ORIGINAL ARTICLE

ANTIBIOFILM ACTIVITY OF THE ETHANOLIC EXTRACT OF THE LEAVES OF *EUGENIA KLOTZSCHIANA* O. BERG AGAINST GRAM-POSITIVE BACTERIA

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Highlights: (1) The pulverized leaves of *Eugenia klotzschiana* yielded 40,61 g of ethanolic extract. (2) The ethanolic extract of *E. klotzschiana* (EEEk) showed slight antimicrobial activity, showing moderate to weak inhibitory activity against the Gram-positive bacteria tested, and no bactericidal activity. (3) EEEk inhibited biofilm formation at subinhibitory concentrations for most Gram-positive bacteria. (4) Biofilm formation by the two biggest formers (*Staphylococcus aureus* ATCC 29213 and *Staphylococcus aureus* ATCC 6538) was intensively inhibited by 1250 $mg.mL^{-1}$ of EEEk.

PRE-PROOF
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ABSTRACT

Eugenia klotzschiana O. Berg is an endangered species endemic to the Cerrado whose therapeutic potential has yet to be fully elucidated. Few studies have shown this plant has antimicrobial, antiparasitic, and antioxidant activity. In this study, the activity of the ethanolic extract of the leaves of *E. klotzschiana* O. Berg against Gram-positive bacteria was evaluated by determining the antimicrobial and antibiofilm activity. All the bacteria were able to form biofilm, with *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* ATCC 29213 being classified as strong biofilm formers, *Enterococcus faecalis* ATCC 51299, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 19433 and *Staphylococcus epidermidis* ATCC 14990 as moderate biofilm formers. The ethanolic extract showed slight antimicrobial activity, but was active against biofilm formation at concentrations ranging from 0.039 to 1.250 $mg.mL^{-1}$. We conclude that the ethanolic extract was active against the biofilm formation of Gram-positive bacteria, and that further studies with *E. klotzschiana* O. Berg should be carried out to confirm the plant's therapeutic potential.

Keywords: Medicinal plants, Biofilms, Antimicrobial, Natural Resource, Bioproducts.

RESUMO

Eugenia klotzschiana O. Berg é uma espécie endêmica do Cerrado ameaçada de extinção e cuja potencialidade terapêutica ainda não está totalmente elucidada. Poucos estudos foram realizados e demonstraram que essa planta possui atividade antimicrobiana, antiparasitária e antioxidante. Neste estudo foi avaliada a atividade do extrato etanólico das folhas de *E. klotzschiana* O. Berg contra bactérias Gram-positivas com a determinação da atividade

antimicrobiana e antibiofilme. Todas as bactérias foram capazes de formar biofilme, sendo classificadas como fortes formadores de biofilme *Staphylococcus aureus* ATCC 6538 e *Staphylococcus aureus* ATCC 29213, como moderados *Enterococcus faecalis* ATCC 51299, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 19433 e *Staphylococcus epidermidis* ATCC 14990. O extrato etanólico apresentou discreta atividade antimicrobiana, contudo foi ativo contra a formação de biofilme com concentrações variando entre 0,039 e 1,250 mg.mL^{-1}. Concluímos que o extrato etanólico foi ativo contra a formação de biofilme das bactérias Gram-positivas e que novos estudos com *E. klotzschiana* O. Berg devem ser realizados para confirmar as potencialidades terapêuticas da planta.

Palavras-chave: Plantas medicinais, Biofilmes, Antimicrobiano, Recurso Natural, Bioprodutos.

1. INTRODUCTION

Eugenia klotzschiana O. Berg is an endemic plant of the Brazilian Savanna (Cerrado) is popularly known as Pêra-do-Cerrado, Cabacinha-do-Cerrado, Perinha-do-Cerrado, or Pereira-do-campo[1]. This plant is part of the Myrtaceae family, and has a rich history of traditional medicinal use. It is used as an anti-inflammatory, antihypertensive, and in treating gastrointestinal disorders[2, 3].

However, despite its popular medicinal use, *E. klotzschiana* O. Berg is on the list of endangered species, and there are few studies on the phytochemical screening of its leaves, fruits, stems, roots and describing its biological activities[4-7]. Although some studies have shown that its leaves and flowers have a high presence of phenolic compounds, there are few reports on the antimicrobial and cytotoxic activity of *Eugenia klotzschiana* O. Berg[8].

Specifically, *Eugenia klotzschiana* O. Berg leaves contain compounds with bioactive properties, such as: anthraquinones, saponins, cardioactive heterosides, tannins, flavonoids[9]. In addition to the significant phenolic content of the leaves, its fruits have ascorbic acid, flavonoids, phenolic compounds, and carotenoids[10].

Microbial biofilms are structured communities of bacteria wrapped in a self-produced extracellular matrix that protects the host's immune system and antibiotics[11]. Biofilm formation is a critical virulence factor for many bacterial pathogens, including Gram-positive bacteria. Biofilm increases bacteria's ability to colonize hosts, impacts the persistence and chronicity of infections, and increases resistance to antimicrobials[12,13].

In clinical settings, microbial biofilms are responsible for infections related to medical devices, such as catheters and prostheses, and tend to cause chronic infections that are difficult to eradicate and resistant to conventional antimicrobial therapies, especially dangerous for immunocompromised patients[14].

The control of biofilms is an ongoing challenge and alternatives to the use of antibiotics such as nanoparticles, antimicrobial peptides and plant extracts are being investigated to combat these microbial aggregates[14,15]. In this context, products of plant origin have a range of bioactive compounds described with antibiofilm activity[16].

Understanding the role of biofilm formation in the virulence of Gram-positive bacteria is essential for developing effective therapeutic strategies[17]. In this context, the present study evaluates the biofilm formation inhibitory activity of the ethanolic extract of the leaves of *E. klotzschiana* O. Berg against Gram-positive bacteria.

2. MATERIAL AND METHODS

2.1 Plant material and extract preparation

Eugenia klotzschiana O. Berg is a plant species endemic to the Cerrado biome and is characterized as a shrubby plant with a clump habit, with hairy leaves, crossed and opposite phyllotaxis, its fruits are pubescent, simple, berry-like and green-yellow-orange in color, depending on their stage of ripeness[18]. The leaves of *E. klotzschiana* O. Berg were collected in the rural area of the municipality of Silvânia-GO, Brazil, at the geographical coordinates: -16.697976S, -48.63388W; -16.698048S, -48.633466W; -16.698009S, -48.633386W and an

altitude of 1020 m. The State University of Goiás Herbarium team carried out botanical identification, under 14911 (Figure 1).

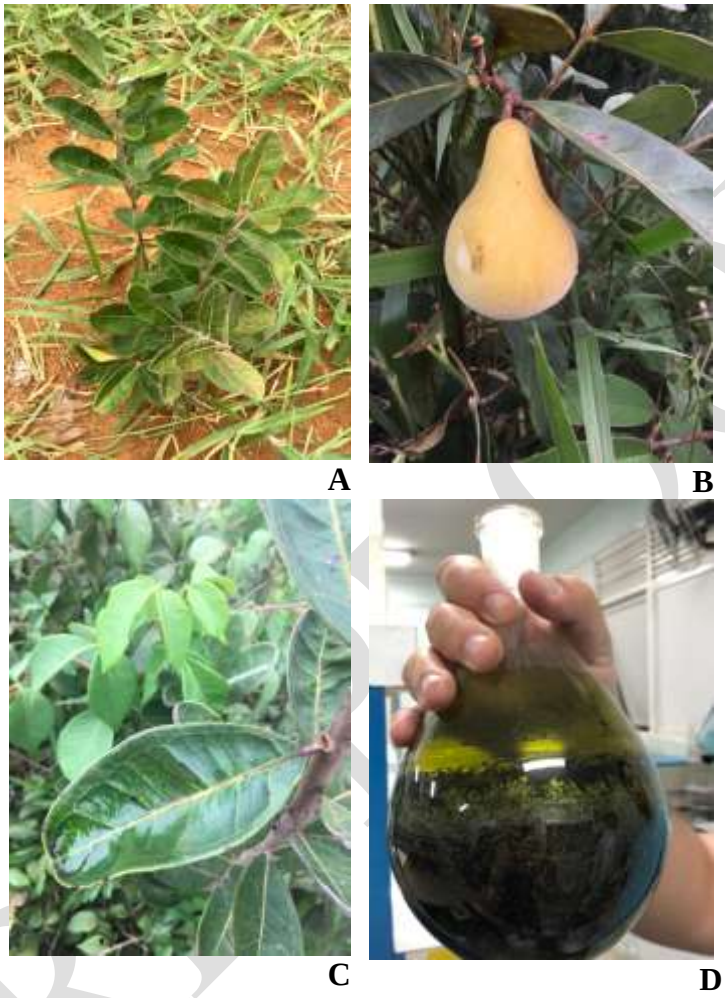

Figure 1. Morphological aspects of *Eugenia klotzschiana* O. Berg and ethanolic extract of leaves. **A**- Clump, **B**- Fruit, **C**- Leaf, **D**- Ethanolic extract. Source: the authors

After collection, the leaves were dried at room temperature - RT (25 °C) at the Biodiversity Research Laboratory (LAPBIO) of the Evangelical University of Goiás for 14 days and ground in a Tecnal knife mill grinder. The leaf powder was weighed and stored in amber glass containers, protected from light, properly sealed, and kept at room temperature.

The extraction was carried out with 250 g of dried and crushed leaves using the cold dynamic maceration technique in 700 mL of ethanol to obtain the ethanolic extract of the leaves

of *E. klotzschiana* O. Berg (EEEk) with an interval of 72 hours and a drug: solvent ratio of 0.35 $g.mL^{-1}$ (m/v). Each extract was then concentrated at reduced pressure in a 45° inclined rotary-evaporator (Fisaton, model 802), water bath (Fisaton, model 553), and hydro vacuum pump (Quimis, model Q355A2). The extract obtained was stored in a freezer (- 10 °C) until used in the tests[19].

2.2 Biofilm Formation Assay

Six bacteria from the microorganism collection of the Bioassay Laboratory of the Research and Postgraduate Center (LabBio-CPPG) of the State University of Goiás identified as *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 51299 and *Enterococcus faecalis* ATCC 19433 were used in this study.

Biofilm formation assays were assessed by detecting total biomass using the crystal violet method[20]. Briefly, bacterial inocula were obtained by suspending colonies grown in a sterile physiological solution (SPS) and adjusting the turbidity using the 0.5 McFarland scale. Subsequently, 500 µL of the inocula were transferred to tubes containing 4500 µL of bovine heart and brain infusion broth with 2% sucrose (BHIS), then 200 µL of the inocula were aseptically transferred to the wells of flat-bottomed polystyrene microplates (Cral, Brazil) which were incubated at 35.5 °C for 48 hours.

After incubation, the microplates were processed by removing the broth with total growth and washing the wells three times with distilled water in an Aquari® automatic microplate washer (MA 615, Brazil). Then 200 µl of 96º ethanol was added to the washed wells, and the microplates were incubated at RT for 20 minutes. The ethanol was removed, and the microplates were dried under the same conditions. Subsequently, 200 µl of 0.1 % crystal violet was added to each well, the microplate was incubated for 20 min at RT, and the excess dye was removed by washing three times with distilled water in the automatic microplate washer. The microplates were dried in the inverted position for 20 minutes at RT, and the staining of the biofilms was solubilized by adding 200 µl of 33 % glacial acetic acid to each well for 20 minutes.

The optical density readings (OD_{620nm}) were taken on a microplate spectrophotometer (Multiskan FC, Thermo Scientific, USA). The average OD_{620nm} value obtained in the uninoculated control wells was used as a reference for classifying biofilm formation as strong, moderate, weak, or non-forming[20]. All the bacteria were tested in independent triplicates totalizing 24 wells, and the results obtained were organized as means and standard deviations.

2.3 Antimicrobial Activity of the Extract

The bacteria were subjected to the broth microdilution test to determine the plant extracts' minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), adapting the method recommended by the Clinical and Laboratory Standard Institute for antimicrobial susceptibility tests[21]. Antibacterial activity was assessed by weighing 400 mg and dissolving the extract in 20 mL of absolute ethanol. The concentrations tested in round-bottomed microplates (Olen, China) were 10000, 5000, 2500, 1250, 0.625, 0.312, 0.156, and 0.078 $mg.mL^{-1}$.

The bacterial suspensions were prepared by dissolving typically isolated colonies in SPS, and the inoculum density was adjusted using the 0.5 McFarland scale. After adjustment, a 1:10 dilution was made in BHIS broth, and then 20 µL of the inocula were transferred to the wells of the microplates.

The MIC was determined by visually reading the lowest concentration of the extract capable of inhibiting bacterial growth after incubation at 35.5 °C for 24 h. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of the extract capable of inhibiting the recovery of viable bacteria after transferring 100 µL of BHIS broth from the wells without detectable turbidity to plates containing CPS agar (Biomérieux, Brazil) after incubation at 35.5 °C for 48 hours. All the tests were carried out in independent triplicates, and the MIC and MBC values were obtained.

2.4 Activity of the Extract Against Biofilm Formation

The activity of EEEk against the biofilm formation of Gram-positive bacteria was carried out as previously described in the total biofilm biomass assay[20]. Concentrations of 5000,

2500, 1250, 0.625, 0.312, 0.156, 0.078, and 0.039 $mg.mL^{-1}$ of the extracts were tested in flat-bottomed microplates (CralPlast, Brazil), and the DO_{620nm} of the biofilms formed in the presence of the extract concentrations were compared with untreated controls lowest concentrations that inhibit biofilm formation by at least 50% were considered the Minimum Biofilm Formation Inhibitory Concentration ($MBIC_{50}$)[22].

3. RESULTS

3.1 Obtaining the extract

The pulverized leaves of *E. klotzschiana* O. Berg yielded 81.24 g. From this total, 40.61 g of the Ethanolic Extract of *Eugenia klotzschiana* O. Berg (EEEk) was obtained.

3.2 Biofilm formation

All the strains tested were able to form biofilm under the conditions tested. *Staphylococcus aureus* ATCC 6538 was the strongest biofilm former, followed by *Staphylococcus aureus* ATCC 29213, *both were* classified as strong biofilm formers. *Enterococcus faecalis* ATCC 51299, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 19433, and *Staphylococcus epidermidis* ATCC 14990 formed biofilms classified as moderate (Figure 2).

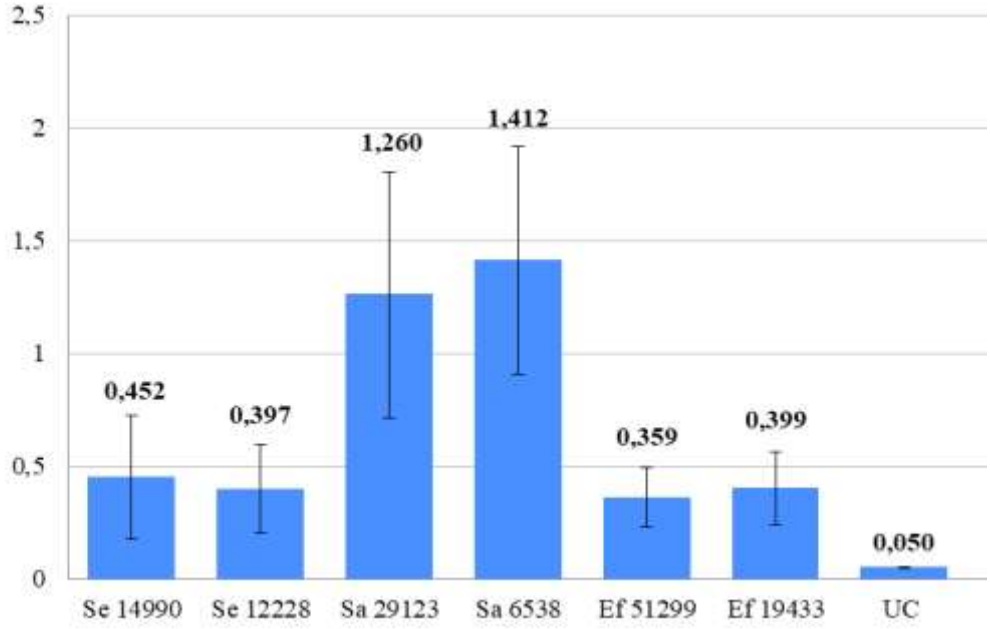

Figure 2. OD_{620nm} of Gram-positive bacteria biofilms detected by the crystal violet method. Se 14990 - *Staphylococcus epidermidis* ATCC 14990, Se 12228 - *Staphylococcus epidermidis* ATCC 12228, Sa 29213 - *Staphylococcus aureus* ATCC 29213, Sa 6538 - *Staphylococcus aureus* ATCC 6538, Ef 51299 - *Enterococcus faecalis* ATCC 51299, Ef 19433 - *Enterococcus faecalis* ATCC 19433, and UC - Uninoculated control.

3.3 Antimicrobial Activity of the Extracts

The ethanolic extract of the leaves of *Eugenia klotzschiana* O. Berg (EEEk) showed activity against Gram-positive bacteria with MICs between 0.312 and 0.625 $mg.mL^{-1}$ (Table 1). Plant extracts with MIC of 0.100 $mg.mL^{-1}$ are considered very active, with 0.100 to 0.500 $mg.mL^{-1}$ having moderate antimicrobial activity, 0.500 to 1 $mg.mL^{-1}$ having weak activity, and above 1 $mg.mL^{-1}$ being inactive[23].

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Table 1. Antimicrobial activity (MIC and MBC) of the ethanolic extract of E. klotzschiana *O. Berg leaves against Gram-positive bacteria*

Ethanolic Extract of *Eugenia klotzschiana* O. Berg (mg.mL^{-1})		
	MIC	**MBC**
Staphylococcus epidermidis ATCC 14990	0.312	> 10
Staphylococcus epidermidis ATCC 12228	0.625	> 10
Staphylococcus aureus ATCC 29213	0.312	> 10
Staphylococcus aureus ATCC 6538	0.625	> 10
Enterococcus faecalis ATCC 51299	0.312	> 10
Enterococcus faecalis ATCC 19433	0.625	> 10

MIC - Minimum Inhibitory Concentration and MBC - Minimum Bactericidal Concentration

According to this criterion, the extract showed moderate to weak antibacterial activity against the Gram-positive bacteria tested, being moderately active against *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 51299, with MIC = 0.312 mg.mL^{-1} and with weak activity against *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 6538, and *Enterococcus faecalis* ATCC 19433 with MIC = 0.625 mg.mL^{-1}, no bactericidal activity was detected, with MIC > 10 mg.mL^{-1}.

The essential oils from the leaves and flowers of *E. klotzschiana* O. Berg showed MICs of between 0.050 mg.mL^{-1} and 0.400 mg.mL^{-1} against bacteria in the oral cavity. They were considered promising compounds against cariogenic bacteria such as *Prevotella nigrescens* and *Streptococcus mutans*[7].

3.4 Antibiofilm Activity of the Extract

The antibiofilm activity of the ethanolic extract of *E. klotzschiana* O. Berg leaves was variable among the Gram-positive bacteria tested, and the inhibition of biofilm formation by EEEk ranged from 0.039 to 1250 $mg.mL^{-1}$. The most sensitive bacteria were *Staphylococcus epidermidis* ATCC 14990 and *Staphylococcus aureus* ATCC 6538 with $MBIC_{50}$ = 0.039 $mg.mL^{-1}$. *Staphylococcus epidermidis* ATCC 12228 was the most resistant with $MBIC_{50}$ = 1250 $mg.mL^{-1}$ (Figure 3).

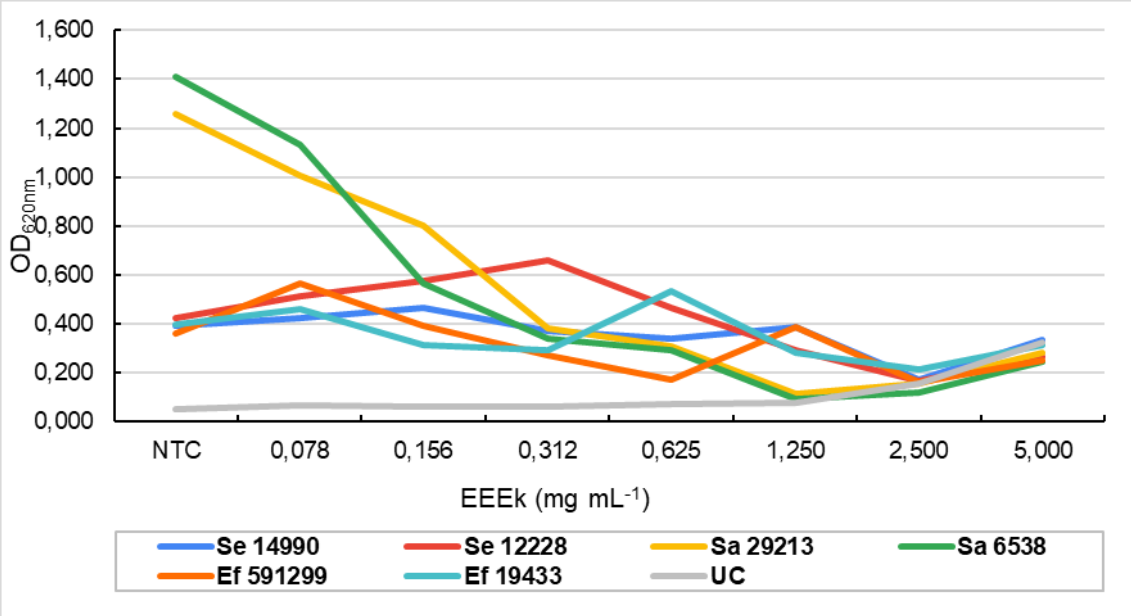

Figure 3. OD_{620nm} of Gram-positive bacteria biofilms in the presence of concentrations of the ethanolic extract of *E. klotzschiana* O. Berg leaves. NTC – non-treated control, Se 14990 - *Staphylococcus epidermidis* ATCC 14990, Se 12228 - *Staphylococcus epidermidis* ATCC 12228, Sa 29213 - *Staphylococcus aureus* ATCC 29213, Sa 6538 - *Staphylococcus aureus* ATCC 6538, Ef 51299 - *Enterococcus faecalis* ATCC 51299 e Ef 19433 - *Enterococcus faecalis* ATCC 19433, and UC - Uninoculated control.

Interestingly, biofilm formation by the two biggest formers (*Staphylococcus aureus* ATCC 29213 and *Staphylococcus aureus* ATCC 6538) was intensively inhibited by 1250 $mg.mL^{-1}$ of EEEk.

EEEk was able to inhibit biofilm formation at subinhibitory concentrations for most bacteria. The most sensitive strain was *S. aureus* ATCC 6538, which had its biofilm formation

inhibited by 0.062 MIC, followed by *S. epidermidis* ATCC 14990 with 0.125 MIC and *S. aureus* ATCC 2913 and *E. faecalis* ATCC 19433 with 0.250 MIC each. On the other hand, *S. epidermidis* ATCC 12228 and *E. faecalis* ATCC 51299 had biofilm formation inhibited only at concentrations close to the MIC, which may indicate that the effect was due to bacteriostatic activity and not antibiofilm activity (Table 2).

Table 2. Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$) of the ethanolic extract of E. klotzschiana *O. Berg leaves against Gram-positive bacteria.*

Ethanolic Extract of *Eugenia klotzschiana* O. Berg (mg.mL^{-1})			
	$MBIC_{50}$	**MIC**	**Relationship $MBIC_{50}$/MIC**
Staphylococcus epidermidis ATCC 14990	0.039	0.312	0.125
Staphylococcus epidermidis ATCC 12228	1.250	0.625	2
Staphylococcus aureus ATCC 29213	0.078	0.312	0.250
Staphylococcus aureus ATCC 6538	0.039	0.625	0.062
Enterococcus faecalis ATCC 51299	0.312	0.312	1
Enterococcus faecalis ATCC 19433	0.156	0.625	0.250

$MBIC_{50}$ - Minimum Biofilm Formation Inhibitory Concentration, MIC - Minimum Inhibitory Concentration.

4. DISCUSSION

Infections caused by bacterial biofilms are typically chronic, as the bacteria in this type of microbiological community are usually resistant to the immune system's defense mechanisms and a wide range of antibiotics and chemotherapy drugs used in clinical treatments[24].

Staphylococcus aureus causes skin infections, endocarditis, lethal pneumonia, osteomyelitis, bacteremia, and soft tissue infections. These infections result in high morbidity and mortality, mainly due to acquired resistance to conventional drugs, caused mainly by the

formation of bacterial biofilm, which gives microorganisms 1000 times greater resistance than planktonic ones[25-28].

The *Staphylococcus epidermidis* species has been responsible for increased clinical infections, mainly caused by its potential to colonize and form an opportunistic biofilm on different surfaces. It is one of the most abundant bacteria on healthy human skin and can contribute to the pathogenesis of skin diseases[29, 30].

Enterococcus faecalis are relevant opportunistic pathogens in the hospital environment and are recurrent causes of outbreaks[31]. They are characterized by expressing virulence factors, antimicrobial resistance, and robust biofilm formation, which contribute significantly to their pathogenicity in persistent infections[32, 33].

The formation and persistence of bacterial biofilms increase the chronicity of infectious processes. This self-produced biological community protects the bacteria in a polymeric matrix that hinders the penetration of antimicrobials[34]. In this context, strategies that seek to inhibit and destroy biofilm formation have a positive impact on reducing morbidity and mortality rates that biofilm-associated microorganisms can cause in public health worldwide[24].

The bacteriostatic activity of EEEk may be related to the presence of major compounds such as catechin, epicatechin, and rutin. In addition, saponins and flavonoids have been identified in the leaves of *Eugenia klotzschiana* O. Berg[35].

Catechins and epicatechins are flavonoids with antiviral, antitumor, anti-inflammatory, and antioxidant activities, while rutin is a flavonoid with antioxidant, anticancer and anti-inflammatory activity. Antioxidant capacity is an identifying characteristic of species from the Myrtaceae family[9, 36, 37].

Flavonoids are compounds produced in different plant organs that have a variety of pharmacological actions ranging from antioxidant to antimicrobial; plants synthesize these compounds in response to microbial infection and have the ability to act as antibacterials, inhibiting virulence factors, forms of microbial resistance such as biofilms; increasing the action of current antibiotics and decreasing antibiotic resistance, which is why they are increasingly attracting the attention of the pharmaceutical industry[38].

In the fraction of essential oil from the leaves of *E. klotzschiana* O. Berg, β-bisabolene, germacrene-D, α-caryophyllene and α-(E)-bergamotene were identified, which demonstrated antimicrobial activity against *Prevotella nigrescens* ATCC 33563, *Streptococcus sanguinis* ATCC 10556, *Streptococcus mitis* ATCC 49452, *Streptococcus salivarius* ATCC 25975, *Streptococcus mutans* ATCC 25175 and *Streptococcus sobrinus* ATCC 33478[7].

In a previous study by our group[39], the presence of catechin, epicatechin, and rutin was detected in the ethanolic extract of *E. klotzschiana* O. Berg leaves. Catechin was active against methicillin-resistant *Staphylococcus aureus* biofilm[40]. Rutin showed antimicrobial and antibiofilm activities and was able to interfere in a series of mechanisms that can be used for the development of new antimicrobials[41].

The decreasing effectiveness of antimicrobials in controlling *E. faecalis* biofilms justifies the investigation of new compounds that inhibit biofilm formation. Due to the diversity of chemical structure and multiple mechanisms of action, phytochemicals are potential sources of anti-biofilm compounds[32].

Plants from the *Eugenia* genus have demonstrated antimicrobial potential. Fractions from extracts of *Eugenia anomala*, *Eugenia arenosa*, *Eugenia hiemalis*, and *Eugenia pitanga* were active against *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923[42]. Crude and fractionated aqueous extracts of *Eugenia pitanga* have been described as active against *Listeria monocytogenes* biofilm formation[43].

Plant-based products and their derivatives are promising compounds with antimicrobial and antibiofilm potential for inhibiting biofilms and controlling associated infections[44,45].

5. CONCLUSION

The ethanolic extract of the leaves of *E. klotzschiana* O. Berg showed little antimicrobial activity against Gram-positive bacteria, being moderately or weakly bacteriostatic and not bactericidal at the concentrations tested. Despite the slight antimicrobial activity, EEEk inhibited biofilm formation at subinhibitory concentrations for most bacteria. The major biofilm formers were very sensitive to the extract, even at low concentrations. Our results reinforce that

the ethanolic extract *E. klotzschiana* O. Berg has antibiofilm potential and that further studies should be carried out to understand the full potential for developing of future drugs to inhibit virulence factors of Gram-positive bacteria.

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