

## *In-vitro* Antiurolithiatic Activity of *Kalanchoe pinnata* Extract

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### ABSTRACT

Background: Upto date, the scientific documentation regarding *in-vivo* antiurolithiatic activity of *Kalanchoe pinnata* has been reported, although it has not reported for *in-vitro* antiurolithiatic activity up till now.

Objective: To explore anti-urolithiatic activities of *Kalanchoe pinnata* leaves extract by utilizing different *in-vitro* models. To investigate the inhibitory effect of extract on *in vitro* crystallization through analyzing nucleation and aggregation assays. Material and Methods: Aqueous extract of *Kalanchoe pinnata* fresh leaves was prepared and arranged in the different concentrations. Homogenous precipitation method was used to prepare artificial stones such as calcium oxalate and calcium phosphate and semi-permeable membrane of eggs was used as dissolution bags. Dissolution models were incubated in 72hrs and after that, the entire content in dissolution bags was estimated spectrophotometrically. The inhibitory activity of *Kalanchoe pinnata* leaves extract on the nucleation of calcium oxalate crystals and the rate of aggregation in calcium oxalate crystals was determined by spectrophotometric assay. Results: In dissolution models, the extract of *Kalanchoe pinnata* has greater capability to dissolve calcium oxalate while Cystone standard has shown better demineralization for calcium phosphate rather than extract of *Kalanchoe pinnata*. Cystone exhibited strongly inhibitory action in the nucleation assay rather than aggregation assay. The extract of *Kalanchoe pinnata* exhibited inhibitory action in both of nucleation and aggregation assays to significant level. Discussion: The present investigation will be supportive to the scientific documentation related *in-vitro* studies. Correlation between *in-vitro* and *in-vivo* studies may be helpful to understand the molecular mechanism of litholysis process and to reveal phytochemicals of the extract responsible for dissolving or disintegrating renal calculi. Conclusion: *Kalanchoe pinnata* extract exhibited significant *in-vitro* anti-urolithiatic activity.

**Keywords:** *Kalanchoe pinnata*, Cystone, Anti-urolithiatic activity

### INTRODUCTION

*Etiology and pathogenesis of kidney stones formation:*

Nowadays stone formation is the oldest and serious painful urologic disease with significant prevalence in the population due to change in lifestyle and dietary factors. Stone formation or lithiasis is characterized by calculi formation. It has two main types such as nephrolithiasis and urolithiasis. Calculi formation in urinary bladder, ureter or any part of urinary tract rather than kidney is known as urolithiasis while nephrolithiasis is characterized calculi formation in kidney<sup>1</sup>. Generally, calcification for the formation of bone and teeth takes place in controlled biological situations. Uncontrolled pathological crystallization occurs when solvent becomes supersaturated leading to the formation of precipitates in the body called as kidney stones<sup>2</sup>.

*Possible mechanism of kidney stone formation in the metabolism:*

Oxalic acid is biosynthesized from ascorbic acid, glycolate and glyoxylate in the metabolism of higher plants. A significant loss of minerals is more prevalent in the body when it is consumed in large content of oxalate rich foods<sup>3</sup>. When calcium ions present in the body bind with free oxalic acid/oxalate it precipitate as insoluble

calcium oxalate crystals and may lead to hypocalcaemia and urolithiasis<sup>4</sup>. Generally kidney stones are comprised of high concentration of calcium oxalate<sup>5</sup> with subsequent minute amount of calcium carbonate, calcium phosphate<sup>3</sup>. The pathogenesis of calcium oxalate stone formation is involved nucleation, crystal growth, crystal aggregation, and crystal retention in multistep process<sup>6</sup>.

*Strategies of dietary supplementation for preventing calcium oxalate stones formation*

Intake of oxalate-rich foods should be limited. Some items are oxalate rich in food such as spinach, rhubarb, beets, nuts, chocolate, tea, wheat bran, and strawberries which have been shown influence for raising oxalate levels and significant increase in urinary oxalate excretion<sup>7</sup>. It is well-known that vitamin C can convert to oxalate therefore; supplementation of higher dose vitamin C may induce to increase oxaluria and increased risk of stone formation. Excess of fluid intake, restricted sodium and protein intake are advisable<sup>8</sup>. Calcium intake during meal time is advised in order to avoid calcium oxalate formation<sup>9</sup>.

*Urolitholytic plants:*

Several medicinal plant extracts have been reported for *in vitro* anti-crystallization activities till date such as

*Herniaria hirsuta*<sup>10</sup>, *Tribulus terrestris*<sup>11</sup>, *Bergenia ciliata*<sup>12</sup>, *Piper nigrum*<sup>13</sup>, *Dolichos biflorus*<sup>14</sup>, *Bergenia ligulata*<sup>15</sup>, *Plantago major*<sup>16</sup>. *Kalanchoe pinnata* or *Bryophyllum pinnatum* is one of many medicinal plants as anti-urolitholytic known as traditionally folk medicine for *Pashanabheda*<sup>17</sup>. *Kalanchoe pinnata* herb has different common synonyms like panfuti (Hindi), life plant, air plant (Mexican), resurrection plant, miracle plant while *Bryophyllum Calycinum*, *Bryophyllum pinnatum* in Latin<sup>18</sup>. It belongs to family Crassulaceae. *Pashanabheda* is a scientific term used in the Ayurveda system of medicine describing the potency of medicinal plant for breaking up and disintegrating renal and urinary calculi i.e., kidney stones and diuretic capacity<sup>19</sup>.

*Need to investigate on the plant by in-vitro studies*

Till to date the cited scientific evidences supporting to pharmacological effect of *Kalanchoe pinnata* against renal calculi has been reported as *in-vivo* studies<sup>16,20</sup> although it has not reported by using *in-vitro* dissolution models. The present study was undertaken to investigate the effect of aqueous extract of *Kalanchoe pinnata* leaves on *in-vitro* urolithiasis using semi-permeable membrane of eggs in dissolution model and its anti-crystallization capacity through analyzing nucleation and aggregation assays.

## MATERIAL AND METHODS

### Chemicals

Calcium chloride dihydrate, sodium oxalate, p-phenylene diamine were purchased from Sigma-Aldrich Ltd.

Potassium permanganate, sodium meta-bisulfite, and tris-buffer were purchased from Loba chemicals Ltd.

Cystone® was purchased from Himalaya Drug Company.

### Collection of Plant

Plant material was identified and authenticated from the Dept of Botany, Yashwantrao Chavan College of Sciences, Karad and fresh leaves were collected in July 2014.

### Extraction

Fresh leaves of *Kalanchoe pinnata* were chopped into small pieces by hand and put into a conical flask. 100ml of distilled water was added to the conical flask and boiled for a while in order to maximize the extraction. After cooling it was filtered through Whatmann filter paper and as aqueous extract stock solution transferred to a suitable container.

### Preparation of the semi-permeable membrane from eggs

Apex of eggs was punctured by a glass rod in order to squeeze out the entire content. Empty eggs were washed thoroughly with distilled water and placed in a beaker consisting 4ml concentrated HCl in 200ml distilled water. It was kept for overnight which led to the complete decalcification of semi permeable membrane. On the next day, semi permeable membranes were removed carefully from egg shells; washed thoroughly with distilled water and placed it in ammonia solution for neutralization of acid traces, and then rinsed it with distilled water. It was stored in refrigerator at a pH of 7-7.4 in the moistened condition.

10mg of the calcium oxalate was suspended in 10ml of distilled water as negative control. 5ml of hot aqueous extract of fresh leaves of *Kalanchoe pinnata* was taken. 500mg tablet of Cystone® was placed in absolute ethanol for removing colour coating and 400mg was obtained. Cystone® tablet was crushed into powder form and dispersed into 100ml of distilled water and filtered. Filtrate of Cystone® was used as positive control for *in vitro* anti-urolithiatic activity.

### Spectrophotometric estimation of calcium oxalate by using dissolution model

#### Synthesis of calcium oxalate by homogenous precipitation

1.47gm of calcium chloride dihydrate was dissolved in 100ml distilled water and 1.34gm of sodium oxalate was dissolved in 100 ml of 2N H<sub>2</sub>SO<sub>4</sub>. Both were mixed equally in a beaker to precipitate out calcium oxalate with stirring. The resultant calcium oxalate was freed from traces of sulfuric acid by ammonia solution; washed with distilled water and dried at a temperature 60 °C for 2hours.

#### Preparation of 0.02M KMnO<sub>4</sub> solution

0.32gm of KMnO<sub>4</sub> was dissolved in 100ml of distilled water. It was boiled for 30min. After cooling, excess of MnO<sub>4</sub> was removed by filtration.

## METHOD

Group I: 1ml of calcium oxalate (1mg/ml) + 1ml of distilled water

Group II: 1ml of calcium oxalate (1mg/ml) + 1ml of Cystone solution (400mg/ml)

Group III: 1ml of calcium oxalate (1mg/ml) + 1ml of hot aqueous extract of *Kalanchoe pinnata* (20mg/ml)

All groups were packed it together in egg semi permeable membrane tied with thread at one end and were suspended in a conical flask containing 150 ml 0.1 M Tris buffer each. At another end of thread tied by a stick placed on the mouth of conical flask and covered with aluminum foil. All groups were kept in an incubator, pre heated to 37°C for 4 hours, kept for three days. The entire content of each group was removed from sutured semi permeable membrane and was transferred into test tube individually. 4ml of 1N H<sub>2</sub>SO<sub>4</sub> and 60-80µl of 0.02M KMnO<sub>4</sub> were added and kept aside for 2 hours. Colour change from dark pink to colourless was observed after 2 hours. Change of colour intensity was measured against 620nm spectrophotometrically. Concentration of undissolved calcium was determined from standard calibration curve of calcium oxalate by using the measured absorbance readings as shown in Fig.1.

### Spectrophotometric estimation of calcium phosphate by using dissolution model

#### Synthesis of calcium phosphate by homogenous precipitation

1.47gm of calcium chloride dihydrate was dissolved in 100ml distilled water and 1.42gm of disodium hydrogen phosphate was dissolved in 100 ml of 2N H<sub>2</sub>SO<sub>4</sub>. Both were mixed equally in a beaker to precipitate out calcium phosphate with stirring. The resultant calcium phosphate was freed from traces of sulfuric acid by ammonia

solution; washed with distilled water and dried at a temperature 60 °C for 2 hours.

#### Preparation of molybdate-sulphuric acid reagent

Molybdate-sulphuric acid reagent was prepared by 5% w/v of sodium molybdate solution, 13ml of conc. H<sub>2</sub>SO<sub>4</sub> in 80ml of distilled water. Finally, volume was adjusted to 100ml with distilled water.

#### Preparation of reducing solution

1gm of p-phenylene diamine was dissolved in 100 ml of 3 % w/v of sodium meta-bisulfite solution.

#### Method

Group I: 1ml of calcium phosphate (1mg/ml) + 1ml of distilled water

Group II: 1ml of calcium phosphate (1mg/ml) + 1ml of Cystone® solution (400mg/ml)

Group III: 1ml of calcium phosphate (1mg/ml) + 1ml of hot aqueous extract of *Kalanchoe pinnata* (20mg/ml)

All groups were packed it together in egg semi permeable membrane tied with thread at one end and were suspended in a conical flask containing 150 ml 0.1 M Tris buffer each. At another end of thread tied by a stick placed on the mouth of conical flask and covered with aluminum foil. All groups were kept in an incubator, pre heated to 37°C for 4 hours, kept for three days. The entire content of each group was removed from sutured semi permeable membrane and was transferred into test tube individually. 4ml of 1N H<sub>2</sub>SO<sub>4</sub> and 3ml of molybdate-sulphuric acid reagent, 1 ml of reducing solution were added and kept aside for 2 hours. Colour change from dark pink to colourless was observed after 2 hours. Change of colour intensity was measured against 620nm spectrophotometrically. Concentration of undissolved calcium was determined from standard calibration curve of calcium phosphate by using the measured absorbance readings as shown in Fig.2.

In a pilot study, we found that the inhibition of nucleation and aggregation by the aqueous extract was not effective at below > 5mg/ml. Aqueous extract exhibited good inhibition activity above at >10mg/ml, and thus concentrations 10-50mg/ml have prepared in the present study. Cystone® was dissolved in distilled water to give concentrations of 10-50mg/ml for both the nucleation and aggregation assays.

#### Nucleation assay (Turbidity method)

The inhibitory activity of the extracts on the nucleation of calcium oxalate crystals was determined by a spectrophotometric assay<sup>12</sup>. Crystallization was initiated by adding 100µl of 4 mM calcium chloride and 100µl of 50 mM sodium oxalate solutions to 0.5ml of human normal urine, both prepared in a buffer containing 0.5ml of 0.05 mM Tris buffer and 0.5ml of 0.15mM NaCl solution at pH 6.5 and 37°C and adjusted to volume by adding 1.5ml of distilled water. The rate of nucleation was determined by comparing the induction time of crystals (time of appearance of crystals that reached a critical size and thus became optically detectable) in the presence of the extract and that of the control with no extract. The optical density (OD) was recorded at 620nm, and the percentage inhibition calculated as (1-OD (experimental)/OD (control))/100.

#### Aggregation assay

The rate of aggregation of the calcium oxalate crystals was determined by a spectrophotometric assay<sup>12</sup> with slight modifications. The calcium oxalate monohydrate (COM) crystals were prepared by mixing both the solutions of calcium chloride and sodium oxalate of 50 mM each. Both solutions were then equilibrated. The solutions were then cooled to 37°C and then evaporated. The COM crystals were then dissolved with 0.5ml of 0.05mM Tris buffer and 0.5ml of 0.15mM NaCl solution at pH 6.5 to a final concentration of 1 mg/ml. Absorbance at 620 nm was recorded. The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the extract against control.

## RESULTS & DISCUSSION

In kidney stones formation, calcium oxalate and calcium phosphate or other chemicals in the urine form crystals on the inner surfaces of kidneys. This stage is called as initial mineral phase formation. Over the period of time crystals may combine to form a small, hard mass called as stones and stage is referred as crystal growth. Calcium oxalate stones have classified into two types i.e., calcium oxalate monohydrate stones (COM) and calcium oxalate dihydrate (COD)<sup>12</sup>.

#### Spectrophotometric estimation of calcium oxalate

The extract of *Kalanchoe pinnata* has greater capability to dissolve calcium oxalate as foremost element for stone forming in urinary tract. Lower percentage indicates more potency in dissolution of calcium oxalate crystals as shown in Table 1.

#### Spectrophotometric estimation of calcium phosphate

Cystone standard has shown better demineralization for calcium phosphate as secondary element for stone forming in urinary tract compared to the extract of *Kalanchoe pinnata* as shown in Table 2.

#### Nucleation assay

Urine supersaturation attributes to calcium oxalate particles crystallization within the urinary tract. This is nucleation process where stone forming salts begins unite into clusters with addition of new constituents<sup>13</sup>. Cystone standard solution exhibited stronger inhibition activity than the extract of *Kalanchoe pinnata* in the nucleation of calcium oxalate salts.

As *in vitro* crystallization study was performed, since nucleation is an important first step for the initiation of crystals, which then grow and form aggregates. Extract of *Kalanchoe pinnata* inhibited the crystallization by inhibiting nucleation of calcium oxalate through disintegrating into smaller particles with increasing concentrations of the fraction. From the results of the nucleation assay confirmed that the extract contained nucleation-preventing agents.

#### Aggregation assay

Calcium oxalate crystals begin grow; aggregate with other crystals and retained in the kidney. This is aggregation process which causes renal injury<sup>13</sup>. The extract of *Kalanchoe pinnata* demonstrated slightly better compared to Cystone standard solution to inhibit

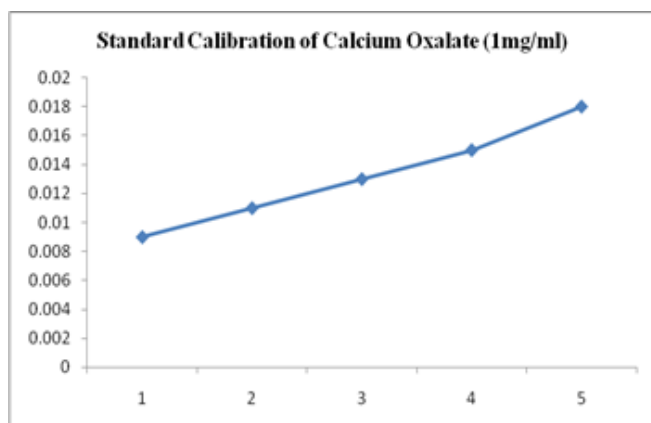


Fig. 1: Standard calibration of calcium oxalate

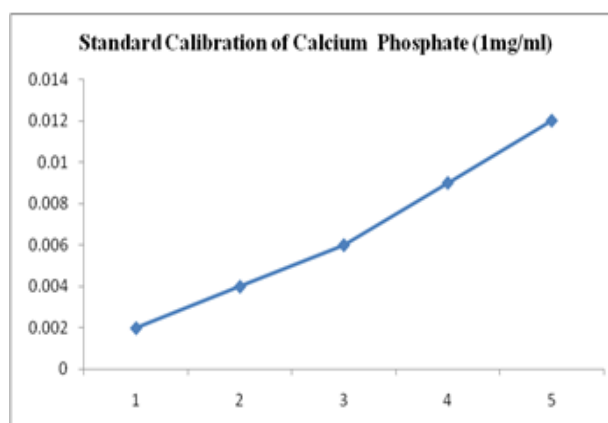


Fig. 2: Standard calibration of calcium phosphate

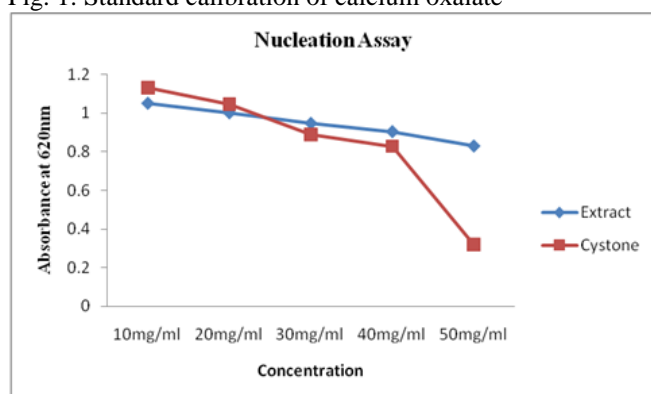
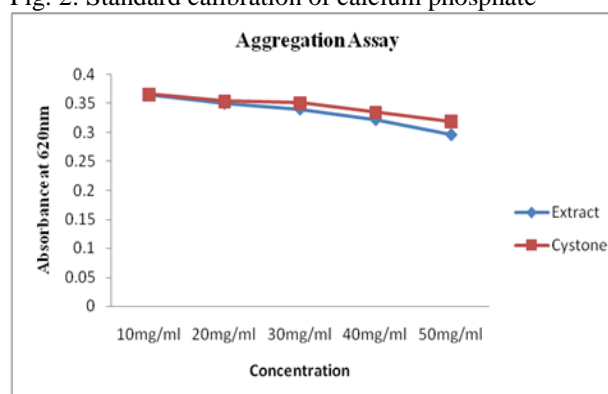
Fig. 3: Nucleation assay showed that the various concentration of *Kalanchoe pinnata* extract and Cystone against absorbance at 620nmFig. 4: Aggregation assay showed that the various concentration of *Kalanchoe pinnata* extract and Cystone against absorbance at 620nm

Table 1: Dissolution of calcium oxalate

Group	Mean $\pm$ SD	Weight of calcium reduced	Dissolution Percentage
Group-I	0.128 $\pm$ 0.0005	--	--
Group-II	0.054 $\pm$ 0.0005	0.074	57.81
Group-III	0.067 $\pm$ 0.073	0.061	47.65

Table 2: Dissolution of calcium phosphate

Group	Mean $\pm$ SD	Weight of calcium reduced	Dissolution Percentage
Group-I	0.109 $\pm$ 0.0005	--	--
Group-II	0.077 $\pm$ 0.001	0.032	29.35
Group-III	0.027 $\pm$ 0.011	0.082	75.22

promoted the formation of COD crystals. COM has a stronger affinity with cell membranes; it may lead to become higher potential risk for renal calculi formation. This is may be due to high content of saponins present in *Kalanchoe pinnata*. *Kalanchoe pinnata* has several polyphenolics, e.g; alkaloids, saponins, phenolics, flavonoids, and other phytoconstituents<sup>17</sup>.

Saponins are well known to have anti-crystallization properties by disaggregating the suspension of mucoproteins as crystallization promoters<sup>12</sup>. Saponin-rich butanol fraction along with crude aqueous extract of *Terminalia arjuna* proved to inhibit initial mineral phase formation of calcium phosphate and growth of COM crystals<sup>20</sup>.

No pharmacologic intervention has definitively been shown to be effective for lithiasis. The present investigation will be supportive as additional information

to the scientific evidences regarding *in-vitro* studies. Since mechanism of anti-urolitholytic activity in the extract is exact unknown till date, correlation between *in-vitro* and *in-vivo* studies should be further investigated to reveal the phytochemicals of the extract are responsible for dissolving or disintegrating renal calculi and to know better understanding in the molecular mechanism of litholysis.

## CONCLUSION

*Kalanchoe pinnata* extract exhibited significant *in-vitro* anti-urolithiatic activity.

## ACKNOWLEDGEMENT

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**CONFLICT OF INTEREST**

We declare that we have no conflict of interest.

**REFERENCES**

1. Colella J, Kochis E, Galli B, Munver R. Urolithiasis/nephrolithiasis: what's it all about? *Urol Nurs* 2005; 25(6):427-48, 475, 449.
2. Konigsberger E, Konigsberger LC. Thermodynamic modeling of crystal deposition in humans. *Pure Appl Chem* 2001; 73:785-797.
3. Noonan SC, Savage GP. Oxalate content of foods and its effect on humans. *Asia Pacific J Clin Nutr* 1998; 8(1): 64-74.
4. Huges J, Norman RW. Diet and calcium stones. *Can Med Asso J* 1992; 146: 137-143.
5. Durgawale P, Shariff A, Hendre A, Patil S, Sontakke A. Chemical analysis of stones and its significance in urolithiasis. *Biomedical Research* 2010; 21(3):305-310.
6. Kanu Priya Aggarwal, Shifa Narula, Monica Kakkar, and Chandereep Tandon. Nephrolithiasis: molecular mechanism of renal stone formation and the critical role played by modulators. *BioMed Research International* 2013; Article ID 292953, 21 pages, 2013. doi:10.1155/2013/292953
7. Massey LK, Roman-Smith H, Sutton RA. Effect of dietary oxalate and calcium on urinary oxalate and risk of formation of calcium oxalate kidney stones. *J Am Diet Assoc* 1993; 93(8):901-906.
8. Finkelstein VA, Goldfarb DS. Strategies for preventing calcium oxalate stones. *CMAJ* 2006; 174(10):1407-1409.
9. Domrongkitchaiporn S, Sopassathit W, Stitchantrakul W, Prapaipanich S, Ingsathit A, Rajatanavin R. Schedule of taking calcium supplement and the risk of nephrolithiasis. *Kidney Int* 2004; 65(5):1835-1841.
10. Atmani F, Khan SR. Effects of an extract from *Herniaria hirsuta* on calcium oxalate crystallization *in vitro*. *BJU Int* 2000; 85(6):621-625.
11. Aggarwal A, Tandon S, Singla SK, Tandon C. Diminution of oxalate induced renal tubular epithelial cell injury and inhibition of calcium oxalate crystallization *in vitro* by aqueous extract of *Tribulus terrestris*. *Int Braz J Urol* 2010; 36(4):480-488.
12. Saha S, Verma RJ. Inhibition of calcium oxalate crystallization *in vitro* by an extract of *Bergenia ciliata*. *Arab Journal of Urology* 2013; 11: 187-192.
13. Manish A. Patel, Paras K. Patel, Avinash K Seth. Inhibition of calcium oxalate crystallization by the fruit extracts of *Piper nigrum* L. *Pharmacologyonline* 2011; 2: 1169-1177.
14. Garimella TS, Jolly CI, Narayanan S. *In vitro* studies on antilithiatic activity of seeds of *Dolichos biflorus* Linn and rhizomes of *Bergenia ligulata* Wall. *Phytother Res* 2001; 15(4):351-355.
15. Aziz SA, See TL, Khuay LY, Osman K, Abu Bakar MA. *In vitro* effects of *Plantago major* extract on urolithiasis. *Malays J Med Sci* 2005; 12(2):22-26.
16. Verma P, Gauttam V, Kalia AN. Comparative Pharmacognosy of Pashanbhed. *J Ayurveda Integr Med* 2014; 5(2):104-108.
17. Kamboj A, Saluja AK. *Bryophyllum pinnatum* (Lam.) Kurz.: Phytochemical and pharmacological profile : A review. *Phcog Rev* 2009; 3:364-74.
18. Shashi Alok, Sanjay Kumar Jain, Amita Verma, Mayank Kumar, Monika Sabharwal. Pathophysiology of kidney, gallbladder and urinary stones treatment with herbal and allopathic medicine: A review. *Asian Pac J Trop Dis* 2013; 3(6): 496-504.
19. Shukla AB, Mandavia DR, Barvaliya MJ, Baxi SN, Tripathi CR. Evaluation of anti-urolithiatic effect of aqueous extract of *Bryophyllum pinnatum* (Lam.) leaves using ethylene glycol-induced renal calculi. *Avicenna J Phytomed* 2014; 4(3):151-159.
20. Chaudhary A, Singla SK, Tandon C. *In vitro* evaluation of *Terminalia arjuna* on calcium phosphate and calcium oxalate crystallization. *Indian J Pharm Sci* 2010; 72(3):340-345.