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CYTOTOXIC POTENTIAL OF FUNGI ASSOCIATED WITH RHIZOSPHERE AND RHIZOPLANE OF WILD AND CULTIVATED PLANTS

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Abstract

Discovery of anticancer drugs that must kill or disable tumor cells in the presence of normal cells without undue toxicity is an extraordinary challenge. In the past 50 years, number of highly successful drugs based upon fungal metabolites was discovered. Beside producing antibiotics, fungi have a much greater potential for producing other medicinally useful compounds including antitumor agents and immunoregulators. Toxicity of plant or microbial material is considered as the presence of antitumor compounds. In this study culture filtrates of 51 fungal isolates, belonging to 15 genera viz., *Alternaria, Aspergillus, Cephalosporium, Chaetomium, Cladosporium, Drechslera, Fusarium, Macrophomina, Memnoniella, Myrothecium, Paecilomyces, Penicillium, Rhizoctonia, Trichoderma* and *Verticillium* (*Pochonia*) isolated from rhizosphere and rhizoplane of cultivated and wild plants showed significant toxicity on brine shrimp (LC_{50} 3.3-116 µl/ml). *Aspergillus niger* (LC_{50} 3.7µl/ml), *Penicillium citrinum* (LC_{50} 3.7µl/ml), *P. purpurescens* (LC_{50} 3.3µl/ml) *P. rugulosum* (LC_{50} 6.3 µl/ml) and *Penicillium* sp., (LC_{50} 4.3 µl/ml) showing highest mortality of brine shrimp. Fungi associated with rhizosphere and rhizoplane of wild and cultivated plants offer a unexhousted source of antitumour agent.

Introduction

Rhizosphere is the soil surrounding the rhizoplane (root surface) and the term was firstly introduced by Hiltner in 1904 (Brimecombe *et al.,* 2001; Lynch, 1990). The loss of organic materials from roots provide the driving force for the development of active microbial populations around the root (Whipps, 2001, Morgan & Whipps, 2001) support higher microbial biomass and microbial activity than in the bulk soil (Nannipieri *et al.,* 2007). Another area recognized as intense microbial activity is rhizoplane, the root surface, which also include strongly adhering soil particles (Barea *et al.,* 2005). Antagonistic activities of numerous microbial populations in the rhizosphere influence plant growth and health (Berg *et al.,* 2005; Weller, 1988; Weller *et al.,* 2002). Among the rhizosphere microorganisms, fungi play an important role in the rhizosphere, they mediate many ecological processes and are responsible for plant growth and health (Hawksworth & Rossmann, 1997). Competition between the saprophytic fungi with soilborne plant pathogens for space or nutrients has been known to exist as biocontrol mechanisms (Whipps, 1997, 2001). Production of antibiotics, toxins, biosurfactants and cell wall degrading enzymes are the weapons used by the fungi against competitors in the rhizosphere and rhizoplane (Whipps, 2001; Berg *et al.,* 2005).

Toxicity of plant or microbial material is considered as the presence of antitumor compounds. Brine shrimp bioassay has successfully been used as prescreening of bioactive compounds having antitumor activity (McLaughlin *et al*., 1993). This test has been established as a safe, practical and economic method for the determination of the bioactivity of synthetic compound (Almeida *et al*., 2002), mycotoxins of fungal pathogens (Schmidt *et al*., 1995; Favilla *et al.,* 2006), marine products (Ara *et al*., 1999, Manilal *et al*., 2009; Ayesha *et al.,* 2010) as well as higher plant products (Stefanello *et al*., 2006; Nino *et al*., 2006). National Cancer Institute (NCI, USA) has found a significant correlation between the brine shrimp assay and *in vitro* growth inhibition of human solid tumor cell lines (Silva *et al*., 2007). Considering the importance of fungal metabolites as an inexhaustible source of new antimicrobial, antiviral and antitumor agents, the present study was undertaken to evaluate the cytotoxic activity of culture filtrates of fungi isolated from rhizoplane and rhizosphere of some wild and cultivated plant species, using brine shrimp bioassay.

Materials and Methods

Fungal cultures: For the isolation of fungi from rhizosphere and rhizoplane, plant samples were collected from different locations like Darsano Chano, Gharo, Karachi University Campus, Kathor, Memon Goth, and Thatta from Sindh and Hub from Baluchistan. Healthy cultivated plants and some wild plants were dug out carefully and root samples with adhering soil were collected in polyethylene bags, brought to laboratory and stored in refrigerator. Isolation of fungi were made within 24 hours of collection.

Isolation of fungi from rhizosphere: For the isolation of fungi from rhizosphere, Volume Displacement Technique as suggested by Reyes & Mitchell (1962) was used, where root pieces with adhering soil were placed in graduated cylinder containing 18 ml of sterilized distilled water and was shaken vigorously. The remaining roots were added and shaken until the total volume of soil and water become 20 ml and assumed it as 1:10 dilution. The amount of rhizosphere soil sample was thus determined by volume displacement. A dilution of soil (v/v) was prepared from 1:10 to 1:10,000. One ml aliquot of the two highest soil dilutions were poured in sterilized Petri dishes containing Potato Dextrose Agar supplemented with penicillin (100,000 units/litres), streptomycin (0.2 g/litres) to prevent bacterial growth. Plates were incubated for 5 days at 28°C. Fungi grown on plates were identified after reference to Barnett & Hunter (1998); Booth (1971); Domsch *et al.,* (1980); Dugan (2006), Ellis (1971); Gilman (1957); Nelson *et al*., (1983); Raper & Fennel (1965); Raper & Thom (1949) and Thom & Raper (1945).

Isolation of fungi from rhizoplane: Roots were washed in running tap water and 1 cm long root pieces from tap and lateral roots were cut and then washed in sterilized distilled water. Root pieces were transferred on PDA plates containing penicillin (100,000 units/litre) and streptomycin (0.2 g/litre). Dishes were incubated for 5 days at 28°C. Grown fungi were identified as mentioned above.

Preparation of culture filtrates of fungi: Test fungi were grown in conical flasks (500 ml) containing 200 ml Czapek's Dox Broth, plugged with cotton wool and autoclaved at 121°C for 20 minutes. After cooling the medium, each flask was inoculated with 5 mm disc, cut from the margin of vigorously growing culture of test fungi. Each test fungus had 5 flasks. These flasks were incubated for 15 days at room temperature (25-30°C). After 15 days, test fungi were filtered through Whattman No.1 filter paper. The culture filtrates were separated whereas mycelium were dried under Laminar flow hood and weighed.

***In vitro* cytotoxicity of culture filtrates on brine shrimp (*Artemia salina*):** Brine shrimp lethality test for larvae nauplii was used to determine the toxicity of culture filtrates (Mclaughlin *et al*., 1993). Brine shrimp eggs (Carolina Biological Supply Company Burlington, NC. USA) were hatched in shallow rectangular container (60x30 cm) filled one fourth with artificial seawater (prepared with commercial sea salt and distilled water). A plastic divider hole was placed in the container to make two unequal compartments. The eggs were sprinkled into the large compartment, which was darkened, while the smaller compartment was illuminated. After 48 hours, the phototropic nauplii were collected from the lighted side.

The brine shrimp bioassay was performed according to the procedure described by McLaughlin *et al.,* (1993). Culture filtrate was used undiluted and diluted to 1:10 by transferring 1 ml culture filtrate with sterile pipette into 9 ml artificial seawater. Further dilution (v/v) of culture filtrate was prepared from 1:10 to 1:1000. Five ml of each dilution (freshly prepared) was transferred into glass vials. Three replicates were used for each dose level. Ten shrimps were transferred to each glass vial containing 5 ml undiluted and diluted culture filtrates. Control glass vial was prepared using 5 ml artificial seawater. A drop of yeast suspension (3mg/5ml) was added as food supplement. The vials were maintained under illumination. Survivors were counted with the aid of a stereomicroscope after 24 hours and the percent death at each dose level and control was determined. LC_{50} was determined from the 24 hours count using the probit analysis method (Finney, 1971).

Results

Most of the test fungi showed significant cytotoxic effect on brine shrimp by killing them at varying degrees (Table 1). Of the species of *Chaetomium* tested, *C. globosum* (S-1, S-2 & S-4) showed significant mortalities (LC_{50} < 19.5-30.6 µl/ml), while *C. indicum* showed mortality only in its undiluted filtrate @ 90% with LC_{50} < 131µl /ml. *Alternaria alternata* showed 36, 50 and 100% death of brime shrimp @ 10, 100, and 1000 µl/ml dilutions respectively with LC_{50} < 30 µl/ml whereas species of *Cephalosporium*, *Cladosporium*, *Curvularia*, *Rhizoctonia*, *Stachybotrys* and *Talaromyces* showed LC_{50} < 13-74 µl/ml. On the other hand, LC_{50} < 100 µl/ml was observed by *Macrophomina phaseolina*, *Memnoniella echinata* and *Scopulariopsis brumptii* towards brine shrimp (Table 1).

Of the Aspergilli tested, maximum activity was shown by *A. niger* (LC_{50}< 3.7 µl/ml), followed by *A. fumigatus*, *A. flavus*, *A. ustus*, *A. sulphureus*, and *A. nidulans* (LC_{50}< 20-88 µl/ml). Significant cytotoxicity was also shown by species of *Drechslera* in their undiluted and diluted culture filtrates. All the three species of *Drechslera* tested demonstrated LC_{50} <15-30.6 µl/ml. Maximum mortality was observed by *Myrothecium cinctum* (LC_{50} <75 µl/ml) as compared to *M. roridum* (LC_{50} <116 µl/ml). Variable cytotoxic activity was shown by three strains of *Paecilomyces lilacinus* (LC_{50} <40-74 µl/ml). Among the *Fusarium* species, *F. oxysporum* showed greater cytotoxic activity (LC_{50} <25.2 µl/ml) followed by *F. solani* (S-1) (LC_{50} <37.8 µl/ml) (Table 1).

Of the genus *Penicillium* tested, highly significant cytotoxic activity was observed by *P. citrinum*, *P. purpurescens*, *P. rugulosum* and *Penicillium* sp., (LC_{50} < 5.4, 3.3, 6.3, and 4.3 µl/ml respectively), while other *Penicillium* species viz., *P. aspermum*, *P. brefeldianum* and *P. purpurogenum* demonstrated LC_{50} < 42-86 µl/ml. Among the species of *Trichoderma* tested, *T. harzianum* and *T. viride* (S-1) showed maximum mortalities of brine shrimp (LC_{50} <21-29 µl/ml). *Verticillium chlamydosporium* (*Pochonia chlamydosporia*) also showed cytotoxic effect on brine shrimp (LC_{50} <100 µl /ml) (Table 1).

Discussion

Discovery of anticancer drugs that must kill or disable tumor cells in the presence of normal cells without undue toxicity is an extraordinary challenge (Hameed *et al*., 2009). In this study fungi isolated from rhizosphere and rhizoplane of different cultivated and wild plants have shown significant cytotoxic activity on brine shrimp. Natural products especially of higher plants and microbial origins have served as rich source of novel drugs. In the past 50 years, number of highly successful drugs based upon fungal metabolites was discovered. Beside producing antibiotics fungi have a much greater potential for producing other medicinally useful compounds including ergots alkaloids, steroid derivatives, antitumor agents and immunoregulators (Wainwright, 1992). In this study some species of *Penicillium* have shown significant activity by causing death of brine shrimp at very low concentration. There is report that the antitumor antibiotic GKK1032 (GKK1032A1, A2, A3 and B) are manufactured with *Penicillium* species (Koizumi *et al*., 2001). Similarly, a novel antitumor antibiotic, methylenolactocin isolated from culture filtrate of *Penicillium* sp., was also found active against Gram-positive bacteria (Park *et al*., 1988). In this study besides, *Penicillium* spp., *Aspergillus niger* also showed significant activity at low concentration. Many antimicrobial and antitumor quionones have been reported from *Aspergillus* species (Remers, 1979; Thomson, 1971). In this study *Myrothecium cinctum* and *M. roridum* also caused brine shrimp death (LC_{50} 75.2 and 116 µl/ml respectively). Two new roridins, having cytotoxic activity against HCT-116 human colon tumor cell line, have been reported from *Myrothecium* sp., (Wagenaar & Clardy, 2001). *Fusarium solani* produced fusarubin showed to have antitumor activity (Issaq *et al*., 1977). In this study

Fusarium oxysporum and *F.solani* also showed potent cytotoxic effect on brine shrimp. Hameed *et al.,* (2009) has been reported the cytotoxicity of strains of seedborne *Fusarium solani.*

The microbial interactions in the rhizosphere are mostly viewed from the perspective of how beneficial microorganisms inhibit the growth or activity of pathogenic microorganisms (Raaijmakers *et al.,* 2009). Some of the species present in rhizosphere or rhizoplane exhibit a range of antagonistic activities including production of nematotoxic compounds (Kerry, 2000; Lopez- Llorca & Jansson, 2006; Berg *et al.,* 2005). However, investigation on the rhizosphere and rhizoplane fungi as a source of antitumor agent is generally neglected. The warfare between the fungi and pathogenic microorganisms in rhizosphere and on rhizoplane for occupancy of space and nutrients is unexhausted source of valuable metabolites for agricultural and pharmaceutical uses.

Table 1. Percent death of *Artemia salina* at different concentrations of culture filtrates of fungi, isolated from rhizosphere and rhizoplane of some wild and cultivated plant species.

S. No.	Isolated fungi	Host	Region	Locality	(10µl)	(100µl)	(1000µl)	LC_{50}
1.	*Alternaria alternata*	*Luffa aegyptiaca*	Rhizosphere	Malir	36	50	100	<30.0
2.	*Aspergillus candidus*	*Leucaena leucocephala*	"	Hub	0	16	100	<116
3.	*A. flavus* (S-3)	*Cynodon dactylon*	"	"	46.6	63.3	86	<19.5
4.	*A. fumigatus*	*Phaseolus vulgaris*	"	Gharo	10	50	80	<18.1
5.	*A. glaucus*	*Citrullus lanatus*	"	Hub	10	10	100	<100
6.	*A. nidulans* (S-1)	*Solanum melongena*	"	"	0	50	100	<88.2
7.	*A. niger* (S-1)	*Cyperus rotundus*	"	"	73	100	100	<3.7
8.	*A. ochraceus*	*Solanum surranttence*	"	"	0	0	100	<116
9.	*A. restrictus*	*S. melongena*	"	Malir	0	0	100	<116
10.	*A. sulphureus* (S-1)	*Lagenaria siceraria*	"	Hub	0	60	100	<72.4
11.	*A. sulphureus* (S-2)	*Gossypium arboreum*	"	KU	0	50	100	<73.9
12.	*A. terreus*	*L. aegyptiaca*	"	Malir	0	0	100	<116
13.	*A. ustus*	*S. surranttence*	"	"	30	36	100	<31.8
14.	*Cephalosporium* sp.	*L. siceraria*	Rhizoplane	"	50	56	100	<20.7
15.	*C.haetomium globosum* (S-1)	*Vigna radiata*	Rhizoplane	KU	43	53	100	<30.6
16.	*C. globosum* (S-2)	*Chenopodium album*	"	Gharo	50	53	100	<20.0
17.	*C. globosum* (S-3)	*Cyamopsis tetragonoloba*	Rhizoplane	Kathor	0	3.3	100	<116
18.	*C. globosum* (S-4)	*Melilotus alba*	Rhizosphere	Malir	45	62	85	<19.5
19.	*C. indicum*	*Solanum surranttense*	"	Hub	0	0	90	<131
20.	*Cladosporium* sp.	*Digera muricata*	Rhizoplane	KU	46	50	100	<13.2
21.	*Curvularia clavata*	*Cenchrno setigerus*	Rhizosphere	Hub	13	46	100	<73.2
22.	*Drechslera australiensis* (S-1)	*Citrullus lanatus*	Rhizoplane	"	40	56	100	<30.6
23.	*D. australiensis* (S-2)	*Launea nudicaulis*	Rhizosphere	KU	50	70	100	<15.0
24.	*D. hawaiiensis*	*Medicago sativa*	Rhizoplane	Malir	53	76	100	<23.5
25.	*Fusarium oxysporum*	*Arachis hypogaea*	"	KU	43	43	100	<25.2
26.	*F. solani* (S-1)	*Luffa aegyptiaca*	"	"	10	10	100	<100
27.	*F. solani* (S-2)	*A. hypogaea*	"	"	26	56	100	<37.8
28.	*Macrophomina phaseolina*	*Abutilon indicum*	Rhizosphere	Malir	10	10	100	<100
29.	*Memnoniella echinata*	*Sorghum bicolor*	Rhizosphere	Kathor	0	26	100	<86.5
30.	*Myrothecium cinctum*	*Citrullus lanatus*	"	Hub	40	50	100	<75.2
31.	*M. roridum*	*Vigna mungo*	Rhizoplane	KU	0	0	100	<116
32.	*Paecilomyces lilacinus* (S-1)	*C. lanatus*	Rhizosphere	Hub	3.3	50	100	<73.9
33.	*P. lilacinus* (S-2)	*Cynodon dactylon*	"	"	0	70	100	<47.7
34.	*P. lilacinus* (S-4)	*Luffa aegyptiaca*	"	Malir	16	46	100	<39.9
35.	*Penicillium asperum*	*Daucus carota*	"	KU	23	43	100	<41.5
36.	*P. brefeldianum*	*C. dactylon*	"	Malir	3.3	6.6	100	<85.8
37.	*P. citrinum*	*Cyamopsis tetragonoloba*	"	Kathor	96	100	100	<5.4
38.	*P. luteum*	*Gossypium arboreum*	"	KU	0	40	100	<100
39.	*P. purpurrescens*	*Raphanus sativus*	"	Malir	26	100	100	<3.3
40.	*P. purpurogenum*	*Vigna mungo*	"	KU	0.06	26	100	<85.8
41.	*P. raistrickii*	*Pennisetum americanum*	"	Malir	0	20	100	<116
42.	*P. rugulosum*	*P. americanum*	"	KU	63	80	100	<6.3
43.	*Penicillium* sp.	*Cyperus rotundus*	Rhizosphere	Hub	100	100	100	<4.3
44.	*Rhizoctonia solani*	*Convolvulus arvensis*	Rhizoplane	Gharo	40	70	100	<20.4
45.	*Scopulariopsis brumptti*	*C. lanatus*	Rhizosphere	Hub	0.06	36	100	<100
46.	*T. harzianum*	*Glycine max*	"	KU	53	56	100	<20.7
47.	*T. koningii*	*Phaseolus vulgaris*	"	Kathor	0	43	100	<100
49.	*T. viride* (S-1)	*Gossypium arboreum*	"	KU	36	60	100	<28.3
50.	*T. viride* (S-2)	*Cyperus rotundus*	"	Hub	40	40	100	<100
51.	*Verticillium chlamydosporium*	*S. melongena*	Rhizoplane	KU	3.3	13.3	100	<100

KU = Karachi University

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