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PHYTOCHEMICAL SCREENING AND *IN-VITRO* ANTIUROLITHIATIC ACTIVITY OF *PUNICA GRANATUM* AQUEOUS LEAF EXTRACT

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Keywords:

Punica granatum, Antiurolithiatic activity, Aggregation assay, Nucleation assay

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ABSTRACT: Pashanabheda is used as antiurolithiatic in Ayurveda. In the present study, *Punica granatum* is used for screening in-vitro antiurolithiatic potentials. Screening of *in-vitro* antiurolithiatic potentials of aqueous leaf extract of *Punica granatum*. The extract was subjected to Preliminary phytochemical analysis. *In-vitro* anti-urolithiatic activity was screened by nucleation and aggregation assay in which the formation and growth of CaOx crystals were quantified. The extract exhibited inhibitory action in both nucleation and aggregation assays to a significant level. In the aggregation assay gradual decrease in the CaOx crystal nucleation, as well as growth, was observed by light microscopy. The findings of the nucleation assay indicate that the extract inhibited the crystallization of CaOx in the solution. There were less and smaller particles with increasing concentration of the extract. The increasing concentrations of extract (100, 200, 300, 400 and 500 µg/ml) inhibited the CaOx crystal growth. *Punica granatum* extract demonstrated slightly better results compared to Cystone standard solution to inhibit the formation of calcium oxalate dihydrate crystals in the nucleation assay. The aqueous leaf extract of *Punica granatum* has shown anti-urolithiatic effect by significantly reducing the size and growth of calculi in the *in-vitro* assays.

INTRODUCTION: The problems related to the kidney are the major problem for human beings throughout the world because the kidney is the major excretory organ in animals and humans. Urolithiasis is characterized by the formation of a stone in the kidneys or urinary tracts.

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In India, approximately 5-7 million patients suffer from stone disease and at least 1/1000 of Indian population needs hospitalization due to kidney stone disease. Currently urinary stone formation affects 10% to 12% of the population in industrialized countries and the peak incidence seems to be at ages 20 to 40 years [1, 7].

Urinary calculi are the third most prevalent disorder of the urinary system. Approximately 80% of these calculi are composed of calcium oxalate [2, 4]. Urine is normally a supersaturated solution and only some individuals are prone to this disease. One reason for this is the presence of inhibitors of

lithogenesis in urine, including macromolecules, citrate and magnesium [4, 5]. Thus, an imbalance between the promoters like low urine volume, calcium, oxalate, uric acid, phosphate and inhibitors may represent a potential factor in lithogenesis. Nowadays, stone formation is the oldest and serious painful urologic disease with significant prevalence in the population due to changes in lifestyle and dietary factors. Stone formation or lithiasis is characterized by calculi formation.

It has two main types such as nephrolithiasis and urolithiasis. Calculi formation in urinary bladder, ureter or any part of urinary tract rather than kidney is known as urolithiasis, while nephrolithiasis is characterized by calculi formation in the kidney [6, 10]. Generally, calcification for the formation of bone and teeth takes place in controlled biological situations. Uncontrolled pathological crystallization occurs when solvent becomes supersaturated, leading to the formation of precipitates in the body called as kidney stones. Calcium-containing stones, especially calcium oxalate monohydrate, calcium oxalate dihydrate are the most commonly occurring ones to an extent of 75-90% followed by magnesium ammonium phosphate to an extent of 10-15%, uric acid 3-10% and cystine 0.5-1%. In most of cases, the commonly occurring stones are calcium oxalate type.

The pathogenesis of calcium oxalate stone formation is a multi-step process and in essence, includes nucleation, crystal growth, crystal aggregation, and crystal retention. The stone formation requires supersaturated urine. Supersaturation also depends on urinary pH, ionic strength, solute concentration, and complexations [8]. In spite of substantial progress in the pathophysiology and treatment of urolithiasis, there is no satisfactory drug being used in clinical therapy. Endoscopic stone removal and extracorporeal shock wave lithotripsy are prohibitively costly, and recurrence is quite common with these procedures [9]. Thus a drug for the prevention of this disease or its recurrence would be of great interest. Oxalic acid is biosynthesized from ascorbic acid, glycolate, and glyoxylate in the metabolism of higher plants. A significant loss of minerals is more prevalent in the body when it is consumed in large content of oxalate rich foods. When calcium ions present in the body bind with free oxalic acid/oxalate it precipitate as insoluble crystals [12]. A large number of plants have been used in India since ancient times, which claim the efficient cure of urinary stone. Medicinal plants have played a significant role in various ancient traditional systems of medication. Even today, plants provide a cheap source of drugs for majority of world's population. Several pharmacological investigations on the medicinal plants used in traditional antiurolithic therapy have revealed their therapeutic potential in the in vitro or in vivo models.

Therefore, it is worthwhile to look for alternative means such as medicinal plants or phytotherapy [11, 12]. Data from *in-vitro*, *in-vivo* studies and clinical trials reveal that phytotherapeutic agents could be useful as either an alternative or an adjunctive therapy in the management of urolithiasis [13, 15]. *Punica granatum* L. commonly known as pomegranate is a fruit-bearing deciduous shrub or small tree, native to Asia and belongs to the family Lathraceae (Altuner, 2011) [14]. The leaves are shiny and about 7.6 cm long [15]. Different parts of plants such as leaves, bark, and fruit have medicinal significance [16]. *Punica granatum* has been used as traditional medicine in many countries for the treatment of dysentery, diarrhoea, helminthiasis, acidosis, hemorrhage *etc.* [17]. Numerous phytochemical constituents have been reported in different parts of the Punica granatum plant, making it medicinally important. Owing to the above apprehensions, the present study was undertaken to screen the phytochemical constituents and antiurolithiatic activity of aqueous leaf extract of *Punica granatum*.

MATERIALS AND METHODS:

Collection and Authentication of Plant: The leaves of *Punica granatum* were collected from Medchal District, Hyderabad, Telangana. The plant was authenticated by the Botanical Survey of India, Deccan Regional center, Sai Hill colony, Attapur, Hyderabad, Telangana 500030.

Extraction Methods[14, 16]: The fresh tender leaves of *Punica granatum* were collected, and the leaves were washed with deionized water and disinfected with 0.1% $HgCl_2$ solution for 5 min and dried in the shade away from light for 15 days and ground

to a fine powder using an electric grinder and sieved [18, 19]. The fine powder of *Punica granatum* was made into a thimble for loading in the Soxhlet apparatus, and extraction was done continuously for 72 h. The extract thus obtained was concentrated under a vacuum rotary evaporator and extracts were kept in desiccators until used [20, 21].

Preliminary Phytochemical Screening: Phytochemical screening of the *Punica granatum* leaf extracts with various solvents was performed to detect different classes of constituents, such as alkaloids, phenolics, flavonoids, tannin, saponins, terpenes, phlorotannins, and coumarins [21, 22].

***In-vitro* Antiurolithiatic Activity:**

Aggregation Assay: The rate of aggregation of the calcium oxalate crystals was determined by a spectrophotometric assay with slight modifications. The calcium oxalate monohydrate (COM) crystals were prepared by mixing calcium chloride and sodium oxalate of 50 mM each. Both solutions were then equilibrated. The solutions were then cooled to 37 ℃ and then evaporated. The COM crystals were then dissolved with 0.5 ml of 0.05 mM Tris buffer and 0.5ml of 0.15 mM NaCl solution at pH 6.5 to a final concentration of 1 mg/ml. Absorbance at 620 nm was recorded. The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the extract against control [23, 24].

Nucleation Assay (Turbidity Method): The inhibitory activity of the extracts on the nucleation of calcium oxalate crystals was determined by a spectrophotometric assay. Crystallization was initiated by adding 100 µl of 4 mM calcium chloride and 100 µl of 50 mM sodium oxalate solutions to 0.5 ml of normal human urine, both prepared in a buffer containing 0.5 ml of 0.05 mM Tris buffer and 0.5 ml of 0.15 mM NaCl solution at pH 6.5 and 37 °C and adjusted to volume by adding 1.5 ml of distilled water. The rate of nucleation was determined by comparing the induction time of crystals (time of appearance of crystals that reached a critical size and thus became optically detectable) in the presence of the extract and that of the control with no extract. The optical density (OD) was recorded at 620 nm- and the percentage inhibition calculated as (1-OD (experimental)/OD (control))/100 [24, 28].

RESULTS:

Preliminary Phytochemical Screening: The phytoconstituents present in the plant are found to be tannins, flavonoids, phenolic compounds, amino acids, and alkaloids. The results are summarized in **Table 1.**

TABLE 1: PRECLINICAL PHYTOCHEMICAL SCREENING

Type of Extract Tests	Petroleum ether	Ethyl acetate	Chloroform	Ethanol	Aqueous
Test for Carbohydrates					
Fehling's test	-	-	-	-	+
Benedicts test	-	+	-	+	+
Barfoed's test	-	-	+	+	-
Pentose sugar	-	-	-	+	+
Test for Amino acids					
Ninhydrin reaction	-	-	-	-	-
Tyrosine test	-	+	+	+	+
Test for Flavonoids					
Shinoda test	-	-	-	-	+
Lead acetate test	-	-	-	-	+
Test for Tannins and Phenolic compounds					
5% $FeCl_3$	-	+	-	+	+
Lead acetate	-	+	-	-	+
Acetic acid	-	-	-	-	+
Dil.Iodine	+	+	-	+	+
Bromine water	+	+	+	+	+
Test for Glycosides					
Legal's test	-	+	+	+	+
Keller-Killiani test	+	+	-	-	+
Foam test		With dry powder +			
Iodine test	-	-	-	-	+
Salkowski reaction	+	+	-	-	+

Aggregation Assay: Calcium oxalate crystals begin grow; aggregate with other crystals, and retained in the kidney. This is an aggregation process which causes renal injury. Aqueous leaf extract of *Punica granatum* demonstrated slightly better compared to Cystone standard solution to inhibit promoted the formation of COD crystals **Fig. 1** [29]**.**

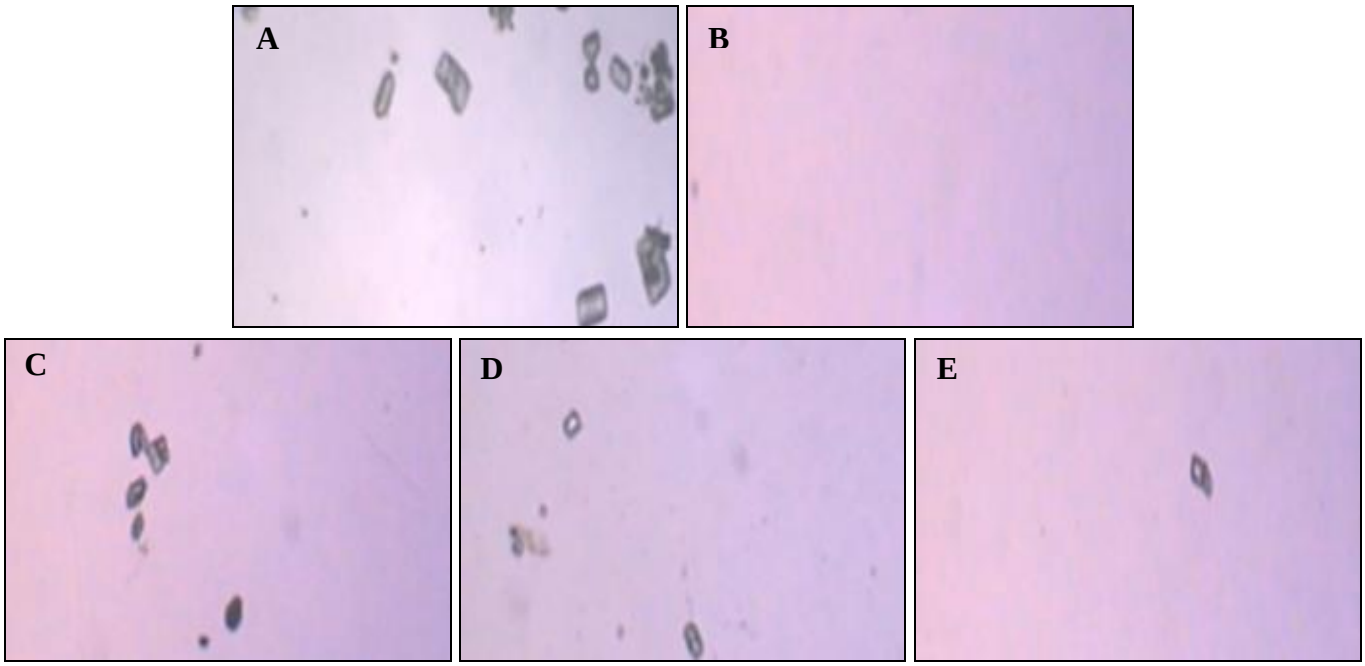
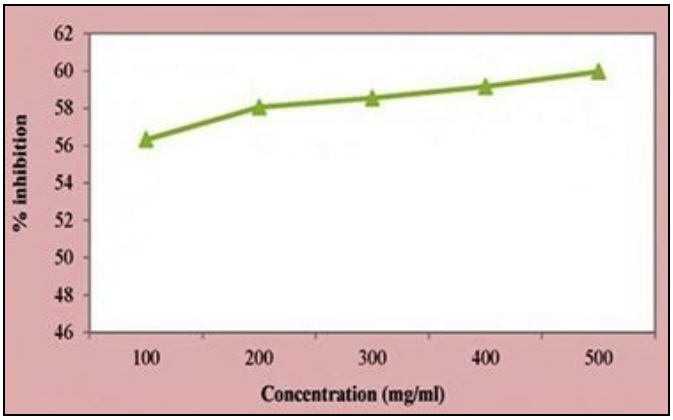
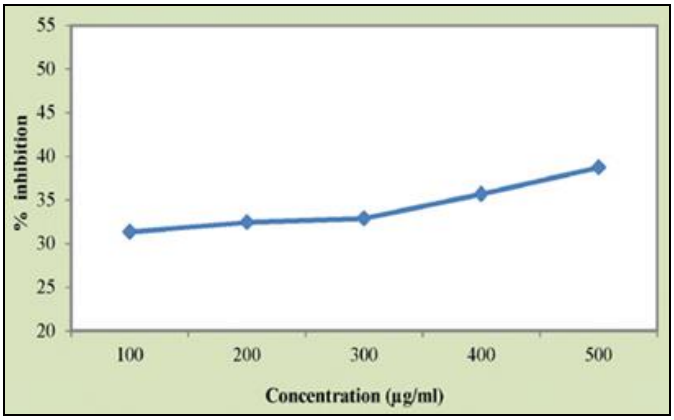


FIG. 1: CAOX CRYSTALS, OBSERVED UNDER LIGHT MICROSCOPE (100X), FORMED IN THE METASTABLE SOLUTION OF CAOX IN THE ABSENCE (A) CONTROL WHICH SHOWS A LARGE NUMBER OF CRYSTALS (B) CYSTONE GROUP WHICH SHOWS NO CRYSTALS (C) 100 MG/ML, (D) 200 MG/ML, (E) 400 MG/ML OF THE TEST EXTRACT SHOWING GRADUALLY DECREASE IN THE CAOX CRYSTALS NUCLEATION AS WELL AS GROWTH

Nucleation Assay: *Punica granatum* inhibited the crystallization by inhibiting nucleation of calcium oxalate through disintegrating into smaller particles with increasing concentrations of the fraction. The results of the nucleation, assay confirmed that the extract contained nucleation-preventing agents **Fig. 2.**



EFFECT OF CYSTONE ON CAOX NUCLEATION



CRYSTAL EFFECT *OF PUNICA GRANATUM* ON CAOX CRYSTAL NUCLEATION

FIG. 2: EFFECT OF CYSTONE AND AQUEOUS LEAF EXTRACT OF *PUNICA GRANATUM* ON CAOX CRYSTAL NUCLEATION

DISCUSSION: Kidney stones are reportedly affecting mankind for a long time and have been one of the causes of renal failure [24, 25]. AS there is no single effective drug available for urolithiasis today, surgery is considered to be the best option, especially when other alternatives fail. However, it

is expensive and not affordable for the common man. Hence the natural drugs are considered to be next alternative. Pashanabheda plants are a group of medicinal plants used in the Indian traditional medicinal system by Ayurveda practitioners as antiurolithiatic drugs and Punica granatum are used conventionally antiurolithiatic and diuretic. In the Aggregation assay, calcium oxalate crystals begin to grow, aggregate with other crystals, and are retained in the kidney [26]. This is an aggregation process that causes renal injury. Aqueous leaf extract of *Punica granatum*. Demonstrated slightly better compared to Cystone standard solution to inhibit promoted the formation of COD crystals. COM has a stronger affinity with cell membranes; it may lead to become a higher potential risk for renal calculi formation.

An *in-vitro* crystallization study was performed since nucleation is an important first step for the initiation of crystals, which then grow and form aggregates. The main findings of the present study were that saponin and flavonoid rich fractions from plants inhibited the crystallization by inhibiting nucleation of CaOx in solution; less and smaller particles were formed with increasing concentrations of the fraction. The results of the nucleation assay confirmed that the extract contained nucleation-preventing agents. The limiting factors in stone formation could be those processes that affect crystal growth because particles may become large enough to occlude the urinary tract, leading to stone formation [27, 31]. The herb extracts may contain substances that inhibit the growth of CaOx crystals. This property of plants may be important in preventing the growth of kidney stone. Aggregation may be an important factor in the genesis of stones [32, 33]. Recurrent calcium stone formers excrete clusters of crystals in their urine, caused by aggregation, also named agglomeration, whereas urine from normal people contains mainly single crystals [34, 36]. Again, the percentage inhibition of crystals aggregation increased as the concentration of aqueous leaf extract of *Punica granatum* increased.

CONCLUSION: Antiurolithiatic activity of *Punica granatum* is mediated through inhibition of CaOx crystal formation and its effect on the urinary concentration of stone-forming constituents. The phytochemical screening conclusively demonstrates that *Punica granatum* leaves are a good source of various phytoconstituents like alkaloids, flavonoids, carbohydrates, glycosides, saponins, tannins, terpenoids, among which saponins and flavonoids are responsible for the significant antiurolithiatic activity. This study rationalizes *Punica granatum* leaves medicinal use in urolithiasis.

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CONFLICTS OF INTEREST: The authors declare no conflicts of interest.

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