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# Evaluation of in-vitro antiurolithiatic activity of ethanolic extract of Delonix regia, and its comparison with marketed formulations.

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**ABSTRACT:**

Urolithiasis, commonly known as kidney stones, is a global health problem. This study investigated the in vitro anti-urolithiatic activity of Delonix regia ethanolic extract (EEDR) compared to F1 and F2. established formulations for urolithiasis. Nucleation and aggregation assays were conducted to assess the effects on calcium oxalate (CaOx) crystal formation. EEDR exhibited dose-dependent inhibition of both nucleation and aggregation, showing highest inhibition at 1000 µg/ml. Polyherbal formulation F1 displayed sustained inhibition throughout the observed time points, F2 showed a significant effect primarily within the first hour. These findings suggest that Delonix regia has potential as a therapeutic intervention for urolithiasis. Further research, including in vivo studies, is warranted to explore its clinical applications.

**Key words: Delonix regia, nucleation assay, aggregation assay, calcium oxalate, kidney stone.**

## 1.INTRODUCTION

Urolithiasis, commonly known as kidney stone disease, is a global health problem characterized by the formation of calculi in the urinary tract. These stones, which can develop in the kidneys, bladder, or urethra, consist mainly of calcium oxalate, calcium phosphate, uric acid, and cystine. As of 2019, the global burden of urolithiasis remains substantial. The age-standardized incidence rate (ASIR) for urolithiasis globally was approximately 1,394 per 100,000 population. While the ASIR has shown a slight decrease over the past few decades, the absolute number of cases has increased significantly, with a 48.57% rise in new cases since 1990[1]. This increase is primarily attributed to population growth, aging, and changes in lifestyle and dietary habits. The number of deaths associated with urolithiasis also increased by 17.12% from 1990 to 2019, reflecting the growing impact of the disease on global health. In India, the incidence of urolithiasis is particularly high, with an estimated two million new cases reported each year [2]. The management of urolithiasis typically involves pharmacological treatment, dietary modifications, and in more severe cases, surgical interventions. However, these approaches often come with side effects, high costs, and the risk of recurrence, necessitating the exploration of alternative therapies [3].

Herbal remedies have been used for centuries in traditional systems of medicine to prevent and treat urolithiasis. Several medicinal plants are reputed to have anti-urolithiatic properties, including Phyllanthus niruri, Tribulus terrestris, and Cratoxylum formosum. These plants are believed to exert their effects through various mechanisms, such as diuretic action, inhibition of stone-forming substances like calcium and oxalate, dissolution of preformed stones, and antioxidant activity that protects renal tissues from oxidative stress [4,5].

The bioactive compounds in these herbal extracts, such as flavonoids, saponins, alkaloids, and phenolic acids, play a significant role in their anti-urolithiatic activity. For example, flavonoids have been shown to reduce the formation of calcium oxalate crystals, while saponins may inhibit stone growth by interacting with stone-forming ions. Additionally, phenolic compounds in these plants exhibit strong antioxidant properties, which can help prevent oxidative damage to the renal epithelium, a key factor in stone formation [4-7].

Recent trends in medical research have shifted towards exploring alternative and complementary therapies, especially those derived from traditional medicine. Among these, Delonix regia, family Fabacaea commonly known as the Royal Poinciana, Flame Tree or Gulmohar, has gained attention for its potential therapeutic benefits. Traditionally used in various cultures for treating a range of ailments, Delonix regia is reported to possess anti-inflammatory, antioxidant, and diuretic properties. Delonix trees, have been used in traditional medicine for centuries. These trees have been used to treat ailments like rheumatism and stomach problems. Additionally, their leaves are believed to be effective in treating bronchitis and pneumonia in infants. This demonstrates that Delonix trees are not only beautiful but also have valuable medicinal properties. Its phytochemical profile, which includes flavonoids, tannins, and saponins, suggests a potential role in mitigating urinary stone formation [8].

In this context, evaluating the in vitro antiurolithic activity of Delonix regia is of particular interest. In vitro studies offer valuable insights into the efficacy of herbal extracts in preventing or dissolving urinary stones before clinical trials are conducted. Such studies can elucidate the mechanisms by which these extracts influence stone formation and contribute to developing effective and safer treatment alternatives.

To establish a comparative framework, this study also examines two marketed formulations F1 and F2. F1 is an Ayurvedic polyherbal formulation traditionally used to address urinary tract disorders and has shown promise in managing urolithiasis. Its composition typically includes over 15 medicinal herbs namely Bergenia ligulata, Tribulus terrestris, Boerhaavia diffusa, Berberis aristata, Crataeva nurvala, Asparagus racemosus, and Physalis alkekengi which exhibit antiurolithiatic action by inhibiting crystallisation, promote kidney stone dissolution and have anti-inflammatory, antioxidant and diuretic actions.[9]. F2, another formulation aimed at treating urinary stones, includes a unique blend of herbal and mineral ingredients which act as urine alkalizers inhibiting growth of calcium oxalate stones [10]. Comparing the antiurolithic efficacy of Delonix regia with these established formulations can provide a comprehensive understanding of its potential benefits.

This research aims to assess the antiurolithiatic activity of ethanolic extract of Delonix regia by in- vitro nucleation and aggregation assay and compare its efficacy with F1 and F2. By evaluating these interventions in a controlled laboratory setting, we seek to contribute to the growing body of evidence supporting the use of herbal remedies in urolithiasis management and potentially offer new avenues for patient care.

## 2. MATERIALS AND METHODS

### *2.1. Preparation of crude drug (D.regia leaves)*

The leaves were collected from the vicinity in Chembur, cleaned, to remove any dirt or impurities and shade dried. The dried leaves were then finely ground or powdered to increase the surface area and facilitate efficient extraction of the bioactive compounds.

### *2.2. Extraction*

Ethanol (AR grade) was used for the extraction. The powdered leaves are soaked and macerated in ethanol for 3 days with intermittent stirring and shaking. The mixture was then filtered through muslin cloth and passed through Whatman filter paper to obtain the filtrate. The filtrate was concentrated by solvent evaporation to obtain the concentrate of ethanolic extract of D.regia (EEDR) and stored under refrigeration in air tight container until further use.

### *2.3. F1 and F2*

Marketed polyherbal formulation F1 and allopathic formulation F2 were bought from local pharmacy store. Different concentrations of the formulations with respect to the major active constituent were prepared for the study by dilution.

## 3. EXPERIMENTAL

### *3.1 Preliminary phytochemical analysis*

The EEDR was subjected to qualitative analysis for phytoconstituents using well established procedures [11]. The plant extract was screened for presence of alkaloids, flavonoids, phytosterols, and terpenoids.

### *3.1.1 For alkaloids:*

3.1.1.1 Wagner's Test: A fraction of extract was treated with 3-5 drops of Wagner's reagent and observed for the formation of reddish brown precipitate (or colouration).

3.1.1.2. Hager's Test: A fraction of extract was treated with an equal volume of Picric Acid (2 ml) and observed

for the formation of a bright yellow precipitate.

3.1.1.3. Dragendroff's Test: A fraction of extract was treated with 2 ml of Dragendorff's reagent and observed for the formation of an orange-red precipitate.

3.1.1.4. Mayer's Test: A fraction of extract was treated with 2 ml of Mayer's reagent and observed for the formation of a bright yellow precipitate.

***3.1.2 For Saponins:***

Foam test: To 2 ml of extract was added 6ml of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.

***3.1.3. For Flavonoids:***

3.1.3.1. Lead Acetate Test: A fraction of extract was treated with 2 ml of 10% lead acetate solution and observed for the formation of a white precipitate.

3.1.3.2. Shinoda Test: Few magnesium turnings and a few drops of concentrated hydrochloric acid were added to the extract and boiled for five minutes. Red coloration confirms the presence of flavonoids.

***3.1.4. For Phytosterols:***

Liebermann-Burchard test: 1 ml of extract was treated with drops of chloroform, acetic anhydride and conc. H2SO4 and observed for the formation of dark pink or red color.

***3.1.5. For Terpenoids:***

Salkowski's Test: 1 ml of chloroform was added to 2 ml of each extract followed by a few drops of concentrated sulphuric acid. A reddish-brown precipitate produced immediately indicated the presence of terpenoid.

***3.2 In vitro anti-urolithiatic activity by Nucleation and Aggregation assay.***

Various stages of calcium oxalate (CaOx) crystal formation were investigated by in-vitro methods, both with and without the presence of the plant extract or drug. This was assessed through nucleation and aggregation assays.

***3.2.1. Nucleation assay:***

Occurrence of nuclei marks the stone formation. The inhibitory activity of the extract (200, 400, 600, 800 and 1000 micrograms/ml) and marketed formulations; F1 and F2 (20, 40, 60, 80 and 100 micrograms/ml) on nucleation of Calcium oxalate crystals was determined by spectrophotometric assay using method described by Bawari et al[11]. Solution of Calcium Chloride (5mmol/L) and Sodium Oxalate (7.5mmol/L) were prepared in buffer containing Tris (0.05 mol/L) and NaCl (0.15 mol/L) buffer at pH 6.5). 1ml of each concentration of extract was mixed with 1 ml of Calcium Chloride solution followed by addition of 1ml of sodium oxalate solution. Final mixtures were incubated for at 37°C for 30 min. The optical density (O.D) was measured at 620 nm with an UV- visible spectrophotometer.

Percentage inhibition of nucleation was calculated using the following formula:

% Nucleation inhibition = {1-(Optical density of Test/ Optical density of Control)} X 100

***3.2.2. Aggregation assay:***

In the assay, crystals are formed under controlled conditions, and the Drug extract being tested is added to see if it prevents the crystals from clumping together (aggregating). This inhibition of aggregation is indicative of the compound's potential to prevent the formation or growth of kidney stones.

When crystals in solution adhere to one another, larger particulate aggregates are formed. Aggregation inhibition by the extract was assessed using the method described by Bawari et al [12]. Solutions of calcium chloride (CaCl2 and Na2C2O4 (each at 50 mmol/L) were mixed, warmed at 60°C in a water bath for 1 hour, and then incubated overnight at 37°C to produce calcium oxalate (CaOx) seed crystals. After drying, a CaOx crystal solution (0.8 mg/mL) was prepared in a buffer containing 0.05 mol/L Tris-HCl and 0.15 mol/L NaCl at pH 6.5. Two milliliter of EEDR (at concentrations of 200, 400, 600, 800, and 1000 µg/mL) or marketed formulations F1 and F2 (20, 40, 60, 80 and 100 micrograms/ml) was added to 2 mL of the calcium oxalate solution, mixed, and incubated for 30 minutes at 37°C. The optical density of the mixtures was measured at 620 nm, and the percentage inhibition of aggregation was calculated as above.

## 4. RESULTS AND DISCUSSION

***4.1. Preliminary phytochemical analysis***

Qualitative phytochemical analysis of EEDR showed presence of alkaloids, saponins, flavonoids, phytosterols and terpenoids. Saponins inhibit calcium oxalate crystal growth and aggregation due to their detergent-like action, helping dissolve kidney stones [13]. Terpenoids by virtue of their anti-inflammatory and antioxidant properties that protect renal cells from oxidative damage, reducing conditions that favour stone formation [14] while flavonoids prevent calcium

oxalate crystal adhesion and promote the dissolution of small crystals by their antioxidant and anti-inflammatory actions [15]. The phytosterols in EEDR inhibit nucleation and calcium oxalate crystal growth, reducing aggregation thus promoting excretion of smaller particles. Alkaloids possess anti-inflammatory and analgesic properties thus reduce renal inflammation and inhibit calcium oxalate crystal formation, decreasing their adhesion to renal cells[16].

### *4.2. In vitro nucleation assay*

There was a gradual increase in % nucleation inhibition observed for EEDR from 200 µg/ml to 1000 µg/ml. The highest inhibition of 67.27 % was observed at 1000 µg/ml (Table 1). The Herbal formulation F1, showed highest % nucleation inhibition of 23.696±0.009% at 100 µg/ml while F2 showed 24.487±0.001% nucleation inhibition at 100 µg/ml (Table 2). The reduction in nucleation suggests that the bioactive compounds in EEDR and F1, such as flavonoids, saponins, terpenoids, and phytosterols, may interfere with the supersaturation process of calcium oxalate crystals. These compounds likely bind to free calcium and oxalate ions, preventing their aggregation into larger crystals. Urine pH is a key factor influencing the formation of kidney stones, with varying pH levels being linked to different types of renal calculi. Alkaline pH as it reduces the crystallisation of calcium oxalate crystals, F2 demonstrated a significant % nucleation inhibition.

### *4.3. In vitro aggregation assay*

EEDR depicted increasing % inhibition with increasing doses in aggregation assay showing highest inhibition of 60.205±0.001 at 1000 µg/ml for 60-minute incubation. The % inhibition for 61-120th min was 36.392±0.002 and 36.125±0.002% for 121-180th min which was significantly lower than that observed in the first hour of incubation (Table 3). The similar trend was observed for F2 where 100 µg/ml of formulation showed % inhibition of 85.677±0.001%, 35.517±0.004% and 38±0.018% in the 0-60th min, 61st -120th min and 121st-180th min respectively (Table 4). F1 exhibited a dose-dependent inhibitory effect on aggregation. As the concentration of F1 increased, the percentage inhibition of aggregation also rose. The inhibitory effect of F1 was observed across all time points (0-60th, 61-120th, and 121-180th minutes). The highest percentage inhibition was achieved at 100 µg/ml concentration, especially within the 61-120th minute time frame (Table 5). Kidney stones often form when particles clump together and grow larger. This process, called aggregation, is crucial in determining the size, composition, and structure of these stones. Once crystals stick together, they become difficult to separate and can form larger, more solid stones. This aggregation plays an essential role in the development of kidney stones. EEDR and F2 have demonstrated significant inhibition in the first one hour while the polyhedral formulation displayed sustained inhibition throughput all the time points this could be due to synergistic actions of phytoconstituents present in F1.

**Table 1: Nucleation assay of Delonix regia ethanolic extract at different concentrations.**

| EEDR (µg/ml) | % Inhibition |
|---|---|
| 0 | 0 |
| 200 | 1.01% |
| 400 | 5.15% |
| 600 | 19.60% |
| 800 | 36.06% |
| 1000 | 67.27% |

**Table 2: Nucleation Assay of F1 and F2**

| Concentration (µg/ml) | % Inhibition (F1) | % Inhibition (F2) |
|---|---|---|
| 0 | - | - |
| 20 | 2.894±0.005 | 12.167±0.002 |
| 40 | 6.499±0.009 | 21.569±0.001 |
| 60 | 12.746±0.006 | 23.644±0.001 |
| 80 | 19.83±0.008 | 22.585±0.002 |
| 100 | 23.696±0.009 | 24.487±0.001 |

**Table 3: Aggregation Assay of Delonix regia ethanolic extract at different concentrations.**

| EEDR | % Inhibition | | |
|---|---|---|---|
| | 0-60$^{th}$ min | 61-120$^{th}$ min | 121-180$^{th}$ min |
| Control | - | - | - |
| 200 µg/ml | 31.732±0.002 | 11.901±0 | 21.177±0.003 |
| 400 µg/ml | 34.223±0.002 | 22.122±0.002 | 20.065±0.004 |
| 600µg/ml | 34.79±0 | 24.836±0.001 | 22.378±0 |
| 800 µg/ml | 37.782±0.001 | 27.172±0 | 23.601±0.001 |
| 1000 µg/ml | 60.205±0.001 | 36.392±0.002 | 36.125±0.002 |

**Table 4: Aggregation Assay of F2 at different concentrations**

| F2 | % Inhibition | | |
|---|---|---|---|
| | 0-60$^{th}$ min | 61-120$^{th}$ min | 121-180$^{th}$ min |
| Control | - | - | - |
| 20 µg/ml | 11.784±0.001 | 1.092±0.004 | 7.333±0.028 |
| 40 µg/ml | 21.484±0.004 | 4.367±0.003 | 18.667±0.011 |
| 60µg/ml | 79.036±0.001 | 5.845±0.001 | 25.333±0.021 |
| 80 µg/ml | 82.747±0.001 | 14.965±0.007 | 30.667±0.011 |
| 100 µg/ml | 85.677±0.001 | 35.517±0.004 | 38±0.018 |

**Table 5: Aggregation Assay of F1 at different concentrations**

| F1 | % Inhibition | | |
|---|---|---|---|
| | 0-60th min | 61-120th min | 121-180th min |
| Control | - | - | - |
| 20 µg/ml | 12.898±0.002 | 18.68±0.001 | 41.363±0.001 |
| 40 µg/ml | 14.782±0.001 | 54.242±0.002 | 42.204±0.004 |
| 60 µg/ml | 30.153±0.002 | 56.727±0.001 | 49.767±0.001 |
| 80 µg/ml | 44.111±0.001 | 61.782±0.001 | 55.509±0.002 |
| 100 µg/ml | 53.121±0.01 | 66.067±0.002 | 90.71±0.001 |

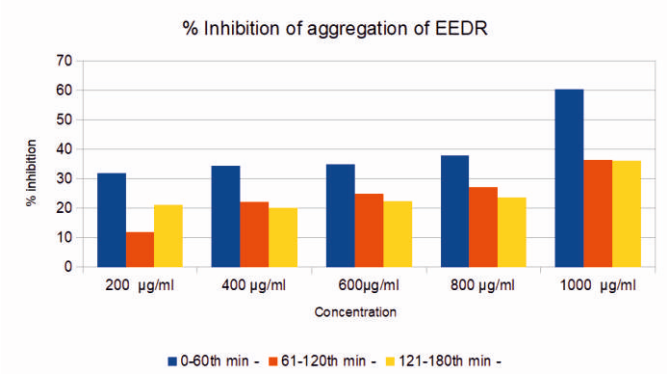


**Figure 1: Percent inhibition of aggregation of EEDR**

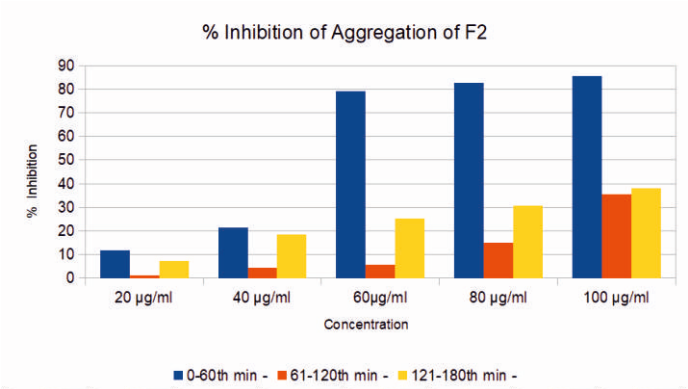


**Figure 2: Percent inhibition of aggregation of F2**

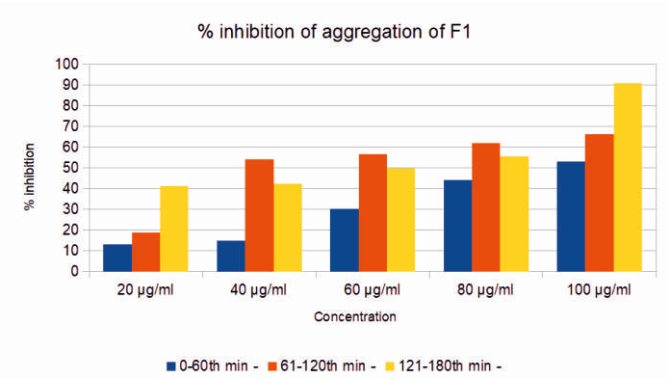


**Figure 3: Percent inhibition of aggregation of F1**

## 5. CONCLUSION

Ethanolic extract of Delonix regia exhibited inhibition of nucleation as well as aggregation comparable to F1 and F2 at all the concentrations and time points. Highest nucleation inhibition was obtained at 1000 µg/ml, in comparison to 100 µg/ml of polyherbal F1 and allopathic F2 formulations. This displays the potential of incorporating Delonix regia as a component of polyherbal formulations which can exhibit synergistic antiurolithiatic activity clinically. In conclusion, our study underscores the significant potential of Delonix regia ethanolic extract as a therapeutic intervention against urolithiasis. Through rigorous examination of critical stone formation processes, including nucleation and aggregation, our findings reveal a dose-dependent effectiveness of the extract in mitigating these pathological events in vitro. Comparative analyses against established herbal and allopathic formulations further validate its efficacy, positioning our extract as a promising alternative treatment option.

## 6. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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