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In vitro antimicrobial activity and formulation of herbal anti-acne gel containing *Rhizophora stylosa* fruits extract

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ABSTRACT

Mangrove fruits have a variety of bioactive metabolites that may control microbial growth. The present study was conducted to formulate and evaluate the antibacterial activities of *Rhizophora stylosa* fruit extract. The antibacterial activity of *R. stylosa* crude extract was investigated against *Staphylococcus aureus, Staphylococcus epidermidis*, and *Propionibacterium acnes* by disc diffusion method. The herbal gel formulations were developed using different concentrations of kappa carrageenan and Carbomer 940 as the gelling agent. The physicochemical characteristics and in vitro antibacterial activity of the gel were evaluated. The results revealed that the extract had a minimum inhibitory concentration value of 0.05 mg/ml against *S. aureus* and *P. acne*, and 0.1 mg/mL against *S. epidermidis*. All herbal gel formulations with 0.5% w/w of the extract showed a strong inhibitory activity against *P. acnes*. The herbal gel showed a good homogeneity, pH, viscosity, spreadability, and physical stability in an accelerated condition. Among all formulation studied, F1 was considered as the optimized formulation which contains 0.5% of the *R. stylosa* fruit extract with 0.25% kappa carrageenan and 0.5% Carbomer 940. It can be concluded that the developed gel formulation has potential against *Acne vulgaris*. Further research is needed to determine the safety and efficacy so *R. stylosa* can be developed as an alternative natural anti-acne agent.

Keywords: antibacterial, herbal gel, red mangrove fruits, *Rhizophora stylosa*

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INTRODUCTION

Acne is one of the most common skin disorders in young adults especially during puberty. Acne pathogenesis involves defects in epidermal keratinization, androgen secretion, sebaceous function, bacterial growth, inflammation, and immune response (Wells et al., 2009). The main microorganisms responsible for these the conditions are *Propionibacterium acnes* (anaerobic bacteria), *Staphylococcus aureus*, and *Staphylococcus epidermidis* (aerobic bacteria). These microorganisms proliferate rapidly which ultimately result in the growth of acne. The widespread and long-term use of antibiotics for acne treatment has resulted in the spread of resistant bacterial strains and treatment failure (Dhillon & Varshney, 2013). The risk-to-benefit ratio of long-term antibiotic use should be carefully considered and generally, its usage should be avoided whenever possible. Thus, effective alternatives to antibiotics for acne treatment are needed to reduce the likelihood of antibiotic resistance (Walsh et al., 2016). Systematic clinical studies that investigated the use of plant extracts, phytochemicals, and herbal formulations for acne treatment reported favorable results. Several studies showed that they are equal or superior to the standard antibiotic therapy with no serious adverse effects (Fisk et al., 2014).

Mangroves are known as rich sources of various secondary metabolites. These higher plants are widely used for traditional medicinal practices that have significant importance in the pharmacological field (Seepana et al., 2016). Indonesia has the widest mangrove area in the world with 3,112,989 hectares or 22.6% of the global area. In addition, Indonesia has the highest variety of species worldwide, accounting for 45 out of the 75 species (Susilo et al., 2018). Rhizophora is one of the genera of mangroves found in Indonesia which contains secondary metabolites that can be used as an alternative drug. The extract of some species of this genus is reported to have antifungal, antibacterial, and anti-inflammatory activity, gastric antiulcer properties, and efficacy in wound healing (Takara et al., 2008). One of those species, the red mangrove (*Rhizophora stylosa*), contains tannins (procyanidin and prodelphinidin), saponins, flavonoids, phenol hydroquinone, triterpenoids, and alkaloids in its fruit. It is found to have inhibited the growth of *Staphylococcus aureus* (2 mg/mL) with a mean diameter of the inhibition zones of 13 mm (Wulandari et al., 2014). Condensed tannins were reported to be responsible for the antioxidant activities of tea mangrove extract or mangrove fruit extract (Miranti et al., 2018). In addition, the black mangrove (*Rhizophora mucronata*), leaf extract with a concentration of 2 mg/ml is shown to have inhibited two types of acne-causing bacteria i.e. *Staphylococcus epidermidis* and *Staphylococcus aureus* by 11.5 mm and 13 mm, respectively (Utami, 2016).

Compared with *R. apiculata*, *R. mucronata*, and *R. mangle*, the phytochemistry and pharmacological activities of *R. stylosa* have not been extensively investigated (Kainuma et al., 2015). Hence, in the present study, we developed its fruit extract as natural active ingredient in preparing a herbal anti-acne gel. Carbomer 940 is the most widely used gelling agent that can produce a gel with a high viscosity with a relatively low concentration (Lu & Jun, 1998). Gel formulation can also be combined with the addition of natural excipients such as kappa carrageenan. Kappa carrageenan can be used as a natural thickening, stabilizing, and gelling agent that is relatively compatible with other cosmetic ingredients. However, kappa carrageenan as a gelling agent has a few disadvantages including rigidity and brittleness. Thus, it needs to be combined with another gelling agent (Rowe et al., 2006). Gel is suitable for topical acne treatment in patients with oily skin, as the climate of Indonesia causes most people to have oily skin (Diharmi et al., 2011). Therefore, this study was conducted to evaluate the anti-acne potential of the red mangrove fruit extract as well as to develop and characterize a gel formulation using a combination of Carbomer 940 and kappa carrageenan as a natural gelling agent.

MATERIALS AND METHOD

Materials

Red mangrove fruits were collected from Untung Jawa Island in the Thousand Islands archipelago (Jakarta, Indonesia) and identified by Herbarium Bogoriense Indonesian Institute of

Sciences (Bogor, Indonesia). *P. acnes*, *S. aureus*, and *S. epidermidis* were obtained from the Microbiology Laboratory, Faculty of Medicine, University of Indonesia. The Nutrient Agar (NA-OXOID), Nutrient Broth (NB-OXOID), Mueller Hinton Agar (MHA-OXOID), Brain Heart Infusion (BH-OXOID), and Blood Agar (BA-OXOID) were used mediums. Kappa carrageenan, Carbomer 940, triethanolamine, glycerine, and sodium benzoate were used as gel excipients.

Methods

Extraction of red mangrove fruits

Fresh samples of red mangrove fruits were washed thoroughly with water. The hypocotyl part of the fruits (Figure 1) was sliced into small pieces and dried until a constant weight was achieved.

Figure 1. Photographs of *Rhizophora stylosa* fruits

The dried fruits were grounded into powder. The powder was extracted by single maceration method using an orbital shaker in 70% ethanol solution with one-sixth (w/v) powder to solvent ratio for 48 hours. The extracts were separated from the residue by filtration with Whatman No 42. Afterward, the extracts were concentrated using a vacuum rotary evaporator. Phytochemical screening was carried out using the color reaction to the presence of the secondary metabolites such us alkaloids, flavonoids, saponins, tannins, phenolic, steroids, and triterpenoids (Fansworth, 1966). The water content of the extract was determined by drying 1 g of the extract in the oven at 105^{o}C for 5 hours.

Determination of antimicrobial activity

Antibacterial activity of red mangrove fruit extract

The antibacterial activity was determined by agar well diffusion method. The cultures of *S. aureus* and *S. epidermidis* were prepared in NB agar medium at 37^{0}C for 24 h under aerobic conditions. Lyophilized culture of *P. acnes* was revived in BHI broth at 37^{0}C for 48 h under anaerobic condition (Moorthy et al., 2007).

The cultures of *S. epidermidis* and *S. aureus* were standardized by UV-Vis Spectrophotometric method at λ = 600 nm and 0.5-0.8 of OD. *P. acnes* was standardized using 0.5 McFarland turbidity standard (1×10^{6} cells per mL) (Moorthy et al., 2007).

P. acnes inoculum was spread on the surface of sheep blood agar with the help of a sterile swab stick and was left to dry. *S. aureus* and *S. epidermidis* were dispensed into an MHA medium. A well with a diameter of 6 mm was made on the surface of solid agar using a sterile cork. The extracts with various concentrations (0.5 mg/mL, 1 mg/mL, and 2 mg/mL) were added into the well. Then, the agar plates of *P. acnes* were incubated in an aerobic environment. The test samples of these aerobic bacteria were incubated at 37^{o}C for 24 hours under an aerobic condition. Standard antibiotic clindamycin at 1 mg/mL concentration was used as the positive control, and 70% ethanol solution was used as the negative control. The antibacterial activity was estimated by measuring the diameter of inhibition zones (Moorthy et al., 2007).

Minimum inhibitory concentration (MIC) of red mangrove fruit extract

The minimum inhibitory concentration (MIC) value was determined by the agar dilution method. Each tube was filled with 5 mL of Nutrient Broth (NB) media for *S. aureus* and *S. epidermidis*, and Brain Heart Infusion (BHI) media for *P. acnes*. Six tubes were sequentially added with the test sample containing the extract with a concentration of 0.4 mg/mL, 0.3 mg/mL, 0.2 mg/mL, 0.1 mg/mL, 0.05 mg/mL, and 0.025 mg/mL, respectively, while the seventh tube was used as the positive control containing the test media and bacterial suspension. The tubes were incubated at 37ºC for 24 hours. Microbial growth was observed for turbidity in the media following the incubation period. The tube with the lowest concentration, where the absence of bacterial growth was observed, was determined as MIC (Mazzola et al., 2009).

Formulation of gels

The anti-acne gel formula from the extract is based on a modified formula from previous studies with various concentrations of kappa carrageenan (Miranti et al., 2018). The selection of excipients was based on the concentration ranges allowed in the Handbook of Pharmaceutical Excipients (Kainuma et al., 2015). Carbomer 940 and kappa carrageenan were used as gelling agents, and the concentration of the extract used in the formula was based on preliminary tests. Triethanolamine acted as a stabilizer and neutralizing agent for acidic Carbomer 940. Glycerin was used as a humectant, and sodium benzoate as a preservative.

Gel formulation was carried out by dissolving Carbomer 940 with distilled water (1:20 w/w) in a mortar at 70℃ for 30 minutes and continuously ground until it completely dissolved. Triethanolamine (TEA) was added to obtain the desired gel consistency (mixture 1). Kappa carrageenan with various concentrations (Table 1) was dissolved in the remaining distilled water at 80℃ for about 25 minutes.

Table 1. Anti-acne gel formulation red mangrove *(Rizophora stylosa) fruit* extract

Ingredients	Concentration (%)		
	Formula A	Formula B	Formula C
Kappa carrageenan	0.25	0.50	0.75
Extract	0.50	0.50	0.50
Carbomer 940p	0.50	0.50	0.50
Triethanolamine (TEA)	qs*	qs*	qs*
Parfume	0.10	0.10	0.10
Glycerin	1.00	1.00	1.00
Sodium benzoate	0.10	0.10	0.10
Distilled water	ad 100	ad 100	ad 100

*qs : quantum satis

Sodium benzoate was dissolved in glycerin and then mixed into the kappa carrageenan solution at 65-68℃. The mixture was stirred until homogeneous (mixture 2). Mixture 2 was added to mixture 1 and stirred until homogeneous, and a gel mass was formed. The gel base was then mixed with the extract, which was dissolved in distilled water (1:2). Additional perfume was added and stirred until homogeneous.

Determination of the concentration of the red mangrove fruit extract for anti-acne gel preparation

The concentration of the red mangrove fruit extract for anti-acne gel preparations was determined by a preliminary test. The series of concentrations of the extract was made based on the MIC value of *P. acnes* bacteria, which is 0.05 mg/mL or equal to 0.005% (w/w). The extract concentration was then varied to 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% (w/w). The extract

concentration in the gel preparations, which has the largest inhibitory zone, was selected for anti-acne gel preparations.

Antibacterial assay of anti-acne gel preparation

An antibacterial activity test for gel preparation was carried out against *P. acnes*. The antibacterial activity test was determined by the agar well diffusion method. In this assay, the gel used had various kappa carrageenan concentrations, which were 0.25% (1), 0.50% (2), and 0.75% (3).

P. acnes bacteria were scratched onto the surface of BA media using a sterile cotton bud. A well with a diameter of 6 mm was made on the media. A weighted amount of 0.5 g of gel preparations 1, 2, and 3 were put into the well. Media containing gel preparations was incubated at 37ºC for 24 hours, and the diameters of the inhibitory zones formed around the well were measured.

Chemical analysis and physical preparation of anti-acne gel

Viscosity

Viscosity measurement was carried out on 100 g of gel preparation using a Brookfield viscometer with spindle No. 7 at 100 rpm.

Homogeneity

The gel preparation was applied to glass to observe its homogeneity. A good gel shows a homogeneous arrangement and does not show coarse grain.

Spreadability

One gram of gel was placed in between two watch glasses. The upper watch glass was given a load of up to 150 g and left to stand for 60 seconds. Measurements were made by measuring the diameter of the dispersed gel using a caliper log.

pH

The pH was determined using a digital pH meter that was calibrated with suitable buffer solutions at a constant temperature.

Physical properties

The color of the gel formulations containing the extracts was observed visually. The consistency and greasiness were characterized by directly applying the gel to the skin. The odor of the gel was checked by mixing the gel with water.

Stability study

The gel formulations were subjected to accelerated stability tests by six freeze-thaw cycles. For each cycle, the samples that were packed in the plastic tube were stored at 4ºC for 24 hours, and then at 30 ºC for another 24 hours. After the completion of six cycles, the physicochemical properties of the gel formulations were evaluated and compared with the freshly prepared formulations.

RESULT AND DISCUSSION

Extraction of red mangrove fruits

The extract has a blackish brown color in the form of paste and has a yield of 3.08%. The water content of the extract was 4.31%. Phytochemical screening showed that the ethanol extract of red mangrove fruits contains tannin, saponin, phenol, flavonoid, and triterpenoid. The results of the phytochemical screening of the ethanol extract of red mangrove fruits are presented in Table 2. Flavonoids, tannin, and phenol have been reported to have antibacterial activity (Wells et al., 2009).

In this study, the fruits were used because they were reported to have a higher amount of tannin, flavonoid, saponin, and terpenoid than the leaves (Wahyuni et al., 2015). The amount of extract yield can be affected by solvent, particle size, and extraction time. The water content of the extract must not

exceed 10%. Otherwise, the extract can be easily damaged due to the growth of microorganisms and the overgrowth of fungi (European, 2007). The *R. stylosa* fruit extract contains various phytochemical constituents such as tannin, saponin, terpenoid, and flavonoid.

Table 2. Phytochemical screening of ethanol extract of red mangrove *(Rizophora stylosa)* fruit

Phytochemical screening	Result
Alkaloid	-
Tannin	+
Saponin	+
Phenol	+
Flavonoid	+
Steroid	-
Triterpenoid	+

Determination of antimicrobial activity

Antibacterial activity of red mangrove fruit extract

The result of the antibacterial activity of the red mangrove fruit extract is presented in Figure 2. Antibacterial activity assay was determined by agar well diffusion method against *S. aureus*, *S. epidermidis*, and *P. acnes*. Clindamycin and 70% ethanol solution were used as positive and negative controls, respectively. All negative controls did not show any inhibitory activity, while the positive controls produced inhibitory zones of 32.00 mm, 13.50 mm, and 29.00 mm against *S. aureus*, *S. epidermidis*, and *P. acnes*, respectively.

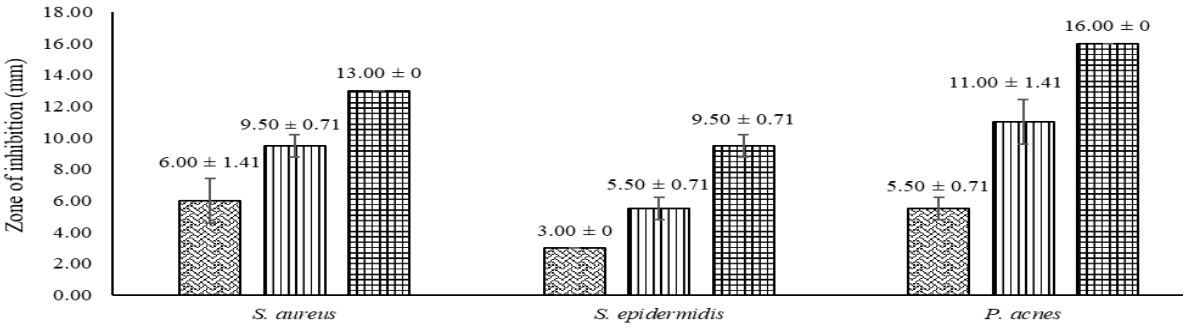

Figure 2. Antibacterial activity test results of the extract at various concentrations
= 0.5 mg/mL, = 1 mg/mL, = 2 mg/mL

The antibacterial activity test of the extract showed that the highest inhibitory zone against all bacteria was at a concentration of 2 mg/mL. In a previous study, the red mangrove fruit extract inhibited the activity of *S. aureus* with an inhibition zone of 14 mm at a concentration of 2 mg/mL (Wulandari et al., 2014). The black mangrove (*R. mucronata*) leaf extract with the same concentration, inhibited two types of acne-causing bacteria i.e. *S. epidermidis* and *S. aureus*, by 11.5 mm and 13 mm, respectively (Utami, 2016).

The antibacterial activity of the extract was determined by several factors, including the type of microbial test, the volume of inoculum, the composition of the culture medium, the incubation time, and the concentration of active substances (Madigan et al., 2012). The antibacterial activity was increased in accordance with the increasing extract concentration. The red mangrove fruit extract contains tannin, which can inhibit the growth of bacteria by contracting the bacterial cell walls, thereby disrupting its permeability (Scalbert, 1991).

The extract with a concentration of 2 mg/mL was classified as a strong inhibitor against *S. aureus* and *P. acnes*, and as a moderate inhibitor against *S. epidermidis*. The positive control, clindamycin, was also classified as a very strong inhibitor against *S. aureus* and *P. acnes* and as a strong inhibitor against *S. epidermidis* because it is effective against Gram-positive and anaerobic bacteria (Chadwick, 1971).

Minimum inhibitory concentration (MIC)

The result of MIC of red mangrove fruit extract is presented in Table 3. The red mangrove fruit extract showed MIC values of 0.05, 0.10, and 0.05 mg/mL against *S. aureus*, *S. epidermidis*, and *P. acnes*, respectively.

Table 3. MIC test results of red mangrove *(Rizophora stylosa)* fruit extract

Test sample	MIC (mg/mL)		
	S. aureus	***S. epidermidis***	***P. acnes***
Red mangrove fruit extract	0.05	0.10	0.05

The extracts with MIC value of < 0.1 mg/mL have strong antimicrobial activity, while the ones with MIC values between 0.1 and 0.5 mg/ml have moderate activity. In addition, the extracts with MIC value of 0.5-1 mg/mL have weak antimicrobial activity, while the ones with MIC value of > 1 mg/mL have no activity or are unable to inhibit bacterial growth (Holetz et al., 2002). In this study, the MIC value of the red mangrove fruit extract was 0.05 mg/mL against *S. aureus* and *P. acnes*, as well as 0.1 mg/mL against *S. epidermidis*, which can be considered a strong inhibitory activity.

Determining red mangrove fruit extract concentration for anti-acne gel preparation

The extract concentration for the anti-acne gel formulation was determined through a preliminary test. Serial dilution was made based on the MIC value of *P. acnes* bacteria that is 0.05 mg/ml or equal to 0.005% (w/w) The extract concentration was then increased to 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5% (w/w). The extract with a concentration of 0.05% did not show an inhibitory zone, while the others (0.1, 0.2, 0.3, 0.4, and 0.5%) produced 2, 10, 16, 19, and 24 mm, respectively. The gel with 0.5% extract has the highest inhibition zone value and is classified as a very strong inhibition zone (Davis & Stout, 1971). Furthermore, 0.5% extract concentration was used for the anti-acne gel formulation.

The results of the antibacterial activity assay of gel preparations showed that the inhibitory zones formed by the three formulas were categorized as very strong. The resulting inhibition zones were affected by the release of the active substance from the gel preparation, which might be affected by the gel's viscosity. High viscosity will make the diffusion of the active substance from the basis more difficult (Chandira et al., 2010). Therefore, the addition of kappa carrageenan will increase the viscosity while decreasing the release of the active substance from the gel preparation. Nevertheless, the antibacterial activities of all formulas were not significantly different.

Gel formulation containing red mangrove fruit extract

Gel formulations containing red mangrove fruit extract in three formulations with different kappa carrageenan concentrations (F1, F2, and F3) were successfully prepared (see Table 4). The resulting gels were clear and translucent with brownish colors. The pH of the formulations was between 5.87 and 6.03. The pH value of all formulations was acceptable for facial skin, which is in the range of 4.9-6.3 (Thune et al., 1988). Antibacterial activity for all formulations was not significantly different. The viscosity of the formulations was increased with increasing concentration of kappa carrageenan.

The gel formulations were physically stable in accelerated testing. After six freeze-thaw cycles, the physicochemical properties of the gel formulations in terms of appearance, color, odor, homogeneity, and pH were unchanged.

Notably, kappa carrageenan has several disadvantages as a gelling agent because it is originally derived from a natural polymer. For instance, it typically shows syneresis that increases over time as the gel contracts (Hotchkiss et al., 2016). Kappa carrageenan has a high gelling capacity to form a brittle gel with high strength. On the other hand, Carbomer 940 can readily absorb water due to its hydrophilic nature. Therefore, kappa carrageenan and Carbomer 940 can be combined to decrease syneresis and obtain better gel properties. In this study, the addition of a different concentration of kappa carrageenan affected only the viscosity without any significant changes in the gel's spreadability. The higher concentration of kappa carrageenan increased the gel's viscosity due to the increase in the formation of three-dimensional cross-linking structure (Datar, 2013). A gel can be considered to have a good spreadability if it can be dispersed in 5-7 cm (Garg et al., 2002). Our study, which contained 0.25% kappa carrageenan, had a very strong inhibition zone, with good spreadability. Thus, it can be concluded that the herbal gel containing *Rhizophora stylosa* fruit extract can be used effectively as an alternative treatment for acne. However, further clinical evaluation should be conducted to confirm its safety and efficacy.

Table 4. Characteristics of anti-acne gel preparation of fruit red mangrove *(Rizophora stylosa)* extract

Parameter	Kappa carrageenan concentration		
	F 1 (0.25%)	**F2 (0.50%)**	**F3 (0.75%)**
Antibacterial (mm)	23.50 ± 0.35^{a}	22.75 ± 0.00^{a}	22.12 ± 0.53^{a}
Viscosity (cPs)	7580.00 ± 127.28^{a}	10760.00 ± 678.82^{b}	16305.00 ± 473.76^{c}
Spreadability (cm)	7.05 ± 0.07^{a}	6.66 ± 0.09^{a}	5.40 ± 0.21^{a}
pH	5.87 ± 0.02^{a}	5.98 ± 0.00^{b}	6.03 ± 0.02^{b}
Homogeneity	homogeneous and having no coarse grains	homogeneous and having no coarse grains	homogeneous and having no coarse grains

Description: different superscript font indicates significant differences ($p < 0.05$). Significance differences were observed between F1, F2, and F3 for viscosity parameter (a,b,c: $p < 0.05$), between F1 and F2 for pH parameter (a,b: $p < 0.05$).

CONCLUSION

The red mangrove fruit extract investigated in this research showed a strong inhibitory activity with the value of MIC of 0.05 mg/ml against *S. aureus* and *P. acnes*, and 0.1 mg/mL against *S. epidermidis*. The concentration of the extract which was selected for the anti-acne gel preparations was 0.5 % (w/w). The formulated gel with 0.5 % (w/w) extract showed the highest inhibition zone value. The herbal gel had a very strong inhibition zone with good viscosity, homogeneity, and spreadability. On the one hand, it was detected that the pH value of the gel is also suitable for topical preparations. Research findings have proven that the combination of 0.25% kappa carrageenan and 0.5% Carbomer 940 is the best gel formula (F1) for red mangrove fruit extract to be used as an anti-acne agent.

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