

Effect of the Application of *Chlorella Vulgaris* Ointment to the Number of Fibroblast Cells as an Indicator of Wound Healing in the Soft Tissue of Pig Ears

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Abstract

Objective: To analyze the clinical and histological condition of *Chlorella vulgaris* ointment extract smearing on wound healing. **Material and Methods:** The sample consisted of 9 pigs by making 4 incisions on the pig's right ear measuring 2cm x 1cm and applying *Chlorella vulgaris* ointment in 3 concentrations namely 5%, 10%, 15% and without ointment application as the control group. Furthermore, the observation of the clinical condition of wounds divided into 4 time periods (day 1, 3, 7 and 14). After completing clinical observations based on time period, the skin of the pig's ear was taken by cutting in the area that had been made injured. Skin tissue extraction carried out under inhalation and local anesthesia. The tissue pieces are then processed in the anatomical pathology laboratory to become 36 slide preparations. The clinical trial assessment was done using parameters of wound moisture, color of wound and scab wound. Whereas for histological examination was done by looking at the number of fibroblast cells in the CX31 light microscope with 40x magnification. ANOVA and Kruskal Wallis tests were used. **Results:** In the clinical observation conditions there were significant differences between the treatment group and the control group as well as the number of fibroblast cells there were significant differences between the 3 concentrations of *Chlorella vulgaris* extract ointment and the per time period control group. **Conclusion:** *Chlorella vulgaris* extract ointment containing 15% extract gives the best results in accelerating the wound healing process in the pig's ear seen from wound healing and increased number of fibroblast cells.

Keywords: *Chlorella vulgaris*; Fibroblasts; Wound Healing; Ointments.

Introduction

In dentistry, many treatments will cause injury to the oral mucosa, which then requires good treatment to prevent serious infections. Handling wounds can be done by using antiseptics, antibiotics and general wound care. Wound healing is an essential thing to restore the continuity of broken tissue and return the functional status of the affected skin. Wound healing involves continuous interactions between cells and cells-matrix seen in four phases of healing overlapping wounds; they are coagulation, inflammatory, proliferation - migration and remodeling phase [1].

The best and optimal treatment in handling open wounds is to use materials and methods that can accelerate wound contractions, prevent excessive granulation tissue formation, prevent bacterial growth and be able to maintain normal pH as an appropriate moisturizer to accelerate wound closure [2].

In cases of tooth extraction, inflammation can occur due to trauma extraction. Tooth extraction can result in disruption of tissue continuity and tissue damage called injury. The wound healing after tooth extraction involves the healing process in soft tissues; they are connective tissue, gingival epithelium and in the hard tissue of the alveolar bone. In the proliferation phase of the wound healing, two things occur, the proliferation and migration of fibroblasts and endothelial cells to the injured area. Fibroblasts will produce collagen and extracellular matrix components so that blood clots will begin to be replaced by granulation tissue. In the process of wound healing, collagen is needed to repair damage and restore anatomical structure and tissue function [3].

Some natural resources have biological activities with potential therapeutic applications. Existing natural resources have several ingredients that contribute as wound healing factors. One of the natural resources currently being developed is *Chlorella vulgaris*. *Chlorella vulgaris* is a green algae that has been widely used as a health food and consists of 4 components that have real health effects, such as chlorophyll, cell wall, beta-carotene and Chlorella Growth Factor (CGF). *Chlorella vulgaris* has some properties that are beneficial to the injured organs and tissues with a variety of causes, it is called "great normalizer" which means the ability of *Chlorella vulgaris* to restore various bodily functions to normal levels [4,5].

Chlorella vulgaris is thought to act as an anti-inflammatory agent because of its ability to reduce the secretion of cytokines associated with inflammatory activity, such as some types of interleukin (IL) and also matrix metalloproteinase (MMP), a matrix which damage the tissue. Somebody activities induced by the administration of *Chlorella vulgaris* can also be related to the growth and development of fibroblasts [4,6].

Fibroblasts play an important role in the process of tissue repair, which is responsible for the preparation of producing protein products that will be used in tissue reconstruction. Therefore, the number of fibroblast cells is one of the parameters to assess the wound healing process.

A previous study evaluated all *Chlorella vulgaris* ointments, explained that the 5% ointment by mouth showed the best result in the formulation test with good dispersion, the most normal pH and the viscosity that met the standard. Meanwhile, the 15% *Chlorella vulgaris* extract ointment produces the best results comparing other concentrations after application in mice's oral mucosa [7].

The application of the *Chlorella vulgaris* form in that study was carried out on the oral mucosa without injury so that further research is needed to make injury in order to assess the effectiveness of each concentration of *Chlorella vulgaris* extract and obtain a more appropriate type of preparation and concentration for oral cavity wound healing in extracted wounds and post-implantation remodeling. Therefore, we wanted to prove the effectiveness of *Chlorella vulgaris* extract ointment on wound healing performed on animal ears through clinical

and histological examination which is expected to be a reference in the selection of *Chlorella vulgaris* ointment as an alternative ingredient that plays a role in the natural healing process after implantation.

Material and Methods

Study Design

This research is an experimental laboratory study, using a post-test only group research design. The extracts were made at the Biopharmaceutical Laboratory of the Faculty of Pharmacy, Hasanuddin University. The treatment of the animals and clinical observations was done at Hewan Sahabat Satwa Celebes Clinic Makassar and Moncongloe Animal Husbandry in Maros Regency. The examination of fibroblast cells number was carried out at the Pathology Laboratory Anatomy of the Rumah Sakit Pendidikan UNHAS.

Experimental Phase

This research was started with the preparation of the sample by making the *Chlorella vulgaris* extract in the form of ointment. The pigs were adapted for 7 days in a cage with 3 pigs in each. Furthermore, treatment was carried out in all samples (9 pigs) making four injuries to the right ear measuring 2 cm x 1cm under inhalation and local anesthesia. After making the injury, the *Chlorella vulgaris* extract was applied to the wound with different concentrations of ointment for each wound: 5%, 10%, 15% and one wound with no application. Sorted from ear tip to base of pig ear.

The pigs were returned to the cage and continued with the application of ointment per 12 hours (2x1) every day and clinical observations were done from day 1 (immediately after application), then on day 3 for 3 first pigs were underwent the incision of the wound area. Each area was placed in a container containing 10% of formalin solution. The sample was sent to an anatomical pathology laboratory for the slide preparations. The observations were carried out on day 7 for 3 pigs and were done on day 14 for 3 pigs and tissue extraction as well as 3 pigs observed at the third day.

Table 1. Wound healing parameters for clinical observation.

Wound Morphology	Category	Score
Humidity	Wet Wound	+3
	Moist Wound	+2
	Dry Wound	+1
Wound Color	Red	+4
	Light Red	+3
	Brownish Red	+2
	White/normal	+1
Wound Scars	Open Wound	+4
	Scab	+3
	Close Wound	+2
	Hairy Wound	+1

Preparations were processed in the anatomical pathology laboratory with preparations stained using the general coloring Hemotoxylin Eosin (HE) to see neovascularization, epithelialization, and fibroblast cells using a CX31 light microscope with 10x and 40x magnification.

Data Analysis

Data were analyzed statistically using ANOVA statistical test to see homogeneity, distribution of the samples and the differences in clinical observation results between treatment groups. The Kruskal-Wallis statistical test used to see the difference in the number of fibroblast cells between the treatment groups per time period.

Results

Clinical observations showed the differences in humidity, wound color and wound scars based on the treatment group in 4 time periods.

Table 2 and Figure 1 shows differences in moisture parameters, wound color, and scab scars based on the length of observation. The highest humidity parameters is on day 3 (1.75) and the lowest is on day 7 and day 14 (1.17) ($p=0.029$), which means that there are differences in the average value of humidity between one observation day to another. The highest color parameters of the wound is seen on day 7 (3.33) and the lowest is on day 14 (1.00) ($p<0.001$), which means that there are differences in the mean color values of injuries between observation days with each other. The highest scar wound indicator is on day 7 (2.92) and the lowest is on day 14 (2.00) ($p<0.001$), there are differences in the mean score of the scar between the observation days with each other.

Table 2. Differences in moisture parameters, wound colors, and wound scars based on length of observation.

Observation		Humidity	Wound Colors	Wound Scars
Day 1	Mean	3.00	4.00	4.00
	SD	0.00	0.00	0.00
Day 3	Mean	1.75	2.25	2.50
	SD	0.75	0.45	0.52
Day 7	Mean	1.17	3.33	2.92
	SD	0.39	0.49	0.29
Day 14	Mean	1.17	1.00	2.00
	SD	0.39	0.00	0.00
p-value		<0.001	<0.001	<0.001

*Anova Test ($p<0.05$).

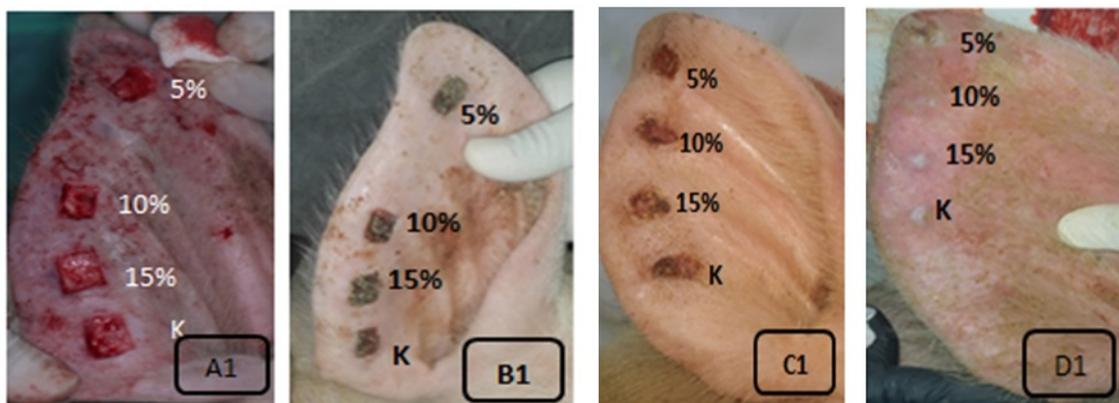


Figure 1. Differences in moistures parameters, wound colors, and wound scars based on length of observation.

The result of analysis of fibroblast cells number showed that there are significant differences between the increase and the decrease in the number of fibroblast cells in the calculation of the number of fibroblast cells between time periods (Figure 2).

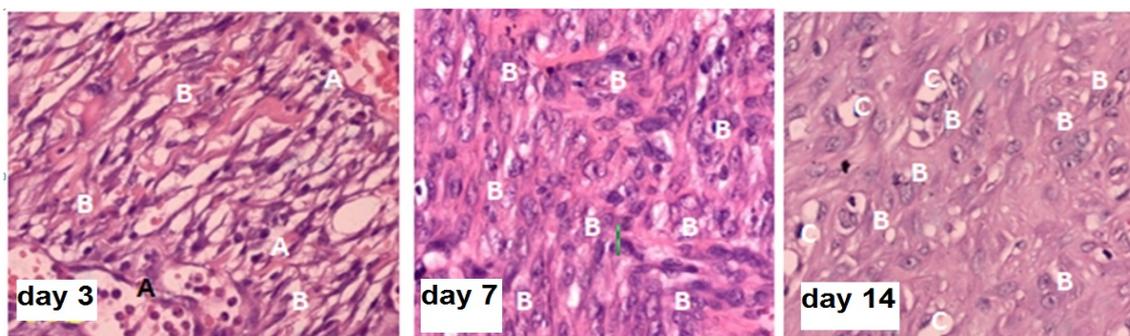


Figure 2. Wound healing phase after application of *Chlorella vulgaris* extract ointment. It appears on day 3 (H3) that there is a picture of the nucleus of dark lymphocyte cells (A) and fibroblast cells (B) in the form of encapsulated cells that contain 1 cell nucleus. On Day 7 (H7) there is an increase in fibroblast cells marked by the wound surface that almost covered by fibroblast cells (B). On day 14 (H14) some fibroblast cells have been lost and replaced by the collagen tissue (C).

In Table 3, the examination and calculation of treatment test 5% of fibroblast cells showed an increase in the number of fibroblast cells on day 3 with an average of 69 experimental animals and decreased on day 7 to 67 and increased again on day 14 to 87. For test animals treated 10% showed an increase in the number of fibroblast cells higher than 5%, on day 3 with an average number of 120 then increasing on day 7 to 128 and decreasing on day 14 with an average of 119. In the treatment group of 15%, there was also an increase in the number of fibroblast cells with an average that was almost equal to 5%, 68, and had a very high increase on day 7, 173 and decreased on day 14 to 45. The observation of the control group showed an increase in fibroblast cells until day 14, whereas 31 in day 3, 89 in day 7 and greatly increases with 141 on day 14.

Table 3. Differences of the fibroblast cells number based on the concentration of *Chlorella vulgaris* ointment.

Groups		Day 3	Day 7	Day 14	p-value
Cream Application 5%	Mean	69.00	67.00	4.00	0.044*
	SD	2.00	1.00	0.00	
Cream Application 10%	Mean	120.00	128.00	2.50	0.048*
	SD	1.00	2.00	0.52	
Cream Application 15%	Mean	68.00	173.00	2.92	0.027*
	SD	3.00	1.00	0.29	
Without Cream Application	Mean	31.00	89.00	2.00	0.027*
	SD	1.00	1.00	0.00	
p-value		0.024*	0.016*	0.016*	

*Kruskal-Wallis test (p<0.05).

In the group that received the application of ointment, the fibroblast cells increased on day 3 and 7 and decreased on day 14. While for the control group there was an increase until the 14th day (Figure 3).

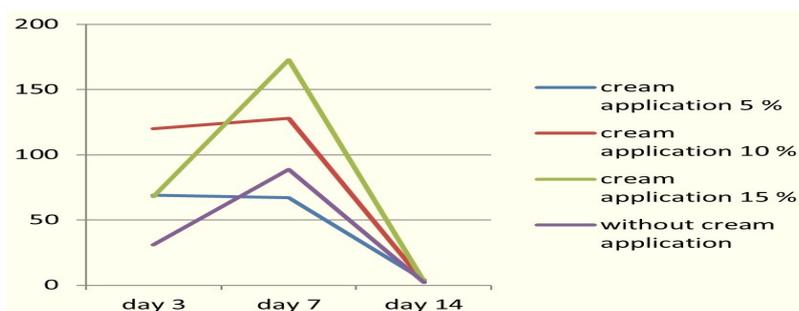


Figure 3. Difference of the number of fibroblast cells based on the concentration of *Chlorella vulgaris* ointment.

Discussion

The administration of *Chlorella vulgaris* extract ointment of 5%, 10% and 15% in each group of animals were analysed by observing the moisture parameters, wound color and scar based on the results of data analysis which showed that the 15% *Chlorella vulgaris* extract ointment showed the best changes in wound parameters among all groups.

At the administration of 15% *Chlorella vulgaris* ointment tends to cover the wound faster than the *Chlorella vulgaris* ointment with concentrations of 10% and 5%. This can be affected by the stability of the ointment formulation itself. In general, *Chlorella vulgaris* 15% ointment appears to be more stable which is indicated by more concentrated color and changes in color to light which tends to be slower than in 10% and 5% of *Chlorella vulgaris* ointment. The right formulation of ointment with the stability of the active ingredient in a good ointment can support more optimal wound healing. In addition to good drug absorption, the content of *Chlorella vulgaris* is the most influential factor in the speed of wound healing in test animals.

Chlorella vulgaris has four main ingredients, namely chlorophyll, carotenoids, phycobilin, and *Chlorella* growth factor (CGF), which have a significant effect on health, including in the process of wound healing. Chlorophyll concentrations of 0.05% - 0.5% found in *Chlorella vulgaris* can invade and multiply fibroblasts, which are useful in the process of wound healing [6]. Fibroblasts will produce collagen, which forms part of the granulation tissue formed in the area of injury. Beta-carotene, which is a component of *Chlorella vulgaris*, also increases immunity by increasing the tissue integrity and enhancing defense cell activity. *Chlorella* growth factor (CGF) contains growth-promoting factors in organisms, including various nutritional elements such as amino acids, sugars, vitamins, minerals, and nucleic acids. The CGF can support the wound healing process through optimizing the regeneration of skin cells. In addition, to stimulate cell regeneration, CGF also has an important role in the activity of inflammatory cells that support the speed of wound healing [6].

Clinical observations showed that the wounds smeared by *Chlorella vulgaris* extract ointment tend to heal quickly and are covered by *Chlorella* deposits. In the early days, the wound closed and formed a kind of dark green layer that is the condition that might accelerate the healing process [6].

The result of the wound moisture parameters between 3 days, 7 days and 14 days of observation showed a significant difference. The average humidity parameters of wounds on day 3 is alkali. The incision causes the skin to lose its retraction so that open sores form a gap. The incision causes inflammation because it causes larger changes in capillary shape. Wet-looking wounds are caused by prolonged dilation of blood vessels so that the amount of extracellular compartment fluid increases abnormally [8-10].

The wound dries and forms a scar on the next day until the wound closes and the scar detaches from the wound. In this phase angiogenesis also occurs, which is a process of newly developing blood vessel capillaries or the formation of new tissue (tissue granulation). In addition, the fat content of the ointment and the base of the ointment used can attract more water so that the wound dries faster [6].

The dried wound then thickens, forming a scar on the surface of the wound. Tissue repair continues to occur until it forms new skin tissue in a wound covered by a scab. When the wound closes completely, the scab will be attracted and detached from the wound so that new skin tissue appears. Scab is released after the wound, closes followed by hair growth on new skin tissue. Hair growth in the wound area shows the regeneration process and the skin condition has begun to return to normal [8].

On day 3, the average condition for all treatments showed a moist wound until it began to dry out. The wound becomes moist due to the release of platelets and proteins that form fibrous tissue and fibrin formation on the wound surface. The wound that begins to dry is caused by an improvement in the circulatory

system so that the hydrostatic pressure is balanced. On the wound smeared by ointment and stale ointment, the wound appears to be drier. This can be influenced by the fat content on the base, which can attract water so that the wound dries faster [8-10].

The basic principle of optimal wound healing is to minimize tissue damage by providing adequate tissue perfusion and oxygenation, providing proper nutrition with humid environmental conditions to restore anatomic continuity and damaged tissue function in a short time. The latest wound management aims to create a moist wound environment to accelerate wound healing (moist wound healing). This concept was explained previously, so that epithelialization occurs twice as fast in moist conditions compared to dry conditions. In addition to proper moisture, the formation of granulation tissue in the wound is a sign of an ongoing wound healing process [11,12].

On the 14th day, the wound treated with several concentration of *Chlorella vulgaris* ointment was closed. The scab that had previously covered the wound was released. Scab is released when the wound closes followed by hair growth a few days after the wound closes. The basic principle of optimal wound healing is to minimize tissue damage by providing adequate tissue perfusion and oxygenation, providing proper nutrition with humid environmental conditions to restore anatomic continuity and damaged tissue function in a short time.

Chlorella vulgaris contains many phytochemical compounds such as sterols, flavonoids, tannins, phenols, terpenoids, saponins, sulfur compounds, and other nutrients that have a significant effect on health, including for wound healing in animals. One of the contents of *Chlorella vulgaris* that affects wound healing, especially for wound moisture is flavonoids and tannins. Flavonoids can stop bleeding in the wound and act as an anti-inflammatory agent that will affect the production of inflammatory cells in the wound healing phase. The presence of flavonoids in ointment can affect changes in the condition of wet wounds to become moist faster. The content of tannin as an astringent can affect the permeability reduction of the mucosa and the bond between the mucosa becomes strong so that it prevents irritation. Thus, indirectly tannin affects the changes in each level of humidity. Apart from having an effect on mucosal permeability, tannins can also affect the permeability of the wall or membrane of bacteria so that bacteria shrink and die. This antibacterial property can prevent wound infections [13,14].

Saponins contained in *Chlorella vulgaris* can affect the production of collagen in the early stages of tissue repair and stimulate the regeneration of epithelial cells in the skin thereby accelerating the process of wound healing in animals. Phenol compounds play a role in preventing cell damage due to free radicals thus preventing inflammation and inflammation. *Chlorella vulgaris* also contains terpenoids, which are useful in reducing inflammatory activity. The anti-inflammatory property of *Chlorella vulgaris* causes the inflammatory process in the wound in the treated pig's ear to be inhibited and the healing process of the wound can occur more quickly [7,14].

Several types of sterols extracted from *Chlorella vulgaris* can inhibit certain inflammation of the skin. Sterol can prevent inflammation and prevent prolonged allergies and regenerate skin cells [15]. The sterol content of *Chlorella vulgaris* can support deep wound healing in pigs, the treatment of which is given *Chlorella vulgaris* ointment so that the effect is noticeable compared to the control group [14].

The diversity of individual results in the same or different groups can be influenced by the content of the compounds that interfere with the wound. The response can be caused by physical abrasion by particles even though the contact time is relatively short and small. Chemical interactions of foreign or solid foreign substances on the skin can also cause a response to the skin. The resulting response will be different between

individuals, depending on the sensitivity of each individual. This is known as the individual variation of each treatment [14].

Chlorella vulgaris can increase the activity of natural killer cells. It can also prevent psychological stress and maintain homeostasis against external stress stimuli through increasing corticosteroids in the blood. The speed of healing of wounds given by *Chlorella vulgaris* ointment can be affected by increased activity of natural killer cells and inflammatory processes. *Chlorella vulgaris* also contains an antibiotic called chlorellin. The antibiotic content in *Chlorella vulgaris* extract can prevent infection so that it supports wound healing in treated animals [4].

The use of *Chlorella vulgaris* extract ointment that has various active ingredients can support the wound healing process in the skin of the pig ear progressively. Topical dosage forms that are easily applied on a good basis can influence the achievement of the optimal cure rate for treatment every day [10].

All specimens were carried out histopathologically by hematoxylin-eosin staining using a light microscope with 400 times magnification. Each specimen was examined for the number of fibroblasts. The number of fibroblasts increased in the 7th day observation in all treatment groups and the control group. But the number of fibroblasts was more dense in the treatment group of 15% compared to the treatment group of 5% and 10% in the 7th day observation.

The increase of fibroblast density is caused by increased stimulation of FGF secretory proteins as a result of platelet degranulation. Microscopic results lead to the possibility of *Chlorella vulgaris* activity against fibroblasts as an intermediary for the effects of accelerated wound healing performed by *Chlorella vulgaris*. *Chlorella vulgaris* will modulate the early days of injury so that fibroblasts multiply faster and more [15].

Macrophages begin to gather in the wound area 24 hours to 48 hours after injury, as well as fibroblasts. The gathering of a large number of macrophages, which is sufficient to have a real effect, begins to occur on the second day and in line with that there is an increase in the production of growth factors. The increase in cytokines will affect the number of fibroblasts so that the increase in number can be seen in histopathological observation on the 3rd day and continues to increase on day 7 [4].

On day 14, the number of fibroblasts in the *Chlorella vulgaris* extract ointment group tended to be lower and most noticeable in the group with a concentration of 15%. At least the number of fibroblasts shows that connective tissue has been formed in the wound that has been extracted. When the wound surface is closed, the fibroplasia process with the formation of granulation tissue will also stop and the ripening process begins. This explains that on the 14th day observation the number of fibroblasts in the treatment wound group decreased compared to the third day because on the 7th day (proliferation phase) the wounds in the treatment group were all covered by the epithelium so that fibroblasts were not synthesized and the number decreased. It can be concluded that the wound healing process works well because *Chlorella vulgaris* extract accelerates epithelialization [16].

Fibroblasts produce collagen, which links the edges of the wound so that the scar tissue becomes strong. Furthermore, the body tries to normalize everything that becomes abnormal because of the healing process. Edema and inflammatory cells, young cells mature, excess collagen and newly closed capillaries are reabsorbed, and the other shrinks according to the strain [16].

The more complete the healing of a wound, the less the number of fibroblasts, because the presence of fibroblasts shows the process of wound healing. This is different in the control group where the observation of day 14 showed an increase of fibroblast cells compared to the 7th and 3rd day of the treatment group. Thus, the large number of fibroblast cells indicates the incomplete formation of collagen, which will form connective

tissue that will link the wound. So even though clinically, the wound in the control group is closed but histologically, there is an increasing process of fibroblasts and collagen synthesis, which is still low [15].

The role of fibroblasts is very important in the repair process, which is responsible for the preparation of producing protein structure products that will be used during the process of tissue reconstruction. Under normal circumstances, fibroblast cleavage activity is rarely seen, but when an injury occurs this cell looks more active in producing extracellular matrices. The proliferation of fibroblasts in the wound healing process is naturally stimulated by interleukin-1b (IL-1b), platelet derived growth factor (PDGF), and fibroblast growth factor (FGF). The wound healing process is strongly influenced by the role of fibroblasts migration and proliferation in the area of injury. The content of *Chlorella vulgaris* extract ointment applied to animal wounds stimulates the synthesis of growth factors including fibroblast growth factor (FGF), which increases the activity of fibroblast cells to produce collagen and forms connective tissue so that the wound heals quickly [15,17,18].

Conclusion

The *Chlorella vulgaris* extract ointment containing 15% concentration extract is the best in accelerating the wound healing process in pig ears compared to other concentrations of 5% and 10% based on healing parameters wound and the increase of the fibroblast cells number.

Authors' Contributions

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All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest

The authors declare no conflicts of interest.

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