of area stained blue was measured per unit tuft area in 10 randomly selected glomeruli per animal.

(2) Cortical interstitial fibrosis: Cortical interstitium was defined as the peritubular space and included the tubular basement membrane and peritubular capillaries. Interstitial fibrosis was estimated by measuring the percentage of area occupied by the Masson’s trichrome-positive interstitium in 10 randomly selected non-overlapping fields per animal.

(3) Medullary fibrosis: It was estimated by measuring the percentage of Masson’s trichrome-positive area in 10 randomly selected non-overlapping fields of the renal medulla per animal.

(4) Immunoreactive area % of alkaline phosphatase of proximal convoluted tubules (PCTS) in 10 randomly selected nonoverlapping fields per animal at magnification ×400.

(5) Immunoreactive iNOS area %: It was determined randomly in the cortex and the medulla. Measurements were taken in 10 randomly selected nonoverlapping fields at magnification ×400 from each animal.

Statistical Analysis

Statistical analysis was performed on Excel software. Data were presented as mean ± standard deviation. Differences among the study groups were detected by one way analysis of variance as the global test to determine any differences in data prior to comparing pairs of groups using t-test. The results were considered statistically significant and highly significant with P < 0.05 and 0.001, respectively [22].

RESULTS

There was no significant difference between subgroups Ia-c in all the outcomes at each time point used in the study; therefore, these three subgroups were pooled in one group (control).

Biochemical Results

As illustrated in Table 1, results of this study showed that NA treatment (in Group II), compared with the control group, resulted in a highly significant increase in the mean values of serum urea and creatinine. All previous results were significantly reversed in the protected group (Group III) compared with NA treated group (Group II).

Histological Results

H and E stained sections

Control animals (Group I) showed a normal appearance for the glomeruli composed of capillary tufts surrounded by Bowman’s capsule with space between the visceral layer adherent to the capillary tuft and the parietal layer, as well as PCTS with characteristic narrow lumen lined by a few cuboidal epithelial cells and distal wider tubules lined by more low cuboidal epithelial cells and the medulla containing wider collecting tubules with a wide lumen and thin wall lined by cubical cells. Glomeruli and tubules were crowded in the kidney with minimal interstitial tissue in between [Figure 1].

NA treated animals (Group II) showed variable histological parenchymatous changes. They demonstrated glomerular congestion in most of the glomeruli. Some glomeruli demonstrated marked mesangial expansion and hence that Bowman’s spaces were almost completely obliterated. Shrinkage of renal glomeruli with widening of Bowman’s spaces could also be seen. Tubulointerstitial changes were present, in the form of focal tubular dilatation with appearance of casts inside the tubules. Congesteb peritubular blood vessels and interstitial hemorrhage were also seen. The medullary region demonstrates

Table 1: The mean value±SD of the biochemical assessment of renal function

<table>
<thead>
<tr>
<th></th>
<th>Control (Group I)</th>
<th>NA treated (Group II)</th>
<th>Protected (Group III)</th>
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<tr>
<td>Serum urea level (mg/dl)</td>
<td>29.22±1.2802</td>
<td>59.82±2.3881</td>
<td>32.66±1.1266***</td>
</tr>
<tr>
<td>Serum creatinine level (mg/dl)</td>
<td>0.34±0.0231</td>
<td>1.98±0.0402</td>
<td>0.58±0.3911*</td>
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*P<0.05 and **P<0.001, compared with control; *P<0.05 and ***P<0.001, compared with the NA treated group, NA: Naphthalene, SD: Standard deviation
vascular congestion among the collecting tubules. Focal cellular infiltration was observed in the interstitium [Figure 2].

Protected animals (Group III) demonstrated less severe changes. Few glomeruli were still congested. Some convoluted tubules appeared dilated. Less vascular congestion was seen in the medulla [Figure 3].

**PAS Stained Sections**

With respect to PAS staining, the control group showed PAS-positive reaction in the mesangial matrix in the glomeruli, Bowman’s capsules, proximal, distal, and collecting tubules. The NA treated group demonstrated increased PAS-positive reaction of a glomerulus, convoluted tubules and collecting tubules in the medulla. The protected group demonstrated an increase in the staining intensity of the glomerular tuft and the tubules appeared normal [Figure 4].

**Masson’s Trichrome Stained Sections**

With respect to Masson’s trichrome staining, the control group showed staining reaction in the mesangial matrix in the glomeruli and boundary of Bowman’s capsule and staining of

![Figure 2](image1)

**Figure 2:** (a-f) Photomicrographs of sections from the naphthalene treated group showing congested capillary tuft of the glomeruli (G), almost obliterating the Bowman’s space with tuft adhesion to the Bowman’s capsule (notched arrow), atrophied glomeruli (AG) with widened Bowman’s space (*). Tubules appeared dilated (D) with intratubular casts (arrow). Note the interstitial hemorrhage (H) and vascular congestion between tubules (arrow head). Focal interstitial cellular infiltration (I) can be clearly seen (H&E, × 400).

![Figure 3](image2)

**Figure 3:** (a,b) Photomicrographs of sections from protected group showing glomerulus (G) with tuft congestion. The cortical convoluted tubules still appeared dilated (D). The collecting tubules in the medulla showed less vascular congestion (arrow head) (H&E, × 400).

![Figure 4](image3)

**Figure 4:** (a-f) Photomicrographs of PAS-stained sections from: (a,b) Control group demonstrating PAS-positive reaction of glomerulus (G) and boundaries of Bowman’s capsule (thin arrow). The renal tubules show PAS-positive reaction in the cytoplasm (thick arrow) and in the collecting tubules of the medulla (curved arrow). (c,d) Naphthalene treated group demonstrating increased PAS-positive reaction of glomeruli (G), proximal, distal convoluted tubules (thick arrow) and collecting tubules in the medulla (curved arrow). (e,f) Protected group demonstrating increased reaction in some glomeruli (G). Normal reaction is seen in proximal and distal convoluted tubules (thick arrow) and around the collecting tubules in the medulla (curved arrow) (Periodic acid-Schiff, × 400).
the basement membrane of proximal, distal, and collecting tubules. In NA treated group, connective tissue fibers appeared increased in the glomeruli, with thickened parietal layer of Bowman’s capsule in some areas. The collagen fibers around the convoluted and collecting tubules were increased as well. The protected group demonstrated a slight increase in the connective tissue content [Figure 5].

**Immunohistochemical Results**

Positive immunohistochemical staining of alkaline phosphatase enzyme revealed an intense positive reaction for alkaline phosphatase in the PCTs in the control rat renal cortex (Group I) [Figure 6]. The renal cortex of NA treated rats (Group II) showed a noticeable reduction in alkaline phosphatase positive reaction in some PCTs [Figure 7]. Sections of the renal cortex of protected rats (Group III) revealed increased reaction for alkaline phosphatase of many PCTs compared with Group II [Figure 8].

*Figure 6: Photomicrograph of section of renal cortex of control group showing intense positive reaction for alkaline phosphatase in the proximal convoluted tubules (arrow) (Alkaline phosphatase immunostaining, × 400).*

*Figure 7: Photomicrograph of section of renal cortex of naphthalene treated group showing a noticeable reduction in alkaline phosphatase-positive reaction in some proximal convoluted tubules (arrow) (Alkaline phosphatase immunostaining, × 400).*

*Figure 8: Photomicrograph of section of renal cortex of protected group showing increased reaction for alkaline phosphatase in many proximal convoluted tubules (arrow) (Alkaline phosphatase immunostaining, × 400).*
Positive immunohistochemical staining of iNOS demonstrated brown cytoplasmic staining (index for the oxidative stress). Negative cytoplasmic staining for iNOS was found in the control group. In the NA treated group, iNOS was highly expressed in the cytoplasm of the cell lining the proximal and distal tubules. In the prophylactic group, iNOS was moderately expressed in the cytoplasm of tubular cells [Figure 9].

**Morphometric Study and Statistical Analysis**

NA treated group demonstrated a highly significant increase in the glomerular matrix index; significant increase in glomerular collagen fibers, interstitial fibrosis, and medullary fibrosis, and in the area % of iNOS immunostaining in both the cortex and the medulla when compared with the control group. The protected group demonstrated a significant reduction in these parameters when compared with the NA treated group. On the other hand, NA treated group demonstrated a highly significant decrease in area % of alkaline phosphatase of PCTS when compared with the control group. The protected group demonstrated a highly significant increase in area % when compared with the NA treated group [Table 2].

**DISCUSSION**

Kidney is a dynamic organ and represents the major control system maintaining body homeostasis. It possesses an impressive regeneration capacity, and it is the most efficient performer among all tissues in the body. It is affected by many chemicals and drugs [6].

NA is a bicyclic aromatic hydrocarbon that has wide industrial and commercial applications. It is a white crystalline solid that has aromatic odor of mothballs [19]. NA does not occur naturally in the environment, it is released into the environment due to burning organic material. Smoking cigarettes and tobacco can also release large quantities into the environment [9]. NA is rapidly absorbed into the systemic circulation following ingestion and may result in systemic toxicity [2].

Our study was designed to demonstrate the renal effect of NA exposure in adult male albino rat and to evaluate the possible protective role of rosemary.

The glomerular congestion and enlargement that was observed in this study may be attributed to structural and functional adaptations to the functioning nephrons. This causes hyperfiltration in the remnant nephrons, which is the most common feature of focal segmental glomerulosclerosis [23]. Accumulation of extracellular matrix could be due to an imbalance between its synthesis and degradation. An imbalance in this dynamic process can result in the accumulation of this matrix and even progressive glomerulosclerosis [24]. With respect to PAS staining, the extracellular matrix of NA treated group appeared to be increased in the mesangium in the glomeruli with thickened parietal layer of Bowman’s capsule in some areas. The basement membranes of the convoluted and collecting tubules appeared thickened as well. Recent advances in renal pathophysiology strongly suggest that the diffuse expansion of the mesangial region may play a critical role in the obliteration of the capillary lumen, leading to a reduction

![Figure 9: (a-f) Photomicrographs of inducible nitric oxide synthase (iNOS) immunostained sections from: (a,b) control group showing negative iNOS reaction of the tubular cell cytoplasm. (c,d) NA treated group showing highly expressed iNOS reaction in the cytoplasm of tubular cells (arrow). (e,f) protected group showing minimally expressed iNOS reaction in cytoplasm of tubular cells (arrow) (iNOS immunostaining, × 400).](image)

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<th>Table 2: Morphometric analysis in the studied groups</th>
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<td><strong>Mean±SD</strong></td>
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<td>Glomerular matrix index (area%)</td>
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<td>Glomerular fibrosis (area%)</td>
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<tr>
<td>Interstitial fibrosis (area%)</td>
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<td>Medullary fibrosis (area%)</td>
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<tr>
<td>Alkaline phosphatase immunoreactivity (area%)</td>
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<td>iNOS immunoreactivity (area%)</td>
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*P<0.05 and **P<0.001, compared with control, *P<0.05 and ***P<0.001, compared with the NA treated group, iNOS: Inducible nitric oxide synthase, NA: Naphthalene, SD: Standard deviation
in the surface area available for filtration and ultimate cessation of glomerular function in various forms of glomerulopathy [25]. On the other hand, some glomeruli were atrophied with a dilated capsular space; this was in agreement with the result obtained by other authors [26], who described this shrunken glomerulus as a sclerotic one.

Tubulointerstitial changes were present in this study such as the presence of casts in the tubular lumen. These signs suggest the presence of lipid peroxidation and the production of free radicals, which destroy the lipid and protein structure of intracellular membranes and hydrolyze the cytoplasm [27]. It has been reported that, when tubular cells die, they slough off into the tubular lumen and contribute to cast formation. The casts obstruct the tubular lumen and increase intratubular pressure, which causes a back leak of ultrafiltrate across the tubular basement membrane [28].

Cellular infiltration was observed in this study. This result is in accordance with those of previous investigators [29], who stated that NA evokes an inflammatory response in experimental animals and in humans with cell infiltration, increased cytokine production and increased capillary permeability. They added that the inflammatory response initially appears as a defense mechanism, but after that it contributes to tissue damage progression. The direct effect of NA also led to tubulointerstitial nephritis, which was manifested by inflammatory cellular infiltration, attributed by some researchers [30] to chemokine release by injured kidney cells, with subsequent interstitial fibrosis as was evident by a significant increase in area percentage of collagen, in the nephropathy group in this study, compared with the control group.

In NA treated group, connective tissue fibers appeared increased in the glomeruli, with thickened parietal layer of Bowman’s capsule in some areas. The basement membranes of the convoluted and collecting tubules appeared thickened as well. Collagen fibrils are not present in normal glomeruli but occur in the mesangium or capillary walls as a secondary event to various glomerular injuries, including some forms of glomerulonephritis, diabetic glomerulopathy, and experimental glomerular disease. It was found that the glomerular lesion may be a result of unusual production of Type-III collagen by mesangial cells [31]. This result agreed with that obtained by other authors [32], who reported that accumulation of collagen fibers within the glomerular tuft has been reported in patients with proteinuria and this is one of the characteristic features of glomerulo-sclerosis that predispose to glomerular degeneration. Moreover, recent reports [33] show that, in sclerotic lesions, few glomerular capillaries were present with mesangial matrix accumulation and collagen deposition.

The renal cortex of NA treated rats showed a noticeable reduction in alkaline phosphatase positive immunoreaction in some PCTs. These results were in agreement with previous studies which stated that, the most remarkable structural change in PCT cells was the loss of integrity and distortion of their brush border, which was evidenced by reduced PAS and alkaline phosphatase reaction. Damage to PCT cells could result in increased urinary excretion of the key brush border membrane enzyme, alkaline phosphatase [34].

Our study demonstrated that, in the NA treated group, iNOS was highly expressed in the cytoplasm of the cell lining the proximal and distal tubules. NO is considered as a proinflammatory mediator that induces inflammation because of overproduction under abnormal conditions [35]. Oxidative stress is believed to occur in a tissue or an organ when the normal balance between oxidants and antioxidants shifts in favor of oxidants, from either an excess of oxidants and/or a depletion of antioxidants [36]. To determine the role of oxidative stress in NA cytotoxicity, renal tissues were immunostained against iNOS. In this study, the iNOS activity was elevated after NA exposure, which was in agreement with the result of Belvisi et al. [37].

In the current work, the blood urea and creatinine levels were estimated to assess the extent of nephrotoxicity. All the biochemical changes in the current work were concomitant with histological changes in the renal glomeruli.

Several mechanisms for NA toxicity have been postulated: Formation of free radicals, disturbed mitochondrial metabolism and a direct toxic effect on tissue; however, the mechanism of formation of free radicals was the most accepted one [38]. NA is a potential source of free radicals [39]. The formation of these radicals is considered to be the rate-limiting step in lipid peroxidation [40]. Some researchers [41] reported that oxidation of lipids generates lipid radicals that can, in turn, initiate, and self-sustain lipid oxidation. Thus, cell membranes and basement membranes that depend on the integration of non-oxidized lipids, to maintain their orderly architecture, may be deranged, a process that could be important in the induction of glomerular proteinuria because it affects the capillary basement membrane, the main factor in glomerular filtration barrier. The generation of reactive oxygen species also leads to mitochondrial DNA damage, with subsequent respiratory chain dysfunction [42]. Thus, the delicate balance between antioxidant defenses and the production of reactive oxygen species may be disrupted, leading to oxidative insult that causes tissue damage and eventually cell death [43]. Oxidative stress, produced by NA could induce the development of nephropathy [44]. The direct acute cytotoxicity of NA is thought to be secondary to DNA intercalation, cross-linking or binding, free radical generation with subsequent induction of DNA damage and cell death by means of necrosis or apoptosis [45]. This acute cytotoxicity affects the tubular epithelial cells, which are particularly vulnerable to toxic injuries [19]; therefore, they showed structural alterations, in response to NA.

The current study revealed that rosemary aqueous extract alleviated the renal toxicity of NA. This was manifested by nearly normal appearance of kidney tissues and decreased levels of creatinine and urea. Similarly, Rašković et al. [46] reported that rosemary prevented histopathological lesions and oxidative stress induced by doxorubicin in liver, kidney and heart of mice. Rosemary was also found to have a therapeutic potential in treatment or prevention of inflammatory nephrotoxicity [47].

The biological activities of rosemary aqueous extracts are mainly ascribed to their high concentration of phenolic constituents namely carnosic and rosmarinic acids that are recognized as natural
antioxidants [48,49]. Moreover, rosemary is rich in phytochemical derivatives such as triterpenes, flavonoids or polyphenols. These polyphenols have shown biological activities in vitro as anti-tumor, chemopreventive and anti-inflammatory agents [50]. Many studies reported that the preventive effects of rosemary and its extracts are attributed to its antioxidant activity [51]. Rosemary extracts are able to donate electrons to reactive radicals, converting them to more stable and on reactive species, therefore preventing them from reaching biomolecules, such as lipoproteins, polyunsaturated fatty acids, DNA, amino acids, proteins and sugars, in susceptible biological systems [52]. Furthermore, it was concluded that rosemary extracts have a high scavenging capacity of different types of reactive oxygen and nitrogen species, mostly free radicals, is thought to be one of the main mechanisms of the antioxidant action exhibited by phenolic phytochemicals [53].

The present results showed that rosemary aqueous extract alleviates NA nephrotoxicity in albino rats.

REFERENCES


39. Akhgari M, Abdollahi M, Kebreayezadeh A, Hosseini R, Sabzevari O. Biochemical evidence for free radical-induced lipid peroxidation as...
43. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol 2003;552:335-44.

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