

Full Length Research Paper

Antibacterial activity of extracts of *Stevia rebaudiana* Bertoni against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*

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Plants have been an important source of secondary metabolites known for their diverse biological activities; some have been shown to inhibit the development of certain pathogenic microorganisms. Herein, the antimicrobial activity of the carbon tetrachloride, hexane, ethanol, and aqueous extracts of leaves of *Stevia rebaudiana* Bertoni against *Staphylococcus aureus* (strain 921), *Staphylococcus epidermidis* (strains 965, 982, and 735), and *Pseudomonas aeruginosa* (strains RO3 and RO4) was presented. The antibacterial activity was evaluated using the disk diffusion method, and the minimal inhibitory concentration (MIC) was determined. The results show that, even at the lowest evaluated concentration (1.06 mg/mL), the hexane extract had an inhibitory effect for all the studied microorganisms. The aqueous extract exhibited high inhibition values (84.4%), on *S. epidermidis* (strain 965). These results indicate that compounds contained in non-polar extracts of *S. rebaudiana* could be potential candidates as conventional pharmaceutical drugs against bacteria, resistant to conventional antibiotics.

Key words: *Stevia rebaudiana*, antimicrobial properties, plant extracts.

INTRODUCTION

One of the most important challenges for public health is the increasing threat posed by infectious diseases caused by bacteria resistant to conventional antibiotic

treatments (Brown, 2004). As examples of these, one may mention Gram-positive bacteria such as *Staphylococcus* spp. and Gram-negative bacteria such

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as *Pseudomonas* spp. (Zaborina et al., 2006). These kinds of bacteria can be found in the environment, and they have been detected in different kinds of surfaces and materials, such as cheeses, sauces and meats, among others (Azelmad et al., 2017).

It is well known that in different places around the world, different plant parts are used in health treatments. Diverse studies have shown that plants are a valuable source of secondary metabolites with biological activity that may be used as medicines. These have several additionally advantages: they are specific and biodegradable; and they do not promote bacterial resistance and do not leave toxic residues in the environment (Siddique et al., 2014). Some plant species contain glucosides that are pharmacologically interesting because of their anti-obesity, anticancer or antibacterial activity (Lemus-Mondaca et al., 2012). *Stevia rebaudiana* B. is a plant species with valuable biological properties, most notably a microbial activity against several microorganisms like *Escherichia coli*, *Lactobacillus acidophilus*, *Streptococcus mutants*, *Corynebacterium ditteriae* and *Candida albicans*, among others (Gamboa and Chaves, 2012; Mali et al., 2015). Some environmental and clinically important microorganisms such as *Staphylococcus epidermidis*, *Staphylococcus aureginosa* and *Pseudomonas aureginosa* have been reported to show resistance to conventional drugs (Claessens et al., 2015; Maliniak et al., 2016). The aim of this study was to evaluate the antimicrobial activity of four vegetable extracts from the leaves of *S. rebaudiana* against microorganisms isolated from the environment (*Staphylococcus aureus*, strain 921; *S. epidermidis*, strains 965, 982, and 735; and *P. aeruginosa*, strains RO3 and RO4).

MATERIALS AND METHODS

Preparation of the vegetable material

Fresh leaves of *S. rebaudiana* were collected in March 2015, from young plants kept under greenhouse conditions at $28 \pm 2^\circ\text{C}$ and a relative humidity of 50% in the Research Center in Applied Biotechnology, National Polytechnique Institute (CIBA-IPN), in March 2014, Mexico. These leaves were later dried in a stove oven at $60 \pm 1^\circ\text{C}$ for 48 h; after this time, they were separated and ground to obtain a vegetable biomass (powder), which was stored in polyethylene bags at -18°C until its use.

Microorganisms

Bacterial strains isolated from the air were obtained from the strain collection of the Microbiology Department of the Chemical Sciences, Faculty of the Meritorious Autonomous, University of Puebla, Mexico. The microorganisms tested in the present work were *S. aureus* (strain 921), *S. epidermidis* (strains 965, 982, and 735), and *P. aeruginosa* (strains RO3 y RO4). Additionally, the reference strains *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 80299 were also used. All the bacterial strains were cultured and maintained on Mueller Hinton agar at 37°C . The susceptibility tests were made by the Kirby-Bauer method on Petri plates with Mueller

Hinton agar. The antibiotics used were piperacillin-tazobactam, cefepime, gatifloxacin, vancomycin, tetracycline, ofloxacin, ampicillin and dicloxacillin, for *Staphylococcus*; and piperacillin, cefepime, meropenem, ofloxacin, gatifloxacin, piperacillin-tazobactam, aztreonam, imipenem and gentamicin, for *Pseudomonas*. The plates were incubated for 18 to 24 h at 37°C . All tests were done in triplicate.

Preparation of the extracts

The extracts were obtained with upward polarity. The aqueous extract was obtained by the maceration technique, and 25 g of leave powder was added in 100 mL of distilled water. It was then maintained in mechanical agitation at approximately 220 rpm for 24 h; the supernatant was used as extract. The crude extract was filtered twice, first with a Whatman No. 5 paper filter, and then with a Whatman No. 1 paper filter. The organic extracts were obtained with hexane, carbon tetrachloride, and ethanol in a Soxhlet extractor for 5 h. The solvent was evaporate and the extracts concentrated by evaporation to dryness under reduced pressure using a rotavap. All the extracts were stored in tagged, sterile amber flasks at -20°C for their later analysis.

Antimicrobial activity bioassay

All the extracts were subjected to an antimicrobial bioassay using the well diffusion technique on (BD Difco) Mueller Hinton medium and measuring the inhibition zone diameter (IZD) (Abdollahzadeh et al., 2014). Bacterial solutions of *Pseudomonas* sp. or *Staphylococcus* spp. aged 24 h and having a cell density equivalent to that of the 0.5 tube in the McFarland turbidity standards were massively seeded on Petri dishes with four wells; the extracts of *Stevia rebaudiana* were later added to the wells. Isotonic saline solution (ISS) was used as negative control. Imipenem® (10 µg) impregnated disks were used as positive control, for *Pseudomonas*, and vancomycin (30 µg) impregnated disks, for *Staphylococcus*. Incubation at 37°C proceeded for 24 h, and at the end of this period, the inhibition zones formed in the medium were measured in millimeters using a scale. All the experiments were done in triplicate.

Minimal inhibitory concentration (MIC)

MIC assays (NCCLS, 2003) were carried out for those extracts that presented inhibiting halos in the well diffusion tests. Each extracts were evaluated by adding it at different concentrations (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) to Mueller Hinton agar in Petri plates, 2 µl of bacterial solution (of *Pseudomonas* sp. or *Staphylococcus* spp.) having a cell density equivalent to that of the 0.5 tube in the McFarland turbidity standards was seeded (dropped) on each plate. ISS was used as positive control. Plates with Mueller Hinton agar without extract were used as negative control (CLSI, 2015). The plates were incubated at 37°C for 24 h. The growth zones were measured at the end of the incubation period and the test was interpreted as positive if no growth was observed in the inoculation spot. The lowest concentration able to inhibit bacterial growth, relative to the positive control, was identified as the MIC.

Statistical analysis

The data obtained was evaluated by means of a variance analysis (ANOVA) and Tuckey's test was done with a $p > 0.05$. The data was interpreted using version 8 of the SPSS software.

Table 1. Effect (as a percentage of the result for the positive control) of extracts of *S. rebaudiana* against different species of environmental bacteria.

Strain	Aqueous extract	Ethanol extract	Tetrachloride extract	Hexane extract
<i>S. aureus</i> ATCC 25923	58.45	55.71	32.88	51.14
<i>S. aureus</i> 921	37.04	22.79	29.91	61.25
<i>S. epidermidis</i> 982	54.63	50.73	32.52	50.73
<i>S. epidermidis</i> 735	14.29	0.00	14.29	44.44
<i>S. epidermidis</i> 965	84.44	54.81	31.11	42.96
<i>P. aeruginosa</i> ATCC 27853	29.11	46.58	0.00	45.12
<i>P. aeruginosa</i> RO 3	0.00	16.51	0.00	22.86
<i>P. aeruginosa</i> RO 4	0.00	0.00	12.50	43.75

Table 2. Minimal inhibitory concentration (MIC) in mg/mL of extracts of *S. rebaudiana* Bertoni for different species of environmental bacteria.

Strain	Aqueous extract	Ethanol extract	Tetrachloride extract	Hexane extract
<i>S. aureus</i> ATCC 25923	40	5.36	2.3	1.06
<i>S. aureus</i> 921	4	0.161	0.23	0.011
<i>S. epidermidis</i> 982	40	5.36	2.3	1.06
<i>S. epidermidis</i> 735	40	*	2.3	1.06
<i>S. epidermidis</i> 965	1.2	0.429	2.3	1.06
<i>P. aeruginosa</i> ATCC 27853	40	5.36	*	1.06
<i>P. aeruginosa</i> RO 3	*	5.36	*	1.06
<i>P. aeruginosa</i> RO 4	*	*	2.3	1.06

*There is no antimicrobial activity.

RESULTS AND DISCUSSION

Table 1 shows the antimicrobial effects of *S. rebaudiana* leave extracts on strains of different environmental bacteria. By analyzing the obtained results, one can see that the hexane extract was inhibited with diverse percentages (from 22.86 to 61.25%), the growth of all the microorganisms was subjected to the tests. For the strains, *S. epidermidis* 965, *S. epidermidis* 982, *S. aureus* ATCC25923 and *S. aureus* 921, all the extracts showed an inhibition effect. The aqueous extract exhibited the maximum inhibition value, which was 84.4% on *S. epidermidis* 965. Regarding the MIC, the values for the strain *S. aureus* 921 were the lowest (0.011-4 mg/mL) for all the extracts evaluated. The MIC for the hexane extract (1.06 mg/mL) was the lowest for all the strains (Table 2). Phytochemical studies have shown that *S. rebaudiana* possesses a great diversity of chemical constituents; some authors have pointed out the presence, in *S. rebaudiana* leave extracts, of alkaloids, flavonoids, phenols, steroids, essential oils and in lower concentration, tannins (Preethi et al., 2011; Lemus-Mondaca et al., 2012). These compounds have been described in several works as having antibacterial activity; with flavonoids, aromatic acids, and terpenoids mentioned as being responsible for that activity (Choi et

al., 2006; Abou-Arab and Abu-Salem, 2010).

It is worth emphasizing that the aqueous extract presented the greatest inhibition percentage and this occurred for some of the strains of *S. epidermidis* and *S. aureus*. These results show that the aqueous extract, having greater polarity, presents greater solubility, and its effects may be seen from reported studies on several medicinal plants (Das et al., 2009; De Boer, 2005). However, other works report that aqueous extracts do not present antimicrobial activity against *S. aureus* (Jayaraman et al., 2008), nor against *P. aeruginosa* (Tadhani and Subhash, 2006).

Recently, Molina-Calle et al. (2017) carried out the characterization and analysis of compounds present in polar and non-polar extracts of leaves of *S. rebaudiana*. Their report highlights the finding, in the polar extracts, of phenolic compounds, which were classified into two families: flavonoids and quinones. Several caffeoylquinic acids were found among the quinones. Jin et al. (2014) reported the antibacterial activity of di-O-caffeoylquinic acid against *Bacillus shigae*. Furthermore, Molina-Calle et al. (2017) reported also that the most important constituents of non-polar extracts were amides, fatty acids and its derivatives, and, remarkably, glycerolipids which are poorly studied.

Muanda et al. (2011) reported a test of the activity of

aqueous, methanol-water, and essential oil extracts against several strains of *S. aureus*, *Bacillus subtilis*, *Escherichia coli*, *P. aeruginosa*, *Aspergillus niger*, and *Candida albicans*; only the aqueous and essential oil extracts do not present activity against *Aspergillus niger* and *P. aeruginosa*. Gamboa et al. (2012) used ethanol, methanol, and hexane extracts to evaluate activity against 16 bacterial strains of the genera *Streptococcus* and *Lactobacillus*, bacteria associated with caries production, and the best results in the MIC test were obtained for the hexane extract. Siddique et al. (2014), using a partitioning extraction method with several solvents, from non-polar to polar, starting with n-hexane, and followed by dichloromethane, acetone and ethanol, successively, tested the extracts obtained against 13 plant- and animal-pathogenic strains implicated in food spoilage.

According to their results, the polar extracts do not show antimicrobial activity, which contrasts with the activity shown by the non-polar extracts obtained using n-hexane and dichloromethane. Dhouioui et al. (2016) reported the antimicrobial activity of several fatty acids, including palmitic and stearic acids (also present in *Stevia*), found in non-polar extracts of aerial parts and roots of *Aristolochia longa* and *Bryonia dioica*; these acids showed antibacterial activity against *E. coli* and *Enterococcus faecium*. Tadhani and Subhash (2006) reported the antimicrobial activity of non-polar extracts of *S. rebaudiana* leaves against *S. aureus* and *P. aeruginosa*; their findings coincide with those reported in the present work.

The search, in plants, of chemical compounds with antimicrobial activity represents a promising alternative for the development of new pharmaceutical drugs. In the case of *S. rebaudiana*, this plant is not only the source of a natural sweetener, but also, because of its strong antimicrobial activity against a wide spectrum of pathogenic microorganisms combined with its lack of adverse effects on human health, which has been shown by several studies, it may be a potential natural antibiotic.

Conclusions

The hexane extract showed an inhibitory effect at the lowest concentration evaluated and it could inhibit the growth of all the microorganisms subjected to tests in this study. The MIC values for the *S. aureus* 921 strain were the lowest (0.011 to 4 mg / mL) for all the extracts evaluated, the MIC for the hexane extract (1.06 mg/mL) was the lowest for all the strains. This study shows *S. rebaudiana* as an alternative for the control of *S. epidermidis*, microorganism that has shown resistance to some conventional antibiotics.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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