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In Vitro Wound Healing Potential of Stem Extract of *Spatholobus littoralis* Hassk

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Abstract

The post-surgical wound healing phase involves an inflammatory response and fibroblast migration. The stem of Spatholobus littoralis Hassk. has been well known to play a role in wound healing. The objective of this study was to examine the stem extract of S. littoralis as an anti-inflammatory agent and accelerator of fibroblast migration. Protein denaturation inhibition method for the anti-inflammatory on 7 groups in ppm concentration: 250, 500, 750, 1000, 1600, negative and positive control. The wound scratch assay method for fibroblast migration on 10 groups: 1, 2.5, 5, 10, 50, 100, 500, 1000, negative and positive control. Statistical analysis showed that there was a significant difference ($p < 0.05$) in the percentage of the protein denaturation inhibition in the studied groups ($p = 0.000$). Potential anti-inflammatory agents are 750, 1000, and 1600.

There was a significant difference ($p < 0.05$) in the percentage of wound closure according to the concentration, the observation time, between the concentration and the observation time ($p = 0.000$). The most effective are 500 and 1000 with wound closure reaching 100% at 22 hours. S. littoralis is potential for wound healing as an anti-inflammatory agent and acceleration of fibroblast migration.

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Introduction

The surgical extraction is a method of extracting teeth which is employed when normal forceps extraction is not possible due to various difficulties.[1] This process may result in injury to the area surrounding the extraction.[2] Wound healing of the skin or oral mucosa occurs in several stages, starting with a haemostatic event followed by an inflammatory response, a proliferative phase with the formation of new and extracellular matrix components, and ended with a remodelling phase involving the reorganization of the matrix to provide functional tissue.[3] The acute inflammatory phase occurs between the first 24 hours and the third day.[4] This period is characterized by the presence of cellular activity, namely the movement of neutrophils from blood vessels to the wound site, resulting in an increase between 24 and 48 hours and then a decrease after the third day.[4]

The surgical extraction of teeth needs a flap removal procedure and bone cutting that causes a severe inflammatory response accompanied by pain.[5] There are 63.5% of patients experience severe pain in the first 24 hours after surgery.[5] Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly used drugs to treat pain and inflammation.[6] NSAIDs have anti-inflammatory, analgesic, and antipyretic effects.[7] However, their use has side effects on the cardiovascular, renal, and gastrointestinal systems.[6,7]

In the proliferative phase, fibroblasts are the main component in the wound healing process.[8] Fibroblasts play a role in the synthesis of collagen, elastin, glycosaminoglycan, proteoglycans, and multi-adhesive glycoproteins.[9] Fibroblasts begin to appear in the injured area after the third day of the injury.[9] Migration of fibroblasts will occur between 48 and 72 hours after the injury.[10] Fibroblasts will replace the damaged tissue and regenerate tissue from the wound edges.[11]

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Medicines from natural ingredients are still often used by Indonesian people to reduce the side effects that cause by chemical drugs.[12] One of the natural ingredients that can be used for wound healing is the stem of *Spatholobus littoralis* Hassk.[13] *S. littoralis* is a plant that can be found in the hinterland of Central Kalimantan Province, Indonesia.[13] Based on a study conducted by Saputera et al., the stem extract of *S. littoralis* has been proven to have an effect on the wound healing process in the back area, parallel to the vertebrae of white male rats.[13] The purpose of this study was to determine the effectiveness of the stem extract of *S. littoralis* as an anti-inflammatory agent and accelerator of fibroblast migration in the in vitro wound healing process.

Materials and methods

Plant Collection and Extract Preparation

The stem of *S. littoralis* were obtained from Muara Teweh, Central Kalimantan, and tested for determination at the Laboratory of the Research Institute for Spices and Medicinal Plants, Bogor (Letter no. B-926/IPH.3/KS/VIII/2020). The extract of *S. littoralis* stem was processed at the BioCORE Laboratory, Faculty of Dentistry, Universitas Trisakti. The stem of *S. littoralis* were dried under indirect sunlight for 20 days and then crushed until becoming powder using a blender. Furthermore, it was sieved using a 60-mesh sieve. After that, the powdered stem of *S. littoralis* was macerated using ethanol 70% for 3 days. The result of the extraction was evaporated using the Buchi R-215 Rotavator System (Germany) to obtain a thick extract of *S. littoralis* stem which was then stored at -20°C.

In vitro Anti-Inflammation with Protein Denaturation Inhibition Method

The anti-inflammatory effect of the stem extract of *S. littoralis* was assessed by protein denaturation inhibition method using bovine serum albumin 5% (Sigma-Aldrich). In this study, researchers used 7 groups, namely the stem extract of *S. littoralis* with concentrations of 250 ppm, 500 ppm, 750 ppm, 1000 ppm, 1600 ppm, positive control, and negative control. In this method, the product control group was used, which was the group with the concentration adjusted according to the concentration in the studied group to be used in the percent inhibition formula.[14] The treatment in each studied group was repeated 5 times. 0.45 mL of bovine serum albumin 5% (Sigma-Aldrich) was put into the test tube of the studied groups, positive control group, and negative control group. After that, 0.05 mL of stem extract of *S. littoralis* was added to each studied group. The positive control test tube was filled with 0.05 mL of 250 ppm diclofenac sodium and the negative control test tube was filled with 0.05 mL of distilled water. The product control test tubes were filled with 0.45 mL of distilled water and refilled with a solution that will be adjusted to the studied groups, namely 0.05 mL of the stem extract of *S. littoralis* (its concentration was adjusted with the studied groups), 250 ppm diclofenac sodium, or distilled water. All test tubes were incubated at 37°C for 20 minutes. After that, the temperature was increased to 57°C and then the tubes were incubated again for 3 minutes. Furthermore, the solution was cooled at room temperature and given 2.5 mL of phosphate buffer saline (pH 6.3), and then vortexed.

The researchers then conducted the measurement of the absorbance of the samples at a wavelength of 415 nm using the iMark™ Microplate Absorbance Reader. After that, the percentage of protein denaturation inhibition was calculated using the formula of %inhibition.[14]

$$100 - \frac{\text{AB test solution} - \text{AB product control}}{\text{AB negative control}} \times 100$$

In vitro Wound Scratch Assay Method

The migration rates of fibroblast cells were assessed by the scratch assay method. Fibroblasts were harvested from the culture flask and then transferred to a 35-mm dish. Each well contained 300,000 cells in 1 mL of culture media of Dulbecco's Modified Fetal Medium (DMEM), Fetal Bovine Serum (FBS) 10%, and Amphotericin-Penicillin-Streptomycin 1%. They were then incubated using an incubator with 5% CO_2 content at 37°C to 80% confluence. The fibroblast migration test was carried out by making a scratch on each well that contained fibroblasts using a 200-μL yellow pipette tip. After that, the medium was discarded and washed with PBS. Each well was given the stem extract of *S. littoralis* at concentrations of 1 ppm, 2.5 ppm, 5 ppm, 10 ppm, 50 ppm, 100 ppm, 500 ppm, and

1000 ppm, while Aloclair™ served as a positive control, and cells without treatment served as a negative control. The treatment for all groups was reduplicated 3 times. Fibroblasts were observed at 0, 4, 8, 12, 22, 24, and 48 hours after being given the treatment, using an inverted microscope (ZEISS) with a magnification of 5x (100 μm scale). Wound closure was measured using ImageJ software and then input into the formula of %Wound closure[15] to calculate the percentage of wound closure.

$$\%\textit{Wound closure} = [(A_{t=0h} - A_{t=4h \text{ or } 8h \text{ or } 12h \text{ or } 22h \text{ or } 24h \text{ or } 48\ h}) / A_{t=0h}] \times 100$$

Where: $A_{t=0h}$: The total area of wound closure after 0 hour. $A_{t=4h \text{ or } 8h \text{ or } 12h \text{ or } 22h \text{ or } 24h \text{ or } 48\ h}$: The total area of wound closure after 4, 8, 12, 22, 24 or 48 hours.

Statistical Analysis

In this study, researchers performed an anti-inflammatory test using one-way ANOVA, followed by the Tuckey Post-Hoc test. Furthermore, for examining the fibroblast migration, researchers employed two-way ANOVA to determine the effect of extract concentration, observation time, and between extract concentration and observation time on the acceleration of fibroblast migration. The analysis was further conducted by the Tuckey Post-Hoc test to find out the presence of significant differences from each group.

Results

Results of the Phytochemical Test

The phytochemical test of stem extract of *S. littoralis* was conducted at the Laboratory of the Research Institute for Spices and Medicinal Plants, Bogor, Indonesia. The results can seen in Table 1.

No.	Samples	Types of Phytochemical Test	Results	Method
1.	The stem extract of *S. littoralis*	a. Alkaloids b. Saponins c. Tannins d. Phenolics e. Flavonoids f. Triterpenoids g. Glycosides	+ + + + + + +	Qualitative

Table 1. Results of the Phytochemical Test.

The effect of *S. littoralis* extract on protein denaturation inhibition

Analysis using one-way ANOVA showed a significant difference in the percentage of protein denaturation inhibition in the studied groups, p= 0.000 ($p < 0.05$). Tuckey Post-Hoc test on the negative control group with the percentage of protein denaturation inhibition of 6.09%, indicating a significant difference with the *S. littoralis* stem extract groups at concentrations of 500 ppm p= 0.039 ($p < 0.05$), 750 ppm, 1000 ppm, and 1600 ppm p = 0.000, in which the percentages of protein denaturation inhibition were 16.50%, 24.78%, 32.20%, and 41.21%, respectively. *S. littoralis* stem extract group at 250 ppm with a percentage of protein denaturation inhibition of 11.28%, showed no significant difference with negative control p = 0.662 ($p > 0.05$). However, the percentage of protein denaturation inhibition at 250 ppm was higher compared to the negative control. The result of data analysis on the positive control group with the percentage of protein denaturation inhibition of 81.50%, indicating a significant difference with the *S. littoralis* stem extract groups at 250 ppm, 500 ppm, 750 ppm, 1000 ppm, and 1600 ppm p= 0.000 ($p < 0.05$). The percentage of protein denaturation inhibition of *S. littoralis* stem extract at 250 ppm and 500 ppm showed no significant difference p= 0.657 ($p > 0.05$). However, the *S. littoralis* stem extract group with a concentration of 500 ppm had a higher percentage of protein denaturation inhibition.

The results of the percentage of protein denaturation inhibition in the studied group showed in Table 2.

Groups	Percentage of protein denaturation inhibition (%)	*p*-value
Extract with 250 ppm	**11.28 ± 3.02**	0.000
Extract with 500 ppm	**16.50 ± 0.27**	
Extract with 750 ppm	**24.78 ± 3.19**	
Extract with 1000 ppm	**32.20 ± 7.15**	
Extract with 1600 ppm	**41.21 ± 7.39***	
Positive control	**81.50 ± 6.99***	
Negative control	**6.09 ± 1.44***	

Table 2. The average percentage of protein denaturation inhibition.

*In the same column, it shows a significant difference ($p < 0.05$).

Extract Concentration	Fibroblast migration (%)					
	Hour 4	Hour 8	Hour 12	Hour 22	Hour 24	Hour 48
1 ppm	2.3 ± 1.3*	12.7 ± 2.7	20.4 ± 3.3	31.5 ± 1.5	45.1 ± 14.7	70.5 ± 27.0
2.5 ppm	3.8 ± 1.6	14.1 ± 3.4	22.3 ± 4.7	40.9 ± 9.1	45.8 ± 5.3	74.5 ± 13.5
5 ppm	7.3 ± 0.4	16.0 ± 8.9	23.5 ± 7.5	45.9 ± 19.5	61.7 ± 18.6	88.3 ± 16.6
10 ppm	9.4 ± 3.2	18.0 ± 2.9	24.7 ± 0.5	52.3 ± 12.4	69.5 ± 4.7	100.0 ± 0.0*
50 ppm	10.2 ± 1.0	18.7 ± 2.8	30.7 ± 12.9	83.3 ± 14.7	92.3 ± 13.3	100.0 ± 0.0*
100 ppm	12.4 ± 2.2	24.8 ± 6.4	39.8 ± 10.4	84.2 ± 17.9	93.0 ± 6.9	100.0 ± 0.0*
500 ppm	13.6 ± 2.4	31.4 ± 6.8	50.2 ± 13.3	100.0 ± 0.0*	100.0 ± 0.0*	100.0 ± 0.0
1000 ppm	16.5 ± 1.1	40.1 ± 14.4	55.4 ± 15.3	100.0 ± 0.0 *	100.0 ± 0.0 *	100.0 ± 0.0*
C (-)	2.4 ± 1.0	6.5 ± 1.0	11.5 ± 4.7	30.5 ± 6.8	60.0 ± 11.6	96.4 ± 6.2
C (+)	17.6 ± 3.0	43.7 ± 7.3	57.0 ± 5.5	100.0 ± 0.0*	100.0 ± 0.0*	100.0 ± 0.0*

Table 3. Average fibroblast migration at different concentrations and observation times.

*In the same row and column, it shows a significant difference ($p < 0.05$).

The effect of *S. littoralis* extract on fibroblast migration

The observation of fibroblast migration on 10 groups with concentrations: 1 ppm, 2.5 ppm, 5 ppm, 10 ppm, 50 ppm, 100 ppm, 500 ppm, and 1000 ppm, negative, and positive control conducted at 0, 4, 8, 12, 22, 24, and 48 hours. The observation is carried out using an inverted microscope (ZEISS) with a magnification of 5x (100 μm scale) in each studied group (Figure 1).

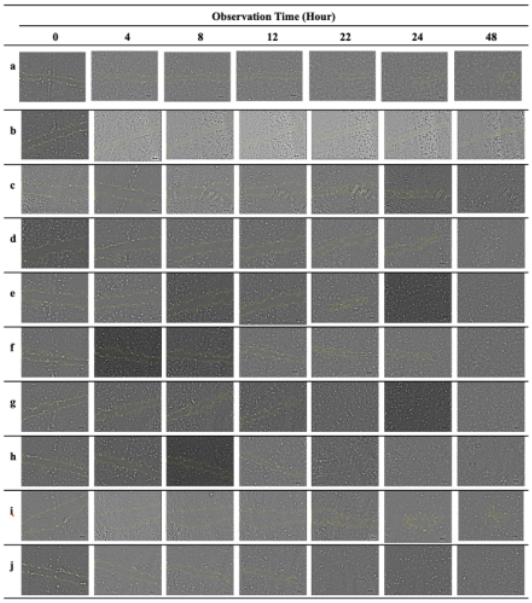

Figure 1. The results of the observation of the fibroblasts migration using the scratch assay method in the *S. littoralis* stem extract groups at concentrations of 1 ppm (a), 2.5 ppm (b), 5 ppm (c), 10 ppm (d), 50 ppm (e), 100 ppm (f), 500 ppm (g), 1000 ppm (h), with the negative control (i), and the positive control (j) with observation time at hours 0, 4, 8, 12, 22, 24, and 48 with a magnification of 5x (100 μm scale)

In this study, the observation of fibroblast migration in the *S. littoralis* stem extract with concentrations of 1 ppm, 2.5 ppm, 5 ppm, 10 ppm, 50 ppm, 100 ppm, 500 ppm, and 1000 ppm, negative, and positive control is carried out with an observation time of 0, 4, 8, 12, 22, 24, and 48 hours. Data analysis using two-way ANOVA showed that there was a significant difference in the concentration of the *S. littoralis* stem extract, the observation time, and between the concentration of the *S. littoralis* stem extract and the time of observation p=0.000 ($p < 0.05$). The description of fibroblast migration in all studied groups can seen in Figure 1.

Tuckey Post-Hoc test on the *S. littoralis* stem extract when compared to the negative control showed a significant difference in the *S. littoralis* stem extract at 5 ppm with the observation time at 48 hours, p=0.048 ($p < 0.05$); 50 ppm with the observation time at 22 hours, p=0.000; 500 ppm with the observation time at 22 hours, p=0.000 and p= 0.019 at 24 hours, and 1000 ppm with the observation time at 12 hours p=0.003; p=0.000 at 22 hours, and p=0.019 at 24 hours. Furthermore, all concentrations of the stem extract *S. littoralis* at the time observation at 4 hours, p=1.000 ($p > 0.05$), indicating no significant difference. In addition, for all concentrations of the stem extract *S. littoralis* at the time observation at 8 hours, there was no significant difference found in which the obtained p=1.000 for concentrations of 1 ppm, 2.5 ppm, 5 ppm, 10 ppm, and 50 ppm; p=0.999 for 100 ppm; p=0.853 for 500 ppm, and p=0.173 for 1000 ppm. Meanwhile, the stem extract of *S. littoralis* 500 ppm with an observation time of 12 hours had no significant difference, p=0.313.

Meanwhile, when the *S. littoralis* stem extract compared to the positive control, there was a significant difference in the *S. littoralis* stem extract groups of 1 ppm and 2.5 ppm with the observation time at 22 and 24 hours, p= 0.000 ($p < 0.05$), the concentration of 5 ppm with the observation time at 22 hours, p=0.000; at 24 hours, p=0.037; and at 48 hours, p= 0.012 and

the concentration of 10 ppm with the observation time at 22 hours, p= 0.000. The results of observations of fibroblast migration in all studied groups can be seen in Table 3.

On the migration of fibroblasts when compared between the study groups and time observations, based on the results from an inverted microscope (ZEISS) showed the percentage of wound closure reaching 100% in the *S. littoralis* extract group concentrations of 500 ppm, 1000 ppm and positive control at 22 hours. The results can be seen in Figure 2.

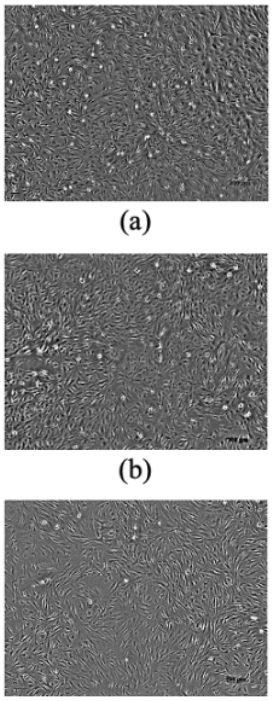

Figure 2. Fibroblast migration in the *S. littoralis* stem extract groups at concentrations of 500 ppm (a), 1000 ppm (b), and the positive control (c) at 22 hours.

Discussion

This study showed that the stem extract of *S. littoralis* at all concentrations could inhibit protein denaturation with an average increase in the percentage of inhibition. That is due to the occurrence of activity in natural ingredients that affect protein denaturation, in which the bigger of the concentration in these natural ingredients, the higher the number of active compounds will be.[16] Although all concentration groups of the stem extract of *S. littoralis* in this study can inhibit protein denaturation, only extract groups with concentrations of ≥ 750 ppm are proven to have an anti-inflammatory effects. This is based on the previous theory, that a compound is considered to have an anti-inflammatory effect if it has a protein denaturation inhibition percentage of more than 20%.[17] That is supported by a similar statement from Williams et al. that a compound that can inhibit protein denaturation of more than 20% is considered to be effective as an anti-inflammatory agent and can be developed as a potential anti-inflammatory.[18] However, the extract with the highest concentration in this study cannot match the anti-inflammatory potential of the diclofenac sodium used as the comparison. That is similar to the study conducted by Aditya et al., in which the concentration of natural ingredients used in their study has an anti-inflammatory effect through inhibiting protein denaturation, however the inhibition of protein denaturation produced is smaller than the protein denaturation inhibition of diclofenac sodium.[19] The result presumably due to differences in the working point of compounds between natural ingredients used and diclofenac sodium.[19] Furthermore, it is also due to low concentrations of active compounds that have anti-inflammatory effects on the natural ingredients utilized.[19] It takes larger concentration of extract to get a higher percentage of protein denaturation inhibition.[20] In other words, the higher the percentage of protein denaturation inhibition is the higher the anti-inflammatory potential possessed will be.[20]

The stem extract of *S. littoralis* has an anti-inflammatory effect presence of alkaloids, saponin, tannins, phenolic, flavonoids, and triterpenoids. That is in line with a previous study that showed the natural ingredients used in their study have potential as anti-inflammatory agents because they contain strong tannins, saponin, phenolic, and flavonoids.[21] Alkaloids, such as bisbenzylisoquinoline are known to be able to inhibit the synthesis of pro-inflammatory cytokines to restrain the inflammatory response.[22] Saponins, such as glycyrrhizin are known to be able to inhibit hyaluronidase, an enzyme that may cause the degradation of hyaluronic acid.[23] Excessive degradation of

hyaluronic acid has been reported to contribute to the progression of inflammation.[23] Tannins are well known to provide anti-inflammatory effects through inhibition of inflammatory mediators, such as cytokines, inducible nitric oxide synthase (iNOs), and COX-2.[22] Flavonoids have been proved to have anti-inflammatory abilities by inhibiting the production of pro-inflammatory cytokines and inducing anti-inflammatory cytokines, such as interleukin-1 receptor antagonists (IL-1RA).[24] In addition, flavonoids are also known to be able to inhibit the peroxidase active site on COX-2 and NF-κB, which increase the expression of COX-2.[24] Triterpenoids and alkaloids have also been reported to have anti-inflammatory.[22] Triterpenoids are known to have the ability to inhibit the release of histamine to suppress the inflammatory process.[25]

Based on the results of this study, the effect of 2.5 ppm concentration of the stem extract of *S. littoralis* on the acceleration of fibroblast migration was not significantly different from the negative control group. That findings contradict the finding from Berbudi et al., that the natural ingredients used in their study were effective in accelerating the growth of fibroblasts with an extract concentration of 2.5 ppm but caused cell death in the concentration of ≥ 10 ppm.[26]

The results of this study indicated that the stem extract of *S. littoralis* have an effect on the acceleration of fibroblast migration, namely at concentrations of 10 ppm, 50 ppm, 100 ppm, 500 ppm, and 1000 ppm, in which concentrations of 500 ppm and 1000 ppm had the most effective effect on the percentage of wound closure, reaching 100% at 22 hours. These results are in line with a study conducted by Zubair et al. on fibroblast migration showed the effective concentration of 1000 ppm at 24 hours.[27]

The ability of the stem extract of *S. littoralis* in accelerating fibroblast migration is due to the presence of alkaloids, saponins, tannins, phenolics, flavonoids, and triterpenoids. Alkaloids play a role in the process of strengthening collagen fibrils, which will affect the growth of new tissue in the wound quickly, densely, and strongly.[28] Furthermore, saponins can activate the TGF-b signal which will increase the fibroblast proliferation process.[29] In addition, saponins will also increase the number of macrophages that will secrete growth factors, such as FGF, PDGF, TGF-b, and EGF, which can stimulate fibroblast migration to the wound area, then synthesize collagen and increase the proliferation of capillary blood vessels.[30]

Tannins in the stem extract of *S. littoralis* serve as an accelerator for the closure of wound tissue because tannins have a role in increasing the formation of capillary blood vessels and fibroblast activation.[31] Phenolics can also stimulate collagen synthesis and cell proliferation.[32] Flavonoids have been proven to be able to accelerate wound healing by increasing the epithelialization process, which may cause the proliferation and migration of fibroblast cells to the wound area.[31] Triterpenoids have been proven to have an effect on wound healing by stimulating the proliferation and migration of fibroblasts.[33]

Conclusions

Based on the results of this research, it can be concluded that the stem extract of *S. littoralis* is a potential ingredient for wound healing because it has effectiveness as an anti-inflammatory agent and affects the acceleration of fibroblast migration.

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Declaration of Interest

The author declares that there is no conflict of interest.

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