Abrahamia
An International Journal of Plant Sciences
Vol. 7 (2)
91-98
2021
ISSN: 2395-4108

Antiurolithiatic potential of selected fruit extracts – an *in vitro* study

Sreeja Parvathy & Bindu R. Nair*
Department of Botany, University of Kerala, Kariavattom P. O., Thiruvananthapuram- 695581, Kerala, India
*Corresponding author: bindunair_r@yahoo.com

Abstract

Urolithiasis is a urologic disorder with complicated treatment outcomes. A complete cure for urolithiasis in terms of medicine or surgery appears futile. Herbal remedies have been used widely for both the treatment and prevention of this disorder. The current study aimed to investigate the effect of selected fruit extracts on calcium oxalate urolith formation and crystallization by *in vitro* assays, namely nucleation, aggregation and oxalate depletion assays. The results of the study highlight the significant inhibition potential of *Carica papaya* L. fruit extract at all the stages of urolith crystal formation. The fruit extracts of *Punica granatum* L. and *Psidium guajava* L. were found to inhibit the aggregation stage of crystallization significantly. However, none of the extracts could inhibit the final growth stages of crystallization relative to the standard drug. The inhibitory activity of the tested extracts could possibly be due to the presence of phenolic compounds and flavonoids.

Keywords: Calcium oxalate crystals, *in vitro* assays, Urolithiasis

Introduction

Urolithiasis is a painful and recurring disease condition affecting the urinary tract. It is characterized by the production of calculi (small stone crystals) in the urine. Dysuria, burning and painful micturition, and pain in the pelvic and lumbo-sacral area are some of the symptoms (Moe, 2006).This process may arise due to genetic anomalies, irregular diet habits and decreased intake of water or some other similar conditions. Approximately 80 % of urinary calculi are composed of calcium oxalate. Urolithiasis occurs commonly and has been recorded in about 1-5 percent of the world's population. In India, the incidence rate of urolithiasis is higher (10-12%), and is a cause for concern (Thomas and Hall, 2005).

Herbal medicine appears to be a viable treatment option for urolithiasis (Butterweck and Khan, 2009). Botanicals exhibit many pharmacological properties and among these; flavonoids are most effective for urolithiasis. It is assumed that, flavonoids successfully prevent the formation of calcium oxalate stones mainly due to their combined diuretic, antioxidant, anti-inflammatory, antibacterial, and other preventive qualities (Zeng *et al.*, 2019). Catechin is a flavonoid that reduces renal calcium crystallization, renal papillary calcification, calcium oxalate monohydrate formation, and the production of papillary calculus. Two other flavonoids, Rutin and

Quercetin, are considered to have antiurolithiatic properties, as they both prevent and inhibit stone formation.

Materials and Methods

Processing, extraction and phytochemical screening

Samples of *Carica papaya* L.*, Psidium guajava* L.*, Punica granatum* L.*, Mangifera indica* L. and *Musa paradisiaca* L. were procured from the local market. The materials were sliced into pieces, dried in the shade, and finely powdered. Powdered materials were extracted with 70 percent ethanol using the cold maceration process. The resulting extract was dried in a rotary evaporator and stored in a desiccator (Aqil and Ahmad, 2003). Preliminary phytochemical evaluation of fruit extracts was carried out (qualitative estimation) as per standard procedures (Thilagavathi *et al.,* 2015).The HPTLC analysis was carried out to verify the presence of detected phytocompounds.

Preparation and characterization of calcium oxalate crystal

$CaCl_2$ (5 mmol/l) and $Na_2C_2O_4$ solution (7.5 mmol/l) were prepared separately in Tris-HCl (0.05 mol/l) and NaCl (0.15 mol/l) buffer (pH 6.5). The seed calcium oxalate (CaOx) crystals, were prepared using calcium chloride ($CaCl_2$) and sodium oxalate ($Na_2C_2O_4$) solutions (50 mmol/l each) were mixed together, heated to 60°C in a water bath for 1 hour, and then incubated overnight at 37°C. FT-IR analysis was used to confirm the CaOx crystals prepared *in vitro* (Bawari *et al.,* 2018).

The nucleation, aggregation and oxalate depletion assays were used to investigate the effect of plant extract on the production (nucleation), growth (aggregation) and degradation of CaOx crystals.

Nucleation assay

Extract dilutions ranging from 100 to 1000 µg/ml were prepared in distilled water and about 1 ml of each extract concentration was combined with 3 ml of $CaCl_2$ solution, followed by 3 ml of $Na_2C_2O_4$ solution. The final combinations were incubated for 30 minutes at 37°C. The optical density (OD) of the mixtures was measured at a wavelength of 620 nm**.** The percentage inhibition of nucleation by extract was estimated and compared to the percentage inhibition of nucleation by the standard polyherbal medicine, Cystone (Bawari *et al.,* 2018).

Aggregation assay

CaOx crystal solution (0.8 mg/ml) was made in a 0.05 mol/l Tris-HCl and 0.15 mol/l NaCl buffer (pH 6.5). About 1 ml of extract aliquots (100-1000 µg/ml) were added to 3 ml of CaOx solution, vortexed, and incubated for 30 minutes at 37°C. After reading the OD of the final mixes at 620 nm, the percent reduction of aggregation was determined as specified in the nucleation assay (Bawari *et al.,* 2018).

Oxalate depletion assay

Different concentrations of extract (500 µg/ml and 1000 µg/ml) were prepared in distilled water. CaOx crystal slurry (1.5 mg/ml) was prepared in 50 mM sodium acetate buffer (pH 5.7). About 1.5 ml Tris-HCl (10 mM) and NaCl (90 mM) buffer were added to 4 mM $CaCl_2$ solution and 4 mM $Na_2C_2O_4$ solution (1 ml each) (pH 7.4). About 30 µl of CaOx crystal slurry were added to this. The rate of oxalate depletion from the solution was measured at a wavelength of 214 nm for 600 seconds to determine the formation of CaOx crystals. The effect of each concentration of plant extract on crystal development was next tested by adding 1 ml of extract to the reaction mixture (500 µg/ml and 1000 µg/ml) and measuring the change in OD recorded. The percentage of crystal growth inhibition was then calculated as described for the nucleation assay (Bawari *et al.,* 2018).

Statistical analysis

All quantitative results from triplicate trials are given as mean ± SEM (standard error of the mean). Graph Pad Prism 6 software was used to perform statistical calculations, which included one-way analysis of variance (ANOVA) and Tukey Kramer's multiple comparison test. Statistical significance was defined as $P <= 0.05$.

Results

Crystal characterization by FT-IR spectroscopy

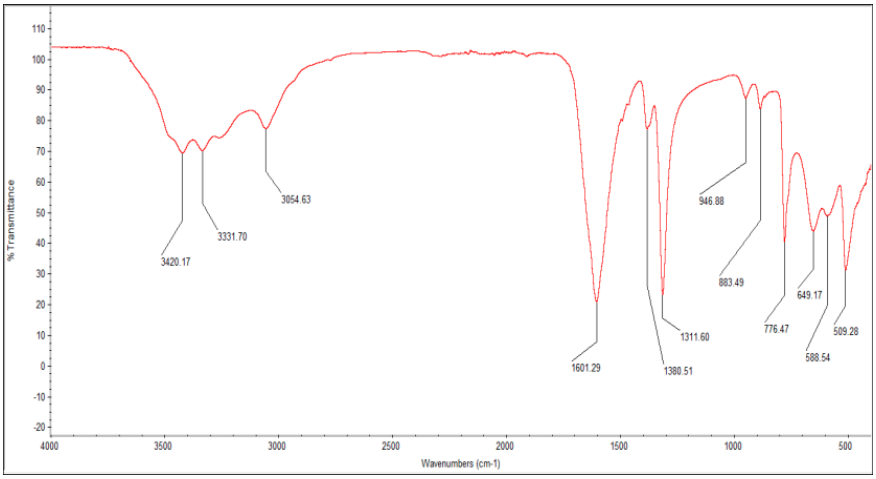
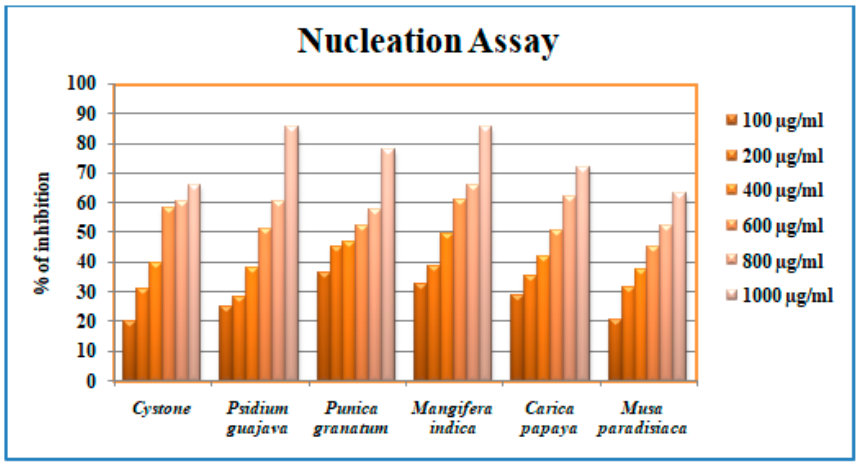


Figure 1: FT-IR Spectra of synthesised CaOx crystal

CaOx crystals synthesized in the laboratory were characterized by FT-IR spectroscopy.The spectrum showed a broad O-H stretching band at 3331.70 cm^{-1}; a strong band at 1609.29 cm^{-1} representing C=O; another band with high absorbance at 1311.60 cm^{-1} representing C-O stretch; C-H bending at 776.47 cm^{-1}. As compared to the data of standard FT-IR spectrum of pure CaOx from the literature (Karahaliloglu *et al.*, 2016), spectrum of *in vitro* synthesized CaOx showed higher absorbance within the same wavelength region. HHHhhhssesweae The spectrum of the synthetic CaOx showed fewer disturbances, which indicates the presence of pure crystal without impurities.

Nucleation assay

The addition of $Na_2C_2O_4$ to a reaction mixture including fruit extract and $CaCl_2$ resulted in the development of CaOx crystals as indicated by turbidity. Fruit extracts in the reaction mixture when observed under the light microscope reduced the nucleation of crystals. Fruit extracts (1000 µg/ml) of *Psidium guajava* and *Mangifera indica* reduced nucleation to a greater extent (85.85±0.74% and85.39±0.79% respectively) than cystone (65.76 ± 0%)($P < 0.05$).*Punica granatum* fruit extract showed a relatively high percent reduction (36.53±0.79 %) even at the lowest concentration studied (100µg/ml)(Table 1, Graph 1). Except for *Musa paradisiaca* (45.21±0 %), all of the extracts tested exhibited a percent reduction greater than 50% at a concentration of 600 µg/ml. When compared to the polyhedral medicine -cystone, fruit extracts from *Psidium guajava*, *Punica granatum*, *Mangifera indica*, and *Carica papaya* showed a higher percent reduction.



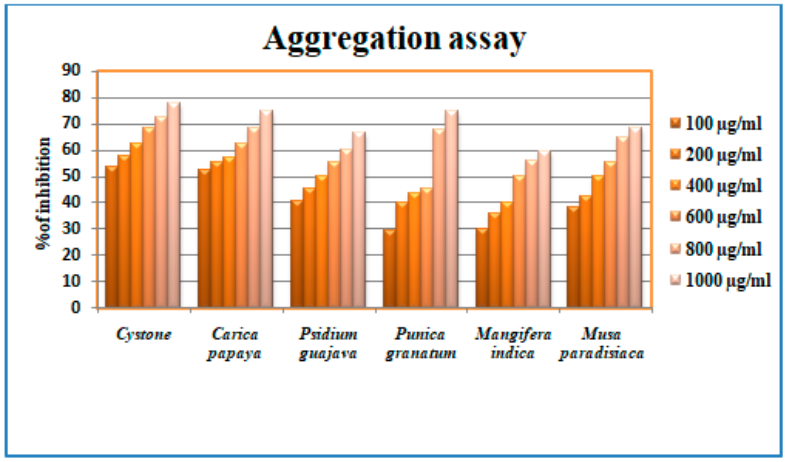
Graph1: Comparative effect of different fruit extracts on nucleation stage of CaOx crystallization

Table 1
Effect of different fruit extracts on nucleation stage of CaOx crystallization

Concentration (µg/ml)	Percentage of inhibition					
	Cystone	*Carica Papaya*	*Psidium guajava*	*Punica Granatum*	*Mangifera indica*	*Musa paradisiaca*
100	20.32±0.38	28.77 ± 0	25.11 ±0.79	36.53 ± 0.79	32.88 ± 0	20.55 ± 0
200	31.05±0.79	35.16 ±0.79	28.31 ±0.79	45.07 ± 1.30	38.81 ± 0.79	31.51 ± 0
400	39.73 ±0	42.01 ±0.79	38.06 ± 0	46.58 ± 0	49.32 ± 0	37.44 ± 0.79
600	58.45±0.79	50.69 ± 0	51.14 ±0.79	52.06 ± 0	61.19 ± 0.79	45.21 ± 0
800	60.28 ±0	61.97 ±0.09	60.28 ± 0	57.99 ± 0.79	65.76 ± 0	52.51 ± 0.79
1000	65.76 ± 0	72.15 ±1.58	85.85 ±0.74	78.09 ± 0	85.39 ± 0.79	63.07 ± 2.32

Aggregation assay

When compared to other test extracts, *Carica papaya* (75.00 ±0) fruit extracts exhibited the highest percentage of inhibition at all concentrations tested (100-1000 µg/ml), however it was less than the positive control cystone (77.00 ±0) at $P<0.05$. Even at the lowest dose tested (100 µg/ml), the percent inhibition values for *Carica papaya* fruit extract (52.50 ± 0%) and cystone (53.33±1.44 %) were comparable. *Punica granatum* (67.50 ± 0and 75.00 ±0) showed better results for higher concentrations only (800 and1000 µg/ml) (Table 2, Graph 2).



Graph 2: Comparative effect of different fruit extracts on aggregation stage of CaOx crystallization

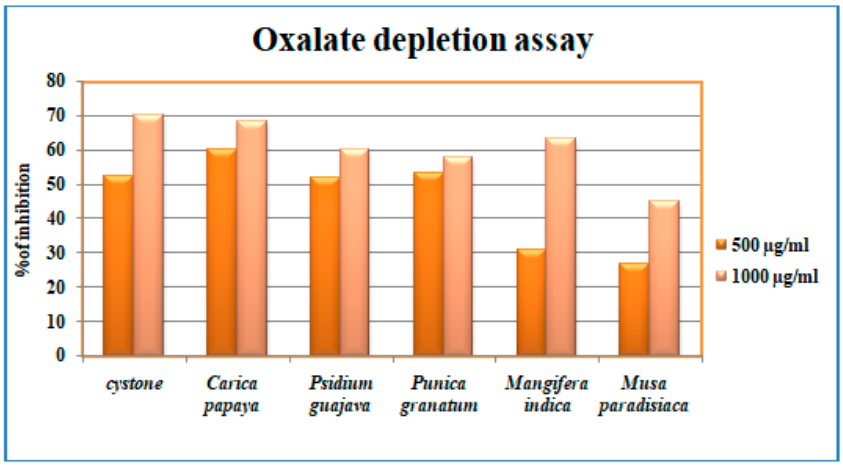
Table 2
Effect of different fruit extracts on aggregation stage of CaOx crystallization

Concen-trations (µg/ml)	Percentage of inhibition					
	Cystone	*Carica papaya*	*Psidium guajava*	*Punica granatum*	*Mangifera indica*	*Musa paradisiaca*
100	53.33±1.44	52.50 ± 0	40.83 ±1.44	29.16±1.44	30.00 ± 0	38.33 ± 1.44
200	57.50 ± 0	55.00 ± 0	45.00 ± 0	40.00 ± 0	35.83± 1.44	42.50 ± 0
400	62.50 ± 0	57.33±0.28	50.00 ± 0	43.40±1.82	40.00 ± 0	50.00 ± 0

600	68.33±1.44	62.50 ± 0	55.00 ± 0	45.00 ± 0	50.00± 0	55.00 ± 0
800	72.50 ± 0	68.33±1.44	60.00 ± 0	67.50 ± 0	55.83± 1.44	65.00 ± 0
1000	77.50 ± 0	75.00 ± 0	66.66 ±1.44	75.00 ± 0	59.16± 1.44	68.33 ± 2.88

Oxalate depletion assay

Oxalate depletion assay was used to monitor the growth of the CaOx crystal. In the presence of fruit extracts, growth was reduced (range 26.57±2.12% to 60.16±3.16% at 500 µg/ml concentration). *Carica papaya,* showed the highest percent decrease (60.16±3.16%) at 500 µg/ml while *Psidium guajava* and *Punica granatum* showed comparable values with that of the standard cystone. At 1000 µg/ml, *Carica papaya* and *Mangifera indica* showed comparable values with cystone. *Musa paradisiaca* showed least percent reduction in both concentrations (26.57±2.12% in 500 µg/ml and 45.10±5.09% in 1000 µg/ml) (Table 3, Graph 3).



Graph 3: Comparative effect of different fruit extracts on growth of CaOx crystallization

Table 3
Effect of different fruit extracts on growth of CaOx crystallization

Concentration (µg/ml)	Percentage of inhibition					
	Cystone	*Carica papaya*	*Psidium guajava*	*Punica Granatum*	*Mangifera indica*	*Musa paradisiaca*
500	52.43±4.40	60.16±3.16	52.11±4.98	53.51±3.96	30.70±2.06	26.57±2.12
1000	70.28±1.92	68.14±6.01	60.07±2.05	57.93±2.07	63.22±3.09	45.10±5.09

It is evident that the fruit extracts possess phytocompounds with inhibition potential. Presence of bioactive compounds in the fruit extracts was evaluated (16 parameters) as per standard procedures and the results are given below

Table 4
Preliminary phytochemical screening of ethanolic fruit extracts

Phytomolecule	*Carica papaya*	*Psidium guajava*	*Punica granatum*	*Mangifera indica*	*Musa Paradisiaca*
Carbohydrates	+	+	+	-	+
Proteins & Amino acids	+	+	+	+	-

Fixed oils and fats	+	-	+	+	-
Gums &mucilage	-	-	-	-	+
Phenols	+	+	+	+	+
Terpenoids	+	+	+	-	+
Flavonoids	+	+	+	+	+
Flavanol	-	-	-	+	-
Tannins	-	+	+	+	+
Phlobatannin	-	-	-	-	-
Alkaloids	+	-	+	+	+
Glycosides	+	-	+	-	-
Anthocyanin	-	-	+	-	-
Quinones	-	-	+	-	-
Phytosterols	+	+	-	-	-
Saponins	+	+	+	-	+

(+: Indicates the presence; - Indicates the absence)

HPTLC analysis

Since the all the tested fruit extracts showed the ubiquitous presence of phenols and flavonoids during preliminary evaluation, existence of flavonoids was confirmed by HPTLC analysis using quercetin as standard.

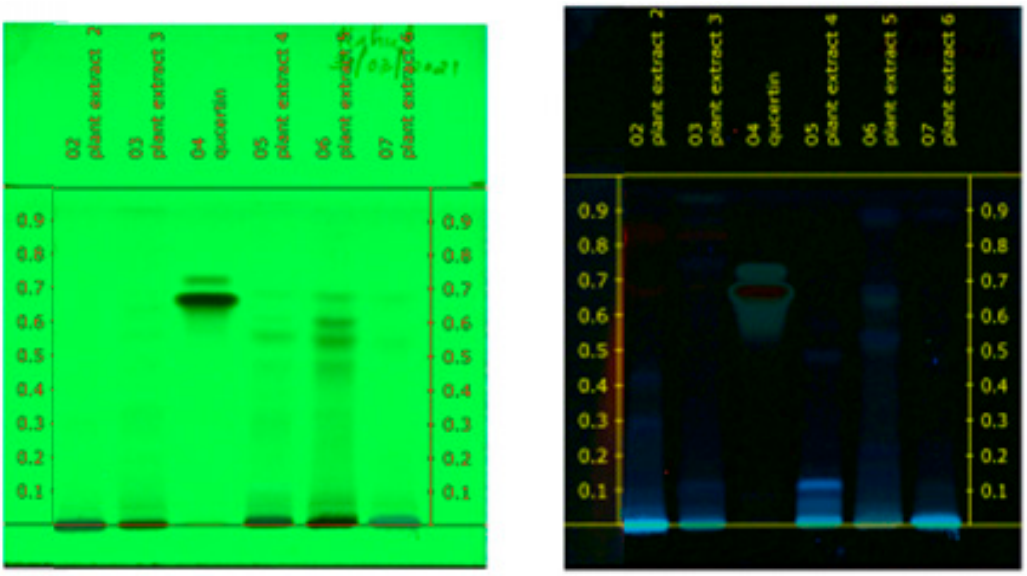


Figure 1: HPTLC Profile of ethanolic fruit extracts under254 nm and 366 nm
(Band labeled in circles represents quercetin)
Figure 4.2.1: HPTLC Profile of ethanolic fruit extracts under RT white light, 254 nm,
Figure 4.2.1: HPTLC Profile of ethanolic fruit extracts under RT white light, 254 nm,

Discussion

Urolithiasis is a complicated condition that involves a number of sequential biological events, including urinary supersaturation, oxidative stress, cell injury, membrane rupture, nucleation, crystal development, crystal aggregation, crystal cell interaction, crystal retention/adhesion, and stone formation (Alelign and Petros, 2018). Supersaturation of the urine with calcium and oxalate salts is the major reason behind kidney stone formation. The stone formation includes nucleation of crystal fractions, growth or gathering of these crystals to a size that can interact with some intra-renal structure(s), confinement of these crystals inside the kidney or renal collecting system succeeded by further aggregation and/ or secondary nucleation ultimately forming the clinical stone (Ratkalkar and Kleinman, 2011).

Pathogenesis of CaOx crystal formation begins with nucleation. Nucleation is a phase change wherein supersaturated substances are impulsively crystallized. Nucleation assay is used to screen the anti-nucleation activity of plant extracts. The anticrystallization activity of plant extract could be related to its ability to complex with free calcium and oxalate ions, thus preventing the formation of CaOx complexes, as has also been suggested for *Sarghassum wightti* (Sujatha *et al*., 2015). The extracts of *Psidium guajava, Mangifera indica, Punica granatum*, and *Carica papaya* inhibited the nucleation of CaOx crystals significantly. These extracts (with the exception of *Musa paradisiaca*) have a stronger inhibitory effect than cystone. The nucleation assay revealed that all of the studied fruit extracts contain some anti-nucleation compounds.

Aggregation is a key determinant in crystal retention which includes numerous crystals in the solution joining together and forming large crystal agglomerates. These agglomerates produce renal tubular obstruction and finally lead to stone formation (Aggarwal *et al.*, 2013). Among tested extracts, *Punica granatum* and *Carica papaya* showed the highest percentage of inhibition on the aggregation of the crystals.

Growth is an important stage in crystal stone formation, wherein agglomerates attain a large enough structure which could make obstruction in renal tubular areas. The result obtained from the present study proved that among tested fruit extracts, *Carica papaya* extract is the best. *Carica papaya* showed a relatively high percent reduction (68.14±6.01) in 1000 µg/ml which is almost comparable to the percent reduction of cystone (70.28±1.92).

Preliminary phytochemical analysis showed the existence of different phytocompounds in tested extracts such as, phenols, tannins, saponins, carbohydrates, proteins and amino acids. All the extracts possess flavonoids as common phytomolecules. Phytocompounds having utmost importance in prevention of urinary calculi includes saponins, tannins, flavonoids and plant phenolics (Bawari *et al.*, 2018).

Oxidative stress is one of the main reasons for many pathological conditions. Likewise, oxidative stress also plays a vital role urinary calculi formation. Plant phenolics and flavonoids also possess antioxidant properties and crystal dissolution properties (Sikarwar *et al.,* 2017). Anti-crystallization, anti-aggregatory, and crystal growth-resisting properties of tested extracts may have resulted from these phytoconstituents in the fruit extracts.

The ethanolic fruit extract of *Carica papaya* could effectively prevent the stone formation at all stages (including nucleation, aggregation, and growth). The current study's findings strongly suggest that *Carica papaya* may be used to prevent and treat calcium oxalate urolithiasis. Fruit extracts of *Psidium guajava* and *Mangifera indica* performed better in the preventative regimen, thus it may be suggested that consuming *Psidium guajava* and *Mangifera indica* can assist to avoid kidney stone formation. *Punica granatum* fruit extract has the capacity to prevent crystal component aggregation. Only in the oxalate depletion assay, cystone gave better results. *Carica papaya* fruit extract had inhibitory activity that was almost identical to that of the standard medication. On the basis of these results, it may be suggested that inclusion of fruits in the diet may help alleviate the discomfort due to urolithiasis.

Although *in vitro* data cannot be directly extrapolated to infer outcomes from more complicated *in vivo* systems, *in vitro* research can provide insight into the activity-related efficacy of investigated drugs or extracts. The present study points out the efficacy of these selected materials to inhibit crystallization of CaOx stones.

Conclusion

It is noticed that four of the five tested fruit extracts (*Carica papaya*, *Psidium guajava, Mangifera indica* and *Punica granatum*) possess inhibitory activity almost identical to the standard medication and may be subjected to further studies.

Acknowledgements

The authors are grateful to the Head, Department of Botany, University of Kerala to the facilities provided.

Reference

Aggarwal, K.P., Narula, S., Kakkar, M. & Tandon, C. (2013) Nephrolithiasis: molecular mechanism of renal stone formation and the critical role played by modulators. *Biomed Res Int.*1(1)1-22

Alelign, T. & Petros, B. (2018). Kidney stone disease: an update on current concepts. *Adv Uro.*1(1),1-13

Aqil, F. & Ahmad, I. (2003) Broad-spectrum antibacterial and antifungal properties of certain traditionally used Indian medicinal plants. *World J Microbiol Biotechnol*. 19(6):653-657

Bawari, S., Sah, A. N. & Tewari, D. (2018) Antiurolithiatic activity of *Daucus carota*: an *in vitro* study. *Pharmacog J.* 10(5):880-884

Butterweck, V. & Khan, S.R. (2009) Herbal medicines in the management of urolithiasis: alternative or complementary? *Planta Med*. 75(10):1095-1103.

Karahaliloglu, Z., Demirbilek, M., Sam, M., Saglam, N., Mızrak, A. K. & Denkbas, E. B. (2016). Surface-modified bacterial nanofibrillar PHB scaffolds for bladder tissue repair. *Artif Cells Nanomed Biotechnol.* 44(1), 74-82.

Moe, O.W.(2006) Kidney stones; pathophysiology and medical management.*Thelancet.* 367(9507):333-344

Ratkalkar, V.N. & Kleinman, J.G.(2011) Mechanisms of Stone Formation. *Clin Rev Bone Miner Metab.* 9(1):187-197

Sikarwar, I., Dey, Y.N., Wanjari, M.M., Sharma, A., Gaidhani, S.N. & Jadhav, A.D.(2017) *Chenopodium album* Linn. leaves prevent ethylene glycol-induced urolithiasis in rats. *J Ethnopharmacol.* 195(4):275-282.

Sujatha, D., Singh, K., Vohra, M., Kumar, K.V. & Sunitha, S.(2015) Anti-lithiatic activity of phlorotannin rich extract of *Sarghassum wightii* on calcium oxalate urolithiasis– *in vitro* and *in vivo* evaluation. *IntBraz J Urol.* 41(3):511-520.

Thilagavathi, T., Arvindganth, R., Vidhya, D. & Dhivya, R. (2015). Preliminary phytochemical screening of different solvent mediated medicinal plant extracts evaluated. *Int Res J Pharmacol.* 6(4), 246-248.

Thomas, B. & Hall, J. (2005). Urolithiasis. *Surgery (Oxford)*. *23*(4), 129-133.

Zeng, X., Xi. Y. & Jiang, W. (2019). Protective roles of flavonoids and flavonoid-rich plant extracts against urolithiasis: a review. *Crit Rev Food Sci Nutr.* 59(13), 2125-2135.

Received: 2 August 2021
Revised & Accepted: 27 October 2021