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ORIGINAL RESEARCH

Anticariogenicity of *Stevia rebaudiana* Extract when used as a Mouthwash in High Caries Risk Patients: Randomized Controlled Clinical Trial

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ABSTRACT

Aim: To assess the anticariogenicity of microwave-assisted 0.5% extract of *Stevia rebaudiana* leaves in high caries risk patients.

Materials and methods: Forty-six patients with high risk for caries were selected. They were randomly assigned to two groups; group I was allocated for chlorhexidine (CHX) mouthwash (0.12% Perio-Aid) and group II for *S. rebaudiana* (0.5% extract of *Stevia* bio) mouthwash. Salivary pH, buffer, and microbial count were assessed before the patients were asked to use the mouthwashes. Patients were prescribed the mouthwash/extract twice a day for 7 days. On the 8th day, postrinse salivary pH, buffer analysis and *Streptococcus mutans* and *Lactobacilli* count were done.

Results: Significant difference in pre- and postrinse values of pH and buffer was found in both groups. However, no difference was noted between groups. Both groups decreased the bacterial count to $<10^5$ colony-forming units (CFUs) in all the patients.

Conclusion: *Stevia rebaudiana* extract in 0.5% concentration improved the pH and buffering capacity of the saliva in a high caries risk patient. It also reduced cariogenic organisms in saliva.

Clinical significance: *Stevia rebaudiana* extract in 0.5% concentration can be used as a mouthwash for moderate-to-high caries risk patients. However, long-term clinical studies are required to prove its substantivity like that of CHX.

Keywords: Anticariogenicity, *Lactobacilli*, Mouthrinse, Randomized controlled clinical trial, *Stevia rebaudiana*, *Streptococcus mutans*.

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INTRODUCTION

Dental caries is a disease, i.e., characterized by the localized destruction of susceptible dental hard tissue by acidic by-products from bacterial fermentation of dietary carbohydrates.[1] The significance of microorganisms in the etiology of dental caries has been highlighted in the ecological plaque hypothesis.[2] Although *Streptococcus mutans* and *Lactobacilli* have been identified as the main cariogenic organisms, the Keyes' circle effectively describes the interaction of the causative factors in dental caries, namely, host, diet, microbes, and time.[3] A prolonged interplay of these factors results in loss of tooth structure in the form of a carious lesion.

These incipient lesions are managed by restorative or by nonoperative treatments.[4] Nonoperative treatments act at various levels against dental caries: At tooth level, microbial level, and salivary level. One of the nonoperative treatments is the control of the cariogenic microorganisms by antimicrobial therapy, in the form of mouthwashes, sprays, dentifrices, gels, varnishes, chewing gums, and lozenges. Chemical/synthetic agents, such as CHX, triclosan, and xylitol are some commonly used antimicrobial agents in caries prevention.

The CHX is a *bis*-biguanide, which is bactericidal as well as fungicidal. It is a broad-spectrum agent targeting both Gram-positive and Gram-negative organisms. The agent is very effective against *S. mutans*. The CHX has high substantivity, which is a main reason for its superior antimicrobial effect.[5] Since it is a broad-spectrum antimicrobial, its routine use can alter the microbial equilibrium in the mouth. Therefore, it should be prescribed in appropriate doses only in selected high-risk patients for a short time.

In contrast to the synthetic chemicals, natural products, such as herbs and herbal extracts have been found to be biocompatible with the tissues and the body. Herbal medicines have been tested over time as a solution for all oral health problems. Extracts of tulsi, neem, green tea, and *Terminalia chebula* have been tested for their anticariogenicity, in both *in vivo* and *in vitro* studies.[6,7]

Stevia rebaudiana (bertoni leaves) is a herbaceous perennial plant of the family *Asteraceae*, and is indigenous to Paraguay and Brazil. It is cultivated in parts of Asia, Europe, and Canada. In India, the cultivation of *S. rebaudiana* herbal plant is mostly seen in Gujarat and it is commercially available in powder form. The leaf extract of *S. rebaudiana* is used to sweeten foods and is also

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used as a dietary supplement. The major components are glycosides, namely, stevioside and rebaudioside-A. These compounds exhibit characteristic organoleptic properties and have sweetness intensities more than 300 times that of sucrose. Leaf extract of this plant has been used traditionally for the treatment of diabetes. It has been investigated as an antihypertensive, antihyperglycemic, and an antioxidant.[8] In 1992, Das et al[9] have assessed the cariogenic potential of the extracts and found them not to be cariogenic.

Studies have evaluated the antimicrobial potential of the extracts of *S. rebaudiana* against many pathogens.[10-12] Few *in vitro* studies have assessed their antibacterial action on *S. mutans*.[13,14] However, only few clinical studies have been reported on its impact on the salivary level of *S. mutans*.[15,16] As there is insufficient evidence available regarding *S. rebaudiana* as an anticariogenic substance, the following research question was raised.

"In high caries risk patients of 18 to 25 years' age group, with use of 0.5% stevia, microwave-assisted extract as a mouthrinse when compared with 0.12% commercially available CHX mouthwash, will there be a reduction in pH, improvement in buffering capacity, and reduction in cariogenic organisms in the saliva?"

The null hypothesis formed was as follows: "There will be no significant difference between 0.5% stevia and 0.12% CHX mouthwash in any salivary parameter".

MATERIALS AND METHODS

Patients between the age group 18 and 25 years attending the outpatient Department of Conservative Dentistry and Endodontics of the institute were included for the study. Patients were categorized as high risk using caries risk assessment sheet. A total of 46 (n = 46) such patients were randomly allocated to both groups using simple random sampling as shown in the flow diagram (Flow Chart 1).

Group I: (n = 23) patients who were given 0.12% CHX mouthwash (Perio-Aid, Dubai).

Group II: (n = 23) patients who were given 0.5% Stevia mouthwash (aqueous extract of Stevia Bio, India).

Patients who were already on any mouthwash regime were excluded from the study. In addition, those who were on any medication that would alter the salivary flow were also not included in the study. The presence of systemic disease was another exclusion criteria.

Procedure for Salivary Analysis of pH, Buffer, and Bacterial Count

Unstimulated saliva was taken for pH analysis and stimulated saliva was obtained for buffer and microbial analysis. The GC saliva kit (GC, Asia) was used for the analysis of pH and buffer. This chairside kit uses a traffic light model color coding for the changes in theses parameters. The caries risk test (CRT) bacteria kit (Ivoclar Vivadent) was used for salivary analysis for the cariogenic bacteria. It consists of agar culture media plate where *S. mutans* can be cultured on one side and the *Lactobacilli* on the other side. A master chart is also provided to compare and grade the CFUs.

Salivary pH Analysis

The patient was asked to spit the saliva collected in the mouth without any stimulation into the beaker provided. The amount collected and the viscosity of the saliva were noted. The pH strip of the GC saliva kit was dipped into the saliva. After 10 seconds, the color change was compared

Flow Chart 1: Consort diagram—allocation of groups

with the chart provided in the kit. Red change denotes highly acidic resting saliva with pH 5.0 to 5.8. Yellow change indicates moderately acidic saliva with pH of 6.0 to 6.6. Green denotes healthy saliva with pH 6.8 to 7.8.

Salivary Buffer Analysis

Stimulated saliva was collected by asking the patient to chew on the paraffin wax provided in the kit. The saliva collected in the first 30 seconds was discarded. The patient was instructed to continue to chew on the paraffin wax and periodically expectorate the saliva into the beaker. After 5 minutes, the amount collected was noted. The buffer strip from the GC saliva kit was used to assess the buffer. The saliva was taken with a dropper and placed on the buffer strip in three different areas. After 2 minutes, the color change is observed. They were again matched with the chart provided. Green is given 4 points. Blue was given 2 points and red 0 point. The values for three color changes in the strip were added and averaged. If the score is 0 to 5, it is categorized as poor buffer, 6 to 9 as moderate buffering capacity, and if it is 10 to 12, it is a good buffering capacity.

Salivary Microbial Analysis

The stimulated saliva was taken in the pipette and dispensed onto the culture plates provided by the CRT bacteria kit. One side is meant for *S. mutans* growth and the other side is for the *Lactobacilli* growth. After placing the saliva, excess was removed and the plate was incubated for 48 hours in a microbiological laboratory. The growth on the plates was compared with the chart provided and graded in CFU/mL. About $<10^5$ CFU/mL of *S. mutans* and *Lactobacilli* in saliva is considered low caries index and $\geq 10^5$ CFU/mL is considered as high caries index.

Stevia Preparation

Preparation of the mouthwash was done with commercially available, partly powdered, dried leaves, Stevia Bio. About 5 gm of herbal powder was weighed in a weighing machine. It was mixed with 1 L of double-distilled water. Beaker was placed inside the microwave for 5 minutes at 1350 W for extraction of the stevia extract.[17] For further purification, the extract is centrifuged. Centrifugations were done at 100c at 10,000 rpm for 20 minutes. Centrifuged solutions were immediately dispensed into amber-colored bottles. Solutions were prepared freshly whenever required.

Prescription of Mouthwash

The patients in group I were given 10 mL of 0.12% and were asked to rinse morning after breakfast and night after dinner for 1 week. The patients in group II were given the 0.5% stevia extract and provided the same instructions to rinse as group I patients. They were blinded to the mouthwash they were prescribed to. They were asked not to eat or drink anything for half-an-hour after using the rinse. Patients were asked to report on the 8th day after breakfast, and they were asked to use the mouthwash in the author's presence. They were asked to wait for half-an-hour and then the postrinse analysis was done. A measuring cup is given for the patients to measure the quantity of the mouthwash to use.

Monitoring

Participants and the operator were blinded in the study. Each patient was assigned two calibrated people to monitor the usage. Phone calls were done twice a day, after breakfast and after dinner to remind and ensure the usage. These phone calls were made on the 2nd, 4th, and the 6th day. In case a patient misses a call, a reminder call was made in 30 minutes and then 45 minutes and then after an hour. Even after all the reminder calls if the patient fails to attend, a reminder in the form of text message was sent.

Evaluation of Outcomes

The salivary pH, buffer, and microbial counts were analyzed on the 8th day of postrinse using the same protocols as in the prerinse analysis. The data were statistically analyzed using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com.

RESULTS

For both groups, mean and standard deviation (SD) of the pre and postrinse pH and buffer have been calculated (Tables 1 and 2). As the data had a nonnormal

Table 1: Mean and SD of pre and postrinse pH of CHX and stevia mouthwash groups

Groups	*Prerinse pH Mean ± SD*	*Postrinse pH Mean ± SD*	*Difference in pH postrinse Mean ± SD*
Group I (0.12% CHX)	6.33 ± 0.24	6.8 ± 0.28	0.47 ± 0.04
Group II (0.5% Stevia)	6.38 ± 0.22	6.9 ± 0.32	0.52 ± 0.10

Table 2: Mean and SD of pre and postrinse buffer of CHX and stevia mouthwash groups

Groups	*Prerinse buffer Mean ± SD*	*Postrinse buffer Mean ± SD*	*Difference in buffer postrinse Mean ± SD*
Group I (0.12% CHX)	3.35 ± 1.03	9.60 ± 1.61	6.25 ± 0.58
Group II (0.5% stevia)	3.40 ± 0.25	9.43 ± 0.31	6.03 ± 0.06

Table 3: Wilcoxon matched-pairs signed-rank test comparing the means between the pre and postrinse values of pH and buffer for both groups, at $p < 0.0001$

	Group I (0.12% CHX)		*Group II (0.5% Stevia)*	
	pH	*Buffer*	*pH*	*Buffer*
Signed rank	−216.00	−276.00	−270.00	−276.00
Sum of positive ranks	18.500	0.000	3.000	0.000
Sum of negative ranks	−234.50	−276.00	−273.00	−276.00
Number of pairs	23	23	23	23
One tailed p-value	<0.0001*	<0.0001*	<0.0001*	<0.0001*

*Highly significant

Table 4: Mann–Whitney test comparing the mean difference in pH and buffer between CHX group and stevia group

pH	*Buffer*
Mann–Whitney U-statistic = 247.00	Mann–Whitney U-statistic = 243.50
U′ = 282.00	U′ = 285.50
Sum of ranks in CHX = 523.00	Sum of ranks in CHX = 561.50
Sum of ranks in stevia = 558.00	Sum of ranks in stevia = 519.50

p-value is 0.3491, considered not significant; p-value is 0.3117, considered not significant

Table 5: Reduction in cariogenic organisms postrinse

Groups	*S. mutans*	*Lactobacilli*
Group I (0.12% CHX)	100% reduction	100% reduction
Group II (0.5% stevia)	100% reduction	100% reduction

distribution, Wilcoxon matched-pairs signed-rank test was done to compare the means of pre and postrinse pH and buffer values within the groups. The difference for both pre and postrinse salivary parameters in both CHX and Stevia group is highly significant at $p < 0.0001$ (Table 3). Mean and SD of the pH change and buffer change after the rinse were analyzed between the groups with Mann–Whitney test. There was no statistically significant difference between the stevia group and CHX group for pH change or for buffering capacity (Table 4). There was 100% reduction in microbial colony count in groups I and II after the rinse on the 8th day (Table 5).

DISCUSSION

As per the Caries Management by Risk Assessment (CAMBRA) guidelines, antibacterial mouthwash is an important caries preventive therapy for prevention or control of dental caries in high-risk individuals.[18,19] Apart from the use of well-known antibacterial mouthwashes, such as CHX, there is a recent surge in the use of natural products as oral care therapies. The antibacterial property of the phytochemicals, essential oils, and flavonoids, extracted from plant or food products, against *S. mutans*, have been established by many *in vitro* and *in vivo* studies.[20]

The hypothesis generated in this study was based on the established antibacterial properties of the glycosides from plant products on microorganisms. The leaves of *S. rebaudiana* are a natural sweetener. It has around 100 phytochemicals. Stevia has two main glycosides that are Stevioside (110–270 times sweeter than sugar) and Rebaudioside A (180–400 times sweeter than sugar). The leaves also contain 80 to 85% water. Ethanolic and methanolic extracts of stevia leaves have been found to be effective against the Gram-negative and Gram-positive organisms. In few *in vitro* studies, aqueous extracts of stevia have been shown to be ineffective against *S. mutans*.[13,14] Despite such an evidence, an aqueous extract of the stevia leaves was planned to be used as mouthwash in this study, for safety and palatability of patient's use.

Several extraction methods are available to extract the content from plant products. Modern methods were optimized in terms of temperature, duration of the process, stability, and quantity of molecules extracted. One such method is microwave-assisted extraction (MAE). The MAE uses pressurized and/or supercritical fluids or microwaves to reduce extraction time. Moreover, in MAE, the solvent volume used is much less than that required in conventional extraction methods. The microwave extraction method is more effective in terms of yield, time, and energy consumption in comparison with conventional and ultrasound techniques.

In this study, to enhance the solubility and extraction of the components of the leaf in an aqueous medium, MAE was used.[17] Extraction was carried out at different power levels ranging from 20 to 160 W with extraction time ranging between 30 seconds and 5 minutes with a temperature range of 10 to 90°C.

Salivary pH and buffer are biological indicators for high caries risk.[21] The *S. mutans* metabolizes the easily fermentable sugars to produce lactic acid that results in the acidic pH of the saliva/biofilm. This, in turn, aids in proliferation of aciduric and acidogenic organisms and results in the demineralization of the tooth structure. Polyol-containing sugar substitutes, such as xylitol are nonfermentable sugars; thus, they have been proven to be anticariogenic.[22] However, very less evidence is present on the *in vivo* effect of the stevia extract on pH and buffer of saliva.[16]

In this study, there was a statistically significant difference in the mean difference between pre and postrinse parameters of pH and buffer in the stevia group as well as in the CHX group. This means that both mouthwashes were effective in favorably altering the pH and the buffer. While comparing the efficacy of stevia mouthwash over

CHX mouthwash, it was found that stevia mouthwash performed as good as the CHX mouthwash in altering the salivary parameters. This is in accordance to a study by Brambilla et al,[16] where 20 volunteers rinsed with stevia solutions and sucrose solutions. Postrinse, the plaque pH was measured at 7 time points after each rinse. It was found that the pH dropped to acidic with sucrose solutions and not with stevia solution, concluding that stevia is nonacidogenic. The prerinse pH of the plaque collected from the volunteers in the study were normal, whereas in our study, the prerinse salivary pH was acidic, as it was collected from high caries risk patients, which became normal after the rinse with stevia extract. Thus, it can be inferred from the current study that stevia extract is not only nonacidogenic but also tends to lower the acidic pH of saliva.

The result of the current study also shows both extract of stevia and CHX mouthwash were effective in reducing the cariogenic microbial count over duration of 1 week. This is also in accordance with previous *in vitro* and *in vivo* studies. In 2012, Gamboa and Chaves[11] evaluated the antibacterial activity of *S. rebaudiana* leaf extracts against cariogenic bacteria in an *in vitro* study. It was reported to have inhibitory effect on both *S. mutans* and *Lactobacilli*, with the latter being more sensitive. An *in vitro* study by Giacaman et al[23] evaluated the effect of several sweeteners on enamel demineralization and on the cariogenic properties of *S. mutans* biofilms in an artificial caries model. It concluded by stating that stevia reduced the number of viable cells and the extracellular polysaccharide formation when compared with sucrose. Similarly, Brambilla et al[16] had performed *in vitro* analysis with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on biofilm formation and found stevia to form lesser biofilm than sucrose. Thus, it can be inferred that stevioside is not only antibacterial but also a nonfermentable sugar compared with sucrose, which is a probable reason for reduction in the biomass in our study.

The presence of numerous essential oils is attributed to be one of the reasons for the antibacterial cation.[24] It also contains nonglycosidic labdane diterpenes, flavonoids, phenolic compounds, vitamins, nutrients, phytosterols, and triterpenes. Steviol glycosides have also shown to be potent antioxidants, which might retard the growth of cariogenic organisms. This has high correlation to the total phenolic and flavonoid contents of the extract.[25] Steviosides offer several advantages over other noncaloric sucrose substitutes: They are heat-stable, resistant to acid hydrolysis, and nonfermentable. Further *in vivo* research has been recommended to make the best use of this plant extract.[26]

Within the limitations of this study and from the evidence obtained, it can be concluded that *S. rebaudiana* mouthwash decreased the acidic pH and improved the buffer in high-risk individuals. Its antimicrobial activity against the cariogenic organisms was also like that of CHX.

CONCLUSION

Microwave-assisted *S. rebaudiana* extract when used as a mouthwash in high caries risk individuals had exhibited the following:

- Improved the pH and buffering capacity of the saliva in a high-risk patient.
- Antimicrobial efficacy against *S. mutans* and *Lactobacilli* like that of CHX.

However, long-term clinical studies are required to prove its substantivity like that of CHX.

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