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Article

Inhibition of calculi forming oxalate by dietary *Basella rubra* organs: Litholytic activity

Roan Ngoc Anh Huy[1], Ramu Govindan[1*], Navanita Sivaramakumar[1], Rajesh Raman[2], Saravanan Jayaraman[3], Duraiswamy Basavan[1], Dhanabal S Palanisamy[1]

[1]Department of Pharmacognosy, JSS College of Pharmacy, JSS Academy of Higher Education & Research, Ooty, Nilgiris, Tamilnadu, India, [2]Department of Pharmaceutical Biotechnology, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Ooty, Nilgiris, Tamilnadu, India, [3]Department of Pharmacology, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Ooty, Nilgiris, Tamilnadu, India

The inhibition of calculi forming oxalate by dietary *Basella rubra* plant organs leaf and stem pod has been investigated. The weight reduction assay was studied. Also a concoction of the plant organs was tested. Leaf extract was found with considerable activity whereas the concoction seems to be not much active as the stem pod extract. Soluble oxalate of the plant organs are partially removed prior to extraction of active constituents. The active component/s seem to be a non-protein and non-tannin molecule/s that may act through inhibition of calcium accumulation there by proving the positive activity against the calculi or kidney stone. Regular consumption of leaf and stem pod extracts of our plant would be helpful in calculi prophylaxis.

Keywords: Calculi. Litholytic.*Basella rubra.*Plant organs.

INTRODUCTION

Calculi (urinary stone) is a common disease in the world. Urinary stone disease has a great influence on human health and is a disease with a high likelihood of recurrence of more than 10% within a year. In India, about 12% of the population has Urolithiasis, of which 50% may suffer kidney loss or kidney damage. The incidence of Urolithiasis is prevalent in the North of India (nearly 15% of the population), while this disease is less commonly found in the South. (Haneefa*et al.,*2012). People who live in dry hot climate and rocky areas are more likely to develop stones. (Alelign, Petros, 2018). Dietary changes can also be the major drivers of this trend. (Romero, Akpinar, Assimos, 2010). The incidence of urinary stones is related to age, gender, race, geographical environment and eating habits. (Ferraro *et al*, 2012). In India, calcium oxalate stones are seen as the most significant part of Urolithiasis. Calcium oxalate stones make up 70-80% of the examined stones. Calcium phosphate stones represent 15-30%, while 15-20% is blended stones. The others are struvite with 15-30%, cystine with 6-10%, and uric acid stones with 2-10%. (Alkhunaizi, 2016). Epidemiology reveals occurrence in men (12%) and in women (6%) between the ages of 20 and 40.

Therefore recent scenario demonstrates that there is a need of natural herbal preparation to treat the urinary stonewhich is increasingly predominant. High-power laser techniques, Surgery and Lithotripsy are the most widely used treatment to eliminate calculi but the system is more expensive and the repeat is also common. There are no engineered medications which hinder the development of kidney stone in the conceivable age gatherings. So there is a need of new preparation which removes or eliminates kidney stone without surgery.

***Correspondence:** R. Govindan. Department of Pharmacognosy. JSS College of Pharmacy, Ooty. JSS Academy of Higher Education &Research, Mysuru. Phone: 0423 - 2443847. E mail: ramupharmu@jssuni.edu.in. ORCID: https://orcid.org/0000-0002-0572-9517

Basella rubra Linn, is a glabrous herb with fleshy twinning stems often tinged with red colour. Leaves alternate, petioled, broadly ovate to orbicular, entire, base often cordate, shining. 2-7 in diameter, narrowed into the petioles. Spikes - 1-6 in axillary, peduncled, simple or branched; flowers - spicale, sessible, 2-sexual, red in cymose clusters. Fruit of the utricle size of a small pea included within the fleshy perianth which is shining black with reddish juice. Seed - white, erect, subglobose, testa crustaceous, albumen scanty, embryo piano-spiral, cotyledons large thin involute. Fresh young leaves of this highly nutritious plant can be used in salads and older leaves may be cooked with dhal, prawn, meat, chicken and soups. It is cooked with fish in Bengali cuisine, and in stir fries and soups in Chinese or Vietnamese food. *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, Saroja, Gayathri Devi, 2013).Diuretic, Wound healing, Antimicrobial, Antiviral (Siriwatanametanon *et al*., 2010), Anticancer (Kumar*et al*., 2015), Antiulcer, Analgesic, Anti inflammatory, CNS depressant, Hepatoprotective (Shu-Mei L*et al*., 2010). Antidiabetic and Antioxidant activities were reported earlier(Nandini, Sudarshana, Rajashekar, 2013).The highest oxalic acid concentration commonly occurs in the leaves and lowest in all other parts (Caliskan, 2000). It exerts its effects by binding calcium (Ca), magnesium (Mg) and other trace minerals such as iron (Fe), making them unavailable for assimilation. The calcium ions bind with free oxalic acid/oxalate and precipitates as insoluble crystals of calcium oxalate which may lead to hypocalcaemia and urolithiasis. In human being, it is considered that <0.5% soluble oxalate in a diet may be acceptable. Plants accumulate oxalate in high proportion only during the young stage of growing and the content decreases with maturity and drying of the plant. Matured plant organs of *Basella rubra* contains very low amounts of soluble oxalate (Franceschi, Nakata, 2005; Stamatelou *et al*., 2003) and were used traditionally in treating kidney stones.In these views, the present study aimed to investigate the dietary and matured *Basella rubra* plant organs such as leaf, stem pod and its concoction which have considerable calcium and poor in soluble oxalate for its Anti-urolithiatic or Litholytic activity by inhibition of calculi forming calcium oxalate crystallization.

MATERIAL AND METHODS

Plant materials and Preparation of Extracts

Basella rubra at an matured stage of growing was procured from the local region of Ooty and authenticated by Dr. B Duraiswamy, following identification a voucher specimen of the plant with accession number Pharmacog./1055 was deposited in the Herbarium of JSS College of Pharmacy, Ooty (JSSCPO). Leaves were then segregated completely from the stem and pod and refrigerated separately one day prior to extraction at 4°C. The extracts used in experiment were

1. An aqueous macerated extracts of the fresh matured leaves and stem pod separately and removal and determination of water soluble oxalate with potassium permanganate.
2. An hydroalcohol macerated extract of dried and powdered leaf marc after removal of oxalate.
3. An hydroalcohol macerated extract of dried and powdered stem pod marc after removal of oxalate
4. An concoction of decalcified and hydroalcohol extract of the dried powdered leaves and stem pod after removal of tannins and proteins.

The standard method (da Costa*et al*., 2009) was primarily carried out for the extraction and removal of water soluble oxalate from fresh plant materials. The later stage of the identification of oxalate removed was carried out by titration with potassium permanganate (Moir, 1953). The hydroalcoholic extracts were prepared from the well dried marc. Leaves and stem pod were subjected to extraction with 70% ethanol separately by maceration for five days. The concentrated extract was

obtained by evaporating the solvent. The same procedure was followed for preparing concoction where powdered leaves and stem pod were mixed together in equal proportion but after detannation and deproteinization of the plant materials. The aqueous macerated concoction was detannated by dropwise addition of lead acetate, until all tannins were precipitated. The excess lead was removed by treating with sulphuric acid. The concoction was further deproteinized by addition of saturated ammonium sulphate. The 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 (Jha*et al*., 2016; Mittal *et al*., 2015).

Phytochemical Analysis

The preliminary phytochemical screening was performed by standard methods (Boxi M *et al*., 2010; Obianime, Uche, 2008; Adegoke *et al*., 2010; Sahid-ud-daula, Basher, 2009). Scavenging activity of hydrogen peroxideby the extracts and concoction were determined by reported method (Ruch, Cheng, Klaunig, 1998). The effects of extracts and concoction on DPPH radicals were measured by standard method (YehLin*et al*., 2012) with minor modifications

Litholytic activities: Preparation of semi-permeable membrane

The semi-permeable membrane was prepared by placing egg in 2M Hcl for one night to cause decalcification of egg shell (Figure 1a & 1b). Then the apex of the egg was punctured to squeeze out the entire content and the outer semipermeable membrane was collected (Figure 2). 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 (Figure 3). (Makwana, Devkar, Setty, 2017; Varicola *et al*., 2018; Niharika *et al*., 2018)

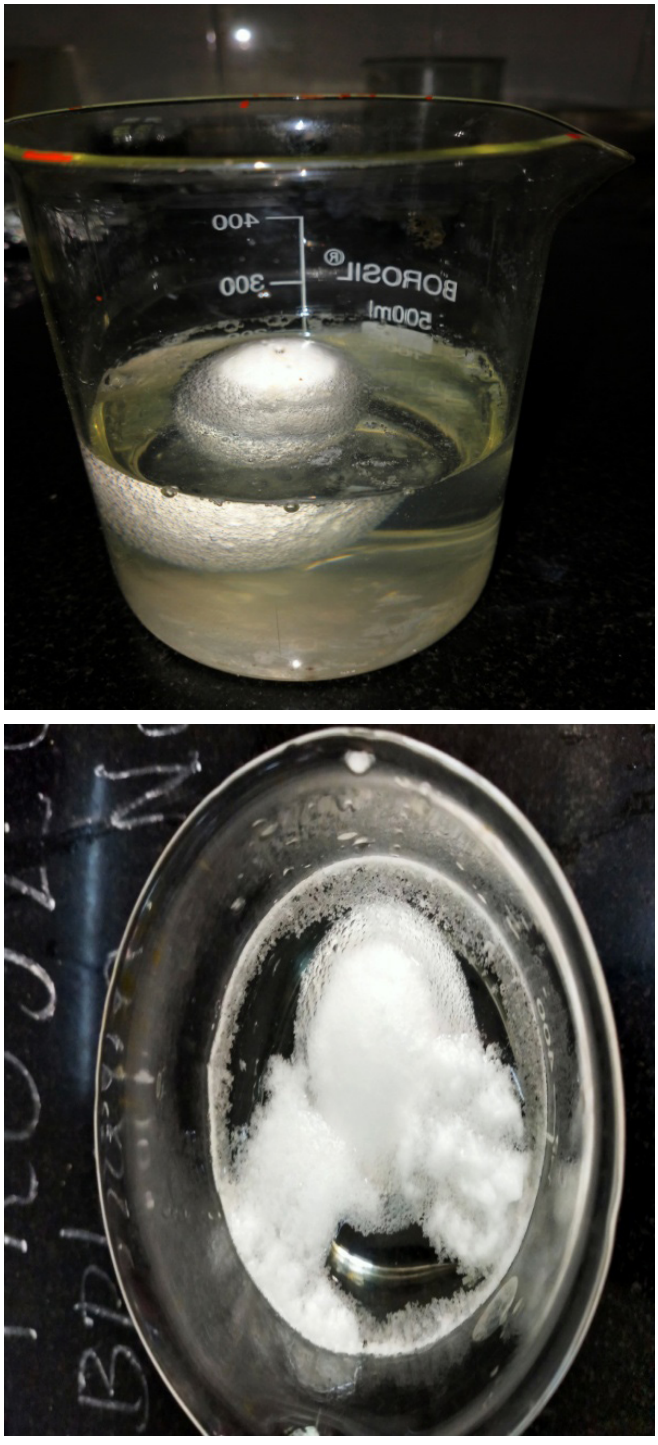

FIGURE 1(a) & 1(b) - Process of Decalcification of egg.

FIGURE 2 - Semipermeable membrane.

FIGURE 3 - Membrane in buffer.

Invitro Calcium oxalate synthesis

A 20 ml of buffer solution of pH 7 (0.66M sodium acetate solution added by a little quantity of acetic acid) and 40 ml of water was allowed to react with 20 ml of 0.05 M oxalic acid in a beaker. Sodium citrate 1.5 g was added to the solution and heated at 80º C. To the mixed solution, 20 ml of 0.05 M calcium solution was added drop by drop with continuous stirring. The resulting white calcium oxalate precipitate (Figure 3) was neutralized by washing with ammonia solution followed by distilled water and then dried completely at 40°C (Aggarwal *et al*., 2012).The characteristic functional groups present in oxalate crystals were analyzed using Fourier Transform-Infra Red (FTIR) spectroscopy using Spectrometer. The sample was mixed with KBr (binding agent) and made into discs at high pressure using a hydraulic press. The disc was scanned in the range of 400-4000 cm to obtain a FTIR spectrum. Then the synthesized calcium oxalate crystals were punched to tabletsand also confirmed by FTIR analysis (Benramdane*et.al*., 2008). These tablets were considered as stones in the study.

Inhibition of calcium oxalate crystallization

Inhibitory activity *in vitro* of the crude extracts and the concoction has been evaluated by weight variation and dissolution of prepared calcium oxalate tablets. Exactly weighed calcium oxalate tablets were placed with 5 ml of extracts and 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 oxalate 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 ± 10º C for 5 weeks. For each bag, the weight loss of calcium oxalate tablet at an interval of two weeks have been evaluated after incubation and complete drying in an oven for 5 hours at 40º C. The calcium oxalate dissolution was estimated by calculating the initial weight and final weight of the tablets using the formula % Dissolution = (W initial – W final) × 100 / W initial where, W – weights of calcium oxalate tablets before and after the incubation with the extracts and concoction (Yachi *et al*., 2018; Suresh *et al*., 2018).

Statistical analysis

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

RESULTS AND DISCUSSION

Extraction and Phytochemical Analysis

The hydroaloholic extracts were prepared from the decalcified leaves and stem pod (Figures 4 & 5) of *Basella rubra* by maceration method. An attempt is also made to prepare a detannated, deproteinized and decalcified extract from the concoction of the plant materials. Phytochemical screening showed the existence of many phytoconstituents including polyphenols, flavonoids and saponins in all the extracts and concoction and steroids were found to be absent (Table I). DPPH and Hydrogen peroxide methods were carried out for all the three extracts to know free radical scavenging properties. In the DPPH method, the results shows that leaf of *Basella rubra* had the highest antioxidant activity with IC50 value of 54.2±3.11 µg/ml followed by concoction (180.1±1.10 µg/ml) and stem pod (286.8±0.29 µg/ml) but significantly lower than the value obtained for the standard ascorbic acid,(40.50±0.30 µg/ml). Hydrogen peroxide is a reactive oxygen species generated *in vivo* by oxidase enzyme like superoxide dismutase. It is a strong oxidizing agent but either directly or indirectly via its reduction product hydroxyl radical causes severe damage to biological systems. Hydrogen Peroxide radical scavenging assay results also indicates that the leaf extract had a highest antioxidant activity by inhibiting H_2O_2 radicals with IC50 value of 28.19±0.17 µg/ml followed by concoction (60.3±2.31 µg/ml**)** and stem pod (118.3±1.17 µg/ml) but significantly lower than the value obtained for the standard rutin, (15.60±1.51 µg/ml**).** The results are shown in figure 6.

FIGURE 4 – *Basella rubra* leaf.

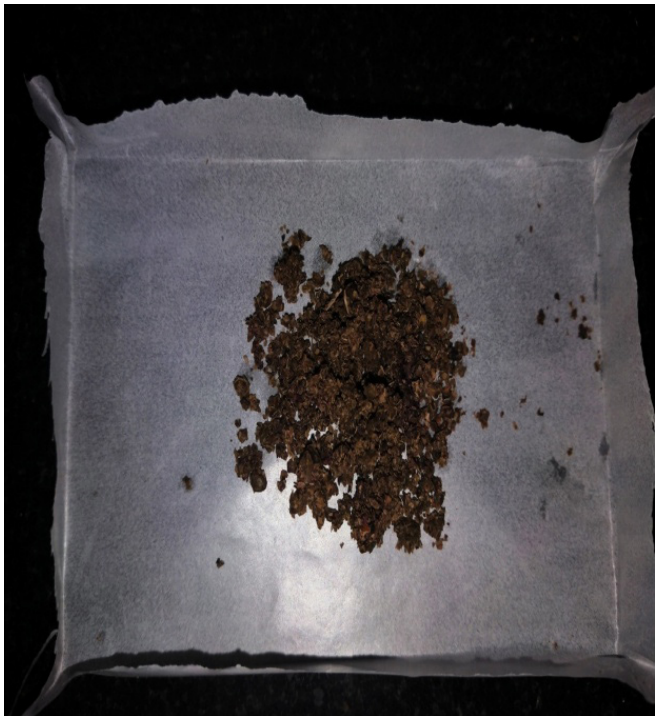

FIGURE 5 - *Basella rubra* stem pod.

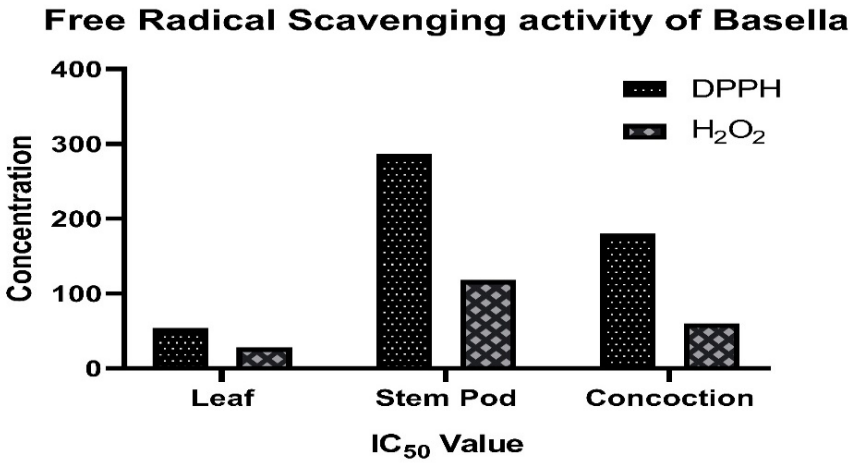

FIGURE 6 - Antioxidant activities of *Basella rubra*.

TABLE I - Phytochemical screening of extracts of *Basella rubra*

CONSTITUENTS	TEST	LEAF	STEM POD	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 CHL0RIDE	+	+	-
	POTASSIUM DICHROMATE	-	-	-
	LEAD ACETATE TEST	+	+	-
GLYCOSIDES	BALJET TEST	-	+	-
	KELLER KILLIANI	-	-	-
	LEGAL TEST	-	-	-
PROTEINS	BIURET TEST	-	-	-
	BIURET TEST	+	+	-
SAPONINS	FOAM TEST	+	+	+
STEROIDS & TERPENOIDS	LIBERMANN TEST	-	-	-

(+) indicates presence and (–) indicates absence of phytochemicals.

Litholytic activities of *Basella rubra* plant organs

In vitro Calcium oxalate synthesis

The calcium oxalate crystals were synthesized in vitro (Figure 7 & 8) and confirmed by FTIR technique (Figure 9 & 10). In FTIR spectrum of calcium oxalate crystals, the band at 3410 cm−1 is related to the stretching vibration of OH group of coordinated water molecules. The band at 1614 cm−1 is related to the stretching frequency of phenolic C=O group connected to calcium ion. An absorbance at 889 cm−1 characterizes the stretching vibration of C-C, which verify calcium oxalate crystals. In FTIR spectrum of calcium oxalate tablets, the band at 3377 cm−1 is related to OH group of coordinated water molecules. The band at 1154 cm−1 is related to the stretching frequency of phenolic C=O group connected to calcium ion. An absorbance at 875 cm−1 characterizes the stretching vibration of C-C, which in general calcium oxalate crystals.

FIGURE 7 - Calcium oxalate crystals.

FIGURE 8 - Calcium oxalate tablets.

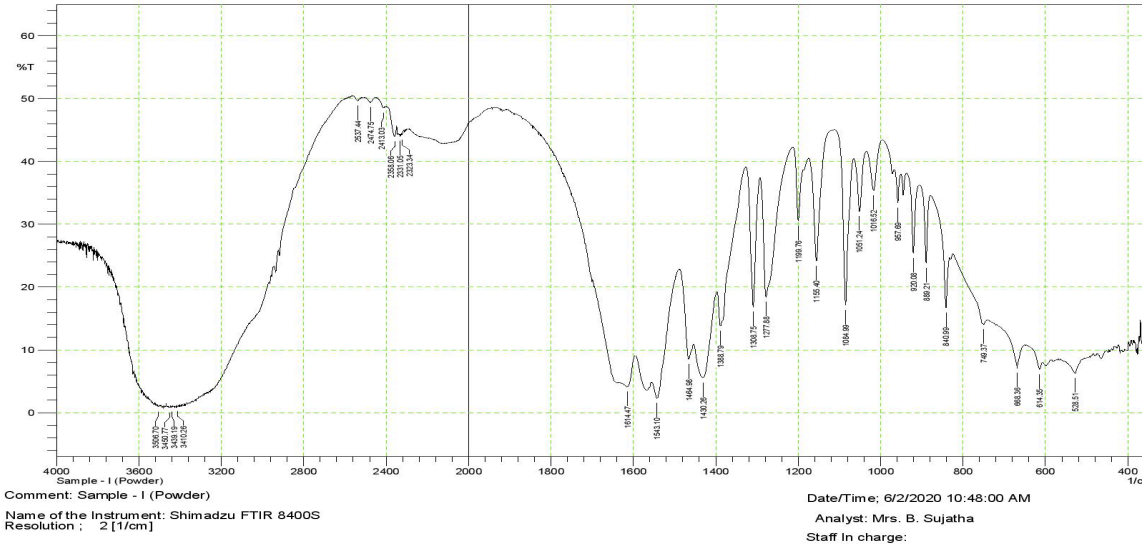

FIGURE 9 - Fourier-transform infrared spectrum of calcium oxalate crystals.

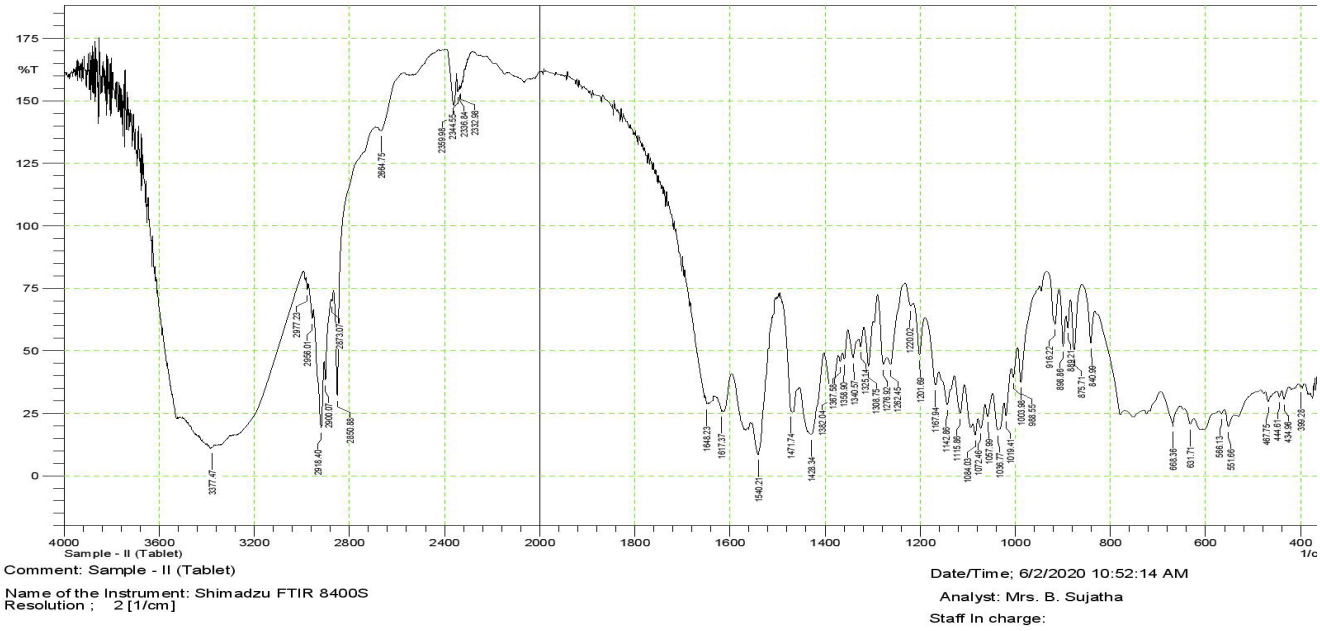

FIGURE 10 - Fourier-transform infrared spectrum of calcium oxalate tablets.

Inhibition of calcium oxalate crystallization

Results revealed that the extracts and the concoction showed considerable reduction in weight of calcium oxalate tablet at a regular interval of two weeks during five weeks (Table II). Stem pod extract was found to have the highest % solubility of the tablet at both the concentrations, followed by the maximum dissolution of concoction and leaf extract after five weeks (Table III). Concoction has shown good reduction after the first week and three weeks at 50 mg concentration but the rate of reduction was slow than stem pod after five weeks. At 100 mg concentration, the stem pod extract has shown good reduction after five weeks. The rate of solubility of the tablet was more in concoction followed by stem pod and leaf at 50 mg after three weeks while it was more in stem pod followed by concoction after five weeks. The dissolution percentage is higher in stem pod extract followed by concoction and leaf after five weeks at both the concentrations used. The solubility in grams and the percentage solubility at regular intervals at both the concentrations were summarized.

TABLE II - Weight of calcium oxalate tablet before and after treatment

S.No	Extract	Concentration	Initial wt of CaOxT (g)	Wt of CaOxT (g) after one week	Wt of CaOxT (g) after three weeks	Wt of CaOxT (g) after five weeks	Reduction in wt after five weeks (g)
1	Control	50 mg	0.1552±0.004	0.1326±0.003	0.1300±0.006	0.1224±0.003	0.0328
		100 mg	0.1562±0.004	0.1376±0.003	0.1382±0.006	0.1381±0.004	0.0181
2	Standard	50 mg	0.1542±0.001	0.1053 ±0.003*	0.0617 ±0.007*	0.0177 ±0.002*	0.1365
		100 mg	0.1569±0.003	0.0657 ±0.006*	0.0542 ±0.005*	0.0136 ±0.005*	0.1433
3	Leaf	50 mg	0.1544 ±0.002	0.1031±0.003*	0.0854±0.006*	0.0735 ±0.001*	0.0809
		100 mg	0.1534 ±0.002	0.0962±0.002*	0.0750±0.004*	0.044 ±0.003*	0.1094

TABLE II - Weight of calcium oxalate tablet before and after treatment

S.No	Extract	Concentration	Initial wt of CaOxT (g)	Wt of CaOxT (g) after one week	Wt of CaOxT (g) after three weeks	Wt of CaOxT (g) after five weeks	Reduction in wt after five weeks (g)
4	Stem pod	50 mg	0.1532±0.004	0.1055 ±0.005*	0.0701±0.003*	0.0389±0.001*	0.1143
		100 mg	0.1517±0.003	0.0863 ±0.004*	0.0640±0.005*	0.0254±0.005*	0.1263
5	Concoction	50 mg	0.1552 ±0.004	0.0943 ±0.005*	0.0675 ±0.004*	0.0489 ±0.001*	0.1063
		100 mg	0.1562 ±0.004	0.0912 ±0.001*	0.0650 ±0.005*	0.036 ±0.0031*	0.1202

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

TABLE III - % Solubility of calcium oxalate tablet during treatment

S.No	Extract	Concentration	% Solubility after one week	% Solubility after three weeks	% Solubility after five weeks
1	Control	50 mg	14.56	16.237	21.134
		100 mg	11.907	11.523	11.587
2	Standard	50 mg	31.712	59.987	88.521
		100 mg	59.143	65.455	91.332
3	Leaf	50 mg	33.225	44.689	52.396
		100 mg	37.288	51.108	71.316
4	Stem pod	50 mg	31.135	54.242	74.608
		100 mg	43.111	57.811	83.256
5	Concoction	50 mg	39.239	56.507	68.492
		100 mg	41.613	58.386	76.952

Anti-urolithiatic effect of the extracts and concoction using *in vitro* model showed that, the stem pod extract of *Basella rubra* exhibited prominent Lithiolytic activity. The percentage dissolution of calcium oxalate tablet by stem pod extract was 74.60% at 50 mg and 83.25% at 100 mg, similarly concoction also showed marginal *anti urolithiatic* activity with percentage dissolution of68.49% to 76.952% at 50 mg and 100mg concentration followed by leaf extract with percentage dissolution of 52.39% to 71.31% at 50 mg and 100 mg concentration respectively. The highest percentage of oxalate tablet dissolution was observed by stem pod extract at the highest concentration 100 mg which was 83.25%which is near potent value to standard i.e. 88.52%.It was found that increasing the concentration of plant extracts resulted in the increase in percentage inhibition of calcium oxalate crystallization.

In Nilgiris, *Basella rubra* is used for the 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. Similar methods of traditional practices were also identified in literature (Prachi *et al*., 2009; Gaur, Sharma, Painuli, 2010). From phytochemical screening, we observed that the hydroalcoholic extract gave a positive result with the Molish test for all the extracts and the Barfoed's test for stem pod which indicated the presence of monosaccharides and carbohydrates. The Fehling and Benedict's test confirmed the presence of carbohydrates in all the extracts. The Dragendorff's reagent showed the presence of alkaloids in all the extracts. The Hagers test gave positive test for alkaloids in the leaf extract and in the concoction whereas Wagner's test confirmed the presence of alkaloids. The Frothing test confirmed the presence of saponins in all the extracts. The Baljet test gave a positive test for glycosides in the stem pod. Terpenes were found to be absent in all the extracts. The ferric chloride test for phenolic compounds gave positive results in both extracts. Test for tannins and phenolic compounds gave positive results with all the extracts. Biuret test gave positive results, which indicates the presence of proteins in the extracts of leaf and stem pod. It was found that the hydroalcoholic extracts and the concoction of *Basella rubra* were capable of scavenging hydrogen peroxide which can be attributed to their phenolic content that donated electrons to hydrogen peroxide thus reducing it to water. All the extracts possess considerable DPPH radical scavenging activities. Phenols are very important plant constituents because of their scavenging activity on free radicals due to their hydroxyl groups. The antioxidant studies carried out also showed good results due to the presence of various chemical constituents present in all the extracts and the concoction.

Since nucleation is the first step in stone formation, our extracts and 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 stem pod extract showed increased activity at the highest concentration used. Whereas concoction and leaf showed nominal activity which can be compared to stem pod extract. The results indicate that the litholytic activity exhibited by all the extracts may be due to their calcium oxalate solubilising capacity of phytoconstituents present in them.

Several phytochemicals like flavonoids, saponins, tannins and glycoside derivatives are reported to be responsible for antiurolithiatic effect by either inhibiting the formation of calcium oxalate crystals, preventing their attachment to renal cells or by their calcium channel blocking activity (Singla *et al*., 2012), Leafy vegetables like *Basella* which contains potassium is a good source in the control of diuretic and hypertensive implications (George, 2003). It was reported that the plants containing saponins and flavonoids as chemical constituents has high possibility to possess solubility of calcium oxalate crystallization which works towards Urolithiasis (Zhong *et al*., 2012). *Basella rubra* contain total flavonoid content of 0.500 g/100g and saponins content of 1.0 g/100g of powder mass (Boham, Koupai-Abyazani, 1974). Thus, flavonoids and saponins of *Basella rubra* may be responsible for their inhibition potential of calculi forming oxalatae. The report of earlier work (Fouada *et al*., 2006) was found to be similar to the present work. The antioxidants might have enhanced the litholytic activities of the extracts tested. But leaf extract did not show much activities than stem pod and concoction even though it has good antioxidant potential than the other extracts. Nonetheless, further studies on isolation and purification of compounds that are responsible for their litholytic and antioxidant activities are needed.

CONCLUSIONS

The study confirmed non-tannin and nonprotein nature of the active ingredient of *Basella rubra* where the detannated and deproteinized concoction also showed good activity. The exact mechanism of action of the plant is not known and the active component/s seem to be a nonprotein, non-tannin molecule/s that may act through inhibition of calcium and phosphate accumulation thereby proving the positive activity against the kidneys stones. The results of our study

clearly indicate the potential of the leaf and stem pod extract before and after removal of the tannins and proteins as therapy for lithiasis.

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