

# The effect of giving extract of Giwang ferns (*Euphorbia milii*) cactus leaves on the number of fibroblast white rats burn infected with *Pseudomonas aeruginosa*



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## ABSTRACT

**Background:** Burns is one of society's most common types of trauma. It is estimated that around 1.2 million people experience burns each year and 100,000 of them require hospitalization. The giwang fern cactus leaf (*Euphorbia milii*) extract contains many antimicrobial compounds in almost all plant parts. This study determined the effect of giwang fern cactus leaf extract (*Euphorbia milii*) on the number of fibroblasts in burnt white rats infected with *Pseudomonas aeruginosa*.

**Methods:** This experimental study uses a randomized post-test research design with a control group using three groups of rats. The data results were analyzed statistically using a parametric test with Anova (One Way Anova). Data were analyzed using SPSS version 20.0 for Windows.

**Results:** This study showed that the 50% giwang fern cactus leaf (*Euphorbia milii*) extract had the highest average number of fibroblasts (38.3±8.77), compared with the control (23.3±1.86) and P1 group (25%) (27.6±6.65). Based on the analysis test, it is known that there is a significant comparison between groups ( $p < 0.05$ ).

**Conclusion:** There is a significant difference in the number of fibroblast cells among groups after providing giwang fern cactus leaf (*Euphorbia milii*) extract.

Keywords: *Euphorbia milii*, *Pseudomonas aeruginosa*, Fibroblast

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## INTRODUCTION

Burns are one of the most common types of traumas in society. It is estimated that around 1.2 million people experience burns each year and 100,000 of them require hospitalization.<sup>1</sup> The cases that occur also vary in cause and level of severity. Generally, the severity level is influenced by the area and depth of the body surface that has been burned.<sup>2</sup> The wider and deeper the body's surface involved, the more difficult and longer the healing process. In addition, deep and extensive wounds will also increase the risk of infection, both local and systemic.<sup>3</sup>

If not cared for properly, Burns will be susceptible to bacterial and fungal infections.<sup>4</sup> The loss of skin and tissue continuity allows many germs and microorganisms to enter and form colonies where they shouldn't.<sup>5</sup> It is estimated that

75 percent of burns deaths are caused by systemic and local infection. Therefore, the control of bacteria in the treatment of burns is a vital thing to do. Even though the wound surface is sterile immediately after a burn, several microorganisms will quickly form colonies if proper treatment is not carried out.<sup>5</sup> Further studies have found certain patterns of germs that are usually the cause of infection in burns.<sup>5,6</sup> The normal flora on the skin, respiratory tract, and human gastrointestinal tract mainly forms colonies on external wounds, including burns.<sup>7</sup> Several aerobic bacteria, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella spp.*, and *Enterococcus spp.*, are known to be the main contaminants in burns besides *Candida spp.*, *Aspergillus*, and *Fusarium fungi*.<sup>7</sup>

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a gram-negative bacterium

that is pathogenic for humans, so that it can cause various infections; these infections are difficult to treat because *P. aeruginosa* is a bacterium that is resistant to most antibiotics.<sup>8</sup> This is caused by biofilms on the *P. aeruginosa* bacteria. Infections caused by *P. aeruginosa* are often associated with a low patient's immune system, such as neutropenia, burns, or cystic fibrosis.<sup>8</sup>

Based on data from Lyczak JB et al. in 2000, *P. aeruginosa* generally causes soft tissue infections, urinary tract infections, bacteremia, respiratory tract infections (pneumonia), otitis externa, keratitis, and otitis media folliculitis.<sup>9</sup> In the last decade, *P. aeruginosa* bacterial infection is most often associated with Healthcare-associated infections (HAIs).<sup>9</sup>

Antibiotics that are currently available are indeed able to treat burns well and can prevent infection. Still, it is also undeniable

that the tendency to use traditional plants as medicine among Indonesian people, especially in rural areas, is still high. This is caused by various factors, including access and costs for modern medicine, which are relatively more expensive and difficult for them to reach.<sup>10</sup> One of the traditional plants that are often used as medicine is *Euphorbia milii* or what the people call the Leaves of the Giwang Fern Cactus/*Ekorbia*.<sup>11</sup>

The crushed Giwang Fern Cactus leaves are believed to be efficacious in preventing infection in burns, so they can indirectly speed up the healing process of burns.<sup>11</sup> Based on the background of the problem above, coupled with studies on chemical compounds which show the presence of flavonoids as antibacterial substances, researchers will assess the minimum killing concentration of the leaf extract of the cactus fern giwang leaves against the bacterium *Pseudomonas aeruginosa* which is often the cause of infection in burns.<sup>9</sup>

Based on those mentioned above, this study aims to determine the effect of giving Giwang Fern Cactus Leaf Extract (*Euphorbia milii*) on the number of fibroblasts in white rats burnt and infected with *Pseudomonas aeruginosa*.

## METHODS

This study approach is experimental research with a Post Test Only Control Group Design, which only makes observations of the control and treatment groups after being given an intervention. This research was conducted experimentally to test the effectiveness of several concentrations of *Euphorbia milii* leaf extract, which could affect the number of fibroblasts and the inhibition of the growth of *Pseudomonas aeruginosa*. Previously, plant determination was carried out to standardize the plants to be used.

The independent variable in this study was the administration of giwang fern cactus leaf extract in various concentrations of 25% and 50%, capable of inhibiting the growth activity of the *Pseudomonas aeruginosa* bacteria with an increase in the number of fibroblasts. The dependent variable in this study was the increase in the number of fibroblasts in white mice. The control variables in this

study were the process of making extracts, the cage temperature, light, humidity, type of rats, rat food, rat body weight, rat age, and rat sex.

The tools used in this study were Erlenmeyer flasks, measuring cups, microscopes, micropipette, round loops, tweezers, tube racks, rotary evaporators, 1 ml syringes and 10 ml syringes, analytical scales, gas stoves, sample drying cabinets, test tubes, glass jar, Urine pot, and metal coin.

The materials used in this study were the Giwang fern cactus leaves (*Euphorbia milii*), pure culture of *Pseudomonas aeruginosa* bacteria, ethyl acetate, filter paper, 10% formalin, and Hematoxylin Eosin color. The research material used was the leaves of the giwang fern cactus (*Euphorbia milii*) obtained in the Denpasar area of Bali. The population of 35 rats has received as many as 27 rats according to the inclusion criteria, with each treatment group consisting of 9 white male rats.

## Research Protocols

### Making *Simplicia* of Giwang Fern Cactus Leaves

Old Giwang fern cactus leaves are washed thoroughly with running water. Then it is placed in a baking dish. Then it is dried using an oven at 50°C for several days until it becomes dry. The dry Giwang fern cactus leaves are then crushed into powder using a blender.

### Making of Giwang Fern Cactus Extract (*Euphorbia milii*)

The dried leaves were crushed into powder using a blender and collected up to 20 grams. The leaf powder is mixed with 250 ml of ethyl acetate solvent and stored in a glass jar. The glass jar was closed and stirred every 30 minutes for 6 hours and then the solution was left (macerated) for 48 hours. After that, the solution was filtered using Whatman No.1 filter paper. Then, the solvent was evaporated using a rotary evaporator to obtain a crude ethyl acetate extract. The extracts obtained were then collected and the total weight was calculated. The extract is assumed to have concentrated 100% and is ready to be diluted into various concentrations using ethyl acetate as a solvent. Furthermore, the extract is stored in a dark or clear

aluminum foil bottle at 5°C to prevent chemical changes.

Preparation of the concentration of the cactus fern giwang leaf extract with 25% and 50%, using ethyl acetate. Make the concentration of a solution with the number of grams of substance in 100 ml of ethyl acetate as follows:

1. The concentration of 25% = 25 grams of cactus fern giwang leaf extract in a 100 ml volumetric flask added ethyl acetate to the mark.
2. The concentration of 50% = 50 grams of cactus fern giwang leaf extract in a 100 ml volumetric flask added ethyl acetate to the mark.
3. Extract yield is calculated by:  $\text{extract weight (final) / Simplicia weight (initial)} \times 100\%$ .

## Phytochemical Screening Test

**Alkaloid Test** A total of 1 ml of giwang fern leaf extract was taken and 5 ml of 1% HCL was stirred in a water bath at 60°C for 15 minutes and then filtered. The filtrate obtained was then added with 1 ml of Dragendorff reagent. The formation of an orange precipitate indicates a positive sample containing alkaloids. **Flavonoid Test** As much as 1 mL of each test solution was put into 2 test tubes. Tube 1 is used as a blank solution. Tube 2 is added with 1 mL of 10% Pb acetate (lead acetate) solution. The formation of a yellow precipitate indicates positive results of flavonoids.

**Tannin Test** As much as 1 ml of earlobe fern leaves was put into a test tube, then 1 ml of 3% FeCl<sub>3</sub> solution was added and then shaken. The formation of a green-black color indicates a positive sample containing tannin compounds. **Saponin Test** Saponin compounds can be identified using the Foth method. As much as 2 ml of giwang fern leaf extract was put into a test tube, added with 10 ml of distilled water, and shaken for 30 seconds. If the consistent foam is formed (lasts for 30 seconds), then the sample is positive for saponins.

## Making mouse models (wounded and infected with bacteria)

Burns were made on the back of a rat about 3 cm, which had been shaved using Veet®. Furthermore, the rats were anesthetized using a mixture of ketamine (40 mg/

kg BW) and xylazine (5 mg/Kg BW) intramuscularly. Moreover, the rats were attached to a metal coin for 10 seconds and heated in a flame for 5 minutes.

Using a sterile cotton swab, giwang fern cactus leaf extract was tested on burnt white rats infected with *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* was smeared evenly on the white rat media. Then the changes were observed for 7 days, 14 days and 21 days. The ability of the cactus fern giwang leaf extract (*Euphorbia milii*) to grow the *Pseudomonas aeruginosa* was assessed by measuring the visible zones on the burns and the number of white rat fibroblasts. Data processing techniques are carried out by presenting models in tables, graphs and images. The study's results were obtained by measuring the diameter of the wound first in three repetitions shown in tabular form.

Specimen collection & observation of fibroblast cells in white rat specimens taken with a size of 2 x 2 cm in the wound treated by excision. Skin tissue was cut crosswise to see the number of fibroblast cells. The skin tissue of the Wistar rats was stored in a urine pot and soaked in 10% formalin, then stained with Hematoxylin Eosin at the Histology Laboratory, Faculty of Medicine, Udayana University. This research was conducted at the Integrated Laboratory of the Faculty of Pharmacy, Mahasaraswati University Denpasar and the Histology Laboratory of Universitas Udayana, Bali, Indonesia.

### Data Analysis

The study results were statistically analyzed using the One Way ANOVA test from SPSS version 20.0 for Windows.

## RESULTS

The phytochemical screening test showed that the ethanol extract of the cactus fern giwang leaves (*Euphorbia milii*) had saponins and flavonoids, as evidenced by the positive test results (Table 1).

The number of fibroblasts was counted at 200 times and 400 times magnification of light microscopy from each tissue preparation stained with Hematoxylin Eosin staining, which had previously been stored in a urine pot. Observations were carried out in 10 visual fields; each field of view was observed and counted fibroblast

**Table 1. Screening results**

Secondary Metabolite	Observation Result	Conclusion
Alkaloids	No orange precipitate formed	(+)
Saponin	Steady foam is formed for not less than 10 minutes, 1-10 cm high. On the addition of HCL 2N, the foam did not disappear.	(+)
Polyphenols and tannins	The appearance of greenish-black color indicates the presence of compounds	(+)
Flavonoids	A yellow amyl alcohol layer is formed	(+)

**Table 2. Histological data on the number of fibroblast cells between treatment groups**

No.	C	P1	P2	p
1	21	33	40	
2	22	30	39	
3	22	28	39	
4	28	26	38	
5	25	23	41	
6	27	28	38	
7	21	28	39	
8	21	27	37	
9	23	26	38	
Mean ± SEM	23.3 ± 1.86	27.6 ± 6.65	38.3 ± 8.77	<0.05*

SEM: Standard Error of Mean; C: Control; P1: Intervention Group 1; P2: Intervention Group 2; \*One Way ANOVA: statistically significant if p-value less than 0.05.

cells with the characteristics of being large, oval in shape and cytoplasm stained purple. There was a significant number of burn wound fibroblast cells infected with *Pseudomonas aeruginosa* in rats among groups ( $p < 0.05$ ) (Table 2).

## DISCUSSION

This study observed the number of burn wound fibroblast cells infected with *Pseudomonas aeruginosa* in rats with the same treatment frequency but with different sample concentrations among groups. From observations of burns with *Pseudomonas aeruginosa* bacterial infection given to the backs of rats every week, it showed a significant change, where the wound was covered first on top by congealed blood, which formed a crust layer. It is proven that, seen from recent findings, the average number of fibroblasts is the highest in the 50% extract. This crust or scab layer prevents oxidation of the wound so that infection

of microorganisms around and causes the wound cannot develop to infect the wound and the wound healing process will go well.<sup>12</sup>

The results of the phytochemical screening test showed that the ethanol extract of the giwang fern cactus leaves (*Euphorbia milii*) had saponins and flavonoids, as evidenced by the positive results of the test. The tannin content is efficacious as an antiseptic, especially in wounds infected with *Pseudomonas sp* bacteria, with tannins preventing wound infection.<sup>13-15</sup> Flavonoids have anti-inflammatory effects by inhibiting the action of inflammatory mediators such as histamine, bradykinin, and leukotrienes. These three substances play a role in increasing capillary permeability during inflammation.<sup>16</sup> Saponins act as antibacterial and stimulate the growth of new cells in wounds.<sup>17</sup>

Compounds can be said to be antimicrobial if they can inhibit the growth

of microorganisms. Giwang cactus fern (*Euphorbia milii*) contains anti-microbial saponin compounds. Saponin compounds are polar compounds that can only dissolve in polar solvents. Saponin compounds can work as antimicrobials by damaging the cytoplasmic membrane, causing cells to die.<sup>18</sup> There are five mechanisms of antibacterial action, inhibiting microbial cell metabolism, inhibiting microbial cell wall synthesis, interfering with the permeability of microbial cell membranes, inhibiting microbial cell protein synthesis and inhibiting synthesis or damaging microbial cell nucleic acids.<sup>19</sup>

In a previous study, these compounds could be bacteriostatic, namely having the ability to inhibit but not kill, or bacteriocidal, namely having the properties of killing microorganisms.<sup>20</sup> These antimicrobials work by damaging the DNA structure, denaturing proteins, disrupting the cell wall membrane, removing free sulfhydryl groups, and chemical antagonism of the bacterial cells.<sup>20</sup>

Extraction was carried out to obtain the desired compounds in *Euphorbia milii* leaves. The selection of the right solvent can increase extraction efficiency. Selectivity, toxicity, polarity, and ease of evaporation must be considered when choosing a solvent.<sup>21</sup> This research is an experimental study that aims to determine the ratio of the number of fibroblast cells in the wounds of Wistar rats with the leaf extract of the cactus fern giwang (*Euphorbia milii*) leaf. The results of this study showed that the control group had an average number of fibroblast cells, namely  $23.3\% \pm 1.86\%$ , the treatment of cactus fern giwang leaf extract (*Euphorbia milii*) was 25%, had an average of  $27.6\% \pm 6.65\%$ , the treatment 50% leaf extract of the cactus fern giwang (*Euphorbia milii*) had the highest average of  $38.3\% \pm 8.77\%$ . A previous study showed that the leaves of the *Euphorbia milii* plant contained terpenoid and saponin compounds which had toxic effects.<sup>22</sup> It was said that the saponin compounds found in *Euphorbia milii* leaf inhibited the growth of the *Pseudomonas aeruginosa* bacteria.<sup>22</sup> There are five mechanisms of antibacterial action, inhibiting microbial cell metabolism, inhibiting microbial cell wall synthesis, interfering with the

permeability of microbial cell membranes, inhibiting microbial cell protein synthesis and inhibiting synthesis or damaging microbial cell nucleic acids.<sup>20,22</sup>

In this study, it was seen that the 50% concentration extract treatment experienced the fastest wound healing when compared to the other treatment groups; this can be seen from the data with an average wound diameter of 0.655 in the 1<sup>st</sup> week, 0.422 in the 2<sup>nd</sup> week, and 0.233 in the 3<sup>rd</sup> week. The wound healing process consists of 3 phases, namely the inflammatory phase, the proliferative phase, and the healing phase. The inflammatory phase is marked by swelling. The proliferative phase is characterized by the formation of exudate and fibroblasts that look like a crust on the top of the wound, and the healing phase is marked by the formation of new tissue, which means the wound has shrunk or healed. The inflammatory process occurs up to 7 days after the injury. Without inflammation, there will be no wound-healing process. Wounds will still be a source of pain, so inflammation and wound healing tend to cause pain.<sup>19-22</sup>

Inflammation controls bleeding, prevents bacteria entry, removes dirt from injured tissue, and prepares for further healing. The proliferative stage of healing is characterized by the formation of granulation tissue in the wound. The inflammatory phase is short if there is no contamination or significant infection.<sup>19-22</sup> After the wound has been successfully cleaned from the tissue, the proliferation phase begins. In this study, it is estimated that the proliferative phase begins in the 2<sup>nd</sup> week when all the treatment groups and the control group have started the wound-healing process, characterized by fibroblasts after the 1<sup>st</sup> and 2<sup>nd</sup> weeks. It is estimated that they will experience an inflammatory phase.

It is said that there is an overlap between the inflammatory and proliferative phases, the inflammatory phase lasts up to 7 days and the proliferative phase occurs for 7-14 days. The number of fibroblasts in the proliferative phase peaked on day 21—the fibroblast increase in the wound area combined proliferation and migration.

Fibroblasts can function as paracrine and autocrine because they can send

paracrine signals such as IGFBP-3 and -5, IGF-II, Connective Tissue Growth Factor (CTGF), IL-33, CXC chemokines, CC chemokines and reactive oxygen species (ROS).<sup>23</sup> CTGF is very important in wound healing, angiogenesis and fibrosis.<sup>24</sup> CTGF is highly expressed by fibroblasts and endothelial cells. CTGF can bind to a variety of receptors, extracellular ligands and ECM proteins.<sup>24</sup>

This study found that the optimal dose of cactus fern giwang leaf extract (*Euphorbia milii*) in fibroblast cell proliferation was 50%. In the previous study, the optimal extract dose was found at a concentration of 50%.<sup>25</sup> The tannin content is efficacious as an antiseptic, especially in wounds infected with *Pseudomonas aeruginosa*. The presence of tannins prevents infection of the wound. Flavonoids are compounds that have anti-inflammatory effects by inhibiting the action of inflammatory mediators such as histamine, bradykinin, and leukotrienes. These three substances play a role in increasing capillary permeability during inflammation.<sup>16</sup>

There was a decrease in the number of fibroblasts in the rat group treated with 25% extract from week 1 to week 3. This is possibly caused by several factors, namely because the rats were infected with the *Pseudomonas aeruginosa* bacterium, which has superantigens and other influences, namely the behavior of each rat. In contrast, the wound area is likely to be contaminated in a different cage, for example, between mice on top of each other by other microorganisms causing the inflammatory period to increase in length and the process of forming new fibroblasts beginning to increase in the last week. It is indicated that fibroblasts have accelerated the epithelialization process by keratinocytes, so this group experiences epithelialization faster than the other groups.<sup>26</sup> Besides, fibroblasts also secrete the *Keratinocyte Growth Factor* (KGF), which contributes to the re-epithelialization process.<sup>26</sup>

## CONCLUSION

Based on the research that has been done, it can be concluded that there is a significant difference in the number of fibroblast cells among groups. Giving giwang fern cactus

leaf extract (*Euphorbia milli*) affects the number of fibroblasts in the skin of white male rats that have suffered burns infected with *Pseudomonas aeruginosa*.

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## CONFLICT OF INTEREST

There is no competing interest regarding the manuscript.

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None.

## ETHICS CONSIDERATION

Ethics approval was obtained from the Ethics Committee, Faculty of Pharmacy, Universitas Maharaswati, Denpasar, Bali, Indonesia before the study was conducted.

## AUTHOR CONTRIBUTIONS

All authors equally contribute to the study from the conceptual framework, data acquisition, and data analysis until reporting the study results through publication.

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