

Solubility of Calcium Phosphate Crystallization *in vitro* in presence of *Basella rubra* Deproteinized Concoction used in non-codified medicine for Urinary stones

ABSTRACT

The solubility of calcium phosphate crystallization *in vitro* by an deproteinized concoction of *Basella rubra* plant was investigated by weight reduction assay. Soluble oxalate of the plant are partially removed prior to extraction of plant material. The weight variation and dissolution results reveals that the concoction showed considerable increased activity at the highest concentration used. The results indicate that the calcium phosphate crystal inhibition activity exhibited by the plant material may be due to their calcium phosphate solubilising capacity of phytoconstituents present in them. Regular consumption of an concoction of our plant would be helpful in calculi prophylaxis.

Keywords: Calculi, *Basella rubra*, concoction, plant organs, phosphate inhibition.

1. INTRODUCTION

Urinary stones are solid masses composed of a collection of small crystals (Figure 1) which are formed and present in the urinary tract, due to the agglomeration of some components in the urine under certain physicochemical conditions. Urinary stone disease has a great influence on human health and is a disease with a high likelihood of recurrence (or recurrent at a rate of more than 10% within a year).



Figure 1. Kidney stones

Most urinary stones are formed in kidneys (80%), then migrate in the urine stream to other places of the urinary tract. When a stone appears at any part of the urinary system, it means a urinary stone disease. Thus, urinary stones include kidney stones, ureteral stones, bladder stones and urethral stones.

According to research, among cases of urinary stones in an average, kidney stones account for the highest proportion (40%), ureteral stones account for 28%; bladder stones account for 26% and urethral stones account for 4% (Kirkaly Z *et.al*)

Basella rubra Linn. is a rich source of nutrients and minerals. Per 100 grams (g) edible portion, alugbati leaves contain Water (g) – 92.5; Energy (kcal) – 23.0; Protein (g) – 2.0; Fat (g) – 0.3; Carbohydrates (g) – 3.0; Fiber (g) – 0.9; Ash (g) – 2.2; Calcium (mg) – 128.0; Phosphorous (mg) – 40.0; Iron (mg) – 4.9; Vitamin A (ug) – 456.0; Thiamine (mg) – 0.04; Riboflavin (mg) – 0.12; Niacin (mg) – 0.5; Ascorbic acid (mg) – 89.027. It also contains calcium 2.32, potassium 5.8, magnesium 0.06, sodium 5.11, iron 0.04mg/100gm. (Nirmala S, Saroja G, Gayathri Devi). Diuretic, Wound healing, Antimicrobial (Cai-Xia Dong *et al.*), Antiviral (Nisarath Siriwatanametanon *et al.*). Anticancer (Sandopu Sravan Kumar, Richa Shrivastava, Mausumi Bharadwaj). Antiulcer, Analgesic, Anti inflammatory, CNS depressant, Hepatoprotective (Shu-Mei Lin *et al.*), Antidiabetic and Antioxidant activities were reported earlier (Besagarahalli Padmanabh Nandini, Mysore Shankar Singh Sudarshana, Nanjaiah Rajashekar). In the present study, inhibition of calcium phosphate crystals *in vitro* by an deproteinized concoction of *Basella rubra* was investigated to confirm its anti-urolithiatic activity.

2. MATERIALS AND METHODS

2.1 Plant material

In Nilgiris, *Basella rubra* plant is used for treatment of urinary stones. Around 50 ml of aqueous leaf juice with one teaspoon of *Piper nigrum* powder is given before breakfast till stone expulsion or stem pod infusion prepared in half a litre of water and *Piper nigrum* powder for 1 hour is given for 7 days in non codified medicinal practices

2.2 Collection of the plant material

The sample identification and authentication was done by Dr. B Duraiswamy, The Head, Department of Pharmacognosy and Phytopharmacy, JSS College of Pharmacy, Ooty (JSSCPO) and the voucher specimen with accession number Pharmacog./1055 was deposited at the Herbarium of JSSCPO. Leaves were segregated completely from the stem and pod and all the parts were refrigerated separately at 4°C.

2.3 Preparation of deproteinized concoction

Stem and pod (50 g) which were refrigerated one day prior to extraction was stirred with distilled water (500 ml) using a electric stirrer. The aqueous extract which contains calcium oxalate was gathered and disposed of. The plant marc was subject to aqueous extraction again and again to collect and dispose of the filtrate containing calcium oxalate for the subsequent times. The method was repeated until the plant juice turns out to be clear (Al-Wahsh *et al*). The materials were then shade dried, pulverized, passed through a 60 mesh and used further for the extraction. The same procedure was followed for isolation of calcium oxalate crystals from *Basella rubra* leaves.

A hydroalcoholic concoction of the decalcified leaf, stem and pod were prepared using 70% ethanol by maceration for five days. Prior to preparation of concoction, the aqueous extracts of plant materials were detannated by dropwise addition of lead acetate, until all tannins were precipitated. The excess lead was removed by treating with sulphuric acid. The extract was further deproteinized by addition of saturated ammonium sulphate. The hydroalcoholic concoction was centrifuged and the supernatant was considered as the detannated and deproteinized concoction. The concentrated concoction was obtained by evaporating the solvent

using a rotary evaporator. The concoction obtained was dried completely to constant weight at room temperature (Sweta *et al*).

2.4 Preliminary Phytochemical Screening (Boxi *et al*).

Carbohydrates tests

2 ml of concoction was warmed with Barfoed's reagent (2ml), monosaccharide is present if red cupric oxide forms and disaccharides on prolong heating causes reduction owing to hydrolysis to monosaccharides.

Fehling test:

Equal volume concoction was boiled separately with dilute hydrochloric acid over the flame in a test tube. This solution was neutralized by adding 3 ml of NaOH, added equal volume of Fehling' A and B (2ml) and boiled (reduction due to galactose or other reducing sugar).

Yellow colour turning to brick red precipitate (cuprous oxide) was considered as the positive result for reducing sugars.

Molisch test:

To the concoction added 3ml Molisch reagent with shaking followed by conc. sulphuric acid to observe violet ring in the test tube between the two liquids which was considered to be carbohydrate.

Benedict's test

The concoction was treated with Benedict's reagent and boiled to observe red colour to confirm carbohydrates.

Test for alkaloids

Few grams of the concoction was mixed with 1ml of Potassium bismuth iodide solution (Dragendorff's reagent) separately in a test tube. Red to brown precipitate formation was considered to have alkaloids.

Mayer's test:

Mayer's reagent mixed with the concoction gives cream colour precipitate to confirm alkaloids.

Hager's test:

Picric acid also known as Hager's reagent mixed with the concoction to observe cream colour precipitate and to confirm if alkaloids present.

Wagner's test:

Wagner's reagent treated with the concoction to observe brown precipitate which indicates alkaloids.

Test for proteins

Biuret test

To 2-3 ml of concoction, few drops of 5% sodium hydroxide and 1% copper sulphate solutions were added. Violet or pink colour indicates the presence of proteins

Test for steroids and triterpenoids

Liebermann–Burchard reaction

To 4-5 ml of the concoction, 2ml each of acetic anhydride and concentrated sulphuric acid were added and the colour from violet to blue was considered to confirm steroids

Salkowski's Test.

The concoction was treated with conc. sulphuric acid where red shading at lower layer shows steroids and presence of yellow shading demonstrates triterpenoids.

To 2 ml of chloroform and 3 ml of concentrated sulphuric acid added to the concoction and appearance of red brown ring indicates terpenes

Test for glycosides

Keller-Killiani Test

The concoction was dissolved in chloroform and then the chloroform layer was evaporated. Added glacial acetic acid containing few drops of ferric chloride followed by addition of conc. sulphuric acid. Blue colour appears in the acetic acid layer to confirm glycosides.

Legal test

2 ml of pyridine and alkaline sodium nitroprusside solutions was treated with the extracts and concoction in the test tubes separately and then added NaOH solution. Red to pink colour appear to indicate glycosides.

Baljet test

To the extracts and the concoction sodium picrate were added and presence of yellow to orange colour were considered for glycosides.

Borntrager's test

1ml organic solvent (benzene) was added to the test residue and upon addition of diluted ammonia, pink to red colour was formed due to the presence of glycoside.

Test for Saponins

Frothing test

Few grams of the concoction was dissolved in distilled water separately and frothing which persists was taken as evidence for saponins

Test for flavonoids

To 2-3 ml of the concoction, concentrated hydrochloric acid (3ml) was added along the sides of the test tube. Red colour appear within less than 1 minute was considered as flavonoids present.

Test for Phenolic compounds

Ferric chloride test

Formation of green or blue colour was considered for phenolic compounds (tannins) for condensed and hydrolysable tannins when 5% w/v ferric chloride solution was mixed with the concoction.

Lead acetate test

The concoction was mixed with basic lead acetate (10% w/v) and formation of flocculent white precipitate indicates tannins.

Potassium dichomate test

A solution of potassium dichromate with the concoction gives dark brown colour was considered for the presence of phenolic compounds.

3. *In vitro* Calcium phosphate crystal solubility potential

3.1 Semipermeable membrane preparation

The semi-permeable membrane was prepared by placing egg in 2M HCl for one night to cause decalcification of egg shell (Figure 2a & 2b). Then the apex of the egg was punctured to squeeze out the entire content and the outer semipermeable membrane was collected (Figure 3). The egg membrane was washed with ammonia followed by distilled water for neutralization of acid traces and stored in refrigerator in Tris buffer maintaining a pH 7-7.4 in the moistened condition (Rohan et. al).



Fig 2 (a) & (b). Process of Decalcification of egg

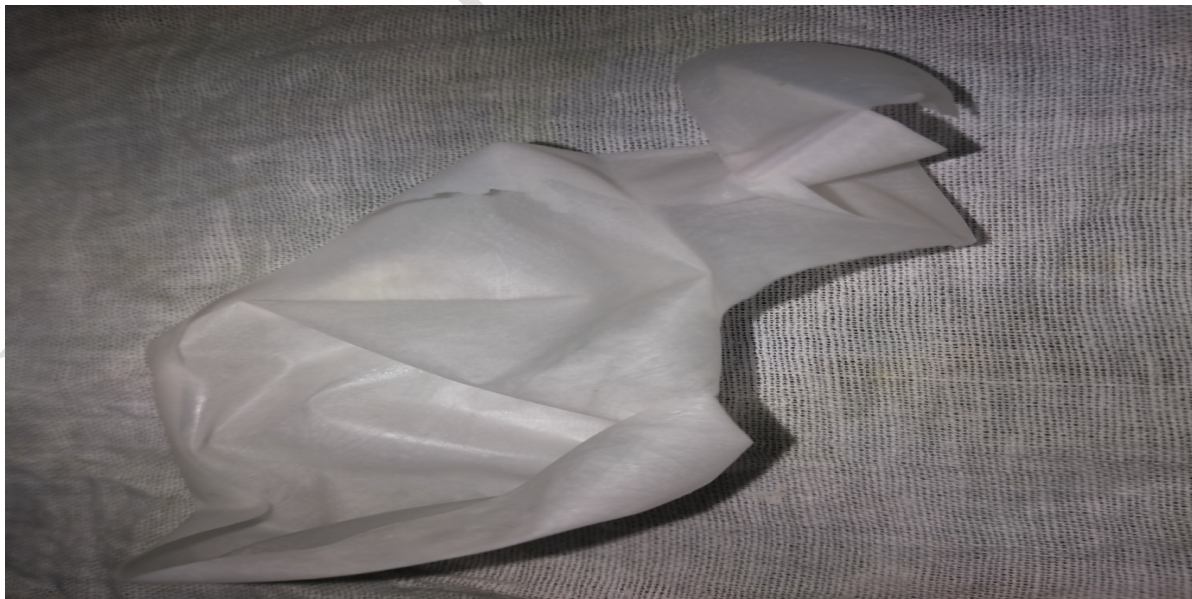


Fig 3. Prepared Semipermeable membrane from egg

3.2 *Invitro* Calcium phosphate synthesis

1.47g of calcium chloride dihydrate was dissolved in 100 ml distilled water and 1.42 gm of disodium hydrogen phosphate was dissolved in 100 ml of 2N H₂SO₄. Both were mixed equally in a beaker to precipitate out calcium phosphate with stirring. The resultant calcium phosphate (Figure 4) was freed from traces of sulfuric acid by ammonia solution, washed with distilled water and dried at a temperature 60° C for 2 hours (Chaudhary et.al)

Then the synthesized calcium phosphate stone were punched to tablets (Figure 5) and these tablets were considered as stones in the study.



Fig 4. Calcium phosphate crystals

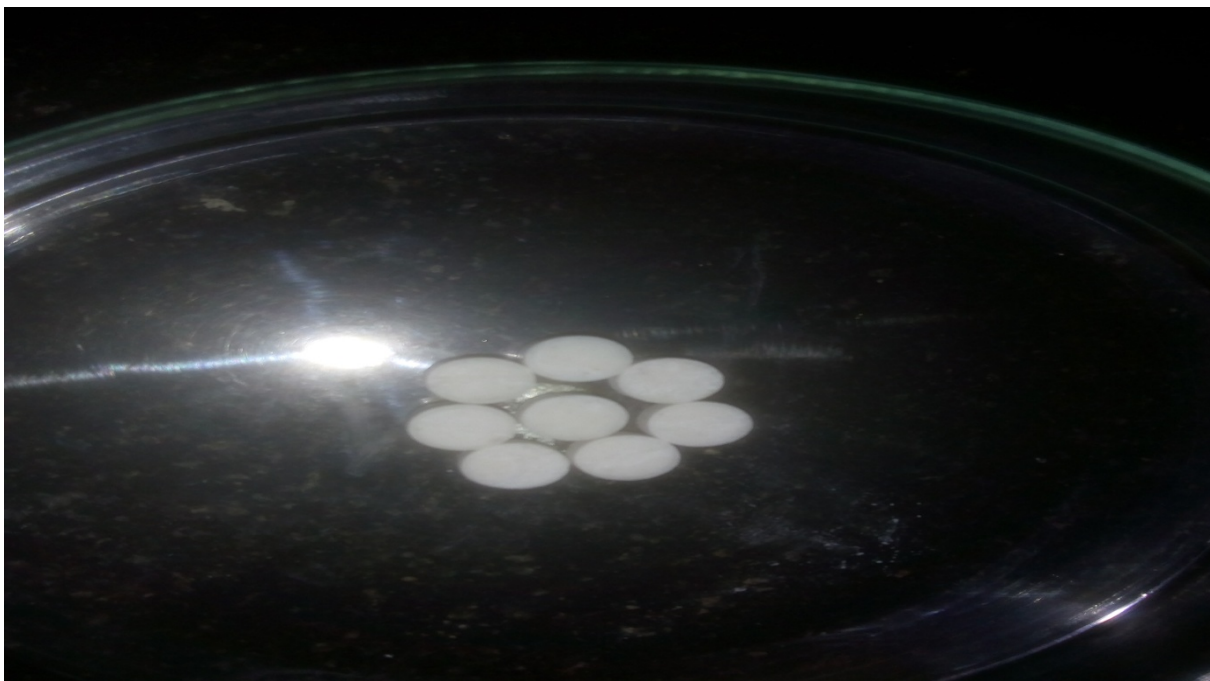


Fig 5. Calcium phosphate tablets

3.2.1 Solubility effect on calcium phosphate

In vitro activity of the concoction has been evaluated by weight variation and dissolution of prepared calcium phosphate tablets. Exactly weighed calcium phosphate tablets were placed with 5 ml of concoction prepared in DMSO solution (50 mg/ml and 100 mg/ml concentrations of each) and packed in the semipermeable bag separately and sutured. Calculax was also prepared in DMSO and used as standard. The semi permeable bags were allowed to suspend in 100 ml tris buffer (0.1 M) separately in conical flasks. Similarly, negative control (one exactly weighed calcium phosphate tablet) has been placed in the semi-permeable bag with 10 ml of water. Each sample treatment was carried out twice and average was taken. All the flasks were subjected for incubation at $37 \pm 10^\circ \text{C}$ for 5 weeks. For each bag, the weight loss of calcium phosphate tablet at an interval of two weeks have been evaluated after incubation and complete drying in an oven for 5 hours at 40°C . The calcium phosphate dissolution was estimated by calculating the initial weight and final weight of the tablets using the formula

$$\% \text{ Dissolution} = (W \text{ initial} - W \text{ final}) \times 100 / W \text{ initial}$$

W – weights of calcium phosphate tablets before and after the incubation with the concoction (Yachi et.al)

Statistical analysis

Values are expressed as mean \pm SD. Statistical analysis was done by one way analysis of variation (ANOVA) followed by Dunnett's test

3. RESULTS AND DISCUSSION

Phytochemical screening showed the existence of many phytoconstituents including flavonoids and saponins in the concoction and steroids were found to be absent (Table 1). Results revealed that the concoction showed considerable reduction in weight of calcium phosphate tablet at a regular interval of two weeks during five weeks (Tablet 2). Standard was found to have the

highest % solubility of the tablet at both the concentrations, followed by maximum dissolution of concoction after five weeks (Table 3). The concoction has shown slow reduction after first week at both the concentrations but the rate of reduction was more after an interval of two weeks. The solubility in grams and the percentage dissolution at both the concentrations were summarized. Anti urolithiatic effects using *in vitro* model showed that, the concoction of *Basella rubra* exhibited prominent Lithiolytic activity. The percentage dissolution of calcium phosphate tablet by concoction was 41.36% at 50 mg and 42.53% at 100 mg. The highest percentage dissolution of calcium phosphate crystals was observed by standard at 100 mg concentration which was 48.92% followed by concoction. It was found that increasing the concentration of plant extracts resulted in the increase in percentage inhibition of calcium phosphate crystallization.

Table 1 : Phytochemical screening of concoction of *Basella rubra*

CONSTITUENTS	TEST	CONCOCTION
ALKALOIDS	WAGNER'S TEST	+
	MAYER'S TEST	-
	HAGER'S TEST	+
	DRAGENDORFF'S	+
CARBOHYDRATES	MOLISCH'S TEST	+
	BENEDICT'S TEST	+
	FEHLING'S TEST	+
	BARFOED'S TEST	+
PHENOLS & TANNINS	FERRIC CHLORIDE	-
	POTASSIUM DICHROMATE	-
	LEAD ACETATE TEST	-
GLYCOSIDES	BALJET TEST	-
	KELLER KILLIANI	-
	LEGAL TEST	-
PROTEINS	BIURET TEST	-
FLAVONOIDS	HCL TEST	+
SAPONINS	FOAM TEST	+

STEROIDS & TERPENES	LIBERMANN TEST	-
	SALKOWSKI TEST	-

(+) indicates presence and (-) indicates absence of phytochemicals

Table 2: Weights of calcium phosphate tablets before and after treatment

S.No	Extracts	Concentration	Initial wt of CaP (g)	Wt of CaP (g) after one week	Wt of CaP(g) after three weeks	Wt of CaP(g) after five weeks	Reduction in wt after five weeks (g)
1	Std calculax	50 mg	0.1425±0.003	0.1029±0.004*	0.0900±0.002*	0.0777±0.003*	0.0675
		100 mg	0.1445±0.002	0.0996±0.002*	0.0825±0.003*	0.0738±0.003*	0.0707
2	Negative Control	50 mg	0.1436±0.0042	0.1256±0.001	0.1225±0.003	0.1219±0.004	0.0217
		100 mg	0.1471±0.002	0.1270±0.002	0.1265±0.002	0.1260±0.002	0.0211
3	Concoction	50 mg	0.14310±0.004	0.1209±0.004*	0.0986±0.002*	0.0839±0.003*	0.0592
		100 mg	0.1453±0.002	0.1101.1±0.001*	0.0985±0.004*	0.08350±0.003*	0.0618

Values are expressed as mean ± SD. Statistical analysis was done by one way analysis of variation (ANOVA) followed by Dunnett's test. n=2; *P<0.05 was considered significant. All groups were compared with control.

Table 3 : % Solubility of calcium phosphate tablet during treatment

S.No	Extract	Concentration	% Solubility after one week	% Solubility after three weeks	% Solubility after five weeks
1	Std calculax	50 mg	27.7894	36.8421	45.4736
		100 mg	31.0726	42.9065	48.9273
2	Negative Control	50 mg	12.5348	14.6935	15.114
		100 mg	13.0726	14.0040	14.3439
3	Concoction	50 mg	15.5136	31.0971	41.3696
		100 mg	24.2257	32.2092	42.5326

In phytochemical screening, the hydroalcoholic concoction gave a positive result with the Wagner's, Hager's and Dragendorff's which indicated the presence of alkaloids. All the tests

confirmed the presence of carbohydrates in the concoction. The Frothing test confirmed the presence of saponins in the concoction. No glycosides and terpenes were identified in the concoction tested by the tests. The ferric chloride test for phenolic compounds and test for tannins compounds gave negative results with the concoction. Biuret test gave negative result, which indicates the absence of proteins in the concoction.

Since nucleation is the first step in stone formation, our concoction inhibited the stone proving anti nucleation properties by disintegrating into small particles. The present study has given primary evidence for *Basella rubra* plant used for kidney stone in traditional medicine

The weight variation and dissolution results reveals that the concoction showed increased activity at the highest concentration used. The results indicate that the *in vitro* anti urolithiatic activity exhibited by the plant material may be due to their calcium phosphate solubilising capacity of phytoconstituents present in them.

4. CONCLUSION

Several phytochemicals like flavonoids and saponins are reported to be responsible for anti urolithiatic effect by either inhibiting the formation of calcium phosphate crystals, preventing their attachment to renal cells or by their calcium channel blocking activity (Agarwal et.al). It was reported that the plants containing saponins as chemical constituents has high possibility to possess solubility of calcium phosphate crystallization which works towards Urolithiasis (Saha et.al).

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