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Research Article

INHIBITION OF CALCIUM OXALATE (CAOX) CRYSTALLIZATION *IN VITRO* BY THE EXTRACT OF BEET ROOT (*BETA VULGAIS L.*)

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ABSTRACT

Objective: To study the inhibitory potential of *Beta vulgaris L.* leaf and root aqueous extracts against calcium oxalate crystallization under *in vitro* condition.

Methods: Under *in vitro* condition, kidney stone formation was studied using three assays such as nucleation, aggregation and growth. Nucleation was studied by adding calcium chloride and sodium oxalate solution in the presence (0.1 to 9 mg/ml) and absence of aqueous extracts at 37˚C. For aggregation and growth calcium oxalate monohydrate crystals were prepared and studied. The effect of extracts on the formation and inhibition of stone forming stages were observed spectrophotometrically and analyzed through phase contrast microscope at 40X magnification. The obtained results are presented in this paper.

Results: The result obtained showed that aqueous extracts of the leaves and root of *Beta vulgaris L.* have higher capacity to inhibit the crystal nucleation, aggregation and growth. When compared with leaf aqueous extract, root aqueous extract of beet root showed better inhibitory activity. Extracts inhibited the crystallization in solution; less and smaller particles were observed in the presence of extracts. These results were further confirmed with the aggregation and growth assay, the extracts prevented the aggregation and growth of formed CaOx particles and it kept the crystals as dispersed.

Conclusion: From this *in vitro* study we can conclude that beet root extract has excellent therapeutic potential as diuretic and promoting the inhibition of the formation of CaOx crystals. Also the phytocompounds present in this plant may be responsible for the anticrystallization activity. To find out the exact bioactive compounds against anticrystalization activity, further elaborate experimentations are needed.

Keywords: CaOx, kidney stone, Urolithiasis, Nucleation, Aggregation, Growth and *Beta vulgaris L.*

INTRODUCTION

Urolithiasis or kidney stone formation is a painful disease affecting human beings for several centuries, occurring in about 1-5% of the world population with recurrence rate of 50-80% [1]. Kidney stones are hard, solid particles, originated and located anywhere in the urinary system [2]. The stone formation occurs due to several factors like excess amount of stone forming constituents, imbalance between promoters (e.g., sodium, urates, etc.) and inhibitors (e.g., citrate, glycosaminoglycans, etc.) [3]. When the stone forming constituents are excess in urine it becomes supersaturated, leads to the precipitation and finally it grow as stones.

Calcium containing stones (either with oxalate or phosphate) [4] are the commonly occurring ones to an extent of 75-90% followed by magnesium ammonium phosphate (10-15%), uric acid (3-10%) and cystine (0.5-1%) [5] Calcium oxalate stones are found in two different varieties namely calcium oxalate monohydrate (Whewellite) and calcium oxalate dihydrate (Weddellite). It has been suggested that calcium oxalate monohydrate has a stronger affinity for cell membranes than calcium oxalate dihydrate and thus Calcium oxalate monohydrate crystals may constitute the higher potential risk for stone formation [6]. The biochemical processes involved in calcium oxalate stone formation are supersaturation, nucleation, aggregation, crystal growth, crystal retention, formation of stone nidus and finally development of stone [7]. Supersaturation of the urine with calcium oxalate crystals creates a suitable environment for crystallization [8]. The stone formation begins from the formation of nuclei from super saturated urine. The process of crystals in solution attracted each other to form larger particles called as aggregation. Newly aggregated crystals may combine to form a small, hard mass called as stones and the stage is referred to as subsequent growth of crystals [9]. Association of grown crystals with the epithelial cells lining in the renal tubules are called as crystal retention. The retained crystals are combined and form a nidus which finally develops as stones [10, 11].

Supersaturation of urine depends on urinary pH, ionic strength, solute concentration and complex ions. The management of kidney stone event mainly involves techniques like extracorporeal shock wave lithotripsy (ESWL) and percutaneous nephrolithotomy (PCNL) and other surgical procedures. The draw back in these procedures include renal injury, decreased renal function and increased incidence in stone recurrence along with the possibility of infection [12]. Therefore it is useful to look for alternate therapy, plant based therapy are reported to be effective at different stages of stone pathophysiology. Several phytochemicals like flavonoids, triterpenes, saponins and tannins are responsible for antiurolithiatic effect. A saponin rich fraction of *Herniaria hirsute* has been found to be a great inhibitor of calcium stone formation. Butanol fraction containing high amount of saponins was able to inhibit initial mineral phase formation of calcium phosphate and growth of calcium oxalate monohydrate crystals along with the crude aqueous extract of *Terminalia arjuna* [13-18]. Patel *et al* [19] reported that *Solanum xanthocarpum* fruits saponin rich fraction has antiurolithiatic activity. In the present investigation the plant *Beta vulgaris L.* (Beet root - Amaranthacea family) has been taken to evaluate the inhibitory potential of calcium oxalate crystallization under *in vitro* conditions. This plant has been documented recently for its therapeutic effects in controlling kidney stone disease [20, 21]. Mroczek *et al* [22] reported that the roots of *Beta vulgaris L.* (three cultivars) contain 11 types of saponins. Till date there is no scientific validation for the use of beet root against urolithiasis. Therefore, present study intends to establish the scientific rationality of the antiurolithiatic activity of beet root leaf and root aqueous extracts using *in vitro* calcium oxalate crystallization assays.

MATERIALS AND METHODS

Collection and Preparation of aqueous extracts

Leaves and roots of *Beta vulgaris L.* was collected from local cultivation areas of kodaikanal. One gram of each sample was ground thoroughly with 10ml of distilled water. Then the extract

was centrifuged, the supernatant was transferred into a fresh sterile tube and stored at 4˚C until use.

Nucleation assay

The stone formation starts from the nuclei, which means the process of new crystal formation. The percentage inhibition of nucleation of calcium oxalate crystals by the leaf and root aqueous extracts of *Beta vulgaris L.* was evaluated by the modified method of Atmani and Khan (2000) [23]. 3 mMol/L and 0.5 mMol/L of calcium chloride and sodium oxalate solutions were prepared in a buffer containing NaCl 0.15 mMol/L and Tris 0.5 mMol/L at pH 6.5. 100 µl of the leaf and root extract at different concentrations (0.5, 1.0, 2.0, 3.0, 6.0and 9.0 mg/ml) were mixed with 950 µl of calcium chloride solution. Crystallization was initiated by the addition of 950 µl of sodium oxalate solution. 100 µl of distilled water without extract served as control.

The tubes were maintained at 37˚C. After incubation the optical density of the solutions were monitored at 620 nm. The rate of nucleation was estimated by comparing the induction time for calcium oxalate crystal formation in the presence and absence of the extracts. Percentage inhibition of nucleation = [(C-S)/C] x 100, Where, C is the turbidity without plant extract. S is the turbidity with plant extract.

Aggregation assay

The aggregation inhibition in presence and absence of extracts were determined by the method of Hess *et* al [24]. 50 mMol/L of calcium chloride and sodium oxalate solution was mixed and equilibrated at 60˚C in a water bath for 1 hour and then cooled to 37˚C overnight. The harvested crystals were used for aggregation studies. Calcium oxalate monohydrate crystals were used at a final concentration of 0.8 mg/ml, buffered with Tris 0.5 mMol/L and NaCl 0.15 mMol/L at pH 6.5. At 37˚C in the absence and presence of the leaf and root aqueous extracts the experiments were conducted. The percentage aggregation inhibition rate (Ir) was calculated by comparing the turbidity in the presence of the aqueous extracts with that obtained in the control using the following formula : Ir = (1-turbidity sample/turbidity control) x 100.

Growth assay

The percentage inhibition of calcium oxalate monohydrate crystal growth was evaluated in the presence and absence of leaf and root aqueous extract of beet root by the procedure described by the method of Aggarwal *et al* (2010) [25]. In 50 mMol/L sodium acetate buffer (pH 5.7) 1.5mg/ml of stone slurry was prepared. 1 mMol/L calcium chloride and 1 mMol/L sodium oxalate solution were prepared in a buffer containing 10 mMol/L Tris-HCl and 90 mMol/L NaCl (pH 7.2). In 1 ml of calcium oxalate monohydrate crystals, 1ml of calcium chloride and sodium oxalate solutions were added. The reaction of calcium chloride and sodium oxalate with calcium oxalate monohydrate crystals led to the deposition of calcium oxalate on the seed crystal surfaces, thereby decreasing free oxalate that was detectable in spectrophotometry at 214 nm. During the addition of leaf and root aqueous extracts, if the test samples inhibit calcium oxalate crystal growth, depletion of free oxalate ions will decrease. The percentage inhibitory activity was calculated as follows: Percentage inhibitory activity = [(C-S)/C] x 100, where, C is the rate reduction of free oxalate without plant extract. S is the rate reduction of free oxalate with plant extract.

RESULTS

Fig. 1 explains the percentage inhibition of the leaf and root aqueous extracts of beet root against the CaOx crystal nucleation. Maximum inhibition percentage was observed at 3 mg/ml concentration. Root aqueous extract of beet root showed better inhibitory activity (75%) when compared with leaf aqueous extract (63%) of beet root. When used lower (0.5 mg/ml) and higher concentrations (6.0 mg/ml and 9.0 mg/ml) of leaf and root aqueous extracts, the dissolutions of the CaOx crystals was found to be low.

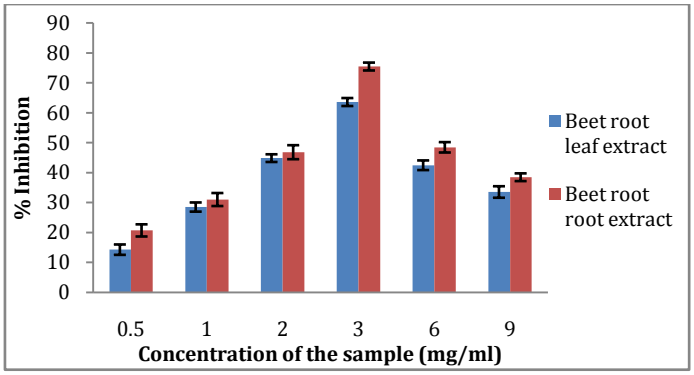
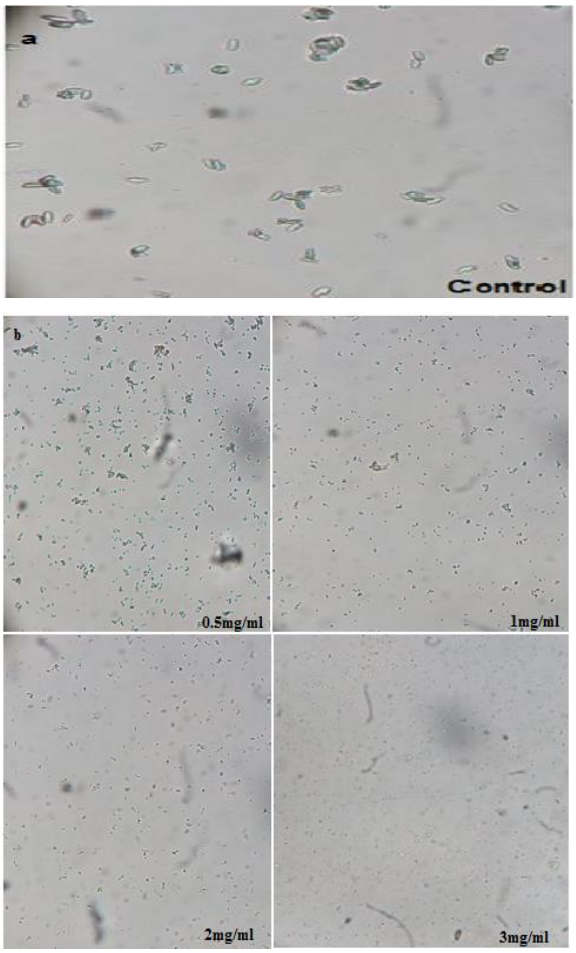


Fig. 1: Effect of beet root leaf and root aqueous extracts on CaOx nucleation

Microscopic examination (fig. 2) reveals the inhibition of nucleation induced by the leaf and root aqueous extract of beet root. Usually calcium oxalate monohydrate crystals are hexagonal in shape; calcium oxalate dihydrate crystals are octahedral in shape. Microscopic result indicates that the crystals present in the control had the hexagonal shape. When the beet root leaves and root extracts of varying concentrations (0.5 to 9 mg/ml) were added, the calcium oxalate monohydrate crystals lost their crystalline nature and it was converted to octahedral shape, as indicated by the dispersed, lesser, very smaller octahedral shape calcium oxalate dihydrate particles compared with control in 40X magnification.

a. Control, b. Microscopic view of leaf aqueous extract on CaOx nucleation, c. Microscopic view of root aqueous extract on CaOx nucleation



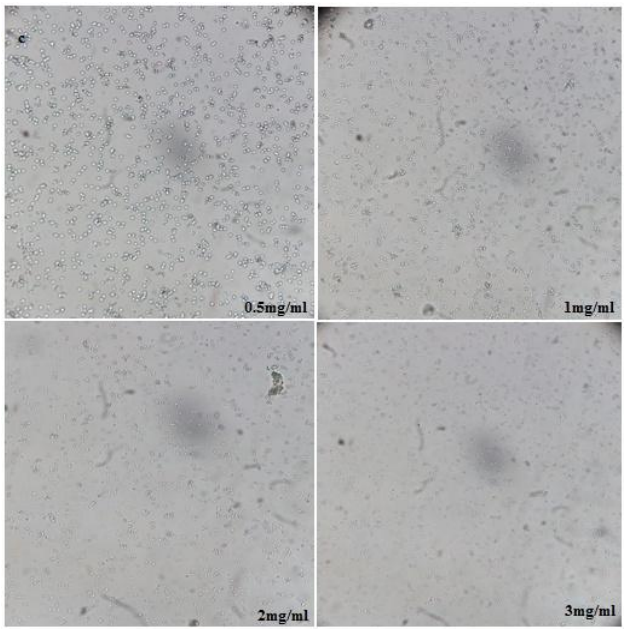


Fig.2: Microscopic evaluation of beet root leaf and root aqueous extracts on CaOx nucleation

The next step in calculus formation is aggregation that constitutes the most effective mechanism to increase the size of particle and structure of urinary stones [26]. Fig. 3 indicates the percentage inhibition of the leaf and root aqueous extract of beet root against CaOx crystal aggregation. Maximum inhibition was observed at 3 mg/ml concentration. When compared with leaf extract, root aqueous extract of beet root showed better activity against CaOx crystal aggregation (81% in leaf; 96% in root). Crystal aggregation inhibition was found low at either lower concentrations or higher concentrations of the extracts.

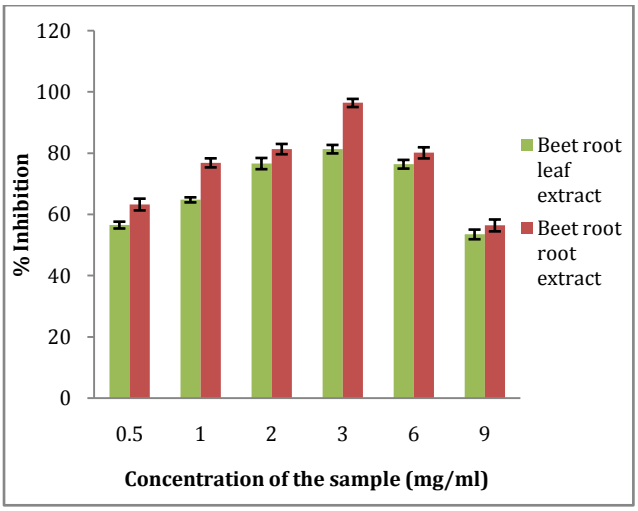


Fig. 3: Effect of beet root leaf and root aqueous extracts on CaOx aggregation

The microscopic examination of aggregation inhibitory potential of the leaf and root aqueous extracts of beet root was shown in fig. 4. Addition of leaf and root extracts in the reaction mixture inhibited the crystal aggregation drastically. The aggregated clusters of crystals were observed in the control group. Maximum crystal aggregation inhibition was found at 3.0 mg/ml compared to other concentrations of leaf and root aqueous extracts.

a. Control, b. Microscopic view of leaf aqueous extract on CaOx aggregation, c. Microscopic view of root aqueous extract on CaOx aggregation.

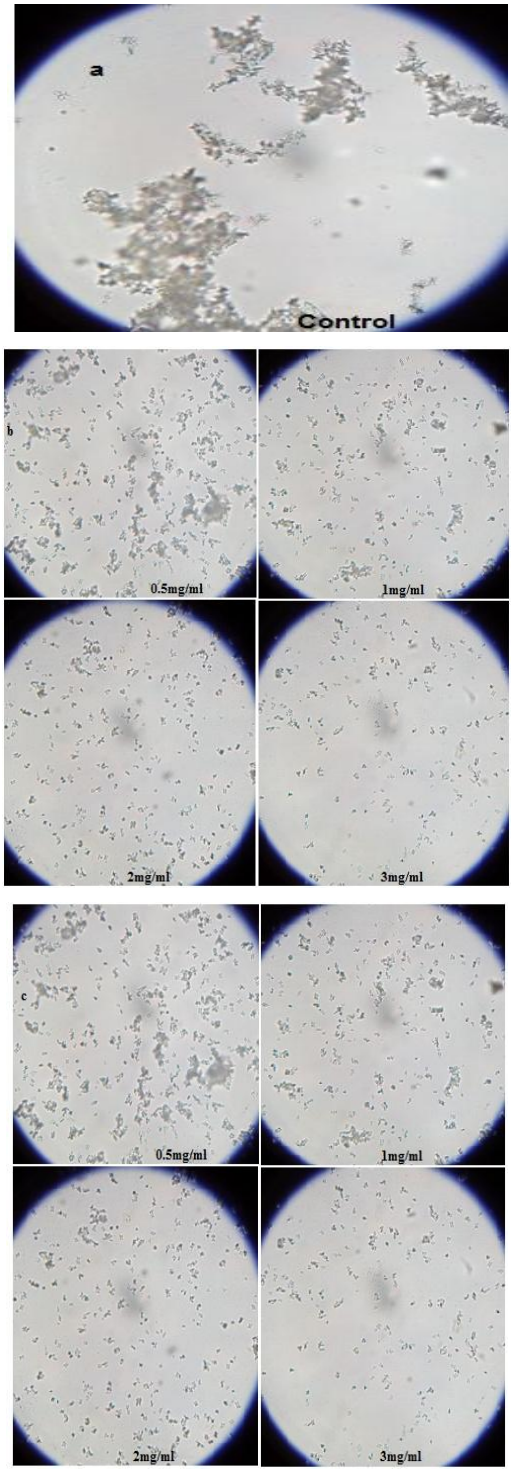


Fig.4: Microscopic evaluation of beet root leaf and root aqueous extracts on CaOx aggregation.

Fig. 5 illustrates the percentage inhibition of the growth of the calcium oxalate crystals in the presence of the leaf and root aqueous extracts of beet root. Maximum inhibition was observed at 3 mg/ml concentration. When compared with leaf aqueous extract of beet root, root aqueous extract showed better activity against CaOx monohydrate crystal growth. It was found that calcium oxalate monohydrate crystal growth was steadily decreased by the increased concentration of leaf and root extracts up to 3.0 mg/ml. The growth of the crystal was considerably reduced when used higher concentrations (6.0 and 9.0 mg/ml). The maximum inhibition of crystal growth was obtained using root extract (85%).

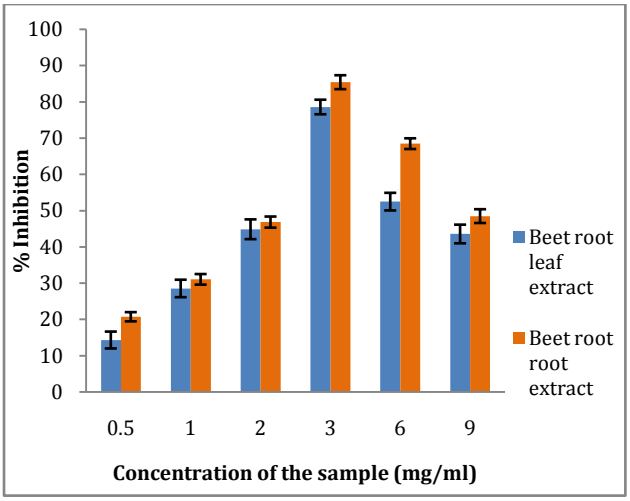


Fig. 5: Effect of beet root leaf and root aqueous extracts on CaOx growth

DISCUSSION

In day to day life, we are consuming oxalate in regular diet, in that only small amounts of dietary oxalate are absorbed, the ingested oxalate binds with the calcium if available in the intestinal tract otherwise it may be degraded by oxalate degrading bacteria present in the gut [27].

If the stone forming components exceeds their limit it leads to the formation of stone. In India, phytotherapy is a primary health care, because from ancient time itself we are using plants as a medicine, it provides apparently effective remedies for many diseases and also pharmaceutical products are expensive compared to folk medicines [23]. Large numbers of plant products already exist in market as a prophylactic or curative agent. Development of standard drugs has some drawback because kidney stone disease is multifactorial in nature and it has different chemical composition [28].

The main findings of the present study were that extracts inhibited the crystallization of calcium oxalate monohydrate and promoted calcium oxalate dihydrate formation in solution; there were less and smaller particles with the presence of extracts. When compared with calcium oxalate dihydrate, calcium oxalate monohydrate has a stronger affinity with cell membranes; it may constitute the form of higher potential risk for stone formation.

Moreover, the most common form of calcium oxalate crystals found in kidney stones is calcium oxalate monohydrate, although many stones contain both crystal forms. This can lead to a reduced supersaturation of the particles [29, 30]. The phytocompounds present in the beet root extract is therefore advantageous; it prevents the stone formation; can promote the excretion of small particles from the kidney and reducing the chance of crystal retention in the urinary tract.

Similar results were obtained in plants like *Tetraclinis articulate* [31], *Bergenia ligulata* [32], *Tachyspermum ammi* [33], *Tribulus* terrestris [25], *Hibiscus sabdariffa* [34], *Achyrantes* indica [35] and *Achyranthus aspera* [36]. The inhibitory nature of beet root plant extracts on kidney stone formation may be due to higher content of saponins present in the plant. The inhibitory activity of saponin fraction against urolithiasis was already reported in *Solanum* xanthocarpum [19].

CONCLUSION

The findings of the present study support the ethanobotanical information of the use of *Beta vulgaris L* on kidney stone treatment. The saponins present in the beet root may be the responsible for this antiurilithiatic activity. Further work will be continued to identify and isolate the potential bioactive compound against crystallization from this plant.

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