ORIGINAL ARTICLE

In vitro effect of hydro alcoholic extract of *Adiantum capillus-veneris* Linn. on calcium oxalate crystallization

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Background: *Adiantum capillus-veneris* Linn. is widely used in the management of urolithiasis in Unani system of medicine. **Aim:** To evaluate the effect of the hydro alcoholic extract of *A. capillus-veneris* Linn. on calcium oxalate crystallisation by *in vitro* study. **Materials and Methods:** The study includes crystallization, nucleation and aggregation assay. Crystallization was induced by addition of 50 µl of 0.1 M sodium oxalate in whole urine in the absence and the presence of extract at different concentrations (0.50 mg, 0.75 mg and 1 mg). The nucleation and aggregation rates were followed at 620 nm after mixing calcium chloride and sodium oxalate solution and in a buffered solution containing calcium oxalate monohydrate crystals, respectively. The rate was evaluated by comparing the slope of turbidity in the presence of extract with that of control using the spectrophotometer. Crystals in the urine were also analysed by light microscopy. **Results and Conclusion:** Extract of the test drug inhibited the crystallization in solution; less and smaller particles were observed in the presence of extract. These results were further confirmed in the nucleation assay, though the rate of nucleation was not inhibited but number of crystals was found to be decreased. The test drug also inhibited crystal aggregation. It can be concluded therefore, that the test drug possesses significant antilithiasic activity.

Key words: Calcium oxalate crystallization, *in vitro*, lithotriptic activity, unani medicine, urolithiasis

INTRODUCTION

Urolithiasis is a complex process that is a consequence of an imbalance between promoters and inhibitors in the kidneys.[1] Nearly 90% of kidney stone patients form stones composed of calcium oxalate (CaOx), calcium phosphate, or mixture of the two. CaOx crystals are the typical type of stone that are found in three hydrate forms of CaOx; calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD), and calcium oxalate trihydrate (COT). COM is the most common form found in the inorganic matrix of a kidney stone, because it is the most thermodynamically stable form. COD is also found as the metastable state of a stone, but COT is rarely found.[2] Kidney stone disease is a common disorder estimated to occur in approximately 12% of the population. Lithiasis is a male predominanl disorder, with a recurrence rate of 70-80% in male and 47-60% in female.[3] Besides dietary factor, the most common cause of kidney stone is drinking insufficient amount of water. Excessive consumption of meat protein also leads to marked increase in kidney stones, because meat causes over acidification of urine, which leads to increased excretion of oxalates, calcium and uric acid, whereas the excretion of citrate, provides protection and reduction in stone formation.[4] The aetiology of this disorder is multifactorial and is strongly related to dietary life-style habits or practices.[5] *Adiantum capillus-veneris* Linn. is an important drug widely used in patients of urolithiasis and is included as an important ingredient in many formulations used for litholytic activity. It has been described to possess lithotriptic, resolvent, deobstruent and diuretic actions and its decoction is frequently used for its lithotriptic effect moreover, it is considered that it is capable of expelling stones from kidney and bladder.[6-10] Many scientific studies have been carried out on *A. capillus-veneris Linn* and it has been reported to possess antifungal,[11] antibacterial,[12] antiviral,[13] and antioxidant[14] activities but the drug has not been studied for its lithotriptic activity. Hence, this drug was taken up to evaluate its antilithiasic activity in *in vitro* models.

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MATERIALS AND METHODS

Plant Materials

The plant of *A. capillus-veneris* Linn. were purchased from an authentic herb supplier in the local market of

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Bangalore, India. They were identified at National Ayurveda Dietetics Research Institute, Department of Ayush, Ministry of Health and Family Welfare, Government of India, Ashoka Pillar, Jayanagar, Bangalore by Dr. Siddamallayya N. A voucher specimen (Ref. no. Drug Authentication/SMPU/NADRI/BNG/2010-11/45a) was deposited there and in the Department of Ilmul Advia, NIUM, Bangalore for future reference.

Preparation of Plant Extract

The dried plant was made free of dirt and ground to powder, using commercial mill. It was then extracted with soxhlet extraction in 50% distilled water and 50% ethanol (1:6, drug: Solvent) for about 6 h. The liquid extract was filtered using a filter paper (Whatman No-40) and the filtrate was then concentrated in a carefully regulated water bath maintained at temperature of 80ºC. The yield of the hydro alcoholic extract was found to be 11% w/w. The extract was stored in a refrigerator pending the time of *in vitro* investigations.

Chemicals

Sodium azide of analytical grade was procured from Fischer Scientific Ltd. Bangalore. Sodium oxalate of analytical grade was purchased from Merck Ltd. Bangalore. Calcium chloride, Tris buffer and sodium chloride of analytical grade were procured from RFCL Ltd. Bangalore and Millipore filter paper of 0.22 µm from the precision scientific company, Bangalore.

Crystallization Assay in Whole Urine

This test was carried out by the method of Atmani *et al.*,[15] 24 h urine sample was collected in propylene bottle, from healthy rats kept in metabolic cages. Prior to collection, 1 ml of 20% sodium azide,[16] an antibacterial agent, was placed in the collection bottles. Aliquots of 2 ml of urine was distributed in to the tubes and allowed to get warm up to 37ºC. Thereafter, 50 µl fraction of hydro alcoholic extract solution was added to the tubes. Tubes with no extract added were used as control. Finally, 50 µl of 0.1 M sodium oxalate solution was added and tubes were incubated at 37ºC for 30 min. At the end of the experiment, optical density of the solution was determined at 620 nm by UV-spectrophotometer. There after the samples were examined by light microscope at ×45 for the presence of crystals.

Nucleation Assay

This test was carried out by the method of Patel *et al.*,[17] solution of calcium chloride and sodium oxalate, prepared at a final concentration of 5 mmol/L and 7.5 mmol/L respectively, in a buffer containing Tris 0.05 mol/L and Nacl 0.15 mol/L at pH 6.5 (calcium chloride solution 950 µl) was mixed with 100 µl of the herb extract at different concentrations (0.5 mg/ml, 0.75 mg/ml and 1 mg/ml). Crystallization was started by adding 950 µl of sodium oxalate solution. The final solution was magnetically stirred at 800 rpm using a polytetrafluroethylene coated stirred bar. The temperature was maintained at 37ºC. The optical density of the solution was monitored at 620 nm. The rate of nucleation were estimated by comparing the induction time (the delay before the appearance of crystals that have reached a critical size and thus, becomes optically detectable) in the presence of the extract with that of the control (no extract).

Aggregation Assay

This test was carried out by the method of Atmani *et al.*,[18] seed of COM crystals were prepared by mixing calcium chloride and sodium oxalate at 50 mmol/L. Both solutions were equilibrated to 60ºC in a water bath for 1 h and then cooled to 37ºC overnight. The crystals were invested by centrifugation and then evaporated at 37ºC. COM crystals were used at a final concentration of 0.8 mg/ml, buffered with Tris 0.05 mol/L at pH 6.5. Experiments were conducted at 37ºC in the absence or presence of the plant extract after stopping the stirring. The rate of aggregation was estimated by comparing the slope of the turbidity in the presence of the extract with that obtained in the control.

RESULTS

Effect on Crystallization in Whole Urine

In the first group where only plain urine was examined by light microscope, only a few minute crystals were observed that is normally found in urine due to supersaturation. In second group where crystallization was induced by addition of 0.1 M sodium oxalate solution, a number of large crystals were observed, most of which were found to be COD [Figure 1a], while in presence of plant extract at different doses, the numbers of crystals, its length and width were found to be reduced [Figure 1b-d]. The same samples were examined for their optical density. The optical density of plain urine sample was found to be 0.685 ± 0.199 while in negative group it was found to be too high, i.e., 1.39 ± 0.0002 ($P < 0.05$) when compared with plain group. The different concentration of test drug extract showed a significant reduction; at 0.50 mg/ml it was observed to be 1.052 ± 4.84 ($P < 0.001$), at 0.75 mg/ml it was found to be 1.0696 ± 0.00047 ($P < 0.05$) whereas, 1 mg/ml showed 1.072 ± 4.84, when compared with negative control [Table 1]. However, a significant reduction was observed at low dose. Examination of crystals by light microscopy also showed much reduction in the number and size of crystals in all test groups when compared with negative control.

Effect on Nucleation

In nucleation assay, the optical density at the same concentration of drug extract was measured over 50 min

at 620 nm. Sharp increase in optical density was observed followed by progressive decrease with time. The maximum slope of decrease in optical density with time therefore, reflects the rate of decrease in particle number due to crystal aggregation. Time course measurement of optical density of different groups was illustrated [Figure 2]. Figure showed sudden increase in the absorbance in all the groups which clearly indicate that there is no effect of test extract on the rate of nucleation or growth of crystals, but the number of particles was found to be reduced in comparison to negative control [Figure 3a] when examined by light microscopy. Further, the higher concentration of drug extract was associated with fewer crystals [Figure 3b-d].

Table 1: Effect of hydro alcoholic extract of *Adiantum capillus-veneris* Linn. on "crystallization assay in whole urine"

Groups	Optical density at 620 nm (mean±SEM)
Plain control (whole urine)	0.685±0.199
Negative control whole urine+0.1 M NaOx	1.39±0.0002*
Test group 1 drug extract 0.5 mg/ml	1.052±4.84***
Test group 2 drug extract 0.75 mg/ml	1.0696±0.00047*
Test group 3 drug extract 1 mg/ml	1.072±4.84

Kruskal-Wallis test with dunn comparision of all columns, Plain versus negative $P<0.05$, Negative versus test 1 $P<0.001$, Negative versus test 2 $P<0.05$, Plain vs negative and test 2 *$P<0.05$, Negative vs test 1 ***$P<0.001$

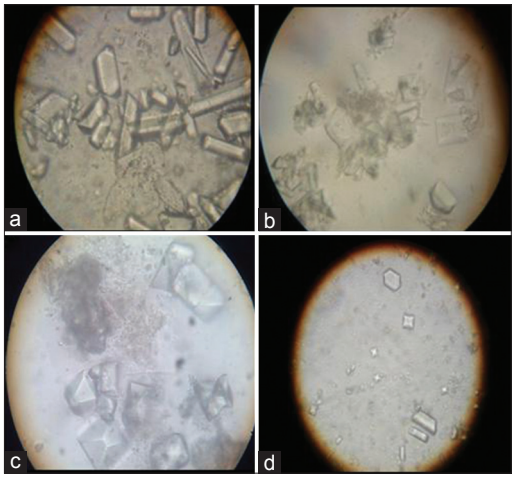

Figure 1: Effect of hydro alcoholic extract of *Adiantum capillus-veneris* Linn. on "crystallization assay in whole urine" by light microscopy (×45) (a) Negative control; (b) Test group 1 (0.5 mg/ml); (c) Test group 2 (0.75 mg/ml); (d) Test group 3 (1 mg/ml)

Effect on Aggregation

In Aggregation assay, Figures 4a-d showed that crystals were less aggregated in treated groups while more inhibition in aggregation was observed at higher dose. The result was also supported by the optical density [Figure 5] as it was found to be increased in test and standard groups (except in group II) in comparison of negative control.

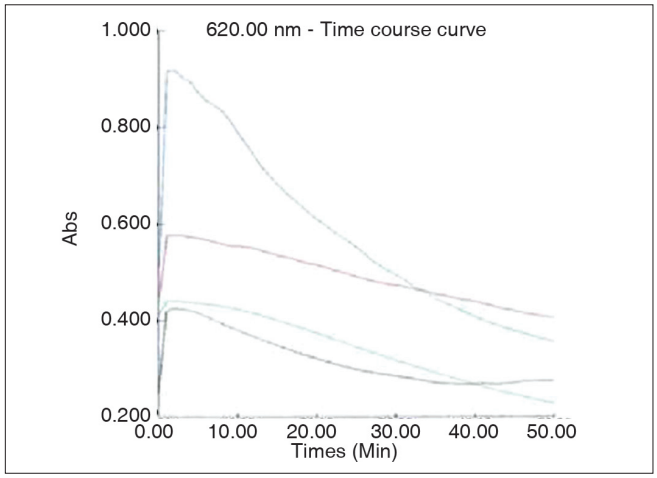

Figure 2: Effect of hydro alcoholic extract of *Adiantum capillus-veneris* Linn. on nucleation with respect to time Test group 1 (red), Test group 2 (blue), Test group 3 (black), Negative control (green)

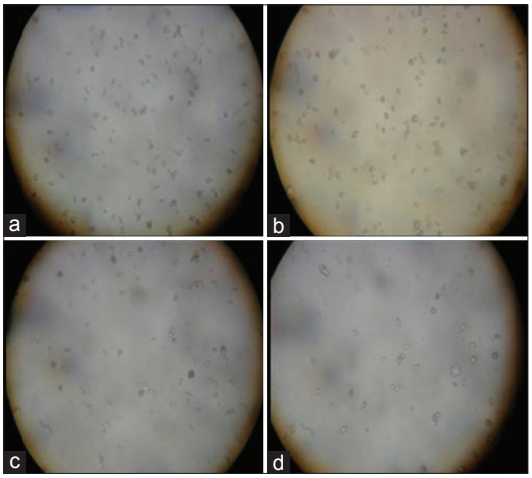

Figure 3: Effect of hydro alcoholic extract of *Adiantum capillus-veneris* Linn. on "nucleation" by light microscopy (×45) (a) Negative control; (b) Test group 1 (0.5 mg/ml); (c) Test group 2 (0.75 mg/ml); (d) Test group 3 (1 mg/ml)

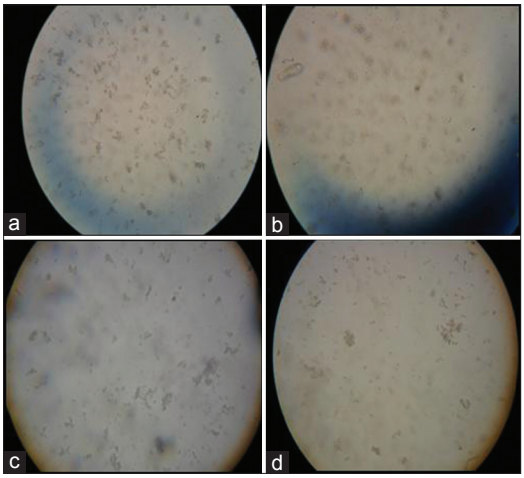

Figure 4: Effect of hydro alcoholic extract of *Adiantum capillus -veneris* Linn. on "aggregation of crystals" by light microscopy (×45) (a) Negative control; (b) Test group 1 (0.5 mg/ml); (c) Test group 2 (0.75 mg/ml); (d) Test group 3 (1 mg/ml)

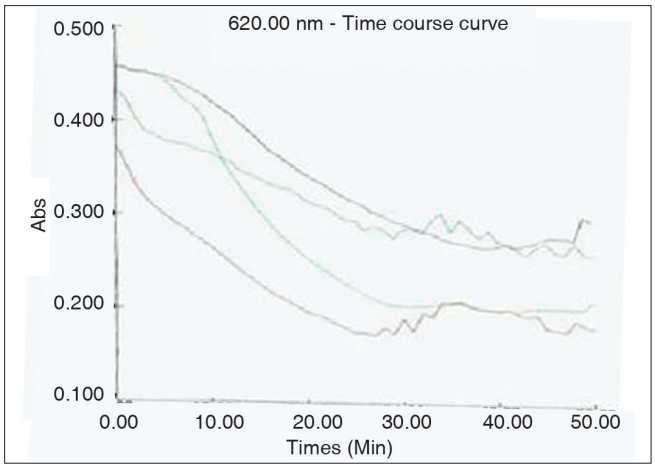

Figure 5: Effect of hydro alcoholic extract of *Adiuntum capillus veneris* Linn. on aggregation with respect to time Test group 1 (black), Test group 2 (red), Test group 3 (green), Negative control (blue)

DISCUSSION

The supersaturation of urine with CaOx, the most common component of renal stone,[19] and an important factor in crystallization with later factors being nucleation, growth and aggregation is responsible for stone formation. Thus, if supersaturation or later steps in crystallization is prevented, lithiasis can be avoided. In the present study, therefore, three experimental models were chosen to find out the efficacy of test drug on each phase. In first test, i.e., "crystallization assay in whole urine" the main findings were inhibition of crystallization of CaOx in solution. However, crystalluria is not a risk factor for lithiasis because it is common in both healthy subjects and stone formers.[20] The limiting factors in stone formers could be those processes that affect the size of the particles formed, because particles may become large enough to occlude the urinary tract leading to stone formation. In the present study, less and smaller particles in the presence of extract were observed. These results were further confirmed by the nucleation assay, though the rate of growth of crystals was not inhibited but number of crystals was found to be decreased, which showed that the extract contains nucleation preventing agents.

Hydro alcoholic extract of the test drug at different concentrations decreased number of crystals in solution there by reduced supersaturation and the size of the particles. This property of the test drug is therefore, advantageous in preventing urinary stone formation by inducing the excretion of small particles from the kidney and reducing the chance of retention in urinary tract.

The test drug may also contain substances that inhibit CaOx crystal aggregation. The agglomeration of particles is a critical step in urinary stone formation as larger crystals are less likely to pass spontaneously in urinary tract.[21] If the extract keeps CaOx particles dispersed in solution they are more easily eliminated. The inhibition of turbidity (aggregation) in the presence of drug extract was lower than that in the control, showing that crystals were less aggregated. The inhibited aggregation is associated with the increased concentration of extract. CaOx crystals are found in urine under several forms including monohydrate (COM) and dihydrate (COD), which is predominantly found in normal individuals. COM crystals have higher capacity to aggregate and adhere and it is the main form excreted by nephrolithitic patients.[22,23] It was observed that test drug reduced mainly the amount of COM crystals, which is responsible for higher potential risk for stone formation.

In the light of the findings and the discussion, it can be concluded that the test drug possesses significant anti lithiasic effect. Thus, it validated the claims of Unani physicians that *A. capillus-veneris* Linn. is useful in treatment of urolithiasis.

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