Antibacterial Activity of *Annona squamosa* Linnaeus (Annonaceae)

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Abstract

Annona squamosa Linnaeus (Annonaceae) is a plant commonly found in Malaysia and used in folk medicine against skin infections, diarrhoea, dysentery and urinary tract infections. Ethanol crude extract of the fruit of *Annona squamosa* was screened for antimicrobial activity against some pathogenic microorganisms, which represent the different existing groups of bacteria and yeast. These organisms were the Gram-positive bacteria, such as *S. aureus* ATCC 25923; *E. faecalis* ATCC 29212 and *S. pneumoniae* ATCC 49619. The Gram-negative bacteria including *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *H. influenzae* ATCC 49247; yeast, such as *C. albicans* ATCC 90028. Twenty clinical strains of *S. aureus* (both methicllin-sensitive (MSSA) and methicillin-resistant (MRSA)) and ten clinical strains of *S. pneumoniae* isolated from patients hospitalized in the University of Malaya Medical Center (UMMC) were also included. The disc diffusion method that followed the National Committee for Clinical Laboratory Standards protocol was used to evaluate antimicrobial activities. *Annona squamosa* fruit extract showed inhibitory activity against *S. aureus* ATCC 25923 and *S. pneumoniae* ATCC 49619. Mean diameters of inhibition zones were 14 mm and 13 mm respectively with disc concentration of 5 mg/disc. Antibacterial activity against clinical strains of *S. aureus* (both methicllin-sensitive (MSSA) and methicillin-resistant (MRSA) and *S. pneumoniae* ranged from 13-15 mm and 12-14 mm. The minimum inhibitory concentration determined by broth dilution method for *S. aureus* ATCC 25923 was 0.25 mg/ml and for *S. pneumoniae* ATCC 49619 was 0.5 mg/ml. The minimum inhibitory concentration reached by 50% ( ) and 90% ( ) of the clinical strains of *S. aureus* (both methicllin-sensitive (MSSA) and methicillin-resistant (MRSA)) and *S. pneumoniae* ranged from 0.25-0.5 mg/ml and 0.5-1 mg/ml respectively. It is concluded that the plant extract may serve as a valuable source for compounds with therapeutic antibiotic potentials.

Introduction

One of the major concerns in healthcare today is the ineffectiveness of antibiotics towards new and re-emerging diseases due to the developed resistance in many pathogens (1). Thus, there is an urgent need to identify novel anti-microbial molecules as leads for effective drug development. These active molecules may be synthesized chemically or isolated from natural origins such as bacteria, fungi, animals or plants.

Man has manipulated the medicinal properties of plants for treatment of diseases since prehistoric times. Currently, there are several plant products in pre-clinical evaluation while others show promising anti-microbial activities in *in vitro* and *in vivo* assays. Therefore, plants are considered the most potential and preferred choice in developing alternative antibiotics of natural origin (2).

The family Annonaceae is a primitive and well-represented group of about 130 genera and over 2000 species of trees, shrubs or climbers mainly growing in the subtropics and tropics regions (3). The family Annonaceae contains a large number of pharmacologically active substances, which are antibacterial (4), antifungal (5) and antiviral (6). They are used in over-the-counter medicine to treat a number of bacterial diseases (7, 8). The active ingredients that contribute to its antimicrobial property are believed to be acetogenin, alkaloid, flavanoid and diterpenes. For example, liriodenine, an alkaloid isolated from *Cananga odorata* appears to be a potent inhibitor of the topoisomerase II, causing the cleavage of the DNA backbone that results in the antibacterial effect (9). Terpenes and flavonoids are

in a lesser manner involved in the Annonaceae biological properties due to their low concentrations in the plants. However, some of the flavanones isolated from species of this family, for example *Desmos chinensis* accumulates 5-methoxy-7-hydroxyflavanone and 6-hydroxydehydrouvaretin which both negated the growth of *B. subtilis* and *S. boydii*. Furthermore, 5-methoxy-7-hydroxyflavanone strongly negated *S. aureus* (8).

Annona squamosa, locally named 'nona' in Malay, is a small deciduous tree that mainly grows in the tropics. It only reaches the height of about 20 to 25 feet. Fruits are as big as 3 to 5 inches in diameter with a lumpy green skin and upon maturity the fruit has a bluish or white blush. The fruits of *Annona squamosa* are used in folk medicine as a remedy to treat several microbial diseases (10). In view of the fruit of *Annona squamosa* being used in folkloric medicine particularly as a potent anti-microbial agent, the present study was undertaken to further characterize the biological activities of *Annona squamosa.*

Methodology

Plant material was collected from Ayer Hitam, Pulau Pinang on September 2002 and was identified by Assc. Prof. Dr. Christophe Wiart (Lecturer of Pharmacognosy and Medicinal Plants, Department of Pharmacy, Faculty of Medicine, University of Malaya). Fruits were separated from the plant. The separated parts were labeled accordingly and cut into small pieces, which were then air-dried at room temperature for a week. The dried material was ground to fine powder (mesh size 1 mm) and stored in sterile airtight plastic bag at 4 °C in the dark until extraction.

100 g of the powder were extracted twice with l L of EtOH (95% w/v) in a reflux extractor for six hours at a temperature not exceeding the boiling points of the solvent. The ethanolic extract was filtered through Schleicher & Schuell 595 filter paper and was evaporated under reduced pressure at 40°C using the Rotary evaporator. The residues obtained were designated as ethanolic extract and stored in the freezer at -20°C until assayed.

Microorganisms

In vitro antimircobial studies were carried out against three Gram-positive (*S. aureus* ATCC 25923; *E. faecalis* ATCC 29212; *S. pneumoniae* ATCC 49619) and three Gram-negative bacteria (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853; *H. influenzae* ATCC 49247). *C. albicans* ATCC 90028 was used as a yeast species. The strains were purchased from the American Type Culture Collection (ATCC). Twenty clinical strains of *S. aureus* (both methicillin-sensitive (MSSA) and methicillin-resistant (MRSA)) and ten clinical strains of *S. pneumoniae* isolated from patients hospitalized in the University of Malaya Medical Center (UMMC) were also included.

All bacteria were cultured on Mueller-Hinton agar. The Mueller-Hinton agar was supplemented with 5% defibrinated sheep blood for *S. pneumoniae* and with 5% defibrinated lysed sheep blood for *H. influenzae*. *C. albicans* was cultured on Sabouraud's dextrose agar. Plates of *S. pneumoniae* and *H. influenzae* were incubated at 35ºC in an incubator for 24 h in 5% . For other bacteria and yeast, plates were incubated in air at 35ºC for 24 h and 48 h respectively.

Disc diffusion Method

The disc diffusion method that followed the National Committee for Clinical Laboratory Standards (11) protocol was used to evaluate antimicrobial activities. For susceptibility testing, 100 mg/ml crude ethanolic extract was prepared in dimethyl sulfoxide (DMSO). Sterile Antibiotic Assay (AA) Discs (Whatman, 6 mm) were impregnated with 50 µL of the reconstituted extract and were dried completely. The discs were placed on the surface of agar dispersion plates inoculated with microbes that was adjusted to the 0.5 McFarland standard equivalent to 1-2 x CFU. Extract was tested in triplicate. DMSO saturated assay discs and blank assay discs were used as negative controls. Standard antibiotics disc such as Vancomycin (5 µg $disc^{-1}$), Penicillin G (1 unit $disc^{-1}$), Ciprofloxacin (5 µg $disc^{-1}$) and Fluconazole (25 µg $disc^{-1}$) were used as positive controls. Plates of *S. pneumoniae* and *H. influenzae* were then incubated at 35ºC in an incubator for 24 h in 5% . For other bacteria and yeast, plates were incubated in air at 35ºC for 24 h and 48 h respectively. Inhibition zones were recorded as the diameter of growth-free zones, including the diameter of the disc, at the end of the incubation period.

Minimum Inhibitory Concentration (MIC) determination

The broth dilution method that followed the National Committee for Clinical Laboratory Standards (11) protocol was used to determine the MIC. The crude ethanolic extract was dissolved in 25% dimethyl sulfoxide (DMSO): 25% Tween 80 and 50% sterile distilled water. Two-fold serial dilution of plant extract from 64 to 0.0156 mg/ml was prepared. 96-well plates of *S. pneumoniae* were then

incubated at 35ºC in 5% for 24 h. Plates of *S. aureus* were incubated in air at 35ºC for 24 h. After 24 h, 5 μL of culture from each well was inoculated onto pre-dried Mueller-Hinton agar or Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. These plates were incubated at similar conditions as before. MIC values were taken as the lowest concentration of extract that completely inhibited bacterial growth after 24 hours. The MICs of vancomycin and penicillin G were also performed concomitantly to validate the methodology and used as controls.

Results and Discussion

Annona squamosa fruit extract showed significant inhibitory activity against *S. aureus* ATCC 25923 and *S. pneumoniae* ATCC 49619 giving mean diameters of inhibition zones of 14 mm and 13 mm respectively with disc concentration 5 mg/disc. All the Gram-negative bacteria as well as *E. faecalis* and *C. albicans* were not inhibited by the plant extract. This is in agreement with previous reports that plant extracts are more active against Gram-positive bacteria than Gram-negative bacteria (12). Antibacterial activity against clinical strains of *S. aureus* (both methicillin-sensitive (MSSA) and methicillin-resistant (MRSA)) and *S. pneumoniae* ranged from 13-15 mm and 12-14 mm respectively.

MIC determination for *S. aureus* ATCC 25923 was 0.25m/ml and for *S. pneumoniae* ATCC 49619 was 0.5mg/ml. The minimum inhibitory concentration reached by 50% ( ) and 90% ( ) of the clinical strains of *S. aureus* (both methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) and *S. pneumoniae* confirmed the high antibacterial activity of *Annona squamosa* fruit extract (Table 1).

Table 1 The and determination of *Annona squamosa* fruit extract against the clinical strains of *S. aureus* and *S. pneumoniae.*

Strains tested	Number tested	MIC (mg/ml)		
				Range
S. aureus				
Methicillin-sensitive (MSSA)	10	0.25	0.5	0.25 - 0.5
Methicillin-resistant (MRSA)	10	0.25	0.5	0.25 - 0.5
S. pneumoniae	10	0.5	1.0	0.5 - 1.0

These results are of interest since they have been obtained from the crude fraction, which may exhibit a lower activity than the purified active compounds. Thus *Annona squamosa* may be a potential antibacterial agent. Further studies are required to discover the active compound(s) of this plant. These findings therefore support the usage of this plant as an alternative treatment of common ailments such as skin infections.

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