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Validating the Traditional Uses of *Kalanchoe pinnata* (Lam.) Pers.: Integrating Anti-inflammatory/Antioxidant Activity with HPTLC Profiling and Computational Toxicity Screening

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Abstract: *Kalanchoe pinnata* (Lam.) Pers. is widely used in traditional medicine for treating inflammation, wounds, and oxidative stress–related disorders. Despite its extensive ethno medicinal relevance, scientific validation integrating phytochemical, biological, and toxicity assessments remains limited. The present study evaluated the High performance thin layer chromatography (HPTLC) profiling integrated with the computational toxicity screening and *in vitro* ABTS antioxidant and anti-inflammatory assays. Ethanolic extract of *Kalanchoe pinnata* (Lam.) Pers. were used for the anti-inflammatory activity, using the inhibition of albumin denaturation test and their percentage of the inhibition were found with PD_{50} values, respectively. The anti-oxidant property of the extract were evaluated with ABTS assay, ability of the plant extract to scavenge the $ABTS^{+}$ free radical showcasing the inhibition percentage with the IC_{50}. The ability of the plant to inhibit the inflammatory markers and solid free-radical scavenging capacity make it support the plant's traditional value. HPTLC profiling revealed the presence of phytoconstituents particularly of flavonoids, and phenolic derivatives. Furtherly *in silico* toxicity studies were conducted to evaluate the ADMET profiling using software tools to predict the hepatotoxicity, and cytotoxicity. In essence the data confirms the traditional uses of *Kalanchoe pinnata* (Lam.) Pers. linking with the richness of phytoconstituents to its efficiency in pharmacology thereby, establishing a foundation for the herbal drug formulation in further research.

Keywords: *Kalanchoe pinnata*, Anti-inflammatory activity, ABTS assay, HPTLC profiling, ADMET, Phytoconstituents

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Introduction

Folk medicine commonly employs *Kalanchoe pinnata* (Lam.) Pers. for treating kidney stones, gastric ulcers, pulmonary infections, and rheumatoid arthritis. It is found throughout India, cultivated in gardens and occurring naturally in the hills of North-Western India, the Deccan, and

Bengal (Nadkarni, 2005). *Kalanchoe pinnata* (Lam.) Pers. is a succulent perennial shrub that grows to about 1.5 metres tall and reproduces both vegetatively and by seed. It has tall, hollow stems, black pendulous bell-shaped flowers, and fresh dark green leaves with scalloped, red-tinged edges. The plant can be easily propagated from stem or leaf cuttings. Mainly the leaves of the plant are commonly used for the pharmacological studies apart from the flowers, stem, or bark. *Kalanchoe pinnata* (Lam.) Pers. is rich in bufadienolides (steroidal compounds) as well as kaempferol, quercetin, alkaloids, lipids, triterpenes, and cardenolide glycosides. These types of phytochemicals are mainly responsible for the various pharmacological activties. Research has shown that the plant may possess strong antitumor, anti-ulcer, and anti-rheumatoid arthritis activities, as well as potential benefits in treating intestinal worms, peripheral neuropathy, burns, and bruises (Silva *et al.*, 2018).

Phytochemicals are the organic compounds produced from the plants. Compounds like carbohydrate, protein, alkaloids and flavonoids are utilized by people in many ways for foods as well as medicine. Identification and standardization of these compounds is a challenge due to the variation in environmental, and processing factors. Differences can be observed in the same plant material and across various parts of the same plant. In contrary, it can be mixture of active, inactive, and unknown phytoconstituents, several of which function more as dietary components than therapeutic agents (Gupta, 2003).Therefore, techniques that produce a fingerprint for each extract in large sets are useful for assessing extract stability over time. Preferably, these methods should support electronic data storage, retrieval, and analysis (Pandey, 2004). To investigate plant-derived active compounds, numerous extraction procedures and analytical techniques—such as spectrophotometry, HPTLC, HPLC, GC–MS, and FT-IR—have been established (Kirtikar and Basu, 2005). HPTLC-based methods serve as a valuable alternative, given their growing role in routine drug analysis.

Inflammation represents a defensive immune response activated by the presence of foreign organisms such as bacteria, viruses, and parasites (Pendota *et al*., 2014). Research on inflammation is a significant domain in global healthcare, largely because commercially available NSAIDs can cause notable side effects, including renal suppression and gastrointestinal issues like ulcer formation (Ashraf *et al*., 2016). Anti-oxidants can be synthesized in vivo or acquired through dietary intake, with the plants serving as a reliable source (Kasote *et al*., 2015). The antioxidant potential of plants has emerged as a promising approach for counteracting the harmful effects of oxidative stress. The most important contributors to this activity are carotenoids, flavonoids, and other phenolic compounds (Balasundram *et al*., 2006).

Modern people increasingly prefer natural drugs, primarily derived from plants, due to their wide availability and lower risk of side effects. In contrast, synthetic drugs and antibiotics often cause widespread toxicity and harmful side effects in users, affecting more than just the targeted health condition or pathogen. Novel bioactive compounds derived from plants have shown therapeutic efficacy with minimal side effects (Gaddaguti *et al*., 2012). Molecular docking is a technique used to evaluate new drugs against their target proteins. Active compounds interact with the target protein in various orientations, and this method predicts the optimal binding orientation by estimating binding affinity and potential biological activity (Ramírez and Caballero, 2018).This method depends on the 3D structure of the target and the electronic properties of the ligand for binding (Pinzi and Rastelli, 2019).The present study aims to validate the traditional uses of *Kalanchoe pinnata* (Lam.) Pers. by integrating assessments of its anti-inflammatory and antioxidant activities with HPTLC profiling and computational toxicity analysis.

Materials and Methods

Collection of plant, identification and extraction

The leaves of *Kalanchoe pinnata* (Lam.) Pers. were collected from the botanical garden of Centre for Bioscience and Nano Science Research (CBNR), Echanari, Coimbatore and were authenticated by Botanical Survey of India at Coimbatore (BSI/SRC/5/23/2023/Tech-475). The identified specimen is deposited for the further studies. The leaves were cleaned with distilled water to remove dirt. Before pulverizing into fine powder the washed leaves were shade dried at room temperature. The powdered leaves were sequentially extracted with ethanol.

High Performance Thin Layer Chromatography (HPTLC) Profiling

The phytochemical content of the ethanol extract was evaluated using previously reported protocols with minor modifications (Casanova *et al.*, 2022) Briefly, HPLC analysis was carried out using an Agilent Technologies 1200 Series system equipped with an automatic detector employing reagents of HPLC grade (Merck), TLC Silica gel $60F_{254}$ 8.0 mm length, 8.0 mm width was employed as stationary phase, while Hexane: Ethyl acetate in the ratio 7:3 was used as mobile phase. To prepare the sample 2 mg of squalene was dissolved in methanol. For the analysis, 0.2 µl of the sample was run at various concentration. Absorbance was monitored at 254 and 366 nm.

Antioxidant Activity - ABTS Assay

Antioxidant activity of the given plant extract was analysed by investigating their ability to scavenge the ABTS+ free radical (Ozgen *et al.*, 2006). The ABTS assay was performed by generating the $ABTS^{+}$ radical cation through the reaction of ABTS (7 mmol/L) with potassium persulfate (2.45 mmol/L), followed by incubation in the dark at 4 °C for 12-16 h. The resulting blue-green radical solution was diluted to an absorbance of 0.70 ± 0.01 at 734 nm to prepare the working reagent. Then, 20 µl of the sample at five concentrations (10, 20, 30, 40, and 50 µg/ml) was mixed with the reagent and incubated at 30 °C for 30 min. Radical scavenging activity was determined by measuring the decrease in absorbance at 734 nm, with greater decolourization indicating stronger antioxidant activity.

The percentage antioxidant activity was calculated using the following equation:

$$I\ (\%) = 100\times (A_0-A_1)/A_o$$

Where, A_0 is the absorbance of the control, A_1 is the absorbance in the presence of the sample of extract.

Anti-Inflammatory Activity – Inhibition of Albumin Denaturation Test

Anti-inflammatory activity of the given plant extract sample was studied using inhibition of albumin denaturation test (Kumari *et al.*, 2015). A reaction mixture containing 0.2 ml of hen egg albumin, 2.8 ml of isotonic phosphate buffer (10 mM, pH 7.4), and 2 ml of the extract at various concentrations (10–50 µg/ml) was prepared. The mixture was incubated at 37 °C for 20 min, followed by heating at 70 °C for 5 min. After cooling, absorbance was recorded at 660 nm. Protein denaturation inhibition (%) was calculated using the formula:

Protection (%) = 1 – (Sample absorbance/ Negative control absorbance) × 100

Sodium diclofenac (100 µg/ml) served as the positive control, while distilled water acted as the negative control. The protective dose (PD_{50}) of the extract was then determined.

In Silico Toxicity Prediction Study and ADMET Profiling

The four compounds showing the highest peaks from the GC-MS analysis, Diethyl Phthalate, Dibutyl Phthalate, Phytol and Squalene and the drug Gemcitabine were selected for *in silico* toxicity assessment. The crystal structures of cancer-related proteins, namely GOT1 (PDB-3II0), c MET (PDB ID- 6SD9) were sourced from the RCSB Protein Data Bank (PDB) (https://www.rcsb.org/) to facilitate docking studies.

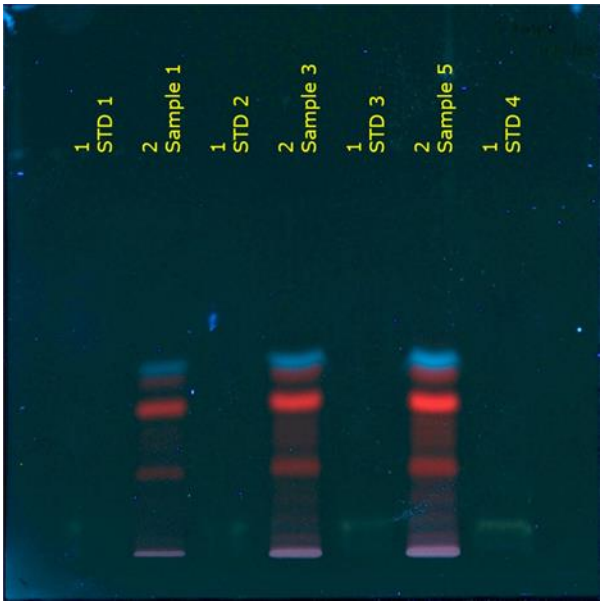

Fig. 1: HPTLC Chromatogram of *Kalanchoe pinnata* (Lam.) Pers.

Drug-likeness and ADMET profiling

Evaluating the drug-likeness and ADMET profile of selected compounds at an early stage is essential to avoid unnecessary time and resource expenditure. The drug-likeness properties of the selected ligand molecules were assessed using the *in silico* tool SwissADME. Key parameters analysed included molecular weight (≤500 Da), octanol–water partition coefficient (iLOGP), number of hydrogen bond donors (≤5), number of hydrogen bond acceptors (≤10), topological polar surface area (TPSA), and number of rotatable bonds (nROT). SwissADME determines these properties by converting the uploaded ligand structure, provided as an SDF file, into SMILES format and generating the corresponding predictions. The molecular properties were evaluated according to Lipinski's Rule of Five (RO5) to determine the drug-likeness of each compound.The toxicity of the compounds were predicted using ProTox-II, a freely available web server (http://tox.charite.de/protox_II/). The tool uses machine learning algorithm-based models to predict toxicological endpoints, pathways, and targets, as well as acute (oral) and organ (liver) toxicities, based on the 2D chemical structures of the compounds.

In Silico Pass Prediction Study

In silico PASS prediction is a computational approach that estimates the potential biological activities of a compound using the Prediction of Activity Spectra for Substances (PASS) algorithm. This method aids in prioritizing drug development and identifying the primary bioactivities of chemical compounds. PASS analyses a molecule's chemical structure to predict up to 3750 possible activities, providing results as 'probable activity' (Pa) and 'probable inactivity' (Pi), with values ranging from 0.000 to 1.000. A compound is considered biologically active if Pa > Pi, and activities with Pa > 0.700 are regarded as highly probable.

Results and Discussion

High Performance Thin Layer Chromatography (HPTLC) Profiling

HPTLC Finger print shows several lanes: standard ("STD") lanes and "Sample" lanes (Fig. 1). After development and visualization, distinct coloured bands appear at different heights in each lane. Table 1 depicts Rf values for certain sample lanes (e.g. "Sample 2", "Sample 3") and compares them with standard compounds. Sample 3 shows an

Table 1: Interpretation of chromatogram *Kalanchoe pinnata* (Lam.) Pers.

Lane	Rf Value	Observation	Interpretation
Sample 3	0.49	Upper blue/red band	Presents the band matching squalene
Sample 2	0.24	Lower pink/red band	Triterpenoids
STD 3	0.53	Upper red band	Shows the presence of squalene
STD 4	0.08	Lowest visible spot (pink/red)	Unknown compound

Table 2: ABTS Assay of *Kalanchoe pinnata* (Lam.) Pers.

S. No.	**Concentration of Sample**	**Inhibition (%)**	**IC_{50}**
1	10 µg/ml	24.6 ± 0.75	IC_{50} = 34.2 µg/ml
2	20 µg/ml	35.9 ± 0.57	
3	30 µg/ml	45.6 ± 0.75	
4	40 µg/ml	55.3 ± 1.05	
5	50 µg/ml	64.9 ± 0.57	
6	Control (Trolox)	69.6 ± 0.75%	

upper blue/red band with Rf ≈ 0.49, which interpret as matching a band from a standard (STD) that corresponds to squalene. Sample 2 shows a lower pink/red band with Rf ≈ 0.24, interpreted as indicating triterpenoids. Another standard, "STD 4," has a very low Rf (0.08) and shows the "lowest visible spot (pink/red)," described as an unknown compound.

When comparing the chromatogram and the tabulated Rf-data, it becomes evident that at least two distinct types of constituents are present in the tested plant fraction. One component (Sample 3) migrates to an Rf value similar to that of a squalene standard, supporting the conclusion that squalene is present. Another component (Sample 2) exhibits a much lower Rf value and displays a different coloured band, consistent with the presence of more polar triterpenoid compounds. Therefore, the TLC profile supports a preliminary identification of both squalene and other triterpenoids in the extract.

Antioxidant Activity - ABTS Assay

The DMSO extracts of all five different concentrations of sample were evaluated for their antioxidant potential by ABTS assay and the inhibition percentage and the IC_{50} values are presented in Table 2 and Figure 2.

The obtained values when compared to control trolox revealed that increase in antioxidant potential was significantly observed with respective to increase in concentration of samples. On the graph, a vertical line marks the concentration at which 50% inhibition (IC_{50}) is reached that is here indicated as ~ 34.2 µg/ml. The IC_{50} is a standard metric: the concentration of the extract required to scavenge (inhibit) 50% of the initial ABTS radical population. The IC_{50} value of ≈ 34.2 µg/ml quantifies the potency: that concentration is sufficient to reduce the ABTS radical by half, indicating the sample has significant free-radical scavenging potential.

Anti-Inflammatory Activity – Inhibition of Albumin Denaturation Test

Kalanchoe pinnata (Lam.) Pers. was evaluated for the activity of inhibition of albumin denaturation. The efficacy of the produced formulation to

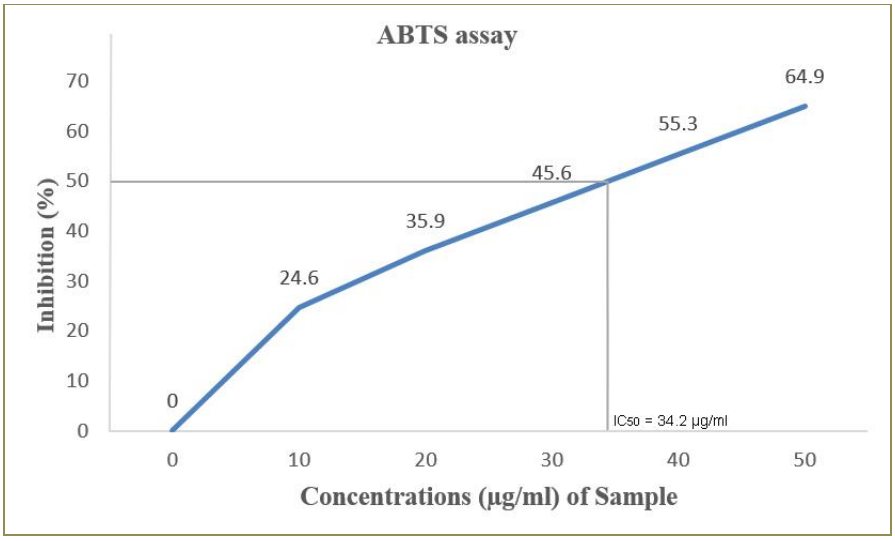

Fig. 2: ABTS Assay of *Kalanchoe pinnata* (Lam.) Pers.

Table 3: Inhibition of Albumin denaturation test of the *Kalanchoe pinnata* (Lam.) Pers.

S. No.	Concentration of Sample	Protection (%)	PD_{50}
1	10 µg/ml	10.9 ± 0.57	PD_{50} = 41.5 µg/ml
2	20 µg/ml	22.6 ± 0.75	
3	30 µg/ml	34.3 ± 1.05	
4	40 µg/ml	48.6 ± 0.75	
5	50 µg/ml	56.3 ± 1.05	
6	Control (Sodium diclofenac)	63.9 ± 0.57%	

prevent the denaturation was evaluated through its anti-inflammatory activity. Table 3 depicts results of an Albumin-Denaturation Inhibition Assay, a commonly used *in vitro* test to screen a sample's potential anti-inflammatory activity. Relative to the positive control, the sample's anti-inflammatory activity increased significantly as sample concentration increased. Figure 3 illustrates the dose-dependent protective effect of a sample against the denaturation of albumin, demonstrating a positive correlation between concentration and effect. The sample exerts concentration-dependent protection against thermal denaturation of albumin; the PD_{50} value of 41.5 µg/ml indicates the concentration required to achieve 50% of the maximal protective effect.

In Silico Toxicity Prediction Study and ADMET Profiling

Drug-likeness and ADMET profiling

The drug-likeness properties of the selected ligand molecules were determined using *in silico* Swiss ADME. These properties include molecular weight (MW≤500 Dalton), octanol–water partition coefficient (iLOGP), and number of hydrogen donors (HBD≤5), number of hydrogen acceptors (HBA≤10), Topological Polar Surface Area (TPSA), and number of rotatable bonds (nROT) are presented in Table 4. These crucial molecular properties was checked according to Lipinski Rules of five (RO5).

In Silico Pass Prediction Study

The toxicity of the compounds were predicted using ProTox-II, a freely available web server. The tool uses machine learning algorithm-based models to predict toxicological endpoints,

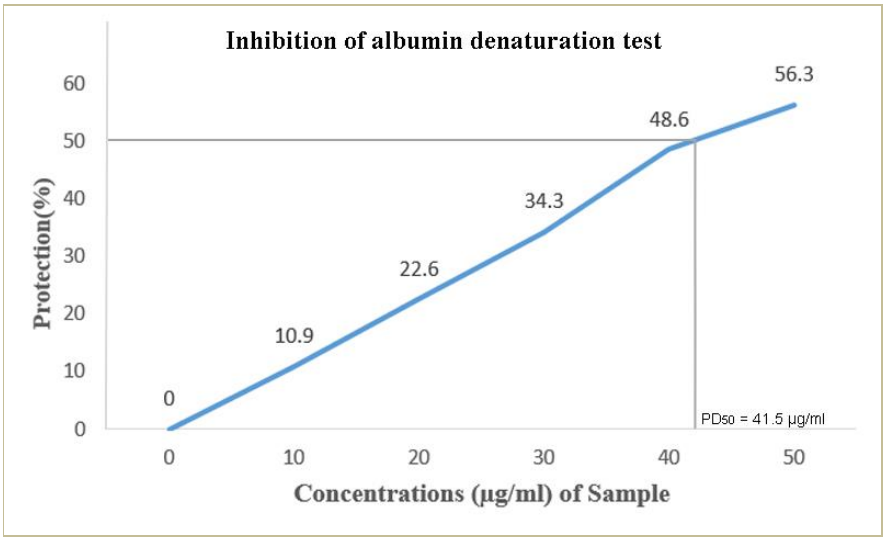

Fig. 3: Inhibition of albumin denaturation test of the *Kalanchoe pinnata* (Lam.) Pers.

Table 4: ADMET properties of four molecules Diethyl Phthalate, Di butyl Phthalate, Phytol and Squalene

Ligands	Formula	Molecular Weight	iLOGP	HBD	HBA	TPSA	nROT	Lipinski Rule violation
L1	$C_{12}H_{14}O_4$	222.24	2.26	0	4	52.6	6	0
L2	$C_{16}H_{22}O_4$	278.34	2.97	0	4	52.6	10	0
L3	$C_{20}H_{40}O$	296.53	4.85	1	1	20.23	13	1
L4	$C_{30}H_{50}$	410.72	6.37	0	0	0	15	1

Table 5: Comparison of Pa and Pi values and toxicity of Diethyl Phthalate

Compound	Pa	Pi	Biological Activity
Diethyl Phthalate	0,941	0,002	2-Hydroxymuconate-semialdehyde hydrolase inhibitor
	0,936	0,003	Prolyl aminopeptidase inhibitor
	0,934	0,002	5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase inhibitor

pathways, and targets, as well as acute (oral) and organ (liver) toxicities, based on the 2D chemical structures of the compounds. Diethyl Phthalate, Dibutyl Phthalate, Phytol, and Squalene are all projected to have multiple biological activities notably enzyme-inhibitory, antioxidant, anti-bacterial, and antidiabetic effects while exhibiting low acute oral toxicity risk (Tables 5,6,7,8).

Conclusion

The succulent species *Kalanchoe pinnata* (Lam.) Pers has been widely introduced into various temperate and tropical regions globally for ornamental purposes. The present study evaluated the Free-radical scavenging and Inflammation-modulating effects and have proved them making it useful for further investigations. The HPTLC analysis of *Kalanchoe pinnata* (Lam.) Pers. yielded a definitive phytoconstituent profile, offering essential information for its subsequent use in herbal drug formulation. The four compounds from HPTLC were assessed to identify their toxicity with selected proteins like C-Met and GOT1; all predicted their biological activity that were related to enzyme inhibition, anti-oxidant,

Table 6: Comparison of Pa and Pi values and toxicity of Dibutyl Phthalate

Compound	Pa	Pi	Biological Activity
Dibutyl Phthalate	0,459	0,035	Pancreatic elastase inhibitor
	0,263	0,030	Pancreatic disorders treatment
	0,248	0,021	Pancreatic endopeptidase E inhibitor
	0,285	0,091	Antimycobacterial
	0,196	0,012	Bacterial leucyl aminopeptidase inhibitor
	0,162	0,040	Antibacterial, ophthalmic
	0,221	0,100	Antibacterial
	0,142	0,112	Antioxidant

Table 7: Comparison of Pa and Pi values and toxicity of Phytol

Compound	Pa	Pi	Biological Activity
Phytol	0,130	0,099	Antibacterial, ophthalmic
	0,417	0,026	Antibacterial
	0,475	0,008	Antioxidant
	0,458	0,070	Anti-inflammatory
	0,305	0,067	Anti-inflammatory, intestinal
	0,299	0,080	Anti-inflammatory, ophthalmic

Table 8: Comparison of Pa and Pi values and toxicity of Squalene

Compound	Pa	Pi	Biological Activity
Squalene	0,657	0,004	Antioxidant
	0,397	0,031	Antibacterial
	0,183	0,023	Antibacterial, ophthalmic
	0,674	0,040	Ocular toxicity
	0,544	0,011	Pancreatic elastase inhibitor

anti-inflammatory and anti-bacterial/microbial activities, while simultaneously posing a low acute oral toxicity risk. The identified compounds in the plant contribute to its antioxidant capacity and anti-inflammatory properties, making it a promising source for medicinal applications. These findings pave the way for *in vivo* and *in vitro* studies to assess the potential of these compounds in treating cancer-related diseases.

Ethical Statement

No animal or microbes have been used or sacrificed for this study, hence Ethical Approval not required.

Author Contributions

Each author has contributed equally to the study's planning, analysis, writing, and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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