



Investigation Of Plausible Anti-Urolithiatic Potential Of Methanolic And Aqueous Extracts Of *Delonix Regia* Leaves

Aayush Sen*, Vishal Shrivastava

School of Pharmacy, LNCT University, Bhopal

Abstract

In the present work, the effect of methanolic and aqueous extracts of leaves of *Delonix regia* on nucleation and aggregation of calcium oxalate crystals and on ethylene glycol induced lithiasis was evaluated. The yield of the methanolic extract was found to be 17.2 % whereas the yield of the aqueous extract was obtained to be 11.7%. The findings of phytochemical analysis suggest the presence of alkaloids, saponin glycosides, phenolics, terpenoids, sterols, and flavonoids in the leaf of the plant. The phenolic content in the methanolic and aqueous extracts was found to be 38.5 ± 1.804 and 17.43 ± 1.054 % w/w respectively. Both the extracts of *Delonix regia* were able to inhibit the rate of nucleation as well as aggregation of calcium oxalate crystals in the in vitro experiment. The methanolic extract presented better inhibition of crystal formation and hence it was used for in vivo experimentation in ethylene glycol induced lithiasis. The methanolic extract was able to exert diuretic action in rats and also decreased the activity lactate dehydrogenase and alkaline phosphatase, the biomarkers of the biomarkers of lithiasis.

Keywords

Delonix regia, lithiasis, calcium oxalate, ethylene glycol, methanolic extract

Introduction

Urinary calculi buildup in the renal tubules causes urolithiasis, a complex illness. The presence or lack of endogenous inhibitors and complex formers, as well as elevated electrolyte concentration (calcium and phosphorous ions), are some of the anatomical anomalies that contribute to its occurrence. [1]

The prevalence of kidney stones in the general population ranges from 7 to 17 percent, making them the most prevalent urologic condition. For men and women, the lifetime risk of developing stones is greater than 12%. [2] Urinary stones are predicted to affect 12% of Indians, with 50% of those people potentially developing serious renal impairment or kidney loss. Additionally, kidney stones affect almost 15% of the population of northern India. Southern India had fewer cases of urinary calculi than northern India, which may be because tamarind is regularly consumed there. [3]

Oxidative stress is frequently brought on by reactive oxygen species (ROS), and this might result in oxidative tubular damage. The production of stones is accelerated by this damage, which also increases crystal retention and crystal nucleation and aggregation in urine. [4] According to the literature, an imbalance between the formation of reactive oxygen species (ROS) and antioxidant capacity results in oxidative stress, which may harm biomolecules and cause cell damage or death. ROS cause oxidative stress, which plays a significant role in the emergence of many disorders, including urolithiasis (UL). Oxidative stress unquestionably plays a significant part in the development of UL, and antioxidant treatment effectively lowers oxidative damage and crystal formation. [4]

The present study was therefore undertaken to investigate the role of methanolic and aqueous extracts of leaves of *Delonix regia* in prevention of urolithiasis.

Material and Methods

Material

Calcium chloride dihydrate, sodium oxalate, sodium chloride and all other reagents and chemicals were purchased from Oxford Fine Chemicals, Mumbai. All chemicals were of analytical grade and used as obtained without any purification.

Methods

The leaves of *Delonix regia* have been collected from the surroundings of Bhopal, Madhya Pradesh and the preliminary identification of the plant was done at RB Science, Bhopal wide letter number RBS/Plant/2023/001 dated 22/02/2023 using previously submitted voucher specimen authenticated by MFP-PARC, Bhopal.

The leaves of the plant were washed with distilled water and dried in shade (preventing from direct sunlight). The dried leaf has been powdered using slow speed blender and is kept in closed airtight container.

Extraction of the plant material

125 g of the leaf powder was equitably pressed in the extractor of the soxhlet apparatus and extracted with methanol by hot consistent extraction process for around 8 h. The extracted material was filtered while hot through Whatman filter paper to eliminate any contamination. The extract was evaporated on a water bath under careful observation to obtain a thick syrupy concentrate which was then allowed to dry in air. [5] The aqueous extraction was carried out using maceration process. The leaf powder after extraction with methanol was dried and weighed. 110 g of this powder was kept with 500 mL distilled water in a jar. The jar was closed and shaken vigorously followed by intermittent shaking for first 6 hours. The jar was allowed to stand overnight and the contents were mixed well and filtered using muslin cloth. The water was evaporated on a water bath to obtain the extract. The phytochemical screening and total phenolic content of the extracts was determined as per reported procedures. [6]

In vitro inhibition of calcium oxalate crystallization [7, 8]

By measuring the turbidity of the solution at 620 nm, it has been possible to study the precipitation of calcium oxalate at 37 °C and pH 5.7. The turbidity that resulted from the production of calcium oxalate was measured using a UV-Visible spectrophotometer (Labtronics, LT2201).

Stock solution

Solution A: 200 mM sodium chloride (NaCl), 10 mM sodium acetate, and 10.0 mM calcium chloride (CaCl₂), pH 5.7

Solution B: 200 mM sodium chloride (NaCl), 10 mM sodium acetate, and 1.0 mM sodium oxalate (Na₂C₂O₄), pH 5.7.

Before use, solution A and B were warmed to 37°C and filtered through a 0.22 m cellulose acetate filter.

Sample preparation

Delonix regia extracts in methanolic and aqueous form were dissolved in 200 mM sodium chloride, and concentrations of 10% were made by appropriately diluting the solution with sodium chloride.

Experimental protocol

To promote the development of calcium oxalate crystals and investigate the impact of *Delonix regia* extracts on crystallisation, the study was conducted both with and without the extracts.

Without extract (Control)

To produce concentrations of 5.0 mM for calcium and 0.5 mM for oxalate, respectively, 1.0 mL of sodium oxalate solution and 1.0 mL of calcium chloride dihydrate were introduced to the quartz cuvette. For a period of 10 minutes, turbidity was measured every 30 seconds by measuring the absorbance at 620 nm with a UV-Visible spectrophotometer. Three duplicates of each observation were made.

With Extract

1.0 mL of sodium oxalate solution (1.0 mM) in 200 mM NaCl and 10 mM sodium acetate, pH 5.7, was added to the quartz cuvette to create a concentration of 5.0 mM for calcium and 0.5 mM for oxalate, respectively. The calcium chloride dehydrate solution contained 10.0 mM of calcium chloride and was transferred to the quartz cuvette. The cuvette received 1.0 mL of a sample solution of extract in 200 mM NaCl. For a period of 10 minutes, turbidity was measured every 30 seconds by measuring the absorbance at 620 nm with a UV-Visible spectrophotometer. A duplicate of each observation was made.

Percentage inhibition produced by the extract was calculated by the formula

$$[1 - (T_{Ni}/T_{Nc})] \times 100 \text{ for the rate of nucleation;}$$

$$\& [1 - (T_{Ai}/T_{Ac})] \times 100 \text{ for the rate of aggregation}$$

Where, i stand for slope of inhibitor (extract) and c for slope of control.

4.2.3 *In vivo antiurolithiatic action***4.2.3.1 *Experimental Animals***

In the animal home, 150–200g Wistar rats of either sex were kept. The chosen animals were kept in groups and housed in polypropylene cages under typical environmental conditions at 23 °C with a 12-hour cycle of darkness and light. The animal was given unlimited access to food and water. One week before testing, all animals were kept in sterile laboratory conditions.

4.2.3.2 *Experimental Design*

Nine groups of three rats each were formed using rats (n = 5). Rats were given 15 days of drinking water containing 1% w/v of AC and 0.75% v/v of EG to induce CaOx stones. Cystone (750 mg/kg) was used as the normal medication, and 200 mg/kg of extract was given. For 28 days, commercial pellet diets were fed to all groups. Table 1 shows the animal grouping and dosing regimen.

Table 1 Experiment design

Group	Treatment	No. of animals
I	Normal Control	5
II	Ethylene Glycol (0.75% v/v) for 28 days	5
III	Ethylene Glycol (0.75% v/v) for 28 days + standard drug Cystone 750mg/kg, p.o (15-28 th day)	5
IV	Ethylene Glycol (0.75% v/v) for 28 days + Methanolic extract 200mg/kg, p.o (15-28 th day)	5
V	Ethylene Glycol (0.75% v/v) for 28 days + Methanolic extract 400mg/kg, p.o (15-28 th day)	5

Induction of urolithiasis

Oral dosing of ethylene glycol (0.75% v/v) in drinking water caused urolithiasis.

Determination of antiurolithiatic activity

On the 28th day, urine samples were gathered from each animal that had been housed in a metabolic cage. Water was available to animals at all times when pee was being collected. The urine was mixed with a drop of strong hydrochloric acid and kept at 4 degrees Celsius. The volume, pH, lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) of the collected urine samples were all measured.

Determination of LDH activity

The Vassault method was used to measure Lactate dehydrogenase (LDH) activity. [9].

Reagents:

1. Substrate - In 450 mL of distilled water, 3.5 g of K₂HPO₄, 0.45 g of KH₂PO₄, 5.35 g of NaCl (pH 7.2), and 31 mg of sodium pyruvate were dissolved.
2. NADH - 42 mg of NADH were solubilized in 4.5 mL of 1% NaHCO₃.

Procedure:

3 mL of substrate, 50 mL of NADH, and 200 mL of sample were put to a cuvette. Rapid mixing of the solution resulted in a drop in absorbance at 340 nm.

The activity of Lactate Dehydrogenase was calculated using the following formula:

$$\text{LDH Activity (Units/min/mg protein)} = \frac{\Delta A_{340} / \text{min}}{6.22 \times \text{mg protein/ml sample}}$$

Determination of ALP activity

The activity of enzyme alkaline phosphatase (ALP) was measured by the method of Bessey et al. [10].

Reagents:

1. ALP Reagent (*Reagent 1*) – Contains p - nitrophenyl phosphate, Mg^{2+} in Tris/Carbonate buffer (pH 10.2)

Procedure:

Add 1.0 mL of Reagent 1 to the 20 L sample. Mix thoroughly and assess the rise in absorbance at 405 nm over time. The ALP activity was determined by the following formula

$$\text{ALP Activity (IU/L)} = \frac{(\Delta A_{405} / \text{min}) \times \text{T.V.} \times 10^3}{\text{S.V.} \times \text{Absorbivity} \times \text{P}}$$

Where, T.V. – total reaction volume in μL ; S.V. – sample volume in μL ; Absorbivity – 18.8; P – cuvette path length (1 cm)

Results and Discussion

The leaves of *Delonix regia* were found to grow alternately on the stem, biparipinnate with stout petiole. The leaf was light green in color with oblong-obtuse leaflets. The yield of the methanolic extract was found to be 17.2 % whereas the yield of the aqueous extract was obtained to be 11.7%. The findings of phytochemical analysis suggest the presence of alkaloids, saponin glycosides, phenolics, terpenoids, sterols, and flavonoids in the leaf of the plant.

Total Phenolic Content

The results of the total phenolic content of the extracts examined, using Folin-Ciocalteu method. The total phenolic content in extracts, expressed as percent w/w. The phenolic content in the methanolic and aqueous extracts was found to be 38.5 ± 1.804 and 17.43 ± 1.054 % w/w respectively.

In vitro inhibition of calcium oxalate crystallization

Turbidity in the sodium chloride solution was measured over time to examine the influence of the extracts on different phases of calcium oxalate crystallisation. Plotting the time against the absorbance represented the absorbance according to the time in the absence or presence of extracts. Figure 1 shows the rate of oxalate crystallisation.

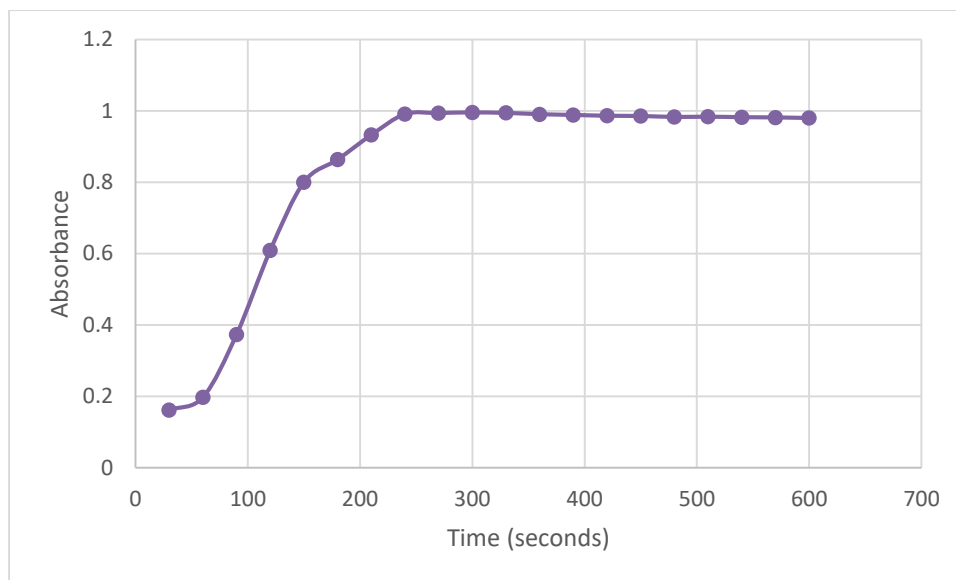


Figure 1 Rate of formation of calcium oxalate crystals (control)

Through the use of linear regression analysis, the maximum slope of the absorbance rise with time was identified. It exhibits a time-dependent increase in particle number. As an indicator of particle concentration, absorbance can also show an increase in particle size. As a result, crystal nucleation is represented as the highest slope of the rise in absorbance with time. Once saturation is reached, crystals are unable to form new crystals or expand, leading to an overall drop in absorbance over time, the slope of which shows the rate of decrease in particle number as a result of crystal aggregation. [11, 12]

The administration of methanolic and aqueous extracts of *Delonix regia* caused an inhibition of the slope of rate of nucleation as well as the rate of aggregation of calcium oxalate crystallization (figure 2 and 3).

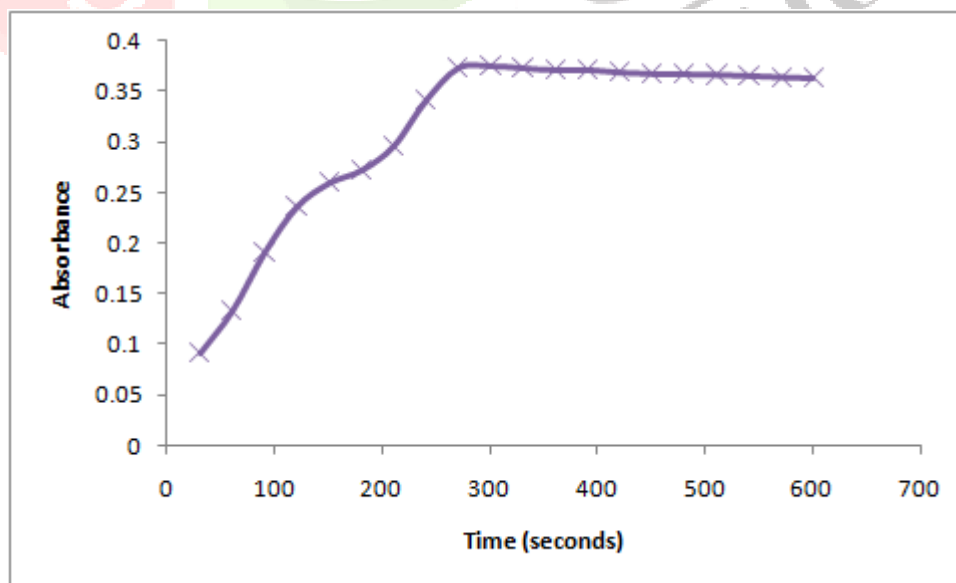


Figure 2 Rate of formation of calcium oxalate crystals (Methanolic extract)

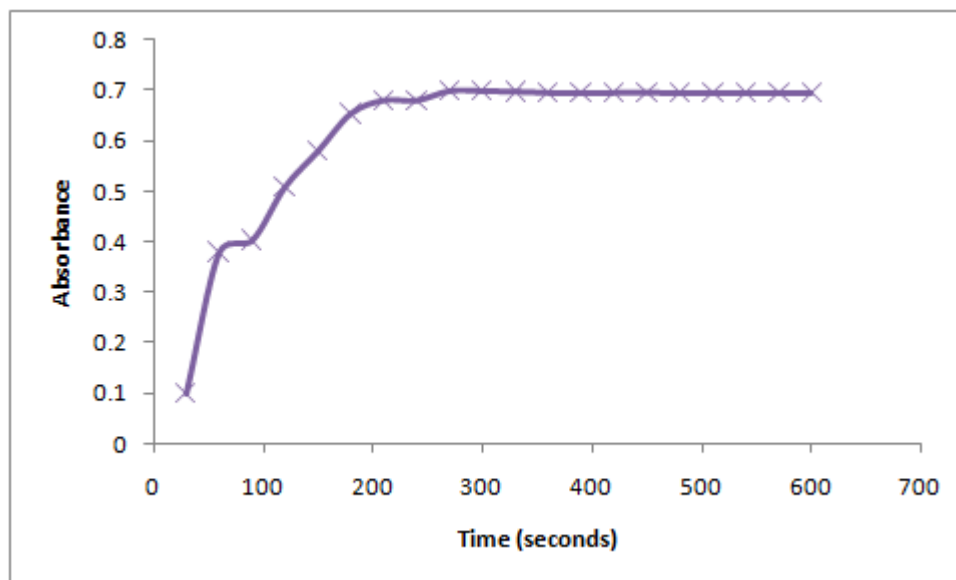


Figure 3 Rate of formation of calcium oxalate crystals (Aqueous extract)

The findings show that when both extracts were administered in comparison to the control, the rate of calcium oxalate crystal nucleation decreased, indicating an anti-urolithiatic action.

***In vivo* antiurolithiatic action**

The methanolic extract was significant in preventing the formation of crystal. Hence it was used for the in vivo studies. Because it causes Calcium oxalate crystalluria in rats without causing serious renal injury and because it matches the aetiology of stone formation in humans, ethylene glycol is employed as a urolithiasis induction agent.

A significant factor in the production of calcium oxalate stones is urine volume. In this study, the presence of calcium oxalate crystals caused a decrease in urine production in the ethylene glycol treated group (Lithiatic), indicating an obstruction in the urinary flow. On treatment with cystone and extract, an increase in urine output was seen, indicating their diuretic activity. Additionally, additional pee could dilute the urinary electrolytes and lessen the likelihood of stone formation (table 2).

Table 2 Effect of treatment of Urine volume and pH

Group	Urine volume	pH
I	2.068 ± 0.043	7.14 ± 0.0547
II	1.2 ± 0.070	6.48 ± 0.130
III	2.66 ± 0.054	7.42 ± 0.109
IV	2.3 ± 0.1	7.08 ± 0.045
V	2.2 ± 0.122	7.04 ± 0.054

Values are expressed as mean ± SD; n = 5

LDH activity

A biomarker for kidney damage is lactate dehydrogenase (LDH), a cytosolic enzyme produced upon cell injury. In lithiatic group II, the activity of urine LDH was extremely raised and reached 386.37%. LDH activity significantly increased in Cystone (positive control), with a rise of 84.42% as compared to group I animals. A drop in LDH activity was also seen after treatment with methanolic extract, with an increase of up to 180.81% at higher extract doses (400 mg/kg) compared to the control group.

ALP activity

Alkaline phosphatase (ALP), a strong biomarker, and other enzymes are released when renal calculi injure the renal epithelium. When compared to the control group, the treatment of ethylene glycol boosted ALP activity by roughly 433.01%. When cystone was administered, the activity of ALP was dramatically reduced, increasing by 136.42% compared to group I mice. At the higher dose compared to control, there was a 297.31% increase in ALP activity in rats treated with methanolic extract.

Table 3 Effect of treatment on ALP and LDH activity

Group	LDH	% increase in LDH*	ALP	% increase in ALP*
I	0.023 ± 0.0003	-	181.75 ± 5.075	-
II	0.112 ± 0.0074	386.37	968.78 ± 13.556	433.01
III	0.042 ± 0.0199	84.42	429.70 ± 20.89	136.42
IV	0.086 ± 0.0098	272	863.20 ± 12.34	374.93
V	0.065 ± 0.0003	180.81	722.13 ± 3.537	297.31

Values are expressed as mean ± SD; n = 5. *compared to control (group I)

Conclusion

Delonix regia has demonstrated promising anti-urolithiatic potential by *in vitro* method suggesting the possible mechanism of interfering with the nucleation of calcium oxalate crystallization. It also decreased the occurrence of calcium oxalate crystals in urine of experimental animals as witnessed from increased urine volume and normalized pH of the urine. It could be concluded from the study that methanolic extract of *Delonix regia* is potent anti urolithiatic and can be optimized for treatment of kidney stones.

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