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Biochemical Effects of Quercetin on MMP-2, BAP and TRAP 5b During Orthodontic Retention Period

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ABSTRACT

The objectives of this study were to evaluate clinically and biochemichally Quercetin administration on orthodontic relapse after orthodontic retention period. So as to, present a supplementary strategy based on dietary recourses that may help to maintain orthodontic retention results and reduce orthodontic relapse tendency. Thirty apparently healthy female albino rabbits, were used in this study. The animals were grouped randomly, into 6 groups of 5 animals in each (2 control groups and 4 Quercetin groups). A modified fixed orthodontic appliances were fixed to rabbit lower central incisors and exerted a reciprocal lateral force of 40±2 gm. Each rabbit received orthodontic intervention for one week followed by six weeks retention period. The relative amount of tooth movement were taken at time of orthodontic appliance insertion, end of orthodontic retention period and at the end of four weeks after retention. At the end of the experiments, the blood samples were collected for serum samples. Rabbit Matrix metalloproteinase-2, Rabbit Bone Specific Alkaline Phosphatase and Rabbit Tartrate Resistance Acid Phosphatase 5b ELISA kits were used for biochemical assessments. Data analyses were performed at level of *P<0.05* for the statistically significant difference. Biochemically, the lowest Bone Specific Alkaline Phosphatase value was for C4 group. The highest Tartrate Resistance Acid Phosphatase 5b value was found in Quercetin H0 group, whereas the lowest Tartrate Resistance Acid Phosphatase 5b value was found in Quercetin L0 group. Supplementation with quercetin during orthodontic retention period, reduced orthodontic relapse by biochemically increasing Matrix metalloproteinase-2, increased Bone Specific Alkaline Phosphatase and reduced Tartrate Resistance Acid Phosphatase 5b activities after orthodontic retention period.

Key words: Bone specific alkaline phosphatase, Matrix metalloproteinases,Orthodontic relapse,Orthodontic retention,Quercetin, Tartrate Resistant Acid Phosphatase 5b.

INTRODUCTION

One of the most challenging problems facing the orthodontists and orthodontic patients is orthodonticrelapse. The stability of the outcome yielded from an orthodontic treatment (OT) is always vital issue. After OT, there are both a retention phase and a post retention phase of OT therapy. Orthodontic relapse is multifactorial problem [(1)]. However, various methods were attempted to decrease orthodontic relapse [(2)]. Retention is the phase of which maintains the teeth in their orthodontically corrected positions following the cessation of active orthodontic tooth movement for an extended period to help stabilize the corrections achieved [(1)].

Biomarker is a substance that measured and evaluated independently as an indicator of either biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention[(3)]. Matrix Metalloproteinase-2 (MMP-2), also known as collagenase type IV-A; CLG4A collagenase type IV, 72-KD; gelatinase, 72-KD; gelatinase A; gelatinase neutrophil [(4)]. Matrix metalloproteinase-2 is a member of calcium-dependent zinc-containing endopeptidases which belong to a larger family of proteases (the metzincin superfamily) [(5)]. Matrix metalloproteinase-2 , due to its unique properties in that it functions as both gelatinase and collagenase[(4)], play an important role in the degradation of extracellular matrix, a process that takes place during processes involved connective tissue degradation such as that accompanied histological reactions in periodontal ligament[(6)] during the course of OT by hydrolyzing denatured collagens (gelatin), native types IV,V and XI collagens, and elastin, acting at regulation of vascularization, the inflammatory response and basement membrane breakdown [(7)].

A demanding issue in orthodontics, is the determination of appropriate orthodontic tooth movement process biomarkers.

Ariffin *et al.*[8] proposed a potential marker for each phase during the course of orthodontic tooth movement, that is bone specific alkaline phosphatase (BAP) (bone formation) and tartrate-resistant acid phosphatase 5 TRAP5 (bone resorption). Alkaline phosphatase is the most frequently used biochemical marker of osteoblastic bone formation [9].Bone specific alkaline phosphatase was chosen as potential biomarker for bone formation during the first three months of OT Asma *et al.*[10], throughout OT [11], and as soon as the tooth movement stops [12]. According to Haima [13], BAP was demonstrated to be one of the most attractive bone turnover biomarker to date in comparison to other bone formation biomarkers. Other cause for choosing BAP is the fact that in both tissues, bone and calcifying cartilage, BAP is expressed early in development. Later in the developmental program, BAP expression declines [14].

Other potential biochemical marker that may be useful during osteoclastic activity to describe the number and activity of osteoclasts and to monitor bone resorption, is Serum TRAP 5b which is derived exclusively from bone-resorbing osteoclasts. TRAP 5b appears to be a highly specific and sensitive marker of bone resorption [15]. The biological and analytical variability of TRAP 5b was lower than those of the other markers, making TRAP 5b more reliable and allowing a smaller decrease to be significant. The clinical performance of TRAP 5b for treatment monitoring is among the best of known bone turnover markers [16].

Recent advances have been made in the scientific understanding of how diet and specific foods within a balanced diet promote human health and prevent illnesses. A natural product is, a secondary metabolite, small molecule produced by an organism (plant or animal) but that is not strictly essential for the survival of the organism [17]. One of natural products is Quercetin (Qu) (3, 3', 4', 5, 7-pentahydroxyflavone) which belongs to the flavonoids and is present ubiquitously in fruits and vegetables especially in onion, broccoli, apple and tea [18]. Due to their high affinity for binding to estrogen receptors (ERs: ER-α and ER-β), flavnoids, are also known as phytoestrogens [19]. Quercetin is mainly present as its glycosylated forms such as quercitrin (3-rhamnosylquercetin) also named rutin, or aglycone (sugarless form of rutin) which is named rutoside (3-rhamnosy-glucosyl quercetin) [20].

Quercetin has been discussed for several decades as a multipotent bioflavonoid showing different activities [21]. The vast majority of studies emphasizing positive roles of Qu on bone. Quercetin is believed to inhibit bone loss by regulating many systemic and local factors including hormones and cytokines[22]. Quercetin enhances osteoblastogenesis[23] and it inhibits osteoclastic differentiation [24].

To the best of our knowledge, there has been no even single clinical or pre-clinical study that has previously evaluated the impact of Qu during orthodontic retention period.

The main objective of the current study was to investigate Qu biological effect during orthodontic retention period in the hope to search for natural sources to enhance alveolar bone (AB) remodeling and mineralization during this period in order to shorten this period and subsequently overall OT time.

Hypotheses:

1. There is a difference between clinical orthodontic relapse of the control groups on one side and that of Qu groups on the other side.
2. Biochemically there is a difference between MMP-2 enzyme, BAP, and TRAP 5b of the control groups on one side and that of Qu groups on the other side.

Other aims were to evaluate clinically the effects of Qu administration on teeth relapse after orthodontic retention period and specify biochemically the influence of Qu on MMP-2 enzyme, BAP, and TRAP 5b activity after orthodontic retention period.

EXPERIMENTAL

The Sample: The ethical approval was obtained from the university authorities (No.351 in 27.1.2013). Thirty apparently healthy female albino rabbits, weighting more than 1000 gm each were used in this study. The rabbits were kept in metallic cages in a well-ventilated room in the animal house of the College of Dentistry, University of Mosul. They were kept in the animal house for about two wks for acclimatization before their use in the experiments. Diet adjustment for the total sample to exclude the possible effects of food's type on the rate of tooth movement. Water was available for the animals at any time. The animals were grouped randomly, into 6 groups of 5 animals in each. Animal were grouped as follows**:** 1- C0 group (the relapse was estimated at zero day after orthodontic retention period), 2- C4 group (the relapse was estimated at end of fourth week after orthodontic retention period), 3- Qu L0 group (Qu was given in 231.5 mg/Kg b.w./day, the relapse was estimated at zero day after orthodontic retention period), 4- Qu L4 group (231.5 mg/Kg/ b.w. day, the relapse was estimated at end of fourth week after retention period), 5-Qu H0 group (463 mg/Kg b.w./day, the relapse was estimated at zero day after retention period) and 6- Qu H4 (463 mg/Kg b.w./day, the relapse was estimated at end of fourth week after retention period).

Quercetin Administration: Quercetin,2-(4,4-Dihydroxyphenyl)-3,5,7-trihydro- xy- 4H-1- benzobyran 4-one,3, 3'4'5,6-pentahydroxyflavone, Quercetin -3-O-rhamnoside. HPLC≥95%, (Sigma Aldrich, USA) was administered to the Qu groups via oral route through mixing to animal diet, and all the doses mentioned above were scheduled based on study of Harwood et *al*. [25] and according to guidelines set by the U.S. Food and Drug Administration [26] and dose conversion method of Shin*et al.*[27].

Orthodontic Intervention: A modified fixed orthodontic appliances were fixed to rabbit lower central incisors and exerted a reciprocal lateral force of 40±2 gm. Each rabbit received orthodontic intervention for one week followed by six weeks retention period.

Clinical Assessments: The relative amount of tooth movement was measured directly using a digital vernier caliper. The measurements were taken at time of orthodontic appliance insertion, end of orthodontic retention period and at the end of four weeks after retention. At the end of the experiments, the rabbits were sacrificed and blood samples were collected for serum samples.

Biochemical Assessments: Rabbit MMP-2 kit was a 1.5 hr solid-phase ELISA which applied the competitive enzyme immunoassay technique utilizing a highly specific monoclonal anti-MMP2 antibody and MMP2-HRP (Horse Raddish Peroxidase) conjugate for quantitative determination of rabbit MMP-2. To reveal the osteoblastic activity, the Rabbit BAP ELISA kit (MyBioSources Company, USA), which applies the competitive enzyme immunoassay technique utilizing a highly specific monoclonal anti-ALPL antibody and ALPL-HRP conjugate was used. To reveal osteoclastic activity the Rabbit TRAP5b ELISA kit (MyBioSources Company, USA) ,which applies the sandwich enzyme immunoassay technique utilizing a highly specific monoclonal anti-TRAP5b antibody and TRAP5b-HRP conjugate was used.

Data analyses: were performed using the Statistical Package for Social Sciences Software(SPSS) for Windows (19.0) (SPSS Incorporated, Chicago, IL) .

- Inter and Intra-examiner calibration were done at level of $P<0.05$ using paired sample t-test.
- The data were checked for their normal distribution.
- Descriptive statistics to show mean, standard deviation for each variable.
- Non-parametric statistical approach were used. Median, interquartile range Mann-Whitney test was performed to analyze the differences among the study groups concerning clinical, biochemical parameters.
- P-Value of less than 0.05 was considered a statistically significant difference.

RESULTS

Clinical Findings: Amount of orthodontic relapse was checked according to time scheduled for each group. Measurement was done at the level of upper mesial wing of bracket of lower central incisor bands. Relapse tendency illustrated significant difference among groups (Tables 1&2). A significant difference was found between C0 and C4, Qu H0 and Qu H4, Qu L0 and Qu L4, C0 and Qu L0 and C0 and Qu H0 groups from comparisons at $p< 0.05$.

Biochemical Findings: Table 3 shows the mean values for MMP2, BAP and TRAP5b with various mean values among different groups.

Matrix Metalloproteinase-2: The result of this study showed no significant difference in majority of the values for group comparisons in statistical analysis (Table 4).

Table 1: Mean and standard deviation for the relapse of the study groups.

Group	N	Mean	Standard deviation
C0	5	0.00	0.00
C4	5	2.11	0.59

Qu L0	5	0.00	0.00
Qu L4	5	1.95	0.12
Qu H0	5	0.00	0.00
Qu H4	5	2.06	0.01

C 0: control zero time, C 4 : control 4 wks, Qu H0 : quercetin high dose zero time, Qu H4 : quercetin high dose 4 wks, Qu L0 : quercetin low dose zero time, Qu L4 : quercetin low dose 4 wks. N=number. Variable unit for relapse is in mm.

Table 2: Comparison of the relapse findings for the study groups.

Groups	Median	Interquartile Range	Mann Whitneytest		Significance
			Cal.Z	*P*-value	
C 0	0.00	0.00	2.79	0.005	S
C 4	2.00	1.49			
Qu L0	0.00	0.00	2.79	0.005	S
Qu L4	1.90	0.60			
Qu H0	0.00	0.00	2.79	0.005	S
Qu H4	2.09	0.21			
C 0	0.00	0.00	0.01	0.001	S
Qu L0	0.00	0.00			
C 0	0.00	0.00	0.01	0.001	S
Qu H0	0.00	0.00			
C 4	2.00	1.49	0.31	0.754	NS
Qu L4	1.90	0.60			
C 4	2.00	1.49	0.31	0.754	NS
Qu H4	2.09	0.21			

C 0: control zero time, C 4 : control 4 wks, Qu H0 : quercetin high dose zero time, Qu H4 : quercetin high dose 4 wks, Qu L0 : quercetin low dose zero time, Qu L4 : quercetin low dose 4 wks. Cal.Z is Calculated Z. ; Significant level is at (p<0.05), NS is non-significant ,S is significant.; Variable unit for relapse is in mm.

Table 3: Mean and standard deviation for the biochemical findings of the study groups.

up	Variable	N	Mean	Standard deviation
C0	MMP-2	5	241.80	79.49
	BAP	5	0.40	0.11
	TRAP5b	5	1073.30	273.54
C4	MMP-2	5	475.98	132.57
	BAP	5	0.34	0.09
	TRAP5b	5	819.98	159.02
Qu L0	MMP-2	5	0.25	44.74

	BAP	5	111.20	0.06
	TRAP5b	5	0.35	55.83
Qu L4	MMP-2	5	214.10	69.25
	BAP	5	0.28	0.12
	TRAP5b	5	154.85	95.75
Qu H0	MMP-2	5	158.88	49.73
	BAP	5	0.34	0.11
	TRAP5b	5	1405.61	195.42
Qu H4	MMP-2	5	212.10	106.98
	BAP	5	0.35	0.15
	TRAP5b	5	741.85	145.87

C 0: control zero time, C 4 : control 4 wks, Qu H0 : quercetin high dose zero time, Qu H4 : quercetin high dose 4 wks, Qu L0 : quercetin low dose zero time, Qu L4 : quercetin low dose 4 wks.; N=number. Variable unit for MMP2=pg/mL ; BAP= ng/mL;TRAP5b= µU/ml.

Bone Specific Alkaline Phosphatase: For BAP, no significant difference was found between values for the various groups (Table 5).

Tartrate Resistance Acid Phosphatase 5b: Significant difference was found for most of the values in group comparison regarding TRAP5b, except for Qu H4 group (p=0.028) and Qu L4 group (P=0.009) (Table 6).

Table 4: Comparison of the Matrix Metalloproteinase-2 findings for the study groups.

Groups	**Median**	**Interquartile Range**	**Mann Whitneytest**		**Significance**
			Cal.Z	***P*- value**	
C 0	293.78	337.12	1.15	0.251	NS
C 4	373.30	457.77			
Qu L0	169.92	186.26	1.57	0.117	NS
Qu L4	354.09	287.98			
Qu H0	214.88	207.98	0.31	0.754	NS
Qu H4	121.45	407.35			
C 0	293.78	337.12	0.10	0.917	NS
Qu L0	169.92	186.26			
C 0	293.78	337.12	0.94	0.347	NS
Qu H0	214.88	207.98			
C 4	373.30	457.77	0.52	0.602	NS
Qu L4	354.09	287.98			
C 4	373.30	457.77	1.57	0.117	NS
Qu H4	121.45	407.35			

C 0: control zero time, C 4 : control 4 wks, Qu H0 : quercetin high dose zero time, Qu H4 : quercetin high dose 4 wks, Qu L0 : quercetin low dose zero time, Qu L4 : quercetin low dose 4 wks. Cal.Z is Calculated Z; NS is non significant; Variable unit for MMP2=pg/mL**.**

DISCUSSION

One of the most commonly encountered problem in orthodontics is relapse after accomplishment of orthodontic retention period. Many authors tried and suggested different methods to limit or solve this problem [2,28]. So, the search for supplementary aid(s) that might influence AB remodeling during the orthodontic retention period, and subsequently overall OT time, is thus of paramount importance.

Among various comparisons done, a major significant difference was found between C0 and Qu L0 and between C0 and Qu H0 groups. It stands to reason that the significant lower relapse tendency of Qu groups in comparison to control groups may be due to the nutritional factors represented by Qu may be especially important in the reduction of relapse tendency after completion of orthodontic retention period possibly by affecting AB remodeling and mineralization. This result is supported by previous studies about Qu effects on bone health [23,29, 30].

Table 5: Comparison of the Bone Specific Alkaline Phosphatase findings for the study groups.

Groups	Median	Interquartile Range	Mann Whitneytest	Significance	
			Cal.Z	*P*-value	
C 0	0.46	0.45	0.31	0.754	NS
C 4	0.27	0.39			
Qu L0	0.53	0.29	0.31	0.754	NS
Qu L4	0.50	0.43			
Qu H0	0.32	0.48	0.31	0.754	NS
Qu H4	0.27	0.46			
C 0	0.46	0.45	0.94	0.347	NS
Qu L0	0.53	0.29			
C 0	0.46	0.45	0.31	0.754	NS
Qu H0	0.32	0.48			
C 4	0.27	0.39	1.15	0.251	NS
Qu L4	0.50	0.43			
C 4	0.27	0.39	0.31	0.754	NS
Qu H4	0.27	0.46			

C 0: control zero time, C 4 : control 4 wks, Qu H0 : quercetin high dose zero time, Qu H4 : quercetin high dose 4 wks, Qu L0 : quercetin low dose zero time, Qu L4 : quercetin low dose 4 wks. Cal.Z is Calculated Z.; Significant level is at (p<0.05); NS is non significant ; Variable unit for BAP is in ng/mL.

In the present study, MMP-2, BAP and TRAP 5b are selected as biomarkers. In investigating biomarkers, the rate, amount, and activity of the released substances not only reflect the activity of individual cells but also indicate the metabolic activity in the involved tissues or organs [31].

Regarding MMP-2, the present study was conducted in the aim to search for a natural inhibitor of MMP-2 activity during orthodontic retention period. So, in this study, the rate and amount of inhibitory effect of Qu on MMP-2 activity were determined using serum Rabbit MMP-2 ELISA detection kit.

In QuL0 and QuL4 groups, the MMP-2 values were non-significantly lower than control group values. The possible explanation to this non-significant difference could be that Qu need to be administered for longer period beyond retention period to gain its beneficial role, or may be due to the process of conjugation of Qu, during its metabolism, prior to passage into the blood stream. Quercetin undergo structural modifications due to the conjugation process that takes place in the small intestine and, mostly,

Table 6: Comparison of the Tartrate Resistance Acid Phosphatase 5b findings for the study groups.

Groups	Median	Interquartile Range	Mann Whitneytest		Significance
			Cal.Z	*P*- value	
C 0	1153.44	1151.72	0.73	0.465	NS
C 4	824.70	614.92			
Qu L0	282.18	240.79	2.61	0.009	S
Qu L4	745.79	394.48			
Qu H0	1387.69	719.27	2.19	0.028	S
Qu H4	603.51	620.83			
C 0	1153.44	1151.72	1.98	0.047	NS
Qu L0	282.18	240.79			
C 0	1153.44	1151.72	0.94	0.347	NS
Qu H0	1387.69	719.27			
C 4	824.70	614.92	0.94	0.347	NS
Qu L4	745.79	394.48			
C 4	824.70	614.92	0.52	0.602	NS
Qu H4	603.51	620.83			

C 0: control zero time, C 4 : control 4 wks, Qu H0 : quercetin high dose zero time, Qu H4 : quercetin high dose 4 wks, Qu L0 : quercetin low dose zero time, Qu L4 : quercetin low dose 4 wks.
Cal.Z is Calculated Z..; Significant level is at (p<0.05); NS is non-significant ;S is significant.; Variable unit for TRAP5b is in µU/ml.

in the liver. The conjugation represents a metabolic detoxication process common to Qu. Although it produces active metabolites from Qu, it reduces the total amount of Qu in the blood stream, increasing its excretion [32]. These modifications deeply affect the bioavailability and the biological activity of Qu.

Concerning BAP, to determine the effect of Qu or Ol on serum BAP, a well-known biomarker of osteoblastic differentiation activity and to assess whether Qu can promote the differentiation of osteoblasts, a serum Rabbit BAP ELISA detection kit was used.

Concerning the effects of Qu on serum BAP, the results showed non-significantly higher value in comparison to control groups, this gave clue to beneficial role of Qu, although no significant, on osteoblast differentiation and function. This was supported by the findings of previous studies concerning the role of Qu on bone tissue, as in studies of Trivedi *et al*.[24] and Siddiqui *et al*.[20] who found that flavonoids including qu may be associated with increases in bone mineral density and have been reported to counteract the bone deleterious effects and to inhibit ovariectomy -induced osteopenia and inhibits bone loss.
The current result also agreed with that of Yin*et al*.[33]; Trivedi *et al*.[24] and Sharan *et al*.[19] who indicated the capability of flavonols, as Qu, to promote osteoblast proliferation, differentiation and mineralization as well as increasing production of osteoprogenitors.

Veerawattanatigul *et al*.[34] agreed with our findings, they found that Qu increased the growth response of sphenooccipital synchondrosis through significant increasing the expressions of transcription factor Sox9(acting during chondrocyte differentiation) and type II collagen (stimulating osteoblasts). Also, Wangwarunyoo *et al*.[35] supported results of the present study ,they found that 1 µM of Qu increased significantly the expressions of bone morphogentic proteins(BMP2)(which is type of cytokines have the ability to induce the formation of bone and cartilage) and PTHrP(Parathyroid hormone-related protein (or PTHrP) is a protein member of the parathyroid hormone family that regulates endochondral bone development) during growth in spheno-occipital synchondroses in a mouse model *in vitro*. Moreover, Siddiqui *et al*.[23] anchored our result. Their findings suggested a significant bone anabolic effect of Qu C-glucoside (extracts of *Ulmus wallichiana*) on osteoblast function and bone formationin osteopenic rats.

On the other hand, several studies that tested the combined effect of Qu with other bioflavnoids on bone tissue, in

addition, agreed with the findings of the present study. Quercetin with other flavnoids restored bone mass in aged ovariectomized rats [36], showed more potent ER agonist activity by activating ER-mediated activator protein 1 (AP-1)(which is a transcription factor that controls a number of cellular processes including differentiation, proliferation, and apoptosis) reporter expression [37] and up regulated the osteoblast differentiation in dose dependent manner by increasing BAP , osteopontin (which is an extracellular structural protein and therefore an organic component of bone), osterix, RunX2 (both are transcription factors that required for the expression of osteopontin), Osteoprotegerin (OPG)(which is a cytokine receptor, and a member of the tumor necrosis factor receptor superfamily) and osteocalcin (that is a noncollagenous protein found in bone and dentin and is secreted solely by osteoblasts and thought to play a role in the body's metabolic regulation and is pro-osteoblastic, or bone-building, by nature) as compared to control [29].

However, only QuH0 group in our study showed reduced BAP activity, although non-significantly, in comparison to control group. This result came in accordance to Son*et al*.[22] who revealed that Qu accelerates tumor necrosis factor alpha (TNF-α)(which is a cell signaling protein cytokine involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction)-mediated apoptosis of MC3T3-E1 osteoblastic cells(which is an osteoblast precursor cell line derived from Mus musculus (mouse) calvaria) through both the mitochondrial-mediated and caspases(which are a family of protease enzymes playing essential roles in programmed cell death (including apoptosis, pyroptosis and necroptosis) and inflammation.)-dependent mechanisms. Quercetin also augmented the TNF-α-mediated apoptosis by activating c-Jun N-terminal kinase (JNK) with the attendant activation of AP-1, where the nuclear translocation of c-Jun protein appeared to be a critical event responsible for the accelerating action of Qu. Thus Qu reduced osteoblastic function.

Despite the fact that the above mentioned studies about the effect of Qu on bone tissue agreed with the results of our study, however those studies used diverse Qu concentrations and combinations, various *in vitro* and *in vivo* experimental models or even human subjects, different natural product concentrations and administration routes, different measuring techniques for Qu activities, various bone formation biomarkers and different observational periods in comparison to the present study.

For serum TRAP 5b values, in the present study, no significant difference was found for most of the values in group comparison regarding TRAP 5b. The use of TRAP as bone resorption biomarker was in concordance to Abdul Wahab *et al*. [11] ;Abu Kasim *et al*.[38] and Abdul Wahab *et al*.[39] observation of TRAP activity accompanied with OT. Although those studies utilized salivary or gingival cervicular fluid TRAP rather than serum TRAP as a bone resorption biomarker, used various experimental models or even human subjects, used various orthodontic appliances with varying applied forces for different observational periods in comparison to the present study.

However, the activity of osteoclasts differentiation maker, TRAP 5b, significantly decreased in Qu L0 group at the concentration of 7.7 mg/kg b.w. daily, suggesting a line of evidence that Qu, the most widespread naturally occurring flavnoids regularly consumed by humans, is a potent *in vivo* inhibitor of osteoclast differentiation. This was in agreement to Trivedi *et al*.[24] who observed that Qu with other flavnoids inhibit osteoclast promoting cytokine production from osteoblasts. Tsuji *et al*.[40] anchored our result. They demonstrated that dietary Qu dose-dependently inhibited receptor activator of nuclear factor-$_{\beta}$ ligand (RANKL)(which is a member of the tumor necrosis factor (TNF) superfamily and has been identified to affect the immune system and control bone regeneration and remodeling.)-induced osteoclast differentiation and the RANKL-stimulated expression of osteoclast related genes in ovariectomized-female mice. Furthermore the result of effect of Qu on bone tissue in this study was in agreement with Napimoga *et al*.[30] who demonstrated that Qu reduced periodontitis-induced bone loss and Interleukin-1 beta (IL-1β)(which is a cytokine, that plays a central role in the regulation of immune and inflammatory responses to infections or sterile insults), TNF-α, IL-17, and RANKL production in the gingival tissue.

Whilst the significantly highest TRAP 5b value is present in Qu H0 group, indicating increase in osteoclast differentiation, a possible interpretation to this high value could be that the Qu in concentration of 15.4mg/kg bw daily may be too high and caused unfavorable outcomes, or indicating early stages of AB turnover ([41].

In spite of the fact that the above mentioned studies about the effect of Qu on bone tissue agreed with the results of our study, however those studies used diverse Qu concentrations and combinations, various *in vitro* and *in vivo* experimental models or even human subjects, different Qu concentrations and administration routes, different measuring techniques for Qu activities, various bone resorption biomarkers and different observational periods in comparison to the present study.

Collectively, one suggestive cause behind the obtained results in the present study may be due to anti-inflammatory [42 ,43] behavior of Qu. Both *in vitro* and *in vivo* studies suggest that Qu ameliorates inflammation by inhibition of the secretion of different inflammatory mediators [42]. In addition, Qu also inhibits the enzymes like cyclooxygenase (COX) and Lipooxygenase which catalyses the conversion of arachidonic acid to its metabolites [44]. Cyclooxygenase-2 (COX-2) has been reported to up-regulate MMP-2 [45] which is a vital enzyme degrading extracellular matrix proteins and also thought to induce bone cell proliferation, differentiation and migration[46].

The antioxidant [43] behavior of Qu is other main reason responsible for effects of Qu in the current study. Quercetin is thought to play an important role in protecting cells from oxidative stress and DNA damage induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) [47]. A biological role has been suggested for osteoclasts which have ROS-generating activity. This ROS generated by TRAP 5b have been suggested to participate in finalizing degradation of bone matrix components in transcytotic vesicles of osteoclasts [48].

Quercetin causes scavenging of free radicals; resulting in less cellular damage. Free radicals can activate transcription factors that generate pro-inflammatory cytokines [42]. Quercetin also modules nitrous oxide (NO) production [43]. Nitrous oxide is known to be a potent stimulator of osteoclasts differentiation through its activation of RANKL [49].

Quercetin significantly inhibited transcription factors production and gene expression in a dose-dependent manner resulting in the stimulation of anti-inflammatory cytokines via inhibiting the activation of receptor activator of nuclear factor beta (NF-κβ) [43]. Napimoga *et al.*[30] demonstrated that Qu reduced periodontitis-induced bone loss, inflammatory cytokines, and RANKL production in the gingival tissue.

CONCLUSIONS

Supplementation with Qu during orthodontic retention period, reduced orthodontic relapse tendency clinically after four wks following orthodontic retention period. Supplementation with Qu especially Qu H0 increased MMP-2, increased BAP and reduced TRAP 5b activities following orthodontic retention period. Supplementation with Qu especially Qu L0 increased ALP, reduced MMP-2 and reduced TRAP 5b activities following orthodontic retention period.

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