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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.

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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,

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Indonesia), adeps lanae (Merck, Germany) were used in this study. Furthermore, the tools used for the experiment included homogenizers (Hsiang Tai, Taiwan), spreadability test tools (Yuyang Industrial, China), UV-Vis spectrophotometers (Hirayana, Japan), brookfield viscometers (Brookfield Engineering, US), particle size analyzers (Beckman Coulter, USA), spray dryers (Buchi, UK).

Blimbi extraction

Two kilograms of fresh Blimbi were washed to prevent any contamination that would influence the pure extract. Afterwards, they were sliced into pieces with approximately 2 mm in thickness, dried under the sun and covered with black cloth. In the next step, the pieces were mashed using a blender until they became powder. The powder was sifted through a No.60 sieve to obtain the powder of simplicia. A kilogram of Blimbi powder was extracted using heat digested with 10 liters demineralized water for about 1.5 h at 40 °C while stirring occasionally, and then it filtered. The filtered substance was evaporated with vacuum or low-pressure evaporator at 60 °C and rotation speed of 50 rpm until a thick extract was obtained followed by storage in the refrigerator overnight. After a freeze, it was put into freeze drying and the condenser was set to -40 °C. The drying process lasted 20 h.

Extract characterization

The extract characterization or organoleptic testing aimed to introduce extracts with subjective characteristics. In addition, its purpose was to indicate the specific parameters to describe shape, color, smell, and flavor through sensing.

Nano preparation of extract

Two grams of Blimbi extract was added to 200 ml aquadest. The extract concentration was 1% b/v and it was stirred using a magnetic stirrer at 1300 rpm followed by filtration. A gram of PAA was dissolved in 100 ml of water using a magnetic stirrer to obtain 1% PAA concentration. Afterwards, 10 ml of 1% PAA solvent was taken and put into a glass breaker which contained the filtered substances of Blimbi extract. It was stirred about 5 min till the pH reached 8, and it was stirred again about 5 min. Thereafter, $CaCl_2$ was added drop by drop until the nanoparticles suspension was obtained (approximately 25 drop $CaCl_2$).The $CaCl_2$ was dropped every 3 seconds in a magnetic stirrer at 400 rpm to form nanoparticles which were characterized by homogenous turbidity. Further, the nanoparticles Blimbi extract was observed for five days to assess the stability of color, turbidity, and sediment.

Nanoparticles characterization

Delsa™Nano (LIPI Cibinong, Indonesia) was used to 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 anhydrate, stearyl alcohol, and vaseline. It was melted in the steam cup in a water bath, stirred until homogenous. After that, propylparaben and alpha-tocopherol 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 approximately 10×15 cm. The shaving process was started from shoulder blades area to the waist bone and half down to the body on each side. Afterward, the ointment was applied to the gauze and placed on the skin, 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

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
B	8	8	8	8	8	8

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 I

Table 6 Results of irritation testing on ointment preparation in rabbits

Hours after applying	Rabbit	Ointment Extract		Nanoparticles Ointment	
		Erythema	Edema	Erythema	Edema
1	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
24	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
48	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
72	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-

Note: + = Irritation happen, -= No Irritation

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

Sample	Concentration	*S. aureus*			*E. coli*		
	(%)	PD1	PD2	Average	PD1	PD2	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

Furthermore, the differences can be observed in the inhibition zones of different extract concentrations against bacteria. Therefore, the extract of Blimbi with 20% concentration had 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* (14.5mm) 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 bacterial 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).

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.

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: Nanoparticles Ointment using Blimbi Extract, OB: Ointment Base

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.

Table 10 Results of spreadability of Blimbi extract ointment evaluation

Formula	**Total Average Spread ability (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

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.

Figure 1 illustrates the results of fluidity testing on all preparations (F1, F2, and OB) with plastic shaped curves. It is shown from the figure that the falling sign of the curve is on the left side of the escalade curve. In a plastic curve, the line does not pass the 0, 0 points, rather it passes the shearing stress.

Table 11 Results of viscosities evaluation of Blimbi Extract Ointment

RPM	Week 0			Week 1		
	Viscosities (cps)			Viscosities (cps)		
	OB	F2	F1	OB	F2	F1
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

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	80222	88575	129144	80582
2	60097	87729	54374	60504	88216	54743	60940	88852	55137
2,5	38696	56488	34883	39031	56907	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

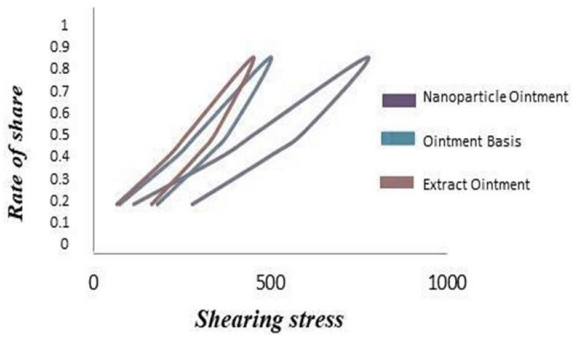

Figure 1 Fluidity of ointment preparation in F1, F2, and OB. **Note:** F1: Blimbi Extract Ointment

liquid can flow if the yield value is already passed.
its are generally the oldest source of
rmacologically active compounds and have provided
ankind with many medicinally useful compounds
enturies. Today more than two-thirds of the world's
ulation rely on plant-derived drugs (12). Numerous
lies have been conducted with the extracts of the
ous plant. Nworu *et al.* conducted a study on an
al ointment containing *Dissotis theifolia* extract for
nd healing and antibacterial activities against clinical
nd 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

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 anti-bacterial and anti-fungal 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* on. 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.

Financial Declaration

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Conflicts of interest

Authors declare no conflicts of interest.

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