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Research article

Cucumis callosus (Rottl.) Cogn. fruit extract ameliorates calcium oxalate urolithiasis in ethylene glycol induced hyperoxaluric Rat model

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ABSTRACT

Cucumis callosus dry fruits are traditionally used as folk remedy to treat conditions like urethral irritations, urine stoppage or dribbling and other urinary ailments of man in north-west India. But no study is reported to validate this ethnic practice of using *Cucumis* fruit in urolithiasis. To evaluate anti-urolithiatic potential of *Cucumis*, hyperoxaluria was induced in rats by supplying 0.75% ethylene glycol (EG) + 1% ammonium chloride (AC) in drinking water for 14 days. Anti-urolithiatic activity of *Cucumis callosus* hydro-ethanolic extract (CCHEE) was assessed by measuring blood and urine biochemical parameters, oxidative stress indices, histopathology and osteopontin (OPN) expression. Administration of EG-AC to rats caused hyperoxaluria, crystalluria, azotaemia, oxidant/antioxidant imbalance (increase in lipid peroxidation (LPO), and decrease in glutathione (GSH) and catalase (CAT)), up-regulation of OPN and calcium oxalate (CaOx) crystal deposition in kidney. Treatment of afflicted rats with *Cucumis* fruits extract restored renal function to a great extent (CCHEE group), testified by improvement of stated parameters. Findings demonstrate curative efficacy of *Cucumis* fruit extract in EG induced urolithiasis of rats. The restoration of renal function was possibly by regulating renal stone formation via reducing urinary oxalate excretion, correcting oxidant/antioxidant imbalances, and reduced expression of OPN. Hence, results of this study validate the ethnic practice of using *Cucumis* fruit and conclude that fruit extracts have beneficial effects on CaOx urolithiasis and renal function.

1. Introduction

Urolithiasis (kidney or renal stone disease) is a process of crystal deposition in any part of renal tract, and in recent years, it has

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become the third most prevalent problem of urinary system [1]. It has been recorded in Egyptian mummies and has found a mention in *Asutu in Mesopotamia, Sushruta Samhita, Aphorisms of Hippocrates* [2,3]. Urolithiasis is one of the most challenging problems in human population, affecting approximately 1–19% in Asia, 7–13% in North America and 5–10% in Europe, 4% in South America and 20–25% in the Middle East countries [4–6]. Calcium oxalate urolithiasis account approximately 75–80% of total urolithiasis [7,8]. Incidence of it is on the rise exponentially due to rapid change in food habit of the people. Though certain advanced surgical interventions (such as ureteroscopy, percutaneous extraction and external shockwave lithotripsy) are available providing immediate relief, but those techniques prove to be insufficient, due to lifetime medical complications (hypertension, chronic kidney disease etc.) and higher rate of reoccurrence (about 50% in 5–10 years and 75% in 20 years) of the disease [9–11]. The incidence varies geographically and influenced by number of factors such as, high environmental temperature, sun exposure and genetic variability, dietary and lifestyle [12,13].

In addition to employing multiple surgical techniques, various therapeutic agents like diuretics, urinary alkalizer (citrate) are commonly used to treat urolith with limited success [14–16]. Till date there is no satisfactory treatment available for urolithiasis due to its complex pathophysiology and multi-facet etiology. Ethnic people across the world use various medicinal plants since antiquity and many of these plants seem to have beneficial effects in managing the urolith. In most cases, medicinal plants are cheap, and are considered to be safe alternative. Multiple plants have been evaluated recently for their antiurolithiatic efficacy [11,17–23]. Cucurbitaceae family has been reported for its efficacy against urolithiasis [24,25].

Cucumis callosus (Rottl.) Cogn is an herb of family *Cucurbitaceae* and it is grown during the rainy season in all dry regions, particularly in the northwestern part of India. Locals refer this herb as Kachari (also spelt as Kachari) in the state of Rajasthan and grown as a perennial herb. Its fruits is used in preparation of pickles, chutneys and delicious vegetable dish like Panchkuta [26,27]. *Cucumis callosus* fruits contain around 32 compounds including alkaloids, flavonoids, tannins, glycosides, carbohydrates and proteins etc [28,29]. The majority of these phytochemical substances have therapeutic effects (eg. Antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, antitumor, and hypocholesterolemic). Various parts of this herb are traditionally recognized for medicinal values against different clinical afflictions. For example, the seeds are useful in bilious abnormalities and have astringent or cooling effects to mucosa [30]. The herb is beneficial in skin disease, snake bite and has anthelminthic, anticancer, antioxidant, antidiabetic and diuretic activities [27,29,31,32]. Also reported to be effective in tumpley disease of cattle [33].

Cucumis callosus (Rottl.) Cogn fruits are used as traditional remedy by ethnic people in Rajasthan and Gujarat states, particularly for urethral irritations, urine stoppage or dribbling, and other urinary ailments. These signs are observed in kidney stone disease or urolithiasis of man. Based on this, we hypothesize that Hydro-ethanolic fruit extract of *Cucumis callosus* may be effective in amelioration of Calcium oxalate urolithiasis. However, to the best of our knowledge, no report is available in experimental model animals to test the hypothesis. Therefore, this study was designed for evaluation of usage of *Cucumis* fruit extracts against urolithiasis in EG-AC induced rats.

2. Materials and methods

2.1. Collection of Cucumis dry fruits

In Indian state of Rajasthan, close to Bikaner, dried fruits of *Cucumis callosus* (Rottl.) Cogn were collected. The fruits were recognized and validated from 'Central National Herbarium' of the 'Botanical Survey of India' in Howrah, West Bengal, India, and the specimen was preserved in Herbarium for further references (Specimen voucher no: CNH/I-I/2016/Tech -23).

2.2. Hydro-ethanolic extraction

The Kachri dried fruits were evenly crushed using an electrical kitchen grinder. The powdered fruits were soaked in equal amount of distilled water and ethanol solvent mixture on a magnetic stirrer for 6 h at room temperature to prepare *Cucumis callosus* hydro-ethanolic extract (CCHEE). Thereafter, extract purification was carried by filter paper (Whatman 40), dried under vacuum, and kept at 4 °C until use [34]. Total of 31.4% (w/w) of the extract was recovered.

2.3. Cucumis callosus phytochemical screening

Cucumis callosus hydro-ethanolic extract was preliminarily screened for phytochemical constituents as per standard methods [35]. For qualitative screening of alkaloids, dragendroff and mayer's; for saponin, foam; for carbohydrate, benedict; for protein, biuret; for flavonoids, alkaline reagent; for phenolics/tannin, lead acetate; for glycoside, cardiac glycoside and coumarin glycoside test were carried out.

2.4. Animals

For present study, we used male healthy Wistar rats (12–13 week, 150–200 g) bred at Institute's "Laboratory Animal Research Division". Throughout experiment, rats were housed and maintained in clean polypropylene cages (12 h light -12 h dark cycle) at room temperature (25 ± 2 °C). Standard ration and *ad libitum* water was given to them. Guidelines prescribed by "Committee for the Purpose of Control and Supervision of Experiment on Animals" (CPCSEA) were followed for maintenance and housing of rats and they were well adapted prior to starting of experiment. Prior permission was obtained from the "Institute Animal Ethics Committee" (IAEC) with Approval no-F.26-1/2015-16/JDR.

2.5. In vivo induction of CaOx urolithiasis and Cucumis treatment

Cucumis anti-urolithiatic efficacy was examined using a previously established hyperoxaluric rat model of calcium oxalate urolithiasis [19,36–39]. Thirty Wistar rats (male) were sub-divided into 5 groups (Healthy control, Lithiatic control, Vehicle control, Cystone treatment and CCHEE treatment) and except for Healthy control, all rats were supplied with 0.75% ethylene glycol (EG) + 1% ammonium chloride (AC) in drinking water for duration of 14 days. Day 14 onwards, rats were provided with respective treatments as indicated in group names. Healthy control, not receiving any treatment or untreated; Lithiatic control, 0.75% EG alone for the remaining 14 days of experiment; Vehicle control, *ad libitum* distilled water till day 28; Cystone treatment, Cystone (commonly used polyherbal formulation, Himalaya Drug Company, Bangalore, India) at 100 mg/kg through gastric gavages till day 28; CCHEE treatment, *Cucumis callosus* hydro-ethanolic extract at 250 mg/kg (EC_{50}) through gastric gavage up to day 28. We have previously estimated EC_{50} dose of *Cucumis* fruit extract (with 3 dosages, 100, 250 and 500 mg/kg through gastric gavage) for anti-urolithiatic effect in EG induced hyperoxaluric rat model (Data not shown).

2.6. Biochemical analysis of urine

During the entire experimental period, urine was analyzed thrice (at days 0, 14 and 28). For the purpose of urine collection, rats were housed for 24 h duration each time in individual metabolic cages. Urine pH and volume were assessed immediately after collection. Samples were centrifuged at 1500 rpm for 10 min to remove extraneous objects. Precipitates were used for microscopic examination of urinary crystals. The clear supernatants were preserved in two halves at −20 °C. Before storage, one half was acidified with 1–2 drops of 3 N hydrochloric acid, while the other was left non-acidified. Urine urea nitrogen and creatinine parameters were analyzed from non-acidified urine [19]. The acidified sample was used to estimate urinary biochemical like calcium, phosphorus, potassium and sodium using a standard commercial kit (Tulip Diagnostics (P) Ltd, Goa, India). Urinary oxalate was estimated by using quantitative rat oxalic acid ELISA kit (Qayee Biotechnology Co., Ltd. Shanghai, China). This colorimetric assay at 550 nm wavelength has sensitivity of 0.02 mmol/L and detection range of 0.02–1 mmol/L for urinary oxalate analysis.

2.7. Biochemical analysis of blood

Blood samples were collected on days 0, 14, and 28 in heparinized sterile vials from rats (retro-orbital venous plexus) with help of micro hematocrit capillaries [40]. Samples were centrifuged at 3000rpm for 20min; plasma was recovered and preserved until usage at −20 °C. Samples were processed for estimation of biochemical parameters like blood urea nitrogen (BUN), creatinine, total protein, albumin, calcium, and phosphorous as per manufacturer's protocol (Tulip Diagnostics (P) Ltd, Goa, India).

2.8. Oxidative stress indices

Sediment erythrocytes/RBCs in above processed blood sample were washed (three times) using isotonic phosphate buffer solution. To create 10% RBC hemolysate, washed erythrocytes were hemolyzed with a nine-fold amount of triple-distilled water. By using the cyanomethaemoglobin technique, haemoglobin in 10% RBC haemolysate was spectrophotometrically measured [41]. The previously established spectrophotometric method was used to estimate lipid peroxidation (LPO) in 10% hemolysate [42]. Malondialdehyde (MDA) was used to measure lipid peroxidation activity and was quantified as nM per mg of haemoglobin, considering extinction coefficient value as 1.56×10^5 [43]. The level of glutathione (GSH) in packed erythrocytes (RBCs) was determined using the DNTB (di-thiobis2-nitro benzoic acid) method [44]. GSH activity was expressed in mM of per mg haemoglobin. Catalase activity (CAT) was estimated in 10% hemolysate at 240 nm wave length and measured in units per mg of haemoglobin [45].

2.9. Histopathology of kidney tissue

Samples of kidney tissue from each rat on day 28 of experiment were collected and fixed with buffered solution containing 10% formalin (pH, 7.4). Graded alcohol was used to dehydrate sample tissue and then paraffin-embedded. Tissue section of 5 μm thickness was prepared using a microtome (Ergostar HM200, Microm, Germany) and thereafter, stained using Hematoxylin-Eosin (H&E) combination dye to investigate kidney histopathological changes [46]. The calcium oxalate crystal deposits in kidney sections were identified after 10 randomly chosen microscopic fields were inspected at 10× magnification using techniques described previously [19].

2.10. Immunohistochemical staining

Expression of osteopontin (OPN) protein was examined in kidney tissue section by immunohistochemical staining [47]. Briefly, the paraffinized kidney tissue section (5 μm thickness) was placed on coated glass slide (P0425 Sigma Poly-Prep Slides, Sigma-Aldrich, USA). To remove paraffin, slide was submerged in xylene and then immersed in graded alcohol (50%–70% - 80%–90% -100%) for removal of water. For antigenic retrieval of tissue sections, sample was placed on a slide and boiled in a buffered solution containing sodium citrate (10 mM) at pH of 6.0 for 10 min, and then allowed to cool at room temperature. To prevent endogenous peroxidase activity, tissues section were incubated for 10min in solution of 0.3% hydrogen peroxide and to check non-specific antibody binding, incubated for 60 min in 3% goat serum. The tissue sections were then incubated and treated with osteopontin primary antibody for

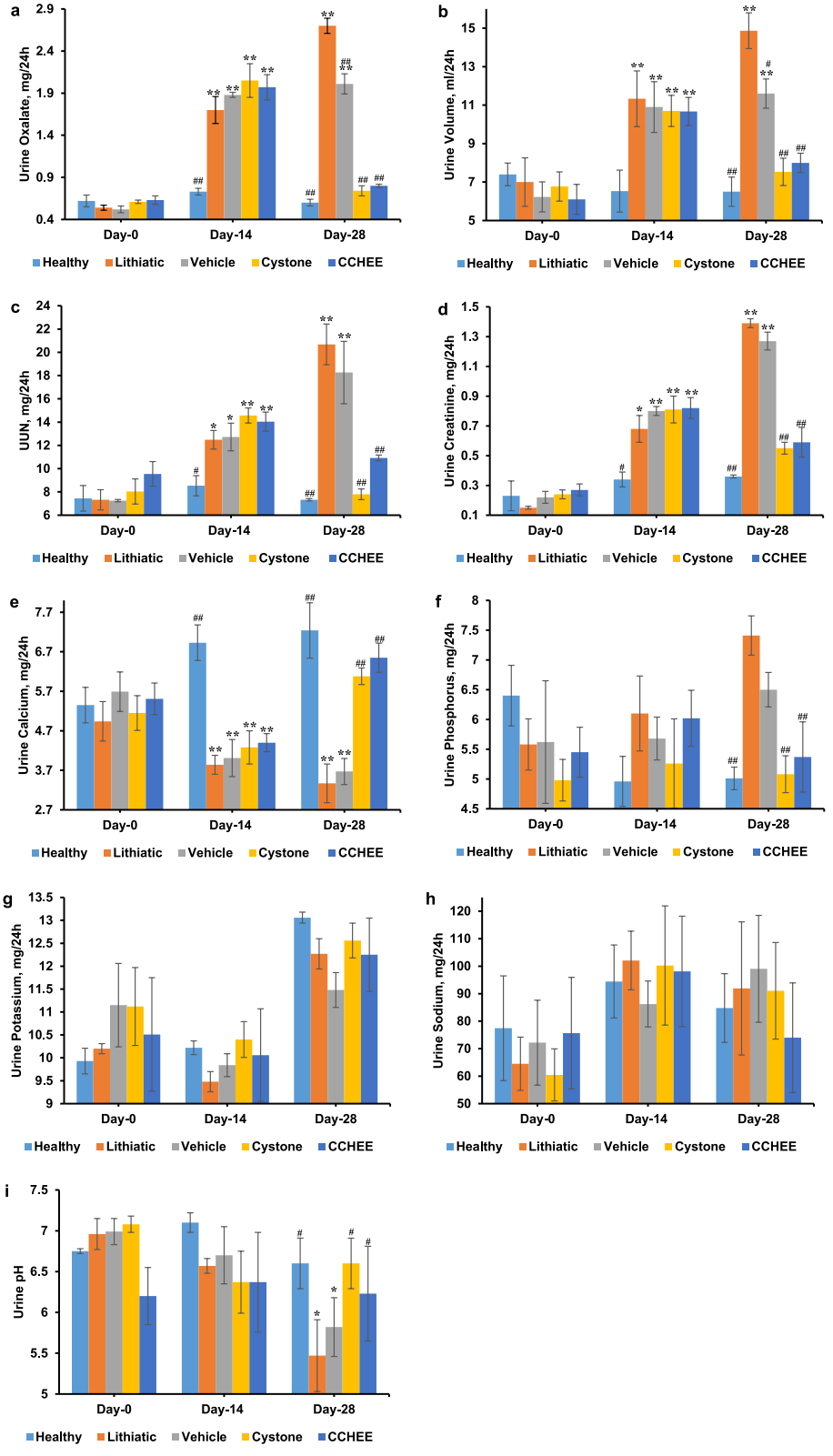

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Fig. 1. Cucumis callosus (Rottl.) Cogn. hydro-ethanolic extract (CCHEE) improves biochemical parameters of urine in ethylene glycol (EG) induced hyperoxaluric rats. As described in methods section urine samples were collected for 24 h in a metabolic cage on days 0, 14, and 28 and analyzed. The values are expressed as mean ± SE in bar diagram. (a) Cystone and CCHEE treated groups significantly reduced urine oxalate (mg/24 h) excretion at day 28 ($p < 0.01$), compared to lithiatic as well as vehicle control; (b) CCHEE significantly ($p < 0.01$) reduced urine volume (ml/24 h); (c) CCHEE treated rats shows significant decrease ($p < 0.01$) in urine urea nitrogen (UUN) concentration (mg/24 h); (d) CCHEE also significantly reduced ($p < 0.01$) urine creatinine excretion (mg/24 h), compared to lithiatic control; (e) CCHEE elevated calcium excretion (mg/24 h), towards normal level ($0 < 0.05$); (f) urine inorganic phosphorus (mg/24 h), (g) Urine sodium (mg/24 h), (h) urine potassium (mg/24 h) did not change significantly in all rats throughout the study period; (i) CCHEE treated group showed improvement in urine pH values compared to lithiatic control ($p < 0.05$). *$p < 0.05$ and **$p < 0.01$ versus Healthy control, #$p < 0.05$ and ##$p < 0.01$ versus Lithiatic control, one-way analysis of variance followed by post hoc Dunnett's test.

overnight at 4 °C (mouse monoclonal to osteopontin, 1: 50 dilutions, Santa Cruz Biotechnology, Texas, United States). Next day, these sections were washed thrice in PBS (Phosphate Buffered Solution), each time for 10min and further, incubated for 1 h with horse radish peroxidase conjugated goat-anti mouse Ig-G secondary antibody (1: 200 dilutions, Santa Cruz Biotechnology, Texas, United States). The sections were then once again washed with PBS three times, each time for 10 min, and stained by 3,3′-Diamino-benzidine (DAB)

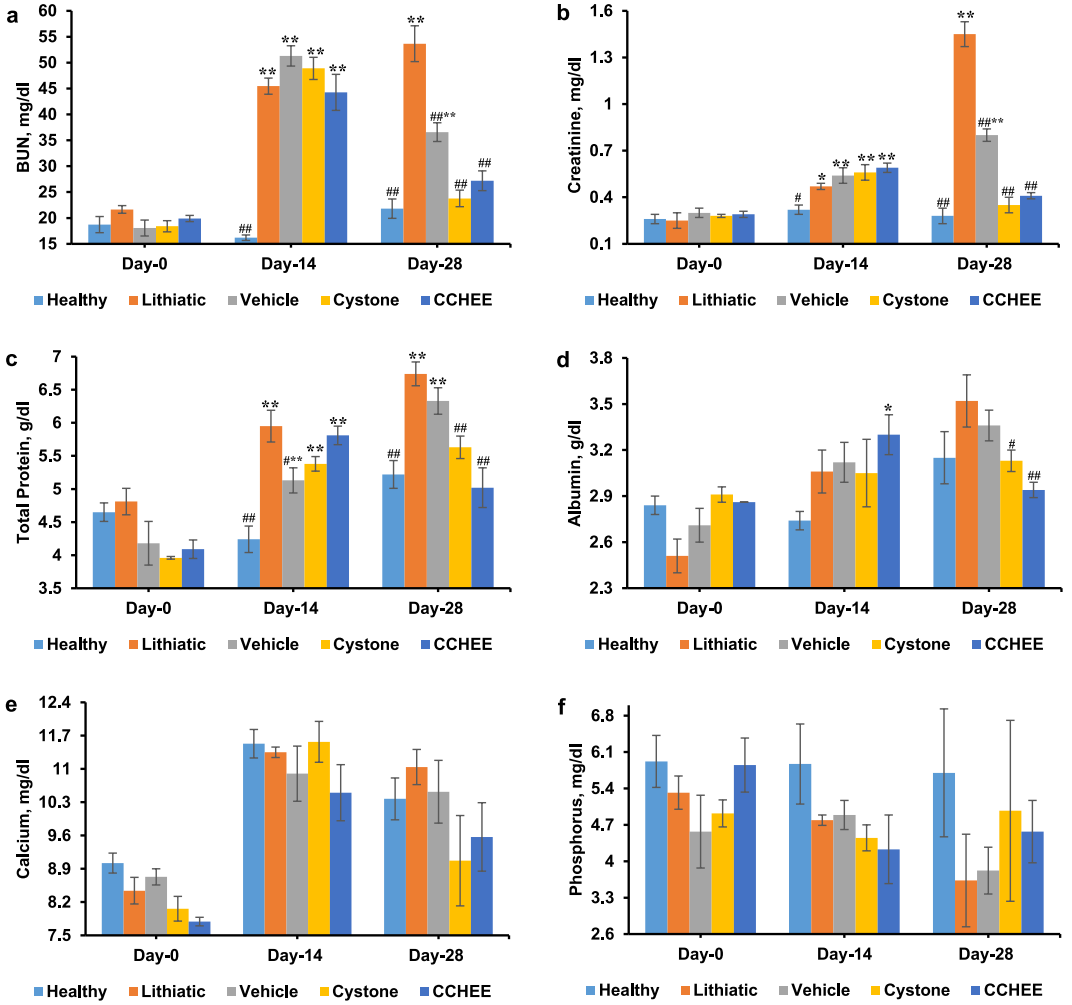

Fig. 2. Cucumis callosus (Rottl.) Cogn. hydro-ethanolic extract (CCHEE) improves biochemical parameters of blood in ethylene glycol (EG) induced hyperoxaluric rats. As described in methods section blood samples were collected on days 0, 14 and 28 in heparinized sterile tube from retro orbital venous plexus of rat using micro-hematocrit capillaries and plasma were used for analyses. The values are expressed as mean ± SE in bar diagram. On day 0 all biochemical values were statistically similar between different groups. (a) Cystone and CCHEE treated groups significantly reduced BUN (blood urea nitrogen, mg/dl) at day 28 ($p < 0.01$), compared to lithiatic as well as vehicle control; (b) CCHEE significantly ($p < 0.01$) reduced creatinine (mg/dl); (c) CCHEE treated rats also shows significant decrease ($p < 0.01$) in total protein (g/dl) level as compared to lithiatic control; (d) CCHEE also significantly reduced ($p < 0.01$) albumin (g/dl) compared to lithiatic control on day 28; (e) calcium (mg/dl) and (f) phosphorus (mg/dl) values did not change significantly throughout the study. *$p < 0.05$ and **$p < 0.01$ versus Healthy control, #$p < 0.05$ and ##$p < 0.01$ versus Lithiatic control, one-way analysis of variance followed by post hoc Dunnett's test.

(Abcam, USA). After counterstaining using Mayer's hematoxylin (Sigma Aldrich, USA), mounting of sections carried out with CC/mount (Sigma Aldrich, USA). Under microscopic examination, cells expressing OPN took brown coloration in cytoplasm.

2.11. Statistical analyses

The IBM SPSS software (Version 20) was used for the statistical analyses. The mean and standard error of the mean was used to express all quantitative data (SEM). When $p < 0.05$, values were considered significant. The post-hoc Dunnett's test was used to compare the group means when an analysis of variance revealed a significant treatment effect.

3. Results

3.1. Cucumis callosus phytochemical screening

Cucumis callosus hydro-ethanolic extract was positive for alkaloids, saponin, carbohydrate, protein, flavonoids, phenolic/tannin, and negative for glycosides like cardiac glycoside and coumarin glycoside.

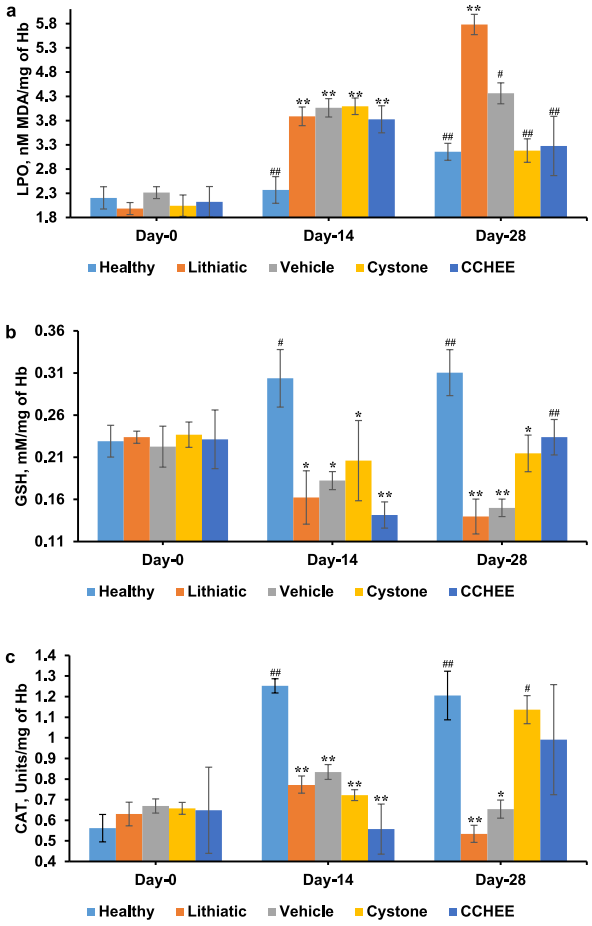

Fig. 3. Cucumis callosus (Rottl.) Cogn. hydro-ethanolic extract (CCHEE) improves oxidative stress indices in ethylene glycol (EG) induced hyperoxaluric rats. As described in methods section blood samples were collected on days 0, 14 and 28 in heparinized sterile tube from retro orbital venous plexus of rat using micro-hematocrit capillaries and packed RBCs (Red blood cells) are used for oxidant/antioxidant analyses. The values are expressed as mean ± SE in line diagram. On day 0 of study, values of oxidant/antioxidant parameters were statistically similar between different groups. (a) Cystone and CCHEE treated groups significantly reduced LPO (lipid peroxidation, nM MDA/mg of Hb) activity urea nitrogen, mg/dl) at day 28 ($p < 0.01$), compared to lithiatic as well as vehicle control; The values of antioxidants (b) GSH (glutathione, mM/mg of Hb) and (c) CAT (catalase, units/mg of Hb) were increased significantly ($p < 0.05$) in CCHEE treatment group as compared to lithiatic control on day 28. *$p < 0.05$ and **$p < 0.01$ versus Healthy control, #$p < 0.05$ and ##$p < 0.01$ versus Lithiatic control, one-way analysis of variance followed by post hoc Dunnett's test.

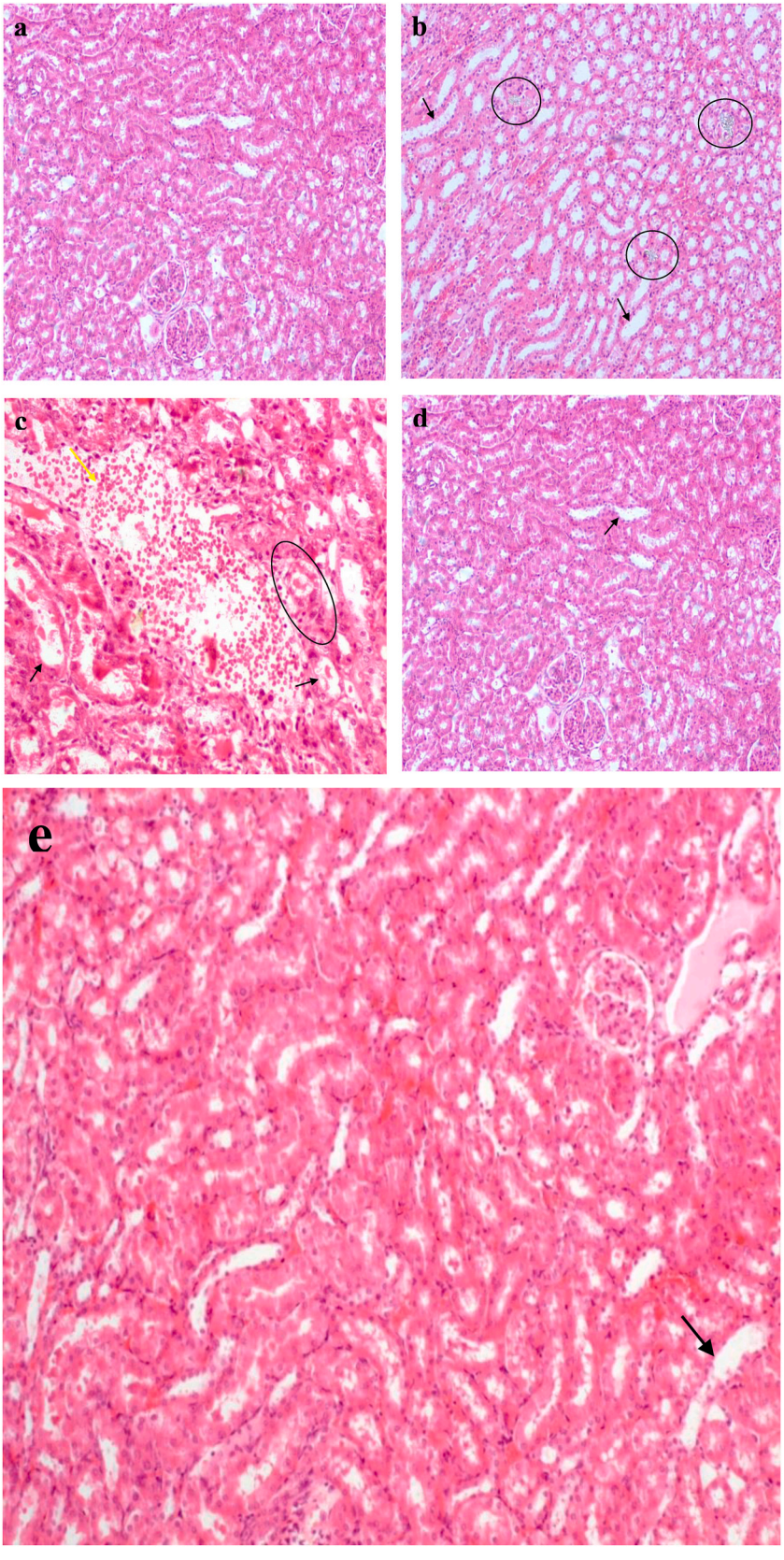

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Fig. 4. Cucumis callosus (Rottl.) Cogn. hydro-ethanolic extract (CCHEE) reduces crystal deposition, inflammation and renal damage in ethylene glycol (EG) induced hyperoxaluric rat kidneys. (a–e) Micrographs showing kidney section of rats stained with hematoxylin and eosin performed on day 28 (×100). (a) Kidney section of healthy control rat showing normal architect; (b) Kidney section of lithiatic control rat showing crystals (encircled) and mononuclear cells infiltration with dilation of tubules (black arrow); (c) Kidney section of vehicle control rat showing hemorrhages (yellow arrow) and hyaline casts with tubular dilatation (black arrow) and degeneration (encircled); (d) Kidney section of cystone treated rat showing almost normal architecture; (e) Kidney section of CCHEE treated rat showing almost normal architecture with mild mononuclear cells infiltration and tubular dilatations (black arrow).

3.2. Cucumis fruit extract reduces urine oxalate, volume, urea nitrogen and creatinine but increases urine calcium and pH in EG induced hyperoxaluric rats

On day 0 of study, values of all biochemical parameters in urine were statistically similar between different groups (Fig. 1). At day 14, rats of all four groups that supplied with the stone-producing treatment (EG-AC) showed significantly ($p < 0.01$) higher levels of urine oxalate content, volume, urea nitrogen, and creatinine as compared to normal healthy control rats (Fig. 1a–d). Significant ($p < 0.01$) decrease in urine calcium concentration and urine pH were recorded on day 14 of the experiment (Fig. 1e,i). On day 28, further increase of urea nitrogen, creatinine levels and decrease of urinary calcium concentration in lithiatic and vehicle control rats were recorded because of ongoing kidney damage. On day 28, after treatment with Cucumis extract (CCHEE) these parameters were restored statistically similar to treatment with medication Cystone (Fig. 1). Urine inorganic phosphorus, potassium and sodium values did not change significantly ($p > 0.05$) throughout the experiment (Fig. 1f–h).

3.3. Cucumis fruit extract reduces BUN, creatinine and total protein in EG induced hyperoxaluric rats

On day 0 of study, values of all biochemical parameters in blood were statistically similar between different groups (Fig. 2). Rats in all four groups receiving lithiatic treatment had significantly higher blood urea nitrogen (BUN), creatinine, and total protein levels on day 14 compared to untreated rats in the healthy control group ($p < 0.01$). Further, these biochemical values were increased significantly ($p < 0.01$) in the rats belongs to lithiatic and vehicle control on day 28 of the trial. Treatment with Cucumis fruit extract significantly ($p < 0.05$) decreased these parameters and values were similar ($p > 0.05$) to that of observed in Cystone treated group on day 28 (Fig. 2a–c). Plasma albumin, calcium, phosphorus values did not change significantly throughout the study (Fig. 2d–f).

3.4. Cucumis fruit extract decreases oxidant enzyme (lipid peroxidase) and boost antioxidant enzymes (GSH and CAT) in EG induced hyperoxaluric rats

On day 0 of study, values of oxidant/antioxidant parameters were statistically similar between different groups. On day 14, the LPO activity was elevated, and the GSH and CAT activities were significantly ($p < 0.01$) reduced in the all four groups that had supplied therapy to induce stone formation (Fig. 3). Further, at day 28, in the lithiatic rats lipid peroxidation activity was significantly ($p < 0.01$) higher than in normal healthy rats. But therapy with Cucumis extract (CCHEE) significantly ($p < 0.01$) decreased LPO activities and restored the activities of GSH and CAT almost similar ($p > 0.05$) to the values recorded in healthy control group (Fig. 3).

3.5. Cucumis fruit extract decreases CaOx crystal deposition and reduces inflammation and renal damages in hyperoxaluric rat kidneys

The healthy control group's kidney tissue segment revealed the organ's normal architecture under microscopic histopathological examination. Crystals were absent and no renal damage was detected (Fig. 4a). Numerous calcium oxalate crystals were deposited in different parts of kidney (tubules, glomeruli, proximal and distal duct of renal cortex or medulla) as a result of stone induction (EG-AC) in the lithiatic and vehicle control rats (Fig. 4b and c). In the kidney of rats in lithiatic and vehicle groups, there were degeneration and dilatation of tubules, necrosis with mononuclear cells infiltrations, severe haemorrhages, and casts or fibrin deposits. Renal tubules were severely damaged by crystal depositions, which led to cellular infiltration in the lumen. The number of infiltrating mononuclear cells and number of crystal deposits were significantly reduced in the group of rats treated with CCHEE and Cystone. After receiving Cucumis fruit extract (CCHEE) treatment, the kidney damage was significantly repaired, much like with Cystone treatment. (Fig. 4d and e).

3.6. Cucumis fruit extract downregulates osteopontin expression in EG induced rat kidneys

The immunohistochemistry of renal tissue showed significant ($p < 0.01$) increase in osteopontin expression after renal stone formation in the lithiatic and vehicle groups rats (Fig. 5b and c). The kidneys of lithiatic control rats displayed enhanced expression in glomeruli, tubules, and convoluted tubules etc. When compared to rats in lithiatic control group, treatment of animals with *Cucumis callosus* extract (CCHEE) significantly reduced OPN expression (Fig. 5e). A similar lower OPN expression was also observed in rats treated with cystone (Fig. 5d). Renal portion of healthy control Rats showed no OPN expression (Fig. 5a). These data corroborated well with number of cells positive/negative for OPN in kidney. It was found significant ($p < 0.01$) reduction (compared to data from lithiatic or vehicle only groups) in percent of OPN positive cells in rats under CCHEE treatment group, and this data was similar to that of observed in cystone treated group rats (Fig. 5f).

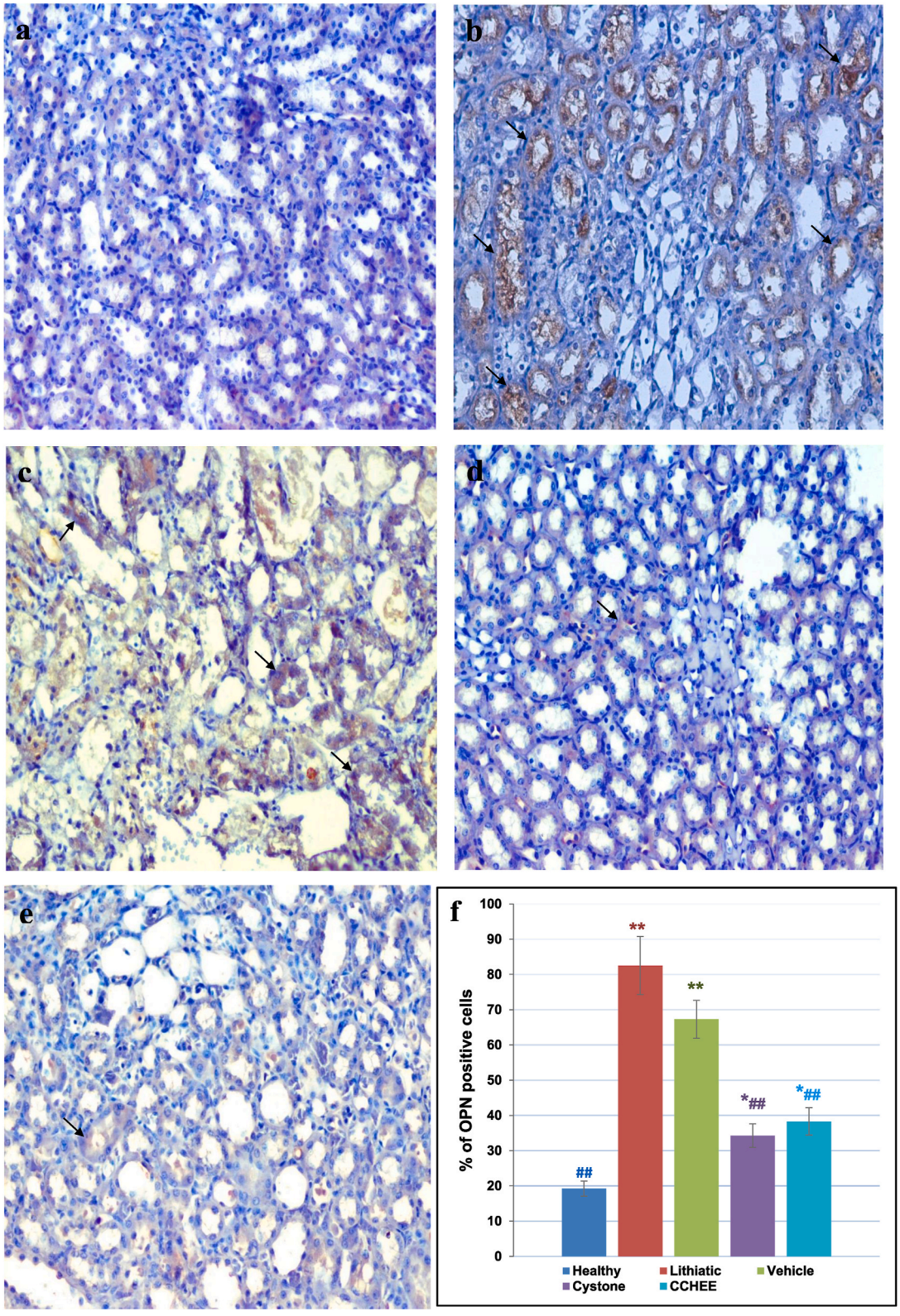

Fig. 5. Cucumis callosus (Rottl.) Cogn. hydro-ethanolic extract (CCHEE) downregulates immunohistochemical expression of osteopontin (OPN) in ethylene glycol (EG) induced hyperoxaluric rat kidneys. (a–e) Micrographs showing kidney section of rats stained for OPN in EG-ammonium chloride (AC) treated rats (n = 6/group) performed on day 28 (×100). (a) Kidney section of healthy control rat showing mild expression; (b)

Kidney section of lithiatic control rat showing increased expression (black arrow); (c) Kidney section of vehicle control rat showing increased expression (black arrow); (d) Kidney section of commonly used poly-herbal drug cystone treated rat showing mild expression (black arrow); (e) Kidney section of CCHEE treated rat showing mild expression (black arrow). (f) Quantitative analysis of immunohistochemical expression OPN in kidney section of EG-AC treated rats. The average number of stained (OPN positive) cells/100cells were counted across 10 fields/section. *$p < 0.05$ and **$p < 0.01$ versus Healthy control, #$p < 0.05$ and ##$p < 0.01$ versus Lithiatic control, one-way analysis of variance followed by post hoc Dunnett's test.

4. Discussion

In many ancient cultures, plants are traditionally employed as folk remedy to treat different ailments and such knowledge is passed on verbally from generation to generation. These remedies are formulated on empirical knowledge, and "experience driven" gathered over many years, sometimes spanned over centuries. In most cases, these approaches have no backing of data from controlled experiments. We have recorded one such plant (*Cucumis callosus* Rottl. Cogn), used as a traditional remedy to cure urinary tract ailments in humans. We have used the hydroethanolic fruit extract of this plant in designed experiment and demonstrated improvement of renal oxaluria condition in experimental rats. Hydroethanolic solvent (1:1 distilled water: ethanol) allowed to dissolve both polar and non-polar compounds of dry fruit for administering to animals. To our knowledge, this is first report that attempted to validate anti-urolithiatic activities of *Cucumis* dry fruits in any experimental model.

The renal oxaluria can be initiated by oral administration of ethylene glycol (EG) alone, or in combined with ammonium chloride (AC) in rat model [36]. We adopted EG-AC method for generation of hyperoxaluria in rat model [36–39]. Here, we specifically used male rats for ensuring formation of calcium oxalate stones with greater efficiency. Previous reports suggested that male sex hormone testosterone enhances the formation of calcium oxalate crystals in EG treated adult rats. Whereas EG mediated crystal formation was inhibited in the presence of female sex hormone, estrogen [48,49].

Urinary stone formation involves number of events involving metabolic, cellular and endocrine pathways [50,51]. In addition, interaction among essential minerals has a significant role for onset and progression of urolithiasis [52]. During this, it affects primarily the following: 1) Glyco-oxalate pathways in liver [53]; 2) Deposition of calcium oxalate in damaged cells [54]; 3) Alteration in expression of extracellular matrix protein [55]. These abnormalities may lead to: 1) Alteration of pH towards acidic range (<7.0); 2) reduction in urine volume; 3) lower GFR (Glomerular filtration rate), and 4) aggravation of crystal deposition in renal tissues.

Any alteration in Glyco-oxalate pathway at initial stage usually enhances the capacity of renal excretion of crystals through urine with increased GFR. This occurs to counteract the azotemia mediated general toxicity, to a certain extent, in the affected individual. Also, excessive oxaluria in conjunction with alteration of urine pH (<7.0) results in deposition of crystals in renal tissue. Further, deposition of oxalate crystals triggers oxidative cell injury in renal parenchyma, causing reactive oxygen species generation. ROS-mediated oxidative damage leads to peroxidation of cell membrane phospholipids, allowing the influx of excessive calcium into damaged cells [54,56]. This is reflected by the presence of uroliths in renal system and also by altered expression of several extracellular matrix proteins (such as, osteopontin) [47,57]. Therefore, pathobiology of urolithiasis should require look at multiple parameters such as, urine oxalate excretion, urine volume, urine pH, creatinine, urea nitrogen, calcium/phosphorus, total crystal content, lipid peroxidation (LPO), GSH, and Catalase. In addition, one should carefully look at the expression of matrix protein mentioned previously. Therefore, in the current study we looked into all these parameters to understand the role of the *Cucumis* fruit extracts in mitigating urolithiasis, if any.

We observed that treatment of lithiatic animals with *Cucumis* fruit extracts restored Oxalate content and urine volume (Fig. 1a and b). We also failed to detect renal crystal in the treated animals (Fig. 4e). Therefore, this data suggest that *Cucumis* fruit extract has litholytic effect mediated via Glyco-oxalate pathways, similar to medicinal herbs or natural compounds described previously [16,19,21,58,59].

Further, treated animals showed restoration of pH, urea nitrogen and creatinine level in urine (Fig. 1c,d,i) and also in blood (Fig. 2a and b). Similar effects were also reported by other researchers [20,21,39]. Under hostile condition such as in the presence of ROS, cell membrane phospholipid is converted into malondialdehyde (MDA), indicating peroxidative damage of cells in the body. We detected higher MDA (significant $p < 0.01$) in lithiatic animals and the values returned to normalcy when animals were treated with herbal extract (Fig. 3a). Moreover, treatment of lithiatic animals also brought back both glutathione peroxidase (GSH) and catalase (CAT) activities (Fig. 3b and c). These enzymes are considered to be good antioxidant indicator, demonstrating antioxidant effect of the plant probably via free radical scavenging ability. Our study demonstrated similar results in oxidant/antioxidant indices in hyperoxaluric rat model as stated by various reports [5,19,21]. Taken together, the data clearly demonstrate that metabolic pathways are intervened by the fruit extracts. Thus, the fruit extract has reno-protective effect and protects the cells/tissues from oxidative cell injury. This may be because Cucumis extract contains alkaloids and flavonoids, which have antioxidant properties.

As stated before, mineral interaction is usually involved during urolithiasis. This is also found in the current study with alteration of calcium (low in urine) and phosphorus (low in blood) level (Figs. 1e and 2e). However, these changes were brought to physiological range in animals treated with the test extract (Figs. 1e and 2e). We did not note any detectable changes of potassium and sodium level in urine though (Fig. 1g and h).

We found presence of crystals in renal tissues (Fig. 4b), presumably causing physical damage to kidney. Physical damage in kidney of experimental rats was ascertained by detection of higher creatinine and urea nitrogen in urine and blood (Figs. 1c, d and 2a, b). Increment in total protein, creatinine and urea nitrogen in blood is considered to indicate tubular and glomerular damage [59]. Our results clearly indicate that herbal extract has protective effects against physical damage both glomeruli and renal tubules (Figs. 1c, d,

2a, c and 4e). This is might be due to extract capability to clear urinary path by dissolving calcium oxalate crystal. It is clearly indicating Cucumis is having lithotriptic activity as well as inhibitory potential on crystal growth and its aggregation. Similar kind of effects were evidenced by various report on herbs [20,21,39].

Matrix proteins (crystallization regulatory molecules) are also good indicator of urolithiasis [47,57]. In the current study we found that ethylene glycol treatment resulted in up-regulation of osteopontin in kidney cells (Fig. 5b), compared to the untreated healthy control (Fig. 5a). However, treatment of animals with both cystone and test plant extract significantly ($p < 0.05$) down-regulated osteopontin expression in renal cells (Fig. 5d and e). In addition, there was reduction of number of cells that expressed OPN (Fig. 5f). Therefore, these data indicate that treatment with *Cucumis* fruit extract has a strong beneficial effect in bringing down the expression of OPN in affected animals. It also suggests that the test extract has anti-crystallization and anti-inflammatory effects, similar to other reports published previously [19,59–61].

The overall limitation of our study is that quantitative phytochemical analysis of *Cucumis callosus* extract has been not done, which is required for identifying candidate compound (s) responsible for the antilithiatic activity. We did not conducted any *in vitro* studies on nucleation and aggregation before *in vivo* experiment in oxaluric rat model of CaOx urolithiasis. As pathogenesis of urolithasis is too complex and these *in vitro* studies cannot be extrapolated for medicinal effect. The majority of the studies of herbal antilithiatic effect have utilized the ethylene glycol induced rat model [11,62,63]. The ethnic people use the whole fruit that contains both polar and nonpolar compounds. To simulate the ethnic practice, we have selected Hydro-ethanolic (Distilled water: Ethanol 1:1) as solvent for extraction of both polar and nonpolar constituents. Other solvents extracts will be studied for their anti-urolithiatic efficacy in future.

Lastly, it is to note that discoveries on use of herbs have been based on either available data base (with certain documentations, such as Ayurveda), or clinical experience (undocumented, and no database available, such as in folk medicine). The present study has been based on ethnic practice and but, it has an advantage that the plant extract has already been tested in hundreds and thousands of clinical cases spread over centuries. Thus, our experiment validates beneficial effects of *Cucumis* in urolithiasis. Therefore, the current study has got implication and in sync towards drug development of herbal origin for biomedical applications and it carries potential of being successful similar to previous reports with anti-malarial drugs from *Artemisia* sp. And antihepatitis drug from *Schisandra* sp [64, 65]. Those successes (with antimalarial and antihepatitic drugs) and current guidelines also emphasize that each of the final compounds needs to undergo rigorous experiments for evaluation of efficacy and toxicity [66] (Pan et al., 2013). So far we have completed the studies on effect of *Cucumis* extract on renal system biology and acute toxicity (OECD-425 guidelines, data on safety study not shown). We are yet to take up studies on pharmacokinetics of active principle needed for identifying candidate compound (s). Even without these data on pharmacokinetics, since the use of *Cucumis* extract has been proven useful over centuries, our study adds an alternative to the existing options available for treating clinical cases of urolithiasis.

5. Conclusion

Taken together, it is evident that *Cucumis* fruit extract has got beneficial effects in urolithiasis and this herbal extract exerts its anti-urolithiatic as well as reno-protective effects at multiple targets involving Glyco-oxalate pathways, oxidant/antioxidant balance, metabolic pathway, mineral interaction and regulation of extracellular matrix protein. In short, *Cucumis* fruit extract corrected hyperoxaluria, inhibited crystal formation, reduced renal crystal deposition, decreased lipid peroxidation (LPO), increased antioxidant enzymes (GSH, CAT) and downregulated OPN expression.

Author contribution statement

Shyam Sundar Choudhary: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Padma Nibash Panigrahi: Performed the experiments; Analyzed and interpreted the data.
Sujoy K. Dhara: Analyzed and interpreted the data; Wrote the paper.
Monalisa Sahoo: Performed the experiments; Contributed reagents, materials, analysis tools or data.
Ananya Dan, Neeraj Thakur, Aron Jacob: Performed the experiments.
Sahadeb Dey: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

None.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e14043.

Abbreviations

CCHEE	*Cucumis callosus* hydro-ethanolic extract
EG	Ethylene glycol
AC	Ammonium chloride
OPN	Osteopontin
LPO	Lipid peroxidation
CAT	catalase
GSH	Glutathione
CaOx	Calcium oxalate

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