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Diabetic Wound Healing Activity of *Anabas testudineus* Fish Scales

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Abstract

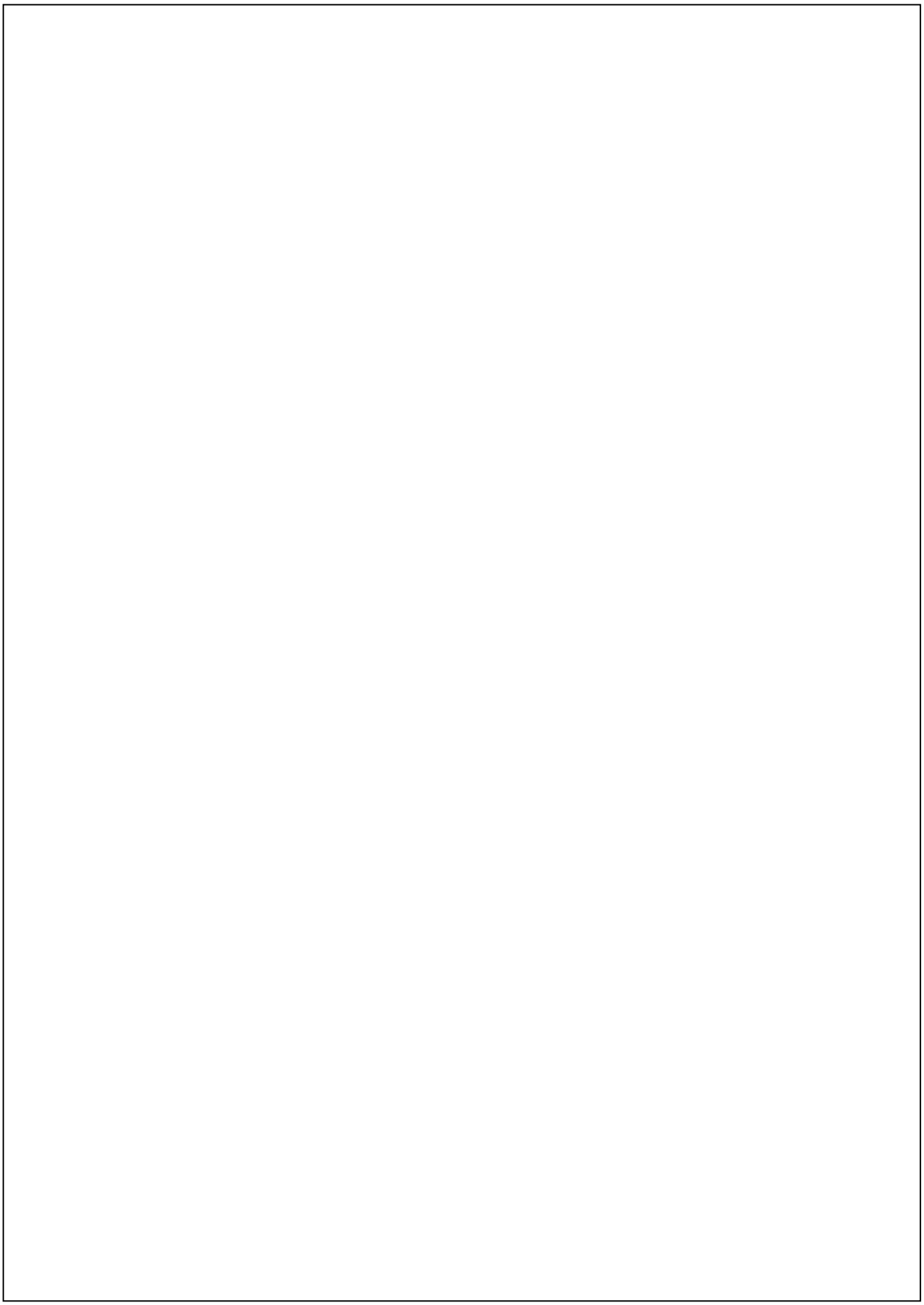
Background: *Anabas testudineus* fish scales are part of the fish that has not been used optimally. Fish scales contain chitin. Chitin which undergoes deacetylation, produces chitosan. Chitosan is an efficacious compound for antibacterial and diabetic wound healing.

Objective: This study aimed to extract chitosan from fish scales and test the diabetic wound healing activity of fish scale extract gel

Methods: Extraction of fish scales using 0.3 M NaOH, 0.55 M HCl, and 50% NaOH. FTIR will analyze the extraction results to see the functional groups of the extract. Activity test of fish scale extracts diabetic wound healing gel was carried out on *Rattus novergicus* Wistar strain for 14 days. There were five treatments: negative control, positive control given octenillin^{R}, and fish scale extract gel conc. 5%, 10%, and 15%.

Results: The results showed that the fish scales extract contained chitosan, and gel extract fish scale conc 5% have diabetic wound healing activity better than 10% and 15%.

Conclusion: *A. testudineus* fish scale extract contains chitosan, as seen from the FTIR results.



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INTRODUCTION

Anabas testudineus fish (*A. testudineus*), besides being edible, this fish also has antibacterial activity.[1,2] Mucus produced by *A. testudineus* fish is antibacterial for *S. aureus* dan *Escherichia coli*, *Pseudomonas aeruginosa* bacteria.[2,3] Fish mucus also has diabetic wound-healing activity. In administering fish mucus gel with a concentration of 40%, the wound area is smaller than the positive control.[4] Apart from fish mucus, *A. testudineus* fish also have the potential to produce chitosan from *A. testudineus* fish scales.[5]

Chitosan is a deacetylated form of chitin. Chitin is the second most abundant biopolymer after cellulose. Sources of chitin: shells of crustaceans, cell walls of fungi and algae, exoskeletons of insects, and mollusks. Commercial chitin, (1,4)-2-(acetylamino)-2-deoxy-beta-D-glucan, is obtained by demineralization and deproteinization processes, mainly from crustacean shells. Commercial chitin, (1,4)-2-(acetylamino)-2-deoxy-beta-D-glucan, are a by-product of the seafood processing industry. The seafood processing industry creates a tremendous amount of waste each year. About 75% of the total body mass of crustaceans ends up as by-products (head, tail, backbone, and shell). Due to a lack of better waste management, raw seafood materials are often dumped back into the sea, incinerated, or left to rot, causing environmental problems. Therefore, the extraction of chitin and other valuable substances from crustacean waste is an alternative that can reduce this waste and is an essential step in producing valuable compounds with critical biological features and applications in various fields. According to the 2018 State of World Fisheries and Aquaculture Report by FAO (Food and Agriculture Organization of the United Nations), the global production of agricultural food fish includes about 7 million tons. Once the edible parts are removed, the remaining material is an essential source of chitin as well as proteins, lipids, pigments, and other molecules.[6]

Chitosan has antimicrobial, hemostatic, biocompatible, and biodegradable effects, which are essential in acute wound healing.[7] One of the tests of wound healing activity is testing on rat models of diabetes mellitus, which are wound-initiated. Until now, no study that tested the diabetic wound healing activity of *A. testudineus* fish scales extracts containing chitosan in rats with diabetes mellitus that were initiated by injury. This study aims to determine the diabetic wound healing activity of fish scale extract *A. testudineus*.

METHODS

Research Design

This study used the true experimental method with a posttest-only with a control group design. The study consisted of 5 treatments: negative control (gel-based), positive control (octenilin®), 5%, 10%, and 15% chitosan gel from fish scale.

Material

A. testudineus fish was obtained from the Martapura market, Banjar Regency, South Kalimantan, Indonesia, and identified at the Faculty of Fisheries and Marine ULM Banjarbaru

Extraction of A. testudineus fish scales

Extracting *A. testudineus* fish scales uses the Djaenudin et al., 2019 method with modifications. The process of extracting chitosan from

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A. testudineus fish scales was carried out in 3 stages: deproteination, demineralization, and deacetylation. The deproteination step used 0.3 M NaOH at 1: 15 (w/v) for 1 hour at 80^0C. When finished, the material will be washed until the pH is neutral using aquadest and can proceed to the demineralization stage. The demineralization stage used 0.55 M HCl in a 1: 10 (w/v) ratio for 1 hour at room temperature. After completing the demineralization stage, the material obtained is washed with distilled water until the pH is neutral, and then proceed to the deacetylation stage. The deacetylation step used 50% NaOH with a 1: 20 (w/v) ratio for 4 hours at 120^0C.[8]

Determination of the Degree of Chitosan Deacetylation.

The degree of deacetylation (DD) of the chitosan samples was calculated using the FTIR results. The DD calculation was carried out by calculating the ratio between the absorbance bands in the FTIR spectrum of the amide I band at a wavelength of 1655 cm^{-1} with the hydroxyl group at a wavelength of 3450 cm^{-1}. The DD calculation formula can be seen below:[5]

$$DA\ (\%) = \frac{A1655}{A3450} \times \frac{100}{1{,}33}$$

$$DD\ (\%) = 100 - DA$$

Making Diabetes Mellitus Rat Models

In this study, the *Rattus novergicus* Wistar strain was used with the criteria of male rats weighing 200-300 grams. The mice were obtained from the UGM Integrated Research and Testing Laboratory, Yogyakarta. The rats were acclimatized for at least seven days. On the 8th, the rats were checked for blood glucose levels, and 45 mg/Kg BW was injected with Streptozotocin (STZ). Blood glucose levels were checked on the third day after STZ administration. Rats were declared diabetes mellitus when their blood glucose level were at least 150 mg/dL. Furthermore, rats that meet the criteria will be continued on the wound healing test. [9]

Treatment of mice

The treatment method on rats followed the research of Rupina et al. (2016); rats with blood glucose levels> 150 mg/dL were treated using ketamine-xylazine 40 mg/Kg BW anesthetic and a wound area of 1.5 cm in diameter on the rat's back. Every day the rats will be smeared until the 14th day. After the 14th day, a skin sample will be taken. The skin sample will be made into a slide, and the skin reepithelialization will be seen.[10]

RESULTS

The results of the calculation of the DD of *A. testudineus* extract were 18.28%. This study was conducted on rats diagnosed with diabetes mellitus by giving STZ. The treatment was given for 14 days. The results of the histopathology of rat skin can be seen in the image in figure 2

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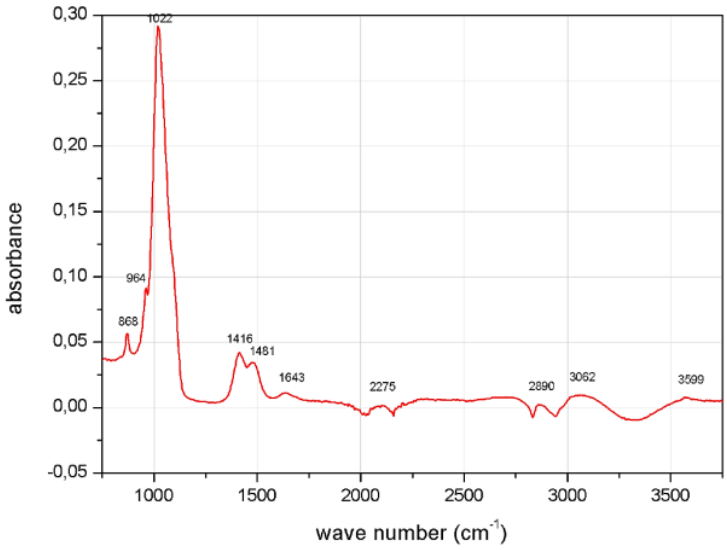

Figure 1. FTIR results of *A. testudineus* fish scale extract

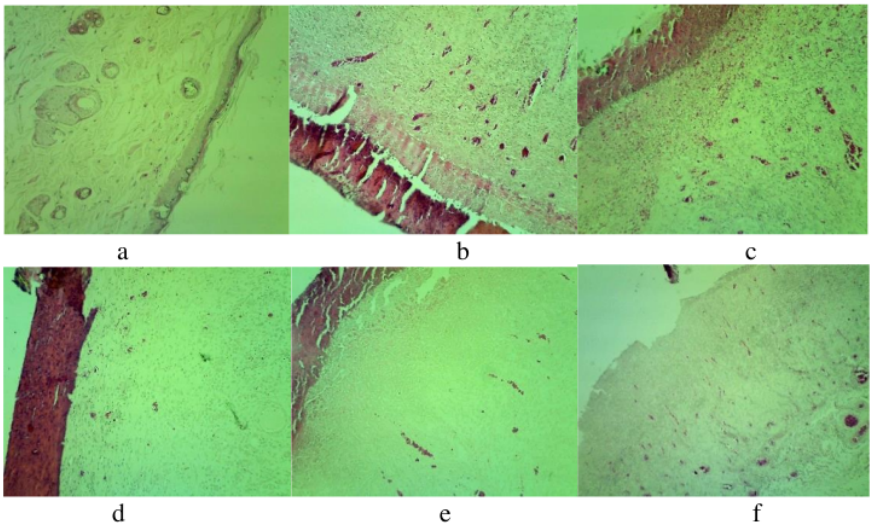

Figure 2. Histology of rats' skin after 14 days of treatment at 100 x magnification (a) normal skin of diabetic rats without treatment, (b) Skin of diabetic rats given a gel base, (c) Skin of diabetic rats given octenellin gel, (d) Diabetic rat skin given 5% chitosan gel, (e) Diabetic rat skin given 10% chitosan gel, (f) Diabetic rat skin given 15% chitosan gel

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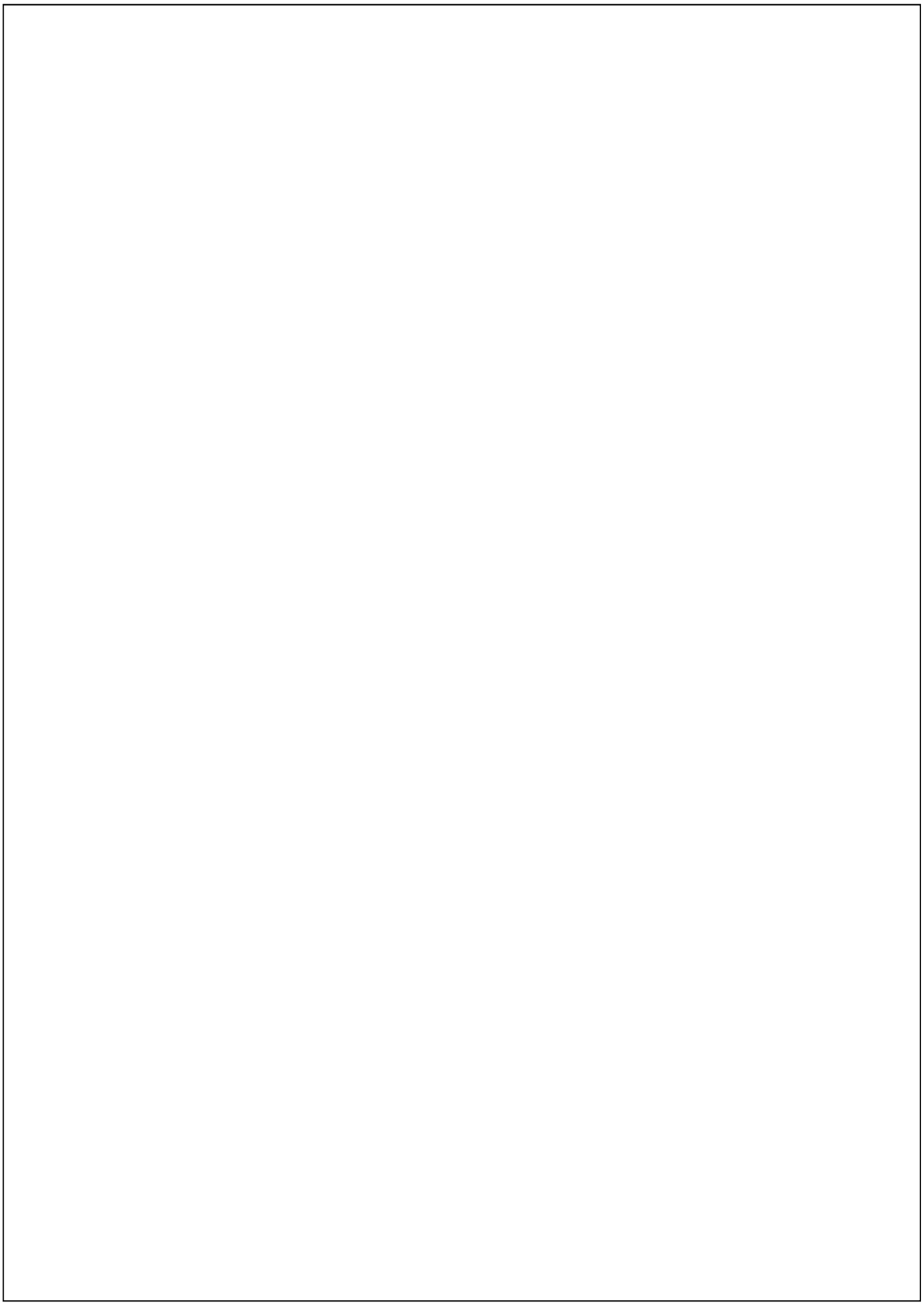

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DISCUSSION

A. testudineus fish is a native Indonesian fish that lives in fresh and brackish water habitats. Fish scales consist of the thin outer layer and the epidermis formed by epithelial cells. The layers beneath are the dermis, cutin, and corium. Under the dermis, there is a layer of cells containing chitin.[11]

The proportion of *A. testudineus* fish scales compared to the whole body weight of the fish is 1.67%. The scales of *A. testudineus* in a dry state had an ash content of 34.97 ± 2.01 %, a fat content of 3.85 ± 0.28 %, and protein content of 54.31 ± 2.05 %.[12] *A. testudineus* fish scales also contain Calcium (Ca) 26.74%, Phosphorus (P) 10.73%, Iron (Fe) 42.63 mg/Kg, Zinc (Zn) 19.63 mg/Kg, Potassium (K) 3658.02 mg/Kg, Sodium 278.56 mg/Kg,[12] carbohydrates 2.0-5.7%, chitin 0.4-3.7%.[11] Based on research by Irawan et al. (2018), fish scales of *A. testudineus* contained 14.0% SiO2, 5.0% Al2O3, 25.0% Fe2O3, 44.9% CaO, 6.8% P2O5, 1.9% ZnO and another 2.4%.[5]

Structure of chitin and chitosan is shown at figure 3. Chitin which is deacetylated, will produce chitosan. Chitosan is a biodegradable poly-saccharide and is considered biocompatible and non-toxic. Chitosan is the second most abundant polysaccharide compound after cellulose. Chitosan has been used in various applications ranging from cosmetics, artificial skin, wound healing, antimicrobial, photography, food, and nutrition. [11] Sources of chitosan come from mushrooms, shrimp shells, shells, insects, and fish scales.[13,14]

The chitosan extraction process goes through 3 stages. The first stage is the deproteinization process, which helps remove the high protein content in the cell wall. The reagent that is often used for the deproteinization process is NaOH. The second stage is the demineralization process. In this process, calcium carbonate minerals are removed from the chitin. At this stage, it will decompose calcium carbonate into water-soluble calcium salts with the help of acids. The acid commonly used is HCl. Residual salt is removed by filtering and washing with deionized water.[11,13] In the final stage, the process of deacetylating chitin to chitosan with the help of NaOH. The sodium hydroxide breaks the bond between the carbon contained in the acetyl group (-CH3COO) and the nitrogen in chitin so that the acetyl group will be released, and then the formation of an amine group (-NH2) occurs.[11] The results of the chitosan obtained will be analyzed using FTIR.

The FTIR results showed a peak at a wavelength of 964 cm-1, indicating the C-O-C bridge and glucosidic linkage of amides. At a wavelength of 1022 cm-1, it indicates the presence of C-O-C stretching bands, and at a wavelength of 1643 cm-1, it indicates the presence of an amide group. These three things are specific groups only in chitosan and not in chitin. In commercial chitosan, the C-O-C stretching bond is only present in chitosan, whereas, in chitin, there is no such bond. However, chitosan produced by insects, fungi, and fish shows the existence of this bond in both chitosan and chitin.[14]

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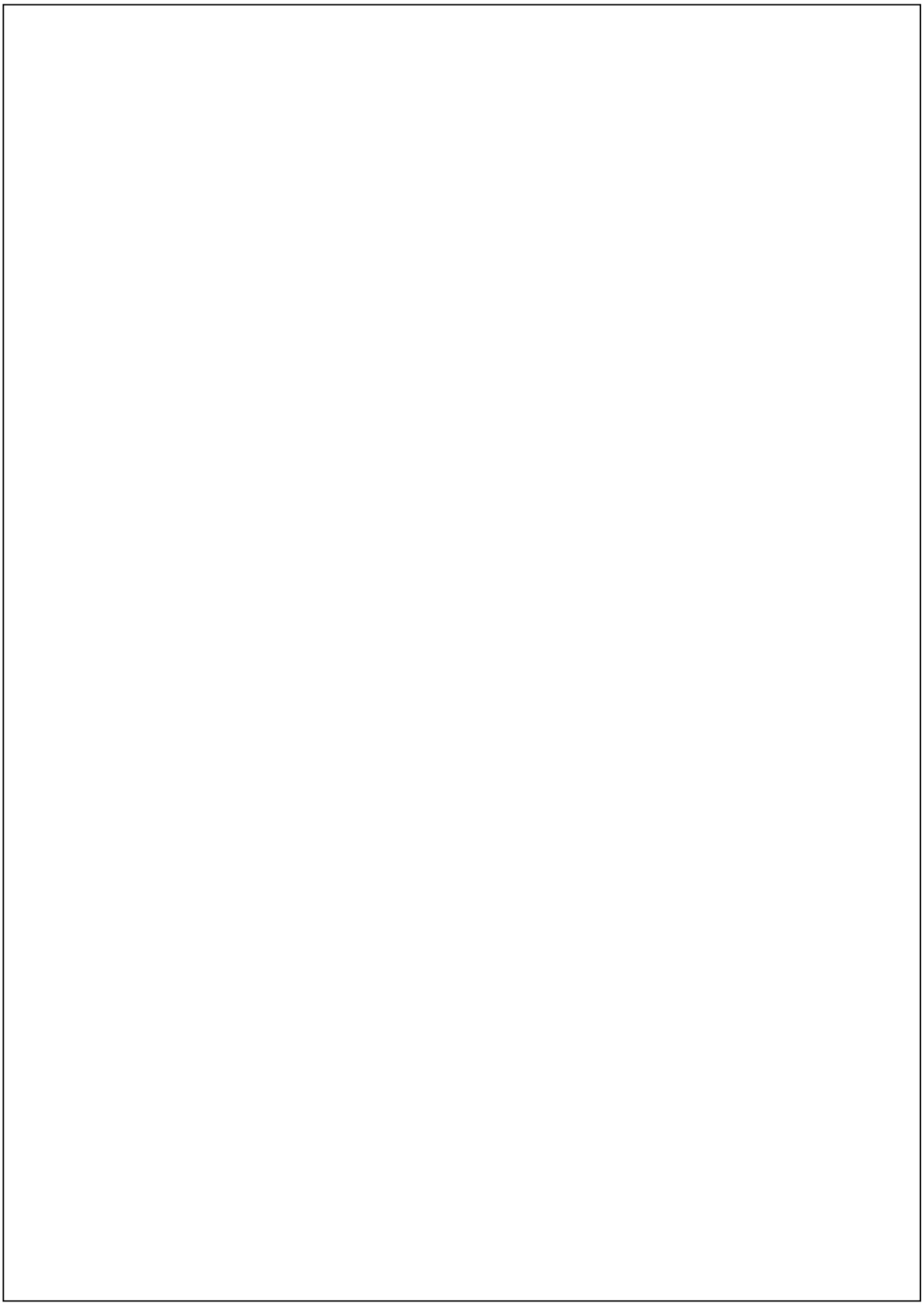

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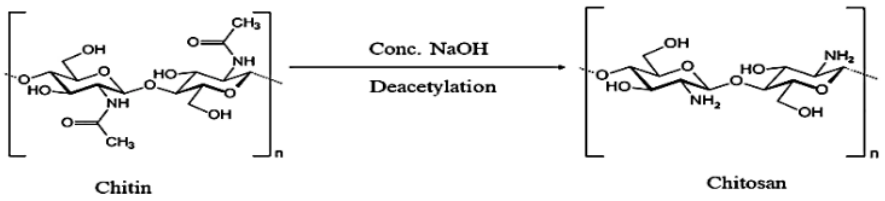

Figure 3. Structure of chitin and chitosan

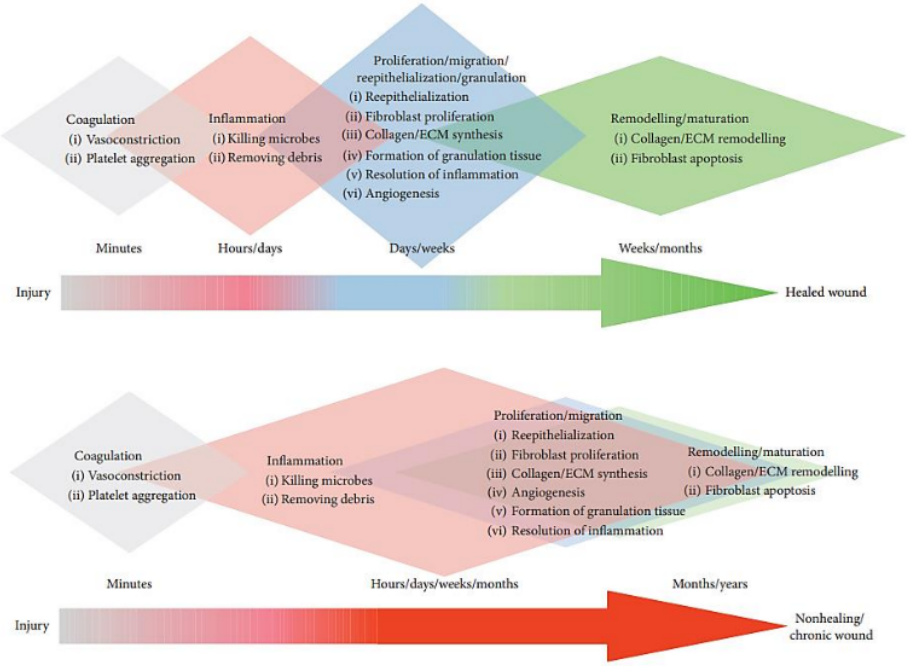

Figure 4. Wound healing cascade, (a) Normal wound healing cascade, (b) Nonhealing/chronic cascade.[18]

The results of the diabetic wound healing test of *A. testudineus* fish scales extract in rats seen from the histological results of the rat skin showed that bleeding, necrosis, and inflammatory cell debris were still occurring, and no epidermal epithelium was visible in all treatments. The wound healing cascade is shown at figure 4. In this research, all treatment epidermis and adnexia have not yet formed on the skin. and this condition is due to the lack of research time. In some studies, the wound healing test was carried out for 14 days with a wound size of 5-10 mm, while in this study, the wound area was 15 mm, so a more prolonged study was needed.[15] But if we look at the size of the wound, it can be seen that the concentration of 5% has a smaller wound area. This result is because chitosan is a compound that dissolves in

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acids [16] while the gel base used is neutral, so the more significant the concentration of the extract, the less it dissolves. The reduced amount of soluble extract causes a decrease in diabetic wound healing activity.[17]

There are four wound healing phases: the coagulation phase, the inflammatory phase, the proliferation/migration/reepithelialization/ granulation phase, and the remodeling/maturation phase (figure 4). In patients with diabetes mellitus, nonhealing/chronic wounds occur; in this condition, the inflammatory phase is prolonged. Diabetic wounds exhibit a persistent inflammatory phase associated with an impediment in forming mature granulation tissue and a reduction in wound tensile strength. This prolongation of the inflammatory phase results in a longer healing time.[18, 19]

CONCLUSION

The FTIR results show that *A. testudineus* fish scale extract contains chitosan. It is necessary to optimize the extraction and wound healing test of fish scales *A. testudineus*.

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