ANTIMICROBIAL POTENTIAL OF EXTRACTS FROM *STEVIA REBAUDIANA* LEAVES AGAINST BACTERIA OF IMPORTANCE IN DENTAL CARIES

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ABSTRACT

In recent years, the antimicrobial activity of Stevia rebaudiana Bertoni leaf extracts against a large number of microorganisms has been evaluated, but not its activity against microorganisms of importance in dental caries. The aim of this study was to evaluate the antibacterial activity of Stevia rebaudiana Bertoni leaf extracts against cariogenic bacteria. Extracts were obtained from the dried Stevia rebaudiana Bertoni leaves in hexane, methanol, ethanol, ethyl acetate and chloroform. The antimicrobial activity of the 5 extracts against 16 bacterial strains of the genera Streptococcus (n= 12) and Lactobacillus (n= 4) was evaluated by the well diffusion method. Minimal inhibitory concentrations (MIC) of the extracts in hexane, methanol, ethanol, ethyl acetate and chloroform on the 16 bacterial strains were respectively 30 mg/ml, 120 mg/ml, 120 mg/ml, 60 mg/ml and 60 mg/ml. The zones of inhibition present at the MIC were variable, ranging from 9 mm to 17.3 mm. Our results suggest that inhibition zones with a hexane extract are similar to those obtained with ethanol and methanol, but the minimal inhibitory concentration (30 mg/ml) is lower. For the four Lactobacillus species, the inhibition zones obtained between 12.3 and 17.3 mm were somewhat larger with ethyl acetate and chloroform extracts, suggesting they were the most susceptible microorganisms.

Keywords: *antiInfective agents - stevia - dental caries*

POTENCIAL ANTIMICROBIANO DE EXTRACTOS OBTENIDOS DE HOJAS DE *STEVIA REBAUDIANA* BERTONI SOBRE BACTERIAS DE IMPORTANCIA EN CARIES DENTAL

RESUMEN

En los últimos años se ha evaluado la actividad antimicrobiana de extractos obtenidos de hojas de Stevia rebaudiana Bertoni sobre un gran numero de microorganismos. Sin embargo, no existen evaluaciones en microorganismos de importancia en caries dental. El objetivo de este estudio fue evaluar la actividad antibacteriana de extractos de hojas de Stevia rebaudiana Bertoni sobre microorganismos cariogénicos. A partir de hojas secas de Stevia rebaudiana Bertoni convertidas en polvillo se obtuvieron los extractos en dichos solventes. La evaluación de la actividad antimicrobiana de los 5 extractos sobre las 16 cepas bacterianas de los géneros Streptococcus (n=12) y Lactobacillus (n=4) se realizó por el método de difusión en pozo. Las concentraciones mínimas inhibitorias (CMI) de los extractos de hexano, metanol, etanol, acetato de etilo y cloroformo, sobre las 16 cepas bacterianas fueron respectivamente de 30 mg/ml, 120 mg/ml, 120 mg/ml, 60 mg/ml y 60 mg/ml, respectivamente. Los halos de inhibición determinados a las CMI fueron variables, el de menor valor fue 9 mm y el de mayor fue de 17.3 mm. Nuestros resultados, sugieren que los halos de inhibición en el extracto de hexano son semejantes a los obtenidos para el etanol y metanol, sin embargo, la CMI (30 mg/ml) es menor. En las 4 especies de Lactobacillus los halos de inhibición obtenidos entre 13 y 17.3 mm, son ligeramente mayores en los extractos de acetato de etilo y cloroformo, sugiriendo que fueron los microorganismos más susceptibles.

Palabras claves: *agentes antiInfecciosos - stevia - caries dental*

INTRODUCTION

In physiological conditions, most bacteria in the oral cavity are compatible with health. However, under certain circumstances of the oral environment and the condition and behavior of the host, these bacteria may reveal their potential virulence and cause disease[1]**.** Dental caries is considered to be an alteration of the oral ecosystem with predominance of pathogenic flora. The main microorganisms associated to caries production are, in order of frequency: 1) *Streptococcus mutans* (mainly serotype c) and to a lesser extent *S. sobrinus* and *S. gordonii* and 2) *Lactobacillus* and *Actinomyces* species[1,2]. Their participation in the dental caries generating process has led to the development and implementation of measures for prevention and/or control[1,3]. Among other strategies implemented to eliminate and/or control the microorganisms involved in dental caries, chemical and antimicrobial substances have been used. Antimicrobial biomolecules of

natural origin are also currently being explored for use in adjuvant therapy[4].

Plants have been widely used around the world as traditional remedies in the treatment of diseases. It is estimated that 66% to 75% of the world population currently uses plant-based medicines[5]. The main aim of research into medicinal plants is to identify plants that possess pharmacological activity, and thus discover new substances or molecules having antimicrobial activity that could be transformed into medications by different chemical processes and used to control or prevent infectious diseases[6].

Research by Katsura et al.[7] reports the bactericidal activity of bakuchiol against *S. mutans, S. sanguis, S. salivarius, S. sobrinus, L. acidophilus, L. casei* among other microorganisms of the oral cavity. Another study reports the high inhibitory capacity of isopanduratin A against *S. mutans, S. sobrinus, S. sanguinis* and *S. salivarius* [8].

Stevia rebaudiana (Bertoni), one of the 407 species of the genus *Stevia* and one of the only two whose leaves contain a sweetening substance, has been used by native people as a sweetener and for medicinal purposes. It is a shrub originally from Paraguay and Brazil, which occasionally grows wild[9]. It was described botanically in 1905 by naturalist Moisés Santiago Bertoni, as an herbaceous plant 40 to 80 cm tall[9] of the family Compositae[10]. In addition to being a non-caloric sweetener known in many parts of the world, it has hypoglycemiant, antioxidant and antihypertensive action[11-14]. Another advantage is that no toxic or genotoxic activity has been found in the complete extracts obtained from *Stevia rebaudiana* Bertoni leaves[15].

There are studies reporting antimicrobial activity of extracts obtained from *Stevia rebaudiana* on fungi and Gram-positive and Gram-negative bacteria[11,16,17]. None of these studies includes an evaluation of *Stevia rebaudiana* extracts on the microorganisms involved in dental caries. The aim of this study was to evaluate the antibacterial activity of extracts in hexane, methanol, ethanol, ethyl acetate and chloroform from *Stevia rebaudiana* Bertoni leaves against bacteria that are important in dental caries and oral health.

MATERIALS AND METHODS

Bacteria

The following 16 bacterial strains were used to evaluate the antimicrobial activity of the extracts obtained from *Stevia rebaudina*: *Streptococcus mutans* ATCC 25175, *Streptococcus mutans* ATCC 31989, *Streptococcus mitis* 804 NCTC 3165, *Streptococcus salivarius* NCTC 8606, *Lactobacillus acidophilus* ATCC 4365, *Streptococcus rattus* FA 1 (G), *Streptococcus mutans* C67-1, *Streptococcus cricetus* AHT, *Streptococcus mutans Ingbritt, Lactobacillus plantarum* 748, *Lactobacillus casei* 475, *Lactobacillus brevis*, *Streptococcus mutans* 35FS3, *Streptococcus mutans* 35FS1, *Streptococcus mutans* 29FS2 and *Streptococcus sobrinus* CIO 428. In order to reconstitute and confirm their viability for the assays, the lyophilized strains were re-suspended in brain heart infusion (BHI) broth and incubated at 37 ºC for 48 hours in anaerobic conditions (H_2:CO_2:N_2, 10:10:80) so that they would grow well, after which they were transferred to BHI agar for isolating.

Extracts

Dry *Stevia rebaudiana* leaves (Agricultura Colombiana) were powdered in a mill until 800 grams were collected. Extracts were obtained in ethanol, methanol, ethyl acetate, chloroform and hexane using the cold soaking technique. Sixty grams of powdered *Stevia rebaudiana* leaves were soaked in 250 ml of each solvent and placed in a mechanical shaker (Precision Reciprocal Shaking Bath, Precision Scientific; USA) at 150 rpm and 37ºC for 24 hours. Then they were filtered through filter paper (Spezialpapier Filtrak, GMBH; Germany) to remove any leaf residue. The extracts were immediately filtered again through 0.45 µm filters (Filter Bottle Top, Sigma Chemical Company; USA) to remove bacteria and ensure that they were free from contamination. Then they were concentrated at low pressure in a rotary evaporator (Buchi B-169, Vacuum-System; Germany) until they were dry. A microbiological test was performed on each concentrated extract to ensure that there was no bacterial contamination at the end of the process. Finally, the concentrated extracts were used to prepare 4 concentrations (15 mg/ml, 30 mg/ml, 60 mg/ml and 120 mg/ml).

Microbiological Assays

The antimicrobial activity of the extracts on bacteria was tested using the well diffusion method described by Dobner et al.[18]. A pure culture of each bacterium to be tested was used to prepare a suspension in trypticase soy broth and adjusted by tur-

bidimetry to 0.5 on the Mac Farland scale. From this suspension, 100 µl were taken and added to 20 ml of Mueller Hinton agar (liquid and sterile), mixed and poured into Petri dishes. The agar was allowed to solidify, and after waiting 20 - 30 minutes, a sterile Pasteur pipette was used to make 4 to 5 0.5 cm wells in the agar in each dish. Thirty µl of each of the 5 extracts was placed in the wells (this volume fits exactly into the wells without overflowing onto the surface of the culture medium). Vancomycin 180 µg/ml and Azithromycin 150 µg/ml were used as positive controls and each of the solvents as negative controls. The dishes were incubated at 37 ºC for 24-72 hours under anaerobic conditions (H_2:CO_2:N_2, 10:10:80).

After the incubation, the presence or absence of inhibition zones was determined. Their diameter was measured and the minimal inhibitory concentrations (MIC: lowest concentration of the extract that produces an inhibition zone of at least 6 mm) determined. Each assay was performed in triplicate and the average reported in mm.

RESULTS

Final yield (dry weight -expressed as a percentage- that was finally obtained from processing 60 grams of *S. rebaudiana* leaves) for each extract in ethanol, methanol, ethyl acetate, chloroform and hexane was, respectively, 22%, 16%, 10%, 14 % and 0.9%. None of the 5 negative controls had antimicrobial activity. The positive controls (Vancomycin 180µg/ml and Azithromycin 150 µg/ml) had variable inhibitory activity on the 16 strains included in the study, with values ranging from 18 mm to 25 mm.

The MIC of the extracts in hexane, methanol, ethanol, ethyl acetate and chloroform on the 16 bacterial strains of the genera *Streptococcus* and *Lactobacillus* were, respectively 30 mg/ml, 120 mg/ml, 120 mg/ml, 60 mg/ml and 60 mg/ml.

Table 1 shows the results obtained with extracts in hexane, methanol, ethanol, ethyl acetate and chloroform on the 16 microorganisms studied. The inhibition zones of the MIC for the 5 extracts on the bacterial strains were variable, ranging from 9 mm to 17.3 mm. The hexane extract, which had the lowest MIC, produced the following inhibition zones, in mm: 10.0, 11.0, 12.0, 10.0, 9.3, 10.6, 10.3, 10.6, 10.3, 9.6, 12.6, 9.6, 13.6, 14.3, 14.0 and 13.3. The inhibition zones of the 5 extracts were slightly higher for the 4 *Lactobacillus* strains than for the 12 *Streptococcus* strains, as the lowest value for inhibition zone was 12.3 mm and the highest was 17.3 mm.

Table 1: Antibacterial activity of the *Stevia rebaudiana* Bertoni leaf extracts against the 16 bacterial strains.

	Extracts				
	Hexane 30 mg/ml	**Methanol 120 mg/ml**	**Ethanol 120 mg/ml**	**Ethyl acetate 60 mg/ml**	**Chloroform 60 mg/ml**
Bacteria					
S. mutans ATCC 25175	10.0	9.3	9.0	10.0	12.0
S. mutans ATCC 31989	11.0	9.0	10.3	10.3	11.6
S. mutans C67-1	12.0	9.3	10.0	10.6	12.3
S. mutans Ingbritt	10.0	9.0	9.3	11.0	12.3
S. mutans 35FS3	9.3	9.0	9.3	11.3	10.3
S. mutans 35FS1	10.6	9.0	9.3	10.3	11.0
S. mutans 29FS2	10.3	10.0	11.0	10.6	9.3
S. sobrinus CIO 428	10.6	9.3	10.3	11.0	10.6
S. mitis 804 NCTC 3165	10.3	8.6	9.3	9.0	10.3
S. salivrius NCTC 8606	9.6	9.0	10.0	9.3	11.6
S. rattus FA1(G)	12.6	13.0	13.0	12.6	13.3
S. cricetus AHT	9.6	9.0	9.6	10.3	11.3
L. acidophilus ATCC 4365	13.6	13.0	14.0	15.0	17.3
L. plantarum 748	14.3	12.3	13.3	15.3	16.0
L. casei 475	14.0	13.0	14.0	15.3	14.3
L. brevis	13.3	13.0	13.6	14.6	14.6

The values of the inhibition zones at the minimal inhibitory concentration are expressed in mm.

DISCUSSION

New substances with pharmacological potential have been searched for and applied since ancient times. During the last twenty years, there has been a revival of interest in research into natural products, as they make up 50% of the drugs used clinically in developed countries, of which 25% come from higher plants[5].

This is the first study to determine the antimicrobial activity of 5 extracts obtained from *Stevia rebaudiana* leaves on 16 Gram-positive bacterial species of the genera *Streptococcus* and *Lactobacillus*, which are important for dental caries and oral health. Existing research to date has only proved the antimicrobial activity of extracts of *Stevia rebaudiana* leaves on fungi, rotavirus virus and very different bacteria from those evaluated in this study[11,16,17,19-23].

The results of this study match those of Tadhani et al.[16], Ghosh et al.[17] and Taware et al.[19] regarding the activity of extracts in hexane, methanol, ethanol, ethyl acetate and chloroform on Gram-positive and Gram-negative bacterial species. In the study by Tadhani[16], the antimicrobial activity of the extracts in water, methanol, ethyl acetate and hexane is highly variable and broad against *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Serratia marcescens, Pseudomonas aeruginosa, Bacillus megaterium, Escherichia coli, Proteus vulgaris, Rhizopus oligosporus* and *Aspergillus niger*. The extract in water is only active against *B. subtilis* and *S. aureus,* and the other three extracts are highly active against all the microorganisms evaluated. The extract in hexane has the greatest activity, including the Gram-positive (*B. subtilis, S. aureus, M. luteus*) with inhibition zones ranging from 8.67 to 35.3 mm. The study by Ghosh[17] reports the antimicrobial potential of extracts in 6 solvents against 4 fungi and 6 bacterial species: *Alternaria solani, Helminthosporium solani, A. niger, Penicillium chrysogenum, E. coli, B. subtilis, Enterococcus faecalis, Proteus mirabilis, P. aeruginosa* and *S. aureus*; the Gram-positive species (*B. subtilis, Enterococcus faecalis* y *S. aureus*) were susceptible to all 6 extracts (petroleum ether, cyclohexane, chloroform, water, acetone and ethanol) at a concentration of 250 µg/ml using the plate dilution method. The study by Taware[19] reports inhibitory activity against *S. aureus* of extracts in ethyl acetate obtained from *S. rebaudiana* callus.

Noticeable in the study by Abou-Arab et al.[11] is the lack of antimicrobial activity of the extracts in hexane, water, ethyl acetate and chloroform against three Gram-positive microorganisms (*Listeria monocytogenes, S. aureus* and *Bacillus cereus*). In the study by Jayaraman et al.[21], the extracts in water and chloroform obtained form *S. rebaudiana* leaves have no antimicrobial activity against *S. aureus*. The differences in the susceptibility of Gram-positive microorganisms to *Stevia rebaudiana* leaf extracts might be due to particularities of the genus and species and to differences in the organization of the cell wall[1,2].

The results of this study show antimicrobial activity of all the extracts at MICs between 30 mg/ml and 120 mg/ml. The ethanol and methanol extracts which have the same MIC (120 mg/ml) have similar inhibitory activity. The inhibition zones for the hexane extract are similar to those for ethanol and methanol, nevertheless, the MIC (30 mg/ml) is lower. The inhibition zones for the 4 *Lactobacillus* species are slightly higher in the ethyl acetate and chloroform extracts (60 mg/ml), suggesting that they are the most susceptible microorganisms. Their greater susceptibility to the different extracts may be due to their cell wall structure or to the presence of a substance or synergic mixture in the extracts that can penetrate the bacteria easily and produce greater damage[24,25].

Further studies are needed on the isolation, characterization and identification of substances present in the extracts, and to determine the antibacterial activity against a wide range of microorganisms that are important in other oral infections. These substances could subsequently be used in toothpastes, mouthwashes or other oral products with antibacterial potential. In addition, before any extracts or substances obtained from *S. rebaudiana* leaves are used therapeutically, it must be ensured that they are not toxic to eukaryotic cells.

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