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Antimicrobial Activity of Terpenoids Extracted from *Annona muricata* Seeds and its Endophytic *Aspergillus niger* Strain SH3 Either Singly or in Combination

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Keywords: Annona muricate; Antimicrobial Activity; Combined extract; Endophytic Fungi and Terpenoids

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Abstract

BACKGROUND: *Annona muricata* (Soursop) has an antimicrobial activity toward various pathogenic microorganisms which support its ethnomedicinal for the treatment of many infectious diseases.

AIM: Aim of the present study to evaluate the relation between antimicrobial activities of terpenoids extracted from different soursop parts with the isolated endophytic fungi.

METHODS: Endophytic fungal species of pulp and peel of Annona fruit along with those of seeds were isolated. Salkowski test was used for qualitative screening of terpenoids in plant and the isolated endophytic *Aspergillus niger* strain SH3.

RESULTS: Endophytic *A. niger strain* SH3 and Annona seed extract showed high terpenoid content indicated by the high intensity of reddish-brown colour. GC/Mass analysis revealed six compounds of terpenoids from endophytic *A. niger* strain SH3 extract and four compounds from seed extract with different retention times. The antimicrobial assay was performed using *A. niger* strain SH3 extract and Annona seed extract singly or in combinations against *S. aureus, P. aeruginosa, E. coli* and *C. albicans*.

CONCLUSION: The results revealed the significant antimicrobial activity of both extracts. However, the combined extract showed some reduction in antimicrobial activity which could be attributed to the antagonistic effect exhibited by their constituents.

Introduction

Annona muricata (Annonaceae) is a tropical plant species known for its edible fruits. It is called soursop, which has some medicinal merits and some toxic effects. Extracts of *A. muricata* have been famous for their antimicrobial, anti-inflammatory, anti-protozoan, antioxidant, insecticide, larvicide, and cytotoxic activities. Mechanisms of action of some pharmacological effects have been declared, such as cytotoxic, antioxidant, antimicrobial and anti-hypertensive activities [1]. *Annona* extracts from its leaves, roots, and seeds have shown antibacterial activity against a plethora of microorganisms. Endophytic fungi from medicinal plants can be considered as a reservoir of bioactive metabolites which include terpenoids, alkaloids, flavonoids, phenolic acids, quinones, steroids, tetralones and xanthones [2], [3].

Dicotyledonous plants such as soursop are proposed to have endophytic microorganisms which are a potential medicinal source. Endophytic microorganisms usually create symbiotic interactions with plant tissues. Several plant endophytic fungi have been shown to have antimicrobial activity [4]. The present study was performed to evaluate the antimicrobial activity of terpenoids in *Annona* seed and its fungal extracts. The combination of *Annona* seed extract and endophytic *A. niger* strain *SH3* has studied also.

Material and Methods

Annona muricata (Fruits and seeds) were collected from Abo-Rawash farms, Egypt. The seeds were surface-sterilised then air-dried before grinding into powder at room temperature and weighed.

Isolation and identification of endophytic fungi from seed, pulp and peel of Annona fruit

Plant parts were separated, two fresh fruits were washed thoroughly with tap water, surface sterilised with 70% ethanol for 1 min, 4% sodium hypochlorite for 3 min and again with 70% ethanol for 1 min, then rinsed twice with sterile distilled water. Samples were dried with sterile filter paper and cut into small pieces with sterile forceps and sterile gloves worn [5]. The fruits were then peeled, and small strips of each were obtained aseptically and plated on potato dextrose agar medium (PDA) containing chloramphenicol to suppress bacterial growth. Plates were then incubated at 25-27°C until the outgrowths of fungi from the explants were observed. The fungal growths were subcultured to produce pure culture on Cazpex-dox's plates. All isolates were maintained in Cazpex-dox's slants and kept at 4°C. The same procedure was applied to pulp and peel of rotten fruits where the fruits were placed in sterile polyethylene bags and stored for one week to allow deterioration. Isolation of endophytic fungi from *Annona* seeds was done by adding seed powder on the plate's surfaces, then was incubated. The endophytic fungal isolates were identified morphologically and microscopically according to Moubasher [6].

Extraction of terpenoids from Annona seeds

Terpenoids extraction from seeds were performed by agitation with ethanol (250 mL / 20 g of seed powder) three times for 48 hours on an orbital shaker. The extract was concentrated under vacuum till dryness. The concentrated extract was then stored in a vacuum desiccator at room temperature for further use.

Extraction of terpenoids from Annona endophytic fungi

The isolated fungi were grown in 2-litre standard flasks containing 500 ml of Potato Dextrose Broth. After 3 weeks of culturing at 25°C, the culture fluids were passed through four layers of cheesecloth to remove solids. To the culture filtrate, 0.25 g sodium carbonate was added with frequent shaking to reduce the number of fatty acids that may contaminate the culture; then terpenoids were extracted with two equal volumes of ethyl acetate solvent. Ethyl acetate layers were collected, concentrated and evaporated to dryness. Residues were stored for subsequent analysis [7].

Qualitative assay of terpenoids

Test for terpenoids (Salkowski test): Five ml of each extract was mixed with 2 ml of chloroform, then concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish-brown colouration at the interface was formed, indicating positive results for the presence of terpenoids [8].

The GC-MS analysis of fungal and seed terpenoids

The analysis was carried out using a GC/MS (Agilent Technologies 7890A) interfaced with a mass-selective detector (MSD Agilent 7000) equipped with an apolar Agilent HP- 5 ms (5%-phenyl methyl polysiloxane) capillary column (30 m × 0.25 mm i.d. and 0.25 µm film thickness). The carrier gas was helium with a linear velocity of 1 ml/min. The identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching with NIST and WILEY library as well as by comparison of the fragmentation pattern of the mass spectral data with those reported in the literature [9].

Assay of antimicrobial activity

Antimicrobial activities of the terpenoids extracted from fungal *Aspergillus niger* SH3 and seed extract singly and in combination were tested against one Gram +ve bacterial species (*Staphylococcus aureus*), two Gram -ve bacterial species (*Escherichia coli* and *Pseudomonas aeruginosa*) and one fungal species (*Candida albicans*). The assay was performed using the Kirby- Bauer disc diffusion method [10], [11]. Nutrient agar (NA) medium was used for testing of bacteria, while candida agar (CA) medium was used for fungi.

The pathogens were inoculated by streaking over the surface of the sterilised media. Fungal and *Annona* extracts were applied at the surfaces of plates at 5 mg/disc dissolved in DMSO. Petri dishes were incubated at 37°c for 48 h for bacterial species and 25°C for 72 h for *Candida*. Sensitivity was then determined by measuring the mean diameter of the inhibition zones in mm. Ampicillin (5 mg/disc) was used as a positive control for bacteria. Amphotericin B (5 mg/disc) was used as a positive control for *C. albicans*. A control test for the solvent only was also performed.

Determination of relative activity

The relative activity of the tested extract concerning positive control was calculated by using

the following formula [12].

$$\text{Relative activity of the test extract} = \frac{100 \times (x-y)}{(z-y)}$$

Where,

x: total area of inhibition of the test extract;

y: total area of inhibition of the solvent;

z: total area of inhibition of the standard drug.

The total area of the inhibition was calculated by using area = πr2; where, r = radius of zone of inhibition.

Statistical analysis

The results were expressed as mean ± standard deviations (mean ± SD). Data were analysed by one-way analysis of variance (ANOVA).

Results

Isolation and identification of endophytic fungi

In the current study, endophytic fungi were isolated from different parts of *A. muricata* (Fresh, rotten fruits and seeds) (Table 1). A total of 65 fungal isolates were detected in *A. muricata* plant constituting 6 endophytic fungal species represented by 3 genera. *Aspergillus* was the most frequent genus represented by 37 isolates and 2 species, followed by *Penicillium* with 20 isolates and 3 species. The least dominant genus was *Rhizoctonia* which constituted one species with 8 isolates.

Table 1: Isolation of endophytic fungi from different parts of A. muricata

Source	Fruit				Seed	TC and Fr (%)							
	Pulp		Peel										
Fruit nature	F	R	F	R	S	TI	Fr	TI	Fr	TI	Fr	TC	Fr
Organism						(F)	(%) (F)	(R)	(%) (R)	(S)	(%) (S)		(%)
Aspergillus niger	0	8	1	3	0	1	3.33	11	39.28	0	0	12	18.4
Aspergillus niger strain *SH3*	5	6	4	6	4	9	30	12	42.86	4	57.14	25	38.5
Penicilium glabrum	3	0	1	0	2	4	13.33	0	0	2	28.57	6	9.23
Penicillium jensenii	2	0	1	0	0	3	10	0	0	0	0	3	4.61
Penicillium sclerotium	2	2	4	3	0	6	20	5	17.86	0	0	11	16.9
Rhizoctonia solani	5	0	2	0	1	7	23.33	0	0	1	14.28	8	12.4
Total count	17	16	13	12	7	30	46.15	28	43.08	7	10.77	65	100

F = fresh fruit; R = rotten fruit; S = Seed; TC = total count (cfu/ml); TI = total isolates; Fr (%) = frequency.

The fresh fruit was colonised with the highest endophytic count with frequency (46.15%) followed by the rotten fruit (43.08%) while seeds reported a frequency of only 10.77%. Concerning fungal species, *A. niger* strain *SH3* was the most dominant species represented by 25 isolates with frequency 38.5 % of the total isolates. *A. niger* came in the second rank with 12 isolates and 18.4 % frequency. *P.sclerotium, R. solani, P. glabrum* and *P. jensenii* came next with frequencies 16.9%, 12.4%, 9.23% and 4.61%, respectively (Table 1).

Terpenoids determination

Salkowski test was used for qualitative screening of terpenoids for both plant parts and endophytic fungal extracts. *A. niger* strain *SH3* and seed extract showed the high intensity of reddish-brown colour indicating high terpenoids concentration. Furthermore, the combination between seed and *A. niger* strain *SH3* extracts showed high terpenoids (Table 2).

Table 2: Qualitative assay of terpenoids produced by endophytic fungal species isolated from *A. Muricata* and Seed extract

Extract Salkowski test	Colour intensity
Pulp extract	-
Seed extract	++
Fungal extract	
A.niger	++
A. niger strain *SH3*	+++
P. glabrum	-
P. jensenii	+
P. sclerotium	-
R. solani	+
Combined extract *(A. niger* strain SH3 extract and Seed extract)	+++

+ = mild amount; ++ =moderate amount; +++= intense amount; - completely absent.

GC-MS of terpenoids in A. niger strain SH3 and Annona Seed extracts

Ten terpenoid compounds were detected among them 6 compounds from the extract of *A. niger* strain SH3 with different retention times (Table 3).

Table 3: GC-MS analysis of terpenoids in A. niger strain SH3 and Seed extracts of A. muricata

Terpenoids	Area %	Retention time (min)
A.niger strain *SH3* extract	1.55	30.98
1',1'.Dicarboxy.1á,2á.dihydro.3'H.cycloprop[1,2]cholesta.1,4,6.trien.3.one		
Tetra.tert.but	1.82	31.98
2,6.di(3.propenyl).3,7.imethoxybiycyclo(3.3.0)o cta.3,7.diene.2,4,6,8dicarboxylate		
25-Norisopropyl-9,19-cyclolanostan-22-en-24-one,3-acetoxy-24-phenyl-4,4,14-trimethyl	2.17	34.35
Silane,[(3a',5a',11a',20S)-pregnane-3,11,17,20,21-pentayl]pentakis(oxy)]pentakis.trimethyl	1.77	38.59
Anodendroside G, monoacetate (CAS)	1.38	39.29
3-[(Z)-2-Phenylethenyl]cholestan-2-one	1.54	45.70
Seed extract 2,4,6,8,10-Tetradecapentaenoic acid,9a(acetyloxy)-1a,1b,4,4a,5,7a,7b,8,9,9a-decahydro-4a,7b-dihydroxy-3-(hydroxymethyl)1,1,6,8-tetramethyl-5-oxo-1H- Cyclopropa[3,4]benz[1,2-e]azulen-9-yl.ester	0.24	33.50
4,6,8(14)-Cholestatriene	0.14	39.00
4-O-Methylphorbol 12,13-didecanoate	0.23	45.01
Pregnan-18-oic acid 3,9,11,20-tetrol, 3,11-diacetate, 18,20-lactone	0.23	46.75

Antimicrobial activity

Antimicrobial activity of seed and fungal extracts singly or in combination were assayed. Data

in Table 4 revealed that the two extracts had antimicrobial activity against gram +ve bacterial species and gram -ve bacterial species, but *C. albicans* was resistant towards any of them singly and in combination. *P. aeruginosa* was strongly susceptible to the inhibitory action of both extracts. Moreover, *E. coli* and *S. aureus* were very sensitive, respectively. The combined extract showed a reduction in antimicrobial activity which could be attributed to the antagonistic effect between both seed and endophytic *A. niger* strain *SH3* extracts. It is worth noting that the inhibitory action of the seed endophytic fungal species was more than that of the seed extract itself which clarifies that the endophytic microorganisms may be the source of the biological activity of the higher plant by its 2ry metabolites or at least intensify these activities.

Table 4: Antimicrobial activities of extracts of A. niger strain SH3 isolated from A. muricata and seed extract (singly and in combination)

Pathogenic	Inhibition zone diameter (mm)				
Microorganism	Negative Control	Positive Control	*A. niger* strain *SH3* extract	Seed extract	Combined Extract
Gram +ve bacteria					
S. aureus	$0^{a} \pm 0.0$	$21^{a} \pm 0.1$	$12^{b} \pm 0.5$	$11^{a} \pm 0.0$	$10^{a} \pm 0.0$
Gram –ve bacteria					
E. coli	$0^{a} \pm 0.0$	$25^{b} \pm 0.0$	$14^{a} \pm 0.0$	$12^{a} \pm 0.0$	$11^{b} \pm 0.5$
P. aeruginosa	$0^{a} \pm 0.0$	$25^{a} \pm 0.1$	$15^{a} \pm 0.0$	$13^{b} \pm 1.5$	$12^{a} \pm 0.0$
Yeast					
C. albicans	$0^{a} \pm 0.0$	$21^{b} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$

The study supports the ethnomedicinal use of *A. muricata* for treatment of many infections. The results of antimicrobial activity of *A. niger* strain *SH3* extract, seed extract and in combination were compared with positive control either Ampicillin or Amphotericin B for evaluating their relative percentage inhibition, where *A. niger* strain *SH3* extract exhibits maximum relative percentage inhibition against *P. aeruginosa* (36%), (32.65%) against *S. aureus* (31.37%) against *E. coli* followed by seed extract and combined extract showed the least percentage (Table 5).

Table 5: Relative activity compared to the standard positive control

Pathogenic	Relative activity (%)		
Microorganism	*A. niger* strain *SH3* Extract	Seed Extract	Combined Extract
S. aureus	32.65	27.43	22.67
E. coli	31.37	23.04	19.36
P. aeruginosa	36.00	27.05	23.05
C. albicans	0.00	0.00	0.00

Mathew *et al.,* [20] proved the effectiveness of *A. muricata* leaf extract as an antibacterial agent against *Enterococcus faecalis*.

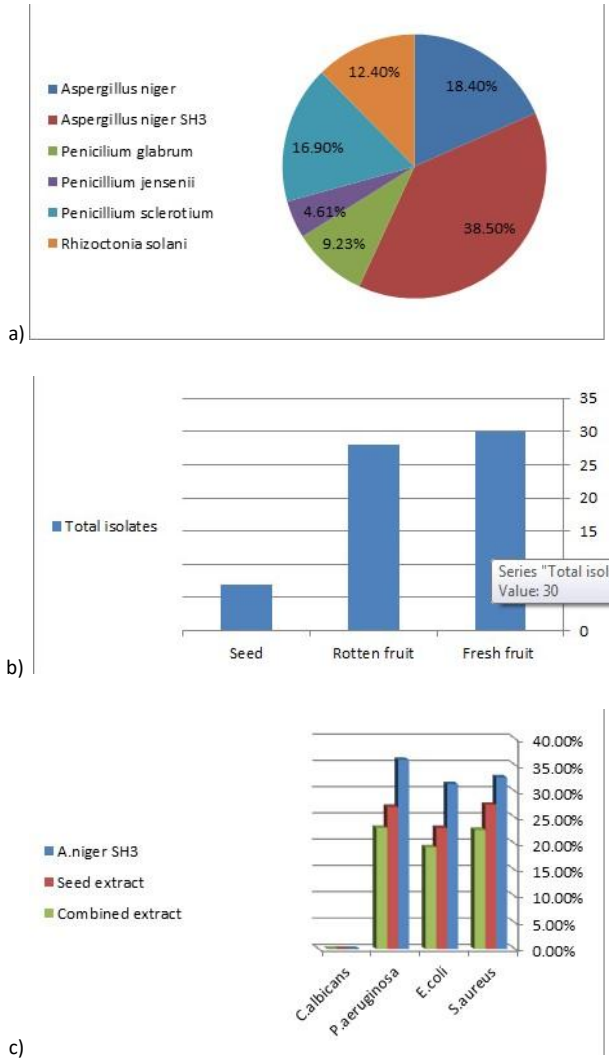

Figure 1: a) Frequency percentage of endophytic fungal species isolated from different parts of A. muricate; b) Total isolates of endophytic fungi isolated from different parts of A.muricata; c) Relative activity % of A. niger strain SH3 and seed extracts either singly or in combination

Discussion

In relations to our study, the fungal genera *Aspergillus, Penicillium, Mucor* and *Rhizopus* were isolated from *A. muricata* as endophytes [13], [14]. We can conclude that among the endophytic flora, *Aspergillus* was found to be the core group fungus from *Annona* with a colonisation frequency of 56.9%.

Salkowski test was more precise for checking terpenoids [15]. The appreciable amount of terpenoids may be due to the precise extraction process which influences the number of secondary metabolites [16].

Abdelhamid *et al*., [17] reported that GC-MS chromatogram of the ethanolic extract of Nelumbo nucifera seed showed thirty-eight peaks which indicates the presence of thirty-eight phytochemical

constituents including [[(trimethylsilyl)oxy] methyl]ethyl ester, Anodendroside E2 monoacetate, Betulin and Cholestan-3-one,cyclic 1,2-ethanediylaetal, (5á) exhibited various biological activities including Antiinflammatory, Antitumor, Antiviral, Cytotoxic and Hypolipemic [18]. Venkatachalam and Jyothiprabha, 2016 also reported antimicrobial activity of cinnamon (*Cinnamomum Verum*) extracts against Vancomycin-Resistant Enterococcus due to the presence of thirty major antimicrobial compounds identified by GC-MS analysis including Anodendroside F.

In this field, *Annona muricata* leaf extracts at a potency 20 mg/ml showed antimicrobial activity when tested against *P. aeruginosa, E. coli, S. aureus* and *C. albicans* with inhibition zones ranging from 20 to 42 mm [19].

The synergism of flavonoids, terpenoids, and alkaloids found in the extracts of *A. muricata* explains its antibacterial activity [1], [21]. It is reported that endophytic fungi from the same host plant could contain identical bioactive compounds but showed different activity [22]. Also, the combination of ethanolic extract of sour soup with antibiotic treatment increased the effectiveness of the antibiotic against multidrug-resistant strains of *E. coli* and *S. aureus* [2], [16].

In conclusion, *Annona muricata* with its endophytic fungi have an important role as antimicrobial agents against certain microorganisms. So, *Annona muricata* can be used for treatment of many infections which could be attributed to presence of terpenoids.

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