The effect of Cinnamon extract mouthwash on *streptococcus mutans* cariogenicity by real-time polymerase chain reaction in high caries risk patients: A randomized clinical trial

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Abstract

Aim: This trial was conducted to evaluate the effect of cinnamon extract or chlorohexidine-based mouthwashes on the percentage of *Streptococcus mutans* recovery detected by real-time polymerase chain reaction (PCR).

Materials and methods: A total number of 74 patients were assigned in this study. Patients were randomly divided into two groups (n=37) according to type of used mouthwash **(M)** where group **(M_1)** used Cinnamon mouthwash while group **(M_2)** used the chlorohexidine mouthwash. Then each group was subdivided into three subgroups according to time **(T)** where **(T_0)** the base line, **(T_1)** after 2 weeks, and **(T_2)** after one month. For each salivary sample, Streptococcus mutans count was detected by the traditional culture method and real-time polymerase chain reaction (qPCR).

Results: regarding the bacterial counts, after one month there was a statistically significant decrease in *Streptococcus mutans* counts detected by qPCR than the culture method ($p<0.001$).

Conclusions: Cinnamon mouthwash affords an effective alternative to chlorhexidine mouthwash in reducing bacterial count in high caries-risk patients. While real-time polymerase chain reaction (qPCR) provides an accurate way for bacterial identification.

Keywords: Cinnamon extract mouthwash, chlorhexidine mouthwash *streptococcus mutans*, real time PCR, High caries risk.

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INTRODUCTION:

Earlier, *Streptococcus mutans* attracted a lot of attention and has been identified as the principal root of dental caries. **Oda et al. (2015).** It had been assumed that there is a positive association between the level of *Streptococcus mutans* in saliva and the instances of dental caries, and there was a limited information on this possible association. **Alhasani et al. (2020).**

Standard medium used for the isolation of *Streptococcus mutans*, Mitis Salivarius Bacitracin (MSB) agar, is not selective. Therefore, it is necessary to develop a specific way to detect these bacteria as a new caries risk index. Recently, quantitative real-time Polymerase chain reaction (qPCR) provides a specific tool for the detection and quantification of *Streptococcus mutans.* **Tjandrawinata et al. (2019).**

To be more accurate for salivary *Streptococcus mutans* detection, its DNA must be targeted. Quantitative real-time Polymerase Chain Reaction (qPCR) is an in vitro technique used to extract, amplify the DNA, and represent the amount of amplified DNA. This allows for a more accurate count of bacterial DNA **Tjandrawinata et al. (2019).**

Mouthwashes are one of the non-surgical preventive models to manage and prevent the tooth decay. These rinses are either synthetic containing agents such as chlorhexidine gluconate (CHX), hydrogen peroxide and fluoride, or natural rinses contain natural herbal products **Abdulkarim et al. (2019) and Nový et al. (2020)**.

Although CHX is considered the gold standard antibacterial agent, it has significant side effects **Haydari et al. (2017) and Polizzi et al. (2020).** Therefore, it had become a necessity to obtain natural antimicrobial products that are safe for humans and aid in oral hygiene care. Among these natural products, cinnamon extract as an essential oil can be considered as an antimicrobial agent against *Streptococcus mutans* that were involved in dental plaque formation and caries development. **Nabavi et al. (2015)**

Therefore, this randomized clinical trial was conducted to evaluate the effect of cinnamon extract or chlorohexidine-based mouthwashes on the percentage of *Streptococcus mutans* recovery detected by real-time polymerase chain reaction (PCR). The null hypothesis was that there was no

difference regarding the percentage of Streptococcus mutans recovery among the different types of used mouthwashes.

MATERIAL & METHODS:

Materials used in this study were natural prepared Cinnamon mouthwash, chlorohexidine (Hexitol) mouthwash, Mitis Salivarius agar (MSA) for *Streptococcus mutans* culture and Quick-DNA™ Miniprep Plus Kit for DNA Extraction.

Patient recruitment, study design and grouping:

74 patients who were medically free healthy adults, from 20-50 years old, high caries risk subjects according to ADA caries risk assessment model, high plaque index (>score 2), non-smoking subjects and have a normal salivary flow rate (0.3-0.4 ml/min) were included in this trial.

A total number of 74 subjects were assigned in this study. subjects were randomly divided into two groups (n=37) according to type of mouth wash (**M)** where group **(M_1)** used Cinnamon mouthwash while group **(M_2)** used the chlorohexidine (Hexitol) mouthwash. Then each group was further subdivided into three subgroups according to time **(T)** where **(T_0)** the base line **(T_1)** after 2 weeks rinsing with the mouthwash, and **(T_2)** after one month rinsing to get 111 sample from each group through one month test period**.**

Clinical Sample Collection:

All subjects of both groups were asked to rinse with 10 ml of the mouthwashes in their respective groups for 1 min twice daily for one month, then they were instructed to spit in a sterile container. At baseline, two weeks later, and one month later, saliva samples were taken from each patient. The subjects were instructed not to eat or drink anything (except water) before to saliva collection to reduce the possibility of food debris and saliva stimulation. Saliva was collected early on the day of collection. The individuals received chewable paraffin wax while sitting upright in the dentist chair. Following two minutes of chewing paraffin wax, the stimulated saliva was collected in sterile containers held close to the mouth **Sharma et al. (2018)**.

Total bacterial count:

The collected salivary samples were transferred immediately for bacterial culturing. The test tubes were homogenized by vortex for shaking (*Paramix II, Vortex Mixer, Medical Trade Center, Hamburg, Germany)* for one minute. A ten-fold (1:10 or 10^1) dilution was produced by adding 1 ml of the homogenised saliva to a tube containing 9 ml of sterile phosphate buffer saline (pH 7.0) to create a serial dilution. It was further serially diluted (1:100 or 10^2). The same process was used to achieve dilutions of 1:1000 (10^3), 1:10,000 (10^4) and 1:10,0000 (10^5) **Salama and Alsughier (2019).** Afterwards, 0.1 ml of each dilution were transferred to the plate using micropipette and cultured on Mitis salivarius agar (MSA). The Mitis Salivarius Agar plates were placed in a micro-aerophilic environment (5-10% CO2) created by using a candle jar and incubated at 37°C for 48 hours **Shah et al. (2018).** Then the jar was immediately closed tightly and placed in the incubator. After incubation, microbial counts to the number of colonies that grew were performed by a single examiner to calculate total colony forming unit (CFUs)**.**

Bacterial Identification:

DNA was isolated from all the samples. DNA extraction protocol was performed according to the manufacturer's instructions. The samples were dissolved, homogenized, and run for 3 times. One run on the first day and two on the following day to account for daily variance. DNA extraction protocol is based on 6 steps: (1) homogenization, (2) mechanical and chemical lysis by bead beating, (3) chaotropic agents and detergents, (4) inhibitor removal, (5) binding of the DNA to the silica membrane, wash process, and (6) an elution step. In this study, DTlite 4 real-time qPCR device *(DNA- Technology, Research and production, Moscow, Russia)* was used. Primer set suitable for real time qPCR was selected for *Streptococcus mutans*. Forward and reverse prime were the oligonucleotide primers utilized in this study.

Statistical analysis:

Categorical data were presented as frequency and percentage values and were analyzed using chi-square test for intergroup comparisons and McNemar's test for intragroup comparisons. Numerical data were presented as mean, standard deviation values. They were checked for normality using 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:

- Bacterial count Colony Forming unit (CFU):

At baseline **(T_0)**, Cinnamon-based mouthwash **(M_1)** showed a lower bacterial count value (6.05±0.45) than CHX-based mouthwash **(M_2)** (6.20±0.19), but the difference was not statistically significant (p=0.079). After 2 weeks **(T_1)** and 1 month **(T_2),** Cinnamon-based mouthwash **(M_1)** bacterial counts (5.59±0.14) and (5.47±0.20) decreased respectively, compared to CHX-based mouthwash **(M_2)** bacterial counts (5.79±0.06) and (5.48±0.15) respectively. This decrease was only statistically significant after 2 weeks (T1) (p=0.001) **(Table 1).**

- Bacterial count using qPCR:

Even though the PCR showed a higher bacterial count when Cinnamon-based mouthwash **(M_1)** was used compared to CHX-based mouthwash **(M_2)** throughout the follow-up intervals, the difference was not statistically significant.

At baseline **(T_0)**, after 2 weeks **(T_1)** and 1 month **(T_2)**, Cinnamon-based mouthwash **(M_1)** showed bacterial count values (6.81±0.58), (5.65±0.14) and (4.97±0.58) respectively, and CHX-based mouthwash **(M_2)** showed bacterial count values (6.37±0.58), (5.45±0.38) and (4.62±0.61) respectively; (p=0.106), (p=0.132) and (p=0.207) respectively **(Table 2).**

- Comparison between traditional culture method and real time PCR:

At baseline **(T_0)**, PCR (6.59±1.61) had a higher bacterial count value than CFU (5.76±1.46), but the difference was not statistically significant (p=0.243). After 2 weeks **(T_1)** and 1 month **(T_2)**, PCR bacterial counts (5.55±0.30) and (4.80±0.60) decreased respectively, compared to CFU bacterial counts (5.69±0.15) and (5.47±0.17) respectively. This decrease was only statistically significant after 1 month **(T_2)** ($p<0.001$) **(Table 3).**

Table (1): Inter, intragroup comparisons, mean and standard deviation (SD) values of log bacterial count (CFU) for the different mouthwash groups:

Interval	Log bacterial count (CFU) (mean±SD)		p-value
	CHX (M_2)	Cinnamon (M_1)	
T_0	6.20 ± 0.19^{A}	6.05 ± 0.45^{A}	**0.079ns**
T_1	5.79 ± 0.06^{B}	5.59 ± 0.14^{A}	**0.001***
T_2	5.48 ± 0.15^{C}	5.47 ± 0.20^{B}	**0.826ns**
p-value	**<0.001***	**<0.001***	

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

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

Interval	Log bacterial count (PCR) (mean±SD)		p-value
	CHX (M_2)	Cinnamon (M_1)	
T_0	6.37 ± 0.58^{A}	6.81 ± 0.58^{A}	**0.106ns**
T_1	5.45 ± 0.38^{B}	5.65 ± 0.14^{B}	**0.132ns**
T_2	4.62 ± 0.61^{C}	4.97 ± 0.58^{C}	**0.207ns**
p-value	**<0.001***	**<0.001***	

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

Table (3): Mean and standard deviation (SD) values of log bacterial count (CFU and PCR):

Interval	Log bacterial count (mean±SD)		p-value
	CFU	PCR	
T_0	5.76±1.46	6.59±1.61	**0.243ns**
T_1	5.69±0.15	5.55±0.30	**0.111ns**
T_2	5.47±0.17	4.80±0.60	**<0.001***

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

DISCUSSION:

The results of this study showed higher *Streptococcus mutans* count using culturing method than real time qPCR. This was in accordance with **Kim and Lee (2015),** the possible explanation is that some non-streptococcal bacterial species as Actinomyces can overgrow on the Mitis Salivarius (MS) agar media. Because of the poor selectivity of Mitis Salivarius (MS) agar media, making this media unsuitable for specific identification of *Streptococcus mutans*. Prolonged exposure to bacitracin increases the bacterial resistance. *Streptococcus mutans* produce exopolysaccharides or *Xanthomonas campestris* have membrane-bound cell-surface phospholipids, which could bind bacitracin and retain it extracellular to be not effective against the bacterium **Matsumoto-Nakano (2018).**

In addition, other bacterial species as *Streptococcus sobrinus, Streptococcus anginosus* and *Phytobacter* have the same morphological appearance as *Streptococcus mutans* on the Mitis Salivarius agar (MSA) media. This may lead to false positive differentiation for these species as *Streptococcus mutans* in the clinical studies **Zeng et al. (2020)**.

The results of this study that showed higher *Streptococcus mutans* count using culturing method were contradicted by **Bersy et al. (2021**) this may be attributed to that they collected non-stimulated saliva samples from child's patients with low caries index.

Another contradiction was found with **Ferdose et al. (2022).** This may be due to their use of selective Mitis Salivarius (MS) media in comparison to non-selective conventional Blood Agar (BA) media. Also, the sample collected was plaque swab not saliva sample.

Chlorhexidine was used as the comparator in this trial as it is known as the gold standard antibacterial agent by binding to the bacterial cell walls. At low concentrations (0.12 %), it has a bacteriostatic effect by altering the bacterial osmotic balance, and release of cytoplasmic components. While at high concentrations (0.2 %), it has a bactericidal effect by enhancing the coagulation of cytoplasmic proteins. Also, it has the ability of substantivity by adsorption on the tissues in the oral cavity. Although its great effect in management of dental caries, it has many side effects as; numbness, taste alteration, teeth discoloration and bacterial resistance if used for a prolonged time **Badri et al. (2021).**

On the other hand, cinnamon was used as an intervention natural mouthwash. The main ingredients of cinnamon oil are cinnamaldehyde, eugenol, phenol, β-caryophyllene and cinnamic acid. According to the used concentration, cinnamon oil may be either bactericidal or bacteriostatic agent **Winska et al. (2019).**

In addition, Eugenol is concentration-dependent antibacterial agent. its effect ranges from destroying the cell membrane, leading to cell shrinkage and death (bactericidal). It can also reduce the biofilm formation and suppress the synthesis of water-insoluble glucans and the adherence of *Streptococcus mutans* to the enamel pellicle (bacteriostatic) **Yanakiev (2020).**

During the study period **(T_0-T_2)** each mouthwash was able to reduce the *Streptococcus mutans* count either when detected by qPCR or CFU. This indicates that mouthwashes either synthetic or natural have bacteriostatic antibacterial effect against *Streptococcus mutans*. This was in accordance with **Pribadi et al. (2017), Gartika et al. (2019), Jeffrey et al. (2020)** and **Yuanita et al. (2020)**.

The results of this study highlight that although the traditional culture methods are considered a less complicated technique for bacterial detection with affordable cost, but they can lead to over bacterial count estimation in comparison to the recent molecular techniques. Moreover, the use of natural mouth washes to control dental caries may be more effective than synthetic mouthwash with lower side effects.

CONCLUSIONS:

Real-time polymerase chain reaction (qPCR) provides an accurate way for bacterial identification. Cinnamon mouthwash affords an effective alternative to chlorhexidine mouthwash in high caries-risk patients.

Statement of ethics

The trial was registered in Clinical Trials Registry ***(www.clinicaltrials.gov)*** with number (NCT04566120). It was reviewed and approved by the Research Ethics Committee (REC), Faculty of Dentistry, Cairo University in February 2020 (Approval no. 8220). 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.

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Author Contributions

Possy Mostafa, Mona Fadel, Nayera Shaker and Amira Farid are the co-first author.

This study was the postgraduate thesis work of Dr. Mostafa El-Ghazali.

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.

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