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Evaluation of nano-sized virgin coconut oil (VCO)-loaded liposomes for enhancing mushroom and B16-F10 tyrosinase activity

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ABSTRACT

This study evaluated the potential impact of VCO-loaded liposomes, particularly on the activation of tyrosinase. Optimized liposomes containing 1 % (w/w) VCO were prepared using the film deposition on carrier method, resulting in a particle size of 84.02 ± 5.00 nm and a zeta potential of -68.40 ± 2.78 mV. Encapsulation of VCO enhanced mushroom tyrosinase activity by 3-fold and exhibited lower cytotoxicity to B16-F10 cells compared to VCO alone. Moreover, a positive correlation was observed between the increase in intracellular tyrosinase activity and the concentrations of VCO ($r = 0.8366$) and VCO-loaded liposomes ($r = 0.4794$) in B16-F10 cells, while a negative correlation ($r = -0.0545$) was found for liposomes without VCO. A hair and eyebrow-darkening gel containing both VCO and VCO-loaded liposomes further enhanced mushroom tyrosinase activity by 283.33 ± 26.58 %. These findings suggest that VCO-loaded liposomes may serve as novel and effective nano-scale carriers for VCO in cosmetic applications.

1. Introduction

Melanogenesis, the complicated process that controls melanin production, occurs mostly within melanosomes found in melanocytes. This intricate pathway involves various paracrine cytokines, including the melanocyte-stimulating hormone (MSH), stem cell factor, endothelin-1, nitric oxide, adrenocorticotropic hormone, prostaglandins, thymidine dinucleotide, histamine, and several enzymes such as tyrosinase, tyrosinase-related protein-1, and tyrosinase-related protein-2. Among these enzymes, tyrosinase plays a major role in melanogenesis by converting tyrosine into melanin pigments [1].

Virgin coconut oil (VCO) is extracted from fresh and mature coconut kernels (*Cocos nucifera*) using a wet processing method that avoids the use of heat. This method is designed to preserve the integrity of biologically active components, such as vitamins and polyphenols, preventing their degradation during extraction. In the cosmetics industry, coconut oil, especially VCO, has numerous benefits, such as antibacterial, anti-collagenase, antioxidant, sunscreen, and lubricating properties [2–9]. VCO has a long history of being used as a natural topical moisturizer in tropical regions. Its emollient properties and occlusive effects prevent transepidermal water loss and enhance skin moisture [10,11]. However, applying VCO directly on the skin remains limited due to its undesirable

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properties, including a strong smell and cloudy appearance at ambient temperature. When applied topically, VCO mostly remains at the skin's surface, resulting in greasiness in the applied areas. Thus, VCO-loaded carriers have attracted interest in overcoming these unfavourable characteristics and enhancing the efficiency of VCO. Noor et al. (2013) prepared VCO-loaded solid lipid particles, which were later incorporated into a moisturizing base lotion. It was found that the lotion with VCO-loaded solid lipid particles (20 %) exhibited a higher occlusive effect and better skin elasticity than that without VCO-loaded solid lipid particles [12]. However, the preparation of solid lipid particles involves using solid lipids with high melting points, requiring high temperatures to melt the lipid phase. This process can negatively impact thermolabile substances, such as certain bioactive compounds. Additionally, solid lipid particles have inherent limitations, including low loading capacities for certain active compounds and the necessity of adequate water content to dissolve the particles [13].

Liposomes are small amphiphilic lipid bilayer vesicles that range from 30 nm to several micrometres. Liposome technology is extensively employed to encapsulate active ingredients in lipid vesicles to enhance penetration into target sites, increase bioactivity and stability, and reduce the toxicity of the encapsulated substances [14,15]. Liposomes have been reported as potential carriers of essential oils for topical applications and transfollicular systems [16]. Unlike solid lipid particles, liposomes can be prepared without high-temperature processing, thereby preserving thermolabile substances. Furthermore, proliposome formulations address challenges related to water sensitivity, such as the autoxidation and hydrolysis of bioactive compounds, while offering a higher loading capacity for active compounds [17].

Both saturated and unsaturated fatty acids, including palmitic acid, myristic acid, lauric acid, and oleic acid, can activate mushroom tyrosinase activity *in vitro*. Saturated fatty acids are more potent tyrosinase activators than unsaturated fatty acids [18,19]. VCO contains high levels of saturated fatty acids (90.896 %) [20], which might enhance tyrosinase activity. This research introduces a novel carrier for VCO to address its undesirable properties, improving its bioactivity, stability, and skin penetration, which will be a potential breakthrough for skin and hair care products.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine from soybean, cholesterol from sheep wool, L-DOPA, palmitic acid, and mushroom tyrosinase were purchased from Sigma-Aldrich, USA. Mannitol, Tween® 80 (polyoxyethylene sorbitan monooleate), and oleic acid were purchased from PC Drug Center Co. Ltd, Thailand. Lauric acid was purchased from Chemipan Corporation, Co. Ltd, Thailand. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-ethylenediaminetetraacetic acid were purchased from Gibco, USA. The Bradford reagent and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from PanReac AppliChem, USA. B16-F10 mouse melanoma cells were purchased from Biomedia, Thailand and used between passages 5 and 12 for all experiments.

2.2. VCO sample

Organic cold-pressed VCO was obtained from Tropicana Oil Co. Ltd, Thailand. VCO was extracted from coconut grown in the Suratthani Province, Thailand. VCO was used as obtained; its total phenolic content, as determined using the Folin-Ciocalteu reagent, was 69.57 ± 0.05 mg gallic acid equivalents (GAE) per 100 g of VCO.

2.3. Preparation of VCO-loaded liposomes

This study utilized two distinct methods for liposome preparation: the first involved ethanol injection [21], while the second employed the film deposition on carrier method [17]. The weight ratio of phosphatidylcholine: cholesterol: Tween® 80 was maintained at 2:1:0.4. The weight of VCO was systematically varied to determine the optimum amount required for liposome formation. The detailed compositions of the prepared liposomes are provided in Table 1.

The ethanol injection method was applied to prepare VCO-loaded liposomes. VCO, phosphatidylcholine, and cholesterol were dissolved in 95 % ethanol at 60 °C. Subsequently, the ethanol phase was injected into the aqueous phase containing Tween 80,

Table 1
The compositions of liposome formulation.

Ethanol injection method			Film deposition on carrier method		
No.	VCO:PC:Cho: Tw80 ratio	VCO (% w/w)	No.	VCO:PC:Cho: Tw80:M ratio	VCO (% w/w)
Base	0:2:1:0.4	0	BasePro	0:2:1:0.4:5.4	0
C1	2:2:1:0.4	1	ProC1-1	2:2:1:0.4:5.4	18.52
C5	5:2:1:0.4	5	ProC1-2	2:2:1:0.4:10.8	12.35
			ProC5-1	5:2:1:0.4:5.4	36.23
			ProC5-2	5:2:1:0.4:10.8	26.04

Note: VCO = virgin coconut oil, PC = phosphatidylcholine, Cho = cholesterol, Tw80 = Tween 80, M = mannitol.

maintaining a steady flow rate of 20 mL/min while agitating at the same temperature. The resulting liposome dispersions appeared after the controlled evaporation of ethanol using a rotary evaporator (Eyela, Japan) under reduced pressure at 40 °C. These refined VCO-loaded liposome dispersions were then stored in an aqueous form at a temperature of 4 °C until their intended use.

Proliposomes containing VCO were prepared using the film deposition on carrier method, as described by Gupta et al. (2008), with minor modifications [17]. Mannitol served as a microporous carrier. VCO, phosphatidylcholine, cholesterol, and Tween 80 were dissolved in 95 % ethanol, maintaining a precise 1:20 ratio (total liposome composition to the volume of ethanol) at an elevated temperature of 60 °C. Subsequently, the ethanol solution was gradually added to a round bottom flask containing dried mannitol at 40 °C. After the evaporation of ethanol under reduced pressure at 40 °C, dried proliposomes were obtained. These proliposomes were kept in a solid form at 4 °C until required. The conversion of these proliposomes into VCO-loaded liposome dispersions was accomplished through a homogenization process involving the blending of prepared proliposomes with water.

2.4. Characterization of liposomes

The physical appearance of formulations was observed by visual inspection. The liposome characteristics, including size, size distribution, and zeta potential, were determined using a photon correlation spectroscopy (Nanosizer ZS, Malvern Instrument, UK). These characteristics were assessed at two different time points: (i) after liposome preparation and (ii) after storage at 4 ± 1 °C and 30 ± 1 °C, under 75 % RH in a constant climate chamber (Memmert, Germany) for one month. All tests were performed in triplicate at 25 °C. Moreover, the VCO-loaded proliposomes, prepared by the film deposition on carrier method, were initially photographed using a scanning electron microscope (SEM). After they were homogenized in water, the size and morphology of the dispersed liposomes were assessed by a transmission electron microscope (TEM).

2.5. Analysis of fatty-acid content by gas chromatography (GC)

The amount of fatty acids in VCO, phosphatidylcholine, and liposome formulations was determined by GC following European Standard EN14103:2003. Briefly, 5 mL of 10 mg/mL heptadecanoate in heptane was added to 250 mg of samples to obtain fatty acid methyl ester (FAME). The FAME was detected using a GC equipped with a flame ionization detector. The separation was performed in a Select Biodiesel column for the FAME type (30 m × 0.32 mm × 0.25 μm). Helium was used as the carrier gas at flow rates of 1.0 mL/min and a split ratio of 50:1. Fatty acids were identified by comparing retention times with an external standard mixture of fatty acid methyl esters (C6-C24).

2.6. Preparation of treatment solutions for melanogenesis studies

The treatment solutions for melanogenesis studies were prepared as follows: (i) VCO-loaded proliposomes were homogenized in water to create a 100 mg/mL stock solution, then diluted with phosphate buffer (pH 7.4) or cell culture media to the desired concentrations; (ii) fatty acids were dissolved in absolute ethanol to prepare a 100 mg/mL stock solution. They were diluted with phosphate buffer (pH 7.4) or cell culture media to the desired concentrations and sonicated for 15 min to ensure the fully dissolved fatty acids.

2.7. In vitro mushroom tyrosinase activity assay

Mushroom tyrosinase activity was estimated by the oxidation of L-DOPA. The assay was modified from the method by Guan et al. (2008) [22]. The experimental mixtures consisted of 50 μL of 3 mM L-DOPA in phosphate buffer (pH 7.4), 50 μL of samples, 50 μL of phosphate buffer (pH 7.4), and 50 μL of tyrosinase (108 U/mL). The 96-well plates were incubated at room temperature for 20 min, and the reaction was subsequently observed at 492 nm using a microplate reader (Spectrostar Nano, BMG Labtech, Germany). Data were collected from three replicates (mean ± s.d.). The activation of tyrosinase was expressed as Equation (1):

$$\text{Tyrosinase activation} = (A - B)/B \times 100 \quad (1)$$

where A is the absorbance of the tested compound, and B is the absorbance of the control.

2.8. Melanogenesis studies in B16-F10 cultured cells

2.8.1. Viability of B16-F10 mouse melanoma cells

The effect of VCO, the liposome formulation, palmitic acid, lauric acid, or oleic acid on the viability of B16-F10 cells was investigated. The melanoma cells were cultured in DMEM supplemented with 10 %v/v FBS and 100 U/mL penicillin-streptomycin at 37 °C, 5 % CO_2. The cells at a density of 1×10^4 cells per well were grown for 24 h. Then, samples at concentrations up to 5,000 μg/mL were added and incubated for 48 h. Next, the cells were incubated with the MTT solution (0.5 mg/mL) for 4 h. Dimethyl sulfoxide was used to solubilize the formazan crystals. The absorbance at 562 nm was measured. Three replicates were performed at each concentration (mean ± s.d.). The relative cell viability (%) was expressed as the ratio between the absorbance value of the treated cells and that of the untreated cells.

2.8.2. Intracellular tyrosinase activity assay and measurement of intracellular melanin content

The intracellular tyrosinase activity assay was performed as described by Lee et al. [23] with slight modifications. Briefly, the melanoma cells were incubated at a density of 2×10^5 cells per 60 mm dish for 24 h. Then, the cells were exposed to the samples or α-MSH (500 nM) for 48 h. Cell pellets (1×10^6 cells) were harvested and mixed with a mild cell lysis buffer containing a cocktail of protease inhibitors. The cells were then lyzed *via* a freeze-thaw cycle for three cycles (each cycle: -80 °C for 30 min and 37 °C for 30 min). Cell debris was eliminated by centrifugation at 13,000 rpm for 15 min. Protein content was determined using the Bradford reagent and compared with a standard curve of bovine serum albumin. The cell lysates were diluted with lysis buffer to obtain a protein concentration of 40 μg/well. Twenty microliters of 5 mM L-DOPA in PBS and 80 μL of PBS were added. A further incubation was performed for 1 h at room temperature. Absorbance was measured at 450 nm using a microplate reader. Three replicates were carried out for each concentration (mean ± s.d.).

For measurement of intracellular melanin content, after exposed to the samples for 48 h, cell pellets (1×10^6 cells) were harvested and subsequently dissolved in 1 M NaOH at 80 °C for 2 h. The melanin content was determined by recording the absorbance at 405 nm using a microplate reader. The total melanin content was calculated by comparison with a standard curve of synthetic melanin and expressed as pg melanin per a cell.

2.9. Formulation development and in vitro mushroom tyrosinase activity of hair and eyebrow-darkening product

VCO and VCO-loaded liposomes were incorporated into hair and eyebrow-darkening products. Table 2 shows the compositions of an optimal hair and eyebrow-darkening gel. A low content of unencapsulated VCO (5 % w/w) was incorporated into the gel formulation as a skin moisturizer and carrier of coloring agents. One hundred grams of product were produced by cold process. The chemicals were separated into 2 phases. The constituents in each phase were mixed using a homogenizer at 10,000 rpm at room temperature. Then, phase A was poured into phase B with constant stirring until uniform. The stability of the product was tested under 6 heating/cooling cycles (1 cycle; 45 °C for 24 h and 4 °C for 24 h). Physical characteristics and *in vitro* mushroom tyrosinase activity of the products were evaluated. pH values were determined using a digital pH meter (SevenCompact, Mettler Toledo, Switzerland). The viscosity at 25 °C was measured using a viscometer (DV-III Ultra, Brookfield, USA) with spindle number SC4-31 at 250 rpm. The color was determined using a spectrophotometer (CM-700d, Konica Minolta, Japan). The color value (ΔE) was calculated as follows:

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

Where ΔL is brightness, Δ*a*is the red-green proportion, and Δb is the yellow-blue proportion.

2.10. Statistical analysis

The experimental data were replicated at least three times and expressed as the mean ± standard deviation (s.d.). Statistical analysis was performed using the Student-paired t-test and one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (GraphPad Prism version 8.3.0) at a significant level of $p < 0.05$.

3. Results and discussion

This research attempted to develop a cosmetic system leveraging the melanogenesis-stimulating properties of VCO, offering a natural alternative for VCO delivery. In this study, VCO has been shown to be a potent tyrosinase stimulator. However, the undesirable properties of VCO, such as greasiness, strong smell, and handling problems, are still a limitation for cosmetic applications. Therefore, VCO-loaded liposomes will be a promising delivery system for utilizing VCO in the cosmetic industry.

3.1. Preparation and characterization of VCO-loaded liposomes

The preparation method significantly influences the types and characteristics of liposomes. In this study, the liposome preparation method was systematically optimized to align with the desired properties of liposomes, encompassing size, stability, and production feasibility. Phosphatidylcholine, cholesterol, and Tween 80 were used at a fixed ratio. Cholesterol served the purpose of stabilizing the

Table 2
Compositions of an optimal hair and eyebrow-darkening product.

Phase	Ingredients	%w/w
A	Distilled water	34.5
	Proliposomes	5
	Phenonip® P4	0.5
	Butylene glycol	10
B	Black Iron Oxide EasyMix™	10
	Virgin coconut oil	5
	Sepigel® 305	5
	Skycore® PT018	30

lipid bilayer, whereas the addition of Tween 80 aimed to enhance the loading efficiency of the liposomes.

The ethanol injection method has been popularly used for preparing liposomes because the solvent is non-harmful, and the method is easy to scale up. However, liposomes produced through this method exist in the form of an aqueous suspension. Consequently, the exposure of liposomes to an aqueous medium upon formation may influence the stability of liposomes in this study, in which the oil component could be susceptible to hydrolysis during storage [24]. The film deposition on carrier method has gained widespread acceptance for the preparation of proliposomes due to the short preparation time and homogeneous distribution of lipids. Notably, proliposomes exist as dry and free-flowing granules; therefore, the problems caused by water, such as autoxidation and hydrolysis, are avoided [25]. Mannitol was selected as the carrier in this study because it is microporous and highly water-soluble. These properties enable easy hydration of the proliposomes upon reconstitution in water.

The characteristics of VCO-loaded liposomes are shown in Table 3. The appearance of VCO-loaded liposomes was determined by visual inspection. Interestingly, liposomes prepared by the ethanol injection method were not observed in the formulation without VCO (Base), while VCO-loaded liposome dispersions (C1 and C5) appeared as a white and milky liquid. The incorporation of VCO resulted in distinctive liposome formation, indicating that VCO might integrate into the liposomal membrane. Previous research has shown that essential oils can influence the liposome size and fluidity of the liposome membrane [16]. Our findings suggest that VCO may indeed contribute to the composition of the liposomal membrane. For the film deposition on carrier method, ProC1-1 and ProC1-2 were pale yellow free-flowing granules, while ProC5-1 and ProC5-2 were yellow semisolids. Since proliposomes should ideally be free-flowing granules, ProC1-1 and ProC1-2 are more suitable for employing as VCO carriers than ProC5-1 and ProC5-2 which are semisolids due to their high coconut oil content. Reconstitution of proliposomes with water resulted in a white and milky liquid similar to the liposome dispersions prepared by the ethanol injection method, suggesting the complete formation of liposomes after reconstitution.

The content of VCO directly impacted the size and zeta-potential value of the liposomes. Specifically, liposomes, prepared by the ethanol-injection method with a low VCO content (C1, 1 %w/w), exhibited a size 1.6 times smaller than formulations with a higher VCO content (C5, 5 %w/w). Also, liposomes, prepared by the film-deposition on carrier method with a high VCO content (ProC5-1, ProC5-2), demonstrated a larger size and size distribution than those with a lower VCO content (ProC1-1, ProC1-2). All formulations displayed a high negative zeta-potential value (> -50 mV), indicating a stable system [26]. Proliposomes of VCO prepared by the film-deposition on carrier method with the same ratio of VCO and phospholipid exhibited a significantly smaller size than VCO-loaded liposomes prepared by the ethanol injection method ($p < 0.05$). This result might be due to the effective re-dispersion of the proliposomes. However, the size distributions were not significantly different between the two formulations ($p>0.05$). This result was consistent with a previous report that the characteristics of liposomes, particularly size and size distribution, are influenced by the preparation method and composition of the liposome membrane [16]. Therefore, from these results, only proliposomes with the VCO content of 18.52 % w/w (ProC1-1) and 12.35 % w/w (ProC1-2) were chosen for stability testing.

The stability of proliposomes was investigated after a 1-month storage period at 4 °C and 30 °C. All liposome formulations displayed an increase in size after storage at ambient temperature, as shown in Table 4. Only ProC1-1 exhibited no significant differences in size, size distribution, and zeta potential after storage at 4 °C ($p>0.05$). These results suggested that the proliposomes should be stored at 4 °C to maintain their characteristics. From these results, the ProC1-1 formulation was selected for subsequent experiments due to its optimal properties, including small size, acceptable size distribution, high negative zeta potential, and reduced amount of tween 80 and mannitol compared to ProC1-2.

Fig. 1A shows that the appearance of ProC1-1 was pale-yellow free-flowing granules. The surface morphology of ProC1-1, which was analyzed by SEM, is illustrated in Fig. 1B. It showed that the proliposome components, including VCO, phosphatidylcholine, cholesterol, and Tween 80, were thoroughly deposited on mannitol surfaces. Upon dispersion of the proliposomes in water, the resulting liposome dispersion manifested as a white and milky liquid (Fig. 1C), indicating liposome formation. As imaged by TEM, the liposomes were spherical and can be classified as unilamellar vesicles (Fig. 1D). These findings suggest that the inherent limitations of VCO for cosmetic applications, such as greasiness, strong smell, and handling problems, could potentially be mitigated by utilizing

Table 3
Characteristics of liposomes and proliposomes after preparation.

Formulations	Physical appearance	Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
Ethanol injection method				
Base	Separation liquid	n.d.	n.d.	n.d.
C1	White and milky liquid	97.65 ± 5.55	0.29 ± 0.11	-62.87 ± 1.45
C5	White and milky liquid	154.30 ± 1.76[a]	0.42 ± 0.02	-54.13 ± 0.74[a]
Film deposition on carrier method				
BasePro	Pale yellow granules	191.43 ± 10.66	0.58 ± 0.04	-59.40 ± 0.78
ProC1-1	Pale yellow granules	84.02 ± 5.00[a,c]	0.37 ± 0.04[c]	-68.40 ± 2.78[a,c]
ProC1-2	Pale yellow granules	87.05 ± 1.32[a,c]	0.34 ± 0.03[c]	-75.30 ± 2.59[a,c]
ProC5-1	Yellow semisolids	125.93 ± 2.23[b,c]	0.60 ± 0.05[b]	-78.70 ± 2.18[b,c]
ProC5-2	Yellow semisolids	147.23 ± 16.18[c]	0.60 ± 0.10[b,c]	-93.20 ± 2.26[b,c]

Note: n.d. = not detectable. Data are presented as the mean ± s.d., n = 3. Data were analyzed using the ANOVA,
[a] : $p < 0.05$, compared with C1;
[b] : $p < 0.05$, compared with C5;
[c] : $p < 0.05$, compared with BasePro.

Table 4
Characteristics of VCO-loaded proliposomes before and after 1-month storage at 4 °C and 30 °C.

Characteristics	Formulations	Initial	1-month storage	
			4 °C	30 °C
Sizes (nm)	BasePro	191.43 ± 10.66	141.97 ± 8.53*	315.10 ± 28.69*
	ProC1-1	84.02 ± 5.00	88.43 ± 0.67	195.40 ± 5.67*
	ProC1-2	87.05 ± 1.32	86.44 ± 1.50	211.20 ± 4.84*
Polydispersity Index (PDI)	BasePro	0.58 ± 0.04	0.77 ± 0.07*	0.58 ± 0.09
	ProC1-1	0.37 ± 0.04	0.39 ± 0.01	0.39 ± 0.01
	ProC1-2	0.34 ± 0.03	0.29 ± 0.02*	0.57 ± 0.02*
Zeta-potential (mV)	BasePro	-59.40 ± 0.78	-55.87 ± 0.32*	-63.23 ± 12.46
	ProC1-1	-68.40 ± 2.78	-67.10 ± 1.97	-71.57 ± 2.25
	ProC1-2	-75.30 ± 2.60	-74.37 ± 1.27	-67.03 ± 0.80*

Note: PDI = polydispersity index. Data are presented as the mean ± s.d., n = 3. Data were analyzed using the paired t-test.
* $p < 0.05$ compared to the initial time.

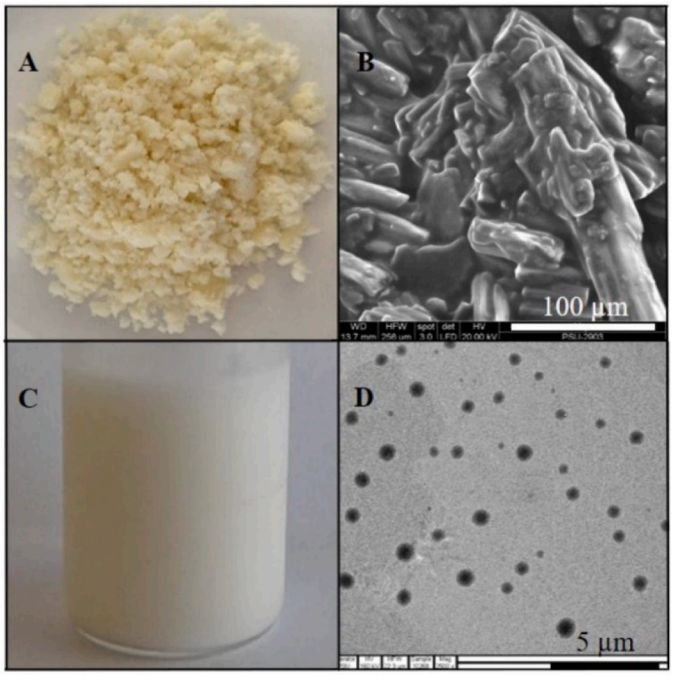

Fig. 1. Appearance of ProC1-1 proliposomes (VCO:Phosphatidylcholine:Cholesterol: Tween 80:Mannitol ratio = 2:2:1:0.4:5.4) (A) and captured by SEM 500× magnification; bar = 100 µm (B), liposomes generated from ProC1-1 proliposomes after dispersion in purified water (C), and captured by TEM 2500× magnification; bar = 5 µm (D).

VCO within proliposome formulations, serving as novel VCO products for a wide range of applications due to their characteristics.

3.2. Fatty acid profile of VCO and liposomes

The fatty acid analysis was employed to identify the distribution of fatty acid in VCO and liposome compositions, as shown in Fig. 2. Our results showed that VCO predominantly comprised fatty acids, accounting for 83.91 ± 0.11 %w/w, with variations in saturation levels and carbon chain lengths. Among the saturated fatty acids, lauric acid (40.59 ± 0.15 %w/w), myristic acid (16.61 ± 0.03 %w/w), and palmitic acid (8.45 ± 0.01 %w/w) emerged as the major constituents of our VCO sample (Fig. 2A). These results were in agreement with other studies showing that lauric acid is a predominant medium-chain saturated fatty acid found in VCO, reported as 46.36 to 48.42 % by Mansor et al. (2012) [27] and 45.567 % by Sabahannur and Alimuddin (2022) [20]. Phosphatidylcholine, a key component of the liposomal membrane, comprises long-chain unsaturated fatty acids, including oleic acid, linoleic acid, and linolenic acid, as principal components (Fig. 2A). Consequently, the composition of fatty acids in liposome formulations depended on the constituents of VCO and phosphatidylcholine. The ratios between total saturated and unsaturated fatty acids (Sat/Unsat ratio) of VCO, ProC1-1, BasePro, and PC were ~9.94, 0.60, 0.27, and 0.27, respectively (Fig. 2B). The highest Sat/Unsat ratio was observed for VCO, followed by that for ProC1-1. While these ratios of BasePro and PC were comparable. These results reflected the highest saturated fatty

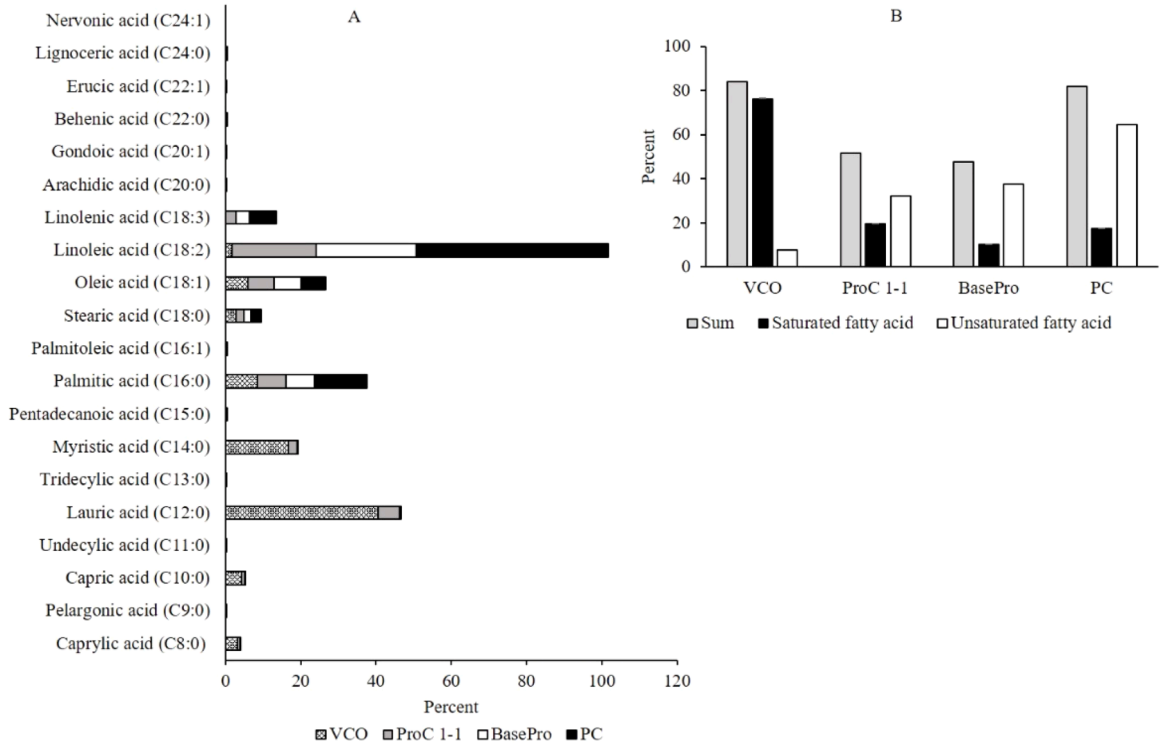

Fig. 2. Fatty acid profile of VCO and liposome compositions
Note: PC = Phosphatidylcholine; data are presented as the mean ± s.d., n =3.

acids found in VCO, whereas ProC1-1, BasePro, and PC resulted in higher unsaturated fatty acids than the saturated ones. Studies indicate that saturated and monounsaturated fatty acids can permeate the hair shaft, contributing to strengthening and moisturizing effects [6,28]. These characteristics might be due to their low molecular weight, straight linear chain, and polar head group, which result in a strong affinity with protein molecules in the cell membrane complex of the hair.

3.3. Mushroom tyrosinase activity assay

Mushroom tyrosinase has been widely used as the target enzyme for screening potential tyrosinase regulators. Initially, the relationship between varying concentrations of fatty acids and VCO and their impact on mushroom tyrosinase activity was investigated

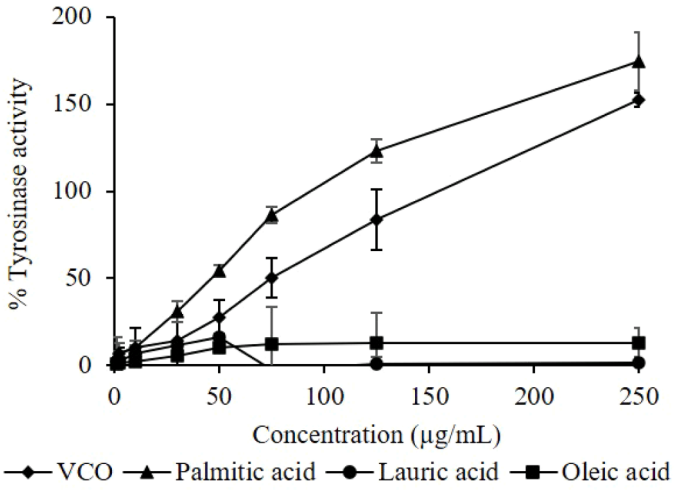

Fig. 3. Effects of VCO, palmitic acid, lauric acid, and oleic acid on mushroom tyrosinase activity at concentrations of 2.5–250 µg/mL. Data are expressed as the mean ± s.d. (n = 3).

(Fig. 3). Then, the percentage of tyrosinase activation for different fatty acids and formulations at the same concentration (40 μg/mL) was evaluated (Table 5).

VCO demonstrated a dose-dependent stimulation of mushroom tyrosinase activity, with palmitic acid, a major long-chain saturated fatty acid present in VCO (~8 %w/w), identified as a potent mushroom tyrosinase stimulator (Fig. 3). In contrast, lauric acid, a principal saturated fatty acid in VCO (~40 %w/w), and oleic acid, a major unsaturated fatty acid in VCO (~5 %w/w), exhibited only slight effects on mushroom tyrosinase activation. These results implied that the tyrosinase activation of VCO might be from the palmitic acid component. Previous research has shown that saturated fatty acids, especially palmitic acid, induced tyrosinase activity, whereas unsaturated fatty acids, particularly linoleic acid, resulted in tyrosinase inhibition in B16 murine melanoma cells [29]. Another previous study has ranked fatty acids in decreasing order of tyrosinase activation as follows: palmitic acid (C16:0) > myristic acid (C14:0) > stearic acid (C18:0) > lauric acid (C12:0) > oleic acid (C18:1) [18].

Effects of VCO, palmitic acid, lauric acid, and oleic acid on mushroom tyrosinase activity were repeated using the same concentration (40 μg/mL) to ensure the attainment of clear solutions (Table 5). The mushroom tyrosinase activation of fatty acids was in descending order as follows: palmitic acid (47.01 ± 2.26 %) > oleic acid (19.41 ± 3.53 %) > lauric acid (9.15 ± 1.13 %). Also, palmitic acid has been shown to be the most potent tyrosinase activator [18]. At 40 μg/mL, VCO (26.17 ± 6.39 %) exhibited lower mushroom tyrosinase stimulation when compared to palmitic acid. Since palmitic acid showed the highest tyrosinase stimulation, this result implied that palmitic acid might be the key tyrosinase-activating component in VCO.

Due to the desirable properties of VCO on tyrosinase stimulation, successfully prepared liposomes containing VCO were also determined for their mushroom tyrosinase activation. The tyrosinase activity of liposome formulations (BasePro and ProC1-1) was investigated at a concentration of 40 μg/mL. As shown in Table 5, the liposomes exhibited an enhancement in mushroom tyrosinase activity. As expected, the liposomes without VCO (BasePro) also demonstrated tyrosinase activation, which is attributed to the high content of fatty acids in phosphatidylcholine. Tyrosinase activation by ProC1-1 (76.20 ± 1.72 %) was two-fold higher than that by BasePro (36.78 ± 2.73 %). Despite both formulations containing approximately the same amount of total fatty acids, they exhibited distinct fatty acid compositions. ProC1-1 contained a higher proportion of saturated fatty acids than BasePro, resulting in a stronger tyrosinase activation. Consistent with previous reports, saturated fatty acids exhibit a more potent tyrosinase stimulation than unsaturated fatty acids [18]. Notably, phosphatidylcholine and Tween 80, which served as amphoteric molecules, can improve the dispersibility of VCO in an aqueous system during the mushroom tyrosinase assay. Additionally, surfactants such as sodium dodecyl sulfate and Tween 80 have been reported to act as tyrosinase activators [30]. Thus, the mushroom tyrosinase activation observed in BasePro and ProC1-1 may be attributed to the combined effects of fatty acids and Tween 80.

3.4. Melanogenesis studies in B16-F10 cultured cells

The effects of the following agents on melanogenesis were investigated in B16-F10 mouse melanoma cells, including VCO, base liposomes (BasePro), VCO-loaded liposomes (ProC1-1), lauric acid, and palmitic acid.

3.4.1. In vitro cytotoxicity in B16-F10 cells

The cytotoxicity of VCO, BasePro, ProC1-1, and fatty acids was evaluated by measuring the cellular mitochondrial activity across varying concentrations. VCO, BasePro, ProC1-1, lauric acid, and palmitic acid exhibited no or negligible cytotoxic effects to B16-F10 cells at concentrations less than 1500, 1500, 1500, 50, and 50 μg/mL, respectively (Fig. 4). The IC_{50} value of ProC1-1 was higher than that of VCO and BasePro. This observation suggested that encapsulating VCO in liposomes could potentially decrease its cytotoxicity. Moreover, lauric and palmitic acids exhibited more cytotoxicity than VCO and liposomes. Similar to our results, higher toxicity of lauric acid than VCO has been reported on human neuroblastoma cells (SH-SY5Y) [31]. Also, many researchers have reported a higher cytotoxic effect of the major components than their parent oil [32–34]. For instance, linalyl acetate, the primary constituent of lavender oil (51 %), demonstrated greater toxicity than the whole oil in 153BR and HNDF cells. This difference might be because of the

Table 5
Mushroom tyrosinase activation by different fatty acids and formulations at a concentration of 40 μg/mL.

Sample	Palmitic acid[a]	Lauric acid[a]	Oleic acid[a]	VCO[b]	BasePro[b]	ProC1-1[b]
Fatty acid (μg/mL)						
Sat	39.68	39.92	-	30.5	4.02	7.79
Unsat	-	-	36.4	3.07	14.98	12.88
Lauric acid	-	39.64	-	16.24	0.04	2.28
Palmitic acid	39.68	-	-	3.38	3.04	3.05
Oleic acid	-	-	36.4	2.38	2.86	2.76
Linoleic acid	-	-	-	0.66	10.67	8.96
Linolenic acid	-	-	-	0	1.42	1.15
Total fatty acid (%)	99.2	99.8	91	83.91	47.48	51.67
% Tyrosinase activation[c]	47.01 ± 2.26*	9.15 ± 1.13*	19.41 ± 3.53	26.17 ± 6.39	36.78 ± 2.73*	76.20 ± 1.72*

Note: Sat = Saturated fatty acid, Unsat = Unsaturated fatty acid.
[a] calculated from certificate of analysis provided by the manufacturer.
[b] determined by GC.
[c] Data are presented as the mean ± s.d., n = 3. Data were analyzed using the ANOVA.
* $p < 0.05$ compared to VCO.

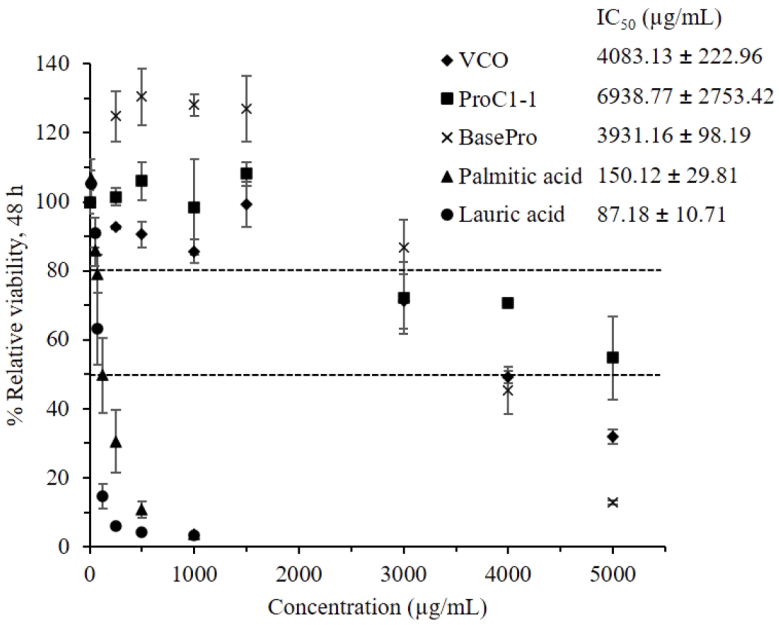

Fig. 4. The effects of VCO, BasePro, ProC1-1, palmitic acid, and lauric acid on the viability of B16-F10 cells. Each value is expressed as the mean ± s.d. (n = 3).

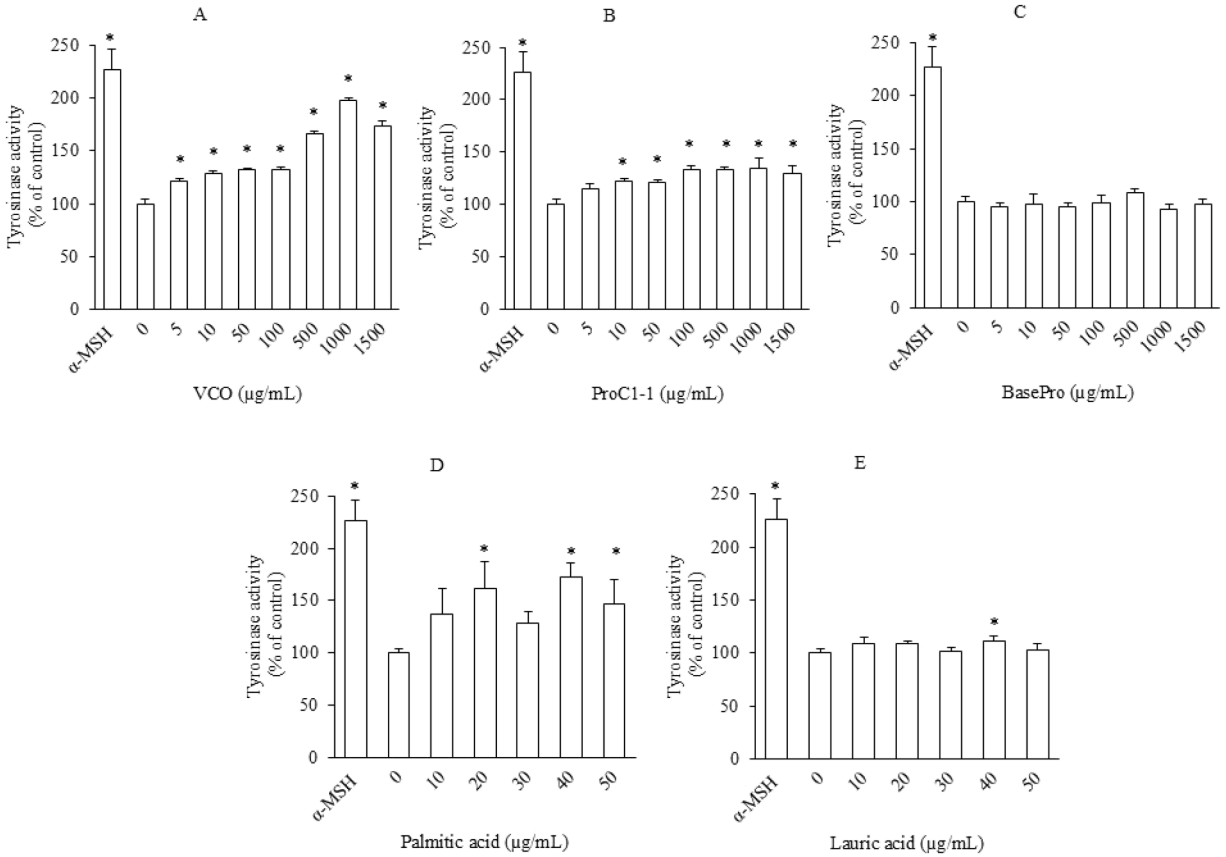

Fig. 5. Intracellular tyrosinase activity in B16-F10 melanoma cells after 48 h exposure to the samples; VCO (A), ProC1-1 (B), BasePro (C), Palmitic acid (D) and Lauric acid (E). Each value is expressed as the mean ± s.d. (n = 3). Data were analyzed using the ANOVA, * $p < 0.05$ compared with the untreated cells.

suppression of its activity by other components within the oil [32]. VCO is a complex mixture of fatty acids and other phytochemicals, such as polyphenols, tocopherols, and phytosterols [7,9]. Even though the major constituent of our VCO is lauric acid (approximately 40 %), it comprises other minor bioactive components, especially phenolic compounds. Our VCO showed a total phenolic content of 69.57 ± 0.05 mg GAE per 100 g VCO. Phenolic compounds have been shown to reduce fatty acid-induced toxicity through antioxidant activity [35]. Therefore, these compounds may interact with lauric acid, palmitic acid and other fatty acids in VCO, potentially modulating the cytotoxic effects of VCO. Apart from this, VCO contains specific forms of fatty acids. They exist as triacylglycerols and glycerol esters with three fatty acids, which exhibit different properties than free fatty acids. This explanation agrees with a previous report, indicating that palmitic acid methyl ester exhibits lower tyrosinase activity and less cytotoxicity than palmitic acid at the same dosage in human melanocytes [36].

3.4.2. *Intracellular tyrosinase activity assay*

The activity of B16-F10 tyrosinase after sample treatment was evaluated. Alpha-MSH, an important cytokine regulating melanin formation by stimulating the tyrosinase enzyme, was used as a positive control. After 48-h exposure of B16-F10 cells to various concentrations of the test compounds, cellular proteins were extracted to analyze the intracellular tyrosinase activity. As shown in Fig. 5, treatment of the melanoma cells with 500 nM α-MSH resulted in a two-fold increase in intracellular tyrosinase activity. For VCO, a positive correlation between VCO ($r = 0.8366$), palmitic acid ($r = 0.6381$), and ProC1-1 ($r = 0.4794$) concentrations and an overall increase in tyrosinase activity was observed. Significant increases in tyrosinase activity were found at concentrations starting from 5 μg/mL for VCO and ProC1-1 ($p < 0.05$). On the other hand, lauric acid ($r = 0.1932$) and BasePro ($r = -0.0545$) demonstrated no significant impact on intracellular tyrosinase activity.

VCO and palmitic acid stimulated tyrosinase activity without significantly affecting cell proliferation. VCO and palmitic acid proved to be potent stimulators of melanogenesis by activating intracellular tyrosinase in B16-F10 melanoma cells. They also showed a positive correlation between its concentrations and an overall increase in intracellular melanin content per cell (Fig. 6). Previous research has revealed that palmitic acid enhances tyrosinase activity by retarding its degradation [19]. Moreover, our finding showed that ProC1-1 (VCO-loaded liposomes) exhibited a stimulatory effect on the B16-F10 tyrosinase, consistent with its effect on mushroom tyrosinase. However, the intracellular tyrosinase-activating effect of ProC1-1 was weaker than that of VCO alone. This result was

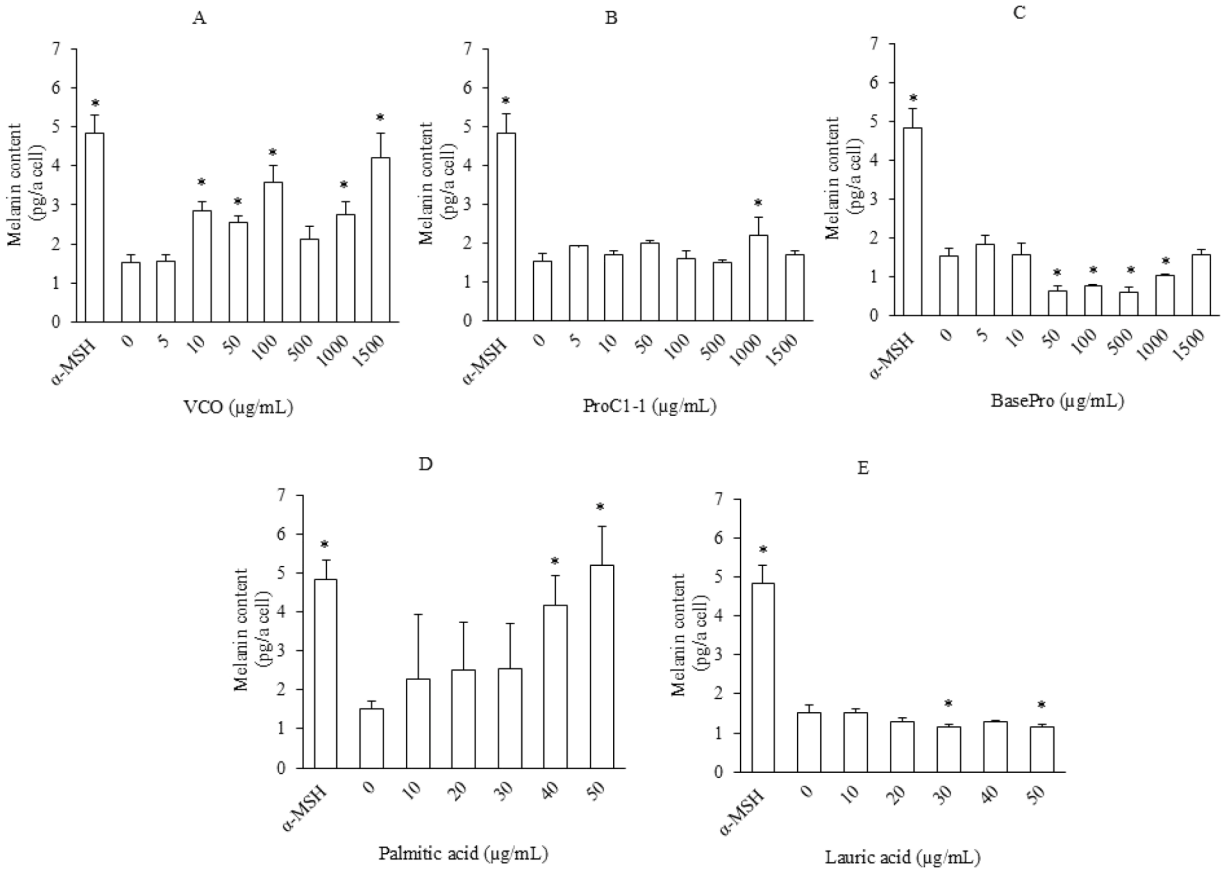

Fig. 6. Intracellular melanin content in B16-F10 melanoma cells after 48 h exposure to the samples; VCO (A), ProC1-1 (B), BasePro (C), Palmitic acid (D) and Lauric acid (E). Each value is expressed as the mean ± s.d. (n = 3). Data were analyzed using the ANOVA, * $p < 0.05$ compared with the untreated cells.

similar to the melanin-producing effect of ProC1-1 and VCO on B16-F10 cells (Fig. 6). This observation may be attributed to the presence of linoleic acid, linolenic acid, and oleic acid, major unsaturated fatty acids in the phosphatidylcholine from soybean, which is the main component of the liposomal membrane. Linoleic acid and α-linolenic acid have shown efficient tyrosinase-inhibiting effects on B16-F10 melanoma cells. The tyrosinase activity after 72-h exposure to linoleic acid and α-linolenic acid was 31.9 % and 19.5 %, respectively, compared to untreated cells [37]. A previous report suggested that the tyrosinase-inhibiting effect of linoleic acid might be due to its ability to accelerate the spontaneous degradation of tyrosinase [19]. Also, linolenic acid and linoleic acid have been shown to decrease intracellular melanin content and enhance melanin secretion to the extracellular fluid in B16-F1 and B16-F10 cells [38]. Although the melanin content in the extracellular fluid was not determined in this study, the unsaturated fatty acids present in the liposome bilayer might enhance melanin secretion [38], resulting in lower melanin production after exposure to ProC1-1 than VCO.

3.5. Formulation development and in vitro mushroom tyrosinase activity of hair and eyebrow-darkening product

Our current results revealed that VCO is a potent mushroom and B16-F10 tyrosinase activator, inducing melanin production in B16-F10 melanoma cells. Similarly, ProC1-1 could activate mushroom and B16-F10 tyrosinase activity. Thus, a synergistic combination of VCO and ProC1-1 was incorporated into our hair and eyebrow-darkening formulation to enhance melanogenesis-stimulating activity. Apart from this, VCO can be utilised as a skin moisturizer and carrier for coloring agents. The formulation of the hair and eyebrow-darkening product was developed in a gel form because of its non-greasy characteristics, ease of application, and suitability for shaping eyebrows. The formulation was dark gel with a faint odour of VCO. The result showed that hair and eyebrow-darkening gel demonstrated high mushroom tyrosinase activation (~280 %), and this product under the heating/cooling cycle has no significant difference in viscosity, pH, color, and tyrosinase activation activity, as shown in Table 6. This finding is in agreement with previous studies demonstrating that liposomal gels are ideal carriers for topical drug delivery. The gel matrix protects bioactive compounds from environmental degradation, thereby maintaining their stability and efficacy [39,40]. Moreover, studies have shown that liposomal gels exhibit better stability than liposomal suspensions, with reduced drug leakage during storage [41,42]. The high viscosity of the gel minimizes liposome movement, agglomeration, and fusion, which are the key factors contributing to drug leakage [42].

4. Conclusion

Nano-sized VCO-loaded liposomes present a promising innovation for cosmetic applications, offering enhanced biocompatibility and several beneficial properties, such as enhanced tyrosinase activity, improved fatty acid penetration, reduced greasiness, and decreased strong odor typically associated with VCO. VCO-loaded proliposomes in dry powder form emerged as a versatile and easily manageable option, indicating their potential as a commercially viable VCO-loaded material. Our findings highlight the strong effectiveness of VCO and VCO-loaded liposomes as activators of both mushroom and B16-F10 tyrosinase, suggesting their potential utility in formulations designed to stimulate melanogenesis for hair and eyebrow darkening. The formulated product, which contained 5 % w/w of VCO and 5 % w/w of VCO-loaded liposomes, demonstrated high mushroom tyrosinase activation. Overall, it was expected that this comprehensive approach would find some valuable insights, and the groundwork could be guided for further research and development, highlighting the potential of VCO-loaded liposomes in new cosmetic formulations.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to improve the clarity and grammar of the text. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Suwipa Ungphaiboon: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sutasinee Ardhanwanich:** Investigation, Formal analysis, Data curation. **Duangkhae Maneenuan:** Investigation, Formal analysis. **Sirirat Pinsuwan:** Formal analysis, Conceptualization. **Pawika Mahasawat:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Table 6
The properties of hair and eyebrow-darkening products containing 5 % w/w of ProC1-1 and 5 %w/w of VCO after preparation and stability studies. The data were expressed as mean ± s.d., n = 3.

Time	Viscosity (P)	pH	Color difference (ΔE)	Tyrosinase activation* (%)	Physical observation
Initial time	583.93 ± 14.87	5.4 ± 0.17	26.86 ± 2.25	283.33 ± 26.58	Dark gel without phase separation
6 heating-cooling cycles	587.06 ± 10.66	5.1 ± 0.02	26.12 ± 0.01	272.37 ± 25.27	Dark gel without phase separation

* Mushroom tyrosinase activation by the hair and eyebrow-darkening product containing 5 % w/w of ProC1-1 and 5 %w/w of VCO at a concentration of 40 μg/mL. Data were analyzed using the paired t-test, and no significance was observed compared to the initial time ($p > 0.05$).

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