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Modulation of the norfloxacin resistance in *Staphylococcus aureus* by *Croton campestris A.* and *Ocimum gratissimum L.*

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Introduction: Some species of *Staphylococcus* are often recognized as etiological agents of many animal and human opportunistic infections. This study is the first test of change in resistance of antibiotic activity by *Croton campestris A.* and *Ocimum gratissimum L.* against multiresistant strains of *Staphylococcus aureus*.
Objective: In this study, the hexane and methanol extract of *Croton campestris A.* and *Ocimum gratissimum L.* was tested for antibacterial activity alone and in combination with norfloxacin against the strain SA1199B.
Materials and methods: The minimum inhibitory concentration (MIC) and the modulatory effect of extracts was assayed using microtitre assay.
Results: By the fact of the MIC observed was not clinically relevant (MIC= 512 to ≥1.024 µg/ml), the antibiotic activity of norfloxacin was enhanced when this antibiotic was combined with sub-inhibitory concentrations of extracts, mainly the hexane extracts.
Conclusions: These results indicate that the assayed extracts present compounds that can be used as a putative efflux pump inhibitor, indicating that *Croton campestris A.* and *Ocimum gratissimum L.* can be a source of plant derived products with antibiotic modifier activity.

Key words: *Staphylococcus aureus, Croton, Ocimum;* drug resistance, microbial; anti-bacterial agents, norfloxacin.

Modulación de la resistencia a norfloxacina de *Staphylococcus aureus* por *Croton A. campestris* y *Ocimum gratissimum L.*

Introducción. Algunas especies de *Staphylococcus* suelen ser reconocidas como agentes etiológicos de muchas infecciones oportunistas en animales y em humanos. Este estudio es la primera prueba del cambio en la resistencia de la actividad antibiótica por *Croton campestris A.* y *Ocimum gratissimum L.* contra cepas multirresistentes de *Staphylococcus aureus*.
Objetivo. Ensayar la actividad antibacteriana de los extractos hexánicos y metanólicos de *Croton campestris A.* y *Ocimum gratissimum L.* sola y en combinación con norfloxacina sobre la cepa SA1199B.
Materiales y métodos. Se analizó la concentración inhibitoria mínima (CIM) y el efecto modulador de los extractos usando el ensayo de microtitulación.
Resultados. Por el hecho de que la CIM observada no era clínicamente relevante (CIM: 512 ≥ 1.024 mg/ml), la actividad antibiótica de norfloxacina fue potenciada cuando se combinó este antibiótico con concentraciones subinhibitorias de los extractos, principalmente con los extractos hexánicos.
Conclusiones. Estos resultados indican que los extractos ensayados presentan compuestos que pueden ser utilizados como inhibidores putativos de la bomba de eflujo, lo que indica que *Croton campestris A.* y *Ocimum gratissimum L.* pueden ser una fuente de productos derivados de plantas con actividad modificadora de antibióticos.

Palabras clave: *Croton campestres A.*; *Ocimum gratissimum L.*, modificación de la resistencia; antibióticos; Sistema de eflujo de NorA; *Staphylococcus aureus.*

Bacteria of the genus *Staphylococcus* are distributed in nature, as well as being part of the normal microbiota of the skin and of the mucosa of animals including birds. Some specimens of *Staphylococcus* are frequently recognized as etiologic agents of opportunistic infections in many animals and humans (1,2). S. *aureus, S. epidermidis, S. saprophyticus* and *S. haemolyticus* are the most important causative species of human infections. Besides causing different types of intoxications, *S. aureus* represents the most common etiologic agent of purulent infections (for example, osteomyelitis, furuncle, carbuncle, abscess, myocarditis, endocarditis, pneumonia, shunt-associated meningitis, bacterial arthritis)(3).

In the last years, there has been great scientific interest in chemical and pharmacological investigations of the biological properties of medicinal plants (4-7). Medicinal plants have been the source of many medications that are now applied in clinical practice. The use of extracts as antimicrobial agents shows a low risk of increasing resistance to their action, because they are complex mixtures, making microbial adaptability very difficult (8).

The family Euphorbiaceae is made up of 317 genera and about 7,500 species. The genus *Croton*, which has 700 species, is widely distributed in warm regions of the world (9). The species *Croton campestris A.*, popularly known as velame-do-campo, is a shrub originally from Brazil, occurring mainly in the southeast and northeast regions, and it is widely used in traditional medicine as a powerful depurative against scrophulosis, venereal diseases, tumors, skin diseases, rheumatism, ulcers of the uterus, diarrhea and arthritis (10).

Ocimum gratissimum L. is an aromatic shrub originally from Asia and Africa (11,12). Popularly known as alfavaca and manjericão, the leaves are used on traditional medicine. The antimicrobial activity of essential oil from these leaves was observed against several pathogenic microrganisms as *Staphylococcus aureus*, *Bacillus* spp, *Pseudomonas aeruginosae*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Leishmania amazonensis* (12-14).

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The aim of this study was to do a phytochemical screening of the methanol and hexane extracts of *Croton campestris A* and *Ocimum gratissimum L.* and to determine their potentiation of antibiotic activity of norfloxacin, using for this one strain with an efflux pump that extrude this antibiotic.

Materials and methods

Bacterial material

The bacterial strains utilized were the clinical isolate *S. aureus* 358 (SA358), the standard strain *S. aureus* ATCC25923 (SA-ATCC25923) and the strain *S. aureus* 1199B (SA1199B), overexpressing the norA, encoding the NorA efflux protein, the main component of the complex that extrude norfloxacin and other biocides (15,16). All strains were maintained on slants with heart infusion agar (HIA, Difco Laboratories Ltda.). Before the assay, the cells were grown overnight at 37 °C in brain heart infusion broth (BHI, Difco Laboratories Ltd.).

Plant material

Leaves of *Croton campestris A.* and *Ocimum gratissimum L.* were collected in the municipality of Crato, Ceará, Brazil. The plants materials were identified and dried and pressed specimens were deposited in the Herbarium of UFRN and Herbarium Dárdano de Andrade Lima – URCA, with numbers 7095 and 3978, respectively.

Preparation of methanol and hexane extracts of Croton campestris A. and Ocimum gratissimum L.

For the preparation of the extracts, leaves were collected which were kept submersed in methanol and hexane separately for 72 h; afterward, the extract was filtered and concentrated using a rotary vacuum evaporator (model Q-344B – Quimis, Brazil) and ultrathermal bath (model Q-214M2 – Quimis, Brazil), obtaining the yields presented on the Table 1. The solutions utilized in the tests was prepared at a concentration of 10 mg/ml, dissolved in DMSO and then diluted with distilled water to obtain a concentration of 1024 µg/ml.

Phytochemical prospecting

The phytochemical tests to detect the presence of heterosides, saponins, tannins, flavonoids, steroids, triterpenes, cumarins, quinones, organic acids and alkaloids were performed according to the method described by Matos (17). The tests were based on the visual observation of a change in color or formation of precipitate after the addition

of specific reagents, and the results for the extracts studied are shown in table 1.

Drugs

Norfloxacin was from Bayer S.A., Brazil and all the other drugs were from Sigma Chemical Co., USA.

Antibacterial assay

Minimal inhibitory concentration (MIC) was determined in a microdillution assay utilizing an inoculum of 100 µl of each strain, suspended in brain heart infusion (BHI) broth up to a final concentration of 10^5 CFU/ml in 96-well microtiter plates. Each well 100 µl received 100 µl of each extract solution, performing a twofold dilution. The final concentrations of the extracts varied 512 - 8 µg/ml. Minimal inhibitory concentrations were recorded as the lowest concentrations required to inhibit bacterial growth. The minimal inhibitory concentration for the antibiotics was determined in BHI by the microdillution assay utilizing suspensions of 10^5 CFU/ml and a drug concentration range of 2.5 to 0.0012 mg/ml (twofold serial dilutions)(18). MIC was defined as the lowest concentration at which no growth was observed. For the evaluation of the extracts as modulators of resistance to the antibiotics, MIC of the antibiotics was determined in the presence or absence of the extracts at sub-inhibitory concentrations (32 µg/ml) and the plates were incubated for 24 h at 37 °C. Each antibacterial assay for MIC determination was carried out in triplicate.

Modulation assay

To evaluate the extracts as a modulator of norfloxacin resistance, the modulation assay was realized following the procedure cited by Stavri, *et al.* (19), using sub-inhibitory concentrations of the extracts. All experiments were realized on triplicate. We used as controls blank discs with no compounds and discs with DMSO, the solvent used on the preparation of extracts concentrations.

Results

With the increase in the incidence of resistance to antibiotics, alternative natural products of plants could be of interest (1). Some plant extracts and phytochemicals are known to have antimicrobial properties, which could be of great importance in the therapeutic treatments. In the last years, various studies have been conducted in different countries, demonstrating the efficacy of this type of treatment (20). Many natural products isolated from plants or animals have been evaluated not only for direct antimicrobial activity but also as resistance modifying agents (21-24). Various chemical compounds, synthetic or from natural sources, have direct activity against many species of bacteria, enhancing the activity of a specific antibiotic, reversing the natural resistance of bacteria to specific antibiotics, causing the elimination of plasmids and inhibiting the active efflux of antibiotics through the plasma membrane (4). The potentiation of antibiotic activity or the reversal of antibiotic resistance allows the classification of these compounds as modifiers of antibiotic activity (1,4).

Tables 2 and 3 show the phytochemical screening and the antibacterial activity of the methanol and hexane extracts, respectively. The results shown a antibacterial activity clinically non relevant for the both plants. However, table 4 shows the modulatory antibiotic activity of the extracts against norfloxacin. When the extracts were incorporated into the growth medium with a sub-inhibitory concentration of 32 µg/ml (1/8 MIC), was detected a enhancement of the inhibition zone varying between 0 and 50%, if compared with the norfloxacin inhibition zone alone. This results can indicates that these extracts affected the efflux capacity of the pump NorA, identified as the cause of the resistance of the strain SA1199B against norfloxacin.

Discussion

The mechanisms by which extracts can inhibit the growth of microorganisms are varied, and can be due in part to the hydrophobic nature of some components. As a result, they can show greater interaction with the lipid bilayer of the cell membrane, affecting the respiratory chain and the

Table 1. Dry mass and yield of methanol and hexane extracts (g)

Species	Solvent used	Leaves	Yield
Croton campestris A.	Methanol (MECC)	31,2	1,74
	Hexane (HECC)	72,38	0,87
Ocimum gratissimum L.	Methanol (MEOG)	58,82	2,49
	Hexane (HEOG)	115,28	1,49

MECC: Methanol extract of *Croton campestris*; HECC: Hexane extract of *Croton campestris*; MEOG: Methanol extract of *Ocimum gratissimum*; HEOG: Hexane extract of *Ocimum gratissimum*

Table 2. Phytochemical prospection of methanol and hexane extracts of *C. campestris A. and O. gratissimum L.*

Metabolites extracts	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
MECC	-	-	+	-	-	+	+	+	+	+	+	-	-	+	+	+
HECC	-	-	+	-	-	+	+	+	+	+	+	-	+	+	-	+
MEOG	-	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+
HEOG	-	-	+	-	-	+	+	+	+	+	+	+	+	-	-	+

1: phenols; 2: tannin pyrogallates; 3: tannin phlobaphenes; 4: anthocyanins; 5: anthocyanidins; 6: flavones; 7: flavonols; 8: xanthones; 9: chalcones; 10: aurones; 11: flavononols; 12: leucoanthocyanidins; 13: catechins; 14: flavonones; 15: alkaloids; 16: terpenes, (+) presence,(-) absence

MECC: Methanol extract of *Croton campestris*; HECC: Hexane extract of *Croton campestris*; MEOG: Methanol extract of *Ocimum gratissimum*; HEOG: Hexane extract of *Ocimum gratissimum*

Table 3. Minimum inhibitory concentration values (µg/ml) of extracts against *Staphylococcus aureus* ATCC25923 and *Staphylococcus aureus* 358

Extracts	SA358	SA-ATCC25923
MECC	≥1024	512
HECC	≥1024	512
MEOG	≥1024	512
HEOG	≥1024	≥1024

MECC: Methanol extract of *Croton campestris*; HECC: Hexane extract of *Croton campestris*; MEOG: Methanol extract of *Ocimum gratissimum*; HEOG: Hexane extract of *Ocimum gratissimum*

Table 4. Enhancement of antibiotic activity of norfloxacin by the extracts of *C. campestris A. and O. gratissimum L.* (32 µg/mL)

	S. aureus 1199B (mm ± SD)	
	Norfloxacin	Enhancement (%)
No treatment	8 ±0	ND
DMSO	8 ± 0	0
MECC	9 ± 0	12,5
HECC	12 ± 0	50
MEOG	9,5 ± 0,5	12,5
HEOG	10 ± 0	25

MECC: Methanol extract of *Croton campestris*; HECC: Hexane extract of *Croton campestris*; MEOG: Methanol extract of *Ocimum gratissimum*; HEOG: Hexane extract of *Ocimum gratissimum*

production of energy (25) or even make the cell more permeable to antibiotics, leading to the interruption of vital cellular activity (26,27). Various components of extracts can permeate the cell membrane, increasing the penetration of antibiotics (28). The interference with bacterial enzyme systems can also be a potential mechanism of action (29). These mechanisms of action can be obtained by the combination of antibiotic with extract at a sub-inhibitory concentration applied directly to the culture medium (1,4).

This strategy is called "herbal shotgun" or "potentiation of activity 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 various targets, where the different therapeutic components collaborate in a potential mechanism of action manner. This approach is not only for combinations of extracts; combinations between natural products or extracts and synthetic products or antibiotics are also possible (30).

The results obtained indicate that *Croton campestris A.* and *Ocimum gratissimum L.* could serve as a source of plant-derived natural products that modify antibiotic resistance, maybe affecting the efflux pump NorA, for use against multidrug-resistant bacteria.

Conflict of interest

The authors have not conflict of interest to disclose.

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