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Effect of *Murraya koenigii* Aqueous Extract as Mouthrinse on Salivary pH, Tongue pH and Glucosyl Transferase Enzyme Inhibition: A Double-Blind Randomized Controlled Trial

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KEYWORDS

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ABSTRACT:

Introduction: The aim of this clinical trial was to comparatively evaluate the salivary pH, tongue pH and glucosyl transferase enzyme inhibition potential in patients with high caries index following the use of *Murraya koenigii* (MK) aqueous extract and 0.2% chlorhexidine (CHX) as mouthwash.

Methods: MK mouthwash was prepared by cold maceration technique and its minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against *Streptococcus mutans (S mutans)* were determined. 36 human participants were randomized to CHX, MK and placebo groups (n=12). Assessment of pre- and post-rinse salivary pH and tongue pH was done along with percentage inhibition of glucosyltransferase enzyme (Gtf). Salivary pH, tongue pH and Gtf inhibition were measured using pH meter, pH indicator strips and colorimetry respectively.

Results: Results showed that the MIC and MBC of MK extract were 1000 µg/mL and 5000 µg/mL respectively. The post-rinse salivary and tongue pH values were significantly higher than placebo group and their respective pre-rinse values in CHX and MK groups ($p<0.05$). No significant difference existed in the post-rinse salivary pH between both the groups at 10 min ($p=0.05$) and 30 min ($p=0.63$). Gtf inhibition of CHX and MK were 81% and 64% respectively ($p<0.05$).

Conclusion: It could be concluded that MK possessed adequate requirements of a potential mouthwash by favorably altering the tongue pH and salivary pH, along with effective Gtf enzyme inhibition in high caries risk patients.

1. Introduction

Dental caries is a multifactorial disease which occurs because of the interaction of cariogenic pathogens, complex biofilm on the tooth surface and fermentable dietary carbohydrates. [1] A shift in the metabolic activity within the microbial biomass results in a localized chemical dissolution of minerals from the tooth surface leading to the formation of a carious lesion. 85% of the restorative procedures are done to rehabilitate the teeth from the consequences of the damage caused by dental caries. [2] This increases the financial and psychological burden on the patients. Hence, a great deal of research focuses on prevention of dental caries globally. The interface between the teeth and the plaque biofilm is highly dynamic and offers wide possibilities of intervention for preventive strategies. Microbial composition, salivary flow rate, its composition and buffering capacity, availability of fluoride, consumption of

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dietary sugars, their frequency of consumption and rate of clearance play a crucial role in determining the balance between the net mineral loss or gain at the tooth-biofilm interface. [3]

Formation of salivary pellicle is the prelude to biofilm formation. The ability of *Streptococcus mutans* to adhere to the pellicle through sucrose-dependent or -independent mechanism makes it one of the main etiologic factors of dental caries. Sucrose-dependent adhesion contributes to the virulence of *S. mutans*. Glucan formation from sucrose is facilitated by the enzyme glucosyltransferases (Gtfs) secreted by *S. mutans*. [4] The synthesized glucans enable stepwise bacterial adhesion to tooth surface as well as to each other thereby, structurally strengthening the biofilm. Novel concepts of caries prevention are directed specifically towards suppression of virulence factors rather than just bacterial viability. Thus, suppression of Gtfs remains one of the strategies for biofilm disruption and caries prevention. [5,6]

The most widely practiced caries-preventive approach has been mechanical plaque removal, aided by chemical agents such as dentifrices or oral rinses. Fluoride has remained the cornerstone of caries prevention for more than a century. Shortcomings of momentary fluoride exposure and concerns of fluorosis in children drove researchers to explore chemical agents as stand-alone or auxiliary aids to fluoride therapy. One such commonly used chemical agent is chlorhexidine (CHX) in the form of chlorhexidine gluconate at a concentration of 0.2%. Though effectively used as an antimicrobial mouth rinse over the years, there is a concern of emerging microbial resistance to CHX. Reports could also be found regarding unpleasant side effects like tooth discoloration, altered taste sensation, dryness and soreness of oral cavity and oral desquamation in children. [7] Absence of microbial resistance, surplus availability and cost-effectiveness have encouraged researchers worldwide to explore natural products as alternatives to existing synthetic antimicrobials. The varying degree of success shown by phytochemicals procured from food sources has made ethnobotanical approach a promising and an effective strategy for disease management. [8]

Murraya koeinigii (MK), commonly known as curry leaf, is an aromatic tree, allied to family Rutaceae, found in India, Bangladesh, Nepal, Malaysia, Sri Lanka and Burma. The MK leaves possess antimicrobial, antioxidant, anti-inflammatory, anti-carcinogenic, anti-diabetic and hepato-protective properties. [9] Previous research has confirmed the antibacterial efficacy of MK extract against *S mutans* and has suggested its use as a mouthwash as it freshens the breath along with antibacterial activity. [10,11]

2. Objectives

Considering the desirable properties of MK, it is intriguing to explore its efficacy as a mouthwash in high risk caries patients. Hence the aim of this double-blind randomized controlled trial was to comparatively evaluate the salivary pH, tongue pH, and Gtf inhibition of MK extract in high-risk caries patients compared to CHX. The null hypothesis was that no significant difference would exist between CHX and MK under the tested parameters.

3. Methods

Preparation of MK solution

Fresh MK leaves were collected from a local organic plantation nursery in Ramapuram, Chennai and were authenticated as organic by a taxonomist. The leaves were thoroughly rinsed using reverse osmosis-treated water. Extraction was done by cold maceration technique. MK leaves were soaked in distilled water within a closed container at room temperature for three days with frequent agitation. The mixture was then press strained by filtration to obtain a pure form of the extract. The prepared MK solution was filled in separate opaque bottles and were sequentially numbered. Green food colouring agent mixed with distilled water served as the placebo.

In vitro antimicrobial activity

Determination of MIC

Streptococcus mutans (MTCC 497) strain was procured from CSIR-IMTECH, Chandigarh, India and inoculated in brain heart infusion (BHI) broth. The broth was incubated at 37°C until the growth reached turbidity equal to or greater than 0.5 McFarland standard. A volume of 20 mg of the MK extract was dissolved in 1 mL of distilled water to prepare the main stock (MS) solution. 0.2 % w/v of CHX was diluted with distilled water to a concentration of 500 µg/mL. The main stock (MS) solution was mixed with BHI broth to obtain the working stock (WS) in dilutions of 3.125 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL, 2500 ug/mL and 5000 ug/mL. A volume of 100 µL of

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inoculum was added to 100 µL of each dilution to make a final volume of 5 mL test sample. Negative control containing 5000 µL of broth only and positive control containing 1000 µL of standard drug containing broth were used for the study. The tubes were incubated under CO_2 at 37 °C for 24 h. Results were evaluated when there was definite turbidity in the positive control. MIC was recorded as the lowest concentration of the agent at which there was visible reduction in the turbidity.

Determination of MBC

Subsequently, a loop full of broth from each test tube showing no visible growth was taken and inoculated in a nutrient agar plate. The agar plates were examined for growth after 24 h to validate the presence of any microbial growth. The lowest concentration of the test sample that showed 99.9% eradication of the bacterial population was determined as the MBC of the sample.

***In vivo* analysis**

Selection of patients and randomization

The study followed the guidelines detailed in the Declaration of Helsinki (1975) and its later amendments. Ethical approval for the study was obtained from the Institutional Review Board of SRM Dental College, Ramapuram, Chennai (SRMDC/IRB/2019/MDS/No.301). The study has been registered under Clinical Trials Registry-India (CTRI/2020/04/024685, https://www.ctri.nic.in)

For the determination of sample size, a pilot study with 5 samples from each group (0.2% CHX, MK and placebo) were taken and analysis of salivary pH was done. Based on the results obtained from the pilot study, sample size estimation was calculated using G*Power 3.1.2 software. The minimum sample size of each group was calculated, following these input conditions: power of 0.95 and $p < 0.05$ and sample size arrived was 12 per group.

36 healthy patients (ASA category I) in the age group of 13-21 years with high caries index (DMFT>5) were recruited from the out-patient wing of the department of Conservative Dentistry and Endodontics, SRM Dental college, Chennai after obtaining their informed consent. Patients undergoing fixed orthodontic therapy and those who had taken antibiotics in the past 2 months were excluded.

Patients were randomized into 3 groups (n=12) based on the mouthrinse used namely, 0.2% CHX, MK and placebo using computer generated randomization on www.sealedenvelope.com (block randomization technique). The allocation concealment was done by the SNOSE (sequentially numbered, opaque, sealed envelopes) method. The patients and assessor were blinded.

Analysis of salivary pH and tongue pH

Complete oral prophylaxis was administered to the patients 4 h prior to the procedure. The baseline (pre-rinse) salivary and tongue pH were evaluated. Unstimulated saliva was collected from the floor of the mouth. The patients were then asked to chew on paraffin wax (0.5X0.5cm) for 3 minutes to stimulate salivary flow, which was then collected using a 2.5 mL sterile disposable syringe for evaluating salivary pH. Salivary pH was determined using a pH meter. Tongue pH was measured with the patient's mouth open, and tongue stuck out, by placing a sterile pH indicator strip over the surface of the tongue for 3 seconds and removing it when a color change from orange to dark green was noted. According to their group allocation, 10 mL of the respective mouth rinse was given to the patients and they were advised to swish the solution in the mouth for 30 s. Post-rinse salivary and tongue pH samples were collected at 5 min, 10 min and 30 min time intervals.

Ex vivo* analysis of Gtf inhibitory activity of *S mutans

Production and partial purification of Gtf

A partially purified Gtf required for enzyme inhibition was prepared according to the modified method stated by Tomita et.al. *S mutans* isolated from the patient's plaque sample (pre- and post-rinse) was inoculated into the media containing BHI broth with 5% sucrose. [12] 1 mg/mL of Tween 80 solution was added to the media and incubated at 37°C for 24 h. Bacteria grown was further centrifuged for 30 min (1800xg) and the obtained supernatant fluid was precipitated using 2/3 volume of ammonium sulfate (60%). The precipitate was further centrifuged and dissolved in a 30 mL phosphate buffer (pH 7.4) and dialyzed for 24 h against the same solution. The insoluble material was then removed by centrifugation and the dialysate obtained was preserved at 0°C until further use.

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Preparation of the experimental and control samples

Experimental test tubes were filled with a mixture of 0.25 M sucrose, Gtf enzyme solution and phosphate buffer (pH 7). 0.025 mL of MK extract, sterile water (negative control) or 0.025mL of 0.2% CHX (positive control) added to this mixture formed the respective groups. The test tubes were then incubated at 37ºC for 2 h.

Analysis of Gtf enzyme inhibition activity

In this method, Gtf activity was determined based on the amount of glucans formed, which is expressed as glucose content per minute. The reaction mixture (50 µL) was incubated at 37°C for 2 h. The reaction was terminated by the addition of 0.6 M sodium dodecyl sulfate, and the formed water insoluble glucan (WIG) pellet was precipitated by centrifugation at 10,000 x g for 5 min. The supernatant was transferred to another tube and water soluble glucan (WSG) was precipitated with 3 volumes of ethanol at 4°C overnight. The washed polysaccharide (WIG & WSG) was quantified based on the glucose content using phenol-sulfuric acid method and a rapid calorimetric assay was performed to analyze formation of WSG and WIG.

Statistical Analysis

SPSS V 20.0 (IBM Corp, NY, USA) was used for data analysis. One-way ANOVA was used for intergroup comparison. Intragroup comparison was done by using repeated measures ANOVA and multiple comparison was done by using Tukey's post-hoc test.

4. Results

Table 1: Salivary pH values (pre- and post-rinse) of all the groups

Groups	Salivary pH			
	Pre-rinse	Post-Rinse		
		5 min	10 min	30 min
CHX	6.6±0.75[a]	8.28±0.59[a]	7.81±0.48[a]	7.73±0.31[a]
MK	7.15±0.72[a]	7.44±0.38[b]	7.47±0.31[a]	7.37±0.33[a]
Placebo	6.54±0.06[a]	6.48±0.04[c]	6.47±0.06[b]	6.49±0.05[b]

*Under each column, different superscript alphabets indicate significant difference between the groups ($p<0.05$).

Table 2: Tongue pH values (pre- and post-rinse) of all the groups

Groups	Tongue pH			
	Pre-Rinse	Post-Rinse		
		5 min	10 min	30 min
CHX	5.93±0.57[a]	7.87±0.61[a]	7.43±0.51[a]	7.18±0.40[a]
MK	6.35±0.49[a]	7.05±0.42[b]	7.05±0.42[b]	7.05±0.42[b]
Placebo	6.0±0.0[a]	6.0±0.0[c]	6.0±0.0[c]	6.0±0.0[c]

*Under each column, different superscript alphabets indicate significant difference between the groups ($p<0.05$).

Table 3: percentage of glucosyltransferase enzyme (Gtf) inhibition of all the groups

Groups	Gtf inhibition (in %)
CHX	81.04±2.91[a]
MK	64.19±5.52[b]
Placebo	5.41±0.96[c]

*Under each column, different superscript alphabets indicate significant difference between the groups ($p<0.05$).

The MIC and MBC of MK extract against *S mutans* were 1000 µg/mL and 5000 µg/mL respectively. The salivary and tongue pH values (pre- and post-rinse) and percentage of Gtf inhibition of all the groups are given in tables 1, 2 and 3 respectively. No significant difference existed between the groups in the pre-rinse salivary and tongue pH values ($p<0.05$). The post-rinse salivary and tongue pH values were significantly higher than the respective pre-rinse values in CHX and MK groups ($p<0.05$). The mean post-rinse salivary and tongue pH in the placebo group was significantly lesser than CHX and MK at all tested time intervals ($p<0.05$). Post-rinse salivary pH in CHX was significantly higher than MK at 5 min ($p=0.00$), but no significant difference existed between the both the groups at 10 min ($p=0.05$) and 30 min ($p=0.63$). CHX showed significantly higher post-rinse tongue pH values compared to MK at all time intervals ($p<0.05$). Spectrophotometric analysis showed that the Gtf inhibition of CHX and MK were 80% and 64% respectively which was statistically significant ($p<0.05$).

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5. Discussion

Oral malodor develops from plaque, breakdown products of food or byproducts of intraoral bacterial metabolism. Mouthwashes can reach areas in the mouth which are inaccessible to the bristles of a toothbrush. Hence, they are excellent means of providing therapeutic benefits for oral diseases like dental caries and gingivitis. Fluorides, CHX and cetylpyridinium chloride and essential oils are some of the active ingredients that are used in therapeutic mouthwashes. [13] CHX, owing to its antimicrobial potential, substantivity and antibiofilm properties is widely prescribed as a mouthrinse at lower concentrations to treat malodor and gingivitis. But, CHX has reportedly caused xerostomia, hypoguesia, glossodynia, coated tongue, oral mucosal desquamation, and staining of teeth, tongue and restorations in the oral cavity. [14]

Humans have sought cure for diseases from nature and hence herbal medicine is gradually spreading into dentistry. Dental researchers have done research on various herbal alternatives like ginger, tulsi, neem, cloves, cardamom and eucalyptus as a remedy for oral diseases as these are anti-inflammatory, antimicrobial and antibiofilm in nature. [15] Tea tree oil, papaya leaves and curcumin when used as herbal alternatives for chlorhexidine present with several inadequacies such as oral mucosal burning sensation, water insolubility and poor bioavailability necessitating the need to explore newer natural products with suitable properties and their diffusible components must be critically evaluated for the successful development of sustainable products. [16-18]

Murraya koenigii (MK) or curry leaves have been proven to have a wide range of antimicrobial, anti-inflammatory, antiemetic, antidiabetic, antiulcer and antioxidative properties with reported use in medicine for various ailments such as leucoderma and blood disorders. [19] Fresh MK leaves contain 2.6% volatile essential oils, sesquiterpenes and monoterpenes which are sufficiently soluble in water. The major constituents identified are D-α-pinene (51.7%), D-sabinene (10.5%), β-pinene (9.8%), β-caryophyllene (5.5%), limonene (5.4%), bornyl acetate (1.8%), terpinene-4-ol (1.3%), γ-terpinene (1.2%), and α-humulene (1.2%). [10] The carbazole alkaloids in curry leaves have been reported to be a potent anticariogenic agent. Hence in this study MK was used as a mouthwash and its effect on salivary pH, tongue pH and glucosyltransferase enzyme inhibition were evaluated.

Tongue coating is a major indicating factor for halitosis causing elements. Even with proper brushing and flossing, mechanical plaque control alone is insufficient to manage halitosis because complete removal of oral bacteria is not achievable. Adjunctive use of antiseptic mouthrinses can more effectively suppress microbial growth. Chlorhexidine (CHX) exhibits strong substantivity, binding to oral tissues and remaining active for approximately 8–12 hours after a single rinse. This prolonged antimicrobial action helps reduce volatile sulfur compound–producing bacteria and contributes to improved control of halitosis. MK has antimicrobial properties as the leaves contain essential oils and terpenoids that target odor causing bacteria and reduce plaque formation and gingivitis. [20]

Saliva is a complex body fluid which plays a pivotal role in the balanced maintenance of oral health. Saliva is required for protecting the oral mucosa, tooth remineralisation, digestion, phonation and taste sensation. It possesses a variety of electrolytes, peptides, glycoproteins and lipids which have anti-microbial, antioxidant, tissue repair and buffering properties. Salivary function is predominantly regulated by its pH. A salivary pH of 7.0 usually indicates a healthy dentition and periodontal situation. At this pH, there is a low incidence of dental caries. [21] CHX was more effective in increasing post-rinse salivary pH at 5 min. The ability of CHX to reduce acidogenic and aciduric potential of plaque has been supported. [22] For MK group, the change in the pH might be justified by the presence of abundant bicarbonates in the stimulated saliva and increased buffering capacity caused by various mineral components present in the MK mouthwash. [23] The bicarbonates possess buffering action that neutralizes the salivary pH, thereby reducing the tongue coating pH, which depletes the formation of odoriferous compounds and thus reducing halitosis. In addition, an increased salivary pH offers therapeutic anti-caries benefit by actively reducing demineralization and increasing remineralization of the enamel crystals which are damaged by acid attacks. [23]

S. mutans are the principal cariogenic bacteria in the human oral flora. It possesses several virulence factors that are associated with its cariogenicity with sucrose-dependent and glucan-mediated colonization of tooth surfaces being among the most important factors. Glucans, either water insoluble glucans (WIG) or water-soluble

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glucans (WSG) are glucose polymers synthesized by extracellular glucosyltransferase enzymes (Gtfs) and are vital in the processes of biofilm formation and stabilization. [24,25] Targeting Gtf enzymes prevents the synthesis of extracellular polysaccharides (glucans) and is an attractive strategy for the development of anti-biofilm compounds. The ability to inhibit Gtf enzyme will confirm the efficacy of an agent as a potent anti-cariogenic agent or vice versa. [25] Phytochemical rich extracts and their associated compounds have repeatedly shown inhibitory effects against microbial adhesion, plaque and biofilm formation of *S mutans* on teeth. [26] As inhibition of essential virulence factors is the primary goal for the prevention of dental caries and possibly other plaque related disease, the evaluation of the percentage of inhibition of Gtf is of great value.

The Gtf activity can be indirectly determined by rapid luminescence, spectrophotometric analysis, colorimetric analysis and ELISA methods. A partially purified Gtf required for enzyme inhibition was used for the study. [27] A significant Gtf inhibition by CHX in the present study is in accordance with previous studies. [28] Chlorhexidine has multiple modes of action that substantially reduce GTF activity by prevention of the synthesis of glucans, non-competitive inhibition and disruption of biofilm architecture. The percentage of gtf inhibition was 64% for the MK group. This could be due to the presence of mahanine, mahanimbine or murrayanol present in MK, which has proven efficacy against dental caries and periodontal disorders. There have been various studies to prove the effect of these components against *S mutans* with favorable results. [29,30]

The results of the present study showed that MK possesses adequate requirements of a potent mouthwash by altering the tongue and salivary pH, with effective inhibition of Gtf enzyme and reduction in plaque biofilm. Thus, *Murraya koenigii* can be used as an alternate herbal mouthwash. Recent studies stating the potential of CHX to induce oral dysbiosis further reiterates the need to develop natural alternatives as potential therapeutic agents. Larger, long-term clinical trials and phytochemical analyses are warranted to standardize formulations and clarify mechanisms of action. The effectiveness of alcoholic and ethanolic extracts of MK needs to be studied. Future studies should evaluate MK in association with other ingredients added to commercial mouthwashes for providing anti-bacterial, anti-inflammatory, anti-oxidant and breath freshener effects. Within the limitations of the present study, it can be concluded that CHX was effective in increasing the salivary pH immediately within 5 min. MK extract was equally effective as CHX in increasing salivary pH at 10 min and 30 min and tongue pH at all tested time intervals. GTF inhibition of CHX was superior compared to MK.

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7. Conflict of Interest

The authors declare that there are no conflicts of interest related to this work.

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