



Antilithiatic activity of poly-herbal formulation tablets by *in-vitro* method

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ABSTRACT

Hydroalcoholic extracts of *Kalanchoe pinnata* and *Rotula aquatica* and its combination were formulated herbal tablets, evaluated for antilithiatic *in vitro* method. The homogenous precipitation method was used. The study was carried out in glass tubes. The buffer system used was TRIS buffer pH 7.4. The experiment consists of the following tubes for control and test, 25 ml each of 25 mM CaCl₂ · 2H₂O, 25 mM Na₂HPO₄ · 2H₂O or 25mM Na₂C₂O₄. To the tubes of each set, tablet formulation or an equal amount of vehicle was added. The tubes were incubated at 37°C for 4 h. The precipitate of *calcium phosphate* was generated by mixing 1 ml of solution from the tubes having calcium chloride dihydrate and disodium hydrogen phosphate monohydrate and *Calcium oxalate precipitate* was generated by mixing 1 ml of solutions from the tubes having calcium chloride dihydrate and sodium oxalate solutions. Calcium was estimated using titrimetry and phosphorus was estimated using colorimetric analysis. Appropriate standard curves were done with each set of experiments. The amounts of precipitate of calcium and phosphate were determined in each of the respectively. The percent inhibition of the test was calculated in comparison with the control samples. Herbal tablet formulation showed antilithiatic activity to the marketed formulation in terms of inhibiting the formation of phosphate precipitate but showed a significantly better potential in preventing the formation of the calcium precipitate. The herbal tablet formulation of *Kalanchoe pinnata* and *Rotula aquatica* have inhibitory effect on calcium oxalate crystallization thus may be beneficial in the treatment of renal lithiasis.

INTRODUCTION

Renal lithiasis is one of the oldest disease known to human beings and has been documented in ancient Greek. Urinary stones have been found in the remains of Egyptian mummies dating back as far as 7000 years (Butt *et al.*, 1956). Renal lithiasis is defined as the consequence of an alteration of the normal crystallization conditions of urine in the urinary tract (Mute *et al.*, 2008). Stone disease is common with the lifetime risk of stone formation exceeding 6-9% in men and 3-4% in women (Curhan *et al.*, 2007). The overall probability of forming stones differs in various parts of the world 1-5% in Asia, 5-9% in Europe, 13% in North America, 20% in Saudi Arabia (Adriano *et al.*, 2000). Kidney stone formation is the result of a physicochemical process that involves nucleation of crystals from a supersaturated solution.

The common constituents of kidney stones are calcium, phosphate and oxalate. The factors that influence crystal generation are urine volume, concentration of stone constituents (a function of urine volume), the presence of a nidus and the balance among various physicochemical factors that inhibit or promote stone formation. Kidney plays an important role in water conservation, but at the same time, minerals with low solubility are excreted. Urinary stones or kidney stone are formed when the normal balance of water, salt, minerals and other things found in the urine is altered. A number of different conditions can lead to kidney stones like gout, hypercalciuria, hyperparathyroidism, renal tubular acidosis and certain inherited metabolic condition like cystinuria, hyperoxaluria.

Some Medications like diuretics, calcium-containing antacids, ephedrine, guaifenesin and protease inhibitors Indinavir, a drug used treat HIV infection are likely to cause kidney stone (Parmar *et al.*, 2004).

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A treatment of renal lithiasis is to relieve symptoms, deal with complications and prevent the formation of more stones. Stones larger than 5mm or stones that fail to pass through should be treated by some interventional procedures such as extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS), or percutaneous nephrolithotomy (PNL) (Coll *et al.*, 2002). Unfortunately, the propensity for stone recurrence is not altered by removal of stones with ESWL and stone recurrence is still about 50% (Nabi *et al.*, 2007). In addition, ESWL might show some significant side effects such as renal damage, ESWL induced hypertension or renal impairment (Tombolini *et al.*, 2000). Although there are a few recent reports of beneficial effects of medical treatments in enhancing clearance of stones in the distal ureters (Dellabella *et al.*, 2005) de facto there is still no satisfactory drug to use in clinical therapy, especially for the prevention of the recurrence of stones. Medicinal Plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of various ailments. Medicinal plants are used from centuries due to its safety, efficacy, cultural acceptability and lesser side effects as compared to synthetic drugs. Today around 50% of world population is totally depends upon the plant derived products as a primary health care with no side effects. Various types of plants and its species are used in the treatment of kidney stones (Choubhay *et al.*, 2010). In present study the two ayurvedic medicinal plants parts such as *Kalanchoe pinnata* leaves and *Rotula aquatica* roots and its combination were selected for designing the modern formulations and determination of *in-vitro* antilithiatic potential comparison with standard antilithiatic tablet formulation 'Cystone'.

MATERIALS AND METHODS

The collection of the plant materials of *Kalanchoe pinnata* and *Rotula aquatica* were done in the month of April in the medicinal garden of Hanumangarh and Salasar of the Rajasthan. The identification and authentication of the plant was carried out by Dr. D. Stephan, Ph.D, Department of Botany, American College, and Madurai-Tamilnadu. (Voucher specimen ACM/09/B-416). Polyvinyl pyrrolidine, Microcrystalline Cellulose, Cross Povidone, Sodium Starch Glycolate, Cross-linked Sodium Carmellose, Aerosil, calcium chloride and calcium phosphate were purchased from Loba chemicals Ltd. Mumbai. All other chemicals used were of analytical grade.

Preparation of extract

The leaves of *Kalanchoe pinnata* and root of *Rotula aquatica* were washed and dried under shade for 15 days on fresh cotton cloth. After 15 days the dry weight is taken. Cleaned and mixer was used for grinding the dried leaves and roots. After proper grinding, the weight of the powder was obtained. These powders were used for hot extraction. The flask with the given solvent is heated to a particular temperature. The vapour produced passes through the siphon tube into the thimble kept above where

it is condensed and tickles down into the flask again through the thimble dissolving the active constituents in it. The method is described as the continuous extraction. The process is continued until all the soluble constituents get separated. The extract at the bottom was collected and dried under reduced temperature and pressure. Each time, before the extraction with other solvents, the powdered substance is air dried. About 200 gm of dried powder was properly packed in Whatmann filter paper (grade no.1) and kept in thimble and the soxhlet apparatus was set up. The extraction of powder was done with different solvents with solvents of increasing polarities like petroleum ether (60-80° C), benzene, acetone, ethyl acetate, chloroform, and hydro alcohol. Here temperature maintenance is based on the solvents used for extraction. The solvents were removed under reduced pressure using rotary evaporator and stored in desiccators (Sim *et al.*, 1968). The consistency of the extract is semi solid.

Preparation of mixed blend of drug and excipients

Powdered form of *Kalanchoe pinnata*, *Rotula aquatica*, microcrystalline cellulose, cross povidone, sodium starch glycolate, cross-linked sodium carmellose, poly vinyl pyrrolidine and aerosil were passed through mesh no. 60. Required quantity of each ingredient was taken for each specified formulation (depicted in the table 1) and all the ingredients were subjected to a required degree of fineness. The powder blend was evaluated for flow properties as follows.

Angle of Repose

Angle of repose was determined using funnel method (Lachman *et al.*, 1987). The blend was poured through a funnel that can be raised vertically until a maximum cone height (h) was obtained. Radius of the heap (r) was measured and the angle of repose (q) was calculated using the formula.

$$\bullet = \tan^{-1}(h/r) \quad (1)$$

Bulk Density

Apparent bulk density (\bullet_b) was determined by pouring the blend into a graduated cylinder. The bulk volume (V') and weight of the powder (M) was determined. The bulk density was calculated using the formula-

$$\text{Bulk Density } (\bullet_b) = M / V' \quad (2)$$

Tapped Density

The measuring cylinder containing a known mass of blend was tapped for a fixed time. The minimum volume (V_T) occupied in the cylinder and weight (M) of the blend was determined. The tapped density (\bullet_t) was calculated using the following formula-

$$\text{Tapped Density } (\bullet_t) = M / V_T \quad (3)$$

Compressibility Index

The simplest way for measurement of free flow of powder is compressibility, a indication of the ease with which a

material can be induced to flow is given by compressibility index (I) which is calculated as follows,

$$I = \frac{V_0 - V_t}{V_t} \times 100 \quad (4)$$

Where V_0 is the bulk volume and V_t is tapped volume. The value below 15% indicates a powder with usually give rise to good flow characteristics; whereas above 25% indicate poor flowability.

Hausner Ratio

Hausner ratio is an indirect index of ease of powder flow. It is calculated by the following formula-

$$\text{Hausner ratio} = \frac{\rho_t}{\rho_d}$$

Where ρ_t is tapped density and ρ_d is bulk density, Lower hausner ratio (<1.25) indicates better flow properties than higher ones (>1.25).

Formulation of Tablets

The ingredients depicted in table I (except aerosil) were mixed homogenously and required degree of fineness was attained. Finally aerosol was added and mixed. The mixed blend of drug and excipients was compressed using a single punch tablet punching machine to produce tablets, weighing 700 mg each with a diameter 8 mm (Khandelwal *et al.*, 2002 and Lachman *et al.*, 1987).

Drug Excipient Compatibility Test

Compatibility of the drug with excipients was determined by FT-IR spectral analysis, this study was carried to detect any changes on chemical constitution of the drug after combining it with the excipients. The samples were taken for FT-IR study. IR spectra of drug in KBr pellets at moderate scanning speed between 4000-400 cm^{-1} was carried out using FTIR.

The peak values (wave number) and the possibility of functional group are shown in spectra which compare with standard value. The comparison of these results with chemical structure shows that the sample was pure aqueous extract.

Evaluation of Tablets

The Three forms of Tablet (T_1 , T_2 , and T_3) were evaluated for general appearance, friability test, hardness test, weight variation test and disintegration test.

In-vitro Antilithiatic activity of herbal tablet formulation

The aqueous extract of Cystone was prepared by grinding a tablet to powder. This powder was mixed with 5 ml water and kept for 2–3 h and then centrifuged at 1000 rpm. The clear supernatant was used for the study. Tablet formulation was compared with the aqueous extract of cystone (a marketed herbal formulation for urolithiasis) for their antilithiatic activity. The homogenous precipitation method was used to study the antilithiatic activity (Singla *et al.*, 1981).

The homogenous precipitation (i.e. in the absence of external matrices) was modified by keeping the pH and isotonicity of the medium constant. The study was carried out in glass tubes instead of collecting the precipitate over filter papers. The buffer system used was TRIS buffer pH 7.4

The buffer composition was: 0.1 M TRIS buffer; Solution A was 0.4 M TRIS [48.4 g of Tris (trihydroxymethyl) amino methane per 1000 ml]; Solution B was 0.4 M hydrochloric acid. [33.6 ml of concentrated hydrochloric acid per 1000ml]; A working solution was made up of 25 ml solution A, 20.7 ml solution B made up to 100 ml, the pH was 7.4.

Table. 1: Formulae for Preparing Tablet Dosage Forms.

Formulation	Kalanchoe pinnata	Rotula aquatica	MCC	CP	SSG	CLSC	PVP	Aerosil
T_1 (mg)	250	250	130	20	20	15	10	5
T_2 (mg)	250	250	120	15	20	20	20	5
T_3 (mg)	250	250	100	25	25	25	20	5

MCC- Microcrystalline Cellulose, CP- Cross Povidone, SSG- Sodium Starch Glycolate, CLSC- Cross-Linked Sodium Carmellose, PVP- Poly Vinyl Pyrolidone

Table. 2: Percentage inhibition using herbal tablet formulation.

Groups	Control	KPE Extract	RAE Extract	Combined Tablet	Cystone tablet
Amount of Precipitate (μmol)					
Calcium	7.13 \pm 0.11	5.90 \pm 0.06	5.10 \pm 0.02	4.60 \pm 0.01	4.00 \pm 0.02
Phosphate	7.43 \pm 0.022	5.7 \pm 0.01	5.52 \pm 0.15	4.15 \pm 0.01	2.22 \pm 0.01
Percentage of Inhibition					
Calcium	-	17.25 \pm 0.3**	28.47 \pm 1.05**	35.48 \pm 0.51***	43.19 \pm 1.70
Phosphate	-	23.28 \pm 0.12**	25.70 \pm 0.06**	43.74 \pm 0.50***	70.12 \pm 1.32

G7- Lithiatic Control; **G8-** Tablet formulation of *K. pinnata* Plant extract; **G9-** Tablet formulation of *Rotula aquatica* Plant extract; **G10-** Herbal tablet Formulation of combined Plant extract. **G11-** Cystone Tablet Treatment.

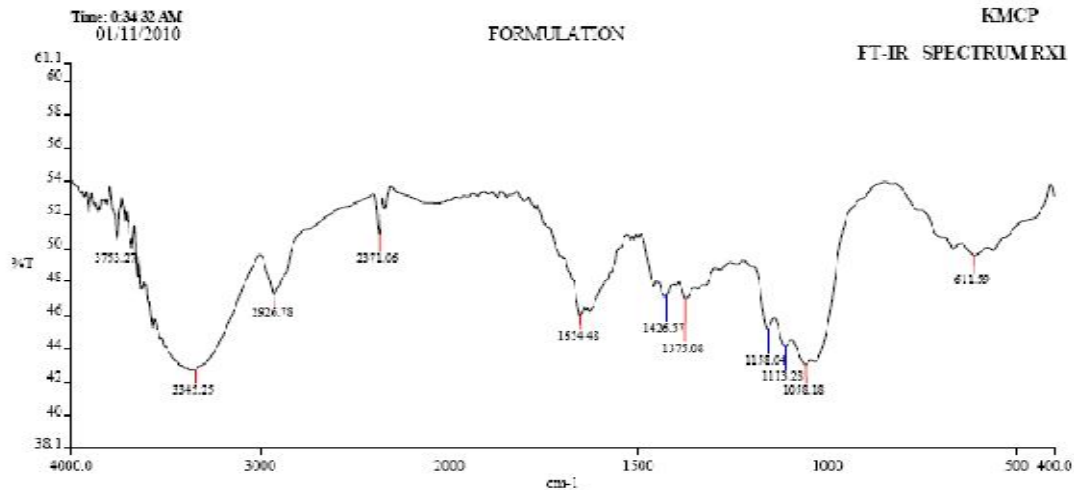
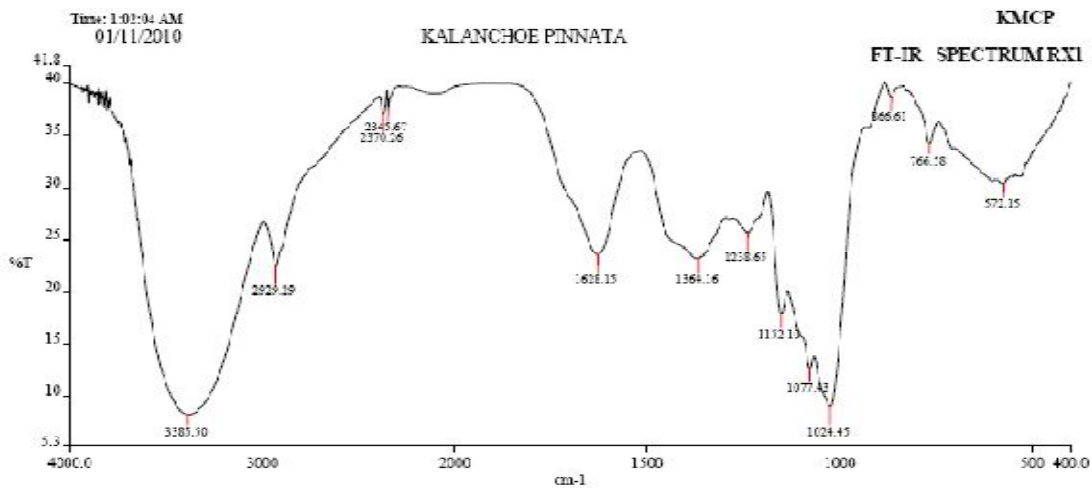
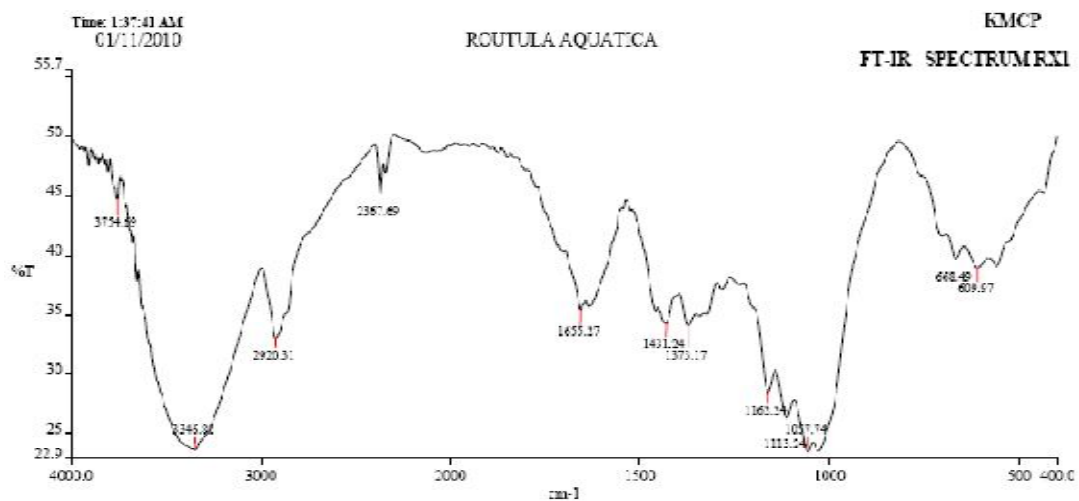
- Values are expressed as Mean \pm SEM
- Values were evaluated by using ONE WAY ANOVA Followed by Newman Keul's multiple range test
- **values were significantly different from Cystone treatment (G11) at (P< 0.01)
- ***values were significantly different from Cystone treatment (G11) at (P< 0.001)

Table. 3: Evaluation of Powder Blend.

Batch	Angle of repose	Carr's index (%)	Bulk density (gm/ml)	Tapped density (gm/ml)	Hausner's ratio
T_1 (mg)	28.79	17	0.42	0.51	1.13
T_2 (mg)	30.96	16.98	0.44	0.53	1.20
T_3 (mg)	27.54	15.07	0.41	0.54	1.31

Table 4: Evaluation of Prepared tablet.

Batch	Hardness (Kg/cm ²)	Thickness (mm ²)	% weight Variation	% Friability	Disintegration
T ₁ (mg)	4.2	3.6	2.48	0.81	15
T ₂ (mg)	4.1	3.6	2.51	0.93	14
T ₃ (mg)	4.4	3.6	2.21	0.72	13

Fig. 1: I. R. Spectra of Formulation Tablet of *K. pinnata* and *R. aquatica*.Fig. 2: I. R. Spectra of Formulation Tablet of *K. pinnata*.Fig. 3: I. R. Spectra of Formulation Tablet of *R. aquatica*

Experimental Procedure

The experiment consists of the following tubes for control and test, 25 ml each of 25 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 25 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ or 25 mM $\text{Na}_2\text{C}_2\text{O}_4$. To the tubes of each set, tablet formulation or an equal amount of vehicle was added. The tubes were incubated at 37°C for 4 h. The precipitates of calcium phosphate and calcium oxalate were generated as follows.

(i) *Calcium oxalate precipitate* was generated by mixing 1 ml of solutions from the tubes having calcium chloride dihydrate and sodium oxalate solutions.

(ii) *Calcium phosphate precipitate* was generated by mixing 1 ml of solution from the tubes having calcium chloride dihydrate and disodium hydrogen phosphate monohydrate.

Calcium was estimated using titrimetry and phosphorus was estimated using colorimetric analysis. Appropriate standard curves were done with each set of experiments. The amounts of precipitate of calcium and phosphate were determined in each of the respectively (Peshin *et al.*, 1994; Varley *et al.*, 1996 and Gomri *et al.*, 1941). The percent inhibition of the test was calculated in comparison with the control samples.

RESULTS AND DISCUSSION

The primary objective of this work was to develop oral herbal dosage formulation of *Kalanchoe pinnata* and *Rotula aquatica* along with evaluated of antilithiatic activity by *in-vitro* method. The development of such formulations will mark an important advancement in the area of phytopharmaceuticals. The present investigation examines design & development of solid oral herbal dosage form. The solid oral herbal dosage form, tablets were prepared using poly vinyl pyrrolidone as binder, three super disintegrate in varying concentration such as croscopovidone, sodium starch glycoate, cross-linked sodium carmellose, and microcrystalline cellulose as filler and aerosil as glidants. For each formulation, blend of drug and excipients were prepared and evaluated for various parameters as explained earlier. The powder blend was compressed using direct compression technique. Bulk density was found in the range of $0.41\text{-}0.44\text{g/cm}^3$ and the tapped density was found in the range $0.51\text{-}0.54\text{g/cm}^3$. Using these two-density data Hausner's ratio and compressibility index was calculated. The powder blends of all the formulations had Hausner's ratio of 1.2 or less indicating good flowability (Tang *et al.*, 2001). The compressibility index was found between 15.07 - 17 and the compressibility- flowability correlation data (Lachman *et al.*, 1987) indicated a fairly flowability of the powder blend. The good flowability of the powder blend was also evidenced with angle of repose (range of $24\text{-}29^\circ$) which is below 40° indicating good flowability.

Tablet was prepared using direct compression technique (Madosiya *et al.*, 2009). Since the powder material was free flowing, tablets were obtained of uniform weight variation as per pharmacopoeial specifications. The weight variation was found

between 2.21 mm -2.51 mm and the hardness of the tablets between $4.1\text{-}4.4\text{kg/cm}^2$. Friability of the tablets was found below 1% indicating a good mechanical resistance of tablets. The disintegration time of the tablets was found between 13-15 minutes. All the parameters were found well within the specified limit for uncoated tablets.

The prepared tablets were brown colour with smooth surface having acceptable elegance. T_3 form of tablets was good quality with regard to hardness, friability & weight variation.

The *in-vitro* antilithiatic activity which has been carried, where the Herbal tablet formulation showed antilithiatic activity to the marketed formulation in terms of inhibiting the formation of phosphate precipitate but showed a significantly better potential in preventing the formation of the calcium precipitate. Tablet formulation of hydroalcoholic extracts of both plants also showed inhibiting the formation of calcium and phosphate precipitate (See Table 2 and figure 4 & 5). Cystone (as positive control) which is a prescribed for treatment for urinary and renal calculi, showed a good inhibitory effect on the formation of the precipitates of calcium and phosphate. These results showed that herbal tablet formulation contained crystallization preventing agents. This property of plants may be an important in preventing the growth of renal lithiasis.

In GC-MS Spectra tyrosol, pyrogallol identified in hydro-alcoholic extract of *Kalanchoe pinnata* and squaline also identified in *Kalanchoe pinnata* and *Rotula aquatica* hydro-alcoholic extracts, are having antioxidant properties, (Loru *et al.*, 2009; Hande *et al.*, 2010; Waterman *et al.*, 2007; Renana *et al.*, 1979 and Gregory *et al.*, 2007) So these components may be responsible for antilithiatic activity.

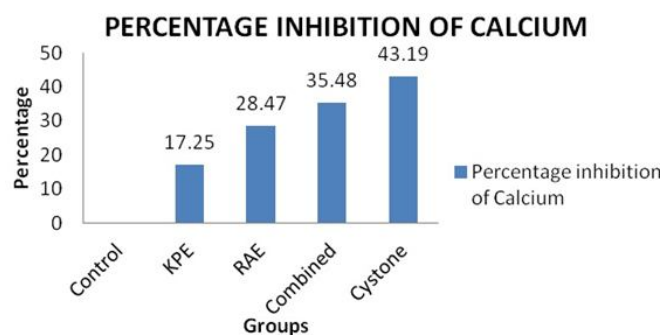


Fig. 4: Percentage inhibition of calcium.

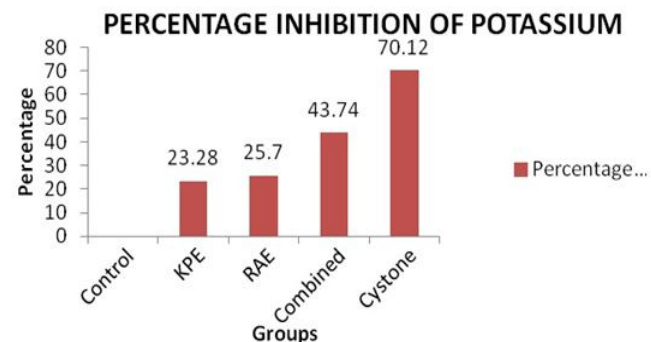


Fig. 5: Percentage inhibition of potassium.

CONCLUSION

The herbal tablet formulation of *Kalanchoe pinnata* and *Rotula aquatica* have inhibitory effect on calcium oxalate crystallization thus may be beneficial in the treatment of renal lithiasis. Further evaluations of this formulation with clinical trials may yield a solution for this urinary problem.

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