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Effect of Ethanol Percentage on Phytochemical Constituent, Antioxidant Activity, and Dermatological Potential of *Cayratia trifolia* (L.) Domin

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ABSTRACT

This study investigated the effect of different ethanol concentrations [0% (i.e., water), 50% ethanol, 70% ethanol, and 99.5% pure ethanol] on their ability to extract total phenolic (TP) and total flavonoid (TF) compounds, as well as their antioxidant activities and dermatological potential from the leaves of *Cayratia trifolia* (L.) Domin. The dried leaves were macerated for 24 hours at room temperature, followed by filtration and concentration of the extracts. The TP and TF contents were quantified using the Folin–Ciocalteu and $AlCl_3$ methods, respectively. Antioxidant activity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) assays. Additionally, it evaluated the in vitro dermatological potential through tyrosinase and elastase inhibitory activities using colorimetric methods. The extract obtained with pure ethanol exhibited the most potent antioxidant activity, with IC_{50} values of 16.60 ± 0.62 µg/mL and 27.53 ± 0.69 µg/mL for the DPPH and FRAP assays, respectively. This extract also demonstrated the highest TP (3.82 ± 0.15 mg GAE/g DW) and TF (3.23 ± 0.09 mg QE/g DW) content. Furthermore, pure ethanol provided the highest extraction yield. However, the 70% ethanol extract emerged as a good source of tyrosinase (IC_{50}, 60.49 ± 7.73 µg/mL) and elastase inhibitors (IC_{50}, 45.49 ± 0.37 µg/mL). Overall, the results revealed that *C. trifolia* leaves are rich in polyphenols and flavonoids, demonstrating significant antioxidant activity when pure ethanol was used as the extraction solvent. Meanwhile, 70% ethanol showed the highest inhibitory activity against skin-related enzymes, tyrosinase and elastase. Future studies should aim to identify bioactive metabolites for use in cosmetic and medicinal formulations to address skin aging and hyperpigmentation.

Keywords: Antioxidant, *Cayratia trifolia* (L.) Domin, Elastase, Phytochemical, Tyrosinase

INTRODUCTION

The skin, the body's largest organ, is highly susceptible to oxidative stress, which has been linked to various cutaneous disorders. It serves as a continuous interface and protective barrier against chemical, physical, and biological invasions (Quan & Fisher, 2015). Maintaining cellular integrity and immune mechanisms, whether innate or adaptive, involves chemical reactions that generate reactive oxygen species (ROS). These highly reactive molecules can rapidly disrupt essential components of cutaneous homeostasis, such as proteins, lipids, and plasma membranes. Antioxidant systems, both endogenous and exogenous, play a critical role in neutralizing these reactive chemicals (Addor, 2017; Checa & Aran, 2020; Chen *et al*., 2021).

Antioxidants are compounds that work synergistically to neutralize ROS, protecting cells and tissues from oxidative damage. The cutaneous antioxidant system consists of enzymatic components (e.g., superoxide dismutase (SOD) and glutathione peroxidase) and non-enzymatic compounds (e.g., vitamins C and E, and β-carotene) (Ighodaro & Akinloye, 2018). While hydroxyl radicals ($HO^{\bullet}$) and superoxide ($O^{\bullet}{}_2^-$) are the most

prevalent ROS, other forms include peroxyl and alkoxyl radicals ($RO_2^{\bullet}$ and $RO^{\bullet}$), singlet oxygen, hydrogen peroxide (H_2O_2), and organic peroxides (ROOH) (Collin, 2019). Upon exposure to photoaging stimuli, ROS accumulation can indirectly activate dermal enzymes such as collagenase and elastase, which degrade collagen and elastin, leading to skin damage (Jiratchayamaethasakul *et al.*, 2020). Additionally, melanin plays a role in maintaining tissue homeostasis under such conditions (Dumbuya *et al.*, 2020).

Melanin, a black or brown pigment produced via the melanogenesis pathway in melanocytes, is essential for determining the color of the skin, hair, and eyes. It enhances the skin's protective barrier against environmental stresses such as ultraviolet (UV) radiation and hormonal factors, including cytokines. Tyrosinase, a key enzyme in melanin biosynthesis, serves as a rate-limiting step in the pathway (Solano, 2020). However, unregulated or excessive melanin production can result in skin conditions such as freckles, melasma, age spots, sun spots, and post-inflammatory hyperpigmentation, leading to visible imperfections and premature aging. Natural antioxidants have shown promise as anti-tyrosinase agents, offering skin protection while preventing dermatological diseases and disorders (Skoczyńska *et al*., 2017). Current research continues to explore safe and effective natural compounds with antioxidant and dermatological potential.

Cayratia trifolia (L.) Domin, known locally as galing-galing, belongs to the Vitaceae family and is native to India, Asia, and Australia. In Indonesia, it has been traditionally used for treating headaches, boils, wound healing, muscle pain, and dandruff (Fajrin *et al.,* 2015; Mangiwa *et al*., 2023). Preliminary phytochemical screening indicates that the entire plant contains yellow waxy oil, steroids/terpenoids, flavonoids, and tannins. Its leaves are rich in stilbenes (e.g., piceid, resveratrol, viniferin, and ampelopsin), while the stems, leaves, and roots contain hydrocyanic acid, delphinidin, and various flavonoids such as cyanidin. Additional compounds such as kaempferol, myricetin, quercetin, triterpenes, and epifriedelanol have also been identified in the plant (Kumar *et al*., 2011). Ethanol and chloroform fractions of *C. trifolia* have demonstrated antioxidant and anticancer properties, respectively (Yunus *et al*., 2021). Meganathan *et al*. reported a dose-dependent DPPH scavenging activity of *C. trifolia* extract, with a strong IC_{50} value of 19.86 ± 0.21 µg/mL (Meganathan *et al*., 2021).

Plant antioxidants can be extracted using various methods, including maceration, digestion, subcritical water extraction, and ultrasound-assisted extraction. Solvent choice significantly affects biological activities such as antioxidant and enzyme inhibition due to potential synergistic or antagonistic interactions among metabolites. Polar solvents, particularly aqueous mixtures of ethanol, methanol, acetone, and ethyl acetate, are commonly used for recovering polyphenols and flavonoids from plant matrices (Pawar *et al*., 2020; Rangasamy *et al*., 2019; Samydurai & Saradha, 2016). Among these, ethanol is widely recognized as an excellent solvent for polyphenol extraction and is safe for consumption (Xu *et al*., 2017). This study aimed to investigate the effects of varying ethanol concentrations on the extraction yield, total phenolics (TP), total flavonoids (TF), and the antioxidant and dermatological properties of *C. trifolia* extracts.

MATERIAL AND METHODS

Aluminum chloride, DPPH, elastase (from porcine pancreas), ethanol, ferricyanide–ferric chloride, Folin-Ciocalteu reagent, phosphate-buffered saline (PBS), sodium acetate, tyrosinase (from mushrooms), L-tyrosine, and water were obtained from Merck Indonesia. All chemicals and reagents used in this experiment were of analytical grade.

Identification of plant material

The leaves of *Cayratia trifolia* were collected from the Kampili Dam area in February 2017, during the rainy season, in Pallangga, Gowa, South Sulawesi, Indonesia. The plants were taxonomically identified by the Indonesian Institute of Sciences (Voucher No. 1541/IPH.1.01/If.07/VI/2017). The collected leaves were processed by removing branches and twigs, washing them under running tap water, cutting them into small pieces, and drying them at 40°C for 72 hours. The dried leaves were then milled and used for further research.

Preparation of plant extracts

The influence of different solvents—0% (water), 50% ethanol, 70% ethanol, and 99.5% (pure) ethanol-on extraction efficiency was evaluated through comparative research. One gram of plant material was mixed with each solvent at a sample-to-solvent ratio of 1:20 (w/v) and left to macerate for 24 hours at room temperature with

occasional stirring. The resulting extracts were filtered using a vacuum filter, evaporated at 60°C using a rotary evaporator (Buchi, Germany), freeze-dried for 24 hours, and stored at 4°C until further analysis. The extraction yield (%) was calculated as follows:

$$\text{Yield} = \frac{\text{dry weight of leaves}}{\text{weight of extract}} \times 100 \ldots\ldots\ldots\ldots(1)$$

Total phenolic (TP)

The modified Folin–Ciocalteu method, as described by Guemari *et al*. (2020), was used to determine the total phenolic (TP) content. One milliliter of each extract (10 mg/mL) was mixed with 1.0 mL of 10% Folin–Ciocalteu reagent, followed by the addition of 3.0 mL of 2% Na_2CO_3. The mixture was incubated at room temperature for 60 minutes, and the absorbance was measured at 765 nm using an 8453 UV-Vis spectrophotometer (Agilent, USA). A calibration curve for gallic acid (2 - 10 µg/mL) was prepared to quantify the TP content. The results were expressed as gallic acid equivalents (GAE) per gram of dry weight (DW). The calibration curve equation was as follows y = 0.0707 × 0.1174, with R^2 = 0.9959.

Total flavonoid (TF)

Total flavonoid (TF) content was determined using the modified aluminum chloride colorimetric method described by Amina *et al*. (2020). Briefly, 1 mL of each extract (10 mg/mL) was mixed with 1 mL of 5% $AlCl_3$ and 1 mL of 1 M sodium acetate solution. The mixture was incubated at room temperature for 15 minutes, and the absorbance was measured at 415 nm using an 8453 UV-Vis spectrophotometer (Agilent, USA). Quercetin was used as the reference standard, and TF content was expressed as milligrams of quercetin equivalent (QE) per gram of dry weight (DW). The standard curve's equation was as follows y = 0.0167 × + 0.1554, with R^2 = 0.9864.

Antioxidant activities assay

DPPH-free radical scavenging

The antioxidant activity of *C. trifolia* leaf extracts was assessed using the DPPH free radical scavenging assay, as described by Pakki *et al*. and Potbhare & Khobragade with slight modifications (Pakki *et al*., 2020; Potbhare & Khobragade, 2017). The extracts were diluted to serial concentrations ranging from 10 to 100 µg/mL. One milliliter of each dilution was mixed with 1 mL of 0.004 M DPPH dissolved in ethanol and 3 mL of ethanol, then incubated for 30 minutes at 37°C. Vitamin C, at five different concentrations, was used as the positive control. The reaction was monitored by measuring absorbance at 515 nm using an 8453 UV-Vis spectrophotometer (Agilent, USA). Absolute ethanol was used as the negative control. The DPPH scavenging activity was calculated as follows:

$$\text{DPPH scavenging activity} = \frac{Ao - Aa}{Ao} \text{x } 100 \ldots\ldots\ldots(2)$$

Where A_0 represents the absorbance of the negative control (0.004 M DPPH), A_a represents the absorbance of the extract-containing sample. Plotting the percentage of inhibition against the log concentration of the sample yields the concentration required for a 50% inhibition of DPPH (IC_{50}).

FRAP assay

The ferric-reducing antioxidant power (FRAP) of *C. trifolia* leaf extracts was determined using the potassium ferricyanide–ferric chloride method, as described by Guediri *et al*. with some modifications (Guediri *et al*., 2021). The antioxidant capacity of the extracts was compared to that of vitamin C. A 100 µL aliquot of each extract was added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 minutes, followed by the addition of 2.5 mL of 10% trichloroacetic acid. After standing for 30 minutes, the absorbance of the solution was measured at 593 nm.

Dermatological potential assay

Tyrosinase inhibitory assay

The ability of the extracts to inhibit tyrosinase activity was determined following the method reported by Indrisari *et al*. (2021). In a 96-well plate, the following reagents were mixed: 50 µL of 0.1 M sodium phosphate buffer (pH 6.8), 50 µL of each sample, 10 µL of tyrosinase solution (1500 units/mL) in phosphate buffer, and 40 µL of 1.5 mM L-tyrosine. After incubating the mixture for 12 minutes at 37°C, the reaction was stopped by placing the plate on ice for 1 minute. The absorbance was measured at 490 nm using a microplate reader (BioTek Instruments ELx808, USA). Kojic acid was used as the reference in this assay.

Elastase inhibitory effect assay

Elastase inhibition was determined using the colorimetric method described by Jiratchayamaethasakul *et al.* (2020) with slight modifications. The elastase substrate was mixed

with 10 µL of the sample in a 96-well plate and preincubated at 25°C for 10 minutes. To initiate the reaction, 10 µL of elastase from porcine pancreas (7.5 units/mL) in tris buffer solution was added. The absorbance of the final mixture was measured at 410 nm using a microplate reader (BioTek Instruments ELx808, USA). Epigallocatechin gallate was used as the positive control.

Statistical analysis

Triplicates were used for all yield, phenolic, flavonoid, antioxidant (using various assays), and dermatological activity tests. The mean and standard deviation (SD) were calculated for each sample. Data comparisons were performed using a one-way analysis of variance (ANOVA), followed by a post-hoc Tukey's honest significant difference (HSD) test for pairwise comparisons. A p-value of < 0.05 was considered statistically significant.

RESULT AND DISCUSSION

Natural products have served as alternatives for preventing and treating human ailments for thousands of years and continue to play a significant role in drug development. Bioactive natural compounds are typically present in modest concentrations in natural therapies. Therefore, it is crucial to develop effective and selective methods for extracting and isolating these bioactive compounds (Janghel *et al*., 2015; Kaur & Gupta, 2017; Zhang *et al*., 2018).

Extraction is the first step in isolating the desired natural products from raw plant materials. Extraction methods include solvent extraction, distillation, pressing, and sublimation, with solvent extraction being the most widely used (Bahadur *et al*., 2016; Zhang *et al*., 2018). In this study, water, 50% ethanol, 70% ethanol, and pure ethanol were used to extract compounds from plant samples. The results showed a significant difference in extraction yields when different solvents were used. Pure ethanol produced the highest extraction yield, followed by 70% ethanol, 50% ethanol, and water (Table 1). This suggests that highly polar solvents are less efficient at extracting compounds compared to semi-polar solvents. Yunus *et al*. (2021) reported an ethanol extract yield of 24.52% from *C. trifolia*, which was higher than the 32.90±0.42% obtained in this study.

The variation in extraction yields can be attributed to differences in the polarity of the solvents, which influence the extraction efficiency of bioactive compounds. Pure ethanol extracts yielded a higher amount of material compared to water, 50% ethanol, and 70% ethanol, indicating that semi-polar solvents are more efficient. This finding aligns with studies on the extraction yield of propolis (Sun *et al*., 2015) and other medicinal plants (Truong *et al*., 2019). This could be due to the higher solubility of semi-polar compounds in solvents like ethanol.

Further analysis was conducted to better understand the effect of solvent polarity on the concentration of bioactive compounds. The concentration of total phenolics (TP) and total flavonoids (TF) in the extracts varied in relation to the extraction yields. The data in Table 1 shows that pure ethanol was the most efficient solvent for extracting phenolic and flavonoid compounds. Pure ethanol extracted 3.82±0.15 mg GAE/g DW of phenolics and 3.23±0.09 mg QE/g DW of flavonoids. In contrast, water extracts contained significantly lower levels of phenolic and flavonoid compounds. These results suggest that phenolics and flavonoids are more soluble in pure ethanol than in other solvents. This supports the conclusion that pure ethanol is the optimal solvent for extracting bioactive compounds from *C. trifolia* leaves.

A previous study by Hikmawanti *et al*. (2021) reported a TF content of 27.95±0.62 mg QE/g in *C. trifolia* leaves cultivated in Tuban, Indonesia, further validating the bioactive potential of this plant.

Extraction solvents significantly influence extraction yield, bioactive chemical content, and the biological activity of the extract (Truong *et al*., 2019). This study evaluated the antioxidant activity of extracts obtained from various solvents using the DPPH and FRAP assays. The DPPH method measures the ability of antioxidants to donate electrons, while the FRAP method assesses antioxidants based on their ability to reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions through electron donation (Baliyan *et al*., 2022).

The antioxidant activities were tested at final concentrations of 10, 50, and 100 µg/mL for both assays. The DPPH assay revealed that all extracts exhibited antioxidant activity to varying degrees, with values ranging from 7.34±0.05% to 37.10±1.12% for water extract, 12.26±1.63% to 51.25±1.69% for 50% ethanol, 23.08±1.31% to 72.13±8.52% for 70% ethanol, and 35.24±1.54% to 95.76±1.38% for pure ethanol (Figure 1A). Among the extracts tested, pure ethanol extract demonstrated the strongest antioxidant activity.

Table I. Effect of different solvents on yield, TP, and TF of leaves of *C. trifolia* extracts.

Solvent	Extraction yield (%)	TP (mg GAE/g DW)	TF (mg QE/g DW)
Water	6.40±0.14	2.12±0.09	0.99±0.04
50% ethanol	9.75±0.07	2.79±0.24	1.2±0.02
70% ethanol	17.95±0.78	3.07±0.07	2.68±0.09
Pure ethanol	32.90±0.42*	3.82±0.15*	3.23±0.09*

Note: *Characters represent significant differences from all groups at $p<0.05$ by one-way ANOVA.

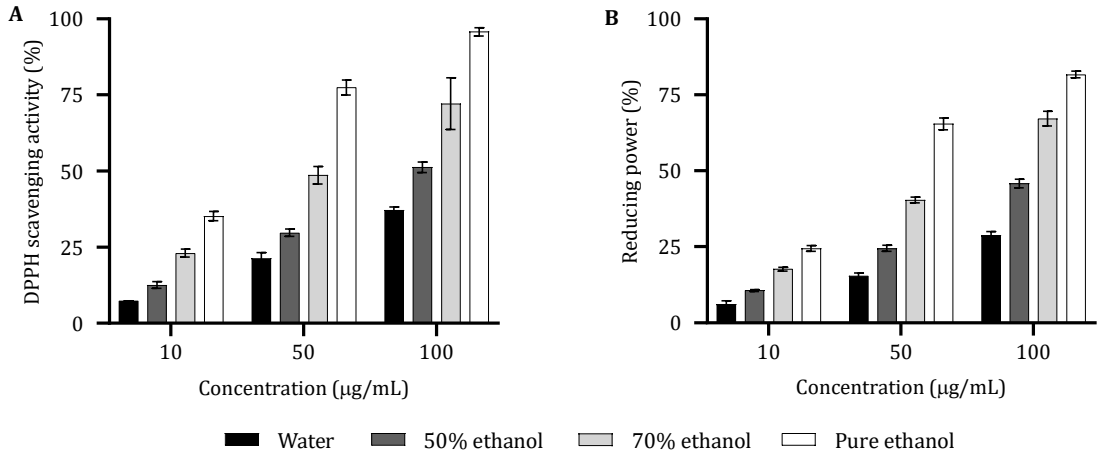

Figure 1. Antioxidant activity of different extracts of leaves of *C. trifolia*: (A) DPPH scavenging activity; (B) FRAP.

A similar trend was observed in the FRAP assay, with all extracts exhibiting reducing power in the following order: pure ethanol > 70% ethanol > 50% ethanol > water (Fig. 1B). The reducing power ranged from 6.02±1.19% to 81.65±1.11%, with the highest activity observed in the pure ethanol extract and the lowest in the water extract. These results suggest that the TP and TF components in *C. trifolia* leaves contribute to its antioxidant activity.

The pure ethanol extract was the most potent among all the extracts tested, as indicated by its IC_{50} values in the DPPH and FRAP assays. This is likely due to its high concentration of total phenolics (TP) and total flavonoids (TF), which are known for their strong antioxidant properties. These compounds protect the human body from oxidative damage by scavenging various reactive oxygen species (ROS), both internally and externally (Collin, 2019; Ighodaro & Akinloye, 2018). These findings suggest that the pure ethanol extract of *C. trifolia* leaves could be a promising antioxidant for future applications.

The skin, which serves as the body's protective barrier, is vulnerable to damage when unprotected, leading to issues like wrinkles, decreased elasticity, uneven pigmentation, and increased roughness and dryness. Plants containing natural antioxidant compounds with anti-aging properties are thus valuable in cosmetic formulations. Both TP and TF have antioxidant effects and are associated with various biological processes, including the inhibition of aging-related enzymes, such as tyrosinase and elastase, in the skin (Indrisari *et al*., 2021; Muslimin *et al.*, 2023).

As seen in Fig. 2A, the dried leaf extract obtained with 70% ethanol exhibited significant anti-tyrosinase activity, outperforming extracts made with water, 50% ethanol, and pure ethanol. This suggests that an increased water ratio does not enhance anti-tyrosinase activity. A similar trend was observed for anti-elastase activity (Figure 2B), with the 70% ethanol extract demonstrating the strongest inhibitory effect against elastase.

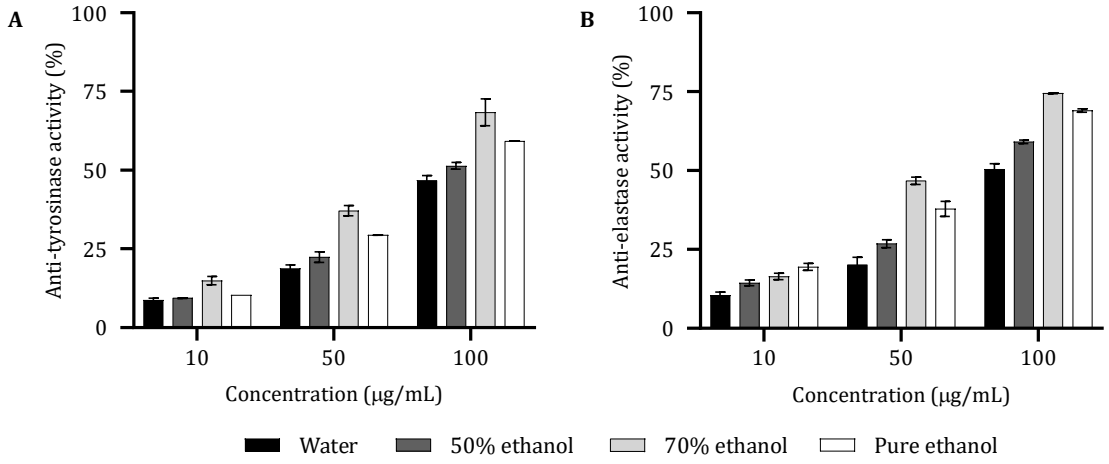

Figure 2. Dermatological activity of different extracts of leaves of *C. trifolia*: (A) anti-tyrosinase activity; (B) anti-elastase activity.

Table II. Half maximal inhibitory concentration (IC_{50}) for DPPH scavenging activity, reducing power, anti-tyrosinase, and anti-elastase of various extracts obtained from leaves of *C. trifolia*

Solvent	IC_{50} (µg/mL)			
	DPPH	FRAP	Anti-tyrosinase	Anti-elastase
Water	218.42±23.92	429.66±69.94	153.88±10.30	136.95±17.74
50% ethanol	111.61±11.12	151.32±13.08	121.30±6.30	92.15±2.31
70% ethanol	42.46±7.14	57.19±2.81	60.49±7.73*	45.49±0.37*
Pure ethanol	16.60±0.62*	27.53±0.69*	85.29±0.15	56.37±1.60
Vitamin C	5.03±0.2	7.27±0.9		
Kojic acid	-	-	17.46±2.16	-
Epigallocatechin gallate	-	-	-	25.94±2.16

Note: *Characters represent significant differences from all groups at $p<0.05$ by one-way ANOVA

These results imply that the TP and TF components in *C. trifolia* leaves may not significantly contribute to anti-tyrosinase and anti-elastase activities. However, the present study indicates that extraction with 70% ethanol is the optimal method for obtaining these bioactive compounds, based on their IC_{50} values. Additionally, an in vitro experiment previously showed that *C. trifolia* leaf extract inhibited epidermal growth factor protein kinase and tyrosine-specific protein kinase (Kumar *et al.*, 2011). This study also reveals for the first time that this plant has elastase-inhibiting properties.

The half-maximum inhibitory concentration (IC_{50}) measures a substance's ability to inhibit a biological or metabolic function. Varieties with the lowest IC_{50} values exhibit the highest biological activity. A sample with an IC_{50} < 50 µg/mL is considered a very strong antioxidant, 50-100 µg/mL is strong, 101-150 µg/mL is medium, and >150 µg/mL is weak in activity (Fidrianny *et al.*, 2018). All extracts generally demonstrated biological activity in the DPPH scavenging, reducing power, anti-tyrosinase, and anti-elastase assays (Table II). The water and 50% ethanol extracts exhibited IC_{50} values above 100 µg/mL (medium activity) for all tests, except the 50% ethanol extract in the anti-elastase assay (strong activity). Both the 50% ethanol and pure ethanol extracts showed strong activity in the anti-tyrosinase assay. However, 70% ethanol performed better in anti-tyrosinase, and anti-elastase activities compared to pure ethanol, although this contradicts the observed antioxidant activities. Both 70% ethanol

and pure ethanol extracts exhibited strong antioxidant activity in the DPPH assay, with pure ethanol being categorized as having very strong activity in the FRAP assay. Overall, all biological activities were still lower than those of the positive controls.

The highest antioxidant activity in the pure ethanol extract is correlated with its higher phenolic and flavonoid content. During reactions with radical compounds, the hydroxyl groups on phenolics and flavonoids donate hydrogen atoms through an electron transfer mechanism, effectively inhibiting oxidation (Platzer *et al.*, 2022).

Positive controls serve as benchmarks to measure the inhibitory strength of potential inhibitors. Kojic acid, a well-known depigmenting agent and tyrosinase inhibitor, has been shown to be effective in reducing melanogenesis in cultured B16F10 cells (Wang *et al*., 2022). Additionally, recent studies have reported that various phenolic compounds, such as catechin and epigallocatechin gallate, also act as elastase inhibitors. Lee *et al.* and Shirzad *et al*. used epigallocatechin gallate as a positive control in anti-elastase activity tests (Lee *et al.*, 2023; Shirzad *et al.*, 2018).

On the other hand, the anti-tyrosinase and anti-elastase activities of the 70% ethanol extract do not correlate with the concentrations of TP and TF in the extract. Several studies have shown that enzyme inhibition can also be attributed to other compounds, such as alkaloids and saponins, in addition to phenolics and flavonoids (Arianti & Elya, 2020; Cespedes *et al.*, 2017; Sun *et al.*, 2017; Yu *et al.*, 2020). These findings suggest that the 70% ethanol extract from the leaves of *C. trifolia* is the most potent and a promising source of skin protection agents. According to Chen *et al*., plant extracts obtained using different solvents can exhibit varying effects. For instance, a coffee pulp extract obtained with 70% ethanol showed the highest tyrosinase inhibition compared to those extracted with water, 50%, and pure ethanol (Chen *et al*., 2021).

Both antioxidant and enzyme inhibition tests rely on colorimetric methods. At high concentrations, the color of the extract can interfere with the color change in the final reaction result, which is a limitation of this study.

CONCLUSION

We concluded that the extract of *C. trifolia* leaves exhibited notable antioxidant activity and dermatological protection. Pure ethanol was the most effective solvent for extraction, yielding the highest phenolic (3.82±0.15 mg GAE/g DW) and flavonoid content (3.23±0.09 mg QE/g DW), along with strong antioxidant activity (DPPH, IC_{50} 16.60±0.62 µg/mL; FRAP, IC_{50} 27.53±0.69 µg/mL). However, 70% ethanol was the preferred solvent for enzyme inhibition, showing potent tyrosinase (IC_{50} 60.49±7.73 µg/mL) and elastase (IC_{50} 45.49±0.37 µg/mL) inhibition. Based on these results, we recommend using *C. trifolia* leaves as an ingredient in functional cosmetic products, particularly for anti-wrinkle and skin whitening applications, to enhance protection against UV rays. Further research is needed to explore the potential of this plant in cancer chemotherapeutics and advanced cosmetic formulations.

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