

Antiuro lithiatic Activity of *Daucus carota*: An *in vitro* Study

Sweta Bawari, Archana Negi Sah*, Devesh Tewari

ABSTRACT

Context: Urolithiasis is a polygenic disorder with complex etiology and even complicated treatment outcomes. *Daucus carota* is a widely cultivated crop with traditional claims for its antiuro lithiatic potential. **Aim:** Present study was an attempt to investigate the antilithic potential of *D. carota* root extract (DCRE) against calcium oxalate (CaOx) urolithiasis by employing *in vitro* methods. **Methods:** Nucleation, growth and aggregation assay of CaOx crystallization were used. FT-IR analysis was used for characterizing CaOx crystals. **Results:** DCRE exhibited significant inhibition of nucleation, growth and aggregation of CaOx crystals. It produced a favorable morphological transformation of CaOx crystals from calcium oxalate monohydrate to calcium oxalate dihydrate. FT-IR analysis confirmed formation of calcium oxalate monohydrate crystals to be utilized for growth and aggregation assays. **Conclusion:** DCRE possesses significant antiuro lithiatic activity against CaOx urolithiasis *in vitro* which could be attributed to its saponins, tannins, flavonoids and polyphenolic content.

Key words: Aggregation, Flavonoids, FT-IR, Nucleation, Urolithiasis.

INTRODUCTION

Urolithiasis is a multifaceted urologic condition that requires frequent emergency department visits and immediate urological intervention.¹ Technological advancement witnessed for the removal of stones has been outstanding, but the associated complexities like, their tendency to enhance stone recurrence is a major limiting factor.²

Development of plant-based medicine as alternative or complementary to the conventional system of medicine have drawn tremendous attention and serves as an immense source of new drug entities.³ Based on these grounds, carrots (*Daucus carota* L., family Apiaceae) were selected for the present study. Carrots are considered functional food and are widely cultivated all around the globe as well as are extensively exploited commercially. Carrots are of high nutritional value and are a rich source of phenolics, polyacetylenes and carotenoids.⁴ Despite their established nephroprotective role⁵ as well as traditional claims for their antilithic potential,⁶ no screening has been carried out so far to establish the antiuro lithiatic efficacy of *D. carota*. Therefore, current investigation was carried out for the screening of the antilithic potential of *D. carota* against calcium oxalate crystallization in an *in vitro* setting.

MATERIALS AND METHODS

Plant collection

Daucus carota L. roots (carrots) were procured from a grocery store of Bhimtal, Uttarakhand and were identified and authenticated from Botanical Survey of India (BSI), Dehradun. A voucher specimen of *D.*

carota (accession number 116593) was also deposited in the herbarium of BSI.

Processing and extraction

D. carota roots were cut into thin slices, shade dried, and finely powdered. Cold maceration method was employed for the extraction of powdered carrot roots with 70% v/v ethanol. *D. carota* root extract (DCRE) thus obtained was dried in a rotary evaporator under reduced temperature and pressure.⁷

Preliminary phytochemical screening

Preliminary phytochemical evaluation of DCRE was carried out for qualitative estimation of phytoconstituents.⁸

Nucleation assay

Effect of DCRE on calcium oxalate (CaOx) crystal formation was determined by means of nucleation assay. Calcium chloride (CaCl₂) (5 mmol/l) and sodium oxalate (Na₂C₂O₄) solution (7.5 mmol/l) were prepared in Tris-HCl (0.05 mol/l) and NaCl (0.15 mol/l) buffer (pH 6.5). Dilutions of DCRE ranging from 100-1000 µg/ml were prepared in distilled water. One milliliter of each DCRE concentration was mixed with 3 ml CaCl₂ solution followed by the addition of 3 ml Na₂C₂O₄ solution. Final mixtures were incubated for 30 min at 37°C. The optical density (OD) of the mixtures was then measured at 620 nm wavelength. Percent inhibition of nucleation by DCRE was calculated using the under mentioned formula and compared to that calculated for the standard polyherbal drug, Cystone.⁹

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$$\% \text{ Inhibition} = \left(1 - \frac{\text{OD}_{\text{Test}}}{\text{OD}_{\text{Control}}} \right) \times 100$$

Microscopic evaluation

Number, size and morphology of CaOx crystals formed in absence or presence of DCRE were determined using a Leica DM 2500 LED microscope at 1000× magnification.

Aggregation assay

Effect of DCRE on CaOx crystal aggregation was determined by means of aggregation assay. CaCl₂ and Na₂C₂O₄ solutions (50 mmol/l each) were mixed together, heated to 60 °C in a water bath for 1 h and then incubated overnight at 37°C to prepare seed CaOx crystals. After drying, CaOx crystal solution (0.8 mg/ml) was prepared in a 0.05 mol/l Tris-HCl and 0.15 mol/l NaCl buffer (pH 6.5). One milliliter of aliquots (100-1000 µg/ml) of DCRE were added to 3 ml CaOx solution, vortexed and then incubated at 37°C for 30 min. OD of the final mixtures was then read at 620 nm wavelength and percent inhibition of aggregation was then calculated as described for nucleation assay.⁹

Oxalate depletion assay

Effect of DCRE on the growth of CaOx crystals was determined by oxalate depletion assay. Varying concentrations of DCRE (100 µg/ml, 500 µg/ml and 1000 µg/ml) were prepared in distilled water. CaOx crystal slurry at a concentration of 1.5 mg/ml was prepared in a 50 mM sodium acetate buffer (pH 5.7). 4 mM CaCl₂ solution and 4 mM Na₂C₂O₄ solution (1 ml each) were added to 1.5 ml Tris-HCl (10 mM) and NaCl (90 mM) buffer (pH 7.4). To this was added 30 µl of CaOx crystal slurry. The growth of CaOx crystals was then determined by measuring the rate of oxalate depletion from the solution at 214 nm wavelength for 600 s. Effect of each concentration of DCRE on crystal growth was then determined by addition of 1 ml of DCRE (100 µg/ml, 500 µg/ml and 1000 µg/ml) to the reaction mixture and change in OD was again recorded. Percent inhibition of crystal growth was then calculated as described for nucleation assay.¹⁰

CaOx crystal characterization by FT-IR

FT-IR analysis was used to confirm the CaOx crystals prepared *in vitro* using attenuated total reflectance (ATR) technique.

Statistical analysis

Quantitative results of all the experiments performed in triplicates were expressed as mean ± S.E.M. (Standard Error of Mean). Statistical computations were performed on GraphPad Prism 6 software using one-way analysis of variance (ANOVA) followed by Tukey Kramer's multiple comparison test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Preliminary phytochemical screening

Percent yield of DCRE was 23.73%. Qualitative phytoconstituent determination in DCRE showed the presence of carbohydrates, saponins, flavonoids, tannins and phenols.

Nucleation Assay

Addition of Na₂C₂O₄ solution to the reaction mixture consisting of CaCl₂ resulted in the formation of numerous CaOx crystals. Presence of DCRE (1000 µg/ml) in the reaction mixture produced a percent reduction in nucleation of 56.1±1.55% which was significantly higher (*P*<0.01) than that produced by Cystone (41.67±1.03%) (Figure 1).

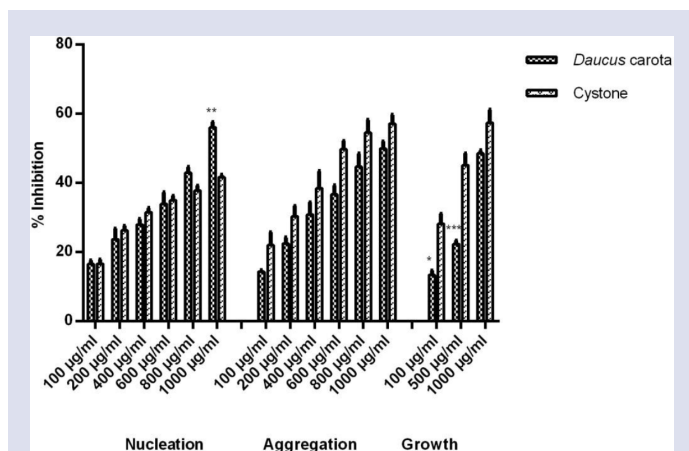


Figure 1: Effect of DCRE and Cystone on nucleation, aggregation and growth of CaOx crystals.

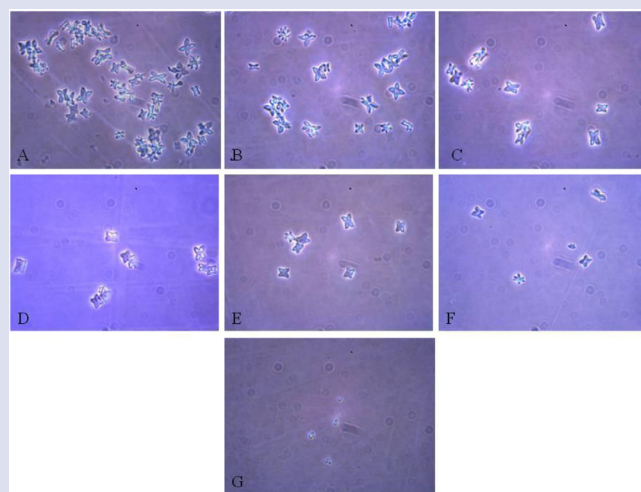


Figure 2: Representative photographs of CaOx crystals as observed under light microscope (x1000) in the absence A, and in the presence of DCRE 100 µg/ml B, 200 µg/ml C, 400 µg/ml D, 600 µg/ml E, 800 µg/ml F, 1000 µg/ml G.

Microscopy of CaOx crystals

Numerous large CaOx monohydrate (COM) crystals of either rectangular habit or dendrites with sharp edges were predominant in the control group. DCRE at higher concentrations (Figure 2) and Cystone from lower concentrations itself (Figure 3) favored the formation of tetrahedral shaped calcium oxalate dihydrate (COD) crystals with smoother morphology. DCRE and Cystone also reduced the size and number of CaOx crystals. Percent reduction in size of CaOx crystals produced by DCRE (62.34%) was comparable to that produced by Cystone (70.10%) (Figure 4). Number of CaOx crystals was reduced to a far greater extent by DCRE (84.71%) than Cystone (58.51%) (Figure 4).

Aggregation Assay

DCRE produced a significant reduction (*P*<0.0001) in aggregation of preformed CaOx crystals. Percent reduction in aggregation produced by DCRE was found to be 49.94±2.07% comparable to that of Cystone (57.15±2.53%) at 1000 µg/ml concentration (Figure 1).

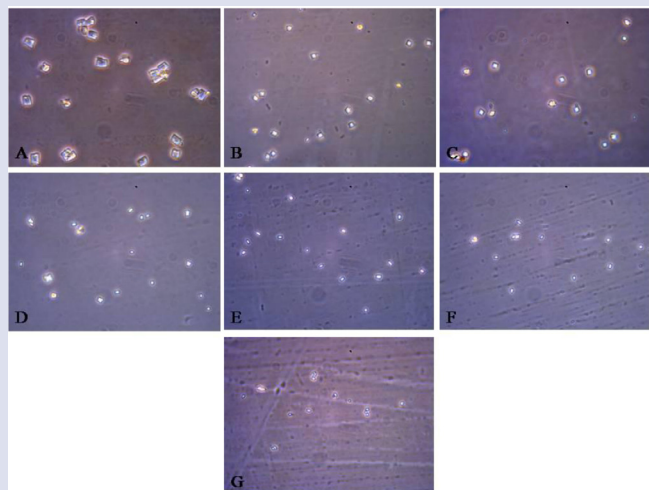


Figure 3: Representative photographs of CaOx crystals as observed under light microscope (x1000) in the absence A, and in the presence of Cystone 100 µg/ml B, 200 µg/ml C, 400 µg/ml D, 600 µg/ml E, 800 µg/ml F, 1000 µg/ml G.

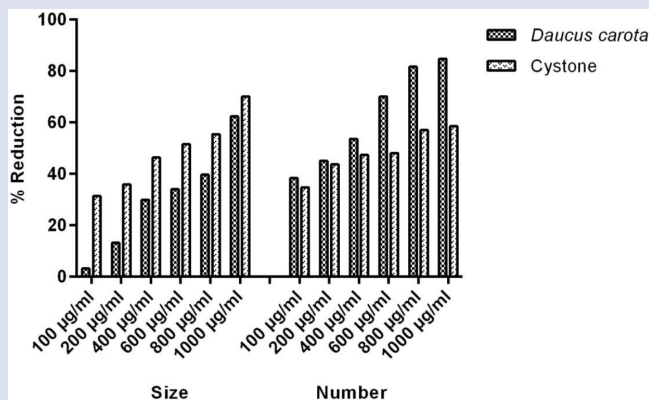


Figure 4: Effect of DCRE and Cystone on size and number of CaOx crystals.

Oxalate depletion Assay

Percent reduction in growth in the presence of DCRE was found to be $48.58 \pm 0.88\%$ while, $57.44 \pm 3.7\%$ with Cystone at 1000 µg/ml. CaOx crystal growth inhibitory effect of DCRE was significantly lower than Cystone ($P < 0.001$) at lower concentrations but was comparable to Cystone at highest concentration (Figure 1).

FT-IR characterization of CaOx crystal

FT-IR analysis of CaOx crystals produced seven bands (Figure 5). Spectrum showed a broad O—H stretching band at 3330.42 cm^{-1} ; a strong band at 1614.47 cm^{-1} representing C=O; another band with high absorbance at 1316.74 cm^{-1} representing C—O stretch; C—H bending at 778.65 cm^{-1} ; out-of-plane O—H bending at 651.68 cm^{-1} ; and O=C=O in-plane bending at 511.79 cm^{-1} as has also been reported in previous studies.¹¹⁻¹²

DISCUSSION

CaOx urolithiasis is the most prevalent type of all urinary stone diseases. Key events involved in its pathological biomineralization include crystal

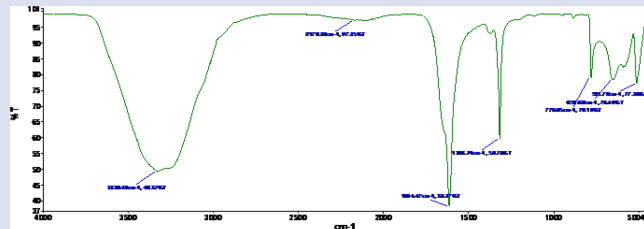


Figure 5: FT-IR spectra of seed CaOx crystals.

nucleation, growth and aggregation.¹³ Present study was designed to address these key events involved in CaOx stone formation as a means to investigate the efficacy of *D. carota* roots as an antiurolithiatic.

Nucleation is a prerequisite in the pathogenesis of CaOx urolithiasis. Nucleation basically marks a thermodynamically driven event of phase change wherein dissolved substances in a supersaturated solution spontaneously crystallize.¹³⁻¹⁴ Similar phase change and formation of CaOx crystals was witnessed while carrying out nucleation assay. Significant inhibition in the nucleation of CaOx crystals was observed in the presence of DCRE which was even better than in the presence of Cystone. This suggests the anticrystallization activity of DCRE against CaOx crystallization. One possible mechanism of anticrystallization activity of DCRE could be its ability to complex with free calcium and oxalate ions, thus preventing the formation of CaOx complexes, as has also been suggested for *Sorghassum wightii*.¹⁵

CaOx polymorphism is a common phenomenon and of utmost significance in urolithiasis. COM and COD crystals are commonly found in CaOx uroliths.¹⁶ Of the two polymorphs, COM is thermodynamically more stable with more aggregatory and adhesive tendency. Hence, COM tends to form large crystal aggregates and adheres strongly to renal epithelial tissue, injuring the same. Therefore, of the two polymorphs, COM significantly promotes crystal retention and eventual stone formation.¹⁷ Therefore, a transformation from COM to COD is advocated as a crucial step in inhibition of calculi formation.¹⁶ In present study, DCRE also promoted transformation of pointy edged dendritic COM crystals to smoother edged COD crystals of extremely reduced size and number. Reduction in size of CaOx crystals is critical as smaller crystals tend to spontaneously pass out in urine.¹⁸

Growth of CaOx crystals marks the event of deposition of crystal forming ions present in the supersaturated solution on preformed CaOx crystal lattice.^{13,19} This event of growth of CaOx crystals was also tracked in the present study. DCRE exhibited growth inhibitory activity as was also confirmed from the crystals of reduced size produced in the presence of DCRE.

Aggregation of crystals marks the process wherein numerous crystals in the solution come together and adhere forming large crystal agglomerates. Aggregation is a key determinant of crystal retention as large crystal agglomerates are the ones that produce renal tubular obstruction thereby promoting stone formation.¹³ DCRE showed significant inhibitory effect on CaOx crystal aggregation.

Qualitative phytochemical estimation of DCRE revealed the presence of flavonoids, phenolic compounds, saponins and tannins. These phytoconstituents are of utmost significance for inhibiting urinary stone formation. Saponins possess antilithic properties⁹ and are known to disintegrate mucoproteins that are crucial components of stone matrix.²⁰ Tannins and polyphenols inhibit CaOx crystal formation as well as dissolve the preformed CaOx crystals by aiding calcium complexation.²¹

Flavonoids also possess CaOx crystal dissolution potency. Phenolics and flavonoids exhibit antioxidant activity.²⁰ Therefore, the anti-crystallization, anti-aggregatory and crystal growth defying activity of DCRE would have been an outcome of these phytoconstituents present in DCRE.

As COM crystals are the most predominant form of all the polymorphs of CaOx found in kidney stones, formation of COM crystals was a mandatory requirement to test the efficacy of DCRE against COM crystallization. In order to confirm the formation of COM crystals to be utilized for aggregation and growth assays, CaOx crystal characterization was carried out by FT-IR spectrophotometry. Formation of COM crystals was confirmed from the sharp peak at 778.65 cm⁻¹ and a comparatively broader peak at 651.68 cm⁻¹ which are characteristic of COM crystals. Whereas, a sharp peak at about 910 cm⁻¹ is known to be specific to COD crystals.²² In addition, peaks at 3330.42, 1614.47, 778.65 and 511.79 cm⁻¹ that were prominent in the FT-IR spectrum of CaOx crystals closely correlate to the typical COM identification bands.²³

It is true that *in vitro* data cannot be simply extrapolated to infer results of more complex *in vivo* systems, but *in vitro* studies give an insight into the activity related efficacy of tested compounds or extracts. Present study also demonstrated prominent inhibitory activity of DCRE against CaOx crystallization.

CONCLUSION

Findings of the present study clearly demonstrate antiurolithiatic potential of DCRE against CaOx urolithiasis *in vitro*. DCRE showed prominent inhibition of all the phases of CaOx stone formation viz. nucleation, growth and aggregation, and favored the formation of more amenable COD crystals. Although further *in vivo* and clinical explorations are required to confirm the efficacy of *D. carota* as an antiurolithiatic, still, considering the vast consumption and availability of *D. carota* all around the globe together with its antilithic potential, *D. carota* could serve as an easily accessible and beneficial alternative or adjunctive treatment for CaOx urolithiasis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

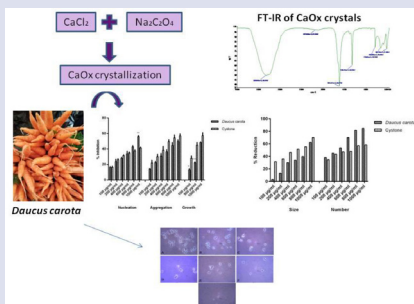
ATR: Attenuated total reflectance; **CaOx:** Calcium oxalate; **COD:** Calcium oxalate dihydrate; **COM:** Calcium oxalate monohydrate; **DCRE:** *Daucus carota* root extract; **FT-IR:** Fourier transform infrared spectroscopy; **OD:** Optical density.

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GRAPHICAL ABSTRACT



SUMMARY

- Hydroethanolic extract of *Daucus carota* roots produced significant inhibition of nucleation, growth and aggregation of calcium oxalate crystals.
- Possible mechanism involved could be complexation of calcium and oxalate ions by *D. carota* extract and anticrystallization and antioxidant actions of the phytoconstituents of *D. carota*.

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