Antibacterial effectiveness of hemolymph-chitosan from cockroach (*Periplaneta americana*) and bandotan leaf (*Ageratum conyzoides* L.) extract

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Abstract. The effectiveness of commonly used antibacterial is increasingly challenged by the global rise in antimicrobial resistance (AMR). Natural resources, such as plants and insects, offer promising potential as novel antibacterial agents. This study aimed to assess the antibacterial effectiveness of the combined extract of hemolymph-chitosan from cockroaches *(Periplaneta americana)* and extract of bandotan leaves (*Ageratum conyzoides* L.), as well as to determine the optimal gel formulation for antibacterial topical application of these ingredients. The methodology included extraction of materials, characterization of cockroach-derived chitosan via Fourier-transform infrared spectroscopy (FTIR), phytochemical screening of bandotan leaves, formulation of gels with three different ingredient concentrations, and *in vitro* antibacterial activity testing. FTIR confirmed the characterization of cockroach chitosan. The presence of flavonoids and alkaloids in bandotan leaves was verified through phytochemical analysis. *In vitro*, antibacterial activity tests showed that all gel formulations displayed moderate antibacterial inhibition properties. This study demonstrates the potential of hemolymph-chitosan derived from cockroaches and extracts from bandotan leaves as effective antibacterial agents. Further research, including optimization of gel formulations and *in vivo* studies, is recommended to validate and enhance the efficacy of these natural antimicrobials.

1 Introduction

The rise in antimicrobial resistance (AMR) has increasingly challenged the effectiveness of commonly used antibacterial. Open wounds are particularly vulnerable to secondary

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infections, with half of these infections showing resistance to antimicrobials [1]. Common multidrug-resistant microorganisms identified from wounds include *Proteus mirabilis* (21%), *Staphylococcus aureus* (19%), and *Escherichia coli* (13%) [1]. Although beta-lactam antimicrobials are frequently used to treat bacterial infection, their widespread usage has caused bacteria like *Klebsiella pneumoniae* and *E. coli* to become resistant to them [1, 2]. This highlights the need for natural antimicrobial alternatives, like from plants and insects.

Research on natural products has made considerable advancements in uncovering new compounds with antimicrobial properties. Plants possess enzymes and secondary metabolites as a source of new antimicrobial compounds [3]. Some plants have been traditionally utilized for wound healing, for instance *Achillea millefolium* roots contain flavonoids which aid in treating superficial wounds. Similarly, *Avena sativa* fruit is used to alleviate mild inflammatory skin symptoms and support the healing of minor wounds [4]. Insects are another potential source of antimicrobial agents because they contain bioactive substances including chitin biopolymers, lauric acid, and defensins [5]. The antimicrobial potential of plants and insects is highly promising and may also exist in various species [4, 5].

The American cockroach (*Periplaneta americana*) is a species that is found in temperate, tropical, and subtropical climates worldwide [5]. Cockroaches produce antibacterial substances as part of their defense mechanism, enabling them to thrive in unsanitary environments [6]. Their exoskeletons contain chitosan, which can promote tissue regeneration and minimize the risk of wound infection [7]. Furthermore, the addition of chitosan to feed has been found to improve digestibility in ruminants by altering the microbial population structure [8]. Chitosan exhibits greater activity than chitin due to its higher degree of acetylation [8].

Open wounds are prone to friction, which can lead to rebleeding. To prevent recurrent bleeding, medications containing hemostatic agents are often used. Hemostatic substances such as alkaloids, flavonoids, saponins, and terpenoids are abundant in bandotan leaves (*Ageratum conyzoides* L.) [3, 9]. These leaves are traditionally utilized for wound healing due to their anti-inflammatory properties [9]. Additionally, bandotan leaf extract has been reported to exhibit antibacterial and antifungal activity *in vitro* [3, 9].

The combination of American cockroach hemolymph and chitosan (HC) extract with bandotan leaves extract has promising potential as a novel antimicrobial agent. However, according to literature reviews, no studies have specifically explored the effectiveness of this combination as an antibacterial agent for some bacteria. This research is significant in the field of animal biomedicine, as it investigates the properties of cockroach HC extract and bandotan leaves against bacteria, such as *Staphylococcus aureus* and *Escherichia coli,* and aims to identify the optimal formulation for therapeutic applications.

2 Methods

2.1 Time and place of research

This study was carried out from May to August 2024. The study was conducted in the Pharmacy Laboratory, the Bacteriology Laboratory of the School of Veterinary Medicine and Biomedicine (SVMBS), and the Biochemistry Laboratory of the Faculty of Mathematics and Natural Sciences, IPB University, Indonesia.

2.2 Materials and tools

The materials used in this research include American cockroaches (*P. americana*) obtained from the Unit of Pest Control Study of SVMBS IPB, bandotan leaves (*Ageratum conyzoides*

L.) obtained from the All Herbal Bogor store, 70% ethanol, Whatman 40 filter paper, 10 mM phosphate buffer pH 6.9, KBr, Mg powder, 1M HCl, Dragendorff reagent, 0.9% NaCl, Mueller Hinton Agar (MHA), Tryptone Soya Agar (TSA), Octadin® gel, pure cultures of *S. aureus*, *E. coli*, and *Micrococcus* sp., acquired from Bacteriology Laboratory SVMBS IPB University. The tools used in this research include NT-S10 1-10 µL micropipette, heparin tube, oven, blender, Mesh 20, Mesh 50, rotary vacuum evaporator, spectrophotometer, fourier transform infrared (FTIR), and gel container.

2.3 Cockroach hemolymph-chitosan and bandotan leaf extraction

Hemolymph extraction is performed by cutting the cockroach's antennae and extremities. The collected hemolymph is stored in a heparin tube to prevent clotting. The cockroaches were dried in an oven (50°C, 24 hours), grounded using a blender, and filtered through a 20-mesh sieve. The transformation of chitin into chitosan involves demineralization and deproteinization processes [7]. The extracted chitosan appears as a black powder [7].

Bandotan leaves were thoroughly washed then dried in an oven (50°C, 24 hours). A 50-mesh sieve was then used to mix and sift the dry leaves. A total of 50 grams of bandotan leaves powder was macerated in 70% ethanol for three days. A rotating vacuum evaporator was used to concentrate the resultant filtrate until a thick, dark green extract was created [10].

2.4 Cockroach hemolymph lysozyme activity test

A 100 µL sample of hemolymph was combined with 10 mM phosphate buffer (pH 6.9) and *Micrococcus sp.* in a microplate and thoroughly mixed. A blank solution was prepared by mixing 100 µL of a bacterial culture combined with 100 µL of phosphate buffer at a concentration of 10^7-10^8 CFU/mL. The reduction in *Micrococcus sp.* cell count was assessed using a spectrophotometer (wavelength 450 nm) and measured every minute up to 9 minutes. Lysozyme activity as an antibacterial was calculated by dividing the total measurement duration by the difference between the starting and final absorbance. A decrease in cell absorbance indicated the antibacterial activity of the hemolymph [11].

2.5 Characterization of cockroach chitosan

Cockroach chitosan powder was combined with KBr and analyzed using Fourier Transform Infrared (FTIR) spectroscopy. The mixture was formed into pellets and placed in a Tensor 37 FTIR spectrophotometer to identify functional groups within the wavelength range of 3500-500 cm^{-1} [7]. The functional groups present in the cockroach chitosan were compared to those found in commercial chitosan [7].

2.6 Phytochemical screening test of bandotan leaves

The flavonoid test and alkaloid test was performed using published methods [10]. For the flavonoid test, 1 g of bandotan leaves extract was dissolved with 2 ml 70% ethanol, followed by addition of Mg and HCl. The development of a red or orange hue suggested the presence of flavonoids [10]. One gram of leaf extract was dissolved in 3 ml 70% ethanol, and two ml of this solution were combined with 2 ml of concentrated HCl and three drops of

Dragendorff's reagent in order to test for alkaloids. The presence of alkaloids was indicated by an orange precipitate [10].

2.7 Gel formulation

The gel base was prepared by dispersing Carbopol 940 into 30 mL of water at 70°C and stirring until the gel formed. Triethanolamine (TEA) was added gradually while stirring. Separately, methyl paraben was dissolved in propylene glycol until completely mixed, and the resulting solution was added slowly to the gel base with continuous stirring. The base was then divided into four containers, each containing 25 g, and the active ingredients were added in proportions as specified in Table 1. The cockroach HC and bandotan leaves extract were incorporated into gel formulations at varying concentrations and stirred until a homogeneous mixture was obtained.

Table 1. Gel formula composition of cockroach hemolymph-chitosan and bandotan leaves extract

Formula	Hemolymph (µL/g)	Chitosan (mg/g)	Bandotan leaf (mg/g)
F1	1.32	10	10
F2	1.32	6.68	13.32
F3	1.32	7.72	3.86

2.8 *In vitro* antibacterial activity test

The well diffusion method was used to conduct the antimicrobial test [7, 12]. *Staphylococcus aureus* and *Escherichia coli* bacteria from TSA media were diluted with a 0.9% NaCl solution until the turbidity reached 0.5 McFarland 1 (10^7-10^8 CFU/mL) [7, 12]. The bacterial suspension (100 µL) was then spread onto MHA media using a spreader [12]. Five wells, each 5 mm in diameter, were created in the media. Each well was filled with gel of the three tested formulas (Table 1), Octadin gel® as a positive control, and the gel base as the negative control. Incubation was conducted at 37°C for 24 hours aerobically [12]. Clear zone around the well indicating bacterial growth inhibition was measured [7, 12].

3 Results and discussion

3.1 Extraction of hemolymph, chitosan, and bandotan

Hemolymph was collected from 80 cockroaches, yielding 0.3 mL of a dark yellow liquid (Figure 1A). After the decalcification and deproteinization process, 24.8 g of dried cockroach powder was obtained from the 80 cockroaches. From this, 0.61 g of shiny brown chitosan (2.4%) was extracted (Figure 1B). This yield is lower than the 5.8% chitosan yield reported in a previous study [7], which extracted chitosan from 3 g of dried *Periplaneta americana*. The reduced yield observed in the present study is likely due to prolonged deacetylation (over three hours) and fluctuations in oven temperature during the drying process, both of which impacted the final extract quantity.

The extraction of 1.5 kg of fresh bandotan leaves produced a 2.8% yield of dark brown extract (Figure 1C). Elevated temperatures are known to degrade key bioactive compounds

in bandotan leaves, including flavonoids and alkaloids. Specifically, temperatures above 50°C cause significant flavonoid degradation, with complete loss occurring at 54°C [13].

Fig. 1. Extraction results of (A) hemolymph; (B) chitosan; (C) bandotan leaves.

3.2 Hemolymph lysozyme activity test

The lysozyme activity of hemolymph exhibited a progressive reduction in absorbance, reaching zero after 5.5 minutes (Figure 2). The observed decline in absorbance in the hemolymph-treated sample indicates a reduction in the number of *Micrococcus* sp. cells, resulting from lysozyme-induced bacterial cell wall degradation. Spectrophotometric analysis revealed that over a 9-minute period, cell absorbance decreased by 0.078, corresponding to an enzymatic activity of 8.6 units per minute (Figure 2). This reduction in absorbance confirms the presence and functionality of lysozyme in cockroach hemolymph. Lysozyme is an enzyme that hydrolyzes peptidoglycan of *Micrococcus* sp., particularly the β-1,4-glycosidic linkages between N-acetyl glucosamine and N-acetyl muramic acid [11]. Lysozyme breaks down bacterial cell walls, leading to bacterial cell death. Another study reported that 1.21 mg/ml hemolymph demonstrated antibacterial effects against *M. luteus* and *E. coli* [6].

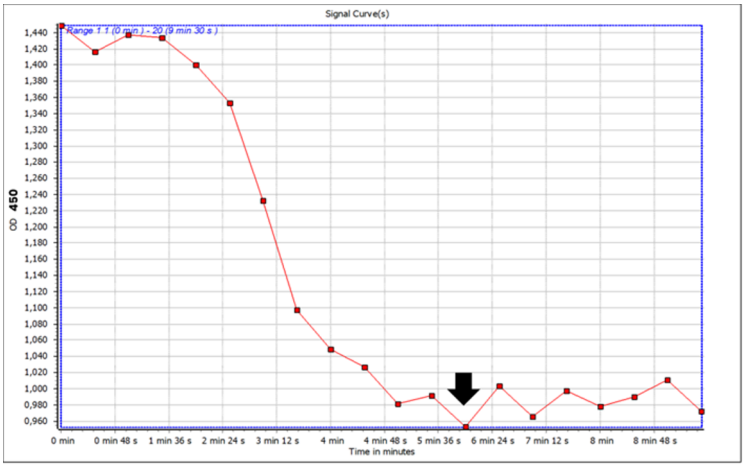

Fig. 2. Decrease of hemolymph absorbance.

3.3 Chitosan characterization test

The Fourier-transform infrared (FTIR) analysis of cockroach-derived chitosan showed functional group characteristics consistent with those of commercial chitosan [5, 7]. The results confirmed that the wave numbers for key functional groups in cockroach chitosan fell

within the standard ranges for commercial chitosan (Figure 3). The OH group in cockroach chitosan was observed at 2922.83 cm^{-1}, within the commercial range of 2900–3250 cm^{-1}. The amine group (-NH_2) appeared at 1374.75 cm^{-1}, also within the commercial range of 1370–1400 cm^{-1}. These functional groups are critical markers of chitosan compounds [7]. The alignment of these spectral characteristics confirms that the composition of cockroach-derived chitosan is similar to that of commercial chitosan.

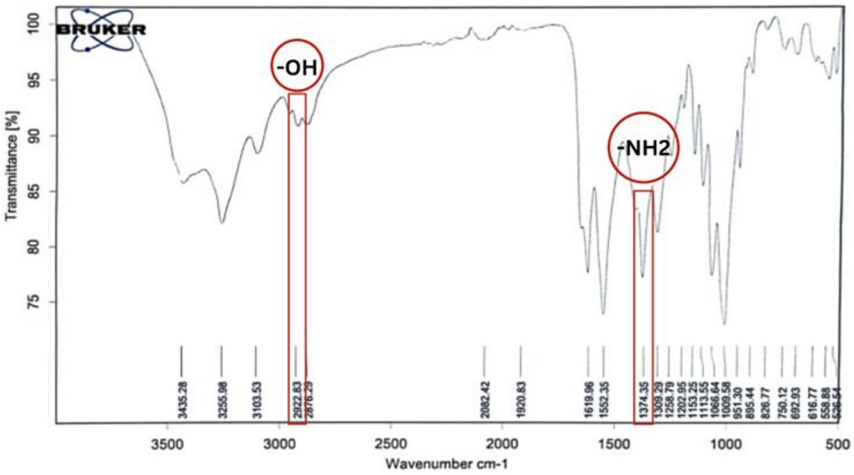

Fig. 3. Results of FTIR analysis of cockroach chitosan.

3.4 Phytochemical test of Bandotan leaves

The phytochemical test for alkaloids in bandotan leaf extract produced favorable outcomes, as evidenced by the sample turning yellow. Similarly, the test for flavonoids produced a yellow-orange precipitate, confirming their presence (Figure 4). These results are consistent with a prior study [10] that found a color change to orange in phytochemical tests signifies the presence of alkaloids and flavonoids. Flavonoids act as hemostatic agents by increasing platelet production to stop bleeding, while alkaloids help inhibit prostaglandin formation by releasing histamine [10], therefore these components are essential for wound healing.

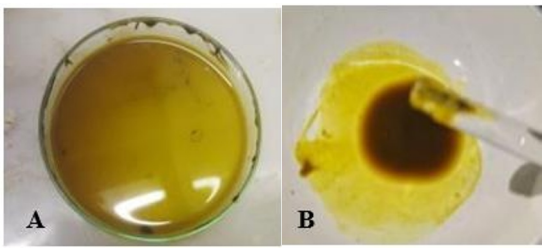

Fig. 4. Results of the alkaloid (A) and flavonoid (B) tests on bandotan leaves extract.

3.5 Gel formulation

The gel containing HC cockroach extract and bandotan leaves extract exhibits a semi-solid consistency, green color, fine brown chitosan granules, a distinctive bandotan leaf-like aroma, and a smooth texture. A noticeable color variation exists among the three formulations, with F2 having the deepest color intensity, followed by F1 and F3 (Figure 5).

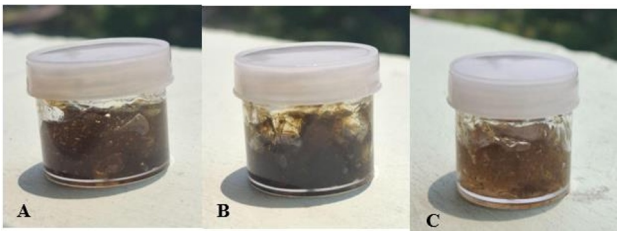

Fig. 5. Cockroach derived hemolymph-chitosan and bandotan leaves extract gel: formula F1 (A), formula F2 (B), formula F3 (C)

3.6 *In vitro* antibacterial activity test

A distinct inhibition zone surrounding the well, signifying the suppression of bacterial growth, was used to determine the antibacterial activity. For every formulation that was tested, the inhibition zone's diameter was measured. Based on the diameter, antibacterial activity was categorized as very strong (>20 mm), strong (10–20 mm), moderate (5–10 mm), or weak (<5 mm). Each formulation was tested in duplicate, with the results averaged and standard deviations calculated (Table 2).

Gel formulations F1, F2, and F3 exhibited antibacterial activity, with moderate inhibition zones against *S. aureus* and *Escherichia coli*. Formulations F1 and F2 demonstrated larger inhibition zones against *S. aureus* compared to the positive control. Formula F1, which contains an equal composition of chitosan and bandotan leaves, produced a larger inhibition zone against *S. aureus* compared to the positive control. Meanwhile, formula F2, with a higher concentration of bandotan leaves than chitosan, exhibited a larger inhibition zone than the positive control for both bacteria. The inhibition zone of F1 and F2 for *S. aureus* was 8.5 mm and 8 mm, respectively, outperforming the positive control (7 mm) (Figure 6). The inhibition zone of F1 and F2 for *E. coli* were 4.5 mm and 6 mm, respectively. Previous studies reported that bandotan leaves exhibit antimicrobial properties *in vitro* as demonstrated by the development of a transparent zone in the culture medium [9]. Our findings align with research by Basseri *et al*. (2019) [14], which demonstrated that chitosan can inhibit *S. aureus* and *E. coli* with similar strength. Furthermore, cockroach-derived chitosan at a 1% concentration was reported able to inhibit the growth of *S. aureus* [7]. Our study indicates that the combination of cockroach HC extract and bandotan leaves extract possesses effective antibacterial properties against both Gram-positive and Gram-negative bacteria.

Table 2. Inhibition zone of each formula against *Staphylococcus aureus* and *Escherichia coli*

Bacteria sample	Inhibition zone diameter (mm)*				
	F1	F2	F3	Positive control	Negative control
Staphylococcus aureus	8.5±0.07	8±0.14	6.5±0.07	7±0.28	0±0.00
Escherichia coli	4.5±0.07	6±0.14	5±0.07	5±0.00	0±0.00

*Explanation for Table 2.
F1: hemolymph 1.32 µL/g; chitosan 10 mg/g; bandotan leaves 10 mg/g
F2: hemolymph 1.32 µL/g; chitosan: 6.68 mg/g; bandotan leaves: 13.32 mg/g
F3: hemolymph 1.32 µL/g; chitosan 7.72 mg/g; bandotan leaves: 3.86 mg/g
Positive Control: Octadine® gel
Negative Control: Gel base

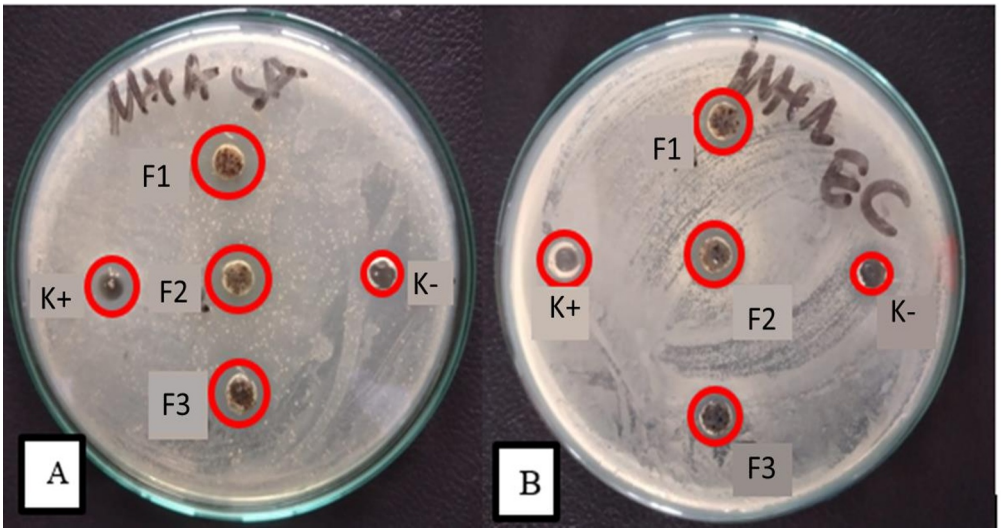

Fig. 6. The test for antibacterial activity against *E. coli* (B) and *S. aureus* (A)

4 Conclusion

Both *S. aureus* and *E. coli* were well inhibited by the combination of cockroach HC extract and bandotan leaf extract, with gel F1 and F2 showing moderate inhibition zones. These findings highlight the synergistic potential of cockroach HC extract and bandotan leaves extract as antibacterial substances against Gram-positive and Gram-negative bacteria. This study supports further investigation into the optimization for practical applications in wound care and bacterial infection treatment from natural sources.

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