European Review for Medical and Pharmacological Sciences 2023; 27: 10642-10650

The effect of *Nigella sativa* extracts against *Porphyromonas gingivalis* isolated from periodontitis patients

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Abstract. – OBJECTIVE: Periodontitis is an inflammatory condition that results in pocket formation, gingival recession, and tooth loss by gradually destroying the periodontium. An alternate therapeutic approach that can address these problems is required due to the prohibitive cost of periodontal therapy, unfavorable antibiotic side effects, the advent of novel bacterial strains, and the resistance of those strains. The primary goal of our study was to assess *Nigella sativa*'s (*N. sativa*) antibacterial effectiveness against *Porphyromonas gingivalis* (*P. gingivalis*) utilizing seed extract.

PATIENTS AND METHODS: Six individuals with periodontitis, both male and female, between the ages of 30 and 50, were enrolled in the study. Each patient's medical and dental histories were documented. Then, anaerobic procedures were conducted in the microbiology lab to find *P. gingivalis* development. The specimens were all then cultured.

RESULTS: At 12.5 mg/ml concentration, *P. gingivalis* did not show any zone of inhibition (ZOI). However, *N. sativa*, at a concentration of 25 mg/ml, demonstrated a ZOI of 6.2 mm against *P. gingivalis*. Similarly, at 50 mg/ml, it showed a ZOI of 8.4 mm. Tetracycline as a positive control demonstrated a ZOI of 14.1 mm against *P. gingivalis*. Although *N. sativa* samples had somewhat less antibacterial activity than tetracycline samples, it was discovered that *N. sativa* had noticeable antibacterial activity against *P. gingivalis*.

CONCLUSIONS: This study's findings suggest that *N. sativa* can be utilized against periodontitis as an adjunct to scaling since it has high antibacterial action against *P. gingivalis*.

Key Words:
Antibacterial activity, Microbiology, *Nigella sativa, Porphyromonas. gingivalis*, Periodontitis.

Introduction

Periodontitis is a chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms and characterized by increasing destruction of the tooth-supporting apparatus. Its primary features include the loss of periodontal tissue support, displayed through clinical attachment loss (CAL) and radiographically assessed alveolar bone loss, the presence of periodontal pocketing, and gingival bleeding. Periodontitis is a significant public health concern owing to its widespread occurrence and potential to result in tooth loss and disability, adversely impact masticatory function and aesthetics, social inequality, and compromise the quality of life. Furthermore, it should be noted that periodontitis constitutes a considerable portion of edentulism and impaired chewing ability, leading to high costs in dental treatment and potentially exerting adverse effects on overall health[1].

The new periodontitis classification combines chronic and aggressive forms into a single category, periodontitis. A multi-dimensional staging and grading system further distinguishes this classification system[1].

Periodontitis is a condition mainly caused by infection and is associated with inflammation of the gingiva and loss of bone tissue. Periodontitis is linked to many systemic conditions, such as cardiovascular, respiratory, musculoskeletal, and reproductive abnormalities. The development of periodontitis is linked to disturbances in oral microbial composition, known as dysbiosis. This dysbiosis is thought to be instigated by the actions of *P. gingivalis*, which disrupts the immune homeostasis of the host and plays an essential role in the pathogenetic mechanism of the disease. *P. gingivalis* produces lipopolysaccharides, proteases, and fimbriae to enhance bacterial colonization and promote the growth of the neighboring microbial community. Furthermore, the virulence factors facilitate *P. gingivalis* coaggregation with different microorganisms, promoting dental biofilm formation[2]. Conventional methods for managing periodontal disease entail the eradication of pathobionts through direct means and eliminating or interfering with the biofilm, with minimal consideration for specificity[3].

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Administering locally active drugs can overcome many drawbacks of systemic delivery, precluding gut microbiome disturbances and patient compliance issues. The tetracycline-infused fibers and sustained-release devices, such as films, fibers, strips, gels, injectable devices, and micro-and nanoparticles, have been used to treat periodontal disease[4].

Providing a continuous release of medication within the periodontal pocket, located beneath the area of bacterial infiltration, appears to be a logical approach supported by a growing body of evidence. According to a systematic review, the combination of mechanical debridement and local drug delivery involving minocycline hydrochloride (Arestin, Laval, Quebec Canada), Chlorhexidine gluconate (Periochip, Alzenau, Germany), 10% doxycycline hyclate (Atridox, Fort Collins, CO, USA), and tetracycline hydrochloride (Periodontal Plus AB, Ashok Nagar, Chenni, India) resulted in an additional decrease in plaque scores, depth of probing, gingival inflammation, and bleeding scores when compared to scaling and root planing (SRP) alone[5]. In addition, statin, specifically atorvastatin and rosuvastatin in conjunction with SRP, significantly improved clinical attachment loss gain and probing depth more than SRP alone[6]. Similarly, a single application of subgingival minocycline hydrochloride delivery was as effective as nonsurgical mechanical debridement alone for treating peri-implantitis in cigarette smokers and non-smokers[7].

In middle eastern countries, the use of medicinal plant species goes back thousands of years and forms an important part of their culture. A large segment of the population in these areas still relies on it to treat serious diseases, including local and systemic conditions[8]. The seed of *N. sativa* has been used worldwide for centuries to treat various animal and human ailments. To date, multiple studies[9-14] have provided evidence of the medicinal efficacy of the seed of *N. sativa* and its primary active constituent, thymoquinone, in treating various illnesses.

The antioxidant action of thymoquinone and its 5-lipoxygenase inhibition may explain the different anti-inflammatory effects of these seeds. Interestingly, it was found that the oil of *N. sativa* had both antioxidant and anti-eicosanoid effects greater than thymoquinone, which is its active constituent[15,16]. A study conducted by Türe et al[17] revealed that the active component of black cumin α-pinene reached the sinus mucosa of the Rats significantly more than the control group.

To this end, the essential oil showed an antibacterial effect against many strains of Gram-positive and Gram-negative bacteria. Therefore, the current research project focused on assessing the antibacterial activity of *N. sativa L.* against *P. gingivalis* as it plays a role in human periodontal diseases. Hence, the research study's main objective was to evaluate the antibacterial activity of *N. sativa L.* against *P. gingivalis* using seed extract. This was accomplished by extracting the seed using methanolic or ether extraction based on the protocol published[18].

Patients And Methods

Ethical Considerations

The study proposal was submitted to the research and innovation center of Riyadh Elm University Riyadh, Saudi Arabia, for ethical approval (FRP/2023/515). Written informed consent to participate in the study was obtained from all the patients. This study followed the declaration of Helsinki. All the patient identifier information was coded, and data was collected anonymously.

Selection of Patients

Inclusion criteria

Six adults were enrolled in the study, including males and females aged 30 to 50 years suffering from periodontitis, attending the dental clinic at Riyadh Elm University, Saudi Arabia. Patients with periodontitis with 5-7 mm true periodontal pockets were included in the study.

Exclusion criteria

Patients with the following conditions were excluded from the study: patients consuming antibiotics during the last six months, pregnancy, patients with systemic diseases that influence periodontal condition or interfere with examination or sampling, smoking, patients with anti-inflammatory drugs during the last two months, and history of periodontal disease during last six months.

Sampling

Each patient's medical and dental history was recorded in the patient file. The following parameters were measured according to (A) Gingival index of Loe and Silness[19] (1963), which assesses the gingivitis severity according to color, consistency, and bleeding and classified into four codes: (0)=normal; (1)=mild inflammation, slight color change and edema, no bleeding; (2)=moderate inflammation, redness, edema, bleeding on probing; (3)=severe

inflammation, marked redness and edema, ulceration, spontaneous bleeding.

(B) Plaque Index of Silness and Loe[20]: this index is obtained in the same way as the Gingival Index (GI) except that it records plaque instead of gingival bleeding using the following criteria: (0)=no plaque in the gingival area; (1)=a thin film of plaque adhering to the free gingival margin and adjacent area of the tooth. Therefore, the plaque may be recognized only by running a probe across the tooth surface; (2)=moderate accumulation of soft deposits within the gingival pocket and on the gingival margin and/or adjacent tooth surface, which the naked eye can see; (3)=abundance of soft matter within the gingival pocket and/or the gingival margin and adjacent tooth surface.

(C) Probing pocket: probing pocket depth measurement used based on 12 University of North Carolina probe (UNC) color-coded probes (Hu-Freidy, Chicago, IL, USA). Patients with the abovementioned characteristics were selected, cotton roll isolation of the sample sites was carried out, and supragingival plaque removal was performed with sterile cotton pellets. Following this step, gingival crevicular fluid was collected by inserting sterile endodontic paper points of size 35 subgingivally into the base of the pocket for 30 seconds (Johnson and Johnson, East Windsor, NJ, USA). Paper points were transferred quickly into a sterile vial containing 5 ml of normal saline. Then, the vials were sent to the microbiology lab no later than 2 hours of sample collection for isolation.

Isolation of P. gingivalis

In six patients, each with two bacterial samples from the pocket depth was evaluated for the presence of associated anaerobic or facultatively anaerobic. First, an anaerobic procedure was accomplished in the microbiology lab to detect the growth of *P. gingivalis* and other bacteria. Then, all specimens were cultured.

The anaerobic Schaedler agar media (Oxide Ltd, Basingstoke, Hampshire, England, supplemented with vancomycin and 5% defibrinated horse blood) was used. The composition of media contains (tryptone soy broth 10.0 µl/ml, special peptone 5.0 µl/ml, yeast extract 5.0 µg/ml, glucose 5.0 µg/ml, cysteine HCL 0.4 µg/ml, hemin 0.1%, tris buffer 0.75 µl/ml, and Ager 13.5 mg/ml) and the composition of the selective medium used (ingredient mg per liter): schaedler agar 40 g/l, vancomycin 7.5 mg/l, horse blood 5% and distilled water up to 1 ml.

After autoclaving, anaerobic media was cultivated with specimens and incubated anaerobically using Gas Pak (Gas generating kit anaerobic system, Oxid Ltd, Altrincham, Cheshire, England) at 37°C for three days (Figure 1). Another media named Columbia Nutrient Agar (CAN) (Oxide LTD, Basingstoke, and Hampshire, England) was also used to isolate *P. gingivalis*. The composition of media contains Columbia nutrient agar powder g/l, colistin 10 mg/l, nalidixic acid 10 mg/l, hemin 0.1% (1 ml/l), vitamin K1 0.1% (1 ml/l), 5% sheep blood, distilled water up to 1l. The incubation was done as mentioned above. Following incubation, the bacteria were identified based on size, color, shape, staining, and biochemical tests.

Rapid Identification of Anaerobic Bacteria Using API 20A

The API 20A strip consists of 20 microtubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension, which reconstitutes the media during incubation. The color differences were identified by adding reagents based on the kit provided after incubation. The API 20A system facilitates 20 tests for expeditious and easy biochemical identification of anaerobic microorganisms. Procedural steps

Figure 1. Anaerobic jar with plated samples from different patients.

of API 20A identifications were carried out according to the manufacturer's instructions. Each ampule of API 20A Medium was used for one isolated bacterium and inoculated from pure young cultures (18-24 hours old) using a swab, harvesting all the growth obtained on blood agar in anaerobic conditions. The final turbidity is greater than or equal to 3 McFarland. This suspension is used immediately after preparation.

A tray and lid were prepared for the incubation box, and approximately 5 ml of distilled water was evenly distributed into the tray to create a humid atmosphere and labeled with strain references. An API 20A strip was removed from its packaging and placed in the incubation tray. Each strip well was inoculated with bacterial suspension using a sterile 5 ml syringe. The tube and cupule were filled for the gel test, and for the Indole test, the cupule was covered using mineral oil to prevent the indole from evaporating as instructed. Then, the strip was covered with the lid on the tray and incubated for 48 hours at 37°C in an anaerobic chamber bag. Following incubation, the results were recorded (Figure 2).

Examination of media: the cultural plates were examined for beta hemolysis and black-pigmented colonies. However, mixed colonies of different bacteria were obtained in each plate, and each beta-hemolytic with and without black pigmentation was selected and identified based on Gram staining morphology and catalase test (Figure 3).

The black pigmentation of *P. gingivalis* is from the accumulation of hemin used as an iron source for bacterial growth. Those colonies that showed Gram-negative rod or cocco-bacilli were sub-cultured on Colombia blood agar to obtain a pure, single colony for further biochemical identification (Figure 4).

Preparation of *N. sativa* extract: *N. sativa* seeds were procured and ground into a fine powder using a blender, then prepared by percolation method as follows. One hundred grams of *N. sativa* powder was soaked in 300 ml of methanol for seven days, filtered using (Whatman® No. 1 filter paper, Darmstadt, Germany), and evaporated using a rotary evaporation apparatus. The extract was dried in a hot air oven at 50°C for 24 h and finally kept at 4°C until further testing. One Gram of each extract was dissolved separately in 1 ml of 10% dimethyl sulfoxide (DMSO) to give a stock solution of 1,000 mg/ml. Three different concentrations of 50, 25, and 12.5 mg/ml were prepared from the stock solution.

Antimicrobial Activity

An antimicrobial agent or antibiotics' ability to inhibit the growth of bacteria is measured by an antimicrobial susceptibility test. A 100 µl of bacterial suspension was spread on each nutrient agar plate. Different concentrations of the extract (12.5-50 mg/ml) (25 µl) were utilized to impregnate in sterile 6-mm blank discs. Ten percent of Dimethyl Sulfoxide (DMSO) loaded discs were utilized as negative controls for the extract. All the impregnated discs were thoroughly dried in a 45°C incubator for 18-24 h before bacteria application. Tetracycline 30 µg was used as a positive control for all strains. After drying completely, the discs impregnated with the extract were applied to the inoculated Muller-Hinton agar. The discs were delicately pressed to ensure consistent and even contact with the agar surface. Within 15 minutes of application, the plates were shifted to an anaerobic jar and kept in an incubator for 48 h. After incubation, the plates were read-only if the lawn of growth was confluent or nearly confluent. The diameter of the inhibition zone around the discs was measured for antibacterial activity. If present, their diameters were measured to the nearest whole millimeter with a ruler.

Figure 2. API 20 A strip after inoculation with different bacterial suspensions.

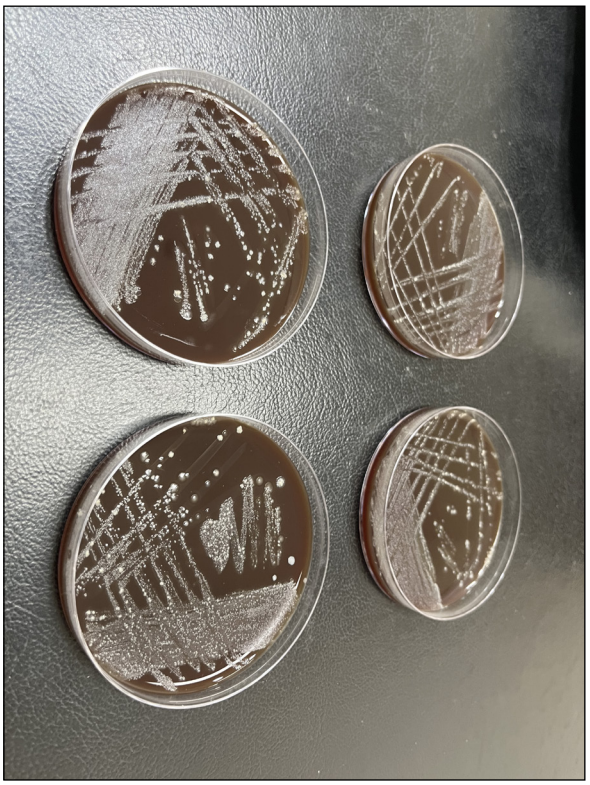

Figure 3. Mixed bacterial growth in Schaedler agar. The beta-hemolytic and brown-pigmented colonies were selected for further analysis.

Statistical Analysis

Descriptive frequency distribution and percentages statistics were calculated for the study participant's brushing frequency and periodontal parameters. The mean zone of inhibition (ZOI) of *P. gingivalis* produced by *N. sativa* extracts at different concentrations (12.5 mg/ml, 25 mg/ml, and 50 mg/ml), positive control, and negative control were calculated and compared using Kruskal-Wallis and Mann-Whitney U tests. A value of $p<0.05$ was considered significant for all the statistical tests. Data analysis was performed using a statistical package for social sciences (SPSS) version 25, (IBM Corp., Armonk, NY, USA).

Results

A total of six patients with varying frequencies of toothbrushing, periodontal pocket depth, and bleeding on probing were considered in this study. Half the participants brushed their teeth once daily, 2 (33%) brushed twice daily, while 1 (17%) patient brushed sometimes. Half of the patients had a periodontal pocket depth of 7 mm, followed by 5 mm in 2 (33%) and 6 mm in 1 (17%) patient. Almost 5 (83%) of patients demonstrated bleeding on probing, and only 1 (17%) did not show any bleeding on probing (Table I).

Figure 4. Sub-culture of *P. gingivalis*.

This study tested the antibacterial effects of *N. sativa* seeds against the periodontal pathogen *P. gingivalis* in different concentrations. The antibacterial effect of *N. sativa* seeds was estimated by measuring the mean ZOI. At 12.5 mg/ml concentration, *P. gingivalis* did not show any ZOI. However, *N. sativa*, at a concentration of 25 mg/ml, demonstrated a ZOI of 6.2 mm against *P. gingivalis*. Similarly, at 50 mg/ml, it showed a ZOI of 8.4 mm for *P. gingivalis*. Tetracycline as a positive control demonstrated a ZOI of 14.1 mm against *P. gingivalis*. In contrast, ten percent of DMSO-loaded discs used as a negative control showed no ZOI. Although *N. sativa* produced

Table I. Study participant's brushing frequency and periodontal parameters (N=6).

Variable analyzed	Variable type	n	%
Brushing	Never	0	0%
	Sometimes	1	17%
	Daily (Once)	3	50%
	Daily (Twice)	2	33%
Periodontal depth	5 mm	2	33%
	6 mm	1	17%
	7 mm	3	50%
Bleeding on probing	Present	5	83%
	Absent	1	17%

slightly less ZOI than tetracycline, it demonstrated highly significant antibacterial activity against *P. gingivalis* ($p<0.01$), as indicated in Table II.

Discussion

Among the many herbal treatments reported worldwide, *N. sativa* is a well-known cultural and religious remedy for various medical ailments[21]. Due to its extensive cultural influence worldwide, its seeds are referred to linguistically by various names from various language origins. One of the well-known names for this plant is "black seed", as the seeds turn black when exposed to air[22]. *N. sativa* is also known as Alhabattul Sawada (black seed) or Habbatul barakah (blessed seed) among the Muslim and Arabic communities[23]. It is also known as black caraway, black cumin, kalonji, shun, or kalonji in various regions of the world[24]. Numerous studies[25,26] have been conducted since the late 20th century to assess the therapeutic properties of *N. sativa* and its diverse bioactive constituents across various medical disciplines. These studies aim to elucidate the potential roles that *N. sativa* may have in clinical therapies and disease prevention strategies in the future.

Periodontitis is a complex disease influenced by multiple factors, with microorganisms playing a significant role in its development and progression. The subgingival bacteria must be eliminated or suppressed for periodontal therapy to be effective. The microorganisms implicated in periodontitis are anaerobic bacteria. Antimicrobial agents try to diminish the pocket microbiota when used with mechanical debridement directly. The primary microbe believed to be responsible for the start and progression of chronic periodontitis is *P. gingivalis*[27].

The present study evaluated the antibacterial activity of *N. sativa* against *P. gingivalis*. The results showed that *N. sativa* possesses significant bactericidal activity against *P. gingivalis* at 25 and 50 mg/ml concentrations, respectively. The mean ZOI was 6.2±0.2 mm and 8.4±0.3 mm at 25 and 50 mg/ml concentrations. While at 12.5 mg/ml concentration, *N. sativa* showed no ZOI. This ZOI at 25 mg/m and 50 mg/ml differed significantly compared to the tetracycline positive control 14.1±2.3 mm. This finding is in line with a previous study[28] in which *N. sativa* at concentrations of 25 mg/ml and 50 mg/ml demonstrated the mean ZOEs of 5.4±0.03 mm and 9.6±0.86 mm, which differed significantly with tetracycline positive control.

Table II. Disc diffusion method showing zone of inhibition for *Porphyromonas gingivalis*.

Test compounds	Zone of inhibition (mm)
Nigella sativa Extract (12.5 mg/ml)	NI
Nigella sativa Extract (25 mg/ml)	6.2±0.2**
Nigella sativa Extract (50 mg/ml)	8.4±0.3**
Tetracycline (positive control)	14.1±2.3**
DMSO (Negative control)	NI

**$p<0.01$, DMSO=Dimethyl Sulfoxide 10%, NI=No Inhibition

In another similar study[27], the growth of *Staphylococcus aureus* was inhibited by ground seeds of *N. sativa* sourced from Hadramout and Ethiopia. The growth inhibition observed in *N. sativa* ground seeds from Ethiopia was slightly lower than the positive control. The results were statistically significant, with a ZOI of 20 mm for the *N. sativa* seeds and 22 mm for the positive control. Our study observed that the inhibition of the zone of *N. sativa* was slightly lower compared to the positive control Tetracycline. As a result, the findings were deemed significant regarding their effectiveness against *P. gingivalis*. This finding is in line with the other research[16,28]. Morsi[29] investigated the antibacterial properties of *N. sativa* (methanolic extract) at a concentration of 100 mg/ml. The study evaluated its effects against various bacteria, including *S. aureus*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli,* and *Bacillus cereus*. The results revealed an inhibition zone of 9 mm in E. coli and 25 mm in *S. aureus*.

Abd-Awn et al[30] examined the impact of an ethanolic extract of black seed (*N. sativa*) oil at a concentration of 20% compared to 0.2% chlorhexidine gluconate on *Streptococcus mutans*. The findings demonstrated that *N. sativa* exhibited a more significant inhibition zone than chlorhexidine. In a similar study conducted by Halawani[31], the bioactive components of *N. sativa*, namely thymoquinone and thymohydroquinone, were utilized to assess their antibacterial properties against *E. coli, Salmonella typhimurium, Salmonella enteritidis, Shigella flexneri, P. aeruginosa*, and *S. aureus*. In addition, their potential interactions with other antibiotics were investigated. *Staphylococcus aureus* was determined to have the highest susceptibility to thymoquinone, with a minimum inhibitory concentration (MIC) of 3 µg/ml. On the other hand, Shigella flexneri exhibited greater susceptibility to both thymoquinone and thymohydroquinone[31]. Al-Bayaty et al[32] employed a biodegradable

periodontal chip containing thymoquinone to significantly reduce plaque index, bleeding on probing, and periodontal pocket depth.

When applied locally, a thymoquinone containing collagen membrane promoted bone repair in bone defects infected with *P. gingivalis*. Thymoquinone improved the rate of angiogenesis and showed promise in reducing the time for bone repair[33]. More cellular and molecular level studies are required to investigate the specific mechanisms of action of *N. sativa* and its components, particularly Thymoquinone, in the context of periodontal treatment or regeneration[34].

Based on our current understanding, this study represents a preliminary evaluation of the antibacterial properties of *N. sativa* against the Gram-negative pathogen *P. gingivalis* associated with periodontal disease. The initial research demonstrated significant antibacterial activity of *N. sativa* against *P. gingivalis*. In light of our findings, *N. sativa* exhibits a slightly lower antibacterial effect than the positive control tetracycline. However, it is essential to emphasize that tetracycline is exclusively suitable for therapeutic use in the nonsurgical treatment of periodontitis. In contrast, *N. sativa* can be utilized for prophylactic and therapeutic purposes, owing to its ready availability and status as a natural product commonly consumed without any documented adverse effects.

Limitations

The present study has certain limitations that should be acknowledged. Firstly, no comparison was made with widely used positive control chlorhexidine. Secondly, the antibacterial activity of *N. sativa* at higher concentrations was not investigated. Lastly, the effectiveness of *N. sativa* against other important periodontal pathogens was not conducted. Additional clinical trials will contribute to advancing *N. sativa* as a natural herbal alternative for chemical plaque control in conjunction with periodontal therapy.

Conclusions

It can be concluded that *P. gingivalis* is susceptible to the antibacterial effects of *N. sativa*, and it may, therefore, provide additional and complementary benefits in the preservation of oral health and the reduction of periodontal disorders such as periodontitis when added to oral hygiene products like toothpaste and mouthwashes. Further research is needed to examine its antibacterial effectiveness against periodontitis-related subgingival microorganisms.

Funding

This research received no external funding.

Ethics Approval

The study proposal was submitted to the research and innovation center of Riyadh Elm University Riyadh, Saudi Arabia, for ethical approval (FRP/2023/515). This study was conducted according to the guidelines of the Declaration of Helsinki.

Informed Consent

Written informed consent was obtained from all the individual patients to participate in the study.

Acknowledgments

The author would like to thank the Research and Innovation Center of Riyadh Elm University for supporting this study.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The author declares no conflict of interest.

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