

EFFECT OF DECOCTION EXTRACTS OF SOME MEDICINAL PLANTS ON CALCIUM OXALATE CRYSTALLIZATION

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ABSTRACT

Objective: To study the inhibitory effect of decoction extracts of some medicinal plants on the crystallization of calcium oxalate.

Methods: The extraction was done by decoction method and *in-vitro* anti-urolithiatic activity was evaluated by the turbidometric method.

Results: *Terminalia* species could be a promising source for curing urinary stone disease.

Conclusion: Further, these *in-vitro* results should be confirmed by *in-vivo* studies to develop a potent antilithiatic agent from these plant species.

Keywords: *Terminalia* species, Medicinal plants, Anti-urolithiatic activity, Nucleation, Calcium oxalate crystallization, Microscopy.

INTRODUCTION

Urolithiasis is the formation of urinary calculi or urinary stones in any part of the urinary tract such as kidneys, ureters, urinary bladder, and urethra [1]. Stone formation is a complex process which occurs due to the successive physicochemical events such as supersaturation, nucleation, growth, aggregation, and retention within the renal tubules [2]. As far as the urinary system is concerned, it is a third prevalent disorder which frequently starts with obstruction and if left untreated results in severe complications like multiple infections, hemorrhage, etc., suggesting need of medical care [3]. In India, 12% of the populations are predicted to have urolithiasis and nearly 15% of the population of North India suffers from kidney stones [4].

There are several types of stones, but the most common ones are calcium oxalate (Ca-oxalate) accounting for more than 80% of stone [5] followed by calcium phosphate [6]. The remaining 20% are composed of struvite, cystine, uric acid, and other stones [7]. Kidney stones cause serious health problems such as severe pain, urinary tract obstruction, and infection that adversely affect comfort of individuals. Treatment options are extracorporeal shock wave lithotripsy (ESWL), ureteroscopy, percutaneous nephrolithotomy, open or laparoscopic stone removal, etc., are costly and painful. ESWL may cause acute renal injury, decrease in renal function, and an increase in stone recurrence. Many synthetic drugs like diuretics and narcotic analgesics are used in the treatment of kidney stone but excess intake of synthetic drugs results in the higher occurrence of adverse drug reactions and people tend to go for natural remedies which are considered as safe, easily available, and recurrence chances are less [8,9]. Thus, phytotherapy has a promising role in the prevention and cure of this renal disorder. There are a number of plants which show promising anti-urolithiatic activity [10].

The extract mechanisms of formation of kidney stone are not fully understood but there are series of physicochemical events which lead to stone formation, which includes super-saturation, nucleation, growth, aggregation, and retention within the renal tubules [11]. Urine is normally super-saturated with most stone forming salt components as well as chemicals that prevent or inhibit crystal development in urinary tract. In many of the individuals, the crystallization inhibiting capacity of urine does not allow urolithiasis but in stone forming individuals the inhibition capacity is deficient [12]. Thus, an imbalance

between promoters and inhibitors of crystallization in urine causes stone formation [13]. Low urine volume, low urine pH, Ca-oxalate, sodium oxalate (Na-oxalate) are some of the promoters of stone formation while citrate, Mg, inorganic inhibitors, organic inhibitors, prothrombin fragment, glycosaminoglycans, and osteopontin are some of the inhibitors of stone formation [9,14-15]. One of the reasons for this imbalance is oxidative stress which generates free radicals or reactive oxygen species which damages epithelium of kidney or bladder, thereby producing a favorable environment for crystal attachment to surface. This does not allow the stone to be discharged from the ureter causing pain, discomfort and block the urine flow from the kidney [16].

Considering the above, in the present work, some traditionally useful medicinal plants were selected to evaluate the inhibitory potential of Ca-oxalate crystallization in *in-vitro* conditions. The selected plants and their uses are given in Table 1.

METHODS

Plant collection

Aerva lanata was collected from sea-shore region of Veraval; *Terminalia* species were collected from Jamnagar; *Zea mays* corn hair was collected from the local market of Rajkot, Gujarat, India. *Tribulus terrestris* and *Boerhaavia diffusa* were purchased from Rajkot, Gujarat, India. They were thoroughly washed, separated, and dried under shade. The dried plant parts were homogenized to fine powder and stored in air tight bottles which were later used for extraction.

Extraction

The extraction was done by decoction extraction method [17]. The details are as described earlier [18].

In-vitro anti-urolithiatic activity by turbidometric method

The effect of different plant extracts on Ca-oxalate crystallization was determined by the time course measurement of turbidity changes due to crystal formation and aggregation in the metastable solutions of Ca-oxalate. The precipitation of Ca-oxalate was studied by the measurement of turbidity at 620 nm [19]. A spectrophotometer ultraviolet/visible was employed to measure the turbidity of the formation of Ca-oxalate. Pure chemicals including calcium chloride dehydrate $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (SRL), sodium chloride NaCl (SRL), sodium

acetate anhydrous $C_2H_3NaO_2$ (SRL), and sodium oxalate $C_2O_4Na_2$ (SRL) were used for this study.

RESULTS AND DISCUSSION

Urolithiasis is a major problem affecting many people since ages. The main cause of stone formation is Ca-oxalate and calcium phosphate accumulation. The various stages or steps involved in the accumulation of these two substances include nucleation, crystal growth, crystal

aggregation, and crystal retention [20]. The nucleation occurs because of supersaturation and this is the first step in the formation of a renal stone in the form of a solid crystal. The nucleation is the establishment of the smallest unit lattice of a crystal species, the first step in crystal formation. There are two types of nucleation, viz., homogeneous nucleation and heterogeneous or secondary nucleation [21]. The former occurs because of nucleation in pure solution, whereas the latter is because of accumulation of new crystals on pre-existing crystals.

Table 1: Name of the selected medicinal plants, part used and its traditional uses

No	Name (family)	Part used	Traditional use
1	<i>A. lanata</i> L. (Amaranthaceae)	Leaf and stem	Treatment of headache, lithiasis, cough, removal of kidney stone. It maintains blood sugar to optimum level
2	<i>T. bellirica</i> (gaertn.) roxb. (Combretaceae)	Leaf and stem	Bark is useful in leucoderma. The fruits are useful in vitiated condition of vata and pitta, cough, bronchitis, dropsy, vomiting, skin diseases, leprosy, ulcer, fever and general debility. Oil obtained from the seeds useful in skin diseases, leucoderma, and grayness of hair
3	<i>T. chebula</i> retz. (Combretaceae)	Leaf and stem	Useful in vitiated condition of tridoshas, wounds, ulcers, inflammations, hepatopathy, gastropathy, jaundice, cough, hiccough, uropathy and general debility
4	<i>Z. mays</i> L. (poaceae)	Corn hairs	Useful in anorexia, general debility, haemorrhoids, vitiated conditions of kapha and pitta. It is valuable article of diet for invalid and children
5	<i>T. catappa</i> L. (Combretaceae)	Leaf	Fruit is useful in-biliusness, bronchitis and bowels. Juice of leaves is used in the preparation of the ointment for scabies, leprosy and also useful in headache and colic. The root bark is given in diarrhea. The bark cures bilious fevers
6	<i>T. terrestris</i> L. (zygophyllaceae)	Fruit	Used in vitiated condition of vata and pitta, rinal and vasicular calculi, asthma, consumption, cardiopathy, anaemia and general weakness. The leaves are useful in ulitis, gonnorrhoea, leprosy, skin diseases, and general weakness. Seeds are useful in haemorrhages and ulcerative stomatitis. The ash of whole plant is good for external application in rheumar arthritis
7	<i>B. diffusa</i> Linn. (Nyctaginaceae)	Root	Used in asthma, kidney ailments, dropsy, jaundice, enlargement of liver

A. lanata: *Aerva lanata*, *T. bellirica*: *Terminalia bellirica*, *T. chebula*: *Terminalia chebula*, *Z. mays*: *Zea mays*, *T. catappa*: *Terminalia catappa*, *T. terrestris*: *Tribulus terrestris*, *B. diffusa*: *Boerhaavia diffusa*

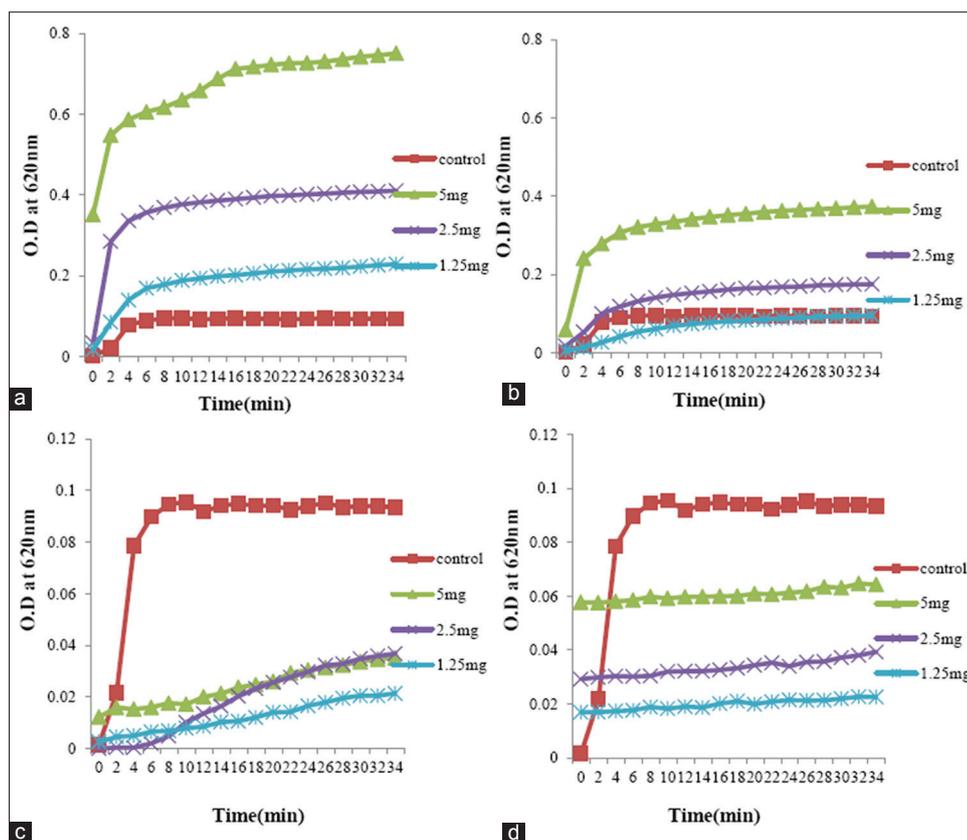


Fig. 1: The effect of different concentrations of *Aerva lanata* leaf (a), and stem (b), *Terminalia bellirica* leaf (c), and stem (d) extract on calcium oxalate crystal inhibition by nucleation assay

The second stage is crystal growth which begins with the nucleation stage which is followed by crystal aggregation or crystal agglomeration where the crystals stick together and form a large particle. Finally, bridge formation occurs among the crystals and makes them stable. The crystals so formed adhere with epithelial cell line and are retained in kidney.

The best way to prevent or treat urolithiasis is to control the process of crystallization events, and the most important step is to control the initial step, i.e. nucleation step. This is best achieved by the use of herbal extracts since they have been widely used in folk medicine to treat kidney stones. The idea to use herbal extract in the first step is that if nucleation itself is stopped or controlled, the next step which leads to the formation, aggregation and retention of crystal do not occur at all.

In this study, the formation of Ca-oxalate crystals was followed after mixing solution of Ca-chloride and Na-oxalate without or with plant extract which served as a control and test, respectively. According to Hennequin *et al.*, [22] increase in optical density at 620 nm with time is related essentially to crystal growth. Optical density is an exact measure of particle concentration per unit volume [23]. In this work, extracts of different parts of seven plants were evaluated for their effect on nucleation process. The results of which are presented in Figs. 1-3. Each extract was prepared in three different concentrations (5 mg/mL, 2.5 mg/mL and 1.25 mg/mL) and evaluated for its effect on nucleation process.

The effect of three different concentrations of *A. lanata* leaf and stem extract and *Terminalia bellirica* leaf and stem extracts is given in Fig. 1. The three different concentrations of *A. lanata*, leaf had no effect, i.e. did not inhibit Ca-oxalate crystal formation (Fig. 1a). In fact as the concentration of extracts increased, crystal formation also increased. *A. lanata* stem also showed similar effect (Fig. 1b). The only difference between leaf and stem extracts was, stem extract did not

show a concentration effect on crystal formation. The effect of lowest concentration, i.e. 1.25 mg/mL was similar to that of control. All the three concentrations of stem extract showed less crystal formation than the three concentrations of leaf extract. The results suggest that the aqueous extract of *A. lanata* leaf and stem do not show anti-urolithiatic activity since it had no effect on nucleation process.

An entirely different trend was observed with leaf and stem extracts of *T. bellirica* (Fig. 1c and d). All the three different concentrations of leaf extract showed inhibitory effect on Ca-oxalate crystal formation evaluated by nucleation assay. However, no concentration effect was observed (Fig. 1c). *T. bellirica* stem extract showed a trend similar to that of *T. bellirica* leaf extract (Fig. 1d). Unlike leaf extract, the stem extract showed a clear concentration effect. The lowest concentration showed the best effect.

The same trend was observed in leaf and stem extracts of *Terminalia chebula* (Fig. 2a and b). All the three concentrations of leaf extract showed inhibitory effect on Ca-oxalate crystal formation evaluated by nucleation assay. However, slight concentration effect was observed with three concentrations of *T. chebula* leaf extract (Fig. 2a). *T. chebula* stem extract showed a trend similar to that of *T. chebula* leaf extract (Fig. 2a). Unlike *T. bellirica* leaf extract, *T. chebula* stem extract showed a clear concentration effect. The lowest concentration showed the best effect.

The effect of different concentrations of young and old corn hair extract of *Z. mays* is given in Fig. 3. All the three concentrations of young corn hair extract inhibited Ca-oxalate crystal formation, significantly lower than that of control. The concentration effect was not found; all the three concentrations showed an exactly similar trend (Fig. 2c). Old corn hair extracts showed a slightly different trend. The lowest concentration was very promising since it inhibited Ca-oxalate crystal formation to a large extent (Fig. 2d). The other two concentrations were not effective.

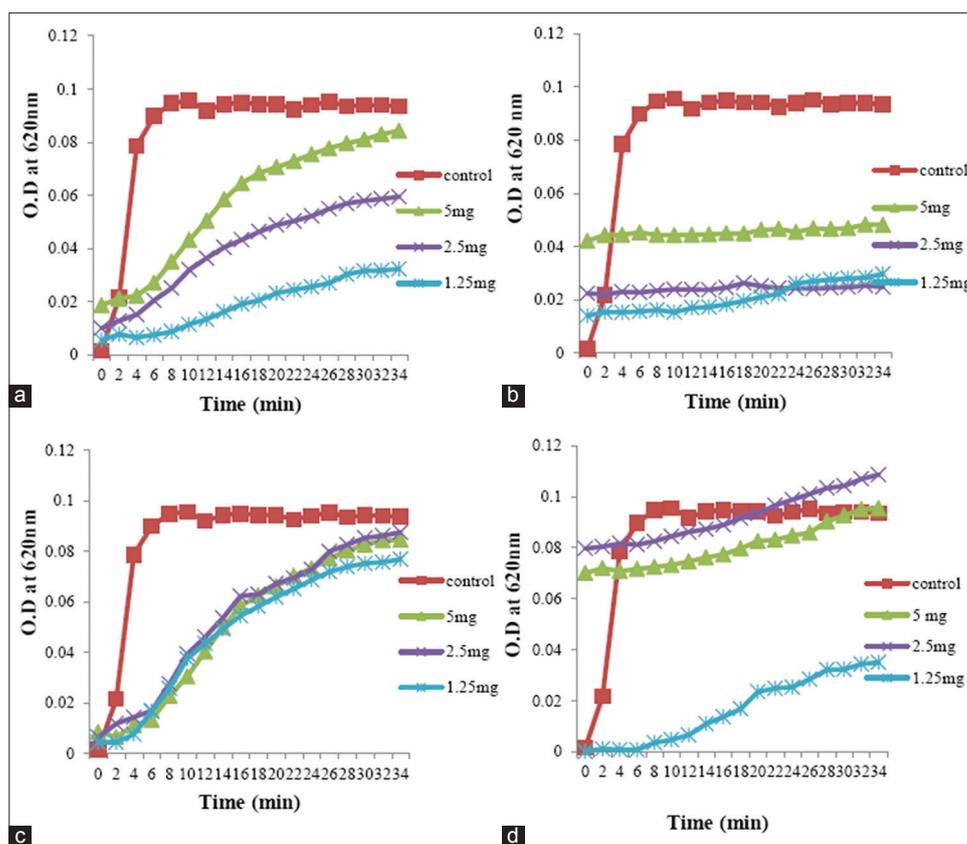


Fig. 2: The effect of different concentrations of *Terminalia chebula* leaf (a), and stem (b), *Zea mays* hair young (c), and old (d) extract on calcium oxalate crystal inhibition by nucleation assay

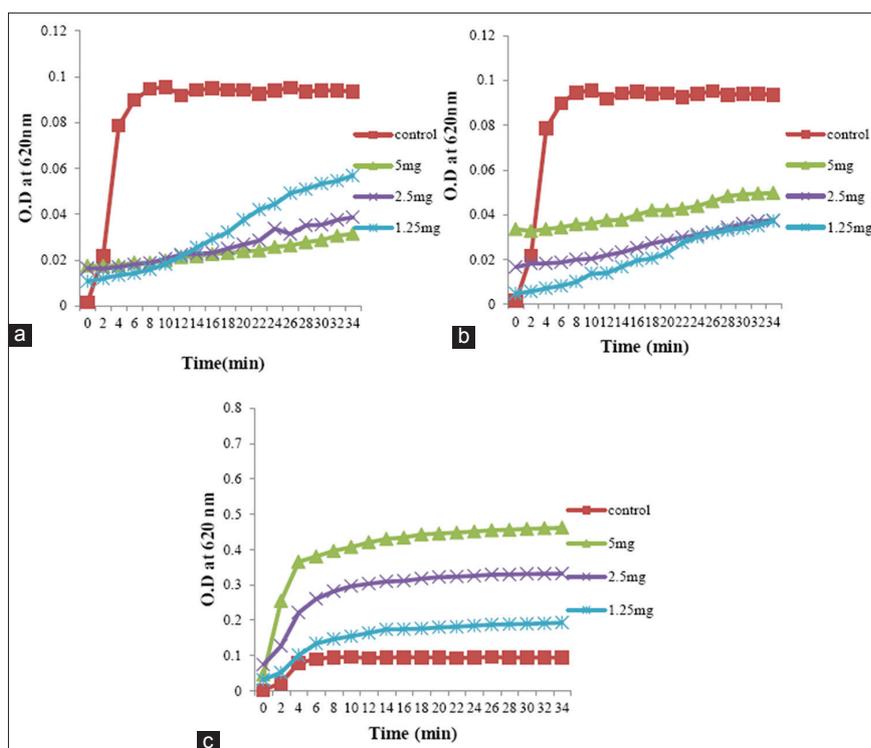


Fig. 3: The effect of different concentrations of *Terminalia catappa* leaf (a), *Tribulus terrestris* fruit (b), and *Boerhavia diffusa* root (c) extract on calcium oxalate crystal inhibition by nucleation assay

The effect of three different concentrations of *Terminalia catappa* leaf, *T. terrestris* fruit and *B. diffusa* is given in Fig. 3. All the three different concentrations of *T. catappa* leaf and *T. terrestris* fruit extract showed inhibitory effect on Ca-oxalate crystal formation evaluated by nucleation assay; however, no concentration effect was observed (Fig. 3a and b). An entirely different trend was observed with *B. diffusa* root extract. The three different concentrations of *B. diffusa* root extract had no effect, i.e. did not inhibit Ca-oxalate crystal formation (Fig. 3c). All the three concentrations showed more crystal formation than control. The results suggest that the aqueous extract of *B. diffusa* root extract did not show anti-urolithiatic activity.

This whole *in-vitro* nucleation assay is based on turbidity changes due to the formation of Ca-oxalate crystals. In this work, out of seven plant extracts, two plant extracts, i.e. *A. lanata* and *B. diffusa* aqueous extracts did not show anti-urolithiatic activity since the plant extract itself was turbid because in this work aqueous extracts by decoction were used which may not have removed the lipophilic components which hindered the assay while other five plant extracts showed inhibitory effect on Ca-oxalate crystallization but effect varied with different plant extracts. The plant extracts may contain phytoconstituents that may inhibit the formation and growth of Ca-oxalate crystals. When crystal growth is restricted, they do not grow to large particles; the small particles are easily excreted from the kidney and reduce the chance of their retention in the urinary tract. This is in agreement with other researchers [24,25]. The inhibition of *in-vitro* Ca-oxalate crystal formation by *Convolvulus arvensis* leaf and flower extracts was reported by Rajeshwari *et al.* [26]; while the beneficial effect of *Ocimum gratissimum* extract on Ca-oxalate crystallization evaluated by turbidity changes at 620 nm was reported by Agarwal and Varma [27]. Crystal agglomeration is the most important process which leads to crystal retention and thus perhaps, interference or inhibition of crystal growth and aggregation is the best therapeutic strategy for the prevention of stone formation as also suggested by [28].

Microscopic study

Several studies are carried out using microscope to validate the results obtained by turbidimetric method [19,25]. Microscopic study of control

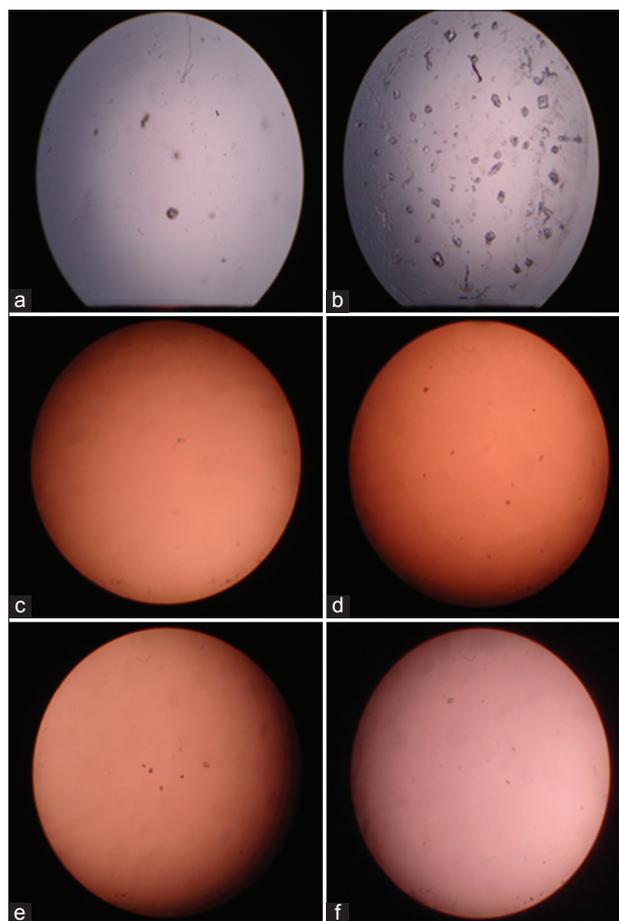


Fig. 4: Control at 0 minute and 30 minutes (a and b), *Tribulus terrestris* leaf at 0 minute and 30 minutes (c and d), and stem at 0 minute and 30 minutes (e and f)

and *T. bellirica* leaf and stem extracts at 1.25 mg/mL concentration is shown in Fig. 4. Control at 0 minute and 30 minutes is shown in Fig. 4a and b, respectively. Crystal number and size was considerably more at 30 minutes than at 0 minute.

T. bellirica leaf extract at 0 minute and 30 minutes is shown in Fig. 4c and d, respectively. Crystal number and size was less at 0 minute and 30 minutes when compared with control. In *T. bellirica* stem extract, crystal number was more than *T. bellirica* leaf extract and less than that of control at 0 minute (Fig. 4e). At 30 minutes, *T. bellirica* stem extract showed a similar trend like *T. bellirica* leaf extract, i.e. the number and size of crystals were very much less than that of control (Fig. 4f).

Microscopic study of control and *T. chebula* leaf and stem extracts at 1.25 mg/mL and 2.5 mg/mL concentration is shown in Fig. 5. Control at 0 minute and 30 minutes is shown in Fig. 5a and b, respectively. Crystal number and size was considerably more at 30 minutes than at 0 minute. *T. chebula* leaf extract at 0 minute and 30 minutes is shown in Fig. 5c and d, respectively. Crystal number and size was less both at 0 minute to 30 minutes as compared to control. In *T. chebula* stem extract, crystal number was more than *T. chebula* leaf extract and less than that of control at 0 minute (Fig. 5e). But in *T. chebula* stem extract at 30 minutes, crystal number was similar with *T. chebula* leaf extract but less than that of control at 30 minutes (Fig. 5f).

Microscopic study of control and *Z. mays* young and old corn hair extracts at 1.25 mg/mL and 2.5 mg/mL concentration is shown in Fig. 6. Control at 0 minute and 30 minutes is shown in Fig. 6a and b, respectively. Crystal number and size was considerably more at 30 minutes than at 0 minute. *Z. mays* young corn hair extract at 0 minute and 30 minutes is shown in Fig. 7c and d, respectively.

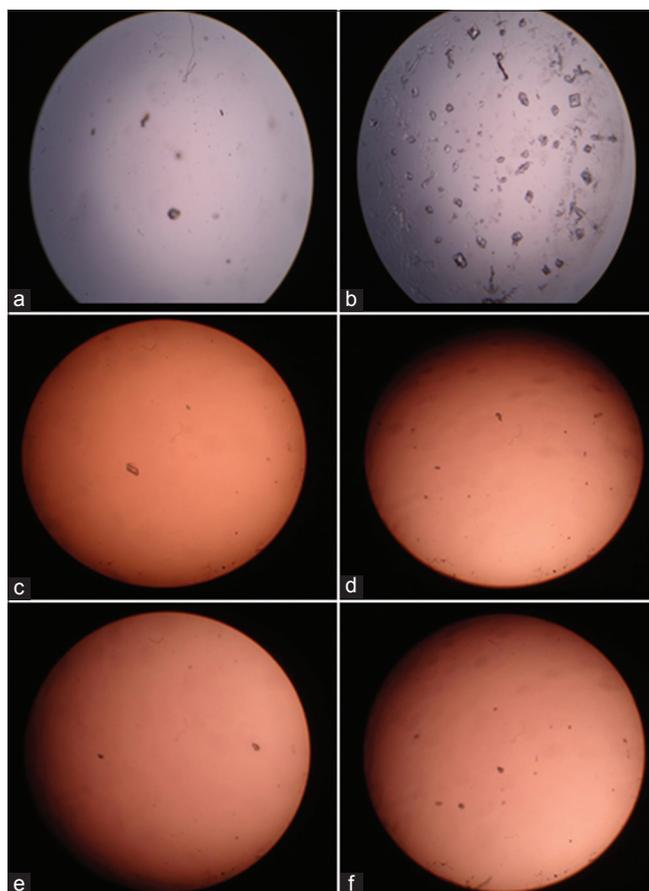


Fig. 5: Control at 0 minute and 30 minutes (a and b), *Terminalia chebula* leaf at 0 minute and 30 minutes (c and d), and stem at 0 minute and 30 minutes (e and f)

Crystal number and size was less at 0 minute (Fig. 6c). However, there was a slight difference between control and *Z. mays* young corn hair at 30 minutes; crystal size and number slightly decreased in *Z. mays* young corn hair at 30 minutes (Fig. 6d). *Z. mays* old corn hair extract at 0 minute and 30 minutes is shown in Fig. 6e and f, respectively. Crystal number and size was less in *Z. mays* old corn hair as compared to control and young corn hair at 0 minute and 30 minutes (Fig. 6a-d).

Microscopic study of control and *T. chebula* leaf and *T. terrestris* fruit extracts at 5 mg/mL and 1.25 mg/mL and 2.5 mg/mL concentration is shown in Fig. 7. Control at 0 minute and 30 minutes is shown in Fig. 7a and b, respectively. Crystal number and size was considerably more at 30 minutes than at 0 minute. *T. chebula* leaf extract at 0 minute and 30 minutes is shown in Fig. 7c and d, respectively. Crystal number and size was less at 0 minute to 30 minutes as compared to control (Fig. 7e). In *T. terrestris* fruit extract, crystal size increased but the crystal number decreased at 30 minutes (Fig. 7f).

The microscopic result indicates that the crystals present in the control were reduced when plant extract was added to the solution. Both crystal number and crystal size gradually decreased when plant extracts were added into solution. The microscopy study supported the results obtained by turbidimetric method in nucleation assay. It indicates that *T. bellirica*, *T. chebula*, *Z. mays*, *T. catappa*, and *T. terrestris* are promising plants with anti-urolithiatic activity.

The *in-vitro* results revealed that *T. bellirica* leaf and stem extracts at 1.25 mg/mL concentration showed potent anti-urolithiatic ability in nucleation assay. *T. bellirica* extracts showed the best activity followed by *T. catappa* and *T. chebula* extracts. There are many reports where different plant extracts showed anti-urolithiatic activity evaluated by *in-vitro* nucleation assay [29-32].

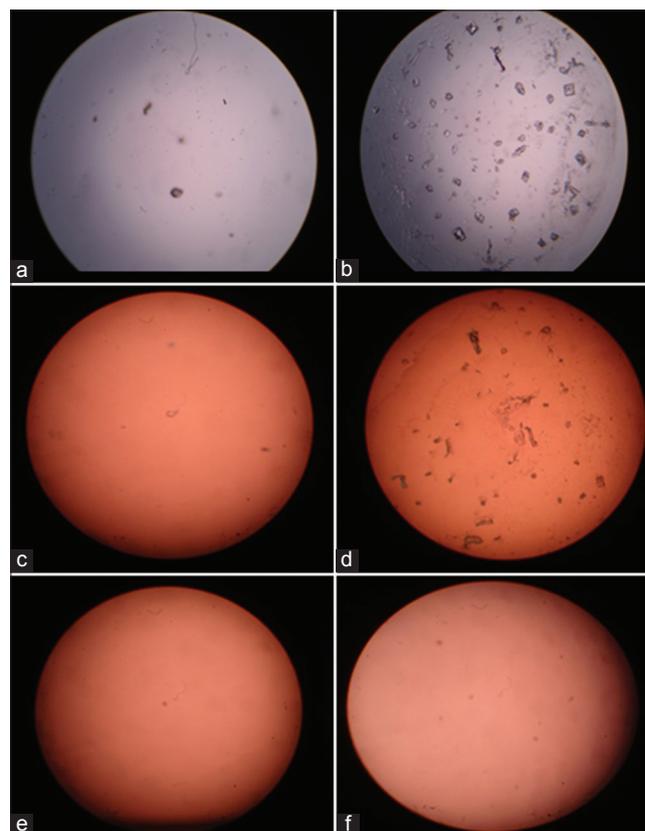


Fig. 6: Control at 0 minute and 30 minutes (a and b), Young corn hair at 0 minute and 30 minutes (c and d), and old corn hair at 0 and 30 minutes (e and f)

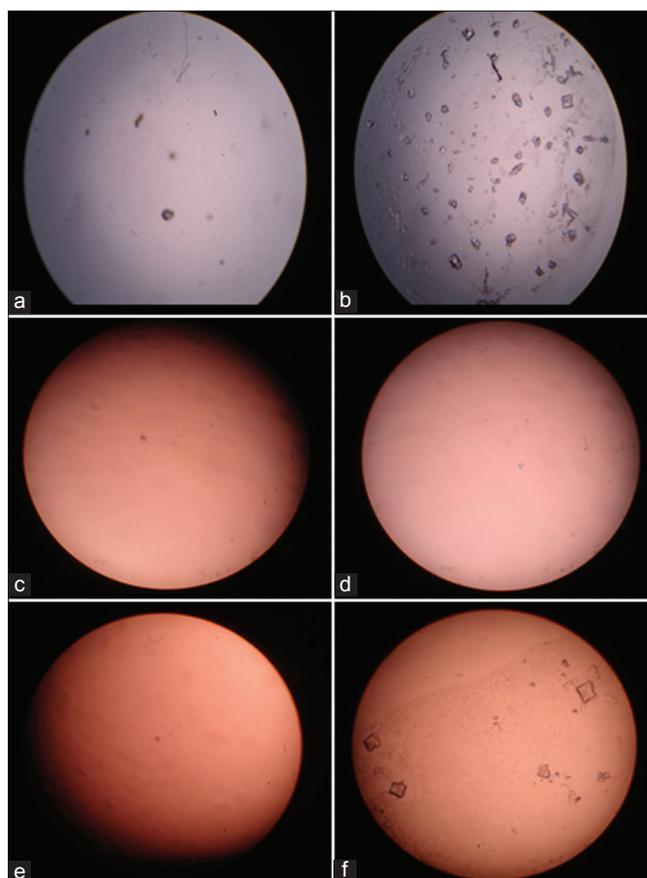


Fig. 7: Control at 0 minute and 30 minutes (a and b), Terminalia chebula leaf at 0 minute and 30 minutes (c and d), and Tribulus terrestris fruit at 0 minute and 30 minutes (e and f)

CONCLUSION

It can be concluded that all the three *Terminalia* species have the ability to prevent urinary stone formation so that small particles do not form into large particles, and they are excreted from the kidney and reduce the chance of their retention in the urinary tract. However, these *in-vitro* results should be confirmed by *in-vivo* studies to develop a potent antilithiatic agent from these plant species. *Terminalia* plant species showed best activity may be because of the phytoconstituents present in them which led to it showing best anti-oxidant activity. These plant species prevent oxidative stress environment, which are responsible for crystal formation and growth. The natural phyto-compounds present in them can prevent supersaturation of urine and the growth of the crystals. Hence, they can be considered as good anti-oxidant and anti-uro lithiatic plants. Hence, these two plants can be considered as best plants for further work in evaluating its role as anti-uro lithiatic agents.

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