



Phytochemical composition, antibacterial and modulatory of antibiotic activity of the extract and fractions from *Annona squamosa* L.

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Abstract

The medicinal plants and natural products are products with a great scientific interest due their possible use like phytodrugs. In this context, the phytochemical composition and the antibacterial and antibiotic modulatory activity of the extract and fractions of *Annona squamosa* against *Staphylococcus aureus* and *Escherichia coli* were performed. All natural products have not showed clinically relevant antibacterial activity, with MIC \geq 1024 mg/mL. However, the extract combined with some aminoglycoside showed a synergistic effect against multiresistant strains *E. coli* 27 and *S. aureus* 358. Due these results, *A. squamosa* (Annonaceae) must be more studied to be used as a source of natural products with adjuvant potential to enhance the antibiotic activity, combating by this way the microbial antibiotic resistance.

Keywords: Antibacterial activity - *Escherichia coli* - Modulator activity - *Staphylococcus aureus*

Introduction

The indiscriminate use of antibiotics has increased of the microbial resistance to these drugs, indicating the necessity of the research for new antibiotics. The study of the bacterial resistance is often based in microorganism with epidemiological importance and responsible for different infectious process (Moellering 2000). In the search of new therapeutic alternatives, natural products and remedies has been the target of a great scientific interest due their pharmacological activities and chemical characteristics to be used as drugs (Michelin et al. 2005; Lima et al. 2006).

Annona squamosa L. (Annonaceae) is known popularly like “pinha”, “ata” or “fruta-do-conde”. In Brazil, there are almost 250 species of the family Annonaceae (Souza and Lorenzi 2008). This plant is native from Central America and is cultivated in the northeastern region of Brazil due its value as a food “*in natura*” (Souza and Lorenzi 2008). In the traditional medicine, parts of this plant are used as insecticide, antispasmodic, antidiarrheal, analgesic and anti-inflammatory (Vohora et al. 1975; Womg et al. 1993; Brito et al. 2008; Roig 1988; Robineau 1995; Amador et al. 2006).

Due this importance, the aim of this work has was analyze perform the phytochemical prospecting and evaluate the antimicrobial and modulatory antibiotic activity of the ethanol extract *Annona squamosa* (EEAS) and hexane (HFAS), ethyl acetate (EAFAS) and methanolic (MFAS) fractions against the multiresistent strains of *Staphylococcus aureus* and *Escherichia coli*.

Material and Methods

Plant Material

Leaves of ten different individuals of *Annona squamosa* were collected in the County of Crato, Ceará, Brazil. The plant was identified by Dr. Maria Arlene Pessoa da Silva and deposited at Herbarium Caririense Dárdano de Andrade-Lima of the Regional University of Cariri – URCA, under number 5539 HCDAL.

Preparation of Ethanol Extract and Fractions of the Annona squamosa L. (EEAS)

Fresh leaves of *Annona squamosa* (275g) were weighted and macerated using the extraction with ethanol for a period 72 hours. The solvent was distilled in a rotary evaporator at 80°C under low pressure, obtaining 10.2g of the extract. The extract was stored at room temperature using amber glass. The fractioning protocol used 5g of the extract. The extract was mixed with silica and filtered with vacuum using the follow solvents P.A: Hexane (HFAS), ethyl acetate (EAFAS) and methanolic (MFAS).

Phytochemical prospecting

The phytochemical test to detect the presence of tannins, flavonoids and alkaloids were performed according to the classic method described by Matos (1997). The tests were based on the visual observation of a change in color or formation of precipitate after the addition of specific reagents.

Drugs

Gentamicin, Kanamycin, amikacin and neomycin were obtained through Sigma Chemical Co. All drugs were dissolved in sterile water.

Bacterial strains

The bacteria used in the Minimal Inhibitory Concentration (MIC) test were the standard strains of *Escherichia coli* ATCC 10536, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC15442 and *Klebsiella pneumoniae* ATCC 4362. The evaluation of the modulatory activity of the ethanol extract and fractions was assayed against multiresistant bacterial strains isolated of clinical environments: *E. coli* 27 and *S.aureus* 358, with the resistance profile demonstrated in Table 1. All strains were obtained through the Laboratory of Clinical Mycology – UFPB.

Table 1. Source of the bacterial strains and resistance profile to antibiotics. Legend: Ast-Aztreonan; Ax- Amoxicillin; Amp-Ampicillin; Ami-Amicilina; Amox-Amoxillin, Ca-Cefadroxil; Cfc-cefador; Cf-Cephalothin; Caz-Ceftazidima; Cip-Ciprofloxacin; Clo-Chloramphenicol; Imi-Imipenem; Can-Kanamycin; Szt-Sulphametrim; Tet-Tetracycline; Tob-Tobramycin; Oxa-Oxacillin; Gen-Gentamicin; Neo- Neomycin; Para-Paramomicina; But-Butirosina; Sis-Sisomicin; Net-Netilmicin.

Bacterium	Origin	Profile of resistance
<i>Escherichia coli</i> 27	Surgical wound	Ast, Ax, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Clo, Imi, Can, Szt, Tet, Tob
<i>Staphylococcus aureus</i> 358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net

Minimal Inhibitory Concentration

Broth microdilution was the method used. The substances to be assayed were dissolved using DMSO and diluted to 1024 µg/mL with sterile water. The bacterial inoculum was diluted using BHI to a final concentration of 10⁵ UFC/mL. A total of 100 µL relating to each inoculum was distributed in each well of a microtiter plate with 96 wells, and then submitted to a double serial dilution using 100 µL of extract and fractions, with concentrations varying between 512 - 8 µg/mL. The plates were incubated during 24 hs at 35°C (Javadpour et al. 1996). MIC was determined using resazurin. The MIC was defined as the lowest concentration where no growth can be observed according to NCCLS (2003).

Modulatory activity assay

The determination of the antibiotic modulatory activity of the extract and fractions of *A. squamosa* was realized according the method proposed by Coutinho et al. (2008). The extract and fractions were assayed in sub-inhibitory concentration (MIC/8 = 128µg/mL). A sample with 100µL of a solution containing BHI, the microbial inoculums and a sub-inhibitory concentration of

the extract or fractions was placed in each well. 100 µL of the antimicrobial drug (f.c. 1024 g/mL) was placed in the first well. This solution was serially two fold diluted, obtaining concentrations ranging between 2500 – 2.44 µg/mL.

Results and Discussion

The use of natural products like antimicrobial agents show as low risk to increase the microbial resistance due the complex mixtures of these compounds, resulting in a lower adaptative capacity (Daferera et al. 2003; Matias et al. 2010).

The Table 2 shows the presence of many complexes such as condensed tannins, flavones, flavonols, xanthonones, chalcones, flavononols, catechins, flavonones and alkaloids, being correlated with the phytochemicals demonstrated by Brito et al (2008). The secondary metabolites identified in the extract of *A. Squamosa* are reported as a source of several biological activities: antimicrobial (Kotkar et al. 2001), antioxidant (Shirwaikar et al. 2004) and antiulcerogenic (Zayachkivska et al. 2005).

Table 2. Phytochemical prospection of the ethanol extract of *Annona squamosa* L. (EEAS). Legend: 1- Phenols; 2- Pirogalic tannins; 3- Condensed tannins; 4- Anthocyanins; 5- Anthocyanidins; 6- Flavones; 7- Flavonols; 8-Xanthonones; 9- Chalcones; 10- Auronas; 11- Flavononols; 12- Leucoanthocyanidins; 13- Catechins; 14- Flavonones; 15- Alkaloids; (+) presence; (-) absence.

Metabolites															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
EEAS	+	-	+	-	-	+	+	+	+	+	+	-	+	+	+

Phenolic compounds, as tannins and flavonoids, have demonstrated several bioactivities as antiinflammatory, antifungal, antimicrobial, antioxidant, and wound-healing agents (Santos and Mello 2004). Synergism between flavonoids and antibiotics against resistant bacterial strains and the bacteriostatic or bactericidal activity of flavonols have been demonstrated by (Cushnie and Lamb 2005).

The extract and fractions did not presented a clinically relevant antibacterial activity, presenting MIC ≥ 1024 µg/mL (Coutinho et al. 2008). However, the extract and the hexane fraction combined with neomycin showed a synergistic effect against the multiresistant strains of *E. coli* 27 and *S. aureus* 358. A synergism was also verified when associated the acetate ethyl fraction with Amikacin and Gentamicin against *E. coli* 27 (Tables 3 and 4).

Studies done by Kotkar et al. (2001), demonstrated the antimicrobial activity of the flavonoid component of leaves from the *A. squamosa* against strains of *Pseudomonas*, *Bacillus*, *Cellulomonas* and *Aspergillus* due alterations in the structure of the cell membrane of the assayed microorganisms. These fact is according with the observations of Helander et al. (1998) and Matias et al. (2011). These works reported that many phytoconstituents of this extract can increase the cell membrane permeability, potentiating the effect of antibiotics by an uptake increase.

Table 3. Modulatory antibiotic activity of natural products of *Annona squamosa* against *E. coli* ($\mu\text{g/mL}$). Legend: EEAS – Ethanol Extract of *Annona squamosa*; HFAS – Hexane Fraction of *Annona squamosa*; EAFAS – Ethyl-acetate fraction of *Annona squamosa*; MFAS – Methanol Fraction of *Annona squamosa*.

Antibiotic	EC27				
	Alone	+EEAS (128 $\mu\text{g/mL}$)	+HFAS (128 $\mu\text{g/mL}$)	+EAFAS (128 $\mu\text{g/mL}$)	+MFAS (128 $\mu\text{g/mL}$)
Kanamycin	1250	1250	1250	1250	1250
Neomycin	312.5	39.06	312.5	312.5	312.5
Amikacin	2500	2500	2500	625	2500
Gentamicin	625	625	625	156.25	625

Table 4. Modulatory antibiotic activity of natural products of *Annona squamosa* against *S. aureus* ($\mu\text{g/mL}$). Legend: EEAS – Ethanol Extract of *Annona squamosa*; HFAS – Hexane Fraction of *Annona squamosa*; EAFAS – Ethyl-acetate fraction of *Annona squamosa*; MFAS – Methanol Fraction of *Annona squamosa*.

Antibiotic	SA358				
	Alone	+EEAS (128 $\mu\text{g/mL}$)	+HFAS (128 $\mu\text{g/mL}$)	+EAFAS (128 $\mu\text{g/mL}$)	+MFAS (128 $\mu\text{g/mL}$)
Kanamycin	39.06	39.06	39.06	39.06	39.06
Neomycin	19.53	4.88	4.88	19.53	19.53
Amikacin	39.06	39.06	39.06	39.06	39.06
Gentamicin	4.88	4.88	4.88	4.88	4.88

The combination of natural products with antibiotics can result in a potentiating effect that must be investigated in others plants of Annonaceae family. Leaves of *Annona muricata* (Annonaceae) demonstrated a synergistic effect associated with aminoglycosides (Kanamycin, Gentamicin, Amikacin and Neomycin) against bacterial strains of *S. aureus* and *E. coli* (Bento 2010). These results of the modulatory antibiotic activity demonstrated the synergistic potential of the Annonaceae family.

Natural products can present an antibacterial activity or resistance-modifying activity, due the presence of differences in polarity and secondary metabolites, which are related to affinities for biological action (Matias et al. 2010). As a result, they can demonstrate a greater interaction with the lipid bilayer of the cell membrane, affecting the respiratory chain and 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; Juven et al. 1994). These mechanisms of action can be due to the combination of antibiotic with extracts and fractions at a subinhibitory concentration added directly to the culture medium Coutinho et al. (2009).

This strategy is called “herbal shotgun” or “synergistic multieffect targeting” and refers to the utilization of plants and drugs in combination,

affecting several targets at once and acting in a synergistic or antagonistic way. This procedure is not only through the combinations of extracts, but also due to combinations between natural products or extracts and synthetic products or antibiotics (Wagner and Ulrich-Merzenich 2009).

The results of this work demonstrated that *A. squamosa* (Annonaceae) must be studied more deeply about the possible usage as an adjuvant to the antibiotic therapy as a manner to combat the bacterial resistance to antibiotic. However, more assays are necessary to investigate this activity using *in vivo* models.

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