Urol Res (2012) 40:151–161
DOI 10.1007/s00240-011-0401-y

ORIGINAL PAPER

Evaluation of antiurolithic effect and the possible mechanisms of *Desmodium styracifolium* and *Pyrrosiae petiolosa* in rats

Jun Mi · Jianmin Duan · Jun Zhang · Jianzhong Lu · Hanzhang Wang · Zhiping Wang

Received: 6 April 2010 / Accepted: 8 July 2011 / Published online: 6 August 2011
© Springer-Verlag 2011

Abstract Chinese herbs *Desmodium styracifolium* (Ds) and *Pyrrosiae petiolosa* (Pp) have been widely used to treat urolithiasis with few side effects in traditional Chinese medicine (TCM). In vitro crystallization study has confirmed their prophylaxis of Calcium oxalate (CaO_x) stones formation. However, little is known on their possible mechanisms in vivo. In the present study, we proposed to systematically evaluate their antilithic effects and clarify the underlying mechanism of Ds and Pp using a rat nephrolithiasis model and administering the aqueous extracts. Adult male Wistar rats were fed with 5% ammonium oxalate (AmO_x) forage to induce nephrolithiasis. After 1 week, the rats were randomly divided into eight groups, and given low, medium, and high dose of each herb treatments (275, 550 and 1,100 mg/kg for Ds and 150, 300 and 600 mg/kg for Pp) by gavage for 3 weeks. In contrast to urolithic rats, urinary oxalate significantly reduced, and urinary calcium increased in medium and high dose Ds groups. Few depositions were observed in kidney with slight dilation of tubules and inflammatory infiltration. Osteopontin (OPN), nitric oxide (NO), and malondialdehyde (MDA) levels significantly decreased, but Superoxide dismutase (SOD) activities were enhanced in kidneys. In high dose Pp group, crystals were found in the dilation of tubules with slight inflammatory infiltration. Partial serum and urinary variables returned to the normal range. In conclusion, medium and high dose Ds has beneficial effect in preventing CaO_x stone formation by rising urinary Citrate excretion, decreasing urinary calcium, diuresis, and antioxidative effects. Although high dose Pp has relatively weak prophylaxis, it indicates significant anti-inflammatory and antioxidant effects.

Keywords Calcium oxalate · Urolithiasis · Antiurolithic effect · *Desmodium styracifolium* · *Pyrrosiae petiolosa* · Rats

J. Mi · J. Duan · J. Zhang · J. Lu · H. Wang · Z. Wang (✉)
Institute of Urology, Gansu Nephro-Urological Clinical Center, The Second Hospital of Lanzhou University, Lanzhou 730030, China
e-mail: erywzp@lzu.edu.cn

J. Mi · J. Duan · J. Zhang · J. Lu · H. Wang · Z. Wang
Institute of Urology, Gansu Key Laboratory of Urology, The Second Hospital of Lanzhou University, Lanzhou 730030, China

Introduction

Urolithiasis is one of the world's major public health burdens. The prevalence of urinary stone range from 1 to 20% (average 10%) world wide due to various factors, such as geography, climate, race and diet [1, 2]. Calcium oxalate (CaO_x) as the major constituent is present in 80% of renal calculi [2]. Although the mechanisms are completely unclear, it is generally considered as a multistep process involving crystal nucleation, growth, formation and aggregation [3]. The disorder of urinary promoters and inhibitors in function and/or concentration play more important roles than a disturbance of any single substance. These substances including inorganic ions and macromolecules can worsen physiochemical conditions and saturation of the urine so as to promote stone formation [4]. Recently, the evidence from renal stone patients and rat nephrolithiasis model studies revealed that both CaO_x and oxalate (O_x) crystal deposition could cause lipid peroxidation and oxygen free radical generation, which mediated renal epithelial cell injury and induced stone formation [5–7]. In addition, injured renal epithelial cells markedly up-regulate expression of osteopontin (OPN) both in vitro

and in vivo, which has been confirmed as one of the secreted crystallization inhibitor protein [8–10].

In the past two decades, minimally invasive techniques such as extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS) and percutaneous nephrolithotomy (PCNL) were widely applied in clinical practice so that many patients with urolithiasis gained an effective management. However, the high recurrence rate is still a serious problem and remains to be decreased [11]. It is necessary to build an effective strategy (e.g. fluid intake increase, dietary modification and drug therapy) in stone prophylaxis. Thiazide diuretics and alkali-citrate have been proven to be effective in reducing calciuria and stone recurrence, they are especially important to urolithic patients who failed to respond to non-drug treatment. Nevertheless, undesirable side-effects limit their use in long-term medical treatment [12]. Therefore, finding an effective alternative with few adverse-effects is becoming a new tendency. Currently, there has been a sustained concern on medicinal plants to prevent stone recurrence owing to their efficiency, economy, and safety [13].

Chinese herbs *Desmodium styracifolium* (Ds) and *Pyrrosiae petiolosa* (Pp) have been widely used to treat urolithiasis with few side effects in traditional Chinese medicine(TCM), and are officially recorded in the Chinese Pharmacopoeia [14]. In vitro crystallization study confirmed that their prophylaxis of CaO_x stones could be achieved through promoting nucleation and inhibition of growth and aggregation of crystals [15]. However, little is known on their possible mechanisms in vivo. Recently,evidence shows that Ds and Pp contain abundant flavonoids [16, 17], which are considered to have antioxidant properties [18].

What role do these plants play in the antilithic process, and whether their antilithic effects are related to antioxidant effect and then modulate OPN expression? Accordingly, we proposed the present study to systematically evaluate antilithic effects and clarify the underlying mechanism of Ds and Pp using a rat nephrolithiasis model by administering the aqueous extracts.

Materials and methods

Plant materials

Both Ds and Pp were purchased from Beijing Tongrentang Co., Ltd (Beijing, China), and authenticated in accordance with the standard of the Chinese pharmacopoeia by Professor Ma (School of Pharmacognosy, Lanzhou University). Since Chinese traditional medicines are used this way with long time decoction, our study used the same method to examine the effect of the aqueous extracts. Meanwhile, given that the aqueous extracts do not decrease the extraction of antioxidants from medicinal plants, the traditional decoction method was applied [19]. For each herb, 1 kg of raw material was immersed in 5 L distilled water for 2 h, and decocted for 30 min. The supernatant was collected by filtering. Subsequently, the residue was added with 3 L fresh distilled water and boiled again. Finally, two portions of aqueous extracts were put together, vacuum filtered, concentrated, and lyophilized. Freeze-dried powder was stored at −80°C. The extraction yield of Ds and Pp was approximately 14.6 and 19.8%, respectively. The powder of each herb was adjusted to low, medium, and high concentrations (275, 550 and 1100 mg/kg for Ds and 150, 300 and 600 mg/kg for Pp) with distilled water as listed in Table 1.

Animals and experimental protocols

Eighty adult male Wistar rats (180–220 g) were provided by the Experimental Animal Center of Lanzhou University (Certificate No.0000782). All animals had free access to drinking water and regular chow with a standard commercial rat feed every day, and were housed in plastic cages under controlled conditions (temperature 22–25°C, humidity 50–60%, light illumination 12 h/d). Rat nephrolithiasis model was induced by calculi producing diet (CPD),which was made from the standard rat feed mixed with 5% ammonium oxalate(AmO_x) [20]. CPD was fed to rats to induce hyperoxaluria and CaOx crystal in 3 weeks. After 1 week of acclimatization, the rats were randomly divided into eight groups each containing 10 rats, and were given different treatments for 3 weeks, as shown in Table 1. The experiment protocol as well as all the procedure was approved by the Institutional Animal Ethical Committee of Lanzhou University.

Sample collection

On the 21st day of experimental period, the rats were placed in separate metabolic cages to collect 24-h urine and 0.02% sodium azide was added into urine to prevent bacterial growth. After determining urinary volume, urine was aliquoted and stored at −80°C for various assays. An aliquot of 24-h urine samples was acidified by addition of 1 M HCL (20:1) for the determination of O_x. The rats were anesthetized intraperitoneally with 10% Chloral Hydrate. Blood was obtained via inferior vena cava and then the kidneys were immediately excised. Serum was transferred into clean tubes and stored at −20°C until analysis. One kidney was fixed with 10% formaldehyde for hematoxylin and eosin (H and E) and immunohistochemistry staining,

Table 1 Experimental schema for evaluating the antiurolithic activity of Ds and Pp

Groups	Dose (mg/5 ml/kg)	Treatment
Normal	Vehicle	Rats received only normal feeds, distilled water and gavage distilled water (2 ml/animal/day)
Control	Vehicle	Rats received CPD, distilled water and gavage distilled water (2 ml/animal/day)
LDs	275	Rats received CPD, distilled water and gavage low dose extract of Ds
MDs	550	Rats received CPD, distilled water and gavage medium dose extract of Ds
HDs	1,100	Rats received CPD, distilled water and gavage high dose extract of Ds
LPp	150	Rats received CPD, distilled water and gavage low dose extract of Pp
MPp	300	Rats received CPD, distilled water and gavage medium dose extract of Pp
HPp	600	Rats received CPD, distilled water and gavage high dose extract of Pp

Ds Desmodium styracifolium, *Pp Pyrrosiae petiolosa*, *CPD* calculi producing diet, *LDs* low dose Ds, *MDs* medium dose Ds, *HDs* high dose Ds, *LPp* low dose Pp, *MPp* medium dose Pp, *HPp* high dose Pp

Table 2 Serum and urinary parameters in control and various treatment groups

	Normal	Control	LDs	MDs	HDs	LPp	MPp	HPp
Serum parameters								
Calcium (mmol/L)	2.49 ± 0.47	2.69 ± 0.49	2.30 ± 0.48	2.31 ± 0.32	2.15 ± 0.60	2.23 ± 0.25	1.93 ± 0.53*	1.76 ± 0.55*
Magnesium (umol/L)	1.40 ± 0.31	1.52 ± 0.54	1.34 ± 0.38	1.52 ± 0.39	1.69 ± 0.75	1.58 ± 0.42	1.38 ± 0.08	1.30 ± 0.23
Potassium (mmol/L)	2.61 ± 0.55	2.54 ± 0.38	2.72 ± 0.71	2.40 ± 0.40	2.63 ± 0.69	3.08 ± 0.73	2.68 ± 0.54	2.95 ± 0.41
Urine values								
pH	6.34 ± 0.35	6.49 ± 0.23	6.62 ± 0.38	6.64 ± 0.37	6.76 ± 0.37	6.64 ± 0.60	6.70 ± 0.43	6.72 ± 0.32
Urine Volume (ml)	9.38 ± 1.09	10.06 ± 1.97	12.23 ± 1.85	19.20 ± 1.60*	18.13 ± 2.64*	10.51 ± 2.90	8.14 ± 2.20	9.11 ± 1.84
Calcium (mmol/L)	2.38 ± 0.34	0.52 ± 0.19**	0.84 ± 0.45	1.80 ± 0.33*	1.96 ± 0.34*	0.95 ± 0.29	1.04 ± 0.33	1.47 ± 0.49*
Magnesium (umol/L)	5.36 ± 0.62	5.12 ± 0.88	4.53 ± 0.70	4.97 ± 0.64	5.26 ± 0.55	4.81 ± 0.73	5.47 ± 0.42	5.16 ± 0.54
Uric Acid (mmol/L)	1.53 ± 0.43	1.65 ± 0.25	1.56 ± 0.43	1.59 ± 0.39	1.46 ± 0.24	1.44 ± 0.22	1.64 ± 0.45	1.51 ± 0.23
Creatinine (mmol/L)	2.66 ± 0.39	2.94 ± 0.47	3.16 ± 0.66	2.41 ± 1.03	2.47 ± 0.65	2.41 ± 0.53	2.90 ± 0.50	2.56 ± 0.65
Oxalate (mmol/L)	0.86 ± 0.29	2.51 ± 0.65**	2.01 ± 0.77	1.22 ± 0.50*	1.13 ± 0.46*	1.75 ± 0.64	1.78 ± 0.57	1.08 ± 0.55*
Citrate (mmol/L)	0.71 ± 0.32	0.54 ± 0.29	0.91 ± 0.19	1.67 ± 0.39*	1.60 ± 0.44*	1.03 ± 0.67	0.75 ± 0.38	0.92 ± 0.42

Values are expressed as mean ± SD, * $P < 0.05$ when compared to the control, ** $P < 0.05$ when compared to the normal

and the other was stored at −80°C for oxidative stress studies.

Biochemistry examination

Serum and urinary variables (shown in Table 2) were determined by an automatic clinical chemistry analyzer (Hitachi7600, Tokyo, Japan). Urinary O_x and citrate (Cit) were measured by the colorimetric method of Hodgkinsona and Jacobs [21, 22]. The pH values were measured by a pH meter (Mettler Toledo 320-s, Shanghai, China).

Crystalluria and CaO_x deposition evaluation

A small amount of urine specimen was dripped on to slides without cover slips and examined for the presence of crystals under the polarizing microscope (Zeiss Axioskop40, Jena, Germany). Crystalluria were analyzed in 10 separate high-power fields (×40), and the highest number of crystals in a single field was recorded [23]. The numbers of crystal deposition in renal tubules were determined by assessing randomly selected 10 fields per kidney (×200). The scoring of crystal deposition included the following:

no deposition = 0 point; crystal deposition in papillary tip = 1 point; crystal deposition in cortico-medullary junction = 2 points; crystal deposition in cortex = 3 points. If crystal depositions were observed at multiple sites, points were combined together for total score per kidney.

Pathological examination

The pathological alterations were semiquantified according to the area of injury from 0 to 3 as follows: 0 = invisible lesions; 1 = mild dilation of tubules and tubulointerstitial inflammatory infiltration with lesion area <20%; 2 = obvious dilation of tubules and tubulointerstitial inflammatory infiltration with lesion area <40%; 3 = severe dilation tubules and massive tubulointerstitial inflammatory infiltration with lesion area >40%.

Immunohistochemical staining

For the immunohistochemical examination, we applied OPN monoclonal antibody against rat (Santa Cruz, USA. Cat: sc-21742) by a two-step method. The primary antibody (200× dilution) was used as the optimal Concentration.

Both Morphologic changes of kidney and crystalluria were independently assessed by two pathologists blinded to treatment groups.

Oxidative stress studies

One gram renal tissue was cut into small pieces with scissors and then homogenized with 9 volumes cold phosphate buffer (pH 7.4) using electric homogenizer. The homogenates were centrifuged at 8,000× *g* for 10 min at 4°C. The supernatants were assayed for nitric oxide (NO), Superoxide dismutase (SOD), malondialdehyde (MDA), and OPN. NO, SOD and MDA were determined spectrophotometrically using three kits (Nanjing Jiancheng Biochemistry, China. Cat: A001, A003 and A012) according to the manufacturer's protocol. OPN was examined by enzyme-linked immunosorbent assay (ELISA) kit (Quantikine assay, R&D Systems, USA. Cat: MOST00) following instructions provided by the manufacturer.

Statistical analysis

The data was analyzed using the SPSS 14.0 software and expressed as mean ± standard deviation (SD). Differences between groups were examined for statistical significance using one-way ANOVA. *P* value less than 0.05 was considered significant.

Results

Serum and urinary biochemical variables

As shown in Table 2, there were 3-fold increase in urinary O_x level and 5-fold decrease in Ca excretion in the urolithic rats. However, urinary volume, Ca, and Cit level increased differently, but O_x concentration decreased in the MDs and HDs group (550 and 1100 mg/kg). Both serum and urinary Ca were decreased in HPp group (600 mg/kg). Nevertheless, in MPp group (300 mg/kg) only hypocalcemia was found without reduction of urinary Ca ($P < 0.05$). No significant difference was observed in all other serum and urinary parameters compared to control.

Urinary crystals and CaO_x crystal accumulation

The analysis of crystalluria showed more whewellite and weddellite crystals existed in control, LDs and Pp groups

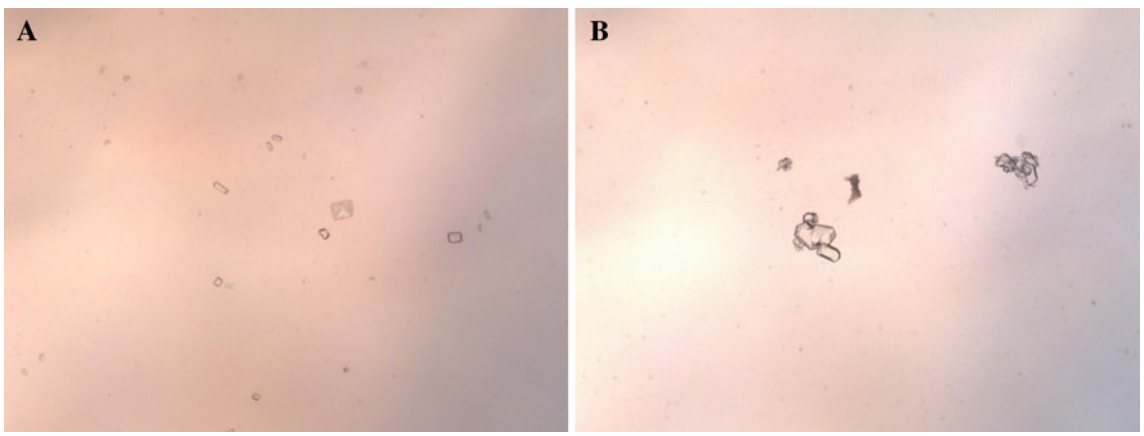

Fig. 1 Photomicrographs of urinary crystals **a** more columnar calcium oxalate monohydrates (COM) and less pyramidal calcium oxalate dehydrates (COD) crystals in urine of the urolithic rats, **b** enlarged COM and COD crystals (magnification: **a** ×100, **b** ×200)

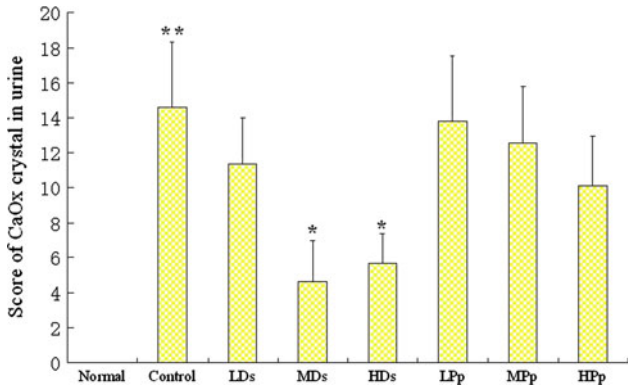

Fig. 2 Score of CaO_x crystal in urine at 21st day. Columns and bars represent mean ± SD (*$P < 0.05$ compared with the control, **$P < 0.05$ compared with the normal)

(Fig. 1). Few CaO_x crystals were observed in the urine of normal rats. The number of crystals in MDs and HDs groups (550 and 1100 mg/kg) was distinctly reduced compared to the control group (MDs, HDs vs. control: 4.63 ± 2.33, 5.67 ± 1.73 vs. 14.6 ± 3.72, $P < 0.001$) (Fig. 2).

The kidney sections stained by H and E were observed by polarizing microscope. No CaO_x deposition was detected in the normal group,but more crystals were observed in all parts of the kidney in the control group , especially in cortico-medullary junction. Scoring of crystal deposition showed a significantly lower number of deposits in MDs and HDs groups (550 and 1100 mg/kg), but no statistical difference was concluded between two groups. CaO_x Crystal deposition in the kidney obviously decreased in high dose Pp group (600 mg/kg), too (Fig. 3).

Histological examination

In the normal group pathological injury was invisible, but there were severe dilations of tubules and massive inflammatory infiltration in the control group. The pathological score of MDs, HDs and HPp groups (550 and 1100 mg/kg for Ds and 600 mg/kg for Pp) were significantly lower compared to the control group (Fig. 4). Pathological alteration in the above three groups reduced and has slight dilation of tubules and inflammatory infiltration.

Osteopontin expression

The concentration of OPN in the urine and renal homogenate were detected by ELISA method. Both renal and urinary OPN protein level were significantly elevated in urolithiatic rats, while it was obviously down-regulated in treated groups except for LDs and LPp (275 mg/kg for Ds and 150 mg/kg for Pp) ($P < 0.05$). However, no statistical difference was observed in a dose-dependent manner of the two herbs, respectively (Fig. 5). As shown in Fig. 6, OPN protein expression was observed in the kidney of all eight groups. The expression of OPN was weak in the normal group and limited to the loop of Henle and papillary surface epithelium, whereas in the kidney of urolithiatic rats OPN staining was enhanced and mostly located to the distal and proximal convoluted tubules, the thin limb and collecting ducts and even the whole kidneys. As expected, OPN in rats treated by Ds and Pp showed significantly lower expression than those in the urolithiatic rats.

Oxidative studies

As shown in Table 3, NO and MDA level rose rapidly, but SOD activities were inhibited in the control group ($P < 0.05$). Although significantly lower NO and higher SOD levels were observed in HDs and MPp groups, there was no Statistical significance in MDA. However, in MDs and HPp groups the above three indicators restored to near normal levels.

Discussion

Due to the complexity of stone formation and high recurrence rate of urolithiasis, more attention was paid to medicinal plants to look for an effective anti-urolithic in the recent decade. In China, DS and Pp were usually combined to eliminate renal stones and clear urinary tract infection [14]. An in vitro crystallization study indicated that Ds and Pp have antiurolithic effect through promoting nucleation and inhibiting growth and aggregation of CaO_x crystals [15]. However, little is known about their respective potential mechanisms in vivo, especially in stone prophylaxis.

In the present study, we chose the rat urolithiatic model induced by 5% AmO_x owing to its high O_x excretion and uniform depositions in renal tissue [24]. To assess the prevention of CaO_x renal stones, the rats in Ds and Pp groups were administered with CPD and simultaneously given different dose Ds or Pp extracts by gastric intubation. Three different dosages were also designed to evaluate the dose-dependent effect on CaO_x stone formation. The rat equivalent doses were selected based on the clinical dosage provided by the Chinese Pharmacopoeia, and calculated according to the ratio of body-surface area between human and rat.

The pathogenesis of CaO_x stone formation is a gradual process including crystal nucleation, growth, aggregation and retention. The abnormal metabolism in serum and urine plays a crucial role during these processes such as hypercalcaemia, hypercalciuria, hyperoxaluria, hypocitraturia and

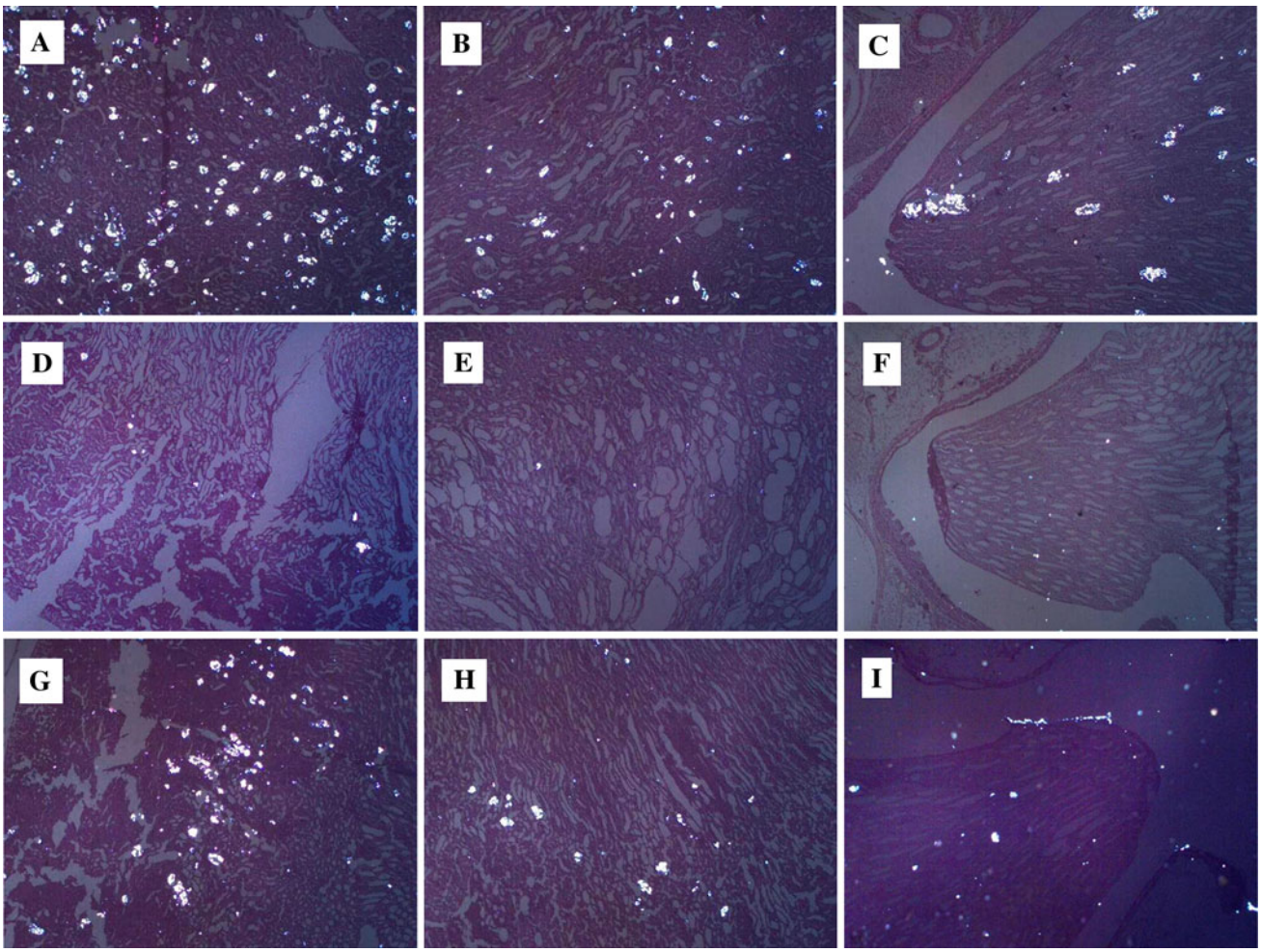

Fig. 3 CaO_x depositions of rat kidney under polarized light microscopy at 21st day. A large number of CaO_x crystals were observed in the cortex (**a**), medulla (**b**), papilla (**c**) of urolithic rat renal section. However, few depositions were found in the cortex (**d**), medulla (**e**) and papilla (**f**) in the rats treated with MD. Crystal accumulations were significantly reduced in cortex (**g**), medulla (**h**), papilla (**i**) of rats treated with HPp compared with the urolithic rats (magnification: ×100)

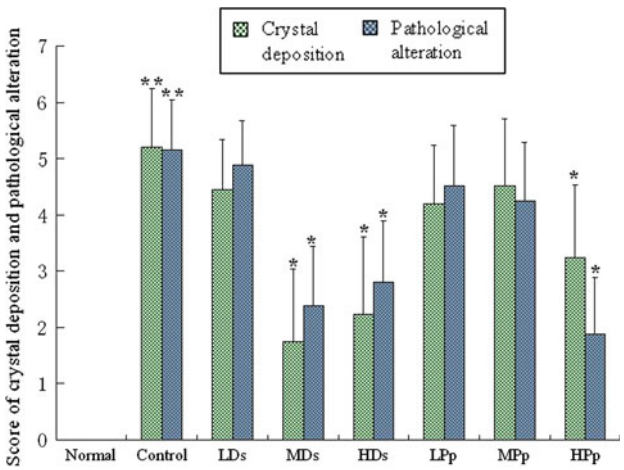

Fig. 4 Score of crystal depositions and pathological alterations at 21st day. Columns and bars represent mean ± SD (*$P < 0.05$ compared with the control, **$P < 0.05$ compared with the normal)

so on [25]. Extensive studies have suggested that hypercalciuria is the result of inhibition of net tubular calcium (Ca) reabsorption rather than glomerular ultrafiltration, and even may be familial. Continuing high level of urinary calcium promotes the nucleation and precipitation of CaO_x from urine and subsequent crystal growth [26]. O_x, a far more significant promoter, has 15-fold greater effective in urinary saturation of CaO_x than Ca [27]. In addition, Cit as an effective complexing agent, is the important inhibitor, which can easily make complex with calcium to form soluble calcium citrate [25]. In our study, urinary Ca in urolithic rats was markedly reduced, but O_x excretion increased. There was no difference in other variables compared with normal rats. Urinary volume and Cit excretion were significantly elevated, and the level of urinary Ca and O_x were decreased in the MDs and HDs group. However, there were no changes observed in serum variables. The similar outcomes were confirmed on urinary Ca and Cit in one previous study from

Japan. They studied the inhibitory effects of triterpenoid extracted from Ds, and also found that the increase of serum Ca was manifested in their research [28]. It is worthwhile to mention that we did not observe the elevation of Ca in serum and urine. On the contrary, hypocalciuria was found. A possible explanation is that CaO_x crystal formation consumes numerous free Ca and result in the reduction of urinary calcium. The above process was suppressed by medium and high dose Ds, and subsequently partially recovered O_x and Ca excretion. The increase of Cit excretion plays an important role in inhibiting CaO_x crystal formation, too. Additionally, 2-fold Urinary volume increase in above two groups showed distinct diuresis, which effectively reduced the concentration of Ca and O_x and increased urine flow avoiding crystal retention. The similar results were also found in HPp groups. Interestingly, the slight decrease of serum Ca only observed only in MPp and HPp groups. The reason is unclear and further analysis is needed to clarify.

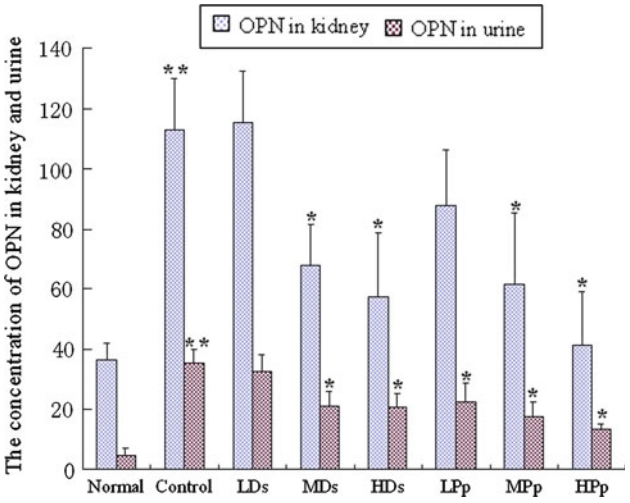

Fig. 5 Quantitative OPN level in kidney and urine at 21st day. Values are expressed as ng/mg protein and ng/ml, respectively. *Columns* and *bars* represent mean ± SD (*$P < 0.05$ compared with the control, **$P < 0.05$ compared with the normal)

Continuous irregular deposits of CaO_x crystal in the kidney not only cause the inflammatory cells to infiltrate around dilated tubules, but also lead to crystal formation in urine. Urinary crystals are closely associated with the extent of urinary stone formation in vivo [29]. Raising urinary Cit or treating with thiazide and cellulose phosphate could lower crystal extent in urine [30, 31]. In crystalluria, there are three different hydrated form, CaO_x

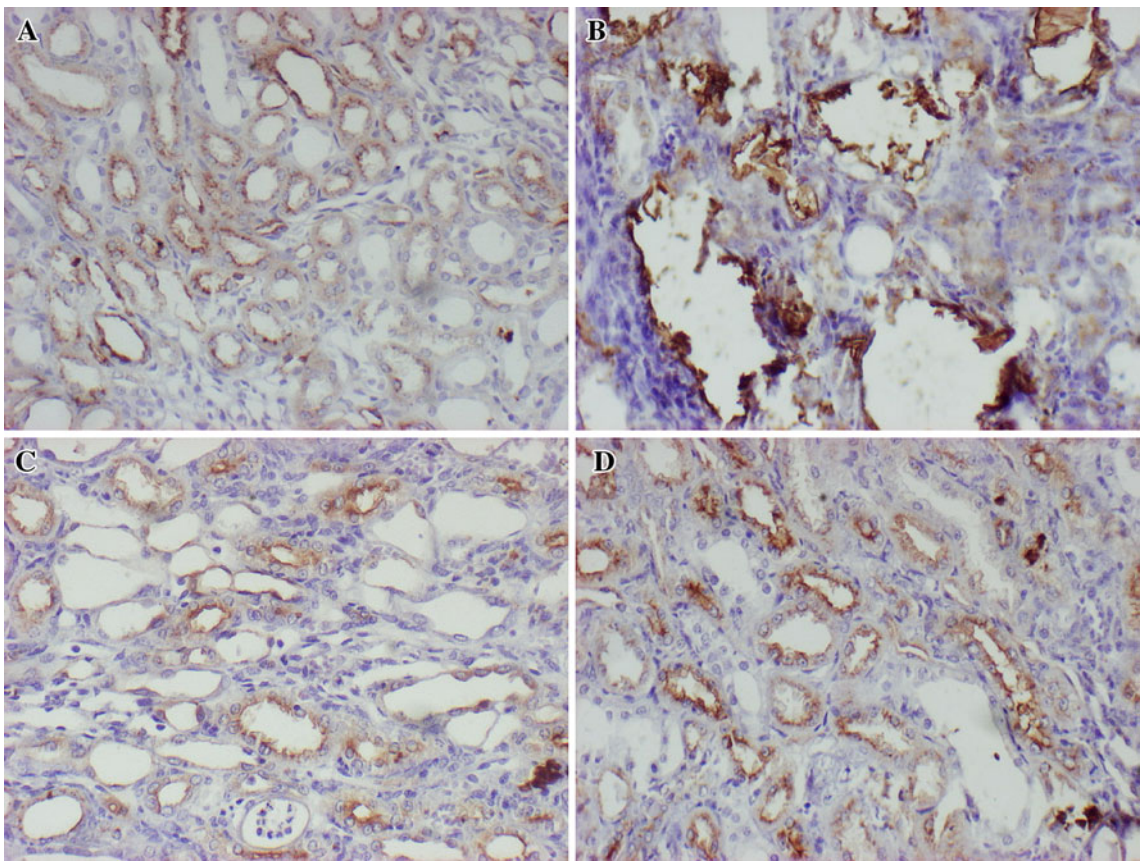

Fig. 6 Immunohistochemical staining revealed expression and location of OPN protein. **a** Weak expression of OPN in normal renal tubular cells. **b** Enhanced OPN expression and obvious dilation of tubules in control rats. **c** Weaker OPN expression and mild dilation of tubules in rats treated with MDs. **d** Weaker OPN expression and mild dilation of tubules in rats treated with HPp (magnification: ×200)

Table 3 Oxidative stress parameters in urolithiasis rats treated with Ds and Pp extract

	Normal	Control	LDs	MDs	HDs	LPp	MPp	HPp
NO (umol/mg protein)	3.26 ± 0.70	6.68 ± 1.29**	5.52 ± 0.86	3.63 ± 1.15*	3.26 ± 1.10*	4.08 ± 0.74*	2.37 ± 1.22*	2.13 ± 0.81*
MDA (nmol/mg protein)	1.13 ± 0.56	3.08 ± 0.61**	2.82 ± 0.97	1.32 ± 0.76*	1.48 ± 0.68*	3.50 ± 0.69	2.13 ± 0.90	1.06 ± 0.61*
SOD (U/mg protein)	216.46 ± 47.70	135.04 ± 26.61**	174.55 ± 42.96	213.66 ± 38.86*	223.31 ± 31.30*	189.14 ± 40.21	245.71 ± 45.73*	236.30 ± 25.29*

Values are expressed as mean ± SD, * $P < 0.05$ when compared to the control, ** $P < 0.05$ when compared to the normal

monohydrate (COM), dihydtate (COD) and trihydrate (COT) [32]. COM and COD are the major components in urines and in renal stones, but COT is rarely observed and seen as a possible precursor in crystal formation [33]. Of three hydrated forms, the monoclinic COM is the most stable phase of thermodynamics and has stronger affinity for renal tubular cell membrane. In addition, dominant COM is closely related to hyperoxaluria whereas COD is mainly associated with hypercalciuria [29]. More COMs mixed with fewer CODs in urine were found in control group by polarizing microscope in our study. Excretion of CaO_x crystal was inhibited in MDs and HDs group and few COMs were observed. With continuous accumulation of CaO_x crystal in the kidney, numerous inflammatory cells infiltrate around dilated tubules, and vice versa. However, a relatively small number of inflammatory cells aggregate around dilated tubules, which suggests that Pp has potential anti-inflammatory effect. It is important to mention that although high dose Pp has significantly inhibited crystal deposition and pathological alteration, crystal level in urine was not attenuated but still high. This phenomenon may be associated with no increase of urine output.

Osteopontin is a multifunctional phosphoprotein with extensive tissue distribution, and a confirmed component of stone organic matrix and a strong inhibitor in CaO_x formation [34]. Free OPN in solution not only inhibits the whole process of nucleation [35], growth [36, 37] and aggregation [38] of CaO_x crystals, but also impede the adhesion of COM crystals to renal epithelial cells [39]. Although OPN is a secreted as a soluble protein, the extracellular matrix can immobilize OPN by cross-linking reaction. Surface-immobilized OPN by collagen granules promotes aggregation and adhesion of CaO_x crystals in artificial urine [40]. Additionally, Hyperoxaluria could significantly up-regulate OPN expression and further promote its secretion after crystal deposition in kidneys. Simultaneously, a concomitant increase was found in urine [41]. In current study, even though the model induced by 5%AmO_x differed from by ethylene glycol, significant elevation of OPN in both urine and kidney was observed in the control group. We also find weak OPN expression in urine and kidney of the normal rat. This confirms that high level urinary oxalic acid induces OPN expression and increases its excretion. Urinary OPN excretion in most treatment groups was suppressed with its weakened expression in kidney when the rats were treated by different doses of Ds or Pp, but it was an exception in low dose Pp. It suggests that OPN excretion may be mainly influenced by the expression in kidney and detection of urinary OPN level could indirectly reflect expression of OPN in kidney. In spite of conspicuous down-regulation of OPN expression, dose-dependent effect was not observed in Ds and Pp groups. The underlying mechanisms may be

related to their abundant flavonoid constituents, which as antioxidants inhibit oxidative stress response so as to down-regulate OPN expression indirectly. Khan SR et al. [41] revealed that with the CaO_x crystal formation, particularly after crystal deposition, OPN expression in the kidney gradually expanded from the loop of Henle and papillary surface epithelium to the whole renal tissue section. We also observed OPN weak expression in normal rats and strong expression in urolithiatic rats. OPN expression was weakened by treatment with Ds and Pp. The present data suggests elevation of OPN expression is achieved through continuously up-regulating renal epithelium cells which does not express or weakly express OPN protein in normal kidney.

Renal epithelial cells injured by O_x and/or CaO_x crystals lead to the overproduction of reactive oxygen species (ROS), which consequently enhance crystallization at physiologic pH and CaO_x crystal deposits in the kidneys [42]. Recent studies have demonstrated oxidant stress in experimental urolithiatic rats would promote the synthesis and release of NO and caused the elevation of MDA secondarily. Meanwhile, activated antioxidant system upregulated SOD activities to scavenge free radical in early stage, but in late stage excessive oxidative stress decreased antioxidant enzyme activities [7, 43, 44]. Therefore, NO, MDA and SOD as the good indicators are widely used to estimate oxidant-antioxidant status both in vitro and in vivo. Vitamin E, an antioxidant, can effectively inhibit CaO_x accumulation by attenuation of tubular cell death and the regulation of OPN and Tamm-Horsfall protein [45]. Our data indicates NO mediated by hyperoxaluria were suppressed by MDs, HDs and different dose of Pp, and MDA level ensues changes. However, Ho-Shiang H and colleagues noted that although production of ROS was signicantly increased in the kidney, NO production remained unchanged. The imbalance of iNOS and eNOS expression in renal medualla is a possible explanation [46]. SOD activities are improvement in MDs, HDs, MPp, and HPp groups. Especially in MDs and HPp groups three markers restore to near normal level and demonstrates optimal antioxidative effects. This may be due to flavonoids in the extracts,which protect cells against the damaging effects of ROS including singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite [18].

On the basis of the results presented here, DS as a traditional antiurolithic herb indicates the beneficial effect on preventing renal stone formation at medium and high dose. However, given their similar effects, MDs is superior to HDs. The underlying mechanism may be concluded as follows: First, Ds up-regulates urinary Cit excretion, and further promotes the formation of soluble calcium citrate, which consume the large number of calcium ions and secondarily result in the reduction of urinary calcium. Second, diuretic action attenuates the concentration of Ca and O_x and inhibits crystal retention. Last but not the least, flavonoid components of Ds, as an antioxidant, protect renal epithelium cells against peroxidative injury, and the increased expression of OPN

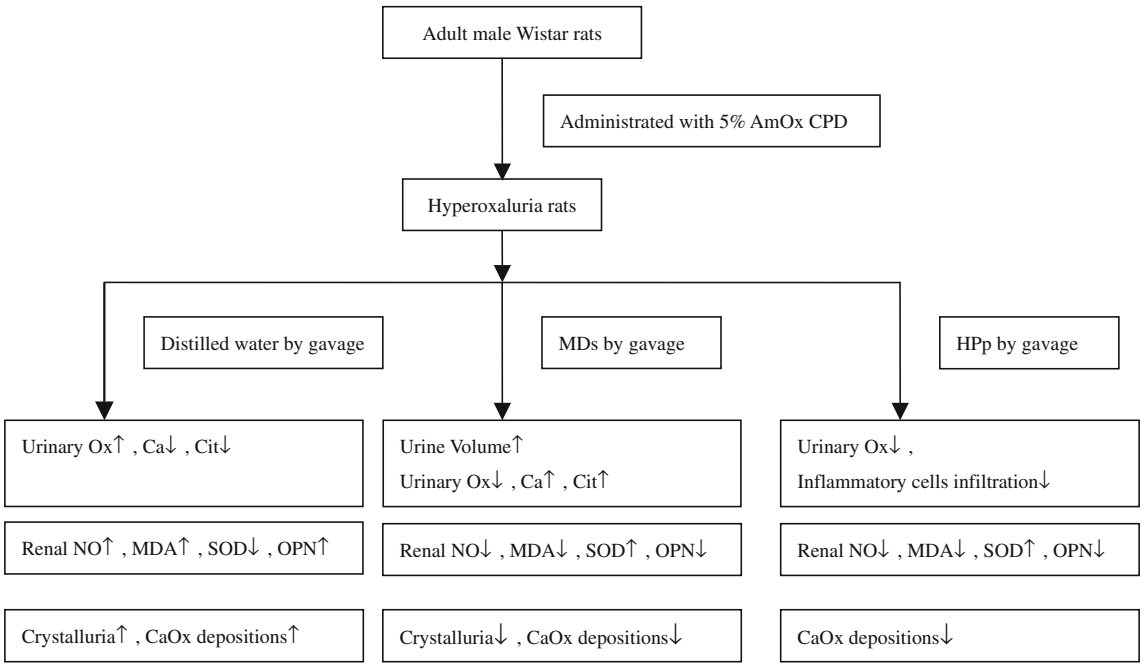

Fig. 7 The underingly mechanisms of antiurolithic effect of *Desmodium styracifolium* and *Pyrrosiae petiolosa*

further suppress renal crystal formation. Meanwhile, high dose Pp shows significant anti-inflammatory and antioxidant effects, and relatively weak prophylaxis of CaO_x stone formation (Fig. 7). However, the active constituents of two herbs remain enigmatic, and further research is needed to identify their biological activity and interaction.

It is well known that herbal therapy emphasizes the combination of herbs to enhance therapeutic efficacy and to reduce adverse effects in theory and practice [47]. Considering CaO_x stone formation as a multifaceted and complex process, we speculate that the combination of Ds and Pp will play a synergistic role in anti-urolithic formula. Currently, we only report their monotherapy effects rather than the combination therapy. The research on their interaction is underway in our laboratory and will be reported in the near future.

References

1. Amato M, Lusini ML, Nelli F (2004) Epidemiology of nephrolithiasis today. Urol Int 72(Suppl 1):1–5. doi:10.1159/000076582
2. Tiselius HG (2003) Epidemiology and medical management of stone disease. BJU Int 91(8):758–767. doi:10.1046/j.1464-410X.2003.04208.x
3. Baumann JM (1998) Stone prevention: why so little progress? Urol Res 26(2):77–81. doi:10.1007/s002400050027
4. Doddametikurke RB, Chandra SB, Anthony JB et al (2007) The Role of Urinary Kidney Stone Inhibitors and Promoters in the Pathogenesis of Calcium Containing Renal Stones. EAU-EBU update series 5(3):126–136. doi:10.1016/j.eeus.2007.03.002
5. Tungsanga K, Sriboonlue P, Futrakul P et al (2005) Renal tubular cell damage and oxidative stress in renal stone patients and the effect of potassium citrate treatment. Urol Res 33(1):65–69. doi:10.1007/s00240-004-0444-4
6. Huang HS, Ma MC, Chen CF et al (2003) Lipid peroxidation and its correlations with urinary levels of oxalate, citric acid, and osteopontin in patients with renal calcium oxalate stones. Urology 62(6):1123–1128. doi:10.1016/S0090-4295(03)00764-7
7. Huang HS, Ma MC, Chen J et al (2002) Changes in the oxidant-antioxidant balance in the kidney of rats with nephrolithiasis induced by ethylene glycol. J Urol 167(6):2584–2593. doi:10.1016/S0022-5347(05)65042-2
8. Madsen KM, Zhang L, Abu Shamat AR et al (1997) Ultrastructural localization of osteopontin in the kidney: induction by lipopolysaccharide. J Am Soc Nephrol 8(7):1043–1053. doi:1046-6673/0807-1043$03.00/0. http://jasn.asnjournals.org/content/8/7/1043.full.pdf+html
9. Lieske JC, Hammes MS, Hoyer JR et al (1997) Renal cell osteopontin production is stimulated by CaOx monohydrate crystals. Kidney Int 51(3):679–686. doi:10.1097/00005392-199805000-00136
10. Kohri K, Nomura S, Kitamura Y et al (1993) Structure and expression of the mRNA encoding urinary stone protein (osteopontin). J Biol Chem 268(20):15180–15184
11. Strohmaier WL (2000) Course of calcium stone disease without treatment. What can we expect? Eur Urol 37(3):339–344. doi:10.1159/000052367
12. Hess B (2003) Pathophysiology, diagnosis and conservative therapy in calcium kidney calculi. Ther Umsch 60(2):79–87
13. Gürocak S, Küpeli B (2006) Consumption of historical and current phytotherapeutic agents for urolithiasis: a critical review. J Urol 176(2):450–455. doi:10.1016/j.juro.2006.03.034
14. The State Pharmacopoeia Committee of China (2005) Pharmacopoeia Commission of the People's Republic of China, vol vol.1. Chemical Industry Press, Beijing
15. Gohel MD, Wong SP (2006) Chinese herbal medicines and their efficacy in treating renal stones. Urol Res 34(6):365–372. doi:10.1007/s00240-006-0068-y
16. Zhao M, Duan JA, Che CT (2007) Isoflavanones and their O-glycosides from Desmodium styracifolium. Phytochemistry 68(10):1471–1479. doi:10.1016/j.phytochem.2007.02.015
17. Wang N, Wang JH, Li X et al (2006) Flavonoids from Pyrrosia petiolosa (Christ) Ching. J Asian Nat Prod Res 8(8):753–756. doi:10.1080/10286020500246550
18. Pietta PG (2000) Flavonoids as antioxidants. J Nat Prod 63(7):1035–1042. doi:10.1021/np9904509
19. Li HB, Jiang Y, Wong CC et al (2007) Evaluation of two methods for the extraction of antioxidants from medicinal plants. Anal Bioanal Chem 388(2):483–488. doi:10.1007/s00216-007-1235-x
20. Kumar S, Sigmon D, Miller T et al (1991) A new model of nephrolithiasis involving tubular dysfunction/injury. J Urol 146(5):1384–1389
21. Hodgkinsona A, Williamsa Ann (1972) An improved colorimetric procedure for urine oxalate. Clinica Chimica Acta 36(1):127–132
22. Jacobs SL, Lee ND (1964) Determination of Citric Acid in Serum and Urine Using Br82. J Nucl Med 5:297–301
23. LaGrange CA, Lele SM, Pais VM Jr (2009) The effect of sodium thiosulfate administration on nephrocalcinosis in a rat model. J Endourol 23(3):529–533. doi:10.1089/end.2008.0204
24. Khan SR (1997) Animal models of kidney stone formation: an analysis. World J Urol 15(4):236–243. doi:10.1007/BF01367661
25. Doddametikurke RB, Chandra SB, Anthony JB et al (2007) The Role of Urinary Kidney Stone Inhibitors and Promoters in the Pathogenesis of Calcium Containing Renal Stones. EAU-EBU update series 5(3):126–136
26. Lemann JJ, Worcester EM, Gray RW (1991) Hypercalciuria and stones. Am J Kidney Dis 17(4):386–391
27. Borghi L, Meschi T, Amato F et al (1996) Urinary volume, water and recurrences in idiopathic calcium nephrolithiasis: a 5-year randomized prospective study. J Urol 155(3):839–843
28. Hirayama H, Wang Z, Nishi K et al (1993) Effect of Desmodium styracifolium-triterpenoid on calcium oxalate renal stones. Br J Urol 71(2):143–147
29. Daudon M, Jungers P, Lacour B (2004) Clinical value of crystalluria study. Ann Biol Clin 62(4):379–393
30. Hallson PC, Rose GA, Sulaiman S (1983) Raising urinary citrate lowers calcium oxalate and calcium phosphate crystal formation in whole urine. Urol Int 38(3):179–181
31. Hallson PC, Rose GA (1976) Crystalluria in normal subjects and in stone formers with and without thiazide and cellulose phosphate treatment. Br J Urol 48(7):515–524
32. Ouyang JM, Deng SP (2003) Controlled and uncontrolled crystallization of calcium oxalate monohydrate in the presence of citric acid. Dalton Trans 63(14):2846–2851. doi:10.1039/b304319c
33. Opalko FJ, Adair JH, Khan SR (1997) Heterogeneous nucleation of calcium oxalate trihydrate in artificial urine by constant composition. J Crystal Growth 181(44):410–417. doi:10.1016/S0022-0248(97)00222-4

34. Kleinman JG, Wesson JA, Hughes J (2004) Osteopontin and calcium stone formation. Nephron Physiol 98(2):43–47. doi:10.1159/000080263
35. Worcester EM, Beshensky AM (1995) Osteopontin inhibits nucleation of calcium oxalate crystals. Ann N Y Acad Sci 760:375–377
36. Hoyer JR (1994) Uropontin in urinary calcium stone formation. Miner Electrolyte Metab 20(6):385–392
37. Shiraga H, Min W, Van Dusen WJ et al (1992) Inhibition of calcium oxalate crystal growth in vitro by uropontin: Another member of the aspartic acid-rich protein superfamily. Proc Natl Acad Sci USA 89(1):426–430
38. Asplin JR, Arsenault D, Parks JH et al (1998) Contribution of human uropontin to inhibition of calcium oxalate crystallization. Kidney Int 53(1):194–199. doi:10.1046/j.1523-1755.1998.00739.x
39. Lieske JC, Leonard R, Toback FG (1995) Adhesion of calcium oxalate monohydrate crystals to renal epithelial cells is inhibited by specific anions. Am J Physiol 268(4 Pt 2):604–612
40. Umekawa T, Iguchi M, Kurita T (2001) The effect of osteopontin immobilized collagen granules in the seed crystal method. Urol Res 29(4):282–286. doi:10.1007/s002400100190
41. Khan SR, Johnson JM, Peck AB et al (2002) Expression of osteopontin in rat kidneys: induction during ethylene glycol induced calcium oxalate nephrolithiasis. J Urol 168(3):1173–1181. doi:10.1016/S0022-5347(05)64621-6
42. Khan SR (2005) Hyperoxaluria-induced oxidative stress and antioxidants for renal protection. Urol Res 33(5):349–357. doi:10.1007/s00240-005-0492-4
43. Volkan T, Eray Kemahli EO, Yasar Volkan A et al (2008) Protective Effect of a Potent Antioxidant, Pomegranate Juice, in the Kidney of Rats with Nephrolithiasis Induced by Ethylene Glycol. J Endourol 22(12):2723–2732. doi:10.1089/end.2008.0357
44. Itoh Y, Yasui T, Okada A et al (2005) Preventive effects of green tea on renal stone formation and the role of oxidative stress in nephrolithiasis. J Urol 173(1):271–275. doi:10.1097/01.ju.0000141311.51003.87
45. Huang HS, Chen J, Chen CF et al (2006) Vitamin E attenuates crystal formation in rat kidneys: Roles of renal tubular cell death and crystallization inhibitors. Kidney Int 71(7):699–710. doi:10.1038/sj.ki.5002148
46. Ho-Shiang H, Ming-Chieh M, Chau-Fong C et al (2006) Changes in nitric oxide production in the rat kidney due to CaOx nephrolithiasis. Neurourol Urodyn 25(3):252–258. doi:10.1002/nau.20152
47. Gilani AH, Rahman A (2005) Trends in ethnopharmacology. J Ethnopharmacol 100(1–2):43–49. doi:10.1016/j.jep.2005.06.001