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Research Article

Comparison of matrix metalloproteinase levels in periodontal pockets treated with chlorhexidine and propolis

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Abstract: Natural antimicrobial products are attracting interest in the treatment of periodontal diseases due to their minimal or nonexistent side effects. Propolis, a complex resin content produced by bees, has drawn particular interest for its antibacterial effects, including inhibitory effects on periodontopathogenic bacterial species. This study compared the effects of propolis, a natural product, and chlorhexidine, the gold standard antimicrobial agent, on MMP-1 and MMP-9 levels in gingival crevicular fluid (GCF). Subgingival irrigations of chlorhexidine and propolis solutions were performed in selected deep periodontal pockets of periodontitis patients along with scaling and root planing. The treatment protocol was administered three times at one-month intervals starting from the initial day of the study. GCF MMP-1 and MMP-9 levels, along with clinical periodontal parameters, were evaluated before and after treatment. The study results showed that a statistically significant decrease in clinical periodontal parameters in both groups (p<0.001). Biochemical analysis revealed a statistically significant decrease in levels of GCF MMP-1 and MMP-9 in both groups (p<0.005). Additionally, the decrease in GCF MMP-1 levels was found to be significantly greater in the propolis group compared to the chlorhexidine group (p<0.001).

1. INTRODUCTION

Periodontitis is a bacterial infectious disease that may lead to destruction of gingival fibers, destruction of the alveolar bone, and, if untreated, eventual tooth loss. The condition arises from an inflammatory response triggered by enzymes produced by bacteria that accumulate in the dental and gingival sulcus, subsequently spreading to the surrounding tissues that support the tooth (Teles *et al*., 2018). Clinical attachment loss, radiographic alveolar bone loss, the presence of periodontal pockets and bleeding on probing are some of the key diagnostic indicators of of periodontitis (Papapanou *et al*., 2018). These conditions are caused by periodontopathogenic bacteria within the periodontal pocket and tissue-destructive enzymes secreted as part of the host's immune response (Vitkov *et al*., 2021). The subgingival plaque and calculus in the

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periodontal pocket are the most important local factors in the development and progression of periodontal disease. Therefore, the primary objective of non-surgical periodontal treatment is to eliminate microbial deposits and debris from the periodontal pocket and root surface (Yan *et al*., 2020). Scaling and root planing (SRP) to remove subgingival plaque and calculus is the gold standard for mechanical debridement of periodontal pockets (Shrivastava *et al*., 2021). However, SRP may not be effective in the removal of all periodontopathogenic bacteria in deep periodontal pockets (Nakao *et al.,* 2020). Antimicrobial agents can therefore be used in combination with mechanical treatment (van Winkelhoff *et al.,* 2000).

Chlorhexidine is the most effective broad-spectrum antimicrobial agent clinically used for oral hygiene maintenance and therapeutic chemical plaque control due to its substantivity and antibacterial properties (Jones, 1997; Sajjan *et al.,* 2016). Chlorhexidine is recognised as the gold standard because of its ability to bind to the dental pellicle and oral mucosa, thereby, enhances its anti-plaque effect (Jenkins *et al.,* 1988). It also has anti-gingivitis properties. Several clinical studies have shown that chlorhexidine inhibits the development of microbial plaque, calculus and gingivitis (Kolahi & Soolari, 2006; Paraskevas, 2005). Chlorhexidine is used for oral hygiene and professional prophylaxis and is available as a mouthwash, gel, tablet, varnish, chewing gum, toothpaste, spray, and sustained-release tablet (Paraskevas, 2005). Despite its many benefits, chlorhexidine has many reported side effects, including browning of tooth surfaces and restorations, altered taste, enlarged parotid glands, mucosal erosions, and allergic reactions (Addy, 1995; Mariotti&Rumpf, 1999). It is essential to investigate alternative approaches to avoid the potential adverse effects of chlorhexidine (Bush *et al.,* 2011). One of these alternative agents is propolis. Propolis is a product with a complex resin content produced by honeybees. The composition of propolis can be derived from a variety of plant sources. However, antibacterial activity has been reported for all types of propolis (Kujumgiev *et al.,* 1999; Khurshid *et al.,* 2017). Studies have reported that propolis has a strong inhibitory effect on periodontopathogenic bacterial species, some fungi, viruses, and protozoan species (Kosalec *et al.,* 2005; Yoshimasu *et al.,* 2018). The significant anti-inflammatory properties of propolis have made it a natural antimicrobial agent. In addition to its immunomodulatory and local anesthetic effects and its contribution to wound healing, propolis also reduces the prevalence of dental caries and pulpal inflammation (Rufatto *et al*., 2018; Eslami *et al.,* 2016). Studies have reported that the topical use of propolis contributes to the protection of periodontal tissue health (Sanghani *et al*., 2014; Nakao *et al*., 2020).

Matrix metalloproteinases (MMPs) are a group of enzymes responsible for degrading extracellular matrix proteins during organogenesis, growth, and normal tissue regeneration. There is a balance between MMP activity and their specific endogenous tissue inhibitors in maintaining physiological events in the organism. Shifting this balance towards MMP activity leads to the destruction of the matrix and the formation of pathological events. In healthy tissues, the synthesis and activity of matrix metalloproteinases (MMPs) are typically low. However, their levels are elevated in various pathological conditions, including inflammation, tumor formation, and metastasis, where they contribute to tissue destruction (Sekhon, 2010). In periodontitis, MMP levels are elevated due to gingival inflammation. Periodontal pathogens contribute to the imbalance between MMPs and MMP inhibitors. Studies have reported a significant correlation between MMP levels in saliva and gingival crevicular fluid (GCF) and clinical parameters of periodontal disease. Increased levels of GCF MMP-1, MMP-8, MMP-9, MMP-12, and MMP-13 have been observed in periodontitis, in particular (de Morais *et al.,* 2018; Séguier *et al.,* 2001; Romano *et al.,* 2019). Among these enzymes, MMP-1 and MMP-9, released by gingival fibroblasts and neutrophils during bacterial infection, cause the breakdown of collagen in periodontal tissue (Romano *et al.,* 2019).

Our study compared the effects of chlorhexidine, used as an antimicrobial and antiplaque agent, and natural propolis that is produced by bees through enzymatic processing of the substances collected from plants and trees with the enzymes secreted from the glands in their

heads, on GCF MMP-1 and MMP-9 levels in periodontal pockets. It was therefore investigated whether natural propolis, which does not have the side effects of chlorhexidine such as staining of oral tissues, could be an alternative for the protection and treatment of periodontal tissues.

2. MATERIAL and METHODS

2.1. Study Population and Ethical Approval

Our study was designed in accordance with the principles of the Declaration of Helsinki. Ethical approval was obtained from Muğla Sıtkı Koçman University Clinical Research Ethics Committee with decision number 12/VI dated 01.06.2023. Informed consent was obtained from all subjects included in the study. Clinical and radiographic examinations were performed at the Department of Periodontology, Faculty of Dentistry, Muğla Sıtkı Koçman University. The study sample consists of 14 patients diagnosed with periodontitis, including 7 female and 7 male patients. For each patient, two teeth were selected, with one tooth randomly assigned to the chlorhexidine group and the other to the propolis group. In total, 28 teeth from 14 patients were included in the study.

Patients with periodontitis were selected for this study based on the following criteria: 1) patients aged between 25 and 40 years; (2) systemically healthy patients confirmed by health checkup results; (3) patients who do not take any regular medication; (4) patients who had not used antibiotics in the previous three months; (5) patients who had not received any periodontal treatment in the prior six months; (6) non-smokers; and (7) patients who had at least two deep periodontal pockets of 5 mm or more. Patients with allergies to any of the study components were excluded.

Each patient's two randomly assigned periodontal pockets were divided into two treatment groups. Subgingival irrigation with two different bioactive agents was performed during periodontal treatment: 0.12% chlorhexidine gluconate subgingival irrigation (control group, n=14) and propolis extract subgingival irrigation (test group, n=14). Thus, from the same patient, one periodontal pocket was assigned to the control group and the other to the test group. In total, 28 periodontal pockets from 14 patients constituted the study groups.

2.2. Study Design

The study used a randomized, double-blind, controlled design. A randomisation programme was used to prevent bias in sample collection and analysis. A total of 28 samples were randomly assigned to one of two different treatment groups, the chlorhexidine group and the propolis group.

The study protocol included four visits for each patient over a three-month period. Clinical periodontal parameters were obtained at the first periodontal assessment. Gingival crevicular fluid (GCF) samples were collected from patients diagnosed with periodontitis using blinded fashion. Subgingival irrigation was then applied to the periodontal pockets of patients undergoing initial scaling and root planing (SRP). Patients who received two additional sessions of scaling and root planing (SRP) and subgingival irrigation at one-month intervals were invited for a control assessment at the third month of treatment. For post-treatment evaluation, clinical periodontal parameters and GCF samples were collected from selected teeth at this session.

2.3. Gingival Crevicular Fluid Sample Collection

Gingival crevicular fluid (GCF) samples were collected from an identified tooth site with a probing pocket depth (PPD) ≥ 5 mm in all participants. GCF samples were collected at baseline (month 0) and at the final follow-up visit (month 3). Before collecting the GCF samples, supragingival plaque was removed with a sterile scaler without touching the gingival margin. The tooth was isolated with cotton roll tampons and saliva absorbents and dried with sterile 2x2 gauze. Paper strips (Proflow Inc., Amityville, NY, USA) of standard size and absorbency (2 x 14 mm) were used for the collection of GCF. Special strips of paper were placed in the periodontal pocket and held in place for 30 seconds. Paper strips contaminated with saliva or

blood were discarded. The paper strips were then placed in sterile Eppendorf tubes containing 500 µL of PBS. Paper strips, samples were centrifuged at 10,000xg for 10 min (Hettich Mikro 200, Andreas Hettich Co., Tuttlingen, Germany). The supernatants obtained from the gingival crevicular fluid were isolated for analysis in the study. The samples were mixed thoroughly and stored at -80°C until the day of the study.

2.4. Subgingival Irrigation

For subgingival irrigation, the two deepest periodontal pockets of each participant were selected. After mechanical periodontal treatment, subgingival irrigation was performed with 0.12% chlorhexidine gluconate (Kloroben, Drogsan, Turkey) in the control group, and propolis extract solution in the test group. 2 mL Chlorhexidine and propolis doses were applied to the periodontal pockets by irrigation using 2 mL syringes. These procedures were performed to the periodontal pockets on three occasions at intervals of one month (month 0, month 1 and month 2).

2.5. Preparation of Propolis Extracts

Propolis can be stored in its raw form at a room temperature of less than 25°C. However, it is preferable to keep it refrigerated at 4°C to avoid mottling. The raw propolis was stored at +4°C until extraction. Propolis samples were first frozen using liquid nitrogen. They were then ground using a mill. Propolis extracts were prepared following the established protocols from previous literature, with modifications when necessary (Bankova *et al.*, 2021). 1 kg of the propolis powder sample was mixed with 4 kg of 99.5 % ethanol in a glass extraction vessel using a mechanical mixer for 21 days, and a 20 % extract of propolis was obtained. The ethanol extracts obtained were filtered through Whatman filter paper and combined. A low-pressure evaporator was then used to remove the ethyl alcohol in the combined solution. The 100% propolis extracts obtained were stored at -18^{o} C until use. To prepare the subgingival irrigation solution, 2 g of propolis extract was dissolved in 10 mL of 80% ethanol.

2.6. Clinical Parameters

Clinical and radiological examinations of all volunteers were performed. The periodontal examination included plaque index (PI), gingival index (GI), bleeding on probing (BOP), probing pocket depth (PPD), and clinical attachment level (CAL). Measurements were taken at six sites per tooth. During these measurements, a Williams periodontal probe (Hu-Friedy, Chicago, IL, USA) was used. All measurements were performed by a single examiner who was blinded to the type of treatment. The examiner was calibrated against an expert periodontist. Calibration was validated if 90% of the recordings could be reproduced within a 1 mm difference. Diagnosis of periodontal diseases was done according to the 2017 American Academy of Periodontology and European Federation of Periodontology classification (Papapanou *et al*., 2018). Participants were diagnosed with periodontitis based on the following criteria: interdental CAL is detectable at ≥2 non-adjacent teeth, or buccal or oral CAL ≥3 mm with pocketing >3 mm is detectable at ≥2 teeth (Tonetti *et al.*, 2018).

2.7. Biochemical Evaluation

The GCF concentrations of MMP-1 and MMP-9 were quantified by ELISA technique (BT-laboratory, Shanghai, China; Cat No: E0916Hu and E0936Hu, respectively) at Muğla Sıtkı Koçman University Faculty of Medicine Medical Biochemistry Research Laboratory based on the manufacturer's instructions. Enzymatic reactions were quantified in an automated microplate photometer (absorbance at 450 nm using Thermo Scientific Multiskan GO, Thermo Fisher Scientific, USA). Sample concentrations were calculated from the standard curve generated using calibrators for each antimicrobial peptide. The kit sensitivity was 0.05 ng/mL, and the assay range was 0.1 ng/mL-10 ng/mL. Upper and lower outliers were examined in duplicate.

2.8. Statistical Analysis

The statistical analyses of the study were carried out using the SPSS software package (IBM Corp. Chicago, 2020. IBM SPSS Statistics for Windows, version 27.0. Armonk, NY: IBM Corp) and Minitab 21.1 software. The Shapiro-Wilk test was used to determine whether the quantitative variables were appropriate for a normal distribution. Independent groups were compared using the Mann-Whitney U test for non-normally distributed variables, and an independent samples t-test was employed for the analysis of normally distributed quantitative variables. Paired samples t-tests were used to compare related samples that conformed to a normal distribution, while the Wilcoxon signed-rank test was used for non-normally distributed variables. The Chi-Square test was used to compare study groups based on gender. Mean ± standard deviation, median (minimum - maximum) were used for descriptive statistics. Descriptive statistics for qualitative variables were expressed as frequencies (%). $p<0.05$ values were considered statistically significant.

3. RESULTS

The gender distribution in the chlorhexidine and propolis groups was homogeneous, with each group comprising 7 males and 7 females. This distribution was statistically non-significant ($p > 0.999$). The mean age of the patients was 36.79±1.81 years. There was no statistical difference ($p>0.05$) between the two groups in terms of age or gender. Demographics are shown in Table 1.

Table 1. Descriptive statistics and comparative outcomes of age variables in men, women, and the sample.

	Total (n=14)	Male (n=7)	Female (n=7)	*p*-value
Age	36.79±1.81	35.86±1.57	37.71±1.60	0.052[t]

t: Independent samples *t*-test

Descriptive statistics are shown as mean±standard deviation.

Table 2. Assessment of clinical periodontal parameters and biochemical parameters in the groups.

Groups	Parameters	Baseline	Post-treatment	*p*-value
Chlorhexidine group (n=14)	MMP-1	26.90±1.11	22.73±1.04	<0.001[s]
	MMP-9	2.96±0.14	2.61±0.16	<0.001[s]
	PI	2.22±0.44	1.11±0.27	<0.001[s]
	GI	1.98±0.60	0.73±0.29	<0.001[s]
	BOP (%)	0.53±0.28	0.16±0.14	<0.001[s]
	PPD (mm)	4.98±0.87	4.44±0.78	<0.001[s]
	CAL (mm)	5.92±0.90	5.45±0.89	<0.001[s]
Propolis group (n=14)	MMP-1	27.34±1.39	21.05±1.51	<0.001[s]
	MMP-9	3.07 (2.60-3.32)	2.47 (2.39-2.97)	0.001[w]
	PI	2.21±0.31	1.17±0.29	<0.001[s]
	GI	2.05±0.34	0.67±0.25	<0.001[s]
	BOP (%)	0.50 (0.17-0.67)	0.17 (0.00-0.17)	0.001[w]
	PPD (mm)	4.75±0.51	4.07±0.48	<0.001[s]
	CAL (mm)	5.46±0.53	5.05±0.54	<0.001[s]

[s]: Paired samples t-test, [w]: Wilcoxon signed-rank test.

Descriptive statistics are shown as median (minimum-maximum) and mean±standard deviation. Statistically significant *p* values are given in bold.

Chlorhexidine group, periodontal pockets treated with subgingival irrigation of chlorhexidine; propolis group, periodontal pockets treated with subgingival irrigation of propolis.

Abbreviations: MMP-1, matrix metalloproteinase-1; MMP-9, matrix metalloproteinase-1; PI, plaque index; GI, gingival index; PPD, probing pocket depth; CAL, clinical attachment loss; BOP, bleeding on probing.

The improvement in all clinical periodontal parameters (PI, GI, BOP, PPD, CAL) following the treatment was statistically significant in both groups (p<0.001) (Table 2). However, no statistically significant difference was found between the chlorhexidine and propolis groups (p>0.05) (Table 3). The decrease in MMP-1 levels after treatment was found to be statistically significant for both groups (p<0.001) (Table 2). The decrease in MMP-1 levels was found to be statistically significantly higher in the propolis group compared to the control group (p<0.001) (Table 3). The decrease in MMP-9 levels after treatment was statistically significant in both groups (p<0.01) (Table 2), this decrease however showed no statistical difference in the comparison between groups (p=0.051) (Table 3).

Table 3. Comparison of differences between groups in clinical periodontal parameters and biochemical parameters before and after treatment.

Parameters	Chlorhexidine group (n=14)	Propolis group (n=14)	*p*-value
MMP-1	-4.07 (-5.77- -2.61)	-6.34 (-8.43- -3.54)	**<0.001**[m]
MMP-9	-0.35±0.13	-0.47±0.18	0.051[t]
PI	-1.12±0.43	-1.05±0.40	0.683[t]
GI	-1.25±0.39	-1.38±0.31	0.330[t]
BOP (%)	-0.34 (-0.67- -0.17)	-0.34 (-0.50- -0.17)	0.839[m]
PPD (mm)	-0.53±0.25	-0.67±0.27	0.150[t]
CAL (mm)	-0.34 (--1.00- -0.17)	-0.34 (-0.67- -0.17)	0.946[m]

[m]: Mann Whitney U test, [t]: Independent samples t-test.

Descriptive statistics are shown as median (minimum-maximum) and mean±standard deviation. The statistically significant *p*-value is given bold.

Chlorhexidine group, periodontal pockets treated with subgingival irrigation of chlorhexidine; propolis group, periodontal pockets treated with subgingival irrigation of propolis.

Abbreviations: MMP-1, matrix metalloproteinase-1; MMP-9, matrix metalloproteinase-1; PI, plaque index; GI, gingival index; PPD, probing pocket depth; CAL, clinical attachment loss; BOP, bleeding on probing.

4. DISCUSSION and CONCLUSION

The treatment of periodontitis includes scaling and root planning (SRP). Mechanical treatment is the gold standard to eliminate periodontal pockets and to remove microbial plaque and calculus. However, antimicrobial agents are used to eliminate subgingival microflora (Nakao *et al.*, 2020; Zarch *et al*., 2021). Chlorhexidine mouthwash is one of the prominent agents in periodontal therapy and is considered the gold standard due to its effective anti-plaque properties. However, chlorhexidine mouthwash alone is not sufficient to eliminate subgingival microflora. Subgingival irrigation with chlorhexidine, on the other hand, is effective for microflora in the periodontal pocket (Sajjan, 2016) but the use of chlorhexidine has potential side effects (Poppolo & Ouanounou, 2022). For this reason, researchers have become increasingly interested in alternative agents that target inflammation in the periodontal pocket. The fact that propolis is a natural product and is biologically active has made it one of these alternatives (Kujumgiev *et al.*, 1999).

In our study, subgingival irrigation of periodontal pockets with chlorhexidine and propolis in combination with mechanical periodontal treatment resulted in a significant improvement in clinical periodontal parameters (PI, GI, BOP, PPD and CAL) at three months in both groups. Sanghani *et al.* (2014) reported a greater improvement in GI, PPD and CAL values as a result of subgingival propolis application after SRP than in the control group. In a similarly designed study by Coutinho *et al.* (2012), periodontal pockets treated with subgingival propolis showed greater BOP improvement and PPD reduction compared to control groups. Nakao *et al.* (2020) investigated the effect of various bioactive agents on periodontopathogens in selected deep periodontal pockets and reported an improvement in BOP, CAL and PPD levels as a result of propolis ointment application. In their study of patients with periodontitis, Zarch *et al*. (2021) used chlorhexidine mouthwash and propolis subgingival irrigation in addition to mechanical

treatment. As a result, both treatments demonstrated improvements in clinical periodontal parameters, including Bleeding on Probing (BOP), Probing Pocket Depth (PPD), Clinical Attachment Level (CAL), and Gingival Index (GI). Seth *et al.* (2022), in their study designed similarly to ours, reported a significant decrease in GI, PI and PPD values in the first month of initial treatment with subgingival irrigation with chlorhexidine and propolis solutions as an adjunct to SRP treatment. Studies on the topical effects of chlorhexidine and propolis consistent with those observed in our study.

MMP-1 and MMP-9 are important biomarkers of periodontitis (Ertugrul *et al.*, 2013). Kubota *et al*. (2008) reported that MMP levels increased in gingival tissue samples taken during the transition from healthy periodontium to periodontitis. Romano *et al.* (2019) reported that GCF MMP-1 and MMP-9 levels significantly increased during the active and progressive stages of periodontitis. Therefore, these markers were selected to assess the effect of the antimicrobials used in our study on GCF. Biochemical findings of this current study revealed a reduction in MMP-1 and MMP-9 levels in both groups after treatment. Vitt *et al.* (2020) observed a decrease in GCF MMP-8 levels as a result of chlorhexidine irrigation of periodontal pockets after SRP. Azmak *et al.* (2002) reported that GCF MMP-8 levels decreased more in periodontal pockets treated with chlorhexidine chips after SRP than in periodontal pockets treated with SRP alone. Yuan *et al.* (2022) reported that the addition of chlorhexidine subgingival irrigation to SRP resulted in a greater reduction of inflammatory mediators, specifically IL-6 and MMP-8, in GCF compared to SRP alone. In a study conducted by Zarch *et al*. (2021), salivary MMP-8 levels were reported to decrease in both groups following treatment. Kale *et al.* (2022) found that propolis reduced MMP-2 and MMP-9 levels in their study on stem cells isolated from human exfoliated deciduous teeth. In another review study, the possibility of using caffeic acid phenethyl ester (CAPE) from propolis as an alternative treatment for oral cancer was discussed. As a result of this study, CAPE was shown to reduce the protein expression and enzymatic activity of MMP-2 (Kuo *et al.*, 2015). In another experimental animal study conduted by Liberio *et al*. (2011), it was reported that the application of oral propolis gel led to an increase in anti-inflammatory serum levels of IL-4 and IL-10. The results of these studies are consistent with the results of our study.

When MMP levels were compared between the groups in our study, a more significant reduction in GCF MMP-1 levels was observed in the propolis group following treatment compared to the control group. Although there was no statistically significant difference in GCF MMP-9 levels, a greater decrease was observed in the propolis group after treatment. Zarch *et al*. (2021) reported that although the difference was not statistically significant, salivary MMP-8 levels decreased more in the propolis group than in the chlorhexidine group.

To the best of our knowledge, this is the first study in which chlorhexidine and propolis subgingival irrigation with SRP was performed in periodontitis patients and GCF MMP-1 and MMP-9 levels were biochemically assessed at the end of treatment. Comparison with other data in the literature is therefore difficult. A current limitation of the study may be the small sample size and short follow-up period. However, given that our study employed gingival crevicular fluid analysis instead of saliva and serum samples, we contend that this methodology has enabled a standardized approach for comparing the results across the treatment groups within the same patient cohort.

The results of our study showed that subgingival irrigation with propolis and chlorhexidine together with SRP led to a decrease in GCF MMP-1 and MMP-9 levels, which are clinical periodontal parameters and biomarkers of periodontal disease, in periodontal pockets. In addition, the effect of propolis on GCF MMP-1 was found to be greater than that of chlorhexidine. Further research with larger sample sizes and extended follow-up periods is required to establish the efficacy of propolis, which is devoid of known side effects, as a subgingival irrigation solution, especially as an alternative to chlorhexidine.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Muğla Sıtkı Koçman University, Clinical Research Ethics Committee, E-72855364-050.01.04-616130.

Authorship Contribution Statement

Ali Batuhan Bayırlı: Conception, Materials, Data collection and processing, Analysis and Interpretation, Writing the Original Draft, and Manuscript Review. **İbrahim Kıvrak:** Materials, Analysis and Interpretation, Supervision, and Writing. **Ercan Saruhan:** Design, Analysis and Interpretation and Writing. **Fulden Cantaş Türkiş:** Analysis and Interpretation and Writing.

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