Research, Society and Development, v. 9, n. 9, e346997410, 2020
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v9i9.7410

# Atividade antimicrobiana e hemolítica de produtos obtidos de *Piper montealegreanum* Yuncker e efeito *in vitro* no crescimento de *Staphylococcus aureus*

# Antimicrobial screening and hemolytic activity of products obtained from *Piper montealegreanum* Yuncker and effect *in vitro* on growth of *Staphylococcus aureus*

# Evaluación de actividad antimicrobiana y actividad hemolítica de los produtos obtenidos de *Piper montealegreanum* Yuncker y efecto *in vitro* sobre crecimiento de *Staphylococcus aureus*

Received: 07/08/2020 | Reviewed: 14/08/2020 | Accept: 17/08/2020 | Published: 30/08/2020

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**Resumo**

Este estudo teve como objetivo determinar atividade antimicrobiana do extrato etanólico bruto (EEB) e das frações clorofórmica ($CHCl_3$) e acetato de etila (EtOAc), determinar a

influência da fração $CHCl_3$ no crescimento de *Staphylococcus aureus* e atividade hemolítica desta fração. Atividade antimicrobiana dos produtos foi realizada pela técnica de microdiluição em caldo diante de: *Staphyloccocus aureus* ATCC25923, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853 e *Candida albicans* ATCC76645 para determinar a concentração bacteriana mínima (CBM) e concentração inibitória mínima (CIM). Curvas de morte foram realizadas para determinar a influência da fração $CHCl_3$ no crescimento de *S. aureus*. A atividade hemolítica do $CHCl_3$ foi realizada em eritrócitos humanos para relatar o efeito citotóxico. A fração $CHCl_3$ mostrou atividade contra *S. aureus* com CIM=1024 µg $mL^{-1}$ e CBM=2048 µg $mL^{-1}$. Essa fração não mostrou atividade contra outras cepas testadas, bem como o EEB e fração EtOAc. A curva de morte de *S. aureus* com a fração $CHCl_3$ mostrou que na CIM essa fração possui atividade bacteriostática, não sendo observado esse efeito na ½ CIM. A atividade hemolítica demonstrou que a CIM da fração $CHCl_3$ não apresenta dano à membrana dos eritrócitos. Estudos devem ser realizados para avaliar a atividade da fração $CHCl_3$ diante de *S. aureus* com diferentes perfis de resistência, além de avaliar o potencial de reversão da resistência por estudos de combinação. O estudo demonstrou que *P. montealegreanum* pode ser uma fonte importante de compostos de importância no combate a micro-organismos de importância clínica.

**Palavras-chave:** Piperaceae; *S. aureus*; Antimicrobiano; Curva de morte; Atividade hemolítica.

## Abstract

This study aimed to determine spectrum of antimicrobial activity of crude ethanolic extract (CEE), chloroform ($CHCl_3$) and ethyl acetate (EtOAc) fractions, to determine influence of $CHCl_3$ fraction on *Staphylococcus aureus* growth and hemolytic activity. Antimicrobial screening of CEE, $CHCl_3$ and EtOAc fractions was carried out using broth microdilution technique against standard strains: *Staphyloccocus aureus* ATCC25923, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853 and *Candida albicans* ATCC76645 to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Time kill curves were performed to determine influence of $CHCl_3$ fraction on MIC and ½ MIC concentrations on *S. aureus* growth. Hemolytic activity of $CHCl_3$ was performed in human erythrocytes to report the cytotoxic effect. $CHCl_3$ fraction showed activity against *S. aureus* with MIC=1024 µg $mL^{-1}$ and MBC=2048 µg $mL^{-1}$. This fraction did not show activity against other tested strains, as well as the CEE and EtOAc fraction. Time kill curve of *S. aureus* treated with $CHCl_3$ fraction showed that at MIC this fraction has

bacteriostatic activity, which was not observed with the ½ MIC. Hemolytic activity demonstrated that when $CHCl_3$ fraction in MIC does not present membrane damage of erythrocytes, since it did not cause hemolysis. Studies should be conducted to evaluate the activity of $CHCl_3$ fraction against *S. aureus* with different antimicrobial resistance profiles, in addition to evaluating the potential for resistance reversion by *in vitro* combination studies. The study demonstrated that the species *P. montealegreanum* can be an important source of compounds of importance for combating microorganisms of clinical importance.

**Keywords:** Piperaceae; *S. aureus*; Antimicrobial; Kill curve; Hemolytic activity.

**Resumen**

Este estudio tuvo como objetivo determinar la actividad antimicrobiana del extracto etanólico crudo (EEC) y las fracciones de cloroformo ($CHCl_3$) y acetato de etilo (EtOAc), para determinar influencia de la fracción de $CHCl_3$ en el crecimiento de *Staphylococcus aureus* y la actividad hemolítica de esta fracción. La actividad antimicrobiana de los productos se realizó mediante la técnica de microdilución de caldo frente: *Staphyloccocus aureus* ATCC25923, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853 y *Candida albicans* ATCC76645 para determinar concentración bacteriana mínima (CBM) y concentración inhibitoria mínima (CIM). Se realizaron curvas de muerte para determinar influencia de la fracción $CHCl_3$ en crecimiento de *S. aureus*. La actividad hemolítica de $CHCl_3$ se realizó en eritrocitos humanos para informar el efecto citotóxico. La fracción $CHCl_3$ mostró actividad contra *S. aureus* con CIM=1024 µg $mL^{-1}$ y CBM=2048 µg $mL^{-1}$. Esta fracción no mostró actividad contra otras cepas probadas, así como la fracción EEC y EtOAc. La curva de muerte de *S. aureus* con la fracción $CHCl_3$ mostró que esta fracción tiene actividad bacteriostática en CIM, sin efecto en ½ CIM. La actividad hemolítica demostró que la CIM de la fracción $CHCl_3$ no tiene daño en la membrana de los eritrocitos. Se deben realizar estudios para evaluar la actividad de la fracción de $CHCl_3$ contra *S. aureus* con diferentes perfiles de resistencia, además de evaluar el potencial de reversión de resistencia mediante estudios combinados. El estudio demostró que *P. montealegreanum* puede ser fuente importante de compuestos de importancia en la lucha contra microorganismos de importancia clínica.

**Palabras clave:** Piperaceae; *S. aureus*; Antimicrobiano; Curva de muerte; Actividad hemolítica.

## 1. Introduction

Microbial resistance to antimicrobials from community and nosocomial isolates represents a serious public health problem. Microorganisms that were previously susceptible to antimicrobial agents, started to show resistance and this selection is mainly due to the indiscriminate use of these drugs (World Health Organization, 2020). The increase in microbial resistance over the years, driven by the indiscriminate use of antibiotics and the emergence of new species of microorganisms not yet reported and with important resistance profiles, resulting on reduction in the clinical treatment options for infections caused by these microorganisms (Roca, et al., 2015), increasing the interest in the search for molecules and new treatment alternatives.

The emergence of multi-drug resistant (MDR) microorganisms is cited as a global health problem (Roca, et al., 2015). According to *Brazilian Surveillance and Control of Pathogens of Epidemiological Importance* (BrSCOPE), 14 % of infections by microorganisms are caused by *Staphylococcus aureus* and of these 43,7 % correspond to methicillin resistant *Staphylococcus aureus* strains (MRSA) (Marra, 2011). In Brazil, 37-57 % of *Pseudomonas aeruginosa* isolates have been reported to be resistant to carbapenems, which is the most effective class of antibiotics against Gram-negative (Hong et al., 2015). A recent study also showed that there was an increase in the isolation of species of yeast-like fungi such as species of *Candida* that are resistant to azoles making treatment approaches more difficult (Castanheira, Messer, Rhomberg & Pfaller, 2016).

In this context, the genus *Piper* has been mentioned for the biological activities attributed to several species, among them the antimicrobial activity (Salehi et al., 2019). This genus comprises about 2000 species distributed mainly in the pantropical region. They are characterized by being aromatic plants and well applied in culinary use, their essential oils are composed of monoterpene hydrocarbons, oxygenated monoterpenoids, sesquiterpene hydrocarbons, oxygenated sesquiterpenoids and large amounts of phenylpropanoids (Silva et al., 2017; Salehi et al. 2019).

Among the biological activities attributed to the genus *Piper* over the years are the antimicrobial activity against bacteria Gram positive, Gram negative and yeast and leishmanicidal activity of *Piper regnellii* (Nakamura et al., 2006; Pessini et al., 2003), the trypanocidal activity of *Piper arboreum* e *Piper tuberculatum* (Regasini, 2009), the activity on growth and metabolism of *Streptococcus mutans* and *Streptococcus sanguis* of *Piper aduncum* (Magalhães, 2010), the potent antiplasmodial activity of flavonoids of *Piper*

*hostmannianum* (Portet et al., 2007), in addition to the insecticidal activity of *Piper hispidinervum* (Lima et al., 2009).

*Piper montealegreanum* has already been reported for antibacterial activity against certain species. Pinto et al. (2012) reported that the ethyl acetate (EtOAc) fraction of the branches from *P. montealegreanum* has antibacterial activity by broth microdilution technique, against strains of *Bacillus subtillis*, *Pseudomonas aeruginosa* and *Escherichia coli.* Reports of antibacterial activity of this species by the Agar diffusion methodology have been previously published, reporting activity of the crude ethanolic extract (CEE), chloroform fraction and EtOAc fraction against *Staphyloccus aureus* strain (Alves et al., 2016; Rocha et al., 2018).

Therefore, this study aimed to verify the antimicrobial activity of CEE and chloroform and ethyl acetate fractions of the branches from *Piper montelagreanum*, as well as to define the action of this species of *Piper* through the Time-kill curve against *Staphylococcus aureus*, in addition to evaluating the toxicity of these products determining their hemolytic potential in human red blood cells.

## 2. Materials and Methods

### *2.1 Study delimitations*

This article presents the results of a research carried out in a laboratory, developed at the Antimicrobial Activity Laboratory located at Campus I of the State University of Paraíba, Campina Grande, Paraíba, Brazil. The research was of the quantitative type, presenting numerical results of antimicrobial activity (Pereira, Shitsuka, Parreira & Shitsuka, 2018). Metodologia da pesquisa científica..

### *2.2 Plant material*

The botanical material from *P. montealegreanum Yuncker* was collected in Belém (Pará State, Brazil; latitude 14° 10' 00" S, longitude 53° 05' 00" W) and was identified by Dra. Elsie F. Guimarães, at the Botanical Department of UFRJ. A specimen voucher was deposited at Emilio Goeldi Museum, Belém, under serial number MSP-010.

### *2.3 Products tested*

For this study, the crude ethanolic extract (CEE), chloroform ($CHCl_3$) and ethyl acetate (EtOAc) fractions were previously obtained of the branches from *Piper montealegreanum* (Alves, Rocha, Braz-Filho & Chaves, 2017). The products obtained from *P. montealegreanum* were solubilized in a solution containing DMSO/Tween 80/distilled water (2/0.5/7.5) and all were prepared in an initial stock solution containing 4096 µg $mL^{-1}$. The stock solution was sterilized through membrane filtration (0.22 µm) in order to guarantee the sterility.

### *2.4 Microorganisms, Inoculum preparation and Culture media*

The microbial strains used in the study were obtained from *American Type Culture Collection* (ATCC): *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 76645, recommended by M100S document from *Clinical Laboratory Standards International* to antimicrobial susceptibility testing (CLSI, 2016). Inoculum were prepared from seeding on Mueller Hinton (MH) agar for bacteria and Sabouraud agar for yeast and incubated at 37 °C/24h and 30 °C/24-48h, respectively. After growth, some colonies were diluted to sterile saline solution (NaCl 0.85 %) to produce a slight turbidity corresponding 0.5 tube of McFarland scale, obtaining the final inoculum of 1.8 x $10^6$ CFU/mL (CLSI, 2016).

For the broth microdilution technique, MH broth was used for the cultivation of bacteria and RPMI 1640 broth for yeast, as recommended for antimicrobial susceptibility testing. All were prepared according to the DIFCO® manufacturer's instructions and recommendations.

### *2.5 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)*

The CEE, $CHCl_3$ and EtOAc fractions from *P. montealegreanum* were diluted to an initial concentration of 4096 µg $mL^{-1}$ in aqueous solution containing 0.5 % Tween 80 and 2.0 % DMSO. 100 µL of the appropriate broth was dispensed to each test strain, followed by addition of 100 µL of product solution to be tested, performing serial dilutions varying the concentration of 2048 to 1.0 µg $mL^{-1}$ of products to be tested, to which was added 10 µL of

microbial inoculum study. The positive controls were included in the tests (presence of inoculum), negative (no inoculation), a pattern of tetracycline (SIGMA™) for bacteria and fluconazole (SIGMA™) for yeast, besides the control of the diluent (DMSO/Tween 80/distilled water) of the products to discard their activity. The plates were incubated at 37 °C/24 h for bacteria and at 30 °C/24-48 hours for yeast.

To determine the MBC, the wells that showed visible inhibition with reduced turbidity had a 10 µL aliquot collected and seeded in plates containing MH agar or Sabouraud agar, which were incubated in the conditions mentioned above, in order to perform the viable cell count after the treatment. MBC was the lowest concentration of antibacterial agent that inhibited 99.9% the microorganism growth.

The MIC reading was performed dispensing 20 µL of resazurin solution (0.01%) in the wells of the plates after expiry of the incubation time (CLSI, 2016). The MIC was defined as the lowest sample concentration able to inhibit microbial growth evidenced by blue staining after addition of resazurin 0.01 %, indicating the absence of viable cells. The experiments were performed in triplicate and the results expressed by the mean.

### *2.6 Effect of $CHCl_3$ fraction on bacterial Time-kill curves*

Time-kill curves were used to test the bactericidal activity of $CHCl_3$ fraction from *P. montealegreanum* against *S. aureus* ATCC 25923 was performed as described to Krogstad, Moellering and Lorian (1986) and Noviello, Ianniello, Leone and Esposito (2002) with some modifications. Time-kill curves were determinate using two different concentrations of $CHCl_3$ fraction: the MIC (1024 µg $mL^{-1}$) and the ½ MIC (512 µg $mL^{-1}$), as well as the use of tetracycline at a concentration of 30 µg $mL^{-1}$ which was used as positive control.

*S. aureus* ATCC 25923 was inoculated on MH agar and incubated at 37° C for 18-24 hours. After this period, 50 µL was transferred into 50 mL of MH broth, incubating at 37 °C/1h in order to produce a slight turbidity also equivalent to 0.5 McFarland standard, with a final concentration of 5 x $10^6$ CFU/mL. There were separate three sterile tubes, the first called test tube (T), the second called positive growth control tube (P) and the third control tube (C). It was transferred 9.0 mL of this bacterial culture and added 1.0 mL of product (MIC and ½ MIC) in the T tube, 1.0 mL of sterile distilled water in the positive growth control tube (P) and 1.0 mL of tetracycline (30 µg $mL^{-1}$) in the C tube. The tubes were kept in incubation at 37 °C for 24 hours and aliquots were removed after 2, 4, 6, 8, 10 and 24 hours incubation and plated on MH agar, using the pour-plate technique, after serial dilutions in saline sterile.

Reading of the plates was done after incubation for 24-48 hours using colony counter CP600 Plus (PHOENIX®). The definition of bactericidal activity was a decrease of ≥ 3 $\log_{10}$ in CFU/mL corresponding to inhibited of 99.9 % of growth and bacteriostatic effect was assessed a decrease of ≤ 2 $\log_{10}$ in CFU/mL comparing to curve control growth (Boswell, Andrews, Wise & Dalhoff, 1999).

### *2.7 Determination of hemolytic effects*

The cytotoxic effects were evaluated by hemolytic activity in human erythrocytes, which were obtained of the blood dropped from the Clinical Analysis Laboratory of the State University of Paraíba (CAL/UEPB). The experimental procedures were reviewed and approved by the Ethics Committee in Human Research UEPB (Certificate/CEP/UEPB No. 42778115.7.000.5187). Aliquots of human blood (type A, B and O) were mixed with 0.9 % NaCl at a ratio of 1:30 under stirring slowly and constantly. Then, the samples were centrifuged to 3000 rpm for 5 minutes to obtain the erythrocytes. This procedure was repeated twice and the sediment from the last centrifugation was resuspended in 0.9 % NaCl to a final concentration of 0.5 % of erythrocytes. 0.5 mL of $CHCl_3$ fraction was added to 2 mL of erythrocytes suspension for final concentrations of 1024 µg $mL^{-1}$ (MIC) and 2048 µg $mL^{-1}$ (MBC). The erythrocytes suspension was the negative control (0 % hemolysis) and the erythrocytes suspension plus 0.5 mL of Triton X-100 (SIGMA™) 1 % was the positive control to 100 % hemolysis. The samples were incubated for 1 hour at room temperature under slow (100 rpm) and constant agitation. After this time, they were centrifuged at 3000 rpm for 5 min and hemolysis was quantified by spectrophotometer at 540 nm (Bozi et al., 2004; Brandão et al., 2005; Eisele et al., 2006; Pinto et al., 2012). The tests were performed in triplicate. Results were expressed as a percentage representing the arithmetic mean of three measurements

## 3. Results and Discussion

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was observed that only $CHCl_3$ fraction from *P. montealegreanum* was able to inhibit the growth of *S. aureus* ATCC 25923 strain. This fact is expected since Gram-positive bacteria are generally more sensitive to antibiotics than Gram negative (Madigan & Martinko, 2000) because of the external membrane of Gram negative bacteria prevent the entry of

antibiotic molecules and of the periplasmic space that contain enzymes that are able to break foreign molecules that are exposed to the microorganism (Holetz et al., 2002).

The CEE and EtOAc fraction no showed activity against any of the strains tested (Table 1), evidenced by the pink color after revelation by resazurin 0.01 %, indicating the presence of viable cells.

**Table 1.** Antimicrobial activity of CEE and $CHCl_3$ and EtOAc fractions from *Piper montealegreanum*

| **Products tested**<br><br>*Piper montealegreanum* (branches) | **Microorganisms/MIC and MBC (µg mL$^{-1}$)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *S. aureus* ATCC 25923 | | *E. coli* ATCC 25922 | | *P. aeruginosa* ATCC 27486 | | *C. albicans* ATCC 76645 | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| **CEE** | >2048 | >2048 | >2048 | >2048 | >2048 | >2048 | >2048 | >2048 |
| **$CHCl_3$** | 1024 | 2048 | >2048 | >2048 | >2048 | >2048 | >2048 | >2048 |
| **EtOAc** | >2048 | >2048 | >2048 | >2048 | >2048 | >2048 | >2048 | >2048 |

Legend – ATCC: American Type Culture Collection; MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration; CEE: Crude ethanolic extract; $CHCl_3$: Chloroformic fraction; EtOAc: Ethyl acetate fraction. Source: Elaborated by the Authors, 2020.

As shown in Figure 1, it was found that the $CHCl_3$ layer was able to inhibit the growth of *S. aureus* ATCC 25923, as evidenced by blue color on the first two plate holes, the first of which had 2048 µg mL$^{-1}$ and the following 1024 µg mL$^{-1}$. These wells had an aliquot sown on Mueller Hinton Agar in order to define the MBC, the colony count after the incubation period determined that the concentration capable of eliminating 99.9 % of microorganism growth was 2048 µg mL$^{-1}$ and was determined as MBC. The MIC was defined as 1024 µg mL$^{-1}$ that was capable to inhibit the *S. aureus* growth.

**Figure 1.** Demonstration of the experiment of antimicrobial activity of the $CHCl_3$ fraction of *Piper montelagreanum* branches against *Staphylococcus aureus* ATCC 25923


Legend - ATCC: American Type Culture Collection; MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration; $CHCl_3$: Chloroformic fraction. Source: Elaborated by the Authors, 2020.

Previously published studies determined results similar to those found in this study regarding antibacterial activity against *S. aureus, E. coli* and *P. aeruginosa* from extracts and fractions of the branches from *P. montealegreanum*. Alves et al. (2016) determined that the $CHCl_3$ fraction showed activity in front *S. aureus* only by the agar diffusion methodology with a higher MIC (MIC = 1024 µg $mL^{-1}$) than that defined in this study, which can be explained by difference in *in vitro* study methods for determining antimicrobial activity. Pinto et al. (2012) determined that the EtOAc fraction showed no activity against standard strains of *S. aureus* ATCC 25925 e *S. aureus* ATCC 29213 which is in accordance with our results.

In contrast, antibacterial activity of the EtOAc fraction obtained of the branches from *P. montealegreanum* has previously been reported, using the same methodology chosen in this study, against *P. aeruginosa* (ATCC 25619, ATCC 8027), *E. coli* strain (ATCC 2536) and *Bacillus subtilis* (CCT 0516) standard strains, these strains being different from those used in this study. It was also reported that the EtOAc fraction did not show the same activity in relation to another strain of *E. coli* (ATCC 10536) (Pinto et al., 2012). Thus, we can evidence that even though the microorganisms are of the same species, the characteristics of each strain can influence the antibacterial activity of a given product.

Although the CEE from *P. montealegreanum* does not show activity against *E. coli* ATCC 25922, a previous study demonstrated that this extract has the ability to increase the diameter of the antibiotic inhibition halos for clinical use when combined *in vitro*, indicating that the CEE has synergistic action when combined with ampicillin, ciprofloxacin, chloramphenicol, sulfamethoxazole and tetracycline against *E. coli* (Rocha et al., 2018). No studies were found to determine the anti-candida activity of the tested products.

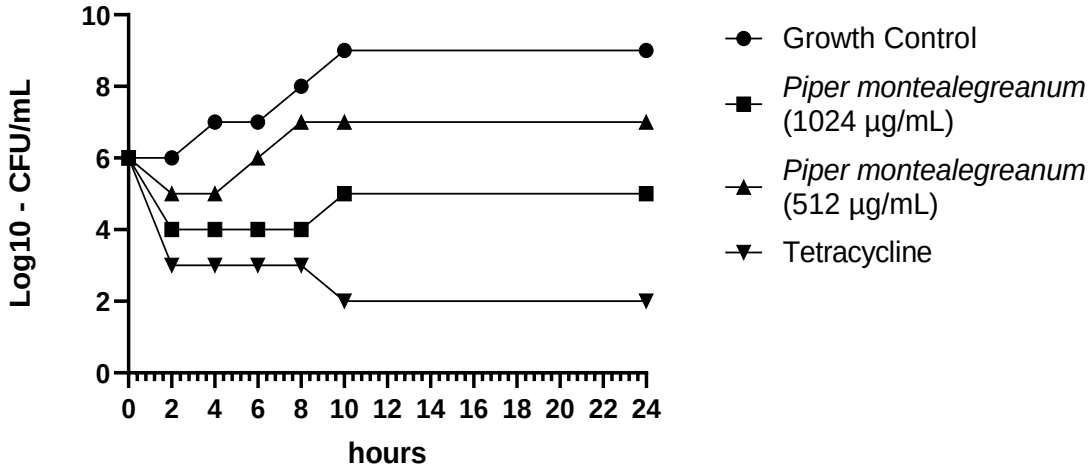
To evaluate the kinetic of inhibition through of time-kill curves of the *S. aureus* ATCC 25923 was used the $CHCl_3$ fraction, because it was the only one that showed antibacterial activity by the microdilution technique in broth. It´s possible to emphasize the effect of the mean value of the $CHCl_3$ fraction determined by the number of viable cells per colony-forming units per mL (CFU/mL) as a function of microorganism exposure time to $CHCl_3$ fraction in your MIC and ½ MIC.

Important differences in antibacterial activity of there were observed $CHCl_3$ fraction of *P. montealegreanum* against *S. aureus* ATCC 25923 with the time-kill curves assay (Figure 2). As expected, the $CHCl_3$ fractions of *Piper montealegreanum* at MIC and ½ MIC concentrations resulted in different effects on the inhibition of growth of *S. aureus*. It is important to highlight that the growth curves of the *S. aureus* strain treated with the two

concentrations (MIC and ½ MIC) of the $CHCl_3$ fraction of *P. montealegreanum* showed that the action of the products was dependent on time and concentration.

The best effect of the $CHCl_3$ fraction in MIC concentration (1024 µg $mL^{-1}$) was found in the interval of 8 and 24 hours of treatment, in which a reduction of 4 log10 of CFU/mL was observed, when compared to the control (Figure 2). By the way, a reduction of 3 log10 of CFU/mL was observed in the interval of 4 and 6 hours of treatment, when compared to the control.

**Figure 2.** Kill curve of *Staphylococcus aureus* ATCC 25923 after treatment with $CHCl_3$ fraction from *Piper montealegreanum*




Legend - CFU: colony formation units. Graph plotted by the GraphPad Prism 8.4.3 program. Source: Elaborated by the Authors, 2020.

The kill curve demonstrated by treatment with the concentration of ½ MIC was similar to treatment with MIC concentration, presenting a better treatment interval in 10 to 24 hours with a greater reduction of CFU/mL when compared with the control. However, the treatment with ½ MIC demonstrated smaller reduction in *log* when compared with MIC kill curve and with growth control curve (without treatment) (Figure 2).

The $CHCl_3$ fraction from *P. montealegreanum* in MIC concentration (1024 µg $mL^{-1}$) and ½ MIC concentration (512 µg $mL^{-1}$), presented bactericidal and bacteriostatic action, respectively, because it inhibited to the growth of the strain of *S. aureus* ATCC 25923, since reduced the number of viable cells when compared to the growth control. The bactericidal effect $CHCl_3$ fraction in MIC can be seen by the reduction higher of 3 log10 CFU/mL or 99 % of cell death, starting from the initial inoculum on a certain time (May, Chan, King, Williams

& French, 2000). Bactericidal action of a product is considering significantly satisfactory when it´s able to reduce an initial inoculum concentration to values equal or lower than 2 log10 CFU/mL in a shorter time or equal to 24 hours of incubation and consider lesser degrees of cell death as bacteriostatic effect (Jones, Anderegg & Deshpande, 2002; Shelburne et al., 2004).

Previous study by Pessini et al. (2003) demonstrated findings similar to that found in this study. A compound isolated from *Piper regnellii* was able to reduce the growth of *S. aureus* over time, having better activity with 9 hours of treatment with 3 log10 CFU/mL, it also demonstrated that the activity before *S. aureus* is dependent on exposure time and concentration of the antimicrobial agent.

Silva et al. (2014) indicated that the mechanism of antimicrobial action of the hydroalcoholic extract of *Piper umbellatum* against *S. aureus* and other species of bacteria, associated with the change in the permeability of the cell wall and cytoplasmic membrane, which can be associated with the presence of flavonoids in the hydroalcoholic extract gross.

Phytochemical study of *P. montealegreanum* Yuncker determined the isolation of five substances, with emphasis on two new flavonoids with characteristic skeleton of monoterpene dihydrochalcones, called claricine and maisine (Alves et al., 2017). Flavonoids are compounds that naturally appear in plants and can also be obtained synthetically, they are reported for their innumerable biological activities, such as anti-oxidant, anti-inflammatory, cardioprotective, anti-Parkinson, antidepressants, spasmolytic activity, antidiabetic, anticancer, in addition to antibacterial activity (Rana & Gulliya, 2019; Sarbu, Bahrin, Babii, Stefan & Birsa, 2019).

Segundo Kumar and Pandey (2013), there are different forms of action of flavonoids in the action on microbial cells that promote activity in front of microorganisms. Flavonoids have the ability to form complexes with proteins, constitutive membrane proteins, cytoplasmic enzymes and adhesins, interacting through hydrogen bonding as well as covalent bonds. They can also interact with membrane lipids.

The $CHCl_3$ fraction revealed no hemolytic activity in human erythrocytes until 1024 µg $mL^{-1}$ and at 2048 µg $mL^{-1}$ presented low cytotoxic effect against these cells (Table 2).

**Table 2.** Hemolytic activity of $CHCl_3$ fraction on human erythrocytes

| Erythrocytes Type (ABO/Rh) | Hemolytic Activity (%) | |
|---|---|---|
| | $CHCl_3$ Fraction (µg $mL^{-1}$) | |
| | 1024 µg $mL^{-1}$ (CIM) | 2048 µg $mL^{-1}$ (CBM) |
| A + | 3.4 | 15.5 |
| B + | 0.0 | 18.1 |
| O + | 0.0 | 38.0 |

Legend - MIC: Minimal inhibitory concentration; MBC: Minimal inhibitory concentration; $CHCl_3$: Chloroformic fraction. Triton X-100 was applied as hemolysis positive control (100 % hemolysis). Source: Elaborated by the Authors, 2020.

It's observed that the concentration at which $CHCl_3$ fraction showed antibacterial activity (1024 µg $mL^{-1}$) cause no damage to the human membrane erythrocytes. Thus, such fraction may have potential for therapeutic use, since it's able to inhibit bacterial growth and does not cause cell effects, characteristic for an agent which present antimicrobial activity with none or low toxicity.

A previous study demonstrated a behavior similar to that observed in the results of this work for hemolytic activity of products of *P. montealegreanum*, where the EtOAc fraction did not show a cytotoxic effect against human erythrocytes (type A, B and O) at a concentration of 1000 µg $mL^{-1}$, in addition, the EtOAc fraction demonstrated a similar effect also when used in a concentration greater than 2000 µg $mL^{-1}$, presenting a higher percentage of hemolysis in relation to blood type O when compared with the other two blood types tested (A and B) (Pinto et al., 2012).

Studies over the years have been conducted in order to demonstrate the antimicrobial activity of natural products, with the aim of investigating new sources of bioactive molecules that have low toxicity (Oliveira et al., 2020; Silva et al., 2020). The results of this study contribute to enrich the sources of knowledge about plants of the genus *Piper* that present important biological activities.

The bactericidal action of the MIC and the bacteriostatic action of the ½ MIC of the $CHCl_3$ fraction of *Piper montealegreanum* branches against *Staphylococcus aureus*, as well as the low toxicity of this fraction against human erythrocytes represent the main findings of this study.

## 4. Conclusion

Bacterial kinetics showed a bacteriostatic effect of the chloroform fraction against *Staphylococcus aureus*, an important agent in hospital infections and low degree of toxicity of

the fraction in human erythrocytes. It is recommended to evaluate the antibacterial activity of the chloroform fraction against *S. aureus* with different profiles of resistance to antibiotics for clinical use. Combination studies *in vitro* with the other fractions aimed at reversing the resistance of *S. aureus* and decreasing the MIC of both the antibiotic and extracts and fractions are recommended too. These data corroborate the continuation of the studies with the species in order to evaluate the biological properties of the isolated products.

## References

Alves, H. S., Rocha, W. R. V., Braz-Filho, R., & Chaves, M. C. D. O. (2017). Isolation of monoterpene dihydrochalcones from *Piper montealegreanum* Yuncker (Piperaceae). *Molecules, 22(6),* 874. https://doi.org/10.3390/molecules22060874

Alves, H. S., Rocha, W. R. V., Fernandes, A. F. C., Nunes, L. E., Pinto, D. S., Costa, J. I. V., & Catão, R. M. R. (2016). Antimicrobial activity of products obtained from *Piper* species (Piperaceae). *Revista Cubana de Plantas Medicinales, 21(2),* 168-180. Retrieved from: https://www.medigraphic.com/cgi-bin/new/resumenI.cgi?IDREVISTA=262&IDARTICULO=67094&IDPUBLICACION=6574

Boswell, F. J., Andrews, J. M., Wise, R., & Dalhoff, A. (1999). Bactericidal properties of moxifloxacin and post-antibiotic effect. *Journal of Antimicrobial Chemotherapy, 43(suppl_2),* 43-49. https://doi.org/10.1093/jac/43.suppl_2.43

Bozi, S., Feruglio, L., Daros, T., Spalluto, G., Gregoretti, B., Terdoslavich, M., Decorti, G., Passmonti, S., Moro, S., & Prato, M. J. (2004). Hemolytic effects of water-soluble fullerene derivatives. *Journal of medicinal chemistry, 47(27),* 6711-6715. https://doi.org/10.1021/jm0497489

Brandão, R., Lara, F. S., Pagliosa, L. B., Soares, F. A., Rocha, J. B. T., Nogueira, C. W., & Farina, M. (2005). Hemolytic effects of sodium selenite and mercuric chloride in human blood. *Drug and chemical toxicology, 28(4),* 397-407. https://doi.org/10.1080/01480540500262763

Castanheira, M., Messer, S. A., Rhomberg, P. R., & Pfaller, M. A. (2016). Antifungal susceptibility patterns of a global collection of fungal isolates: results of the SENTRY Antifungal Surveillance Program (2013). *Diagnostic Microbiology and Infectious Disease, 85*, 200-204. https://doi.org/10.1016/j.diagmicrobio.2016.02.009

CLSI. Wayne, P. A. (2016). Clinical and laboratory standards institute. Performance standards for antimicrobial susceptibility testing. Retrieved from: https://clsi.org/

Eisele, K., Lang, P. A., Kempe, D. S., Klarl, B. A., Niemoller, O., Wieder, T., Huber, S. M., Duranton, C., & Lang, F. (2006). Stimulation of erythrocyte phosphatidylserine exposure by mercury ions. *Toxicology and applied pharmacology, 210(1-2),* 116-122. https://doi.org/10.1016/j.taap.2005.07.022

Holetz, F. B., Pessini, G., Sanches, N.; Cortez, D. A. G., Nakamura, C. V., & Dias Filho, B. P. (2002). Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. *Memórias do Instituto Oswaldo Cruz, 97(7),* 1027-1031. https://doi.org/10.1590/S0074-02762002000700017

Hong, D. J., Bae, I. K., Jang, I. H., Jeong, S. H., Kang, H. K., & Lee, K. (2015). Epidemiology and characteristics of metallo-β-lactamase-producing *Pseudomonas aeruginosa*. *Infection & chemotherapy, 47(2),* 81-97. https://doi.org/10.3947 / ic.2015.47.2.81

Jones, R. N., Anderegg, T. R., & Deshpande, L. M. (2002). AZD2563, a new oxazolidinone: bactericidal activity and synergy studies combined with gentamicin or vancomycin against *Staphylococci* and *Streptococcal* strains. *Diagnostic microbiology and infectious disease, 43(1),* 87-90. https://doi.org/10.1016/S0732-8893(02)00360-7

Krogstad, D. J., Moellering, R. C., & Lorian, V. (1986). Antibiotics in laboratory medicine. *Baltimore: Williams and Wilkins.* Retrieved from: https://books.google.com.br/

Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: an overview. *The scientific world journal, 2013*. https://doi.org/10.1155/2013/162750

Lima, R. K., Cardoso, M. G., Moraes, J. C., Melo, B. A., Rodrigues, V. G., & Guimarães, P. L. (2009). Atividade inseticida do óleo essencial de pimenta longa (*Piper hispidinervum* C. DC.) sobre lagarta-do-cartucho do milho *Spodoptera frugiperda* (JE Smith, 1797)(Lepidoptera: Noctuidae). *Acta amazônica, 39(2),* 377-382. https://doi.org/10.1590/S0044-59672009000200016

Madigan, M., & Martinko, J. (2000). The Bacteria In: Brock-Biology of Microorganisms. Retrieved from: https://books.google.com.br/

Magalhães, C. F. (2010). *Efeito de extratos e frações de Piper aduncum sobre o crescimento e metabolismo dos Streptococcus mutans e Streptococcus sanguis* (Doctoral dissertation, Dissertação (Mestrado-Imunopatologia das Doenças Infecciosas e Parasitárias)-Universidade Vale do Rio Doce, Governador Valadares). Retrieved from: http://www.dominiopublico.gov.br/pesquisa/DetalheObraForm.do?select_action=&co_obra=198347

Marra, A. R., Camargo, L. F. A., Pignatari, A. C. C., Sukiennik, T., Behar, P. R. P., Medeiros, E. A. S., & Brites, C. (2011). Nosocomial bloodstream infections in Brazilian hospitals: analysis of 2,563 cases from a prospective nationwide surveillance study. *Journal of clinical microbiology, 49(5),* 1866-1871. https://doi.org/10.1128/JCM.00376-11

May, J., Chan, C. H., King, A., Williams, L., & French, G. L. (2000). Time–kill studies of tea tree oils on clinical isolates. *Journal of Antimicrobial Chemotherapy, 45(5),* 639-643. https://doi.org/10.1093/jac/45.5.639

Nakamura, C. V., Santos, A. O., Vendrametto, M. C., Luize, P. S., Dias Filho, B. P., Cortez, D. A., & Ueda-Nakamura, T. (2006). Atividade antileishmania do extrato hidroalcoólico e de frações obtidas de folhas de *Piper regnellii* (Miq.) C. DC. var. pallescens (C. DC.) Yunck. *Revista Brasileira de Farmacognosia, 16(1),* 61-66. https://doi.org/10.1590/S0102-695X2006000100011

Noviello, S., Ianniello, F., Leone, S., & Esposito, S. (2002). Comparative *in vitro* bacteriostatic and bactericidal activity of levofloxacin and ciprofloxacin against urinary tract

pathogens determined by MIC, MBC, Time-Kill curves and bactericidal index analysis. *Infez Med, 10*, 100-106. Retrieved from: https://pubmed.ncbi.nlm.nih.gov/12702887/

Oliveira, E. H. de, Gonçalves, T. V. da S. R., Verde, R. M. C. L., Holanda, E. C., Pereira, S. T., & Nunes, L. C. C. (2020). Avaliação antimicrobiana do extrato seco obtido da casca e arilo da Punica granatum. *Research, Society and Development*, *9*(8), e461985941. https://doi.org/10.33448/rsd-v9i8.5941

Pessini, G. L., Holetz, F. B., Sanches, N. R., Cortez, D. A. G., Dias Filho, B. P., & Nakamura, C. V. (2003). Avaliação da atividade antibacteriana e antifúngica de extratos de plantas utilizados na medicina popular. *Revista Brasileira de Farmacognosia, 13,* 21-24. https://doi.org/10.1590/S0102-695X2003000300009

Pereira, A. S., Shitsuka, D. M., Parreira, F. J., & Shitsuka, R. (2018). Metodologia da pesquisa científica. Retrieved from: https://repositorio.ufsm.br/bitstream/handle/1/15824/Lic_Computacao_Metodologia-Pesquisa-Cientifica.pdf?sequence=1.

Pinto, D. S., Duarte, F. M., Costa, J. I. V., Almeida Filho, G. G., Alves, H. S., Chaves, M. C. O., & Pessoa, H, L. F. (2012). Antibacterial and hemolytic activities from *Piper montealegreanum* Yuncker (Piperaceae). *Anti-Infective Agents, 10(1),* 1-5. https://doi.org/10.2174/2211362611201010001

Portet, B., Fabre, N., Roumy, V., Gornitzka, H., Bourdy, G., Chevalley, S., Sauvain, M., Valentin, A., & Moulis, C. (2007). Activity-guided isolation of antiplasmodial dihydrochalcones and flavanones from *Piper hostmannianum* var. berbicense. *Phytochemistry, 68(9),* 1312-1320. https://doi.org/ 10.1016/j.phytochem.2007.02.006

Rana, A. C., & Gulliya, B. (2019). Chemistry and pharmacology of flavonoids - A review. *Indian Journal of Pharmaceutical Education and Research, 53(1),* 8-20. https://doi.org/ 10.5530/ijper.53.1.3

Regasini, L. O., Cotinguiba, F., Passerini, G. D., Bolzani, V. D. S., Cicarelli, R. M. B., Kato, M. J., & Furlan, M. (2009). Trypanocidal activity of *Piper arboreum* and *Piper tuberculatum*

(Piperaceae). *Revista Brasileira de Farmacognosia, 19(1b),* 199-203. https://doi.org/10.1590/S0102-695X2009000200003

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., Cohen, J., Findlay, D, Gyssens, I., Heuer, O. E., Kahlmeter, G., Kruse, H, Laxminarayan, R, Liébana, E., López-Cerero, L., MacGowan, A., Martins, M., Rodríguez-Baño, J., Rolain J.M., Segovia, C., Sigauque, B., Tacconelli, E., Wellington, E., Vila, J. (2015). The global threat of antimicrobial resistance: science for intervention. *New microbes and new infections, 6,* 22-29. https://doi.org/10.1016/j.nmni.2015.02.007

Rocha, W. R. V., Alves, H. S., Nunes, L. E., Nóbrega, B. S., Chaves, M. C. O., & Catão, R. M. R. (2018). Antibacterial activity and interaction in vitro of extract from *Piper montealegreanum* Yuncker on antibiotics of clinical use. *Revista Cubana de Plantas Medicinales, 23(2).* Retrieved from: http://revplantasmedicinales.sld.cu/index.php/pla/article/view/552

Sarbu, L. G., Bahrin, L. G., Babii, C., Stefan, M., & Birsa, M. L. (2019). Synthetic flavonoids with antimicrobial activity: a review. *Journal of applied microbiology, 127(5),* 1282-1290. https://doi.org/10.1111/jam.14271

Salehi, B., Zakaria, Z. A., Gyawali, R., Ibrahim, S. A., Rajkovic, J., Shinwari, Z. K., & Valussi, M. (2019). *Piper* species: a comprehensive review on their phytochemistry, biological activities and applications. *Molecules, 24(7),* 1364. https://doi.org/10.3390/molecules24071364

Shelburne, S. A., Musher, D. M., Hulten, K., Ceasar, H., Lu, M. Y., Bhaila, I., & Hamill, R. J. (2004). *In vitro* killing of community-associated methicillin-resistant *Staphylococcus aureus* with drug combinations. *Antimicrobial agents and chemotherapy, 48(10),* 4016-4019. https://doi.org/10.1128/AAC.48.10.4016-4019.2004

Silva, A. C., Paulo, M. C. da S., Silva, M. J. O., Machado, R. S., Rocha, G. M. de M., & Oliveira, G. A. L. de. (2020). Atividade antimicrobiana e toxicidade dos méis das abelhas sem ferrão Melipona rufiventris e Melipona fasciculata: uma revisão. *Research, Society and Development*, 9(8), e897986325. https://doi.org/10.33448/rsd-v9i8.6325

Silva, J. K., Trindade, R., Alves, N. S., Figueiredo, P. L., Maia, J. G. S., & Setzer, W. N. (2017). Essential oils from neotropical *Piper* species and their biological activities. *International Journal of Molecular Sciences, 18(12),* 2571. https://doi.org/10.3390/ijms18122571

Silva, I. F. Jr., Oliveira, R. G., Soares, I. M., Costa Alvim, T., Ascêncio, S. D., & Oliveira Martins, D. T. (2014). Evaluation of acute toxicity, antibacterial activity, and mode of action of the hydroethanolic extract of *Piper umbellatum* L. *Journal of ethnopharmacology, 151(1),* 137-143. https://doi.org/10.1016/j.jep.2013.10.011

World Health Organization (WHO). (2020). *Antimicrobial resistance.* Retrieved from: http://www.who.int/mediacentre/factsheets/fs194/en/

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