Kurdistan Journal of Applied Research (KJAR)
Print-ISSN: 2411-7684 | Electronic-ISSN: 2411-7706

Original Article

Check for updates

Antibacterial and Antibiofilm Effects of Green Tea and *Salvadora Persica L.* Extracts Against Clinically Isolated *Porphyromonas gingivalis*: An *in Vitro* Study

Murtadha Imad Abdulkareem [a], **Hayder Raad Abdulbaqi** [b], **Aram Mohammed Sha** [c*], **Firas Bashir Hashim Al-Taweel** [b], **Muhammad Saad Shaikh** [d]

[a] Department of Periodontics, College of Dentistry, University of Basrah, Basrah, Iraq.
[b] Department of Periodontics, College of Dentistry, University of Baghdad, Baghdad, Iraq.
[c] Department of Periodontics, College of Dentistry, University of Sulaimani, Sulaymaniyah, Iraq.
[d] Department of Oral Biology, Sindh Institute of Oral Health Sciences, Jinnah Sindh Medical University, Karachi, Pakistan.

Submitted: 15 March 2023
Revised: 25 April 2023
Accepted: 15 May 2023

*** Corresponding Author**:
aram.hamad@univsul.edu.iq

Keywords: Antibacterial, Antibiofilm, Green tea, Salvadora, *P. gingivalis.*

How to cite this paper: M. I. Abdulkareem, H. R. Abdulbaqi, F. B. Al-Taweel, A.M. Sha, M. S. Shaikh,” Antibacterial and Antibiofilm Effects of Green Tea and *Salvadora Persica L.* Extracts Against Clinically Isolated *Porphyromonas gingivalis*: An *in Vitro* Study’ KJAR, vol. 8, no. 1, pp. 73-86, December. 2023, doi: 10.24017/science.2023.1.8

Abstract: The combination of an aqueous extract of green tea (GT) and *Salvadora persica L.* (SP) was proved to prevent the growth of Gram +ve facultative anaerobes and reduce biofilm formation on teeth surfaces. *Porphyromonas gingivalis* (*P. gingivalis)* is amongst the red complex pathogenic bacteria that cause periodontal disease. Thus, the aim of the current study was to examine the antibacterial and antibiofilm effects of the above combination in reducing the growth of *P. gingivalis*. Subgingival dental biofilm samples were collected from patients with severe periodontitis to isolate and confirm the presence of *P. gingivalis*. Gas mass chromatography-mass spectrometry was used for phytochemical analysis. The maceration method was used to extract the GT and SP. Disc diffusion and broth dilution methods were performed to determine antibacterial and minimum inhibitory concentrations (MIC) of SP, GT aqueous extract, and their combination in contrast to clinically isolated *P. gingivalis*. Further, the antibiofilm activity of the extracts and their combination was assessed using the tube adhesion technique. The findings showed that only the GT aqueous extract was effective against *P. gingivalis*, while the SP aqueous extract demonstrated no effectiveness. The MIC of GT was 6.25mg/mL. The aqueous extract of SP showed a greater antibiofilm effect than the aqueous extract of GT at the lowest concentrations of 6.25mg/mL and 12.5mg/mL, respectively. In conclusion, the antibacterial property of the SP and GT extracts combination against *P. gingivalis* was attributed to GT only. While SP extract displayed no inhibitory role against *P. gingivalis*, it could potentially reduce the biofilm attachment of *P. gingivalis* better than GT extracts.

1. Introduction

Periodontitis is a chronic inflammatory disease of the tooth-surrounding structures caused by dysbiotic dental biofilm. If left untreated, permanent destruction of periodontal tissues is an unavoidable consequence, which in turn impacts chewing function and esthetics, social inequity, and quality of life [1]. *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola* and *Tannerella forsythia* were acknowledged as red complex bacteria and secondary colonizers are considered as the main periodontal pathogens in the initiation and progression of periodontitis following colonization in subgingival tissues [2]. *P. gingivalis* is infrequently isolated in individuals with healthy periodontium. To the contrary, saliva from periodontitis patients harbors a significant level of this bacterium [3], and *P. gingivalis*

load within subgingival biofilm in deep periodontal sites around teeth is correlated to the amount of *P. gingivalis* in saliva [4]. *P. gingivalis* is the main pathogen that is strongly associated with severe destruction of the periodontal tissues when its level in subgingival biofilm is beyond the host defense ability [2]. Many virulence factors of *P. gingivalis* enable its invasion into host tissues, such as fimbriae, lipopolysaccharides, and gingipains [5]. Accordingly, *P. gingivalis* can adhere to oral epithelial cells and deteriorate the junction between these cells; hence, it can invade gingival tissues [6, 7]. Therefore, therapeutic attempts should be directed toward controlling the load of *P. gingivalis* around periodontal tissues to a level compatible with the state of symbiosis.

In the past few years, extracts from traditional plants have received much attention due to their biological activities and safety. *Camellia sinensis var. assamica* or green tea (GT) is one of the most commonly consumed beverages worldwide with a well-known impact in promoting periodontal health [8]. Extracts from GT have been reported to potentially minimize the harmful effect of *P. gingivalis* on periodontal tissues. For example, GT extracts were found to reduce *P. gingivalis* adherence to oral epithelial cells and enhance the tight junction recovery between spaced epithelial cells despite being stimulated by *P. gingivalis*, thus decreasing the permeability of the epithelial cells [7]. Furthermore, GT extracts have the potential to attenuate lipopolysaccharide-induced gingival inflammation and reduce tumor necrosis factor (TNF)α, interleukin (IL)-1β, and IL-6, and lipopolysaccharide is a well-known toxin of *P. gingivalis* [9]. Such an effect might be attributed to catechins, constituents of GT extract, which are found to downregulate the production of IL-1β by *P. gingivalis*-infected macrophages and interfere with toll-like receptor signaling and nuclear factor-kB (NF-kB), accordingly reducing alveolar bone loss [10].

On the other hand, *Salvadora persica L.* (SP) root sticks (commonly known as miswak, sewak, and siwaki) are natural oral hygiene tools for dental biofilm control. Their beneficial effects in enhancing oral health exceed their role in the mechanical removal of dental biofilm due to their constituents' broad range of biological properties [11]. For example, a product containing SP was reported to show an inhibitory effect on *P. gingivalis* clinical isolate [12]. Further, Benzyl Isothiocyanate, a component of SP extracts, is bactericidal to Gram-negative periodontal microorganisms such as *gingivalis* and *Aggregatibacter actinomycetemcomitans* [13]. However, it has been reported that using SP sticks as a toothbrush for three weeks does not decrease the levels of *P. gingivalis* in pockets of periodontitis patients [14]. Adam *et al.* [15], in a new systematic review and meta-analysis, showed that tooth brushing utilizing SP sticks has a comparable anti-dental biofilm effect to standard toothbrushes with better periodontal health outcomes. Moreover, many biological activities of SP extracts, such as anti-inflammatory, antioxidant, anticonvulsant, anti-osteoporosis, antidepressant, antitumor, and analgesic, were reported [16].

The combination of GT and SP aqueous extracts has displayed anti-adherence and antibacterial efficacy against dental biofilm of primary colonizer bacteria such as *Streptococcus mitis*, *Streptococcus sanguinis*, and *Actinomyces viscosus* [17]. For example, a randomized controlled trial has shown that rinsing with this combination significantly reduces dental biofilm buildup after 24 hrs of regrowth [18] and after 4 days of abstaining oral hygiene measures [19]. Moreover, rinsing with this combination resulted in fewer primary colonizer bacteria in saliva [19]. Although the above evidence showed the antibacterial activity of this combination against primary colonizer bacteria, the effect of GT and SP combination against the secondary colonizer bacteria such as *P. gingivalis* has yet to be investigated. Thus, the current study aims to evaluate the antibacterial and antibiofilm effects of combining GT and SP aqueous extracts against *P. gingivalis.*

2. Materials and Methods

2.1 Plants Extraction

The plants' extract were prepared as previously described [17]. Briefly, 100 gm of GT from ready-made tea packs (*Camellia sinensis var. assamica* leaves; BOH, B. NO. 80619, Malaysia) was dipped in non-ionized distilled water (1000 ml) (DEMI WATER, Iraq) for three days with intermittent manual shaking every 2 hrs. After three days, the extracts were sieved through muslin fabric cloth to get rid of undissolved coarse plant remnants. Then, the extracts were centrifuged for 10 mins at 4000 rpm and filtered by filter paper (Cat No 1001 150, England; Whatman No 1, 150 mm diameter). Later, the extracts were freeze-dried (using the CRIST ALPHA 2-4 LD plus Germany freeze dryer) to produce extract powder,

which was kept in germ-free containers at room temperature to be instantly obtainable when needed. Similarly, the same procedure was applied for the extraction of SP root sticks (obtained in sealed wrappers from the local markets, B. NO. AK. 108/140222 SP, AL KHAIR) after cutting and blending.

2.2 Detection of Compounds in SP and GT Extracts and their Combination

Gas chromatography–mass spectrometry (GC-MS) analysis was carried out to determine the ingredients of the extracts (Agilent Technologies 7820A GC System, USA). For both extracts, 1µL of sample solution was manually added into the GC-MS system. The detector and the injection port temperatures were 230°C and 220°C, respectively. After being kept at 100°C for 5 mins, the column temperature was steadily increased to 250°C at a rate of 10°C per minute, followed by a 10-minute isothermal hold. The mass spectrum quad and the ion source temperatures were set at 150°C and 240°C, respectively, and the electron impact ionization mode was set at 1500V. Mass spectral data was collected using GC-MSD Agilent Chem Station Software. By comparing their mass spectrum values to those in the National Institute of Standards and Technology collection, the extracted components were recognized.

2.3 Dental Biofilm Sampling

Subgingival dental biofilm samples were collected from volunteers after receiving their signed consent. The volunteers were systemically healthy and had periodontitis with at least one periodontal pocket of ≥6mm as inclusion criteria. The volunteers were clinically examined and were diagnosed with periodontitis with interdental clinical attachment loss at ≥ two non-adjacent teeth [20]. The selected pockets were isolated by a cotton roll, and a sterile periodontal curette was used to remove supragingival dental biofilm and calculus. Afterward, a germ-free paper point (F1 Dia- ProTTM) was gently inserted into the periodontal pocket base and maintained in place for one minute. Then, after careful removal of the paper point, it was streaked over Columbia agar plates enriched with 5% human blood (25mL), distal water, bacitracin (5mg) (Himedia), vitamin K1 (1µg/mL, 0.5mg, Himedia), and hemin (5µg/mL, Sigma-Aldrich, China) [21]. All plates were incubated in an anaerobic jar with an anaerobic gas pack (THERMO scientific, AnaeroGen 2.5L) at 37°C for 7 days.

2.4 P. gingivalis Isolation and Identification

After numerous subcultures based on colony morphology, pigment synthesis, Gram staining, and aerobic control, *P. gingivalis* was identified using the PCR method as outlined below:

- DNA isolation: from each sample, a colony was collected in an Eppendorf test tube with 50µL ultrapure sterilized deionized distilled water and vortexed thoroughly to achieve proper homogenization, afterward incubated at 95°C in a heat block (Thermo Fisher Scientific) for 10 mins. The samples were then disseminated, and the remaining (DNA) was utilized as a template [22].
- PCR analysis: *P. gingivalis*-specific primers were used to verify the presence of *P. gingivalis* that targeted the 16S rDNA gene, and then DNA amplification was performed [23]:

"Forward primer 5′-AGGCAGCTTGCCATACTGCG-3′

reverse primer 5′- ACTGTTAGCAACTACCGATGT-3′".

By using the final volume of 20µL (that consisted of 2µL of the forward primer (10pmol/µL), 2µL of the reverse primer (10pmol/µL), 1µL of ddH2O, 10µL of 2XPrime Taq Premix (GeNet BioG 2000), and 5µL of the DNA template) the amplification was carried out. The PCR cycle (Applied Biosystems, VeritiTM 96-well thermal cycler, USA) included the first denaturation at 95°C for 5 mins for one cycle. Amplification for 35 cycles included denaturation for 30 sec at 95°C of the DNA template, annealing for 30 sec at 62°C, and extension of primers for 30 sec at 72°C. The final extension was carried out at 72°C for 5 mins for one cycle. Later, 2% agarose gel electrophoresis for 35 mins at 80V was used to examine the PCR product, followed by adding ethidium bromide (3µL) to dye the gel. Finally, the PCR product's routine sequencing was finalized (Macrogen, South Korea).

2.5. Standard Bacterial Cell Suspension Preparation

A 3mL Mueller Hinton broth (MHB) enriched with 5μg/mL of hemin (Sigma-Aldrich, China) was inoculated with pure black colonies identified as *P. gingivalis* by sterile loops. The bacterial suspension was adjusted at an optical density (OD) 660 of 0.5 McFarland's turbidity standard, which is equal to 5×10^5 CFU/mL, by using a spectrophotometer [24].

2.6. Antimicrobial Evaluation of the Extracts

All tests for the antimicrobial evaluation of the extracts were carried out in triplicate at three different times.

2.6.1. Agar Well Diffusion Assays

To evaluate the antibacterial activities of the GT and SP against *P.*, the agar well diffusion technique was used [25]. Over the surface of Mueller Hinton agar media, 50μL of the prepared bacterial suspension was spread by using an L-shaped loop and allowed to dry. Later, punch holes of 6 mm diameter were created using sterile micropipette tips through the agar plate surfaces. For each type of herbal extract, one agar plate was used, and a volume of 200μL of the extract's solutions (at concentrations of 100, 75, 50, and, 25mg/mL for both SP and GT extracts, respectively) was introduced into the wells. In addition, Chlorhexidine (CHX) was used as a positive control and sterilized distilled water as a negative control. Finally, the plates were incubated for 48 hrs at $37C^0$ under strict anaerobic conditions. With a millimeter ruler, the mean diameter of the inhibition zone around the well containing the extracts was determined.

2.6.2. Minimum Inhibitory and Bactericidal Concentrations of GT and SP Extracts

To determine the minimum inhibitory concentration (MIC) of the GT and SP extracts, two-fold serial macro-dilution was performed [26]. To start, 900μL of MHB was added into ten tubes. For MIC of GT determination, 900μL of 50mg/mL aqueous extract of GT was added into the first tube and two-fold serially diluted. The negative control tube contained only MHB, while the positive control tube contained 900μL of a mixture of CHX with MHB. All tubes were incubated for 48 hrs at 37°C in an anaerobic jar with an anaerobic gas pack (THERMO scientific, AnaeroGen 2.5L) after being inoculated with 100μL of *P. gingivalis* suspension (5×10^5 CFU/mL). The MIC was determined by the naked eye after incubation, which represented the least concentration of GT extracts in a tube with loss of turbidity. Additionally, this was further confirmed by using a spectrophotometer (V-1100 Digital Spectrophotometer, EMC LAB, Germany) to record the OD values of the suspensions in the tubes. The procedure was repeated in triplicate at three different times.

Similarly, the MIC of SP extracts consisted of 900μL of 200mg/mL aqueous extract of SP added to the first tube, and the procedure was continued in the same manner as for GT extract. Following the MIC procedure, 50μL was taken from the tubes that showed no turbidity and subcultured onto MH agar plates and incubated anaerobically for 48 hrs at 37°C. The MBC value was calculated as the concentration at which there was no growth or less than three colonies were counted, showing a bactericidal activity of 99% to 99.5% [26].

2.6.3. MIC of the Combination of SP and GT Extracts

A combination of 50mg of GT and 50mg of SP extracts per 1mL of sterile MHB was formulated to be a reference concentration for the determination of GT and SP extracts. First, 900μL of the MHB was added into sterile tubes to assess the antimicrobial activity of the test combination. Next, 900μL of the mixture was added to 1mL and two-fold serially diluted. Two tubes, one containing MHB only and the other MHB plus CHX, were used as the negative and positive control, respectively. This was followed by the inoculation of all test tubes with 100μL of *P. gingivalis* suspension. Then, the tubes were incubated as mentioned above. After incubation, the bacterial growth inhibition was evaluated by the naked eye through the observation of loss of turbidity and confirmed by using a spectrophotometer to measure OD values of the bacterial suspensions.

2.7. Antibiofilm Assays of GT and SP Extracts and their Combination

The tube adhesion method was used to assess the anti-biofilm activities of the extracts separately as well as their combination. Briefly, and as described by Eladawy *et al.* [27], the contents of tubes used to determine the MIC of GT, SP, and GT+SP extracts with different extract concentrations were carefully discarded. Later, phosphate buffer saline (pH 7.3, Biochem, Cosne-Cours-sur-Loire, France) was used to wash the used tubes and remove the planktonic bacteria and they were allowed to dry in an inverted position for 45 mins. Then, the tubes were stained with 0.1% crystal violet and the extra stain was washed out using deionized water. Afterward, the tubes were left in an inverted position for 24 hrs to dry, and biofilm formation was monitored. When the tube's wall and bottom were lined with a visible film, this was considered a valid sign of biofilm development. The degree of biofilm formation in the tubes was graded as 0 (absent), 1 (weak stain), 2 (moderate stain), and 3 (strong stain), the latter indicating a weak antibiofilm effect of the extracts [28]. The experiment was repeated three times in triplicate to guarantee the accuracy and repeatability of the results.

2.8. Statistical Analysis

The data were presented as median and interquartile range and analyzed by IBM SPSS Statistics software version 26.0. Mann-Whitney U and Kruskal-Wallis tests were used to compare the data between the two or the three groups, respectively. The significance threshold was set at ≤0.05.

3. Results

3.1. GC-MS Analysis of GT, SP Extracts, and their Combination

Figure 1 shows GC-MS analysis of GT, SP aqueous extracts, and their combination of 12, 9 and 18 peaks, respectively. The number and nature of extract ingredients revealed by the peaks were categorized and identified by comparison of the mass spectra of the components with the National Institute of Standards and Technology (Table 1). Accordingly, the main compounds of GT were Caffeine (60.299%), followed by Oleic Acid (14.978%) and Butanoic acid (8.789%), whereas Glycerin (56.238%), Oleic acid (20.858%) and Hexadecanoic acid (5.816%) were the main compounds of SP. Finally, Caffeine (40.140%), 2-Methyl-Z, Z-3,13-octadecadienol (27.999%) and Hexadecanoic acid (7.929%) were the main compounds of the GT and SP combination.

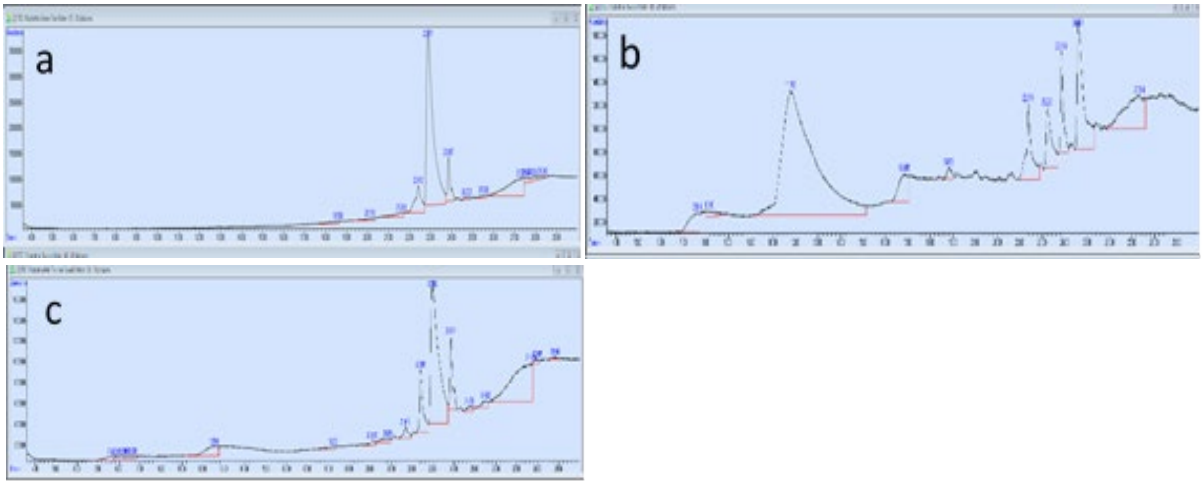

Figure 1: GC-MS chromatogram of (a) GT aqueous extract, (b) SP aqueous extract, and (c) combination of GT and SP extracts.

Table 1: GC-MS analysis of the GT, SP and GT+SP aqueous extracts.

Extracts	Peak	Compounds	Chemical formula	Retention Time (mins)	% of total
GT aqueous extracts	1	2-Propenoic acid, 2-(acetylamino)-	C5H7NO3	18.590	1.174%
	2	9-Octadecenoic acid, (E)-	C18H34O2	20.110	1.317%
	3	Benzothiazole, 2-methyl-	C8H7NS	21.614	1.945%
	4	Butanoic acid, methyl ester	C5H10O2	22.412	8.789%
	5	Caffeine	C8H10N4O2	22.871	60.299%
	6	cis-13-Octadecenoic acid, methyl ester	C19H36O2	23.857	6.749%
	7	Z,E-2,13-Octadecadien-1-ol	C18H34O	24.723	0.728%
	8	E,E-2,13-Octadecadien-1-ol	C18H34O	25.530	0.665%
	9	Oleic Acid	C18H34O2	27.331	14.978%
	10	2-Methyl-Z,Z-3,13-octadecadienol	C19H36O	27.544	1.081%
	11	9-Octadecenal, (Z)-	C18H34O	27.824	0.627%
	12	2-Methyl-Z,Z-3,13-octadecadienol	C19H36O	28.342	1.647%
SP aqueous extracts	1	Ethyl (trimethylsilyl)acetate	C7H16O2Si	7.614	2.954%
	2	Ethyl (trimethylsilyl)acetate	C7H16O2Si	8.140	0.719%
	3	Glycerin	C3H8O3	11.785	56.328%
	4	Benzene, 1-chloro-2-methyl-	C7H7Cl	16.865	3.696%
	5	Piperidine, 1,4-dimethyl-	C7H15N	18.819	0.754%
	6	Hexadecanoic acid, methyl ester	C17H34O	22.379	5.816%
	7	n-Hexadecanoic acid	C16H32O	23.220	4.886%
	8	6-Octadecenoic acid, methyl ester	C19H36O	23.848	3.989%
	9	Oleic Acid	C18H34O2	24.604	20.858%
Combination of GT and SP aqueous extracts	1	Diglycolamine	C4H11NO2	7.622	1.292%
	2	Butanedioic acid, 2,3-dimethoxy-	C8H14O	7.919	0.410%
	3	Ethyl (trimethylsilyl)acetate	C7H16O2Si	8.166	0.573%
	4	Butyl-dimethyl-propoxysilane	C9H22OSi	8.361	0.513%
	5	Cyclopentane, 1,1,2-trimethyl-.	C8H16	8.650	0.442%
	6	1,2,3,4-Butanetetrol, [S-(R*, R*)]-	C4H10O	12.541	4.665%
	7	Erythritol	C4H10O	18.233	0.970%
	8	1-Nitro-beta-d-arabinofuranose, tetraacetate	C13H17NO11	20.102	0.904%
	9	2-Hydroxy-5-nitropyridine	C5H4N2O3	20.858	1.322%
	10	alpha. -Ketostearic acid	C18H34O3	21.673	1.862%
	11	Hexadecanoic acid, methyl ester	C17H34O	22.395	7.929%
	12	Caffeine	C8H10N4O2	22.982	40.140%
	13	cis-13-Octadecenoic acid, methyl	C19H36O	23.857	7.185%
	14	2-Methyl-Z,Z-3,13-octadecadienol	C19H36O	24.732	0.911%
	15	-Octadecenoic acid (Z)-, methyl ester	C19H36O	25.522	1.842%
	16	2-Methyl-Z,Z-3,13-octadecadienol	C19H36O	27.679	27.999%
	17	9-Octadecenal, (Z)-	C18H34O	27.985	0.638%
	18	9-Octadecenal, (Z)- · Formula:	C18H34O	28.843	0.404%

3.2. Isolation and Identification of P. gingivalis

Black-pigmented anaerobic bacteria were identified in the dental biofilm samples. The colonies were round, convex, small, and opaque. Black-colored colonies appeared after 7–10 days on lysed blood (Figure 2A). Later, further confirmation of *P. gingivalis* was carried out by PCR technique and DNA sequencing (Figure 2B). DNA sequencing demonstrated that all pure black colonies were identical genetically to the ATCC 33277 strain (Figure 2C).

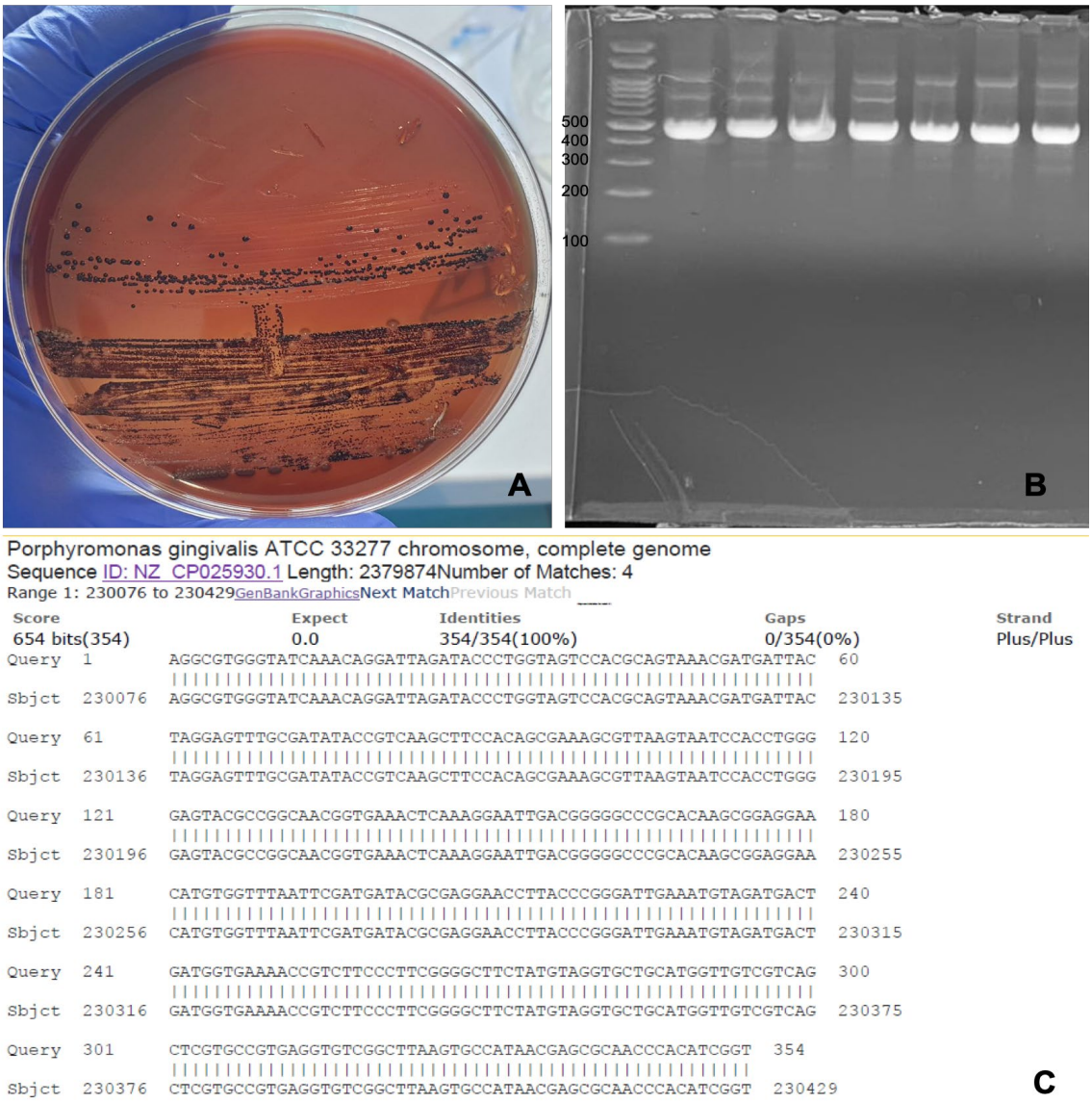

Figure 2: (A): Black pigmented colonies of *P. gingivalis*, (B): Agarose gel electrophoresis of PCR products showing multiple bands similar to template size of *P. gingivalis* (575 base pairs) using 100 base pairs ladder, and (C): 16S rDNA gene sequence of the purified colonies of *P. gingivalis*.

3.2 Antibacterial Assays of the Extracts

The GT aqueous extracts had antibacterial activity at different concentrations, while SP aqueous extracts exhibited no antibacterial effect, as shown in figures 3, figure 4A and figure 4B. The lowest concentration of GT extracts that showed antibacterial activity was 6.25mg/mL; meanwhile, no antibacterial effects were observed for SP extracts at its highest concentration (200mg/mL). On the other hand, the test combination of the two extracts exhibited antibacterial effects at a concentration of 6.25mg/mL. The analyses of the OD values at MIC levels of both GT and SP and their combinations are illustrated in table 2 and figure 4C. Finally, the bactericidal activity of GT aqueous extracts at a minimum concentration was 12.5mg/mL.

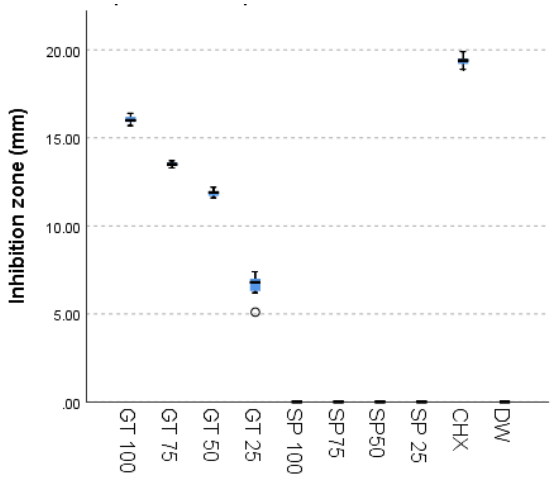

Figure 3: Inhibition zone of *P. gingivalis* for various concentrations of GT and SP aqueous extracts. *P. gingivalis* was sensitive to GT extract in a dose-dependent manner, while not to SP extracts, even in the highest dose (100%). The highest inhibition zone of *P. gingivalis* was observed for 0.12% CHX as the positive control and distilled water (DW) used as the negative control.

Table 2: MIC levels of both GT and SP extracts and their combinations.

Extract	Concentration (MIC) mg/mL	OD (SD)	Versus	P value*
SP	200	-	-	-
GT	6.25	0.0338 (0.0023)	Combination	0.605
			Broth	0.000
			CHX	0.002
Combination	6.25 (GT)+ 6.25 (SP)	0.0331 (0.0018)	Broth	0.000
			CHX	0.003
Broth	-	0.5107 (0.0076)	CHX	0.000
CHX	0.06%	0.0297 (0.0018)		

* Comparison by Mann-Whitney test, significant at $p < 0.05$., showed SP exerted no antibacterial effect against *P. gingivalis*. CHX: chlorhexidine 0.06%; OD: optical density; MIC: minimum inhibition concentration.

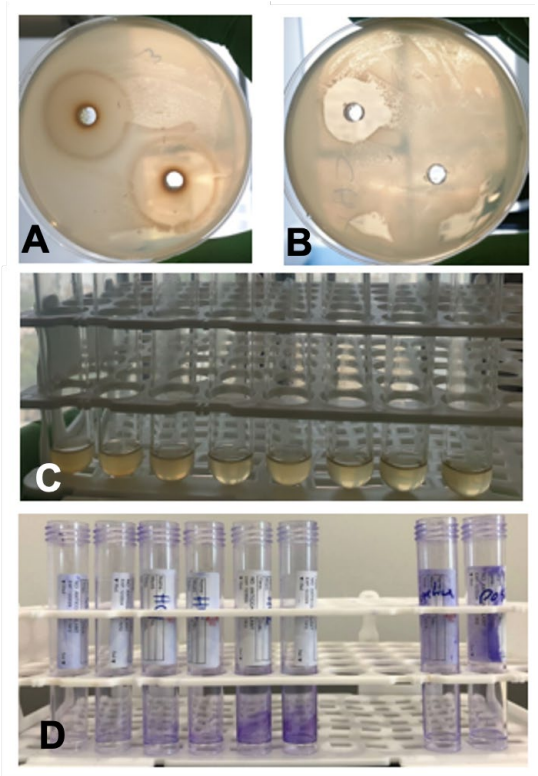

Figure 4: (A) Inhibition zone of GT, (B) Inhibition zone of SP, (C) MIC for GT and (D) antibiofilm activity of GT.

3.3 Antibiofilm Activity of GT, SP, and their Combination

The antibiofilm activity results showed a superior antibiofilm effect of SP aqueous extracts at a concentration of 6.25mg/mL equal to 0.06% CHX. While the GT aqueous extracts showed antibiofilm activity at a minimum concentration of 12.5mg/mL, the antibiofilm effect of the extract combination was shown at a concentration of 6.25mg/mL for each extract, as illustrated in figures 4D and figure 5.

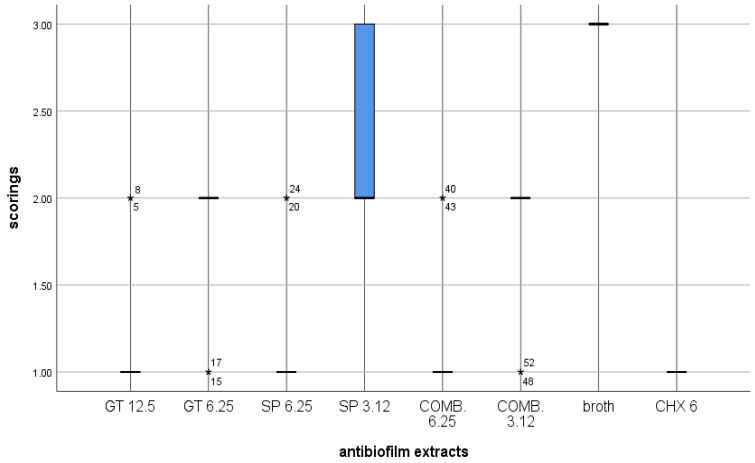

Figure 5: Antibiofilm activity of MIC of GT, SP, and their combination. The SP aqueous extracts had an antibiofilm effect at 6.25 mg/mL, similar to 0.06% CHX. Meanwhile, 12.5 mg/mL of GT aqueous extracts exhibited a comparable effect. On the other hand, the antibiofilm effect of the combination of the two extracts was found at 6.25 mg/mL for each extract. The antibiofilm tests for SP 3.12 mg/ml showed different scores for each test, ranging from score 2 to score 3 as represented by the blue line.

4. Discussion

Herbal medicine has been utilized in healthcare therapy in many developing countries for thousands of years, including use of natural products for periodontal therapy [29]. *P. gingivalis* is thought to be the key pathogen responsible for periodontal disease initiation and progression [30]. Therefore, determining the antibacterial efficacy of different local products against *P. gingivalis* has been the objective of many studies. There is encouraging data to support use of GT and SP extracts in the treatment of periodontal diseases. GT extracts in the form of mouthwash, toothpaste, chewing gum, strips or capsules have shown a positive impact in reducing periodontal pockets and gingival inflammation [31, 32]. On the other hand, oral rinsing with SP extracts exerts antibiofilm and anticarcinogenic effects as well as reduces gingival inflammation [33]. However, using these extracts as alternatives to the synthetic gold-standard CHX for treating periodontal diseases needs more solid studies. In this context, there is limited data regarding the antibacterial properties of GT and SP aqueous extracts against clinically isolated *P. gingivalis*. Thus, the aim of the present study was to assess the antibacterial effects of GT, SP extracts and their combination against this periodontal pathogen.

The main finding of the present study is that the antibacterial effect of the combination of GT and SP against *P. gingivalis* was attributed to GT only. SP extract showed no inhibitory effect against *P. gingivalis.* In contrast, GT extract exhibited high antibacterial activity against *P. gingivalis,* a result consistent with the outcomes of other studies [6, 34]. However, there is inconsistency in the MIC values of GT aqueous extract among the latter studies. This could be due to the phytochemical differences between the tested products as different methods of herbal extraction were used that may yield different active ingredients.

The antibacterial effects of GT aqueous extract may be related to its high caffeine content, as revealed by GC-MS analysis, which was reported to have antibacterial activities against Gram-negative bacteria [35]. Further, it has been reported that the capacity of *Pseudomonas aeruginosa* cells to produce biofilms was dramatically decreased when treated with caffeine [36]. In literature, GT catechins are the most commonly reported phytochemical components that have antibacterial activity against *P. gingivalis* [6, 34]. Both high-performance liquid chromatography and GC–MS are capable of detecting traces

of these chemicals in complex biological matrices [37]. However, catechins were not detected by GC-MS analysis in this study for unknown reasons. The use of water as solvent at an ambient temperature during maceration might negatively affect the extraction of catechins which may need heat to be properly extracted [38]. Hence, contents other than catechins in GT extracts, such as caffeine, might potentially have a role in the inhibition of the growth of *P. gingivalis.*

On the other hand, up to 100 mg/mL of the aqueous extracts of SP showed no effect against *P. gingivalis*, a finding that disagrees with what was previously reported [12, 13]. Other studies concluded that using SP sticks as toothbrushes showed no inhibitory effect against *P. gingivalis* [14, 39]. This could be explained by the heterogeneity of the bioactive ingredients of SP extracts and the methodologies used in the studies. Despite the lack of the antibacterial effect of SP aqueous extracts, a combination in a ratio of 1:1 between GT and SP extracts was made to test whether there would be an interaction between the two extracts. However, the results showed no change in the MIC value of GT aqueous extracts in this combination (i.e., 6.25mg/mL), and, therefore, SP extracts neither enhance nor inhibit the antibacterial activity of GT extract.

SP aqueous extracts demonstrated antibiofilm properties without showing significant antibacterial activity; the divergence can be clarified by the means through which the SP targeted the biofilm formation process without killing *P. gingivalis.* This could be due to the presence of compounds such as polyphenols and flavonoids that prevent bacterial adhesion to the surfaces. Further, the SP aqueous extract also has anti-quorum sensing properties because it stops bacteria from sending signals, which lowers the formation of biofilm. Finally, the extract could deteriorate current biofilms by breaking matrix integrity, which is important for biofilm protection and stability [40-42].

Microorganisms may create a multispecies community adhering to both biotic and abiotic surfaces, which is recognized as a biofilm. The biofilms developed on hard and soft tissues are responsible for various oral infections, such as dental caries, periodontal, and mucosal diseases. Generally, they exhibit antibiotic resistance, which is a main factor in the ineffectiveness of chemicals such as CHX for biofilm infections [43]. Thus, testing the antibiofilm properties of SP, GT extracts and their combination may further enhance our study concerning the biological properties of these extracts. The tubes used for MIC determination were utilized to estimate the antibiofilm effect by tube adhesion method. The results of this test suggested a superior antibiofilm influence of SP over the GT. These findings agree with the anti-adherence data reported by Abdulbaqi *et al*. [17], where SP was more efficient than GT at decreasing the number of bacterial cell attachments to glass beads after 30 sec of therapy with SP and GT aqueous extracts.

GC-MS analysis was performed for both extracts and their combinations to recognize the phytochemical constituents of the tested extracts. Analysis of GT aqueous extracts showed 12 peaks containing a high percentage of caffeine (60.299%), followed by 14.978%, 8.789%, and 6.749% of oleic acid, butanoic acid, and cis-13-octadecenoic acid, respectively. All of these components were found to have various biological activities according to databases by Dr. Duke's Phytochemical (https://phytochem.nal.usda.gov/phytochem/search). On the other hand, the GC-MS analysis of SP extracts showed 9 peaks containing a high percentage of glycerin (56.328%) and up to 20% of oleic acid and 5.816% of hexadecanoic acid. Benzyl isothiocyanate, salvadorine and thiocyanate were not detected in the SP extracts. Those chemicals were previously identified in SP extracts and were reported to contribute the inhibitory effect of the SP extracts against *P. gingivalis* [13, 44]. The finding that SP extract exhibited no inhibitory effect against *P. gingivalis* might be explained by the lack of those chemicals. However, extracting the abovementioned chemicals from SP sticks might need extraction processes or solvents other than those used in this study.

Finally, GC-MS analysis detected 18 peaks of GT and SP extracts combination, with caffeine constituting the highest percentage (40.14%), followed by about 28% of 2-methyl-Z, Z-3,13-octadecadienol. Such results may explain why there was no difference when comparing the antibacterial effects of GT aqueous extract alone and its combination with SP as the interaction between the two extracts in combination did not show any effective phytochemical changes.

It is important to acknowledge that water solvent was used to prepare GT and SP extracts in the current study. Water-based extracts of GT and SP were reported to exert profound antibacterial

activities against primary dental biofilm colonizers [17]. Although the use of alcohol (i.e., ethanol and methanol) as a solvent showed superior antibacterial effects compared to aqueous extracts, the use of alcohol was avoided. It is important to mention that the rationale of the present study was to estimate the antibacterial properties of a formulation that could be potentially used as an oral health product. For instance, it could be incorporated in toothpastes and mouthwashes as adjunctive chemicals to control dental biofilm daily. Alcohol-based oral health products have many limitations in some individuals, such as pregnant and nursing women, alcoholics, diabetics, youngsters, and xerostomia patients [45-47]. Therefore, a decision was taken to use the safer water solvent to prepare the extracts. Further, maceration was used for extracting GT and SP crude plants in this experiment. Heat was avoided in maceration as the components of solid plant mass were extracted by steeping the product in water at room temperature. The authors tried to protect the plant extracts' possibly thermolabile components, which could be impaired by other heat-dependent extraction methods [48].

The main limitation of the current study is that some biologically active ingredients of GT and SP were not detected by GC-MS analysis. It may be the case that those ingredients were not extracted by the used solvent and extraction protocol. However, alternative methods for phytochemical screening such as high-performance liquid chromatography should be considered in future research, which could be used for detecting chemicals that cannot vaporize or decompose at high temperatures [49]. Furthermore, a difficulty was noticed in scoring for the antibiofilm effect by tube adhesion method, especially between weak and negative biofilm scores. Thus, more accurate and reproducible methods for verification of the antibiofilm activity of herbal extracts are suggested for future studies, such as the tissue culture plate method [50]. Nevertheless, this is the first study to examine the antibacterial and antibiofilm effects of SP, GT and their combination against *P. gingivalis* which is considered as a keystone pathogen for periodontal disease. Further, the fact that clinically isolated *P. gingivalis* was used is another strong point of the present study as clinically isolated species are more representative of the clinical conditions compared to the laboratory reference of *P. gingivalis* which might have lost its pathophysiological features due to continuous subculturing over the decades since its isolation and exposure to various laboratories' tests [51].

5. Conclusions

The antibacterial activity of the combination of GT and SP extracts against clinically isolated *P. gingivalis* could be attributed to GT only. At MIC of 6.25 mg/ml, GT aqueous extracts had an antibacterial activity against clinically isolated *P. gingivalis*. SP demonstrated no inhibitory effect against clinically isolated *P. gingivalis,* while SP could potentially reduce the biofilm attachment of clinically isolated *P. gingivalis* better than GT extracts. However, further researches on the present topic are therefore recommended to validate the outcomes of this study, particularly the antibiofilm effect of the combination of both GT and SP, which could be used as natural herbal products to be incorporated in oral health products.

Acknoaledgements: The authors would like to acknowledge Sarhang S. Gul for providing technical assistance.

Author contributions: Murtadha Imad Abdulkareem: Investigation, Methodology, Writing – original draft. **Hayder Raad Abdulbaqi**: Conceptualization, Data curation, Formal analysis, Validation. **Aram Mohammed Sha**: Project administration, Visualization, Writing – review & editing. **Firas Bashir Hashim Al-Taweel:** Conceptualization, Invesitigtion, Writing – original draft. **Muhammad Saad Shaikh:** Project administration, Validation, Writing – Review & editing.

Data availability: Data will be available upon reasonable request.

Conflicts of 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.

Funding: The authors did not receive support from any organization for the submitted work.

References

[1] S. Murakami, B. L. Mealey, A. Mariotti, and I. L. Chapple, "Dental plaque–induced gingival conditions," *J. Clin. Periodontol.,* vol. 45, pp. S17-S27, 2018. doi: 10.1002/jper.17-0095.

[2] S. S. Socransky and A. D. Haffajee, "Dental biofilms: difficult therapeutic targets," *Periodontol. 2000,* vol. 28, no. 1, pp. 12-55, 2002. doi: 10.1034/j.1600-0757.2002.280102.x.

[3] J. Mättö, M. Saarela, S. Alaluusua, V. Oja, H. Jousimies-Somer, and S. Asikainen, "Detection of Porphyromonas gingivalis from saliva by PCR by using a simple sample-processing method," *J. Clin. Microbiol.,* vol. 36, no. 1, pp. 157-160, 1998. doi: 10.1128/jcm.36.1.157-160.1998.

[4] N. O'Brien-Simpson, K. Burgess, J. Lenzo, G. Brammar, I. Darby, and E. Reynolds, "Rapid chair-side test for detection of Porphyromonas gingivalis," *J. Dent. Res.,* vol. 96, no. 6, pp. 618-625, 2017. doi: 10.1177/0022034517691720.

[5] G. Hajishengallis and R. J. Lamont, "Breaking bad: Manipulation of the host response by P orphyromonas gingivalis," *Eur. J. Immunol.,* vol. 44, no. 2, pp. 328-338, 2014. doi: 10.1002/eji.201344202.

[6] J. Fournier-Larente, M.-P. Morin, and D. Grenier, "Green tea catechins potentiate the effect of antibiotics and modulate adherence and gene expression in Porphyromonas gingivalis," *Arch. Oral Biol.,* vol. 65, pp. 35-43, 2016. doi: 10.1016/j.archoralbio.2016.01.014.

[7] A. B. Lagha, S. Groeger, J. Meyle, and D. Grenier, "Green tea polyphenols enhance gingival keratinocyte integrity and protect against invasion by Porphyromonas gingivalis," *Pathog. Dis.,* vol. 76, no. 4, p. fty030, 2018. doi: 10.1093/femspd/fty030.

[8] M. Kushiyama, Y. Shimazaki, M. Murakami, and Y. Yamashita, "Relationship between intake of green tea and periodontal disease," *J. Periodontol.,* vol. 80, no. 3, pp. 372-377, 2009. doi: 10.1902/jop.2009.080510.

[9] A. Hagiu, T. Attin, P. R. Schmidlin, and L. L. Ramenzoni, "Dose-dependent green tea effect on decrease of inflammation in human oral gingival epithelial keratinocytes: in vitro study," *Clin. Oral Iinvestig.,* vol. 24, pp. 2375-2383, 2020. doi: 10.1007/s00784-019-03096-4.

[10] H. A. Lee, Y. R. Song, M. H. Park, H. Y. Chung, H. S. Na, and J. Chung, "Catechin ameliorates Porphyromonas gingivalis-induced inflammation via the regulation of TLR2/4 and inflammasome signaling," *J. Periodontol.,* vol. 91, no. 5, pp. 661-670, 2020. doi: 10.1002/jper.18-0004.

[11] M. M. Haque and S. A. Alsareii, "A review of the therapeutic effects of using miswak (Salvadora Persica) on oral health," *Saudi Med. J.,* vol. 36, no. 5, p. 530, 2015. doi: 10.15537/smj.2015.5.10785.

[12] Z. J. Esfahani, Z. Kadkhoda, S. S. Eshraghi, and M. H. S. Surmaghi, "Antibacterial effect of an herbal product persica on porphyromonas gingivalis and aggregatibacter actinomycetemcomitans: an in-vitro study," *J. Dent. (Tehran),* vol. 11, no. 4, p. 464, 2014.

[13] A. Sofrata, E. M. Santangelo, M. Azeem, A.-K. Borg-Karlson, A. Gustafsson, and K. Pütsep, "Benzyl isothiocyanate, a major component from the roots of Salvadora persica is highly active against Gram-negative bacteria," *PloS one,* vol. 6, no. 8, p. e23045, 2011. doi: 10.1371/journal.pone.0023045.

[14] R. Albabtain, L. Ibrahim, S. Bhangra, A. Rosengren, and A. Gustafsson, "Chemical effects of chewing sticks made of Salvadora persica," *Int. J. Dent. Hyg.,* vol. 16, no. 4, pp. 535-540, 2018. doi: 10.1111/idh.12359.

[15] F. A. Adam, N. Mohd, H. Rani, B. Baharin, and M. Y. P. M. Yusof, "Salvadora persica L. chewing stick and standard toothbrush as anti-plaque and anti-gingivitis tool: A systematic review and meta-analysis," *J. Ethnopharmacol.,* vol. 274, p. 113882, 2021. doi: 10.1016/j.jep.2021.113882.

[16] M. Farag, W. M. Abdel-Mageed, A. A. El Gamal, and O. A. Basudan, "Salvadora persica L.: Toothbrush tree with health benefits and industrial applications–An updated evidence-based review," *Saudi Pharm. J.,* vol. 29, no. 7, pp. 751-763, 2021. doi: 10.1016/j.jsps.2021.05.007.

[17] H. R. Abdulbaqi, W. H. Himratul-Aznita, and N. A. Baharuddin, "Anti-plaque effect of a synergistic combination of green tea and Salvadora persica L. against primary colonizers of dental plaque," *Arch. Oral Biol.,* vol. 70, pp. 117-124, 2016. doi: 10.1016/j.archoralbio.2016.06.011.

[18] H. R. Abdulbaqi, W. H. Himratul-Aznita, and N. A. Baharuddin, "Evaluation of Salvadora persica L. and green tea antiplaque effect: a randomized controlled crossover clinical trial," *BMC Complement. Altern. Med.,* vol. 16, pp. 1-7, 2016. doi: 10.1186/s12906-016-1487-0.

[19] R. Salah, H. R. Abdulbaqi, A. Mohammed, and A. A. Abdulkareem, "Four-day randomized controlled crossover trial evaluating the antiplaque effect of a combination of green tea and Salvadora persica L. mouthwash," *J. Herb. Med.,* vol. 23, p. 100357, 2020. doi: 10.1016/j.hermed.2020.100357.

[20] P. N. Papapanou *et al.*, "Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions," *J. Periodontol.,* vol. 89, pp. S173-S182, 2018. doi: 10.1002/JPER.17-0721.

[21] A. M. Sha and B. T. Garib, "Antibacterial effect of curcumin against clinically isolated Porphyromonas gingivalis and connective tissue reactions to curcumin gel in the subcutaneous tissue of rats," *BioMed Res. Int.,* vol. 2019, no. 1, p. 6810936, 2019. doi: 10.1155/2019/6810936.

[22] N. Gebhardt, *Fluorescence in Situ Hybridization (fish): Application Guide*. Springer, 2010.

[23] M. Sakamoto, Y. Takeuchi, M. Umeda, I. Ishikawa, and Y. Benno, "Rapid detection and quantification of five periodontopathic bacteria by real-time PCR," *Microbiol. Immunol.,* vol. 45, no. 1, pp. 39-44, 2001. doi: 10.1111/j.1348-0421.2001.tb01272.x.

[24] C. M. Wunsch and J. P. Lewis, "Porphyromonas gingivalis as a model organism for assessing interaction of anaerobic Bacteria with host cells," *J. Vis. Exp.,* no. 106, 2015. doi: 10.3791/53408.
[25] M. Balouiri, M. Sadiki, and S. K. Ibnsouda, "Methods for in vitro evaluating antimicrobial activity: A review," *J. Pharm. Anal.,* vol. 6, no. 2, pp. 71-79, 2016. doi: 10.1016/j.jpha.2015.11.005.
[26] S. Cavalieri *et al.*, "Manual of antimicrobial susceptibility testing. American Society for Microbiology," *Pan American Health Organization,* 2005.
[27] M. Eladawy, M. El-Mowafy, M. M. A. El-Sokkary, and R. Barwa, "Effects of lysozyme, proteinase K, and cephalosporins on biofilm formation by clinical isolates of Pseudomonas aeruginosa," *Interdiscip. Perspect. Infect. Dis.,* vol. 2020, no. 1, p. 6156720, 2020. doi: 10.1155/2020/6156720.
[28] K. Harika, V. P. Shenoy, N. Narasimhaswamy, and K. Chawla, "Detection of biofilm production and its impact on antibiotic resistance profile of bacterial isolates from chronic wound infections," *J. Glob. Infect. Dise.,* vol. 12, no. 3, pp. 129-134, 2020. doi: 10.4103/jgid.jgid_150_19.
[29] B. Anand, "Herbal therapy in periodontics: a review," *J. Res. Pharm. Sci.,* vol. 3, no. 5, pp. 1-7, 2017.
[30] S. C. Holt and J. L. Ebersole, "Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: the 'red complex', a prototype polybacterial pathogenic consortium in periodontitis," *Periodontol. 2000,* vol. 38, no. 1, pp. 72-122, 2005. doi: 10.1111/j.1600-0757.2005.00113.x.
[31] M. Mazur, A. Ndokaj, M. Jedlinski, R. Ardan, S. Bietolini, and L. Ottolenghi, "Impact of Green Tea (Camellia Sinensis) on periodontitis and caries. Systematic review and meta-analysis," *Jpn. Dent. Sci. Rev.,* vol. 57, pp. 1-11, 2021. doi: 10.1016/j.jdsr.2020.11.003.
[32] S. J. Gartenmann, Y. v. Weydlich, S. L. Steppacher, C. Heumann, T. Attin, and P. R. Schmidlin, "The effect of green tea as an adjunct to scaling and root planing in non-surgical periodontitis therapy: a systematic review," *Clin. Oral Investig.,* vol. 23, no. 1, pp. 1-20, 2019. doi: 10.1007/s00784-018-2684-7.
[33] E. Jassoma, L. Baeesa, and H. Sabbagh, "The antiplaque/anticariogenic efficacy of Salvadora persica (Miswak) mouthrinse in comparison to that of chlorhexidine: a systematic review and meta-analysis," *BMC Oral Health,* vol. 19, no. 1, pp. 64, 2019. doi: 10.1186/s12903-019-0741-5.
[34] S. Sakanaka, M. Aizawa, M. Kim, and T. Yamamoto, "Inhibitory effects of green tea polyphenols on growth and cellular adherence of an oral bacterium, Porphyromonas gingivalis," *Biosci. Biotechnol. Biochem.,* vol. 60, no. 5, pp. 745-749, 1996. doi: 10.1271/bbb.60.745.
[35] P. Pruthviraj, B. Suchita, K. Shital, and K. Shilpa, " Evaluation of antibacterial activity of caffeine," *Int. J. Res. Ayurveda Pharm.,* vol. 2, no. 4, pp. 1354-1357, 2011. https://ijrap.net/admin/php/uploads/604_pdf.pdf.
[36] P. Chakraborty *et al.*, "Inhibition of biofilm formation of Pseudomonas aeruginosa by caffeine: A potential approach for sustainable management of biofilm," *Arch. Microbiol.,* vol. 202, no. 3, pp. 623-635, 2020. doi: 10.1007/s00203-019-01775-0.
[37] J. L. Donovan, D. L. Luthria, P. Stremple, and A. L. Waterhouse, "Analysis of (+)-catechin,(−)-epicatechin and their 3'-and 4'-O-methylated analogs: A comparison of sensitive methods," *J. Chromatogr. B Biomed. Sci. Appl.,* vol. 726, no. 1-2, pp. 277-283, 1999. doi: 10.1016/s0378-4347(99)00019-5.
[38] S. Sakanaka and Y. Okada, "Inhibitory effects of green tea polyphenols on the production of a virulence factor of the periodontal-disease-causing anaerobic bacterium Porphyromonas gingivalis," *J. Agric. Food Chem.,* vol. 52, no. 6, pp. 1688-1692, 2004. doi:10.1021/jf0302815.
[39] M. Al-Otaibi, M. Al-Harthy, A. Gustafsson, A. Johansson, R. Claesson, and B. Angmar-Månsson, "Subgingival plaque microbiota in Saudi Arabians after use of miswak chewing stick and toothbrush," *J. Clin. Periodontol.,* vol. 31, no. 12, pp. 1048-1053, 2004. doi. 10.1111/j.1600-051X.2004.00618.
[40] T. AL Lafi and H. Ababneh, "The effect of the extract of the miswak (chewing sticks) used in Jordan and the Middle East on oral bacteria," *Int. Dent. J.,* vol. 45, no. 3, pp. 218-222, 1995.
[41] M. Khan, H. Z. Alkhathlan, and S. T. Khan, "Antibiotic and antibiofilm activities of Salvadora persica L. essential oils against Streptococcus mutans: A detailed comparative study with chlorhexidine digluconate," *Pathogens,* vol. 9, no. 1, p. 66, 2020. doi: 10.3390/pathogens9010066.
[42] E. Noumi *et al.*, "Phytochemical composition, anti-biofilm and anti-quorum sensing potential of fruit, stem and leaves of Salvadora persica L. methanolic extracts," *Microb. Pathog.,* vol. 109, pp. 169-176, 2017. doi: 10.1016/j.micpath.2017.05.036.
[43] A. S. Lynch and G. T. Robertson, "Bacterial and fungal biofilm infections," *Annu. Rev. Med.,* vol. 59, pp. 415-428, 2008. doi: 10.1146/annurev.med.59.110106.132000.
[44] S. Vahabi, E. Najafi, and S. Alizadeh, "In vitro antimicrobial effects of some herbal essences against oral pathogens," *J. Med. Plant. Res.,* vol. 5, no. 19, pp. 4870-4878, 2011. doi: 10.5897/JMPR.9000744.
[45] S. Mankodi *et al.*, "A 6-month clinical trial to study the effects of a cetylpyridinium chloride mouthrinse on gingivitis and plaque," *Am. J. Dent.,* vol. 18, pp. 9A-14A, 2005.
[46] D. Van Strydonck, M. Timmerman, U. Van Der Velden, and G. Van Der Weijden, "Plaque inhibition of two commercially available chlorhexidine mouthrinses," *J. Clin. Periodontol.,* vol. 32, no. 3, pp. 305-309, 2005. doi: 10.1111/j.1600-051X.2005.00681.x.
[47] J. Witt, P. Walters, S. Bsoul, R. Gibb, J. Dunavent, and M. Putt, "Comparative clinical trial of two antigingivitis mouthrinses," *Am. J. Dent.,* vol. 18, 2005.
[48] V. Seidel, "Initial and bulk extraction of natural products isolation," in *Natural products isolation*: Springer, 2012, pp. 27-41. doi: 10.1007/978-1-61779-624-1_2.
[49] K. S. Banu and L. Cathrine, "General techniques involved in phytochemical analysis," *Int. J. Adv. Res. Chem. Sci.,* vol. 2, no. 4, pp. 25-32, 2015.

[50] T. Mathur, S. Singhal, S. Khan, D. Upadhyay, T. Fatma, and A. Rattan, "Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods," *Indian J. Med. Microbiol.,* vol. 24, no. 1, pp. 25-29, 2006. doi: 10.4103/0255-0857.19890.

[51] N. E. Head and H. Yu, "Cross-sectional analysis of clinical and environmental isolates of Pseudomonas aeruginosa: biofilm formation, virulence, and genome diversity," *Infect. Immun.,* vol. 72, no. 1, pp. 133-144, 2004. doi: 10.1128/iai.72.1.133-144.2004.