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Cytotoxicity Assay of Some Fungal Filtrates Using *Artemia salina* Leach (Brine Shrimp)

Imran Ali Siddiqui, [1]Amer-Zareen and [1]S. Shahid Shaukat
PAF Intermediate College, Korangi-Creek, Karachi, Pakistan
[1]Department of Botany, University of Karachi, Karachi-75270, Pakistan

Abstract: Cytotoxic activity of six fungi namely *Aspergillus niger. A. terreus, A. nidulans, Trichocierma harnatum, Fusarium pallicioroseam* and *Alterrierla alternate* was tested using Brine shrimp bioassay. Pure culture filtrates of *A. niger* and *F. pallidoroseum* caused 100 percent mortality of Brine shrimp larvae within 24 h while rest of the filtrates caused >30% mortality. Boiling the filtrates retained their cytotoxicity suggesting that the active compounds were thermostable. Water-soluble fraction of *A. niger* was much more cytotoxic (LC_{50} 31 μg/ml) compared to ethyl acetate LC_{50} = 663 μg/ml) and hexane extract (LC_{50} = 981 μg/ml). These results are discussed with respect to their pharmacological utility.

Key words: Cytotoxicity, fungal filtrates, *Aspergillus*, *Fuserium*, *Alternaria*, *Triehoderma*

Introduction

The discovery of penicillin in 1928 from *Penicillium notatum* has lead to the isolation of thousands of antibiotics and therapeutic drugs from microorganisms (Tyler *et al*., 1988). Antibiotics have found success as antimicrobial and antieoplastic agents (Evans, 1989) in both human medicine and animal health care as growth stimulants and also in the control of plant diseases. The efficacy of griseofulvin, a metabolic product of *Penicillium aigricans, P. utricae* and *P. raistrickii as* a therapeutic agent in the treatment of dermatomycosis is well known (Brain, 1960). Brine shrimp lethality test for larvae (napulii) has used as bioassay for a variety of toxic substances and also as antitumor prescreening test that facilitates the isolation of biologically active compounds for the development of drugs (Meyer *et al*., 1982; Ara *et al*., 1999). The present paper describes the cytotoxic activity of the fungal culture filtrates against Brine shrimp (*Arterrda saline* Leach) larvae.

Materials and Methods

Fungal isolates: *Aspergillus niger* (ZCC-5). isolated from *Meloidogyne javaoica,* the root-knot nematode larvae, *A. nidulans* (ZCC-7), *A. ferret's* (ZCC-8) from *M. javanica* female, *Trichoderma hamatum* (Karachi University Culture Collection-29) *Fusarium pailidoroseurn* (from soil) and *Alrernaria alternate* from rape seeds were maintained on PDA at 28°C before use.

Preparation of the culture filtrate (CF): The fungi were grown in Erlenmeyer flasks containing Czapek's Dox liquid medium. The phi of the medium was adjusted to 6.8 before autoclaving. inoculation was done by adding a 5 mm block from a 5-dayold culture of the fungus. The flasks were closed with cling film and kept on a rotary shaker at 100 r.p.m. at 24°C with 12 h light per day for one week. The liquid was passed through Whatman no. 1 filter paper several times to remove any spores.

Preparation of dilutions from culture filtrate: A 1:1. 1:10, 1:100 and 1:1000 dilutions of the culture filtrates were prepared in artificial saline water. Freshly prepared liquid media without the fungus served as a control. Thermostability of the extract was tested after boiling the culture filtrate for 5 min and then bioassaying against Brine shrimps, Four replicates were prepared for each treatment.

Fractionation of culture filtrate: Of the fungi tested, culture filtrate of *A. niger* exhibited highest cytotoxic activity in Brine shrimps, was selected for further fractionation. Culture filtrate of *A. niger* was extracted with ethyl acetate or hexane (1:2) and concentrated to dryness on a rotary vacuum evaporator and weighed. The remaining water phase of the filtrate was lyophilized.

Preparation of dilutions from ethyl acetate. hexane and water extracts: Samples were prepared by dissolving a 0.1 g of ethyl acetate extract in an appropriate volume of ethyl acetate. An appropriate amount of solution was transferred into vials to make concentrations of 5, 10, 100 and 1000 μg/ml. Similarly, dilutions of 5, 10, 100 and 1000 μg/ml from hexane and water extracts were prepared in hexane and artificial sea water respectively. A 2-ml of each dilution was transferred in a 1-cm-diam., cavity glass slides and left for 48 h to evaporate the organic solvent.

Brine shrimp bioassay: Freshly hatched shrimps (20-25/cavity-glass-slide) were transferred to each glass slide and 2 ml artificial sea water was added 1where the extract was made in organic solvent). Survivors were counted with the aid of low power microscope (×6) and percentage of deaths at each dose was recorded.

Statistical analysis: Data were analyzed following one way analysis of variance (ANOVA). LC_{50}'s and 95% confidence intervals were determined from the 24 h counts using the probit analysis method described by Finney (1971).

Results and Discussion

Of the 6 fungi tested, pure culture filtrates of *Aspergillus niger* and *Fusarium pallitioroseum* caused 100% deaths in Brine shrimps, Pure culture filtrate of *A. niger* showed >97% mortality within 15 min., when culture filtrate was subjected to boiling, it retained its effectiveness and caused >92% mortality of the brine shrimps. Similarly, unboiled and boiled

Table 1: Cytotoxic activity of fungal culture filtrates against *Artemia salina* Leach

Treatments	Concentration	Percentage deaths Exposure time			
		15 mins.	1hr.	3 hr.	24 hr.
Czapek's dox broth	100%	0	0	0	0
Czapek's dox broth	100% (boiled)	0	0	0	0
Aspergillus niger	100%	97	100	100	100
	100% (boiled)	92	100	100	100
	1 : 10	75	89	98	100
	1 : 100	11	14	19	35
	1 : 1000	2	8	15	34
A. terreus	100%	0	9	9	30
	100% (boiled)	0	0	0	11
	1 : 10	1	6	15	22
	1 : 100	0	3	9	15
	1 : 1000	0	0	7	14
A. nidulans	100%	0	1	3	38
	100% (boiled)	0	1	1	9
	1 : 10	4	13	26	36
	1 : 100	5	11	24	36
	1:1000	3	9	24	35
Fusarium pallidoroseum	100%		4	80	100
	100% (boiled)	0	0	77	87
	1 : 10	5	8	26	35
	1 : 100	3	9	20	33
	1 : 1000	0	5	17	32
Trichoderma hamaturn	100%	0	0	1	31
	100% (boiled)	0	0	0	0
	1 : 10	0	3	17	22
	1 : 100	0	4	12	21
	1 : 1000	0	2	11	19
Alternaria alternata	100%	7	12	17	38
	100% (boiled)	0	1	1	8
	1:10	3	6	10	22
	1:100	1	2	3	10
	1:1000	1	2	4	9
$LSD_{0.05}$					
Treatment		2.34			
Concentration		1.97			
Exposure time		1.77			

Analysis performed using arcsin transformed values

Table 2: Cytotoxic activity of different fractions of *Aspergillus niger* against *Artemia salina*

Fungal extract	Percent deaths at 24 hr							
	0 µg/ml	5 µg/ml	10 µg/ml	100 µg/ml	500 µg/ml	1000 µg/ml	LC_{50} µg/ml	Confidence interval at 95%
Ethyl acetate	2	3	12	32	42	56	663	419.45-1205.49
Hexane	1	1	6	18	37	53	981	643.57-1724.27
water	0	13	32	77	85	100	31	24.02-413

culture filtrates respectively of *F. pallidoroseurn* also resulted in 80 and 77% mortality of the Brine shrimps after 3 h exposure (Table 1). *Fusarium* species have been reported to produce naphthaquinone type pigments including fusarubin, an hydrofusarubin, ethyl ether-fusarubin, solaniol, nectriafusarubin, dihydrofusarubin lactone (Kurobane *et al*., 1980; Baker *et al*., 1981; Tatum and Baker, 1983; Tatum *et al*., 1985). Some naphtaquinones have antimicrobial, cytotoxic, insecticidal and antitumor properties (Mokhtar *et al*., 1979). In monitoring fractionation studies, Brine shrimp previously shown utility with fungal extracts directed at isolation of mycotoxins (Eppley and Bailey, 1973).

Oxalic acid and citric acid have previously been reported as being an exometabolite of *A. niger* (Mankau, 1969; Zuckerman *et al*., 1994). Mankau (1969), also noted the thermostability of the *A. niger* toxic principel and its nematicidal action both *in vitro* and in soil against *Aphelnchus avenae* Bastin. Thermostability of the cytotoxic constituent

indicates the non-proteinaceous nature of the compoundls(s). Water-soluble fraction of *A. niger* showed (LC_{50} <32 μg/ml) better results as compered to its ethyl acetate or hexane fractions. When ethyl acetate or hexane extracts were used, a lower LC_{50} (663 μg/ml and 98 μg/ml respectively) was observed (Table 2). This indicates the polar nature of the cytotoxic compoundls). However, it is not known whether the cytotoxic activity demostrated *by A. niger* is due to s single or a number of oompoundls) responsible for the cytotoxic activity in Brine shrimps.

The result of the present study would suggest that *A. urger* could be exploited for the isolation of cytotoxic compounds. Furthermore, once the active compounds are isolated, could be subjected to more elaborate bioassay for specific pharmacological activities.

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