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Research article

Development of a serum for melasma treatment based on the biological activities of *Euphorbia thymifolia* L. extract

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Abstract: *Euphorbia thymifolia* Linn is a medicinal plant widely used in traditional Asian medicine due to its bioactive constituents, which exhibit antioxidant, antimicrobial, antifungal, anticancer, and especially, antityrosinase activities. The aim of this study was to assess the antioxidant capacity, antityrosinase activity, and melanin inhibition potential of *E. thymifolia* L. extract and a serum prototype containing 2% of the extract. Antioxidant activity was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) radical scavenging capacity, and ferric reducing antioxidant power (FRAP) assays. Melanin production inhibition and cytotoxicity of the extract and serum were investigated using the B16-F10 mouse melanoma cell line. The *E. thymifolia* L. extract scavenged free radicals, with half-maximal inhibitory concentration (IC_{50}) values of 63.50 µg/mL in the DPPH assay and 168.55 µg/mL in the ABTS assay, and concentration at absorbance 0.5 ($Abs_{0.5}$) values of 90.99 µg/mL in the FRAP assay. Both the extract and the serum showed significant antityrosinase activity, with IC_{50} values of 3.92 and 14.07 µg/mL, respectively. Moreover, the extract and serum inhibited melanin production in

B16-F10 melanoma cells by 26.35% and 27.23%, respectively, at a concentration of 20 μg/mL, with relatively low cytotoxicity (46.33% and 46.87%, respectively) even at the high concentration of 100 μg/mL. The developed serum had a pH of 6.17 and high uniformity and was nontoxic to melanocytes. These promising results concerning the beneficial activities of the *E. thymifolia* extract and serum containing the extract suggest that this medicinal plant may be a potential source for the development of pharmaceutical and cosmetic products.

Keywords: *Euphorbia thymifolia* Linn; antioxidant; antityrosinase; melanin inhibition; serum

1. Introduction

Herbal medicines include herbal materials, herbal preparations, and products that contain plant materials or their combinations as bioactive ingredients. Currently, more than 80% of the global population prefers to use natural health and beauty care products as a first option before turning to modern medical treatments [1]. Among the various aspects of beauty, skin quality is crucial, and achieving flawless skin has become a universal aspiration. Healthy skin is typically characterized by an even tone, the absence of melasma and dark spots, adequate hydration, and a natural rosy glow. These attributes are regulated by skin cells through antioxidant processes and the inhibition of tyrosinase activity, which effectively reduces melanin production in pigment cells [2–4]. Tyrosinase, an enzyme present in living organisms, catalyzes melanin biosynthesis from tyrosine, causing undesirable browning in the melanocytes of the skin through a process called melanogenesis [5–7]. Melanin contributes to skin, hair, and fur pigmentation in humans and animals but can also lead to several conditions, such as Parkinson's disease, premature hair graying, skin pigmentation disorders, and cancer [8–10]. Melanin also plays a crucial role in protecting the skin from UV radiation, and melanin production has been linked to mitigating UV-induced skin damage [11]. The frequent exposure of human skin to UV radiation is recognized as a potential cause of skin cancer [12,13]. Thus, melanin biosynthesis can be inhibited by minimizing UV exposure, tyrosinase inhibitor supplementation, or promoting melanin removal [14–16].

Serums are formulations that contain concentrated active ingredients and are characterized by excellent skin absorption properties [17]. Owing to their effectiveness and high level of customer satisfaction, serums are widely utilized in cosmetic and cosmeceutical products. However, antioxidants commonly used in the cosmetic industry, such as propyl gallate, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), have raised concerns because of their potential to induce liver damage and carcinogenicity [17,18]. Consequently, there is an urgent need to identify safer, natural alternatives to these chemical agents. Notably, there is great potential for the development of skin care products supplemented with natural extracts, as studies on serum formulations supplemented with natural bioactive compounds have shown that these compounds enhance skin conditions by increasing the degree of complexion and reducing the appearance of wrinkles [17–19].

Euphorbia thymifolia Linn is a small herb from the Euphorbiaceae family known in Vietnam as small-leaved milk grass [20]. In traditional medicine, whole plants, leaves, and seeds have been used to treat intestinal and skin diseases because they exhibit various therapeutic properties, such as wound healing promotion and antimicrobial, antifungal, and antioxidant activities [21,22]. *E.*

thymifolia L. is commonly found in Kien Giang Province, Vietnam, which is often referred to as the country's National Medicinal Garden, where the climate and soil conditions are harsh. Plants growing in regions with challenging environmental conditions have to adapt to stress by activating defense mechanisms that initiate complex biochemical processes. Stress-induced responses at the gene or protein level can result in substantial alterations to a plant's metabolite composition [23]. Thus, the aim of this study was to investigate the antioxidant and antityrosinase activities of the extract, along with its melanin-inhibitory effects on the B16-F10 mouse melanoma cell line. Additionally, the efficacy and toxicity of the serum formulation containing the extract were evaluated in melanocyte lines. These findings provide a basis for further clinical studies, paving the way for the development of cosmetic products that target melasma skin care, with the goal of introducing these products to the consumer market.

2. Materials and methods

2.1. Chemicals

Analytical-grade chemicals, including anhydrous Na_2SO_4, $FeCl_3 \cdot 6H_2O$, $Pb(CH_3COO)_2$, Na_2CO_3, K_2HPO_4, CH_3COOK, I_2, KI, $AlCl_3 \cdot 6H_2O$, $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$ 30%, NaOH, concentrated H_2SO_4, and 99% ethanol, were purchased from chemical companies in Vietnam.

Other reagents, including 2,2-diphenyl-1-picrylhydrazyl (DPPH; 99%), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid ($ABTS^{\bullet+}$; 98%), gallic acid (99%), tyrosinase enzyme, levodopa (L-DOPA) (99%), and kojic acid (99%), were purchased from Sigma (USA), while Dulbecco's modified Eagle's medium (DMEM) and Eagle's minimum essential medium (EMEM) supplemented with L-glutamine, sodium pyruvate, $NaHCO_3$, penicillin/streptomycin, 10% fetal bovine serum (FBS), trypsin-EDTA (0.05%), trichloroacetic acid (TCA), dimethyl sulfoxide (DMSO), Tris base, phosphate-buffered saline (PBS), and sulforhodamine B (SRB) were purchased from Sigma (USA) and Merck (Darmstadt, Germany).

2.2. Plant material and extraction process

Sample collection and extraction were performed according to Khuu (2023) [20] and Chintong (2019) [24], with slight modifications. Mature *E. thymifolia* samples (roots, stems, leaves, and flowers) were collected from the Mekong Delta region of Vietnam. All the samples were collected from 8 am to 10 am in the summer. *E. thymifolia* samples were then washed with tap water, thoroughly drained, and dried at temperatures ranging from 40 to 47 °C to a moisture content of less than 13%. Afterward, the dried *E. thymifolia* was ground into powder, tightly packed in plastic bags, frozen, and stored at −18℃ until use.

To obtain the extract, the dried powder was soaked in ethanol at a ratio of 1:4 (g/mL) for 72 h with ultrasonic assistance (120 W) using an S100H ultrasonic bath (ELMA, Germany) during the first hour. The liquid extract was then obtained by filtration, and the residue was extracted three more times to maximize compound recovery. The solvent from the extracts was removed under vacuum using an RE 301 rotary evaporator (Biobase, Shandong, China) before analysis.

2.3. Analytical methods

2.3.1. Antioxidant activity assays

DPPH free-radical scavenging assay: The extracts at concentrations ranging from 0 to 125 μg/mL were added to a DPPH solution prepared in methanol (0.1 mM) and sodium acetate buffer (pH 5.5) [25]. The DPPH radical scavenging activity was calculated as a percentage of inhibition on the basis of the absorbance at 517 nm of the solution after the reaction, considering the absorbance of the blank using a spectrophotometer (Shimadzu 1800, Japan).

Ferric reducing antioxidant power (FRAP) assay: This experiment was performed as described by Sharma et al. (2014) [21]. The *E. thymifolia* ethanol extract was tested at concentrations ranging from 0 to 100 μg/mL. Ascorbic acid solutions (2–12 μg/mL) were used as standards, and the absorbance of each standard and test solution was measured at 700 nm.

ABTS radical scavenging assay: Solutions of the *E. thymifolia* extract at concentrations of 0, 100, 300, 500, 600, and 800 μg/mL were treated with ABTS solution for 60 min, after which the absorbance of each solution was measured at 734 nm. Solutions of gallic acid in ethanol at final concentrations of 0–3.5 μg/mL were used as positive controls. The antioxidant activity of the *E. thymifolia* ethanol extract is expressed as an IC_{50} value determined from the linear regression equation on the basis of an antioxidant content equivalent to that of gallic acid (μg/mL) [26].

2.3.2. Tyrosinase inhibition assay

This experiment was carried out on the basis of the methods reported by Khuu (2023) [20] and Chintong et al. (2019) [24] with modifications. The extract was first dissolved in DMSO to generate a 2 mg/mL stock solution, which was subsequently diluted in phosphate buffer (pH 6.8) to obtain working extract concentrations of 0, 4.375, 8.75, 17.5, 35, and 70 μg/mL; working solutions of the serum were prepared at concentrations of 0, 1.25, 2.5, 5, 10, and 20 μg/mL. Kojic acid was used as a positive control at concentrations of 2, 4, 6, 8, 10, and 12 μg/mL.

The tyrosinase enzyme and L-DOPA were diluted in phosphate buffer (pH 6.8) to final concentrations of 250 U/mL and 1 mg/mL, respectively. The total reaction volume was maintained at 1 mL, comprising 25 μL of tyrosinase and 200 μL of extract, and each sample was incubated for 15 min at 37°C. Then, 50 μL of L-DOPA was added, and the mixture was incubated for an additional 15 minutes at 37°C. The absorbance of the solution at 475 nm was measured using a spectrophotometer (Shimadzu 1800, Japan).

Tyrosinase inhibition was calculated according to the following formula:

$$Tyrosinase\ inhibition(\%) = \frac{(A-B)-(C-D)}{(A-B)} \times 100.$$

Where, A: absorbance of the reaction solution containing the enzyme and substrate but not the extract.
B: absorbance of the substrate solution without the extract or enzyme.
C: absorbance of the reaction solution containing the enzyme, substrate, and extract.
D: absorbance of the reaction solution containing extract and substrate but not the enzyme.

2.3.3. Melanin inhibition assay

These experiments were carried out following the protocols of Kim and Kim (2023) [27] and Le

et al. (2018) [28] with modifications. B16-F10 melanoma cells, a subclone derived from the 10th serial passage of the B16 parent tumor line in C57BL/6 mice, were used in these experiments. B16-F10 cells were cultured in DMEM supplemented with 1% antibiotics and 10% FBS. After incubation at 37°C with 5% CO_2 for 2–3 days, cells were detached with trypsin-EDTA, plated in 6-well plates at a density of 1×10^5 cells/mL, and allowed to adhere overnight. The cells were subsequently exposed to different concentrations of the test samples in the presence of α-MSH (10 nM) for 48 h. After treatment, cells were collected and washed with PBS, and the cell pellets were lysed in 1 N NaOH containing 10% DMSO before incubation at 80°C for 1 h. Optical density (OD) measurements were taken at 405 and 450 nm to assess melanin inhibition. Melanin inhibition by the test samples was compared to that of the negative controls (untreated cells) using the following formula.

$$Melanin\ inhibition(\%) = \frac{(\mathrm{OD(sample)-OD(-\alpha MSH)})}{(\mathrm{OD(MSH)-OD(+\alpha MSH)})} \times 100.$$

2.4. Serum preparation

The serum was prepared using substrates described by Khuu (2023) [20], Thakre (2017) [21], and Amnuaikit et al. (2022) [29] with minor modifications. The test serum formulation consisted of a water phase, composed of distilled water and EDTA, hyaluronic acid, and carbomer 94, and an active ingredient phase, which included avocado oil, triethanolamine, Tween 80, vitamin E, and *E. thymifolia* extract at predetermined concentrations. The complete list of the serum ingredients and their quantities is detailed in Table 1.

Analysis of the serum physical characteristics: The color and appearance of the serum were visually evaluated. The pH of the formulation was assessed using a pH meter (pH510, Mettler Toledo GmbH, Urdorf, Switzerland), and the viscosity was evaluated with a viscometer (Viscolite VL7-100B-d21, Hydramotion, UK).

Table 1. Composition of the serum prepared with the *Euphorbia thymifolia* extract.

Numerical order	Ingredient	Amount (%, w/w)
1	RO water	47.00
2	Carbomer	0.16
3	Water RO	Customized according to the remaining ingredients
4	Sodium hyaluronate	0.50
5	Phenoxyethanol	0.40
6	Glycerine	3.00
7	Propylene glycol	5.00
8	Panthenol	2.00
9	Water RO	2.00
10	Triethanolamine 99%	0.80
11	*Euphorbia thymifolia* extract	2.00
12	**Total**	**100.00**

2.5. Cytotoxicity of the extract and serum

The serum was prepared using substrates based on the methods described by Thakre (2017) [18] and Khuu (2023) [20].

The testing procedure was adapted from the methods outlined by Skehan et al. (1990) [30] and Vo et al. (2022) [22] with modifications. The in vitro cytotoxicity assay employed was certified by the National Cancer Institute (NCI) as a standard method for evaluating cytotoxicity. After staining with SRB, the OD of the B16-F10 cells (University of Milan, Italy) was measured to determine the total cellular protein content.

The cells were trypsinized, counted using a hemocytometer, and adjusted to the appropriate density (1×10^5 cells/mL) for testing. Then, 190 µL of cell suspension was seeded into a 96-well plate. The test substance was dissolved in 100% DMSO to prepare a 20 mM stock solution, which was diluted with cell culture medium (without FBS) to four different concentrations, and 10 µL of each diluted sample was added to wells. A control well containing only B16-F10 cells (190 µL) and 1% DMSO (10 µL) was designated as the day 0 control.

Cells were incubated at 37°C for 72 h, fixed with TCA for 1 h, and stained with SRB for 30 min. The samples were then washed three times with acetic acid before air drying at room temperature. SRB was dissolved in 10 mM unbuffered Tris base with gentle agitation for 10 min. OD measurements at 540 nm were obtained using an ELISA plate reader (BioTek).

Solutions of ellipticine (10, 2, 0.4, and 0.08 µg/mL) were used as positive controls, whereas 1% DMSO served as the negative control. The percent cell growth inhibition caused by the test substance was calculated according to the following equation.

$$\%Inhibition = 100\% - \frac{OD(sample) - OD(day\ 0)}{OD(DMSO) - OD(day\ 0)}.$$

2.6. Statistical analysis

All the experiments were repeated three times. The results are shown as the mean values ± standard deviations. Differences between groups were evaluated using analysis of variance (ANOVA), the coefficient of variation (CV), and Tukey's test ($p < 0.05$) using Minitab 1.6 software.

3. Results

3.1. Antioxidant activities of the E. thymifolia *extract*

The antioxidant activities of the *E. thymifolia* ethanol extract made from samples collected in Kien Giang Province, Vietnam, are summarized in Table 2. The *E. thymifolia* extract exhibited DPPH free radical scavenging activity in a concentration-dependent manner. The average IC_{50} value was determined to be 63.50 µg/mL, which is approximately 12 times greater than that of standard gallic acid (IC_{50} value of 5.21 µg/mL). Additionally, the average IC_{50} value for ABTS radical scavenging was 168.55 µg/mL.

Table 2. Antioxidant activities of the *Euphorbia thymifolia* extract.

Test method	Substrate	Concentration (µg/mL)	Percentage of inhibition (%)	Regression equation (R^2)	IC_{50}/$Abs_{0.5}$ value (µg/mL)
PPH radical scavenging activity	*Euphorbia thymifolia* extract	15.625	15.43 ± 0.89[e]	y = 0.7083x + 5.6245 R^2 = 0.9868	63.50 ± 0.084[a]
		31.25	30.93 ± 1.53[d]		
		62.5	54.13 ± 1.49[c]		
		90	77.27 ± 0.54[b]		
		125	83.06 ± 2.13[a]		
	Gallic acid	2	28.80 ± 1.98[e]	y = 8.1261x + 6.971 R^2 = 0.9866	5.21 ± 0.084[b]
		4	44.52 ± 0.91[d]		
		6	54.76 ± 0.40[c]		
		8	71.33 ± 0.59[b]		
		10	86.20 ± 0.64[a]		
$ABTS^+$ radical scavenging activity	*Euphorbia thymifolia* extract	50	17.47 ± 1.50[e]	y = 0.2862x + 2.0853 R^2 = 0.9893	168.55 ± 9.15[a]
		100	31.07 ± 0.70[d]		
		150	46.37 ± 5.50[c]		
		200	55.64 ± 0.44[b]		
		250	74.50 ± 1.41[a]		
	Gallic acid	1	17.90 ± 0.05	y = 28.287x − 3.4091 R^2 = 0.997	1.89 ± 0.05[b]
		1.5	39.92 ± 0.10		
		2	54.83 ± 0.01		
		2.5	68.21 ± 0.01		
		3	82.12 ± 0.02		
		3.5	95.05 ± 0.02		
Reducing power (FRAP)	*Euphorbia thymifolia* extract	20	14.47 ± 0.40[e]	y = 0.5513x + 0.0202 R^2 = 0.9893	90.99 ± 1.26[a]
		30	18.50 ± 0.53[d]		
		50	22.30 ± 0.17[c]		
		80	42.63 ± 0.29[b]		
		100	57.63 ± 1.25[a]		
	Gallic acid	2	24.90 ± 0.85[g]	y = 5.5199 + 7.8236 R^2 = 0.9948	7.65 ± 0.07[b]
		4	35.44 ± 0.21[e]		
		6	41.72 ± 0.26[d]		
		8	49.94 ± 1.87[c]		
		10	58.19 ± 1.24[b]		
		12	75.89 ± 0.54[a]		

Note: In the same column, values followed by at least 1 similar letter are not significantly different ($p < 0.05$).

3.2. Tyrosinase inhibitory activity of the E. thymifolia *extract and serum containing the extract*

The tyrosinase inhibitory effects of the *E. thymifolia* ethanol extract and the serum supplemented with the extract are presented in Table 3. The *E. thymifolia* ethanol extract inhibited tyrosinase activity in a concentration-dependent manner, as higher concentrations resulted in greater inhibition. Thus, the ethanol extract of *E. thymifolia* has potent tyrosinase inhibitory activity with a low IC_{50} value (3.92 µg/mL), which is notably lower than that of the standard reference substance kojic acid (IC_{50} = 19.10 µg/mL).

The serum supplemented with *E. thymifolia* extract presented in vitro tyrosinase inhibition that increased with increasing extract concentration in the range of 1.25–20 µg/mL (Table 3). At a

concentration of 1.25 µg/mL, tyrosinase inhibition was 11.89%, and at a concentration of 2.5 µg/mL, the inhibition percentage was greater (16.49%). At the maximum tested concentration of 20 µg/mL, tyrosinase inhibition was 77.60%, highlighting a strong concentration-dependent inhibitory effect. The data also indicate that *E. thymifolia* serum exhibits stronger tyrosinase inhibitory activity (IC_{50} value of 14.07 µg/mL) than the positive control, kojic acid, which has an IC_{50} value of 19.10 µg/mL. Importantly, kojic acid, a highly pure standard substance, lacks a serum base and therefore inherently possesses superior inhibitory activity, whereas the *E. thymifolia* serum formulation combines multiple components, which may influence its overall performance.

Table 3. Tyrosinase inhibitory activity of the *Euphorbia thymifolia* extract and serum.

Euphorbia thymifolia extract	Concentration (µg/mL)	Tyrosinase inhibition (%)	Tyrosinase inhibitor content	IC_{50} (µg/mL)
	4.38	27.62 ± 1.65^{c}	10.41 ± 0.68^{c}	3.92 ± 0.11^{c}
	8.75	35.24 ± 7.19^{c}	13.56 ± 2.97^{c}	
	17.50	52.38 ± 6.60^{b}	20.64 ± 2.73^{b}	
	35.00	60.95 ± 1.65^{b}	24.18 ± 0.68^{b}	
	70.00	84.76 ± 1.65^{a}	34.02 ± 0.68^{a}	
Serum containing the extract	1.25	11.89 ± 1.76d	3.49 ± 0.52^{d}	14.07 ± 0.32^{b}
	2.50	16.49 ± 1.12d	5.21 ± 0.49^{d}	
	5.00	29.64 ± 1.97c	11.74 ± 1.21^{c}	
	10.00	42.56 ± 1.82b	16.58 ± 0.76^{b}	
	20.00	77.60 ± 1.49a	31.06 ± 0.62^{a}	
Kojic acid	5.00	19.61 ± 0.67^{c}	5.48 ± 1.40^{e}	19.10 ± 0.50^{a}
	10.00	29.41 ± 0.58^{bc}	10.07 ± 1.24^{d}	
	15.00	39.22 ± 0.34^{b}	13.85 ± 0.47^{c}	
	20.00	52.94 ± 2.41^{a}	19.79 ± 1.24^{b}	
	25.00	62.75 ± 3.40^{a}	24.65 ± 1.24^{a}	

Note: In the same column, values followed by at least 1 similar letter are not significantly different ($p < 0.05$).

3.3. Toxicity of the E. thymifolia *extract and serum to B16-F10 cells*

The results from the B16-F10 cell growth inhibition experiment are presented in Table 4. To evaluate the ability of the *E. thymifolia* extract to restrict B16F10 cell growth upon stimulation with α-MSH, the extract was diluted in 0.1% DMSO to concentrations ranging from 0.8 to 100 µg/mL. Ellipticine at concentrations of 0.08–10 µg/mL was used as the positive control, while culture medium without ellipticine or the extract was used as the negative control. The data in Table 4 show that the *E. thymifolia* extract did not exhibit significant toxicity to B16-F10 cells ($IC_{50} > 100$ µg/mL). In contrast, the positive control, ellipticine, resulted in a strongly linear increase in cytotoxicity across the tested concentration range ($IC_{50} = 0.38$ µg/mL).

An increase in the concentration of serum resulted in significantly greater cell growth inhibitory effects. However, at a very high concentration (100 µg/mL), the serum inhibited less than 50% of melanoma cell growth. The serum prepared with the *E. thymifolia* extract showed toxicity to B16-F10 cells. According to the National Cancer Institute (NCI) guidelines, an extract is considered cytotoxic to B16-F10 cells when the IC_{50} value is ≤ 20 µg/mL.

Table 4. B16-F10 cell growth inhibitory effects of the *Euphorbia thymifolia* extract, the serum, and ellipticine.

Euphorbia thymifolia extract			Serum			Ellipticine	
Conc. (μg/mL)	Living cells (%)	Growth inhibition (%)	Conc. (μg/mL)	Living cells (%)	Growth inhibition (%)	Conc. (μg/mL)	Growth inhibition (%)
0.8	99.92 ± 0.07^{a}	7.78 ± 0.50^{d}	0.8	101.62 ± 3.42^{a}	5.29 ± 0.36^{d}	0.08	21.22 ± 0.95^{d}
4	96.17 ± 0.13^{b}	16.17 ± 0.99^{c}	4	95.58 ± 2.94^{b}	10.02 ± 0.67^{c}	0.4	50.33 ± 1.26^{c}
20	93.36 ± 2.45^{c}	31.93 ± 1.40^{b}	20	94.46 ± 2.44^{c}	23.92 ± 1.40^{b}	2	76.27 ± 0.94^{b}
100	83.19 ± 0.39^{d}	46.33 ± 2.47^{a}	100	82.20 ± 3.53^{d}	46.87 ± 1.43^{a}	10	91.22 ± 6.63^{a}
IC_{50} cytotoxicity > 100 μg/mL			IC_{50} cytotoxicity > 100 μg/mL			IC_{50} = 0.38 μg/mL	

Note: In the same column, values followed by at least 1 similar letter are not significantly different ($p < 0.05$).

3.4. Melanin production inhibition by E. thymifolia *extract and serum*

Subsequently, the ability of the *E. thymifolia* extract to inhibit melanin production in B16-F10 cells was evaluated. B16-F10 melanocytes are known to produce melanin, which contributes to pigmentation in the skin, hair, retina, bone marrow, and certain central gray nuclei of the nervous system. The ability of the extract to inhibit melanin production is illustrated in Figure 1.

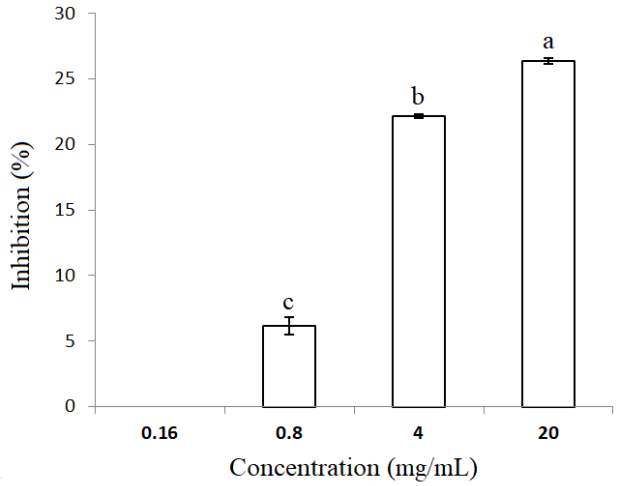

Figure 1. Percent inhibition of melanin production in B16-F10 cells treated with the *Euphorbia thymifolia* ethanol extract. [Note: Different letters above the columns indicate significant differences ($p < 0.05$) among the corresponding mean values.]

To investigate the effect of the *E. thymifolia* extract on melanin synthesis, B16-F10 melanoma cells were exposed to different concentrations of the extract (ranging from 0.16 to 20 μg/mL) for 48 h. At a concentration of 0.8 μg/mL, the extract reduced melanin production by 6.17% compared with that of the untreated control. Increasing the extract concentration to 4 and 20 μg/mL further increased the melanin production inhibition rates to 22.16% and 26.35%, respectively. However, at the lowest concentration of 0.16 μg/mL, no inhibition of melanin production was observed.

The melanin inhibition and toxicity to B16-F10 cells data for the serum are presented in Table 5.

At concentrations of 20 and 4 μg/mL, the serum demonstrated melanin production inhibition rates of 27.23% and 16.86%, respectively. At 100 μg/mL, the inhibitory effect did not increase further, which may be due to a saturation effect. This suggests that while the serum exhibits melanin-inhibitory properties at lower concentrations, its effect does not scale proportionally at higher doses.

Table 5. Inhibition of melanin production by the *Euphorbia thymifolia* serum.

Concentration (μg/mL)	Melanin inhibition (%)
0.8	8.65 ± 0.66^{c}
4	16.86 ± 1.16^{b}
20	27.23 ± 1.18^{a}
100	-3.02 ± 0.67^{d}

Note: In the same column, values followed by at least 1 similar letter are not significantly different ($p < 0.05$).

3.5. Physical characteristics of the developed serum

Images of the serum samples are presented in Figure 2. The serum formulation was developed on the basis of the ability of the *E. thymifolia* extract to inhibit melanin production in B16-F10 melanocytes. The pH of the prepared serum was 6.17, which aligns with the natural pH of human skin. The formulated product was subjected to centrifugation at 5000 rpm to evaluate phase homogeneity, yielding a phase separation time of 71.67 min. These findings suggest that the serum has good stability and durability.

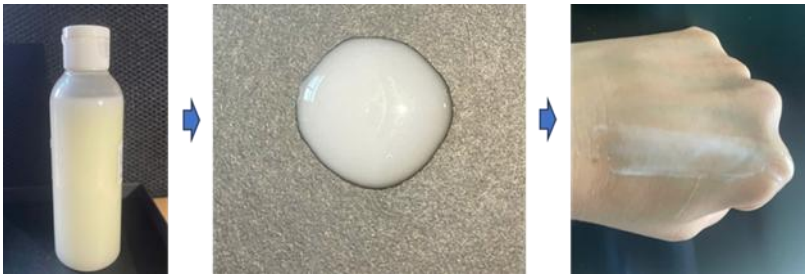

Figure 2. The serum prepared in the laboratory.

4. Discussion

In this study, the antioxidant activity of the *E. thymifolia* ethanol extract was comprehensively evaluated using the DPPH, ABTS, and FRAP assays, and the extract exhibited the best antioxidant activity in the DPPH assay (IC_{50} = 63.50 μg/mL). Studies on the antioxidant activity of plant extracts have demonstrated that an extract may exhibit varying strengths of antioxidant capacity across different assays. This variation can be attributed to the distinct antioxidant mechanisms of the major bioactive compounds present in the extract. A substance may exhibit strong antioxidant activity in one assay but show weaker activity in another assay. Polyphenols and flavonoids are the major chemical composition in *E. thymifolia* extract [22,31], containing hydroxyl groups that readily donate hydrogen atoms that have been demonstrated to exhibit strong activity in the DPPH free radical

scavenging assay.

Previously, Muthumani and Manikandan (2013) [31] investigated the antioxidant capacity of the *E. thymifolia* ethanol extract by examining nitric oxide neutralization, which yielded an IC_{50} value of 638.36 μg/mL. However, a study by Sharma et al. (2014) [21] reported an IC_{50} value of 175 μg/mL for the in vitro DPPH scavenging activity of the *Euphorbia hirta* aqueous extract. The difference in antioxidant capacity between these two extracts may be the result of many factors, such as plant species (*E. thymifolia* versus *E. hirta*), extraction solvent (water versus ethanol), soil conditions, harvest season, age of the samples, and testing method.

The tyrosinase inhibition results obtained in this study exceeded those reported by Rauf et al. (2022) [32], where *E. thymifolia* demonstrated an IC_{50} of 48.7 μM, whereas kojic acid had an IC_{50} of 47.6 μM. Previous studies have indicated that the *E. thymifolia* contains a significant number of polyphenols and flavonoids [22,31]. These compounds have been reported to exhibit tyrosinase inhibitory activity and reduce melanin synthesis by either binding to the active site of the tyrosinase enzyme or interfering with the oxidation process of L-DOPA. Previous studies on the biological activities of plants have demonstrated that these bioactivities may vary significantly depending on the collection site environment, such as the climate conditions or soil composition, or the pre-experimental sample processing conditions [23,33]. Plants are immobile living organisms that are constantly influenced by dynamic biotic and abiotic factors. As a result, they have evolved adaptive biochemical responses by producing secondary metabolites. The levels of these metabolites may fluctuate in response to various environmental factors [34]. Kien Giang Province, a coastal region of Vietnam, is significantly affected by saline intrusion and possesses soil that is both acidic and saline [20]. The unique environmental conditions of this humid tropical coastal area may explain the 12.4 times greater tyrosinase inhibitory effect of the *E. thymifolia* crude extract collected in Vietnam in this study than that of the extract obtained from the same plant species collected in Pakistan, as reported by Rauf et al. (2022) [32].

The tyrosinase inhibitor content increased linearly with extract concentration, as shown in Table 3, highlighting the strong correlation between extract concentration and inhibitory activity. Many skin-whitening agents have been screened for their tyrosinase inhibitory potential, as tyrosinase catalyzes the initial oxidation step of L-DOPA [25,27]. The results of this study demonstrate that ethanol extracts of Vietnamese *E. thymifolia* exhibit promising inhibitory effects on the oxidase activity of tyrosinase. The tyrosinase inhibitory activity of the extract in this study surpasses that reported by Kim and Kim (2023) for the methanol and ethanol extracts of Korean fir needles (*Abies koreana* Wilson), which showed inhibition percentages of 65.4% and 59.0%, respectively, at 2 mg/mL [27]. *Abies koreana* belongs to the Pinaceae family, whereas *Euphorbia thymifolia* in this study belongs to the Euphorbiaceae family. Despite their taxonomic differences, both species produce bioactive secondary metabolites, particularly phenolic compounds, which may contribute to their tyrosinase inhibitory activity. This comparison highlights the potential of *Euphorbia thymifolia* as a natural source of skin-whitening agents.

The melanin production inhibition results obtained in this study align with previous findings by Vo et al. (2022) [22], who reported that *E. thymifolia* extract modulates the activities of the enzymes tyrosinase and acetylcholinesterase (AChE) and reduces motor activity in *Drosophila melanogaster*. *E. thymifolia* extract was found to inhibit the activity of AChE, an enzyme involved in the breakdown of the neurotransmitter acetylcholine in the central nervous system. Specifically, at 50 mg/mL, the extract demonstrated AChE 62.67% inhibition compared with that of the control. In

addition, evaluation of the toxicity of the *E. thymifolia* extract to second-stage fruit fly larvae revealed 53.33% lethality at a concentration of 15 mg/mL.

Antityrosinase, antioxidant, and melanin inhibitory activities are three critical biological properties of plant extracts that are widely pursued for applications in medicine, pharmaceuticals, and cosmetics [34]. *E. thymifolia* extract has been reported to possess strong antityrosinase activity, effectively inhibiting melanin production in melanocytes. This plant contains a diverse array of bioactive compounds, including quercetin, thymol, salicylic acid, carvacrol, glycosides, 2-sesquiterpenes, steroids, terpenoids, tannins, flavonoids, various phenolics, essential oils, and minerals [20,24]. Consequently, further research into extract standardization, the mechanisms of action, formulation development, and clinical and toxicological efficiency is warranted. *E. thymifolia* represents a cost-effective source of therapeutic agents, given the widespread distribution of phenolic compounds across plant sources and their diverse molecular structures and biological activities. These compounds, characterized by aromatic rings with hydroxyl groups, act as melanogenesis inhibitors by suppressing tyrosinase expression [20,35].

E. thymifolia serum is considered noncytotoxic on the basis of the American National Cancer Institute (NCI) standards. The extract demonstrated strong activity, with an $IC_{50} \leq 20$ μg/mL, whereas the pure substances showed more potent activity, with an $IC_{50} \leq 5$ μM [36]. Additionally, Vo et al. (2022) [22] identified flavonoids, polyphenols, tannins, and alkaloids in the *E. thymifolia* extract when evaluating the toxicity of the extract to second-stage fruit fly larvae, reporting 53.33% lethality at a concentration of 15 mg/mL. In contrast, the experimental serum prepared from the *E. thymifolia* ethanol extract in this study was noncytotoxic, potentially because of the lower concentration of the extract used (20 μg/mL).

5. Conclusions

In this study, the extract of *E. thymifolia*, which was collected from the Mekong Delta of Vietnam, was shown to have significant antioxidant activity via DPPH, ABTS, and FRAP assays. The *E. thymifolia* extract and the serum containing the extract also exhibit strong, concentration-dependent tyrosinase inhibitory activity. The IC_{50} values of the extract (3.92 μg/mL) and serum (14.07 μg/mL) indicate that their tyrosinase inhibitory activities were much stronger than that of kojic acid (IC_{50} = 19.10 μg/mL). Both the extract and the serum effectively reduced melanin production in B16-F10 melanoma cells without significant cytotoxicity. In addition, the serum prepared with the *E. thymifolia* extract exhibited the desired uniformity and insignificant toxicity to melanocytes. These findings suggest that *E. thymifolia* extract holds promise as an effective ingredient for the development of skincare products.

Author contributions

Conceptualization, T.T.H.N.; methodology, T.T.H.N., V.C.H., V.B.H., K.Y.H. and T.Y.V.; investigation, T.T.H.N., V.K.D.; data curration T.T.H.N., V.C.H., V.K.D., K.Y.H. and T.Y.V.; writing-original draft, T.T.H.N., V.C.H. and T.T.H.; writing-review and editing, T.T.H.N., V.C.H., V.B.H. and T.T.H.; funding acquisition T.T.H.N. All authors have read and agreed to the published version of the manuscript.

Use of Generative-AI tools declaration

The authors declare that they did not use artificial intelligence (AI) tools in the creation of this article.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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