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Effects of Roselle (*Hibiscus sabdariffa Linn.)* flower extracts on various inflammatory and bone apposition biomarkers during orthodontic tooth movement: An experimental animal study

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ABSTRACT

Background: Roselle (*Hibiscus sabdariffa*) flower extract (RFE) can potentially be an adjuvant in orthodontic tooth movement (OTM) in the alveolar bone (AB) by regulating inflammatory response, and bone remodeling through tumor necrosis factor-α (TNF-α), nuclear factor kappa beta (NF-κB), heat shock protein (HSP)-10, HSP-70, alkaline phosphatase (ALP), and osteocalcin expression.
Objective: To investigate TNF-α, NF-κB, HSP-10, HSP-70, ALP, and osteocalcin expression after RFE administration during OTM in the AB *in vivo*.
Materials and methods: Forty healthy male Wistar rats (Rattus norvegicus) were randomly divided into two groups, OTM group (K) and OTM with RFE group (KP). An 8-mm nickel-titanium closed coil spring and a ligature wire linked from the first upper left molar to the central incisor at a light force of 10 g. The rats received RFE administration using a blunt microneedle and a 0.01-ml dosage on the gingiva sulcus of a molar on days 1, 7, 14, and 21 and were then sacrificed, respectively. The ALP, osteocalcin, NFκβ, TNF-α, HSP-10, and HSP-70 expressions were analyzed immunohistochemically.
Result: The highest HSP-70, NFκβ, and TNF-α expressions on the compression side of the AB found on day 1 were significantly different between the groups ($p \leq 0.05$). The highest expressions of ALP and HSP-10 on the tension side were found on day 7. Meanwhile, the highest osteocalcin expression found on day 21 was significantly different between the groups ($p \leq 0.05$).
Conclusion: RFE decreased TNF-α, NF-κB, and HSP-70 expression and enhanced osteocalcin and ALP expression in the AB during OTM in Wistar rats (*R. novergicus*).

1. Introduction

In the dentoalveolar complex, orthodontic tooth movement (OTM) is the result of a synergistic interaction between biological reactions in alveolar bone (AB) tissue and orthodontic mechanical compressions to treat malocclusion. Following the application of orthodontic force, two mechanisms take place: Bone formation occurs on the tension side, and bone resorption occurs on the compression side. Osteoclastogenesis, or bone resorption, by osteoclasts and the production of new bone by osteoblasts are the two mechano-sensing processes that make up the

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process of bone remodeling in OTM.[1]

The potential indicators for modeling bone development during OTM have been investigated. Alkaline phosphatase (ALP) and osteocalcin are two frequent bone biomarkers that are linked to the creation of new bone tissue during OTM.[2] The findings from research examining ALP expression and osteoblast counts in relation to OTM, which demonstrate that an increase in osteoblast counts also boosts ALP expression, corroborate this.[2] Furthermore, an increase in the rate of the osteogenic differentiation of mesenchymal stem cells is correlated with a higher expression of osteocalcin, a biomarker of bone formation.[3]

The rate-limiting stage of OTM occurs on the compression side when multinucleated osteoclasts start bone resorption, enabling tooth movement in the direction of the applied force. Moreover, the production of pro-inflammatory cytokines triggers a sterile inflammatory response.4 One of the chemical mediators, tumor necrosis factor-α (TNF-α), is regarded as the master regulator of the cytokines involved in innate immunity and inflammatory responses.[4] Nuclear factor kappa beta (NF-κB) is induced to become activated by TNF-α. After translocating from the cytoplasm to the cell nucleus, NF-κB produces many genes linked to inflammation, which promotes bone resorption during OTM.[5]

Heat shock protein (HSP), which is associated with pro-inflammatory cytokines, is another component that plays a function in preserving homeostasis and shielding periodontal ligament fibroblast cells from the draw and strain of orthodontic mechanical forces.[6] One of the HSPs, HSP-70, is highly concentrated on the compression side of the AB and is correlated with pro-inflammatory cytokines.[7] As a component of the damage-associated molecular pattern, HSP-70 plays a role in starting a sterile inflammatory response that allows AB resorption to take place.[8]

Pro-inflammatory cytokine release is inhibited by HSP-10, which is a direct antagonist of HSP-70, an anti-inflammatory protein. AB formation is anticipated because HSP-10 functions as one of the components of the resolution-associated molecular pattern, which sets off the resolution of inflammation and the initiation of cell regeneration.[9] Aligning teeth in an optimal arch with the least amount of inflammation and flaws in the teeth and surrounding periodontal tissues is the primary objective of OTM.[9] A balanced AB remodeling process between resorption and formation is necessary for effective OTM. Adjuvant therapies, such as anti-inflammatories and antioxidants, can promote bone formation and reduce the adverse effects of orthodontic force-induced resorption of the AB.[10]

The plant known as roselle (*Hibiscus sabdariffa*) is widely grown and simple to cultivate in tropical regions, such as Indonesia. For thousands of years, roselle flowers have been utilized as a medical remedy and traditional herbal beverage with various health benefits.[11] In the realm of dentistry, a 10 % roselle flower extract (RFE) has been demonstrated to both quickly speed up the rebuilding of the AB and efficiently minimize resorption. This is due to the high concentration of anti-inflammatories and antioxidants in RFE, such as anthocyanins, quercetin, and gossypetin.[12] These compounds can decrease the synthesis of inflammatory mediators, inhibit osteoclastogenesis, and may speed up the process of inflammatory resolution by boosting osteoblastogenesis during OTM.[12,13] One flavonoid molecule from the flavonol subclass, quercetin, has pharmacological actions that manifest as anti-inflammatory properties. Quercetin can block the action of the enzyme histone acetyltransferase on inflammatory gene promoters.[14] By blocking the NF-κB pathway's regulation, the anthocyanin derivative delphinidin 3-sambubioside can lower inflammatory mediators, such as interleukin, protein-1 monocyte C, TNF-α, and cyclooxygenase-2.[15] By controlling the signaling pathways that improve osteoblast activity and lessen the impacts of low-level chronic oxidative or inflammatory stress, flavonoids may be able to prevent bone loss.[16] Furthermore, this study aims to observe the effect of RFE on the expression of ALP and osteocalcin as biomarkers of bone formation, as well as TNF-α, NF-κB, HSP-70, and HSP-10, which are biomarkers of the inflammatory process during OTM *in vivo*.

2. Material and methods

2.1. Experimental study design and ethical clearance

The Health Committee of Research Ethical Clearance of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia, confirmed this study design and gave permission to conduct this study with an animal model with permit number 578/HRECC. FODM/VIII/2022.

This study used a true experimental laboratory-based approach with a randomized post-test-only control group design. This study used male Wistar rats (*Rattus norvegicus*) aged 1–2 months and weighing 250–300 g. The age selection of the Wistar rats was based on the size of the jaw, which was large enough so that RFE could be applied to the experimental animals. The sample was selected with a blind random sampling method. The sample size for this study was determined using Lemeshow's minimum sample size for each group, examined variables and as many as four samples per group. The total sample size was 40 male Wistar rats, which were divided into two groups: OTM no treatment group (K) and OTM with RFE group (KP) (10 μl intra-sulcus application, every day). Each group was divided into subgroups for observation time on days 1, 7, 14, and 21, with five samples in each group.

2.2. Orthodontic tooth movement animal model

The rats were anesthetized with a solution consisting of a mixture of ketamine (100 mg/ml) and xylazine (0.03 ml/100 g body weight (BW)). Before insertion, the force of a nickel-titanium (NiTi) closed coil spring was measured using a tension gauge to produce a strength of 10 g. The NiTi closed coil spring was placed between the maxillary central incisor and the maxillary left first molar to move the molar mesially. The device was fixed using a 0.007-in. stainless steel ligature wire around the maxillary central incisor. The posterior portion of the device was also ligated with a 0.007-in. stainless steel ligature archwire placed around the first molar. Retention was ligated using glass ionomer cement.[17]

2.3. Preparation and administration of roselle flower extract

RFE was dried and then extracted with ethanol. The extract was diluted in a 10 % solution for testing on the experimental animal groups.[12] RFE was administered to the OTM and RFE groups and the RFE-only group by injection into the gingival sulcus. RFE was injected once a day using a Hamilton microliter syringe that was blunted with as much as 0.01 cc (10 μl) phosphate buffer saline (PBS) in the gingival sulcus of the maxillary left first molar after tooth movement by the NiTi closed coil spring.[1,17] OTM was performed on days 7, 14, and 21 after the administration of RFE to the experimental animals, and the animals were immediately euthanized using a lethal dose of rodent anesthesia, respectively. The study samples were dissected on the maxilla (first molar area) and then immersed for 3 days in 10 % formalin for fixation for sample collection.[1,2]

2.4. Histopathological preparation

To prepare the histological samples from the Wistar rat periodontal tissues, the tissues were cleaned with PBS, fixed in 10 % formalin, and decalcified in 10 % Ethylenediaminetetraacetic acid for 2–3 months. The tissues were dehydrated in graded alcohol, cleared with xylol, and infiltrated with soft paraffin. The blocks were formed in hard paraffin and sectioned using a rotary microtome. The sections were mounted on glass slides with 5 % gelatin. Deparaffinization involved immersing the sections in xylol, rehydrating them with alcohol, and rinsing them in water, resulting in histological preparations ready for analysis.[1,2]

2.5. Immunohistochemical staining analysis of ALP, osteocalcin, TNF-α, NF-κB, HSP-70, and HSP-10 expressions

The first step was to rehydrate the samples prepared with 100 %, 95 %, and 70 % ethanol for 2 min and then rinse them with water for 1 min. They were immersed in peroxidase-blocking solution at room temperature for 10 min, followed by incubation in blocking serum at 25 °C for 10 min. Next, the samples were immersed in monoclonal antibodies of ALP, osteocalcin, TNF-α, NF-κB, HSP-70, and HSP-10 with a dilution of 1:100 with PBS for 5 min and then incubated with a secondary antibody (conjugated horseradish peroxidase) at 25 °C for 10 min. They were washed with PBS for 5 min, reincubated with peroxidase at 25 °C for 10 min, washed with PBS, and then incubated with diaminobenzidine at 25 °C for 10 min. Then, the samples were incubated with hematoxylin-eosin for 3 min, washed, cleaned, fixed with mounting media, and covered with glass. The ALP, osteocalcin, TNF-α, NF-κB, HSP-70, and HSP-10 expressions were observed through immunohistochemical staining under a microscope at 400× and 1,000× magnification and documented with a digital camera.[1,2]

2.6. Statistical data analysis

The results of the ALP, osteocalcin, TNF-α, NF-κB, HSP-70, and HSP-10 expressions from the sample groups were tabulated and tested using the Shapiro–Wilk test for data normality ($p > 0.05$). Then, Levene's test was used to test the homogeneity of the data ($p > 0.05$). The normal and homogeneous data were tested using a one-way analysis of variance to examine the differences between the treatments, and Tukey's honestly significant different post hoc test was used to compare the treatment groups ($p < 0.05$). The non-normal and inhomogeneous data were analyzed utilizing the Kruskal–Wallis test for differences between the treatments, and the Mann–Whitney test was used for comparison between the groups ($p < 0.05$).

3. Result

The highest NFκβ and TNF-α expressions on the compression side of the AB were found on day 1, with significant differences between the groups ($p \leq 0.05$). The NFκβ and TNF-α expressions significantly decreased after RFE local application from days 1–21 ($p \leq 0.05$) (see Figs. 1 and 2). The highest HSP-70 expression on the compression side of the AB was found on day 1, with significant differences between the groups ($p \leq 0.05$). HSP-70 expression significantly decreased eventually after RFE local application from days 1–21 ($p \leq 0.05$) (see Fig. 3). Meanwhile, the highest HSP-10 and ALP expressions on the tension side of the AB were found on day 7, with significant differences between the groups ($p \leq 0.05$). The HSP-10 and ALP expressions significantly increased after RFE local application from day 1–7 ($p \leq 0.05$) (see Figs. 4 and 5). By contrast, the highest osteocalcin expression on the tension side of the AB was found on day 21, with significant differences between the groups ($p \leq 0.05$). Osteocalcin expression significantly increased after RFE local application from day 14–21 ($p \leq 0.05$) (see Fig. 6). The Expression of various pro-inflammatory cytokines in OTM no treatment group (K) and RFE group (KP) Wistar rats at various time points of force application shown in Tables 1 and 2. In addition, the comparison of expression of various pro-inflammatory cytokines between the groups at various time points of force application can be seen in Table 3.

4. Discussion

In this present study, we found that RFE affected ALP, osteocalcin, TNF-α, NF-κB, HSP-70, and HSP-10 expressions during OTM in the Wistar rats. According to the study's findings, ALP activity was high on day 7–14, followed by a reduction on days 14–21. ALP activity reached its peak on day 7 but dramatically decreased thereafter. Seven days after OTM with RFE (KP), ALP activity dramatically increased in the late phase of bone deposition, indicating osteoblast activity. Following OTM, ALP activity notably increased throughout the late period of bone deposition (7–14 days). This was in line with research demonstrating that an increase in ALP expression, a marker of osteoblast activity during OTM, occurs at this time. Low ALP activity in the compressed hyaline zone of the periodontal ligament indicates the removal of the hyalinized zone and the ensuing decrease in ALP activity.[2,18] However, on day 21, the OTM group treated with RFE (KP) had the greatest expression of osteocalcin. Between the OTM group that received RFE (KP) and the OTM-only group (K), a noticeable difference was seen. From days 7–21,

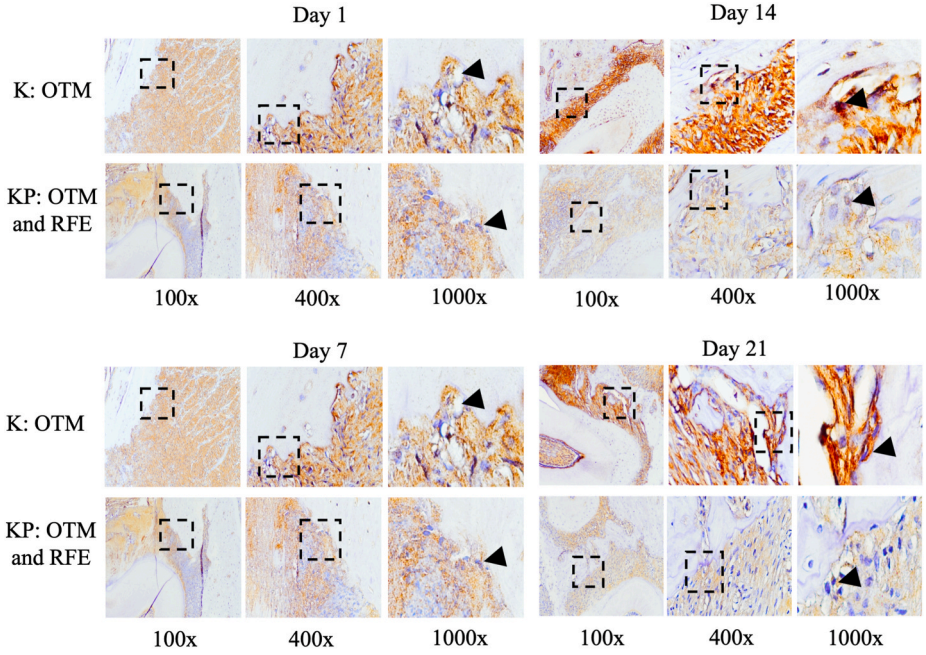

Fig. 1. Comparison of NF-κB expression in RFE treated (KP) and untreated groups (K) on each observation day. Information: K1: OTM untreated with RFE day 1; K7: OTM untreated with RFE day 7; K14: OTM untreated with RFE day 14; K21: OTM untreated with RFE day 21, KP1: OTM treated with RFE day 1; KP7: OTM treated with RFE day 7; KP14: OTM treated with RFE day 14; K21: OTM treated with RFE day 21.

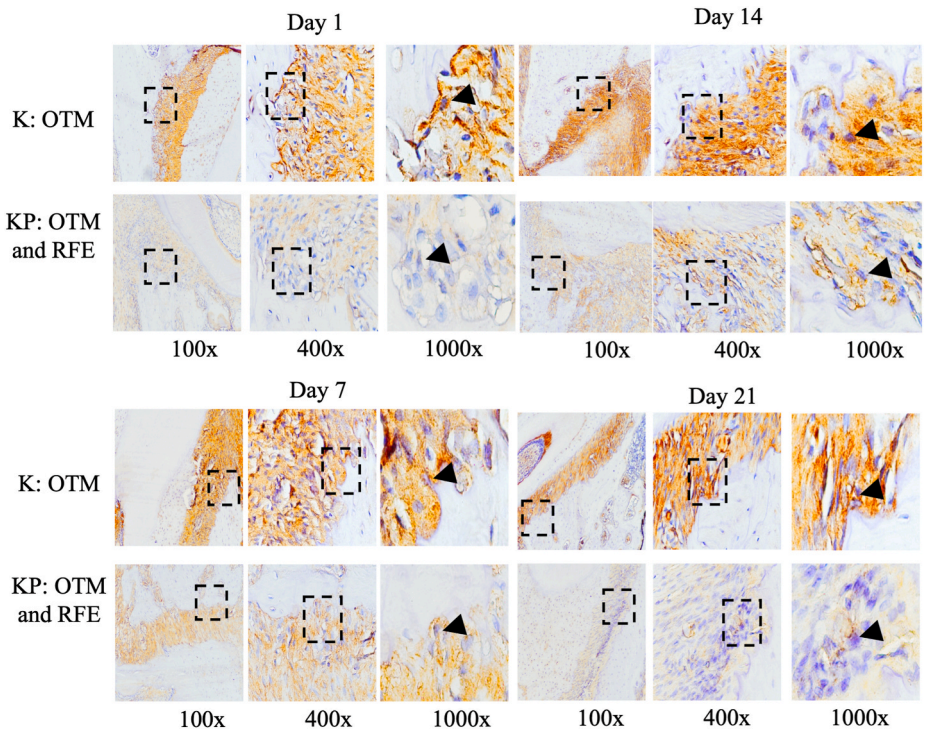

Fig. 2. Comparison of TNF-α expression in RFE treated (KP) and untreated groups (K) on each observation day. Information: K1: OTM untreated with RFE day 1; K7: OTM untreated with RFE day 7; K14: OTM untreated with RFE day 14; K21: OTM untreated with RFE day 21, KP1: OTM treated with RFE day 1; KP7: OTM treated with RFE day 7; KP14: OTM treated with RFE day 14; K21: OTM treated with RFE day 21.

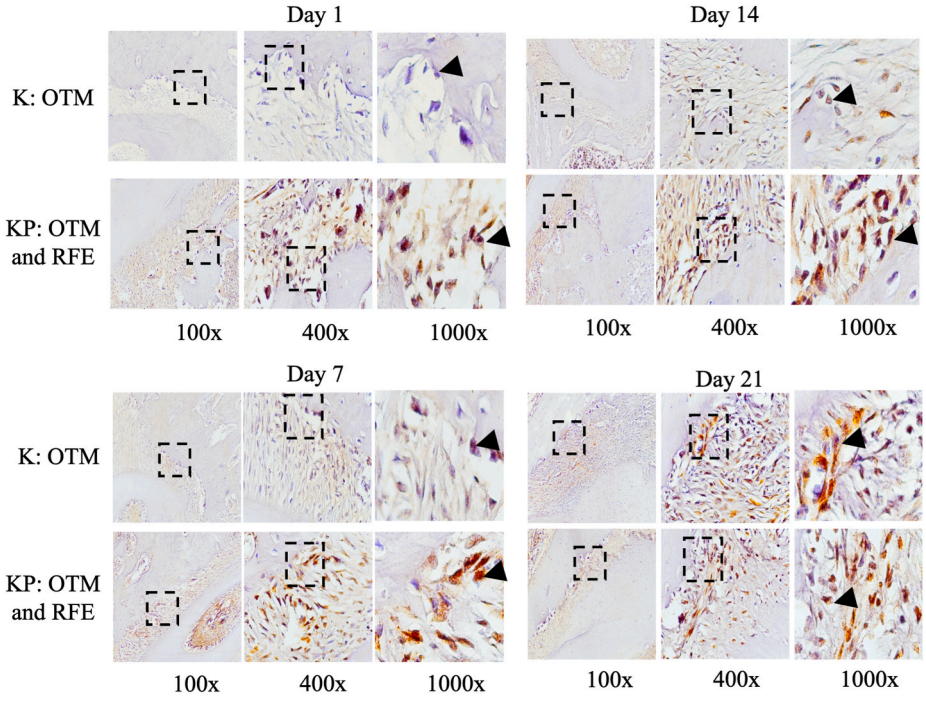

Fig. 3. Comparison of HSP-70 expression in RFE treated and untreated groups on each observation day. Information: K1: OTM untreated with RFE day 1; K7: OTM untreated with RFE day 7; K14: OTM untreated with RFE day 14; K21: OTM untreated with RFE day 21, KP1: OTM treated with RFE day 1; KP7: OTM treated with RFE day 7; KP14: OTM treated with RFE day 14; K21: OTM treated with RFE day 21.

RFE treatment (KP) increased the expression of osteocalcin. This was consistent with other studies investigating the effects of sodium fluoride treatment on osteocalcin and osteonectin expression, which found that osteocalcin increased significantly after day 7.[19]

Biological reactions resulting from OTM occur following the application of orthodontic compression.[9,20] The results of this study showed that from days 7–21, TNF-α expression was reduced in the both control group (K) and treatment group (KP). A prior study produced comparable findings to support these findings.[4] This may be because of the first chemotactic activity increase that occurs promptly after

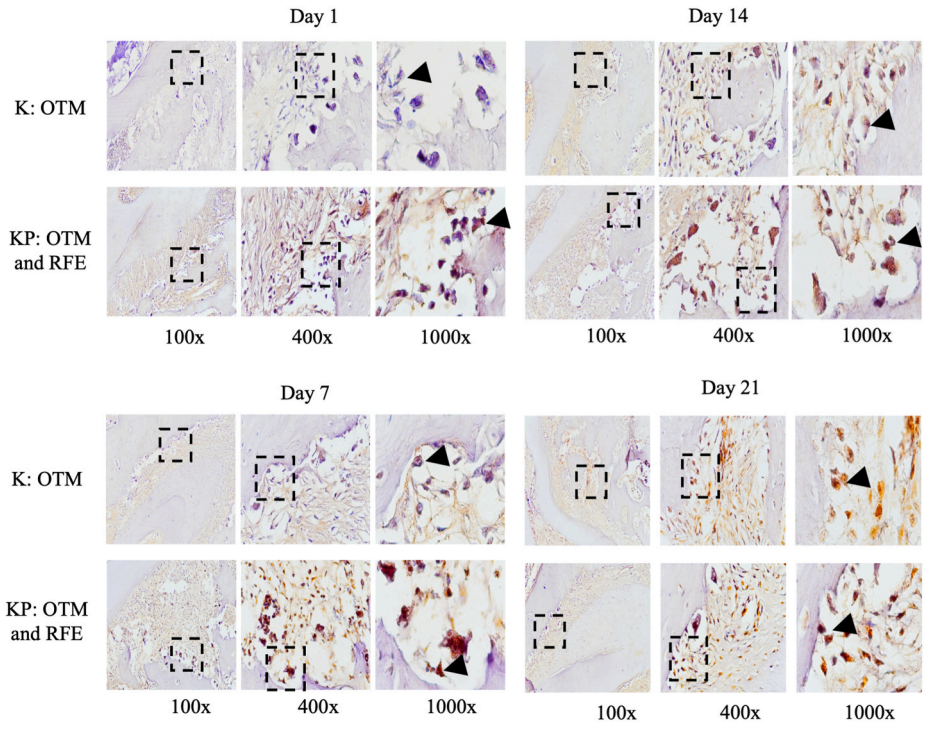

Fig. 4. Comparison of HSP-10 expression in rosella extract treated and untreated groups on each observation day. Information: K1: OTM untreated with RFE day 1; K7: OTM untreated with RFE day 7; K14: OTM untreated with RFE day 14; K21: OTM untreated with RFE day 21, KP1: OTM treated with RFE day 1; KP7: OTM treated with RFE day 7; KP14: OTM treated with RFE day 14; K21: OTM treated with RFE day 21.

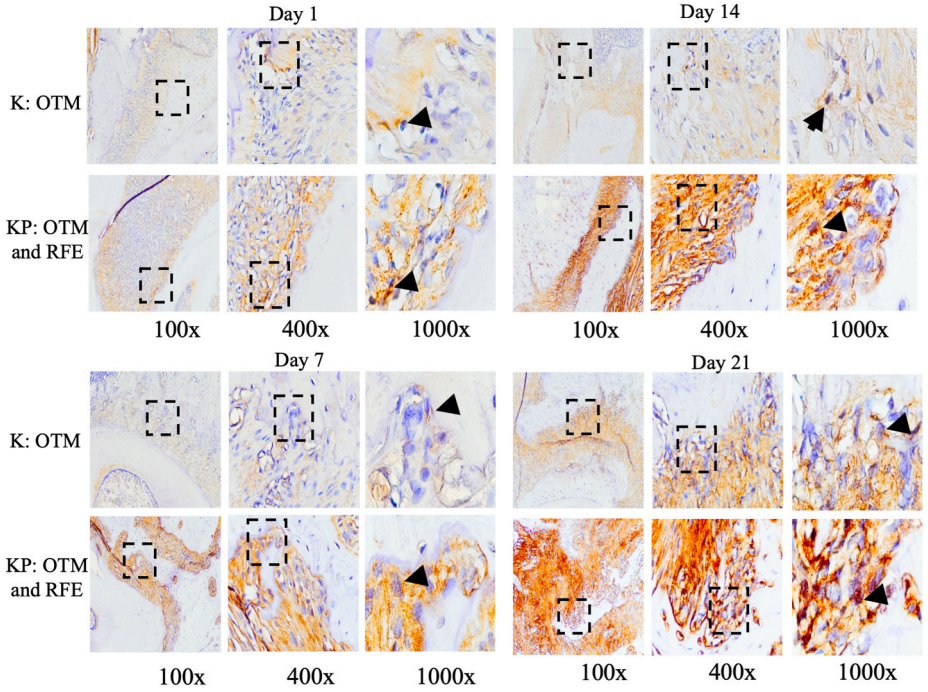

Fig. 5. Comparison of ALP expression in RFE treated (KP) and untreated groups (K) on each observation day. Information: K1: OTM untreated with RFE day 1; K7: OTM untreated with RFE day 7; K14: OTM untreated with RFE day 14; K21: OTM untreated with RFE day 21, KP1: OTM treated with RFE day 1; KP7: OTM treated with RFE day 7; KP14: OTM treated with RFE day 14; K21: OTM treated with RFE day 21.

orthodontic mechanical force application. Studies that demonstrate an immediate inflammatory response during the early stages of tooth movement are also compatible with this. TNF-α reaches a peak after 3 days and starts to decrease from days 7–10 in rats.[21] Additionally, previous research demonstrated that TNF-α concentrations rise noticeably at 24 h and fall nearly to baseline levels throughout OTM's linear phase.[21]

The study's findings also showed that from days 1–14, NF-κB expression fell in the K group. Although NF-κB expression was reduced in the treatment group (KP), it was more pronounced from days 7–21 in

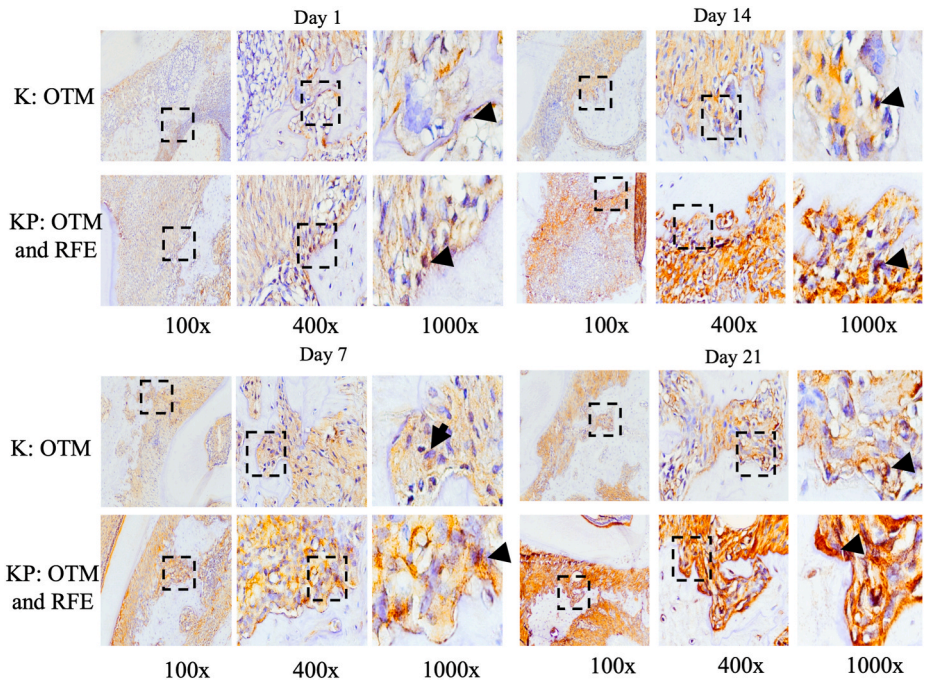

Fig. 6. Comparison of Osteocalcin expression in RFE treated (KP) and untreated groups (K) on each observation day. Information: K1: OTM untreated with RFE day 1; K7: OTM untreated with RFE day 7; K14: OTM untreated with RFE day 14; K21: OTM untreated with RFE day 21, KP1: OTM treated with RFE day 1; KP7: OTM treated with RFE day 7; KP14: OTM treated with RFE day 14; K21: OTM treated with RFE day 21.

Table 1
Expression of various pro-inflammatory cytokines in OTM no treatment group (K) Wistar rats at various time points of force application.

Molecular Marker	Group (Mean ± Standar Deviation (SD))				P-value for "within the group" comparison		
	OTM Day 1	OTM Day 7	OTM Day 14	OTM Day 21		p-value	
NFκβ	12.8 ± 1.48	9.8 ± 1.9	7.2 ± 1.3	4.2 ± 0.84	0.001*	0.27	OTM Day 1 vs. OTM Day 7
						0.01*	OTM Day 1 vs. OTM Day 14
						0.01*	OTM Day 1 vs. OTM Day 21
						0.08	OTM Day 7 vs. OTM Day 14
						0.01*	OTM Day 7 vs. OTM Day 21
						0.03*	OTM Day 14 v.s OTM Day 21
TNF-α	13 ± 1.58	9.2 ± 1.79	8.4 ± 1.67	4.6 ± 1.14	0.001*	0.01*	Day-1 Vs Day-7,
						0.01*	Day-1 Vs Day-14,
						0.01*	Day-1 Vs Day-21,
						0.99	Day-7 Vs Day-14,
						0.01*	Day-7 Vs Day-21
						0.01*	Day-14 Vs Day-21
HSP-70	12 ± 1.58	10 ± 1.58	8.5 ± 0.79	5.6 ± 1.14	0.001*	0.12	Day-1 Vs Day-7,
						0.01*	Day-1 Vs Day-14,
						0.01*	Day-1 Vs Day-21,
						0.41	Day-7 Vs Day-14,
						0.01*	Day-7 Vs Day-21
						0.01*	Day-14 Vs Day-21
HSP-10	4 ± 1.58	6.3 ± 1.2	8.7 ± 1.2	4.2 ± 0.84	0.001*	0.07*	Day-1 Vs Day-7,
						0.01*	Day-1 Vs Day-14,
						>0.99	Day-1 Vs Day-21,
						0.049*	Day-7 Vs Day-14,
						0.12	Day-7 Vs Day-21
						0.01*	Day-14 Vs Day-21
ALP	2.8 ± 0.84	2.8 ± 0.84	8.8 ± 1.48	7 ± 1.23	0.001*	0.99	Day-1 Vs Day-7,
						0.01*	Day-1 Vs Day-14,
						0.01*	Day-1 Vs Day-21,
						0.01*	Day-7 Vs Day-14,
						0.01*	Day-7 Vs Day-21
						0.41	Day-14 Vs Day-21
Osteocalcin	2.6 ± 0.89	4 ± 1.58	7.2 ± 1.3	9 ± 1.58	0.001*	0.75	Day-1 Vs Day-7,
						0.01*	Day-1 Vs Day-14,
						0.01*	Day-1 Vs Day-21,
						0.02*	Day-7 Vs Day-14,
						0.01*	Day-7 Vs Day-21
						0.47	Day-14 Vs Day-21

Information: Sample for each group in every variable was 5 samples. *significant different between groups at $p < 0.05$.

Table 2
Expression of various pro-inflammatory cytokines in OTM with RFE group (KP) Wistar rats at various time points of force application.

Molecular Marker	Group (Mean ± Standar Deviation (SD))				P-value for "within the group" comparison		
	OTM and RFE Day 1	OTM and RFE Day 7	OTM and RFE Day 14	OTM and RFE Day 21		p-value	
NFκβ	10 ± 1.58	6.6 ± 1.4	3.4 ± 1.14	2.6 ± 1.14	0.001*	0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 7
						0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 14
						0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 21
						0.01*	OTM + RFE Day 7 vs. OTM + RFE Day 14
						0.01*	OTM + RFE Day 7 vs. OTM + RFE Day 21
						0.98	OTM + RFE Day 14 vs. OTM + RFE Day 21
TNF-α	9.0 ± 1.58	5.4 ± 1.4	4 ± 1.58	3.6 ± 1.14	9.0 ± 1.58	0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 7
						0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 14
						0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 21
						0.8	OTM + RFE Day 7 vs. OTM + RFE Day 14
						0.54	OTM + RFE Day 7 vs. OTM + RFE Day 21
						0.99	OTM + RFE Day 14 vs. OTM + RFE Day 21
HSP-70	9 ± 0.79	7 ± 0.79	4.4 ± 0.96	2.8 ± 0.84	9 ± 0.79	0.12	OTM + RFE Day 1 vs. OTM + RFE Day 7
						0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 14
						0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 21
						0.01*	OTM + RFE Day 7 vs. OTM + RFE Day 14
						0.01*	OTM + RFE Day 7 vs. OTM + RFE Day 21
						0.33	OTM + RFE Day 14 vs. OTM + RFE Day 21
HSP-10	8.8 ± 1.48	11.3 ± 1.2	8.4 ± 0.82	3 ± 0.71	8.8 ± 1.48	0.04*	OTM + RFE Day 1 vs. OTM + RFE Day 7
						0.99	OTM + RFE Day 1 vs. OTM + RFE Day 14
						0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 21
						0.01*	OTM + RFE Day 7 vs. OTM + RFE Day 14
						0.01*	OTM + RFE Day 7 vs. OTM + RFE Day 21
						0.01*	OTM + RFE Day 14 vs. OTM + RFE Day 21
ALP	8.4 ± 1.14	9 ± 1.58	12.2 ± 1.64	10 ± 1.58	0.001*	0.99	OTM + RFE Day 1 vs. OTM + RFE Day 7
						0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 14
						0.56	OTM + RFE Day 1 vs. OTM + RFE Day 21
						0.01*	OTM + RFE Day 7 vs. OTM + RFE Day 14
						0.93	OTM + RFE Day 7 vs. OTM + RFE Day 21
						0.18	OTM + RFE Day 14 vs. OTM + RFE Day 21
Osteocalcin	4.4 ± 1.14	6.4 ± 1.67	10.6 ± 1.14	13 ± 1.58	0.001*	0.34	OTM + RFE Day 1 vs. OTM + RFE Day 7
						0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 14
						0.01*	OTM + RFE Day 1 vs. OTM + RFE Day 21
						0.01*	OTM + RFE Day 7 vs. OTM + RFE Day 14
						0.01*	OTM + RFE Day 7 vs. OTM + RFE Day 21
						0.15	OTM + RFE Day 14 vs. OTM + RFE Day 21

Table 3
Comparison of expression of various pro-inflammatory cytokines between the groups at various time points of force application.

Group	P-value for "between groups" comparison					
	Molecular Marker					
	NFκβ	TNF-α	HSP-70	HSP-10	ALP	Osteocalcin
OTM Day 1 vs. OTM + RFE Day 1	0.047*	0.01*	0.01*	0.01*	0.01*	0.47
OTM Day 1 vs. OTM + RFE Day 7	0.01*	0.01*	0.01*	0.01*	0.01*	0.01*
OTM Day 1 vs. OTM + RFE Day 14	0.01*	0.01*	0.01*	0.01*	0.01*	0.01*
OTM Day 1 vs. OTM + RFE Day 21	0.01*	0.01*	0.01*	0.87	0.01*	0.01*
OTM Day 7 vs. OTM + RFE Day 1	0.99	0.99	0.84	0.036*	0.01*	0.99
OTM Day 7 vs. OTM + RFE Day 7	0.02	0.01*	0.01*	0.01*	0.01*	0.15
OTM Day 7 vs. OTM + RFE Day 14	0.01*	0.01*	0.01*	0.12	0.01*	0.01*
OTM Day 7 vs. OTM + RFE Day 21	0.01*	0.01*	0.01*	0.01*	0.01*	0.01*
OTM Day 14 vs. OTM + RFE Day 1	0.047*	0.99	0.99	0.99	0.99	0.06
OTM Day 14 vs. OTM + RFE Day 7	0.99	0.052	0.41	0.01*	0.99	0.98
OTM Day 14 vs. OTM + RFE Day 14	0.01*	0.01*	0.01*	0.99	0.01*	0.01*
OTM Day 14 vs. OTM + RFE Day 21	0.01*	0.01*	0.01*	0.01*.	0.84	0.01*
OTM Day 21 vs. OTM + RFE Day 1	0.01*	0.01*	0.01*	0.01*	0.71	0.01*
OTM Day 21 vs. OTM + RFE Day 7	0.13	0.99	0.5	0.01*	0.28	0.09
OTM Day 21 vs. OTM + RFE Day 14	0.98	0.99	0.6784	0.01*	0.01*	0.61
OTM Day 21 vs. OTM + RFE Day 21	0.58	0.96	0.01*	0.73	0.02*	0.01*

*Information: there was significant different between groups analyzed with Tukey HSD at $p < 0.05$.

comparison to the control group (K). This was caused by anthocyanin, which is present in RFE and can lower the production of various cytokines and pro-inflammatory mediators, as well as the activation of the transcription factor NF-κB.[12] An anthocyanin flavonoid compound in RFE has been shown in certain studies to have antioxidant properties and to suppress NF-κB activation due to its phenolic content. By preventing the breakdown of Iκβ and the activation of the Iκβ kinase, which stops NF-κB from being phosphorylated and inhibits translocation into the nucleus, anthocyanins can block the activation of NF-κB.[15]

When cells experience stress, including mechanical stress from orthodontic forces, they release HSP, which is an immediate reaction. In response to compression from orthodontic mechanical forces, fibroblast cells in the periodontal ligament release histone-sensitive protein as a self-defense mechanism and maintain cell homeostasis.[8] HSP-70 on the compression side, which has been linked to inflammatory activity, and HSP-10 on the tension side, which has been linked to anti-inflammatory activity, are the two HSPs that exhibit the most prominent expressions.[7–9] The study's findings demonstrated that from days 1–7, HSP-70 expression was higher in the K group than in the KP group, with significant differences. The release of HSP-70 coincided with the acute phase of the inflammatory reaction that followed the application of the orthodontic mechanical compression.[7] As a result, the expression of HSP-70 in the control group (K) gradually decreased after day 7. The anti-inflammatory properties of RFE, namely quercetin, reduced HSP-70 expression in the treatment group (KP) from days 1–14. The findings of this investigation align with hypotheses derived from earlier *in silico* experiments indicating quercetin's potential involvement in suppressing HSP-70 expression.[13] Further investigation revealed that from days 7–14, HSP-10 expression steadily increased in the treatment-free group (K). This was because HSP-10 is a molecule that starts an ongoing immune response against inflammation. Moreover, the higher HSP-10 expression demonstrated that it functioned as a direct antagonist of the control group's HSP-70 expression.[22] Administering RFE to the KP group had the effect of increasing HSP-10 expression on day 7, with a substantial reduction on days 14–21. The findings of this investigation support the hypothesis that quercetin contributes to elevated HSP-10 expression, which was predicted by earlier *in silico* experiments.[13,23]

Overall, merits the study's findings indicated a rise in the biomarkers for bone formation, osteocalcin, and ALP; a fall in pro-inflammatory variables, including NF-kB, TNF-α, and HSP-70; and a rise in HSP-10, an antagonist of HSP-70. Roselle (*H. sabdariffa*) daily consumption may benefit for periodontal tissue during light force OTM by regulating inflammatory and bone apposition biomolecular markers due to possess active compound such as quercetin and anthocyanin.[12,13] Quercetin and the anthocyanins found in roselle (*H. sabdariffa*) may have an impact on bone development. Prior research demonstrated that quercetin increases ALP activity in human osteoblasts within the 1–50 mM range without appreciably harming the cells.[24] The majority of anthocyanins that can promote osteogenesis also enhance the control of gene expression in at least one of these transcription factors, as well as in osteoblastic markers, including ALP, type 1a collagen, osteopontin, and osteocalcin.[25,26] Nevertheless, this study's demerits were not investigated in the OTM rates after RFE local administration, and exact molecular pathway or the mRNA fold changes of the inflammatory response and bone apposition after RFE local administration in the alveolar bone during OTM *in vivo*. In addition, this study only examined several molecular markers of inflammatory response and bone apposition with the immunohistochemical method.

5. Conclusion

The aforementioned present study result revealed that.

- Local administration of RFE decreased NFκβ, TNF-α, HSP-70 expressions on the compression side of the AB in the Day 1–21 eventually with significant different between groups during light force OTM application in Wistar rats (*R. novergicus*).
- RFE local administration enhanced HSP-10 and ALP expression on the tension side of the AB enhanced significantly between groups in the Day 1–7 during light force OTM application in Wistar rats (*R. novergicus*).
- Osteocalcin expression stimulated in Day 21 significant differences between the groups after RFE local administration during light force OTM application in Wistar rats (*R. novergicus*).

Patient's/Guardian's consent

This study conducted in animal model (*in vivo*) study. Thus, Patient's/Guardian's consent is unnecessary.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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