Evidence-Based Complementary and Alternative Medicine

Innovations, Developments, and Applications of Natural Products in Oral Care

Lead Guest Editor: Lidia Audrey Rocha Valadas
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Research Article

GC/MS Profiling and *Ex Vivo* Antibacterial Activity of *Salvadora persica* (Siwak) against *Enterococcus faecalis* as Intracanal Medicament

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Introduction. *Salvadora persica* L. (*S. persica*, Siwak) has been used for many centuries as oral hygiene tools, particularly in Saudi Arabia. This study aimed to assess the effectiveness of *S. persica* petroleum ether extract (SPE) as an intracanal bactericidal for endodontic treatment against *Enterococcus faecalis*. Calcium hydroxide $Ca(OH)_2$ gold standard intracanal medicament was used for comparison. *Methods*. The gas chromatography mass spectrometry (GC/MS) analysis was carried out to identify the components of SPE. First, the consistency of SPE was accomplished according to ANSI/ADA specification no 57. Forty-five single-rooted mandibular premolars were infected with that of *E. faecalis* suspension. Colony-forming units (CFU) were counted before the medicaments' application (CFU-1) and after seven days of their applications (CFU-2). Group I: SPE, Group II: positive control $Ca(OH)_2$, and Group III: saline solution negative control. The microdilution method was applied to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of SPE. *Results*. Thirty-two compounds were identified (89.09%), with main components of benzyl isothiocyanate (BITC) (33.32%) and steroids (34%). CFU before and after using SPE and $Ca(OH)_2$ recorded a statistically significant reduction in bacterial count ($P = 0.006$) and ($P = 0.01$), respectively. There was an insignificant difference between CFU after using SPE and $Ca(OH)_2$ ($P = 0.210$). On the contrary, comparing both medicaments with the negative control saline group resulted in significant differences, ($P = 0.001$) and ($P = 0.007$), respectively. Moreover, the equality of minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of SPE is recorded. *Conclusion*. This finding could be referred to the high content of bactericidal BITC in synergism with other antimicrobial components, representing 70.71% of SPE. Thus, SPE is a good candidate as an intracanal medicament, which warrants further investigation.

1. Introduction

The ultimate goal of an infected root canal preparation, including cleaning, shaping, and using the disinfectant solution, is to entirely remove bacteria, their by-product, and pulpal remnants. The microorganisms in the root canal of the teeth are principally responsible for pulpal/periapical diseases. The primary intraradicular infections have been caused by many members of endodontic bacterial communities [1]. Some of them are persistent pathological bacteria, including *Streptococcus sanguinis, Streptococcus mutans, Enterococcus faecalis, Fusobacterium nucleatum,*

Porphyromonas gingivalis, and Prevotella intermedia [2]. However, *E. faecalis* is significantly more associated with asymptomatic cases of primary endodontic infections. Furthermore, *E. faecalis* is much more likely to be found in cases of failed endodontic therapy [3]. The antibacterial intracanal medication is used to eradicate bacteria in adults and children's root canal systems. Accordingly, the pain and inflammation of pulpal and periapical tissues are reduced [4, 5]. This represents an optimal root canal disinfection protocol that guarantees a high success rate of root canal treatment [6–8]. The most commonly used intracanal medicament is $Ca(OH)_2$ because of its significant bactericidal effect and inhibition of inflammatory exudates [8].

The foremost commonly utilized irrigants in endodontics are sodium hypochlorite (NaOCl), ethylenediamine-tetra-acetic acid (EDTA), and chlorhexidine (CHX), which can cause destructive side effects. Intracanal medicament such as $Ca(OH)_2$ causes collagen breakdown and leads to weakening of radicular dentin. Numerous plants are utilized as phytomedicines in dentistry since they have biological and antibacterial impacts. In endodontics, plants and their extracts can be utilized as irrigant and intracanal medicament to avoid the potential side effects caused by routine chemical agents [9].

Salvadora persica L. (*S. persica*. Siwak) is a plant whose roots, twigs, or stems have been used for many centuries as oral hygiene tools, particularly in Saudi Arabia. Using Siwak (tooth stick) for cleaning of mouth is advocated Islamic well-established beliefs [10].

Many studies have been demonstrated that extracts of *S. persica* possess various antiplaque, antiperiopathic, anticaries, anti-inflammatory, and antimycotic effects [11]. Extracts of *S. persica* as intracanal irrigant also proved significant antimicrobial activity on the oral pathogens in both *in vitro* and *in vivo* [12–14]. The present *in vitro* study was designed to assess the effect of an experimental intracanal medicament based on SPE against the most causative bacteria for endodontic pulpitis/periodontitis; *E. faecalis*. The gold standard intracanal medicament $Ca(OH)_2$ was employed as positive control. The null hypothesis: there would be no difference between $Ca(OH)_2$, the gold standard endodontic medicament, and SPE as an experimental intracanal medicament in bactericidal activity against *E. faecalis* for 7 days.

2. Materials and Methods

2.1. Materials. The materials used in this study were *S. persica* sticks brought from Riyadh region in Saudi Arabia, zinc oxide powder (zinc oxide; Prevest DenPro, Bari Brahmana, India), and $Ca(OH)_2$ endodontic medicament (MetaPaste; META BIOMED, Chungcheongbuk-do, Republic of Korea).

The plant was identified by Dr. Talal Dahan, assistant professor of plant classification at Bisha University, Saudi Arabia, and a voucher specimen (# 19750) was deposited in properly labeled polythene bags for future reference at the Herbarium Centre, College of Pharmacy, Umm Al-Qura University (UQU), Saudi Arabia. The authors followed IUCN Policy Statement on Research Involving Species at Risk of Extinction.

2.2. Preparation of SPE. The fresh plant sticks of *S. persica* were freeze-dried and then ground to a fine powder using a commercially available food blender. The ground sample (500 g) was used to prepare the extract. Consequently, petroleum ether extract was prepared by cold percolating 500 g of dried powder of the plant sticks in one liter of petroleum ether for 72 h, and every 24 h fresh solvent was used. The solvent was removed and recovered in a rotary evaporator (BÜCHI Rotavapor RII; Büchi Labortechnik, Flawil, Switzerland) at 40°C using a BÜCHI vacuum pump. At the last stage, the oily extract was freeze-dried to ensure that solvents were removed to yield 20 ml of an oily material (SPE) with a powerful aromatic odor. SPE was kept in a brown screw-capped tube in a −20°C freezer until further analysis.

2.3. Gas Chromatography-Mass Spectrometry GC/MS Analysis. Mass spectra were recorded using Shimadzu GCMS-QP2010 (Kyoto, Japan) equipped with Rtx-5MS fused bonded column (30 m × 0.25 mm internal diameter i.d ×0.25 μm film thickness) (Restek, USA) and a split-splitless injector. The initial column temperature was kept at 50°C for 3 min (isothermal), programmed to 300°C at a rate of 5°C/min, and kept constant at 300°C for 10 min (isothermal). Injector temperature was 280°C. Helium carrier gas flow rate was 1.37 ml/min. All the mass spectra were recorded applying the following conditions: (equipment current) filament emission current, 60 mA; ionization voltage, 70 eV; and ion source, 220°C. Diluted samples (1% v/v) were injected with split mode (split ratio) 1 : 15.

2.3.1. Compounds Identification. The identification of compounds was performed based on their retention indices relative to a homologous series of *n*-alkanes (C8–C28) injected under the same conditions and matching their mass spectra with National Institute of Standards and Technology (NIST) (Gaithersburg, United States) and Wiley Library database (John Wiley & Sons, Hoboken, New Jersey, United States) as well as literature [15–19].

2.4. Collection of Extracted Teeth. A total sample size of 15 samples for each group was sufficient to reject the null hypothesis that the bactericidal activity for $Ca(OH)_2$ and SPE was equal with probability (power) 0.8 as the true probability of success among the intervention group is 0.65. The Type I error probability associated with this test of this null hypothesis was 0.05. The sample size was calculated by G power program 3.1.9.2 (University of Düsseldorf, Düsseldorf, Germany). Forty-five single root premolars extracted teeth for periodontal or orthodontic reasons were obtained from the department of oral surgery at a different governmental and private hospital in Makkah city, Saudi Arabia. Teeth with previous endodontic therapy, fracture, crack, resorption, and root caries were excluded from the

study. The present study followed the ethical guidelines for clinical investigation: ethical policy of the American Dental Association (ADA) regarding the use of human subjects in clinical research and approved by the Institutional Review Board (IRB), 21 January 2019, Faculty of Dentistry, Umm Al-Qura University, Saudi Arabia (IRB # 122-19).

2.5. Preparation of the Experimental Siwak-Based Extract Endodontic Medicament

2.5.1. Determination of Consistency. The first intended procedure was to determine the appropriate powder/liquid mixing ratio (zinc oxide powder/SPE) for the experimental endodontic medicament. The purpose was to obtain a smooth and homogeneous material whose consistency is acceptable for clinical application. As well, the consistency should be analogous to the control endodontic medicament, commercial $Ca(OH)_2$ paste (MetaPaste). The consistency was accomplished according to ANSI/ADA specification no# 57 [20]. Using a sterile stainless spatula and glass slab, the powder was added to the SPE in small increments until the cement was of ideal consistency: that is, until it formed a 2.5 cm string to connect the spatula to the glass plate when the spatula is lifted from the mix (one-inch string method). The paste was creamy in consistency but quite heavy. On obtaining the adequate consistency, the constituents' powder and liquid were quantified. It was found that when one scoop provided with zinc oxide powder mixed with one drop of SPE resulted in the required consistency [21].

2.5.2. Preparation of Extracted Tooth. All forty-five single-rooted premolars were decoronated to standardize their root length. The working length was established to be 14-16 mm, and the roots was undergone rotary standardized instrumentation up to X3-ProTaper Next (DENTSPLY Tulsa Dental Specialties, Dentsply-Maillefer, Switzerland). An irrigant 2.5% sodium hypochlorite NaOCl (HYPOSOL; Prevest DenPro, Bari Brahmana, India) was used in each instrument and finally dried with size 25 absorbent paper points. Each root was placed in a closed test tube containing 3 ml of brain heart infusion (BHI) broth (BHI BROTH; SPML—Saudi Prepared Media Laboratory, Riyadh, Saudi Arabia) and sterilized by autoclaving at 121°C for 20 min and then incubated for 24 h at 37°C to confirm sterility by the absence of turbidity.

2.5.3. Preparation of E. faecalis Suspension. *E faecalis* ATCC 29212 strain was grown on Bile Esculin agar media (BILE AESCULIN AGAR; SPML—Saudi Prepared Media Laboratory, Riyadh, Saudi Arabia) at 37°C for 24 h. After growth, bacterial suspension was prepared from grown bacterial colonies by inoculation in brain heart infusion (BHI) broth and adjusted to the optical density of approximately 1.5×10^8 CFU/ml by comparing its turbidity to a 0.5 McFarland standard spectrophotometrically.

2.5.4. Infection of Root Canals. Two ml of sterile BHI broth was removed from each root containing tube and replaced by 2 ml of the prepared bacterial suspension. The tubes were then closed and incubated at 37°C for 48 h.

2.5.5. Colony-Forming Unit-1 (CFU-1). After 48 h, predetermined contamination period of root canals was elapsed, each root was removed from the tube under complete aseptic precautions, and irrigated with sterile saline (100 μl). Later, a dry sterile absorbent paper of point size 25 was inserted into the root canal and left for 5 min. Afterwards, these paper points were transferred individually to sterile test tubes, each containing 1 ml of sterile saline solution, vortexed for 30 seconds, and then four serial dilutions were made for each tube. Aliquots of 10 μl of each dilution were plated onto Bile Esculin (BE) agar plates and incubated at 37°C for 24 h. The grown bacterial colonies were counted and multiplied by their dilution factor to represent CFU-1/ml.

2.5.6. Ex Vivo Testing of Antimicrobial Activity of the Experimental Medicaments. The 45 decoronated teeth were randomly allocated into 3 equal groups ($n = 15$) by simple random sampling using random digit table depending on medicament used:

Group I: experimental Siwak extract-based medicament

Group II: $Ca(OH)_2$ paste (positive control)

Group III: sterile physiological saline as negative control group

Medicaments were placed into root canals with lentulo spiral (Dentsply-Maillefer, Swaziland) inserted to the full working length. The root canal foramina were sealed by sterile cotton pellets and the orifices closed by temporary restoration; then, the samples were kept in an incubator at 37°C for seven days.

2.5.7. Colony-Forming Unit-2 (CFU-2). Seven days later, each root was irrigated with sterile normal saline, and then the previous technique was repeated to obtain CFU-2.

2.5.8. The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of SPE. The MIC of the SPE was determined in 96 multiwell microtiter plates using microdilution method [22] with minor modifications. SPE was adjusted to a concentration of 50 mg/ml in cation-adjusted Mueller Hinton broth medium, then pipetted 50 μl of cation-adjusted Mueller Hinton broth medium into the first well of the plate, and 50 μl of broth medium was distributed from the 1st to the 12th well of each row. Twofold serial dilution was achieved by transferring 50 μl of scalar dilution from the first to the subsequent wells of each row. The final concentration of SPE adopted to evaluate antibacterial activity was included from 25 mg/ml to 0.003 mg/ml. Finally, 10 μL of *E. faecalis* suspension was

added to each well. Two row lines in each plate were used as controls: one row line with $Ca(OH)_2$ as a positive control (in a serial dilution of 25–0.003 mg/mL). Plates were incubated at 37°C for 18–24 h. The lowest concentration at which no turbidity occurred was taken as the MIC value. Plates were analyzed individually to determine MIC, and the average MIC values from three repeats were taken in determination of the final MIC values for each extract to ensure accuracy and reproducibility. To determine the MBC of SPE, 50 μl of the solution was removed from the well before the MIC well and the well after the MIC well. The solution was inoculated into tryptic soy agar plate and incubated at 37°C for 24 h.

2.6. Statistical Analysis. Data were collected, tabulated, and statistically analyzed using Statistical Package for Social Science (SPSS v.20, IBM. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM). $P < 0.05$ was considered as a level of significance. The mean and standard deviation were tabulated and statistically analyzed using one-way ANOVA test regarding the CFU-1 of the three tested groups before using intracanal medication. Regarding the CFU-2 of three groups, the mean and standard deviation were analyzed using one-way nonparametric ANOVA (Kruskal–Wallis). Finally, the antimicrobial effect of different tested medications on *E. faecalis* was tabulated and statistically analyzed using paired *t*-test.

3. Results

3.1. Chemical Composition of SPE. Analysis of SPE by GC and GC/MS revealed that SPE contained 32 compounds, of which benzyl isothiocyanate (33.32%), γ-sitosterol (25.76%), stigmasterol (5.92%), β-sitosterol acetate (2.28%), *n*-hexadecanoic acid (4.27%), and (*Z*)-11-octadecenoic acid (3.16%) were found to be the major constituents. Compounds are listed in order of their elution times on Rtx-5MS column in Table 1.

3.2. SPE Endodontic Therapy. The statistical analysis of the CFU-1 of three tested groups before using intracanal medications (SPE and $Ca(OH)_2$) was tabulated in Table 2. It was evident that there were no significant differences in bacterial counts among them ($P = 0.359$). Table 2 shows the statistical analysis using paired *t*-test of the number of the CFU of *E. faecalis* before and after applying the investigated medicaments at the root canal lumens; CFU-1 and CFU-2, respectively. The results showed a significant difference between CFU-1 and CFU-2 either in using SPE ($P = 0.006$) or $Ca(OH)_2$ ($P = 0.011$). On the other hand, the bacterial count of the negative control group showed a nonsignificant difference between CFU-1 and CFU-2 ($P = 0.438$). Finally, the bacterial count's significant difference among the three tested groups was compared using one-way nonparametric ANOVA. The values were insignificantly different between bacterial counts after using SPE and $Ca(OH)_2$ ($P = 0.210$). On the contrary, comparing both medicaments with the negative control saline group resulted in significant differences ($P = 0.001$) and ($P = 0.007$), respectively.

3.3. MIC and MBC of SPE. The MIC of SPE and $Ca(OH)_2$ were measured to be 3.5 and 4.2 mg/ml, respectively. No growth in the MIC well and the well before that and bacterial growth in the well after the MIC well indicated equality of MBC and MIC of SPE.

4. Discussion

The chemomechanical cleaning and shaping of the root canals of the teeth are essential for the success of endodontic therapy [23, 24]. Yet, intracanal medication is a crucial procedure during root canal treatment where the probability of complete eradication of bacteria is questionable [25].

It is well documented that the use of biocompatible intracanal medications possessing antimicrobial properties between appointments destroys and eradicates bacteria in the root canal system [26]. It was proven that *E. faecalis* is the most predominant bacteria found in the infected root canal [27]. Unfortunately, this species has the ability to survive in hard environment with deprived nutrients and alkaline pH reaching up to 11.5 that implies a challenge to be completely exterminated from root canal system [28, 29].

Calcium hydroxide $Ca(OH)_2$ is the gold standard intracanal medication used in the treatment of infected root canals, referring to its well-known antibacterial features and ease of application [30, 31]. The effect of both SPE and $Ca(OH)_2$ against *E. faecalis* was considered in the presence of a negative control group saline, to specify the antimicrobial effect. The antimicrobial effect of the experimental medicament SPE insignificantly surpasses that of $Ca(OH)_2$ ($P = 0.210$), as shown in Table 2. In the present investigation, SPE experimental intracanal medicament (Group l) proved an effective eradication of *E. faecalis*. This finding was in agreement with the results reported about the usefulness of SPE as endodontic irrigant against *E. faecalis* [32]. As well, it was evidenced that *S. persica* has an inhibitory effect on the growth of the oral pathogen [33]. The MIC value of SPE was in agreement with those reported for aqueous and methanol extracts of *S. persica* against *E. faecalis* [34] and for $Ca(OH)_2$ [35]. MIC of SPE is recorded in the current study for the first time.

In this study, the chemical profile representing 32 compounds of SPE was identified by GC/MS (89.09%) and listed in Table 1. The major component was found to be benzyl isothiocyanate. This compound was reported as the most robust antibacterial component with high bactericidal activity against Gram-negative periodontal pathogens [36]. The potent antibacterial effect of the experimental medicament, as shown in Table 2, suggested that SPE targeted the bacterial membrane, whereas BITC has both lipophilic and electrophilic properties. It is reported that BITC penetrates through the outer bacterial membrane and possibly interfered with the bacterial redox systems, thus hampering the bacterium's ability to maintain its membrane potential. Such an effect of BITC has been demonstrated for mitochondrial membranes [37].

In addition, there are γ-sitosterol (25.76%), *n*-hexadecanoic acid (4.27%), and (*Z*)-11-octadecenoic acid (3.16%) that are reported as potent broad-spectrum

Table 1: The chemical profile of SPE.

Peak #	R_t	Component	Molecular formula	RI_{exp}[a]	RI_{lit}[b]	Content (%)	Identification[c]
1.	18.35	Carvacrol	$C_{10}H_{14}O$	1288	1288	0.57	MS, KI
2.	20.47	Benzyl isothiocyanate	C_8H_7NS	1361	1359	**33.32**	MS, KI
3.	21.14	*n*-Tetradecane	$C_{14}H_{30}$	1384	1400	0.21	MS, KI
4.	23.76	*n*-Pentadecane	$C_{15}H_{32}$	1485	1500	0.38	MS, KI
5.	26.24	*n*-Hexadecane	$C_{16}H_{34}$	1582	1600	0.13	MS, KI
6.	34.40	*n*-Hexadecanoic acid (palmitic acid)	$C_{16}H_{32}O_2$	1948	1946	**4.27**	MS, KI
7.	34.91	Hexadecanoic acid ethyl ester	$C_{18}H_{36}O_2$	1972	1975	0.13	MS, KI
8.	36.12	Cyclic octaatomic sulphur	S_8	2034	2055	0.99	MS, KI
9.	37.86	Oleic acid	$C_{18}H_{34}O_2$	2130	2130	**1.95**	MS, KI
10.	37.92	(*Z*)-11-Octadecenoic acid	$C_{18}H_{34}O_2$	2133	2117	**3.16**	MS, KI
11.	38.15	(*Z*,*Z*)-9,12-Octadecadienoyl chloride (linoleic acid chloride)	$C_{18}H_{31}ClO$	2146	2139	0.37	MS, KI
12.	38.24	(*Z*)-9-Octadecenoic acid ethyl ester (oleic acid ethyl ester)	$C_{20}H_{38}O_2$	2151	2168	0.45	MS, KI
13.	38.36	9-Octadecenoic acid ethyl ester	$C_{20}H_{38}O_2$	2157	2171	0.14	MS, KI
14.	42.84	*p*-Cresol, 2,2′-methylene bis [6-tert-butyl-	$C_{23}H_{32}O_2$	2403	2398	0.13	MS, KI
15.	48.95	Squalene	$C_{30}H_{50}$	2796	2814	0.17	MS, KI
16.	49.51	N-Benzylpalmitamide	$C_{23}H_{39}NO$	2832	2880	0.36	MS
17.	51.09	Stigmasta-3,5-diene	$C_{29}H_{48}$	2934	—	0.77	MS
18.	51.69	Stigmasterol acetate	$C_{31}H_{50}O_2$	2972	2879	0.12	MS, KI
19.	51.87	3β-Acetoxystigmasta-4,6,22-triene	$C_{31}H_{48}O_2$	2984	-	0.24	MS
20.	52.06	*n*-Benzyloctadecenamide (*n*-benzyloleamide)	$C_{25}H_{41}NO$	2996	2991	0.53	MS, KI
21.	52.17	Stigmasta-5,22-dien-3-ol, acetate	$C_{31}H_{50}O_2$	3003	-	0.65	MS
22.	52.47	Clionasterol acetate	$C_{31}H_{52}O_2$	3022	-	0.50	MS
23.	52.66	Stigmastan-3,5,22-triene	$C_{29}H_{46}$	3034	2981	1.89	MS, KI
24.	52.94	β-Sitosterol acetate	$C_{31}H_{52}O_2$	3052	-	**2.28**	MS
25.	53.28	Cholesterol	$C_{27}H_{46}O$	3074	3075	0.66	MS, KI
26.	54.89	Campesterol	$C_{28}H_{48}O$	3177	3131	0.70	MS
27.	55.40	Stigmasterol	$C_{29}H_{48}O$	3210	3213	**5.92**	MS, KI
28.	56.44	γ-Sitosterol	$C_{29}H_{50}O$	3277	3290	**25.76**	MS, KI
29.	57.47	Unidentified	-	3344	-	**3.57**	-
30.	58.21	3,5-Stigmastadien-7-one	$C_{29}H_{46}O$	3391	-	0.56	MS
31.	59.04	Sitostenone	$C_{29}H_{48}O$	3444	3435	0.54	MS, KI
32.	60.19	Lupeol	$C_{30}H_{50}O$	3518	3451	1.24	MS
		% total identified				89.09	

Compounds are listed in order of their elution times on Rtx-5MS column. [a]RI_{exp}, retention index determined experimentally relative to C8 – C28 *n*-alkanes on Rtx-5MS column; [b]RI_{lit}, published retention indices; [c]Identification, was based on comparison of the compounds' mass spectral data (MS) and retention indices (RI) with those of NIST Mass Spectral Library (2017), Wiley Registry of Mass Spectral Data 8th edition and literature. Bold shows major components.

Table 2: The descriptive statistical analysis of the *E. faecalis* count before (CFU-1) and after (CFU-2) the application of the medicaments.

Bacterial count groups	CFU-1 Mean ± SD	CFU-2 Mean ± SD
Group I Siwak	$48.9 \pm 53.7 * 10^4$	$0.0250 \pm 0.1 * 10^4$
Group II $Ca(OH)_2$ (+ve control)	$51.6 \pm 68.4 * 10^4$	$0.07 \pm 0.25 * 10^4$
Group III Saline (−ve control)	$56.3 \pm 59 * 10^4$	$50 \pm 115 * 10^4$

antimicrobials [38–42]. Moreover, SPE has antimicrobial effect against both Gram-positive and Gram-negative bacteria due to the presence of carvacrol (0.57%) confirmed by Memar *et al*. (2017) [43]. SPE components in Table 1 show the presence of *n*-tetradecane (0.21%), *n*-hexadecane (0.13%), 11-octadecenoic acid (3.16%), squalene (0.17%), stigmasterol (0.12%), sitosterol (2.28%), and sitostenone (0.54%), and these components are potent antibacterial agents [44–48]. Therefore, SPE's strong bactericidal effect is attributed to the antimicrobial components that represents a percentage of 70.71% of the extract. It is worthy to mention that some components with anti-inflammatory and antioxidant effects are identified as oleic acid (1.95%), 11-octadecenoic acid (3.16%), 2,2′-methylenebis (6-*tert*-butyl-4-methylphenol) (0.13%), squalene (0.17%), *n*-benzyloleamide (0.53%), *n*-benzylpalmitamide (0.36%) stigmasta-5, 22-dien-3-ol (0.65%) lupeol (1.24%), and hexadecanoic acid, ethyl ester (0.13%) [46, 47, 49].

Based on the obtained results, the null hypothesis is accepted as the experimental intracanal medicament SPE, a novel trial in the field of endodontic treatment, has non-significant difference in antimicrobial activity comparing to the gold standard; $Ca(OH)_2$ against *E. faecalis*. However, further studies are suggested to explicate SPE effect against

other reported endodontic pathogens. Moreover, the limitations of this study as the assessment of the activity against the biofilm formation that resembles the clinical situation should be investigated after the evaluation of the effect of SPE in an in *vivo* study. SPE comprises potent antimicrobial and anti-inflammatory components as identified by GC/MS. Thus, it could be claimed that it will be a robust intracanal medicament. However, its anti-inflammatory effect is still under investigation by the authors.

5. Conclusion

Within the limitations of this *Ex vivo* study, SPE can efficiently eradicate *E. faecalis* from the root canal system by many antimicrobial components. It could be used as a good alternative to calcium hydroxide in endodontic therapy; however, further studies are recommended to investigate its efficacy in an *in vivo* assessment and inhibitory effects on biofilm formation before engaging it in regular dental practice.

Data Availability

The data that support the findings of this study are openly available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

N. A., N. B., and S. A. conceptualized the study; A. A., R. A., R. Q., N. S., and I. A. were responsible for methodology; A. N. was responsible for software; N. S., N. A., and N. B. validated the data; N. S. performed formal analysis and visualized the study; N. A. investigated the data and contributed resources and was involved in project administration and funding acquisition; A. A. and R. A. contributed to data curation; A. A., R. A., N. S., and I. A. prepared the original draft; N. A., N. S., and N. B. reviewed and edited the manuscript; N. B. performed study supervision. All authors have read and agreed to the published version of the manuscript.

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Supplementary Materials

S1: *Salvadora persica plants*' sticks. S2: *Salvadora persica* pet-ether extract. S3: prepared 45 extracted teeth. S4: prepared 45 extracted teeth in BHI tubes and prepared for incubation at 37°C for 48 hours. Two ml of sterile BHI broth was removed from each root-containing tube and replaced by 2 ml of the prepared bacterial suspension. The tubes were then closed and incubated at 37°C for 48 h. S5: bile esculin (BE) agar showing a high number of growing colonies of *E. faecalis* before application of medicaments. S6: BE agar showing no growing colonies of *E. faecalis* after application of Siwak as a medicament (CFU2). S7: BE agar showing a few number of growing colonies of *E. faecalis* after application of $Ca(OH)_2$ (CFU2). S8: BE agar showing a high number of growing colonies of *E. faecalis* after using saline (CFU2). S9: GC/MS total ion chromatogram (TIC) of *Salvadora sp.* petroleum ether extract. GCMS revealed the identification of 32 compounds from SPE by comparing their mass spectra with the National Institute of Standards and Technology (NIST) (Gaithersburg, United States) and Wiley library database (John Wiley & Sons, Hoboken, New Jersey, United States), as well as the literature. (*Supplementary Materials*)

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Research Article

Evaluation of Cytotoxic and Antimicrobial Properties of Iranian Sea Salts: An *In Vitro* Study

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Background. Dental caries is known as a multimicrobial disease. Caries are very prevalent in numerous countries, and the incidence is higher in underdeveloped countries than in developed countries. Dental caries is a major public health problem, and it is the most prevalent health problem across the world, affecting 2.4 billion people. Natural mouthwashes can be beneficial in the prevention of dental caries and oral infections without the side effects of synthetic mouthwashes. *Aim*. The aim of the present study was to investigate the antibacterial, antifungal, and cytotoxicity properties of sea salt from different areas of Iran. *Methods and Materials*. Sea salts from different areas (Urmia, Qom, and Jarquyeh) of Iran were collected. In order to define the elemental and mineralogical features of different salt samples, X-ray powder diffraction (XRD) was employed. Different concentrations (0.19–50 mg/mL) of sea salt were used in the antimicrobial and antibiofilm tests. The antimicrobial (MIC, MBC, MFC, and DAD tests) and antibiofilm (formation and degradation tests) effects were evaluated against *L. acidophilus, S. aureus, E. coli, S. mitis, S. mutans, S. salivarius*, and *C. albicans*. The cytotoxic effect of salts was evaluated on human gingival fibroblasts by the MTT test. *Results*. The range of MIC values in mg ml^{-1} was as follows: *S. salivarius* (50), *S. mutans* (50), *S. mitis* (50), L. *acidophilus* (12.5 to >50), *C. albicans* (50), *E. coli* (12.5 to 25), and *S. aureus* (12.5 to 25), while MBC values were, *S. mutans* (>50), S. salivarius (>50), *S. mitis* (>50), *L. acidophilus* (50 to >50), *C. albicans* (>50), *E. coli* (50), and *S. aureus* (50). MTT results showed that more than 50% of cell viability depends on decreasing the salt concentration (<1.56 mg/ml). *Conclusion*. Sea salts had significant antimicrobial effects on cariogenic bacteria and *C. albicans*. Therefore, sea salts can be a suitable candidate for mouthwash.

1. Introduction

Dental caries is a multifactorial disease which is considered by local destruction of the tooth. Dental biofilm plays an important role in the progression of periodontal diseases and caries [1]. Evidence from the decayed, missing, and filled index (DMF), published by FDI (Federation Dental International), presented that carious lesions are highly prevalent in many countries and are higher in underdeveloped countries than in developed countries. Therefore, dental

caries is a major public health problem [2]. A microbial biofilm contains a microbiological community. They are organized into an extracellular matrix. They stick to tooth surfaces. Poor oral hygiene and high consumption of fermentable carbohydrates cause an imbalance in the biofilm. These situations cause demineralization [3]. An important factor to initiate carious is the presence of bacteria, mostly *Streptococcus mutans*, though it is not sufficient for caries development. In carious lesions, additional microbial species were also isolated such as Gram-negative bacteria *lactobacilli*. They have been related to the dental caries [1, 4–6]. The metabolism of these species of bacteria produces acids, which decrease pH and result in demineralization of the tooth tissue [6]. *Streptococcus mutans*'s capability to make extracellular polysaccharides (mainly glucans) is an essential factor for tooth caries [1, 3, 5]. The production of acids, especially lactic acid, is a very significant virulence factor of *S. mutans* which leads to caries and an acidic environment [5, 7]. The acidic environment determined by the bacterial plaque decalcifies the enamel and/or dentine [6, 7]. *Lactobacilli* should be considered as "secondary invaders." They are not capable of producing caries [3, 5–7]. Bacteria produce glucans from nutritional carbohydrates by glucosyltransferases (GTFs). Glucans are important for oral pathogens to stick to and accumulate on the tooth surface [1]. Many efforts have been made to remove cariogenic microorganisms from the oral cavity. In preventing dental caries antimicrobials such as ampicillin, penicillin, and tetracycline have been very effective. However, prolonged application of these substances causes unwanted side effects such as microorganisms' susceptibility, diarrhea, vomiting, and tooth staining. Sanguinarine harvest from the *Sanguinaria canadensis*. It has an extensive range of action against several oral bacteria. It has been used in different oral care products due to its robust antibacterial usefulness. It was reported to be related with oral leukoplakia, therefore its application had been decreased. These complications necessitate more research for natural antibacterial materials which are specific for oral microorganisms and safe for humans [8, 9]. Chemical agents have potential side effects. For example, chlorhexidine gluconate (0.12%) could result in dryness, staining of teeth, loss of taste sensation, and allergies. Therefore, there is a need for tissue friendly and cost-effective rinses. Saltwater rinses have been used after extractions, alveolar osteitis, and oral infections [10]. Saltwater rinses inducing vasodilation and lowering the bacterial load, simplifying phagocytes to the injury site, alkalizing saliva, and acting as an astringent [10]. Furthermore, saltwater improves wound healing through reducing inflammation and contracting the tissues [11]. There is very little available data on the efficiency of sea salt. Sea salt is produced via evaporation of saltwater lakes or oceans with very little processing, but table salt is typically gained from underground salt deposits and severely processed [10, 12, 13]. Refined salt typically contains about 99.5–99.9% NaCl and some additives such as anticaking agents and whitening. "Natural" sea salt contains no additives and is not chemically processed. This salt is naturally evaporated by the sun allowing the sea salt to hold its natural mineral content and some trace heavy metals [10, 12, 13]. The aim of this study was to evaluate the efficacy of sea salts from different origins in Iran in controlling some oral microorganisms and their effect on normal human gingival fibroblast cells.

2. Materials and Methods

2.1. Materials. *Streptococcus salivarius*, *Streptococcus mutans*, *Streptococcus mitis*, *Candida albicans*, *Lactobacillus acidophilus*, *Staphylococcus aureus*, *and* Escherichia coli, and human gingival fibroblast cells were gifted. Brain heart infusion (BHI) broth and agar and yeast peptone dextrose (YPD) broth and agar and crystal violet were provided from Merck (Darmstadt, Germany). The MTT Kit was purchased from Bioidea (Iran). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Dulbecco's modified Eagle's medium (DMEM), trypsin, fetal bovine serum (FBS), phosphate-buffered saline (PBS), antistreptomycin, and betaglycerol were bought from Gibco (New York, USA).

2.2. Sea Salt Sampling

2.2.1. Sea Salt Sampling. Sea salts were collected in 2020 from Urmia (West Azerbaijan) (37.6822° N, 45.3943° E), Qom (34.5239° N, 51.8946° E), and Jarquyeh (Isfahan Province) (32.09548° N, 52.74346° E) (Figure 1).

2.2.2. Identification of Sea Salt Mineral Elements. Elemental characteristics of salt samples were characterized by X-ray powder diffraction (XRD) (using a Philips PW3710 X-ray equipped with Cu Kα radiation and a secondary graphite monochromator at 40 kV and a current of 30 mA in a range from 2 to 70° 2θ/degree, with a speed of 3.0 2 θ/min). Patterns were recognized by X'Pert HighScore Plus with a PAN-ICSD database [12].

2.3. Cytotoxic Evaluation. Human gingival fibroblasts were incubated in 96-well plates with different concentrations (0.19–50 mg/mL) of sea salt in the growth medium. Cell survival was analyzed in this test containing 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide. 2×10^5 cell/ml of the fibroblast cells were plated in each well. Then, different concentrations of sea salt samples (0.19–50 mg ml^{-1}) with DMEM (serum-free) were added (100 µl/well). Then, the cultures were incubated (37°C) (moistened atmosphere of 5% CO_2) for 24 h. In the next step, using the MTT solution (5 mg/ml), cell growth induction activity was assessed. MTT solutions were added to each well, and the plates were incubated (4 h at 37°C) (moistened atmosphere of 5% CO_2). Dimethylsulfoxide (DMSO) (1000 µl) was used to change the well's medium. DMSO dissolves the dark blue crystals. The plates were read with an ELISA reader (EL *X* 808) after 10 min (room temperature) at 570 nm as the test wavelength and 630 nm as the reference wavelength. An MTT-based method was used to analyse cell mitochondrial activity after 24 h and 48 h of conditioning. Data were changed into percentages of viable cells [14, 15]. The MTT assay measures the mitochondrial and metabolic

Figure 1: Three samples were collected from different regions of Iran.

activity of treated cells. The MTT assay is broadly known as a reliable method to study cell viability [14, 15]. The results were presented as percentages (control value = 100%). All tests were done three times. The percentages of cell viability were measured using the following equation:

$$\text{the percentage of cell viability} = \frac{\text{samples (OD)}}{\text{control (OD)}} \times 100. \quad (1)$$

2.4. Antimicrobial Activity of the Sea Salts

2.4.1. Bacterial Strain and Inoculum Preparation for Evaluation of Minimum Inhibitory (MIC), Minimum Bactericidal Concentration (MBC), and Minimum Fungicidal Concentration (MFC). The bacterial and fungal strains used in this experiment were *Streptococcus mitis, Streptococcus mutans, Streptococcus salivarius, Lactobacillus acidophilus, Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans.* The bacterial strains were reactivated in brain heart infusion agar (BHI) medium ((37°C, (5% CO_2) (48 h)). Then, a loopful of bacteria were suspended in BHI Broth medium (25 mL) (Merck, Darmstadt, Germany). After incubation, the concentration of cells was determined (37°C for 24 h). In a spectrophotometer (at 625 nm) (absorbance of 0.18), a cell density equivalent of 1.0×10^8 CFU/mL was obtained [16]. A concentration of 1.0×10^5 CFU/mL was used for the MIC assay [4]. The stock culture of *C. albicans* was inoculated initially into sterilized yeast peptone dextrose (YPD) broth to form the *C. albicans* suspension. A concentration of 1.0×10^5 CFU/mL was used for the MIC assay (18, 19).

2.4.2. Determination of MIC, MBC, and MFC Tests. BHI and YPD broth (100 μL) was injected into the wells of 96-well microtiter plates to assess MIC. In the next stage, sea salts (100 μL) were inserted into the first column of the wells at their primary concentration (50 mg/ml). Then, by transferring well content (100 μL) from the highest concentrated to the least concentrated, the sea salt solution was sequentially diluted (from 50 to 0.19 mg/mL) (1 : 1 v/v) [16]. In the next stage, 100 μL of the well contents of the last column were thrown away. At the final stage, bacterial and fungal inoculums were inserted (100 μL) (1.0×10^5 CFU/mL). The control groups were used as follows: (1) growth control (only microbial content without any antimicrobials); (2) antimicrobial control (Chlorhexidine (CHX) 0.2%); and (3) sterility control (only sterile culture medium). The microplates were incubated (37°C, 5% CO_2, and 24 h) [4].

2.4.3. Disk Agar Diffusion Test (DAD). The antimicrobial activity of sea salt solutions was determined by DAD using brain-heart infusion. Colonies of different strains grown on BHI agar were suspended in NaCl solution (145 mM). Then, they were adjusted to the McFarland 0.5 scale by a spectrophotometer. Sea salt solutions (400 ml) were mixed with BHI agar (40 ml) (at 45°C). Then, they poured on a set layer of BHI agar [17, 18]. Then, strains were inoculated on plates by sterile swabs [17, 18]. 0.5 ml of suspension of inoculums having 3×10^8/ml of strains was streaked on BHI agar. By the DAD test, the antibacterial activity of sea salt solutions was measured. The plates of sea salt solutions were filled with 0.08 ml each of 2× MBC of each sea salt and 0.2% CHX (positive control). Then, plates were incubated (for 48 h) (at 37°C). The inhibition zone around the wells was measured and noted [17, 18].

2.4.4. Biofilm Formation and Degradation Evaluation. Biofilm growth was assessed by the crystal violet staining method. The strains were cultured in microplates with sucrose (1%) and sterile BHI agar. Microplates were cultured with 2×MBC of each sea salt solution, under anaerobic conditions at 37°C with CO_2 5% for 48 h. Then, the broth was eliminated and the microplates were washed with PBS 3 times to remove nonadherent bacteria. Then, microplates were dried for 45 minutes at 60°C. Then, crystal violet (1% (w/v)) (100 μL) solution was added to each well. Then, microplates were incubated for 15 minutes. Using PBS, the microplates were washed. By adding ethanol (95%) (125 μL) to each well, biofilm formation was determined. With a microplate reader, the optical density of wells (OD) was measured in comparison to the control biofilm (without sea salt) at 590 nm [19]. The mean absorbances of the samples were assessed, and by the following formula, the percentage inhibition gained for the sea salt solutions at different concentrations was measured:

$$\text{the biofilm formation rate} = \frac{\text{samples (OD)}}{\text{control (OD)}} \times 100,$$

$$\text{the biofilm reduction rate} = 100 - \left(\frac{\text{samples (OD)}}{\text{control (OD)}}\right) \times 100, \quad (2)$$

where $\text{OD}_{\text{treatment}}$ and $\text{OD}_{\text{control}}$ refer to the absorbance at 570 nm in each well with and without the samples, respectively, after the addition of the dissolving solution.

2.5. Statistical Analysis. The results were analyzed by Tukey post hoc test and one-way ANOVA to compare means among groups. Statistical evaluation becomes carried out with SPSS statistics model 20.

3. Results

3.1. Identification of Sea Salt Mineral Elements. The results for the mineral elements of the sea salt samples are found in Table 1.

3.2. Cell Viability Evaluation. To determine the possible effect of sea salt samples on cell growth, cells were incubated with different extracts (0.19 to 50 mg/ml) (for 24 and 48 h). Using samples, a significant reduction of viable cells was observed in a dose and time-dependent pattern. By determination of the optical density of vital cells after treatment with the samples for incubation of 24 and 48 h, the cell viability percentage for both cell lines was calculated and compared to the percentage of the control group. The cell viability was increased by decreasing the concentration of salt, and the 1.56 mg/ml was considered as more than 50 percentage of cell viability. Results are shown in Figure 2.

3.3. Antimicrobial Analysis

3.3.1. MIC. MIC values of the sea salt solutions against strains were determined by the broth microdilution method (Table 2). The range of MIC values in mg ml^{-1} was as follows: *S. salivarius* (50); *S. mutans* (50); *S. mitis* (50); L. *acidophilus* (12.5 to >50); *C. albicans* (50); *E. coli* (12.5 to 25); and *S. aureus* (12.5 to 25) (Table 2).

3.3.2. MBC and MFC. The range of MFC and MBC values in mg mL^{-1} were *S. mutans* (>50); *S. salivarius* (>50); *S. mitis* (>50); *L. acidophilus* (50 and > 50); *C. albicans* (>50); *E. coli* (50); and *S. aureus* (50) (Table 2).

3.3.3. Disk Agar Diffusion Analysis. The means of microbial growth inhibition zones. Results were depended on the samples and on the strains. The range of zones of microbial growth inhibition by sea salt samples was (mm) as follows: *S. mutans* (5 to 6 mm); *S. salivarius* (4 to 6 mm); *S. mitis* (4 to 6 mm); *L. acidophilus* (4 to 5 mm);*C. albicans* (4 to 5 mm); *E. coli* (3 to 4 mm); and *S. aureus* (4 to 5 mm) (Table 3).

3.3.4. The Effect of Sea Salt Solutions on the Formation of Microbial Biofilm (Crystal Violet Staining Assay). The effect of samples on the prevention of biofilm formation was tested using the microdilution method. Biofilm formation rates of samples are reported in percentages in Table 4. These percentages compare the biofilm formation of the tested microorganisms during exposure of different samples with the control group by measuring the OD of each well (at 570 nm wavelength) (Table 4). All salt solutions had antibiofilm properties. Sea salt solutions had relatively similar results.

Table 1: Sea salt mineral elements.

Elements (ppm)	S1	S2	S3
Ag	<0.1	<0.1	<0.1
Al	<0.1	<0.1	0.6165
As	2.292	3.404	1.8495
Be	<0.1	<0.1	<0.1
Ca	814.424	1615.198	2681.159
Cd	<0.1	<0.1	<0.1
Co	<0.1	<0.1	<0.1
Cr	<0.1	<0.1	<0.1
Cu	<0.1	<0.1	<0.1
Fe	<0.1	<0.1	<0.1
Hg	<0.1	<0.1	<0.1
K	640.232	349.761	1557.896
Mg	871.724	645.909	3685.437
Mn	<0.1	<0.1	<0.1
Ni	<0.1	<0.1	<0.1
P	6.876	7.659	5.5485
Pb	1.528	<0.1	<0.1
S	507.296	717.393	1131.278
F	<0.1	<0.1	<0.1
Sr	4.584	17.02	51.1695
V	0.764	<0.1	<0.1
Zn	<0.1	<0.1	<0.1

Urmia (S1), Qom (S2), and Jarquyeh (S3).

3.3.5. The Effect of Sea Salt Solutions on the Degradation of Microbial Biofilm (Crystal Violet Staining Assay). The effect of treatment with samples on biofilms which had already been formed was tested using the same method. In this case, the biofilm reduction rate in percentage was calculated (Table 5). All salt solutions had antibiofilm properties. Sea salt solutions had relatively similar results.

4. Discussion

Some methods to control dental caries include the use of varnish, fluoride gel, professional tooth cleaning, antimicrobial agents, fissure sealant, fluoride mouthwashes, fluoride gels for personal use, fluoride supplements, oral hygiene, diet control, and noncariogenic sweeteners like xylitol. Natural products can also be used as coadjutant factors for caries control. Natural materials are a useful substitute to synthetic antimicrobials [16]. Many bacteria are involved in the carious procedure. The beginning of the caries is not dependent on the presence of *S. mutans* only. The *S. mutans*' biofilm is commonly selected to assess the antibacterial activities of various materials. Sucrose and *S. mutans* are the main factors of the growth of biofilms [5, 16]. Mechanical plaque control is the main method for plaque control, but it needs patient motivation and cooperation, so chemical agents can be useful adjutants for achieving plaque control. Chloride helps the host's defense against infection. It serves as a substrate for the production of chlorine bleach (microbicide) by stimulated neutrophils and helps maintain ionic homeostasis for antimicrobial activity inside phagosomes [11].

Sea salt makes the environment alkaline, thereby accelerating the healing of the surgical wound [7]. Sea salt

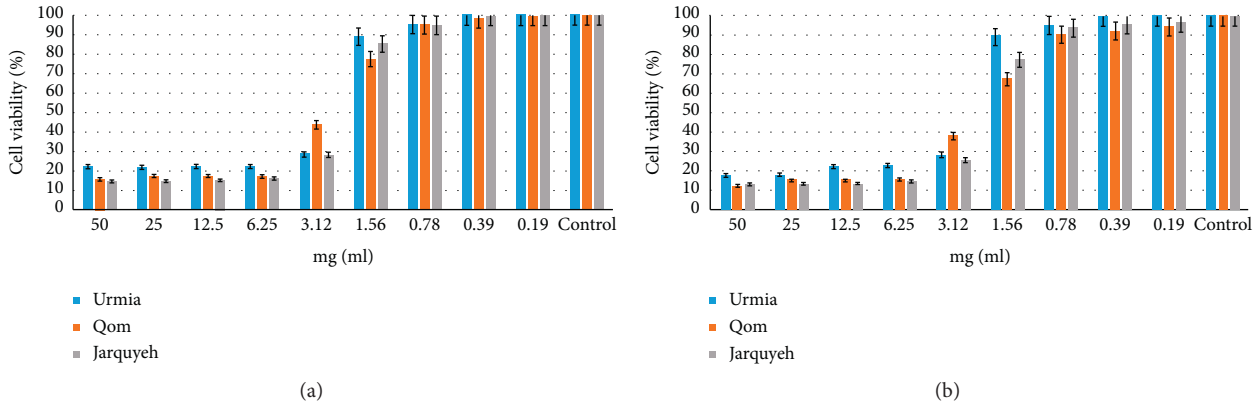

Figure 2: The percentage of cell viability by MTT exclusion on fibroblast cell lines. (a) 24 h and (b) 48 h. Data are expressed as mean ± SD ($n = 3$).

Table 2: MIC, MBC, and MFC in mg·mL^{-1} of sea salts obtained using the broth microdilution method.

Bacteria	Sample 1		Sample 2		Sample 3	
	MIC (mg/ml)	MBC/MFC (mg/ml)	MIC (mg/ml)	MBC/MFC (mg/ml)	MIC (mg/ml)	MBC/MFC (mg/ml)
E. coli	25	50	25	50	12.5	50
S. aureus	12.5	50	25	50	25	50
S. mutans	50	>50	50	>50	50	>50
S. salivarius	50	>50	50	>50	50	>50
S. mitis	50	>50	50	>50	50	>50
L. acidophilus	12.5	50	50	>50	25	50
C. albicans	50	>50	50	>50	50	>50

All samples were tested three times in independent experiments. Results show the insignificant difference between samples. Urmia (sample 1), Qom (sample 2), and Jarquyeh (sample 3).

Table 3: Mean area of microbial growth inhibition zones in mm ($n = 3$) provided by the sea salt samples.

Bacteria	MBC/MFC concentrations			CHX 0.2%
	Sample 1	Sample 2	Sample 3	
E. coli	4	3	4	15
S. aureus	4	4.5	5	17
S. mutans	6	5	5	18
S. salivarius	5.5	6	4	17
S. mitis	5	6	4	17
L. acidophilus	4	5	4	16
C. albicans	5	5	4	16

The difference was significant between the samples and control (CHX 0.2%) ($p < 0.01$). Results show the insignificant difference between samples. Urmia (sample 1), Qom (sample 2), and Jarquyeh (sample 3).

Table 4: The effect of sea salts on the biofilm formation of microbial biofilms (percentages).

Bacteria	OD (570 nm)			CHX 0.2 (%)
	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)	
E. coli	85	90	85	20
S. aureus	85	90	85	22
S. mutans	90	85	80	20
S. salivarius	90	90	85	25
S. mitis	85	90	85	25
L. acidophilus	85	90	80	20
C. albicans	85	85	80	22

All samples were tested three times in independent experiments. The difference was significant between the samples and control (CHX 0.2%) ($p < 0.01$). Results show the insignificant difference between samples. Urmia (sample 1), Qom (sample 2), and Jarquyeh (sample 3).

increases the pH of the oral environment and helps reduce the acidity created by bacteria [7, 10, 11]. Also, saltwater decreases inflammation and contracts the tissues [11]. Saltwater induces vasodilation and helps phagocytes, lowering the bacterial load. Salt is recognized as osmosis kills some types of bacteria, effectively by sucking water out of them. Bacterial enzymes cannot function without water, and eventually, the cell can be collapsed [10].

In this study, the range of zones of microbial growth inhibition by sea salt samples was as follows: *S. mutans* (5 to 6 mm); *S. salivarius* (4 to 6 mm); *S. mitis* (4 to 6 mm); *L. acidophilus* (4 to 5 mm); *C. albicans* (4 to 5 mm); *E. coli* (3 to 4 mm); and *S. aureus* (4 to 5 mm). The best results were, respectively, related to Urmia, Qom, and Jarquyeh. In this study, we evaluate the effect of sea salts on the formation and degradation of microbial biofilm. Sample 2 (Qom) had the highest effect on the formation of biofilm, and sample 3 (Jarquyeh) had the lowest effect on the formation of biofilm. Sample 3 had the highest effect on the degradation of biofilm, and sample 2 had the lowest effect on the degradation of biofilm.

Table 5: The effect of extracts on the degradation of microbial biofilms (percentages).

Bacteria	OD (570 nm)			CHX 0.2 (%)
	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)	
E. coli	11	8	10	70
S. aureus	12	9	11	70
S. mutans	8	10	10	72
S. salivarius	10	10	11	75
S. mitis	11	10	11	73
L. acidophilus	12	9*	13**	71
C. albicans	7	10	11	70

All samples were tested three times in independent experiments. The difference was significant between the samples and control (CHX 0.2%) ($p < 0.01$). Results show the insignificant difference between samples. Urmia (sample 1), Qom (sample 2), and Jarquyeh (sample 3).

MTT results showed that more than 50 percentage of cell viability depends on decreasing the salt concentration (≤1.56 mg/ml).

In this study, sea salts were collected from different regions in Iran. In this study, sea salts were effective against the tested microorganisms. Michel et al. assessed the usefulness of the application of sea salt rinse (each child rinsed with a solution containing 2.5 grams of sea salt in 20 ml of water) on periodontitis in street children of Manila [20]. Mani et al. studied 30 adults with gingivitis at a dental college in Maharashtra (India). There was a significant decrease in clinical indices in the group using sea salt rinse for three months compared to the other groups [13]. However, one study conducted by Hoover J et al. reported that application of sea salt rinse for 30 days was not useful for the treatment of periodontitis and gingivitis. This may be contributed to the fact that the study had a short trial period and a small sample size [10]. Further studies are needed to investigate the effects of sea salt. The limitations of this study are that it is an in vitro study and sea salt rinse needs to be verified clinically in future in vivo research studies. Although sea salt rinse has a significant effect on microbial organisms, it was less effective than chlorhexidine 0.2% mouthwash. Nevertheless, CHX mouthwash has many side effects, especially when used for a long time, causing taste changes, burning mouth, dry mouth, tooth staining, and side effects from probable swallowing. Using natural mouthwash without any significant side effects can be a helpful substitute, even with a lower biofilm impact and antimicrobial effect [21].

5. Conclusion

Antimicrobial studies specifically demonstrate that sea salt solutions are potent plaque inhibitors that could be used as a mouthwash. The solutions blocked plaque formation and degraded biofilm formation. However, further long-term clinical trials are required to include the much needed standardization and certification of the mouthwash in order to overcome the drawbacks of the gold standard chlorhexidine.

Data Availability

All the data generated or analyzed during this study are included in this published article, and also the datasets analyzed to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Comparison of Herbal Agents with Sodium Hypochlorite as Root Canal Irrigant: A Systematic Review of *In Vitro* Studies

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During endodontic treatment, eliminating microorganisms from the root canals should be considered with utmost importance. Before filling the canal, every effort should be made to ensure optimal shaping and adequate disinfection of the root canal system. This systematic review aimed to compare the efficacy of herbal agents with sodium hypochlorite (NaOCl) in reducing the microbial load while used as a root canal irrigant. The research question in the present study was to assess "Is there a significant difference in reducing microbial load comparing sodium hypochlorite (NaOCl) and herbal agents." Electronic databases (PubMed, Scopus, and Web of Science) were searched from their start dates to November 2020 using strict inclusion and exclusion criteria and reviewed following PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. Only *in vitro* studies comparing herbal agents with NaOCl regarding antimicrobial efficiency were included. Two reviewers independently assessed the included article. 825 articles were obtained from an electronic database. Twenty papers were included for review of the full text. Eleven papers were excluded because they did not meet the inclusion criteria. Finally, nine articles were included in the systematic review. The present systematic review was at the *in vitro* level; therefore, the result cannot translate the exact clinical conditions. This systematic review concludes that herbal agents cannot be used as a main irrigant for canal disinfection.

1. Introduction

The root canal of infected teeth is usually polymicrobial, existing as a biofilm. The ideal outcome of an endodontic treatment should target the elimination of microorganisms and their byproducts established within the root canal system [1]. Cases with persistent infection where the microorganisms are not completely eliminated need more attention as in the majority of cases it leads to reinfection [2]. To attain this, mechanical preparation of the root canal alone may not be sufficient. A disinfection regimen should emphasize optimal shaping such that the disinfectant reaches inaccessible areas such as anastomosis, isthmus, and the lateral canal [3].

An ideal irrigant should possess antimicrobial properties, an ability to dissolve the remnant necrotic pulp tissue, and should cause minimal irritation to the periapical tissue [4]. Different concentrations of sodium hypochlorite (NaOCl) are commonly used as root canal irrigants as they possess the majority of the properties mentioned above for an ideal root canal irrigant. Its uniqueness among available root canal irrigants is that it can dissolve the necrotic and the remnant vital pulp tissue [4, 5]. However, it irritates the periapical tissues when extruded beyond the apex, eventually leading to pain during root canal treatment.

In order to avoid complications associated with the usage of NaOCl, many authors have suggested the usage of herbal agents as an alternative or as an adjunct to the conventional root canal irrigants [6, 7]. Several herbal agents such as essential oil, triphala, green tea polyphenol, *Morinda citrifolia*, neem, tulsi, German chamomile, orange peel extract, and essential oregano oil have been studied to assess the antibacterial properties against the most resistant endodontic and periodontal pathogen [8–10]. Therefore, this systematic review was undertaken to investigate the effect of herbal irrigants in comparison with NaOCl.

2. Materials and Methods

2.1. Rationale of Systematic Review. This systematic review aimed to assess the antimicrobial efficiency of herbal agents with sodium hypochlorite. This systematic review was conducted following the PRISMA 2020 (Preferred Reporting Items for Systematic Reviews and Meta-Analyses).

2.2. Objectives. The present systematic review included articles published on the conventional agent (NaOCl) and herbal agents. The search was performed using multiple terms till November 2020.

2.3. PICOS Question. The research question was constructed based on the following:

Population: extracted human teeth infected with *Enterococcus faecalis* and *Candida albicans*

Intervention: all herbal irrigants

Comparison: 1–5.25% sodium hypochlorite (NaOCl)

Outcome: assessment of microbial reduction

Studies: *in vitro* studies

Principle: do the herbal irrigants differ in antimicrobial efficiency compared to NaOCl

2.4. Search Strategy. Search terms related to root canal dentin, endodontic treatment, irrigants, herbal agents, and antimicrobial efficiency were searched for potential articles until November 2020. The databases used for the search were PubMed, Scopus, and Web of Science. The search strategy was modified based on the database used. A representative search strategy (used for PubMed) has been shown in Figure 1.

2.5. Eligibility Criteria.

Type of studies:

(i) *In vitro* studies and (ii) root canal irrigation performed on extracted teeth.

2.5.1. Inclusion Criteria. The herbal agents were compared with NaOCl for antimicrobial efficiency while performing an *in vitro* root canal treatment.

Full-text articles in English were selected.

The search was performed from different databases and duplicates were removed. Based on the eligibility criteria, the title and abstract of the article were carefully appraised to include the articles that matched the scope of the systematic review. Full-text articles were assessed for further analysis. Two independent reviewers performed the analysis as abovementioned and in the situation of any discrepancies, it was sought by the third reviewer.

2.5.2. Exclusion Criteria.

Animal studies and review articles

Full-text articles in languages other than English were excluded.

2.6. Data Extraction. Two independent reviewers performed the data extraction from the full text article included in the review. The outcome measure of this review compared the antimicrobial efficiency of herbal irrigants with NaOCl. Variables such as sample size, choice of irrigants, volume and concentration of irrigant, choice of the needle, and the method of sample collection were assessed.

3. Results

A total of 825 articles were obtained from an electronic database, out of which 34 were duplicate records and hence were removed. Out of 791 articles, 771 articles were excluded following the title search. Twenty papers were included for the full-text review. Another eleven papers were excluded because they did not meet the inclusion criteria. Finally, nine articles were included in the systematic review (Table 1). A summary of article selection is presented as a flowchart, based on PRISMA guidelines (Figure 1). The general characteristics of the included article were tabulated separately for antimicrobial efficiency (Table 2).

3.1. Selection of Teeth and Sample Size. All the studies included for the review have chosen only single-rooted teeth for analysis with a sample size ranging from of 40 to 180 (Table 2).

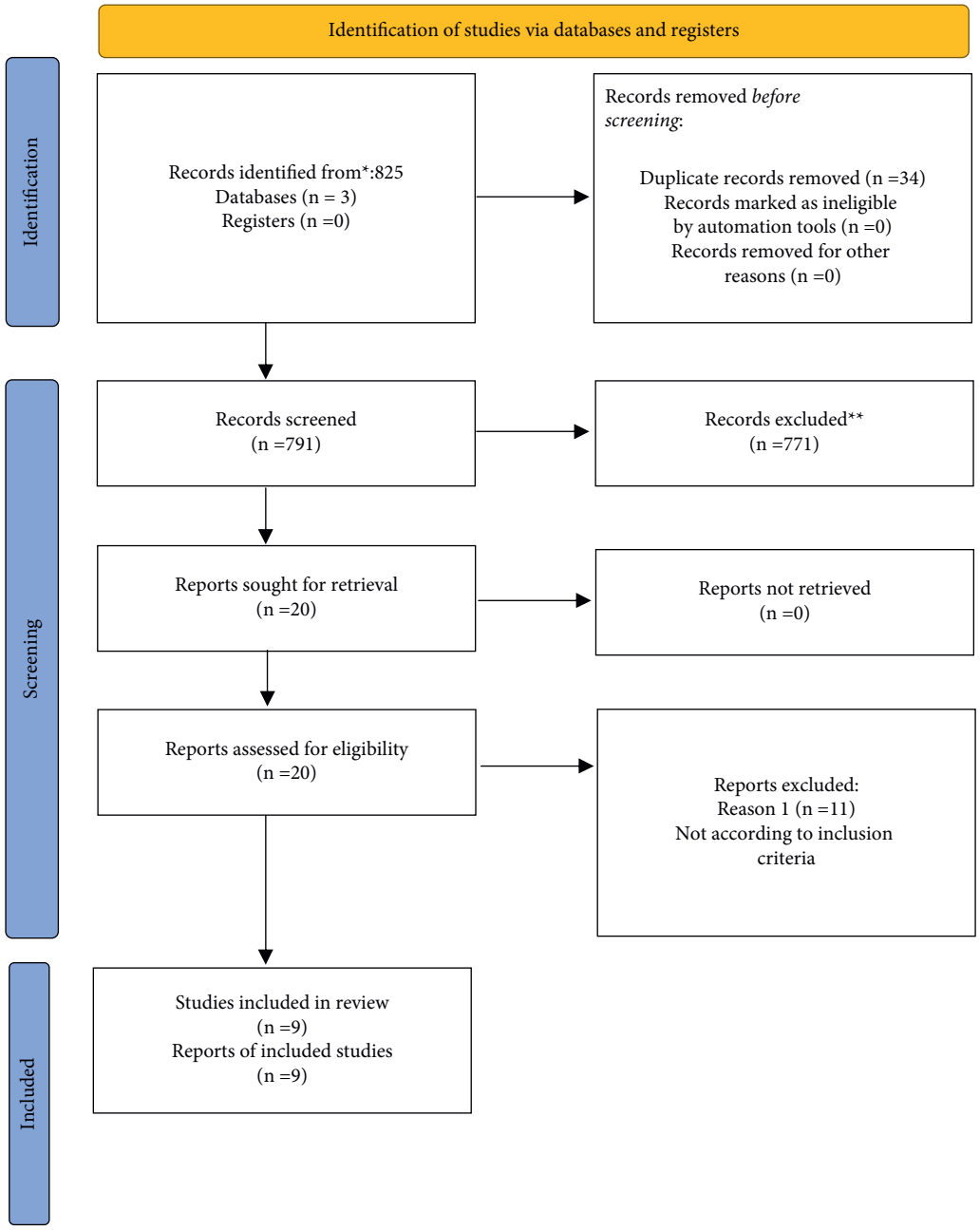

Figure 1: PRISMA flow diagram. * Consider, if feasible to do so, reporting the number of records identified from each database or register searched (rather than the total number across all databases/registers). ** If automation tools were used, indicate how many records were excluded by a human and how many were excluded by automation tools.

The risk of bias was high in all the papers for blinding and randomization as these details were not mentioned. All papers had a low risk of bias in data reporting; four out of nine studies reported low risk for standardization of protocol. The overall risk of bias was considered high.

3.2. Antimicrobial Efficiency (Tables 3 and 4)

3.2.1. Size of Apical Preparation. Four studies have performed the root canal preparation using a K file [11, 12, 17, 18]. In the study performed by Rosaline et al. [14], the size of root canal preparation was not mentioned. Sharifian et al. [16], in their study, used low-speed round bur for canal preparation, whereas a rotary file system was used for the preparation of root canals in the remaining studies [5, 13, 15].

3.2.2. Microbial Inoculation. Out of the nine studies included in the review, two studies did not mention the strain of *Enterococcus faecalis* used for their study [11, 18]. Two studies did not mention the adjusted suspension of colony forming units (CFU) of the strain [16, 17].

Table 1: Included article.

S. No	Author	Title of included article
1	Ok et al. [5]	Antibacterial and smear layer removal capabilities of oregano extract solution
2	Pujar et al. [11]	Comparison of antimicrobial efficacy of triphala, green tea polyphenols (GTP), and 3% of sodium hypochlorite on *Enterococcus faecalis* biofilms formed on tooth substrate: in vitro
3	Choudhary et al. [12]	Exploring the role of *Morinda citrifolia* and triphala juice in root canal irrigation: an ex vivo study
4	Sedigh-Shams et al. [13]	In vitro comparison of antimicrobial effects of sodium hypochlorite solution and *Zataria multiflora* essential oil as irrigants in root canals contaminated with *Candida albicans*
5	Rosaline et al. [14]	Influence of various herbal irrigants as a final rinse on the adherence of *Enterococcus faecalis* by fluorescence confocal laser scanning microscope
6	Gupta-Wadhwa et al. [15]	Comparative evaluation of the antimicrobial efficacy of three herbal irrigants in reducing intracanal *E. faecalis* populations: an in vitro study
7	Sharifian et al. [16]	Antibacterial substantivity of carvacrol and sodium hypochlorite in infected bovine root dentin
8	Divia et al. [17]	A comparative evaluation of *Morinda citrifolia*, green tea polyphenols, and triphala with 5% sodium hypochlorite as an endodontic irrigant against *Enterococcus faecalis*: an in vitro study
9	Arvind Kumar et al. [18]	Comparative evaluation of antibacterial and smear layer removal efficacy of two different herbal irrigants: an in vitro study

Table 2: General information of included article.

Author and year	Selection of teeth	Sample size	Herbal irrigant	Positive control (% NaOCl)	Negative control	Other irrigants
OK et al. 2015 [5]	Permanent maxillary central incisors	$N = 180$	Group 3: 1% oregano extract solution, group4 : 2% oregano extract solution, and group5: 5% oregano extract solution	Group 6: 5.25% NaOCl	Group 7: sterile saline	Group 1: 17% EDTA and group2: 2% chlorhexidine
Pujar et al. 2011 [11]	Permanent single rooted premolar	$N = 40$	Group 1: 60 mg/ml of triphala in 10% DMSO and group 2: 60 mg/ml of green tea polyphenol in 10% DMSO	Group 3: 3% NaOCl	Group 4: sterile saline	
Choudhary et al. 2018 [12]	Permanent single rooted teeth	$N = 84$	Group 1 ($N = 16$): MCJ and group2 ($N = 16$): triphala juice	Group 3: 1% NaOCl	Group 6: sterile distilled water	Group 4: 2% chlorhexidine and group 5: preservative control group
Sedigh-Shams et al. 2015 [13]	Permanent mandibular premolar	$N = 60$	Group 1: a minimum fungicidal concentration (MFC) of *Z. multiflora* EO (1 mg/ml) of 1 : 1024 and group 2: twice the MFC of *Z. multiflora*	Group 3: 1: 16 MFC of 5% NaOCl (3 mg/ml)	Group 4: distilled water	
Rosaline et al. 2013 [14]	Permanent single rooted teeth	$N = 50$	Group 3: 1.25 mg/ml of *Morinda citrifolia*, group 4 : 0.33 mg/ml of *Azadiracta indica*, and group 5 : 0.33 mg/ml of green tea polyphenol	Group 2: 5.25% NaOCl	Group 1: sterile saline	
Gupta-Wadhwa et al. 2016 [15]	Permanent maxillary and mandibular single rooted teeth	$N = 40$	Group A: 40% O. sanctum, group B: 10% S. aromaticum, and group C: 10% C. zeylanicum	Group D: 3% NaOCl	Group E: distilled water	
Sharifian et al. 2009 [16]	Bovine incisors	$N = 120$	Group 2: carvacrol 10%	Group 1: NaOCl 5.25%	Group 3: infected dentin tube	Group 4: sterile dentin tube

Table 2: Continued.

Author and year	Selection of teeth	Sample size	Herbal irrigant	Positive control (% NaOCl)	Negative control	Other irrigants
Divia et al. 2018 [17]	Permanent premolar teeth	$N = 60$	Group 3: *Morinda citrifolia* (64 mg/ml in 10% DMSO), group 4: triphala (64 mg/ml in 10% DMSO), and group 5: green tea polyphenols (64 mg/ml in 10% DMSO)	Group 2: 5% NaOCl	Group 1: distilled water	
Kumar et al. 2018 [18]	Maxillary central incisors	$N = 120$ antimicrobial efficacy ($N = 60$) and smear layer removal efficacy ($N = 60$)	Group IB: 25% neem extract ($N = 20$) and group IC: 25% tulsi Extract ($N = 20$)	Group IA: 3% NaOCl ($N = 20$)		

Table 3: Methodology assessment.

Author and year	Microbial inoculation	Root canal preparation (instruments used and size of preparation)	Irrigation protocol	Volume of irrigant	Time of irrigation	Needle used for irrigation	Irrigant activation devices used
Ok et al. 2015 [5]	*E. faecalis* (ATCC 29212) cultured in a BHI agar suspension adjusted to 1×10^8 CFU	ProTaper NiTi rotary files 30.06% taper	No protocol mentioned	6 ml of each irrigant	2 min	Not mentioned in the study	Nil
Pujar et al. 2011 [11]	*E. faecalis* cultured in a BHI agar (strain not mentioned)	Step back upto 40 K file	No protocol mentioned	3 ml of each irrigant	10 mins	Not mentioned in the study	Nil
Choudhary et al. 2018 [12]	*E. faecalis* (MTCC 2729) and *C. albicans* (MTCC 1637) in a BHI agar is inoculated in 5 mL of suspension to obtain 1 : 1010 dilution	Step back upto 40 K file	During canal preparation, 3 mL of respective irrigant was used for 15 mins after enlargement, 2 mL of irrigant solution was used to rinse debris in the canals for another 5 min. Sterile normal saline (2 mL) was used as a final rinse	5 ml of each irrigant	20 mins	30-gauge needle	Nil
Sedigh-Shams et al. 2015 [13]	*C. albicans* in sabouraud dextrose agar suspensions adjusted to 1.5×10^8 CFU	ProTaper NiTi rotary files 30.06% taper	During canal preparation, 10 ml of respective irrigants were used. Groups 1 and 2 were irrigated with 2 ml of sterile distilled water to remove the remaining *Z. multiflora* EO. Group 3 were irrigated with 2 ml of 4% sterile sodium thiosulfate solution to neutralize the remaining NaOCl	10 ml of each irrigant	12–14 mins	27-gauge needle	Nil

Table 3: Continued.

Author and year	Microbial inoculation	Root canal preparation (instruments used and size of preparation)	Irrigation protocol	Volume of irrigant	Time of irrigation	Needle used for irrigation	Irrigant activation devices used
Rosaline et al. 2013 [14]	*E. faecalis* (ATCC 29212) cultured in tryptone bile X-glucuronide agar suspensions adjusted to 1×10^6 cells/ml	Not mentioned in the study	All the specimens were treated with 5.25% NaOCl for 30 min followed by 5 mmol/L 17% EDTA for 5 mins. After which, the final irrigants were used	Not mentioned about the volume of final rinse	Final irrigation for 30 mins	Not mentioned in study	Nil
Gupta–Wadhwa et al. 2016 [15]	*E. faecalis* (ATCC 29212) suspensions adjusted to 1.5×10^8 CFU	ProTaper NiTi rotary files 30.06% taper	Initially, 2 mL of experimental extract for 30 s; during instrumentation, the canal was irrigated with 2 mL of the tested extract. After instrumentation experimental, extract was left undisturbed for 60 s and then finally irrigated with 2 mL of 3% NaOCl followed by 5 mL of 17% EDTA for 1 min and again with 2 mL of experimental extract	20 ml used in each canal	6mins 30 s approx.	30-gauge needle	Nil
Sharifian et al. 2009 [16]	*E. faecalis* (ATCC 29212)	Specimens enlarged low-speed round burs of ISO sizes 025, 027, 029, 031, and 033	No protocol was mentioned	Not mentioned	20 mins contact time of irrigant	Not mentioned	Nil
Divia et al. 2018 [17]	*E. faecalis* (ATCC 29212)	Step back upto 50 k file	No protocol was mentioned	Not mentioned	Not mentioned	Not mentioned	Nil
Kumar et al. 2018 [18]	*E. faecalis* (strain not mentioned)	Step back up to 30K size	No protocol was mentioned	6 ml of irrigants	At the rate of 2 ml/15 seconds	25-gauge needle	Nil

Table 4: Methodology assessment.

Author and year	Sample collection	Culture plate	Assessment method	Statistical analysis performed
Ok et al. 2015 [5]	The sample was collected in an Eppendorf tube containing BHI broth	BHI and blood agar broth	Colony forming units	Kruskal–Wallis test and mann–Whitney *U* test
Pujar et al. 2011 [11]	The sample was scraped from the root canal	BHI broth	Colony forming units	One-way analysis of variance with post hoc tukey tests
Choudhary et al. 2018 [12]	Sterile paper points were inserted into the canal to collect samples	BHI agar plate and SDA	Colony forming units	Intragroup comparison of Friedman's two-way analysis of variance by ranks and post hoc Wilcoxon signed-rank test. Intergroup comparison of Kruskal–Wallis test
Sedigh-Shams et al. 2015 [13]	Sterile paper points were inserted into the canal to collect samples	SDA agar plate	Colony forming units	Kruskal–Wallis and Mann–Whitney tests
Rosaline et al. 2013 [14]	Dentin specimen was spread on the slide. Stained with BacLight	Confocal laser scanning microscopy	Bacteria counted by a manual digital counter	One-way ANOVA

Table 4: Continued.

Author and year	Sample collection	Culture plate	Assessment method	Statistical analysis performed
Gupta-Wadhwa et al. 2016 [15]	Paper point was used to the collect sample	PCR BHI agar plate	Bacterial DNA isolation and detection colony forming units	Student's *t*-test, the mann–whitney test, Kruskal–Wallis test, and Dunn's multiple comparison test
Sharifian et al. 2009 [16]	Dentin chips were collected from the bur	BHI agar plate	Colony forming units	One-way analysis of variance and the post hoc test (Tukey)
Divia et al. 2018 [17]	Not mentioned in the study	Not mentioned in the study	Colony forming units	Kruskal–Wallis test and student's *t*-test
Kumar et al. 2018 [18]	Sample collected using H file	Real time quantitative PCR	Bacterial DNA isolation and detection	One-way analysis of variance with post hoc test (Tukey)

3.2.3. Irrigation Protocol. Five studies did not mention the protocol followed for root canal irrigation [5, 11, 16–18]. The remaining four studies mentioned the irrigation protocol which they followed in their studies [12–15].

3.2.4. Volume of Irrigant and Time of Irrigation. The other important aspect for a successful outcome of disinfection is the volume of the irrigant used and its time of contact within the root canal system. Out of included studies, two studies have not mentioned the volume of the irrigant used [14, 16, 17]. Concerning the contact time of the irrigant, no details were mentioned in the study conducted by Divia et al. 2018 [17].

3.2.5. Choice of Irrigation Needle and Irrigant Activation. The choice of needle used for root canal disinfection has a considerable impact on the penetration depth of the irrigant, irrigant flow pattern, and shear stress exerted on the canal wall. Five studies have not mentioned the gauge of the needle and tip design [5, 11, 14, 16, 17]. Two studies have performed irrigation with a 30-gauge needle [12, 15]. Sedigh-Shams et al. [13] used a 27-gauge needle, whereas Arvind Kumar et al. [18] used a 25-gauge needle. However, the remaining studies have not mentioned the choice of the needle. Among the included articles, none of the studies has mentioned the agitation device used for disinfection.

3.2.6. Sample Collection. Most of the included articles used sterile paper points to collect the samples from the canal. Out of all the studies, only one study has not mentioned the sample collection method [17]. In the study by Kumar et al. [18], an H file was used to collect the sample from the root canal.

3.2.7. Culture Method. Rosaline et al. [14] used confocal laser scanning microscopy to evaluate the remaining bacterial adhesion to root dentin. On the other hand, Gupta–Wadhwa et al. [15] and Kumar et al. [18] used polymerase chain reaction (PCR). Remaining studies performed CFU's assessment to assess antimicrobial efficiency. The assessment of antimicrobial activity is mentioned in Table 5.

3.3. Risk of Bias. Cochrane criteria for risk of bias were modified according to *in vitro* studies by evaluating the domain about present reviews such as randomization, standardized operator protocol, blinding, and data reporting. Blinding in these studies implies blinding of the evaluator. The risk of bias was scored as low when the details of the parameters as mentioned earlier were mentioned with no ambiguity, but when there was ambiguity, they were scored as unclear. When no details were mentioned, it was scored as high.

Cochrane criteria were modified by taking into consideration a few parameters for the quality assessment [19]. For the antimicrobial studies, all the included articles reported about the type and number of canals. None of the studies mentioned whether a single operator performed all the experimental procedures. Four studies reported a high risk of bias as they did not mention the volume of the irrigant used, and five studies did not mention the type of needle used for the irrigation—none of the studies mentioned the blinding of the evaluator (Table 6).

The authors of this review take the standpoint that all the study details need to be mentioned and focused on keeping the risk of bias low. Furthermore, the authors of this review believe that the experiments were performed according to a standardized protocol but might not have reported the intricate details since these are *in vitro* studies.

4. Discussion

It is well documented that primary endodontic infections are dominated by obligate anaerobic microorganisms detached from the root canal system compared to facultative bacteria [20]. It can be substantiated by the ability of flora to colonize predominantly into the main canal and least colonization into the complex areas of the root canal system [21]. Once established within the root canal system, these bacteria are difficult to eradicate as they can survive in extreme conditions. Attaining three-dimensional disinfection becomes more complex, predominantly when the microorganisms get colonized into intercanal communication, fins, cul-de-sac, and isthmus [22]. Mechanical enlargement of the root canal alone cannot eradicate the microorganisms from the root canal system, which eventually emphasizes disinfection.

4.1. Call for Action. Considering the disinfection for the root canal system, various factors need to be taken into account. It depends on various factors such as endodontic microbiota, access cavity design, canal preparation technique, property of the agents used for canal disinfection, the volume and

Table 5: The results of the assessment.

Author and year	Reduction in bacterial load	Outcome
Ok et al. 2015 [5]	Mean of the Log_{10} CFU and SD: 1% oregano extract—1.404 ± 1.803 CFU/ml and 5.25% NaOCl—2.308 ± 1.739 CFU/ml	1% oregano extract solution showed a similar result to 5.25% NaOCl
Pujar et al. 2011 [11]	Mean of the Log_{10} CFU and SD: triphala 2.3 ± 0.59 × 104 CFU/ml, green tea 3.8 ± 0.79 × 104 CFU/ml, and 3% NaOCl 0.00	Hypochlorite exhibited the maximum bacterial inhibition compared to triphala and green tea extract
Choudhary et al. 2018 [12]	Mean of the Log_{10} CFU and SD: *Morinda citrifolia* 3.51 ± 0.29, triphala 3.37 ± 0.56, and 1% NaOCl 1.43 ± 0.53	Compared to 1% NaOCl, morinda and triphala showed reduced effectiveness
Sedigh-Shams et al. 2015 [13]	% bacterial CFU reduction: *Z. multiflora* (2 times of MFC)—200,000 ± 0 5% NaOCl- 200,000 ± 0 *Z. multiflora* MFC—199,540 ± 313	Both *Z. multiflora* (2 times of MFC) and 5% NaOCl showed similar antimicrobial efficiency
Rosaline et al. 2013 [14]	% remaining bacterial adhesion to dentin: *Azadiracta indica* 9.30% and 5.25%, NaOCl 12.50%, green tea 27.30%, and *Morinda citrifolia* 44.20%	Compared to 5.25% NaOCl, *Azadiracta indica* showed reduced bacterial adherence
Gupta-Wadhwa et al. 2016 [15]	*Ocimum sanctum*— 2.65×10^5, *Cinnamomum zeylanicum*—1.535×10^5, *Syzygium aromaticum*—1.425×10^5, and 3% NaOCl—1.402×10^4	Compared to other herbal agents, 3% NaOCl showed better antimicrobial efficiency
Sharifian et al. 2009 [16]	Substantivity was assessed over a period of 28 days. CFU reduction (%) ±SD: carvacrol 99.3 ± 1.54 5.25% and NaOCl 99.98 ± 0.04	5.25% NaOCl showed significantly better results than carvacrol
Divia et al. 2018 [17]	CFU reduction ± SD: 5% NaOCl 0.67 ± 0.78, *Morinda citrifolia* 158.17 ± 19.83, triphala 15.92 ± 2.87, and green tea polyphenol 56.67 ± 7.18	NaOCl showed the maximum antibacterial effect, followed by triphala, green tea polyphenol, and least was *Morinda citrifolia*
Kumar et al. 2018 [18]	PCR mean value of antimicrobial efficiency: 3% NaOCl—34.20, neem—33.93, and tulsi—31.86	NaOCl showed the maximum antibacterial activity, followed by neem leaf extract. The least effect was seen with tulsi

Table 6: Risk of bias assessment.

Author and year	Randomization	Allocation concealment	Blinding	Standardized preparation	Reporting data
Ok et al. 2015 [5]	Unclear (teeth were randomly divided)	High (not mentioned)	High (not mentioned)	Low	Low
Pujar et al. 2011 [11]	Unclear (the samples were divided.)	High (not mentioned)	High (not mentioned)	High (strain not mentioned, irrigation protocol not mentioned, also the type of needle used)	Low
Choudhary et al. 2018 [12]	Low	High (not mentioned)	High (not mentioned)	Low	Low
Sedigh-Shams et al. 2015 [13]	High (not mentioned)	High (not mentioned)	High (not mentioned)	Low	Low
Rosaline et al. 2013 [14]	Unclear (teeth were randomly divided)	High (not mentioned)	High (not mentioned)	High (canal preparation not mentioned, the volume of irrigant used not mentioned, and the type of needle used not mentioned)	Low
Gupta-Wadhwa et al. 2016 [15]	Unclear (samples were randomly divided)	High (not mentioned)	High (not mentioned)	Low	Low
Sharifian et al. 2009 [16]	Unclear (samples were randomly divided)	High (not mentioned)	High (not mentioned)	High (irrigation protocol not mentioned, the volume of irrigant and type of needle used not mentioned)	Low
Divia et al. 2018 [17]	High (not mentioned)	High (not mentioned)	High (not mentioned)	High (irrigation protocol not mentioned, the volume of irrigant, time of irrigation, type of needle used, sample collection, and culture medium were not mentioned)	High
Kumar et al. 2018 [18]	High	High (not mentioned)	High (not mentioned)	Unclear (irrigation protocol not mentioned)	Low

contact time of the disinfectant, and the choice of the irrigant needle used for disinfection. Assessing the antimicrobial efficiency, eight out of nine studies showed a low risk of bias in reporting the outcome data [5, 11, 16, 18].

Studies have shown that nearly 70% of bacterial species invade the dentinal tubule, predominantly in apical periodontitis, discussing the microflora. The notable point was that more bacterial invasion was dominant in the coronal

and middle third of the root canal system [23]. With the reduction in the oxygen potential in the apical third area, it creates a favourable environment for these organisms to establish and further colonize [24]. The virulence factors produced by these bacteria play a critical determinant role in the sustainability of these organisms [25]. Therefore, considering the abovementioned factors, the endodontic treatment should be carried out with the rationale of attaining the eradication of microbial species, although complete elimination is not possible [26].

Access cavity design plays an essential role in the disinfection of the root canal system. The cavity design should aim to provide straight-line access, facilitating the ease of instrumentation and allowing the irrigant flow to reach the working length [27]. The critical factor that plays a paramount role in conservative endodontic access is the incomplete elimination of necrotic tissue remnants. In such instances, the absolute eradication of microorganisms is absurd [28]. The previous study reported that even when a traditional endodontic access cavity was performed, the remaining pulp tissue was inevitable in the isthmus region [29]. Neelakantan et al. reported that complete debridement of the root canal was not achievable with contracted endodontic access as the entire pulp horn was not included in the cavity design, and deroofing of the pulp chamber was not performed [30].

Preparation of the root canal system is another crucial confounding factor. Preparation of the canal should be directed toward optimal in order to facilitate maximum disinfection [31]. Studies in literature have reported a larger preparation size with increased taper shown to exhibit better disinfection when compared to a lesser preparation size [32]. Maintaining the apical terminus diameter is a prime concern as the preparation taper and diameter of the apical terminus are interconnected [29]. It is noteworthy to mention that emphasize should be laid on circumventing overzealous preparation regarding respect for the canal morphology. Comparing the hand and rotary instruments, more uninstrumented areas were evident with hand instruments than with the rotary instruments for canal preparation [33, 34]. Apart from this, the instruments used for the canal preparation also play an essential role in accumulating dentinal mud into the apical ramification and lateral canal. It generally scraps more dentin when used with radial land and gets plugged into the isthmus, fins, and lateral canal. Therefore, the choice of canal preparation instruments should be considered as a contributing factor in attaining disinfection.

Over the years, the widely used agents for the disinfection of the root canal include sodium hypochlorite as the primary root canal irrigant and EDTA as a chelating agent. Sodium hypochlorite is a nonspecific proteolytic agent possessing antibacterial properties and dissolves the remnant pulp tissue [35]. Apart from this, it causes the dissolution of organic components of dentin. In an attempt to achieve the primary goal of eliminating the microorganisms, a higher concentration of sodium hypochlorite is used which can effectively eliminate microbes and indirectly affect the structural aspect of the root canal dentin. Due to the proteolytic action of NaOCl at a higher concentration, it promotes more removal of type 1 collagen, thus declining the tooth's strength when the contact time was more than 1 hour [36]. It is also evident that the concentration of the NaOCl influences the dissolving property. Periodic replenishment of NaOCl during instrumentation also plays a significant role in maintaining the concentration throughout the treatment procedure.

The contact time of the irrigant and its volume can be increased by lowering the concentration of NaOCl so as to reduce the cytotoxic effects. However, the studies have shown that a contact time of not less than 40 minutes with 5.25% NaOCl showed to be more effective when compared to lesser concentration for the same period with frequent replenishment [20]. The authors strongly make a standpoint that studies must adhere to the standardized irrigation regimen when they comparatively evaluate the antibacterial activity of NaOCl with herbal agents. None of the studies included in the systematic review maintained the contact time of 40 minutes for NaOCl. They concern herbal agents; there is no randomized clinical trial evidence to recommend contact time. There is no clear-cut recommendation exists regarding the volume of the irrigant. However, Zehnder suggested a beneficial role of copious amount of hypochlorite [20, 37].

It is a well-known fact that the needle's design and its insertion depth play a prime role in flow rate, which in turn influences the disinfection of the root canal system [38]. A 30-gauge side-vented needle allows the operator to place it up to 1 mm short of working length without binding. It also facilitates the ease of back and forth movement in the canal in order to reduce the exerted apical pressure and prevent the vapor lock effect. Discussing fluid dynamics, another aspect that needs to be taken into consideration is the flow rate of the irrigant. The effective disinfection of the root canal is related to the flow rate of the irrigant. The confounding factor directly related to the flow rate depends on the intrabarrel pressure, operator fatigue, thumb control of the operator, and gender of the operator [39].

4.2. Quantitative Review. The present systematic review discussed the studies that compared hypochlorite with herbal agents on antimicrobial efficiency. Efforts are going on in the field of research to minimize the antimicrobial resistance developed using conventional chemical agents by investigating the efficiency of herbal agents to replace the existing gold-standard agents. The authors of this review put forth a standpoint on whether these agents can become an alternative for canal disinfection.

Studies' included in the current systematic review used various herbal agents as a root canal irrigant. They included herbal agents such as oregano extract, triphala, green tea polyphenol, *Morinda citrifolia*, neem, carvacrol, tulsi, *Ocimum sanctum*, *Cinnamomum zeylanicum*, *Syzygium aromaticum*, and *Zataria multiflora*.

Discussing the individual agents, among all the herbal agents used, triphala, green tea polyphenol, and *Morinda citrifolia* were used in 4 studies [11, 12, 14, 17]. From the

abovementioned herbal agents, triphala was found to be the most effective antimicrobial agent, followed by green tea polyphenol, and *Morinda citrifolia* was found to be the least effective. The included studies which used oregano extract and neem showed conflicting results. Since different herbal agents were used in previous studies, there is no homogeneity of the variables in the included articles. Hence, it is impossible to conclude with a single herbal agent as an effective irrigant for canal disinfection. However, its antimicrobial activity was the least when compared with hypochlorite.

Previous studies have used different concentrations of hypochlorite. This variation could be a significant confounding factor in the outcome results, which should also be taken into consideration. Therefore, it is not possible to conclude that one herbal agent is a better alternative irrigant for smear layer removal as there are variables in the included studies.

4.3. Qualitative Review. Meta-analysis could not be performed due to the heterogeneity of the included articles and variation in the included studies.

4.4. Inference. The authors of this review infer that the herbal agents cannot be a substitute for hypochlorite concerning the antimicrobial properties. All the included studies in this review showed inferior antimicrobial efficacy of the herbal agents (triphala, green tea extract, *Morinda citrifolia*, neem, carvacrol, tulsi, *Ocimum sanctum*, *Cinnamomum zeylanicum*, and Syzygium aromaticum). Only oregano oil and *Zataria multiflora* showed similar antimicrobial efficiency as sodium hypochlorite [5, 13].

4.5. Future Inference. The present systematic review was at the *in vitro* level; therefore, the results cannot be translated into the exact clinical conditions. Studies should concentrate on the concentration, type, volume, and contact time of these herbal agents such that they can either be used as an alternative to minimize antimicrobial resistance or as an adjunct to the already existing conventional agents. Furthermore, future studies can concentrate on whether there is a difference in the efficiency of these herbal when used freshly after the extract preparation compared to stored extract. More clinical trials should be performed to uncover any precipitate formation using these agents when used in combination and to assess if any discoloration is observed.

5. Conclusion

Within the limitations of the study, taking into consideration various factors, herbal agents showed less efficiency than different concentrations of sodium hypochlorite regarding the antimicrobial property. The authors of this systematic review put forth the standpoint that though results do not translate the clinical scenario, they cannot furnish definitive evidence in reporting the outcome. However, future studies can focus on the use of these herbal agents in attaining optimal disinfection.

Data Availability

The data set used in the current study will be made available at a reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest to report regarding the present study.

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Research Article

A Two-Year Longitudinal Study of the Effectiveness of the CRT® Bacteria Test in Evaluating Caries Risk in Three-Year-Old Children

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Objective. To study the correlation between the level of infection with *Streptococcus mutans* (SM) and lactobacilli (LB) in saliva with existing status and the development of primary dental caries in 3-year-old children and to evaluate the results of CRT® bacteria as a Caries Risk Test. *Methods*. A total of 140 3-year-old children were selected for the study. Oral examination was conducted and the levels of infection with SM and LB in saliva were measured using a CRT® bacteria test. Oral reexamination was conducted after two years. The prevalence rate of caries, the decayed-missing-filled tooth (dmft) and decayed-missing-filled surface (dmfs) indices, and Caries Severity Index (CSI) were calculated at the start and end of the two years. The indices were statistically analyzed. *Results*. The caries prevalence rate, dmft, dmfs, and CSI increased with increasing levels of CRT-SM and CRT-LB at the start and end of the two years; the increases in dmft, dmfs, and CSI over the period were consistent with the increases in CRT-SM and CRT-LB levels, with all differences being highly statistically significant. The increase in caries prevalence rate over the two years was not statistically different for different CRT-SM and CRT-LB levels. CRT-SM and CRT-LB levels were highly positively correlated with dmft, dmfs, CSI and their increases over the two years. Levels of infection with oral SM and LB were found to be independent risk factors for primary dental caries, respectively. For an SM concentration in saliva of $\geq 10^4$ CFU/mL and an LB concentration of $<10^4$ CFU/mL, the risk of caries increased by approximately 2.8-fold. When the concentration of LB in saliva was $\geq 10^4$ CFU/mL and that of SM $<10^4$ CFU/mL, the risk of caries increased by approximately 3.9-fold. When the concentration of both SM and LB was $\geq 10^4$ CFU/mL, the risk increased by approximately 10.9-fold. *Conclusions*. Significant positive correlations were found between the level of infection with oral SM and LB and existing oral decay status and the trend in the development of primary dental caries. Infection with SM and LB significantly increased the risk of caries in primary teeth. The CRT® bacteria is a simple, convenient, reliable, and effective Caries Risk Test.

1. Introduction

The most common chronic illness among children globally in both developed and developing countries is caries [1, 2]. The prevalence of childhood caries remains the highest of all childhood diseases, five times greater than that of asthma and seven times that of hay fever [1]. Untreated primary dental caries affected 621 million children worldwide in 2010, representing approximately 9% of the global population [2]. In 2005 and 2015, the 3rd and 4th national oral epidemiological surveys were conducted in China, and the results revealed that the prevalence rate of caries and the mean value of the decayed-missing-filled tooth (dmft) index in 5-year-old children were 66.0% and 3.5 in 2005 and 71.9% and 4.24 in 2015, respectively, reflecting a significant deterioration in oral health [3, 4]. The 3rd oral epidemiological survey in China found that 79.3% of caries in children aged 5 years was concentrated in one-third of the population with a mean dmft value of 8.33, and the 4th survey reported that 75.4% of caries in 5-year-old children could be found in one-third of the population with a mean dmft value of 9.61 [4]. An oral epidemiological

survey conducted in the US also found that 20% of the population suffered from approximately 60% of all caries [5]. Evidence indicates a skewed distribution of caries in the population, with a particular subpopulation susceptible to severe caries [3–5].

Caries are a chronic infectious illness resulting from the interaction of multiple factors, such as varying microorganisms, the host, and dietary factors [6, 7]. A wide range of bacteria grow in the oral cavity and the population undergoes pathological evolution and change during the development of caries in children, from an equilibrium state with multiple bacteria to one in which a few cariogenic bacteria predominate [7–9]. Furthermore, cariogenic bacteria decompose and ferment food to produce acid which gradually erodes the teeth, eventually leading to demineralization and so caries [6]. In particular, *Streptococcus mutans* (SM) and lactobacilli (LB) are considered the primary cariogenic bacteria [6, 9].

Caries risk refers to the sensitivity of a host to caries, reflecting their susceptibility and propensity to develop caries [6]. A Caries Risk Test (CRT) aims to detect risk factors for the occurrence of caries by objectively evaluating the risk of caries or the level of caries activity in an individual which is significant for the prevention and control of caries in high-risk populations [6].

In the present study, we conducted an investigation of 3-year-old children from the Shenzhen Kindergarten, Guangdong Province, China, in which oral examinations were performed and tests for levels of infection with SM and LB in saliva using a CRT® bacteria test, from which the correlation between the level of SM and LB infection and the development and status of primary dental caries was calculated, as well as the ability of the CRT® bacteria test to function as a CRT during surveillance of the occurrence and development of caries in children.

2. Materials and Method

2.1. Subjects. The present study was approved by the Medical Ethics Committee of Shenzhen Maternity and Child Healthcare Hospital (Clinical Trial Registration Number: [2018]106). 3-year-old children in Shenzhen Kindergarten were recruited to the study and followed up for two years. The inclusion criteria for participants were as follows:

(i) Healthy children with no systemic disease, 3 years of age at the time of screening

(ii) Willing to accept oral examination and collection of a saliva sample stimulated using paraffin

(iii) No antibiotics use for two weeks prior to saliva collection

(iv) No professional fluoride treatment within the 48 hours prior to saliva collection

(v) No use of an antimicrobial mouth rinse for 12 hours prior to saliva collection

(vi) Signed parental consent or that of a legal guardian or a family member that is the primary care provider when the primary caregiver is not the parent

The results of the first clinical examination and reexamination after two years were provided to the parents in written form. Parents were provided oral healthcare guidance each year, principally relating to the children's diet, and oral cleaning and healthcare, such as the impact of sugar consumption and the frequency of its consumption on oral health, the need to gargle after eating, the use of fluoride toothpaste, teaching parents how to brush and floss for children, and the importance of regular oral examination.

2.2. Research Method. Oral examination and the relative measurement of SM and LB infection levels in saliva were conducted for each individual at the initial clinical examination. Only an oral examination was conducted at the clinical reexamination after two years.

2.2.1. Oral Examination. The initial and follow-up oral examinations were conducted by the same senior pediatric dentist. The kappa statistic was calculated for both examinations, the resultant values were found to be greater than 0.9, indicating that the results were reliable [10, 11].

The dentist performed a diagnosis both visually in natural light and with probing using a disposable mirror and probe, with all examination results recorded contemporaneously.

The diagnostic criteria of caries as described in the *Oral Health Surveys: Basic Methods* by the World Health Organization (WHO) [12] was used, any uncertain cases being excluded. The type of caries was also recorded, such as secondary caries, enamel caries, dentin caries, or that of the residual crown or residual root.

2.2.2. Indicators of Caries Status. Based on the oral examination results, the prevalence rate of caries, dmft, decayed-missing-filled surface (dmfs), and Caries Severity Index (CSI) were calculated [6, 13]. CSI was scored using the caries criteria developed by Shimono et al. [13]: 0 if a tooth was sound; 0.5 where a filling was present; 1 if secondary caries was present after the filling had been placed, enamel caries, or superficial dentin caries; and 2 when deep caries of the dentin was present, or exposure of the endodontium, or a residual crown, or root was observed. The highest score was recorded if multiple decayed surfaces were detected on one tooth.

Caries prevalence rate = (number of cases of caries/total number of participants) × 100%.

dmft = numbers of decayed teeth (dt) + missing teeth (mt) + filled teeth (ft).

dmfs = numbers of decayed surfaces (ds) + missing surfaces (ms) + filled surfaces (fs).

CSI = [sum of caries scores ÷ (number of teeth × 2)] × 100.

2.2.3. Measurement of Levels of Infection of Oral SM and LB. A standard CRT® bacteria kit (Ivoclar Vivadent Inc., Liechtenstein) was adopted in the present study, containing

paraffin pellets and two special plates. One side of the special plate was covered with black SM selective culture medium (MSB) and the other side coated with green LB selective culture medium (Rogosa Agar). The test was performed between 9 and 10 a.m. Participants fasted for one hour prior to the examination. The specific collection procedure was as follows: stimulated saliva was obtained from the children by asking them to chew the paraffin pellets prior to its collection in a sterile sputum cup. The agar was entirely covered with saliva. The carrier was then held slightly obliquely to allow excess saliva to flow out. The agar was held upright and placed tightly to form a seal. No contact by the researcher was permitted with the surface of agar during the entire process. The agar plates were incubated at 37°C for 48 h and the colony density of SM and LB was recorded. The numbers of SM and LB on the agar were compared with a standard plate and the results recorded accordingly.

Numbers of CRT-SM and CRT-LB observed were categorized into the following levels: level 0: bacterial count $<10^4$ CFU/mL; level 1: 10^4 CFU/mL $\leq$ bacterial count $<10^5$ CFU/mL; level 2: 10^5 CFU/mL $\leq$ bacterial count $<10^6$ CFU/mL; and level 3: bacterial count $\geq 10^6$ CFU/mL.

2.3. Sample Size. This was a prospective cohort study. From the data in a previous report [14], the two-year increment for decayed-filled surface (dfs) of CRT-SM at levels 0, 1, 2, and 3 were 1.53 ± 2.43, 2.75 ± 3.12, 6.91 ± 6.49, and 9.61 ± 6.19, and CRT-LB at levels 0, 1, 2, and 3 were 1.54 ± 3.21, 3.80 ± 4.82, 6.82 ± 4.83, and 10.36 ± 7.52, respectively. For a two-sided test where $\alpha = 0.05$ and $\beta = 0.10$ with power = 90%, the sample size N for a study of SM was 64 cases, and 80 cases for a study of LB, as calculated using PASS 15 software. Considering a loss to follow-up of 15%, at least 92 cases should be included.

In the present study, all the 3-year-old children in the same kindergarten in Shenzhen were selected. A total of 143 3-year-old children were included in the first clinical examination. After two years, 3 children had withdrawn from the kindergarten due to the family moving away, a rate of loss in the follow-up of 2.10%. A total of 140 children completed the two-year follow-up, half male and half female.

2.4. Statistical Analysis. A normality test, Chi-square test, Kruskal–Wallis test, Wilcoxon signed-rank test, and logistic regression with Spearman's rank correlation coefficient were calculated in the present study. All statistical analyses were conducted using SAS 8.02 software. The results of two-sided tests were considered statistically significant where $P < 0.05$ and highly significant at $P < 0.01$.

3. Results

3.1. Oral Examinations for Caries. In the initial oral examination, the prevalence of caries in the 140 children was 34.29%, with dmft, dmfs, and CSI values of 1.48, 2.23, and 4.71, respectively. In the follow-up oral examination two years later, the prevalence was 66.43%, and the values of dmft, dmfs, and CSI were 3.81, 6.08, and 11.87, respectively. No significant difference between the genders in the indicators of caries status at the initial exam and after two years was observed, nor an increase in indicator values over the two years (Table 1).

3.2. Correlation between the Caries Status and CRT-SM Levels. Table 2 displays the caries status and statistical analysis of the two oral examinations of the 140 children at each CRT-SM level. The prevalence of caries, dmft, dmfs, and CSI at each CRT-SM level at both the initial and the follow-up examinations and the increase in dmft, dmfs, and CSI over the two years increased with increasing CRT-SM level, at high levels of statistical significance. The prevalence of caries, dmft, dmfs, and CSI was statistically different when comparing CRT-SM levels 0, 2, and 3 at the initial oral examination. At the follow-up examination, the prevalence of caries was statistically different between CRT-SM levels 0 and 3, in addition to between levels 1 and 3, and dmft, dmfs, and CSI were statistically different between CRT-SM levels 0, 2, and 3, between levels 1 and 3, and between levels 2 and 3. The increase in dmft, dmfs, and CSI during the two years was found to be statistically different between CRT-SM level 3 and all other levels. No statistical difference was found for the increase over two years of caries prevalence rate between each CRT-SM level.

Table 3 displays the results of statistical and correlation analysis between the CRT-SM levels and indicators of caries status, such as dmft, dmfs, and CSI in the two oral examinations and the increase in values of indicators over the two years. All were positive correlations with coefficients ranging from 0.30 to 0.41 ($P < 0.01$).

3.3. Correlation between Caries Status and CRT-LB Levels. Statistical analysis of caries status at each CRT-LB level of the two oral examinations of the 140 children is presented in Table 4. The prevalence of caries, dmft, dmfs, and CSI increased with increased CRT-LB level at both the initial and follow-up examinations, with dmft, dmfs, and CSI increasing over the two years with increased CRT-LB level, at a high level of statistical significance. In the initial oral examination, the prevalence of caries was significantly different between CRT-LB level 0 and all other levels, and dmft, dmfs, and CSI were statistically different between CRT-LB levels 0, 2, and 3. In the follow-up examination after two years, the prevalence was statistically different between CRT-LB levels 0 and 2, with dmft, dmfs, and CSI significantly different between CRT-LB levels 0, 2, and 3, and levels 1, 2, and 3. The increase in dmft and dmfs over the two years was statistically different between CRT-LB levels 0 and 2, with CSI significantly different between CRT-LB levels 0, 2, and 3, and between levels 1 and 2.

Table 5 displays the results of statistical and correlation analysis between CRT-LB levels and the indicators of caries status, such as dmft, dmfs, and CSI in the two examinations and the increase in values of indicators over the two years, all positively correlated with coefficients ranging from 0.26 to 0.39 ($P < 0.01$).

Table 1: Caries status of 140 children of both genders over two years.

Examination	Gender	Number of participants	Number of caries cases	Caries prevalence (%)	χ^2	P	dmft ($\overline{x} \pm s$)	Z	P	dmfs ($\overline{x} \pm s$)	Z	P	CSI ($\overline{x} \pm s$)	Z	P
Initial exam	Boy	70	28	40.00			1.46 ± 2.18			2.07 ± 4.28			4.23 ± 6.54		
	Girl	70	20	28.57	2.03	>0.05	1.50 ± 2.91	1.00	>0.05	2.39 ± 5.40	0.92	>0.05	5.20 ± 11.14	0.99	>0.05
	Total	140	48	34.29			1.48 ± 2.56			2.23 ± 4.86			4.71 ± 9.11		
Follow-up exam after two years	Boy	70	46	65.71			3.73 ± 4.11			5.93 ± 7.60			11.43 ± 14.19		
	Girl	70	47	67.14	0.03	>0.05	3.90 ± 4.39	−0.10	>0.05	6.23 ± 8.29	−0.10	>0.05	12.30 ± 15.82	−0.17	>0.05
	Total	140	93	66.43			3.81 ± 4.24			6.08 ± 7.93			11.87 ± 14.98		
Increase over two years	Boy	70	18	25.71			2.27 ± 2.83			3.86 ± 5.14			7.20 ± 9.88		
	Girl	70	27	38.57	2.65	>0.05	2.40 ± 2.79	−0.46	>0.05	3.84 ± 4.85	−0.39	>0.05	7.11 ± 8.55	−0.41	>0.05
	Total	140	45	32.14			2.34 ± 2.80			3.85 ± 4.98			7.15 ± 9.20		

Table 2: Caries status of the 140 children in different CRT-SM levels over two years.

Examination	SM level	Number of participants	Number of caries cases	Caries prevalence (%)	SNK	χ^2	P	dmft ($\bar{x} \pm s$)	SNK	χ^2	P	dmfs ($\bar{x} \pm s$)	SNK	χ^2	P	CSI ($\bar{x} \pm s$)	SNK	χ^2	P
Initial exam	0	49	8	16.33	B	12.66	<0.001	0.45 ± 1.08	B	15.63	<0.01	0.53 ± 1.32	B	15.14	<0.01	1.22 ± 2.93	B	16.40	<0.001
	1	33	11	33.33	B A			1.39 ± 2.50	B A			2.15 ± 4.18	B A			4.28 ± 9.67	B A		
	2	31	15	48.39	A			2.19 ± 2.97	A			2.77 ± 4.43	A			6.45 ± 9.78	A		
	3	27	14	51.85	A			2.63 ± 3.33	A			4.78 ± 8.14	A			9.58 ± 12.30	A		
Follow-up exam after two years	0	49	25	51.02	B	11.07	<0.001	1.98 ± 2.76	C	23.71	<0.0001	2.82 ± 4.34	C	23.35	<0.0001	5.56 ± 8.49	C	24.69	<0.0001
	1	33	22	66.67	B			3.30 ± 3.87	C B			5.27 ± 7.07	C B			9.81 ± 13.39	C B		
	2	31	22	70.97	B A			4.52 ± 4.55	B			6.61 ± 7.44	B			13.83 ± 15.78	B		
	3	27	24	88.89	A			6.96 ± 4.69	A			12.37 ± 10.65	A			23.56 ± 18.20	A		
Increase over two years	0	49	17	34.69	A	0.06	>0.05	1.53 ± 2.27	B	14.74	<0.01	2.29 ± 3.83	B	19.03	<0.001	4.34 ± 7.42	B	17.93	<0.001
	1	33	11	33.33	A			1.91 ± 2.43	B			3.12 ± 4.23	B			5.53 ± 6.78	B		
	2	31	7	22.58	A			2.32 ± 2.59	B			3.84 ± 4.36	B			7.38 ± 8.11	B		
	3	27	10	37.04	A			4.33 ± 3.43	A			7.59 ± 6.45	A			13.98 ± 12.32	A		

Note. Differences between groups with identical letters denoting SNK ranking are not statistically significant, while differences between groups with different letters are statistically significant. The level of SNK is ranked alphabetically.

Table 3: Correlation between CRT-SM levels and caries status.

Indicators of caries status	SM level and indicators at the initial exam		SM level and indicators in the follow-up exam after two years		SM level and the increase in indicators over the two years	
	r	P	r	P	r	P
dmft	0.3323	<0.0001	0.4030	<0.0001	0.3047	0.0003
dmfs	0.3281		0.3983		0.3489	<0.0001
CSI	0.3426		0.4099		0.3444	

3.4. Logistic Regression Model for Caries Status at Different Levels of Infection with Cariogenic Bacteria

3.4.1. Single-Factor Logistic Regression Model for Caries Status at Different Levels of Infection with Cariogenic Bacteria. The single-factor logistic regression analysis of the impact of various levels of infection with oral cariogenic bacteria on caries status is displayed in Table 6, suggesting that both SM and LB infections are independent risk factors for caries in primary teeth.

3.4.2. Multivariate Logistic Regression Model for Caries Status at Different Levels of Infection with Cariogenic Bacteria. The results of multivariate logistic regression analysis of the impact of different levels of infection of oral SM and LB on caries status found $\chi^2 = 19.9783$ with $P < 0.0001$, a highly significant result (Table 7). The parameters obtained and the statistical analysis are shown in Table 8. Furthermore, the resultant probability of caries was calculated to be $p = \Pr(\text{caries} = 1) = e^{-2.2740+1.0442*\text{SM}+1.3482*\text{LB}}/(1 + e^{-2.2740+1.0442*\text{SM}+1.3482*\text{LB}})$, with the odds of caries increasing 2.8-fold when $\text{SM} \geq 10^4$ CFU/mL and $\text{LB} < 10^4$ CFU/mL in saliva, 3.9-fold when $\text{LB} \geq 10^4$ CFU/mL and $\text{SM} < 10^4$ CFU/mL in saliva, and 10.9-fold when both SM and LB were equal to or greater than 10^4 CFU/mL.

4. Discussion

Both SM and LB are naturally present within the human oral microbiota [6–8]. SM is a chain-like coccus 0.5~0.8 μm in length and can be observed everywhere in the human mouth [15]. LB is rod-shaped bacteria, not generally abundant in the oral cavity, accounting for approximately 1% of the total salivary flora, and can often be obtained from the surface of the tongue, oral saliva, and decayed teeth [16–18]. SM and LB share the following biological characteristics: they are Gram-positive; they are acidogenic and aciduric bacteria that can survive in a strongly acidic environment and continue to ferment sugars to produce lactic acid; they rely on glycolysis for energy; and they are microaerophiles and require similar nutrition [18, 19]. Hence, both SM and LB can survive and thrive in low pH in addition to environments with inadequate oxygen or nutrition [18, 19]. Furthermore, SM-derived glucosyltransferase can synthesize glucans by fermenting sucrose [20, 21]. Glucan is a high-molecular weight polymer that can be both water-soluble and water-insoluble. Soluble glucan can act as a reserve source of energy, while insoluble glucan is highly viscous and plays an important role in SM adhesion and aggregation to the surface of teeth [20, 21]. Additionally, surface proteins on SM are also important factors for adhesion, which can selectively attach the bacteria to the surface of tooth enamel to form dental plaque [20, 22]. Unlike SM, LB have no adherent surface proteins because they do not produce large quantities of extracellular polysaccharides to promote adhesion, and therefore have a low affinity for dental tissue, thereby often presenting at low levels in plaques [16].

A clinical study investigating changes in the proportion of cariogenic bacteria in dental plaques during the development of caries in children's primary teeth indicated that the percentage of SM in a complete plaque was 16.35%, 26.10%, and 37.24% in precaries, enamel caries, and superficial dentin caries, respectively, and the proportion of LB was extremely low, 0.02%, and 7.17% in precaries, enamel caries, and superficial dentin caries, respectively. The increase in both SM and LB was statistically significant, indicating that SM was the primary cariogenic bacteria and that LB was not the initiating factor in the development of caries but the driving factor in its progression [23].

In the results of the present study, the prevalence of caries, dmft, dmfs, and CSI significantly increased with the increasing CRT-SM and CRT-LB levels at both the initial and follow-up examinations ($P < 0.01$), suggesting that the children with different levels of infection of oral SM and LB had significant differences in caries status, with caries severity increasing as concentration levels of SM and LB increased in the saliva. In children with different levels of CRT-SM and CRT-LB, although there was no statistical difference in the increase in caries prevalence over two years, the increase in dmft, dmfs, and CSI over the two years was highly significant ($P < 0.01$). Increasing evidence has emphasized the contribution of SM and LB to caries. Beighton et al. [24] demonstrated that SM and LB are detected in children with caries significantly more frequently than in caries-free children. Lin et al. [25] studied children aged 3 to 4 years and found that, in the caries group with mean dmft of 9.00 and caries-free group, SM was present in 95.0% and 65.0% of cases, respectively, and LB in 42.5% and 10.0%, respectively, differences that were significant in each case. Matee et al. [26] discovered that the mean SM and LB counts in dental plaque in children with rampant caries were 100-fold higher than in caries-free children, indicating that the level of infection with salivary SM is directly related to rampant caries status. Mattos-Graner et al. [27] studied children aged 1 to 2.5 years and established that children with high levels of

Table 4: Caries status of 140 children at the different CRT-LB levels over two years.

Examination	LB level	Number of participants	Number of caries cases	Caries prevalence (%)	SNK	χ^2	P	dmft ($\bar{x} \pm s$)	SNK	χ^2	P	dmfs ($\bar{x} \pm s$)	SNK	χ^2	P	CSI ($\bar{x} \pm s$)	SNK	χ^2	P
Initial exam	0	58	9	15.52	B	17.98	<0.0001	0.52 ± 1.35	B	21.58	<0.0001	0.67 ± 1.84	B	21.16	<0.0001	1.38 ± 3.60	B	21.97	<0.0001
	1	39	14	35.90	A			1.44 ± 2.52	B A			1.85 ± 3.44	B A			4.84 ± 9.98	B A		
	2	27	16	59.26	A			2.70 ± 3.07	A			4.59 ± 6.78	A			8.75 ± 10.57	A		
	3	16	9	56.25	A			3.00 ± 3.56	A			4.81 ± 8.31	A			9.69 ± 13.26	A		
Follow-up exam after two years	0	58	32	55.17	B	7.97	<0.01	2.24 ± 2.96	B	20.83	<0.0001	3.31 ± 4.63	B	21.16	<0.0001	5.86 ± 8.30	B	24.08	<0.0001
	1	39	25	64.10	B A			3.54 ± 4.42	B			5.41 ± 7.97	B			11.22 ± 15.98	B		
	2	27	23	85.19	A			6.56 ± 4.71	A			11.00 ± 9.52	A			21.99 ± 17.17	A		
	3	16	13	81.25	B A			5.56 ± 4.29	A			9.44 ± 9.80	A			18.13 ± 17.11	A		
Increase over the two years	0	58	23	39.66	A	2.12	>0.05	1.72 ± 2.59	B	13.29	<0.01	2.64 ± 4.10	B	15.94	<0.01	4.48 ± 7.00	C	20.477	<0.0001
	1	39	11	28.21	A			2.10 ± 2.83	B			3.56 ± 5.61	B			6.38 ± 10.08	C B		
	2	27	7	25.93	A			3.85 ± 2.96	A			6.41 ± 5.32	A			13.24 ± 9.94	A		
	3	16	4	25.00	A			2.56 ± 2.42	B A			4.63 ± 4.32	B A			8.44 ± 8.52	B A		

Differences between groups with identical letters denoting SNK ranking are not statistically significant, while differences between groups with different letters are statistically significant. The level of SNK is ranked alphabetically.

Table 5: Correlation between CRT-LB levels and caries status.

Indicators of caries status	LB level and indicators at the initial exam		LB level and indicators in the follow-up exam after two years		LB level and the increase in indicators over the two years	
	r	P	r	P	r	P
dmft	0.3870		0.3598		0.2645	<0.01
dmfs	0.3804	<0.0001	0.3553	<0.0001	0.2931	<0.001
CSI	0.3892		0.3842		0.3243	<0.0001

Table 6: Single-factor logistic regression analysis of cariogenic bacterial infection.

Factors	Total cases	Caries cases	Caries-free cases	X^2	P	β	OR	95% confidence interval
SM ≥ 10^4 CFU/mL	91	40	51	10.79	<0.001	1.3911	4.019	1.695–9.530
SM <10^4 CFU/mL	49	8	41					
LB ≥ 10^4 CFU/mL	82	39	43	15.48	<0.0001	1.5969	4.938	2.148–11.352
LB < 10^4 CFU/mL	58	9	49					

Table 7: Influence of cariogenic bacterial infection on caries status.

SM	LB	Caries cases (caries = 1)	Caries-free cases (caries = 0)	Total
SM ≥ 10^4 CFU/mL(SM = 1)	LB ≥ 10^4 CFU/mL (LB = 1)	35	29	64
	LB < 10^4 CFU/mL (LB = 0)	5	22	27
SM <10^4 CFU/mL(SM = 0)	LB ≥ 10^4 CFU/mL (LB = 1)	4	14	18
	LB < 10^4 CFU/mL (LB = 0)	4	27	31

Table 8: Multivariate logistic regression model for cariogenic bacterial infection.

Factors	Parameters	χ^2	P	OR	95% confidence interval
Intercept	−2.2740	23.00	<0.0001		
SM/β1	1.0442	5.08	<0.05	2.841	1.146–7.043
LB/β2	1.3482	9.40	<0.01	3.850	1.627–9.115

infection of salivary SM had a higher prevalence of caries than those with low infection levels. Additionally, Wu et al. [28] observed 8-month-old infants and conducted caries and LB tests in their plaque every 6 months until 32 months of age, revealing that measurements of LB were significantly higher in all age groups than in caries-free infants.

The levels of CRT-SM and CRT-LB were highly positively correlated with dmft, dmfs, and CSI in the two oral examinations and their increase over the two years, further demonstrating that the levels of infection of oral SM and LB are associated with the severity and activity of caries in children [6]. SM and LB can colonize the mouth in early infancy [26]. Teanpaisan et al. [29] conducted a longitudinal study of 169 infants aged 3 to 24 months and found that the detection rates of SM and LB in the saliva of 3-month-olds were 1.78% and 8.88%, respectively, and 86.98% and 66.86% by 24-months, respectively. Moreover, the detection rate of LB in children aged 3–9 months was evidently higher than that of SM, and the rate of SM in children aged 18–24 months was considerably higher than that of LB [29]. The risk of caries in children aged 12–24 months with an SM count >50 CFU/1.5 cm^2 in the saliva was found to be 7.5–13.0-fold higher than in children without SM infection, and the risk of caries was 3.1- and 13.3-fold higher in children aged 24 months with salivary LB counts of 1–50 and >50 CFU/1.5 cm^2, respectively, compared with children without LB infection. Importantly, children in whom SM and LB had colonized the oral cavity at early time point were more susceptible to caries, the level of infection with SM and LB positively correlated with the caries status of the children [29]. Kanasi et al. [30] also reported that the level of infection with oral SM and LB was positively correlated with caries in children, and a risk marker for early childhood caries. The results of the present study confirmed that infection with SM and LB are independent risk factors for caries in primary teeth, with the risk of caries increasing approximately 10.9-fold when both SM and LB counts are ≥10^4 CFU/mL in saliva. Li et al. [31] researched 3- and 5-year-old children and found that the risk of caries increased 6-8-fold when SM were present at >10^6 CFU/mL in saliva. Hong et al. [32] investigated the association between the concentration of salivary SM in children aged 11 to 12 years and caries; their findings demonstrate that the concentration of salivary SM in children with caries was significantly higher than that of caries-free children, with a highly positive correlation between the concentration of SM in saliva and caries. Moreover, Hong and Hu [32] also concluded that the prevalence of caries in children increases exponentially at an SM concentration of 8.64×10^7/L in saliva.

High levels of infection with SM and LB in childhood caries and their capacity to generate a low pH environment, in addition to their pathogenicity and aciduric properties, indicate that they are key determinants of the development and progression of caries [16]. In the present study, we found

that the level of infection of oral SM and LB reflects the caries status and its progression in primary teeth, while a high concentration of SM and LB in saliva predicts an increasing trend for caries in the future. Matee et al. [26] pointed out that there is no difference in SM count in dental plaque and healthy enamel surfaces, but the LB count in dental plaque is 100-fold higher than on a healthy tooth surface, and the proliferation of LB in caries lesions suggests that LB is associated with the progression and severity of caries. Studies have revealed that SM is the bacteria that is cariogenic rather than LB, whereas LB is involved in the development of caries. Specifically, LB counts gradually increase in dental plaque after caries has occurred in normal plaque, lowering the pH and thus affecting the development of caries [17, 18]. It has also been concluded that LB and SM interact and operate in combination during the development and progression of caries. LB is an indirect indicator of fermentable carbohydrate [16, 18].

Caries is a chronic infectious disease that is affected by multiple factors. In addition to microbiological factors, children's feeding and oral hygiene habits are also closely related to the occurrence and development of caries [6, 7]. Studies have demonstrated that the risk factors for caries in young children are a delay to start to brush their teeth, the absence of toothpaste, a high frequency of sweets, and their frequent consumption [6, 33]. All subjects in the present study were from the same kindergarten in Shenzhen. The composition of the diet and the frequency of its consumption in the kindergarten were identical for each child. The children were from civil servants' families living close to the kindergarten, with relatively little mobility. The parents that had been provided with oral healthcare guidance every year were relatively consistent in how they had educated their children and the habits they had retained, reducing the impact of host and dietary factors on the research results to the greatest extent, although this was also a limitation of the study. The caries diagnostic criteria as described in the *Oral Health Surveys: Basic Methods (5th Edition)* formulated by the WHO [12] in 2013 were used in the present study. Caries with cavitated lesions were examined and recorded, but early caries of the enamel with initial noncavitated lesions were not observed and evaluated, a limitation of the WHO caries diagnostic criteria and also the present study. In addition, if follow-up observation data a year after initial examination and additional follow-up examinations over a longer duration had been available, such as three years, the research results would be more complete and the data more convincing.

A Caries Risk Test contributes to the identification of populations with high caries activity or at high risk of causing caries [6]. An ideal Caries Risk Test has the following attributes: consistency with clinical findings; high reproducibility; the ability to reflect current caries status and predict caries trends; ease of use; short test duration with high accuracy; and the capacity to present individual characteristics [34]. At present, no Caries Risk Test fully meets the aforementioned criteria [34]. Tests often require sampling from dental plaque, saliva, and teeth [25–28, 35–37]. Saliva is a bridge between different tissues and structures in the oral cavity and serves as an oral microecological medium with a large number of microorganisms that remain relatively stable [36]. The collection of saliva is a simple, noninvasive, and acceptable approach and a common source for oral clinical research [18, 32, 36]. Evidence has shown that a dynamic balance exists between the bacteria in saliva and dental plaque, with SM and LB counts in saliva highly correlated with the number of corresponding cariogenic bacteria in dental plaque [18]. Motisuki et al. [36] compared the influence of different sample types and collection methods on SM and LB counts and found that the number of SM identified in whole saliva and in dental plaque were similar, whereas the number of LB detected using a whole saliva method was superior to the dental plaque method, suggesting that whole saliva is sensitive to LB measurements. The present study utilized stimulated whole saliva as the sample collected from 3-year old children, who showed a high level of cooperation. The results of the study demonstrated that levels of infection with SM and LB in saliva can be used to predict caries risk in children [37–41].

In the present study, we leveraged the CRT® bacteria test for semiquantitative measurement of SM and LB in saliva; the results showed that the test represents a simple, convenient, reliable, and effective method of conducting a Caries Risk Test, consistent with the findings of Liang and Xu et al. [14, 42]. Tanabe et al. [43] found satisfactory consistency in terms of outcomes between the CRT® bacteria method and conventional methods of selective microbial culture and counting.

The CRT® bacteria kit contains a special plate preprepared with MSB and Rogosa agar on different sides; thus, no special preparation is required; and it is characterized by its simple operation and measurement, with high reproducibility and feasibility, low technical requirements, ability to be used in large sample testing, and easy generalization [14, 42, 43]. However, this method requires incubation for 48 hours after sample collection and manual comparison of results rather than precise quantification. Therefore, the development of easy-to-use, fast, and accurate quantification methods would be a significant step forward for Caries Risk Testing.

5. Conclusions

The level of infection with oral SM and LB was positively correlated with caries status in children's primary teeth and the development and progression of caries. A high level of infection with oral SM and LB suggests a high prevalence of caries and predicts an increasing trend in the future, with a large number of decayed teeth and surfaces indicating more severe caries. Furthermore, infections with oral SM and LB are independent risk factors for caries in primary teeth, the risk of caries increasing approximately 10.9-fold when both salivary SM and LB counts $>10^4$ CFU/mL. Finally, the CRT® bacteria test is a facile yet effective form of the Caries Risk Test.

Data Availability

The data used to support the findings of this study are restricted by the Ethics Committee of the Shenzhen Maternity

and Child Healthcare Hospital Affiliated to Southern Medical University in order to protect children's privacy. The data that support the findings of this study are available from the corresponding author for researchers who meet the criteria for access to confidential data upon reasonable request.

Conflicts of Interest

The authors have declared that no competing interest exists.

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Research Article

Antimicrobial Activity against Oral Pathogens Confirms the Use of *Musa paradisiaca* Fruit Stalk in Ethnodentistry

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Background. Ethnodentistry is the use of indigenous oral cleansing agents such as plant parts by local folks not only to maintain oral hygiene but also to treat oral infections. Mostly, ethnodentistry is inspired by traditions and belief systems of local communities. *Musa paradisiaca* is extensively cultivated and used in many cultures for its nutritional and medicinal values. In Ghana, the fruit stalk of *Musa paradisiaca* is used as an oral cleansing agent to maintain oral hygiene; yet this folk claim remains to be ascertained scientifically. *Objective*. The study assessed the antibacterial and antifungal effects of three extract fractions (aqueous, ethanol, and ethyl acetate fractions) of *Musa paradisiaca* fruit stalk against *Lactobacillus acidophilus*, *Aggregatibacter actinomycetemcomitans*, and *Candida albicans*, common oral pathogens implicated in dental caries and periodontitis. *Materials and Methods*. Aqueous, ethanol, and ethyl acetate fractions of *Musa paradisiaca* fruit stalk were prepared by cold maceration and qualitatively screened for their phytochemical composition. Antimicrobial effects of the three extract fractions were assessed by using serial broth dilutions at increasing concentrations (62.5, 125, 250, 500, and 1000 μg/ml) and compared to standard antimicrobial agents (erythromycin, doxycycline, and fluconazole). Subsequently, the absorbances of the microbial suspensions treated with increasing concentrations of the extract fractions were measured at 450 nm, and the cell densities were determined. *Results*. Except for the aqueous extract, which was less effective in decreasing microbial growth, the ethyl acetate and the ethanol extract fractions demonstrated antimicrobial efficacies comparable to those of the standard drugs. All three extract fractions demonstrated concentration-dependent growth inhibitory effects on the tested oral pathogens although not as effective as the standard drugs used. *Conclusion*. *Musa paradisiaca* fruit stalk has demonstrated antimicrobial effects against *Lactobacillus acidophilus*, *Aggregatibacter actinomycetemcomitans*, and *Candida albicans*, common oral pathogens implicated in dental caries and periodontitis, and this finding confirms in part folk use of *Musa paradisiaca* fruit stalk as a traditional dental care agent. Thus, the fruit stalk of *Musa paradisiaca* could be explored for use as a cheap and readily available dental care agent for people entrapped in the poverty line.

1. Introduction

Despite advancements in dentistry, oral diseases (dental caries, periodontitis, bleeding gums, toothache, oral sores, bad breath, tooth sensitivity, tooth loss, and oral cancer) remain a major health problem worldwide [1, 2]. Over 2.3 billion people are reported suffering from caries of permanent teeth and more than 530 million children are suffering from caries of primary teeth [3]. Evidence abounds linking risk of colorectal cancer, gum bleeding, toothache, preterm birth among pregnant mothers, chronic kidney disease, myocardial infarction, and stroke to oral diseases [4–9]. It is reported that most forms of periodontal diseases such as plaque, dental caries, halitosis, gingivitis, periodontitis, and toothache are caused by a complex and elusive activity of over 600 polymicrobial species inhabiting the oral cavity [10]. Common among these oral pathobionts are Gram-positive and -negative bacteria such as *Veillonella*

species, *Atopobium* species, *Prevotella* species, *Streptococcus mutans*, *Lactobacillus* species, *Enterococcus faecalis* and some nonmutant streptococci, which are associated with caries formation and progression [10–12]. Also, common Gram-negative bacteria, such as *Aggregatibacter actinomycetemcomitans*, have been associated with aggressive periodontitis [13], while commensal yeasts, such as *Candida albicans*, are also implicated in oral candidiasis [14], requiring holistic treatments.

Treatment of oral diseases involves the use of various agents having demonstrable antimicrobial, antioxidant, anti-inflammatory, antifungal, and analgesic effects. Risk of urticaria, taste masking, increased calculus formation, stained teeth and mucous membranes, oral mucosa desquamation, and parotid swelling are associated with long-term use of some conventional oral care agents, such as chlorhexidine [11, 15, 16]. Given the incidence of oral disease, increased microbial resistance, adverse effects associated with some conventional oral care agents currently used in oral care, and the high cost of these conventional oral care agents, it has become necessary to prospect for alternatives treatments which are relatively cheaper, readily available, effective, and safe.

Use of plants and plant-derived products to improve human health has been in existence for centuries even before the dawn of modern medicine. Most of the curative effects of medicinal plants have been determined over hundreds of years through centuries of uneventful use [17, 18]. The World Health Organization (WHO) has reported that in developing countries, 65–80% of populations depend on traditional therapies, mostly plant-based therapies [17]. Thus, the WHO has recommended that countries should formulate national policies and regulations to integrate plant-based therapies into their healthcare systems [19].

Interestingly, use of plant-based therapies to improve general health is quite common in Ghana [20]. For instance, *Musa paradisiaca* fruit stalk is commonly used in rural Ghana to maintain oral hygiene; however, this folk claim remains to be ascertained scientifically. Ecologically, *M. paradisiaca* (family, Musaceae) is distributed in the tropical and subtropical regions of the world. Traditionally, *Musa paradisiaca* is used for treatment of diarrhea, dysentery, intestinal lesions in ulcerative colitis, diabetes, in sprue, uremia, nephritis, gout, hypertension, and cardiac disease [21, 22]. In view of the ethnobotanical importance of *M. paradisiaca*, many studies have investigated some of its traditional uses. For example, the peels of the fruit have demonstrated antifungal activity against *C. albicans* [23]. Extracts of unripe fruit peels and leaves of *Musa paradisiaca* have demonstrated antimicrobial activity against *Pseudomonas* species, *Staphylococcus aureus, Escherichia coli*, and *Proteus mirabilis* [23, 24]. Also, extracts from various parts of *M. paradisiaca* have demonstrated antidiarrheal, hypoglycemic, antioxidant, antihypertensive, wound healing, antiallergic, antimalarial, leishmanicidal, and anti-snake venom activities [21, 25, 26]. The observed pharmacological properties were attributed to organic and inorganic components of *M. paradisiaca* including vitamins, lutein, carotene, potassium, and magnesium [23, 27] as well as phytochemicals such as flavonoids, serotonin, tryptophan, indole, tannins, and triterpenes [21, 22]. The present study assessed the antimicrobial effect of *M. paradisiaca* fruit stalk extracts against three common oral pathogens implicated in dental caries and periodontitis.

2. Materials and Methods

2.1. Chemicals and Reagents. The materials used in the study included Sabouraud Dextrose agar (SDA), peptone water, blood agar, doxycycline (Actavis, Barnstaple, UK), erythromycin (Concordia International, Capital House, UK), and fluconazole (FDC International, Fareham Harts, UK). Also, ethyl acetate, ethanol, sodium hydroxide (VWR Prolab Chemicals, France), hydrogen peroxide, Wagner's reagent, Mayer's reagent, and ferric chloride chemicals used were of analytical grade. Equipment such as a gas bath oscillator, dry-air oven, aerobic incubator, and other apparatus such as Petri dishes, beakers, and pipettes were also used.

2.2. Plant Collection, Identification, and Authentication. Fresh *M. paradisiaca* fruit stalks (red arrows in Figure 1) were collected from Abura market in the Cape Coast Metropolis, Ghana. Identification and authentication were done by Mr. Francis Otoo, the curator at the Herbarium Unit, School of Biological Sciences, University of Cape Coast. The green outer coverings of the fruit stalk were removed with a clean sharp knife and discarded. The remains were then chopped into small pieces and shade dried for two weeks. The dried fruit stalk remains were pulverized manually by pounding with mortar and pestle. The powdered samples were packed into transparent plastic sample bags, labeled, and stored at room temperature.

2.3. Preparation of Musa paradisiaca Fruit Stalk Extracts. Three flat-bottomed flasks were each filled with 20 g of the powdered sample. A 200 ml each of distilled water, ethanol, and ethyl acetate were added to each of the flasks and appropriately labeled. The flasks were corked with clean cotton wool and aluminum foil and allowed to stand with intermittent shaking on a mechanical shaker for 72 hours. After shaking, all four samples were vacuum filtered through filter paper (Whatman No. 54) separately with a Buckner funnel and a suction pump. The filtrates were each collected into separate conical flasks and labeled. The four samples were then concentrated using a rotary evaporator and the concentrates transferred into weighed and labeled crucibles for further evaporation on a water bath. All four samples were then placed into a desiccator and allowed to dry. The resultant fractions were weighed and labeled according to the solvent used to extract them as aqueous (AEMP), ethanol (EEMP), and ethyl acetate fractions (EAMP). Five different concentrations (62.5, 125, 250, 500, and 1000 μg/ml) were prepared from each of the extracts by serial dilution.

2.4. Preliminary Phytochemical Screening. The phytochemicals screened for included alkaloids, flavonoids, tannins, terpenoids, saponins, and phenols as follows.

Figure 1: Areal part of *Musa paradisiaca* (plantain) showing fruit stalk (red arrow) and fruits.

2.4.1. Test for Alkaloids. Presence of alkaloids was determined as previously described [28]. Briefly, 0.4 g of the dried extracts was dissolved in 5 ml of 1% HCl and the mixture warmed and filtered. 1 ml of filtrate was treated separately with (a) a few drops of potassium mercuric iodide (Mayer's reagent) and (b) potassium bismuth (Dragendorff's reagent). Turbidity or precipitation with either of these reagents was taken as evidence for the existence of alkaloids.

2.4.2. Test for Flavonoids. The presence of flavonoids was assessed by using a previously described method. Briefly, 0.5 g of each extract fraction was suspended in 1 ml of distilled water stirred and filtered to obtain a filtrate. A 0.5 ml of dilute ammonia solution was added to 0.5 ml of the filtrate followed by the addition of few drops of concentrated sulphuric acid. Appearance of yellow coloration confirmed the presence of flavonoids.

2.4.3. Test for Tannins. Tannins were detected using a modified version of the Ferric chloride test as described previously [28]. Briefly, 2 drops of 1% aqueous ferric chloride reagent were added to 0.5 ml of crude extract and observed for the formation of blue-black or green coloration, which indicated the presence of tannins.

2.4.4. Test for Phenolic Compounds. Phenolic compounds were detected using a modified version of the Folin–Ciocalteu procedure [29]. Briefly, 200 μl of crude extract was added to 2 ml of 3% aqueous sodium carbonate, followed by the addition of 200 μl Folin–Ciocalteu reagent. The mixture was allowed to stand for 30 minutes at room temperature. The formation of blue/gray colour indicated the presence of phenolic groups.

2.4.5. Test for Triterpenoids. Triterpenoids were detected using a modified version of the Salkowski test [30]. Briefly, 1 ml of extract was slowly added to 400 μl chloroform, followed by the careful addition of 400 μl concentrated sulphuric acid. Formation of a red/brown/purple colour at the interface indicated the presence of triterpenoids.

2.4.6. Test for Cardiac Glycosides. Cardiac glycosides were detected using a modified version of the Keller–Kiliani test [29]. 500 μl of extract was added to 500 μl glacial acetic acid. A few drops of 1% aqueous iron chloride and concentrated sulphuric acid were then carefully added. The presence of a red/brown ring of the interface indicated deoxy sugar characteristic of cardiac glycosides.

2.4.7. Test for Anthraquinones. Anthraquinones were detected using modified versions of the Kumar and Ajaiyeoba tests [28, 29]. The modified Kumar test involved the addition of a few drops of concentrated sulphuric acid to 500 μl pure extract, followed by the careful addition of 500 μl of ammonia. A rose-pink colour indicated the presence of free anthraquinones. For the Ajaiyeoba test, 450 μl of crude extract was added to 50 μl concentrated HCl and allowed to stand at room temperature for several minutes. 500 μl of chloroform was then carefully added. The formation of a rose-pink colour indicated the presence of free hydroxyl anthraquinones.

2.5. Ethical Clearance. The study was approved (UCCIRB/CHAS/2016/29) by the University of Cape Coast Institutional Review Board (UCCIRB) and the management of University of Cape Coast Dental Clinic. Also, patients' consent was sought for by means of a verbal or written agreement after the aim of the study was clearly explained to the patients.

2.6. Acquisition, Culturing, and Identification of Test Organisms. This was a laboratory experimental study to determine the antibacterial and antifungal activities of aqueous, ethanol, and ethyl acetate extracts of *Musa paradisiaca* fruit stalk against three common oral pathogens. The test bacteria were acquired from the Dental Clinic of University of Cape Coast. The research was carried out in the laboratories at the Department of Medical Laboratory Science, University of Cape Coast and Cape Coast Teaching Hospital. The plant extracts were screened for phytochemicals present and then assayed for the antibacterial activity using serial broth dilution. Test fungal strains, *Candida albicans*, that were used in the study are clinical isolates and were provided by the Microbiology Laboratory of Cape Coast Teaching hospital. These species were cultured on Sabouraud Dextrose agar (SDA) plates and incubated at room temperature (28 ± 3°C) for 3 days. *Candida* species were subcultured unto SDA supplemented with chloramphenicol and incubated at 37°C for 72 hours to obtain pure isolates. Identification of *Candida albicans* was done using germ tube test. Bacterial strains, *Lactobacillus acidophilus* and *Aggregatibacter actinomycetemcomitans*, were obtained by taking dental swabs from the patients who visited the University of Cape Coast Dental Clinic. Using sterile cotton swab sticks, swabs were taken from deep cavities in the teeth and also from plaques on the teeth. The swabs were placed in Bijou bottles containing sterile peptone water and incubated overnight at 35°C. Bacterial colonies were subcultured on

blood agar and incubated at 35°C for 24 hours. After incubation, colony appearance, biochemical tests as previously performed [31], and Gram staining to confirm the presence of *Lactobacillus acidophilus* and *Aggregatibacter actinomycetemcomitans* were performed. Table 1 shows a summary of results of biochemical tests that were conducted to confirm or otherwise the identity of the test organisms.

2.7. Serial Broth Dilution. Each fraction of the extract was dissolved in sterile distilled water to form stock concentrations of 10380 μg/ml, 10230 μg/ml, and 10300 μg/ml for ethanol, aqueous, and ethyl acetate fractions, respectively. From these stock solutions, working concentrations of 1000, 500, 250, 125, and 62.5 μg/ml were prepared and used for the tests for antimicrobial activity. Fluconazole (FDC International, Fareham Harts, UK), doxycycline (Actavis, Barnstaple, UK), and erythromycin (Concordia International, Capital House, UK) were used as positive control drugs for *Candida albicans*, *Aggregatibacter actinomycetemcomitans*, and *Lactobacillus acidophilus*, respectively, with the same concentration as the fractions. A 0.5 McFarland standard was used to standardize each of the three freshly cultured isolates to a density of 1.5×10^8 CFU/ml in sterile peptone water. 0.5 ml of this inoculum was added to sterile test tubes with the label of the fraction and the concentration. 1 ml of each concentration of the fractions was added to the corresponding test tubes containing the inoculum. The same was done for the drug control with the drug replacing the fractions instead. Turbidity standard for the bacteria was prepared by adding 0.5 ml of the inoculum to 1 ml of sterile peptone water whereas sterility standard was prepared by using sterile peptone without any additions. Bacterial and fungal suspensions were incubated at 35°C and 25°C, respectively, for 24 hours. Absorbance of the content of each of the test tubes was determined at a wavelength of 450 nm. The density of each suspension after incubation was determined by proportion as it was compared to the density and absorbance of the 0.5 McFarland. Each experiment was repeated at least three times.

2.8. Statistical Analysis. Data generated from the antibacterial effect of the three plant extracts of *Musa paradisiaca* on the 2 test bacteria and 1 fungi specie were entered and organized in Microsoft Excel. These data were then exported to IBM SPSS version 24.0 (SPSS Inc., Chicago, IL, USA) to compute descriptive statistics of the mean and standard deviations. Data were also analyzed with GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA) to perform one-way analysis of variance (ANOVA) using Tukey's multiple comparisons to compare between different antibacterial activities of different extract concentrations against each test bacteria versus controls. Two-way ANOVA was used to determine if there were significant differences in the antibacterial activities between the aqueous, ethanol, and ethyl acetate extracts at varying concentrations against the test bacteria. P values ≤ 0.05 were considered statistically significant in all analyses.

Table 1: Results of biochemical tests to confirm *Lactobacillus acidophilus* and *Aggregatibacter actinomycetemcomitans*.

Test	Reaction
Lactobacillus acidophilus	
Gram reaction	+
2% NaCl tolerance	+
2% bile tolerance	+
Glucose fermentation	+
Galactose	+
Sucrose	+
Lactose	+
Mannose	+
Catalase	−
Motility	−
Indole	−
TSI	−
Citrate	−
Aggregatibacter actinomycetemcomitans	
Gram reaction	−
Glucose fermentation	+
Motility	−
Fructose	+
Maltose	+
Indole	−
Catalase	+
Oxidase	−

(+) indicates positive reaction; (−) indicates negative reaction.

3. Results

3.1. Yield of Extracts and Phytochemical Screening. The aqueous extract yielded the highest fraction of crude extract of *M. paradisiaca* stalk. Upon phytochemical screening of *M. paradisiaca* fruit stalk extracts (MPFSEs), it was observed that phenols were present in all the three extracts fractions (Table 2). Triterpenoids and cardiac glycosides were detected in the ethyl acetate fraction. All fractions except the aqueous fractions showed presence of alkaloids (Table 2).

3.2. Effect of Extracts on Mean Cell Density (CFU/mL) of L. acidophilus. Erythromycin demonstrated concentration-dependent decrease in mean cell density of *L. acidophilus.* Although the extracts fractions demonstrated inhibitory effects on mean cell density of *L. acidophilus*, this was not comparable to that of erythromycin (Figure 2). Among the three extract fractions, the ethyl acetate fraction demonstrated significant inhibitory effects on cell density, and this inhibitory effect was concentration dependent.

3.3. Effect of Extracts on Mean Cell Density (CFU m/L) of A. actinomycetemcomitans. Both doxycycline, aqueous and ethanol fractions did not demonstrate significant inhibitory effects on mean cell density of *A. actinomycetemcomitans*. The ethyl acetate fraction demonstrated concentration-dependent inhibitory effect on mean cell density (Figure 3).

3.4. Effect of Extracts on Mean Cell Density (CFU m/L) of Candida albicans. Aqueous fractions of the extract had no inhibitory effect on mean cell density of *C. albicans*

Table 2: Percentage yield and qualitative phytochemical profile of the three extract fractions of *Musa paradisiaca* fruit stalk.

	Solvents for extraction		
	Aqueous	Ethanol	Ethyl acetate
Yield* N (%)	1.82 (9.10)	0.53 (2.65)	0.24 (1.20)
Phytochemicals			
Phenols	+	+	+
Flavonoids	−	−	−
Anthraquinones	−	−	−
Tannins	−	−	−
Triterpenoids	+	−	+
Alkaloids	−	−	+
Cardiac glycosides	−	+	+

+ = present; − = absent; and $^{*}N/Y \times 100$, where N = mass of the extract and Y = mass of dry powdered fruit stalk (20 g).

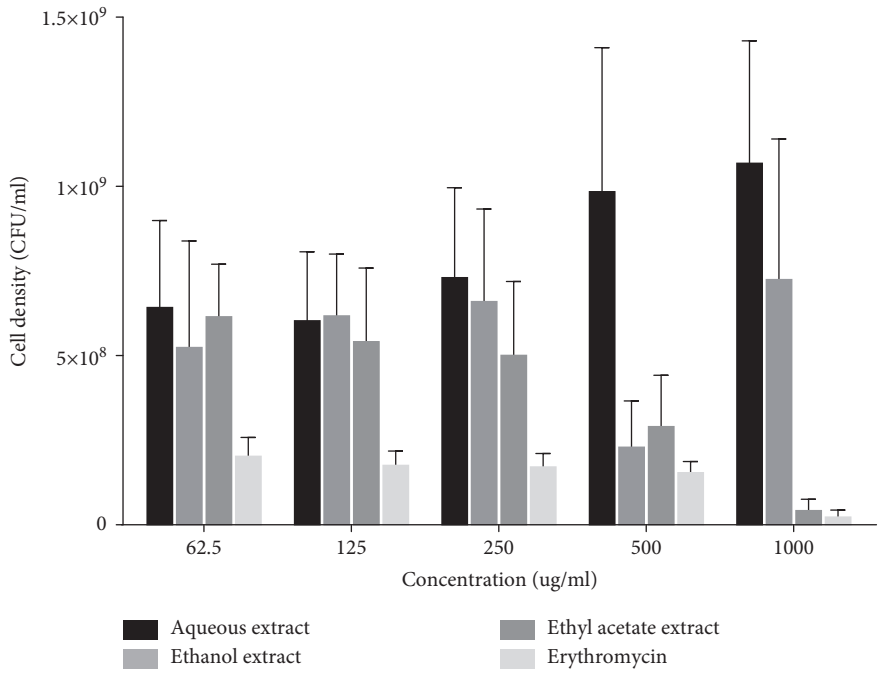

Figure 2: Effect of extracts on microbial cell density of *Lactobacillus acidophilus*. Each bar is the mean cell density ± SD, $n = 3$.

compared to that of fluconazole. Ethyl acetate and ethanol fractions of the extract demonstrated concentration-dependent inhibitory effects on the mean cell density of *C. albicans* comparable to that of fluconazole (Figure 4). At the highest equipotent concentrations, the ethyl acetate fraction demonstrated more inhibitory potency than fluconazole (Figure 4).

3.5. Effect of Extracts on Growth of Test Organisms. All the three fractions (aqueous, ethanol, and ethyl acetate) demonstrated some degree of growth inhibition against the three test organisms (*L. acidophilus, A. actinomycetemcomitans*, and *C. albicans*). Among the fractions, ethyl acetate fraction was the most potent against all the test organisms (Figure 5).

4. Discussion

This study has demonstrated that extracts from *M. paradisiaca* fruit stalk have antimicrobial effects against common oral pathogens (*Lactobacillus acidophilus*, *Aggregatibacter actinomycetemcomitans*, and *Candida albicans*), mostly implicated in oral infections. For instance, these pathobionts have long been linked to the initiation and progression of dental caries, oral thrush, and periodontitis [12, 14, 32]. Antimicrobial property is considered as one of

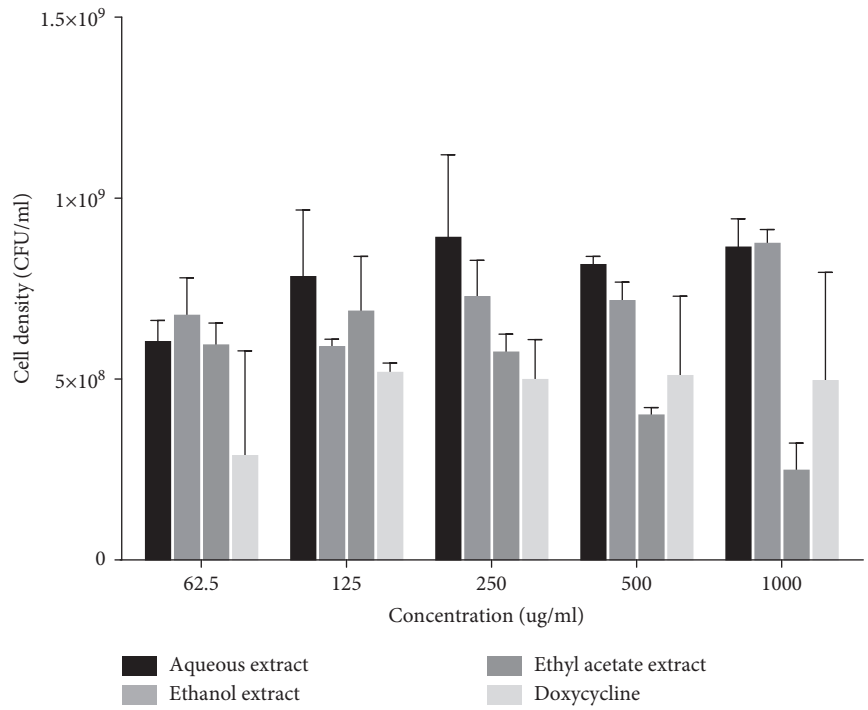

Figure 3: Effect of extracts on microbial cell density of *A. actinomycetemcomitans*. Each bar is the mean cell density ± SD, $n = 3$.

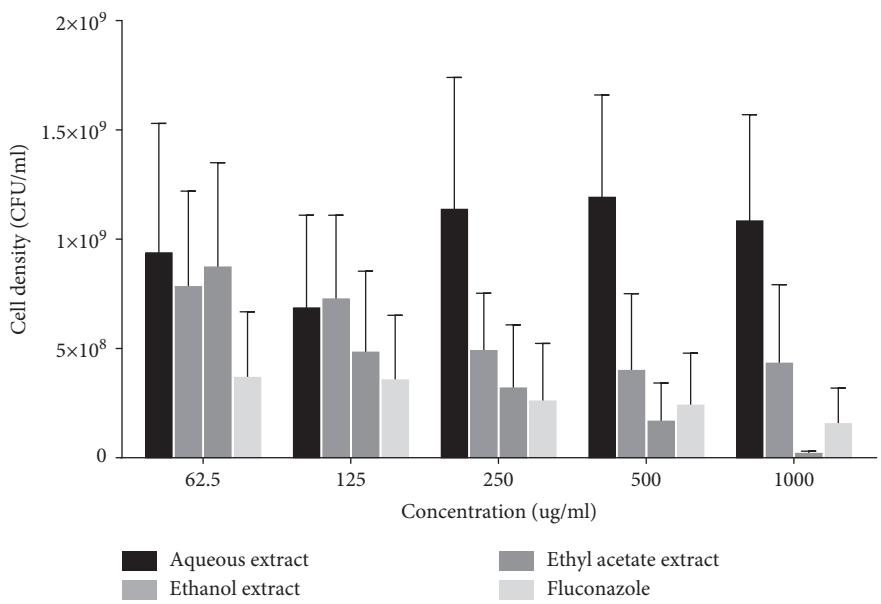

Figure 4: Effect of extracts on microbial cell density of *C. albicans*. Each bar is the mean cell density ± SD, $n = 3$.

the key hallmarks of oral care agents, in view of the involvement of pathogenic bacteria and fungal species in oral infections. The fruit stalk of *Musa paradisiaca* is used in rural Ghana to maintain oral hygiene. Specifically, a pulverized preparation of the fruit stalk of *Musa paradisiaca* with or without charcoal is used to clean the teeth and also to maintain oral hygiene. Use of *M. paradisiaca* fruit stalk by rural folks has been handed down many generations, and this age-old practice has been uneventful. At a time, that use of conventional oral cleansing agents has not only received a backlash for claimed adverse effects but also has been seen as expensive yet ineffective; it is refreshing to learn of readily available alternative or complementary oral care therapies. However, such alternative oral care therapies need to be verified scientifically to substantiate these claims.

In this study, three (aqueous, ethanol, and ethyl acetate) extract fractions of *M. paradisiaca* produced concentration-

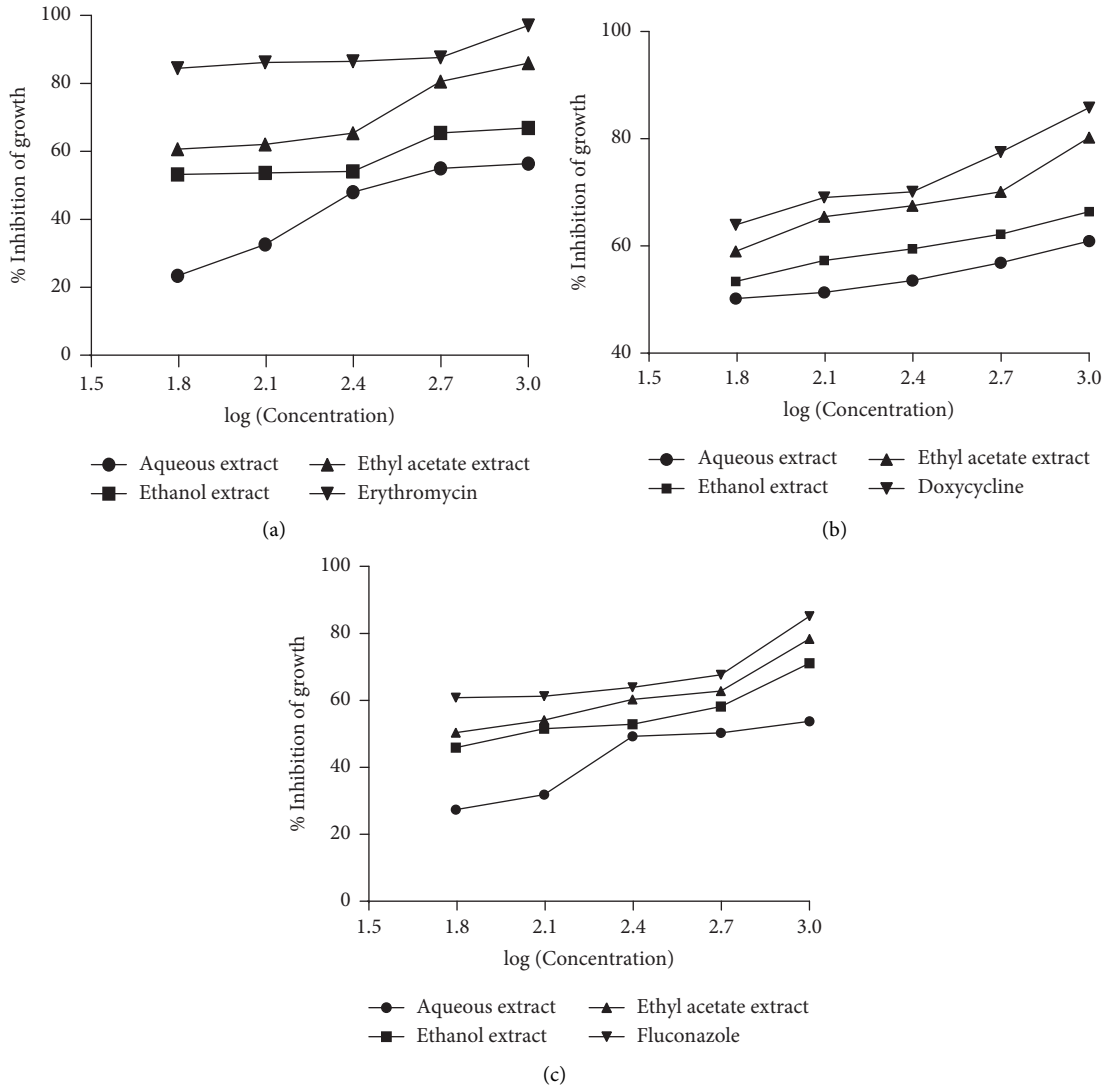

Figure 5: Inhibitory effects of extract fractions of *M. paradisiaca* fruit stalk on the of growth of (a) *Lactobacillus acidophilus*, (b) *Aggregatibacter actinomycetemcomitans*, and (c) *Candida albicans*.

dependent inhibition of growth of the test oral pathogens; in particular, the ethyl acetate extract fraction demonstrated significant antimicrobial activity compared to the other extract fractions. Except for the aqueous extract, which did not demonstrate significant decrease in cell density of the test pathogens, the ethyl acetate and ethanol fractions demonstrated significant antimicrobial activity. The ethyl acetate extract demonstrated concentration-dependent broad-spectrum decrease in cell densities of the three test pathogens. In the case of the ethanol extract, antifungal activity against *Candida albicans* was observed. The current observed antimicrobial activity of *M. paradisiaca* fruit stalk extracts against oral pathogens implicated in oral infections complements that of other studies which showed that extracts from fruits, florets, root, leaves, and peels of *M. paradisiaca* possess antimicrobial properties [23, 27, 33, 34]. The current observation together with that of previous studies which demonstrated that *M. paradisiaca* has antimicrobial activity against *A. actinomycetemcomitans* [35] and *C. albicans* [23] clearly shows the possible prospects of *M. paradisiaca* as an alternative or complementary oral therapy that can be exploited for use by indigenous people.

The bioactivity of medicinal plants has always been linked to their phytochemical composition and functional group enrichment [36]. Antioxidant, antiproliferative, chemopreventive, and antimicrobial properties demonstrated by synergistic interactions of naturally occurring phytocompounds including alkaloids, phenolic compounds, tannins, saponins, and terpenoids have been reported in oral health [16, 37]. The present study showed that *Musa paradisiaca* fruit stalk contains alkaloids, terpenoids, cardiac glycosides, and phenols, which corroborates earlier reports on *Musa paradisiaca* [21, 23] and these phytocompounds possibly may account for the observed antimicrobial activity in the current study.

In increasing order, the antimicrobial activity of the three extract fractions of *M. paradisiaca* fruit stalk was in the order aqueous < ethanol < ethyl acetate, and this order of

antimicrobial activity reflected their phytochemical composition and probably solvent system dependent. It was not surprising to observe that ethyl acetate fraction demonstrated significant antimicrobial activity as it extracted most of the phytocompounds, including alkaloids and cardiac glycosides, which were absent in the aqueous fraction (Table 2). Cardiac glycosides from other medicinal plants have demonstrated antifungal properties [38]. Thus, it is also possible that the antifungal activity demonstrated by the ethyl acetate and ethanol extract fractions of *M. paradisiaca* fruit stalk could be due to their cardiac glycoside components. Surprisingly, alkaloids and cardiac glycosides were absent in the aqueous extract fraction of *M. paradisiaca* fruit stalk in the current study. However, in a study involving fruit peel of *M. sapientum*, it was shown that the aqueous fraction demonstrated significant antibacterial effect compared to that of absolute ethanol fraction and this was linked to its alkaloidal components [39] and this observation stands at variance with the current observation where the aqueous fraction of *M. paradisiaca* fruit stalk demonstrated the least antimicrobial activity. In summary, the observed antimicrobial activity of the fractions (aqueous, ethanol, and ethyl acetate) directly relates to their ability to extract diverse compounds from the fruit stalk; however, disparity between the current results and that of other species in the genus *Musa* could be due to the differences in both species and the part of plant investigated.

5. Conclusion

M. paradisiaca fruit stalk extracts have demonstrated antimicrobial effects against *Lactobacillus acidophilus, Aggregatibacter actinomycetemcomitans*, and *Candida albicans*, and this observation perhaps adds credence to its folk use in maintaining oral hygiene. The observed antimicrobial activity is attributable to the phytochemical composition of *M. paradisiaca* fruit stalk; therefore, future studies should focus on bio-assay guided extraction, identification, and purification of specific antimicrobial phytocompounds with specific antimicrobial activity against oral pathogens.

Data Availability

All data used to support the findings of this study are available within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Alex Boye conceived, designed, and wrote and critically reviewed the final manuscript for intellectual content. Ernest Owusu-Boadi, Gabriel Mensah, and Emmanuel Ayamba Ayimbissa performed experiments. Mainprice Akuoko Essuman analyzed the results and retrieved literature information.

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Research Article

Evodiae fructus Extract Inhibits Interleukin-1β-Induced MMP-1, MMP-3, and Inflammatory Cytokine Expression by Suppressing the Activation of MAPK and STAT-3 in Human Gingival Fibroblasts In Vitro

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Periodontitis is a Gram-negative bacterial infectious disease. Numerous inflammatory cytokines, including interleukin-1β (IL-1β), regulate periodontitis pathophysiology and cause periodontal tissue destruction. In human gingival fibroblasts (HGFs), IL-1β stimulates the production of matrix metalloproteinases (MMPs) and proinflammatory cytokines via various mechanisms. Several transcription factors, such as signal transducer and activator of transcription 3 (STAT-3), activator protein 1 (AP-1), and nuclear factor-κB (NF-κB), regulate gene expression. Mitogen-activated protein kinases (MAPKs) regulate these transcription factors. However, the MAPK/STAT-3 activation signal in HGFs is unknown. We investigated the potential inhibitory effects of the extract of *Evodiae fructus* (EFE), the dried, ripe fruit of *Evodia rutaecarpa*, on MMP and proinflammatory cytokine expression in IL-1β-stimulated HGFs. EFE inhibited the expression of MMP-1, MMP-3, and proinflammatory cytokines (TNF-α, IL-6, and IL-8) in IL-1β-stimulated HGFs through the inhibition of IL-1β-induced MAPK/STAT-3 activation. Also, these results suggest that the EFE may be a useful for the bioactive material for oral care.

1. Introduction

Periodontitis is a serious inflammatory disease of the gums. It is most commonly caused by periodontal Gram-negative bacterial infections such as *Porphyromonas gingivalis* infection, causes connective tissue destruction and bone resorption around the teeth, and is the leading cause of tooth loss [1, 2]. *P. gingivalis* produces lipopolysaccharide (LPS), which is a major virulence factor, to induce inflammation [3]. LPS induces the expression of proinflammatory cytokines and inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-8, E-prostaglandins (PGEs), and nitric oxide (NO) [4]. Among these, IL-1 plays a major role in periodontal tissue destruction by enhancing the expression of collagenic enzymes, including matrix metalloproteinases (MMPs) [5, 6].

Gingival connective tissue mainly consists of stromal cells, such as fibroblasts, and extracellular matrix (ECM). ECM is a complex structure of mucopolysaccharides and fibrin, including collagens, elastin, fibronectin, glycosaminoglycans, and

laminins, which structurally supports the cells and mechanical strength of tissues [7, 8]. MMPs are a family of structurally related ECM-degrading enzymes and are associated with several destructive processes, including inflammation, tumor invasion, and periodontitis [9, 10]. Inflamed periodontal tissue, including epithelial cells and fibroblasts, expresses various types of MMPs [11]. Especially, gingival fibroblasts are known to produce MMP-1, -2, -3, -7, -8, -9, and -13 and to be involved in periodontal tissue destruction [12–14]. MMP-1 (collagenase I) and MMP-3 (stromelysin) play important roles in periodontal diseases [6, 12, 15]. MMP-1 mainly degrades the collagen present in periodontal tissues during periodontal disease progression [16]. MMP-3 disassembles ECM structural substances and induces the release of some proenzymatic MMP forms [16–18]. Human gingival fibroblasts (HGFs) are known to secrete MMP-1 and MMP-3 in response to stimulation with IL-1 [6, 19, 20].

IL-1 is an important and multifunctional cytokine associated with host immune and inflammatory reactions [21]. Previous studies have suggested the importance of IL-1 in the development of periodontal diseases [15, 22, 23]. Through its various biological activities, IL-1 induces a variety of inflammatory reactions, including the synthesis of NO and reactive oxygen species (ROS) and the expression of PGEs, MMPs, and inflammation-related cytokines [24]. In addition, it is one of the most powerful inducers of MMPs in HGFs [25]. IL-1 activates MMP expression-related signaling pathways, including mitogen-activated protein kinase (MAPK), and transcription factors, such as signal transducer and activator of transcription 3 (STAT-3), activator protein 1 (AP-1), and nuclear factor-κB (NF-κB) [26–28]. In HGFs, IL-1β induces TNF-α, IL-6, IL-8, and MMP expression via the MAPK/NF-κB/AP-1 signaling axis [20] and causes STAT-3 phosphorylation [29].

Evodiae fructus (EF) is the dried, ripe fruit of *Evodia rutaecarpa*, which has been used as an analgesic, antidiarrheal, antiemetic, and anti-inflammatory drug in Chinese traditional medicine [30, 31]. Alkaloids are the main active ingredients of EF, and recent studies have investigated the pharmacological activities of alkaloids such as evodiamine, hydroxyevocarpine, hydroxyevodiamine, evocarpine, isoevodiamine, higenamine, and rutaecarpine [31]. Especially, EF exerts anti-inflammatory effects by inhibiting proinflammatory cytokine production, ROS generation, and MAPK activation [32]. However, the anti-inflammatory effects of EF on HGFs have not yet been investigated.

Therefore, the hypothesis of the present study was that EFE could inhibit inflammatory reactions in IL-1β-stimulated HGFs. In this study, we investigated the effects of the EF extract (EFE) on IL-1β-induced proinflammatory cytokine and MMP expression in HGFs. Furthermore, we demonstrated the signal pathways of anti-inflammatory effects of the EFE by detecting several inflammatory mediators such as MAPK, NF-κB, AP-1, and STAT-3.

2. Materials and Methods

2.1. HGF Culture. HGFs were obtained from Lifeline Cell Technology (Walkersville, MD, USA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin 100 units/mL, streptomycin 100 μg/mL, and Fungizone® (amphotericin B) 0.25 μg/mL at 37°C in a 5% CO_2 atmosphere. Cells were passaged at a rate of one-third at regular intervals so that the monolayers did not exceed 70–80% confluence.

2.2. Reagents. EF water extract was purchased from the Korean Plant Extract Bank (CW02-075; Daejeon, Korea), and a 50 mg/ml stock was prepared in distilled water. Human MMP-1 and MMP-3 antibodies and recombinant human IL-1β were obtained from R&D Systems (Minneapolis, MN, USA). Anti-β-actin antibody, p38 inhibitor SB203580, JNK inhibitor SP600125, and ERK inhibitor PD98059 were purchased from Sigma-Aldrich (St. Louis, MO, USA). p-c-Jun antibody was purchased from Abcam (Cambridge, MA, USA). PCNA, p65, and p50 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p-STAT3, STAT-3, p-JNK, JNK, p-p38, p38, p-ERK, and ERK antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). High-glucose DMEM was purchased from HyClone (Logan, UT, USA). FBS and phosphate-buffered saline (PBS) were purchased from Gibco BRL (Gaithersburg, MD, USA).

2.3. Cell Viability Assay. The effect of the EFE on HGF viability was determined using the Cellrix® Viability Assay Kit (Medifab, Seoul, Korea). Cells were seeded in each well of a 96-well plate and incubated for 24 h. EFE was added at various concentrations (0–100 μg/ml), and the plate was further incubated for 24 h. Then, 10 μl of assay reagent was added in each well, and the plate was incubated in the dark at 37°C for 1 h. After the incubation, the optical density at 450 nm was read on a microplate reader (Sunrise™, Tecan, Mannedorf, Switzerland).

2.4. Western Blot Analysis. HGFs were pretreated with 25 or 50 μg/ml EFE for 1 h and then incubated with IL-1β at 37°C for 24 h. Collected cell pellets were lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA, USA) on ice. Total proteins were quantified using a BioSpec-nano instrument (Shimadzu, Kyoto, Japan). Proteins (25 μg) were electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel at 100 V and transferred onto blotting membranes (GE Healthcare Life Sciences, Little Chalfont, UK). After blocking for 2 h, the membranes were incubated with primary antibodies diluted (1 : 1000) in blocking solution (5% skim milk or bovine serum albumin in TBS with Tween 20) at 4°C overnight and then with secondary antibodies for 1 h under gentle agitation. Protein bands were visualized using a Mini HD6 image analyzer (UVITEC, Cambridge, UK).

2.5. Enzyme-Linked Immunosorbent Assay (ELISA). Cells were pretreated with the EFE for 1 h and then treated with IL-1β at 37°C for 24 h. Cell culture medium was collected after removing the particulates. MMP-1 and MMP-3 levels

in the culture supernatants were determined using a Human Active MMP-1 Fluorokine E Kit and Human MMP-3 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.

2.6. Quantitative Reverse Transcription (RT-q) PCR. RNA was extracted from cultured cells using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). RNA concentrations were determined on the BioSpec-nano instrument. cDNA was synthesized from total mRNA (1 μg) using the PrimeScript™ RT Reagent Kit (Takara, Shiga, Japan). mRNA levels of MMP-1, MMP-3, TNF-α, IL-6, IL-8, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined by qPCR using SYBR® Green reagent and a StepOnePlus Real-Time PCR System (both from Applied Biosystems, Foster City, CA, USA). Target mRNA levels were normalized to those of GAPDH as an internal control (Table 1).

2.7. Nuclear Fractionation. HGFs were prestimulated with EFE or MAPK inhibitors (SB203580, PD98059, and SP600125) for 1 h and then incubated with IL-1β for 3 h. Then, the cells were immediately washed twice with PBS. Nuclear proteins were isolated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA) per the manufacturer's protocol.

2.8. RNA Interference. Negative control small interfering RNA (siRNA) and STAT-3-specific siRNA were purchased from BIONEER (Daejeon, Korea). In brief, HGFs were transfected with 200 pmol siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions in 60 mm dishes at 37°C for 48 h.

2.9. Statistical Analysis. All experiments were performed in triplicate. Statistical significance was evaluated using the analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test. $p < 0.01$ was considered significant.

3. Results

3.1. Effect of the EFE on IL-1β-Induced MMP-1 and MMP-3 Expression in HGFs. First, to evaluate the cytotoxicity of the EFE on HGFs, the cells were incubated with various concentrations of the EFE for 24 h. EFE treatment for 24 h did not significantly affect cell viability (Figure 1(a)). Thus, nontoxic concentrations (25 and 50 μg/ml) of the EFE were used in subsequent experiments. To evaluate the effects of the EFE on IL-1β-induced MMP expression, MMP protein and mRNA levels were determined using western blotting and RT-qPCR, respectively. IL-1β significantly increased MMP-1 and MMP-3 protein expression, whereas the increases were significantly suppressed by the EFE (Figure 1(b)). Similarly, IL-1β-induced increases in mRNA levels were significantly suppressed by pretreatment with the EFE (Figures 1(c) and 1(d)). In addition, we evaluated the effects of the EFE on MMP-1 and MMP-3 secretion using ELISA, and IL-1β-induced MMP secretion was inhibited by the EFE (Figures 2(a) and 2(b)).

Table 1: Primer sequences.

Gene name	Primer sequences
TNF-α	Forward: 5′-CTGCTGCACTTTGGAGTGAT-3′ Reverse: 5′-AGATGATCTGACTGCCTGGG-3′
IL-6	Forward: 5′-TACCCCCAGGAGAAGATTCC-3′ Reverse: 5′-GCCATCTTTGGAAGGTTCAG-3′
IL-8	Forward: 5′-AGACAGCAGAGCACACAAGC-3′ Reverse: 5′-ATGGTTCCTTCCGGTGGT-3′
MMP-1	Forward: 5′-AGTGACTGGGAACCGATGCTGA-3′ Reverse: 5′-CTCTTGGCAAATCTGGCCTGTAA-3′
MMP-3	Forward: 5′-ATTCCATGGAGCCAGGCTTTC-3′ Reverse: 5′-CATTTGGGTCAA ACTCCAACTGTG-3′
GAPDH	Forward: 5′-ATGGAAATCCCATCACCATCTT-3′ Reverse: 5′-CGCCCCACTTGATTTTGG-3′

3.2. Effect of the EFE on IL-1β-Induced Proinflammatory Cytokine Expression in HGFs. To evaluate the effects of the EFE on proinflammatory cytokine expression in IL-1β-stimulated HGFs, cells were pretreated with 50 μg/ml EFE for 1 h and then stimulated with IL-1β at 37°C for 0, 2, 4, 6, or 8 h. The mRNA levels of TNF-α, IL-6, and IL-8 were estimated by RT-qPCR. As shown in Figure 3, EFE suppressed the increases in TNF-α, IL-6, and IL-8 mRNA levels induced by IL-1β stimulation at several time points.

3.3. Effect of the EFE on IL-1β-Induced MAPK/NF-κB/AP-1 Activation in HGFs. To investigate the signaling pathway involved in EFE-mediated suppression of IL-1β-induced proinflammatory cytokine and MMP expression, we examined the effects of the EFE on the activation of MAPK and several transcriptional factors. In HGFs, MMP expression is regulated by the MAPK/NF-κB or MAPK/AP-1 pathway [48]. Therefore, we confirmed that the EFE inhibited IL-1β-induced phosphorylation of MAPKs (p38, ERK, and JNK) (Figure 4(a)). To investigate the effects of the EFE on NF-κB and AP-1 activation by IL-1β, we examined the nuclear translocation of p65, p50, and p-c-Jun by western blotting, which confirmed that the EFE did not affect IL-1β-induced activation of NF-κB and AP-1 (Figure 4(b)). These results indicated that the EFE inhibits IL-1β-induced TNF-α, IL-6, IL-8, MMP-1, and MMP-3 expression through the inhibition of MAPK activation but does not involve the NF-κB and AP-1 pathways in HGFs.

3.4. Effect of MAPK/STAT-3 Activation on IL-1β-Induced MMP-1 and MMP-3 Expression. As the EFE did not inhibit either of the MAPK/NF-κB and MAPK/AP-1 pathways and STAT-3 activation reportedly induced MMP expression and was affected by MAPK activation [33, 34], we investigated the potential association with STAT-3. As shown in

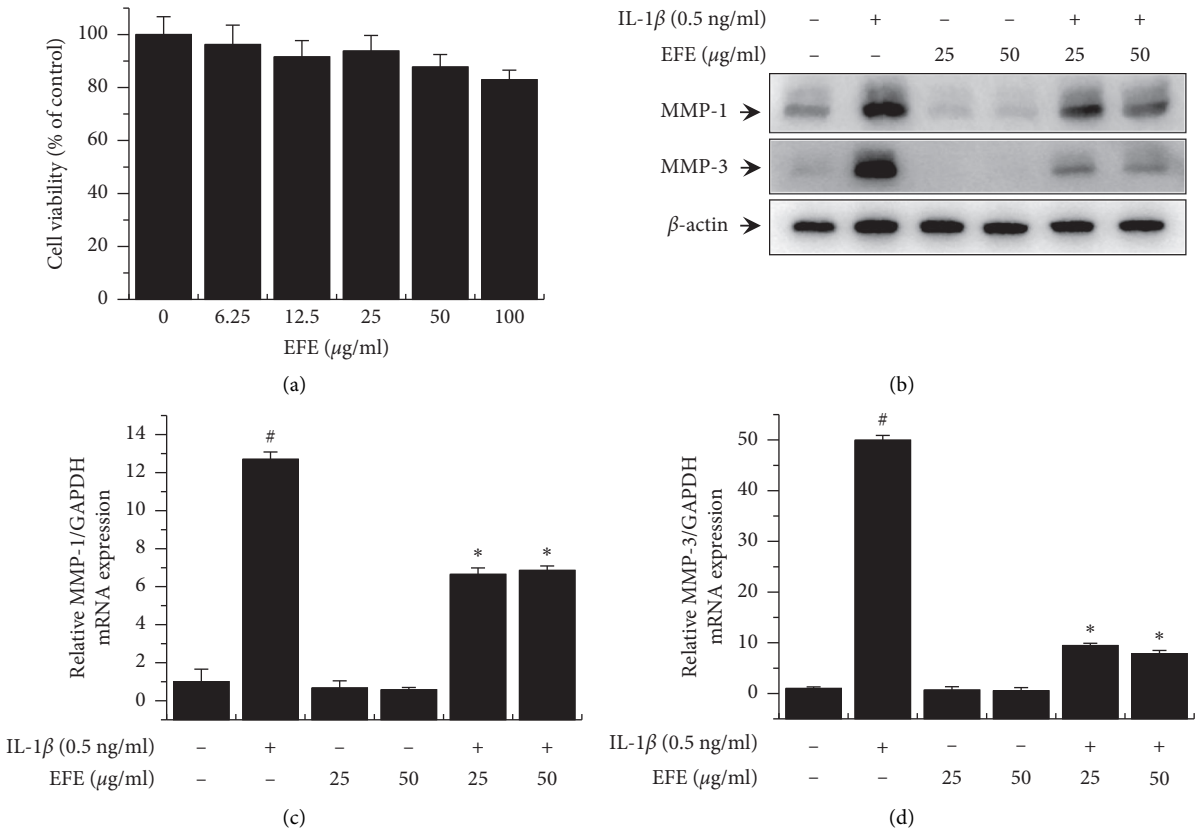

Figure 1: Effect of the EFE on the viability of HGFs. Cells were seeded, incubated for 24 h, and treated with the EFE at 0, 6.25, 12.5, 25, 50, and 100 μg/ml. Cytotoxicity of the EFE was investigated after treatment for 24 h (a). Cells were preincubated with 25 and 50 μg/ml of the EFE for 1 h and then treated with IL-1β for 24 h. Western blot analysis was performed to determine MMP-1 and MMP-3 protein levels in HGF lysates (b). The MMP-1 and MMP-3 expression was analyzed using RT-qPCR (c, d). Data were presented as the mean ± SEM of three independent experiments. $^{\#}p < 0.01$ vs. control; $^{*}p < 0.01$ vs. IL-1β.

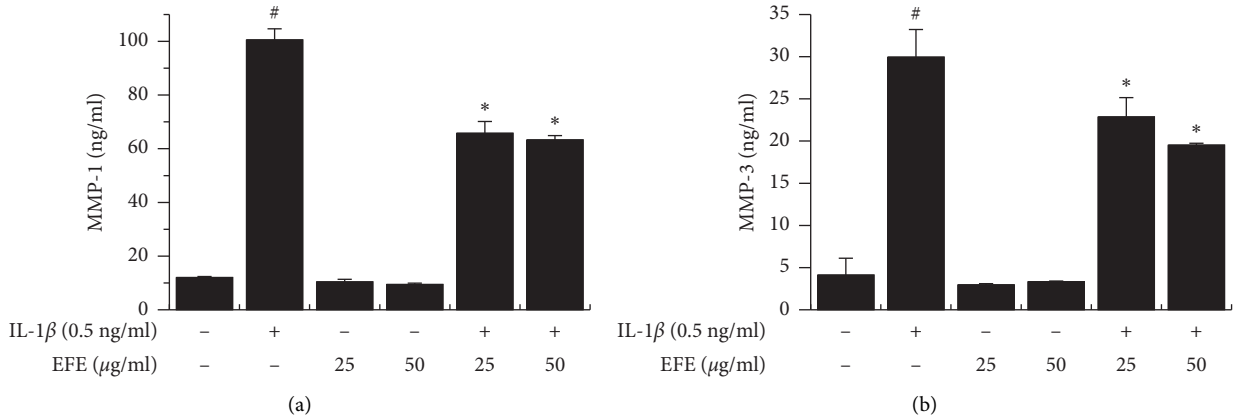

Figure 2: EFE inhibits IL-1β-induced MMP secretion. Cells were pretreated with the EFE for 1 h and then incubated with IL-1β for 24 h. Secreted MMP-1 (a) and MMP-3 (b) proteins were detected using ELISA. Data were presented as the mean ± SEM of three independent experiments. $^{\#}p < 0.01$ vs. control; $^{*}p < 0.01$ vs. IL-1β.

Figure 5(a), IL-1β increased STAT-3 phosphorylation in the nuclear fraction of HGFs, which was inhibited by the EFE. To investigate whether MAPK signaling is directly involved in IL-1β-induced STAT-3 phosphorylation in HGFs, we evaluated the effects of MAPK inhibitors (p38: SB203580, ERK: PD98059, and JNK: SP600125) on IL-1β-induced activation of STAT-3 phosphorylation in HGFs. All three MAPK inhibitors inhibited STAT-3 phosphorylation at 3 h after IL-1β treatment (Figure 5(b)). Next, to investigate the association between STAT-3 and IL-1β-

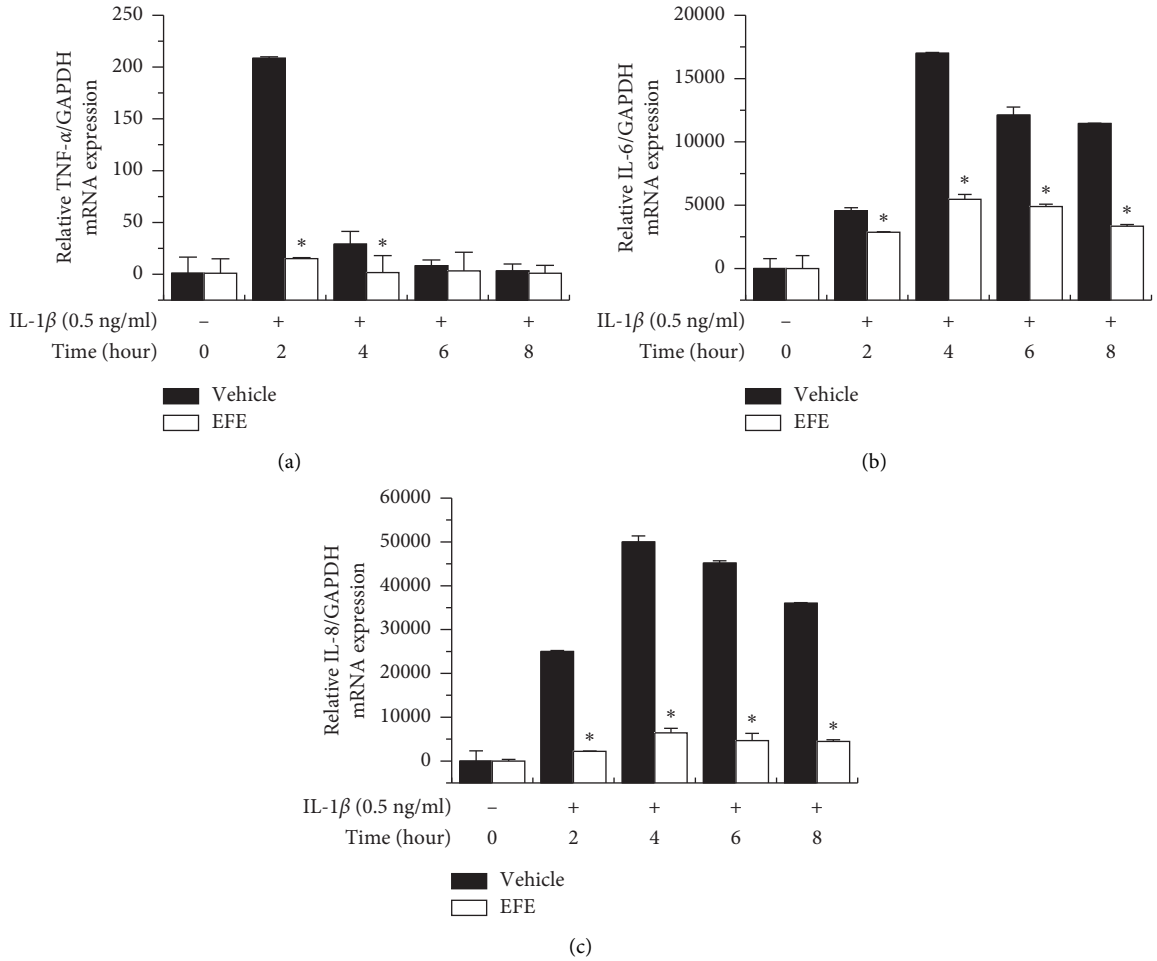

Figure 3: EFE inhibits IL-1β-induced proinflammatory cytokine mRNA expression in HGFs. HGFs were prestimulated with 50 μg/ml of the EFE for 1 h and then incubated with IL-1β various times. Total cellular mRNA levels of TNF-α (a), IL-6 (b), and IL-8 (c) were analyzed by RT-qPCR. Data were presented as the mean ± SEM of three independent experiments. $^{*}p < 0.01$ vs. vehicle.

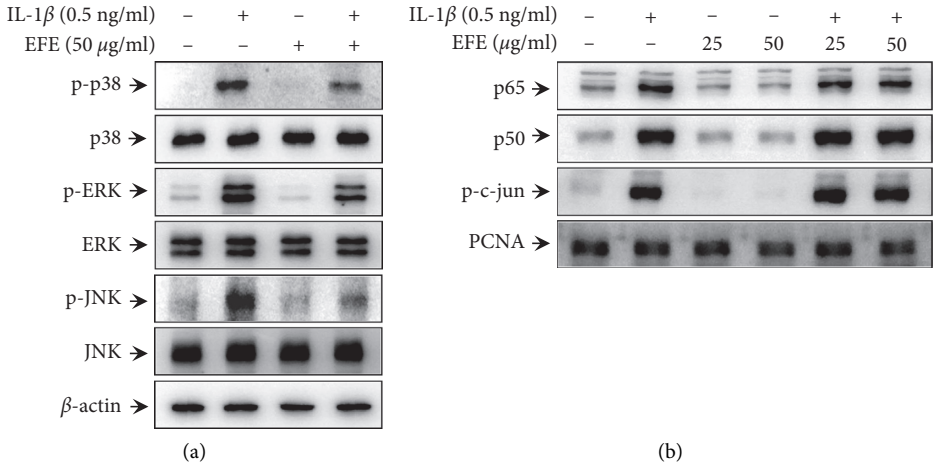

Figure 4: EFE inhibits MAPK activation by IL-1β in HGFs. Cells were preincubated with the EFE for 1 h and then stimulated with IL-1β for 30 min. Using western blotting, the phosphorylated and total MAPK (p38, ERK, and JNK) proteins were quantified (a). Cells were prestimulated with 25 and 50 μg/ml of the EFE for 1 h and then incubated with 0.5 ng/ml of IL-1β for 3 h. After nuclear fractionation, western blot analysis was performed to quantify nuclear NF-κB (p65 and p50) and AP-1 (p-c-Jun) subunits (b).

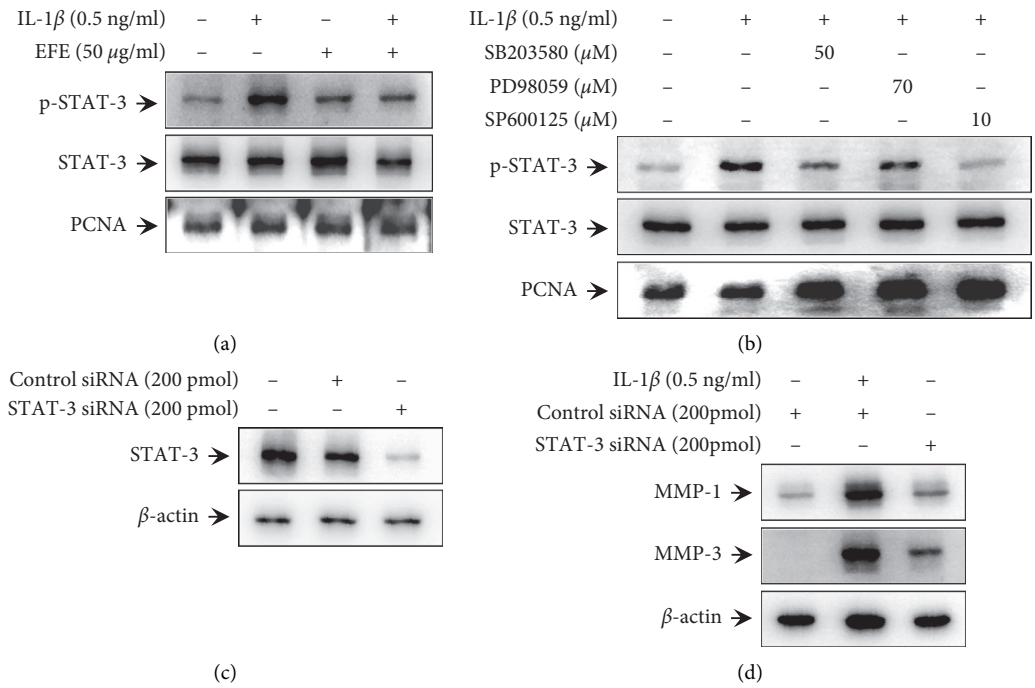

Figure 5: EFE inhibits IL-1β-induced MAPK/STAT-3 activation in HGFs. Cells were pretreated with 50 μg/ml of the EFE for 1 h and then incubated with IL-1β for 3 h. Western blot analysis was performed to confirm the presence of p-STAT-3 and STAT-3 in the nucleus (a). HGFs were pretreated with MAPK inhibitors (p38: SB203580, ERK: PD98059, and JNK: SP600125) for 1 h and then stimulated with IL-1β for 3 h. The levels of p-STAT-3 and STAT-3 in nuclear extracts were analyzed by western blotting (b). Cells were transfected with STAT-3 or negative control siRNA for 48 h. STAT-3 protein levels were analyzed by western blot analysis (c). HGFs were transfected for 48 h and then incubated with IL-1β for 24 h. Western blot analysis was performed to determine MMP-1 and MMP-3 protein expression (d).

induced MMP-1 and MMP-3 expression, we knocked down STAT-3 expression using STAT-3 siRNA. STAT-3 knockdown reduced IL-1β-induced MMP-1 and MMP-3 protein expression (Figures 5(c) and 5(d)). This result indicated that IL-1β-induced MMP-1 and MMP-3 expression is regulated through MAPK/STAT-3 activation in HGFs.

4. Discussion

Periodontitis is the most prevalent inflammatory disease and is caused by periodontal Gram-negative bacterial infection [1]. Such infections induce an inflammatory reaction, leading to periodontal tissue destruction and bone resorption [35]. *P. gingivalis*, one of the most important bacteria in periodontal disease, produces LPS, a major toxin that induces inflammatory responses. LPS induces the production of various proinflammatory cytokines and mediators, such as IL-1, TNF-α, NO, and PGE2, in immune cells, which trigger the destruction of periodontal tissue by MMPs produced by gingival fibroblasts or inflammatory cells, including osteoclasts [11, 36]. MMPs play important roles in the degradation of the ECM and bone collagen matrix in periodontitis [37–39]. In active periodontitis, periodontal tissue loss and alveolar bone destruction are increased by MMPs and inflammatory cytokines [2, 38]. Various types of MMPs, including MMP-1, -2, -3, -8, -9, and -13, are involved in periodontal tissue destruction [12–14, 40]. Especially, MMP-1 and MMP-3 are important in periodontal diseases [12, 15, 41]. Therefore, unraveling the regulation of MMP-1 and MMP-3 may contribute to the development of treatments for periodontitis. Therefore, we investigated the potential inhibitory effects of the EFE on MMP-1 and MMP-3 expression in HGFs (Figures 1 and 2).

HGFs are a major cell type in periodontal tissue and secrete various inflammatory cytokines upon inflammatory stimuli, including bacteria and their pathogenicity factors [42–44]. IL-1 is an important proinflammatory cytokine present in inflammatory gingiva and plays essential roles in the pathogenesis of periodontitis [45, 46]. IL-1 is involved in the inflammatory response and ECM remodeling through the induction of various factors, including ROS, NO synthase, PGEs, cytokines, and MMPs [24]. Upon stimulation with IL-1β, HGFs produce TNF-α, IL-6, and IL-8 [47]. TNF-α is primarily secreted by immune cells such as fibroblasts and is a potent proinflammatory cytokine that induces the production of MMP, cytokines, PGE2, cell adhesion molecules, and molecules related to bone resorption [20, 48, 49]. IL-6 plays a crucial role in infected periodontal tissue as well as in bone resorption, osteoclast differentiation, and continuous tissue destruction [50]. IL-8 induces the migration of neutrophils to periodontal lesions, weakening periodontal tissue due to intracellular ROS production, MMP expression, and lysosomal enzyme release [51, 52]. This cytokine is highly expressed in periodontal tissue [53]. Our study findings suggest that the EFE may improve periodontal inflammation by inhibiting the expression of proinflammatory cytokines in HGFs (Figure 3).

IL-1β reportedly induces MAPK/NF-κB and AP-1 signaling in HGFs [20, 54]. Furthermore, the MAPK/AP-1 and NF-κB cascades mediate IL-1β-stimulated cytokine and MMP-1 expression in HGFs [20, 55]. To evaluate the mechanism underlying the inhibitory effect of the EFE on IL-1β-induced MMP and proinflammatory cytokine expression, we examined the effects of the EFE on MAPK/NF-κB/AP-1 activation. EFE did not regulate MMPs through MAPK/NF-κB/AP-1 activation (Figure 4). Therefore, we next investigated a potential association with STAT-3. It has been reported that intracellular, activated STAT-3 regulates the expression of MMPs and proinflammatory cytokines [33, 34, 56]. However, the association between STAT-3 and cytokine and MMP expression induced by IL-1 in HGFs had not been investigated. To evaluate the association between MAPK and STAT-3, we used inhibitors of p38 (SB203580), ERK (PD98059), and JNK (SP600125) and determined STAT-3 phosphorylation induced by IL-1β. While p-STAT-3 levels were increased in the IL-1β-treated group, the MAPK inhibitors were found to inhibit STAT-3 phosphorylation, suggesting an interaction between MAPK and STAT-3 (Figure 5(b)). Additionally, we confirmed that STAT-3 knockdown suppressed IL-1β-induced MMP-1 and MMP-3 expression (Figures 5(c) and 5(d)). These results are particularly relevant as this is the first report of MMP expression regulation by IL-1β-induced MAPK/STAT-3 activity in HGFs. Finally, we confirmed that the EFE inhibits IL-1β-induced STAT-3 phosphorylation (Figure 5(a)). However, since the EFE is an extract, not a single compound, further investigation is required to analyze the active compounds that exhibit the anti-inflammatory effects of the EFE. Additional studies will be necessary to confirm the anti-inflammatory effects of the EFE in periodontitis using an in vivo experimental model.

5. Conclusion

Taken together, this study demonstrated that the EFE inhibits IL-1β-induced MAPK/STAT-3 activation in HGFs and the expression of MMP-1 and MMP-3, which decompose various substrates present in periodontal tissue. The extract also inhibits several increased proinflammatory cytokines, such as TNF-α, IL-6, and IL-8. These results suggest that the EFE may be a useful bioactive material for oral care.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

Hyun-Kyung Song and Eun-Mi Noh are the co-first authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Kang-Beom Kwon was responsible for project administration. Young-Rae Lee was responsible for conceptualization and project administration. Hyun-Kyung Song and Eun-Mi Noh contributed equally to this work. Jeong-Mi Kim analyzed the data. Yong-Ouk You provided comments and editorial review of the manuscript.

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Review Article

Camellia sinensis in Dentistry: Technological Prospection and Scientific Evidence

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Purpose. This study aimed to evaluate reports of patents for oral care formulations, based on *Camellia sinensis* (*C. sinensis*), deposited and granted in intellectual property banks. *Methods*. A survey was conducted through collection, treatment, and analysis of extracted information from patent reports selected. The documentary research was conducted in January 2021 on formulations with *C. sinensis* for dental applications, including since the first patent deposits until the current time. The risk of bias of clinical trials with these formulations was analyzed to verify the scientific evidence. The data extracted represent the distribution of the number of patents by banks, annual evolution of patent deposits, applicant of patents by country, distribution of patents according to International Patent Classification codes, and the types of patented products. *Results*. Data and information from 20 selected patents were extracted. The United States Patent and Trademark Office (USPTO) and World Intellectual Property Organization (WIPO) were the banks with the largest number of patents for products/formulations with *C. sinensis* for oral care applications with 7 (35%) and 6 (30%) patent registrations, respectively. Other banks did not provide patents related to the search. Patents of compositions were the largest with 14 filings, and the remainder of formulations are represented specially by mouthwashes and toothpastes. As for clinical application, 18 patents were filed as products with antimicrobial and antibiofilm action, while 2 patents are directed to the treatment of xerostomia. In general, the aspects of the studies of clinical efficacy pointed to a low risk of bias. *Conclusion*. The study pointed out a small number of products protected by patents for *Camellia sinensis* for oral care indication, highlighting mainly mouthwash compositions and formulations. In the methodological parameters of clinical trials carried out with the formulations, the majority pointed out a low risk of bias.

1. Introduction

The practice of using medicinal plants with pharmacological activities for the treatment of diseases is ancient, including for conditions related to oral health. The use of natural products as medicinal therapy existed long before the pharmaceutical industry and after the emergence of these products has been incorporated into the development of formulations to the present day [1–3].

Herbs and medicinal plants can be used in different ways, including whole herb, leaves, roots, essential oils, and prepared as teas, syrups, creams, ointments, and even

capsules or pills that contain a powdered form of the plant [4]. Tea constitutes an infusion prepared from dry leaves and is the second most consumed drink in the world, its consumption being surpassed only by water, in addition to having great cultural and economic relevance in several countries. Among the most varied types of teas, *Camellia sinensis* (*C. sinensis*) teas stand out as one of the most popular and used worldwide [5–7].

C. sinensis is a plant rich in polysaccharides, caffeine, polyphenols, amino acids, and antioxidants, in addition to micro and macronutrients beneficial to human health [8]. It has different important pharmacological properties such as antimicrobial, antioxidant, antidiabetic, and anti-inflammatory activities, in addition to its consumption presenting several documented benefits related to oral health, especially regarding caries and periodontal diseases [3, 6].

This plant is a species belonging to the Theaceae family and has small perennial shrubs, widely used to produce teas, especially green and black [9]. These teas have several pharmacological properties, with their production being carried out mainly in regions of tropical and subtropical climate, with abundant and regular rainfall [9, 10]. According to its fermentation and maturation process, *C. sinensis* tea can be classified as green, white, yellow, red, and black, where important differences can be verified according to its cultivation and leaf processing [6, 11].

Green tea from *C. sinensis* has a strong antioxidant power through its polyphenolic chemical constituents, beneficial in several clinical conditions such as dental caries, gingivitis, periodontitis, and halitosis, in addition to neuroprotection in the oral cavity [1]. Since it has a high concentration of fluoride in its nutrient composition, the consumption of this tea is extremely beneficial in repairing dental tissue in an acidic environment and improving resistance to demineralization [12, 13]. Furthermore, it has been shown that this tea has other properties, such as antiviral action against influenza viruses, herpes viruses, and antifungal action against *Candida albicans*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum* [14].

Black tea is a product of *C. sinensis* treated with an oxidation process that requires longer steps and contains a greater amount of caffeine when compared to other teas from this plant. Its continued use can reduce blood pressure, the risks of type 2 diabetes, and improve the lipid profile. Its polyphenolic constituents promote health benefits, mainly in obesity, diabetes, cancer, atherosclerosis, inflammatory diseases, and osteoporosis [15–18].

In recent years, the search for new substances and formulations with pharmacological potential and biocompatibility has increased, which is revealed by the growing number of studies on the use of natural products. Often these studies seek to generate new technologies for society with a natural raw material traditionally used, giving rise to the development of innovations and patents [19, 20].

The elaboration of an invention patent or utility model is an extremely relevant indicator to mainly evaluate the level of development and technological innovation in industries and research institutions. Therefore, natural products are sources of biomolecules or therapeutic complexes that can be used for technological innovation and maintain competition in the market in several areas, including products for dental applications [21, 22]. Given the economic importance and medicinal products of *C. sinensis*, as well as its several benefits to systemic and mainly oral health, this study aimed to evaluate reports of patents deposited and granted on dental formulations based on *C. sinensis* in intellectual property banks.

2. Materials and Methods

2.1. Elaboration of the Technological Prospective Study. The survey was conducted through collection and analysis of extracted information from patent reports selected. The documentary research was conducted in January 2021 about formulations with *C. sinensis* for dental applications, including since the first patent deposit in 2004 until all the year of 2020. The searches were direct with access to reports of patents deposited and granted in the following intellectual property banks of worldwide references:

(1) Canadian Intellectual Property Office (CIPO)—Canada
(2) China National Intellectual Property Administration (CNIPA)—China
(3) Espacenet—European Patent Office (EPO)—Europe
(4) German Patent and Trademark Office (DPMA)—Germany
(5) Intellectual Property India—India
(6) Japanese Patent Office (JPO)—Japan
(7) National Institute of Intellectual Property (INPI)—Brazil
(8) Swiss Federal Institute of Patent Office (IGE-IPI)—Switzerland
(9) United States Patent and Trademark Office (USPTO)—United States
(10) World Intellectual Property Organization (WIPO)—Europe

2.2. Search Strategy and Data Extraction. For the preparation of the study, we conducted a mapping of patent applications using the keyword "*Camellia sinensis*" in the search field. All patent documents that included this term were initially considered in the search with the exploratory reading of titles and summaries, as a criterion for inclusion of the patents found. Then, only the active reports related to dentistry were selected; expired, abandoned, or denied patents were not included. Relevant information that describes the invention in the patent reports was selected and organized in graphics in GraphPad Prism 6 program to analyze descriptive statistics.

The data extracted represent the distribution of the number of patents by banks, annual evolution of patent

deposits, applicant of patents by country, distribution of patents according to International Patent Classification (IPC) codes, and the types of patented products.

2.3. Scientific Evidence. According to the patents selected at the end of the search, the described inventions and their purposes with dental applications were evaluated and were searched clinical trials related to each selected patent in PubMed.

2.4. Risk of Bias Assessment. Trials were assessed using Cochrane's tool for assessing the risk of bias in randomized trials [23]. The tool includes the following domains: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other sources of bias. We rated each domain as low risk, unclear risk, or high risk of bias.

We classified the overall risk of bias as low if all domains were at low risk of bias, as high if at least one domain was at high risk of bias, or as unclear if at least one domain was at unclear risk of bias, and no domain was at high risk. This rule is specified by the Cochrane tool for assessing the risk of bias in randomized controlled trials because any source of bias in a trial is problematic, and there is a paucity of empirical research to prioritize one domain over the other.

3. Results

The initial search resulted in 5126 patents found with the term "*Camellia sinensis*" in the intellectual property banks selected, followed by the stage title and abstract read targeting dental applications from the first patent deposited until the last one that had a total of 28 patent registrations. Then, the repeated records (8) were deleted. At the end of the search, data and information from 20 selected patents were extracted (Table 1).

According to the searches, the United States Patent and Trademark Office (USPTO) and World Intellectual Property Organization (WIPO) were the banks with the largest number of filing patents for products/formulations with *C. sinensis* for dental applications with 7 (35%) and 6 (30%) patent registrations, respectively. The German Patent and Trade Mark Office (DPMA), Japanese Patent Office (JPO), and Swiss Federal Institute of Intellectual Property (IGE-IPI) did not provide patents related to the search.

Figure 1 shows the annual evolution of patent filings according to the number of patent registrations. There is an increase in the number of deposits, with a highlight from 2005 to 2006 with a total of 6 patents, also in 2016 with 3 registrations, and no deposits were found between 2017 and 2019. It is noteworthy that as expected in the last 18 months, the number of patents submitted found in the search is less than reality since many deposits are still in the period of confidentiality.

Figure 2 shows the origin of the patent applicants with a wide variety of countries, highlighting the United States as the largest applicant with 8 (40%), followed by South Korea with 4 (20%) and Brazil with 2 (10%) patent filings. Other countries, such as the United Kingdom, Italy, Germany, India, China, and Japan had only one patent registration, representing 5% each.

There were different types of patent applicants according to the findings. We observed that half of the patents filed (10) were inventions developed and registered by companies and industries. Also, the Colgate–Palmolive Company (US) showed most patents with three filings and the others with only one registration, among them the Nippon Zettoc Company, Ltd. (JP), Indena SpA Company (IT), and Amorepacific Corporation (KR). As well as, the applicants were represented by universities and research institutes with seven filings, among them Kingston University (UK), São Paulo University (BR), and Georgia Health Sciences University Research Institute (US). The other patents (3) had their applicants represented by people (inventor) and researchers.

In Figure 3, there is the distribution of classification of patented products indicating that the patent filings are more focused on human need section (A), and the classification A61K presented the largest number of patents (19), a category which includes patents for the preparations for medical, dental, or hygienic purposes, followed by A61Q with cosmetic products or formulations for personal hygiene (10), A61P with specific therapeutic activity of chemical compounds or medicinal preparations (7), and A01N with preservation of organisms of humans or animals or plants or their parts (3). As we can observe, some reports have more than one classification.

Figure 4 shows that the types of patented dental products with the term "*Camelia sinensis*" were the largest in composition form with 14 filings, and the remainder of the formulations is represented in mouthwash (3), toothpaste (2), and bagged tea (1). As for clinical application, 18 patents were filed as products with antimicrobial and antibiofilm action, while two patents are directed to the treatment of xerostomia.

Figure 5 shows the level of evidence of clinical trials studies about the efficacy of these formulations, found in the PubMed database. For the risk of bias, factors are random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other bias. In general, the risk of bias of the studies was low, reinforcing that the formulations were effective.

4. Discussion

In the last decades, the scientific community and industries have shown a growing interest regarding the technological innovation for new products in dentistry, mainly based on the development of patents as intellectual property, which is a response to market demands [20]. Many of these inventions have been developed using natural products, such as *C. sinensis* extract, with different potential benefits for human oral health. Formulations containing *C. sinensis* in its composition look for advantages for treatment of oral

Table 1: Study.

	Title	Publication number	Product	Country	Year	Classification IPC	Inventor
1	Oral care composition containing extract of unoxidized *Camellia*	4852/DELNP/2007	Composition	US	2005	A61K	Colgate–Palmolive®
2	Oral compositions containing oxidized *Camellia*	4860/DELNP/2007	Composition	US	2005	A61K/A61Q	Colgate–Palmolive®
3	Oral composition comprising *Camellia* extract of semioxidized tissue from a member of the genus *Camellia* and an enhancing agent	2387/KOLNP/2009	Composition	UK	2006	A01N	Kingston University
4	Composição de higiene oral, método de tratamento ou prevenção de xerostomia e uso de polifenol de chá verde (gtp) (in Portuguese)	BRPI1008380	Composition	US	2008	A01N/A61K	Georgia Health Sciences University Research Institute, Inc.
5	Composição oral, e, métodos para a promoção da saúde oral de um paciente animal, e para a redução da extensão de descoloração em uma pasta de dentes (in Portuguese)	BRPI0519427 (A2)	Toothpaste	US	2004	A61K/A61Q	Colgate–Palmolive®
6	Formulação tópica de uso bucal e seu uso (in Portuguese)	BR102016237505	Composition	BR	2016	A61K/A61P	University of São Paulo
7	Composições odontológicas contendo inibidores de metaloproteinases e seus usos (in Portuguese)	PI 1003771-3	Composition	BR	2010	A61K/A61Q	University of São Paulo
8	Oral care composition	WO2017/199453	Toothpaste	IN	2012	A61K/A61Q	Nippon Zettoc Co., Ltd.
9	Oral care compositions for treating xerostomia	PCT/US2010/024906	Composition	US	2009	A01N/A61K	University of São Paulo
10	Compositions for the treatment and prevention of infections of the oral cavity	PCT/EP2009/002515	Composition	IT	2009	A61K/A61P	Indena SpA Company
11	Noncarious material and anticarious agent containing rare sugar	US 20100166678 A1	Composition	JP	2006	A61K/A61P	Matsutani Chemical Industry Co., Ltd./National University Corporation Kagawa University
12	Antibacterial oral rinse formulation for preventing coronary artery disease	US 20070154414 A1	Mouthwash	US	2005	A61K	Richard Paul Bonfiglio
13	Epigallocatechin-3-gallate crystal compositions	WO2008/153938	Composition	US	2007	C09K/A61P/C07D/A61K	University of South Florida
14	Oral care compositions containing combinations of antibacterial and host-response modulating agents	US 20070053849 A1	Composition	US	2006	A61K/A61P/A61Q	The Procter & Gamble Company
15	Use of a phenol-containing extract from *Camellia sinensis*in oral and dental cleaning agents for improving the visual appearance of the gums	WO/2016/062449	Composition	DE	2016	A61K/A61Q	Henkel Ag & Co., KGaA
16	Oral composition containing saponin extracted from the root of *Camellia sinensis* for effectively preventing or treating periodontal diseases	KR1020130035323	Composition	KR	2013	A61K/A61Q/Y10S	Jeong Kee Kim; Su Kyung Kim; Dae Bang Seo; Seok Sik Moon
17	Composition for enhancing oral hygiene comprising natural extract as active ingredient and use thereof	KR1020160050108	Composition	KR	2016	A61K/A61P/A61Q	Dongguk University Gyeongju Campus Industry-Academy Cooperation Foundation
18	Effervescent mouthwash	US20130149359	Mouthwash	KR	2011	A61K/A61Q	Eric M. Sanders
19	Health care buccal bag for refreshing mouth smell	CN101015516	Bagged tea	CN	2007	A61K/A61Q/A61P/A23F	Zhu Huagang
20	Foamable mouthwash solid formulation and preparation method therefor	WO/2020/054996	Mouthwash	KR	2020	A61K	Eun Sang Lee

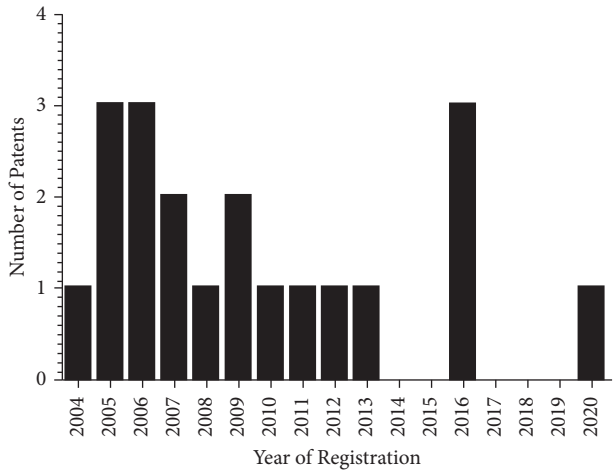

Figure 1: Annual evolution number of patent filings per year with the term *Camellia sinensis* in formulations for oral care applications in the intellectual property banks accessed, 2021.

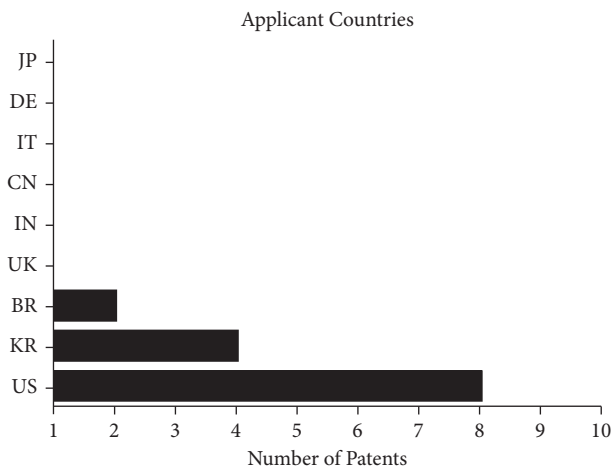

Figure 2: Applicant of patents by country with term "*Camellia sinensis*" in formulations for oral care applications in the intellectual property banks accessed, 2021.

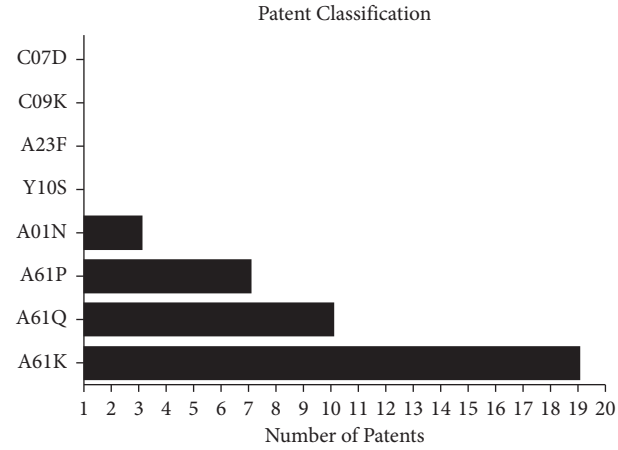

Figure 3: Distribution of patents deposited with term "*Camellia sinensis*" for dental applications by International Patent Classification Codes (IPC). A61K: prescriptions for medical, dental, or hygienic purposes; A61Q: cosmetic products or formulations for personal hygiene; A61P: specific therapeutic activity of chemical compounds or medicinal presets; A01N: preservation of organisms of humans or animals or plants or their parts; A23F: coffee, tea, their substitutes, manufacture, preparation, or infusion thereof; C09K: materials for applications not otherwise provided for; applications of materials not otherwise provided for; C07D: heterocyclic compounds; Y10S: technical subjects covered by former USPC cross-reference art collections (XRACs) and digests.

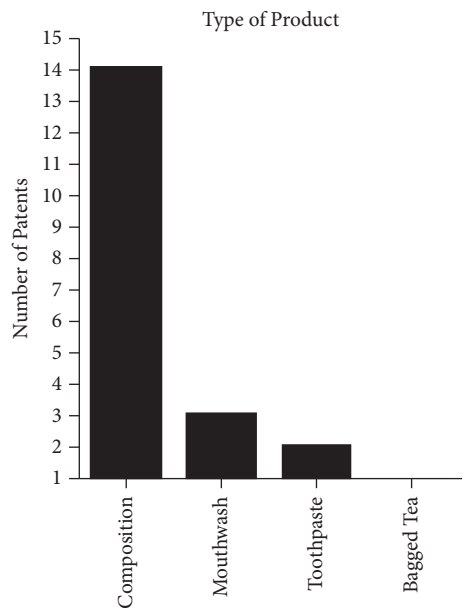

Figure 4: Types of dental formulations deposited with the term "*Camellia sinensis*." Applicant of patents by country with term "*Camellia sinensis*" in formulations for dental applications in the intellectual property banks accessed, 2021.

biofilm, helps promote the enamel and dentin remineralization by the fluoride component, and bad breath treatment [3, 6, 7, 12, 13, 15, 18].

Two reviews highlight and discuss patents that used *C. sinensis* and its derivatives for different applications according to the available literature. The first review highlighted that several patents were developed to improve tea processing methods for different purposes, including promoting changes in the composition of tea products, improving their sensory properties and stability, and increasing production yield [15]. The second reported invention patents using trihydroxybenzoate derivatives present in the tea composition of *C. sinensis* with antiviral, antifungal, and antibacterial properties related to different diseases, not only to oral health [14]. However, our study highlights recent patents that employed *C. sinensis* in formulations focused exclusively on dental applications, being the first review of patents for oral care on this topic.

The present study observed that the majority of patent deposits with *C. sinensis* belonged to the United States, both as an applicant country and as an intellectual property bank. Therefore, following the ranking of the WIPO statistical base, that when considering the technology areas, the United States is in first place in the medical field with patent

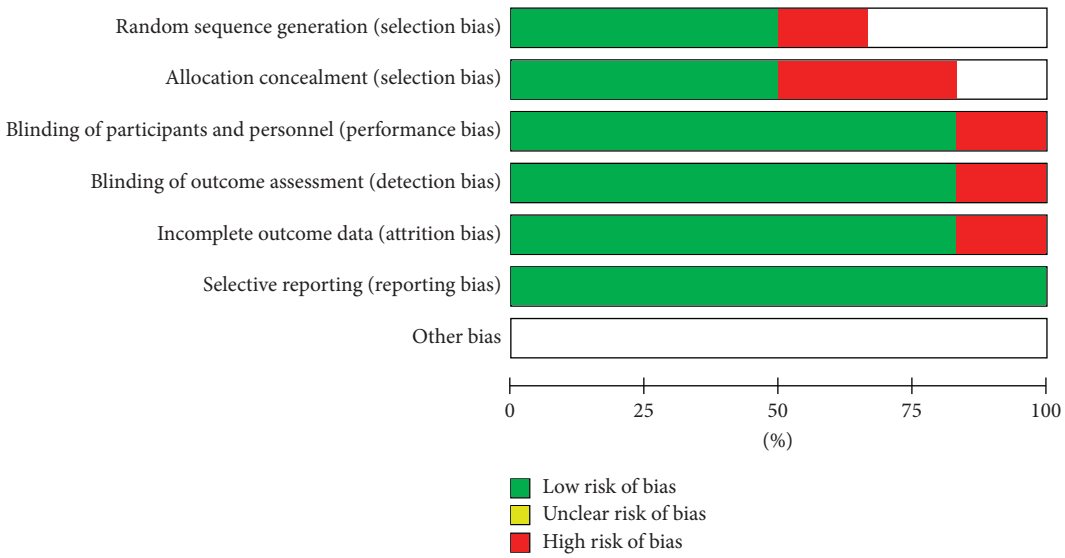

Figure 5: Review authors' judgements about each risk of bias item presented as percentages across all included clinical studies.

deposits, including value-added dental equipment in the economic sector [24]. Among the types of applicants, the Colgate–Palmolive company was already expected to hold of a greater number of deposits, although the amount is not yet significant. Today, it represents a pioneering brand in the production of toothpaste, founded in New York (US) with more than 200 years of existence in the global market and later started to add the soap brand Palmolive.

The first patent registration (PI0519427-0) [25] entitled "Oral composition and methods for promoting the oral health of an animal patient, and for reducing the extension of discoloration in a toothpaste" was deposited in 2004 at the intellectual property bank Espacenet by the Colgate–Palmolive Company. The invention describes low water content toothpaste containing a variety of plant extracts, including *C. sinensis*, humectants, and abrasive compounds, together with an additional antioxidant component. Also, it proves methods for promoting the oral health of an animal patient.

In 2010, Buzalaf et al. [26] developed a BR patent (PI 1003771–3 A2) filed by the University of São Paulo for dental compositions (gel and/or varnish) containing metalloproteinase inhibitors, such as epigallocatechin-3-galate (EGCC) obtained from *C. sinensis* to be applied in the demineralization of the dental surface, caused by tooth decay or noncarious lesions, as well as preventing the progression of periodontal disease. More recently, in 2020, Juhwan Bio.Cell Company patented a foamable mouthwash solid formulation (WO/2020/054996) [27] which comprises a mixture of green tea and other extracts as an active ingredient. The inventors concluded that the product exhibits an excellent effect on dentin remineralization and dentinal tubule occlusion. Also, the invention has anti-inflammatory, antibacterial, dental caries-preventing, halitosis-eliminating, and scaling effects.

The consumption of *C. sinensis* is a promising agent in maintaining oral health, especially in relation to periodontal disease and caries [1, 7]. Its benefits concerning the prevention of dental caries are due to the leaves being accumulators of fluorine, with antimicrobial action, and the catechins (polyphenols) present have a protective effect on the dental tissue [1, 9, 12, 28]. In addition, the use of *C. sinensis* products for halitosis treatment suggests that this clinical condition can also benefit from these formulations. This fact is due to catechins capable of chemically reacting with sulfur compounds that promote bad breath, through a methylation reaction with orthoquinone, decreasing volatility, and neutralizing the chemical compound, and the antimicrobial activity decreasing the fermentation of sulfur compounds [7, 29]. There is also evidence that these polyphenolic catechins are active in preventing oral cancer and reducing bleeding after tooth extraction [7, 28, 30].

Mouthwash formulations stood out in the present study as a strategy for oral care because of their antimicrobial and anti-inflammatory properties, both in relation to patents and clinical trials found. Radafshar et al. observed the effects of a mouthwash containing 1% green tea tannins on dental biofilm and chronic gingivitis, comparing chlorhexidine and finding similar results [31]. Another clinical trial verified the effect of *C. sinensis* 5% mouthwash on gingivitis induced by biofilm accumulation for five weeks, observing positive effects and no adverse effects [32]. Sarin et al. also evaluated the effectiveness of a mouthwash containing 2% green tea compared to a placebo mouthwash for controlling plaque and gingivitis for four weeks, noting a significant reduction in plaque and gingival index in the group treated with *C. sinensis* mouthwash compared to placebo [33]. Another clinical trial evaluated the effectiveness of a mouthwash with 5% green tea to control pain and trismus associated with acute pericoronitis and compared it to the mouthwash with chlorhexidine, obtaining better results than the same [34]. According to Ardakani et al., *C. sinensis* becomes an alternative to a therapeutic agent carried in mouthwash, given that it has several other therapeutic properties differently from chlorhexidine [35].

In a recent systematic review published, it was found that there is clinical evidence and a favorable safety profile for the use of *C. sinensis* in the form of mouthwash; these formulations are being able to act as antiseptic, antibiofilm, and anti-inflammatory agents. Thus, *C. sinensis* has a favorable phytochemical and pharmacological profile, making it a promising incorporation agent in mouthwashes [3]. These findings justify the highlight of the rinsing formulations in this study.

In view of all these therapeutic benefits of the constituents of *C. sinensis*, despite the small number of patents, it appears that this natural product has great potential for incorporation in dental products for oral care, which can be an attractive cost-effective alternative for consumers.

5. Conclusions

The study pointed out a small number of products protected by patents for *Camellia sinensis* for oral care indication, highlighting compositions to be incorporated in formulations, mainly mouthwash. In the methodological parameters of clinical trials carried out with the formulations, the majority pointed out a low risk of bias.

Data Availability

This study used data available in the banks on intellectual property cited on the methods.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

The Current Strategies in Controlling Oral Diseases by Herbal and Chemical Materials

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Dental plaque is a biofilm composed of complex microbial communities. It is the main cause of major dental diseases such as caries and periodontal diseases. In a healthy state, there is a delicate balance between the dental biofilm and host tissues. Nevertheless, due to the oral cavity changes, this biofilm can become pathogenic. The pathogenic biofilm shifts the balance from demineralization-remineralization to demineralization and results in dental caries. Dentists should consider caries as a result of biological processes of dental plaque and seek treatments for the etiologic factors, not merely look for the treatment of the outcome caused by biofilm, i.e., dental caries. Caries prevention strategies can be classified into three groups based on the role and responsibility of the individuals doing them: (1) community-based strategy, (2) dental professionals-based strategy, and (3) individual-based strategy. The community-based methods include fluoridation of water, salt, and milk. The dental professionals-based methods include professional tooth cleaning and use of varnish, fluoride gel and foam, fissure sealant, and antimicrobial agents. The individual-based (self-care) methods include the use of fluoride toothpaste, fluoride supplements, fluoride mouthwashes, fluoride gels, chlorhexidine gels and mouthwashes, slow-release fluoride devices, oral hygiene, diet control, and noncariogenic sweeteners such as xylitol. This study aimed to study the research in the recent five years (2015–2020) to identify the characteristics of dental biofilm and its role in dental caries and explore the employed approaches to prevent the related infections.

1. Introduction

Oral cavity provides an environment that leads to the colonization and growth of an extensive range of microorganisms. Bacteria are the most prevalent of them. The highest bacterial accumulation is found as a biofilm on the tooth surface (dental plaque). The loss of mucosal surface reduces the microbial load on the mucosal surfaces [1, 2]. These microbes normally exist on all oral surfaces and are necessary for the normal physiological growth of the oral cavity [3]. Static microflora contributes to the host health by preventing the establishment and colonization of exogenous microorganisms and potential pathogens and regulating the inflammatory response of the host to oral commensal bacteria [4]. The oral bacterial population with almost 1000 species is very complicated [5]. The clinical treatment of caries usually deals with the restoration of hard tissues considering the functional and aesthetic needs. However,

this does not treat the illness but treats its outcomes. In clinical settings, a common method for the management of dental caries is the elimination of contaminated tissues to prevent the further progress of the disease. The areas surrounding the restoration gradually undergo caries, so a small restoration must be substituted by a greater restoration and dental tissues are increasingly eliminated. Repetition of this cycle leads to extensive loss of dental tissue, pulpal involvement, and finally tooth loss [6]. Admittedly, an inadequate understanding of dental caries as a biological process leads dentist to such a problem. Considering the developments in genetics, particularly molecular biology, findings make it possible for us to replace the old paradigms with new ways of caries prevention and treatment. Dental decay is caused by an ecological imbalance in mouth microflora. These imbalances can be influenced by environmental and biological reasons. Dental caries can be controlled by managing the above mentioned changes [7, 8]. In this review, we aimed to study the recent research to identify the characteristics of dental biofilm and its role in dental caries and also explore the employed methods to prevent the related infections.

2. Caries and Its Stages

Dental caries is a chronic and preventable disease caused by dental biofilm activity. This disease is multifactorial and is initially caused by an imbalance of mouth microflora as a result of existence of carbohydrates on the tooth. Caries is characterized by local demineralization of the teeth and loss of dental structure. Some biofilm bacteria metabolize the existing carbohydrates and produce acids. These acids can reduce the pH and reach lower than the critical limit (5.5 for enamel and 6.2 for dentine) if they remain in the biofilm for a long time. This acidic environment affects the biofilm composition and tooth surface. Therefore, acidogenic and acidophilic bacteria increase, which results in a more acidic environment. Calcium and phosphate are removed from the tooth surface, as a result of which demineralization occurs due to the loss of minerals. If pH returns to its normal level, calcium and phosphate can return to the tooth structure and cause remineralization [9]. Dental caries is created on the surface and subsurface areas of the tooth following a dynamic trend of demineralization and remineralization. These events occur several times over time and are influenced by several factors like the type and number of biofilm microorganisms, diet, oral hygiene, genetics, tooth anatomy, fluoride consumption, and salivary content and composition. These factors are specific to each individual and vary from tooth to tooth and from place to place [9]. As demineralization continues, primary caries occurs, a lesion with no cavities called white spot lesion (WSL) on enamel. Most primary caries can be terminated or remineralized; i.e., they are reversible. Dental caries becomes cavitated as it progresses. Cavitated lesions often need restorative intervention and are irreversible. Moderate lesions are lesions that have not extended the internal one-third of the dentin, and advanced or deep lesions are those that have extended the internal one-third of the dentin [9].

3. Dental Plaque

Dental plaque is a soft and sticky layer accumulated on the tooth surface. Dental plaque, also called biofilm, mostly consists of bacteria and their products, extracellular matrix, and water. Biofilm is not sticky food debris or accidental accumulation of opportunistic microorganisms; it is the accumulation of a series of organized events. The formation of dental plaque involves the formation of the pellicle, initial attachment, and plaque maturity [10]. Plaque forms as follows: a clean tooth surface is immediately exposed to the salivary byproducts, gingival crevice fluid (GCF), and some compounds resulting from bacteria. These products are absorbed by the surface with a negatively charged hydroxyapatite, creating a layer called acquired pellicle. The dental pellicle is covered with positively charged molecules, which contain more than 180 proteins, peptides, and glycoproteins such as proline-rich proteins, histidine-rich proteins, phosphoproteins (e.g., statherin), creatine, mucin, and other molecules that act as the binding sites for bacteria [10]. Some bacterial products like glycosyltransferases and glucans are also found in the pellicles. Interestingly, the main compound of a pellicle is stable in different areas of the oral cavity and among individuals. The bacteria attached to the tooth surface are not directly connected to the enamel; they are bound to the enamel through pellicle [10]. Primary bonding is initiated a few minutes after brushing the colonization. A cell wall with negatively charged bacteria facilitates its connection to the positively charged receptors of the pellicle. The early stages of transfer and interference with the surface are similar and nonspecific for all bacteria. Specific involvement of the binding molecules of the bacterial cell wall and pellicle receptors determines whether the bacteria remain in contact with the surface or not. Only a small part of oral bacteria can bind to the pellicle receptors, which constitutes the most common biofilm bacteria over a short time after tooth cleaning. During the early hours, streptococci form over 60–80% of biofilm bacteria. These bacteria along with *Haemophilus*, *Neisseria*, *Actinomyces*, and *Veillonella* species are known as primary colonizers. They initially create a wide range of nonspecific and reversible Van der Waals connections with pellicle (more than 50 nm). Then, they make stronger and irreversible short-range connections (10–20 nm) with pellicle receptors by their specific surface molecules [10].

Streptococci create different adhesion mechanisms. They have different adhesion mechanisms such as products of glycosyltransferases, glucan-binding proteins, and pili, while other bacteria such as actinomycetes bind to the surface using their fimbriae [11]. These bacteria provide new adhesion sites for other bacteria and change the environment with their metabolic activity, thereby affecting the biofilm viability (e.g., reducing the ambient oxygen) [11]. Plaque matures as follows: the primary colonizing bacteria bound to the tooth surface provide new receptors for the bacteria to help induce coadhesion via binding. The bacteria of different species or even strains of a species can specifically bind to specific bacteria. *Fusobacterium* coaggregates with all oral bacteria, while *Veillonella*, *Capnocytophaga*, and *Prevotella*

coaggregate with streptococci or actinomycetes. Most of this adhesion between various bacteria is performed through lectin-like receptors (proteins that identify the carbohydrates). Thus, they can be inhibited by lactose or other galactosidase or amino acids like L-arginine [10, 12]. Secondary colonizers like *P.Int*, *FN*, *Prevotella loescheii*, *Capnocytophaga*, and *PG* cannot bind to the clean tooth surface but can bind to bacteria in the dental plaque. Some specific structures in dental biofilm such as corn cob and test-tube brush are created due to the adhesion of cocci to filamentous bacteria. In this stage of plaque maturity, the bacteria secret extracellular polysaccharide, which constitutes the biofilm scaffold [10, 13]. If the dental plaque remains for about 7 days, it provides a favorable environment for the colonization of some aerobic Gram-negative bacteria, which are called secondary colonizers, including *PG*, *Aggregatibacter actinomycetemcomitans*, and *Treponema denticola* [10, 13].

4. Dental Biofilm

A healthy dental biofilm is mostly composed of commensal nonpathogenic microbes. These microbes are not completely independent and are regularly linked with each other and host tissues like gingiva even in a healthy state. The host provides the surfaces for colonization, and beneficial bacteria prevent the colonization of pathogenic bacteria [4, 14]. The advantages of this connection are manifested in conditions such as antibiotic sore mouth when inhibition of normal flora leads to the growth of opportunistic pathogens [4]. Studies have shown that commensal bacterial species such as *S. salivarius*, *S. sanguis*, and *Atopobium parvulum* can induce biofilm health-related conditions. Nevertheless, further research is needed to explore this issue. It has been shown that *S. salivarius* inhibits the quorum sensing process and mutans biofilm formation, which results in its anticaries properties [15–17]. The commensal bacteria in biofilm are involved in the immune system development. They do this by presenting diverse antigens to the host immune system. The commensal bacteria create a cascade of signals that induce the host resistance, while pathogenic bacteria induce severe inflammation in the host. Hence, proinflammatory cytokines are produced in a low number in oral epithelial cells, which induces the expression of E-selection in vascular endothelial tissues and produce IL8 [10]. The commensal bacteria induce the innate immune response of the host, which strategically juxtaposes the neutrophils with subgingival bacteria and junctional epithelium. The main bacteria with the highest variation in the oral cavity include *S*, *Staphylococcus*, *Peptostreptococcus*, *P*, *Haemophilus*, *Veillonella*, *Leptotrichia*, *Treponema*, *Propionibacterium*, *Actinomyces*, *Fusobacterium*, *Corynebacterium*, *Eikenella*, *Gemella*, *Granulicatella*, *Rothia*, *Porphyromonas*, *Capnocytophaga*, *L*, *Neisseria*, and *Eubacteria* [18–20]. Oral health depends on the conservation of its normal microflora. A disease occurs once the species are imbalanced and pathogens become dominant. Oral health and disease are dynamic processes in which the ecology of communities is a determinant factor, not an organism. Understanding the meaning of and identifying the molecular changes among the disease and health conditions provide the clinicians with the ability to diagnose and reverse the disease in early stages [21].

5. Bacterial Groups in the Caries Process

The newest molecular biology methods of microorganisms related to caries are *B. dentium*, *B. adolescentis*, *SM*, Scardovia wiggsiae, *B. longum*, *Selenomonas* spp., *P.* spp., and *L.* spp. [16]. Studies have indicated that early streptococci on the newly cleaned tooth surface mostly include *S. sanguinis*, *S. oralis*, and *S. mitis* strains, and the content of their mutans is only 2% or less. It has also been shown that most primary colonizers belong to the *S. mitis* group. The amount of microflora actinomycetes increases over time so that the smooth surfaces of most bacteria in the mature plaque include actinomycetes and nonmutans streptococci. Mutans are found in small numbers [22] and are more abundant in white spot lesions (WSLs) than in healthy regions. However, nonmutans streptococci still constitute most WSL bacteria. It has been shown that the initial members of microflora can singly dissolve enamel in the absence of mutans and lactobacilli [22]. In cavitated dentinal lesions, including rampant caries, mutans constitute about 30% of microflora, which indicates that mutans are linked with progressive carious lesions. Nevertheless, some studies have shown that the dentinal caries of mutans is less frequent in deep lesions, and lactobacilli, *Prevotella*, and bifidobacteria are more prominent. These results indicate that microflora on the tooth surfaces change as caries progresses [22]. *S. mutans* is Gram-positive cocci. The oral *S* spp. are normal flora but are also opportunistic and can cause dental caries [23]. They include *SM*, *S. sobrinus*, *S. rattus*, *S. cricetus*, *S. ferus*, *S. downei*, and *S. macacae*. Virulence has numerous factors that induce its demineralization [23] (Table 1).

6. Extracellular Polymeric Substance [24]

The extracellular polymeric substance [24] of the biofilm plays a pivotal role in maintaining the bacterial integrity and adhesion. New studies on the biology of EPS have reported several roles for the scaffold resulting from EPS, which are vital for the biofilm [25, 26]. Some of these roles include surface adhesion, spatial and chemical heterogeneity in biofilm, competitive or collaborative interactions, and improved resistance to antimicrobials [25]. The construction of EPS matrix relates to the existing substrates, production and secretion of e-materials, and shear forces. The key part of EPS content in oral biofilms which is related to caries is polysaccharides, especially the glucans derived from *SM* [27]. In addition to these, polysaccharides result from other bacteria (e.g., actinomycetes, *Streptococcus salivarius*, and *Streptococcus gordonii*), and combination compounds of starch glucan exist in this matrix. Further, this matrix contains eDNA [28] (proteins derived from bacteria with properties similar to amyloid), host GP which are able to participate in the scaffold with G such as glucan-eDNA compounds [29]. The function and structure of this class of extracellular polymers are still not known completely and

Table 1: Virulence factors of *SM*.

Property	Description
Making acid	Capability to make lactic acid
Aciduricity	Capability to tolerate low pH
IPS	Capability to consume IPS to continue making lactic acids with the lack of fermentable carbohydrates
EPS	Making matrix of the biofilm

require further studies. Glucans are composed of glucose components, which are connected byα 1–4 and α 1–6 glycosidic bonds and are created by the coordinated activity of streptococcal exoenzymes called glucosyltransferase [3, 27, 30]. Interestingly, these extracellular enzymes can bind to the tooth structure in the active state and produce glucans locally, thereby providing new binding sites for the bacteria. In addition to these, glucosyltransferase enzymes bind to other oral microorganisms (e.g., commensal *S*, actinomycetes, *L*, and even *Candida albicans*) and make them G producers [3, 27, 30].

The G produced on the tooth surface increase the surface bacterial aggregation. They also cause new interspecies interaction and increase cell-cell adhesion [3]. These extracellular polymers aggregate on different areas of tooth surface on the cell membrane, each having a complementary role in the EPS matrix formation and biofilm development. Some of these roles are surface adhesion, cell-cell adhesion, and cell cluster development found in the biofilm systems [31]. The biofilm matrix is developed three-dimensionally with biofilm maturation and production of extracellular polymers, covering cell clusters, building bridges between them, and creating a firmly divided structure. This heterogeneous structure resulting from EPS explains the presence of microbial clusters in various sizes and composition in the human oral biofilm [32–34]. The EPS sediment on the surface and its development affect the mechanical properties of biofilm like the binding strength of biofilm to the surface and its integrity [35]. A well-formed biofilm is hardly removed from the tooth surface and has reinforced viscoelastic properties that help it to remain on the surface during the shear stresses exerted by liquids [35]. The structure of the EPS matrix can be locally altered by dextranases, DNAs, and protolithic enzymes to create new binding sites for the following bacteria that have not been able to join the biofilm. The stiffness of the biofilm matrix is increased over time. The physicochemical properties of biofilm protect the buried bacteria by inhibiting the access of drugs and increasing their AM resistance. For instance, EPS can bind to cationic AM like CHX and AM peptides and prevent their permeation into the depth of biofilm, thus preventing their toxic effect [3, 34].

7. Fighting Dental Caries

The caries-preventive strategies can be classified into three groups depending on the role and responsibility of the individuals doing them (Figure 1): (1) community-based strategies, (2) dental professionals-based strategies, and (3) individual-based strategies. The dental professionals-based strategies include professional tooth cleaning and use of varnish, fluoride gel and foam, fissure sealant, and antimicrobial agents. The individual-based (self-care) strategies consist of using fluoride toothpaste, fluoride supplements, fluoride mouthwashes, fluoride gels for personal use, chlorhexidine gels and mouthwashes for personal use, slow-release fluoride devices, oral health, diet control, and noncariogenic sweeteners like xylitol [36]. Recent studies (2015–2020) are mentioned in Table 2.

8. Communal Activity-Based Methods

8.1. Fluoridation of Water, Salt, and Milk. Fluoridation of drinking water has been used as an affordable practical way to reduce socioeconomic inequalities related to dental caries. Fluoridation of drinking water decreases dental caries by 30–50%, and termination of water fluoridation in conditions with inadequate alternative sources of fluoride increases caries by 18% [149]. Fluoridation of salt includes many advantages of water fluoridation, and consumers can choose to use it. The fluoride-containing salt will have different effects depending on the type of consumer. In high-consumption families, it can cause fluorosis [150]. Fluoridation of milk is beneficial for school children, especially for their permanent teeth. An advantage of this method is that it can be used in the high-risk group. Its consumption rate is also controllable. Moreover, individuals have the right to choose it or not. Fluoride in milk is absorbed more slowly than the fluoride in water due to the presence of calcium in milk [150, 151].

9. Dental Professionals-Based Methods

9.1. Professional Tooth Cleaning. Karlstad program can be used for biofilm control. In this program, the application of topical fluoride and diet, daily oral hygiene, and tooth cleaning with certain intervals are presented by a trained specialist. Further, this is done every two weeks in this program because studies have shown that the biofilm remaining for two to three weeks leads to the formation of WSL and caries. These intervals can be increased to three months in people with good oral health [152].

9.2. Use of Varnish, Gel, and Fluoride. The individuals aged <18 years are advised to use fluoride varnish every 3–6 months. Tooth caries due to the application of fluoride varnish has been reported to reduce 46% in permanent dentition and 33% in primary dentition [153, 154]. APF and NaF gels are used professionally. Studies have reported 26% reduction in caries in permanent dentition and 20% in

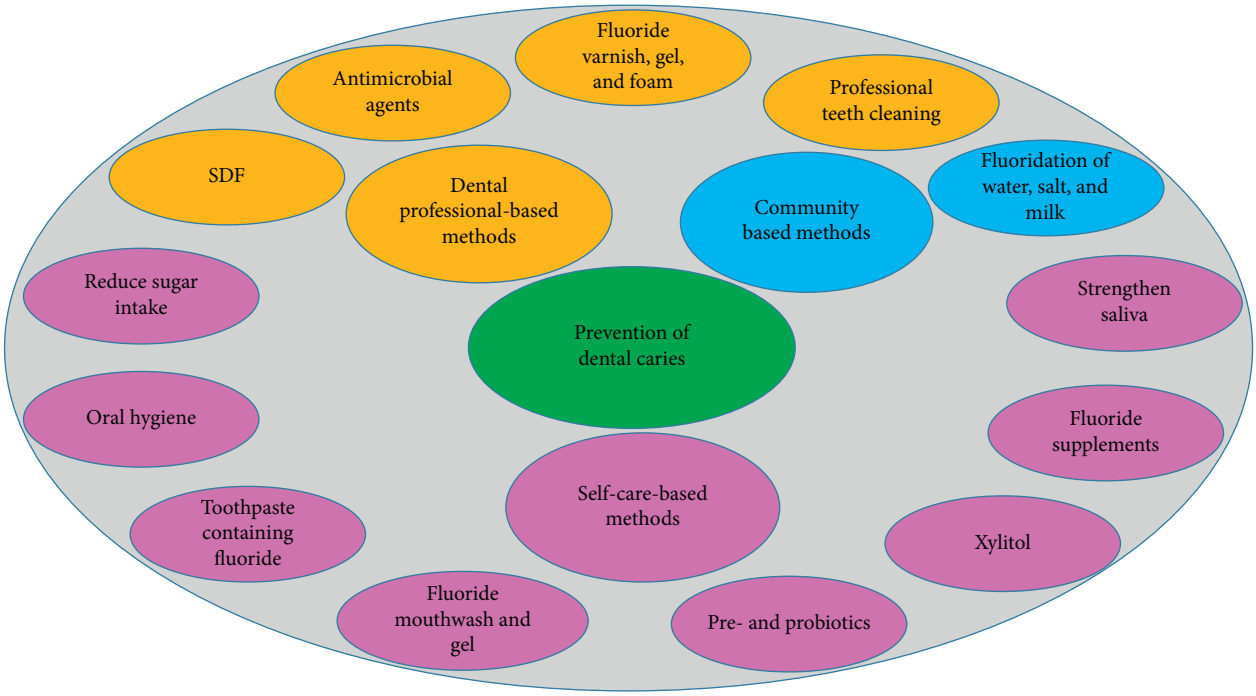

Figure 1: Prevention strategies of dental caries.

Table 2: Recent strategies in dental caries prevention.

Treatment	Type of study	Methods	Outcomes	Ref/year
Stannous fluoride products	In vivo	The groups were as follows: the test group using stannous fluoride products and the control group.	The analysis showed no effect of stannous fluoride products.	[37]/2018
Sodium fluoride dental protective agent combined with pit and fissure sealant	In vivo	The groups were as follows: in the control group pit and fissure sealant was used, and in the test group sodium fluoride tooth protector joined with pit and fissure sealant was used.	The combined group had better results.	[38]/2019
GI and RBS	In vivo	The groups were as follows: GI and RBS.	Groups were the same in the survival of GI and RBS.	[39]/2017
Ozone, sealant, and fluoride varnish	In vivo	The groups were as follows: (1) control; (2) fluoride varnish; (3) sealant; (4) ozone.	The use of fissure sealant, fluoride varnish, and ozone is suggested for prevention of occlusal pit and fissure caries.	[40]/2016
Silver diamine fluoride (SDF)	In vivo	SDF (38%) or Pl was applied topically. The primary outcome was caries arrest (Nyvad criteria).	Topical 38% SDF was effective in arresting cavities.	[41]/2018
RBS and GI	In vivo	The groups were as follows: RBS, GI sealant, and control.	The RBS was higher than the GI sealant in prevention of caries.	[42]/2018
F coating joined with PRF or PTS	In vivo	The groups were as follows: group A, PTS; B, PRF; C, 0.5% F coating + pit and fissure sealing; D, 0.5% fluoride + preventive resin filling; E, control.	Groups C and A had a certain effect on prevention of dental caries, but group D was better.	[43]/2018
Lactobacillus paracasei	In vivo	The groups were as follows: probiotic milk or control (standard milk).	Probiotic milk reduced salivary *S. mutans* count.	[44]/2018
MIV and MIPP	In vivo	The groups were as follows: FTP, using MIV + MIPP application, and control.	Groups were the same in ICDAS scores and EDI sum.	[45]/2018

Table 2: Continued.

Treatment	Type of study	Methods	Outcomes	Ref/year
CXT and FJ	In vivo	The groups were as follows: CXT or FJ.	GIC sealants were effective in preventing caries.	[46]/2018
PTS and FV	In vivo	The groups were as follows: resin-based fissure sealant or FV was applied.	Groups were the same in caries prevention.	[47]/2017
Xylitol-containing chewing gum	In vivo	The groups were as follows: in the test group xylitol gum + oral health education were used, and in control group oral health education alone was used.	Both groups had a reduction in the caries rate.	[48]/2017
MIn	In vivo	The groups were as follows: CE or MIn.	MIn group was better in prevention of caries.	[49]/2019
PTS combined with fluorine protective paint	In vivo	The groups were as follows: control, PTS, and PTS + fluorine protective paint.	Pit and fissure sealant + fluoride protective paint can decrease the incidence of dental caries.	[50]/2019
MIn	In vivo	The groups were as follows: HE, MIn, and MI + RA.	MIn group had higher preventive effects against caries than HE group.	[51]/2017
TiF_4 varnish	In vivo/in situ	The groups were as follows: TiF_4 varnish, Duraphat, Pl varnish, and no treatment.	F-varnishes showed caries-preventive effect.	[52]/2019
Silver NP + PTS	In vivo/in situ	The groups were as follows: conventional and silver NP combined with PTS.	Silver NP mixed sealant was more effective than conventional sealant in reducing tooth demineralization.	[53]/2017
Fluoride varnish	In vivo	The groups were as follows: fluoride varnish or Pl.	Quarterly applications of fluoride varnish were not effective in preventing development of dental caries.	[54]/2016
Different fluoride regimens	In vivo	The groups were as follows: G1: control group, fluoride (F) TP (1450 ppm); G2: FTP (1450 ppm) + 0.2% F oral rinse; G3: TP (5000 ppm).	The recommendation was application of FTP (5000 ppm F) or oral rinse (0.2% NaF) + usual TPs.	[55]/2019
Moisture tolerant RBS and GIS	In vivo	The groups were as follows: moisture tolerant RBS or GIS was placed on one side of the mouth, and the other one was placed on the opposing side. DIAGNOdent readings were taken.	Both materials were effective in arresting enamel caries.	[56]/2019
Fluoride varnish Duraphat	In vivo	The groups were as follows: fluoride varnish and control.	Fluoride varnish Duraphat was effective in decreasing caries incidence.	[57]/2019
Low-dose xylitol chewing gum	In vivo	The groups were as follows: xylitol and polyols.	Xylitol group showed a significantly lower increment of dental caries.	[58]/2017
RBS with and without F	In vivo	The groups were as follows: sealants with or without fluoride and control.	The effects of the sealants were similar.	[59]/2018
Xylitol and polyol chewing gum	In vivo	The groups were as follows: xylitol chewing gum, polyol chewing gum, and control group.	Xylitol-containing chewing gum was effective in decreasing caries incidence.	[60]/2018
Oral health education (OHE) and FV	In vivo	The groups were as follows: control, OHE, and OHE + FV.	OHE or OHE + FV reduced the caries incidence.	[61]/2016
FM and D	In vivo	The groups were as follows: participants brushed their teeth with either a D (1150 ppm) or a Pl D without F and either daily application of FM (220 ppm) or not.	FM was effective in remineralization.	[62]/2018
Biannual treatment with FV	In vivo	The groups were as follows: standard yearly intervention with or without FV.	Biannual treatment with FV was not effective in preventing dental caries.	[63]/2017
STB and S	In vivo	The groups were as follows: STB, CR sealant, and ART-GIC sealant.	The groups were the same in preventing caries.	[64]/2015

Table 2: Continued.

Treatment	Type of study	Methods	Outcomes	Ref/year
SDF 12% and SDF 38%	In vivo	The groups were as follows: 12% SDF applied yearly, 12% SDF applied twice a year, 38% SDF applied yearly, and 38% SDF applied twice a year.	Higher concentration or frequency of SDF had more effect in arresting active tooth caries.	[65]/2018
FV and peptide P_{11}-4	In vivo	The groups were as follows: P_{11}-4 + FV or FV.	P_{11}-4+FV was effective in early carious lesions.	[66]/2018
CHX/thymol V or FV	In vivo	The groups were as follows: three-time monthly use of CHX/thymol varnish or semiannual use of FV + semiannual use of Pl V.	The groups were the same in dental caries development.	[67]/2015
FM, EO, and CHX oral rinses	In vivo	The groups were as follows: FM; EO; CHX; control (saline).	FM and CHX had more effect than EO mouth rinse.	[68]/2015
F, CPP-ACP, IR	In vivo	The groups were as follows: A, control (blank); B, control (Irr); C, Irr + F; D, Irr + CPP-ACP, E, Irr + CPP-ACP + F; F, Irr + IR; G, Irr + IR + F; H, Irr + IR + CPP-ACP.	IR + CPP-ACP, IR + F, CPP-ACP + F, and IR were the best effective methods to prevent Irr-dentin-destructions.	[69]/2019
TP containing Arg	In vivo/in vitro	Individuals wearing a dental device: the studies stages were lead-in, Arg-free, washout, and Arg-active stages.	Arg-containing TP can significantly decrease the LA construction.	[70]/2017
Varnish containing chlorhexidine	In vivo	The groups were as follows: Cervitec Plus® or Pl varnishes.	Application of Cervitec Plus® had a significant advancement in patients' oral health.	[71]/2018
Herbal extracts (Tulsi and Black myrobalan) and sodium fluoride	In vivo	The groups were as follows: (1) FM, (2) Tulsi mouth rinse, and (3) Black myrobalan mouth rinse.	Herbal mouth rinses could be tried as an anticaries agent for dental caries.	[72]/2018
FV	In vivo	FV applied every three months.	The use of fluoride varnish every three months prevented the incidence of caries.	[73]/2019
Infiltrant application	In vivo	The groups were as follows: icon infiltrant (DMG) and PFS (Alpha Seal-DFL).	The infiltrant was effective in preventing the caries progression comparable with the conventional sealant.	[74]/2017
Fluoridated milk	In vivo	The groups were as follows: fluoridated milk and nonintervention.	Consumption of fluoridated milk could significantly (34%) reduce the caries.	[75]/2018
PRG filler-containing sealant placed with a self-etching primer/adhesive	In vivo	The groups were as follows: self-etch primed sealant (BeautiSealant, Shofu) or the etch and rinse sealant (Seal it, Spident).	The groups were the same in caries prevention.	[76]/2018
High-fluoride toothpaste	In vivo	The groups were as follows: 5,000 ppm F toothpaste or 1,450 ppm F toothpaste.	High-fluoride toothpaste had more effects than control toothpaste in preventing caries.	[77]/2019
FV	In vivo	The groups were as follows: FV or Pl.	FV application was not effective in children.	[78]/2018
MIn	In vivo	The groups were as follows: HE and MIn.	MIn had more effect than HE in reducing caries.	[79]/2018
Hydrophilic F-releasing sealant and ACP sealant	In vivo	The groups were as follows: Aegis™ or Embrace WetBond™ sealant.	Aegis™ was more effective than Embrace WetBond™ sealant as Aegis™ demonstrated lower caries scores.	[80]/2019
School-based fluoride varnish program	In vivo	Volunteers used FTP at home.	The school-based fluoride varnish program prevented progression of caries.	[81]/2016

Table 2: Continued.

Treatment	Type of study	Methods	Outcomes	Ref/year
Topical F	In vivo	The groups were as follows: (1) annual use of SDF solution (30%); (2) three-time use of SDF (30%) per week; (3) three-time use of 5% FV per week.	Yearly use of SDF solution had more effect than three-time use of FV or SDF solution.	[82]/2018
Nutrition and hygiene education	In vivo	The groups were as follows: intervention and control.	The education intervention reduced the progression of caries.	[83]/2018
Organoselenium-containing pit/fissure sealant (DenteShield™ (DS)) and UltraSeal™ XT Plus (UXT)	In vivo	The groups were as follows: DS and UXT.	The groups had the same results for Caries prevention.	[84]/2019
Fluoride TP	In vivo/in vitro	Volunteers used FD or not.	FD group had lower demineralization.	[85]/2016
Toothpastes with fluoride and hydroxyapatite	In vivo	The groups were as follows: toothpastes with hydroxyapatite and fluoride.	Observation group had significantly higher ($p > 0.05$) acid resistance compared with the group of patients using fluoride toothpaste.	[86]/2018
Ordinary and PB cake (*Bacillus coagulans*)	In vivo	The groups were as follows: (1) 1-week consumption of PB cake, then 4-week washout period, and 1-week consumption of regular cake; (2) consumption of the cakes was reversed.	The addition of PB bacteria led to a slight increase in the number of *SM* bacteria in the saliva.	[87]/2019
Ordinary TB and an interactive power TB	In vivo	The groups were as follows: power TB with Bluetooth technology or an ordinary handy TB.	An interactive power TB was more effective in plaque removal versus a handy TB.	[88]/2019
Food enriched with probiotics	In vivo	The groups were as follows: PB milk and standard milk.	The groups were the same in the incidence of caries.	[89]/2018
Resin infiltration	In vivo	The groups were as follows: 1) FTP + flossing + infiltration; 2) control group (FTP + flossing).	Infiltration group had better results than control group.	[90]/2018
GI sand resin s	In vivo	The groups were as follows: GIS and RS.	GISs presented effective prevention of caries development.	[91]/2016
PB yogurt and gums with xylitol	In vivo	The groups were as follows: PB yogurt or gums with xylitol.	The groups were the same in reduction of *SM* counts.	[92]/2017
Fissurit FX sealant and Grandio Seal nanofilled fissure sealant	In vivo	The groups were as follows: Fissurit FX sealant and Grandio Seal nanofilled fissure sealant.	Fissurit FX and Grandio Seal pit and fissure sealants were similar in caries prevention.	[93]/2019
Photodynamic therapy and US	In vivo	The groups were as follows: PDT with MB and US.	PDT or US postponed side effects.	[94]/2018
Fluoride varnish or fluoride mouth rinse	In vivo	The groups were as follows: semiannual fluoride varnish applications (FV) and fluoride mouth rinses once per week (FMR).	The groups had the same results in dental caries progress.	[95]/2016
PB and normal milk	In vivo	The groups were as follows: PB milk and standard milk.	Long-term drinking of probiotic milk may decrease caries progress.	[96]/2016
Intensive FV	In vivo	The groups were as follows: 3 applications of FV in 2 weeks and extra applications at 1 and 3 months; FV treatment twice a year.	The intensive FV application had no adequate effect to prevent dental caries.	[97]/2018
Erythritol	In vivo	The groups were as follows: erythritol, xylitol, or sorbitol (control) group.	Erythritol consumption had caries-preventive effect.	[98]/2016
Interdental cleaning device	In vivo	The groups were as follows: manual toothbrush + mechanical interdental device or manual toothbrush alone.	The combination group had a superior plaque removal compared to manual brushing alone.	[99]/2018

Table 2: Continued.

Treatment	Type of study	Methods	Outcomes	Ref/year
FTP containing zinc ions	In situ	The groups were as follows: F, F/ZN/ phytate, F/Zn, and F Pl.	Phytate had slight effect on capability of fluoride to prevent more advanced lesion demineralization. Moreover, zinc ions had no bad effect on fluoride ability.	[99]/2018
High-fluoride varnish	In vivo	The groups were as follows: differing frequencies of Duraphat varnish application.	Periodic application of fluoride varnish could be useful in prevention of white spots.	[100]/ 2016
Fluoride varnish	In vivo	The groups were as follows: control and use of FV (every 3 or 6 months).	Results suggested using FV with three-month intervals for prevention of caries.	[101]/ 2019
Fluoride varnish	In vivo	The groups were as follows: (1) dental hygiene + FTP and one-time use of three varnishes: Fluor Protector S, Elmex® fluid, or control (Pl).	FV application had no extra protective benefit.	[102]/ 2016
Toothpaste with nanosized sodium hexametaphosphate	In vivo/in vitro	The groups were as follows: conventional fluoride TP, fluoride TP (1100 ppm), fluoride TP (1100F + micro HMP), and fluoride TP (1100F + nano HMP).	1100F/HMPnano revealed a superior protective effect against enamel demineralization.	[15]/2019
Three different compositions of topical fluoride varnishes	In vivo	The groups were as follows: FV having CPP-ACP; FV having xylitol; FV with 0.9% difluorosilane.	FV having CPP-ACP showed higher decrease in *SM* count.	[103]/ 2019
CHX MR, combination MR, and green tea extract MR	In vivo	Volunteers used different MR.	Green tea mouth rinse was effective in prevention of caries.	[104]/ 2017
Povidone-iodine (PI), CHX, or FV (fluor protector)	In vivo	The groups were as follows: PI, CHX V, or FV and control.	Fluoride varnish showed higher decrease in S. *mutans* count.	[105]/ 2017
Probiotic milk and fluoride mouth rinse	In vivo	The groups were as follows: probiotic milk and fluoride mouthwash.	Groups were the same in reduction of *S. mutans* and PI scores.	[106]/ 2019
Topical fluorides	In vivo	The groups were as follows: group 1, 30% SDF solution yearly; group 2, 30% SDF solution per week; group 3, 5% FV per week.	Application of SDF had more effect on arresting caries than FV.	[107]/ 2016
Milk sweetened with xylitol	In vivo	The groups were as follows: (a) xylitol milk, 8 g/200 ml, one time daily; (b) xylitol milk, 4 g/100 ml, two times daily; (c) sorbitol milk, 8 g/200 ml, one time daily; (d) sorbitol milk, 4 g/100 ml, two times daily; or (e) sucrose milk 8 g/200 ml, one time daily.	There were no significant differences in caries incidence between groups.	[108]/ 2016
Probiotic chewing tablets	In vivo	The groups were as follows: the test group got chewing probiotic tablet and the Pl group got the same tablets without bacteria.	Probiotic chewing tablets could be helpful in reducing caries.	[109]/ 2015
Fluoride TP	In vivo	The selected product was brushed twice daily for 4 months.	Clinpro 5000, Clinpro Crème, and MI paste Plus all could be helpful in reducing white spot lesions.	[110]/ 2019
Probiotic lozenge	In vivo	The groups were as follows: probiotic lozenge and Pl lozenge.	Probiotic group had significantly lower *S. mutans*.	[111]/ 2019
Self-etching adhesives having an AB agent and/or F	In vitro/ in vivo	The groups were as follows: fluoride-containing (One-Up Bond F Plus, OP), MDPB and fluoride-containing adhesive (Clearfil Protect Bond, PB).	The AB group had lower demineralization adjacent to restorations.	[112]/ 2015
CPP-ACP and xylitol gum	In vivo	The groups were as follows: gum containing CPP-ACP and xylitol.	Both gums increase saliva's properties.	[113]/ 2017
Resin infiltration	In vivo	The groups were as follows: test group lesions were treated with resin infiltration + 5% topical NaF application and control group with 5% NaF alone.	Resin infiltration was more effective in reducing the development of initial proximal enamel lesions compared with the other group.	[114]/ 2018

Table 2: Continued.

Treatment	Type of study	Methods	Outcomes	Ref/year
Salt fluoridation	In vivo	The groups were as follows: salt containing fluoride and control.	Salt containing fluoride was more effective in prevention of caries.	[115]/ 2018
Sodium fluoride varnish	In vivo	The groups were as follows: intervention group (fluoride varnish) and control group.	Significant caries reversal was seen in primary dentition after intensive fluoride application after 1 year of study.	[116]/ 2017
Sour cherry extract	In vivo	The groups were as follows: gum with cherry extract or control.	Sour cherry extract may have effect on prevention of caries.	[117]/ 2018
Arginine-containing TP	In vivo/in vitro	The groups were as follows: fluoridated TPs (FD) and arginine-containing fluoridated TPs (AFD).	AFD had an anticaries effect like that of ordered fluoridated TPs.	[118]/ 2018
PBM of major salivary glands	In vivo	The groups were as follows: continuous mode LED light, pulsed mode LED light, and control group.	Results suggested that PBM of salivary glands reduces risk of caries.	[119]/ 2020
Herbal mouthwash	In vivo	The groups were as follows: herbal mouthwash, chlorhexidine mouthwash, or Pl mouthwash.	The effectiveness of herbal mouthwash in decreasing plaque formation was similar to chlorhexidine.	[120]/ 2018
Filling intervention health education	In vivo	The groups were as follows: intervention group receiving filling of teeth; and health education group.	Intervention group had better results.	[121]/ 2015
CHX and F MR	In vivo	The groups were as follows: (a) CHX (0.12%) + NaF (0.2%); (b) NaF (0.2%); (c) CHX (0.12%); (d) control.	Groups a and c had similar plaque formation.	[89]/2018
Resin infiltration	In vivo	The groups were as follows: resin infiltration or control.	Progression of caries was significantly higher in control versus infiltration group.	[122]/ 2018
Probiotic *Lactobacillus reuteri*	In vivo	The groups were as follows: probiotic lozenges and Pl lozenges.	Probiotic lozenges reduced bacterial counts significantly.	[123]/ 2018
GIC sealant	In vivo	The groups were as follows: sealant application with or without extra light curing.	Caries prevention in both groups was similar.	[124]/ 2019
Propolis dental varnish	In vivo	Propolis varnishes were used in different concentrations (1%, 2.5%, 5%, and 10%).	Propolis V has AM activity.	[125]/ 2020
Resin infiltration	In vivo	The groups were as follows: infiltration and control.	Resin infiltration was more effective in reducing caries progression.	[126]/ 2016
GIS covered with resin-based agents	In vivo	Fuji VII was used and covered with G-Coat Plus or Heliobond.	The results were the same in both groups in incidence of caries.	[127]/ 2017
CPP-ACP	In vivo	The groups were as follows: stannous F gel (0.4%) with or without CPP-ACP.	CPP-ACP was not effective in decreasing caries development.	[128]/ 2015
New sealant	In vivo	The groups were as follows: Select Defense™ sealant; control.	Test group had lower incidence of WSLs.	[129]/ 2016
Atraumatic restorative treatment by chlorhexidine: disinfection or incorporation	In vivo	The groups were as follows: group (a) CHX having GIC; group (b) CHX; group (c) regular GIC.	Both chlorhexidine disinfection and incorporation showed higher efficacy in inhibiting residual microbes compared to conventional ART.	[130]/ 2017
Fluoride-releasing resin composite	In vitro	The groups were as follows: intervention group (F having adhesive resin) and control.	The materials used in test group were not effective in prevention of WSL.	[131]/ 2017
Fluoridated milk	In vivo/in vitro	Volunteers used an intraoral appliance. They dipped it in fluoridated milk for 5 minutes and once every other day drank the same milk.	Drinking fluoridated milk once per day prevented enamel demineralization.	[132]/ 2018
Toothbrush with paste and Munident	In vivo	The groups were as follows: normal TP and Munident.	Munident (herbal) TP group had significantly lower *S. mutans*.	[133]/ 2017

Table 2: Continued.

Treatment	Type of study	Methods	Outcomes	Ref/year
Fluoride TP and GC Tooth Mousse	In vivo	The groups were as follows: fluoride TP, CPP-ACP crème, and fluoride TP + CPP-ACP crème.	All groups had the same results; combination groups did not have additive benefits.	[134]/ 2020
MIPP and Er: YAG laser	In vitro	The groups were as follows: (a) MIPP; (b) Er: YAG laser; (c) MIPP + Er: YAG laser; (d) saliva; (e) control.	Group c was the most effective group in the treatment of WSLs.	[135]/ 2020
Probiotic bacterium *Lactobacillus reuteri*	In vivo	The groups were as follows: probiotic lozenge and Pl lozenge.	Probiotic lozenges did not prevent progressing of WSL.	[136]/ 2016
RMGI cement varnish	In vivo	The varnish was applied to teeth.	Application of RMGI cement varnish could be useful in preventing WSLs.	[137]/ 2015
Probiotic *Streptococcus dentisani*	In vivo	The probiotic was applied in a buccoadhesive gel.	*S. dentisani* was able to buffer oral pH, especially after multiple dosing.	[138]/ 2020
Semiannual fluoride varnish application	In vivo	The groups were as follows: typical oral health program with or without FV twice a year.	Applications of FV + typical oral health program did not decrease caries progress.	[139]/ 2016
CPP-ACP	In vivo	The groups were as follows: test group receiving CPP-ACP paste monthly and control group.	Test group had lower WSL compared to the control patients.	[140]/ 2016
Peptide P_{11}-4	In vivo	The groups were as follows: P_{11}-4 or FV.	Application of P_{11}-4 significantly reduced the size of early carious lesions. This reduction was higher than fluoride varnish application.	[24]/2020
CHX MR and neem MR	In vivo	The groups were as follows: group a: CHX MR; group b: neem MR; group c: control.	Both MR significantly decreased PI index.	[141]/ 2017
TiF_4 V	In vivo/in vitro	TiF_4, NaF (2.45% F), or control (Pl V).	TiF_4 V was the only treatment able to improve enamel remineralization.	[142]/ 2017
Fluoride and sodium hexametaphosphate in toothpaste	In vivo/in vitro	TP having 1100 ppm F and 1100F + HMP1% and Pl.	TP containing HMP1% was more effective than TP containing 1100F in decreasing demineralization.	[143]/ 2015
Toothpaste Apadent Total Care medical nanohydroxyapatite	In vivo	Volunteers used Apadent Total Care toothpaste with nano-calcium hydroxyapatite.	Application of toothpaste with nanohydroxyapatite showed the improvement of all indices.	[144]/ 2016
Protective chlorhexidine varnish layer over resin-infiltrated proximal carious lesions	In vivo	The groups were as follows: in the test group infiltration + double layer of chlorhexidine varnish was used and in the control group only infiltration was used.	Results suggest application of chlorhexidine varnish layer on resin infiltration when surface had microcavitation.	[145]/ 2016
AgNO3 solution and FV	In vivo	The groups were as follows: (1) $AgNO_3$ solution (25%) + FV; (2) SDF (38%) + Pl V.	Results suggest application of AgNO3/NaF for management of ECC.	[146]/ 2015
Tooth Mousse		The groups were as follows: CPP-ACP (daily) and control.	CPP-ACP reduced *Streptococcus mutans* in test group.	[147]/ 2016
Fluoride rinse		The groups were as follows: sodium F + amine F; control.	Application of fluoride rinse helps prevent demineralization.	[148]/ 2015

primary dentition as a result of the application of 1% sodium fluoride twice per year. Fluoride foam has the same advantages and density as fluoride gel and releases the equivalent fluoride [153]. Fluoride exerts its anticaries effects by three different ways. In the first way, fluoride ion in dental tissues reinforces the fluorapatite deposition from the salivary phosphate and calcium ions. This insoluble deposition occupies the soluble salts including magnesium and carbonate lost during demineralization by the bacteria. This process makes the enamel more resistant to acid [9]. In the second way, caries becomes remineralized without the formation of the cavity using a similar process [9]. In the third way, fluoride ion has AM activity. At low concentrations, fluoride obstructs the construction of glycosyltransferase enzyme. Glycosyltransferase gets the glucose involved in the formation of extracellular polysaccharides and enhances the bacterial adhesion. The formation of extracellular polysaccharides is inhibited by limited bacterial metabolism during the meal time, which prevents the aggregation and maintenance of carbohydrates. Therefore, the

duration of the attack of caries is limited to the period of eating and after it. High concentrations of fluoride ions (12000 ppm) are directly toxic for some oral microorganisms like *SM*. Fluoride has a wide range of activities and has long stability in the oral cavity. It reduces the acid production at 1–10 ppm concentration; it is bacteriostatic at 250 ppm and bactericidal at 1000 ppm [9]. The iodine group is bactericidal and has a wide range of antibacterial activities and short-term stability in the mouth [9]. Many studies have documented the efficacy of fissure sealant treatment in reducing occlusal caries in permanent dentition in children and adults with a high risk of caries [36]. The population-based studies have confirmed the cost-effectiveness of fissure sealant and its long-term effects. Since most caries in the current population occurs in the pits and fissures, fissure sealant seems to be beneficial [150].

9.3. Silver Diamine Fluoride (SDF). Systematic reviews have recommended the application of SDF for termination or prevention of caries in children and adults and root caries in the aged people [155, 156]. This solution is used locally, and silver ions exert their antibacterial effects by breaking the bacterial membrane, denaturing the proteins, and preventing the DNA proliferation [157]. Silver and fluoride both have a key role in the termination of caries progress and sensitivity of tooth [158–161]. Silver reduces the demineralization speed and boosts the remineralization process [162].

9.4. Antimicrobial Agents. Numerous antimicrobial factors have been introduced to decrease the number of bacteria and disturb the biofilm structure. Dental decay is a biofilm associated disease which changes with regimen. Hence, changing the number of bacteria does not have a long-term effect on it. If a remarkable reduction does not occur in the consumption of fermentable carbohydrates, the microbiome in biofilm will adapt to the acidogenic environment and the uric acid produced by the cariogenic diets; thus, AM will have slight effect on the outcome of dental caries [9].

Principally, most antimicrobial agents used for prophylaxis contain a wide range of antimicrobials and provide a ground for the growth of opportunistic factors by eliminating the normal flora. Therefore, except for the consumers of fluoride toothpaste, all other chemical agents should not be used routinely in the daily schedule of the patients. These agents are used as an auxiliary aid when the routine prevention is not effective in individuals with a mental or physical disability, people with reduced salivary secretion, or cases with difficult mechanical removal of plaque such as conditions associated with orthodontic treatment, before and after oral surgeries, and frequent use of crowns [9]. Some antimicrobial agents along with their mechanism of effect are presented in Table 3.

10. Self-Care Methods for Caries Prevention

10.1. Reducing the Consumption of Fermentable Carbohydrates. The main reason for caries-induced dysbiosis is the overuse of fermentable carbohydrates [179]. The diet mechanism has significant effects on the impact of biofilm on dental caries. Frequent consumption of foods having sucrose changes the biofilm from a noncariogenic state to a cariogenic state. Established biofilm, which is repeatedly exposed to sucrose, quickly makes acids from it, thereby creating an acidic environment [22]. Dental caries is mostly caused by the frequency of sucrose consumption, not its amount [22]. The commensal bacteria use the sugar and make acid when a person has a low sugar diet; however, pH is quickly recovered by the mechanisms present in mouth. Frequent consumption of sugars disturbs the balance, and fully reciprocal acidogenic and aciduric strains appear in pathogenic amounts [180, 181]. WHO has seriously recommended restraining the intake of free sugars to <10% of the entire energy intake to prevent weight gain and dental caries [182]. This refers to the <50 g/day consumption of free sugars. Natural sugars such as sugars in honey and added monosaccharides to the foods are free sugars [182]. Cohort studies with quality evidence have reported 15% sugar as the moderate level. Other studies have also recommended the reduction of free sugars to 5% of total energy. Sugar consumption below 5% seems unlikely to cause any caries [183]. Diet with high amount of sugar causes the aggregation of acidogenic and acid-tolerant bacteria and protects them by increasing the production of EPS [27, 174]. Indirect evidence shows caries reduction by decreasing the intake of free sugars. For example, a significant decrease of caries during five years has been reported in the Iraqi children with reduced sugar intake due to the sanctions of the United Nations [184]. Starch as well as sucrose is known as a cariogenic agent. Starch's metabolism causes the long-term acidity in the pits and interdental spaces vulnerable to decay [180].

11. Oral Hygiene

The tooth surfaces free of biofilm are not decayed. Patients should regularly eliminate biofilm by brushing with fluoride-containing toothpaste and dental floss [9, 159]. The oral biofilm composition changes over time following oral hygiene by regular brushing twice a day, and oral microbiome is maintained in the healthy state [160]. The brushing does not remove oral bacteria totally but rather eliminates them from the tooth surface. A massive amount of them is removed from the mouth after swallowing and/or rinsing after brushing and flossing; however, an enough number of them stay for proliferation. Cleaning and exposing to oxygen may kill anaerobic organisms; however, no species is eliminated. Accurate mechanical tooth cleaning disrupts the dental biofilm and cleans tooth surface. While all bacteria that constitute established biofilm still do not exist, most of them are not able to bind to the clean tooth surface [9]. Brushing and flossing are advantageous in that they do not destroy the oral normal flora. Frequent mechanical removal of biofilm does not cause the risk of opportunist infection but rather changes the biofilm's composition. Patients with good oral hygiene have a high percentage of *S. mitis* or *S. sanguis* in their teeth biofilm and have a smaller amount of cariogenic bacteria than the more developed biofilm having a high

Table 3: Some antimicrobial agents and their mechanisms.

Antimicrobial agents	Combinations	Mechanisms	Ref
Antibiotics	Aminoglycosides	Inhibiting protein synthesis	[9, 163]
	Glycopeptides	Interfering with the construction of the cell wall	
	Penicillins	Interfering with the construction of the cell wall	
	Quinolones	Preventing DNA replication and transcription	
	Rifamycins	Inhibiting transcription	
	Tetracyclines	Inhibiting protein synthesis	
	Actinobolin	Obstructing protein synthesis	
AMEs*	Lysozyme	Catalyzing glycosidic bond hydrolysis in bacterial cell wall peptidoglycans	[9, 164, 165]
	Acylase	Quorum-quenching	
AMPs*	Natural	Pore construction in membrane Inhibition of metabolism	[19, 166]
	Synthetic AMPs	Pore construction in membrane Inhibition of metabolism	
Cationic compounds	Chitosan	Disruption of cell membrane	[9, 167]
	Chlorhexidine	Disruption of cell walls	
	Poly(ε-lysine)	Destroying the outer membrane	
	QACs*	Interference with enzymes	
Metal and metal oxides	Ag nanoparticles	Making oxidative stresses Disabling enzymes of bacteria Affecting the permeability of the cell membranes	[168–170]
	Cu nanoparticles	ROS* construction Lipid peroxidation in membranes	
	TiO_2 nanoparticles	ROS* construction Disrupting phosphorylation	
	ZnO nanoparticles	Making ROS* Making membrane more permeable	
Other noncationic compounds	Nitric oxide givers	Making nitrosative stresses Making oxidative stresses	[9, 13, 171]
	Triclosan	Disruption in signaling Disrupting synthesis of fatty acid	
Natural materials	Tea	Disruption of membrane Inhibiting salivary amylase activity	[26, 172, 173]
	Propolis	Interaction with bacterial membrane	
	Cranberry	Inhibition of biofilm formation	
Amino acids	Arginine	Keeping a well dental biofilm	[70, 118, 157, 174–177]
Antioxidants	Antioxidants	Disrupting proteins, decreasing bacterial EPS	[178]

*AMEs: antimicrobial enzymes; AMPs: antimicrobial peptides; QACs: quaternary ammonium compounds; ROS: reactive oxygen species.

amount of *S. mutans* [9]. Nevertheless, it should be emphasized that the former concepts of plaque removal or accurate control of another plaque are not considered for the management of caries. Proper oral hygiene is certainly important for biofilm control, but systemic review studies have shown that mechanical removal of biofilm alone, in the absence of fluoride, is not enough to manage caries [158].

11.1. Fluoride Toothpaste. One of the main causes of caries reduction in developed countries is the extensive application of fluoride TP. Easy and extensive application, low cost, and cultural acceptance have turned fluoride toothpaste into an ideal method for the promotion of general health, and brushing twice a day using fluoride toothpaste has been recommended as a powerful preventive strategy [185].

11.2. Fluoride Supplements. Fluoride supplements are capable of decreasing dental caries by about 20–30% and apply to populations with no access to fluoridated water. These supplements, due to the risk of fluorosis, should be used cautiously and can be used in kids who are at high risk of dental caries and committed parents [153].

11.3. Fluoride Mouthwashes and Gels for Personal Use. Fluoride mouthwashes with 0.2 and 0.05% concentrations are available for daily and weekly use and have been reported to prevent caries by 26%. The fluoride gels for personal use are available as APF, neutral NaF, and stannous fluoride and have been found to reduce caries by 32% in communities with inadequate fluoridated water [36, 153].

11.4. Noncariogenic Sweeteners. Numerous studies have reported the caries-preventive effect for xylitol and to a lesser extent for sorbitol. Xylitol can decrease biofilm [186]. Xylitol is a five-carbon sweetener that has beneficial effects on oral health. Most clinical studies have shown that daily consumption of ≥5 g xylitol gum is efficacious in decreasing caries [187, 188]. Use of xylitol decreases the *SM* amount [189]. Different mechanisms that can reduce mutans are growth inhibition, plaque reduction, oral pH increase, reduction of sticky polysaccharides produced by mutants, and inhibition of stress proteins [48, 58, 60, 92, 98, 108, 186–190].

11.5. Strategies to Boost Saliva. Salivary secretion plays a pivotal role in preventing dysbiosis and maintaining oral health. In addition to the mechanical deletion and buffering capability of saliva, it has enzymes, GP, salts, immunoglobulins, and AM peptides which help with the biofilm stability and control [162]. Although there are clear salivary stimulants, salivary induction is difficult in practice. In patients who are still able to produce saliva, local methods like increasing water consumption and chewing gums and salivary alternatives regularly can enhance saliva making. The moisturizers and enzymes in the products can decrease the symptoms of patients with dry mouth and maintain healthy biofilm [4, 191, 192].

11.6. Prebiotics and Probiotics. Prebiotics are oligosaccharides and nutritional fibers which contribute to useful bacteria's growth while probiotics are defined as living microbes which have advantages for patients [155]. There are not many studies on prebiotics in dentistry, while there are more studies on Arg. Arg is an aminoacid that is found in saliva. The bacteria in healthy biofilm could use Arg through ADS. There is high activity of ADS in noncarious places rather than places with caries lesions [157, 193]. Moreover, studies [157, 194] have shown that adding Arg to fluoride TP can enhance the ADS activity, increase the pH, and help to make a healthy biofilm [157, 175, 193].

The analysis of clinical trials has shown the synergistic effect of arginine and fluoride on the primary caries [176]. Another interesting aspect of prebiotics is breastfeeding. Breast milk is rich in complex biotic oligosaccharides. Systemic studies have shown that breastfeeding in the first year has protective effects against ECC, and bottle-fed newborns had four times more caries than breastfed newborns [195, 196]. Recently, application of probiotics to fight harmful bacteria has increased [156]. Probiotics are dietary supplements that are principally consumed through fermented vegetables, dairy products, and bread. Additionally, there are different forms of these materials such as tablets, drops, and lozenges that have been commercialized. Their mechanism is not known completely. They have effects on biofilms directly and on the immune system [197]. Studies have concluded that therapeutic probiotics can especially reduce the number of *S. mutans* [198]. However, there are very few studies on the effect of oral microbiome composition. Moreover, an increase in nonmutans streptococci and a decrease in the count of *S. mutans*, *Fusobacterium*, and *Prevotella* have been reported after using a supplement for 12 weeks [199]. In contrast, another study has found that the use of lozenges containing *Lactobacillus rhamnosus* and *Brevibacillus brevis* has no significant effect on the oral microbiome in adults after 4 weeks. These conflicting results may be partly due to different molecular and sequencing technologies used in various studies. The results of these studies have shown a 33% reduction in caries in preschool children [200]. Nevertheless, there is insufficient evidence for their overall recommendation. Probiotic treatment for oral health is one of the highly advanced emerging concepts.

12. Conclusion

One of the basic aspects of general health is oral health. Oral diseases cause many problems for individuals and society. They are very common all over the world. While there are effective methods for and adequate information about the prevention of oral diseases, they are still one of the most prevalent health problems. The best strategy to fight caries is the use of a combination of community-based strategies, oral health professionals-based strategies, and self-care strategies. The most important strategies to fight against caries are paying attention to the oral health in macroeconomic policies of countries to create a healthy society, changing the attitude of dental professionals from a treatment-centered approach to a preventive approach, and empowering the individuals by enhancing their knowledge, attitude, and performance in line with maintaining and promoting their oral health.

13. Future Directions

Despite many attempts made to control caries, these efforts have not been effective in controlling caries. Further studies are suggested to identify the confounding factors of biofilm such as natural antimicrobial materials that, unlike antibiotics, do not cause bacterial resistance and are cheap and available to everyone. Moreover, more studies are required on probiotics and production of *S. mutans* strains that are not able to produce lactic acid by recombinant DNA technology or other bacteria that competitively eliminate the microbial cariogenic agents of the oral cavity and manufacture of slow-release devices for antimicrobials such as fluoride in the oral cavity and silver nanoparticle pins that prevent caries by releasing antimicrobial agents. Furthermore, we suggest conducting future studies on the production of caries vaccine to induce biologic defense against caries in the oral cavity and creation of protective layers on the tooth that act beyond the current sealants in terms of gear, duration, and caries prevention.

Abbreviations

AB: Antibacterial
ACP: Amorphous calcium phosphate
ADS: Arginine deiminase system

AM: Antimicrobials
AMEs: Antimicrobial enzymes
AMPs: Antimicrobial peptides
Arg: Arginine
ART-GIC: Atraumatic restorative treatment-high-viscosity glass-ionomer cement
B: Bifidobacterium
CE: Conventional oral health education
CHX: Chlorhexidine
CPP-ACP: Casein phosphate polypeptide-amorphous calcium phosphate
CR: Composite resin
CXT: Clinpro XT
Dl: Deciliters
E: Extracellular
ECC: Early childhood caries
EDI: Enamel decalcification index
EO: Essential oil
EPS: Extracellular polysaccharide production
F: Fluoride
J: Fuji IX GP FAST
FN: *Fusobacterium nucleatum*
FV: Fluoride varnish
G: Glucans
GI: Glass ionomer
GIS: Glass-ionomer sealant
GP: Glycoproteins
HE: Health education
ICDAS: International caries detection and assessment system
IPS: Intracellular polysaccharide production
IR: Infiltration resin
Irr: Irradiation
L: *Lactobacterium*
LA: Lactic acid
MB: Methylene blue
MIn: Motivational interviewing
MIPP: MI paste plus
MR: Mouth rinse
NP: Nanoparticles
P: *Prevotella*
PB: Clearfil Protect Bond
PBM: Photobiomodulation
PFS: Pit and fissure sealing
PG: *Porphyromonas gingivalis*
P.Int: *Prevotella intermedia*
Pl: Placebo
PRF: Preventive resin filling
PRG: Prereacted glass
QACs: Quaternary ammonium compounds
RA: Risk assessment
RBS: Resin-based sealants
Ref: Reference
ROS: Reactive oxygen species
RMGI: Resin-modified glass ionomer
S: *Streptococcus*
SDF: Silver diamine fluoride
SM: *Streptococcus mutans*
STB: Supervised tooth brushing
TB: Toothbrush
TP: Toothpaste
US: Ultrasonic scaler
V: Varnish
WHO: World Health Organization
WSLs: White spot lesions
Xy: Xylitol.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

The authors declare that this work was done by the authors named in this article. Mohammad Nima Motallaei, Mohsen Yazdanian, Hamid Tebyanian, Elahe Tahmasebi, Reza Ranjbar, and Alireza Yazdanian were involved in study design and data collection. Mohsen Yazdanian, Hamid Tebyanian, Elahe Tahmasebi, and Alexander Seifalian critically reviewed the data and wrote the review article.

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Research Article

Antimicrobial Efficacy of Propolis-Containing Varnish in Children: A Randomized and Double-Blind Clinical Trial

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Dental caries is a sugar-dependent condition common in childhood, which causes microbiological imbalance in dental biofilm. The present study evaluated the antimicrobial efficacy of a 2.5% Brazilian Red Propolis (BRP) dental varnish to prevent caries in children. Seventy-five children with high caries risk, aged between 36 and 71 months and with no caries, were assigned to three groups to receive varnish treatment containing 2.5% BRP, 1% chlorhexidine, or 5% fluoride. The varnish was applied to the occlusal surfaces of the deciduous second molars on the first day of treatment (D1), after 90 days (D90), and 180 days of the start of treatment (D180). Saliva was collected to assess *S. mutans* before each varnish application and 180 days at the end of treatment (D360). Values were expressed in log10 (CFU/mL). Statistics were performed by applying repeated measures of variance analysis, Tukey's multiple comparisons test, and paired *t*-test. In the first dilution (1 : 10), there was microbial load reduction at the following periods: BRP in D0-D90 ($p < 0.05$) and D0-D180 ($p < 0.01$); fluoride in D0-D90 ($p < 0.001$); and chlorhexidine in D0-D180 ($p < 0.05$). In the second dilution (1 : 100), there was microbial load reduction in the groups at the following periods: BRP in D0-D90 ($p < 0.05$) and D0-D180 ($p < 0.01$); fluoride in D0-D180 ($p < 0.05$), and chlorhexidine in D0-180 ($p < 0.01$) and D0-360 ($p < 0.05$). The 2.5% BRP dental varnish was effective in decreasing *S. mutans* colonies in saliva when used within 90 days.

1. Introduction

Dental caries is a sugar-dependent condition that can be defined as the process of demineralization of dental enamel and/or dentin induced by acids released by bacteria, presenting multiple factors that can modulate this pathway [1, 2]. Early childhood caries (ECC) can be defined as a condition that affects the deciduous dentition. It is characterized by the presence of at least one decayed tooth (injury with or without cavitation), absence of a tooth (by caries), or the existence of a provisional restoration in a tooth in a child aged 0 to 71 months [3, 4].

Bacterial biofilm consists of a microbiological community organized in an adhesive extracellular matrix that adheres to dental surfaces. Under ideal conditions such as high consumption of fermentable carbohydrates, there may be imbalance in this microsystem, culminating in biochemical and microbiological alterations that favor the demineralization process [5, 6].

In recent years, different formulations have been associated with natural products. Propolis is a resinous complex responsible for sealing bee hives (*Apis mellifera*) and comes from collecting it from different types and different parts of plants. At present, a variety of compounds have been identified in propolis from different geographic samples and botanical diversity. The chemical characterization of brazilian propolis includes prenylated phenolic acids, lignans, terpenes, and terpene alcohols, in addition to p-coumaric acid derivatives [7, 8]. The Brazilian Red Propolis (BRP) differs from other varieties due to the presence of the isoflavonoids vestitol and neovestitoll. [9, 10].

The following can be listed in relation to the pharmacological effects of propolis: antimicrobial activity against bacteria, fungi and viruses, as well as anti-inflammatory, antioxidant, immunomodulatory, and healing activities [7, 11]. Among the chemical constituents present in the BRP, we can mention vestitol and neovestitol, isoflavonoids which demonstrate antimicrobial activity against *Streptococcus mutans (SM)*, *Streptococcus sobrinus*, *Staphylococcus aureus*, and *Actinomyces naeslundii* and anti-inflammatory activity [12].

Dental varnishes are pharmaceutical forms for dentistry use. They are generally composed of a polymer matrix, pharmaceutical excipients, and an active ingredient, usually fluoride, xylitol, or chlorhexidine. The most commonly used polymers are ethylcellulose, chitosan, and acrylate or vinyl acetate, which may be used in the form of polymers alone or in combination [13].

Such formulations have the advantages of high adhesion to the occlusal dental face and the slow and gradual release of the active principles; thus, an extension of the effect of the therapeutic agent conveyed in this pharmaceutical form is either an antimicrobial or anticariogenic agent [14]. In addition, the pharmaceutical form of dental varnish allows a greater contact surface between the active substance and the dental plaque, thus favoring greater interaction between the active substance and the tooth [15, 16].

No BRP dental varnishes were found in intellectual property banks, and the application for a patent of invention under protocol BR1020160190142 was filed. A pilot study was conducted, and the results encouraged further research [17]. The objective of this work was to perform a clinical and microbiological evaluation of 2.5% BRP dental varnish in children.

2. Materials and Methods

2.1. Ethical Aspects. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Research Ethics Committee of the Federal University of Ceara (no. 195,096). Parents were invited to participate in the research and were informed about it and later signed the Written Informed Consent Form (WICF).

2.2. Characterization of Propolis Extract. The BRP extract was collected from the city of Marechal Deodoro (9° 44.555′ Latitude South, 35° 52.080′ Latitude West, and altitude of 18.1 m above sea level), a region with a geographical indication granted by the National Institute of Industrial Property, in the state of Alagoas, Brazil. One hundred and fifty grams (150 g) of the red propolis extract was used and dissolved in 1 L of cereal alcohol of greater graduation. A sample of the absolute alcoholic extract was submitted to chemical identification of its constituents at the Pharmacotechnique's Laboratory of the Pharmacy College of the Federal University of Ceara, Brazil, by High-Performance Liquid Chromatography (HPLC) with the main constituents of quercetin, vestitol, and neovestitol being identified. Identification was performed by comparing the chromatographic profile of the BRP samples in relation to the standards of the isolated chemical constituents subjected to the same analysis conditions.

2.3. Manipulation of Dental Varnishes. Propolis extract and chlorhexidine were incorporated into the varnishes in the Pharmacotechnical Laboratory of the Pharmacy course of the Federal University of Ceara, Brazil. A commercial fluoride varnish was applied (Fluorniz®-SS White, São Cristovão, Brazil).

2.4. Study Design. This is a longitudinal, parallel, randomized, double-blind, controlled clinical trial and adhered to the CONSORT checklist. The clinical phase occurred in the city of Aracati, CE, Brazil, a city where only 0.8% of the population has fluoridated water coverage [18].

Seventy-five caries-free (ICDAS II 0) and healthy children enrolled in public daycare centers were selected to participate in the study. Screening was performed by means of a dental clinic examination performed in a specific clinic by two calibrated professionals (kappa = 0.88). The high-risk caries classification was performed according to the American Academy of Pediatric Dentistry (AAPD) criteria, for example, sugar consumption more than three times a day, lack of access to fluoridated water, presence of visible plaque, poor oral hygiene, and the absence of a professional dentist. The children were divided into 3 groups of 25 participants each. Group I received application of 2.5% BRP varnish (G1), group II received 1% chlorhexidine varnish (G2), and group III received 5% fluoride varnish (G3). Each patient received the varnish application corresponding to their group in the four second deciduous molars. The varnish was applied 1 time for each tooth at three different times: at the first day of treatment (D1), after 90 days (D90), and 180 days after the start of treatment (D180). The presence or absence of caries lesions in the evaluated teeth was also recorded during all clinical evaluations.

Inclusion criteria were children aged 36 to 71 months, the absence of caries, both genders, belonging to public daycare centers, and who had erupted primary molars, as well as the absence of carious lesions (ICDAS II 0). Exclusion criteria were the presence of any systemic disease, use or application of any antibiotic or anti-infective chemotherapy three months prior to the beginning of the study, history of any allergy, or the presence of any active carious lesion.

2.5. Saliva Collection. For the clinical trial, the stimulated saliva from each patient was collected at four moments: baseline (D0), after 90 days (D90), 180 days (D180), and 360 days from the start of treatment (D360). Each patient initially chewed a piece of 3 cm plastic film (Parafilm) for 60 s to stimulate the production of saliva and release the bacteria from the dental biofilm. Saliva was collected using a plastic device and stored in sterile microcentrifuge tubes (Eppendorf), which were then stored in a polystyrene box containing ice. All samples were collected in the same session and conditions by the same operator between 9:00 and 11:00 AM to minimize the influence of the circadian rhythms on salivary flow. Thereafter, the varnish was applied by the same operator to the deciduous molars with relative insulation of each patient after Robinson prophylaxis using brushes and pumice. A triple syringe was then used after 10 s to gently dry the varnish.

Each patient received application of the varnish corresponding to their group in the four primary second molars at times D1, D90, and D180. The teeth were professionally cleaned with a Robinson brush and pumice prior to the varnish application. The varnishes were applied with relative insulation in the selected molars using a microbrush. After 10 s, the varnish was subtly air-dried by using the triple syringe. The cotton rolls were removed after 25 s to avoid saliva contamination. The presence or absence of caries was also recorded in all teeth during each evaluation.

2.6. Microbiological Analysis. A volume of 0.1 mL of each sample was transferred to a sterile hemolysis tube containing 0.9 mL of saline. This process was repeated twice, establishing dilutions of 1 : 10 and 1 : 100. A volume corresponding to 10 μL of each dilution was seeded in a medium of *Agar mitis salivarius bacitracin* (MSB) in triplicate. The plates were incubated at 37°C for 48 hours in microaerophilic jars and placed in an oven. Colonies with morphological characteristics of *S. mutans* were then counted after this period. Bacteria were expressed as CFU/mL of saliva.

2.7. Statistical Analysis. The transformed values of the CFU number were initially analyzed by the Kolmogorov–Smirnov test to verify the normality of the distribution. Thus, mean and standard deviation were calculated for the descriptive statistics, as well as parametric tests were used to analyze the data. Analysis of variance (ANOVA) was used to compare the three groups at each time (intergroup analysis), associated with Tukey's multiple comparison test to verify differences between the paired groups. Comparisons between the different times within each group (intragroup analysis) were performed by repeated measures analysis of variance (ANOVA), associated with the Tukey multiple comparisons test in order to verify differences between paired times. The level of significance was set at 0.05 (5%) in all analyzes, with a p value less than 0.05 being considered as statistically significant. GraphPad Prism® software version 5.00 for Windows® (GraphPad Software, San Diego, California, USA, 2007) was used for both statistical procedures and graphing.

3. Results

3.1. First Dilution 1 : 10. In the first dilution, there was a statistically significant difference in D180 compared to the baseline in the chlorhexidine-treated group (Table 1). A significant difference was observed in the reduction of the number of CFUs of *S. mutans*, in all posttreatment periods with fluoride varnish compared to baseline. Although the fluoride varnish showed a statistically significant reduction only in the first period, this reduction was maintained until the end of the study (Table 1). The BRP varnish group showed a statistically significant reduction of microbial load in the periods of D0-D90 ($p < 0.05$) and D0-D180 ($p < 0.01$) (Table 1).

Therefore, when comparing the reduction of the microbial load generated by the treatment with the varnishes tested, a satisfactory performance of BRP varnish treatment is observed, in the reduction of the CFU of *S. mutans*, especially in the periods intermediates D90 and D180, due to the statistically significant reduction in saliva analysis, at the dilution of 1 : 10.

3.2. Second Dilution 1 : 100. In the second dilution, the fluoride varnish showed reduction in microbial load in D180 compared to baseline ($p < 0.05$). In the group treated with the chlorhexidine varnish, there was a significant reduction of CFU in D180 ($p < 0.01$) and D360 ($p < 0.05$) compared to baseline (Table 2).

Table 2 shows, graphically, the evolution of the microbial counts in saliva samples at a dilution of 1 : 100 during the treatment with BRP varnish. There was a decrease in all periods compared to the baseline, but it was only statistically significant in the period D90 ($p < 0.05$) and D180 ($p < 0.01$). In the D360, there was an increase in the CFU analyzed, being statistically significant in relation to D180 ($p < 0.05$).

At the end of the study, the presence of caries was verified in five children treated with the chlorhexidine varnish; in the group treated with BRP varnish, carious lesions were evidenced in a child, although it was not localized in the molars. In the group treated with fluoride varnish, no carious lesions were developed.

4. Discussion

In the present study, we evaluated the antimicrobial efficacy of a new varnish containing brazilian red propolis along 360 days and compared with dental varnishes with fluoride and chlorhexidine, in order to prevent dental caries in a high-risk group of children.

Studies in dentistry with natural products especially occur in relation to cariogenic and periodontal biofilm [7, 12–14, 16, 17, 19]. Several studies have validated the use of BRP. An in vitro study which verified the efficacy of 80% alcoholic extract of BRP against *S. mutans* and *S. sobrinus* also verified the ability of the extract to inhibit acid production by microorganisms. This decrease in acid production was attributed to the enzymatic inhibition of cytosolic F-ATPase [20].

Table 1: Amount of *Streptococcus mutans*, expressed as the logarithm of the number of colony forming units (CFUs) per ml of saliva, measured in saliva samples with a dilution of 1 : 10 on days 0 (pretreatment), 90, 180, and 360 in patients treated with chlorhexidine, fluoride, and propolis varnishes. The data correspond to the mean and standard deviation of the logarithm of the number of CFUs verified in the saliva samples of 25 patients treated with chlorhexidine and fluoride varnish and 24 subjects treated with propolis varnish.

Day	Chlorhexidine Mean ± SD	Fluoride Mean ± SD	Propolis Mean ± SD	Significance (ANOVA)
0	0.58 ± 0.43	0.86 ± 0.37^{a}	1.01 ± 0.40^{c}	$p = 0.0010$
90	0.38 ± 0.23	0.51 ± 0.33^{z}	$0.64 \pm 0.45^{a,x}$	$p = 0.0403$
180	0.33 ± 0.14^{x}	0.41 ± 0.24^{z}	$0.60 \pm 0.40^{b,y}$	$p = 0.0042$
360	0.55 ± 0.52	0.53 ± 0.44^{z}	0.71 ± 0.35	$p = 0.2869$
Significance (repeated measures ANOVA)	$p = 0.0107$	$p < 0.0001$	$p = 0.0036$	

SD: standard deviation; ANOVA: analysis of variance; $^{a}(p < 0.05)$, $^{b}(p < 0.01)$, and $^{c}(p < 0.001)$ denote statistically significant differences in relation to the chlorhexidine varnish on the same day (Tukey's test); $^{x}(p < 0.05)$, $^{y}(p < 0.01)$, $^{z}(p < 0.001)$ indicate statistically significant differences in relation to day 0 in the same group (Tukey's test).

Table 2: Amount of *Streptococcus mutans*, expressed as the logarithm of the number of colony forming units (CFUs) per ml of saliva, measured in saliva samples with dilution of 1 : 100 on days 0 (pretreatment), 90, 180, and 360 in patients treated with chlorhexidine, fluoride, and propolis varnishes. The data correspond to the mean and standard deviation of the logarithm of the number of CFUs verified in the saliva samples of 25 patients treated with chlorhexidine and fluoride varnish and 24 subjects treated with propolis varnish.

Day	Chlorhexidine Mean ± SD	Fluoride Mean ± SD	Propolis Mean ± SD	Significance (ANOVA)
0	0.45 ± 0.26	0.48 ± 0.20	$0.68 \pm 0.24^{a,c}$	$p = 0.0023$
90	0.33 ± 0.13	0.36 ± 0.11	0.45 ± 0.25^{x}	$p = 0.0766$
180	0.30 ± 0.00^{y}	0.34 ± 0.07^{x}	$0.40 \pm 0.18^{a,y}$	$p = 0.0120$
360	0.31 ± 0.18^{x}	0.40 ± 0.28	$0.64 \pm 0.36^{b,c,z}$	$p = 0.0004$
Significance (repeated measures ANOVA)	$p = 0.0037$	$p = 0.0417$	$p = 0.0021$	

SD: standard deviation; ANOVA: analysis of variance; $^{a}(p < 0.01)$ and $^{b}(p < 0.001)$ denote statistically significant differences in relation to the chlorhexidine varnish on the same day, while $^{c}(p < 0.05)$ indicates a statistically significant difference in relation to the fluoride varnish on the same day (Tukey's test); $^{x}(p < 0.05)$ and $^{y}(p < 0.01)$ designate statistically significant differences in relation to day 0 in the same group, while $^{z}(p < 0.05)$ denotes a statistically significant difference in relation to day 180 in the same group (Tukey's test).

Dental varnishes are pharmaceutical forms of dental use that are well accepted by pediatric patients. The advantage is that it is a relatively quick, simple, and safe professional application [16, 17, 21]. The BRP varnish in the present study was able to act inreducing the salivary CFU of *S. mutans*, being effective in the periods of 90 and 180 days after its application. In this context, 2.5% BRP dental varnish applications would demonstrate greater efficacy if they were repeated with a shorter time period. From the data presented in the study, an application every 3 months would be more interesting to include the pharmaceutical product in dental practice.

However, the index of patients who presented dental caries in the group treated with BRP varnish was relatively low, even with the elevation in the CFU analyzed at D360. This fact can be partly explained by the presence of at least two chemical constituents in BRP: vestitol and neovestitol [12, 22] These isoflavonoids have great antioxidant and antimicrobial effects. In an in vitro study, the two chemical constituents were isolated and showed to have the ability to decrease the production of soluble and insoluble extracellular polysaccharides. Thus, the production and adhesiveness of the bacterial biofilm in the dental matrix is decreased, which hinders the cariogenesis process [12]. Another result of the study by Bueno-Silva et al. [12] revealed that these compounds have the capacity to generate stress in the metabolism of *S. mutans*, thereby diminishing the activities of several enzymes, glycosyltransferases being among them, which help in the formation of biofilm, and also of other metabolic routes involved in the production of acid by microorganisms.

An in vitro study evaluated the antimicrobial activity of green propolis varnishes at concentrations of 5, 10, and 15% with chitosan. The varnishes were tested against *S. mutans* microorganisms and other microorganisms being compared with chlorhexidine and nystatin, obtaining superior results [14]. In addition, this study also verified that propolis can be released by the pharmaceutical form of dental varnish for up to nine weeks, releasing 20 to 30% of propolis in the first 24 hours. Therefore, an activity profile similar to that found in the propolis varnish studied in our present study was verified, which was able to present satisfactory results even within 90 and 180 days of the application.

Another in vitro study also evaluated the action of green propolis varnishes at concentrations of 5, 10, and 15% with chitosan against cariogenic microorganisms. *S. mutans*, *S. sanguinis*, *S. salivarius,* and *Lactobacillus casei* microorganisms were tested. Green propolis and chitosan varnishes of different concentrations significantly inhibited the growth of microorganisms, similar to the pure propolis extract, demonstrating that there is no possible incompatibility in the release of propolis from the varnish. In addition, propolis varnishes at all concentrations showed greater inhibition of microbial growth than chlorhexidine [13]. The work of De Luca et al. [13] also conducted a cytotoxicity test of varnishes in different concentrations, wherein it was verified that the

green propolis and chitosan varnish showed very low cytotoxicity according to ISO 10993-5 of 2009, which governs the cytotoxicity assays of clinical devices.

James et al. [23] described that the application of chlorhexidine varnish to pediatric patients should occur every 2 to 3 months. In another study, it was verified that the chlorhexidine varnish only had an effect on the prevention of dental caries when applied at intervals of 3 to 4 months [24]. The group treated with chlorhexidine varnish, as expected, showed a statistically significant reduction of the CFU of *S. mutans* in the saliva samples at day 180 at the dilution of 1 : 10 and at days 180 and 360 at the dilution of 1 : 100. However, five children were detected as having dental caries at the end of the study.

There is currently in the literature a weak evidence of using chlorhexidine varnish in the prevention of dental caries. A systematic review evaluated the efficacy of chlorhexidine varnishes in the prevention of dental caries in children, in which it was found that the efficacy of its use is inconclusive [23]. Although chlorhexidine effectively decreases microbial load in the short-term dental biofilm, it is again taken into account that caries is a sugar-dependent disease mediated by multiple factors. Thus, a broader approach is needed to better control this clinical condition [1, 2].

The group treated with fluoride varnish showed a significant reduction of the microbial load in a sustained way during the study on days 90, 180, and 360 when verified in the 1 : 10 dilution. However, a statistically significant reduction of microbial load was only observed at day 180 when counting CFU at the 1 : 100 dilution of saliva. This decrease can be explained by the residual effect of fluoride in the oral cavity and would not specifically present an antimicrobial effect, but it controls the microorganism population in the oral cavity because of the capacity to cause stress in the metabolism of *S. mutans* [25].

5. Conclusions

The 2.5% BRP dental varnish was effective in controlling the formation of *S. mutans* colonies in the oral cavity when used within 90 days, thus being a complementary strategy to assist in the control of dental caries.

Data Availability

The datasets used in the present study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Clinical and Antimicrobial Evaluation of *Copaifera langsdorffii* Desf. Dental Varnish in Children: A Clinical Study

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Background. The objective of this study was to evaluate the clinical and microbiological efficacies of (*C. langsdorffii*) dental varnish in children at high risk of dental caries.. *Methods*. This is a longitudinal, randomized, controlled clinical trial. Ninety high-risk caries-free children (ICDAS II = 0) were recruited and randomly divided into three groups: *C. langsdorffii*, chlorhexidine, or fluoride. The varnishes were applied on the second deciduous molars for three times: baseline (D0), after 90 days (D90), and after 180 days (D180). Saliva was collected on D0, D90, D180, and D360 to evaluate *S. mutans* reduction. Statistics were carried out by ANOVA, Tukey's test, and the paired *t*-test. *Results*. Copaiba varnish demonstrated significant *S. mutans* reduction: D360 versus D0 ($p < 0.0001$), D180 versus D0 ($p < 0.001$), D360 versus D90 ($p < 0.001$), D180 versus D90 ($p < 0.001$), and D360 versus D180 ($p < 0.05$). Chlorhexidine varnish significantly reduced *S. mutans* at D180 versus D0 ($p < 0.05$). Fluoride reduced at D180 versus D0 ($p < 0.001$). *Conclusions*. Three annual applications of this varnish showed substantial antimicrobial activity against *S. mutans* and caries prevention for up to 12 months.

1. Introduction

Medicinal plants have great biological and pharmacological diversities, being great targets in drug development [1]. There are more than 100 million bioactive molecules cataloged, and this number can be considered unlimited due to still unexplored possible chemical arrangements and resources [2].

Copaiba tree belongs to the Leguminosae family, Caesalpinioideae subfamily, and *Copaifera* genus, and the trees can reach up to 400 years of age. The oilresin is yellow-brown with several active components such as sesquiterpenes and diterpenes, which have anti-inflammatory, analgesic, antimicrobial, and antitumor properties [3]. Copaiba oilresin has been widely used and especially found in neotropical regions where bees of the *Apis mellifera* species are the main pollinating agents. There are records of copaiba oilresin use for almost 400 years, with several studies proving its innumerable biological activities, being effective against several microorganisms and commonly used in traditional medicine against various diseases [3, 4].

The genus *Copaifera* is widely found in South and Central America, India, and West Africa. The greatest species richness is found in Brazil, where trees can especially be found in the Southeast, Midwest, and Amazon regions. Among the 72 cataloged species, 20 have already been found in Brazil, with 16 being exclusive to the country and considered a food, thus presenting its safety proven by the wide traditional popular use. Among them are *Copaifera officinalis* L., *Copaifera guianensis* Desf., *Copaifera reticulata*

Ducke, *Copaifera multijuga* Hayne, *Copaifera confertiflora* Benth., *Copaifera langsdorffii* Desf. (*C. langsdorffii*), *Copaifera coriacea* Mart., and *Copaifera cearensis* [3, 5, 6].

C. langsdorffii oilresin is effective against Gram-positive and Gram-negative bacteria, especially for topical use. Its effectiveness against the cariogenic bacterial is also emphasized [7]. Copaiba oilresin has high activity against oral bacteria and can be used in appropriate formulations, since the main oral diseases, caries, and periodontal diseases are strongly related to the dental biofilm. However, in vitro and in vivo assays of these formulations must be well studied [5].

Dental caries is the most prevalent disease in the world and is called early childhood caries (ECC) when it occurs in children under 6 years of age. This condition specially affects the deciduous second molars due to their occlusal morphology which favors accumulating bacterial plaque [8, 9]. Even with the expansion of access to health services, early childhood caries (ECC) is still a public health problem [10], being the main cause of losing deciduous teeth early, negatively influencing speech, aesthetics, the masticatory system, and the dental arches [11].

The main products in preventing oral diseases are fluoride, chlorhexidine, triclosan, cetylpyridinium chloride, and natural products, especially extracts and essential oils which have attracted attention due to antimicrobial activity. These have also been used as therapeutic alternatives against dental caries [12–14].

Dental biofilm is the main biological determinant in the development of dental caries, and salivary microbiota is related to tooth decay [15, 16]. Several microorganisms colonize dental biofilms, where *S. mutans* are strongly associated with dental caries, and are found in all niches such as saliva, tongue, oral mucosa, and dental plaque [17]. After consumption of sucrose, *S. mutans* produce extracellular polysaccharides and are acidogenic and aciduric, being able to survive in adverse conditions. The oral cavity presents several genotypes of *Streptococcus mutans* (*S. mutans*) with different virulence capacities [10, 18]. Thus, although they are not the only ones involved in the process, they are a key contributor in forming dental biofilms and can be considered a salivary biomarker [19, 20].

Healthy behavioral approaches and promotion should be implemented for the prevention of dental caries in public health, in addition to policies such as public water fluoridation and strategies in high-risk groups with restricted access to dental and fluoride services [9]. It is also known that preventing decay in primary teeth will prevent permanent dentition. In the current growing model of minimally invasive dentistry, it is argued that dental caries can be controlled and prevented in a noninvasive way through several products, among which varnishes can be mentioned [17, 21].

As children under six years of age do not yet have the proper habit of "rinsing and spitting," the most appropriate formulations for preventing caries disease at this age would be varnishes rather than a rinse aid or gel and also because of their high retentive capacity and the slow release of the active principle.

No reports have been found in the literature on the use of varnishes based on copaiba, and thus, the application of a patent of invention was deposited under protocol BR 1020160212628. The objective of this study was to evaluate the clinical and microbiological efficacies of *C. langsdorffii* dental varnish in children at high risk of caries.

2. Materials and Methods

2.1. Copaiba Oilresin. Samples of copaiba oilresin obtained from *Copaifera langsdorffii* Desf. (Fabaceae: Caesalpinioideae) plant material deposited in the herbarium of the Federal University of Mato Grosso, voucher Silva, R. R. et al. 1749, were received from the Federal University of Mato Grosso and originally obtained from Juruena Valle (region: midwest, latitude: 10° 19' 05" S, longitude: 58° 21' 32" W, and height: 300 m). Chemical constituents were identified by specialists at the Department of Chemistry in the Federal University of Ceara (GC–MS QP 5050, Shimadzu, Japan). The total content of the bioactive constituents was 84.69%. The main compounds were caryophylene oxide (54.2%), β-caryophylene (6.08%), β-element (4.43%), α-cis-bergamotene (4.56%), and ar-curcumene (4.63%).

2.2. Clinical Study. This is a longitudinal, parallel, randomized, double-blind controlled clinical trial. The rules of the CONSORT checklist were followed in order to improve the study methodology.

2.3. Local and Population/Ethical Aspects. This study was approved by the Ethics Committee of the Federal University of Ceara (UFC), with number 195.096. The clinical phase occurred in the city of Aracati-CE-Brazil, a city in which only 0.8% of the population has fluoridated public water coverage. The parents were invited to participate in their search and then informed so as to sign the clear and informed consent form. The population selection (90 children) was carried out by means of a clinical examination of the patients in public schools and daycare centers, where children who were free of caries (ICDAS II 0) with 4 erupted primary second molars, aged between 36 and 71 months and of both genders, were included. The detection was performed by a single researcher calibrated for ICDAS II (kappa index 0.78). The high-risk caries classification was performed according to the criteria of the American Academy of Pediatric Dentistry (AAPD, 2014) [22], for example, consumption of sugar more than three times a day, lack of access to fluoridated water, presence of visible plaque, poor oral hygiene, and absence of visits to a professional dentist. Exclusion criteria were presence of any buccalor systemic disease or the application or use of any antibiotic or antimicrobial three months prior to starting the study.

2.4. Varnish Preparation. Copaiba oilresin was formulated as a varnish in the pharmacotechnical laboratory of the pharmacy course of the Faculty of Pharmacy, Dentistry, and Nursing of the Federal University of Ceara, in a standardized

way in order to obtain similarity of color, odor, consistency, and flavour.

A pilot study was initially performed to obtain the dose-response curve [23]. The concentration (1%) used in the main study was first checked as having the greatest relative reduction capacity of bacteria (%). All varnishes were stored in tubes and encoded with letters of the alphabet to ensure blinding in this study.

2.5. Grouping, Application of Varnishes, and Saliva Collection. After randomization in the Excel program, children were divided into 3 groups, with 30 participants each. The sample for each group was calculated considering a power of 90% and a significance level of 5%. The sample size needed to satisfy the requirements of this study was calculated as being 24 subjects in each group. However, 25% was added to this value in order to cover possible follow-up losses; then, the final sample size was estimated as 30 patients in each group. All participants received a toothbrush of the same brand with a straight handle, small head, and soft bristles and fluoridated toothpaste to use thrice a day.

Group I received application of 1% chlorhexidine varnish, 5% fluoride varnish group II, and 1% *C. langsdorffii* oilresin group (copaiba). Each patient initially chewed a piece of 3 × 3 cm plastic film (Parafilm®) for 60 s to stimulate the production of saliva and release the bacteria from the dental biofilm. All participants received the same type of toothbrush and fluoridated toothpaste. Standardized oral hygiene instruction was conducted through a single instructor for all parents that received the recommendations to be followed in writing to reinforce the instructions.

Saliva was collected using a plastic device and stored in sterile microcentrifuge tubes (Eppendorf®), which were stored in polystyrene box containing ice. To minimize the influence of the circadian rhythms on salivary flow, all samples were collected in the same session and conditions by the same operator between 9 : 00 and 11 : 00 AM. After the collection of saliva, each patient received an application of the varnish corresponding to their group in the four second deciduous molars. Prior to an application of varnishes, the teeth were professionally cleaned with a Robinson brush and pumice. The varnishes were applied with relative insulation on to the selected molars using a microbrush. After 10 s, the varnish was subtly dried by air from a triple syringe. The cotton rolls were removed after 25 seconds to avoid saliva contamination. The varnish was applied 3 times for each tooth: at the baseline, after 90 days, and after 180 days of starting treatment. The presence or absence of caries was also recorded in the evaluated teeth as well as in others during each evaluation and after the baseline. The saliva of each patient was collected at 4 moments: at the baseline, after 90 days, after 180 days, and after 360 days of starting treatment.

2.6. Microbiological Analysis. Samples were transported to the laboratory for microbiological analysis in a hermetically sealed case containing ice.

Saliva was homogenized on a tube shaker for 30 seconds. A volume of 0.1 mL of each sample was aseptically drawn and transferred into one sterile test tube containing 0.9 mL of saline. The procedure was repeated twice, establishing dilutions of 1 : 10 and 1 : 100. A corresponding volume of 10 μL of each dilution was plated onto mitis salivarius-bacitracin (MSB) agar medium in triplicates. The plates were then incubated at 37°C during 48 h in jars under microaerophilic conditions. Bacterial counts were expressed as colony forming units (CFU)/mL of saliva and followed by phenotypical colony identification, as described elsewhere.

2.7. Clinical Evaluation. The children were evaluated for monitoring carious lesions by the ICDAS II method in the same saliva collection periods.

2.8. Statistical Analysis. The data regarding the number of CFU were initially transformed in order to achieve normal distribution, using a logarithmic transformation (log10). The transformed values of the CFU number were initially analyzed by the Kolmogorov–Smirnov test to verify the normality of the distribution. Thus, mean and standard deviation were calculated for the descriptive statistics, as well as parametric tests were used for data analysis. Analysis of variance (ANOVA) was used to compare the three groups at each time (intergroup analysis), associated with Tukey's multiple comparisons test to verify differences between the paired groups. Comparisons between the different times within each group (intragroup analysis) were performed by repeated measures analysis of variance (ANOVA), associated with Tukey's multiple comparisons test in order to verify differences between paired times. The level of significance was set at 0.05 (5%) in all analyzes, with a p value less than 0.05 being considered as statistically significant. GraphPad Prism® software version 5.00 for Windows® (GraphPad Software, San Diego, California, USA, 2007) was used for both statistical procedures and graphing.

3. Results

We have randomized 90 children in three groups (Figure 1). All the participants completed the study.

Table 1 shows the amount of *S. mutans*, expressed as the logarithm of the number of colony forming units (CFU) per mL of saliva, measured in saliva samples with dilution of 1 : 10 on days 0 (baseline), 90, 180, and 360 in patients treated with chlorhexidine, fluoride, and copaiba varnishes on the first dilution. The data correspond to the mean and standard deviation of the logarithm of the number of CFU verified in the saliva samples of the patients in each treatment group. At the end of treatment, the groups treated with copaiba varnish ($p < 0.0001$) and fluoride ($p < 0.0001$) had higher statistical difference in relation to the start of treatment compared with the chlorhexidine group ($p = 0.0107$).

Table 2 shows the amount of *S. mutans*, expressed as the logarithm of the number of colony forming units (CFU) per ml of saliva, measured in saliva samples with dilution of 1 : 100 on days 0 (pretreatment), 90, 180, and 360 in patients treated with chlorhexidine, fluoride, and copaiba varnishes, on the second

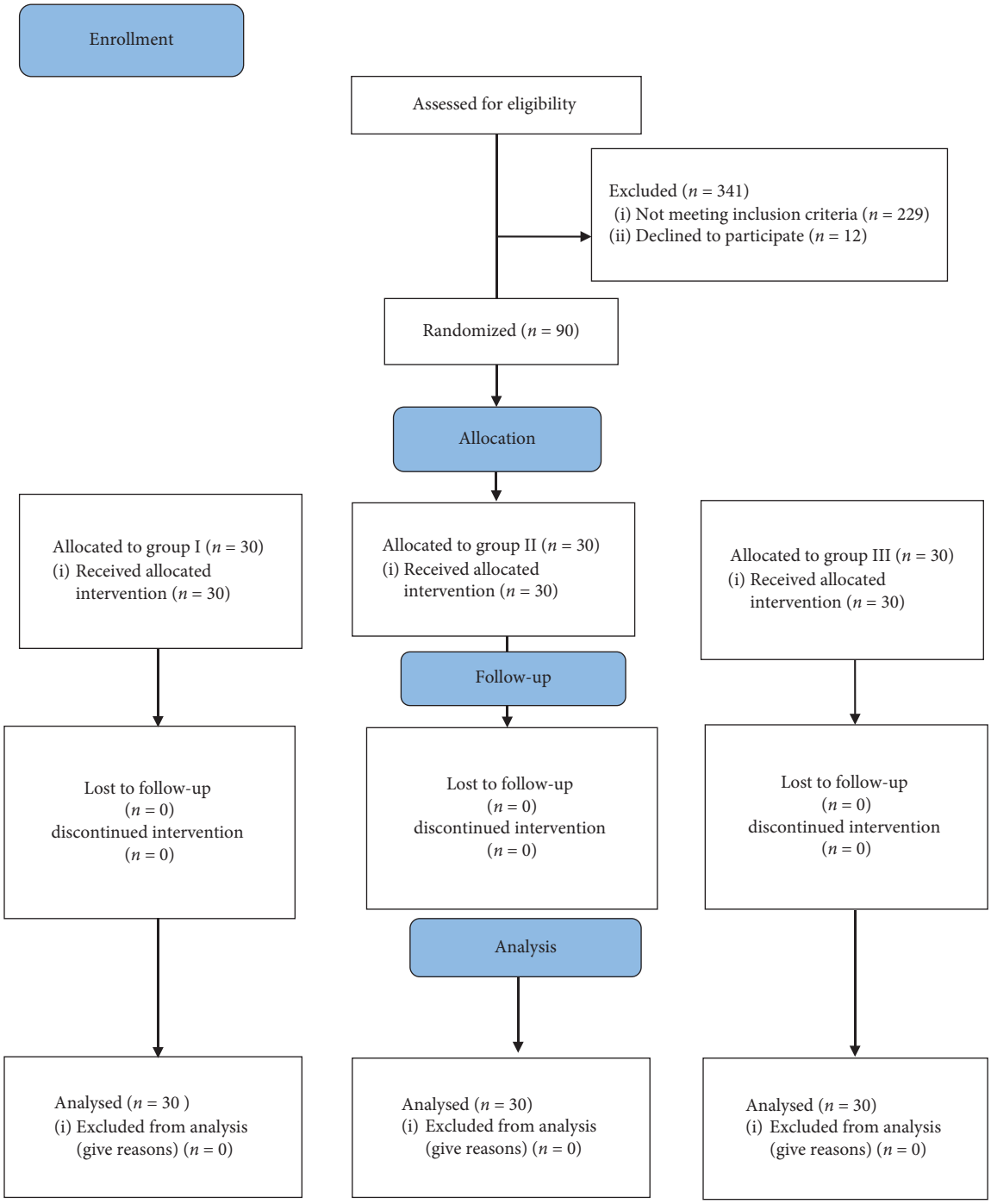

Figure 1: CONSORT 2010 flow diagram of the study.

dilution (1 : 100). The data correspond to the mean and standard deviation of the logarithm of the number of CFU verified in the saliva samples of the patients in each treatment group. At the end of treatment, the group treated with chlorhexidine ($p < 0.05$) and copaiba ($p < 0.001$) varnishes had a statistical difference in relation to D0.

Regarding the clinical data at the end of the groups with different treatment of dental varnishes (Table 3), the appearance of 5 initial carious lesions in the group treated by the chlorhexidine varnish was observed in 2 patients. The group treated with copaiba and fluoride varnishes registered no caries lesion using ICDAS II scores.

4. Discussion

In the present study, the clinical and antimicrobial efficacies of a new varnish containing copaiba oilresin were evaluated along 360 days and compared with dental varnishes with fluoride and chlorhexidine, in order to prevent dental caries in a high-risk group of children.

Table 1: Amount of *S. mutans*, expressed as the logarithm of the number of colony forming units (CFU) per mL of saliva, measured in saliva samples with dilution of 1 : 10 on days 0 (pretreatment), 90, 180, and 360 in patients treated with chlorhexidine, fluoride, and copaiba varnishes.

	Chlorhexidine Mean ± SD	Fluoride Mean ± SD	Copaiba Mean ± SD	Significance (ANOVA)
0	0.58 ± 0.43	0.86 ± 0.37	$1.32 \pm 0.61^{a,d}$	
90	0.38 ± 0.23	0.51 ± 0.33^{x}	$0.99 \pm 0.57^{a,c,y}$	
180	0.33 ± 0.14^{y}	0.41 ± 0.24^{x}	$0.39 \pm 0.22^{x,z}$	$P = 0.3580$
360	0.55 ± 0.52	0.53 ± 0.44^{x}	$0.12 \pm 0.19^{b,d,x,z}$	
Significance (repeated measures ANOVA)				—

SD, standard deviation; ANOVA, analysis of variance. The letters ^{a} ($P < 0.001$) and ^{b} ($P < 0.01$) denote statistically significant differences in relation to the chlorhexidine varnish on the same day, while the letters ^{c} ($P < 0.001$) and ^{d} ($P < 0.01$) indicate statistically significant differences in relation to the fluoride varnish on the same day (Tukey test). The letters ^{x} ($P < 0.001$) and ^{y} ($P < 0.05$) designate statistically significant differences in relation to day 0 in the same group, while the letter ^{z} ($P < 0.001$) denotes statistically significant difference in relation to day 90 in the same group (Tukey's test).

Table 2: Amount of *Streptococcus mutans*, expressed as the logarithm of the number of CFU per mL of saliva, measured in saliva samples with dilution of 1 : 100 on days 0 (pretreatment), 90, 180, and 360 in patients treated with chlorhexidine, fluoride, and copaiba varnishes.

Day	Chlorhexidine Mean ± SD	Fluoride Mean ± SD	Copaiba Mean ± SD	Significance (ANOVA)
0	0.45 ± 0.26	0.48 ± 0.20	$0.89 \pm 0.55^{a,c}$	
90	0.33 ± 0.13	0.36 ± 0.11	$0.66 \pm 0.44^{a,c}$	
180	0.30 ± 0.00^{v}	0.34 ± 0.07^{w}	$0.38 \pm 0.19^{u,y}$	$P = 0.0723$
360	0.31 ± 0.18^{w}	0.40 ± 0.28	$0.10 \pm 0.19^{b,c,u,x,z}$	
Significance (repeated measures ANOVA)				—

SD, standard deviation; ANOVA, analysis of variance. The letters ^{a} ($P < 0.001$) and ^{b} ($P < 0.01$) denote statistically significant differences in relation to the chlorhexidine varnish on the same day, while the letter ^{c} ($P < 0.001$) indicates statistically significant difference in relation to the fluoride varnish on the same day (Tukey test). The letters ^{u} ($P < 0.001$), ^{v} ($P < 0.01$), and ^{w} ($P < 0.05$) designate statistically significant differences in relation to day 0 in the same group; the letters ^{x} ($P < 0.001$) and ^{y} ($P < 0.05$) denote statistically significant differences in relation to day 90 in the same group, while the letter ^{z} ($P < 0.05$) indicates statistically significant difference in relation to day 180 in the same group (Tukey test).

Table 3: Distribution of the lesions on the molars (scores ICDAS II) of different groups treated with dental varnishes at the end of the study.

Tooth	Copaiba	Chlorhexidine	Fluoride
Score 0	0	0	0
Score 1	0	4	0
Score 2	0	1	0
Score 3	0	0	0
Score 4	0	0	0
Score 5	0	0	0
Score 6	0	0	0

Among the risk markers for ECC are the *S. mutans* and *Lactobacillus* species, which are part of the oral microbiome [22]. These may reflect different stages of the caries process and reveal changes in the oral microbiota [15]. Although several species are involved in dental caries, *S. mutans* are still strongly associated with the disease; its high colonization in the oral cavity may be associated with the disease, since they are an indicator of microbial disequilibrium [12, 13, 24]. Streptococci, although not the only ones involved in dental caries, are one of the major colonizers of the oral cavity, initiating this colonization soon after tooth eruption [17]. Saliva is a representative medium of the oral microbiota, which may reflect the changes in it, and was chosen because it is an accessible medium [15].

Studies evaluating the use of chlorhexidine for a period of 6 months were insufficient to verify the effect on dental caries, as most do not show any effect on disease control [25]. Vale et al. (2014) [26] evaluated the time of recolonization of *S. mutans* after two consecutive days of treatment with 1% chlorhexidine gel. Saliva was collected before the study and at days 1, 7, 14, 21, and 28 for evaluation of *S. mutans* levels. The levels decreased but were not statistically significant. In this present study, the chlorhexidine varnish reduced the CFU for a period of six months ($p = 0.0107$), but in the last analysis (D360), it was observed that the CFU returned to the same levels of the initial period.

The topical use of fluoride products in high concentrations (>2,500 ppm) creates fluoride reservoirs, providing fluoride to the dental surface and promotes its penetration into the biofilm, being effective in reducing demineralization and increasing remineralization. Fluoride may present bactericidal activity with frequent professional applications. The group treated with fluoride varnish in the present study showed a reduction of CFU throughout the study period ($p < 0.0001$).

The antimicrobial activity of copaiba oilresin may be related to the combination of sesquiterpenes and diterpenes, thus affecting the integrity of the bacterial cell wall. The oil has scientifically proven activity against several pathogens, especially Gram-positive bacteria such as *Staphylococcus* spp. and *Streptococcus* spp. It is important to use a suitable methodology for the dilution of oilresin in research and

validation by gas chromatography [3]. In the present study, copaiba showed a significant reduction of CFU throughout all the periods ($p < 0.0001$). In the intergroup analysis in each period studied, the group treated with copaiba varnish was the only one to show statistically significant results for the two dilutions.

According to Diefenbach et al. (2018) [5], most of the studies which evaluate the antimicrobial activity of copaiba oilresin compared it with chlorhexidine, which is the positive control, where *S. mutans* are the most studied organisms, as well as the other studies with natural products in Dentistry [14].

Pieri et al. (2010) [27] evaluated the action of β-caryophyllene isolated from copaiba oilresin on the adhesion of *S. mutans* bacteria, in which it had better action than chlorhexidine. Pieri et al. (2016) [28] evaluated the antimicrobial activity of β-caryophyllene isolated from copaiba oilresin against dental plaque bacteria in vitro. The results demonstrated that β-caryophyllene prevented plaque-forming bacteria from proliferating.

Dental varnishes stand out in preventing dental caries and are widely accepted by pediatric patients, especially children under 6 years of age. Patients in this age group do not have adequate capacity to eject saliva, so the varnishes were the chosen formulations for the use of copaiba oilresin [29, 30]. They are composed of polymer matrices, excipients, and active principle. In the case of the present varnish, the chosen matrix was insoluble (in this case, ethylcellulose), used to modulate the release of the active principle, and thus, its substantivity was higher [21, 23]. This type of formulation adheres to dental scars and fissures, gradually releasing the active principle, disrupting the dental biofilm, and becoming a long-term therapeutic agent which is suitable for antimicrobial formulation [29, 31].

Most of the randomized clinical trials with outcomes in dental caries are currently focused on the performance of restorative materials and with many biases in the sample. In the current phase of minimally invasive dentistry, studies with materials and preventive alternatives are important [32].

In the pilot study of copaiba dental varnish, all oilresin concentrations showed antimicrobial activity; however, only 1% showed a reduction in *S. mutans* colony forming units [23]. It is believed that the greater complexity of the chemical constituents present in copaiba, a pharmaceutical form with lower concentration, presents a smaller interaction between the pharmaceutical excipients used in the formulation. In addition, the active ingredients of copaiba were probably retained in the varnish matrix and were released locally. It was also observed that higher concentrations of copaiba oilresin lost the ability to retain its active principle, and in these situations, the active principle was released so quickly that the varnish partially lost its antimicrobial activity.

For clinical applications, copaiba and fluoride varnishes showed similar results in preventing dental caries. However, it is important to consider that it is a study with small sample and only one year of follow-up was considered.

5. Conclusions

After three annual applications, copaiba varnish demonstrated significant antimicrobial activity against *S. mutans* for up to 12 months in children with high risk of caries. The fluoride and copaiba varnishes had good results regarding dental caries prevention. Future studies are needed to identify anticaries effects to establish the use of varnish in caries prevention.

Data Availability

The data used to support the findings of this study are available at the repository http://repositorio.ufc.br/bitstream/riufc/44474/1/2019_tese_larvmarques.pdf.

Disclosure

The data are part of Ph.D thesis of the main author, Federal University of Ceara.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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