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The Effect of *α-Mangosteen* on Runt-Related Transcription Factor 2and Tartrate-Resistant Acid Phosphatase 5b Expressions on Bone Remodeling in Periodontitis (An Experimental Research on Wistar Rats)

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ABSTRACT
The alteration of supporting structures of the teeth in periodontitis results from the complex interaction between colonies of microorganisms and the immune-inflammatory host response. α-mangosteen contains xanthone which has antioxidant, anti-inflammatory, anti-fungal, and osteoinductive properties and has the potential as an alternative treatment to promote bone remodeling of the damaged bone in periodontitis. This study aims to investigate the effect of α-mangosteen on RUNX2, and TRAP5b. Forty-eight Wistar rats aged 2- 3 months with bodyweight 250-300 g were used in this study. Rats were divided randomly into 8 groups (4 groups in 2-day-periods). Group 1 as the negative control, group 2,3, and 4 were given α- mangosteen hydrogel with the concentration of 0.5%, 1%, and 2% respectively. The alveolar bone on the left mandibleof the rats was removed by using low-speed inverted bur with the size of 3mm in the mesiodistal direction, 2mm in thecrown-root direction, and 1mm at the buccolingual direction. The α-mangosteen was applied after on the site of the removed bone in the subject groups. At the end of each treatment period (day 7 and day 14), the examination of RUNX2 and TRAP5b expression with immunohistochemical analysis was done. The results of this study showed that on day 7 and day 14, α-mangosteen had an effect on RUNX2. Meanwhile, the TRAP5b examination showed that there was an effect of α-mangosteen on day 7, but not on day 14. α-mangosteen as an antioxidant has a role in bone healing. RUNX2 plays an essential role in osteoblast differentiation, while TRAP5b was found necessary for osteoclast differentiation. The conclusion of this study shows that α-mangosteen affect RUNX2 and TRAP5b.

Keywords: *periodontitis, α-mangosteen, bone remodeling, RUNX2, TRAP5b*

1. INTRODUCTION

Periodontitis is the most common degenerative disease that often occurs in adults and the elderly. The main aetiology of this disease is initiated by dental plaque which results from the accumulation of subgingival biofilm bacteria on the tooth surface due to changes in the balance between the *host* and bacteria [17]. This is an immune-inflammatory condition that causes damage to the soft and hard tissues of the supporting tissue of the

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teeth, resulting from a complex interaction between the colony of microorganisms and the *host* immune-inflammatory response. The characteristics of periodontitis are the irreversible histopathological changes such as recession and alteration of the gingiva, periodontal ligament, cementum, and alveolar bone which will lead to tooth mobility and tooth loss [1][2][3]. Increased secretion of pro-inflammatory cytokines such as *tumor necrosis factor-α* (TNF-α), *prostaglandin E2* (PGE2) and *interleukin 1β* (IL-1β) as well as *receptor activator of nuclear factor-κβ ligand* (RANKL) and *receptor activator of nuclear factor κβ* (RANK) disrupts the balance between protective and destructive processes, causing alveolar bone resorption through osteoclasts. *Tumor necrosis factor-α* inhibits the differentiation of osteoblast precursor cells and the activity of the differentiation of *Runt-related transcription factor 2* (RUNX2) whereas a number of bone matrix coding genes require RUNX2 and its expression, including *alkaline phosphatase* (ALP), *osteopontin*, *bone sialoprotein*, and *collagen type 1α* [4]. Cells involved in the process of bone remodeling are osteoclasts and osteoblasts. Osteoclast cells secrete *tartrate-resistant acid phosphatase* 5b (TRAP5b) during the process of bone differentiation and resorption. TRAP5b is a protease enzyme that is expressed in high concentrations by lysosomes osteoclast precursors and mature osteoclasts and is often used as a biomarker of bone resorption [5][6].

Periodontal treatment in advanced periodontal tissue damage aims to induce regeneration such as the use of tissue regeneration scaffold material [7][8]. The standard treatment in the condition of bone damage includes removing necrotic bone fragments, administering local and/or systemic antibiotics, bone grafting, and application of enamel matrix derivatives. Treatment of bone damage requires special properties of materials that include osteoconductive and osteoinductive. The potential of alternative new materials that have osteoinductive properties becomes very promising to support bone remodeling [9][10].

The results of the study of Hong *et al*., (2018) demonstrated that α-mangosteen affects osteoclastogenesis by inhibiting ERK and JNK phosphorylation, without affecting p38 signaling and NF-κB signaling [11]. Another study stated that α-mangosteen promotes *myoblast* differentiation by modulating gene expression. Transdifferentiation of *myoblasts* into osteoblasts allows it to be used for bone therapy [12]. α-mangosteen is the active compound and the most abundant of the xanthone group (75-85%) [13][14]. All these studies show that α-mangosteen extracted from mangosteen peel extract can be an alternative material in the process of bone remodeling. The bone remodeling process is followed by the resorption process and the bone formation process using the biomarkers RUNX2 and TRAPb [15][16][17].

2. METHODS

2.1 Ethical Procedures

The tested-animals condition in this study has been approved by the Research Ethics Commission, Faculty of Medicine, Andalas University, Padang, West Sumatra, Indonesia, 569/KEP/FK/2018.

The research population was the male Wistar rats (Rattus norvegicus) obtained from the Pharmacology Laboratory, Faculty of Pharmacy, Andalas University. The criteria for the tested-animal samples were male Wistar rats (Rattus norvegicus), 2-3 months of age, and 250-300 grams of body weight. A total of 48 rats were divided into 8 groups (4 groups in 2-day-periods). Mice were kept in cages by acclimatization with the laboratory environment for 1 week before being given treatment to adapt the mice to place and food. Mice were given standard feed and drank boiled water ad libitum. Mice were grouped randomly. One cage consisted of 2 rats with clean and well-ventilated conditions, size 50 cm, width 40 cm, and height 40 cm, temperature 25 - 27°C, humidity 7-75%, with bright light for 12 hours, and 12 hours in dark. Bedding in experimental animals with sterile husks previously sterilized by autoclave. The bedding was changed every 3 days and mice were fed standard food and water ad libitum [18][19].

2.2 Plant Material

The plant as an assay material was obtained from Sumatra Barat Province, Indonesia. *Garcinia mangostana L.* was purified α-mangosteen from Batu Sangkar, Kubang Landau (-0.4555771, 100.6409525), certificate of analysis No: 11/RC-FP/2017 by Andalas Sitawa Fitolab Andalas University, Padang, Sumatera Barat, Indonesia (GPS Coordinate, -0.9476875,100.4529375). The preparation of α-mangosteen was made in the form of hydrogel which was carried out at the Biota Laboratory of Sumatra, Andalas University with concentrations of 2%, 1%, and 0.5%.

2.3 Periodontitis Model

The rats were anesthetized with general anesthesia which was injected intramuscularly (IM) the left thigh of the rat, xylazine base was injected at a dose of 0.05 ml per 100 g of body weight to cause a sedative effect, ± 5 minutes later, the rats were injected with ketamine HCl 0.1 ml per 100 g body weight [18][20]. The anesthetized mice were incised using a scalpel knife no. 15 with a full-thickness flap opening through the masseter muscle and periosteum until it reaches the alveolar bone in the M1 and M2 root areas. Bone destruction was performed at the left mandible of each rat. The area of bone damage was measured using a periodontal probe with a size of 3mm in the mesiodistal, 2mm in the crown-root aspect, and 1mm in the buccolingual aspect starting from the mesial M1 to the distal M2.

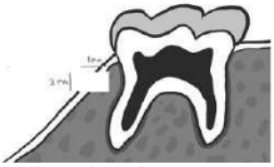


Figure 1. Illustration of Bone Damage Area

α-mangosteen was placed on the bone damaged area until it covered all damaged areas (± 6µl). Masseter muscle and skin were repositioned and sutured using absorbable surgical 6.0 suture. After surgery, the rats were given an injection of buprenorphine HCl 0.01 mg/kg BW subcutaneously to reduce postoperative pain [21][22]. The end of the treatment period was carried out 2 times, namely on the 7th and 14th days.
Furthermore, rats were euthanized and the mandibles were taken and fixated in 10% formalin buffer solution for 48 hours [19][20].

2.4 Immunohistochemistry of RUNX2

RUNX2 staining using the avidin-biotin complex method with Rabbit polyclonal anti-RUNX primary antibody. Goat anti-rabbit IgG, a laboratory vector with a dilution of 1:200 was used as a secondary antibody. The chromogen uses 3-3' diaminobenzidine (DAB) (Dojindo Laboratories). Furthermore, the preparations were placed into a glass box containing citrate buffer, then put into an autoclave for 15 minutes to optimize its antigenicity. The preparation was cooled at room temperature for 1 hour, and after being dried for a while, the tissue was demarcated using a pap pen. Preparations were washed with dH2O for 5 min and PBS for 5 minutes before incubation with 0.3% hydrogen peroxide for 15 minutes. After the endogenous peroxidase was blocked, the preparation was incubated with the blocking solution for 30 minutes to block the avidin present in the tissue. Then the preparations were incubated overnight at -4°C with the RUNX2 antibody primer diluted 1:200. The preparations were washed 3 times with dH2O before being incubated with secondary antibodies and streptavidin for 30 minutes each. Then the preparation was dehydrated using alcohol which concentration was gradually increased from 70%, 80%, 90% to 100% for 2 minutes each. After that, the preparation was immersed in xylene for 5 minutes. Finally, the preparation was given malinol before being covered with a deg glass and dripped with Canadian balsam.

The assessment was carried out on a photomicrograph with a magnification of 400x, by isolating the brown-colored areas and converting them to black and white. The immunohistochemical assessment was assessed by measuring the proportion of positively stained areas using software; Image J (ImageJ 1.49v software, National Institute of Health, Bethesda, MD, USA), and values are reported in percentage area. The results of the immunohistochemical examination will show the yellow-brown granules which indicate positive cells. Samples were counted in 5 fields of view.

2.5 Immunohistochemistry of TRAP5b

Immunohistochemical examination of TRAP5busing rabbit polyclonal anti-TRAP with a dilution of 1:100 and staining using the avidin-biotin complex method. Goat anti-rabbit IgG, a laboratory vector with a dilution of 1:200 was used as a secondary antibody. Chromogen using 3-3' diaminobenzidine (DAB) (Dojindo Laboratories), and detected using streptavidin- biotin (Histostain-SP Kits, Invitrogen Ltd, UK). After deparaffination, staining TRAP5b was done. TRAP staining is performed to examine the state of bone cells and can serve as a specific marker for osteoclasts and preosteoclasts. Staining using ASBI phosphate naphthol solution as a substrate. Substrate and coupler solution (Tri HCL, Sodium Nitrite which has been dripped with pararosalini). Non-osteoclastic acid phosphatase inhibition (non-osteoclast acid phosphatase inhibitor) using 50 mML (+) tartaric acid. Slices of specimens are dripped with 2-3 drops of dye. The specimens were then incubated for 20-30 minutes at 37°C, then immediately washed again using distilled water for 2 minutes twice.

The stained preparations were observed using a light microscope to see the number of osteoclasts. Using TRAP staining, positive osteoclasts appear brown. The assessment was carried out on a photomicrograph with a magnification of 400x, by isolating the brown-colored areas and converting them to black and white. The immunohistochemical assessment was done by measuring the proportion of stained black positive areas using software; Image J (ImageJ 1.49v software, National Institute of Health, Bethesda, MD, USA), and values are reported in percentage area. Calculations were carried out in 5 fields of view taken at random on the slices of the preparation [23].

2.6 Statistical analysis

The data obtained were analyzed by multivariate using the *Shapiro-Wilk test* to determine whether the data obtained were normally distributed. To determine the interaction between two factors (concentration and time) a two-*way ANOVA* test was conducted with a significance limit ($p < 0.05$). Then, a *multiple comparison* test was done to determine which groups had differences.

3. RESULTS

The percentage area of the average value of RUNX2 showed the highest value on the 7th day of the treatment groups α-mangosteen 1% and the lowest rate in the negative group. On day 14, the highest score in the treatment group α- mangosteen 2% and the lowest in the negative group as shown in Figure 1.

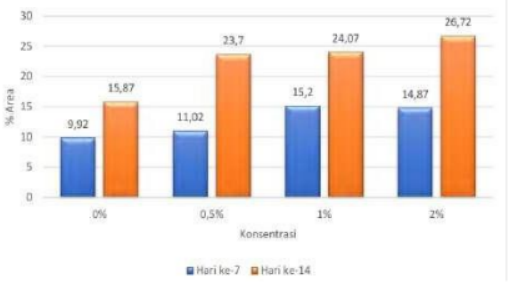


Figure 1. Average RUNX2 on day 7 and 14

The RUNX2 normality test was carried out to select the statistical test method according to the obtained data distribution. On the RUNX2 value, the *Shapiro-Wilk* test was carried out and the data on the 7th day was normally distributed (p>0.05), while on the 14th day it was not normally distributed (p<0.05). Based on the results of the normality test, the statistical test carried out on day 7, namely the *One-way ANOVA* test, is shown in table 2.

Table 2	**One Way Anova Test Results for Each Treatment Group Against RUNX2 on day 7**
Day	**p-value**
Day 7	0.00*

Note: * p<0.05 significant

The results of the One-way ANOVA test, the p-value is 0.00, which means that there is an effect of α-mangosteen on RUNX2 on day 7, then the Bonferroni test is carried out as shown in table 3.

Table 3	**Bonferroni Test Results of Each Treatment Group Against RUNX2 on Day 7**	
Group	**Group**	**p-value**
Negative Group	α-mangosteen 0,5%	1.00
	α-mangosteen 1%	0.00*
	α-mangosteen 2%	0.00*
α-mangosteen 0.5%	α-mangosteen 1%	0.00*
	α-mangosteen 2%	0.00*
α-mangosteen 1%	α-mangosteen 2%	1.00

Note: * p<0.05 significant

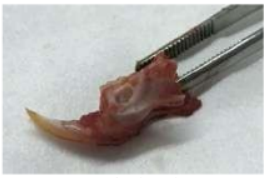


Figure 2. Areas of Alveolar Bone Damage in Periodontitis Rat Model

Bonferroni's test showed that there was a difference in RUNX2 between the 0.5% α-mangosteen treatment group and the 1% α-mangosteen group and the 2% α-mangosteen group (p<0.05).

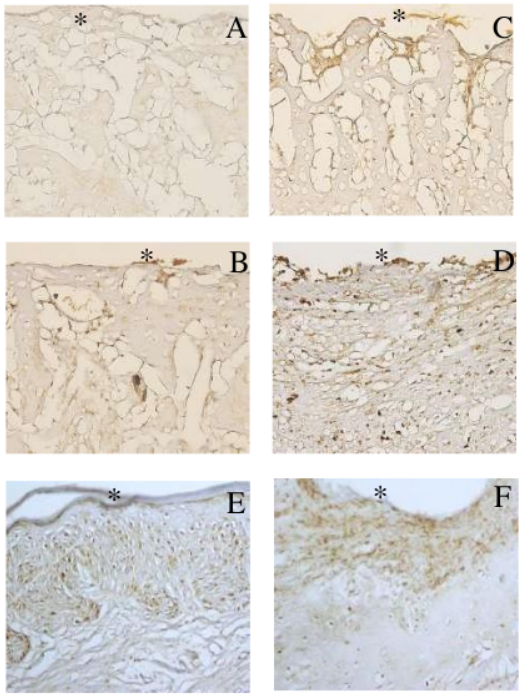
Table 4	**Kruskal-Wallis Test Results of Each Treatment Group Against RUNX2 on Day 14**
Day	**p-value**
Day 14	0,03*

Note: * p<0.05 significant

The statistical test that was carried out on day 14 was the Kruskal-Wallis test which was based on an abnormal data distribution on the Shapiro-Wilk test. The results of the Kruskal-Wallis test showed that the p-value was 0.03 which means that there was an effect of α-mangosteen on RUNX2 on day 14, then the Mann-Whitney test was performed (Table 5).

Table 5	***Mann-Whitney* Test Results *of* Each Treatment Group Against RUNX2 on Day 14**	
Group	**Group**	**p-value**
Negative group	α-mangosteen 0,5%	0,06
	α-mangosteen 1%	0,03*
	α-mangosteen 2%	0,01*
α-mangosteen 0,5%	α-mangosteen 0,5%	0,69
	α-mangosteen 2%	0,26
α-mangosteen 1%	α-mangosteen 2%	0,42

Note: * p<0.05 significant



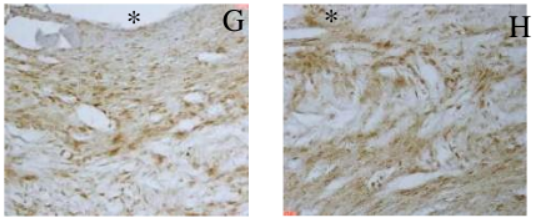


Figure 3. Immunohistochemistry of RUNX2 on the left mandible in the area of the 1st and 2nd molars. Negative Control Group (A – day 7, B - day 14), α-mangosteen group 0.5% (C – day 7, D - day 14). Group α-mangosteen 1% (E - day 7, F - day 14), and Group α-mangosteen 2% (G - day 7, H - day 14). (*): shows the bone formation area at the edge of the excavation cavity. Immunoperoxidase, 100µm scale

Figure 3 shows that there are excavation cavity and bone formation areas at the edge of the cavity on the7th and 14th days. RUNX2 was detected as brown staining on the tissue. There is an impression of an increase in RUNX2 expression in treated tissues compared to control, RUNX2 expression was higher. Theresults of the *Mann-Whitney* RUNX2 test 24 day 14 showed that there was a difference in RUNX2 between the negative group and the 1% α-mangosteen treatment group and the 2% α-mangosteen group (p<0.05).

The results of TRAP5b average percentage area showed the highest value was on day 7 in the 0.5% α-mangosteen treatment groups shown in figure 4.

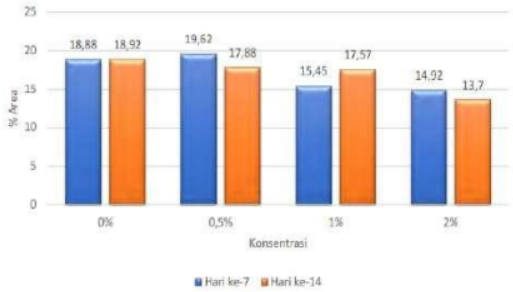


Figure 4. Average TRAP5b on day 7 and day 14

The TRAP5b normality test was conducted toselect a statistical test method that was in accordance with the distribution of the 36 a obtained. For the TRAP5b value, the Shapiro-Wilk test was performed and the data were normally distributed (p>0.05). The statistical test carried 46 out is the One-way ANOVA test which is based on the normal distribution of data on the Shapiro-Wilk test.

Table 7	***One Way Anova* Test Results for EachTRAP5b - Treatment Group**
Day	**p-value**
Day 7	0,01*
Day 14	0,47

Note: * p<0.05 significant

Based on 69 e 7, there was an effect of α-mangosteen on TRAP5b on day 7 (p<0.05), while on day 14 there was no effect of α-mangosteen on TRAP5b (p>0.05). To see differences between 61 ups on day 7, the *Bonferroni* test was done as shown in table 8.

Table 8	***Bonferroni* Test Results of Each Treatment Group Against TRAP5b Day 7**	
Group	**Group**	**p-value**
Negative Group	α-mangosteen 0,5%	1,00
	α-mangosteen 1%	0,18
	α-mangosteen 2%	0,08
α-mangosteen 0,5%	α-mangosteen 1%	0,06
	α-mangosteen 2%	0,03*
α-mangosteen 1%	α-mangosteen 2%	1,00

Note: * p<0.05 significant

47

The results of the Bonferroni test shown in table 8 showed that there was a difference in TRAP5b between the treatment group α-mangosteen 0.5% and α-mangosteen 2% (p<0.05). Immunohistochemical staining of TRAP5b is shown in Figure 5, which shows that on day 7 and day 14 there was an area of bone formation at the edge of the excavation cavity. TRAP5b was detected as brown staining of the tissue. There was an impression of decreased TRAP5b expression in treated tissues compared to the control group.

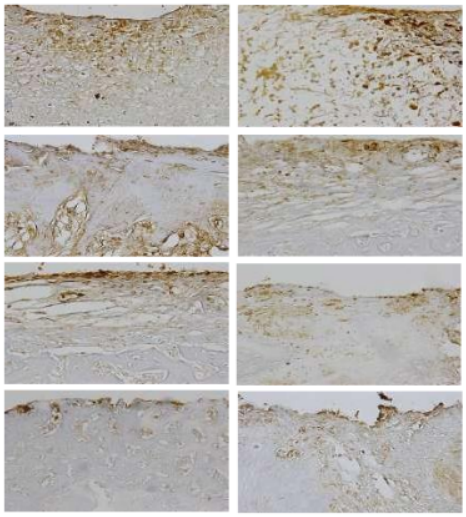


Figure 4. Immunohistochemistry of TRAP5b in the left mandible in the area of the 1st and 2nd molars. Negative Control Group (A – day 7, B – day 14), α-mangosteen group 0.5% (C – day 7, D - day 14), Group α-mangosteen 1% (E - day 7, F - day 14), and Group α mangosteen 2% (G – day 7, H - day 14). (*): shows the area of bone formation at the edge of the excavation cavity. Immunoperoxidase, 100µm scale

5. DISCUSSION

Antioxidants can be obtained exogenously and endogenously, in this study antioxidants were obtained from xanthone α-mangosteen which acts as an antioxidant. α-mangosteen has strong antioxidant activity and has been gradually confirmed in recent years. The researchers found that α-mangosteen was able to clear oxygen, superoxide anion, and peroxynitrite anion in a concentration-dependent manner. It was concluded that α-mangosteen can directly scavenge reactive oxygen species (ROS) and has a neuroprotective effect against 3-NP primary cultures of CGNs, which is attributed to its ability to increase ROS production of 3-NP cells. α-mangosteen exhibits biocompatibility, immunomodulation, antimicrobial, wound healing, and osteogenic activity so that it has the potential to be used as a periodontal regenerative material when α-mangosteen is introduced into areas of bone damage. The application of α-mangosteen to bone damage after a few days gradually releases bioactive molecules to increase the regeneration of surrounding tissues [24][25]. Previous research conducted by Ioyah (2019) also showed that the active content of xanthones as antioxidants plays a role in healing bone fractures. In immunoassay also shows an increase or decrease of biomarkers in tissue damage induced by ROS in a number of periodontal diseases [26].

5.1 RUNX2

Based on the results of this study, RUNX2 increased in line with the level of α-mangosteen concentration on day 7 and day 14. The test results showed a significant effect of various concentrations of α-mangosteen on day 7 and day 14, where RUNX2 plays an important role in osteoblast differentiation. Assessment of RUNX2 using the immunohistochemical method with the results of the brown staining image being converted to grayscale. The color intensity of the brown staining was identified as a combination of red, green, and blue (RGB) activity.

Theoretically, RUNX2 is a major regulation of osteoblast and chondrocyte differentiation, a marker of tissue expression and osteoblast function. In vitro and in vivo reported that osteogenic activity in spinal stromal cells showed an increase during RUNX2 expression. RUNX2 is also a component of BMP and TGF-β that plays a role in osteogenesis [27]. The results of research by Lim et al (2019) in vitro with mangosteen extract showed that RUNX2 expression tended to increase on day 4 and day 8.

Based on the time interval showed an increase in expression on day 14 compared to day 7. The longer the time and the higher the concentration of α-mangosteen used in the bone remodeling process observation, the higher the healing rate of the mandibular alveolar bone. It can be seen that the highest RUNX2 value in the 2% α-mangosteen treatment group. Immunohistochemistry of RUNX2 showed an area of ossification at the edge of the excavation cavity. RUNX2was detected as brown staining of the tissue. There was an impression of an increase in RUNX2 expression in the treated tissue compared to the control, RUNX2expression was higher in the treatment with high concentrations. RUNX2 staining was stained in part of the cell cytoplasm in the granulation and ossification area and stained in the extracellular matrix.

Another study showed that RUNX2 wasexpressed in osteoblasts near the alveolar bone and fibroblasts in the periodontal ligament. RUNX2 is a positive regulator that can regulate the gene expression of bone matrix proteins, including Col1a1, Spp1, Ibsp, Bglap2, and Fn1. In addition, the assays report revealed that RUNX2 activates bone matrix protein gene promoters, including Col1a1, Col1a2, Spp1, and Bglap2 [28]. RUNX2 expression levels are important for normal bone development. Decreased RUNX2 expression leads to abnormal bone development. These data suggest that the expression level of RUNX2 is very important for maintaining the balance between bone formation and bone resorption processes. Overall, RUNX2 is a major transcription factor for bone formation. RUNX2 is a transcription factor important for osteoblast commitment and early stages of osteoblast differentiation, which activity is tightly controlled by transcription factors via protein-DNA interactions or proteins [29].

5.2 TRAP5b

The study showed a decrease in TRAP5b on day 7 and day 14. The lowest value of TRAP5b was seen in the 2% α-mangosteen treatment group. TRAP5b was detected as brown staining in the tissue, there was an impression of a decrease in TRAP expression in the treated tissue compared to the control. TRAP5b staining part of the cell cytoplasm in the granulation and ossification area and stained in the extracellular matrix. TRAP5b plays an important role in osteoclast differentiation which is secreted by osteoclasts during the process of differentiation and bone resorption. Previous studies have also suggested TRAP5b as a biomarker of osteoclast activity, macrophages, and a biomarker of bone resorption. TRAP5b also produces ROS and then destroys degradation products that indicate the degree of bone resorption [5][30][31]. Hartiningsih's study also showed an increase in the expression of TRAP5b as osteoclasts resorb bone during the consumption of the combination drug raloxifene and calcitriol. This study was also supported by research on the application of G mangostana extract after 5 days which showed a decrease in TRAP5b expression in bone damage [32].

Hartiningsih's study also showed an increase in the expression of TRAP5b as osteoclasts resorb bone during the consumption of the combination drug raloxifene and calcitriol. This study is also supported by other studies which showed a significant reduction in osteoclasts through the identification of the TRAP biomarker on the fifth day after administration of α- mangosteen with a concentration of 0.25% [32]. Other

studies have also shown that α-mangosteen can suppress osteoclastogenesis at day 4 by inhibiting osteoclast-specific genes, such as TRAP, CTR, CTSK DC-STAMP, V-ATPase a3, and V-ATPase d2, resulting in blocking of osteoclast formation and bone resorption ability [11].

Osteoclasts produce large amounts of the TRAP enzyme which is an enzyme produced in osteoclast precursors namely osteoclast markers. Research results Amin et al., (2010) showed that decreased osteoclast formation around the alveolar bone was characterized by reduced TRAP expression [33]. This study showed a decrease in TRAP5b on the 14th day compared to the 7th day. TRAP5b on day 7 showed significant results with a higher value than on day 14 which was probably due to the bone resorption process still occurring. On day 14 TRAP5b tended to decrease but the results were not significant. The preparation of α-mangosteen in the form of a gel can cause the gel to not last in the damaged area for a long time so that the drug dose is unstable. Drug delivery currently being developed is in the form of in situ gel, a solution with low viscosity so that the drug will be in contact for a longer time [34].

6. CONCLUSION

1. It is proven that there is an effect of α-mangosteen at a concentration of 0.5% on the expression of RUNX2 on day 7 and day 14 in the bone remodeling process of periodontitis.

2. It was proven that there was an effect of α-mangosteen at a concentration of 0.5% on the expression of TRAP5b on the bone remodeling periodontitis on day 7, while on day 14 it was not proven to have a significant effect of α-mangosteen in all concentrations.

AUTHOR CONTRIBUTION

The contributions of each author are as follows conceived and designed the analysis, collect the data, contributed data or analysis tools, performed the analysis, and wrote the paper.

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