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Antibacterial and Antioxidant Activity of Trigona Sp Nanopropolis

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Abstract: ***Propolis is one of the important by-products of bees used as a component of the body's defense, external immune system and antimicrobial. Trigona Sp bees are a type of bee native to Asia that has the characteristic that the honey produced has a sour taste but is resistant to fermentation and rarely moves, and the haractivity of its honey products is higher than that of Apis genus honey. In this study, particle size modification of propolis from Trigona Sp spider honey into nano-sized particles was carried out using the ionic gelation method. The resulting nanopropolis was tested for antioxidant activity using the DPPH shift method and tested for antibacterial activity against Staphylococcus Aureus bacteria. Through phytochemical tests, it was found that propolis was positive for tannins, flavonoids, and alkaloids, but negative for saponins. Antioxidant activity test showed that nanopropolis had weak antioxidant activity (500-650 mg/L) while propolis in bulk form had very weak antioxidant activity (2092.2 mg/L). The inhibition against Staphylococcus Aureus bacteria of propolis extract, NP1:5, NP1;10, and NP 1:15 were 0 mm, 18 mm, 12 mm, and 8 mm, respectively.***

Keywords: Propolis, Trigona Sp bees, nanopropolis, antioxidant activity, antibacterial activity

1. Background

Propolis is a by-product of bees in the form of a thin greenish-brown layer that covers honey sacs and bee pollen sacs. Propolis is often expressed as bee glue which is used as a self-defense agent of Trigona sp to protect themselves from predators. In Trigona sp bee communities, propolis is produced more than honey (Hasan, et. al., 2016). Propolis as a by-product in the form of sap has very high benefits. Honey bees will collect sap from various plants to fill gaps and holes in their nests. This is done to protect the hive from external threats, such as microbes and predatory animals. Basically, the chemical composition of propolis can vary depending on where the material is collected by the bees. From several studies on bees, it was concluded that propolis consists of several components such as pinocembrin, pinobanksin and 3-O-acetate, chrysin, galangin, prenyl esters of caffeic and ferulic acids, and others (Anjum et. al., 2019).

The utilization of nanotechnology in the pharmaceutical and healthcare fields promises drug efficiency. The distribution of the delivery system can be controlled through the regulation of its size and surface properties. Drug-carrying particulate systems are characterized by considering the amount of drug absorbed, so that the effect of controlled drug release is as good as the effect of protecting the drug from degradation. The main objective in designing nanoparticles as drug delivery systems is to control the particle size, surface properties, and release of the active substance to obtain the pharmacologically specific action of the drug at its optimum dose. The advantages of using nanoparticles as drug delivery systems include particle size and surface characteristics of nanoparticles can be manipulated easily to obtain both active and passive drug targeting after parenteral administration. Nanomaterials control and release drugs slowly during distribution and modify drug distribution in organs and slow down drug clearance so that drug therapy and minimize side effects.

Ionic gelation method is one of the methods of nanoparticle synthesis. This method is used to produce crosslinked nanoparticles which are nanoparticles formed from the crosslinking process between electrolytes and their ionic partners. This crosslinking occurs ionically or covalently. The ionic gelation method is relatively easy to perform because it uses ion pairs that are more suitable for proteins and avoids excessive stirring, high heat, and the use of organic solvents (Park and Yeo, 2007).

Chitosan is a natural cationic polysaccharide that can serve as a biomaterial in drug delivery systems and nanocareer formulations due to its biocompatible, biodegradable, and low toxicity properties (Detsi, et al., 2020). Chitosan is widely used as a matrix for encapsulation of valuable compounds. In this ionic gelation method, chitosan is used as a precursor where the mechanism of chitosan nanoparticle formation is based on the electrostatic interaction between the amine of chitosan and the negative charge of the polyanion. Chitosan can be dissolved with acetic acid, polyanions are then added so that nanoparticles are formed spontaneously with magnetic stirrer stirring at room temperature. Chitosan which is a cationic polymer will react with multivalent anions such as tripolyphosphate. The formation of nanoparticles by ionic gelation method can be done by hardening of liquid droplets dispersed in oil or organic phase. The formation of these cross-links will increase the mechanical strength of the particles formed (Vaugh and Williams, 2007).

2. Methods

This research is a descriptive-explorative research that aims to modify the particle size of propolis into nanopropolis by ionic gelation method using chitosan and Tripolyphosphate as precursors. Furthermore, the resulting nanopropolis was tested for antioxidant activity using the DPPH method and its antibacterial activity against Staphylococcus Aureus. The research was conducted at Udayana Chemistry Research Laboratory, Joint Laboratory FMIPA, and Analytical Unit

and microbiology laboratory of Biology PS FMIPA Udayana University.

Research Materials
The materials used in this study include Trigona Sp bee propolis from the Probolinggo area, acetic acid, ethanol, chitosan, NaTPP, distilled water, DPPH, Staphylococcus aureus bacterial preparations, Whatman filter paper.

Research Equipment
The tools used in this research are volume pipette, dropper, measuring flask, measuring cup, goblet, Erlenmeyer flask, stirring rod, watch glass, porcelain cup, ball filler, magnetic stirrer, analytical balance, furnace, Buchner funnel, separating funnel, rotary vacuum evaporator, UV Vis spectrophotometer, Particle Size Analyzer (PSA), Fourier Transform Infra-Red Spectroscopy (FTIR).

Propolis Preparation
A total of 500 grams of raw propolis was washed with distilled water and then dried. After drying, it was cut into small pieces and then crushed into powder using a blender.

Preparation of propolis extract
Five hundred grams of propolis powder was macerated with ethanol for 3-7 days. The yield was then filtered using filter paper to obtain the filtrate. The filtrate obtained was collected to be concentrated using vacuum rotary evaporation until a thick propolis extract was formed.

Preparation of Nanopropolis
Thick ethanol extract of propolis weighing 1 gram was dissolved in 35 mL of ethanol and added with 15 mL of distilled water. In a different container, a solution of chitosan in acetic acid with varying composition with NaTPP was made. In this study, variations of chitosan and NaTPP were made as follows: (1: 5), (1:10). (1:15). Propolis extract that has been dissolved in ethanol is mixed with NaTPP chitosan solution, stirred with a magnetic stirrer for 2 hours. The resulting colloid was separated by centrifugation. The solid obtained was cooled in a freezer (temperature approximately -4°C) for 2 days, then further storage at 3°C until dry (Hariyanto, 2017). The resulting colloidal nanoparticles were characterized for particle size and Zeta potential by Particle Size Analyzer and their functional groups by FTIR spectrophotometer.

Antibacterial activity test
The method used in testing antibacterial activity is agar diffusion wells. A bacterial suspension of 1 mL was added to 20 mL of nutrient agar (NA) media. The mixture was made homogeneous using a vortex, then cooled and solidified in a sterile petri dish. Furthermore, the test media made wells measuring ± 6 mm using a preforator and then incubated for 30 minutes at 37°C. The test extract, positive control (antibiotic) and negative control (distilled water) as much as 20µL was put into the well and then incubated for 24 hours. The observation results obtained are in the form of whether or not there is a clear area formed around the disc paper which shows the inhibition zone on bacterial growth.

Antioxidant Activity Test
Antioxidant activity test with DPPH method begins with making a standard curve using ascorbic acid with a concentration of 100, 200, 300, 400, 500 ppm. A total of 2 mL of acetate buffer solution (pH = 5.5) was mixed with 3.75 mL of methanol 200 µL DPPH then vortexed. Standard and sample solutions of 50 µL each were vortexed again and incubated at room temperature in a dark place for 20 minutes. Then the absorbance was measured with a UV-Vis spectrophotometer at a wavelength of 517 nm.

3. Results and Discussion

Phytochemical tests were conducted to determine the content of secondary metabolites contained in propolis. From the tests carried out, it was found that propolis was positive for tannins, flavonoids and alkaloids but negative for saponins. The complete results of the phytochemical test are presented in Table 1.

Table 1: Phytochemical Test Results of ethanol extract of propolis

Phytochemical Test	Reagent	Result	Description
Phenolic	FeCl3	Color change from brownish red to black	Positive Phenolic
Saponin	Plus water and then shaken then added HCl1%	No strong foam formed and lasted for 30 seconds	Negative saponin
Alkaloids	Wigner reagent	Formed a red precipitate	Positive alkaloids
Steroids/ terpenoids	Anhydrous acetic acid and concentrated sulfuric acid	Color change from brownish red to dark brown	Positive steroids
Flavonoids	Mg powder and HCl	Color change from brownish red then formed a brown precipitate	Positive flavonoids

Phenolic, terpenoid and flavonoid groups are active compounds found in propolis. The flavonoid groups commonly found in propolis are chrysin, pinocembrin, apigenin, galangin, kaempferol, quercetin, tectochrysin, pinostrobin and others (Przybyłek and Karpiński, 2019).

Particle size and zeta potential of nanopropolis
Particle size and zeta potential were measured using a Particle Size Analyzer. The results obtained showed that there was a change in particle size after propolis was modified into nanopropolis. In this study, it was found that the difference in precursor composition, namely chitosan and polyphosphate, did not greatly affect the particle size of the resulting nanopropolis. There is an increase in zeta potential as the particle size of propolis decreases. The smallest particle size and the largest zeta potential are owned by nanopropolis with the composition of Chitosan: Tropolyphosphate = 1:15

Table 2: Particle Size and Zeta Potential of Propolis and Nanopropolis

Sample	Partikel size (nm)	Zeta Potensial (mV)
Propolis	329,73	-8,25
Nanopropolis 1: 5	136,067	-18,525
Nanopropolis 1:10	151,833	-13,175
Nanopropolis 1:15	105,707	-25,075

Antibacterial activity

The antibacterial activity of propolis can be studied from two perspectives: the first is related to direct activity against the microorganism and the second is the stimulation of the immune system resulting in the activation of the natural defenses of the microorganism. The mechanism of propolis as an antibacterial compound is through its effect on cell membrane permeability, disruption of membrane potential and adenosine triphosphate (ATP) production and decreased bacterial mobility (Bankova, et al., 2011). The antibacterial activity test was carried out using the well or disc diffusion method using Staphylococcus Aureus test bacteria. The results of the activity test are shown in Table 3.

Table 3: Inhibition of nanopropolis against Staphylococcus Aureus

Sample	Inhibition power (mm)
Propolis (crude extract)	0
Nanopropolis 1:5	18
Nanopropolis 1:10	12
Nanopropolis 1:15	8

Table 3 shows that overall nanopropolis has better antibacterial activity than propolis. There is a tendency of decreasing antibacterial activity with increasing tripolyphosphate concentration where the composition of Chitosan precursor: NaTPP = 1:5 had the highest antibacterial activity of 18 mm. According to Izabela Przybyłek and Tomasz M. Karpiński 2019 the antimicrobial activity of propolis is higher on Gram-positive than Gram-negative bacteria. This is explained by the species-specific structure of the outer membrane of Gram-negative bacteria and the production of hydrolytic enzymes that break down the active ingredients of propolis.

Antioxidant Activity

The antioxidant activity of nanopropolis was tested in vitro using the DPPH shift method. The magnitude of antioxidant activity is shown in Table 4. It can be seen that after being modified into nanomaterials, propolis in nano form or nanopropolis has a stronger antioxidant activity compared to its bulk form although it is still in the range of weak antioxidants where the IC_{50} value of nanopropolis is much lower than its bulk form.

Table 4: Antioxidant activity of Propolis and Nanopropolis

Sample	IC_{50}(mg/L)	Antioksidan activities
Propolis	2092,21	Very weak
Nanopropolis 1:5	6,44,211	Weak
Nanopropolis 1:10	5,08,901	Weak
Nanopropolis 1:15	5,21,115	Weak

The antioxidant activity of propolis is largely determined by the place from which it is obtained.

Characterization of Propolis and nanopropolis with IR spectrophotometer

IR spectra showed no significant difference in spectra between propolis and nanopropolis. Each functional group still appears at adjacent vibrational frequencies (wave numbers). The slight shift in wave numbers that occurs is due to the slightly different atomic environment and energy that can be caused by particle size.

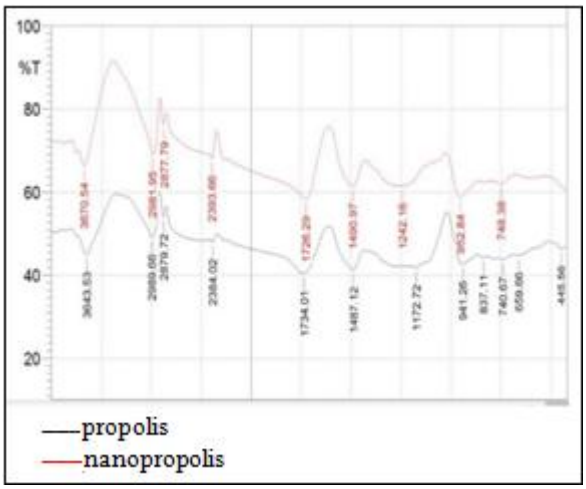

Figure 1: IR spectra of Propolis and nanopropolis

IR spectra of propolis and nanopropolis with vibrational frequencies at wave numbers 3643.53cm-1 and 3670.54 cm-1 respectively indicate stretching of O-H groups of phenolic compounds such as cinnamic acid, caffeic acid, ferulic acid (Villalobos, et.al., 2017). Absorption at 2989.66 cm-1, 1734.01 cm-1 in propolis and 2981.95 cm-1, 1726.29 cm-1 in nanopropolis indicate CH2 and C-O stretching vibrations (Bilia et al, 2014). Vibration peaks at 1487.12 cm-1 and 1490.97 cm-1 are associated as C=O and C=C functional groups. Vibrations at 1400-1500 cm-1 are associated with C-O stretching of ester groups (Villalobos, et.al., 2017).

4. Conclusion

1) Nanopropolis has antibacterial activity against Staphylococcus Aureus greater than propolis.
2) Nanopropolis has weak antioxidant activity with IC_{50} of 500-650 ppm.
3) There is no significant difference in the vibrational frequency of the IR spectra of propolis and nanopropolis.

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