Antibacterial Activity Against Acne-Causing Bacteria and Compound Profiling of Bacteria Associated with the Black Gum Sea Cucumber (*Holothuria leucospilota*)

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Abstract

Sea cucumbers (Holothuria leucospilota) have significant potential as sources of antibacterial compounds. These organisms live in association with bacteria that produce secondary metabolites exhibiting biological activities similar to those of their hosts, including antibacterial effects. Acne is an inflammatory condition that can be exacerbated by acne-causing bacteria such as Staphylococcus epidermidis and Propionibacterium acnes. Although antibiotics are commonly used for treatment, their effectiveness has increasingly been compromised by the emergence of antibiotic resistance. Bacteria associated with H. leucospilota therefore represent a promising alternative source of antibacterial agents due to their ability to produce bioactive secondary metabolites. This study aimed to characterize the macroscopic and microscopic diversity of these bacteria, evaluate their antibacterial activity against S. epidermidis and P. acnes, and analyze the chemical profiles of their ethyl acetate fractions. Bacterial isolation was conducted using Sea Water Complete Agar (SWCA), and antibacterial activity was assessed using the TLC bioautography method with iodonitrotetrazolium chloride (INT) as a growth indicator. A total of 12 bacterial isolates were obtained, comprising 10 isolates from the internal tissues and 2 isolates from the external surface of H. leucospilota. Antibacterial screening showed that 11 of the 12 isolates were active against S. epidermidis, while 8 isolates exhibited activity against P. acnes. Phytochemical screening indicated that phenolic and terpenoid compounds were the bioactive constituents responsible for the observed antibacterial activity.

Keywords: *antiacne, antibacterial, H. leucospilota, marine bacteria, TLC bioautography.*

Introduction

Acne is a chronic inflammatory disorder of the pilosebaceous follicles, primarily driven by increased sebum production in the skin. In addition to sebum overproduction, the condition is often exacerbated by bacterial infection. Acne is commonly associated with the colonization of acne-related bacteria, particularly *Staphylococcus epidermidis* and *Propionibacterium acnes*. These microorganisms are part of the normal skin microbiota inhabiting pilosebaceous follicles, and their populations tend to proliferate in response to elevated sebum levels.

Antibiotics are commonly used to treat infections caused by these bacteria. However, inappropriate or excessive antibiotic use has contributed to the development of bacterial resistance. In Indonesia, resistance to methicillin, which is commonly used to treat *Staphylococcus* infections, was reported to be 38% in 2017. *Staphylococcus epidermidis* has also demonstrated resistance to several other antibiotics, including rifamycins, fluoroquinolones, gentamicin, tetracycline, chloramphenicol, clindamycin, and sulfonamides (Rogers *et al*., 2009). According to a study by Hindritiani *et al.* (2017), *Propionibacterium acnes* showed resistance to clindamycin (43%), erythromycin (32%), minocycline (23%), tetracycline (16%), and doxycycline (<10%).

Sea cucumbers represent a promising natural source of bioactive compounds with pharmaceutical and cosmetic potential, particularly due to their demonstrated antibacterial properties, including anti-acne activity. The bioactive compound composition of sea cucumbers can vary across different seas due to differences in nutrient availability and the ecological conditions of their habitats. (Gao *et al*., 2014). However, the growing demand for sea cucumbers has led to unsustainable harvesting and population decline, resulting in limited availability and increased market value.

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The scarcity of sea cucumbers has prompted efforts to identify alternative sources with comparable bioactive properties. Sea cucumbers are known to live in association with a wide range of microorganisms, including bacteria and fungi, which inhabit their environment as a result of the cucumbers' feeding habits—either through ingestion of marine sediments or filtration of seawater. These interactions create favorable conditions for the development of diverse microbial communities (Gao *et al*., 2014). Associated bacteria are currently being explored for their ability to produce secondary metabolites with antibacterial activity, potentially serving as substitutes for those produced by their sea cucumber hosts (Yuliana, 2014). These bacteria offer practical advantages for utilization, as their shorter life cycles compared to their hosts can significantly reduce the production time of bioactive compounds in large-scale applications (Janatiningrum *et al*., 2021). Given the diversity of associated bacteria, there is a corresponding diversity in the secondary metabolites they produce. Therefore, comprehensive investigations at both macroscopic and microscopic levels, along with analyses of the secondary metabolites they contain, are essential to establish a basis for understanding their antibacterial activity.

Research by Ardiansyah *et al*. (2021) reported that *Holothuria* sp. exhibits antibacterial activity against three acne-causing bacteria: *Staphylococcus aureus*, *S. epidermidis*, and *P. acnes*. Sea cucumbers from Bastiong Island, Maluku are known to contain a variety of bioactive compounds, including alkaloids, saponins, flavonoids, steroids, and triterpenoids (Pringgenies, 2010). Because differences in the habitat of sea cucumbers can lead to variations in their compound composition and biological activity, this study aims to examine the chemical constituents and antibacterial activity of *H. leucospilota* from Pramuka Island, and to determine whether they exhibit antibacterial activity and compound profiles similar to those of sea cucumbers inhabiting other marine environments.

Accordingly, the present study aims to identify the macroscopic and microscopic diversity of bacteria associated with the black gum sea cucumber (*H. leucospilota*) from Pramuka Island, evaluate the antibacterial activity of ethyl acetate extracts from these bacteria against *S. epidermidis* and *P. acnes*, and characterize the chemical profiles of these extracts using thin-layer chromatography (TLC).

Materials and Methods

Sample collection and identification

Specimens of the sea cucumber *H. leucospilota* were collected from the waters surrounding Pramuka Island, Seribu Islands, DKI Jakarta, Indonesia, at depths of approximately 5–20 m on August 15, 2023. The collected specimens were placed in sterile plastic bags with ice to minimize stress during transport. Taxonomic identification of *H. leucospilota* was performed at the Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. Wet-preserved samples in 70% ethanol were submitted to confirm species identification.

Isolation of bacteria associated with sea cucumbers

The sea cucumbers were rinsed with seawater, and their skin, flesh, and internal organs were separated. The skin was classified as the external part, while the flesh and internal organs were grouped as the internal part. Each processed portion (1 g) was serially diluted in 9 ml of physiological saline to obtain concentrations ranging from 10^{-2} to 10^{-8}. From each dilution, 0.1 ml was evenly spread onto Sea Water Complete (SWC) agar plates using the spread plate method. The plates were incubated at 25–30°C for 7–30 d. Colonies that appeared were subsequently purified by streaking onto Sea Water Complete Agar (SWCA) plates using the four-quadrant method (Vishwakarma, 2017).

Macroscopic and microscopic characterization

After 24 h of incubation on SWCA, bacterial colonies were characterized macroscopically based on shape, margin, elevation, and color (Zuraidah *et al*., 2020). Microscopic examination was performed using Gram staining to determine cell morphology and Gram reaction. Gram-positive bacteria appeared purplish blue, whereas Gram-negative bacteria appeared red under microscopic observation (Wulandari and Purwaningsih, 2019).

Fermentation and extraction of bacterial isolates

A loopful of each bacterial isolate was inoculated into 5 ml of Sea Water Complete Broth (SWCB) in an Erlenmeyer flask and incubated at 28°C for 24 h. The culture was subsequently transferred into 200 ml of SWCB and incubated under the same conditions. Extraction was carried out using the sonication method with ethyl acetate at a 1:1 (v/v) ratio. The resulting mixture was separated using a separatory funnel to obtain an ethyl acetate fraction and an aqueous fraction. The ethyl acetate fraction was then concentrated under vacuum using a rotary evaporator at 28–30°C.

Antibacterial screening

Pathogenic bacteria (*S. epidermidis* and *P. acnes*) were standardized to a turbidity equivalent to McFarland 0.5 (absorbance range 0.08–0.13, corresponding to $1–2 \times 10^{8}$ CFU.ml^{-1}) using a UV–Vis spectrophotometer. Ten microliters of bacterial extract (10 mg.ml^{-1}) were spotted onto silica gel GF_{254} TLC plates. After drying, the plates were immersed in suspensions of the test bacteria and incubated at 37°C for 18–24 h. Following incubation, the plates were sprayed with iodonitrotetrazolium chloride (INT, 4 mg.ml^{-1}). The presence of a clear inhibition zone indicated antibacterial activity against the respective bacteria (Praptiwi *et al*., 2019). Erythromycin (100 mg.ml^{-1}) for *S. epidermidis* and doxycycline (100 mg.ml^{-1}) for *P. acnes* were used as positive controls, while ethyl acetate served as the negative control. The length and clarity of inhibition zones were measured, and all assays were performed in triplicate. The isolate exhibiting the largest and clearest inhibition zone was selected for further analysis.

Separation and phytochemical screening by Thin Layer Chromatography

The ethyl acetate fraction of the selected isolate was applied to a silica gel GF_{254} TLC plate and developed using an n-hexane:ethyl acetate solvent system (7:3, v/v). The separated spots were visualized under UV light at 254 nm and 366 nm. The plate was subsequently sprayed with ferric chloride ($FeCl_3$) and vanillin–sulfuric acid reagents to detect phenolic and terpenoid compounds, respectively, which are commonly associated with antibacterial activity (Mulyndin and Kovalev, 2001).

Results and Discussion

Sample collection and identification

Sample identification involved comparing the collected specimens with reference material of known species to ensure accurate taxonomic classification. During transport, the specimens were kept on ice to minimize stress, as evidenced by their normal movement and responsiveness to tactile stimulation. This approach maintained an environmental temperature close to their natural seawater habitat, where average sea surface temperatures in Indonesian waters range from 28–29°C. Identification occurred at the Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. Samples were preserved in 70% ethanol to prevent microbial decay. Based on these results, the specimens belonged to the family Holothuriidae, genus *Holothuria*, species *H. leucospilota*—commonly known as the black gum sea cucumber (Figure 1).

Isolation of bacteria associated with sea cucumbers

Bacteria were isolated from both the internal and external parts of *H. leucospilota*. The internal parts included the stomach, intestine, Cuvierian tubules, and body wall, while the external part consisted of the skin. This separation followed previous findings that bioactive compound distribution varies across sea cucumber body parts (Farjami *et al*., 2013).

Using SWCA medium, we obtained 10 isolates from the internal parts (coded WI-1 to WI-10) and 2 from the external parts (coded WE-1 and WE-2) (Figure 2). Previous studies have reported greater bacterial diversity in the internal versus external parts of *H. leucospilota* from Indonesian waters (Wibowo *et al*., 2019).

Macroscopic and microscopic characterization

Table 1 summarizes the morphological characteristics of bacterial isolates. All isolates formed circular colonies with convex elevation and entire margins. Macroscopic characterization involved observing colonies grown on SWCA medium for 3–7 d, assessing shape, elevation, margin, and color. As shown in Table 1, all 10 internal isolates (WI-1 to WI-10) and 2 external isolates (WE-1 and WE-2) shared similar traits: circular shape, convex elevation, and entire margins. Several isolates also displayed comparable colony colors (Figure 2).

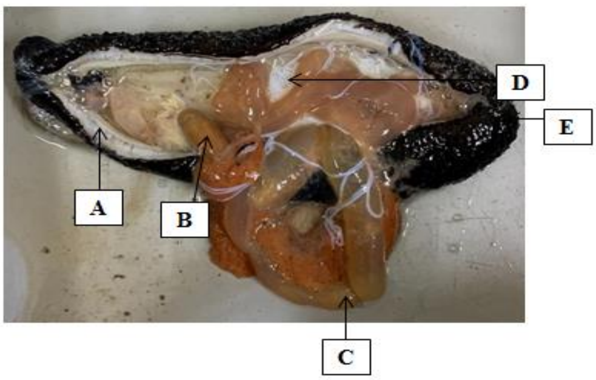

Figure 1. *Holothuria leucospilota*: (A) Body Wall; (B) Stomach; (C) Intestine; (D) Cuvierian tubules; (E) Skin

Table 1. Morphological Characterization of Bacteria Associated with *H. leucospilota*

Sea Cucumber Parts Taken	Bacterial Code	Shape	Elevation	Margin	Color	Pigmen-tation	Gram Staining	Gram Type	Cell Shape
Internal	WI-1	Circular	Convex	Entire	Brownish white	No	Purple	+	*Coccus*
	WI-2	Circular	Convex	Entire	Pink	Yes	Purple	+	*Coccus*
	WI-3	Circular	Convex	Entire	Dark red	Yes	Purple	+	*Coccus*
	WI-4	Circular	Convex	Entire	Pink	Yes	Purple	+	*Coccus*
	WI-5	Circular	Convex	Entire	Pink	Yes	Purple	+	*Coccus*
	WI-6	Circular	Convex	Entire	Brown	No	Purple	+	*Coccus*
	WI-7	Circular	Convex	Entire	Brownish white	No	Red	-	*Coccus*
	WI-8	Circular	Convex	Entire	Pink	Yes	Purple	+	*Coccus*
	WI-9	Circular	Convex	Entire	Brown	No	Red	-	*Coccus*
	WI-10	Circular	Convex	Entire	Red	Yes	Red	-	*Coccus*
External	WE-1	Circular	Convex	Entire	Brown	No	Red	-	*Coccus*
	WE-2	Circular	Convex	Entire	Brownish white	No	Red	-	*Coccus*

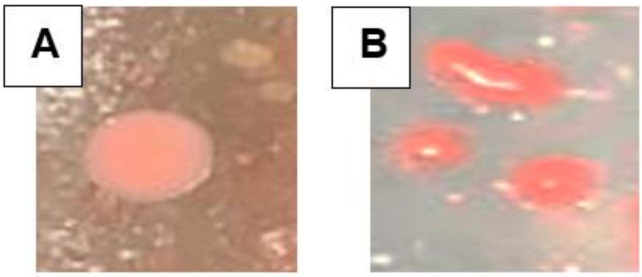

Figure 2. Macroscopic Bacterial Isolates WI-5 and WI-10 from Bacteria Associated with *H. leucospilota:* (A) WI-5; (B) WI-10

Some colonies produced pigments. Initially, all appeared white-brown to brown, but after three days, certain isolates developed pink, red, or dark red pigmentation. Bacterial pigment production serves as an adaptive response to environmental stressors like light exposure, temperature fluctuations, oxidative stress, and heavy metals (Wang *et al*., 2019). Common red pigments include melanin, anthraquinones, carotenoids, astaxanthin, canthaxanthin, prodigiosin, lycopene, and β-carotene (Venil *et al*., 2013). The red coloration in these sea cucumber-associated bacteria likely stems from one or more of these compounds.

Microscopic examination under a light microscope at 100× magnification after Gram staining (Figure 3) classified 58.3% (7 isolates) as Gram-positive and 41.7% (5 isolates) as Gram-negative. This also revealed bacterial morphology, which macroscopic analysis cannot detect—all isolates from both internal and external parts were cocci (spherical). Previous studies support this: Kamarudin and Maryam (2018) identified Bacillus, Brevibacillus, Lysinibacillus, Staphylococcus, Dermacoccus, and Micrococcus in the gut of *H. leucospilota* from Teluk Nipah, Malaysia. Similarly, Lukman *et al*. (2014) isolated Bacillus, Exiguobacterium, Pseudomonas, Stenotrophomonas, and Vibrio from its coelomic fluid at the same site.

Antibacterial screening

Antibacterial activity was assessed using TLC bioautography with iodonitrotetrazolium chloride (INT) as a microbial growth indicator. Clear inhibition zones on TLC plates signified antibacterial effects (Praptiwi *et al*., 2023). As shown in Table 2, 11 of 12 ethyl acetate fractions from *H. leucospilota*-associated bacterial isolates inhibited *S. epidermidis*, while 8 showed activity against *P. acnes*. Since both are Gram-positive bacteria, this likely does not involve cell wall synthesis inhibition. Sea cucumbers produce phenoloxidase, an oxidative immune enzyme that catalyzes phenolic substrates into quinones and ultimately melanin. These compounds and their intermediates disrupt bacterial membranes, exerting agglutinative and bactericidal effects (Jiang *et al*., 2014).

Positive activity appeared as inhibition zones surrounded by a purple background after INT spraying (Figures 4 and 5). The purple hue arises from

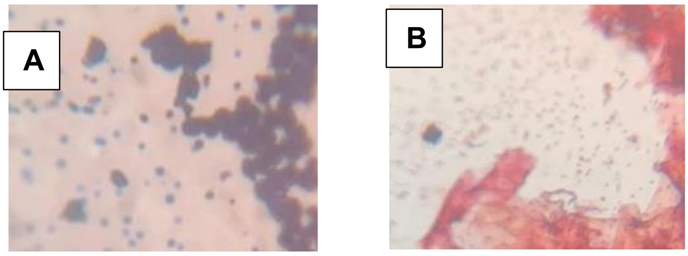

Figure 3. Microscopic Bacterial Isolates WI-5 and WI-10 from Bacteria Associated with *H. leucospilota* at 100× magnification: (A) WI-5; (B) WI-10

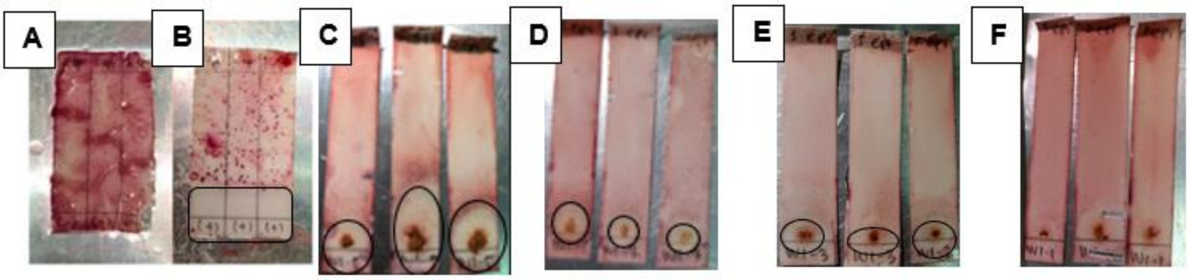

Figure 4. Antibacterial Screening Against *S. epidermidis:* (A) Negative Control; (B) Positive Control; (C) Strong Antibacterial Isolate; (D) Moderate Antibacterial Isolate; (E) Weak Antibacterial Isolate; (F) Not Have Antibacterial Activity

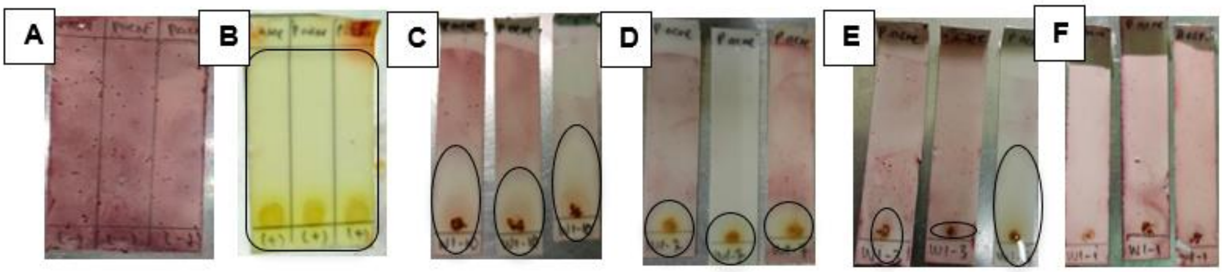

Figure 5. Antibacterial Screening Against *P. acnes:* (A) Negative Control; (B) Positive Control; (C) Very Strong Antibacterial Isolate; (D) Moderate Antibacterial Isolate; (E) Weak Antibacterial Isolate; (F) Not Have Antibacterial Activity

tetrazolium salts reduced to formazan by dehydrogenase enzymes in viable cells (Villegas-Mendoza *et al*., 2019). Formazan production rises with bacterial growth (Cretu and Morlock, 2014), but antibacterial compounds prevent this reduction, creating white inhibition zones (Aslam *et al*., 2018).

Among the isolates, WI-5 yielded the largest average zone against *S. epidermidis* (12 mm), though faint purple spots suggested partial survival. WI-10 produced the largest, cleanest zone against *P. acnes* (17 mm) with no spots, indicating superior potency. WI-5 and WI-10 were thus selected for further compound separation and TLC-based chemical profiling.

Compound separation and phytochemical screening

Thin-layer chromatography (TLC) separated compounds and revealed chromatographic profiles for isolates WI-5 and WI-10 using silica gel GF254 plates as the polar stationary phase. The mobile phase was an n-hexane:ethyl acetate mixture (7:3, v/v), chosen for its nonpolar nature, which aligns with reports that *H. leucospilota* bioactive compounds are predominantly nonpolar (Mulyndin and Kovalev, 2001).

Optimal retention factors (Rf) for separation range from 0.2–0.8; values below indicate poor migration, while those above suggest excessive movement (Samosir *et al*., 2018). As shown in Table 3, under UV light at 366 nm, WI-5 showed one fluorescent spot (Rf 0.33) and WI-10 one (Rf 0.30) (Figure 6). Fluorescence at this wavelength signals compounds with extended conjugated double bonds (Ambarwati *et al*., 2015).

Phytochemical tests confirmed the WI-5 spot positive for phenolics (blackish-blue after $FeCl_3$

Table 2. Antibacterial Screening Against *S. epidermidis* and *P. acnes*

Sea Cucumber Parts Taken	Bacterial Code	*S. epidermidis*		*P. acnes*	
		Antibacterial Activity	Estimated Length of Inhibition Zone (mm)	Antibacterial Activity	Estimated Length of Inhibition Zone (mm)
Positive Control		++++	12	++++	64.6
Internal	WI-1	-	0	-	0
	WI-2	++	7.3	++	6.7
	WI-3	+	6.7	+	6.3
	WI-4	+	6.3	-	0
	WI-5	+++	12	++++	15.3
	WI-6	+	8	++	9
	WI-7	++	5	++	7.3
	WI-8	+	9.6	++	10
	WI-9	++	5.6	++	6.7
	WI-10	+	7	++++	17
External	WE-1	+	5	-	0
	WE-2	+++	10.6	-	0

Notes : (-) = negative ; (+) = weakly positive for antibacterial with an inhibition zone length of 1-10 mm and there are still purple spots in the inhibition zone ; (++) = moderate positive for antibacterial with an inhibition zone length of 1-10 mm and no purple spots in the inhibition zone ; (+++) = strong positive for antibacterial with an inhibition zone length of > 10 mm and there are still purple spots in the inhibition zone ; (++++) = very strong positive for antibacterial with an inhibition zone length of > 10 mm and no purple spots in the inhibition zone

Table 3. Bioautography TLC Phytochemical Screening

Bacterial Code	Rf value at UV 254 nm	Rf value at UV 366 nm	$FeCl_3$	Vanilin - Sulfate
WI-5	0.33 (spot A)	0.33 (spot A)	+ (phenolic positive)	-
	0.69 (spot B)	-	+ (phenolic positive)	-
WI-10	0.3 (spot C)	0.3 (spot C)	-	+ (terpenoid positive)

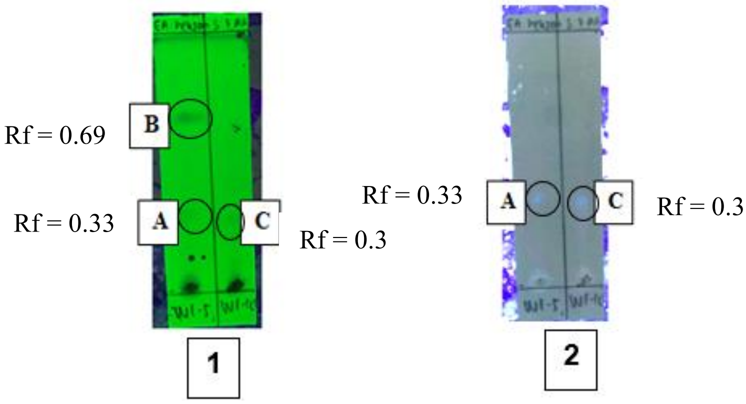

Figure 6. Chromatogram Observation: (1) At UV 254 nm ; (2) at UV 366 nm ; (A) Spot A ; (B) Spot B ; (C) Spot C

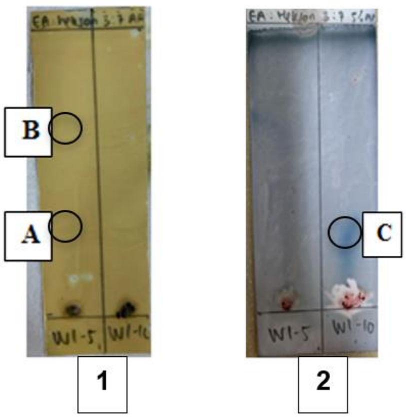

Figure 7. Phytochemical Screening of Separated Fractions with TLC: (1) Test for the presence of phenolics by spraying with $FeCl_3$; (2) Test for the presence of terpenoids by spraying with vanillin-sulfate; (A) Spot A; (B) Spot B; (C) Spot C

spraying) and the WI-10 spot for terpenoids (blue after vanillin–sulfuric acid spraying and 100°C heating) (Figure 7). These findings link WI-5's antibacterial activity to phenolics and WI-10's to terpenoids (Han *et al*., 2010). Phenolics, abundant in nature and *H. leucospilota* (Ceesay *et al*., 2019), drive activity in plants like *Rhanterium adpressum* (Boussoussa *et al*., 2016) and *Scirpus holoschoenus* against *S. aureus* and *B. subtilis* (Oussaid *et al*., 2017); here, 2,4-bis(1,1-dimethyl ethyl)-phenol was identified (Ceesay *et al*., 2019).

Beyond antibacterial effects, phenolics offer antioxidant and anti-inflammatory benefits by curbing lipid peroxidation and DNA damage (Martins *et al*., 2016; Velmurugan *et al*., 2018). Terpenoids provide antibacterial, anti-inflammatory, antitumor, and antiviral properties (Yang *et al*., 2020). Thus, WI-5 and WI-10 hold promise as sources of bioactive compounds for skincare, especially acne treatments.

Conclusion

Twelve bacterial isolates were successfully obtained from *H. leucospilota*—10 from internal parts and 2 from external parts—with 7 Gram-positive and 5 Gram-negative, all exhibiting coccus morphology. TLC bioautography screening revealed that 11 isolates inhibited *S. epidermidis* and 8 inhibited *P. acnes*. Further TLC analysis of selected ethyl acetate fractions (WI-5 and WI-10) under UV light at 254 and 366 nm showed two spots in WI-5 (Rf 0.33 and 0.69) and one in WI-10 (Rf 0.30), identified as phenolics and terpenoids, respectively. These findings attribute the antibacterial activity of WI-5 and WI-10 to phenolic and terpenoid compounds, underscoring their potential for pharmaceutical or cosmetic applications.

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