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BIOLOGICAL SCREENING OF THE CRUDE EXTRACT ISOLATED FROM A SOIL BORN FUNGI *Cladosporium carrionii*

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ABSTRACT

Cladosporium carrionii were isolated from soil and were grown in Czapek yeast extract broth (CYB) for the production of metabolites. The study was conducted in 2010 at Centre of Biotechnology and Microbiology, University of Peshawar with the objectives to discover some biologically active secondary metabolites. The crude extract and its fractions were tested against the pathogenic fungi namely Aspergillus flavus, Candida albicans, Candida glabrata, Fusarium solani, Microsporum canis and Trichophyton longifusus, including brine shrimps and Lemna aequinoctialis. The ethyl acetate extract showed highest inhibition of 66 mm against C. albicans, with MIC of 0.25 mg/mL, n-Hexane fraction showed highest inhibition of 63 mm against C. glabrata, with MIC of 0.50 mg/mL and aqueous fraction showed highest inhibition of 56 mm against M. cannis, with MIC of 0.25 mg/mL. The chemical investigation of the extract led to the isolation of pure antibacterial compounds and their structure was confirmed by 1D and 2D spectroscopic techniques. The pure compounds [2-(4,6-dihydroxy-3-oxo-1,3-dihydroisobenzofuran-1-yl) acetic acid (1) and 2-(4-hydroxy-1,3-dihydroisobenzofuran-1-yl) acetic acid (2)] were isolated from C. carrionii and were tested only against the pathogenic bacteria, which showed growth inhibition activities with minimum inhibitory concentration (MIC) value of 0.10 mg/mL for compound (1) and 0.50 mg/mL for compound (2).

Keywords: Antifungal, *Cladosporium carrionii*, cytotoxic, duckweed, phytotoxic activities.

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INTRODUCTION

In the developing countries the misuse of antibiotics are progressively developing resistant to the available drugs, therefore the antibiotic-resistant bacteria and fungi is a major health problem throughout the world (Hsueh *et al.,* 2005). Consequently, scientific efforts have been made to study and develop new compounds to be used beyond conventional antibiotic therapy. These comprise probiotic strains (Reid *et al.,* 2001; Petti *et al.,* 2008).

Fungi; the eukaryotes are important for producing a wide-range of biologically active secondary metabolites (Cox, 2007). Fungi are using these metabolites for their protection and survival in the neighbourhood of other micro-organisms (Chalfoun *et al.,* 2010). Therefore, by knowing the nature of metabolites as "antibiosis", the human is dependent on the natural metabolites for their health care. Because these natural metabolites have been remained the most successful platform for the discovery of new drugs, e.g. penicillin has been produced by fungi. In the last thirty years, many valuable bioactive secondary metabolites have been produced by endophytic fungi, including alkaloids, lactone, lignans, phenols, quinones, steroids and terpenoids with anticancer, antimicrobial, insecticidal and cytotoxic activities (Zhang *et al.,* 2006; Schulz *et al.,* 1995).

The genus Cladosporium is consisting of 1500 species (Kirk *et al.,* 2002), which have the potential of producing antibacterial metabolites (Holler *et al.,* 2002). While this is also common that fungal infections remain a major cause of severe diseases. Meningitis is a serious disease which is caused by fungi (*Cryptococcus neoformans*) in HIV-positive patients (Powderly *et al.,* 1999). Thus the main objectives of the present study were to investigate the potential of extract and isolated secondary metabolites from *C. carrionii* for in vitro biological activities.

MATERIALS AND METHODS

Fungal strain and growth condition

Study was conducted at Centre of Biotechnology and Microbiology, University of Peshawar, KPK, Pakistan during April, 2010. Deionized water (10 mL) was added to plates having 15 days old growing spores of C*. carrionii*. The surface was scratched carefully with sterilized loop to pass the spores into the deionized water. The spore suspension (1 mL) was transferred to each 100 mL of the culture media in 250 mL baffled erlenmeyer flasks i.e. Czapek Dox Broth (CB) Sigma-Aldrich®, Czapek Yeast-extract Broth (CYB), Yeast Extract Sucrose (YES), Potato Dextrose Broth (PDB) Acumedia®, and Czapek-dox Sigma-Aldrich®, (supplemented with glucose & starch) Broth (CGSB) for maximum production of metabolites. The spores were

allowed to grow on shaking incubator at 150 rpm and 30 °C for 12 days.

Extraction and Fractionation of metabolites from liquid culture

After the incubation, the media containing mycelia were grinded and then acidified to pH 4.0-5.0 by using concentrated 37% HCl. Then equal amount of ethyl acetate was added and stirrered gently for 10-15 minutes. The mixture was then filtered using filter paper, the organic layer was separated from the mixture using separating funnel. The organic layer was washed with 2M brine solution and dried with Na_2SO_4 and then was concentrated by rotary evaporator at 45 °C with a slight rotation of 150 rpm. The crude metabolites (500 mg) were recovered from rotary evaporator in methanol. Metabolites were dissolved in 200 mL distilled water and then fractionized into ethyl acetate, *n*-Hexane and aqueous fractions. These fractions were used for antibacterial activities as per protocol used by Zhang *et al.* (2012).

Isolation of metabolites

Column chromatography technique was used, started with (5:95V/V of Ethyl acetate-n-Hexane 5 L). This mobile phase isolated none of the compounds as previously reported by Hostettmann *et al.* (1997). However, the polarity was gradually increased and at 40:60, 45:55 V/V of Ethyl acetate-n-Hexane 5 L, 5 mg of the compound was obtained. The structure of the pure isolated compound was confirmed by using and comparing 1D and 2D NMR spectroscopic data.

Antibacterial activities

Modified agar well diffusion method was used for antibacterial activities of the crude extract and pure isolated compounds from *C. carrionii* using Nutrient agar (NA) media. The experimental organisms (pathogenic bacteria) were inoculated at 37 °C for 24 hours to get the fresh culture. Then 20 mL of NA were taken in each plate and allowed to cool. Then 0.2 mL of each experimental organism were taken from broth culture and poured onto NA media to make homogenous lawn. The test sample of both the extract and compounds were prepared in di-methyl sulfoxide (DMSO). Different concentrations were used to find the minimum inhibitory concentration (MIC). Carbenicillin was used as reference antibiotic. The plates were kept at 25 °C for 2-3 hours for diffusion of the samples and then transferred to incubator for 24 hours at 37 °C (Benkeblia *et al.* 2004; Gulluce *et al.* 2003).

Antifungal activities

Modified method was used for antifungal testing of the crude extract and metabolites/compounds from *C. carrionii* using potato dextrose agar (PDA) media. The experimental organisms (pathogenic fungi) were grown at 28 °C for 3 days for fresh culture. Then 10 mL PDA were taken in each test tube and test samples were added with concentrations 10, 20, 50, 100, 250, 500 and 1000 µg/mL from stock

solution prepared in sterile di-methyl sulfoxide (DMSO) and allowed to cool in slanted position. A small piece of about 4 mm in diameter was detached from 3 days old culture of fungi and implanted. Meconazol was used as a standard drug with concentration of 100 µg/ mL. The test tubes were transferred to incubator for 7 days and 28 ^{o}C (Gulluce *et al.,* 2003).

Brine shrimp lethality assay

Artificial hatching media or sea water was made by dissolving 3.8 g of sea salt in 1000 mL of de-ionized water. One milligram (1 mg) of brine-shrimp eggs were transferred to the hatching media (sea water) into a small tank and were covered with aluminum foil. It was then left for 24 hours at 25 ^{o}C which hatched and thus produced numerous larvae. Stock solution was prepared in ethanol (30 mg/1.5 mL). The test samples were transferred to sea water with concentration of 1000, 100 and 10 µL/mL. Then ten (10) shrimps were transferred to each vial and were left for 24 hours, the surviving shrimps were recorded as per previous studies (Meyer *et al.,* 1982).

Phytotoxic activity

The artificial medium was prepared by mixing various inorganic ingredients in de-ionize water (1000 mL) and pH was adjusted (5.5-6.5) by two molar solution of potassium hydroxide (2M KOH). Stock solution was prepared in ethanol (30 mg/ 1.5 mL). The test samples were transferred to E-medium with concentration of 1000, 100 and 10 µL/mL. The solution was allowed to evaporate the excessive solvent under aseptic condition for 24 hours. After 24 hours 20 mL of the medium was added to all of the flasks and ten healthy plants of *Lemna aequinoctialis*, having three fronds each were transferred to it. Temperature was maintained at 30 ^{o}C and light intensity was 9000 lux, where humidity were maintained around 60% by keeping water in open beaker in the cabinet/chamber. The flasks containing *L. aequinoctialis* were plug with cotton cloth and were left in the growth cabinet/chamber for seven days. On eighth day the number of fronds was measured. Parquet was used as positive control (McLaughlin, 1988; De-Almeida *et al.,* 2010).

RESULTS AND DISCUSSION

Antifungal activities

The ethyl acetate extract (crude), *n*-Hexane and aqueous fractions of *C. carrionii* were tested against six pathogenic fungi namely; *Aspergillus flavus, Candida albicans, Candida glabrata, Fusarium solani, Microsporum canis* and *Trichophyton longifusus.* Significant results were observed (Table-1), hence ethyl acetate extract showed 60% inhibitions against *C. albicans*, *n*-Hexane showed 53% inhibitions against *C. glabrata.* Whereas, aqueous fractions

showed 36% inhibitions against *M. canis*. Different concentrations of metabolites were used to calculate the MIC, therefore, ethyl acetate extract, *n*-Hexane and aqueous fractions showed MIC of 0.10, 0.25 and 0.25 mg/mL against C*. glabrata*, *F. solani* and *M. canis,* respectively.

Table-1. Antifungal activities of the crude extract of *C. carrionii*

Pathogenic Fungi	DMSO Linear growth (mm)	Ethyl acetate fraction		*n*-Hexane fraction		Aqueous fraction	
		% Inhibition (mm)	MIC (mg/mL)	% Inhibition (mm)	MIC (mg/mL)	% Inhibition (mm)	MIC (mg/mL)
A. flavus	100	47	0.25	19	0.50	20	0.50
C. albicans	100	60	0.25	37	1.00	10	0.50
C. glabrata	100	54	0.10	53	0.50	5	1.00
F. solani	100	12	0.50	25	0.25	6	0.50
M. canis	100	28	0.50	0	NI*	66	0.25
T. longifusus	100	36	0.25	0	NI*	2	1.00

NI* **=** No Inhibition

Cytotoxic and Phytotoxic activities

The ethyl acetate extract, *n*-Hexane and aqueous fractions of *C. carrionii* were tested for cytotoxic and phytotoxic activities as shown in Table-2. All the fractions showed high level of activities. Where 1000 µg/ mL of *n*-Hexane fraction depicted 90% death of the shrimps and 87% mortality for *L. aequinoctialis*.

Table-2. Cytotoxic and phytotoxic activities of the crude extract of *C. carrionii*

Does (µg/ mL)	Cytotoxic activities				Phytotoxic activities			
	Number of Shrimps	% Death in ethyl acetate	%Death in *n*-Hexane	% Death in Aqueous	Number of fronds	% Death in ethyl acetate	%Death in *n*-Hexane	% Death in Aqueous
1000	30	87	90	82	30	80	87	75
100	30	64	68	50	30	57	64	55
10	30	14	15	11	30	24	37	10

Description of the compounds

2-(4,6-dihydroxy-3-oxo-1,3-dihydroisobenzofuran-1-yl) acetic acid (1)

The 2-(4,6-dihydroxy-3-oxo-1,3-dihydroisobenzofuran-1-yl) acetic acid (1) was produced by *C. carrionii* and was identified by

comparing its H^+NMR/^{13}CNMR data with those in the literature (Choi *et al.* 2010) as shwon in Fig. 1.

2-(4-hydroxy-1,3-dihydroisobenzofuran-1-yl) acetic acid (2)

The 2-(4-hydroxy-1,3-dihydroisobenzofuran-1-yl) acetic acid (2) was produced by *C. carrionii,* identified by comparing its H^+NMR/^{13}CNMR data with those in the literature (Holler *et al.* 2002) as shwon in Fig. 1.

The isolated pure compounds elucidated by 1D and 2D NMR techniques as shown in Fig. 1, also showed significant activities against the pathogenic bacteria (Table-3). Compound (1) displayed no activities against *B. subtilis*, *K. pneumoniae* and *S. typhi* while slight inhibitions of 24% against S. flexneri were observed. Compound (2) displayed 69% inhibition against *S. flexneri* while compound (1) was less potent with MIC value of 1.0 mg/ mL. Compounds (2) remained more potent as compared to compound (1) with MIC value of 0.10 mg/ mL.

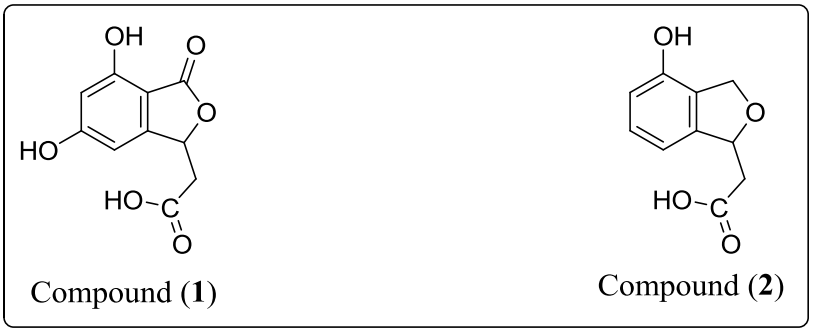

Figure 1. Compounds isolated from the ethyl acetate fraction of *C. carrionii*, 2-(4,6-dihydroxy-3-oxo-1,3-dihydroisobenzofuran-1-yl) acetic acid (1) and 2-(4-hydroxy-1,3-dihydroisobenzofuran-1-yl) acetic acid (2).

Table-3. Antibacterial activities of the compounds isolated from ethyl acetate fraction of *C. carrionii*

Pathogenic Bacteria	Carbenicillin Inhibition Zone (mm)	Compounds (1)		Compounds (2)	
B. subtilis	30± 0.028	NI*	NI*	48	0.25
E. coli	33± 0.057	19	1.00	47	0.50
K. pneumoniae	28± 0.050	NI*	NI*	60	0.10
S. typhi	26± 0.028	NI*	NI*	36	0.50
S. flexneri	33± 0.028	24	1.00	69	0.10
S. aureus	30± 0.057	22	1.00	25	0.50

NI* = No Inhibition

In the instant study we investigated the crude extract of *C. carrionii* for biological activities and isolation of metabolites. Both the crude extracts and pure compounds from *C. carrionii* showed significant biological properties. Ethyl acetate extract, *n*-Hexane and aqueous fractions showed MIC of 0.10, 0.25 and 0.25 mg/mL against C*. glabrata*, *F. solani* and *M. canis*, respectively. Which shows the presence of some active constituents in the extracts because several studies have reported that compounds having quaternary amines possess more antimicrobial properties (Thorsteinsson *et al.,* 2003); whereas, for antifungal activities sulfur and other numerous phenolic compounds have been reported earlier (Griffiths *et al.,* 2002). Beside this 1000 *µ*g/mL of *n*-Hexane fraction displayed 90% death of the shrimps and 87% mortality for *L. aequinoctialis*. The extract showed the presence of a different groups, which has already been reported that the properties of the natural products are due to the presence of many well known metabolites like alkaloids, flavonoids, tannins and phenolic compounds (Hill, 1992). Upon exploitation of the extract two known secondary metabolites namely, 2-(4,6-dihydroxy-3-oxo-1,3-dihydroisobenzofuran-1-yl) acetic acid (Choi *et al.,* 2010) and 2-(4-hydroxy-1,3-dihydroisobenzofuran-1-yl) acetic acid (Holler *et al.,* 2002) were isolated for the first time from the extract of *C. carrionii*. The structures of the biologically active secondary metabolites were elucidated by comparing different 1D and 2D NMR spectroscopic data with the data available in literature. Compounds (1 and 2) remained more potent with MIC value of 0.10 mg/ mL and compound (3) was less potent with MIC value of 1.0 mg/ mL.

CONCLUSION

The experimental observations of the biological activities of the crude extract and of known pure compounds revealed that nitrogenous compounds from *C. carrionii* were involved in the biological activities. Hence further exploitation is suggested to confirm the presence of sulfur containing compounds from this fungus.

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