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Phytochemical richness and anti-aging properties of *Kalanchoe pinnata*: Insights from antioxidant and elastase inhibition assays

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Abstract

Objective

This study aimed to evaluate the phytochemical composition and biological activities of *Kalanchoe pinnata* leaf and stem extracts. The specific objectives were to quantify the contents of phenolics, flavonoids, and alkaloids, assess antioxidant potential using multiple in vitro assays, and determine anti-aging activity via elastase inhibition.

Materials and methods

Leaves and stems of *K. pinnata* were collected from South Sulawesi, Indonesia, dried, powdered, and extracted with 96% ethanol by maceration. Phytochemical screening was performed using standard qualitative tests, while quantitative analysis of phenolics, flavonoids, and alkaloids was conducted with colorimetric assays. Antioxidant activity was evaluated using total antioxidant capacity (TAC), ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity

(CUPRAC), ABTS radical scavenging, and β-carotene bleaching assays. Anti-elastase activity was determined using porcine pancreatic elastase with N-succinyl-(Ala-Ala-Ala)-p-nitroanilide as substrate. All experiments were performed in triplicate, and data were analyzed using descriptive statistics, correlation analysis, and principal component analysis (PCA).

Results

Both leaf (KPLE) and stem (KPSE) extracts contained phenolics, flavonoids, and alkaloids, with terpenoids detected only in leaves and saponins only in stems. Quantitative analysis revealed higher levels of phenolics (36.73 mg GAE/g), flavonoids (20.06 mg QE/g), and alkaloids (11.42 mg Quinin Equivalen/g) in stems compared with leaves. KPSE exhibited more potent antioxidant activity, with higher TAC (66.78 µM GAEAC/g), FRAP (122.26 µM FeEAC/g), and CUPRAC (31.60 µM QEAC/g) values, as well as potent ABTS radical scavenging and lipid peroxidation inhibition (IC_{50} < 50 µg/mL). In contrast, KPLE showed moderate antioxidant capacity (IC_{50} values 50-100 µg/mL). Elastase inhibition was moderate for KPSE (IC_{50} = 44 µg/mL) and weak for KPLE (IC_{50} = 680 µg/mL). PCA and correlation analyses confirmed strong associations between phytochemical richness and both antioxidant and anti-elastase activities.

Conclusion

The stem extract of *K. pinnata* demonstrated superior phytochemical content, antioxidant capacity, and elastase inhibition compared with the leaf extract. These findings highlight *K. pinnata* stems as a promising natural source of antioxidant and anti-aging compounds, supporting their potential application in herbal-based cosmetics and therapeutic formulations.

Keywords: Antioxidant, anti-aging, anti-elastase**,** *Kalanchoe pinnata* extract, phytoconstituent

Paper Type: Research Paper.

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Introduction

Chemical entities possessing an unpaired or odd number of electrons are classified as free radicals. Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), play advantageous roles in the body by participating in several metabolic processes, specifically generated as components of cellular defense systems against pathogens, and regulating multiple aspects of glucose metabolism, cellular development, and proliferation.

Superoxide radicals and NO, for example, which are reactive species most frequently synthesized and produced by NADPH oxidase and NO synthase, can increase intracellular Ca^{2+} concentrations, specific protein phosphorylation, activate specific transcription factors, modulate eicosanoid metabolism, stimulate cell growth and control physiological mediators for several transcription factors (Hunyadi, 2019). Conditions of oxidative stress can affect cellular and extracellular macromolecules (proteins, lipids, nucleic acids, and DNA) so that they can experience oxidative damage to body tissues, which triggers degenerative diseases through several oxidation pathways in the body which can cause gene mutations, disruption of enzyme activity or changes cell membrane permeability (Gulcin, 2020; Nimse & Pal, 2015; Oroian & Escriche, 2015). The human body has a natural defence against exposure to free radicals such as enzymes (Glutathione peroxidase, Superoxide Dismutase, Glucose-6-phosphate Dehydronegase, Catalase, and Glutathione-S-Transferase), which play an essential role in the body as initial protection against free radicals. These enzymes can convert reactive free radicals to non-reactive ones, thereby preventing an increase in ROS, which can cause damage to body tissues (Apak et al., 2008, 2017; Phaniendra et al., 2015). Continuous activation of ROS formation will have negative impacts and cause various diseases. One of the harmful effects that can occur is premature aging. The increase in ROS in the body will mediate an increase in neutrophil elastase, which can degrade the elastin matrix protein in tissues, causing wrinkles. The unprogrammed formation of wrinkles accelerates the aging process and will cause various other accompanying diseases. However, exposure to excess free radicals in the body requires an exogenous antioxidant derived from natural ingredients that effectively reduce free radicals to produce stable complexes and low reactivity (Nur et al., 2021). Phytobiotics and medicinal plants have attracted considerable attention in recent years, largely due to their potential as natural alternatives to medicine (Amirteymoori et al., 2021; Mohammadabadi et al., 2022). These plant-rich contain bioactive compounds-such as essential oils, alkaloids, flavonoids, and phenolic acids-that contribute to a wide range of biological activities, including antibacterial, antioxidant, anticancer, and anti-inflammatory effects (Mohammadabadi et al., 2025). Consequently, phytobiotics play a crucial role in improving health, performance, and immunity (Safaei et al., 2025). he use of phytobiotics and medicinal plants as natural antimicrobial growth promoters in place of antibiotics in feed offers numerous advantages.These advantages encompass enhanced zootechnical efficiency metrics, inhibition of particular diseases (Mohammadabadi et al., 2023), antimicrobial and antioxidant properties, hypocholesterolemic effects, augmentation of digestive enzymes, anticancer functions, and improved hepatic performance (Roudbar et al., 2014). So that, phytobiotics have demonstrated the ability to modify gut microbiota, hence improving nutrition absorption and bolstering overall immunological function (Vahabzadeh et al., 2020). Studies have demonstrated that incorporating these plants into the diets can increase feed consumption, improve feed conversion to muscle, and enhance physical ability (Vahabzadeh et al., 2020). Furthermore, phytobiotics are associated with reducing stress-related impacts, improving meat quality, regulating gene expression involved in cancer, and decreasing the environmental impact of animal production systems by optimizing nutrient utilization (Mohammadabadi et al., 2024). One of the plants that can act as an exogenous antioxidant is *Kalanchoe pinnata* (Lam.) per or by

another name, Bryophyllum calycinum, and in Indonesia, it is known as Cocor Bebek. In addition to having a function as an antioxidant, traditionally, this plant has been widely used by the broader community as a traditional medicine, as has been reported. This is related to compounds in the *K. pinnata* plant, which have been analysed in the methanol extract of *K. pinnata* roots containing sterols, flavonoids, chlorides, nitrates, and potassium. Meanwhile, the leaves of this plant contain phenols, flavonoids, lycopene, and β-carotene (in ether solvents), as well as tannins and alkaloids (Hernández-Caballero et al., 2022; Majaz et al., 2011; Mora-Pérez & Hernández-Medel, 2016; Okwu & Josiah, 2006). Leaves contain primary metabolites, including amino acids, carbohydrates, proteins, lipids, ascorbic acid, thiamine, pyridoxine, and minerals such as sodium, calcium, potassium, magnesium, iron, copper, zinc, and sugars (Alabi et al., 2005; Majaz et al., 2011) (Alabi et al., 2005; Majaz et al., 2011). These compounds have stimulated comprehensive investigation into their bioactivities, such as anticancer (SUPRATMAN et al., 2001; Ueda et al., 2002), antidiabetic (Ogbonnia et al., 2008), antifungal (Ogbonnia et al., 2008), hepatoprotective (Yadav & Dixit, 2003), and immunomodulatory effects (Cruz et al., 2008). This study aims to evaluated the phytochemical constituents of *Kalanchoe pinnata* extract, and to know its antioxidant activity through various methodologies, and examine its anti-aging potential by elastase inhibition. Previous studies on the antioxidant properties of *K. pinnata* are predominantly limited to a specific methodology or focus on a particular plant component. This gap opens up new ways to ask questions. This study introduces a novel methodology by simultaneously examining leaf and stem extracts through various analytical techniques and correlating the results to the anti-aging mechanism via the elastase enzymatic pathway. The goal of this study is to provide a more in-depth understanding of *K. pinnata's* bioactivity and to strengthen the scientific basis for its development as a plant with nutritional and medicinal value.

Materials and Methods

The reagents and solvents used in this study were analytical grade, such as distilled water obtained from OneMed (Indonesia). In addition, several reagents used were obtained from Merck (Germany): sulfuric acid, sodium phosphate, sodium carbonate, ammonium sulfate, potassium persulfate, ABTS, and Folin-Ciocalteu reagent. Compounds such as gallic acid, quercetin, $CuCl_2$, neocuproine, ammonium molybdate, bromocresol green, $AlCl_3$, potassium acetate, Trizma base, porcine elastase (SLBV 9311), and N-succinyl-(Ala-Ala-Ala)-p-nitroanilide (Sigma-Aldrich, Germany) were used as standards and assay reagents. Ethanol (96%) and chloroform (JT-Baker) as solvents and β-carotene was purchased from TCI (Japan).

Plant material and extraction: The leaves and stems of *Kalanchoe pinnata* was obtained from Soppeng Regency, South Sulawesi, Indonesia. The samples were dried in an oven at 40°C for four days, then ground into a fine powder. Extraction was done by cold maceration with 96% ethanol at a 1:10 (w/v) ratio for 72 hours at room temperature. The residue was re-extracted until the solvent became clear, and the filtrate was separated. We combined all the filtrates and concentrated them using a rotary evaporator under low pressure. This approach gave us leaf (KPLE) and stem (KPSE) extracts.

Phytochemical profiling: Preliminary screening of secondary metabolites was carried out following Harborne's procedure, using specific reagents to detect alkaloids, flavonoids, terpenoids, and saponins (Harborne, 1998).

Determination alkaloids content: Alkaloid content was determined using a colorimetric method described by Sharma et al. with slight modifications(Sharma et al., 2021). Alkaloid levels were quantified using a modified colorimetric method with caffeine as the standard (2-15 µg/mL). For analysis, 100 mg of each extract (KPLE and KPSE) was dissolved in 10 mL of ethanol to obtain a 10,000 µg/mL solution. A 0.5 mL aliquot was mixed with 1 mL of 2 N HCl and extracted three times with 1 mL of chloroform. After discarding the chloroform phase, 1 mL of 0.1 N NaOH, 0.5 mL of bromocresol green, and 1 mL of phosphate buffer (pH 4.7) were added. The mixture was re-extracted with 1 mL of chloroform, and the chloroform phase was collected in a 10 mL volumetric flask and diluted to volume. Absorbance was measured at 273 nm using a UV-Vis spectrophotometer. A quinine calibration curve (3; 6; 9; 12; and 15 µg/mL) was used to determine the total alkaloid content in the extract, expressed as quinin equivalent/g extract.

Determination of phenolic content: The total phenolic content was determined using a colorimetric method as described by Nur et al. (2022) and Nur et al. (2023) with minor modifications. The modified Folin-Ciocalteu method was used to determine total phenolic content. Ten milligrams of each extract (KPLE and KPSE) were dissolved in 10 mL of ethanol. One mL of the sample was mixed with 0.2 mL of Folin-Ciocalteu reagent and incubated for 5 minutes. Then, 2 mL of sodium carbonate solution (7% w/v) was added. The mixture was topped off to 10 mL with distilled water and allowed to sit for 30 minutes, during which a blue complex formed. The absorbance was measured at 769 nm, and the results were reported as gallic acid equivalents. The gallic acid calibration curve (2, 4, 6, 8, and 10 µg/mL) was used to determine the phenolic content of the extract (GAE/g extract).

Determination of flavonoid total: The colorimetric method with minor modifications was used to determine the total flavonoid content, as described by Nur et al. (2022). Flavonoid content was measured using an aluminum chloride colorimetric assay. Ten milligrams of each extract were dissolved in ethanol and diluted to 10 mL. A 1 mL aliquot was mixed with 0.1 mL of 10% $AlCl_3$ and 0.1 mL of 1 M potassium acetate, homogenized, and left to stand for 3 minutes at room temperature. The solution was then adjusted to 5 mL with ethanol and incubated for 30 minutes. Absorbance was measured at 382.7 nm using a UV-Vis spectrophotometer (Shimadzu UV-1900, Japan), and results were expressed as quercetin equivalents. A quercetin calibration curve (2, 4, 6, 8, and 10 µg/mL) was used to determine the total quercetin content in the extract (µM QE/g of final extract).

Antioxidant capacity assay-Total Antioxidant Capacity (TAC) Assay: The total antioxidant capacity (TAC) was assessed using the methodology outlined by Nur et al. (2022). For the TAC test, 1 mL of each extract solution was combined with 1.5 mL of a reagent containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 1% ammonium molybdate. The mixture was then diluted to 5 mL with distilled water. The mixture was put in an incubator at 95°C for 10 minutes, then cooled down, and the absorbance was measured at 695 nm using a Spectrophotometer Simadzu UV-1900 from Japan. The standard was gallic acid at 20, 40, 60, 80,

and 100 µg/mL, and the results were given as gallic acid equivalent antioxidant capacity (µM GAEAC/g) final extract.

FRAP assay: The FRAP assay was performed according to the method described by Nur et al. (2023b). In the FRAP test, 0.5 mL of extract solution (0.1% w/v) was combined with 1 mL of FRAP reagent, which consisted of acetate buffer (pH 3.6), one mM TPTZ in 0.05 M HCl, and three mM $FeCl_3$ in 5 mM citric acid (10:1:1, v/v/v). Distilled water was added to bring the total volume to 5 mL, and the mixture was incubated at 37°C for 30 minutes. The absorbance was then measured at 595 nm using a Shimadzu UV-1900 spectrophotometer from Japan. Quercetine (2, 4, 6, 8, and 10 µg/mL) was used as the standard and antioxidant capacity was expressed as quercetin equivalent antioxidant capacity (µM QEAC/g final extract).

CUPRAC assay: The cupric reducing antioxidant capacity (CUPRAC) experiment was conducted in accordance with the modified protocols established by Apak et al. (2008) and Nur et al. (2023a). For the CUPRAC assay, 0.5 mL of extract solution was combined with 1 mL of a reagent solution containing 10 mM $CuCl_2$, 7.5 mM neocuproine, and 1 M ammonium acetate buffer (pH 7.0) in equal volumes (1:1:1, v/v/v). The mixture was diluted to 5 mL with distilled water and left at room temperature for 30 minutes. The absorbance was then measured at 442 nm using a Spectrophotometer Simadzu UV-1900 from Japan. The standard was gallic acid (2, 4, 6, 8, and 10 µg/mL), and the results were given as µM GAEAC/g final extract.

ABTS assay: The ABTS free radical scavenging activity was assessed by evaluating the extracts' capacity to neutralize ABTS radicals, as outlined by Nursamsiar et al. (2025). ABTS radicals were generated by mixing 2.75 mM ABTS with 2.59 mM potassium persulfate and incubating for 12 to 16 minutes hours. Extracts at concentrations of 20, 40, 60, 80, and 100 µg/mL were combined with 1 mL of ABTS solution and subsequently diluted to 5 mL using ethanol. Following a 30-minute incubation in darkness, absorbance was assessed at 751 nm utilizing a Shimadzu UV-1900 spectrophotometer (Japan). Radical scavenging activity was determined as percentage inhibition compared to the blank, using quercetin (2, 4, 6, 8, and 10 µg/mL) as the standard. Radical scavenging activity was measured as percentage inhibition (%) using the following equation:

$$\text{Inhibition (\%)} = \frac{Blank\ Abs - Sample\ Abs}{Blank\ Abs} \times 100 \quad \text{..... Eqn 1}$$

β-Carotene bleaching assay: The lipid peroxidation-inhibitory efficacy of ethanol extracts from *Kalanchoe pinnata* leaves (KPLE) and stems (KPSE) was assessed using the methods outlined by Nur et al. (2017) and Sulistyowati et al. (2023). In the β-carotene bleaching assay, extracts at final concentrations of 20, 40, 60, 80, and 100 µg/mL were combined with 1 mL of a β-carotene emulsion, which was prepared from 2 mg β-carotene, 0.2 mL chloroform, 2 mL Tween 20, 0.2 mL linoleic acid, and distilled water to a total volume of 100 mL. The mixture was diluted to 5 mL in a vial and incubated at 50 °C for 20 minutes, with absorbance recorded at 458 nm (Spectrophotometer, Shimadzu UV-1900 Japan). Incubation lasted 120 minutes, and antioxidant activity was quantified as percentage inhibition and IC_{50} values, with BHT as the positive control. The antioxidant activity was measured as percentage inhibition (%), and IC_{50} values were calculated from the degradation rate relative to the blank using the following equation:

% degradation rate inhibition = ln (a/b) × (1/t)..... **Eqn 2**

where **a** is the absorbance of the sample after 20 min of incubation, **b** is the absorbance after 120 min of incubation, and ***t*** is the incubation time (120 minutes). The percentage inhibition (%) was then determined using the equation:

$$\% \text{ Inhibition} = \frac{blank\ degradation\ rate - sample\ degradation\ rate}{blank\ degradation\ rate} \times 100$$ ..... **Eqn 3**

Anti-elastase assay: The anti-elastase activity of *Kalanchoe pinnata* extracts was evaluated using porcine pancreatic elastase (Sigma-Aldrich, SLBV 9311) as the enzyme and 30 µL of N-succinyl-(Ala-Ala-Ala)-p-nitroanilide (Sigma-Aldrich, S4760) as the substrate Inhibition assays for both leaf (KPLE) and stem (KPSE) extracts were performed following the methodology outlined by Nur et al., with slight modifications (Nur et al., 2023b).

Data analysis: Total phenolic, flavonoid, and alkaloid contents, as well as antioxidant and anti-elastase activities, were expressed as mean ± standard deviation (SD) and analyzed using Microsoft Excel 2019. Similarity analysis and correlation of bioactivity data across assays were performed using the MetaboAnalyst platform (version 5.0).

Result and discussion

Extraction and phytochemical screening of the extract: In the preliminary phase of this study, KPSE were macerated with 96% ethanol to extracted secondary metabolites with potential bioactivity. The extraction method, solvent type, and plant material are essential factors affecting the efficacy of compound recovery. Maceration was selected to preserve thermolabile compounds by circumventing high temperatures during extraction. Luliana et al. report that the maceration method yields a greater extract compared to other conventional extraction techniques (Luliana et al., 2019). This study reported a high extraction yield (>10%) (Table 1), with *K. pinnata* leaf extract (KPLE) yielding 13.90% and stem extract (KPSE) yielding 12.22%. The effective use of maceration with 96% ethanol on *K. pinnata* indicates that the extracted compounds are primarily polar, aligning with the principle of "like dissolves like," which states that compounds are preferentially solubilized in solvents of analogous polarity. Polar constituents may encompass phenolics, flavonoids, and alkaloids, which are extensively documented to demonstrate a range of biological activities pertinent to the prevention and management of various diseases.

Table 1. Percentage of yield of ethanol extract of leaves and stems of *K. pinnata* extract

Sample	Sample Weight (g)	Extract Weight (g)	Yield (%)
K. pinnata Leave Extract (KPLE)	200	27.80	13.90 %
K. pinnata Stem Extract (KPSE)	200	24.44	12.22 %

Phytochemical screening: Ethanol extracts from the leaves (KPLE) and stems (KPSE) of *Kalanchoe pinnata* were subjected to phytochemical screening using specific colorimetric reagents. Table 2 indicates that both extracts tested positive for flavonoids, phenolics, and alkaloids. Terpenoids were colorimetrically identified in KPLE, whereas saponins were

exclusively detected in KPSE. The phytochemical profiles of KPLE and KPSE were analogous, consistently yielding positive results for flavonoids, phenolics, and alkaloids. The compound profile of this plant is thoroughly documented for its diverse pharmacological effects, suggesting the potential of *K. pinnata* as a candidate for standardized traditional medicine. Prior research has recognized flavonoid compounds in K. pinnata, such as quercetin, myricetin, kaempferol, apigenin, avicularin, luteolin-7-glucoside, taxifolin, baicalin, and genistin (Ramon et al., 2023). The identified phenolic compounds comprise epigallocatechin gallate, 4-hydroxy-3-methoxycinnamic acid, ferulic acid, and caffeic acid (Pereira et al., 2018; Uthman et al., 2023).

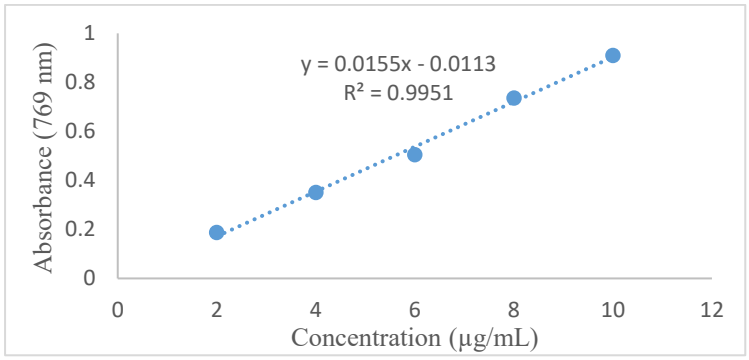
Table 2. Results of phytochemical screening of leaves and stems of *K. pinnata* extract

Sample	Compound Test	Information
K. pinnata Leave Extract (KPLE)	Alkaloid	+
	Flavonoid	+
	Phenolic	+
	Terpenoid	+
	Saponin	-
K. pinnata Stem Extract (KPSE)	Alkaloid	+
	Flavonoid	+
	Phenolic	+
	Terpenoid	-
	Saponin	+

Note: (-) absence in extract, (+) present in extract

This study also showed that the leaves extract of *K. pinnata* were positive for terpenoids while negative for saponins. In contrast, the *K. pinnata* stem extract tested positive for saponins and negative for terpenoids. Leaves are typically abundant in flavonoids and terpenoids, which provide protection against UV radiation and pests. Stems contain more saponins/alkaloids, which function as structural defense and internal metabolism. Furthermore, the cultivation site, climatic factors, and soil characteristics affect the biosynthesis of secondary metabolites (Saini et al., 2024; Shakeel et al., 2025; Shen et al., 2022). This study is further corroborated by data from research conducted by (Purwanti et al., 2025) and (Qomaliyah et al., 2023), which showed similar results.

Phenolic, flavonoid and alkaloid total of leave and stem *K. pinnata*: Phytochemical screening of *Kalanchoe pinnata* leaf (KPLE) and stem (KPSE) extracts confirmed the presence of phenolic, flavonoid, and alkaloid compounds. Quantitative analysis of these secondary metabolites was subsequently performed to support the potential development of *K. pinnata* extracts as natural medicines and to provide an estimate of their therapeutic dosage. The total contents of phenolics, flavonoids, and alkaloids in each extract were determined using the corresponding standard calibration curves (Figure 1). The results were quantified as gallic acid equivalents (GAE) for phenolics, quercetin equivalents (QE) for flavonoids, and quinine equivalents (QE) for alkaloids, per gram of extract. Gallic acid, quercetin, and quinine were chosen as standards due to their representation as secondary metabolites frequently present in plant extracts, thereby reflecting the principal bioactive components of *K. pinnata.*



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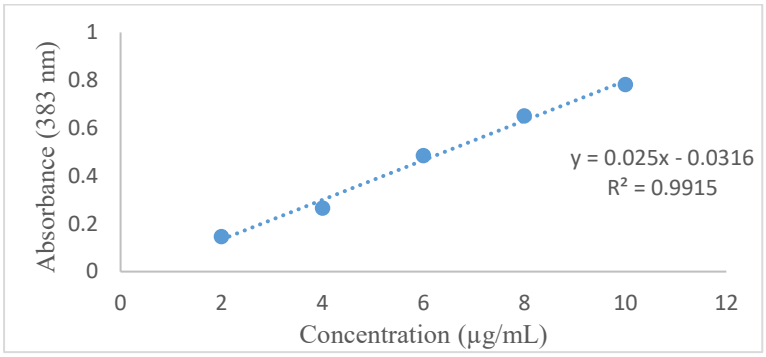

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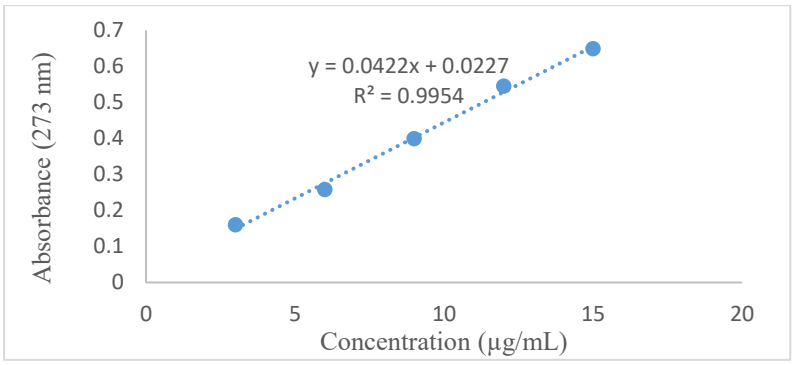

C

Figure 1. Standard calibration curves for (A) gallic acid in the determination of total phenolic content, (B) quercetin in the determination of total flavonoid content, and (C) quinine in the determination of total alkaloid content. All measurements were performed in triplicate (n = 3)

The results of total phenolic, flavonoid, and alkaloid content analysis are presented in Table 3. The analysis results showed that the total phenolic, flavonoid, and alkaloid content in *Kalanchoe pinnata* stem extract (KPSE) was higher than that in KPLE extract. In the KPSE extract, the levels of phenolic, flavonoid, and alkaloid compounds were recorded as 36.729±0.744, 20.064±1.386, and 11.687±0.068 mg/g extract, respectively. Among the three groups of compounds, phenolics were the dominant group, with a gallic acid equivalent (GAE) content of 36.729±0.744 mg per gram of extract. This relatively high phenolic yield indicates that

K. pinnata stem extract has potential as a raw material for traditional medicine. Phenolic derivatives are known for their therapeutic applications in disease prevention and treatment (Rahman et al., 2021), and their quantification is crucial for standardizing natural products as medicinal raw materials (Shi et al., 2022). Based on the results, the total alkaloid content in the stem extract was significant, with the value of 11.419±0.468 Quinine Equivalent per gram of extract. Alkaloids also contribute significantly to pharmacological activities, such us antibacterial, anti-inflammatory, anticancer, and anti-degenerative effects. This study demonstrates that KPSE contains elevated concentrations of phenolics, flavonoids, and alkaloids compared to leaves, as the distribution of secondary metabolites varies among plant organs and is affected by the physiological role of the stem, extraction techniques, and environmental conditions. Stems frequently accumulate significant quantities of polar and semi-polar compounds as components of the defense and metabolite transport mechanisms. The distribution of secondary metabolite content may vary due to the impact of geographical conditions on plants (Palazon & Alcalde, 2025). Consequently, assessing the concentrations of these compounds and pinpointing specific biomarkers is essential for establishing *K. pinnata* as a scientifically substantiated herbal remedy.

Table 3. Total phenolic, flavonoid, and alkaloid levels of *K. pinnata* extract

Content Parameters	*K. pinnata* Leave Extract (KPLE)	*K. pinnata* Stem Extract (KPSE)
	Average (mean±SD)	Average (mean±SD)
Alkaloid (mg Quinin Equivalent±SD/g extract)	8.834±0.253*	11.419±0.468*
Phenolic (mg GAE+SD)/g extract	27.439±0.129*	36.729±0.744*
Flavonoid (mg QE±SD)/ g extract	10.397±0.401*	20.064±1.386*

* The symbol indicates that there is a significant difference between the compound content of the leaf and stem extracts of the plant (LSD, p-value <0.05, n=SD).

Antioxidant activity: The antioxidant effectiveness of *Kalanchoe pinnata* leaf (KPLE) and stem (KPSE) extracts was assessed using various tests that reflect the main antioxidant mechanisms in biological systems. Metal-reducing capacity was evaluated using total antioxidant capacity (TAC), copper-reducing antioxidant capacity (CUPRAC), and iron-reducing antioxidant power (FRAP) assays. Free radical scavenging activity was evaluated using the ABTS radical assay, which, in this mechanism, can be seen as the ability of compounds in *K. pinnata* extract to inhibit ABTS radical activity, which works by donating electrons or hydrogen from bioactive compounds to $ABTS^{\bullet+}$ radicals. This process neutralizes the radicals, reduces absorbance, and reflects the antioxidant capacity of the extract (Griffiths, 2016). At the same time the β-carotene bleaching (BCB) assay, which assesses inhibition of reactive oxygen species (ROS) radicals. Antioxidant capacity in the $ABTS^{\bullet+}$ and BCB assays was expressed as IC_{50} values, representing the concentration of extract required to inhibit 50% of radical activity. Together, these assays provided a comprehensive profile of the antioxidant mechanisms of the bioactive compounds present in the extracts. The results of antioxidant quantification are presented in Table 4 and

Figure 2. Notably, data in Table 4 indicate that the stem extract (KPSE) exhibited greater metal-reducing activity compared with the leaf extract (KPLE).

Table 4. Antioxidant quantification by reducing metal method

Antioxidant assay	*K. pinnata* Leave Extract (KPLE)	*K. pinnata* Stem Extract (KPSE)
	Average (mean±SD)	Average (mean±SD)
TAC (µM GAEAC±SD)	7,435±4,555*	66,783±2,671*
CUPRAC (µM QEAC±SD)	19,792±1,523*	31,597±1,633*
FRAP (µM QEAC±SD)	48,348±7,786*	122,261±8,348*

* The symbol indicates that there is a significant difference between the antioxidant capacity of the leaf and stem extracts of the plant (LSD, p-value <0.05, n=SD).

The antioxidant activity profiles of *Kalanchoe pinnata* leaf and stem extracts showed variations in quantification results between methods. The FRAP test produced the highest values among the methods tested. However, this difference cannot be directly interpreted as a preference for bioactive compounds in reducing Fe^{3+} over Cu^{2+}. Higher FRAP values likely reflect the sensitivity of the method, reaction conditions, and standards used in the test (Cerretani & Bendini, 2010; Wojtunik-Kulesza, 2020). Therefore, in developing a quantitative method, especially for antioxidants, it is hoped that the results will be interpreted with consideration of each analytical method's characteristics. This preferential reduction of iron ions parallels antioxidant mechanisms in vivo, particularly the Fenton reaction (Urbański & Beresewicz, 2000), in which hydrogen peroxide (H_2O_2), a metabolic by-product, reacts with Fe^{3+} to generate highly reactive radical species. By reducing Fe^{3+} to Fe^{2+}, antioxidant compounds can interrupt this process, thereby preventing lipid peroxidation and subsequent oxidative damage(Rabeta & Nur Faraniza, 2013a).

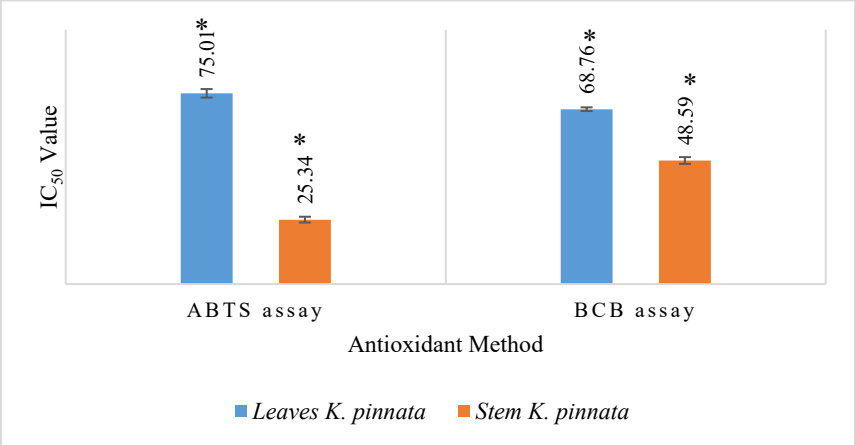

Figure 2. Antioxidant activity in scavenging ABTS radicals (A) and BCB assay (B) from ethanol extract of *K. pinnata* leaves and ethanol extract of *K. pinnata* stems

The antioxidant activity of *Kalanchoe pinnata* leaf (KPLE) and stem (KPSE) extracts was also determined for their ability to scavenge ABTS radicals and inhibit lipid peroxidation through

β-carotene bleaching (BCB). The results showed that the stem extract (KPSE) exhibited very strong antioxidant potential, with IC_{50} values < 50 µg/mL for both ABTS radical scavenging and lipid peroxidation inhibition. While, in the leaf extract (KPLE) showed strong activity, with IC_{50} values ranging from 50 to 100 µg/mL in both assays (Figure 2). The antioxidant efficacy of an extract is typically evaluated by its IC_{50} value, which denotes the concentration necessary to inhibit 50% of free radical activity. The classification employed adheres to (Molyneux (2004), categorizing activity as very strong (<50 µg/mL), strong (50-100 µg/mL), moderate (100-150 µg/mL), or weak (>150 µg/mL). This classification is a crucial reference for evaluating the biological potential of a natural ingredient. The test results indicated that KPSE possesses greater antioxidant potential than the leaf extract. In the ABTS assay, the stem exhibited an IC_{50} of 25.34 µg/mL, classified as very strong, whereas the leaf demonstrated an IC_{50} of 75.01 µg/mL, categorized as strong. In the interim, the BCB method revealed that the stems exhibited enhanced activity, with an IC_{50} of 48.59 µg/mL, whereas the leaves presented an IC_{50} of 68.76 µg/mL. Both methods consistently exhibited statistically significant differences between leaves and stems, thereby reinforcing the conclusion that stems possess enhanced antioxidant capacity.The observed differences in potency not only reflect biological variations between plant parts but are also closely related to the results of phytochemical screening. The KPSE were shown to contain higher concentrations of phenolics and flavonoids, two groups of secondary metabolites that play an important role in neutralizing free radicals. Furthermore, the group compounds of tannins and saponins in the stems also have a role to the detected antioxidant activity. Conversely, although the leaves also contain flavonoids and alkaloids, their phytochemical profile is relatively mild. This results in antioxidant activity that remains strong, although not as intense as that of the stems. Interestingly, despite the clear differences between the leaves and stems, both extracts still exhibited high antioxidant bioactivity, with activity ranging from strong to very strong. This finding was consistent even though the tests targeted different radical species: reactive nitrogen radicals (RNS) using the ABTS method and reactive oxygen radicals (ROS) using the BCB method (Pratama & Busman, 2020; Rabeta & Nur Faraniza, 2013b). These differences in radical targets confirm that the compounds in the leaf and stem extracts act through different mechanisms. Based on its chemical mechanism, the ABTS method works by means of electron transport, while the BCB method involves the transfer of hydrogen atoms (Munteanu & Apetrei, 2021). In all antioxidant activities carried out, the stem extract (KPSE) consistently shows a more potent antioxidant effect than the leaf extract (KPLE). This increased activity is consistent with the significantly higher levels of phenolics, flavonoids, and alkaloids in the stems. The correlation between phenolic and flavonoid content and antioxidant capacity strengthens the existing theory that hydroxyl groups in chemical compounds are able to donate electrons and hydrogen atoms to stabilize radicals. In addition, their metal-reducing activity toward Fe^{3+} and Cu^{2+} ions occur through redox reactions, whereby hydroxyl groups reduce Fe^{3+}/Cu^{2+} to Fe^{2+}/Cu^{+}, accompanied by oxidation of the antioxidant compound itself (Spiegel, 2023). This oxidation involves hydrogen release and electron transfer, ultimately stabilizing the antioxidant molecule and preventing further radical propagation (Aisyah et al., 2022; Nur, Aswad, Yulianti, et al., 2022).

Anti-elastase activity: The inhibition of elastase enzyme activity by KPLE and KPSE extracts was assessed to demonstrate the potential of anti-aging properties. An excessive concentration of elastase is closely correlated to the skin-aging process, will be facilitates the breakdown of elastin, a structural protein crucial for preserving skin elasticity and inhibiting wrinkle formation (Eun et al., 2020; Nur, Aisyah, et al., 2022, 2023; Nur, et al., 2023b; Tsuji et al., 2001). As shown in Figure 3, both extracts inhibited elastase activity in a dose-dependent manner, with higher concentrations producing greater levels of inhibition. The stem extract showed moderate inhibitory effect with an IC_{50} value of 243.94 ± 2.63 µg/mL, while the leaf extract showed weak inhibition with an IC_{50} value of 679.50 ± 5.71 µg/mL. Since a lower IC_{50} value reflects a stronger inhibitory activity, this finding confirms that the stem extract of *K. pinnata* has superior elastase inhibitory potential compared to the leaf extract.

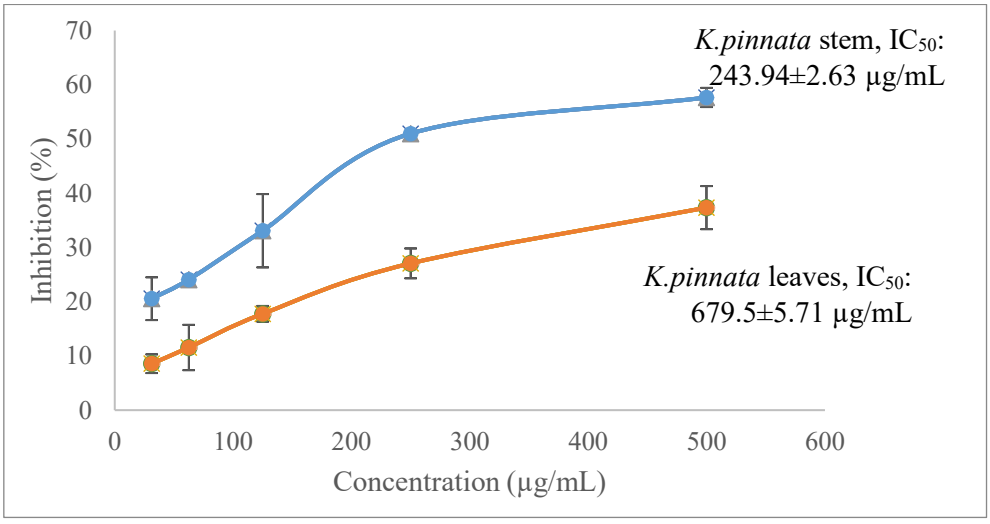

Figure 3. Anti-elastase effect of *Kalanchoe pinnata* leaf (KPLE) and stem (KPSE) extracts. The elastase inhibition assays were employed using extract concentrations of 3.125, 6.25, 12.5, 25, and 50 µg/mL. Data represent mean values ± standard deviation (SD) of triplicate measurements (n = 3)

Compounds with antioxidant properties are closely linked to their ability to inhibit elastase activity. Overexpression of elastase is one of the downstream consequences of reactive oxygen species (ROS) formation. Thus, it can be assumed that antioxidant compounds-particularly phenolic and flavonoid derivatives-have the potential to suppress elastase activity through two main mechanisms. First, indirectly by reducing oxidative stress levels, thereby reducing the availability of free radicals that can activate or enhance elastase activity. Second, directly through possible interactions with the enzyme's active site. This is in line with previous reports that flavonoids/phenolics can interact with serine or histidine catalytic residues in proteases, as well as with metalloenzyme centers that serve as cofactors (Xue et al., 2017). This hypothesis emphasizes that the Effectiveness of elastase inhibition by bioactive compounds depends not only on their general antioxidant capacity but also on the molecule's ability to interact specifically with the enzyme's catalytic components (Jakimiuk et al., 2021; Sianipar et al., 2023). Phenolic and flavonoid derivatives are capable of forming hydrogen bonds with targeted amino acid residues,

as well as engaging in ionic and van der Waals interactions with the metalloenzyme region, thereby reducing enzymatic activity. To further explore these relationships, principal component analysis (PCA) (Figure 4) was performed to visualize similarities and differences between the extracts and to examine associations among phytoconstituent levels, antioxidant activity, and elastase inhibition. In addition, heatmap correlation analysis (Figure 5) was conducted to provide a complementary overview of these interactions (Pinteus et al., 2017). The first two principal components accounted for 73.5% (PC1) and 26.5% (PC2) of the total variance, respectively. As illustrated in Figure 4, total phenolic, flavonoid, and alkaloid contents exhibited strong correlations with antioxidant activity across all assay methods, as well as with elastase inhibition in both extracts. Similarly, antioxidant activity data were strongly correlated with elastase inhibitory activity, suggesting that the same phytochemical constituents are likely responsible for both bioactivities.

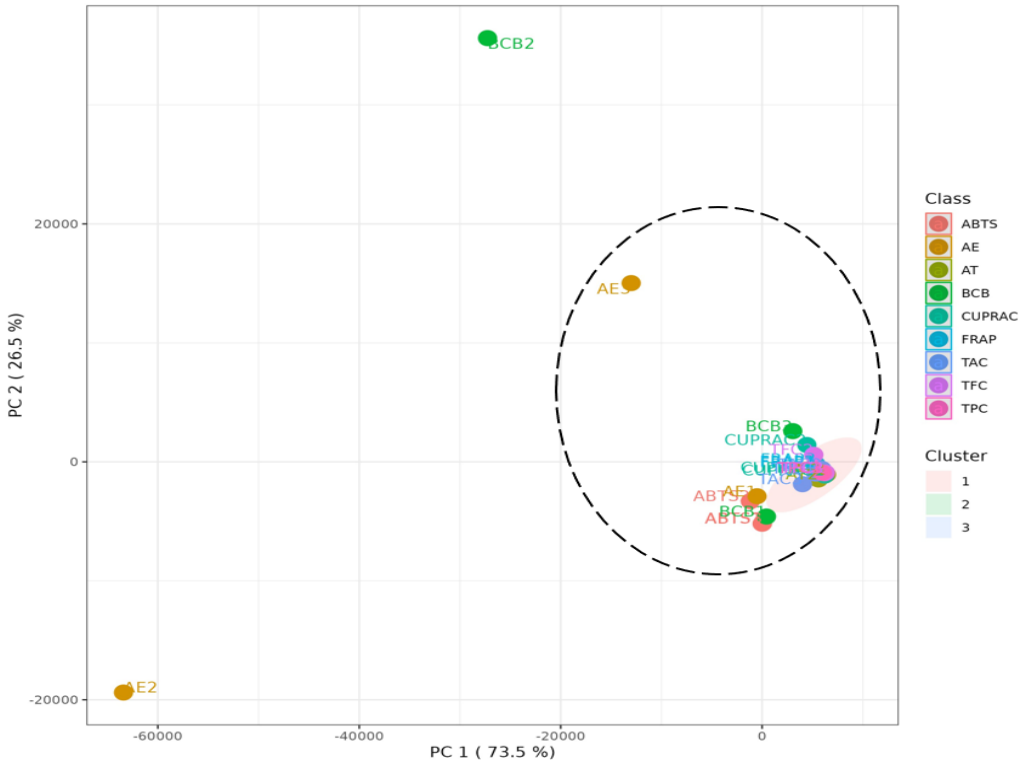

Figure 4. The principal component analysis (PCA) profile between the phytochemical identification, antioxidant, and anti-elastase effect in KPLE and KPSE extracts. The visualization showed that the first two principal components (PC1 and PC2) together explained 73.5% and 26.5% of the total variance, respectively. This means that they captured important trends in the dataset. The PCA biplot showed, the KPLE and KPSE extract were clearly grouped. This analysis also revealed how phytoconstituent levels (phenolics, flavonoids, and alkaloids) were related to antioxidant activity (TAC, FRAP, CUPRAC, ABTS, BCB) and elastase-inhibitory effects

Heatmap analysis (Figure 5) confirmed a clear correlation between the evaluated parameters. The phytoconstituent levels correlated with a consistent positive relationship between bioactive

compound content, antioxidant and anti-elastase activities in both extracts. KPSE extract, which has the highest levels of phenolics, flavonoids, and alkaloids, appeared to be closely associated with robust antioxidant capacity and elastase inhibition. On the other hand, the leaf extract (KPLE), with its lower phytoconstituent, exhibited reduced radical and elastase activities. These findings suggested that the importance of phytochemical richness-especially phenolics and flavonoids-as a key factor driving antioxidant potential and elastase inhibition.

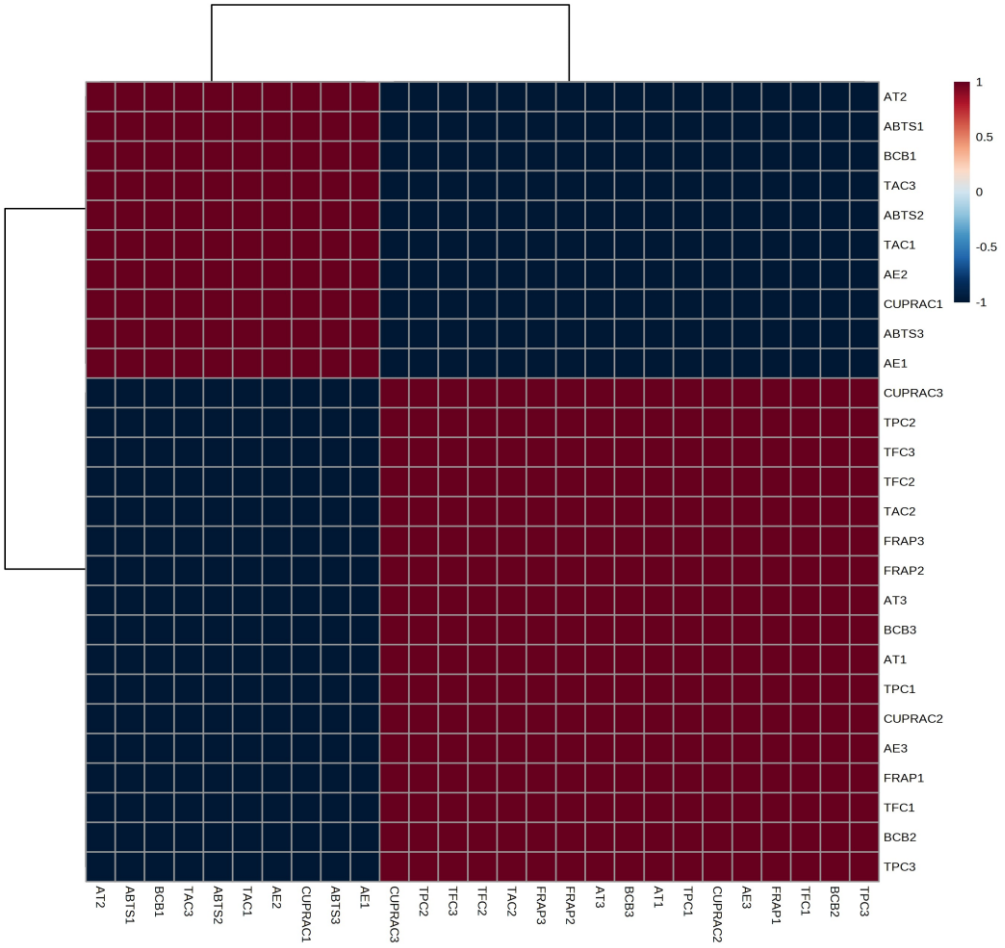

Figure 5. Heatmap analysis revealed a clear correlation between phytochemical composition, antioxidant activity, and anti-elastase activity in leaf (*Kalanchoe pinnata*; KPLE) and stem (KPSE) extracts. The color scale depicts the correlation coefficient from -1 (blue, strong negative correlation) to +1 (red, strong positive correlation). Hierarchical clustering revealed a correlation between phytoconstituent levels, such as total phenolics, flavonoids, and alkaloids, with various antioxidant assays (TAC, FRAP, CUPRAC, ABTS, BCB) and elastase inhibitory activity. A consistent positive correlation was found between phytochemical richness and both antioxidant and anti-elastase potential, with stem extracts showing the strongest association.

Conclusion: The ethanol extract of *Kalanchoe pinnata* stems (KPSE) showed higher levels of phenolics, flavonoids, and alkaloids than the leaf extract. This high concentration of

phytoconstituents is consistent with its strong antioxidant capacity, particularly in metal-reduction assays, as well as its significant ability to neutralize ABTS radicals and inhibit lipid peroxidation. Furthermore, the stem extract exhibited moderate, yet superior, elastase-inhibitory activity compared to the leaf extract. Overall, this study's results confirm that KPSE is a potential source of natural antioxidants, with possible applications as a raw material in anti-aging formulations.

Author Contributions

Conceptualization, F.J.S. and S.N.; methodology, F.J.S.; software, H.Z.; validation, F.J.S., H.Z. and S.N.; formal analysis, F.J.S.; investigation, F.J.S., M., and R.R.; resources, A.K.R. and S.N.; data curation, F.J.S. and M.; writing-original draft preparation, F.J.S.; writing-review and editing, H.Z., R.R., and S.N.; visualization, A.K.R.; supervision, S.N.; project administration, F.J.S. and H.Z.; funding acquisition, F.J.S and S.N. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Data available on request from the authors

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Conflict of Interest

The authors declare no conflict of interest.

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غنای فیتوشیمیایی و خواص ضدپیری گیاه *Kalanchoe pinnata*: بینش‌هایی حاصل از آزمون‌های آنتی‌اکسیدانی و مهار الاستاز

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چکیده

هدف: این مطالعه با هدف ارزیابی ترکیبات فیتوشیمیایی و فعالیت‌های زیستی عصاره‌های برگ و ساقه گیاه *Kalanchoe pinnata* انجام شد. اهداف اختصاصی شامل تعیین مقدار ترکیبات فنولی، فلاونوئیدی و آلکالوئیدی، بررسی توان آنتی‌اکسیدانی با استفاده از چندین آزمون *in vitro* و تعیین فعالیت ضدپیری از طریق مهار آنزیم الاستاز بود.

مواد و روش‌ها: برگ‌ها و ساقه‌های *K. pinnata* از استان سولاوسی جنوبی اندونزی جمع‌آوری، خشک، پودر و با استفاده از اتانول ۹۶٪ به روش ماسراسیون استخراج شدند. غربالگری فیتوشیمیایی با آزمون‌های کیفی استاندارد انجام گرفت و اندازه‌گیری کمی ترکیبات فنولی، فلاونوئیدی و آلکالوئیدی با روش‌های رنگ‌سنجی صورت پذیرفت. فعالیت آنتی‌اکسیدانی با استفاده از آزمون‌های ظرفیت کل آنتی‌اکسیدانی (TAC)، قدرت احیای آهن (FRAP)، ظرفیت احیای مس (CUPRAC)، مهار رادیکال ABTS و آزمون سفیدشدگی بتاکاروتن ارزیابی شد. فعالیت مهار الاستاز با استفاده از الاستاز پانکراس خوک و بستر-N-succinyl-(Ala-Ala-Ala)-p-nitroanilide تعیین گردید. تمامی آزمایش‌ها در سه تکرار انجام شد و داده‌ها با استفاده از آمار توصیفی، تحلیل همبستگی و تحلیل مؤلفه‌های اصلی (PCA) مورد بررسی قرار گرفتند.

نتایج: هر دو عصاره برگ (KPLE) و ساقه (KPSE) حاوی ترکیبات فنولی، فلاونوئیدی و آلکالوئیدی بودند؛ در حالی که ترپنوئیدها تنها در برگ‌ها و ساپونین‌ها فقط در ساقه‌ها شناسایی شدند. آنالیز کمی نشان داد که میزان ترکیبات فنولی (۳۶.۷۳ میلی‌گرم معادل اسید گالیک بر گرم)، فلاونوئیدی (۲۰/۰۶ میلی‌گرم معادل کوئرستین بر گرم) و آلکالوئیدی (۱۱/۴۲ میلی‌گرم معادل کینین بر گرم) در عصاره ساقه بیشتر از برگ بود. عصاره ساقه (KPSE) فعالیت آنتی‌اکسیدانی قوی‌تری نشان داد و مقادیر بالاتری از TAC (66.78 µM GAEAC/g)، FRAP (122.26 µM FeEAC/g) و CUPRAC (31.60 µM QEAC/g) را ثبت کرد و همچنین توان بالایی در مهار رادیکال ABTS و پراکسیداسیون لیپیدی) (IC_{50} کمتر از ۵۰ میکروگرم بر میلی‌لیتر) داشت. در مقابل، عصاره برگ (KPLE) ظرفیت آنتی‌اکسیدانی متوسطی (IC_{50} در بازه ۵۰ تا ۱۰۰ میکروگرم بر میلی‌لیتر) نشان داد. مهار الاستاز برای KPSE متوسط (IC_{50} = 44 µg/mL) و ضعیف برای KPLE (IC_{50} = 680 µg/mL) بود. تحلیل PCA و همبستگی، ارتباط قوی بین غنای فیتوشیمیایی و فعالیت‌های آنتی‌اکسیدانی و ضدالاستازی را تأیید کرد.

نتیجه‌گیری: عصاره ساقه *K. pinnata* در مقایسه با عصاره برگ، محتوای فیتوشیمیایی بالاتر، ظرفیت آنتی‌اکسیدانی قوی‌تر و مهار مؤثرتری بر الاستاز نشان داد. این نتایج، ساقه‌های *K. pinnata* را به‌عنوان منبع طبیعی امیدبخش ترکیبات آنتی‌اکسیدانی و ضدپیری معرفی می‌کند و از پتانسیل کاربرد آن‌ها در فرمولاسیون‌های آرایشی–بهداشتی گیاهی و فرآورده‌های درمانی حمایت می‌نماید.

کلمات کلیدی: آنتی‌اکسیدان، ترکیبات فیتوشیمیایی، ضدپیری، عصاره *Kalanchoe pinnata*، مهار الاستاز

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