



# An insight investigation to the antiurolithic activity of *Trachyspermum ammi* using the in vitro and in vivo experiments

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## Abstract

The crude extract of *Trachyspermum ammi* seeds (Ta.Cr) was studied for its antiurolithic activity using the in vivo and in vitro experiments. In the in vivo experiments, Ta.Cr treatment showed a diuretic activity at the dose of 30 and 100 mg/kg and exhibited curative effect in male hyperoxaluric Wistar rats, which received 0.75% ethylene glycol (EG) in drinking water given for 3 weeks, with 1% ammonium chloride (AC) for initial three days. In the in vitro experiments, Ta.Cr delayed the slopes of nucleation and inhibited the calcium oxalate (CaOx) crystal aggregation in a concentration-dependent manner like that of potassium citrate. Ta.Cr also inhibited DPPH free radicals like standard antioxidant drug butylated hydroxytoluene (BHT), and significantly reduced cell toxicity and LDH release in Madin–Darby canine kidney (MDCK) cells, exposed to oxalate (0.5 mM) and COM (66 µg/cm<sup>2</sup>) crystals. In isolated rabbit urinary bladder strips, Ta.Cr relaxed high K<sup>+</sup> (80 mM) and CCh (1 µM)-induced contractions, showing antispasmodic activity. The findings of this study suggest that the antiurolithic activity of crude extract of *Trachyspermum ammi* seeds may be mediated by a number of mechanisms, including a diuretic, an inhibitor of CaOx crystal aggregation, an antioxidant, renal epithelial cell protection, and an antispasmodic, thus, showing the therapeutic potential in urolithiasis, for which there is no viable non-invasive option in modern medicine.

**Keywords** Urolithiasis · *Trachyspermum ammi* · In vitro · In vivo · Rats · MDCK cell line

## Introduction

Urinary stone formation is one of the earliest disorders. Its incidence and prevalence is constantly increasing, with no regard for geographical, cultural, or racial boundaries. Calcium oxalate (CaOx) alone or in combination with the nucleus of calcium phosphate accounts for approximately 80% of kidney stones, while uric acid and struvite stones account for 9 and 10%, respectively, leaving only 1% for

all others such as cystine, ammonium acid urate and others [1, 2].

Clinical and experimental data provide strong evidence for the development of reactive oxygen species (ROS) production and oxidative stress (OS) in the kidneys of stone-forming patients and rats, respectively. Initially, ROS prevents stone formation by the production of crystallization inhibitors. However, decreased antioxidant capacity or persistently supersaturated urine, may result in OS and urolithiasis [3]. It is also evident that OS and inflammation are associated with both nephrolithiasis and cardiovascular diseases. In addition, kidney stone formation can lead to chronic kidney disease, hypertension, diabetes and myocardial infarction (MI) [4]. Antilipidemic drug; atorvastatin, reduce in renal epithelial injury and CaOx crystal deposition in hyperoxaluric rats [5]. Renal cells exposed to CaOx crystals secrete superoxide in real-time as measured by an electrochemical superoxide biosensor [6] while the presence of antioxidants considerably reduced the cell injury and improved the antioxidant status of the cells when exposed to Ox, CaOx or CaP crystals [7]. There is limited success in the treatment with pharmaceutical medicine while

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surgical procedure is expensive and beyond the reach of a common man, particularly in developing countries. According to WHO, traditional medicine comprising mainly herbs provides an economical and affordable source of drugs for three-quarters of the world population and its therapeutic use is increasing day by day [8, 9]. In this study, we selected the seeds of *Trachyspermum ammi* (L.), which is traditionally used as diuretic, litholytic, antiseptic, in stomachache, colic among other multiple uses [10–12]. The phytochemical constituents are carbohydrates, calcium, iron, carotene, nicotinic acid, riboflavin, beta-pinene, alpha-pinene, potassium, protein, thiamine and thymol [12].

*Trachyspermum ammi* has been scientifically evaluated for its anthelmintic, antiaggregatory of human platelets [13], antihistaminic [14], dislipidemic [15], hepatoprotective, bronchodialatory, antihypertensive, antispasmodic [16], antioxidant and antimutagenic [17] activities. In 1990, a study was conducted to see the antiurolithic effect of different plants including *Trachyspermum ammi*. At the end of the experiment, it was concluded that all other plants were quite effective, *Trachyspermum ammi*, which give only 29% protection as opposed to others [18], the authors, therefore, concluded that their data did not support the traditional use of *Trachyspermum ammi* in the treatment of urolithiasis. In this study, the *Trachyspermum ammi* was used in the powder form, with oral gavages and 3% glycolic acid and measured calcium and oxalate content in kidney tissue, which is not a very sensitive approach to evaluate antilithiatic properties. Kaur, T. has isolated anticalcifying protein from *Trachyspermum ammi* and study its antiurolithic action [19]. Therefore, this study has been undertaken to investigate anti-urolithic activities of the crude extract of *Trachyspermum ammi* seeds (Ta.Cr) using the in vivo calcium oxalate and diuretic activity in animal models and several in vitro experiments including antioxidant, crystallization assays, renal epithelial (MDCK) cells protective and spasmolytic using rabbit urinary bladder strips, to investigate the mechanisms likely to contribute in its potential antiurolithic effect and to rationalize medicinal use of Ta.Cr in the management of kidney stones.

## Methodology

### Chemicals

Chemicals were obtained from the following specified sources: 1,1,3,3,-tetraethoxy propane, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5-5'-dithiobis, ammonium chloride, bovine serum albumin, calcium oxalate monohydrate crystals (COM), butylated hydroxyl toluene (BHT), sodium oxalate, ethylene glycol (EG), carbachol (carbamylocholine chloride, CCh), ferrous sulfate heptahydrate, Folin–Ciocalteu's

phenol reagent, Hydrogen peroxide ( $H_2O_2$ ), petroleum spirit and n-butanol (Merck, Darmstadt, Germany). Nuclear fast red, potassium citrate tribasic hydrate, silver nitrate ( $AgNO_3$ ), thiobarbituric acid, potassium chloride, verapamil hydrochloride, 5–5'-dithiobis, thymol, trichloroacetic acid, 2-nitrobenzoic acid (DTNB), (Sigma Chemical Company, St. Louis, MO, USA). For the estimation of blood urea nitrogen, calcium and magnesium Randox Kits (Randox Laboratories Ltd. Ardmore, Diamond Road, Crumlin, Co. Antrim, UK) were used. Trinity Biotech (Plc, IDA business park, Bray, Co. Wicklow, Ireland) kit was used to estimate oxalate while citrate kit was obtained from R-Biopharm AG, D-64293 Darmstadt. Reagents of histopathology: eosin, hematoxylin, xylene, paraffin wax, and chemicals for physiological salt solutions were calcium chloride ( $CaCl_2$ ), glucose, ethylenediamine tetraacetic acid (EDTA), magnesium chloride ( $MgCl_2$ ), potassium dihydrogen phosphate ( $KH_2PO_4$ ), magnesium sulfate ( $MgSO_4$ ), potassium chloride (KCl), sodium bicarbonate ( $NaHCO_3$ ), sodium dihydrogen phosphate ( $NaH_2PO_4$ ), were obtained from Merck, Darmstadt, Germany. Sodium chloride (NaCl) was obtained from BDH Laboratory Supplies, Poole, England, and potassium chloride (KCl) was from Sigma Chemical Co, St Louis, MO, USA.

### Animals

Wistar rats (190–220 g) of either sex were housed at the animal house of the Aga Khan University and kept in cages ( $47 \times 34 \times 18 \text{ cm}^3$ ) containing saw dust (renewed after every 2 days). Controlled temperature of 23–25 °C and 12 h light–dark cycles were maintained in the animal house. A standard diet containing (Kg/10 kg): bran (3.85 kg), flour (3.85 kg), molasses (115 g), salt (57.7 g), nutrivet L (19.25 g), potassium meta bisulphate (11.5 g), oil (385 g), fishmeal (1.73 kg), and powdered milk (1.54 kg), and tap water was given ad libitum to animals. However, no food was given 24 h before and during 6 h of diuretic study, and during collecting 24 h urine samples.

The study was approved by the Board of Advance Studies & Research, University of Karachi through reference No. BASR/No./2274-A/Sc, Resol. No. 09 (08) as part of the research proposal for PhD research. All experiments were performed in agreement with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, [20]. Research Council (1996) and all efforts were made to minimize suffering for animals used in this research.

### Extraction method

Dried seeds of *Trachyspermum ammi* were purchased from a local herbal store and identified by Prof. Dr. Jhandar Shah, Taxonomist/Ex Vice-chancellor Shaheed Benazir Bhutto

University, Sheringal, Pakistan. The sample of the seeds was submitted to the herbarium of the Department of Biological and Biomedical Sciences (DBBS), the Aga Khan University, Karachi, with voucher specimen No. TA-SE-06–08-73. The clean seeds were extracted with 70% methanol (70% methanol and 30% water, v/v) and stored for three days, with intermittent shaking. It was filtered twice: once with a muslin cloth and once with Whatman qualitative grade 1 filter paper. This procedure was repeated three times, and then the combined filtrate was evaporated on a rotary evaporator at low pressure (60 mmHg) to a thick, semi-solid brown mass, i.e. the crude extract of *Trachyspermum ammi* seeds (Ta.Cr). The final yield of the crude extract was approximately 17% w/w [24].

### In vivo diuretic activity

Wistar rats (180–220 g) of either sex were used for the diuretic activity of Ta.Cr according to the method described previously [21]. Briefly, animals were divided into six groups, each having 6–8 rats. Normal saline and positive control groups were given saline (20 ml/kg of body weight) by gavage and hydrochlorothiazide (HCT; 10 mg/kg), respectively. While groups 3–6 were given 10, 30, 100 and 300 mg/kg body weight of Ta.Cr in saline. Afterward, each rat was individually placed in the metabolic and diuretic cage. At 2-h interval, the urine was collected in graduated cylinders for 6 h. The volume of the total excreted urine for 6 h was determined. Then its pH was determined using pH meter (Orion® 320 PerpHecT™ Thermo Scientific), while Na<sup>+</sup> and K<sup>+</sup> concentrations were measured on Corning's flame photometer (Flame Photometer 410).

### Studies on animal models of urolithiasis

Anti-urolithic activity of the extract was determined using the rat model of CaOx urolithiasis as mentioned in our previous studies [22, 23]. Male Wistar rats weighing 180–220 g were divided into 8 groups each containing 6 rats to receive various treatments for preventive and curative effects of Ta.Cr.

In preventive study model, normal water was given to rats of group I, which served as normal control. Whereas Group II; served as the diseased control group, were given water containing EG (0.75% w/v) for 21 days along with NH<sub>4</sub>Cl (AC) (1% w/v) for the first 5 days. Group I and II received i.p injection of normal saline (2.5/kg) while group number III and IV, received i.p injections of Ta.Cr 30 and 100 mg/kg, respectively, once in a day along with stone-inducing treatment (EG + AC), like that of group II (Disease control).

In the curative study, CaOx stones were induced in the kidneys of both untreated control (Group VI) and treated (Groups VII and VIII) rats with EG (0.75%) and NH<sub>4</sub>Cl (1%) administration for 21 days, while the group V received

normal tap water for the first three weeks. Thereafter, stone-inducing treatment was withdrawn and, normal and diseased control groups were given normal saline 2.5 ml/kg, while group number VII and VIII received Ta.Cr 30 and 100 mg/kg, respectively, for another 2 weeks.

Animal weights and their activity were observed regularly in both the preventive and curative study models, whereas 24 h urine samples were collected at the start day 0, 21 and 35. 3-h morning urine sample was collected after both 21 and 35 days, for crystalluria measurement.

24 h urine samples were stored at –20 °C after volume and pH were determined. Blood was taken from rats under ether anesthesia via cardiac puncture to separate serum creatinine and blood urea nitrogen (BUN). Then experimental rats were sacrificed and their kidneys were fixed in 10% neutral-buffered formalin, processed, embedded in paraffin wax, sectioned at 5 m, and stained for microscopic examination with haematoxylin and eosin (H&E) and Pizzolato's method for calcium oxalate crystals [24].

### Deposition of calcium oxalate crystal in kidneys

Crystal distribution within the kidneys was determined by using the semi-quantitative scoring methods used previously [22, 23]. Briefly, the crystal deposits in stained sections with visible in a field of 10× magnification were counted, and severity grades were assigned as 0=no crystals, 1=1–10 crystals, 2=11–30, 3=31–50, 4=51–75 and 5=more than 75 crystals. Most of the crystals were located in the cortical and outer modularly region of the kidney.

### Urine and serum analyses

Urine samples for Ca<sup>++</sup>, citrate, Ox, Mg<sup>++</sup>, and uric acid (UA) and serum samples for and blood urea nitrogen (BUN) and creatinine contents were measured through commercially available kits, while urinary protein and inorganic phosphate were measured through Lowry's [25] and molybdenum blue (MB) reaction [26], respectively.

### In vitro crystallization assays

#### Kinetic study

The effects of Ta.Cr on calcium oxalate (CaOx) crystallization was verified by the measurement of the change in turbidity with time, due to the crystal nucleation and aggregation of Ca<sup>++</sup> and Ox after mixing metastable solutions of Ca<sup>++</sup> and Ox. Stock solutions of CaCl<sub>2</sub> (8.5 mM) and Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub> (1.5 mM), containing sodium acetate (10 mM) and NaCl (200 mM) were adjusted to pH 5.7 [27]. To inspect the event of CaOx crystallization, platelet aggregometer (Chrono-Log Corporation, USA), was used [28]. CaCl<sub>2</sub> solution (0.5 ml)

was stirred continuously both in the absence and presence of different concentrations of standard drug (potassium citrate) or extracts at 37 °C. Crystallization was induced by the addition of Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub> solution (0.5 ml) to get the final concentration of 0.75 mM of Ox and 4.25 mM of Ca<sup>++</sup>. The change in turbidity of the solution with time was concurrently started on a chart, which was moving for around 15 min, at the speed of 30 mm/h with constant stirring of the solutions. All the experiments were repeated three times. Linear regression analysis was used to calculate the slopes of aggregation phases (S<sub>A</sub>) and nucleation (S<sub>N</sub>). The percentage inhibition was calculated as [(1-Se/Sc) × 100], where Se is the slope in the presence of plant extract and Sc is the slope of the control experiment.

### Incubation assay

To examine the effect of incubation of extract or control drug; potassium citrate, on CaOx crystal formation, stock solutions of Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub> and CaCl<sub>2</sub>; similar to that used in kinetic study, were used. CaCl<sub>2</sub> solutions, containing various concentrations of potassium citrate or Ta.Cr, were aliquoted (0.5 ml) in a 24 well (flat-bottomed) plate. (Iwaki Microplate with lid, Scitech Div., Asahi Techno Glass, Japan). Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub> solution (0.5 ml) was added to each well [29]. Three concentration of each test material was prepared. The plates were then incubated for 45 min. at a temperature of 37 °C in a shaking water bath. Abundance and the morphology of the crystals in each tube were then examined under an inverted microscope (Nikon Corporation, Tokyo, Japan).

### Antioxidant activity

Antioxidant activity of the Ta.Cr was evaluated *in vitro* by lipid peroxidation and free radical scavenging activities.

### Lipid peroxidation inhibitory activity

Lipid peroxidation inhibitory potential was evaluated in homogenized kidney tissues of Wistar rats (Zero-Max<sup>®</sup>). The homogenate was prepared in ice-cold PBS (50 mmol/l, pH 7.4) as reported earlier [30]. Briefly, the homogenate was centrifuged for 10 min at 10,000 × g and the supernatant was incubated for 01 h in 10 μM FeSO<sub>4</sub> and 0.1 mM ascorbic acid at 37 °C, in the absence (control) or presence of different concentrations (50 & 150 μg/ml) of the test material. The reaction was blocked/stopped by adding 0.5 ml TCA (28%) and 0.75 ml TBA (1%) in succession and then heated for 20 min at 90 °C. This incubation caused the formation of pink-colored malondialdehyde–thiobarbituric acid (MDA–TBA), which was extracted with n-butanol (3 ml). Then the color intensity of the MDA-TBA complex was measured by spectrophotometer (DU 730, Beckman

Coulter) at 532 nm. The same formula, given above for the free radical scavenging activity, was used to determine the inhibition ratio.

### Free radical scavenging activity

To determine the free radical scavenging potential, 1 ml of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical solution prepared in methanol, was added to 3 ml of the different concentrations of test material [31]. These solutions were then incubated for 30 min at room temperature. Then absorbance was evaluated at 517 nm. A rise in DPPH radical-scavenging activity was indicated by a decrease in the absorbance of DPPH solution. This activity is shown as % DPPH radical-scavenging and calculated by using the following equation keeping DPPH solution as control:

% DPPH radical - scavenging

$$= 1 - \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control}} \times 100.$$

### Effect on MDCK (Madin–Darby canine kidney) cell lines

MDCK cells were purchased from ATCC (Manassa, VA, U.S.A.) and maintained as sub-confluent monolayers in 5% CO<sub>2</sub> at 37 °C. 75 cm<sup>2</sup> Falcon tissue culture flasks were used for cell cultures growth, in a 1:1 mixture of modified Eagle's medium (DMEM) nutrient mixture and F-12 medium (DMEM/F-12) having 10% fetal bovine serum (Gibco BRL), 2% streptomycin/penicillin, pH 7.4. The medium was changed regularly at every 3 to 4 days.

### Cell viability assay

Media was aliquoted to selected wells of a 96 well plate, containing MDCK cells and viability of the cells were assessed by XTT cell viability Assay Kit obtained from Biotium, Inc., Hayward, CA, USA). Cells were incubated for 24 h with different concentration of Ta.Cr. Then 50 μL of the working activation solution, prepared by mixing of one bottle of XTT solution and one of the activation Reagent PMS, was added to all the wells and incubated for 2–4 h. Optical density was read at 450–490 nm on a Bio-Rad 3550 microplate reader.

### Lactate dehydrogenase (LDH) assay

Media was aliquoted to selected wells of a 96-well plate. CytoTox 96 Non-Radioactive Cytotoxicity assay kit was used for determination of percent LDH release. The



substrate, supplied with the kit, was added to the blanks (acclimatization media), positive control (MDCK cells containing lysis solution-only), and all samples. The plate was incubated for 30 min at normal temperature in the dark. Stop solution, supplied with kit, was added to blanks, positive control and all samples. Bio-Rad 3550 microplate reader (Bio-Rad, Hercules, CA) was used to read the optical density at 490 nm.

### Antispasmodic activity in the urinary bladder

Antispasmodic activity of Ta.Cr was assessed against high  $K^+$  (80 mM) and carbachol (CCh; 1  $\mu$ M)-induced contractions in rabbit's urinary bladder strips [32]. Each strip of the bladder was mounted in a 10–15 ml tissue bath containing Krebs–Henseleit solution. Tissue baths were continuously bubbled with carbogen and maintained at 37 °C. Throughout the experiment, a tension of 1 gm was applied to each tissue. Power lab (AD Instruments Australia) was used to record the isometric responses of the tissues. Before the addition of any drug, the tissues were kept for a period of about 01 h to equilibrate. During this period, the isolated tissues were washed with fresh bathing fluid at an interval of every 20–25 min.

After equilibrium, the reparations were stabilized, by treating with 1  $\mu$ M CCh repeatedly, until constant responses were obtained. Then the spasmolytic activity of the Ta.Cr was determined by adding Ta.Cr on CCh and high  $K^+$ -induced sustained contraction in rabbit bladder, in a cumulative way to get the concentration-dependent inhibitory response [33].  $IC_{50}$  values (concentration causing 50% inhibition) were calculated from these curves and calculated as a measure of the spasmolytic potency of the extract.

$Ca^{++}$  CRCs were made in the absence (control curve) and the presence of increasing concentrations of Ta.Cr, for confirmation of the  $Ca^{++}$  channel blocking (CCB) activity. For this purpose, the tissues were stabilized in a normal Krebs–Henseleit solution, which was then replaced with  $Ca^{++}$ -free Krebs–Henseleit solution containing EGTA (0.1 mM) to remove  $Ca^{++}$  from the tissues. Then this solution was replaced by  $K^+$ -rich and  $Ca^{++}$ -free Krebs–Henseleit solution. This solution was refilled 3–5 times with 10 min intervals and then, the  $Ca^{++}$  CRCs were constructed in the absence (control curve) and the presence of different concentrations of the verapamil or Ta.Cr [34]. Isometric responses were recorded on the PowerLab 4/24 data acquisition system attached to the computer, running Lab Chart 6 (AD Instruments, Sydney, Australia).

Similarly, for the determination of anticholinergic activity, the cumulative CCh-CRCs were constructed in

the presence of the increasing concentration of the test material (Gilani et al., 2008).

### Data analysis

All these data are expressed as mean  $\pm$  standard error of mean (SEM,  $n$  = number of experiments) and the median effective concentration ( $EC_{50}$  value) with 95% confidence intervals (CI). The statistical comparisons between the two groups are made through Student's  $t$  test, while one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test, was used for comparisons among more than two groups.  $p$  value less than 0.05 ( $p < 0.05$ ) is considered as significant. Concentration–response curves (CRCs) were analyzed by non-linear regression using GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA, USA).

## Results

### In vivo diuretic activity

Ta.Cr (30 and 100 mg/kg) increased the urine output in Wistar rats, whereas, HCT (10 mg/kg) was used as a standard drug (Fig. 1A). Also, Ta.Cr also increased ( $p < 0.05$ ) urine excretion of  $Na^+$  at the dose of 100 mg/kg; however, slight but no significant increase was observed in the  $K^+$  excretion (Fig. 5C and D). No significant change was observed in the urine pH of any group (Fig. 1B).

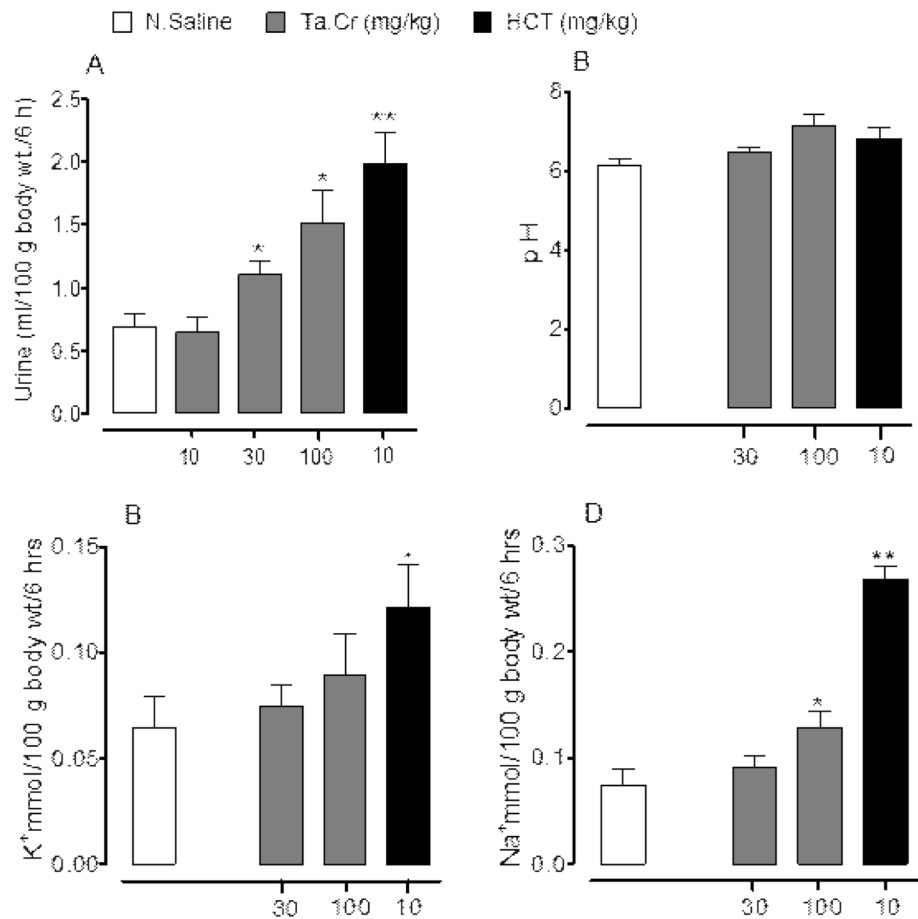
### Effect on rat model of urolithiasis

#### Preventive study

In the preventive study, all the parameters were recorded before (day 0) and 3 weeks after the treatment. The parameters recorded before the treatments were not different ( $p > 0.05$ ) among the groups. After the treatment, the stone-forming group (diseased control) was compared with normal control and also with the treated groups.

At the end of the 3 weeks' treatment, a significant loss in body weights was caused by the lithogenic treatment (EG and AC) in the stone-forming group (group II) as compared to saline (normal control). The co-administration of Ta.Cr (30 and 100 mg/kg) prevented the net loss in body weight, 24 h urine volume and water intake were elevated in the stone-forming (lithogenic) group compared to that of saline (Normal control) animals. Urine pH was also reduced ( $p < 0.05$ ) in the stone-forming group. A co-treatment with Ta.Cr prevented these urinary changes compared to that of saline (Normal control) group (Table 1). In 3 h morning urine, significantly more and bigger CaOx crystals mostly of COD were observed in the stone-forming group as compared

**Fig. 1** Effect of the crude extract of *Trachyspermum ammi* (Ta.Cr) and hydrochlorothiazide (HCT) on urine volume (A), pH (B),  $K^+$  (C),  $Na^+$  contents (D), collected in 6 h (values shown are means  $\pm$  SEM,  $n=6$ ). \* $p<0.05$ , \*\* $p<0.01$  compared with saline-treated group



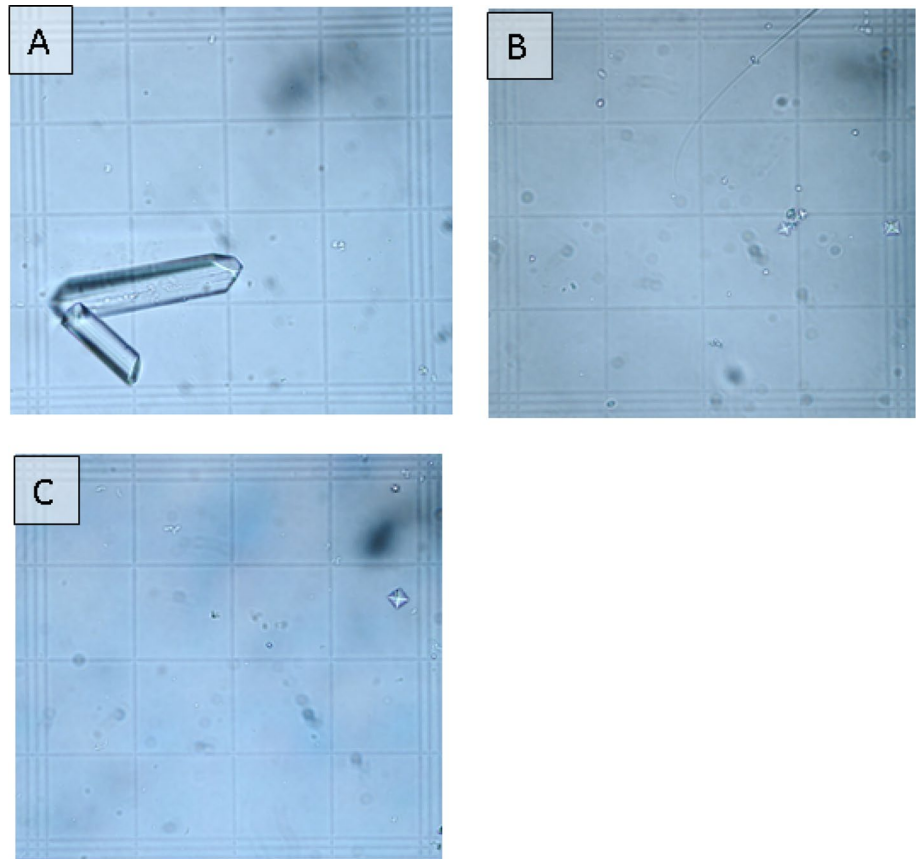
**Table 1** Various parameters from different groups of rats recorded after 21 days, to study the preventive effect in animal model of urolithiasis

	Baseline Mean $\pm$ SEM	N. Saline Mean $\pm$ SEM	Lithogenic group (EG) Mean $\pm$ SEM	EG + Ta.Cr (30 mg/kg) Mean $\pm$ SEM	EG + Ta.Cr (100 mg/kg) Mean $\pm$ SEM
% Change in BW		17 $\pm$ 1.5	-3.8 $\pm$ 2.3**	8.7 $\pm$ 2.4##	9.2 $\pm$ 2.6##
Urine vol ml/24 h	6.7 $\pm$ 1	7.7 $\pm$ 1.2	15 $\pm$ 1.5**	9.1 $\pm$ 1.6#	9 $\pm$ 0.88#
Water intake ml/24 h	6.6 $\pm$ 0.9	7.6 $\pm$ 0.6	14.4 $\pm$ 0.9**	8.8 $\pm$ 0.12#	8 $\pm$ 0.9#
Urinary pH	6.6 $\pm$ 0.08	6.5 $\pm$ 0.21	6.4 $\pm$ 0.12	6.5 $\pm$ 0.1	6.7 $\pm$ 0.1
<b>Urine mg/24 h</b>					
Oxalate	0.79 $\pm$ 0.19	0.72 $\pm$ 0.18	2.49 $\pm$ 0.37**	1.40 $\pm$ 0.29	1.2# $\pm$ 0.20
Citrate	12.68 $\pm$ 2.13	15 $\pm$ 1.74	16.35 $\pm$ 1.38	18.5 $\pm$ 1.92	19.1 $\pm$ 2.74
Phosphate	4.00 $\pm$ 0.71	4.41 $\pm$ 0.52	4.47 $\pm$ 0.59	5.05 $\pm$ 0.62	4.99 $\pm$ 0.35
Uric acid	0.618 $\pm$ 0.08	0.72 $\pm$ 0.08	0.63 $\pm$ 0.049	0.63 $\pm$ 0.12	0.70 $\pm$ 0.12
Calcium	4.05 $\pm$ 0.48	5.0 $\pm$ 0.52	2.77 $\pm$ 0.42*	4.52 $\pm$ 0.70	3.97 $\pm$ 0.71
Mg <sup>++</sup>	4.15 $\pm$ 0.67	5.15 $\pm$ 0.52	4.28 $\pm$ 0.814	5.04 $\pm$ 0.88	5.24 $\pm$ 0.48
Na <sup>+</sup>	0.25 $\pm$ 0.03	0.24 $\pm$ 0.02	0.23 $\pm$ 0.018	0.227 $\pm$ 0.03	0.12 $\pm$ 0.015**
K <sup>+</sup>	0.80 $\pm$ 0.08	0.77 $\pm$ 0.07	0.73 $\pm$ 0.07	0.725 $\pm$ 0.06	0.47 $\pm$ 0.04**
Total urinary protein	5.27 $\pm$ 1.25	4.6 $\pm$ 1.20	12.00 $\pm$ 1.56**	7.72 $\pm$ 0.54#	7.69 $\pm$ 1.18#
Serum creatinine mg/dl		0.75 $\pm$ 0.11	1.50 $\pm$ 0.13**	0.88 $\pm$ 0.12#	0.86 $\pm$ 0.12#
BUN mg/ml		22.9 $\pm$ 2.80	40.20 $\pm$ 3.5**	31.5 $\pm$ 2.69#	28.1 $\pm$ 3.0#

Data are expressed as mean  $\pm$  SEM

\* $p<0.05$ , \*\* $p<0.05$  and \*\*\* $p<0.001$  vs. Normal, # $p<0.05$ , ## $p<0.01$  vs. Lithogenic group

**Fig. 2** Images of calcium oxalate crystals in 3 h morning urine collected from Normal control (A), untreated control (B) and treated with crude extract of *Trachyspermum ammi* (Ta.Cr) (C), under light microscope at 400× magnification



to the saline control, whereas Ta.Cr significantly reduced urinary crystal count (Fig. 2). Similarly, oxalate excretion was also raised significantly in stone-forming rats while calcium excretion was decreased. Ta.Cr significantly decreased the urinary oxalate and restored the urinary calcium. Urinary contents of phosphate, citrate, UA and magnesium remained unchanged; however, Ta.Cr at a dose of 100 mg/kg significantly reduced urinary  $\text{Na}^+$  and  $\text{K}^+$  concentration (Table 1).

Ethylene glycol treatment damaged renal function as evident from increased serum creatinine, BUN and total urinary protein loss. However, Ta.Cr significantly reduced the increased serum creatinine, BUN and total protein loss with respect to disease control group (Table 1).

In histological preparations of H and E staining, many crystalline deposits were observed under polarized light in almost all regions of the kidneys, especially in the cortex and papilla of rat's kidneys tissues in the untreated control group. However, in Ta.Cr-treated groups (30–100 mg/kg), the densities of crystals were much lower than that of the untreated control group (Figs. 3 and 4).

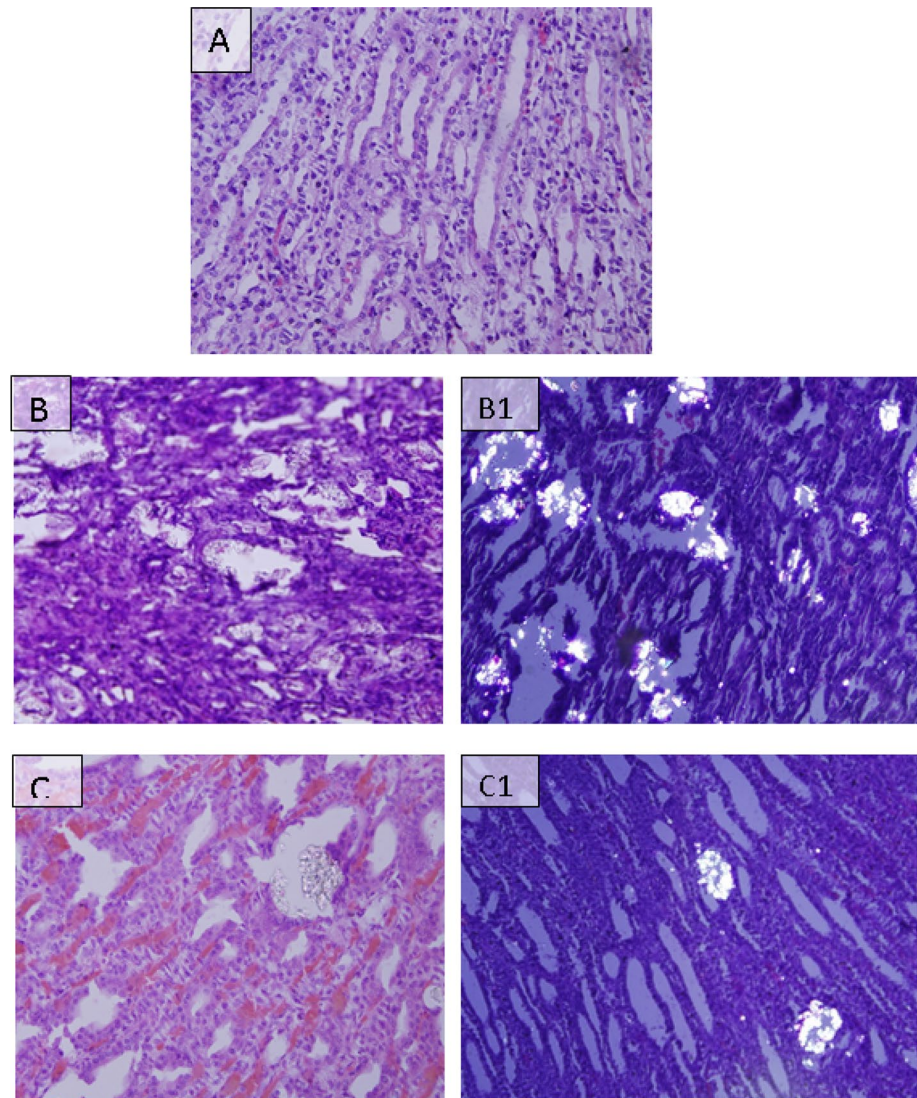
### Curative study model

In the curative study, all the parameters were recorded three times during the experimental protocol; once at day 0, then at

day 21, and lastly at day 35. Before the treatment, parameters were compared with each other. After 21 days of stone induction, the parameters were compared with the Saline (Normal control). After 35 days, when the stone-inducing treatment was withdrawn, the diseased control (negative control) was compared with Saline (Normal control) and also with the treated groups.

On day 0, all the parameters were similar in all the groups. The EG (0.75%) and AC (1%) consumption for the first 21 days initiated the lithogenic parameters in the animals in untreated groups as compared to the saline group like in the preventive study. This was suggested by a net loss in the body weights and a significant increase in urine volume, water intake, oxalate, and uric acid while a decrease in urine pH and calcium content of the urine. Serum creatinine, BUN and total serum protein were also increased significantly compared to the normal saline group. Post-induction treatment with Ta.Cr (10 and 30 mg/kg) reversed the loss in body weights, unpaired urinary and serum functions (Table 2), crystal deposition in the kidneys more quickly than in the untreated group (Figs. 5 and 6).

**Fig. 3** Representative pictures (100×) of H and E stained kidney sections from normal (A), stone-forming (B) and treated with Ta.Cr (C) rats in the preventive study. B1 and C1 are the respective sections in polarized light



## In vitro experiment

### Effect on in vitro crystallization

**Kinetic study** Effect of Ta.Cr on different phases of CaOx crystallization as revealed by time-course measurement of turbidity under standard conditions (4.25 mM  $\text{Ca}^{++}$  and 0.75 mM Ox) is given in Fig. 7A while Fig. 7B shows representative tracing of the experiment. After induction of the crystallization with disodium oxalate, an initial increase in the turbidity was observed in the control curve, reached its maximum at about  $150 \pm 15$  s, followed by a slow decrease, which is typical of the pattern reported in other studies [9, 25]. Ta.Cr decreased both slopes of nucleation (1–4 mg/ml) and inhibits the crystal aggregation (0.5–4 mg/ml) in a concentration-dependent manner, while potassium citrate (2–4 mM) increased nuclei induction time and also slow down the crystals nucleation and aggregation (Fig. 7C).

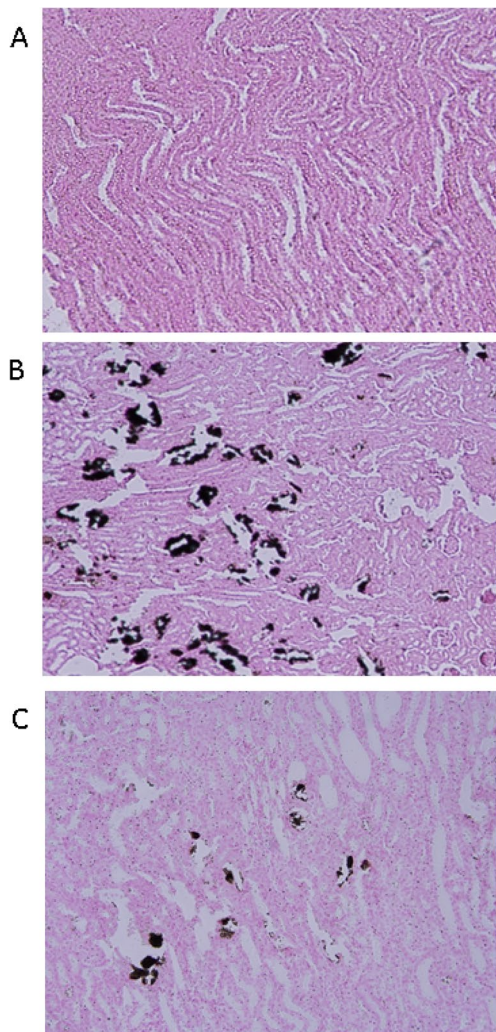
**Incubation study** In the incubation study, mixing the metastable solutions of  $\text{Ca}^{++}$  and Ox resulted in the formation of CaOx crystals predominately of calcium oxalate monohydrate (COM) (Fig. 8A). Ta.Cr caused a concentration-dependent (1–4 mg/ml) decrease in the CaOx crystal formation and also decreased the number and size of COM (Fig. 8B), similar to potassium citrate (Fig. 8C and D).

### Antioxidant effect

#### Effect on free radicals

Ta.Cr caused inhibition of DPPH free radicals with  $\text{IC}_{50}$  value of 3.4  $\mu\text{g}/\text{ml}$  (95% CI, 3.166 to 3.692) and while the control drug BHT inhibited DPPH with  $\text{IC}_{50}$  value of 3.2  $\mu\text{g}/\text{ml}$  (95% CI, 2.7 to 3.8) (Fig. 9).





**Fig. 4** Representative pictures (100×) of Pizzolato's stained kidney sections from normal (A), stone-forming (B) and treated with Ta.Cr (C) rats in the preventive study

#### Lipid peroxidation inhibitory activity

Ta.Cr inhibited lipid peroxidation, induced in rat's kidney tissue homogenate by  $20.33 \pm 4.7\%$  and  $73.07 \pm 5.03\%$  while BHT caused  $30.85 \pm 2.60$  and  $71.60 \pm 3.84\%$  inhibition of lipid peroxidation at 50 and 150  $\mu\text{g/ml}$ , respectively (Fig. 9).

#### Effect on kidney epithelial cell lines (MDCK)

Ta.Cr had no toxic effect on MDCK cells up to a concentration of 0.1 mg/ml. However, it induced significant cell viability inhibition at and after increasing the concentration beyond 0.3 mg/ml (Fig. 10A).

#### Effect of Ox and COMs

The cell viability was significantly decreased ( $p < 0.001$ ) after exposure to 0.5 mM oxalate and  $66 \mu\text{g/cm}^2$  of COM vs. untreated control. However, the co-exposure of Ta.Cr increased ( $p < 0.05$ ) cell viability at a dose of 0.1 mg/ml (Fig. 10B and C).

#### Effect on LDH release

LDH release has significantly increased after exposure to 0.5 mM oxalate or  $66 \mu\text{g/cm}^2$  COM vs. untreated control. However, after co-exposure of Ta.Cr at the concentration of 0.1 mg/ml, the LDH decreased significantly (Fig. 11).

#### Effect on urinary bladder

Ta.Cr relaxed both CCh (1  $\mu\text{M}$ ) and high  $\text{K}^+$  (80 mM)-induced contractions in rabbit's isolated urinary bladder strips at a concentration of 1 mg/ml with an  $\text{EC}_{50}$  values of 0.17 (0.14–0.20, 95% CI) and 0.13 mg/ml (0.10–0.16, 95% CI) (Fig. 12A), similarly, verapamil, relaxed both high  $\text{K}^+$  and CCh induced contractions (Fig. 12C) with  $\text{IC}_{50}$  values of 0.08  $\mu\text{M}$  (0.06–0.12) and 0.34  $\mu\text{M}$  (0.25–0.47), respectively, and found more potent against the high  $\text{K}^+$  than the CCh-induced contraction. Ta.Cr (0.03–0.1 mg/ml) caused a rightward shift of the  $\text{Ca}^{++}$ -CRCs accompanied by suppression of the maximum contractile effect, like that caused by verapamil (0.01–0.03  $\mu\text{M}$ ) as shown in Fig. 12B and D, respectively.

## Discussion

The crude extract of *Trachyspermum ammi* (Ta.Cr) was evaluated for its possible diuretic and antiurolithic activities using the in vivo and in vitro experimental models to rationalize its traditional use as antiurolithic agent.

In the in vivo diuretic experiment Ta.Cr significantly increased urine output at a dose of 30 and 100 mg/kg similar to positive control; hydrochlorothiazide (10 mg/kg), while, administration of the next higher dose (300 mg/kg) did not cause any significant change in the diuretic effect, compared to the saline control, which could be due to the possible co-existence of anti-diuretic component(s) in Ta.Cr. The presence of synergistic and/or side effect neutralizing properties in plants is very well studied [9] which is probably meant by nature not to allow the therapeutic effect to go beyond a certain limit beyond which it could have caused adverse effects, as is the case with chemical drugs [9, 35]. Diuretics increase urine volume, which results in the reduction of the supersaturation of stone-forming salts, and also help in the expulsion of crystals [36, 37].

**Table 2** Various parameters from different groups of rats, recorded after 21 and 35 days, to study the curative effect in animal model of urolithiasis

	Day	Baseline Mean $\pm$ SEM	N. Saline Mean $\pm$ SEM	Lithogenic group (EG) Mean $\pm$ SEM	EG + Ta.Cr 30 (mg/kg) Mean $\pm$ SEM	EG + Ta.Cr 100 (mg/kg) Mean $\pm$ SEM
% Change in BW	21		15.1 $\pm$ 1.2	-2.15 $\pm$ 3.2**	-1.25 $\pm$ 3.0**	-2.5 $\pm$ 3.0**
	35		26 $\pm$ 1.9	-0.3 $\pm$ 2.43*	8.49 $\pm$ 1.85#	9.25 $\pm$ 1.89#
Urine vol ml/24 h	21	8.6 $\pm$ 1.4	8.5 $\pm$ 1.1	15 $\pm$ 1.15**	16 $\pm$ 1.83*	15.8 $\pm$ 1.49*
	35		10.8 $\pm$ 0.8	16.6 $\pm$ 0.93*	10.7 $\pm$ 2.05#	11.2 $\pm$ 1.64#
Water intake ml/24 h	21	9.39 $\pm$ 1.08	9.17 $\pm$ 1.35	17.4 $\pm$ 1.86**	17.6 $\pm$ 3.2*	17.6 $\pm$ 3.2*
	35		10.2 $\pm$ 1.58	18.5 $\pm$ 1.44*	11 $\pm$ 1.65#	10.3 $\pm$ 1.95#
pH	21	6.5 $\pm$ 0.05	6.6 $\pm$ 0.06	5.82 $\pm$ 0.15**	6.05 $\pm$ 0.06*	6.14 $\pm$ 0.15*
	35		6.34 $\pm$ 0.09	5.53 $\pm$ 0.09*	6.19 $\pm$ 0.16	6.49 $\pm$ 0.21
Urine mg/24 h						
Oxalate	0	0.76 $\pm$ 0.07				
	21		0.69 $\pm$ 0.10	2.60 $\pm$ 0.21**	2.65 $\pm$ 0.27**	2.95 $\pm$ 0.29**
	35		0.95 $\pm$ 0.19	2.29 $\pm$ 0.24**	1.36 $\pm$ 0.15#	1.30 $\pm$ 0.13#
Citrate	0	25 $\pm$ 3.13				
	21		23 $\pm$ 2.80	25.50 $\pm$ 2.43	28.20 $\pm$ 4	27.60 $\pm$ 3.87
	35		22.5 $\pm$ 2.52	26.70 $\pm$ 2.51	29.00 $\pm$ 4.6	27.00 $\pm$ 1.8
Phosphate	0	3.98 $\pm$ 0.55				
	21		4.22 $\pm$ 0.60	4.31 $\pm$ 0.66	4.12 $\pm$ 0.77	4.30 $\pm$ 0.8
	35		4.15 $\pm$ 0.91	4.91 $\pm$ 0.6	4.97 $\pm$ 52	4.85 $\pm$ 0.70
Uric acid	0	0.77 $\pm$ 0.17				
	21		0.72 $\pm$ 0.16	1.32 $\pm$ 0.17*	1.29 $\pm$ 0.12*	1.33 $\pm$ 0.11*
	35		0.9 $\pm$ 0.08	1.35 $\pm$ 0.06**	0.93 $\pm$ 0.112#	0.89 $\pm$ 0.08##
Ca <sup>++</sup>	0	12.4 $\pm$ 1.76				
	21		11.2 $\pm$ 1.50	4.57 $\pm$ 0.8*	4.45 $\pm$ 0.7**	4.50 $\pm$ 0.5**
	35		12.3 $\pm$ 1.47	6.29 $\pm$ 0.56*	10.80 $\pm$ 0.72	11.60 $\pm$ 0.59#
Mg <sup>++</sup>	0	3.2 $\pm$ 0.41				
	21		3.4 $\pm$ 0.53	3.16 $\pm$ 0.56	3.60 $\pm$ 0.43	3.57 $\pm$ 0.67
	35		3.08 $\pm$ 0.66	3.85 $\pm$ 0.7	4.30 $\pm$ 0.4	3.88 $\pm$ 0.63
Na <sup>+</sup>	0	0.25 $\pm$ 0.03				
	21		0.23 $\pm$ 0.04	0.35 $\pm$ 0.02	0.36 $\pm$ 0.03	0.39 $\pm$ 0.04
	35		0.28 $\pm$ 0.02	0.39 $\pm$ 0.02	0.29 $\pm$ 0.0155	0.25 $\pm$ 0.03
K <sup>+</sup>	0	0.84 $\pm$ 0.08				
	21		0.97 $\pm$ 0.06	1.10 $\pm$ 0.01	0.99 $\pm$ 0.16	0.97 $\pm$ 0.1
	35		0.781 $\pm$ 0.10	0.88 $\pm$ 0.08	0.82 $\pm$ 0.06	0.77 $\pm$ 0.11
Total protein	0	6.45 $\pm$ 0.60				
	21		7.6 $\pm$ 1.02	16.10 $\pm$ 1.56**	14.50 $\pm$ 0.67*	13.80 $\pm$ 1.34*
	35		9.99 $\pm$ 1	18 $\pm$ 2.45*	13.2 $\pm$ 1.06	11.6 $\pm$ 1.3#
Serum creatinine (mg/dl)	35		0.523 $\pm$ 0.07	0.75 $\pm$ 0.03*	0.65 $\pm$ 0.04	0.52 $\pm$ 0.04#
BUN (mg/dl)	35		28.7 $\pm$ 3.13	50.70 $\pm$ 5.4**	33.90 $\pm$ 2.87#	23.90 $\pm$ 4.35##

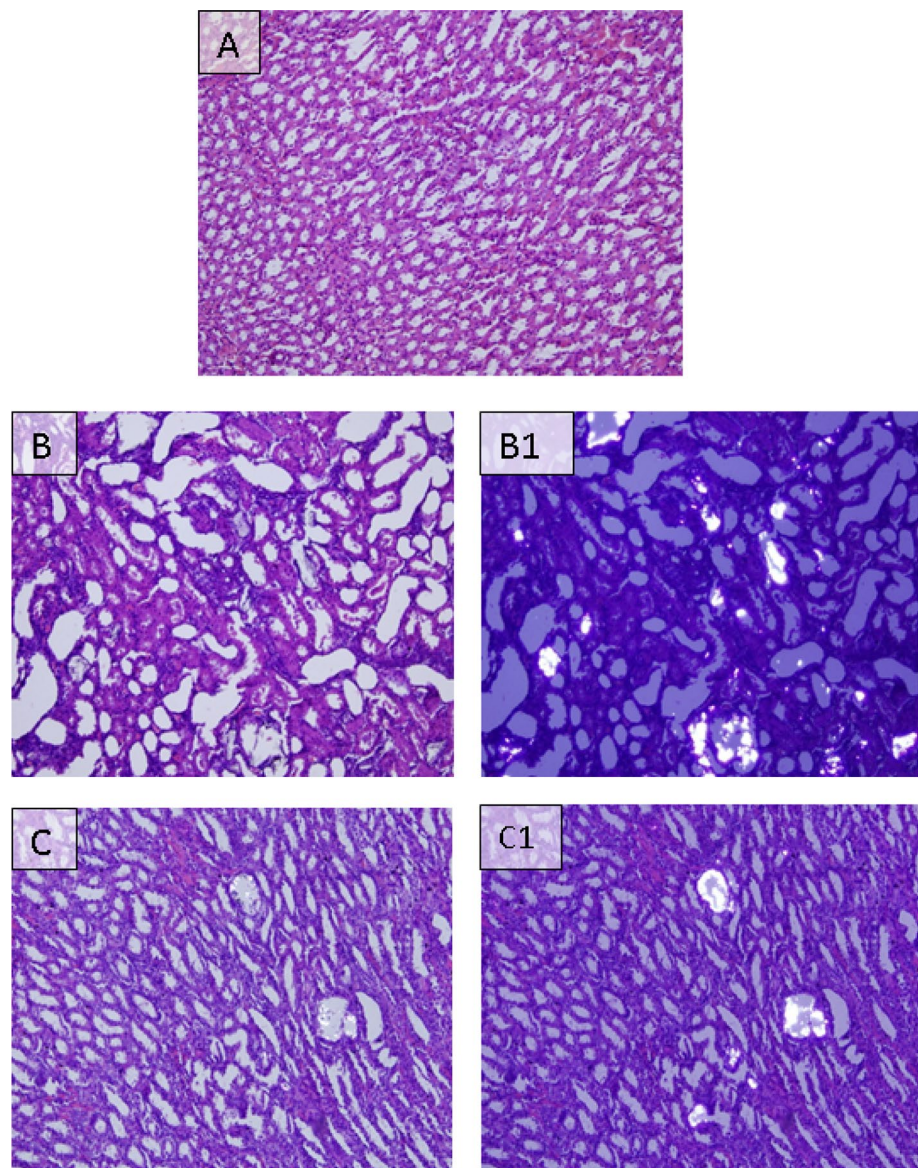
Data are expressed as mean  $\pm$  SEM

\* $p < 0.05$ , \*\* $p < 0.05$  and \*\*\* $p < 0.001$  vs. Normal, # $p < 0.05$ , ## $p < 0.01$  vs. Lithogenic group

The antiurolithic effect of Ta.Cr was evaluated in the in vivo rat model by using ethylene Glycol (EG) in drinking water [46–48]. Male Wistar rats were selected because they are more sensitive to ethylene glycol toxicity; therefore, changes in their urine electrolytes and CaOx supersaturation occur to a greater extent than in other strains [38].

In this study, hyperoxaluria was induced by administration of EG (0.75%) for 21 days, and 1% ammonium chloride (AC) for the first 5 days, as the addition of 1% AC for more than 5 days lead to extreme weight loss and ultimately death of rats, which is consistent with the other studies [39].

**Fig. 5** Representative pictures (100×) of H and E stained kidney sections from normal (A), stone forming (B) and treated with Ta.Cr (C) rats in the curative study. B1 and C1 are the respective sections in polarized light



In the preventive study, the administration of EG and AC resulted in crystalluria with larger crystals due to hyperoxaluria; an increase in water intake and urine output might be due to renal impairment [40], as there was a significant increase in serum creatinine, blood urea nitrogen and total urinary protein loss in the control group as compared to normal, which has been restored by the Ta.Cr treatment. These data suggest that lithogenic treatment (EG) increases oxalate and decreases  $\text{Ca}^{++}$  excretion in urine [39, 41], which was prevented by Ta.Cr.

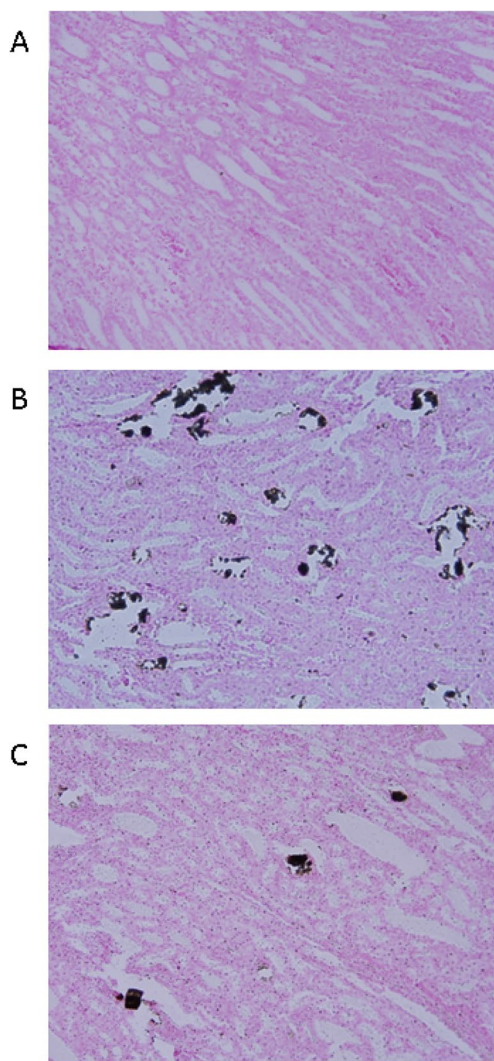
There were hypertrophy and widespread deposition of CaOx crystals in the kidneys of untreated rats. The renal tubules were markedly dilated, which might be due to the obstruction in distal renal tubular flow by large crystals [42]. Several *in vivo* and *in vitro* studies have revealed that hyperoxaluria; a key risk factor for CaOx stone formation, results

in greater production of hydroxyl free radicals and superoxide, leading to oxidative stress, cell membrane rupture and cell death [42, 43] which leads to CaOx crystal adherence and retention in renal tubules [25, 42]. Thus, it can be hypothesized that the antioxidant activity of Ta.Cr may be contributing factor to its inhibitory effect against CaOx crystal deposition in renal tubules.

In the curative study, Ta.Cr treatment was started after 21 days of lithogenic period. Ta.Cr increased the recovery in the treated group as compared to the untreated group, which was clearly shown by the significant decrease in urinary oxalate, gain in body weight, renal crystal deposition and improvement in renal functions compared to the untreated rats.

In further possible mechanisms, which might be contributing to the antiurolithic activity of the Ta.Cr, we performed





**Fig. 6** Representative pictures (100×) of Pizzolato's stained kidney sections from normal (A), stone forming (B) and treated with Ta.Cr (C) rats in curative study

a number of in vitro experiments, which include crystallization studies, anti-oxidant studies and spasmolytic effect on urinary bladder.

The effect of Ta.Cr on CaOx crystallization kinetics was studied by the time course measurement of turbidity using an aggregometer, like that of the previous studies [25, 44]. Ta.Cr inhibited CaOx crystal nucleation and aggregation in a concentration-dependent manner, similar to K<sup>+</sup> citrate, an inhibitor of CaOx crystallization and clinically used for the treatment of urolithiasis [45]. Similarly in the incubation study, Ta.Cr decreased crystal count and changed CaOx monohydrate crystals, which are more likely to attach with the kidney epithelial cells than CaOx dihydrate, resulting in the formation of kidney stones [46, 47] like that of citrate and Mg<sup>2+</sup> [48]. Calcification is a multifactorial phenomenon [48, 49], arising as a result of a series of proceedings

initiated by supersaturation, including crystal nucleation, growth, aggregation and retention [50]. Although supersaturation is not the only critical step evolved in the formation of kidney stones and several studies has identified many inhibitors of calcium oxalate and calcium phosphate crystallization, characterized as ionic or macromolecular inhibitors [51], yet it is the primary prerequisite for the formation of the crystals in the urinary tract [52]. Various crystal inhibitors like potassium-sodium citrate and magnesium oxide are known to decrease the CaOx supersaturation and inhibit “crystal nucleation, growth and aggregation” causing a decrease in urinary crystallization of stone forming patients [53]. Therefore, agents causing inhibition of crystal growth and aggregation can serve as possible therapeutic options for the management of recurrent kidney stones formation. Ta.Cr inhibits CaOx crystal nucleation and aggregation along with a decrease in the count and morphological change in crystals. These results indicate crystallization inhibitory of activity of Ta.Cr extract could be one of the contributing factors in its antiurolithic activity.

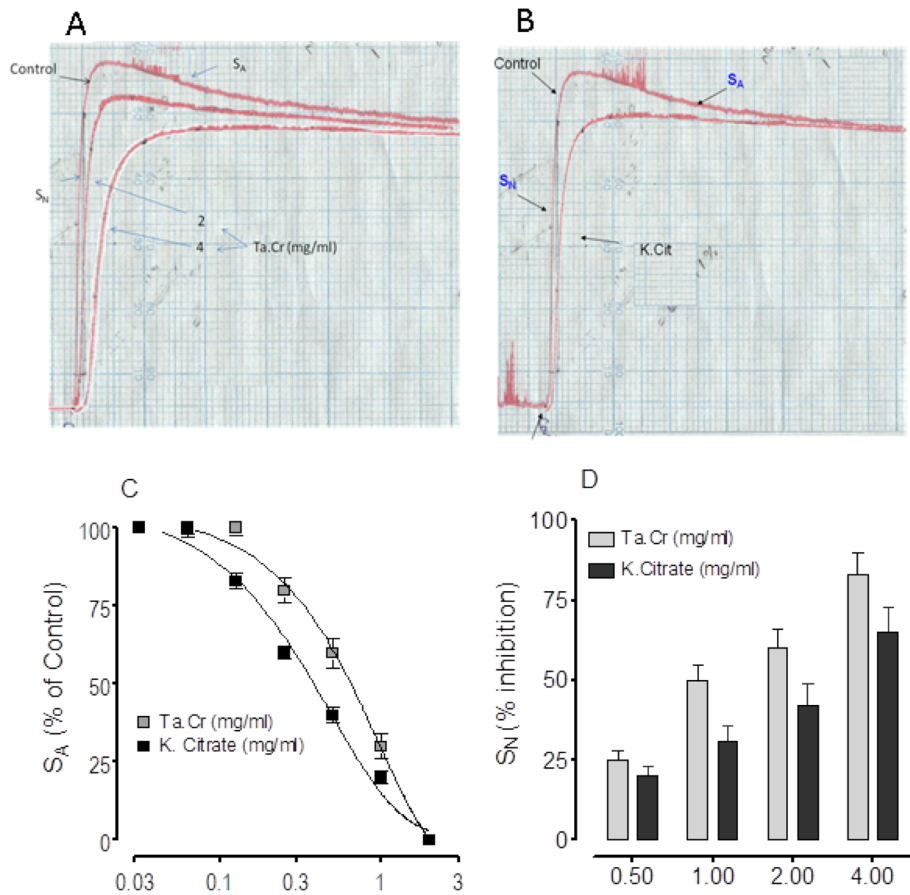
The in vitro and in vivo studies revealed that oxalate, calcium oxalate and hydroxy apatite crystals cause injury in kidney cells [54, 55]. This injury is caused by the production of ROS and is considered to be the risk factor for the crystallization and crystals deposition in the kidney as it promotes sites for crystal nucleation, aggregation, retention and stone development [53, 56]. Therefore, antioxidants such as catechin, vitamin E and selenium can protect against oxalate and crystal deposition-induced oxidative injury [42, 57]. Antioxidant activity of Ta.Cr was measured by free radical scavenging and lipid peroxidation inhibitory activity, where Ta.Cr showed of DPPH free radicals scavenging activity and inhibited ferrous-ascorbate-induced lipid peroxidation of rat kidney tissue homogenate like BHT, a standard antioxidant [41].

Several studies revealed that oxalate and COM crystals are not only the major constituent of oxalate stones but also have cytotoxic effect on renal epithelial cells [42, 58]. Here pretreatment with the Ta.Cr significantly increases the survival rate and decreased LDH release in MDCK cells, when exposed to Ox and COM crystals. This protective effect could be the result of its antioxidant activity.

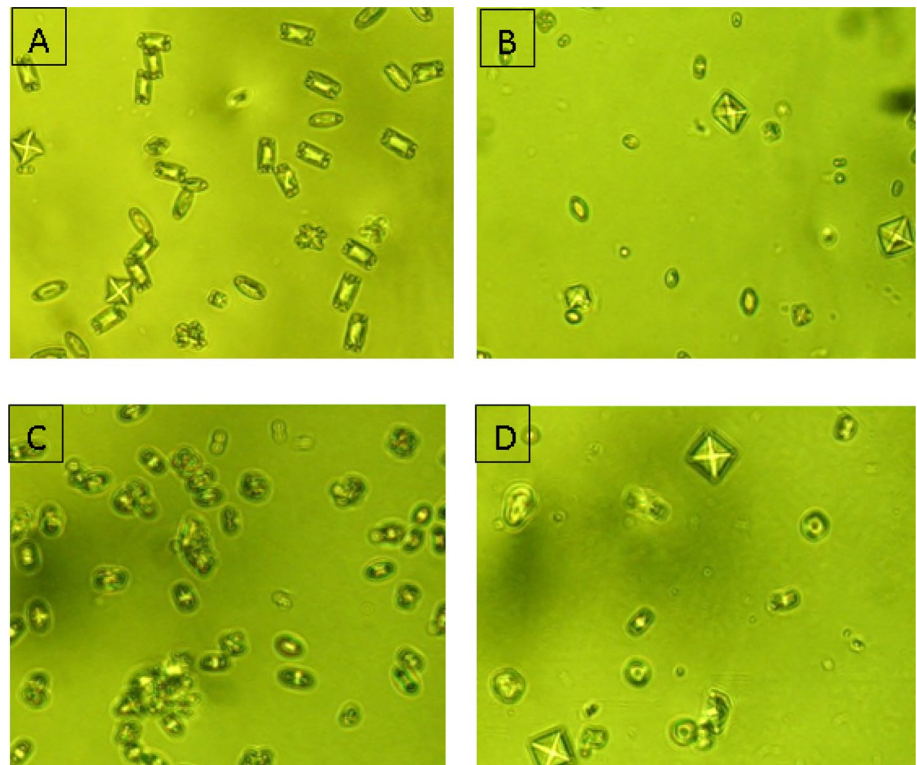
In Medical Expulsive Therapy (MET) for kidney stones expulsion, spasmolytics drugs are more commonly used [59]. Alpha-adrenergic antagonists (alpha-blockers) or Ca<sup>++</sup> channel blockers (CCB) increases stone expulsion rates and decreases colic event [60]. Therefore, we evaluate the anti-spasmodic effect of Ta.Cr, on K<sup>+</sup> (80 mM) and CCh (1 μM) induced contraction, using rabbit urinary bladder. K<sup>+</sup> concentration greater than 30 mM, is known to cause smooth muscle relaxation through the opening of voltage-dependent L-type calcium channels, thus allowing an influx of extracellular Ca<sup>++</sup>, resulting in the contraction of the smooth



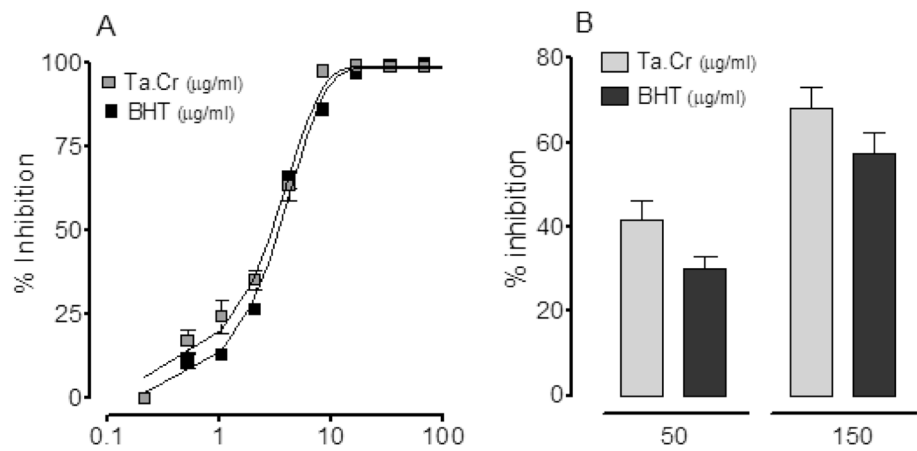
**Fig. 7** Effect *Trachyspermum ammi* (Ta.Cr) and potassium citrate (K-Cit) on calcium oxalate crystallization. **A** and **B** are the typical tracing of the control and in the presence of Ta.Cr and K.Cit. Panel **(C)** is the concentration–response curves of Ta.Cr and potassium citrate on  $S_A$  of the turbidity curves, while **(D)** shows the % inhibition on the  $S_N$ . Symbols shown are mean  $\pm$  S.E.M. ( $n=3$ ).  $S_N$  and  $S_A$  represent the slope of nucleation and slope of aggregation, respectively



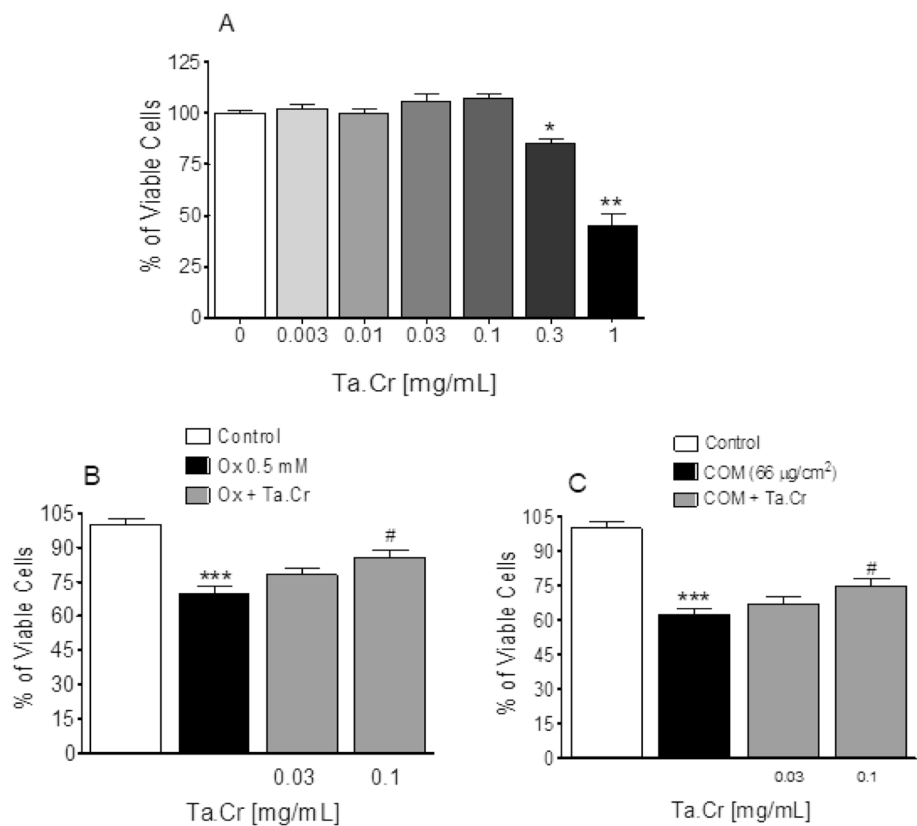
**Fig. 8** Representative photographs, under inverted microscope (200 $\times$ ), of CaOx crystals developed in the metastable solutions in the absence (**A** and **C**) and in the presence of crude extract of Ta.Cr 2 mg/ml (**B**) and 2 mM K-Citrate (**D**)



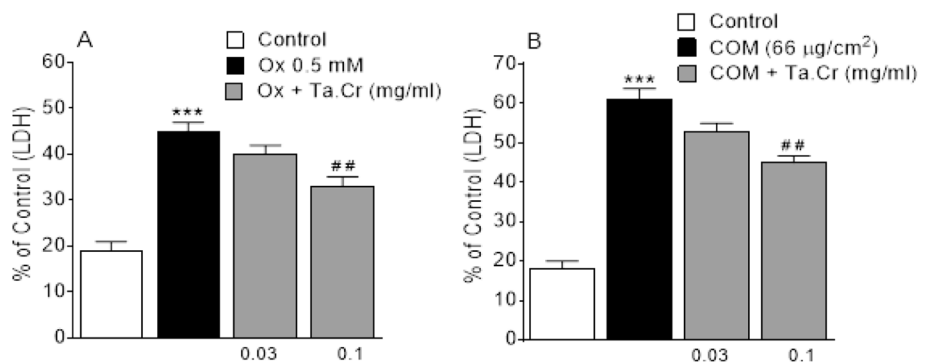
**Fig. 9** Concentration–response curves of the free radical scavenging activity of the butylated hydroxytoluene (BHT) and the crude extract of *Trachyspermum ammi* (Ta.Cr) while bar-chart representing lipid peroxidation inhibitory activity of two different concentrations of Ta.Cr and butylated hydroxytoluene (BHT). Inhibition is measured as % of the respective control experiments. The values shown are mean ± SEM ( $n = 3$ )



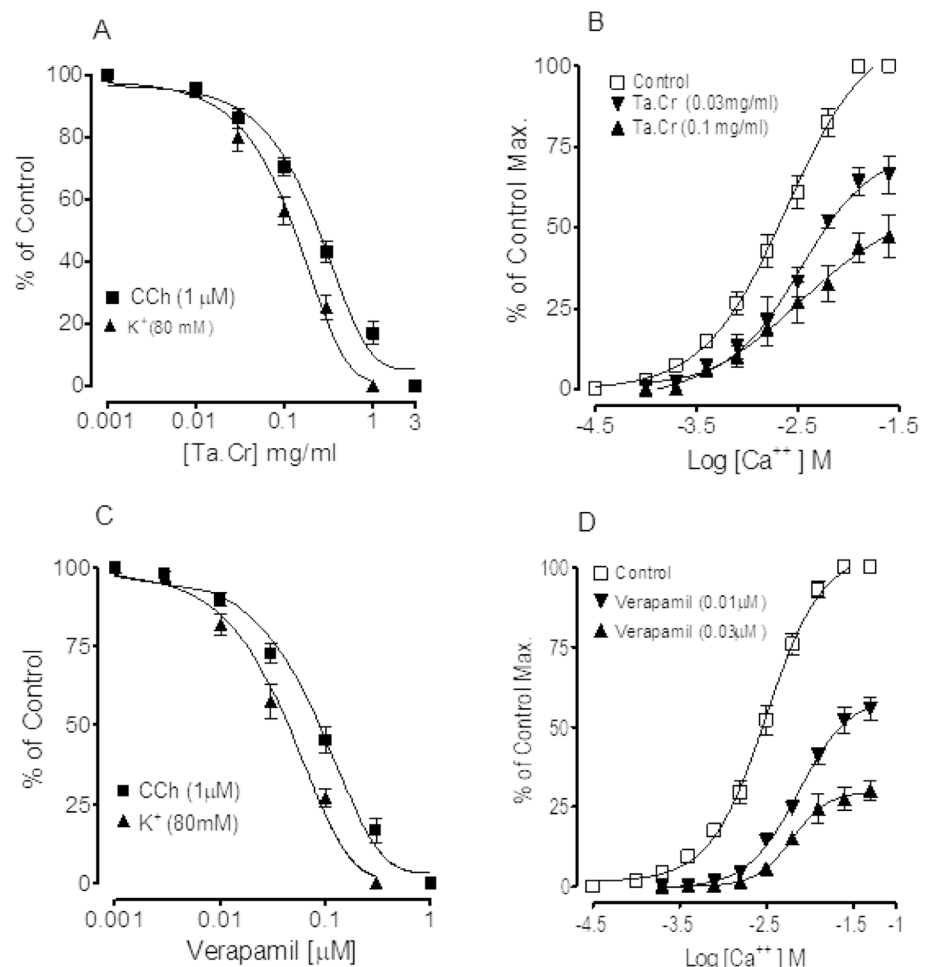
**Fig. 10** Effects of various concentrations of *Trachyspermum ammi* MDCK cell survival in acclimatization media. Ta.Cr has no toxic effect on MDCK cells at up to 0.3 mg/ml for 24 h (A). MDCK cell survival fraction decreased significantly vs. that in untreated controls after exposure to 0.5 mM oxalate (B) and 66 μg/cm<sup>2</sup> COM (C). Ta.Cr increases the cell survival fraction of MDCK cells at 0.03 and 0.1 mg/ml concentration



**Fig. 11** Percent increase in lactate dehydrogenase (LDH) release against control by MDCK cells exposed to A Ox. 0.5 mM and B COM 66 μg/cm<sup>2</sup> alone and with 0.03 and 0.1 mg/ml of Ta.Cr for 24 h. Data shown are mean plus or minus SEM of two separate experiments with 3 independent replicates. \*\*\* $p < 0.05$ , \*\* $p < 0.05$  and \*\*\* $p < 0.001$  control versus exposed groups



**Fig. 12** Concentration–response curves of crude extract of Ta.Cr (A) and verapamil (B) on  $K^+$ (80 mM) and CCh (1  $\mu$ M)-induced contractions and  $Ca^{++}$  CRCs constructed in the absence (control curve) and presence of increasing concentrations of Ta.Cr (C) and verapamil (D) in isolated rabbit urinary bladder. The symbols represent mean  $\pm$  SEM ( $n=4-6$ )



muscles [61]. A substance that inhibits the high  $K^+$  induced contraction is considered to be CCB [62], whereas, CCh is a cholinergic drug that can induce contraction in the urinary bladder through activation of muscarinic (predominately M3) receptors [63]. Ta.Cr inhibited the high  $K^+$  and CCh induced contraction at similar doses like that of verapamil, as standard CCB [63], indicating CCB activity. To confirm the CCB action, CRCs of  $Ca^{++}$  were constructed, using the rabbit urinary bladder, in the absence (control curve) and presence of the increasing concentration of Ta.Cr. It shifted the CRC to the right with suppression of the maximum response, like standard calcium channel blocker (verapamil), confirming the presence of CCB constituent in Ta.Cr.

## Conclusion

Results of our study suggest the presence of antiurolithic effects in Ta.Cr against  $CaOx$  kidney stone formation may be mediated through multiple pathways, which rationalize its traditional medicinal use in the management of

urolithiasis. However, more studies may be required to investigate the possible molecular pathways and establish its in-depth safety and efficacy for clinical use.

Future studies should focus on the activity-guided fractionation for the isolation of respective compounds for different activities, which can help resolve the dilemma that why crude extracts of the plants are more effective in these kinds of complex disorders than the pure single molecule.

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**Author contributions** Dr. Anwarul-Hassan Gilani, supervised the research work and critically revise and correct the manuscript. Dr. Aslam Khan, did the experimental work and wrote the first draft of the manuscript.

**Data availability** The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

## Declarations

**Conflict of interest** The authors declare that they have no competing interests.

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