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HPTLC ANALYSIS AND IN-VITRO ANTI-LITHIATIC ACTIVITY OF THE KALANCHOE PINNATA LEAVES EXTRACT

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Abstract— Kalanchoe Pinnata is very popularly used for the treatment of lithiasis based on its traditional use for kidney stones. Kalanchoe is a perennial plant found in plains, forests & hills of southern & eastern India. A study was undertaken to evaluate the phytochemical characteristics (identification tests that confirm the presence of chemicals in the extract), chromatographic techniques such as TLC, HPTLC, and In vitro antidiabetic efficacy of the ethanol extract of Kalanchoe Pinnata leaves. The in vitro activity was evaluated by measuring the inhibition of calcium precipitation using titrimetric analysis and phosphate precipitation via colorimetric assessment. Cystone, a commercial product, served as a reference medication for assessing various concentrations of ethanol extracts (0.2 to 0.7 g/ml) for calcium and phosphate estimation, yielding results of 41.11% to 67.11% and 22.88% to 36.88%, respectively. Additionally, a 1 ml aqueous extract of Cystone demonstrated 68.21% and 38.09% for calcium and phosphate estimation, respectively.

Many disorders can still be effectively treated with traditional medicine, which has been used for many years. Phytochemicals were used in this investigation to screen for the principal secondary metabolites. The study found that the plant leaf extracts included phenols, terpenoids, tannins, saponins, steroids, and flavonoid compounds. The plant's pharmacological and therapeutic potential is enhanced by the presence of these important phytochemicals.

Keywords*— Kalanchoe Pinnata, phytochemical prescreening, anti-lithiasis activity, pharmacological study, Cystone, HPTLC*

I. INTRODUCTION

Herbs and other natural resources have many medical applications. Every ancient civilization recorded the use of medicinal herbs in its ethnobotanical works. There is a long list of drugs made from plants. Numerous drugs have been used to treat hepatic disease over the years. Many herbs are taken from plants and appear to be beneficial, but science has not yet demonstrated exactly what they do, with the exception of a few plants and certain herbal drugs. (1, 2)

Kalanchoe Pinnata leaves have anti-hepatic, anti-gastric, anti-asthmatic, anti-inflammatory, anti-diarrheal, anti-spermatogenic, and anxiolytic properties, among other uses in traditional medicine. Nonetheless, numerous therapeutic benefits have been demonstrated. To get more clinical data to support the drug's efficacy, controlled clinical investigations are critically important in animal testing models. (3, 4)

Due to their potential therapeutic use, medicinal plants are currently undergoing extensive examination, with a significant focus on their chemistry. The minerals found in medicinal plants have a major impact on raw material identification. Treating infectious and other diseases is a major and difficult problem because of the combination of newly emerging infectious diseases and the increase in bacteria that are resistant to many treatments. (5, 6)

The plant thrives in hot, humid regions of India, particularly Bengal. Growing to a height of one and a half meters, it is a succulent perennial. Its opposing, succulent, decussate leaves are 10 to 20 cm long. The upper leaves have long petioles that are 3–7 axial, while the lower leaves are simple. They have a noticeable scalloped and red-trimmed look and have a considerable dark green tinge. Three to five 10–30 cm long leaflets and 2–4 cm long petioles make up the pinnate leaf-blade complex. A latent bud that may grow into a healthy plantlet with an obtuse apex is located in each notch on the oblong to

elliptic leaflet blades, which have a crenate edge and measure 6-8 x 3-5 cm. (7-10)

Bryophyllum Pinnatum is abundant in lipids, triterpenes, alkaloids, glycosides, flavonoids, steroids, cardenolides, and bufadienolides. Fresh *Bryophyllum pinnatum* leaves have been found to contain three new compounds: bryophyllol, bryophollone, and bryophollenone. Two insecticidal bufadienolides, bryophyllin A and bryophyllin C, were found when *Kalanchoe Pinnata* leaves were extracted in methanol. The foliage of the Kalanchoe The Pinnata plant has many enzymes, including Rubisco, pyruvate orthophosphate dikinase (PPDK), and phosphoenolpyruvate carboxylase (PEPC). The leaf comprises amino acids, carbohydrates, proteins, lipids, p-hydroxycinnamic acid, 4-hydroxybenzoic acid, 4-hydroxy-3-methoxy-cinnamic acid, ferulic acid, para-coumaric acid, and caffeic acid. (11-14)

An adult kidney measures around 3 cm in thickness, 5 to 7 cm in width, and 10 to 12 cm in length. The spinal column is located on the inside of the concave medial margin of each kidney. Each kidney is encased in three layers of tissue.

The thick, asymmetric connective tissue that constitutes the deep layer of the renal capsule is translucent and smooth. The mass of fatty tissue that envelops the renal capsule is called the adipose capsule, which is the intermediate layer. The extra layer of thick, asymmetrical connective tissue is called the superficial layer, or renal fascia. (15, 16)

There are several things that may affect stone formation, such as the amount of urine, the pH level, and natural calcium inhibitors like citrate, magnesium, and bikunin. The concentrations of calcium, phosphate, oxalate, sodium, and uric acid ions are all significant factors. Reduced urine volume, decreased pH, elevated ion concentrations, and diminished citrate levels all facilitate the formation of calculi. (17, 18) **(Table 1)**

Table 1: Common types of stones

Name of stone	**Constituents**	**Incidence**
Calcium oxalate	Calcium, oxalate	34%
Calcium phosphate	Calcium, phosphate	33%
Struvite	Ammonium, magnesium, phosphate	15%
Uric acid	Uric acid	8%
Cystine	Cystine	3%
Medication-induced stones	Depends on the medication (eg, Indinavir, ephedrine, Guaifenesin, silica, etc.)	1%

II. MATERIALS AND METHODS

Extraction of plant material

The leaves of *Kalanchoe Pinnata* were cleaned with filtered water and then stacked for a week. It was allowed to dry at room temperature. A mortar and pestle were then used to grind it into a powder. Using a Soxhlet device, the continuous solvent extraction method was used to extract the powdered medication. Solvent evaporation was used to concentrate the ethanol extract. An extractor tube containing 250 g of the crude drug sample was filled with 1000 mL of 70% ethanol. The extract was taken out after thirty cycles were finished. Acetone was used to defeat the extract. For future research, the concentrated crude extracts were kept in a refrigerator at 4°C. (19)

Phytochemical screening

The extract of the species underwent preliminary phytochemical testing to identify the presence of several phytoconstituents, including alkaloids, glycosides, starches, terpenoids, polyphenols, tannins, sugars, steroids, flavonoids, amino acids, and oils. The following tests were performed on the selected plant in order to screen for phytochemicals.

- Protein and Amino Acids through the Biuret and Ninhydrin Test
- Carbohydrates through molish’s, fehling’s solution, and Benedict’s test
- Alkaloids through Dragendorff’s, Mayer's, Hager's, and Wagner’s tests
- Glycosides through borntrager's and Keller-Killani tests
- Triterpenoids
- Saponin through the foam test
- Steroids through the salkowski test
- Flavonoids through the ammonia test and the Shinoda test
- Phenolic compounds and tannins (20-23)

Thin Layer Chromatography

On the white surface of a TLC plate, draw two straight lines with a pencil: one at the top, 1 cm from the bottom, and one at the bottom, 2 cm from the top. Put 1 µl of each test sample at the bottom of the TLC plate. Let the plate air dry for fifteen minutes. To further dry the TLC plate, place it in an incubator or hot air oven set to 70°C for two to three minutes. After covering the TLC chamber, add 10 milliliters of the solvent solution and let it rest at room temperature for 10 minutes. Use sterilized forceps to put the TLC plate in the chamber. Let the solvent front go all the way to the top of the plate. The plates can be left at 70°C for two minutes to dry further. After applying 1 milliliter of the developing agent, carefully spin the plate. Examine the development of the colour spots on the test sample.

The distance traveled by the solvent front and the substance was determined using the formula: Rf = distance

traveled by the substance divided by distance traveled by the solvent. (24)

High-performance thin-layer chromatography (HPTLC)

Mobile Phase: Toluene: Ethyl Acetate: Formic Acid (7:3:0.2 v/v/v)

Saturation Time: 20 minutes

Procedure: On a pre-coated silica gel 60F254 TLC plate (Merck) of uniform thickness (0.2 mm), apply 5 µL, 7.5 µL, and 10 µL of test solution onto three distinct tracks: T1, T2, and T3. Subsequently, develop the plate in the mobile phase for a distance of up to 7 cm. Examine the plate at wavelengths of 254 nm and 366 nm after it has dried. To complete the derivatization process, immerse the plate in the Anisaldehyde Sulfuric Acid Reagent (ASR) along with heat to 105 °C until the spots or bands exhibit a color change.

Visualization: To record the fingerprint profile, view the plate (both before and after derivatization) at various wavelengths, such as UV-254 nm, UV-366 nm, and white light.

In vitro anti-lithiasis activity

Cystone: We prepared the aqueous extract by pulverizing a tablet into a fine powder. The powder was combined with 5 mL of water, allowed to rest for 2–3 hours, and then centrifuged at 1000 rpm. The study used the transparent supernatant.

0.1 M TRIS Buffer (pH 7.4)

Answer Resolution A contained a 0.4 M solution of TRIS (trihydroxymethyl) aminomethane, equivalent to 48.4 g per 1000 mL. Solution B contained 0.4 M hydrochloric acid, equivalent to 33.6 mL of concentrated hydrochloric acid per 1000 mL. A viable solution was achieved by combining 25 mL of solution A with 20.7 mL of solution B, followed by the addition of sufficient water to reach a total volume of 100 mL. The pH level was 7.4. (25)

Experimental setup

The experiment included control and test tubes, each containing 1 mL of 25 mM $CaCl_2 \cdot 2H_2$.

0.25 mM $Na_2HPO_4 \cdot 2H_2O$ or 25 mM $Na_2C_2O_4$. Subsequently, three pairs of test tubes were prepared, each containing 2 mL of the buffer Tris-HCl and 1 mL of 105 mM NaCl. The pH level remained at 7.4. Subsequently, 2 mL of the extract and the tested vehicle were incorporated. The tubes were maintained at 37°C for four hours. The calcium both calcium and phosphate oxalate precipitate was synthesized in this manner. (25)

Calcium oxalate

When you mixed 1 mL of solution from the tube with $CaCl_2 \cdot 2H_2O$ and $Na_2C_2O_4$, calcium oxalate precipitate formed.(26)

Calcium phosphate

Calcium phosphate was produced by combining 1 milliliter of the tube containing the $CaCl_2 \cdot 2H_2O$ and the $Na_2HPO_4 \cdot 2H_2O$ solutions. (27)

Titrimetric analysis was used to determine calcium, while colorimetric analysis was used to estimate phosphorus. For every set of experiments, the appropriate standard curves were created. The techniques of Clark and Collip and Fiske and Subbarrow, respectively, were used to calculate the amount of calcium and phosphate precipitate in each set. **(Figure 1)**

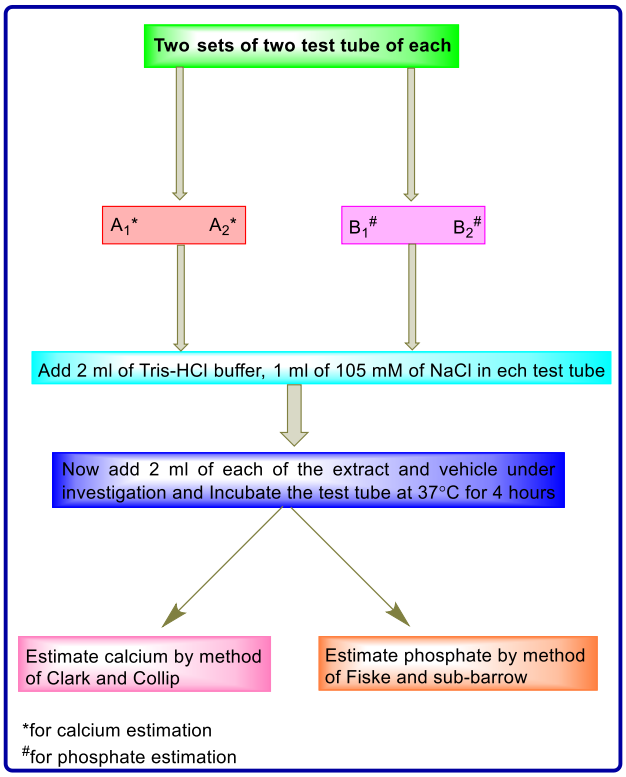

Figure 1 Methodology of calcium and phosphate estimations

III. RESULTS AND DISCUSSION

Extraction yield

250 g of the plant leaf extract was obtained using 70% ethyl alcohol solvent as mentioned in the methodology part, and the mass of the pulverized plant leaf extracts was 15 grams, and the extraction yields (w/w) were calculated as:

Percentage yields = Weight of dry extract / Weight of dry material X 100

The Percentage yields of the plant leave extracts isolated by using the hot percolation method are 6 % with greenish-brown colour.

Preliminary phytochemical analysis

Plant secondary metabolites, including tannins, terpenes, alkaloids, flavonoids, steroids, glycosides, and others, are essential for physiological processes. The results of the phytochemical screening tests performed to examine the plant leaf extracts of *Kalanchoe Pinnata* showed that the phytochemicals included in ethanolic extracts included alkaloids, carbohydrates, flavonoids, terpenoids, steroids, and phenols. The ethyl alcohol crude extract contains most of the secondary metabolites, except for tests for proteins, lipids, tannins, and fixed oil. **(Table 2)**

Table 2: Phytochemical investigation of *Kalanchoe Pinnata*

S.No.	Tests	Ethanolic extract
1	Test for Alkaloids	Present
2	Test for Glycosides	Present
3	Test for Triterpenoids	Present
4	Test for Flavones and Flavonoids	Present
5	Test for Phenolic and Tannins	Absent
6	Test for Saponins	Absent
7	Test for Carbohydrates	Present
8	Test for Proteins and Amino Acids	Absent
9	Test for Steroids	Present

TLC Study of Ethyl Alcohol Extract of *Kalanchoe Pinnata*:

The thin-layer chromatography analysis of the ethanol extract of *Kalanchoe Pinnata* showed the presence of flavonoids, terpenes, and alkaloids. TLC also shows the colour and Rf value of the flavonoids, terpenes, and alkaloids present in an extract with suitable spraying agents.

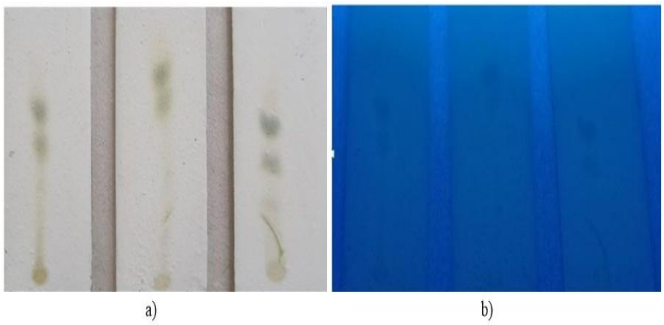

Figure 2: a) TLC of an isolated compound in the visible region, **b)** TLC of the isolated compound in the Ultraviolet region (UV 366 nm)

The TLC data, such as the solvent system, colour of the spot, R_f values, etc., are mentioned in

Table 3: TLC Study of Ethyl Alcohol Extract of *Kalanchoe Pinnata.*

Solvent systems	Spraying reagent	Color of spot	R_f value	Inference
Ethyl acetate: formic acid: acetic acid: water (5:3:2:1)	Iodine vapors	Dark color	0.98	Presence of flavonoids
Ethyl acetate: toluene: formic acid (5:5:1.5)	Anisaldehyde HCL	Red-violet color	0.67	Presence of terpenes
Dioxane: ammonia (25%)(9:1)	Ninhydrin	Gray color	0.79	Presence of alkaloids

HPTLC Study:

Visualization: When you look at the plate at other wavelengths, such UV-254 nm and UV-366 nm, you can see multiple clear bands of different colors, as shown in Figure 3.

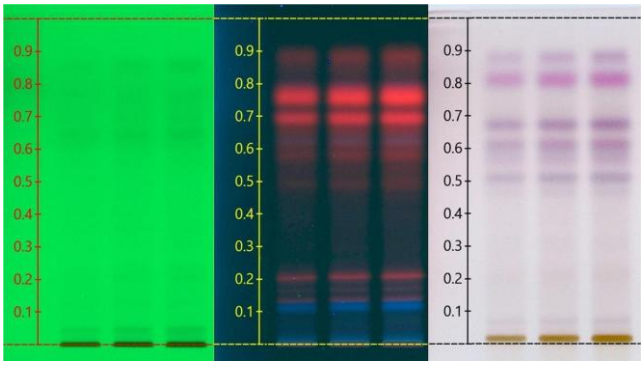

Figure 3: HPTLC chromatograms of *Kalanchoe pinnata* (leaves extract)

HPTLC is the easiest and quickest way to separate things nowadays. It also delivers more accurate and precise results with a lot of freedom for different processes. Table 4 presents the results, including the quantity of peaks with the Rf values. We utilized HPTLC spectral analysis to examine both plant extracts at three specific wavelengths: UV at 254 nm, UV at 366 nm, and white light **(Figure 3).**

Table 4: R_f values of *Kalanchoe pinnata* (Leaves) under UV-254 nm, UV-366 nm, and White light

S. No.	254 nm			366 nm			White light (Derivatized with ASR)		
	5 µl	7.5 µl	10 µl	5 µl	7.5 µl	10 µl	5 µl	7.5 µl	10 µl
1.	0.004	0.004	0.004	0.008	0.010	0.010	0.017	0.018	0.019
2.	0.041	0.043	0.046	0.112	0.115	0.118	0.065	0.066	0.068
3.	0.644	0.647	0.644	0.164	0.165	0.167	0.211	0.210	0.213
4.	0.854	0.852	0.856	0.202	0.205	0.208	0.507	0.509	0.509
5.	-	-	-	0.623	0.622	0.624	0.608	0.608	0.609
6.	-	-	-	0.693	0.693	0.693	0.672	0.671	0.672
7.	-	-	-	0.759	0.757	0.759	0.809	0.809	0.808
8.	-	-	-	0.881	0.880	0.882	0.880	0.880	0.882

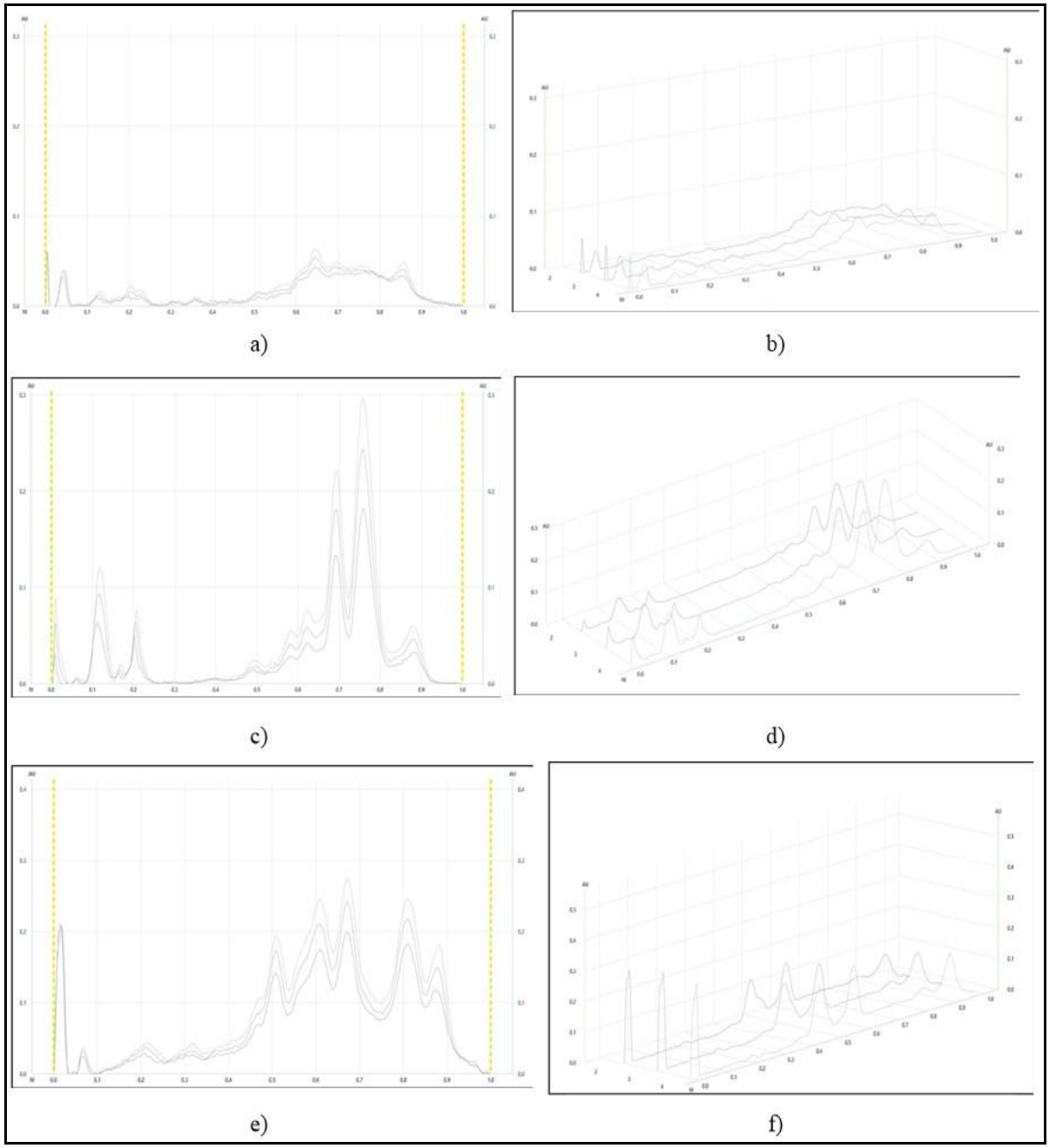

Figure 4 a) 2D Densitogram at UV 254 nm. **b).** 3D Densitogram at UV 254 nm. **c).** 2D Densitogram at UV 366 nm **d)** 3D Densitogram at UV 366 nm. **e)** 2D Densitogram after derivatization. **f).** 3D Densitogram after derivatization.

In vitro anti-lithiasis activity:

Effect of *Kalanchoe Pinnata* leaves extracts on the inhibition of Calcium & Phosphate:

Various quantities of Kalanchoe Pinnata leaf extract shown similar efficacy to the commercial product in inhibiting the development of calcium and phosphate precipitates. Cystone, a prescription therapy for renal calculi, had a significant inhibitory impact on the production of calcium and phosphate precipitates.

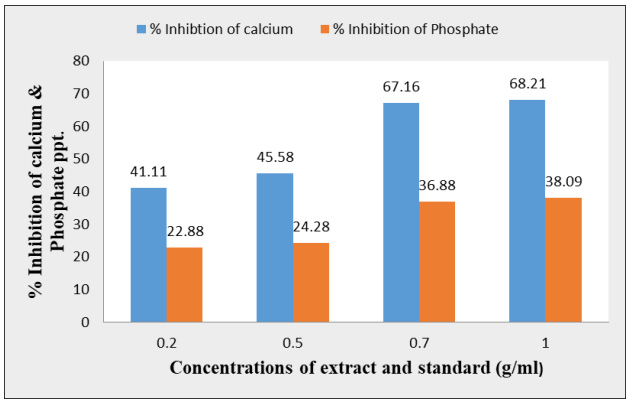

Figure 5: Percent inhibition of calcium & Phosphate using different concentrations of extract

Estimation of Calcium

Table 5. Percent inhibition of calcium using different concentrations of the extract of *Kalanchoe Pinnata*

Parameters	Ethanol extract (0.2 g/ml)		Ethanol extract (0.5 g/ml)		Ethanol extract (0.7 g/ml)		Aqueous extract (1ml 1tab./5ml)	
	Control	Test	Control	Test	Control	Test	Control	Test
% Inhibition	-	41.11	-	45.58	-	67.16	-	68.21

I. [A]P<0.05, [B]P<0.01, [C]P<0.001, STATISTICALLY ANALYSED BY STUDENT'S T-TEST

Estimation of phosphate

II. TABLE 6. PERCENT INHIBITION OF PHOSPHATE USING DIFFERENT CONCENTRATIONS OF THE EXTRACT OF *KALANCHOE PINNATA*

Parameters	Ethanol extract (0.2 g/ml)		Ethanol extract (0.5 g/ml)		Ethanol extract (0.7 g/ml)		Aqueous extract (1ml 1tab./5ml)	
	Control	Test	Control	Test	Control	Test	Control	Test
% Inhibition	-	22.88	-	24.28	-	36.88	-	38.09

[a]P<0.05, [b]P<0. 01, [c]P<0.001, *P>0.05, statistically analyzed by student's t-test

IV. CONCLUSION

Many disorders can still be effectively treated with traditional medicine, which has been used for many years. Phytochemicals were used in this investigation to screen for the principal secondary metabolites. The study found that the plant leaf extracts included phenols, terpenoids, tannins, saponins, steroids, and flavonoid compounds. The plant's pharmacological and therapeutic potential is enhanced by the presence of these important phytochemicals, which also lend scientific credence to the use of the plant in traditional medicine to treat a range of human and animal illnesses. The plant has a range of polar chemical compounds, according to TLC studies; however, due to budgetary and schedule limitations, these components were not isolated for this study.

Therefore, any researcher interested in studying these plant leaves should focus on the methanol extract, which contains polar compounds, instead of using non-polar extracts. The presence of bioactive chemicals that might be employed as conventional pharmaceuticals was established by the discovery of a few chemical components from plant leaves in this study. Therefore, the continued traditional therapeutic use of these plants is supported, even though more research is advised to extract, purify, and maybe define more bioactive components from them. To boost the yields of high-polarity crude extracts and separate more chemical components from the plant leaves, the extraction solvent should be made as readily available as possible. Further research on the identification of high-extracting solvents would be desirable to elucidate the complete and precise structure of pure chemicals and to explore the potential of these plant extracts in the treatment of infectious disorders. Similar research should be conducted on other plant portions, including the root, stem, and bark, to identify more bioactive components. Toxicological investigations of the plant should also be carried out to determine the safety indices of the extracts. Sophisticated chromatographic techniques, including GC-MS and HPLC procedures, were recommended to isolate and characterize pure compounds from the plant body.

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Declaration of Competing Interest

The authors have affirmed that they do not have any financial or interpersonal conflicts that would have looked to influence the study disclosed in this publication.

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