Antimicrobial activity of *Anonna mucosa* (Jacq.) grown *in vivo* and obtained by *in vitro* culture

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Abstract

Brazilian flora includes numerous species of medicinal importance that can be used to develop new drugs. Plant tissue culture offers strategies for conservation and use of these species allowing continuous production of plants and bioactive substances. *Annona mucosa* has produced substances such as acetogenins and alkaloids that exhibit antimicrobial activities. The widespread use of antibiotics has led to an increase in multidrug-resistant bacteria, which represents a serious risk of infection. In view of this problem, the aim of this work was to evaluate the antibacterial potential of extracts of *A. mucosa* obtained by *in vitro* techniques and also cultured under *in vivo* conditions. Segments from seedlings were inoculated onto different culture media containing the auxin picloram and the cytokinin kinetin at different concentrations. The calluses obtained were used to produce cell suspension cultures. The materials were subjected to methanol extraction and subsequent fractionation in hexane and dichloromethane. The antimicrobial activity against 20 strains of clinical relevance was evaluated by the macrodilution method at minimum inhibitory and minimum bactericidal concentrations. The extracts showed selective antimicrobial activity, inhibiting the growth of *Streptococcus pyogenes* and *Bacillus thuringiensis* at different concentrations. The plant tissue culture methods produced plant materials with antibacterial properties, as well as *in vivo* grown plants. The antibacterial activity of material obtained through biotechnological procedures of *A. mucosa* is reported here for the first time.

Key words: antibacterial properties, Annonaceae, medicinal plant, callus cultures, cell suspension cultures.

Introduction

*Annona mucosa* (Jacq.), a tree species belonging to the Annonaceae family, has great economic value associated with its medicinal properties and food (Lorenzi, 2002). Trees in the Annonaceae family have several groups of metabolites with medicinal activities. Among these, we highlight acetogenins, alkaloids, and lignans (Figueiredo et al., 1999; Reyes et al., 2002). Alkaloids of the Annonaceae species have been isolated and evaluated for their medicinal properties, showing antibacterial modulation (Costa et al., 2013), antiplasmodial (Osorio et al., 2006) and antileishmanial (Vila-Nova et al., 2011) activities, and tumor cell cytotoxicity (Costa et al., 2012).

The indiscriminate use of antibiotics to treat infections has led to an increase in antimicrobial resistance to different drugs. Brazil has a large number of resistant bacteria among most of its key pathogens (Rossi, 2011); such a loss of effective antibiotics leads to complications in the treatment of vulnerable patients. With the decreasing effectiveness of antibiotics to control bacterial infections, the search for new alternatives to the use of synthetic substances is necessary. Plants are the greatest source of substances with pharmaceutical potential on the planet and a
significant source of natural antimicrobial compounds (Abdallah, 2011)

The use of plant extracts or their compounds represents an important alternative to synthetic antimicrobials. However, when the species of medicinal interest is a tree, the exploitation for medicinal purposes becomes limited due to the long life cycle, time to produce a determined metabolite, and difficulties in growing and handling (Assis and Teixeira, 1998). The use of biotechnological approaches such as plant tissue culture techniques to improve the exploitation of the medicinal potential of plants makes the continuous and large-scale production of bioactive substances possible (Lourenço, 2003).

Among these techniques, callogenesis is a process that involves the use of certain plant growth regulators in order to induce dedifferentiation, proliferation, and formation of cell masses from an organ or tissue cultured in vitro on a specific medium. The plant segments, called explants, used in in vitro responses, can be obtained from any part of the plant (Torres et al., 2000).

The cellular mass called callus can be used for various purposes, such as a source of inoculum to obtain cell suspensions in liquid culture medium (Andrade, 2002). These cultures can provide significant amounts of bioactive substances in a few weeks, which enable quick and economic production (Amaral and Silva, 2003).

Thus, the aim of this study was to investigate the antimicrobial activity of methanol extracts of A. mucosa grown in vivo and obtained by in vitro culture protocols against 20 different bacterial strains. This is the first study focusing on this evaluation for A. mucosa.

Material and Methods

In vitro culture of A. mucosa

Hypocotyl and leaf explants obtained from seedlings grown under in vivo conditions were inoculated onto culture media in order to induce callus formation. Disinfection procedures and culture conditions were conducted according to Figueiredo et al. (2000).

Hypocotyl explants were inoculated onto Wood Plant Medium (WPM) (Lloyd and McCown, 1980) supplemented with 2 μM of the auxin picloram and 0.02 μM of the cytokinin kinetin. For leaf explants, culturing was performed on Murashige and Skoog Medium (MS) (Murashige and Skoog, 1962) supplemented with 10 μM of picloram and 0.1 μM of kinetin. The media were prepared with 30 g.L⁻¹ of sucrose and solidified with 8 g.L⁻¹ of agar, with the pH adjusted to 5.8. The cultivation was carried out in the absence of light at 26 ± 2 °C for 90 days, with monthly subculture.

The cell suspension cultures were prepared by inoculating 2 g of the callus mass obtained from hypocotyl explants in liquid WPM medium with the same composition of growth regulators. Cultures were maintained in the dark in the culture room at 26 ± 2 °C under constant shaking at 114 rpm for five weeks.

Extracts preparation

Leaves and stems of specimens grown in vivo, as well as callus and cell suspension cultures, were fragmented and dried at 40 °C for 48 h. Extraction with methanol (MeOH) was performed by incubation for 14 days on a gyratory shaker (100 rpm) in darkness, at 26 ± 2 °C. The samples were filtered and evaporated to dryness under reduced pressure using a rotary vacuum evaporator (Marconi MA 120, São Paulo, Brazil) at 40 °C. The samples were diluted in sterile distilled water and stored at -20 °C. Residual solvent was evaporated using a chamber containing silica.

For the fractionation, 500 mg of the crude extract were suspended in 50 mL of methanol and transferred to a separation funnel. Then, 50 mL of hexane were added during the methanolic phase, removing the hexane fraction, repeating the process three times. In the residual fraction of methanol 50 mL of Dichloromethane was added. The process was performed three times, following the above procedure. Aiming to further elucidate the results, the extracts were submitted to a qualitative phytochemical analysis following the protocol used by Mattos (1988). The selected tests comprise the identification of general alkaloids, flavonoids (flavones and isoflavonoids), phenolics, tannins, and saponins.

Antimicrobial assay

To evaluate antimicrobial activity, extracts from A. mucosa were solubilized in dimethylsulfoxide (DMSO), obtaining a working solution at a concentration of 250 mg.mL⁻¹.

For the validation of tests, reference strains were used, mostly obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and others from the collection of the Department of Microbiology, Immunology, and Parasitology, from the State University of Rio de Janeiro. Twenty bacterial strains encompassing Gram-positive and Gram-negative (Table 1) were used, with most of these representing strains of clinical interest in public health.

The testing of the antimicrobial potential of extracts was performed using the agar dilution method (macro-dilution), described by Soberón et al. (2007), with modifications.

The bacterial strains were cultivated in Mueller-Hinton Broth (MHB, Oxoid, Ltda.) and kept at 37 °C for 18 h for growth. The extracts of A. mucosa were solubilized in 20 mL of pre-warmed Mueller-Hinton Agar (MHA) and the final content was poured into petri dishes. The final concentration of the extracts was 500 mg.mL⁻¹.

For each treatment, 2 μL of each bacterial suspension were cultivated in duplicate. After 24 h of incubation at 37 °C, the growth of the colonies was observed.
Table 1 - Bacterial strains used for the evaluation of antibacterial potential of A. mucosa extracts.

| Gram-positive | Strains                  |
|---------------|--------------------------|
| Bacillus thuringiensis | ATCC 33679               |
| Enterococcus faecalis     | ATCC 29212               |
| Staphylococcus aureus     | ATCC 25923               |
| Staphylococcus saprophyticus | ATCC 15305          |
| Staphylococcus simulans   | ATCC 27851               |
| Streptococcus pyogenes    | ATCC 8668                |

| Gram-negative | Strains                  |
|---------------|--------------------------|
| Aeromonas caviae     | ATCC 15468               |
| Aeromonas hydrophila | ATCC 7966               |
| Aeromonas media      | ATCC 33907               |
| Citrobacter freundii | ATCC 12241              |
| Escherichia coli     | ATCC 25922               |
| Escherichia coli K-12 | C600                      |
| Escherichia coli K-12 | HB 100                   |
| Klebsiella pneumoniae | ATCC 700603             |
| Pseudomonas aeruginosa | ATCC 27853             |
| Salmonella Typhimurium | C20                     |
| Serratia marcescens   | 7145                     |
| Shigella sonnei      | ATCC 25931               |

Evaluation of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and the inhibitory concentration of 50% of the population (IC50) were determined by the dilution method in 96-well plates. Only the strains that showed inhibition of growth by the macrodilution method were evaluated. The bacterial suspensions, adjusted to 0.5 on the McFarland scale, were cultured in triplicate for 18 h in the presence of the extract at concentrations of 500, 250, 125, 62.5 and 31.25 μg.mL⁻¹. After the incubation period, an aliquot of 2 μL of each well was plated onto MHA medium and grown for 18 h at 37 °C for proof of the MBC.

The experimental controls used were the Mueller-Hinton medium without extracts and the same medium supplemented with 0.2% of DMSO, which is the residual concentration after solubilization of extracts. All the materials and culture media were previously sterilized at 121 °C for 20 min.

Results

The analysis of extracts of the in vivo and in vitro cultures of A. mucosa at a concentration of 500 μg.mL⁻¹ showed selective antimicrobial activity, inhibiting the growth of only two strains (Table 2).

For the in vivo grown material, crude methanol extracts of the stem (S) and leaves (L) and their dichloromethane fraction (L2) showed inhibitory activity on the growth of Streptococcus pyogenes (ATCC 8668). The stem fractions in dichloromethane (S2) and hexane (S1) were able to reduce the growth of this strain. Despite the inhibition of the strain, the dichloromethane fraction of the leaf extract (L2) led to the increased growth of 10 other strains evaluated in relation to controls.

The biotechnological material demonstrated inhibitory activity to the growth of Bacillus thuringiensis (ATCC 33679). This selective effect was observed in methanol crude extracts of the callus of leaves (CL) and cell suspension cultures of the callus of hypocotyls (CCS). The dichloromethane fraction of this (CCS2) was capable of reducing the growth of the strain.

A reduction in the growth of Pseudomonas aeruginosa (S1, S2, L, CH, and CCS2), Shigella sonnei (CL), and Klebsiella pneumoniae (L) (Table 2) was observed.

The methanol extracts of leaves showed IC50 for S. pyogenes at a concentration of 203.1 μg.mL⁻¹ and MIC and MBC above of 250 and 500 μg.mL⁻¹, respectively. For biotechnological material, the leaf callus exhibited IC50, MIC, and MBC values of 44.33, 62.5, and 125 μg.mL⁻¹, respectively, for the cultivation of B. thuringiensis.

The preliminary phytochemical analysis of the methanol extracts revealed the presence of various compounds (Table 3). The in vitro culture modulates the production of these compounds, as observed in extracts CCS, which mostly showed alkaloids in its composition.

Discussion

Methanol extracts of A. mucosa are rich in alkaloids such as Romucosina I, obtained from methanolic stem extracts (Kuo et al., 2004), which are related to the antibacterial effect observed in the present work.

Similar results to those obtained with extracts from A. mucosa were observed from methanol extracts of A. muricata, which was active in inhibiting the growth of Bacillus subtilis (in the present work, Bacillus thuringiensis), and Streptococcus pyogenes, with results comparable to streptomycin (Pathak et al., 2010). Likewise, hexane and dichloromethane extracts from the stems of Polyalthia cerasoides (Annonaceae), when evaluated on 27 strains, were active only on Gram-positive bacteria, having great inhibitory potential in B. subtilis and S. pyogenes (Treeratanabipoon et al., 2011).

Dichloromethane extracts of two species of Annonaceae, Guatteria schomburgkiana and G. riparia, as well as 20 other species from the Atlantic Forest and Amazon Rainforest, showed growth inhibitory activity only on...
Gram-positive strains, while Gram-negative strains were not sensitive (Suffredini et al., 2006). Their selective potential with Gram-positive strains was also evaluated for methanolic and aqueous extracts of the roots of Annona senegalensis, which showed activity in the growth of Staphylococcus aureus, but not for Escherichia coli (Gram-negative); they were also less active with Pseudomonas aeruginosa (Lino and Deogracious, 2006).

The lack of inhibitory activity against Gram-negative bacteria may be related to inherent characteristics of this taxonomic group. The limited number of effective antibiotics against Gram-negative bacteria may be due to the permeability barrier of their outer membrane, which reduces the number of active substances as compared to Gram-positive bacteria (Savage, 2001).

Although antimicrobial activity was not found against some strains, extracts of Annonaceae species may also act in the modulation of drug resistance, as in Staphylococcus aureus strains resistant to norfloxacin (Costa et al., 2008).

The antibacterial activity of material obtained through biotechnological procedures of A. mucosa is re-

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**Table 2** - Antimicrobial activity against 20 bacterial strains from methanol extracts of *A. mucosa* obtained by *in vivo* and *in vitro* conditions at 500 μg.mL⁻¹, determined by Agar Dilution Method assay.

| Strains                          | Extracts     |
|---------------------------------|--------------|
|                                 | S | S1 | S2 | L | L1 | L2 | CH | CL | CCS | CCS1 | CCS2 |
| 1 - *P. aeruginosa* (ATCC 27853) |   | R  | R  | R | -  | -  | R  | -  | -   | R    |      |
| 2 - *C. freundii* (ATCC 12241)  |   | -  | -  | - | -  | I  | -  | -  | -   | -    |      |
| 3 - *A. caviae* (ATCC 15468)    |   | -  | -  | - | -  | I  | -  | -  | -   | -    |      |
| 4 - *E. coli* (17-2)            |   | -  | -  | - | -  | I  | -  | -  | -   | -    |      |
| 5 - *E. coli* (ATCC 25922)      |   | -  | -  | - | -  | I  | -  | -  | -   | -    |      |
| 6 - *S. aureus* (ATCC 25923)    |   | -  | -  | - | -  | I  | -  | -  | -   | -    |      |
| 7 - *S. marcescens* (7145)      |   | -  | -  | - | -  | I  | -  | -  | -   | -    |      |
| 8 - *S. simulans* (ATCC 27851)  |   | -  | -  | - | -  | I  | -  | -  | -   | -    |      |
| 9 - *B. thuringiensis* (ATCC 33679) | - | -  | R  | -  | -  | IH | IH | -  | R    |      |
| 10 - *S. saprophyticus* (ATCC 15305) | - | -  | -  | - | -  | -  | -  | -  |      |      |      |
| 11 - *S. Typhimurium* (C20)     | -  | -  | -  | - | -  | -  | -  | -  |      |      |      |
| 12 - *E. faecalis* (29212)      | -  | -  | -  | - | -  | -  | -  | -  |      |      |      |
| 13 - *E. coli* K-12 (C600)      | -  | -  | -  | - | -  | -  | -  | -  |      |      |      |
| 14 - *S. sonnet* (ATCC 25931)   | -  | -  | -  | - | -  | -  | -  | R  |      |      |      |
| 15 - *E. coli* (ATCC 35218)     | -  | -  | -  | - | -  | -  | -  | -  |      |      |      |
| 16 - *S. pyogenes* (ATCC 8668)  | IH | -  | R  | IH | R  | IH | -  | -  |      |      |      |
| 17 - *E. coli* K-12 (HB 101)    | -  | -  | -  | - | -  | I  | -  | -  |      |      |      |
| 18 - *K. pneumoniae* (ATCC 700603) | - | -  | R  | -  | -  | -  | -  | -  |      |      |      |
| 19 - *A. media* (ATCC 33907)    | -  | -  | -  | - | -  | I  | -  | -  |      |      |      |
| 20 - *A. hydrophila* (ATCC 7966) | -  | -  | -  | - | -  | I  | -  | -  |      |      |      |

S: Crude methanol extract of stem; S1: hexane fraction; S2: dichloromethane fraction; L: Crude methanol extract of leaf; L1: hexane fraction; L2: dichloromethane fraction; CH: Crude methanol extract of callus from hypocotyl; CL: Crude methanol extract of callus from leaves; CCS: Crude methanol extract of cell suspension culture; CCS1: hexane fraction; CCS2: dichloromethane fraction.

I = increase of growth; R = reduction; IH = inhibition; Trace = growth is similar of the control in Mueller-Hinton Agar without extract.

**Table 3** - Phytochemical analysis of methanolic extracts of *Annona mucosa* cultivated *in vivo* and obtained by plant tissue culture techniques.

| Extracts       | Alkaloids¹ | Flavonoids² | Phenols | Tannins | Saponins |
|----------------|------------|-------------|---------|---------|----------|
| Leaf           | +          | +           | +       | +       | +        |
| Steam          | +          | +           | -       | -       | -        |
| Callus from leaves | +          | +           | +       | +       | -        |
| Callus from hypocotyls | +         | +           | -       | +       | -        |
| Cell suspension culture | +          | -           | -       | -       | -        |

(+): presence; (-): absence. ¹ Tests with the reative of Meyer and Bouchardat. ² Tests for flavone, flavonol and xanthone.
ported here for the first time. Potential antimicrobial activity was also observed in another species investigated by our group, obtained through biotechnological procedures, where methanol extracts were also active against several strains (Simões-Gurgel et al., 2012; Mello et al., 2014).

The present work revealed the presence of secondary metabolites with antibacterial activity from different extracts of *Annona mucosa*, cultivated under *in vivo* and *in vitro* conditions. The material produced could be used for phytochemical characterization leading to their use in the treatment of infectious diseases.

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**References**

Abdallah EM (2011) Plants: an alternative source for antimicrobials. J Appl Pharmaceut Sci 6:16-20.

Amaral CLF, Silva AB (2003) Melhoramento biotecnológico de plantas medicinais. Rev Biotec Ciência e Desenv 30:55-59.

Andrade SRM (2002) *Princípios da cultura de tecidos vegetais*. Embrapa, Planaltina.

Assis TF, Teixeira SL (1998) Enraizamento de plantas lenhosas. In: Torres AC, Caldas LS, Buso IA (eds) Cultura de Tecidos e Transformação Genética em Plantas. Embrapa, Brasília, pp 331-353.

Costa EV, Da Cruz PE, De Lourenço CC et al. (2013) Antioxidant and antimicrobial activities of aporphinoids and other alkaloids from the bark of *Annona salzmannii* A. DC. (Annonaceae). Nat Prod Res 27:1002-1006.

Costa VCO, Tavares JF, Agra MF et al. (2008) Composição química e modulação da resistência bacteriana a drogas do óleo essencial das folhas de *Rollinia leptopetala* R. E. Fries. Rev Bras Farmacognos 8:245-248.

Costa VCO, Tavares JF, Queiroga CS et al. (2012) Constituintes químicos das folhas de *Rollinia leptopetala* R. E. Fries. Quim Nova, 35:138-142.

Figueiredo SFL, Simões C, Albarello N et al. (2000) *Rollinia mucosa* cell suspension cultures: establishment and growth conditions. Plant Cell Tiss Org Cult 63:85-92.

Figueiredo SFL, Viana VRC, Simões C et al. (1999) Lignans from leaves, seedlings and micropropagated plants of *Rollinia mucosa* (Jacq.) Baiill. - Annonaceae. Plant Cell Tiss Org Cult 56:121-124.

Kuo RY, Chen CY, Lin AS et al. (2004) A new phenanthrene alkaloid, Romucosine I, form *Rollinia mucosa* Baiill. Z Naturforsch 59:334-336.

Lino A, Deogracious O (2006) The in-vitro antibacterial activity of *Annona senegalensis*, *Securidaca longipendiculata* and *Steganotaenia aralaiaceae* - Ugandan medicinal plants. Afr Health Sci 6:31-35.

Lloyd G, Mcclown B (1980) Commercially - feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Int Plant Prop Soc Proc 30:421-427.

Lorenzi H (2002) Árvores Brasileiras: Manual de Identificação e Cultivo de Plantas Arbóreas Bativas do Brasil, vol. 4. Plantarum, Nova Odessa.

Lourenço MV (2003) Biotecnologia de plantas medicinais: produção de biomoléculas. Biólogo 65:63-65.

Mattos FJA (1988) Introdução à Fitoquímica Experimental. Fortaleza Edições, UFC, 128p.

Mello GS, Ignacio ACPR, Albarello N (2014) Avaliação do potencial anticancerígeno de *Schwartzia brasiliensis* (Choisy) Bedell ex Giralda-Cañas cultivada *in vivo* e *in vitro*. Novas Edições Acadêmicas, OmniScriptum GmbH & Co. Saarbrücken, Deutschland/Alemanha. 52 p.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-479.

Oskirio EJ, Montoya GL, Arango GJ (2006) Productos naturales alcaloidiales con actividad antiprototrozoaria. Vitae 13:61-84.

Pathak P, Saraswathy D, Vora A et al. (2010) *In vitro* antimicrobial activity and phytochemical analysis of the leaves of *Annona muricata*. Int J Pharma Res Dev 2:1-6.

Reyes RE, Alvarez AL, Lopez-Rubalcava C et al. (2002) Lignans from leaves of *Rollinia mucosa*. Z Naturforsch 57:29-32.

Rossi F (2011) The challenges of antimicrobial resistance in Brazil. Antimicrobial Resist 52:1138-1143.

Savage PB (2001) Multidrug-resistant bacteria: overcoming antibiotic permeability barriers of Gram-negative bacteria. Ann Med 33:167-171.

Simões-Gurgel C, Rocha AS, Cordeiro LS et al. (2012) Antibacterial activity of field-grown plants, *in vitro* propagated plants, callus and cell suspension cultures of *Cleome rosea* Vahl. J Pharm Res 5:3304-3308.

Soberón JR, Sgariglia MA, Sampietro DA et al. (2007) Antibacterial activity of plant extracts from northwestern Argentina. J Appl Microbiol 102:1450-1461.

Suffredini IB, Paciencia MLB, Varella AD et al. (2006) Antibacterial of brazilian amazon plan extracts. Braz J Infect Dis 10:400-402.

Torres AC, Ferreira AT, Sá FG et al. (2000) Glossário de Biotecnologia Plantarum Vegetal. Embrapa, Brasília.

Treeratanabipoon L, Worachartcheewan A, Suksrichavalit T et al. (2011) Bioactive 4-hydroxycinnamide and bioactivities of *Polyalthia cerasoides*. Excli J 10:16-22.

Vila-Nova NS, Morais SM, Falcão MJC et al. (2011) Leishmanial activity and cytoxicity of compounds from two Annonaceae species cultivated in Northeastern Brazil. Rev Soc Bras Med Trop 44:567-571.

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