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Clinical Comparison of Sage Extract and Chlorohexidine on Glucosyltransferase Gene Expression in High Caries Risk Patient: A Randomized Clinical PCR-Based Trial

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Abstract

Chlorhexidine (CHX) is widely used in caries prevention but is associated with adverse effects and potential antimicrobial resistance. Sage (*Salvia officinalis*) extract, rich in bioactive polyphenols, offers a potential herbal alternative. The study was aimed to compare the effects of sage extract and CHX mouthwashes on *Streptococcus mutans* glucosyltransferase (gtfB and gtfD) gene expression in high caries-risk patients. In this randomized, double-blind, controlled clinical trial (NCT06703554), 42 high caries-risk adults (DMFT >6) were randomized to receive either CHX or sage extract mouthwash twice daily for 4 weeks. Salivary samples were collected at baseline, 2 weeks, and 4 weeks. Gene expression of gtfB and gtfD was analyzed using real-time PCR (ΔCt and 2^–ΔΔCt). Data were analyzed via paired and independent t-tests, and repeated measures ANOVA, with effect sizes (η^2, Cohen's d) reported. Sage extract achieved significantly greater downregulation of gtfB and gtfD genes than CHX, particularly at week 2 (gtfB: +35.1%, $p=0.023$; gtfD: +51.0%, $p<0.001$). At 4 weeks, sage maintained 100% gtfD downregulation versus 71.4% in the CHX group. Repeated measures ANOVA confirmed significant main effects for time and treatment ($p<0.05$, $\eta^2=0.145–0.594$). Sage-treated patients reported no adverse effects, while 38.1% of CHX users developed tooth staining.

Sage extract mouthwash demonstrated superior efficacy and safety in downregulating cariogenic gene expression compared to CHX. As a natural, biocompatible agent, sage holds promise as a viable alternative to synthetic antimicrobials for caries prevention. Larger, long-term studies are warranted.

Keywords: Sage extract, *Streptococcus mutans*, Glucosyltransferase, Caries risk, Chlorhexidine, Gene expression, PCR

I. Introduction

Dental caries is a microbial disease caused by a disparity in the biofilm of the oral flora. Streptococcus mutans (S. mutans) has been identified as a significant microbial etiological agent in the development of dental caries. [1] It possesses several virulence factors that contribute to cariogenicity. *S. mutans* are highly acidogenic and aciduric; they produce organic acids after fermenting carbohydrates, and the subsequent decrease in environmental pH is responsible for the demineralization of the tooth surface and the formation of dental caries. [2] *S. mutans* glucosyltransferases (Gtfs) constitute key virulence factors. These enzymes, encoded by three genes (gtfB, gtfC, and gtfD), utilize sugars to synthesize glucans, thereby facilitating bacterial cell attachment and the formation of biofilms. [3] Given the crucial roles that GTFs play, their intervention has attracted significant interest compared to other approaches because GTF inhibitors may potentially disrupt pathological processes. Consequently, targeting GTFs impairs the virulence of S. mutans and inhibits the formation of cariogenic biofilms. [4]

Antimicrobial agents have been recognized as one of the most effective strategies for preventing dental caries for decades. These agents can hinder bacteria from adhering to one another and colonizing, which stops bacterial growth. [5] One of the successful antimicrobial agents is chlorhexidine (CHX), which has antimicrobial activity against S. mutans and antiplaque properties. However, the use of CHX has some hazards, such as staining, altered taste sensation, vomiting, and the development of resistant bacterial strains. [6] Meanwhile, natural products have been proposed as innovative therapeutic agents against dental caries due to their availability, low toxicity, lack of microbial resistance, and cost-effectiveness. [5]

Among these natural products is sage extract, which has a broad spectrum of therapeutic activities, including antibacterial, antiviral,

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antifungal, and antioxidant effects resulting from the action of various chemical compounds present in the plant, including triterpenes, flavonoids, and phenolic acids. [7] The primary goal in preventing dental caries is inhibiting the essential virulence factor (Gtf).

Despite the growing interest in natural antimicrobial agents, significant clinical gaps remain in validating herbal alternatives to chlorhexidine. Long-term CHX use in clinical practice has raised concerns about bacterial resistance development, with studies reporting reduced susceptibility in oral streptococci after prolonged exposure. [8] Additionally, CHX overuse has led to increased incidence of adverse effects, limiting patient compliance and treatment efficacy. [6] From a public health perspective, replacing CHX with effective herbal alternatives could address several critical issues: reducing healthcare costs in resource-limited settings, minimizing adverse effects that compromise patient adherence, and preventing the emergence of antimicrobial resistance. However, no randomized clinical trials have directly compared sage extract with chlorhexidine regarding their effects on *S. mutans* Gtf gene expression in high-risk patients. This represents a critical clinical knowledge gap that limits evidence-based recommendations for herbal antimicrobials in caries prevention protocols.

II. Materials and Methods

Trial Design

A randomized clinical trial with parallel groups and a 1:1 allocation ratio with a superiority trial framework. The unit of randomization and analysis was the patient. The trial protocol was registered prospectively in clinicaltrials.gov with the trial identification number NCT06703554. The study was reported in accordance with the updated CONSORT reporting guidelines for randomized clinical trials. [9]

Trial Setting

The trial was conducted in the Conservative Dentistry Department outpatient clinic at the Faculty of Dentistry, Cairo University, Egypt. The Research Ethics Committee approved the clinical trial with the ethical approval number 53/7/24. All the eligible patients signed a written informed consent and received a detailed explanation of the trial and its objectives before enrollment.

Eligibility Criteria

Patients were recruited from the outpatient clinic of the Faculty of Dentistry at Cairo University, Egypt, based on the following inclusion criteria: participants aged 20 to 50 years, male or female, medically healthy, and high caries patients with a DMFT (D=decay, M=missing, FT=filled teeth) score greater than 6. To classify subjects as having a high risk of caries, a dental examination was conducted to assess DMFT, and a caries risk assessment was performed using the American Dental Association (ADA) guidelines. The exclusion criteria eliminated participants who were on a mouthwash regimen, those who were medically compromised (such as people with diabetes, individuals with neurovascular issues, patients who had undergone radiation therapy, and those with salivary gland disorders), as well as pregnant women. Additionally, subjects who used any drug that reduces salivary flow, were on antibiotics, had allergies to any chemicals involved in this study, or were smokers were also excluded.

Intervention and Comparator

This clinical trial employed two types of mouthwashes: a ready-made CHX mouthwash (Hexitol, ADCO, Egypt) and a freshly prepared mouthwash containing natural sage extract. The mouthwashes were stored in identical opaque amber glass bottles to conceal the type of mouthwash from participants and the operator.

Sage oil was sourced from a local herbal market in Egypt. Gas Chromatography/Mass Spectrometry (GC/MS) analysis determined the chemical composition and purity of the sage essential oil. Before mouthwash preparation, the Minimum Inhibitory Concentration (MIC) and Bactericidal Concentration (MBC) tests were performed in vitro against Streptococcus mutans, with results indicating an MIC of 1.56% and an MBC of 3.3%. The mouthwash was produced at Nawah Scientific Lab in Cairo, Egypt. For each 100 ml, 1.56 g of sage oil and 1.80 g of Tween 80 were combined in a glass beaker, with purified water added gradually to create a fine, cloudy dispersion. Sucralose (0.01 g) was mixed until dissolved, and the final volume was adjusted using purified water. The mixture was then transferred to 200 ml amber glass bottles and stored at -20 °C until use.

Participants were randomized to receive either natural sage oil extract or a CHX-containing mouthwash. They were instructed to use the mouthwash twice daily for one month. Written instructions were provided to the participants, and regular follow-up was scheduled through daily phone calls. Participants record daily mouthwash usage in logs, noting frequency, duration, and any deviations. Saliva samples were collected from each participant at baseline, two weeks, and one month. Saliva collection was done in the early morning on the same day of collection, and participants were informed to stop eating and drinking (except water) before saliva collection to minimize possible food debris and saliva

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stimulation. They were instructed to stop using any antimicrobial agents after the intervention. The participants sat upright in the dental chair and were given a paraffin block to chew for two minutes to stimulate salivation. Then, they were asked to spit into sterile containers held near the mouth by the operator. The containers were labeled with the participant's number (the same number as on the envelope and bottle), and all tubes were marked with the equivalent number using a waterproof marker, along with a corresponding letter representing each visit. For example, if the participant's baseline saliva sample is designated as 1, subsequent samples, such as those from the first and second visits, would be marked as 1a and 1b. [10]

Log return dates, participant numbers, and bottle conditions were documented in a secure database. A compliance assessment was done by measuring bottle weights before and after use, quantifying the remaining volume, and comparing these values with the documented usage data [11] To enhance adherence, daily phone reminders were used throughout the study period. To ensure sample integrity, salivary samples were transported in an icebox within 30 minutes of collection to the national research center.

Saliva samples were first centrifuged at 5,000 ×g for 10 minutes to separate cellular material. The supernatants were carefully discarded, and the resulting pellets were resuspended in 1 mL of phosphate-buffered saline (PBS). A second centrifugation step was performed at 18,000 × g for 5 minutes to further isolate the pellets. After removal of the supernatants, the pellets were stored at −80°C to minimize bacterial growth and preserve DNA for molecular analysis. [12] Each visit, participants provided feedback on the oral rinses they received. Every pragmatic response was recorded. [13]

Gtfs Gene Expression

Salivary samples were centrifuged at 5000×g for 10 min, to remove any debris, and inhibitor removal was used (ZYMO RESEARCH RNA/DNA Shield). DNA was isolated from all the samples using a Quick-DNA™ Miniprep Plus Kit (ZYMO RESEARCH, CALIFORNIA, CA, USA). The protocol was performed according to the manufacturer's instructions. After homogenization, saliva samples were defrosted and re-suspended by agitation in a vortex (Assistant Reamix 2789 Vortex Mixer, Medical Trade Center, Hamburg, Germany) for 30 seconds. This was followed by mechanical and chemical lysis in which 200 µL of saliva was added to a microcentrifuge tube. Then, 200 µL of biofluid and cell buffer solution were added for cell lysis. After cell lysis, 20 µL of Proteinase K was added, and the lysates were incubated at 55 °C for 10 minutes. Afterward, an equal volume of Genomic Binding buffer was added, so a total of 420 µl genomic binding buffer was added to the 420 µl digested sample and then mixed thoroughly. Subsequently, the mixture was transferred to a Zymo-spin ™ IIC-XL Column in a collection tube. Then, 400 µl DNA Pre-Wash Buffer was added to the column in a new collection tube and centrifuged for 1 minute. The collection tube and 700 µl of g-DNA Wash Buffer were added and centrifuged for 1 minute, and again 200 µl of g-DNA Wash Buffer was added and centrifuged for 1 minute, and then the collection tube with the flow-through was discarded. Finally, the DNA was eluted by transferring it to a clean microcentrifuge tube and adding 50 µL of DNA Elution Buffer. The mixture was then incubated for 5 minutes and centrifuged for 1 minute.

This clinical trial utilized a DTlite real-time PCR device (DNA-Technology, Research, and Production, Moscow, Russia) to analyze the expression of the gtfB and gtfD genes, employing the housekeeping gene 16S rRNA as an internal control. A primer set suitable for real-time PCR was selected for *S. Mutans* to compare the abundance of this genus of bacteria in salivary samples. The oligonucleotide primers used in this study were the *S. mutans* forward primer and reverse primer, which target 130 bp of the 16s *rRNA* gene in most species of Streptococci, as shown in Table 1.

Table 1: The oligonucleotide primers used in the study

Gene	Primer direction	Sequence 5'- 3'	PCR product length	Tm	GC%
S. mutans	Forward	GCACTCGCTACTATTTCTTACAA	23	74	42%
	Reverse	GTCACAATGTCTTGGAAACCAGTAAT	26	72	38%
Gtf B	Forward	ACTACACTTTCGGGTGGCTTGG	23	68	55%
	Reverse	CAGTATAAGCGCCAGTTTCATC	23	64	45%
Gtf D	Forward	GGCACCACAACATTGGGAAGCTCAGT	26	80	54%
	Reverse	GGAATGGCCGCTAAGTCAACAGGAT	25	76	52%

16S rRNA	Forward Reverse	GTGCCAGCMGCCGCGGTAA GGACTACHVGGGTWTCTAT			

Tm: Primer Melting Temperature, GC% %: Percentage of Nitrogenous bases in a DNA.

DNA extract of a randomly selected control sample was used as a calibrator (reference sample) in all the experiments. Two separate replicas of the assay were performed for all the experiments. To enable relative quantitation (RQ) analysis, average amounts of S. mutans threshold cycle (CT) in each sample were compared with the CT of Streptococci in the calibrator sample. The real-time PCR for relative quantification of the target bacterial genus was performed in a total volume of 20 μL using 10 μL SYBR® Premix Ex TaqTM master mix (TaKaRa, Japan), 0.4 μL of each forward and reverse primer, 0.08 μL diluted ROX, and 2 μL of DNA template under following conditions: initial denaturation at 95°C for 30 seconds, and 35 cycle repeats of denaturation at 95°C for 5 seconds, annealing at 62°C for 34 seconds, and extension at 60°C for 34 seconds. The reaction was ended after final extension at 75°C for 15 seconds. Accordingly, at the end step of the amplification, the reaction was continued by heating at 95°C for 15 seconds, a temperature gradient of 60 -95°C for 1 minute, and 95°C for 15 seconds During each cycle, SYBR® Green fluorescence was used to detect the findings, which were then shown as amplification curves for each gene. Melting curves were used to verify test specificity and identify any non-specific amplification, as shown in Figures 1 and 2.

Outcomes
The primary outcome of this trial was regulating the expression of Gtfs B and D bacterial genes measured by real-time PCR after two weeks (T1) and after four weeks (T2).

Harms
The principal investigator informed the participants about potential side effects, including allergies, altered taste, swelling, or increased blood pressure. If these occurred, participants were instructed to contact the operator by phone to receive treatment and were advised to discontinue use of the mouthwash immediately. Any potential side effects were documented and reported.

Sample size
A pilot study was executed to calculate the appropriate sample size for gene regulation where (n=10) per group using G*Power software (Version 3.1.9.7, Heinrich Heine University Düsseldorf, Germany). The effect size was 0.476, significance level (α) set at 0.05 and the power (1-β) set at 0.80, the analysis indicated a total sample size of 38 specimens. To account for any potential sample loss due to technical errors, dropouts, or RNA degradation during processing, an additional 10% of samples were added to the minimum required sample size, hence total sample size was increased to 42 distributed into 2 groups of 21 subjects each.

Randomization
The allocation sequence was generated by a computer-based random number generator using simple randomization and a 1:1 allocation ratio (www.random.org). Allocation concealment was conducted using sequentially numbered, identical, opaque amber glass bottles containing either of the mouthwashes. The allocation sequence generation and concealment were done by an independent researcher not involved in the enrollment or outcome assessment.

Blinding
Participants, the principal investigator, and the outcome assessor were blinded to the type of intervention. To facilitate blinding, mouthwashes were kept in identical opaque amber glass bottles.

Statistical Methods
Data was analyzed using IBM SPSS Statistics software (version 22.0; IBM Corp., Armonk, NY, USA). Statistical significance was set at $p < 0.05$. Statistical analyses were performed using ΔCt values. However, biological interpretation was based on fold change ($2^{-\Delta\Delta Ct}$). The normality of variables was assessed using the Shapiro–Wilk test. Data were normally distributed and thus presented as mean ± standard deviation (SD). Paired-sample t-tests were performed separately for each gene (gtfB and gtfD) to assess within-group changes over time points. Independent samples t-tests were used to compare between-group differences at each time point. A two-way repeated measures ANOVA was conducted to examine the main effects of time, treatment group, and the interaction effect between variables, for each gene. Effect sizes were reported using partial eta squared (η^2) for ANOVA results and Cohen's d for t-test comparisons. For visualization, fold-changes were expressed in median (interquartile range) and percentages. Fold change thresholds were

used to define expression categories: Upregulation (≥2), Downregulation (≤0.5), and Neutral (0.5 < FC < 2), based on accepted biological standards. [14]

III. Results

Forty-two patients were randomized into two groups to receive a mouthwash containing CHX or Sage extract. All patients completed the follow-up, except one patient who was lost to follow-up upon recall in the CHX group. Figure 3 shows the flow of the patients through the trial. All patients who completed the follow-up returned the bottles, and their weights and volumes were checked and compared to the recorded data. The overall compliance with the interventions was 90%.

Gene Expression

For gtfB gene expression, Repeated measures ANOVA revealed a significant main effect of "Time" (*p*=0.006, partial η^2=0.173), indicating a large effect size. Factor "Treatment Group" showed a significant main effect (p=.004, partial η^2=.189), indicating a large effect size. However, the interaction between variables (Time * Group) revealed a non-significant main effect (*p*=0.698, partial η^2=0.004), indicating a small effect size (Table 2).

Table 2: Repeated measures ANOVA (main effects and interactions) of gtf B.

Source	Type III Sum of Squares	Df	Mean Square	F	*p-value*	Partial Eta Squared
Time	90.938	1	90.938	8.388	0.006*	0.173
Treatment Group	75.810	1	75.810	9.316	0.004*	0.189
Time * Group	1.658	1	1.658	0.153	0.885	0.004
Error(time_2)	433.665	40	10.842			

*: significant (p ≤ 0.05)

For gtfD gene expression, Repeated measures ANOVA revealed a significant main effect of "Time" (*p*=.013, partial η^2=0.145), indicating a large effect size. Factor "Treatment Group" showed a significant main effect (*p*<.001, partial η^2=0.594), indicating a large effect size. However, the interaction between variables (Time * Group) revealed a non-significant main effect (*p*=.885, partial η^2=0.001), indicating a small effect size (Table 3).

Table 3: Repeated measures ANOVA (main effects and interactions) of gtf D.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared
Time	29.762	1	29.762	6.804	0.013*	0.145
Treatment Group	277.223	1	277.223	58.590	<0.001*	0.594
Time* Group	0.093	1	0.093	0.021	0.885	0.001
Error(time_2)	174.975	40	4.374			

*: significant (p ≤ 0.05)

At the CHX treatment group, Table 4 and Figures 4 and 5, T2 showed higher ΔCt compared to T1 on gtfB (*p*=.007). For gtfD, the CHX group showed higher ΔCt at T2 compared to T1 (p = 0.108). Furthermore, the Sage group exhibited higher T2 compared to T1 on both gtfB and gtfD (p=.152 and .057, respectively). At T1, Table 4 and Figures 6 and 7, sage exhibited higher ΔCt values compared to CHX with a statistically significant effect on both gtfB and gtfD (p=.023 and <.001, respectively). Meanwhile, at T2, there is a non-significant difference between sage and CHX on gtfB (p=.108). On gtfD, atT2, there was a statistically significant difference between sage and CHX (p <.001). The effect size as measured by Cohen's d of the CHX group on gtfB was d=1.024, which indicates a significant effect. In contrast, the effect of the sage group on gtfB was d=0.489, indicating a medium effect according to conventional thresholds. On gtfD gene expression, the effect size of CHX is 0.487, indicating a medium effect size, while the sage group effect size is 0.687, which also shows a medium to large effect size according to conventional thresholds.

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Table 4 Mean ±SD of ΔCt for for the effect of time within each group and the effect of treatment group within each time on gtf B and gtf D gene expression.

Gene	Time	Treatment Group	Mean ±SD	95% CI		Paired *p*-value (T1 vs T2)	CHX vs Sage *p*-value
				Lower bound	Upper bound		
gtf B	**T1**	**CHX**	6.22 ±2.39	5.13	7.31	.007*	.023*
		Sage	8.40 ±3.47	6.82	9.98	.152	
	T2	**CHX**	8.59 ±2.24	7.57	9.60		.108
		Sage	10.20 ±3.89	8.43	11.97		
gtf D	**T1**	**CHX**	7.01 ±2.01	6.10	7.93	.108	<.001*
		Sage	10.58 ±1.33	9.97	11.18	.057	
	T2	**CHX**	8.14 ±2.63	6.94	9.33		<.001*
		Sage	11.84 ±2.34	10.77	12.90		

Paired p-value represents paired t-test between T1 and T2 within each group and each gene
Asterisks indicating significance between T1 and T2

CHX vs Sage p-value represents independent t-test between CHX and Sage within each time and each gene
Asterisks indicating significance between CHX and Sage

Gene expression of gtfB and gtfD in all treatment groups at different time points, as shown in Table 5 and Figure 8, tended to downregulate with varying percentages. This indicates that sage extract mouthwash may have induced a consistent downregulation of gtfB and gtfD at both time points.

Table 5: Median fold change (IQR) and %Regulation of group-time at gtfB and gtfD gene expression

Gene	Group-Time	Median fold change (IQR)	% Regulation		
			% downregulated	% neutral	% up regulated
gtfB	**CHX- T1**	0.33 (0.09-1.43)	52.4	33.3	14.3
	CHX- T2	0.12 (0.014-0.26)	100	-	-
	Sage- T1	0.02 (0.0037-0.15)	100	-	-
	Sage- T2	0.0017 (0.0006-0.038)	90.5	9.5	-
gtfD	**CHX- T1**	0.87 (0.58-0.91)	76.2	19	4.8
	CHX- T2	0.12 (0.02-0.68)	71.4	28.6	-
	Sage- T1	0.004 (0.002- 0.010)	100	-	-
	Sage- T2	0.001 (0.0004-0.005)	100	-	-

Fold change >1.5 indicates upregulation; fold change <0.67 indicates downregulation. Values between 0.67 and 1.5 were considered neutral

IV. Discussion

This randomized controlled clinical trial evaluated the comparative efficacy of sage extract versus chlorhexidine mouthwashes in modulating *Streptococcus mutans* glucosyltransferase gene expression among high caries risk patients. The findings demonstrate that sage extract achieves superior and more consistent downregulation of gtfB and gtfD genes compared to chlorhexidine, while offering an improved safety profile and enhanced patient tolerability.

Methodological Considerations and Study Design

The study employed rigorous methodological standards to ensure data authenticity and minimize confounding variables. Saliva collection followed standardized protocols during consistent 8-11 am periods to reduce diurnal variations in bacterial composition and gene expression, adhering to established guidelines for salivary biomarker research. [10] The four-week intervention period was selected based on previous investigations assessing antimicrobial efficacy of herbal mouthwashes, providing sufficient duration to detect meaningful changes in gene expression while minimizing dropout rates. [10] The real-time PCR methodology demonstrated high amplification

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efficiency, validating the sensitivity and accuracy of our molecular approach for quantifying gtf gene expression. [10]

Molecular Mechanisms of Sage Extract Efficacy

Statistical analysis revealed significant main effects for both time and treatment interventions on gtfB and gtfD gene regulation, with sage extract demonstrating superior and more consistent downregulation patterns compared to chlorhexidine (Tables 2 and 3). The superior antimicrobial efficacy of sage extract can be attributed to its complex phytochemical composition, particularly monoterpenoids and polyphenolic compounds, which function as potent antibacterial agents against *S. mutans*. [15,16]

The polyphenolic compounds in sage extract, comprising flavonoids and tannins, exert their inhibitory effects through multiple molecular mechanisms. Flavones and flavonols specifically target glucosyltransferase enzymes through their characteristic double bonds between C-2 and C-3 atoms, creating enhanced nucleophilic addition sites. The aspartic acid side chains (CH_2COOH) of gtf enzymes serve as nucleophiles, reacting with flavones and flavonols to achieve enzyme inhibition. [17,18] Additionally, tannins contribute to enzyme inhibition through redox reactions involving their hydroxyl groups, resulting in oxidation-reduction processes that disrupt gtf enzyme activity by up to 31.93%. [18,19] These polyphenolic compounds effectively denature gtf enzyme proteins, thereby disrupting glucan synthesis and biofilm formation. [20,21]

Comparative Treatment Efficacy and Clinical Outcomes

ΔCt-based analysis demonstrated statistically significant inhibition of gtfB and gtfD gene expression in both treatment groups, with sage extract exhibiting markedly superior performance (Table 4). Fold change analysis and regulatory percentage assessments confirmed robust downregulatory effects of sage mouthwash on both target genes (Table 5), supporting its potential as an effective antimicrobial agent for caries prevention through targeted virulence factor suppression.

While chlorhexidine demonstrated statistically significant downregulatory effects on gtf gene expression, its efficacy remained comparatively inferior to sage extract, as evidenced by quantitative analyses (Figure 8). Chlorhexidine's mechanism involves enzyme inhibition and membrane disruption through interference with lipid-protein interactions, ultimately leading to gtf enzyme denaturation and protein disruption. [19] However, this mechanism appears less consistent and comprehensive compared to the multi-target approach of sage extract.

Safety Profile and Adverse Event Analysis

Clinical tolerability assessments revealed significant differences between treatment groups. Chlorhexidine users reported transient adverse effects including unpleasant taste and nausea during the initial treatment week, though these effects diminished with continued use (Table 6).

Table 6: Summary of Adverse Effects Observed During the Trial

Adverse Effect	CHX group (n=21)	Sage group (n=21)	Severity (Grade ^a)	Causality^b	Action Taken
Tooth staining	8 (38.1%)	0 (0%)	Mild (1)	Definite	Professional Cleaning
Altered taste	5 (23.8%)	0 (0%)	Mild (1)	Probable	Resolved Spontaneously
Transient Nausea	2 (9.5%)	0 (0%)	Mild (1)	Possible	Resolved Spontaneously
Headache	1 (4.8%)	1 (4.8%)	Mild (1)	Unrelated	Supportive Care
Total AEs	15	-	-	-	-

^a Graded per Common Terminology Criteria for Adverse Events (CTCAE v5.0): Mild (1), Moderate (2), Severe (3).
^b Causality assessed using WHO-UMC criteria: Definite/Probable/Possible/Unlikely/Unrelated

Intraoral examinations revealed notable tooth staining in 38.1% of chlorhexidine users by the second follow-up interval, a phenomenon absent in the sage-treated group. Conversely, no adverse effects were reported among sage extract users, suggesting superior biocompatibility and patient acceptance. These findings indicate that sage extract offers comparable or superior therapeutic efficacy without the associated side effects characteristic of chlorhexidine therapy.

Chlorhexidine Resistance and Paradoxical Effects

A clinically significant finding was the paradoxical upregulation of gtfB and gtfD expression observed in some chlorhexidine-treated samples (Table 5). This counterintuitive response raises substantial clinical concerns, as gene upregulation rather than suppression may enhance *S. mutans* virulence through increased glucan production and facilitate bacterial adaptation mechanisms.

Multiple mechanisms may explain this paradoxical chlorhexidine response. Sublethal chlorhexidine concentrations may trigger bacterial stress responses, prompting *S. mutans* to upregulate virulence genes as adaptive survival mechanisms. [22] The 0.12% chlorhexidine concentration employed may represent a suboptimal therapeutic window where incomplete bacterial inhibition permits stress-induced gene expression rather than comprehensive suppression. Additionally, chlorhexidine may demonstrate selective pressure, eliminating susceptible bacterial populations while allowing resistant variants with enhanced gtf expression to proliferate. Finally, the observed upregulation may reflect transient stress responses occurring between sampling intervals, necessitating more frequent monitoring and dose-response optimization studies.

Recent molecular investigations support these observations. Muehler et al. (2022) demonstrated that sublethal chlorhexidine exposure induces comprehensive transcriptomic stress responses in *S. mutans*, including upregulation of genes associated with purine nucleotide synthesis, biofilm formation, and stress adaptation, while simultaneously downregulating other protective mechanisms. [22] These findings confirm that even subinhibitory chlorhexidine concentrations can significantly alter bacterial gene expression patterns, potentially fostering antimicrobial resistance development.

Antimicrobial Resistance and Microbiome Implications

Emerging evidence increasingly links chlorhexidine usage to antimicrobial resistance development, particularly through efflux pump mechanisms and cross-resistance to antibiotics including tetracycline. Bartsch et al. (2024) revealed that prolonged chlorhexidine use substantially alters oral microbiota composition, reducing microbial diversity while favoring caries-associated streptococci and increasing prevalence of tetracycline resistance genes. [23] These findings indicate that chlorhexidine disrupts microbial homeostasis, promotes pathogenic shifts, and may drive resistance mechanisms that compromise long-term oral health outcomes.

Multiple systematic reviews have documented chlorhexidine's association with antimicrobial resistance, microbiome imbalance, and ecological disruption. While chlorhexidine remains clinically valuable for specific short-term indications, its indiscriminate use poses significant risks for antimicrobial resistance development and microbiome stability. [24–26]

Comparison with Current Literature and Evidence Base

Our findings align with recent systematic reviews evaluating herbal alternatives to chlorhexidine in caries prevention. Saikia et al. (2024) emphasized that herbal mouthwashes demonstrate comparable anti-*S. mutans* efficacy to chlorhexidine while exhibiting fewer adverse effects, a trend consistent with our results where sage achieved 100% gtfB downregulation at two weeks compared to chlorhexidine's more variable response. [Reference] The observed 95.2% gtfD inhibition substantially exceeds the 31-89% *S. mutans* reduction reported for other polyphenol-rich extracts in systematic analyses. [8]

Shamim et al. (2023) noted that polyphenolic compounds in herbal extracts disrupt biofilm formation through glucosyltransferase enzyme inhibition, a mechanism directly consistent with our observed sage efficacy. [16]

Furthermore, multiple systematic reviews have underscored the advantages of natural antimicrobials, including reduced microbial resistance development and fewer adverse effects compared to chlorhexidine, supporting our clinical findings. [5,18]

Regulatory and Global Health Perspectives

The World Health Organization's 2025 guidelines on oral health interventions maintain chlorhexidine 0.12-0.2% as the standard antimicrobial for short-term caries prevention in high-risk populations, while acknowledging limitations regarding long-term safety and microbial resistance. [27] Simultaneously, the WHO Traditional

Medicine Strategy 2025-2034 specifically encourages rigorous evaluation of plant-derived oral care products, particularly for resource-limited settings where conventional treatments may be inaccessible. [28] Current public health trends increasingly incorporate herbal mouthwashes into prevention programs due to their affordability, cultural acceptability, and sustainability profiles.

Clinical Implications and Public Health Impact
The multifaceted etiology of dental caries necessitates comprehensive preventive strategies addressing biological, socioeconomic, and environmental determinants. Sustainable prevention requires eco-friendly and cost-effective solutions that can be implemented across diverse populations. Incorporating evidence-based natural compounds into oral care protocols, combined with oral health education, offers a holistic approach particularly valuable for underserved populations and developing regions. [29]

Statistical analysis revealed medium-to-large effect sizes for sage extract's downregulation of both gtfB and gtfD genes, demonstrating clinically meaningful impacts comparable to or exceeding chlorhexidine performance. These findings support sage extract's potential as an effective alternative to chlorhexidine for caries prevention, particularly given its superior safety profile and patient acceptance.

Study Limitations and Behavioral Considerations
Although mouthwash efficacy is typically attributed to active antimicrobial ingredients, patient behavior and adherence significantly influence clinical outcomes. This study achieved high compliance (90%) through daily monitoring and objective bottle weight measurements. However, clinical trial participation, particularly with novel herbal interventions, may enhance oral hygiene behaviors through increased motivation and attention, potentially contributing to improved outcomes independent of the active agent. [30] The placebo effect may similarly influence self-care habits and subjective reporting. [31] Although both groups remained blinded to treatment assignment, the novelty of sage extract may have subtly improved user engagement. Future investigations should incorporate inactive placebo controls to isolate pharmacological effects from behavioral influences.

Limitations and Future Research Directions
This study has several important limitations that affect result interpretation and generalizability. The short follow-up period, single-center design, and modest sample size limit external validity. Additional constraints include unassessed batch-to-batch variability in sage extract composition and lack of comprehensive microbiome analysis. Future investigations should incorporate multiple extract batches with standardized GC/MS profiling and employ 16S rRNA sequencing to evaluate ecological impacts on oral microbiota diversity and composition.

Extended follow-up studies are essential to establish long-term safety profiles, monitor sustained gene expression changes, and identify potential adverse effects associated with prolonged sage extract use. Multi-center trials with larger, more diverse populations are needed to confirm generalizability across different demographic groups and caries risk categories. Additionally, dose-response studies should optimize sage extract concentrations to maximize therapeutic efficacy while maintaining safety margins. Future research should incorporate false discovery rate (FDR) correction or Bonferroni adjustment when conducting multiple statistical comparisons to enhance the robustness of findings and reduce the risk of Type I errors.

V. Conclusions

Under the current study's limitations, sage extract mouthwash significantly outperformed chlorhexidine in downregulating gtfB and gtfD expression in *S. mutans* from high caries risk patients, demonstrating superior magnitude and consistency of gene suppression. As a natural product with minimal adverse effects, sage extract represents a promising alternative to synthetic antimicrobials for caries prevention. The observed paradoxical effects of chlorhexidine, combined with growing concerns about antimicrobial resistance, further support the clinical potential of herbal alternatives. However, extended randomized clinical trials with larger populations and longer follow-up periods are essential for comprehensive safety and efficacy validation before widespread clinical implementation.

Conflict of interests
The authors declare no conflicts of interest related to the study.

Contributions
Both authors contributed equally in design, conduct, analysis, writing and reviewing the manuscript.

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Figure legends

Figure 1: S. mutans samples 1-23 amplification curve using df/dT (derivative of fluorescence over temperature), a standard way to plot melting curves in qPCR to assess amplicon specificity and purity.

Figure 2: The qPCR amplification plot illustrates the threshold cycle (Ct) analysis used for relative quantification of target DNA/RNA. The plot is labeled as 'Relative', indicating that the quantification is compared to a reference gene, such as a housekeeping gene or calibrator sample. The threshold cycle (Ct) method utilized is represented by the acronym 'Q', with a threshold set at 13.9 fluorescence units. This aids in the interpretation of the amplification efficiency and the starting quantity of the target nucleic acid.

Figure 3: CONSORT flow chart showing the patients' flow during the study.

Figure 4: Bar chart showing the mean and SE of ΔCt values for gtfB gene expression at T1 and T2 of treatment with CHX and Sage mouthwashes.

Figure 5: Bar chart showing the mean and SE of ΔCt values for gtfD gene expression at T1 and T2 of treatment with CHX and Sage mouthwashes

Figure 6: Bar chart showing the mean ±SD of ΔCt values for gtfB gene expression at different Treatment Groups-Time

Figure 7: Bar chart showing the mean ±SD of ΔCt values for gtfD gene expression at different Treatment Groups-Time

Figure 8: Stacked bar chart showing downregulation percentage comparison between gtfB and gtfD gene expression on different Treatment Groups-Time

Tables

Table 1: The oligonucleotide primers used in the study

Table 2: Repeated measures ANOVA (main effects and interactions) of gtf B.

Table 3: Repeated measures ANOVA (main effects and interactions) of gtf D.

Table 4 Mean ±SD of ΔCt for for the effect of time within each group and the effect of treatment group within each time on gtf B and gtf D gene expression.

Table 5: Median fold change (IQR) and %Regulation of group-time at gtfB and gtfD gene expression

Table 6: Summary of Adverse Effects Observed During the Trial