

Antibacterial and antibiofilm activity of *Physalis peruviana* calyx extract**Atividade antibacteriana e antibiofilme do extrato do cálice de *Physalis peruviana***

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ABSTRACT

This study aimed to evaluate the antibacterial and antibiofilm activity of a *P. peruviana* calyx extract. Goldenberry calyx extracts were prepared with 60% (v/v) ethanol. Minimum inhibitory concentration (MIC) analyses were performed by the 96-well plate microdilution method together with the minimum bactericidal concentration (MBC). Biofilm inhibition and destruction was performed in microdilution plates. The *P. peruviana* calyx extract presented antibacterial activity against the pathogens analyzed (*Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC 1705, *Staphylococcus epidermidis* (clinical isolate), *Streptococcus pneumoniae* ATCC 99619, *Pseudomonas aeruginosa* PA01, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* (clinical isolate), *Shigella sonnei* (clinical isolate), *Acinetobacter baumannii* ATCC 19606, *Streptococcus agalactiae* (clinical isolate), *Acinetobacter baumannii* (clinical isolate), *Salmonella* sp. (clinical isolate), *Salmonella enteritidis* (clinical isolate), and *Staphylococcus aureus* (clinical isolate)). MIC varied from 3.15 to 30 mg/mL extract and showed bacteriostatic activity against eight pathogens and bactericidal activity at 30 mg/mL concentration against six strains. Biofilm tests revealed biofilm formation inhibition, although there was no destruction. According to these results, the potential antibacterial activity of *P. peruviana* calyx extract was verified. This will enable further studies to be carried out to contribute to its use in the food industry as a preservative of natural origin and other clinical applications.

Keywords: biofilm, goldenberry, minimum inhibitory concentration, minimum bactericidal concentration.

RESUMO

Este estudo teve como objetivos avaliar a atividade antibacteriana e antibiofilme do extrato do cálice de *P. peruviana*. Os extratos do cálice de goldenberry foram preparados com etanol 60% (v/v). As análises de concentração inibitória mínima (CIM) foram realizadas pelo método de microdiluição em placas de 96 poços, juntamente com a concentração bactericida mínima (CBM). A inibição e destruição de biofilme foi realizada em placas de microdiluição. O extrato do cálice de *P. peruviana*

apresentou atividade antibacteriana frente aos patógenos analisados (*Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC 1705, *Staphylococcus epidermidis* (isolado clínico), *Streptococcus pneumoniae* ATCC 99619, *Pseudomonas aeruginosa* PA01, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* (isolado clínico), *Shigella sonnei* (isolado clínico), *Acinetobacter baumannii* ATCC 19606, *Streptococcus agalactiae* (isolado clínico), *Acinetobacter baumannii* (isolado clínico), *Salmonella* sp. (isolado clínico), *Salmonella enteritidis* (isolado clínico), e *Staphylococcus aureus* (isolado clínico)). A CIM variou de 3,15 a 30 mg/mL de extrato, demonstrando atividade bacteriostática frente a oito patógenos e atividade bactericida na concentração de 30 mg/mL frente a seis cepas. Os testes de biofilme revelaram inibição da formação de biofilme, embora não tenha havido destruição. De acordo com esses resultados, foi verificada a potencial atividade antibacteriana do extrato do cálice de *P. peruviana*. Isto possibilitará que novos estudos sejam realizados para contribuir na sua utilização na indústria de alimentos, como um conservante de origem natural e outras aplicações clínicas.

Palavras-chave: biofilme, goldenberry, concentração inibitória mínima, concentração bactericida mínima.

1 INTRODUCTION

Among the new natural sources, *Physalis peruviana* has been studied for its antimicrobial potential. ERTÜRK et al. (2017) [1] evaluated the antioxidant and antimicrobial activity of ethanolic extracts from different parts of *P. peruviana* (fruit, seed, root, body and leaf). Seeds and fruit were the most effective of the plant in terms of its antimicrobial activity. In another study, the dimethyl sulfoxide extract of *P. peruviana* fruit inhibited the growth of microorganisms used in the test [2]. All parts of the *P. peruviana* plant may be potential sources of antimicrobial agents, the most potent being leaf extracts in dichloromethane [3]. In addition, the ripe berries and leaves of *P. peruviana* were evaluated and the ethanolic extracts were more effective than the aqueous ones [4].

P. peruviana fruit is usually marketed without its calyx; consequently, the waste generated by its trade is substantial. Colombia is the world's leading producer of *P. peruviana*, which represents the country's second largest exported product. However, waste generated by the agro-industrial sector, particularly fruit and vegetable waste, is a major economic and environmental issue for many companies, as it is up to them to assume the cost of their management [5].

The bacteria used in this study were chosen due to their clinical and food industry interest. Some strains used were based on the World Health Organization's list of priority microorganisms for research and development of new antibiotics. Among them, Priority 1: CRITICAL (*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and the *Enterobacteriaceae* family). Priority 2: HIGH (*Staphylococcus aureus* and the *Salmonellae* family). Priority 3: MEDIUM (*Streptococcus pneumoniae* and *Shigella* spp. [6]. In addition to these, *Staphylococcus epidermidis* was also studied because of its clinical interest [7]. The other studied foodborne pathogens that can cause foodborne diseases are (*Salmonella* sp., *Salmonella enteritidis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*,

Pseudomonas aeruginosa, *Enterococcus faecalis*, *Escherichia coli*, *Shigella sonnei*, and *Streptococcus agalactiae*) [8].

P. aeruginosa PA01 is a Gram-negative rod and considered a standard biofilm-producing strain and important pathogen for this study. A biofilm is a complex of bacterial cells lined with a polysaccharide layer that acts as a protective factor for bacteria against antimicrobial attacks and the host immune system. The formation of biofilms causes considerable problems in the medical and industrial area, as these structures cause greater resistance to treatment with antibiotics and biocides. Resistance to antibiotic therapy and emergence of multiresistant strains is worrisome, and new therapeutic approaches are required [9].

Several chemical preservatives are used to control these foodborne pathogens [10]. However, due to the increased awareness of the side effects of these additives, there is a growing consumer demand for natural products in place of chemical preservatives. In recent years, research on natural antimicrobial compounds has increased at a rapid pace [11].

There are a variety of natural preservatives from different sources and with different preservation properties, such as plant extracts, essential oils, organic acids (acetic, ascorbic, tartaric, malic, and citric), lactic acid bacteria and bacteriocins from microbiological sources, and chitosan of animal origin [12]. Plants that possess these properties have bioactive compounds that act to protect plants from microbiological attacks, although they can also be exploited and employed by humans as sources of food and medicine [13].

Thus, *P. peruviana* calyces are an important by-product that deserves further study in order that different sources of reuse can be explored and the database on their antimicrobial activity expanded. Therefore, the aim of this study was to evaluate, for the first time, the antibacterial activity against different microorganisms and antibiofilm activity against *Pseudomonas aeruginosa* of *P. peruviana* calyx extract for therapeutic and food application.

2 MATERIAL AND METHODS

Material: *P. peruviana* calyces were obtained from Italbraz (Vacaria, Brazil - 28:0:44 S and 50:56:02 W) in the 2016/2017 harvest. Mueller Hinton broth (Himedia®) and Mueller-Hinton agar (Sigma-Aldrich®) and crystal violet (Sigma Chemical®) were used.

Microorganisms and inoculum: American Type Culture Collection (ATCC) bacterial strains and clinical isolates provided by the UFSM Department of Microbiology and Parasitology were used [*Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC 1705, *Escherichia coli* (clinical isolate), *Staphylococcus epidermidis* (clinical isolate), *Streptococcus pneumoniae* ATCC 99619, *Shigella sonnei* (clinical isolate), *Acinetobacter baumannii* ATCC 19606,

Streptococcus agalactiae (clinical isolate), *Acinetobacter baumannii* (clinical isolate), *Pseudomonas aeruginosa* PA01, *Enterococcus faecalis* ATCC 29212, *Salmonella sp.* (clinical isolate), *Salmonella enteritidis* (clinical isolate), and *Staphylococcus aureus* (clinical isolate)]. The bacterial inoculum sizes were standardized according to CLSI guidelines (2017) [14]. Isolated colonies were grown for 18 and 24 h in Mueller Hinton Agar and the suspension prepared in saline solution (NaCl 0.85%) with density adjusted to 0.5 on the McFarland scale (1.5×10^8 CFU/mL).

Extraction: The calyx extracts were prepared following Bazana et al. (2019) [15]. The *P. peruviana* calyces (3 g) were separated from the fruit, submitted to knife milling (Mill MA 630/1-Marconi) (speed = 5 rpm, time = 10s), added with 50 mL of 60% ethanol (v/v), and submitted to stirring in a homogenizer (Shaker model TE 421-Tecnal, Piracicaba, SP, Brazil) at 200 rpm for 2 h. Afterwards, the extracts were filtered in a qualitative filter (Fitec) for coarse separation of the calyx. Subsequently, the extract was subjected to filtration in a polypropylene syringe (0.22- μ m pore size) (Chromfilter) and kept in a refrigerated environment.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): The MICs were determined by the microdilution method according to Clinical and Laboratory Standards Institute [14]. The assay was carried out in 96-well-microplates using Mueller Hinton broth (MHB). Several concentrations of *P. peruviana* calyx extract (60; 30; 15; 7.5; 3.75; and 1.87 mg/mL) was diluted in ethanol 60% (v/v) and added in wells with MHB and the suspension with microorganism (0.5 MacFarland scale). The positive control was considered the well with the bacterial suspension and MHB while the negative control was MHB and extract. A control with ethanol 60% (v/v) was performed to discard the diluent activity. The plates were incubated at 37 °C and the minimum inhibitory concentration (MIC) was recorded after 24 h of incubation. 2,3,5-triphenyltetrazolium chloride was used as an indicator which develop a red colour in the microbial grown. The assays were performed in triplicate. The MICs were defined as the lowest concentration that inhibits visible bacterial growth. The determination of the MBC was performed in conjunction with the MIC, using the method proposed by Courvalin (1985) [16]. After the determination of MIC, wells containing visible or non-visible growth were transferred to Petri dishes containing the solid Mueller-Hinton agar and incubated at 37 °C for 18-24 hours. After this period, the number of colonies per plaque was determined and the MBC was defined as the lowest concentration of the extract that presented 0.01% viable bacteria.

Biofilm inhibition: For this analysis, 15 μ L of inoculum were pipetted with 100 μ L MHB and 100 μ L of extract, in MIC and subinhibitory concentrations into four wells of a sterile flat-bottomed microtiter plate. After incubation for 24 h at 37 °C, all of the planktonic microorganisms were removed and washed with distilled water three times. The biofilm was visualised by adding 200

μL of 0.1% crystal violet solution to each well. The microplates were washed with distilled water and air-dried. To solubilize the biofilm, 200 μL of 95% ethanol was added. The solution was transferred to a new microtiter plate and the biofilm formation was revealed by measuring the absorbance at 570 nm in a microplate reader. For the control culture, broth was used for negative control and only *P. aeruginosa* PA01 inoculum for positive control [17].

Biofilm destruction: 15 μL of inoculum with 185 μL of MHB was pipetted into four wells of a sterile flat-bottomed microtiter plate. After incubation for 24 h at 37 °C, the planktonic microorganisms were removed from the wells and the extracts added in MIC and sub-inhibitory concentrations. After incubation for 24 h at 37 °C, all of the planktonic microorganisms were removed and washed with distilled water three times. The biofilm was visualised by adding 200 μL of 0.1% crystal violet solution to each well. The microplates were washed with distilled water and air-dried. To solubilize the biofilm, 200 μL of 95% ethanol was added. The solution was transferred to a new microtiter plate and the biofilm formation was revealed by measuring the absorbance at 570 nm in a microplate reader. For the controls, culture broth was used as the negative control and only *P. aeruginosa* PA01 inoculum was used as the positive control [17].

Statistical analyses: The experiments were performed in triplicate. The results were evaluated by analysis of variance (ANOVA), and the means were compared by the Tukey test, considering the level of significance at 5% ($p < 0.05$).

3 RESULTS AND DISCUSSION

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The antimicrobial activity of the ethanolic extracts of *P. peruviana* calyx is shown in Table 1. The lowest minimum inhibitory concentration observed was against *Enterococcus faecalis* bacteria, in which the extract concentration of 3.75 mg/mL was able to inhibit growth of this bacterium. MBC = 30 mg/mL was found for *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* PA01e *Enterococcus faecalis*, indicating that for these bacteria the 30 mg/mL *P. peruviana* calyx extract has a bactericidal action. For the other microorganisms, $\text{MBC} \geq 30$ mg/mL indicated that the extract has bacteriostatic action. These results suggest that the bioactive compounds of calyx are responsible for antimicrobial action. According to Ballesteros-Vivas et al. (2019) [18], several compounds were identified in the *P. peruviana* calyx as phytochemicals (terpenoids, phytosterols, and phytol derivatives), flavonoids, phenolic acids, and withanolides. Phytosterols are reported as bioactive compounds of great interest due to their antioxidant capacity and health impact. They are referred to as anti-inflammatory, antitumor, antibacterial, antifungal, and hypocholesterolemic compounds. Its presence in significant

levels in oil extracted from the skin and pulp of *P. peruviana* has already been found [19]. According to these values, the calyx extract can be considered promising for use as a preservative of natural origin or to be part of a sanitizer for use in the food industry.

Table 1. MIC and MBC values of *P. peruviana* calyx extract against bacterial strains

Bacterial strains	* MIC (mg/mL)	** MBC (mg/mL)
<i>Enterobacter aerogenes</i> ATCC 13048	30	30
<i>Klebsiella pneumoniae</i> ATCC 1705	30	30
<i>Escherichia coli</i> (Clinical isolate)	7.5	≥ 30
<i>Staphylococcus epidermidis</i> (Clinical isolate)	15	30
<i>Streptococcus pneumoniae</i> ATCC 99619	30	30
<i>Shigella sonnei</i> (Clinical isolate)	15	≥ 30
<i>Acinetobacter baumannii</i> ATCC 19606	7.5	≥ 30
<i>Streptococcus agalactiae</i> (Clinical isolate)	7.5	≥ 30
<i>Acinetobacter baumannii</i> (Clinical isolate)	30	≥ 30
<i>Pseudomonas aeruginosa</i> PA01	7.5	30
<i>Enterococcus faecalis</i> ATCC 29212	3.75	30
<i>Salmonella sp.</i> (Clinical isolate)	15	≥ 30
<i>Salmonella enteritidis</i> (Clinical isolate)	7.5	≥ 30
<i>Staphylococcus aureus</i> (Clinical isolate)	7.5	≥ 30

* Minimum Inhibitory Concentration

** Minimum Bactericidal Concentration

Biofilm inhibition and destruction

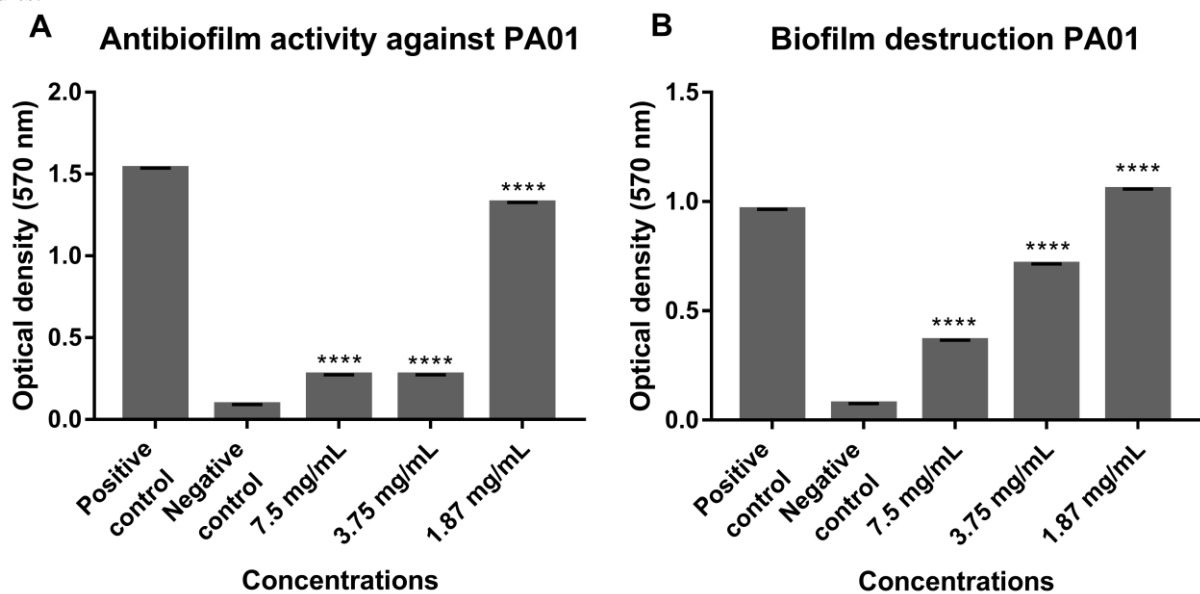
The results regarding biofilm are shown in Figure 1. The biofilm growth in our study was confirmed by the standard *P. aeruginosa* strain PA01 (control) with 100% growth. *P. aeruginosa* is known to be an opportunistic human pathogen capable of causing a wide range of acute and chronic infections that can be life threatening, particularly in immunocompromised patients. *P. aeruginosa* has been historically associated with lung infections in cystic fibrosis patients and is one of the major nosocomial pathogens that affects hospitalized patients, while being intrinsically resistant to a wide range of antibiotics [20]. In addition, the presence of *Pseudomonas* biofilms is also found in food production and its facilities, causing food spoilage and generating various problems [21].

Food contamination is quite recurrent, for example, Crippa et al. (2020) [22] analyzed minimally processed fruits and vegetables that presented values outside the acceptable limits for thermotolerant coliforms and found enterotoxin-producing strains in vitro and biofilm-producing strains on stainless steel and glass surfaces. This demonstrates that the analyzed products were

considered unfit for consumption and presented levels of contamination above the recommended by legislation [22]. Therefore, plant extracts and essential oils have been studied as promising alternatives due to their actions to inhibit bacterial growth [23, 24].

P. peruviana calyx extract concentrations of 3.75 and 7.5 mg/mL inhibited biofilm formation in the early stages (Figure 1A). Biofilm destruction testing showed that no condition led to total destruction of the microorganism (Figure 1B). The lowest extract concentration (1.87 mg/mL) did not have the ability to inhibit biofilm formation, i.e. the most effective concentrations to inhibit *P. aeruginosa* PA01 biofilm formation were 3.75 and 7.5 mg/mL of calyx extract.

Figure 1. A: Inhibition of *P. aeruginosa* PA01 biofilm formation. B: Destruction of *P. aeruginosa* biofilm. Error bars are shown as one standard deviation and differences were considered statistically significant when $p < 0.0001$ between the positive control and the corresponding concentration. Each experiment was carried out three times, yielding similar results.



4 CONCLUSION

The *P. peruviana* calyx extract showed antibacterial activity against the pathogens analyzed. The minimum inhibitory concentration ranged from 3.15 to 30 mg/mL extract. The highest activity demonstrated was bacteriostatic, but also had bactericidal activity at a concentration of 30 mg/mL against *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* PA01, and *Enterococcus faecalis*, indicating that, at this concentration, it acts as a bactericidal agent against these pathogens. Regarding the biofilm tests, there was inhibition of biofilm formation at the concentrations of 3.75 and 7.5 mg/mL of the calyx extract and partial destruction at the same concentrations. These results may be used in future research with emphasis on the use of *P. peruviana* calyx extract in therapeutic approaches and the food industry as a preservative of natural origin.

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DECLARATION OF CONFLICTING INTERESTS

We have no conflicts of interest to declare.

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