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Ointment Formulation of Arumanis Mango (*Mangifera indica* L.) Leaf Extract with Chitosan Tripoliphosphate Matrix as Antibacterial

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ABSTRACT. This report presented the synthesis of Arumanis mango (*Mangifera indica* L.) leaf extract with chitosan tripolyphosphate matrix and its antibacterial activity. This research aimed to obtain an ointment formulation from mango leaf extract with chitosan tripolyphosphate matrix, to figure out the characteristics, including the particle morphology, and to determine the optimum formulation and the characterization of the antibacterial ointment. The research showed that extract morphology with chitosan tripolyphosphate was uneven-edge aggregates. Antibacterial tests were conducted on *P. acnes* and *E. coli bacteria*. The formula giving the greatest antibacterial activity was further utilized for the ointment preparations and then was characterized for 16 days. Formula C (chitosan and NaTPP 1: 0.0992(%)) gave the most excellent inhibition zone for *P. acnes* and *E. Coli* bacteria, at 7.94 mm and 10.02 mm, respectively. The obtained ointment preparation was white color homogeneous semi-solid with protective properties. The spreading power of the ointment was 5.25 – 6.25 cm, with the adhesive power of 1 – 5 seconds and pH of 6.0 – 6.4. The ointment's antibacterial activity was tested against *P. acnes* and *E. coli* bacteria using the formation of inhibition zone method. The activity of ointment prepared on day one against *P. acnes* and *E. coli* was at 14.03 mm and 14.24 mm, respectively, while the activity on day 16 against *P. acnes* and *E. coli* was at 9.33 mm and 9.98 mm, respectively.

Keywords: chitosan, *Escherichia coli, Mangifera indica* L, *Propionibacterium acnes*, Tripolyphosphate

INTRODUCTION

Infection is one main disease existing in the developing countries, such as Indonesia. Infection is caused by bacteria, viruses, protozoa, fungi, and several other minor groups, such as chlamydia, microplasma, and rickettsiae (Nugraha et al., 2017) Some infections are caused by human- pathogenic bacteria. Those bacteria causing infection include *Propionibacterium acnes* and *Escherichia coli*. *P. acnes* is a pathogenic bacterium easily found anywhere, rod in shape, and without spores (Aida et al., 2016) *P. acnes* produces lipase enzymes hydrolyzing the unsaturated fatty acids into saturated fats resulting in acnes (Hafsari et al., 2015) *E. coli* is a group of Gram-negative bacteria, which become the intestine's normal flora possibly causing infection. *E. coli* can cause diseases in different places, such as urinary tract infection (UTI) and diarrhea (Alharbi et al., 2019).

Antibiotic is the best medicine to treat infection. Antibiotic is a chemical substance killing or inhibiting the growth of microorganisms (Castanon, 2007). Continuous use of antibiotics can lead to bacterial resistance (Rahman et al., 2015). Thus, it is necessary to find an alternative treatment by utilizing a plant potential becoming antibacterial.

One medicinal plant having the characters as antibacterial is arumanis mango (*Mangifera indica L.*) leaves. The largest content of mango leaf extract is mangiferin known having many functions, including as antimicrobe (Parvez, 2016) The methanol extract of arumanis mango leaves also shows the presence of alkaloids, flavonoids, steroids, polyphenols, tannins, and saponins. The methanol extract of arumanis mango leaves was able to inhibit the *Candida albicans* with the minimum growth inhibitory concentration of 65 ppm with the inhibition zone of 0.64 mm (Ningsih et al., 2017). The methanol extract of arumanis mango leaves can be formulated into the ointment preparations and inhibit *C. albicans* at the concentration of 125 ppm with the inhibition zone of 9.51 mm (Ningsih et al., 2019). The arumanis mango leaf extract can be formulated into ointment and inhibit *Propionebacterium acnes* at the concentration of 15 ppm with the inhibition zone of 21.88 mm (Ningsih et al., 2020). The use of ointment made from the mango leaf extract as antibacterial can increase its potential application in the form of biomaterials. The application of biomaterials in ointment preparations can increase the preparation stability and eventually give the maximum effect (Setyowati & Setyani, 2015).

The biopolymer used in this research was chitosan reacted with the tripolyphosphate crosslinker. Chitosan has several advantages, such as possibly used as matrix for plant extract or medicinal preparation, having antimicrobial properties, biodegradable, inexpensive, biocompatible, and non-toxic (Agnihotri et al., 2004) TPP was chosen as the crosslinker since it is a non-toxic material which does not change the biocompatibility of chitosan and can be applied for biomedical applications (Alauhdin & Widiarti, 2014). Based on the explanations above, research on making and characterization of ointment made from arumanis mango leaf extract with chitosan tripolyphosphate matrix as antibacterial is necessary to be conducted.

EXPERIMENTAL SECTION

Research Instruments

The equipment required in this research included pH meter (pHep by HANNA®), vacuum rotary evaporator, oven, autoclave, drigalski spatula, crock drill, hot plate, magnetic stirrer, mortar, Heraus incubator, spectrophotometer of Thermo Scientific Genesys 20, caliper, centrifuge (Hitachi), and SEM (Scanning Electron Microscope) Hitachi TM 3000.

Research Materials

The materials used in this research were arumanis mango (*Mangifera indica L.*) leaf extract, *P. acnes* bacterial isolate, *E. coli* bacterial isolate (InaCCB5), tetracycline, distilled water, Nutrient Agar (NA) (Merck), Nutrient Broth (NB) (Merck), alcohol 70% (technical), chitosan (Sigma-Aldrich), $Na_5P_3O_{10}$ (technical), CH_3COOH (Merck), CH_3OH (technical), stearyl alcohol, vaseline, glycerol, nipagin, nipasol, and wrapping.

Formulation of Mango Leaf Methanol Extract Using Chitosan Tripolyphosphate Matrix (Kurniasih & Atun, 2017)

The preparation of mango leaf extract with chitosan tripolyphosphate matrix was by weighing 1 gram of mango leaf extract. The extract was then dissolved in the distilled water 50 mL. The kitosan solution 100 mL was then added. NaTPP pH 3 350 mL was also gradually added while stirring with a magnetic stirrer at the speed of 1000 rpm for 2 hours. The NaTPP chitosan colloid was then separated with the centrifugation at 12,000 rpm for 15 minutes at 10 °C. The obtained solid was then washed using distilled water and then dried at the temperature of 40 °C. The concentration comparison between chitosan and NaTPP in (%) is presented in **Table 1.**

Antibacterial Activity Test (Ningsih et al., 2020)

The antibacterial activity test was conducted using agar diffusion method (well). The sample was mango leaf extract-chitosan biomaterial. Each 0.01 gram of formula A, B, and C was dissolved in 10 mL of acetic acid 1% (v/v) and then stirred until homogeneous using a stirrer. The homogeneous solution was then made to a concentration of 5 ppm. One ose of respectively *P. acnes* and *E. coli* bacteria from the culture stock was taken and then incubated in 10 mL of liquid medium (Nutrient Broth) for 18-24 hours at the temperature of 37 °C. 2 mL of bacterial culture was taken and its OD (absorbance) was then measured at the wavelength of 600 nm. If the OD value is > 1 with the maximum limit of 1.2, 50 L of culture is then taken, while if OD is < 1 with the maximum limit of 0.8, 100 L of culture is then taken. 15 mL of Nutrient Agar medium was poured into a petri dish and allowed to solidify. The culture was plate-like spread on the

Table 1. Concentration comparison between chitosan and NaTPP in (%)

Formula	Chitosan (%)	NaTPP (%)
A	0.1	0.0992
B	0.5	0.0992
C	1.0	0.0992

Table 2. The characterization of mango leaf extract with chitosan tripolyphosphate matrix. *Making the oil-in-water ointment preparations (*Ningsih et al., 2018*)*

Material	F1 (g)	F2 (g)	F3 (g)
Distilled water	Added up to 100 mL		
Glycerol	9.98	9.98	9.98
Tween 80	5	5	5
Nipagin	0.0025	0.0025	0.0025
Stearyl alcohol	9.98	9.98	9.98
Nipasol	0.0025	0.0025	0.0025
Vaseline	24.96	24.96	24.96
Formula C	-	0.5 mL	-
Acetic Acid	0.5 mL		
Tetracycline			0.5 mL

Information: F1 = Negative control, F2 = Sample (arumanis mango leaf extract with the addition of chitosan 1%), F3 = Positive control.

media using a drigalski spatula. The agar media was perforated with the diameter of ± 8 mm using a crock drill. The sample was inserted into the media hole at 50 µL, and then incubated for 1 x 24 hours at the temperature of 37 °C.

Characterization of Ointment Preparations

Homogeneity test

Ointment 1 gram was weighed, applied on a glass plate, rubbed, and then palpated. The ointment preparation is said homogeneous if the ointment structure is flat or the ointment mass does not remain solid. The test was performed 3 times with different ointment preparations.

pH test

Ointment 1 gram was diluted with 20 mL of distilled water, then immersed in the pH meter for one minute. A good pH for ointment preparations is in accordance with the skin pH at between 4.5-7.0.

Dispersion power test

Ointment 0.5 gram was placed on a petri dish in inverted position, then another petri dish was placed on top of the ointment and then left for one minute. The diameter of the spreading ointment was measured, added with additional load 150 gram, and then allowed to stand for one minute. The diameter of the spreading ointment was measured horizontally, vertically, and diagonally and then averaged. The test was performed 3 times with different ointment preparations.

Adhesive power test

Ointment 0.1 mg was weighed and placed on an object glass with the previously determined area. Another object glass was placed on top of the ointment and then pressed with a weight of 1 kg for 5 minutes. The object glass was set on the test equipment, the load was removed, and the time was recorded until the two object glasses were released. The test was performed 3 times with different preparations.

RESULTS AND DISCUSSION

Formulation of Arumanis Mango Leaf Extract with Chitosan Tripolyphosphate Matrix

The preparation of arumanis mango leaf extract with chitosan tripolyphosphate matrix was conducted using the ionic gelation method. Ionic gelation is a method involving a cross-linking process between polyelectrolytes and their multivalent ion pairs (Abdassah, 2017) This is the most widely used method since the process is simple and does not use many organic solvents (Fabregas et al., 2013) Making biomaterials was performed by mixing this with chitosan, NaTPP pH 3, and distilled water stirred using a magnetic stirrer for 2 hours with the speed of 1000 rpm. This research used chitosan due to its advantages, such as biocompatible, antibacterial, biodegradable, and having bioactive properties.

Tripolyphosphate (TPP) is a crosslinker and non-toxic material which does not change the chitosan biocompatibility and can be applied for biomedical applications (Alauhdin & Widiarti, 2014) The use of pH 3 aimed to obtain TPP cross-linked chitosan because if the solution pH is acidic, the ions contained in the solution are only TPP ions. In addition, acidic pH conditions will increase amine ionization in chitosan to increase the potential for bonding with TPP (Alauhdin & Widiarti, 2014).

Based on the research results on making the chitosan biomaterial, arumanis mango leaf extract produced a green colloid color. The green colloid was then centrifuged at 12,000 rpm for 15 minutes and produced a wet precipitate and was further dried using an oven at the temperature of 40 °C to form a solid which was then characterized using SEM, tested for antibacterial activity and made into ointment preparations.

Characteristics of Arumanis Mango Leaf Extract with Chitosan Tripolyphosphate Matrix

Mango leaf extract with chitosan tripolyphosphate matrix was then dried and characterized using the Scanning Electron Microscopy (SEM). SEM characterization aimed to figure out the particles' surface morphology. The obtained data were in the form of two-dimensional photos displaying the samples' surface. SEM analysis was performed on all formulas using the magnification of 3000x with chitosan control as the comparison as shown in Figure 1.

Based on the research results, it can be seen that the surface morphology of chitosan biomaterial has a rough and uneven surface without pores, agglomeration, and not spherical shape. the chitosan's surface morphology will change if there is a filler in chitosan. All formulas A, B, and C tended to have a rougher surface morphology with irregular shape and agglomeration, while chitosan without treatment had a smoother surface morphology. The results of this characterization also described the condition of arumanis mango leaf extract in having the uneven surface and non-uniform size.

The range of particle size from those three treatments was not uniform and there were many agglomerating particles. The particle size is between 4-15.6 µm, while the untreated chitosan has a smoother surface morphology with the largest particle size of around 31µm. A large concentration of chitosan with a constant NaTPP concentration will increase the tendency of particles to agglomerate higher. This is in accordance with the research conducted by (Mardliyati et al., 2012) stating that at a high concentration, the particles formed from chitosan and NaTPP became more numerous and denser forming clusters and aggregates into micro-sized particles.

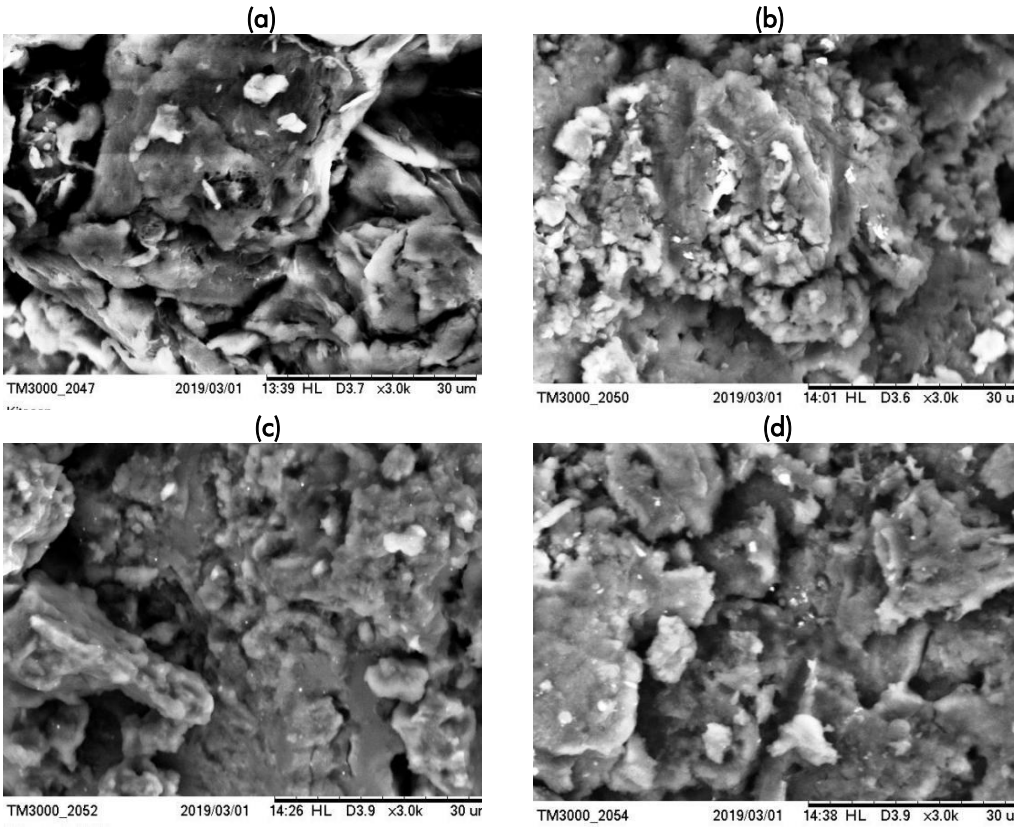

Figure 1. The SEM of chitosan (**a**), Formula A (**b**), Formula B (**c**), and Formula C (**d**)

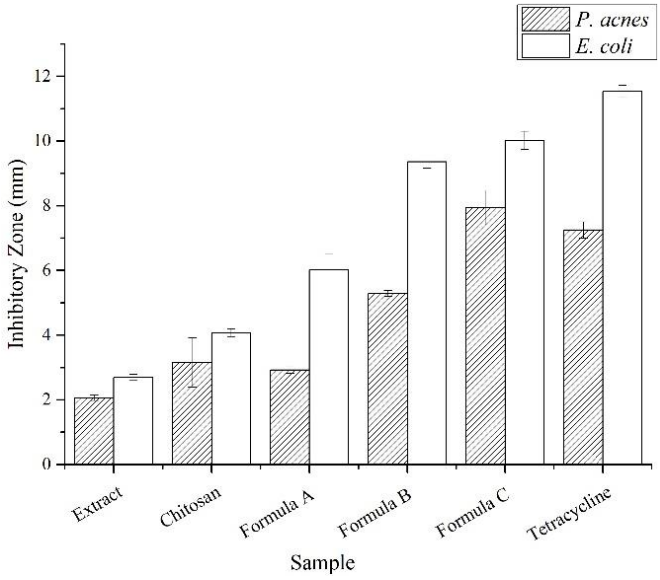

Figure 2. Graph of antibacterial activity test results on samples and chitosan

Antibacterial Activity

The samples used as antimicrobial substances were arumanis mango leaf extract, chitosan, formula A, formula B, and formula C with the same concentration of 5 ppm. The negative control was 1% acetic acid which has the ability to inhibit the bacterial growth, while the positive control was 500 mg tetracycline with the concentration of 5 ppm. The antibacterial activity test results can be seen in **Figure 2**.

Based on **Figure 2**, the antimicrobial substances used in this research could inhibit the growth of *P. acnes* and *E. coli*. *P. acnes* and *E. coli* are bacteria that can cause infection. The resulting inhibition zone against *E. coli* was larger than that against *P. acnes*. The inhibition zone produced by arumanis mango leaf extract against *P. acnes* and *E. coli* was respectively 2.07 mm and 2.67 mm. Based on the research conducted by (Ningsih et al., 2017), the compounds contained in arumanis mango leaf extract included flavonoids, alkaloids, saponins, steroids, and tannins.

The inhibition zone produced by formula A against *P. acnes* and *E. coli* was respectively 2.92 mm and

6.05 mm. The inhibition zone of formula B against *P. acnes* and *E. coli* was respectively 5.29 mm and 9.36 mm. The inhibition zone of formula C against *P. acnes* and *E. coli* was respectively 7.94 mm and 10.02 mm considered the largest inhibition zone of those 3 treatments. This shows that the greater the concentration of the chitosan used, the larger the resulted inhibition zone. These results are in accordance with the results of research conducted by (Suptijah et al., 2008) mentioning that the higher the concentration of chitosan, the higher the inhibition will be. The resulting inhibition zone against *E. coli* was larger than that of *P. acnes*. The difference in inhibition of test bacteria in general by antimicrobial substances is due to differences in the cell walls of the two types of Gram positive and Gram negative bacteria. The greater inhibition in Gram-negative bacteria was due to the thinner cell walls of Gram-negative bacteria with 10% peptidoglycan composition and high lipid content (11-22%). While the cell wall of Gram-positive bacteria is thicker which consists of more than 50% peptidoglycan and low lipid content (1-4%) (Damayanti et al., 2016).

The research results show that mango leaf extract with chitosan tripolyphosphate matrix was able to provide greater inhibition when compared to the extract and chitosan treatments for both Gram-positive and Gram-negative bacteria. This indicates that mango leaf extract with a low concentration of chitosan tripolyphosphate matrix was able to provide greater inhibition than that using extract and chitosan. Chitosan matrix has not only biocompatible properties but also antibacterial properties, while arumanis mango leaf extract was proven inhibiting the growth of both Gram-positive and Gram-negative bacteria. The antibacterial mechanism of arumanis mango leaf extract with chitosan tripolyphosphate matrix was generally supported by the antibacterial mechanism of chitosan and arumanis mango leaf extract. The antibacterial mechanism of chitosan is generally through cell wall interaction and destruction. Gram-positive bacteria have a cell membrane covered by a cell wall composed of 30-40 peptidoglycan layers. The positive charge in chitosan is able to bind and cause cell wall distortion and breakdown. Gram-negative bacteria will experience a bacterial nutrient flow blocking process causes the cell death. This causes the cells died faster. Gram-negative bacteria have the ability to interact and absorb greater chitosan to more easily enter and damage the cell walls of Gram-negative bacteria compared to those of Gram-positive bacteria (Damayanti et al., 2016).

Oil-in-Water Ointment Preparation

Preparation A was in the form of water phase made by heating the distilled water, glycerol, polysorbate 80, nipagin at the temperature of 70 °C and then added formula C with the concentration of 5 ppm, positive control in the form of tetracycline 5 ppm, and acetic acid as the negative control. Preparation B was in the form of oil phase made by melting the stearyl alcohol, white vaseline, and nipasol into the heated mortar containing the water phase. White Vaseline had the function as emollient. It was added to reduce the base hardness. The stearyl alcohol had the function as emulsifier. The emulsifier bridged the water phase and the oil phase to prevent phase separation in which oil would be above the liquid and water would be at the bottom. Nipasol was used as the preservative. The addition of preservative was the most important thing since the mixture of oil and water in contact frequently allowed the growth of microorganisms. The use of parabens was because the preservative had low toxicity, odorless, and did not cause irritation. The concentration of the commonly used nipasol was 0.01% -0.6% (Syamsul et al., 2015). The oil phase was then poured and stirred in a mortar until the preparation became solid. The ointment preparation was the oil-in-water type. The ointment preparation made were then characterized using homogeneity, pH, Dispersion power, adhesion, protection, and antibacterial activity against *P. acnes* and *E. coli*.

Ointment Preparation Characteristics

The physical property testing of ointment preparation was performed to change the ointment preparation physical properties during storage for 16 days with the testing time on days 1, 6, 11, and 16.

Homogeneity

The homogeneity test was conducted to figure out whether the ointment preparation was evenly mixed (between active substances with ointment base and other required additives). The ointment preparation was considered homogeneous if no lumps nor coarse granules found in the preparation and the particles were evenly distributed at the top, middle, and bottom parts. Based on the conducted research, it shows that the ointment preparation was homogeneous during the storage for 16 days since there were no lumps nor coarse granules.

The ointment preparation had to be homogeneous because the medicinal ingredients would be evenly dispersed in the ointment base and each part would contain the same number of medicinal ingredients, so that the ointment preparation had good results. If the medicinal ingredients were not evenly dispersed, then the medicinal ingredients would not provide the expected therapeutic effect (Ulaen *et al*., 2004). The preparation homogeneity depended on the similarity of ingredients used in the base and active substance. When there were property differences between the base and active substance, clumping might occur and result in larger preparation particle forms (Ningsih et al., 2017). The results of this research were in accordance with SNI since there are no lumps nor coarse granules in the ointment preparation.

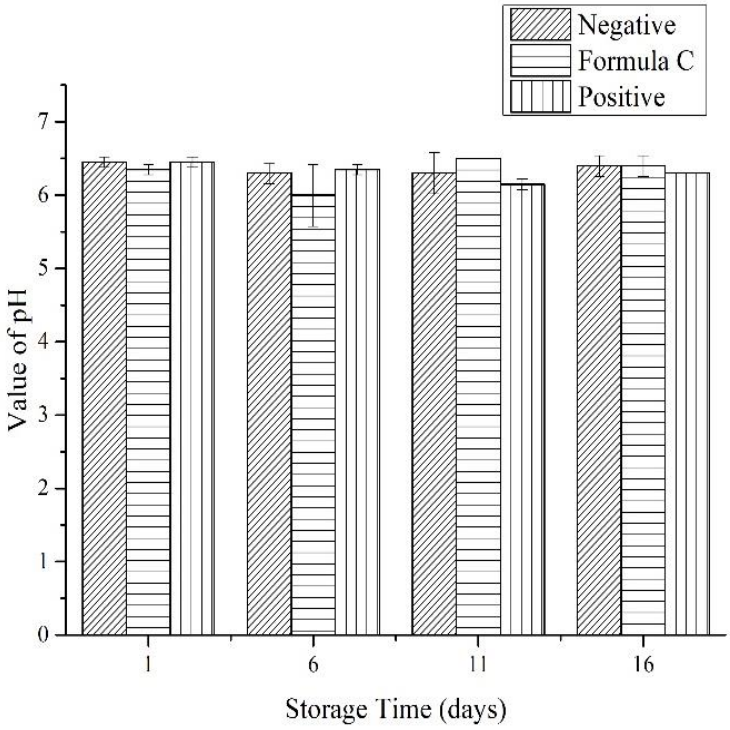

Figure 3. Ointment preparation pH value Graph

Ph Value

The pH test was conducted to figure out the acidity or alkalinity level of ointment preparation made. A good ointment preparation has the pH range appropriate for the skin's pH, at 4.5 - 7.0 without irritating the skin. The pH test results were presented in **Figure 3**.

Based on the research result graph presented in **Figure 3**, the pH values obtained were in the SNI pH range appropriate for skin, at 4.5 - 7.0. the too acidic pH could cause skin irritation, while too alkaline pH could cause dry skin (Swatika et al., 2013).The ointment preparation with acetic acid additives had the pH value of 6.3 – 6.45 and the ointment formula C had the pH value of 6.0 – 6.4, while the ointment with tetracycline additives had the pH value of 6.15 – 6.45.

Dispersion power

The dispersion power test was intended to figure out the ability of ointment preparation to spread on skin. Good dispersion of ointment preparations will promote a better substance absorption. Preparation dispersion differences greatly affected the substance diffusion rate as they pass through the skin membrane. The greater the Dispersion power the better the preparation because the larger the membrane where the preparation site is spread, the medicinal diffusion will increase due to the greater distribution coefficient (Novita & Hayati, 2017). The results of Dispersion power measurement of ointment preparation could be seen in **Figure 4**. Based on the research results, **Figure 4** ointment preparation with the additional ingredient of acetic acid had the dispersion value of 5.48 – 6.40 cm, while that of formula C chitosan respectively with mango leaf extract 5 ppm had the dispersion value of 5.25 – 6.3 cm, and tetracycline 5 ppm had the dispersion value of 5.45 – 6.32 cm. The produced dispersion value was in accordance with the standard for semi-solid preparation of 5-7 cm (Ulaen et al., 2004). Dispersion differences could be caused by various factors, such as the added active substance, stirring method, and also where the ointment was stored (Widyaningrum *et al*., 2012).

Adhesive Power

The adhesive power test was conducted to figure out how long the ointment preparation attached to the skin surface, so that the active substance could be absorbed. The greater the ointment preparation adhesive power to the skin, the more optimal the active substance absorption will be. On the other hand, if the ointment adhesive power is low, the preparation will be easily separated so that the active substance absorption is not optimal. Good adhesive power will have optimal antibacterial properties during the application time (Wibowo et al., 2017). The adhesive power test results could be seen in **Figure 5**.

Based on the graph of research results in Figure 5, the preparation adhesive power tended to unstably fluctuate. Nevertheless, within the range of good adhesion of semi-solid formulations of over one second (Garg et al., 2002). The ointment preparation respectively with the addition of acetic acid had the adhesive power value of 1 – 4.50 seconds, formula C of 1 – 5 seconds, and tetracycline of 1 – 4 seconds.

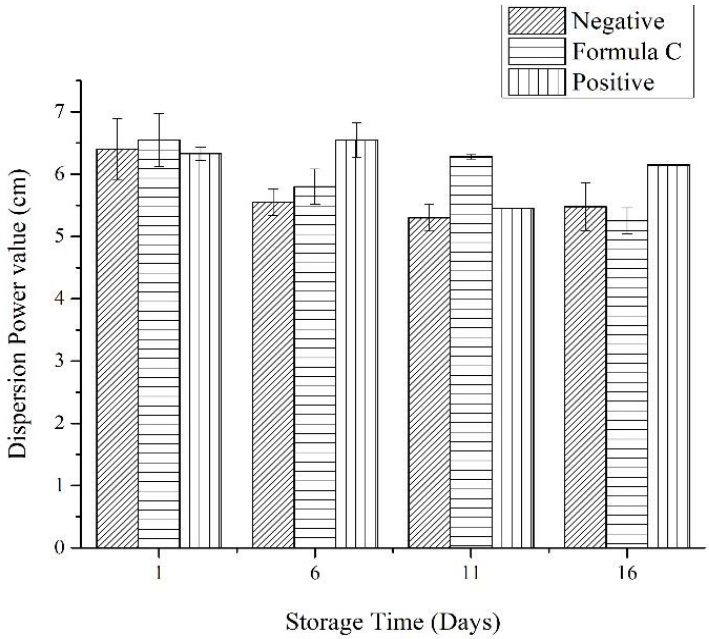

Figure 4. The ointment preparation distribution value graph

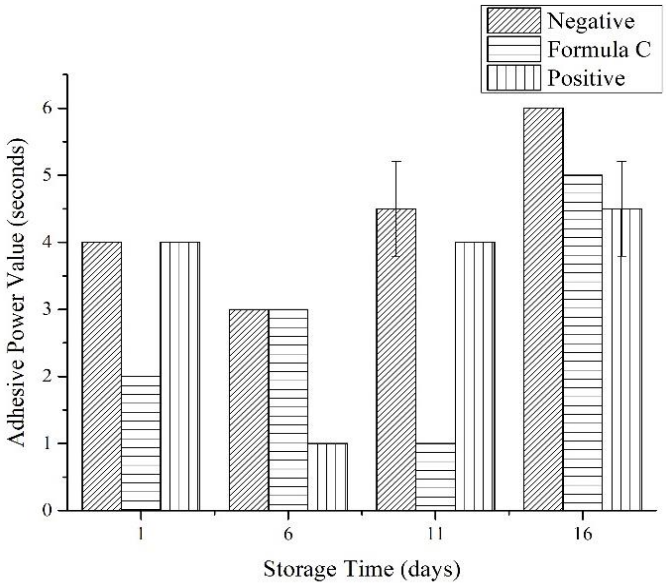

Figure 5. The ointment preparation adhesive power value graph.

Ointment Preparation Antibacterial Activity

The ointment preparation antibacterial activity test was conducted, such as the antibacterial activity test, using the well diffusion method. The relationship between ointment storage time and activity value could be seen in **Figure 6**.

Based on the research results, the ointment preparation activity was indicated by the formation of clear zone (inhibition zone) around the hole. The inhibition zone produced by the ointment formula C on day 1 with the concentration of 5 ppm against *P. acnes* and *E. coli* bacteria, was respectively 14.03 mm and 14.24 mm, while that produced by the biomaterial ointment preparation on day 16 with the concentration of 5 ppm on *P. acnes* and *E. coli* bacteria was respectively 9.33 mm and 9.98 mm. The results of ointment preparation activity decreased on day 16. The inhibition zone produced by the positive control in the form of 5 ppm teracycline on day 1 against *P. acnes* and *E. coli* bacteria was respectively 23.26 mm and 24.22 mm, while that produced on day 16 against *P. acnes* and *E. coli* bacteria was respectively 7.22 mm and 8.19 mm. This shows that the longer the activity, the ointment preparation will decrease against *P. acnes* and *E. coli bacteria*. This was also in accordance with the research conducted by (Zalizar, 2011) stating that the longer the ointment is stored, the produced inhibition will decrease. The decreasing antibacterial activity was predictively caused by the longer the material is used, so that the diffused substance will continuously decrease and the antimicrobial activity in inhibiting the bacterial growth also continuously decreases.

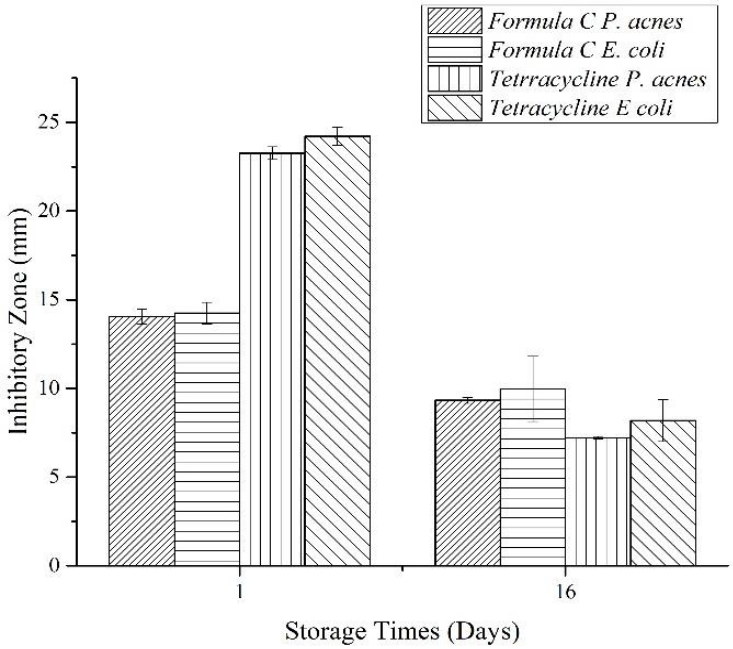

Figure 6. The ointment preparation antibacterial activity test result graph.

Based on the antibacterial activity test results, the ointment formulation could increase the inhibition zone of bacterial growth in formula C and tetracycline as the positive control. The increasing inhibition zone in the ointment preparation form predictively came from the increasing penetration of antibacterial compound diffused into the testing media to produce a larger inhibition zone. In addition, the increasing inhibition zone in the ointment preparation form could also be influenced by the carrier or the ointment base used. The ointment base used in this research was O/W or water-washed ointment containing much water. The presence of this hydration effect could lead to the increasing medicinal penetration into the skin. If water saturates the skin, the skin tissue will soften and expand. Meanwhile, the skin permeability will increase and cause the medicinal penetration also increases resulting in a larger inhibition zone when compared to the extract form (Dermawan et al., 2015).

CONCLUSIONS

Making arumanis mango leaf extract with chitosan tripolyphosphate matrix can be performed using the ionic gelation method with various chitosan compositions. The results of morphological characterization using SEM on various chitosan compositions show that all morphology was in the form of uneven-surface aggregates. The formula C activity test results showed the greatest activity against *P. acnes* and *E. coli* respectively at 7.94 mm and 10.02 mm. The obtained ointment preparation was white in color, smelled ointment preparation, semi solid in shape, spreading power at 5.25 – 6.3 cm, adhesive power at 1 – 5 seconds, pH at 6.0 – 6.4, homogeneous. The activity of ointment preparation on day 1 against *P. acnes* was respectively at 14.03 mm and *E. coli* at 14.24 mm, while the activity on day 16 on *P. acnes* was respectively at 9.33 mm and *E. coli* at 9.98 mm.

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