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Production of Nanopropolis Using High Pressure Ball Mill Homogenizer

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Abstract. Propolis is one of the product that produces by bees. Propolis commonly used as a supplement because it has a bioactive compound. Propolis is hydrophobic, so it is not absorbed by the body very well, that's why it needs other technologies to solve this problem. Encapsulation is one of the answers because this technology has "drug delivery systems." The purpose of this study is to look at the efficiency of coating, particle size of nanopropolis and antibacterial activity. The result of this research shows that the efficiency of propolis coatings by flavonoids is 94,71%. Particle Size Analyzer (PSA) is used to measure the particle size. The result is nanopropolis has an average diameter 75,7 nm and 83,9 nm. The antibacterial activity of propolis against *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* was detected but not in nanopropolis.

Keyword: encapsulation, nanopropolis, casein micelle

1. Introduction

Biodiversity of Indonesia in the world is the second largest after Brazil. That diversity is a potential for a variety of products as well. The product produced with bees is affected by biological life around, such as propolis. Not only propolis, but bees also produce honey, royal jelly, and pollen. Propolis is a bee product of a complex resin that has a variety of physical properties, depends on many factors [1]. The word propolis is taken from the Greek language, "pro" means guards and the "polis" means a city. Generally, the propolis works maintain bee colonies and its products from invading microorganisms [2].

People often consume propolis because it is various of primacy. It is a natural product with antiseptics, antimycotics, bacteriostatic, astringent, spasmolytic, anti-inflammatory, anesthetic, antioxidant [3,4]. Propolis also has been used as an anti-inflammatory and antibacterial drug traditionally for centuries [5].

Propolis contains various compounds with bioactive components. The main compound of propolis is flavonoids and polyphenols. There are also other compounds such as terpenes [6]. Recently, most manufacturers are using ethanol as a solvent for propolis extract because it is more economical and easy to get. Currently, research is very much associated with propolis, starting from the content of propolis, propolis plant sources and it benefits. Propolis had antioxidant activity and its components can be used as sunscreen formulations (sunscreen). This development could be used as one of the propolis products.

Another use of propolis as a supplement, whether liquid or solid. There's one issue about propolis, and that is the nature of propolis properties can't dissolve in water (hydrophobic), and it causes the body human being cannot absorbed well because propolis properties has substance like oil [7]. Because of this matters, it need to be solve quickly in order to make propolis useful for bodies. To cause the bioactive compounds in the body can be optimally absorbed, there has been a solution in the processing propolis that can solve the problem. There is one technology that has existed is encapsulated with a coating casein micelle. These technologies apply the technique of encapsulation or coating using casein micelle. This encapsulation can be optimized change into nano-particles, it is called nanoencapsulation. Encapsulation is a technique which of a substance or mixture of coated



materials is trapped in the system [8]. A coated material called active or core material and coating material called the shell, wall material, carrier or encapsulant [8]. Thus this technique will be made nanopropolis coated with casein micelle using high-pressure ball mill homogenizer for making nano-sized particles.

Point of view from potential nanopropolis researchers, and that was research from Supardi (2011), the resulting product nanopropolis has a diameter of 316.1 nm [9]. Then the coating efficiency of the existing levels of flavonoids in propolis by 93.9%. The researcher will comparison the result from another research on this subject.

The main purpose of production nanopropolis made using high-pressure ball mill homogenizer is to obtain manufacturing methods that can produce potential benefits that can be used as an active ingredient for the design of future products, like sunscreen and other supplements. Based on these reasons the study of nanopropolis needs to be developed in a larger scale in Indonesia to obtain more value of propolis product.

2. Material and Methods

2.1. Materials

Cow's milk casein, rennet, ethanol 96%, extract of propolis (EEP).

2.2. Isolation of Casein from Cow's Milk

At this stage aims to gain casein from cow's milk. A total of 8 liters of pasteurized milk a cow that has been heated to a temperature of 35° C. When milk is heated, weighed 264 mg rennet but must be destroyed first become more refined as an originally shaped tablet. Rennet dissolved in 10 ml distilled water and wait as long as 20 minutes. This function can precipitate casein in milk. When the milk reaches a temperature of 35° C, rennet is mixed into the milk. To produce the maximum sediment milk has a stirring speed of 200 rpm/min for 45 minutes. Then milk was added distilled water with a temperature of 60° C to deactivate chymosin (rennet enzyme). Then allowed it to stand for 30-45 minutes, about room temperature. After that, the milk was put in the refrigerator for one night. After one night, casein in the sediment has done decantation and then washed with distilled water three times, each wash use 8 liters of distilled water and then do again, filtered and decantation. After that, we will get sediment that is casein. Casein obtained will be calculated dry weight.

2.3. Extraction of Propolis

A total of 2 kg of raw materials propolis were weighed, then crushed and added 10 liters of 96% ethanol. After that the maceration while in use agitator is stirring for 16 hours. Permeate Propolis Extract 96% ethanol (EEP 96%) and the resulting resin was filtered using a Vacuum Filters with ordinary filter paper (pore diameter ten µm). EEP permeate diluted 96% to 70% by adding distilled water, the precipitate formed when the addition of propolis wax. Then EEP 70% is heated at 50 ° C for 30 minutes, then left at room temperature for 15 minutes, then stored in the freezer for one night. Once the wax deposition were incubated overnight at room temperature, until the solution becomes clear and is formed of two layers, the clear top layer and the bottom layer is thick [10,11]. After that, the solution is filtered using a vacuum filter, and 70% EEP pH was raised to 6.4 by the addition of Na₂CO₃. Then take as much as 10 liters of a solution of 70% EEP had raised the pH and then mixed with 2 liters of glycerol, after which the solution in the distillation at a temperature of 65 ° C to obtain as much as 5 liters of ethanol. After getting as much as 5 liters of ethanol the temperature is raised to 80 ° C to remove the water content up to 1-2 liter until it reaches desired consistency.

2.4. Antibacterial Activity Assay

The method used is the disc diffusion method Disc diffusion method with paper discs prepared to accommodate a small sample of 40 ml. Each paper discs or holes filled with samples of propolis and nanopropolis. Nanopropolis have previously been dispersed in the supernatant ePCM (encapsulation propolis with casein micelle). Positive controls used were antibiotics chloramphenicol 30µg/disc [12]. Petri dish allowed to stand in the incubator for 24 hours at a temperature of 37° C. After incubation, the antibacterial activity can be observed with the formation of a clear zone in around of paper sample disc.

2.5. Extraction of Propolis

At this stage of manufacture nanopropolis, first of all that is casein as much as 1.3 kg wet weight was added a solution of phosphate buffer pH 10 in 5 liters, then stirred for 15 minutes, then add 1 liter of propolis, and then CaCl_2 10% was added 100 ml every 5 minutes in six times, during the process of adding CaCl_2 a pH is maintained at pH 7 using 0.1 N HCl or 0.1 N KOH. Then the mixture solution was centrifuged so supernatant and sediment yield, the process is at the stage before encapsulation can be used as nano-sized particles called encapsulation with casein micelle (ePCM). The resulting precipitate propolis encapsulation will be made into nano-sized particles in crushed by using a High-Pressure Ball Mill Homogenizer conducted in Nanotech Laboratory, Agency for the Assessment and Application of Technology (BPPT), Serpong. Then the precipitate which had become a nanopropolis with casein micelle (NePCM). NePCM re-dispersed in the resulting supernatant was 2.5 liters. Finally, the sample and the supernatant encapsulation nanopropolis analyzed total polyphenols, total flavonoids, particle size, and the antibacterial activity assay.

2.6. Coating Efficiency on Flavonoid and Polyphenol

The coating efficiency performed to determine the ability of casein coating of the active compounds from propolis extract. Therefore, some tests that the test is active compounds flavonoids and polyphenols.

2.6.1 Flavonoid Test

Method of aluminum chloride (AlCl_3) is used for determination of total flavonoids in propolis and the supernatant nanopropolis [13]. Standards used were quercetin, first made a calibration curve for quercetin (at concentrations of 12.5: 25.0: 50.0: 80.0; and 100 $\mu\text{g mL}^{-1}$ in methanol). EEP sample and the supernatant was pipetted nanopropolis of 0.5 mL and 1.5 mL methanol was added, 0.1 mL of 10% AlCl_3 (m/v), 0.1 mL of 1 M CH_3COOK and 2.8 mL distilled water. After the incubation for 30 min at room temperature, then the absorbance of the sample can be measured using a spectrophotometer at a wavelength of 415 nm.

2.6.2 Polyphenols Test

Determination of total polyphenol content of Follin-Ciocalteu method [14]. Standards used were a gallic acid with a concentration of 0; 50; 100; 150; 200; 250; $\mu\text{g mL}^{-1}$ was dissolved in methanol: distilled water (50:50, v/v). For the measurement of the sample was added 0.5 mL of reagent Follin samples were 5 mL (1:10 diluted with distilled water) and 4 mL of 1 M Na_2CO_3 , then stirred and allowed to stand for 15 minutes at room temperature, then absorbance was measured using a spectrophotometer at a wavelength of 765 nm. The test is then determined the total levels of the active compounds by using the following equation.

$$\text{Total levels of Compounds Active} = V \times C \quad (1)$$

By:

Levels of total active compounds [flavonoid/polyphenols] (μg)

V = Volume of the final sample (mL); C = Concentration of test samples ($\mu\text{g/mL}$)

2.7. Nanopropolis Size Analysis

Determination of size nanoparticles is determined using Particle Size Analyzer (PSA), Delta™ Nano C, Beckman Coulter, and the results of the expected size of the nanoparticles formed 100-500 nm size.

3. Results and Discussion

3.1. Isolation of Casein from Cow Milk

Casein is derived from cow's milk because according to Park et al. (2007) content of casein in cow's milk is greater than the milk of goats [15]. Higher casein content (2.6%) compared with the content of goat's milk (2.4%) and men (0.4%) than that of cow's milk is more easily obtained. Isolation of casein begins with the mixing of milk and rennet at a temperature of 35° C. This is because enzymes work optimally at a temperature of 35° - 40° C. The first isolation of casein by mixing milk and rennet at a temperature of 35° C so that the mixing process can take place quickly, rennet will work evenly to form a precipitated. There is a protease enzyme in rennet which is in charge of deciding ties on casein.

These enzymes are hydrolyzed specific binding chymosin on kappa-casein milk, resulting in termination of the bond, the milk, kappa-casein acts as a stabilizer [16]. After the activity is destroyed by chymosin, coagulation will occur so that the casein can be precipitated and separated by decantation and filtered. The resulting sediment is stored in the fridge and closed the meeting, because it is easily attacked by bacteria.

This method is made simpler in isolating casein. The result in a larger scale production is faster in producing casein. Casein is obtained to be tested dry weight. Casein was obtained in the wet weight of the final stage of the process of decantation while filtered. Weighed 1 gram wet weight and then put in the oven to 110 °C. Weighing is done once every 1 hour 3 data to obtain a constant weight.

3.2. Extraction of Propolis

At this stage of propolis extraction method used is a method of maceration. Maceration is one of the extraction method used for materials that are not heat resistant. This method of soaking the material with a specific solvent and a period at room temperature. In this study, the solvent used is ethanol 96%, which is semi-polar, so that the active compounds with a different polarity is expected to be extracted perfectly. Maceration method was used while doing the stirring (mixing) so that the active compounds are extracted and a lot faster. After maceration, 96% ethanol extract of propolis (EEP 96%) resulting in resin and wax (wax) is also participating extracted that were deemed to be a lot of impurities in the extract of propolis, so one needs to separate to obtain more pure propolis without impurities. On the separation of wax takes the optimal separation.

To achieve optimal separation between propolis with its impurities, performed by diluting using distilled water. Dilution was done using distilled water until the concentration of ethanol 70% (EEP 70%). Ethanol 70% is the optimum conditions for extracting propolis bioactive extract of propolis [10]. The purpose of this separation is that the solubility of wax on decreases, so the ethanol contained in propolis wax will precipitate and then separated by filtered. After that, the bottom layer of thick white candle that is separated using a vacuum filter. Propolis is already separated from the wax and resin. Then propolis diluted with glycerol (food grade) as a solvent that is safe for human consumption. Later stages of propolis have evaporated to remove ethanol and water levels. Propolis will look viscous after a process of evaporation because of there a solvent glycerol propolis. Ethanol was removed for safe human consumption. Water content was removed to more pure propolis and improve quality.

3.3. Antibacterial Activity Assay

Determination of antibacterial properties of propolis samples and nanopropolis performed by disc diffusion method against Gram-positive bacterium *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*. Concentration adjusted to concentrations of propolis samples were obtained for 40 µl of disk space. As a positive control used at a dose of antibiotics chloramphenicol 30µg/disc. Tests performed are limited to a qualitative test. By looking at whether or not to form the inhibitory zone around a paper disc that had been spilled samples. After it was incubated for 24 hours. The test is performed as triple (n = 3) for each type of test bacteria (Figure 1).

In the antibacterial test using the disc diffusion method was aware that the propolis samples occurs barrier against bacteria *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* bacteria, although the visible zone of inhibition was small. This suggests that propolis raw materials derived from the "Madu Pramuka" have antibacterial activity against all three bacteria. For casein and nanopropolis both methods are not visible on the antibacterial activity of gram-positive bacteria tested. In the sample of casein itself seemed no inhibitory zone it shows no antibacterial activity of casein. At nanopropolis well as barriers that did not happen, because the active substances contained in it is still enveloped in / stuck with casein micelle so that the active ingredient of propolis had no activity in inhibiting the bacteria tested. Looking at the results, it can be concluded that nanopropolis nanopropolis well as in consumption. Casein as a carrier that is able to protect up to reach the small intestine. Nanopropolis are coated by casein will reach the small intestine first. Active substances contained in the propolis did not spread the way to the small intestine so that the active ingredient of propolis will be absorbed when it reaches there.

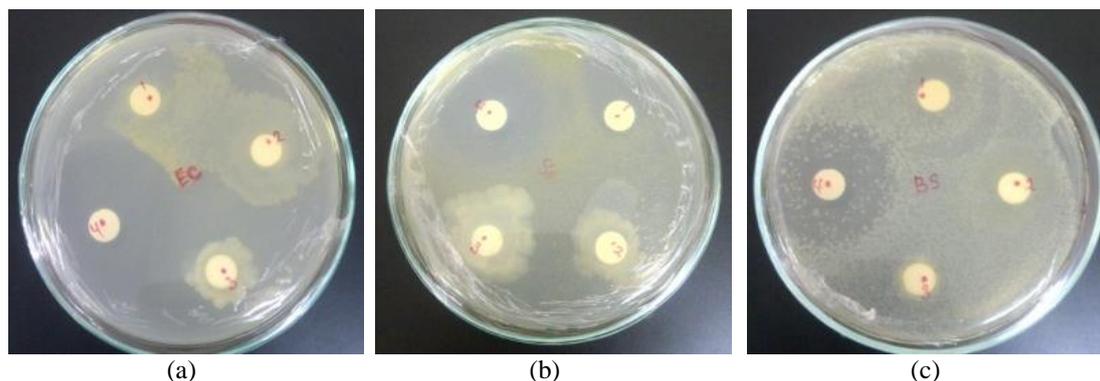


Figure 1. Propolis Antibacterial Activity Test Results, casein and nanopropolis by disc diffusion method. Propolis (1), Casein (2), Nanopropolis (3) Positive Control (4) Chloramphenicol 30 μ g/disc. 1(a) *Escherichia coli*, 1(b) *Staphylococcus aureus*. 1(c) *Bacillus subtilis*.

In previous research conducted by Dewi et al. (2018) propolis contains various compounds with antimicrobial properties for *Candida albicans*. In this study, phenolic content plays a major role in antifungal cases [17].

3.4. Encapsulation of Propolis Extract with Casein

In this process, the solution used for the coating process is a phosphate buffer. Phosphate buffer pH serves to maintain and to reshape the bridge of calcium phosphate, with the addition of CaCl_2 in stages so that casein can coat propolis. At the time of the addition of CaCl_2 pH is also maintained by adding a solution of 0.1 M HCl, 0.1 M KOH. After the coating of casein with propolis, further to reduce the size of a nano particle tools used "High-Pressure Ball Mill Homogenizer." Samples will be destroyed by ground first, then with high pressure in the particle push and hit the ring in the instrument, so that a nano-sized particle distributions. In large-scale manufacture of these devices suitable for use because in first running its use is 1 kg sample that can be used as nano-particles. In the study conducted Semo et al. (2007), the process used to form the nano-size high-pressure centrifugation. In the present study, performed using the High-Pressure Ball Mill Homogenizer and the result does not affect the activity of the resulting product [18].

3.5. Coating Efficiency

Tests were performed using the spectrophotometric analysis of two parameters, namely the levels of polyphenols and flavonoids content. The method used to measure the polyphenols is a method Follin-ciocalteau, in the sample when there are polyphenolic compounds will give a blue color after addition of reagent Follin and absorbance was measured with a spectrophotometer, the results obtained compared with the standard test used, namely gallic acid.

For flavonoid analysis using aluminum chloride (AlCl_3), in the sample when there are flavonoid compounds will give a greenish yellow color after the addition of AlCl_3 , the results are compared with the standard test used, namely quercetin. After the measurements performed calculations to determine the levels of each test. Here is the Table 1. Spectrophotometer analysis results for these tests.

Tabel 1. Spectrophotometric Assay Results

No	Sample	The content of Flavonoid (μg)	The content of Polyphenol (μg)
1	Propolis	2.028.571,4	1.406.667
2	Supernatant ePCM	107.142,8	10.633.333
	Coating Efficiency	94,71%	0%

In Table 1. can be seen levels of total flavonoids of propolis 2.028.571,4 mg and total flavonoid content of 107,142.8 mg supernatant EPCM. From these results can be calculated coating efficiency. From the experimental results, the coating efficiency of flavonoids by 94.71%. For polyphenolic compounds do not occur because the casein coating is covered to the maximum flavonoid compounds that are no longer any room for coated casein.

According to Chen et al. (2006), a good coating efficiency of at least 80%, because it shows the process does not eliminate the existing active substances [7]. In the study Semo et al. (2007) obtained 85% efficiency with the casein micelle coating to coated single compound, that is vitamin D2 [18]. In a previous study that Supardi (2011) obtained 93.9% efficiency using casein micelle coating with propolis "Cibubur" active ingredient. Supardi (2011) also have levels of flavonoids from propolis cibubur of 1846.15 ppm, while the results of my research of propolis flavonoid content of raw materials derived from the "Madu Pramuka" has levels of 2028.57 ppm [9]. This research is very good for getting levels of flavonoids and flavonoid bioactive coating efficiency is higher than previous studies.

3.6. Nanoparticle Size Analysis

Measurement of nanoparticles using particle size analyzer (PSA). Particle measurements carried out to see the particle size of casein micelle nanopropolis with the coating. Measurements were made on the final product that is nanopropolis which is a result made into the size of the nanoparticle size using a high-pressure ball mill homogenizer. In the test particle size is expected to form the nano-sized particles. The importance of the results obtained in the form of nanoparticles due to the nano-sized products, the drug delivery process to be more selective in maximal and specific areas of the body and minimize the occurrence of side effects [7].

From the results of particle measurements using the PSA, in samples taken twice nanopropolis measurements. In the first measurements of the samples had an average diameter of 75.7 nm, 86.3 nm of the largest and smallest measurement of 68.9 nm, while for the latter having an average diameter of 83.9 nm, 94.1 nm of the largest and smallest 72 nm. Based on these results, to produce nanoparticles (<1000 nm) [7,19]. Also affects the particle size to the nano-delivery system can be developed when NePCM for the design of such supplements and other products consumed by the body, it is very advantageous because it is easily absorbed. To assure again that these nano-sized particles should be analyzed SEM (Scanning Electron Microscope) or TEM (Transmission electron microscopy), but because it is still constrained by the tools that have not been found in Indonesia, this analysis can not be done.

In the previous study Supardi (2011) that the particle size can be 316,1 nm [9]. Particles to this size are still quite large compared with the results i get. Particle-making process on research Supardi (2011) is sonication. This technique uses a tool that makes sonicator nano-sized particles due to ultrasonic waves [11]. The smaller particle size of a material which has a nanoparticle size will be more easily absorbed by the body [20]. High-pressure ball mill homogenizer tools has proven the resulting particle size is smaller.

4. Conclusion

From this research there are several conclusions can be drawn that propolis extracts may be made by coating nanopropolis casein micelle. Coating efficiency of propolis by the casein micelle to 94.71% flavonoids. The compound of flavonoids coated in the casein represents 5576.95 gram in every 1 gram dry weight of casein. Propolis shows antibacterial activity against the bacteria *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*. Nanopropolis particle measurements have an average diameter of 75.7 nm, and the second measurement of particles having an average diameter of 83.9 nm. The result from Particle Size Analyzer (PSA) indicate that smaller is better, it means the body can absorbed nanopropolis easily.

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