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Evaluation of the antimicrobial and resistance-modulating activities of extracts and fractions of *Piper mollicomum* (Piperaceae)

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Resumen

Evaluación de las actividades antimicrobianas y moduladoras de resistencia de extractos y fracciones de Piper mollicomum *(Piperaceae)*

Este estudio tuvo como objetivo evaluar las actividades antimicrobianas de diferentes extractos de *Piper mollicomum* Kunth ex Steud. contra cepas estándar y resistentes a múltiples fármacos de bacterias y hongos, así como analizar su potencial para modular la resistencia a los antimicrobianos. Los datos obtenidos en este trabajo demostraron una actividad potenciadora de antibióticos prometedora por parte de *P. mollicomum*, alentando el desarrollo de nuevas investigaciones para caracterizar las propiedades toxicológicas y farmacológicas de los extractos y compuestos aislados de esta especie, que pueden contribuir al desarrollo de nuevos agentes terapéuticos para combatir la resistencia a los antimicrobianos.

Palabras clave: Actividad antimicrobiana; Resistencia antimicrobiana; *Piper mollicomum*.

Abstract

This study aimed to evaluate the antimicrobial activities of different extracts of *Piper mollicomum* Kunth ex Steud. against standard and multidrug resistant strains of bacteria and fungi, as well as to analyze their potential to modulate antimicrobial resistance. The data obtained in this work demonstrated promising antibiotic-enhancing activity by *P. mollicomum*, encouraging the development of further research to characterize the toxicological and pharmacological properties of the extracts and isolated compounds of this species, which may contribute to the development of new therapeutic agents to combat antibacterial resistance.

Key words: Antimicrobial activity; Antimicrobial resistance; *Piper mollicomum*.

Introduction

In a worldwide context, the sustainable medicinal exploration of plant species has represented a promising alternative in the search for new bioactive compounds (Mendes *et al.* 2011). In particular, the search for new antimicrobial agents has been increasingly urgent as the emergence of resistant microorganisms has been directly correlated with increased mortality rates for infectious diseases. Therefore, antimicrobial resistance is currently a major public health problem worldwide (Pena *et al.* 2001, Duarte 2006, Coutinho *et al.* 2008).

In this context, bioprospecting for natural products capable of combating infections caused by resistant bacteria has had a significant impact on antimicrobial drug development. Accordingly, consistent evidence has demonstrated that natural products have immense potential to combat bacterial resistance because, due to their chemical complexity, extracts, fractions and some isolated compounds can impair the development of resistance mechanisms (Daferera *et al.* 2003, Coutinho *et al.* 2008).

The family Piperaceae (order Piperales) constitutes one of the most primitive families of angiosperms. Studies have demonstrated that the species in this family have a peculiar metabolism leading to the generation of several secondary metabolites with remarkable biological activities, and as such, have many medicinal applications (Wanke *et al.* 2007). The genus *Piper* L. is the largest of this family with more than 1000 species, distributed especially in tropical and subtropical regions of Asia and America (Nunes *et al.* 2007), among which *Piper mollicomum* Kunth ex Steud. (popularly known as "Pariparoba", "Jaguarandi", "Jaborandi" and "Jaborandi-Manso") has notable pharmacological potential (Magevski, 2012). Accordingly, studies have demonstrated this species has several biological activities, including antimicrobial (Barbosa *et al.* 1999, Duarte *et al.* 2007, Da Silva Alves *et al.* 2016), larvicidal (Gonche *et al.* 2005) and antifungal (Lago *et al.* 2007).

Therefore, this study aimed to evaluate the antimicrobial activities of different extracts and fractions of *P. mollicomum* against standard and multidrug resistant strains of bacterial and fungi, as well as to analyze their potential to mo-dulate antimicrobial resistance.

Materials and methods

Plant material

The leaves of *P. mollicomum* were collected in the forest garden of the Federal University of Paraíba (Areia, PB, Brazil), and identified by Prof. Leonardo Félix. After identification, a voucher specimen was registered in the herbarium of the same University (registry code EAN 16120).

Preparation of extracts and fractions from the leaves of *Piper mollicomum*

The leaves of *P. mollicomum* were dehydrated in a greenhouse at 40 °C for 72 h and then, mechanically crushed. The resulting powder was subjected to 5 cycles of extraction by maceration with ethanol for three days each. The solution obtained was concentrated by evaporation, resulting in the crude ethanolic extract. An aliquot of 190 g of the crude ethanolic extract was solubilized in MeOH: H2O (8:2), and subjected to liquid-liquid partition using chloroform ($CHCl_3$) and ethyl acetate (AcOEt) separately. The solutions obtained after this process were treated with anhydrous sodium sulfate (Na_2SO_4) and subjected to filtration. After these procedures, the solvents were evaporated in a rotary evaporator under reduced pressure (temperature $\leq$ 50 ºC), resulting two corresponding phases: Chloroform (76.11 g) and ethyl acetate (4.22 g).

Preparation of solutions from the extract and fractions of *Piper mollicomum*

The solutions used in the tests were prepared as follows: 200 mg of the extract and fractions were dissolved in 1 mL of dimethyl sulfoxide (DMSO), resulting in solutions with an initial concentration of 200 mg/mL each. These solutions were diluted (1:20) in sterile distilled water to 10 mg/mL, and prior to the tests, diluted again in sterile water to a concentration of 1024 μg/mL.

Microorganisms

The following microorganisms were throughout this study: standard fungal strains of *Candida albicans* (C.P. Robin) Berkhout (ATCC 40006), *Candida tropicalis* (Castellani) Berkhout (ATCC 40042) and *Candida krusei* (Castellani) Berkhout (ATCC 40147); standard bacterial strains of *Staphylococcus aureus* Rosenbach (ATCC 25923), *Escherichia coli* (Migula) Castellani and

Chalmers (ATCC 10536), *Pseudomonas aeruginosa* (Schröter) Migula (ATCC 15442), and multiresistant strains of *S. aureus* (SA 358), *E. coli* (EC 27) and *P. aeruginosa* (P 03). All strains were provided by the Laboratory of Mycology of the Federal University of Paraiba (UFPB) and maintained in Heart Infusion Agar (HIA, Difco Laboratories Ltda.). Prior to the assays, the microorganisms were cultivated for 24h at 37 °C in brain and heart infusion broth (BHI, Difco Laboratories LTDA).

Drugs

The following antimicrobial drugs were used in the tests: kanamycin, amikacin, neomycin, gentamicin, amphotericin B, benzoylmethronidazole, mebendazole and nystatin. All of them were purchased from Sigma Chemical Co. (St. Louis, USA).

Determination of Minimim Inhibitory Concentration (MIC) and analysis of antimicrobial resistance modulation

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method. Briefly, an inoculum of 100 µL of each lineage was diluted in 10% Brain Heart Infusion (BHI) broth to a final concentration of 10^5 CFU/mL and transferred to the wells of 96-well microdilution plates. Each well was added with 100 µL of the solution of the extract or fractions, serially diluted to reach concentrations ranging from 512 to 8 µg/mL. The MIC of each extract was defined as the lowest concentrations capable of inhibiting microbial growth. Alternatively, in the antifungal activity analysis, the treatments were added at concentrations ranging from 1024 to 2 µg/mL. To analyze the effects of *P. mollicomum* on the modulation of antimicrobial resistance, the MICs of conventional drugs (section 2.5) were determined in the presence or absence of subinhibitory concentrations (MIC÷8) of the ethanolic extract (EEPM), chloroform fraction (FCPM) or ethyl acetate fraction (FAEPM) of *P. mollicomum*. After treatments, the plates were incubated for 24 h at 37 °C and the readings were performed as previously described (Javadpour *et al.*, 1996). All tests were performed in duplicate and data were expressed as a mean of the replicates.

Results and Discussion

The ethanolic extract, as well as the chloroform and ethyl acetate fractions of *P. mollicomum* presented MIC values above ≥1024 µg/mL against all bacterial and fungal strains evaluated. Similar findings were obtained in the study carried out by Souto (2014), which evaluated the effect of the chloroform fraction and the crude ethanolic extract of P. mollicomum, using the Bioautography method. The authors demonstrated that none of these treatments exhibited clinically effective antimicrobial activity against the strains S. aureus ATCC 25923, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922 and C. albicans ATCC 76615.

On the other hand, studies using crude extracts of other species belonging to the same genus, including *Piper caldense* C. DC., *Piper cernuum* Vell. and *Piper lindbergii* C. DC., found significant antibacterial activity against Gram-positive

Bacteria	Origin	Resistance profile
***Escherichia coli* 27**	Surgical wound	Ast, Ax, Amp, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Clo, Im, Can, Szt, Tet, Tob
***Escherichia coli* ATCC10536**	—	—
***Staphylococcus aureus* 358**	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net
***Staphylococcus aureus* ATCC25923**	—	—
***Pseudomonas aeruginosa* ATCC 15442**	—	—
***Pseudomona aeruginosa* 03**	—	Cpm,Ctz,Imi,Cip,Ptz,Lev, Mer,Ami

Tabla 1. Origen de las cepas bacterianas. Ast: Aztreonan; Ax: Amoxacilina; AMP-ampicilina; Ami: Amikacina; Amox: Amoxilina; Ca: Cefadroxilo; CFC: Cefaclor; Cf: cefaloxina; Caz: Ceftazidima; Cip: Ciprofloxacina; CLO: cloranfenicol; Im: Imipenem; Can: Kanamicina; Szt: Sulfametrim; Tet: tetraciclina; Tob: tobramicina; Oxa: Oxacilina; Gen: gentamicina; Neo: Neomicina; Para: paramomicina; Pero: butirosina; Sis: Sisomicina; Net: Netilmycin; CPM: Cfepime; CTZ: Ceftazidima; Ptz: Piperacilina-Tazobactam; Lev: levofloxacina; Mer: Meropenem; (—) Sin resistencia o resistencia sin relevancia.

Table 1. Origin of bacterial strains. Ast: Aztreonan; Ax: Amoxacillin; AMP-ampicillin; Ami: Amikacin; Amox: Amoxilina; Ca: Cefadroxil; CFC: Cefaclor; Cf: Cephaloxin; Caz: Ceftazidima; Cip: Ciprofloxacin; CLO: chloramphenicol; Im: Imipenem; Can: Kanamycin; Szt: Sulfametrim; Tet: tetracycline; Tob: tobramycin; Oxa: Oxacillin; Gen: gentamicin; Neo: Neomycin; For: Paramomycin; But: Butyrosine; Sis: Sisomycin; Net: Netilmycin; CPM: Cfepime; CTZ: Ceftazidime; Ptz: Piperacillin-Tazobactam; Lev: levofloxacin; Mer: Meropenem; (—) No resistance or resistance without relevance.

bacteria (Cordova *et al.* 2010). Moreover, previous research has demonstrated that the leaves of *P. mollicomum* is rich essential oils, which can potentially concentrate the constituents with antibacterial action. This phenomenon could justify the absence of significant antibacterial activity demonstrated by the extract and fractions evaluated by the present study (Guimarães & Valente 2001, Bardelli *et al.* 2008).

The analysis of the antibiotic-enhancing activity demonstrated that *P. mollicomum* has the potential to modulate the activity of antibiotics against resistant bacteria. As shown in table 2, the combination of amikacin the ethyl acetate fraction against *E. coli* reduced the MIC of the antibiotic from 312.5 to 78.12 µg/mL, indicating synergism. Synergistic effects were also obtained with the combination of kanamycin with the ethanolic extract and the chloroform fraction, as well as when the ethanolic extract was associated with neomycin against the same strain. In all these combinations, the MIC of the antibiotic was significantly reduced in the presence of the natural product, indicating enhanced antibiotic activity.

With regard to the tests with *S. aureus*, synergistic interactions were obtained from the combination of amikacin with both the chloroform and ethyl acetate fractions, as well as from the combination between neomycin and the chloroform fraction. However, the combination of neomycin with the ethyl acetate fraction caused an antagonistic effect, as observed through an increase in the MIC of the antibiotic (Table 2).

The antibiotic-enhancing effect exhibited by both the extract and fractions of *P. mollicomum* evaluated by this study may have resulted from the action of secondary metabolites such as flavonoids and chalcones, which have been previously identified as antimicrobial compounds in this species (Santos *et al.* 2015 Almeida *et al.*, 2019).

Finally, the extract and fractions obtained from *P. mollicomum* showed no significant antibiotic-enhancing effect against *P. aeruginosa* (Table 2), as well as did not affect the activity of antifungal agents against *Candida* strains (all cases presented MIC values above ≥1024 µg/mL against all bacterial and fungal strains evaluated.).

Despite the lack of significant antifungal activity demonstrated in the present research, previous studies have identified the antifungal potential of *Piper* species. In this context, the essential oil of *Piper hispidonervum* C.DC. was found to inhibit the growth of phytopathogenic fungi. In addition, different extracts of *Piper arboreum* Aubl. presented antifungal activity against *C. krusei*, *Candida parapsilosis* Langeron & Talice and *Cryptococcus neoformans* (San Felice) Vuill. strains (Zacaroni 2009, Regasini 2009, Da Silva Alves *et al.* 2016).

	EC 27				SA 358				PA03			
	MIC	MIC in the presence of			MIC	MIC in the presence of			MIC	MIC in the presence of		
Antibiotics		**EEPM**	**FCPM**	**FAEPM**		**EEPM**	**FCPM**	**FAEPM**		**EEPM**	**FCPM**	**FAEPM**
Kanamycin	2500	312.5	312.5	1250	312.5	312.5	625	312.5	1250	625	625	625
Amikacin	312.5	156.25	156.25	78.12	312.5	312.5	78.12	78.12	1250	625	625	1250
Neomycin	1250	39.06	625	156.25	625	625	156.25	1250	625	312.5	312.5	312.5
Gentamicin	78.12	39.06	39.06	39.06	39.06	39.06	39.06	39.06	1250	625	1250	1250

Tabla 2. Valores de las MIC (µg/mL) de los antibióticos en ausencia y presencia del extracto y fracciones obtenidas de *Piper mollicomum* contra *E. coli* 27, *S. aureus* 358 y *P. aeruginosa* 03

Table 4. MIC values (µg/mL) of antibiotic in the presence or absence of the extract and fractions obtained from *Piper mollicomum* against *E. coli* 27, *S. aureus* 358 and *P. aeruginosa* 03.

Conclusion

The analysis of the antimicrobial properties of the ethanolic extract and chloroform and ethyl acetate fractions of *P. mollicomum* revealed that these products had no clinically relevant antibacterial and antifungal activities. In addition, they did not enhance the activity of conventional antifungal drugs against *Candida* strains. However, both the extract and fractions obtained from this plant exhibited significant antibiotic-enhancing effects against both Gram-negative and Gram-positive bacterial strains.

In conclusion, the data obtained in this study encourage the development of further research to characterize the toxicological and pharmacological properties of the extracts and isolated compounds of this species, which may contribute to the development of new therapeutic agents to combat antibacterial resistance.

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