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Research Report

Star fruit leaves (*Averrhoa bilimbi*) extract and shrimp shell chitosan gel improves neovascularization in gingival wound healing *in vivo*

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ABSTRACT

Background: Gingival incision is a procedure widely used in dentistry that can potentially cause infection. Star fruit leaves (*Averrhoa bilimbi*) extract contains saponins, tannins, flavonoids, and phenols that can act as antibacterials. Shrimp shells chitosan have good biocompatibility, biodegradability, non-toxicity, and antimicrobial properties. Star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan are made in gel preparations to accelerate drug delivery to target cells. **Purpose:** To specify the effect of applying a gel extract of star fruit leaves (*A. bilimbi*) and chitosan shrimp shells on the formation of new blood vessels in the healing process of gingival incision wounds. **Methods:** 25 males *Rattus norvegicus* needed to make an incision in the labial gingiva and were divided into five groups, namely the K-group (without any application), K+ (*aloe vera* gel application), and three treatment groups PI, PII, and PIII (5%, 10%, and 15% gel extract of Star fruit leaves (*A. bilimbi*) and shrimp shell chitosan). Topical application is carried out twice a day for seven days. Microscopic observations with hematoxylin-eosin staining were used to count the number of new blood vessels. The analysis used the one-way analysis of variance and the post-hoc Tukey Honest Significant Different method. **Results:** The treatment in the PII group showed significantly ($p < 0.05$) higher results than other groups. **Conclusion:** The formulation of 10% gel Star fruit leaves (*A. bilimbi*) extract and shrimp shells chitosan has increased blood vessels in the healing process of gingival incision wounds.

Keywords: Averrhoa bilimbi L.; chitosan; gel; neovascularization; wound healing; medicine

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INTRODUCTION

Gingival incisions are often performed in dentistry treatment, for example, in endodontic surgery, alveolar ridge augmentation, implants, odontectomy, dental resection, etc.[1] Incisions or postoperative wounds often cause complications, with a ratio of 14%–16%.[2] Physiologically, the injury will be accompanied process. und healing process. The wound healing process consists of several interrelated stages: hemostasis and coagulation, inflammation, inflammation, proliferation, and remodeling. remodeling.[3,4] Using wound medications can improve wound healing and reduce the risk of bacterial infection. infection.[5] One of the most common topical medications used during wound healing after an incision is verified as *aloe vera* gel or povidone-iodine.[6]

Povidone-iodine is antibacterial, and prevents inflammation.[6] The use of povidone-iodine 10% as an antiseptic has irritative and toxic weaknesses if it enters the blood vessels and, if used excessively, will cause the granulation process to be inhibited, itching, swelling, and dermatitis in the wound area.[7] The content of *aloe vera* gel, that is acid, if ingested more than 10 mg/day, can interfere metabolism.[8] Thus, many alternative biomaterials have been developed to overcome wound healing after prolonged gingival incisions.

Indonesia is rich in biodiversity and animals, such as Star fruit leaves (*Averrhoa bilimbi*) and shrimp. Star fruit leaves contain flavonoids, phenols, saponins, triterpenoids, alkaloids, tannins, and coumarins.[9] Flavonoids, phenols, tannins, and saponins are active substances that act antibacterially by affecting permeability and disrupting the formation of bacterial cell walls, even causing bacterial lysis. The antibacterial ability of the active compounds in star fruit leaves (*A. bilimbi*) is essential to eliminating bacteria or microorganisms in the wound area and preventing infection.[10] The compounds in star fruit leaves are classified as 10-100 mg/L or toxicity in water, which has a moderate toxic level.[11] Suppose a toxicity test is carried out using a fixed dose method with blood biochemical parameters in male white rats of the Wistar strain (*Rattus*

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novergicus). In that case, the toxicity results of star fruit leaves (*A. bilimbi*) extract will be obtained, including acute toxicity, which means it has no effect on the liver, kidneys, or heart if consumed by the organism.[12]

Shrimp is a type of fish that lives in brackish water, shelled, and if it goes through the deacetylation process, it will produce chitosan. Chitosan contains lysozyme groups and aminopolysaccharide groups that inhibit microbial growth. Chitosan can inhibit bacterial growth because it has a positively charged polycation.[13] In addition, chitosan has good biocompatibility during wound healing due to its ability to stimulate cell proliferation, increase collagenization, and accelerate cell regeneration.[7]

Chitosan is a non-toxic polysaccharide that is quickly biodegradable. Chitosan has a structure similar to cellulose and can form a gel in an acidic atmosphere. Thus, chitosan has properties as a matrix for drug delivery systems. Chitosan is a bioadhesive that readily adheres to negatively charged surfaces, including membranes. Non-viral gene transfer using trimethylchitosan as a derivative. Chitosan also protects against fungal infections and can clot blood quickly.[14–16] Based on the description above, researchers are interested in using Star fruit leaves (*A. bilimbi*) extract and shrimp shells chitosan as an alternative herbal medicines to enhance new blood vessel formation or neovascularization during the healing process of gingival incision wounds*, in vivo.*

MATERIALS AND METHODS

This research uses laboratory experimental research with true experimental *in vivo* with a randomized post-test-only control group that has been ethically tested with number 082-KEP-UB-2021. This study was divided into five groups, namely K- (control group without treatment), K+ (control group that used verified *aloe vera* gel), and three treatment groups (PI, PII, and PIII) (star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel with concentrations of 5%, 10%, and 15%).

Materials used in this research are electric scales, refrigerators (2°–8°C), freezers (-14°–24°C), autoclaves, rotary evaporators, ovens, grinders, measuring cups, Buchner funnels, sonicators, homogenizers, blades No. 11, handle scalpels No. 3, Erlenmeyer flasks, watches, vials, incubators, rat cages, drinking bottles and mouse feed containers, tweezers, syringes, one cc, chloroform, cotton buds, chitosan shrimp shell powder, formalin buffer 10%, NS, alcohol 70%, ethanol 96%, HPMC,propylene glycol, methylparaben, aloclair gel, sterile gauze, xylazine, wood shaving, ketamine, and aquades.

Star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel are made according to the formulation design shows in Table 1. The base used in making gels of Star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan is hydroxypropyl methylcellulose (HPMC). The aqua-distillate was heated to 80°C, and HPMC was added little by little, stirred until fluffy, and then crushed until homogeneous to develop HPMC (phase I). In separate containers, nipagin and nipasol were dissolved in propyleneglycol, and then nanoparticles of star fruit leaves extract and chitosan shrimp shells were added (phase II). Little by little, phase II is inserted into phase I while being stirred continuously. Then, the remaining aqua-distillate is added and stirred until homogeneous.[17]

This study used male Wistar Rats (*R. norvegicus)* 150 to 200 grams body weight, 2 to 3 months old, and up to twenty-five animal models had undergone acclimation for one week before receiving treatment. All samples were anesthetized using a single intramuscular injection of 0.2 ml of ketamine before incision. The gingiva of the sample was smeared with betadine, and an incision using scalpel number 11, 5 mm long, was made on the labial gingiva under the two anterior teeth of the mandible as deep as the alveolar bone. After incision, aquades irrigation is carried out. Each group consists of 5 rats. Topical application Star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel were applied to the gingival incision wounds and given twice daily for six days.[18]

The gingival labial mucosal tissue was taken and fixed using 10% formalin on the seventh day after the incision, with all rats being decapitated by cervical dislocation. Histological preparations are made from tissue samples by cutting tissue 5–6 µm thick and staining with Hematoxylin Eosin (HE). Histopathological observations are carried out using a light microscope to observe and count the number of blood vessels in as much as a 400x 8 field of view. Microscopically observed identification of blood vessels by HE staining, that is, a thin and slightly elongated picture with dimensions of about 30–50 mm, width of 10–30 mm, and thickness of 0.1–10 mm. In addition, the shape is a layer of endothelial cells forming a circle with a lumen containing red erythrocytes in the layer of endothelial cells on the preparation slide.[19] The statistical analysis by means of the statistical package for social science (SPSS) (IBM Corporation, Illinois, Chicago) was done by performing the one-way analysis of variance (ANOVA) and the post-hoc Tukey Honest Significant Difference (HSD) ($p<0.05$).

Table 1. Gel formulation of Star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan

Material	FI (g)	FII (g)	FIII (g)	Information
Star fruit leaves extract nanopartcles-chitosan shrimp shells	5	10	15	Active Ingredients
HPMC	10	10	10	Gel Base
Nipagin	0.075	0.075	0.075	Preservatives
Nipasol	0.025	0.025	0.025	Preservatives
Propylene Glycol	15	15	15	Humectants
Aqua-distillate		Ad 100 ml		Solvent

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RESULTS

Figure 1 shows an image of blood vessels in the gingiva of rat's post-incision seen with a 400x magnification microscope. Figure 2 shows that the average number of blood vessels is highest in the PII group, then decreases successively in the PIII, PI, K+, and K- groups. Data on the number of neovascularities were analyzed using the Shapiro-Wilk normality test to see whether or not the distribution of the data used in this study was normal. On the other hand, testing for homogeneity is performed using the Levene test to see whether the variance of the data is homogenous or not. Then, we continued the analysis of the one-way ANOVA test. Based on one-way ANOVA analysis, it is known that there are significant differences between these groups. Then, we proceeded to use Tukey HSD post-hoc test to determine whether one group had significant differences from others.

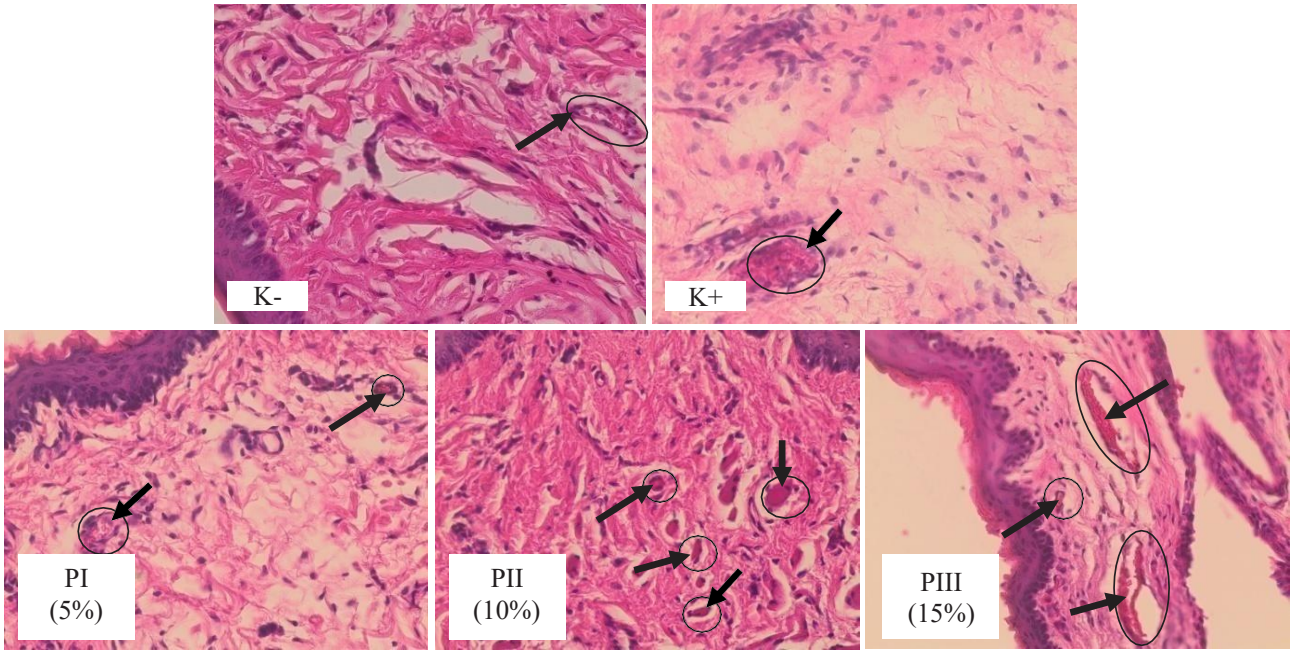

Figure 1. Neovascularization in gingiva of the sample after treatmentInformation: K- (control group without treatment), K+ (control group that used verified *aloe vera* gel), and three treatment groups (PI, PII, and PIII) (star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel with concentrations of 5%, 10%, and 15%).

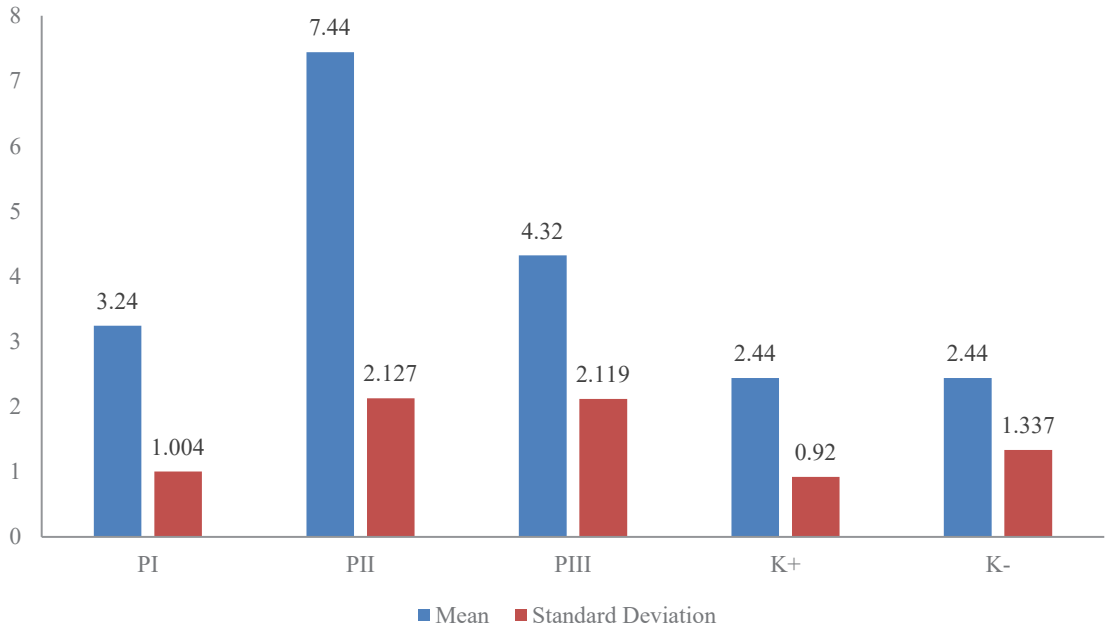

Figure 2. Graph of the calculation of the average number of neovascularizations in each group.

Table 2. Post-Hoc Tukey HSD test results with negative control group comparison (K-) and positive control group comparison (K+)

Tukey HSD Post-Hoc Test	Average	P value
K- vs PI	-0.800	1.000
K- vs PII	-5.000*	0.010
K- vs PIII	-1.880	0.765
K- vs K+	0.000	1.000
K+ vs PI	-0.800	1.000
K+ vs PII	-5.000*	0.001
K+ vs PIII	-1.880	0.765
K+ vs K-	0.000	1.000

Table 3. Post-Hoc Tukey HSD test results with a comparison of the treatment group with a concentration of 5% (PI), 10% (PII) and 15% (PIII) of star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel

Tukey's HSD Post-Hoc Test	Concentration	Average	P value
PI vs PII	5%	-4.200*	0.005
PI vs PIII		-1.080	1.000
PI vs K+		0.800	1.000
PI vs K-		0.800	1.000
PII vs PI	10%	4.200*	0.005
PII vs PIII		3.120	0.056
PII vs K+		5.000*	0.001
PII vs K-		5.000*	0.001
PIII vs PI	15%	1.080	1.000
PIII vs PII		-3.120	0.056
PIII vs K+		1.880	0.765
PIII vs K-		1.880	0.765

Information: *: there is a significant difference ($p<0.05$).

The Table 2 and 3 shows that the PII group improved significantly compared to other groups. Meanwhile, the PI, PIII, K+, and K-groups did not have significant differences. However, when compared to the treatment group with a 15% concentration of star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel (PIII), no group had a significant difference from the PIII group.

DISCUSSION

The results of gingival incision wound observations *in vivo* showed that star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel could accelerate wound healing. Wound healing will occur faster if the neovascularization process or blood vessel formation occurs quickly. This is due to the active substances in star fruit leaves extract, namely flavonoids, alkaloids, phenols, and steroids, which can inhibit the growth of bacteria.[10] The ethanol extract of star fruit leaves has vigorous antioxidant activity and high anti-inflammatory activity.[20,21] These antioxidants and anti-inflammatory effects prevent bacteria from entering the wound, thus accelerating wound healing.

In this study, star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel application was carried out from the first day to the seventh day, estimated to have reached the proliferation phase.[22] In histopathological observations, it was found that the average number of neovascularizations was most significant in the second treatment, where the treatment was given star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel with a concentration of 10%. From the results of the analysis, it was found that the average number of neovascularizations was most significant in the second treatment (PII). Indeed, at a 10% gel concentration extracted from star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel showed significant differences compared to the control group and other treatment groups. In addition, high concentrations have a high level of toxicity as well.[20]

Statistical analysis results by one-way ANOVA test show that treatment with 10% concentration of PII group gives better results than PI, PIII, positive control, and negative control groups. This is consistent with research conducted by Hartini, showing that the 10% concentration group of star fruit leaves (*A. bilimbi*) extract has the best healing ability compared to the 20% concentration group of star fruit leaves (*A. bilimbi*) extract, in which the concentration of the star fruit leaves (*A. bilimbi*) extract group has the best healing ability. A 10% concentration indicates a higher number of fibroblasts in the wound area.[23]

In addition, applying ointment with a concentration of 10% resulted in faster wound healing without signs of inflammation or wounds in the maturation phase on the eighth day compared to other treatment groups and control groups. Indeed, the flavonoid, saponin, and tannin content can accelerate the inflammatory and proliferative phases as well as the maturation phase.[24] The most effective dose for wound healing, such as increasing fibroblast cell count and collagen fiber density score on the seventh day, was a 10% concentration compared with verified *aloe vera* gel.[25]

CONCLUSION

The most effective gel application of star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel with a 10% concentration. It also showed significant differences among the three treatment groups, namely 5%, 10%, and 15% concentrations, and the control groups, namely positive and negative control. Of the three treatment groups, the treatment group with a 10% concentration of star fruit leaves (*A. bilimbi*) extract and shrimp shell chitosan gel has the

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concentration that can increase neovascularization most significantly, so that the inflammatory phase will run faster and the wound-healing process will occur more quickly.

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