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Wound-Healing effects of the combination of cockroach (*Periplaneta americana*) hemolymph–chitosan and bandotan leaf (*Ageratum conyzoides* L.) extract in mice (*Mus musculus*)

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Abstract. Open wounds are highly susceptible to secondary infections and rebleeding, necessitating the use of antibacterial and hemostatic agents to promote effective healing. Cockroach (*Periplaneta americana*) hemolymph and chitosan have been reported to possess antibacterial properties, while bandotan (*Ageratum conyzoides* L.) is a wild plant known for its hemostatic activity. This study aimed to evaluate the wound-healing efficacy of the gel containing combinations of cockroach hemolymph and chitosan extract, and *A. conyzoides* leaves extract, for treating open wounds in mice (*Mus musculus*). Hemolymph was collected from cockroaches, while chitosan from cockroach and bandotan leaves were extracted. Three gel combinations were prepared with different compositions: F1 (1.32 µL/g hemolymph, 10 mg/g chitosan, 10 mg/g bandotan extract), F2 (1.32 µL/g hemolymph, 6.68 mg/g chitosan, 13.32 mg/g bandotan extract), and F3 (1.32 µL/g hemolymph, 7.72 mg/g chitosan, 3.86 mg/g bandotan extract). An *in vivo* study was conducted by applying the gel combinations to open wounds in mice. The *in vivo* wound-healing results revealed that F1 accelerated wound closure and tissue regeneration compared with the other formulations. These findings suggest that the F1 gel formulation has potential as a topical treatment for open wounds.

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1 Introduction

The skin is the largest organ and outermost layer of the body, serving as the primary physical barrier against the invasion of pathogenic microorganisms into deeper tissues [1]. Open wounds can damage skin structures, and without proper treatment, they can lead to rebleeding, secondary infection, inflammation, and complications in tissue regeneration [2]. Treatment of an open wound usually involves antiseptics to prevent the skin from further infection [2]. However, antimicrobial resistance could complicate the treatment of bacterial infection [3], including in open wounds. Furthermore, antiseptics should be combined with wound-healing agents, such as fibroblast proliferation stimulators and hemostatic agents, to accelerate wound closure [4]. Commercially available open wound medications generally combine antiseptics with wound healing agents such as allantoin. Allantoin can accelerate wound healing by modulating the inflammatory response, stimulating fibroblast proliferation, and promoting extracellular matrix synthesis [4].

The American cockroach (*Periplaneta americana*) contains antibacterial substances and can survive in unsanitary environments [5]. These antibacterial substances can be utilized to aid in wound healing. Additionally, chitosan from cockroaches may support tissue regeneration and reduce wound infection [6].

Hemostatic compounds can prevent recurrent bleeding. Bandotan leaves (*Ageratum conyzoides* L.) contain hemostatic compounds such as alkaloids that can accelerate blood clotting and flavonoids that can stimulate fibroblast formation [7]. Traditionally, these leaves are widely used by communities to heal wounds [7]. Combining extracts of hemolymph and chitosan from American cockroaches and bandotan leaves has the potential to be a solution for healing open wounds in animals. However, based on a literature review, to our knowledge, no study has evaluated the effectiveness of this triple-combination extract gel in mice.

2 Methods

2.1 Ethical approval and location

This study was approved by the Animal Ethics Committee, School of Veterinary Medicine and Biomedical Sciences (SVMBS), IPB University, Indonesia, number No. 226 KEH/SKE/VII/2024. The study was conducted at IPB University, Bogor, Indonesia.

2.2 Cockroach hemolymph-chitosan and bandotan leaf extraction and gel formulation

Eighty American cockroaches were obtained from the Unit of Pest Control Study of SVMBS IPB, while bandotan leaves were obtained from AllHerbal Bogor. Hemolymph was obtained by cutting the antennae and extremities of the cockroaches and gently pressing their abdomen and head [8]. This yields 0.3 mL hemolymph. Chitosan from cockroaches was extracted using a decalcification and deproteinization process, as described in our previous study [8], yielding 0.61 g of chitosan from 80 cockroaches. Bandotan leaves were extracted by the maceration method [8].

The gel base was prepared as reported in our previous study [8]. The gel base was supplemented with cockroach hemolymph-chitosan and bandotan leaves extract, as shown in Table 1 for three formulas: F1, F2, and F3.

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

Formula	Hemolymph (µL/g)	Chitosan (mg/g)	Bandotan leaf (mg/g)	Ratio of chitosan: bandotan
F1	1.32	10	10	1 :1
F2	1.32	6.68	13.32	1 :2
F3	1.32	7.72	3.86	2 :1

2.3 *In Vivo* test in mice

Twenty-five mice were divided into three treatment groups (F1, F2, and F3) (Table 1), along with two control groups: a positive control treated with Octadin gel® (containing Allantoin 0,20% and Octenidine hydrochloride 0,15%) and a negative control treated with the gel base. Each group consisted of five mice. The mice were acclimatized for 2 weeks and housed in separate cages according to treatment at room temperature of ±25 °C, with standard feed and water provided *ad libitum*.

Table 2. Parameters of wound healing in mice

Score	Parameters
Wetness	
0	Dry wound
1	Slightly moist wound
2	Moist wound
3	Very wet wound with a small amount of exudate
4	Wound flooded with abundant purulent or serosanguinous exudate.
Redness	
0	No redness; wound color identical to the surrounding skin
1	Mild redness (<0.1 mm from the wound margin)
2	Moderate redness (0.2–0.4 mm from the wound margin)
3	Extensive redness (>0.5 mm from the wound margin)
4	Very intense redness accompanied by other signs of inflammation, such as edema and increased temperature
Scab formation	
0	No scab formation observed
1	A thin scab is present only along a small portion of the wound margin.
2	Moderate scab covering part of the wound
3	A thick scab covering almost the entire wound
4	Thick, dry scab completely covering the wound.

Before wound modeling, the mice were anesthetized with a combination of *xylazine* (16 mg/kg body weigth) and *ketamine* (80 mg/kg body weight) via intraperitoneal injection. An open wound model was created by making a 1.5 cm long, 0.15 cm wide, and subcutaneous-depth incision along the dorsal midline of each mouse. All procedures were performed aseptically. Following wound induction, 0.5 g of gel was applied to each wound, as specified for the treatment group, evenly spreading it over the wound area. Treatments were administered once daily for 11 days. Before each treatment, the wounds were cleaned with 0.9% NaCl solution to remove residual treatment and exudate that could interfere with the wound healing process [9]. Wound area measurements (length x width of the wound) and macroscopic observations were conducted daily in the afternoon and reported on days 0, 4, 7, and 11. Observed parameters included the degree of wound wetness, redness, and scab formation. Each parameter was scored according to the criteria presented in Table 2. The scores for each group were averaged, and standard deviations were calculated.

2.4 Statistical analysis

The data from wound-healing assays in mice were analyzed quantitatively. The wound area per day was statistically analyzed using the non-parametric Kruskal-Wallis test. Significant differences ($p < 0.05$) were further analyzed using a post hoc multiple comparison test with a 95% confidence interval. Statistical analyses were performed using IBM SPSS Statistics version 21.

3 Results and discussion

The gel was administered topically to the induced wounds of mice for 11 days of observation. Macroscopic parameters included the degree of wetness, redness, and scab formation (Table 3). On day 1, macroscopic parameters indicated that all treatment groups were in the inflammatory phase, characterized by wet and erythematous (rubor) wound conditions. Inflammation serves as a critical defensive mechanism that eliminates pathogenic microorganisms and neutralizes irritants. This process triggers alterations in local blood flow and enhances vascular permeability, facilitating the migration of essential fluids, proteins, and leukocytes into the wound area [10]. Based on its duration, inflammation is classified into two types: acute and chronic. Acute inflammation is typically of rapid onset and short duration, while chronic inflammation is characterized by a sustained and long-term course. The classic hallmarks of inflammation include five cardinal signs: redness (*rubor*), swelling (*tumor*), increased temperature (*calor*), pain (*dolor*), and impaired function (*functio laesa*) [11].

In this study, wound wetness was attributed to the accumulation of exudate, a plasma-like fluid originating from the vasculature. During the inflammatory phase, inflammatory mediators, such as histamine, increase capillary permeability, allowing leukocytes to migrate out of the vessels and promoting increased fluid release into the damaged tissue, resulting in exudate formation. Under normal conditions, exudate production gradually decreases as the wound healing process progresses [12].

On day 4, wound wetness decreased in all treatment groups, redness increased, and scab formation became more pronounced. The increased redness was attributed to an enhanced blood supply to the wound area [13]. This increased blood flow also delivers soluble mediators such as prostaglandins and nitric oxide (NO), as well as inflammatory cells, including neutrophils and macrophages, which function to remove damaged tissue and prevent the spread of infection at the wound site [10, 13].

The drying of previously wet wounds was further facilitated by the formation of scabs on the surface, marking the onset of the early proliferative phase. These scabs, resulting from

blood coagulation, serve to protect the wound and maintain an optimal moisture environment. The detachment of the scab signifies that new cell growth has occurred, facilitating scab removal and allowing the wound edges to approximate. Scab detachment occurs when the underlying tissue dries and the wound margins are gradually pulled toward the center.

Table 3. Macroscopic parameters of wound healing in mice in the treatment and control groups on days 1, 4, 7, and 11

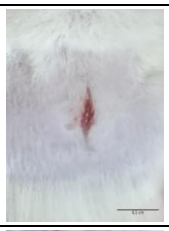
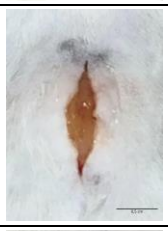
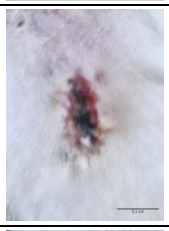
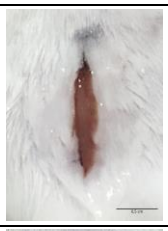
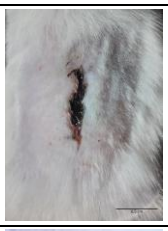
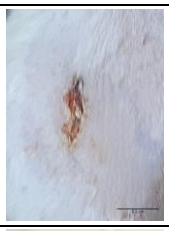
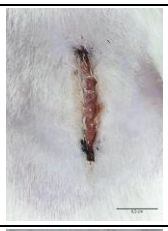
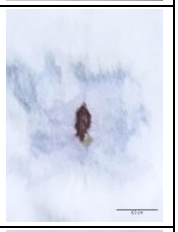
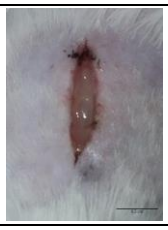
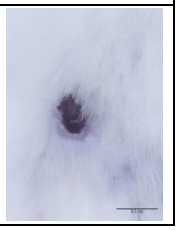
Treatment	**Average scores of macroscopic parameters over several days of observation**			
	1	**4**	**7**	**11**
	Wetness			
Positive Control	3.0±1.2	1.0±1.2	0.0±0.0	0.0±0.0
Negative Control	2.8±0.8	0.8±1.0	0.5±0.6	0.0±0.0
F1	2.0±1.6	0.8±0.5	0.0±0.0	0.0±0.0
F2	1.8±0.8	0.8±1.0	0.0±0.0	0.0±0.0
F3	2.8±1.3	0.5±0.6	0.3±0.6	0.0±0.0
	Redness			
Positive Control	2.6±0.9	2.0±0.8	1.0±0.8	0.0±0.0
Negative Control	1.0±1.0	2.3±1.0	1.5±1.0	1.0±0.0
F1	1.4±0.6	1.5±0.6	0.8±0.5	0.3±0.6
F2	2.0±0.7	2.3±0.5	2.0±0.8	0.5±0.7
F3	1.8±0.5	2.0±0.8	1.5±0.6	0.5±0.7
	Scab Formation			
Positive Control	0.2±0.5	1.5±0.6	0.8±1.5	0.0±0.0
Negative Control	0.4±0.6	2.3±1.5	2.8±1.3	0.5±0.7
F1	0.8±1.1	2.5±1.3	3.0±1.4	0.3±0.6
F2	0.8±0.5	2.3±1.5	2.8±1.3	1.0±1.0
F3	0.6±0.6	2.8±1.0	2.5±1.7	1.0±1.0

By day 7, all treatment groups demonstrated a nearly identical wound-healing process, characterized by fading redness, increased scab formation, and the regeneration of new skin tissue. By day 11, the wounds in all treatment groups had dried, and the redness had significantly reduced, especially in the positive control and F1 groups. While most groups still retained minor residual scabs, the positive control exhibited a small amount of residual scab, indicating a more advanced stage of tissue remodeling.

The macroscopic progression of wound healing in mice across treatment and control groups on days 1, 4, 7, and 11 is presented in Table 4. On day 1, wound healing in the negative control, F2, and F3 groups showed inflammatory responses characterized as both redness and swelling (edema). In contrast, the positive control and F1 groups exhibited localized redness

without significant swelling. Wound swelling occurs as a result of vasodilation of blood vessels, a protective response to foreign substances [13]. On day 4, all treatment groups exhibited swelling, and accompanied by the initiation of scab formation that began to cover the entire wound surface area by day 7, clinical signs of tissue swelling had resolved in all treatment groups, suggesting that the combination of cockroach hemolymph-chitosan and bandotan leaf extract effectively supports the resolution of the inflammatory phase, allowing the healing process to proceed toward mature tissue regeneration.

Table 4. Macroscopic appearance of wound healing in mice in the treatment and control groups on days 1, 4, 7, and 11 (Scale bar = 0.5 cm)

Treatment	Day 1	Day 4	Day 7	Day 11
Positive Control				
Negative Control				
F1				
F2				
F3				

Over the 11-day observation period, the F1 group demonstrated wound-healing outcomes most comparable to the positive control among the treatments. Wounds in the F1 group dried more rapidly, exhibited milder inflammation, and showed markedly reduced redness by the

final day of observation. This significant reduction in redness was associated with decreased inflammatory cell infiltration, while the antibacterial properties of hemolymph–chitosan may have modestly contributed as a supportive factor [8]. In addition, the wound area in the F1 group decreased more rapidly, presumably due to the flavonoid content of the gel. This finding is consistent with the study [14], which reported the role of flavonoids in wound healing, particularly in stimulating fibroblast formation. Fibroblasts are elastic connective tissue cells that function to contract and close open wounds. Furthermore, flavonoids may prevent excessive scar formation and fibrosis by reducing the production of TGF-β1 and IL-1β, inhibiting extracellular matrix (ECM) deposition, and preventing excessive accumulation of fibrous connective tissue [14].

The wound area decreased in all treatment groups over the course of healing; however, the rate of reduction varied among groups. The fastest decrease, observed on day 11, occurred in the positive control group, followed by the F1 extract combination: the negative control, F2, and F3 groups. The graph showing wound area measurements for each treatment group on days 0, 4, 7, and 11 is presented in Figure 1.

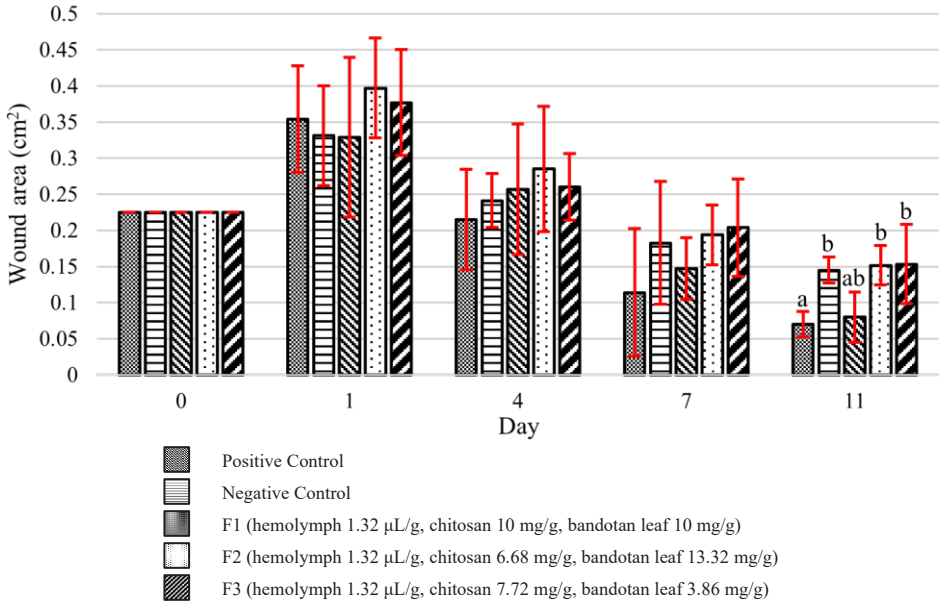


Fig. 1. Graph of the average wound area in mice on days 1, 4, 7, and 11

[a]Bars on the same day followed by the same letter are not significantly different at the 5% significance level (Kruskal–Wallis post hoc test).

The Kruskal–Wallis test of wound area on days 0, 1, 4, and 7 indicated no statistically significant differences among treatment groups ($p > 0.05$). However, on day 11, a significant difference in at least one median among the treatment groups ($p = 0.046 < 0.05$). Subsequent *post hoc* analysis revealed that F1 did not differ significantly from any of the treatment groups ($p > 0.05$). Similarly, the positive control group showed no significant difference compared with F1 ($p > 0.05$), but it was significantly different from the negative control, F2, and F3 groups ($p < 0.05$). Additionally, no significant differences were found among the F2, F3, and negative control groups ($p > 0.05$).

The good wound-healing outcomes observed in the F1 group are consistent with previous findings of our study [8], which established that the gel formulations F1, F2, and F3 possess moderate antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Specifically, F1 and F2 demonstrated larger inhibition zones against *S. aureus* than the

positive control [8]. This efficacy is presumed to be due to the adequate composition of chitosan and *A. conyzoides* leaf extract used in this formulation. The F1 group contained chitosan at a concentration of 10 mg/g gel, which is comparable to another study [5] that used cockroach chitosan extract at 10 mg/mL. *A. conyzoides* leaf extract in the F1 group had a lower concentration than in another study [15], which applied *A. conyzoides* at a concentration of 50 mg/mL to inhibit bacterial growth.

The gel combination evaluated in this study is proposed to accelerate the resolution of the inflammatory phase by preventing bacterial infection and reducing inflammatory cell infiltration. As inflammatory cells decrease during the proliferative stage, flavonoids derived from *A. conyzoides* leaf extract stimulate fibroblast formation, thereby accelerating epithelial tissue formation [12]. Administration of the combined extracts of cockroach hemolymph, cockroach chitosan, and *A. conyzoides* leaf extract demonstrated an accelerated wound healing process.

A limitation of this study is that the macroscopic evaluation of wound healing in mice was conducted qualitatively, potentially introducing subjectivity bias into the data. In addition, the 11-day observation period was also relatively short and did not fully represent the tissue remodeling phase.

Although the results indicate the therapeutic potential of the extract combination for wound healing, further studies are required to compare its performance with commercially available gel formulations that possess similar mechanisms of action, including antibacterial activity, stimulation of fibroblast proliferation, and hemostatic efficacy, to ensure a more comprehensive assessment of its clinical viability.

4 Conclusion

The combination of cockroach hemolymph, cockroach chitosan, and bandotan (*A. conyzoides*) leaf extract accelerates wound healing in mice. A gel formulation containing 1.32 µL/g hemolymph, 10 mg/g cockroach chitosan, and 10 mg/g bandotan leaf extract was the most effective in promoting wound healing and demonstrates potential for the development of topical preparations for open wound treatment.

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