The efficacy taro leaf extract on wound healing contaminated with *Staphylococcus aureus*

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ABSTRACT

Introduction: Taro (*Colocasia esculenta* (L.) Schott) leaves have been reported to possess wound healing, anti-inflammatory, antibacterial and antioxidant activities. One of the factors that affect wound healing is infection in the wound, wherein the wound is infected by bacteria, especially *Staphylococcus aureus* that is mostly found in the oral cavity, the wound healing process will be hampered and become longer healed. The objective of this research was to analyse the efficacy of *Colocasia esculenta* (L.) Schott extract on the healing process of wounds contaminated with *Staphylococcus aureus*. Methods: This experimental study on *Sprague dawley* rats was carried out at the Veterinary Medical Teaching Hospital of the Faculty of Veterinary Medicine of IPB University. Circular excision wounds were applied on 24 *Sprague dawley* rats with a diameter of 2 cm on the dorsum, and *Staphylococcus aureus* specimens were contaminated with a dose of 3.4x10⁸ LAC. Rats were randomly divided into 2 groups. Group I (control group) was given a placebo, and group II (treatment group) was given a 25% concentration of *Colocasia esculenta* (L.) Schott extract on the wound once a day. Four rats from each group were euthanized on the 3rd, 7th, and 14th days. The number of neutrophils, macrophages, re-epithelialization and FGF-2 expression were measured by microscopic observation with visual field control, and comparative data were analysed using the Kruskal-Wallis statistical test. Results: The number of neutrophils on the treatment group on the 7th day was less than the control group (p=0.040). The number of macrophages on the 3rd day was found more in the treatment group than the control group (p=0.032), and on the 14th day, the treatment group was less than the control group (p=0.040). Epithelial cells on the 14th day of the treatment group was found more than the control group (p=.0.017). The level of FGF-2 expression of the treatment group on the 7th day was higher than the control group (p=0.044). Conclusion: Application of *Colocasia esculenta* (L.) Schott leaf extract is efficacious for healing wounds contaminated with *Staphylococcus aureus* in the proliferative phase.

Keywords: taro; *Colocasia esculenta* (L.) Schott; wound healing; *Staphylococcus aureus*; re-epithelialization; FGF-2 expression

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INTRODUCTION

Colocasia esculenta (L.) Schott is an ancient plant that grows throughout the world; Africa, Asia, West India and South America. Ancient plants that have existed since long ago have flourished in tropical and subtropical regions. Colocasia esculenta (L.) Schott is included in the Araceae family and is known as taro. By the Sasak tribe of Lombok, in addition to being a source of nutrition, taro plants are traditionally used as medicine for scarring, from various studies it is said that taro leaves contain bioactive components with ingredients important in it, including anticancer, antihyperlipidemic, anti melanogenic, wound healing, anti-inflammatory, probiotics, antihypertensive, antioxidants, antimicrobials. The contents of the leaves of Colocasia esculenta (L.) Schott are saponins, flavonoids, tannins, riboflavin, alkaloids, terpenoids, steroids that play a role in healing wounds.2,3

When there is injury to the skin tissue, the process of wound healing and cell regeneration occurs automatically as the body's physiological response to the healing process through three interconnected phases, namely the inflammatory phase (from 1 to 3rd day), the proliferation phase (day 4th to 2-3 weeks) and the remodeling phase (after 3 weeks until 6-12 months).4,5 In the inflammatory phase, neutrophils are one of the inflammatory cells that are mobilized to the injured area. Under normal circumstances neutrophils are rarely found on the skin, but the amount will increase after tissue damage occurs.

Besides being responsible for cleaning the wound area of bacteria, neutrophils also play an important role in antigen presentation, phagocytosis, production of growth factors and also the production of cytokines which are useful for the process of re epithelialization and wound closure.6 After performing its function in the wound area during the wound healing process, neutrophils undergo the apoptosis process. Then the apoptosis of these neutrophils is ingested by macrophages.7

The collection of apoptotic cells by macrophages provides a strong signal for inflammatory resolution to proceed to the next phase. Macrophages are phagocytic cells that are almost found in every organ throughout the body. The function of macrophages, namely phagocytosis, produces and releases cytokines and pro angiogenic, inflammation and fibrogenic factors and releases free radicals. Macrophages also produce prostaglandin, Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor (TGF) Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF) which are the main cytokines that can stimulate the formation of granulation tissue. The release of EGF, FGF TGF-a and TGF-b is thought to stimulate the migration and proliferation of epithelial cells, through keratinocytes which then migrate in the area of the extracellular matrix. When wound closure is reached, keratinocytes form a layer and differentiate to form a barrier as reepithelialization.5,6,9

In the proliferation phase, fibroblasts are found to play a role in collagen synthesis and forming growth factors. One of them is FGF-2 which is a potent angiogenic stimulator. FGF-2 has the function of helping to repair tissue by stimulating cellular differentiation and proliferation, increasing neovascularization formation, mitogenesis, stimulating epithelium and collagen synthesis in the wound healing process.8 The replacement of damaged and dead tissue by new and healthy tissue through a process called reepithelialization.

Reepithelialization began to occur at the wound edge 24 hours post-trauma and granulation in the wound starting on the 5th post-traumatic day. Within 24 hours post-trauma, at the wound edge, the undamaged keratinocyte epithelial cells began to migrate to form new epithelial cells which then migrate. Migration of epithelial cells in the wound area as a monolayer. Approximately 2-3 days post-trauma, keratinocytes in the basal layer of the wound margins and epithelial stem cells of the nearest hair follicle or sweat gland begin to proliferate. Migration continues until there is overlap with epithelial cells originating from other migration directions.6,10

One of the factors that affect wound healing is infection in the wound, wherein the wound is infected by bacteria, especially Staphylococcus aureus that is mostly found in the oral cavity, the wound healing process will be hampered and become longer healed. There are two types of infections in the wound, namely primary infection,
and secondary infection. Primary infection is that there is contamination of germs immediately after the injury, this is because the object that causes the wound contains pathogenic microorganisms and secondary infections are conditions of infection that arise after the occurrence of a wound, this condition is caused by bacteria originating from outside the wound.\textsuperscript{11,12}

Examples of primary infection wounds are injuries due to odontogenic infections. In odontogenic infection wounds, pathogenic microorganisms that are often found are \textit{Staphylococcus aureus}, which we can encounter in normal conditions in the oral cavity.\textsuperscript{11,13}

Treatment of infected wounds is currently using antibiotics or synthetic antimicrobials. Some wound dressings contain antibiotics or antimicrobials. The ideal wound care material or a drug other than controlling infection must also protect standard network and should not interfere wound healing. Various wound care modalities has been used for many years in various types of wound dressings such as cream, ointment, solution. Several types of wound dressings such as occlusive dressing, non-occlusive dressing, absorbent dressing, skin substitute, and negative suction vacuum dress.\textsuperscript{14}

The use of antibiotics or synthetic antimicrobials in the treatment of infected wounds increases the incidence of bacterial resistance and allergic reactions.\textsuperscript{15,16} Research on the use of natural ingredients is currently being developed to address these problems in treating infected wounds. \textit{Colocasia esculenta} (L.) Schott taro leaf has the potential to be researched and developed as a natural ingredient for the treatment of infected wounds. The objective of this research was to analyse the efficacy of \textit{Colocasia esculenta} (L.) Schott extract on the healing process of wounds contaminated with \textit{Staphylococcus aureus}.

**METHODS**

This experimental study on \textit{Sprague dawley} rats was carried out at the Veterinary Medical Teaching Hospital of the Faculty of Veterinary Medicine IPB University. The extract gel of leaves of \textit{Colocasia esculenta} (L.) Schott was made in the Department of Pharmacy Faculty of Veterinary Medicine IPB University. The leaves are washed under running water, then aerated. The sample is then chopped into small pieces. The sample extract was made using the maceration method. The solvent used was 96% ethanol with a ratio of 1:10 simplicia and irradiance.

The maceration process is carried out for 3x24 hours. The macerated filtrate is then evaporated using a rotary evaporator to produce a thick extract. The plant extract gel preparation had a concentration of 25%. The preparation of the gel was carried out by adding 10 grams of plant extract to the gel base (90 grams). The mixture is then stirred until homogeneous. The extract obtained is stored in a sterile container then stored in a refrigerator at 60-70°C, and is used for treatment.

Observations were carried out on 24 \textit{Sprague dawley} rats aged 8 weeks with a weight of 200-300 grams which had been acclimatized for 2 weeks. All \textit{Sprague dawley} rats were incised on the back and contaminated with a dose of 3.4x10\textsuperscript{8} CFU / ml \textit{Staphylococcus aureus} bacteria obtained from the Department of Microbiology Faculty of Veterinary Medicine IPB University, and left for 3 days without antibiotics, and given analgesics for three days.

The twenty-four SD white rats were divided into two groups, each group consisted of 12 individuals, namely the group that received the application of \textit{Colocasia esculenta} L. Schott leaf extract gel in his incision and the untreated control group. The application of \textit{Colocasia esculenta} L. Schott taro leaf extract gel was applied everyday to the group that received the application, a day after incised wounds were made and were contaminated with \textit{S. aureus}. And for the control group, they were left untreated. Then each group was divided into 3 time observations, with necropsy on day 3, day 7 and day 14 to see the presence of research variables namely the number of neutrophil cells, macrophages, reepithelialization and expression of FGF-2.

Examination of neutrophil cells, macrophage cells and reepithelialization amounts were observed through Hematoxylin eosin (HE), and FGF-2 expression examination were observed with Immunohistochemical examination (IHC) using FGF-2 antibody reagents from Santa Cruz Biotechnology. The HE staining procedure
begins with planting the specimen in a paraffin block that has previously been fixed in 10% BNF, decalcification, trimming, dehydration and embedding. The tissue was cut with a microtome with a thickness of 3-5 µ. Then the tissue is put into a water bath with a temperature of 35°C for about 20 seconds.

Continued to take the preparation with a glass object, drain it and put it in an incubator for 2 hours at a temperature of 45°C. The xylol I was administered on the slide for 2 minutes, followed by giving xylol II for 2 minutes. Followed by giving absolute alcohol for 2 minutes, 95% alcohol for 1 minute, 80% alcohol for 1 minute. Wash the slides under running water for 1 minute until the tissue is colorless. Stained with Mayer’s Haematoxyllin for 8 minutes.

Wash the slides under running water for 30 seconds. Give lithium carbonate for 15 to 30 seconds. The next step is to wash the slide under running water for 2 minutes. Eosin staining for 2 - 3 minutes, then wash the slides in running water for 30 - 60 minutes. Followed by immersing 10 dyes of 95% alcohol, absolute alcohol I of 10, absolute alcohol II for 2 minutes. The administration of xylol 1 for 1 minute, xylol 2 for 1 minute and finally cover the slide with a cover glass.

The IHC procedure performed with fixation, tissue creation, embedding, then the tissue was cut using a 3-5mm thick microtome. The tissue was placed in the object glass and deparaffinized and rehydrated with 96%, 80%, 70% absolute alcohol, then was being washed under the running water. Giving endogenous 0.5% peroxide blocking and retrieval decloaking chamber (diva) antigen. Furthermore, the pieces of tissue are cooled for 20 minutes. Wash in PBS pH 7.4 and block with a proxy block. Administration of FGF-2 primary antibody with a 1: 500 dilution in 1% BSA, then the tissue pieces are cooled for 4 °C overnight (±14 hours), then washed in PBS. The pieces of tissue are reacted with a secondary antibody in the form of chromogen DAB + buffer substrate. The complex formed between the primary antibody and the antigen will be bound by the secondary antibody. Washing with running water. Positive reactions were observed with the appearance of a brown color after dropping the pieces of tissue with chromogen in the form of 3-diaminobenzidine (DAB) solution.

The DAB reaction was stopped by washing the tissue pieces in running water and soaking in PBS (5 minutes, 3 times). Then dehydration of tissue preparations at graded alcohol concentration and mounting by attaching the cover glass using Entellan adhesive. Then looking at the stain under a microscope, which on the CPI will give a brown color.

The scoring method used is ordinal type by means of blind scoring. Scoring of neutrophil, macrophage and re epithelialization cell distribution in the HE examination was obtained through the Gibson-Corley et al system. And for immunohistochemical results FGF-2 was measured based on a semiquantitative approach, which was determined by cell distribution and color intensity, then converted into histopathologic calculations via the scoring criteria of The Immunoreactive Score (IRS).

The data analysis was determined by looking at the mean and standard deviation. Comparison of statistical results was carried out using a bivariable difference test to look for differences between the immunohistochemical expression of FGF-2 control samples and experimental animal samples. Because the distribution is not normal, the Kruskal-Wallis statistical hypothesis test was performed. Prior to the study, an ethical clearance approval had been obtained, with the number of 124-2018 IPB.

RESULTS

The macroscopic observation of the wound was 2 cm in size in the dorsal region of Sprague dawley rats in the treatment group application of Colocasia esculenta (L.) Schott leaf extract and control, as presented in Figure 1 to Figure 4.

Neutrophils

Microscopic observations with HE staining of neutrophil counts are by finding neutrophil cells depicting three nuclei of bluish red cells with granules in the cytoplasm in Sprague dawley rats in the application group of Colocasia esculenta (L.) Schott leaf extract and visibility control (100x), as presented in Figure 5 and Table 1.

Table 1 presented the number of reepithelizations on the 3rd day in the treatment group. The reepithelialized cells were found in the
Figure 1. Injuries of Sprague Dawley rat in the dorsal area with 2 cm in diameter.

Figure 2. A. Sprague Dawley rat in the non-treated group of gels which was euthanized on the 3rd day; B. Sprague Dawley rat in the treatment group application of *Colocasia esculenta* L. Schott leaf extract on the 3rd day.

Figure 3. A. Sprague Dawley rat in the non-treated group of gels that was euthanized on the 7th day; B. Sample of wound in the treatment group application of *Colocasia esculenta* L. Schott leaf extract on the 7th day.

Figure 4. A. Sample of wound in the non-gel treatment group recommended on the 14th day; B. Sample of wound in the treatment group application of *Colocasia esculenta* (L.) Schott leaf extract on the 14th day.

Figure 5. Neutrophil cells (arrows) in the group who were given *Colocasia esculenta* (L.) Schott leaf extract: A. on the 3rd day; B. on the 7th; C. on the 14th day after injury; HE staining, magnification 100x.
The efficacy taro leaf extract on wound healing contaminated with Staphylococcus aureus (Sjamsudin et al.)

The efficacy taro leaf extract on wound healing contaminated with Staphylococcus aureus (Sjamsudin et al.)

category of less than 25% to 50%, whereas in the control group re epithelial cells were found not including less than 25%. On the 7th day in the group using Colocasia esculenta (L.) Schott leaf extract, epithelial cells were found in the category of less than 25% to 50%, as well as in the control group re epithelial cells were found to be less than 25% to 50%. On the 14th day in the group using Colocasia esculenta (L.) Schott leaf extract, epithelial cells were found in the 51% category to more than 75%, whereas in the control group re epithelial cells were found to be less than 25% to 50%. Epithelial cells on the 14th day in the treatment group were more numerous than the control group (p=.0.017).

**FGF-2**

Microscopic observations of FGF-2 expression was performed with IHC staining and visual control

**Table 1.** The amount of neutrophils in the wound healing process contaminated with Staphylococcus aureus bacteria on days 3rd, 7th and 14th

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Description: *Significant differences (p <0.05), Kruskal-Wallis*

Figure 7. Re-epithelialization (arrow) in the group given the 7th day Colocasia esculenta (L.) Schott leaf extract after injury, HE staining, magnification 100x.

Figure 8. Expression of FGF-2 with immunohistochemical staining seen in brown with color intensity: A. Strong; B. Medium (on the 3rd, 7th and 14th day in observation); C. Weak.
under the magnification of 100x, as presented in Figure 8 and Table 4. Table 4 explains that the FGF-2 expression level on the 3rd day, in the group administered with Colocasia esculenta (L.) Schott leaf extract, was found in moderate to strong expression, while at the 7th day and 14th day observation were found on medium to strong expressions. The level of expression of FGF-2 on the 7th day of the treatment group was higher than the control group (p=0.044); the level of expression was moderate to strong. In the control group, on the 3rd day until the 14th day of observation, the FGF-2 expression was found in the medium expression category.

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<td>Moderate expression</td>
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<tr>
<td>Strong expression</td>
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Description: The p value is calculated based on the numerical expression FGF-2, Kruskal-Wallis

**DISCUSSION**

**Neutrophils**

The data in Table 1 showed that the number of neutrophils on the 7th day in the Colocasia esculenta (L.) Schott leaf extract group (treatment) was less than the control group (p=0.040). Gibson-Corley et al. investigated the anti-inflammatory activity of the ethanolic extract of Colocasia esculenta leaves extract in Wistar rats. They concluded that the extract are able to inhibit leukocyte migration, reduce pleural exudate, and reduce granuloma weight.17,18,19,20 Neutrophils are one of the most abundant types of leukocytes, accounting for 60-70%.21 Neutrophils are chemotaxic and infiltrate inflammation faster, therefore neutrophils are often referred to as the first cellular defense.11

During the histopathological observations, the two groups showed an average pattern of almost the same number of neutrophils which was high in the early days and decreased gradually in the following days. All groups experienced a decrease in the number of neutrophils from the 7th day until the 14th day, along with the drying process of wounds due to the presence of several inflammatory mediators that have been released by neutrophils such as histamine, lysosomal enzymes, and platelet activating factors. This condition showed that neutrophil cells performed their roles as defense cells only at the beginning of post injury because their task will be replaced by macrophage cells as the second cellular defense cell.6,22

The main functions of neutrophils are phagocytic and microbicidal. Neutrophils are the first leukocytes to respond to the presence of foreign objects in the wound, the way neutrophils work in providing an immune response is to use lysosome enzymes that can digest several bacterial cell walls, proteolytic enzymes, ribonuclease and phospholipase together which can destroy several bacteria. Neutrophils when entering the tissue are mature cells that can immediately start phagocytosis. A neutrophil cell can phagocyte 5-20 bacteria before the neutrophil cells themselves become inactive and die.6,11,22
Macrophages

The analysis of the effect of *Colocasia esculenta* (L.) Schott leaf extract showed that the number of macrophages on the 3rd day was more in the treatment group than the control group (p=0.032). Meanwhile, on day 14, the treatment group was less than the control group (p=0.040). It was suspected that the administration of *Colocasia esculenta* (L.) Schott leaf extract had an effect on the migration of macrophages to the wound area when compared to the control group on the 3rd day after injury. Macrophages play an important role in the wound healing process since they produce growth factors as well as trigger angiogenesis and fibro genesis.

The excreted macrophages can even phagocytize bacteria and clean out tissue debris. During the transition from inflammatory processes to wound repair, macrophages can stimulate cell migration, proliferation, and tissue matrix formation. Growth factors involved in angiogenesis are transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and fibroblast growth factors-2 (FGF-2). This is probably due to alkaloid compounds stimulating the migration and proliferation of fibroblasts, stimulating macrophages and the process of angiogenesis.

The content of vitamin C stimulates the migration of monocytes to the wound area which then transforms into macrophages through modulation of the production of several cytokines during the inflammatory phase in the wound healing process.

Within a period of 6 hours after a wound occurs, the circulating defense cells will enter the wound area. PMN is the first cell, and the number will increase until it reaches a peak at 24-48 hours after injury. Short life time and the number begins to decrease on the 4th or 5th day after injury. The next stage, macrophages will migrate to the injured area. These cells come from monocytes that undergo migration and chemotaxis. Macrophages appear first in the wound area about 48 hours after injury and reach a peak on the 3rd day after injury. Macrophages begin to disappear from wounds in resolution/repair, around the 5th day after injury. Some macrophages are destroyed in the injured area and some migrate to the lymph nodes for drainage.

Re-epithelialization

Epithelial cells on the 14th day in the treatment group were found more than the control group (p=0.017). In the process of reepithelialization, epithelial cells undergo gradual migration in layers. Keratinocytes migrate to the wound area around 24 hours after injury. On the 1st and 2nd day, migration of epithelial surface closure is only two to three cells thick and forms a basal layer. The peak of proliferation occurs after the 3rd day and continues until the epithelialization process is complete. The wound will be seen with granulation tissue marked by the appearance of scab. Wound healing is strongly influenced by reepithelialization, because the faster the process of reepithelialization, the faster the wound is closed so that the wound heals faster. This shows that the administration of leaf extract gel has the ability to accelerate wound closure with a faster reepithelialization process compared to the control group.

Reepithelialization is the process of repairing skin epithelial cells that leads to wound closure. Reepithelialization consists of stages of wound repair which include mobilization, migration, mitosis and differentiation of epithelial cells. These stages will restore the integrity of the lost skin. Mitosis and migration of epithelial cells will function to restore the integrity of the skin. On the skin surface, reepithelialization will occur through the movement of epithelial cells from the edge of the free tissue to damaged tissue. Fibronectin is an important matrix component that supports keratinocyte adhesion and guides cell movement in crossing wounds. At the beginning of epithelialization, temporary matrix formation is carried out by fibronectin together with fibrin. The matrix acts as a cellular anchor and the way for the epithelium to migrate on its own. Fibronectin is an important matrix component that supports keratinocyte adhesion and guides cell movement in crossing wounds. At the beginning of epithelialization, temporary matrix formation is carried out by fibronectin together with fibrin. The matrix acts as a cellular anchor and the way for the epithelium to migrate on its own.

FGF-2

The data in Table 4 showed that the expression level of FGF-2 on the 7th day of the treatment group was higher than the control group (p=0.044), and the expression level was moderate to strong. Protein is an important factor of cell communication that works locally and chemically, which is needed in the integration of cell functions for development and homeostasis.
FGF-2 is a single chain protein composed of amino acids acting as signal transmitters secreted to tyrosine kinase and protein receptors intrasel non signaling, which is produced by almost all cells in the body. FGF-2 signals control from embryonic development, maintain tissue homeostasis, improve wound healing and tissue regeneration and regulate organ function.

Colocasia esculenta L. Schott leaf extract compound has antibacterial, antifungal, anti-inflammatory, and antimicrobial effects. Saponins and tannins stimulate the secretion of growth factors that can affect fibroblast proliferation. FGF2 is the key factor in promoting fibroblast proliferation. The FGF main pathway is RAS/ MAP kinase which contains many protein signaling. An important event of FGF signal pathway is tyrosine residue phosphorylation on protein docking.

FGF-2 is produced by inflammatory cells, vascular endothelial cells, fibroblasts and keratinocytes. They play a role in epithelialization, angiogenesis and granulation tissue formation. FGF-2 also stimulates the production of decreased ECM and enzyme matrices, thus contributing to matrix synthesis and remodeling which is very important for wound healing. FGF-2 is expressed by fibroblasts and keratinocyte proliferation. These factors are mitogenic and motogenic for keratinocytes and induce enzymes that are important for nucleotide synthesis, as well as MMP production. Apart from playing a direct role in wound healing, FGF-2 also stimulates the production of TGF-α and other ligands by dermal keratinocytes thus contributing to epithelialization.

FGF-2 is extensively developed to maximize its role in wound healing. FGF-2 stimulates cell proliferation in the injured area and increases cells to produce cytokines and other growth factors that stimulate the migration of macrophages and monocytes to the injured area to destroy damaged or dead cells. FGF-2 also stimulates epithelial cells and endothelial cells for migration to tissue healing. In accordance with its original name, FGF-2 increases the growth and differentiation of fibroblasts, and stimulates cells to release collagenase and plasminogen activator to improve angiogenesis in wound tissue. FGF-2 also regulates procollagen I expression and suppresses collagen production and stores it in fibroblasts thus preventing scar tissue formation.

FGF-2 is formed by fibroblasts, blood vessel smooth muscle cells, adrenocortical cells, chondrocytes and osteoblasts that help improve new vascularization formation, mitogenesis, stimulates epithelium and collagen synthesis. Further research is needed to test the effectiveness of the leaf extract concentration of Colocasia esculenta (L.) Schott in healing wounds contaminated with Staphylococcus aureus bacteria. It is necessary to do a culture test before and after the contamination of the Staphylococcus aureus culture to determine the condition of the infected tissue.

CONCLUSION

Application of Colocasia esculenta (L.) Schott leaf extract is efficacious for healing wounds contaminated with Staphylococcus aureus through examination of neutrophils, macrophages, re-epithelialization, and expression of fibroblast growth factor-2. The Colocasia esculenta (L.) Schott leaf extract affects the proliferative phase of wound healing.

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