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Indonesian honey as a natural remedy: Accelerating wound healing in Staphylococcus-infected mice

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Abstract: Honey derived from different geographical and floral origins exhibits varying antibacterial properties, likely influenced by differences in chemical composition. Honey contains flavonoids, phenols, and various minerals that act as antioxidants, anti-inflammatory agents, antibacterial agents, and immune system boosters, promoting wound healing. In this study, Balb/c mice with normal blood glucose levels were inflicted with two circular full-thickness skin wounds and subsequently infected with *Staphylococcus aureus*. The wounds were treated with the following: hydrocolloid dressing alone as a control (Control group), hydrocolloid dressing with Java honey (Java group), hydrocolloid dressing with Kalimantan honey (Borneo group), and hydrocolloid dressing with East Nusa Tenggara (NTT) honey (NTT group). The results indicated that body weights were higher in the NTT and Borneo groups compared to other groups, though the differences were not statistically significant ($p > 0.05$). Exudate weight measurements revealed that the NTT group had the lowest exudate weight, while the control group had the highest. Daily wound area ratio measurements showed that the NTT group exhibited the smallest wound area ratio, whereas the control group had the largest ($p < 0.05$ on days 1–7). Leukocyte counts were lowest in the NTT group, but no significant differences were observed among the honey-treated groups ($p > 0.05$). However, a significant difference was observed when comparing the NTT group to the control group ($p = 0.037$). The NTT group also demonstrated superior performance in terms of re-epithelialization percentage and collagen deposition. Although no significant differences were found among the honey-treated groups on days 3 and 7 ($p > 0.05$), the NTT group showed statistically significant advantages compared to the control group ($p < 0.05$). These findings highlight the benefits of honey in wound treatment, with East Nusa Tenggara honey demonstrating the most significant advantages across multiple parameters.

Keywords: Honey; Infected Wound; Wound Healing; Indonesian Honey.

INTRODUCTION

Wound healing consists of 3 stages: the inflammatory phase, the proliferation phase, and the remodelling phase.[1] and infected wounds would take a longer time to heal than uninfected wounds as intrinsic and extrinsic factors cause a longer inflammatory phase. Honey has been used in traditional medicine since ancient times and has recently been rediscovered by medical researchers for its use in dressing acute and chronic wounds.[2,3] Honey also has been known for its properties to promote wound healing, both in sterilized and infected wounds.

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Honey contains flavonoids, phenols, and various minerals which serve as antioxidants, anti-inflammatory agents, antibacterial agents, and strengthen the immune system to promote wound healing.[4]

Recently, there has been a major improvement in the management of wound healing due to the presence of several drug-resistant bacteria that can interfere with the wound repair process. Therefore, compounds from natural ingredients continue to be sought. One of them is honey. The use of honey has recently gained clinical popularity due to its possible use in wound treatment and regenerative medicine.[5] There is a lot of evidence from published studies, where honey is recommended as a clinical treatment. Research by Haryanto, et al states that honey from Indonesia has the same effectiveness as manuka honey and hydrocolloid dressings in the wound healing process.[6] Apart from that, honey is also reported to be used as an alternative wound dressing for patients with diabetic foot ulcers.[7–9]. Honey has antioxidants, antibacterial and anti-inflammatory agents so it can be used as a wound dressing to speed up healing and improve healing.[10] Honey from NTT has been proven to increase the stimulation of angiogenesis in acute wounds.[11]

Honey is produced from nectar gathered and modified by the honeybees. It is in a form of carbohydrate-rich syrup originated from floral and other plants nectars and secretions. Indonesia has the highest diversity of honey bees in the world, almost all species in the world occur in Indonesia, and most of them are island endemic.[12,13] Indonesia is a vast archipelago comprising more than 17,000 islands, has the highest diversity of honeybees in the world, with almost all global present, many of which are island endemics. Honey of different geographical and floral origins may possess differences in antibacterial properties, which may be related to different chemical compositions of honeys.[14–16] The study aimed to determine the effectiveness of three Indonesian honeys from Java, Borneo, and East Nusa Tenggara on the antimicrobial effects in Staphylococcus-infected wounds in mice. Indicators of effectiveness included exudate production during the inflammatory phase, and wound size, wound ratio, re-epithelialization, and collagen deposition during the proliferation phase.

MATERIAL AND METHOD

1. Animals

Forty eight 10-week-old male balb/c mice with normal blood glucose levels (Integrated Biomedical Laboratory Unissula, Semarang, Indonesia) were used in this study. Mice were caged individually in a temperature-controlled room (25.0 ± 2.0˚C) and free access to water and chow. All animal experiments conducted in the present study were reviewed and approved by the Animal Experiment and Use Committee of No. 0235/EA/KEPK/2022

2. Wounding And Treatment

Mice were kept and fed for acclimatization until 10 weeks of age. One day before wounding, mice were anesthetized by Ketamine-Xylazine (50mg/kg dan 5 mg/kg) intraperitoneally and the dorsum was shaved. Mice were divided into the following groups: Natural healing for normal mice treated only with a hydrocolloid dressing (Control group), mice treated with honey from Java and a hydrocolloid dressing (Java Group), mice treated with honey from Borneo/Kalimantan and a hydrocolloid dressing (Borneo Group), mice treated with honey from East Nusa Tengara and a hydrocolloid dressing (NTT Group).

On the wounding day, the dorsum was disinfected with 70% ethanol, and two circular full-thickness skin wounds (Ø4 mm), including the panniculus carnosus muscle, were made on both sides of the dorsum using a Kai sterile disposable biopsy punch (Kai Industries Co., Ltd., Gifu, Japan) under anesthesia. Each wound in treatment group received the honey treatment (75µl each wound). This treatment was performed once daily. Wounds were covered by a hydrocolloid dressing

(Tegaderm; 3M Health Care, Tokyo, Japan) to maintain a moist environment and wrapped with sticky bandages (Leukoplast Hypafix™; BSN Medical GmbH, Hamburg, Germany), which were replaced every day. Body weights and hydrocolloid dressing weights were measured daily before and after dressing to assess the weight of exudate produced from the wound[17].

3. Wound Observations

The day when the wound was made was designated as day 0. The wound healing was monitored daily until day 7. Wound tracing was done by making a drawing of the wound area pattern on the skin on the polypropylene sheet using a permanent marker, and images of wounds were also taken. Traces on the sheets were moved onto a personal computer using a scanner and Adobe Photoshop Elements 11.0 software (Adobe System Inc., Tokyo, Japan), and wound areas were calculated using ImageJ (National Institutes of Health, Bethesda, Maryland, USA) image analysis software. The wound area was expressed as the daily ratio of the wound area to the initial wound area on day 0 when the wound was made[17].

4. Sample Collection

Mice were anesthetized by Ketamine-Xylazine (50mg/kg dan 5 mg/kg) intraperitoneally, on days 3 and 7 after wounding. Before the euthanasia, the skin swab by using sterile cotton bud from each wound was collected and put in 0.89% NaCl on a tube to count the bacteria. The bacteria count was using.

Blood sample to count the leukocytes was collected by using a heart puncture technique (euthanasia). The leukocyte count was done by an analyser.

The wound and surrounding skin were harvested, and each wound sample was bisected at the wound center. Wound tissue was spread on and stapled to polypropylene sheets, and tissue processing was done to prepare 5-μm-thick serial sections.

5. Histological Observations

Five-micrometer-thick paraffin sections from days 3 and 7 were stained with hematoxylin and eosin or subjected to Masson's Trichrome staining. The hematoxylin and eosin staining result was used for the re-epithelialization percentage calculation was done by measuring the length of the new epithelium covering the wound from the edge of the wound and divided by the length of the wound from the wound edges. In the other hand, the Masson's Trichrome staining results to calculate the collagen deposition. The collagen deposition was assessed at five wound sites of granulation tissue, two sites near the two wound edges, and three sites around the center of granulation tissue using the 40× objective magnification, and the value was divided by the whole area of these five sites.

6. Statistical Analysis

Data are shown as the mean ± standard deviation (SD) and the statistical analysis was performed by a paired t-test or one-way ANOVA and Tukey–Kramer multiple comparison test with SPSS 25.0 (SPSS Inc., Chicago, USA). $p < 0.05$ was considered to be significant

RESULTS AND DISCUSSION

The Honey Origins

In this study, the honey was collected from three different islands from Indonesia. The honeys were directly sent from Java islands, Kalimantan islands, and East Nusa Tenggara islands (figure 1). Each island has its own characteristic in weather, topography, and vegetation. These unique characteristics result in varied honey production.

Java is a highly volcanic island, with many volcanoes. Its climate is generally hot and humid throughout the year, often debilitating due to high humidity. Java's rich vegetation is southern Asian, with Australian affinities; more than 5,000 species of plants are known.

Borneo lies astride the Equator. Borneo's climate is equatorial—hot and humid with a fairly distinct division into two seasons. Borneo is largely covered in dense rainforest, and both the floral and the faunal populations of the island are extremely varied. There are extensive stands of teak, oak, conifers, and hardwoods.

The East Nusa Tenggara islands present a splintered topography of volcanic mountains. The mountain peaks are lower on the islands in the north-eastern part of the province. Coral atolls and reefs border much of the narrow coastal lowland. The islands have a long dry season, and there are few perennial streams and no major rivers. Sandalwood and eucalyptus woodlands, scrub, and grasslands are common.

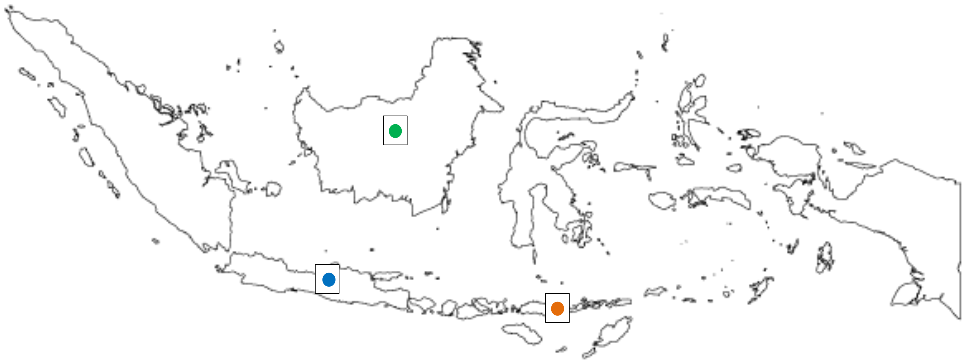

Figure 1. The map Indonesian islands and the origins of honey. Blue dot with square marks the Java islands, green dot with square marks the Kalimantan islands, orange dot with square marks the East Nusa Tenggara islands.

Body Weights, Exudate Weights, Wound Size Observations And Wound Areas Ratio

Body weight was measured every day during the study period and compared to that before wounding (Fig 2). After wounding, the body weight in all group were stable until days 7, only control group which slightly decreased and was lower than the initial body weight on the last measurement day. The difference in the body weight was not statistically different (p>0.05)

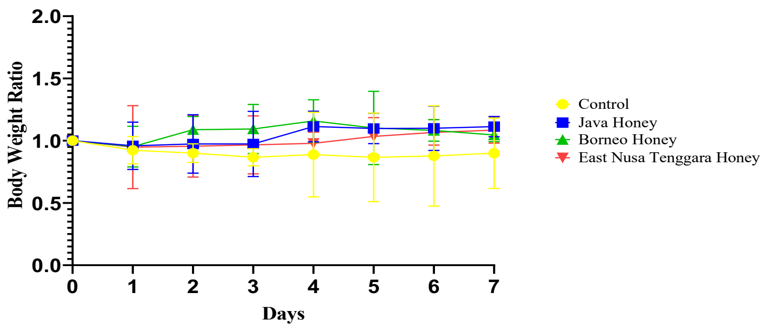

Figure 2. The daily body weight ratio comparison. Values are presented as the mean ± SD of six mice

Exudate weights varied in all groups but showed the same pattern (Fig 3). The exudate weights were the highest on day 2 and then decreased. In the control group, exudate weights has a highest increase compared to day 1, also on the end observation day the control group has the highest monitored exudate weight compared to honey treatment group. The statistic test showed a difference between control group and honey treatment group on day 1 and days 6.

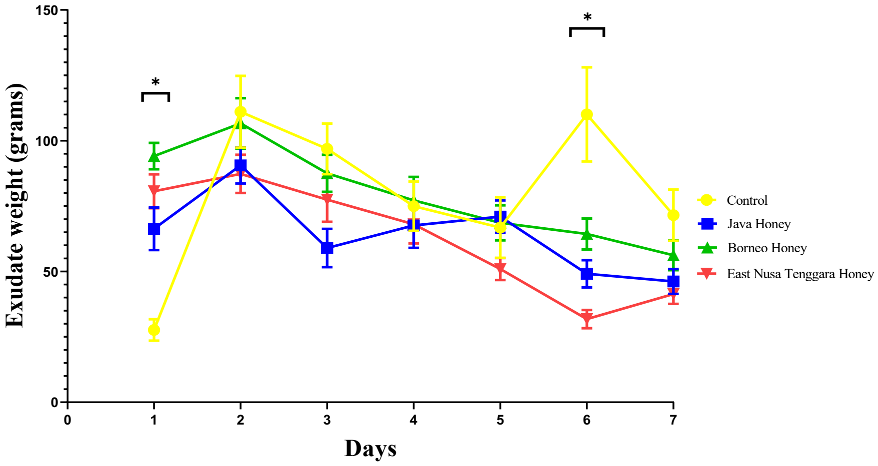

Figure 3: Exudate weights. Exudate weights in each group were recorded daily by measuring the weight (grams) of the hydrocolloid dressing. Values are expressed as the mean ± SD, ANOVA, Tukey-Kramer *p<0.05

Wound size evaluations were performed in every group until days 7, as shown in Figure 4. Wound areas generally increased during the inflammation stage (early days) and then decreased until the end of the observation period. Wound sizes markedly increased until days 2.

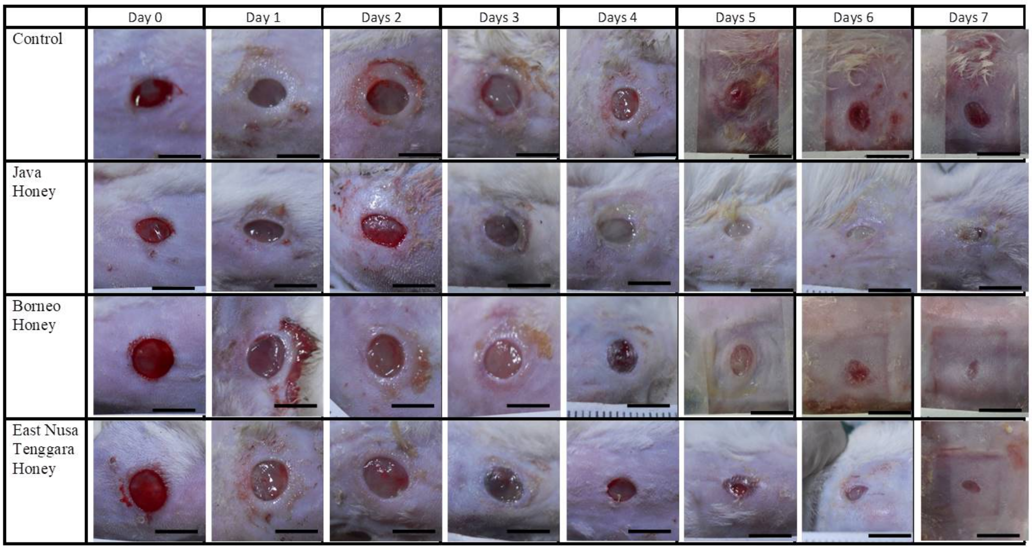

Figure 4: Daily wound healing observations by photograph images. Wounds of 4 mm in diameter were made on the dorsum, and healing was recorded by photography from the same wound of a mouse in each group. The wound edge is indicated by arrows. Bar, 5 mm

Figure 5 shows that the wound area ratio comparison in control and honey treatment group. There was significantly higher wound ratio in the control group than in the Borneo and East Nusa Tenggara honey treatment group on days 1–7 ($p<0.01$). Daily wound area ratios were smaller in the Java honey group than in the control group on day 3,5, 6 and 7 with significant differences being observed ($p<0.05$).

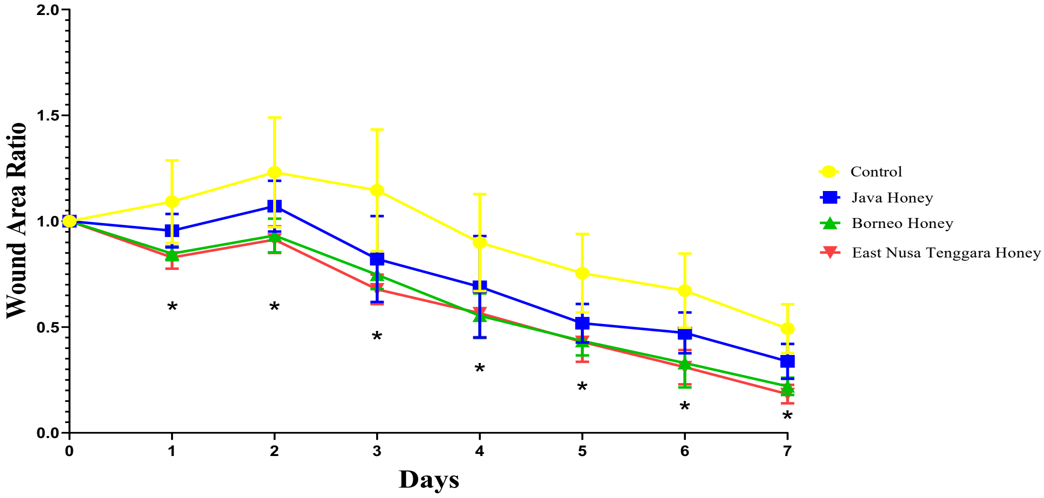

Figure 5: Comparison of wound area ratios. The wound area is presented as the mean ± SD of twelve wounds. Values are expressed as the mean ± SD, ANOVA, Tukey-Kramer *p<0.05

Leukocyte Number

The numbers of leukocyte counts were measured on day 3 and 7. The highest leukocyte count on day 3 was observed on control group with average leukocyte count of 7450,5/mm3, and the lowest was on Borneo Honey group (4633,1/mm3). Similar with days 3, on days 7 the highest leukocyte counts was on control group with average leukocyte counts of 4716,7/mm3, and the lowest was on East Nusa Tenggara honey treatment group with 3575/mm3. The statistical analysis comparison for honey treatment group showed no difference among honey group ($p>0.05$), and showed a difference when compared East Nusa Tenggara group to control group ($p=0.037$).

Histological Analyses

The new epithelium extended from the wound edges and covered the wound. The percentage of re-epithelialization during wound healing was evaluated on days 3 and 7 (Figure 6). On day 3, the wound surfaces were partially covered by an epithelium in both control and honey treatment group. On day 7, the epithelium was almost fully covered the wound on honey treatment group. Furthermore, in comparisons of the percentage of re-epithelialization, no significant differences were observed among the honey treatment group on days 3 and 7 ($p>0.05$), and statistically different compared to control group ($p<0.05$).

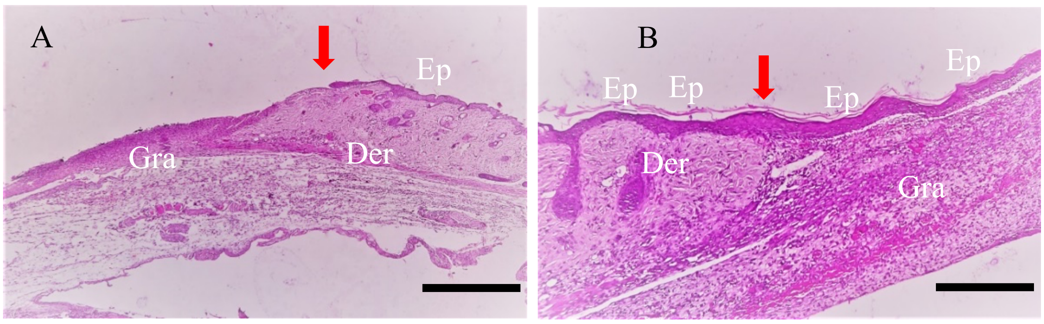

Figure 6: Re-epithelialization in wound healing. (A) Partially covered epithelium (B) Fully covered epithelium. Histological features of wounds stained by hematoxylin and eosin. Red arrows indicate the tip of the re-epithelium (Ep) covering the wound surface. Block arrows indicate the edge of the wound, the boundary between the normal dermis (Der) and granular tissue (Gra).
Scale bar: 200 μm.

Collagen depositions were observed in the wound area on days 3 and 7. In all honey treatment groups, the collagen depositions were denser in the honey treatment groups compared to the control group. A significant difference was observed in the control group and all honey treatment on days 3 and 7 ($p<0.05$), while there is no significant difference among honey treatment group ($p>0.05$).

A B

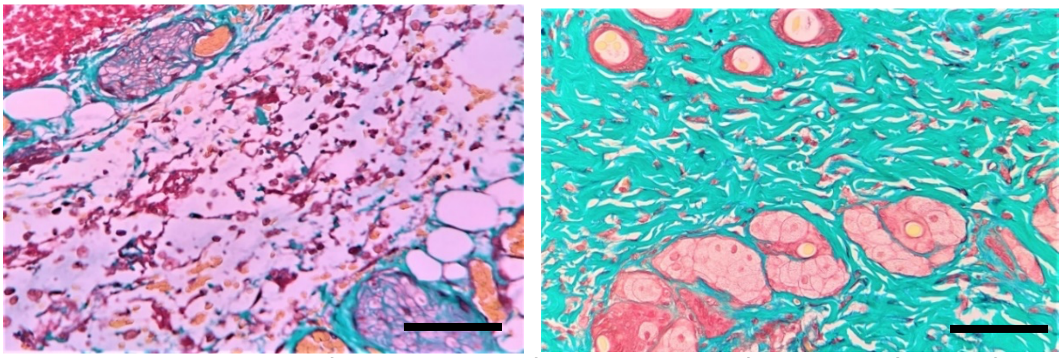

Figure 7: Microscopic view of new blood vessel formation in areas of granulation. Collagen fibers were stained greenish blue on Masson's trichrome satining. (A) The Collagen deposition in the East Nusa Tenggara honey group on day 3. (B) The Collagen deposition in the East Nusa Tenggara honey group on day 7. Scale bar: 50 µm.

Discussion

The result on exudate and leukocyte counts indicates the honey's ability to prevent wound infections and promote wound healing through its natural antimicrobial properties (Hydrogene peroxide (H_2O_2) production, osmotic effect, polyphenols, etc.) and by acting as a physical barrier to the wound site has been extensively explored.[18,19] The antimicrobial properties of honey are crucial for the response to tissue damage. Protein-digesting enzymes produced by bacteria are harmful to tissues and are detrimental to the growth factors and extracellular matrix (ECM) to stimulate tissue regeneration.[20–22] Lower exudate weight counts in honey treatment group may due to honey's anti-inflammatory ability which plays a crucial role in tissue regeneration. During haemostasis, blood flow can be restricted through the capillaries (ischaemia) causing oxygen starvation (hypoxia), along with a lack of nutrients, both of which are vital for cell proliferation, which is required to repair tissue damage. Honey's high phenolic content supports its antioxidative and anti-inflammatory effects, which are crucial for tissue regeneration. These compounds exhibit radical scavenging properties due to the high reactivity of their hydroxyl radicals, clearing the free radicals formed due to inflammation.[23–25]

Wound closure was also observed faster in honey treatment group, since honey has a low water activity which provides a moist environment to the wound bed. This is effectively provides a barrier that prevents formation of dead tissue and mitigates dermal necrosis, which is often observed in wounds exposed to air. The wound closure was also supported by a faster re-epithelialization and better collagen deposition in this research. Faster Re-epithelialization and a scab avoiding on skin wounds that are kept moist under a dressing, in contrast to wounds exposed to air. Further supported this claim and showed that granulation tissue develops faster in moist conditions, when compared to dry, and even wet conditions.[26] Moreover, the moist wound surface enhances the migration of epidermal cells, compared to migration under the scab. An additional benefit of applying honey is the osmotic effect and subsequent drawing of water and lymph to the wound environment, which helps the oxygenation and nutrition of damaged tissue.[18] Furthermore, the creation of a mixture of diluted honey and drawn lymph under the dressing prevents it from adhering to the wound bed, minimizing the risk of tearing newly formed tissue when changing the dressing. The high sugar content on honey contributes to the high osmolarity of honey and has been suggested to provide localized nutrition to the wound site.[27] The application of honey provides a low pH environment, which promote epithelialisation and wound closure. This low pH also may reduce the activity of proteases and limit ECM removal.[28] Moreover, this acidification promotes oxygen dissociation from hemoglobin, which results in improved tissue oxygenation which lead to collagen deposition to support the wound healing

Honey also works in wound healing with reactive oxygen species (ROS) in cells was seen as a consequence of an anaerobic environment. Moreover, ROS such as H_2O_2 have been classed as harmful and responsible for molecular damage such as DNA mutation and protein oxidation. Hence, it was believed that it was imperative for cells to eliminate these oxidising species.[29] A more important and complex role for ROS in biological functions such as wound healing and growth regulation has been demonstrated by Dunnill, et al.[30] The production of H_2O_2 is induced when the cells are exposed to epidermal growth factor. The ROS produced activates signalling pathways that lead to cell proliferation and differentiation. Furthermore, a clear correlation between the increase in ROS production and increase in mitogenic rate has been identified.[29–31]

The role of H2O2 generation in honey is crucial for tissue regeneration applications. ROS levels influence the different stages of wound healing.[30] For example, H_2O_2 released from honey has been shown to stimulate the proliferation of fibroblasts when used in a time- and dose-dependent manner[32]. However, prolonged exposure to high concentrations of H_2O_2 can exhibit a cytotoxic effect. Moreover, honey's phenolic content and its antioxidant properties can counteract this toxic effect, rendering protection to cells and enhancing their growth.[32,33] Honey has the potential to supply the levels of H_2O_2 required for the Wnt signalling pathway, which is widely implicated in regenerative processes.[29,34]

The wound ratio in honey treatment was lower compared to control group, this result might be from the aid of ROS which can help the wound healing through the activation of neutrophil protease. This enzyme is inactive inside neutrophil granules until stimulated by the inactivation of its inhibitor. This required inhibitor inactivation occurs as a result of ROS oxidation, hence releasing neutrophil protease to carry out the proteolytic removal of damaged wound tissue, which can potentially simplify debridement in chronic wounds. The regulation of matrix metalloproteinases (MMPs), crucial to the healing process in chronic wounds, can be influenced by honey.[35–38] Supported by Sanchez, ROS in skin wounds promote the activation of nuclear factor erythroid derived 2-like 3 (Nrf2), which, in turn, increased the activity of MMPs in fibroblasts.[39] Both the up- and downregulation of MMPs in keratinocytes have been observed when cultured with honey and honey-derived flavonoids, which provides contradictory conclusions.[37] The use of several different honey types may contribute to the discrepancies, and the amount of ROS generated has not been adequately quantified. ROS may be involved in the regulation of MMPs; however, further research is required.

The H_2O_2 from honey to the wound will influence multiple wound healing pathways and have complex effects on cellular behaviour, including proliferation, signalling, metabolism, and migration. Maintaining a low level of ROS is likely to promote tissue regeneration and wound healing, in the other hand, the high and excessive production of ROS can lead to oxidative stress and impaired wound healing.[17,40] The antibacterial peptide, Defensin-1 (Def-1), has been shown to be responsible for promoting re-epithelialisation in vivo in a study using royal jelly.[41] Def-1 will increase the keratinocyte production of MMP-9 and enhances keratinocyte migration, which produce a significant increase in wound closure rates.

CONCLUSION

Honey treatment demonstrates significant advantages in wound healing compared to natural healing, as indicated by lower exudate counts, reduced wound area ratios, lower leukocyte counts, higher re-epithelialization percentages, and increased collagen deposition. These findings highlight the potential of honey, particularly from East Nusa Tenggara, as a natural therapeutic agent for wound care.

The study suggests that honey's bioactive components, including flavonoids and phenols, contribute to its antibacterial, anti-inflammatory, and tissue-repair properties, making it a promising alternative to conventional wound treatments. Future research should explore the clinical application of honey in diverse wound types and patient populations to validate its efficacy and safety. Additionally, further studies could investigate the specific chemical compositions of different honey varieties to identify the active compounds responsible for their wound-healing properties. Developing honey-based wound care products, such as medicated dressings or gels, could also bridge the gap between laboratory findings and practical applications, offering an accessible and cost-effective solution for wound management globally.

AUTHORS' CONTRIBUTIONS

Eko Naning Sofyanita: Conceptualization, Methodology, Project administration. **Arya Iswara:** Software, Data Curation, Writing - Original Draft. **Rima Munawaroh:** Original draft preparation, Formal Analysis. **Mochamad Rizal Maulana:** Investigation, Writing - Review & Editing. **Ririh Jatmi Wikandari**: Resources, Visualization. **Rachmad Bayu Kuncara:** Validation, Supervision.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute in this researchare available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The authors have declared that no competing interests exist.

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