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Effect of Herbal Toothpaste Without Fluoride Against *Candida Albicans* and *Streptococcus Mutans*: An In Vitro Study

Natrapee Akarathanakrit, M.Sc., Nattapon Rotpenpian, DDS, Ph.D.,
Suwanna Jitpukdeebodintra, DDS, Ph.D.

Department of Oral Biology and Occlusion, Faculty of Dentistry, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand.

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Abstract:

Objective: This study aimed to evaluate the antimicrobial effects of herbal toothpaste without fluoride on *Candida albican*s ATCC 90028 (*C. albicans* ATCC 90028) and *Streptococcus mutans* ATCC 25175 (*S. mutans* ATCC 25175). Despite limitations, such as the discrepancy between *in vitro* conditions and the human oral environment, the findings have provided valuable insights for consumers and dental professionals, guiding informed decisions in toothpaste selection.

Material and Methods: *C. albicans* ATCC 90028 was cultured in Sabouraud's dextrose agar (SDA), and *S. mutans* ATCC 25175 was cultured in brain heart infusion broth (BHI) at 37 °C with 5% CO_2 for 18 hours. The bacteria suspension was adjusted to the turbidity standard according to the 0.5 McFarland standard (1.5×10^8 colony forming unit (CFU)/ml). Twenty-seven commercially available herbal toothpaste formulations were diluted with 2% Dimethyl Sulfoxide at a 1:1 ratio and mixed using a vortex mixer. Antimicrobial sensitivity testing was performed using SDA for *C. albicans* and BHI for *S. mutans*. The results were recorded by measuring the diameter of the clear zone. The minimum inhibitory concentration (MIC) was determined using a 96-well plate with resazurin, while the minimum bactericidal concentration (MBC) was assessed by culturing on agar and counting colony forming units. Biofilm formation was analyzed using crystal violet staining and the optical density measurement at 560 nm. All tests were performed in triplicate.

Results: All 27 herbal toothpaste formulations tested at 1 g/ml effectively inhibited and killed *C. albicans* ATCC 90028 and *S. mutans* ATCC 25175 in the MIC and MBC tests. In the antimicrobial sensitivity testing, 5 formulations (A005, A018, A025, A004, and A023) showed notable inhibition zones against *C. albicans*, with A005, A018, and A025 demonstrating superior antifungal activity. The biofilm formation inhibition assay indicated that 15 formulations completely inhibited biofilm formation. All formulations successfully inhibited acid production by *S. mutans*. In patient saliva tests, most herbal

Contact: Suwanna Jitpukdeebodintra, DDS, Ph.D.
Department of Oral Biology and Occlusion, Faculty of Dentistry,
Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand.
E-mail: suwanna.ji@psu.ac.th

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Fluoride-free formulations effectively inhibited *S. mutans* growth, while some demonstrated reduced antibacterial and antifungal activity. Formulations A018 and A025 showed the highest overall efficacy.

Conclusion: The study confirms the antifungal potential of herbal toothpaste formulations against *C. albicans* ATCC 90028 and *S. mutans* ATCC 25175. Some formulations were more effective than others, highlighting the need for further investigation into their active ingredients and mechanisms of action. The ability to inhibit biofilm formation is particularly valuable in preventing persistent infections. These findings support the potential use of herbal toothpaste as a preventive measure against *C. albicans* and *S. mutans*-related oral infections.

Keywords: *Candida albicans,* fluoride, herbal toothpaste, *Streptococcus mutans*

Introduction

Dental caries and oral candidiasis are major global public health issues that significantly[1] impact quality of life and work productivity, resulting in substantial medical costs. These conditions are primarily caused by disruptions in the oral microbial ecosystem[2]. Identified risk factors include frequent consumption of sugar-rich foods or beverages, tobacco use, and poor oral hygiene practices, such as inadequate brushing and infrequent scaling[3].

Toothpaste is a key oral hygiene product used to remove plaque and food debris while inhibiting microbial growth. Its essential components typically include fluoride, antimicrobial agents, and whitening agents[4]. In recent years, a growing number of studies have examined the incorporation of herbal ingredients into toothpaste formulations. Various herbs—such as licorice (*Glycyrrhiza glabra*), aloe vera (*Aloe vera*), clove (*Syzygium aromaticum*), mangosteen peel (*Garcinia mangostana*)[5], and peppermint oil (*Mentha piperita*)—have demonstrated antimicrobial properties, plaque reduction, and other oral health benefits[6]. Although international research has reported the antimicrobial efficacy of herbal toothpaste, studies conducted in Thailand remain limited. Meanwhile, manufacturers continue to introduce new formulations to the market[7]. According to the Thai Industrial Standard (TIS 41-2562), herbal toothpaste must be free from fluoride and synthetic coloring agents, which may influence its antimicrobial performance[8].

Given the importance of maintaining the oral microbial balance and the widespread use of toothpaste in daily oral hygiene, this study was conducted to evaluate the antimicrobial properties of herbal toothpastes without fluoride available in Thailand. The investigation specifically focused on *Candida albicans* (*C. albicans*) and *Streptococcus mutans* (*S. mutans*), 2 key pathogens associated with oral diseases. The findings aim to provide fundamental data to assist consumers in making informed product choices and to support dental professionals in offering targeted recommendations to patients[9-15].

This study was conducted in vitro and is subject to certain limitations, including the inability to fully replicate the complexity of the oral environment and potential variability in microbial inhibition due to biofilm formation. These factors are further discussed in the full research report.

Material and Methods

Ethics consideration

The study protocol and informed consent were approved by the Ethics Committee of the Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand (EC6702-008).

Microorganisms

The microorganisms used in this study included *C. albicans* ATCC 90028 and *S. mutans* ATCC 25175.

Culture preparation

Standard cultures of *C. albicans* ATCC 90028 and *S. mutans* ATCC 25175 were obtained from the Faculty of Dentistry laboratory, Prince of Songkla University. Each strain was cultivated in 50 ml culture flasks containing 30 ml of the appropriate broth media, Sabouraud's Dextrose Broth for *C. albicans* and brain heart infusion broth (BHI) for *S. mutans*. The cultures were incubated at 37 °C in a 5% CO_2 atmosphere for 18–24 hours. After incubation, the turbidity of each culture was adjusted to the 0.5 McFarland standard,[16] equivalent to approximately 1.5×10^8 CFU/ml[17]. Turbidity adjustment was performed using a spectrophotometer at a wavelength of 600 nm. The adjusted suspensions were prepared in BHI for *S. mutans* and Sabouraud dextrose agar (SDA) for *C. albicans* to ensure consistency and reproducibility in subsequent antimicrobial testing.

Toothpaste preparation

Twenty-seven herbal toothpaste formulations (Table 1) were diluted with 2% Dimethyl sulfoxide (DMSO) by mixing 1 g of toothpaste and 1 ml of DMSO in a ratio of 1:1. The mixture was homogenized using a vortex mixer for 5 min[18].

Table 1 The 27 toothpaste formulas studied

No	Types of toothpaste
A001	Twin lotus herbal toothpaste original formula
A002	Twin lotus herbal toothpaste everfresh formula
A003	Goodgarnic herbal toothpaste
A004	Threeherb herbal toothpaste
A005	Sparkle triple white toothpaste
A006	Sparkle double white toothpaste lemon soda
A007	Sparkle white toothpaste
A008	Sparkle naturals himalaya pink salt toothpaste
A009	Sparkle natural complete care toothpaste
A010	Twin lotus herbalist active charcoal toothpaste
A011	Doctor V whitening system toothpaste-diavee 01
A012	I-Vish gentle powerful whitening and anti-cavity toothpaste
A013	Toothpaste wisut pure extra gum protection & gentle powerful whitening toothpaste
A014	MYSEPTIC MYBACIN® toothpaste with andrographis paniculata formula
A015	Denticon Q10 total care toothpaste
A016	Denticon Q10 rose refresh toothpaste
A017	Blooms nature love natural fresh & gum care toothpaste
A018	Herbal den plus neem toothpaste
A019	Veldent extreme awake tooth paste
A020	Thep Thai herbal toothpaste, concentrated formula, spearmint flavor
A021	Thep Thai herbal toothpaste, concentrated formula, mixed fruit flavor
A022	Thep Thai Herbal toothpaste, concentrated formula, original flavor
A023	Thep Thai Herbal toothpaste, concentrated formula, salt flavor
A024	Dentiste toothpaste original formula
A025	Dentamate herbals toothpaste
A026	Colgate total advanced fresh toothpaste
A027	Colgate total 12 professional clean toothpaste

Antimicrobial sensitivity testing of each toothpaste type

The antimicrobial activity test was conducted using 2 fluoride-containing toothpaste formulations as positive controls: Colgate total advanced fresh and Colgate total 12 professional clean, both of which are known for their antimicrobial properties. Sterile distilled water was used as the negative control.

Culture media, including BHI agar (BHIA), were used for *S. Mutans,* and SDA was used for *C. albicans*. Using a sterile biopsy punch, 4 wells with a diameter of 9 mm were punched into each agar plate at equal distances from one another. Separate plates were prepared for both the positive and negative controls. To inoculate the plates, a sterile cotton swab was used to evenly spread microbial suspensions that had been adjusted to the 0.5 McFarland turbidity standard across the surface of the agar. This process was repeated 3 times for consistency. After allowing the plates to dry for 5 minutes, 50 μL of each toothpaste sample was dispensed into the wells.

All plates were incubated in a 5% CO_2 incubator at 37 °C for 24 hours. Following incubation, the diameter of the inhibition zones around each well (indicating bacterial or fungal growth inhibition) was measured using a millimeter-scale ruler. All measurements were conducted by a single researcher to ensure consistency, and each measurement was performed 3 times to confirm reliability and repeatability.

Minimum inhibition concentration (MIC) testing

The first step was to transfer 100 μl of culture medium into the wells containing 100 μl of 1:1 toothpaste in a 96-well plate. Sterile distilled water was used as a negative control. Chlorhexidine (CHX) was used as a positive control. The toothpaste was mixed with the culture medium as a control for each toothpaste because each toothpaste has a different color. After that, the wells were mixed thoroughly and incubated at 37 °C for 24 hours. Then 30 μl of 0.015% resazurin was added. The result was recorded after 2-4 hours of incubation by observing the change in the color of the resazurin solution. The concentration indicated is the MIC value. The color of the resazurin solution will not change from the original color (blue) when there has not been any organism growth. However, the original color will not be read as the MIC value if the color changes from the original color, indicating that the organism has grown. The experiment was repeated 3 times[20].

Minimum bactericidal concentration (MBC) testing

After obtaining the MIC value, the wells with minimum and maximum concentrations were spread so as to inhibit *C. albicans* onto SDA and *S. mutans* onto BHIA. After that, the concentrations were incubated at 37 °C for 48 hours. Then, the number of colonies growing on the medium was counted and calculated in CFU/ml. The number of bacteria that decreased by more than or equal to 3 log (99.9%) was indicated as the MBC value compared to the control[21].

Anti-biofilm formation activity of toothpaste (biofilm formation assay)

First, 100 μl of culture was transferred to wells containing 100 μl of 1:1 toothpaste in a 96-well plate. Then sterile distilled water was used as a negative control, and CHX was used as a positive control. Next, the cultures were mixed and incubated at 37 °C with 5% CO_2 for 24 hours. After which, all the cultures in the wells were aspirated, and care was taken not to touch the biofilm. The cultures were washed twice with distilled water, 150 μl per well, and 125 μl of 0.1 crystal violet was added to each well. The cultures were left for 15 minutes. Finally, the wells were aspirated again, washed twice with distilled water, 150 μl per well, and 250 μl of 90% ethanol was added. The optical density (OD) of the cultures was measured at 560 nm. The experiment was repeated 3 times.

Inhibition of the acid production test for the toothpaste

First, the cultures were centrifuged at 8000 rpm at 4 °C for 30 min. Then the cells were washed twice with potassium chloride and homogenized using a vortex mixer. Next, 40 mg/ml of potassium chloride was added, and the cultures were incubated at 37 °C with 5% CO_2 for 3 hours. After that, the prepared toothpaste with a concentration of 1:1 was adjusted to achieve the exact pH with potassium chloride (concentration of 0.02 mg/ml). Next, 500 μl of 10% glucose was added and incubated at 37 °C with 5% carbon dioxide for 0, 30, 60, 90, 120, and 150 min. Lastly, the pH was measured at the specified incubation times, and each measurement was repeated 3 times[22].

Testing the effectiveness of toothpaste in killing bacteria

Saliva samples were collected from patients and mixed with 1 g of toothpaste in test tubes containing 900 µl of culture and incubated at 37 °C with 5% CO_2. Bacterial growth was then assessed for 2 min by aspirating 100 µl of culture and quantifying the bacterial count by serial dilution. In the control group, phosphate-buffered saline solution was used instead of toothpaste, and the number of colonies formed in the plates was counted. The experiments were repeated 3 times[23].

Statistical analysis

The data from all experimental results were analyzed and presented using inferential statistics, expressed as mean±standard deviation (Mean±S.D.). Differences between the groups were evaluated using one-way ANOVA, followed by post-hoc comparisons with Tukey's test to determine statistically significant differences among the tested groups.

Results

MIC and MBC

All 27 herbal toothpaste formulations, at a concentration of 1 g/ml, effectively inhibited and killed *C. albicans* and *S. mutans.* No color change was observed, indicating microbial inhibition, and no microbial growth was detected, confirming both bactericidal and fungicidal activity across all formulations.

Antimicrobial sensitivity testing

The findings collectively identified specific toothpaste formulations exhibiting strong antimicrobial activity. For *C. albicans*, 5 formulations A005, A018, A025, A004, and A023 demonstrated notable inhibitory effects. Statistically significant differences were observed among these, with formulations A005, A018, and A025 showing significantly greater inhibition than A004 and A023 (p-value<0.05).

In the case of *S. mutans*, 5 formulations—A023, A018, A021, A027, and A002—also exhibited notable inhibitory effects. However, no statistically significant differences were found among these formulations (p-value>0.05).

Notably, formulations A018 and A023 demonstrated consistent antimicrobial efficacy against both *C. albicans* and *S. mutans*, suggesting their potential as broad-spectrum antimicrobial agents in toothpaste formulations, as shown in Table 2.

Biofilm formation inhibition assay

All toothpaste formulations showed the ability to inhibit biofilm formation of *C. albicans* when compared to the negative control. Notably, 15 out of the 27 formulations completely inhibited biofilm formation, suggesting strong anti-biofilm activity in more than half of the tested samples. These results are presented in Figure 1.

Inhibition of acid production

All 27 herbal toothpaste formulations were able to inhibit acid production by *S. mutans*, as evidenced by the maintenance of a neutral pH throughout the incubation period. The positive control, CHX, also maintained a pH range of 7.11–7.20, consistent with its known efficacy. These results are presented in Figure 2.

Effectiveness in killing bacteria in patient saliva

When tested directly in patient saliva, most herbal fluoride-free toothpaste samples effectively inhibited the growth of *S. mutans*, with the majority showing no bacterial growth. However, formulas A005, A015, A016, and A024 exhibited moderate growth ("++"), while A007 and A023 showed heavy growth ("+++"), indicating reduced antibacterial efficacy.

For *C. albicans*, most toothpaste samples again showed no growth, suggesting good antifungal properties.

However, samples A005, A006, A015, and A024 exhibited mild to moderate growth (“+” or “++”), and sample A023 showed heavy fungal growth (“+++”). This indicates a reduced ability of these formulas to inhibit fungal growth.

Overall, toothpaste formulations A005, A007, A015, A023, and A024 demonstrated relatively low effectiveness against both *S. mutans* and *C. albicans*, whereas formulations such as A018 and A025 consistently showed high efficacy across multiple assays.

Table 2 The Inhibitory Effectiveness of 27 Types of Toothpaste Against *S. mutans* and *C. albicans*

Herbal toothpaste	Median CA	Median SM
A001 Twin lotus herbal toothpaste original formula	15.5	35.5
A002 Twin lotus herbal toothpaste everfresh formula	15.25	35.75
A003 Goodgarnic herbal toothpaste	11.5	32.0
A004 Threeherb herbal toothpaste	18	33.5
A005 Sparkle triple white toothpaste	26	0.00
A006 Sparkle double white toothpaste lemon soda	13.25	26.5
A007 Sparkle white toothpaste	14.25	29.5
A008 Sparkle naturals himalaya pink salt toothpaste	12	29.5
A009 Sparkle natural complete care toothpaste	17.25	0.00
A010 Twin lotus herbalist active charcoal toothpaste	0	33.25
A011 Doctor V whitening system toothpaste - diavee 01	15.5	25.50
A012 I-Vish gentle powerful whitening and anti-cavity toothpaste	0	20.25
A013 Toothpaste wisut pure extra gum protection & gentle powerful whitening toothpaste	0	33.25
A014 MYSEPTIC MYBACIN® paniculata formula	11.5	33.25
A015 Denticon Q10 total care toothpaste	0	31.75
A016 Denticon Q10 rose refresh toothpaste	16	33.00
A017 Blooms nature love natural fresh & gum care tooth paste	16.5	37.00
A018 Herbal Den plus neem toothpaste	23.75	32.00
A019 Veldent extreme awake tooth paste	17.75	35.50
A020 Thep Thai herbal toothpaste concentrated formula spearmint flavor	16.25	37.25
A021 Thep Thai herbal toothpaste, concentrated formula, mixed fruit flavor	12.5	37.00
A022 Thep Thai herbal toothpaste, concentrated formula, original flavor	13	40.50
A023 Thep Thai herbal toothpaste, concentrated formula, salt flavor	15.25	29.75
A024 Dentiste toothpaste original formula	12.5	30.00
A025 Dentamate herbals toothpaste	22.5	33.50
A026 Colgate total advanced fresh toothpaste	0	36.50
A027 Colgate total 12 professional clean toothpaste	0	33.75

CA=Candida albicans, SM=Streptococcus mutans

Table 3 Results of testing the effectiveness of toothpaste in killing bacteria in patient saliva

No. sample	Pure saliva on BHI	Pure saliva on SDA	Toothpaste	Saliva+toothpaste on BHI	Saliva+toothpaste on SDA
1	+++	+++	A001	-	-
2	+++	+++	A002	-	-
3	+++	+++	A003	-	-
4	+++	+++	A004	-	-
5	+++	+++	A005	++	+

Table 3 (Continued)

No. sample	Pure saliva on BHI	Pure saliva on SDA	Toothpaste	Saliva+toothpaste on BHI	Saliva+toothpaste on SDA
6	+++	+++	A006	–	++
7	+++	+++	A007	+++	+++
8	+++	+++	A008	–	–
9	+++	+++	A009	–	–
10	+++	+++	A010	–	–
11	+++	+++	A011	–	–
12	+++	+++	A012	–	–
13	+++	+++	A013	–	–
14	+++	+++	A014	–	–
15	+++	+++	A015	++	++
16	+++	+++	A016	++	–
17	+++	+++	A017	–	–
18	+++	+++	A018	–	–
19	+++	+++	A019	–	–
20	+++	+++	A020	–	–
21	+++	+++	A021	–	–
22	+++	+++	A022	–	–
23	+++	+++	A023	+++	+++
24	+++	+++	A024	++	++
25	+++	+++	A025	–	–
26	+++	+++	A026	–	–
27	+++	+++	A027	–	–

SDA=sabouraud dextrose agar, BHI=brain heart infusion, +++heavy growth, ++moderate growth, +mild growth, –no growth

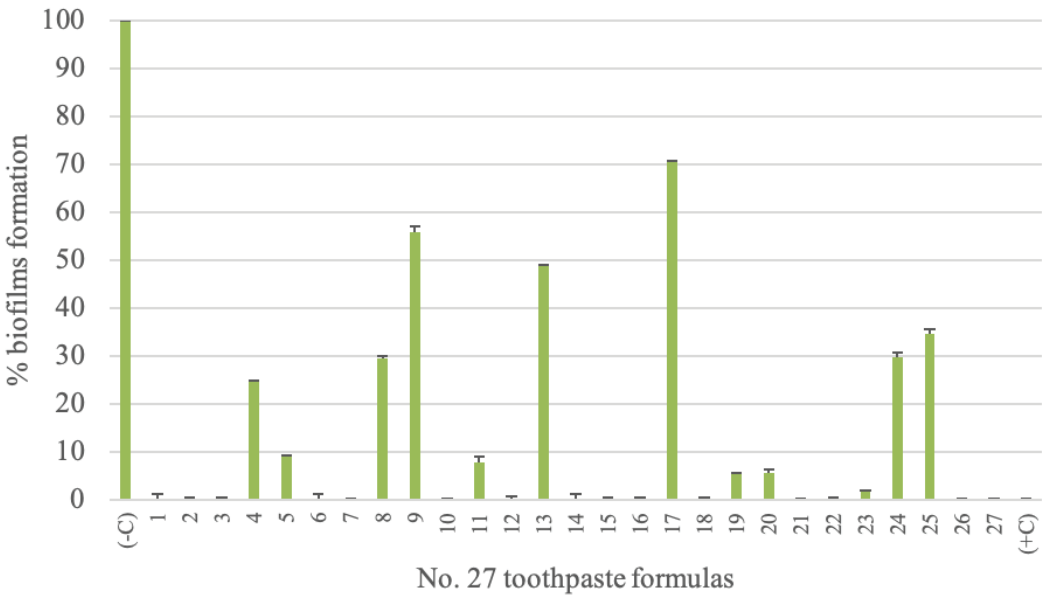
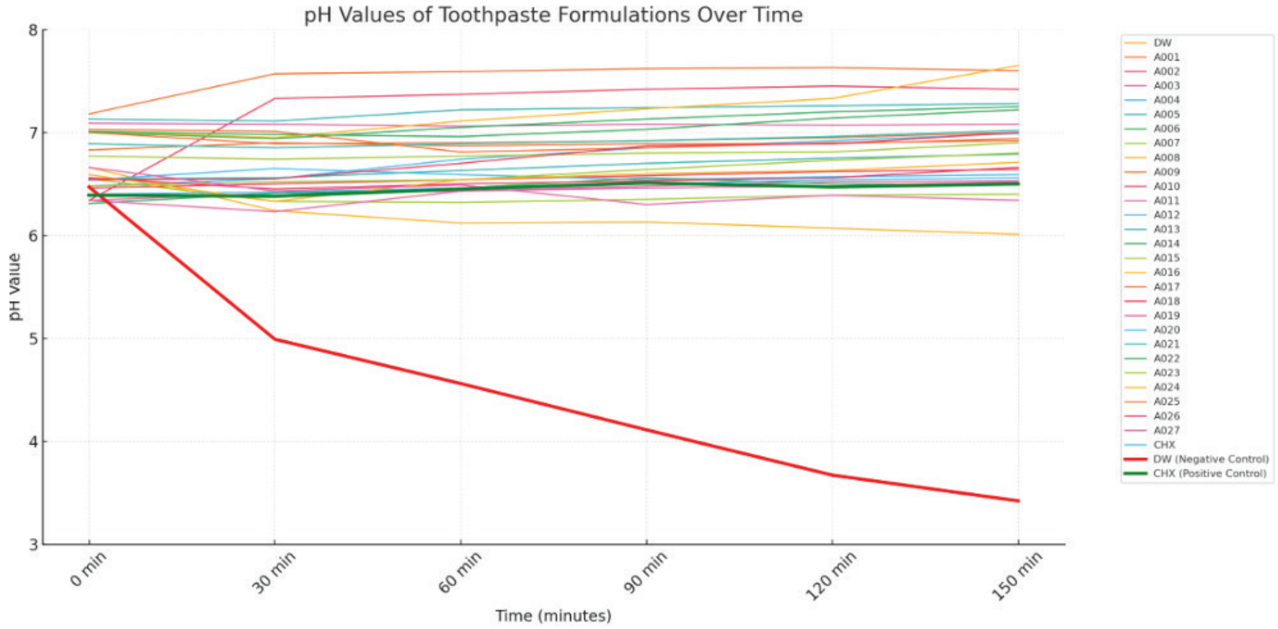


Figure 1 The graph shows % biofilm formation compared to the negative control and positive control



DW=distilled water, CHX=chlorhexidine

Figure 2 The graph shows the reduction of acid production by *S. mutans* in response to 27 different toothpaste formulations

Discussion

This study evaluated the antimicrobial properties of 27 herbal fluoride-free toothpaste formulations against *S. mutans* and *C. albicans* using a comprehensive set of assays. The findings demonstrate that a substantial number of the formulations possessed strong antibacterial and antifungal properties, though varying degrees of effectiveness were observed among different samples.

The MIC and MBC tests confirmed that all toothpaste formulations were capable of inhibiting and killing both *S. mutans* and *C. albicans* at a concentration of 1 g/ml. The absence of color change in the resazurin-based MIC assay and the lack of microbial growth in the MBC assay indicate that the active components in these formulations are both bacteriostatic and bactericidal/fungicidal at this concentration. These outcomes suggest that the toothpaste formulas contain sufficient concentrations of antimicrobial agents, potentially derived from herbal extracts known for their bioactive properties, such as clove oil, tea tree oil, neem, or miswak. The present findings are consistent with previous research, which demonstrated that herbal toothpastes possess significantly stronger antimicrobial and antifungal activities compared to conventional formulations and negative controls[16]. These results reinforce the potential of herbal toothpaste as an effective alternative in oral microbial management.

In the antimicrobial sensitivity test, formulations A005, A018, A025, A004, and A023 demonstrated distinct zones of inhibition against *C. albicans*, with A018 and A025 showing the most pronounced effects. These results may be attributed to differences in the types or concentrations of essential oils or other plant-based compounds, such as phenolics, flavonoids, or alkaloids, known to disrupt fungal cell walls or inhibit enzymatic function. This study is consistent with previous research demonstrating that herbal toothpaste containing guava extract exhibits antibacterial efficacy comparable to that of commercial toothpastes, as evaluated by the well-diffusion method[24].

More than half of the tested toothpaste samples completely inhibited *C. albicans* biofilm formation, which is a critical virulence factor in persistent infections. The strong anti-biofilm activity of certain formulations could be due to the presence of compounds that interfere with cell adhesion or quorum sensing mechanisms, which are essential for biofilm maturation.

The acid production assay further supported the antimicrobial efficacy of the toothpaste formulations. The maintenance of near-neutral pH across all samples over a 150-minute period suggests effective inhibition of acidogenic activity by *S. mutans*. This is particularly significant for caries prevention, as acid production by *S. mutans* is a key contributor to tooth enamel demineralization. The comparable performance of several formulations to CHX (positive control) highlights their potential as natural alternatives for controlling acidogenic oral bacteria.

Direct testing in patient saliva revealed that while most formulations effectively suppressed microbial growth, a few, specifically A005, A007, A015, A023, and A024, exhibited reduced effectiveness. This inconsistency might be due to lower concentrations of active antimicrobial ingredients, formulation instability, or interactions with salivary proteins and enzymes that reduce their efficacy in real biological conditions.

In contrast, A018 and A025 consistently demonstrated superior performance across all assays, indicating that these formulations may possess a well-balanced and potent combination of antimicrobial agents with broad-spectrum activity. The ingredients in these toothpastes warrant further phytochemical analysis to identify the compounds responsible for the observed antimicrobial effects.

Overall, the variation in efficacy among formulations underscores the importance of ingredient selection, concentration, and formulation stability in developing effective herbal toothpaste. These findings support the potential use of certain herbal formulations as alternative or complementary agents in oral hygiene, particularly for individuals seeking options without fluoride.

Conclusion

This study demonstrated that most fluoride-free herbal toothpaste formulations possess effective antimicrobial properties against *S. mutans* and *C. albicans*. All 27 samples were capable of inhibiting microbial growth, killing pathogens, and suppressing acid production and biofilm formation. Notably, formulas A018 and A025 exhibited consistently superior performance across all assays. In contrast, formulas A005, A007, A015, A023, and A024 showed reduced efficacy. These findings highlight the potential of certain herbal toothpaste formulations as effective alternatives for oral hygiene, especially in fluoride-sensitive populations.

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Conflict of interest

The authors have no conflict of interest.

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