Haya: The Saudi Journal of Life Sciences (SJLS)
Scholars Middle East Publishers
Dubai, United Arab Emirates
Website: http://scholarsmepub.com/

ISSN 2415-623X (Print)
ISSN 2415-6221 (Online)

Evaluation of *in-vitro* Anti-Urolithiatic Activity of *Citrus limetta* Fruit Peel Extract

Mosrur Ahmed Laskar[1*], Rajeswari CH[2], Shirisha K[3], Dr. M. Chinna Eswaraiah[4]
[1-3]Department of Pharmacology, Anurag Pharmacy College, Kodad, Telangana, India
[4]Principal & Professor, Department of Pharmacognosy, Anurag Pharmacy College, Kodad, Telangana, India

Original Research Article

***Corresponding author**
Mosrur Ahmed Laskar

Article History
Received: 10.03.2018
Accepted: 20.03.2018
Published: 30.03.2018

DOI:
10.21276/haya.2018.3.3.16

Abstract: Urolithiasis is the process of forming stones in the kidney, bladder, and urethra. The prevalence of urolithiasis is approximately 2 to 3 percent in the general population. The development of the stones is related to decreased urine volume or increased excretion of stone-forming components such as calcium, xanthine, oxalate, cystine, urate and phosphate. Herbal plants have been the basis for medical treatments since ancient time, and such traditional medicines still widely practiced today. The scope of herbal medicine is sometimes extended to include fungal and bee products, as well as minerals, shells and certain animal parts. In this study in vitro anti-urolithiatic activity of Citrus limetta was done by two methods. Method A. was test by turbidity method in which method activity of the extract was tested in terms of inhibition of calcium oxalate formation by the method of Prachi Khare et al. with modification and Method B.was test by calcium oxalate dissolution method in this second method the role of plant extract in dissolving the already formed calcium oxalate stones nucleus in artificial system. The experiment was carried out in four arrangements according to the method of Unnati Atodariya et al. with modification. In the method A it was observed that the extract with 100 mg/ml concentration has shown higher percentage of inhibition (35.29%) of calcium oxalate formation than the extract with 250 mg/ml concentration. While the standard drug has shown percentage inhibition of 58.82% and in Method B the Low Dose of the Extract (50 mg/ml) had shown 15.2% of dissolution and High Dose of the Extract (100 mg/ml) had shown 27.7%, while the standard cystone (50 mg/ml) had shown 68% of dissolution of calcium oxalate. From the study it can be concluded that the hydroethanolic extract of Citrus limetta possesses in-vitro anti urolithiatic activity which can be due to the presence of phyto-constituent such as flavonoids present in it.
Keywords: Urolithiasis, kidney, Citrus limetta, hydroethanolic, anti-urolithiatic, turbidity, calcium oxalate, flavonoids.

INTRODUCTION

Herbal medicines are also known as Herbalism or herbology. Herbal medicines are used for medicinal purposes, and the study of such use. Plants have been the basis for medical treatments since ancient time, and such traditional medicines still widely practiced today. Modern medicine recognizes herbalism as a form of alternative medicine, as the practice of herbalism is not strictly based on evidence gathered using the scientific method. The scope of herbal medicine is sometimes extended to include fungal and bee products, as well as minerals, shells and certain animal parts [1]. According to World Health Organization (WHO) 80 percent of the population of some African and Asian countries presently utilize herbal drugs for some aspect of primary health care [2]. Pharmaceuticals are prohibitively expensive for most of the world's population, half of whom lived on less than $2 U.S. per day in 2002 [3]. 'Traditional' use of herbal medicines suggested essential historical use, and this is certainly true for many products which are available as 'traditional herbal medicines'. In many developing countries large proportion of the population depend on traditional practitioners and use herbal medicine for their health care needs. Modern medicine also may exist side-by-side with such traditional practice. These products have become more widely available commercially, especially in developed countries. In this modern medicine system, ingredients are sometimes marketed for uses which were never use in the traditional systems from which they appeared. An example ephedra is use for the weight loss or athletic performance enhancement but in some countries, herbal medicines are subject to strict in manufacturing it , this is not so everywhere. The pharmacological treatment of disease began since ago with the use of herbal medicines.

Urolithiasis is the process of forming stones in the kidney, bladder, and/or urethra (urinary tract). The

prevalence is approximately 2 to 3 percent in general population, and the estimated lifetime risk of developing a kidney stone is about 12 percent for white males [4]. Approximately 50 percent of patients with previous urinary calculi have a recurrence within 10 years [5]. Kidney stones occur in 1 in 20 people at some time in their life. The common signs and symptoms of kidney stones are flank, or groin, blood in the urine and pain in the abdomen. The development of the stones is related to decreased urine volume or increased excretion of stone-forming components such as calcium, xanthine, oxalate, cystine, urate and phosphate. The stones form in the pelvis of the kidney and may range in size from tiny to staghorn stones the size of the renal pelvis itself. The cystine stones compared with the size to a quarter of a U.S. $0.25 coin were obtained from the kidney of a young woman by percutaneous nephrolithotripsy (PNL), cystinuria is characteristic of a procedure for crushing and removing the dense stubborn stones. Certain medications, such as triamterene (Dyrenium), indinavir (Crixivan) and acetazolamide (Diamox), are also associated with urolithiasis. Dietary oxalate is another possible cause, but the role of dietary calcium is less clear, and calcium restriction is no longer universally recommended [6]. Some plants having anti-urolithiatic activity are *Plectranthu-smollisSpreng* ,*Didymocarpuspedicellata, Teraxacumofficinale*, *Dendrophthoe elastic desr* etc. [7]

MATERIALS AND METHODS

Material collection and extract preparation

The fruit peels of *Citrus limetta* were collected from the local juice shop of Kodad Township. The fruit peels were cleaned and dried under sun-shade under standard conditions. Then it was grinded to obtain coarse powder of standard size suitable for extraction. The peel powder was subjected to hydro ethanolic (Water: Ethanol= 60: 40) extraction by Soxhlet apparatus. After that the hydro ethanolic extract was defatted with petroleum ether to remove the chlorophylls. Finally the extracts were air dried. The practical percentage yield will be calculated.

Methods for evaluation of in-vitro anti-urolithiatic activity

Method A: In vitro anti-urolithiatic activity test by turbidity method

In this method the vitro anti-urolithiatic activity of the extract was tested in terms of inhibition of calcium oxalate formation by the method of PrachiKhare*et al*. with modification [8]. The inhibition of calcium oxalate formation in the presence of the extract was compared with the inhibition of calcium oxalate formation in the presence of the standard (Cystone). The precipitation of calcium oxalate at 37°C and pH 6.5 was studied by the measurement of turbidity at 620nm using UV/Vis spectrophotometer. The turbidity caused due to formation of calcium oxalate by the reaction of calcium chloride ($CaCl_2$) with sodium oxalate.

In the control, turbidity due to the formation of calcium oxalate was determined in the absence of any inhibitor. For this, a volume of 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of water were added in a test tube. Then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. Formation of the turbidity results immediately after mixing of above chemicals. The measurement of turbidity was done by measuring the absorption by UV/Vis spectrophotometer at 620 nm after shaking the mixture for 1 min. Then the measurement of the absorbance was carried out after 1 min interval upto a period of 5 min. Absorptions were noted down. The study was continued to know the effect of plants extracts against stone nucleus formation (formation of calcium oxalate) in vitro. In this experiment the effect of the extract on inhibition was carried out in two concentrations of the extract. For this, in one test tube a volume of 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of 100 µg/ml extract in water were added, and then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. In another test tube a volume of 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of 250 µg/ml extract water were added, and then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. The measurement of turbidity was done by measuring the absorption by UV/Vis spectrophotometer at 620 nm after shaking the mixture for 1 min. Then the measurement of the absorbance was carried out after 1 min interval upto a period of 5 min. Absorptions were noted down. After that the effect of the standard (cystone) on the inhibition of the formation of calcium oxalate was studied. The standard drug is a poly herbal formulation. For this a volume of 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of 100 µg/ml of standard in water were added, and then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. The measurement of turbidity was done by measuring the absorption by UV/Vis spectrophotometer at 620 nm after shaking the mixture for 1 min. Then the measurement of the absorbance was carried out after 1 min interval upto a period of 5 min. Absorptions were noted down.

Inhibition in stone nucleus formation was calculated by the graphical method using the following mathematical formula:

$$\text{Inhibition \%} = \{1-[Si / Sc]\} \times 100$$

Where;Si: slope of graph in the presence of inhibitor (drugs/extracts)
Sc: slope of without Inhibitor (Control).

Method B: In vitro anti-urolithiatic activity test by calcium oxalate dissolution method

In this second method the role of plant extract in dissolving the already formed calcium oxalate stones

nucleus in artificial system. For this artificial calcium oxalate crystal were prepare in the laboratory by standard method.

The experiment was carried out in four arrangements as given below according to the method of UnnatiAtodariya*et al.* [9] with modification.

Control

10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2 times and average was taken. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

Extract low dose

10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of 50 mg/ml of the hyroethanolic extract in water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2 times and average was taken. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

Extract high dose

10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of 100 mg/ml of the hyroethanolic extract in water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2 times and average was taken.. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

Standard

10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of 50 mg/ml of the cystone in water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2 times and average was taken.. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

All the flasks were placed for 24 hours. After that membranes were taken out of the flask and content of each membrane was collected in different test tubes. To each test tube 2 ml of 1 N sulphuric acid was added and titrated with 0.9494 N $KMnO_4$ till a light pink colour end point obtained.1ml of 0.9494 N $KMnO_4$ equivalent to 0.1898mg of Calcium.

RESULTS

Method A: In vitro anti-urolithiatic activity test by turbidity method

Table-1: Reading of absorption in the method of inhibition of calcium oxalate formation

Sample	Absorbance After 1 min	Absorbance After 2 min	Absorbance After 3 min	Absorbance After 4 min	Absorbance After 5 min
Control	0.202	0.249	0.262	0.265	0.280
Extract 100µg/ml (A1)	0.043	0.076	0.085	0.089	0.094
Extract 250µg/ml (A2)	0.131	0.152	0.171	0.182	0.193
Standard (Cystone) (A3)	0.036	0.042	0.048	0.056	0.070
Product Control 100µg/ml (A4)	0.001	0.001	0.001	0.001	0.001
Product Control 250µg/ml (A5)	0.003	0.003	0.003	0.003	0.003
Product Control Standard (A6)	0.004	0.004	0.004	0.005	0.005
Corrected absorbance for Extract 100µg/ml (A1-A4)	0.42	0.075	0.084	0.088	0.093
Corrected absorbance for Extract 250µg/ml (A2-A5)	0.128	0.149	0.168	0.179	0.190
Corrected absorbance for standard (A3-A6)	0.032	0.038	0.044	0.051	0.065

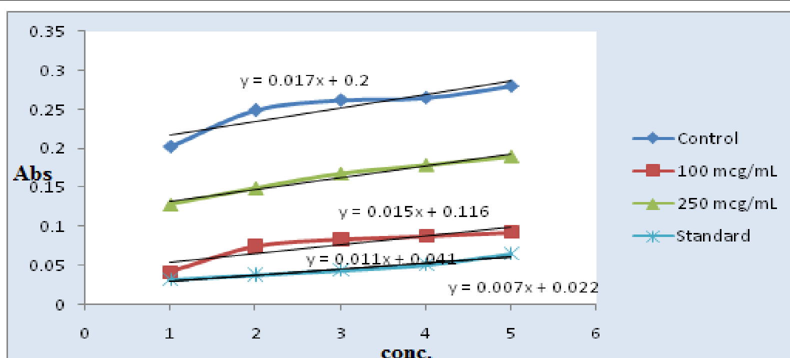

Fig-1: Shows change in turbidity without and with plant extracts and standard at 620nm

Table-2: Calculation of the percentage inhibition from the regression equation

Sample	Regression equation	Slope	% Inhibition
Control	y = 0.017x + 0.2	0.017	-
100μg/ml Extract	y = 0.011x + 0.041	0.011	35.29
250μg/ml Extract	y = 0.015x + 0.116	0.015	11.76
Standard	y = 0.007x + 0.022	0.007	58.82

Method B: In vitro anti-lithiatic activity test by calcium oxalate dissolution method

Table-3: Comparision of percentage dissolution of the calcium oxalate by the extract and the standard

Sample	% of dissolution
Control	-
Low Dose of the Extract (50 mg/ml)	15.2%
High Dose of the Extract (100 mg/ml)	27.7%
Standard (50 mg/ml)	68.0%

Fig-2: In vitro Experimental Dissolution model set-up to evaluate anti-urolithiatic activity

Fig-3: Determination of end point by titration

DISCUSSION

The dried fruit peel of *Citrus limetta* was powdered and grinded to coarse powder. The powder was packed in to Soxhlet apparatus and was extracted with hydroethanolic solution (water: ethanol: 60:40). The hydroethanolic solution is high polar and it is efficient to extract maximum of the components from the substance. After complete extraction (when the eluting solvent becomes colourless) the extract was fractionated with petroleum ether to remove the fatty components. As petroleum ether is a low polar solvent, it removes the fats which are also low polar. The fractionation was repeated till the petroleum ether becomes colourless. After fractionation is over the remainghydroethanolic solution was kept apart for air drying. It took almost fourteen days for complete drying of the extract. The dried extract was weighed. The colour, odour and consistency were recorded. For determining the pH of the extract 1g of the extract was dissolved in 100 ml of water and pH was determined by using pH meter. The pH was found to be acidic (5.05). In order to determine the specific gravity, 1g of the extract was dissolved in 100 ml water, using 25ml specific gravity bottle. The specific gravity was found to be 1.229 g/ml. The in-vitro anti-urolithiatic activity of the extract was evaluated by using two models namely inhibition of calcium oxalate formation (turbidity method) and dissolution of calcium oxalate model.

Inhibition of calcium oxalate formation (turbidity method)

In the first model i.e; inhibition of calcium oxalate formation, the inhibition was measured in terms of turbidity by using UV-Visible spectrophotometer. The turbidity caused due to formation of calcium oxalate. More is the inhibition less will be the turbidity and less absorbance will be observed. The inhibition of calcium oxalate formation in the presence of the extract was compared with the inhibition of calcium oxalate formation in the presence of the standard (Cystone). The study carried out at 37°C and pH 6.5. The measurement of turbidity was done at 620nm using UV/Vis spectrophotometer.

In the control, turbidity due to the formation of calcium oxalate was determined in the absence of any inhibitor (absence of test drug and standard). To study the effect of the extract on inhibition, two concentrations of the extract were used i.e.; 100 µg/ml and 250 µg/ml. For comparison the standard (cystone) was used. The measurement of turbidity was done by measuring the absorption by UV/Vis spectrophotometer at 620 nm after shaking the mixture for 1 min. Then the measurement of the absorbance was carried out after 1 min interval upto a period of 5 min. Similarly for necessary corrections, product control for 100 µg/ml and 250 µg/ml of extracts, product control of standard and the blank absorbance were determined.

Graphs are plotted by taking corrected absorbance in the X-axis and time (in minutes) in the Y-axis. For each graph i.e.; control, corrected absorbance for Extract 100µg/ml (A1-A4), corrected absorbance for Extract 250µg/ml (A2-A5) and corrected absorbance for standard (A3-A6) versus time. Regression equation for each graph was calculated (y=mx+c). From the regression equation the slope of each graph was determined. The the percentage inhibition was calculated by using the formula: % Inhibition= {1-(Si/Sc)} x100. It was observed that the extract with 100 mg/ml concentration has shown higher percentage of inhibition (35.29%) of calcium oxalate formation than the extract with 250 mg/ml concentration. While the standard drug has shown percentage inhibition of 58.82%

Calcium oxalate dissolution method

In the second model the role of plant extract in dissolving the already formed calcium oxalate was evaluated in artificial system. For this artificial calcium oxalate crystal were prepare in the laboratory by standard method. Im this model semipermeable membrane of the farm hen egg was used. 10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of extract (or) standard and packed in semi permeable egg membranes by suturing. For the extracts two concentrations were used 50mg/ml and 100 mg/ml. While for the standard 2ml of 50mg/ml was used. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer. All the flasks were placed for 24 hours. After that membranes were taken out of the flask and content of each membrane was collected in different test tubes. To each test tube 2 ml of 1 N sulphuric acid was added and titrated with 0.9494 N $KMnO_4$ till a light pink colour end point obtained.1ml of 0.9494 N $KMnO_4$ equivalent to 0.1898mg of Calcium. After that the percentage of dissolution was calculated. It was observed that the Low Dose of the Extract (50 mg/ml) had shown 15.2% of dissolution and High Dose of the Extract (100 mg/ml) had shown 27.7%, while the standard cystone (50 mg/ml) had shown 68% of dissolution of calcium oxalate.

The observed anti-urolithiatic activity of the hydroehanolic extract of the fruit peel of *Citeuslimetta* might be due to the various phytocostituents present in it. Flavonoids are reported to be present in the peel [34] and flavonoids containg plant have been reported to be associated with anti-urolithiatic activity [9,10]

CONCLUSION

From the study it can be concluded that the hydroethanolic extract of *Citrus limetta* possesses in-vitro anti urolithiatic activity which can be due to the presence of phyto-constituent such as flavonoids present in it. Further in-vivo study can be suggested to investigate the potential in detail.

REFERENCES

1. Winslow, L. C., & Kroll, D. J. (1998). Herbs as medicine. *Arch Intern Med., 15*, 219-299.
2. Medicinal and aromatic plants trade programme, Available from http://www.traffic.org/medicinal-plants.
3. DaSilva, E. J., Baydoun, E., & Badran, A. (2002). Biotechnology and the developing world. *Electronic Journal of Biotechnology, 5*(1).
4. Menon, M. (1998). *Urinary lithiasis*: etiology, diagnosis, and medical management. Campbell's urology, Ch-91.
5. Uribarri, J., Oh, M. S., & Carroll, H. J. (1989). The first kidney stone. *Annals of internal medicine, 111*(12), 1006-9.
6. Curhan, G. C., Willett, W. C., Rimm, E. B., & Stampfer, M. J. (1993). A prospective study of dietary calcium and other nutrients and the risk of symptomatic kidney stones. *New England Journal of Medicine, 328*(12), 833-8.
7. Baheti, D. G., Kadam, S. S. (2013). Antiurolithiatic activity of some traditional medicinal plants against calcium oxalate induced urolithiasis in rats. *International Journal of Pharmaceutical, Chemical and Biological Sciences, 3*(4), 1276-1285.
8. Khare, P., Mishra, V. K., Kakkar, A., Bais, N., Singh, R. (2014). Study on in vitro anti-lithiatic activity of Phyllanthusniruri Linn. Leaves by homogenous precipitation and turbiditory method. *International Journal of Pharmacy And Pharmaceutical Sciences, 6*(4), 124-127
9. Barad, U., Upadhyay, R., Upadhyay, S. (2013). Anti-urolithiatic activity of Dolichosbiflorus seeds. *Journal of Pharmacognosyand Phytochemistry, 2*(2), 209-213.
10. Phatak, R. S., & Hendre, A. S. (2015). In-vitro Antiurolithiatic Activity of *Kalanchoepinnata* Extract. *International Journal of Pharmacognosy and Phytochemical Research, 7*(2), 275-279.