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Research Paper

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

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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. Disinfestation 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 isoflavonols), 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 (macrodilution), 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	
Species	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	
Species	Strains
Aeromonas caviae	ATCC 15468
Aeromonas hydrophila	ATCC 7966
Aeromonas media	ATCC 33907
Citrobacter freundii	ATCC 12241
Escherichia coli	EAEC 17-2
Escherichia coli	ATCC 25922
Escherichia coli	ATCC 35218
Escherichia coli K-12	C600
Escherichia coli K-12	HB 101
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 (IC_{50}) 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 $\mu g.mL^{-1}$. 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 $\mu g.mL^{-1}$ 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 IC_{50} for *S. pyogenes* at a concentration of 203.1 $\mu g.mL^{-1}$ and MIC and MBC above of 250 and 500 $\mu g.mL^{-1}$, respectively. For biotechnological material, the leaf callus exhibited IC_{50}, MIC, and MBC values of 44.33, 62.5, and 125 $\mu g.mL^{-1}$, 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 Anonnaceae, *Guatteria schomburgkiana* and *G. riparia*, as well as 20 other species from the Atlantic Forest and Amazon Rainforest, showed growth inhibitory activity only on

Table 2 - Antimicrobial activity against 20 bacterial strains from methanol extracts of *A. mucosa* obtained by *in vivo* and *in vitro* conditions at 500 $\mu g.mL^{-1}$, 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. sonnei* (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	Alkaloids[1]	Flavonoids[2]	Phenols	Tanins	Saponins
Leaf	+	+	+	+	+
Steam	+	+	-	-	-
Callus from leaves	+	+	+	+	-
Callus from hypocotyls	+	+	-	+	-
Cell suspension culture	+	-	-	-	-

(+) presence; (-) absence. [1] Tests with the reactive of Meyer and Bouchardat. [2] Tests for flavone, flavonol and xanthone.

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-

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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