Stevia Rebaudiana fraction presents broad-spectrum antibacterial action and antibiofilm action for Staphylococcus aureus

Fração Stevia Rebaudiana apresenta ação antibacteriana de amplo espectro e ação antibiótica para Staphylococcus aureus

DOI:10.34117/bjdv8n7-057

Recebimento dos originais: 23/05/2022
Aceitação para publicação: 30/06/2022

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ABSTRACT
Fractions of S. rebaudiana leaves, extracted in Ethyl Acetate (EAF), rich in antioxidant compounds and Isobutanolic fraction (IF), with a high content of glycosides, were evaluated for antibacterial and antibiofilm potential against reference strains of Gram-positive and Gram-negative bacteria of clinical interest. EAF had bactericidal action against Gram-positive strains evaluated and against most Gram-negative strains, with the exception of Salmonella enteritidis. Gram-positive species showed greater sensitivity to EAF. For IF, up to the maximum concentration, there was no bactericidal effect for the strains. The pretreatment of planktonic cells of S. aureus with subinhibitory concentrations of EAF determined a reduction in the potential of structuring of the biofilm by up to 71%. The fraction showed effectiveness in biofilm treatments formed by S. aureus, with a 99% reduction in cell viability compared to the control. In addition to the antibacterial action on planktonic cells, EAF has promising effects against S. aureus biofilms.

Keywords: phytotherapy, microbial resistance, biofilm, Stevia fraction, antimicrobial action.

RESUMO
Frações de folhas de S. rebaudiana, extraídas em acetato de etilo (EAF), ricas em compostos antioxidantes e fração isobutanólica (IF), com alto teor de glicosídeos, foram avaliadas quanto ao potencial antibacteriano e antibiótico contra cepas de referência de bactérias Gram-positivas e Gram-negativas de interesse clínico. EAF teve ação bactericida contra as cepas Gram-positivas avaliadas e contra a maioria das cepas Gram-

negativas, com exceção da Salmonella enteritidis. As espécies Gram-positivas mostraram maior sensibilidade à FEA. Para IF, até a concentração máxima, não houve efeito bactericida para as cepas. O pré-tratamento das células planctônicas de S. aureus com concentrações subinibitórias de FEA determinou uma redução no potencial de estruturação do biofilme em até 71%. A fração mostrou eficácia no tratamento do biofilme formado por S. aureus, com uma redução de 99% na viabilidade celular em comparação com o controle. Além da ação antibacteriana sobre as células planctônicas, a FEA tem efeitos promissores contra os biofilmes de S. aureus.

Palavras-chave: fitoterapia, resistência microbiana, biofilme, Fração de Stevia, ação antimicrobiana.

1 INTRODUCTION

Antimicrobial resistance is one of the greatest global threats to public health and the World Health Organization recognizes investment in research and development of new antimicrobials as one of the pillars for controlling the spread of resistant microorganisms (WHO, 2020).

In addition to the selection of resistance genes, the structure in biofilms can provide microorganisms the ability to withstand high concentrations of antimicrobials. These sessile communities are particularly important in the hospital context, where it is estimated that between 60 to 70% of nosocomial infections are related to the structuring of biofilms in implants and devices, such as catheters (Paraje, 2011; César et al., 2013; Lebeaux et al., 2014; Rodis et al., 2020).

The secondary metabolites of plants can have several pharmacological properties, especially phytoactive compounds with antimicrobial potential, which inserts traditional medicine as a possible ally in combating microbial resistance (Gupta; Birdi, 2017).

Stevia rebaudiana Bertoni (*S. rebaudiana*) is a shrub that has recognized medicinal properties and has been used in traditional medicine in Brazil and Paraguay for hundreds of years, in the treatment and prevention of diseases. Among the phytochemical constituents already described in the plant are antimicrobial compounds such as flavonoids and terpenoids and antibacterial and antifungal actions from Stevia extracts have already been reported. However, there is still a need for further exploration and understanding about the antimicrobial potential of stevia and its mechanism of action in inhibiting microbial growth (Joseph; George, 2019; Latarissa et al., 2020).

Although the antibiofilm effect of stevia leaf extract has recently been reported on biofilms formed of *Bacillus* sp. and *Enterobacter* sp. (Hastuty, 2019), in general, the few

studies that address plant activity in biofilms are restricted to cariogenic bacteria, particularly *Streptococcus mutans*, in dental plaques or artificial caries models (Mohine; Yadav, 2010; De Slavutzky, 2010; Giacaman et al., 2013; Brambilla et al., 2014).

Considering the above, this study aimed to evaluate the antimicrobial effect of fractions of *S. rebaudiana* leaves on Gram-positive and Gram-negative bacterial species of clinical interest, as well as to evaluate the action of the ethyl acetate fraction both in the structuring of *Staphylococcus aureus* biofilms and in the viability of biofilms of the species, established on an abiotic surface.

2 METHODOLOGY

2.1 MICROBIAL STRAINS AND CULTURE MEDIA

In this study, reference strains of *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* ATCC 700603), *Proteus mirabilis* (ATCC 7002), *Salmonella enteritidis* (ATCC 13076) e *Salmonella typhimurium* (UK-1) were used, which were maintained at -20 ° C in glycerol (20%). Tryptic Soy Broth (TSB) was used for the cultivation of the strains. TSB medium plus 0.025% dimethyl sulfoxide (DMSO) was used to elute *Stevia rebaudiana* fractions while TSB medium plus glucose (1%) and DMSO (0.025%) was used in biofilm formation analysis. For cultures in solidified medium, Tryptic Soy Agar (TSA) was used, obtained by adding agar (1.5%) to the TSB.

2.2 STEVIA REBAUDIANA BERTONI FRACTIONS

Stevia rebaudiana fractions were obtained from the methanolic extract of stevia leaves of the UEM-13 variety, according to Milani et al., 2017. The extracted fractions were called hexane fraction (FH), chloroform fraction (FC), ethyl acetate fraction (EAF) and isobutanolic fraction (IF), based on the solvents used to obtain each of the fractions. The effects of the EAF and IF fractions were assessed. The hexane and chloroform fractions were selected, however, they were shown to not be viable for the evaluations tested in this study.

2.3 ANTIBACTERIAL ACTIVITY

The antimicrobial activity of the Ethyl Acetate (EAF) and Isobutanolic (IF) fractions was evaluated by microdilution in broth, with concentrations of 20 to 100

mg/mL of each fraction of *S. rebaudiana* being tested. The bacterial strains were reactivated in TSB broth (18 h/37°C). After centrifugation (10 min/1800g) the supernatant was discarded, the pelleted cells were resuspended in TSB and the cell concentration adjusted according to the Mc Farland scale (tube 0.5) to reach a concentration of approximately $1.5x10^8$ colony forming unit/mL (CFU/mL). In 96-well microplates, 100 µL of each bacterial suspension were added to the wells followed by 100 µL of the preparations of each fraction, to obtain the evaluated concentrations. Positive controls consisted of 100 µL of the bacterial suspension plus 100 µL of TSB supplemented with DMSO (0.025%). The sterility controls consisted of 100 µL of TSB plus 100 µL of TSB supplemented with DMSO (0.025%) (sterility of the culture medium) and 100 µL of TSB plus 100 µL of TSB plus the fractions in the respective concentrations evaluated (sterility of fractions). The microplates were incubated (24h/37°C), without shaking. Then, 50 µL of each culture was inoculated in TSA and the plates incubated for 24 hours at 37°C. MBC was defined as the lowest concentration tested in which there was no microbial growth in the subculture (Yamaguchi et al., 2009).

2.4 EFFECT OF THE ETHYL ACETATE FRACTION ON THE POTENTIAL OF BIOFILM FORMATION

The strain *S. aureus* 25923 was used for the analysis in biofilms. Initially, was evaluated the effect of pretreatment with subinhibitory concentrations of EAF on the potential of formation of biofilm of the strain. The strain was grown (37°C/18h) from the stock in TSB broth plus glucose (1%). After centrifugation (10 min/1800g) the supernatant was discarded, the pelleted cells were resuspended in TSB and the cell concentration adjusted to approximately $1.5x10^8$ $CFU.mL^{-1}$. The cell suspensions were added with subinhibitory concentrations of EAF (5, 10, 15 and 20 $mg.mL^{-1}$) and incubated (37°C/24h). The resulting cells were pelleted (10 min/1800g), washed twice in saline (0.9% NaCl), pelleted again and the cell concentration was adjusted to approximately $1.5x10^8$ CFU/mL^{-1}. In sequence, 200 µL of these cell suspensions were deposited in 96-well flat-bottomed microplates and incubated (37°C/48h). For the positive control, cells from the bacterial strain were used, grown under the same conditions, but not treated with EAF. For the negative control, glycated TSB medium (1%) was used. The wells were gently washed with saline, fixed (37°C/1:30h), stained with crystal violet solution (1% / 15 min), washed with saline and dried in a bacteriological oven (30 min). Then 100 µL of acetic acid (33%) was added to each well and a spectrophotometric reading was

performed at 490nm. The results were expressed as percentage of inhibition of biofilm formation compared to the positive control, which was considered to be 100%. The experiment was carried out in triplicate.

2.5 ANTIBACTERIAL ACTIVITY OF THE ETHYL ACETATE FRACTION IN BIOFILM

The EAF antibacterial activity in biofilms formed of *S. aureus* was also evaluated. The strain was grown (37°C/18h) from the stock in TSB broth plus glucose (1%). After centrifugation (10 min/1800g) the supernatant was discarded, the pelleted cells were resuspended in TSB and the cell concentration adjusted to approximately $1.5x10^8$ $CFU.mL^{-1}$. Two hundred microliters of the cell suspension were inoculated into 96-well flat-bottomed microplate wells; the plates were incubated (37°C/24h). Afterwards, the supernatant was discarded, the wells were washed with saline solution and 200 µL of glycated TSB medium (1%) plus EAF in concentrations 25, 50 or 100 $mg.mL^{-1}$ were inoculated. The positive control consisted of the strain grown under the same conditions, except for the absence of EAF. The microplates were incubated (37°C/24h) and, subsequently, the wells were washed with saline solution and the bottom of the wells were scraped so that the adhered cells were released. The biofilm cells were resuspended in saline and plated in TSA. After incubation (37°C/24h), the $CFU.mL^{-1}$ was counted. The results were expressed as percentage of reduction in cell viability. The experiment was carried out in triplicate.

2.6 STATISTICAL ANALYSIS

The statistical analyses of the experiments were performed using STATISTICA7 software, using Analysis of Variance (ANOVA) and Tukey's test to compare the means between the tests, with a 5% significance level.

3 RESULTS AND DISCUSSION

In the present study, the antimicrobial action of two stevia leaf extract fractions was evaluated – EAF and IF. The fraction obtained in ethyl acetate (EAF) has antioxidant compounds (phenolic and flavonoids) and proteins that together add up to more than 90% of its composition. The isobutanolic fraction is rich in steviol glycosides (70.8%) and also has low concentration phenolic compounds (Milani et al., 2017).

The results obtained in this study for the EAF can be attributed to phenolic compounds, flavonoids and proteins present, since it practically has no sweeteners in its composition (less than 1%) and for the fact that IF, rich in glycosides, have not presented such effects. The obtaining of the ethyl acetate fraction has been studied by our research group regarding its beneficial effects on the metabolic control of diabetic animals and the results show considerable antioxidants effects, effects in the control of insulin secretion, in the reduction of hyperglycemia and in restoration of pancreatic function (Milani et al, 2017b; Milani et al., 2020).

The antimicrobial and bactericidal effects found against the microorganisms evaluated in this study show the importance of carrying out more studies that improve the parameters of obtaining yield, solubility and conservation of the EAF. Also it is important that more antimicrobial effects are tested in deteriorating and pathogenic microorganisms and, still, as adjuvants in the control of metabolic syndromes, so that this fraction can be added in foods, supplements or even in natural medicines for the prevention or treatment of diseases (Piovan et al., 2018; Zorzenon et al., 2019).

EAF was found to have a bactericidal effect against all the Gram-positive strains evaluated and for the majority (4/5) of the Gram-negative strains. Gram-positive strains showed higher sensitivity to the fraction, with MBCs between 25 to 30 $mg.mL^{-1}$, while for Gram-negative strains the lowest MBC was 40 $mg.mL^{-1}$. On the other hand, for IF there was no bactericidal action against any of the strains evaluated up to the maximum concentration tested, as shown in Table 1.

Table 1. Minimum bactericidal concentration of the Ethyl Acetate (EAF) and Isobutanolic fractions (IF).

		Minimum Bactericidal Concentration ($mg.mL^{-1}$)	
Gram classification	**Bacteria / Fraction**	**EAF**	**IF**
Gram positives	*S. aureus* 25923	25	*
	E. faecalis 29212	27.5	*
	S. epidermidis 12228	30	*
Gram negatives	*E. coli* 25922	40	*
	K. pneumoniae 700603	40	*
	P. mirabilis 7002	45	*
	S. typhimurium UK-1	50	*
	S. enteritidis 13076	*	*

* there was no inhibition at a concentration equal to or less than 100 $mg.mL^{-1}$

The greater efficiency in the antimicrobial action of stevia against Gram-positive bacteria compared to Gram-negative bacteria has also been reported by other researchers (Jayaraman et al., 2008; Singh et al., 2012; Noor et al., 2014). The difference in the

structure of the cell wall of these bacterial groups possibly contributes to the profile of greater sensitivity of Gram-positives, either by the action of stevia components on the cell wall or by the barrier provided by the external membrane of Gram-negatives, which could prevent access of stevia's active components to the cell.

In a recent study that evaluated the chemical composition of different fractions of a variety of *S. rebaudiana*, it was demonstrated that EAF had the highest concentrations of phenolic and flavonoid compounds, which were respectively 7 and 1.6 times higher than those found in IF (Milani et al., 2017). These components exhibit important biological properties and several studies have highlighted their antimicrobial potential (Choi et al., 2006; Aktar et al., 2014; Singh et al., 2018).

Among the bacteria evaluated in the present study, *S. aureus* 25923 showed the highest sensitivity to EAF. The World Health Organization classifies *S. aureus* as one of the most important human pathogens and its clinical impacts are due not only to its prevalence and pathogenicity, but also to the emergence of resistant strains, which reinforces the importance of studies on compounds that can assist in containing its dissemination (O´Gara et al., 2017; WHO, 2020).

The resistance to antimicrobials by *S. aureus* can be established not only by selection and dissemination of resistance genes, but also by their ability to structure themselves in Biofilms. These sessile communities are often established by *S. aureus* on invasive medical devices, such as catheters and prostheses, and are recalcitrant to antimicrobial treatment and therefore being related to therapeutic problems such as treatment failure and recurrent infections (Jones et al., 2001; Archer et al., 2011; Suresh et al., 2018).

This study evaluated the effect of EAF on the formation of biofilms by *S. aureus* and on the viability of established biofilms. The previous exposure of *S. aureus* 25923 planktonic cells to sub-bactericidal concentrations of the EAF significantly reduced ($p < 0.05$) their biofilm formation potential. For this analysis, concentrations of 5 to 1.25 times lower than the MBC previously determined for the strain were used and the reduction in biofilm formation varied from 53.1% to 71% compared to the control (Figure 1). These results demonstrate that stevia can be an ally in preventing the structuring of biofilms by *S. aureus*.

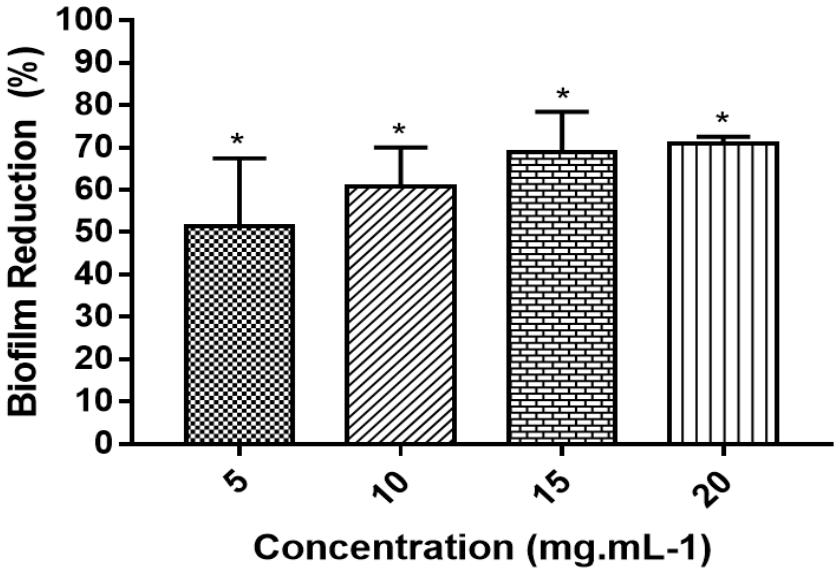

Figure 1: Percentage of reduction in biofilm formation by *S. aureus* after pretreatment with the Ethyl Acetate fraction. * indicates a significant difference in relation to the control ($p < 0.05$).

In addition, significant antibacterial action of stevia was also observed in *S. aureus* 25923 biofilms established on an abiotic surface atthree concentrations of the fraction, starting from the MBC for planktonic cells (25 $mg.mL^{-1}$) and concentrations 2 and 4 times higher. The cell viability in biofilms after treatment with the fraction showed a reduction greater than 99% for all concentrations evaluated, differing significantly from the control ($p < 0.05$). However, up to the maximum concentration evaluated (100 $mg.\ mL^{-1}$) it was not possible to detect the MBC for *S. aureus* biofilms, since there was growth of colonies from the subcultures (Figure 2).

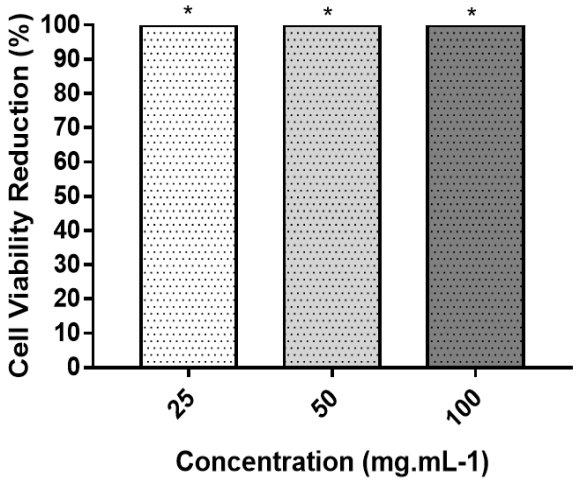

Figure 2: Percentage of reduced viability of *S. aureus* biofilm cells after treatment with the Ethyl Acetate fraction. * indicates a significant difference in relation to the control ($p < 0.05$).

Although stevia's performance was not effective for eradicating *S. aureus* biofilms, the data from the present study demonstrate a significant effect in reducing both the structuring and viability of established *S. aureus* biofilms.

4 CONCLUSION

This study confirms antibacterial action of a broad spectrum of *S. rebaudiana* fractions and demonstrates that the plant has an antibiofilm effect on *S. aureus*, acting preventively in reducing the structuring and interfering in the viability of established biofilms. Considering that the structure in biofilms contributes extensively to bacterial resistance, we emphasize that the antibiofilm potential of stevia should be better explored, including the possible synergistic effects of the plant extracts with other antimicrobials.

ACKNOWLEDGMENT

This study was partially financed by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, Brazilian National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq), Secretaria de Estado da Ciência, Tecnologia e Ensino Superior (SETI), and Fundação de Apoio ao Desenvolvimento Científico (Foundation for the Support of Scientific Development) (FADEC).

Professor Tamires Cruz Idiomas for revising the language of the article.

A STATEMENT ON CONFLICT OF INTEREST

The author and co-authors declare that there is no conflict of interest in the submitted manuscript.

A STATEMENT ABOUT THE ORIGINALITY OF WORK

The author and co-authors declare that the work is original and that it is not being submitted to another journal.

The work is a continuation of our group's research and studies already published in this journal.

Thus the authors consider the publication in the Brazilian Journal of Development

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