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Research Article

Characterization of antifungal metabolites of C*haetomium globosum* Kunze and their antagonism against fungal plant pathogens

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ABSTRACT: *Chaetomium* species which are normally found in soil and organic compost are noted for the presence of secondary metabolite with biological activities. Secondary metabolites from culture filtrate of *Chaetomium globosum* Kunze were extracted by solvent extraction method using ethyl acetate and separated by thin layer chromatography in five major bands of different R_f values. These compounds were further purified and fractioned with column chromatography. Compound '1' eluted with hexane (fraction 1-18) as color less liquid (R_f 0.84), and other five compounds (2,3,4,5,6) with different ratio of hexane, benzene and acetone as solvent in different fractions as: 34-63 (R_f- 0.46), 71-75 (R_f 0.31), 78 (R_f 0.58), 76-77 (R_f 0.58) and 85-89 (R_f 0.47) were eluted and purified. GC- MS and NMR studies revealed that compounds 2-6 were identical to spectral data of metabolites, chaetomin, BHT, mollicelin G, isomer of mollicelin G and cochiliodinol respectively. One more elution with benzene: acetone (95 : 5 v / v) gave a pale yellow crystalline compound of R_f 0.37 identified as chaetoglobosin. Bioassay studies with two compounds i.e., chaetoglobosin and chaetomin revealed significant growth inhibitory activity against various plant pathogens such as *Bipolaris sorokiniana, Macrophomina phaseolina, Rhizoctonia solani* and *Pythium ultimum* under *in vitro* conditions.

KEY WORDS: C*haetomium globosum*; Biocontrol; Secondary metabolites; HPLC; GC- MS.

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INTRODUCTION

Chaetomium globosum Kunze Fr., an Ascomycete has been identified as potential biocontrol agent against a number of plant pathogens (Vannacci and Harman, 1987; Walther and Gindrat, 1988; Di Pietro *et al*., 1992). Earlier workers found that seed borne *C. globosum* and *C. cochlioides* impart natural resistance to *Helminthosporium victoriae* in oat varieties. Seed coatings with selected isolates of *C. globosum* protected corn and oat from seedling blight caused by *Fusarium* spp. (Tveit and Moore, 1954**;** Tveit and Wood, 1955; Chang and Kommedahl, 1968**;** Kommedahl and Mew, 1975) and barley from *Drechslera sorokiniana* (Vannaccai and Pecchia, 1986). Antagonistic effect of *C. globosum* to rice blast (*Pyricularia oryzae*) was reported by Soytong and Quimino (1989) and Kommedahl and Mew (1975) observed increased field stands of maize hybrids when seeds were coated with *C. globosum*. Apple scab disease caused by *Venturia inaequalis* has been significantly controlled by foliar spray of *C. globosum* ascospore suspension (Andrews *et al*., 1983; Boudreau and Andrews, 1989). Studies in our laboratory have proved the potentiality of *C. globosum* for the biological control of spot blotch disease of wheat caused by *Bipolaris sorokiniana* and ascochyta blight of chickpea (Mandal *et al.,* 1999; Biswas *et al.,* 2000; Rajkumar *et al*., 2007). Different isolates showed different mechanisms of antagonism against this pathogen and they also have been reported to produce different antifungal metabolites (Aggarwal *et al.,* 2004; Aggarwal *et al*., 2007a). There are a few previous reports on production of various metabolites by *Chaetomium* spp. (Di Pietro *et al*., 1992), BHT (Brewer *et al*., 1972) and benzoquinone derivatives (Brewer *et al*., 1968). Keeping these points in view, present investigations on purification and characterization of the metabolites from Indian strain of *C. globosum* and their bioefficacy under *in vitro* and *in vivo* were undertaken.

MATERIALS AND METHODS

Culturing of *Chaetomium globosum* and pathogenic fungi

The strain of *C. globosum* which was isolated earlier from wheat leaf surface (Mandal *et al*., 1999) was used

for the present study. Pure culture of the fungus was sub-cultured and multiplied in sterilized Petri plates containing potato dextrose agar (PDA) medium. The inoculated plates were incubated for seven days under continuous fluorescent light at 25±1°C. Cultures of plant pathogenic fungi namely *Bipolaris sorokiniana* was wheat (Wellington), *Pythium ultimum* from ginger, (Assam) and *Rhizoctonia solani* from maize (New Delhi) from maize pathology laboratory (New Delhi) *Macrophomina phaseolina* from soybean (Delhi) and *Fusarium graminearum* from wheat (Delhi) from Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, were procured and maintained on PDA slants. Uredospore inoculum of *Puccinia triticina* (race 77-5) was produced from Directorate of Wheat Research, Regional Station, Flowerdale, Shimla, Himachal Pradesh, India was also evaluated.

Extraction of antifungal compounds

Raising culture filtrate of *C. globosum*

C. globosum was grown in 1000 ml conical flasks containing 400 ml of potato–dextrose broth (PDB) medium. Two agar plugs from actively growing colony of *C. globosum* were transferred to a flask aseptically in a laminar flow chamber. The flasks were incubated at 25±1°C for 28 days. Around 30 L of culture filtrate was raised and collected by passing the fluid through three layers of cheese cloth. This culture filtrate was used for the extraction of metabolites by solvent extraction procedure.

Solvent extraction and concentration of antifungal compounds

The antifungal compounds from culture filtrate of *C. globosum* were extracted with ethyl acetate. The culture filtrate was taken in a separating funnel to which ethyl acetate was added in 1:2 ratio. The suspension of mixture was shaken vigorously for 15 minutes and allowed to stand undisturbed for at least 15 minutes. The upper layer of the solvent containing the antifungal compound was separated by taking out lower layer, which was again subjected to extraction procedure extracting with fresh ethyl acetate. The ethyl acetate extract was dried over anhydrous sodium sulfate. Finally, the solvent was evaporated on water bath through distillation process. The crude extract (oily residue) was collected in small glass vials for further studies.

Purification of secondary metabolites from crude extract with column chromatography

The secondary metabolites from crude extract were purified by column chromatography using a glass column (50 cm x 1 cm) packed with slurry of pre activated silica gel (60-120 mesh, 50 gm). Column was successfully eluted with hexane; hexane: benzene (5%); hexane: benzene (10%); hexane: benzene (25%); hexane: benzene (50%); hexane: benzene (75%); benzene: acetone (1%); benzene: acetone (2%); benzene: acetone (5%) and fractions of 20-25 ml of each were collected in 50ml conical flasks. These fractions were concentrated on a rotary evaporator. Each fraction was chromatographed on thin layer chromatographic plate (TLC). Slica-gel TLC plates were prepared by spreading a slurry of silica gel (60 + 120 mesh) containing 10 per cent binder (gypsum) in distilled water on glass plates (20 x 20 cm and 20 x 5 cm) uniformly by using a TLC applicator. The thickness of silica gel layer on plate was maintained at 0.25 mm or 0.55 mm. These silica gel plates were activated at 100–120°C for 2–3 hrs in an oven before use. The sample solutions were spotted on activated TLC plates using capillary tubes. The plates were developed in number of different ratios of benzene and methanol solvent systems. Iodine vapor was used as a visualizing agent. The spots were marked on the TLC plates and their Rf values were determined using following formula:

Fractions containing similar R_f values were considered as same compounds and were mixed together and further purified by preparative TLC and by crystallization from appropriate solvents.

$$R_f = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent front}}$$

Fourier Transform Infra-red spectroscopy (FT-IR)

The purified compounds were analyzed by FT–IR spectroscopy. Infra red spectra (IR) were recorded on a Nicolet Fourier Transform Infra-red spectrophotometer (Model Impact-700 FT-IR spectrophotometer). The liquid samples were analysed as thin films and solid samples as KBr disc and a nujol mull.

Proton Magnetic Resonance Spectroscopy (H-NMR)

The proton nuclear magnetic resonance spectra of purified compounds were recorded on a varian EM 360 L (60 MHz) and on a Bruker 300 AC (300 MHz) instrument. The solvent used was carbon tetrachloride (CCl_4) and deuterio chloroform ($CDCl_3$) containing tetramethylsilane (TMS) as the internal standard. The chemical shifts were expresed in values and coupling constant (J) were given in hertz (Hz). The notations used for spotting pattern were, S = singlet, d = doublet, t = triplet, q = quarlet and m = multiplet.

***In vitro* bioassay**

The inhibitory effect of two compounds purified from culture filtrate of *C. globosum* on growth of *Bipolaris sorokiniana, Macrophomina phaseolina, Phythium ultimum*, *Rhizoctonia solani* and *Fusarium gaminearum* were studied by food poisoning technique (Aggarwal *et al*., 2004). The bioassay was done taking two concentrations of compounds *viz.,* 1000ppm and 500ppm and three

replicates were kept for each treatment. The data were collected as colony diameter (mm) after 3 days of inoculation and subsequently observed until control plate was grown almost full. The treatment data was analyzed through ANOVA using SPSS version 17.0.

RESULTS AND DISCUSSION

Characterization of purified compounds

Elution of column with hexane (Fraction 1–18) gave a colorless liquid of R_f 0.84. The ^{1}H–NMR spectrams showed the presence of a doublet at δ 0.79 and a singlet at δ 1.16. The FT–IR spectra did not show the presence of a carbonyl or carboxylic group. Thus, the structure of this compound could not be established. The elution with benzene (fraction 34–63) resolved a colorless liquid which on purification by preparative TLC gave a amorphous solid of R_f 0.46. The spectral data recorded with ^{1}H–NMR were (CD Cl_3) δ. 7.5 – 7.11 (8 H, aromatic); 5.35 (d, 2, H–CH_2). These details proved to be identical to spectral data related with metabolite 'chetomin' (Safe and Taylor, 1972) (Fig. 1). Further elution of column with benzene (fraction 71–75) gave a colorless liquid of R_f 0.31. The FT–IR did not show the presence of a carbonyl group. On the basis of spectral data, recorded as ^{1}H-NMR ($CDCl_3$); 7.5 (d, 1H), 7.35 (s 1H), 7.25 (s 1H), 7.13 – 7.10 (dd – 1H), the metabolite was identified as 2–(buta- 1, 3– dienyl) –3 hydroxy – 4 – (penta – 1, 3 – dienyl) – tetra hydrofuran (Fig. 1). Further elution with benzene (fraction 78, R_f 0.58) was analyzed and the spectral data ^{1}H-NMR (CD Cl_3) δ; 12.49 (1H, aldehydic proton CHO), 10.19 (s 1H), 7.28 (s 1H), 5.36 (s 1H), 5.30 (s 1H), 5.08 (m, 1H), 3.72 (d, 2H), 2.37 (S 3H), 2.10 (S 3H), 1.63(s, 3H) was obtained which matched with metabolite mollicelin G (Silverton *et al*.,1976) (Fig. 1). Fraction 76–77 which was eluted with benzene, gave a colorless solid. The ^{1}H–NMR spectrum of this compound showed similarity with the spectra of mollicelin G (Fig. 1) but the retardation factor value of the tested fraction was different (R_f 0.58), therefore, the compound was identified as an isomer of mollicelin G. The elution of column (fraction 85-89, R_f 0.47) with benzene: acetone (1% v / v) resulted into a crystalline solid having m.p. 206-208^{0}C and ^{1}H-NMR (CD Cl_3) δ ; 10.30 (d, 1H), 8.78 (s 1H), 7.54 (d), 7.35 (d), 7.14 (m), 5.42 (t, 2H), 3.68 (bd, 4H), 1.70 (bs 12H) was obtained (Fig. 1). This ^{1}H-NMR spectrum of the fraction matched which cochliodinol (Brewer *et al*., 1968). Another elution with benzene : acetone (95 : 5 v / v) gave a pale yellow crystalline compound of R_f 0.37. The ^{1}H-NMR showed the presence of an aromatic ring as multiplate at δ 7.54 – 7.51 (4H) and a NH proton at δ 8.35 (91H). Beside, the ^{1}H-NMR spectrum showed protons at δ 2.66, 3.81, 3.03, 6.94 and 5.85 (Fig. 1). Thus, the product was identified as chaetoglobosin A (Silverton *et al*., 1976).

In vitro bioassay

Both purified secondary metabolites chaetomin and chaetoglobosin significantly suppressed the radial growth of all fungal plant pathogens tested (Table 1). Chaetoglobosin @ 1000 ppm and 500 ppm produced 13mm and 17 mm of colony *B. sorokiniana* after 3 days of inoculation, which was significantly suppressed as compared to 69 mm in control. Similarly, this secondary metabolite significantly reduced the growth of *Fusarium graminearum, Pythium ultimum*, *Macrophomina phaseolina* and *Rhizoctonia solani* at both concentrations (Table 1). Chaetomin also suppressed the growth of all fungal pathogens tested at 3 days post inoculation (Table 1). Maximum inhibition was observed in *B. sorokiniana* @1000ppm (13mm) followed by 500ppm (17mm). This antifungal metabolite also suppressed growth of *F. graminearum*, *P. ultimum, M. phaseolina* and *R. solani* significantly in comparison to control at both concentrations. However, overall chaetoglobosin proved more effective than chaetomin in suppressing the growth

Table 1. *In vitro* bioassay showing bioefficacy of Chaetoglobosin and Chetomin purified from *Chaetomium globosum* against fungal plant pathogens

Secondary metabolites	Colony diameter of plant pathogens (mm)*				
Chaetoglobosin (ppm)					
	Bipolaris sorokiniana	*Fusarium graminearum*	*Pythium ultimum*	*Macrophomina phaseolena*	*Rhizoctonia solani*
500	22^b	23^b	55^b	69^b	59^b
1000	13^a	1.6^a	42^a	54^a	32^a
control	66^c	33^c	80^c	89^c	88^c
CD (5%)	3.5	1.8	3.4	10.7	2.8
Chetomin (ppm)					
500	17^a	32^b	59^b	71^b	63^b
1000	13^a	25^a	48^a	57^a	41^a
control	69^b	36^c	89^c	89^c	86^c
CD (*P*=0.05)	4.7	1.4	2.8	4.1	3.0

*Average of three replications; values followed by same letter were not significantly different (*P*=0.05)

of these pathogenic fungi (Fig. 5). It was observed that after 5-7 days of incubation, chaetoglobosin retained its bioefficacy against *B. sorokiniana, F. graminearum* and *P. ultimum* but, lost its effectiveness against *M. phaseolina* and *R. solani*. Further, after 10 days of incubation, this secondary metabolite still remained effective against *B. sorokiniana* and *F. graminearum*. Chaetomin was not found effective after 5 days of incubation against all tested fungal pathogens.

The genus *Chaetomium* is known to produce a number of biological active metabolites, possibly due to heterothalism in various species of the fungus (Tveit *et al*., 1955). Toxic metabolites like chetomin, cochliodinol, mollicellin, oosporein, sterigmatosystin, chetoglobosin etc. were characterized from different isolates of *C. globosum* (Powell and Whalley, 1969; Brewer *et al*., 1970; Udagawa *et al*., 1979; Sekita *et al*., 1981; Amemiya *et al*., 1994). Anthraquinone-chromanone compound named chaetomanone and seven known compounds, ergosterol, ergosteryl palmitate, chrysophanol, chaetoglobosin C, alternariol monomethyl ether, echinuline and isochaetoglobosin D were characterised from *C. globosum (*KMITL-N0802) and also reported that chaetomanone and echinulin (Kanokmedhakul *et al*., 2001) having antifungal properties used for the biocontrol of phytopathogenic fungi. Considering the involvement of toxic antifungal compounds with *Chaetomium* sp. our earlier studies under *in vitro* and *in vivo* have shown bioefficacy of *C. globosum* which has been correlated with strainal differences for the production of secondary metabolites (Aggarwal *et al*., 2004; 2007a). Biswas *et al*., (2000) conducted the bioassay test using crude extracts of *C. globosum* against *B. sorokiniana.* The isolated extract succeeded in protecting wheat from spot blotch infection. Further, scanning electron microscopy showed the distortion in conidial wall and disordered mycelial growth of *B. sorokiniana* as a result of post application of crude extract on plant. In continuation of our earlier studies, we have been successful in purifying and characterizing the antifungal metabolites from potential strain in the present studies. Five metabolites like chaetoglobosin, chaetomin, BHT, mollicelin G and cochliodinol from culture filtrate of *C. globosum* have been purified and characterized, out of which two metabolites viz., chaetoglobosin and chaetomin proved effective in suppressing the growth of *B. sorokiniana*, *F. graminearum*, *P. ultimum*, *M. phaseolina* and *R. solani* under *in vitro*. Bioactive compounds from different fungi have been reported to inhibit many plant pathogenic fungi (Amemiya *et al*., 1994). The research on biocontrol agents against plant pathogens have become increasingly interesting among the scientists in recent years, more so with many recent reports on production of antifungal secondary metabolites from various antagonists (Aggarwal *et al.,* 2007b). Kanokmedhakul *et al*., (2007) reported that bioactive constituents from *Chaetomium* sp., *Emericella* sp. like indol-3-yl-[13] cytochalasans, azaphilones, xanthones, xanthoquinodines, diterpenoids and diketopiperazines have been found effective against many fungi. Some of these compounds exhibited activity towards *Plasmodium falciparum*, *Mycobacterium tuberculosis*, *Candida albicans* and cancer cell lines. These compounds also showed activity against plant diseases like *Phytophthora* sp. causing root rot of plants and *Colletotrichum gloeosporioides* causing anthracnose disease. Our earlier work has shown that partially purified secondary metabolites impaired the conidial germination and hampered the ramification of mycelium of *B. sorokiniana* infecting wheat (Aggarwal *et al*., 1996; Biswas *et al.,* 2000). Consequently, the effect of biocontrol agent was manifested as suppression of spot blotch lesions in wheat (Aggarwal *et al.,* 2004). But, in all these studies the metabolites were not purified and characterized. Our present study confirms the role of antibiosis in biological control of spot blotch of wheat and further indicates the possibilities of using this biocontrol agent for the management of diseases caused by many soil borne fungi like *F. graminearum*, *P. ultimum*, *R. solani* and *M. phaseolina*. The role of antibiosis in the antagonistic activity of *C. globosum* against *Venturia inaequalis*, *Fusarium nivale*, *Pythium ultimum* and *Helminthosporium* sp. has been reported by earlier workers (Tveit and Wood, 1955; Hubbard *et al.,* 1982; Cullen and Andrews, 1984; Walther and Gindrat, 1988). Motoo *et al.,* (2005) isolated an antifungal antibiotic, FR207944, from the culture broth of a fungal strain *Chaetomium* sp. 217, which isolate a triterpene glucoside with antifungal activity against *Aspergillus fumigatus* and *Candida albicans.*

In conclusion, *C. globosum* can be used as a non-chemical alternative treatment for the biological control of spot blotch of wheat and other soil borne diseases where the mechanism of antagonism is antibiosis and through production of antifungal metabolites.

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