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Nanochitosan-Propolis And Its Activity Test Of Invitro Cell Proliferation And In Vivo Wound Healing

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Abstract:
Background:*Propolis is a product of honey bees (Apismellifera) containing many secondary metabolite compounds in the form of phenols and flavonoids, those which make propolis has antibacterial and antioxidant effects in the wound healing process. Apart from propolis, chitosan is also a natural ingredient having an antimicrobial effect and ability to modulate the function of inflammatory cells. This study aimed to see the synergy between chitosan and propolis with nanoencapsulation technology and to test it in vitro cell proliferation and in vivo wound healing.*
Materials and Methode*: Nanopropolis was synthesized using high-speed homogenizer technology and then encapsulated using nanochitosan which was synthesized using the ionic gelation method through the adsorption process. Nanopropolis particle size and encapsulation results were analyzed using PSA (Particle Size Analyzer). The coated propolis (nanochitosan-propolis) was then tested for in vitro cytotoxicity and cell proliferation using the MTT method against NIH3T3 cells (mouse fibroblast cell line). Cell proliferation observation was carried out at 0, 24, and 48 hours and then tested in vivo on Sprague dawley rats to observe the macroscopic wound healing (the presence of erythema and edema).*
Resulsts:*The results of the analysis showed that the average size of propolis nanoparticles was 74.63 ± 24.50 nm and the nanopropolis encapsulated by nanochitosan was 754.76 nm ± 54.097 nm. The results of the cytotoxicity test for nanopropolis and nanochitosan-propolis showed IC10 values of 0.023% and 0.00093%. In vitro cell proliferation observation showed that nanochitosan-propolis required a concentration of 1/20 smaller than the concentration of nanopropolis to exert the same proliferative effect against the growth of NIH3T3 cells. In vivo results in Sprague Dawley rat did not show erythema or edema in either the group given nanochitosan-propolis or nanopropolis.*
Conclusion: *Nanochitosan-propolis required a 1/20 concentration of nanopropolis to exert the same proliferative effect on NIH3T3 cells.*
Key Word: *Nanochitosan, nanopropolis,proliferation,propolis , NIH3T3 cell, wound healing*

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I. Introduction

Propolis has a complex chemical composition which has been used extensively in traditional medicine for many years. In addition, propolis has many biological and pharmacological properties and the mechanism of its activity has been extensively investigated in recent years, some of its medical properties include antibacterial, antiviral, anti-inflammatory, anti-cancer and immunomodulatory using various experimental models in vitro and in vivo[1]. Several research results confirm the benefits of propolis therapy through qualitative and quantitative analysis of collagen type 1 and III expression as well as its derivation in the wound matrix, indicating that propolis has good biochemical compounds to support epithelialization[2]. The ability of propolis to repair wound and regenerate tissue is correlated with the ability of propolis as an antimicrobial, anti-inflammatory and immunomodulatory[2].To improve the function of propolis as a wound healer, there has been done an experiment using a nanoparticle encapsulation technique. Nanoparticles are a formulation of a dispersed particle at the nanometer size or scale per thousand microns. Nanoparticles begin to be developed in the pharmaceutical field, especially in connection with drug delivery system. Nanoparticle formation can increase system affinity due to an increase in the contact surface area by an equal amount[3]. In addition, nanoparticles can increase the solubility of compounds, reduce treatment doses, and increase absorption of drug targets. Some of these advantages underlie the use of nanoparticle technology as a drug delivery system in various medicinal, cosmetic, or dermatological dosage form[4]. Encapsulation is a process of coating a core in the form of a solid, liquid or gas

with a polymer as a microcapsule forming wall[5], aiming to transport functional materials to reach the desired state and maintain the stability of a compound.

One of the materials used as the encapsulant in the encapsulation process is chitosan. Chitosan is a natural polymer which is widely used in various fields such as agriculture, water treatment, food, cosmetics, biomedicine and pharmaceuticals. Chitosan is obtained from processing animal waste from shellfish such as shrimp, clams, crabs and so on. The large supply of marine waste in Indonesia makes chitosan easy to obtain in nature. Chitosan was chosen because of its properties, such as being biocompatible, biodegradable, and non-toxic[6]. Chitosan also has several biochemical activities such as anti-infection, stimulation of angiogenesis, and activation of growth factors[7]. Chitosan also has the ability to increase the permeation of substances across biological membranes, so it has the potential to be used as a matrix in drug delivery systems. In connection with the use of chitosan in the pharmaceutical and cosmetic fields, both as an active ingredient and as a carrier in drug delivery systems, chitosan in the form of nanoparticles has many advantages compared to chitosan solutions or chitosan microparticles, such as protective property, release controller agent, and having better diffusion and penetration rate[8].Based onthis background, this study aimed to synergize propolis and chitosan as encapsulants in the size of nanoparticles and to test their activity on in vitro cell proliferation and in vivo wound healing in *Sprague dawley* rat.

II. Materialsand Methods

The research was started from July to December 2020, conducted at the Laboratory of Nano Herbaltama Internasional Inc, Serpong, Center for Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology, PUSPIPTEK Serpong, and Tropical Biopharmaca Research Center (TropBRC), IPB University.

2.1 Materials

Chitosan powder with 94.82% deacetylation degree (pharmaceutical grade), sodium tripolyphosphate (TPP) (pharmaceutical grade)acetic acid glacial (Merck)were purchased from local industries., ketamine, xylazine, bioplacenton, MTT [3- (4,5-dimethylazole-2-il) -2,5-diphenyl-tetrazolium bromide], Penicillin-streptomycin, Fetal bovine serum, RPMI/DMEM medium, Sodium bicarbonate, Fungizone, trypsin-EDTA, DMSO, NIH3T3 cells (mouse fibroblasts cell line), sterile water, *Sprague dawley* male rats, 70% alcohol and distilled water.propolis extract (Nano Herbaltama Internasional Inc.)propolis extract (Nano Herbaltama Internasional Inc.)

2.2 Preparation ofNanopropolis

Propolis extract was obtained from Nano Herbaltama Internasional Inc. Propolis extract was dissolved in distilled water at a concentration of 10% (w/v) using a high-speed homogenizer at room temperature for 24 hours, and then filtered using a filter. The particle size and the polydispersity index value of the propolis solution were analyzed using Particle Size Analyzer (PSA).

2.3 Synthesisof Nanochitosan

Nanochitosan was synthesized by ionic gelation method[9].A 0.25% chitosan solution was prepared by dissolving 25g of powdered chitosan into 1L of 1.5% acetic acid solution. A 0.4% TPP solution was prepared by dissolving 2g of sodium tripolyphosphate in 500 mL of distilled water.0.4% TPP solution was added slowly into0.25% chitosan solution at various chitosan and TPP ratio (Table 1). The mixture was stirred at 100 rpm for 30 minutes at room temperature. The particles formed were then analyzed using the PSA. The particle of nanochitosan was separated by centrifugation at 10,000 rpm for 30 minutes at 4°C.

Table no1: Nanochitosan composition

No	Formulation	Chitosan :TPP ratio
1	F1	2.5:1
2	F2	3:1
3	F3	4:1

2.4 Encapsulation of Nanopropoliswith Nanochitosan (Nanochitosan –propolis)

Nanopropolis encapsulation was carried out using the adsorption method[10]. The nanopropolis liquid was added to the nanochitosan paste under stirring at 250 rpm for 1 hour at room temperature according to the formulation in Table 2. By this process, nanopropolis was expected to be entrapped within nanochitosan. The encapsulated particles were measured using PSA and stored at 4°C.

Table no2: Encapsulation formula of nanochitosan-propolis

No	Formulation	Nanochitosan: Propolis Ratio
1	G1	2:1
2	G2	3:1
3	G3	4:1
4	G4	5:1

2.5 Encapsulation Efficiency

The encapsulation efficiency (EE) was determined by measuring the amount of flavonoid content in the formulated nanochitosan-propolis. The number of flavonoids in propolis before and after encapsulation was analyzed using a spectrophotometer, so that the EE of nanochitosan-propolis can be calculated using the formula:

$$\text{Encapsulation efficiency (\%)} = \left(\frac{(Total\ amount\ of\ flavonoids\ -Free\ flavonoid\ s}{Total\ amount\ of\ propolis}\right) x100\%$$

2.6 Particle Size Analysis and Polydispersity Index

This analytical work procedure was carried out based on the procedure carried out by Suciati*et al* (2014)[11]. The mean diameter and polydispersity index (IP) of nanochitosan-propolis were measured using photon correlation spectroscopy (PCS) onDelsa^{TM}Nano C particles size analyzer (Beckman Coulter) at a fixed angle of 90° and a temperature of 25°C. A 2 mL of sample had been made with a concentration of 0.1%. Particle size data was read by the computer on the PSA.

2.7 Morphology Analysis of Nanocitosan-propolis.

Analysis of the morphology of nanochitosan-propolis was observed using a Transmission Electron Microscopy (TEM). This analytical work procedure was carried out based on the procedure of Almoussalam*et al*(2015) with slight modification[12]. Nanochitosan-propolis samples were diluted with distilled water at a concentration of 0.1%. Each sample droplet was applied to a copper grid coated with carbon film and air dried. The grid was then stained with 2% (w/v) phosphotungstic acid solution and dried below room temperature. The particles were examined using TEM JEOL 1400 at an operating voltage of 100 kV with a magnification of 20,000 times.

2.8 Cytotoxicity Assay

Cytotoxicity assay was performed on NIH3T3 cells (mouse fibroblast cell line) with MTT [3- (4,5-dimethylazol-2-il) -2,5-diphenyl-tetrazolium bromide)] assay. Cells were grown on DMEM media containing 0.1% Penicillin-streptomycin and 10% FBS then sown into 96 well plates and incubated at 37°C for 24 hours in a 5% CO_2 incubator. After the desired cell growth was obtained, the samples of nanochitosan-propolis and nanopropolis were added to the well. The test was carried out 3 times with 6 concentration ranges (0.010313 to 0.33 %). At the end of the incubation time, MTT dye was added andwas re-incubated until formazone crystals were formed. The reaction was stopped by adding 10% SDS in 0.1 N HCl solution. The absorbance of the well was read with an ELISA reader at a wavelength of 570 nm.

2.9 Cell Proliferation Assay

The activity of encapsulated propolis towards cell proliferation was tested in vitro using NIH3T3 cells with MTT assay. Nanopropolis, nanochitosan-propolis, and NIH3T3 cell culture media (negative control) were prepared at various concentrations. The samples were dispersed in fibroblast culture media containing serum and antibiotics to accelerate cell growth. The cells were incubated at 0, 24 and 48 hours. At the end of the incubation time, MTT dye was added. The absorbance of the well was read with an ELISA reader at a wavelength of 570 nm. Data analysis was performed statistically with the one-way ANOVA test.

2.10 In vivo Wound Healing Assay

The procedure of in vivo assay was approved by Ethics Committee of TropBRC, IPB university for animal use in research.The animal used were 5 male rats SD (*Sprague dawley*) strain, 12 weeks old with the weight of ± 200 grams. The rats were housed in individual cages. Before testing, the rats were acclimated for 1 week, fed with 15 g/head/day of adlibitum, and treatedwith 12:12-h light-dark cycle. Then the rats were weighed and anesthetized with a combination ofketamine and xylazine (80+10 mg/kg body weight). The incision wound was made in the back of the rats by dividing it into 4 quadrants with each incision length of 1.5 cm and incision distance of 1 cm (Figure 1). The process of making the incision wound was carried out in a sterile manner. Each

wound was treated with 0.1 mL of nanochitosan-propolis, nanopropolis, bioplasenton (positive control), and no treatment(negative control), respectively. Observation of wound was carried out macroscopically on days 2,4,6,8,10,12, and 14, including redness (erythema) and swelling (edema). Erythema was evaluated by looking for signs - reddish color marks of the wound, and edema wasobserved by looking for signs of wound swelling.

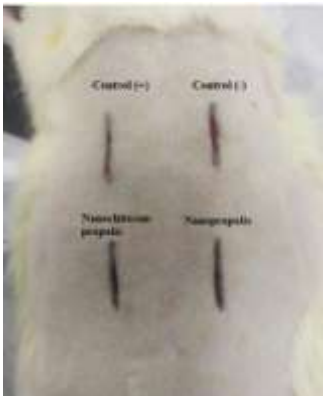

Figure no1: Wound treatment on rat

Table no3: Erythemaandedema score value[13]

Criteria	Score	Explanation
Redness (Erythema)	0	No Erythema
	+1	A LittleErythema (almost invisible)
	+2	Clearly Visible Erythema (25,11-3mm)
	+3	Moderate to Heavy Erythema (30,1-35 mm)
	+4	Heavy Erythema (Visible Wound)
Swelling (Edema)	0	No Edema
	+1	Light Edema (Invisible adsorben)
	+2	Light Edema (Clearly Visible with the width of < 1mm
	+3	Moderate Edema (The width of ± 1mm)
	+4	Heavy Edema (The width of > 1 mm)

III. Result

The result of particle size analysis was shown in Table 4.The average of nanopropolis particle size was 74.63 ± 24.50 nm with a polydispersity index value ranging from 0.3 to 0.493.

Table no4. Data of nanopropolis particle size

No	Replication	PSA (nm)	*Polydipersity Index*
1	I	48.9	0.493
2	II	77.3	0.315
3	III	97.7	0.491

In this study,the synthesis of nanochitosanwas carried out by a combination of chitosan and TPP with various formulations (Table 1).The results were shown in Figure 2 and Table 5.

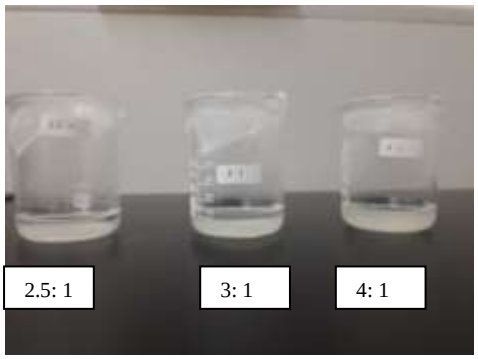

Figure no2. Formulation result of nanochitosan synthesis on ionic gelation method

Table no 5. Data of nanochitosan particle size

No	Formulation	Replication	Particle Size Analyzier (nm)	Polydispersity Index (PI)
1	F1 (2,5:1)	1	2,007.5	0.332
		2	2,694.3	3.422
		3	7,731.3	6.56
2	F2 (3:1)	1	861	6.282
		2	588.7	4.940
		3	2,649.8	2.971
3	F3 (4:1)	1	92.5	0.305
		2	92.1	0.442
		3	84.6	0.425

The result of formulation F3 was then centrifuged at 13.000 rpm for 30 minutes to separate the particles from the suspension.

Figure no3. Nanochitosan paste obtained after centrifugation

Table no 6. Data of nanochitosan-propolis particle size

No	Replication	PSA (nm)	*Polydipersity Index* (PI)
1	I	786.0	0.273
2	II	692.3	0.342
3	III	786.0	0.273

Nanopropolis encapsulated in nanochitosan (i.e, nanochitosan-propolis) with various composition was shown in Figure 4.

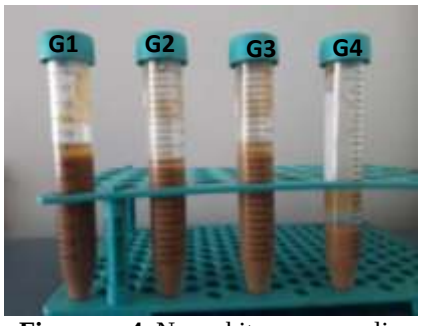

Figure no4. Nanochitosan-propolis

The TEM morphology of nanochitosan-propolis were spherical with many aggregating particles (Figure 5).

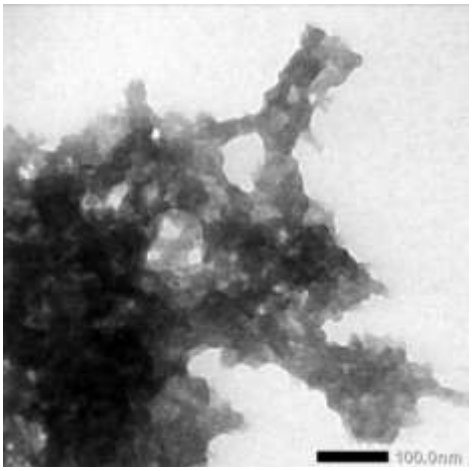

Figure no5. TEM morphology of nanochitosan-propolis

Table no 7. Analysis result of polyphenols dan flavonoids levels on nanopropolis and nanochitosan-propolis

No	Sample	Polyphenol mg GAE/100g	Flavonoid mg/100 g
1	Nanopropolis	644.14	92.7
2	Nanochitosan-propolis	22.73	0.85

Table no 8. IC_{10} value of nanopropolisandnanochitosan-propolis on NIH3T3 cell

No	Sample	IC_{10}
1	Nanopropolis	0.023%
2	Nanochitosan-propolis	0.00093%

Note: The test was done three times with 6 concentrations

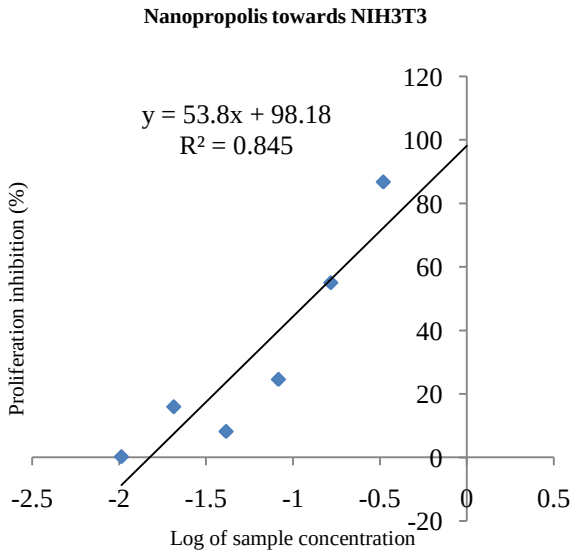

Figure no6. The relationship between nanopropolisconcentration with the percentage of proliferation inhibition onNIH3T3 cell

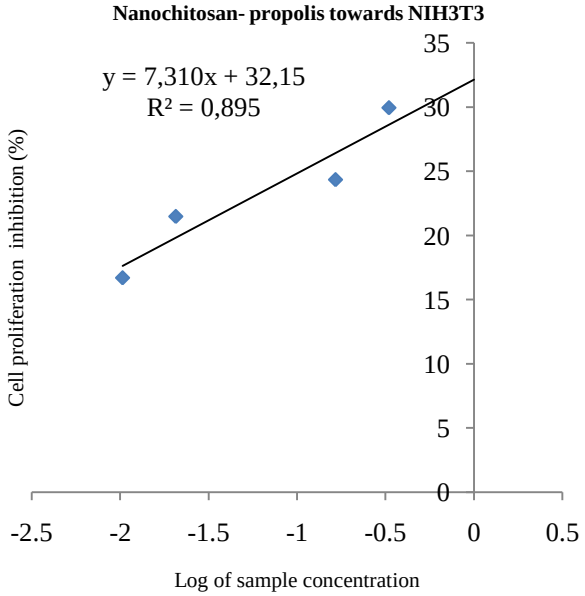

Figure no7. The relationship between nanochitosan-propolisconcentration with the percentage of proliferation inhibition onNIH3T3 cell

Table no9. The percentage of average living cells for *in vitro*test during 0, 24 dan 48 hours incubation time

No	Incubation Time	% average living cells			
			0 hour	24 hours	48 hours
	Sample	Concentration			
1		0.000625 %	100	98.681	93.054
		0.00125 %	100	98.852	96.840
	Nanopropolis	0.0025 %	100	102.127	96.151
		0.005 %	100	99.277	94.869
		0.01 %	100	97.065	92.616
		0.02 %	100	97.746	86.577
2		0.00003125 %	100	97.533	93.023
		0.0000625 %	100	94.513	94.086
	Nanochitosan-propolis	0.000125 %	100	94.258	95.275
		0.00025 %	100	94.173	96.370
		0.0005 %	100	99.532	103.411
		0.001 %	100	95.236	98.029

Table no 10. One way ANNOVA table of nanopropolis sample

Hour	Replication number	Average cell growth
0	3	100 ± 1.326[a]
24	3	99.27±1.626 [a]
48	3	94.87± 3.13[a]

Table no11 One way ANNOVA table of nanochitosan-propolis

Hour	Replication number	Average cell growth
0	3	100± 1.326 [a]
24	3	95.24±4.60 [a]
48	3	95.24± 3.45[a]

IV. Discussion

The use of natural ingredients in the wound healing process has been widely studied by researchers. One of natural ingredients having the potential to provide benefits in the wound healing process ispropolis. Propolis has active compounds, including vitamins, minerals, enzymes, flavonoids,[14] terpenoids, steroids, amino acids, caffeine and its derivatives caffeic acid phenyl ester-caffeic acid phenylethyl ester (CAPE)[15] . These active compounds enable propolis to have a wide range of biological and pharmacological activities, such as anti-cancer, antibacterial, anti-inflammatory, tumoricidal and antifungal[16], antihistamine, antasis, antiprotozoa, anti-helicobacter pylori (ulceration treatment), and as a therapeutic agent[17], in which the function of propolis can be associated with the accelerated wound healing process.

In this study, the synthesis of nanopropolis was carried out using a high-speed homogenizer, inwhich the propolis extract was dissolved with water first. The average nanopropolis particle size obtained was 74.63 ± 24.50 nm with a polydispersity index value ranging from 0.3 to 0.493 as shown in Table 4. Particle measurement was carried out using the principle of dynamic light scattering using PSA. The particle size of the nanopropolis obtained is close to the average size of the nanopropolis synthesized using a high-pressure ball mill homogenizer with an average small particle diameter of 100 nm[18]. The particle size of the nanoparticles is closely related to their absorption in the body, increasing bioavailability, thereby increasing the effectiveness of treatment[19].There are two main processes used to make particles into small sizes (nano) which are commonly used to make drug particles with high purification tools, namely wet-grinding in agitated grinding mediamills[20]and highpressure homogenization [21,22].

Nanochitosan synthesis was carried out using the ionic gelation method. Ionic gelation is a method based on the ability of polyelectrolyte ions to cross bonds to form hydrogel beads which are also known as gel balls or gelisphers. Hydrogel beads are produced by dropping a drug-filled polymer solution into an aqueous solution of polyvalent cations. The cations diffuse into the drug-filled polymer drops, forming the three-dimensional side of the ionic bond groups. Molecules can also be inserted into this gel. Molecules can also be inserted into this gel under light conditions to maintain a three-dimensional structure[23,24] namely the complexation of polylelectrolytes between positively charged chitosan and negatively charged tripolyphosphates[9.] In this study, the ionic gelation method for optimizing the synthesis of chitosan nanoparticles was carried out by a combination of chitosan and sodium tripolyphosphate (TPP) with various formulations with a concentration of 0.25% chitosan and 0.4% TPP.Based on observations, there were deposits in the F1 and F2 formulas (Figure 2). This precipitate shows that the particles formed in this formulation had a large size, this was evidenced by the results of the PSA analysis which showed the size of the particles formed was larger than 2,000 nm with a polydispersity index value of 0.7. The large polydispersity index value could be caused by the high amount of TPP used, resulting in swelling or enlargement. Swelling occurs due to electrostatic repulsion between ionic groups that have a charge, causing the particles to enlarge and the particles to form aggregates[25].The polydipersity index describes the level of uniformity in a system where the smaller the polydispersity index value, the more uniform distribution of particles in a monodispersion system [26]. The polydispersity index value <0.7 is said to have a uniform particle distribution. Then this indicates a stable nanoparticle system due to the fact that fewer particles aggregate[27.] Formulation F3 showed that chitosan with a concentration of 0.25% and TPP 0.4% produced an average particle size below 100 nm with a PI value of 0.3 - 0.425. The result of formulation F3 was then centrifuged at 13,000 rpm for 30 minutes to separate the particles from the solvent. The particles formed were in the form of a semi-solid paste with a brownish white color as shown in Figure 3 with a process yield of 8%. The yield obtained was very small. This was due to the low concentration of chitosan and TPP solution to obtain nanochitosan particle sizes below 100 nm so that the particles formed were also small. The F3 formulation would then be reproduced as an encapsulant material for nanopropolis.

Protection and controlled release of bioactive compounds at the time and place according to the target can be done by encapsulation. Nanoencapsulation is a promising technology that has the ability to trap bioactive compounds[28]. The encapsulation process of nanopropolis with nanochitosan was carried out using the adsorption method. This method refers to chitosan in the form of nanochitosan which has a better function as an adsorbent of Cd heavy metal[10]in which the matrices formed on the nanochitosan can adsorb the nanopropolis. In this method, a slight modification is made to the adsorption process, so that the adsorption process is carried out with several formulations by stirring slowly for 1 hour using a magnetic stirrer at a speed of 200 rpm. The results of the formulations were stored at 4 °C and observed for 4 weeks. The observation results showed that of the 4 formulas, the G4 formula revealed stable results seen from the absence of a layer on the surface. The particle size of the encapsulated G4 was then analyzed using PSA. The average particle size formed was 754.76 nm ± 54.097 nm with PI of 0.2-0.342. The PI value shows that the nanochitosan-propolis particles were formed uniformly.

Analysis of flavonoids and polyphenols level in nanopropolis and nanochitosan-propolis was done to calculate the encapsulation efficiency (EE) on the results of nanochitosan-propolis encapsulation by

centrifugation. The EE value of the nanochitosan-propolis sample was 96.47% for the polyphenol parameter and 99.08% for the flavonoid parameter. The value of the encapsulation efficiency shows how efficient the nanochitosan is used to encapsulate the nanopropolis (nanochitosan-propolis). The greater the encapsulation efficiency, the better the concentration of nanochitosan used as a coating for nanopropolis. The morphological examination of nanochitosan-propolis particles was carried out with a TEM at an operating voltage of 200 kV with a magnification of 20,000 times. The morphology of nanochitosan-propolis were spherical with many aggregating particles (Figure 5). This aggregation is due to the high concentration of the sample used during preparation. The results obtained are in accordance with research conducted by Ong *et al* 2017[29]. In his research, the morphology of propolis encapsulatedin chitosan was spheric at the average particle size of 107, 74 nm (Figure 8).

Before proliferation assay,the nanochitosan-propolis cytotoxicity test was carried out on NIH3T3 cells (mice fibroblast cell line). The in vitro cytotoxicity method is an important test method in assessing the biocompatibility of new formulations. This aims to ensure that the experiments carried out do not pose a risk or acute toxicity[30]. The selection of NIH3T3 mice fibroblast cells in this test is because these fibroblast cells are found in the matrices and connective tissue of the body and are being widely used to identify cellular cytotoxicity and genotoctotic formulations[31,32]. In this study, cell cytotoxicity and in vitro tests were carried out by the staining method[33] by adding MTT [3- (4,5-dimethyl-2-thiazolyl) -2, 5-diphenyl-2H-tetrazolium bromide] aimed to determine the cytotoxic activity of the sample by observing cell viability[34]. MTT will react with the enzyme succinate dehydrogenase in the mitochondria of living cells and form blue formazan crystals that can be observed at a wavelength of 570 nm using the Elisa Reader.

In this cytotoxicity test, 6 concentrations were used for both nanopropolis and nanochitosan-propolis samples. The higher the concentration used, the percentage of proliferation inhibition also increased for the two samples tested (Figure 6 and Figure 7). The results showed that the IC10 value for nanochitosan-propolis was 0.00093% and nanopropolis was 0.023% (Table 8), indicating that the high cytotoxicity of nanochitosan-propolis and nanopropolis, in which IC10 shows a sample concentration that inhibits cell population growth by 10%. This concentration is considered safe. for cells which will then be used as a reference in determining the concentration for proliferation test on NIH3T3 cells with incubation times of 0, 24, and 48 hours.

The in vitro method was used to measure the migration of cells which could be affected by gene expression, and the extracellular matrix. The ability of certain cells to migrate is critical in many physiological processes, such as tissue repair, regeneration, and immune system response. Test cell viability, proliferation activity, cell migration, and protein expression will increase in the presence of test materials that have wound healing activity. This test usually uses the MTT assay method, scratch wound healing assay, observation of protein and gene expression using immunocytochemical methods and Polymerase Chain Reaction (PCR). Protein expression such as COX-2 and VEGF affects neovascularization and fibroblast development[35]. Important data obtained from the wound healing test is the rate of gap closure which is a measure of the movement velocity of cell groups[36]. In addition, fast and functional wound closure is the main target in wound care[37]. The results of the invitro test of the proliferation of nanopropolis and nanochitosan-propolis samples towards NIH3T3 cells with incubation times of 0, 24 and 48 hours using the MTT method are presented in Table 9.The data obtained were then followed by statistical analysis with the one way ANNOVA test to see the effect of differences in each sample against NIH3T3 cell proliferation at 0, 24, 48 hours. The results of statistical analysis showed that there was no significant difference in the mean NIH3T3 cell proliferation that occurred at 0, 24 and 48 hours both in nanopropolis at a concentration of 0.01% and nanochitosan-propolis at a concentration of 0.001% at the 0.05 level with a P value of $\geq$ 0.05 (Table 10 and Table 11). The effect of proliferation could be obtained when viewed from the data on the average percentage of living cells (Table 9). It can be seen that the use of the concentration for the in vitro test of the proliferation of nanopropolis samples was 20 times higher than the concentration of the nanochitosan-propolis sample. It can be said that nanochitosan-propolis had the same proliferative effect with a concentration of 1/20 of the concentration of nanopropolis, so that with a very small concentration, nanopropolis-chitosan was able to provide a high proliferative effect.

In vivo wound healing test was performed on male *Sprague dawley* rats. Before carrying out the in vivo test, the authors submitted an ethical clearance (EC). Ethical clearance was obtained from the Ethical Commission of Tropical Biopharmaca Research Center, IPB University. Ethical clearance was a written statement provided by the Research Ethics Commission for research involving living things, stating that a research proposal was feasible to be carried out after meeting certain requirements Annex 1. Observation of wound healing were carried out on days 2, 4, 6, 8, 10, 12, 14,macroscopically including observations of skin redness (erythema) and swelling (edema) (scoring) as in Table 4. Macroscopic observation was made to determine the activity of using test preparations that were administered topically. Macroscopic observation of in vivo study on day 0, it was found that there was a little erythema, but edema was not observed in all samples. Observationon day 1-14 did not reveal any erythema or edema in either all samples. Observation of erythema was carried out by looking for signs of redness in the wound, while observation of edema was carried out by

looking for signs of swelling in the wound. Signs of erythema and edema are common signs of an inflammatory response given by the body when experiencing an injury. When an injury occurs, the body responds with vasoconstriction of blood vessels to stop bleeding. This is followed by the release of inflammatory mediators causing vasodilation of the arterioles and venules that supply the inflamed area. As a result of this reaction, the inflamed area becomes congested, causing the tissue to become red and hot. At the same time, capillary permeability will increase, causing fluid to move into the tissues and affecting swelling and pain[38]. Such body response is called an inflammatory response which usually occurs between 24-48 hours, and it can last for more than 2 weeks in some cases[39].

V. Conclusion

Synthesis of nanopropolis and nanochitosan-propolis was successfully performed with the average particle size of 74.63 ± 24.50 and 754.76 nm ± 54.097 nm, respectively. The polydispersity index of nanopropolis and nanaochitosan-propolis was below 0.7, indicated that the particles uniformly distributed. In the in vitro proliferation study, nanochitosan-propolis required a 1/20 concentration of nanopropolis to obtained the same proliferative effect on NIH3T3 cells. In the in vivo study, there was noerythema or edema observatedduring wound healing process in *Sprague dawley* rats.

References

[1]. Sforcin and Bankova 2010. Propolis: Is there a Potential for The Depelopment of New Drugs?, Journal of Enthropharmacology 133 (2011) 253-260

[2]. Olczyk P, Komosinska-Vassev K, Winsz-Szczotka K, Stojko J, Klimek K, Kozma EM. Propolis induceschondroitin/dermatan sulphate and hyaluronic acidaccumulation in the skin of burned wound. *Evidence-BasedComplementary and Alternative Medicine,* 2013; 86(3):345-391

[3]. Martien, R., Adhyatmika, A., Irianto, I.D., Farida, V. and Sari, D.P., 2012. Development of Nanoparticle Technology as A Drug Delivery System. Pharmaceutic Magazine, 8(1), pp.133-144

[4]. Rismana, E., Kusumaningrum, S., Bunga, P.O., Rosidah, I. and Marhamah, 2013. Syinthesis and Characterization of Chitosan Nanoparticles- Mangosteen (Garcinia mangostana Extract). J. Sains and Technology. Indonesia, 14, pp.189-196.

[5]. Senatore, D. 2008. Microencapsulationfor Controlled Relase ofLiquid Crosslinker.Geboren te Cava de Tirreni.Ital.

[6]. Sandeep A, Sangameshwar K, Mukesh G, Chandrakant R, Avinash D. 2013. A Brief Overview on Chitosan Application. Indo Am. J. Pharm. Res. 2013 3, 1564–1574

[7]. Nguyen VQ, I. M. Kinoda, H Hattori, S Nakamura, T Ono, Y Miyahira, TMatsui. 2014. Development of Antimicrobial Biomaterials Produced from Chitin-nanofiber Sheet/ Silver Nanoparticle Composites. *Journal of Nanobiotechnology*

[8]. Agnihotri, S.A., Mallikarjuna, N.N., and Aminabhavi, T.M. Recent advances on chitosan-based microandnanoparticles in drug delivery. *J. of ControlledRelease* 100: 5–28 (2004)

[9]. Mardliyati *et al*. 2012. Sintesis Nanopartikel Kitosan-Trypoly Phosphate Dengan Met ode Gelasi Ionik: Pengaruh Konsentrasi Dan Rasio Volume Terhadap Karakteristik Partikel. ISSN 1411-2213

[10]. Yudha S. Nugroho AP. 2017. Synthesis and Application Of Chitosan Nanopaticles as Cd Adsorbents and Coliform Antibacterials Biogenesis. Vol 5, No. 1, Juni 2017, hal 42-48

[11]. Suciati, T. Aliyandi, A. Satrialdi. 2014. Development of Transdermal Nanoemulsion Formulation for Simultaneous Delivery of Protein Vaccine and Artin-M Adjuvant, International Journal of Pharmacy and Pharmaceutical Science, 6(6): 536-541

[12]. Almoussalam *et al*, 2015. Characterization, purification and stability of gold nanoparticles. *International Journal of Pharmaceutical Sciences*, 7 (2), 211-220

[13]. Kuncari, E. S., Iskandarsyah, dan Praptiwi. 2015, Irritation Test and Hair Growth Activity of White rats: Effects of Apigenin Gel and The juice of Celery (Apium graveolens L.) , *Media Litbangkes*, 25: 15 22

[14]. Jose MS, Vassya B.2011. Propolis is there a potential for development of new drugs?. J Ethnopharmacol. 133(2):253-60

[15]. Lawal, Bashir, O.K Shittu, AN. Abubakar, G.M. Haruna, S. Saidu dan P.C. Ossai. 20155. Haematopoetic effect of methanol extract of Nigerian honey bee (Apis mellifera) propolis in mice. Journal of Coastal Life Medicine. 3(8): 648-651

[16]. Safari, Manouchehr, Hamid Reza Sameni, Leila badban, Ahmad Raze Bandegi, Abbas Ali Vafaei, Ali Rashidy Pour dan Laya Ghahari. 2015. Protective Effects of Water Ekstract of Propolis on Dopaminergic Neurons, Brain Derived Neurotrophic Factor and Stress Oxydative Factor in the Rat Model of Parkinson's Disease. International Journal of Pharmacology. 11(4):300-308. ISSN 1811-7775

[17]. Pasupuleti, Viswesware Rao, Lakhsmi Sammugam, Nagesvari RAmesh, dan Siew Hua Gan. 2017. Honey, Propolis, and Royal Jelly: A Comprehensive Review of Their Biological Actions and Health Benefits. Hindawi Oxidative Medicine and Cellular Longevity. Volume 2017. Article ID 1259510.

[18]. Hamdi. D, Wijanarko A, Hermansyah H, Asih SC, M Sahlan. 2019. Production of Nanopropolis Using High Pressure Ball MillHomogenizer. Earth and Enviromental Science 217

[19]. Madane, R.G. and H.S. Mahajan. 2016. Curcumin-loaded nanostructured lipid carries (NLCs) for nasal administration: Design, characteritation, and invivo study. Drug Delivery. 23(4): 1326-1334,

[20]. Merisko-Liversidge, E., Liversidge, G. G., & Cooper, E. R. (2003). Nanosizing: a formulation approach for poorly-water-soluble compounds. European Journal of Pharmaceutical Sciences, *18(2), 113–120*. doi:10.1016/s0928-0987(02)00251-8

[21]. Muller RH, Krause K. & Mader K, penemu; PCT PatentApplication. Method for controlled production ofultrafine microparticles and nanoparticles.2001/03670 A1 July 10, 2000

[22]. Muller RH, Jacobs C. & Kayser O. 2001. Nanosuspensionsas particulate drug formulation in therapy rationalfor development and what we can expect for thefuture. *Advanced Drug Delivery Reviews* 47:3-19.

[23]. Hemalatha K, Lathaeswari R, Suganeswari M, Senthil Kumar V, Anto SM. Formulation And Evaluation Of Metoclopramide Hydrochloride Microbeads By Ionotropic Gelation Method. International Journal of Pharmaceutical & Biological Archives 2011; 2 suppl 3: 921-925.

[24]. Patil JS, Kamalapur MV, Marapur SC, Kadam DV. Ionotropic gelation and polyelectrolyte complexation: the novel techniques to design hydrogel particulate sustained, modulated drug delivery system: a review. Digest Journal of Nanomaterials and Biostructures 2010; 5(1): 241-248.
[25]. Yosmarina H. Preparation and Characterization of Chitosan nanoparticles with Acid Variation. Universitas Indonesia, Depok, Skripsi, 2012
[26]. Luo, X. Zhou, Y. Bai, L. Liu, F. Deng, Y. McClements, D.J. 2017. Fabrication of β-carotene Nanoemulsion-based Delivery Systems Using DualchannelMicrofluidization: Physical and Chemical Stability. *Journal of Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 504: 312-319
[27]. Nidhin, M. Indumathy, R. Sreeram, K.J. dan Nair, B.U. 2008. Synthesis of Iron Oxide Nanoparticles of Narrow Size Distribution on Polysaccharide Templates. *Bulletin of Materials Science*. 31: 93-96
[28]. Ezhilasari.PN, karthik P. Chhanwal N. 2013, Nanoencapsulation Techniques for Food Bioactive Compounds, Food Bioprocess Technol 2013, 6:628-647.
[29]. Ong TelkHwa, Chitra Ebenezer, Ramamurthy S, Siddalingam R P, Yuen Kah hay, Ambu SP, Davamani F. 2017. Chitosan-propolis nanoparticle formulation demonstrares anti-bacterial activity against *Enterococcus faecalis*bioflms.. PLoS ONE 12(3):e0174888.
[30]. Li, W., Zhou, J., Xu, Y., 2015. Study of the in vitro cytotoxicity testing of medical devices.Biomed. Rep. 3(5), 617-620.
[31]. Coradeghini, R., Gioria, S., García, C.P., Nativo, P., Franchini, F., Gilliland, D., ... & Rossi, F.,2013. Size-dependent toxicity and cell interaction mechanisms of gold nanoparticles on mousefibroblasts. Toxicol. Lett. 217(3), 205-216.
[32]. Ridolfi, D.M., Marcato, P.D., Machado, D., Silva, R.A., Justo, G.Z., Durán, N., 2011. In vitrocytotoxicity assays of solid lipid nanoparticles in epithelial and dermal cells. In Journal of Physics:Conference Series (Vol. 304, No. 1, p. 012032). IOP Publishing.
[33]. D. Hughes dan Mehmet. 2003. Cell Proliferation and Apoptosis. BIOS. Scientific publisher
[34]. The Effectiveness of Snail Mucus (Achatina Fulicia) and Chitosan Toward Limfosit proliferation In vitro. Asian Journal of Pharmaceutical And clinical Research. 2018. Vol 11(3) 2018.
[35]. Pusnik, M., Imeri, M., Deppierraz, G., Bruinink, A., and Zinn, M. (2016). The Agar Diffusion Scratch Assay - A Novel Method to Assess the Bioactive and Cytotoxic Potential of New Materials and Compounds. *Scientific Reports*. 6: 1-10. Raghavendra, S.N., and Raghavarao
[36]. Jonkman, J.E., Cathcart, J.A., Xu, F., Bartolini, M.E., Amon, J.E., Stevens, K.M., Colarusso, P.,2014. An introduction to the wound healing assay using live-cell microscopy. Cell. Adh. Migr. 8(5),440-451.
[37]. Bektas, N., Şenel, B., Yenilmez, E., Özatik, O., Arslan, R., Evaluation of woundhealing effect of chitosan-based gel formulation containing vitexin,*Saudi Pharmaceutical Journal* (2019).
[38]. Celloti, F. And Laufer S., 2001, Inflammation, Healing and Repair Synopsis, *J. Phar.*, 43(5): 2001,
[39]. Li, J., J. Chen, R. Kirsner, Pathophysiology of acute wound healing, *Clinics in Dermatology.* 25: 9-18 (2007)

NofaMardiaNingsihKaswati, et. al. "Nanochitosan-Propolis And Its Activity Test Of Invitro Cell Proliferation And In Vivo Wound Healing." *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)*, 16(2), (2021): pp. 01-12.