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ISOLATION AND IDENTIFICATION OF NATURAL HERBICIDAL COMPOUND FROM A PLANT PATHOGENIC FUNGUS, *DRECHSLERA BISEPTATA*

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Abstract

Weeds incur heavy losses in crop production. Use of agrochemicals is wide spread in weed management all over the world but all of these agrochemicals are associated with numerous undesired side effects. In recent years, there is emerging trend of exploring natural herbicides from plants and microbes. In this study, phytotoxic metabolites produced by phytopathogenic fungus *Drechslera biseptata* (Sacc. & Roum.) Richardson & Fraser were investigated for their herbicidal activity against *Rumex dentatus* L., a cosmopolitan noxious weed that causes enormous yield losses in different wheat varieties. For this aim, extracts (Ethanolic extract and partitioned ethanolic extracts), chromatographic fractions and main secondary metabolite, obtained from culture of *D. biseptata* were tested on *R. dentatus*. A phytotoxic pure compound was identified as di-(2-ethyl-hexyl)-phthalate (DEHP), by spectroscopic techniques [Mass Spectrometry (MS) and Nuclear Magnetic Resonance Spectrometry (NMR)]. In leaf disk assay, DEHP at 0.5 µg μL^{-1} produced necrosis on weed but not on wheat leaves. On the other hand, synthetic herbicidal compound, 2,4-dichlorophenoxyacetic acid, used as positive control, produced necrotic spots at leaf discs of *R. dentatus* at concentration of 0.25 µg μL^{-1}. The present investigation suggests the use of DEHP as natural herbicidal compound.

Key words: *Drechslera,* Fungal metabolite, Natural herbicide, Phytotoxins, *Rumex dentatus*.

Introduction

Rumex dentatus L. is cosmopolitan noxious weed that can cause reduction in grain yield by 60% and total dry matter yield by 74% (Umm-e-Kulsoom *et al*., 2018). Chemical herbicides are still considered as the most effective approach to combat this weed (Usman *et al*., 2010; Hulting *et al*., 2012). However, there are numerous environmental and public health concerns associated with these agrochemicals (Van Bruggen *et al*., 2018), including the evolution of herbicide resistance in weeds (Moss *et al*., 2019), as well as deleterious effects on soil biology and functions (Rose *et al*., 2016). So, there is dire need to explore newer eco-friendly herbicides from natural resources.

Currently, some herbicide ingredients based on natural products are available. These ingredients having herbicidal effects include rice straw extract (Afridi *et al*., 2014), essential oils (citrus, pine, clove), organic acids (pelargonic or acetic acids), corn gluten, and L-alanyl-L-alanyl-phosphinothricin (Duke *et al*., 2018). An interesting alternative in weed management consists the use of phytotoxins produced by microbes. In fact, there is strong evidence that much of the activity of phytopathogens is due to secondary metabolites. Many fungal phytopathogens produce phytotoxins which can be exploited as natural herbicides (Varejão *et al*., 2013; Todero *et al*., 2018). Furthermore, in order to identify new classes of herbicides, action mechanisms of some phytotoxins have also been determined (Duke & Dayan, 2015, Vurro *et al*., 2018).

In this respect, among many genera analyzed, some *Drechslera* species, viz: *D. australiensis, D. hawaiiensis, D. biseptata, D. rostrata,* and *D. holmii*, have shown herbicidal activity (Javaid & Adrees, 2009; Akbar & Javaid, 2010, Javaid *et al*., 2011). *Drechslera gigantea* produces ophiobolin A and other related compounds (6-*epi*-ophiobolin A and 3-anhydro-6-*epi*-ophiobolin A, ophiobolin B, E, I, J and 8-*epi*-ophiobolin J). Among these, ophiobolin A was found to be more toxic when assayed on several grassy and dicotyledonous weeds (Evidente *et al*., 2006a, b). Moreover, its growth-inhibitory activities in plant cells were also reported (Bury *et al*., 2013). Different strains of *Drechslera siccans* have been found to produce phytotoxic compounds. In particular, Hallock *et al*., (1988) identified de-*O*-methyldiaporthin, whose phytotoxicity was recorded regarding necrotic spot area when tested on common oat, barnyard grass, and spiny amaranth. Siccanol, isolated from the same species, completely arrested the root growth of Italian ryegrass (*Lolium multiflorum* Lam.) seedlings (Lim *et al*., 1996). Evidente *et al*., (2005) identified phytotoxic trisubstituted naphthofuroazepinone, named drazepinone, from another strain of *D. siccans*. This compound exhibited broad-spectrum phytotoxic activity and low toxicity to *Artenia salina*. Cytochalasin B and dihydrocytochalasins with phytotoxic properties have been isolated from *Drechslera wirreganensis* and *Drechslera campanulata* (Capio *et al*., 2004). Likewise, phytopathogenic fungus *Drechslera australiensis* produced holadysenterine, a phytotoxic compound against *R. dentatus* (Akbar *et al*., 2014). Although several phytotoxins have been identified from many species of *Drechslera*, there is not any such report from *D. biseptata*.–The present study was, therefore, undertaken to identify phytotoxic compounds from this fungal species for control of *Rumex dentatus*.

Materials and Methods

Fungal isolate and weed: A species of *D. biseptata* [FCBP accession No. FCBP-PTF- 1073 and FCBP-DNA-1073] was acquired from Culture Bank, University of the Punjab, Lahore, Pakistan. Single spore subculture of *D. biseptata* was accomplished by serial dilution method on potato dextrose agar (PDA) at 28°C. Ripened seeds of *R. dentatus* were collected from plants growing in wheat fields of Gujrat, Punjab, Pakistan. These seeds were sun-dried for 7 days, stored in paper bags and kept at 4°C.

Fungal culture production: Minimal medium (M-1-D) was prepared according to Evidente *et al*., (2006a). M-1-D (400 mL) was transferred into 1000 mL conical flasks, autoclaved at 121°C for 20 minutes and cooled down to 25°C. The flasks were inoculated with bits of *D. biseptata* mycelia and spores, and kept at 28 ± 2°C for 28 days. After completion of the incubation period, the cultures were stored at 4°C (Javaid & Adrees, 2009).

Extraction and purification process: Fungal culture broth (5 L) with fungal mycelia was adjusted to pH 4.5 with HCl 0.1 M and homogenized in a mixer with 5 L of ethanol (HPLC grade). The suspension was centrifuged (40 min at 7000 rpm, 10°C) and separated from the supernatant. The residue was extracted with 3 L of fresh ethanol overnight. The suspension was centrifuged, and supernatants were combined and evaporated under reduced pressure yielding ethanolic extract (EE) as brown oil (630 mg). This extract was reconstituted in double distilled water (1 L) and extracted successively three times with the same volume of *n*-hexane, chloroform and ethyl acetate (EtOAc). Organic phases were dried on anhydrous Na_2SO_4, and evaporated *in vacuo* yielding three partitioned ethanolic extracts (PEEs) viz. *n*-hexane (36.8 mg), chloroform (280 mg) and ethyl acetate (225 mg).

Bioactive EtOAc extract was purified by TLC on silica gel eluted with *n*-hexane/EtOAc/CH_3CN (10:15:75, v/v/v) to afford eight chromatographic fractions (CFr): A (R_f 0.13) 20 mg, B (R_f 0.14) 12 mg, C (R_f 0.25) 35 mg, D (R_f 0.4) 19.2 mg, E (R_f 0.54) 30 mg, F (R_f 0.65) 24.3 mg, G (R_f 0.7) 14.6 mg, H (R_f 0.87) 17 mg. Fraction E, obtained as yellowish oil, was identified as di-2-(ethyl-hexyl)-phthalate (DEHP).

Chemical analysis procedures: Thin Layer Chromatography (TLC) was performed on silica gel plates (Kieselgel 60, F254, 0.25 and 0.5 mm, respectively) (Merck). The spots were visualized by UV light. Column chromatography was carried out using silica gel (Merck, Kieselgel 60, 0.063–0.200 mm).

^1H and ^{13}C Nuclear Magnetic Resonance Spectroscopy (NMR) spectra were recorded at 400/100 MHz in deuterated methanol (CD_3OD) on Bruker spectrometers. CD_3OD was kept as internal standard. 2D NMR spectra were recorded using Bruker microprograms. ESI-TOF mass spectrum was recorded on Agilent Technologies QTOF 6230 in +ive ion mode.

Di-2-(ethyl-hexyl)-phthalate (DEHP): ^1H NMR (400 MHz, CD_3OD, *J* in Hz) δ: 7.72 (dd, 7.2, 2.7, H-11, H-11'), 7.63 (dd, 7.2, 2.7, H-12, H-12'),), 4.25-4.19 (m, H_2-6, H_2-6'), 1.72-1.66 (m, H-5, H-5'), 1.48-1.42 (m, H_2-8, H_2-8'), 1.39-1.29 (m, H_2-2, H_2-3, H_2-4, H_2-2', H_2-3', H_2-4'), 0.95 (t, 4.5, H_3-9, H_3-9'), 0.93 (t, 5.4, H_3-1, H_3-1'); ^{13}C NMR (100 MHz, CD_3OD) δ: 169.4 (C-7, C-7'), 133.7 (C-12, C-12'), 132.5 (C-11, C11'), 130.0 (C-10, C-10'), 69.2 (C-6, C-6'), 40.3 (C-5, C-5'), 31.8 (C-8, C-8'), 30.3 (C-4, C-4'), 25.1 (C-3, C-3'), 24.2 (C-2, C-2'), 14.5 (C-9, C-9'), 11.5 (C-1, C-1'); HR ESI MS (+) spectrum *m/z*: 819 $[2M+K]^+$, 803 $[2M+Na]^+$, 429 $[M+K]^+$, 413 $[M+Na]^+$, 391.2778 [$C_{24}H_{39}O_4$, calcd. 391.2848, $M+H]^+$.

Phytotoxicity assays: Ethanolic extract (EE), partitioned ethanolic extracts (PEEs), chromatographic fractions [(CFr) (A-H)] obtained from culture broth + fungal mycelia of test fungus, *D. biseptata* were assayed by leaf disk puncture assay on detached leaves of *R. dentatus* following technique explained by Akbar *et al*., (2014). Fresh leaves from 20 days old *R. dentatus* plants were collected and leaf discs having size of 1-cm diameter were cut out from these *R. dentatus* leaves. These discs were positioned on glass slide.

In total 7 concentrations of ethanolic extract (4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 µg μL^{-1}) were prepared following serial double dilution in sterile water as described by Akbar *et al*., (2014). The highest concentration of 4.0 µg μL^{-1} also contained 100 µL of dimethylsulfoxide (DMSO) used to dissolve EE, and solutions of lower concentrations accordingly comprised lower concentrations of DMSO.

Two milli grams of PEEs were dissolved in 50 µL of dimethylsulfoxide (DMSO). Final volume of each fraction was raised to 1.0 mL with dH_2O to prepare a stock solution at the concentration of 2 µg μL^{-1}. The stock solution was serially double diluted by adding dH_2O to prepare lower concentrations of 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 µg μL^{-1}.

Chromatographic fractions and DEHP were also tested on wheat leaves. The leaves of wheat used for testing bioactivity were of same age as of weed. In this case, fractions were investigated at the highest concentration of 1.0 µg μL^{-1}. 2,4-D was used as reference compound.

Twenty micro liters of each test solution, prepared as previously described, were applied by micropipette on ten *R. dentatus* leaf discs, detached from different plants. Blank control treatments were obtained by diluting 20 µL of DMSO at 100 µL mL^{-1} of dH_2O at the highest concentration, and serial lower concentrations were made by double diluting it with dH_2O. Treatment with pure dH_2O was also performed. After application of solutions of test concentrations, leaf discs were placed at 20°C and 60% humidity under continuous light in a growth chamber. Necrotic spot formation on leaf discs was recorded after 72 h. The following necrotic spot scale 0-7 was used to study the herbicidal effect of the treatments [modified from Akbar *et al*., (2014)]: 0 = No Necrotic spot (NS); **1** = NS ≤ 1 mm; **2** = NS ≤ 2 > 1 mm; **3** = NS ≤ 3 > 2 mm; **4** = NS ≤ 4 > 3 mm, **5** = NS ≤ 5 > 4 mm; **6** = NS ≤ 6 > 5 mm; **7** = NS ≤ 7 > 6 mm.

Results and Discussion

Phytotoxicity of ethanolic extract (EE) and partitioned ethanolic extracts (PEEs): Ethanolic extract (EE) obtained from culture broth + mycelia of *D. biseptata* produced necrotic spots on leaf discs of *R. dentatus* at the highest concentration investigated (4.0 µg µL^{-1}), while lower concentrations did not reveal any necrotic spot on leaf discs of *R. dentatus*. Among the partitioned ethanol extracts (PEEs), only EtOAc PEE produced necrotic spots at the concentrations of 2.0 and 1.0 µg µL^{-1}, while *n*-hexane PEE, and chloroform PEE did not produce any necrotic spot at all concentrations tested. DMSO at different concentrations and dH_2O used as control treatments did not produce any necrotic spots on leaf discs of *R. dentatus* (Tables 1 & 2). Earlier investigations also support our findings as the ability to produce phytotoxic extracts by another fungus, *Trichoderma* spp. against *R. dentatus* has also been reported (Javaid & Ali, 2011).

Table 1. Phytotoxic activity* of ethanolic extract (EE) of *Drechslera biseptata* on leaf surface of *Rumex dentatus*

DMSO Conc. (µL mL^{-1})	DMSO effect	EE Conc. (µg µL^{-1})	EE effect
Distilled water	0	Distilled water	0
1.560	0	0.0625	0
3.125	0	0.1250	0
6.250	0	0.2500	0
12.500	0	0.5000	0
25.000	0	1.0000	0
50.000	0	2.0000	0
100.000	0	4.0000	**1**

*Necrotic spot (NS) scale: **0** = No N.S, **1** = NS ≤ 1 mm, **2** = NS ≤ 2 > 1 mm, **3** = NS ≤ 3 > 2 mm, **4** = NS ≤ 4 > 3 mm, **5** = NS ≤ 5 > 4 mm, **6** = NS ≤ 6 > 5 mm, **7** = NS ≤ 7 > 6 mm

Table 2. Phytotoxic activity* of partitioned ethanolic extract (PEE) on leaf surface of *Rumex dentatus*.

DMSO Conc. (µL mL^{-1})	DMSO effect	PEE Conc. (µg µL^{-1})	PEE effect		
			n-hexane	Chloroform	Ethyl acetate
Distilled water	0	Distilled water	0	0	0
0.780	0	0.03125	0	0	0
1.560	0	0.0625	0	0	0
3.125	0	0.1250	0	0	0
6.250	0	0.2500	0	0	0
12.500	0	0.5000	0	0	0
25.000	0	1.0000	0	0	**1-2**
50.000	0	2.0000	0	0	**3**

*Necrotic spot (NS) scale: **0** = No N.S, **1** = NS ≤ 1 mm, **2** = NS ≤ 2 > 1 mm, **3** = NS ≤ 3 > 2 mm, **4** = NS ≤ 4 > 3 mm, **5** = NS ≤ 5 > 4 mm, **6** = NS ≤ 6 > 5 mm, **7** = NS ≤ 7 > 6 mm

Structural elucidation of DEHP: Structural elucidation of the active chromatographic fraction E was achieved by comparing the data obtained by NMR and ESI-TOF MS with those reported earlier for di-(2-ethyl-hexyl)-phthalate (DEHP). The active compound had a molecular formula $C_{24}H_{38}O_4$ as deduced from its ESI-TOF MS spectrum which depicted protonated molecular ion $[M+H]^+$ at *m/z* 391.2778 ($C_{24}H_{39}O_4$, calcd. 391.2848). The same spectrum showed the dimeric potassium and sodium clusters $[2M+K]^+$ and $[2M+Na]^+$ at *m/z* 819 and 803, and potassium and sodium clusters $[M+K]^+$, and $[M+Na]^+$ at *m/z* 429 and 413, respectively. ^1H NMR spectrum showed the presence of two signals typical of 1,2-omodisubstituted benzene at δ 7.72 and 7.63 (*J*=6.2, 2.1 Hz) typical of the protons of ortho-substituted aromatic ring (H-11 and H-12, respectively). The same spectrum displayed two multiplets at δ 4.25-4.19 (H_2-6) and 1.72-1.66 (H-5), assigned to an oxygenated methylene and a methine groups, respectively. COSY spectrum showed homocorrelation between these protons, between H-5 and the proton resonating as multiplet at δ 1.48-1.42, attributed to methylene group H_2-8, and between this latter and proton resonating as triplet at 0.95 (*J*=4.5 Hz) assigned to methyl group H_3-9. Finally the proton spectrum showed multiplets at δ 1.39-1.29 assigned to three methylene groups (H_2-2, H_2-3, H_2-4) and a triplet at δ 0.93 (*J*=5.4 Hz) assigned to methyl group H_3-1. The structure of DEHP was confirmed by analysis of ^{13}C NMR spectrum. In fact this spectrum, confirming the symmetry of the compound, exhibited the expected 12 carbon resonances as reported in the experimental section. In particular, it showed the signals at δ 169.4 assigned to carboxylic group (C-7) that in HMBC spectrum showed correlation between H-11 and H_2-6. By comparing with published spectroscopic data (Lotfy *et al*., 2018; Chua *et al.*, 2015; Rao *et al.*, 2000), the compound was identified as di-(2-ethylhexyl) phthalate (Fig. 1).

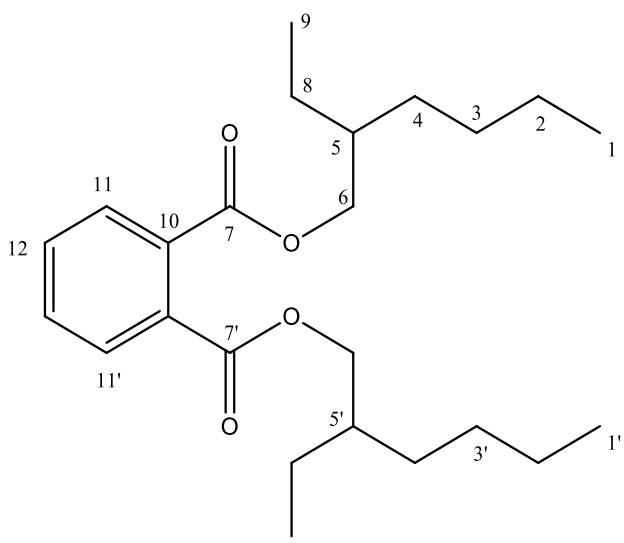

Fig. 1. Chemical structure of di-(2-ethyl-hexyl)-phthalate.

Phytotoxicity of chromatographic fractions (CFrs) and DEHP: In bioassays with chromatographic fractions [CFrs (A-H)] obtained from active ethyl acetate PEE of *D. biseptata,*–fraction E (DEHP) was active in producing necrotic spots on leaf discs of *R. dentatus* at the concentrations of 1.0 and 0.5 µg µL^{-1}. Fractions A, B, C, D, F, G and H did not produce any necrotic spot at all concentrations. In the same assay, 2,4-D used as positive control caused the induction of necrotic spots at the concentrations of 1.0, 0.5 and 0.25 µg µL^{-1}. On the other hand, no necrotic spot formation was observed on wheat leaf sections even at the highest concentration (1 mg mL^{-1}) of 2,4-D solution. Also DEHP isolated from ethyl acetate PEE of *D. biseptata* did not cause any necrotic spot of leaf sections of wheat (Table 3).

Table 3. Phytotoxicity activity* of chromatographic fractions (CFrs) and DEHP on *Rumex dentatus* (R. d) and wheat (W) leaf discs.

DMSO Conc. (µL mL^{-1})	DMSO effect	2,4-D & Cfrs Conc. (µg µL^{-1})	R. d	2,4-D effect	CFrs effect															
					A		*B*		*C*		*D*		*E(DEHP)*		*F*		*G*		*H*	
				W	R. d	W	R. d	W	R. d	W	R. d	W	R. d	W	R. d	W	R. d	W	R. d	W
Distilled water	0	Distilled water	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.3900	0	0.0156	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.7800	0	0.0312	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.5600	0	0.0625	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3.1250	0	0.1250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6.2500	0	0.2500	**2**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12.500	0	0.5000	**4**	0	0	0	0	0	0	0	0	0	**3**	0	0	0	0	0	0	0
25.000	0	1.0000	**6**	0	0	0	0	0	0	0	0	0	**4**	0	0	0	0	0	0	0

*Necrotic spot (N.S) scale: **0** = No NS, **1** = NS ≤ 1 mm, **2** = NS ≤ 2 > 1 mm, **3** = NS ≤ 3 > 2 mm, **4** = NS ≤ 4 > 3 mm, **5** = NS ≤ 5 > 4 mm, **6** = NS ≤ 6 > 5 mm, **7** = NS ≤ 7 > 6 mm

DEHP is a plasticizer and has been persistently found in different terrestrial and aquatic environments (Magdouli *et al.*, 2013). However, it has also been found that DEHP is synthesized by many organisms such as plants, bacteria, fungi and several studies have demonstrated different biological activities of this compound. DEHP isolated from flowers of *Calotropis gigantea* exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria, antifungal activity against *Aspergillus flavus*, and also exhibited toxicity on brine shrimp nauplii (Habib & Karim, 2009). Later on, the same authors explored the anticancer activity of DEHP (Habib & Karim, 2012). This compound was also isolated from roots of *Chlorophytum borivilianum* (Chua *et al.*, 2015), and leaves of *Cassia auriculata* (Rao *et al*., 2000). Some microorganisms, such as *Streptomyces* sp.; *Streptomyces mirabilis* and *Nocardia levis*, have also been reported to produce DEHP, displaying antimicrobial activity (Kavitha *et al*., 2009; Smaoui *et al*., 2011; El-Sayed, 2012). Recently, DEHP was isolated from a strain of fungus *Aspergillus awamori* derived from river Nile, and this compound exhibited antimicrobial and cytotoxic activity (Lotfy *et al.*, 2018). The natural origin of DEHP could be confirmed by biosynthesis studies in filamentous fungi which occur through the shikimic acid pathway, and esterification of phthalic acid with butyl alcohol (Tian *et al.*, 2016). Moreover, the isolation of optically active di-2-*R*-(–)-ethylhexyl-*O*-phthalate from dry cultured cells of *Aconitum baicalense* can confirm its natural origin (Semenov *et al*., 2016).

Conclusion

In conclusion one phytotoxic compound was isolated from EtOAc extract of culture of *Drechslera biseptata* and identified as di-(2-ethyl-hexyl)-phthalate. This compound can be used as structural lead to synthesize phytotoxic compounds that can be used as natural herbicides.

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