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

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Abstract

Wheat incurs heavy losses in crop production. Use of agrochemicals is widespread 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 (Ethanol 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 mL⁻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 mL⁻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 ophiohbin A and other related compounds (6-epi-ophiobolin A and 3-anhydro-6-epi-ophiobolin A, ophiohbin B, E, I and 8-epi-ophiohbin J). Among these, ophiohbin 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-methylidiploarthin, 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 drazipine, from another strain of D. siccans. This compound exhibited broad-spectrum phytotoxic activity and low toxicity to Artemia 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 method: 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 Na₂SO₄, 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/CH₃CN (10:15:75, v/v/v) to afford eight chromatographic fractions (CFr): A (Rf 0.13) 20 mg, B (Rf 0.14) 12 mg, C (Rf 0.25) 35 mg, D (Rf 0.4) 19.2 mg, E (Rf 0.54) 30 mg, F (Rf 0.65) 24.3 mg, G (Rf 0.7) 14.6 mg, H (Rf 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 13C Nuclear Magnetic Resonance Spectroscopy (NMR) spectra were recorded at 400/100 MHz in deuterated methanol (CD₃OD) on Bruker spectrometers. CD₃OD 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, CD₃OD, 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-6, H-6’), 1.72–1.66 (m, H-5, H-5’), 1.48–1.42 (m, H-8, H-8’), 1.39–1.29 (m, H-2, H-3, H-4, H-2’, H-3’, H-4’), 0.95 (t, 4.5, H-3, H-9’), 0.93 (t, 4.5, H-3, H-9’); 13C NMR (100 MHz, CD₃OD) 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 [C₆H₁₃O₄], calcld. 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 L⁻¹) were prepared following serial double dilution in sterile water as described by Akbar et al., (2014). The highest concentration of 4.0 µg L⁻¹ also contained 100 µL of dimethylsulfoxide (DMSO) used to dissolve EE, and solutions of lower concentrations accordingly comprised lower concentrations of DMSO.

Two milligrams of PEEs were dissolved in 50 µL of dimethylsulfoxide (DMSO). Final volume of each fraction was raised to 1.0 mL with dH₂O to prepare a stock solution at the concentration of 2 µg L⁻¹. The stock solution was serially doubly diluted by adding dH₂O to prepare lower concentrations of 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 µg L⁻². 

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 L⁻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 dH₂O at the highest concentration, and serial lower concentrations were made by double diluting it with dH₂O. Treatment with pure dH₂O 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 mL⁻¹), while lower concentrations did not reveal any necrotic spot on leaf discs of *R. dentatus*. Among the partitioned ethanolic extracts (PEEs), only EtOAc PEE produced necrotic spots at the concentrations of 2.0 and 1.0 μg mL⁻¹, while *n*-hexane PEE, and chloroform PEE did not produce any necrotic spot at all concentrations tested. DMSO at different concentrations and dH₂O 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. Phytoxic activity* of ethanolic extract (EE) of *Drechlera biseptata* on leaf surface of Rumex dentatus. |
|---|---|---|---|
| DMSO Conc. (µL mL⁻¹) | DMSO effect | EE Conc. (µg mL⁻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 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

| Table 2. Phytoxic activity* of partitioned ethanolic extract (PEE) on leaf surface of *Rumex dentatus*. |
|---|---|---|---|
| DMSO Conc. (µL mL⁻¹) | DMSO effect | PEE Conc. (µg mL⁻¹) | PEE effect |
| Distilled water | 0 | Distilled water | 0 |
| 0.780 | 0 | 0.03125 | 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 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

Phytoxicity 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 mL⁻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 mL⁻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).

**Fig. 1.** Chemical structure of di-(2-ethyl-hexyl) phthalate.
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 Chlorophyllum bontivillanum (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-Ω-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 EIOAc 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.

**References**


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