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Original Research Article

Sonneratinone: A new antimicrobial benzofuranone derivative from the endophytic fungus *Aspergillus niger* isolated from the mangrove plant *Sonneratia apetala* Buch.-Ham

Tauhidur Rahman nurunnabi[1,2], Shaymaa Al-majmaie[1], Lutfun Nahar[3], Ismini Nakouti[1], S.M. Mahbubur Rahman[2], Md. Hossain Sohrab[4], Md. Morsaline Billah[2], Fyaz md. Ismail[1], George p. Sharples[1] and Satyajit d. Sarker[1*] ✉

[1]Centre for Natural Products Discovery, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, James Parsons Building, Byrom Street, Liverpool L3 3AF, United Kingdom
[2]Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh
[3]Laboratory of Growth Regulators, Institute of Experimental Botany ASCR & Palacký University, Šlechtitelů 27, 78371 Olomouc, Czech Republic
[4]Pharmaceutical Sciences Research Division, BCSIR Laboratories, Dhaka, Bangladesh

ABSTRACT

A new antimicrobial benzofuranone derivative, named, sonneratinone (**1**), was isolated from the endophytic fungus *Aspergillus niger*, obtained from the leaves of *Sonneratia apetala*, a mangrove plant from the Sundarbans. Whilst the fungal strain was identified by macroscopic, microscopic and molecular techniques, the structure of the new compound was elucidated by spectroscopic means, e.g., 1D and 2D NMR, and HRESIMS. Sonneratinone (**1**) showed considerable antimicrobial activity against *Micrococcus luteus*, *Staphylococcus aureus* and *Candida albicans* in the resazurin 96-well microtitre plate antimicrobial assay.

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1. Introduction

The habitat of the mangroves plants is in the tropical and subtropical coastlines (Tomlinson, 1986). Globally, mangrove forests cover 0.6% of all inland forests. Although, mangroves represent only a fraction of the world's forest resources, they are of great economic and ecological importance; mangrove forests is one of the world's most productive ecosystems (Lear and Turner, 1977) and produces numerous renewable resources (Field, 1995). Bangladesh contributes about 4% of the world's mangrove forests covering 5885.40 km^2, of which 5800 km^2 is in the Sundarbans and 85.40 km^2 is in Chakaria (Das and Siddiqi, 1985). Sonneratia apetala Buch.-Ham., commonly known in Bangladesh as "Keora", belongs to the family Lythraceae. It is a fast growing and pioneer tree species in ecological succession in the Sundarbans of Bangladesh (Hossain et al., 2017). Leaves of this plant have been used traditionally to treat cardiac complications, dysentery, hepatitis, sprain and bruises (Hossain et al., 2017). Whilst there are reports on isolation of bioactive compounds from *S. apetala* (Bandaranayake, 1995; Hossain et al., 2017), there is hardly any investigation on the compounds of endophytes associated with this plant. Endophytes are the organisms, which are in an imperceptible relationship with the plant in which they reside for their whole lifetime or a part of their life. These include fungi, bacteria and some algae. They colonize all the plants evaluated to date and isolated from almost all the plant parts like leaves, roots, stems, flowers, barks and even from dry seeds (Tomlinson 1986; Duke et al., 1998; Sette et al., 2006). Endophytic fungi have a capacity to produce diverse class of plant associated secondary metabolites with a wide variety of biological activities, such as taxol (anticancer) (Nurunnabi et al., 2017), rohitukine (anticancer) (Ren et al., 2009), antimicrobial agent hypericin (Bandaranayake, 1995) and acetylcholinesterase inhibitor huperzine A (Hossain et al., 2017). In continuation of our phytochemical and

✉Corresponding author: Satyajit D. Sarker
Tel: +44 (0) 1512312096; Fax: +44 (0) 1512312170
E-mail address: S.Sarker@ljmu.ac.uk

bioactivity studies on endophytic fungi from the plants of the Sundarbans (Nurunnabi et al., 2017; 2018), we now report on the isolation and identification of a new antimicrobial benzofuranone derivative, named, sonneratinone (**1**) (Fig. 1), from the endophytic fungus, *Aspergillus niger*, obtained from the leaves of the mangrove plant S. apetala, growing in the Sundarbans.

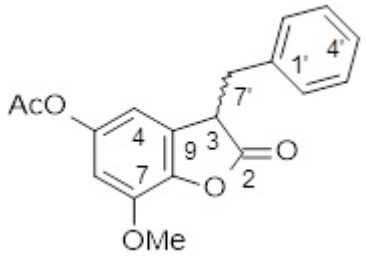

Fig. 1. Molecular structure of sonneratinone (**1**).

2. Experimental

2.1. General instrumentation

An Ultrashield Bruker AMX 600 NMR spectrometer (Coventry, UK) was used to record all 1D and 2D NMR spectra. DEPT-Q experiment was used to distinguish methyl, methylene and methine carbons and on the other hand homonuclear ^{1}H connectivity was determined by using the COSY experiment. ^{1}H-^{13}C one-bond connectivity was established with HSQC gradient pulse factor selection. HMBC experiments successfully reviled two- and three-bond connectivity. Chemical shifts and coupling constant are reported in δ (ppm) and *J* Hz, respectively. Sigma-Aldrich (Dorset,UK) was the supplier of all chemicals used in this work. All solvents for both extraction and chromatography were purchased from Fisher Scientific, Loughborough, UK. NMR solvents were obtained from GOSS Scientific (Crewe, UK). HRMS analyses were performed on an Agilent 6200 Series Accurate-Mass Time-of-Flight (TOF) LC/MS (Santa Clara, California, USA).

2.2. Plant material

Based on the morphological characterization described by Zabala (1990), the plant Sonneratia apetala Buch.-Ham. was identified prior to collection of the leaves and a voucher specimen (TRN-KU-2017012) of this collection was deposited at the Herbarium of the Pharmacy Discipline, Khulna University, Khulna, Bangladesh. Healthy leaves of S. apetala were collected from the strong saline zone of the Sundarbans (N 220 12′ 10.6′′ E 890 10′ 46.9′′), Bangladesh. Tightly sealed polythene bags were used to store the leaves immediately after collection and all leaves were used for isolation of endophytic fungi within 24 h of collection.

2.3. Isolation of endophytic fungi

The endophytes were isolated using water agar method described previously (Nurunnabi et al., 2017). Briefly, to eliminate extraneous substances, plant materials were washed thoroughly in sterile water and then the samples were surface sterilised by sequentially immersing in 70% ethanol for 30 sec and sodium hypochlorite solution (5.0%) for 1 min and finally rinsed with sterile distilled water. Small pieces (1×1) cm of inner tissues and needles were transferred and impregnated to the petri-dishes containing aqueous agar (1.5% agar in distilled water) supplemented with antibiotic streptomycin (3 mg/100 mL) and incubated at 28 ± 2 °C until fungal growth was initiated. The tips of the fungal hyphae were picked up and inoculated on PDA (Potato Dextrose Agar) medium. After five days of incubation at 25 °C, and then five more days at 35 °C, colony morphology was assessed to determine the purity of each fungal culture. The isolates were purified, sub-cultured and maintained using PDA medium.

2.4. Identification of the fungal isolate

Both morphological and molecular techniques were employed to identify fungal endophytes (Nurunnabi et al., 2017). Fungal DNA was isolated using fungal DNA isolation kit (Cat-26200; NORGEN BIOTEK Corp., 3430 Schmon Parkway, Thorold, ON, Canada). Phylogenetic analyses of the endophytes were performed by the acquisition of ITS1-5.8S-ITS2 ribosomal gene sequencing. The internal transcribed spacer (ITS) region of the fungi were amplified using the forward primer ITS4 (5′TCCTCCGCTTATTGATATGC3′) and reverse primer ITS 5 (5′GGAAGTAAAAGTCGTAACAAGG 3′), using the Polymerase Chain Reaction (PCR) (Nurunnabi et al., 2017). The PCR amplified products were sent to Cambridge Genomic Services (The University of Cambridge, UK) for sequencing according to their requirements. Consensus sequence of 500 bp of 5.8S rRNA was generated from forward and reverse sequence data using aligner software. Consensus sequences were submitted in the GenBank. Multiple BLASTN searches against the sequence were made at the National Center for Biotechnology Information (NCBI). Primarily, Clustal Omega was employed for Multiple Sequence Alignment and further followed by trimming using trimAl tool for later alignment. Phylogenetic analysis performed with the neighbour joining method using MEGA 7.0 software.

2.5. Small-scale fermentation for secondary metabolites

The fungal isolate was grown in (5 × 250 mL) conical flasks containing potato dextrose broth (PDB, 150 mL) for 28 d. Culture broth was separated from the mycelium by filtration (Whatman® qualitative filter paper, Grade 1; Sigma-Aldrich, USA) and the culture filtrates were extracted three times with an equal volume of EtOAc in a separating funnel. The EtOAc extract was evaporated under reduced pressure at 40-45 °C using a rotary evaporator to obtain crude EtOAc extract.

2.6. Large-scale fermentation for secondary metabolites

As the EtOAc extract of endophytic fungus showed significant antimicrobial activity, it was subjected to large-scale fermentation. The fungus was grown on PDA medium for 3 d. The mycelium (5 mm) was transferred to 40 × 250 mL conical flasks containing PDB (150 mL). The flasks were placed at room temperature under continuous shaking for around 28 d at 180 rpm. The mycelia were separated from the medium by filtration and extracted by equal volume of EtOAc. Finally, the extracts were dried using a rotary evaporator.

2.7. Fractionation, isolation and structure elucidation of the major bioactive compound

Reversed-phase analytical HPLC analysis of the EtOAc extract was carried out using a Phenomenex C18 reversed-phase column (250 mm × 4.6 mm; particle size 5 µm) on a Dionex Ultimate 3000 analytical HPLC-coupled with a photo-diode-array detector (mobile phase: standard gradient of 30-100% MeOH in water over 30 min, flow rate: 1 mL/min). The crude EtOAc was subjected to preparative HPLC using the same solvent system but with a flow rate of 10 mL/min and using a HiChrom preparative column (250 mm × 20.1 mm; particle size 5 µm) on an Agilent prep-HPLC system to isolate the major new compound (1). The purity of the isolated compound was checked by analytical HPLC, and the structure was confirmed by spectroscopic analyses including MS, and 1D and 2D NMR analyses. Sonneratinone (**1**): Brown amorphous solid; ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD): See Table 1. HRESIMS: m/z 313.1075 $[M+H]^+$ (calcd. 313.1076 for $C_{18}H_{17}O_5^+$).

2.8. Assessment of antimicrobial potential

The EtOAc extract of the endophytic fungus, and its major secondary metabolite, sonneratinone (1), were screened for their potential antimicrobial activity against two Gram-positive, i.e., *Staphylococcus aureus* (NCTC 12981) and *Micrococcus luteus* (NCTC 7508), and two Gram-negative, i.e., *Escherichia coli* (NCTC 12241) and *Pseudomonas aeruginosa* (NCTC 12903) bacterial strains as well as against a fungal strain, *Candida albicans* (ATCC 90028) using the resazurin 96-well microtitre plate based *in vitro* antimicrobial assay (Sarker et al., 2007). Ciprofloxacin was used as a positive control for bacterial strains, and nystatin for *C. albicans*. The average of three values was calculated and that was the MIC for the test material and bacterial strain.

3. Results and Discussion

Microscopic examination followed by molecular characterization confirmed that the fungal isolate was a species of the genus *Aspergillus* (Fig. 2). The phylogenetic tree revealed that the isolate possessed maximum homology with *Aspergillus niger* KF305758.1. The best BLAST matches for endophytic fungus *Aspergillus* sp. with maximum homology. Because of 5.8S rRNA gene homology, the fungal isolate was designated as *Aspergillus* sp. (*A. niger*). Consensus sequences (5.8S rRNA) of isolated fungi were submitted in the GenBank of NCBI and accession numbers MH447401.1 was found for the fungi. Table 2 represents the name of the fungus, accession number, reference accession number, query length and the percentage of similarity. Molecular techniques, as employed in the current study, have significantly improved the delimitation of fungal species that are hard to distinguish based on morphology alone and reveal their phylogenetic relationships. A. niger is a species complex with broad genetic and biological diversity grouped together by similar conidial morphology and ITS sequences. Bevan Weir and Peter Johnston (Landcare Research, Auckland, New Zealand) presented their research on this species complex and possible approaches to species delimitation through the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Damm et al., 2009; 2010).

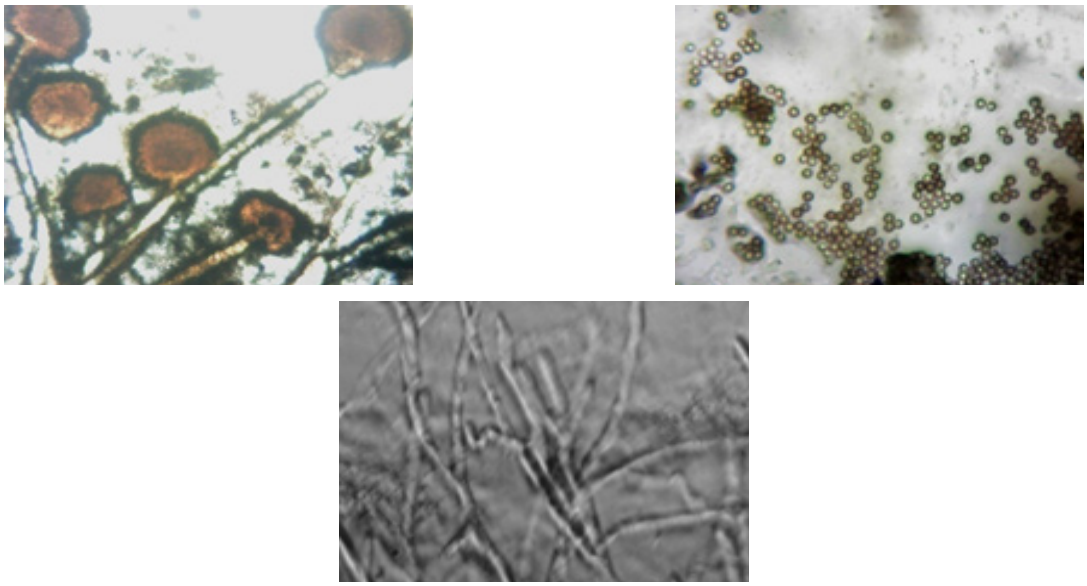

Fig. 2. (a) Aspergillus niger (conidiophores and conidial head); (b) Conidia of the fungus at 40x; (c) Hypha of the fungus at 40x magnification.

Table 1
^{1}H NMR (600 MHz, coupling constant *J* in Hz in parentheses), ^{13}C NMR (150 MHz) data and ^{1}H-^{13}C long-range HMBC correlation for sonneratinone (**1**).

Position	Chemical shift δ in ppm		HMBC*	
	^{1}H NMR*	^{13}C NMR*	^{2}J	^{3}J
2	-	173.1	-	-
3	4.92 dd (9.0, 7.2)	52.5	C-2, C-7′,	C-4, C-8, C-1′
4	6.01 d (1.8)	101.9		C-3, C-6, C-8
5	-	166.9	-	-
5-OAc	1.96 s	173.5, 22.5	C=O	-
6	5.57 d (1.8)	89.2	C-5, C-7	C-4
7	-	138.2	-	-
7-OMe	3.84 s	57.2	-	C-7
8	-	164.2	-	-
9	-	124.0	-	-
1′	-	134.0	-	-
2′	7.27-7.30 m	129.7	C-3′	C-4′, C-7′
3′	7.22-7.25 m	130.3	C-2′, C-4′	C-1′
4′	7.22-7.25 m	128.1	C-3′, C-5′	C-3′, C-6′
5′	7.22-7.25 m	130.3	C-6′, C-4′	C-1′
6′	7.27-7.30 m	129.7	C-5′	C-4′, C-7′
7′	3.00 dd (12.0, 9.0)	39.5	C-3, C-1′	C-2′, C-6′
	3.18 dd (12.0, 7.2)			

*Spectra obtained in CD_3OD

Table 2
Identification of endophytic fungus in *Sonneratia apetala* with NCBI accession number.

Name of the fungus	Genbank Accession Number	BLAST match Sequence		
		Reference Accession No	Query Length	Similarity
Aspergillus niger	MH447401.1	*Aspergillus niger* KF305758.1	602	100%

The *A. niger* on PDB medium was extracted with EtOAc to obtain 320 mg of crude mixture of secondary metabolites. Reversed-phase preparative HPLC of the crude mixture afforded the new compound (1, 22 mg) as the major secondary metabolite present in the EtOAc extract of this fungus. The structure of the new compound (**1**) (Fig. 1) was confirmed by 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR (Table 1) and MS spectroscopic data analyses. The ^{1}H NMR spectrum (Table 1) revealed the signals for seven aromatic protons, assignable to a mono-substituted benzene ring (five overlapped protons in the region δ 7.27-7.30 ppm appeared as multiplets), and a tetra-substituted benzene ring with two protons having meta-couplings (δ 6.01 and 5.57, *J* = 1.8 Hz), signals for two protons of a methylene group (δ 3.00 and 3.18 ppm), and a signal (δ 4.92, dd, *J* = 7.2, 9.0 Hz) assignable to a methine functionality. Additionally, there were signals for a methoxyl group (δ 3.84, s) and an acetyl (δ 1.96, s).The ^{1}H-^{1}H COSY experiment established three

different spin systems: for two aromatic rings, and a CH-CH_2. The ^{13}C NMR spectrum (Table 1) showed the signals for 18 carbons, twelve of which were aromatic carbons (seven methines, two quaternaries, and three oxygenated quaternaries), one methylene, one methine, a methoxyl, an ester (acetate), a lactone carbonyl and methyl of the acetate group. While the HSQC experiment revealed the direct correlations between ^{1}H and ^{13}C, the HMBC (Table 1) experiment established all key ^{1}H-^{13}C 2*J* and 3*J* correlations, and thus established the structure shown in Fig. 1. The HRESIMS data confirmed the molecular formula of this compound as $C_{18}H_{16}O_5$. Thus, the compound was identified as a new benzofuranone derivative, 3-benzyl-7-methoxy-2-oxo-2,3-dihydrobenzofuran-5-yl acetate, and named, sonneratinone (**1**). Various phenolic compounds, malic acid derivatives, terpenoids and sterols were previously reported from *S. apetala* (Cao et al., 2015; Hossain et al., 2016) and the fungus, *A. niger*, was reported to produce mycotoxins and other secondary metabolites (Vadlapudi et al., 2017). Sonneratinone (**1**) displayed antimicrobial activity against tested microorganisms and was most effective against *Micrococcus luteus* and *Staphylococcus aureus* (MIC = 2.5 × 10^{-1} mg/mL) and the least against *Candida albicans* (MIC = 5 × 10^{-1} mg/mL) (Table 3). However, it is noteworthy that the crude EtOAc extract showed remarkable activity against *Pseudomonas aeruginosa* (MIC = 9.7 x 10^{-3} mg/mL), which was just half the potency of the positive control ciprofloxacin MIC = 1.2 × 10^{-4} mg/mL (Table 3). The significant loss of activity in the isolated compound against *Pseudomonas aeruginosa*, compared to that of its parent extract, indicated that there might be other active compounds present in the extract, which could not be detected by the HPLC-PDA because of lack of any chromophores present in those molecules.

Table 3
Antimicrobial activity of the EtOAc extract of *A. niger* and sonneratinone (**1**).

Test organisms	Minimum inhibitory concentration (MIC) in mg/mL			
	Positive controls			Test samples
	Ciprofloxacin	Nystatin	EtOAc extract	1
Escherichia coli (NCTC 12241)	4.9×10^{-4}	NT	2.5×10^{-1}	1.0
Micrococcus luteus (NCTC 7508)	9.8×10^{-4}	NT	6.25×10^{-1}	2.5×10^{-1}
Pseudomonas aeruginosa (NCTC 12903)	1.2×10^{-4}	NT	9.7×10^{-3}	5×10^{-1}
Staphylococcus aureus (NCTC 12981)	9.8×10^{-4}	NT	6.25×10^{-1}	2.5×10^{-1}
Candida albicans (ATCC 14053)	NT	9.8×10^{-4}	1.25×10^{-1}	5×10^{-1}

NT = Not tested

4. Concluding remarks

Endophytic fungi are a good source of bioactive compounds, often with new chemical structures. The present study afforded the isolation and identification of a new natural product, sonneratinone (**1**), from the endophytic fungus, *Aspergillus niger*, obtained from the leaves of *Sonneratia apetala*, a well-known mangrove plant from the Sundarbans. This new benzofuranone derivative showed considerable antimicrobial activity in the resazurin assay.

Conflict of interest

The authors declare that there is no conflict of interest.

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