

Additive effects of *Pityrogramma calomelanos* L. (link.) combined with aminoglycosides against *Staphylococcus aureus*

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Abstract

This study is the first report about the enhancement of additive effect of *Pityrogramma calomelanos* L. (link) associated with aminoglycosides against a multiresistant *Staphylococcus aureus*. In this study, the ethanol extract and the ethyl acetate fraction were assayed to identify the relationship between these natural products and the aminoglycosides using the checkerboard method. The checkerboard assay was realized to verify if the relationship between these drugs and natural products represent a synergistic, additive, indifferent or antagonistic effect. All natural products and antibiotics assayed demonstrated an additive effect, with FIC index = 0.5. These results indicate that the ethanol extract and the ethyl acetate fraction of *P. calomelanos* can be a source of plant derived products with antibiotic modifier activity, affecting positively the antibiotic activity of aminoglycosides.

Keywords: *Pityrogramma calomelanos*; checkerboard method; aminoglycosides; *Staphylococcus aureus*; additive effect.

Introduction

With the increase of the antibiotic resistance, the research about the antimicrobial activities of natural products becomes an interesting alternative [1,2]. Several plant extracts and phytochemicals present important antimicrobial properties, showing a great significance in the therapeutic treatments of several diseases as demonstrated by several scientific reports [3,4].

Pityrogramma calomelanos (Pteridaceae), known in Brazil as "avenca-branca" or "avenca-preta" is used as ornamental and medicinal plant. Natural products isolated from this plant as flavonoids, terpenes, chalcones, phenols, present several biological activities reported in the literature: astringent, analgesic, anti-hemorrhagic, depurative, anti-hypertensive, anti-pyretic and stimulating of blood circulation [5-9].

The aminoglycosides are potent antibiotics that affect the bacterial ribosome, been the enhancement of the resistance to these antibiotics widely recognized as a serious health problem [10]. the main mechanisms of resistance to aminoglycosides in

Staphylococcus aureus is the active efflux and enzymatic inactivation [11].

In this study, we assayed the ethanol extract and acetyl acetate fraction of *P. calomelanos* to identify the relationship between these natural products associated with aminoglycosides against a multiresistant strain of *S. aureus*.

Materials and Methods

Strain

The experiments were performed in the Laboratory of Microbiology and Molecular Biology (Department of Biological Chemistry) in the University of the Region of Cariri, Crato, CE, Brazil. The experiments were performed with clinical *S. aureus* isolate (SA358) presenting multiresistance to antibiotics [12] (Table 1). The strains were maintained in heart infusion agar slants (HIA, Difco, USA), and prior to assay, the cells were grown overnight at 37°C in brain heart infusion agar (BHI, Difco).

Table 1. Origin of bacterial strains and resistance to antibiotics.

Bacteria	Origin	Resistance
<i>Staphylococcus aureus</i> SA358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net

Ami - Amikacin; Can - Kanamycin; Tob - tobramycin; Oxa - Oxacillin; Gen - gentamicin; Neo - neomycin; Para - Paramomicina; But - Butirosin; Sis - sisomicin; Net - Netilmicin



Plant Material

Leaves of *P. calomelanos* were collected in the county of Crato, Ceará State, Brazil. The plant material was identified by Dr. Antonio Álamo Feitosa Saraiva of University of the Region of Cariri, Crato, CE, Brazil, and a 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.

Drugs

The antibiotics used were gentamicin, amikacin and neomycin, and were obtained from Sigma Chemical Co., USA. All drugs were dissolved in sterile water.

Preparation of extracts and fractions

Leaves of the plant (950 g) were dried and kept at room temperature. The powdered material was extracted by maceration using 3 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 760mm/Hg of temperature and pressure, respectively [12]. Each 500 g of aerial parts yielded 26.3 g of ethanol extract. 20 g of ethanol extract of *P. calomelanos*

(EEPC) was fractionated with ethyl acetate, obtained 9.25 g of ethyl acetate fraction of *P. calomelanos* (EAFPC). For the tests, the dry ethanol extract and ethyl acetate fraction were dissolved in dimethylsulphoxide (DMSO).

Checkerboard assay

The SA358 strain was tested by the microdilution checkerboard technique [13]. The antibiotics and natural products assayed were diluted serially 2:1, ranging between 2500 - 19.53 µg/mL and 512 - 4 µg/mL, respectively. In order to evaluate the activity of combinations of drugs, fractional inhibitory concentration (FIC) index was calculated as FIC^A (natural products) + FIC^B (antibiotics), where $FIC^A = MIC^A$ combined / MIC^A alone and $FIC^B = MIC^B$ combined / MIC^B alone. The FIC index was calculated and the interpretation made was follows: synergistic (<0.5), additive (0.5-1.0), indifferent (>1), or antagonistic (>4.0). All tests are realized in triplicate with concordance between the tests 80%.

Result and Discussion

Additive effect aminoglycosides was observed with combination of EEPC and EAFPC and all aminoglycosides tested. The combinations of EEPC and EAFPC and amikacin, gentamicin and neomycin showed a FIC index of 0.5 (Table 2).

Table 2. Fractional Inhibitory Concentration (FIC) of antibiotics aminoglycosides and the effect of combinations with ethanol extract (EE) and ethyl acetate fraction (EAF) against *S. aureus* 358.

MIC alone		MIC combined		MIC alone		MIC combined		FIC index (type of interaction)
Ami	39.06	Ami + EE	19.53	EE	512	EE + Ami	4	0.5 (additive)
Gen	39.06	Gen + EE	19.53	EE	512	EE + Gen	4	0.5 (additive)
Neo	39.06	Neo + EE	19.53	EE	512	EE + Neo	4	0.5 (additive)
Ami	39.06	Ami + EAF	19.53	EAF	64	EAF + Ami	4	0.5 (additive)
Gen	39.06	Gen + EAF	19.53	EAF	128	EAF + Gen	4	0.5 (additive)
Neo	39.06	Neo + EAF	19.53	EAF	64	EAF + Neo	4	0.5 (additive)

Ami - Amikacin; Gen - Gentamicin; Neo - Neomycin; EE – Ethanol Extract; EAF – Ethyl Acetate Fraction

This strategy is named as "herbal shotgun" or "synergistic multi-target effects" and refers to the use of herbals and drugs in a multitargeted approach, due the fact of mono or multi-extract combinations affecting not only a single target, but several ones, co-operating in an agonistic-synergistic way. This approach is not exclusive for extract combinations, but combinations between single natural products or extracts with chemosynthetics or antibiotics are also possible [14-18].

Conclusions

P. calomelanos is used in the folk medicine. However, there is not data in the literature about the combinatory effect of natural products from this plant with synthetic drugs. These are the first

results published with the specie *P. calomelanos* demonstrating the additive effect of the association with aminoglycosides. The additive effect of these natural products with antibiotics may represent an interesting approach in the antibiotic therapy. In conclusion, *P. calomelanos* could represent a source of natural products with modifying antibiotic activity, being an interesting alternative to combat infectious agents with multiresistance to antibiotics.

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