IN VITRO ANTIUROLITHIATIC ACTIVITY OF KALYANA KSHARAM – A HERBO MINERAL FORMULATION

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ABSTRACT

PURPOSE

To evaluate the in vitro antiurolithiatic activity of Kalyana Ksharam (KK).

MATERIALS AND METHODS

Inhibition of calcium oxalate (CaOx) crystal formation by KK was investigated by four different methods, namely, nucleation, aggregation, artificial urine and dissolution method. In nucleation, aggregation and artificial urine assay, the effectiveness of KK at different concentration (100, 200, 400, 600, 800 and 1000 µg/ml) on CaOx crystallization in vitro was evaluated, while in dissolution method the percentage of dissolution of CaOx crystals was investigated.

RESULTS

KK was effective in inhibiting CaOx crystals in dose-dependent manner and was comparable to that of cysteine.

CONCLUSIONS

The present study suggests that KK has potential effect on in vitro inhibition of CaOx crystals.

KEYWORDS: *Calcium oxalate, Kalyana Ksharam, Urolithiasis & Crystallization*

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INTRODUCTION

Urolithiasis is the common disease of the urinary tract and may be formed in any part of the urinary system¹. It affects 12% of population, with a reappearance rate of 70–80% in male and 47–60% in female². Calcium oxalate (CaOx) exists in two crystal forms such as 78% of calcium oxalate monohydrate (COM) and 43% of calcium oxalate dihydrate (COD)¹. For crystallization, the impelling cause is the decline of potential energy of molecules binding to each other³. Gone are days when mankind was looking towards modernization and westernization. Though allopathic drugs are effective in acute situation on chronic use, it exhibits severe adverse effects. A frightening rise in the occurrence of urolithiasis tied with the motivation given by WHO created a need to exploit traditional system of medicines⁴. One such is the ayurvedic formulation Kalyana Ksharam (KK), that is clinically used in the management of urinary calculi. It is prepared by mixing an appropriate concentration of Suthi (*Zingiber officinale*), Maricha (*Piper nigrum*), Pippali (*Piper longum*), Saindhava (*Rock salt*), Vidalavana (*Sal ammoniac*), Samudralavana (*Sea salt*), Hartaki (*Terminalia chebula*), Amalaki (*Phyllanthus emblica*), Vibhitaki (*Terminalia bellirica*), Danti (*Baliospermum montanum*), Arushkara

(*Semecarpusanacardium*), Chitraka (*Plumbagozeylanica*), Gomutram (cow's urine) and Eranda (*Ricinuscommunis*). Clinically, it is used to alleviate in urinary tract infection, dysuria, constipation, bloating, hemorrhoids, sprue, intestinal worm infestation, cough, cold, bronchitis, and asthma. Though being clinically used in ayurveda still no scientific evidence for antiurolithiatic activity of KK has been established. Hence, the study is to document the *in vitro* antiurolithiatic activity of KK by four *in vitro* methods.

MATERIAL AND METHODS

Chemicals: Cystone was procured from Himalaya Drug Company, Karnataka, India and KK was procured from Kottakkal Arya Vaidyasala, Kerala. Calcium chloride, sodium oxalate, Tris buffer, sodium chloride, sodium phosphate, sodium citrate, magnesium sulphate, sodium sulphate, potassium chloride, ammonium hydroxide, ammonium chloride, sulphuric acid, ammonia, hydrochloric acid, potassium permanganate were procured from Merck Specialities Private Limited, Mumbai.

Apparatus: UV Spectrophotometer – Thermos Fisher Scientific, Great Britain

Nucleation Assay

About 5 mmol/L and 7.5 mmol/L of calcium chloride and sodium oxalate solutions were prepared in a buffer with a pH of 6.5 containing Tris 0.05 mmol/L and NaCl 0.15mol/L, respectively. Solution of calcium chloride was mixed with KK and cystone (standard) in a concentration of 100, 200, 400, 600, 800 and 1000 µg/ml. About 950 µl of sodium oxalate solution was added to initiate the crystal formation. Final solutions were incubated for 30 min at 37°C. The absorbance was taken at 620 nm. The rate of nucleation was estimated by comparing the induction time in the presence of the KK and cystone with that of control⁵. The percentage inhibition of CaOx crystal formation was calculated⁶.

Aggregation Assay

Calcium chloride and sodium oxalate at 50 mmol/L concentration were mixed to form COM crystals. Solution were equilibrated to 60°C for 1 h and cooled to 37°C and then evaporated. The formed COM crystals are added to the solution containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5. KK and cystone in the concentration of 100, 200, 400, 600, 800 and 1000 µg/ml were added to the solution. The absorbance at 620 nm was recorded at 30, 60, 90, 180 and 360 min⁷. The percentage inhibition of CaOx crystal formation was calculated⁶.

IN VITRO Crystallization using Artificial Human Urine

Preparation of artificial urine: The artificial urine (AU) was prepared at pH 6.0 in a composition containing calcium chloride (4.5mM), sodium phosphate (32.3 mM), magnesium sulfate (3.85mM), sodium citrate (3.21mM), sodium sulfate (16.95mM), sodium chloride (105.5mM), ammonium hydroxide (17.9mM), potassium chloride (63.7mM), sodium oxalate (0.32mM) and ammonium chloride (0.0028mM). One milliliter of AU was added to 0.5 ml of distilled water to serve as a blank and 0.5 ml of 0.01 M sodium oxalate was added to it and measured in a period of 10 min. KK and cystone at the concentration of 100, 200, 400, 600, 800 and 1000 µg/ml were prepared. Each 0.5 ml of KK was added to 1 ml of AU to measure blank reading and then 0.5 ml of 0.01 M sodium oxalate solution was added and the absorbance at 620 nm were taken for a period of 10 mins. The percentage of inhibition of CaOx crystal formation was calculated³.

Dissolution of Experimental Stone

Preparation of Experimental Kidney Stones (CaOx stones)

Calcium oxalate is precipitated by mixing 1.47 g of calcium chloride dehydrate in 100ml of distilled water and 1.34 g of sodium oxalate in 100 ml of 2N sulphuric acid. The precipitate is freed of sulphuric acid by using ammonia. The precipitate is dried at 60°C for 2 h after washing with distilled water.

Preparation of Semi-Permeable Membrane from Farm Eggs

Empty eggs shell were taken by removing the entire content of egg and kept in a solution of 8 ml concentrated hydrochloric acid in 400ml distilled water for 48h resulting in decalcification of semi-permeable membrane. The semi-permeable membranes were removed after 48h from the egg shells and placed in a solution of ammonia to neutralize. Semi permeable membranes were refrigerated at pH 7.2 in the moistened condition.

Estimation of Calcium Oxalate

The following solutions were loaded in the semi-permeable membrane tied at one end by immersing in 0.1 M Tris buffer.

Solution I: 2 ml of CaOx (1mg/ml) + 2 ml of distilled water

Solution II: 2 ml of CaOx (1mg/ml) +2 ml of cystone solution (10 mg/ml, 20mg/ml, 40 mg/ml)

Solution III: 2 ml of CaOx (1mg/ml) +2 ml of KK (10 mg/ml, 20 mg/ml, 40 mg/ml)

Other end is knotted to the mouth of the container with a stick and incubated for 3 days after heating at 37°C for 4h. Each solution is removed from the semi-permeable membrane after 3 days. Four milliliter of 1N sulphuric acid and 60–80 μ l of 0.02M potassium permanganate were added to the each solution and kept for 2 hours and the absorbance was taken at 620nm⁸.

RESULTS

Figure 1: Percentage Inhibition of CaOx Crystals by Cystone and KK using Artificial Human Urine Assay.

Figure 2: Percentage Inhibition of CaOx crystals by Cystone and KK for Nucleation Assay.

Data were expressed as mean $\pm$ SEM (n=2). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 against 100 µg/ml concentration using Student t-test.

There was no significant difference (Figure 1) between the percentage inhibition of CaOx crystallization at various concentration of cystone and KK and the percentage inhibition increases in dose dependent manner with 100µg/ml. The study showed that KK had more percentage inhibition of crystallization in artificial urine than cystone.

Data were expressed as mean $\pm$ SEM (n=2). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001vs 100 µg/ml concentration using Student t-test.

Figure 2 shows the percentage inhibition of nucleation of CaOx crystals with various concentrations of KK and cystone. There was significant difference in percentage inhibition of cystone and KK at 600, 800 and 1000 µg/ml indicating that cystone had better significant inhibition of nucleation than KK and percentage inhibition increases with 100µg/ml in a dose dependent manner for both the drugs.

Figure 3: Percentage Inhibition of CaOx Crystals by Cystone and KK for Aggregation Assay.

Figure 4: Percentage Dissolution of CaOx Crystals by Cystone and KK for Dissolution Method.

Data were expressed as mean $\pm$ SEM (n=2). Groups were compared using Student t-test.

There was no significant difference (Figure 3) in the percentage inhibition of aggregation of CaOx crystals at various concentrations of both drugs and there were comparable increases in percentage inhibition of CaOx in a dose dependent manner for both the drugs.

Data were expressed as mean $\pm$ SEM (n=2). Groups were compared using Student t-test.

There was a significant difference in percentage dissolution of CaOx crystals for cystone and KK at increasing doses (Figure 4). Also, KK exhibited higher percentage of dissolution than cystone in its lower concentration but at its highest concentration both exhibited equal percent of dissolution.

DISCUSSIONS

Urolithiasis includes supersaturation, nucleation, aggregation, growth and retention of crystals in the renal tubules⁵. In this dissertation work, the properties of KK were explored against kidney stone by *in vitro*.

Artificial human urine assay was used to study the supersaturation in growth of CaOx crystals. Normal human urine is dynamic, because new solutes are added or detached so the artificial urine method is considered⁷. The result from the study shows that KK had more percentage inhibition of crystallization in artificial urine (Figure 1) than cystone but was not statistically significant.

Crystalluria and stone formation is from heterogeneous nucleation induced by crystallization promoters¹⁰. Cystone exhibited better inhibition of nucleation than KK at various concentrations and was statistically significant. There was a significant difference in percentage inhibition of cystone and KK at 600, 800 and 1000 μ g/ml (Figure 2) indicating cystone had better significant inhibition of nucleation than KK.

Once stone formed, the crystalline particles aggregate to form larger particles. Aggregation of particles in solution is a balance of aggregating effects and disaggregation effects¹¹. There was no significant difference (Figure 3) in the percentage inhibition of aggregation of CaOx crystals at various concentrations between the two drugs.

KK has a higher percentage of dissolution than cystone even in its lower doses, but was not statistically significant. Also, KK exhibited higher percentage of dissolution (Figure 4) than cystone in its lower concentration, but in its higher concentration there was no much difference in percentage dissolution between them. Thus, our study shows that KK has significant antiurolithiatic activity based on *in vitro* test.

KK has phytoconstituents such as tannins, terpenoids, saponins, flavonoid and phenolic compounds. Tannin presents in *Piper nigrum*, *Piper longum*, *Terminalia chebula*, *Terminalia belleria*, *Zingiber officinale*, *Emblical officinale*, *Plumbago zeylanica*, *Baliospermum montanum*, *Semecarpus anacardium*, ingredients of KK which might have contributed to the inhibition of calcium oxalate crystals formation and dissolution of formed crystals in semi permeable membrane dissolution test. Flavonoids present in *Baliospermum montanum*, *Semecarpus anacardium*, *Ricinus communis*, *Terminalia chebula*, *Terminalia belleria*, *Zingiber officinale*, *Piper nigrum*, ingredients of KK and possess CaOx crystal dissolution potency and antioxidant activity⁶.

The ingredients of KK such as *Piper nigrum*, *Piper longum*, *Zingiber officinale*, *Baliospermum montanum*, *Ricinus communis*, *Semecarpus anacardium* containing saponins have antilithic properties and fragment mucoproteins in stone matrix⁶. Terpenoids present in *Piper nigrum*, *Piper longum*, *Terminalia chebula*, *Zingiber officinale*, *Plumbago zeylanica*, *Baliospermum montanum*, *Semecarpus anacardium*, ingredients of KK have been found to inhibit the cytotoxicity induced by calcium oxalate, they are also know to normalize the excretion of stone forming constituents⁹. Phenolic constituents in KK such as *Zingiber officinale*, *Piper nigrum*, *Emblical officinale*, *Baliospermum montanum*, *Semecarpus anacardium* exhibit antioxidant activity and prevent the crystal adhesion.¹². Therefore, the anti-nucleation, anti-aggregatory and crystal growth defying activity of KK would have been an outcome of these phytoconstituents present in KK such as tannins, terpenoids, saponins, flavonoid and phenolic compounds.

Percentage inhibition of both drugs increased in a dose dependent manner for nucleation, aggregation, *in vitro* crystallization in artificial urine and dissolution methods. KK has more significant effect at higher doses in inhibiting supersaturation, however, the percentage inhibition of aggregation, nucleation and percentage dissolution was comparable with cystone. Ingredients of both cystone and KK were entirely different. Cystone is given at the dose of 4 tablets per day (each tablet contains 446 mg based on the label claim) for treatment until stone passes out and 2 tablets per day after surgery for 2–3 months to prevent the recurrence of urolithiasis. But based on the case study, KK is prescribed at 500 mg a day for 15 days¹³. Thus, even at lower strength and shorter duration of treatment KK seems to be cost effect and better choice for the treatment of urolithiasis.

CONCLUSIONS

This study has given primary evidence for KK possessing antiurolithiatic property *in vitro*.

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