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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

Objective- 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.*

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, Minimum inhibitory concentration, Plant suspension, *Stevia rebaudiana*, Sugar substitute

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.

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Their participation in dental caries therefore has led to the development and implementation of new and preventive measures in the control of dental caries [1].

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 [2]. Therefore, implementation of new caries preventive strategies can be significantly improved

in less developed countries and people with low socio-economic status [3]. 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 *S. 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. *S. 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 Pharmacopia 2007, 5th edition [6]. For 1% aqueous suspension of *S. rebaudiana,* 1 g 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 200 ml 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, 5 mg 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 100 ml 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 *S. rebaudiana* leaves were subjected to the microbiological assay to determine the MIC and MBC) using broth dilution method against the standard strains *S. mutans* (MTCC 25175) and *L. acidophilus* (MTCC 10307) obtained from PGI Chandigarh, India. The culture media used was Brain heart infusion (BHI) broth and in 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 was 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^{-1} 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.

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 *S. rebaudiana* leaf powder in 100ml suspension. Hence 200 µl of the sample contained 2 mg of *S. 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 the 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 1: MIC of various *S. rebaudiana* preparations against *S. mutans*

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

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

Table 2: Multiple Comparisons Post-hoc Tukey test

(A) Preparation	(B) Preparation	Mean Difference (A-B)	Std. Error	Sig.	95% Confidence Interval	
					Lower bound	Upper bound
1	2	-3.3333*	1.0715	.047	-6.621	-.046
	3	-4.1667*	1.0715	.019	-7.454	-.879
2	1	3.3333*	1.0715	.047	.046	6.621

	3	-.8333	1.0715	.729	-4.121	2.454
3	1	4.1667*	1.0715	.019	.879	7.454
	2	.8333	1.0715	.729	-2.454	4.121

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

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

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

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

Table 4: Multiple comparisons post-hoc Tukey test

(A) Preparation	(B) Preparation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-2.6667	1.0715	0.104	-5.954	0.621
	3	-4.3333*	1.0715	0.016	-7.621	-1.046
2	1	2.6667	1.0715	0.104	-.621	5.954
	3	-1.6667	1.0715	0.333	-4.954	1.621
3	1	4.3333*	1.0715	0.016	1.046	7.621
	2	1.6667	1.0715	0.333	-1.621	4.954

*** 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 has 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 [9,10]. The usage of various sugar substitutes is a common method to reduce caries risk and sweeteners both natural and artificial have been proposed [11,12]. Among the natural high-intensity sweeteners, *Stevia rebaudiana* has been used for several years in South America, Asia, Japan, China, and Europe [13].

S. 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. *S. 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 [5].

A study was carried out to find the antibacterial activity of chloroform, acetone and methanol extracts of *S. rebaudiana* leaves against *S. mutans* and reported that methanolic extracts of *S. rebaudiana* leaves showed best concentration-dependent antibacterial and antifungal activity [14]. Another study evaluated the antibacterial activity of *S. rebaudiana* leaves extract using various solvents against *E. coli*, *B. subtilis*, *S. aureus*, *S. typhi* and *V. cholera* and it was found out that the acetone extract showed greater activity against gram-positive bacteria than gram-negative bacteria [8].

In a study, conducted by Lingaraj *et. al.* [2], the anti-bacterial efficacy of aqueous and ethanolic extract of *S. rebaudiana* was compared with chlorhexidine against *S. mutans* and *L. acidophilus* and it concluded that ethanolic extract of *S. rebaudiana* shown 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 *S. rebaudiana* as compared to its alcoholic extract and their combination, maybe, due to the 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 [4].

Also, the stevioside acts over the enzymes, which are responsible for the decomposition of sugars [15]. Some other compounds identified were 80–85% water, ascorbic acid, beta-carotene, riboflavin, thiamine, gibberellic acid, indole-3-acetonitrile, isoquercitrin, kaempferol, stigmasterol, xanthophyll, umbeliferone, chlorogenic acid, caffeic acid, chromium, cobalt, magnesium, iron, potassium, and phosphorus [16].

However, the MBC was not observed for 1% aqueous suspension, which would have been obtained by 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 *S. 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 a 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.

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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

[1] Gamboa F, Chaves M. Antimicrobial potential of extracts from *Stevia rebaudiana* leaves against bacteria of importance in dental caries. Acta. Odontol. Latinoam., 2012; 25(2): 171–75.

[2] S. Lingaraj et al. Effect of aqueous and alcoholic *Stevia* (*Stevia rebaudiana*) extracts against *Streptococcus mutans* and *Lactobacillus acidophilus* in comparison to chlorhexidine: An *in vitro* study, J. Int. Soc. Prev. Commun. Dent., 2014; 4(5): 116–21.

[3] Ferrazzano GF, Cantile T, Alcidi B, Coda M, Ingenito A, et al. Is *Stevia rebaudiana* bertoni a non cariogenic sweetener? A review. Mol., 2016; 21(1): 01–12.

[4] De Slavutzky SMB. Stevia and sucrose effect on plaque formation. J. Fur. Consumer protection Leb, 2010; 5(2): 213–16.

[5] Brambilla E, Cagetti MG, Ionescu A, Campus G, Lingström P. An *in vitro* and *in vivo* comparison of the effect of *Stevia rebaudiana* extracts on different caries-related variables: A randomized controlled trial pilot study. Caries Res., 2014; 48(1): 19–23.

[7] Marshall TA. Chairside Diet Assessment of Caries Risk. J. Am. Dent. Assoc., 2009; 140(6): 670–74. doi: 10.14219/jada.archive.,2009;pp.0252.

[8] Jayaraman S, Manoharan MS, Illanchezian S. *In-vitro* Antimicrobial and Antitumor Activities of *Stevia rebaudiana* (Asteraceae) Leaf Extracts. Trop. J. Pharm. Res., 2008; 7(4): 1143–49.

[9] Geuns JM. Stevioside. Phytochem., 2003; 64(5): 913–21. doi: 10.1016/s0031-9422(03)00426-6.

[10] Van Loveren C, Broukal Z, Oganessian E. Functional foods/ingredients and dental caries. Eur. J. Nutr., 2012; 51(S2): 15–25.

[11] Pol J, Hohnova B, Hyotylainen T. Characterisation of *Stevia rebaudiana* by comprehensive two dimensional liquid chromatography time-of-flight mass spectrometry. J. Chromatogr. A., 2007; 1150 (1–2): 85–92.

[12] Matsukubo T, Takazoe I. Sucrose substitutes and their role in caries prevention. Int. Dent. J., 2006; 56(3): 119–30. doi: 10.1111/j.1875-595x.2006. tb00083.x.

[13] Chavarria N, Gamboa FO, Escribano S, Vitery R. Inhibitory Activity of 107< Caries Res., 2008; 42(3): 221.

[14] Debnath M. Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*. J. Med. Plants Res., 2008; 2(2): 45-51.

[15] Yabu M, Takase M, Toda K, Tanimoto K, Yasutake A. Studies on Stevioside, natural sweetener. Effect on the growth of some oral microorganisms. Hiroshima Daigaku Shigaku Zasshi, 1977; 9(1): 12–17.

[16] Goyal S, Goyal R. Stevia (*Stevia rebaudiana*) a bio sweetener: A review. Int J Food Sci, 2010; 61: 01–10.

[17] Kim NC, Kinghorn AD. Highly sweet compounds of plant origin. Arch. Pharm. Res., 2002; (6): 725–46. doi: 10.1007/BF02976987.