



Short communication

Herbs in association with drugs: Enhancement of the aminoglycoside-antibiotic activity by *Pityrogramma calomelanos* (L.) LinkTeógenes M. Souza^a, Maria F.B. Morais-Braga^a, José G.M. Costa^b, Antônio A.F. Saraiva^c, Micheline A. Lima^d, Henrique D.M. Coutinho^{a,*}^aLaboratory of Microbiology and Molecular Biology, Department of Biological Chemistry, University of the Region of Cariri, CE/Crato, CE, Brazil^bLaboratory of Natural Products Research, Department of Biological Chemistry, University of the Region of Cariri, CE/Crato, Brazil^cLaboratory of Paleontology, Department of Biological Sciences, University of the Region of Cariri, CE/Crato, Brazil^dLaboratory of Biochemistry and Cellular Biology, Department of Ecology and Environment, Federal University of Paraiba, PB/Rio Tinto, Brazil

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ABSTRACT

Aims: In this work, the ethyl acetate and hexane fractions of *Pityrogramma calomelanos* (L.) were evaluated to antibacterial and antifungal activity against strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Candida krusei* and *Candida tropicalis*.

Methods: The study was performed aiming to assess the antimicrobial effect with the method of dilution in HIA. The hexane and ethyl acetate fractions were named HFPC and EAFPC, respectively.

Results: Both fractions of specie *P. calomelanos* displayed good activity against *S. aureus* when associated with gentamicin. When associated with the antifungal, the fractions did not exhibit relevant activity against species of *Candida*.

Conclusions: These results indicate that this specie can be used as a possible source of natural products of antibacterial interest, mainly when combined with aminoglycosides.

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1. Introduction

Nosocomial infections are a serious problem of public health around the globe, causing significant increases in morbidity, mortality and hospital costs. Among the main pathogens involved in these infections stands *Staphylococcus aureus*. The clinical manifestations of *S. aureus* can be cutaneous or systemic. The cutaneous symptoms include folliculitis, boils, carbuncles and the skin scalded Staphylococci syndrome. *S. aureus* is frequently isolated in post-surgical wounds, and therefore is a serious problem due causing systemic infections. When cause bacteremia, that bacteria can produce endocarditis, osteomyelitis, piocarditis and meningitis.¹

In health field, other bacterium of clinical importance is *Escherichia coli*. In humans, this microorganism has been identified as the primary cause of urinary tract infections, neonatal meningitis, nosocomial septicemia and enteritis. *Pseudomonas aeruginosa* also is found among the bacteria of greater clinical relevance, being the cause of infections in immunocompromised patients, as occurs in cystic fibrosis. Furthermore, this bacterium presents high rate of

spontaneous mutations, resulting in progressive enhancement in the antibiotic resistance, raising difficulties in the anti-infective therapy.²

Another growing problem is the increase of bacterial resistance to antibiotics. The resistances at two or more antimicrobial classes have been reported frequently in human and veterinary medicine, limiting the available therapeutic options. The increasing of the antibiotic resistance enhances the morbidity, mortality and the treatment costs.³

In the traditional medicine, the plants are used together with the conventional medicines. In this association, the medicinal plants or their sub-products can act inhibiting or increasing the therapeutic effect of conventional drugs. The plants with therapeutics properties used in the traditional medicine are an important source of news biologically active compounds. The use of extracts with antimicrobial agents presents a low possibility of resistance development by the microorganisms because these natural products are complex chemical mixtures. Modifier of antibiotic activity is a term used to name substances that modulate or even reverse bacterial resistance to specific antibiotics, such as 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.⁴

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

Bacteria	Source	Resistance profile
<i>Escherichia coli</i> 27	Surgical wound	Ast, Ax, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Clo, Im, Can, Szt, Tet, Tob
<i>Staphylococcus aureus</i> 358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net
<i>Pseudomonas aeruginosa</i> 03	Uroculture	Cpm, Ctz, Imi, Cip, Ptz, Lev, Mer, Ami

Ast – Aztreonam; Ax – Amoxicillin; Amp – Ampicillin; Ami – Amicillin; Amox – Amoxicillin; Ca – Cefadroxil; Cfc – cefaclor; Cf – Cephalothin; Caz – Ceftazidimide; Cip – Ciprofloxacin; Clo – Chlorphenicol; Imi – Imipenem; Can – Kanamycin; Szt – Sulphametrim; Tet – Tetracycline; Tob – Tobramycin; Oxa – Oxacillin; Gen – Gentamicin; Neo – Neomycin; Para – Paromomycin; But – Butirosin; Sis – Sisomicin; Net – Netilmicin; Com – Cefepime; Ctz – Ceftazidime; Ptz – Piperacillin-tazobactam; Lev – Levofloxacin; Mer – Merpenem.

The family Pteridaceae presents a high morphological diversity, several species occurring in the tropical and arid regions. About 50 genus and 950 species are arranged in this family. *Pityrogramma* is a genus with 17 species, occurring mainly in the Tropical America. The species *Pityrogramma calomelanos* (L.) Link is popularly known as “feto-branco”, “avenca-branca” or “avenca-preta” and is used as ornamental and medicinal plant in the traditional medicine.⁵

The objective of the present work was to identify the main classes of chemical compounds and to evaluate the inflection of antimicrobial activity of ethyl acetate and hexane fractions from the leaves of *P. calomelanos*.

2. Material and methods

2.1. Bacterial strains

The bacterial strains used were: *E. coli* (EC-ATCC10536 and EC27), *S. aureus* (SA-ATCC25923 and SA358) and *P. aeruginosa* (ATCC15442 e PSU 03) with profile of resistance showed in Table 1. All the strains were kept in heart infusion Agar slants (HIA; Difco) and before the assays, the cells were cultured during 24 h at 37 °C in brain heart infusion (BHI, Difco). All the strains were obtained from collection microorganisms of mycology laboratory – UFPB.

2.2. Fungal strains

The fungal strains used were: *Candida albicans* ATCC 40227, *Candida krusei* ATCC 6538 and *Candida tropicalis* ATCC 13803. All the strains were kept in heart infusion Agar slants (HIA; Difco) and before of the assays, the cells were cultured during 24 h–37 °C in brain heart infusion (BHI, Difco). The strains were obtained from the Microorganisms Collection of Mycology Laboratory – UFPB.

2.3. Plant material

Leaves of *P. calomelanos* were collected in the rainy season (September, 2009) in the county of Crato, Ceará State, Brazil. The

plant material was identified by Dr. Antonio Álamo Feitosa Saraiva from University of the Region of Cariri, Crato, CE, Brazil, and voucher specimen have been deposited with the identification number 5570 at the Herbarium “Dárdano de Andrade Lima” University of the Region of Cariri, Crato, CE, Brazil.

2.4. Drugs

Solutions with 5.000 µg/mL of amikacin, kanamycin, gentamicin and neomycin were prepared (Sigma Co., St. Louis, USA). The antifungal drugs solutions of 1024 µg/mL to amphotericin B were prepared (Sigma Co., St. Louis, USA), Mebendazole (Lasa – Pharmaceutical Industries LTDA., Brazil), Nystatin (Brazilian Laboratory Teuto S/A, Brazil), e Benzoilmetronidazole (Prati, Donaduzzi & Cia LTDA., Brazil). The antibiotic solutions were prepared allowing the recommendations of NCCLS.⁶

2.5. Preparation of ethyl acetate fraction (EAFPC) and hexane fraction (HFPC) from leaves of *P. calomelanos* (L.) Link

Leaves of the plant (950 g) were dried and kept at room temperature. The powdered material was extracted by maceration using 1 L of 95% ethanol as solvent at room temperature. The homogenate was allowed to stand for 72 h at room temperature. The extract was filtered and concentrated under vacuum in a rotary evaporator (Q-344B – Quimis, Brazil) and ultrathermic bath (Q-214M2 – Quimis, Brazil) under 60 °C and 760 mm/Hg of temperature and pressure, respectively. Each 500 g of aerial parts yielded 26.3 g of ethanol extract. Afterward, 20 g of ethanol extract was fractionated with hexane and ethyl acetate, producing 0.28 g and 9.25 g of material fractionated respectively. The fractions were diluted using DMSO.

2.6. Phytochemical screening

The phytochemical assays is to use for qualitative analysis of the presence secondary metabolites as: heterosides, saponins, tannins, flavonoids, steroids, triperpens, cumarins, quinones, organics acids and alkaloids were performed allowing the method described for Matos.⁷ The tests are analyzed by the color modification and by the formation of precipitate after of the addition of the reagents specific. Table 2 presents the metabolites observes in hexane and ethyl acetate fractions.

2.7. Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined in BHI 10% by method of microtitration, using a suspension of 10⁵ UFC/mL and an initial drug concentration 1024 µg/mL for fungi and 5.000 µg/mL for bacteria.⁶ The MIC was defined as the lowest concentration at which no growth was observed. To the evaluation of the ethyl acetate fraction and hexane fraction as modulators of resistance to antibiotics and antifungal agents, a sub-inhibitory

Table 2
Phytochemical screening of hexane fraction (HFPC) and ethyl acetate fraction (EAFPC) from *Pityrogramma calomelanos* (L.) Link.

Metabolites	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
HFPC					+		+	+	+		+		+		
EAFPC				+		+	+	+	+	+	+		+		

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; HFPC (hexane fraction of *Pityrogramma calomelanos*); EAFPC (ethyl acetate fraction of *Pityrogramma calomelanos*).

Table 3
Evaluation of modulator antifungals effect of hexane fraction (HFPC) and ethyl acetate fraction (EAFPC) of *Pityrogramma calomelanos* (L.) Link.

	<i>C. albicans</i>			<i>C. krusei</i>			<i>C. tropicalis</i>		
	ALONE	+HF	+EAF	ALONE	+HF	+EAF	ALONE	+HF	+EAF
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	128	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024

Hexane fraction of *Pityrogramma calomelanos* (HFPC); ethyl acetate fraction *Pityrogramma calomelanos* (EAFPC).

Table 4
Evaluation of modulator antibacterial effect of hexane fraction (HFPC) and ethyl acetate fraction (EAFPC) from *Pityrogramma calomelanos* (L.) Link.

	<i>E. coli</i>			<i>S. aureus</i>			<i>P. aeruginosa</i>		
	ALONE	+HF	+EAF	ALONE	+HF	+EAF	ALONE	+HF	+EAF
Amikacin	5000	9.765	9.765	78.125	78.125	625	1250	1250	1250
Kanamycin	2500	312.5	2500	156.25	156.25	39.06	2500	2500	2500
Gentamicin	625	625	625	312.5	4.88	4.88	1250	1250	1250
Neomycin	156.25	156.25	156.25	156.25	156.25	19.53	625	625	625

Hexane fraction of *Pityrogramma calomelanos* (HFPC); ethyl acetate fraction *Pityrogramma calomelanos* (EAFPC).

concentration was used (MIC/8 = 128 µg/mL) and assayed using the follow strains: EC27, MRSA 358, *C. albicans*, *C. krusei* and *C. tropicalis*. The plates were incubated for 24 h at 37 °C.

3. Results and discussion

In the antimicrobial assays using the ethyl acetate fraction and hexane fraction, was determined the Minimum Inhibitory Concentration (MIC ≥ 1024 µg/mL). To these fractions, there was not any clinically relevant antimicrobial activity against fungi or bacteria observed.

In the test of modulatory antimicrobial activity against multi-resistant bacteria, was verified in *E. coli* that ethyl acetate fraction potentiated the effect of amikacin. The hexane fraction potentiated the effect of two antibiotics, amikacin and kanamycin. This result indicates the capacity of these fractions to facilitate the action of aminoglycosides on *E. coli*. In the assay with *S. aureus*, was observed that ethyl acetate fraction potentiated the antibiotic activity of kanamycin, gentamicin and neomycin. The hexane fraction potentiated the effect only in the gentamicin. For *P. aeruginosa*, no potentiation of antibacterial activity was observed. In the assay with fungi, any modulatory effect of the natural products over the antifungals against the species of *Candida* test was not observed, with exception of the Benzoilmetronidazole, that was potentiated against *C. albicans* when associated with the hexane fraction. These are the first reports about the antimicrobial and modifying antibiotic and antifungal activity of *P. calomelanos* (Tables 3 and 4).

There are several mechanisms by which the extracts may inhibit the growth of microorganisms and generally are associated with the hydrophobic nature of some components. These components could interact with the cell membrane, affecting the respiratory chain and the energy production, or even make the cell more permeable, leading to disruption of vital cellular activity and enhancing the antibiotic intake. Several compounds of extracts or fractions increase the permeability of the cell, raising the penetration of antibiotics. This effect can be obtained by the combination of antibiotics with extract or fractions in sub-inhibitory concentration applied directly to the culture medium.^{4,8}

This strategy is called “herbal shotgun” and refers to the utilization of plants and drugs in an approach using mono- or multi-extract combinations, affecting various targets.^{9,10}

4. Conclusions

This work revealed that natural products of *P. calomelanos* are effective antimicrobial-modifying agents. This information is promising and can incentive search futures about pharmacological aspects and toxicity of the natural products of *P. calomelanos* with the objective to promote the possible future use of natural products from this fern or from other plants in the antifungal and antibacterial therapy, as well as combat the problem of antibiotic resistance. Future works to identify the chemical compounds that are relevant from these activities are necessary, as well as other studies *in vitro* and *in vivo* before the possible combined use as new drugs.

Conflicts of interest

All authors have none to declare.

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