Bioactive isochromenone isolated from *Aspergillus fumigatus*, endophytic fungus from *Bacopa monnieri*

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Abstract

Fungal endophytes are a significant reservoir of novel bioactive secondary metabolites. Present communication describes isolation and structure determination of isochromenone, from endophytic microorganism *Aspergillus fumigatus* hosted in *Bacopa monnieri* plant. Further, its biological evaluation revealed it as antioxidant and antitubercular. The methanol extract of *A. fumigatus* inhibits the growth of the virulent strain of *Mycobacterium tuberculosis* H_{37}RV with minimum inhibitory concentration 500 µg/mL. This is the first report of isolation of isochromenone from *A. fumigatus*.

Introduction

Endophytes are microorganisms residing in the inter- and intra-cellular living tissues of host plant. These are harbored by the plants. Endophytes are mainly fungi, symbiotically benefit the host plant and adapt the microenvironment. These are considered as a good source of novel bioactive secondary metabolites for the development of pharmaceuticals and agrochemicals.[1] A single endophytic strain can produce multiple bioactives. Diverse natural products are produced by endophytes like antibiotics, anticancer, antioxidants, immunosuppressants and other biologically active substances and many of them have been isolated.[2,3] Brahmi (*Bacopa monnieri*) is an important medicinal herb used in Ayurveda system of medicine in India. The herb was described in Charaka Samhita, Susrutu Samhita and Athar-Ved to sharpen intellect and attenuate mental deficits. It is a perennial, creeping herb native to the wetlands of India. Other than India, it is also found in Europe, Asia, Australia, Africa and Americas. It grows well in marshy areas. Traditionally, this herb is mainly prescribed for neurological disorders like epilepsy. Its extract has shown potential cognitive effects.[4] Bacosides are the main bioactive compounds of Brahmi. Present communication describes isolation and identification of an endophytic fungus from *B. monnieri* plant. Isolation of a fungal metabolite *i.e.* 6-hydroxymellein is the first report from this fungus.[1]

Materials and Methods

General

General laboratory chemicals were procured from Thomas Baker, India. Deuterated solvents were procured from Sigma Aldrich USA. Thin layer chromatography was done on Merck aluminium sheet thin layer chromatography (TLC, UV_{254nm}) plates. NMR spectra were measured on a Bruker Avance 300MHz instrument. Tetramethylsilane (TMS) was used as an internal standard. Electron Impact ionization mass (EI-MS) were recorded on Perkin-Elmer Turbo Mass GC-MS system and ESI-High resolution mass was of compound 1 was done on Agilent 6520 Q-TOF. Biochemicals for bioevaluations were obtained from Sigma-Aldrich, India and Himedia Laboratories, India.

Isolation of endophytic fungus from *Bacopa monnieri*

The isolation was performed as per previously reported method.[5] The healthy *B. monnieri* plant (Brahmi) (Herbarium no. CIMAP-12597) leaves were obtained from Research Farm of CSIR-CIMAP, Lucknow, India. The leaves were properly washed in running tap water, double distilled water and cut into small discs (approximately 0.5 cm^2). Leaf segments were surface sterilized with ethanol (70%) for 5 seconds to eliminate epiphytic microbes. Samples were treated with 4% sodium hypochlorite (NaOCl) for 3-5 minutes, rinsed in sterile saline water for 1 minute (4 times) and inoculated (1 mL) in NB (Nutrient Broth) or TSB (Tryptic Soy Broth) and incubated overnight at 28°C in shaker for sterility check. Samples (5.0 gm) were ground in sterile mortar pestle in sterile saline water (5 mL). Dilutions were prepared to 10^{-6}. 100 µL of aliquot was spread plated on PDA (Potato Dextrose Agar), plates were sealed with parafilm and incubated at 28±1°C till growth in a BOD incubator. The hyphae growth was observed from the tissues on PDA plates, immediately these were transferred into fresh PDA plates

Molecular identification of the endophytic fungal strains

Fungal genomic DNA was isolated from fully grown 4 days old culture as per standard procedures.[6] The quality and quantity of isolated DNA was checked spectrophotometrically (Nanodrop ND 1000) and through agarose gel electrophoresis as well. The universal primers ITSI (5,-TCCGTAGGTGAACCTGCGG-3,) and ITS 4 (5, -TCCTCCGCTTATTGATATGC-3,) were used for amplification of the ITS gene from fungal strain. The fungal genomic DNA (25ng) and each primer (5 pmol) was used for amplification in a Thermocycler programmed as 95°C for 5 min; 32 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, 72°C for 10 min; 4°C for infinite period. The PCR product was purified with a PCR Cleanup Kit (Axygen, Union City, CA, USA) and directly sequenced using the forward universal primer and Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on a 3130×1 Genetic Analyzer (Applied Biosystems) as per manufacturer's protocol. Sequence analysis was carried out using the nucleotide BLAST (BLASTN) (NCBI).

Fungal culturing

The isolates were inoculated in an Erlenmeyer flask (500 mL) containing liquid medium (100 mL) and the cultures were incubated with constant shaking of 200 rpm at 28±1°C for 96-120 h.

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Extract preparation and isolation of pure metabolite

The isolates were harvested by filtration and the filtrates were centrifuged at 6000 rpm for 15 min, the supernatant as well as pellet were used for extract preparation. 40mL of supernatant was mixed with 4.0 g of diaion HP20 (Supelco, Bellefonte, PA, USA) and shacked on a shaker cabinet at 28±1°C for 30 min. The contents were packed in a plastic column. The resin was drained by applying gentle pressure using syringe plunger, washed with distilled water (15 mL), followed by elution with methanol (20 mL), and concentrated *in vacuo* at 40±1°C to get a residue. The residue (5.34 g) thus obtained was charged on a column (glass, 4×80 cm) using silica gel (60-120 mesh) and eluted successively with hexane (1.2 L), then increasing polarity of ethyl acetate 2%, 5%, 10%, and so on each with 1.6 L. The pure fractions 119-125 were mixed together and after evaporation yielded a pure gummy compound (27 mg) at 30% ethyl acetate-hexane. Its structure was established by spectroscopy (IR, ^{1}H NMR, ^{13}C NMR, DEPT etc. and mass) (Table 1 and Supplementary Material) and finally authenticated by X-ray crystallography.

X-ray crystallography of isochromenone 1

Isochromenone 1 ($C_{10}H_{10}O_4$, M=194.2) single crystals were obtained by slow evaporation from ethyl acetate. The compound crystallized in monoclinic space group $P2_1$ with two independent molecules in the asymmetric unit. The unit cell dimensions were as, a=6.4616 (5) Å, b=16.4391 (12) Å, c=8.3548 (6) Å, Z=4, V=887.34 (11) Å^3, D_{calc}=1.454 gm/cm^3, F(000)= 408. X-ray diffraction experiment was performed on a Bruker AXS SMART APEX CCD diffractometer using MoK radiation (λ=0.71073 Å), using combinations of ϕ and ω scans at room temperature (20°C). The structure was solved by direct methods using SHELXS and was refined against F^2 with full-matrix least squares method by using SHELXL.[7,8] Non-hydrogen atoms were refined anisotropically. Hydrogen atoms attached to the hydroxyl groups were located from the difference Fourier maps were refined isotropically. Rest of the hydrogen atoms were geometrically fixed and were refined as riding over the atoms to which they are bonded. 7249 reflections were measured (3930 independent reflections) with R_{int}=0.04. Friedel pairs were merged prior to structure refinement. The final R-value was 0.0388 (wR=0.0865) for 1748 observed reflections with [I>2sigI] and for 269 parameters. The goodness-of-fit was 1.069. The largest difference peak was 0.21eÅ^{-3} and the largest difference hole was −0.27eÅ^{-3}. Absolute configuration of the molecule was not determined in the present study. Crystallographic data (excluding structure factors) has been deposited to the Cambridge Crystallographic Data Centre as supplementary publication number CCDC-998759. Copies of the data can be obtained, free of charge, on application to CCDC, 12-Union Road, Cambridge-CB2 1EZ, UK.

In vitro antimycobacterial assay by BACTEC radiometric susceptibility assay

The BACTEC TB medium (12B) is an enriched Middlebrook 7H9 broth base. Mycobacteria utilize a ^{14}C-labeled substrate (palmitic acid) present in the medium and release $^{14}CO_2$ into the atmosphere above the medium. A test protocol adopted was as per Siddiqi.[9] Isoniazid and rifampicin were used as standard drugs.

Antioxidant activity

Total phenolic estimation

The total phenolic (TP) content was determined by Folin Ciocalteau reagent (Phenol reagent) according to the reported method.[10,11] Presence of total phenolic content was expressed as gallic acid equivalent obtained from standard curve of gallic acid.

Estimation of total flavonoid content

The total flavonoid (TF) content was determined using the spectrophotometrically with minor modifications.[12] The results were expressed in terms of quercetin equivalent using a standard curve of quercetin with different dilutions.

Reducing power assay

The iron reducing power (RP) was established according to the previous method of with some modifications.[13,14] The absorbance was taken at 700 nm against a reagent blank.

Ferric reducing antioxidant power assay

The Ferric Reducing Antioxidant Power Assay (FRAP) assay measures the antioxidant capacity to reduce the Fe^3+/tripyridyl-s-triazine (TPTZ) complex, to the ferrous form. The activity was calculated by comparing the concentration of each compound with the concentration of ferrous sulphate required to give the same absorbance change and results were expressed as FSE value.[15]

DPPH radical scavenging activity

DPPH radical scavenging activity of different concentration of compounds from *Aspergillus fumigatus* was estimated according to method reported earlier.[16] Results were expressed as percent inhibition calculated from reagent control.

Total antioxidant capacity estimation

The total antioxidant capacity (TAC) of different concentration of *Aspergillus fumigatus* was estimated as per previous method with minor amendment.[17,18] Values expressed as ascorbic acid equivalent from standard curve.

Nitric oxide scavenging activity

Nitric oxide (NO) generated from sodium nitroprusside liberates nitric acid at physiological pH. The nitric acid is converted into nitrous acid which on reaction with sulphanilic acid and naphthylethylenediamine (Griess reagent) forming diazo complex. The pink color of the product of the diazo coupling was measured at 546 nm.[19] The nitric oxide radicals scavenging activity was calculated using the equation;

$$\% \text{ Inhibition} = (A_0\text{-}A_1) / A_0 \times 100)$$

where A_0 was the absorbance of the control (without test compound) and A_1 was the absorbance in the presence of the test compound.

Results and Discussion

A fungal endophyte was identified as *A. fumigatus* isolated from the leaves of *B. monnieri* plant.[20,21] Methanol extract of *A. fumigatus* was subjected to repeated column chromatography on silica gel to get compound-1 as a light brown solid with mp 205-7°C (214-217°C) which was identified as isochromenone derivative *i.e.* 6-hydroxymellein (1) by spectroscopy. The yield of this metabolite was about 0.51% in the methanol extract. ^{13}C NMR showed ten distinct carbons in the molecule. DEPT 135 experiment ascertained one methyl (δ20.32 ppm), one methylene (δ34.57), three methines (δ75.85, δ101.46 and 106.97) and five quaternary carbons (δ101.17, 142.68, δ164.79, 164.79 and one lactone at δ170.22). It was further confirmed by ESI-High Resolution Mass (ESI-HRMS) for [M+H]$^+$ observed at 195.0668 against calculated value 195.0657. Based on ^{13}C NMR and HRMS mass its chemical formula was determined as $C_{10}H_{10}O_4$. Presence of six unsaturated carbons at δ101.17 to 164.79 indicated presence of an aromatic ring. Its double bond equivalence (DBE) was six which clearly indicated presence of a bicyclic system. Acetylated product (2) showed ESI mass at 278 [M$^+$] as diacetate derivative, indicating presence of two hydroxyls in the parent compound (1). Two exchangeable protons at δ9.60 and δ11.29 ascertained possibility of two phenolic hydroxyls (In ^{13}C, oxygenated quaternary at δ164.79x2). From HSQC experiment chemical shifts of 3-CH (δ4.68 m, 75.85), 4-CH_2 (δ2.90

bs, 34.57), 5-CH and 7-CH together (δ6.28 d, 106.97) and 11-CH_3 (δ1.53 d, 20.32) were established. In ^{1}H-^{1}H COSY spectrum, 2-CH (δ4.68 m) showed coupling with 3-CH_2 (δ2.90 bs) and also with 11-CH_3 (δ1.53) indicating presence of a –CH_2-CH-CH_3 system.

In HMBC correlations (Figure 1), 4-CH_2 protons (δ2.90 bs) showed long range correlations with C3 (δ75.85), C5 (δ106.97), C9 (101.17), C10 (δ142.68) and C10 (δ20.32) while 5-CH and 7-CH protons (δ6.28 d) showed long range correlations with C4 (δ34.57), C6 (δ164.79), C8 (164.79), C9 (101.17) and C10 (δ142.68). It was established as 3,4,-dihydro-6,8-dihydroxy-3-methylisochromen-1-one (1). The ^{1}H NMR and ^{13}C NMR data were well in agreement with previously reported data.[22] The structure was finally authenticated by X-ray crystallography (Figure 2). Previously, 6-hydroxymellein was isolated from *Aspergillus terreus*, *A. fischeri,* and *A. ochraceus* etc.[23-25] To the best of our knowledge occurrence of 6-hydroxymellein in *A. fumigatus* is the first report from this fungus.

Its diacetyl derivative (2) was synthesized on treating compound with acetic anhydride in dry chloroform and 4,4-diaminopyridine (DMAP) at room temperature in 92% yield. It was confirmed by ^{1}H NMR (2xOAc at δ1.84ppm) and EI mass at 278 [M$^+$] (Table 1). Figure 2 provides a view of the asymmetric unit of compound 1 dimeric crystal. Both the molecules in the asymmetric unit have very similar conformations. There is an intramolecular O-H...O hydrogen bond between the C=O group and one of the hydroxyl groups, resulting in 6-membered hydrogen bonded ring motif, in both the molecules. The two molecules are held together in the asymmetric unit by O-H...O and C-H...O hydrogen bonds.

Methanolic extract of *A. fumigatus* was evaluated for ferric reducing capacity and free radical scavenging activity (Table 1) exhibiting maximum scavenging of DPPH radicals (52.91±2.15%) at 100 µg/mL which was concentration-dependent. Contrary to DPPH scavenging activity, NO radical scavenging was almost half (26.93±0.82%) at 100 µg/mL which further decreases at lower concentrations. Reductions of ferric to ferrous (Iron-free radicals) ions was also noticed and evidenced by increased absorbance at 700 nM in extract

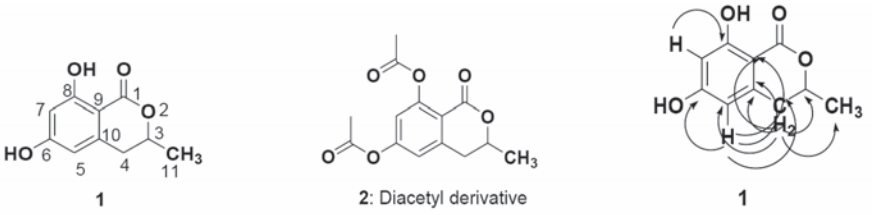

Figure 1. Structures of isochromenone (1), its diacetate derivative (2) and HMBC correlations of 1.

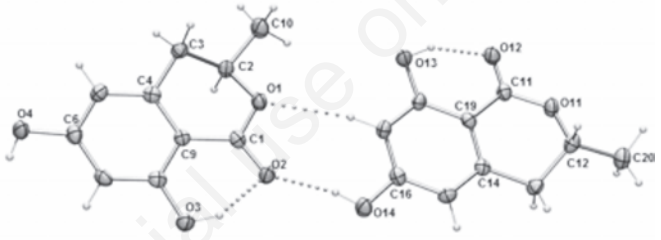

Figure 2. Asymmetric unit of the compound 1 crystals. Hydrogen bonds are indicated by dotted lines.

Table 1. Spectral data of isochromenone (1) and its diacetyl derivative (2).

Assignments	Compound 1				Compound 2	
	^{13}C ($CDCl_3$, 75 MHz) δ values (ppm)	^{1}H ($CDCl_3$, 300 MHz) δ values (ppm)	Mass (ESI mass)/ ESI-HRMS	IR (CCl_4, Cm^{-1})	^{1}H ($CDCl_3$, 300MHz) δ values (ppm)	Mass (EI mass)
C1	170.22 (QC)	-	ESI-MS: 195	3219,	-	278 [M]$^+$
2(O)	-	-	[M+H]$^+$, 217	2925,	-	
C3	75.85 (CH)	4.64-4.73, m, 1H	[M+Na]$^+$, 233	2854,	4.20-4.27, m, 1H	
C4	34.57 (CH2)	2.89-2.94, bs, CH2	[M+K]$^+$;	1735,	2.36, m, CH2	
C5	106.97 (CH)	6.26-6.29, d,CH, aromatic, J=7.2Hz & 1.5Hz.	Negative mode: 192.9 [M-H]$^-$	1636, 1378	7.54, d, 1H, aromatic	
C6	164.7 (QC)	-	ESI-HRMS;			
C7	101.46 (CH)	6.26-6.29, d, CH, aromatic, J=7.2Hz & 1.5Hz.	Calculated for [M+H]$^+$, $C_{10}H_{11}O_4$= 195.0657, Observed,		7.69, d, 1H, aromatic	
C8	164.7 (QC)	-			-	
C9	101.17 (QC)	-	195.0668		-	
C10	143.68 (QC)	1.53, d, 3H, CH3, J=9.6Hz-			-	
C11	20.32 (CH3)				0.98, d, 3H, CH3	
6-OH & 8-OH (1)/ 6-OAc & 8-OAc (2)	-	9.60 (exchangeable), 11.29 (exchangeable)			1.68 (bs, 6H, 2x-COCH3), both acetates	

Assignments are based on ^{1}H, ^{13}C, DEPT 135 & DEPT 90 experiments. QC= quaternary carbon, OH= hydroxyl group, OC-CH3-.

Table 2. Various parameters related to antioxidant profile of endophytic extract, compound 1 and 2.

Antioxidant assay	Methanolic extract					Isochromenone (1)					Isochromenone diacetate (2)				
	10 µg	25 µg	50 µg	100 µg	IC_{50} (µg/mL)[a]	10µg	25 µg	50 µg	100 µg	IC_{50} (µg/mL)[a]	10µg	25 µg	50 µg	100 µg	IC_{50} (µg/mL)[a]
DPPH	33.68±0.95	46.88±3.25	48.28±4.52	52.91±2.15	94.5	16.03±0.82	18.15±0.52	25.45±0.90	26.93±0.82	185.66 (957 M)	36.47±0.67	43.32±1.27	46.11±1.04	46.8±0.89	106.6 (383.4 M)
NO	-29.89±3.20	-10.83±5.21	13.47±1.09	17.04±2.27	293.4	45.77±3.37	41.80±.075	21.43±3.37	-46.56±3.74	ND[b]	19.40±1.73	28.84±0.73	29.01±3.57	34.60±0.64	140.4 (505.0 M)
FRAP	6.40±0.16	11.07±0.06	20.69±0.11	36.85±0.03	ND	0.33±0.02	1.34±0.17	1.52±0.04	2.03±0.02	ND	0.83±0.01	1.78±0.07	4.36±0.29	7.01±0.33	ND
RP	0.07±0.00	0.30±0.02	0.36±0.00	0.68±0.02	ND	0.02±0.01	0.02±0.01	0.03±0.00	0.05±0.01	ND	0.01±0.00	0.01±0.00	0.01±0.00	0.05±0.00	ND
TP	0.16±0.09	7.28±0.26	10.07±0.23	18.13±0.20	ND	1.07±0.15	1.13±0.02	1.37±0.10	2.77±0.10	ND	-0.23±0.00	0.14±0.03	0.32±0.04	1.57±0.20	ND
TF	-0.67±0.12	0.25±0.03	1.48±0.07	16.44±0.61	ND	0.03±0.01	0.45±0.01	0.61±0.06	2.93±0.77	ND	0.01±0.00	0.50±0.01	2.01±0.12	4.24±0.01	ND
TAC	-1.06±0.03	-0.48±0.02	0.34±0.03	2.18±0.09	ND	-0.70±0.00	-0.62±0.01	-0.58±0.00	-0.57±0.00	ND	-0.75±0.00	-0.90±0.00	-0.38±0.02	-0.08±0.02	ND

IC_{50} was calculated from extrapolated values (hypothetical); for ascorbic acid (standard) DPPH=11.5 µg/mL and NO=7.25 g/mL. ND means not determined.

(0.68±0.02 at 100 µg/mL) as well as high FSE value (36.85±0.03 at 100 µg/mL) in a dose-dependent manner, which may be correlated with the presence of high phenolic and flavonoids contents (18.13±0.20 GAE and 16.44±0.61 QE at 100 µg/mL) in the extract. Compound 1 exhibiting 26.93±0.82% DPPH radical inhibition at 100 µg/mL and nitric oxide radical inhibition was found to be 45.77±3.37% at 10 µg/mL (Table 2). Interestingly, DPPH inhibition was decreased as compared to extract whereas NO inhibition increases significantly. The molecule also exhibited positive results for reducing iron radicals (FRAP and RP) but its antioxidant activity was not better than the crude extract. Total antioxidant capacity was not observed in pure molecule striking the fact of the structural difference from ascorbic acid.

Based on these observations, diacetates derivative 2 was also prepared and tested for antioxidant activity. The molecule revealed improved activity for both DPPH and NO inhibition, equivalent to extract *i.e*. 46.8±0.89 and 34.6±0.64% at 100 µg/mL. Iron reducing activity also improved as compared to pure isolated compound (Table 2).

Antitubercular activity from this fungus has not been reported previously. The antitubercular activity of methanolic extract (MIC) was at 500 µg/mL against *Mycobacterium tuberculosis* H37RV strain through BACTEC assay.[9] The minimum inhibitory concentration (MIC) of test compound was noted on the basis of GI (growth index) value. The MIC of standard drugs isoniazid and rifampicin were 0.85 M and 0.28 M respectively.

Conclusions

In conclusion, the present study has described isolation of 6-hydroxy mullein (1) from *A. fumigatus* hosted in *B. monnieri* plant. To the best of our knowledge this is the first report of this metabolite from this fungus. Our study suggests that not only plants but some other fungi may also be a good source of antioxidant compounds and *A. fumigatus* is one such potential candidate presenting a better scope for production bioactive compounds.

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