SHORT REPORT

A C G publications

Rec. Nat. Prod. 3:3 (2009) 161-164

records of natural products

Isolation and Biological Evaluation of Two Bioactive Metabolites from *Aspergillus gorakhpurensis*

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(Received May 1, 2009; Revised June 1, 2009; Accepted June 2, 2009)

Abstract: Fungi are known to produce a vast array of secondary metabolites that are gaining importance for their biotechnological applications. Screening of *Aspergillus gorakhpurensis* for the production of bioactive secondary metabolites results in the production of 4-(*N*-methyl-*N*-phenyl amino) butan-2-one and itaconic acid. The structure of the known compounds was established by ^{1}H-, ^{13}C-NMR and Mass spectral data. Biological evaluation of the two compounds against test microorganisms showed strong inhibitory activity of 4-(*N*-methyl-*N*-phenyl amino) butan-2-one towards bacteria and fungi. Only 4-(*N*-methyl-*N*-phenyl amino)-butan-2-one showed a marked significant activity (LD_{50} = 330.69 µg/mL) in *Spodoptera litura* larvicidal bioassay.

Keywords: *Aspergillus gorakhpurensis;*4-(*N*-methyl-*N*-phenyl amino) butan-2-one; Itaconic acid; Antibacterial; *Spodoptera litura.*

1. Fungal Source

Microbial natural products remain the most promising source of novel secondary metabolites. The impact of microbial biodiversity favours the chance of isolating new antibiotics. Identification of microorganisms that produce bioactive compounds is of great interest in the development of new molecules to fight against many pathogens. Fungi produce a wide range of secondary metabolites with high therapeutic value as antibiotics, cytotoxic substances, insecticides, compounds that promote or

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The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 06 /07/2009 EISSN: 1307-6167

inhibit growth, attractor, repellent etc., [1]. These metabolites are being exploited in different fields of medicine and industries [2]. Among fungi classes, Ascomycetes are reported to be active producers of antimicrobial compounds, which have high therapeutic values [3]. Within our screening program for antimicrobaial and larvicidal fungal secondary metabolites, we investigated an Ascomycetes fungi *Aspergillus gorakhpurensis* (MTCC 547) procured from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India, for chemical and biological studies. The fungus was cultivated for 7 days on potato dextrose broth medium and the culture was extracted with ethyl acetate.

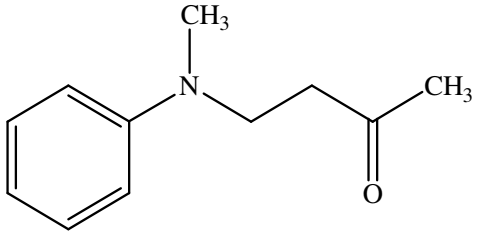
2. Previous Studies

There is no previous studies on the metabolites of this fungus.

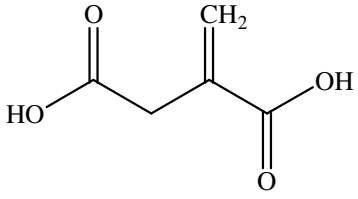
3. Present Study

In the present study *Aspergillus gorakhpurensis* MTCC 547 was procured from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh India and exploited for the production of secondary metabolites. A portion of mature agar slant was inoculated in one liter of potato dextrose broth in 2 liter Erlenmeyer flask and incubated at 27 ± 2 º C as resting cell suspension for 7 days. The fermented broth (8 L) was treated with ethyl acetate (V: V) and incubated overnight. The mixture (fermented broth and solvent) was shaken vigorously for 30 min and kept in stationary condition for another 30 min to separate the solvent from aqueous phase. The organic extract was separated, dried over anhydrous sodium sulfate and concentrated *in vacuo* to yield crude (2.1 g).

Activated silica gel (60–120 mesh) was packed on to a glass column (450 mm × 40 mm) using n-hexane solvent and 2.1 g of crude ethyl acetate extract was loaded on the top of silica gel column. The column was eluted with the mixture of hexane and ethyl acetate (8:2). Fractions that showed homogeneity on TLC plates were combined and concentrated together to give pure compounds. Fraction 1 (54 mg) and Fraction 2 (31 mg) were obtained. The pure fractions were subjected to Chemical characterization using Nuclear magnetic resonance spectroscopy (^{1}H- & ^{13}C-NMR) (Bruker UXNMR at 300MHz in $CDCl_3$) and Mass spectroscopy (Finnigan MAT 1020-B in $CDCl_3$). The metabolites were identified as -(*N*-methyl-*N*-phenyl amino) butan-2-one; $C_{11}H_{15}NO$; Mol.wt.177.0 **(1)** and Itaconic acid; $C_5H_6O_4$; Mol.wt.130.1 **(2)** (**Figure 1**).



4-(*N*-Methyl-*N*-phenyl amino) butan-2-one



Itaconic acid

Figure. 1 Chemical structures of -(*N*-methyl-*N*-phenyl amino) butan-2-one and Itaconic acid

4-(N-methyl-N-phenyl amino) butan-2-one ($C_{11}H_{15}NO$): ^{1}H NMR (300MHz, $CDCl_3$): δ : 2.13 (3H, s), 2.67 (2H, t, J=6.798), 2.91 (2H, s), 3.61 (2H, t, J=6.798), 6.60 (3H, m), 7.12 (2H, t, J=7.554). ^{13}C NMR (300MHz, $CDCl_3$): δ : 30.54, 38.90, 40.28, 47.56, 112.74, 117.05, 129.35, 148.40, 206.29.

Itaconic acid ($C_{13}H_{26}O$): ^{1}H NMR (300MHz, $CDCl_3$): δ: 3.21 (2H, s), 5.65 (1H, s), 6.23 (1H, s), 11.34 (2H, s). ^{13}C NMR (300MHz, $CDCl_3$): δ :36.87, 126.47, 134.47, 167.02, 171.58.

Bioactivity Tests

Antibacterial activity evaluated against Gram-positive organisms and Gram-negative bacteria by well diffusion method [4]. Solutions were prepared (50-150 µg/mL) by dissolving the test compounds in dimethyl sulfoxide (DMSO) and add to appropriate well. The petri dishes with treated and the control cups were incubated at 37°C for 24 h. The zones of inhibition diameters (mm) were measured. Triplicates performed for each treatment. Control experiment carried out with the pure solvent. Antifungal activity was carried out in similar manner using zone of inhibition method against eight fungal strains according to the method of Linday.

The minimum inhibitory concentration was determined according to the method described by Andrews [5]. Different concentrations (200-10 µg/mL) of isolated compounds and 100 µL of the bacterial suspension (10^{-5} CFU/mL) were placed aseptically in10 mL of nutrient broth separately and incubated for 24 h at 37 °C. The growth was observed both visually and by measuring O.D. at 600 nm. The lowest concentration of test sample showed no visible growth was recorded as the minimum inhibitory concentration. Duplicate sets of tubes were maintained for each concentration of test sample**.**

Larvicidal activity (measured as mortality after 24 h) of the compounds was determined by topical application to early fourth instars according to Laurin *et.al* [6]. Lethality was estimated by applying different concentrations (50 to 1000 µg/mL) of the metabolites. Two replicates of 10 larvae were tested per dose. A probit analysis was carried out to calculate LD_{50} and LD_{90} [7].

The antimicrobial activity of the isolated compounds against all the test organisms had shown in the Table. 4-(*N*-methyl-*N*-phenyl amino) butan-2-one showed strong antibacterial activity against gram-positive bacteria i.e. *Staphylococcus aureus*, *Staphylococcus epidermides* and gram-negative bacteria i.e. *Escherichia coli* with zone of inhibition between 15 to 18 mm at a concentration of 100 µg/mL. The MIC value of the 4-(*N*-methyl-*N*-phenyl amino) butan-2-one was 100 µg/mL against *Staphylococcus aureus* and *Staphylococcus epidermides*. 4-(*N*-methyl-*N*-phenyl amino) butan-2-one was tested for anti fungal activity against eight fungi and showed moderate activity against all fungi except *Candida albicans* (15 mm). Whereas Itaconic acid showed antibacterial and antifungal activity at very higher concentrations (150 µg/mL). 4-(*N*-methyl-*N*-phenyl amino) butan-2-one showed potent lethality against *Spodoptera litura* 4^{th} instar larvae (Table). The data was further subjected to probit analysis and the LC_{50} value calculated to be 330.69 µg/mL.

In conclusion, we have reported the isolation of two known compounds from *Aspergillus gorakhpurensis*. The antimicrobial and larvicidal activities of the isolated pure compounds were reported. The isolated compounds showed moderate antimicrobial and insecticidal activities. Microbial secondary metabolites represent a large source of compounds endowed with ingenious structures and potent biological activities. Many of the products currently used for human or animal therapy, in animal husbandry and in agriculture are produced by microbial fermentation, or derived from chemical modification of a microbial product [8]. The present study of screening bioactive secondary metabolites revealed that *Aspergillus gorakhpurensis* as a source for the production of two bioactive metabolites. These metabolites can be further exploited for the biotechnological applications in medicine and agriculture.

Table 1. Antibacterial, antifungal and larvicidal bioassay of 4-(*N*-methyl-*N*- phenyl amino) butan-2-one and Itaconic acid

	Zone of inhibition					MIC (µg/mL)		
	(1)		(2)		Control 30 µg	(1)	(2)	Control
Bacteria	**50 µg**	**100 µg**	**100 µg**	**150 µg**	**Penicillin-G**			**Nitrofurantoin**
Gram Positive Bacteria								
S. aureus MTCC 96	15	18	-	8	18	100	>200	50 µg/mL
S. epidermides MTCC 435	14	16	-	-	18	100	>200	50 µg/mL
B. subtilis MTCC 441	12	14	-	-	20	150	>200	100 µg/mL
B. sphericus MTCC 511	12	14	-	-	20	150	>200	100 µg/mL
Gram Negative Bacteria					Streptomycin			
E. coli MTCC 443	13	15	-	-	29	150	>200	50 µg/mL
P. aeruginosa MTCC 741	12	14	-	-	34	150	>200	75 µg/mL
P. oleovorans MTCC 617	12	14	-	-	30	150	>200	75 µg/mL
K. pneumoniae MTCC 39	12	14	-	-	30	150	>200	50 µg/mL
Fungi							Larvicidal assay	
Filamentous fungi					Clotrimazole		**(1)**	
A. niger MTCC 1344	10	12	-	-	22	LD_{50}	330.69 µg/mL	
A. parasiticus MTCC 411	10	12	-	-	22	LD_{90}	1132.62µg/mL	
R. oryzae MTCC 262	12	14	10	12	23		**(2)**	
C. cladosporides MTCC 2607	10	12	-	-	20	LD_{50}	>1000 µg/mL	
Unicellular fungi						LD_{90}	>1000 µg/mL	
C. albicans MTCC 227	13	15	8	10	18		Control (Pyrethrum)	
C. albicans MTCC 3018	13	15	-	8	18	LD_{50}	1.6 µg/mL	
S. cerevisiae MTCC 170	12	14	-	-	19	LD_{90}	3.0 µg/mL	
S. cerevisiae MTCC 171	12	14	-	-	19			

(**1**)= 4-(*N*-methyl-*N*-phenyl amino) butan-2-one; (**2**)= Itaconic acid. Zone of inhibition was calculated in mm.
LD_{50} = Lethal concentration (µg/mL) at which 50 % of the larvae showed mortality.
Negative control DMSO-No activity.

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