Original Article

Effect of aqueous and alcoholic *Stevia* (*Stevia rebaudiana*) extracts against *Streptococcus mutans* and *Lactobacillus acidophilus* in comparison to chlorhexidine: An *in vitro* study

Sunil Lingaraj Ajagannanavar, Supreetha Shamarao[1], Hemant Battur, Shreyas Tikare[2], Abdulaziz Abdullah Al-Kheraif[3], Mohammed Sayed Al Esawy Al Sayed[4]

Department of Public Health Dentistry, Kurunji Venkatramana Gowda Dental College and Hospital, Kurunjibhag, Sullia, [1]Department of Public Health Dentistry, Coorg Institute of Dental Sciences, Maggula, Virajpet Karnataka, India, [2]Department of Preventive Dental Sciences, Division of Dental Public Health, King Khalid University, College of Dentistry, Abha, Saudi Arabia, [3]Department of Dental Health, Dental Biomaterials Research Chair, College of Applied Medical Sciences, [4]College of Engineering, King Saud University, Riyadh, Kingdom of Saudi Arabia

Corresponding author (email: <sunilajagannavar@gmail.com>)
Dr. Sunil Lingaraj Ajagannanavar, Department of Public Health Dentistry, KVG Dental College and Hospital, Kurunjibhag, Sullia - 574 237, Dakshina Kannada, Karnataka, India.

Abstract

Introduction: *Stevia* (*S. rebaudiana*) a herb which has medicinal value and was used in ancient times as a remedy for a great diversity of ailments and sweetener. Leaves of *Stevia* contain a high concentration of Stevioside and Rebaudioside which are supposed to be sweetening agents. **Aim:** To compare the efficacy of aqueous and alcoholic *S. rebaudiana* extract against *Streptococcus mutans* and *Lactobacillus acidophilus* in comparison to chlorhexidine. **Materials and Methods:** In the first part of the study, various concentrations of aqueous and ethanolic *Stevia* extract were prepared in the laboratory of Pharmacy College. It was then subjected to microbiological assay to determine its zone of inhibition using Agar disk diffusion test and minimum inhibitory concentration (MIC) using serial broth dilution method against *Streptococcus mutans* and *Lactobacillus acidophilus*. Chlorhexidine was used as a positive control. One way Analysis of Variance (ANOVA) test was used for multiple group comparisons followed by Tukey post hoc for group wise comparisons. **Results:** Minimum inhibitory concentration (MIC) of aqueous and ethnolic *Stevia* extract against *Streptococcus mutans* and *Lactobacillus acidophilus* were 25% and 12.5% respectively. Mean zone of inhibition of the aqueous and alcoholic *Stevia* extracts against *Streptococcus mutans* at 48 hours were 22.8 mm and 26.7 mm respectively. Mean zone of inhibition of the aqueous and alcoholic *Stevia* extracts against *Lactobacillus acidophilus* at 48 hours were 14.4 mm and 15.1 mm respectively. Mean zone of inhibition of the chlorhexidine against *Streptococcus mutans* and *Lactobacillus acidophilus* at 48 hours was 20.5 and 13.2 respectively. **Conclusion:** The inhibitory effect shown by alcoholic *Stevia* extract against *Streptococcus mutans* and *Lactobacillus acidophilus* was superior when compared with that of aqueous form and was inferior when compared with Chlorhexidine.

Key words: *Chlorhexidine, Stevia, Stevia rebaudiana*

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INTRODUCTION

Dental caries is a chronic, infectious, transmissible, biobehavioral disease that extends throughout the life span. The essential process of this disease involves bacterial adherence to tooth surfaces, dental plaque formation, and localized demineralization of tooth enamel by acids of bacterial origin produced from the fermentation of dietary carbohydrates.[1]

More than a century back, W. D. Miller had postulated the chemico-parasitic theory for the etiology of dental caries. Until today, the modern concepts of cariogram demonstrate microorganisms as one of the major etiological factors for dental caries. Mutans streptococci are shown to be highly associated with caries in humans. Considerable evidence exists implicating *Streptococcus mutans* as an important etiological agent in initiation of enamel caries, both in laboratory animals and in humans. The presence of $\geq 10^6$ mutans streptococci/ml saliva may indicate a high caries risk or activity.[2]

For preventing this disease in an individual, the focus should be on increasing the ability of the host to respond to the insult, decreasing the cariogenicity of the bacterial agents, and altering the diet to be less caries promoting.

Renewed interest in developing an antimicrobial approach for the management of dental caries has evolved as a result of: (1) Identifying certain members of the oral microflora as major cariogens and (2) increased understanding of the specific ecology of these cariogens. In conjunction with this concept, control and prevention of caries has been sought by reducing the number of colonizing bacteria. Reducing their level in the oral cavity will provide an additional rationale for the prevention of dental caries.[2]

Research in the field of caries prevention has been focusing on ways for reducing or totally eradicating cariogenic flora from the oral cavity. Studies have shown that caries can be prevented by regular tooth brushing and flossing. However, most of the studies have shown it difficult to eliminate *S. mutans* from the pits, fissures, and approximal surfaces by mechanical means alone. For effective caries control, these methods should be combined with the chemoprophylactic agents. These agents, e.g., chlorhexidine and antibiotics, act by lowering the number of microorganisms or inhibiting dental plaque formation. However, they have several undesirable side effects, including tooth staining and emergence of bacterial resistance. These side effects stimulate the search for alternative agents.[1]

Ayurveda is the traditional medicinal form prevalent in India since 2000 BC. The Ayurvedic treatment is entirely based on herbs, which have certain medicinal value or property. In the ancient times, the Indian sages believed that Ayurvedic herbs are one-stop solutions to cure a number of health-related problems and diseases. They studied about the herbs thoroughly and experimented with herbs to arrive at accurate conclusions about the efficacy of different plants and herbs that have medical value. Most of the Ayurvedic herbs, thus formulated, are free of side effects or reactions. This is the reason why Ayurveda is growing in popularity across the globe. The Ayurvedic herbs that have medicinal quality provide rational means for the treatment of many internal diseases which are otherwise considered incurable in other systems of medicine.[3] In 2003, according to the World Health Organization, the use of traditional herbal medicines has spread not only in developing countries but also in the industrialized ones, as a complementary way to treat and prevent illnesses.[1]

Plant extracts constitute rich sources of novel compounds with a variety of pharmacological activities. In many countries, plant extracts have been traditionally used for the treatment of oral mucosal lesions and periodontal diseases without any scientific validation.[1]

Herbal extracts have been successfully used in dentistry as tooth cleaning and antimicrobial plaque agents, as most of the oral diseases are due to bacterial infections and it has been well documented that medicinal plants confer considerable antibacterial activity against various microorganisms including the bacteria responsible for dental caries. The antibacterial activities of some plant species like *Melia azadirachta, Calotropis gigantea, Leucas aspera, Vitex negundo*, and others have been tested. In India, plant wealth is greatly exploited for its therapeutic potential and medicinal efficacy to cure dental caries. It includes *M. azadirachta*, *Moringa pterygosperma*, and *Balsamodendron mukul*. The stem, bark, root, and young fruits of *M. azadirachta* are used for their Bitter, tonic, antiseptic, astringent, and antibacterial properties. In several indigenous tooth powders, toothpastes, and toilet soaps, the extract from various parts of this tree is used. The use of neem twigs as tooth brush has been endorsed by the dentists to prevent caries. *Azadirachta indica* mouth wash is reported to inhibit the growth of *S. mutans* and carious lesions.[4]

The main aim of research into medicinal plants is to identify the plants that can potentially exhibit pharmacological activity and thus discover new substances or molecules having antimicrobial activity that could be modulated as various medications and, therefore, control or prevent infectious diseases.

One such shrub that has medicinal value is *Stevia rebaudiana* (*Stevia*). The leaves have been traditionally used for hundreds of years in both Brazil and Paraguay to sweeten local teas and medicines.[5] In addition to some flavonoids, the leaves of *Stevia* contain a high concentration of stevioside and rebaudioside, the

concentration of which varies between 2.5 and 9% according to the source and work-up of the drug.

With its steviol glycoside extracts having up to 300 times the sweetness of sugar, *Stevia* has attracted attention with the rise in demand for low-carbohydrate, low-sugar sweeteners. Because *Stevia* has a negligible effect on blood glucose, it is attractive to people on carbohydrate-controlled diets.[5]

Studies have reported the antimicrobial effect of *Stevia* extracts on fungi and various bacteria. Hardly few studies have been reported on evaluation of the effect of *Stevia* extracts on *S. mutans* and *Lactobacillus acidophilus* and none of the studies have compared them with chlorhexidine.

The study was conducted with an aim to compare the efficacy of aqueous and alcoholic *Stevia* extracts against *S. mutans* and *L. acidophilus*, in comparison to chlorhexidine.

MATERIALS AND METHODS

A standard procedure was followed for finding the minimum inhibitory concen tration (MIC). Dried *Stevia* leaf extract powder was procured from M/s Amruth Kesari Depot, Bangalore, Karnataka.

The study was carried out for a period of 4 months from June to October 2013. Ethical permission was obtained from Ethical Clearance Committee, KVG Dental College, Sullia.

The materials used in this study were as follows.

- Test materials used
  a. *Stevia* leaf powder
  b. Aqueous solution
  c. Ethanol
  d. Chlorhexidine (0.2%).
- Microorganisms
  a. *S. mutans* ATCC 25175
  b. *L. acidophilus* ATCC 4356

The microbial strains selected for the present study were collected from the American Type Culture Collection (ATCC), USA.

- Brain heart infusion agar
- Vernier caliper.

Preparation of *Stevia* aqueous extract

Stevia powder was identified by a botanist and a pharmacognosist for its authenticity at the Department of Pharmacognosy, Bapuji College of Pharmacy, Davangere. The powder obtained thus was weighed up to 50 g and then mixed with 100 ml of sterile distilled water in a round bottom flask with occasional shaking. The extract was then filtered through a muslin cloth for coarse residue and finally through Whatman No. 1 filter paper and kept in an airtight amber-colored container.

Preparation of *Stevia* ethanolic extract

Stevia extract was prepared by macerating 50 g of dry powder with 100 ml of 70% (w/v) ethyl alcohol for a week in a round bottom flask with occasional shaking. The extract was then filtered through a muslin cloth for coarse residue and finally through Whatman No. 1 filter paper and was stored at 4°C for further use. Stock solutions of the crude extracts were prepared by mixing well appropriate amount of dried extracts with an inert solvent dimethyl sulfoxide to obtain the final concentrations.

Microbial analysis

Revival of the organisms

The bacterial strains from the stock were revived by plating on blood agar medium. After overnight incubation at 37°C, isolated colonies were selected and the identities of the organisms were confirmed. Isolated colonies were transferred to sterile Brain Heart Infusion (BHI) broth for bacteria and once again incubated overnight. The growth concentration was adjusted to 10^5 organisms/ml by using 0.5 McFarland's turbidity standard.

Agar ditch plate method for testing the antibacterial properties

Agar well diffusion assay was used to evaluate the antimicrobial potential of the extracts. Petri dishes containing 18 m1 of BHI agar for *S. mutans* and *L. acidophilus* were inoculated with approximately 100 µl of microbial strain using swab technique.

Wells of 8 mm diameter were cut into solidified agar media using a sterilized standard device. One hundred microliters of each extract was poured in the respective well and the plates were incubated at 37°C for 48 h. To ensure the consistency of all findings, the experiment was performed and repeated under strict aseptic conditions. The antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced by each extract at the end of incubation period.

MIC determination for the aqueous Stevia extract

Two hundred microliters of the BHI broth was added to each of 10 MIC tubes per bacterial strain. In the first MIC tube containing 200 µl broth, 200 µl of stock was

added. After mixing well, 200 µl was transferred to the second MIC tube. This was continued till the last (10th) tube. From the last tube, 200 µl of final solution was discarded. By following this serial dilution, the concentration of the *Stevia* powder achieved was as follows: 50%, 25%, 12.5%, 6.25%, 3.1%, 1.56%, 0.78%, 0.39%, 0.19%, and 0.09%, respectively. To each of these 10 MIC tubes prepared with varying concentrations, 200 µl of the earlier prepared strain of *S. mutans* was added such that the final volume per tube was 400 µl.

After incubation, the MIC values were determined by visual inspection of the tubes. With each batch of tests, positive and negative controls were put up. 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. Turbidity in the MIC tube indicated growth of the bacterial strain, implying that the organisms were resistant to the aqueous *Stevia* extract.

MIC determination for the alcoholic Stevia extract

A solution of 50% concentration was prepared as the stock solution. The working concentration of the extract was as follows: 50%, 25%, 12.5%, 6.25%, 3.1%, 1.56%, 0.78%, 0.39%, 0.19%, and 0.09%, respectively. A similar procedure of serial dilution, as mentioned above, was followed to test the antimicrobial effect of the alcoholic *Stevia* extract.

All measurements of the zone of inhibition were carried out by a single examiner. Calibration of the examiner was done prior to and during the study by re-examining 5% of the samples, to minimize intra-examiner variability. Intra-examiner agreement was determined using kappa statistics (k) and the score thus obtained (k = 0.84) was almost perfect, according to Landis and Koch, thus meeting the scientific requirement for validity and reliability.

Statistical analysis

The collected data were classified and tabulated in Microsoft Office Excel. SPSS for Windows version 17 software (Chicago, IL, USA) was employed for statistical analysis. Frequency distributions of responses to the questions were produced. 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 h, statistically significant antimicrobial activity was demonstrated by all the test specimens used in this study ($P = 0.002$).

Table 1 shows the antimicrobial activity of the extracts against *S. mutans* at 48 h. Chlorhexidine showed the highest inhibition rate against *S. mutans*, compared with alcoholic and aqueous forms of *Stevia*. However, the alcoholic *Stevia* extract was found to be better than the aqueous form and the results were found to be statistically significant.

Table 2 shows the antimicrobial activity of the extracts against *L. acidophilus* at 48 h. Again chlorhexidine was found to be superior in inhibiting *L. acidophilus*, compared with alcoholic and aqueous forms of *Stevia* and the results were found to be statistically significant ($P < 0.003$). However, alcoholic *Stevia* extract was found to be closely competent enough with chlorhexidine but was much superior than the aqueous extract.

Table 3 shows the MIC of aqueous *Stevia* extract against *S. mutans* and *L. acidophilus*. *S. mutans* showed sensitivity to aqueous *Stevia* extract at concentrations of 50% and 25% and demonstrated resistance to concentrations of 12.5%, 6.25%, 3.1%, 1.56%, 0.78%, 0.39%, 0.19%, and 0.09%. *L. acidophilus* showed sensitivity to aqueous *Stevia* extract only at the concentration of 50% and demonstrated resistance to concentrations of 25%, 12.5%, 6.25%, 3.1%, 1.56%, 0.78%, 0.39%, 0.19%, and 0.09%. Hence, the MIC of aqueous *Stevia* extract for *S. mutans* was 25% and for *L. acidophilus* was 50%.

Table 1: Antimicrobial activity of the extracts against *Streptococcus mutans* at 48 h

Sample	Mean zone of inhibition (in mm)±SD	*P*[a]	Tukey *post-hoc*
Stevia extract (aqueous)	22.8±0.27	0.002*	3>2>1
Stevia extract (alcohol)	24.7±0.34		
Chlorhexidine (0.2%)	26.5±0.42		

[a]*ANOVA, *Significant at 0.05 level, SD=Standard deviation*

Table 2: Antimicrobial activity of the extracts against *Lactobacillus acidophilus* at 48 h

Sample	Mean zone of inhibition (in mm)±SD	*P*[a]	Tukey *post-hoc*
Stevia extract (aqueous)	10.8±0.21	0.003*	3>2>1
Stevia extract (alcohol)	12.3±0.12		
Chlorhexidine (0.2%)	13.2±0.24		

[a]*ANOVA, *Significant at 0.05 level, SD=Standard deviation*

Table 4 shows the MIC of alcoholic *Stevia* extract against *S. mutans* and *L. acidophilus*. *S. mutans* showed sensitivity to aqueous *Stevia* extract at concentrations of 50%, 25%, and 12.5% and demonstrated resistance to concentrations of 6.25%, 3.1%, 1.56%, 0.78%, 0.39%, 0.19%, and 0.09%. *L. acidophilus* showed sensitivity to aqueous *Stevia* extract at concentrations of 50%, 25%, 12.5%, and 6.25% and demonstrated resistance to concentrations of 3.1%, 1.56%, 0.78%, 0.39%, 0.19%, and 0.09%. Hence, the MIC of alcoholic *Stevia* extract for *S. mutans* was 12.5% and for *L. acidophilus* was 6.25%.

DISCUSSION

The current antimicrobial strategies used to treat dental caries consist primarily of mechanical removal of dental plaque or generalized killing of oral bacteria with antibacterial compounds. These remove-all, kill-all approaches have shown limited efficacy, since a cleaned tooth surface provides an equal opportunity for commensal and pathogenic bacteria to re-colonize in the non-sterile environment of the oral cavity. Cariogenic bacteria usually re-dominate the dental plaque after the treatment and start another cycle of cariogenesis. This study proposes to develop a targeted antimicrobial therapy against *S. mutans*. By selectively killing or inhibiting the cariogenic bacteria within a pathogenic dental plaque, a non-pathologic, commensal microbial community could be established. This healthy plaque would then serve as an effective barrier to prevent the subsequent colonization of cariogenic bacteria on the tooth surface, leading to a sustained anti-caries therapeutic effect.

Stevia is a green leafy plant that is native to South America. Whole or crushed *Stevia* leaves, or extract (either liquid or powder), or a refined version of the plant's isolated compounds can be used as a sweetener and they have medicinal value too. The two major sweet compounds that are isolated from the *Stevia* leaves, called as stevioside and rebaudioside, are hundred times sweeter than sugar.

A study was carried out to find the antibacterial activity of chloroform and methanol extracts of *S. rebaudiana* leaves against *S. mutans* and reported that the chloroform and methanol extracts of *S. rebaudiana* leaves exhibited a concentration-dependent antibacterial and antifungal activity. Acetone and ethanol extracts of *S. rebaudiana* leaves presented a better inhibitory effect on *S. mutans*.[6]

Another study evaluated the antibacterial activity of *S. rebaudiana* leaves extracted using various solvents against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, and *Vibrio cholera*, and it was found in the study that the acetone extract showed greater activity against Gram-positive bacteria than Gram-negative bacteria.[7]

In spite its use in medicinal field, less light has been shed for the use of *Stevia* in Dentistry. Therefore, this study was aimed to assess the antimicrobial effect of *Stevia* extracts on caries causative microorganisms, i.e. *S. mutans* and *L. acidophilus*, in comparison to chlorhexidine.

Chlorhexidine was considered as the positive control in this study. The bis-biguanide chlorhexidine (CHX), which has been studied extensively for over 25 years, is currently the most potent antimicrobial agent against mutans streptococci and dental caries. Its method of action has been comprehensively reviewed by Hugo, whose classical studies demonstrated that CHX concentrations is a potent membrane active against both Gram-positive and Gram -negative bacteria, including the release of K^+, 260 nm-absorbing material, and pentose. It is also an inhibitor of adenosine triphosphatase activity. At higher bactericidal concentrations, CHX induces precipitation of cytoplasmic protein and nucleic acids. It blocks the activity of the phosphoenolpyruvate-phosphotransferase sugar transport

Table 3: Minimum inhibitory concentration of aqueous *Stevia* extract against *Streptococcus mutans*

Microorganisms	Concentration of aqueous *Stevia* extract (%)									
	50	25	12.5	6.25	3.1	1.56	0.78	0.39	0.19	0.09
Streptococcus mutans	S	S	R	R	R	R	R	R	R	R
Lactobacillus acidophilus	S	R	R	R	R	R	R	R	R	R

R=Resistant, S=Sensitive

Table 4: Minimum inhibitory concentration of alcoholic *Stevia* extract against *Lactobacillus acidophilus*

Microorganisms	Concentration of alcoholic *Stevia* extract (%)									
	50	25	12.5	6.25	3.1	1.56	0.78	0.39	0.19	0.09
Streptococcus mutans	S	S	S	S	R	R	R	R	R	R
Lactobacillus acidophilus	S	S	S	S	R	R	R	R	R	R

R=Resistant, S=Sensitive

system, and thereby markedly inhibits acid production in oral streptococci cariogenic bacteria in subjects with a high risk of developing caries.[2]

It was unanticipated that chlorhexidine would yield greater inhibition rates than *Stevia* extracts. Particularly, alcoholic *Stevia* extract was superior in inhibiting *S. mutans* and *Lactobacillus*, compared with the aqueous form.

The reason why the mean inhibition rates were more with alcoholic *Stevia* extract than the aqueous form is unknown. However, the reasons may be better solubility of the *Stevia* compound in alcohol or the very presence of alcohol. This finding is in agreement with the findings of other studies.[6,8]

With respect to the MIC of *Stevia* extract, inhibition of *S. mutans* and *L. acidophilus* by alcoholic *Stevia* extract at lower concentration was superior when compared with the aqueous form. This may be due to better dissolving capacity in alcohol, better bioavailability (thus enhancing bioactivity), and polarity of the antibacterial compounds which makes the compounds to be more readily extracted by organic solvents.

However, a limitation of the study is that the study could have been conducted with the other group of 70% ethyl alcohol to clearly state that it is the effect of *Stevia* alone which has led to the inhibition of *S. mutans* and *L. acidophilus* and not that of alcohol.

CONCLUSION

The inhibitory effect shown by chlorhexidine against *S. mutans* and *L. acidophilus* was superior when compared with that of the aqueous extract and chlorhexidine. However, it was also observed that alcoholic *Stevia* extract was competent enough with chlorhexidine and was many times better than the aqueous form in inhibiting *S. mutans* and *L. acidophilus*.

Recommendations

- *Stevia* is a popular alternative sweetener used by those who cannot use cane sugar

Leaves of *Stevia* plants have functional and sensory properties superior to those of many other high-potency sweeteners. *Stevia* is likely to become a major source of high-potency sweetener for the growing natural food market in the future. Although *Stevia* is useful to all, there are certain groups who are more likely to benefit from its remarkable sweetening potential.[9] Also, because of its antimicrobial property, it appears to offer protection against dental caries. For these reasons, cultivation and marketing of *Stevia* should be increased, wherein inclusion of liquorice can be considered in various food stuffs. Health concerns due to sucrose usage, obesity, and diabetes may also increase the demand of such sweeteners. A few developed countries are widely marketing confectionery incorporating this compound and other countries should follow suit.

- If further studies show promise, the *Stevia* compounds could eventually be used as cavity-fighting components in mouthwash and toothpaste. Drug industries can also incorporate such extracts, which can be delivered as syrups or in other products. Stevioside can serve as an efficient vehicle for topical oral medications because of its agreeable sweet taste, excellent dispersing qualities, and its capability to form stable aqueous gels
- Animal studies, *in vivo* studies, and large clinical trials have to be carried out to ascertain the effect of *Stevia* extract on microorganisms. Research assessing the action of *Stevia* extract on periodontal pathogens, other caries-causing microorganisms, and fungal species is recommended.

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