

SUPPLEMENTARY MATERIAL

Herbicidal activity of pure compound isolated from rhizosphere inhabiting *Aspergillus flavus*

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Abstract

In the quest for bioactive natural products of fungal origin *Aspergillus flavus* was isolated from rhizosphere of *Mentha piperita* using Potato Dextrose Agar (PDA) and Czapek Yeast Broth (CYB) nutrient media for metabolites production. In total three different metabolites were purified using HPLC/LCMS and the structures were established using 500 Varian NMR experiments. Further the isolated metabolites in different concentrations (10, 100, 1000 µg/mL) were tested for herbicidal activity using Completely Randomized design (CRD) against the seeds of *Silybum marianum* and *Avena fatua* which are major threats to wheat crop in Pakistan. Among the isolated metabolites one compound was found active against the test weed species whose activity is reported in the present work. The chemical name of the compound is 2-(1, 4-dihydroxybutan-2-yl)-1, 3-dihydroxy-6, 8-dimethoxyanthracene-9, 10(4aH, 9aH)-dione with mass of 388. Results showed that all seeds were germinated in control treatment; however, with the metabolite treated, the growth was retarded to different levels in all parts of the weeds. At a dose of 1000 µg/mL of the pure compound, 100% seeds of *S. marianum* and 60% seeds of *A. fatua* were inhibited. Interestingly the pure compound exhibited less inhibition of 10% towards the seeds of common wheat (*Triticum aestivum*).

Keywords

Aspergillus, herbicidal activities, *Silybum marianum*, *Avena fatua*, *Triticum aestivum*, LCMS, NMR

Experimental

Isolation of fungal strains

Samples were taken from rhizosphere of *Mentha piperita* and inoculated on sterile Potato Dextrose Agar (PDA) medium Acumedia[®] prepared according to the manufacturer's instructions. After inoculation petriplate were incubated for ten (10) days at 28 °C (Onyegeme-Okerenta et al. 2009). After completion of incubation period growth of different fungal strains was observed which were sub-cultured on fresh medium and further purification was performed.

Screening of bioactive fungal strains

Preliminary antibacterial testing of the fungal strains were performed against pathogenic bacteria. Only those fungal strains which showed considerable antibacterial activity were further processed for bioassay screening and for production of bioactive secondary metabolites.

Fungal strains identification

Slide culture technique was utilized for identification of fungi. Purified fungal strains were studied under light microscope (100-200 magnification) for identification. Different characteristics like structure of fruiting body and hyphae, arrangement of spores and pigmentation of the colonies were studied by growing the isolates on both PDA and Czapek Dox Agar (CDA).

Culturing of fungal strains for Production of secondary metabolites

Czapek yeast broth (CYB) medium, containing 1% Peptone, 0.05% KCl, 1% Glucose, 0.05% MgSO₄·7H₂O, and 0.001% FeSO₄·7H₂O; pH 7.3±0.2 was prepared and autoclaved at 128 °C. After autoclaving fungal strains were inoculated in separate flasks containing the media and the flasks were then transferred to shaking incubator for 14 days at 28 °C and 150 rpm.

Extraction of fungal metabolites from broth medium

After completion of incubation period, 250 µl of 40% HCl was added each flask which helps separating the media components. Using electrical blender fungal mycelia were grinded and equal volume of ethyl acetate was added to each flask. After mixing for 40 minutes the mycelia were filtered using cheese cloth. The mixture was transferred to separating funnel to recover the organic layer containing metabolites. In order to remove the impurities the mixture was washed with 2M brine solution. Anhydrous sodium sulphate (Na₂SO₄) was used to dehydrate the organic layer which was again filtered. The recovered organic layer containing the crude metabolites were subjected to rotary evaporator at 45 °C in order to concentrate it.

Isolation of fungal metabolites using column chromatography techniques

For isolation of pure compounds small amount of fungal crude extract was dissolved in an appropriate solvent and allowed it for 10 minutes for complete solubility. Using Morton and piston a slurry of the sample was prepared in small amount of silica gel of 60 mesh size. The sample was mixed thoroughly till the evaporation of solvent and complete dryness of the sample. The glass column was thoroughly washed with water and then with 75% ethanol and allowed to dry. A bed of silica was formed by soaking the silica in 100% *n*-Hexane solution and then by carefully pouring it into the glass column. The silica was allowed to settle at the bottom of column and solvent was collected in a beaker at the bottom of the column. The sample was then carefully poured with the help of a glass funnel on to the silica bed inside the column. After passing 100% *n*-Hexane the polarity of the solvent system was gradually increased by adding ethyl acetate while TLC was regularly performed to check for isolated compounds. Whenever traces of compounds were observed on TLC the polarity of the solvent system was increased in fractions to increase the chances for isolation of pure compounds (Roge et al. 2011; Dane Ganesh et al. 2013).

Mass spectrometry (MS)

i) ESI mass spectra

An Electrospray Ionization (ESI) mass spectrum was measured from an API-150EX mass spectrometer (Applied Biosystems) with a turbo ion spray source.

ii) HR-FTICR-MS spectra

The high resolution positive ion ESI mass spectra were obtained from a Bruker Apex III 70e Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with a 7.0 T superconducting magnet, an RF-only hexapole ion guide and an external electrospray ion source. The sample solutions were introduced continuously via a syringe pump with a flow rate of 120 μ l/h.

Nuclear magnetic resonance (NMR) spectroscopy

1D (^1H , ^{13}C) and 2D (HSQC, HMBC, COSY) NMR spectra were obtained using 500 MHz Varian NMR machine. Chemical shifts in ppm were referenced to the internal tetra methyl cyclane (TMS). The unity of 500 MHz stands 500 for ^1H and 125 for ^{13}C NMR.

Herbicidal activity

The pure compound was screened for herbicidal potential against two common weed species of wheat crop i.e. *Silybum marianum* and *Avena fatua*. This experiment was conducted in Petri plates using Completely Randomized Design (CRD). For this experiment autoclaved Petri plates were used and two folds of a sterile tissue paper were carefully placed in each Petri plate. Seeds of *S. marianum* and *A. fatua* were surface sterilized with 0.1 % solution of mercuric chloride and 10 seeds were carefully placed in each petridish on the tissue paper keeping appropriate distance. Different dose concentrations (10, 100, 1000 µg/ mL) of the compound were prepared and applied to petridishes containing the seeds with the help of a sterile dropper. As per requirement water was provided to seeds each day with the help of a sterile dropper. This experiment was performed in triplicate and a control treatment was used for each set of experiment. In order to test the selective toxicity of the pure compound same method was repeated against the seeds of *T. aestivum*. The seed germination data was recorded on daily basis and the whole experimental setup continued for 18 days. The data was recorded on the parameters such as percentage of seed germination, shoot length (cm), root length (cm), shoot weight (g) and root weight (g) (Hassan et al. 2004; Saeed et al. 2013). The results were analyzed using SPSS software and significant differences and means were calculated by least significant difference (LSD). The values are represented as mean ± standard deviation.

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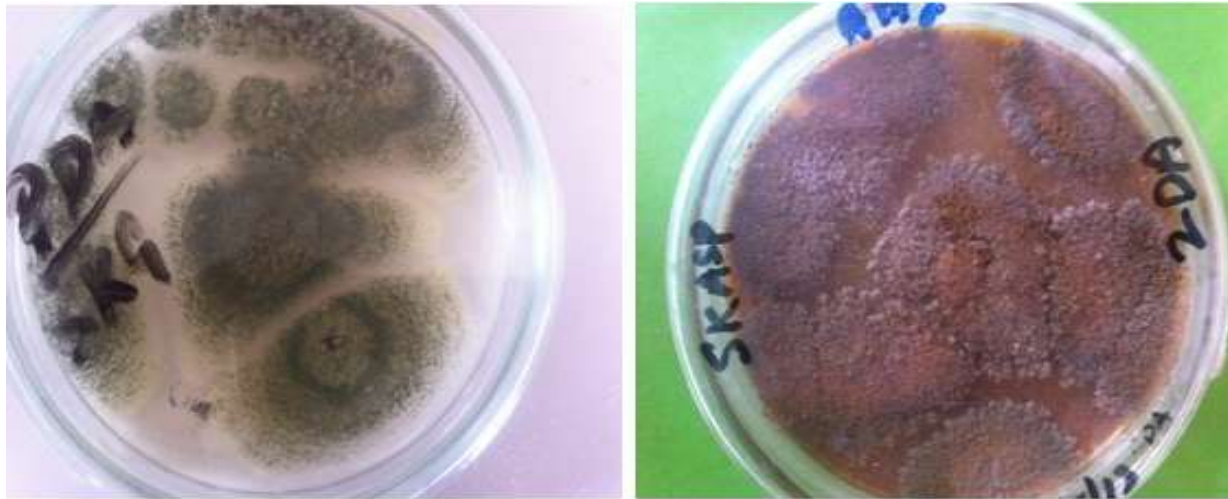


Figure S1. *Aspergillus flavus* growing on PDA medium (left) and on CDA medium (right)



Figure S2. Broth culture of *Aspergillus flavus* used in the study

Table S1. Chemical Shifts of the isolated Compound. Data Obtained from 500 MHz Varian NMR

Carbon No.	Multiplicity (DEPT)	¹³ C-NMR	¹ H (<i>J</i> = Hz)
1	-C-	162.16	-----
2	-C-	124.34	-----
3	-C-	163.00	-----
4	CH	107.78	7.14s
5	CH	105.45	6.99d (<i>J</i> = 10.0Hz)2.4
6	-C-	166.60	-----
7	CH	105.35	6.22d (<i>J</i> = 10.0Hz)
8	-C-	161.80	-----
9	-C-	186.10	-----
10	-C-	182.85	-----
11	-C-	135.56	-----
12	-C-	115.90	-----
13	-C-	109.45	-----
14	-C-	132.05	-----
15	-O-CH ₃	56.53	3.99 s
16	-O-CH ₃	56.53	3.79 s
17	CH ₂	62.78	3.78 dd (<i>J</i> = 5.0, 10.0Hz) 3.66 dd (<i>J</i> = 5.0, 10.0 Hz)
18	CH	34.30	3.50dt (<i>J</i> = 5.0, 10.0Hz)
19	CH ₂	30.65	1.45dd (<i>J</i> = 5.0, 10.0Hz)
20	CH ₂	60.50	3.40dd (<i>J</i> = 5.0, 10.0Hz)

Table S2. Herbicidal activity (Mean \pm SD) of the compound against *S. marianum*

Treatments	Percent Germination	Shoot length (cm)	Root length (cm)	Shoot weight (g)	Root weight (g)
Control	100 \pm 0.0	5.07 \pm 1.2	9.35 \pm 1.8	0.0232 \pm 0.01	0.05 \pm 0.04
10 μg /mL	80 \pm 5.0	2.8 \pm 0.8	4.75 \pm 1.1	0.0084 \pm 0.01	0.023 \pm 0.03
100 μg /mL	30 \pm 8.0	0.0	0.0	0.0	0.0
1000 μg /mL	0.0	0.0	0.0	0.0	0.0

Table S3. Herbicidal activity (Mean \pm SD) of the compound against *A. fatua*

Treatments	Percent Germination	Shoot length (cm)	Root length (cm)	Shoot weight (g)	Root weight (g)
Control	100 \pm 0.0	13.94 \pm 0.0	4.45 \pm 2.0	0.0718 \pm 0.01	0.0158 \pm 0.002
10 μg /mL	90 \pm 8.0	5.65 \pm 1.0	5.17 \pm 1.5	0.0368 \pm 0.004	0.01 \pm 0.001
100 μg /mL	80 \pm 6.0	2.0 \pm 0.7	3.11 \pm 1.0	0.0245 \pm 0.001	0.009
1000 μg /mL	40 \pm 3.0	0.0	0.0	0.0	0.0

Table S4. Herbicidal activity (Mean \pm SD) of the compound against *T. aestivum*

Treatments	Percent Germination	Shoot length (cm)	Root length (cm)	Shoot weight (g)	Root weight (g)
Control	100 \pm 0.0	15.14 \pm 2.0	4.12 \pm 0.7	0.0837 \pm 0.01	0.0189 \pm 0.002
10 μg /mL	100 \pm 0.0	15.08 \pm 3.0	3.96 \pm 0.9	0.0868 \pm 0.2	0.0192 \pm 0.003
100 μg /mL	100 \pm 0.0	14.45 \pm 2.5	3.59 \pm 1.0	0.0800 \pm 0.1	0.0164 \pm 0.001
1000 μg /mL	80 \pm 0.9	12.12 \pm 3.0	3.05 \pm 0.5	0.0604 \pm 0.1	0.0126 \pm 0.001