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COMPARISON STUDY OF IONIC GELATION AND SNEDDs METHOD IN THE PREPARATION OF COCOA PEEL EXTRACT NANOPARTICLES AS ANTIBACTERIAL AGAINST *Klebsiella pneumonia*

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ABSTRACT

Cocoa peel is the primary waste from chocolate production. It contains flavonoids that can inhibit the growth of *Klebsiella pneumonia* which causes chronic bronchitis. This study aims to obtain cocoa peel extract nanoparticles for antibacterial caused by *K. pneumonia*. The isolation of flavonoids was done using the maceration method in 96% ethanol. Phytochemical and Thin Layer Chromatography (TLC) analyses have been used to identify the active compounds contained in the extract. Flavonoid levels have been investigated using UV-Visible spectrophotometry. The preparation of nanoparticles from cocoa peel used ionic gelation and Self-Nano Emulsifying Drug Delivery System (SNEDDS). The nanoparticle obtained has been analyzed for particle size and polydisperse index. In vitro test was carried out to determine the antibacterial activity of *K. pneumonia*. Identification of active compounds using a UV-Visible spectrophotometer showed that cocoa peel extract contained flavonoids of 6.33%. Nanoparticle preparations using the SNEDDS method were more optimal than the ionic gelation method, with particle sizes 135.2 nm (4%), 156.1 nm (12%), and 235.3 (20%). Based on the in vitro test, nanoparticles from cocoa peel extract were able to inhibit the growth of *K. pneumonia*
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Keywords: *bronchitis, flavonoid, ionic gelation, maceration, SNEDDs*

INTRODUCTION

Cocoa (*Theobroma cacao L.*) is one of the export commodities with a high deal. Indonesia is the third largest cocoa producer in the world, with distribution reaching 13.6%. At harvest time, the farmers only harvest cocoa seeds to be processed into various chocolate products, and a large amount of cocoa peel waste is released. Cocoa peel is a waste generated from the processing of cocoa beans. Every ton of dry cocoa beans will produce 10 tons of cocoa peel. If the cocoa peel is not processed, it will cause environmental pollution [1,2].

Previous research has stated that cocoa peel contains flavonoid compounds. Wiyono et al. [3] have investigated that cocoa peel extract contains 344.6 ± 18.7 g/mL of total flavonoids. Cocoa peel extract contained 0.0113% flavonoid [4]. In addition, the cocoa peel contains flavonoids used as an antibacterial against *Escherichia Coli* and *Staphylococcus Aureus* [1]. Flavonoids have also been used as an antibacterial against *K. pneumonia* [5]. Nabi et al. [6] have used leaf extract of *Skimmia anquetilia* containing flavonoid as an antibacterial of *K. pneumonia*. Based on their research, the leaf extract of *Skimmia anquetilia* showed the highest zone of inhibition against K. pneumonia (17 mm). Red galangal rhizome extract containing flavonoids can inhibit *K. pneumonia* growth in rats [7]. *K. pneumonia* is one of the bacteria that cause chronic bronchitis [8].

Clinical therapy to treat bronchitis is the use of antibiotics. The conventional antibiotic used is amoxicillin, amoxicillin-clavulanic acid, azithromycin, cefuroxime, cefprozil, cefdinir, cefditoren, cefixime, cefpodoxime, ceftibuten, clarithromycin, doxycycline, levofloxacin, and moxifloxacin [9]. However, antibiotic use's side effects are allergic, idiosyncratic, and toxic reactions. In addition, antibiotics can change biological and metabolic in the host [10]. Alternatives to solve this problem are utilizing the compounds in the cocoa peel to treat bronchitis which is effective and efficient.

Previous researchers have used cocoa peel extract as an antibacterial, such as *E. Coli*, *Salmonella sp*, and *Staphylococcus aureus* [11]. Santos et al. [12] and Al-Shalah et al. [13] investigated cocoa peel extract's activity against *K. Pneumonia*. However, no research used nanoparticles of cocoa extract as *K. pneumonia* antibacterial. So, the novelty of this research is the use of cocoa peel waste, an environmental problem in Indonesia, in nanoparticle products for chronic bronchitis treatment. There are several advantages of the drug in the nanoparticle form as able to penetrate target cells more quickly and on target. In addition, the size change to nanometer has increased physical and chemical properties compared to bulk size.

Ionic gelation is a drug delivery method to produce particles in nanometer size. For example, the ionic gelation method has used to obtain nanoparticles of *Epilobium parviflorum* extract with a length of 64.47 nm [14]. The ionic gelation method involves a cross-linking between multivalent ion pairs and polyelectrolytes. For example, chitosan is a polyelectrolyte that can react with tripolyphosphate as a multivalent ion. The formation mechanism of nanoparticles includes mixing two liquid phases, one phase containing multivalent ions and the other containing chitosan [15].

Furthermore, the delivery of drug nanoparticles can also use Self-Nano Emulsifying Drug Delivery System (SNEDDs) method. SNEDDs can produce drug particles in nanometer size. The lipid-based drug delivery system of SNEDDs can deliver the active compound in the drug gradually (slow release), facilitate drug dissolution and increase drug absorption in the human body [16]. Therefore, this study aimed to investigate the activity of nanoparticles from the cocoa peel that was synthesized using

the ionic gelation and SNEDDs method as an antibacterial against *K. pneumonia*-causing bronchitis disease.

METHODS

Materials and Instrumentation

The materials used in this study included deionized water; cocoa peel waste obtained from cocoa farmers in Nglanggeran, Pathuk, Gunung Kidul; magnesium/Mg powder, hydrochloric acid/HCl, acetic acid, sodium acetate, and aluminium chloride/AlCl3 purchased from Merck; chitosan, sodium triphosphate (Na-TPP), tween 20, capryol, polyethylene glycol/PEG and rutin obtained from Sigma-Aldrich; 96% ethanol and methanol. A Particle Size Analyzer (Horiba Scientific, Nano Particle Analyzer SZ-100) was used to characterize the particle size and zeta potential. In addition, the UV-Visible spectrophotometer (Hitachi UH 5300) was used to determine the total flavonoid content in the cocoa peel extract.

Plant Materials

The cocoa plant used in this research has been classified as *Theobroma cacao L* based on the determination test. This test was carried out at the Plant Systematics Laboratory, Faculty of Biology, Gadjah Mada University, Yogyakarta. The samples used are the stems, leaves, flowers, and fruit from cocoa plants.

Extraction of Cocoa Peel

Cocoa peel extract using the maceration technique. First, the dried cocoa peel is ground using a grinder. Then, 40 grams of cocoa peel powder was dissolved in 98% ethanol with a ratio of 1:10 between the sample and ethanol. The mixture was stirred. After 24 hours, the mixture was filtered. The filtrate obtained was concentrated using a rotary evaporator to extract cocoa peel. Based on the organoleptic tests, including examination of the shape, colour, and odour of the cocoa peel extract, it is known to have a gel form, a characteristic aroma of cocoa, and dark brown. The % yield of cocoa peel extract obtained was 21.91%.

Phytochemical Screening and Thin Layer Chromatography of Cocoa Peel Extract

Flavonoid Test. The flavonoid test was carried out using the Shinoda test by adding concentrated Mg powder and HCl to the cocoa peel extract in a test tube. The Shinoda test showed positive results if the colour changed to brownish green and yellowish green [17].

Steroid Test.

The steroids were identified by adding 0.5 mL of anhydrous acetic acid and 2 mL of concentrated sulfuric acid into a tube containing cocoa peel extract. This reagent is known as Libbermen-Burchard. The formed colour is red, blue, and green, indicated existing of sterols [18].

Thin Layer Chromatography.

The TLC test was carried out to determine flavonoids contained in cocoa peel extract using 60 F254 silica gel plate as stationary phase and n-butanol: acetic acid: water, 4: 1: 5 (v/v) as mobile phase.

Total Flavonoid Levels of Cocoa Peel Extract

a. Standard solution of rutin (35; 45; 55; 65; 75 ppm)

A total of 1.75; 2.25; 2.75; 3.25; and 3.75 mL of 1000 ppm rutin solution were put into a 50 mL volumetric flask, and methanol was added to the mark. After that, 1 mL of

each was taken, then 3 mL of methanol, 0.2 mL of 10% aluminium chloride, 0.2 mL of sodium acetate (1 M), and 5.6 mL of distilled water were added. Next, it was centrifuged and incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm by a UV-Visible spectrophotometer. The same was also done in filling blank solutions without adding rutin solutions.

b. Sample Solution

100 mg of cocoa peel extract was dissolved in 100 mL methanol in a 50 mL volumetric flask. Next, 1 mL of the solution was taken and added 3 mL of methanol, 0.2 mL of 10% aluminium chloride, 0.2 mL of 1 M sodium acetate, and 5.6 mL of distilled water. Then, it was centrifuged and incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm by a UV-Visible spectrophotometer.

Synthesis of Cocoa Peel Extract Nanoparticles Using Ionic Gelation Method

Variation of chitosan concentration. 0.2 g of cocoa peel extract was dissolved into 5 mL of chitosan solution with various concentrations, as shown in Table 1. Next, 2.5 mL of 0.1% w/v Na-TPP solution was added drop by drop with a syringe pump while homogenized using an aerator and stirrer for 30 minutes.

Variation of extract weight. Cocoa peel extract with weight variations, as shown in Table 1, was dissolved into 5 mL of chitosan solution (0.1% w/v). Next, 2.5 mL of 0.1% w/v Na-TPP solution was added drop by drop with a syringe pump while homogenized using an aerator and a stirrer for 30 minutes.

Table 1. Chitosan concentration and extract mass variations in the preparation of cocoa peel extract nanoparticles using the ionic gelation method

Chitosan Concentration (%)	Extract mass (g)
0.1	0.2
0.2	0.2
0.3	0.2
0.4	0.2
0.1	0.1
0.1	0.3
0.1	0.5

Synthesis of Cocoa Peel Extract Nanoparticles Using SNEDDS Method

The cocoa peel extracts (0.05; 0.1; 0.3; and 0.5 g) were put into a vial, and then 1.25 mL of tween 20 was added and sonicated for 2x2 minutes. Next, 0.5 mL of PEG was added and sonicated for 2x2 minutes. Then 0.75 mL of capryol was added and sonicated for 2x2 minutes. As a result, the cocoa peel extracts nanoparticle 2; 4; 12; and 20 % will be obtained.

In Vitro Test of Cocoa Peel Extract Nanoparticles

The bacterial colonies of *K. pneumonia* were added to 0.9% NaCl solution to equalize the turbidity of the bacterial colonies with the Mc. Farland standard (1.5 x 108 colony form units/mL). Then, it is smeared on a petri dish containing MH nutrient agar media. Then a well is made with a diameter of 6 mm in the test medium. Next, the well is given cocoa peel extract nanoparticles. After that, the Petri dishes were incubated at 37 °C for 48 hours. The observation was done by measuring the inhibition zone diameter of bacterial growth using a colony counter instrument.

RESULTS AND DISCUSSION

Phytochemical Screening and Thin Layer Chromatography of Cocoa Peel Extract

Phytochemical screening has been carried out on the cocoa peel extract. Based on phytochemical screening (Table 2), cocoa peel extract contains flavonoids. It is consistent with the research of Singh et al. [19], Hasanuddin et al. [11], Santos et al. [12], and Al-Shalah et al. [13]. The identification of flavonoids was made using thin-layer chromatography (TLC) to strengthen the allegations of phytochemical screening results. The eluent used was butanol: acetic acid: water (4:1:5). The TLC test result is shown in Figure S1. Four spots are based on the TLC test results (Table 3). Spots 1 and 4 belong to flavonoid compounds.

Table 2. Phytochemical screening results of cocoa peel extract

Phytochemical Test	Reagent	Results	Conclusion
Flavonoid	HCl and Mg	Red	+
Steroid	Liebermann-Burchard	No greenish blue ring	-

Note: (+) = exist; (-) = not exist

Table 3. Identification of compounds in cocoa peel extract using TLC

Spot	Rf	Visible		UV 366 nm		Compound
		Without NH_3	NH_3	Without NH_3	NH_3	
1	0.55	-	Light yellow	Blue	Blue	Flavonoid
2	0.70	-	Light yellow	-	Mauve black	-
3	0.88	-	Light yellow	-	Mauve black	-
4	0.92	-	Light yellow	Blue	Blue	Flavonoid

Total Flavonoid Levels of Cocoa Peel Extract

The standard solution is rutin because most flavonoids are often found in glycoside, such as quercetin 3-rutinoside or rutin [20]. Rutin standards are characterized using a UV-Visible spectrophotometer at a maximum wavelength of 420 nm (Figure 1). Rutin has conjugated π bonds which causes the molecule can absorb light in the visible range [21]. Therefore, the increase in rutin concentration can cause an increase in absorbance (Table 4).

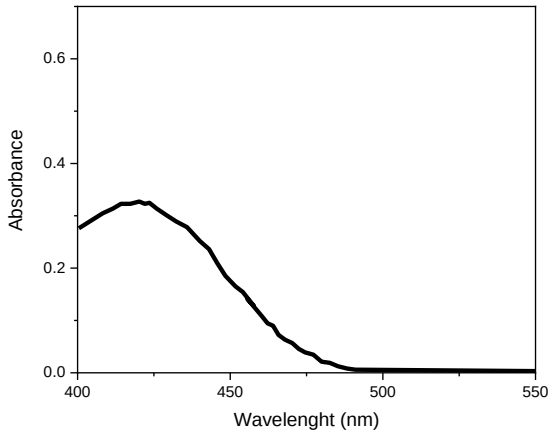

Figure 1. The UV-Visible spectrum of rutin standard

Table 4. Results of rutin standard analysis using UV-Visible spectrophotometer

Rutin Concentration (mg/L)	Absorbance
35	0.092
45	0.127
55	0.189
65	0.214
75	0.281

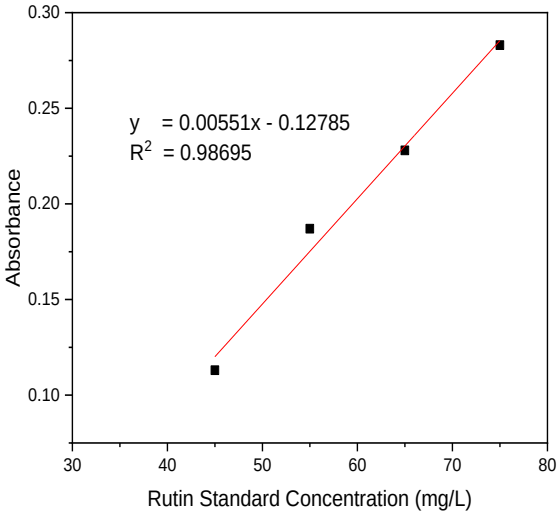

Figure 2. Regression curve of rutin standard

The regression curve of the rutin standard is shown in Figure 2. A linear equation is found y = 0.0046x-0.0752 and R^2 = 0.9833. It indicated that the analytical data obtained have good linearity. This linear equation can be used to determine the flavonoid concentration of cocoa peel extract. Table 5 shows the maximum absorbance and wavelength of the cocoa peel extract. The maximum wavelength of cocoa peel extract is 420 nm, indicating the existence of flavonoid compounds. It is consistent with previous research that conjugated pi bonds (chromophore group) in flavonoid structure cause the molecule to absorb light in the visible range [21]. The flavonoid concentration in the cocoa peel extract has been calculated and found to be 6.33%. This concentration is higher than in the previous report [4]. Therefore, this research's extraction method is more suitable for obtaining flavonoids from the cocoa peel.

Table 5. Results of cocoa peel extract using UV-Visible spectrophotometer

Number	Maximum wavelength (nm)	Absorbance
1	420	0.21
2	420	0.22

Particle Size of Cocoa Peel Extract Using Ionic Gelation Method

Characterization using Particle Size Analyzer (PSA) aims to determine the size of cocoa peel extract nanoparticles. Table 6 shows the PSA characterization result of the cocoa peel extract nanoparticle obtained using the ionic gelation method with varying concentrations of chitosan. Chitosan was chosen in this study because it has some properties such as chelating, biocompatible, and biodegradable. However, chitosan has a high degree of swelling in an aqueous environment, so the drug's application as delivery and release is less profitable. Therefore, it is necessary to add Na-TPP to produce chitosan derivatives with high biocompatibility and low swelling. The formation mechanism of nanoparticles is based on electrostatic interaction between the amine group of chitosan and the negative group of polyanion on the tripolyphosphate [15].

Based on Table 6, the higher concentration of chitosan added caused an increased particle size because agglomeration of the chitosan molecules occurred. The greater chitosan concentration with a constant amount of Na-TPP will also

increase the particle size because of the tendency to agglomerate. At high concentrations, the particles formed from the reaction between chitosan and TPP are very numerous and dense, so they group to form aggregates into micro-sized particles. Based on Table 6, it is known that the smallest particle size obtained using 0.1% chitosan concentration is 464.40 nm. The smaller the extract's particle size, the higher its effectiveness as an antibacterial [22].

The polydispersity index (PI) ranges from 0 to 1. A PI value close to 0 indicates a homogeneous dispersion, whereas a value greater than 0.6 indicates a high heterogeneity. Based on PI obtained from the cocoa peel extract measurement, nanoparticles with chitosan concentration variations do not exceed 0.6, indicating that the particle dispersion is quite homogeneous and stable from the possibility of particle collision and gravity separation [23].

Table 6 also shows the particle size's effect due to the extract mass increase. Increasing extract mass from 0.1 to 0.2 g caused particle size growth from 423.47 to 464.40 nm. Further increase in cocoa peel extract mass to 0.3 g causes particle size to 614.30 nm. The greater the mass of cocoa peel extract, the greater the particle size. The smallest particle size was obtained using 0.1 grams of extract mass.

Table 6. The size characterization of cocoa peel extract nanoparticles obtained using the ionic gelation method

Chitosan Concentration (%)	Extract mass (g)	Particle Size (nm)	Polidispers Index
0.1	0.2	464.40	0.49
0.2	0.2	492.97	0.50
0.3	0.2	519.07	0.46
0.4	0.2	553.70	0.56
0.1	0.1	423.47	0.57
0.1	0.3	614,30	0.33
0.1	0.5	614.60	0.45

Particle Size of Cocoa Peel Extract Using SNEDDS Method

The particle size of cocoa peel extract nanoparticles obtained using the SNEDDS method with extract mass or extract concentration variations (4; 12; and 20%) is 135.2, 156.1; and 235.3 nm, respectively (Table 7). From these data, it is known that there is an effect of particle size due to an increase in extract mass, the same as using the ionic gelation method. The greater mass of cocoa peel extract used, the greater the particle size. The smallest particle size was obtained using an extract mass of 0.1 grams, 135.2 nm. Therefore, the size of cocoa peel extract nanoparticles using the ionic gelation method is smaller than the SNEDDS method. Consequently, it can be concluded that the SNEDDS method is the most effective method for preparing cocoa peel extract nanoparticles. Based on the PI obtained, cocoa peel extract nanoparticles using the SNEDDS method do not exceed 0.6, indicating that the particle dispersion was relatively homogeneous.

Table 7. Cocoa peel extract nanoparticle size with mass variations of cocoa peel extract obtained using SNEDDS method

Extract Mass	Extract Concentration (%)	Particle Size (nm)	*Polidispers Index*
0.1 g	4	135.20	0.39
0.3 g	12	156.10	0.36
0.5 g	20	235.30	0.47

Zeta Potential of Cocoa Peel Extract Nanoparticles

The zeta potential of cocoa peel extract nanoparticles was studied (Figure 3 and Figure 4). Increasing mass of cocoa peel extract in the preparation of cocoa peel extract nanoparticles using the SNEDDs method can shift zeta potential in a positive direction. It indicates that the increased cocoa peel extract mass causes the surface of cocoa peel extract nanoparticles to be positively charged. Although cocoa peel does not contain pure flavonoids, other compounds cause positive surface charge, such as alkaloid compounds [4,24].

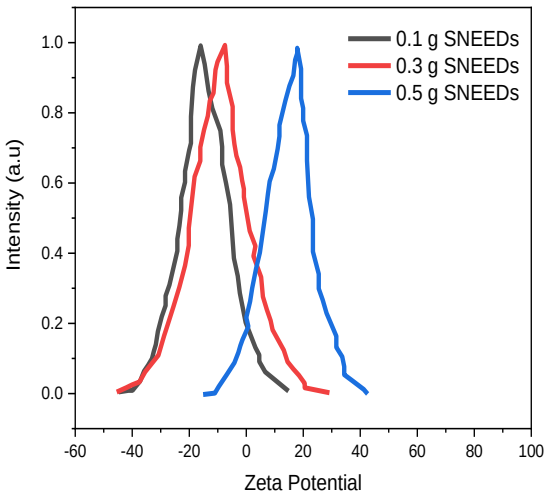

Figure 3. Zeta potential of cocoa peel extract nanoparticles obtained using SNEDDs method

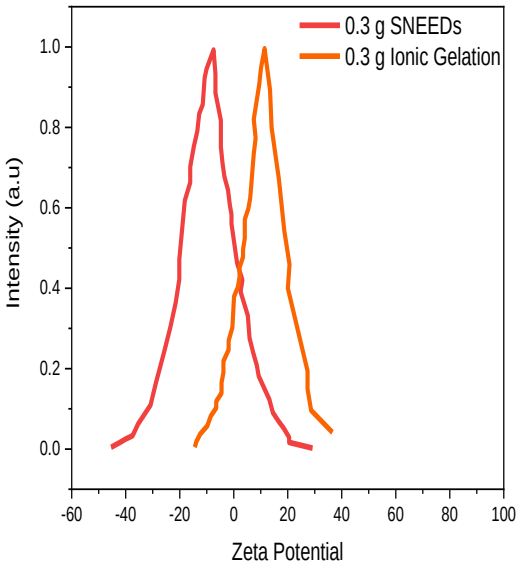

Figure 4. Zeta potential comparison of cocoa peel extract nanoparticles obtained using SNEDDs and ionic gelation method

Compared with the ionic gelation method, the nanoparticles of cocoa peel extract using the SNEDDs method have a less positive zeta potential than the ionic gelation method. It can be attributed to the size of cocoa peel extract nanoparticles. SNEDDs method uses capryol and PEG, which can improve the production of negative charge on the surface so that electrostatic repulsion between particles and particle aggregation does not occur. As a result, n-occurring assembly causes particle size to be smaller [25]. The smaller extract size will increase its effectiveness as an antibacterial agent [22].

In Vitro Test of Cocoa Peel Extract Nanoparticles

In vitro test was conducted to determine the activity of nanoparticles and crude extract of the cocoa peel against *K. pneumonia* bacteria. The diffusion method has carried out the antibacterial activity in this study. Visually, the results of the 48-hour growth inhibition from *K. pneumonia* can be seen in Figure S2.

Table 8. Diameter of the bacterial inhibition zone based on in vitro test

Sample		Inhibition Zone Diameter (mm)		Average (mm)
		I	II	
Cocoa Peel Extract Nanoparticles	2%	9.60	8.90	9.25 ± 0.49
	4%	11.60	10.90	11.25 ± 0.49
	12%	10.70	11.10	10.90 ± 0.28
	20%	11.40	10.70	11.05 ± 0.49
Crude Cocoa Peel Extract	2%	8.50	9.60	9.05 ± 0.77
	4%	9.30	8.90	9.10 ± 0.28
	12%	9.30	9.60	9.45 ± 0.21
	20%	10.00	10.40	10.20 ± 0.28
Negative Control	-	0	0	0

Based on in vitro test data (Table 8), cocoa peel extract nanoparticles with various concentrations of 4, 12, and 20% had potent inhibition against *K. pneumonia* bacteria. Determination of these criteria is based on Darmadi et al. research [26], who reported that the provisions of antibacterial strength were as follows: inhibition zone of 20 mm or more was extreme, 11-20 mm was strong, 6-10 mm was moderate, and less than 5 mm was weak. Cocoa peel extract nanoparticles with various extract concentrations (4; 12; and 20%) can inhibit the growth of *K. pneumonia* bacteria, but it is no significant difference in the zone of inhibition, namely 11.25, 10.90, and 11.05 mm, respectively. Therefore, it was necessary to reduce the concentration of extract to 2%. The purpose is to see whether the inhibition zone of bacterial growth was significantly different at a lower concentration. Based on the results obtained, the inhibition of cocoa peel extract nanoparticles with 2% concentration differed substantially with a concentration variation of 4%; 12% and 20% are 9.25 mm. Therefore, this inhibitory ability is classified as medium.

In vitro test results for cocoa peel extract showed the increase of inhibition zone with extract concentration (Table 8). The inhibition against *K. pneumonia* from crude cocoa peel extract was moderate to strong.

However, the inhibitory strength of crude extract was smaller than cocoa peel extract, which had been modified in nanoparticle size. It is due to the small particle size of the active substance so that it can enter the bacterial body system quickly, causing damage of processes in the body. It is consistent with previous research. The smaller the particle size, the greater the antibacterial activity.

In the preparation of cocoa peel extract nanoparticles using the SNEDDS method, additional chemicals were used, such as tween 20, PEG, and capryol. To ensure that the cocoa peel extract nanoparticle was the inhibitory agent for the growth of *K. pneumonia*, in vitro tests were carried out on tween 20, PEG, and capryol (negative control). Figure S2 also shows the results of in vitro test from the negative control. Tween 20, PEG, and capryol have no activity to inhibit the growth of *K. pneumoniae bacteria.* Therefore, it confirmed that the primary role of an antibacterial agent is the active substance (flavonoid) in cocoa peel extract. It is in line with previous research, which stated that flavonoids have activity as an antibacterial against *K. pneumoniae* [7].

Inhibition Mechanism of Bacterial Growth by Flavonoid

Based on the characterization that has been carried out, cocoa peel extract contains flavonoids as secondary metabolites. These groups of compounds play a role in inhibiting the growth of *K. pneumonia*. The mechanism of flavonoids as an antibacterial is by denaturing bacterial cell proteins and damaging the cytoplasmic membrane. According to Tagousop et al. [27] explained that flavonoid compounds could damage the cytoplasmic membrane, which can cause leakage of essential metabolites and inactivate bacterial enzyme systems. This damage allows nucleotides and amino acids to seep out and prevent the entry of active ingredients into cells. This condition will inhibit growth and cause the death of bacteria.

CONCLUSION

In this research, the preparation of cocoa peel extract nanoparticles has been successfully carried out. Cocoa peel extract contains flavonoid compounds of 6.33%. The cocoa peel extract nanoparticle made using the SNEDDS method has a smaller size than using the ionic gelation method, namely 135.2 nm (4%), 156.1 nm (8%), and 235.3 nm (12%). Based on the in vitro test, the cocoa peel extract nanoparticle was able to inhibit the growth of *K. pneumoniae* bacteria, which causes chronic bronchitis in a strong category.

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