Section A-Research paper

Efficacy of Sage extract versus Chlorohexidine Mouthwashes on Streptococcus mutans using Real-Time Polymerase Chain Reaction (qPCR) in High Caries Risk Patients: A Randomized Quadruple Clinical Trial

Dina Diab [ab], Mona Fadel [c], Dina Mohamed [a], Nayera Shaker [d], Amira El-Zoghby [a]

[a] Conservative Dentistry Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

[b] Conservative Dentistry Department, Faculty of Dentistry, Egyptian Russian University, Cairo, Egypt.

[c] Conservative Dentistry Department, Faculty of Dentistry, October University for Modern Science and Arts, Cairo, Egypt.

[d] Food and Diary Microbiology, National Research Center, Cairo, Egypt.

Short Title: Efficacy of Sage extract on *Streptococcus mutans* using qPCR

Corresponding Author:

Dina Ezz Eldin Mohamed

Conservative Dentistry

Faculty of Dentistry, Cairo University, Cairo, Egypt

Address: 11 El Saraya St., El Manyal, Cairo, Egypt.

Zip code: 11553 Tel: (+202) 23634965 - (+202) 23642938

E-mail: Dina.ezz@dentistry.cu.edu.eg

Number of Tables: 4 tables

Number of Figures: Please indicate the number of figures submitted.

Word count: 4944 excluding references

Keywords: Sage extract mouthwash, Chlorohexidine mouthwash, *Streptococcus mutans*, Real time PCR, High caries risk.

1. Abstract

Aim: To assess the efficacy of Sage extract versus Chlorohexidine-based mouthwashes on the detected percentage of recovery of *Streptococcus mutans* and its pathogenicity by real-time polymerase chain reaction (qPCR) in high caries risk patients over one-month follow-up.

Methodology: A total number of 74 high caries-risk patients were assigned to this study. Participants were randomly divided into two groups (n=37) according to the type of mouthwash (A) where group (A*1*) patients used Sage extract containing mouthwash while group (A*2*) participants used chlorohexidine-containing mouthwash**.** The traditional microbiological cultivation method of *S.mutans* and quantitative real-time polymerase chain reaction was employed to analyze the bacterial count of each salivary sample at baseline (T_0), after two weeks (T_1) and after four weeks (T_2) of using mouthwashes.

Results: Comparison of antimicrobial effectiveness across CHX and sage groups revealed a significant difference concerning oral streptococcus count. Sage extract as a natural product had a significantly higher value than CHX ($p<0.001$) in decreasing log bacterial count. Comparing bacterial cultural method and real-time PCR, real-time PCR was more specific and sensitive in detecting *S.mutans.*

Conclusions: Sage mouthwash as a natural product could be suggested as an effective substitution for chlorhexidine to control caries in high caries-risk patients. Real-time PCR is more specific than the traditional methods for the bacterial culture which provides qualitative and quantitative results.

2. Introduction

Dental caries is a microbial disease that is initially caused by the disparity in the biofilm of the oral flora. *Streptococcus mutans (S.mutans)*, have been implicated as the major microbial etiological agent in the development of dental caries. *S.mutans* is 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 formation of dental caries Zafar et al. (2020) The levels of *S.mutans* in saliva have been shown to be a mean of predicting caries activity Alamoudi et al. (2022). However, the quantification of *S. mutans* requires great effort and the levels of *S. mutans* have not been used as an established index of dental caries diagnosis. The traditional culture methods used for the isolation of *S.muatns* do not have the selectivity, that is necessary for the identification of its species. On the other hand, real-time quantitative polymerase chain reaction (qPCR) technology is very sensitive for the identification of *S.mutans*, revolutionizing the field of quantification methods than traditional culture-based method. DNA and RNA released by microorganisms are also present in saliva because Streptococcus 16S rRNA / rDNA is specified in the liquid phase of saliva Moncada et al. (2016). For ages, the use of antimicrobial mouthwashes has been found one of the effective methods for caries control and prevention. These mouthwashes are able to inhibit the bacterial adhesion and colonization which affect the bacterial growth. Chlorhexidine (CHX) is one of the most successful used mouthwashes due to its antimicrobial activity against *S.mutans* and antiplaque properties as well. However, the use of CHX has some hazards such as staining, altered taste sensation,

vomiting, and development of resistant bacterial strains Deus et al. (2022). Meantime, 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. Among these natural products, is sage extract (*Salvia officinalis*) which has a wide spectrum of therapeutic activities including antibacterial, antiviral, antifungal, and antioxidant effects resulting from the action of various chemical compounds present in the plant including triterpenes, flavonoids, and phenolic acids Almeida et al. (2019). The aim of the study to evaluate the efficacy of Sage extract versus chlorohexidine-based mouthwashes on the detected percentage of recovery of *S.mutans* by real-time polymerase chain reaction (PCR) in high caries risk patients.

Materials and Methods

Study Design

The present study was designed to be randomized, two parallel arms, unicentered, and quadruple-blinded, where the primary investigator (clinician), participants, assessor and data analyzer were blinded. This clinical trial was conducted at the Conservative Dentistry Department, Outpatient Clinic of Faculty of Dentistry— Cairo University, Egypt.

Sample size collection

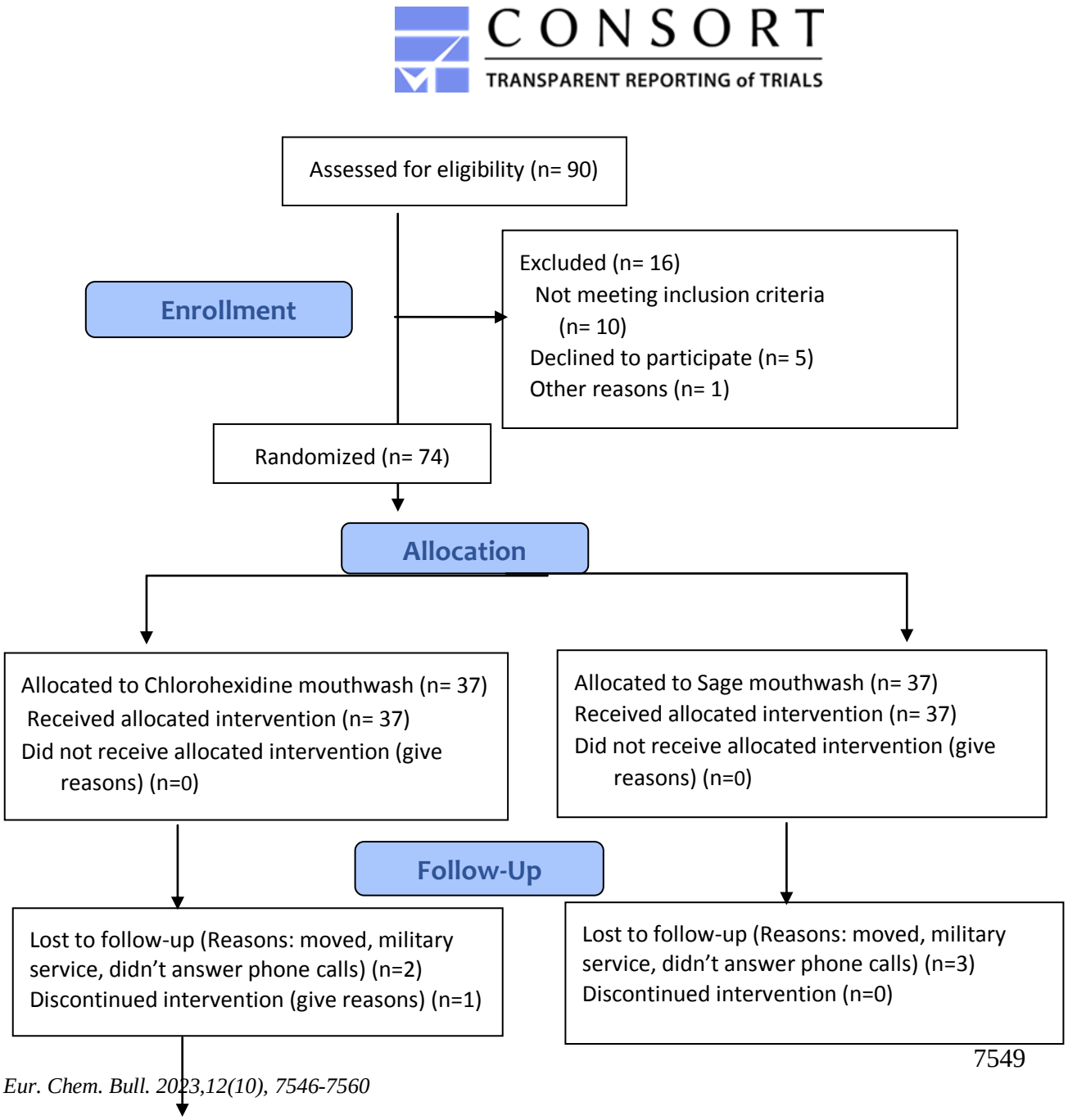
According to a previously published study by Netto et al. (2013) the response within each subject group was normally distributed with a standard deviation of 0.7. If the true difference in the experimental and control means is 0.51, we need to study 31 experimental subjects and 31 control subjects to be able to reject the null hypothesis that the population means of the experimental and control groups are equal with probability (power) 0.8. This was increased to 37 subjects per group to compensate for losses during follow-up. The Type-I error probability associated with this test of this null hypothesis is 0. The sample size was calculated using PS power and sample version 3.1.2 for windows using T-test.

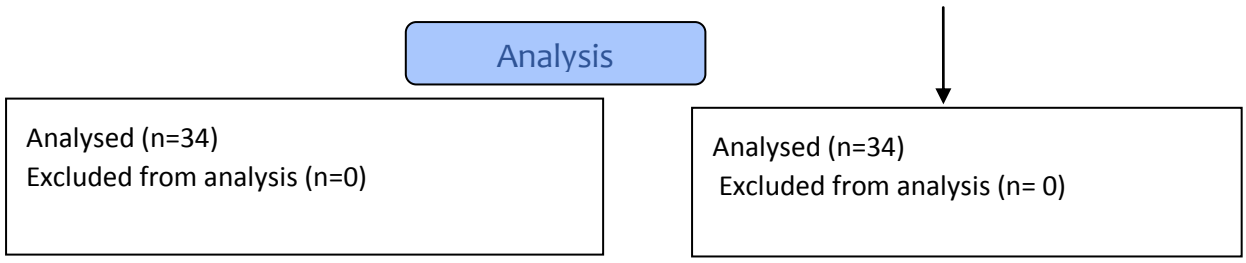
Eligibility Criteria

Patients were recruited from the outpatient clinic of Faculty of Dentistry—Cairo University, Egypt—with the following inclusion criteria: participants age range from 20 to 50 years old, male or female, medically free, high caries patient with DMFT (D=decay, M=missing, FT=filled teeth) >6. In order to classify the subjects as a high caries risk, dental examination was carried out to detect DMFT as well as caries risk assessment was done using the American Dental Association (ADA) caries risk model. The exclusion criteria excluded those who are already on a mouthwash regimen, medically compromised patients (diabetic, neurovascular problem, undergone radiation therapy, and with salivary gland disorders), pregnant women. Subjects who consuming any drug reduce salivary flow, under any antibiotic, allergic from to any chemicals used in this study and smokers were also excluded. The researcher explained well the whole procedure to all eligible participants, including the objective of the study, different procedures, safety precautions, and benefits. An informed consent in simple Arabic language was obtained from each participant prior to the beginning of the trial.

Randomization, Allocation concealment and Blinding

Total of 74 eligible participants were randomly allocated to the two groups through an online randomization Web-based tool (https://www.random.org/). Patients were randomly divided into two groups (*n* = 37) according to the type of mouthwash (G): Group G1 participants used sage extract mouthwash, while group G2 patients used the chlorhexidine-containing mouthwash. Each group was further subdivided into three subgroups according to time (T) where (T_0) represented the baseline (T_1) represented after two weeks of rinsing with the mouthwash, and (T_2) represented after one-month rinsing. Random-generated numbers were placed in opaque-sealed envelopes prepared by a contributor who was not involved in the trial, and the allocation sequence was concealed from the primary investigator. Study timeline from recruitment to follow-up as well as the analysis was demonstrated in CONSORT 2010 flow diagram (Flowchart 1).





Flow chart 1: CONSORT 2010 Flow Diagram

Materials

Two types of mouthwashes were used in this clinical trial: Synthetic chlorhexidine mouthwash and natural sage extract mouthwash. Mouthwashes were stored in unlabeled and amber glass bottles, to conceal the type of mouthwash from the participants and the operator.

Preparation of Sage extract mouthwash

Sage oil was obtained from a local market of herbal products in Egypt. In order to identify the chemical composition of sage essential oils and overall purity, Gas Chromatography/ Mass Spectrometry analysis (GC/MS) was performed. Prior to the preparation of mouthwash, the Minimum Inhibitory Concentration (MIC) and the Bactericidal Inhibitory test (MBC) was done in vitro against *Streptococcus mutans* using the microbroth dilution method. The MIC for *S.mutans* was shown to be 1.56 % and the MBC was 3.3 %. The preparation of mouthwash was carried out at a private lab (Nawah Scientific Lab- Al Mokattam, Cairo, Egypt). For each 100 ml mouthwash preparation, in a glass beaker, 1.56 g of Sage oil (Active ingredient) was mixed with 1.80 g of tween 80 (Solubilizer), and then 100 ml of Purified water (Main vehicle) was added portion-wise to the sage tween mix until a fine cloudy dispersion was obtained. 0.01 g of sucralose (Sweetener) was added under mixing until complete dissolution occurred. Further, the volume was completed in a graduated measuring cylinder using Purified water. Finally, the batch was filled into 200 ml amber glass bottles. The extract was stored in a refrigerator -20 °C until being used.

Mouthwash Administration and Saliva Sampling

Participants were asked to rinse their mouth with 10 ml of the mouthwashes in their respective groups for 1 min, then they were instructed to spit in a labeled sterile container. Written instructions were provided to the participants, and regular follow-up was scheduled through daily phone calls. Saliva samples were collected from each participant at base line, after two weeks and after one month. Saliva collection was done in the early morning on the day of collection and the participants were also informed not to eat or drink anything (except water) before saliva collection to minimize possible food debris and stimulation of saliva. They were also instructed not use any other antimicrobial agents after the intervention. The participants sit in an erect position on the dental chair and were given a paraffin block to chew for two minutes for salivary stimulation. Then, they were asked to spit in sterile containers held near the mouth by the operator. The containers were properly labeled on basis of the group which was allocated to them Sharma et al. (2018). Afterwards, saliva samples were transferred

immediately to the Microbiology Department—Cairo University—for the microbial analysis. The tubes were labeled with nonidentifiable numbers to ensure blinding of the microbiologist.

Microbiological Assessment

Microbiological assessment was done on several steps, it started with the preparation of the fresh selective agar media. Mitis Salivarius Agar (MSA) (Difco Laboratories, Dickinson and Company, France) was used for *S. mutans* culture. The MSA agar was prepared according to the manufacturer's instructions. The MSA agar media was supplemented with 20% sucrose, 0.2 units/mL bacitracin, and 1% potassium tellurite (mitis-salivarius bacitracin).

Dilution of the collected Saliva Samples

Serial dilution of the saliva sample was done in order to all allow an easy and accurate bacterial count. The salivary samples in the test tubes were vortexed for 1 minute and then were serially diluted to 10^3,10^4, and 10^5. The tubes were vigorously shaken before dilution for 30 seconds on vortex (Assistent Reamix 2789 Vortex Mixer, Medical Trade Center, Hamburg, Germany) to obtain homogenous distribution. For the cultivation of the collected salivary samples, 0.1 ml of each dilution was taken using an automatic micropipette and placed on the center of MSB plates. Using a sterile glass rod, the sample was spread on the agar surface in order to provide a smooth surface. Afterwards, the inoculated plates were incubated anaerobically in a gas pack jar and the incubator (Precision dual illuminated program, Germany) was adjusted at 37^{o}c for 48 hours.

Bacterial count

After 48 hours, the most appropriate plates were selected for counting *S. mutans* colonies by a single examiner who was unaware of the treatment of participants and calculated the total colony forming unit (CFUs) on each salivary sample for each mouthwash. The colonies were recognized by their characteristic morphology (0.5-1mm in diameter, convex, raised with rough boarders and opaque with dark blue color. The bacterial count was obtained at baseline, after two weeks and one month for both groups.

qPCR

DNA Extraction. DNA was isolated from all the samples using a Quick-DNA™ Miniprep Plus Kit (ZYMO RESEARCH, CALIFORNIA CA, USA). Protocol was performed according to the manufacturer's instructions. Starting with homogenization saliva samples were defrosted and re-suspended by agitation in a vortex (Assistent Reamix 2789 Vortex Mixer, Medical Trade Center, Hamburg, Germany) for 30 seconds. Followed by mechanical and chemical lysis in which 200 µL of saliva was added to a microcentrifuge tube, then 200 µL biofluid and cell buffer solution was added for cell lysis. After cell lysis, 20 µL of Proteinase K was added and the lysates were incubated at 55 ℃ for 10 minutes. Afterward, an equal volume of Genomic Binding buffer was added so that 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. Emptied the collection tube and 700 µl of g-DNA Wash Buffer was 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, to elute the DNA, transferred to a clean micro centrifuge tube and 50 µl DNA Elution Buffer was added and incubated for 5 minutes and then centrifuged for 1 minute.

Amplification and quantification of *S. mutans*

In this clinical trial, DTlite real-time PCR device (DNA- Technology, Research, and production, Moscow, Russia) was used. 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 *S.mutans* forward primer and reverse primer which targets 130 bp of 16s *rRNA* gene in most species of Streptococci as shown in table 1

Gene	Primer direction	Sequence 5´- 3´	PCR product length	Tm	GC%
S. mutans	**Forward** **Reverse**	**GCACTCGCTACTATTTCTTACAA** **GTCACAATGTCTTGGAAACCAGTAAT**	**23** **26**	**74** **72**	**42%** **38%**

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 (C_T) in each sample were compared with C_T of Streptococci in the calibrator sample.

qPCR Conditions

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.

Statistical Methods

Categorical data were presented as frequency and percentage values and were analyzed using the chi-square test for intergroup comparisons and McNemar's test for intragroup comparisons. Numerical data were presented as mean and, standard deviation values. They were checked for normality using the Shapiro-Wilk test. Data showed parametric distribution and were analyzed using independent t-test for intergroup comparisons and repeated measures ANOVA followed by Bonferroni post hoc test for intragroup comparisons. The significance level was set at $p \leq 0.05$ within all tests. Statistical analysis was performed with R statistical analysis software version 4.1.3 for Windows

Results

According to the sample size calculations, 62 participants were required for this trial, and the number was increased to 74 to compensate for the loss of follow-up. Therefore, a total of 74 participants were recruited at the beginning of the trial. Participants were equally and randomly allocated to a control group (*n*=37) and the intervention group (n=37). Six participants discontinued the intervention during the different follow-up periods.

Bacterial count Colony Forming unit (CFU)

Regarding the bacterial count by the traditional culture method, the results of the intergroup comparison showed that the log bacterial count at baseline using CHX had a higher value than sage herbal yet the difference was not statistically significant (p=0.364) as presented in Table 2. After two weeks as well as four weeks sage herbal mouthwash had a significantly higher value than CHX where (p<0.001). Concerning the intragroup comparison, there was a statistically significant difference between values measured at different follow-up intervals. The highest value was recorded at baseline, followed by the value measured after two weeks of administration, while the lowest value was found after four weeks of administration for both groups (*p* <0.001) (Table 2). For CHX group post hoc pairwise comparisons showed values measured at different intervals to be significantly different from each other (p<0.001), while for the sage group post hoc pairwise comparisons showed the value measured T_0 to be significantly higher than values measured at other intervals (p<0.001).

Bacterial count using qPCR

Regarding the bacterial count by real time PCR, the results of the intergroup comparison showed that the log bacterial count using sage herbal mouthwash had a higher value than CHX at all time intervals yet, the difference was not statistically significant at baseline, after two weeks and after four weeks at (p=0.232), (p=0.118), and (p=0.092), respectively, as listed in Table 3. Concerning the intragroup comparison, there was a statistically significant difference between values measured at different follow-up intervals. The highest value was recorded at baseline, followed by the value measured after two weeks of administration, while the lowest value was found after four weeks of administration for both groups (*p* <0.001) (Table 3). post hoc pairwise comparisons showed the value measured T_0 to be significantly higher than values measured at other intervals (p<0.001).

Comparison between traditional culture method and real time PCR

When comparing the both methods for bacterial count regardless any intervention, the results showed that at the baseline the results of q PCR had a higher value than CFU yet the difference was not statistically significant (p=0.210) as listed in Table 4. While, after two weeks and four weeks the bacterial count CFU had a higher value than PCR yet the difference was not statistically significant where (p=0.132) and (p=0.092), respectively, as listed in (Table 4).

Table (2): Inter, intragroup comparisons, mean and standard deviation (SD) values of log bacterial count (CFU) for different groups

Interval	Log bacterial count (CFU) (mean±SD)		p-value
	CHX	Sage	
T0	6.20±0.19^{A}	6.15±0.25^{A}	0.364ns
T1	5.79±0.06^{B}	5.95±0.11^{B}	<0.001*
T2	5.48±0.15^{C}	5.91±0.14^{B}	<0.001*
p-value	<0.001*	<0.001*	

Means with different superscript letters within the same vertical column are significantly different *; significant (p≤0.05) ns; non-significant (p>0.05)

Table (3): Inter, intragroup comparisons, mean and standard deviation (SD) values of log bacterial count (qPCR) for different groups

Interval	Log bacterial count (PCR) (mean±SD)		p-value
	CHX	Sage	
T0	6.37±0.58^{A}	6.55±0.63^{A}	0.232ns
T1	5.45±0.38^{B}	5.82±0.62^{B}	0.118ns
T2	4.62±0.61^{C}	5.02±0.37^{C}	0.092ns
p-value	<0.001*	<0.001*	

Means with different superscript letters within the same vertical column are significantly different *; significant (p ≤ 0.05) ns; non-significant (p>0.05)

Table (4): Inter, intragroup comparisons, mean and standard deviation (SD) values of log bacterial count (CFU and qPCR) for different groups and intervals

Interval	Log bacterial count (mean±SD)		p-value
	CFU	qPCR	
T0	5.83±1.39	6.71±1.63	0.210ns
T1	5.78±0.18	5.64±0.44	0.132ns
T2	5.62±0.26	4.87±0.54	<0.001*

*; significant ($p \leq 0.05$) ns; non-significant (p>0.05

Discussion

The present randomized controlled clinical trial aimed to evaluate the efficacy of sage extract and chlorohexidine mouthwashes by determining the percentage of *S. mutans* in clinical salivary samples of participants with caries risk at baseline, two weeks, and four weeks. Sage extract is a natural safe herbal extract of phytochemicals derived from plants, had been shown to have an antibacterial activity reducing *S.mutans* levels, and can be considered as a cost-effective alternative to synthetic antimicrobial agents that have led to a rise in prevention as well as controlling of dental caries Ntondini et al. (2021). (standardize) Chlorohexidine mouthwash was used as a comparator to the test group as it is considered one of the best reported commercially and available rinses due to broad-spectrum anti-microbial activity with no adverse systematic side effects, it could be a valuable control group Thomas et al. (2016).

In this clinical trial, the inclusion criteria included patients with high caries risk and of high plaque index as those are the targeted population who are at high caries risk. While for the exclusion criteria, pregnant women were excluded as sage oil can cause uterine contractions and slow down breast milk production due to the presence of thujone, and terpene ketones which are considered the most toxic compounds in sage. These compounds induce toxic effects on the fetus and newborn. Thujone works as a nervous stimulant, patients with a history of seizures were excluded, due to the potential risk of triggering a seizure. Since sage might lower blood sugar levels in people with diabetes, they were also excluded Ghorbani and Esmaeilizadehn (2017).

In this study, saliva collection was done under standardized conditions to provide authenticity along with working out reproducible results Wang et al. (2015). A regular period of 8–11 am was followed for the collection of saliva to reduce diurnal variations. Whole stimulated saliva was collected from the floor of the mouth as it confirms an actual representation of the salivary composition Sharma

et al. (2018). At the baseline evaluation of the saliva samples, the molecular analysis revealed the prevalence of the tested microorganism across all recruited individuals possessing high caries activity. The participants were recalled after two and four weeks, for salivary sample collection and microbiological assessment. This follow-up period was in accordance with a study conducted by Jacob et al. (2021) to assess the antibacterial effect of CHX and natural herbal mouthwash to validate the substantive activities of both types of mouthwash and to be able to detect any side effects.

In this clinical trial, the findings yielded by qPCR amplification exhibited a high percentage of efficiency. Thus, validating the results, sensitivity, and accuracy of this technique, as previously published by Jacob et al. (2021). Consequently, it is possible to declare that molecular identification using qPCR is not only able to detect but also able to quantify *S. mutans* in saliva. Moreover, the traditional methods for microbial identification and quantification used the method of bacterial cultivation using a selective media to detect the presence of *S. mutans*, which implies processing samples immediately without the possibility of storing them for long periods and freezing them for later analysis Moncada et al. (2016).

In addition, the culture method has limitations such as prolonged time for microbiological processing and a lower level of sensitivity. Also, the preservation of culture agents must ensure viability over time; contamination risks should be minimal ensuring that culture purity remains unaffected. These limitations make it laborious for culture methods to provide accurate microbial evaluations in order to identify and quantify *S. mutans* associated with individuals with high caries susceptibility Ezzeldin et al. (2021).

This study showed a significant difference between CHX and sage mouthwashes intergroup against oral streptococcus count. Sage mouthwash had a significantly higher value than CHX in decreasing log bacterial count after two weeks, as well as four weeks, follow up and this was in agreement with Beheshti Rouy et al. (2015) who explained the antibacterial mechanism of sage and stated that the hydrophobic nature of essential oils is well known to target the cell membrane's lipid bilayer, making it penetrable and resulting in leakage or even the loss of vital cell contents. Thus, Sage has been shown to inhibit the growth of the *S. mutans* cell by destroying the cell wall and further leading to a loss of cellular content. The permeability and loss of cellular content characteristics are the cause of cell death.

Intragroup of Sage mouthwash showed the lowest bacterial count after four weeks of application in agreement with in vivo studies by Beheshti Rouy et al. (2015) and Ntondini et al. (2021) who showed that there was a marked decline in the number of *S.mutans* oral bacteria. This also was in accordance with in vitro studies by Almeida et al. (2019), Agrawal et al. (2021), and Tambur et al. (2021) who reported that sage oil showed antibacterial activity against *S. mutans.*

The antibacterial property was due to the essential oils of sage which comprise monoterpenoid major constituents that are known to be effective antibacterial agents as mentioned by Tariq et al. (2019). The monoterpenoid components such as thujone, camphor, 8-cineole, and camphene are known to have antimicrobial activity against human bacterial pathogens. The presence of both camphor and camphene in salvia officinalis provides it with an antibacterial property as mentioned by Ntondini et al. (2021).

Intragroup of chlorhexidine mouthwash showed the lowest bacterial counts values after four weeks of application in accordance with Jacob et al. (2021) which explained chlorhexidine is positively charged and has a high attraction for negative ions found in cell membranes of *S.mutans* bacteria. It is capable of binding onto the microbial cell walls, eventually creating an osmotic imbalance, and finally initiating death. However, Öznurhan et al. (2019) and Brooker et al. (2021) reported that chlorhexidine had some hazards including bitter taste alteration, increased risk of caries due to fermentation and alcohol content, discoloration, altered taste perception, and cytotoxic effects on the cell.

Based on feedback, the CHX group reported a certain level of unpleasant taste was experienced during the first week of application, which eventually subsided on routine usage. For the sage group, none of the candidates announced any known side effects. On intraoral examination, vivid staining of teeth was observed in a few subjects from the CHX group following the second follow-up period which was relatively absent in the sage group.

In this clinical study, when comparing between the colony-forming unit (CFU) and the real-time PCR log bacterial count. The results of qPCR corresponded well to those of the traditional cultural method for *S. mutans* due to the sensitivity and the specificity of this technique. The difference in units between the CFU and the number of DNA should be considered. Generally, one colony is not necessarily derived from one single bacterial cell when the cells are aggregated, and rapidly growing bacterial cells usually contain more than one chromosome. At baseline, the results of qPCR had a higher value than CFU but the difference was not statistically different. This was explained by Cangelosi et al. (2014) that the bacterial cultural method is able to distinguish the living from the dead cells as it depends on selectively detecting viable organisms. However, because only a small percentage of species can be cultured, this strategy underestimates microbial diversity.

In contrast, nucleic acid-based methods cannot discriminate DNA associated with a viable bacterial cell from DNA associated with an inactivated one. qPCR was more sensitive, the detected bacterial count was likely higher owing to a combination of the theoretical sensitivity of the methods, as well as the possibility of decreased cell viability or lack of growth on selective media but still detected by qPCR. Since PCR measures genetic material rather than the viable cells quantified by culture-based methods, it may overestimate oral indicator bacteria because of the inclusion of target DNA from dead or viable cells in the measurement AL-Qazzaz et al. (2014).

In the era of the widespread usage of natural products, sage herbal mouthwash can be suggested as an antibacterial agent for controlling dental caries and substitution for chlorhexidine in high-caries risk patients.

Conclusions

Under the limitations of the current study, it could be concluded that, in high caries risk patients, there was a significant difference between sage extract and chlorhexidine mouthwashes regarding *Streptococcus* bacterial count. Consequently, sage mouthwash as a natural product could be an effective alternative to the current synthetic mouthwashes. Concerning the bacterial count method, real-time PCR is more specific than the traditional methods which provides qualitative and quantitative results.

Statement of ethics

The trial was reviewed and approved by the Research Ethics Committee—Faculty of Dentistry, Cairo University— January 2020 (Approval no.20788) and registered in the Clinical Trials Registry (NCT04565912). Patients were recruited between January 2021 and July 2021 and written informed consent was obtained from each participant.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Funding statement

Self-funded by the main author.

Author Contributions

Dina Mohamed, Mona Fadel, Nayera Shaker and Amira Farid are the co-first author.

This study was the postgraduate thesis work of Dr. Dina Diab.

Data Access statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

References:

Agrawal R, Yusufsaheb YG, Narasimhamurthy N, Itagi AB, Kulshrestha R, Patil SR. Anti-Microbial Activity of Hempseed Oil and Sage Oil against Streptococcus mutans and Candida albicans: An In-Vitro Study. Pesquisa Brasileira em Odontopediatria e Clínica Integrada. 2021 Aug 27;21.

Alamoudi A, Alamoudi R, Gazzaz Y, Alqahtani AM. Role of Salivary Biomarkers in Diagnosis and Detection of Dental Caries: A Systematic Review. Diagnostics. 2022 Dec;12(12):3080.

Almeida CM, de Sousa Sales EA, Alves ÉP, de Freitas Lima R, Cavalcanti YW, Gomes RC, Pereira JV, de Brito Costa EM. In vitro Evaluation of the Antimicrobial Potential of Salvia officinalis L. against Oral Pathogens. Journal of Health Sciences. 2019 Jun 19;21(2):129-33.

Halah Kamal AL, Wia'am Al-Amili A. Comparison Between Traditional and PCR Analysis for Identification of Oral Streptococci with Dental Caries in Iraqi Diabetic Patients. Iraqi journal of biotechnology. 2014;13(2):224-36.

Beheshti-Rouy M, Azarsina M, Rezaie-Soufi L, Alikhani MY, Roshanaie G, Komaki S. The antibacterial effect of sage extract (Salvia officinalis) mouthwash against Streptococcus mutans in dental plaque: a randomized clinical trial. Iranian journal of microbiology. 2015 Jun;7(3):173.

Bescos R, Ashworth A, Cutler C, Brookes ZL, Belfield L, Rodiles A, Casas-Agustench P, Farnham G, Liddle L, Burleigh M, White D. Effects of Chlorhexidine mouthwash on the oral microbiome. Scientific reports. 2020 Mar 24;10(1):5254.

Cangelosi GA, Meschke JS. Dead or alive: molecular assessment of microbial viability. Applied and environmental microbiology. 2014 Oct 1;80(19):5884-91.

Deus FP, Ouanounou A. Chlorhexidine in dentistry: pharmacology, uses, and adverse effects. International dental journal. 2022 Mar 12.

Ezzeldin N, Elshishtawy H, Mohamed M. Molecular assessment of oral Streptococcus mutans isolated from patients with different ages and caries activity via selective media and protein pattern. Egyptian Dental Journal. 2021 Jan 1;67(1-January (Conservative Dentistry and Endodontics)):789-800.

Ghorbani A, Esmaeilizadeh M. Pharmacological properties of Salvia officinalis and its components. Journal of traditional and complementary medicine. 2017 Oct 1;7(4):433-40.

Jacob B, Malli Sureshbabu N, Ranjan M, Ranganath A, Siddique R. The antimicrobial effect of pomegranate peel extract versus chlorhexidine in high caries risk individuals using quantitative real-time polymerase chain reaction: a randomized triple-blind controlled clinical trial. International Journal of Dentistry. 2021 Aug 30;2021.

Moncada G, Duperat LD, Palma P, Corsini G, Neira M, Reyes E, Oliveira Junior OB, Faleiros S, Gordan V, Yévenes I. Real-time quantitative polymerase chain reaction (QPCR) for the identification and quantification of streptococcus mutans in saliva and dental biofilm in children. Revista Facultad de Odontología Universidad de Antioquia. 2016 Dec;28(1):71-94.

Netto, C.A., Marcucci, M.C., Paulino, N., Anido-Anido, A., Amore, R., de Mendonça, S., Neto, L.B. and Bretz, W.A., 2013. Effects of typified propolis on mutans streptococci and lactobacilli: a randomized clinical trial. *Brazilian dental science*, *16*(2), p.31.

Ntondini SS, Lenatha GG, Dzogbewu TC. Inhibitory effect of essentials oil against oral pathogens. Int J Dent Oral Sci. 2021 Jan 8; 8:1018-23.

Öznurhan F, Buldur B, CARTI Ö, Tutar U, Celik C, Hepokur C. Antimicrobial efficacy of chlorhexidine and licorice mouthwashes in children. Meandros Medical and Dental Journal. 2019;20(1).

Tambur Z, Miljković-Selimović B, Opačić D, Vuković B, Malešević A, Ivančajić L, Aleksić E. Inhibitory effects of propolis and essential oils on oral bacteria. The Journal of Infection in Developing Countries. 2021 Jul 31;15(07):1027-31.

Tariq S, Wani S, Rasool W, Shafi K, Bhat MA, Prabhakar A, Shalla AH, Rather MA. A comprehensive review of the antibacterial, antifungal and antiviral potential of essential oils and their chemical constituents against drug-resistant microbial pathogens. Microbial pathogenesis. 2019 Sep 1; 134:103580.

Thomas A, Thakur SR, Shetty SB. Anti-microbial efficacy of green tea and chlorhexidine mouth rinses against Streptococcus mutans, Lactobacilli spp. and Candida albicans in children with severe early childhood caries: A randomized clinical study. Journal of Indian Society of Pedodontics and Preventive Dentistry. 2016 Jan 1;34(1):65-70.

Wang J, Schipper HM, Velly AM, Mohit S, Gornitsky M. Salivary biomarkers of oxidative stress: A critical review. Free Radical Biology and Medicine. 2015 Aug 1; 85:95-104.

Zafar N, Ali A, Yasir Afzal M, Tanveer Q, Bibi S, Basit I, Nasir H, Imtiaz S, Nazir U. Role of Streptococci as etiological agents of dental caries. Novel Research in Microbiology Journal. 2020 Jun 1;4(3):766-78.