Comparison of Antibacterial Efficacy of Aqueous Suspension, Alcoholic Extract and their Combination of *Stevia rebaudiana* against two Cariogenic Organisms—An *in vitro* study

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ABSTRACT

**Objectives:** The purpose of this study was to compare the effectiveness of aqueous suspension, alcoholic extract and their combination of *Stevia rebaudiana* against *Streptococcus mutans* and *Lactobacillus acidophilus*.

**Materials and Methods:** Preparations of aqueous suspension, alcoholic extract and their combination was subjected to microbiological assay to determine the Minimum Inhibitory Concentration (MIC) by broth dilution method and Minimum Bactericidal Concentration (MBC) using agar plate sub-culture Streaking method at various concentrations. One-way analysis of variance (ANOVA) test was used for multiple group comparisons followed by Tukey post hoc for group wise comparisons.

**Results:** MIC test was done in triplicates. The mean MIC of aqueous suspension against *S. mutans* and *L. acidophilus* was 0.83±0.28 and 0.66±0.28 respectively, which was significantly better (p<0.05) than alcoholic extract and the combination which were 4.16±1.44 & 3.33±1.44 and 5±1.73 & 5±1.73 respectively. Post-hoc Tukey group wise comparison test also showed significant mean differences between aqueous suspension and the other two preparations against *S. mutans* and *L. acidophilus*.

**Conclusion:** The inhibitory effect shown by aqueous suspension of *Stevia rebaudiana* against *S. mutans* and *L. acidophilus* was superior when compared with that of the alcoholic extract and their combination.

**Key-words:** Dental caries, *Stevia rebaudiana*, Plant suspension, Sugar substitute, Minimum inhibitory concentration

INTRODUCTION

Dental caries is known to be a chronic and infectious disease which leads to the alteration in oral microflora, of which *Streptococcus mutans* and *Lactobacillus acidophilus* play a major role in caries production.

Their participation in dental caries therefore has led to the development and implementation of new and preventive measures in the control of dental caries. Some studies have shown that caries can be prevented by regular tooth brushing and flossing, but however, for an effective caries control, these methods should be combined with the chemo prophylactic agents such as chlorhexidine mouth rinses and antibiotics which can lower the number of microorganisms or inhibit the dental plaque formation. However, they have several undesirable side effects which include tooth staining and bacterial resistance. Therefore, implementation of new caries preventive strategies can be significantly improved.
in less developed countries and people with low socio-economic status[^1]. Current research is focused on the elaboration of a new methodology that is based on the identification of natural active compounds that have anti-caries activity[^4].

One such plant is *Stevia rebaudiana* Bertoni, a natural sweetener and a perennial shrub of the Asteraceae family, native of Paraguay and Brazil. The glycosides Stevioside and Rebaudioside A are present in the *S. rebaudiana* leaves and they taste approximately 200 and 300 times sweeter than sucrose. *Stevia rebaudiana* sweeteners have also shown to be non-cariogenic[^5].

In recent years, the antimicrobial activity of *Stevia rebaudiana* leaf extracts against microorganisms of importance in dental caries has been evaluated, but till now its activity in Pure Form (Suspension) against *Streptococcus* and *Lactobacillus* has not been proved. Therefore, the aim of this study is to compare the effectiveness of aqueous suspension, alcoholic extract and their combination of *Stevia rebaudiana* against *Streptococcus mutans* and *Lactobacillus acidophilus*.

**MATERIALS AND METHODS**

This was an *in vitro* study carried out at Dr. Prabhakar Kore’s Basic Science Research Center, KLE University with the guidance from the Department of Public Health Dentistry in the month of September, 2016. *Stevia rebaudiana* leaves and its powder were purchased by a company Stevia Zone, Ahmedabad, Gujarat, India. Authentication of the same was done at KLE’s B.M Kankanwadi Ayurveda College, Shahpur, Belagavi, Karnataka, India.

**Method of Preparation of mouth wash**- According to the Standard textbook of Pharmaceutics, Indian Pharmacopoeia 2007, 5th edition[^6]. For 1% aqueous suspension of *Stevia rebaudiana*, 1 gram of *Stevia rebaudiana* leaf powder was dissolved in a 2% hydroxypropyl methylcellulose (suspending agent) dispersed with constant stirring for 2 hours, 0.5 ml of glycerin was added and the volume of 100ml was made up with distilled water. For 5% alcoholic extract, 20 grams of *Stevia* leaf powder was macerated with 95% of 200ml of ethanol for 7 days with occasional shaking. The extract was filtered; the filtrate was evaporated on a water bath under reduced pressure for half an hour. The crude liquid extract was obtained using IKA rotary evaporator at 40°C without traces of alcohol, 5mg of the crude extract was dissolved in water with the aid of Tween 20 (non-ionic surfactant) stirred for half an hour till homogenous solution is formed, 0.5 ml of glycerin was added and the volume of 100ml was made up with distilled water. For 6% of their combination: 1:1 ratio of both suspension and extract was taken to check for any enhanced inhibitory effect of the preparation. The following preparations of *Stevia rebaudiana* leaves were subjected to the microbiological assay to determine the Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) using broth dilution method against the standard strains *Streptococcus mutans* (MTCC 25175) and *Lactobacillus acidophilus* (MTCC 10307) obtained from PGI Chandigarh, India. The culture media used was Brain heart infusion (BHI) broth and for the inoculum preparation, the growth method or the log phase method was performed as follows.

At least three to five well isolated colonies of the same morphological type were selected from an agar culture plate. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4-5 ml of BHI broth. The broth culture was incubated at 35°C for 2–6 h until it achieved the turbidity of the 0.5 McFarland standards. With each batch of tests, positive and negative controls were put up. The positive control containing broth plus bacterial strain showed turbidity and negative control containing the broth only appeared clear. In each series of tubes, the last tube with a clear supernatant was considered to be without any growth and taken as the MIC value.

The turbidity of actively growing broth culture was adjusted with broth to obtain a final turbidity optically comparable to that of the 0.5 McFarland standards, done visually by comparing the inoculum tube and the standard against a white card with contrasting black lines.

**Broth dilution method**- A total of 10 tubes were taken and nine dilutions of the vehicle were done with BHI for MIC. In the initial tube, only 200 μl of vehicle was added. For further dilutions, 200 μl of BHI broth was added to the next nine tubes separately. From the $10^{-3}$ diluted tube, 200 μl was transferred to the second tube to make $10^{-2}$ dilution. The serial dilution was repeated up to $10^{-8}$ dilution for each vehicle.

[^1]: Reference 1
[^2]: Reference 2
[^3]: Reference 3
[^4]: Reference 4
[^5]: Reference 5
[^6]: Reference 6
The tubes were kept for incubation for 24 h at 37°C in bacteriological incubator and observed for turbidity. For determination of MBC, agar plate sub-culture streaking method was used and the least concentration which showed no visible growth on the agar plate after incubation period was considered as the MBC value. MIC, as determined using broth dilution method had 10 tubes of various dilutions to the preparation, starting from 100% concentration and ending at 0.39%. 200 μl was taken from 10 ml of stock solution and the same was diluted to further amounts. To begin with, 1% aqueous suspension had 1 g of Stevia rebaudiana leaf powder in 100ml suspension. Hence 200 μl of the sample contained 2 mg of Stevia rebaudiana. As a result, the concentration resulting from dilutions from tube 2 to tube 9 ranged from 1mg/200 μl to 0.0078 mg/200 μl. Similarly, for 5% ethanolic extract, the concentrations ranged from 5 mg/200 μl to 0.039 mg/ μl and for 6% combinational preparation it ranged from 6 mg/200 μl to 0.046 mg/200 μl respectively.

Statistical Analysis- The experiments were repeated thrice and the data collected was classified and entered in Microsoft Office Excel and SPSS Windows version 17 software (Chicago, IL) was used for statistical analysis. Since the data were of continuous type, parametric tests were used for analysis. Mean (X) and Standard Deviation (SD) were calculated. One-way analysis of variance (ANOVA) test was used for multiple group comparisons, followed by Tukey post-hoc for group-wise comparisons, and p<0.05 was considered statistically significant.

RESULTS
At the end of 48 hours, statistically significant antimicrobial activity was demonstrated by all the test specimens used in this study. Table 1 and 3, shows the mean values of MIC against S. mutans and L. Acidophilus after performing the procedures in triplicates. A statistically significant difference was seen between the mean values of the two preparations. Tukey’s Post hoc ANOVA (Table 2 and 4) indicated that the efficacy of 1% aqueous suspension was better than the other two preparations. The mean difference of MIC’s in case of S. mutans was statistically significant. However, it was not the phenomena in case of MIC against L. acidophilus. No MBC was found for any of the preparations.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Preparation</th>
<th>Mean MIC (mg/200 μl) ± SD</th>
<th>Minimum MIC (mg/200 μl)</th>
<th>Maximum MIC (mg/200 μl)</th>
<th>F ratio and P value</th>
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<tbody>
<tr>
<td>1</td>
<td>1% Aqueous Suspension</td>
<td>0.83±0.28</td>
<td>5</td>
<td>1</td>
<td></td>
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<tr>
<td>2</td>
<td>5% Alcoholic extract</td>
<td>4.16±1.44</td>
<td>2.5</td>
<td>5</td>
<td>8.46 &amp; 0.018</td>
</tr>
<tr>
<td>3</td>
<td>6% (Combination of above two)</td>
<td>5±1.73</td>
<td>3</td>
<td>6</td>
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</tr>
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</table>

(One way Anova test, Level of significance p<0.05)

<table>
<thead>
<tr>
<th>(A) Preparation</th>
<th>(B) Preparation</th>
<th>Mean Difference (A-B)</th>
<th>Std. Error</th>
<th>Sig.</th>
<th>95% Confidence Interval</th>
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<td>Lower Bound</td>
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<td>1.0715</td>
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<td>3.3333</td>
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<td>.047</td>
<td>.046</td>
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</table>

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Table 3: MIC of various *Stevia rebaudiana* preparations against *L. acidophilus*

<table>
<thead>
<tr>
<th>S. No</th>
<th>Preparation</th>
<th>Mean MIC (mg/200 μl) ± SD</th>
<th>Minimum MIC (mg/200 μl)</th>
<th>Maximum MIC (mg/200 μl)</th>
<th>F ratio &amp; P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1% Aqueous Suspension</td>
<td>0.66±0.28</td>
<td>0.5</td>
<td>1</td>
<td>8.32 &amp; 0.019</td>
</tr>
<tr>
<td>2</td>
<td>5% Alcoholic extract</td>
<td>3.33±1.44</td>
<td>2.5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6% (Combination of above two)</td>
<td>5±1.73</td>
<td>3</td>
<td>6</td>
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</table>

(One way Anova test, Level of significance p<0.05)

Table 4: Multiple comparisons post-hoc Tukey test

<table>
<thead>
<tr>
<th>(A) Preparation</th>
<th>(B) Preparation</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>Sig.</th>
<th>95% Confidence Interval</th>
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<tr>
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<td>1</td>
<td>1.6667</td>
<td>1.0715</td>
<td>0.333</td>
<td>-1.621</td>
</tr>
</tbody>
</table>

* The mean difference is significant at the 0.05 level
1: 1% aqueous suspension, 2: 5% alcoholic extract, 3: 6% (combination of 1 and 2)

DISCUSSION

A definite relationship exists between the dietary consumption of sucrose and chronic diseases, such as obesity, diabetes, and heart disease along with the incidence of dental caries, so research for alternatives to sucrose have resulted in the development of artificial sweeteners which are considered safe for teeth but some animal studies have also proven them to cause weight gain, brain tumors, bladder cancer, and many other health hazards [4]. Caries causing bacteria tend to re-dominate the dental plaque after the treatment and start another cycle of carcinogenesis [7,8,17]. New substances with pharmacological potential and effect have been searched for and applied since ancient times. Recently herbal extracts have been successfully used in dentistry as an antimicrobial plaque agent against dental caries.
Therefore, modifications in the diet have been recommended in order to reduce the fermentable carbohydrate intake causing cariogenic microorganisms which produce acids. The usage of various sugar substitutes is a common method to reduce caries risk and sweeteners both natural and artificial have been proposed. Among the natural high-intensity sweeteners, Stevia rebaudiana has been used for several years in South America, Asia, Japan, China and Europe.

Stevia rebaudiana leaves extract have shown health benefits when used as a supplement in the diet. The anti-carcinogenicity of stevioside was presumed; in addition, stevioside, when administered to diabetic patients, produced beneficial effects on glucose metabolism. Stevia rebaudiana leaves extract administered to hypertensive patients reduces blood pressure. Also, a caries-preventive action of Stevia extracts was proposed related to the antibacterial properties and a reduction in the intake of fermentable carbohydrates.

A study was carried out to find the antibacterial activity of chloroform, acetone and methanol extracts of Stevia rebaudiana leaves against Streptococcus mutans and reported that methanolic extracts of Stevia Rebaudiana leaves showed best concentration-dependent antibacterial and antifungal activity. Another study evaluated the antibacterial activity of Stevia rebaudiana leaves extract using various solvents against Escherichia coli, Bacillus subtilis, S. aureus, Salmonella typhi, and Vibrio cholera, and it was found out that the acetone extract showed greater activity against Gram-positive bacteria than Gram-negative bacteria.

In a study conducted by Lingaraj et al., the anti-bacterial efficacy of aqueous and ethanolic extract of Stevia rebaudiana was compared with chlorhexidine against Streptococcus mutans and Lactobacillus acidophilus and it concluded that ethanolic extract of Stevia rebaudiana showed better inhibitory results than the aqueous extract due to better dissolving capacity in alcohol, better bioavailability and polarity of the antibacterial compounds which are readily extracted by organic solvents.

However, in contrast to this study, the present study showed better inhibitory results for aqueous suspension of Stevia rebaudiana as compared to its alcoholic extract and their combination, maybe, due to method of preparation of the suspension where the extraction process was avoided which preserved the active components and anti-oxidants such as tannins, xanthine (theobromine and caffeine) and flavonoids in stevioside.

Also, the stevioside, acts over the enzymes which are responsible for the decomposition of sugars. Some other compounds identified were 80-85% water, ascorbic acid, beta-carotene, riboflavin, thiamine, gibberellic acid, indole-3-acetonitrile, isoorcitrin, kaempferol, stigmasterol, xanthophyll, umbeliferone, chlorogenic acid, caffeic acid, chromium, cobalt, magnesium, iron, potassium and phosphorus.

However, the Minimum Bactericidal Concentration (MBC) was not observed for 1% aqueous suspension which would have been obtained on increasing the concentration and this was the limitation of the study.

Also, in many studies the antibacterial efficacy of the alcoholic extract was superior, maybe, not due to the presence of active ingredients but that of alcohol itself, and as per our knowledge this study is first of its kind where aqueous suspension was tested against cariogenic bacteria and the results showed a remarkable potential of the product to be tested in vivo or in human population at a large scale in order to serve as an alternative to the benchmark oral rinses.

CONCLUSIONS

The inhibitory effect shown by the aqueous suspension of Stevia rebaudiana against S. mutans and L. acidophilus was superior when compared with that of the alcoholic extract and their combination. S. rebaudiana compounds could eventually be used as caries inhibiting agent in mouthwash and toothpaste preparation.

Stevioside can also serve as an efficient vehicle for topical oral medications in gel form due to its sweet taste and easy dispersal. Also, drug industries can incorporate such extracts, which can be delivered as syrups and in other products.

ACKNOWLEDGMENTS

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CONTRIBUTION OF AUTHORS

Dr. Barkha S. Tiwari- Materialised study, participated in study design, performed the experiment, manuscript...
draft preparation, critical review and approved the final manuscript.

Dr. Anil V. Ankola- Participated in study design, supervised, assisted in the draft preparation of the manuscript, and approved the final manuscript.

Mr. U.B. Bolmal- Idea, participated in study design, assisted in the draft preparation of the manuscript, critical review and approved the final manuscript.

Dr. Roopali Sankeshwari- Contributed substantially to the discussion, assisted in the draft preparation of the manuscript, critical review and approved the final manuscript.

Dr. Bhargava Kashyap- Contributed substantially to the discussion, performed the statistical analysis and approved the final manuscript.

REFERENCES

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