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Full Paper

Antioxidant, anti-tyrosinase and anti-collagenase activities of virgin coconut oil and stability of its cream

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Abstract: Virgin coconut oil (VCO) was extracted from coconut meat (*Cocos nucifera* Linn.) by centrifugation technique. The VCO was analysed to investigate its potential as a cosmetic ingredient. Inhibition of free radicals with IC_{50} values of 1.39 ± 0.01, 78.16 ± 0.60, and 27.43 ± 0.37 mg/mL in ABTS, DPPH and superoxide radical scavenging tests was obtained for VCO respectively. The skin-whitening effect was examined with tyrosinase assay using L-DOPA as enzymatic substrate. The anti-aging property was determined by collagenase inhibitory activity. The optimal IC_{50} values for anti-tyrosinase and anti-collagenase activities were 761.89 ± 18.85 mg/mL and 625.93 ± 11.62 mg/mL respectively. These results suggest that VCO has potential as a functional ingredient in both cosmeceutical products (e.g. whitening and anti-aging skincare products), and products that aim to decrease oxidative stress, hyperpigmentation, and wrinkles of facial skin. The optimal concentration of VCO was formulated as a VCO cream and analysed in terms of both its physical characteristics and stability. The results showed that VCO cream has absolute physical stability at different storage conditions which evidences its potential as an anti-aging product.

Keywords: virgin coconut oil, antioxidant activity, anti-collagenase activity, anti-tyrosinase activity, cosmetic ingredient

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INTRODUCTION

Skin aging is a complicated biochemical progression that occurs due to intrinsic and extrinsic factors. Intrinsic factors include telomere shortening, the imbalance between free radicals and antioxidant components and hormonal changes [1]. Extrinsic factors include exposure to ultraviolet (UV) radiation, stress, cigarette smoke, pollution, drugs and food [2]. Skin wrinkles form as a result of natural ageing processes and the presence of excessive amounts of reactive oxygen species (ROS) [3]. ROS are defined as oxygen-containing, highly reactive species. ROS are generated constantly during normal cellular metabolism, which is essential for biological functions. However, excessive ROS causes oxidative stress and damages biological molecules [4]. ROS such as superoxide anion radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide are formed during normal metabolic processes that can result in lipid peroxidation and lead to the accumulation of lipid peroxides [5]. ROS directly influence the process of skin ageing by subjecting the skin's lipids, proteins and DNA to oxidative damage that results in extracellular matrix degradation.

Tyrosinase is a copper-containing enzyme that is usually found in plant and animal tissues. This enzyme is a key regulatory enzyme of melanogenesis that catalyses the production of melanin and other pigments by oxidation from the substrate tyrosine [6]. Melanin is a dark biological pigment found in hair, skin, eyes and other tissues and it plays a crucial role in protecting human skin against UV radiation. The accumulation of tyrosinase causes hyperpigmentation, which is a common problem in middle-aged and elderly individuals [7]. Synthetic and naturally occurring tyrosinase inhibitors can be attractive in medicinal and cosmetic products when used as a depigmentation agent for treating hyperpigmented skin and related skin pigmentation disorders. However, increasing importance is being placed on the development of natural agents due to the disadvantages of synthetics agents such as high cytotoxicity, insufficient penetrating power and low activity [8].

Collagen is the most abundant protein in the extracellular matrix; it functions as an adherent for connective tissues [9] and provides the tensile strength that gives firmness to the skin. The collagenases are zinc endopeptidase enzymes that can degrade molecules such as gelatin, aggrecan, fibronectin, casein, ovostatin, elastin and collagen (types I, II, III). These enzymes typically make the first cleavage of peptide bonds in the triple-helical collagen, allowing its further degradation by other proteases [10]. Accordingly, collagenase inhibitors may have beneficial effects in maintaining healthy skin by preventing dermal matrix degradation.

Virgin coconut oil (VCO) is extracted directly from fresh mature coconut meat (*Cocos nucifera* Linn.) without using chemicals or high heat. Consequently, it has more beneficial effects as it retains most of its unsaponifiable components [11]. VCO is colourless and clear and has an aroma of fresh coconut. While VCO has been traditionally used for skin moisturisation, a recent study has demonstrated its other biological activities, namely anti-inflammatory and skin protective properties [12]. Another study showed that phenolic compounds present in VCO have antioxidant and anti-inflammatory effects [13]. The present study was performed to evaluate the antioxidant, anti-tyrosinase and anti-collagenase properties of VCO, which may have beneficial effects in skincare products.

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MATERIALS AND METHODS

Chemicals, Reagents and Apparatus

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH), sodium phosphate monobasic, sodium phosphate dibasic, tyrosinase and methanol were obtained from Sigma-Aldrich (USA). Gallic acid, 3,4-dihydroxy-L-phenylalanine (L-DOPA), N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS) and kojic acid were purchased from Sigma-Aldrich (Germany). Collagenase type I from *Clostridium histolyticum*, (-)-epigallocatechin gallate (EGCG) and tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) were procured from Merck (USA). All other reagents were of analytical grade. All of the cosmetic ingredients were cosmetic grade purchased from Namsiang (Thailand). The absorbance was measured using a microplate reader (Biochrom EZ Read 400, UK) and spectrophotometer (Thermo Scientific™ Evolution 260 Bio, USA). The colour of cream was measured with a colorimeter (Color CF-18, China). The pH of cream was measured using a pH-meter (SI Analytics®, Germany). The viscosity of cream was measured using a viscometer (Qingtian DV-1, China) with spindle no. 6.

Preparation of VCO

Coconuts were acquired from a local market in Chiang Mai and the fresh coconut meat was shredded and then cold-pressed using a muslin cloth to obtain coconut milk. The extraction method described by Wong and Hartina [14] was used with some modifications. The coconut milk (1 kg) was filtered through Whatman's filter paper No. 4. The filtrate was centrifuged at 5,000 rpm, 30°C for 30 min. The separated VCO (8.8%) was then stored in a glass bottle at room temperature.

Antioxidant Activities of VCO

$ABTS^{+\bullet}$ cation radical scavenging activity

The method described by Re et al. [15] was used with some modifications. ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The mixture was then kept in the dark at room temperature for 16 hr to allow the completion of radical generation. It was then diluted with deionised water to give an absorbance of 0.70 ± 0.05 at 734 nm before use. To determine the scavenging activity, 1 mL of ABTS reagent was mixed with 10 µL of VCO and the absorbance was measured at 734 nm 6 min. after the initial mixing. The scavenging activity of $ABTS^{+\bullet}$ radical was calculated using equation (1). Trolox, a derivative of vitamin E, was employed as a positive control. The $ABTS^{+\bullet}$ radical scavenging activity was expressed as a Trolox equivalent antioxidant capacity (TEAC), which represents the mM concentration of Trolox per gram of sample. The IC_{50} value is the inhibitory concentration at which 50% of $ABTS^{+\bullet}$ radical is scavenged. The experiment was carried out in triplicate.

$$\text{Radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (1)$$

($A_{control}$ = absorbance of control; A_{sample} = absorbance of sample or positive control)

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DPPH radical scavenging activity

DPPH radical scavenging activity of VCO was measured according to the method of Hou et al. [16]. Briefly, 0.3 mL of VCO was added to 0.1 mL Tris-HCl buffer (pH 7.9) and then mixed with 0.6 mL of 0.2 mM DPPH in methanol for 20 min. under light protection. The absorbance at 517 nm was determined. The scavenging activity was calculated using equation (1). Gallic acid was used as a positive control. DPPH radical scavenging activity was expressed as gallic acid equivalent (GAE) in milligrams of gallic acid per gram of sample. The experiment was carried out in triplicate. The IC_{50} value was the inhibitory concentration at which 50% of DPPH radical was scavenged.

Superoxide anion ($O_2^{-\bullet}$) scavenging activity

Measurement of the superoxide anion scavenging activity of VCO was performed by following the method described by Nishikimi et al. [17]. NBT, NADH and PMS solutions at the concentrations of 156 µM, 468 µM and 60 µM respectively were prepared in 0.1 M phosphate buffer (pH 7.4). One mL of NBT solution, 1 mL of NADH solution and 0.1 mL of VCO were mixed. The reaction was then initiated by adding 0.1 mL of PMS solution to the mixture. After 5 min. of incubation at room temperature, the absorbance was measured at 560 nm and the scavenging activity was calculated using equation (1). Gallic acid was used as a positive control. Superoxide anion radical scavenging activity was expressed as GAE in milligrams of gallic acid per gram of sample. The experiment was carried out in triplicate.

Anti-tyrosinase Activity of VCO

The determination of tyrosinase inhibition activity was measured using the method described by Lim et al. [18] with L-DOPA as substrate. Forty µL of 2.5 mM L-DOPA in 0.1 M phosphate buffer (pH 6.8), 80 µL of 0.1 M phosphate buffer (pH 6.8), 40 µL of VCO and 40 µL of tyrosinase (200 units/mL) were mixed and incubated at 37°C for 30 min. The absorbance of the solution was measured at 475 nm. The tyrosinase inhibition activity was determined using equation (2). Kojic acid was used as standard tyrosinase inhibitor control. The experiment was carried out in triplicate.

$$\text{Inhibition activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (2)$$

($A_{control}$ = absorbance of control; A_{sample} = absorbance of sample or positive control)

Anti-collagenase Activity of VCO

Collagenase inhibition activity was determined according to the protocol of Van Wart and Steinbrink [19] with slight modification. Briefly, 10 µL of VCO was mixed with 10 µL of collagenase (125 units/mL) and 190 µL of 0.5 mM of FALGPA. The absorbance at 340 nm was measured immediately and then continuously for 5 min. using a microplate reader.

The inhibition activity on collagenase was determined using equation (2). EGCG was used as a positive control. The experiment was performed in triplicate.

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VCO Cream

A cream containing VCO was formulated using ingredients shown in Table 1. Preservatives and other water-soluble components were dissolved in the aqueous phase (Part A). VCO, emulsifying agent and other oil-soluble components were dissolved in the oil phase (Part B). Then Part A was added in portions to Part B and the mixture was homogenised with a homogeniser set to constant stirring at 2,000 rpm for 10 min.

Table 1. Ingredients used in formulation of VCO cream

Phase	Ingredient	% w/w
Water Phase (Part A)	Dipotassium glycyrrhizate	0.10
	Alkyl acrylate crosspolymer	0.28
	Butylene glycol	1.00
	Propylene glycol	0.50
	Panthenol	1.00
	Caprylhydroxamic acid	1.80
	Water	70.12
Oil Phase (Part B)	VCO	12.20
	Squalane	2.80
	Triethylhexanoin	4.20
	Tocopheryl acetate	2.00
	dl-Alpha tocopherol	0.50
	Phenyl trimethicone	1.00
	Methacrylate crosspolymer	2.00
	Fragrance	0.50

Cream Stability Test

A preliminary estimation of stability of VCO cream was done immediately and then again 24 hr after preparation by centrifuging at 5,000 rpm (25°C) for 30 min. The VCO cream after centrifugation was subjected to different conditions, viz. standing at room temperature (45 days); standing at 4°C (45 days); standing at 45°C (45 days); five cycles of heating/cooling (one cycle consisting of 48 hr at 45°C followed by 48 hr at 4°C). Several physicochemical properties , i.e. colour, pH, viscosity and phase separation were evaluated to assure the desired stability of the cream [20]. Colour measurement was expressed as three values: L* (for lightness from black to white), a* (from green to red) and b* (from blue to yellow).

Statistical Analyses

Statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS), version 17.0 for Windows. Statistical comparisons between groups were carried out using one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) post hoc test. *p*-Values under 0.05 were considered significant.

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RESULTS AND DISCUSSION

Different methods have been used to extract VCO, e.g. chilling-thawing, fermentation and enzymatic techniques [21]. In this study the centrifugation technique was used because of the rapidity in extracting VCO when compared to other techniques. Additionally, the VCO extracted by this technique was found to contain phenolic substances [13], which are known to exhibit antioxidant and anti-inflammatory activities. In our study we investigated whether the VCO extracted by centrifugation would also demonstrate antioxidant, anti-tyrosinase and anti-collagenase activities, properties with potential for use as an anti-aging ingredient.

The percentages of ABTS radical scavenging activity of VCO is shown in Figure 1. Its IC_{50} was determined to be 1.39 ± 0.01 mg/mL and that of Trolox was 0.03 ± 0.01 mg/mL. Figure 1 shows that the inhibitory effect of VCO on ABTS radical is concentration dependent. The TEAC value of VCO was found to be 0.72 ± 0.01 mM.

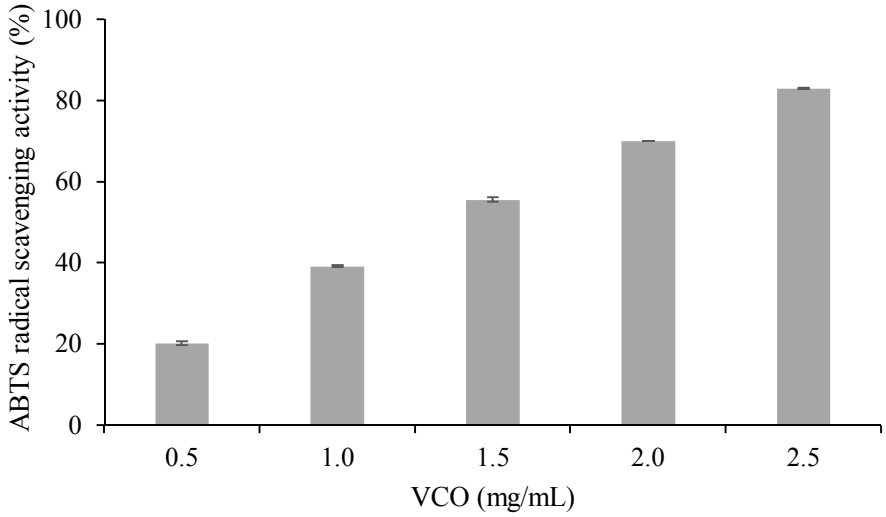

Figure 1. ABTS radical scavenging activity of VCO

Figure 2 shows the DPPH radical scavenging activity of VCO. Applying a linear regression analysis, the IC_{50} values of VCO and gallic acid were found to be 78.16 ± 0.60 and 0.04 ± 0.19 mg/mL respectively. The GAE value of VCO was 0.56 ± 0.01 mg.

Ghani et al. [22] found that the IC_{50} values for DPPH scavenging activity of VCO extracted by chilling-thawing and fermentation techniques were 58.71 and 7.49 mg/mL respectively. Ahmad et al. [23] reported that the antioxidant capacity (EC_{50}) of VCO obtained by chilling-thawing technique was 5.07 ± 0.19 mg/L. According to Arlee et al., the IC_{50} of VCO from fermentation technique was found to be in the range of 0.80-1.27 mg/mL [24]. In this study the IC_{50} value of VCO extracted by centrifugation technique was 78.16 mg/mL.

VCO also scavenges superoxide radicals, as illustrated in Figure 3. The IC_{50} values of VCO and gallic acid, which can be determined from linear regression, were 27.43 ± 0.37 and 0.99 ± 0.02 mg/mL respectively. The GAE value of VCO was found to be 36.32 ± 0.75 mg.

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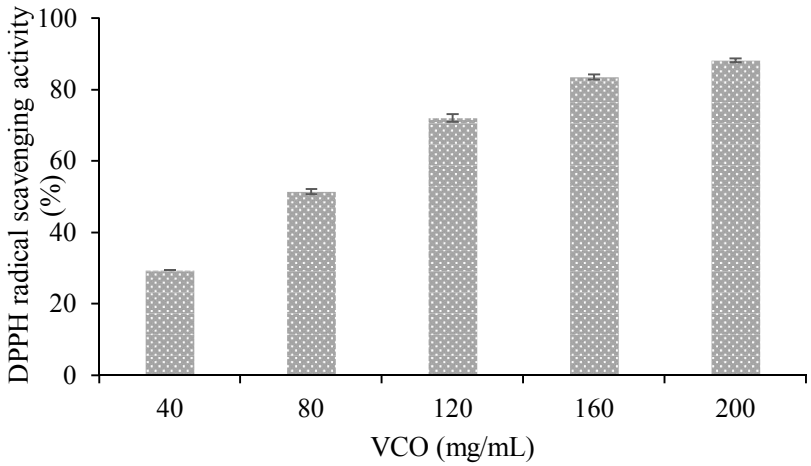

Figure 2. DPPH radical scavenging activity of VCO

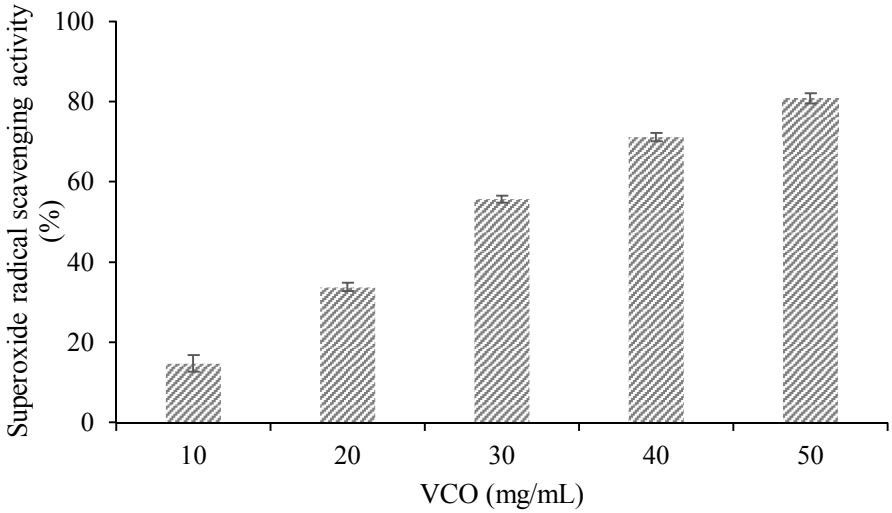

Figure 3. Superoxide radical scavenging activity of VCO

From the results of the three antioxidant assays, VCO obtained by centrifugation technique seems to display antioxidant properties. Its main mechanism may come from its electron-donating ability and its quenching of superoxide anion radicals, evidenced from the more potent ABTS and superoxide radical scavenging activities.

Hyperpigmentation causes human skin aging and occurs as a result of both internal and external factors including those related to hormones, UV exposure, drugs and the presence of various chemicals [25]. Related skin whitening research reveals tyrosinase inhibitors as the most common target for decreasing pigmentation [26]. Tyrosinase is an enzyme involved in the control of melanin production and the inhibition of tyrosinase activity tends to induce skin whitening due to a reduction in melanin synthesis. The tyrosinase inhibitory activity of VCO is shown in Figure 4. The IC_{50} values of VCO and kojic acid determined from linear regression were found to be 761.89 ± 18.85 and 0.39 ± 0.01 mg/mL respectively.

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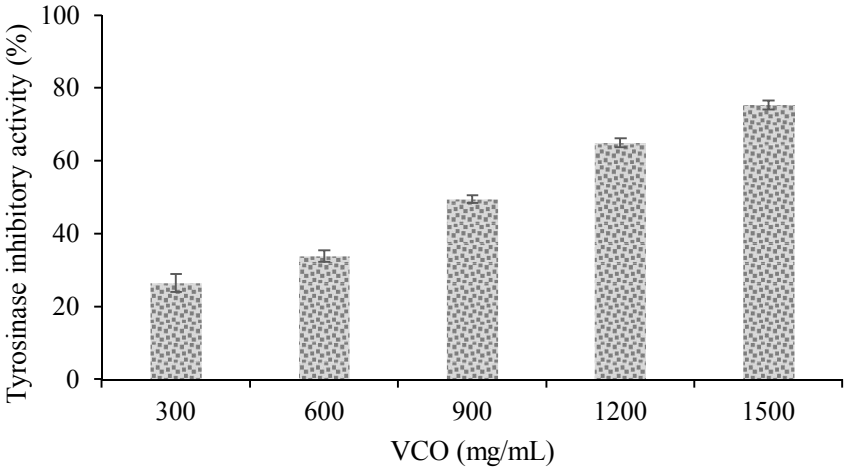

Figure 4. Tyrosinase inhibitory activity of VCO

Collagenase is the enzyme that digests the triple-helix structure of collagen, which is the major component of the extracellular matrix in the dermis layer of the skin [27]. The inhibition of collagenase activity could protect against collagen breakdown and subsequently the wrinkling process. The anti-collagenase activity of VCO is illustrated in Figure 5. EGCG at 0.05 mg/mL resulted in the inhibition of collagenase activity at 65.48 ± 2.70%. VCO at 150, 300 and 600 mg/mL inhibited collagenase activity at 9.51 ± 1.03%, 23.99 ± 1.02% and 47.47 ± 0.89% respectively. The IC_{50} values of VCO and ECGC were found to be 625.93 ± 11.62 and 0.015 ± 0.004 mg/mL respectively.

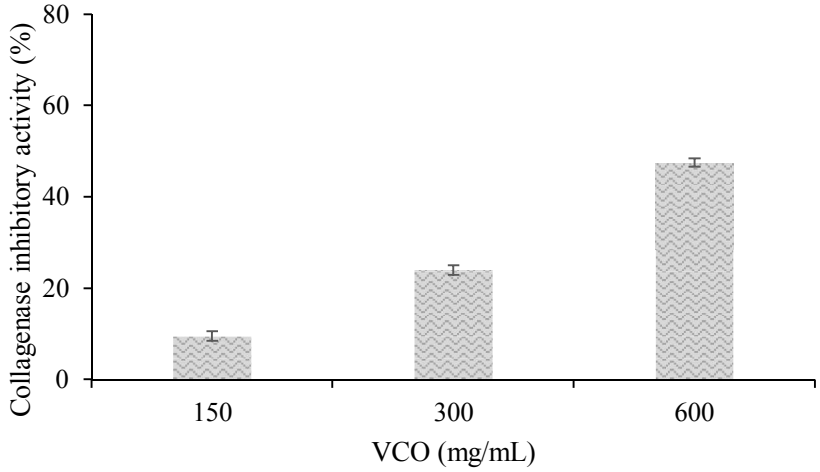

Figure 5. Effect of VCO on collagenase inhibitory activity

The formulated cream containing VCO was studied for its physical characteristics, namely colour, pH and viscosity as shown in Table 2. The cream was found to be white in colour, demonstrated satisfactory spreadability and showed no signs of phase separation when centrifuged at 5,000 rpm for 30 min. While in storage, a cosmetic formulation's thermal stability, colour and viscosity are the primary characteristics that affect the formulation's acceptability. No significant differences in pH, viscosity and colour are found in the creams tested under all conditions.

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Table 2. Characteristics of formulated cream

Condition	Colour			pH	Viscosity (cps)	Phase Separation
	L^*	a^*	b^*			
Initial	62.35±0.04[a]	1.32±0.10[a]	6.35±0.04[a]	8.13±0.06[a]	21962.33±134.75[a]	none
RT	62.09±0.54[a]	1.37±0.08[a]	6.46±0.15[a]	8.11±0.02[a]	21911.94±231.90[a]	none
4 °C	62.28±1.03[a]	1.48±0.16[a]	6.35±0.04[a]	8.12±0.02[a]	21774.00±93.38[a]	none
45 °C	61.67±0.83[a]	1.43±0.21[a]	6.36±0.08[a]	8.11±0.04[a]	21994.22±242.03[a]	none
H/C	61.31±0.80[a]	1.53±0.25[a]	6.37±0.05[a]	8.12±0.06[a]	22202.50±169.92[a]	none

Note: Data are expressed as mean ± standard deviation of triplicate measurements. Averages with the same letters in a column are not significantly different ($p < 0.05$). RT= room temperature, H/C= heating/cooling

CONCLUSIONS

VCO produced by centrifugation technique was found to exhibit inhibitory activities against free radicals (ABTS, DPPH and superoxide radicals), tyrosinase and collagenase, all of which are involved in skin aging. Thus, it is potentially beneficial to cosmeceutical products as a functional ingredient for anti-aging applications such as whitening and anti-aging skincare, which seeks to decrease oxidative stress, hyperpigmentation and wrinkles in the facial skin. VCO cream also has good physical stability at different storage conditions.

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