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A Preliminary phytochemical screening and anti-urolithiatic property of aqueous extract of *Kalanchoe pinnata*

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Abstract

Urolithiasis is one of the major diseases of the urinary tract and is a major source of morbidity. Stone formation is one of the painful urologic disorders that occur in approximately 12% of the global population and its re-occurrence rate in males is 70-81% and 47-60% in female. Urolithiasis has become the third most Prevalent disorder of the urinary tract and nearly 80% of these calculi are composed of calcium oxalate (CaOx). Large sections of the population in developing countries still rely on traditional practitioners and herbal medicines for their primary care. In ancient traditional systems, several medicinal plants have been reported for anti-urolithiatic activity. These plants provide a cheap source of drugs and are regarded as comparatively safe with minimal or no side effects, are readily available, and are affordable. So, the present study was undertaken to investigate the effect of aqueous extract of Kalanchoe pinnata leaves on in-vitro urolithiasis.

Since most of phytochemical compounds are extracted in water, the objective of the present study was to evaluate the diversity of phyto-constituents present in the extract and to determine the in-vitro anti-urolithiatic potential of aqueous extract of leaves of K. Pinnata for the use of these plants in traditional systems of medicine. Phytochemical screening of aqueous extract of Kalanchoe pinnata was analysed for understanding the phytochemicals present in the extract .The test result indicates that the presence of Phenols, Tannins, Carbohydrates, Flavonoids, Alkaloid, Protein, Triterpenoids, Steroids and Phytosteroids in the aqueous extract of pinnata.

Cystone is used as standard for comparative analysis of anti-urolithiatic property of plant extracts. In this study, calcium oxalate was also prepared as per the standard protocol and the synthesized calcium oxalate was used for the analysis of anti-urolithiatic potential of the aqueous extract of K. pinnata. Anti-urolithiatic potential of the aqueous extract of K. pinnata was analysed. The result indicates more potency in dissolution of calcium oxalate crystals.

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Hence the present study demonstrates a significant anti-urolithiatic potential of the aqueous extract of K. pinnata. Hence, the aqueous extract of K. pinnata may be used for the isolation of anti-urolithiatic compound for commercialization.

Keywords: Urolithiasis, Anti- urolithiatic effect, Kalanchoe pinnata, Phytochemicals, Calcium Oxalate

Introduction

The development of stone in the urinary system, i.e. in the kidney, ureter and urinary bladder or in the urethra is called urolithiasis. 'Urolithiasis' = ouron (urine) and lithos (stone). Urolithiasis is one of the major diseases of the urinary tract and is a major source of morbidity. Stone formation is one of the painful urologic disorders that occur in approximately 12% of the global population and its re-occurrence rate in males is 70-81% and 47-60% in female (Phatak and Hendre, 2015). It is assessed that at least 10% of the population in industrialized part of the world are suffering with the problem of urinary stone formation. The occurrence of the renal calculi is less in the southern part when compared with other parts. The rate of occurrence is three times higher in men than women, because of enhancing capacity of testosterone and inhibiting capacity of oestrogen in stone formation. (Vijaya et al. 2013). It has been found that the formation of urinary calculi dates back not only to 4000 B.C in the tombs of Egyptian mummies also in graves of North American Indians from 1500 to1000 B.C. Stone formation is also documented in the early Sanskrit documents during 3000 and 2000 B.C. The problem of stone formation is considered as a medical challenge due to its multifactorial aetiology and high rate of reoccurrence.

Urolithiasis has become the third most Prevalent disorder of the urinary tract and nearly 80% of these calculi are composed of calcium oxalate (CaOx) It estimated to occur in around 12% of the global population worldwide (Alelign and Petros 2018, Patel et al., 2012). The formation of calcified renal stone is a physiochemical event leading to crystal nucleation, aggregation and its growth assisted by many biological processes including urine volume, pH, increased calcium oxalate or sodium oxalate, and urates (Ratkalkar and Kleinman, 2011; Khan et al., 2016). When an increased amount of calcium ions present in the body reacts or binds with oxalic acid/oxalate which are present in oxalate rich foods, they precipitate as calcium oxalate crystals in the body and lead to hypocalcaemia (due to low availability of free calcium ions) and urolithiasis (Evan et al., 2010; Dawson CH 2012). There are multiple mechanisms involved in the pathogenesis of urolithiasis are one of the most important reasons for failure in the development of anti-urolithiatic drugs. At present, over 90% of upper urinary tract stones patients have been treated according to the size, type, and position of the stones with a success rate of 68 to 86% (Alelign and Petros 2018, Bahmani et al., 2016). In these cases, patients have to treat with modern interventional procedures which are not easily assessable, and almost not suitable for patients with high urinary stone recurrence rate.

Thiazide diuretics and alkali citrate are used commonly in the prevention of recurrence of urolithiasis. Stones larger than 5 mm or stones that fail to pass through should be treated using interventional procedures, such as endoscopic stone removal, Extracorporealshock wave lithotripsy (ESWL), Ureteroscopy (URS), or Percutaneous Nephrolithotomy (PNL). These procedures are very expensive for many patients and are associated with recurrence of kidney stones, which is often up to 60%. They also require careful follow-up for many years for possible complications such as acute renal injury (C. T et al. 2022). Therefore, in many countries, phytotherapeutic agents are widely used as complementary and alternative therapies for the management of urolithiasis (C. T et al. 2022). According to World Health Organization (WHO), medicinal plants would be the best source to obtain variety of drugs. Large sections of the population in developing countries still rely on

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traditional practitioners and herbal medicines for their primary care.

In ancient traditional systems, several medicinal plants have been reported for anti-urolithiatic activity. These plants provide a cheap source of drugs and are regarded as comparatively safe with minimal or no side effects, are readily available, and are affordable (Chandra et al., 2014). Medicinal plants are plants in which one or more of their organs contain substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. Such plants should be investigated to better understand their properties, safety and efficacy (World Health Organization (WHO) 2002). On this context, herbal medicines are found to be effective, as well as easily available and economical. Plants of lepidagathis genus were used traditionally for the treatment of urinary calculi, dysuria, polyuria, fever, dysentery and uterine disorders (Madhavan et al., 2009).

Various medicinal plants whose anti-urolithiatic activity is reported till date are Herniaria hirsute, Amni visnaga, Tribulus terrestris, Bergenia ligulata, Dolichos biflorus, Dolichos biflorus, Vediuppu chunnam, Raphanus sativus, Achyranthus aspera, Quercus salicina, Phyllanthus niruri, Cynodon dactylon (Hussain and Nazmeen, N. 2019). Pashanabheda is a scientific term used in the Ayurveda system of medicine describing the potency of medicinal plant for breaking up and disintegrating renal and urinary calculi i.e., kidney stones and diuretic capacity (Hussain and Nazmeen, N. 2019, Kapoor,1990)

Extensive use of plant belonging to Kalanchoe species in complementary and alternative therapy has been widely reported. It is one of the Pashanbhed (meaning ‘stone breaker’) plant mentioned in ancient Ayurvedic literature. Kalanchoe pinnata (Lam.) Pers. (Synonyms: Bryophyllum pinnatum, Bryophyllum calycinum) belongs to family Crassulaceae, and is commonly known as life plant, air plant (Mexican), love plant, Canterbury bells, Cathedral bells etc. It is a perennial herb growing widely and used in folkloric medicine in tropical India, Africa, China, Australia and tropical America (Kamboj and Saluja 2009). In India, the leaves are consumed by various methods of preparations like, drinking leaves juice directly by squeezing them, chewing fresh leaves or eating dried leaf powder followed by drinking water, as a hot decoction of whole or crushed fresh leaves in Fresh or dried plant materials can be used as a source for the extraction.. Till to date only few scientific evidences supporting to pharmacological effect of K. pinnata against renal calculi are reported. However, it has not much report by using in-vitro dissolution methods (Chandra et al., 2014). So, the present study was undertaken to investigate the effect of aqueous extract of K. pinnata leaves on in-vitro urolithiasis. The objective of the present study was to evaluate the diversity of phytoconstituents present in the aqueous extract and to determine the in-vitro anti-urolithiatic potential of aqueous extract of leaves of K. Pinnata for the use of these plants in traditional systems of medicine.

Materials and Methods

Collection of Plant: Whole plant, Kalanchoe pinnata was purchased from nursery garden and brought to laboratory. The species name was confirmed with the authentication certificate obtained from Siddha Central Research Institute, Arumbakkam, Chennai.

Figure 1: The plant Kalanchoe pinnata

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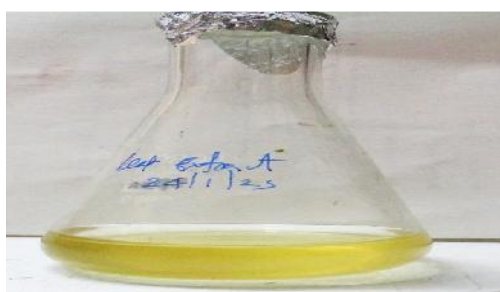
Preparation of extract: Fresh leaves of K. pinnata were collected and washed with distilled water chopped into small pieces and put into a conical flask. 10 grm of fresh leaves with 100 ml of distilled water was added to the conical flask and boiled for a while in order to maximize the extraction. After cooling, it was filtered through Whatmann filter paper No.1 and the aqueous extract stock solution transferred to a suitable container for analysis. 1 ml of the solution was dried in hot air oven and the dried extract was measured. The extract was diluted to in the ratio of 10 mg per ml concentration.(Figure 2)



Phytochemical screening: Preliminary phytochemical screening was carried out for the presence of secondary metabolites; alkaloids, sterols, terpenoids, phenols, flavonoids, glycosides, tannins, saponins, fixed oils and fat by standard methods (Evans, 2009; Kokate, 1991)

Determination of Total Phenolic Content: Total phenolic content was determined in the aqueous extract of K. pinnata by the Folin-Ciocalteau method. A calibration curve was prepared using Gallic acid as standard. The standard/extract solution (1 mL) was mixed with 5 mL Folin-Ciocalteu reagent (diluted ten-fold with water) and 4 mL of 0.7 M sodium carbonate. The absorbance was measured at 765 nm with a UV-spectrophotometer after incubation for 2 hour at room 21 temperature. All determinations were carried out in triplicate. The concentration of phenolic compounds in the extracts was determined from the standard Gallic acid calibration curve. 1mg/ml of Gallic acid used as a standard. The total content of phenolic compounds in the extracts was expressed as mg Gallic acid equivalents (GAE)/g of extract (Slinkard & Singleton, 1977).

Determination of Total Flavonoid Content: The total flavonoid content was determined by the aluminium chloride method. A calibration curve was constructed using quercetin. Different concentrations of aqueous extract (0.5 mL) were mixed with 1.5 mL methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL distilled water. The mixture was incubated for 30 min and the absorbance measured at 415 nm with a UV-spectrophotometer. Aluminium chloride was substituted with distilled water in blank. All determinations were carried out in triplicate. The flavonoid content was determined from the standard quercetin curve. The total flavonoid content in the extract and fractions was expressed as mg quercetin equivalents (QE)/g of extract (Chang et al., 2002)

Determination of Total Tannin Content: Condensed tannins (proanthocyanidins) were determined according to the method of Sun et al. (1998). To 50 µL of diluted sample, 3 mL of 4% vanillin solution in methanol and 1.5 mL of concentrated HCl were added. The mixture was allowed to stand for 15 min, and absorption was measured at 500 nm with a double beam Analykjena UV/Visible 22 spectrophotometer (Model 205, Jena, Germany) against methanol as a blank. The amount of total condensed tannins is expressed as mg (+)-catechin/g DW. All samples were analysed in triplicate

Preparation of Cystone: 500 mg tablet of Cystone was placed in absolute ethanol for removing colour coating. The colour removed 500 mg Cystone tablet was crushed into powder form and dispersed into 100 ml of distilled water and filtered. Filtrate of Cystone was used as positive control for in vitro anti-urolithiatic activity.

Synthesis of Calcium Oxalate by homogenous precipitation: 1 molar calcium chloride: 1.47gm of calcium chloride dihydrate was dissolved in 100 ml distilled water. 1 molar sodium oxalate: 1.34gm of sodium oxalate was dissolved in 100

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ml of 2N H2SO4. Both were mixed equally in a beaker to precipitate out calcium oxalate with stirring. The resultant calcium oxalate was freed from traces of sulphuric acid by washing with ammonia solution; washed with distilled water and dried at a temperature 60 °C for 2 hours.

Fig.2. Synthesized Calcium Oxalate

Spectrophotometric estimation of calcium oxalate by using dissolution model

Group 1: 1ml of calcium oxalate (1mg/ml) + 1ml of distilled water
Group 2: 1ml of calcium oxalate (1mg/ml) + 1ml of Cystone solution (1 mg/ml)
Group 3: 1ml of calcium oxalate (1mg/ml) + 1ml of Cystone solution (2 mg/ml)
Group 4: 1ml of calcium oxalate (1mg/ml) + 1ml of Cystone solution (3 mg/ml)
Group 5: 1ml of calcium oxalate (1mg/ml) + 1ml of Cystone solution (4 mg/ml)
Group 6: 1ml of calcium oxalate (1mg/ml) + 1ml of hot aqueous extract of Kalanchoe pinnata (10 mg/ml).
Group 7: 1ml of calcium oxalate (1mg/ml) + 1ml of hot aqueous extract of Kalanchoe pinnata (20 mg/ml)
Group 8: 1ml of calcium oxalate (1mg/ml) + 1ml of hot aqueous extract of Kalanchoe pinnata (30 mg/ml).
Group 9: 1ml of calcium oxalate (1mg/ml) + 1ml of hot aqueous extract of Kalanchoe pinnata (40 mg/ml).
Group 10: 1ml of calcium oxalate (1mg/ml) + 1ml of hot aqueous extract of Kalanchoe pinnata (50 mg/ml).

All groups were packed it together in porous bag tied with thread at one end and were suspended in a conical flask containing 150 ml 0.1 M Tris buffer each. At another end of thread tied by a stick placed on the mouth of conical flask and covered with aluminium foil. All groups were kept in an incubator, pre heated to 37ºC for 4 hours, kept for three days. The entire content of each group was removed from sutured semi permeable membrane and was transferred into test tube individually. 4 ml of 1N H2SO4 and 60-80ml of 0.1N KMnO4 were added and kept aside for 2 hours. Colour change from dark pink to colourless was observed after 2 hours. Change of colour intensity was measured against 620 nm spectrophotometrically. Concentration of undissolved calcium was determined from standard calibration curve of calcium oxalate. All experiments were performed in triplicate and dissolution percentage was calculated. Dissolution percentage (%) = [calcium content of stone - (calcium content of stone in the presence of extract - calcium content of extract) / stone calcium content (× 100)].

Figure 4: Incubation of calcium oxalate with extract in dialysis membrane

Nucleation assay: Effect of the aqueous extract of Kalanchoe pinnata on calcium oxalate (CaOx) crystal formation was determined by means of nucleation assay. Calcium chloride (CaCl2) (5 mmol/l) were prepared in Tris-HCl (0.05 mol/l) and NaCl (0.15 mol/l) buffer (pH 6.5). The equal volume of extract / cystone with calcium oxalate was incubated for 24 hour and the result was observed under microscope.

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Results

Table 1: Phytochemical analysis of Aqueous extract of K.pinnata

Name of the Phytochemical	Presence/Absence
Phenols	+
Tannins	+
Carbohydrates	+
Saponins	-
Flavanoids	+
Alkaloids	+
Proteins	+
Quinones	-
Glycosides	+
Triterpenoids	+
Coumarins	-
Steroids and Phytosteroids	+
Anthraquinones	-

(+)Positive result, (-) Negative result

Table 2:Total Phenol, Flavonoid and Tannin content in aqueous extract of *K.pinnata*

Name of the Phytochemical	Amount
Total Phenol (µg/ml) (mgGAE /g)	95.44 ± 0.003
Total Flavonoid (µg/ml) (mgQE/g)	40.23 ± 0.02
Total Tannin (mg/10 ml)	12.13±0.04

Values are mean ± SD

Table 3: Dissolution studies of Calcium Oxalate by Standard Cystone and aqueous extract of *K.pinnata* (Values are mean ± SD)

Group	Percentage dissolution
Standard Cystone	
1mg/ml	30.04±1.52
2mg/ml	43.04±2.12
3mg/ml	54.26±1.81
4mg/ml	64±1.90
Aqueous Extract of *K.pinnata*	
10mg/ml	48±1.71
20mg/ml	60±1.18
30mg/ml	70.7±1.9
40mg/ml	82.97±1.87

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Figure 5: Microscopic image of dissolution of Calcium oxalate

A: Crystal formation of calcium oxalate
B: Calcium oxalate with cystone
C: Calcium oxalate with the plant extract

Discussion

The results of Phytoconstituents of aqueous extract of *Kalanchoe pinnata was given in* the **Table 1**. The test result indicates that the presence of Phenols, Tannins, Carbohydrates, Flavonoids, Alkaloid, Protein, Triterpenoids, Steroids and Phytosteroids inthe aqueous extract of *Kalanchoe pinnata.* However, other common phytochemicals suchas Saponins, Quinonoes, Coumarins and Anthraquinones were negative.

The total phenolic, flavonoid and tannin content of aqueous extract of *K. pinnata* were analysed.

The total phenol was 95.44 ± 0.003 (µg/ml), total flavonoid was 40.23 ±0.02 (µg/ml) and total tannin was 12.13 ± 0.04 (mg/10 ml).

In general Calcium oxalate was prepared by using calcium chloride and sodiumOxalate (Sushant, Puspa, and Chhitij 2021). In this study, calcium oxalate was also prepared as per the standard protocol (Sushant, Puspa, and Chhitij 2021) and the synthesized calcium oxalate was used for the analysis of anti-urolithiatic potential of the aqueous extract of *K. pinnata.* Anti-urolithiatic potential of the aqueous extract of *K.pinnata was* analyzed. The result indicates more potency in dissolution of calcium oxalate crystals. The equal volume of plant extract (with various concentrations 10, 20, 30, 40 and50 mg /ml) was incubated with calcium oxalate (1mg per ml). The plant extract at the concentration of 10 mg / ml dissolved 48±1.71 percentage of calcium oxalate. Likewise20, 40, 50 mg / ml of plant extract dissolved the 60±1.18, 70.7±1.9, 82.97±1.87 and89.44±1.98 of calcium oxalate.

Likewise the in vitro inhibitory effect of the plant extracts on CaOx crystallization was analysed. Microscopic photographs showed the formation of CaOx crystals upon incubation of 24 hours. The calcium oxalate incubated with cystone and plant extract up to 24 hours showed the reduction of nucleation or crystal formation. The results showed the presence of Anti-urolithiatic potential of aqueous extract of *K. pinnata.*

Conclusion

The present study demonstrates a significant anti-urolithiatic potential of the aqueous extract of *K. pinnata.* Hence in future the aqueous extract of *K. pinnata* may be used for the isolation of anti-urolithiatic compound for commercialization.

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