

Innovations

The Study on Antioxidant and Dissolution of Calcium Stones by *Kalanchoe Pinnata* Extracts

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Abstract: *Kalanchoe pinnata* is a perennial plant of family belongs to Crassulaceae family having global distribution. These plants are usually found in Madagascar where it grows in appreciable numbers in sandy and granitic soil under subhumid to humid climatic conditions. Some of them are even grown in the malenadu regions of Karnataka in Shivamogga District. These plants are aromatic and have extreme therapeutic potential and high medicinal importance due to the presence of unique chemical constituents such as alkaloids, steroids, triterpenes, glycosides and flavonoids. Also these plant leaves are used in south Asia as natural kidney stone dissolvers and known to exhibit wound healing, insecticidal, anti-inflammatory, anti-allergic, anti-tumor activities and anti-oxidant potentials. The main objective of this study is to identify the artificial calcium stone dissolving properties, antioxidant activity and antimicrobial properties of *K. pinnata* leaf extracts. In vitro studies were conducted to evaluate the calcium stone dissolving properties of *K. Pinnata* leaf extract with 25%, 50%, 75% and 100% ethyl acetate and aqueous solvent extracts on artificially made calcium monohydrate crystals. Further the antioxidant, phytochemicals and antimicrobial properties are carried out against the pathogenic species such as *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Penicillium chrysogenum*, *Sclerotinia sclerotiorum* and *Aspergillus flavus*. Our study concludes that the leaf extracts of *Kalanchoe pinnata* have a potent antioxidants, antimicrobial and calcium stone dissolving properties with 75%, 100% ethyl acetate and aqueous extract samples. From the scientific validations it was lead us to the isolation and determination of the applications of the bioactive compounds from various solvent extracts of the *K. Pinnata* plant. Further research and clinical trials have to be carried out in order to commercialise the potential pharmaceutical uses of the plant for which one should thoroughly know about the pharmacognostical properties of the plant.

Keyword: *Kalanchoe pinnata*; Phytochemicals; Antimicrobial; Calcium stone dissolver.

1. Introduction

Nature uses medicinal plants to toss out the preponderance fatal diseases has been evinced in traditional practice and this practice is more common in remote regions with inadequate infrastructure for health care. However, different scientific literatures and economic reports also indicate, resurgence in the use of medicinal plants in many developed nations. The rapid and increasing rise in the worldwide use of herbal medicines in recent decades suggests botanical medicines; traditional and modern, have been proposed to be the “next choice” to cater the ever demanding therapeutic niche, which sometimes has not adequately addressed in modern health care delivery systems. The relation of traditional knowledge of medicinal plants to contemporary activities confers a new approach, which provides more effective discovery rate in comparison to random selection. Now a day a number of such research work is being carried out with modern tools (Ekor 2014).

Kalanchoe pinnata (Bryophyllum) is a perennial plant belonging to the family Crassulaceae. *K. pinnata* is widely distributed in all tropical regions with various species having structural and botanical variations and unique chemical composition which make it an attractive option to be used medicine. Analysis of various fraction of *K. pinnata* indicated the incidence of alkaloid, diterpenoidal lactones, glycosides, steroids, phenolics and aliphatic compounds. The notable pharmacological properties includes anti-diabetic, antineoplastic, anti-oxidant, anti-allergic and many more events that are controlled by these chemicals (Rafia et al. 2019).

K. pinnata is an erect/upright, succulent, fleshy and hairless stem whose leaves are also succulent and fleshy having simple or compound type such as pinnate leaves or trifoliate leaves. These leaves are often found oppositely arranged having variable number of leaflets with approximate length of 5 to 25 cm and approximate diameter of 2 to 12.5 cm. The leaves are green colored with hairless structure. Leaflets of *K. pinnata* are oval in shape having thick headed rounded tips at the leaflets (Rafia et al. 2019).

This plant is a major source of lipids, alkaloids, bufadienolides, triterpenes, steroids, glycosides, cardienolides and flavonoids. Its leaves are affluent in bufadienolides that consist bryotoxin-A, bryotoxin-B, bryotoxin-C, digitoxin and digoxin that show sturdy chemo-preventive, anti-bacterial and anti-tumour effects and insecticidal potentials. The leaves tastes bitter and were used in various infections include diarrhea, vomiting and flatulence. Also used as painkiller against astringent in bowels. It also has major anti-ulcer chemical constituents in different parts of this plant (Ramon et al. 2013).

This plant is rich in bio organic compounds and essential phytochemical constituents. *Kalanchoe pinnata* has long been used to treat the hemorrhage and

various bleeding disorders because of astringent activities and haemostatic potentials. This plant is considered as cough suppressant, anti-inflammatory agent, diuretic medicine, wound healing remedy and sedative drug. It is also been used for respiratory disorders from cough to asthma. It helps to treat the edema, skin infections, ulcers and kidney stones. Peripheral administrations are found effective for burns, chronic ulcers, insect bites, severe, wounds, headache and toothache. It is useful remedy for prevent liver damages, viral infections, and shattering alcoholic symptoms. Aqueous extract has known to be potential anti-tumor, anti-diabetic and anti-inflammatory agent. It also normalizes the level of nitrogen in urea (Nascimento et al. 2023;Gautam et al. 2023).

Various extracts of *K. pinnata* are known, the mixture of its leaf extract, banaspati ghee and jeera are used to treat food poisoning and infection in stomach that results in bloody diarrhea. Leaf extracts are used to treat cholera and also to treat hemorrhoids and oozing piles. Leaf powder is used with black pepper for blocked, burning urination and Hansen's disease. The roasted leaves extract are applied on skin to prevent the dark scars. Roots extracts are used to reduce the high blood pressure and cardiac problems (Aejazuddin et al. 2011; Anandan and Shanmugam 2024; Assis et al. 2023).

Previous reports motivated us to take up the present study to explore the potential of *Kalanchoe pinnata* for its therapeutic potential in the management of infections and the calcium stone dissolving capacity.



Fig 1: *Kalanchoe Pinnata* plant

2. Materials and Methods

2.1 Materials

2.1.1 Phosphate Buffer pH 7.3 : Sodium phosphate di basic hepta hydrate 20.214gm and sodium phosphate monobasic monohydrate 3.394gm was weighed accurately and dissolved in 200ml of distilled water and made up the final volume to 1000ml (pH 7.3±1) (Lier et al. 2024).

2.1.2 Artificial Kidney stone preparation: Kidney stones are generally composed of calcium and oxalates hence we used some of the similar chemicals

to artificially prepare kidney stones that consist of Calcium carbonate, Sodium oxalate, oxalic acid and urea taken 1gm each or in equal volume, gently mixed with 10ml of distilled water and made into paste and kept overnight in $97^{\circ}\text{C}\pm 5^{\circ}\text{C}$ for formation of crystals and further this crystals weighed in small portions and are taken for the dissolving assay (Ozkan 2024;Gonzalez-Enguita& Garcia-Gimenez 2024; Nyame et al. 2015; Liu et al. 2024).

2.1.3 Nutrient broth preparation: Accurately weigh beef extract of 0.15gm, peptone 0.25gm, and 0.025gm of NaCl and dissolved in 50ml of distilled water and made up the final volume to 500ml with distilled water ($\text{pH } 7.3\pm 1$)(Yang et al. 2023; Jhalora et al. 2024).

2.1.4 SDA (Sabouraud dextrose broth): Peptone 0.5gm and Dextrose 2gm are weighed accurately and dissolved in 50ml of distilled water and made up the final volume to 500ml and adjust the pH to 5.6 ± 1 (Yan et al. 2024).

3. Methods

The leaves of *Kalanchoe pinnata*(Family: Crassulaceae) was collected from Sahyadri Science College campus, and authenticated. The fresh leaves were extracted with ethyl acetate by cold maceration, and the extract was filtered using clean white muslin cloth.

3.1 Extraction of sample

K. Pinnata fresh leaves was collected from the Sahyadri Science College campus botanical garden, Vidyanagara, Shivamogga, washed with distilled water and wiped with clean cotton cloth and placed in dryer for three days at $40\text{-}43^{\circ}\text{C}$ and further made into fine powder using a mixer and taken as sample for the assay. The obtained powder was weighed accurately about 70gm, to this 350ml of ethyl acetate was added, mixed gently and homogenized using homogenizer at 700-900rpm and placed for two days in an covered conical flask. After two days the sample was again agitated for up to two hours and filtered with muslin cloth. The obtained filtrate was kept for solvent evaporation, further the extracted sample was taken in sterilized vial and utilized for the assays. Same process is carried for the aqueous extract (Salehi et al. 2024;Issaet al. 2024; Gebremedhin et al. 2024).

3.2Solubility test for sample

In this test we use different types of solution like phosphate buffer (pH 7), saline (pH 7), 5% NaOH, 10 % NaOH, 5% HCl, 10% HCl, and distilled water to check the solubility of the extracted *K. pinnata* samples (Allay et al. 2024; Huo et al. 2024).

3.3 Kidney stone dissolving activity by *K. pinnata* extracts

The ethyl acetate extract and aqueous extract of *K. Pinnata* was standardized as stock solution with 0.1gm/5ml. From the stock solutions the working standard solutions with concentrations 25%, 50%, 75% and 100% were made in an Eppendorf vials for both ethyl acetate and aqueous extracts. Further these working standards were utilized for the assay. The assay was carried in an Eppendorf vials. The vials were labelled as 25%, 50%, 75% and 100% and added each with 100µl of extracts (4x2 sets for 1hr, 5hr, 16hr and 48hr for both ethyl acetate and aqueous extracts) and to this each vials artificially made calcium stones (50µg) was added and kept for incubation in a room temperature. After the incubation (1hr, 5hr, 16hr and 48hr) the vials were centrifuged, extracts were removed and kept in hot air oven for 5 minutes to evaporate extracts and further the weight of artificially made calcium stones are noted down (Ranaweera et al. 2023; Lu et al. 2024; Raj et al. 2024).

3.4 DPPH Radical Scavenging Assay

DPPH (2, 2-diphenyl -1-picrylhydrazyl) radical scavenging activity will be measured. Briefly, 140µL of DPPH solution (6.2mg in 100mL 100% ethanol) will be incubated with 20µL of extract for 30 minutes in dark at room temperature and the absorbance measured at 536nm. For construction of a calibration curve, increasing concentrations of both the ethyl acetate and aqueous extracts of *K. pinnata* (5-100µg/mL) will be incubated with 140µL of DPPH solution and after 30 minutes the absorbance was read at 536nm and the optical density values are compared with ascorbic acid standards. The results will be expressed as percentage of free radical scavenging activity (Taeri et al. 2021). This method is based on the scavenging of DPPH by antioxidant, which upon a reduction reaction decolorizes the DPPH methanol solution. This assay measure the reducing ability of antioxidants towards the DPPH radical (Yamauchi et al. 2024; Yan et al. 2024).

3.5 Antimicrobial Assay

- ✓ **Nutrient broth composition:** The 0.6gm of beef extract, 1gm of peptone, 0.1gm NaCl were added to 200ml of distilled water with pH 7 and these mix are gently agitated and poured into clean test tube and were closed with cotton plug, packed and sterilized.
- ✓ **SDA broth composition:** The 0.5gm of peptone and 2gm of dextrose were dissolved and made final volume to 50ml with distilled water, pH 5.6 and these mix are gently agitated and poured into clean test tube and were closed with cotton plug, packed and sterilized.

For the NA broth bacterial cultures such as *Escherechia coli*, *Staphylococcus aureus*, *Enterococcus fecalis* and for SD broth fungal cultures such as *Pencillium*, *Sclerotium* and *Aspergillus flavus* are inoculated and different concentration of both sample 1 and 2 are added to different set of broth and for bacteria with sample are incubated at 37°C for 16 hrs and for fungal cultures with sample are incubated at 32°C for 32-48 hrs. After the incubation, the percentage of turbidity was determined interpreting the results that it directly proportion to inhibition of microbes. These results are compared with the positive standards antibiotics (Tajudin et al. 2022).

4. Result and Discussion

4.1 Sample preparation and extraction



Fig 2: Preparation and extraction process of sample

Kalanchoe pinnata extracts was extracted by using ethyl acetate and distilled water, separated and weighed accurately. For 72gm of *Kalanchoe pinnata* powder 10 gm of *Kalanchoe pinnata* extract was obtained for both the extracts.

4.2 Solubility test for sample

Table 1: Solubility tests for ethyl acetate and aqueous extracts samples of *K. pinnata*

Solutions	Ethyl acetate extract	Aqueous extract
Phosphate buffer	Positive	Positive
Saline	Positive	Positive
5% NaOH	Positive	Positive
10%NaOH	Positive	Positive
5% HCl	Positive	Positive
10% HCl	Positive	Positive
Distilled water	Negative	Positive

Although a lot of phytoconstituents that has been found to be a good medicinal property and many of them are not very much stable in water or aqueous solutions. The phytochemicals in the pharmaceutical segment is very limited, because of low water solubility, and that results in major risk of deprived bioavailability, requirement of high dose and side effects. Hence its results in hindering of the utilization of plant assets. The conception of biocompatible solvents with much dissolving powers for phytochemicals as delivery systems is an easy and effective way to increase the solubility and bio availability, which has significant practical implications to satisfy the needs of many applications. In this distinction we check for solubility and our *K. pinnata* extracts dissolves in all the solutions that we used.

4.3 Kidney stone dissolving activity by *K. pinnata* extracts

Table 2: Artificially made calcium stones treated with ethyl acetate and aqueous extracts of *K. pinnata*

Percentage of extracts taken	Artificial made calcium stones								
	Initial weight	Final weight							
		Ethyl acetate extract				Aqueous extract			
		1hr	5hr	16hr	48hr	1hr	5hr	16hr	48hr
25%	50µg	50µg	50µg	50µg	50µg	50µg	50µg	50µg	50µg
50%	50µg	50µg	48µg	48µg	50µg	50µg	49µg	48µg	
75%	50µg	50µg	48µg	47µg	50µg	50µg	47µg	45µg	
100%	50µg	50µg	45µg	42µg	50µg	50µg	43µg	40µg	

The table shows that the artificially made calcium stones immersed with ethyl acetate and aqueous extracts of *K. pinnata* having different percentage of extracts can able to dissolve the stones at higher concentration (100%, 75% and 50%) compared to 25% extracts and the dissolving capacity of stones are very good compared to blank that is phosphate buffer pH 7.2 Table 2. These results lead us to make more concentrated *K. pinnata* extracts, hence we again made another attempt with 0.1gm/2.5ml, 0.2gm/2.5ml and 0.4gm/2.5ml standard stock extract solutions and we directly used this aliquots for the dissolving assay and we got very good results for dissolving calcium stones at higher concentrations. Overall results gives approximately 15-30% dissolves of artificially made stone Table 3.

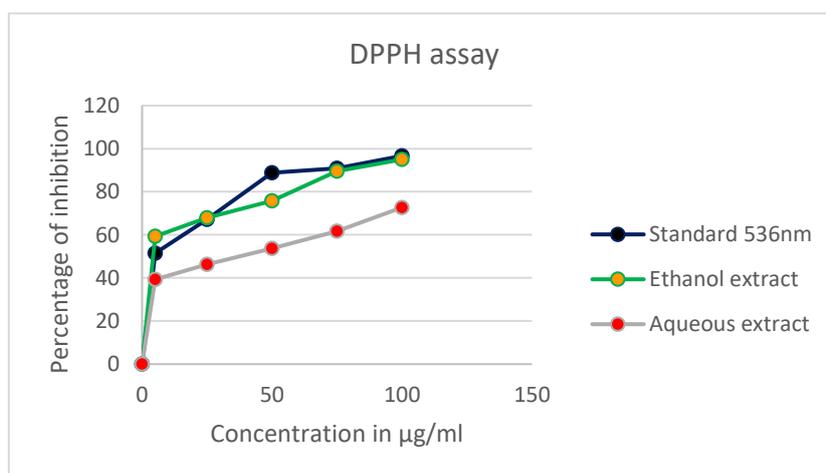
Table 3: Artificially made calcium stones treated with higher concentrations of ethyl acetate and aqueous extracts of *K. pinnata*

Concentration of extracts	Artificially made calcium stones with Ethyl acetate extract		Artificially made calcium stones with Aqueous extract	
	Initial weight	Final weight	Initial weight	Final weight
0.1gm/2.5ml	50µg	37µg	50µg	38µg
0.2gm/2.5ml	50µg	36µg	50µg	35µg
0.4gm/2.5ml	50µg	30µg	50µg	29µg

4.4 Antioxidant activity

DPPH Radical Scavenging Assay

The DPPH radical scavenging activity was carried to evaluate the antioxidant capacity of *K. pinnata* leaf extract. The percentage of DPPH radical scavenging inhibition was calculated for each concentration of the samples that was taken and the results were represented in the graph 1.



Graph 1: The graph represent the DPPH radical scavenging activity of *K. pinnata* extracts. In x axis, the concentration of standard ascorbic acid was taken and in y axis the absorbance was measured at 517nm. Ethyl acetate extract and aqueous extracts radical scavenging activity shown a good results compared to standard.

4.5 Antimicrobial Assay

4.5.1 Anti-bacterial activity

The most prominent activity was shown by ethyl acetate extract, the results shows maximum activity against *Escherichia coli* and *Staphylococcus aureus* at 25%, 50%, 75% and 100% concentrations. Compared to aqueous extract maximum activity was shown by ethyl acetate extract against *Staphylococcus aureus* with 75% and maximum activity of aqueous extract against *Staphylococcus aureus* was seen in 100%, ethyl acetate extract against *Enterococcus faecalis* with 25%, 75% and 100% extracts shown maximum activity, aqueous extract shows the minimum activity against *Enterococcus faecalis* at all concentration. This concludes that the maximum activity was shown by the ethyl acetate extract than that of aqueous extract samples.

4.5.2 Anti-fungal activity

The maximum activity was shown by the sample 1 with 25%, 50% and 100% extracts than that of the aqueous extract. Ethyl acetate extract shown maximum activity against *Pencillium* with 75%, 100% extracts and with 50%, 100% extracts showed activity against *Sclerotium* while aqueous extract shown the maximum activity against *Sclerotium* with 25%, 75%, 100% extract. Ethyl acetate extract shown the maximum activity against *Aspergillus flavus* with 25%, 50%, 75% and 100% extracts, while aqueous extract shown the maximum activity at 75% extract only. This shows that ethyl acetate extract shown the maximum activity against *Aspergillus flavus* and *Pencillium* compared to aqueous extract. Aqueous extract shown the maximum activity against *Sclerotium* than ethyl acetate extract.

By comparing with all the different percentage ethyl acetate extract and aqueous extract we interpret that the results we come to know with the *K. pinnata* extracts is having a potent antimicrobial activity in both the samples and results in inhibiting the growth of the pathogenic microbial mass.

5. Conclusion

Kalanchoe pinnata is a perennial plant of family belongs to *Crassulaceae* family having global distribution. These plants are usually found in Madagascar where it grows in appreciable numbers in sandy and granitic soil under sub humid to humid climatic conditions. Some of them are even grown in the malenadu regions of Karnataka in Shivamogga District. These plants are aromatic and have extreme therapeutic potential and high medicinal importance due to the presence of unique chemical constituents such as alkaloids, steroids, triterpenes, glycosides and flavonoids. Also these plant leaves are used in south Asia as natural kidney stone dissolvers and known to exhibit wound healing, insecticidal, anti-inflammatory, anti-allergic, anti-tumor activities and anti-oxidant potentials.

The main objective of this study is to identify the artificially made calcium stone dissolving properties, antioxidant activity and antimicrobial properties of *K. pinnata* leaf extracts. *In vitro* studies were conducted to evaluate dissolving properties of *K. Pinnata* leaf extract with 25%, 50%, 75% and 100% ethyl acetate and aqueous solvent extracts on artificially made calcium monohydrate crystals. Further the antioxidant properties were identified and antimicrobial properties are carried out against the pathogenic species such as *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Penicillium chrysogenum*, *Sclerotinia sclerotiorum* and *Aspergillus flavus*. Our study concludes that the leaf extracts of *Kalanchoe pinnata* have a potent antioxidants, antimicrobial and calcium stone dissolving properties with 75%, 100% ethyl acetate and aqueous extract samples. From the scientific validations it was lead us to the isolation and determination of the applications of the bioactive compounds from various solvent extracts of the *K. Pinnata* plant. Further research and clinical trials have to be carried out in order to know the potential pharmaceutical uses of the plant for which one should thoroughly know about the pharmacognostical properties of the plant.

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Abbreviations

DPPH :	2,2-diphenyl-1-picrylhydrazyl
gm :	Gram
% :	Percentage
*	: Multiply
/	: Divide
ml :	Millilitre
N :	Normality
°C :	Degree Celsius
hr :	Hours
<i>K. pinnata</i> : <i>Kalanchoe pinnata</i>	

µg/mL: Microgram per milliliter

Declarations:-

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