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Research Article

Comparative analysis of vitamin E extraction methods in *Cleome viscosa* L. and the role of key biosynthesis genes

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Abstract
Vitamin E is a lipid-soluble, potent radical-scavenging antioxidant that inhibits lipid peroxidation, which damages cell membranes, proteins, and DNA in the human body. Currently, it is widely utilized in dietary supplements and cosmetic products and is primarily sourced from plant-derived oils. However, *Cleome viscosa* L. (Asian spider flower) an ethnobotanical species that is not as widely recognized, could provide a different natural source of tocopherols, especially in the early stages of seed development. The present study aimed to evaluate which extraction methods produced the highest vitamin E contents by comparing different extraction techniques. A method was developed in which different solvents and extraction durations were tested to analyze the vitamin E and antioxidant contents in *Cleome viscosa* L. seed from 2-5 weeks after fruit set (WAF). Two methods were used, the Soxhlet method and maceration. The highest amount of gamma-tocopherol was present at 5 WAF and a concentration of 1946.78 µg/100 g fresh weight with an IC_{50} value of 32.02 ± 2.02 µg/ml using the Soxhlet method with ethanol for 6 hours. To gain information on the relationship between vitamin E content and the expression of four genes (*VTE*1-4) involved in vitamin E biosynthesis was examined. The higher expression of the *VTE*1 and *VTE*4 genes from the early seed stage to the mature seed stage. Furthermore, the maximum amount of alpha-tocopherol, 898.44 µg/100 g fresh weight, with an IC_{50} value of 7.46 ± 2.20 µg/ml, was produced by the maceration method using ethanol solution at 3 WAF, and the *VTE*4 gene was more highly expressed compared to other tocopherol biosynthesis genes.

Keywords: Antioxidant activity, *Cleome viscosa* L., Extraction methods, Gene expression, Vitamin E

INTRODUCTION

Vitamin E is a fat-soluble nutrient that does not break down in warm and acidic conditions, but it is sensitive to alkaline conditions. The dietary nutrients of vitamin E act as an antioxidant and prevent lipid peroxidation, which is damaging to membranes, protein, and DNA in the human body (Rychter *et al*., 2022). Vitamin E is a nutrient that most humans need to consume frequently with age-specific daily recommendations: 4 mg/day for infants (0–6 months), 5 mg/day for 7–12 months, 6 mg/day for 1–3 years, 7 mg/day for 4–8 years, 11 mg/day for 9–13 years, and 15 mg/day from age 14 years. Pregnant women require 15 mg/day, while lactating women need 19 mg/day (Rizvi *et al*., 2014). Recent interest has grown in the use of nutraceuticals, including vitamin E, for managing metabolic syndromes, metabolic bone disorders, and both communicable and noncommunicable diseases. This emerging field reflects the advantages of nutraceuticals as cost-effective, easily consumed agents with demonstrated antioxidant, anti-inflammatory, and neuroprotective effects in both animal and human studies (Ara *et al*., 2025). Vitamin E, primarily sourced from vegetable oils, is widely used in cosmetic formulations for its antioxidant and photoprotective properties. It enhances skin health by reducing oxidative stress and improving hydration, making it a key ingredient in many cosmetic products. The growing integration of vitamin E in both nutraceuticals and cosmetics reflects the broader trend of utilizing bioactive compounds to support health and prevent age or lifestyle-related damage (Budzianowska

et al., 2025).

Vitamin E is primarily found in oils extracted from plants, including soybean, olive, sunflower, and corn oil. Interestingly, many varieties of disregarded weeds actually contain significant quantities of oil. *Cleome viscosa* L., commonly known as the Asian spider flower, is predominantly found in shrublands, forests, grasslands, paddy fields, and sugarcane plantations. It has been confirmed to contain high levels of vitamin E, specifically around 0.318 mg per 100 g fresh weight, as noted by Pillai and Nair (2013).

There are several methods for extracting vitamin E from plants, many of which involve traditional extraction techniques. Some examples include the pressurized liquid extraction (PLE) from grape seed oil (Dos Santos Freitas *et al*., 2008), microextraction from coffee beans (Alves *et al*., 2009), PLE from palm oil (Maarasyid *et al*., 2014), Soxhlet extraction from pink powder puff seeds (Irabor *et al*., 2020). Various techniques, including Soxhlet extraction, ultrasonic extraction, saponification, and direct extraction, were employed in a comparison of vitamin E extraction from genetically modified and germinated soybean seeds. It was found that the seeds containing the highest level of vitamin E were extracted using Soxhlet extraction, while the germinated seeds had the highest vitamin E levels with direct extraction (Lee *et al*., 2012). Similarly, in marigold flowers, ultrasound assisted extraction (UAE) achieved higher yields and polyphenol content, whereas Soxhlet remained advantageous for extracting lipophilic pigments such as carotenoids and tocopherols, highlighting the potential of plant matrices as both nutritional ingredients and natural food colorants (Núñez *et al*., 2025).

The present study aimed to investigate various techniques for extracting vitamin E from *Cleome viscosa* L. at different stages of seed development (2, 3, 4, and 5 weeks after fruit set). The present study also investigated the relationship between vitamin E content and the expression of four genes involved in vitamin E biosynthesis: tocopherol cyclase (*VTE*1), homogentisic acid prenyltransferase (*VTE*2), gamma-tocopherolmethyltransferase (*VTE*4), and MPBQ methyltransferase (*VTE*3), as well as their correlations with vitamin E content.

MATERIALS AND METHODS

Plant material

Cleome viscosa L. seeds were collected and separated from the pods and categorized into four stages, 2-5 weeks after fruit set (WAF). All seeds were air-dried at room temperature for 7 days.

Extraction methods

Soxhlet

Soxhlet extraction method was carried out using a Soxhlet apparatus (Gerhardt, Germany) with three types of pure solvents: methanol (RCI-Labscan, Ireland), ethanol (Merck, Germany), and acetonitrile (RCI-Labscan, Ireland). The Soxhlet extraction method was modified from the procedure described by Gopalasatheeskumar (2018). Five grams of the sample were ground using 300 mL of different solvents (methanol, ethanol, and acetonitrile). The extraction was performed for 6 and 24 hours. The solvent was removed by rotary evaporator (Relona, Germany) at 50 °C. The extracted samples were maintained at -20 °C for further analysis.

Maceration

The sample (5 g) was ground and submerged in 100 mL of solvent (methanol, ethanol, and acetonitrile) at room temperature. The mixture was allowed to macerate for 7 days, and after that, it was filtered to remove the solid residue. The solvent was removed by rotary evaporator (Relona, Germany). The extraction samples were maintained at -20 °C for further analysis (Ćujić *et al*., 2016).

Antioxidant assay

Antioxidant activity was determined by 2,2-diphenyl-1-picryl hydrazyl (DPPH). The DPPH antioxidant solution was prepared by dissolving DPPH (Sigma-Aldrich, St. Louis, MO, USA) in methanol. A 100 µL of sample solution was added to 100 µL of buffer solution onto a 96-well microplate and allowed to stand in the dark for 30 min (Baliyan *et al*., 2022). The absorbance of the mixture was measured using a UV-Vis spectrometer (BioTek Synergy H1, Aligent). DPPH radical scavenging activity was calculated using the following equation (Prieto, 2012):

$$\%\ \text{of antioxidant activity} = \frac{(\text{A contol} - \text{A sample})}{\text{A control}} \times 100 \quad \text{Eq. 1}$$

A control = Absorbance of the DPPH solution without the sample

A sample = Absorbance of the DPPH solution after adding the sample

Each sample was analyzed in triplicate (n = 3), and the results were expressed as the mean ± standard deviation (SD).

High performance liquid chromatography (HPLC) analysis

Extraction samples were prepared at a concentration of 250 mg/µL in methanol. The standard solution of tocopherol (Sigma-Aldrich, St. Louis, MO, USA) in methanol was prepared 1, 5, 10, 50, 100, 250, 500, and 1000 µg/mL to create a standard curve. The mobile phase used was a mixture of acetonitrile and methanol (98:2) in an isocratic system, with a flow rate of 1 mL/min and a wavelength of 265 nm. The sample solution (20 µL) was directly injected into a Shimadzu chromatograph consisting of an LC-20 AT pump coupled to an SPD-20A UV-VIS detector, reverse phase column Luna 5µm C18 (2) 100 Å Phenomenex (250 mm × 4.6 mm). The standard curve was constructed using a standard solution of α-, γ-, and δ-tocopherol and was used for quantification. Tocopherol (α-, γ-, δ-) is expressed as micrograms per 100 grams of fresh weight (µg/100 g FW). Tocopherol (α-, γ-, δ-) (Sigma-Aldrich, St. Louis, MO, USA) was of HPLC grade. This RP-HPLC method was adapted and modified based on the approach de-

scribed by Mateeva *et al*. (2023) for simultaneous analysis of fat-soluble vitamins.

Semi-quantitative PCR analysis

Total RNA was isolated from seeds that were harvested 2-5 WAF with LiCl method (Verwoerd *et al*., 1989). RNA was converted to cDNA using BIO-RAD iScript™ cDNA Synthesis Kit. The qRT-PCR was conducted using the SensiFAST SYBR® No-ROX mix (Thermo fisher) on Bio-Rad Launches CFX Duet Real-Time PCR System. The qRT primers (*VTE*1, *VTE*2, *VTE*3, and *VTE*4) were employed from the study of Jai-Uean and Sangin (2021), and actin was used as the reference gene. Relative expression levels were calculated using the $_2$^-ΔΔCt method. All qRT-PCR reactions were performed in triplicate.

Statistical analysis

Expression data were statistically analyzed by the LSD one-way ANOVA test using R Statistical Software (v4.12; R Core Team, 2021). The agricolae R package (de Mendiburu & Yaseen, 2020) was used for analysis to compare the differences between treatments, where all data were normalized, and a significant difference at $P \leq 0.05$ was found.

RESULTS AND DISCUSSION

Seed classification

Cleome viscosa L. seeds were separated into 4 stages from 2-5 weeks after fruit set (Fig. 1). At 2 WAF, seeds were small, round, and green, in an early development stage and still immature. Stage 3 WAF seeds were darker green and slightly larger, indicating early maturation, but they remained in the drying process. Stage 4 WAF seeds were mostly brown, hardened, and significantly dried, suggesting they were highly developed. Stage 5 WAF, seeds were dark brown to black, indicating the maturity of capsule dehiscence.

Tocopherol content

Tocopherols, the main forms of vitamin E, are natural fat-soluble antioxidants. The quantification of tocopherols in *Cleome viscosa* L. seeds were assessed using two different extraction methods: Soxhlet and maceration with acetonitrile, methanol, and ethanol as solvents. Tocopherols were identified by comparing their retention times to those of standard tocopherols (δ, γ, and α), which were about 13.347, 16.004 and 18.626 minutes, respectively (Fig. 2).

The results of the maceration extraction process using acetonitrile showed that the concentration of α-tocopherol increased slightly during the 2-3 WAF, followed by a subsequent decrease at 4 WAF, and then peaked at 5 WAF. In contrast, δ-tocopherol showed the highest concentration at the early stage (2 WAF). After that, the concentration gradually dropped over time, and γ-tocopherol was not detected at any stage (Fig. 3). Ethanol extraction revealed that α-tocopherol levels were of low concentration in 2 WAF. However, 3 WAF showed a significant increase, with the concentration peaking at 898.44 µg/100 g of fresh weight (µg/100 g FW). The highest concentration of γ-tocopherol was detected at 550.85 µg/100 g FW in 2WAF, and γ-tocopherol was not detected in 3-4 WAF. At all stages, no detection of δ-tocopherol was observed (Fig. 4).

The results of a 6-hour Soxhlet extraction with acetonitrile, methanol, and ethanol as solvents demonstrated that the extraction efficiency varied significantly depending on the solvent used (Fig. 5). It was found that the efficiency of acetonitrile extraction in separate tocopherols from the samples was limited. To be more precise, 14.10 µg/100 g FW of δ-tocopherol was present, while α-tocopherol was undetected. Despite the low overall yields, γ-tocopherol was detected at 89.77 µg/100 g FW, suggesting relatively higher extraction efficiency for this isoform than δ-tocopherol. On the other hand, δ-tocopherol was extracted at a significant concentration of 742 µg/100 g FW from methanol, which was demonstrated as a highly selective solvent. However, the absence of α- and γ-tocopherol indicated the low efficiency of this solvent. A moderate level of extraction efficiency was indicated by the concentration of α-tocopherol, which was 309.25 µg/100 g FW with ethanol. The extraction of γ-tocopherol yielded an incredibly high concentration of 1946.78 µg/100 g fresh weight, indicating that ethanol is especially suitable for isolating this isoform. Consistently, analysis of the unsaponifiable fractions of hemp seed oil (HSO) from extra-European and Italian sources indicated γ-tocopherol as the predominant vitamin E isoform, with α-tocopherol represented as a minor component (Blasi *et al*., 2022). Similarly, the extraction of vitamin E from flaxseed showed the same pattern, with γ-tocopherol predominant over α-tocopherol (Gundev *et al*., 2019).

Interestingly, when the extraction duration was extended to 24 hours, the only solvent that could effectively

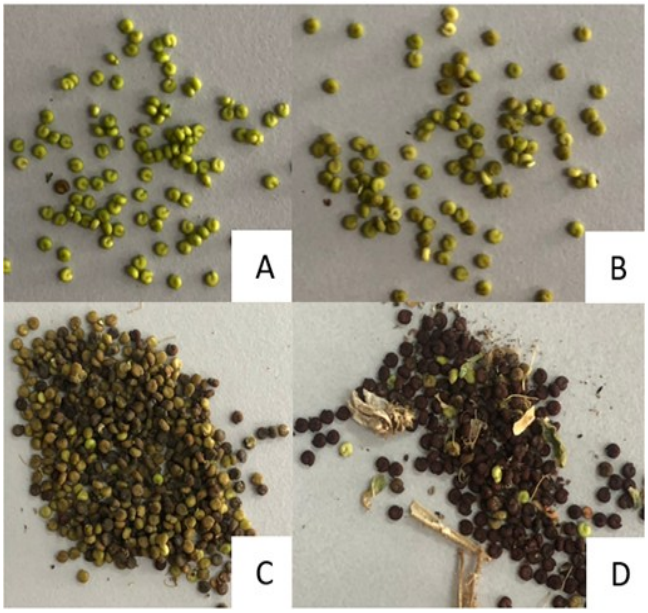


Fig. 1. *Cleome viscosa* L. *seeds at different stages: A = 2 weeks after fruit set (WAF), B = 3 WAF, C = 4 WAF, and D = 5 WAF*

Table 1. Tocopherol contents and IC_{50} of *Cleome viscosa* seed extract with different extraction techniques

Method	Solvent	Stage (WAF)	Vitamin E Composition (µg/100 g FW)			Total	IC_{50} (µg/ml)
			α-tocopherol	δ-tocopherol	γ-tocopherol		
Soxhlet Extraction 24 hr.	Acetonitrile	**5**	39.96	108.21	35.86	184.03	121.42 ±2.26
	Methanol		ND	ND	ND	-	153.10 ± 1.80
	Ethanol		ND	ND	18.57	18.57	51.16 ± 3.80
Soxhlet Extraction 6 hr.	Acetonitrile	5	ND	14.10	89.77	103.87	106.09±2.32
	Methanol		ND	742	ND	742	38.59±4.42
	Ethanol		309.25	ND	1946.78	2256.03	32.02±2.20
Maceration 7 days	Acetonitrile	5	363.58	106.58	ND	470.16	78.25±1.10
	Methanol		ND	ND	ND	-	116.68±1.31
	Ethanol		9	ND	10.89	19.89	17.91±1.91
	Acetonitrile	2	116	302.92	ND	418.92	30.26±2.04
		3	117.03	205.21	ND	322.24	415.67±5.55
		4	72.77	122.37	ND	195.14	307.96±2.42
		5	363.58	106.58	ND	470.16	78.25±1.10
	Ethanol	2	29.21	ND	550.85	580.06	21.48±1.21
		3	898.44	ND	ND	898.44	7.46±2.20
		4	ND	ND	ND	-	76.79±3.65
		5	9	ND	10.89	19.89	17.91±1.91

* ND = not detected

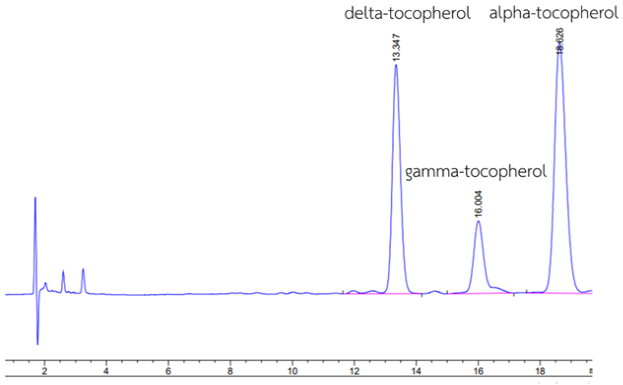
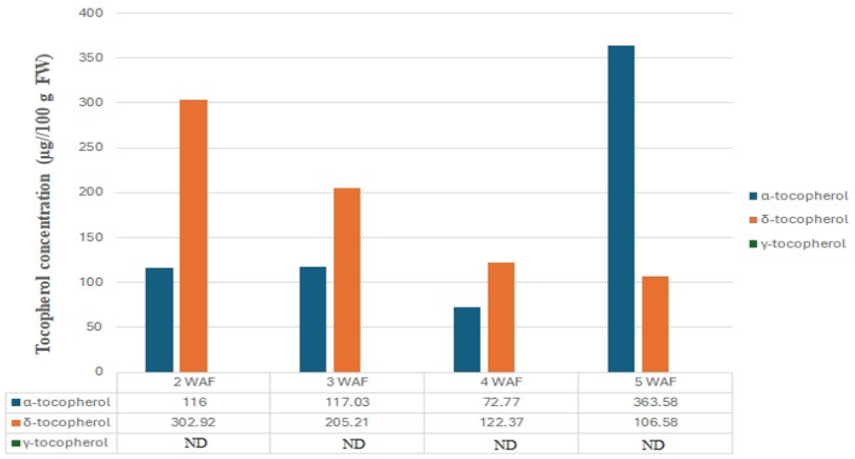


Fig. 2. *High performance liquid chromatography (HPLC) chromatogram of a mixture of tocopherols*

extract all three tocopherol isoforms was acetonitrile. In contrast, the extraction efficiency of ethanol was limited as it only produced 18.57 µg/100 g FW of γ-tocopherol, with α- and δ-tocopherol remaining undetected (Fig. 6). The drop in ethanol's efficiency over longer durations could be explained by oxidation or thermal instability of tocopherols during prolonged solvent exposure (Liu *et al*., 2025). The persistence of γ-tocopherol suggests its relatively higher stability under these conditions.

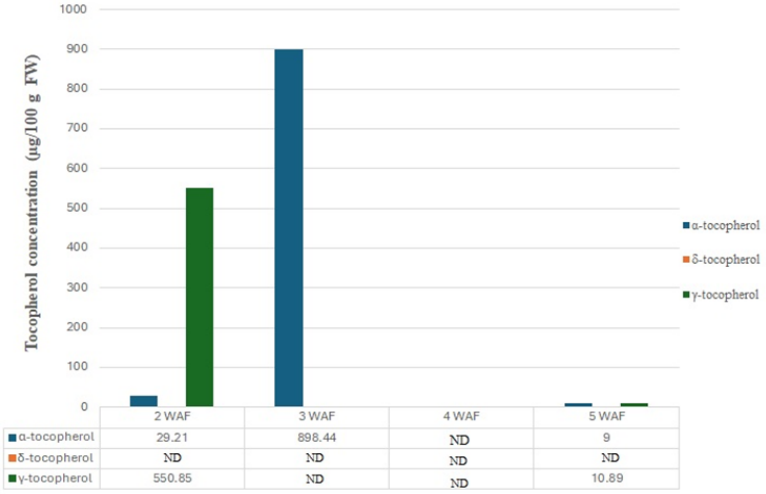
The Soxhlet extraction with ethanol for 6 hours at 5 WAF proved to be the most effective method for extracting total tocopherol content, yielding 2256.03 µg/100 g FW, compared to other extraction methods. The methanol Soxhlet extraction and maceration extraction obtained the lowest values. However, the concentration of tocopherols in both methods varied with the solvent used for the extraction. The ethanol Soxhlet extraction method revealed a high content of γ-tocopherol (1946.78 µg/100 g FW). Similarly, ethanol maceration for 7 days showed a higher concentration of α-tocopherol at 3 WAF. As vitamin E is polar, ethanol or other polar solvents can be used for its extraction. These results are in agreement with those reported by Tourabi *et al*. (2025), which show that solvent polarity and extraction parameters play a critical role in the recovery of bioactive compounds from *Mentha longifolia* (L) Huds extracts. Their optimization study concluded that ethanol enhanced the recovery of phenolics and antioxidant compounds due to its ability to disrupt plant cell walls and solubilize polar constituents efficiently. However, a higher extraction temperature in the Soxhlet method reduced the γ-tocopherol content of palm oil in both oil extraction and seed drying (Wang *et al*., 2010). Similarly, it resulted in lower tocopherol levels in *Cnidoscolus quercifolius* Pohl (faveleira) seed oil compared to cold pressing, most likely due to heat-induced degradation (Ribeiro *et al*., 2019).

The analysis of various vegetable oils using high-performance liquid chromatography (HPLC) revealed differences in tocopherol contents. Rapeseed oil had an α-tocopherol content of 120.3±4.2 and a γ-tocopherol content of 122.0±7.9 mg/kg. Sunflower oil had an α-tocopherol content of 432.3±86.6 and γ-tocopherol at 92.3±9.5 mg/kg. Corn oil had α-tocopherol at 173.0±82.3 and γ-tocopherol at 259.7±43.8 mg/kg. Soybean oil had α-tocopherol at 71.3±6.4 and γ-tocopherol at 273.3±11.1 mg/kg. γ-tocopherol is generally more abundant than α-tocopherol in most seed oils (Grilo *et al*., 2014). Researchers attribute this to the important role of γ-tocopherol as an antioxidant in plants, particularly in protecting plant cells from oxidative stress. This function is crucial for seeds, which need to preserve energy and nutrients for seedling growth. High levels of γ-tocopherol in seeds contribute to their resilience to environmental fluctuations (Abbasi *et al*., 2007). However, the present study found that α-tocopherol was the



	2 WAF	3 WAF	4 WAF	5 WAF
α-tocopherol	116	117.03	72.77	363.58
δ-tocopherol	302.92	205.21	122.37	106.58
γ-tocopherol	ND	ND	ND	ND

Fig. 3. *Tocopherol contents at the 2-5-WAF were extracted using the maceration method for 7 days with acetonitrile as the solvent. (ND = not detected)*



	2 WAF	3 WAF	4 WAF	5 WAF
α-tocopherol	29.21	898.44	ND	9
δ-tocopherol	ND	ND	ND	ND
γ-tocopherol	550.85	ND	ND	10.89

Fig. 4. *Tocopherol content at the 2-5-WAF was extracted using the maceration method for 7 days with ethanol as the solvent (ND = not detected)*

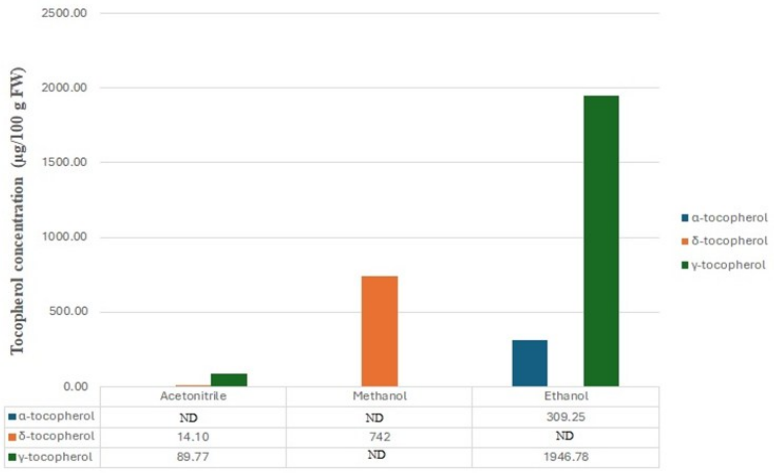
most abundant compound in the maceration extracts, particularly at 3 WAF, suggesting that this method might work with particular effectiveness for isolating α-tocopherol during the early stages of seed development. In contrast, γ-tocopherol was predominantly detected at 5 WAF using the Soxhlet method, indicating that this technique favors the recovery of γ-tocopherol during seed maturation.

Antioxidant assay

Antioxidant activity tests of vitamin E seed extraction were performed using the DPPH free radical inhibition assay (Table 1). A measure used to determine antioxidant activity was the IC50, the concentration of antioxidant at which 50% of DPPH free radical activity was inhibited (Jumina *et al*., 2019). IC50 values ranged from 7.46 ± 2.20 to 415.67 ± 5.55 µg/ml. The extract with the best antioxidant activity, as indicated by the lowest level of IC50 (7.46 ± 2.20 µg/ml), was obtained from maceration with ethanol for 7 days, which also contained the highest concentration of α-tocopherol. In contrast, Soxhlet extraction with ethanol for 24 hours resulted in a low concentration of total tocopherol, and the IC50 value was high (32.02±2.20 µg/mL). This may be attributed to thermal degradation of tocopherols, which are known to be heat-sensitive compounds. Previous studies reported a significant decrease in tocopherol content in oils subjected to prolonged heating at high temperatures (Kmiecik *et al*., 2019). Moreover, the variation in antioxidant activity across extraction methods may reflect differences in tocopherol profiles. Extracts enriched in α-tocopherol tend to demonstrate greater radical scavenging activity than those dominated by δ-tocotrienols and γ-tocopherol. Therefore, selecting the appropriate extraction method and plant part is essential for optimizing the antioxidant potential (Hisatomi *et al*., 2000; Górnaś *et al*., 2025).

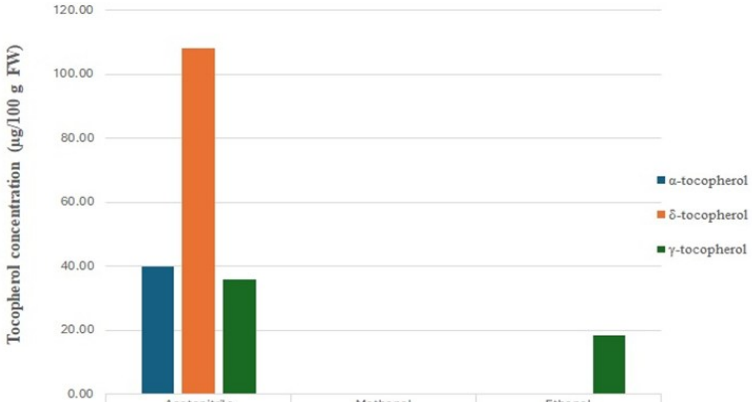
Relationship between the biosynthesis of the vitamin E gene expressions and tocopherol content

The expression levels of genes involved in the biosynthesis of vitamin E enzymes in *Cleome viscosa* L.,



	Acetonitrile	Methanol	Ethanol
α-tocopherol	ND	ND	309.25
δ-tocopherol	14.10	742	ND
γ-tocopherol	89.77	ND	1946.78

Fig. 5. *Tocopherol content at the 5-WAF was extracted using the Soxhlet method for 6 hours with acetonitrile, methanol, and ethanol as solvents. (ND = not detected)*



	Acetonitrile	Methanol	Ethanol
α-tocopherol	39.96	ND	ND
δ-tocopherol	108.21	ND	ND
γ-tocopherol	35.86	ND	18.57

Fig. 6. *Tocopherol content at the 5-WAF was extracted using the Soxhlet method for 24 hours with acetonitrile, methanol and ethanol as solvents. (ND = not detected)*

VTE1, VTE2, VTE3, and VTE4, were analyzed in seeds at 2-5 WAF (Fig. 7). *VTE*1, *VTE*2, and *VTE*3 showed an increasing trend as the seeds matured. However, the highest expression was observed at 4 WAF. This suggests a coordinated upregulation of the early biosynthetic steps involved in γ-tocopherol formation during mid-to-late seed development. In contrast, *VTE*4 had similarly high expression levels throughout all stages with 5 WAF consistently showing higher expression. The high expression levels of *VTE*1-4 at 4-5 WAF may be crucial for the accumulation of tocopherol in the seeds. Previous studies have reported similar patterns, indicating that increased expression of *VTE*2 is associated with oil storage seeds, such as soybeans (Karunanandaa *et al*., 2005). Moreover, mature seeds exhibit lower expression levels than green seeds 3-4 weeks after pollination (Chao et al., 2014), leading to higher tocopherol accumulation.

In the present study, higher levels of γ-tocopherol, compared to other forms of vitamin E, were found to correlate with significant expression of the genes VTE1 and VTE4 in mature seeds, indicating a tendency toward upregulation in these seeds. Extraction by maceration in ethanol also revealed the highest γ-tocopherol levels in seeds at the second week after flowering (2 WAF). This finding is consistent with the highest α-tocopherol levels observed in seeds at the third stage (3 WAF), as γ-tocopherol serves as a precursor for the conversion of α-tocopherol by gamma-tocopherol methyltransferase (*VTE*4) (Guo *et al*., 2022). Similarly, the overexpression of *VTE*4 from *Perilla frutescens* (L) Britton under a seed-specific promoter in soybean resulted in enhanced conversion of γ-tocopherol to α-tocopherol, leading to increased α-tocopherol content in transgenic seeds (Arun *et al*., 2014).

Conclusion

This is the first report on the extraction and characterization of vitamin E from *Cleome viscosa* L. seeds. The study found that the Soxhlet method, using ethanol for 6 hours, was suitable for extracting γ-tocopherol, whereas γ-tocopherol was undetectable when methanol was used. On the other hand, the maceration method using ethanol for 7 days was more suitable for extracting vitamin E in the form of α-tocopherol, whereas

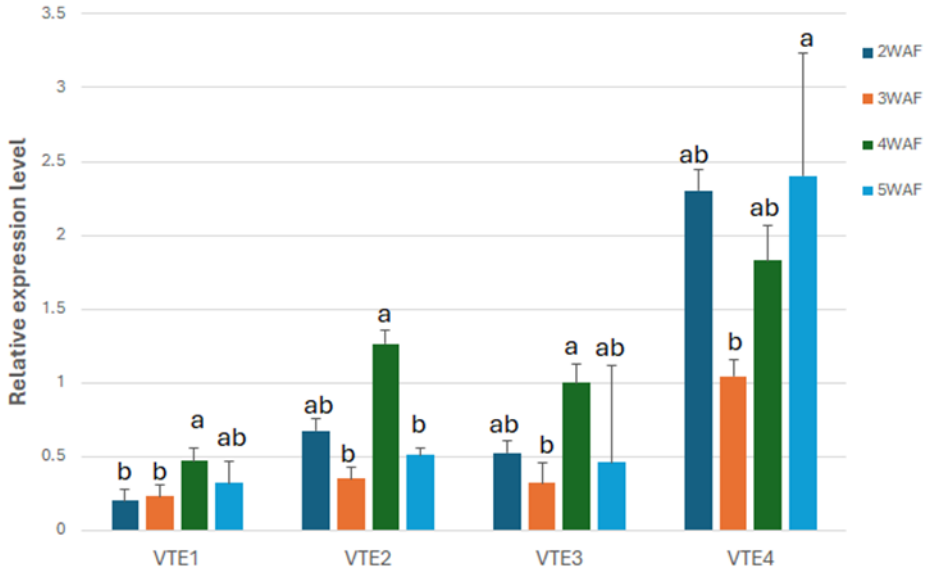


Fig. 7. *Expression levels of VTE1–VTE4 genes across developmental stages (2-4 WAF). Statistical significance was determined by one-way ANOVA followed by LSD post hoc test (α = 0.05). Different letters indicate significant differences among stages for each gene.*

using other solvents, α-tocopherol was undetectable. The present study demonstrated that α-, δ-, and γ-tocopherol present in *Cleome viscosa* L. seed extract varied depending on the solvent and extraction method used. Moreover, the *VTE*1 and *VTE*4 genes were highly expressed during seed development, correlating with the accumulation of vitamin E. Consequently, *Cleome viscosa* L. has the potential to be used as a vitamin E in a wide range of products, including dietary supplements, antioxidant compounds, skin care formulations and natural preservatives.

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Conflict of interest

The authors declare that they have no conflict of interest.

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