Research, Society and Development, v. 10, n. 6, e53610616043, 2021
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v10i6.16043

Antimicrobial screening of extracts and fractions from *Piper* species using bioautografy method

Investigação da atividade antimicrobiana de extratos e frações de espécies de *Piper* usando bioautografia

Detección de la actividad antimicrobiana de extractos y fracciones de especies de *Piper* mediante bioautografía

Received: 05/13/2021 | Reviewed: 05/19/2021 | Accept: 05/24/2021 | Published: 06/09/2021

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Abstract
The development of new drugs used to combat and control microorganism's remains significant in the global scientific context, with natural products, including plant products, being potential sources of new antibacterial and antifungal agents, thus the purpose of this work was to determine the antimicrobial activity of Piper species by bioautography. Antimicrobial activity was performed using the bioautography method, where plates were previously prepared using the thin layer chromatography technique by eluting the crude ethanolic extract (CEE), hexane fraction (HEX) and/or chloroform fraction ($CHCl_3$) of *P. caldense*, *P. arboreum* and *P. mollicomum*, in order to separate the components. The chromatography plates were tested against four standard strains *American Type Culture Collection: Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 76645, the activity being determined by the formation of zones of inhibition in Agar. It was possible to verify that both the CEE and the HEX fraction of *Piper caldense* showed activity against the *S. aureus* strain, presenting the place of activity of the chromatoplate, these products did not show activity in relation to the other strains studied. The extracts and fractions of *Piper arboreum* and *Piper mollicomum* showed no activity in relation to the studied strains. From the results it is possible to conclude that *Piper caldense* has potential antibacterial activity against *S. aureus*. The importance of future studies to identify the active substances and their content in each part of the plant is emphasized.
Keywords: Bioautography; Piperaceae; *Piper caldense; Staphylococcus aureus.*

Resumo
A necessidade do desenvolvimento de novos fármacos utilizados no combate e/ou controle dos micro-organismos permanece significativa no contexto científico mundial, sendo os produtos naturais, dentre eles os produtos vegetais, potenciais fontes de novos agentes antibacterianos e antifúngicos. Desse modo, o objetivo deste trabalho foi

determinar a atividade antimicrobiana de espécies de *Piper* por bioautografia. Atividade antimicrobiana foi realizada pelo método da bioautografia, onde foram preparadas, previamente, cromatogramas pela técnica de cromatografia em camada delgada (CCD) pela eluição do extrato etanólico bruto (EEB), fração hexânica (HEX) e/ou fração clorofórmica ($CHCl_3$) de *P. caldense*, *P. arboreum* e *P. mollicomum*, a fim de separar os metabólitos. As placas de CCD foram testadas diante de quatro cepas padrão *American Type Culture Collection*: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 e *Candida albicans* ATCC 76645, sendo a atividade determinada pela formação de zonas de inibição em Agar. Foi possível verificar que tanto o EEB como a fração HEX de *Piper caldense* foram ativos frente à cepa de *S. aureus*, as zonas de inibição foram observadas na cromatoplaca. No entanto estes produtos não apresentaram atividade diante das demais cepas estudadas. Os extratos e frações de *Piper arboreum* e *Piper mollicomum* não apresentaram atividade frente as cepas testadas. A partir dos resultados é possível concluir que *Piper caldense* possui potencial atividade antibacteriana frente a *S. aureus*. Ressalta-se a importância para que estudos futuros sejam realizados para identificação das substâncias ativas bem como seu teor em cada parte da planta.
Palavras-chave: Bioautografia; Piperaceae; *Piper caldense; Staphylococcus aureus.*

Resumen
La necesidad de desarrollar nuevos fármacos utilizados para combatir y controlar microorganismos sigue siendo significativa en el contexto científico mundial, siendo los productos naturales, incluidos los vegetales, fuentes potenciales de nuevos agentes antibacterianos y antifúngicos, de ahí el propósito de este El trabajo consistió en determinar la actividad antimicrobiana de las especies de Piper mediante bioautografía. La actividad antimicrobiana se realizó mediante el método de bioautografía, donde las placas se prepararon previamente mediante la técnica de cromatografía en capa fina eluyendo el extracto etanólico crudo (EEB), fracción de hexano (HEX) y/o fracción de cloroformo ($CHCl_3$) de *P. caldense*, *P. arboreum* y *P. mollicomum*, con el fin de separar los componentes. Las placas de cromatografía se probaron frente a cuatro cepas estándar *American Type Culture Collection: Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 y *Candida albicans* ATCC 76645, estando determinada la actividad por la formación de zonas de inhibición en Agar. Se pudo comprobar que tanto la BSE como la fracción HEX de *Piper caldense* mostraron actividad contra la cepa *S. aureus*, presentando el lugar de actividad de la cromatoplaca, estos productos no mostraron actividad en relación a las otras cepas estudiadas. Los extractos y fracciones de *Piper arboreum* y Piper *mollicomum* no mostraron actividad en relación con las cepas estudiadas. De los resultados es posible concluir que *Piper caldense* tiene una actividad antibacteriana potencial contra *S. aureus*. Se destaca la importancia de futuros estudios para identificar las sustancias activas y su contenido en cada parte de la planta.
Palabras clave: Bioautografía; Piperaceae; *Piper caldense; Staphylococcus aureus.*

1. Introduction

Piperaceae is a bothanical family formed by five native genera: *Piper*, *Peperomia*, *Pothomorphe*, *Ottoniae* and *Sarcorhachis*, which encompass around 2000 identified species, where in Brazil alone, this family is represented by approximately 460 species (Santos, Moreira, Guimarães & Kaplan, 2001; Rosa & Souza, 2004). The species of this family are generally represented by climbing or erect herbs, shrubs and less often trees, presenting a high commercial, economic and medicinal value (Dominguez & Alcorn, 1985).

Some species are part of the world market because of their economic and medicinal importance, such as black pepper (*Piper nigrum* L.) and others are used empirically by populations in different diseases (Guimarães & Monteiro, 2006). Piperaceae species have been used in food, as insecticides and also in traditional medicine due to the accumulation of different classes of biologically active metabolites (Baldoqui, Bolzani, Furlan, Kato & Marques, 2009).

Therefore, secondary metabolites play a role of significant importance in plant species, because they are being responsible for the aroma, odor and pigments of plants, they serve as defense mechanisms against microorganisms, insects and herbivores. Unlike primary metabolites, they have restricted distribution, they are found in relatively low concentrations in plants and microorganisms, have a complex structure, low molecular weight and are characterized by enormous chemical diversity and remarkable biological activities (Aresi, 2011).

Thus, the search for new substances with antimicrobial activity is evident over the years. According to a report by the World Health Organization, there is an urgent need to research and develop molecules with such activity, especially against

resistant microorganisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and species carbapenema resistants from Enterobacteriaceae family (WHO, 2019). Therefore, different techniques are used to determine the antimicrobial activity *in vitro* of natural or synthetic products, like broth microdilution and Agar diffusion techniques being the most used (CLSI, 2020).

New alternatives of techniques that determine antimicrobial activity of these products are being inserted in research and development of molecules, such as bioautography. This method is efficient in determining the antimicrobial activity, which highlights the high sensitivity of the technique for identifying the fractions/molecules responsible for the antimicrobial action, for this reason it has been widely disseminated (Müller, 2006).

Previous studies have shown the isolation of secondary metabolites in different *Piper* species, such as extracts of *Piper montealegreanum* that showed activity against *Staphylococcus aureus*, *Bacillus subtillis*, *Pseudomonas aeruginosa* and *Escherichia coli*, furthermore isolated molecules of *Piper glandulosissimum* that showed antifungal activity against *Trichophyton mentagrophytes* and *Microsporum canis*, in addition to presenting antibacterial activity against *Staphylococcus aureus* and *S. epidermidis* (Pinto et al., 2012; Cordova, Benfatti, Magina, Guedes & Cordova, 2010). Studies also reporting antibacterial and antifungal activity of products obtained from P. *caldense*, *P. mollicomum* and *P. arboreum* against *S. aureus* and *Candida albicans*, *C. glabrata* and *C. tropicalis* (Rocha, Nunes, Fernandes, Catão & Alves, 2020; Alves et al., 2016). Rocha et al. (2018) report that although the extractive products of *P. montealegreanum* have no activity against *E. coli* ATCC 25922, these extracts when evaluated in association with antimicrobials such as quinolones, tetracyclines and betalactams were able to increase diameter of the halos of inhibition of these substances when compared to the values of antibiotics alone, indicating a possible *in vitro* synergism between the products and antimicrobials agents.

Given the knowledge of the antimicrobial activity of *Piper* species, this work aimed to evaluate the antimicrobial activity of crude ethanolic extracts (CEE), hexane (HEX) and chloroform ($CHCl_3$) fractions of *Piper arboreum*, *Piper caldense* and *Piper mollicomum* by bioautography method.

2. Methodology

2.1 Research design

This original article presents data obtained from an experimental study carried out at the Antimicrobial Research Laboratory of the State University of Paraíba (UEPB), Campus I, Campina Grande, Paraíba, Brazil. The data correspond to a qualitative study with data on antimicrobial activity (Pereira, Shitsuka, Parreira & Shitsuka, 2018).

2.2 Plant material

Piper arboreum was collected in Bananeiras city (Paraíba State, Brazil, latitude 6° 45' 12" S, longitude 35° 37' 59" W) with voucher specimen deposited in the herbarium of Health and Education Center - UFCG under the number C.A Garcia 205. *Piper mollicomum* was collected in Areia city (Paraíba State, Brazil; latitude 6° 58' 20" S, longitude 35° 42' 25" W), and the respective exsicata deposited in the herbarium of the Federal University of Paraíba under number EAN- 16120, samples of the *Piper caldense* species were collected in Santa Rita (Paraíba State, Brazil; latitude 7° 08' 38" S, longitude 35° 00' 42" W) with an voucher specimen deposited at the Herbário Prof. Lauro Pires Xavier - UFPB under number 20,311.

The plant materials were identified by Dra. Elsie Guimarães of the Department of Botany at the Federal University of Rio de Janeiro and sent later to the Laboratory of Pharmaceutical Technology Prof. Delby Fernandes de Medeiros of the Federal University of Paraíba - LTF/UFPB, where the steps involving the ethnobotanical study, collection and processing of plant material, obtaining the crude ethanolic extract and fractioning it were carried out.

2.3 Tested products

The CEE and hexane fraction (HEX) of the *Piper arboreum* leaves and the *Piper caldense* stem , as well the CEE and chloroformic fraction ($CHCl_3$) of the *Piper mollicomum* leaves were used. The extracts were obtained according to the methodology described by Alves et al. (2016). Standard solutions (Table 1) were prepared previously, after obtaining the products the total yield was solubilized in 1.0 mL of the solvent used for extraction and/or partition, and subsequently submitted to an ultrasound bath for 15 minutes at room temperature. Finally, the solutions were sterilized in a 0.22 µm Millipore membrane.

Table 1. Standard solutions for *Piper arboreum, Piper caldense* e *Piper mollicomum.*

***Piper* species**	**Product**	**Standard solution (mg mL^{-1})**
Piper arboreum (leaves)	CEE	400
	HEX	100
Piper caldense (branches)	CEE	400
	HEX	100
Piper mollicomum (leaves)	CEE	200
	$CHCl_3$	400

Legenda: CEE – Crude ethanolic extract; HEX – Hexanic fraction; $CHCl_3$ – Cloroformic fraction. Source: Elaborated by the authors (2021).

2.4 Preparation of the plates by thin-layer chromatography (TLC)

To perform the thin-layer chromatographic test (TLC) previously prepared solutions of the products were applied with the aid of a capillary on the silica gel chromatoplate GF254. Chromatography was performed using the methylene chloride elution system for *Piper arboreum* and *Piper caldense* as mobile phase, and the 2 % ethyl acetate system in methylene chloride for *Piper mollicomum*. After the elution of the solvents, the chromatographic plates were stored in an oven at 37 °C for 24 hours, until the total volatility of the mobile phase. The plates were prepared with the objective of separating by chromatography the components of the products of *Piper* species selected for this study.

2.5 Antimicrobial activity

2.5.1 Microorganisms, Culture Media and standardization of inoculations

Standard strains obtained by American Type Culture Collection (ATCC) of *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* ATCC 76615 were used to determine antimicrobial activity. The selected strains were stored and maintained in appropriate culture media according to the supplier's recommendations. Muller-Hinton agar medium was used for bacterial strains cultivation and for the yeast cultivation the Sabouraud agar medium was used.

The strains were enriched in Brain Heart Infusion (BHI) broth in order to standardize the inoculum. Aliquots of each growth was sown using the streak depletion technique in appropriate culture media and incubated at 37 ºC for 24 hours, thus allowing the microorganisms were growing exponentially. After the incubation period, some colonies were diluted in sterile saline solution (NaCl 0.85 %) until reached the turbidity corresponding to 0.5 McFarland scale, a concentration equivalent to $1.5x10^8$ Colony Forming Units/mL (CFU/mL) (CLSI, 2018).

2.5.2 Assay of Antimicrobial Activity by Direct Bioautography

To determine the antimicrobial activity by bioautography on the surface of chromatoplates, previously prepared and placed in sterile Petri dishes, 20 ml of the culture medium (Muller-Hinton agar or Sabouraud agar) were aseptically poured at

40 °C, containing 1 mL of microorganism suspension (1.8 x 10^6 CFU/mL), so that the entire surface of the chromatoplate was covered with the inoculum. The procedure was carried out for all microorganisms.

The plates containing bacteria were incubated in an oven at 37 °C for 24 hours and the plates containing fungi were incubated at 25 °C for 48 hours, after solidification of médium. The tests were performed in duplicate. Antimicrobial activity was evaluated by the presence of inhibition zones that indicate the existence of active compounds. Thus, after the incubation period, the chromatoplates were developed with the addition of a solution of 2,3,5-triphenyltetrazolium (TTC) (2.5 mg/mL) to highlight the growth inhibition zones. It was possible to verify the location of the chromatographic plaque that showed antimicrobial activity and consequently, the region that contains compounds with antimicrobial activity (Dias, et al., 2006).

3. Results and Discussion

The results obtained from antimicrobial activity of *Piper* species by bioautography are shown in Table 2.

The bioautography of *Piper caldense* showed activity against the strain of *S. aureus* ATCC 25923, represented by the formation of a zone of growth inhibition by treatment with CEE (Figure 1A) and with the HEX fraction (Figure 1B), indicating that the elution of the extracts on the chromatoplate allowed the separation of metabolites with antibacterial activity.

Table 2. Evaluation of antimicrobial activity by bioautography of Piper species

***Piper* species**	**Product**	***Staphylococcus aureus* ATCC 25923**	***Pseudomonas aeruginosa* ATCC 27853**	***Escherichia coli* ATCC 25922**	***Candida albicans* ATCC 76615**
Piper arboreum (leaves)	CEE	Not active	Not active	Not active	Not active
	HEX	Not active	Not active	Not active	Not active
Piper caldense (branches)	CEE	**Active**	Not active	Not active	Not active
	HEX	**Active**	Not active	Not active	Not active
Piper mollicomum (leaves)	CEE	Not active	Not active	Not active	Not active
	$CHCl_3$	Not active	Not active	Not active	Not active

Legend: CEE – Crude ethanolic extract; HEX – Hexanic fraction; $CHCl_3$ – Cloroformic fraction; Active – formation of zone of growth inhibition in the chromatoplate; Not active – no growth inhibition zone on the chromatoplate.
Source: Elaborated by the authors (2021).

However, these products showed no activity against the strains of *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922 and *C. albicans* ATCC 76615, indicated by the non-formation of zones of inhibition in all chromatoplate elution. This result allows the determination of the elution point of the CEE chromatoplate and the HEX fraction that has antibacterial activity, as it specifically indicates the presence of a component with such activity, facilitating its isolation.

Figure 1. Bioautography of *Piper caldense* with emphasis on zones of inhibition against *Staphylococcus aureus* ATCC 25923. (A) Crude ethanolic extract; (B) Hexane fraction.

Legend: Inhibition zones formed by the absence of red pigmentation indicate the formation of growth inhibition zones, revealed by the addition of a solution of 2,3,5-triphenyltetrazolium (TTC) (2.5 mg/mL) in regions of the chromatoplate. Source: Elaborated by the author (2021).

Previous studies have observed CEE and HEX fraction activity obtained from *Piper caldense* leaves in inhibiting the growth of *S. aureus* ATCC 25923 by the Agar diffusion method (Justen, 2007; Cordova et al., 2010; Alves et al., 2016), the tests elucidate that both leaves and the stem of *P. caldense* present substances that perform antimicrobial activity against the species. The CEE and the HEX fraction of *Piper arboreum*, as well as the CEE and the $CHCl_3$ fraction of *P. mollicomum* did not show activity against the strains of *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922 and *C. albicans* ATCC 76615 by the bioautography method, evidenced by the non-formation of growth inhibition zones in all chromatoplate elution.

Previous studies indicated that dichloromethane extracts and $CHCl_3$ fraction from *Piper arboreum* leaves were active against strains of clinical origin of sensitive and methicillin-resistant *S. aureus* - MRSA (Nascimento, Silva, Silva, Araujo & Ramos, 2012), as well as demonstrating the activity of the hexane fraction of this species against *S. aureus* ATCC 25923 (Alves et al., 2016). From these results it can be determined that the antibacterial activity of *Piper arboreum* is related to the activity together of the secondary metabolites found in the extracts, since after the separation of CEE components and HEX fraction in chromatoplate, no results were obtained for growth inhibition zone, as the results of this study indicated.

There are reports of antimicrobial activity of *Piper* species that show activity against *S. aureus*, but demonstrate that the same extracts do not show activity against Gram-negative bacteria (Alves et al., 2016; Rocha et al., 2020) and corroborate with the results found in this study, since the results indicate that the products eluted in chromatoplates did not show activity against *E. coli* and *P. aeruginosa.* Holley and Patel (2005) suggest that the dual membrane presented by Gram-negative bacteria forms a complex envelope, being responsible for the lower sensitivity of these microorganisms to plant extracts.

The extracts and fractions of *Piper arboreum*, *Piper caldense* and *Piper mollicomum* against *C. albicans* strain at bioautographic method did not show antifungal activity. However, a previous study reported antifungal activity of *P. caldense* and *P. arboreum* hexane phases against *C. albicans*, *C. glabrata* and *C. tropicalis* (Alves et al., 2016; Justen, 2007) using disk-diffusion on agar, indicating that hexane fraction has antifungal activity against these species when the extracted components

are together in the treatment preparation, since in this study this activity was not detected with the components separated by thin layer chromatography.

The extracts and fractions that did not show activity in this study cannot have their antimicrobial activity discarded, since the synergism between the substances of plant metabolism can be a determining factor for antimicrobial activity. Thus, the lack of standardized methods for determining the antimicrobial activity of plant derivatives is an obstacle to the direct comparison of results between studies.

The elucidation of antimicrobial activity by bioautographic methods is an analytical field that is expanding due to its practical and agile utility for researching antibiotic substances. Valuable technique, bioautography can quickly and accurately determine the component of a plant derivative responsible for antibiotic activity against a microorganism (Horváth et al., 2010).

In addition, it is important to understand that the bioautographic technique is not a quantitative measure for determining antimicrobial activity, since an indication of the number of compounds that have been separated and capable of inhibiting microbial growth is observed (Shakeri et al., 2018). In addition to the need for further investigation into the complex antimicrobial effects that these compounds present.

4. Conclusion

The crude ethanolic extract and the hexane fraction of the leaves of *Piper caldense* showed antibacterial activity by the bioautography technique against *Staphylococcus aureus*, confirmed by the identification of the growth inhibition zone of this bacterial species caused by the isolated component by means of the chromatographic method. Future studies should be conducted in order to isolate and identify the compound in the leaves of *P. caldense* that has antibacterial activity. The crude ethanolic extract and the hexanic and chloroform fraction of *P. mollicomum* and *P. arboreum* did not show antimicrobial activity of the components isolated by bioautography, through separation by thin layer chromatography.

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