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Full Length Research Paper

Modulatory antibiotic activity and chemical composition of hydroalcoholic extract of *Croton campestris*

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***Croton campestris A.* St.-Hill., known as "Velame do campo" (Euphorbiaceae), native of the Brazilian Northeastern region, is used in the popular medicine as decoction against several diseases as inflammations, hematological disturbances, flu and gastritis. The study analyzed the chemical composition and the antibacterial activity against bacterial strains of *Escherichia coli* and *Staphylococcus aureus* of the hydroalcoholic extract of the leaves of the *C. campestris* (HEFCC). The inhibition of the bacterial growth was demonstrated for the bacteria tested with MIC values ≥ 1024 µg/ml. When combined with aminoglycosides (gentamicin, kanamycin, amikacin and neomycin), the extract demonstrated synergistic effect. This result showed that HEFCC can be used as promisor font of new antimicrobial compounds for pathogenic diseases.**

Key words: *Croton campestris,* antimicrobial activity, modulatory aminoglycoside effect, antimicrobial activity, antibiotic, synergism.

INTRODUCTION

With the increase of the incidence of bacteria resistant to antibiotics, the natural products from medicinal plants can represent an interesting alternative (Lu et al., 2007; Mbwambo et al., 2007). Some extracts and phytocompounds are known by their antimicrobial properties. In the last years several studies were accomplished, in different countries, demonstrating this effectiveness (Singh et al., 2007; Benoit-vical et al., 2006). Several combinations of substances with natural and synthetic chemical compounds, as antibiotics, have been presenting direct activity against a lot of bacteria, thereby increasing the activity of a specific antibiotic and reverting the bacterial resistance to certain antibiotics. The effects of these combinations characterized these compounds as modifiers of antibiotic activity (Wolfart et al., 2006; Molnar et al., 2004).

Among the antibiotic resistant bacteria, *Staphylococcus* was recognized as a preoccupying one (Georgopapadakou, 2002; Nostro et al., 2004). For patients, the bacterial resistance increases the morbidity/ mortality, while it increases the costs for institutions (Dancer, 2001; Coutinho et al., 2005). *Staphylococcus aureus* is a commensal microorganism usually mentioned as a cause of infectious diseases as infected wounds, pneumonias and sepsis (Chambers, 2001; Diekema et

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al., 1999; Hiramatsu et al., 2002). Several hospital infections are correlated with this bacterium, especially in ICUs (Gibbons, 2004).

The diarrheic diseases constitute one of the main problems of public health in the world, with more than 2 million deaths a year (Davidson et al., 2002). This process can be caused by different microorganisms as virus, bacteria (for example, *Escherichia coli*) and parasites (Hien et al., 2008). *E. coli* is typical of the intestinal and commensal of the vaginal flora. The incidence of neonatal of infections for this microorganism is a serious problem. The main cause of these infections is the maternal transmission during the birth (Watt et al., 2003).

The genus *Croton* (Euphorbiaceae) is subdivided in 40 genus and 1,300 different species around of the world. In Brazil were observed approximately 350 species, distributed in 29 different genus (Berry, 2006; Cordeiro and Carneiro, 2006). Several species of *Croton* are used by the traditional medicine in Africa, Asia and South America. Such uses include cancer treatment, intestinal constipation, diarrhea and other digestive problems as ulcer, diabetes, external wounds, fever, hypercholesterolemic, antihypertensive, anti-inflammatories, antimicrobial, intestinal worms, anti-spasmodic, malaria, pain, ulcers and obesity (Salatino et al., 2007). *Croton campestris* is a shrub found mainly in the southeastern and northeastern regions (Santos et al., 2005). This plant is known as “Velame do campo or Curraleira”. Ethnobotanical studies indicate that leaves and roots are used as tea in the popular medicine against inflammatory, parasitic, venereal diseases (Ribeiro Prata et al., 1993), eczemas, tumors, skin diseases, rheumatism, ulcer in the uterus and diarrhoea (Santos et al., 2005).

The objective of this work was to realize the phytochemical screening and evaluate the antibacterial and modulatory activity associated with the aminoglycoside of the hydroethanolic extract of the leaves of the *C. campestris*.

MATERIALS AND METHODS

Drugs, chemical, apparatus and general procedures

Gentamicin, amikacin, kanamycin and neomycin were obtained by SIGMA Co. (St. Louis, MI). The solutions of the antibiotics were prepared with base in the recommendations of the Clinical and Laboratory Standards Institute (NCCLS, 2003).

Bacteria

The bacterial strains used were *E. coli* (EC27) and *S. aureus* (SA358), both clinical isolates with resistance profile identified and showed in the Table 1.

All of the strains were maintained in slants with heart infusion agar (HIA, Difco Laboratories Ltda.). Before the tests, the cells were cultivated overnight at 37°C in brain heart infusion (BHI, Difco Laboratories Ltda.).

Plant material

The leaves of *C. campestris* St.-Hil were collected in the county of Crato, Ceará, Brazil, in the follow GS location: South: 7°22’2,8’’; west: 39°28’42,4’’. The botanical material was identified and a voucher was deposited under the number #7095, in the Herbarium of the Federal University of Rio Grande do Norte - UFRN.

Preparation of hydroalcoholic extract of dried leaves of *Croton campestris*

For preparation of extracts, leaves were collected, dried and weighed. The mass (852 g) was powdered to increase the contact surface and packaged in a container with the solvent (water / ethanol – proportion 1:1). The volume was used to submerge the material, after which it was done for 72 h. After this period, the eluent was filtered through filter paper to separate solid waste and concentrated on rotary vacuum (Brasileiro et al., 2006), condensed (model Q-344B - Quimis, Brazil) with a water bath (model Q-214M2 - Quimis, Brazil) and lyophilized during 48 h. The dry power (70.89 g) was used in preparation of the solutions in the concentration of 10 mg/ml, dissolved in DMSO (dimethyl sulfoxide), then diluted with distilled water to a concentration of 1024 µg/ml.

Phytochemistry

The phytochemical tests to detect the presence of different class of secondary metabolites as: glycosides, saponins, tannins, flavonoids, steroids, triterpenes, coumarins, quinones, organic acids and alkaloids were carried out using the method described by Matos (1997). The tests are based on the visual observation of the color changed or precipitated after the addition of specific reagents, as shown in Table 2.

Antimicrobial activity test

The antibacterial activities of the extracts were investigated by microdilution method, as recommended by Javadpour et al. (1996) using two strains *E. coli* (EC27) and *S. aureus* (SA358). Inoculums of each bacterial strain were suspended in Brain Hear Infusion Broth (BHI 3.8%) and used for bacterial growth (24 h, 35±2°C). After this, the suspension was diluted to 1×10^6 UFC/ml in 10% BHI. 100 µl of each dilution were distributed in 96-well plates plus extracts in different concentrations, obtaining 5×10^5 CFU/ml as the final concentration. The extracts were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1024 µg/ml. Further, serial dilutions were performed to obtain a final concentration in the range of 512^{-8} µg/ml. All experiments were performed in triplicate, and the microdilution trays were incubated at 35±2°C for 24 h. For the evaluation of the modulator of antibiotic resistance, the MICs of the antibiotics were determined in the presence and absence of the extract at a sub-inhibitory concentration (MIC/8). The isolate clinical bacteria strains EC27 and SA358 were assayed with four different aminoglycosides with final concentrations varying of 2500^{-1} µg/ml. Antibacterial activity was detected using a colorimetric method by adding 25 µl of resazurin staining (0.01%) aqueous solution in each well at the end of the incubation period. The minimal inhibitory concentration (MIC) was defined as the lowest, in that the extracts were able to inhibit the bacteria growth, as indicated by resazurin staining.

RESULTS AND DISCUSSION

Several works have been confirming the promising use of natural products as antimicrobial agents (Gibbons, 2004). Also, the clinical importance of the hospital infections was caused by multiresistant infectious agents. This fact has pushed the researchers to investigate on the antimicro

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

Bacteria	Origin	Resistance
Escherichia coli EC27	Surgical wound	Ast, Ax, Amp, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Clo, Im, Can, Szt, Tet, Tob
Staphylococcus aureus SA358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net

Ast - Aztreonan; Ax – Amoxicillin; Amp - Ampicillin; Ami - Amikacin; Amox – Amoxicillin; Ca - Cefadroxil; Cfc - Cefaclor; Cf - Cephalothin; Caz - Ceftazidime, CIP - Ciprofloxacin; Chl - Chloramphenicol; Im - Imipenem; Can - Kanamycin; SZT – Sulphametrim; Tet - Tetracycline; Tob - Tobramycin; Oxa - Oxacillin; Gen - Gentamicin; Neo - Neomycin; To - Paramomicina; But - Butirosin; Sis - Sisomicin; Net – Netilmicin.

Table 2. Phytochemical analysis of hydroalcoholic extracts of stems and leaves from *Croton campestris* A. St.-Hil.

	Metabolite																
Extract	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
HEFCC	-	-	+	-	-	+	+	+	-	-	+	-	-	+	+	+	-

The presence (+) or absence (–) of the different metabolites is indicated. 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 - Leukoanthocyanidins; 13 - Catechins; 14 - Flavonones; 15 - Alkaloids; 16 - Terpenes; 17 - Steroids; and 18 - Flavonones.

bial potential of natural products (Hernandéz et al., 2003; Duarte et al., 2005).

Through phytochemical prospecting of the extracts, it was possible to determine the presence of diverse classes of secondary metabolites (Table 2). Diverse class of secondary metabolites present in the extract have been a wide variety of biological activities such as antimicrobial (Esquenazi et al., 2002), antioxidant (Barreiros and David, 2006), antitumor, anti-ophidic (Okuda et al., 1989) and photosensitizing (Matias et al., 2010a, d). Phytochemical studies indicate the presence of alkaloids in species of the genus *Croton.* These metabolites present a well known antimicrobial activity (Ribeiro et al., 1993; Tsacheva et al., 2004). About the tannins, the antimicrobial properties appear to be associated with the hydrolysis of an ester bond with gallic acid, thereby serving as a mechanism of natural defense against microbial infections. The antimicrobial property of tannic acid can also be utilized in food processing to increase shelf life. The tannin components of epicatechin and catechin (*Vaccinium vitisidaea* L.) demonstrated strong anti-microbial activity against bacteria and fungi (Ho et al., 2001). Flavonoids are synthesized by plants in response to microbial infection (Dixon et al., 1983) and are effective against a broad range of microorganisms. The activity is probably due to their capacity to form complexes with extracellular soluble proteins, which bind to the bacterial cell wall. Some lipophilic flavonoids can also cause rupture of the plasma membrane of microorganisms (Tsuchiya et al., 1996). Terpenes occur in the form of diterpenes, triterpenes, tetraterpenes as well as hemiterpenes and sesquiterpenes. Terpenenes or terpenoids are active against bacteria (Ahamd et al., 1993). The seeds are rich in these active components; for example, volatile oil and thymoquinone afford protection against nephrotoxicity and hepatotoxicity induced by any disease or chemical product (Ali et al., 2003).

HEFCC demonstrated antimicrobial activity against the tested bacterial strains, with values of MIC ≥ 1024 µg/ml (Table 3). However, this result cannot be considered to be clinically significant, due to the fact that a relevant value of MIC is lower than 1 mg/ml (Houghton et al., 2007). However, important modulatory effect aminoglycosides could be observed, being the MIC of the antibiotics in the presence of HEFCC reduced in a range between 87.5 to 99.9%. Several studies have been revealing the interesting antimicrobial activity associated to species of the genus *Croton*, as the antimicrobial activity of the essential oil of the leaves of the *Croton zehntneri* against *E. coli*, *Shigella flexneri* and *S. aureus* (Costa et al., 2006). However, this study was performed using the disk diffusion technique, being the microdilution method employed in this work the more accepted technique to evaluate antibacterial activity (Hadacek and Greger, 2000). The results presented in this study confirm the data found in several works with *C. campestris* about its antibacterial activity (Matias et al., 2010b, c; Coutinho et al., 2010).

Some works have reported synergism between flavonoids and conventional antibacterial agents against resistant strains (Cushnie and Lamb, 2005). According to Cushnie and Lamb (2005), the antibacterial activity of flavonoids has been increasingly documented. Many researchers have isolated and identified flavonoids that present antibacterial activity, such as quercetin, 3-O-methylquercetin and various glycosides of quercetin (Rauha et al., 2000; Basile et al., 2000; Arima and Danno, 2002).

Table 3. MIC values (µg/ml) of aminoglycosides in the absence and presence of HEFCC in *Escherichia coli* 27 and *Staphylococcus aureus* 358.

Antibiotics	EC27			SA358		
	MIC alone	MIC combined	Reduction (%)	MIC alone	MIC combined	Reduction (%)
Gentamicin	2500	2.4	99.9	39	9.7	87.5
Kanamycin	2500	156.2	93.8	2500	156.2	93.8
Amikacin	156.2	19.5	87.5	78.1	9.7	87.5
Neomycin	312.5	9.7	96.9	312.5	39	87.5
Extract						
HEFCC	≥ 1024	-	-	≥ 1024	-	-

The mechanisms by which extracts can inhibit the growth of microorganisms are several, 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 production of energy (Nicolson et al., 1999), or even make the cell more permeable to antibiotics, leading to the interruption of vital cellular activity (Burt, 2004). Several compounds of extracts can permeabilize the cell membrane, increasing the penetration of antibiotics (Helander et al., 1998). The interference with bacterial enzyme systems can also be a potential mechanism of action (Wendakoon and Sakaguchi, 1995). 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 (Coutinho et al., 2008a, b). Comparatively, the natural products may have a different antibacterial activity, when we consider the existence of differences in polarity on the secondary metabolites (Matias et al., 2010b, c). It is necessary to consider that studies have been demonstrating antimicrobial properties attributed to the flavonoids (Rauha et al., 2000; Sohn et al., 2004).

The results obtained in this study revealed the modulatory of antibiotic activity of *C. campestris* St.-Hil., being a promising natural source of antimicrobial products. This indicated the necessity of more projects to evaluate the possibility of the developing drugs when combining the natural product and antibiotics to combat the multiresistant bacterial infectious agents.

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