Effectiveness of Garlic Bulb (Allium Sativum L.) Ethanol Extract Cream on the Number of Fibroblast Cells in the Healing Process of Male White Rat (Rattus norvegicus L.) Wistar Strain Incision Wound

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Abstract

Background: The case of antibiotic resistance in the standard treatment of skin wounds is now a major problem in the medical world, thus herbal preparations are needed as an alternative. However, wound healing creams that are currently available on the market are the result of extraction from Centella asiatica whose insignificant antibacterial and anti-inflammatory activity. Therefore, in this research, another alternative herbal medicine cream from garlic bulb (Allium sativum L.) extract is being examined to see its effectiveness in healing incision wounds.
Methods: The following method is used by the researchers to analyze the effectiveness of topical preparations of garlic bulb ethanol extract on the increase of fibroblast cells in the healing process of incision wounds. This research was conducted in June - August 2019 by scraping incision in the back area of male white rats (Rattus norvegicus L.) Wistar strain with a size of ± 1 cm and a thickness of ± 2 mm. Furthermore, a true experimental design was carried out using a post-test control group design using 5 treatment variables for 7 days into 5 groups of mice randomly selected. The skin tissue that has been given treatment is then extracted and painted using Hematoxylin-eosin (HE) to then be observed the number of fibroblast cells under a microscope with a magnification of 400 times at 5 times the field of view. The data were analyzed using the Post Hoc test which then summarized using the Mann Whitney test ($p < 0.05$).
Results: There is an overall difference in the concentration of garlic bulb (Allium sativum L.) ethanol extracts on the increase of fibroblast cells for the incision wound healing process of the male white rats (Rattus norvegicus L.) Wistar strain.
Conclusion: The results of histopathological observations using fibroblast cell scoring can be concluded that the concentration groups of 20% and 15% have the highest scoring values of 3 with "moderate" interpretation.

Key Words: Garlic Bulb, Fibroblast, Incision Wound.
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Introduction

The incision wound is categorized as an open wound that is caused by tools that have sharp edges. Its visible size of the scar looks longer than the actual depth of the wound [1].
Acid mucopolysaccharide, new capillary vessels, and fibroblasts are essentials for the wound healing process [2]. Fibroblasts are responsible for producing protein structure materials that will be used during the tissue reconstruction process [3].

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Garlic (*Allium sativum L.*) is one of the nutritious herbal plants which has the potential to overcome antibiotic resistance problem in the nowadays medical sector.

Chemical substances contained in garlic bulb are flavonoids, allicin, and essential oils. Allicin has the greatest antibacterial activity, besides the essential oil from the bulb of garlic also has the ability as an antibacterial and antiseptic [4]. The content of flavonoids in garlic can result in the death of bacterial cells in which they also have an anti-inflammatory effect, and affect re-epithelialization. Flavonoids also function as antioxidants and anti-bacterial which can increase the activation and proliferation of fibroblasts, thereby triggering the formation of collagen and accelerating the process of wound healing [5].

In previous studies, the use of garlic extract gel with lower concentrations of 20% was better than the concentrations of 40% and 80% in the healing process of the inflammatory phase wound [6]. In the administration of garlic extract ointment a concentration of 10%, there was no effect on wound healing assessed by the number of lymphocytes compared to the day group [7]. Also, the speed of wound healing carried out within the 14 day limit with the use of garlic extract cream concentration of 1.5% in shallow degree II burns Wistar rats recover faster in ±10 days compared to the concentration 3.0% and 6.0% [8].

Based on the above-explained background, it is necessary to conduct an ongoing study of the effectiveness of ethanol extract cream of garlic tubers (*Allium sativum L.*) on the wound healing process of white rat incision (*Rattus norvegicus L.*) Wistar strains by observing histopathological features on the increasing number of fibroblast cells.

Method

Research Design

This research was conducted with a true experimental design study that uses a post-test control group design. Chart 2.1 explains that in this study, 5 treatment variables were arranged into 5 randomly selected groups. This method the researcher will analyze the effectiveness of topical preparation of garlic (*Allium sativum L.*) ethanol extract cream on the increase of fibroblast cells in the healing process of incision wounds in male white rats (*Rattus norvegicus L.*) Wistar strain.

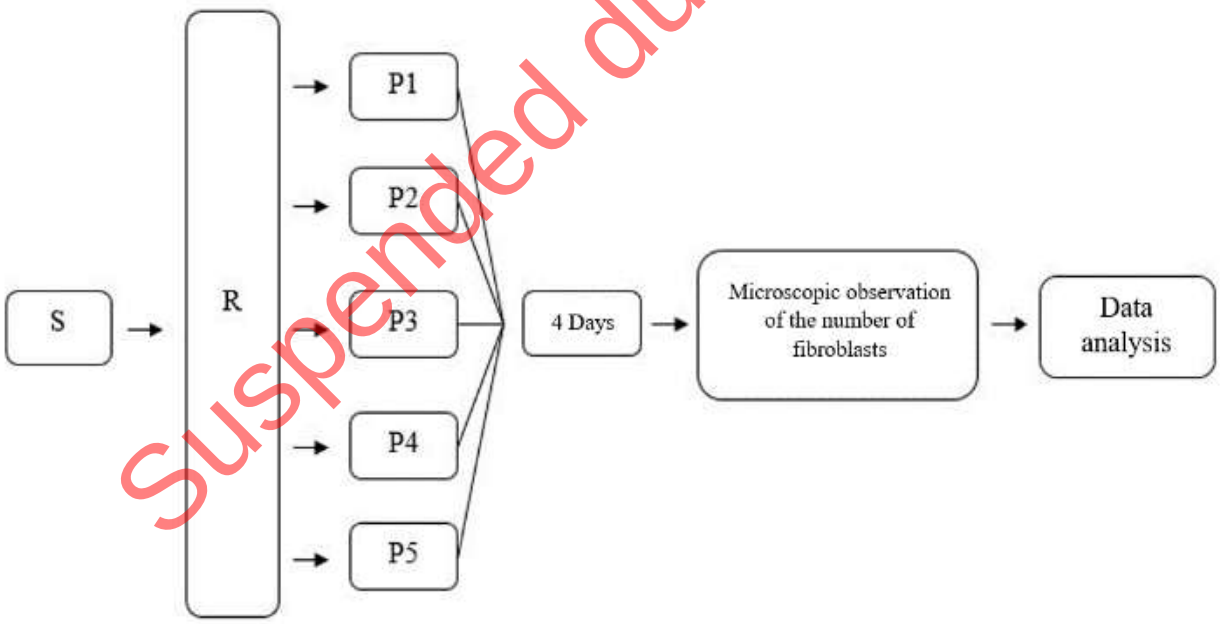

Chart 2.1. Research design scheme

S=Sample; R=Random; P1=Control group, without treatment (-); P2= Control group, Madecassol 1% (+); P3=Treatment group with garlic ethanol extract cream 20%; P4= Treatment group with garlic ethanol extract cream 15%; P5= Treatment group with garlic ethanol extract cream 10%

Samples and Sampling Techniques

The sample studied was male Wistar strain white rats obtained from Banjarmasin city following the inclusion criteria.

This research used a simple random sampling technique by taking sample members from a

population carried out randomly without regard to strata in the population. Sample calculation according to Federer:

$$(t-1)\,(n-1) \geq 15 \qquad (2.1)$$

t = number of treatment groups (5); n = number of samples

Based on the above formula, it is obtained n≥5, so that a sample of at least 5 animals was required for each group. Each group was given a group code P1, P2, P3, P4, and P5. To anticipate the loss of the experimental unit, corrections are carried out using the following formula.

$$N = n / (1-f) \qquad (2.2)$$

N = correction sample size; n = size of initial sample; f = estimated drop out proportion of 10%
So, the sample used by each group was 6 animals and the number of groups used was 5 groups. Thus, this study used 30 rats.

Sample Selection Techniques

The sample selection technique in this study is based on the inclusion, exclusion, and drop-out criteria.

Inclusion Criteria

Wistar strain male white rats that look healthy are agile movements, clear eyes, and fur looks shiny; weight 150-250 grams as it is expected that experimental animals are heavy enough to be able to represent the expected result; and 2-3 months old.

Exclusion Criteria

Experimental animals that were confirmed by a consultant veterinarian as proven to have an illness or physical injury during the period of clinical evaluation under the appropriate environmental conditions; trial animals get sick; mice die during the adaptation period before treatment.

Drop out Criteria

Mice die due to the treatment process.

Research Variables

This study aims to determine the effective concentration of garlic extract on the number of fibroblast cells. It is divided into independent and dependent variables as table 2.1 illustrates the definition of the variables to be used in this study.

Table 2.1. Operational Definition

No.	Variable		Definition
1.	Independent	Topical concentration of garlic ethanol extract (Measuring results in %, scale in ordinal)	The preparations were obtained by extracting plant active substances with ethanol through maceration process so that the extracts would be divided into 3 concentration groups: 20%, 15%, 10%.
2.	Dependent	Increased number of fibroblast cells (Scale in Ratio)	Observation of the fibroblasts level increment on histopathological examination using 400x magnification with hematoxylin-eosin staining

Tools and Materials

The tools used in this study were: Wistar male rats, animal cages, animal feedlot, rat feed, gloves, glassware (pyrex), rotary evaporators, blenders, scissors, animal tissue, scales, markers, minor sets, masks, mouse shavers, microscopes.
The materials used in this study were: garlic bulb, aquades, 96% ethanol, hematoxylin-eosin, biocream, veet®, BNF 10%, madecassol 1%, and hypafix.

Work Procedures

Acclimatization of Experimental Animals and Administration Doses Determination.

All white rats were put in separated cages for adaptation for 7 days. During the adaptation period, all of the white rats were given ad libitum food and drink.

Determination of the garlic bulb extracts concentration was based on the dose that has been used in previous studies: 20%, 40%, 80%. The results of the study showed an effective dose of garlic extract in healing the rapidly epithelialized edge of wounds was 20%. as the minimum dosage [6].

Based on these studies, in this study researchers effective concentrations of 20%, 15%, and 10%.

Making Garlic Bulb Ethanol Extract

The extraction method in this research was maceration method using 96% ethanol solvent. The

extraction process was conducted by drying the ingredients first then smoothing it to a certain degree of fineness. After that, dried powder (*simplisia*) was immersed using 96% ethanol solvent at room temperature for 3x24 hours (filtered every 24 hours). The ethanol solvent liquid will penetrate the cell wall and enter the cell cavity which contains the active substance that will dissolve, because of the difference in concentration between the solution of the active substance inside the cell and outside the cell, the concentrated solution was pushed out [9].

Phytochemical Identification (Flavonoids, Tannins, Saponins, Alkaloids).

The identification process was carried out following standard procedures listed in the Indonesian Pharmacopoeia editions III, IV, V, and Indonesian Herbal Pharmacopoeia [10]–[13]

Treatment of Experimental Animals

Before the treatment was done, the rats were sheared back with scissors, then the remaining hair that had not been perfectly shaved was shaved by using Veet®. Left for 2 days to avoid inflammation caused by shaving and administration of Veet®.

On the 3rd day, the back skin area of the rats was anesthetized using a local anesthetic cream (25mg lidocaine cream and 25mg prilocaine combination). It was applied to the rats' back skin for 2 minutes. The incision wound was done in the back area with a size of ± 1 cm.65 The depth of the incision wound was only to the dermis layer with a size of ± 2 mm (so that it did not penetrate the muscle) using a scalpel blade that has been given a measurement mark of ± 2 mm. After making incision wounds on the backs of rats were rinsed with antiseptic fluid to prevent infection.

Table 2.2 illustrates the treatment on the incised backs of mice in each group treated with different concentrations of 0.1 g per each rat with one smearing on the incision wound area then dressing it using hypafix. The treatment was done once a day, every single morning for 7 days.

7 days after the treatment, on the 8th day, euthanasia of the mice was conducted using a combination of 80 mg/kg body weight of ketamine and 10 mg/kg body weight of xylazine injected intraperitoneally.

The animal was positioned in a dorsal fall, so that back was located in the dorsal section for the sampling. The part of the incised skin was fixed and rinsed using 0.9% physiological NaCl. The skin was cut and then put in a pot containing a 10% BNF solution [14].

The skin that had been placed in a 10% BNF solution is then sent to the Anatomy Pathology Department of Airlangga University to be colored using Hematoxylin-eosin (HE) and observed regarding the number of fibroblast cells under a microscope at 400 times magnification at 5 times the field of vision.

Observation of fibroblast cells used the method of scoring the presence of connective tissue based on the number of fibroblast cells [15].

Table 2.2. Scoring the growth of fibroblast cells

	Score
1/Absent	0-10%
2/Mild	11-40%
3/Moderate	41-70%
4/Severe	71-100%

Data Processing Methods and Data Analysis Techniques

Data obtained from the number of mice fibroblast cells per observation treatment in all groups were tabulated. Observation of histopathological preparations of fibroblasts in the wound area used a light microscope with a magnification of 400x. The calculation was done in 5 fields of view.

Data analysis was done by using SPSS 20 computer software for windows. First, the normality test was conducted, the univariate was done first to see the mean, median, and mode of the data. The distribution normality of bivariate data was analyzed by using the Shapiro-Wilk test. If the data distribution is normal p> 0.05 then the One Way ANOVA statistical test can be used at a 95% confidence level because it is a comparative hypothesis test of numerical variables of the normal distribution, more than two groups. One Way Anova test requirements for >2 unpaired groups include: (1) data distribution must be normal; (2) data variance must be the same.

Data that meets the requirements were analyzed by using the One Way ANOVA parametric test. If the data are not normal or p <0.05 then the Kruskal-Wallis non-parametric test has to be done.

Research Flow

The design of this study consists of the selection of plants, sample distribution, and analysis of the results of the study. The research flow of this study is fully presented in Figure 2.2

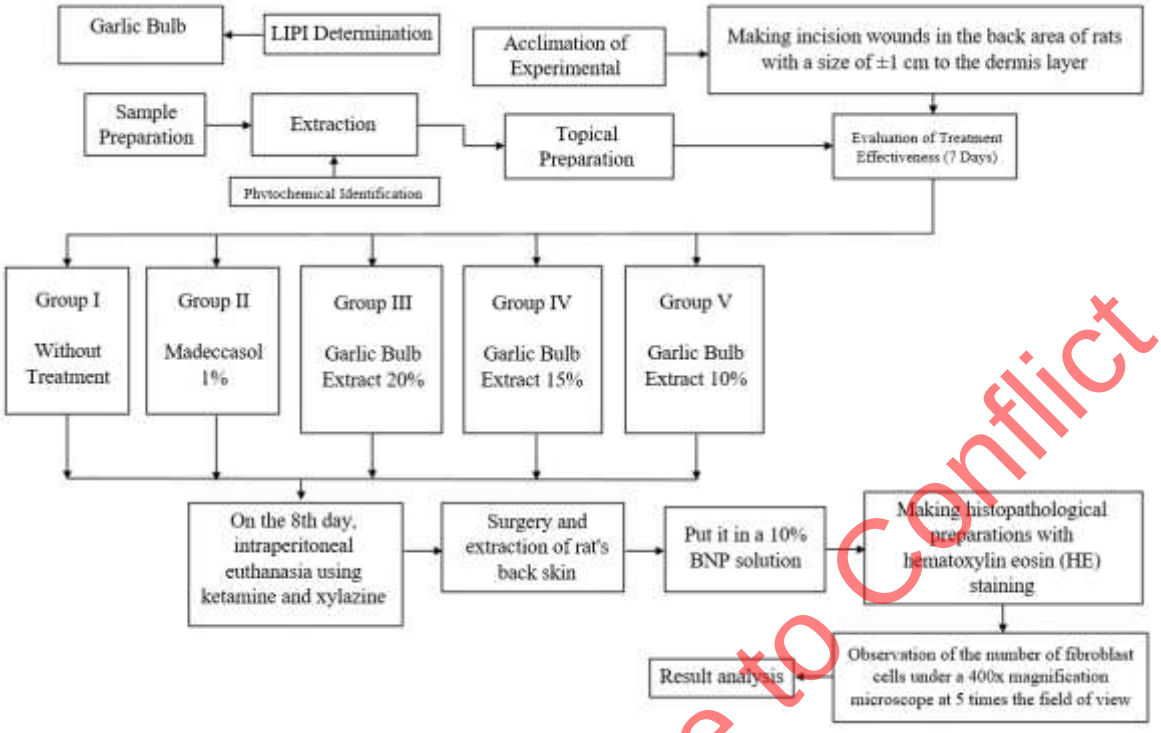

Figure 2.2. Research Flow

Research Place and Time

Garlic sampling in the Tangkiling Area, Palangka Raya City, Central Kalimantan Province and for the white mice Wistar strain sample were taken from Banjarmasin, South Kalimantan. For the garlic (Allium sativum L.) extraction was conducted in the Biology Laboratory of Muhammadyah University, Palangka Raya. The treatment of experimental animals was done in the Biomedical Laboratory of the Faculty of Medicine, Palangka Raya University. Then the observation of the histopathological picture of the sample preparation was done in the Histopathology Laboratory of Airlangga University, Surabaya. This research was conducted in June-August 2019.

Results

Plant Determination

Garlic plant used in this research wad identified at the Botanical Garden Conservation Center Purwodadi - LIPI Jl. Raya Surabaya - Malang Km. 65, Purwodadi, Pasuruan - East Java. The results of the determination state the plant taxonomies.
Kingdom: Plantae
Division: Magnoliophyta
Class: Liliopsida
Subclass: Liliidae
Order: Liliales
Family: Liliaceae
Genus: Allium
Species: Allium sativum L.

Phytochemical Test Results of Garlic Bulb (Allium sativum L.)

Garlic plants were identified by phytochemical method in the Laboratory of the Faculty of Medicine, Chemistry-Biochemistry Division, Lambung Mangkurat University. Phytochemical test results of garlic (*Allium sativum L.*) illustrated in Table 3.1.

Table 3.1. Phytochemical Test Results

Phytochemical Compounds	Content
Saponins (%)	33.681 ± 1.931
Alkaloids (%)	23.420 ± 1.337
Flavonoids (mgEQ/gr)	12.333 ± 0.722
Steroids (mg/mL)	49.375 ± 0.179
Tannins (mg/mL GAE)	2.233 ± 0.027

Yield of Ethanol Extract

Garlic (*Allium sativum L.*) studied were fulfilled predetermined criteria: around 3-5 months old which were marked by the size of 3-5 cm bulb. The garlic was extracted by the maceration method with the results as listed in table 3.2.

Table 3.2. Measurement Results of the various Extraction Stages

Garlic kg(s)	Simplisia gr(s)	Filtrate (ml)	Viscous Extract gr(s)	Yield
2	2820	2250	270	1.00%

Histopathology Overview of Haematoxylin Eosin (HE) Fibroblast Cell Staining

The results of all treatments showed different histopathological pictures in the dermis layer with a magnification of 400x that were visible as fibroblast cells which can be seen in the following figure.

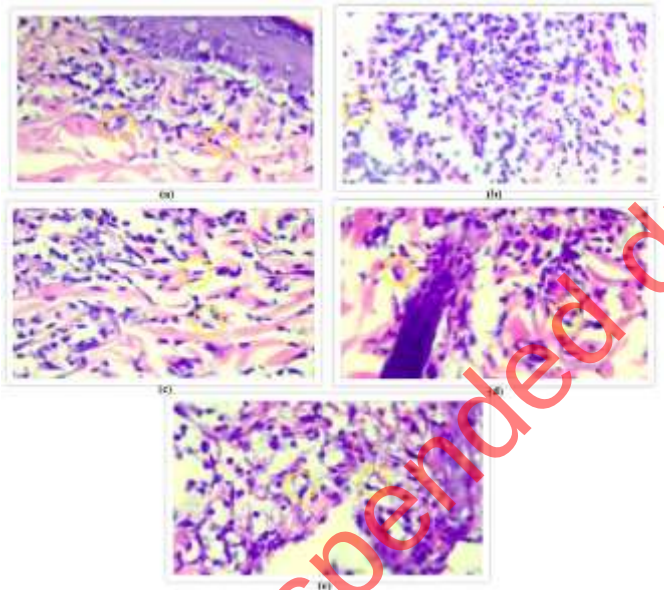

Figure 3.1. (a) Histopathology picture of 400x magnification of the negative group; (b) positive group; (c) the treatment group with extract concentration of 20%; (d) the treatment group with extract concentration of 15%; (e) the treatment group with extract concentration of 10%. Yellow circle shows fibroblast cells in hispatological observations

The Effectiveness of Garlic Bulbs (Allium satvum L.) Ethanol Extract Cream on the Number of Fibroblast Cells in the Healing Process of Incised Male White Mice (Rattus norvegicus L.) Wistar Strains

The effectiveness of garlic ethanol extract cream on the increasing number of fibroblast cells was observed using a microscope and calculated. After the microscopic observations and the numerical calculations of the fibroblast cells, data analysis was performed. The first test conducted was the normality test using Shapiro-Wilk. A normality test was used to see whether the data are normally distributed. Normally distributed data is a requirement of parametric data so that homogeneity analysis can be done with a one-way ANOVA test. If the significant value> 0.05 then the data is normally distributed. Table 3.3 is a normality test before the data is transformed. The normality test results are as follows.

Table 3.3. Results of Shapiro-Wilk Test Analysis

	Shapiro-Wilk
Sig.	0.053

The data were significant as they were >0.05. Thus, the data processing was continued by using the homogeneity test to find out whether the data met the parametric test requirements (one-way ANOVA analysis).

Homogeneity test results of the data using one-way ANOVA analysis are as follows.

Table 3.4. Results of One-Way ANOVA Test Analysis

Sig.
0.000

One of the ANOVA test requirements is that the equal variance or the significant value >0.05. Homogeneity of data can be seen in Table 3.4 which shows a value (P-value) of 0.000 <0.05 which means the data in this study have variants that are not the same or do not meet parametric assumptions so that later using the Kruskal-Wallis non-parametric test with results as follows.

Table 3.5. Results of Kruskal-Wallis Test

Asymp. Sig.	0.000

The table above was used to see the differences or effects of 5 extracts, frog the Sig column table, obtained value (P-Value) = 0.00. 0.00 < 0.05, thus Ho was rejected, so the conclusion was there is an effect of the garlic extract concentration on the increasing number of Wistar strain fibroblast cells.

To find out the effectiveness of garlic bulb ethanol extract on the increasing number of fibroblast cells with significant differences, a Post Hoc test was conducted which can be summarized using Mann Whitney Test. The criteria: if the significant value <alpha (0.05) then it can be stated there are the different effects of the garlic bulb ethanol extract bulb on the increasing number of fibroblast cells in

Wistar strain mice. In this research, it is concluded there are the effectiveness differences as it is illustrated in Table 3.6 below.

Table 3.6. Analysis of Mann Whitney Test Results

	Control (-)	Control (+)	20%	15%	10%
Control (-)		0.002*	0.002*	0.002*	0.002*
Control (+)	0.002*		0.002*	0.002*	0.002*
20%	0.002*	0.002*		0.002*	0.002*
15%	0.002*	0.002*	0.002*		0.002*
10%	0.002*	0.002*	0.002*	0.002*	

Information:
*There are significant differences
Table 3.6 were summarized from the Pos Hoc test. Significant differences among the groups were marked *. Thus, it can be concluded that there are differences in the overall concentration of garlic bulb ethanol extract on increasing the number of fibroblast cells

Table 3.7. Results of Scoring of Fibroblast Cells

Treatment Groups	Total Fibroblast (%)	Scoring
P1 (-)	12	2
P2 (+)	28	2
P3 (20%)	62	3
P4 (15%)	42	3
P5 (10%)	33	2

Table 3.7 can be inferred that the highest number of fibroblasts was 62 in the 20% concentration group with moderate scoring. The lowest number of fibroblasts was in the negative control group, in the number of 12, which was categorized as mild. The scoring result was gained from the total fibroblast cell calculation.

Discussion

In this study, microscopic observations were conducted to determine the effectiveness of ethanol extract of garlic bulb (Allium sativum L.) on the number of fibroblast cells in the wound healing process of incision of male white rats (Rattus norvegicus L.) Wistar strain. Fibroblast cells are one component of wound healing in the form of cells that are widely distributed in connective tissue, producing collagen precursor substances, elastic fibers, and reticular fibers [16]. Fibroblasts first appear on day 3 to 7. The increasing number of fibroblasts in the wound area is a combination of proliferation and migration [17]. The process of wound healing is strongly influenced by the role of migration and proliferation of fibroblasts in the area of injury [18]. Based on the results of statistical tests using the Kruskal-Wallis test, there was an effect of topical administration of ethanol extract of Garlic (*Allium sativum L.*) against the increasing number of fibroblast cells in the back skin of mice.

Negative control group test results (without treatment) showed the lowest number of fibroblast cells in the dermis area compared to other groups. This shows the absence of a combination of vascular response, cellular activity, and the formation of chemical compounds as a mediating substance in the wound area which is an interrelated component of the wound healing process. As a result, fibroblast cells are not widely distributed in connective tissue [19].

The Madeccasol® group showed the second-lowest number of fibroblast cells in the dermis area of all groups. Madeccasol® is an anti-inflammatory cream that is indicated to accelerate the wound healing process. Madeccasol® contains *Centella asiatica* extract which is a medicinal plant that has beneficial health components, but its use as a drug is still limited [9]. This is due to the anti-inflammatory effect of *Centella asiatica* affecting the production of one of the inflammatory mediators, namely prostaglandin so that the anti-inflammatory effect is small and will affect fibroblasts against the formation of collagen which is responsible for the formation of new tissue [20]

In terms of statistical test results in the ethanol extract group of garlic bulbs, all three concentrations showed a higher increase in the number of fibroblasts compared to the positive control group of Madeccasol® due to the small anti-inflammatory effect of *Centella asitica* extract. Besides, the antibacterial activity of *Centella asiatica* essential oil content is small because essential oils are not the main ingredient in it so they are only a small amount [9].

Some plants also have anti-inflammatory effects. The active compounds of garlic compounds such as flavonoids also play an important role in maintaining permeability and increasing capillary blood vessel resistance. Therefore, flavonoids are used in pathological conditions such as impaired vascular wall permeability [21]. Steroids have astringent, antimicrobial, and anti-inflammatory properties, which play a role in the process of wound healing [22], [23]. Anti-inflammatory activity can prevent prolonged inflammation, thereby accelerating the process of wound healing [23]. Tannins play a role in increasing the attractiveness of wounds in the wound healing process. The tannins function as an astringent that can cause shrinking of the skin pores, harden the

skin, stop exudates and mild bleeding so that it can cover the wound and prevent bleeding that usually arises in the wound and accelerate epithelialization [14], [15]. Then it is supported by the presence of saponin compounds with a mechanism which inhibits the increase in vascular permeability so that the mechanism of inflammation is inhibited [15].

The Mann Whitney test was used to determine the treatment group that has a significant difference. All treatment groups showed p-value <0.05 which means there were significant differences in all treatment groups in the increasing number of fibroblast cells. Based on the results of all groups of fibroblasts scoring, in the negative control group, positive control, and the treatment group with a concentration of 10% have the same fibroblast scoring value of 2, it can be stated that all three groups experienced increased fibroblasts in mild scoring. While the treatment group with concentrations of 20% and 15% have the same scoring value of 3, it can be stated that all three groups experienced an increase in fibroblasts in moderate scoring. Increasing the number of fibroblasts in the injured area is a combination of proliferation and migration [24].

In this study, all groups experienced fibroblast cell proliferation. This is because fibroblasts originate from local mesenchymal cells associated with the layer of adventitia, their growth is stimulated by cytokines produced by macrophages and lymphocytes. Fibroblasts are the main element in the repair process for the formation of structural proteins, besides, fibroblasts also produce large amounts of collagen, this collagen in the form of triple chain glycoproteins, which is useful for forming strength in scar tissue. The process of fibroblast proliferation and synthetic activation is known as fibroplasia [25].

There are several factors causing garlic to have a higher increase in fibroblast cells compared to the positive control of Madeccasol®. Based on various sources it can be seen that the garlic plant has many chemical compounds that can work as anti-inflammatory and antibacterial properties that affect the proliferation of fibroblast cells. Thus, the results according to the study of garlic can be said to be more effective than the positive control of Madeccasol®.

Conclusion

Data analysis on the number of fibroblast cells using the Post Hoc test summarized using the Mann Whitney test obtained a p-value of 0.002 (p <0.05). It can be concluded that there are differences in the overall concentration of garlic bulb (Allium sativum L.) ethanol extract on the increase in the number of fibroblast cells in the incision wound healing process of male white mice (Rattus norvegicus L.) Wistar strain. From the histopathology observation using fibroblast cell scoring can be concluded that the concentration groups of 20% and 15% have the highest scoring value of 3 with moderate interpretation.

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