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Full Length Research Paper

Effect of different extracts of *Stevia rebaudiana* leaves on *Streptococcus mutans* growth

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Plants produce a diverse range of bioactive molecules, making them rich source of different type of medicines. In this study, effect of aqueous, methanol, ethanol and acetone extracts of *Stevia rebaudiana* leaves were evaluated against *Streptococcus mutans*. Agar-well diffusion method was used to determine the antibacterial activity of extracts. Minimal inhibitory concentration was obtained by micro-dilution method. The acetone, ethanol and methanol extracts of *S. rebaudiana* leaves exhibited a concentration dependent on antibacterial inhibition. Acetone and ethanol extracts of *Stevia* leaf gave the highest zone of inhibition against *S. mutans*. Aqueous extract of this plant was not effective on *S. mutans*. Therefore, the findings confirmed the idea that plant extracts of *S. rebaudiana* leaves may have a role to be used as pharmaceuticals and/or preservatives.

Key words: Antibacterial activity, leaf extract, *Stevia rebaudiana*, *Streptococcus mutans.*

INTRODUCTION

The plant *Stevia rebaudiana* Bertoni is a perennial herb and belongs to the family Asteraceae. It is a native of certain regions of South America particularly in Paraguay and Brazil. *Stevia* today is grown in other countries because of economic benefits. It is a natural alternative source to traditional sugar (sucrose) obtained from sugarcane, sugar beet and a non-calorie value one. Recently, *S. rebaudiana* has received a greater attention due to its high range of sweet content (75 to 500 times more than cane sugar) and its therapeutic values for inhibiting fat accumulation and lowering blood pressure in human (Brandle et al., 1998; Soejarto et al., 1982). There are growing interest in using natural compounds, especially extracted from plants, for the preservation of foods, drugs and other products. *Stevia* extracts are officially approved as food additives in Brazil, Korea, Japan, the United States and Iran (Debnath, 2008). *S. rebaudiana* is rich in trepenes and flavanoids. The phytochemical presents in *S. rebaudiana* are austroinullin, β-carotene, dulcoside, niacin, rebaudi oxides, riboflavin, steviol, stevioside and tiamin (Crammer and Ikan, 1986). These nutritional substances affects microbial flora of mouth (Soderling et al., 2008). *Stevia* is thought to inhibit the growth of certain bacteria. Some people even claim that using *Stevia* help to prevent the onset of dental carries. *S. mutans* is common flora of human mouth that has polysaccharide capsule and has important rule to produce dental caries (Hamada et al., 1984; Sweeney et al., 2004). No studies on the concentration dependency of the inhibition of *Streptococcus mutans* with *S. rebaudiana* leaf extracts are available. Therefore, the present study was planned to evaluate the antibacterial activity of *S. rebaudiana* leaves extracted using various solvents against *S. mutans*.

MATERIALS AND METHODS

Dried *S. rebaudiana* leaves were supplied by Biotechnology Research Ltd., Esfahan, Iran. Leaves were packed in polyethylene

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Table 1. Antibacterial activity of different extracts of *S. rebaudiana* leaves against *S. mutans* (Inhibition zone in mm).

Extract	Concentration (mg/ml)					
	100	50	25	12.5	6.25	3.13
Acetone	28.7±2.8	22.8±2.2	21.5±2.4	20.0±1.2	17.0±1.2	16.3±0.9
Ethanol	27.0±0.8	26.5±1.3	23.2±0.9	19.2±0.9	17.8±1.0	16.2±0.9
Methanol	21.3±2.2	17.5±0.5	13.8±0.9	12.5±0.6	10.3±0.5	-

*The inhibition zone of Tetracycline was 10.0; *No inhibition zone observed for 0.25% DMSO well.

bags and stored at -20°C until they were used. The *S. mutans* (ATCC: 35668) strain were obtained from Iranian Research Organization for Science and Technology, Tehran, Iran. *S. mutans* was grown and maintained on Brain Heart Infusion agar (BHI, Merck) supplemented with blood.

Preparation of extracts

Dried powdered *Stevia* leaves were extracted separately with aqueous, methanol, ethanol and acetone by Soxhlet apparatus. The extracts were filtered and dried using a rotary evaporator. Residues were stored in labeled sterile screw capped bottle at -20°C (Sathishkumar et al., 2008).

Antimicrobial assay

The dried extracts were dissolved in 0.25% Dimethylsulfoxide (DMSO, Merck). The yield of aqueous, methanol, ethanol and acetone extracts of the leaves were found to be 200, 100, 50, 25, 12.5, 6.25 and 3.13 mg/ml. Screening of extracts for antibacterial activity was done by agar-well diffusion method (Ghosh et al., 2008). The bacterial suspensions, standardized to 10^8 CFU/ml of *S. mutans* were distributed over the Brain Heart Infusion agar using a sterile swab. Wells (6 mm) were made in the agar plate with a sterile cork borer (6 mm). 100 µl of the plant extracts were poured in the wells. Plates were aerobically incubated at 37°C with 5% CO_2 for 48 h. Antibacterial activity of the plant extract was determined by measuring the diameter of the inhibition zone. Extracts were considered active if the inhibition zone diameter was 8 mm or more as recommended (Baker and Silverton, 1985). 100 µl of 0.25% DMSO served as control. Stock solution of tetracycline was prepared as 10.0 mg/ml (w/v) concentration in sterile distilled water and filter sterilized by using syringe filter. The concentration of 1 µg/well was used for the antibacterial assay in each well. The experiment was done thrice and the mean values were presented.

Minimum inhibitory concentration

The MIC evaluation was adapted from the micro-dilution methodology proposed by Clinical and Laboratory Standards Institute (Wiegand et al., 2008). The wells of a 96-well ELISA tray were filled with 100 µl of exponentially growing culture (10^8 CFU/ml) and added with 100 µl of diluted extract. The absorbance of each well was determined using an automatic ELISA tray reader at 630 nm (StatFax 2100, Awareness Technology Inc., USA). The plate was incubated at 37°C with 5% CO_2 for 48 h, agitated and the absorbance was read again in the reader at the same wavelength. These absorbance values were subtracted from those obtained before incubation. This procedure eliminated the interference of the tested substance. All tests were performed in triplicate. The MICs value for an extract was expressed as the lowest concentration that inhibits the bacterial growth.

Minimum bactericidal concentration

After determination and interpretation of the MIC, a 50 µl aliquot of the remaining culture was added to BHI plates, with one plate corresponding to each tube, and spread with a sterile loop. The whole of the previous procedure was carried out with the standardized inoculums of *Streptococcus mutans* as a positive control for MBC, which corresponded to the total number of colonies. The plates were incubated at 37°C with 5% CO_2 for 48 h and examined for growth bacteria. MBC value was defined as the lowest concentration of extracts needed to kill 99% of bacteria. Each experiment was repeated at least twice. Positive and negative controls were considered for all part of experiments.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. The data were subjected to one-way analysis of variance (ANOVA), and differences between samples were determined by Duncan's multiple range tests using the SPSS 17.0 software. P values < 0.05 were regarded as significant.

RESULTS

The antibacterial activity of the solvent extracts of *S. rebaudiana* showed significant variations. To search for evaluated the antibacterial activity of aqueous, methanol, ethanol and acetone extracts of *S. rebaudiana* leaves against *S. mutans*, the extracts showed different effect. *In vitro* antibacterial activity of methanol, ethanol and acetone extracts of *Stevia rebaudiana* is shown in Table 1. Aqueous extract of *S. rebaudiana* had not inhibition activity against *S. mutans*.

All extracts showed dose dependent activity which increases with increase in concentration (Table 1). Methanol, ethanol and acetone extracts were very effective against *S. mutans* but aqueous extract exhibits no activity. Among the four extracts, tested acetone extract had greater antibacterial potential followed by ethanol extract and then methanol extract. Acetone (28.7±2.8) and ethanol (27.0±0.8) extracts showed significantly ($p<0.05$) higher activity compared to

Table 2. MIC and MBC values for different extracts of *S. rebaudiana* leaves.

Extracts	MIC (mg/ml)	MBC (mg/ml)
Acetone	3.13	12.5
Ethanol	6.25	12.5
Methanol	50.00	100.00

methanol extract against *S. mutans* on each concentration. There was not significantly different between acetone and ethanol extract activity ($p<0.05$). Maximum growth inhibition against *S. mutans* was shown by acetone extract (28.7 mm) at 100 mg/ml which was followed by ethanol extract (27.0 mm). Inhibition zone of methanol extract was 21.3 ± 2.2 at 100 mg/ml that was less than other extracts.

S. rebaudiana extracts are considered to have moderate antibacterial activity. The MIC and MBC of these extracts are obtained and reported in Table 2. The activities of the extracts were found to be comparable with 1% concentration of antibiotic used (tetracycline).

DISCUSSION

A renewed interest has occurred in the last decade to search for antibacterial activity and phytochemicals of native plants (Solanki, 2010). *S. rebaudiana* belonging to family Asteriaceae is a natural alternative to artificial sweetener. It contains over 100 phytochemicals (Fazal et al., 2011). *S. rebaudiana* leaf extracts demonstrated antibacterial activity. These extracts showed varying degree of antibacterial activity against *S. mutans* (Table 1). There is no previous reported work on antibacterial activity of this plant against *S. mutans*, except the study reported by Debnath (2008). He worked on antibacterial activity of chloroform and methanol extracts of *S. rebaudiana* leaves against *S. mutans*. Debnath (2008) reported that the chloroform and methanol extracts of *S. rebaudiana* leaves exhibited a concentration dependent on antibacterial and antifungal activity (Debnath, 2008). Acetone and ethanol extracts of *S. rebaudiana* leaves presented a better inhibitory effect on *S. mutans*. Sathishkumar et al. (2008) evaluated antibacterial activity of *S. rebaudiana* leaves extracted using various solvents against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi* and *Vibrio cholera*. They found that the acetone extract showed greater activity against Gram positive bacteria than Gram negative bacteria. The higher antibacterial activity of the acetone and ethanol extracts may be due to the greater solubility of the extract in these organic solvents or this could be attributed to the concentration of the active substances (Malik et al., 2011). It seems the use of acetone and ethanol as extracting solvents proved to be more efficient in extracting the active compounds. These results are in agreement with that of Fazal et al. (2011). The methanol extract of *S. rebaudiana* leaves showed low antibacterial activity against *S. mutans*. A similar result was found by Manish et al. (2006) in their study of *in vitro* antimicrobial activity of *S. rebaudiana* Bertoni leaves. Manish et al. (2006) reported that hexane extracts of *S. rebaudiana* leaves showed higher activity compared to methanol and ethyl acetate extracts against micro-organisms tested. Their results showed that aqueous extract of *Stevia* leaves have no effect on micro-organisms tested. The aqueous extract of *S. rebaudiana* leaves was practically ineffective against *S. mutans*. This finding is similar to that of other researchers (Debnath, 2008, Manish et al., 2006). Tomita et al. (1997) reported bactericidal activity of fermented hot water extract from *S. rebaudiana* towards enterohemorrhagic *E. coli* O157: H7 and other food borne pathogenic bacteria. Methanol extract was found to be the best solvent to result in good antimicrobial activity but they did not examine other extracts against selected bacteria (Tomita et al., 1997).

Conclusion

Acetone and ethanol extracts of *S. rebaudiana* leaves showed the highest activity against *S. mutans*. It may also be concluded that the active metabolites are most soluble and act as antibacterial substance while acetone and ethanol act as solvent systems. Obviously, it needs further researches on this plant and their pharmacological implication of its extracts. Therefore, the present work indicate that *Stevia* leaf extracts may be an ideal candidate for further research into their uses for food preservation as well as, pharmaceutical and natural plant-based products.

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