Protective effect of *Urtica dioica* methanol extract against experimentally induced urinary calculi in rats

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Received November 2, 2013; Accepted May 9, 2014

DOI: 10.3892/mmr.2014.2610

Abstract. Renal calculi formation is one of the most common urological disorders. Urinary stone disease is a common disease, which affects 10-12% of the population in industrialized countries. In males, the highest prevalence of the disease occurs between the age of 20 and 40 years, while in females, the highest incidence of the disease occurs later. Previous studies have shown that long-term exposure to oxalate is toxic to renal epithelial cells and results in oxidative stress. In the present study, a methanolic extract of aerial parts of *Urtica dioica* was screened for antiurolithiatic activity against ethylene glycol and ammonium chloride-induced calcium oxalate renal stones in male rats. In the control rats, ethylene glycol and ammonium chloride administration was observed to cause an increase in urinary calcium, oxalate and creatinine levels, as well as an increase in renal calcium and oxalate deposition. Histopathological observations revealed calcium oxalate microcrystal deposits in the kidney sections of the rats treated with ethylene glycol and ammonium chloride, indicating the induction of lithiasis. In the test rats, treatment with the methanolic extract of *Urtica dioica* was found to decrease the elevated levels of urinary calcium, oxalate and creatinine, and significantly decrease the renal deposition of calcium and oxalate. Furthermore, renal histological observations revealed a significant reduction in calcium oxalate crystal deposition in the test rats. Phytochemical analysis of the *Urtica dioica* extract was also performed using liquid chromatography-electrospray ionization tandem mass spectrometry and high-performance liquid chromatography with photodiode array detection, to determine the chemical composition of the extract. The eight chemical constituents identified in the extract were protocatechuic acid, salicylic acid, luteolin, gossypetin, rutin, kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside and chlorogenic acid. In conclusion, the results of the present study suggest that *Urtica dioica* has strong antiurolithiatic activity and may have potential as a natural therapeutic agent for various urological disorders.

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Key words: Urtica dioica, hyperoxaluria, antiurolithiatic activity, histopathology

Introduction

Kidney stones, also termed renal calculi, are crystal aggregations formed in the kidneys from dietary minerals in the urine. Urolithiasis is the term used for the condition where urinary stones are formed or located in the urinary system. In humans, calcium oxalate is a key component of the majority of urinary stones. Compared with normal subjects, patients with calcium stones excrete significantly more calcium and oxalate. Kidney stones are more prevalent in males than females with ~80% of individuals with kidney stones being male (1). Urinary stone disease is a common disease, which affects 10-12% of the population in industrialized countries (2). In males, the highest prevalence of the disease occurs between the age of 20 and 40 years, while in females the highest incidence of the disease occurs later (3). Numerous treatment modalities are used to treat kidney stones. However, the high recurrence rate is a serious concern in urinary stone disease and in the majority of cases the rate of recurrence may be >50% after 10 years (4). At present, numerous treatment regimens are available to treat kidney stone recurrence, including extracorporeal shock wave lithotripsy (SWL) and endourological procedures, including ureteroscopy, as well as percutaneous extraction procedures. However, these treatments are associated with severe side effects. The side effects associated with SWL include traumatic effects caused by the shock waves, severe hematuria, pancreatitis, infection and continual residual fragments, which may serve as nidi for the formation of further calculi. Moreover, side effects associated with endourological and percutaneous procedures include extravasation of irrigating fluid, urosepsis and ureteral damage (5-10). Various orally administered drugs are used to treat the formation of renal calculi; however, their long-term use is limited by severe side effects and their lack of universal tolerance by patients. Thus, alternative treatment options have arisen, including herbal medicines, which have been used to treat urinary stone disease for hundreds of years without evident harmful side effects. Certain herbal extracts exert their antilithogenic effects through changing the ionic

composition of urine, such as calcium ions and magnesium ions. Furthermore, a number of the herbal extracts are rich in saponins, which disarrange mucoprotein suspensions and promote crystallization (11).

Urtica dioica, commonly termed 'Stinging Nettle,' is a herbaceous perennial flowering plant and the best known member of the nettle genus Urtica. Nettle leaf has a long history of traditional medicinal use for arthritis in Germany (12). *Urtica dioica* has been used in traditional Austrian medicine internally (as tea or fresh leaves), as well as for the treatment of kidney, urinary tract, gastrointestinal tract and locomotor disorders. Furthermore, nettle root extracts have been studied in human clinical trials as a treatment for benign prostatic hyperplasia. In the present study, the antiurolithiatic activity of the methanol extract of *Urtica dioica* leaves was investigated in male rats. Phytochemical analysis of the extract was also performed using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS) and high-performance liquid chromatography with photodiode array detection (HPLC-DAD), which to the best of our knowledge is the first study to do so (13-15).

Materials and methods

Plant material and preparation of the extract. Leaves of *U. dioica* were collected between May and June 2013 from Jiuzhaigou, China. The plant material was confirmed by a taxonomist. The leaves of *U. dioica* were washed with water, shade-dried and homogenized. Methanol (95%) was used for hot extraction, which was performed for 3 h using a Soxhlet extraction apparatus (Benang Instrument Co., Ltd., Shanghai, China). The extract was then concentrated under reduced pressure in a rotary evaporator (Yarong Biochemistry Instrument Factory, Shanghai, China) at 40˚C and was stored at 4˚C prior to use.

LC-ESI-MS-MS-HPLC analysis. An Agilent 6410B Triple quadrupole LC/MS system equipped with a 1260 Infinity chromatographic system coupled with an Agilent triple quadrupole mass spectrometer fitted with an ESI source (Agilent Technologies, Santa Clara, CA, USA) was used for the LC-ESI-MS-MS-HPLC analysis. The MS conditions were as follows: MS range, 100-1,200 Da; nebulizer gas, 45 psi; gas temperature, 325˚C; and capillary voltage, 4,000 V. MSn spectra were obtained using positive and negative modes. HPLC analysis was performed using an Agilent 1260 Infinity series HPLC system (Agilent Technologies). A Chromolith® RP-18e column (internal diameter, 4.6 mm ID; length, 50 mm; Merck & Co., Inc., Readington, NJ, USA) was also used. The mobile phase consisted of aqueous formic acid (A; 0.1%) and methanol (B). The gradient conditions were: 0-8 min, linear gradient from 12 to 25% of B; 8-12 min, isocratic conditions at 25% of B; 12-16 min, linear gradient between 25 and 40% of B; 16-40 min, linear gradient between 40 and 50% of B; and 40-50 min, linear gradient between 50 and 100% of B. The flow rate was 1 ml/min.

Experimental animals. Male Sprague-Dawley rats (Experimental Animal Centre of Sichuan University, Chengdu, China) weighing between 100 and 170 g were used in the present study. Rats were maintained under hygienic conditions in cages in a temperature- and humidity-controlled room (30±2˚C and 50%, respectively), with a 12-h light/dark cycle. Food and water were available *ad libitum*. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health; 1996) and all procedures were approved by the Ethics Committee of the General Hospital of Chengdu Military Region (Chengdu, China).

Experimental induction of urinary stones in rats. To induce calcium oxalate urinary calculi in rats, the rats were allowed free access to drinking water containing 0.55% (v/v) ethylene glycol and 1% (w/v) ammonium chloride for 10 days, as described previously (16). Rats were then divided into four groups containing 10 rats per group, prior to treatment.

Experimental design, acute toxicity study and dose selection. For the *Urtica dioica* extract dose selection, rats were divided into five groups containing 10 rats per group and were fasted overnight with free access to drinking water. Rats in group I received only distilled water (10 ml/kg body weight orally). Rats in groups II, III, IV and V were administered a single oral dose of the *Urtica dioica* extract at 10, 50, 600 or 2,000 mg/kg body weight, respectively, through gastric intubation using a soft catheter. Rats were observed continuously for 4 h after the administration of the extract and then observed at one hour intervals for 48 h and the everyday for 15 days to monitor mortalities. The extract was determined to be non-toxic as no animal mortalities were observed with doses ≤2000 mg/kg body weight. The doses selected for the experimental investigations were 0.7 and 1.4 g/kg body weight. The final experimental groups were as follows: Group I, rats without the experimental induction of urinary stones (normal); group II, urinary stone-induced rats treated with 10 ml/kg body weight distilled water (control); group III, urinary stone-induced rats treated with 0.7 g/kg body weight *Urtica dioica* extract; and group IV, urinary stone-induced rats treated with 1.4 g/kg body weight *Urtica dioica* extract.

Determination of the antiurolithiatic activity of the extract. Following hydration with 10 ml distilled water administered orally, rats were put in separate metabolic cages and urine samples were collected after 48 h from the overnight-fasted rats on day 30. Urine samples were centrifuged at 3,000 rpm at 25±2˚C for 10 min. The supernatants of the urine samples were used to estimate the pH and to quantitatively measure the levels of oxalate, calcium and creatinine, as described previously (17-19). Rats were sacrificed using cervical dissection on day 31. The kidneys were then removed and washed using ice-cold saline solution and were sectioned. Ice-cold 0.10 M KCl solution was used to wash one kidney from each rat, which was then weighed. The kidney was sectioned into two equal halves. One half of the kidney was then homogenized with 5% HCl solution and the homogenate was centrifuged at 3,000 x g at 25±2˚C for 10 min. The supernatant was used to determine the renal deposition of oxalate and calcium as described previously (15,16).

Histopathological studies of the kidneys. The remaining half of the kidney sample was rapidly fixed using 10% neutralized

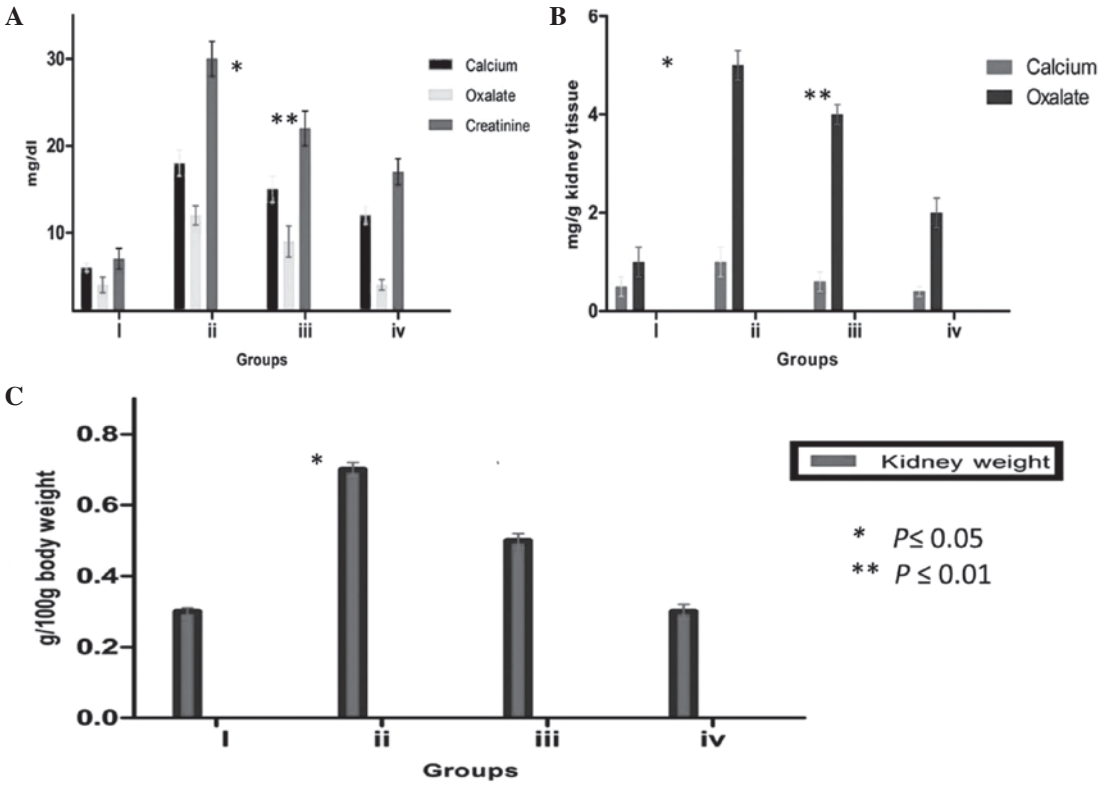

Figure 1. Effect of *Urtica dioica* extract on (A) urinary excretion of calcium, oxalate and creatinine, (B) renal deposition of calcium and oxalate crystals and (C) kidney weight in rats.

formalin (pH 7.4). Paraffin-embedded kidney sections were prepared and stained with hematoxylin and eosin, prior to being histopathologically assessed.

Statistical analysis. Data are presented as the mean ± standard error of the mean. One way analysis of variance was used to measure inter-group variation. P≤0.05 was considered to indicate a statistically significant difference.

Results

Toxicological and histopathological results. The methanol extract was determined to be non-toxic as no animal mortalities were reported upon treatment with doses ≤2,000 mg/kg body weight. In the control rats (group II), following ethylene glycol and ammonium chloride ingestion for 30 days, levels of urinary oxalate, calcium and creatinine were observed to significantly increase compared with the rats in group I (Fig. 1). Furthermore, compared with the normal rats, an increase in the renal deposition of calcium oxalate was found in the rats in the control group, as revealed by phase contrast microscopy (Fig. 2). A significant decrease in urine pH (from 7.0-7.5 to 5.0-5.7, P≤0.01; data not shown) and an increase in kidney weight (Fig. 1) were also observed in the control rats compared with the normal rats. In addition, histopathological studies using phase contrast microscopy revealed inflammation of tubules and atrophy of glomeruli, as well as intra-tubular and interstitial calcium oxalate crystal deposition in the kidneys of the rats in the control group compared with those in the normal group.

Antiurolithiatic effects of the U. dioica extract. Oral administration of the *U. dioica* methanol extract to rats in Group III (0.7 g/kg body weight) and Group IV (1.4 g/kg body weight from day 10-30 was found to induce a significant reduction in urinary excretion (Fig. 1), as well as a reduction in the renal deposition of oxalate and calcium (Fig. 2). Furthermore, the *U. dioica* methanol extract was found to decrease kidney weight and urinary creatinine levels (Fig. 1), and restore urinary pH levels (data not shown). Histopathological analysis using phase contrast microscopy revealed that treatment with the *U. dioica* extract also reduced calcium oxalate crystal deposition and renal damage, and promoted the regeneration of the renal tubules and glomeruli compared with the control group (Fig. 2). The reduction in calcium oxalate crystal deposition and renal injury in the renal tubules and glomeruli suggests that treatment with the *U. dioica* extract promoted the dissolution of preformed calcium oxalate crystals. The decrease in kidney weight observed in the *U. dioica* extract-treated rats supports this hypothesis. Furthermore, the more marked decrease in urinary and renal parameters observed in the high-dose group IV rats compared with the low-dose group III rats, suggests that the *U. dioica* extract had a dose-dependent effect on calcium oxalate stone formation. In the group IV rats, which received 1.4 g/kg body weight *U. dioica* extract, the decrease in the renal deposition of calcium and oxalate and the decrease in urinary excretion were also more pronounced, compared with the group III rats. Moreover, in the group IV rats, the reduction in kidney weight and levels of urinary creatinine were more pronounced, further indicating a dose-dependent effect of the *U. dioica* extract.

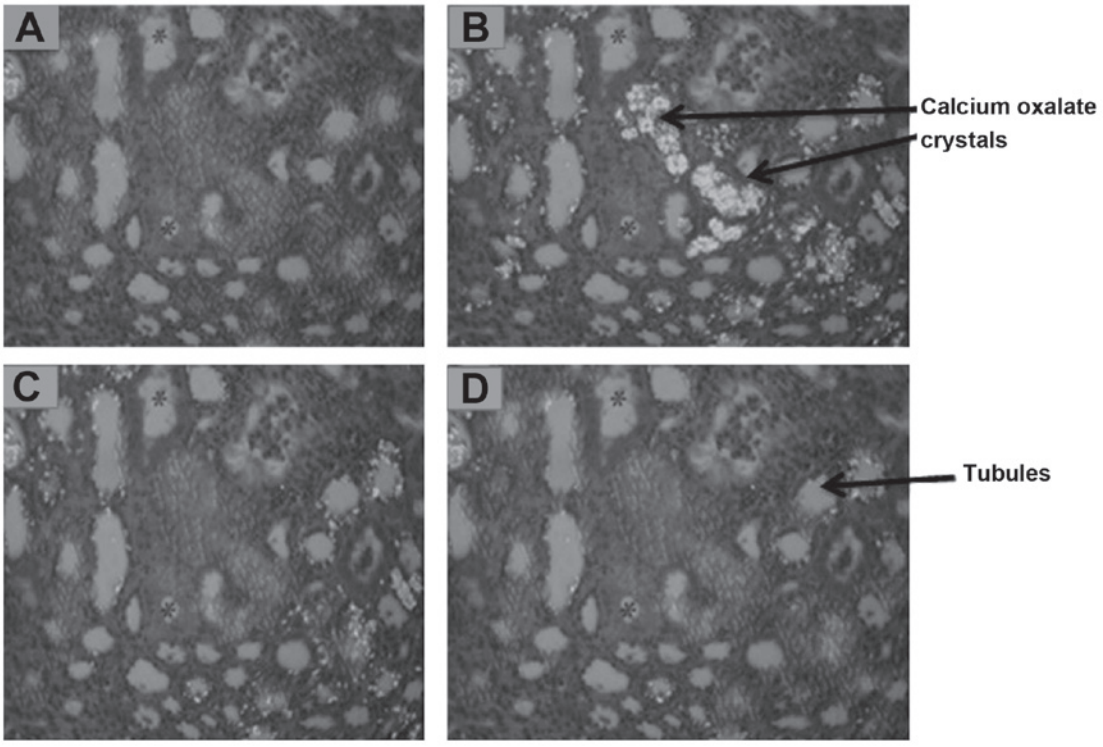

Figure 2. Hematoxylin and eosin-stained sections of (A) normal rat kidney showing normal tubular epithelial cells, (B) control rat kidney showing deposition of numerous calcium oxalate crystals and rat kidney treated with (C) 0.7 and (D) 1.4 g/kg body weight *Urtica dioica* extract, showing regenerative effects and few calcium oxalate crystals (magnification, x60).

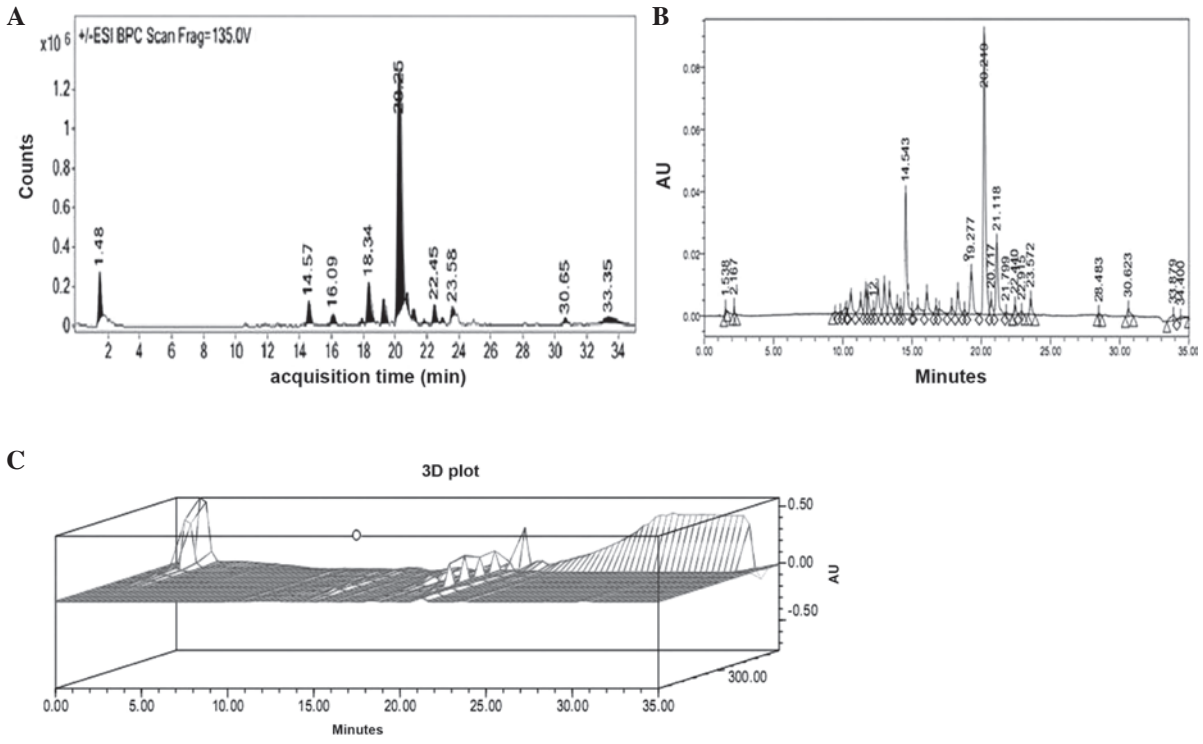

Figure 3. (A) LC-ESI-MS-MS analysis of the methanolic antilithogenic extract of *U. dioica*. (B) HPLC-DAD chromatogram of the *U. dioica* methanol extract. (C) HPLC three dimensional plot of the *U. dioica* methanol extract. LC-ESI-MS-MS, liquid chromatography-electrospray ionization tandem mass spectrometry; HPLC-DAD, high-performance liquid chromatography with photodiode array detection; AU, absorption unit.

LC-ESI-MS-MS-HPLC analysis. Phytochemical analysis of the *U. dioica* methanol extract was performed using LC-ESI-MS-MS and HPLC-DAD. The extract was run under positive and negative ESI-MS conditions and was observed to be composed of several major and minor ionic species. The total ion MS chromatogram, HPLC profile and HPLC 3D plot

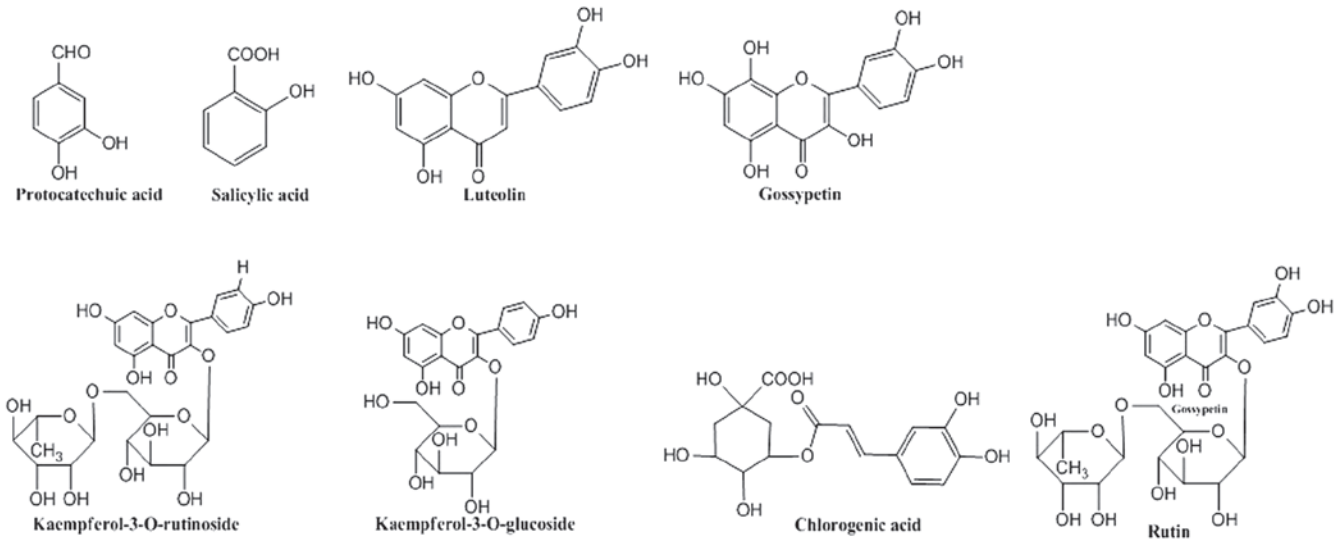

Figure 4. Chemical structures of eight compounds identified in the methanol extract of *U. dioica* using liquid chromatography-electrospray ionization tandem mass spectrometry and high-performance liquid chromatography with photodiode array detection analyses.

are shown in Fig. 3. Fragmentation of the major peaks was used for the identification of the compounds. The identification of the chemical compounds was also performed by comparing the molecular ion peaks and the MS fragmentation pattern with those reported in the literature. The eight chemical constituents identified were protocatechuic acid, salicylic acid, luteolin, gossypetin, rutin, kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside and chlorogenic acid (Fig. 4). The phytochemicals present in the aerial parts of the Urtica species have been found to be primarily phenolic compounds, including caffeic acid, chlorogenic acid, 2-O-caffeoylmalic acid and flavonoids, such as quercetin and isorhamnetin glycosides. Moreover, the biological activities of nettle leaves are often assigned to the flavonoid fraction (20-23).

Discussion

In recent years, there have been marked advances in phytotherapy for urolithiasis, with the United States investing >1.5 billion dollars annually (3). Although phytotherapeutic extracts are popular in certain cultures, their specific mechanism of action in urolithiasis remains unclear. Elucidating the mechanism of action of these herbal extracts in urolithiasis has diagnostic value with regard to the nature of this disease, as well as potential therapeutic implications in this future field of research. Based on the traditional medicinal claims of *Urtica dioica* for the treatment of various urinary disorders, the present study aimed to investigate the antiurolithiatic effect of a methanol extract of the leaves of *Urtica dioica* in male rats and define its chemical composition using LC-ESI-MS-MS and HPLC-DAD, which to the best of our knowledge is the first study to do so.

Numerous studies have reported the presence of flavonoids, saponins and anthocyanins in *U. dioica*, thus the decrease in the renal deposition of calcium and oxalate in the *U. dioica* extract-treated rats observed in the present study, may be induced by these phytochemicals (24-26). Saponins and flavonoids prevent calcium and oxalate deposition through disintegrating mucoproteins, which have a high affinity for calcium oxalate crystal surfaces and thus promote the growth and deposition of crystals (27).

In the present study, a significant increase in urinary creatinine was observed after 48 h in the control rats, suggesting the occurrence of hyperoxaluria-induced renal damage, which may cause decreased urine out-put and subsequent supersaturation of lithogenic promoting agents. Furthermore, hyperoxaluria-induced renal damage and stone formation was found to be associated with calcium oxalate crystal deposition and damage to the kidney. Urinary pH has been reported to affect crystaluria, with alterations to urinary pH found to induce urinary stone dissolution. A urinary pH between 5.0 and 6.3 promotes calcium oxalate stone formation (28). In the present study, the decrease in the urinary pH from 7.0-7.3 to 5.0-5.4 supports the formation of calcium oxalate calculi. Furthermore, restoration of the urinary pH (5.4-7.3) was found to support the dissolution of preformed calcium oxalate crystals.

In conclusion, the present study revealed that the methanol extract of *U. dioica* efficiently dissolves calcium oxalate renal stones in male Sprague-Dawley rats. The extract showed a dose-dependent curative effect on urinary and renal parameters, including calcium oxalate renal stone formation. These findings support previous reports that the extract can be used for the treatment of kidney and urinary tract disorders.

Acknowledgements

This study was supported by grants from the Shandong Province Natural Science Foundation of China (grant no. ZR2009CL027), the Science and Technology Star Plans Foundation of Jinan Technology Bureau of China (grant no. 20100118) and the Medical Foundation of Shandong Academy of Medical Science (grant no. 201023).

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