Urolithiasis is a very painful disease that has afflicted a wide sector of human population since ancient times (1). Calcium-containing stones are the most common comprising about 75% of all urinary calculi, which may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%). Calcium oxalate stones are found in two different varieties, calcium oxalate monohydrate (COM) or Whewellite, and calcium oxalate dihydrate (COD) or Weddellite. COM, the thermodynamically most stable form, is observed more frequently in clinical stones than COD and has a greater affinity for renal tubular cells, thus responsible for the formation of stones in the kidney (2). Crystal growth and agglomeration may be due to supersaturation with respect to stone forming constituents or the
presence of various inhibitory or stimulatory bio-
 molecules or even pH (3).
Renal tubular fluid is supersaturated with
calcium and oxalate ions, which can nucleate to
form crystals of calcium oxalate monohydrate.
Microcrystals of COM, the most common crystal
in renal stones, irreversibly bind to cell surface
microvilli, are subsequently internalized and can
stimulate proliferation (4). Therefore, this indi-
cates that renal epithelial cells can bind and in-
ternalize calcium oxalate crystals.
Extracorporeal shock wave lithotripsy
(ESWL) is currently the first line treatment for
upper urinary tract calculi. This treatment is not
without side effects and kidney damage dur-
ing ESWL is a clinically significant problem (5).
Therefore, it is worthwhile to look for an alter-
native cure and phytotherapy is proving to be a
promising alternative.
Since civilization, medicinal plants are
part of human society to combat diseases. Tra-
ditional knowledge of healers and vendor are in
great demand in the developed as well as devel-
oping countries for primary healthcare because
of their wide biological and medicinal activities,
higher safety margins and lesser cost (6).
Terminalia chebula locally named as
“harad” in India has been extensively used in
ayurveda. It is used to treat urolithiasis and is
actively used in various drug formulations of
kidney stone treatments like neeri (product infor-
mation by AIMIL PHARMACEUTICALS (INDIA)
LTD.). It is extensively explored for antimicrobial
(7), antioxidant (8), anticarcinogenic (9), hypo-
cholesterolemic (10) and diuretic (11,12) activities
by various research groups.
The present study is aimed to investigate
the efficacy of Terminalia chebula on nucle-
atation and growth of calcium oxalate in vitro
and further examining the potency of the same
on oxalate induced injury in MDCK and NRK
52E cells.
MATERIALS AND METHODS

Plant material
The fruits of Terminalia chebula used in
this study were collected from Shimla, India which

were identified and then authenticated by micro-
scopical and physiochemical data.

Preparation of plant extracts
The air-dried fine powdered plant fruits
were infused in distilled water until complete
exhaustion. The extract was then filtered using
Whatman No. 1 filter paper and the filtrate was
evaporated in vacuo and dried using a rotary
evaporator at 40° C. The final dried samples were
stored in labeled sterile bottles and kept at -20° C
and were referred to as aqueous extract (13). The
various concentrations of the plant extract tested
for their inhibitory potency were 25 μg/mL, 50
μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL and
1000 μg/mL, which were prepared at the time of
experiment.

For cell culture studies a stock solution
of the crude extract prepared as defined above
was dissolved in dimethyl sulfoxide (DMSO) (fi-
nal concentration of the DMSO in the highest
concentration of plant extract tested did not ex-
ceed 0.4% (v/v) and did not affect the cell pro-
liferation). Further dilutions of the stock were
done using serum free DMEM (Dulbecco’s Modi-
fied Eagle’s Media) and filtered by 0.3 mm sy-
ringe filter (14).

Nucleation Assay of calcium oxalate
The method used was similar to that de-
scribed by Hennequin et al. with some minor
modifications (15). Solutions of calcium chloride
and sodium oxalate were prepared at the final
concentration of 3mmol/L and 0.5mmol/L, respec-
tively, in a buffer containing Tris-HCl 0.05mol/L
and NaCl 0.15mol/L at pH 6.5.3 mL of calcium
chloride. The solution was mixed with test sample
at different concentrations of 25 μg/mL, 50 μg/
ml, 100 μg/ml, 200 μg/ml, 400 μg/ml and 1000
μg/ml. Crystallization was started by adding 1.5
mL of sodium oxalate solution in 1.5 mL calcium
chloride. The temperature was maintained at 37°
C. The absorbance of the solution was monitored
at 620nm every 1 min. The percentage inhibition
produced by the aqueous extract was calculated
as (1-(Tsi/Tsc)) X 100, where Tsc was the turbidity
slope of the control and Tsi the turbidity slope in
the presence of the inhibitor.
Growth Assay of calcium oxalate
Inhibitory activity against CaOx crystal growth was measured using the seeded solution-depletion assay, described previously by Nakagawa et al (16). Briefly, an aqueous solution of 10mM Tris-HCl containing 90mM NaCl was adjusted to pH 7.2. Stone slurry (1.5 mg/mL) was prepared in 50mM sodium acetate buffer (pH 5.7). CaOx monohydrate crystal seed was added to a solution containing 4mM calcium chloride (CaCl₂) and 4mM sodium oxalate (Na₂C₂O₄). The reaction of CaCl₂ and Na₂C₂O₄ with crystal seed led to deposition of CaOx on the crystal surfaces, thereby decreasing free oxalate that is detectable by spectrophotometry at λ214 nm. When various extracts at different concentrations of 25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL and 1000 μg/mL is added into this solution, depletion of free oxalate ions will decrease if the test sample inhibits CaOx crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after every 1 min. for 20 mins. incubation with or without test sample. The relative inhibitory activity was calculated as follows: % Relative inhibitory activity = ((C-S)/C) × 100, where C is the rate of reduction of free oxalate without any test sample and S is the rate of reduction of free oxalate with a test sample.

Cell Culture
MDCK and NRK 52E cells were obtained from National Centre of Cell Sciences (NCCS, Pune, India). The cells were maintained as monolayer in DMEM with 2.0 mM L-glutamine adjusted to contain 3.7 g/l sodium bicarbonate and 4.5 g/l glucose. Medium was supplemented with 1% penicillin (100 units/mL), streptomycin (10,000 μg/mL) and 10% fetal bovine serum. Cells were cultured in 25 cm² tissue-culture treated flasks at 37°C and 5% CO₂ in humidified chambers.

Oxalate-induced Cell Injury
MDCK and NRK 52E cells were incubated in DMEM containing 1mM sodium oxalate in the presence of different concentrations of the aqueous extract of the test sample (10, 25, 50 and 80 μg/mL) for 48 hours (14,17). Cell injury was assessed by measuring the cell viability through trypan blue and monitoring the lactate dehydrogenase (LDH) leakage into the medium.

Cytotoxicity Trypan blue assay
The cytotoxicity of the crude extract of T. chebula was assessed by determining the cell viability using trypan blue exclusion method. For the determination of cell viability, cells were plated at a density of 4 × 10³ cells/well and cultured for 48 hours. The medium was replaced with serum-free medium and the cells were treated with various concentrations of the plant extracts (10, 25, 50 and 80 μg/mL) for a further 48 hours. The percentage viability for the cells was calculated as (live cells/total cells)*100.

Lactate dehydrogenase leakage assay
LDH leakage assay was performed by the method of Wagner et al. (18). Briefly, 6.6mM NADH and 30mM sodium pyruvate were prepared in Tris (0.2 M, pH 7.3). The reaction was initiated with the addition of 50 μL of the test sample and the disappearance of NADH was monitored at 340 nm for 5 min. at an interval of 1 min. The percentage of LDH release was calculated by dividing the activity of LDH in the supernatant by the LDH activity measured after complete cell lysis achieved by sonication.

Statistical Analysis
Data were expressed as mean values of three independent experiments (each in triplicate) and analyzed by ANOVA (p < 0.05) to estimate the differences between values of extracts tested.

RESULTS
Inhibition of Nucleation of CaOx Crystals by Terminalia chebula Extract:
Figure-1 displays the effect of aqueous extract of Terminalia chebula on the nucleation of calcium oxalate crystals. With respect to the control (with no plant sample), the percentage inhibition shown by aqueous extract at 25 μg/mL was 95.8% with almost constant inhibition at 100 μg/mL, 200 μg/mL and 400 μg/mL in the range of 91-94%.
Inhibition of CaOx Crystal Growth by Terminalia chebula Extract:

Figure-2 displays the effect of Terminalia chebula on the growth of calcium oxalate crystals. The aqueous extract displayed concentration dependent percentage inhibition when compared to the control (with no plant extract). The percentage inhibition with 25 μg/mL was found to be 39.9%, which increased to 105.8% with 1000 μg/mL aqueous extract.

Diminution of Oxalate-Induced Renal Tubular Epithelial Cell Injury by Terminalia chebula Extract:

Figures-3 and 4 depicts the protective effect of the aqueous extract of Terminalia chebula towards the renal tubular epithelial cells, MDCK and NRK-52E respectively with respect to cell viability. The oxalate induced a significant injury to the cells which could be ascertained by a decrease in viability which was greater in MDCK as compared to NRK-52E. However, the injury due to oxalate was significantly reduced in those cells treated with the T. chebula extracts. As the concentration of the extract increased from 10 μg/mL to 80 μg/mL, the percentage viability improved showing that the plant has an inhibitory activity towards the oxalate which caused injury to the renal cells in a concentration dependent manner. The plant extract alone (80 μg/mL, containing 0.4% DMSO) had no effect on the cell injury in the absence of oxalate indicating that even at the highest concentration of DMSO used there was no cytotoxicity to the cells. The concentration dependent percentage viability was seen in both the cell lines. The viability increased from 41.3%, as in oxalate injured cells to 60.4% in the presence of 80 μg/mL plant extract when tested with NRK-52E. A similar pattern was observed with MDCK where the viability increased to 71.3% when treated with 80 μg/mL plant extract as compared to 52.9% viability in oxalate injured cells.
Figure 2 - Effect of crude extract of Terminalia chebula on growth of CaOx. Data are mean ± S.D. of three independent observations. * p < 0.05

Figure 3 – Effect of Terminalia chebula on the viability of MDCK. Data are mean ± S.D. of three independent observations. * p < 0.05 versus untreated control, ** p < 0.05 versus oxalate control.
Figure 4 – Effect of Terminalia chebula on the viability of NRK-52E. Data are mean ± S.D. of three independent observations. * p < 0.05 versus untreated control, ** p < 0.05 versus oxalate control.

Figure 5 – Effect of Terminalia chebula on the % LDH release of MDCK. Data are mean ± S.D. of three independent observations. * p < 0.05 versus untreated control, ** p < 0.05 versus oxalate control.
Lactate dehydrogenase is a stable cytosolic enzyme that is released when the cell is lysed or there is any injury on the cell membrane. A significant increase in LDH release was seen when both the cells were exposed to oxalate alone. When cells were treated with the plant extract at varying concentrations (10, 25, 50 and 80 μg/mL) along with oxalate (1mM) for 48h, a reduction in oxalate-induced cell injury was observed as assessed by a decreased LDH release (Figures 5 and 6). Again it was observed that the plant extract alone had no significant effect on the measures of cell injury in the absence of oxalate. The percentage LDH release for MDCK and NRK-52E is dependent upon concentration after treatment with oxalate and the plant extract with respect to control. The percentage LDH release for MDCK and NRK-52E is dependent upon concentration after treatment with oxalate and the plant extract with respect to control. The percentage LDH release for MDCK and NRK-52E is dependent upon concentration after treatment with oxalate and the plant extract with respect to control. The percentage LDH release for MDCK and NRK-52E is dependent upon concentration after treatment with oxalate and the plant extract with respect to control. The percentage LDH release for MDCK and NRK-52E is dependent upon concentration after treatment with oxalate and the plant extract with respect to control. The percentage LDH release for MDCK and NRK-52E is dependent upon concentration after treatment with oxalate and the plant extract with respect to control. The percentage LDH release for MDCK and NRK-52E is dependent upon concentration after treatment with oxalate and the plant extract with respect to control.

DISCUSSION

There has been a long standing quest for potent inhibitors of calcium oxalate growth as it is the most common urinary stone associated with renal injury. Recent evidence suggests that in many calcium oxalate stone formers the earliest changes may be calcium salt deposition in the medullary interstitium. In marked hyperoxaluric states, primary hyperoxaluria directs calcium oxalate crystal adhesion to renal epithelial cells (19).

Stones larger than 5mm fail to pass through and hence can be treated through ESWL but the chance for stone recurrence is still about 50%. In addition, ESWL might show some significant side effects such as renal damage, hypertension or renal impairment. Therefore, phytotherapeutic agents could be useful as either an alternative or
a complementary therapy in the management of urolithiasis with some possible mechanisms of action including an increased excretion of urinary citrate, decreased excretion of urinary calcium and oxalate or could be attributable to diuretic, antioxidant or antibacterial effects (20).

The present investigation deals with the effects of putative litholytic medicinal plant, Terminalia chebula on CaOx crystals. Fruits of T. chebula are a popular folk medicine and have been studied for its diuretic activity but the scientific basis of its activity was not yet established. In this study, the inhibitory potency of the plant was tested on nucleation and growth of calcium oxalate crystallization in vitro. The aqueous extract inhibited the CaOx growth to the tune of 105.8%. Further, a protective effect on renal epithelial cells was shown by the aqueous plant extract in a concentration-dependent manner. When MDCK and NRK-52E cells were injured by exposure to 1mM oxalate for 48h, the plant extract prevented the injury in a dose-dependent manner. The oxalate injury to MDCK cells were more intense as compared to that of NRK-52E. The percentage LDH release in NRK-52E was less at 80 μg/mL than MDCK. Therefore, T. chebula seems to be more responsive towards NRK-52E than MDCK.

Several traditional Chinese medicines/plants that are used in Kampou medicine also have demonstrated their abilities to inhibit calcium oxalate crystallization. Dietary factors appear to affect the ability of urine to inhibit COM crystallization. In this regard, lemon juice has been found to inhibit the rate of crystal nucleation and aggregation (20).

Various plants are being evaluated for their antiurolithiatic potency through their activity on renal epithelial cell lines which are accepted as a powerful tool to establish the mechanism of nephrolithiasis. Atmani et al. used MDCK cell lines as a model to study the adhesion of radioactive COM crystals in the presence and absence of aqueous extract of Herniaria hirsuta and found that the crystal attachment was inhibited in a concentration dependent manner (19). In vitro effect of an aqueous extract of Phyllanthus niruri L., a plant used in Brazilian folk medicine for the treatment of urolithiasis, on a model of COM crystal endocytosis by MDCK cells, was investigated by Campos and Schor. The extract exhibited a potent and effective non-concentration-dependent inhibitory effect on the COM crystal internalization. This response was present even at very high (pathologic) COM concentrations and no Phyllanthus niruri L. induced toxic effect could be detected (21). The fruits of Ammi visnaga L. commonly called as Khella have traditionally been used in Egypt to relieve pain of kidney stone passage by drinking a tea prepared from the crushed or powdered fruits of khella. A study was undertaken to evaluate its effect on renal epithelial injury using LLC-PK1 and Madin-Darby-canine kidney (MDCK) cells. Over the past few decades two continuous renal epithelial cell lines have been most used or studying nephrolithiasis, the Madin-Darby canine kidney collecting duct tubular epithelial cells (MDCK) and porcine kidney proximal tubular epithelial cells (LLC-PK1). MDCK cells have been widely used as a model system for the distal/collecting duct and LLC-PK1 cells have retained many characteristics of the proximal tubule. Vanachayangkul et al. evaluated the effect of aqueous khella extract on oxalate induced renal injury and found that the cell injury (LDH release) was significantly reduced in cells treated with the extract (22).

The antilithiasic potency of various plants like Dolichos biflorus (23), Trachyspermum ammi (24), Tribulus terrestris (2) and Achyranthes aspera (25) and the inhibitory effect of various biomolecules on renal stones (26) have been evaluated in our lab.

The inhibitory role of various plant species from west and south of Algeria in calcium oxalate growth was investigated in vitro by Beghelia et al. (27). They further postulated that the plant extracts may contain substances that inhibit COM crystal aggregation and also the binding of the crystals to the renal epithelial surface. This could explain a decrease in LDH release observed in the cells treated with the plant extract compared to those treated with oxalate alone. The cDNA microarray was used to evaluate gene expression in urolithiasis by exposing the COM crystals to NRK-52E cells (28).
CONCLUSIONS

This study demonstrated that Terminalia chebula extract showed cytoprotective properties towards the MDCK and NRK-52E cells by reducing the LDH leakage and increasing the cell viability. At the same time, it also has an ability to inhibit the calcium oxalate crystals in vitro. In the light of these studies, it is a valuable candidate for further pharmacological analysis.

ABBREVIATIONS

COM: calcium oxalate monohydrate
COD: calcium oxalate dihydrate
CaOx: calcium oxalate
ESWL: extra corporeal shock wave lithotripsy
MDCK: madin darby canine kidney
NRK-52E: normal rat kidney epithelial
dMso: dimethyl sulfoxide
DMEM: dulbecco’s modified eagle’s media
LDH: lactate dehydrogenase

CONFLICT OF INTEREST

None declared.

REFERENCES

The urolithiasis clinical treatment has been extensively studied with different herbs, otherwise, others studies demonstrated a potent inhibitory effect on CaOx crystal adhesion and endocytosis of folk plants, as Phyllanthus niruri, from South America, Africa in vitro and vivo models (1,2).

This well done paper demonstrated a nice result, but it needs to be done in animal models, human cells to provide a future clinical study to be a consistent and reproducible data.

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EDITORIAL COMMENT

This is a very interesting paper which addresses a subject that is not commonly seen in the urologic literature. It has been written in a satisfactory and scientific way since the type of therapy described is more commonly found in lay press and as such does not contain strong scientific subtract.

I am sure that this study brings scientific information that could lead to further studies in this area similarly as some substances used as cancer latest generation chemotherapeutic agents which were originally found in plant species. The Brazilian species of Phyllanthus niruri L. Popularly known as “chá de quebra pedra” (Ref. 21 in the article) is an example of many plants of our flora that could have been submitted to randomized studies to try to bring answers to the prevention and eventually treatment of stone disease. This management represents a major source of loosing work force and complications for patients without mentioning the great money expenditure with sophisticated equipments like ESWL that time has proven to be not as effective as initially proposed.

The authors must be congratulated for the style and content of this manuscript.

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