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Article

Isolation and Characterization of Natural Herbicidal Compound from *Drechslera rostrata*

Isolamento e Caracterização de Compostos Herbicidas Naturais Derivados de ***Drechslera rostrata***

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ABSTRACT - In agriculture, weeds have inevitable importance because of reducing the crop growth and also affecting food quality. Although, synthetic herbicides are available to combat these weeds but during recent years there is a trend of using ecofriendly herbicides extracted from natural resources. Present investigation is a continuity of the research, which reported a natural herbicidal compound named as Ophiobolin A from a fungus, *Drechslera rostrata*. The fungus was incubated in a growth medium of known composition (minimal medium) up to 28 days and its metabolites were extracted with organic solvents. The compound showing bioactivity was purified with the help of Reversed Phase High Performance Liquid Chromatography (RPHPLC) and identified with the help of Spectroscopic techniques viz. Mass Spectroscopy (MS) (LRESIMS and HRESIMS) and Nuclear Magnetic Resonance Spectroscopy (NMR). The purified compound caused death of superficial leaf cells of *Chenopodium album*, a noxious weed of wheat. Present investigation concludes that the identified compound could be used as structural analogue alternative to synthetic herbicides to synthesize natural herbicides.

Keywords: *Chenopodium album,* natural herbicide, metabolites.

RESUMO - Na agricultura, as plantas daninhas têm uma importância fundamental, por reduzirem o crescimento das culturas e também alterarem a qualidade da comida. Apesar de os herbicidas sintéticos estarem à disposição para combatê-las, nos últimos anos tem-se observado a tendência de usar herbicidas sustentáveis, extraídos de recursos naturais. O presente estudo é uma continuação da pesquisa sobre um composto herbicida natural chamado Ophiobolin A, derivado do fungo ***Drechslera rostrata****. O fungo foi incubado em um meio de cultura de composição conhecida (meio mínimo) por até 28 dias, e seus metabólitos foram extraídos com solventes orgânicos. O composto que apresentou bioatividade foi purificado com a ajuda da Cromatografia Líquida de Alta Eficiência de Fase Reversa (CLAEFR) e identificado com a ajuda das técnicas espectroscópicas, nomeadamente a Espectrometria de Massa (EM) (LRESIMS e HRESIMS) e a Espectroscopia de Ressonância Magnética Nuclear (RMN). O composto purificado causou a morte das células superficiais das folhas de* ***Chenopodium album****, uma planta daninha do trigo. O presente estudo concluiu que o composto identificado poderia ser usado como análogo alternativo estrutural aos herbicidas sintéticos para sintetizar herbicidas naturais.*

Palavras-chave: *Chenopodium album*, herbicida natural, metabólitos.

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INTRODUCTION

Weeds are known as unwanted plants within crops. They diminish the benefits of farm/ agricultural inputs not only by reducing the final crop yield but also by deteriorating the nutritional quality of agricultural products (Jabran et al., 2015). Wheat, the staple food of Pakistan as well as many other countries, faces a hindrance in its normal growth, due to weeds. Among them, four weeds viz. *Chenopodium album*, *Rumex dentatus*, *Phalaris minor* and *Avena fatua* are designated as wheat weeds. Although chemical herbicides are available to eradicate them, all synthetic herbicides have serious environmental and human health concerns (Akbar and Javaid, 2015). Moreover, there is an emerging problem of herbicide resistance in these weeds. *C. album* is the best example of herbicide resistance, since it has become resistant to synthetic herbicides (Aper et al., 2014; Nawaz et al., 2016). Nowadays, there is an emerging trend towards eco-friendly herbicides to combat such issues (Akbar and Javaid, 2015). Natural resources have plethora of novel bioactive compounds that may be used as natural pesticides. e.g. mycotoxins (Cimmino et al., 2015). Mycotoxins have subtle physiological effects and are toxic to plants when applied at very low concentrations; hence, they can be harnessed as natural herbicides (Ismaiel and Papenbrock, 2015). As an example, chaetomugilin D and J have shown to possess herbicidal/ phytotoxic activity on lettuce (*Lactuca sativa*) (Piyasena et al., 2015). Phomentrioloxin is a phytotoxic compound produced by *Phomopsis* sp., a promising natural herbicide for the management of *C. album* and *Carthamus lanatus* weeds (Cimmino et al., 2013a). Phomentrioloxin, along with other phytotoxic metabolites such as gulypyrones A and B and 1, O- and 2, O-dehydro derivatives of phomentrioloxin, named phomentrioloxins B and C, are also reported from *Diaporthe gulyae*. When tested on punctured leaf discs of weed and crop plants, phomentrioloxin B caused necrosis on various plant species (Andolfi et al., 2015). Similarly, chenopodolan B isolated from *Phoma chenopodiicola* showed herbicidal activity against punctured leaves of *Sonchus oleraceus*, *Mercurialis annua* and *C. album* (Cimmino et al., 2013b). Lu et al. (2016) isolated three herbicidal compounds, lunatoic acid A, 5Z-7-oxozeaenol and zeaenol, from the metabolites of *Cladosporium oxysporum* fungus, for a problematic weed, *Amaranthus retroflexus.* The efficacy of these compounds was similar to the synthetic herbicide 2,4-dichlorophenoxyacetic acid.

The *Drechslera* genus is considered incredible for the production of secondary phytotoxic compounds (Strobel et al., 1988). An herbicidal compound, macrodiolide, has already been isolated from the culture of *Drechslera avenae*. This compound was effective against *Avena sativa* (Kastanias and Chrysayi-Tokousbalides, 2005). Evidente et al. (2006b) isolated and identified phytotoxic/ herbicidal constituents (e.g. ophiobolin A) from a fungus, *Drechslera gigantea,* which has herbicidal properties against many monocot and dicot weeds such as *C. album.* Moreover, culture filtrates of *Drechslera* have shown phytotoxic activity on some knotty weeds of wheat and on an invasive weed, *Parthenium hysterophorus* (Akbar and Javaid, 2010; Javaid et al., 2011). However, researches about the isolation and characterization of phytotoxic ingredients from *Drechslera rostrata* are missing. Hence, the present research was a step forward towards characterizing phytotoxic constituents from culture filtrates of *D. rostrata* that can be used as analog to synthesize natural herbicides.

MATERIALS AND METHODS

Procurement and incubation of fungal isolate

An isolate/culture of *D. rostrata* was obtained from the Culture Bank, University of the Punjab, Lahore, Pakistan. The isolate was maintained on potato dextrose agar plates until further processing. Mass culturing of the fungal isolate was carried out in M-1-D broth (growth medium) (Evidente et al., 2006b). This medium was autoclaved and inoculated with bits of mycelia and spores of *D. rostrata* and kept at 25 ^{o}C. After 28 days of growth, fungal cultures and their metabolites were subjected to further processing.

Extraction of phytotoxins from liquid broth

One liter of test fungus culture filtrate with its mycelia was collected. First, it was defatted with *n-hexane* extraction followed by the extraction with chloroform in a separating glass funnel.

The extraction process was repeated thrice, until clear organic solvent phases were obtained. The organic solvent phases were collected, pooled together and extra solvents were removed *in vacuo* in a rotary evaporator (Heidolph). The temperature during this process was maintained at 40 °C. Further evaporation of organic solvents was accomplished in a hood at room temperature. Materials obtained in this manner were combined together to give crude fraction.

In vitro bioassays with crude fractions

In vitro bioassays using crude fractions were performed according to the methodology adopted by Akbar et al. (2014). Tender leaflets from 25 days old plants *(C. album)* were used for this purpose. Small leaf discs (1 cm diameter) were made using a cork borer. Then, the upper surface of these small leaf sections was scratched/punctured by needles and put on microscope slides (5 leaf sections/slide). These glass slides were positioned on Petri dishes having filter paper moistened with 2 mL of water. This whole arrangement was made to provide humid conditions for leaf sections, in order to avoid their drying up.

The crude chloroform fraction (4 mg) was melted in a small quantity of dimethylsulfoxide (DMSO) (100 µL). The final volume was taken to 1.0 mL by adding dH_2O. This stock solution was serially diluted with dH_2O to have lower concentrations (2, 1, ..., 0.0625 mg mL^{-1}). Fifteen µL of each concentration was dropped on each punctured leaf section area. Ten leaf sections of *C. album* weed were used for assessing the bioactivity of fungal metabolites in each concentration. Both positive and negative controls were included for comparisons. Positive control, 100 µL DMSO was dissolved in dH_2O to prepare a 1.0 mL mixture, which was later diluted to prepare corresponding lower concentrations by adding dH_2O. In order to compare the effect of DMSO, another treatment was made, with dH_2O as negative control. These Petri plates were kept under continuous light in a growth chamber at 25 °C and 50% humidity. Symptoms of detectable (visible to naked eye) necrotic spots on leaf discs were observed at regular intervals, recorded after 72 hours. The following parameter was used for comparisons.

Necrotic spot scale (millimeter)

0 = Necrotic spot not detectable

1 = Necrotic spot diameter ≤ 1

2 = Necrotic spot diameter ≤ 2 > 1

3 = Necrotic spot diameter ≤ 3 > 2

4 = Necrotic spot diameter ≤ 4 > 3

Isolation of herbicidal compound

The thin layer chromatography (TLC) of crude fractions was carried out. TLC chromatogram revealed the presence of 3 components in the mixture. These components were partitioned with the help of Preparative Thin Layer Chromatography (PTLC) and Reversed Phase High Pressure Liquid Chromatography (RPHPLC). The solvent system in both TLC and PTLC was Isopropanol (Merck): ethyl acetate (Merck), 81:19 ratio. The final elution was made with methanol (Merck) in case of PTLC. R_f values for the three separated components were: (compound 1, R_f 0.19), (compound 2, R_f 0.265) and (compound 3, R_f 0.4). These fractions were observed in a dark room cabinet equipped with short wave and long wave UV light.

These compounds were finally purified with Reversed Phase High Pressure Liquid Chromatography. (Lambda-Max, Model 481, LC Spectro-photometer) Acetonitrile and dH_2O (both with 0.1% formic acid added) were used for the final elution. Gradient elution was used with an eluting system of acetonitrile and dH_2O as (10:90 as initial ratio). The ratio of acetonitrile to water was increased (100:0 as final ratio). The fractions were evaporated to dryness with a continuous clean airflow in a hood at 25 °C.

Phytotoxicity bioassay measurements with purified compounds

The bioactivity of purified compounds was determined by adopting the same procedure as of the one for crude chloroform fractions, except for the fact that in these experiments a lower concentration of purified compounds was employed. Two mg mL^{-1} solutions of the 3 purified compounds were made by dissolving 2 mg of compound in 50 µL of DMSO. The final volume was taken to 1.0 mL by adding dH_2O. Lower concentrations of 1, 0.5,..., 0.03125 mg mL^{-1} were made by adding dH_2O. Positive control received DMSO in different corresponding concentrations. Moreover, another control sample having various concentrations of 2, 4- dichlorophenoxyacetic acid, corresponding to concentrations of pure compounds, was included to compare the bioactivity of pure isolated compounds from the test fungus. Another treatment with distilled autoclaved water was included in these experiments as negative control. The appearance of symptoms (detectable necrotic spot on leaf discs) were observed every 9 hours and finally recorded after 72 hours. Temperature and humidity conditions were the same as the ones of crude fraction.

Spectroscopic analyses

Electron Spray Ionization Mass Spectrometry (ESIMS) spectra were recorded on a Mariner ™ Biospectrometry Work station by Perseptive Biosystems.

RESULTS AND DISCUSSION

Phytotoxicity measurements with crude toxins

A positive reaction showing necrotic spots was recorded on punctured *C. album* leaf sections. On punctured leaf sections, chloroform fraction exhibited prominent necrotic spots. A 4.0 mg mL^{-1} and 2.0 mg mL^{-1} concentration of crude chloroform fraction produced necrotic spots. No observable effect was noticed with lower concentrations. Leaf sections that received DMSO (positive control) and dH_2O (negative control) remained unaffected with no detectable necrotic spots on their surface.

Phytotoxicity measurements with purified toxin

In these bioassays, three purified chromatographic fractions/compounds viz. A, B and C with as chloroform fraction of culture filtrate of *D. rostrata* were isolated and tested for their necrogenic activity. Among them, compound B was found effective in producing necrotic spots on punctured leaf discs of *C. album*. Compound/fraction B was found active in the minimum concentration of 1.0 mg mL^{-1}. In case of experiments conducted with 2,4-D, biological activity was recorded at the concentration of 0.25 mg mL^{-1}. However, this synthetic herbicide was found inactive below this concentration.

Mass Spectrometry of the isolated purified toxin

Mass Spectrometry of the chromatographic fraction B was carried out, since the other two compounds were found inactive in current bioassays. The chromatographic fraction B was isolated from the white colored compound coming from the chloroform extraction. Its Molecular weight was observed by (Electron Spray Ionization Mass Spectrometry) ESIMS, showing the following peaks:

ESIMS (+) *m/z*: 401 $[M + H]^+$, 801 $[2M + H]^+$, 423 $[M + Na]^+$, 439 $[M + K]^+$.

Molecular formula = $C_{25} H_{36} O_4$

Molecular weight = 400.5501

mp = 183-184.5 °C

$[á]_D^{28}$ +265.5° (*c* 1.0, $CHCl_3$)

^{1}H NMR spectra were comparable with already reported data (Evidente at al., 2006b).

Based on the above spectroscopic data and compared to previously published literature, chromatographic fraction B was identified as Ophiobolin A. Figure 1. In previous studies, some phytotoxic compounds were isolated and characterized from many species of *Drechslera*. Extracts and mycelia of *D. maydis* are known for generating phytotoxins; Drechslerol-A, Drechslerol-B and Drechslerol-C. Drechslerol-A caused necrotic spots on leaf sections of wild species of ginger at 1.6 x 10^{-4} M concentration. Drechslerol-C caused chlorosis and necrotic spots on the leaves of *Costus speciosus* at concentrations from 2.85 x 10^{-5} to 2.28 x 10^{-4} M (Shukla et al., 1987, 1989, 1990). Evidente et al. (2006a, b) isolated and identified many herbicidal compounds from different species of genus *Drechslera*. As an example, the herbicidal compound drazepinone, was isolated from culture extracts of *Drechslera siccans* (Evidente et al., 2005). Curvulin and O-methylcurvulinic acid phytotoxic compounds were isolated from *Drechslera indica*. These toxins caused necrosis on purslane and spiny amaranth (Kenfield et al., 1989). In a recent investigation, chenopodolan B isolated from the fungal species *Phoma chenopodiicola* showed strong herbicidal activity when

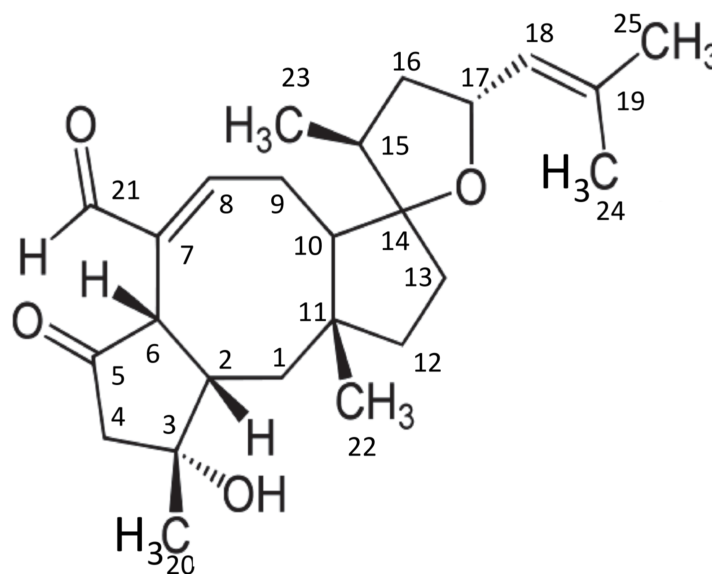

Figure 1 - Structure of Ophiobolin A.

tested on punctured leaves of *C. album* (Cimmino et al., 2013b). Similarly, Phomentrioloxin is a phytotoxic compound produced by *Phomopsis* sp., a potential mycoherbicide for the control of *Chenopodium album* weed (Cimmino et al., 2013a). Akbar et al. (2014) isolated and characterized an herbicidal compound, holadysenterine, from metabolites of *D. australiensis*. This compound caused necrosis and discoloration on leaf discs of *Rumex dentatus*. During this investigation, a natural herbicidal compound was isolated; it caused necrosis on the leaf surface of *C. album*. These results are consistent with the fact that plant pathogenic fungi have natural toxins that act as herbicides. This study concludes that compound isolated from *D. rostrata* have phytotoxic/ herbicidal properties that can be exploited as natural herbicides.

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