Antimicrobial ointment based on *Bacillus subtilis* subsp. *subtilis* HSFI-9 isolated from Sea Cucumber of Kodek Bay Lombok Indonesia

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Abstract

The ethyl acetate extract of *Bacillus subtilis* subsp. *subtilis* HSFI-9, a bacteria isolated from intestinal fermentation of sea cucumbers (*Holothuria scabra*) is known to have antimicrobial properties. This research aimed to determine the activity of HSFI-9 extract ointment against *Staphylococcus aureus* and *Candida albicans*. Extract ointment is a topical preparation for skin infections made from a mixture of Vaseline Alba and Adeps lanae bases. The extract ointment was prepared into four concentrations of 0.003%, 0.03%, 0.3%, and 1% w/v using HSFI-9 as active ingredient. Antimicrobial assay was carried out in vitro using the disc diffusion method. The extract ointment was evaluated based on organoleptic, homogeneity, spreadability, adhesion characteristics, and pH tests. The optimal concentration of the extract ointment to inhibit the growth of *S. aureus* 0.3% (inhibition zone of 11.67±1.26 mm) and against *C. albicans* is 0.03% (inhibition zone of 10.16±1.50 mm). The activity of the extract ointment was categorized as strong although not as strong as the antibiotic control ointment Mupirocin 2% or Ketoconazole 2%. The extract ointment organoleptic indicated a characteristic odor of ethyl acetate, was yellowish-white ointment, and had a homogeneous and smooth consistency. The extract ointment also had properties such as good spreadability but poor adhesion and tended to have an acidic pH (< 4.5). The HSFI-9 extract can be declared feasible for the development of topical antimicrobials. The ointment still needs to be optimized, especially regarding improving the adhesion characteristic and pH to be safe for the skin and mucosa.

Keywords: Anti-infective ointment, Bacillus subtilis subsp. subtilis, Holothuria scabra, skin diseases

Introduction

Infections of the skin and mucosa can be caused by several microbial agents such as *Staphylococcus aureus* and *Candida albicans* (Talapko *et al*., 2021). Normal flora on the skin can prevent infection, however, when the skin surface experiences a traumatic wound or puncture, pathogenic microbes can enter the wound and cause infection (Creech *et al*., 2015). For example, in around 30% of the human population, *S. aureus* colonization can be found in skin and soft tissue areas (Tong *et al*., 2015). This causes infection with various skin disease manifestations such as pyoderma, dermatitis, carbuncle, impetigo, and acne accompanied by the formation of abscesses and pus (Del Giudice, 2020). Otherwise, *C. albicans* colonies on the epithelial surface of the skin can manifest as infections of the oral and vaginal mucosa (Kühbacher *et al*., 2017).

Skin infection therapy caused by *S. aureus* faces the problem of Methicillin-Resistant *S. aureus* (MRSA), even preventive therapy with mupirocin has developed resistance (Chahine & Sucher, 2018; Dadashi *et al*., 2020). Ketoconazole treatment for candidiasis fungal infections caused by *C. albicans* has also been widely reported to be resistant (Xu *et al*., 2021). The development of antimicrobial ointments is needed to overcome this resistance problem. Natural products, especially compounds and essential oils from plants are known to have many benefits as “green” topical antimicrobials (Rybczy´nska-Tkaczyk *et al*., 2023). Microorganisms also play an important role in the discovery of topical antimicrobials, for example, fungi such as *Fusidic coccineum* can produce Fusidic acid which can be used topically against *S. aureus* (Bandyopadhyay, 2021).

Natural antimicrobial ingredients of bacterial origin are also widely known, especially from the genus *Streptomyces* and *Bacillus*. Antimicrobial compounds have been widely studied from secondary metabolites of *Bacillus sp*. Compounds from *Bacillus sp*. that have been developed in the form of topical antimicrobials include Polymyxin-b from *Bacillus polymixia* and Sonorensis from *Bacillus sonorensis* (Stan *et al*., 2021). The ability of secondary metabolites from *Bacillus* sp. have also been proven from several metabolomics studies for treatment targeting infections of the skin and mucosa (Rasyid *et al*., 2018), including the discovery of antifungal compounds from the fermentation of *Bacillus sp*. (Gadhoumi *et al*., 2022). The extract can be developed into a topical dosage form such as antibiotic ointment to be more optimal for treating superficial skin infections (Bandyopadhyay, 2021).

Ointment is a semi-solid preparation that is widely used to treat skin infections caused by *Staphylococcus aureus* and *C. albicans* (Brown *et al*., 2018). Ointments can be made using an inert base formulation in order not to affect the therapeutic effect of the active substance (Naibaho *et al*., 2013). Topical dosage forms with a variety of hydrocarbon and absorption base mixtures are known to be good for developing antimicrobial ointment. The ointment with these two base mixtures has a suitable consistency for easy application on the skin (Tong *et al*., 2018).

In this study, the HSFI-9 extract ointment was made using a mixture of hydrocarbon base (Vaseline alba) and absorption base (Adeps lanae). Ethyl acetate extract from *Bacillus subtilis* subsp. *subtilis* HSFI-9 (hereinafter referred to as HSFI-9 extract) has been proven to detain the growth of *S. aureus* at a concentration of 3.125 µg/mL, which is equivalent to concentration of 0.003% w/v (Rakhmawatie *et al*., 2023a). The aim of this research was to test HSFI-9 extract ointment against the growth of *S. aureus* and *C. albicans*. The ointment was also evaluated to meet the criteria for organoleptic, homogeneity, spreadability, adhesion characteristics, and pH tests (Wardani & Septiarini, 2021).

Materials and Methods

HSFI-9 Secondary Metabolites Extraction

B. subtilis subsp. *subtilis* HSFI-9 was isolated from *Holothuria scabra* which was found in Kodek Bay Lombok, West Nusa Tenggara, and has been molecularly identified from previous research (Rakhmawatie *et al*., 2023a). Bacterial isolates were stored in glycerol in a refrigerator at – 20°C, then re-grown using Brain Heart Infusion Broth media (BHIB, Oxoid CM1135B), with a ratio of bacterial suspension and media, is 1:10. Next, the bacteria in BHIB were incubated in an incubator at 37°C for 24 hours (Rahman *et al*., 2022). As much as 10 µl of *B. subtilis* subsp. *subtilis* HSFI-9 from BHIB were streaked onto Mueller Hinton Agar media (MHA, Merck 103872). Next, the extract was produced by the HSFI-9 culture using Starch Yeast Peptone media. The culture's supernatant was extracted with ethyl acetate, which was then concentrated by evaporating all the ethyl acetate solvent.

Preparation of HSFI-9 Extract Ointment

The ointment is made by mixing all the ointment components (Table 1) until the required amount is achieved. Mixing the ointment base ingredients is done by melting all the ointment components in a water bath at a temperature of 80°C, then cooling with constant stirring using a mixer until it thickens (Nareswari &

Table 1. Base formulation components for the HSFI-9 extract ointment

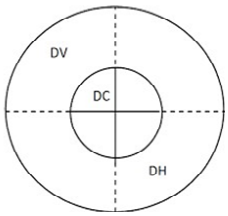
Component	Ointment Formulation				
	Ointment Base	I	II	III	IV
HSFI-9 extract in 0.5% DMSO	-	0.003%	0.03%	0.3%	1%
Vaseline alba (Brataco 982426)	70%	70%	70%	70%	70%
Adeps lanae (Brataco 200315)	10%	10%	10%	10%	10%
Propylene glycol (Sigma Aldrich, PHR1051)	15%	15%	15%	15%	15%
Benzoic acid (Sigma Aldrich 242381)	1%	1%	1%	1%	1%
Butylated hydroxytoluene (Sigma Aldrich PHR1117)	1%	1%	1%	1%	1%
Distilled water (Waterone)	3%	3%	3%	3%	3%

Kuncoro, 2017). The extract was first diluted with 0.5% Dimethyl Sulfoxide (DMSO, Sigma Aldrich D8418). The addition of the HSFI-9 extract and distilled water is carried out after the base components reach room temperature (Khan *et al*., 2015), then all components are homogenized by a vortex. The DMSO 0.5% was used to dissolve the dry concentrated extract of HSFI-9 before mixing with the base, because this concentration is known not to inhibit microbial growth (Liu *et al*., 2023).

Antimicrobial Assay of HSFI-9 Extract Ointment

The *S. aureus* and *C. albicans* wild-type test microbes were from the collection of the Microbiology Laboratory, Faculty of Medicine, Universitas Muhammadiyah Semarang. The antimicrobial assay was carried out using the Kirby Bauer disc diffusion test method. *Staphylococcus aureus* was grown on MHA, while *C. albicans* was grown on Potato Dextrose Agar media (PDA, Oxoid CM0139). A total of 25.0 mL of each sterile MHA and PDA was poured into disposable Petri dishes (90 x 15 mm) for an agar medium thickness of 4 mm. The streak plate technique was used to grow both microbes on each appropriate agar medium. The streak plate is carried out with a sterile cotton bud dipped in a microbial suspension according to the McFarland 0.5 standard which has been diluted to 1.5 x 10^6 CFU/mL (Alghamdi, 2022).

The HSFI-9 extract, ointment base, and antibiotic control ointment were applied to the blank disk in the amount of ± 40.0 mg. The antibiotic control ointment for *S. aureus* was 2% mupirocin (Etercon Company), and for *C. albicans* was 2% ketoconazole (Kimia Farma Company). The antimicrobial activity assay was replicated three times. Petri dishes were incubated for 24 hours at 37°C (Yagnik *et al*., 2018). Antimicrobial activity was interpreted by measuring the inhibition zone formed around the paper disc (Figure 1). Interpretation of the inhibition zone is classified into (a) weak category with an average inhibition zone diameter < 5 mm, (b) medium diameter 5 - 10 mm, (c) strong diameter 11-20 mm, and (4) very strong inhibition zone diameter > 20 mm (Ratu *et al*., 2019).



$$Inhibition\ Zone = \frac{(Dv - Dc) + (Dh - Dc)}{2}$$

Figure 1. Inhibition zone calculation formula (Harti, 2015) for determining the antimicrobial activity of HSFI-9 extract ointment.

Dosage Forms Evaluation of HSFI-9 Extract Ointment

Evaluation of ointment dosage forms carried out during storage includes organoleptic, homogeneity, spreadability, adhesion characteristic, and pH test (Lasut *et al*., 2019). Organoleptic tests are carried out visually on dosage forms by assessing the odor, color, and texture (Sari, 2016). The homogeneity determination test is carried out by applying ointment to a glass object and pressing it with another glass object. A good homogeneous ointment is characterized by the absence of lumps from the start point to the end point of the application (Sari, 2016). The ointment spreadability test was performed by weighing 10 mg of ointment and then applying a gradual load on two round glasses with a diameter of 15 cm for 1 minute. The constant diameter formed in the spreading force test is measured until the total load applied is 750 g. The average of all diameters of ointment spread was calculated (Putranti *et al*., 2019). The adhesion test was carried out by weighing 25 mg of ointment and then smearing it on two glass objects which were then attached until they merged. The attached glass object is then given a load of 1 kg for 5 minutes, and then given a release load of 100 g. The sticking time of the ointment is calculated by recording the time (in seconds) required for the two glass objects to be separated (Naibaho *et al*., 2013). The pH test was carried out using a digital pH meter (Ohaus AB33PH-F) by dipping the pH meter electrode into the ointment (Lasut *et al*., 2019).

Statistical Analysis

The results of organoleptic, homogeneity, the adhesion characteristic, and pH test are described univariately. Differences in the spreadability test results across groups were analyzed using One-Way ANOVA followed by the Duncan Test. Differences in the inhibition zone of the HSFI-9 extract, ointment base,

and the antibiotics control ointment were analyzed using the Kruskal Wallis test followed by Post hoc Mann Whitney-U.

Results and Discussion

Production of the HSFI-9 Extract Ointment

The growth pattern of *B. subtilis* subsp. *subtilis* HSFI-9 taken from glycerol stores until the extract produced from the culture process in SYP media did not experience changes and has been described in previous research (Rakhmawatie et al., 2023b). This shows the stability of the bacterial storage and the culture method used for the production of secondary metabolites. Ointment preparations containing extracts of various concentrations do not have differences in consistency and color when compared with the ointment- base mixture (Figure 2).

Antimicrobial Activity of HSFI-9 Extract Ointment

Ethyl acetate extract ointment of *B. subtilis* subsp. *subtilis* HSFI-9 has inhibitory activity against *S. aureus* and *C. albicans*, although the strength of the inhibition zone (weak to strong) depends on the extract concentration. Ethyl acetate was used in this study because it can extract semi-polar secondary metabolites, and its use was based on optimization in previous studies. Ointment base as a negative control can also cause weak inhibition zones, meanwhile, the positive control, 2% mupirocin, and 2% ketoconazole could very strongly inhibit the growth of *S. aureus* and *C. albicans*, respectively (Table 2).

The extract ointment with concentrations of 0.3% and 1% can strongly inhibit *S. aureus* growth, although it is still below the strength of 2% mupirocin ($p<0.05$).

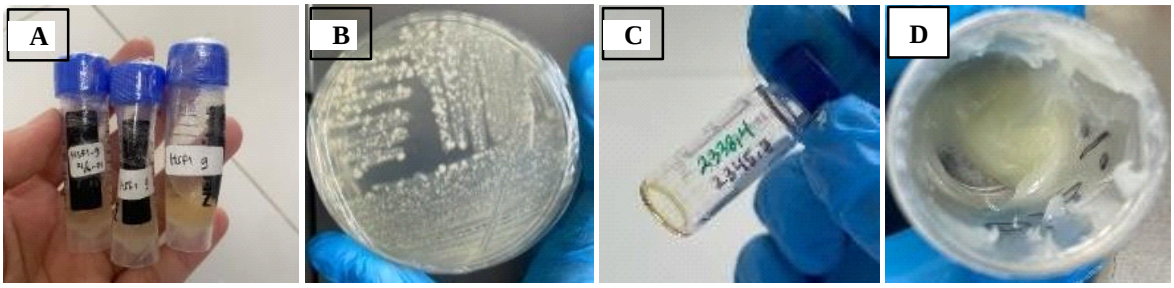


Figure 2. (A) Glycerol stores of *B. subtilis* subsp. *subtilis* HSFI-9, (B) Growth of HSFI-9 on MHA media, (C) Ethyl acetate extract of HSFI-9, (D) HSFI-9 extract ointment.

Table 2. Mean ± standard deviation of the inhibitory zone of the HSFI-9 extract ointment against *S. aureus* and *C. albicans*

Microorganism	Ointment Base	HSFI-9 Extract Ointment				Mupirocin 2%	Ketoconazole 2%
		0.003%	0.03%	0.3%	1%		
S. aureus	3.67 ± 0.57	7.33 ± 1.89^{a,b}	6.00 ± 0.57^{a,b}	11.67 ± 1.26*^{a,b}	15.67 ± 2.25*^{a,b}	30.67 ± 0.76	-
C. albicans	3.83 ± 0.28	4.60 ± 0.76^{b}	10.00 ± 1.52*^{a,b}	8.60 ± 1.25^{a,b}	12.50 ± 2.5^{a,b}	-	31.50 ± 1.00

Although the concentrations of 0.3% and 1% have significant differences, when considering the time needed for extract production, the extract ointment with a concentration of 0.3% can be declared more efficient for further development. Meanwhile, the extract ointment with a concentration of 0.03% can strongly inhibit the growth of *C. albicans*. In this study the positive control used was commercial antibiotic ointment. Topical antibiotics for commercial use have varying concentration ranges depending on the active ingredient, then comparing drug activity does not necessarily require using the same drug strength (Okunye *et al.*, 2020). The concentration of the selected HSFI-9 extract ointment for further development was also determined based on the HSFI-9 extraction process which requires quite a large cost and limited extract production capacity. The application of active substance concentration efficiency for ointment preparations can reduce the costs required for the next drug development steps, because different formulations can cause differences in raw material costs (Fauzi *et al.*, 2024).

The ability of the HSFI-9 extract ointment is caused by the presence of antibacterial secondary metabolite compounds. In previous research, the antibacterial compounds have been identified from the extract of *B. subtilis* subsp. *subtilis* HSFI-9 using Gas Chromatography-Mass Spectroscopy. It was stated that there were findings of compounds that could be responsible for being antibacterial and antifungal, namely 1-butanol 3-methyl formate which is alcohol derivative (Rakhmawatie *et al*., 2023a). Alcohol compounds are known to have an antimicrobial mechanism by damaging bacterial membranes and causing leaks cell contents (Saritha *et al*., 2015). *Bacillus* sp. is also known to produce peptide and polyketide compounds that actively inhibit bacterial growth through mechanisms that inhibit cell membrane formation and protein synthesis. *Bacillus* sp. can also produce triterpenoid and saponin compounds which react with porins on the outer membrane of the bacterial cell wall by forming complex bonds. The damage received by bacteria from these two compounds result in reduced cell wall permeability and inhibits bacterial growth (Rasyid *et al*., 2018).

Inhibition zones are also formed from ointment bases, but the inhibitory power is weak and significantly different from ointment bases containing extracts of various concentrations. The existence of an inhibition zone of the ointment base can be caused by the additional ingredients used such as propylene glycol, Butylated Hydroxytoluene (BHT), and benzoic acid. The addition of 15-30% of propylene glycol serves as an antimicrobial preservative, humectant, and stabilizer. Propylene glycol has no toxic effect and aims to minimize irritation to the skin (Nalawade *et al*., 2015; Tsabitah *et al*., 2020). Benzoic acid as an ointment preservative, has relatively low toxicity and antimicrobial properties (del Olmo *et al*., 2017). The BHT compound is a stabilizer that acts as an antioxidant, is easily soluble in water, and is non-toxic when used at low doses (Mladenovi *et al*., 2023).

In this study, *S. aureus* and *C. albicans* were categorized as sensitive to antibiotic control ointment. The 2 % mupirocin ointment has a mechanism of action that inhibits the action of enzymes needed by bacteria to make proteins (Dadashi *et al*., 2020). Meanwhile, the antifungal activity of 2% ketoconazole ointment is due its ability to inhibit fungal NADH oxidase activity at the mitochondrial level (Tao *et al*., 2022).

Dosage Forms Evaluation of the HSFI-9 Extract Ointment

The ointment base is a mixture of Vaseline alba and Adeps lanae which has a bright white color and the distinctive smell of lanolin. Other ingredients mixed in the ointment base produce a good semi-solid dosage form. A good organoleptic test result is a semi-solid texture, no color change, has a non-pungent odor, and dissolves well (Lumentut *et al*., 2020). The HSFI-9 extract ointment has color, shape, and odor that meets the organoleptic test requirements, except for the concentrations of 0.3% and 1% which produce a distinctive pungent odor of typical sweet ethyl acetate. The greater concentration of the extract, the more distinctive the ethyl acetate smell, that can be caused by solvent residue during the extraction process. Ethyl acetate or its hydrolysis product (acetic acid) can have antimicrobial activity at concentrations >5% for ethyl acetate (Lens *et al*., 2016) and >6% for acetic acid (Cortesia *et al*., 2014). The results of homogeneity showed that ointment in all concentrations was free of particles and lumps, making the smooth texture and meeting the criteria for good homogeneity (Auliafendri & Gee, 2023).

Spreadability and adhesion tests were carried out on ointment base, antibiotic control ointment, and extract ointment concentration of 0.03%. The assessment of spreadability and adhesiveness was only carried out at one concentration of the extract ointment preparation due to limited extract. The spreadability of ointment is considered good if it has an average diameter of 5-7 mm (Sawiji & Sukmadiani, 2021). The evaluation results show that the 0.03% extract ointment has good spreadability compared to the 2% mupirocin and ketoconazole ($p < 0.05$). The spreadability test determines the ability of the ointment to spread well on the skin (Badia *et al*., 2022). A good adhesion test evaluates the ointment's ability to stick for more than 4 seconds (Rajab *et al*., 2021). The HSFI-9 extract ointment had poor adhesion, although it was better than the mupirocin (Table 3). The adhesive test aims to assess the ability of the ointment to stick to the skin surface. The longer the adhesion of the ointment, the greater and optimal drug absorption due to the stronger engagement between the ointment and the skin (Lasut *et al*., 2019).

The pH test is used to determine whether the pH of the ointment is in the normal skin range of 4.5 – 7 and the mucosal range of 6.5 – 7.5 (Lasut *et al*., 2019; Ratu *et al*., 2019). The results of the pH test evaluation stated that the mupirocin, ketoconazole, and ointment base met the requirements for a good pH value. The pH test is used to ensure that ointment does not cause irritation and aggravate complaints on skin and mucosa infected with *S. aureus* and *C. albicans* (Pratimasari *et al*., 2015). The addition of the HSFI-9 extract at higher concentrations caused the pH of the ointment to become more acidic (Table 3), which was probably due to the acidic nature of the compounds in the HSFI-9 extract.

This research proves that the ethyl acetate extract ointment of *B. subtilis* subsp. *subtilis* HSFI-9 can inhibit the growth of *S. aureus* and *C. albicans*. The pH of topical preparations is important to maintain the balance of the stratum corneum. One study stated that pH 4 is actually safe for the skin (Lukic *et al*., 2021), but optimization of the increase in pH of HSFI-9 ointment can also be done for further research. The extract ointment is suitable for next step development into antibiotic ointments by optimizing its pH and improving the adhesion characteristic. The pH of the ointment can be optimized by adding an alkalizing agent such as triethanolamine which has been proven to increase the pH of cream (Sari *et al*., 2021) or lotion preparations based on Vaseline alba and Adeps lanae (Sehro *et al*., 2015). Another ingredient that can be used to optimize the increase in pH of the ointment is sodium bicarbonate (Gibson *et al*, 2023).

The development of pharmaceutical preparations mostly uses secondary metabolite active ingredients from plants. The presence of natural ingredients sourced from marine exploration will increase the development of blue cosmetics. Currently, research is being conducted on pharmaceutical ingredients from secondary metabolites of marine microorganisms such as bacteria, fungi, and algae for antioxidant, vitamin, anti-aging, moisturizing, and hair care activities (Alparslan *et al*., 2018; Gupta *et al*., 2019). Topical pharmaceutical products as antibacterials have not been widely studied, this research could initiate the development of topical antibacterial from natural marine ingredients. In this study, the mechanism of antimicrobial action of HSFI-9 ointment is not yet known, so it needs to be carried out in further research.

Table 3. Dosage forms evaluation results of the HSFI-9 extract ointment

Ointment	Dosage Forms Evaluation Parameters				
	Organoleptic (Color, Odor)	**Homogeneity** (Smooth Texture)	**Spreadability** (Mean ± SD in mm)	**Adhesion** (second)	**pH**
0.003% HSFI-9	Semi-solid, yellow, typical lanolin odor	Yes	ND	ND	3,79
0.03% HSFI-9	Semi-solid, yellow, typical lanolin odor	Yes	5,36 ± 0,36[a]	02:15	4,30
0.3% HSFI-9	Semi-solid, yellow, typical ethyl acetate odor	Yes	ND	ND	4,07
1% HSFI-9	Semi-solid, yellow, typical ethical acetate odor	Yes	ND	ND	4,25
2% Mupirocin	Semi-solid, white, no odor	Yes	3,91 ± 0,33	01:53	6,84
2% Ketoconazole	Semi-solid, white, no odor	Yes	4,78 ± 0,33	04:51	5,90
Ointment Base	Semi-solid, yellow, typical lanolin odor	Yes	5,22 ± 0,37	03:50	6,03

Note: [a]Significant compared to antibiotic control ointment; ND (Not Determined)

Conclusion

Ethyl acetate extract ointment of *B. subtilis* subsp. *subtilis* HSFI-9 can optimally inhibit the growth of *S. aureus* and *C. albicans* at a concentration of 0.3% and 0.03%, respectively. The ointment meets the evaluation of the physical requirements for the homogeneity and spreadability test. The organoleptic test revealed that extract ointment has a pungent odor typical of ethyl acetate and does not meet the requirements for good adhesion characteristics and pH tests. Furthermore, research can be carried out to optimize pH by adding alkalizing agent.

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