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NANOENCAPSULATION OF THE FLAVONOIDS ISOLATED FROM *PHALERIA MACROCARPA* LEAF BY CASEIN MICELLE

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ABSTRACT

Phaleria macrocarpa [Scheff.] Boerl. (PM) is known as “Mahkota Dewa”, which originates from Papua Island, Indonesia and grows in tropical areas. It is potential in treating diabetic, cancer and diuretic patients. Methanol extract with further fractionated by ethyl acetate (EAF) of PM leaf have anti-hyperglycemic activity. Flavonoids, the major compounds in the EAF, suggested responsible for this reported activity. Here, we encapsulate the flavonoids by casein micelle (CM) with a homogenizer followed by sonication. The sample was separated by ultra-filtration system and created nano-particles. Encapsulations capacity of the CM was about one milligram flavonoids per a gram CM. Size of particles was analyzed by particle size analysis (PSA) showed that the average size of particles was 109 nm. The results showed that CM to be potential nano-vehicle for the PM leaf extract.

KEYWORDS: **Anti-hyperglycemic, casein micelle, encapsulation, *Phaleria macrocarpa***

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INTRODUCTION

Phaleria macrocarpa [Scheff.] Boerl. (PM) is known as "Mahkota Dewa", and "Pau". It originates from Papua Island, Indonesia and grows in tropical areas. This plant has been used for traditional medicine in Indonesia [1, 2, 3]. It is potential in treating diabetic, liver, heart disease, kidney disorder, rheumatism, cancer and diuretic patients [1, 2, 4, 5, 6, 7, 8]. It was reported that the secondary metabolites from PM leaf has shown the presence of the flavonoids and it has found anti-hyperglycemic activity [8, 9].

Milk proteins contain 80% of casein, organized in micelles. Casein micelle (CM) naturally was designed for concentrate, stabilize and transport essential nutrients for neonate. Mammalian milk contain CM, Cow's milk contains 24-28 g of casein per liter. CM is spherical colloids which have average diameter of 150 nm, made by four caseins: two alpha casein, beta casein and kappa casein[10]. The caseins join together in the micelle by hydrophobic interactions and bridging of calcium-phosphate cluster bound to serine-phosphate residues present within casein molecules [11]. CM is able to encapsulate vitamin D2, curcumin and propolis form nano-particles [10, 12, 13]. The purpose of our study was to encapsulate the flavonoids from PM leaf and evaluate encapsulation efficiency and encapsulation CM properties.

MATERIALS AND METHODS

Cow milk was obtained commercially from market store around University of Indonesia, Depok, Indonesia. PM leaf was obtained from Tangerang prefecture, Jawa Barat. Indonesia.

(i) Preparation and characterization of the extract.

The extraction of PM leaf was carried out based on Sugiwati *et al.*, with some modification. 200 g PM leaf was weighed and placed into 2 l beaker glass. 1.5 l 0f 95% methanol was added and macerated for 4 days in room temperature. The sample was filtered with a Whatmann No. 1 filtered paper (Whatmann england). Filtrate was concentrated by using a vacuumed rotary evaporator (Buchii, Switzerland) at 40^{o}C. Then, the concentrate was fractionated with water (WF) and ethyl acetate (EAF) (1:1). The EAF was concentrated by using a vacuumed rotary evaporator (Buchii, Switzerland) at 40^{o}C, and dilution with 5 ml ethanol.

(ii) Total flavonoid assay

Total flavonoid was determined according to aluminum chloride ($AlCl_3$) method. 0,5 ml sample was placed in test tube, and added with 1,5 ml methanol, 0,1 ml 10% $AlCl_3$ (w/v), 0,1 ml 1 M potassium acetate and 2,8 ml aquadest, and incubated for 30 min in room temperature. The absorbance was measured at 415 nm by using spectrophotometer (spectroquant® pharo300, Merck). Quercentin was used as standard.

(iii)Anti-hyperglycemic activity measured by α-glucosidase inhibition assay

α-glucosidase inhibitor acts as competitive inhibitors of intestinal α-glucosidase. It can delay the digestion and subsequent

absorption of glucose by intestine. The EAF was pre-incubated with the substrate p-nitrophenyl- α-d-glucopyranoside (PNPG) before adding the enzyme. The enzyme activity was measured by determining the color developed by the product p-nitrophenol arising from the hydrolysis of substrate PNPG using spectrophotometric method.

The α-glucosidase inhibitory activity was measured with a set of test tubes which were labelled as black, controls and samples. To all test tubes 1,47 ml potassium phospate buffer, 3 μl of test sample and 750 μl of 20 mM PNPG was used. The sample was vortexed and incubated at 37 °C for 5 minutes, after incubation 600 μl of 0,04 μg/ml enzyme and incubated at 37 °C for 5, 10, 15 and 20 minutes. Finally the reaction was terminated by adding 3 ml of 200 mM Na_2CO_3. The absorbance of all the sample was measured at 400 nm ((spectroquant® pharo300, Merck))[14].

(iv)Isolation of the casein.

The casein was purified was fresh cow milk. The pH or milk was decreased until 6.4 by adding 1 N HCl. The sample was incubated at 30^{O}C for 1 hour then added rennet with agitation for 15 min at 30^{O}C. To increase size of it, the sample was incubated in same temperature for 15 min. The sample was filtered with a Whatmann No. 1 filtered paper (Whatmann england). The remained rennet was inactivated by incubated the sample by hot water (70 ^{O}C) for 5 mins. The hot water was removed by filtration with a Whatmann No. 1 filtered paper (Whatmann england).

(v) Encapsulation of the EAF by casein micelle.

The non covalent binding between casein with the EAF was achieved by dropewise 3 ml EAF into 30 ml phosphate buffer containing 3 g casein. While stirring the solution added 6 ml of 10% $CaCl_2$ was added. To get nano particle, the solution, was ultrasonicated for 5 minutes. The sample was filtered with a Whatmann No. 42 filtered paper (Whatmann england). The filtrate was collected and filtered again with a Amicon ultra-15 (Millipore). The retentate was suspended with phosphate buffer and analysed the size particle by particle size analyser.

(vi)Encapsulation efficiency.

The efficiency of encapsulation process was calculated as follow:

$$\text{The encapsulation efficiency} = 100\% - \left[\frac{\text{the total flavonoids of unencapsulted EAF}}{\text{The initial total flavonoids of EAF}} \times 100\%\right]$$

(vii) Particle Size analysis analysis.

The nano/micro particles sizes were determined using laser light scattering granularity analyzer (Delsa^{tm} Nano, Beckman Coulter). The sample was analyzed in duplicate.

RESULTS AND DISCUSSIONS

Isolation and anti-hyperglycemic characterization of PM leaf extract. This work was set to encapsulate PM leaf extract which is has anti-hyperglycemic activity and potent to treat diabetic disease. At beginning, we extracted PM leaf based on Sugiwati *et al*.[8]. The PM leaf was macerated with methanol, then, fractionated with water (WF) and ethyl acetate (EAF) (1:1). The EAF was reported have higher anti-hyperglycemic activity compare with others[8]

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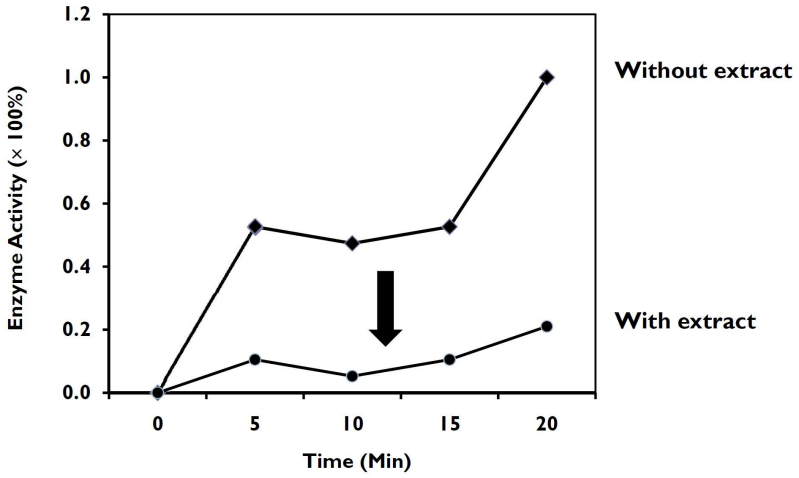

Figure 1

The inhibitory of α-glucosidase activity by the extract.

Furthermore, to confirm the anti-hyperglycemic activity of the EAF, ability of the sample to inhibit α-glucosidase was measured. Fig. 1 shows the inhibitory of α-glucosidase activity by the extract. The enzyme, without extract, hydrolysed PNPG to be p-nitrophenyl and α-d-glucopyranoside. The p-nitrophenyl could adsorb visible wavelengt at 400 nm, act as indicator of the enzyme activity[14]. The enzyme activity was stopped until 20 minutes and measured as 100% activity. The results show that the extract (EAF) could inhibit the enzyme in hydrolysing PNPG.

Encapsulation of the extract by casein micelle

The protein composition of isolated casein was analyzed by SDS-PAGE. Isolated CMs were suspended in phosphate buffer. To encapsulate the extract (EAF), while stirring, the extract was added to the suspension. The CM was stabilized by $CaCl_2$. Ca^{2+} ions were necessary to stabilize CM suspension[11]. To reduce size of particle, the suspension was ultrasonicated for 5 minutes. The unencapsulted EAF was separated by micro- and ultra-filtration. The retentate was resuspended with phosphate buffer. The efficiency of encapsulation process was

measured by comparing the total flavonoids before encapsulation and the total flavonoids of unencapsulated EAF. The results are shown in table 1. The efficiency of CM encapsulation was 42%. The results showed that the CMs had encapsulation capacity of 1000 μg flavonoids per gram casein.

Table 1

The effiency of encapsulation process

	Flavonoids (μg)
Before Encapsulation	7718
Unencapsulated of EAF (after encapsulation)	4478
Encapsulation Efficiency	**42%**

The size of the EAF-CMs particle was measured by determined using laser light scattering granularity analyzer (Delsa^{tm} Nano, Beckman Coulter). The sample was analyzed in duplicate. Fig. 2 shows that the means size of the particle was 99 nm the other one shows 119 nm (data not shown). The mean size of particle was 109 nm.

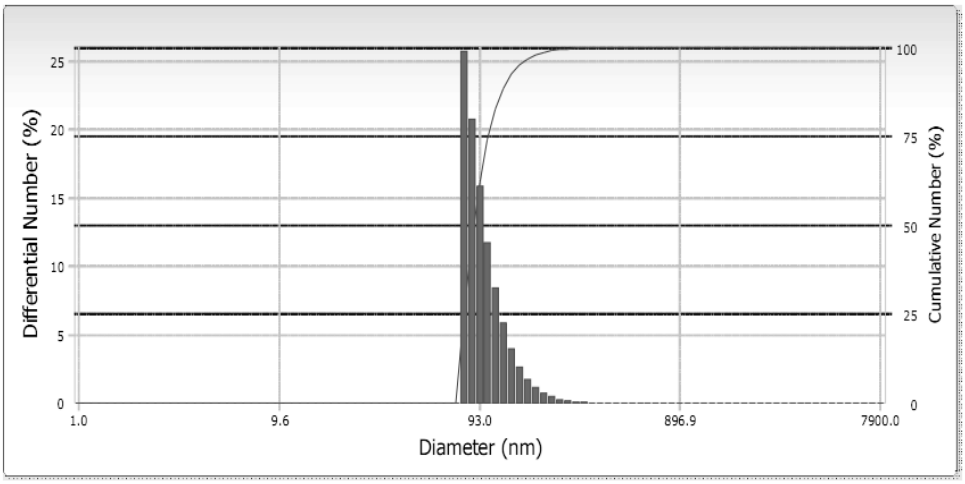

Figure 2

Size distribution of the nanoencapsulated EAF by CM.

As a conclusion, we successfully prepared the flavonoids-CM complex, which could be an alternative drug formulation of the flavonoids isolated from PM leaf for diabetic disease. We report that complex is a nanoformulation which has encapsulation efficiency about 42%.

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