Malva sylvestris L. Protects from Fluoride Nephrotoxicity in Rat

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Received: 5 July 2016 **Accepted:** 6 Nov. 2016

Abstract

Background: ***Malva sylvestris*** **L. (*M. sylvestris*) has antioxidant property and is widely used in the traditional medicine to treat gastrointestinal, respiratory, skin and urological disorders.**

Objective: In this study, the protective effect of *M. sylvestris* against sodium fluoride-induced nephrotoxicity in the rat was evaluated.

Methods: The *M. sylvestris* flower extract was prepared and injected intraperitoneally at the doses of 100, 200, 400 mg/kg/day to rat groups (10 in each group) for 1 week and subsequently 600 ppm sodium fluoride was added to the rats drinking water for 1 additional week. After these steps, the rat serum levels of urea, creatinine, reduced glutathione, catalase and malondialdehyde were determined. The histopathology of the rats' kidney was also studied. In this study, vitamin C (10 mg/kg/day) was used as positive control.

Results: Sodium fluoride administration increased levels of BUN, creatinine, glutathione, catalase activity and decreased malondialdehyde levels indicating induction of nephrotoxicity in the rats. *M. sylvestris* extract pretreatment significantly decreased the BUN and creatinine levels ($P<0.05$). Catalase activity and glutathione levels were significantly increased by *M. sylvestris* ($P<0.05$). All three doses of the *M. sylvestris* decreased the malondialdehyde level, but it was significant only for the dose of 400 mg/kg/day ($P<0.05$). The *Malva sylvestris* effects were comparable with those of vitamin C. Histopathological findings also showed protective effects of *M. sylvestris* against the renal damage induced by sodium fluoride.

Conclusion: The results suggest that *M. sylvestris* has protective effects against sodium fluoride-induced nephrotoxicity which maybe mediated by the antioxidant activity of the plant flavonoids.

Keywords: ***Malva sylvestris*****, Nephrotoxicity, Sodium fluoride, Rat**

Introduction

Fluoride is widely distributed in the environment and can cause fluorosis due to excessive fluoride intake. Besides its well-known effects on the skeleton and teeth, fluorosis can also damage soft tissues such as the liver and kidneys. The oxidative stress is an important mechanism of the fluoride toxicity [1]. Sodium fluoride has been used in different studies to induce nephrotoxicity [2]. Sodium fluoride produces high levels of oxygen and nitrogen species leading to increased oxidative stress which induces renal toxicity [3, 4]. Since the kidneys are the major routes of fluoride excretion, kidneys are important target for fluoride toxicity. Antioxidant agents by modulating oxidant-antioxidant balance can prevent renal toxicity of oxidants

Malva sylvestris (*M. sylvestris*) L. from the family Malvaceae is native to Europe, North Africa, Asia and the Mediterranean regions [5]. The plant has a long history of use in the traditional medicine for the treatment of various diseases including urinary system diseases [6-7]. All parts of the plant are used as a pharmaceutical material, but the flowers and leaves have more applications. In a study, the effectiveness of the plant extract in the treatment of peptic ulcer was compared with that of cimetidine [8]. Recently, the hepatoprotective effect of *Malva sylvestris* was also established [9]. Moreover, the plant protective effect against renal toxicity of vanadium has been reported [10]. Previous studies indicate that the *M. sylvestris* flowers contain mucilage, flavonoids, tannins, phenolics and anthocyanins [5]. Recent studies have shown that the plant has a high antioxidant activity and its biological effects may be mediated by substances such as the vitamins C and E, β carotens and phenolic substances [11-13]. Research also has shown that the best sources of natural antioxidants are phenolic compounds found in plants [14-15]. Nowadays, it is important to find natural antioxidants with fewer side effects. The efficacy of *M. sylvestris* for the prevention of fluoride toxicity has not been evaluated so far. Thus, considering the plant antioxidant activity, previous reports indicating nephroprotective and hepatoprotective effects of the plant and nephrotoxicity of fluoride even at low doses, the present study was conducted to evaluate the protective effect of this plant against sodium fluoride-induced nephrotoxicity in the rat.

Materials and Methods

Chemicals: In this study, sodium fluoride, ethanol, formaldehyde and diethyl ether, acetic acid, sodium acetate, butanol, formic acid, acetonitril, methanol, NaH_2pO_4, H_2sO_4, $KmnO_4$ all from the Merck Company (Germany) and vitamin C from the Daroupaksh Company (Iran) were used. All solutions were prepared using de-ionized water.

***M. sylvestris* extract preparation:** The *M. sylvestris* flowers were collected from the lands of the Iran's Yazd Province (Taft City) in July, identified by the botanist Dr. Yousef Ajanii and the plant voucher specimen (number 168) was deposited in the herbarium of the Pharmacy Faculty of the Yazd

University of Medical Sciences. The plant extract was prepared in collaboration with the Institute of Medicinal Plants by the following method. After carefully weighing the plant powder, extraction was performed with percolator using ethanol: water (80:20) as solvent. The extract was filtered and concentrated by rotary evaporator and then dried [16]. The extraction yield was 5 percent.

Extract Standardization: The extract total flavonoid content was measured by the spectrophotometric method described previously [16].

Animals: In this study, 60 adult male Wistar rats weighing 200 to 250 grams were used. During the study, the rats were given water and food *ad libitum*. The animal house had temperatures between 20°C and 25 °C, and 12 hours of light and dark cycle. Experiments were conducted on animals in accordance with the rules of the Yazd University of Medical Sciences Ethics Committee and the national guidelines.

Induction of nephrotoxicity: Nephrotoxicity was induced as described previously [17-18]. Sodium fluoride was added at the concentration of 600 ppm to the rats' drinking water for 1 week. The volume of water containing sodium fluoride consumed daily by each rat was determined. There was no significant difference between the rats in terms of water intake. Moreover, the levels of blood urea nitrogen (BUN) and creatinine were significantly increased in the rats with nephrotoxicity compared to the normal rats (50.33 ± 1.28 mg/dL and 0.61 ± 0.04 mg/dL *vs.* 36 ± 0.44 mg/dL and 0.43 ± 0.02 mg/dL), indicating induction of nephrotoxicity by sodium fluoride (Table 1). Also, the kidney histopathological changes showed nephrotoxicity (Figure 4).

Evaluation of the plant effects: Rats were divided to six groups, each consisting of 10 rats. The first group was as control and only received 1 mL/kg of normal saline intraperitoneally for 7 days. The second group only received sodium fluoride in drinking water. The third group received sodium fluoride and vitamin C as positive control group. The fourth, fifth and sixth groups received oral sodium fluoride and pretreated with intraperitoneal doses of 100, 200 and 400 mg/kg of *M. sylvestris* extract respectively for 7 days. Injection volume for all rats was 1 ml/kg body weight [9, 10].

Blood samples were taken from the eye, after rats were anesthetized with diethyl ether. Serum samples were prepared by centrifuging the blood at 12000g for 10 minutes and stored at -20 ^{O}C until biochemical assays. Kidney was removed and preserved in formalin for histological studies. Serum creatinine and BUN levels were determined by the commercially available kits in the central laboratory of the Yazd University of Medical Sciences

Serum glutathione level determination: The reduced glutathione level was determined by the Tietze F method. Briefly, 730 mL of the 0.3 mol/L solution of Na2HPO4 and 90 µL of 40 mg/dL 5, 5'-dithio-bis (2-nitrobenzoic acid) DTNB (dissolved in 1 g/dL sodium citrate) as substrate and 180 µL of rat serum were used and the absorbance changes of the above solution were determined at 412 nm for 4 minutes, then the cellular glutathione levels in terms of

μmol/mg of protein were determined using a calibration curve [19].

Serum malondialdehyde (MDA) level determination: The product of lipid peroxidation as MDA was measured using the thiobarbituric acid method. The lipid peroxidation value was determined by measuring the thiobarbituric acid (TBA) reaction with MDA formed during hydrolysis of lipid peroxidase using the modified Uchiyama & Mihara method. Thereafter, 0.1 mL of the serum and 0.375 mL of the 20% acetic acid and 0.375 mL of the 0.6 percent thiobarbituric acid solution were mixed and afterward heated in boiling water bath for 60 minutes. Then, it was placed in cold water for 10 minutes and 1.25 mL of normal butanol /pyridine 1:15 was added to it and centrifuged for 5 minutes at a speed of 2000 rpm. Afterward, the butanol phase absorption was measured at 532 nm. 1,1,3,3-tetra ethoxy propane was used as standard in the same way and MDA concentration was reported as μmol/mL of the sample [20].

Serum catalase activity determination: The catalase activity was measured by determining the amount of H_2O_2 hydrolysis by the enzyme. In this method, H_2O_2 was added to a test tube containing the sample and distilled water and the standard solution of H_2O_2 were used as control. After 3 minutes incubation, enzymatic reaction was stopped by addition of H_2SO_4 and after adding 1.4 mL of $KMnO_4$ to each tube, the absorption was measured at 480 nm [21].

Histological evaluation of the kidney samples: After removing one kidney of each animal, it was fixed in 4% formaldehyde solution for two days. Then, the kidney was divided into two hemispheres and one of the hemispheres was used for the study. Dehydration of tissues was performed by increasing the concentrations of ethanol and then paraffin blocks were prepared. In order to perform histological analysis of the tissue, serial sections of the kidney tissue with thickness of 5 microns were prepared and stained with hematoxylin-eosin. Finally, the samples were examined under an optical microscope at the 400x magnification [22].

Statistical analysis: The one-way analysis of variance followed by the Tukey *post hoc* test was used for data analysis. $P<0.05$ was considered as significant.

Results

Extract Standardization: The extract contained 204.95 ± 2.9 mg flavonoids/g extract.

Effects of *M. sylvestris* on the serum BUN and creatinine levels: Table 1 shows changes of the BUN and creatinine levels in different groups of sodium fluoride-induced nephrotoxic rats pretreated with the *M. sylvestris* flower extract (injected i.p.). Administration of sodium fluoride 600 ppm for 1 week in the drinking water of the rats caused about 28% increase in the BUN level (from 36±0.44 to 50.33 ±1.28) which was statistically significant ($P<0.001$). The changes observed in the creatinine level were also paralleled by the rise in the BUN level. Creatinine increased from the baseline level of 0.43±0.02 mg/dL to 0.61±0.04 mg/dL which was also statistically significant ($P<0.001$). The extract pretreatment decreased the BUN

Table 1- Effects of *M. sylvestris* pretreatment (i.p. injection) on the serum BUN and creatinine levels (mg/dL) in the sodium fluoride-induced nephrotoxic rats

Groups	BUN (mg/dL)	Creatinine (mg/dL)
Normal	36±0.44	0.43±0.02
Control	50.33±1.28**	0.61±0.04**
Vitamin C	42.73±1.83*	0.51±0.01*
M. sylvestris 100 mg/kg	47.83±1.83*	0.58±0.01
M. sylvestris 200 mg/kg	45.16±1.35*	0.55±0.01*
M. sylvestris 400 mg/kg	42±.21*	0.51±0.02*

The BUN and creatinine levels were measured in groups of sodium fluoride-induced nephrotoxic rats (n=10) injected intraperitoneally with 100, 200 and 400 mg/kg/day of the *M. sylvestris* extract for one week. The normal groups only received vehicle (ethanol 80%) and were not treated with sodium fluoride or the extract. The control group only received sodium fluoride (600 ppm) in drinking water for one week. The extract was administered, before sodium fluoride treatment, for one week. Vitamin C was used as positive control and was injected i.p. (10 mg/kg/day) for one week. The data are given as mean ± S.E.
*P<0.05 compared with the control group. **P<0.001 compared with the normal rats.

and creatinine levels dose-dependently which was also statistically significant except the effect of the dose 100 mg/kg of the extract on the creatinine level which was not statistically significant (P> 0.05). The change in the levels of both BUN and creatinine caused by the dose of 400 mg/kg of the extract was similar to the effect of vitamin C which was applied in this study as positive control but these values were significantly more than those observed in the normal rats.

Effects of *M. sylvestris* on the serum catalase activity: As shown in the Figure 1, one week sodium fluoride treatment by adding it (600 ppm) in the drinking water caused about 75% decrease in the serum catalase activity compared with the normal rats. Pretreatment of the sodium fluoride-intoxicated rats with three doses of the *M. sylvestris* extract caused a dose-dependent increase in the catalase activity compared with the control sodium fluoride-intoxicated rats. Increase of the catalase activity by all doses of the extract was statistically significant (P<0.05) compared with the control sodium fluoride-intoxicated rats. The effect produced by vitamin C (10 mg/kg) on catalase activity was less than the effect produced by the 400 mg/kg dose of *M. sylvestris*. But, catalase activity even in the group treated with 400 mg/kg of the extract was about 25% less than that in the normal rats.

Effects of *M. Sylvestris* on the serum glutathione levels: As shown in the Figure 2, one week sodium fluoride treatment by adding it (600 ppm) to the drinking water caused about 60% decrease in the serum glutathione levels. Pretreatment of sodium fluoride-induced nephrotoxic rats with 100, 200, and 400 mg/kg of the *M. sylvestris* extract increased the serum glutathione levels compared with the control groups (P<0.05) which was significant at the doses of 200 and 400 mg/kg but the effect produced by the dose of 100 mg/kg were not statistically significant (P>0.05). The effect of *M. sylvestris* extract on the serum glutathione levels were dose-dependent. The effect produced by vitamin C (10 mg/kg) on the GSH level were comparable with the effect produced by the 400mg/kg dose of *M. sylvestris.*

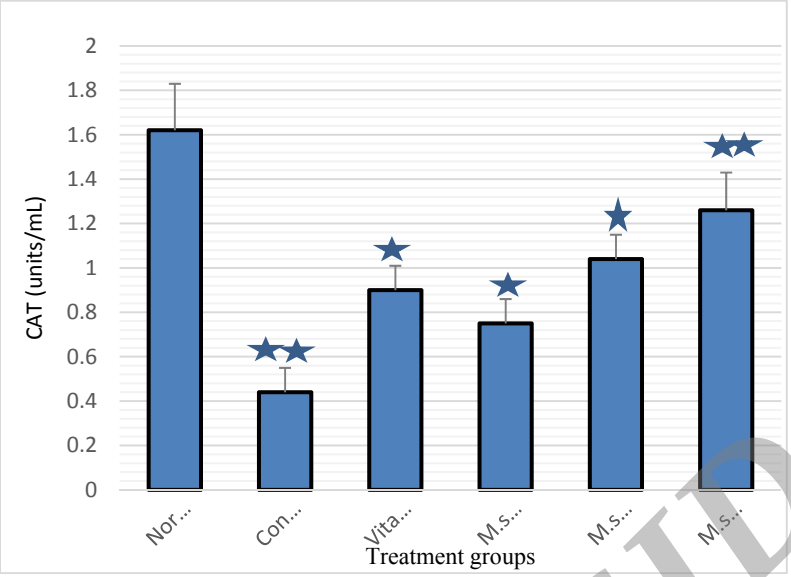

Figure 1- Effects of the *M. sylvestris* extract on the serum catalase activity in the sodium fluoride intoxicated rats. Catalase activity was measured in the sodium fluoride-induced nephrotoxic rats (n=10 in each group) receiving 100, 200 and 400 mg/kg of the extract intraperitoneally. Normal groups received only vehicle (ethanol 80%) and were not treated with sodium fluoride or the extract. The control group was only treated with sodium fluoride 600 ppm in drinking water for one week. Vitamin C was administered i.p. with the dose of 10 mg/kg. The extract was administered, before sodium fluoride intoxication, for one week. The data are presented as mean ± S.E.

☆ *P*<0.05 compared with the control group.

☆☆ *P*<0.05 compared with the normal group.

☆☆☆☆ *P*<0.05 compared with the control group.

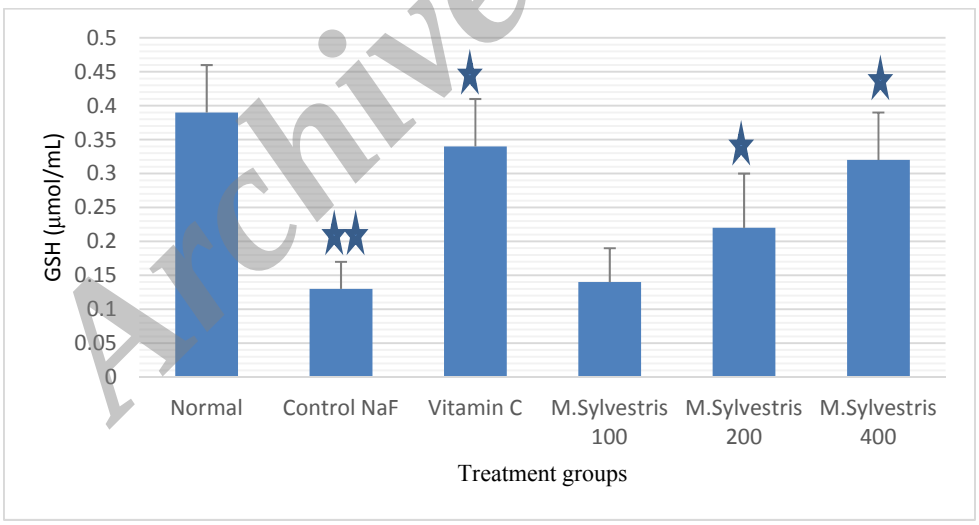

Figure 2- Effects of the *M. sylvestris* extract on the serum reduced glutathione levels of the sodium fluoride-induced nephrotoxicity rats. Glutathione was measured in the sodium fluoride-induced nephrotoxic rats (n=10 in each group) receiving 100, 200 and 400 mg/kg of the extract intraperitoneally. Normal groups received only vehicle (ethanol 80%) and were not treated with sodium fluoride or the extract. The control group was only treated with sodium fluoride 600 ppm in drinking water for one week. Vitamin C was administered i.p. with dose of 10 mg/kg. The extract was administered, before sodium fluoride intoxication, for one week. The data are presented as mean ± S.E.

☆ *P*<0.05 compared with the control group.

☆☆ *P*<0.05 compared with the normal group.

Effects of *M. sylvestris* on the serum malondialdehyde (MDA) levels: As shown in the Figure 3, the MDA levels in the sodium fluoride-induced nephrotoxic rats were about 17 µmol/mL more than those in the normal rats. The MDA increased in the sodium fluoride-induced nephrotoxic groups to levels about 55% more than those of the normal rats which was statistically significant (P<0.001). Pretreatment of sodium fluoride-induced nephrotoxic rats with three different doses of the *M. sylvestris* extract decreased the MDA levels compared with the control group. But, the MDA level decrease was statistically significant only at the doses of 200 and 400 mg/kg (P<0.05) compared with the control sodium fluoride-induced nephrotoxic rats. The MDA levels in the group treated with the dose of 400 mg/kg were about 25% more than those in the control sodium fluoride-induced nephrotoxic rats. The effect produced by vitamin C (10 mg/kg) on the MDA level were comparable with the effect produced by the 200 mg/kg dose of *M. sylvestris.*

Effects of *M. sylvestris* on the histopathology of kidney: Figure 4 shows the micrographs of the tissues prepared from the kidneys of all groups receiving different treatments. As observed in this figure, in the normal group which only received vehicle (ethanol 80%), the kidney tissues did not have any pathological changes (Figure 4A).

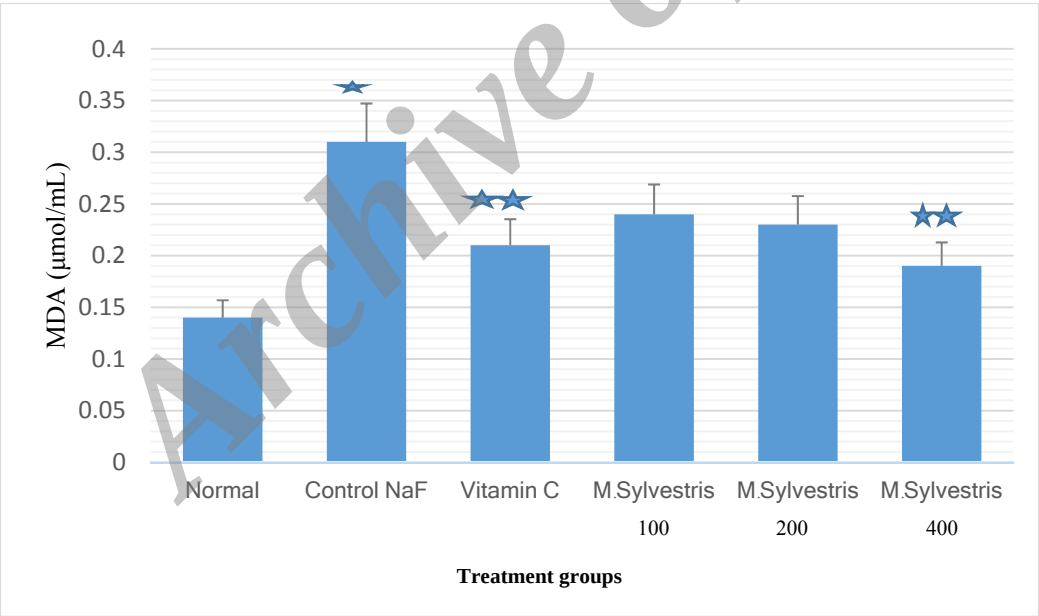

Figure 3- Effects of the *M. sylvestris* extract on the serum malondialdehyde (MDA) levels of the sodium fluoride-induced nephrotoxic rats. The MDA levels were measured in the groups of sodium fluoride-induced nephrotoxic rats (n=10) receiving 100, 200 and 400 mg/kg of the extract. The normal group received only vehicle (ethanol 80%) and was not treated with sodium fluoride or the extract. The control group was treated only with sodium fluoride 600 ppm in drinking water for one week. Vitamin C was administered i.p. with the dose of 10 mg/kg. The extract was administered, before sodium fluoride treatment, for one week. The data are presented as mean ± S.E.

☆ ***P*<0.05 compared with the normal group.**

☆☆ ***P*<0.05 compared with the control group.**

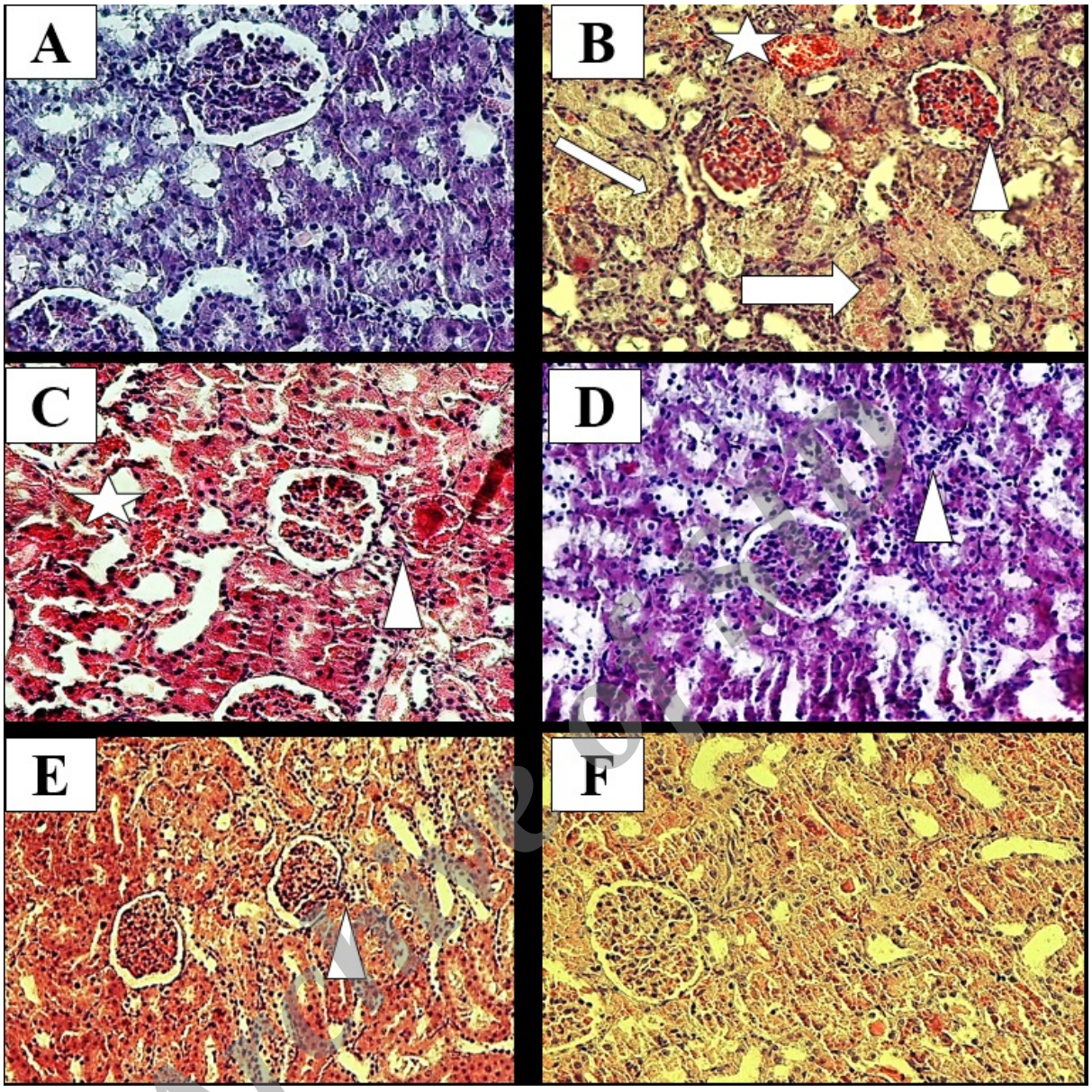

Figure 4- The micrographs of the kidney tissue from the sodium fluoride–induced nephrotoxic rats treated with different doses of the *M. sylvestris* extract. Different groups of the rats (A) the control group without sodium fluoride or the extract treatment; (B) the group receiving sodium fluoride 600 ppm; (C) the group receiving sodium fluoride plus 100 mg/kg of the extract; (D) the group receiving sodium fluoride plus 200 mg/kg of the extract; (E) the group receiving sodium fluoride plus 400 mg/kg of the extract; (F) the group receiving sodium fluoride plus 10 mg/kg of vitamin C (Zoom: 400x).

In the control group not receiving the extract and only treated with sodium fluoride added in the drinking water for 7 days, proximal and distal tubular necrosis (thin arrow), destruction of the proximal tubular epithelial cells and congestion of the glomerular inflammation (arrowhead) and blockage of the tubular lumen (thick arrow) as well as congestion and tissue vacuolization and cell infiltration (star) of the inflamed kidney tissue were observed (Figure 4B). The kidney histopathology of the vitamin C pretreated rats (Figure 4C) was similar to

that of normal rats. Pretreatment of the rats receiving sodium fluoride for nephrotoxicity induction with different doses of the extract injected intraperitoneally decreased the pathological changes dose-dependently (Figure 4CDE). In other words, the extract pretreatment reduced renal tubular tissue changes induced by sodium fluoride and the dose of 400 mg/kg/day was more effective than the doses of 100 and 200 mg/kg in this respect.

Discussion

The aim of the present study was to investigate the protective effects of the *M. sylvestris* extract on the rat model of sodium fluoride-induced nephrotoxicity. In this study, administration of sodium fluoride in drinking water for one week caused a significant increase in the serum BUN and creatinine levels as well as pathological kidney changes, indicating induction of nephrotoxicity in the rats. Furthermore, sodium fluoride significantly increased the MDA level and decreased the glutathione level and catalase activity, indicating induction of oxidative stress. The extract pretreatment with the doses of 100, 200 and 400 mg/kg/day decreased the BUN and creatinine levels siginificantly ($P< 0.05$) and dose-dependently. Pretreatment with all doses of the extract produced dose-dependent effects on the oxidative stress parameters of glutathione, catalase and malondialdehyde. While, the extract effects on the catalase activity were significant ($P<0.05$) at all the three doses, the extract effects on the glutathione and malondialdehyde levels were significant only at the doses of 200 and 400 mg/kg/day ($P<0.05$). The fluoride ion increases the MDA levels and decreases the catalase activity and glutathione levels, leading to increased oxidative stress and serious damage especially in the biological membrane structure and cell activities [23-24]. It has been reported that sodium fluoride intoxication causes renal damage including tubular damage, fibrosis, edema of the interstitial cells, inflammation and glomerular congestion in the rats [3]. According to a study, sodium fluoride can cause glomerular inflammation, hypertrophy and atrophy of the kidney [24]. In the present study, sodium fluoride produced proximal and distal tubular necrosis, destruction of the proximal tubular epithelial cells and glomerular inflammation. The *M. sylvestris* extract pretreatment reduced the pathological renal changes induced by sodium fluoride and the nephroprotective effects of the *M. sylvestris* extract were dose-dependent. Vitamin C, a strong antioxidant, applied as positive control in this study had a protective effect against the fluoride-induced pathological changes in the kidney which was similar to the effects of the *M. sylvestris* extract. The results of the present study also demonstrate that the *M. sylvestris* extract effects at the dose of 400 mg/kg (i.p.) are comparable with the effects produced by vitamin C (10 mg/kg, i.p.). The protective effect of the phenolic compound gallic acid and the flavonoids curcumin and quercetin against sodium fluoride-induced nephrotoxicity through antioxidant effects have been demonstrated in the rats [17-18]. A previous study has also implicated the

antioxidant activity of the *M. sylvestris* phenols in the plant protective effect against vanadium-induced nephrotoxicity in the rat [10]. The protective effect of a flavonoid-rich substance called MPPP (maize purple plant pigment) against sodium fluoride nephrotoxicity has been attributed to the antioxidant activity in mice [25]. Therefore, the results of the present study accord with these studies. Moreover, the antioxidant activity of the high flavonoid content of the extract may be involved in the protective effect of the plant against the sodium fluoride nephrotoxicity. Finally, the bioactive compounds involved in the nephroprotective effect of *M. sylvestris* should be identified. Moreover, further studies particularly clinical trials regarding the nephroprotective effect of *M. sylvestris* are warranted.

Conclusion

M. sylvestris prevents sodium fluoride-induced nephrotoxicity in the rat through the antioxidant activity of the plant flavonoids.

Acknowledgement

This study was conducted as thesis for doctor of pharmacy degree and supported by the ACECR Institute of Medicinal Plants and the Yazd University of Medical sciences.

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