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ORIGINAL PAPER

Protective effect of *Flos carthami* extract against ethylene glycol-induced urolithiasis in rats

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Abstract *Flos carthami* (FC), also known as *Carthamus tinctorius*, is a traditional Chinese herbal plant that has been prescribed since centuries for treating various symptoms related to blood circulation improvement. This study aimed to investigate the effects of FC on calcium oxalate (CaOx) formation in ethylene glycol (EG)-fed rats. A total of 50 male Sprague–Dawley rats were divided into the following 6 groups: group 1, as the normal control ($n = 5$); group 2 received gastric gavages of starch and 0.75% EG (placebo, $n = 5$) as a stone inducer; group 3 ($n = 10$) received EG and potassium citrate as positive controls; group 4 ($n = 10$) received 0.75% EG and 300 mg/day FC; group 5 ($n = 10$) was treated with EG and 600 mg/day FC; group 6 ($n = 10$) received with EG and 1,200 mg/day FC. For all experimental animals, 24-h urine and blood samples were analyzed at the beginning and end of the experiment. Kidney tissue was histopathologically examined using a polarized light microscope, and crystal deposits were evaluated by a semi-quantitative scoring method; these scores were significantly lower in the FC groups (600 and 1,200 mg/day) than in the placebo group. Thus, FC administration appeared to inhibit the deposition of CaOx crystal EG-fed rats. We, therefore, consider that FC may be effective for preventing stone disease, albeit with certain side effects, such as a bleeding tendency. Further clinical trials are needed for evaluating its benefits and possible side effects.

Keywords Calcium oxalate · Ethylene glycol · Traditional Chinese medicine · *Flos carthami*

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Introduction

Nephrolithiasis has a high prevalence worldwide, including Taiwan [1–3]. Changing lifestyles, genetic factors, and climate-related changes are considered to gradually increase the prevalence and decrease the age of onset [4, 5]. However, long-term treatment strategies for preventing the recurrence of urinary stones are few: these include the administration of potassium citrate (K-citrate) [6]. Currently, several herbal drugs have been used in the treatment

of nephrolithiasis. Reported herbal treatments for urolithiasis include single-plant as well as polyherbal formulae such as Wulingsan, Zhulingtang [7, 8], Takusha [9], *Herniaria hirsuta* extract [10], *Phyllanthus niruri* [11], and other kampo medicines [12–14]. Most of these studies have demonstrated several degrees of inhibition of calcium oxalate (CaOx) crystal formation.

We previously performed a quick screening for antilithic Chinese herbal drugs in a *Drosophila* animal model [15, 16]. Eighty candidate herbal extracts with possible effects on the kidneys were chosen from a database. *Flos carthami* (FC, also known as *Carthami tinctorius,* plant family name: Asteraceae) has been found to have the highest efficacy for the inhibition of CaOx crystal deposition in *Drosophila* Malpighian tubules (unpublished data). Therefore, we chose FC for further studying its effects on the inhibition of CaOx crystallization. FC contains carthamin, neocarthamine, and kaempferol 3-rhamnoglucoside; in China, it has been used in the treatment of urological diseases since centuries [17]. Based on an official website of our government and literatures, FC was initially used to improve blood circulation based on its anti-coagulation effects [18, 19]. FC is also used in amelioration of pain, dilation of coronary arteries, lowering blood pressure, and anti-inflammatory effects. Moderate and low doses of FC can improve blood flow [19]. However, a high dose of FC enhances the risk of bleeding tendencies [20]. Li et al. [21] reported that FC decreased blood viscosity, plasma viscosity, and erythrocyte aggregation in a rat model with blood stasis. However, few animal and clinical studies have been conducted to confirm the effects of FC on kidney stone disease. The purpose of this study was to investigate the effects of FC on CaOx formation in ethylene glycol (EG)-fed rats. Therefore, we focused on the in vivo effects of FC in male SD rats by analyzing changes in body weight and the biochemical parameters of urine and serum along with assessment of CaOx deposition in rat kidneys using a polarized light microscope and a computer-assisted image scoring system.

Materials and methods

Experimental animals and designs

Fifty male Sprague–Dawley (SD) rats (6 weeks of age), each weighing approximately 250–300 g, were purchased from BioLASCO Co (Taipei, Taiwan) and acclimated to a room temperature of 25°C with a 12-h light, 12-h dark cycle. They were housed in the animal breeding center of the China Medical University (Taichung, Taiwan) and fed standard commercial rat chow. The rats were randomly divided into six groups. Rats in the experimental groups (groups 2, 3, 4, 5, and 6) were given free access to drinking water containing 0.75% EG as a stone inducer throughout the entire 4-week experimental period (According to the previous studies [7, 8], the CaOx crystals appeared after EG administration for 4 weeks. The incidence of crystal formation in EG group for 4 weeks was about 90%). The commercial herbal powder of FC was furnished by the Koda pharmaceutical company (Taoyun, Taiwan). The quality control of FC was provided by the Koda pharmaceutical company (Fig. 1). The HPLC fingerprint of FC powder was examined by the Medical and Pharmaceutical Industry Technology and Development Center (Taiwan) (data not shown). Briefly, reverse-phase C18 column (Cosmosil 5C18-MS-II Waters 4.6 × 250 mm, 5 μm, NACALAI), and a linear gradient of 0.1% H3PO4 and acetonitrile at a flow rate of 1 ml/min were used. Chromatographed samples were monitored at UV 203 nm. The metal contents of FC powder were analyzed, and the result revealed cadmium $\leq$ 5.0 ppm, mercury $\leq$ 0.1 ppm, arsenic $\leq$ 2.0 ppm and lead $\leq$ 10.0 ppm.

Rats in group 1 ($n = 5$) served as the normal control and were given de-ionized distilled water and fed normal chow. Rats in group 2 ($n = 5$) were treated with gastric gavages of 60 mg starch and 0.75% EG (placebo) as a stone inducer once daily. Rats in group 3 ($n = 10$) were given EG-added drinking water and daily gastric gavages of K-citrate (500 mg/day). The doses of FC were determined by our pilot study and translated from the dosage used for human. The dose of scientific herbal medicine for human is 3–10 g/day. After conversing of animal doses to human equivalent dose based on body surface area [22], 300 mg/day were determined for the study. We further used 600 and 1,200 mg/day to examine the high-dose response. Rats in group 4 ($n = 10$) were given EG-added drinking water and treated with daily 300 mg FC of gastric gavages. Rats in group 5 ($n = 10$) were given EG-added drinking water and treated with daily 600 mg FC of gastric gavages. Rats in group 6 ($n = 10$) were given EG-added drinking water and treated with daily 1,200 mg FC of gastric gavages. Various treatments were administered to the rats in the same volume (1–2 ml). Body weights of rats were recorded before

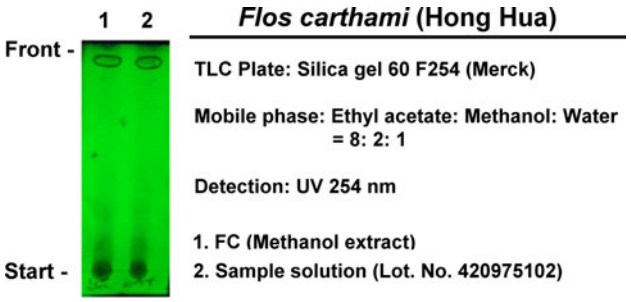

Fig. 1 Thin layer chromatography of *Flos carthami* (Chinese name: Hong Hua) compared with standard

and after the experiment. All rats were sacrificed at the end of the experiment under ether anesthesia. Both kidneys from each rat were harvested and weighed. Right-sided kidneys were fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin Y solution. We then examined the histological sections of kidneys for crystal deposits with a polarized light microscope.

Analysis of serum and urine

Twenty-four-hour urine samples were collected twice, once at the beginning and once at the end of the experiment. For collection of 24-h urine samples, we placed rats individually in metabolic cages. The food and water were given to the rats during 24-h urine collection. Serum was obtained for biochemical analysis at the beginning and end of the experiment. We drew blood samples from the tails at the beginning of the experiment and from the heart at the end. Urine and serum calcium, free calcium, phosphorus, and pH values, and known risk factor parameters such as sodium, potassium, magnesium and citrate for stone disease were measured with a Hitachi-7150 and a Roche-Omnic analyzer. Prothombin time (PT) and activated partial thromboplastin time (APTT) were measured. We checked serum aspartate transaminase (AST), alanine transaminase (ALT), uric acid, and urinary micro-albumin at the end of the experiment.

Evaluation of the severity of renal crystal deposition

We used a polarized light microscope to measure the severity of crystal deposition as previously described. In brief, spotlight the bi-refringent crystals of CaOx with a magnification factor of 100 to evaluate the score. A subjective, semi-quantitative scoring system was used with four grades ranging from 0 to 3+ (where 0 = none, 1+ = few, 2+ = several, and 3+ = many crystal deposits) to evaluate the degree of crystal deposition as previously described [10–12]. The scoring system was according to our computer-assisted image-scoring system *ImageScoring* [7, 8]. This software offers randomized and blinding selection of digital images spontaneously. We then randomly took a digital image of one of the eight regions under a polarized light microscope (100×) using a digital camera mounted onto the microscope. Six independent coworkers used this computer-assisted system to score every individual picture in the database. Finally, all of the personal score files were collected, merged, and sent for statistical analysis.

Statistical methods

Data regarding rat weights and parameters of urine and serum (presented as mean ± SD) were analyzed by one-way ANOVA. The severity of renal crystal deposition was expressed as an ordinal scale using the semi-quantitative scoring method described above. The crystal deposit scores were analyzed by the non-parametric Kruskal–Wallis test for inter-group comparison and by the Wilcoxon rank sum test (Mann–Whitney *U*) for pair wise comparison. A p value of <0.05 denotes the presence of statistical significance. The Statistical Package for the Social Science (SPSS® for Windows, release 11.5) was used for statistical analysis.

Results

There were no differences in the baseline biochemical data among the six groups. No significant differences in body weight, urine volume, pH, micro-albumin, creatinine, uric acid, Na^+, K^+, Cl^+, Mg^{2+}, and Ca^{2+} ions were observed (Table 1). At the end of the 4-week experiment, no significant differences were observed for body weight among the six groups.

At the end of the experimental period, the urine volume in group 2 was significantly increased. By contract, the urine volume in group 6 was significantly decreased after 1,200 mg/day FC administration. Serum creatinine was significantly decreased in the group 3. Serum ALT and AST levels were significantly increased in EG-treated group, whereas FC administration reduced the ALT and AST levels, suggesting a potential hepatoprotective effect of FC. All other urine and serum biochemical data were similar among the six groups.

Histological studies revealed no calcifications in the kidneys of the normal control rats. As expected, several crystal deposits were found in the renal cortex and medulla of the EG-fed rats. Under polarized light microscopy, the crystals exhibited a birefringent appearance as shown in Fig. 2, wherein multiple crystals were found in the renal cortex and the medulla. Analyses of the crystal deposit scores revealed that the scores of the K-citrate- and FC-fed groups (600 and 1,200 mg/day) were significantly lower than those of the placebo group (Fig. 3). Overall, FC administration significantly inhibited CaOx crystal deposition in the kidneys of the experimental rats.

Discussion

The results of the present study were consistent with our hypothesis that FC would inhibit CaOx crystal formation since the severity of renal crystal deposition and the proportion of crystal formation in the 600 and 1,200 mg/day FC treatment groups were significantly lower than those in the placebo group. To the best of our knowledge, this is the

Table 1 Biochemical data of rats

	Group 1 Control	Group 2 EG + starch	Group 3 EG + K-citrate (500 mg/day)	Group 4 EG + FC (300 mg/day)	Group 5 EG + FC (600 mg/day)	Group 6 EG + FC (1,200 mg/day)
Baseline						
Body weight (g)	304.7 ± 12.4	313.2 ± 19.0	302.6 ± 12.3	303.1 ± 17.4	305.4 ± 16.5	302.9 ± 19.6
Urine						
Volume (ml/24 h)	21.2 ± 7.9	25.4 ± 4.9	25.5 ± 11.5	22.8 ± 6.4	20.4 ± 6.	19.2 ± 3.0
pH	7.0 ± 0.1	7.1 ± 0.3	7.0 ± 0.1	7.0 ± 0.2	7.1 ± 0.2	7.2 ± 0.3
Micro-albumin (mg/l)	1.0 ± 1.6	3.1 ± 4.2	3.4 ± 3.6	1.9 ± 2.4	3.5 ± 2.4	2.2 ± 2.4
Creatinine (mg/dl)	39.6 ± 13.7	43.0 ± 11.2	51.1 ± 18.8	51.9 ± 17.2	53.6 ± 17.2	58.5 ± 13.6
Uric acid (mg/dl)	10.6 ± 3.3	9.2 ± 2.4	8.2 ± 2.7	6.9 ± 2.3	11.2 ± 2.3	13.5 ± 3.5
Na^+ (mmol/l)	98.0 ± 17.2	85.7 ± 17.8	111.9 ± 31.6	112.3 ± 34.0	91.9 ± 36.0	98.6 ± 37.9
Mg^{2+} (mmol/l)	20.3 ± 10.4	30.5 ± 15.4	34.0 ± 18.7	29.3 ± 20.4	25.3 ± 20.4	32.1 ± 10.9
Ca^{2+} (mmol/l)	0.51 ± 0.09	0.34 ± 0.04	0.53 ± 0.15	0.54 ± 0.06	0.40 ± 0.15	0.53 ± 0.13
Serum						
pH	7.6 ± 0.2	7.5 ± 0.1	7.4 ± 2.2	7.5 ± 0.1	7.5 ± 0.1	7.5 ± 0.1
Creatinine (mg/dl)	0.63 ± 0.10	0.63 ± 0.11	0.58 ± 0.11	0.63 ± 0.41	0.52 ± 0.09	0.52 ± 0.09
Uric acid (mg/dl)	9.7 ± 0.3	8.9 ± 1.4	8.0 ± 1.6	8.1 ± 1.5	8.1 ± 1.5	7.9 ± 1.7
Na^+ (mmol/l)	120.4 ± 3.9	121.9 ± 2.5	123.1 ± 2.2	111.6 ± 13.1	119.0 ± 4.9	113.3 ± 9.1
K^+ (mmol/l)	17.6 ± 1.6	16.9 ± 4.1	16.9 ± 1.8	16.7 ± 2.2	15.2 ± 2.5	15.5 ± 2.4
Cl^- (mmol/l)	102.6 ± 2.6	103.5 ± 5.2	100.3 ± 1.2	93.7 ± 12.0	97.5 ± 5.5	94.6 ± 7.2
Mg^{2+} (mmol/l)	3.7 ± 0.15	3.1 ± 1.0	3.2 ± 0.3	3.2 ± 1.0	3.1 ± 0.5	3.2 ± 0.7
Ca^{2+} (mmol/l)	0.82 ± 0.09	0.78 ± 0.11	0.87 ± 0.06	0.69 ± 0.17	0.79 ± 0.72	0.74 ± 0.14
4 weeks						
Body weight (g)	439.6 ± 27.5*	441.0 ± 34.0*	405.8 ± 20.3*	417.2 ± 47.4*	423.0 ± 40.8*	409.5 ± 46.3*
Left kidney weight (g)	1.9 ± 0.3	1.9 ± 0.4	1.8 ± 0.2	1.9 ± 0.3	1.7 ± 0.2	1.7 ± 0.1
Right kidney weight (g)	2.0 ± 0.2	1.9 ± 0.2	1.8 ± 0.4	1.8 ± 0.3	1.8 ± 0.3	1.7 ± 0.2
Urine						
Volume (ml/24 h)	26.0 ± 6.0	43.2 ± 15.9#	30.5 ± 13.6	37.0 ± 11.8	27.8 ± 8.3	24.8 ± 8.3†
pH	7.2 ± 0.3	7.0 ± 0.2	7.1 ± 0.4	7.0 ± 0.5	7.0 ± 0.5	7.1 ± 0.4
Micro-albumin (mg/l)	0.5 ± 0.6	2.1 ± 4.2	1.2 ± 3.2	1.1 ± 1.9	1.3 ± 3.8	1.4 ± 2.6
Creatinine (mg/dl)	55.3 ± 16.6	41.9 ± 18.6	50.3 ± 20.2	47.2 ± 19.9	64.0 ± 23.3	58.9 ± 26.8
Uric acid (mg/dl)	7.2 ± 0.6	6.8 ± 3.1	7.3 ± 3.1	6.4 ± 2.8	8.5 ± 2.8	7.7 ± 3.4
Na^+ (mmol/l)	108.9 ± 18.7	78.6 ± 27.7	72.9 ± 16.0	90.9 ± 27.4	109.7 ± 28.5	93.1 ± 41.3
Mg^{2+} (mmol/l)	21.6 ± 15.7	16.1 ± 5.4	14.1 ± 11.8	25.3 ± 7.2	17.6 ± 7.3	12.1 ± 9.6
Ca^{2+} (mmol/l)	0.49 ± 0.24	0.36 ± 0.15	0.43 ± 0.12	0.39 ± 0.26	0.63 ± 0.15	0.53 ± 0.25
Serum						
pH	7.3 ± 0.0	7.4 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.0
Creatinine (mg/dl)	0.45 ± 0.07	0.51 ± 0.11	0.23 ± 0.06†	0.56 ± 0.06	0.51 ± 0.04	0.52 ± 0.04
Uric acid (mg/dl)	2.1 ± 0.5	4.0 ± 1.2#	5.5 ± 0.6	4.5 ± 1.4	3.5 ± 0.7	3.0 ± 0.8
Na^+ (mmol/l)	137.8 ± 1.7	135.6 ± 5.3	135.2 ± 2.1	125.4 ± 38.2	137.4 ± 2.1	137.1 ± 1.2
K^+ (mmol/l)	5.1 ± 0.7	8.1 ± 3.8	7.4 ± 1.3	6.6 ± 0.4	6.2 ± 1.3	6.5 ± 1.7
Cl^- (mmol/l)	100.0 ± 1.0	100.8 ± 1.0	100.1 ± 1.3	99.8 ± 1.6	100.1 ± 0.5	99.6 ± 0.8
Mg^{2+} (mmol/l)	2.2 ± 0.3	2.5 ± 0.5	2.7 ± 0.5	2.7 ± 0.6	2.0 ± 0.4	2.4 ± 0.4
Ca^{2+} (mmol/l)	1.3 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
Albumin (mg/dl)	4.0 ± 0.1	4.0 ± 0.9	4.0 ± 0.3	4.1 ± 0.2	4.0 ± 0.1	4.0 ± 0.2
AST (U/l)	80.8 ± 4.3	153.6 ± 39.7#	185.2 ± 43.1	107.7 ± 25.1†	95.1 ± 10.1†	101.7 ± 20.1†
ALT (U/l)	51.4 ± 5.8	74.6 ± 8.6#	69.8 ± 7.9	64.5 ± 5.1	58.6 ± 7.6†	52.8 ± 13.2†
PT (sec)	10.2 ± 0.3	10.3 ± 0.1	27.9 ± 25.7	10.2 ± 0.3	17.7 ± 19.1	17.4 ± 19.2
APTT (sec)	19.4 ± 2.2	18.6 ± 0.9	75.5 ± 81.0	20.8 ± 3.1	45.5 ± 60.1	43.4 ± 60.3

Values are expressed as mean ± SD

* $p < 0.05$, values are significantly different compared to the relative groups in baseline

# $p < 0.05$, values are significantly different compared to control group

† $p < 0.05$, values are significantly different compared to "EG + starch" group

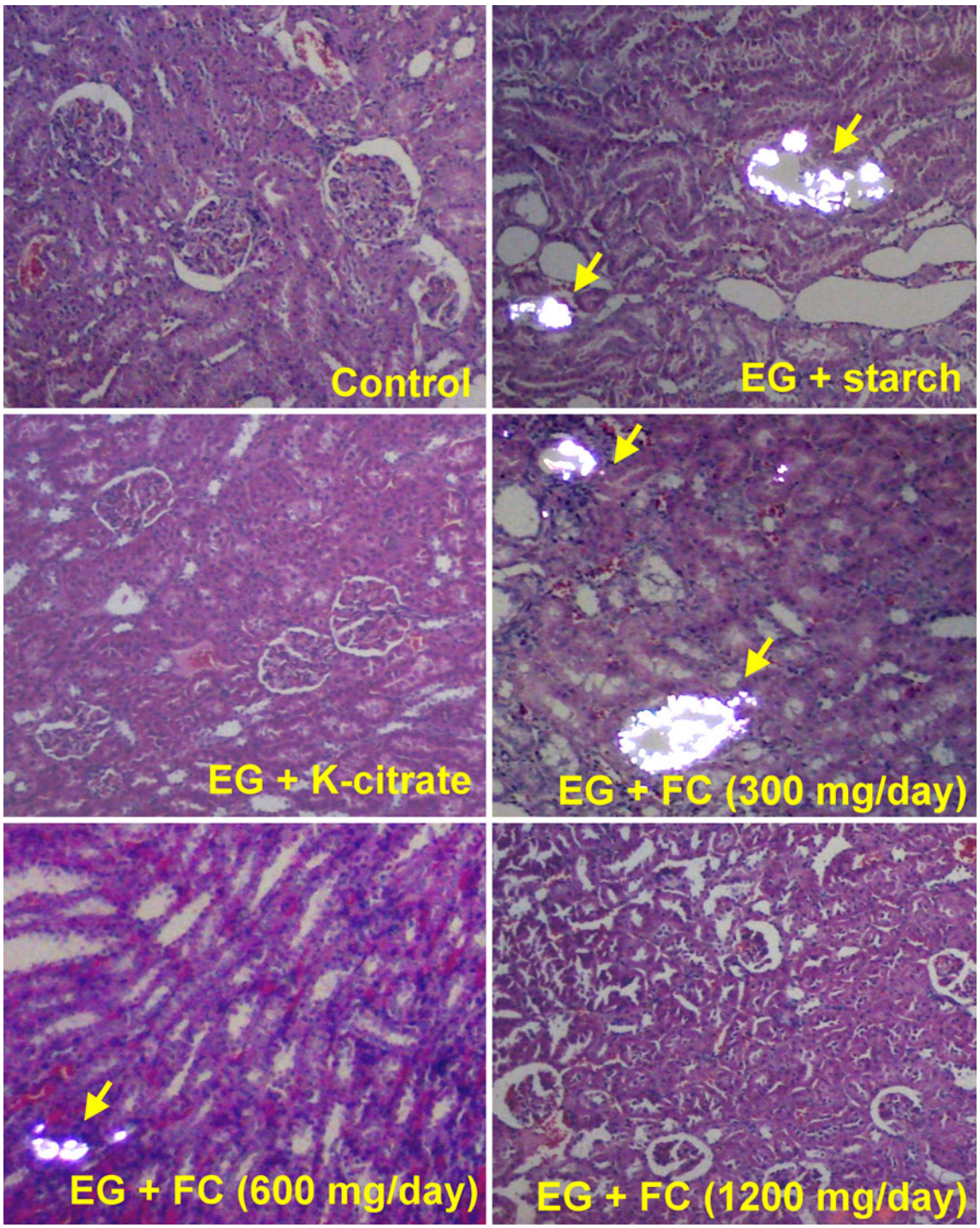

Fig. 2 Represented paraffin sections viewed under polarized light of rat kidneys in each groups. *Arrows* indicate the crystals in the cortex, tubular, and medulla (×100)

first evidence for the inhibitory effect of FC on CaOx crystal formation in EG-fed rats. Based on these preliminary results, we aim to perform further investigations of the prophylactic effects of FC on nephrolithiasis in a larger sample size and different dosages. At least, we can no longer assume that the dose of FC equal to the human daily dose per unit of body weight reveal obvious nephrolithic prophylaxis.

Administering EG to rats is the most common and effective method for inducing CaOx renal stones in vivo, and our present study successfully induced crystal formation using this model [23, 24]. Other methods for

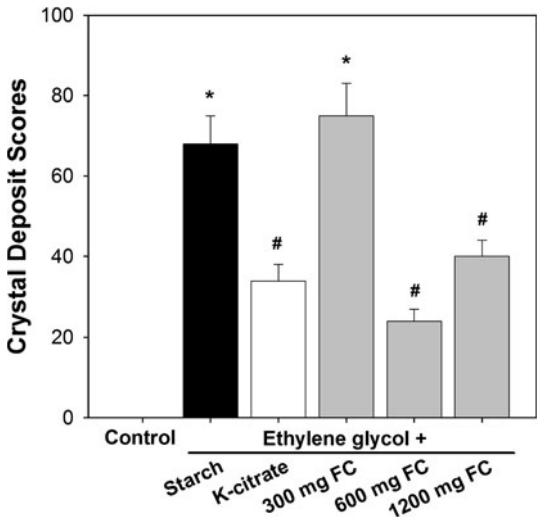

Fig. 3 Quantitative analysis of crystal deposits scores. *Values* represent mean ± SD. *Asterisk* values are significantly different ($p < 0.05$) from the control group, *Hash* Values are significantly different ($p < 0.05$) from the EG + starch group

experimentally inducing CaOx renal stones include the addition of hydroxy-L-proline (HLP) to drinking water or food, implantation of osmotic mini-pumps filled with oxalate, and intraperitoneal administration of oxalate or HLP [25, 26]. However, it has been shown that if rats are made significantly hyperoxaluric via intraperitoneal injections of sodium oxalate or oral intake of EG, CaOx crystalluria occurs rather than the formation of papillary plaques or stones [15, 26, 27]. EG has also been criticized because it induces metabolic acidosis, and some of its metabolites are nephrotoxic in nature [28]. Furthermore, both oxalate and CaOx crystals are known to damage renal epithelial cells. Therefore, it is difficult to distinguish the effects of EG and its metabolites from those induced by oxalate and CaOx crystals. However, Green et al. [29] reported that metabolic acidosis does not always occur if renal function is preserved. Khan et al. reported that HLP, a common ingredient of many diets, is a hyperoxaluria-inducing agent. They further reported that CaOx crystal deposition in animals administered HLP was similar to that observed in other animal hyperoxaluria models of CaOx, although HLP was less nephrotoxic [26]. Therefore, we aim to compare the differences of the effects of FC between HLP and EG-induced nephrolithiasis models in our next investigation.

Many medicinal plants are used to treat urolithiasis worldwide [30–32], of which *Alisma orientalis* and *Poria cocos* Wolf have been shown to inhibit the kidney stone formation process [9, 33–35]. *Alisma orientalis* (also known as Takusha) strongly suppresses each step of crystal formation, growth, and aggregation of CaOx crystals in vitro; furthermore, the renal calcium content has been shown to decrease in *Alisma*-treated rats [33, 36]. In addition, several studies have shown that this herb significantly decreased the formation of CaOx deposits [35, 37, 38]. All of these reports suggest that herbal medicines may be a useful strategy for preventing renal stones. However, it is important to note that FC seems to prolong the PTT and APTT. Since FC was initially used to improve blood circulation in the traditional Chinese medicine, it should be noticed when used in patients with abnormal function of coagulation, although there were no apparent bleeding complications in the animals treated with the FC extract.

The present study has some limitations. The antilithic mechanism of FC remains unclear. Some key urine numbers are not provided, including urine oxalate and citrate. Without this sort of basic information, we have little information on what the extract may or may not be doing. In addition, whether the extract changes EG metabolism to oxalate or oxalate handling, and does it inhibit crystallization, and its cellular responses are unclear. A complete characterization of the model and effects of the extract on urinary components would interpret these results; appropriate evaluation must be further established.

The software (*Image Scoring*) that we created has proved to be a helpful tool, because it could easily reduce the time-consuming process of reviewing a multitude of images. Furthermore, the automatically randomized selection process diminished examination bias and offered a reliable method for blindness toward the samples. Moreover, the format of the scoring files was compatible with the statistical package used; thus, no data transfer was needed, preventing the possibility of transfer errors [7, 8].

In summary, our results revealed that FC administration led to a significant reduction in CaOx crystal formation as compared to that in the placebo group. The herbal medicine FC appeared to effectively prevent kidney stone formation, indicating that this herb might be an effective antilithic drug. The exact mechanism underlying its effects remains unknown, and further experimental and clinical studies are required to elucidate the chemical constituents of the extracts and the mechanism(s) responsible for its pharmacological activities.

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Conflict of interest The authors declare that there are no conflicts of interest.

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