EVALUATION OF STEVIA REBAUDIANA EFFECT ON LACTOBACILLUS PLANTARUM GROWTH: AN IN VITRO STUDY

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Abstract. One way to control dental caries is the use of antibacterial mouthwash. The use of medicinal plants is highly considered, because of side effects and antibacterial resistance of the chemical substances. The aim of this study is to evaluate the antibacterial effects of Stevia Rebaudiana extract on Lactobacillus Plantarum. In this experimental invitro study, the extract of Stevia Rebaudiana was prepared. The indices of antibacterial activity were; minimum inhibitory concentration (MIC), maximum bacterial concentration (MBC) and the zone of growth inhibition of Stevia Rebaudiana against Lactobacillus Plantarum. These criteria were determined by broth macrodilution and agar well diffusion methods. The results was assessed by SPSS20 and one-way ANOVA (α=0.05). Lactobacillus Plantarum showed the most sensitivity to Stevia Rebaudiana extract (p-Value=0.02). MIC and MBC for Stevia was 6.71 µg/ml and 26.87 µg/ml. The more the concentration of Stevia, the greater zone diameter of growth inhibition of bacteria (p-value=0.0). Maximum diameter of growth inhibition was measured for 880 µg/ml of Stevia. Assessment of zone of inhibition in 24 and 48 hours showed no significant difference (p-Value=0.7). The results of this study show that this plant has antibacterial effect, and can be used as a drug to prevent and control the growth of Lactobacillus Plantarum.

Keywords: Caries, Stevia Rebaudiana, Lactobacillus Plantarum.

Introduction. Dental caries is a very common epidemic in the world. Bacteria in the mouth, with the metabolism of carbohydrates, lead to acid production, demineralization and dental caries. The main strains involved in this process are Streptococcus mutans and lactobacilli.[1] Several studies have shown that lactobacilli are more likely to occur in carious lesions, and probably these bacteria are associated with dental caries.[2] The use of multiple mouthwashes to remove dental bacteria and plaque is known as one of the common methods for caries elimination. Due to the beneficial effects of medicinal herbs in the treatment of diseases and due to their greater compatibility with the human immune system, the use of herbs to counteract the side effects of chemical drugs is increasing.[3] Some plants show antibacterial effects against common dental bacteria such as streptococci and lactobacilli.[4] Stevia is a scientific name of the Stevia Rebaudiana. Extract of this plant, has anti-fungal, anti-inflammatory, antimicrobial and antiviral properties.[5] The stevia leaf extract is 300 times sweeter than sugar. Stevia leaves have glycosyids. This product do not produce calories. Stevia has been considered economically and scientifically for being sweet and medicinal.[6] Laboratory studies have shown that the extract of this plant has antibacterial activity on Streptococcus mutans, Streptococcus subrinus, and Lactobacillus acidophilus and common microorganisms in the development of oral caries.[3] Lactobacillus casei and Lactobacillus plantarum are dominant decay carcinogenic lactobacilli in both adults and children groups.[7] Lactobacillus plantarum is one of the common strains of lactobacilli. So far, the effects of Stevia extract on it have not been investigated , The aim of this study was to investigate the antibacterial effects of Stevia extract on Lactobacillus plantarum growth in laboratory. The null hypothesis of this study was that Stevia Rabbadiana does not show the inhibitory effect on Lactobacillus plantarum growth.

Materials and Methods. Preparation of Stevia extract: Stevia extract bought from Golsaran North Company. To prepare the extract, we crushed 50 gr of dried plant in mixer (Pars Khazar Co., Iran) with distilled water. The milled powder was poured into a volumetric flask and then filled, up to two thirds of the balloon volume with distilled water. The Clevenger device was then attached to the balloon containing the material and the Clevenger itself was firmly fixed with the clamp and base (Fig1).

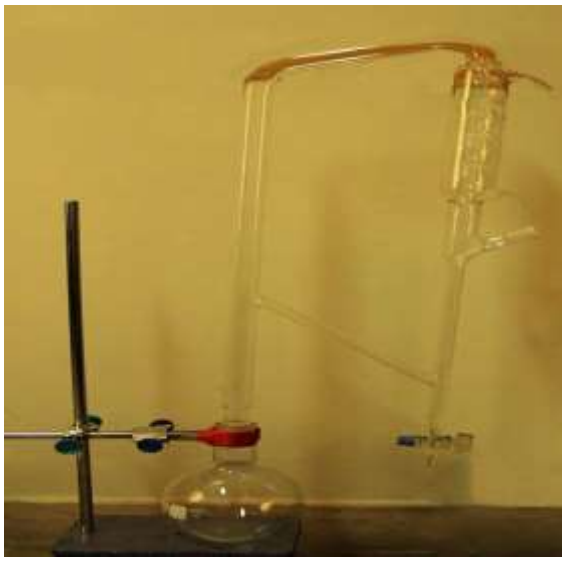

Figure1: *Clevenger device*

Due to its compounds, the extract usually has a lower density than water and therefore is placed on the water. The Clevenger Outlet valve was then carefully opened and the extract was poured into its own container. Since the resulting extract may have water, sodium sulfate was used to absorb water. The extract was stored at sterilized vials at 4 ° C until the test was completed.

Preparation of microbial specimen: Lyophilised Lactobacillus plantarum was prepared from Pasteur Institute of Iran and was grown for 18-24 hours in the culture medium (de Roger & Sharpe broth) (MRS broth) products of Merck, Germany.

Measurement of the effect of Stevia extract on lactobacillus specimens (Macrodilution broth): The determination of MIC and MBC by broth macrodilution was performed according to the protocol (CSLI Clinical and Laboratory).

For this purpose, a series of sterile tubes were prepared with MRS broth culture medium into each tube. Then, the extracts with 100% stevia rhabadian concentration (880 μg/ml) was poured into the first tube .serum macrodilution was performed to produce other concentrations.

Then, from each microbial suspension, 1 cc (equivalent to CF / ml 1.5×10^6 bacteria) was added to each tube, two tubes were considered as positive growth control (culture medium with the desired strain) and another as negative control (culture medium with desired extract). The tubes were incubated for 18-24 hours at 37˚C and then the results were evaluated by turbidity method.

The concentration of the first tube in which growth was not observed was considered as MIC of the bacterial growth by that extract. Following the MIC determination, tubes lacking turbidity were cultured on a solid MRS broth medium (Fig.2).

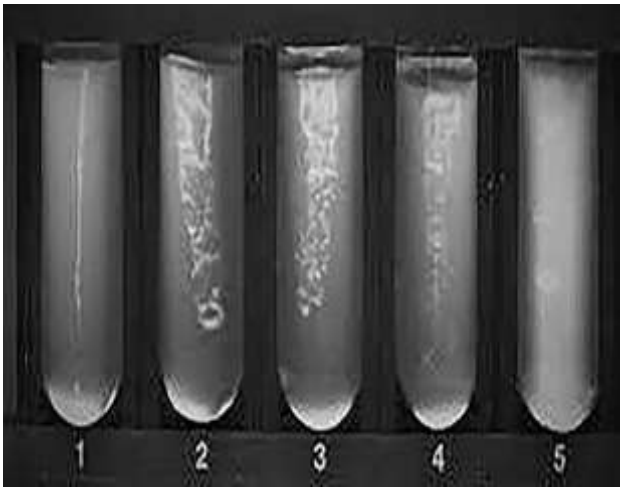

Figure2: *Measurement of the effect of Stevia extract on lactobacillus specimens (Macrodilution broth)*

After incubation at 37 ˚C for 18 to 24 h, growth or non-growth of bacteria was investigated. For the lowest concentration, no bacterial growth was observed, 0 colony count was reported (MBC).

Measurement of the effect of Stevia extract on lactobacillus specimens (well diffusion method): A bacterial culture was prepared On MRS broth culture media, (equivalent to 0.5 MacFarland) with Spread Plate Method, the cultures were prepared and after 5 to 10 minutes in the environment, holes (6 mm in diameter) were prepared, the distance between the holes was 1.5 cm from the edge of the plate and 2.5 cm from each other. 50 µL of various concentrations of the extract poured in the holes. The plates were placed in the refrigerator for 2 hours so that the antimicrobial agent can be released in the environment. The plates were then incubated for 24 hours at 37 ˚C. Subsequently, the inhibition zone were measured under appropriate light using caliper. This measurement was repeated at intervals of 48 hours (Fig.3).

Figure3: *Formation of the inhibition zone*

Data were provided by the SPSS20 statistical program and were evaluated by one-way ANOVA. The meaningful limit was less than 0.05.

Results

The results of the Broth Macrodilution method (percentages of MBC and MIC) for the plant extracts on the bacteria studied in Table 1.

Table1: MBC and MIC of Stevia extracts

MBC	MIC	Herbal extract percent µg/ml
3.12% 26.87 µg/ml	0.78% 71.6µg/ml	

The shorter the MBC and MIC, the higher antibacterial activity. As observed, the extract on the studied bacterial strain had even inhibitory effect at low concentrations. The results of Agar Well Diffusion showed that the extract has a growth inhibitory effect on lactobacillus plantarum. The mean diameter of the inhibition zone (in millimeters) in different concentrations of extract on the studied bacteria is in Table 2.

Table2: Comparison of mean diameter of the inhibition zone (in millimeters) in different concentrations of extract based on the strain of Stevia

p-Value=0.0003 is meaningful	Mean of inhibition zone diameter	Percent of Stevia concentration
	0.60±11.48	100
	0.82±9.45	50
	0.58±8.88	25
	0.24±8.25	12.5
	0.50±6.25	6.25
	0.50±7.55	3.12

there was a positive and significant relationship between the diameter of inhibition zone and the different concentrations of the extract. As the concentration increases, the diameter of the inhibition zone increases (P value <0.01) (Fig.4), so that the maximum diameter of the inhibition zone is with the concentration of 880 µg/ml (Fig.4).

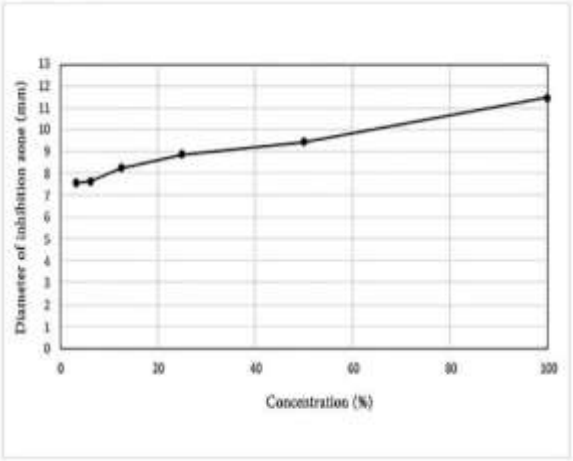

Figure4: *Relationship between concentration and diameter of inhibition zone*

The results of measuring inhibition zone at different times (24 and 48 h) showed that the diameter during the studied periods did not change significantly (p-value -0.78) (Fig.5).

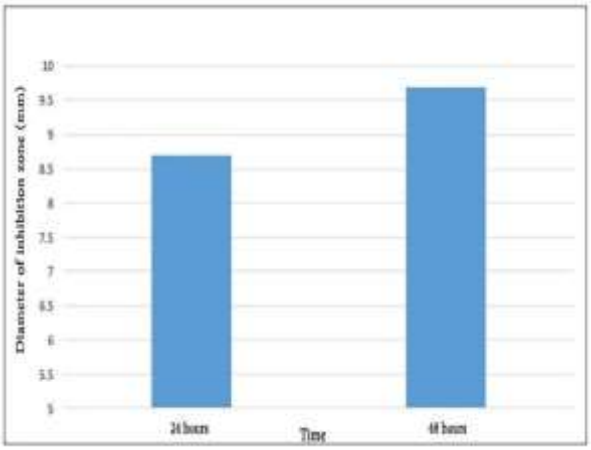

Figure5: *Comparison of mean diameter of inhibition zone in different times*

Discussion

In recent years, bacterial resistance, has identified the need for new antibacterial agents.[8] Stevia is one of the medicinal plants that have been used since the past.[9] It has several antimicrobial and pharmacological effects and reduces the rate of biofilm formation and the progression of caries.[10] Many herbal products have been tested effectively for years to treat gum disease and gastrointestinal problems.[11]

Mohammadi-Sichani et al[5] studied the effect of ethanol and methanol-based Stevia leaf extract on the growth of Streptococcus mutans in 2012. The results of this study confirm the inhibitory effect of Stevia extract on growth of Streptococcus mutans and recommended the use of herbal extracts in toothpastes and mouthwashes. As shown in table 1, the extract had inhibitory effect against Lactobacillus plantarum, and even at low concentrations inhibited the growth of lactobacillus plantarum. The results of MBC also show the effect of stevia in the low concentrations. The results of the Agar Well Diffusion method also confirm the antibiotic effect of stevia. These findings are in agreement with the study of Ghomboa et al.[12] that showed antibiotic activity of stevia against 16 different bacteria including Streptococcus mutans and

lactobacillus. Measured MIC for the stevia in the study of Mohammadi-Sichani et al.[5] against some oral bacterial bacteria, including S. Mutans, was consistent with the results of this study.

In the study of Ajagannanavar et al.,[13] the stevia extract was effective on S. mutans oral streptococci and inhibition zone was observed. In the study of Ajagannanavar[13], the Agar well diffusion method was used because this method is highly accurate. In the present study, unlike the study by Mohammadi-Sichani et al.,[5] the aqueous extract of Stevia prevented the growth of lactobacillus plantarum. In the study of Mohammadi-Sichan,[5] it was shown that only Acetone and Alcohol-based extracts of Stevia caused growth inhibition of Streptococcus mutans, and the aqueous extract of this plant has no inhibitory effects on the bacteria studied. This difference may be attributed to the type of studied bacteria. It has been shown in various studies that lactobacilli are more susceptible than streptococci.[14]

More studies on acetone and alcohol-based solutions is recommended. The data from the measurement of the inhibition zone diameter at different times (24 and 48 h) showed that the diameter of inhibition zone during the studied periods did not change significantly, the reason for this can be attributed to the rapid effect of the extract, which caused a significant difference in the two studied periods. One of the limitations of this study was the use of only one microbial species of lactobacilli[15]. In the future, due to the antimicrobial effects of stevia, it is possible to make the antibacterial composition of the plant through various chemical analysis methods. Also, after examining the effects of the pharmacologic substance of the purified material in vitro and in vitro, the introduction of it for treatment and prevention of dental caries will be provided[16]. In addition, due to the antibacterial effects of Stevia Ribaudiana, the extraction of strong antimicrobial compounds of this plant and the preparation of a new drug formulation for diseases and dental infections are not unexpected.

Conclusion

The results of this study in both Broth Macrodilution and Agar Well Diffusion showed that the plant extract of stevia has the potential to inhibit the growth of lactobacillus plantarum and has an inhibitory effect on it.

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