Optimization of Ethanol Extract Preparation Gel Formula from the Combination of Lime Peel and Neem Leaves : Optimum Physical Properties and Antibacterial Activity

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Abstract

Lime peel (*Citrus aurantifolia*) and neem leaf (*Azadirachta indica*) have antibacterial properties, especially against *Staphylococcus aureus*. These two ingredients can be used to treat various skin problems caused by *Staphylococcus aureus*. This study aimed to determine the optimal ratio of 96% ethanol extract of lime peel and neem leaves to produce a gel formula with optimum physical properties and antibacterial activity. Physical properties tested were pH test, adhesion test, spreadability test, and viscosity test. Antibacterial activity was measured by inhibition zone diameter when tested by the well method. There were 8 gel formula with the various ratio of components of lime peel and neem leaves extracts, FI (8:2), FII (6.5:3.5), FIII (5:5), FIV (3.5:6.5), FV (2:8). The optimization method used was Simplex Lattice Design using the Design Expert 11 application (Trial). The optimum formula obtained from the Design Expert was verified and analyzed using the SPSS one-sample t-test with a 95% confidence level. The optimum formula of lime peel and neem leaves obtained were 8.00%:2.00% with a desirability value of 0.471. The one sample t-test showed that the results were not significantly different between the predicted and verified values.
***Keywords**: antibacterial, lime peel, neem leaf, optimization, Simplex Lattice Design.*

INTRODUCTION

Staphylococcus aureus is one of the most prevalent infectious organisms that cause morbidity and mortality in the world. Numerous diseases, including sepsis, pneumonia, and potentially fatal skin infections, can be caused by this organism. (Cheung *et al.*, 2021). These bacteria are typically responsible for endovascular, skin, respiratory, bone, and joint diseases. (David and Daum, 2017). Numerous skin and soft tissue infections (SSTIs), ranging from harmless (like cellulitis and impetigo) to potentially fatal, are brought on by these bacteria. Surgical wound infections (SSIs), skin abscesses, and purulent cellulitis are the usual sources of this pathogen's isolation. (Tong *et al.*, 2015). Other skin disorders brought on by *Staphylococcus aureus* include purpura, erythema, lymphangitis, and furuncles. (Del Giudice, 2020). According to studies conducted on SSTI patients in the Bali and Java islands, wound cultures from 257 out of 567 patients (45.3%) showed a positive *Staphylococcus aureus* infection, and eight out of 257 patients (3.1%) had an MRSA infection (Santosaningsih *et al.*, 2018).

The antibacterial activity of the lime peel extracted with 25%, 50%, and 75% ethanol against *Staphylococcus aureus* was demonstrated by the inhibitory zones that measured 12.50 mm, 17.33 mm, and 18.33 mm, respectively. The extract's ability to inhibit *Staphylococcus aureus* increases with the increase of ethanol concentration (Wardani *et al*., 2018). Meanwhile, the inhibitory zone of lime peel extracted with 96% ethanol against *Staphylococcus aureus* was 19 mm (Ekawati *et al*., 2019).

In addition, neem leaves also has antibacterial activity against *Staphylococcus aureus*. Neem leaves' minimum inhibitory concentration (MIC) varied from 4 to 8 mg/mL. At 8 mg/mL, the maximum number

Table 1. The extraction yield of lime peel (*Citrus aurantifolia*) and neem leaves (*Azadirachta indica*) ethanol extracts

Ingredients	Simplicia weight (gram)	Extract weight (gram)	Yield (%)
Lime peel	500	51.61	10.322%
Neem leaves	500	43.33	8.666%

of bacteria that were inhibited was 23 out of 32 isolates of *Staphylococcus aureus* (Saeed *et al*., 2019). The minimum bactericidal concentration of *Azadirachta indica* against *Staphylococcus aureus* was 6.25 mg/mL. (Mehrishi *et al.*, 2022). Neem parts (seeds, leaves, bark) have been proven to have effective antibacterial activity against *Staphylococcus aureus*. Meanwhile, the leaves extract showed an inhibition zone of 15 mm against *Staphylococcus aureus* (Yaseen, 2016).

To improve effectiveness and comfort, the lime peel and neem leaf ethanol extracts were combined and made in a gel form. Gel products can be absorbed better than other topicals so their bioavailability increase. Other than that, gel preparations are recommended because they are simple to wash due to their water content, dry quickly, and do not adhere to the skin (Mohiuddin, 2019). In order to evaluate the optimal composition of lime peel and neem leaf extracts as well as the antibacterial activity of a combination of ethanol extracts of neem leaves (*Azadirachta indica*) and lime peel (*Citrus aurantifolia*) formulated in a gel preparation against *Staphylococcus aureus*, this research was carried out.

RESEARCH METHODOLODY

Equipments

Herbs grinder, analytical balance, glassware (Pyrex), maceration vessel, Buchner funnel, rotary evaporator (Heidolph), water bath (Memmert), porcelain cup (Pyrex), magnetic stirrer, mortar, stamper, pH meter (HANNA 211), adhesion tester, spreadability tester, viscometer (RION VT 04-F), LAF cabinet, petri dish (Pyrex), micropipette, vortex.

Ingredients

The ingredients used are lime obtained in Kartasura, Sukoharjo (Indonesia), neem leaves obtained at the Gede market in Surakarta (Indonesia), ethanol 96%, carbopol 940 (technical), propylene glycol (technical), PEG-400 (technical), glycerin (technical), methylparaben (technical), triethanolamine (pro analysis), distilled water (technical), DMSO solvent (Merck pro analysis), Mueller Hinton agar (pro analysis), mannitol salt agar (pro analysis), NaCl (pro analysis).

Sample Extraction

Lime peel and neem leaves were each dried and crushed into fine powder with a grinder. A total of 500 g of fine powder from each plant was added separately to 5 liters of 96% ethanol. The powder has been soaked,

Table 2. Equation of physical properties of gel formulation combining lime peel (*Citrus aurantifolia*) and neem leaves (*Azadirachta indica*) extracts

Response	Graphic Model	Equation	ANOVA (p-value)
pH	*Quartic*	$Y = 5.50(A) +6.00(B) -1.71(AB) -0.3413(AB)(A-B) +4.86(AB)(A-B)^2$	Significant (0.0001)
Adhesion	*Quartic*	$Y = 1.47(A) +1.57(B) +1.67(AB) -0.1187(AB)(A-B) -7.55(AB)(A-B)^2$	Significant (0.0457)
Spreadability	*Quartic*	$Y = 5.21(A) +4.61(B) -0.58(AB) -0.3013(AB)(A-B) +3.78(AB)(A-B)^2$	Significant (0.0003)
Viscosity	*Quadratic*	$Y = 110.75(A) +66.31(B) +230.59(AB)$	Significant (0.0001)

Table 3. Physical properties test results of the gel formulation combining lime peel (*Citrus aurantifolia*) and neem (*Azadirachta indica*) leaves extracts

Formula	Physical Properties Test						
	Organoleptic		Homogeneity	pH	Adhesion time (s)	Dispersion diameter (cm)	Viscosity (dPa.s)
	Consistency	Color					
I	Thick	Blackish moss green	Homogeneous	5.55 ± 0.01	1.48 ± 0.32	5.23 ± 0.24	110 ± 0
I	Thick	Blackish moss green	Homogeneous	5.55 ± 0.01	1.45 ± 0.07	5.2 ± 0	110 ± 0
II	Thick	Blackish moss green	Homogeneous	5.55 ± 0.01	1.44 ± 0.38	5.16 ± 0.08	150 ± 0
III	Thick	Blackish moss green	Homogeneous	5.32 ± 0	2.04 ± 1.00	4.75 ± 0.05	140 ± 0
III	Thick	Blackish moss green	Homogeneous	5.32 ± 0.01	1.83 ± 0.56	4.78 ± 0.03	140 ± 0
IV	Thick	Blackish moss green	Homogeneous	5.81 ± 0	1.51 ± 0.05	4.8 ± 0.09	130 ± 0
V	Slightly liquid	Blackish moss green	Homogeneous	6.00 ± 0.01	1.57 ± 0.14	4.6 ± 0.1	65 ± 0
V	Slightly liquid	Blackish moss green	Homogeneous	6.00 ± 0.01	1.56 ± 0.10	4.62 ± 0.08	65 ± 0

filtered and the filtrate was concentrated using a rotary evaporator. Then the remaining solvent was evaporated on a waterbath until a thick extract was produced that did not smell of alcohol (Safeena, 2019). The thick extract was weighed and the yield was calculated.

Optimization and Formulation of Gel with Simplex Lattice Design

The upper and lower limits of the ethanol extract of the materials used, such as lime peel and neem leaves, were determined. The upper limit and upper limit were chosen based on previous research, which lime peel had an inhibition zone at a concentration of 20 mg/mL while the minimum inhibitory concentration of neem leaves against *S.aureus* was 4-8 mg/mL (Sekar *et al.*, 2015; Saeed *et al*., 2019). Hence, the lower limit for both extracts was set at 2% and the upper limit at 8%. After the lower and upper limits were determined and inputted in the Design Expert version 11.0 application, the composition of the ethanol extract for each extract was obtained (Supplementary Table 1).

Comparisons of the concentrations of lime peel and neem leaves ethanol extracts obtained from Simplex Lattice Design version 11.0 were formulated with the additional ingredients to obtain a gel formula (Supplementary Table 2). The total extract used in each formula was 10%.

Preparation of Gel Containing Combination of Lime Peel and Neem Leaves

Carbopol 940 was mixed with hot distilled water in a mortar for 60 minutes until carbopol 940 dissolves. Methylparaben, glycerin and polyethylene glycol were put into a beaker and stirred until dissolved. Then

Table 4. Results of analysis of the optimum gel formula combining lime peel (*Citrus aurantifolia*) and neem leaves (*Azadirachta indica*) extracts using SPSS

Parameter	Prediction	Verification	Significance	Explanation
pH	5.996	6.00 ± 0.01	0.914	Not significantly different
Daya lekat	1.568 detik	1.57 ± 0.11 detik	0.994	Not significantly different
Daya sebar	4.608 cm	4.61 ± 0.08 cm	0.992	Not significantly different
Viskositas	66.307 dPa.s	65 ± 0 dPa.s	0.014	Significantly different
Daya hambat	14.8 mm	14.83 ± 0.82 mm	0.924	Not significantly different

Table 5. Criteria for the optimum gel formulation combining lime peel (*Citrus aurantifolia*) and neem leaves (*Azadirachta indica*) extracts

Physical properties	Limit	Target	Importance
pH	4.5-6.5	*In range*	+++
Adhesion	1-2.04 s	*Maximize*	+++
Spreadability	4-7 cm	*Maximize*	+++
Viscosity	60-500 dPa.s	*In range*	+++
Inhibitory zone	11.5-15 mm	*Maximize*	+++

the solution of methyl paraben, glycerin and polyethylene glycol were mixed into a mortar containing dissolved carbopol 940 and expands slowly while stirring (Phase I). After dissolving both extracts in propylene glycol (Phase II), Phase I was gradually mixed with the Phase II solution and stirred for five minutes. Finally, triethanolamine was added slowly while stirring until it reached a gel structure with a neutral pH.

Gel Evaluation

Organoleptic Test

Observation of the gel preparation was carried out by observing the odor, color and homogeneity. The gel is good if it is clear in color with a semi-solid consistency (Tjahyadi *et al.*, 2015).

pH Test

The pH test was carried out with a pH meter. The pH meter should be calibrated using pH 4 and pH 7 buffers before use. The pH test was carried out by dipping the electrode directly into the gel formula. The test was carried out 3 times replication (Zhang and Zhou, 2018).

Adhesion Test

A total of 0.25 grams of gel was placed between 2 object glasses and a 1 kg load was placed on the object glass for 5 minutes. After 5 minutes, a 1 kg load was lifted from the object glass, then the object glass was placed on the adhesion tester. At each side of adhesion tester, there was given a load of 80 grams. The 80 gram load was released and the time of glass objects separated was recorded. This test was carried out 3 times (Syarifah *et al*., 2021)

Spreadability Test

On the center of a round glass, 0.5 grams of gel was placed. Then another round glass was placed on top of the gel mass for 1 minute. After that, the glass was given a weight of 150 grams and left to rest for 1 minute. The diameter of the spread gel was measured and repeated three times (Bayan *et al*., 2023).

Viscosity Test

The viscometer rotor was dipped in 50 grams of gel. Then the viscometer was turned on. The viscosity resulted in the form of

Table 6. Inhibitory zone diameter of gel and combination extract of lime peel (*Citrus aurantifolia*) and neem leaves (*Azadirachta indica*) against *Staphylococcus aureus*

No	Variations in concentration of neem leaves: lime peel (%w/w)	Inhibitory diameter (mm)	
		Gel	Extract
1	8 : 2	11.5 ± 0.87	12.75 ± 0.66
1	8 : 2	11.6 ± 0.76	12.83 ± 0.76
2	6.5 : 3.5	12.83 ± 0.29	13.17 ± 0.58
3	5 : 5	12.60 ± 0.58	14.17 ± 1.04
3	5 : 5	12.6 ± 1.15	14 ± 1
4	3.5 : 6.5	14.6 ± 0.29	15.67 ± 0.58
5	2 : 8	14.6 ± 1.15	15.83 ± 1.44
5	2 : 8	15 ± 0.5	16 ± 0

numbers appeared on the screen, wait until the numbers were stable. (Pakan *et al.*, 2023)

Antibacterial Activity Test

On Mueller Hinton agar media, 100 µL *Staphylococcus aureus* bacterial suspension was inoculated and was spreaded evenly with a spreader glass. After that, holes were made in the media with a size of 7 mm using a cork borer. Gel with active ingredients, gel without active ingredients (negative control) and clindamycin gel (positive control) were inserted into the well. The media was incubated at 37°C for 24 hours. Then the diameter of the inhibition zone was measured from the vertical and horizontal positions (Babaei *et al.*, 2018).

Determination and Verification of the Optimum Formula

The responses tested were pH, spreadability, adhesive strength, viscosity and inhibitory power against *Staphylococcus aureus* bacteria. Each response was tested three times and the average of these data was calculated. Then the results were analyzed using the Design Expert application version 11.0 with the Simplex Lattice Design method and the optimum formula was obtained. The optimum formula obtained was followed by formula verification.

Using the Statistical Package for the Social Sciences (SPSS) application, the optimum formula and the verification formula's results were analysed at a 95% confidence level using the one sample t-test if the data was normally distributed and the Wilcoxon signed rank test if it wasn't.

RESULT AND DISCUSSION

Lime Peel and Neem Leaf Extract

Lime peel extract had a thick consistency, greenish black color and a distinct lime peel odor. Neem leaf extract had a thick consistency, black color and a distinct neem leaf odor. Yield value of each extract can be seen in Table 1.

Gel Evaluation

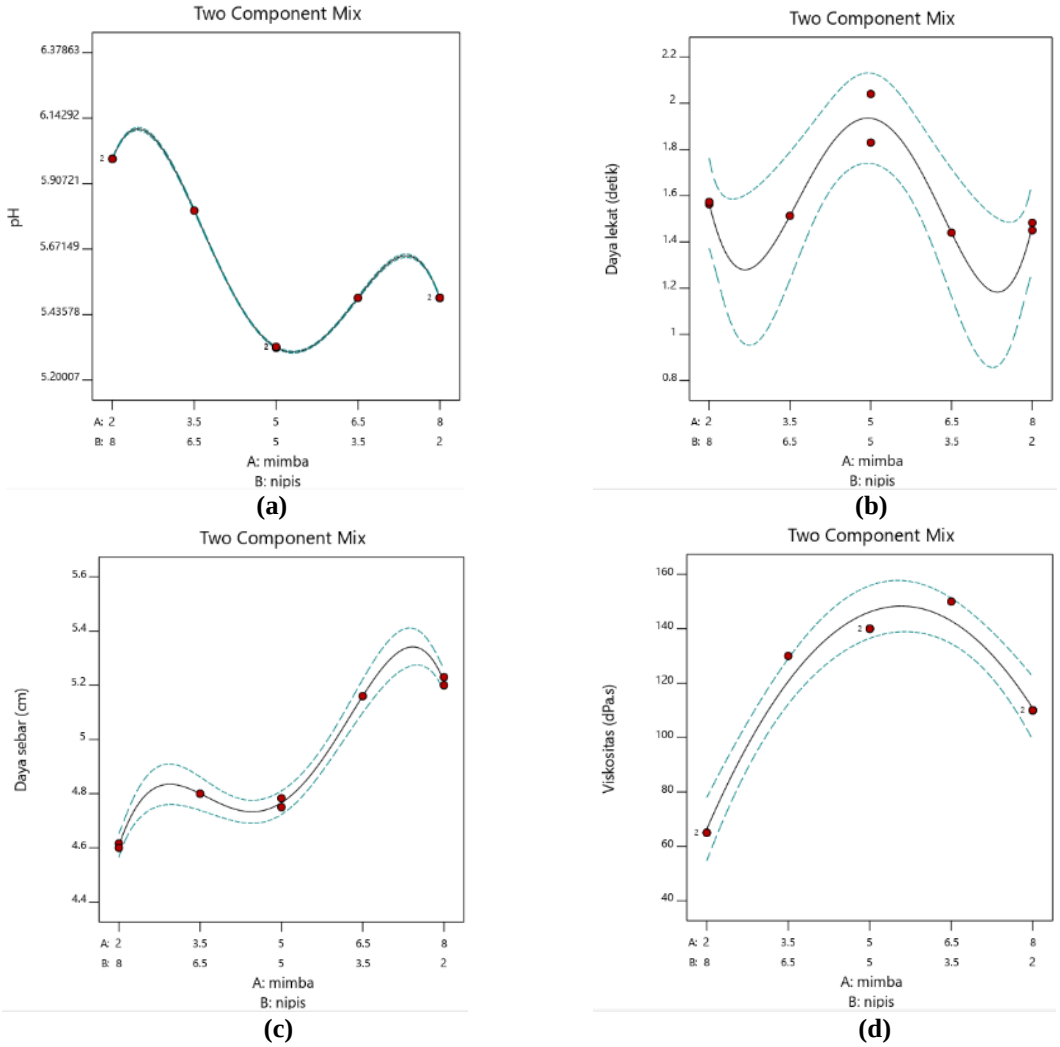
The results of the evaluation of the gel formula can be seen in Table 2 and Table 3. In the organoleptic test, FI, FII, FIII, FIV were produced with a thick consistency, while FV was not as thick as the other formulas. All formulas had a blackish moss green color and a distinct smell of a mixture of lime peel and neem leaves. The gel meet the homogeneity requirements if there are no coarse grains and the color is evenly distributed (Tjahyadi *et al.*, 2015). According to the results, every gel formula was homogeneous.

Testing for pH was done to ensure that the preparation is between 4.5 and 6.5, which is the pH range that is safe for skin (Kuo *et al.*, 2020). All formulas had a safe pH for topical preparations. Based on the Scheffe equation in Table 3, Y is the pH response, A is the neem leaves fraction and B is the lime peel fraction. The combination of neem leaves and lime peel extracts lowers the pH as seen from the negative coefficient results. This can occur due to the antagonistic interaction between lime peel and neem leaves extracts. The coefficient of lime peel extract was greater than that of neem leaves, indicating that lime peel extract had a more significant impact and elevated pH. The results of the ANOVA analysis in Design Expert showed a p-value (0.0001) < 0.05, indicating that the resulting equation was significant in estimating the distribution pattern of pH data.

Adhesion testing was carried out to determine the ability of the gel to stick to the skin immediately after application. The gel should have an adhesion time of more than 1 second. The adhesive power of 8 formulas met the requirements. From Figure 2 it is known that the adhesion test graph is in quartic form. Based on the Scheffe equation in Table 3, Y is the adhesive force response, A is the neem leaves fraction and B is the lime peel fraction. The combination of neem leaves and lime peel extracts increase adhesion as seen from the positive coefficient results. The coefficient of lime peel extract is greater than that of neem leaves, indicating that lime peel extract has a more dominant influence and increases adhesion. The results of the ANOVA analysis in Design Expert showed a

(a) (b) (c) (d) (e)

Figure 1. Homogeneity test results of a gel formulation combining lime peel (*Citrus aurantifolia*) and neem leaves (*Azadirachta indica*) ethanol extracts



(a) (b) (c) (d)

Figure 2. (a). pH response surface graph, (b). Adhesion response surface graph, (c). Spreadability response surface graph, (d). Viscosity response surface graph, A. Neem leaves, B. Lime peel

p-value (0.0457) < 0.05, indicating that the resulting equation was significant in estimating the distribution pattern of adhesive strength data.

The spreadability test measures how quickly the gel would spread across the skin following application. The recommended dispersion for topical treatments is 5-7 cm (Marlina *et al.*, 2023). The spreadability test

results showed that FI and FII were the formulas that fulfil the requirements. The spreadability response is denoted by Y, the neem leaves fraction by A, and the lime peel fraction by B, according to Table 3 Scheffe equation. As can be observed from the negative coefficient values, the combination of lime peel and neem leaves extracts decreased the spreadability. Neem leaf extract has a higher coefficient than lime peel, which indicates that it has a more potent effect and improves spreadability. ANOVA analysis in Design Expert shows a p-value (0.0003) < 0.05, indicating the significance of the resulting equation in estimating distribution pattern of dispersion power data.

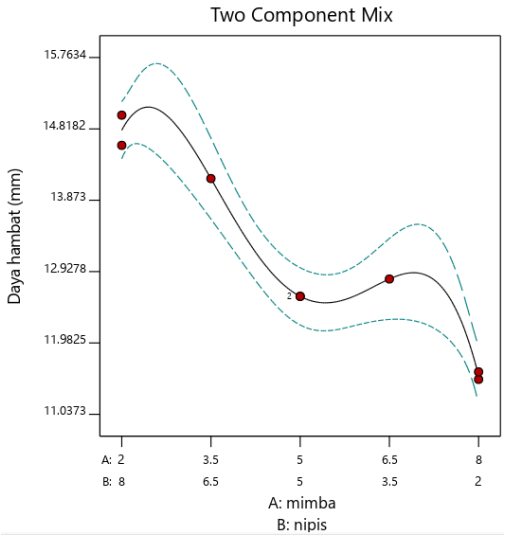


Figure 3. Response surface graph of the inhibitory power of a gel combination of ethanol extract of lime peel (*Citrus aurantifolia*) and neem leaves (*Azadirachta indica*)

The gel's viscosity was determined by the viscosity test. Not too runny is the viscosity of a good gel. A good range for gel viscosity is 60–500 dPa.s or 6–50 Pa.s (Wagoner *et al.*, 2020). Every gel met the acceptable range for gel viscosity. A is the neem leaf fraction, B is the lime peel fraction, and Y is the viscosity response according to the Scheffe equation in Table 3. The positive coefficient results demonstrated that adding lime peel and neem leaf extracts together increased viscosity. Neem leaves extract had a higher coefficient than lime peel, which indicated that it had a more powerful effect and raised the gel's viscosity. ANOVA analysis in Design Expert shows a p-value (0.0001) < 0.05, indicating the significance of the derived equation in calculating the viscosity data distribution pattern.

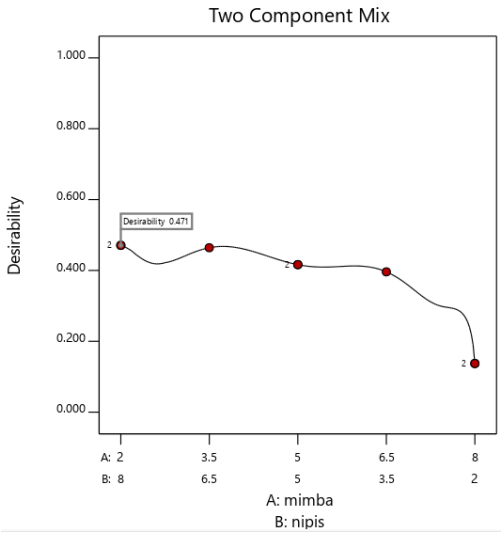


Figure 4. Graph of desirability of optimum formula gel combination of ethanol extract of lime peel (*Citrus aurantifolia*) and neem leaves (*Azadirachta indica*)

Conducting an antibacterial test against *Staphylococcus aureus* is essential following the completion of all physical property tests on the gel, as this is the primary function of each extract used in the formulation of the gel. Lime peel and neem leaves extracts had inhibitory zone diameters of 19 mm and 15 mm, respectively, according to earlier studies (Yaseen, 2016; Ekawati *et al*., 2019). Table 4 shows the average inhibition zone diameter for each formula on comparison with the average inhibition zone diameter of the extract in solvent (DMSO) with the same concentration variations as the formula.

A quartic graph is produced by the inhibitory test results shown in Figure 3. The inhibitory Scheffe equation is Y = 11.55(A) +14.80(B) -2.30(AB) +1.57(AB)(A-B) +16.03(AB)(A-B)2. The inhibitory response

is represented by Y in this equation, the neem leaf fraction by A, and the lime peel fraction by B. The results of the negative coefficient show that the inhibitory power is reduced when neem leaf extract and lime peel are combined. Compared to neem leaves, lime peel extract had a higher coefficient, indicating a more dominant influence and increased inhibitory power. The results of the ANOVA analysis in Design Expert shows a p-value (0.0014) < 0.05, indicating that the resulting equation was significant in estimating the distribution pattern of inhibitory power data.

Determination of the optimum formula

Optimization was carried out on the components of neem leaves and lime peel extracts. The responses used were pH, adhesive power, spreading power, viscosity and inhibitory power. The optimum formula was obtained from the Design Expert application using the Simplex Lattice method. The optimum formula criteria can be seen in Table 5.

The pH test limit was chosen as 4.5-6.5 because it is adjusted to the skin's pH. The adhesion test limit was chosen as 1-2.04 seconds because good gel adhesion is above 1 second, while 2.04 seconds was the result of the greatest adhesion test response. The spreadability test limit was chosen as 4-7 cm because it corresponds to a good gel spreadability range of 5-7 cm. However, 4 cm was chosen for the lower limit because the spreadability results obtained ranged from 4-5 cm. The viscosity limit was chosen to be 60-500 dPa.s according to the literature that the viscosity of a good gel ranges from 60-500 dPa.s. Then there are no rules for the inhibitory limit, but a limit of 11.5-15 mm was chosen because the smallest inhibitory zone diameter was 11.5 mm and the largest was 15 mm. The target for inhibitory power is desired as large as possible so that the gel's antibacterial activity against *Staphylococcus aureus* is maximized. The importance of each test was made the same because each test was considered to have the same level of importance.

The results of analysis using the Design Expert Simplex Lattice method showed that the concentrations of neem leaves and lime peel extracts were 2% and 8% respectively. The optimum formula obtained from these two components would produce a predicted pH of 5.996; sticking power 1.568 seconds; spreadability 4.608 cm; viscosity 66.307 and inhibition zone diameter 14.8 mm. The desirability value obtained was 0.471 and can be seen in Figure 4.

The desirability value shows the ability to fulfill desires based on established criteria. The desirability value ranges between 0 – 1. The desirability value can be said to be good if it is close to 1. A desirability value close to 1 has high conformity to the specified criteria. The results of the pH test verification, adhesion power, spreadability, viscosity and inhibitory power of the optimum formula were respectively 6.00 ± 0.01; 1.57 ± 0.11 seconds; 4.61 ± 0.08 cm; 65 ± 0 and 14.83 ± 0.82. The results of the verification test were then analyzed using the SPSS one sample t-test.

Statistical Test Results

Design Expert's prediction results were compared with the results of the verification formula test using SPSS as in Table 6. The results of the SPSS one sample t-test (normally distributed data) for the adhesive power, spreading power and inhibitory power tests show a significance value of p > 0.05. This shows that the prediction and verification results were not significantly different. The results of the SPSS Wilcoxon signed rank test (data not normally distributed) for the pH test show a significance value of p > 0.05, indicating that the prediction and verification results of the pH test were not significantly different. Meanwhile, the viscosity test showed a significance value of p < 0.05, indicating that the prediction and verification of the viscosity test were significantly different. There was no significant difference, indicating that the

optimization method used could predict the test parameters accurately.

CONCLUSIONS

Based on this research, it was concluded that a 96% ethanol extract of lime peel and neem leaves would yield the best physical qualities and antibacterial activity, specifically 8% of lime peel and 2% of neem leaves. The results of analysis using SPSS showed that the test parameters pH, adhesion, spreadability and antibacterial activity were not significantly different. Meanwhile, the result of the SPSS analysis of the viscosity test was significantly different. So the optimization method could predict almost all parameters accurately. If the responses obtained from the gel formula meet the range requirements given in the literature, the verification formula is considered good.

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