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Evaluation of the antibacterial potency of different new herbal mouthwashes against oral plaque-forming bacteria: A randomized controlled trial

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Abstract— **Aim:** This study aimed to investigate the effectiveness of Star anise mouthwash against S. mutans and to find the most effective concentration of Star anise and Moringa Oleifera mouthwashes, compared to Fluoride mouthwash which has been thoroughly investigated in the literature. **Material and methods:** Seventy children 6-13 years old were randomly selected from outpatient clinics of the Pediatric Dentistry Department. They were randomly assigned to 7 groups according to the type of intervention, which were either Star anise, Moringa Oleifera (5%,10%,15% concentrations), or Fluoride. They were instructed to use the prepared herbal mouthwash for a week and a sample of non-stimulated saliva was obtained before and after an intervention. The preparation of the herbal extracts was performed followed by the mouthwash, then the required media for the microbial cultivation wasmade ready to receive the saliva samples for obtaining the microbial count. Statistical analysis was then done to investigate the bacterial count before and after the use of the mouthwash. **Results:** There was a statistically significant bacterial reduction between all the groups except for Fluoride, 15% Star anise, 10% and 15% Moringa groups with the highest mean percentage bacterial reduction in the Star anise 15% group and the least mean percentage bacterial reduction in the Star Anise 5% group.**Conclusions:**All the herbal groups were effective in bacterial reductionat certainconcentrations;compared to Fluoride, with the Star anise 15% concentrationbeing the most effective.

Keywords: Star anise, Fluoride, Moringa, mouthwash, Streptococcus mutans.

Introduction

Dental caries represents the highest prevalence among oral diseases affecting children, it can start in the form of a white spot lesion which is considered to be the initial stage[1].The caries process involves the formation of an altered bacterial biofilm in the oral cavity, and among the representative bacteria identified to be the causeis the Streptococcus mutans (S.

mutans)[2].Therefore, trials forcontrolling the number of bacteriain dental plaque are regardedas the keystone for proper oralhygiene [3].

Unfortunately, the use of toothbrushes; even incombination with dental floss, did not result in the complete removal of dental plaque [4]. Therefore, several attempts have been made to make a mouthwash with bactericidal or bacteriostatic effects to reduce the caries prevalence[5]. For any mouthwash to be effective, it must be able to reduce the number of harmful bacteria without affecting other beneficial microorganisms present in the oral cavity [6-7].

Chlorhexidine mouthwash was the most commonly studied material and was found to provide significant results[5].Nevertheless, there is a controversy regarding its effectiveness in the prevention of caries, besides the reported side effectsin its long-term use[5].Different types of fluoride mouthwashes were alsoutilized for the prevention of caries in children and resulted in large reductions in caries increment. On the other hand, swallowing large amounts of fluoride proposes a high risk especially in children less than six years[8].

The proposed risks with the use of chemical mouthwashes have aroused the need for new mouthwashes formed from natural ingredients. The advantage of natural products over chemicals lies in the absence of alcohol, their lower toxicity,andtheir non-irritant effect[9].

Moringa Oleifera has been reported to be among the medicinal plants that have antimicrobial effects. The ethanolic extract of Moringa was shown to have the most efficient antimicrobial effect on S. mutans; however, the optimal concentration needs to be investigated[10].Illicium verum known as Star anise was found to be highly effective against oral bacteria responsible for periodontal diseases in many studies[11].This indicates that a potential effect against S. mutans could exist.

Since oral health impacts the quality of life, there is a great demand for the prevention and treatment of oral diseases; especially dental caries[9].Accordingly, this study aimed to investigate the effectiveness of Star anise mouthwash against S. mutans and to find the most effective concentration of Star anise and Moringa Oleifera mouthwashes, compared to Fluoride mouthwash which has been thoroughly investigated in the literature.

Material and methods

This study was a randomized clinical trial registered in clinical trials.gov IDNCT05623605and has an ethical approval number “1434052022”. The permission to collect the herbal plants was taken from the Pharmacognosy Department of Misr University for Science and Technology No. PG7. A voucher specimen of Moringa Oleifera and Star anisewas stored in the Herbarium of the Pharmacognosy Laboratory, Faculty of Pharmacy, Cairo UniversityNo. 4.8.2020/ 5.9.2020 respectively.

Sample size calculation

The sufficient sample size needed to detect an effect size of 0.40, a power (1-β) of 80% ata 5% ($p<0.05$)significant level,was calculated to be 70children divided equally among seven groups. It was calculated using G*Power software; version 3.1.9.4.; where fS=the effect size, $\alpha= 0.05$, $\beta= 0.2$ and Power= $1- \beta = 0.8$.

Patient selection

The sample consisted of 70 childrenbetween the age of 6-13 years old randomly selected from the outpatient clinic of the Pediatric Dentistry Department. Theyhave selected according to the following criteria:no systemic diseases, no history of recent antibiotic administration in the last 2 weeks, antimicrobial mouth-rinse was not used in the last 12 hours, no topical fluoride treatment was done in the last 4 weeks, children with no orthodontic appliances and no previous orthodontic treatment and children low caries index (DMF& def < 4). The treatment plan was explained to the children and their guardiansand written consent was signed by the guardians.

Grouping

The children were randomly divided into seven groups, each group containing 10 children,according to the type of intervention, i.e., the usedmouthwash and the tested concentration.

Group 1: 5% Moringa Oleifera mouthwash
Group 2: 10% Moringa Oleifera mouthwash
Group 3: 15% Moringa Oleifera mouthwash
Group 4: 5% Star anise mouthwash
Group 5: 10% Star anise mouthwash
Group 6: 15% Star anise mouthwash
Group 7: Fluoride mouthwashasthe control group. (DG Wash Fluoride, Al-Esraa pharmaceutical optima)

A sample of at least 1 ml of non-stimulated salivawas collected from all the participating childrenover two intervals: before the intervention and one week after using the mouthwash. The collection of the saliva samples was done in the morning and the children were instructed not to eat or drink anything for one hour prior to the sample collection. Theywere instructed to spit the saliva into a labeled sterile test tube containing transporting media. After collection, the samples were immediately transferred to the microbiology laboratory.

All the children were instructed to rinse their mouths with 5 ml of the given mouthwash twice daily for one week. They were instructed to keep the mouthwash in their mouths for at least 30 seconds before spitting it and not to eat or drink for at least one hour after using the mouthwash.

Randomization and blinding

Randomization was done using the randomization software program (random.org), 70 numbers were randomly and equally divided into 7 groups. The numbers were inserted into an opaque dark envelope and the children were asked to withdraw a number from it. According to the number each child was assigned to its corresponding intervention group. The clinic supervisor conducted this process, which was not part of the investigation. Both the investigators and the children were blinded to the intervention.

Herbal extract preparation:

A 200gm of Moringa Oleifera (Moringa) and Star anisechopped herbs were dried andground into fine powder. After that, 150 gm of each herb was soaked in 96% ethanol for 72 hours. After evaporation of the ethanol, the concentrated extract was stored at 4°C in specialrefrigerators until needed.

Preparation of the mouthwash

In a beaker, both the Moringa and Star aniseextracts weremixed with 5gmof propylene glycol and 2.5gmof Tween. A mixture of sorbitol and tegobetaine was added to the previous preparation. After that, 2.5gm of methylparaben was dissolved in distilled water and added to the mixture. Distilled water was also added to prepare 100ml of mouthwasheswith different concentrations. The PH of the prepared mouthwash was adjusted and tested using a PH meter.

Preparation of MitisSalivariusBacitracin Agar(MSBA) media:

The preparation of MitisSalivariusBacitracinAgar (MSBA) media was done according to the manufacturer's instructions. MSBA powder (9gm) was dissolved in 100ml of distilled water and then autoclaved at 121°C for 15-20 minutes, then it was allowed to cool to 50°C. After that, 0.1 ml of 1% potassium tellurite solution and bacitracin solution were added to the medium. To prepare the bacitracin solution, four bacitracin discs which contain 10IU of bacitracin antimicrobialwere dissolved in 2ml of distilled water, each.Sterile Petri dishes were then filled with 20ml of the medium and dried for 24 hours at room temperature with a 5-10% CO_2 tension [12].

Microbial cultivation of Streptococcus mutans:

In sterile centrifuging tubes, 1 ml of the saliva sample was diluted with4 ml of Brain Heart Infusion broth (BHI). The mixture was vortexed in a cyclomixer to blend the saliva sample with the BHI broth and 0.1 ml of the vortexed sample wastransferred into Streptococcus mutans-selectiveMSBA using an inoculation loop4ml in diameter. The MSBA plates were incubated for 48hoursat 37°C with 5% CO2 in nitrogen. The colonies of Streptococcus mutans were then counted under a microscope depending on their morphological traits on the MSBA. The colonies were identified as being dark blue, elevated, convex, with rough borders, and with a diameter of 0.5 to 1mm. The Streptococcus mutans count was done according to the following equation [12]:

$$\frac{\text{No. of colonies countedxDilution factor}}{\text{The volume of the culture plate}} = \text{numberof colonies/mL(CFU/mL)}$$

Statistical analysis

For each group in each test, the means and standard deviations were calculated. There was a parametric (normal) distribution when the data were explored for normality using

Kolmogorov-Smirnov and Shapiro-Wilk tests.To compare more than two groups in non-related samples,One-way ANOVA followed by the Tukey post hoc test was used. The significance level was set at $P \leq 0.05$. The statistical analysis was performed with IBM®

SPSS® Statistics Version 20 for Windows.

Results

The means of bacterial count and standard deviations of all groups before and after intervention are shown in **Table 1**and **Figure 1**. The results showed that there was a statistically significant difference between all groups;$p<0.001$.

The percentage of bacterial reduction after intervention showed a statistically significant differencebetween the following groups, **Table 2** and **Figure 2**:

- Fluorideand each of Moringa 5%, Star Anise (5%, 10%) groups; p=0.018, p=0.003and p=0.019, respectively.
- Moringa 5%and each of Moringa 10% and 15% and Star Anise 15% groups; p=0.010, p=0.002, and p=0.010, respectively.
- Moringa 10% and each of Moringa 5% andStar Anise (5%,10%) groups; p=0.022 and $p<0.001$.
- Moringa 15% and each of Moringa 5% and Star Anise (5% and 10%) groups; p=0.004and p=0.023.
- Star Anise 5% and each of Moringa (10%, 15%) and Star Anise 15% groups; $p<0.001$.
- Star Anise 10% and each of Moringa (10%,15%) and Star Anise 15% groups; $p<0.001$.
- Star Anise 15% and each of Moringa 5% and Star Anise (5%,10%) groups; $p<0.001$.

While there was no statistically significant differencebetween any other group.
Furthermore, the highest mean percentage of bacterial reduction was found in StarAnise 15% group, while the least mean percentage of bacterial reduction was found in StarAnise 5% group.

Table (1): The means of bacterial count and standard deviations (SD) ofall groups, pre and post intervention

Variables	Bacterial count				p-value
	Pre		Post		
	Mean	SD	Mean	SD	
Fluoride	13300.00	1388.52	7033.33	1398.09	<0.001*
Moringa 5%	17700.00	1912.07	9166.67	750.11	<0.001*
Moringa 10%	12820.00	2279.82	7720.00	1188.95	<0.001*
Moringa 15%	14133.33	1481.44	7466.67	1078.27	<0.001*
Star Anise 5%	12100.00	1405.70	7533.33	1339.65	<0.001*
Star Anise 10%	14600.00	2414.95	8733.33	806.64	<0.001*
Star Anise 15%	15725.00	1680.92	7500.00	782.30	<0.001*

*; significant ($p<0.05$)

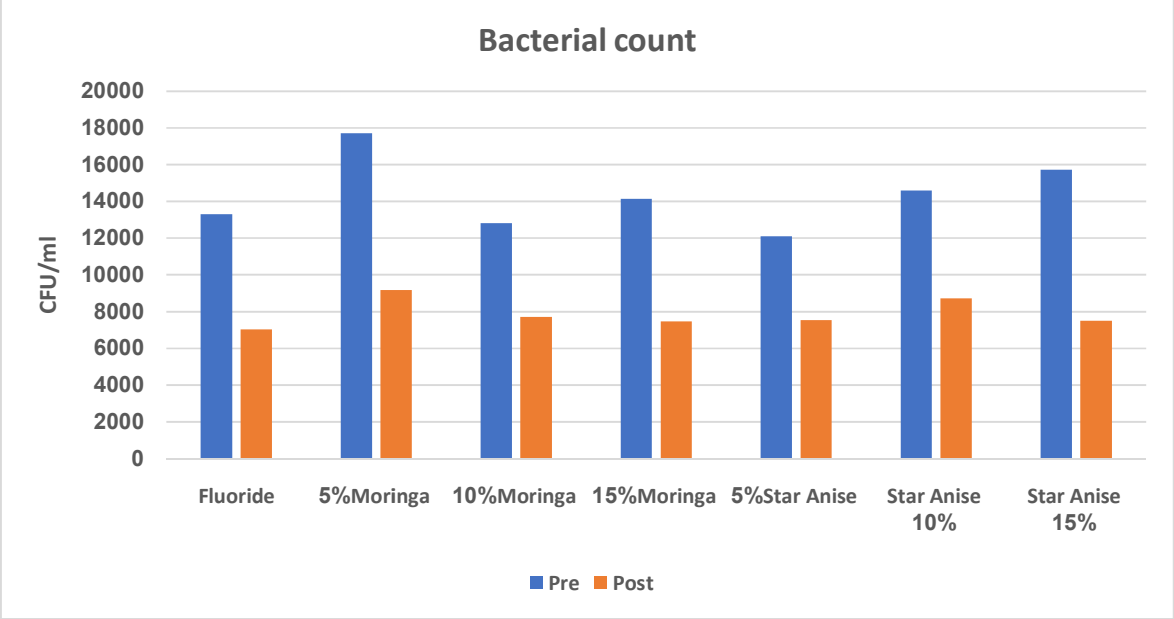

Figure (1): the antibacterial activity in all groups

Table (2): The percentage of bacterial reduction inall groups

Variables	Percentage of bacterial reduction	
	Mean	SD
Fluoride	47.54% [a]	5.06
Moringa 5%	39.53% [b]	4.00
Moringa 10%	47.36% [a]	2.17
Moringa 15%	48.07% [a]	1.94
Star Anise 5%	38.09% [b]	3.73
Star Anise 10%	39.56% [b]	4.50
Star Anise 15%	52.09% [a]	4.80
p-value	**<0.001***	

Means with different small letters in the same column indicate statistically significance differences. *; significant (p<0.05) ns; non-significant (p>0.05)

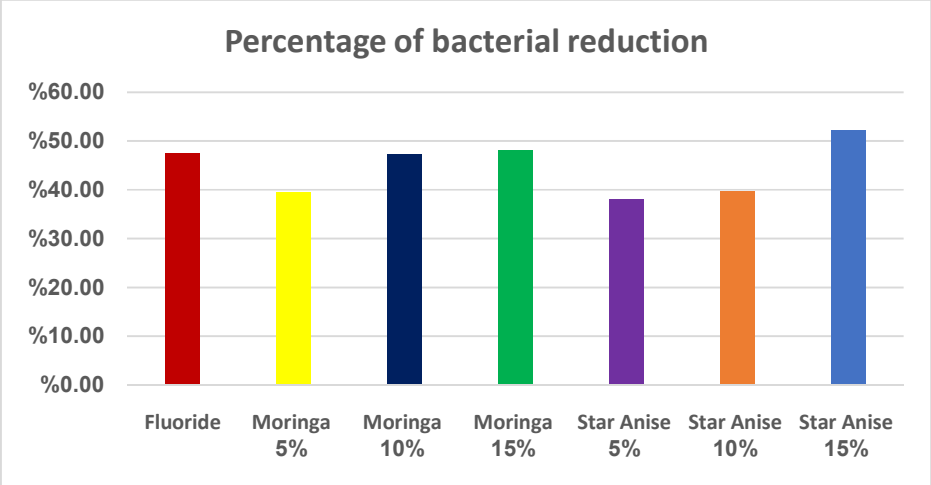

Figure (2):the percentage of bacterial reduction in all groups

Discussion

Dental caries is the most prevalent disease affecting about 60-90% of school children and adults [13-14]. Its levels vary considerably, but they are generally higher in lower socioeconomic classes [15-17]. The oral cavity is a house for over 700 different types of bacteria and Streptococcus mutans (S. mutans) is considered the primary representative of caries [18]. They produce lactic acid through hydrolyzing fructose lowers the oral cavity pH which in turn results in tooth demineralization and caries [19]. Attempting to remove all the bacteria by physical methods like brushing is difficult and requires the additional use of mouthwashes [20].

However, the chemical composition of commercially available mouthwashes and their side effects present a constraint over their long-term use (Kim). As a result, several studies were conducted to find an effective mouthwash with natural constituentsbecause of their reduced potential side effects, broad biological activity, and lower cost [2,11,21].

This randomized clinical trial aimed at investigating the effectiveness of Star Aniseon the reduction of S. mutans bacterial colonies responsible for dental caries, and its most effective concentration along with Moringa Oleifera compared to Fluoride.

The results of this study faced the limitation of being the first study conducted on the effect of Star Anise on S. mutans bacteria.It was also the first to investigate the most effective concentration of either the Star anise or Moringa Oleifera; which presented difficultyin comparing the outcomes of the trial to other studies.

Fluoride mouth rinses were commonly used in public health programs targeting high caries index populations. Their effectiveness in reducing the spread of caries was noted by the stoppage of such programs and the fact that they became part of the personal dental products market[22-24].According to **Whitford**[25], the estimated toxic dose of Fluoride was5 mg/kg body weight, which would be available in 434 ml of the standard daily rinsing solution[8].
The results showed that there was a significant difference in the reduction of bacterial colonies between Fluoride and Moringa 5% and Star anise 5% and 10% groups, with Fluoride being more effective.

Moringa Oleifera has a powerful antibacterial and anti-inflammatory effect (Nurul).**Nuruland Haron**[26] reported that the ethyl alcohol extract of Moringa leaves has potential anti-cariogenic effects against the bacterial biofilm created by S. mutans in the oral cavity. Furthermore, the ethanolic extract of Moringa was found to be more effective against S. mutans,according to **El gamily et al**[10]Nevertheless, the most effective concentration was not identified in their research but was shown to be either 10% or 15%, in this research, with no significant difference either between themselves or Fluoride. The use of Moringa Oleifera though, being a natural substance, presents an edge over the use of Fluoride.

Star Anise was subject to investigation before and was reported to have antibacterial, antifungal, and anti-inflammatory effects [27-30].However, its antibacterial effect against S.

mutans was not reported previously in the literature.From the results, it was observed thatStar anise was efficient in reducing S. mutanscolonies in the oral cavityandthat the 15% concentration was significantly more effectivethan its5% or10% concentration as well as the 5% concentration of Moringa.Interestingly, it was not significantly different from either Fluoride or 10% or 15% Moringa concentrations.

Collectively, it can be said that 15% Star anise, Fluoride, and 10% or 15% Moringa had relatively similar effects as they didn't have any significant differences among them. However, with the 15% concentration of Star anise showing the highest mean percentage of bacterial reduction and being a natural substance; it can be claimed to be the best choice for a new anti-cariogenic mouthwash.

A possible limitation of this study was that monitoring the compliance of the subjects was beyond the control of the investigators.

Conclusions

Star anise was found to be effective against Streptococcus mutans and the most effective concentration was 15%. While the most effective concentration of Moringa Oleifera was found to be either 10% or 15% indifferently.

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