Inhibition of Alveolar Bone Destruction by Roselle Extract (*Hibiscus Sabdariffa L.)*

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Abstract

Alveolar bone destruction in periodontitis causes subsequent pathological conditions, such as tooth mobility and loss; thus, the treatment to prevent and overcome this condition is indispensable. The naturally occurring medicinal plant *Hibiscus sabdariffa* (roselle) contains anti-bacterial and anti-inflammatory compounds; it is expected to be effective in inhibiting bone damage in periodontitis caused by excessive osteoclast activity. This study investigates the inhibitory effect that roselle extract has on bone destruction in periodontitis and in calvaria bone damage.

Alveolar and calvaria bone damage was induced either via ligature wire or lipopolysaccharide (LPS) injection in mice (*Mus musculus*). Roselle extracts in concentrations of 5% and 10% were applied before and after bone destruction. Then, the extent of the bone damage was measured using microCT and histological analysis.

Roselle extract 10% attenuated alveolar bone destruction in the periodontitis and calvaria bone destruction models. The provision of 10% roselle extract before inducing alveolar bone damage also showed inhibitory activity.

Roselle extract 10% effectively diminished alveolar bone destruction. Its inhibitory mechanism may be accomplished through its anti-inflammatory and anti-bacterial activity. These findings demonstrate the potential use of roselle extract as a therapeutic product for periodontitis treatment.

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Introduction

Periodontal disease is characterized by bone damage caused by mechanical trauma, chemicals, and pathogenic microorganisms as the primary etiology of plaque found on the tooth surface. Inflammatory mediators triggered by the presence of bacterial LPS can induce osteoclastogenesis to absorb bone. In severe cases, it can cause tooth mobility and loss, increase the risk of systemic disease, and reduce patients' quality of life.[1,2]

The colonization of Gram-negative anaerobic bacteria initiates periodontitis as subgingival biofilms.[3] Disease development and progression is caused by the virulence factors the bacteria release and the host's response to bacterial colonization, which is destructive to the periodontal tissue. Periodontitis is caused not only by one causative pathogen but rather by an imbalance in the microbial biofilm. Disruption of the balance between osteoblast and osteoclast activity by some bacterial products and inflammatory cytokines is a major cause of bone loss as the result of excessive inflammation.[1,4] Furthermore, bone defects are caused by the inflammatory cells such as T-cells, B-cells, macrophages, and neutrophils. These cells augment bone loss by producing inflammatory mediators such as the cytokines that promote bone resorption via osteoclasts. Moreover, macrophage colony-stimulating factor (M-CSF), which includes cytokines primarily through binding to the c-Fms receptor, can stimulate osteoclast differentiation by regulating the signaling system. Alveolar bone damage is

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triggered by bacteria accumulation in the form of plaque, which can increase the body's inflammatory response to these bacterial endotoxins.[3,5]

Periodontal disease has a relatively high incidence rate and is a common concern in dentistry.[6] The following outlines why superior treatment and prevention efforts are still needed. Research on periodontal disease therapy is still being developed, with one of the goals being to find alternative therapies that are relatively cheap, easy to access, and simple to apply. As per the results of previous studies, Roselle (*Hibiscus sabdariffa* L.) is an herbal plant that has the potential to treat alveolar bone damage because it has anti-inflammatory and antibacterial effects.[7–10] The specific elements within roselle make it highly beneficial to human health, including anthocyanins, polyphenols, niacin, riboflavin, ascorbic acid, calcium, iron, potassium, and magnesium. Roselle also contains delphinidin-3-sambubioside, which can reduce inflammatory mediators' production, inhibiting osteoclastogenesis. Roselle's antibacterial properties imply that it may prevent plaque formation, which is the primary etiology of bone damage.[11–14]

Previous studies using roselle extract report its anti-inflammatory effects.[15–17] However, the effect of roselle in an in vivo model by observing the extent of bone damage has yet to be reported. In this study, the therapeutic effect of roselle petal extract in concentrations of 5% and 10% was tested by applying said concentrations to a model of *Mus musculus* periodontitis induced by ligature insertion of the maxillary left second molar. The experiments on the periodontal model were carried out in two different procedures: roselle application before ligature placement (pre-ligation) to determine the preventive effect and roselle application after ligature placement (post-ligation) to determine the therapeutic effect. Additionally, to roselle's inhibitory effect on the bone damage, a third experiment was carried out in which roselle extract was applied in a calvaria bone damage model via introducing lipopolysaccharide (LPS).

This research hopes to determine the effects that roselle extract has in treating alveolar bone damage in periodontitis. In the future, the results from this study could enhance the development of roselle as a medicinal plant with preventive and therapeutic effects on periodontitis.

Materials and methods

Roselle ethanol extract

The roselle petals (*Hibiscus sabdariffa* L.) were dried and then extracted with ethanol. After the extraction process was completed, phytochemical analysis, an ash content test, a moisture content test, and a density calculation were performed to identify the active compound that was extracted. The extract was then dissolved into 5% and 10% preparations for testing in the experimental mice groups.

Mice

The study used 30 mice aged eight weeks (*Mus Musculus*, Swiss Webster strain) with body weights of 20–25 grams. The mice were divided into five treatment groups, each consisted of 6-7 mice: pre-ligature, post-ligature 5%, post-ligature 10%, and LPS-induced calvaria. The mice were kept in accordance with the applicable standards in animal cages in the experimental animal laboratory at Universitas Indonesia. This study received approval from the Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia (protocol number 18-06-0634).

Periodontitis model

Animal preparation was carried out by performing anesthetic procedures and fixation of the mice jaws with a cheek retractor. Ligation of the maxillary left second molar was performed using a 5-0 ligature silk (Roboz Surgical, MD; USA) around the tooth with a knot on the buccal side. The ligatures were kept intact before being removed on the third day. Roselle extract 5% and 10%, respectively, was injected into the post-ligature groups (50 μl) on the palatal mucosa of the maxillary left second molar after ligature displacement. For the pre-ligature group, the same procedure was performed before ligation, using 10% roselle extract. The control group was given 50 μl of NaCl 0.9% (NS Otsu, Otsuka).

Bone defect observation

After seven days of ligation, all mice were sacrificed by anesthetic overdose intraperitoneal euthanasia. The maxilla was dissected and the soft tissue was separated from the hard tissue and then immersed in 3% hydrogen peroxide. Then, the exposed root area starting from the cemento enamel junction (CEJ) to the alveolar crest was recorded and compared with the

control group. The area of the bone defect was calculated using the ImageJ in μm^2.

LPS-induced bone destruction

LPS injection (LPS; Sigma-Aldrich) was administered in the right hemisphere of the calvaria bone in a dose of 25 µg/g of the mice's body weight. After 24 hours, 10% roselle ethanol extract was injected into the right hemisphere of the calvaria bone for the experimental animals; the controls were injected with 0.9% NaCl (NS Otsu, Otsuka). On the fifth day, all the experimental animals were sacrificed, and the samples were isolated. Specimens of calvaria bone were analyzed using three-dimensional microcomputed tomography (microCT) (SkyScan 1173, Bruker-Micro-CT; Kontich, Belgium); histological preparations were made using hematoxylin and eosin (HE) staining.

Statistical analysis

The data were statistically processed using GraphPad Prism 8 for MacOS X. T-tests and one-way ANOVA were performed to compare the differences between the groups (ns: not significant, *P < 0.05, **P < 0.01, and ***P < 0.001 throughout the paper).

Results

Roselle extract's effectiveness in inhibiting bone destruction was analyzed by measuring and comparing the area of bone destruction among and between the groups. The extent of the bone damage was assessed by measuring the decrease in alveolar bone height at day ten after ligature wire placement for the post-ligature group and day seven for the pre-ligature group. The area extending from the CEJ to the alveolar crest was calculated and compared between the control group and the groups with roselle extract treatments of 5% and 10%. The area of bone destruction was calculated using the Image-J application. Specifically, the area of bone defect was measured three times over by three different examiners and then analyzed via reliability testing. The bone defect data from each group were then statistically analyzed by one-way ANOVA; they revealed that the 10% roselle application exhibited the highest reduction in bone defects of all the groups (P < 0.05) (see Figure 1).

In the pre-ligature experiment, t-tests were carried out to compare the area of bone destruction between the control group and the rosella 10% group. There was a decrease in the rate of bone damage in the treatment group compared to the control, but it was not statistically significant (P > 0.05) (Figure 2).

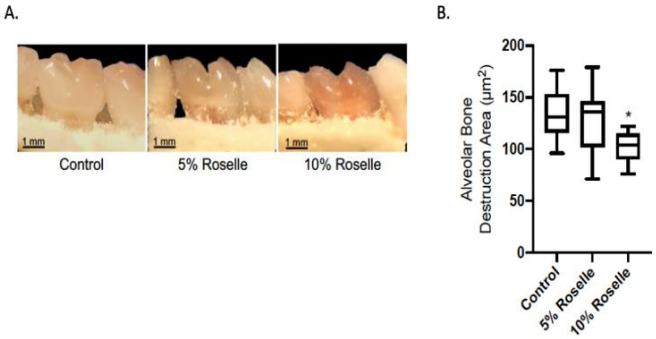

Figure 1. Bone defect analysis from post-ligature experiments. (A) microscopic feature of maxillary left second molar after ligature displacement. (B) Bone destruction area from control 5% and 10% roselle extract group calculated using ImageJ.

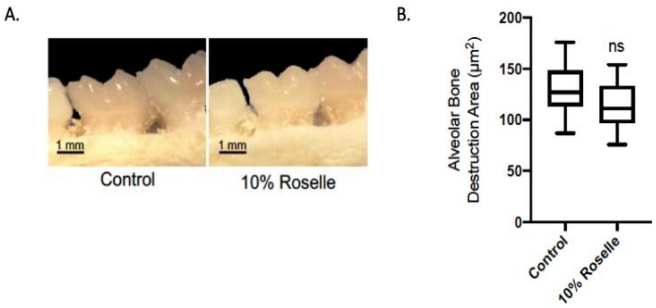

Figure 2. Bone defect analysis from pre-ligature experiments. (A) microscopic feature of maxillary left second molar after ligature displacement. (B) Bone destruction area from control and 10% roselle extract group calculated using ImageJ.

In the LPS-induced calvaria bone damage experiment, on the fifth day after the LPS injection, the mice were sacrificed and the calvaria bone tissue was extracted for analysis by performing a photo scan and a microCT reconstruction. The area of damage to the calvaria bone and the total area of the calvaria bone were calculated using the ImageJ application. The results were then compared to determine the percentage of damage.

The results obtained in the calvaria model with a 10% roselle application showed a lower area of bone damage than the control group.

Bone cavities were seen in the calvaria bone of the control group extending both anteriorally and posteriorally. There was also discontinuity of bone in certain areas. The statistical test results of the t-test in the control group and the roselle 10% group showed a significant difference in results between the two groups ($P < 0.005$) (Figure 3).

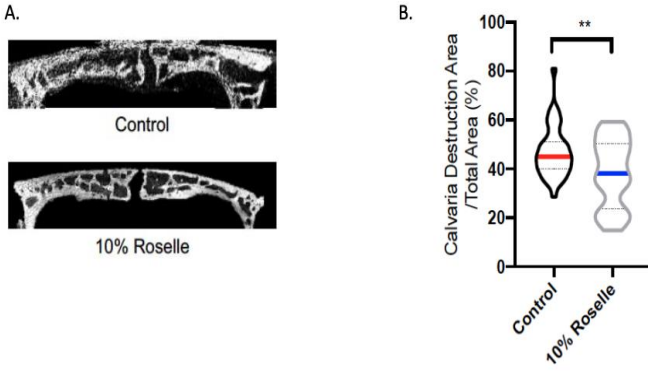

Figure 3. LPS-induced bone destruction in mice calvaria bone with administration of 10% roselle extract. (A) Features of microCT scanning and (B) MicroCT analysis of the calvarial bone from control and 10% roselle extract group.

Observation of bone damage through histological HE staining showed that bone damage in the control group was more extensive than it was in the 10% roselle group. T-tests showed a significant reduction in bone damage in the 10% roselle group compared to the control group ($P < 0.001$).

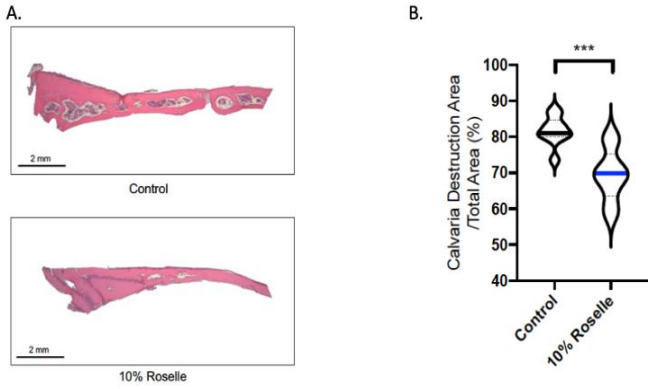

Figure 4. LPS-induced bone destruction in mice calvaria bone with administration of 10% roselle extract. (A) Features of histological preparation and (B) histological analysis of the calvarial bone from control and 10% roselle extract group.

Discussion

This study conducted an in vivo experiment on mice to determine the effect of two concentrations of roselle extract on alveolar bone destruction using a periodontitis model with ligature silk application on maxilla molar teeth and a calvaria bone destruction model with LPS injection on the calvaria. Bone destruction repair in these two models was carried out by applying roselle extract to the damaged bone area before induction of bone destruction with ligature (pre-ligature) as a preventive effect and after three days of ligature placement in a periodontitis model as a therapeutic effect. Concentrations of 5% and 10% of roselle extract were used as per the results from the previous study.[18] The effectiveness of these applications was then evaluated by comparing the bone damage area in the treatment and the control groups.

Alveolar bone destruction in the periodontitis model was due to the accumulation of plaque in the ligature binding area—the ligation in the surrounding the teeth caused the bacterial colonization.[3,19] Bacteria release exotoxins and endotoxins (e.g., LPS), which triggers alveolar bone destruction that binds to TLR4 on the macrophage membrane. In this way, macrophages produce inflammatory mediators such as TNF-α and IL-6 via MAPK signaling pathway. The inflammatory mediator induces RANK-L production by osteoblasts and stromal cells; these bind to the RANK on preosteoclasts to induce osteoclast differentiation. Apart from macrophages, RANK-L expression is carried out by T-lymphocytes and B-lymphocytes after detecting bacterial antigens. B-lymphocytes also secrete IgG antibodies, which bind to C1q to form the complex IgG-IC. This complex interacts with the Fc receptor on the preosteoclast membrane, which triggers the osteoclast differentiation resulting in bone destruction.[2,20]

This study performed the periodontal model in two different roselle extract application procedures to determine both the preventive and therapeutic effects of roselle extract. The 10% roselle extract yielded a decrease in bone damage in the calvaria bone in the post-ligation group, indicating the extract's anti-inflammatory potential (Figure 1). The results of the bone

defects analysis in the pre-ligature 10% roselle extract group also showed the ability to prevent bone destruction—however, the effect was not statistically significant, indicating that roselle extract's antibacterial properties inhibit bone destruction.

The inhibition of alveolar bone destruction by roselle extract may occur because delphinidin-3-sambubioside (Dp3Sam), which is the main component of the anthocyanins in roselle extract, has an anti-inflammatory effect.[21] Specifically, it suppresses the NF-kB and MAPK pathways, reducing inflammatory mediators such as iNOS, NO, IL-6, MCP-1, and TNF-α. In addition, Dp3Sam roselle extract has revealed immunostimulatory activity by increasing the body's production of IL-10, which decreases RANK-L expression and the production of IL-1, IL-6, TNF-α, and NFATc; it also increases OPG production. The polyphenol content in the roselle extract also inhibits COX-2 formation by reducing JNK and p38 MAPK. COX-2 induced by IL-1β plays a vital role in osteoclast differentiation through prostaglandin synthesis (PGE2).[7,21] Due to the decreased amount of inflammatory mediators, the differentiation and activation of osteoclasts would subsequently be inhibited, thereby inhibiting bone destruction. Roselle extract also contains anthocyanins, such as delphinidin-3-glucoside. This stimulates IL-10 production, which is important in reducing the inflammatory effects of cytokines such as IL-1, IL-6, and TNF-α. In sum, this event would inhibit bone destruction.[22,23] Moreover, roselle extract has an anti-microbial effect that works by manipulating the surface of the bacteria so that their permeability increases, causing lysis.[11,24,25]

Based on Kaboosaya et al.'s research, ligation displacement could heal inflammation in two weeks.[26] Thus, in this study, the roselle extract application procedure was performed within a week—before the physiological healing process occurs. Applying roselle extract is expected to enhance recovery by inhibiting osteoclast activity such that it inhibits bone destruction; this allows bone regeneration to occur through the normal remodeling process.

Upon evaluating the results from the periodontitis model in the pre-ligation and post-ligation with 10% roselle extract groups, applying 10% roselle extract post-ligation might perform better than pre-ligation in terms of protection against bone destruction. The extract's protection against alveolar bone destruction in the pre-ligation groups may have occurred through inhibition of dental plaque formation surrounding the ligature silk.[25,27] A single application of roselle extract before ligation might not be enough to inhibit bacteria from accumulating around ligature silk. The liquid form of the extract might not last as long as a gel format in the oral environment. Additionally, multiple instead of singular applications of roselle extract to the surrounding area might increase its protection ability.

The bone destruction mechanism in the periodontal model not only enhances plaque formation but also works via mechanical trauma to the surrounding area, inducing bone destruction through increased osteoclast activity.[19,26] Conversely, the LPS bone destruction model works solely by inducing osteoclast formation and activation through the inflammation mechanism. In comparing the results obtained from the periodontal and LPS models, better bone protection ability was observed in the LPS-induced model. One possible explanation is that roselle extract works predominantly as an anti-inflammatory rather that an anti-bacterial agent, specifically in mice models, which have different oral environments than human.[28,29] In the future, it would be worth investigating the effect of roselle extract on the periodontitis mice model featuring the introduction of periodontitis-inducing bacteria (e.g., *P. gingivalis*).

Conclusions

The inhibition of alveolar bone destruction observed in this study is assumed to have occurred via the anti-inflammatory and anti-bacterial activities of the roselle extract. By administering roselle extract in the defect area, plaque accumulation, which plays a vital role in inflammation and triggers osteoclastogenesis, was reduced. Further research is still needed to analyze the amount of plaque that still accumulates when roselle extract is applied. The present study concludes that roselle extract effectively inhibits bone destruction in both periodontitis and LPS-induced bone destruction. How it works to reduce alveolar bone damage could involve various pathways, including inhibition of plaque accumulation, inhibition of inflammation or differentiation, and inhibition of osteoclast. Further study is needed to explore

and determine the specific pathway for inhibiting alveolar bone damage by roselle extract.

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Declaration of Interest

The authors report no conflict of interest.

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