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RESEARCH ARTICLE

Comparative study of four medicinal plants for their anti-nucleation, anti-aggregation, anti-bacterial, antioxidant and anti-inflammatory properties in urolithiasis

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Abstract

The etiology of urolithiasis is complex with various factors such as supersaturation, inflammation, oxidative stress and microbial infection due to *E. coli*, *Pseudomonas spp.* and urease-splitting bacteria etc., contributing to stone formation. Therefore, present article analyses four medicinal plants viz. *Anneslea fragrans, Mallotus philippensis, Magnolia grandiflora* and *Bauhinia variegata* for their anti-nucleation, anti-aggregation, antioxidant, anti-inflammatory and anti-microbial properties in comparison with Cystone using methanolic extracts. The analysis indicated that *Anneslea fragrans* exhibit significant anti-nucleation, anti-aggregation properties, though marginally weaker than Cystone, but have higher anti-inflammatory and anti-microbial properties as compared to Cystone. All the plant extracts exhibit significant antioxidant properties with IC_{50} values of 1.95, 1.9, 2.14 and 1.85, and ascorbic acid equivalent (AAE in mg/g) values of 0.395, 0.75, 0.349 and 0.72, remarkably higher anti-inflammatory property with IC_{50} values of 420.75, 646.16, 408 and 940.93 for proteinase inhibition assay of *Anneslea fragrans, Mallotus philippensis, Magnolia grandiflora* and *Bauhinia variegata* respectively, and notable antimicrobial properties when compared with Cystone, a polyherbal formulation. The current study demonstrated the therapeutic potentials of the plant extracts in ameliorating factors that cause kidney stones, and can be further investigated to identify their bioactive components.

Keywords: Urolithiasis; Medicinal Plants; *Anneslea fragrans; Mallotus philippensis; Magnolia grandiflora; Bauhinia variegata.*

1. Introduction

Kidney stone, also known as urolithiasis, is a prevalent medical condition that affects large section of global population. Around 12% of the world's population are afflicted with kidney stones at some point in their lives (Sofia et al., 2016). More than 60% of urinary stones are said to contain calcium oxalate (CaOx), more than 40% contain calcium phosphate, and only 4% have magnesium-ammonium-phosphate (struvite) stones, which are an aggregate of crystals, bacteria, and protein matrix (Pak et al., 2003; Flannigan et al., 2014). The prevalence of urolithiasis has dramatically increased recently in both industrialized and developing nations. Urine supersaturation with calcium and oxalate that resulted in crystallization, have been suggested to be one of the main causes of urinary stones (Coe et al., 1992). Research findings have suggested a correlation between microbial infection of kidney and urolithiasis (Flannigan et al., 2014).

The etiology and process involved in renal stone development are multifaceted. It consists of supersaturation of urine, crystal nucleation, growth, aggregation, and renal retention (Tavasoli and Taheri, 2019). Recent studies have suggested that urolithiasis should be considered a systemic disorder as multiple factors interacted and contributed to this underlying medical condition (Cicerello et al., 2021).The molecular process that causes calcium oxalate stone development is currently not fully understood. Various studies have reported that damages to renal tubular cells could take place due to exposure to high oxalate and CaOx crystals (Khan, 1996a; Tsujihata et al., 2003). Injury of renal cells could have resulted in higher affinity for adhesion of crystals (Wiessner, 2001). Macromolecules that led to inflammation and fibrosis were found to be elevated by the deposition of CaOx crystals in the kidney (Hackett et al., 1999; Hackett et al., 1994; Koul et al., 1996). Crystal deposition in the renal tissues has been demonstrated to be closely linked to the generation of free radicals (Trump et al., 1980). Rats supplemented with kidney stone-promoting diets showed rise in lipid peroxidation and decline in antioxidant capability (Karadi et al., 2006). Therefore, it is evident that medicinal plants or its active principles that can prevent these processes can play significant role in bringing about effective medical remedy towards urolithiasis. A number of physiological properties of medicinal plant extracts, including diuretic, lithotriptic, analgesic, and anti-inflammatory properties, may contribute to their capacity to prevent urolithiasis (Joy et al., 2012). As the etiology of urolithiasis is complex, the present study explores different parameters such as inhibition potency on crystal nucleation and aggregation, antimicrobial, anti-inflammatory and antioxidant properties of four medicinal plants viz. *Anneslea fragrans, Mallotus philippensis, Magnolia grandiflora* and *Bauhinia variegata* in comparison with Cystone.

2. Material and method

2.1 Collection of plant materials

The leaves of *Anneslea fragrans*, *Bauhinia variegata* and *Magnolia grandiflora*, and barks of *Mallotus philippensis* were collected from Chandel District, Manipur and authenticated by taxonomist in Manipur University. The materials were properly washed in running water and dried under shade. The dried plant materials were ground and properly stored for extraction of methanolic extracts.

2.2. Preparation of solvent extracts

Methanolic extracts were obtained using Soxhlet apparatus. The total extracting time was adjusted to 24 h, using 250 ml of methanol, at 40°C. The viscous semisolid crude extracts were obtained using a rotary vacuum evaporator. The crude extracts obtained were stored at 4 °C for later use.

2.3. Nucleation assay

Nucleation assay was conducted on the basis of the methodology given by Hennequin et al (1992). A solution of sodium oxalate and calcium chloride was made with final concentrations of 0.5 and 3 mmol/l respectively, in a pH 5.5 buffer that contains 0.15 mol/l of NaCl and 0.05 mol/l of Tris. 200 µl of different concentrations of plant extracts were combined with 1.9 ml of calcium chloride solution and incubation was done at 37 ºC for 30 min in a water bath. Crystallization reaction was triggered by addition of 1.9 ml of sodium oxalate solution. The optical density of the solution was observed at 620 nm, and percentage inhibition was calculated as follow;

% Inhibition = {(Absorbance of control - Absorbance of sample) / Absorbance of control} × 100

2.4. Aggregation assay

The aggregation assay was conducted on the basis of methodology outlined by Hess et al (1989). To prepare seeds of CaOx crystals, 50 mmol/l solutions of $CaCl_2$ and $Na_2C_2O_4$ were combined together, heated to 60 ºC in a water bath for one hour, and then incubated overnight at 37 ºC. Following drying, 0.8 mg/ml of CaOx crystal solution was prepared in a buffer containing 0.05 mol/l Tris-HCl and 0.15 mol/l NaCl at pH 6.5. 3 ml of CaOx solution was mixed with 1 ml of aliquots (100–1000 µg/ml) of the sample, vortexed, and incubated for 30 min at 37 ºC. The optical density of the final mixture was measured at 620 nm, and the percentage inhibition of aggregation was estimated using the following formula;

% Inhibition = {(Absorbance of control - Absorbance of sample) / Absorbance of control} × 100

2.5. Antioxidant study

2.5.1. 2, 2-diphenylpicrylhydrazyl (DPPH) assay

The DPPH assay was performed as per methods given by Blois (1958). Ascorbic acid was used as the reference standard. Using the following formula, the percentage of DPPH radical scavenging activity of plant extracts was determined;

% DPPH radical scavenging activity = $\{(A_0 - A_1)/A_0\} \times 100$

Where, where A_0 represents the absorbance of control and A_1 represents the absorbance of sample.

2.5.1.1. Standard preparation

The quantification of antioxidant activity of plant extracts were determined by 2, 2-diphenylpicrylhydrazyl (DPPH) assay using Ascorbic acid (2 mg/ml) as standard solution. Ascorbic acid in different concentrations (200, 400, 600, 800 & 1000 µl) were prepared using methanol. To 1 ml of all different concentration prepared, 3 ml of DPPH solution (6mg DPPH in 100ml methanol) was added. The mixture was properly mixed, and incubation was done for 30 min in the dark at room temperature. The optical density was measured at 517 nm with spectrophotometer. The antioxidant potential is inversely proportional to the absorbance of the reaction mixture. Finally, calibration curve showing the percentage of DPPH scavenged against the standard concentration was developed.

2.5.1.2 Sample preparation

A 2 mg/ ml of plant extracts were prepared, and different concentrations (200, 500, 1000 µl) of extract were prepared by using methanol. To 1 ml of extracts, 3 ml of DPPH was added, thoroughly mixed, and incubated in the dark at room temperature for 30, and the optical density was recorded at 517nm with spectrophotometer. The inhibition percentage was plotted against concentration, and IC_{50} was calculated using the graph.

2.5.2. Ferric Reducing Antioxidant Potential (FRAP) assay

2.5.2.1. Standard preparation

The Ferric Reducing Power (FRAP) assay was used to measure the antioxidant activity, with ascorbic acid (1 mg/ml) as a standard. Standard was prepared in different concentration (200, 400, 600, 800, 1000 µl) of ascorbic acid. 1 ml of sodium phosphate buffer (0.2 mol; pH=6.6) and 1ml of 1% potassium ferricyanide were added to 0.1 ml of each different concentrations of ascorbic acid prepared, and the mixture was then incubated at 50°C for 20 min. Subsequently, the mixture was mixed with 1 ml of 10% trichloroacetic acid (TCA) and centrifuged for 10 min at 3000 rpm. Then, 1.5 ml of distilled water and 0.1 ml of 0.1% ferric chloride ($FeCl_3$) were combined with 1.5 ml of supernatant. Then, 10 min incubation was done at room temperature, and the optical density was measured at 700 nm with spectrophotometer.

2.5.2.2. Sample preparation

A 2 mg/ ml of extracts was soaked overnight in methanol for the assay. 0.5 ml of extracts was made up to 1 ml with solvent methanol and mixed thoroughly. Then 1 ml of 1% potassium ferricyanide and 1 ml sodium phosphate buffer (0.2 mol; pH=6.6) were added to the mixture, and kept for incubation for 20 min at 50°C. To this, 1 ml of 10% TCA was added, and centrifuged for 10 min at 3000 rpm. To 1.5 ml supernatant, 0.1% ferric chloride ($FeCl_3$) and 1.5 ml of distilled water was added. Then, incubation was done for 10 min at room temperature, and its optical density was measured at 700 nm with spectrophotometer. High absorbance signifies higher reducing potential. The reducing potentials were expressed in mg equivalents of Ascorbic Acid Equivalent per g of sample (mg AAE/g dry weight of samples).

2.6. Antimicrobial study

The antimicrobial activity was analysed by disc diffusion method against two gram positive bacteria viz. *Pseudomonas aeruginosa and Escherichia coli*, and *Staphylococcus aureus,* a gram positive bacterium. Nutrient agar (medium) and plate count agar (medium) were autoclave-sterilized after being dissolved in distilled water at 121°C at 15 psi for 15 min, and then cooled at room temperature. After being poured into petri-plates, the agar medium was left to cool at room temperature until it solidified. The Nutrient agar and Plate count agar plates were inoculated with standardized inoculum of *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. Using sterile disc of filter paper the surface of solidified media was inoculated by soaking each disc onto sample extract having concentration 2 mg/ml. Plates were kept in room temperature for diffusion of sample for 30 min. Standard plate was made with ampicillin, penicillin and vancomycin. Then, incubation was done for 24 h at 37 °C, and the zone of inhibition was assessed.

2.7. Anti-inflammatory activity

2.7.1. Proteinase Inhibition assay

The proteinase inhibition assay was done based on the principle developed by Sakat et al (2010). The reaction mixture consisted of 3 ml of 0.07 mg of trypsin along with 1 ml of 20 mmol Tris HCl buffer of pH 7.4, as well as 1 ml of a test sample with varying concentrations ranging from 50 to 600 µg/ml. The mixture underwent incubation at 37 ºC for 5 min. Following this, 1 mL of 1% (weight/volume) casein solution was introduced. The incubation period was extended by 20 min. A volume of 2 ml perchloric acid with a concentration of 70% was introduced into the reaction mixture in order to halt its progress. After centrifugation, the absorbance was recorded at 210 nm, using buffer as a blank. The experiment was conducted in triplicates to ensure accuracy and reliability of the results. The inhibition percentage of proteinase activity was measured as follows;

% inhibition of denaturation = 100 × (1 – Absorbance of sample/ Absorbance of control)

3. Result

3.1. Anti-nucleation and anti-aggregation studies

The results showed that among the four plants being studied *Anneslea fragrans* showed significant anti-nucleation and anti-aggregation potential with value of 41.86% and 56.67% when compared with Cystone, a polyherbal formulation, as depicted in Table 1, Table2 and Figure 1 and Figure 2 respectively.

Table 1. Percentage CaOx nucleation inhibition activity.

Samples	Percentage (%) inhibition of CaOx nucleation
Anneslea fragrans	41.86%
Mallotus philippensis	1.78%
Magnolia grandiflora	22.78%
Bauhinia variegata	7.62%
Cystone	51.19%

Table 2. Percentage CaOx aggregation inhibition activity of different methanolic extracts.

Samples	Percentage (%) inhibition of CaOx nucleation
Anneslea fragrans	56.67%
Mallotus philippensis	0.60%
Magnolia grandiflora	36%
Bauhinia variegata	15.90%
Cystone	57.51%

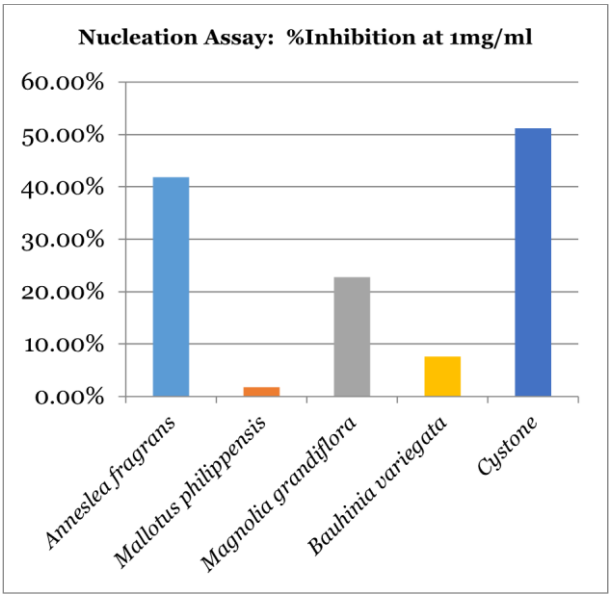

Figure 1. Graphical representation of CaOx nucleation inhibition activity of different plant extracts.

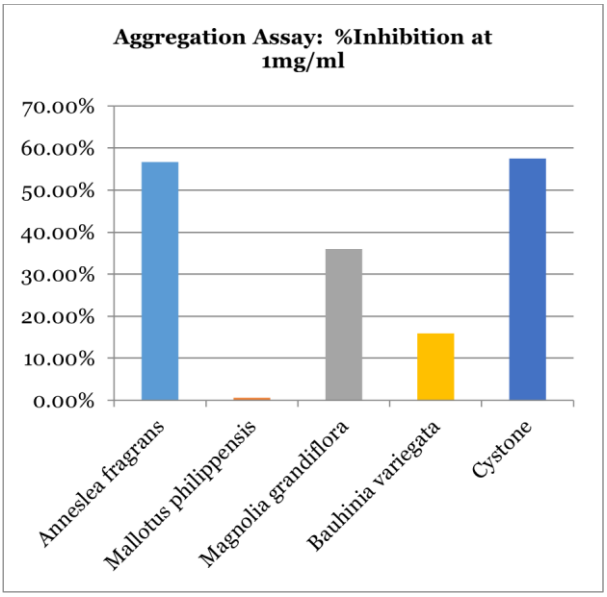

Figure 2. Graphical representation of CaOx aggregation inhibition activity of different extracts.

3.2. Antioxidant studies

3.2.1. 2, 2-diphenylpicrylhydrazyl (DPPH) Assay

The DPPH study using ascorbic acid (2 mg/ml) as standard showed that all the plant extracts showed significant antioxidant properties. Among the plant extracts studied *Bauhinia variegata* showed greater antioxidant property with IC_{50} value of 1.85 as depicted in Table 3 and Figure 3. The remaining other medicinal plants also showed comparable yet lesser antioxidant properties as compared to Cystone.

3.2.2. Ferric Reducing Antioxidant Potential (FRAP) Assay

The FRAP assay showed that *Mallotus philippensis* and *Bauhinia variegata* showed notable antioxidant properties in comparison with Cystone with AAE values of 0.895, 0.95 and 0.92 respectively as depicted in Table 4 and Figure 4.

3.3. Anti-inflammatory study

The anti-inflammatory study indicated that *Magnolia grandiflora* and *Anneslea fragrans* have remarkably higher anti-inflammatory potential as compared to Cystone, as presented by their IC_{50} values of 408 and 420.75 respectively, as shown in Table 5 and Figure 5. Similarly, all the studied medicinal plants showed greater proteinase inhibition potential as compared to Cystone.

3.4. Anti-microbial studies

Anti-microbial studies indicated that all the selected plants showed activities against both gram negative and gram positive bacteria. Among the selected plants *Magnolia grandiflora* appeared to have better anti-microbial activity against *Staphylococcus aureus*, a gram-positive bacterium. The zone of inhibition against gram negative bacterium *Pseudomonous aeruginosa* was of the same range, though it was slightly higher in Cystone as depicted in Table 6 and Figure 6.

4. Discussion

The present study highlighted the medicinal properties of four medicinal plants used in Urolithiasis. The results indicated the important scientific authentication of medicinal plants traditionally used in stone disease. The current study emphasizes on the importance of multifactorial approach in evaluation of medicinal plant extracts for urolithiasis, as the mechanism of stone formation is complex. Urine supersaturation being one of the main prerequisites for crystals formation in the urinary tract, the present study on anti-nucleation and anti-aggregation studies demonstrated that *Anneslea fragrans* has remarkable inhibition properties, having inhibition values of 41.86% and 56.67% respectively when compared with Cystone. These findings suggested that the presence of bioactive components that prevent CaOx crystal nucleation and aggregation. The fact that Cystone contains a variety of therapeutic medicinal plants may account for its marginally greater inhibitory values, each of which contributes to its medical efficacy.

Reactive oxygen species (ROS) are extremely reactive molecules with unpaired electrons that have the ability to damage and alter the chemical composition of nucleotides, proteins, lipids, and carbohydrates (Kamata and Hirata, 1999; Dröge, 2002). Various experimental and clinical data indicated that the renal epithelial cells exposed to elevated concentrations of CaOx and calcium phosphate (CaP) crystals produce ROS, that led to injury and inflammation. Studies have also shown that rats with hyperoxaluria and CaOx nephrolithiasis produce lipid peroxides in

Table 3. IC_{50} values of different plant extracts in comparison with that of Cystone.

Samples	*IC_{50} values*
Anneslea fragrans	1.95
Mallotus philippensis	1.9
Magnolia grandiflora	2.14
Bauhinia variegata	1.85
Cystone	1.4

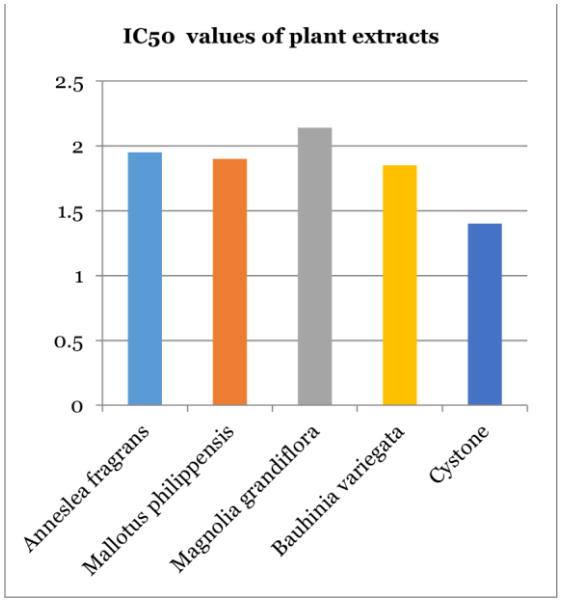

Figure 3. Graphical representation IC_{50} values of different plant extracts in comparison with Cystone.

their urine and renal tissue (Toblli et al., 2002; Thamilselvan et al., 1997). It has been demonstrated that oxalate and crystal deposition-induced oxidative stress can be prevented by antioxidants such vitamin E, selenium and catechin (Santhosh and Selvam, 2003; Khan et al., 2006b). The present studies on 2, 2-diphenylpicrylhydrazyl (DPPH) radical scavenging activity showed that *Bauhinia variegata, Mallotus philippensis and Anneslea fragrans* showed significant antioxidant properties with IC_{50} values of 1.85, 1.9 and 1.95 respectively. The Ferric reducing antioxidant power (FRAP) assay also indicated significant antioxidant properties with AAE values of 0.75 mg/g and 0.72 mg/g for *Mallotus philippensis* and *Bauhinia variegata* respectively, as compared to Cystone with AAE value of 0.895 mg/g, which are indicative of their protective and preventive attributes towards kidney stone formation.

Crystal formation has been suggested to cause activation of inflammation pathways (Mulay et al., 2013). Research has linked kidney cell damage, inflammation, cell loss and fibrosis to crystal deposition (Khan, 2004c). Studies have indicated the crucial role of reactive oxygen species (ROS) in stone formation as both agents of inflammation and injury, and signalling molecules (Khan, 2004d; Khan, 2005e; Khan, 2006f). The anti-inflammatory study demonstrated that *Magnolia grandiflora* and *Anneslea fragrans* showed higher anti-inflammatory potential as compared to Cystone, as showed by their IC_{50} values of 408 and 420.75 respectively. Similarly, all the studied medicinal plants have better proteinase inhibition potential as compared to Cystone.

E. coli and *Pseudomonas spp* were implicated to be the most commonly isolated bacteria from stone cultures, responsible for struvite stone formation (Tavichakorntrakool et al., 2012; Thompson and Stamey, 1973; Golechha and Solanki, 2001). Therefore, the antimicrobial study was conducted to analyze their activities against these microbes. The results on antimicrobial

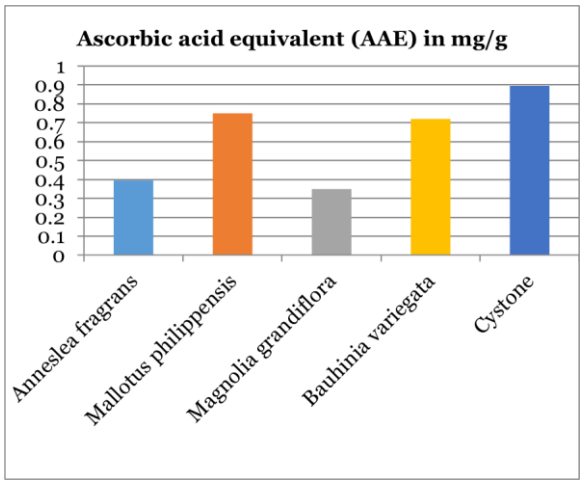

Figure 4. Graphical representation of Ascorbic acid equivalent (AAE) in mg/g for different plant extracts.

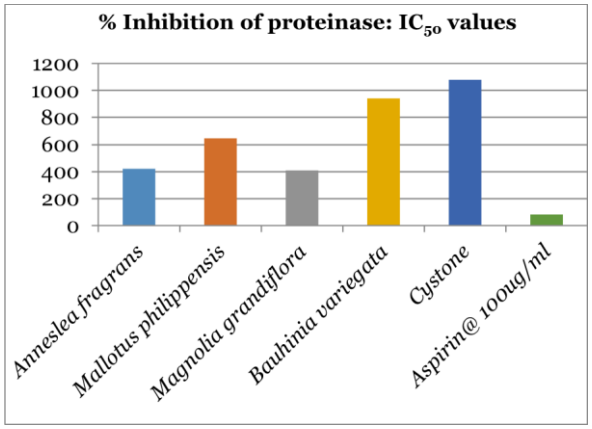

Figure 5. Proteinase inhibition percentage represented in studies using two-gram positive bacteria viz. *Escherichia coli* and *Pseudomonas aeruginosa* and one-gram positive bacterium viz. *Staphylococcus aureus* suggested that all the samples were effective against the tested microbes. The tested samples turned out to be even more effective against *Staphylococcus aureus,* a gram positive bacterium. Among the plant extracts, *Magnolia grandis* was found to be most effective against *Staphylococcus aureus,* when compared to Cystone, whereas *Mallotus philipensis* was found to have similar activity with Cystone.

5. Conclusion

The etiology of stone formation is complex involving many factors, such as urine super saturation, inflammation, oxidative stress and microbial infection. Consequently, it becomes crucial to address each contributing element. Since plant extracts contain different types of phytochemicals, they are one of the best candidates to look for effective bioactive compounds for urolithiasis. Moreover, various medicinal plants, each highly effective against one or more contributing factors, can be properly analysed and used for

Table 4. Data showing Absorbance values at 700 nm and Ascorbic acid equivalent (AAE) in mg/g for FRAP analysis.

Samples	*Volume of sample (µl)*	*Absorbance (700nm)*	*Concentration of test samples (µg)*	*Equivalent concentration of ascorbic acid mg AAE/g of dry weight of sample (AAE mg/g)*
Anneslea fragrans	500	0.0345	395	0.395
Mallotus philippensis	500	0.070	750	0.75
Magnolia grandiflora	500	0.0299	349	0.349
Bauhinia variegata	500	0.067	720	0.72
Cystone	500	0.0845	895	0.895

Table 5. IC_{50} values of different samples.

% Inhibition of Proteinase: IC_{50} values	
	IC $_{50}$ values
Anneslea fragrans	420.75
Mallotus philippensis	646.16
Magnolia grandiflora	408
Bauhinia variegata	940.93
Cystone	1078.95
Aspirin @ 100ug/ml	81.57

Table 6. Zone of inhibition (mm) of different samples.

	Zone of Inhibition (in mm)		
Samples	***Escherichia coli* (-ve)**	***Pseudomonous aeruginosa* (-ve)**	***Staphylococcus aureus* (+ve)**
Anneslea fragrans	8	14	16
Mallotus philippensis	15	12	16
Magnolia grandiflora	13	12	18
Bauhinia variegata	8	13	12
Cystone	6	15	15

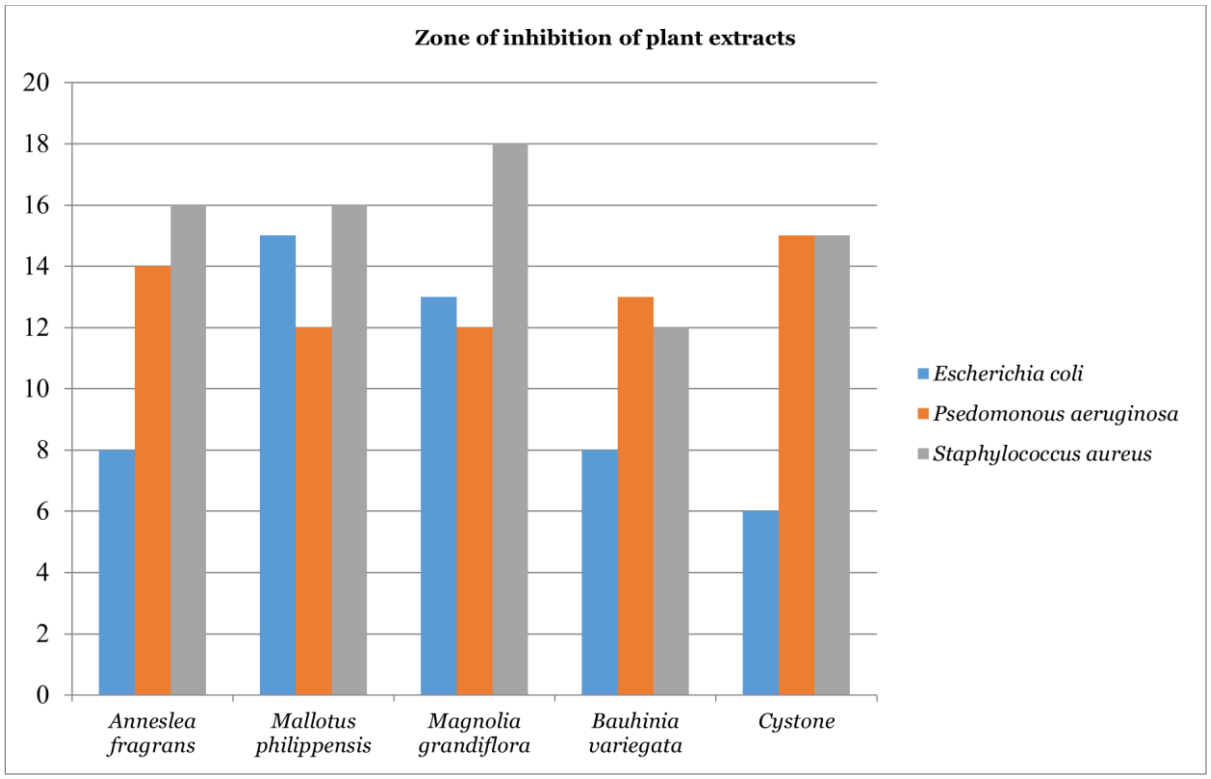

Figure 6. Graphical representation of antimicrobial activities of different samples analyzed.

production of polyherbal formulation like Cystone. In our present study, among the four plants the anti-nucleation and anti-aggregation properties were only slightly lesser than that of

Cystone, which could be due to its additive increase owing to presence of many plants in Cystone. Similar conclusion can be made for antioxidant studies and antimicrobial study. All the plant extracts exhibit greater anti-inflammatory potential than Cystone, however *Anneslea fragrans* and *Magnolia grandiflora* showed significantly greater efficacy among them. It can be concluded that with greater efficiency for screening more traditionally used medicinal plants, more potent bioactive chemicals against urolithiasis could be isolated in future.

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Authors contributions
All the authors have taken equal part in research design, data generation and manuscript preparations.

Conflict of interest
Authors have no conflict of interest.

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