

Research Article



In-vitro and Ex-vivo Studies on Synergistic Effects of *Citrus maxima* on Anti-Urolithiatic Activity

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ABSTRACT

The aggregation of the renal stones in the body are composed of minerals like calcium, oxalate, phosphates, uric acid, cystine etc. which finally end up accumulating in urinary system –especially in the different parts of the kidney, ureter or urethra (urinary tract) thereby obstructing the parts of the system. When the renal stones are not removed or left untreated it can be life threatening, fatal because there is an obstruction in the major route of excretion. Its further results in increasing toxicity levels in the living system leading to long term complications like complete failure of organ, coma and death. Traditionally, pomelo is used in indigenous system of medicine to treat various ailments like epilepsy, convulsive cough, hemorrhage, leprosy, eye related problems, sedative for nervous related problems. The present study was conducted to provide a scientific proof that pomelo belonging to citrus family is abundantly loaded with ascorbic acid, flavonoids, terpenoids, alkaloids, antioxidants, phenolics etc. contain inhibitory properties and Urolithiatic nature it can be used to prevent and treat the kidney stones. Therefore, In-vitro tests for nucleation, aggregation, CaOx crystal growth assay and Kidney stone degradation assay was conducted on pomelo. 10% methanolic extract of pulp and peel were tested for the phytochemical constituents and quantitative estimation of flavonoids, terpenoids and ascorbic acid was performed. Nucleation and aggregation assay, Calcium oxalate crystal growth assay and Kidney stone degradation assay was performed to check the urolithiatic activity. The number of flavonoids, terpenoids and ascorbic acid was more in the methanolic pulp extract at 0.34mg/100mL, 0.192 mg/100mL and 1.70 mE \pm 0.014 mE respectively as compared to the peel. Methanolic pulp extract gave 65% inhibition for nucleation and aggregation assay and 24.26% inhibition for calcium oxalate crystal growth assay whereas methanolic peel extract gave 25% inhibition for nucleation and aggregation assay and 28.47% inhibition for calcium oxalate crystal growth assay. It was the best sample in dissolving the kidney stones (89.47%). The methanolic extract of pulp has more capacity to dissolve the kidney stones as compared to the peel and can be used to dissolve the renal stones.

Keywords: Anti-Urolithiatic activity, *Citrus maxima*, Renal stones, Synergistic effects, Lyophilized Pulp extract, Brushite crystals, Nucleation and Aggregation studies, Pulp methanol, Peel methanol.

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INTRODUCTION

The aggregation of the renal stones in the body are composed of minerals like calcium, oxalate, phosphates, uric acid, cystine etc. which finally end up accumulating in urinary system –especially in the different parts of the kidney, ureter or urethra (urinary tract) thereby obstructing the parts of the system. The process of formation of renal stones in kidney, ureter, urethra, urinary bladder is termed as Urolithiasis. Urolithiasis means ouron (urine) and lithis (stone). When the balance between the precipitation of salts and solubility in kidney or urinary tract is been disturbed results in urolithiasis. Due to enhancing capacity of testosterone and inhibiting capacity of estrogen occurrence of renal stone formation is three times (80%) more in men than compared to women where the occurrence is (60%).¹

Kidney stone are composed of minerals like calcium, phosphate, oxalate, uric acid, cystine and other compounds. Based on mineralogy and pathogenesis kidney stones are classified into five types-Calcium oxalate and calcium phosphate, struvite or magnesium ammonium phosphate, uric acid or urate, cystine and silicate or drug induced stones. Amongst these stone COM, CaOx dihydrate are more prevalent followed by struvite stones, uric acid stones and cystine stones are least common of all types of stones. Kidney stones are treated by surgery, extracorporeal shock wave lithotripsy (ESWL), percutaneous nephrolithotomy (PCNL) and shock wave lithotripsy.²

Citrus maxima commonly known as shaddock, chakotra, pummel or papanus. It belongs to Rutaceae family and genus Citrus. *Citrus maxima* are perennial shrub and edible fruit. This fruit is found in countries like India, Japan, Vietnam, Malaysia, Indonesia, China, Thailand, America and Philippines.³

This fruit is loaded with abundant number of flavonoids, terpenoids, alkaloids, tannins, phenolics. Ascorbic acid is one of the most important components present in abundant amount it has an inhibitory effect, inhibits the growth of kidney stones. All the parts of pomelo tree leaves, flower, fruit, rind, root and bark are used to treat



asthma, cough, epilepsy, cardio tonic, vomiting, diarrhea, headache, eye problems, sedation for problems associated with nervous system. This fruit is also having various activities antioxidant, anti-inflammatory, anti-diabetic, anti-tumor, Hepatoprotective, anti-bacterial, anti-depressant, anti-fungal, Larvicidal activity, Hypcholesterolemic and ACE inhibitory activity.⁴

The present study is dedicated towards identifying the potential of anti-Urolithiatic activity of the fruit pulp and the peel of *Citrus maxima* (Pomelo). Simultaneously following this we are examining for the synergistic /antagonistic effect of the test samples on urolithiasis.

MATERIALS AND METHODS

Ripened organic pomelo was procured from the local area in Bengaluru. The pulp content was directly extracted and the peel was sun dried, powdered and stored. The chemicals and solvents were provided by the laboratory, Department of Biochemistry, Mount Carmel college, Bengaluru, India.

Phytochemical screening:

Phytochemical evaluation of pulp and peel extract with methanol was conducted to check the activity. 10% methanolic extract was prepared by placing the mixture on magnetic stirrer for 20 mins and centrifuged at 8000 rpm, 15 mins, 24°C. The obtained clear supernatants were subjected to various phytochemical tests like carbohydrates, proteins, alkaloids, glycosides, tannins/phenolics, flavonoids and terpenoids in the dilution 2:8.^{5,6,7,8}

Quantitative estimation of flavonoids:

The flavonoid content of the samples was estimated according to the method proposed by Yang *et al.*, (2001). 0.2 to 1.0 mL (10 µg/mL) aliquots of standard quercetin solution was taken in different test tubes which was made up to 2.0 mL of methanol was taken as blank and 2:8 ratio of diluted 1 mL extracts were taken separately for flavonoid estimation. 0.1 mL of 10% AlCl₃ and 0.1 mL of 1M potassium acetate solutions were added to all the test tubes which were incubated at 30 minutes at room temperature. The absorbance was checked at 415 nm for all the test tubes and standard graph was plotted to estimate the flavonoid content of extracts as quercetin equivalents. The test was conducted in duplicates.^{10,11}

Quantitative estimation of terpenoids:

The terpenoid content of the samples were estimated according to the method proposed by Narayan Ghorai *et al.*, (2012). Sample preparations were conducted according to the methods prescribed previously. Aliquots of 1 µM–5 µM of linalool solution were taken in different eppendorf tubes which was made up to 200 µL with methanol. Alternatively, 200 µL of different sample supernatants were taken in the eppendorf tubes. 1.5 mL of chloroform was added to all the tubes and vortexed. The solutions were incubated at room temperature for 3

minutes. 100 µL of concentrated Sulphur acid was added to all the tubes and vortexed. All the tubes were incubated in dark for 1.5 to 2 hours. The supernatant was discarded and 1.5 mL of methanol was added to all the tubes to dissolve the precipitate. Absorbance was noted at 538 nm and the tests were conducted in duplicates.²³

Quantitative estimation of ascorbic acid

10% methanolic extract of the fruit pulp and peel was prepared and filtered and used for titrable acidity estimation. Standard solutions of sodium hydroxide and oxalic acid were prepared with distilled water. NaOH was standardized with 10 mL of 0.05M of oxalic acid. 0.5% phenolphthalein was used as indicator which turned the solution to pale pink in presence of base which was considered as the end point. 25 mL of extract was titrated against standardized 0.05M NaOH in presence of indicator until end point was obtained. All the tests were conducted in duplicates.¹²

Parameters to test for urolithiasis

Nucleation and aggregation assay

Nucleation and aggregation assay were performed as per method previously described by Hess *et al.*, (2000) with minor modifications. Stock solutions of 10 mM CaCl₂ and 1 mM of Sodium oxalate solutions were prepared with buffer solutions containing 200 mM NaCl and 10 mM Sodium acetate (pH 5.7). All the solutions were prepared with filtered Millipore water and again filtered after preparations. 15 mM of CaCl₂ solution was added into a clean beaker and maintained on magnetic stirrer with continuous stirring at 37°C. 1.5 mL of Control (Millipore water/Standard (10 mg/mL of cystone) / 10 mg/mL of methanolic extract of pulp and peel / 1:1 mixture of pulp and peel / Lyophilized sample (10 mg/mL) was added under continuous stirring. Incubation time was started as soon as 15 mL of sodium oxalate solution was added. Absorbance was noted every minute at 620 nm for 30 minutes. The blank was set with Millipore water. All the crystallization experiments were conducted in duplicates. Percentage inhibition of the standard and extracts were calculated as $[1 - (T_{si}/T_{sc})] \times 100$ where T_{sc} indicated the turbidity slope of the control and T_{si} indicates the turbidity slope in presence of inhibitor like cystone and extracts.^{13,15,16}

Calcium oxalate crystal growth assay

Inhibitory activity of the *Citrus maxima* pulp and peel against CaOx crystal growth was conducted according to the previously described methods. 10 mL of 4 mM Calcium chloride and 10 mL of 4 mM Sodium oxalate were added to 15 mL of Tris-NaCl (10 mM pH 7.2) in a 50 mL beaker. 1 mL of distilled water (control) / Cystone-10 mg/mL (standard) / 10 mg/mL of methanolic extract of pulp and peel / 1:1 mixture of pulp and peel / Lyophilized sample (10 mg/mL) was added separately to the above solution. 300 µL of Calcium oxalate monohydrate (COM) crystal slurry (1.5 mg/mL of acetate buffer pH–5.7) was added to the above reaction mixture (maintained on a



magnetic stirrer at 450 rpm). All the solutions were prepared with filtered Millipore water and prepared solutions were filtered. Oxalate consumption by the COM added begins immediately which should be monitored for 10 mins where the absorbance was recorded at 214 nm at every 30 seconds. Rate of the free oxalate consumption decreases if the test samples inhibit CaOx crystal aggregation. The relative inhibitory activity was calculated as $[(C-S/C)] \times 100$ where C is the rate of reduction of free oxalate without any extract and S is the rate of reduction of free oxalate in the presence of test extracts.^{18,21,22}

Kidney stone degradation assay

Surgically removed human kidney stones were procured from M.S. Ramaiah Memorial Hospital, M.S. Ramaiah Nagar, Bengaluru, Karnataka, India. The assay was performed with reference to Rao and Bano method., (2004), with some minor modifications. The length of the kidney stones was measured (cm) and the weight was recorded (in grams) and labeled. 40ml of 0.05M Tris HCl

buffer (pH-5.7) containing 0.15M NaCl was dispensed in various sterile tarson's containers and were labeled accordingly. 10ml of 10 % aqueous extract of cystone/ 10% Methanolic extract of pulp/10% Methanolic extract of peel/1:1 mixture of Methanolic pulp and peel extract / Lyophilized pulp extract was added in respectively labeled containers. The evaluated and labeled kidney stones were inoculated in the respective containers. Control was maintained by inoculating the kidney stone in buffer containing 10 ml of distilled water few kidney stones were inoculated in 1:1 mixture of methanolic pulp and peel extract in the absence of buffer. The containers were vortexed daily to ensure equal distribution of sample in the mixture. The size and weight of the kidney stones was measured every 4 days by drying the washed stones at 100°C in the hot air oven for 5 minutes. The test was conducted in duplicates for all the samples. % weight reduction was calculated as % dissolution = $[(\text{Initial weight} - \text{Final weight}) / \text{Initial weight} \times 100]$.²⁴

RESULTS AND DISCUSSION

Table 1: Phytochemical Screening of *Citrus maxima* fruit extracts

Sl. No	Phytochemical tests	Pulp Aqueous	Peel Aqueous	Methanolic pulp	Methanolic peel
1	Molisch test	-	-	+	-
2	Fehling's test	+	+	+	+
3	Proteins	-	-	-	-
4	Alkaloids	+	+	+	+
5	Glycosides	-	-	-	-
6	Tannins/ Phenolics	-	-	+	+
7	Flavonoids	+	+	+	+
8	Terpenoids	+	+	+	+

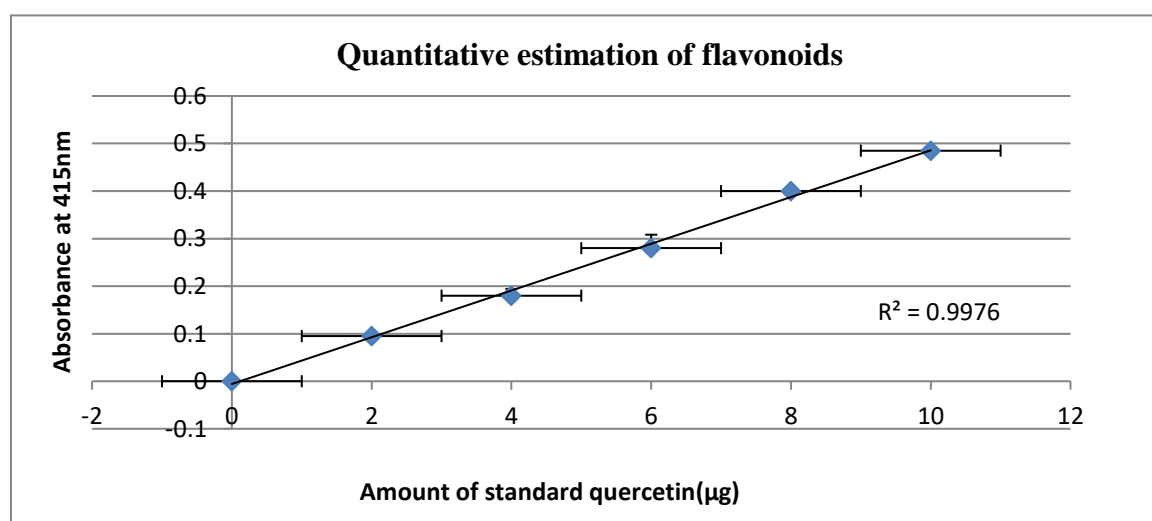


Figure 1: Flavonoid content in *Citrus maxima* (mg/100g or mg/100ml) expressed as Mean \pm SD

The methanolic extract of pulp contained higher amount of flavonoid at 0.34 mg /100mL when compared to methanolic extract of peel which contained 0.32mg /100mL of flavonoid.

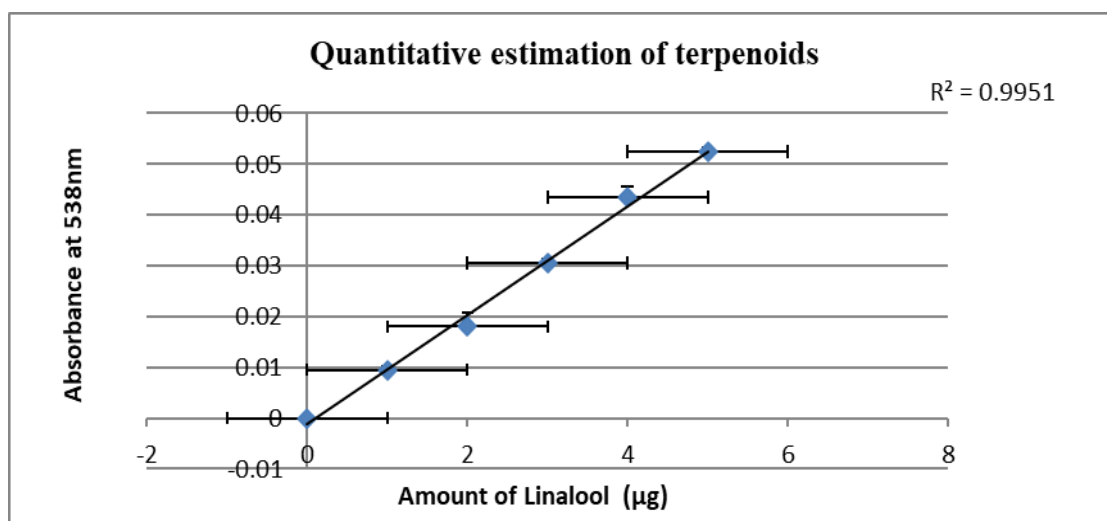


Figure 2: Terpenoid content in *Citrus maxima* (mg/100g or mg/100ml) expressed as Mean \pm SD

The methanolic extract of pulp contained higher amounts of terpenoids at 0.192mg/100mL when compared to methanolic extract of peel which contained 0.185mg/100mL of terpenoids.

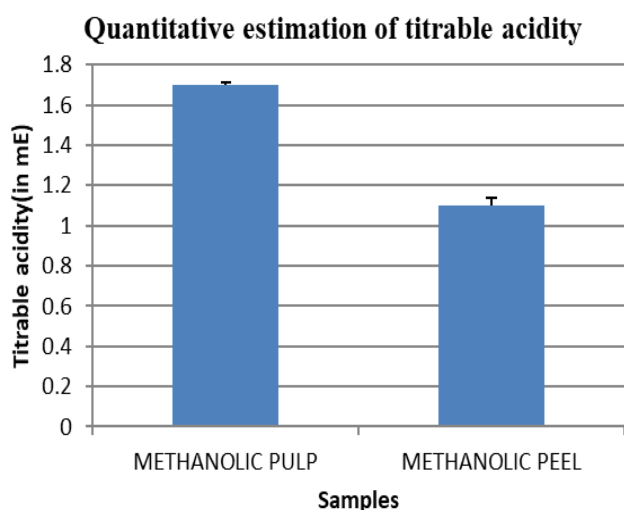


Figure 3: Quantified Titrable acidity of *Citrus maxima* expressed in mE as Mean

The methanolic extract of pulp contained higher amount of titrable acids at 1.70 mE \pm 0.014mE when compared to 10% methanolic extract of peel i.e. 1.10mE \pm 0.035mE.

Table 2: Slope and % Inhibition for Nucleation and Aggregation

Samples	Concentration	Slope	% Inhibition
Standard (Cystone)	10mg/ml	0.0009	35
Methanolic pulp	10mg/ml	0.001	65
Methanolic peel	10mg/ml	0.0004	25
1:1 sample mixture	10mg/ml	0.0008	37.5
Lyophilized	10mg/ml	0.001	25

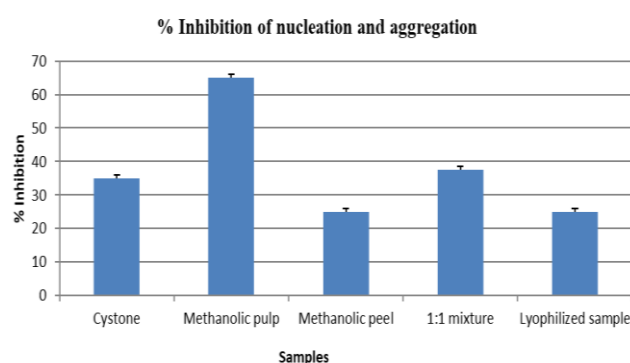


Figure 4: % of Inhibition of Nucleation and Aggregation of CaOx nuclei expressed as Mean \pm SD

Table 3: Slope and % Inhibition for CaOx Crystal growth assay

Samples	Concentration	Slope	% inhibition
Standard (Cystone)	10mg/ml	0.0045	46.525
Methanolic pulp	10mg/ml	0.0075	24.26
Methanolic peel	10mg/ml	0.006	28.47
1:1 mixture	10mg/ml	0.0075	11.805
Lyophilized sample	10mg/ml	0.004	50

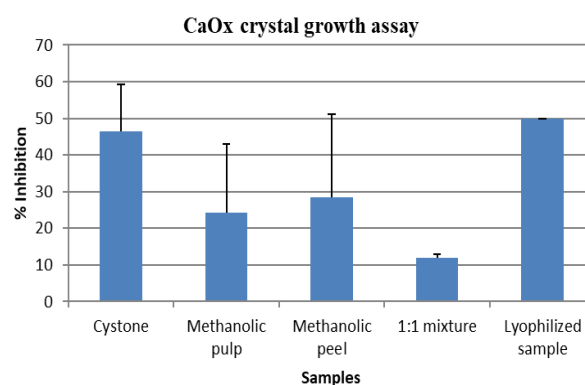
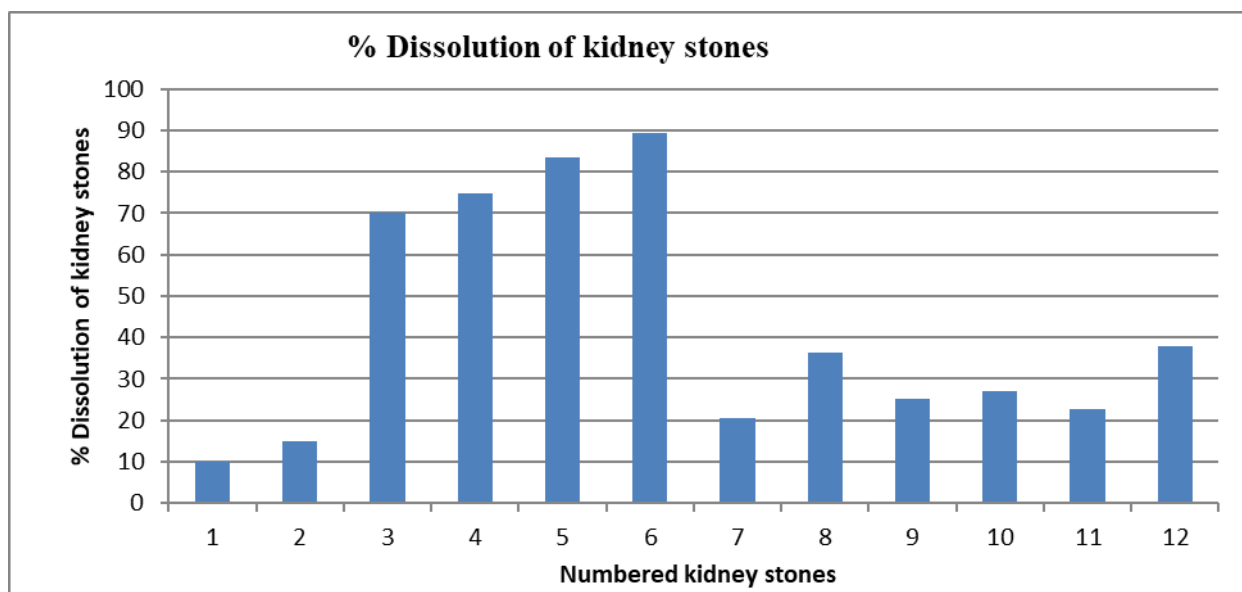


Figure 5: % Inhibition of calcium oxalate crystal growth in the presence of test samples expressed as Mean \pm SD

Table 4: Depiction of difference in the weight of kidney stones based on the initial weight of the stone

Samples	Kidney stone number	Initial Weight	Final Weight	% Dissolution
Control	1	0.10	0.09	10
	2	0.02	0.0170	15
Cystone	3	0.10	0.03	70
	4	0.0159	0.0040	74.84
Methanolic pulp	5	0.06	0.01	83.33
	6	0.0190	0.0020	89.47
Methanolic peel	7	0.39	0.31	20.51
	8	0.0198	0.0126	36.36
1:1 mixture	9	0.12	0.09	25
	10	0.0279	0.0204	26.88
Lyophilized sample	11	0.22	0.17	22.72
	12	0.0349	0.0217	37.8

**Figure 6:** % Dissolution of kidney stones based on the size of the stones

The present study focused on understanding the effect of the samples on the kinetics and the dynamics of kidney stones. Their effect on the formation of stones was studied and the dissolution strength was calculated.

The phytoconstituents of the pulp and peel of pomelo (*Citrus maxima*) was estimated to understand the relation between the concentration and effect on the formation and degradation of kidney stones. The pulp contained high amounts of flavonoids (0.34mg/100ml), terpenoids (0.192mg/100ml) and titrable acids (1.70mE \pm 0.014mE).

Pomelo methanolic pulp extract gave 65% inhibition for nucleation and aggregation assay (Fig 4) and 24.26% inhibition for calcium oxalate crystal growth assay (Fig 5) whereas methanolic peel extract gave 25% inhibition for nucleation and aggregation assay (Fig 4) and 28.47% inhibition for calcium oxalate crystal growth assay (Fig 5). Comparing both the results it can be concluded that

methanolic extract of pulp has better inhibition activity than peel.

The dissolution studies on surgically obtained kidney stones showed better dissolution in the presence of methanolic pulp extract than methanolic peel extract. (Fig 6).

The studies were conducted for the combination of extracts to understand the synergism and antagonism. The synergism was seen in nucleation and aggregation assay which showed second highest inhibition at 37.5%. Antagonism was observed in calcium oxalate crystal growth assay at 11.80% for the combination of extracts whereas highest inhibition was shown by lyophilized pulp extract i.e. 50%.

The effect of test extracts on the biologically obtained kidney stones was also evaluated by incubating them in the presence of buffer (Table 4). It was seen that the methanolic pulp extract gave the highest dissolution

followed by cystone and lyophilized sample. They were also related to the initial weight of the stones. The extent of dissolution was higher in the small stones. The percentage dissolution of stone number 6 was the highest at 89.47% as the initial weight of the stone was low and the effective sample was the methanolic pulp extract. From the above study it can be concluded that the methanolic pulp extract has better anti-urolithiatic activity.

CONCLUSION

It can be concluded that the pulp of citrus maxima has more phytochemical constituents than the peel of the fruit. Therefore, the methanolic extract of pulp has more capacity to dissolve the kidney stones as compared to the peel and can be used to dissolve the renal stones.

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Abbreviations

- AlCl₃ - Aluminum Chloride
- NaOH - Sodium Hydroxide
- CaCl₂ - Calcium chloride
- NaCl -Sodium Chloride
- CaOx- Calcium Oxalate
- HCl- Hydrochloric acid

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