Formulation and evaluation of antibacterial activity of nanoparticles ointment preparation using Blimbi extract

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ABSTRACT
Indonesia has various beneficial plant species which have not been cultivated well yet. Blimbi (Averrhoa bilimbi L.) is among those plants which have antibacterial activities against Staphylococcus aureus and Escherichia coli. As carriers, the nanoparticles of this plant dissolve, trap, encapsulate, and attach the chemical preparation inside its matrix. This study aimed to compare the effect of two forms of the nanoparticles using Blimbi extract (i.e., single state and ointment preparation) on the antibacterial activity against bacteria. In this experimental study, the Blimbi extract changed to nanoparticles followed by a drying process using a drier spray. Afterwards, the nanoparticles were tested for antibacterial activity and mixed with an ointment base. The Blimbi extract was formulated in a form of absorbent ointment preparation with dark brown color and unique fragrance with pH of 6.42-6.80 and spread ability of 6.16-6.90 mm. According to the results, the diameters of the inhibition zone of nanoparticles using Blimbi extract on S. aureus and E. coli were 20 and 19.75 mm, respectively. The nanoparticles levels were higher in E. coli (12.25 mm) than those in S. aureus (15.50 mm). Meanwhile, the nanoparticles levels in ointment preparation were 15.84 and 14.73 mm for S. aureus and E. coli. The nanoparticles using Blimbi extract and the ointment using Blimbi extract were safe and did not irritate the skin.

Introduction
Infection is a medical condition causing different kinds of diseases with increasing rates, especially in Indonesia. It can be transmitted from one person to another or even from animal to human. Skin infections are caused due to various microorganisms, such as bacteria, viruses, rickettsia (bacteria gram negative), fungi, and protozoa. Therefore, topical medications for infected skins lead to decrease systemic side effects and improve healing (1). Blimbi or Averrhoa bilimbi L. is a kind of plant in Indonesia that has a lot of beneficial functions for medication. It can be used for the treatment of cough, rheumatic pain, goiter, oral ulceration, acne, tinea versicolor, high blood pressure, and toothache. Therefore, it cannot be separated from the chemical concentrations in Blimbi antioxidants, such as alkaloid, saponin, and flavonoid (2, 3). The rough extracts of Blimbi can potentially be used as antibacterials against Staphylococcus aureus and Escherichia coli (3). Due to the beneficial effects of Blimbi, the extract was formulated in a form of ointment using absorption base. The ointment had a suitable concentration for the treatment of bacterial skin infections, including ulcer, cellulitis, and urticaria (4).

Furthermore, with regard to the technology applied in the preparation of herbal medication, the use of nanoparticles as a medication medium has been developing for several years. One of the advantageous of the nanoparticles technology is the tiny form of nanoparticles that will cause the increase of the compound solution and decrease the dosage of medication. Moreover, nanoparticles keep the skin hydrated, permeate through the skin, and increase the skin stability (5). Ointments are oil-based preparations that are semi-solid and easy to apply as external medicines. Ointments do not smell rancid; therefore, they should consist of oil substantive or fat emulsion/wax which has high water proportion (6). This study aimed to develop an ointment with nanoparticle formulation containing Blimbi extract and polyacrylic acid (PAA) using ionic glass method. The nanoparticles of Blimbi extract were mixed with the ointment base using various concentrations. The mixture was tested against S. aureus and E. coli to examine the in vitro antibacterial activities.

Materials and Methods
The plant of Blimbi was obtained from BALITRO (Laboratory of Plants, Spices, and Medicine) in Bogor, Indonesia. The sample under study was Blimbi fruit (voucher number: DF 51012). Meanwhile, the Blimbi determination was carried out in World-Class State Research Institute in Cibinong, Indonesia, to ensure the plant was suitable for the research. In addition to Blimbi, nutrient agar (Merck, Germany), peptone water (Merck, Germany), silica gels 60 F254 nm (Merck, Germany), S. Aureus (ATCC 25923) (Laboratory of Plants, Spices, and Medicine, Indonesia), E. Coli (ATCC 29322) (Laboratory of Plants, Spices, and Medicine, Indonesia).
measure 100 µl nanoparticle suspension and the experiment was carried out in triplo. The particle size distribution was obtained from this assay.

**Ointment preparation using Blimbi Extract**

The ointment base was made by smelting the Cera Alba, lanoline hydrate, stearyl alcohol, and vaseline. It was melted in the steam cup in a water bath, stirred until homogenous. After that, propylparaben and alpha-phocopherol were poured while stirring. When it cooled, the extract was poured little by little while being constantly stirred until homogenous and fulfilling the requirement of the ointment. Then, the extract was poured into the container.

**Antibacterial activity Assay**

The experiment was carried out on appropriate germination, containing microbial experiment to determine whether the absorbent ointment base influenced the inhibition toward microbial experiment by the result of diameter in mm. The microbial suspension preparation was taken from 1 test bacterial loop which was inserted into peptone broth at 35-37 °C for 24 h. In antibacterial activity assay toward *S. aureus* and *E. coli*, 15 ml nutrient agar was poured into sterile Petri dishes, and then 0.1 ml of bacterial suspension was poured till homogenized. The nanoparticles Blimbi extract was poured into Sumuran, incubated at 35-37 °C for 24 h. Afterwards, the inhibition diameter was measured (all processes were done aseptically on laminar air flow).

**Irritation testing in rabbits**

Male albino rabbits weight ± 2 Kg were used in this experiment (Ethical code: 16-11-462). A day before the testing, the animals’ fur was shaved on the back area and covered with non-irritant plaster. The rabbits involved in this testing were observed for erythema and edema (n = 3). The response assessment was performed at 1, 24, 48, and 72 h after opened up the plaster. If the skin damage did not be identified as corrosive and irritation after 72 h, the observation was continuously until 14 days. Besides the observation toward irritation, the toxic effect as like defatting of skin and others toxic effect as well as the weight were explained and recorded.

**Statistical analysis**

Data, pH, dispersion, and viscosity were analyzed using paired sample t-test and SPSS (version 24, Chicago, USA) with 95% confidence interval.
Results

Blimbi extract preparation
A kilogram of Blimbi powder was extracted using heat digestion and aquadest. The concentrated extract was obtained after concentrating about 295 grams with a yield amount of 14.75%.

Organoleptic extract testing
Table 1 demonstrates the results of organoleptic testing with regard to Blimbi extract in terms of shape, color, smell, and flavor.

Table 1 Results of Blimbi extract organoleptic testing

| Organoleptic      | Result      |
|-------------------|-------------|
| Shape             | Thick Extract |
| Color             | Dark Brown  |
| Smell             | Aromatic    |
| Flavor            | Acid        |

Preparation of blimbi extract nanoparticles
The results obtained from a five-day observation regarding Blimbi extract nanoparticle solvents are presented in table 2. According to the results, the solvent was stable with brown color containing no sediments. Therefore, it was considered suitable for this experiment.

Table 2 Results of a five-day observation regarding nanoparticles Blimbi extract solvent

| Observation days | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
|------------------|-------|-------|-------|-------|-------|
| Color            | Brown | Brown | Brown | Brown | Brown |
| Turbidity        | Stable | Stable | Stable | Stable | Stable |
| Sediment         | None  | None  | None  | None  | None  |

Nanoparticle characteristic evaluation
Delsa™Nano was applied three times to measure the particle size of nanoparticles Blimbi extract. Consequently, the appropriate size for nanoparticles ranged from 1 to 500 nm. According to the test results of the particle size in the table 3, the desired nanoparticle sizes which fulfilled the requirement criteria were 142.8, 153.1, and 121.2 nm. The appropriate size of the particles depended highly on the method applied. Therefore, ionic glass method was used with a magnetic stirrer in this study. The formation of a tiny size of the nanoparticle using magnetic stirrer in high speed generalized the accepted energy in all sides of the solvent, leading to the homogeneity of the particle size. The appropriate size of particles was required to enable nanoparticles solvent Blimbi extract to proceed further which resulted in the production of nanoparticles ointment using Blimbi extract.

Blimbi extract ointment preparation
The first step in ointment preparation using Blimbi extract was to make the ointment base using the melting method. Table 4 presents the formulations of ointment preparation which included Cera Alba, Lanoline anhydrate, Stearyl alcohol, and Vaseline album melted in a steam dish with a certain speed and stirred until homogenized. When it was cooled, the nanoparticles using powder extract was poured little by little till homogenous using homogenizer for 15 min at 200 rpm.

Table 3 Results of particle size testing

| Testing | Particle Diameter (nm) |
|---------|------------------------|
| 1       | 142.8                  |
| 2       | 153.1                  |
| 3       | 121.2                  |
| Average | 139.03                 |

Table 4 Formulations of ointment preparation

| Formula | I      | II     |
|---------|--------|--------|
| Extract | 20%    | 1Xkhm  |
| Cera Alba | 5%    | 5%    |
| Lanolin anhydrate | 10%   | 10%   |
| Stearyl Alcohol | 10%   | 10%   |
| Alpha Tocopherol | 0.1%  | 0.1%  |
| Propyl Paraben | 0.1%  | 0.1%  |
| Aquadest | -      | 5%    |
| Vaseline album | Ad 100 | Ad 100 |

Note:
Formula I: Blimbi Extract
Formula II: Nanoparticles powder using Blimbi Extract

Antibacterial activity and irritation testing
The result of antibacterial activity testing of ointment preparation using Blimbi extract showed activity status after the second day of observation (48 h). This was due to the diffusion of substance that was beneficial to inhibit bacteria. It can be compared with the single state extract which showed an activity status only after the first day (24 h) of observation. Based on the results of
testing presented in table 5, the largest diameter of the inhibition between nanoparticle extract and nanoparticles using Blimbi extract ointment was nanoparticle extract. The irritation testing results can be observed in table 6 in which negatively erythema and edema are showed in rabbit skin. Therefore, it is confirmed that Blimbi extract ointment and nanoparticles using Blimbi extract ointment is safe or do not cause irritation for the skin.

### Discussion

This study aimed to assess the antibacterial activities of Blimbi extract against the growth of bacteria. Table 7 presents the results of antibacterial activities of Blimbi extract with various concentrations against *S. aureus* and *E. coli* using the diffusion method. As can be seen, the Blimbi extract has antibacterial activities against *S. Aureus* and *E. coli* which resulted from the interactions between active compounds in the extract.

| Table 5 Results of inhibition diameter (mm) of antibacterial activity in ointment preparation against *S. aureus* and *E. coli* |
|---|---|---|---|---|---|
| Formula | *S. aureus* | | | *E. coli* | |
|  | I | II | Average | I | II | Average |
| 1 | 14.50 | 14.42 | 14.46 | 13.90 | 13.33 | 13.62 |
| 2 | 15.87 | 15.80 | 15.84 | 14.74 | 14.72 | 14.73 |
| P | 31.87 | 31.14 | 31.51 | 31.68 | 32.40 | 32.04 |

Note: F1: Blimbi Extract Ointment, F2: Nanoparticles Ointment using Blimbi Extract, OB: Ointment Base
C: Comparison (Positive Control = Chloramphenicol Ointment), I: Petri Dish I, II: Petri Dish II

| Table 6 Results of irritation testing on ointment preparation in rabbits |
|---|---|---|---|
| Hours after applying | Ointment Extract | | Nanoparticles Ointment |
| Rabbit | Erythema | Edema | Erythema | Edema |
|  | | | | | |
| 1 | - | - | - | - |
| 2 | - | - | - | - |
| 3 | - | - | - | - |
| 1 | - | - | - | - |
| 24 | 2 | - | - | - |
| 3 | - | - | - | - |
| 1 | - | - | - | - |
| 48 | 2 | - | - | - |
| 3 | - | - | - | - |
| 1 | - | - | - | - |
| 72 | 2 | - | - | - |
| 3 | - | - | - | - |

Note: + = Irritation happen, - = No Irritation
Furthermore, the differences can be observed in the inhibition zones of different extract concentrations against bacteria. Therefore, the extract of Blimbi with 20% concentration has antibacterial activity against S. aureus and E. coli. However, the results of inhibition diameter from Blimbi extract against S. aureus and E. coli with 20% concentration showed contradictions in diameters. One of the differences dealt with the cell wall composed between a positive and negative gram of bacteria. These varieties made different responses toward inhibition from the antibacterial substance in the extract. Table 7 shows the differences among inhibition diameters of each bacterium in every concentration. The inhibition diameter with 20% concentration in E. coli (14.5 mm) is smaller than S. aureus (15.9 mm). It is due to the antibacterial compounds having difficulties to E. coli cell. This finding is consistent with the results of a study conducted by Lay and Hastowo. They stated that the substance wall of negative bacteria contained some layers, such as lipoprotein, lipopolysaccharide, and peptidoglycan. As a result, the negative bacteria had an elimination system toward different substances in the lipopolysaccharide layer (7). Meanwhile, the inhibition diameter of S. aureus is larger due to the simple structure of the cell wall. Positive bacteria only have one tight layer (i.e., peptidoglycan) in which it will ease the antibacterial compound to enter into the substance. This inhibition zone shows that the extract can diffuse; therefore, it will show the sign of antibacterial activity against S. aureus and E. coli. The compounds which successfully obtained from the filtration of Blimbi extracts are saponins and flavonoids. Saponins are active compounds increasing the permeability of the cell membrane to lead to the formation of cell hemolysis. If saponins interact with bacterial substance, the bacteria will become broken or disrupted (8). The ultimate effect of the saponins toward bacteria is the protein and enzyme release from the cell. Meanwhile, flavonoids are depicted as one of the compounds under phenolic class (9). The characteristic of this antibacterial can cause protein denaturation in a cell. The existence of flavonoids along with saponins causes the cell to break leading to lysis conditions. Furthermore, the ointment preparation included physical and chemical assessments, such as organoleptic, pH, spreadability, viscosities, fluidity, and inhibition activity against S. aureus and E. coli. The organoleptic evaluation aimed to assess the different colors and smells (10). According to the results of the evaluation, no differences were observed in terms of aroma, smell, shape, and homogeneity during the first and the fourth weeks (Table 8).

Table 7 Inhibition diameter of Blimbi extract against S. aureus and E. coli using Sumuran method

| Sample       | Concentration (%) | S. aureus PD1 | S. aureus PD2 | S. aureus Average | E. coli PD1 | E. coli PD2 | E. coli Average |
|--------------|-------------------|---------------|---------------|-------------------|-------------|-------------|----------------|
| Blimbi Extract | 20                | 15.7          | 16.1          | 15.9              | 14.4        | 14.6        | 14.5           |
|              | 40                | 17.5          | 18.4          | 18.0              | 16.6        | 16.1        | 16.4           |
|              | 80                | 22.2          | 22.0          | 22.1              | 18.2        | 18.9        | 18.6           |
| C(+)         |                   | 26.4          | 24.1          | 25.3              | 30.3        | 30.8        | 30.6           |
| C(-)         |                   | 8             | 8             | 8                 | 8           | 8           | 8              |

Note: PD1: Petri Dish 1, PD2: Petri Dish 2, C (+) : Control positive, C (-) : Control negative

Based on the organoleptic evaluation of the ointment, the physical appearance of the ointment was semi-solid and homogenous with Blimbi aroma. The colors of the Blimbi extract ointment, the ointment base, and the comparison were obtained as dark chocolate, cream, and white, respectively. The ointment was homogenous if the base, active materials, and other materials mixed smoothly. The homogeneity evaluation was obtained to get the appropriate result of the ointment preparation. Therefore, with regard to the obtained homogeneity, no irritation occurs when the ointment is administered (11). With reference to pH evaluation of ointment preparation in table 9, formula F1 had the lowest amount, compared to formula F2 and ointment base. Formula 2 had the highest pH value due to the presence of extra materials to maintain pH 8. The appropriate pH for skin regarding the Blimbi extract ointment, nanoparticles using Blimbi extract, and the ointment base were 4, 5-6, and 5, respectively; therefore, there was no harm to the skin. If pH is too acidic, it causes irritation, whereas high levels of alkaline make the skin dry.
According to the results of spreadability evaluation, Blimbi extract ointment (F1), nanoparticles using Blimbi extract ointment (F2), and ointment base (OB) were semi-solid. The observed differences were due to the different concentrations in each of the active substances. Table 10 presents that the levels of spreadability decreased in F1, F2, and OB. This, however, relates to the amount of storage time. When the ointment became thicker, the water was reduced during storage leading to the decrease in the spreadability of preparation. The viscosity is a measure of liquid resistance to flow. The higher the viscosity, the smaller the globule size and vice versa. The non-Newtonian fluid consists of Bingham plastic, pseudoplastic, thixotropic, rheopectic, and dilatant (11). An evaluation was done to investigate the differences in terms of viscosities and fluidities regarding each formula. There are contradictions with respect to the results of viscosities in Blimbi extract ointment preparation and Blimbi extract nanoparticles (Table 11). These varieties are attributed to the different concentrations of each formula. The F2 obtained the highest viscosities, compared to F1 and OB. However, the lowest viscosities assigned to F1 leading to the decrease in viscosities of a preparation.

Table 8 Organoleptic evaluation results of ointment preparation

| Formula | Organoleptic | Shape | Homogeneity | Aroma | Color      |
|---------|--------------|-------|-------------|-------|------------|
| F1      | Semi-solid   | Homogeneous | Blimbi Aroma | Dark Chocolate |
| F2      | Semi-solid   | Homogeneous | Blimbi Aroma | Dark Chocolate |
| OB      | Semi-solid   | Homogeneous | Wax Aroma    | Cream          |
| C       | Semi-solid   | Homogeneous | Ointment Aroma | White          |

Note: F1: Blimbi Extract Ointment, F2: Nanoparticle Ointment using Blimbi Extract, OB: Ointment Base, C: Comparison (Positive Control)

Table 9 Results of pH evaluation in Blimbi ointment

| Formula | Average pH/week |
|---------|-----------------|
|         | 0   | 1   | 2   | 3   | 4   |
| F1      | 4,64 | 4,71 | 4,79 | 4,84 | 4,90 |
| F2      | 6,42 | 6,50 | 6,56 | 6,64 | 6,80 |
| OB      | 4,98 | 5,01 | 5,05 | 5,10 | 5,15 |

Note: F1: Blimbi Extract Ointment, F2: Nanoparticle Ointment using Blimbi Extract, OB: Ointment Base

Table 10 Results of spreadability of Blimbi extract ointment evaluation

| Formula | Total Average Spreadability (cm) / week |
|---------|----------------------------------------|
|         | 0   | 1   | 2   | 3   | 4   |
| F1      | 7,79 | 7,56 | 7,32 | 7,16 | 6,97 |
| F2      | 6,90 | 6,72 | 6,62 | 6,45 | 6,16 |
| OB      | 11,60 | 11,44 | 11,28 | 11,08 | 10,82 |

Note: F1: Blimbi Extract Ointment, F2: Nanoparticle Ointment using Blimbi extract, OB: Ointment Base
Plants are generally the oldest source of pharmacologically active compounds and have provided humankind with many medicinally useful compounds for centuries. Today more than two-thirds of the world’s population rely on plant-derived drugs (12). Numerous studies have been conducted with the extracts of the various plant. Nworu et al. conducted a study on an herbal ointment containing Dissotis theifolia extract for wound healing and antibacterial activities against clinical wound isolates of Staphylococcus aureus and Pseudomonas aeruginosa. The results of the study showed the antibacterial and wound healing effects of D. theifolia when formulated as an ointment. It may explain

| RPM | Viscosities (cps) | Viscosities (cps) |
|-----|------------------|------------------|
|     | OB               | F1               | F2               | OB               | F1               | F2               |
| 1   | 85369            | 126000           | 76624            | 85836            | 126690           | 77043            |
| 2   | 58578            | 86458            | 52577            | 58898            | 86930            | 52865            |
| 2,5 | 37717            | 55668            | 33853            | 37785            | 55769            | 33915            |
| 4   | 26239            | 38728            | 23552            | 26287            | 38798            | 23594            |
| 2,5 | 18598            | 27450            | 16693            | 18632            | 27500            | 16723            |
| 2   | 23151            | 34169            | 20779            | 23193            | 34231            | 20817            |
| 1   | 34465            | 50869            | 30935            | 34528            | 50961            | 30991            |

Table 11 Results of viscosities evaluation of Blimbi Extract Ointment

| RPM | Week 2 | Week 3 | Week 4 |
|-----|--------|--------|--------|
|     | Viscosities (cps) | Viscosities (cps) | Viscosities (cps) |
|     | OB     | F2     | F1     | B     | F2 | F1 | B     | F2 | F1 |
| 1   | 87104  | 127154 | 79244  | 88180 | 128567 | 85022 | 88575 | 129144 | 80582 |
| 2   | 60097  | 87729  | 54374  | 60504 | 88216 | 54743 | 60940 | 88852 | 55137 |
| 2,5 | 38696  | 56488  | 34883  | 39031 | 56970 | 35185 | 39312 | 57317 | 35439 |
| 4   | 26921  | 39299  | 24268  | 27154 | 39590 | 24478 | 27349 | 39876 | 24655 |
| 2,5 | 19081  | 27854  | 17201  | 19246 | 28061 | 17350 | 19385 | 28263 | 17475 |
| 2   | 23752  | 34672  | 21411  | 23957 | 34930 | 21597 | 24130 | 35181 | 21752 |
| 1   | 35360  | 51618  | 31876  | 35666 | 52002 | 32152 | 35923 | 52376 | 32384 |

Note: Viscosities evaluation is measured using Brookfield Viscometer with RV #25 spindles, F1: Blimbi Extract Ointment, F2: Nanoparticles Ointment using Blimbi Extract, OB: Ointment Base
its widespread use in many folk traditions for the treatment of sores, and wounds. Moreover, Pandey et al. (2010) evaluated the antibacterial and antifungal activities of a herbal ointment containing Aloe Vera, Azadirachta indica, and Curcuma-longa. It was found that bacteria were more sensitive to all kinds of the ointments, compared to fungi. In addition, Aloe Vera ointment showed more antibacterial and antifungal activities than those of the other ointments (13).

Meanwhile, the present study tries to formulate and evaluate antibacterial activity among nanoparticles using Blimbi extract. Blimbi, which was utilized as plant extract in this study, is considered one of the plants in Indonesia with a lot of benefits; however, it is not cultivated well. The formulation of antibacterial activities of nanoparticles using Blimbi extract was found to be suitable to be used as an ointment and was reported to be safe for skin.

**Conclusion**

According to the result of the study, Blimbi extract with 20% concentration can inhibit the growth of S. aureus and E. coli. The mixture of PAA and calcium chloride can form nanoparticles with a diameter size of 139.033 nm using ionic glass method. The nanoparticles using Blimbi extract with 17.926% concentration had antibacterial activity against S. aureus and E. coli. In addition, they can be formulated for ointment preparation with basis absorption, brown color, pH of 4.64-6.8, spreadability of 6.16-11.60 mm, and Bingham plastic characteristic. After irritation testing, nanoparticles ointment preparation using Blimbi extract was found safe without skin irritation.

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**Ethical Approval and Consent to Participate**

None.

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**Conflicts of interest**

Authors declare no conflicts of interest.

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