Protective Role of *Urtica dioica* methanol extract in treating experimentally induced urinary calculi in rats.

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**ABSTRACT:** Formation of renal calculi is one of the common urological disorders. Urinary stone disorder has always been a common disease currently affecting 10-12% of the population in industrialized countries. The highest prevalence of this disease seems to be at the age of 20-40 years for men, while for the women, the incidence of this disease is somewhat later. Various 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, 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 resulted in increased urinary calcium, oxalate and creatinine and renal deposition of calcium and oxalate. Histopathological observations confirmed the induction of lithiasis as calcium oxalate microcrystal deposits were observed in sections of kidney from animals treated with ethylene glycol and ammonium chloride. In the test rats, treatment with methanolic extract of *Urtica dioica* decreased the elevated levels of urinary calcium, oxalate and creatinine and significantly decreased renal deposition of calcium and oxalate. Renal histological observations exhibited significant reduction in calcium oxalate crystal depositions. In addition, we also carried out the phytochemical analysis of the extract to determine its chemical composition by using LC-ESI-MS-MS as well as HPLC-DAD analytical techniques. 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, our results suggest that *Urtica dioica* is embellished with potent antiurolithiatic activity and as such may be considered as a potent natural medicine for various urological disorders.

**Key words:** *Urtica dioica*, hyperoxaluria, antiurolithiatic activity, Histopathology.

1. **INTRODUCTION:**

A kidney stone also known as a renal calculus is a crystal aggregation 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 anywhere in the urinary system. In humans, calcium oxalate is a major component of the most urinary stones. As compared to normal subjects, calcium stone patients excrete significantly more calcium and oxalate. This problem is more prevalent in men than women with about 80% of those with kidney stones are men. [1]. Urinary stone disorder has always been a common disease currently affecting 10-12% of the population in industrialized countries. The highest prevalence of this disease seems to be at the age of 20-40 years for men, while for the women, the incidence of this disease is somewhat later. [2]. As far as its treatment is concerned, many modern treatment modalities are used. But the high recurrence rate is a serious concern in urinary stone disease and in most cases it may be more than 50% after 10 years [3]. Currently this serious problem of recurrence can be treated by many modern treatment regimens such as extracorporeal Shock Wave Lithotripsy (SWL), endourological procedures such as ureteroscopy or percutaneous extraction procedures. But these methods are not without significant severe side effects such as the traumatic effects, of shock waves, severe hematuria, pancreatitis, infection and continuous residual fragments that may serve as a nidus for new calculus
formation after SWL. Similarly, extravasation of irrigating fluid, urosepsis, and ureteral damage are the side-effects of endourological and percutaneous procedures [4-9]. Many orally administered drugs are used to curb renal calculi disorder, but severe harmful side-effects along with the fact that they are not tolerated by all patients, hamper their long term use. This situation has given rise alternative treatment options comprising of herbal medicines which have remained the mainstay of medical therapy for urinary stone disease for hundreds of years without evident harmful side-effects. Some of the popularly used herbal extracts exert their antilithogenic effects by changing the ionic composition of urine like calcium ions and magnesium ions. Many of the herbal extracts are rich in saponins which can disarrange mucoprotein suspensions which actually promote crystallization [10].

_Urtica dioica_ commonly known as “Stinging Nettle” is an herbaceous perennial flowering plant, probably the best known member of the nettle genus_Urtica_. Nettle leaf has a long history of traditional medicinal use for arthritis in Germany. Urtica dioica herb has been used in the traditional Austrian medicine internally (as tea or fresh leaves) for the treatment of kidney and urinary tract disorders, gastrointestinal tract, locomotor system etc. Nettle root extracts have been extensively studied in human clinical trials as a treatment for benign prostatic hyperplasia (BPH). So taking cue from the traditional medicinal use of this herb, we undertook the present study to evaluate the antiurolithiatic activity in male rats of the methanol extract from the leaves of _Urtica dioica_, along with phytochemical analysis of the extract using Liquid Chromatography-Mass Spectrometry (LC-ESI-MS-MS) as well as High Performance-Liquid Chromatography (HPLC-DAD), which to the best of our knowledge constitutes the first such report [11-13].

2. MATERIALS AND METHODS:

2.1. Plant Material and preparation of the extract

The leaves of _U. dioica_ were collected during May-June 2013 from Jiuzhaigou, Chengdu, China. The plant material was confirmed by a well-known taxonomist. The leaves of _U. dioica_ were thoroughly washed with tap water, shade dried and then chopped into small pieces. Methanol (95%) was used for hot extraction which was carried out for 3 hours using a soxhlet extraction apparatus. The extract was then concentrated under reduced pressure in a rotary evaporator at 40 °C and was then kept in a refrigerator at 4 °C prior to use.

2.2. Liquid chromatography–tandem mass spectrometry (LC–ESI-MSMS)/HPLC analysis.

LC–MS equipment (LC–MS QqQ-6410B Agilent Technologies) consisted of a chromatographic system (1260 Infinity Agilent Technologies) coupled with an Agilent Triple Quad mass spectrometer fitted with an ESI source. MS conditions were the following: MS range 100–1200 Da, MSn spectra were obtained using both positive and negative modes, nebulizer gas 45 Psi, gas temperature 325 °C, capillary voltage 4000 V.

HPLC analysis was carried out by an Agilent 1260 infinity series. A Chromolith RP-18e column (4.6 mm ID, 50 mm length) (Merck) was used. Mobile phase consisted of (A) aqueous formic acid (0.1%) and (B) methanol. Gradient condition was; 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 from 25% to 40% of B; 16–40 min, linear gradient from 40 to 50% of B, 40–50 min, linear gradient from 50 to 100% of B. Flow rate: 1 ml/min.

2.3. Animals used in the experiment:

Male Sprague-Dawley rats (Experimental Animal Centre of Sichuan University, Chengdu City, P.R, China) weighing 100-170 g were used in the present study. The rats were kept under highly hygienic conditions in cages with temperature and humidity-controlled room (30±2 and 50% respectively), with a 12-hour light-darkness cycles. Food and water were available ad libitum. Animals were treated in accordance with the Guide for Animal Care and Use of
Laboratory Animals (National Institute of Health, 1996), and all procedures were approved by the Ethical Committee of the General Hospital of Chengdu Military Region.

2.4. Experimental Induction of urinary stones in rats:
Free access to drinking water containing 0.55 % v/v ethylene glycol and 1% w/v ammonium chloride for 10 days was used to induce calcium oxalate urinary calculi in rats, as described in a previous report [14]. Rats were divided into four groups consisting of 10 rats per group and were put on treatment.

2.5. Experimental design, Acute Toxicity Studies and dose selection:
The study design consisted of dividing the rats into five groups containing 10 animals in each group and were kept on fast overnight with free access to drinking water. The rats in the Group 1 served as normal subjects and they received only distilled water (10 ml/Kg orally). The rats in Groups 2-5 received a single dose of 10, 50, 600 and 2000 mg/Kg body weight of the extract respectively orally through gastric incubation using a soft catheter. The rats were continuously observed for 4 h after the administration of the extract and then observed intermittently at one hour interval up to 48 hours and everyday up to 15 days to monitor the animal deaths. The extract was found to be safe because no animal death was occurred up to 200 mg/kg body weight by oral administration. The doses selected for the current study were 0.7 and 1.4 g/kg, orally.

2.6. Determination of antiurolithiatic activity of the extract:
Rats after hydration with 10 ml of distilled water oral administration were put in separate metabolic cages and after 48 hours urine samples were collected from overnight fasted rats on day 30. Then the urine samples were centrifuged at 3000 r.p.m at 25 ± 2 °C for 10 min. Supernatant of the urine sample was used to estimate PH and quantitative determination of oxalate, calcium and creatinine according to the previously published reports [15-17]. Then the rats were sacrificed by cervical execution on day 31, kidneys were removed and washed with ice-cold saline solution and were then carefully cut. An ice-cold 0.10 M KCl solution was used to wash one kidney from each rat and then were weighed. Then the kidney was sliced into two equal halves, one part of the kidney was homogenized with 5% HCl solution. Then the homogenate was centrifuged at 3000 r.p.m at 25 ± 2 °C for 10 min and the supernatant was used to determine the renal deposition of oxalate and calcium as per methods already documented in the literature [15, 16].

2.7. Histopathological Studies of the kidneys:
The other half of the kidney sample was fixed rapidly with 10% neutralized formalin (PH 7.4). Sections of kidney fixed in paraffin were prepared and stained with hematoxylin and eosin and then observed for histopathological details.

2.8. Statistical analysis:
All the results were expressed as mean ± SEM. One way analysis of variance was used to measure inter-group variation. Statistical significance was considered at p≤ 0.05.

3. RESULTS AND DISCUSSION:
3.1. Toxicological and Histopathological Results:
From the toxicological studies, methanol extract was found to be safe as no animal death was reported up to 2000 mg/kg body weight. On ethylene glycol and ammonium chloride ingestion in control rats for 30 days, a considerable elevation in urinary oxalate, calcium and creatinine were observed (Fig-1). Also there was a significant increase in the renal deposition of calcium oxalate as revealed by phase contrast microscope (Fig-2). A significant decrease in urine PH (from 7.0-7.5 to 5.0-5.7) and increase in kidney weight were also observed. Histopathological studies under phase contrast
microscope revealed the presence of inflammation of tubules and atrophy of glomeruli accompanied by intra-tubular and interstitial calcium oxalate crystal depositions.

## 3.2. Antiurolithiatic effects of the *U. dioica* extract:

On administration of the methanol extract of *U. dioica* methanol extract, in Group III (0.7 g/kg, oral) and Group IV (1.7 g/kg, oral) rats, from day 10-30, a significant reduction in urinary excretion (Fig-1) and renal deposition of oxalate and calcium (Fig-2) were observed. The extract administration also resulted in decreased kidney weight as well as decreased urinary creatinine (Fig-3), accompanied by restoration of urinary pH. Histopathological studies involving phase contrast microscope revealed a few calcium oxalate crystal depositions in kidney sections with reduced renal damage and regeneration of renal tubules and glomeruli (Fig-4). Decreased calcium oxalate crystal depositions accompanied by minimum renal injury in renal tubules and glomeruli indicates that the extract promoted the dissolution of preformed calcium oxalate crystals. The decrease in kidney weight also supported these observations. The more pronounced decrease in urinary and renal parameters in Group IV rats with increased dose of the extract revealed that the extract exhibited dose-dependent effect on calcium oxalate stone formation. In Group IV rats that received 1g/kg body weight of the extract, the decrease in renal deposition of calcium and oxalate and decrease in urinary excretion were more pronounced. Further, in the same group, the reduction in kidney weight and urinary creatinine were also more pronounced indicating a dose-response relationship between these parameters and the extract concentration used.

## 3.3. LC–ESI-MSMS analysis/HPLC analysis:

The phytochemical analysis of the *U. dioica* methanol extract was carried out by LC–ESI-MS as well as HPLC-DAD techniques. The extract was run under both positive and negative ESI-MS conditions and it showed several major and minor ionic species. The total ion MS chromatogram (TIC), HPLC profile and HPLC 3-D plot are shown in Fig-5a, 5b and 5c. Fragmentation of the major peaks was used for the identification of compounds. The identification of the chemical compounds was also carried out by comparing the molecular ion peaks along with the MS fragmentation pattern with those of 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-6). The phytochemicals present in the aerial parts of most *Urtica* species have been found to be mostly phenolic compounds like caffeic acid, Chlorogenic acid, 2-O-caffeoylmalic acid and flavonoids like quercetin, isorhamnetin glycosides etc. The biological activities of nettle leaves are usually assigned to the flavonoid fraction [18-21].

Recent years have witnessed dramatic advances in phytotherapy for urolithiasis. An unavoidable interest in this area results in an expense of more than 1.5 billion dollars annually in the United States [3]. Although phytotherapeutic extracts are popular in folk cultures, but their exact mechanism of action for urolithiasis is not clearly known. A precise mechanism of the action of these herbal extracts would have a diagnostic value in regard to the nature of this disease, besides the potential therapeutic implications in this future field of research. Keeping in view the traditional medicinal claims of *Urtica dioica* for the use of various urinary problems, we undertook this research proposal to evaluate the antiurolithiatic effects of the methanol extract from the leaves of *Urtica dioica* in male rats along with defining its chemical composition using LC-ESIMS-MS and HPLC-DAD techniques, which to the best of our knowledge is reported for the first time.

Many researchers have reported the presence of flavonoids, saponins and anthocyanins from *U. dioica* and we presume that the decrease in renal deposition of calcium and oxalate in the extract treated rats may be attributed to the presence of these phytochemicals. This statement stems from the fact that these saponins and flavonoids prevent calcium and
oxalate depositions by disintegrating mucoproteins which have a considerable affinity for calcium oxalate crystal surface and hence promote the growth and deposition of crystals [22].

In this study we showed convincingly that in the control group rats, the significant increase in 48 h urinary creatinine is a manifest indication of hyperoxaluria induced renal damage which might have led to decreased urine out-put and subsequent supersaturation of lithogenic promoting agents. Further, hyperoxaluria promoted renal damage and stone formation was substantiated by the presence of calcium oxalate crystal depositions and damaging changes in kidney sections. Further, it has been reported that urinary PH affects crystaluria and by changing urinary PH, urinary stone dissolution can be achieved. Usually PH range of 5.0-6.3 favors the calcium oxalate stone formation [23]. In our study, the decrease in the urine PH from 7.0-7.3 to 5.0-5.4 supports the formation of calcium oxalate calculi. Restoration of urinary PH (5.4 to 7.3) supports the dissolution of preformed calcium oxalate crystals.

4. CONCLUSION:
The results of the current study reveal that the methanol extract of *U. dioica* efficiently dissolves calcium oxalate renal stones in male Sprague-Dawley rats. The extract showed dose-dependent curative effect on urinary and renal parameters including calcium oxalate renal stone formation. The current study lends support to the traditional medicinal claim of this herb, which report that the herb has been used for the treatment of kidney and urinary tract disorders.

REFERENCES


Figure-1: Effect of *Urtica dioica* extract on the urinary excretion of calcium, oxalate and creatinine.

Figure-2: Effect of *Urtica dioica* extract on renal deposition of calcium and oxalate crystals.

Figure-3: Effect of *Urtica dioica* extract on rat kidney weight.
Figure-4: [a] A section of normal rat kidney showing normal tubular epithelial cells (60 x), [b] section of control kidney showing many calcium oxalate crystal depositions (60 x), [c] and [d] sections of extract treated (at 0.7 and 1.4 g/kg respectively) rat kidney showing regenerative effects and only very few calcium oxalate crystals (60 x).

Fig-5a: LC-ESI-MS analysis of the methanolic antilithogenic extract of *U. dioica*.
Fig-5b: HPLC-DAD chromatogram of the *U. dioica* methanol extract.

Fig-5b: HPLC 3-dimensional plot of the *U. dioica* methanol extract.
Fig-6: The chemical structures of 8 compounds identified in the methanol extract of *U. dioica* by LC-ESI-MS and HPLC-DAD analysis.