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Short communication

Modulatory antimicrobial activity of *Piper arboreum* extracts

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Abstract – The side effects of certain antibiotics have been a recent dilemma in the medical arena. Due this fact, the necessity of natural product discovery could provide important indications against several pharmacological targets and combat many infectious agents. *Piper arboreum* Aub. (Piperaceae) has been used by Brazilian traditional communities against several illnesses including rheumatism, bronchitis, sexually transmitted diseases and complaints of the urinary tract. Medicinal plants are a source of several remedies used in clinical practice to combat microbial infections. In this study, ethanol extract and fractions of *Piper arboreum* leaves were used to assay antimicrobial and modulatory activity. The minimum inhibitory concentration (MIC) was determined using microdilution method of ethanol extract and fractions from the leaves of *P. arboreum* ranging between 8 and 1024 μg mL^{-1}. The capacity of these natural products to enhance the activity of antibiotic and antifungal drugs was also assayed. In these tests, natural products were combined with drugs. The natural products assayed did not demonstrate any clinically relevant antimicrobial activity (MIC ≥ 1024 μg mL^{-1}). However, the modulation of antibiotic activity assay observed a synergistic activity of natural products combined with antifungal (such as nystatin and amphotericin B) and antibiotic drugs (such as amikacin, gentamicin and kanamycin). According to these results, these natural products can be an interesting alternative not only to combat infectious diseases caused by bacteria or fungi, but also to combat enhanced resistance of microorganisms to antibiotic and antifungal drugs.

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Keywords: antimicrobial activity, synergism, *Piper arboretum, Staphylococcus aureus*, *Escherichia coli*, *Candida*

Abbreviations: ATCC – American type culture collection; **MIC** – minimum inhibitory concentration

Introduction

The genus *Piper* (Piperaceae) presents almost 1,000 species, distributed mainly in the tropical and subtropical regions of Asia and America. Plants from this genus are used for commercial and medicinal purposes (NUNES et al. 2007) and demonstrate several biological activities (RUIZ and ROQUE 2009). Antibacterial and trypanocidal activities have been reported for *Piper arboreum* Aub. and this plant is used by traditional communities in Brazil in the form of a decoct against rheumatism, bronchitis, and sexually transmitted diseases (AGRA et al. 2007, RAMOS and KATO 2009, REGASINI et al. 2009).

The World Health Organization (WHO 1998) defines a medicinal plant as any plant that contains any active compounds that can be used for therapeutic or chemical purposes. Substances produced by plants are their defence mechanism against insects, herbivores, and phytopathogenic microorganisms (GOTLIEB 1981). Some researchers have named these phytocompounds »antibiotic like-substances« (GEISMANN 1963). The use of plant extracts as antimicrobial agents represents a low risk for the development of resistance by the microorganisms because these products are composed of several phytocompounds of different groups (DAFERERA et al. 2003).

Concerning resistance to antibiotics, many researchers have expressed their opinion that natural products from plants could be interesting alternatives (DANCER 2001, NOSTRO et al. 2004, COUTINHO et al. 2005, GEORGOPAPADAKOU 2005). Several plant extracts and phytochemicals are known for their antimicrobial properties. Many studies have been conducted in different countries to demonstrate such activities (SALVAGNINI et al. 2008, SIMÕES et al. 2008, SILVA et al. 2008).

The *Staphylococcus* genus is found in skin and mucosal microbiota of animals and birds. Some *Staphylococcus* species are etiological agents of many animal and human infections (NOSTRO et al. 2004, COUTINHO et al. 2009a, b). *Staphylococcus aureus*, *S. epidermidis*, *S. saprophyticus* and *S. haemolyticus* are the most important etiological agents of community and nosocomial human infections. Besides, this bacterium is responsible for different types of intoxications, being the most common etiological agent of infections on different tissues and/or organs (e.g. furuncle, carbuncle, abscess, myocarditis, endocarditis, pneumonia, meningitis, bacterial arthritis) (VERHOEFF et al. 1999). *Escherichia coli* is one of the most important human infectious agents. This bacterium is an enterotoxin producer and the causal agents of diarrhoea outbreaks (KONOWALCHUK et al. 1977, SCOTLAND et al. 1980) and urinary tract infections (HUGHES et al. 1982).

Candidiasis is the most frequent mycosis caused by opportunistic fungi. The main species associated with this disease are *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *C. krusei* (COUTINHO 2009). These yeasts are found in skin and in mucosal microbiota, becoming pathogenic in patients with immunodeficiency or immunosuppression (DIGNANI et al. 2003).

Many plants have been evaluated not only for direct antimicrobial activity but also as resistance-modifying agents (GIBBONS 2004). Several chemical compounds, synthetic or from natural sources, have demonstrated a direct activity against many species of bacteria, enhancing the activity of a specific antibiotic, reversing the natural resistance of bacteria to a specific antibiotic, causing the elimination of plasmids and inhibiting the active efflux of antibiotics through the plasma membrane (COUTINHO et al. 2009a, b). The potentiation of antibiotic activity or the reversal of antibiotic resistance allows the classification of these compounds as modifiers of antibiotic activity (COUTINHO et al. 2009a).

The aim of this work was to evaluate the antimicrobial and modulatory activity of ethanol extract and fractions of *Piper arboreum* associated with antibiotic and antifungal drugs against multiresistant bacteria and fungi.

Materials and methods

Microbial strains

The bacteria used in the minimum inhibitory concentration assay were the strains of *E. coli* ATCC25923 and *S. aureus* ATCC10536. In the antifungal assays, the strains of *Candida albicans* ATCC40006, *C. krusei* ATCC2538 and *C. tropicalis* ATCC40042 were used. To evaluate the modulatory activity of the ethanol extract and fractions the multi-resistant bacterial strains isolated from clinical environments: *E. coli* 27 and *S. aureus* 358 with the resistance profile were used (Tab. 1). All strains were obtained from the Laboratory of Clinical Mycology – Universida de Federal da Paraíba.

Tab. 1. Bacterial source and antibiotic resistance profile.

Bacteria	Source	Antibiotic resistance
Escherichia coli 27	Surgical wound	Ast, Ax, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Clo, Im, Can, Szt, Tet, Tob
Escherichia coli ATCC10536	–	
Staphylococcus aureus ATCC25923	–	
Staphylococcus aureus 358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net

Ami – amicilina; Amox – amoxillin, Amp – ampicillin; Ast – aztreonam; Ax – amoxacillin; But – butirosina; Ca – cefadroxil; Can – canamycin; Caz – ceftazinidima; Cfc – cefaclor; Cf – cephalothin; Cip – ciprofloxacin; Clo – chloramphenicol; Com – cefepime; Ctz – ceftazidime; Gen – gentamicin; Imi – imipenem; Lev – levofloxacin; Mer – merpenem; Neo – neomycin; Net – netilmicin; Oxa – oxacillin; Sis – sisomicin; Szt – sulfametrim; Tet – tetracycline; Tob – tobramycin; Para – paramomicina; Ptz – piperacilina-tazobactam

Plant material

Stems of *Piper arboreum* were collected in March/2010 in the Gaiambira reserve, Bananeiras County, Paraíba State (Brazil). The plant material was identified by Prof. Carlos Alberto Garcia and a voucher (C. A. Garcia 205) was deposited in the Education and Health Center Herbarium, Universida de Federal de Campina Grande. The leaves of *Piper arbo-*

reum, after collection, were dried at 40 °C by 72 hours and powdered, obtaining 1.5 kg. The powdered material was extracted by maceration using 1 L of 95% (v/v) methanol as solvent at room temperature by three days, this process being repeated five times. The solution was then filtered and concentrated under vacuum in a rotary evaporator under 60 °C and 760 mm/Hg of temperature and pressure, respectively. This amount of powdered material yielded 350 g of ethanol extract.

The ethanol extract from the leaves (50 g) of *Piper arboreum* was dissolved in a mixture of EtOH:H_2O (7:3), generating a hydroalcohol solution which was submitted to a liquid/liquid partition using the following solvents: hexane, dichloromethane and ethyl acetate. The obtained solutions were mixed with sodium sulphate anhydrous (Na_2SO_4) and filtered. After this process, the solutions were concentrated using a rotary vacuum evaporator at temperatures below 50 °C, yielding the following fractions: hexane fraction (3 g), dichloromethane fraction (3.5 g) and ethyl acetate fraction (9 g).

Drugs

The drugs used in the tests were the aminoglycosides kanamycin, amikacin, neomycin and gentamicin, and the antifungals mebendazole, amphotericin B, nystatin and benzoylmetronidazole (Sigma Co., St. Louis, USA). All drugs were diluted in sterile water.

Minimum inhibitory concentration

The broth microdilution method was used in order to determine minimum inhibitory concentration (MIC). The ethanol extract and fractions were dissolved using dimethylsulfoxide and diluted to 1024 μg mL^{-1} using sterile distilled water. The bacterial inoculum was diluted using brain heart infusion broth to a final concentration of 10^5 colony forming units per mL. Each inoculum (100 μL) was distributed in each well of a microtiter 96 wells plate and then submitted to a twofold serial dilution using 100 μL of the ethanol extract, with concentrations ranging between 8 and 1024 μg mL^{-1}. The plates were incubated for 24 hours at 35 °C (JAVADPOUR et al. 1996). Bacterial MIC was determined using resazurin, with blue changing to red indicating bacterial growth. The fungal MIC was determined by turbidity, observing the growth of fungal strains. The MIC was defined as the lowest concentration in which no growth can be observed, according to CLSI (2008). Positive controls of growth were performed using the culture medium with the microbial inoculum.

Drug modulation test

To observe how the ethanol extract and fractions affect the action of antimicrobial drugs against the assayed strains, the method proposed by COUTINHO et al. (2008) was used. The ethanol extracts and fractions were tested using a sub-inhibitory concentration (MIC/8 = 128 μg mL^{-1}). A sample with 100 μL of the solution containing brain heart infusion broth, the microbial inoculums and the extract or fractions was placed in each well. After this, 100 μL of the antimicrobial drug was mixed in the first well, following the twofold dilution. Concentrations of aminoglycosides and antifungals ranged between 5000 and 2.44 μg mL^{-1} and 1024 and 2 μg mL^{-1}, respectively. The assays were performed in triplicate.

Results and discussion

The MIC results of the extract and fractions alone and combined with antifungals are demonstrated in the table 2. Against all fungal strains assayed, the MIC observed was ≥ 1024 μg mL^{-1}, these results not being clinically relevant. The same results were observed by REGASINI et al. (2009). However, when the antifungal drugs were associated with the extract and fractions, a synergistic activity was observed against *C. krusei*, enhancing the antifungal drug activity. The MIC results of the natural products alone and combined with amino-

Tab. 2. Minimum inhibitory concentration (MIC) of antifungal drugs alone and associated with extract and fractions of *Piper arboreum* against *C. albicans*, *C. krusei* and *C. tropicalis* (μg mL^{-1}).

DFPA	***C. albicans***		***C. krusei***		***C. tropicalis***	
Natural product/antifungal	**Alone**	**+ DFPA**	**Alone**	**+ DFPA**	**Alone**	**+ DFPA**
DFPA	≥ 1024	–	≥ 1024	–	≥ 1024	–
Amphotericin b	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024
Mebendazole	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024
Nystatin	≥ 1024	≥ 1024	64	≥ 1024	≥ 1024	≥ 1024
Benzoilmetronidazole	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024
EEPA						
Natural product/antifungal	**Alone**	**+ EEPA**	**Alone**	**+ EEPA**	**Alone**	**+ EEPA**
EEPA	≥ 1024	–	≥ 1024	–	≥ 1024	–
Amphotericin b	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024
Mebendazole	≥ 1024	≥ 1024	≥ 1024	64	≥ 1024	≥ 1024
Nystatin	≥ 1024	≥ 1024	64	64	≥ 1024	≥ 1024
Benzoilmetronidazole	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024
HFPA						
Natural product/antifungal	**Alone**	**+ HFPA**	**Alone**	**+ HFPA**	**Alone**	**+ HFPA**
HFPA	≥ 1024	–	≥ 1024	–	≥ 1024	–
Amphotericin b	≥ 1024	≥ 1024	≥ 1024	2	≥ 1024	≥ 1024
Mebendazole	≥ 1024	≥ 1024	≥ 1024	2	≥ 1024	≥ 1024
Nystatin	≥ 1024	≥ 1024	64	1	≥ 1024	≥ 1024
Benzoilmetronidazole	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024
EFPA						
Natural product/antifungal	**Alone**	**+ EFPA**	**Alone**	**+ EFPA**	**Alone**	**+ EFPA**
EFPA	≥ 1024	–	≥ 1024	–	≥ 1024	–
Amphotericin b	≥ 1024	≥ 1024	≥ 1024	2	≥ 1024	≥ 1024
Mebendazole	≥ 1024	≥ 1024	≥ 1024	0.5	≥ 1024	≥ 1024
Nystatin	≥ 1024	≥ 1024	64	64	≥ 1024	≥ 1024
Benzoilmetronidazole	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024	≥ 1024

DFPA – dichloromethane fraction; EEPA – ethanol extract from the leaves; HFPA – hexane fraction; EFPA – ethyl acetate fraction

glycosides are shown in the table 3. As observed with the antifungals, the natural products also demonstrated a MIC ≥ 1024 μg mL^{-1}, but when the natural product was associated with the aminoglycosides, a synergistic or antagonistic antibiotic activity was detected against both bacterial strains assayed. When compared with the results of SUFFREDINI et al. (2006), the results observed on this work indicate a better antibacterial activity.

Tab. 3. Minimum inhibitory concentration (MIC) of antibiotics alone and associated with extract and fractions of *Piper arboreum* against *Escherichia coli* strain 27 and *Staphylococcus aureus* strain 358 (μg mL^{-1}).

DFPA	***EC27***		***SA358***	
Natural product/antibiotic	**Alone**	**+ DFPA**	**Alone**	**+ DFPA**
DFPA	≥ 1024	–	≥ 1024	–
Kanamycin	1250	1250	156	39
Amikacin	2500	2500	78	78
				78
Neomycin	1250	1250	78	
Gentamicin	156	625	39	39
EEPA				
Natural product/antibiotic	**Alone**	**+ EEPA**	**Alone**	**+ EEPA**
EEPA	≥ 1024	–	≥ 1024	–
Kanamycin	1250	1250	156	39
Amikacin	2500	625	78	78
Neomycin	1250	1250	78	78
Gentamicin	156	156	39	39
HFPA				
Natural product/antibiotic	**Alone**	**+ HFPA**	**Alone**	**+ HFPA**
HFPA	≥ 1024	–	≥ 1024	–
Kanamycin	1250	≥ 5000	156	39
Amikacin	2500	78	78	78
Neomycin	1250	1250	78	78
Gentamicin	156	39	39	39
EFPA				
Natural product/antibiotic	**Alone**	**+ EFPA**	**Alone**	**+ EFPA**
EFPA	≥ 1024	–	≥ 1024	–
Kanamycin	1250	1250	156	39
Amikacin	2500	312	78	78
Neomycin	1250	1250	78	78
Gentamicin	156	39	39	39

DFPA – dichloromethane fraction; EEPA – ethanol extract from the leaves; HFPA – hexane fraction; EFPA – ethyl acetate fraction

All these different MIC results can be attributed to the environmental conditions related to the differences in the plants studied (CYSNE et al. 2005). However, the natural products of *P. arboreum* enhanced the antimicrobial activity of the assayed drugs, acting in a synergistic form (Tabs. 2, 3). This is the first report of synergism between natural products of *P. arboreum* and antimicrobial agents.

There are several mechanisms involved in the inhibition of microorganism growth, especially due to the hydrophobic nature of some phytocompounds. These compounds may interact with the lipid bilayer of the cell membrane, affecting the respiratory chain and the production of energy (WENDAKOON and SAKAGUCHI 1995) or enhancing the membrane permeability, including permeability to antimicrobials (BURT 2004). This permeability enhancement can be obtained from a combination of antimicrobial drugs with natural products in a sub-inhibitory concentration (COUTINHO et al. 2008, 2009b).

This strategy refers the usage of natural products and drugs in an approach with mono- or multi-extract combinations that will affect not only a single but several targets (HEMAISWARYA et al. 2008, WAGNER et al. 2009).

Conclusions

As demonstrated in our work, natural products of *Piper arboreum* showed an expressive capacity to modulate the activity of antimicrobial drugs in a synergistic or antagonistic manner, representing an alternative to the efforts to control infectious diseases caused by multidrug-resistant strains of fungi and bacteria.

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