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Antimicrobial Activities of Secondary Metabolites Produced by Endophytic Fungi from *Glinus lotoides*

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Abstract: Endophytic fungi were isolated from healthy leaves of a medicinal plant *Glinus lotoides* L. (Molluginaceae) collected at three sites of Wadi Allaqi, the largest wadi in Egypt's South- eastern desert protected area in Aswan. Of 7 strains isolated from the three sites examined, only two, *Aspergillus fumigatus* and *A. terreus* were examined for their bioactive compounds. Culture broth of the two fungal strains were separated by TLC method and tested for biological activities using TLC bioautography technique against Gram positive and Gram negative bacteria (*B. subtilis, E. coli, P. aeruginosa* and *S. aureus*) and filamentous fungi (*A. niger, F. oxysporum, P. chrysogenum* and *B. cinrea*). All fungal fractions effectively inhibited the growth of all test organisms with variable extents, indicating that they may represent a potential for pharmaceutical and/or agricultural applications and are worthy of further study.

Key words: Endophytes · *Aspergillus fumigatus* · *A. terreus* · *Glinus lotoides*

INTRODUCTION

Endophyes are microorganisms that live in the intercellular spaces of stems, petioles roots and leaves of plants causing no discernible manifestation of their presence and have typically gone unnoticed [1]. The symbiosis between plant and endophyte was ascertained, namely, the former protects and feeds the latter which produces "inreturn" bioactive(plant growth regulatory, antibacterial, antifungal, antiviral, insecticidal, etc.) substances to enhance the growth and competitiveness of the host [2]. According to [3], approximately 4,000 secondary metabolites of fungal origin have been described to possess biological activities. The largest part of these metabolites are produced by a group of fungi that [3] called "creative", they include, among others, species of *Acremonium, Aspergillus, Fusarium* and *Penicillium.*

As tropical regions host more than half the number of living worldwide, a large number of biologically active metabolites is probably produced in these ecosystems. This is also supported by the fact that several plant species of the tropics are known to possess medicinal properties and are now actively investigated by ethinobotanists. The genus *Glinus* growing in Egypt comprises two annual species, one of them is *G. lotoides* L. (Syn = *Glinus dictamnoides* = *Mollugo hirta* = *Mollugo glinus*) which grows wild in tropical and subtropical regions worldwide [4]. It is a robust procumbent herb, either green or more often densely grey-canescent known under the Arabic names of "Hasheesh el aqrab" or "Ghobberia" [5]. It is used as a folk medicine against oharrhas bilious attacks and as a purgative, for the treatment of boils, wounds, pains in the limbs and as an antihelmintic [6]. In this study we isolated and identified the fungal endophytes from leaves segments of *G. lotoides* collected at three sites in wadi Allaqi protected area, and to describe the microfermentation, isolation and biological and chemical evaluation of secondary metabolites produced by the endophytes isolated.

MATERIALS AND METHODS

Fungal isolation and identification: The endophytic fungal strains were separated from healthy leaves of *Glinus lotoides* according to procedure described [7]. Specifically, the leaves of *G. lotoides* were washed with running tap water, sterilized with 75% ethanol for 1 min and 2.5% sodium hypochloride for 15 min, then rinsed in sterile water for three times and cut into 1 cm long segments. Both borders of sterilized segments were cut off and the rest was incubated at 28°C on PDA medium supplemented with 200µg/ml ampicilin and 200µg/ml streptomycin to inhibit the bacterial growth until the

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mycelium or colony originating from the newly formed surface of the segments appeared. The mycelium was purified in the same conditions. Another segment of the same origin without surface sterilization was cultured as a negative control to check the presence of contaminated microbes on the segment surface. The purified endophytic fungi were numbered and transferred to fresh PDA slants separately and were kept at 4°C after being cultured at 28°C for 7 days. The fungal taxa were identify on the basis of cultural characteristics and the morphology of fruiting bodies and spores [8-10]. Strains of *Aspergillus fumigatus* and *A. terreus* were then chosen for this study, because of their high frequencies of isolation.

Fermentation and extraction: Twelve Erlenmeyer flasks each conta-ining 100 ml of malt extract broth (ME) (20 g/L malt extract, 1 g/L pept-one, 20 g/L glucose) were inoculated with three fragments taken from the edge of active growing colonies of *Aspergillus fumigatus* and *A. terreus* for seven days at 110 rpm on rotary shaker at room temperature, with 12 h photoperiod under fluorescent light. After fermentation, culture broth was separated from mycelia by filtration, culture filtrate were extracted successively with EtOAc (1000x3) and n- butanol (1000x3) then filtered through a pad anhydrous Na_2SO_4 and evaporated to dryness. We obtained (82 mg, 95 mg) (75 mg, 88mg) for *A.fumigatus and A.terreus* respectively.

A part of the crude extract was used for the biological tests and the remainder was analysed by thin layer chromatography (TLC) to detect fungal secondary metabolites, to determine the ratio of components present in the crude extracts for further purification.

Analytical TLC was performed on silica gel plates with CHCL3 –MeOH (9:1) and CHCL3 –MeOH –HO2 (6:3:1), as the developing system. Spots were visualized by exposure to UV light. Dragendorff reagent was used to detect spots of alkaloids [11]. For detection of non-alkaloids, TLC plates developed were sprayed with P-anisoaldhyde reagent (15 g anisoaldhyde in 250 ml ethanol and 2.5 ml sulfuric acid) and heated at 60°C.

Preparation of spore suspension: The following test microorganisms were used for detecting antimicrobial activities: *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Subtilis aureus, Aspergillus niger, Fusarium oxysporum, Botrytis cinerea* and *penicillium chrysogenum*. Bacteria cultures were grown overnight in nutrient broth and diluted to 10^6 cells/ml by means of serial dilution just before bioautography. Conidial suspensions of the filamentous fungi were obtained from seven days old cultures on malt extract agar by flooding the plates with sterile water. Conidial suspensions were adjusted at 5 X 10^6 by serial dilutions, counting spores /dilution.

Bioautography of TLC: Each Ethyl-acetate and Butanol fractions of *Aspergilus fumigatus* and *A. terreus* dissolved in methanol were spotted on TLC plate. After drying, the plate was sprayed with fungal spore suspension (5 X 10^6 spore ml^{-1}) and bacterial spore suspension (1 X 10^6 spore ml^{-1}). TLC plate sprayed with fungal spore suspension was incubated on a sheet of wet filter paper in a petri dish at 25°C for 8 days. Another TLC plate sprayed with bacterial spore suspension and incubated at 37°C for 24-48 h. the diameter of inhibited zone was measured. For determination of compounds in each fraction 10 µl (20 mg ml^{-1}) each of fraction was applied to another TLC plate as developed one with chloroform, methanol (9:1) and chloroform, methanol, water (6:3:1).

RESULTS AND DISCUSSION

Endophytic fungi were isolated from surface sterilized plant tissues of leaves of *Glinus lotoides*. No isolates were recorded before 24 hours of incubation and most isolates were recorded in the first two weeks of incubation. These results correspond with other result obtained for the rate of isolation of endophytic fungi from others hosts [7]. The three sites, respectively in wadi Quleib, wadi um Arka and wadi Heisurbay, are geographically distant but they both fall within the natural distribution of downstream tributaries of wadi Allaqi. Despite this distance, the fungal species composition of all sites was essentially the same. To our knowledge there have been no published reports of endophytes fungi residing in *G. lotoides* plants. So there is no data available for comparison. The isolation of 7 species, show few diverse nature of endophytic population of leaves of *G. lotoides*. Most species isolated during this study belong to genera which have already been described as endophytes from different hosts at different locations [12]. In addition, some of them are cited as pathogens of many hosts.

Alternaria alternaia exhibited the highest counts which constituted 67.6% of total count (Table 1). It appears in all tested samples. *Alternaria alternaria* has been routinely isolated as endophyte of leaves from wheat and other plants [13], from maize and soybean

Table 1: Actual number of endophytic isolates from leaves of *Glinus lotoides* from three sites in Wadi Allaqi (Aswan area)

Fungal species	S1	S2	S3
Acremonium strictum	3	8	5
Alternaria alternaria	35	15	90
Aspergillus fumigatus	38	14	18
A. niger	2	1	3
A. terreus	9	36	19
Chaetomium globosum	32	6	15
Mucor circinolloides	2	2	2

S_1: Wadi Quleib, S_2: Wadi Um Arka, S_3 Wadi Heisurbay, are all down stream tributaries of Wadi

Table 2: RF values of compounds detected on thin layer chromatography of *Aspergillus fumigatus* and *A. terreus*

	RF values			
	Aspergillus fumigatus		*A. terreus*	
Fungal fraction	A	B	A	B
Ethylacetate fraction	1.4	0.79	1.4	0.05
	1.9	0.87	1.6	0.11
	2.5		1.9	0.58
	3.0		3.0	0.65
	4.0		3.6	
	4.6			
	5.2			
Butanol fraction	1.4	0.11	1.4	0.05
			1.9	0.11
			2.5	0.41
			3.6	0.65
			4.0	0.70
			6.5	0.79
				0.87

A solvent system chloroform : methanol 9 : 1

B: solvent system chloroform : methanol : water 6 : 3 : 1

leaves [14]. In addition *Alternaria* has been described as pathogen of water hyacinth [15] and as laten pathogen in persimmon [16]. Some endophtic spec-ies namely, *Alternaria phragmospora, Drechslera australiensis, Aspergillus niger* and *Fusarium moniliforme* isolated from healthy looking, senescent and decayed leaves of *Eicchornia craspes* [17]. *Aspergillus* contributed three species of which *A. fumigatus* and *A. terreus* were the most frequent. Although *Aspergillus* is ubiquitous and usually epiphytic, it has been known to grow endophytically [18]. *A. fumigatus* isolated as an endophytic fungus in *Cynodon dactylon* for the first time [19]. The possibility that it could be a conta-minating microbe was excluded by the vitality test [20]. As reported [19, 21], an endophyte in one plant could be a pathogen of the other depending on the balance between pathogenicity and endophytism of the microorganism in different hosts.

Table 3: Antimicrobial activity of secondary metabolites from *Aspergillus fumigatus* and *A. terreus* detected by TLC bioautography[a]

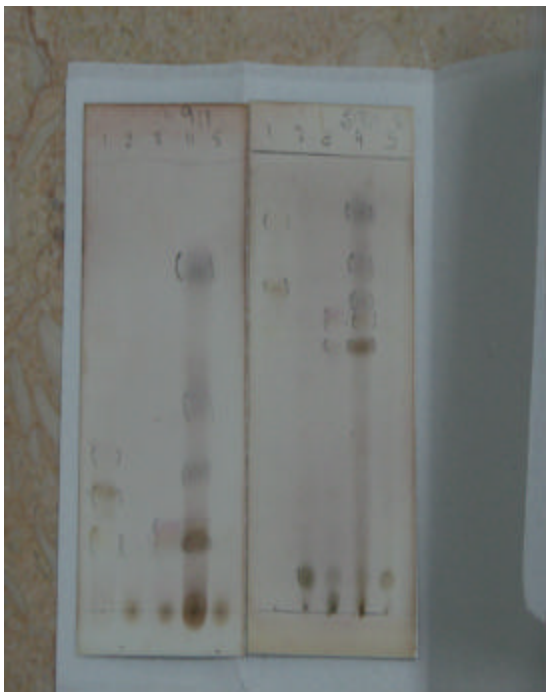
	Aspergillus fumigatus		*A. terreus*	
Test microorganisms	E	B	E	B
Aspergillus niger	0.8	1.2	1.8	0.5
Botrytis cinerea	1.4	0.7	0.0	2.3
Fusarium oxysporum	0.1	0.0	1.4	0.6
Penicillium chrysogenum	0.2	2.5	0.0	2.8
Bacillus subtilis	0.9	0.6	1.0	0.4
Escherichia coli	0.0	0.0	0.5	0.2
Pseudomonas aeruginosa	0.4	0.0	0.6	1.8
Staphylococcus aureus	2.8	0.8	1.5	0.6

a values are diameter of inhibition zone (cm). No inhibition denoted as "0", E, ethylacetate fraction; B, n-butanol fraction

Chaetomium globosum was one of the basic components of endophytic fungi and constituted 17.5% of the total count. Although *Chaetomium gl-obosum* is reported to be a corophilous fungus and a common biodeterioration agent of cotton and plant tissues, it was recorded as endophyte from *Rhizo-phora apiculata* at mangroves in India [22] and from *Kandelia candel* leaves [23]. *Chaetomium cochloides* has been recorded as endophyte from roots *Alnus glutinose* [24]. Although *Acrem-onium strictum*, *Aspergillus niger* and *Mucor circinolloides* were confined to the three sites, they were isolated at very low frequency.

Culture broths of *Aspergillus fum-igatus* and *A. terreus* were separated by filtration followed by extraction with ethylacetate and then with n-butanol. TLC of successive extracts showed that ethylacetate fraction of *A. fumigatus* and *A. terreus* are the same, (Table 2 & Fig. 1), showing 9 spots (RF 1.4, 1.9, 2.5, 3, 4, 4.6, 5.2, 0.79, 0.87 for *A. fumigatus*), (RF 1.4, 1.6, 1.9, 3, 3.6, 0.05, 0.11, 0.58, 0.65 for *A. terreus*), three of which are the same RF. Butanol fraction of *A. terreus* shows 13 spots, 9 of which are similar to those of Et-Ac fraction of both *A. fumigatus* and *A. terreus* (1.4, 1.9, 2.5, 3.6, 4, 0.05, 0.11, 0.79 and 0.87).

The fungal secondary metabolite pro-files obtained during this investigation presented a satisfactory separation with the use of TLC plate using the solvent (A); Chloroform Methanol (9:1) and solvent (B); Chloroform Methanol water (6:3:1), modified Dragendroffis reagent to detect spots of alkaloids and



(1) (2)

Fig. 1: TLC running with solvent system (1) (Chloroform: Methanol) (9:1) and solvent system (2) (chloroform: Methanol: Water) (6:3:1)

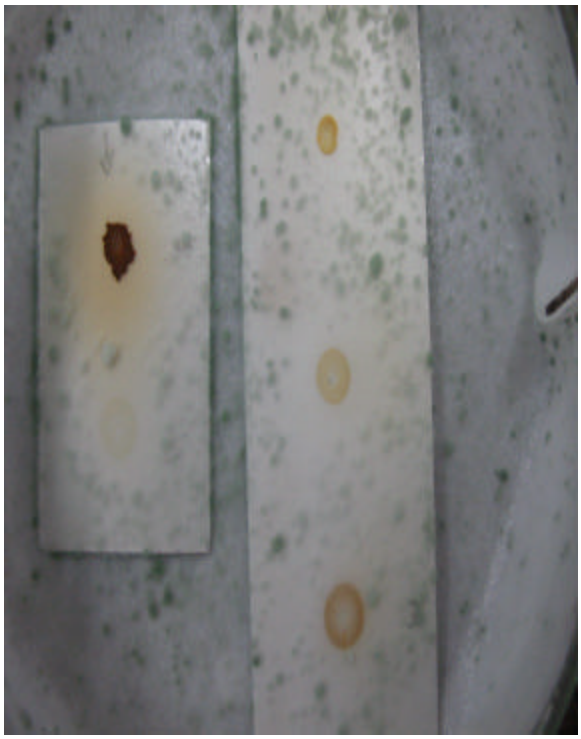


Fig. 2: Zones of fungal inhibition, produced by antifungal metabolites found in *Aspergillus fumigatus* and *A. terreus*, prepared by their *Ethylacetate* extractions (1,3) and *Butanol* extractions (2,4) respectively. Direct bioautography was performed by inculating the plates (with spore suspensions in potato dextrose of either *P. chrysogenoum* (A) which showed high inhibition with Butanol extraction of *A. terreus* (4)

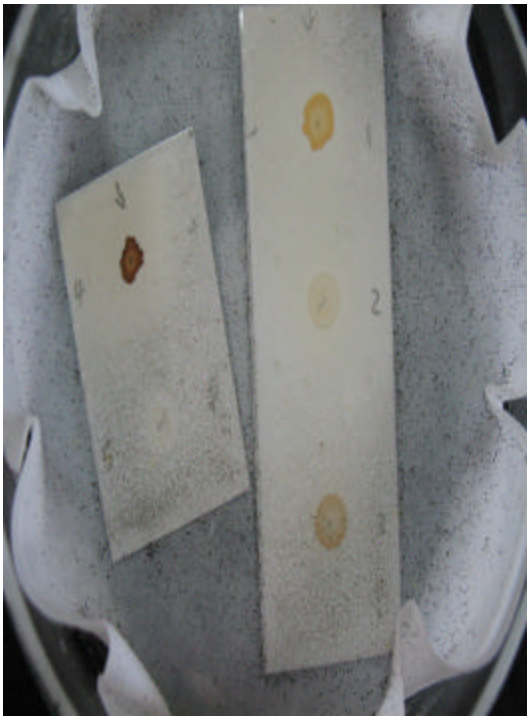


Fig. 2B: *A. niger* gave high inhibition with Butanol extractions of *A. fumigatus* (2) and then *A. terreus* (4)

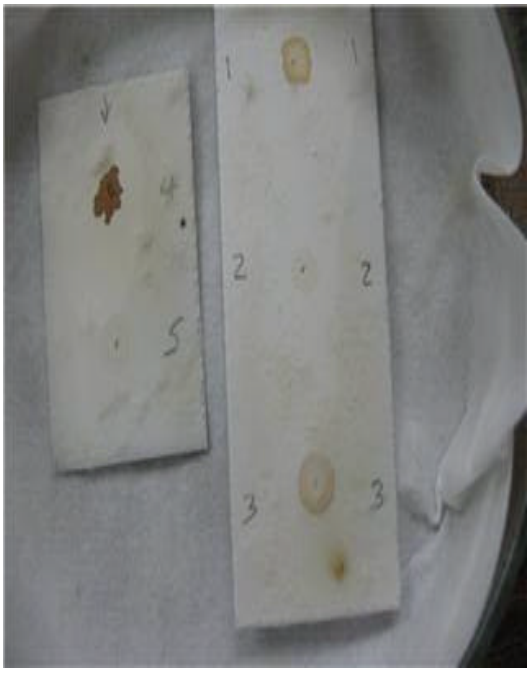


Fig. 2C: *Botrytis cinerea* gave clear inhibition zone with Butanol extraction of *A. terreus* (4) and then with Ethylacetate extraction of *A. fumigatus* (1)

were sprayed with P. anisaldhyde reagent and heated 60°C to detect spots of non-alkaloids, confirming its strength as the most currently used technique for screening plant extracts [25].

Additionally, our results pro-vided preliminary information for pointing out *Aspergillus* as creative organisms according to the classification of Dreyfuss Chapela [3]. *Aspergillus* as genus is of intense biological, industrial, agricultural and medicinal importance. The species responsible for more than 9% of human disease is *Aspergillus fumigatus* [26]. One set putative *A. fumigatus* virulence factors are mycotoxins, secreted fungal secondary metabolites that are harmful to humans and animals [27]. Recently Keller *et al.* [28] identified a nuclear protein, lae A, from *A. fumigatus* that regulates the production of all metabolites. *A. terreus* is an especially prolific producer of secondary metabolites [29]. Lovastatin is clinically useful for reducing serum cholesterol produced by *A. terreus* as secondary metabolite [30].

Fungal extracts tested for antimicrobial activities were examined using TLC- bioautography. Represent-ative TLC- bioautography is shown in (Table 3, and Fig. 2A-C). Butanol fraction of *A. terreus* was the most effective one. It was able to inhibit the growth of *A. niger*, *Botrytis cinerae, Fusarium oxy-sporum, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* but with variable extents, and therefore will be selected for further bioactivity guided fractionation.

Ethylacetate fraction of the two endophytes (*A. fumigatus* and *A. terreus*), also has high antimicrobial activity against fungal and bacterial strains. Endophytic fungi have been to known to produce antifungal comp-ounds extractable with ethylacetate [31]. Liu *et al.* [19] proved that the endophytic fungus *A. fumigatus* Cyo18 inside the healthy leaves of *Cynodon dactylon* was a versatile producer of new metabolites named (asperfumoid and asperfumin). Furthermore, the anti-fungal action of these compounds were confirmed against the human path-ogens *Candida albicans*, *Tricophyton rubrum* and *Aspergillus niger*.

Our investigation supports the claims that bioactive fungal const-ituents produced by tropical endo-phytic fungi are a rich source of novel metabolites exhibiting a wide range of important biological activities [32].

Further investigations on the isola-tion and purification of the active constituents of fungal extracts are in progress in our laboratory.

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