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Studies on the *in vitro* and *in vivo* antiurolithic activity of *Holarrhena antidysenterica*

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Abstract

Background—*Holarrhena antidysenterica* has a traditional use in the treatment of urolithiasis, therefore, its crude extract has been investigated for possible antiurolithic effect.

Materials and methods—The crude aqueous-methanolic extract of *Holarrhena antidysenterica* (Ha.Cr) was studied using the *in vitro* and *in vivo* methods.

Results—In the *in vitro* experiments, Ha.Cr demonstrated a concentration-dependent (0.25–4 mg/ml) inhibitory effect on the slope of aggregation. It decreased the size of crystals and transformed the calcium oxalate monohydrate (COM) to calcium oxalate dehydrate (COD) crystals, in calcium oxalate metastable solutions. It also showed concentration-dependent antioxidant effect against 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) free radicals and lipid peroxidation induced in rat kidney tissue homogenate. Ha.Cr (0.3 mg/ml) reduced ($p < 0.05$) the cell toxicity and LDH release in renal epithelial cells (MDCK) exposed to oxalate (0.5 mM) and COM (66 $\mu g/cm^2$) crystals. In male Wistar rats, receiving 0.75% ethylene glycol (EG) for 21 days along with 1% ammonium chloride (AC) in drinking water, Ha.Cr treatment (30–100 mg/kg) prevented the toxic changes caused by lithogenic agents; EG and AC, like loss of body weight, polyurea, oxaluria, raised serum urea and creatinine levels and crystal deposition in kidneys compared to their respective controls.

Conclusion—These data indicate that *Holarrhena antidysenterica* possesses antiurolithic activity, possibly mediated through inhibition of CaOx crystal aggregation, antioxidant and renal epithelial cell protective activities and may provide base for designing future studies to establish its efficacy and safety for clinical use.

Keywords

Urolithiasis; *Holarrhena antidysenterica*; *In vitro*; *In-vivo*; MDCK cell line; Rats

INTRODUCTION

Urolithiasis, urinary stone formation, is the third most common problem of the urinary tract, sparing no geographical, racial and cultural boundaries and is associated with high rate of recurrence [1, 2]. In Pakistan its prevalence is 10–15%, while its complication can lead to

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acute or chronic renal failure, pyelonephritis, pyonephritis and perinephric abscess, which can present with life threatening situations and even death [3].

Despite the fact that the currently available stone removal techniques like extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS), and percutaneous nephrolithotomy (PNL), are considered to be effective, but they are costly, making them an option of choice for limited number of patients and a compelling data suggest that these techniques have some serious side effects [4]. Moreover, the high rate of recurrence in stone formation, which is around 50% at a 5 years follow-up [3], makes it a chronic condition which underscores the importance of preventive therapy [5]. In spite of substantial progress in the study of the biological and physical manifestation of urolithiasis, its mechanism is still not clearly understood [6] and there is no satisfactory drug available for the treatment of urolithiasis, especially for the prevention of recurrence of the stones [7]. The agents used clinically for prophylactic therapy are primarily aimed to correct the underlying metabolic disorders but the evidence for their effectiveness is still not convincing in addition to their side effects and tolerability [8, 9]. One reason for a limited success of chemical drugs in urolithiasis is that multiple factors are involved in its pathogenesis [10] and thus treatment demands multiple targets, such as antispasmodic, antioxidant, anti-inflammatory activities [11]. The medicinal plants which are known to contain multiple chemical constituents, which could offer a synergistic and/or side-effect neutralizing combinatations [12] are likely to offer more effective and safer remedy particularly in urolithiasis. Therefore, there is a need to look for an alternative therapy, especially herbal remedies, for the management and treatment of urolithiasis, which have the potential to be effective in urolithiasis [13]. In addition, herbal medicine are considered to be relatively safer, economical and accessible to large population of the developing countries like Pakistan [12]. Therefore, we evaluated the seeds of *Holarrhena antidysenterica* Wall (Apocynaceae) for its antiurolithic activity because of its traditional use as a lithotriptic, along with other uses like colic, diarrhea, dysentery, constipation, flatulence, carminative, antispasmodic, astringent, diuretic and antihypertensive [14, 15].

Holarrhena antidysenterica is known to contain conessine, ergostenol, holarrhenine, kurchicine, resin and tannin [16, 17] and has been reported to possess antimutagenic [18], antibacterial [19], immuno-modulatory [20], antispasmodic [21] and diuretic [22] properties.

This study has been undertaken to investigate the antiurolithic activity of the seeds of *Holarrhena antidysenterica* using Ethylene glycol-induced rat model of CaOx urolithiasis and the *in vitro* assays to investigate its calcium oxalate crystallization inhibitory, antioxidant, renal cell protective and antispasmodic activities.

Materials and Methods

Chemicals and reagents

The following chemicals were obtained from the Merck, Darmstadt, Germany, BDH Laboratory supplies, Poole, England and Sigma Chemical Company, St. Louis, MO, USA. Kits used in this study for the determination of calcium, magnesium, and blood urea nitrogen were supplied by Randox Laboratories Ltd. Ardmore, Diamond Road, Crumlin, Co. Antrim, UK. Oxalate estimation was done by the kit from Trinity Biotech Plc, IDA business park, Bray, Co. Wicklow. All the chemicals used were of analytical grade available.

Animals

Experiments were performed in compliance with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (1996) [23]

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and approved by the Ethical Committee for Research on Animals (ECRA) of the Aga Khan University, Karachi, Pakistan.

Plant material and extraction

Dried seeds of *Holarrhena antidysenterica* were taken from local herbal store, identified by a taxonomist, Prof. Dr. Jhandar Shah, University of Malakand, Chakdara, Khyber Pakhtunkhwa, Pakistan and voucher specimen (HA-SE-01-08-71) was submitted to the herbarium of the Department of Biological and Biomedical Sciences, the Aga Khan University, Karachi. The clean seeds were extracted with 70% aqueous-ethanol and kept for three days with occasional shaking. It was filtered through a muslin cloth and then through a Whatman qualitative grade 1 filter paper. This procedure was repeated twice and the combined filtrate was evaporated on rotary evaporator under reduced pressure (–60 mmHg) to a thick, semi-solid mass of brown color i.e. the crude extract of *Holarrhena antidysenterica* seeds (Ha.Cr), yielding approximately 18% w/w [24].

In vitro experiments

Determination of CaOx crystallization—The effect of the Ha.Cr on kinetics of calcium oxalate (CaOx) crystallization was determined by the time course measurement of turbidity changes due to the crystal nucleation and aggregation after mixing metastable solutions of Calcium (Ca^{++}) and Oxalate (Ox). While the effect on the number, size and morphology of the crystals was determined by incubating different concentrations of Ha.Cr with the metastable solutions of Ca^{++} and Ox. as described previously [25].

Determination of antioxidant activity—Antioxidant potential of the Ha.Cr was estimated by its effect on free radical scavenging activity [26] and its inhibitory effect against lipid peroxidation induced in rat kidney homogenate with ferrous-ascorbate system, as described previously [27].

Cell lines (MDCK) Experiment—Madin Darby Canine Kidney (MDCK) cells, obtained from ATCC (Cat.# CRL-34; Manassa, VA, U.S.A), were maintained as sub-confluent monolayers at 37 °C in 5% CO_2. The culture were grown and maintained in 75 cm^2 Falcon tissue culture flasks as discussed previously.

Determination of cell viability and LDH release—After incubating the cells for 24 hrs with Ox (0.5 mM) or COM crystals (67 $\mu g/cm^2$), renal cells toxicity was determined by XTT cell viability Assay Kit (Biotium, Inc., Haward, Ca, USA Cat # 30007), while LDH release was estimated by CytoTox 96 Non-Radioactive Cytotoxicity assay kit (Fisher Scientific, Norcross, GA, Promega Cat # PR-G1780).

In vivo experiments

Study on animal model of urolithiasis—Antiurolithic activity of the test material was determined using hyperoxaluric rat model of CaOx urolithiasis described previously [11, 28, 29]. Male Wistar rats (weighing 180–220 g) were divided with matched body weights into groups of 6–8 animals each, which were then randomly selected to receive various treatments. In this study, rats were divided into five groups. Group 1 serving as normal control, received intraperitoneal (i.p.) injections of normal saline (2.5 ml/kg), once in 24 hrs. Group II serving as the Ha.Cr control received only the Ha.Cr (100mg/kg), Group III, serving as the lithogenic control received stone inducing treatment, while group IV and V received the lithogenic treatment along with the Ha.Cr 30 and 100 mg/kg respectively. Lithogenic treatment was given for 21 days and comprised of 0.75% (w/v) ethylene glycol (EG) with 1% (w/v) ammonium chloride (AC) in drinking water for 5 days, following this

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the water supply was switched to 0.75% EG alone in water [28, 30], along with saline treatment. Animal weight and activity were regularly monitored to assess their overall health. 24 hrs urine samples were collected immediately before the onset and at the end of total 21 days of treatment, for which animals were housed individually in metabolic and diuretic cages. The 3 hrs morning urine for crystalluria study was also collected at the end of 21 days of treatments. The number of the crystals/mm^3 was counted under light microscope using haemocytometer.

Following volume and pH determination, part of each 24 hrs urine sample was acidified to pH 2 with 5M HCl. Both acidified and non-acidified urine samples were then centrifuged at 1500g for 10 min to remove debris and supernatants were stored at −20°C until analyzed. Blood was collected through cardiac puncture from animals under ether anesthesia for serum separation in order to assess serum creatinine and blood urea nitrogen (BUN). Animals were sacrificed and both the kidneys were excised, rinsed in ice cold physiological saline and weighed. The right kidney was fixed in 10% neutral buffered formalin, processed, embedded in paraffin wax, sectioned at 5 μm and stained with Hematoxylin and Eosin (H & E) and by Pizzolato's method, for calcium oxalate crystals [31], for microscopic examination.

Calcium oxalate crystal deposition in kidney

Crystal distribution within the kidneys was determined by using the semiquantitaive scoring by the methods used previously [11, 32]. Briefly the crystal deposits in stained sections with visible in a field of 10X magnification were counted and severity grades were assigned as 0 = < 1 crystals, 1 = 1–10, 2 = 11–30, 3 = 31–50, 4 = 51–75 and 5 = > 75 crystals. Most of the crystals were located in the outer modularly and cortical region of the kidney.

Biochemical analysis of urine and serum

In acidified urine samples, Ox, Ca^{++} and Mg^{++} contents were determined by using commercially available kits. Inorganic phosphate (PO_4^{-2}) excretion was determined by the molybdenum blue reaction [33]. In non-acidified urine samples, citrate, creatinine and uric acid (UA), while in serum, creatinine and BUN were estimated with the help of kit-based methods. Total protein in non-acidified urine was estimated by lowery method [34] based on the development of blue colour as a result of a combination of two reaction involving the interaction Cu^{2+} in the presence of base with peptide bonds and Folin-Ciocalteu phenol reagent with tyrosine and tryptophan residues.

Data Analysis

The data expressed are mean ± standard error of mean (SEM) and the median effective concentration (EC50 value) with 95% confidence intervals (CI). All statistical comparisons between the groups are made by means of t- test (comparison between two groups) or One Way Analysis of Variance (ANOVA) with post hoc Dunnett's test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). p value less than 0.05 is regarded as significant.

RESULTS

In vitro Experiment

Effect on in vitro crystallization—Effect of Ha.Cr on various phases of CaOx crystallization was determined by time course measurement of turbidity under standard conditions (4.25 mM Ca^{++} and 0.75 mM Ox). Typical tracings of the experiment in the presence of Ha.Cr and Potassium Citrate is shown in Fig. 1A and B. Ha.Cr inhibited the S_A with an IC_{50} of 0.27 mg/ml (0.23 to 0.33, 95% CI), similar to potassium citrate, which

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caused dose dependent inhibition with IC_{50} value of 0.31 mM (0.30 to 0.52, 95% CI) as shown in Fig. 1C. Ha.Cr did not significantly decrease the slopes of nucleation (1–4 mg/ml), while potassium citrate caused 31 ± 2.31, 42 ± 4.0, 65 ± 4.2 and 85 ± 5.2% inhibition at a concentration of 0.5, 1, 2 and 4 mM, respectively (Fig. 1D). In the incubation study, mixing the metastable solutions of Ca^{++} and Ox resulted in the formation of CaOx crystals predominately of COM (Fig. 2A). Ha.Cr did not influence the number of crystal formation but significantly decreased the size and morphology of the crystals from COM to COD (Fig. 2B), whereas, potassium citrate reduced the number as well as the size and morphology of the crystals from COM to COD (Fig. 2C).

Antioxidant effect—Ha.Cr caused inhibition of DPPH free radical with IC_{50} value of 14.4 μg/ml (95% CI, 11.21 to 18.35), while the control drug BHT inhibited DPPH with IC_{50} value of 3.42 μg/ml (95% CI, 3.166 to 3.692) as shown in Fig. 3A. Ha.Cr inhibited lipid peroxidation, induced in rat kidney homogenate by 13.40 ± 3.40 and 42.40 ± 3.10, while BHT caused 30.85 ± 2.60 and 71.60 ± 3.84% inhibition of lipid peroxidation at 50 and 150 μg/ml, respectively (Fig. 3B).

Effect on Kidney Epithelial Cell Lines (MDCK)—Ha.Cr had no toxic effect on MDCK cells up to 0.3 mg/kg. However, it reduced significantly the cell viability at a concentration of 1 mg/ml or more (Fig. 4A). The cell viability was decreased significantly ($p < 0.001$) in the untreated control after exposure to 0.5 mM Ox or 66μg/cm^2 of COM. However, the treatment of Ha.Cr significantly ($p < 0.05$) increased the cell viability at a concentration of 0.3 mg/ml compared to untreated control (Fig. 4B and C). LDH release was significantly increased after exposure to 0.5 mM oxalate or 66 μg/cm^2 COM vs. untreated control. However, after pretreatment with Ha.Cr, the LDH decreased was significantly at the concentration of 0.3 mg/ml (Fig. 4D and E).

In vivo experiments

In the *in vivo* study, all the parameters, like body weights, 24 hrs water intake, urine volume, urinary pH and composition, recorded before the treatment were not significantly different among the groups. The parameters recorded at day 0 and at the end of 3 weeks of treatment period are listed in the Table 1.

Ha.Cr had no significant effect on the CaOx crystalluria, however, the crystals (mostly CaOx dehydrate) found in the treated groups were quite smaller than those found in the untreated lithogenic group (Fig. 5). The body weight was significantly ($p < 0.01$ vs. Normal) reduced in stone forming group as compared to the normal saline group. The co-administration of Ha.Cr (30–100 mg/ml) prevented ($p < 0.05$ vs. Stone forming group) the loss in body weight. The 24 hrs urine volume and water intake were higher ($p < 0.01$) in the stone forming group compared to that in the normal saline animals. Urine pH was slightly reduced in the stone forming group, though not to a significant extent. A co-treatment with Ha.Cr significantly reduced ($p < 0.05$) polyurea and water intake compared to stone forming group. Similarly, Oxalate excretion was significantly increased ($P < 0.01$) in stone forming animals, whereas Ca^{++} excretion was decreased ($P < 0.05$). Urine contents of citrate, phosphate, uric acid (UA), Mg^{2+}, Na^+ and K^+ were not altered to a significant level ($p > 0.05$). Co-administration of Ha.Cr (30–100 mg/kg) to EG treatment group, significantly ($p < 0.05$) decreased oxalate excretion, whereas unlike control group, Ca^{2+} excretion remained unchanged in the treated groups as compared to the normal group. EG treatment caused impairment of renal functions of the untreated rats as evident from total protein loss and raised BUN and serum creatinine ($P < 0.05$), which were prevented in the animals treated with Ha.Cr (Table 1).

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In the histological preparations of kidneys, the normal saline group did not show any crystalline deposits. Whereas, a high score of crystal deposits were observed under polarized light in all regions of kidneys in lithogenic group. However, in Ha.Cr treated groups significantly less number of CaOx crystal deposits ($p < 0.5$) were observed as compared to untreated EG (lithopgenic) group (Fig. 6 and 7).

DISCUSSION

In order to investigate the medicinal use of *Holarrhena antidysentrica* in urolithiasis, we evaluated crude extract for its antiurolithic activity using different *in vitro* assays and *in vivo* rat model of urolithiasis.

The effect of Ha.Cr on CaOx crystallization kinetics was studied by the time course measurement of turbidity using aggregometer, as already reported in previous studies [25, 35]. The initial positive slope of the turbidity curve, called the slope of nucleation (S_N), is mainly due to an increase in the particle number resulting from crystal nucleation. After a plateau is achieved, a gradual decrease of absorbance (negative slope) despite continuous stirring reflects the decrease in the particle number, due to crystal aggregation [25, 35]. In this experiment, unlike potassium citrate, Ha.Cr had no effect on the slope of nucleation but inhibited the CaOx crystals aggregation in a concentration-dependent manner, similar to potassium citrate, a well-known inhibitor of CaOx crystallization and clinically used for the management of urolithiasis [36]. Similarly, in the incubation study, Ha.Cr caused a decrease in crystal size and transformed COM to COD crystals, which are less likely to attach with the kidney epithelial cells than COM crystals [38, 39] like that of citrate and Mg^{2+} [37]. Crystal polymorphism plays an important role in calcium oxalates nephrolithiasis. COM crystals are thermodynamically more stable and more frequent in kidney stones as compared to COD. Macromolecules isolated from urine of normal healthy individuals inhibit COM crystals and favors formation of COD, which are less likely to adhere to renal epithelail cells. This suggests that COD formation protects against stone disease because of its reduced capacity to form stable aggregates and strong adhesion contacts to renal epithelial cells and thus potentially inhibiting a critical step in the formation of kidney stones [38, 39].

Calcification is a multifactorial phenomenon [40], developing as a result of a cascade of events initiated by supersaturation, including crystal nucleation, growth, aggregation and retention [41]. Various crystal inhibitors like potassium-sodium citrate and magnesium oxide have been shown to decrease the saturation of CaOx and inhibit crystal nucleation, growth and aggregation, while reduced crystallization in urine of stone forming patients [42]. Interference with crystal growth and aggregation therefore seems a possible therapeutic strategy for the prevention of recurrent stone disease. The effect on CaOx crystal aggregation might be due to the presence of saponins, present in the crude extract, which is evident from the phytochemical screening in our previous study [21], as saponins have been reported to have anti-crystallization property by disaggregating the suspension of mucoproteins, which are promoters of crystallization [43]. This shows that the Ha.Cr may contain substances that inhibit CaOx crystal aggregations and thus preventing a critical step in urinary stone formation, as larger particles are less likely to pass spontaneously in urinary tract. If the extract keeps CaOx particles dispersed in solution they can be easily eliminated [44].

Animal and cellular studies have revealed that oxalate ions, or COM and hydroxy apatite (HA) crystals cause injury to the kidney cells [45, 46], most probably by the production of reactive oxygen species (ROS). Epithelial injury is considered to be a risk factor for the crystallization and crystals deposition in the kidney, as it promotes crystal nucleation, aggregation, retention and stone development [45, 47]. Antioxidants, such as vitamin E,

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catechin and selenium have been shown to provide protection against oxidative injury by oxalate and crystal deposition [48, 49]. When tested for its antioxidant activity Ha.Cr caused scavenging of DPPH free radical and inhibited ferrous-ascorbate-induced lipid peroxidation of rat kidney homogenate similar to BHT, a standard antioxidant [50]. These results, are in line with earlier study [51] which showed the presence of antioxidant activity in *Holarrhena antidysentrica*. As hyperoxaluria and COM crystals are known to cause oxidative stress resulting in toxicity of renal epithelial cells [49, 52], the protective effect of Ha.Cr on renal epithelial cell might be due to its antioxidant activity, as pretreatment with the Ha.Cr, significantly increased the survival rate and reduced the LDH release, a marker of cell membrane damage, in MDCK cells, when exposed to oxalate and COM crystals.

For the *in vivo* antiurolithic effect of Ha.Cr, 0.75% ethylene glycol (EG) and 1% ammonium chloride (AC)-induced hyperoxaluric rat model of urolithiasis was used. Since the stone inducing treatment, Ethylene glycol (EG), was given orally, therefore, the extract was given i.p. in order to prevent any potential interaction of EG with plant constituents inside gut, interfering with absorption of either of the two. We have used intra-peritoneal (i.p) route, which is also commonly used in previous studies [11, 25, 53, 54].

Administration of EG and AC resulted in the increased CaOx crystalluria, with larger crystals due to hyperoxaluria, increase in water intake and urine output, which might be due to the renal impairment [29], as evident by increase in serum creatinine, blood urea nitrogen and total urinary protein loss in lithiatic group as compared to normal. Consistent with some previous reports, stone induction by hyperoxaluria caused an increase in oxalate and decrease in Ca^{2+} excretion in the untreated group [52, 55]. Hyperoxaluria is one of the major risk factor in the pathogenesis of kidney stone formation [56], as it cause oxidative stress and damages the renal epithelial cells thereby providing a nidus for crystals attachment and ultimately cause crystal aggregation retention and deposition in the kidney [45, 47]. Therefore, decrease in oxalate may explain its decrease in oxidative stress and renal crystal deposition. It not uncommon that plant extracts may interfere with oxalate metabolism in animal model of urolithiasis. like extract of *Aerva lananta* decrease the oxalate excretion, in ethylene glycol fed rats, by decreasing the formation of oxalate synthesizing enzymes like glycolic acid oxidase (GAO) in liver and lactate dehydrogenase (LDH) in liver and kidney [57], similar results were found in the extract of *Tribulus terrestris* [58]. This can be speculated that decrease in oxalate might be useful in hyperoxaluric kidney stone formers.

There was hypertrophy and extensive CaOx crystal deposition in 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 [29]. Several *in vivo* and *in vitro* studies have demonstrated that hyperoxaluria, a major risk factor for calcium oxalate nephrolithiasis, results in greater production of superoxide and hydroxyl free radicals, leading to antioxidant imbalance, cell membrane rapture and cell death [49, 59] which leads to CaOx crystal adherence and retention in renal tubules [49, 60]. Thus, it can be speculated that the inhibitory effect of the plant extract on CaOx crystal deposition in renal tubules is possibly caused by its antioxidant activity. The plant is considered relatively safer, as it has been used in different herbal preparations and supplements, such as Kutaj (*Holarrhena antidysenterica*), a herbal supplement, is used in humans for ailment of diarrhea and urinary disorders, which has also undergone clinical trials for several studies, with no reported side effect, for ulcerative colitis and bleeding piles [61–63].

Thus, these data suggest that the preventive effect of *Holarrhena antidysentrica* in urolithiasis is mediated its effect through multiple pathways including inhibition of the CaOx crystal aggregation, antioxidant and epithelial cell protective effects, which provide a

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step forward for designing further studies on *Holarrhena antidysentrica* to establish its safety and efficacy for clinical use.

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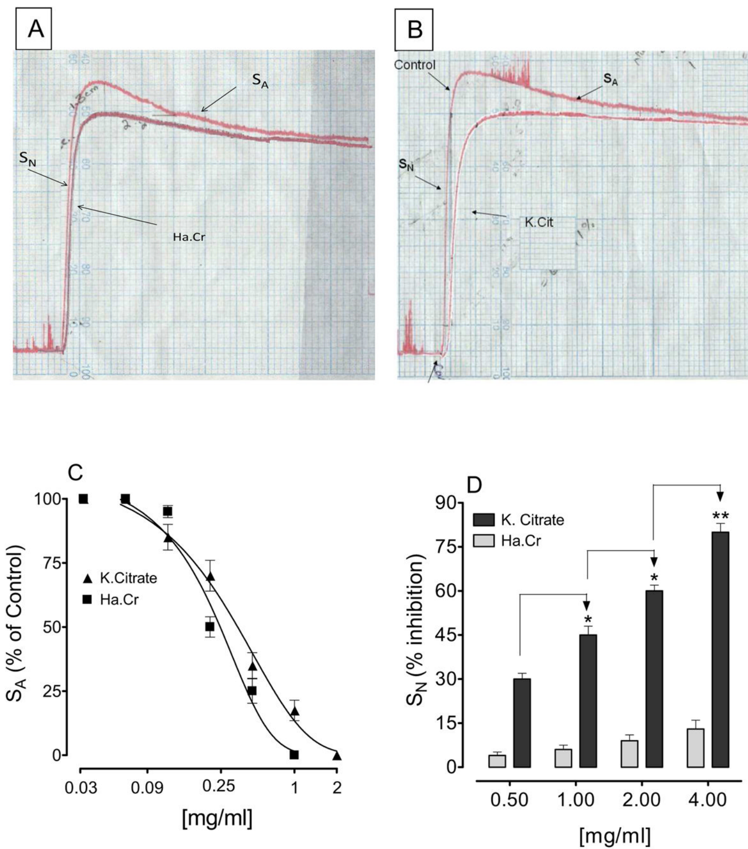

Fig. 1. Calcium Oxalate crystallization study

Effect of *Holarrhena antidysenterica* (Ha.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 Ha.Cr and potassium citrate. Panel (C) is concentration response curves of *Holarrhena antidysenterica* and potassium citrate on S_A of the turbidity curves, while (D) shows the % inhibition on the S_N. Symbols shown are mean ± S.E.M. (n = 3). S_N and S_A represent slope of nucleation and slop of aggregation respectively.

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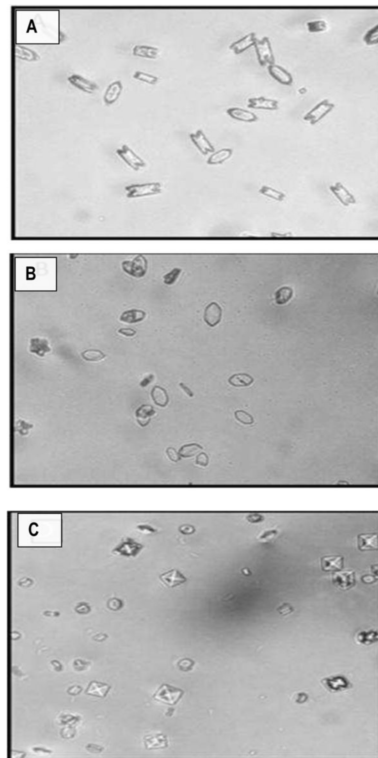

Fig. 2. Calcium Oxalate incubation study

Representative photographs, under inverted microscope (200x), of CaOx crystals developed in the metastable solutions in the absence (A) and in the presence of *Holarrhena antidysenterica* (Ha.Cr) 2 mg/ml (B) and 2 mM K-Citrate (C).

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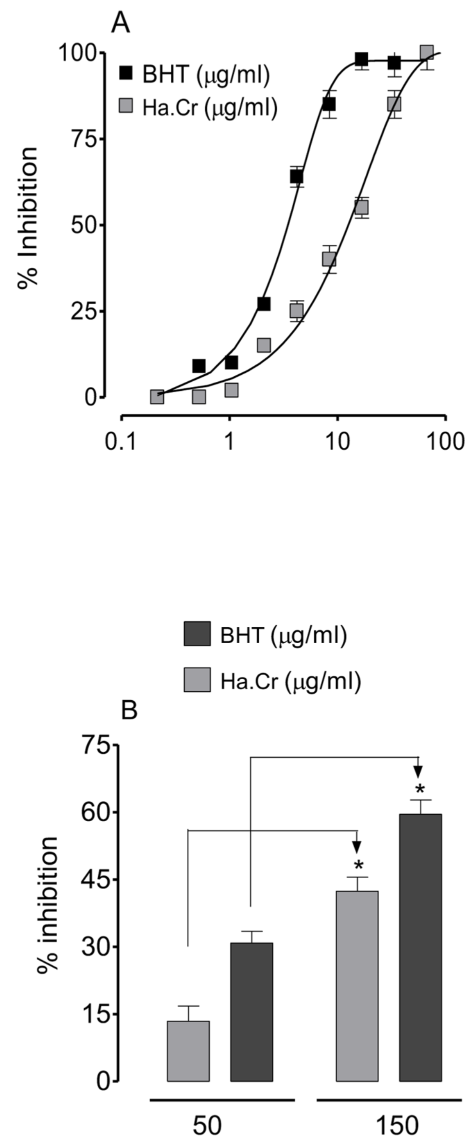

Fig. 3. Antioxidant activity

Concentration response curves of the free radical scavenging activity of the butylated hydroxytoluene (BHT) and *Holarrhena antidysenterica* (Ha.Cr), while bar-chart (B) representing lipid peroxidation inhibitory activity of two different concentrations of Ha.Cr and BHT. Inhibition is measure as % of the respective control experiments. The values shown are mean ± SEM (n=3).

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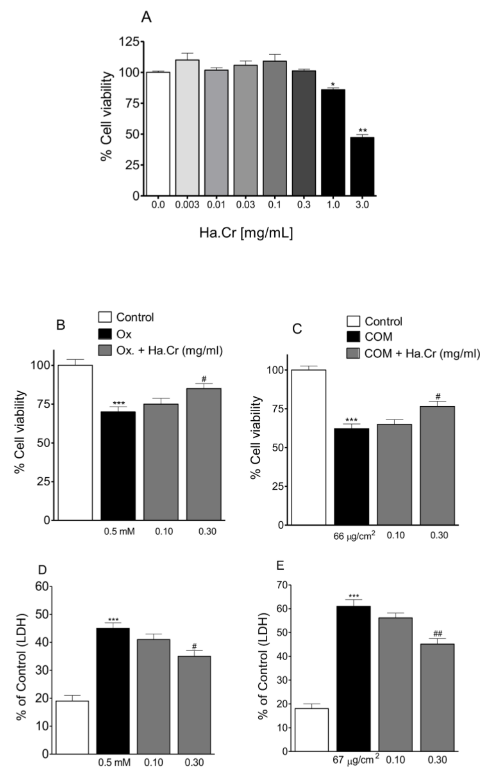

Fig. 4. Effect on MDCK cells

Effects of various concentrations of Ha.Cr on MDCK cell survival in acclimatization media (A). B and C show the protective effect of Ha.Cr after exposure to 0.5mM oxalate or 66 mg/ cm^2 COM respectively. While (D) and (E) shows the percent increase in LDH release against control by MDCK cells exposed Ox. (0.5 mM) and COM (66 $\mu g/cm^2$) for 24 hours. Data shown are mean ± SEM of two separate experiments with 3 independent replicates. $^*P < 0.05$, $^{**}P < 0.05$ and $^{***}P < 0.001$

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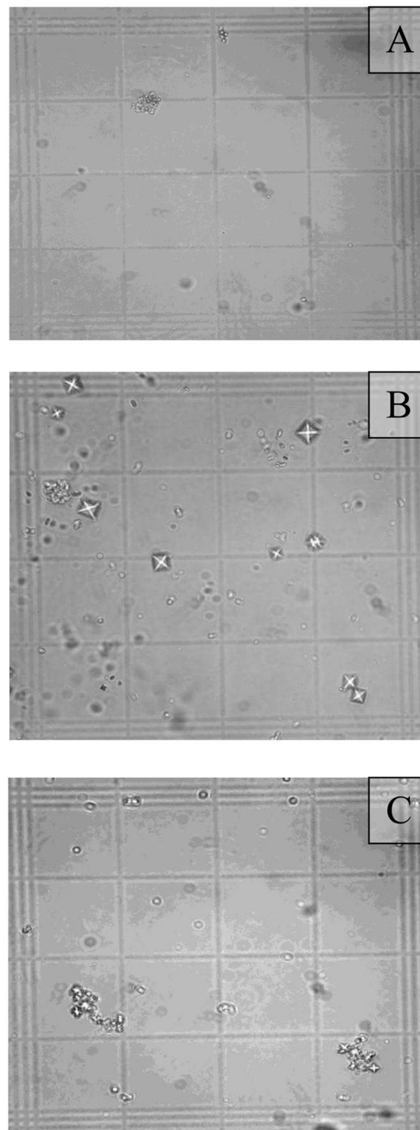

Fig. 5. Images of Crystalluria

Images of calcium oxalate crystals in 3 hrs morning urine collected from Normal control (A), Lithogenic Control (B) and treated with *Holarrhena antidysenterica* (Ha.Cr) (C), under light microscope at 400x magnification.

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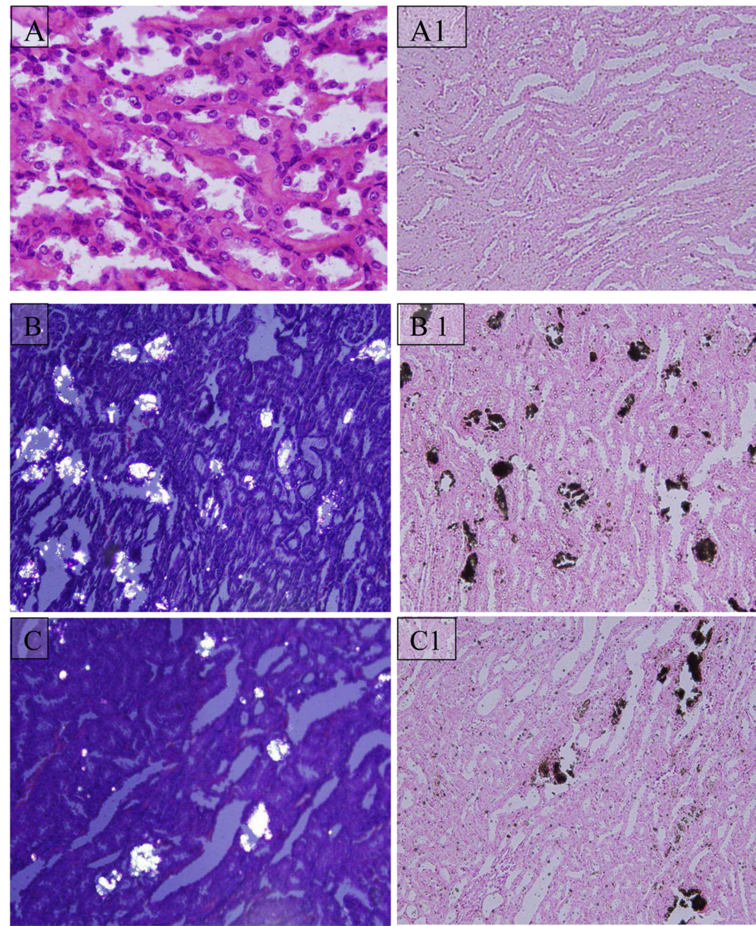

Fig. 6. Microscopic images of Kidney sections in the *in vivo* study

Representative microscopic images of the H and E stain of the kidney sections from normal (A), Lithogenic group (B) and Treated (C) with Ha.Cr. A1, B1 and C1 show the Pizzolato's staining of the respective kidney sections.

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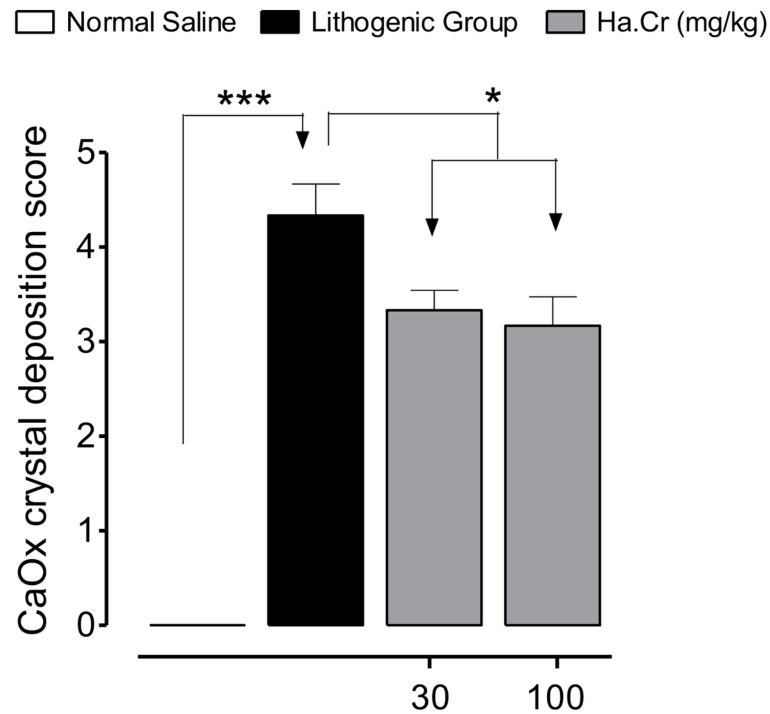

Fig. 7. CaOx crystal deposition score in preventive study;

Calcium oxalate crystal deposition score after treatment with 0.75% EG, 1% NH_4Cl (lithogenic group); Ha.Cr 30 mg/kg and 100 mg/kg. Severity grade were assigned as 0 = < 1 crystals, 1 = 1–10, 2 = 11–30, 3 = 31–50, 4 = 51–75 and 5 = > 75 crystals; data are expressed as mean ± SEM. *p < 0.05, **p < 0.05 and ***p < 0.001

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TABLE

Parameters recorded from urine and blood of groups of rats after 21 days of *in vivo* study.

Parameters	Baseline Mean±SEM	N. Saline Mean±SEM	Ha.Cr 100 Mean ± SEM	EG (0.75%) Mean±SEM	EG+Ha.Cr 30 Mean ± SEM	EG+Ha.Cr 100 Mean ± SEM
change in BW		16.1±1.4	18±3.4	−3.3 ** ±2.3	8.33 ## ±4.1	10.74 ## ±2.8
Urine Vol (ml/24h)	6.5±0.8	7.6±1.2	12.4±1.6	15 ** ±1.5	11.7 # ±1.7	8.5 # ±0.6
Urine Vol (ml/24h)	8.2±0.7	8.5±1.1	10.8±1.3	14.4 ** ±0.9	12.4 # ±1.0	10 # ±0.6
pH	6.6±0.08	6.5±0.21	6.6±0.06	6.4±0.12	6.9±0.2	6.6±0.29
Urinary (mg/24hrs)						
Oxalate	0.80±0.20	0.72±0.19	0.82±0.10	2.34 ** ±0.32	1.39±0.27	1.37 # ±0.15
Citrate	12.21±2.1	13.59±1.3	5.5±0.71	4.47±0.59	4.02±0.54	4.39±0.54
Phosphate	4.01±0.71	4.41±0.52	5.5±0.71	4.47±0.59	4.02±0.54	4.39±0.54
Uric acid	0.62±0.06	0.7±0.07	0.72±0.06	0.63±0.05	0.60±0.08	0.61±0.10
Calcium	4.06±0.48	5.01±0.52	5.75±0.71	2.67 * ±0.41	3.15±0.36	3.28±0.34
Mg^{++}	4.1±0.68	5.05±0.53	4.21±0.53	5.11±0.90	4.03±0.61	4.38±0.87
K^{+}	0.76±0.08	0.77±0.07	0.71±0.10	0.72±0.06	0.78±0.08	0.72±0.09
Total protein	3.5±1.00	4.6±1.20	5.5±0.70	12.4 ** ±1.07	7.48 # ±0.93	9.09 # ±0.57
Serum						
Creatinin mg/dl		0.73±0.11	0.45±0.06	1.4 ** ±0.15	0.81 * ±0.08	0.81 # ±0.08
BUN mg/ml		22.9±3.9	25.14±3.9	40.19 * ±3	26.28±3.9	21.58 # ±3.47

* $p < 0.05$,

** $p < 0.05$ and

*** $p < 0.001$ vs. Normal,

# $p < 0.05$,

$p < 0.01$ and

$p < 0.001$ vs. Lithogenic group