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Antioxidant and tyrosinase inhibitory activity of face serum containing cocoa pod husk phytosome (*Theobroma cacao* L.)

Sani Ega Priani*, Saskara Aprilia, Ratih Aryani, Leni Purwanti
Pharmacy Department, UNISBA, Bandung, West Java, Indonesia.

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ABSTRACT

High polyphenol content of cocoa pod extract causing it potential to be developed as antioxidant and tyrosinase inhibitory agent in cosmetic preparations. Phytosome system known could enhance skin penetration of phytoconstituent like polyphenol-rich extract. The objectives of this research were to formulate phytosome containing cocoa pod extract, develop phytosome complex into face serum preparation, and determine antioxidant and tyrosinase inhibitory activities of the extract and the formulated serum. The cocoa pod extract was developed into phytosome system by thin-layer method using soy-phosphatidylcholine. The phytosome then develop into face serum formulation using Viscolam MAC 10 as a gelling agent. The antioxidant activity was conducted by 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay and tyrosinase inhibitory activity was conducted by colorimetric enzymatic assay. The cocoa pod extract has very strong antioxidant activity with inhibitory concentration (IC_{50}) of 17.21 ppm. The extract also has tyrosinase inhibitory activity with IC_{50} of 199.98 ppm. The phytosome complex containing cocoa pod extract and phosphatidylcholine (1:1) has good entrapment efficiency (90.5%) with an average particle size of 672 nm. The formulated face serum has good physical characteristic and also has antioxidant and tyrosinase inhibitory activities that equal with the marketed product (Hadalabo ultimate whitening milk, Rohto, Indonesia).

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is an important commodity in the world. Cocoa pod husk is a waste of cocoa processing in chocolate production (Fig. 1). The proportion of cocoa pod husk can reach 70%–75% of whole cocoa fruit that could cause an environmental problem (Daud *et al*., 2013; Irwanto *et al*., 2018). The increasing demand for chocolate will cause increasing of cocoa pod husk waste. Some studies have been conducted to explore the benefits of cocoa pod husk. Cocoa pod husk is known to have some pharmacology activities, including antibacterial, antifungal, and antioxidant (Hasanuddin *et al*., 2018; Rachmawati *et al*., 2018; Vega *et al*., 2018).

Cocoa pod extract is known to contain a high amount of polyphenol (about 15 %). The previous research showed that cocoa pod husk extract has antioxidant and tyrosinase inhibitor activities that make cocoa pod extract potential to be used in cosmetic preparations for the treatment of skin hyperpigmentation (Karim *et al*., 2014a; 2014b; Tebouke *et al*., 2018,). Hyperpigmentation is a common skin problem, in which skin become darker in color than normal caused by the increase of skin pigment (melanin) production (Krobotova, 2012).

Biosynthesis of melanin (melanogenesis) involves a series of complex enzymatic and oxidative reactions. Tyrosinase is an enzyme that stimulates melanogenesis process. This enzyme catalyzes the hydroxylation of L-Tyrosinase to L-3,4-dihydroxyphenylalanine (L-DOPA) and oxidation of L-DOPA to DOPA-quinone, at the initial process of melanogenesis. Using inhibitor tyrosinase in cosmetical products can inhibit the melanin production of the skin. Antioxidant compound can help to inhibit melanogenesis at the sequence of oxidation process (Ertam *et al*., 2018; Sun *et al*., 2014; Vashi and Kandu, 2013).

Some studies prove that polyphenol in the herbal extract has poor oral absorption and skin penetration (Scalbert *et al*., 2002; Surini *et al*., 2018). Formulation strategy must be applied to increase the oral absorption or skin penetration of polyphenol compounds. One of the effective strategies to enhance

*Corresponding Author
Sani Ega Priani, Pharmacy Department, UNISBA, Bandung, West Java, Indonesia. E-mail: egapriani @ gmail.com

Figure 1. Theobroma cacao.

skin penetration of phytoconstituent is phytosome formulation. Phytosome is a chemical interaction between water-soluble herbal compounds and phospholipids that can be applied for oral and topical administration. Phytosome is cell-like structures containing standardized extract or polyphenolic constituents (Das and Kalita, 2013; Kadu and Madhavi, 2017; Singh *et al*., 2011). Recent study shows the enhancing of polyphenol penetration through the skin with phytosomal formulation compared with non-phytosomal formulation (Pawar and Bhagyashree , 2015; Surini *et al*., 2018).

In this study, gel-based topical serum was selected as the final dosage form. Serum is a highly concentrated cosmetical product with low viscosity to enhance the effectivity. Gel-based dosage forms are considered convenient to be used because it has a high content of water that can hydrate the skin and easily spread when applied (Makino *et al*, 2017; Sasidaran *et al*., 2014).

The objectives of this research were to formulate phytosome containing cocoa pod husk extract from cocoa plantation in West Java Indonesia. The phytosome then develop into face serum dosage form. Antioxidant and tyrosinase inhibitory activities of the extract and the formulated serum were also determined.

MATERIALS AND METHODS

Reagents

Ethanol (Bratachem, Indonesia), Propylene glycol (Bratachem, Indonesia), Phosphatidylcholine (Lipoid, Switzerland), L-DOPA (Sigma-Aldrich, United State), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, United State), Mushroom Tyrosinase (Sigma-Aldrich, United State), and Viscolam MAC 10 (Lamberti, Italy).

Plant material

The cocoa pod were collected from the cocoa plantation in December 2018, West Java, Indonesia. The sample was identified in Herbarium Bandung Institute of Technology Indonesia.

Preparation and phytochemical screening of cocoa pod extract

Cocoa pod husk were dried (at 40°C) and powdered before extraction. Simplicia powder then extracted by maceration using ethanol 70% at comparison 1:3 for 24 hours (three repetitions). Rotatory vacuum evaporator was used to drying the extract (Rachmawati *et al*., 2018). Phytochemical screenings were conducted in plant simplicia and extract using the standard procedure to detect alkaloids, saponins, quinones, polyphenol, tannin, terpenoid, and steroid. (Franswort, 1966)

Determination total polyphenol content of cocoa pod extract

Polyphenol content was determined using the Folin-Ciocalteu Assay. 1 mL of extract solution (1,000 ppm) and gallic acid standard solution (30, 40, 50, 80, and 100 ppm) in methanol were added with 5 ml Folin-Ciocalteu solution 7.5% then preincubated in room temperature for 8 minutes. After preincubation, 4 ml of NaOH 1% solution was added to the mixture and incubated for 1 hour. The absorbance against the reagent blank was determined at 550 nm with an UV-Visible spectrophotometer. The absorbance of extract solutions was converted to polyphenol content using the regression equation of standard gallic acid. Total phenolics content was expressed as mg gallic acid equivalents (GAE) (John *et al*., 2014).

Antioxidant and inhibitor tyrosinase activity test of cocoa pod extract

Antioxidant activity tests were conducted by DPPH radical scavenging assay. 2 ml of extract solution in ethanol (5, 10, 15, 20, and 25 ppm) were added by 2 ml DPPH solution (60 ppm). The control solution was prepared by mixing ethanol (2 ml) and DPPH radical solution (2 ml). The mixture then mixes vigorously using vortex for 1 minute. The mixture solution incubated for 30 minutes at room temperature. The absorbance was determined at 517 nm. Percent inhibition of DPPH by the extract was then calculated and converted to inhibitory concentration 50 (IC_{50}) using regression equation (Boulanouar *et al*., 2017).

Inhibitor tyrosinase tests were conducted by a colorimetric enzymatic assay using a spectrophotometer. 20 µl of extract solution in dimethyl sulfoxide (6.25, 12.5, 25, 50, 100, 200, and 400 ppm) transfer to 96 well plate. 138 µl phosphate buffer (50 mM) and 2 µl tyrosinase enzyme (2,500 U/ml in phosphate buffer pH 6.5) were added to each plate. Finally, 40 µl L-DOPA (2.5 mM at phosphate buffer). The mixture then incubated for 10 minutes at 37°C. Absorbance in each well was read at 475 nm using microplate reader. The same procedure was conducted to arbutin as a reference.

% inhibition of the samples was calculated using the following equation:

$$\%\ \text{inhibition} = [(A - B)/A] \times 100\ \%$$

where A is the absorbance of mixture at 475 nm without inhibitor and B is the absorbance of mixture at 475 nm with inhibitor. % inhibition value then converts to inhibitory concentration 50 (IC_{50}) using regression equation (Karim *et al*., 2014; Wang *et al*., 2018, Wuttisin *et al*., 2017).

Formulation and characterization of cocoa pod phytosome

Phytosome complex formation was carried out by thin-layer method by combining cocoa pod extract and phosphatidylcholine with weight comparison (1:1) in ethanol. The mixture then sonicated for 30 minutes, followed by the evaporation process using rotary vacuum evaporator at 40 rpm (40°C), and resulting in a thin layer on the bottom of the flask. After overnight storage, the thin layer than solve by double distilled water using rotary vacuum evaporator at 45°C. To decrease the particle size of phytosome system, it was then ultrasonicated for 120 minutes (Maryana *et al*., 2016; Tahir, 2016).

The phytosome suspension then centrifuged at 12,000 rpm for 60 minutes to determine the entrapment efficiency (EE). Polyphenol content of the supernatant was determined using the same procedure with the extract (Folin-Ciocalteu Assay). The EE then calculated with the following equation with Qt is the total polyphenol content in phytosome suspension and Qs is the polyphenol content in the supernatant (Tripathy *et al*., 2018).

$$EE = \frac{Qt - Qs}{Qt} \times 100\ \%$$

Particle size distribution (mean diameter and polydispersity index) of the phytosome was measured by Particle Size Analyzer based on Dynamic Light Scattering (Beckman colter; United State).

Formulation and physical characterization of cocoa pod serum

Formula of face serum was presented in Table 2. The gel was prepared with disperse viscolam on distilled water with adding triethanolamine (TEA) using magnetic stirrer at 300 rpm. Ascorbic acid that has been dissolved in distilled water and methylparaben and propylparaben in propylene glycol were added to the previous mixture. The phytosome complex suspension was then added to the gel. The final mixture was homogenized using magnetic stirrer at 300 minutes for 10 minutes. (Surini *et al*., 2018).

The face serum then submitted to the cycling test. The preparations were placed at 4°C for 24 hours then moved to 40°C storage condition. The process was repeated in six cycles. Physical characteristics of the gel including organoleptic properties, homogeneity, pH, spreadability, viscosity, and rheological properties were compared before and after cycling test procedure. (Krongrawa *et al*., 2018)

Antioxidant and tyrosinase inhibitory activity tests of cocoa pod serum

The antioxidant activity test was carried out with a similar procedure for the extract. Diluted face serum (1,000 times) was submitted to the DPPH radical scavenging assay. Percent inhibition of the face serum was calculated and compared with a marketed cosmetic product containing arbutin, ascorbic acid, and hyaluronic acid as reference. The tyrosinase inhibition was carried out with a similar procedure for the extract (enzymatic colorimetric assay) to dilute face serum containing cocoa pod phytosome (5,000 times). The percent of enzyme inhibition of cocoa pod serum was compared with the marketed product (Hadalabo ultimate whitening milk, Rohto, Indonesia) (Zhao *et al*., 2015).

RESULTS AND DISCUSSION

Preparation and phytochemical characterization of cocoa pod husk extract

Maceration proses using ethanol 70% was done to extract the active compound of cocoa pod husk (semi polar–polar). The yield of the extraction process is 10.25%. Phytochemical screening of herbal simplicia and extract give a positive result for alkaloids, flavonoids, saponins, terpenoids, tannins, quionone, polyphenol, monoterpene, and sesquiterpene.

Determination polyphenol content of cocoa pod husk extract

Since polyphenol is the constituent that gives antioxidant and inhibitor tyrosinase activities, polyphenol content of the extract must be determined. Our study shows that the polyphenol content of the cocoa pod extract from cocoa plantation in Indonesia is 15.56% or 155.6 mg GAE/g. This result was similar with the previous study which states that polyphenol content of cocoa pod extract is 153.51 mg GAE/g (Teboukeu *et al*., 2018). While, in another study, the polyphenol content of cocoa pod extract using ethanol 80% as a solvent is 49.54 mg GAE/g (Karim *et al*., 2014). All the results show that more polar solvent (ethanol 70%) could extract polyphenol compounds better than ethanol 80%. Previous study conducted by Karim *et al*. (2014) using LC-MSMS showed that polyphenol compound of cocoa pod extract contains phenolic acid (protocatechuic acid, salicylic acid), flavonols (kaempferol), and flavons (linarin, apigenin, luteolin), stilbenoid (resveratrol), and crysoplenol (terpenoid).

Antioxidant and inhibitor tyrosinase activity test of cocoa pod husk extract

Cocoa pod extract will develop as an active agent in cosmetic preparation for treatment of hyperpigmentation. Inhibitor tyrosinase and antioxidant compound could prevent melanin production by inhibiting the enzymatic and oxidative reaction, respectively. Inhibitor tyrosinase activity was conducted by a colorimetric enzymatic assay using a spectrophotometer. Tyrosinase is a key enzyme in melanin biosynthesis. Inhibition of the enzyme could decrease the production of DOPA-chrome from L-DOPA as substrate. Decrease of red color intensity of DOPA-

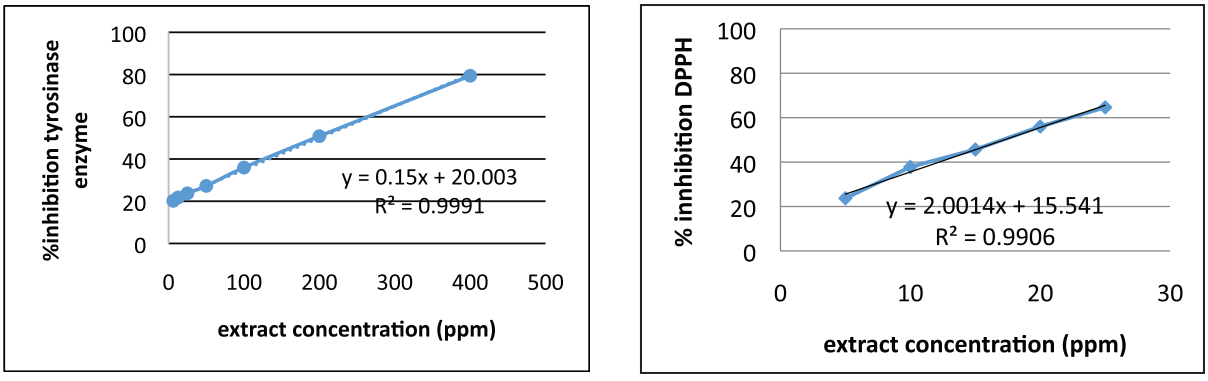


Figure 2. Linear regression curves of % inhibition.

chrome shows the inhibition of the enzyme by the extract (Chang, 2009; Di Petrillo *et al*., 2016). Antioxidant activity was determined by DPPH radical scavenging assay. DPPH is a stable free radical α-diphenyl-β-picrylhydrazyl. The assay based on the measurement of the scavenging capacity of antioxidants toward DPPH. The odd electron of a nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants. Decrease color intensity (violet) of DPPH shows free radical inhibition (Kadare and Singh, 2011). Correlation between percent inhibition (tyrosinase inhibitor and antioxidant) with extract concentration shown at Figure 2. Our study shows that cocoa pod extract has antioxidant and tyrosinase inhibitory activities due to high polyphenol content (Table 1). Study conducted by Karim *et al*. (2014) shows that cocoa pod extract (ethanol 80%) have antioxidant and tyrosinase inhibitory activities with IC50 26.1 ppm and 357.95 ppm, respectively. Our recent study shows stronger antioxidant and tyrosinase inhibitory activities of cocoa pod extract. Based on our analysis, the difference of solvent used at the extraction process causing differences of extract activity. Presence of phenolic acid, citric acid, malic acid, and other polyphenols in cocoa pod husk extract contribute very strong antioxidant activity of it (IC50 < 50 ppm). Stilbenoids, Flavonols, Flavanols, and phenolic acid in cocoa pod extracts contributed to tyrosinase inhibitory activity (Karim *et al*., 2014). The activity of the extract was weaker compared with ascorbic acid and alfa-arbutin due to the purity of extract is lower than references as pure active compounds.

Preparation and evaluation of cocoa pod phytosome

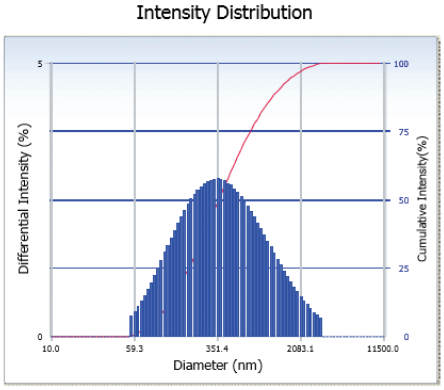
Phytosome formulation of the extract was carried out to improve the skin penetration of the active compounds (polyphenol); that is the reason causing phytosome technology to be widely used in cosmetics preparation (Awasthi *et al*., 2011). In phytosome complex, phytoconstituents are surrounded by phospholipid molecules. Since phosphatidylcholine, being an important component of the cell membrane, phytosome complex regulates the physiology of skin composition without damaging the epidermis and enhance skin penetration (Mazumder *et al*, 2016). Physical characteristic of phytosome was shown in Table 2. Result shows that EE of polyphenol compound in phytosome complex was 90.2%, which means the majority of polyphenol constituent has entrapped in phytosome complex. The result of particle size analysis shows that cocoa pod phytosome was categorized as a fine particle (100–2,500 nm) with narrow particle size distribution since PDI < 0.5 (Fig. 3) (Shailaja and Sugunthan, 2016).

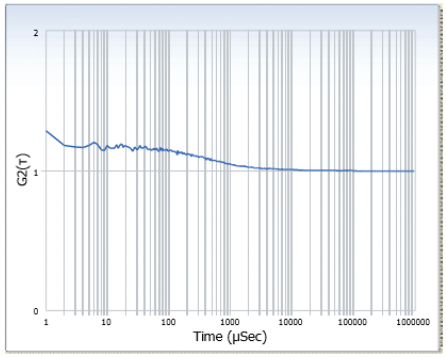
Table 1. Inhibitory concentration (IC_{50}) of cocoa pod extract.

Sample	Activity test	IC_{50} (ppm)
Cocoa pod extract	Antioxidant	17.21
Cocoa pod extract	Tyrosinase inhibitor	199.98
Vitamin C (reference)	Antioxidant	4.29
Arbutin (reference)	Tyrosinase inhibitor	121.33

Table 2. Physical characterization of cocoa pod phytosome.

Parameter	Result
Entrapment efficiency	90.05%
Mean particle diameter	627 nm
Polydipersity index (PDI)	0.259





Distribution Results (Contin)

Peak	Diameter (nm)	Std. Dev.
1	571.2	561.2
2	0.0	0.0
3	0.0	0.0
4	0.0	0.0
5	0.0	0.0
Average	571.2	561.2

Residual : 1.419e-002 (O.K)

Cumulants Results

Diameter	(d)	: 627.2	(nm)
Polydispersity Index	(P.I.)	: 0.259	
Diffusion Const.	(D)	: 8.767e-009	(cm^2/sec)

Measurement Condition

Temperature	: 29.4	(°C)
Diluent Name	: WATER	
Refractive Index	: 1.3323	
Viscosity	: 0.8059	(cP)
Scattering Intensity	: 10056	(cps)

Figure 3. Particle size analyzer result.

Preparation and evaluation of cocoa pod phytosome serum

For topical application, cocoa pod phytosome was then formulated as face serum using viscolam as a gelling agent. Formulation of the serum was described in Table 3. Physical stability of cocoa pod serum was determined by the cycling test. Physical characteristics of the serum were compared before and after cycling test period. The results show that cocoa pod serum has good physical stability at the change of storage temperature (Table 4). The preparation revealed non-Newtonian behavior, pseudoplastic (Fig. 4). Pseudoplastic is a desirable property of topical semisolid preparation since it should thin during application and thicken otherwise. (Shahin *et al*., 2011)

Tyrosinase inhibitory and antioxidant activities test of cocoa pod phytsome serum

Activity test was conducted to cocoa pod serum and compared with the marketed product (Hadalabo ultimate whitening milk, Rohto, Indonesia) containing arbutin, vitamin C, and hyaluronic acid. The result shows that the face serum of cocoa pod phytosome has antioxidant and tyrosinase inhibitory activities that relatively equal with the marketed product (Fig. 5). The cocoa pod face serum contains ascorbic acid that also contributes to antioxidant and tyrosinase inhibitory activities. From this final result, it can be concluded that cocoa pod extract has very potential to develop as an active ingredient of a cosmetic product. For the future, in vivo anti melanogenic activity will be done to the extract and the formulated serum.

Table 3. Formulation of face serum.

Ingredients	Amount (%)	Function
Cocoa pod extract phytosome	1	Active agent
Viscolam MAC 10	3	Gelling agent
Ascorbic acid	0.05	Antioxidant
Propylene glycol	15	Humectant
Methyl Paraben	0.18	Preservatives
Propyl Paraben	0.02	Preservatives
TEA	Qs	Adjustment pH
Distilled water ad	100	Solvent

Table 4. Result of cocoa pod serum physical evaluation.

Parameters	Results	
	Before cycling test	After cycling test
Organoleptic	Homogenous, Brownish, little odor of cocoa pod	Homogenous, Brownish, little odor of cocoa pod
pH	7.32 ± 0.01	7.32 ± 0.01
Spreadability (cm)	5.77 ± 0.06	5.90 ± 0.10
Viscosity (cps)	375.00 ± 5.00	373.33 ± 2.89
Rheology	Pseudoplastic	Pseudoplastic

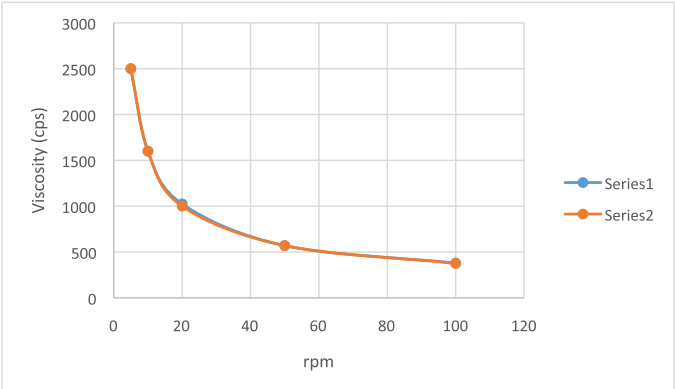


Figure 4. Rheologycal properties of cocoa pod serum.

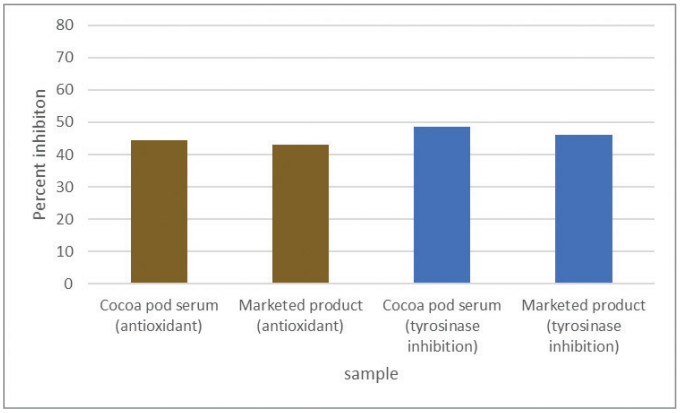


Figure 5. Cocoa pod serum activities.

CONCLUSION

Cocoa pod extract from cocoa plantation in Indonesia has potential antioxidant activity and tyrosinase inhibitory activities with IC_{50} 17.21 and 199.98, respectively. The phytosome complex containing the extract has 90.05% of EE with average globule size of 627 nm with narrow distribution size. The phytosome complex has been developed to be face serum preparation with good physical and stability characteristic. The formulated serum has antioxidant and tyrosinase inhibitory activities that equal with marketed product.

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

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