Revista Internacional de Investigación e Innovación Tecnológica

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Antibacterial effect of ethanolic extract of *Stevia rebaudiana* B. against *Staphylococcus spp.*

Efecto antibacteriano del extracto etanólico de *Stevia rebaudiana* B. frente a *Staphylococcus spp.*

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Technological innovation: New plant extracts to control nosocomial diseases caused by *Staphylococcus.*

Industrial application area: Food biotechnology.

Received: december 03th, 2020
Accepted: october 06th, 2021

Resumen

En la actualidad diversos factores como la automedicación, el incumplimiento de un tratamiento médico y los medicamentos falsos (no poseen la concentración adecuada de la sustancia activa), han provocado un serio problema a la salud provocando la resistencia bacteriana a los antibióticos. El mal uso de antibióticos ha originado que las bacterias (*Staphylococcus spp, Klebsiella pneumoniae, Pseudomonas aeruginosa*), causales de infecciones nosocomiales se vuelvan resistentes a los fármacos que se utilizan para el control de estas. Una de las alternativas verdes es la utilización de extractos vegetales que poseen metabolitos secundarios para los cuales han reportado poseer diversas actividades biológicas. *Stevia rebaudiana* ha demostrado tener diversas funciones biológicas como antitumoral, antihiperglicémica, antidiarreica, diurética y antimicrobiana. Por tanto, el objetivo fue evaluar el efecto antibacteriano in vitro del extracto etanólico de *Stevia rebaudiana* contra bacterias de importancia clínica, la caracterización de los metabolitos secundarios de *S. rebaudiana* y la determinación de la concentración mínima inhibitoria (CMI). El extracto etanólico de hojas fue obtenido por ultrasonido, en la evaluación antibacteriana se utilizó el método de difusión en agar con pozos y para la CMI fue por micro dilución en agar en microplaca. Los grupos funcionales se determinaron por espectroscopía FT-IR y UV-vis. El extracto de *S. rebaudiana* presentó un 40% de inhibición para *Staphylococcus*

saprophyticus salvaje, un 55% contra *S. aureus* ATCC 29213, 60% para *S. epidermidis* ATCC 12228 a una concentración de 40 mg/mL y un 55% contra *S. aureus* salvaje a una concentración de 20 mg/mL. Mientras que la MIC fue de 40 mg/mL para *S. saprophyticus* salvaje y *S. aureus* salvaje, 30 mg/mL frente a *S. epidermidis* ATCC 12228 y 50 mg/mL sobre *S. aureus* ATCC 29213. A partir de la espectroscopía de FT-IR y UV- visible se observaron picos correspondientes a grupos O-H que posiblemente podrían corresponder a grupos funcionales de los polifenoles. Los resultados muestran que *Stevia rebaudiana* tiene la capacidad de inhibir el crecimiento de *S. aureus, S. epidermidis* y *S. saprophyticus.*

Palabras Clave: bacterias nosocomiales, CMI, extractos vegetales, *Staphylococcus, Stevia rebaudiana.*

Abstract

At present, various factors such as self-medication, non-compliance with medical treatment and false medications (they do not have the adequate concentration of the active substance), have caused a serious health problem, causing bacterial resistance to antibiotics. The misuse of antibiotics has caused those bacterial nosocomial infections (*Staphylococcus spp, Klebsiella pneumoniae, Pseudomonas aeruginosa*) to become resistant to the mechanisms of action of drugs used to control them. One of the green alternatives is the use of plant extracts which have secondary metabolites that have been reported to have various biological activities. *Stevia rebaudiana* has been reported to possess various biological functions such as antitumor, antihyperglycemic, antidiarrheal, diuretic, and antimicrobial. Therefore, the objective was to evaluate the in vitro antibacterial effect of the ethanolic extract of *Stevia rebaudiana* against bacteria of clinical importance, the characterization of the secondary metabolites of S. rebaudiana and the determination of the minimum inhibitory concentration (MIC). The ethanolic extract of leaves was obtained by ultrasound, in the antibacterial evaluation the method of diffusion in agar with wells was used and for the MIC it was by micro-dilution in agar in a microplate. The functional groups were determined by FT-IR and UV-vis spectroscopy. The *S. rebaudiana* extract showed 40% inhibition for wild *Staphylococcus saprophyticus*, 55% against *S. aureus* ATCC 29213, 60% against *S. epidermidis* ATCC 12228 at a concentration of 40 mg/mL and 55% against wild *S. aureus* at a concentration of 20 mg/mL. While the MIC was 40 mg /mL for wild *S. saprophyticus* and wild S. aureus, 30 mg/mL against *S. epidermidis* ATCC 12228 and 50 mg/mL for *S. aureus* ATCC 29213. From spectroscopy of FT-IR and UV-visible peaks corresponding to O-H groups that could possibly correspond to functional groups of polyphenols were observed. The results show that *Stevia rebaudiana* could inhibit the growth of *S. aureus, S. epidermidis* and *S. saprophyticus.*

Key Words: MIC, nosocomial bacteria, *Stevia rebaudiana, Staphylococcus,* vegetable extracts.

I. Introduction

In Mexico, it is estimated that every 450,000 cases related to infections in the hospital setting cause 32 deaths per 100,00 inhabitants per year [1]. The inappropriate use of antibiotics has produced the bacteria that originate nosocomial diseases to become resistant to the mechanisms of action of drugs. Infectious diseases are a fundamental reason of morbidity and mortality in the world, one of the motives for this problem is the appearance of microorganisms resistant to antibiotics such as those of the genus *Staphylococcus*. The problem of bacterial

resistance to antibiotics is imminent, so green alternatives are being sought to control them. Plant extracts utilization dates to our ancestors who used them to alleviate the ailments of the population. The use of herbalism to obtain compounds from natural products goes beyond considering it as traditional medicine, it is intended to become complementary medicine [2]. Most plants present allelopathic activity, plants express certain substances to a lesser or greater degree in an attractive or repellent way, when they feel threatened or stressed by climate change, an insect, another plant or by fungal or bacterial pathogens. Plants are evolutionary entities that at the minimum effect on them, they produce secondary metabolites to defend themselves from the threat, a clear example is the Ruda plant (*Ruta graveolens*), which when it encounters something expresses aromatic substances with repellent action towards insects.

There is a great diversity of plant species that have allelopathic activity and one of these plants is *Stevia rebaudiana*, which is characterized by being a plant with many secondary metabolites with various physiological properties such as anti-inflammatory, antitumor, antidiuretic, and antimicrobial. *Stevia rebaudiana* Bertoni, also known as sweet herb, is a natural sweetener, 300 times sweeter than common sugar, *S. rebaudiana* contains the secondary metabolites mostly in the leaves: glycosides, terpenes, diterpenes, flavonoids, carotenoids, polyphenols, vitamins, and minerals [3]. In addition, there are some other reported studies of *S. rebaudiana* and its antibacterial activity against Gram negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Salmonella typhi*) [4]. Likewise, *S. rebaudiana* extracts with antibacterial activity against environmental bacteria *S. aureus, S. epidermidis* and *P. aeruginosa* [5]. Therefore, the objective was to evaluate the *in vitro* antibacterial effect of the ethanolic extract of *Stevia rebaudiana* against bacteria of clinical importance, the characterization of the secondary metabolites of *S. rebaudiana* and the determination of the minimum inhibitory concentration (MIC).

II. Materials and methods

Leaf of Stevia. To assure possible differences in the chemical composition, the dried leaves of *S. rebaudiana* were from the Todo natural® brand, which contains powdered stevia without additives such as maltodextrin, erythritol, natural flavors, vegetable fiber, fillers, binders or artificial ingredients of any kind.

Strains. *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 12228, wild *S. saprophyticus* and wild *S. aureus* were obtained by the Clinical Analysis Laboratory of the specialty medical tower of the Puebla University Hospital (HUP). The strains were kept in 0.9% isotonic saline solution and for bacterial activation it was carried out on TSA agar by striatum incubating them at 37 ° C for 18-24 hours.

2.1 Plant material and obtaining extracts

The ethanolic extract was obtained from dried and pulverized *S. rebaudiana* leaves with absolute ethanol in a 1:10 (m/v) ratio. It was placed in the ultrasonic bath (Branson 3510) of a single frequency (47kHz) for 40 minutes at 40°C. The solvent was then removed by rotary evaporation (VICHI-MNR-250). The extract was dried and stored at −20°C for further analysis.

2.2 Antibacterial evaluation by the well agar diffusion method

A bacterial suspension of 0.5 on the McFarland scale (10^8 CFU/mL) was made of each strain *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *S. saprophyticus* wild, with a previous activation of 24 hours. It was massively

inoculated on Mueller-Hinton agar plates and then 20 µL of the extracts at different concentrations of 20, 40, 60, 80 and 100 mg / mL were placed in each well. The solvent (ethanol 100%) was used as a negative control and vancomycin (Va 30 µg/mL) as a positive control and were incubated at 37 ° C for 24 hours, the bioassays were performed in triplicate. After 24 hours, the inhibition halos were measured with a PRETUL Ver-6px analog vernier and the inhibition percentage was determined using the formula Eq. (1).

$$Inhibition\ (\%) = \frac{mean\ diameter\ inhibition\ halo}{diameter\ of\ the\ positive\ control\ inhibition\ halo}\ x\ 100$$

(Eq. 1)

2.3 Determination of the minimum inhibitory concentration (MIC)

MIC assays [6] were carried out for those extracts that presented inhibiting halos in the well diffusion tests. Each extract was evaluated by adding it at different concentrations (100, 90, 80, 70, 60, 50, 40, 30, 20 and 10 mg/mL) to Mueller Hinton agar in Petri plates, and 20 µl of bacterial solution (*Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *S. saprophyticus* savage) having a cell density equivalent to that of the 0.5 tube in the McFarland turbidity standards was seeded (dropped) on each plate. Isotonic saline solution was used as positive control. Plates with Mueller Hinton agar without extract were used as negative control [7]. 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.

2.4 Characterization of secondary metabolites by FT-IR and UV-vis

The preliminary characterization of the compounds was by the FT-IR technique, using equipment (Digilab from Agilent Technologies, Scimitar model), starting from a solid and dry sample of the ethanolic extract of *S. rebaudiana*, it was placed in the mortar with KBr and it was pulverized until obtaining a homogeneous sample and forming a tablet and placed within the infrared for its reading, then the extract graph was obtained. The UV-vis analysis equipment was used (Varian, Cary 50 model), first blank was run (base line), next the solvent used to obtain the extract (ethanol). Subsequently, 20 µL of the 10 mg/mL extract were placed in the cell and titrated with ethanol. Finally, a graph was obtained**.**

2.5 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.

III. Discussion and results

3.1 Evaluation of the antibacterial activity of the ethanolic extract of *S. rebaudiana* and determination of the MIC

Table 1 shows the antimicrobial effects of *S. rebaudiana* leave ethanolic extracts on strains of different bacteria. By analyzing the obtained results, one can see that *S. rebaudiana* extracts inhibited with diverse percentages (from 40 to 65%). For the strains, *S. saprophyticus* of 40%, for *S. aureus* ATCC 29213 of 55%, in *S. epidermidis* ATCC 12228 of 60%, the lowest concentration with which an inhibitory effect was obtained in all the evaluated strains was 60 mg/mL.

The results for wild *S. aureus* showed a 55% inhibition at a concentration of 20 mg/mL. (Table 1). The extract in none of the

concentrations exceeds the inhibition halo of the commercial antibiotic (Vancomycin).

Recent studies have shown that the application of the ultrasound extraction technique is promising for obtaining a greater quantity of secondary components, as well as a higher yield for some activities including the extraction of proteins, medicinal components, among others [8]. In reported studies, the continuous use of bacteria in a medium to certain specific conditions generates adaptation and mutation, this can be drastically increased in *S. aureus* [9]. Latarissa (2020) mentioned that ethanol extract of *Stevia rebaudiana* leaves had the highest activity against *Staphylococcus albus* [10].

Moreover, Ibrahem (2020), mentioned methanolic, aqueous and chloroformic extracts also have antibacterial activity against various bacteria (*Bacillus cereus, Salmonella typhi, Pseudomonas aeruginosa, Listeria monocytogenes, Escherichia coli, Bacillus cereus, Staphylococcus aurous, Aspergillus flavus*, etc.) [11].

The results obtained from the MIC show that for *S. aureus* ATCC 29213 the MIC is 50 mg/mL, wild *S. saprophyticus* and wild *S. aureus* the MIC is 40 mg/mL and *S. epidermidis* ATCC 12228 is 30 mg/mL. This difference in MIC between wild strains and ATCC can be attributed to the fact that wild strains have been in greater contact with antibiotics and because of the interaction between hosts and their host, they may be less beneficial than ATCC [12, 13]. Phytochemical studies have shown that *S. rebaudiana* has a great diversity of chemical components. Some authors have pointed out the presence, in *S. rebaudiana* leaf extracts, of alkaloids, flavonoids, phenols, steroids, essential oils and, to a lesser extent, tannins [14, 15]. Various authors have mentioned that antibacterial activity may be related to the presence of various compounds such as flavonoids, aromatic acids and terpenoids have been described in several works, mentioned as responsible for the antibacterial activity [16, 17, 18].

Even though the four bacteria are of the same genus, but different species, the *S. rebaudiana* extract shows a superior inhibitory effect on *S. epidermidis* ATCC 12228.

Table 1. Antibacterial activity (%) of the ethanolic extract of *S. rebaudiana* against *Staphylococcus* at different concentrations.

Ethanol extract (mg/mL)	Inhibition (%)				
	***S. aureus* ATCC29213**	***S. epidermidis* ATCC12228**	***S. saprophyticus* Salvaje**	***S. aureus* Salvaje**	
100	55	60	50	65	Positive control Vancomycin (30 µg) 2 cm
80	55	60	50	65	
60	55	60	50	65	
40	55	60	40	60	
20	//	//	//	55	

// Does not present antibacterial activity.

3.2 Characterization of the ethanolic extract of *Stevia rebaudiana*

3.2.1 FT-IR spectroscopy: Structural determination

The FT-IR spectrum of the *S. rebaudiana* extract is presented in figure 1. The corresponding absorption band located at 3402.8 cm^{-1} was probably assigned to the single bond O-H stretching groups from the natural sweetener *Stevia* [18]. The √C-H elongation vibration modes of the C-H chemical species are located at 2923.4 cm-1. The asymmetric √CH elongation vibration mode is located at 2900 cm-1, while the absorption at 2352.1 cm-1 corresponds to the interactions of symmetric CO species in 1950 cm-1, the vibration band corresponding to bending-type interactions √OH of the OH-groups, which are due to the moisture and hydroxyl groups on the surface of the material. The values of 1445.8 cm-1 and 1410 cm-1 correspond to the symmetric elongation vibrations √COO- and deformation √CH3. The vibration bands corresponding to the asymmetric elongation √COO- vibrations of carboxylate ions are located at 1593.0 cm-1. According to phytochemical studies, the antimicrobial activity of *S. rebaudiana* leaves could be due to phenolic compounds, flavonoids, anthocyanins, and tannins [20]. Myint (2020), summarizes that other authors have found more than 30 phenolic compounds in extracts obtained from *Stevia rebaudiana* leaves, chlorogenic acid being one of the main components to which various antimicrobial activities are attributed [21].

The FTIR spectra, coincide with that described by other authors [22].

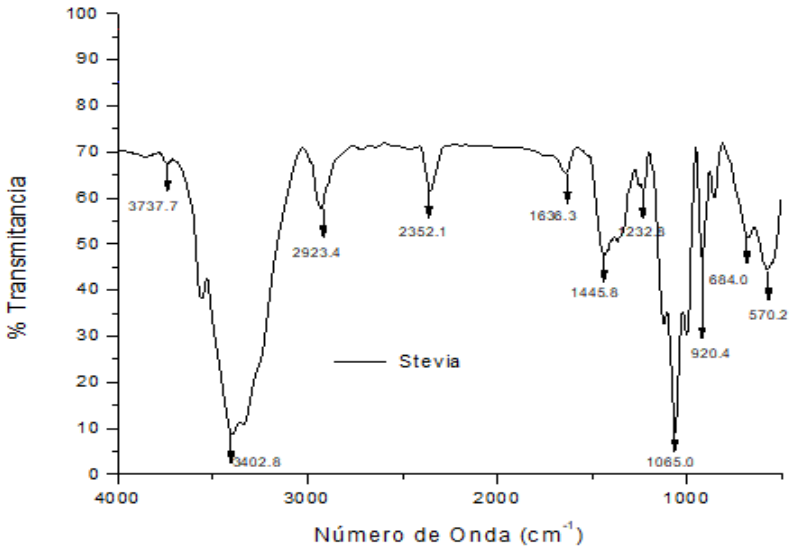


Figure 1. FT-IR spectrum of the ethanol extract of *S. rebaudiana.*

3.2.2 UV-visible spectrophotometry

The spectrum of the *S. rebaudiana* UV-VIS extract (figure 2) shows two absorption bands, the one with the highest intensity is in the median UV region at 328.0 nm, corresponding to the electronic transitions: n→π * of the carbonyls α, β unsaturated and the presence of cyclic oxypolienate groups of stevioside. The absorption band of lower intensity located in the visible region at 672 nm corresponding to the absorption of red color with a frequency of 4.46x1014 Hz. The electronic transitions are of the n→π * type of the steviol molecule.

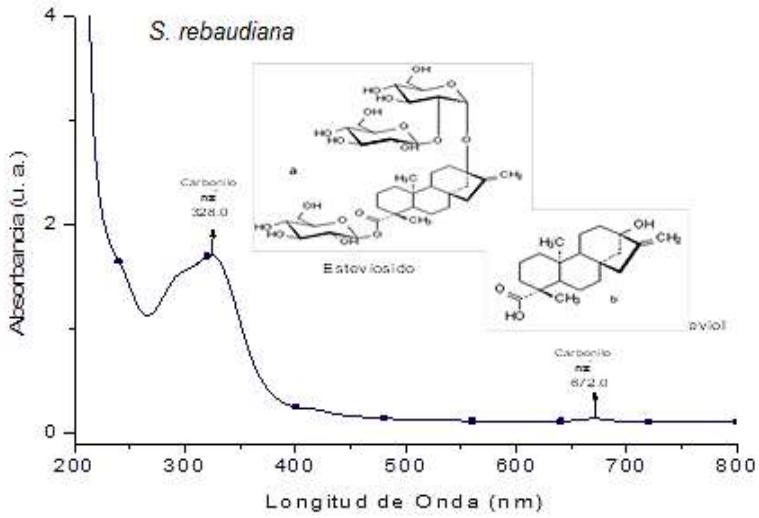


Figure 2. UV-VIS spectrum of the ethanolic extract of *S. rebaudiana*.

V. Conclusions

This work demonstrates that ethanolic extract obtained by ultrasound from *S. rebaudiana* can inhibit the growth of bacteria resistant to antibiotics such as *S. aureus, S. epidermidis* and *S. saprophyticus* compared to the negative control (ethanol).

VI. Acknowledgements

Thanks to M.C. Susana Pérez Fernández (HU-BUAP University Hospital) for providing the *Staphylococcus* strains used in this study.

VII. References

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