IN VITRO CALCIUM OXALATE CRYSTALLIZATION INHIBITION POTENTIAL OF *CHENOPODIUM ALBUM*

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ABSTRACT

Chenopodium album is one of the renowned medicinal plants utilized in the treatment of a variety of metabolic disorders. In the present investigation, the aqueous extracts of fresh leaves Chenopodiumalbum were selected for the phytochemical characterizations and determination of In Vitro calcium oxalate crystallization inhibition potential of Chenopodiumalbum which was evaluated and next compared with the positive control Cystone both in diverse concentration (100µg/ml –500µg/ml). The aqueous extract of fresh leaves was further subjected to phytochemical screening and found to contain alkaloids, tannins, coumarins, flavonoid, Emodins, quinones and saponins. The present research work was executed under In Vitro conditions by using two critical assays Viz. Crystal nucleation Assay and Aggregation assay. The inhibitory potential of four solvent like aqueous, ethanol, chloroform and petroleum ether extracts of the Chenopodium album were tested for the Calcium oxalate crystal formation and aggregation, under In Vitro conditions which are chiefly present in most of the kidney stones. The nucleation and aggregation of calcium oxalate (CaOX) crystals were examined separately using Spectrophotometric methods. A result obtained revealed that aqueous leaves extract of Chenopodium album has the excellent capacity to inhibit the calcium oxalate (CaOX) crystal formation and aggregation.

Keywords: *In Vitro, Chenopodium album, Antilithiatic potential, Crystal nucleation Assay, Aggregation assay.*

Introduction

Stone formation in the kidney is one of the oldest and most commonly spread diseases which are known to man. It is one of the most frequent and painful urologic disorder of the urinary tract affecting millions of people every year (Adhirajan*et al*., 2003).The life span risk of developing lithiasis ranges between 10-12% (Aggarwal *et al*., 2010) and significantly affects the economy and public health as it has a high rate of recurrence (Alok*et al*., 2008). Urinary lithiasis is normally the result of an imbalance between inhibitors and promoters in the kidney (Atmani*et al*., 2004). Promoters of stone formation facilitate stone formation whereas inhibitors prevent it. Approximately 85% of the stones in humans are calcium stones which comprise oxalate and phosphate, either alone or combined (Basavaraj*et al*., 2007). Of these, the crystals of calcium oxalate (CaOX) are the primary constituents of more than 60% of the majority of human kidney stones: they exist in the form of CaOX monohydrate (COM) and CaOXdihydrate (COD) (Beghalia*et al*., 2008). In spite of substantial improvement in the pathophysiology and treatment of urolithiasis, there is no satisfactory drug offered for use in clinical therapy. Endoscopic stone removal and extracorporeal shock wave lithotripsy are prohibitively costly and recurrence is pretty common with these types of procedures (Bensatal, A. and Ouaharani, M.R. 2008). Thus, the search for antilithiatic drugs from natural sources has supposed greater importance (Chauhan, C.K. and Joshi, M. J., 2008).

Medicinal plants are considered as rich resources of ingredients which can be used in drug development either pharmacopeia-non pharmacopeia or synthetic drugs. These plants play a vital role in the development of human cultures around the world. Moreover, some plants are highly considered and are highly recommended for therapeutic value. Over the part of two decades, there has been a tremendous increase in the use of herbal medicine; however, there is still a significant lack of research till date in this field. Plants are a good source of various phytochemicals like alkaloids, flavonoids, glycosides, proteins, phenolic groups and reducing sugars. Such phytochemicals are responsible for medicinal activity of plant species. They have ability for curing various ailments and also possess

potential anti-diabetic, anti-bacterial, antioxidant and anti-fungal properties. Natural products from medicinal plants extracted as pure compounds offer opportunities for a novel drug since plants have unmatched accessibility of chemical diversity. Due to high chemical diversity, screening programmes seeking therapeutic drugs from herbal products are quite interesting throughout the world.

Concerning the treatment of urinary stone diseases, a number of medicinal plants are available (Das, I. and Verma, S., 2008; Daudon, M. and Jungers, P., 2001; Freitas *et al*., 2002). While the plants are claimed to be low-cost, nontoxic, and available in rural areas, culturally acceptable and found to be traditionally effective for the urinary diseases and disorders. The herbal medicines have been utilized to help in urolithiasis through diuretic, anti-inflammatory, antimicrobial and litholytic properties (Atmani, F. and Khan, S.R., 2000).

Chenopodium album L. (family: Chenopodiaceae) is an herbaceous vegetable plant locally acknowledged as Bathua. It is cultivated as pot-herb and frequently grown in gardens, but can be found in the corner of early grain fields and elsewhere. The medicinal property of this plant is chiefly present in leaves. The leaves of *Chenopodium album* are used in ethno-medicinal practices for treatment of kidney diseases and urinary stones. That's why, the present study was undertaken to assess antiurolithiatic activity of the*Chenopodium album.*

2. Materials and Methods

2.1 Materials

All the chemicals employed in the present investigation were of analytical grade. $Na_2C_2O_4$ (Sodium oxalate), NaCl (Sodium chloride) and $CaCl_2$ (Calcium chloride) was purchased from Qualigens, Thermo scientific.

2.2 Drugs and Chemicals

Water, chloroform, ethanol and petroleum ether, etc. chemicals used specifically.

2.3 Collection of plant

The plant material *Chenopodium album* was collected from the local area of Nanded, Maharashtra. Identified parts of the plants viz. leaves of *Chenopodium album* and then shade dried. Dried leaves were cleansed of extraneous matter and then ground to fine powder in a grinder.

2.4 Preparation of Plant Extract

The 50g powdered plant material was extracted by using Hot continuous Soxhlet extraction method. The plant material was extracted with solvents like water, Ethanol (99.9% v/v), Chloroform and Petroleum ether each of 500ml for four days in a Soxhlet apparatus (Muthusamy P. *et al*., 2009).

It is a continuous extraction method in which the solvent can be circulated for several times through the extractor. The vapors from the solvent are taken to the condenser and the condensed liquid is returned to the extract for continuous extraction .The apparatus consist of a body of extractor (thimble) which was attached with a side siphon tube. The lower end connected with distillation flask and the mouth of the extractor is fixed by the standard joints to the condenser.

2.5 Qualitative Phytochemical Characterizations of *Chenopodium album*

Qualitative phytochemical characterizations of *Chenopodium album* was carried out to detect the presence of alkaloids, sterols, saponins, tannins, terpenes, carbohydrates and phenolic substances.

2.6 *In Vitro*Antiurolithiatic Activity of *Chenopodium album* under Study

2.6.1 Preparation of reagents and solution

All the chemicals used were of AR grade. Crystalloid forming solutions, *i.e.* solution of calcium acetate and sodium oxalate for calcium oxalate crystal formation were prepared in distilled water.

2.6.2 Inhibition assay

Antiurolithiatic activity in extract of the medicinal plant *Viz.Chenopodium album* was investigated as per the method of N. A. M. Farrook*et al*., (Sangeetha S. J. and Muniyandi J. 2004) with minor modifications. The whole amount of extract solutions (50mL) was placed in the beaker in the beginning itself and the two salt forming solutions were allowed to run into it drop wise through burettes. As a result, a reservoir of the extract solutions was produced

into which the salt forming solutions ran down. Lastly, the mixture was boiled on a heating mantle for a period of 10min., then allowed cooling at room temperature and the precipitate was collected into a pre-weighed centrifuge tube by centrifuging (Remi centrifuge) small volumes at a time and discarding the supernatant liquid. Further, the tube with the precipitate was dried out in a hot air oven, then cooled to room temperature and weighed till constant weight by using a weighing balance. The Weight of the precipitate was done. Concurrently blank experiments (Control) with water in place of extract were also performed for evaluating the inhibition efficiency of inhibitors when compared to water. All the experimentation was carried out at room temperature (RT).

2.6.3 Nucleation assay

Solution of calcium chloride (5mmol/l) and sodium oxalate (7.5mmol/l) were prepared in a buffer containing Tris–HCl 0.05mol/l and NaCl 0.15mol/l at pH 6.5. Nine milliliter (9mm) of calcium chloride ($CaCl_2$) solution was mixed with 1ml of plant extracts under study at different concentrations (100mg/ml, 200mg/ml, 300mg/ml, 400mg/ml, and 500mg/ml). Crystallization was initiated after adding 950ml of sodium oxalate solution. The temperature was kept at 37^0C. After 30min, the optical density (OD) of the solution was monitored at 620 nm. The degree of nucleation was estimated by comparing the induction time in the presence of extract with that of control (Paras *et al.*, 2012).

$$\text{Percent (\%) inhibition} = \frac{\text{(Absorbance Control – Absorbance Test)}}{\text{Absorbance Control}} \times 100$$

2.6.4 Aggregation assay

The method used for aggregation described by (Atmani*et al.*, 2003; Paras *et al.*, 2012) was modified. Calcium oxalate (CaOX) crystals were made by mixing sodium oxalate and calcium chloride (CaOX) at 50mmol/l. Both solutions were equilibrated to 60^0C in a water bath for 1 hour and then cooled to 37^0C overnight. The crystals formed were harvested by using centrifugation and then evaporated at a temperature of 37^0C. Calcium oxalate crystals were utilized at a final concentration of 0.8mg/ml, buffered with sodium chloride (NaCl) 0.15mol/l, Tris–HCl 0.05mol/l and at pH 6.5. Experiments were carried out at 37^0C in the presence or absence of the plant extract. The percentage aggregation inhibition was calculated by comparing the turbidity in the presence of SXS at different concentrations (100–500µg/ml) with that obtained in the control using following formula:

$$\text{\% inhibition} = \frac{\text{1-Turbidity sample}}{\text{Turbidity control}} \times 100$$

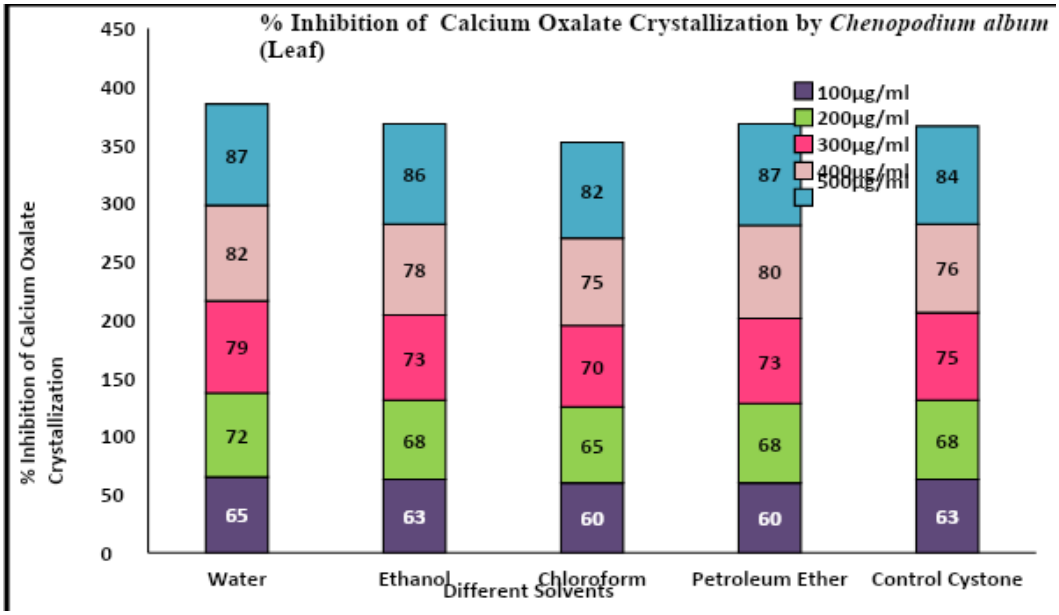
3. Results and Discussions

The % Inhibition of Calcium Oxalate Crystallization by *Chenopodium album* (Leaf) was studied and is depicted in **Figure 1.** The extract was prepared in four different solvents like water, ethanol, chloroform and petroleum ether. The extract inhibited crystal formation in addition to promoting crystal dissolution in a dose dependent mode which was comparable to the activity of Cystone (Control).

From all the extracts, the highest % inhibition of nucleation was obtained from water and petroleum extract (87%) respectively at a concentration of 500µg/ml, the second highest % inhibition of nucleation was found from ethanol (86%), chloroform (82%) at a concentration of 500µg/ml. The water, ethanol, chloroform and petroleum ether extracts (500µg/ml) were found to possess best antiurolithiatic activity when compared to standard Cystone (500µg/ml). The pronounced antiurolithiatic activity shown by the aqueous extract of *Chenopodium album* (Leaf) at a concentration of 100µg/ml to 500µg/ml is

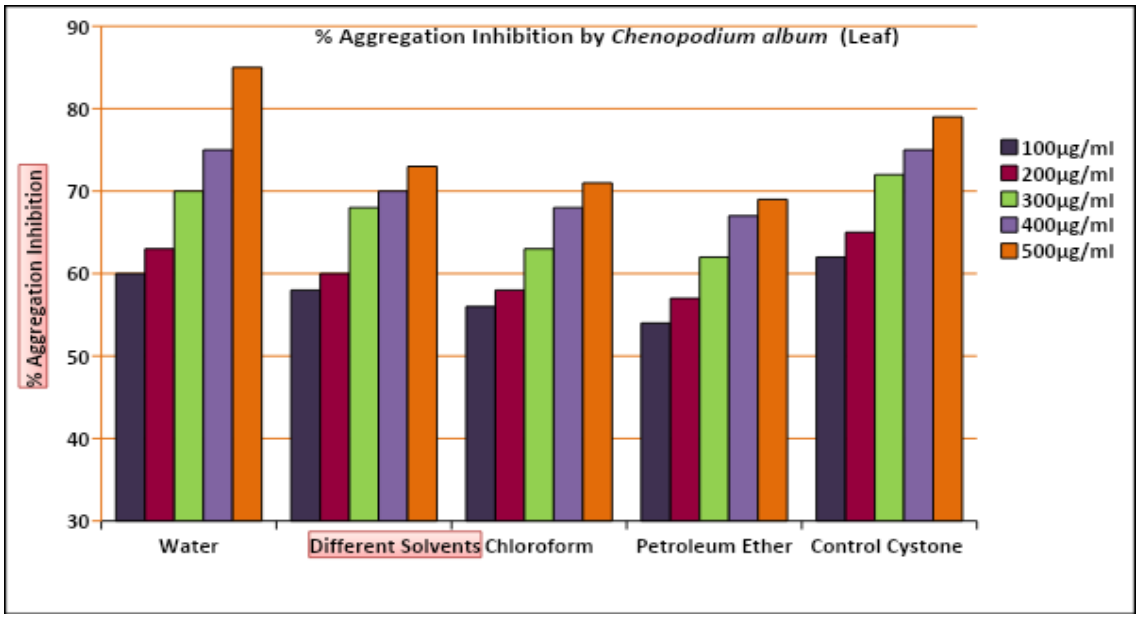
65%, 72%, 79%, 82% and 87% respectively whereas the ethanol extract showed 63%, 68%, 735, 78% and 86% respectively at the above said concentration. The chloroform extract was found to have the % crystallization inhibition is 60%, 65%, 70%, 75% and 82% respectively while the petroleum ether extract exhibited 60%, 68%, 73%, 80% and 87% respectively at the same concentration.

Figure 1: % Inhibition of Calcium Oxalate Crystallization by *Chenopodium album* (Leaf)



The antiurolithiatic activity shown by the Cystone (Control) of *Chenopodium album* (Leaf) at a concentration of 100µg/ml to 500µg/ml is 63%, 68%, 75%, 76% and 84% respectively. Conversely, there was no significant difference in the antiurolithiatic potential when compared with Cystone. Even though, % inhibition of nucleation is found to excellent by water extract (87%) when compared to Cystone (84%) at 500µg/ml concentration (**Figure 1)**

Figure 2: % Aggregation Inhibition by *Chenopodium album* (Leaf)



The % Inhibition of Calcium Oxalate Crystal aggregation by *Chenopodium album* (Leaf) was studied and the results represented in **Figure 2 The** leaf extract were prepared in four different solvents like water, ethanol, chloroform and petroleum ether. The leaf extract inhibited the crystal aggregation while in absence of these leaf extract the crystals were extremely aggregated as shown in **Figure 2.**

It is evident from Figure **2** that the % inhibition of aggregation was highest (85%) in the presence of aqueous leaf extract of *Chenopodium album* and it was lowest (79%) at the highest concentration of control (500µg/ml). The crystals aggregated in the presence of aqueous leaf extract were less than that in the control, indicating that the crystals were less aggregated.

The percent inhibited aggregation associated with the aqueous extract of leaf at concentration of 100µg/ml-500µg/ml was found to be 60%, 63%, 70%, 75% and 85% respectively whereas the percent inhibited aggregation associated with the ethanol extract of leaf at concentration of 100µg/ml-500µg/ml was found to be 58%, 60%, 68%, 70% and 73% respectively. Furthermore, the percent inhibited aggregation associated with the chloroform extract of leaf at concentration of 100µg/ml-500µg/ml was found to be 56%, 58%, 63%, 68% and 71% respectively while the percent inhibited aggregation associated with the petroleum ether extract of leaf at concentration of 100µg/ml-500µg/ml was found to be 54%, 57%, 62%, 67% and 69% respectively. The percent inhibited aggregation associated without the leaf extract (Control) at concentration of 100µg/ml-500µg/ml was found to be 62%, 65%, 72%, 75% and 79% respectively.

It can be concluded from Figure **2** that the % aggregation inhibition of crystal is excellent by *Chenopodium album* aqueous leaf extract (85%) when compared with the control (79%) at 500µg/ml concentration.

These two phenomena represented the entire process of *In-Vitro* crystallization as observed previously by Hess *et al.*, (2000).

Simultaneous addition of CAAE (500 and 1000µg/ml) and cystone (1000µg/ml) in conjunction with salt dihydrate inhibited the nucleation also as aggregation process of CaOx crystallization as indicated by dose-dependent decrease in turbidity of the answer in both phenomena. The inhibition of *In-Vitro* crystallization of CaOX suggests that CAAE has influence on the formation of crystals from sodium oxalate and salt and/or their aggregation. Most of the previous papers (Fouda*et al.*, 2006; Saha S. and Verma R.J., 2013) stated that the test drug or extract inhibited the crystallization by favoring the formation of calcium oxalate dehydrate (COD) crystals instead of calcium oxalate monohydrate (COM).

The results revealed that CAAE has noteworthy influence on the formation, growth and dissolution of the crystals which further suggest that the extract has beneficial effects in preventing the crystals formation and their growth.

Literature of the previous studies is silent on the precise mechanism involved within the inhibition of *In-Vitro* crystallization and stated that the extracts contained some chemical components that inhibited the crystallization. There are reports that flavonoids inhibit calcium oxalate crystallization in human urine also as in animal models (Zhong*et al.*, 2012) and crystal deposition (Noorafshan*et al.*, 2013). Saponins revealed anti-crystallization potential thereby disaggregating the suspension of mucoproteins which are the promoters of crystallization (Gurocak S. and Kupeli B., 2006). The phytochemical screening and estimation of important constituents revealed that CAAE have flavonoids and saponins. It's total flavonoid content of 87.23mg quercetin equivalents/g of extract and total Saponins content of 1.6mg saponins/100g of powder mass. Thus, the flavonoids and saponins present could even be playing a contributing role in the anti-crystallization action of CAAE. Fouda*et al.*, (2006) showed in their investigation that the effect of In-Vitro and In-Vivo antilithiaticpotentials of saponins rich fraction of *Herniariahirsuta* and stated that the fraction contained a substance which promoted the nucleation of COD crystals (Fouda et al., 2006). Finally, the results of these investigations suggest that the leaves of the *C. album* have *In-Vitro* anti-crystallization upshot on calcium oxalate (CaOX) and brushite

crystals. These findings substantiate the normal use of the leaves within the treatment of urinary stones and kidney problems.

4. Conclusions

The results of the present investigation on the evaluation of antiurolithiatic activity of different solvent extracts *Viz*. water, ethanol, chloroform and petroleum ether in Urolithiasis have led to the conclusions that aqueous extract is more potent than the other extract in the urolithiasis. Flavonoids, Polyphenols and saponins present in the extracts may be responsible for the antioxidant and antilithiatic activity of the extracts.

References

1. Adhirajan, N., Kumar, T.R.S., Shanmugasundaram, N. and Babu, M. (2003). In Vivo and In Vitro evaluation of hair growth of Hibiscus rosasinensis. Journal of Ethnopharmacology, 88:235-239.
2. Aggarwal, A., Tandon, S., Singla, S.K. and Tandon, C. (2010). Diminution of oxalate induced renal tubular epithelial cell injury and inhibition of calcium oxalate crystallization in vitro by aqueous extract of Tribulusterrestris, International Braz. J.,36:480-489.
3. Alok, S.H., Subharwal, M., Rawl, S. and Mohor, A. (2008). Herbal drugs in antilithiasis: A review, Int. J. Pharma Res. Dev, 1:1-7.
4. Atmani, F. and Khan, S.R. (2000). Effects of an extract from Herniariahirsuta on calcium oxalate crystallization In Vitro, British Journal of Urology International, 85:621-625.
5. Atmani, F., Farell, G., Lieske, J.C. (2004). Extract from Herniaria hirsute coats calcium oxalate monohydrate crystals and blocks their adhesion to renal epithelial cells, J. Urol., 172:1510–1514.
6. Basavaraj, D.R., Biyani, C.S., Browning A.J. and Cartilage J.J. (2007). The role of urinary kidney stone inhibitors and promoters in the pathogenesis of calcium containing renal stones, EAU-EBU Update Series, 5:126-136.
7. Beghalia, M., Ghalem, S., Allali, H., Belouatek, A. and Marouf (2008). A. Inhibition of calcium oxalate monohydrate crystal growth using Algerian medicinal plants. Journal of Medicinal Plants Research, 2:066-070.
8. Bensatal, A. and Ouaharani, M.R. (2008).Inhibition of crystallization of calcium oxalate by the extraction of Tamariagallica, L. Uol, Res., 36:283 -287.
9. Chauhan, C.K. and Joshi, M. J. (2008). Growth inhibition of Struvite crystals in the presence of Citrus medica Linn. Urol. Res., 36: 265-273.
10. Das, I. and Verma, S. (2008). Human stones: dissolution of calcium phosphate and cholesterol by edible plant extracts and bile acid, Journal of Science and Industrial Research, 67:291-294.
11. Daudon, M. and Jungers, P. (2001). Epidémiologie de la lithiaseurinaire.,Euro. Biologiate, 253:5-15.
12. Fouda A., Yamina S., Nait M.A., Mohammed B., and Abdlekrim R. (2006). In-Vitro and In-Vivo antilithiatic effect of saponin rich fraction isolated from Herniariahirsuta. J Bras Nefrol.,28:199–203.
13. Freitas, A.M., Schor, N. and Boim, M. A. (2002). The effect of Phyllanthusniruri on urinary inhibitors of calcium oxalate crystallization and other factors associated with renal stone formation, BJU Int., 89:829-834.
14. Gurocak S. and Kupeli B. (2006). Consumption of historical and current phytotherapeutic agents for urolithiasis: a critical review. J Urol., 176:450–5.
15. Hess B., Jordi S., Zipperle L., Ettinger E. and Giovanoli R. (2000). Citrate determines calcium oxalate crystallization kinetics and crystal morphology-studies in the presence of Tamm- Horsfall protein of a healthy subject and a severely recurrent calcium stone former. Nephrol Dial Transplant, 15:366–74.
16. Noorafshan A., Doust S.K. and Karimi F. (2013). Diosmin reduces calcium oxalate deposition and tissue degeneration in

nephrolithiasis in rats: a stereological study. Korean J Urol., 54:252–7.

17. Saha S. and Verma R.J. (2013). Inhibition of calcium oxalate crystallization In-Vitro by an extract of Bergenia ciliate. Arab J Urol., 11:187–92.
18. Zhong Y.S., Yu C.H., Ying H.Z.,Wang Z.Y. and Cai H.F. (2012). Prophylactic effects of OrthosiphonstamineusBenth. extracts on experimental induction of calcium oxalate nephrolithiasis in rats. J Ethnopharmacol.,144:761–7.