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Antioxidant Activity of the Peel of Citrus sinensis *(L.) Osbeck on the Histological Features of Second-Degree Burns in Mus musculus*

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Abstract

Burns are injuries to the tissue that not only occur on the skin's surface but can also affect deeper layers. Globally, burns are the fourth most common type of injury, after traffic accidents, falls, and physical abuse. The cost of treatment is relatively high, depending on the area of the burn; the larger the area, the higher the costs. Many researchers have increasingly carried out research on burn treatments using herbal ingredients. One of the typical Indonesian herbal ingredients is the Pacitan orange (*Citrus sinensis* (L.) Osbeck). Correspondingly, this experimental study aimed to investigate the formation of epithelial cells, fibroblasts, and collagen after treatment with Pacitan orange peel extract. The sample in this study consisted of 20 white rats, which were divided into four groups: Group 1 (K1) - the burn group without treatment; Group 2 (K2) - the burn group with Bioplacenton treatment; Group 3 (K3) - the burn group with 0.9% NaCl treatment; and Group 4 (K4) - the burn group treated with 100% Pacitan orange peel extract. The results of this study indicated that the treatment with Pacitan orange peel extract could accelerate the healing process of Grade II burns in the skin of white rats, as evidenced by increased collagen production, epithelial thickness, and fibroblast activity.

Keywords: Pacitan orange peel extract, burns, *Mus musculus*, histopathology

Introduction

Globally, burns are the fourth most common type of injury, after traffic accidents, falls, and physical violence. Until the first half of the 20th century, the treatment of burn patients was very limited, and they often died of hypervolemic shock within the first few days after injury (Markiewicz-Gospodarek et al., 2022).

A recent study demonstrated that burn injuries affect morbidity and mortality for at least 5–10 years after the injury (Mason et al., 2019). A second-degree burn, also known as a superficial partial-thickness burn, affects the superficial layer of the dermis. Blisters are common and may still be intact when first evaluated. Once the blister is unroofed, the underlying wound bed appears homogeneously red or pink and will blanch with pressure. These burns are painful. Healing typically occurs within 2 to 3 weeks with minimal scarring. A deep partial-thickness burn involves the deeper reticular dermis. Similar to superficial partial-

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thickness burns, these can also present with intact blisters. Once the blisters are debrided, the underlying wound bed appears mottled and will sluggishly blanch with pressure (Giretzlehner, Ganitzer, & Haller, 2021).

One cause of skin damage is contact with extreme temperatures. Skin tissue can be damaged at certain temperatures and exposure times, such as high temperatures with short contact times or lower temperatures with long contact times. Heat that impacts the body not only causes local damage but also has systemic effects. These changes are commonly observed in burn injuries.

Epithelialization, which is crucial in the healing process of burns, is often hindered by various factors, including infection or the presence of necrotic tissue. The goal of wound management is to promote rapid healing in a way that is both functionally and aesthetically satisfactory. Physiologically, the wound healing process consists of three phases: the inflammatory phase, the proliferation phase (or fibroplasia), and the maturation (or remodeling) phase. Epithelialization occurs during the proliferation phase of wound healing. It is the process of coating the wound surface with new epithelium derived from the proliferation and migration of keratinocytes at the wound edge and base (Kalaszczynska & Ferdyn, 2015).

Based on the depth of the wound, burns are classified into three degrees: Grade I (epidermal), Grade II (superficial or deep dermal), and Grade III (full thickness). The damage in Grade II burns extends to the dermis layer. The dermis is a skin layer containing connective tissue, which supports the epidermis, fibroblasts, and leukocytes that can migrate from blood vessels. The healing phase for Grade II burns typically lasts 1 to 6 weeks (Evers et al., 2010).

The cost of burn treatment is relatively high, depending on the burn area—the larger the area, the higher the treatment costs. Many researchers have conducted research on the treatment of burns using herbal ingredients. One such herbal ingredient is the Pacitan orange (*Citrus sinensis* (L.) Osbeck), a typical Indonesian plant. In addition to being inexpensive, Pacitan oranges are readily available and easy to cultivate. Given the high potential, the researchers aimed to conduct experimental research on the potential of Pacitan orange peel extract during the inflammatory phase of erythema in white rats with Grade II burns.

Research Methods

This study employed an experimental research method with a post-test-only control group design. The sample consisted of male Wistar rats (*Mus musculus*). The inclusion criteria were male Wistar rats aged 2 months, weighing 150–200 grams, and in healthy condition with no anatomical abnormalities. The exclusion criteria were Wistar rats in a sick condition (not actively moving) or with anatomical abnormalities. The drop-out criterion was the death of a rat during the study.

Tools and Materials

To create burns on the experimental animals, the following equipment was required: A sterile equipment tub containing gloves, gauze, iron plate (diameter 2 cm), syringe, shaver, sterile comb with cotton, lidocaine/other local anesthetic agents, hot water at 100°C, sterile water, 70% alcohol, ruler, waterproof sheet, scissors, tweezers, and practicum suit.

For the treatment of the wounds created on the experimental animals, the following tools and materials were required: A sterile tub containing gloves, gauze, anatomical tweezers (2 pieces), Pacitan orange peel extract (40%, 60%, and 80%), normal saline, aquadest (distilled water), tweezers, waterproof sheet, plaster, scissors, and forceps.

Procedure

Making Pacitan orange peel extract

The steps to prepare the Pacitan orange peel extract were as follows:

1. Remove the white inner part (albedo) of the Pacitan orange peel using a knife, leaving only the outermost layer (flavedo).
2. Cut the Pacitan orange peel into small pieces to facilitate faster drying.
3. Place the cut orange peel pieces in a petri dish, ensuring that the pieces are not stacked to allow for quicker drying.
4. Dry the Pacitan orange peel in an oven for approximately 1 day at a temperature of 60°C.
5. Once dried, grind the Pacitan orange peel using a blender.
6. Extract the dried Pacitan orange peel using a 96% alcohol solvent in an evaporator.

Burn creation procedure

Animal experimental models are important tools for evaluating burn therapeutics. Rats are commonly used in such experiments (Cai et al., 2014). The procedure for creating burns in experimental animals was as follows:

1. Determine the area for the wound, which in this experiment was the upper right back.
2. Clean and shave the area, leaving a margin of approximately 5 cm around the wound site.
3. Disinfect the area to be wounded and wait until the alcohol has dried.
4. Administer anesthesia using lidocaine (1–1.5 cc).
5. Soak a 2x2 gauze block in hot water (approximately 97–100°C) for 1 minute.
6. Attach the gauze to the experimental animal for 40 seconds, then wash the area with sterile water.
7. Dry the wound and close it.
8. Clean and organize the tools, and then wash hands.

Procedure for making histological preparations

Samples for this study were collected through biopsy. Prior to the skin biopsy, the white rats were anesthetized using a combination of ketamine and xylazine administered intramuscularly (De Monte et al., 2015). A skin biopsy was performed on the 14th day after the operation. It was conducted on 20 white rats, which were divided into 4 groups: K1, K2, K3, and K4. The skin biopsy was taken from the injured skin on the back, and a skin sample with a diameter of approximately 1.5 cm was collected. The sample was then placed in an organ tube containing 10% formalin (Giretzlehner, Ganitzer, & Haller, 2021).

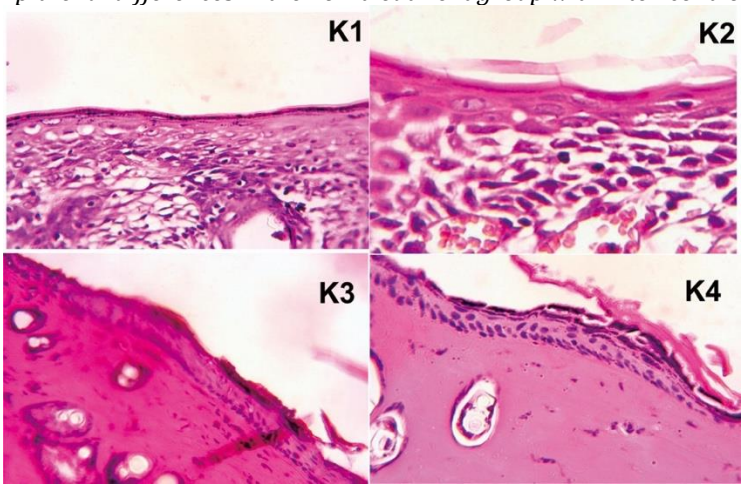
Histological preparations were made according to Gunawan et al. (2019). The samples, which consisted of skin fixed in 10% formalin, were dehydrated and successively cleaned using a series of solutions (3 times with 10% formalin, 70% alcohol, and 96% alcohol; 3 times with absolute alcohol; 3 times with xylol; and 2 times with liquid paraffin) over 23 hours. The samples were then embedded in liquid paraffin. After cooling for 30 minutes, the samples were cut using a microtome. Before mounting, staining was performed using the Hematoxylin and Eosin (HE) method. The samples were soaked in xylol I, II, and III for

5 minutes each. They were then soaked in absolute alcohol I and II for 5 minutes each. After soaking in aquadest for 1 minute, the samples were immersed in the HE solution for 15 minutes. Following this, they were immersed in aquadest again (1 minute), followed by 5-7 minutes in 10% acid alcohol and two washes in aquadest (1 minute and 15 minutes, respectively). The samples were then stained with eosin. Finally, the stained preparations were dehydrated by soaking them in 4 changes of 96% alcohol for 3 minutes each, followed by cleaning in xylol I and II for 5 minutes each.

Results and Discussion

The results of this study demonstrated that the administration of Pacitan orange peel extract could accelerate the healing process in Grade II burns in white rats, as observed through histopathological changes in epithelial thickness, collagen production, and fibroblast activity from day 1 to day 14 after the burn induction. The findings indicated that there were differences between the four groups. In the treatment group, inflammatory cells were absent on days 10 and 15, while in the control group, inflammatory cells were absent only on day 15. Collagen production, as observed during examinations on days 1, 5, 10, and 15, gradually increased, with a mild to noticeable increase over time. Collagen production in the treatment group was faster compared to the control group.

Figure 1. Epithelial differences in the non-treatment group with inter-control clusters.



Discussion

Differences in epithelial thickness were observed across the K1, K2, K3, and K4 groups. The K4 group exhibited a higher epithelial thickness compared to the K2 and K3 groups. The increase in epithelial thickness was particularly noticeable in the treatment group compared to the control group. An increase in the number of new blood vessels was also observed. It can be noted that the treatment group exhibited a gradual increase in the number of new blood vessels compared to the control group.

Overall preparations displayed perfectly stained cell nuclei, cell membranes, and extracellular matrix. The cell nuclei and cell membranes were stained blue due to hematoxylin (HE) staining. Hematoxylin was alkaline and stained the acidic cell nuclei blue-violet. Fibroblast cells, stained with hematoxylin-eosin, appeared purplish-blue. This result aligned with the principle of hematoxylin-eosin staining, where acidic fibroblasts were stained by the alkaline hematoxylin, resulting in a blue-to-purple color. The cytoplasm and extracellular matrix were stained pink by eosin. This staining followed the principle of hematoxylin-eosin, where eosin bound to positively charged protein molecules in the cytoplasm and connective tissue, resulting in a pink color (Alturkistani, Tashkandi, & Mohammedsaleh, 2015).

The progression and overlap of the phases involved in the physiological wound healing process were as follows: (a) Inflammation began with coagulation, platelet aggregation, and fibrin clot formation. Inflammatory events occurred through neutrophil and macrophage infiltration and phagocytosis of debris, apoptotic cells, and pathogens. Anti-inflammatory events occurred through inhibition of the destructive inflammatory process and promotion of proliferation. (b) In the proliferation phase, angiogenesis, reepithelization (epithelial cell mitosis and fibroblast transformation into myofibroblasts), and granulation tissue formation (extracellular matrix composed of collagen, glycoproteins, proteoglycans, fibroblasts, and keratinocytes, under the modulation of MMP-9) occurred. (c) Remodeling was marked by the reorganization of the extracellular matrix (EMC), apoptosis of cells, regression of angiogenesis, and replacement of type III collagen by type I (Serra et al., 2017).

Burns are wounds caused by heat energy, leading to skin damage and protein denaturation (Franck et al., 2019). Cells that are stressed and damaged by burns activate MAPK signaling and produce proinflammatory cytokines, including IL1, IL6, IL8, and TNF-α (Zhang et al., 2020). The wound healing process involves the inflammatory, proliferative, and remodeling phases. It is a biological process aimed at repairing the damage (Kelly & Johnson, 2021).

Besides the epithelium, the main component in the wound-healing process is collagen. Fibroblasts are cells responsible for collagen synthesis. The physiology of wound healing naturally progresses through phases, such as the inflammatory phase, which occurs during vasoconstriction, hemostasis, and the infiltration of inflammatory cells. This phase begins at the occurrence of the wound and lasts until the fifth day. Immediately after the wound occurs, the severed blood vessels undergo constriction and retraction, accompanied by hemostatic reactions due to platelet aggregation. This hemostatic process releases and activates cytokines, including Epidermal Growth Factor (EGF), Insulin-like Growth Factor (IGF), Platelet-Derived Growth Factor (PDGF), and Transforming Growth Factor Beta (TGF-β), which play a role in the chemotaxis of neutrophils, macrophages, mast cells, endothelial cells, and fibroblasts. This state is called the inflammatory phase, in which vasodilation and the accumulation of Polymorphonuclear Leukocytes (PMNs) occur (Darwin, 2016).

Following tissue injury, the inflammatory response plays a critical role in both normal and pathological healing. The body's innate immune system is activated immediately after an injury, initiating a rapid and localized inflammatory reaction. As a result, inflammatory cells are recruited from the circulation to respond to the host tissue damage. However, persistent inflammation leads to the excessive production of reactive oxygen species (ROS), resulting in oxidative stress (Shafiq et al., 2021).

ROS are responsible for regulating the normal healing response and tissue repair process through various mechanisms (Pedro et al., 2022). Nonetheless, under certain pathological conditions, ROS levels can exceed 500 μM in the inflammatory tissues, much higher than the normal tissue level of 1–15 μM (Samadian et al., 2010). Since skin tissues are particularly susceptible to oxidative stress, excessive ROS production can lead to protein dysfunction, abnormal cellular interactions, DNA/RNA damage, and cell apoptosis (Liu et al., 2018).

The proliferative phase occurs during the processes of wound contraction, tissue formation, and re-epithelialization. Tissue formation involves neoangiogenesis, the formation of an active extracellular matrix, and the production of TNF and TGF, which accelerate wound healing. Wound contraction leads to increased migration and fibroblast proliferation, forming new tissue structures that accelerate healing. Re-epithelialization increases the epithelial regeneration, triggering epithelial formation and further accelerating wound healing. Epithelial cells change shape both internally and externally to facilitate movement (Żwierełło et al., 2023).

The proliferative phase is considered complete when the epithelium of the epidermis and the collagen layer have formed. Epithelialization can be determined by measuring the epithelium from the stratum basale to the stratum corneum that has formed. During the proliferative phase, scar tissue forms in the wound. In this process, collagen production in the wound area increases. Collagen is a protein fiber that provides strength and elasticity to the skin. The presence of collagen encourages the edges of the wound to shrink and close. Subsequently, small blood vessels or capillaries form in the wound to supply the newly formed skin with blood.

The remodeling is the final phase in wound healing, commonly referred to as the maturation phase. In this phase, new epithelial cells fully cover the edges of the wound. Collagen is remodeled from type III to type I, and the wound closes completely. Cells involved in wound repair but no longer needed are removed through apoptosis or programmed cell death. Collagen is laid down during the proliferative phase, which is initially irregular, and the wound appears thick. Over time, collagen is reshaped into a more regular structure along the pressure lines, thereby increasing the tensile strength of the healing tissue. Fibroblasts secrete a matrix of metalloproteinases, which facilitate the remodeling of type III collagen into type I collagen (Franck et al., 2019).

Treating burns requires wound care that includes antibacterial agents to prevent the risk of infection, anti-inflammatory agents to accelerate the inflammatory phase, and antioxidants to bind free radicals that can inhibit wound healing. Research conducted by Saleem et al. (2023) exhibited that the content of orange peel extract could effectively accelerate epithelial cell regeneration in grade IIB burns. Membrane edamame contains isoflavones and vitamins A, C, and E. Isoflavones have antioxidant, antibacterial, and anti-inflammatory properties.

Orange peel contains compounds such as catechol, dimethoxy phenol, cyclohexane, coumarin, acetic acid, stigmasterol, sitosterol, and vitamin E, contributing to its antioxidant properties. Orange peel has high antioxidant activity, with the aqueous extract of orange peel powder demonstrating 71.2% antioxidant activity. Orange peels are also rich in flavonoids and vitamin C (110.4–127.70 mg/100 g of orange peel on a dry basis) (Saleem et al., 2023).

Pacitan orange peel extract contains flavonoids known to have anti-inflammatory, antioxidant, hepatoprotective, and anticarcinogenic effects. The extract also contains active compounds such as d-limonene, flavonoids, saponins, and tannins, which can stimulate cell regeneration in healing burns. Additionally, orange peels contain provitamin A, vitamin C, folate, riboflavin, thiamine, vitamin B6, and calcium. Vitamin A plays a critical role in the wound-healing process by promoting epithelial cell differentiation and enhancing immunity.

Vitamin A can accelerate the transition from the inflammatory to the proliferative phase by increasing the number of monocytes and macrophages in the injured area, thus facilitating phagocytosis. Retinoids regulate the growth and

differentiation of many cell types within the skin, and their deficiency leads to abnormal epithelial keratinization. In wounded tissue, vitamin A stimulates epidermal turnover, increases the rate of re-epithelialization, and restores the epithelial structure. Retinoids also have the unique ability to reverse the inhibitory effects of anti-inflammatory steroids on wound healing. In addition to its role in the inflammatory phase of wound healing, retinoic acid has been shown to enhance the production of extracellular matrix components, such as type I collagen and fibronectin, increase the proliferation of keratinocytes and fibroblasts, and decrease levels of matrix metalloproteinases that degrade the matrix (Polcz & Barbul, 2019).

Vitamin C contained in Pacitan orange peel is critical in stimulating collagen formation, which helps new epithelial cells to form. Vitamin A and vitamin C make orange peels natural antioxidants that help boost the immune system and fight germs and viruses. Vitamin C acts as a co-factor for the proline and lysine hydroxylases that stabilize the tertiary structure of the collagen molecule. It also promotes collagen gene expression in the skin. Collagen formation is primarily carried out by fibroblasts in the dermis, generating the basement membrane and dermal collagen matrix. The dependence of the collagen hydroxylase enzymes on vitamin C has been demonstrated in several studies with fibroblast cells in vitro, showing decreased total synthesis and reduced crosslinking when vitamin C is absent. The activity of the hydroxylases is much more difficult to measure in vivo, as the amount of collagen synthesized may vary only slightly (Pullar, Carr, & Vissers, 2017).

Research published in the *Food Science and Human Wellness* journal indicates that orange peels have anti-inflammatory properties that are very beneficial for health. Vitamin B6, or pyridoxine, helps in the formation of amino acids that are important for maintaining healthy skin. It also supports nerve health and is essential for red blood cell production. Additionally, it can increase the production of T lymphocytes and interleukins, which play an important role in fighting disease-causing infections (Parra, Stahl, & Hellmann, 2018). Thus, flavonoids, vitamin A, vitamin C, and vitamin B6 in Pacitan orange peel extract can accelerate wound healing.

Conclusion

Based on the research results, it can be concluded that the administration of Pacitan orange peel extract was proven to accelerate the healing process of Grade II burns on white rat skin, as seen microscopically in the increased collagen production, epithelial thickness, and fibroblast activity.

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