

PHYTOCHEMICAL SCREENING AND IN VITRO EVALUATION OF THE ANTI-UROLITHIASIS ACTIVITY OF ETHANOLIC EXTRACT OF *CROSSANDRA INFUNDIBULIFORMIS* FLOWERS

Samuel S.^{1*}, Balaji P.², Balaji R.³, Sakthikaviyarasan K.⁴, Syfullah S.⁵, Manivannan R.⁶

Department of Pharmacology, Excel College of Pharmacy, Komarapalayam, Namakkal-638183.

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*Corresponding Author

Samuel S.

Department of Pharmacology, Excel
College of Pharmacy,
Komarapalayam, Namakkal-638183.



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ABSTRACT

Urolithiasis is a common urinary tract disorder characterized by the formation and aggregation of crystalline stones, with calcium oxalate being the predominant type of urinary stone. The present study aimed to evaluate the in-vitro anti-urolithiasis activity of the ethanolic flower extract of *Crossandra infundibuliformis* and to investigate its phytochemical constituents. The flowers of *Crossandra infundibuliformis* were collected, authenticated, shade-dried and powdered, followed by Soxhlet extraction using ethanol. The percentage yield of the obtained ethanolic flower extract was found to be 28.6%. Preliminary phytochemical screening revealed the presence of tannins, phenols, reducing sugars, cholesterol, terpenoids, carotenoids and resins, while alkaloids, flavonoids, steroids, carbohydrates, proteins, glycosides, saponins and phytosterols were not detected. The in-vitro anti-urolithiatic activity was evaluated by assessing the inhibition of

calcium oxalate crystal aggregation at different concentrations of the extract (10, 50, 100, 250 and 500 µg/mL) and compared with Cystone as the standard drug. The ethanolic flower extract exhibited concentration-dependent inhibition of calcium oxalate crystal aggregation, with mean percentage inhibition of 30.554%, 43.267%, 49.781%, 55.591% and 61.740% at 10, 50, 100, 250 and 500 µg/mL, respectively. Cystone showed a mean inhibition of 81.259%. The observed anti-urolithiatic activity may be associated with the phytoconstituents present

in the extract, particularly tannins, phenols and terpenoids. Overall, the findings suggest that the ethanolic flower extract of *Crossandra infundibuliformis* possesses promising concentration-dependent in-vitro anti-urolithiatic activity against calcium oxalate crystal aggregation. Further experimental studies are required to validate its therapeutic potential.

KEYWORDS: Urolithiasis; *Crossandra infundibuliformis*; Ethanolic flower extract; Calcium oxalate; Crystal aggregation; Antiurolithiatic activity.

1. INTRODUCTION

1.1 Urolithiasis

Urolithiasis, commonly known as kidney stone disease, is a common disorder of the urinary tract characterized by the formation of crystalline stones in the kidneys or urinary tract. Stone formation occurs when urine becomes supersaturated with stone-forming substances, leading to crystal formation, growth and aggregation. It is an important urological condition because of its prevalence, recurrence and associated complications.

1.2 Calcium Oxalate Stones

Urinary stones can be classified according to their chemical composition, including calcium oxalate, calcium phosphate, uric acid, struvite and cystine stones. Calcium-containing stones are the most common, with calcium oxalate being the predominant type. Approximately 80% of urinary stones are calcium-containing stones.

1.3 Stone Formation

The formation of urinary stones involves several stages, including urinary supersaturation, nucleation, crystal growth, aggregation and crystal retention. When calcium and oxalate concentrations increase in urine, calcium oxalate crystals may form. These crystals can grow and aggregate to produce larger particles, which may be retained in the urinary tract and contribute to stone formation.

1.4 Risk Factors

Urolithiasis is a multifactorial disorder. Important risk factors include inadequate fluid intake, dehydration, dietary habits, metabolic abnormalities, obesity, urinary tract infections, genetic predisposition and environmental conditions. These factors can alter urinary composition and increase the possibility of crystal formation.

1.5 Management Of Urolithiasis

Management of urolithiasis depends on the size and location of the stone. Conservative treatment, pharmacological therapy and surgical procedures may be used. Common procedures include extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS), percutaneous nephrolithotomy (PCNL) and retrograde intrarenal surgery (RIRS). Prevention of recurrence through adequate fluid intake and dietary modification is also important.

1.6 Herbal Approach

Medicinal plants are being investigated as potential sources of anti-urolithiatic agents. Plant extracts may inhibit different stages of stone formation, particularly calcium oxalate crystal nucleation, growth and aggregation. Phytochemicals such as tannins, phenolic compounds, flavonoids and terpenoids have been associated with antioxidant and crystal-inhibitory activities.

1.7 Crossandra Infundibuliformis

Crossandra infundibuliformis belongs to the family Acanthaceae and has been investigated for its phytochemical constituents and medicinal properties. Previous investigations of its flowers have reported the presence of several bioactive phytoconstituents. Therefore, the flower extract was selected for preliminary phytochemical screening and evaluation of its potential anti-urolithiatic activity.

1.8 Rationale of The Study

Calcium oxalate crystal aggregation is an important step in urinary stone formation. Therefore, inhibition of calcium oxalate crystal aggregation can be used as a preliminary method for evaluating anti-urolithiatic activity. The present study was undertaken to evaluate the ethanolic flower extract of *Crossandra infundibuliformis* for its phytochemical constituents and in-vitro anti-urolithiatic activity against calcium oxalate crystal aggregation.

2. AIM

To evaluate the anti-urolithiasis activity of *Crossandra infundibuliformis* plant extract and its potential role in preventing kidney stone formation.

OBJECTIVES

1. To collect, authenticate, and prepare the ethanolic extract of *Crossandra infundibuliformis* flowers.

2. To perform preliminary qualitative phytochemical screening of the *Crossandra infundibuliformis* flower extract.
3. To evaluate the in vitro anti-urolithiasis activity of the flower extract by assessing its ability to inhibit calcium oxalate crystal nucleation, growth, and aggregation.
4. To analyze and compare the inhibitory effect of the extract on calcium oxalate crystal formation as an indicator of its potential role in preventing kidney stone formation.

3. MATERIALS AND METHODOLOGY

3.1 PREPARATION OF FLOWER EXTRACT

Fresh *Crossandra infundibuliformis* flowers were washed thoroughly and shade-dried at room temperature. The dried flowers were powdered and used for extraction.

3.2 SOXHLET EXTRACTION

Soxhlet extraction was used for the preparation of the ethanolic flower extract. About **5 g of powdered flower material** was placed in a Soxhlet thimble, and **200 mL of ethanol** was used as the extraction solvent. The apparatus was assembled with a round-bottom flask, Soxhlet extractor and condenser. The solvent was heated using a heating mantle, allowing repeated extraction cycles through evaporation, condensation and siphoning. The extraction was continued for approximately **4 hours**. The obtained extract was collected for further phytochemical and pharmacological evaluation.

PROCEDURE

1. Test sample (CI) can be fresh or dried. It needs to be crushed, using a pestle and mortar, to provide a greater surface area.
2. The test sample should be sufficient to fill the porous cellulose thimble (in our experiments we use an average of 14 g of *Crossandra infundibuliformis* in a 25- x 80-mm thimble).
3. All equipment should be too assembled. Build a rig using stands and clamps to support the extraction apparatus.
4. Following this, 5g of sample CI, 200ml of Ethanol were added to a round bottom flask, which is attached to a Soxhlet extractor and condenser on heating mantle.
5. The crushed plant material is loaded into the thimble, which is placed inside the Soxhlet extractor.
6. The side arm is lagged with glass wool.
7. The solvent is heated using the heating mantle and will begin to evaporate, moving

through the apparatus to the condenser.

8. The condensate then drips into the reservoir containing the thimble.
9. Once the level of solvent reaches the siphon it pours back into the flask and the cycle begins again.
10. The process should run for a total of 4 hours.
11. Once the extraction set up, it can be left to run without direct supervision.
12. It is not advised to leave the equipment completely alone due to the mix of running water and an electrical appliance, so a technician or other lab user should be made aware.
13. The equipment can be turned off.



Figure 1: Soxhlet extraction of Test sample (CI).



Figure 2: Extraction of CI sample.



Figure 3: Sample Heated at 70°C.

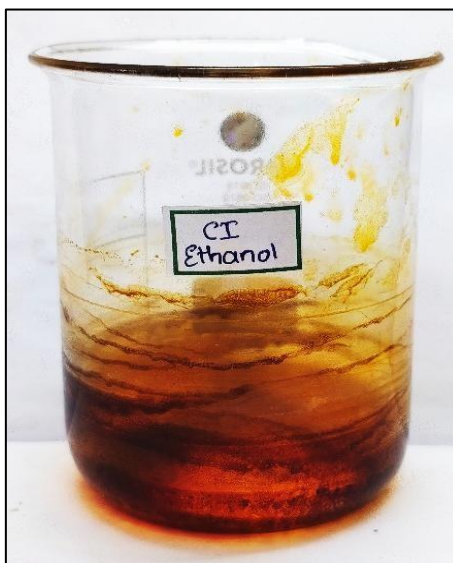


Figure 4: Concentrated sample CI.

3.3 QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

1. Test for Alkaloids - Mayer's test

The extracts are dissolved in dilute hydrochloric acid and filtered. To the extract add two drops of Mayer's reagent along the sides of the test tube. Appearance of creamy white / yellow precipitate indicate the presence of alkaloids.

2. Detection of Flavonoids

To the filtrate of plant extract, 5mL dil. Ammonia solution is added. To it, a few drops of conc. H₂SO₄ is added. Formation of yellow colour indicates the presence of flavonoids.

3. Detection of Steroids

A few drops of acetic anhydride are added to the extracts, and the formation of violet to blue to green in the samples indicates the presence of steroids.

4. Detection of Tannins

3 gm of powdered sample is boiled in 50mL distilled water for 3 min and then filtered. 1 ml of filtrate is mixed with 3mL of distilled water, and 3 drops of 10% Ferric chloride solution is added to it. Development of Blue-green colour indicates the presence of tannins.

5. Detection of Phenol (Ferric Chloride Test)

To 500µl of extract, few drops of 5% ferric chloride solution is added. The formation of dark green / bluish black colour indicates the presence of phenol.

6. Detection of Carbohydrates (Molish's test)

To 2 ml of plant extract 2 drops of alcoholic α -naphthol and 1mL conc. H₂SO₄ is added along the sides of test tube. Formation of a violet ring indicates the presence of carbohydrates.

7. Detection of Proteins (Biuret test)

To 2mL of extract filtrate, 1 drop of 2% copper sulphate sol. Is added. To it 1mL of 95% ethanol and KOH pellets are added. Formation of pink coloured solution in the ethanolic layer indicates the presence of proteins.

8. Detection of reducing sugar

0.5mL of plant extract filtrate and 0.5mL of Benedict's reagent is mixed and boiled for 2 min. Formation of red or green or yellow colour indicate the presence of reducing sugars.

9. Detection of Glycosides

5ml plant extract is mixed with 2mL of glacial acetic acid and a drop of 5% FeCl₃, followed by addition of conc. H₂SO₄. Formation of brown ring indicate the presence of glycosides.

10. Detection of Cholesterol (Lipid)

2mL extract, 2mL chloroform, 10 drops of acetic anhydride and 2-3 drops of conc. H₂SO₄ were mixed. Red-rose colour formation indicate presence of cholesterol.

11. Saponin detection (Foam Test)

To 50 g of extract, 20 ml of distilled water was added and shaken vigorously for 25 minutes.

Formation of 2 cm of thick foam layer indicates the presence of saponins.

12. Detection of Phytosterols (Salkowski's test)

To the plant extract filtrate few drops of conc. H₂SO₄ is added and shaken well and allowed to stand. Formation of red colour in the lower layer indicate the presence of phytosterols.

13. Detection of Terpenoids

5mL of plant extract and 2ml chloroform is mixed and evaporated in a water bath. Then 3 ml of conc. H₂SO₄ is added and boiled in a water bath. Development of A grey coloured solution indicate presence of terpenoids.

14. Detection of carotenoids

10 ml of plant extract is evaporated to dryness, and to it 2 -3 drops of Plant extract is dissolved in distilled water + 3-4 drops of copper acetate solution is added. A blue colour solution that eventually changes to red colour indicate the presence of carotenoids.

15. Detection of resins

To 1mL of plant extract, acetic anhydride solution and 1mL conc. H₂SO₄ is added. Formation of orange to yellow colour indicates the presence of resins.

3.4 *In vitro* ANTIUROLITHIATIC ACTIVITY

Material Required

1. Calcium oxalate,
2. Calcium chloride,
3. Ammonium dihydrogen phosphate,
4. H₂SO₄

Procedure

Calcium oxalate method

The experimental kidney stones of calcium oxalate (CaOx) crystals were determined by the method of Mazni Abu Zarin et al., with a slight modification. The CaOx monohydrate crystals were prepared by mixing equal concentrations of calcium chloride (50 mmol/L) and sodium oxalate (50 mmol/L). Both solutions were equilibrated in a water bath for 1 h at 60 °C for the formation of CaOx monohydrate crystals. The crystals were cooled to 37 °C prior to evaporation. The CaOx monohydrate crystals were prepared at a final concentration of 0.8

mg/mL in a Tris buffer (Tris 0.05 mol/L and NaCl 0.15 mol/L) at pH 6.5. A different concentration of CI samples (500, 250, 100, 50 and 10 µg/ml) were added to the CaOx monohydrate crystals solution and incubated at 37 °C for 24 hrs. Cystone was used as positive control (10 mg/ml). The aggregation activity was estimated by the turbidity using a microplate reader (Thermo fisher scientific, USA) in the presence of the extract compared to the control by measurement at 620 nm. The experiment was done in triplicate.

The percentages inhibition of aggregation by the plant extract was calculated as below:

$$= [(OD \text{ control} - OD \text{ test}) / OD \text{ control}] \times 100$$

Where OD = Optical Density

4. RESULTS AND DISCUSSION

4.1 RESULTS AND DISCUSSION OF FINAL PROUCT OF CI



Figure 5: Final Product of CI Sample.

$$\text{Percentage of Yield obtained} = \frac{\text{Yield in grams}}{\text{Loaded sample in grams}} \times 100$$

$$\text{Percentage of Yield obtained} = \frac{1.43}{5 \text{ gm}} \times 100$$

$$\text{Percentage of Yield obtained} = 28.6 \%$$

The ethanolic extraction of the CI sample produced 1.43 g of extract from 5 g of dried plant material, resulting in a 28.6% percentage yield.

The percentage yield indicates that ethanol was an effective extraction solvent for isolating polar and moderately polar phytoconstituents from the plant material. Ethanol is widely used

in herbal extraction because it efficiently dissolves phenolic compounds, tannins, terpenoids, and other bioactive constituents that contribute to pharmacological activity. A yield of approximately 20–30% is considered satisfactory for many medicinal plants, suggesting that the extraction process successfully recovered a substantial amount of phytochemicals for further investigation.

4.2 QUALITATIVE PHYTOCHEMICAL RESULT

The table showing the qualitative phytochemical screening results

Table 1: Phytochemical screening results.

S. No	Name of the sample / Sample code	Phytochemical compound	Result
1.	CI Ethanol extract	Alkaloids	-
2.		Flavonoids	-
3.		Steroids	-
4.		Tannins	+
5.		Phenol	+
6.		Carbohydrates	-
7.		Proteins	-
8.		Reducing sugar	+
9.		Glycosides	-
10.		Cholesterol	+
11.		Saponin	-
12.		Phytosterols	-
13.		Terpenoids	+
14.		Carotenoids	+
15.		Resins	+

Red (+) = Indicates Present

Black (-) = Indicates Absent

4.3 Images of the qualitative phytochemical screening methods of the sample



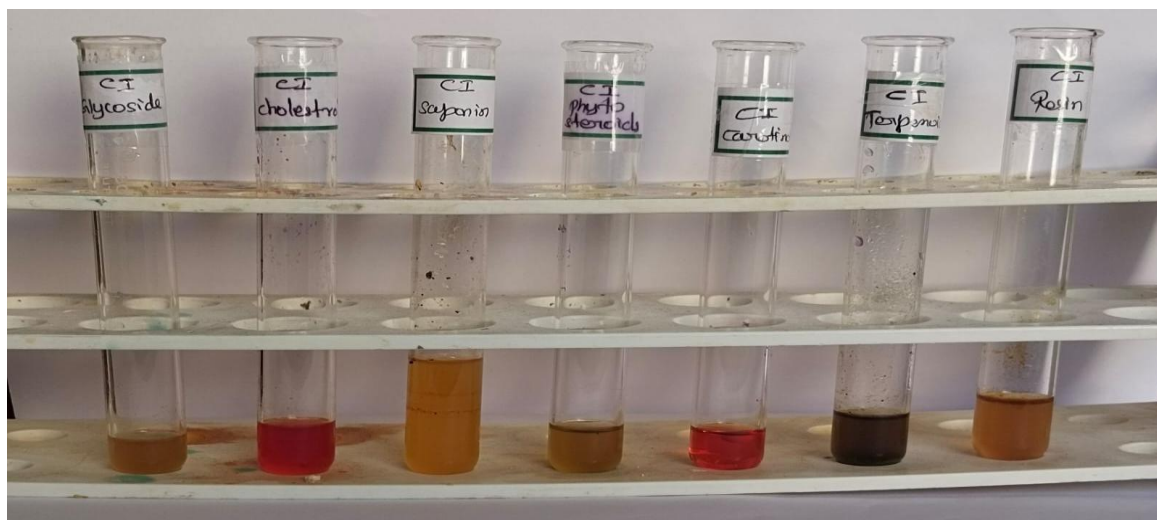


Figure 6: Phytochemical screening methods.

The qualitative phytochemical screening demonstrated the presence of tannins, phenols, reducing sugars, cholesterol, terpenoids, carotenoids, and resins, while alkaloids, flavonoids, steroids, carbohydrates, proteins, glycosides, saponins, and phytosterols were absent.

The presence of phenolic compounds is particularly significant because these compounds possess strong antioxidant activity. Oxidative stress plays an important role in calcium oxalate stone formation by damaging renal epithelial cells and promoting crystal adhesion. Phenolic compounds can neutralize reactive oxygen species, thereby reducing cellular injury and preventing crystal retention within the kidney.

Tannins are another important group of phytochemicals detected in the extract. They possess protein-binding properties that may interfere with crystal aggregation and reduce inflammation associated with renal injury. Their astringent nature may also contribute to protecting epithelial surfaces from crystal-induced damage.

Terpenoids are well-known for their antioxidant and nephroprotective activities. These compounds may inhibit crystal nucleation and aggregation while protecting kidney tissues against oxidative injury. Carotenoids further enhance antioxidant defense by scavenging free radicals and reducing lipid peroxidation.

4.3 IN VITRO ANTIUROLITHIATIC ACTIVITY

The percentages inhibition of aggregation by the plant extract was calculated as below:

$$\% \text{ of inhibition} = [(\text{OD control} - \text{OD test}) / \text{OD control}] \times 100$$

A. OD Value at 620nm

Control Mean OD value – 1.941

S. No.	Tested sample concentration (µg/ml)	OD value at 620 nm (in triplicates)		
1	Control	1.656	1.254	1.204
2	500 µg/ml	0.508	0.530	0.536
3	250 µg/ml	0.610	0.613	0.604
4	100 µg/ml	0.702	0.690	0.674
5	50 µg/ml	0.821	0.774	0.739
6	10 µg/ml	0.951	0.912	0.994
7	Cystone	0.226	0.285	0.260

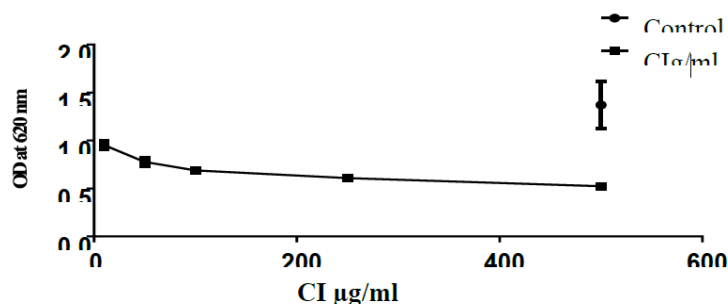
The optical density values measured at 620 nm showed a progressive reduction as the concentration of the extract increased.

The control group exhibited the highest optical density, indicating maximum crystal aggregation. In contrast, the treated groups showed progressively lower OD values with increasing extract concentration, while Cystone produced the lowest optical density among all groups.

Optical density is directly related to the turbidity of the crystal suspension. A lower OD value indicates fewer suspended crystal aggregates, confirming that the extract effectively reduced calcium oxalate crystal formation.

B. Percentage of Inhibition

S. No.	Tested sample concentration (µg/ml)	Percentage of inhibition (%) (in triplicates)			Mean Value (%)
1.	Cystone	83.520	79.217	81.040	81.259
2.	500 µg/ml	62.956	61.351	60.914	61.740
3.	250 µg/ml	55.518	55.299	55.955	55.591
4.	100 µg/ml	48.809	49.684	50.851	49.781
5.	50 µg/ml	40.131	43.559	46.111	43.267
6.	10 µg/ml	30.651	33.495	27.516	30.554



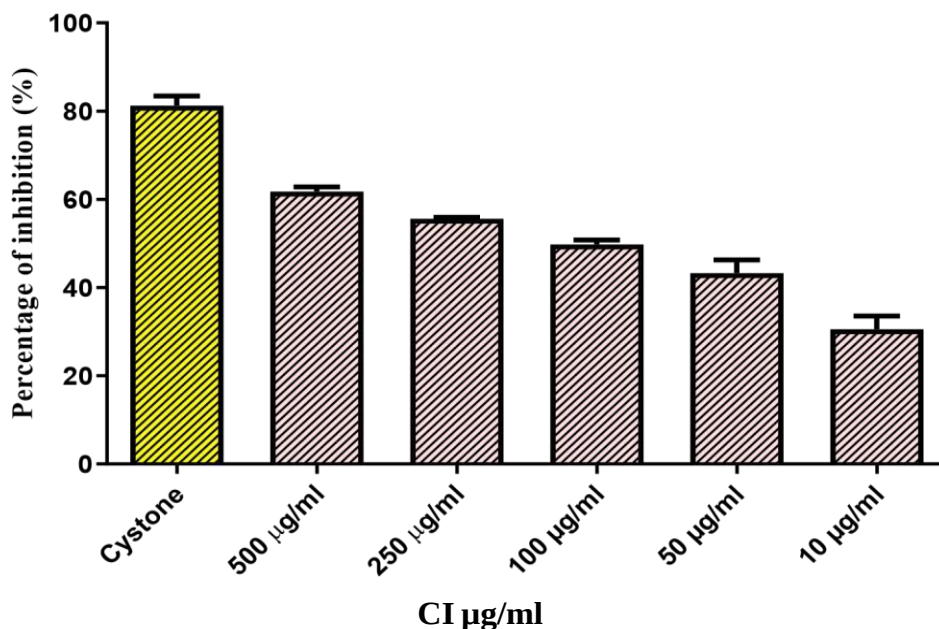


Figure 7: Dose Response Relationship.

As the extract concentration increased from **10 $\mu\text{g/ml}$ to 500 $\mu\text{g/ml}$** , the inhibitory activity increased steadily while optical density decreased. Such a pattern is characteristic of pharmacologically active plant extracts whose bioactive constituents exert greater effects at higher concentrations. A dose-dependent response is considered an important indicator of biological activity because it demonstrates that the observed effects are unlikely to occur randomly. Instead, the increasing inhibition suggests that higher concentrations provide greater availability of active phytochemicals capable of interacting with calcium oxalate crystals.

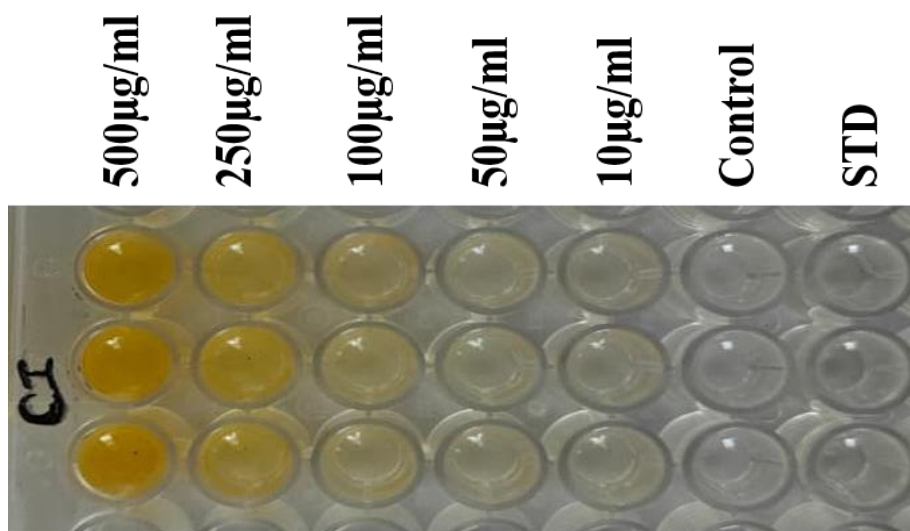
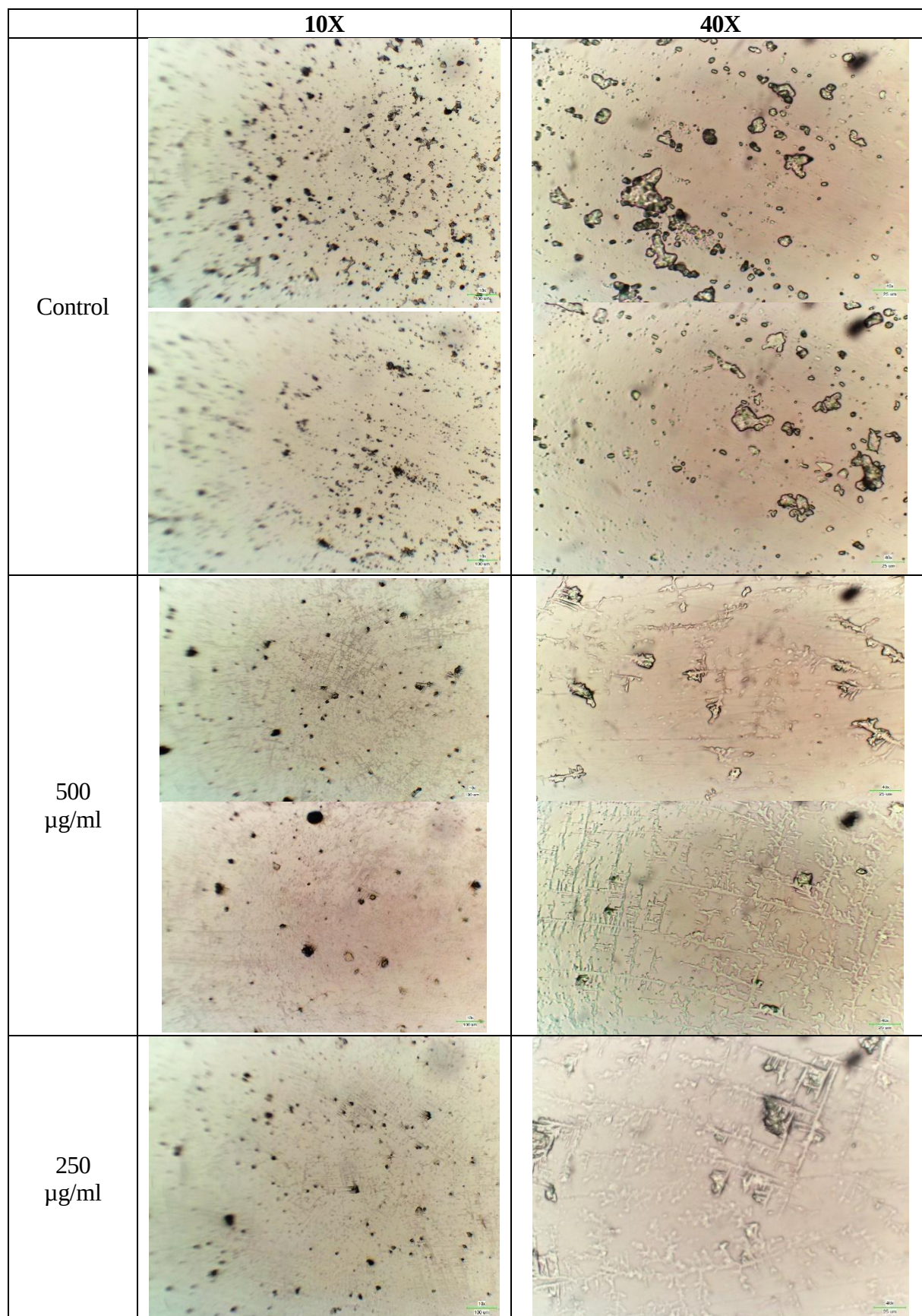
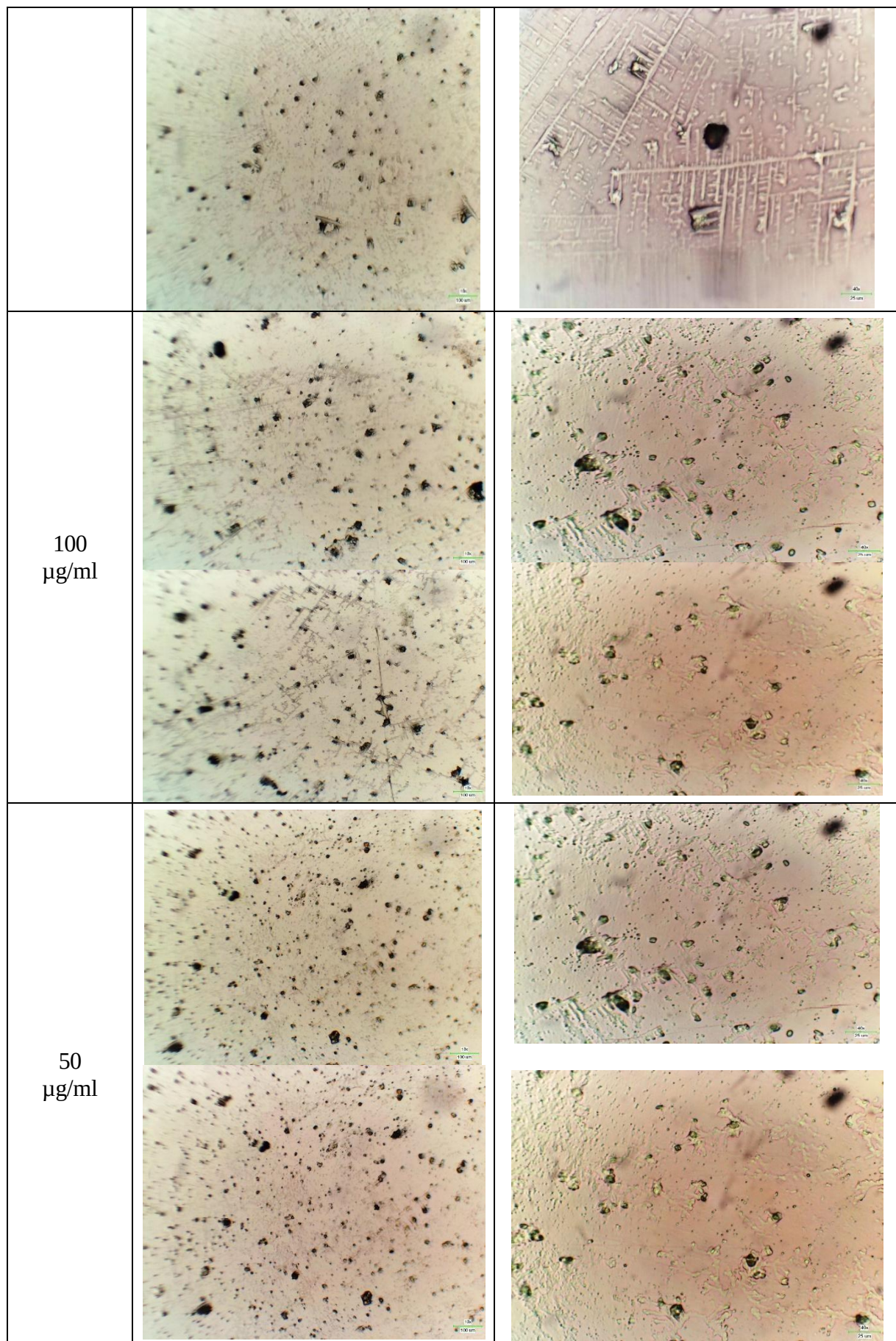


Figure 8: Triplate Test.

4.4 CALCIUM OXALATE CRYSTALS WERE TREATED WITH VARIOUS CONCENTRATIONS OF THE CI (*Crossandra infundibuliformis*) SAMPLES





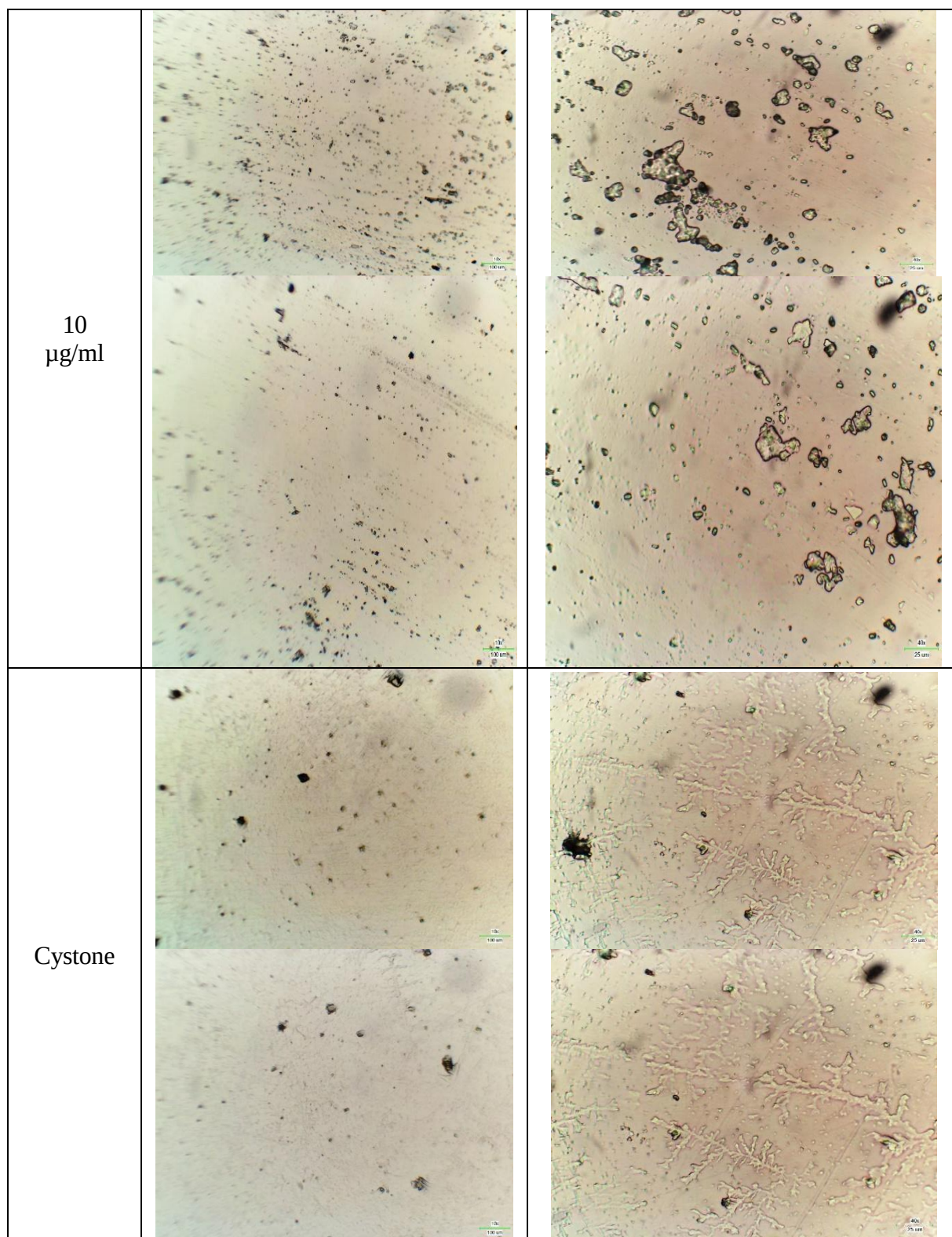


Figure 9: Microscopic Evaluation of Calcium Oxalate Crystals.

The control group exhibited abundant calcium oxalate crystals with marked aggregation. As the extract concentration increased, both the size and aggregation of the crystals decreased noticeably. The highest concentration produced a much greater reduction in crystal

aggregation, while the standard drug showed the most pronounced inhibitory effect.

These observations complement the optical density results and indicate that the extract interferes with several stages of crystal development, including nucleation, crystal growth, aggregation, and adhesion.

The reduction in crystal aggregation observed under the microscope supports the hypothesis that the phytochemicals present in the extract may modify crystal surfaces and prevent their enlargement into clinically significant kidney stones.

5. CONCLUSION

The present study demonstrates that the ethanolic flower extract of *Crossandra infundibuliformis* possesses promising in-vitro anti-urolithiatic activity against calcium oxalate crystal aggregation. The extract showed a concentration-dependent increase in inhibition, with the highest activity observed at 500 µg/mL (61.740%), compared with 81.259% for the standard Cystone.

Preliminary phytochemical screening revealed several secondary metabolites, particularly tannins, phenols, terpenoids, carotenoids and resins, which may contribute to the observed activity. The study therefore provides preliminary scientific evidence supporting the potential of *Crossandra infundibuliformis* flowers as a source of anti-urolithiatic compounds.

However, the present study is limited to preliminary phytochemical screening and an in-vitro calcium oxalate aggregation model. *In-vitro* findings cannot completely reproduce the complex physiological environment of the urinary tract. Therefore, further *in-vivo* studies, isolation and characterization of active phytoconstituents, toxicity studies, mechanistic investigations, and eventually clinical studies are required to establish the therapeutic potential of *Crossandra infundibuliformis*.

Overall, the study concludes that *Crossandra infundibuliformis* flower extract is a promising candidate for further investigation as a natural anti-urolithiatic agent and may contribute to the future development of safer and more affordable approaches for the prevention and management of urolithiasis.

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