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Research Article

Inhibitory Activity Of Roselle Flower (Hibiscus Sabdariffa L.) Aqueous Extract - Sodium Alginate Gel Against Staphylococcus aureus ATCC 25923

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ABSTRACT

Staphylococcus aureus is one type of bacterium that causes skin infections resistant to some antibiotics. This encourages research to find new antibacterials by using substances from natural sources, often called medicinal plants. Aqueous extract of Roselle (Hibiscus sabdariffa L.) flower (AERF) was selected as one of natural ingredients for antibacterial in gel dosage form, in which sodium alginate was used as a matrix base. In this study, determination of the sodium alginate concentration effects (2.5%, 3% and 3.5%) on the properties of the gel was performed. The characteristics evaluated included pH, viscosity, and dispersive power; that should be met standard requirement and the gel ability to inhibit Staphylococcus aureus ATCC 25923 growths. A diffusion test method was used to determine the Minimum Inhibitory Concentration (MIC) of the AERF powder against the test bacteria. The study results showed that AERF gel preparation with a composition of 3% sodium alginate and 3% roselle extract had better properties (pH, viscosity, and dispersive power) than the matrix and extract concentrations of 2.5% and 3.5% respectively. It was found that the optimum formula obtained a MIC of 0.04 mg/ml. In line with these results, it is expected that the AERF gel preparation will be stable, safe, and acceptable when used and might be recommended as antibacterial topical dosage form against Staphylococcus aureus. In the future, inhibitory activity against resistant pathogenic bacteria could be evaluated and developed.

Keywords: inhibitory activity, Staphylococcus aureus ATCC 25923, roselle flower aqueous extract, sodium alginate gel.

Introduction

There are various bacteria on the skin such as commensal bacteria that protect it both directly and indirectly from pathogenic bacteria's interference. The bacteria present on the skin can be Gram-positive and Gram-negative, one of which Staphylococcus aureus that often causes skin infection (Chiller, 2001). The Staphylococcus aureus does not form spores and move, but it can grow on various media under aerobic conditions. These bacteria can ferment some carbohydrates and can produce coloured pigments. The S. aureus can cause infection on the skin and tissue invasively such as pneumonia, osteomyelitis, meningitis, and endocarditis (Barlett et al., 2010).

One of the attempts to fight pathogenic bacteria like S. aureus is by using antibacterials. The working mechanism of antibacterials is as follows: inhibiting the formation of cell walls, inhibiting cell membrane functions, inhibiting protein synthesis, and inhibiting nucleic acid synthesis. However, S. aureus is a bacterium that has been resistant to several antibiotics, such as beta-lactamase, methicillin, nafcillin, oxacillin and vancomycin (Keyser et al., 2005). Resistance to bacteria such as S. aureus has put the discovery of new medicines as a top priority, one of which is by using materials derived from the nature called medicinal plants (Fischbach and Walsh, 2009). One of the medicinal plants which is widely used by people to deal with various diseases is roselle (Hisbiscus sabdariffa L.). This plant belongs to Malvaceae family, otherwise commonly known as roselle, red sorrel or karkaday, is a bush (shrub) species. Water extract of Hibiscus sabdariffa L. is acidic with a pH of about 2.42 ± 0.01 and is known to have contents such as polyphenols and flavonoids that have pharmacological activities as an antibacterial (Da-Costa-Rocha et al., 2014).

The working mechanism of flavonoid as an antibacterial is to form complex compounds with extracellular and dissolved proteins that can damage

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the membrane of bacterial cells and followed by the release of intracellular compounds (Nuria et al., 2009). Plants containing polyphenols are considered to have antibacterial activities by disrupting the function of bacterial cell membranes, i.e. inhibiting bacterial growth or division (Cushnie and Lamb, 2005). However, a high amount of phenol contents found in roselle extract, such as flavonoid, anthocyanin, cyanidin, and delphinidin are not always correlated with their antibacterial activities. The antibacterial activities of roselle extract may be due to the presence of specific phenolic compounds and there may be synergistic effects with the non-phenolic compounds present in the extract. In addition, roselle's calyx water extract can inhibit the growth of bacteria Staphylococcus aureus ATCC 6358 with MIC 0.5 mg/ml (Navarro, 2006).

Judging from its pharmacological effects and viscosity increase, roselle extract has the potential to be developed into a semi-solid topical medicine preparation. The type of semi-solid preparation selected in this study was gel with advantages as follows: it contains high water content, has a cold sensation effect when used, has long-term efficacy and better than conventional therapy, its onset of action is fast, has a good safety profile, and yields high patient satisfaction when using medicine preparation in a gel form (Tan et al., 2012).

The gel preparation of roselle extract was made using sodium alginate as the base. Sodium alginate is a gelling agent which can be used in a variety of topical preparation formulations (Rowe et al., 2009). This base was selected because roselle extract material is acidic and it was expected that the preparation had a pH corresponding to the range of skin pH so that an expandable and stable gelling agent in acidic environment was used.

Evaluation stage of gel preparation of roselle water extract included organoleptic test, preparation's pH test, viscosity test, and dispersive power test. Meanwhile, to evaluate the effectiveness of antibacterial compound of gel preparation of roselle water extract, might used agar diffusion method by measuring the inhibition zones of bacterial growth caused by diffusion of antibacterial compounds in solid media through cultivation (Brooks et al, 2007).

In this study, the concentrations of sodium alginate (2.5%, 3% and 3.5%) in the preparation of roselle water extract gel that met the standard (pH, viscosity, and dispersive power) and able to do antibacterial activities against Staphylococcus aureus ATCC 25923 were determined.

Material and Method

Chemical Materials

The chemicals used in this study were roselle water (PT. Agarius Sidomakmur) extract (dried), sodium alginate, propylene glycol, sodium benzoate, nutrient agar (Ctoid), NaCl p.a (Merck), citric acid monohydrate, sodium citrate dihydrate, aquadestilata (PD. Surabaya Aqua).

Microorganisms

Staphylococcus aureus ATCC 25923 was obtained from the Department of Microbiology, Faculty of Medicine, Universitas Airlangga.

Instruments and equipments

pH-meter SI Analytic, viscometer Brookefield, glass plate scales, micropipette (Eppendorf) and tip, sterile tube Eppendorf, analytical balance (Sartorius), autoclave (Huxley), autoshaker (Gerhardt), vortex (Thermo), UV-Vis (Thermo) spectrophotometer, incubator (Memmert), Öse needles, injection syringe, a Vernier caliper, and sterile glassware for microbiological test.

Characterization of roselle extract

The qualitative test of AERF included: organoleptics (shape, odour, and colour) visually, pH, and chromatogram patterns of flavonoid and polyphenol compounds using TLC and optimized eluents.

pH Test

The AERF with a mass of ± 500 mg was dissolved in CO2-free aquadest. The pH measurement was performed using pH meter SI Analytic. The pH meter electrodes were calibrated in advance with a standard buffer solution pH 2 and rinsed with CO2-free aqua prior to measuring the preparation's pH. After rinsing, the electrodes were dried and then dipped into the diluted preparation and the observed pH was then recorded. This was replicated three times.

TLC of flavonoid compounds

The AERF with a mass of 1 g was shaken with n-hexane repeatedly until became colourless and the residue was dissolved in 5 ml of ethanol. Then the solution was spotted in the stationary phase Kiesel Gel GF 254. Then it was put in a chamber containing mobile phase chloroform : acetone : formic acid (6 : 6 : 1). After perfectly eluation, the plate was sprayed with ammonia vapour. The presence of flavonoid was indicated by the appearance of intensive yellow stain.

TLC of polyphenol compounds

The AERF with a mass of 1 g plus 10 ml of hot aquadest was then stirred, exposed to room temperature, and then filtered. Furthermore, the solution was dripped in the stationary phase Kiesel Gel GF 254 and was put in a chamber containing mobile phase chloroform : ethyl acetate : formic acid (0.5 : 9 : 0.5). After perfectly eluation, the plate was sprayed with stain identifier FeCb reagent. The presence of polyphenols was indicated by the appearance of black spots

Qualitative Test of Sodium Alginate

Qualitative test of sodium alginate involved visual examination of the organoleptics (shape, colour, and

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smell), viscosity examination using Brookfield viscometer, and pH examination.

Viscosity test

The first step was to make a 1% sodium alginate solution which was then measured using the Brookefield viscometer. The preparation was inserted into the container and then the tool was switched on. Spindle was installed according to the viscosity of the prepration to be measured, then the appropriate rpm was selected. Spindle was dipped into the container, and then the button "run" was pressed to initiate the measurement and observation of the preparation's viscosity.

pH Test

A 1% sodium alginate solution was weighed as much as ± 5 g and then diluted with 45 ml of CO2-free distilled water. pH measurement was performed the same way as measuring the pH of the AERF.

Determining MIC of the AERF

As much as 10 ml of nutrient agar medium (temperature: 45-50oC) was poured into a 9 cm-diameter-sterile petri dish aseptically, and then was let to solidify (base layer). Then, 5 ml of inoculum of the S. aureus ATCC 25923 was taken and incorporated in 8 ml nutrient agar medium (temperature: 45-50°C), mixed with vortex, then poured into the base layer that had been formed, and let to solidify. The media were perforated with a sterile hole holder to make six holes (five for the sample and one for the positive control). The positive control used was gentamicin 25 ppm. The sample and positive control were inserted into each of the holes, then incubated at 37°C for 24 hours and observed, and then the inhibition zone formed was measured with the Vernier caliper. The MIC was determined by looking at the smallest concentrations that could still inhibit the growth of test bacteria. The selected roselle concentrations to be included in the formula were above the MIC.

Preparation of AERF Gel

Tabel 1. The Gel of AERF formula

Material	Formula					
	Preparation			Base		
	I	II	III	I	II	III
Sodium alginate	2.5%	3%	3.5%	2.5%	3%	3.5%
Roselle water extract	3%	3%	3%	-	-	-
Propylene glycol	5%	5%	5%	5%	5%	5%
Sodium benzoate	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%
Buffer citrate pH 4.5	89.3%	88.8%	88.3%	92.3%	91.8%	91.3%

The gel of AERF was performed according to the Table 1 formula. A total of 12.5 g of propylene glycol was dissolved into buffer solution until homogenous; then, a total of 7.5 g of dried AERF and 500 mg of sodium benzoate were weighed. After that, the AERF and sodium benzoate were dissolved into the buffer solution and then stirred until homogeneous. Some powder of sodium alginate was weighed and then dispersed into the mixture of propylene glycol, sodium benzoate, AERF and buffer solution; the mixture was stirred slowly and continuously until a homogeneous gel mass was obtained.

Physical Evaluation of the Gel Preparation

The physical evaluation of the gel preparation included the tests of pH, viscosity, and dispersive power. The preparation was weighed as much as ± 5 g and diluted with 45 ml of CO2-free distilled water. The measurement of the preparation's pH and viscosity was conducted as specified in points 2.4, while the measurement of the dispersive power was described in the following paragraph.

Dispersive Power Test

The test was conducted with two glass plate scales. The preparation was weighed as much as ± 1 g and placed at the centre of the glass plate, then covered with another glass plate. Gradual loading was performed starting from 5 g on the upper glass plate. The dispersion diameter was measured at each loading until the preparation's dispersion stopped (± 3 minutes) by which time the diameter was recorded. The curve of the relationships between the dispersion diameter (mm) and the load's weight (g) were plotted. The dispersive power was defined as the slope of the regression equation of dispersion and the load's weight. The dispersive power was determined from the maximum dispersion diameter at the increments of certain loads. Then it was replicated three times.

Evaluation of Antibacterial Activities of the gel preparation

Testing antibacterial activities of the gel preparation

The gel preparation of ± 4 g was diluted using 6 ml of buffer citrate with a pH of 4.5 (solution concentration 12.0 mg/ml). Microbiological test was then conducted as described in point 2.5.

Determining the MIC of Selected Formulas

Concentration solution of 12.0 mg/ml was diluted until solutions of 6.0; 3.0; 1.5; 0.8; 0.4; 0.2; 0.1; and 0.05 mg/ml were obtained. A total of 30 ml of nutrient agar medium (45-50°C) was poured into a 20 cm diameter sterile petri dish aseptically and let to solidify (base layer). After that, 10 µl inoculum of S. aureus ATCC 25923 was taken and inserted into 20 ml nutrient agar medium (temperature 45-50oC), mixed with vortex, and then poured into base layer which was already formed, and then let to solidify. Then the media were perforated with a sterile hole maker of 19 holes (nine for the sample, nine for the negative control, and one for the positive control). The positive control used was gentamicin 25 ppm and the negative control was gel base. The next step was to insert the sample, the positive control, and the negative control into each hole. Then, the media were incubated at 37oC for 24 hours and observed and measured for the inhibitory zones formed using the

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Vernier caliper. The MIC was determined by looking at the smallest concentrations containing inhibitory zones.

Determining the Potential Ratios of the Selected Formulas

The comparative antibiotic used was gentamicin sulphate. Then a solution of gentamicin with a concentration of 100 ppm was made, which was then diluted to obtain solution with concentrations of 25, 20, 15, 10, and 5 ppm. Microbiological test as specified in 2.4.2 was conduced. The next step was making log graphs of concentrations of gentamicin vs. inhibitory diameters against the test bacteria; and then we calculated the regression equation as well as the gentamicin concentrations equivalent to the preparation's inhibitory power.

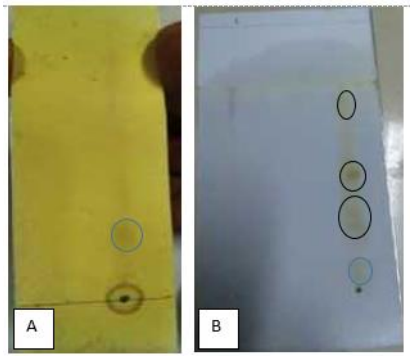
Data Analysis Design

In the data analysis phase, statistical analysis was performed using one-way Analysis of Variance (ANOVA) to know significant differences between sodium alginate concentrations and roselle dry water extract on the physical properties (pH, viscosity, and dispersion) and inhibitory power. From the analysis, F-values were obtained and then compared with the F critical values. If the F value > F critical value, there was a significant difference among the formulas. To identify different formulas, honestly significant difference (HSD) tes was conducted with 0.95 (a = 0.05) confidence interval.

Results and Discussion

Characteristic of the AERF

Organoleptic test found that AERF was hygroscopic powder, blackish red in colour, sour in taste, acid pH in solution (Table 2) and had unique aromatic smell. Chromatogram pattern test using TLC proved the presence of polyphenol and flavonoid in the AERF. Figures 1 present the results of chromatography pattern test of polyphenol and flavonoid compounds in AERF.



Figures 1 the results of chromatography pattern test of polyphenol and flavonoid compounds in AERF.

Table 2 Results of pH test of roselle water extract solution

No.	Weight (mg)	Concentration (% w/v)	pH
1	500	1.000	2.541
2	503	1.006	2.536
3	505	1.010	2.534
Average ± SD			2.537 ± 0.004

Qualitative Test of Sodium Alginate

The organoleptic test found that the sodium alginate was powder in form, white in colour, and had no taste. This is in line with the literature which states that sodium alginate is powder and has a colour of white to brownish yellow.

The viscosity test was performed using a Brookfield viscometer with sodium alginate concentration examined at 1% w/v.

Table 3. Results of pH and viscosity test of 1% sodium alginate solution

Replication	pH	Viscosity (cP)
1	6.914	440
2	6.894	420
3	6.953	396
Average ± SD	6.920 ± 0.03	418.33 ± 22.00

Characteristic of pH and viscosity of 1% sodium alginate base (Table 3) obtained 6.920 ± 0.03 and 418,33 ± 22,00 cP respectively. These results were not differed much from those mentioned in the literature both for pH and the viscosity, i.e. 7.2 and 20-400 cP, a slight difference which was probably caused by the effects of pH and temperature.

Determining the MIC of AERF

The inhibitory activity of AERF against Staphylococcus aureus ATCC 25923 expressed as zone diameter was presented in Table 4.

Table 4. Diameter of inhibition zones of AERF against Staphylococcus aureus ATCC 25923

Replication Level		Inhibition Diameter (mm)			
		1	2	3	Average ± SD
I	0.500	8.80	8.65	8.00	8.48 ± 0.42
II	0.250	8.20	7.70	7.55	7.82 ± 0.34
III	0.125	7.70	7.35	7.20	7.42 ± 0.26
IV	0.100	6.65	-	6.60	6.42 ± 0.36
V	0.050	-	-	-	-
VI	0.025	-	-	-	-

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The extract at a concentration of 0.05 mg/ml showed no inhibition zone against Staphylococcus aureus ATCC 25923. Thus, the degree to which roselle water extract still provided inhibition zones against Staphylococcus aureus ATCC 25923, Moreover, concentration of 0.1 mg/ml, was set as the MIC of the aqueous extract.

Evaluation of Physical Preparation

The effect of extract addition in the formula showed increasing viscosity of the gel formula compared to the gel base without extract (Table 5).

Table 5. Viscosity test results of base and preparation of roselle water extract gel

Formula	Viscosity	Base	Viscosity
I	48,240	I	14,320
II	64,400	II	28,080
III	200,400	III	110,400

The results of pH tests on the gel preparation and bases presented in Table 6 and Table 7 showed that adding roselle (Hibiscus sabdariffa L.) extract caused the decrease of pH of the gel preparation.

Table 6. Results of pH Tests of Roselle Water Extract Gel

Replication	pH			
Formula	1	2	3	Average±SD
I	3.621	3.601	3.613	3.611 ±0.010
II	3.689	3.679	3.690	3.686 ±0.006
III	3.732	3.725	3.732	3.729 ±0.004

Table 7. Results of pH Tests of Gel Bases

Replication	pH			
Base	1	2	3	Average±SD
I	4.879	4.889	4.857	4.875 ±0.016
II	4.894	4.881	4.856	4.877 ±0.019
III	4.931	4.914	4.883	4.909 ±0.024

This phenomenone was caused by the roselle water extract that had acidic pH. To evaluate the effects of the roselle (Hibiscus sabdariffa L.) water extract addition, statistical analysis of One-Way Anova post hoc Tukey was performed, by which an F-value of (6159.101) > F critical value (3.106) with a confidence interval $\alpha = 0.05$, indicating a significant difference. The difference significance of each formula was observed by an HSD test. It was found that Formulas I and II, I and III, II and III had significant differences. These significant differences may not only be influenced by the addition of roselle extract, but also probably by the amount of gelling agent added to the preparation Formulas. Formulas I, II, and III had significant differences compared with Bases I, II, and III, whereas Bases I and II, Bases I and III, and Bases II and III had no significant differences.

Dispersive Power Test of the Preparation

Table 8. Dispersive power (g/cm) of AERF gel preparation and its base

Inhibition Diameter (mm)					
		Replication			Average±SD
		1	2	3	
Formula	I	0.018	0.025	0.025	0.027±0.004
	II	0.014	0.014	0.018	0.015±0.002
	III	0.012	0.020	0.012	0.014±0.005

Table 8 presented the results of the preparation's dispersive power of the extract gel. Base I reached the maximum dispersive power at a 55 g of load addition, whereas Formula I reached the maximum dispersive power at a 25 g of load addition. Base II achieved maximum dispersive power at 30 g of load addition, whereas Formula II achieved maximum dispersive power at 20 g of load addition. Base III achieved maximum dispersive power at 50 g of load addition, whereas Formula III achieved maximum dispersive power at 15 g of load addition. To know the effects of roselle water extract addition on the preparation towards dispersive power, a statistical test of One-Way Anova was conducted, a test which obtained an F value of 3.778 > F critical value of 3.106, indicating a significant difference. As for the difference significance of each formula, the HSD test results showed that Formulas I and II, Formulas I and III, and Formulas II and III had no significant differences.

Evaluation of antibacterial activities of the gel preparation

A test of antibacterial activities of roselle extract gel preparation was performed to obtain appropriate formulas between Formula I, Formula II, and Formula III at a 12 mg/ml solution concentration. Table 9 presents the observed inhibition diameters of roselle water gel.

Table 9. Diameters of the inhibition zones of roselle water extract preparation with a concentration of 12 mg/ml on Staphylococcus aureus ATCC 25923

Inhibition Diameter (mm)					
		Replication			Average±SD
		1	2	3	
Formula	I	0.018	0.025	0.025	0.027±0.004
	II	0.014	0.014	0.018	0.015±0.002
	III	0.012	0.020	0.012	0.014±0.005

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Table 9 shows that the inhibitory power of Formula II was higher than that of Formulas I and III. Bases I, II, and III did not show any inhibition power towards the bacteria Staphylococcus aureus ATCC 25923. This was because the sodium benzoate that served as the preservatived in Bases I, II and III became inactive because the pH level of the three bases was close to 5. To know the effect of addition of roselle water extract in the preparation on its dispersive power, a statistical test One Way Anova was conducted, a test which obtained an F value of 22.465 > F critical value of 5.143, indicating a significant difference. As for the difference significance of each formula, the HSD test results showed that Formulas I and II, Formulas I and III had significant differences, wheras Formulas II and III had no significant difference.

Determining the Minimum Inhibitory Concentration (MIC) of Roselle Extract Gel Preparation

After physical properties were evaluated and antibacterial activities of the three formulas of roselle extract gel preparation were tested, Formula II was chosen in order to determine its MIC. The observed results of the inhibition zone diameters of Formula II towards Staphylococcus aureus ATCC 25923 are presented in Table 11.

Table 10. Diameters of inhibitory zones of Formula II towards Staphylococcus aureus ATCC 25923

Inhibitory zone diameter (mm)				
Concentration (mg/ml)	Replication			Average±SD
	1	2	3	
12.00	11.40	11.20	11.05	11.22±0.17
6.00	10.75	10.25	9.85	10.28±0.45
3.00	10.55	9.75	9.60	9.96±0.49
1.50	10.35	9.40	9.40	9.72±0.54
0.80	9.80	8.70	8.80	9.10±0.60
0.40	8.70	8.50	7.15	8.11±0.84
0.20	-	-	-	-
0.10	-	-	-	-
0.05	-	-	-	-

Table 10 shows that Formula II at a concentration of 12.00-0.040 mg/ml still had an inhibitory zone against Staphylococcus aureus ATCC 25923, whereas a concentration of 0.020-0.005 mg/ml did not show any inhibitory zones against Staphylococcus aureus ATCC 25923. Thus, the concentration in which Formula II still provided inhibitory power towards Staphylococcus aureus ATCC 25923 was at a 0.040 mg/ml concentration, henceforth defined as the MIC of Formula II of roselle water extract gel preparation.

Potential Ratio of gel preparation as Antibacterial

The test was performed to determine the potential of Formula II of roselle water extract gel preparation, compared with gentamicin, as an antibacterial against Staphylococcus aureus ATCC 25923. The results of the potential test of Formula II of roselle gel preparation are presented in Table 12.

Table 11. Diameters of Inhibitory Zones of Gentamicin and Formula II against Staphylococcus aureus ATCC 25923

Inhibition Diameter (mm)				
Sample	Replication			Average±SD
	1	2	3	
Gentamicin 25 ppm	20.90	20.90	19.70	20.50±0.69
Gentamicin 20 ppm	19.20	19.60	18.70	19.17±0.45
Gentamicin 15 ppm	18.05	18.55	17.80	18.13±0.38
Gentamicin 10 ppm	17.30	17.05	16.30	16.88±0.52
Gentamicin 5 ppm	14.60	15.55	14.80	14.65±0.50
Formula II (12.0 mg/ml)	13.00	13.10	13.50	13.20±0.26

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Table 11 shows that the inhibitory diameter of roselle extract gel preparation of Formula II was 13.20, which was subsequently substituted into the regression equation y=8,0529x+33,046 as a value of y. The regression equation was obtained from concentration log vs. gentamicin's inhibition diameter. From the substitution results, it was revealed that a solution concentration of 12 mg/ml (roselle concentration in 3% w/w gel) had an inhibitory power equivalent to 3.432 ppm of gentamicin as an antibacterial.

Conclusions

The aqueous extract of Roselle (Hibiscus sabdariffa L.) flower gel preparation Formula II with 3% (w/w) sodium alginate concentration had better properties (pH, viscosity, and dispersive power) than Formula I and Formula III with 2.5% and 3.5% sodium alginate concentrations respectively. At 3% (w/w) of sodium alginate concentration, the extract gel preparation containing 3% (w/w) of the aqueous extract exhibited Minimum Inhibitory Concentration of 0.40 mg/mL against Staphylococcus aureus ATCC 25923.

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