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The Effect of *Amphidromus palaceus* and *Lissachatina fulica* land snail mucus on collagen density in white rats (*Rattus norvegicus*)

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Abstract

Treatment with natural ingredients is essential for healing incision wounds. One promising natural remedy is land snail mucus, which has been used for centuries to address various health conditions, including wounds. This study aimed to evaluate the effects of mucus from *Amphidromus palaceus* and *Lissachatina fulica* on collagen density during the healing process of incision wounds in *Rattus norvegicus*. The in vivo study included 36 rats, divided into four cages with nine rats per cage. Each of rats subjected to approximately 1 cm incision wounds on their backs. The rats were divided into four groups: a negative control group (KN) receiving aquades, a positive control group (KP) treated with 1% bioplacenton, treatment group 1 (P1) receiving 25 µL of *A. palaceus* mucus, and treatment group 2 (P2) receiving 25 µL of *L. fulica* mucus. The experiment concluded on days H3, H5, H7, and H10. Histological preparations were made using MT staining to assess collagen density. The results showed both species of snail mucus significantly increased collagen density compared to the negative control (aquades). However, there were no statistically significant differences when comparing L. fulica, A. palaceus, and bioplacenton. Among all the treatment groups, L. fulica mucus (P2) produced the highest collagen density. These findings suggest that both types of snail mucus hold promising potential in promoting the wound healing process.

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Keywords: *Gastropoda; In vivo; Incision wound; Masson trichome; Wound healing.*

Introduction

An incision or cut wound is created by a sharp and clean object. Examples of this type of wound include cuts from surgical procedures or injuries caused by knives, razors, or broken glass [1]. The body naturally heals these wounds through a physiological process that responds to tissue damage. Wound healing is a complex phenomenon involving interactions between various types of cells, cytokines, mediators, and the blood vessel system [2]. The goal of this healing process is to restore the skin's functional integrity and structure. However, larger and chronic wounds tend to take longer to heal, making them more difficult to manage due to their susceptibility to infection and fluid loss. While the body can heal wounds on its own, this process can be accelerated through the use of medications or other treatments [3].

Chemical drugs frequently used for wound healing are often less effective than alternative medicines. As a result, many people are turning to these alternatives, which are typically more effective, affordable, and accessible. This shift has led to an increased interest in natural ingredients, including various plants and animals. A potential alternative for wound healing is land snail mucus. The medicinal use of land snails dates back a long time. It is believed that Hippocrates, the father of medicine, utilized land snail mucus to treat protocoele [4]. Moreover, research has shown that land snail mucus is effective for wound healing [5] [6] and possesses antibacterial properties [7][8]. The heparan sulfate content in this mucus aids in accelerating the wound healing process, while achatin isolates provide antibacterial effects. This antibacterial property also enhances the inflammation phase, leading to a faster progression to the proliferation phase in wound healing [9].

Collagen is an extracellular matrix protein that provides structural support to the skin, joints, and bones of the human body. The high collagen content found in animals is considered the primary source of this protein. Collagen plays a crucial role in regulating the wound-healing process by attracting fibroblasts and stimulating the formation of new collagen in the wound area. [10]. As a result, collagen can be used as a supportive material in wound treatment to accelerate healing. Additionally, collagen is present in land snail mucus. The production and accumulation of collagen are essential for regeneration, helping to repair the skin after damage caused by mechanical friction or sun exposure [11].

Indonesia is renowned for its high diversity of land snails, particularly across the islands of Java (263) [12], Sumatra (276), Bali and Nusa Penida (126), Borneo (558), and Sulawesi (253) [13]. While numerous studies in Indonesia have explored the wound healing and

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antibacterial properties of land snail mucus, the majority focus solely on the species *Lissachatina fulica* [14][15][16]. There is limited research on *Amphidromus palaceus* in Indonesia. The distribution of *A. palaceus* is not as extensive as that of *L. fulica*. Previous study utilizing LC-MS have compared the biologically active compounds from *A. palaceus* and *L. fulica*, revealing 28 biologically active substances in *A. palaceus* compared to 16 in *L. fulica* (Pertiwi *et al.* – in prep). However, ethnomalacological research conducted in Gunungkelir Hamlet, Yogyakarta, reveals that local people prefer two species for wound treatment: *L. fulica* and *Amphidromus palaceus* (Pertiwi *et al*. - in prep*). Amphidromus palaceus* is a native land snail species in Indonesia [17], suggesting it may also serve as a potential wound medicine based on the empirical knowledge of the community. Despite this promising insight, scientific evidence supporting the efficacy of *A. palaceus* is currently limited. Therefore, the objective of this study is to investigate the effects of mucus from both *Amphidromus palac*eus and *Lissachatina fulica* on collagen density during the healing process of incision wounds in *Rattus norvegicus*. By undertaking this research, we hope to bridge the gap between traditional practices and scientifically validated treatments, unlocking new potential in the medicinal properties of these snails.

Materials and methods

1. Land snail slime collection

The land snails used in the study are two species: *Amphidromus palaceus* and *Lissachatina fulica*. Both species were collected from Dusun Gunungkelir in Yogyakarta, Indonesia. *Amphidromus palaceus* has the following characteristics like a dextral/sinistral shell shape, tall and conical shape, and yellowish-green shell color. The shell features an oval opening, a slightly rounded shell edge, and a slit-like navel. Typically, it has 6.5 to 7 whorls and is decorated with axial striae that vary in strength along the growth line. The average size of this snail is approximately 50 mm [18]. This species is native to Indonesia.

The shell is large, solid, and pyramidal, featuring a rounded spire and base. Its base color ranges from light yellow to yellowish brown and is adorned with irregular vertical lines, streaks, or spots in brown or light purple beneath a greenish-yellow epidermis. The shell exhibits coarse vertical striations that become rib-like as they approach the sutures and aperture. Many specimens also display finer spiral carvings, particularly on the whorls of the spire, which can make this part of the shell appear less distinct. The entire shell is covered by a yellowish or brownish periostracum, which can be easily peeled off. The seventh to ninth whorls expand rapidly in size and become more convex, with the final whorl being large and

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somewhat inflated. The upper section of the whorls is smooth, and the umbilicus is closed, even in younger individuals [19].

The number of snails required for this study varies by species due to differences in size. For *A. palaceus*, 25 snails are needed, while only five *L. fulica snails* are necessary. The difference in mucus production is attributed to the varying amounts produced by each species. Based on empirical observations, *L. fulica* generates a large quantity of mucus from a single individual. In contrast, *A. palaceus* may produce only a drop of mucus from one individual, after which its soft body retracts into the shell, making it impossible to stimulate mucus production again. Consequently, more individuals of *A. palaceus* are required to provide additional opportunities for testing. The collected snails were housed in a transparent cage equipped with an air hole at the top. Each cage contained only one species of snail. During maintenance, routine feeding consists of white mustard greens and cucumbers. The base of the cage was covered with sand and crushed eggshells ($CaCO_3$ powder). Every day, water was sprayed into the cage to maintain humidity, and the cage was cleaned twice daily.

The method for collecting snail mucus involves a natural technique known as "milking," which stimulates the snail's soft body. A spatula is used to collect the mucus secretion into a vial [20]. The collected mucus was then stored in a -20°C freezer for one to three months. The mucus production from 4 to 5 *A. palaceus* snails yielded approximately 2 to 3 ml, while 1 *L. fulica* snail also produces about 2 to 3 ml of mucus. The milking process lasted between 30 to 45 minutes and was performed every 2 days for a duration of 1 month. The milking process was performed under aseptic conditions. This involved using tools such as spatulas and petri dishes that were sprayed with alcohol before they were used. Additionally, candles were lit around the milking area, and the hands of the milking operators were covered with gloves.

2. Rat Maintenance

The collagen density experiment on white rats (*Rattus norvegicus*) has been approved by the Animal Ethics Commission of the IPB School of Veterinary Medicine and Biomedicine under code No. 048/KEH/SKE/V/2023. A total of 36 male Sprague-Dawley rats, each weighing approximately 150 grams with a standard deviation of 20%, were utilized. All rats were in good health with no signs of dull fur, baldness, or infection, and exhibited normal behavior. They were allowed to acclimate in the laboratory for one week under a 12-hour light and 12-hour dark cycle. Food and water were provided *ad libitum* and the rats were divided into four groups of nine rats per cage.

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3. Making an Incision Wound

Once all groups were ready, the incision on the back of the rat was made using a surgical blade. All tools and the area where the wound was executed were sterilized with alcohol. Prior to the procedure, the rats were anesthetized with a combination of 100 mg/kg of ketamine and 3 mg/kg of xylazine. The fur around the incision area was shaved and cleaned to ensure sterility. A mark of 1 cm was made on the rats' backs to guide the incision. Each wound was approximately 1 cm long and deep enough to cut through the skin layer until the fascia beneath was visible. The incision was performed on the back to make it difficult for the rats to reach, ensuring the area was more extensive, with fewer muscles, blood vessels, and nerves present. Each rat received four wounds as part of the experimental procedure.

4. Application of Land Snail Mucus

All groups were treated for a duration of 10 days. The treatments included a negative control (KN) using aquades, a positive control (KP) with 1% bioplacenton -bioplacenton is a product derived from animal sources, similar to land snail mucus treatments-, treatment 1 (P1) involving the application of 25 µL of *Amphidromus palaceus* snail mucus, and treatment 2 (P2) using 25 µL of *Lissachatina fulica* snail mucus. Treatment was administered once daily at 10:00. The snail mucus was applied by dripping it onto the wound area. Rat were euthanized by cervical dislocation on days 3, 5, 7, and 10 and wound skin area samples were collected for histological preparation. The selected termination days of 3, 5, 7, and 10 are based on the observation of collagen regeneration. On day 3, the inflammatory phase begins, showing slight formation of type III collagen. By day 5, this formation increases. On day 7, type I collagen starts to form, and its prevalence continues to grow by day 10 [21].

5. Animal histological preparation

The skin samples were placed in a 10% neutralized buffered formalin (10% NBF) preservative solution for 24 hours. The samples were then trimmed to fit the size of the cassette and soaked in a series of alcohol solutions: 70% alcohol, 80% alcohol, 90% alcohol, and 96% alcohol, each for 2 hours. Following this, the samples were immersed in two 100% ethanol solutions for 2 hours each. The immersion process continued with xylene I, xylene II, and xylene III solutions for 45 minutes each. Afterward, the samples were placed in paraffin 1 and paraffin 2 solutions for 1 hour each. The samples were then arranged and embedded in a paraffin block, which was stored in the refrigerator for 24 hours. Next, the samples were cut using a microtome, placed on microscope slides, and adhered with adhesive. A cover glass was

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added, and the slides were left at room temperature for 48 hours to prepare for Masson's trichrome (MT) staining.

6. Masson's Trichome (MT) staining

Masson's Trichrome staining is a three-color histological staining procedure. In this technique, collagen fibers are stained blue, the nuclei appear black, and the background is red. The steps were started by immersing the tissue in Bouin's fluid for 30-40 minutes. Wash the tissue two times with distilled water. Then, hydrate the tissue with hematoxylin (Part A) for 20 minutes. Wash again two times with distilled water and hydrate with hematoxylin (part B) for 20 minutes. Continue washing again with distilled water two times, then hydrate with acid fuchsin for 2-5 minutes. Dip the tissue two times in 1% acetic acid. Then, hydrate in phosphomolybdic acid for 10-15 minutes. Again, dip two times in 1% acetic acid. Continue hydrate the tissue in an amino blue solution for 15 minutes. Wash two times in 1% acetic acid. Dehydrate the tissue with 96% alcohol for 5 minutes, followed by two steps of absolute alcohol (first for 10 minutes and second for another 10 minutes). Finally, clear the tissue with xylene. After completing the Masson's Trichrome staining, the relative density of the blue-stained collagen was quantified in the wound area using ImageJ. The field of view was selected to include the entire wound bed at an optical magnification of 10x.

7. *Data analysis*

The collagen density data were analyzed using statistical methods. The analysis included the Kolmogorov-Smirnov test for normality, Levene's test for homogenity, a two-way ANOVA, and post hoc Tukey's tests. This analysis used R version 4.3.3.

Results and Discussion

Wound healing is a natural process that occurs when the skin is injured. One important parameter studied is collagen density. The following graph illustrates this parameter.

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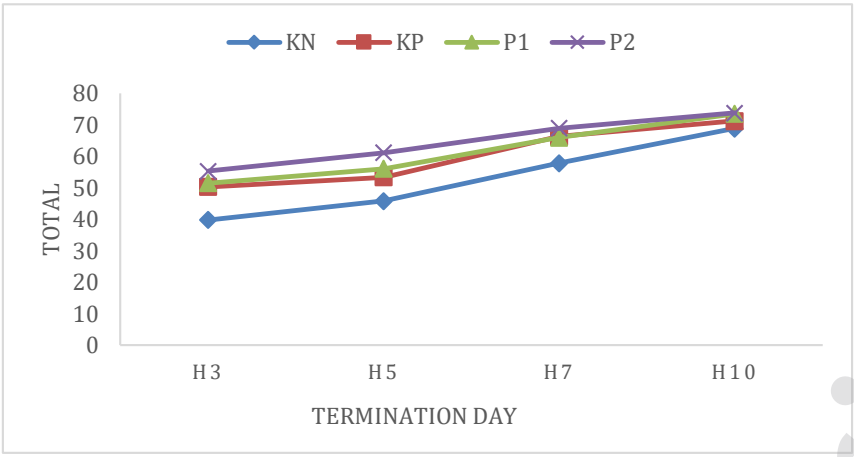

Figure 1. Collagen density in *Rattus norvegicus* at H3, H5, H7, and H10 after treatment of *A.palaceus* and *L. fulica* mucus

According to Figure 1, both *A. palaceus* and *L. fulica* mucus enhance collagen density in wound healing on the backs of rat. Both outperform bioplacenton, a commercial drug. Several factors indicate the success of this process, including the speed of healing, the formation of new blood vessels, the production of collagen, and the absence of infection. The collagen density in *Rattus norvegicus* after 10 days of treatment with mucus from *Amphidromus palaceus*, a native Indonesian land snail, and *Lissachatina fulica*, an invasive alien species (IAS) revealed that both *A. palaceus* and *L. fulica* mucus showed better collagen formation than positive treatmeant using bioplacenton. Similar studies indicate that land snail mucus contains the highest collagen density [22][23] [24]. The high collagen density found in the mucus of both *A. palaceus* and *L. fulica* can trigger markers that promote accelerated wound healing [25]. Notably, collagen formation reaches its peak around the seventh day and continues to develop beyond that point. On day 7, many fibroblasts become activated and start producing significant amounts of collagen. Following this, the tissue enters the remodeling or maturation phase, where type I collagen is formed. This type of collagen offers improved mechanical strength to the healing wound, though the rate of healing slows compared to the proliferation phase [21].This progression can be observed in the images below at H3, H5, H7, and H10 with P2.

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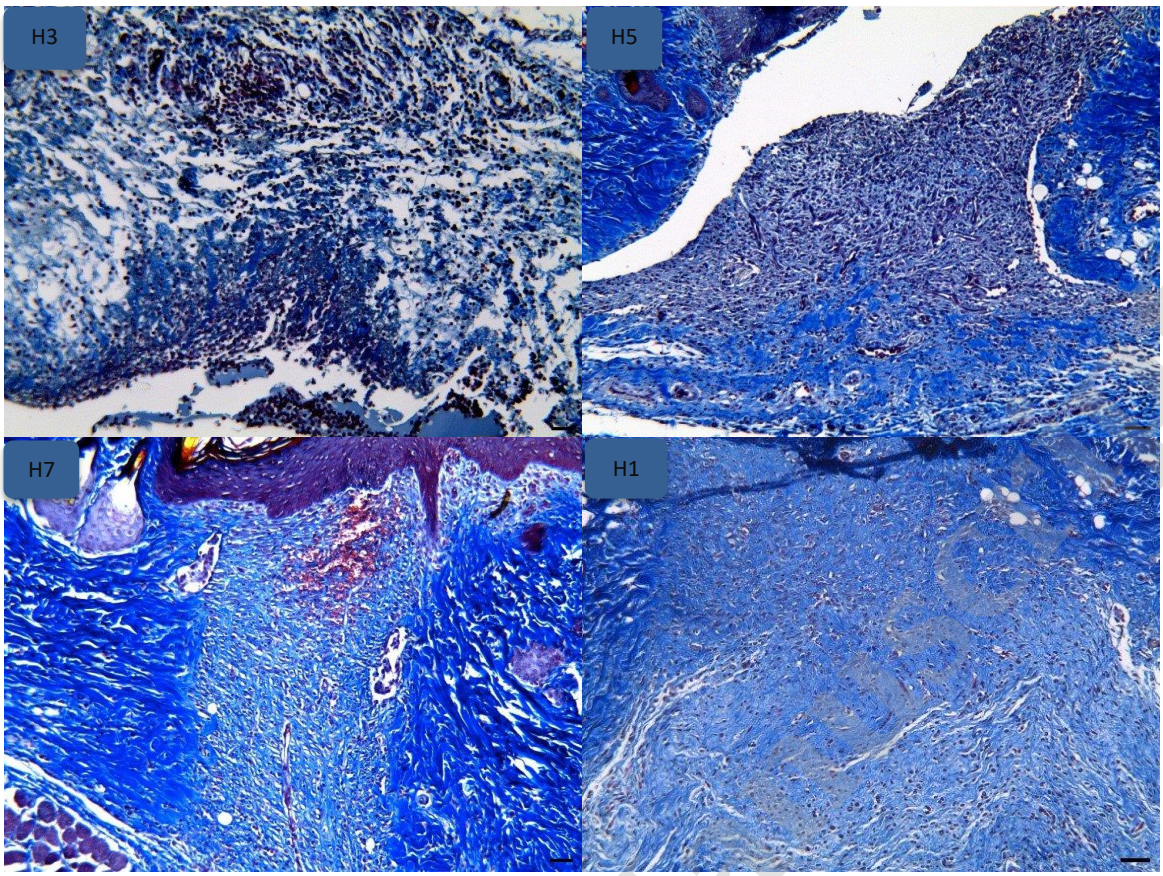

Figure 2. Anatomy of the incision wound area in *Rattus norvegicus* after treatment of *L. fulica* mucus (P2) on H3, H5, H7, and H10 (light blue: collagen; dark blue: dense connective tissue; scale bar: 5 mm, 10x magnification).

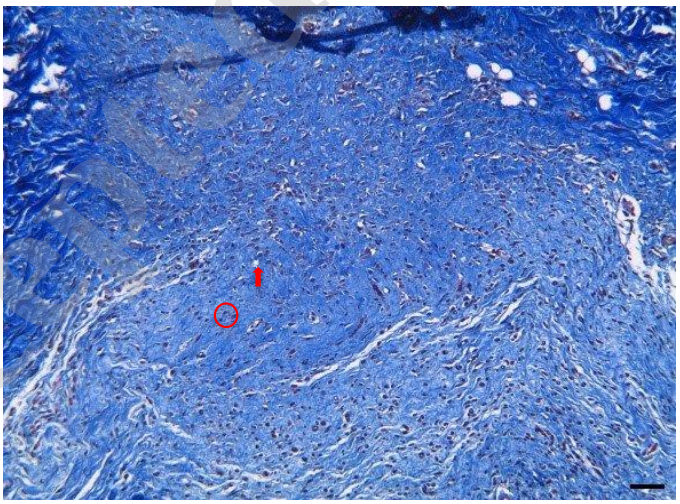

Figure 3. Anatomy of the incision wound area in *Rattus norvegicus* with P2 at H10 (red arrow = sebaceous gland; red circle = hair follicle; light blue: collagen; dark blue: dense connective tissue; scale bar = 5 mm; magnification 10x).

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The data obtained were analyzed using the Kolmogorov-Smirnov test and Levene's test. Both tests indicated that collagen density data met normality and homogeneity (p-value < 0.05). The analysis continued with a two-way ANOVA, yielding the following results (Table 1).

Table 1. Statistical results of collagen density analyzed through two-way ANOVA testing

SK	db	JK	KT	F statistic	P-value
Treatment	3	1182	394.1	21.211	0.0000***
Day	3	5033	1677.6	90.284	0.0000***
Treatment:Day	9	174	19.3	1.037	0.425
Residuals	48	892	18.6		

From the table above, only the Treatment: Day variable is not different (p-value > 0.05). Therefore, further tests using Tukey's method were conducted for both treatment and day. The results of the Tukey test are presented in table 2 and table 3 as follows

Table 2. Statistical results of collagen density for treatment parameter analyzed using the Tukey test

Day	*diff*	*lower*	*upper*	*p-adj*
H3-H10	-22.6387	-26.6948	-18.5827	0.0000
H5-H10	-17.7547	-21.8107	-13.6987	0.0000
H7-H10	-7.0681	-11.1241	-3.0120	0.0002
H5-H3	4.8840	0.8280	8.9401	0.0124
H7-H3	15.5707	11.5147	19.6267	0.0000
H7-H5	10.6866	6.6306	14.7426	0.0000

Table 2 demonstrates a statistically significant difference in collagen production between the following group pairs: KP and KN, P1 and KN, P2 and KN, as well as P1 and KP (pvalue < 0.05). These results demonstrate that the treatments, including the commercial drugs, bioplacenton, and mucus from *A. palaceus* and *L. fulica*, enhance collagen density, thereby promoting faster wound healing compared to the control group that received only distilled water. Notably, the treatment with *A. palaceus* mucus was found to be more effective in increasing collagen density than bioplacenton. This study is the first to investigate the use of *A. palaceus* mucus for wound treatment in rat, so no previous studies directly compare it to bioplacenton. The observed effectiveness may be attributed to the glycoprotein and hyaluronic acid content in mucus, which supports collagen production and skin tissue repair [26]. This

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research provides scientific evidence that highlights the superior performance of *A. palaceus* mucus compared to commercial drugs.

Table 3. Statistical results of collagen density for day parameter analyzed using the Tukey test

Treatment	*diff*	*lower*	*upper*	*p-adj*
KP-KN	11.7056	7.6495	15.7616	0.0000
P1-KN	7.2020	3.1459	11.2580	0.0001
P2-KN	8.6617	4.6057	12.7177	0.0000
P1-KP	-4.5036	-8.5596	-0.4475	0.0241
P2-KP	-3.0439	-7.0998	1.0121	0.2033
P2-P1	1.4597	-2.5963	5.5157	0.7738

Unfortunately, the mucus from *L. fulica* (P2) did not show a significant difference compared to control group that using commercial drug (p-value > 0.05). This result contrasts with the other research which indicated a significant difference between *A. fulica* mucus and the control group. However, it is important to note that their control group used CMC Na [27]. The other study has also demonstrated that *A. fulica* mucus significantly increases fibroblast cells, which are essential for collagen synthesis in post-tooth extraction sockets [28]. The discrepancies in results between this study and previous research may be attributable to variations in mucus concentrations, the types of wounds examined, the target organs affected, and the methods of application used. Therefore, while snail mucus shows potential for increasing collagen density, further research with consistent designs and strict controls is necessary to definitively assess its effectiveness.

As mentioned earlier, both types of mucus were effective in enhancing collagen density, evenmore the density of P2 collagen is the highest compared to the other treatments. However, statistical analysis did not reveal a significant difference between P2-P1 (0.7738 > 0.05). This finding does not align with the study referenced in [27], which indicated that higher concentrations of *L. fulica* mucus resulted in increased collagen density, the difference was statistically significant when compared to lower concentrations. However, the study found no significant difference in collagen density between the low (24%) and medium (48%) concentrations of snail mucus gel. Furthermore, the effects of *A. palaceus* mucus cannot be compared to existing literature, as it is the first study to investigate its use in an *in vivo* model for *Rattus norvegicus* incision wound closure. The variations in collagen deposits between the two types of mucus may be attributed to several factors, one of which is the presence of the metalloproteinase (MMP) family. These MMPs play a crucial role in every phase of wound

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healing by modulating the wound matrix [29]. However, this study did not test the MMP content in the mucus samples.

All termination days showed significant differences in collagen density. As illustrated in Figure 2, collagen density increased from the H3 to H10 stages, indicating that higher collagen density correlates with these later stages. Similar results were noted from the second to the seventh termination days, where collagen density also increased [27]. Notably, the high collagen density observed on the 7th and 14th days, following land snail mucus treatment, yielded results resembling normal skin [30]. Collagen provides essential structural support for various components of the skin, including sebaceous glands and hair follicles, which are encased in collagen-rich connective tissue (see Figure 3). It plays a crucial role across different layers of the skin, particularly in forming the connective tissue around hair follicles and sebaceous glands, while also contributing to the skin's strength and elasticity [31][32].

Based on the study's findings, both types of land snail mucus contribute to collagen formation, deposition, and maturation. The composition of land snail mucus, which includes proteins and glycosaminoglycans, resembles the extracellular matrix, with collagen being the most significant component of this matrix [30]. This composition also supports the structural proteins in the skin [26]. The advantages of land snail mucus include its biodegradable properties, strong adhesive capabilities, and excellent compatibility. Consequently, the mucus from both *A. palaceus* and *L. fulica* demonstrates significant potential to enhance collagen density, making it a promising option for wound healing. These characteristics are essential for providing a superior alternative treatment. This potential opening could lead to further opportunities in both the medical and cosmetic industries. The implications of this development can enhance the local economy while also protecting Indonesia's biodiversity.

Conclusions

The treatment using mucus from the native Indonesian land snail *A. palaceus* and the invasive alien species *L. fulica* had a positive impact on collagen density parameters. Mucus from both species showed significant different effect on collagen density when compared to aquades (negative control). However, this difference was not statiscally significant when compared between *L. fulica* with *A. palaceus* and bioplacenton. Among the treatments, *L. fulica* mucus (P2) demonstrated the highest collagen density. Therefore, both types of mucus show promising potential for enhancing the wound healing process. Further research is needed to explore the factors that contribute to the varying collagen densities between the two mucus types and how these differences influence the rate of wound healing.

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Conflict of interest

We declare that there is no conflict of interest.

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