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Potency of Nanopropolis Stinglessbee *Trigona* spp Indonesia as Antibacterial Agent

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ABSTRACT: *Propolis is one of the natural produced by honey bee which have many benefits, due to its properties as antibacterial, antivirus, and anticancer. The aim of this study is to determine potency of nanopropolis honey bee from Pandeglang Banten Indonesia as antibacterial agent. Encapsulated nanopropolis was prepared by high speed homogenization technique followed by encapsulation using maltodekstrin with solvent evaporation technique. The size of nanopropolis was measured with SEM. The result of SEM show that size of nanopropolis is 175 nm. The antibacterial activity test was performed by agar well diffusion. Nanopropolis have activtiy as antibacterial agent for Gram positive and Gram negative bacteria. The nanopropolis showed higher bacterial activity than extract propolis were 205.86% for B. subtilis, 211.83% for S. aureus, 227.01% for E. coli, and 230.29% for Salmonella sp. While compared to amphicillin 10 mg/ml equel to 43.87%, 49.12%, 42.35%, and 37.58%. Minimum Inhibitory Concentration (MIC) of nanoproplis were 0.313%, 0.15%, 0.313%, and 0.625% for B. subtilis, S. aureus, E.coli, dan Salmonella sp respectively.*

KEY WORDS: *propolis, nanopropolis, antibacterial, Trigona* spp*, Indonesia*

I. INTRODUCTION

Indonesia is a tropical country which has a flora and fauna are very diverse or megadiversity. In addition to producing honey, bees also produce other products that can be utilized, such as propolis. Though propolis in the beehive can be extracted into pharmaceutical products, cosmetics, and functional food. Propolis is a resin adhesive material collected from the worker bees from the buds, bark, and other plant parts (Chintalapally *et al*. 1993; Gojmerac 1983; Kuropatnicki *et al*. 2013). Collected resins mixed with bee enzymes so different from the resin plant origin. Propolis yellow to dark brown, even transparent. An important component in the form of resin propolis (benzoic acid derivatives and flavonoids), waxes and fatty acids, essential oils, pollen, and minerals. The flavonoids contained in propolis respond to antibacterial activity (Barud *et al*., 2013; Muli and Maingi 2017) as well as anticancer and immunomodulatory role in (Sforcin 2007; Burdock 1998).

Previous studies on the information that propolis has potential for very profitable. Schmidt et al. (2014) and Kathai and Jayanthi (2014) showed that propolis has efficacy against the inhibitory effects of free radicals and as an antibacterial. Research conducted Barud et al. (2013) explain that the ethanol extract of propolis 70% effective at inhibiting the growth of bacteria both Gram-positive and Gram-negative. Dziedzic *et al*. (2013) and De Luca *et al*. (2014) showed propolis can be used as an alternative anticaries in reducing the number and growth of cariogenic bacteria (Streptococcus mutans), a bacteria that causes dental caries. Even Al Jumaily *et al*. (2014) showed propolis can be used as an agent antihepatoprotektif when used in the treatment. Hasan *et al*. (2011) found that propolis can be used as an antibacterial agent *Salmonella thipymurium*. Research conducted by Popova *et al*. (2013) showed that propolis is able to inhibit *S. aureus* and *E. coli*. Trusheva *et al*. (2010) found no activity of Iranian propolis against *Candida albicans* and *E. coli* but active against bacteria *Staphilococcus aureus*. According Yaghoubi *et al*. (2007) Iranian propolis can act as an antibacterial. Propolis can serve as natural antibiotics as antimicrobial capabilities with the benefits more secure, do not cause resistance, and side effects are small and have a high selectivity (Winingsih 2004).

Nanoparticles is one result of the rapid development technological. Nano technology is widely used in industry (nanocomposite, nanotubes), pharmaceutical (drug manufacturing), and food (manufacture of nano vitamine A) (Aitken *et al.* 2004). The advantage of using nanoparticles in medicine, among others: the particle size and surface characteristics make it easy to be manipulated in order to achieve the effect of passive and active against the target; enhance the therapeutic effects of the drug; can use a variety of channels such as oral, nasal, parenteral, or intraocular; expenditure control and adjustable surface degradation of the matrix composition; and can be attached to specific targets via ligand or with the help of magnetic (Mohanraj and Chen 2006).

Propolis has a small solubility in water. Preparation of propolis preparations in the form of nano which would increase the ability to dissolve a surface so that the better. Nano size in propolis can pass through the outer membrane of the bacteria so that the active antibacterial compounds can damage the bacterial cell wall. Use the form nanopropolis expected to provide a better antibacterial activity compared to the usual forms of propolis.

Propolis then be coated with a microencapsulation technique. Microencapsulation is a technique for coating a compound (may be, for solids, liquids, and gases) with a polymer coating that is very small (micron) (Yoshizawa 2004). The advantage of using microencapsulation is to protect an active compound of decomposition and controlling the release of an active compound so as to prevent an increase in the concentration of drug in the gastrointestinal tract of a sudden. Controlled drug release makes more efficient use of the drug, reduce side effects, as well as reducing the frequency of drug use (Sutriyo *et al*. 2004). Hasan *et al*. (2012) and Prasetiyorini *et al*. (2011) conducted a study and test nanopropolis mengkapsulasi propolis against bacteria *Escherichia coli* showed that the nanopropolis inhibit the growth of *E. coli* at very low concentrations. Sahlan and Supardi (2013) have done studies that show that propolis has antibacterial activity encapsulation of *Bacillus substillis*. The difference between the real activity of propolis and nanopropolis Iranian origin have been proved by Afrouzan et al (2012) against *Candida albicans* and *Staphilococcus aureus*. Currently, the use nanopropolis as antibacterial substances have never been scientifically studied further.

Testing of antibacterial activity of nanopropolis using Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli* and *Salmonella thipymurium*). All four of these bacteria may represent other bacteria that cause disease. In addition, bacteria are easy to grow, easy to obtain, and is a common bacteria found in the body and the environment.

II. METHODS

Propolis Extraction

Propolis extracted from the honeycomb stinglessbee origin Pandeglang (Banten Province, Indonesia) with method of Harbone (1987); Hasan *et al*. (2006) and Matienzo & Lamonera (2004). Extraction was carried out by maceration with 70% ethanol. A total of approximately 150 grams of propolis obtained honeycomb *Trigona* spp origin Pandeglang (Banten Indonesia) soaked with 70% ethanol, and then stored in a closed dark room for 1 week. Furthermore, the filtrate taken daily for one week to clear solvent. After the filtrate obtained propolis extracts, concentration is done by using a rotary evaporator at a temperature of ± 40^{o}C. This extract was dissolved in 70% ethanol as one volume.

Nanopropolis 10% Production (Modifikation from Bhaskar *et al*. 2009; Hasan *et al*. 2012 and Sutriyo *et al*. 2004)

A total of 20 grams of ethanol extract of propolis was added 120 ml of 70% ethanol. Coating material as much as 85 grams of maltodextrin dissolved in 80 mL of distilled water and added 5 grams of Mg-stearate and mix with a stirrer until well blended. Maltodextrin mixture was homogenized at a speed of 22 000 rpm for 30 minutes. Propolis dissolved in 70% ethanol mixed with maltodextrin mixture and homogenized at a speed of 22000 rpm back for 30 minutes. The solution was dried with a vacuum dryer at a temperature of ≤ 50 ° C. The powder is formed and then smoothed and generalized by High Energy Milling (HEM) with a speed of 915 rpm and frequency ± 28.8 Hz for 15 minutes. Results nanopropolis identified using Scanning Electron Microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR).

Antibacterial Activity Test

Antibacterial activity test using the well diffusion method. Positive controls used were ampicillin 500 mg tablets with a concentration of 10 mg / ml and negative control used distilled water. Regeneration Bacteria Test. Before use, the bacteria that will be used must be regenerated first. Bacteria derived from primary cultures initially cultured in agar slant Agar Nutrient (NA). A total of one loop of bacteria streaked to agar slant NA and incubated at 37° C for 24 hours. This is the initial activity of cultured bacterial stock stored there 4-5^{o}C temperature. Introduction Antibacterial Activity Test. Preliminary test of antibacterial activity performed by the well diffusion method. A total of one loop of bacteria from stock cultures were taken and incubated in 10 ml of Nutrient Broth (NB) liquid medium for 18-24 h at 37 ° C and shaken using a water bath while shaking. After the cultures taken from a number of bacteria that is spread in a petri dish, then poured 20 ml of PYG agar ± 45oC temperature, and then the cup is shaken so that the bacteria are spread evenly. Furthermore, settling at room temperature until the agar solidifies. Once solid, so riddled with ± 5 mm in diameter. Is inserted into the hole nanopropolis extract as much as 50 mL and incubated at 37 ° C for 24 hours. Clear the area around the hole is visible indicates the antibacterial activity of the samples.

Determination of Minimum Inhibitory Concentration (MIC)

Determination MIC done after it emerged that nanopropolis extracts have antibacterial activity. The first stage is nanopropolis dilution with distilled water to obtain 10 concentrations (of 0.02% to 10% v / v). Each concentration of 50 mL is inserted into the hole solid PYG medium containing test bacteria and incubated at 37°C for 24 hours. Antibacterial activity was obtained by measuring the diameter of a clear zone around the hole samples using calipers.

Statistical Analysis

The statistical analysis used in the processing of the data is two-factor experimental design in a completely randomized design. Here's a model of the design (Mattjik and Sumertajaya 2002). $Y_{ij} = +_i + _{ij}$; Y_{ij} = Observations on the treatment of the i-th and j-th repetition; = The influence of the average general; = The effect of the i-th treatment; = Random effects in the treatment of the i-th and j-th replicates.

Data were analyzed by ANOVA (analysis of variance) at the 95% confidence level and standard of $\alpha <$ 0:05. Further test used is the Duncan test. All data were analyzed using SPSS 16.0.

III. RESULTS AND DISCUSSION

Solvents used for meceration is 70% ethanol. Ethanol 70% is semipolar so as to extract the active compounds with different polarity in propolis. According to Harborne (1987), 70% ethanol is a good solvent to extract flavonoids. Ethanol also has a low boiling point and evaporate easily, thus minimizing the amount carried in the extract. The use of 70% ethanol also reduces extracted beeswax is regarded as a nuisance in the extraction of propolis (Cunha *et al*. 2004). Beeswax is composed of fatty acid esters and alcohols with long carbon chains are not soluble in ethanol 70% (Fearnley 2005). Krell (2004) states that 70% ethanol is the best solvent for propolis as an antibacterial agent. Propolis is produced brown with a yield of 13.33% (Figure 1). The colour of propolis depends on the phenolic compounds contained in the extract, the flavonoid compounds. Propolis contains flavonoids darker more, so it has a yield that is more than the younger coloured propolis (Woo 2004). Propolis has thermostable nature, hard, and clay at a temperature of 15^{o}C with a boiling point of 60-69^{o}C (Woo 2004). Propolis is stored at temperatures less than 25°C and placed in a dark place that is not exposed to direct sunlight as it will damage the active compounds in propolis (Krell 2004).

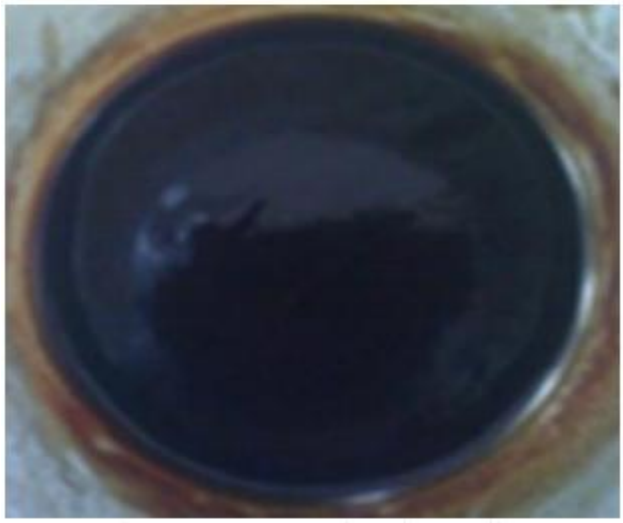

Figure 1. Extract Ethanol Propolis.

Nanopropolis particle formation in this study using the merger method of homogenization Bhaskar *et al*. (2009) by solvent evaporation microencapsulation method (Sutriyo *et al*. 2004). Formation begins with the manufacture of the coating nanopropolis propolis using microencapsulation techniques. Microencapsulation is a technique for coating a compound (may be, for solids, liquids, and gases) with a polymer coating that is very small (micron) (Yoshizawa 2004). The advantage of using microencapsulation technique is able to protect an active compound of decomposition and controlling the release of an active compound so as to prevent an increase in the concentration of drug in the gastrointestinal tract of a sudden. Controlled drug release makes more efficient use of the drug, reduce side effects, as well as reducing the frequency of drug use (Sutriyo *et al*. 2004). Microencapsulated component consists of core material and coating material. The core material is a material that is trapped, while the coating material is a material that protects the core material in the process of microencapsulation.

Figure 2. Nanopropolis Powder

Coating material used is maltodextrin (MDE). The use of MDE in the pharmaceutical industry is still very limited or no popular than its use in the food industry is very broad. Maltodextrin is a starch derivative products produced from the partial hydrolysis by the enzyme α-amylase with a value of dextrose equivalent (DE) of less than 20. DE describes the percentage of hydrolysis of glycosidic bonds and a decrease in strength. Maltodextrin $(C_6H_{10}O_5)nH_2O$ is a polymer of D-glucose binds to the α-1,4 glycosidic bond. Ties contained in this maltodextrin are very weak and easily disconnected (Sukamdiyah 2009). The reason for the selection of maltodextrin as the coating is water soluble, colorless, odorless, and non-toxic (Sukamdiyah 2009). Anwar *et al*. (2004) stated that starch derivatives such as maltodextrin is to increase the viscosity to form a hydrogel matrix, and has the sticking power. MDE structure is shorter than the starch so that when microencapsulated produce microcapsules were dry, uniform size, and not sticky (Suseno 2009). In microencapsulation, MDE structure which will be filled by a hollow core that propolis as the active compounds in propolis can be protected by the MDE.

Magnesium stearate ($C_{36}H_{70}MgO_4$) is commonly used in the pharmaceutical world as a lubricant with a concentration of 0.25 to 5.0% (Maziyyah 2010). Magnesium stearate has the ability to reduce friction between the tablet with the mold wall when removed from the machine. Excessive use of magnesium stearate causes a decrease in tablet hardness and prolonged the time integrated. The addition of 5% magnesium stearate in nanopropolis coating to reduce the sticking of the granules in the mixing container and vacuum dryer so that the coating is more perfect and improve the appearance of powder nanopropolis. Selection of the use of magnesium stearate in nanopropolis because magnesium stearate did not interact with the active compounds in nanopropolis. The use of magnesium stearate in small amounts for disintergrasi nanopropolis not too long so that the dissolution of the active compound is not disturbed (Barra and Somma 1996).

Sutriyo *et al*. (2004) stated perfection coating is influenced by the speed and duration of stirring. A very high rate of stirring produces droplets of very small particles, and vice versa. Maltodektrin and magnesium stearate homogenized at a speed of 22,000 rpm for 30 minutes. Coating formed then homogenized with EEP 100% with pace and stirring the same time. Homogenization is an important factor in order to perfect the active components can be coated by the coating material. The speed is much higher than the study Sutriyo *et al*. (2004), which is 3,000 rpm which resulted in a particle size distribution between 425 to greater than 850 microns.

After the coating material and EEP 100% homogenized, the next step is drying. Drying technique used is vacuum drying. Drying is carried out at 40°C. The purpose of drying is to remove the solvent. The use of 40°C to protect the active compounds in propolis, such as flavonoids as antimicrobial materials to prevent damage due to flavonid not stand the heat. Nanopropolis produced a very dry powder form, but it is still very rough and clustered. Nanopropolis smoothed and generalized back with HEM (High Energy Milling). HEM will soften and flatten a particle of a 3-way, ie vertical, horizontal, and rotation. Nanopropolis smoothed using an iron ball (ball mill) were included in the HEM tube. HEM tube will rotate vertically, horizontally, and rotate. The resulting powder nanopropolis brownish white and very smooth (Figure 2). Brownish color indicates the presence of propolis were coated in the coating material.

Characterization by SEM (Scanning Electron Microscope) to determine the morphology and size nanopropolis. Nanopropolis characterized using a JSA-65 10LA Analytical Scanning Electron Microscope (JEOL) at BATAN, Serpong Indonesia. The results of the SEM observation nanopropolis nanopropolis seen that the particles have a uniform shape and uneven edges. Particles nanopropolis apparent with the spread fairly evenly (not clustered). Observations by Anwar *et al*. (2004) to show maltodextrin particle shape is not uniform. Nanopropolis made by Coneac *et al.* (2009) with cyclodextrin coating material derived from starch derivatives

also show the shape of the particles are not uniform and uneven edges. According Coneac *et al*. (2009), the use of an appropriate extraction solvent will result nanopropolis with a smaller size.

Observation of nanopropolis 10% done randomly with a magnification of 3,000 times. The smallest size that can still be measured at 175 nm, 197 nm, and 307 nm (Figure 3). In this study, the particle size distribution was not analyzed specifically. Some memeiliki particle size larger than 500 nm. Sutriyo *et al*. (2004) stated that the difference of particle size distribution is influenced by the amount of coating material used as a wall-forming.

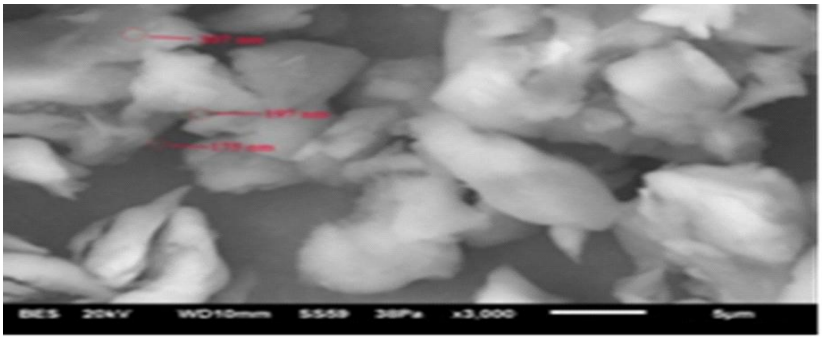

Figure 3. SEM Morphology Nanopropolis

Nanopropolis visible spectrum profile to see where propolis in the coating. Martos et al. (2008) stated that the components in propolis have antibacterial activity in the form of phenolic compounds in the form of flavonoids (pinocembrin, naringin, and hesperidin).

The results indicate that the FTIR profile analysis nanopropolis has a cluster C = C (aliphatic) with the absorption peak 1542.38 cm-1, with a -CH2- group absorption peak 2919.07 cm-1, and the OH group absorption peak 3394.80 cm-1 (Figure 4). C = C group suspected of propolis because many compounds in propolis in the form of phenolic compounds (benzene cicncin existence). -CH2- Group probably derived from maltodextrin, which is derived from starch. OH group probably derived from maltodextrin and propolis. Propolis has many compounds in the form of phenol while maltodextrin having aldehyde group in its structure.

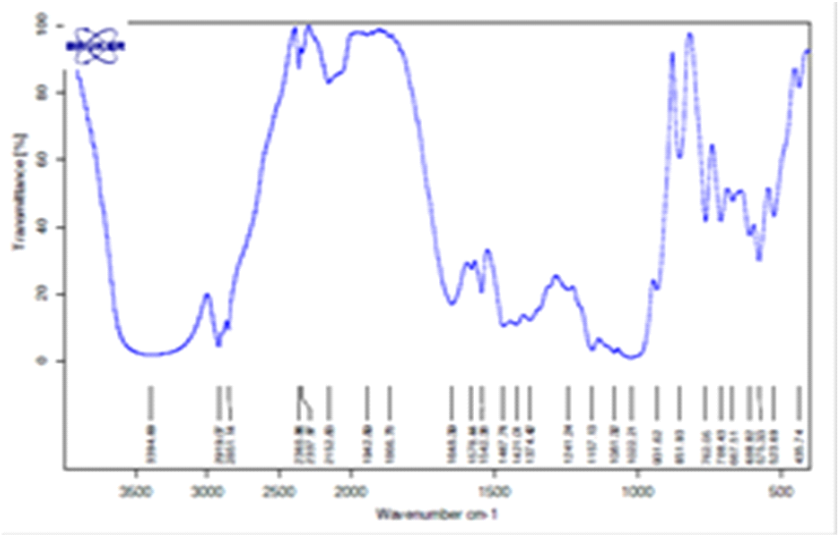

Figure 4. Profile spectrum nanopropolis

The method used to evaluate the antibacterial activity of nanopropolis is well diffusion method. This method is general and easy to use for a couple of test bacteria. The presence of a clear zone around the well indicates nanopropolis has potential as an antibacterial. Pelczar and Chan (1988) states that a compound has the ability as influenced antibacterial antibacterial concentration, the number of bacteria, and the type of bacteria used. The greater the concentration of antibacterial is used, then the inhibitory power will also increase.

In general, based on the results of the study showed a clear zone that forms the greater following the increasing concentration of propolis. The mechanism of propolis in inhibiting the growth of bacteria is not fully known. Fatoni (2008) suggested that the active compound that acts as a antibacterial components are flavonoids and tannins. Hydroxyl group of flavonoids cause changes of organic components and the transport of nutrients which ultimately resulted in toxic effects for bacteria (Sabir, 2005).

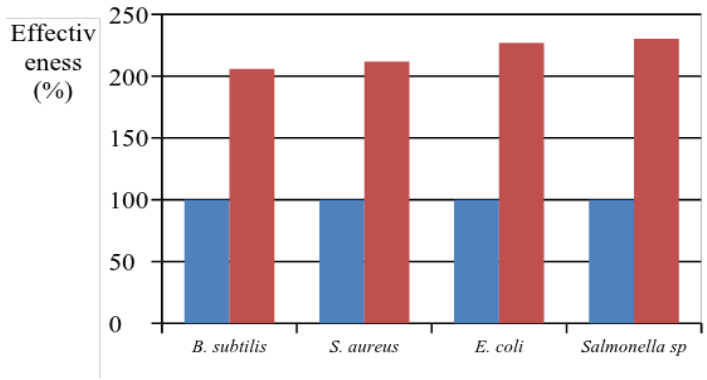

Figure 5 Effectiveness nanopropolis inhibition of propolis extract.
EEP ( ), Nanopropolis ( )

Sabir 2005 states several components in propolis could inhibit the bacterial enzyme RNA polymerase to attach to the DNA that bacterial DNA replication does not occur. Moreover, these components are also capable of inhibiting the action of the enzyme restriction endonucleases that do not occur in RNA transcription resulting in impaired cell division.

Nanopropolis have higher efficacy when compared with the propolis extract of 205.86%, 211.83%, 227.01%, and 230.29% respectively against *Bacilus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* sp (Figure 5). Statistical analysis showed that the inhibition zone diameter of nanopropolis significantly different with EEP for all bacteria tested.

Mohanraj and Chen (2006) stated that the nanoparticles have a very small size so that the greater surface area. Kim *et al*. (2008) also stated that nanopropolis propolis is more effective than usual because nanopropolis more soluble form and has higher permeability than the usual propolis. Therefore, the process of release of the active compound from its protective material more quickly.

Positive controls were used to evaluate the antibacterial activity is ampicillin. The use of ampicillin as a positive control for a broad-spectrum antibiotic, meaning that it can inhibit bacteria Gram positive and Gram negative (Siswandono and Soekarjo 1995). The concentration of ampicillin were used at 10 mg / ml. Effectiveness nanopropolis to ampicillin 10 mg / ml respectively for *Bacilus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* sp was 43.87%, 49.12%, 42.35%, and 37.58% (Figure 6). Based on statistical analysis, the diameter of clear zone nanopropolis significantly different with ampicillin 10 mg / ml for all test bacteria. Ampicillin is a β-lactam antibiotic and belongs to the class of semisynthetic penicillins. Activity of ampicillin interfere with the process of transpeptidation. Ampicillin nucleophilic attack of the serine hydroxyl group on the carbonyl carbon transpeptidase enzyme β-lactam ring is positively charged so that the biosynthesis of the peptidoglycan cell wall becomes disrupted as a result become weak. Weak cell walls will not be able to withstand the turgor pressure from inside so that the cell will rupture and lead to bacterial death (Siswandono and Soekarjo 1995). Mechanism of action of ampicillin ampicillin cause has a great antibacterial and are bacteriocidal.

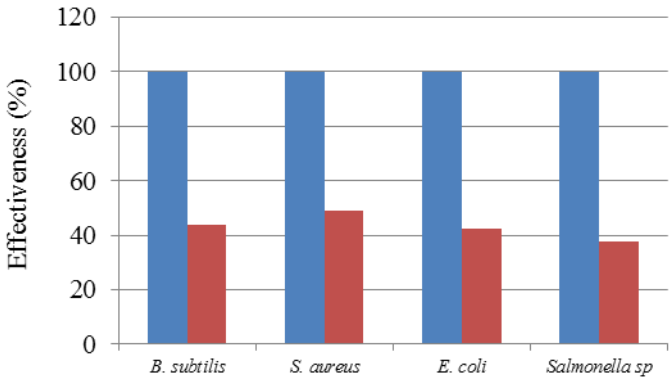

Figure 6 The effectiveness of inhibition nanopropolis to ampicillin 10 mg / ml
Ampisilin ( ), nanopropolis ( ).

Determination of the MIC conducted to determine the lowest concentration of nanopropolis which can inhibit the growth of test bacteria. Variations in the concentration used nanopropolis than 0.02% to 10%. Selection is expected to give a concentration lower than the value MIC Hasan *et al.* (2006) who use propolis extract. The results showed that MIC nanopropolis against Gram-positive bacteria of *B. subtilis* (0313%) and *S. aureus* (12:15%) and Gram-negative bacteria of *E. coli* (0313%) and *Salmonella* sp (0.625%) (Figure 7). These results have MIC lower than Hasan *et al*. (2006) who use propolis extract. These results are also in line with the statement Silici and Kaftanoglu (2003) which states that the active compounds in propolis give MIC lower for Gram-positive bacteria than Gram-negative bacteria.

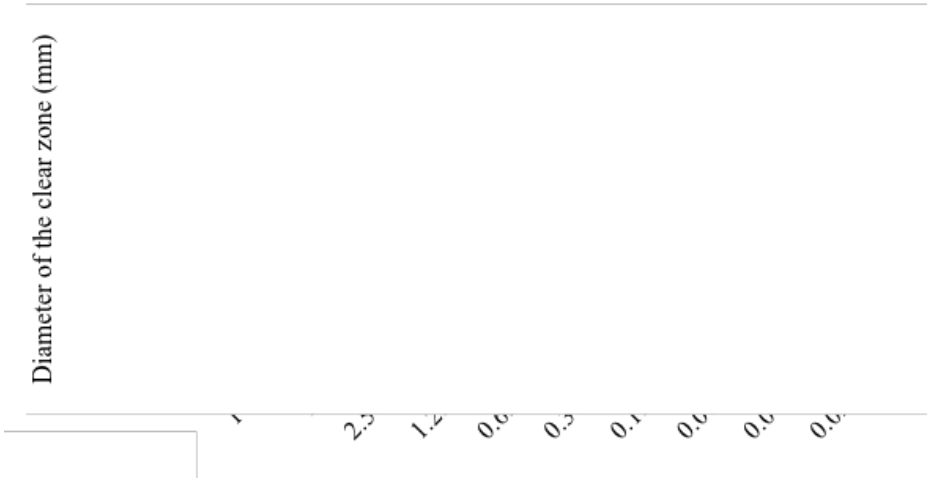

Figure 7. MIC of *B. subtilis* ( ), *S. aureus* ( ), *E. coli* ( ), *Salmonella* sp ( ).

Resistance of Gram positive and Gram negative influenced compilers and different cell wall structure. Gram-negative bacteria have more complex structures than Gram-positive bacteria to Gram-negative bacteria more resistant to antibacterial compounds. Outer membrane of Gram-positive bacteria have a high content of peptidoglycan and single-layered and does not have a polysaccharide layer. Therefore, Gram-positive bacteria are stronger to withstand the osmotic pressure of the (Greenwood *et al.* 1995). Outer membrane of Gram-negative bacteria such as *E. coli* have lipids, polysaccharides, lipoproteins, and lipopolysaccharides which gives 80% weight walls. The thick layers of peptidoglycan protects the work of lysozyme, and blocking antibiotic compounds. Lipopolysaccharide layer is semi-permeable, can not be by passed large molecules, so it can only pass small molecules such as nucleosides, oligosaccharides, and amino acids.

Nanopropolis lowest concentration that still inhibits the growth of *B. subtilis* is 0313%, while S. aureus was not inhibited at lower concentrations of 0.15% with a clear zone diameter of 5:24 mm and 5.93 mm. Research conducted Hasan *et al.* (2006) uses bee propolis extract Pandeglang origin can inhibit the growth of *B. subtilis* and *S. aureus* at concentrations of 0.78% and 0:39% with a diameter of 6,450 mm and a clear zone of 6.142 mm. Hasan et al. (2006) estimated the active compounds from propolis inhibited the growth of *S. aureus* in a way attached to the cytoplasmic membrane constituent teicoic acid because the teicoic acid negatively charged. The surface of the cytoplasmic membrane can attract antibacteria compounds that are polar as tannins and flavonoids (Lay & Hastowo 1992)

Nanopropolis have KHTM 0313% against E. coli with 7:25 mm diameter clear zone. KHTM nanopropolis against Salmonella sp of 0.625% with a diameter of 2.66 mm clear zone. KHTM E. coli obtained Hasan et al. (2006) used a propolis extract Pandeglang origin of 0.7812% with a diameter of 6.042 mm clear zone. MIC of propolis extracts against Salmonella sp at 1:04% with a diameter of 1.62 mm clear zone (Tukan 2008). Khismatullina (2005) stated that the addition of propolis extract may increase the antibiotic effect of *E. coli*. Propolis is bacteriostatic in vitro cultures of E. coli (Woo 2005). Tukan (2008) surmised that propolis acts as polymyxin or streptomiksin. Polymyxin is able to destroy the wall of Gram negative bacteria in particular. Polymyxin interacts strongly with phospholipids of cell membranes, resulting in loss of osmotic control, resulting in the leakage of K^+ ions and other vital components of bacteria. Easy penetration into and damage the structure of cell membranes. Work of this kind is antibiotics damage cell membranes react with phosphate in cell membrane phospholipids (Tukan 2008).

IV. Conclusion

Propolis obtained brown. SEM results showed the smallest particle size of 175 nm. Results of FTIR analysis showed that nanopropolis have C = C group, -CH2-, and OH. Nanopropolis antibacterial effect against four bacterial test (*Bacilus subtilis, Staphylococcus aureus, Escherichia coli*, and *Salmonella* sp). Nanopropolis more effective at inhibiting the growth of all test bacteria compared with propolis extract. MIC of *B. subtilis* and *E. coli* was 0313%, 0.15% and *S. aureus* and *Salmonella* sp of 0.625% repectively.

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