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Antifungal Potential of Extracellular Metabolites Produced by *Drechslera* spp. against Phytopathogenic Fungi

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ABSTRACT

Culture filtrates of nine *Drechslera* isolates (*D. australiensis*, *D. cactivora*, *D. cynodontis*, *D. ellisii*, *D. hawaiensis*, *D. maydis*, *D. neergaardii*, *D. poae* and *D. spicifera*), used at 30, 50 and 70%, were evaluated *in-vitro* against mycelial growth and spore germination of eight plant pathogenic fungi (*Alternaria solani*, *Botrytis cinerea*, *Botrytis fabae*, *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Sclerotium cepivorum*). Among the tested culture filtrates, only *D. cynodontis* was a highly effective growth inhibitor against all tested fungi; it reducing the fungal growth from 51.1 to 86.7%, and was the strongest inhibitor of spore germination, inhibiting spore germination of all tested fungi by 92 to 98%. The chloroform extract of *D. cynodontis* was the best growth inhibitor against all tested fungi: it inhibited fungal growth from 66.7% to 88.9% at 30 mg/ml and also highly effectively suppressed spore germination of all tested fungi at all concentrations. In greenhouse experiments, the chloroform extract was most effectively controlled damping-off disease caused by *F. solani* and *S. sclerotiorum* on bean. The ethyl acetate extract was the second best for *S. sclerotiorum* and *R. solani*. Two sesquiterpenes, dihydrobipolaroxin (1) and sorokinianin (2), were isolated from the chloroform extract of *D. cynodontis* and identified by EI-MS, ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HMQC and HMBC experiments. Compound (1) was a highly effective growth inhibitor against *A. solani*, *F. oxysporum* and *S. sclerotiorum* at 100 µg/ml. Compound (2) decreased the growth of all tested fungi between 22.2 and 61.1%. Compound (1) greatly decreased spore germination of *A. solani* and *F. solani*. Compound (2) highly effectively suppressed spore germination of *F. solani* at 100 µg/ml.

Keywords: antifungal activity, biological control, plant pathogenic fungi, sesquiterpenes

INTRODUCTION

Phytopathogenic fungi cause serious damage to crops and threaten global food security. Amongst the most widespread and important soil-borne pathogens are *Rhizoctonia solani*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum* (Grosch *et al.* 2005; Leslie and Summerell 2006; Saharan and Mehta 2008). In addition to root pathogens, there are many phytopathogenic fungi that affect shoots. One of the most serious of these for human food production is *Botrytis cinerea* and other *Botrytis* species which affect nursery plants, vegetables, ornamental, field and orchard crops and stored and transported agricultural products (Elad *et al.* 2007).

An important strategy to develop new anti-fungal compounds is to seek to identify and exploit compounds that are naturally produced by organisms that suppress growth of fungi and which are produced and biodegraded in natural ecosystems and to screen organisms with known anti-fungal action for the production of such compounds (Wolpert *et al.* 2002). One complication in selection of antimicrobial compounds is that fungal metabolites often include phytotoxins, so their use may cause damage to plants (Ahmad *et al.* 2008; Mobius and Hertweck 2009). Phytotoxins are typically produced by pathogens in infected plant tissues, and in cultures of these organisms (Joaquin *et al.* 2009). The metabolites facilitate invasion of plant tissue by penetration and colonization (Heiser *et al.* 1998). The chemical nature of phytotoxins ranges from low molecular weight compounds includes all classes of natural products such as terpenes, chromanones, butenolides, pyrones, macrolides, aromatic derivatives, amino acids etc., to high molecular compounds such as proteins, glycoproteins and polysaccharides (Berestetskiy 2008).

Drechslera is a well-known genus producing numerous phytotoxic metabolites in the Eremophilane and Ophiobolin families. They are secondary metabolites produced by the pathogenic fungi that attack some crops (Au *et al.* 2000; Capio *et al.* 2004; Evidente *et al.* 2006). Phytotoxins may be useful tools for the study of particular physiological processes occurring in plants, since a better understanding of their mode of action can reveal new and unexpected physiological aspects and biochemical mechanisms (Daub *et al.* 2005; Dayan *et al.* 2008). It was shown that the virulence (severity of disease symptoms) of plant pathogens, and sometimes their pathogenicity (ability to produce disease symptoms) may depend on their ability to produce phytotoxins (Baidyaroy *et al.* 2002). Studies on the mechanisms of toxin production can lead to a better understanding of the role of phytotoxins in disease development (Dayan *et al.* 2000; Stephen *et al.* 2000).

Because phytotoxins are biologically active compounds, various applications employing these metabolites have been attempted. Due to their role in disease development, some phytotoxins have been proposed as potential markers for screening disease resistant plants (Feys and Parker 2000; El Hadrami *et al.* 2005). Phytotoxins could also be used as potential fungicides and herbicides (El-Sayed 2005; Hoagland *et al.* 2007). Since phytotoxins are toxic not only to crop plants, but also to weeds, herbs, etc. Overall, phytotoxins appear to have an important role in the invasion of the plant tissue, how they are produced, how they interact with the plant defense mechanisms (Martinez-Luis *et al.* 2007), will bring understanding of the plant-pathogen relationship that would ultimately lead to designing better methods to increase plant resistance against fungal pathogens (Slavov 2005; Amusa 2006).

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Original Research Paper

During our screening program to find new natural fungicides, culture filtrates of nine *Drechslera* isolates were evaluated *in-vitro* against mycelial growth and spore germination of eight plant pathogenic fungi. Only one of the selected fungal strains (*D. cynodontis*) was able to produce a highly phytotoxic culture filtrate. Bioactivity-guided fractionation leads to the isolation of two active sesquiterpenes compounds, Dihydrobipolaroxin and sorokinianin.

MATERIALS AND METHODS

Drechslera isolates

Drechslera isolates, except for *D. maydis*, were purchased from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands and Assiut University Mycological Centre (AUMC), Egypt.

Isolation of *Drechslera maydis*

D. maydis was isolated from naturally infected leaves collected from the field of maize. Diseased leaf tissue was cut into small bits (3 mm diam.) with a sterilized scalpel. These bits surface-sterilized in 96% ethanol (v/v) for 30 sec, then in 14% sodium hypochlorite (v/v) for 30 sec, followed by another 30 sec in 96% ethanol and washed further in repeated changes of sterile distilled water. The leaf bits were aseptically transferred to 20 ml of cool potato dextrose agar (PDA) in Petri dishes (90 mm diam), which were sealed with Parafilm. The stock cultures of the fungus on PDA slants were stored in a refrigerator at 4ºC before being use for toxin production. The fungus was identified based on the morphological characteristics.

Plant pathogens

Botrytis fabae, *Fusarium oxysporum*, *Fusarium solani*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Sclerotium cepivorum* were isolated from different crops (broad bean, squash, cowpea, cotton, pea and onion) on PDA and Czapek agar media. The plates were incubated at 26 ± 2°C for 7 days. Cultures of the tested fungi were purified using single spore or hyphal tip techniques. Identification of the pure cultures was accomplished according to Barnett and Hunter (1979). Isolates were grown on PDA medium in Petri dishes, then transferred to PDA slants and kept in a refrigerator at 4°C as stock cultures. Fungal strains of *Alternaria solani* and *Botrytis cinerea* were obtained from the Plant Pathology Institute, Agricultural Research Center, Giza, Egypt.

Production of culture filtrates

For toxin production, small pieces from mycelial mats of 10-day-old cultures of *D. cynodontis*, grown on PDA Petri dishes, were transferred to 500 ml flasks containing 200 ml of modified M-1-D medium (Pinkerton and Strobel 1976).

Isolation and purification of the phytotoxin of *D. cynodontis*

The culture filtrate of *D. cynodontis*, showed the highest phytotoxicity against mycelial growth and spore germination of the eight plant pathogenic fungi tested. Bioactivity-guided fractionation revealed that the chloroform extract of *D. cynodontis* was the best growth inhibitor against all tested fungi. Therefore trials to isolate and identify compounds that are naturally produced by the organism that suppress growth of fungi were done.

1. Thin layer chromatography (TLC)

TLC was performed on a silica gel, GF_{254} (E-Merk, Germany). Developed chromatograms were visualized under UV light and by spraying with 5% H_2SO_4 in methanol, followed by heating at 110°C for 10 min till the colors developed. For TLC analysis, the following solvent systems were used: chloroform-methanol (9.5: 0.5, system I), chloroform-methanol (9: 1, system II) and petroleum ether-ethyl acetate (9.5: 0.5, system III) (Sherma and Fried 2003; Deinstrop 2007).

2. Column Chromatography (CC)

The stationary phase is silica gel (E-Merck, Germany) which is placed in a vertical glass column. The mixture to be analyzed is loaded on top of this column. The mobile phase is a solvent with increasing polarity poured on top of the loaded column. Elution was performed using chloroform followed by chloroform-methanol gradient. The solvent flows down the column, causing the components of the mixture to distribute between the powdered adsorbent and the solvent, thus separating the components of the mixture so that as the solvent flows out of the bottom of the column, some components elute with early collections and other components elute with late fractions (Brown *et al*. 2005). The eluted fractions were collected, concentrated and screened by TLC. Similar fractions were pooled together, concentrated to dryness, placed in a dessiccator and weighed.

3. Preparative TLC

The glass plates were coated with silica gel GF 254 LR (E-Merck, Germany). The developed plates were air dried and the plates were visualized under UV light. Each band was carefully cut off and extracted with hot methanol. Chloroform-methanol (9: 1, system II) was used for preparative TLC (Sherma and Fried 2003; Deinstrop 2007).

Identification of bioactive compounds

IR spectra were measured on a Nicolet MX-1 FT-IR spectrometer. 1D and 2D NMR spectra were recorded on Bruker AVANCE DPX300 FT NMR at 500 MHz for ^{1}H and 125 MHz for ^{13}C in DMSO, using TMS as internal standard. EI-MS (70 ev) were recorded on a JOEL JMS-GCMATE mass spectrometer, Japan. Ultra-Violet Lamp 254 and 366 nm (Desaga, Germany).

Extraction of *D. cynodontis* metabolites

D. cynodontis culture filtrates were concentrated in a rotary evaporator at 45°C to a small volume (10% of original volume). Successive extraction was done in a separating funnel with chloroform, ethyl acetate and *n*-butanol respectively till complete exhaustion. Each fraction was dried over anhydrous sodium sulphate, concentrated in a rotary evaporator, placed in a dessiccator and weighed.

Investigation of the chloroform extract

The chloroform extract of *D. cynodontis* (9.5 g) was chromatographed on a silica gel column. Elution was performed using chloroform followed by chloroform-methanol gradient. The eluted fractions (100 ml each) were collected, concentrated and screened by TLC using solvent systems (I, II, III). Similar fractions were pooled together, concentrated to dryness, placed in a dessiccator and weighed.

1. Fractions 5-14

Eluted with chloroform, were purified by silica gel column using chloroform, then chloroform-methanol gradient. Fractions of 20 ml were collected and monitored by TLC using solvent systems I and II and 5% H_2SO_4 as a spray reagent. Subfractions 6-8 eluted with chloroform-methanol (9.5:0.5) were pooled together and crystallized from methanol to afford pure compound 1 (200 mg).

2. Combined fractions 21-26

Eluted with chloroform-methanol (2%), were rechromatographed on silica gel column using chloroform, then mixture of chloroform-methanol with increasing polarity. Fractions of 20 ml were collected and monitored by TLC using the same solvent systems and spray reagent previously used. Subfractions 3-6 eluted with chloroform-methanol (9: 1) were further purified on preparative TLC plates developed in $CHCl_3$–MeOH 9:1. The band of the major component was located using 254 nm UV light and scraped

with a spatula. The component was extracted from powdered silica with hot methanol and the solvent evaporated to give a pure compound 2 (60 mg).

Physical and spectral data of the isolated compounds

1. Compound 1

(Dihydrobipolaroxin) was obtained as yellow amorphous powder; R_f = 0.37 (system II); EI-MS m/z 264 $[M]^+$; ^1H and ^{13}C NMR (DMSO-d_6, 500 MHz).

2. Compound 2

(Sorokinianin) was obtained as yellow amorphous powder; R_f = 0.48 (system III); IR (KBr, γcm^{-1}): 3430 (alcoholic OH), 1770 (γ–lactone); EI-MS m/z 309 $[M+1]^+$; ^1H and ^{13}C NMR (DMSO-d_6, 500 MHz).

Antifungal activity against some important phytopathogens

The antifungal activity of culture filtrates, organic solvent extracts and isolated compounds to tested fungi was investigated using the following mycelial radial growth bioassay. Concentrations were prepared in the range 30–70% from culture filtrates. The test was carried out by growing each fungal species in Petri dishes containing 20 ml of PDA amended with concentrations of 30, 50 and 70% of each culture filtrates. Concentrations 10, 20 and 30 mg/ml from chloroform, ethyl acetate and butanol extracts were prepared similarly, and concentrations 50, 75 and 100 µg/ml from isolated compounds (control plates containing only DMSO). The centre of each PDA plate was inoculated with one fungal disc (5 mm diameter) from edges of fungal solid cultures of the respective fungus. The plates were incubated at 24 ± 2°C for 1-2 weeks, depending on the fungal species. The antifungal activity was evaluated by measuring the diameter of test and control colonies in millimeter. The percentage of inhibition was calculated on the basis of growth in the control plates using the expression:

% Mycelial inhibition = (X - Y / X) ×100

where X and Y are the average diameters of mycelial colonies in control and treated fungi sets (Raghavendra *et al.* 2009).

Effects on spore germination of various phytopathogenic fungi

The assays were carried out with *A. solani*, *B. cinerea*, *B. fabae*, *F. oxysporum* and *F. solani*, as test fungi. Cultures of the fungi were grown on PDA and/or potato carrot agar (PCA) media for 7–10 days at 25 ± 2°C until well sporulated except *A. solani*. The spores were harvested by adding 10 ml of sterile water and aseptically dislodging the spores with a sterile inoculating loop. Spores suspensions were aseptically filtered through sterile cheesecloth to remove mycelial debris. Culture filtrates of each *Drechslera* isolate were diluted in sterile distilled water to produce twofold serial concentrations 30, 50 and 70%, and 1-ml portions of each concentration were add to test tubes. Spores suspended in distilled water were diluted in potato dextrose broth to produce the inoculum of 1.0×10^5 spores/ml by using haemocytometer slide. Portions of 1 ml of a particular fungal spore suspension were added to the tubes that contained culture filtrates or distilled water (control). These tubes were then incubated at 24 ± 2°C for 24 hrs. The percentage of spore germination was calculated in 100 spores from 5 microscopic fields. Three tubes were prepared for each treatment and the data was recorded as the mean of three replicates. Similar experiment was carried out separately with the chloroform, ethyl acetate and butanol extracts (concentrations 10, 20 and 30 mg/ml) and isolated compounds at concentrations 50, 75 and 100 µg/ml (control tubes containing only DMSO). Sporulation was induced in *A. solani* by growing it in culture plates of PMDA medium (Dhingra and Sinclair 1995).

Determination of the minimum inhibitory concentrations (MIC)

1. The MIC for fungal growth

The MIC was determined as the lowest concentration of culture filtrates, organic solvent extracts and isolated compounds that completely inhibited visible fungal growth (Webster *et al.* 2008).

2. The MIC for spore germination

The MIC was determined as the lowest concentration of culture filtrates, organic solvent extracts and isolated compounds that induced no germination of fungal spores or inhibited 90% or more of spore germination (Maregesi *et al.* 2008).

Greenhouse experiments

1. Pathogenicity tests

Cultures of soil-borne fungi (*F. oxysporum*, *F. solani*, *R. solani* and *S. sclerotiorum*) were grown 5-10 days on PDA, and a 5 to 10 mm block of agar with hyphal tips was transferred to 150 ml of 3% cornmeal sand in 250 ml Erlenmeyer flasks. Cultures were grown 2 to 3 weeks on cornmeal sand at 22 ± 2°C. Soil was infested separately with fungal cultures (0.1 to 1% v/v). Controls were non infested soil. A randomized complete block design with three replications was used in all experiments. Seeds of cowpea, pea, squash, bean and pepper were surface-sterilized with 3% sodium hypochlorite solution for 3 min, then washed with tap water. Five seeds were sown in each pot and the pre and post emergence damping off were recorded 20 and 40 days after planting, respectively (Ganesan *et al.* 2007).

2. Effect on the development of the damping-off disease

The soil used in this experiment was sterilized before being infested with fungal isolates (*F. oxysporum*, *F. solani*, *R. solani* and *S. sclerotiorum*) at the rate of 1% by weight. The inoculums of the pathogen was prepared by growing each tested fungus on 3% cornmeal sand medium for 2 to 3 weeks at 22 ± 2°C. Surface sterilized seeds of bean were soaked for 1-2 hrs in crude organic solvent extracts (chloroform extract, ethyl acetate extract and butanol extract). Seeds were sown at the rate of 5 seeds/pot, and three pots were used for each treatment.

Disease assessment

Percentage of pre-emergence damping off was determined after 20 days as (Loganathan *et al.* 2004):

$$\%\,\text{Pre-emergence} = \frac{\text{No. of ungerminated seeds/pot}}{\text{No. of sown seeds/pot}} \times 100$$

Percentage of post-emergence damping off was determined after 40 days as:

$$\%\text{Post-emergence} = \frac{\text{No. of dead seedlings/pot}}{\text{No. of surviving seedlings/pot}} \times 100 \cdot$$

RESULTS

Antifungal activity against some important phytopathogens

1. Culture filtrates of Drechslera isolates

Data in **Tables 1** and **2** show that among the tested culture filtrates, only *D. cynodontis* culture filtrate was highly effective growth inhibitor against all tested fungi, it reducing the fungal growth from 51.1% (on *R. solani*) to 86.7% (on *Sclerotium cepivorum*) at concentration of 70%. Also, *D. ellisii* culture filtrates exhibited high inhibition on the fungal growth of the tested fungi from 22.2% (on *R. solani*) to 77.8% (on *A. solani*) at the same concentration.

Table 1 Antifungal activity of culture filtrates of *Drechslera* isolates on the mycelial growth of tested fungi.

Fungi	%Inhibition											
	Alternaria solani			*Botrytis cinerea*			*Botrytis fabae*			*Fusarium oxysporum*		
	30%	50%	70%	30%	50%	70%	30%	50%	70%	30%	50%	70%
D. australiensis	27.8	61.1	75.6	33.3	44.4	55.6	0	11.1	33.3	16.7	33.3	44.4
D. cactivora	33.3	50	66.7	0	0	11.1	0	11.1	22.2	16.7	38.9	50
D. cynodontis	63.3	72.2	82.2	66.7	71.1	75.6	48.9	55.6	72.2	36.7	48.9	61.1
D. ellisii	61.1	72.2	77.8	27.8	33.3	50	46.7	55.6	66.7	22.2	27.8	50
D. hawaiensis	22.2	42.2	50	0	11.1	44.4	0	2.2	15.6	16.7	22.2	44.4
D. maydis	30	38.9	60	22.2	30	55.6	11.1	20	55.6	22.2	27.8	44.4
D. neergaardii	33.3	48.9	55.6	33.3	44.4	50	11.1	50	61.1	11.1	20	33.3
D. poae	16.7	33.3	33.3	0	11.1	11.1	0	11.1	11.1	16.7	22.2	22.2
D. spicifera	11.1	17.8	22.2	0	11.1	11.1	0	11.1	11.1	11.1	22.2	22.2

Table 2 Antifungal activity of culture filtrates of *Drechslera* isolates on the mycelial growth of tested fungi.

Fungi	%Inhibition											
	Fusarium solani			*Rhizoctonia solani*			*Sclerotinia sclerotiorum*			*Sclerotium cepivorum*		
	30%	50%	70%	30%	50%	70%	30%	50%	70%	30%	50%	70%
D. australiensis	33.3	44.4	50	0	5.6	11.1	0	11.1	13.3	0	33.3	55.6
D. cactivora	44.4	44.4	44.4	0	5.6	11.1	0	5.6	18.9	0	33.3	51.1
D. cynodontis	44.4	53.3	62.2	10	13.3	51.1	41.1	57.8	66.7	71.1	74.4	86.7
D. ellisii	44.4	48.9	55.6	7.8	11.1	22.2	16.7	24.4	33.3	60	64.4	70
D. hawaiensis	33.3	36.7	44.4	0	0	6.7	11.1	11.1	11.1	11.1	22.2	33.3
D. maydis	33.3	55.6	58.9	4.4	12.2	33.3	11.1	22.2	44.4	66.7	75.6	81.1
D. neergaardii	33.3	41.1	51.1	0	0	5.6	0	11.1	11.1	53.3	58.9	66.7
D. poae	27.8	27.8	33.3	0	5.6	5.6	0	6.7	12.2	0	22.2	33.3
D. spicifera	11.1	20	22.2	0	5.6	10	0	6.7	11.1	0	25.6	33.3

Table 3 Antifungal activity of organic solvent extracts of *D. cynodontis* on the mycelial growth of tested fungi.

Extracts	%Inhibition											
	Alternaria solani			*Botrytis cinerea*			*Botrytis fabae*			*Fusarium oxysporum*		
	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml
Chloroform	33.3	71.1	86.7	27.8	72.2	81.1	44.4	58.9	87.7	38.9	55.6	77.8
Ethyl acetate	11.1	53.3	66.7	27.8	44.4	61.1	44.4	55.6	87.7	27.8	50	60
Butanol	11.1	22.2	51.1	22.2	33.3	50	27.8	41.1	70	11.1	20	48.9

Table 4 Antifungal activity of organic solvent extracts of *D. cynodontis* on the mycelial growth of tested fungi.

Extracts	%Inhibition											
	Fusarium solani			*Rhizoctonia solani*			*Sclerotinia sclerotiorum*			*Sclerotium cepivorum*		
	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml
Chloroform	55.6	71.1	80	22.2	50	66.7	48.9	53.3	74.4	50	72.2	88.9
Ethyl acetate	33.3	50	70	22.2	50	60	36.7	41.1	55.6	44.4	61.1	81.1
Butanol	15.6	27.8	50	5.6	22.2	44.4	22.2	27.8	33.3	33.3	55.6	70

Table 5 Antifungal activity of culture filtrates of *Drechslera* isolates on spore germination of tested fungi.

Fungi	%Inhibition														
	Alternaria solani			*Botrytis cinerea*			*Botrytis fabae*			*Fusarium oxysporum*			*Fusarium solani*		
	30%	50%	70%	30%	50%	70%	30%	50%	70%	30%	50%	70%	30%	50%	70%
D. australiensis	74	82	88	31	44	57	12	21	36	16	30	42	39	59	85
D. cactivora	61	77	80	20	30	43	18	23	39	20	31	50	30	48	80
D. cynodontis	88	95	98	76	84	94	74	83	92	74	80	92	82	89	98
D. ellisii	84	88	94	48	61	80	59	73	82	67	79	90	50	77	90
D. hawaiensis	31	48	63	23	40	56	11	19	30	23	31	48	29	50	61
D. maydis	49	61	75	34	57	71	49	63	76	60	73	81	64	73	82
D. neergaardii	28	50	68	30	48	69	25	47	59	9	15	28	20	38	56
D. poae	23	31	48	16	27	34	11	24	33	34	51	68	13	23	36
D. spicifera	11	22	34	19	21	27	10	22	30	11	27	39	51	62	74

2. Organic solvent extracts of Drechslera cynodontis

Data in **Tables 3** and **4** show that chloroform extract was the supreme growth inhibitor against all tested fungi, whereas it inhibited the fungal growth from 66.7% (*R. solani*) to 88.9% (*S. cepivorum)* at concentration of 30 mg/ml. Also, ethyl acetate extract exhibited high inhibition on the fungal growth from 55.6% (*S. sclerotiorum*) to 87.7% (*B. fabae*) at this concentration, while butanol extract decreased the fungal growth by 33.3-70%.

Effects on spore germination of various phytopathogenic fungi

1. Culture filtrates of Drechslera isolates

The strongest inhibitors to spore germination among the tested culture filtrates were *D. cynodontis*, which inhibited spore germination of all tested fungi by 92 to 98%. The culture filtrates of *D. ellisii* were the second best in this regard where it reduced spore germination by 80% (*B. cinerea*) to 94% (*A. solani*) at concentration of 70%. The culture fil-

trates of *D. maydis* were the third best in terms of suppressing germination of fungal spores. It strongly inhibited spore germination of all tested fungi by 71 82% (**Table 5**).

2. Organic solvent extracts of Drechslera cynodontis

Data in **Table 6** shows that chloroform extract was highly effective in suppressing spore germination of all tested fungi at all concentrations, whereas it inhibited the germination of spores from 88% (*B. fabae*) to 99% (*A. solani*) at concentrations of 30 mg/ml. Extract of ethyl acetate highly inhibited spore germination from 70% (*B. fabae*) to 96% (*A. solani*) at the same concentration, while butanol extract induced 50 and 85% inhibition for spore germination of *F. oxysporum* and *A. solani*, respectively at the maximum concentration (30 mg/ml).

Determination of the minimum inhibitory concentrations MIC

1. The MIC for fungal growth

Culture filtrates of *Drechslera* isolates. Even using the maximum concentration (70%) of all culture filtrates of *Drechslera* isolates did not inhibit completely the growth of tested fungi. All the culture filtrates exhibited higher values of MIC (>70%).

Organic solvent extracts of *Drechslera cynodontis*. The maximum concentration (30 mg/ml) of any organic solvent extracts of *Drechslera cynodontis* culture filtrates was not sufficient for inhibiting completely the growth of tested fungi. All tested extracts recorded higher MIC values (>30 mg/ml).

2. The MIC for spore germination

Culture filtrates of *Drechslera* isolates. Data in **Table 7** shows that the culture filtrates of *D. cynodontis* had the highest level of inhibition to spore germination of *A. solani* when used at concentration of 50%, and inhibited 90% or more of spore germination of *B. cinerea*, *B. fabae*, *F. oxysporum* and *F. solani* at concentration of 70%. This was followed by culture filtrates of *D. ellisii*. It inhibited the spore germination of *A. solani*, *F. oxysporum* and *F. solani* at concentration of 70%, while recorded higher values of MIC (>70%) for spore germination of *B. cinerea* and *B. fabae*. The other culture filtrates of *Drechslera* isolates had less inhibitory effect on spore germination of all tested fungi (MIC >70%).

Organic solvent extracts of *Drechslera cynodontis*. Data in **Table 8** shows that the chloroform extract was the strongest inhibitor for spore germination of *A. solani*, *B. cinerea* and *F. solani*, its MIC was 20 mg/ml, and 30 mg/ml for *F. oxysporum*, while MIC for *B. fabae* was >30 mg/ml. It was followed by the ethyl acetate extract, which recorded the lowest MIC against spore germination of *A. solani* and *F. oxysporum* (30 mg/ml), while MIC for *B. cinerea*, *B. fabae* and *F. solani* was >30 mg/ml. Butanol extract had less inhibitory effect on spore germination of tested fungi, It had high values of MIC (>30 mg/ml).

Greenhouse experiments

1. Pathogenicity tests

Pathogenicity tests proved that all tested fungi were able to cause damping-off infection on bean plants with different degrees at both pre- and post-emergence stages. Results in **Table 9** show that *F. oxysporum* and *R. solani* were the most fungi isolates caused damping-off disease to bean plants. Isolates of *F. solani* and *S. sclerotiorum* caused a highly significantly effect at pre- and post-emergence stages at the rate of 86.6 and 6.6%, respectively. As the least percent of survival plants were recorded with *F. oxysporum* and *R. solani* (0%) followed by 6.8% with *F. solani* and *S. sclerotiorum* under greenhouse conditions. *F. solani* had a pathogenic effect on squash causing 100% pre-emergence damping-off. However, it was less effective against pepper, pea and cowpea inducing 86.6, 73.3 and 60% pre-emergence damping-off, respectively. *R. solani* caused 100% pre-emergence damping-off on cowpea, while pepper and pea plants exhibited low level of pre-emergence damping-off (66.6 and 46.6%). Also, *S. sclerotiorum* caused 100% pre-emergence damping-off only on pea.

Table 6 Antifungal activity of organic solvent extracts of *D. cynodontis* on spore germination of tested fungi.

Extracts	%Inhibition														
	Alternaria solani			*Botrytis cinerea*			*Botrytis fabae*			*Fusarium oxysporum*			*Fusarium solani*		
	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml	10 mg/ml	20 mg/ml	30 mg/ml
Chloroform	88	95	99	76	91	98	65	79	88	75	84	93	75	95	98
Ethyl acetate	77	84	96	50	63	80	30	53	70	60	77	90	64	77	87
Butanol	63	75	85	27	50	67	34	51	70	20	32	50	34	51	74

Table 7 The MIC of culture filtrates of *Drechslera* isolates on germination of fungal spores.

Fungi	*Alternaria solani*	*Botrytis cinerea*	*Botrytis fabae*	*Fusarium oxysporum*	*Fusarium solani*
D. australiensis	>70	>70	>70	>70	>70
D. cactivora	>70	>70	>70	>70	>70
D. cynodontis	50	70	70	70	70
D. ellisii	70	>70	>70	70	70
D. hawaiensis	>70	>70	>70	>70	>70
D. maydis	>70	>70	>70	>70	>70
D. neergaardii	>70	>70	>70	>70	>70
D. poae	>70	>70	>70	>70	>70
D. spicifera	>70	>70	>70	>70	>70

Table 8 The MIC of organic solvent extracts of *Drechslera cynodontis* on spore germination of the tested fungi

Extracts	*Alternaria solani*	*Botrytis cinerea*	*Botrytis fabae*	*Fusarium oxysporum*	*Fusarium solani*
Chloroform	20*	20	>30	30	20
Ethyl acetate	30	>30	>30	30	>30
Butanol	>30	>30	>30	>30	>30

* Concentration = 20 mg/ml.

Table 9 Pathogenicity tests of selected fungi against some plants.

Plant species	*Fusarium oxysporum*			*Fusarium solani*			*Rhizoctonia solani*			*Sclerotinia sclerotiorum*		
	Pre -	**Post -**	***Survival***	**Pre -**	**Post -**	***Survival***	**Pre -**	**Post -**	***Survival***	**Pre -**	**Post -**	***Survival***
Bean	100	0	0	86.6	6.6	6.8	100	0	0	86.6	6.6	6.8
Cowpea	86.6	6.6	6.8	60	13.3	26.7	100	0	0	66.6	0	33.4
Pea	40	13.3	46.7	73.3	13.3	13.4	46.6	0	53.4	100	0	0
Pepper	73.3	13.3	13.4	86.6	6.6	6.8	66.6	0	33.4	73.3	13.3	13.4
Squash	46.6	0	53.4	100	0	0	60	13.3	26.7	46.6	13.3	40.1

Pre - = Pre-emergence damping-off.
Post - = Post-emergence damping-off.
Pre -, post -emergence damping-off and survival are expressed as percentage.

Table 10 Effect of bean seed treatment with the solvent extracts of *D. cynodontis* on damping-off incidence.

Extracts	Pre-emergence damping-off %				Post-emergence damping-off %				Survival %			
	F. oxysporum	***F. solani***	***R. solani***	***S. sclerotiorum***	***F. oxysporum***	***F. solani***	***R. solani***	***S. sclerotiorum***	***F. oxysporum***	***F. solani***	***R. solani***	***S. sclerotiorum***
Chloroform	40	6.6	26.6	6.6	0	0	13.3	0	60	93.4	60.1	93.4
Ethyl acetate	46.6	33.3	66.6	13.3	0	0	6.6	6.7	53.4	66.7	26.8	80
Butanol	66.6	53.3	80	53.3	13.3	13.3	6.6	13.3	20.1	33.4	13.4	33.4

2. Effect of bean seeds treatment with organic solvent extracts of Drechslera cynodontis on the development of the damping-off disease

Results in **Table 10** show that chloroform extract had the highest effect in controlling the damping-off disease caused by *F. solani* and *S. sclerotiorum* (93.4% control), and (60.1 and 60% control) for the damping-off disease caused by *R. solani* and *F. oxysporum* respectively. Ethyl acetate extract was the second best (80% control for *S. sclerotiorum* and 66.7% for *R. solani*). On the other hand, the least effective in this respect was butanol extract which gave only 33.4% control for *S. sclerotiorum*.

Identification of isolated compounds

1. Compound (1)

The EI-MS showed a molecular ion peak $[M]^+$ at m/z 264 and thus suggested the molecular formula $C_{15}H_{20}O_4$. ^{13}C-NMR (**Table 11**) and DEPT spectrum confirmed the presence of 2 methyl groups at δ_C 11.2 and 23.1 ppm, 3 methylene at δ_C 46.5, 61.3 and 109.8 ppm, 5 methine at δ_C 42.3, 66.5, 123.4, 127.9 and 139.5 ppm and 5 quaternary carbons at δ_C 36.4, 76.4, 154.2, 163.6 and 197.5 ppm. The structure was deduced to be bicyclic based on the calculated double bond equivalence (DBE = 6) and the presence of 3 double bonds and one carbonyl carbon. ^{13}C-NMR signal at δ 197.5 confirmed the presence of a keto group. In ^{13}C-NMR spectrum six signals were observed in the olefinic region. Two carbon signals at δ_C 127.9 and 139.5 were attributed to C-1 and C-2 respectively, signals at δ_C 123.4 and 163.6 were assigned to C-9 and C-10 respectively and the characteristic signals for exomethylene group at δC 154.2 and 109.8 were assigned to C-11 and C-13 respectively. Besides, DEPT spectrum confirmed the presence of a primary hydroxyl at δ_C 61.3 (C-12), a secondary hydroxyl at δ_C 66.5 (C-3) and a tertiary hydroxyl at δ_C 76.4 (C-7). ^{1}H-NMR and ^{13}C-NMR were in full agreement with those reported for dihydrobipolaroxin, the eremophilane-type sesquiterpene (Sugawara *et al.* 1985). The structure of compound 1 (dihydrobipolaroxin) was further substantiated by complete assignments of HMBC, HMQC and COSY experiments spectra, which have not been previously reported for it. In HMBC spectrum the proton at δ_H 6.23 (1 H, br.s., H-1) showed cross peaks with C-3, C-5, C-9 and C-10, the proton at δ_H 5.7 (1 H, S, H-9) showed cross peaks with C-5, C-7 and C-1. The presence of the exomethylene group in the side chain was confirmed from COSY and HMBC (**Fig. 1**) as its two protons at δ_H 5.15 and 5.18 ppm showed cross peaks with the proton at δ_H 3.78 (2 H, S, H-12) (COSY) and with C-7, C-11 and C-12 (HMBC). This is the first report of separation of dihydrobipolaroxin from this fungus. It was separated before from *Bipolaris cynodontis* (Sugawara *et al.* 1985).

Table 11 NMR spectral data of compound 1.

Carbon number	δ ^{13}C-NMR (*J* H_Z)	δ ^{1}H (multiplicity, *J* H_Z)
1	127.9 CH	6.23 br.s.
2	139.5 CH	6.23 br.s.
3	66.5 CH	(3.95 d, *J*=14.4)
4	42.3 CH	1.52 m
5	36.4 C	----------
6	46.5 CH_2	1.86 m
7	76.4 C	----------
8	197.5 C	----------
9	123.4 CH	5.7 s
10	163.6 C	----------
11	154.2 C	----------
12	61.3 CH_2	3.78 s
13	109.8 CH_2	5.15 s, 5.18 s
14	23.1 CH_3	1.32 s
15	11.2 CH_3	0.93 s

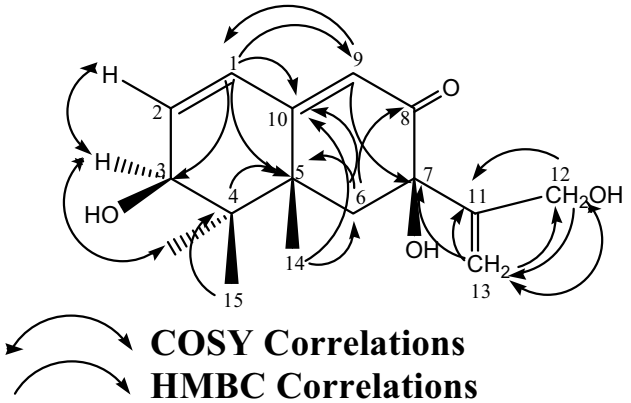
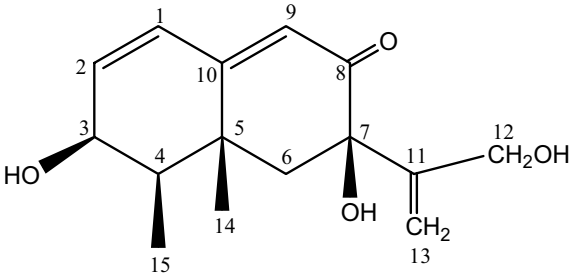


Fig. 1 Important COSY and HMBC correlations of compound 1.



Compound 1 (dihydrobipolaroxin)

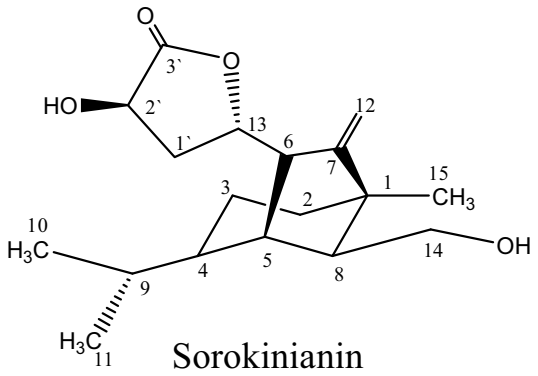
2. Compound (2)

The EI-MS showed a molecular ion peak $[M+1]^+$ at m/z 309 suggesting the molecular formula $C_{18}H_{28}O_4$. The structure was suggested to be tricyclic based on the calculated double bond equivalence (DBE = 5) and the presence of one double bond and one carbonyl carbon (**Table 12**). An absorption band at 1770 cm^{-1} (IR spectrum) and a C=O signal at δ_C 177.1 ppm indicated a γ–lactone ring. Besides the characteristic signals for the exomethylene group at δ_C 159.2 and 107.3 were assigned to C-7 and C-12 respectively. The presence of isopropyl group was confirmed from the two methyl signals at δ_H 0.77 (3 H, d, J = 6.9, H-10) and δ_H 0.88 (3 H, d, J = 6.9, H-11), both have the same J value i.e. most possibly split by the same proton. The detailed ^{1}H-NMR and ^{13}C-NMR data were consistent with those of sorokinianin (Nakajima *et al*. 1994). This is the first report for the isolation of sorokinianin from *D. cynodontis*. It was isolated before from *Bipolaris sorokiniana* (Nakajima *et al.* 1994).

Table 12 ^{1}H- and ^{13}C-NMR spectral data of compound 2.

Carbon number	δ ^{13}C-NMR (J Hz)	δ ^{1}H (multiplicity, J Hz)
1	48.3	----------
2	44.2	----------
3	24.7	----------
4	49.4	----------
5	39.2[a]	2.6 br.s.
6	48.3	----------
7	159.2	----------
8	59.6	----------
9	31.2	----------
10	20.6[b]	0.77 (d, J=6.9)
11	20.9[b]	0.88 (d, J=6.9)
12	107.3	4.78 s, 5.1 s
13	82.3	4.36 ddd (J=6.0, 6.9, 10)
14	61.5	----------
15	22.1	0.91 s
1¥	37.8	----------
2¥	67.0	----------
3¥	177.1	----------

[a] - under solvent peak
[b] - may be interchanged



Compound 2 (sorokinianin)

Antifungal activity of isolated compounds from *Drechslera cynodontis* on the mycelial growth

Data in **Tables 13** and **14** show that compound 1 (Dihydrobipolaroxin) was a highly effective growth inhibitor against *A. solani*, *F. oxysporum* and *S. sclerotiorum* (66.7%), while decreased the other fungal growth by 44.4–51.1% at a concentration of 100 µg/ml. Compound 2 (Sorokinianin) decreased the fungal growth of all tested fungi which ranged from 22.2% (on *R. solani*) to 61.1% (on *F. solani*) at concentration of 100 µg/ml.

Antifungal activity of isolated compounds from *Drechslera cynodontis* on spore germination

Data in **Table 15** shows that compound 1 (dihydrobipolaroxin) highly decreased spore germination of *A. solani* and *F. solani* by 80 and 77%, while it inhibited spore germination of the other fungi by 34 to 61% at a concentration of 100 µg/ml. Compound 2 (sorokinianin) was highly effective in suppressing spore germination of *F. solani* (75%) at a concentration of 100 µg/ml, while it inhibited the spore germination of the other four fungi by 43 to 60%.

Determination of the minimum inhibitory concentrations MIC

1. The MIC for fungal growth

The maximum concentration (100 µg/ml) of any compounds was not sufficient for inhibiting completely the growth of tested fungi. All tested compounds recorded higher MIC values (>100 µg/ml).

2. The MIC for spore germination

Compounds (1) and (2) had less inhibitory effect on spore germination of all tested fungi, they recorded higher MIC values (>100 µg/ml).

DISCUSSION

Several fungal pathogens, especially those belonging to the genera *Drechslera* (Capio *et al.* 2004; Evidente *et al.* 2005), *Alternaria* (Liebermann *et al.* 2000; Andersen *et al.* 2008) and *Cochliobolus* (Nakajima *et al.* 1992, 1997) produce phytotoxins that are virulence and/or pathogenicity factors. These compounds are active against the same plant species as the fungal pathogens and low (physiological) concentrations of the toxin are able to reproduce symptoms of the natural infections. These plant-specific metabolites have received attention as models for new herbicides (Hoagland *et al.* 2007).

There are many reasons why natural products might be good sources of molecules or molecular templates for pesticides or at least lead to new targets of action (Rimando and Duke 2006; Thines *et al.* 2006). New mechanisms of action for pesticides are highly desirable to fight the evolution of resistance in the target pests, to create or exploit unique market niches, and to cope with new regulatory legislation (Dayan *et al.* 2008).

Our interest to find a new natural fungitoxic compounds from the genus *Drechslera* led us to examine culture filtrates of nine *Drechslera* isolates *in-vitro* against mycelial growth and spore germination of eight plant pathogenic fungi (*A. solani*, *B. cinerea*, *B. fabae*, *F. oxysporum*, *F. solani*, *R. solani*, *S. sclerotiorum* and *S. cepivorum*). Among the tested culture filtrates, only *D. cynodontis* was a highly effective growth inhibitor against all tested fungi and the strongest inhibitor to the spore germination at concentration of 70%. The chloroform extract of *D. cynodontis* was the strongest inhibitor for fungal growth and spore germination of all tested fungi comparable to the ethyl acetate and the butanol extracts of the same fungus. Moreover, in greenhouse experiments, the chloroform extract also had the highest effect in controlling the damping-off disease caused by *F. solani* and *S. sclerotiorum* on bean.

Bioactivity-guided fractionation led to the isolation of two sesquiterpenoid compounds, dihydrobipolaroxin and sorokinianin from the chloroform extract of *D. cynodontis*. This is the first report for the isolation of these compounds from *D. cynodontis*. Dihydrobipolaroxin was separated before from *Bipolaris cynodontis* (Sugawara *et al.* 1985) and this is the first time to carry out 2D-NMR experiments for this compound while sorokinianin was isolated before from *Bipolaris sorokiniana* (Nakajima *et al.* 1994).

Dihydrobipolaroxin and sorokinianin were tested for

Table 13 Antifungal activity of isolated compounds from *D. cynodontis* on the mycelial growth of tested fungi.

Compound	%Inhibition											
	Alternaria solani			*Botrytis cinerea*			*Botrytis fabae*			*Fusarium oxysporum*		
	50 µg/ml	75 µg/ml	100 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
Compound 1	36.7	55.6	66.7	16.7	38.9	50	5.6	33.3	51.1	11.1	33.3	66.7
Compound 2	22.2	33.3	55.6	11.1	20	44.4	5.6	22.2	44.4	11.1	20	55.6
Control	0	0	0	0	0	0	0	0	0	0	0	0
DMSO	0	0	0	0	0	0	0	0	0	0	0	0

Table 14 Antifungal activity of isolated compounds from *D. cynodontis* on the mycelial growth of tested fungi.

Compound	%Inhibition											
	Fusarium solani			*Rhizoctonia solani*			*Sclerotinia sclerotiorum*			*Sclerotium cepivorum*		
	50 µg/ml	75 µg/ml	100 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
Compound 1	16.7	38.9	50	0	44.4	44.4	41.1	51.1	66.7	33.3	36.7	48.9
Compound 2	20	50	61.1	0	11.1	22.2	11.1	33.3	57.8	33.3	55.6	58.9
Control	0	0	0	0	0	0	0	0	0	0	0	0
DMSO	0	0	0	0	0	0	0	0	0	0	0	0

Table 15 Antifungal activity of isolated compounds from *D. cynodontis* on spore germination of tested fungi.

Compound	%Inhibition														
	Alternaria solani			*Botrytis cinerea*			*Botrytis fabae*			*Fusarium oxysporum*			*Fusarium solani*		
	50 µg/ml	75 µg/ml	100 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
Compound 1	60	75	80	19	21	34	28	49	61	10	23	56	50	61	77
Compound 2	20	30	60	26	45	59	40	40	50	11	27	43	50	60	75
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DMSO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

their inhibitory activity against some pathogenic fungi. It was found that Compound 1 (dihydrobipolaroxin) was a highly effective growth inhibitor against *A. solani*, *F. oxysporum* and *S. sclerotiorum* (66.7%) while compound 2 (sorokinianin) decreased the fungal growth of all tested fungi which ranged from 22.2% (on *R. solani*) to 61.1% (on *F. solani*) at concentration of 100 µg/ml. Compound 1 highly decreased spore germination of *A. solani* and *F. solani* by 80 and 77%, while compound 2 was highly effective in suppressing spore germination of *F. solani* (75%) at concentration of 100 µg/ml, (inhibiting the spore germination of other fungi by 43 to 60%).

Species of *Bipolaris*, *Drechslera*, *Exserohilum*, and *Curvularia* constitute a group of taxonomically related and ecologically similar deuteromycetes (mitosporic fungi) that are important plant pathogens or common saprophytes throughout the world (Zhong and Steffenson 2001; Kodsueb *et al.* 2006; Schoch *et al.* 2006). The first three of these genera were segregated from *Helminthosporium* in several revisions from 1930 to 1974, and some species of *Bipolaris* and *Curvularia* share the same teleomorph (Pratt 2006). The generic name *Bipolaris* for the *Helminthosporium* species with fusoid, straight, or curved conidia germinating by one germ tube from each end (bipolar germination) (Motlagh and Kaviani 2008). The former genus *Helminthosporium* was divided into three anamorphic genera: *Bipolaris*, *Drechslera*, and *Exserohilum* with the teleomorphic stages *Cochliobolus*, *Pyrenophora*, and *Setosphaeria*, respectively. *B. sorokiniana* is differentiated from other members of the *Bipolaris* genus on the basis of morphological features of conidiophores and conidiospores (Kumar *et al.* 2002; Müller *et al.* 2005; Asad *et al.* 2009).

In this report, it has clearly been demonstrated the role of the culture filtrates of some plant pathogenic fungi and their organic solvent extracts as a source of fungitoxic chemicals and their importance in controlling different plant pathogens, which has not been previously reported. The most of the scientific literature were focused on the phytotoxicity of these culture filtrates to some plants or weeds and their relationship to disease symptomology and their toxins proposed as potential natural herbicides. Therefore, we can use the culture filter of *D. cynodontis* as a potential source of natural fungitoxic compounds.

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