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Protective effect of *Ferula gummosa* hydroalcoholic extract against nitric oxide deficiency-induced oxidative stress and inflammation in rats renal tissues

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Abstract

Nitric oxide (NO) synthase inhibition increases hypertension and causes renal injury. *Ferula gummosa* is used in Iranian traditional medicine for treatment of several diseases and has been reported to exert a potent anti-inflammatory and antioxidant action. The aim of this investigation was to evaluate the renoprotective effects of hydroalcoholic extract of *Ferula gummosa* (HEG) on Nω-nitro-L-arginine methyl ester (L-NAME)-induced oxidative stress and inflammation and explore the mechanisms that link NO deficiency with altered renal heat shock protein (HSP70). Rats were injected intraperitoneally with L-NAME (10 mg/kg) to induce renal injury. Simultaneously, HEG (90 mg/kg) was administered by gastric gavage to L-NAME-treated rats for 6 days/week during an 8-week period. Renal thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), HSP70, plasma NO and total antioxidant capacity (TAC) were evaluated. The administration of L-NAME significantly increased renal TBARS, TNF-α, IL-6, HSP70 levels and decreased renal SOD activity, that these changes were accompanied by the reduced plasma NO and TAC levels. HEG administration decreased TBARS, HSP70, TNF-α and IL-6 levels and increased SOD activity in the kidney tissues of L-NAME treated rats ($p < 0.05$). Also, plasma TAC level and NO bioavailability have been elevated after administration of HEG ($p < 0.05$). These findings support that NO deficiency induces renal stress oxidative and inflammation, which markedly increased renal HSP70 and HEG could protect kidney against these damaging effects via its anti-oxidative, anti-inflammatory action and modulate renal HSP70.

Keywords

Antioxidant, nitric oxide, pro-inflammatory cytokines, stress protein

History

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Introduction

Hypertension, is known as a major universal health problem due to its high prevalence and concomitant complications of cardiovascular and kidney disease (1). Essential hypertension is a consequence of pressure natriuresis malfunction, which can be induced through a relative impairment in sodium excretion by the kidney. Renal injury such as afferent arteriole arteriolosclerosis and the loss of peritubular capillaries plays an important role in mediating the impaired pressure natriuresis (2). Growing evidence suggests that renal oxidative stress is critically involved in the initiation and progress of hypertension (3). Oxidative stress can also increase afferent arteriolar tone, decrease endothelium relaxation, enhance inflammation and lipid peroxidation and down regulate the antioxidant defense systems, such as antioxidant enzyme activities in the renal tissues (2).

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In addition, chronic low-grade inflammation is one of the hallmarks of hypertension (3). Several studies have shown that renal proinflammatory cytokines (PICs), such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), are increased in hypertensive rats (4,5). TNF-α and IL-6 are cytotoxic for renal cells and could induce renal injuries such as renal hypertrophy, sodium retention and alterations in endothelial permeability (6). Therefore, a part of the endothelial dysfunction and hypertension-induced renal damage may be mediated by oxidative stress and proinflammatory cytokines (7).

It is well documented that the immune system and the invasion of lymphocytes, macrophages and T cells have important roles in hypertension-induced pathophysiological changes in the renal tissues (5,8,9). Heat shock proteins or stress proteins are induced in response to the wide variety of adverse environmental factors and unfavorable physiological conditions in all of the cells. Among the most prominent proteins, the inducible HSP70 family members are up-regulated under the heat stress and toxic chemicals (10). Intracellular HSP70 members play essential roles as

molecular chaperones under both normal and stressful conditions through folding of nascent proteins, refolding denatured proteins and re-solubilizing protein aggregates. However, the exact role of renal HSP70 overproduction in autoimmunity induction and/or tissue protection in hypertension conditioning is not yet fully understood (11). Due to these paradoxical and contradictory effects of HSP70, optimal modulation of intracellular HSP70 concentration levels will be potential drug targets in inflammation (12).

Recently, the use of natural antioxidant agents, especially those derived from plants, in the mitigation and/or treatment of hypertension and renal injuries has been increased (13). *Ferula gummosa* Boiss is a perennial plant which is native to central Asia, also called ''Barijeh'' by the nomads of southwest Iran. The gum obtained from the aerial parts of this plant was used in Iranian folk medicine for the treatment of many diseases such as stomachache, chorea, epilepsy and even as a poultice to heal wounds (14). Several compounds such as gumosin (a sesquiterpene coumarin), gumosides (sesquiterpene coumarin glycoside), cauferoside, feselol, conferoside, ferilin, ferocaulidin, ligupersin A, conferol, daucosterol and acantrifoside E and 4-hydroxybenzoic acid 4-(6-*O*-sulfo)glucopyranoside were reported from *Ferula gummosa* root and could be responsible of protective properties (15). In fact, anti-inflammatory and antinociceptive activities of flowers, stem and leaf extracts of *Ferula gummosa* have also been previously reported (14). There is negligible report on renoprotective activity and possible mechanisms herbal plant. Long-term L-NAME administration and blockade of NO synthesis causes systemic hypertension and glomerular injury which is largely utilized as a model for study hypertension-induced renal injuries (16,17).

The aim of this study was to investigate the protective role of the hydroalcoholic extract of *Ferula gummosa* against Nω-nitro-L-arginine methyl ester (L-NAME)-induced oxidative stress and inflammation through the measurement of malondialdehyde (MDA) levels as an index of lipid peroxidation, superoxide dismutase activity (SOD), HSP70 and proinflammatory cytokines (IL-6 and TNF-α) in renal tissues of the rats. In addition, the change of plasma nitric oxide (NO) and total antioxidant capacity (TAC) levels were also assessed.

Materials and methods

Chemicals

L-NAME, ketamine, xylazine, NaCl, Tris–HCl, NP40, glycerol, PMSF, leupeptin, sodium vanadate, AEBSF, tetrazolium salt, tripyridyltriazine 1,1,3,3-tetra-ethoxypropane, were obtained from Sigma Chemical Company (St. Louis, MO) and Kits for estimation of HSP70, IL-6, TNF-α were purchased from R&D Systems (Minneapolis, MN). Also, kites for determination of SOD and TBARS were purchased from Cayman Chemical Company (Ann Arbor, MI) and Randox kits (Randox Laboratories, Crumlin, UK) were used for determination of TAC.

Preparations of hydroalcoholic extract of *Ferula gummosa*

Aerial parts of *Ferula gummosa* were collected from Central Elburz Mountains, north of Iran. The samples were dried at room temperature and then powdered. Plant powder (100 g) was extracted with ethanol/water (70/30 v/v) three times at 25 °C for 24 h by the percolation method (18). The extract was filtrated with Whatman No. 1 filter paper. The solvents were removed by rotary vacuum for the preparation of crude plant extract.

Animals

Male Wistar rats (8 weeks old, initially weighing 200–250 g) were used throughout the study and were obtained from the Pasture Institute of Iran (Tehran, Iran). All procedures were performed according to institutional guidelines procedures in the Care and Use of Animals.

Rats were housed in the cages of polycarbonate ($20 \times 15 \times 15$) under a constant temperature (22 ± 2 °C) with a 12-h light/dark cycle and $60 \pm 5\%$ humidity. A standard laboratory feed manufactured by the Pasture Institute of Iran was used while water was provided *ad libitum*.

Animal and experimental design

Following 1-week familiarization with lab environment, the animals ($n = 40$) were divided into four groups, each of which contained 10 rats. The first group (control) did not receive any treatment. The second group (sham) was daily injected with a physiological salt (1 ml/kg body weight, i.p.) during 8 weeks. The third group (L-NAME) was injected with L-NAME (10 mg/kg body weight, i.p.) for 6 days/week during 8 weeks. The fourth group (*Ferula gummosa* group) was treated with L-NAME as explained above and was simultaneous treated with *Ferula gummosa* extract (90 mg/kg body weight, through gavage) 6 days/week during 8-week period.

After the last application (48 h) and after overnight fasting (12 h), the animals were sacrificed under anesthesia by intraperitoneally administration of the mixture of ketamine (60 mg/kg) and xylazine (5 mg/kg). The blood samples were collected through cardiac puncture and then centrifuged at 3000 rpm for 15 min (at −4 °C) for measurement of nitrite/nitrate and TAC levels. Kidneys were carefully removed, cleaned of excess fat, and quickly frozen in liquid nitrogen and stored at −70 °C until biochemical analyses.

Preparation of tissue homogenate

The frozen kidney was ground to a powder and then homogenized in ice-cold lysis buffer (PBS, pH 7.4) containing 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM PMSF, leupeptin (1 μg/ml), sodium vanadate (0.5 mM, AEBSF (100 mg/ml) and centrifuged (12 000 rpm, 30 min, 4 °C). Supernatants were removed and used for assays (19).

Measurement of renal IL-6, TNF-α, HSP70, MDA and SOD

Renal IL-6 and TNF-α were measured using Rat ELISA kit (sensitivity 5.3 pg/ml, Intra CV%: 6.1 and 5.2, respectively, Assaypro Co., Brooklyn, NY) from R & D Systems according to the manufacturer's instructions. Enzyme immunoassay (ELISA) was performed for HSP70 according to the manufacturer's manual (R&D Systems, Rat Eliza kit, Assaypro

Co.). The lower detection limits were 15.6 pg/ml (Intra CV%: 7.3). Superoxide dismutase (SOD) activity was measured spectrophotometrically in renal tissue homogenate by the inhibition rate of a tetrazolium salt (water-soluble tetrazolium salt-1; WST-1) using a SOD assay kit (Cayman Chemical Co.) following the manufacturer's manual instructions. Enzyme activity was reported as U/mg protein. One unit SOD is defined as a point where a sample gives 50% inhibition of a colorimetric reaction between superoxide anion and reactive dye (WST-1) (20). Thiobarbituric acid reactive substances (TBARS), as a marker of lipid peroxidation index, react with thiobarbituric acid (TBA) and produce a red colored complex that has a peak absorbance at 532 nm. The levels of TBARS were measured by TBARS kit (Cayman Chemicals Co.) and using 1,1,3,3-tetra-ethoxypropane as a standard in accordance with the method previously described (21).

Measurement of plasma nitrite/nitrate and TAC levels

Plasma TAC was measured using a commercially available kit (Randox Laboratories, Crumlin, UK) method. The plasma concentration of NO, due to its rapid oxidization into nitrate and nitrite, was determined by converting the nitrate to nitrite using the Griess reagent (9).

Statistical analysis

Data are expressed as mean ± SEM. Statistically significant differences among groups were tested using one-way analysis of variance (ANOVA) and followed by Tukey–Kramer *post hoc* test for multiple comparisons. The p values <0.05 were considered statistically significant.

Results

No significant differences in body and kidney weights were observed between all animal groups at the end of the experimental period (Table 1).

Renal TBARS levels were significantly increased in L-NAME-treated rats compared to sham and control groups ~21% and 27%, respectively ($p<0.05$). The treatment with *Ferula gummosa* extract significantly reduced lipid peroxidation index as it was evidenced by a decrease in the TBARS levels from both sham (25%, $p<0.05$) and L-NAME (38%, $p<0.001$) groups (Figure 1A). Moreover, the L-NAME-administered rats had a decrease in activity of the enzyme SOD in renal tissue homogenate compared to sham and control groups (24% and 27%, respectively, $p<0.001$), and this alteration was markedly reversed by simultaneous administration of *Ferula gummosa* extract (Figure 1B).

The IL-6 and TNF-α levels in the rats of the L-NAME-treated group were higher than sham and control groups (82% and 91% for IL-6 and 104% and 105% for TNF-α, $p<0.001$) and *Ferula gummosa* treatment attenuated this increase, although did not completely reestablish the normal values (Figure 2A and B).

As shown in Figure 3, chronic L-NAME administration increased HSP70 levels in the kidney tissue homogenate compared to the sham (138%) and control (127%) groups, respectively ($p<0.001$). The administration of *Ferula gummosa* appeared to cause a reduction in the elevated renal HSP70 levels (42%, $p<0.001$), although it was still significantly higher than both the sham group and the control group ($p<0.05$).

Plasma TAC was significantly reduced in the L-NAME-treated group when compared to sham and control groups (26%, $p<0.001$). As it can be seen in Table 2, *Ferula gummosa* extract administration restored the plasma antioxidant capacity towards normal levels ($p<0.001$).

Nitrite/nitrate levels of plasma were significantly decreased in the rats exposed to chronic L-NAME administration when compared to the sham (45%) and control (47%) groups. The *Ferula gummosa* treatment increased plasma nitrite/nitrate levels in rats (47%), although this difference was not statistically significant (Table 2).

Discussion

In recent years, a general interest about the use of herbal extracts for the treatment of different types of renal injuries has been developed (13). There are several treatment

Table 1. Effect of *Ferula gummosa* extract on body and kidney tissue weights during chronic L-NAME administration in rats.

Variables	Control	Sham	L-NAME	*Ferula gummosa*
Body weight (g)	375 ± 20	400 ± 13	387 ± 18	380 ± 11
Kidney weight (g)	1.32 ± 0.07	1.29 ± 0.04	1.35 ± 0.07	1.36 ± 0.07

Values are expressed mean ± SEM for 10 rats. L-NAME, Nω-nitro-L-arginine methyl ester.

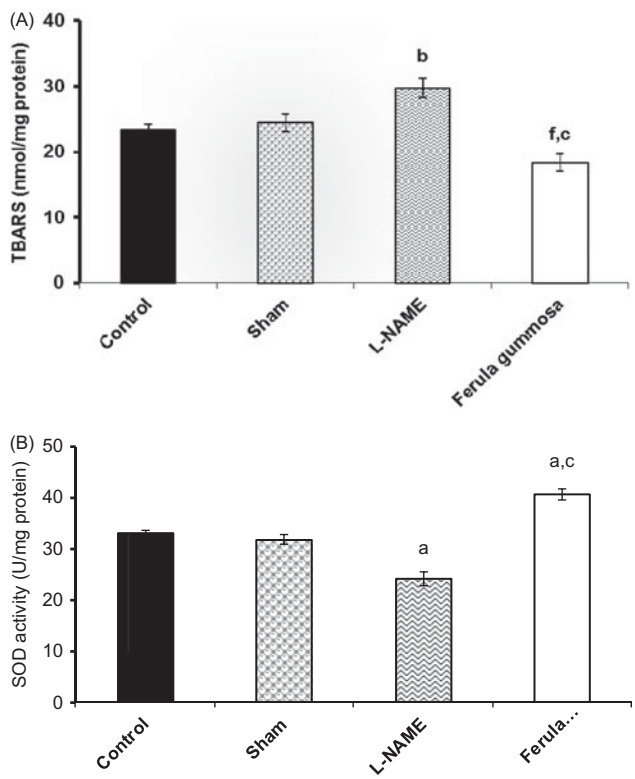

Figure 1. Effect of *Ferula gummosa* extract on renal TBARS levels (A) and SOD activity (B) during chronic L-NAME administration in rats. [a]Statistical significant at $p<0.001$ versus control and sham groups, [b]Statistical significant at $p<0.05$ versus control and sham groups, [c]Statistical significant at $p<0.001$ versus L-NAME group, [f]Statistical significant at $p<0.05$ versus sham group. SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

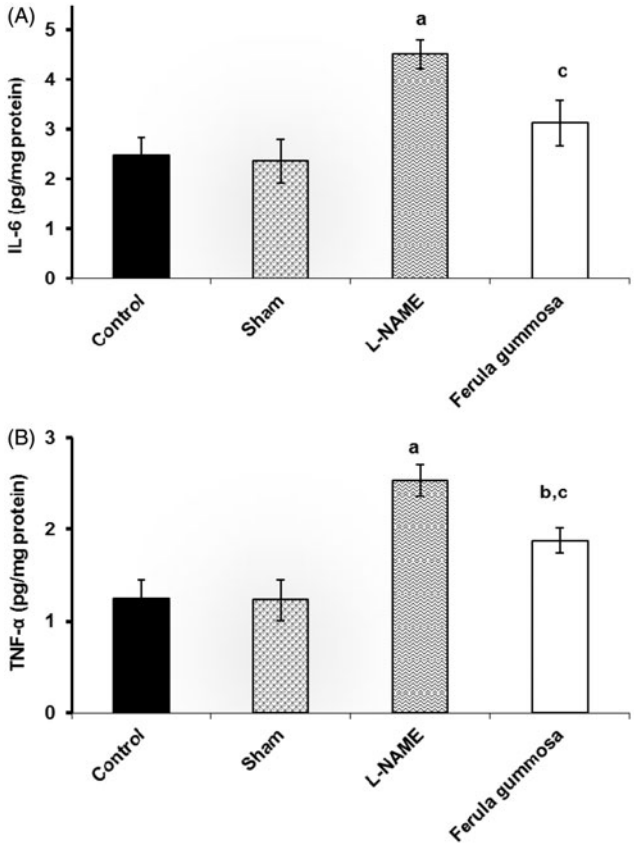

Figure 2. Effect of *Ferula gummosa* extract on renal IL-6 (A) and TNF-α (B) levels during chronic L-NAME administration in rats kidney. [a]Statistical significant at $p<0.001$ versus control and sham groups, [b]Statistical significant at $p<0.05$ versus control and sham groups, [c]Statistical significant at $p<0.05$ versus L-NAME group. IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha.

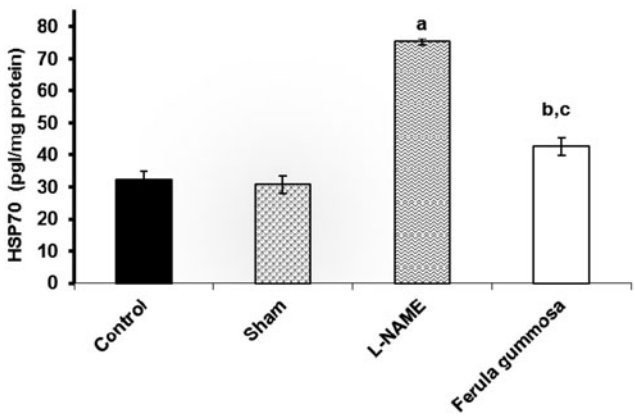

Figure 3. Effect of *Ferula gummosa* extract on renal HSP70 levels during chronic L-NAME administration in rats kidney. [a]Statistical significant at $p<0.001$ versus control and sham groups, [b]Statistical significant at $p<0.05$ versus control and sham groups, [c]Statistical significant at $p<0.001$ versus L-NAME group. HSP70, heat shock protein70.

protocols for hypertension and derived complications including changes in the lifestyle, diet and, or even pharmacological therapy. The main findings of this study were the evidence of the renoprotective effects of *Ferula gummosa* extract when was chronically administered on injuries caused by L-NAME-induced oxidative stress and inflammation. Chronic blockade

Table 2. Effect of *Ferula gummosa* extract on plasma NO and TAC levels during chronic L-NAME administration in rats.

Variables	Control	Sham	L-NAME	*Ferula gummosa*
TAC (mmol/l)	583 ± 12	576 ± 9	429 ± 9[a]	560 ± 16[b]
Nitrite/nitrate (μmol/l)	33.0 ± 1.0	32.1 ± 0.9	17.5 ± 2.9[a]	25.7 ± 1.1

Values are expressed mean ± SEM for 10 rats. [a]Statistical significant at $p<0.001$ versus control and sham groups, [b]Statistical significant at $p<0.001$ versus L-NAME group. L-NAME, Nω-nitro-L-arginine methyl ester; TAC, total antioxidant capacity.

of NO synthesis induced by L-NAME treatment is an excellent experimental model of salt-sensitive hypertension and shares many common features of human essential hypertension (20,22). In this experiment, we found that chronic L-NAME administration in the rats resulted in a significant increase in the renal HSP70, proinflammatory factors (IL-6, TNF-α) and TBARS levels. These physiological changes were accompanied by a decrease of serum nitrite/nitrate and by a down-regulation of the renal SOD activity, indicating a state of enhanced oxidative stress and impaired antioxidant defenses.

In a previous study, it was demonstrated that the family of NADPH oxidase enzymes are the main source of ROS generation in the kidney (2). NADPH oxidase expression was regulated by TNF-α via p38 mitogen-activated protein kinase (MAPK) in the kidney tissues of hypertensive rats (23). Furthermore, intrinsic renal cells including mesangial, glomerular, endothelial, dendritic and renal tubular cells are able to produce TNF-α, and an increased expression of IL-6 mRNA has been found in glomerular cells (24). We found significant increases in proinflammatory factors and in oxidative stress markers and, a decrease in renal antioxidant levels which are able to cause severe renal damage similar to that found in experimental hypertension-induced renal damage (25,26). These inflammatory changes were ameliorated by administration of *Ferula gummosa* extract. Thus, it is possible that the protective role of the hydroalcoholic extract of *Ferula gummosa* was able to attenuate renal inflammation via direct superoxide anion scavenging and via induction of the endogenous antioxidant systems in L-NAME-treated rats. These observed renoprotective effects of *Ferula agummosa* extract may be attributed to its potent antioxidant potential related to the presence of phenol and flavonoid components in the extract (19).

Tian et al. (5) reported that treatment with antioxidant vitamins C and E in high-Na Dahl S rats during a 5-week period decreased renal inflammatory cytokines (IL-6 and TNF-α) and the activation of the nuclear factor κB (NF-κB) and also improved renal functions accompanied with tubulointerstitial damage and glomerular necrosis. NF-κB is a key transcription factor in the regulation of proinflammatory response such as IL-6 and TNF-α (27). A close relationship between the activation of renal NF-κB and an increased oxidative stress has been also shown in high-Na diet Dahl S rats (4). Furthermore, numerous literature has reported that several therapeutic strategies such as antioxidant diets, superoxide dismutase mimetic (TEMPOL) and immunosuppressive drugs (mycophenolate mofetil) administrations

mitigated renal oxidative stress and inflammation and modified antioxidant enzymes activity in hypertensive animals (8,28,29).

The data indicated that chronic administration of *Ferula gummosa* extract during 8 weeks significantly ameliorated the increased renal HSP70 levels in the L-NAME-treated rats, together with a decreased oxidative stress and inflammation. Parra et al. (21) have shown that renal expression of HSP70 significantly increased in different types of salt-sensitive hypertension (such as angiotensin II or L-NAME) in accordance with the present results. Upregulation of the HSP70 expression in renal endothelium and medial smooth muscle artery (30), proximal tubules and collecting ducts in the cortex of hypertensive rats (31) have also been reported in previous studies. These abnormalities occur in the tubulointerstitial areas and, according to evidences inflammation and oxidative stress have been linked to the development of salt sensitive hypertension (30,31). In agreement with previous studies and our findings, increasing hemodynamic stress may play a key role in the enhancement of renal HSP70. Another possibility is that the induction of renal HSP70 may be due to oxidative stress and its concomitant inflammation. It is well known that the tubulointerstitial infiltration of immune-competent cells have commonly been identified in experimental models of hypertension. Suppression of the inflammation process was also achieved with immunosuppressive drugs (3,4,8).

In addition, several experimental studies suggested that autoimmunity to heat shock protein (HSP70) is involved in persistent low-grade inflammatory infiltration induced in the tubulointerstitial areas of the hypertensive kidney (22,30,31). Bae et al. (32) have demonstrated that the elevated levels of HSP70 protein expression in the kidneys of hypertensive rats were attenuated by the antihypertensive effects of Rosiglitazone treatment. Under stressful conditions, moderate expression of intracellular HSP70 developed a protective role against cell damage, while over expressed HSP70 or HSP70 released directly from the cell act as danger signals that might trigger cell necrosis (3,12). L-NAME administration has cytotoxic effect through increasing of renal HSP70 level and therefore *Ferula gummosa* treatment mollifies this abnormality by modulation of HSP70 production.

Moreover, we found that *Ferula gummosa* extract administration leaded to a significant increase in TAC and restored NO levels. These findings support the idea that this extract possesses significant antioxidant effects and also has a reinforcing role on endothelial function. NO released from endothelial cells is a main regulator of vascular tone and exerts a tonic inhibition on sympathetic nerve system activity. NO deficiency is the main factor responsible for the development of L-NAME-induced hypertension (25,33,34) and increased systemic oxidative stress, accompanied with decreases in antioxidant defenses (35) that may activate the sympathetic nervous system and Tyrosine hydroxylase (33) through enhanced oxidation/inactivation of NO (25). Mate et al. (36) have reported that chronic treatment with L-carnitine diminished the systemic oxidative stress by enhanced plasma NO and TAC in L-NAME-administered rats, in accordance with our results. Thus, increases in the antioxidant capacity and NO bioavailability caused by the administration of *Ferula gummosa* extract will eventually produced a reduced the levels of arterial blood pressure (25,34).

In conclusion, the present results reported that treatment with hydroalcoholic extract of *Ferula gummosa* ameliorates L-NAME-induced renal inflammation and oxidative stress in rats. In addition, *Ferula gummosa* extract attenuated renal inflammation via modulation of HSP70, decreased proinflammatory cytokines such as TNF-α and IL-6 in rat renal tissues and modified plasma antioxidant capacity and NO bioavailability due to its anti-inflammatory and antioxidant abilities. Collectively, *Ferula gummosa* extract is a multifaceted compound and its pharmacological properties can help to mitigate and/or protect against renal failure in multiple diseases that are coupled to NO deficiency, such as hypertension.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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