

SUPPLEMENTARY MATERIAL

Antifungal activity of compounds isolated from *Aspergillus niger* and their molecular docking studies with tomatinase

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Using a dual culture antagonism assay, *Aspergillus niger* exhibited 51.5 ± 1.1 % growth inhibition against *Fusarium oxysporum* f.sp. *lycopersici*, the wilt pathogen of tomato. For enhanced production of antifungal metabolites, nutrient optimization was performed and *in vitro* well-diffusion antifungal assays demonstrated that crude extract obtained from GPYB culture showed a maximum zone of inhibition (8.8 ± 0.4 mm) against the wilt pathogen, which is corroborated by the comparative LCMS profiles of the extracts from all three media *i.e.* GPYB, YEB and PDB. Two known compounds, Asperazine (m/z 665 $[M+H]^+$) and Nigerone (m/z 571 $[M+H]^+$), were isolated from *A. niger* and their antifungal activity is reported here for the first time. In MIC experiments, Asperazine and Nigerone inhibited the pathogen at 60 and 80 $\mu\text{g}\cdot\text{mL}^{-1}$ respectively. Molecular docking studies of Nigerone and Asperazine with *F. oxysporum* tomatinase showed five and six binding interactions respectively.

Keywords: dual culture assay; growth inhibition; LCMS; antifungal docking; fungicides

Experimental

Culturing of fungal species

Fusarium oxysporum f.sp. *lycopersici* (Wilt causing pathogen) was isolated from infected parts of tomato plant as described by Singha et al. (2011). Soil serial dilution (Warcup 1950) was used to isolate *Aspergillus niger* at the Laboratory of Natural Product Chemistry, Department of Agricultural Chemistry, The University of Agriculture, Peshawar, Pakistan. Fungal culture was identified from its morphological characteristics and examining the colonies under microscope (Siameto et al. 2010).

Dual culture assay

Dual culture antagonism assays were used to test inhibition potential of *Aspergillus niger* against the wilt pathogen (Mishra 2010). Mycelial plug (5 mm) of *Fo* f.sp. *lycopersici*. were placed at the periphery of culture plates and incubated for 2 days at 27 °C. After two days the plates were doubly-inoculated with mycelial plugs (5 mm) of *A. niger*. The plates were

incubated for an additional 7 days at 27 °C. Growth pattern of both fungi; in dual culture and control were recorded. Percentage inhibition of radial growth was calculated as

$$\% \text{ Inhibition} = \frac{R1 - R2}{R1} \times 100$$

where R1 = radial growth of the pathogen in control and R2 = radial growth of the pathogen in dual culture with antagonist.

Nutrient optimization

Optimization of nutrients was carried out for enhancing metabolite production and antifungal activity of *A. niger* (VanderMolen et al. 2013). Three different media were tested: Potato Dextrose Broth (PDB); Glucose Peptone Yeast Broth (GPYB) and Yeast Extract Broth (YEB). Fungal crude extraction was carried out as described by Joel and Bhimba (2013). The whole cell culture of *A. niger* (1 L, 6-10 days) was filtered and mycelia was homogenized in 0.3 L solution of ethyl acetate (EtOAc) to extract overnight. The extract was dried over anhydrous MgSO₄, then filtered and the organic filtrate was concentrated *in vacuo* to collect 300 mg of crude material.

In vitro antifungal activity

Spore suspension (1x10⁸ spores·mL⁻¹) of the pathogenic fungi from a ten day old culture was prepared into distilled water using Haemocytometer. Stock solutions of the crude extract and standard (Mancozeb) were prepared by dissolving 5 mg·mL⁻¹ in methanol. *In vitro* antifungal assay of crude extracts was carried out using the well diffusion method as previously described (Sonawane et al. 2015). A sterilized cork borer was used to prepare wells (5 mm) and 20 µL of the crude extract and Mancozeb (5 mg·mL⁻¹ in methanol) were applied to the wells. The plates were incubated at 27 °C for 7 days and inhibition zones were measured in millimeters (mm).

In vivo pot experiment

In vivo antifungal activity of crude extract obtained from *Aspergillus niger* was carried out in a screen-house as described (Lee et al. 2001). After 10 days of cultivation, 5 mL of the treatment suspension was applied using hypodermic syringe to tomato plants as *A. niger*

Extract (AE), *Fusarium* (Fus), *A. niger* Extract (AE) + *Fusarium* (Fus), Mancozeb + *Fusarium* (Man+Fus), *A. niger* Extract (Sprayed) + *Fusarium* (AEsp + Fus) and Mancozeb (Sprayed) + *Fusarium* (Mansp + Fus). Disease severity scale (Grattidge and O'Brien, 1982) was given to the wilted and the non-wilted plants as; 0 = no symptom, 1 = slight yellowing or wilting, 2 = moderate yellowing or wilting, 3 = complete wilting and 4 for 100% leaves yellowed and wilted.

Identification and purification of antifungal compounds

Analytical LCMS

Samples were analysed on a Waters HPLC system equipped with Waters 2998 photodiode array detector between 200 and 600 nm and Waters 2424 evaporative light scattering detector (ELSD) coupled to a Waters SQD-2 mass detector. Samples were prepared as 1 mg·mL⁻¹ of extract in LCMS grade CH₃CN and then centrifuged for removal of solid particles prior to injection. 20 µL of this solution was injected through Waters 2767 autosampler to LCMS equipped with Phenomenex Kinetex Column (2.6 µm, C₁₈, 100 Å, 4.6 × 100 mm).

Preparative LCMS

Preparative LCMS was carried out for identification and isolation of compounds using a Waters 2767 auto sampler and auto purification system eluted at 20 mL·min⁻¹ at ambient temperature. HPLC grade H₂O and CH₃CN + 0.05% formic acid were used as Solvent A and Solvent B respectively. Waters photodiode array detector (PDA) was used to detect the peaks. Waters SQD-2 operating in both positive and negative modes was used to measure the mass of compounds. Detected peaks (purified compounds) were collected into glass tubes and were tested against *Fusarium oxysporum* f.sp. *lycopersici* in 96 well plates.

Nuclear magnetic resonance (NMR) spectroscopy

NMR experiments were recorded on a Bruker Ascend NMR-400 and UltraShield 500 spectrometers at 25 °C. Purified compounds with potent antifungal activity were dissolved in deuterated Methanol (CD₃OD) and structures were confirmed using NMR spectroscopy.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of the purified compounds was determined using 96 well plates. Eight distinct concentrations (10, 20, 40, 60, 80, 100, 120 and 140 $\mu\text{g}\cdot\text{mL}^{-1}$) were prepared in methanol and applied in 96 well plates with spores suspension of *Fusarium oxysporum* f.sp. *lycopersici* (Fol). The plate was incubated at 27 °C for 4 days.

Computational studies

The protein sequence of the enzyme Tomatinase with accession number Q8TGC1_FUSOX was acquired from UniProt in FASTA format. The x-ray crystallographic (3D) structure of this protein was not available, therefore computer based 3D structure determination was achieved through homology modelling using MOE 2014 software. The 3D structure was produced using MOE 2014 software. A template (Accession No. P55330) was selected from the Protein Data Bank (PDB). Both the sequences, target sequence and the template were superimposed to build the homology model. Refinement and validation of the 3D structure was performed. The active sites were predicted for the tomatinase using site finder tool of MOE. It is an energy based method for the prediction of protein-ligand binding sites.

MOE (Molecular Operating Environment) was used to dock compounds in crystal structure of the targeted protein. Before docking, hydrogen atoms were added and charges were corrected. For every inhibitor multiple conformations were produced through selection of torsion angles to all rotatable bonds. A total of thirty conformations were generated for each inhibitor and to accept the conformations London dock scoring function was used which explicate the free energy of binding (Kumar et al. 2012).

The MOE software Generalized-Born volume integral/Weighted surface area (GBVI/WSA) method was utilized for finding binding affinities for each ligand docking. Its energy associated with non-bonded interactions like columbic electrostatic interaction, Van der Waal and implicit solvent interactions. For each hit binding energy was calculated and reported in $\text{kcal}\cdot\text{mol}^{-1}$ (Labute 2008).

Data analysis

In vitro dual culture and well diffusion assays were statistically analysed using Microsoft Excel 2013 for calculating the mean and standard deviation.

Table S1. Mean zone of inhibition (Mean \pm SD) of three different media against *Fusarium oxysporum* (Fol)

Media	Zone of Inhibition (mm)	Positive Control (Mancozeb)	Negative Control (Methanol Only)
PDB	0.0 \pm 0.0	11.7 \pm 0.6	0.0 \pm 0.0
YEB	5.0 \pm 0.2		
GPYB	8.8 \pm 0.4		

Table S2. Comparison of ^1H and ^{13}C NMR of Asperazine

Position	Assigned in Literature		Current Assignment	
	δ_{C}	δ_{H} m (J in Hz)	δ_{C}	δ_{H} m (J in Hz)
1 (NH)		6.14 br s		6.05 br s
2	84.20	5.71 d (1)	83.06	5.69 d (0.9)
3	59.28		57.06	
4	134.16		132.01	
5	121.48	6.82 dd (7, 1)	122.86	6.91 dd (7, 1)
6	120.93	6.66 dd (7, 1)	119.25	6.51 m
7	130.45	7.12 m	128.71	7.00 m
8	112.41	6.86 d (7.5)	110.21	6.67 d (8.0)
9	148.57		147.13	
11	57.79	3.32 dd (7.5, 9.5)	56.71	3.07 dd (8.0, 9.5)
12	39.55	3.25 dd (14, 7.5) 2.40 dd (14, 9.5)	38.31	2.90 dd (13.9, 5.0)
13	169.98		169.93	
14 (NH)		6.18 br s		6.05 br s
15	57.14	3.59 ddd (4.5, 5, 5.5)	55.21	2.96 m
16	168.89		168.16	
17	40.40	3.01 dd (13.5, 5.5) 2.95 dd (13.5, 5)	38.22	2.83 dd (13.9, 4.7)
18	137.72		135.05	
19	131.46	7.12 m	129.65	7.00 m
20	130.09	7.12 m	129.85	7.00 m
21	128.98	7.20 m	127.10	7.13 m
22	130.09	7.12 m	129.85	7.00 m
23	131.46	7.12 m	129.65	7.00 m
24	126.31		124.63	
25	120.24	7.12 m	118.74	7.00 m
26	120.61	7.12 m	118.96	7.00 m
27	119.02	7.48 dd (7, 1)	118.34	7.48 dd (8.0, 0.9)
28	130.45		128.98	
29	135.45		133.71	
30 (NH)		8.61 br s		8.41 br s
31	125.20	6.92 d (2.5)	126.81	6.67 d (3.0)
32	111.15		108.51	
33	30.20	3.05 dd (3.5, 14.5) 3.09 dd (5.5, 14.5)	28.74	2.73 dd (13.9, 4.7)
34	56.36	3.51 ddd (3.5, 5.5, 6)	54.59	2.90 dd (13.9, 5.0)
35 (NH)		6.16 br s		6.05 br s
36	169.64		167.61	
37	60.46	4.12 ddd (5, 6, 4.5)	58.53	4.15 t (4.5, 0.7)
38 (NH)		6.49 d (4.5)		6.51 d (5.0)
39	168.84		169.12	
40	40.72	3.06 dd (14, 6) 2.93 dd (14, 5)	39.02	2.90 dd (13.9, 5.0)
41	137.72		135.05	
42	131.63	7.12 m	128.02	7.00 m
43	130.15	7.12 m	127.69	7.00 m
44	128.72	7.27 m	127.44	7.13 m
45	130.15	7.12 m	127.69	7.00 m
46	131.63	7.12 m	128.02	7.00 m

Table S3. Comparison of ^1H and ^{13}C NMR of Nigerone

Position	Assigned in Literature		Current Assignment	
	δ_{C}	δ_{H} m (J in Hz)	δ_{C}	δ_{H} m (J in Hz)
2	167.6		169.2	
3	107.2	5.98 s	112.4	6.21 s
4	184.3		174.3	
5	162.0		161.2	
6	160.5		162.4	
7	96.9	6.24 d (1.80)	92.3	6.53 d (1.80)
8	161.8		160.0	
9	96.3	6.07 d (1.80)	93.7	6.27 d (1.80)
10	107.2		106.3	
11	151.2		160.0	
12	103.9		102.3	
13	108.2		111.2	
14	140.7		136.5	
2-Me	20.7	2.02 s	22.30	2.38 s
5-OH		15.22 br s		8.49 br s
6-MeO	55.6	3.67 s	54.4	3.83 s
8-MeO	56.3	4.09 s	55.0	3.86 s
2'	168.3		169.2	
3'	106.8	5.89 s	111.1	6.12 s
4'	184.5		174.1	
5'	163.4		162.3	
6'	161.7		162.8	
7'	96.8	6.50 d (1.80)	92.1	6.63 d (1.80)
8'	159.1		159.6	
9'	100.4	6.41 d (1.80)	93.7	6.35 d (1.80)
10'	104.6		103.3	
11'	151.1		159.5	
12'	103.9		102.3	
13'	108.2		110.6	
14'	140.8		136.5	
2'-Me	20.6	2.05 s	22.30	2.40 s
5'-OH		15.43 br s		8.49 br s
6'-MeO	55.2	3.51 s	54.2	3.71 s
8'-MeO	56.1	4.15 s	54.9	3.92 s

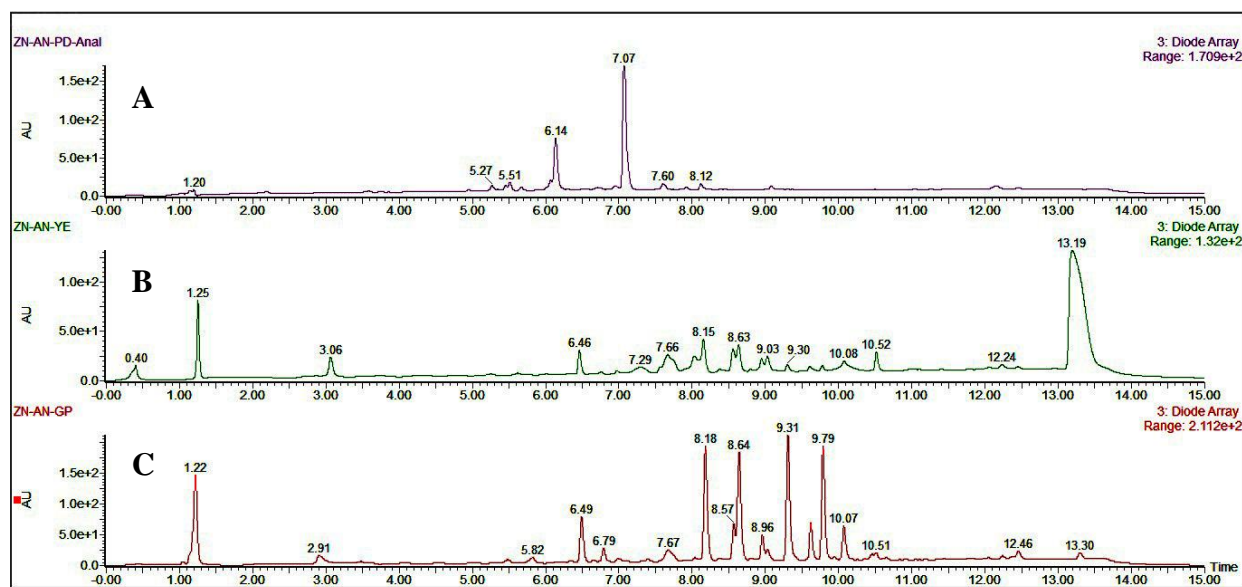


Figure S1. HPLC chromatogram (Diode array traces, 210-600 nm) of *Aspergillus niger* grown in three distinct media (A) PDB (B) YEB (C) GPYB.

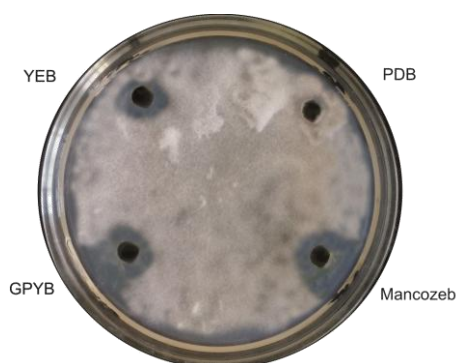


Figure S2. Well diffusion method for *in vitro* antifungal activity of extracts obtained from GPYB, YEB and PDB. Mancozeb as positive control.

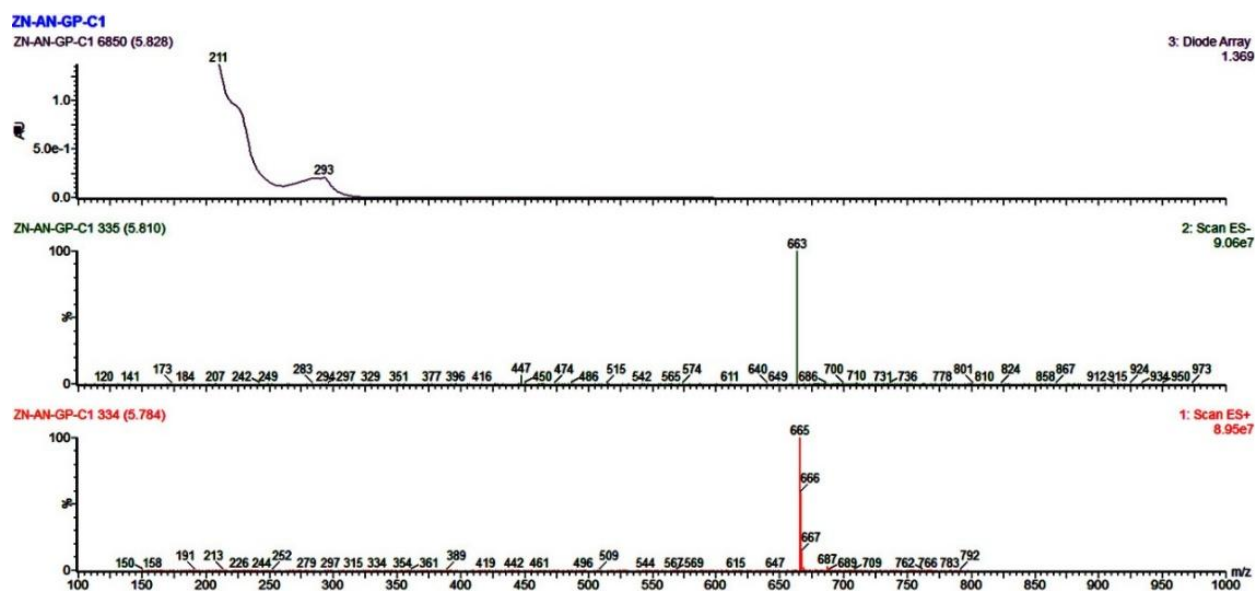


Figure S3. UV_{max}, ES (+) and ES (-) of the purified compound eluted at 6.5 min (t_R).

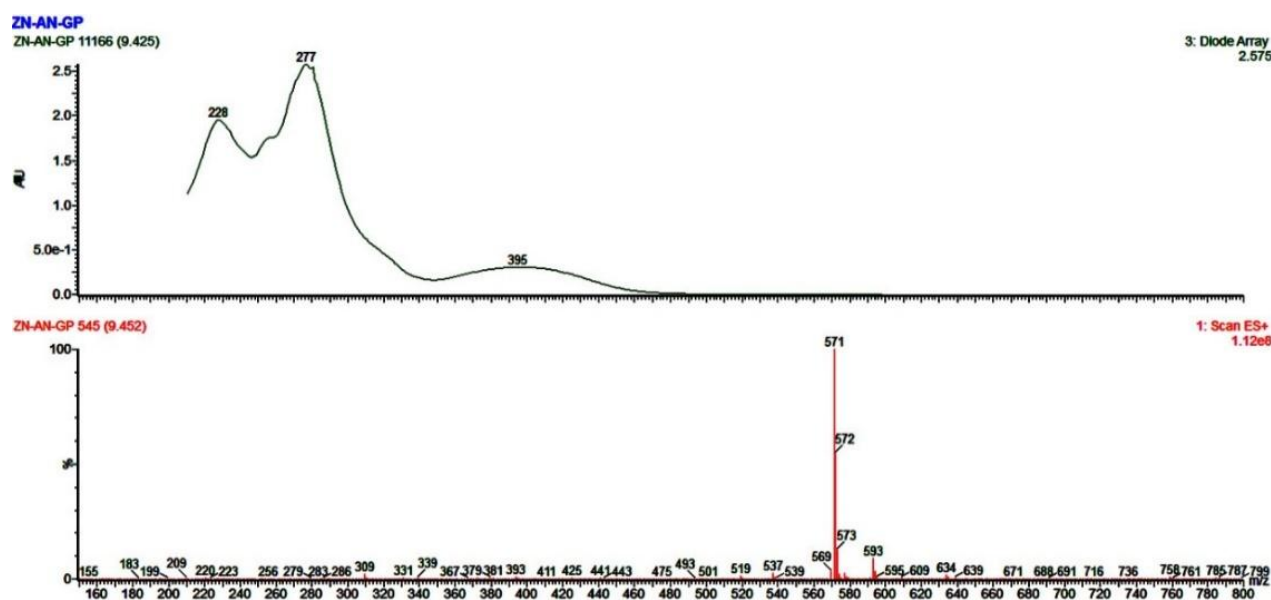


Figure S4. UV_{max} and ES (+) of the purified compound eluted at 9.3 min (t_R).

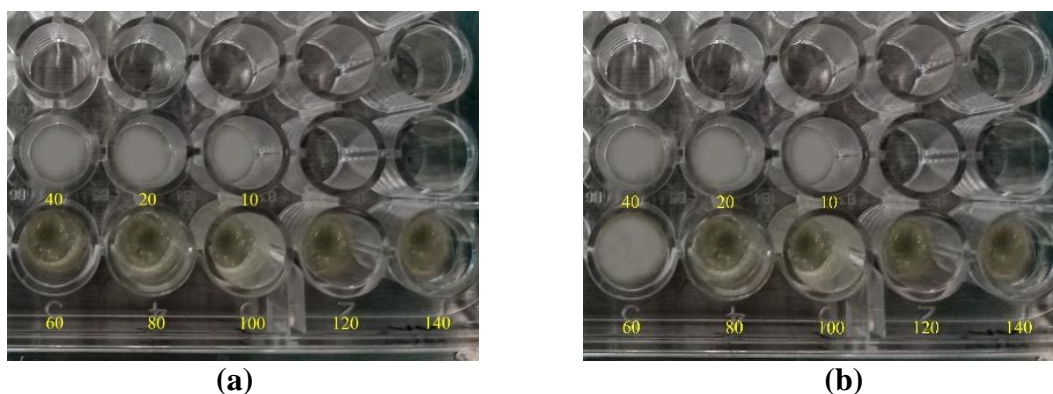


Figure S5. Different concentrations ($\mu\text{g}\cdot\text{mL}^{-1}$) of compounds isolated from *Aspergillus niger* (a) eluted at t_R 6.5 min (b) eluted at t_R 9.3 min in 96 well plate.

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