Isolation and characterization of antibacterial naphthalene derivative from *Phoma herbarum*, an endophytic fungus of *Aegle marmelos*

Endophytes receive their nutrition and shelter from plants and in response provide various benefits to the plants in the form of growth stimulation, pest resistance and stress tolerance. The mission of novel drug discovery to fulfil the growing demand worldwide, led to the identification and characterization of more than 100 anticancer compounds only from endophytic fungi, a relatively less explored microbe after 1993 (ref. 1). The endophytic fungi *Muscodor albus*, *M. roseus*, *M. vitigenus*, *M. crispans* and *Gliocladium* sp. produce a mixture of volatile antimicrobial compounds, including naphthalene[2–5]. *Hypoxylon* sp., isolated from *Persea indica*, is known to produce so many volatile antimicrobial compounds like 1,8-cineole, 1-methyl-1,4-cyclohexadiene and tentatively identified (+)-alpha-methylene-alpha-fenchocamphorone including some unidentified ones[6].

The present study is based on endophytic fungus isolated from *Aegle marmelos* Corr. growing wildly in forest Hathinala of Vindhyan Plateau, India. *A. marmelos* is commonly known as 'bael', a holy and medicinal plant with a long history of antifungal and antibacterial activity[7,8]. Here we report the significant antimicrobial activity and identification of 1-iodo-naphthalene from the active extract of endophytic fungus *Phoma herbarum* isolated from *A. marmelos*.

Healthy leaves of *A. marmelos* were collected in the month of March–April 2009 from Hathinala forest (24°18′N, 83°6′E; elevation 315–485 m amsl), Vindhyan Plateau, Sonebhadra district, Uttar Pradesh, India. The samples collected in sterile polythene bags were brought to laboratory in an icebox. Samples stored at 4°C were used to isolate endophytic fungi within 48 h of collection.

Surface sterilization was done by immersing the plant tissues in 70% ethanol for 2 min and in aqueous solution of sodium hypochlorite (4% available chlorine) for 4 min followed by washing with 70% ethanol for 10 sec. The tissues were then rinsed in sterile distilled water and allowed to surface dry under sterile conditions. Leaves were cut into small pieces of 5×5 mm^2 with a sterile blade. The pieces were placed on petri plates containing potato dextrose agar (PDA) medium supplemented with streptomycin (200 mg/l) and incubated for 21 days at 26 ± 2°C in BOD-cum-humidity incubator (Calton, NSW, New Delhi). Tissues were observed for fungal growth every 2 days for 20 days. Actively growing fungal tips emerging from the plant tissues were sub-cultured on PDA Petri plates for identification and enumeration.

The emergence of fungi was suppressed with increasing time length of surface sterilization treatment with sodium hypochlorite. Hence, the leaves were treated with sodium hypochlorite for 4 min as optimized time for surface sterilization. Some imprints of surface-sterilized leaf pieces were taken on the PDA plate as control to check the efficacy of surface sterilization. The control PDA plate was also run parallel to the experiment to examine the contamination[9].

The endophytic fungus was cultured in 1 litre Erlenmeyer flask containing 500 ml of potato dextrose broth in triplicate and incubated at 26 ± 2°C for 21 days. Each of these broths was filtered collectively and extracted thrice with the same amount of ethyl acetate. The extracts were combined and evaporated to dryness *in vacuo* by a rotary evaporator (Rotary vacuum, Perfit India Ltd). Antibacterial activity of crude extract of endophytic fungus was observed against six clinical isolates of human pathogenic bacteria – *Shigella flexnii* IMS/GN1, *Shigella boydii* IMS/GN2, *Salmonella enteritidis* IMS/GN3, *Salmonella paratyphi* IMS/GN4, *Pseudomonas aeruginosa* ATCC 27853 and *Morganella morganii* IMS/GN6.

The fungal extract was diluted at various concentrations (20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml) in 5 ml culture tubes containing 2 ml Mueller-Hinton broth[10]. One drop of exponentially growing culture of test bacterium was inoculated in each concentration of test sample and incubated at 37 ± 2°C for 24 h. A control was prepared using test bacterium in Mueller-Hinton broth without test sample. After the incubation period, the viability of test bacteria was tested on Mueller-Hinton agar plates. The test procedure was repeated thrice to check the reproducibility of the results. The lowest concentration of test sample which gave no viability of test bacterium was considered as minimum inhibitory concentration (MIC).

The bioactive crude extract was separated into various fractions by column chromatography (CC). The column was packed with silica gel (mesh 60–120) and run with *n*-hexane : EtOAc (8 : 2). Each fraction separated by CC was observed for antimicrobial activity against the above-mentioned bacterial pathogens by disc diffusion assay. The most active fraction separated by CC was analysed under GCMS. Gas chromatography attached with mass spectrometry was performed on Shimadzu QP-5000. The mass spectra were recorded on EI mode from MW (molecular weight) 50 to 700.

The genomic DNA of endophytic fungus was extracted and amplified following the protocol of Sim *et al.*[11]. The universal primers ITS1 and ITS4 were used to amplify 5.8S rDNA and ITS2 regions flanked between the 18S and 28S rRNA genes. Amplified PCR product was sent to First BASE Laboratories (Malaysia) for sequencing (Figure 1). The obtained sequences were compared by BLAST search at NCBI GenBank for identification.

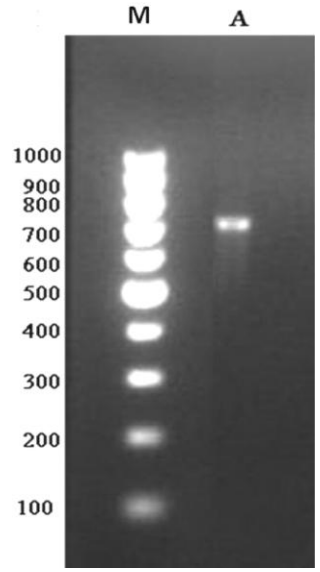

Figure 1. Gel photograph of PCR-amplified DNA of *P. herbarum* (lane A). Lane M, marker.

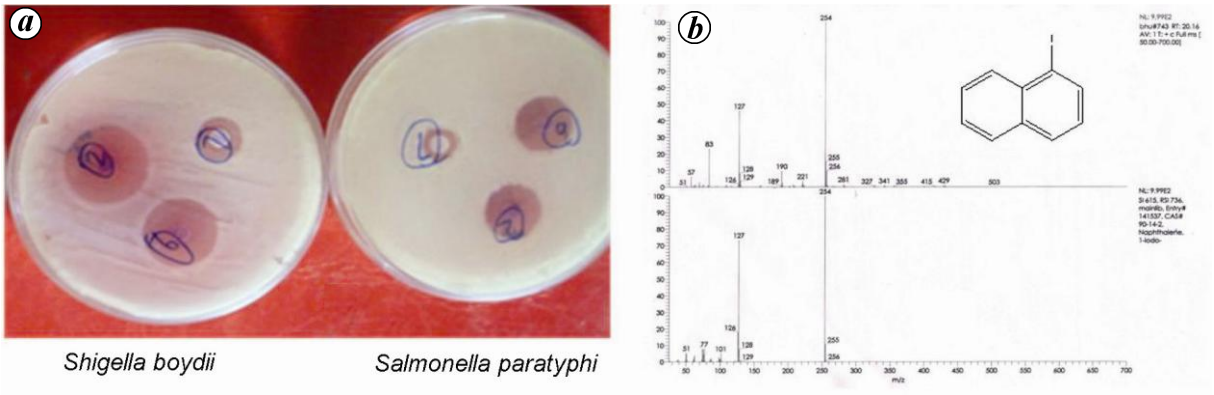

Figure 2. ***a***, Inhibitory activity of column chromatographic fractions of crude extract of *Phoma herbarum*. ***b***, MS chromatogram of fungal and authentic 1-iodo naphthalene.

Table 1. Antibacterial activity of crude extract of *Phoma herbarum*

		MIC (µg/ml)					
Endophytic fungus	Host	A	B	C	D	E	F
P. herbarum	*Aegle marmelos*	30	30	50	50	60	50

A, *Shigella flexnii* IMS/GN1; B, *Shigella boydii* IMS/GN2; C, *Salmonella enteritidis* IMS/GN3; D, *S. paratyphi* IMS/GN4; E, *Pseudomonas aeruginosa* ATCC 27853; F, *Morganella morganii* IMS/GN6.

Table 2. Antibacterial activity of column chromatographic (CC) fractions of crude extract of *P. herbarum*

	Antibacterial activity*					
CC fraction no.	A	B	C	D	E	F
1	+	+	–	–	–	–
2	+++	+++	++	++	++	+
3	++	++	+	+	–	–
4	+	+	–	–	–	–
5	–	+	–	–	–	–
6	+	+++	–	++	+	–
7	+	+	+	+	–	+
8	–	+	–	–	–	+
9	–	–	–	–	–	–
10	–	+	–	–	–	–
11	–	+	–	–	–	–

*Inhibition zone less than 10 mm (+), 10–20 mm (++), more than 20 mm (+++) and no inhibition (–). A–F, same as in Table 1.

The crude extract of *P. herbarum* gave the least MIC (30 µg/ml) against *S. flexnii* and *S. boydii*. The MIC of extract of *P. herbarum* against *S. enteritidis* and *S. paratyphi* was observed at 50 µg/ml (Table 1). The crude extract of *P. herbarum* was fractionated by silica gel CC with *n*-hexane : EtOAc (8 : 2), which gave 11 fractions. Each fraction was evaluated for antibacterial activity via disc diffusion assay. Ten out of eleven fractions showed antibacterial activity against one or more bacteria. Fraction no. 2 gave maximum inhibitory activity against all bacteria tested (Table 2, Figure 2 *a*). The purified compound was brown-coloured, semi-solid and oily, having an aromatic odour. This fraction was further analysed by GCMS analysis. The library search (mainlib) identified the major compound as 1-iodo-naphthalene (MW 254) at retention time 20.16 min. The identified fungal 1-iodo-naphthalene had similar mass spectrum as the authentic one (Figure 2 *b*).

Naphthalene is used as an antimicrobial, insecticide, insect repellent, anthelmintic and vermicide. The production of insect-repellent naphthalene by endophytic *M. vitigenus* was recorded by direct gas chromatography/mass spectrometry[3]. Fungi are also reported for the biotransformation of naphthalene. The species of *Cunninghamella*, *Syncephalastrum* and *Mucor* transformed naphthalene into 1-naphthol and several other derivatives like 4-hydroxy-1-tetralone, *trans*-1,2-dihydroxy-1,2-dihydronaphthalene, 2-naphthol, 1,2-and 1,4-naphthoquinone[12]. Two novel naphthalene-containing compounds, colelomycerones A and B, have been isolated from a freshwater fungus YMF 1.01029 with antifungal and antibacterial activity[13].

In this study, fungal extract containing 1-iodo-naphthalene gave significant antibacterial activity against *S. flexnii* and *S. boydii* (Table 1). The different species of *Shigella* are known to cause shigellosis infecting the digestive tract with various symptoms like diarrhoea, cramping, vomiting and nausea. The extract was also active against typhoid-causing agent *Salmonella* spp. Volatile organic compounds (VOC) containing naphthalene derivatives produced by endophytic *Muscodor albus* exhibited maximum antibiotic activity against many plant pathogenic fungi and various bacteria, including *Escherichia coli*[2]. An endophytic fungus *Oidium* sp. isolated from *Terminalia catappa* produced VOCs primarily consisting of esters of propanoic acid, 2-methyl-, butanoic acid, 2-methyl-, and butanoic acid, 3-methyl-

with inhibitory activity against pathogenic fungi[14]. An antimicrobial naphthaquinone javanicin has been identified from the endophytic fungus *Chloridium* sp. isolated from *Azadirachta indica*[15]. The fungal naphthalene is synthesized by polyketide synthases (PKSs). *Aspergillus nidulans* wA and *Colletotrichum lagenarium pks*1 coded for iterative type I PKSs and synthesized heptaketide naphthopyrone and pentaketide naphthalene respectively[16]. Recently, two new naphthalene derivatives, 1-(4′-hydroxy-3′,5′-dimethoxy-phenyl)-1,8-dimethoxynaphthalene-2(1H)-one and 1,8-dimethoxynaphthalene-2-ol have been identified from an endolichenic fungal strain *Scopulariopsis* sp.[17]. The production of naphthalene by endophytic fungus may be related to restrict the growth of other fungal competitors in its natural environment[3].

The amplified ITS region of rDNA of endophytic fungus produced 727 bp long sequence (Figure 1). The BLAST search of this ITS sequence revealed 99% similarity with the deposited sequence of *P. herbarum*. This ITS sequence was deposited at NCBI GeneBank with accession no. JN163857. The fungus was deposited at the Culture Bank of Department of Botany, Banaras Hindu University (BHU), Varanasi with isolate no. MMTL/AMH 350.

The species of *Phoma* are common in fungal endophytic census and a gibberellin-producing endophytic *P. herbarum* was isolated from roots of salt-stressed soybean (*Glycine max*) plant. Interestingly, almost all forms of gibberellin, especially GA4 and GA7, were found in higher amounts in conjunction with physiologically inactive GA9, GA12, GA15, GA19 and GA20 in culture of *P. herbarum*[18].

This report suggests that naphthalene derivative produced by endophytic fungus may be warding off the plants from pathogens of *A. marmelos*. Future research on this fungus may provide clues on the biosynthetic mechanism of naphthalene and may enhance the production of naphthalene for commercial purpose.

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Resource partitioning between two 'near threatened' heronry species – a case of nest sharing between Painted stork (*Mycteria leucocephala*) and Black-headed ibis (*Threskiornis melanocephalus*)

Selection of a nest site can be viewed as an adaptive trade-off between the cost of searching and defending the site and the reproductive benefits of selecting a site enhancing reproductive success[1,2]. Differential resource selection is also one of the major factors allowing the coexistence of species[3,4]. However, when a species has a specific nesting requirement, suitable nesting locations may be difficult to obtain[5,6], which may cause the overlap of nesting sites[7].

Members of the order Ciconiformes (storks, ibises, egrets, etc.) are well known as heronry-forming species that nest communally during their breeding season, which is more or less the same for heronry-forming species. Black-headed ibis (BHI) and Painted stork (PS) are also heronry species and are classified as 'Near Threatened' according to the IUCN Red list of species (Birdlife International, 2001).

Oriental white ibis, now also referred to as black-headed ibis (*Threskiornis melanocephalus* Latham), is a nomadic, ciconiiformes that frequents shallow wetland habitats in India[8–10]. Its breeding season is from June to August in North India, November to February in South