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ENHANCEMENT OF ANTIMICROBIAL ACTIVITY OF ANTIBIOTICS AND ANTIFUNGALS BY THE USE OF NATURAL PRODUCTS FROM *PITYROGRAMMA CALOMELANOS* (L.) LINK.

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Abstract - The ethanol extract and methanol fraction of *Pityrogramma calomelanos* (L.) link were evaluated for antibacterial, antifungal and modulatory activities against strains of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans, C. krusei and C. tropicalis*. The antimicrobial activity of the natural products was evaluated by the microdilution method associated or not with aminoglycosides and antifungals. The ethanol extract and methanol fraction of *P. calomelanos* showed good activity against *S. aureus* when associated with aminoglycosides and with benzoilmetronidazol against species of the genus *Candida*. These results indicate that *P. calomelanos* should be studied as a possible source of natural products to combat bacteria and fungi either directly or by modulating the mechanisms of resistance of these microorganisms, enhancing the antimicrobial activity of these drugs and combating microbial resistance.

Key words: Antimicrobial activity, ethanol extract, methanol fraction, *Pityrogramma calomelanos*, modifying antibiotic activity

INTRODUCTION

Nosocomial infections represent a serious problem to public health, causing a significant increase in morbidity, mortality and hospital costs (Boyce, 2001). Among the major pathogens involved in these infections, *Staphylococcus aureus* is one of the most important causal agents (Moraes et al., 2000). The clinical manifestations of *S. aureus* can be cutaneous or systemic (Ferreira and Ávila, 2001). The cutaneous symptoms include folliculitis, boils, carbuncles and the staphylococcal scalded skin syndrome. *S. aureus* is frequently isolated in post-surgical wounds and is therefore a serious risk to systemic infections. When it causes bacteremia, this can result in endocarditis, osteomyelitis, penumonia and meningitis (Koneman et al., 2001).

Other clinically important bacteria are *Escherichia coli* and *Pseudomonas aeruginosa. E. coli* has been identified as the primary cause of infections of the urinary tract, neonatal meningitis, nosocomial septicemia and enteritis. *P. aeruginosa* is also among the bacteria with considerable clinical relevance, causing infections in immune compromised patients as occurs in cystic fibrosis where the secretion stasis make possible the colonization by this bacterium. Furthermore, this bacterium presents a high rate of mutations that results in progressive resistance to antibiotics and difficulties with anti-infective therapy (Oliver et al., 2000).

In this context, a growing and important problem is the increase of bacterial resistance to antibiotics (Georgopapadakou, 2005). The resistance against

two or more classes of antimicrobials that has been a common finding reported in human and veterinary medicine, limits the available therapeutic options (Von Baum and Marre, 2005). For patients, the resistance to antibiotics increases morbidity and mortality, which in turn increases the cost to health institutions (Dancer, 2001).

In folk medicine, the plants are used either alone or together with conventional medicines (Amorim, 1999). In this association, the medicinal plants or their sub-products can inhibit or increase the therapeutic effect of conventional drugs (Nascimento et al., 2000). The plants with therapeutic properties used in folk medicine are an important source of new biologically active compounds. Being complex mixtures, extracts with antimicrobial agents present a low possibility for the microorganisms to acquire resistance (Daferera et al., 2003). The expression "modifiers of antibiotic activity" refers to substances that modulate or even reverse bacterial resistance to specific antibiotics, as is the case of several natural products of plant origin (extracts and phytoconstituents) that change the microbial susceptibility to antibiotics for inhibition of efflux of pumps (Piddock, 2006; Gibbons, 2004).

The family Pteridaceae presents a considerable morphological diversity. Most of the species occur in the tropics and arid regions. There are 50 genera and 950 species (Prado, 2005). *Pityrogramma* is a genus with about 17 species occurring mainly in tropical America (Smith et al., 2006). The species *Pityrogramma calomelanos* (L.) Link is used as an ornamental and medicinal plant (Ambrósio and Barros, 1997; Corrêa, 1984).

Due to the lack of data about the biological activity of Brazilian ferns and due to serious problems of resistance to antibiotics, the objective of this work is qualitatively identify the chemical composition of *P. calomelanos* and to evaluate the antimicrobial and modulatory antibiotic activity of the ethanol extract and methanol fraction from leaves of *P. calomelanos*.

MATERIALS AND METHODS

Bacterial and fungal strains

The bacterial strains used were *E. coli* (EC-ATCC10536 and EC27), *S. aureus* (SA-ATCC25923 and SA358) and *P. aeruginosa* (ATCC15442 e PSU03), and the profiles of resistance are given in Table 1. The fungal strains used were *Candida albicans* ATCC 40227, *C. krusei* ATCC 6538 and *C. tropicalis* ATCC 13803. All the strains were kept on heart infusion Agar slants (HIA; Difco) and before the assays, the cells were cultured for 24 h at 37°C in brain heart infusion (BHI, Difco). All the strains were obtained from the collection of microorganisms of the Laboratory of Clinical Mycology - UFPB.

Plant material

Leaves of *P. calomelanos* were collected in the city of Crato, Ceará, Brazil. The plant material was identified by Dr. Antônio Álamo Feitosa Saraiva of the Regional University of Cariri, Brazil, and a voucher specimen has been deposited with the identification number 5570 at the Herbarium "Dárdano de Andrade Lima" of the Regional University of Cariri – URCA.

Drugs

Solutions with 5 mg/mL of the antibiotics amikacin, kanamycin, gentamicin and neomycin were obtained from Sigma Co. (St. Louis, USA). Antifungal drugs were prepared as follows: solutions of 1024 μg/ml of Amphotericin B (Sigma Co., St. Louis, USA), Mebendazole (Lasa – Pharmaceutical Industries LTDA., Brazil), Nystatin (Brazilian Laboratory Teuto S/A, Brazil), and Benzoilmetronidazol (Prati, Donaduzzi and Cia LTDA., Brazil). The solutions of antibiotic and antifungals were prepared according to the recommendations of NCCLS (2003).

Preparation of the ethanol extract (EEPC) and methanol fraction (MFPC) of P. calomelanos leaves

Leaves (950 g) were dried and kept at room temperature. The powdered material was extracted by mac-

Table 1. Origin of bacterial strains and profile of resistance at antibiotics.

Bacteria	Origin	Resistance Profile
Escherichia coli 27	Surgical wound	Ast,Ax,Ami,Amox,Ca,Cfc,Cf, Caz,Cip,Clo,Im,Can,Szt,Tet,Tob
Staphaylococcus aureus 358	Surgical wound	Oxa,Gen,Tob,Ami,Can,Neo,Para, But,Sis,Net
Pseudomonas Aeruginosa 03	Uroculture	Cpm,Ctz,Imi,Cip,Ptz,Lev,Mer,Ami

Ast-Aztreonan; Ax-Amoxacillin; Amp-Ampicillin; Ami-Amicillin; Amox-Amoxillin, Ca-Cefadroxil; Cfc-cefaclor; Cf-Cphalothin; Caz-Ceftazinidima; Cip-Ciprofloxacin; Clo-Chloraphenicol; Imi-Imipenem; Can-Kanamycin; Szt-Sulphametrim; Tet-Tetracycline; Tob-Tobramycin; Oxa-Oxacillin; Gen-Gentamicin; Neo-Neomycin; Para-Paramomicin; But-Butirosin; Sis-Sisomicin; Net-Netilmicin; Com-Cfepime; Ctz-Ceftazidime; Ptz-Piperacilin-tazobactam; Lev-Levofloxacin; Mer-Merpenem.

Table 2. Phytochemical screnning of ethanol extract (EEPC) and methanol fraction (FMPC) of Pityrogramma calomelanos (L.) Link.

	METABOLITES														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
EE	+			+	+	+	+	+							+
MF	+			+	+	+	+	+							+

Alkaloids; 2 – Antocianidins; 3 – Antocianins; 4 – Aurones; 5 – Catequines; 6 – Chalcones; 7 - Flavones; 8 – Flavonones; 9 – Flavonols; 10 – Flavononols; 11 – Phenols; 12 – Leucoantocianidins; 13 – Xanthones 14 – Tannins Pyrogallics; 15 – Saponins; (+) presence; (-) ausence; EEPC (ethanol extract of *Pityrogramma calomelanos*); HFPC (hexane fraction of *Pityrogramma calomelanos*).

Table 3. Evaluation of antifungal and antibacterial activity of *Pityrogramma calomelanos* (L.) Link.

Minimum Inhibitory Concentration (MIC) (µg/mL)		
Strains	EEPC	MFPC
C. albicans (ATCC40227)	≥ 1024	≥ 1024
C. krusei (ATCC6538)	≥ 1024	≥ 1024
C. tropicalis (ATCC13803	≥ 1024	≥ 1024
E. coli (ATCC10536)	≥ 1024	≥ 1024
S.aureus (ATCC25923)	≥ 1024	≥ 1024
Pseudomonas aeruginosa (ATCC15442)	≥ 1024	≥ 1024

Table 4. Evaluation of modulatory antifungal effect of ethanol extract (EE) and methanol fraction (MF) of *Pityrogramma calomelanos* (L.) Link.

	C. albicans			*C.krusei*			*C.tropicalis*		
	ALONE	+ EE	+ FM	ALONE	+ EE	+ FM	ALONE	+ EE	+ FM
Anphotericin B	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024
Mebendazole	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024
Nystatin	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024
Benzoilmetronidazole	≥1024	≥1024	128	≥1024	256	≥1024	≥1024	256	≥1024

Ethanol Extract (EE) and Methanol Fraction (MF)

Table 5. Evaluation of modulatory antibacterial effect of ethanol extract (EE) and methanol fraction (MF) of *Pityrogramma calomelanos* (L.) Link.

	***E. coli* 27**			***S. aureus* 358**			***Paeruginosa* 03**		
	Alone	+ EE	+ FM	Alone	+ EE	+ FM	Alone	+ EE	+ FM
Amikacin	≥5000	312,5	1250	78,125	9,765	78,125	1250	1250	1250
Kanamycin	2500	625	2500	156,25	39,06	39,06	2500	2500	2500
Gentamicin	625	625	625	312,5	4,88	4,88	1250	2500	2500
Neomycin	156,25	156,25	156,25	156,25	156,25	39,06	625	625	625

Ethanol Extract (EE) and Methanol Fraction (MF)

eration using 1 L of ethanol 95% as a solvent at room temperature. The mixture was allowed to stand for 72 h at room temperature. The extract was filtered and concentrated under vacuum in a rotary evaporator at 60ºC and 760 mm/Hg of temperature and pressure, respectively (Brasileiro et al., 2006). Aerial parts (950 g) yielded 50 g of an ethanol extract. Forty g of EEPC were fractionated with methanol producing 14.3 g of a methanol fraction. The ethanol extract and methanol fraction were diluted using DMSO.

Phytochemical Screening

Phytochemical assays are used for the qualitative analysis of the presence of secondary metabolites such as heterosides, saponins, tannins, flavonoids, steroids, triperpens, coumarins, quinones, organics acids and alkaloids, and were performed according to the method described by Matos (2009). The tests are based on the observation of color modification and precipitate formation after the addition of specific reagents. Table 2 presents the metabolites observed in the ethanol extract and methanol fraction.

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined in BHI 10% by the method of microdilution, using a suspension of 10^5 UFC/mL and an initial drug concentration of 1024 µg/mL for fungi and 5000 µg/mL for bacteria (Javadpour et al., 1996). The MIC was defined as the lowest concentration at which no growth was observed. To evaluate the ethanol extract and methanol fraction as modulators of resistance to antibiotics and antifungal agents, the sub-inhibitory concentration was used with the antibiotic solution (MIC/8 = 128 µg/mL). The plates were incubated for 24 h at 37°C. To visualize bacterial growth, resazurine was used. No dye was used in the fungal assay.

RESULTS AND DISCUSSION

Neither EE nor MF demonstrated an antimicrobial activity clinically relevant to fungi or bacteria. The values of MIC are presented in Table 3. However, in the modulation assay it was verified that the ethanol extract and methanol fraction modulated the most of the antibiotics tested against *S. aureus*, with the exception to amikacin associated with MF and neomycin with EE, indicating relevant properties of the fraction and extract to enhance the action of aminoglycosides against *S. aureus*. Against *E. coli*, the ethanol extract promoted modulation of the action of amikacin and kanamycin. Against *P. aeruginosa* no modulatory activity was observed. In the modulation of antifungal activity, it was observed that MF modulated the antifungal action of benzoilmetronidazol against *C. albicans*. Against *C. krusei* and *C. tropicalis*, the EE promoted modulation in the effect of benzoilmetronidazol. These results are the first reports about the antimicrobial or modulatory activities of *P. calomelanos* (Tables 4 and 5).

The mechanisms through which the extracts could inhibit the growth of microorganisms are several and can due at hydrophobic nature of some components. These components can interact with the lipid layer

of the cell membrane, affecting the respiratory chain and energy production, or even making the cell more permeable to antibiotics, thus disrupting vital cellular processes (Nicolson et al., 1999; Burt, 2004). Various components of the extracts or fractions increase the permeability of the cell, increasing the entry of antibiotics (Helander et al., 1998). This mechanism can be obtained by the combination of antibiotics with extracts or fractions at sub-inhibitory concentrations when applied directly to the culture medium (Coutinho et al., 2010a). No modulatory activity was found to be associated with the fern. However, several natural products from plants and animals have been studied (Ferreira et al., 2009; Coutinho et al., 2010b; Rodrigues et al., 2009).

This strategy is called "herbal shotgun" or "synergistic multi-effect targeting" and refers to the utilization of plants and drugs in an approach using mono- or multi-extract combinations, which can affect not only a single target but also various targets, where the different therapeutic components collaborate in a synergistic-agonistic manner. This approach is not only meant for combinations of extracts. Combinations of natural products or extracts and synthetic products or antibiotics are also possible (Coutinho et al., 2008; Wagner and Ulrich-Merzenich, 2009).

The results obtained in this study are promising and are an incentive for future research into the pharmacological aspects and toxicity of sub-products of *P. calomelanos* with the objective of promoting their possible rational use in antifungal and antibacterial therapy, as well as for resolving some of the questions of resistance to antibiotics.

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