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Artigo

Antibacterial and cytotoxic properties of some plant crude extracts used in Northeastern folk medicine

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RESUMO: "Propriedades antibacterianas e citotóxicas de alguns extratos de plantas usados na medicina popular nordestina". No presente estudo, 32 extratos hexânicos e etanólicos de *Protium bahianum, P. heptaphyllum, Croton sellowii, C. rhamnifolius, C. jacobinensis, C. micans* e *Muntingia calabura,* foram avaliados para atividade antibacteriana, pelo método de difusão em disco. Ensaios de citoxicidade foram realizados com o modelo do microcrustáceo *Artemia salina* Leach para determinar a concentração letal para 50% dos indivíduos (CL_{50} µg/mL). A presença de atividade antibacteriana foi observada com os extratos hexânicos das flores de *M. calabura* contra *B. subtilis*, e extratos etanólicos das folhas contra *S. aureus* and *B. subtilis* na concentração de 1 mg/mL. Dentre os 32 extratos, 19 apresentaram toxicidade baixa ou ausente (CL_{50} > 250 µg/mL), 6 mostraram toxicidade moderada (CL_{50} entre 80 µg/mL e 250 µg/mL) e 7 foram muito tóxicos (CL_{50} < 80 µg/mL).

Unitermos: *Artemia salina*, citotoxicidade, atividade antibacteriana, *Croton*, *Protium*, *Muntingia calabura*.

ABSTRACT: In the present study, 32 hexane and ethanol extracts of *Protium bahianum, P. heptaphyllum, Croton sellowii, C. rhamnifolius, C. jacobinensis, C. micans* and *Muntingia calabura* were screened for antibacterial activity by the disc-diffusion method. Cytotoxicity assays using the brine shrimp *Artemia salina* Leach as a model were performed to determine lethal doses for 50% of individuals (LC_{50} µg/mL). Antibacterial activity was found in flowers hexane extracts of *M. calabura* against *B. subtilis*, and leaves ethanol extracts against *S. aureus* and *B. subtilis* at concentration of 1mg/mL. Among 32 extracts, 19 showed low or no toxicity (LC_{50} > 250 µg/mL), 6 showed moderate toxicity (LC_{50} between 80 µg/mL and 250µg/mL), and 7 were highly toxic (LC_{50} < 80 µg/mL).

Keywords: *Artemia salina,* cytotoxicity, antibacterial activity, *Croton*, *Protium*, *Muntingia calabura*.

INTRODUCTION

Brazilian flora is worldwide known to be a source of chemical substances with biological activity (Barbosa-Filho et al., 2007; Corrêa et al., 2008; Sousa et al., 2008). Plant crude extracts have been used in folk medicine for treatment of abscesses, insect bite, mycosis, genital infections, anti-inflammatory, antihelminthic, diarrhea and cough (Nakamura et al., 1999; Leal-Cardoso et al., 1999; Holetz et al., 2002). This potential has been exploited by the pharmacological industry on the production of new analgesic, anticarcinogenic and antimicrobial drugs (Bruneton, 1999). The search for new antimicrobial compounds is particularly motivated by the great diversity of Brazilian plant species whose biological activities are not completely known (Alves et al., 2000; Cardoso-Lopes et al., 2008).

Plant extracts and essential oils have been broadly reported to inhibit growth of both fungi and bacteria (Adam et al., 1998; Bara; Vanetti., 1998; Nascimento, 2000; Volpato et al., 2001; Vieira et al., 2001; Holetz et al., 2002; Loguercio et al., 2005; Costa

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et al., 2008). For example, Suffredini et al (2004) have shown among 705 organic and aqueous extracts of plants obtained from Amazon Rain Forest and Atlantic Forest, many were active against *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli*. As well, antibacterial and antifungal activities against pathogenic microorganisms of human medical importance such as *Staphylococcus aureus*, *Streptococcus sobrinus*, *Porphyromonas gingivalis, Streptococcus mutans, Candida albicans* and *Cryptococcus neoformans* were recently described for essential oil of *Croton* species (Alviano et al., 2005; Fontenelle et al., 2008). Similar studies have lead to identification of antimicrobial compounds in *Croton* species, such as hardwickic and 3,4-secotrachylobanoic acids, linalool, alpha-phellandrene, alpha-pinene and p-cymene (McChesney et al. 1991; Martins et al. 2000; Alviano et al. 2005).

The genus *Croton* (Euphorbiaceae) comprises about 1,000 species widespread in the Northeastern Region of Brazil with many species used in folk medicine for treatment of inflammations, wound infections, hypertension, ulcers, cancer, rheumatism and malaria (Marini-Bettolo; Scarpati, 1979; Burke et al., 1981; Peres et al., 1997; Bighetti et al., 1999; Hiruma-Lima et al., 2002; Agra et al., 2007; 2008). Likewise, genus *Protium* (Burseraceae) has a broad range use in folk medicine as tonic, stimulant, anti-inflammatory, and analgesic, for treatment of cough, ulcer and hemorrhages (Costa, 1975; Correa, 1978; Pott & Pott, 1994; Siani et al., 1999; Brandão et al., 2008). Moreover, flowers of *M. calabura* have been used as anti-septic, anti-spasmodic, antidyspeptic, diaphoretic, tranquilizer, tonic and for the treatment of headache, whereas roots are employed as emmenagogue and abortifacient (Correa, 1978; Kaneda et al., 1991).

Considering *in vitro* tests are the first step on elucidation of biological properties of plant crude extracts and for selection of substances of pharmacological interest, in the present study we evaluated 32 plant extracts of *Croton* and *Protium* species, and also *M. calabura*, ordinarily used in the Northeastern folk medicine.

MATERIAL AND METHODS

Plant material

Table 1 shows the plant species used in this study. The botanical identification was provided by Dr Carmen Zickel from the Universidade Federal Rural de Pernambuco (UFRPE). Each plant species received a voucher number and was deposited in the Vasconcelos Sobrinho Herbarium at the Biology Department.

Obtaining plant extracts

The methodology used was adapted from Matos (1988). Leaves and stem of *Croton* and *Protium* species, and flowers, fruits, leaves and stem of *Muntingia calabura* were selected and dried in oven at 45 ºC. Dried parts were ground to a fine powder and submitted to successive extractions with hexane or ethanol during 24 h. Then, the solvents were evaporated at reduced pressure and the extracts were weighed and suspended in chloroform to obtain a stock solution of 50 mg/mL (w/v).

Microbial strains

The gram-negative bacterial strains *Escherichia coli, Salmonella enterica* Subsp. *enterica* Var. Typhi (ATCC - 6534) and *Pseudomonas aeruginosa* (ATCC - 27853) and Gram-positive *Staphylococcus aureus* (ATCC - 6538) and *Bacillus subtilis* (ATCC - 6633) were used as test organisms. All bacteria were grown in Nutrient broth at 37 °C and maintained in Nutritive agar at 4 C until use.

Antibacterial tests

Antibacterial tests were carried out by the disc diffusion method (Bauer et al., 1966). Briefly, bacterial strains were grown in Brain Heart Infusion broth (Oxoide) for 24 h at 37 °C. Cultures were adjusted to 10^8 ml^{-1} cells (0.5 of the MacFarland Standard) and added to Petri dishes containing Müeller Hinton agar (Oxoide) using a sterile swab. Sterile filter paper disks, 6 mm in diameter, were embedded with 20 µL of plant extracts (corresponding to 1 mg from extract stock solution) and added onto plates. Before incubation, Petri dishes were left at room temperature for 30 min to facilitate diffusion of extracts on medium. All assays were carried out in duplicate. Discs with 20 µL chloroform were used as control. Diameter inhibition zone was measured in millimeters, using a a caliper, after 18 h incubation at 37 ºC. In all experiments a diameter zone inhibition of 10 mm or smaller was not considered.

Cytotoxicity assay

Citotoxicity evaluation of plant extracts was carried out using the brine shrimp *Artemia salina* Leach as model (Carvalho et al., 1988; Meyer et al., 1982; Silva et al., 2007; Nunes et al., 2008). Eggs of *A. salina* were incubated in Marine Salt solution (2.5%) and exposed to a lamp of 45 W by three days for egg's hatching. Then, metanauplii were collected and transferred to tubes containing 5 mL Marine Salt solution 2.5% (10 nauplii/tube) containing plant extracts in several concentrations (25, 50, 75, 100, 150, 200 e 250 µg/mL) plus 1% DMSO (dimethyl-sulfoxide). Tubes were incubated in a dark

Table 1. Plants selected for antimicrobial and toxicity evaluation.

Scientific name (Voucher number)	Popular name	Family	Popular use	Part tested
Protium bahianum Daly (46380)	Breu/Almécega	Burseraceae	Analgesic	Leaves, stem
Protium heptaphyllum March. (46329)	Almécega	Burseraceae	Antioxidant	Leaves, stem
Croton sellowii Baill (45622)	Marmeleiro	Euphorbiaceae	Anti-inflammatory	Leaves, stem
Croton rhamnifolius Kunth. (45552)	Velame	Euphorbiaceae	Ulcer treatment	Leaves, stem
Croton jacobinensis Baill (45553)	Marmeleiro branco	Euphorbiaceae	Anti-hypertensive	Leaves, stem
Croton micans Sw. (45553)	Umbuzeiro	Euphorbiaceae	Diarrhoea control	Leaves, stem
Muntingia calabura L. (2816)	Pau de Seda	Muntingiaceae	Antispasmodic	Flower, fruit, leaves, stem

*References included in the text.

room at 28 °C and *A. salina* survival was observed 24 h later. Control tubes with 1% DMSO did not contain plant extracts. Experiments were carried out in triplicates.

Toxicity was measured in terms of LC_{50} (lethal concentration for 50% of metanauplii) and according to Dolabela (1997): LC_{50} < 80 µg/mL, was considered highly toxic; between 80 µg/mL and 250 µg/mL, moderately toxic; LC_{50} > 250 µg/mL, low toxicity or non toxic. Extracts that have not show 50% mortality in any of the tested concentration were considered non toxic.

Statistical analysis

Lethal doses of the plant extracts were determined using LC_{50} software program of E-Views version 4.1, based on Finney's Probit Analysis Statistical method (Finney, 1971).

RESULTS AND DISCUSSION

Our data have shown *Croton* and *Protium* extracts did not carry inhibitory activity against the tested bacteria at concentration of 1 mg per disc. This high tested concentration was useful considering that bioactive compounds in plant extracts are present at distinct proportions. Although a small inhibitory halo (smaller than 10 mm in diameter) was observed by some *Croton* and *Protium* species against the *B. subtilis* and *S. aureus* (data not shown), it was not considered for further data analyses. On the other hand, a growth inhibition zone was measured in flowers hexane extracts of *M. calabura* against *B. subtilis* (11.0 mm ± 0.5), and leaves ethanol extracts against *S. aureus* (13.0 mm ± 0.5) and *B. subtilis* (11.0 mm ± 0.5). Although it was not the aim of the present study to evaluate the effect of extracts against yeasts, we have found flower hexane extracts of *M. calabura* also inhibited *in vitro* growth of *Candida albicans* (19.5 mm ± 0.5) and *Cryptococcus neoformans* (12.5 mm ± 0.5).

The lack of antimicrobial activity in the *Croton* and *Protium* species evaluated here could be attributed to the presence of distinct compounds present at different amounts in plant extracts. For example, Palmeira et al. (2003) have shown caryophyllene oxide (46.8%) and *trans*-caryophyllene (40.8%) were the most abundant compound in the leaves, whereas caryophyllene oxide (26.5%) and cubenol (16.7%) were the main constituents in the stems of *C. sellowii*. On the other hand, antimicrobial activity for *Croton* species have been previously reported for *C. nepetaefolius* containing methyl-eugenol (15.7%) and bicyclogermacrene (14.1%) as main constituents, and *C. argyrophylloides* containing spathulenol (20.3%) and bicyclogermacrene (11.7%) (Fontenelle et al., 2008). Thus, antimicrobial activity in plant species depends on part of the plant studied as well as on solvent used for the extraction procedures.

Cytotoxicity assays revealed nineteen extracts have shown low or no toxicity (LC_{50} > 250 µg/L) whereas thirteen showed toxicity ranging from moderate (LC_{50} between 80 and 250 µg/L) to highly toxic (LC_{50} < 80 µg/L). The LC_{50} for each extract are shown in table 2. There was a high toxicity in stem ethanol extracts of *C. jacobinensis* and *C. rhamnifolius*. Stem hexane and ethanol extracts and leaves ethanol extract from *C. selowii* were also highly toxic as well as flowers and leaves hexane extract of *M. calabura* (Table 2). *Croton rhamnifolius* (leaves and stem hexane extracts) and *C. micans* (leaves ethanol and stem hexane extracts) showed moderate toxicity whereas *Protium bahianum* (leaves ethanol extract), *C. jacobinensis* (stem hexane extract) and *C. micans* (leaves hexane extract) showed low toxicity. *P. heptaphyllum* and all the other extracts have shown low or no toxicity regardless of the concentrations tested.

The brine shrimp *Artemia salina* Leach is a useful tool for toxicity evaluation of plant extracts (Carvalho et al., 1988; Alves et al., 2000). This *in vitro* model has a good correlation between tests using mice (Logarto et al., 2001). Among 32 plant extracts tested in this study, extracts from *Croton* species were the most toxic (Table 2). Toxicity in some *Croton* species was reported and seems to be due to the presence of diterpenoids (Rodriguez et al., 2004; Giang et al., 2005). Toxicity was not limited to a specific anatomic part of the plant or a particular secondary metabolite. On the other hand, *Muntingia calabura* toxicity to *A.*

Table 2. Toxicity of plant extracts against nauplii of *Artemia salina* Leach.

Plant species	Extract	LC_{50} µg/mL	Toxicity classification
Protium bahianum	Leaves hexane	> 250	NT* or low
	Leaves ethanol	282.36	NT or low
	Stem hexane	> 250	NT or low
	Stem ethanol	> 250	NT or low
Protium heptaphyllum	Leaves hexane	> 250	NT or low
	Leaves ethanol	> 250	NT or low
	Stem hexane	> 250	NT or low
	Stem ethanol	> 250	NT or low
Croton sellowii	Leaves hexane	> 250	NT or low
	Leaves ethanol	32.28	High
	Stem hexane	31.25	High
	Stem ethanol	53.84	High
Croton rhamnifolius	Leaves hexane	215.47	Moderate
	Leaves ethanol	> 250	NT or low
	Stem hexane	148.32	Moderate
	Stem ethanol	0.15	High
Croton jacobinensis	Leaves hexane	> 250	NT or low
	Leaves ethanol	> 250	NT or low
	Stem hexane	322.97	NT or low
	Stem ethanol	6.37	High
Croton micans	Leaves hexane	333.71	NT or low
	Leaves ethanol	203.78	Moderate
	Stem hexane	238,27	Moderate
	Stem ethanol	> 250	NT or low
Muntingia calabura	Flowers hexane	41.2	High
	Flowers ethanol	244.5	Moderate
	Fruits hexane	> 250	NT or low
	Fruits ethanol	169.96	Moderate
	Leaves hexane	66.44	High
	Leaves ethanol	> 250	NT or low
	Stem hexane	> 250	NT or low
	Stem ethanol	> 250	NT or low

* NT = non toxic

salina varied from low to high, depending on the part of the plant used in assay. Previously, Kaneda et al. (1991) reported cytotoxicity to cultured P-388 cells and some human cancer cell lines for *M. calabura* flavonoids.

Taken together, our data revealed flower hexane extracts of *M. calabura* seem to correlate in cytotoxicity and antimicrobial activity against *B. subtilis*. On the other hand, leaves ethanol extracts of *M. calabura* were not toxic, but inhibited *in vitro* growth of *S. aureus* and *B. subtilis* as well. However, at the present we cannot indicate the significance of such data; this work highlights the potential of *M. calabura* for new pharmacological studies.

CONCLUSION

In the present work, we reported new data about the antibacterial activity and toxicity of plant extracts not reported before. The results here are a starting point for new studies concerning the knowledge of the biological activities of plants from Northeastern Brazil.

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