Research Article

Sadia Noor, Mst. Nadira Begum, Satyajit Roy Rony, Mohammad Zashim Uddin, Md. Hossain Sohrab* and Md. Abdul Mazid*

Bioactivity and chemical screening of endophytic fungi associated with the seaweed *Ulva* sp. of the Bay of Bengal, Bangladesh

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Abstract: Several studies have shown that endophytic fungal metabolites possess vital biological activities; nevertheless, there is a lack of knowledge regarding the medicinally important marine endophytic fungi associated with the seaweeds mainly found in the Bay of Bengal, Bangladesh. In this study, six endophytic fungi, belonging to five genera and four classes, were isolated from the well-known chlorophyte, *Ulva* sp. and were most closely related to *Chaetomium globosum*, *Nigrospora magnoliae*, *Curvularia* sp., *Curvularia moringae*, *Aspergillus terreus* and *Collariella* sp. This is the first report of these fungi as endophytes associated with *Ulva* sp. from the Bay of Bengal, Bangladesh. A preliminary biological evaluation of the ethyl acetate extract of each endophytic fungal crude extract was the prime objective of this research, e.g., antimicrobial assay, 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity and brine shrimp lethality bioassay. Evaluation of test results revealed that each fungal crude extract possessed one or more relevant biological activities. Preliminary chemical screening using TLC and NMR spectroscopic analysis revealed the presence of several secondary metabolites in the crude fungal extracts. These findings suggest that the marine endophytic fungus may be a valuable source for investigating potentially bioactive chemicals or leads for novel drug candidates.

Keywords: seaweed; marine endophytic fungi; antimicrobial; DPPH scavenging activity; bioassay

***Corresponding authors: Md. Hossain Sohrab**, Pharmaceutical Sciences Research Division, BCSIR Dhaka Laboratories, Dhaka, Bangladesh, E-mail: mhsohrab@bcsir.gov.bd; and **Md. Abdul Mazid**, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka, Bangladesh, E-mail: ma.mazid@du.ac.bd. https://orcid.org/0000-0003-2190-2591
Sadia Noor, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka, Bangladesh; and Department of Pharmacy, University of Asia Pacific, Dhaka, Bangladesh, E-mail: noorsadia.du@uap-bd.edu
Mst. Nadira Begum, Biological Research Division, BCSIR Dhaka Laboratories, Dhaka, Bangladesh, E-mail: nbegum470@gmail.com
Satyajit Roy Rony, Pharmaceutical Sciences Research Division, BCSIR Dhaka Laboratories, Dhaka, Bangladesh, E-mail: satyajit_pharm@yahoo.com. https://orcid.org/0000-0002-6884-2904
Mohammad Zashim Uddin, Department of Botany, University of Dhaka, Dhaka, Bangladesh, E-mail: zashim@du.ac.bd

1 Introduction

Seaweeds (marine macroalgae) have been found to be a favoured host for some fungal species to such an extent that approximately one-third of all known filamentous marine fungi have been isolated from seaweeds (Teuscher et al. 2006). The potential of seaweed-associated endophytic fungi as a plentiful source of structurally novel secondary metabolites with diverse biological activities is undeniable (Debbab et al. 2012; Zhang et al. 2016) and, over the past few decades, the previously untapped marine endophytic fungi have drawn considerable attention (Flewelling et al. 2013). A wide range of seaweeds, including green species in the genus *Ulva* (family: Ulvaceae), are found naturally in the coastal areas of Bangladesh.

It is commonly recognized that *Ulva*-associated bacteria have been extensively studied with functions related to host growth and morphological development (Dhanya et al. 2016; Ghaderiardakani et al. 2017; Habbu et al. 2016; Wichard 2023), yet species of *Ulva* also harbour a rich diversity of endophytic fungi like many other seaweeds. Endophytic fungi can colonize the inner tissues of algae without causing any visible damage or disease symptoms (Zhang et al. 2016). These asymptomatic marine fungi and their environmental roles remain mostly underexplored (Uzor et al. 2015; Vallet et al. 2018). A mutual interaction between endophytic fungi and host seaweeds may alter according to the habitat and eco-geographical conditions, eventually enhancing the

host's nutrition, improving its growth development, and affecting secondary metabolism (Patyshakuliyeva et al. 2019; Radu and Kqueen 2002; Smrutirekha et al. 2021). These secondary metabolites allows for chemical adaptability to surrounding factors (such as changes in nutrients, temperature or salt levels). It also helps in competition for substratum and serves as a defence mechanism against attacks from pathogens, thus protecting the host (Glombitza et al. 2004; Teixeira et al. 2019; Yu and Keller 2005). Deutsch et al. (2021) revealed that the number of fungal endophytes isolated from the same genus of alga varied across different locations. In all algae at all locations, they found a much lower number of fungal isolates than of bacterial isolates. Vallet et al. (2018) demonstrated that fungal endophytes, which are found in association with macroalgae, could protect their host by producing bioactive metabolites. Recent research has focused on secondary metabolites produced by endophytic fungi because of their potential for antibacterial, antifungal, antioxidant, and anticancer activities. To defend against newly emerging pathogens and stimulants, the pharmaceutical and agriculture sectors are always searching for novel, biologically active compounds. To meet this requirement, it is critical to choose an appropriate starting source for natural products (Schulz et al. 2008). The current investigation aims to identify new sources of endophytic fungal-derived bioactive compounds. To serve this purpose, endophytic fungi were isolated from the marine macroalga *Ulva* sp. The prominent chemical constituents present in ethyl acetate extracts of the isolated endophytic fungi were screened by thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR) spectroscopic analysis, widely used as a convenient tool for initial qualitative and quantitative analysis of complex plant and fungal extracts (Chowdhury et al. 2016). The fungal crude extracts were also examined for antibacterial, antifungal, and antioxidant activities, as well as brine shrimp lethality bioassay.

2 Materials and methods

2.1 Collection, identification and extraction of seaweed

Fresh seaweed samples were collected in December 2020 from the coastline of Saint Martin's Island, Bangladesh, where *Ulva* sp. grows abundantly. The identification of the seaweed as *Ulva* sp. was based on the morphology. A sample specimen has been deposited at the Bangladesh National Herbarium (Accession no. DACB-64245). The air-dried seaweeds were dried at 40 °C for 24 h to reduce moisture content. The dried samples were ground and soaked in 100 % methanol for 7 days at room temperature to extract soluble materials, followed by another 5 days of extraction.

2.2 Isolation of endophytic fungi associated with *Ulva* sp.

The technique used by Chowdhury et al. (2016) was modified to isolate endophytic fungi from *Ulva* sp. To remove sand and adhering debris, the seaweed samples were coarsely washed with seawater from the collection site before being transported and processed in the lab within the shortest possible time. The seaweed samples were then subjected to surface sterilization, i.e., immersion of samples in ethanol (EtOH, 70 %), sodium hypochlorite solution (NaOCl, 5 %), and finally in EtOH (70 %) sequentially taking 1–2 min in each solution. To eliminate EtOH residues, all algal samples were then washed three times with sterile water. The selected samples were divided aseptically into small pieces (1–1.5 cm long), which were then put on the culture media supplemented with streptomycin sulphate (100 mg l^{-1}, to suppress bacterial contamination), and then incubated at room temperature in the dark. A few positive controls (media with unsterilized algal samples), a few negative controls (media without any algal samples) and some imprints of sterilized algal samples on the culture media were also incorporated to detect endophytic fungi and to test the effectiveness of the surface sterilization process. The culture medium was prepared by dissolving agar (16 g l^{-1}) in artificial seawater (Nagano et al. 2009; Robinson 1954) and autoclaving at 121 °C for 15 min. The hyphal tips that developed on the initial cultures were transferred to potato dextrose agar (PDA). Pure cultures of the isolated endophytic fungi were then obtained by serial dilution or streaking methods (Fergus 1964).

2.3 Identification of endophytic fungi associated with *Ulva* sp.

Endophytic fungi were identified taxonomically based on macroscopic and microscopic morphological characterization and molecular identification. The morphological characteristics of each isolate, including the rate of growth, mycelium depth and hyphal orientation, colour, texture, elevation, margin, diameter and form of the colony, as well as spore view, were observed after 3, 6, 9 and 12 days. For molecular identification of fungi, genomic DNA was extracted and the entire Internal Transcribed Spacer region (ITS1, 5.8S, ITS2) was amplified and sequenced (Martin and Rygiewicz 2005). At first, a colony from 4 to 7 days of pure culture of each fungus was selected, scratched with a sterile surgical blade and ground to make powder with liquid nitrogen using a pestle and mortar. Then, DNA was isolated using the Maxwell® 16 LEV Plant DNA Kit (AS1420, Promega, USA) (Cappuccino and Sherman 1996). DNase-free RNase (30 min at 37 °C) was used to remove RNA contamination from the isolated DNA, and this was then stored at −20 °C for further analysis. Then, the ITS region from the isolated DNA was amplified by PCR (Raja et al. 2017), followed by nucleotide sequencing of the amplicons by the Sanger dideoxy sequencing method.

Raw sequence data was processed using BioEdit 7.2 and then compared to those available in the rRNA/ITS database of the National Center for Biotechnology Information (NCBI) GenBank. The nucleotide sequence of each isolate of the present study was tested for its relatedness with similar sequences using the Basic Local Alignment Search Tool (BLAST). Based on total score, query cover and percent identity, the selected sequences were grouped into distinct clades by constructing the phylogenetic tree. The phylogenetic tree was constructed using the Maximum Likelihood method and the Tamura-Nei model (Tamura and Nei 1993) conducted in MEGA-X software (Kumar et al. 2018). The support of each node was assessed using a bootstrap technique with 1000

iterations, and the tree was scaled with branch lengths denoting the number of substitutions per site. A subculture of each isolate has been deposited at the BCSIR Laboratories, Dhaka, Bangladesh.

2.4 Preparation of fungal crude extracts associated with *Ulva* sp.

Each endophytic fungal isolate was cultured and incubated at 28 ± 2 °C in the dark. The culture medium was prepared by dissolving PDA (39 g l^{-1}) in artificial seawater and autoclaving at 121 °C for 15 min. After 21–28 days, the media with fungal metabolites were frozen at −20 °C after observing the highest mycelial growth. When thawed, the aqueous part of the fungal culture was separated from the fungal mycelia by filtration and then extracted with chloroform three times using a separating funnel. The fungal mycelia were soaked with ethyl acetate. The EtOAc extract (organic part) was prepared by filtration after 7 days and subsequent solvent evaporation at 5-day intervals using a rotary evaporator at 40 °C (Chowdhury et al. 2017; Khan et al. 2016). The dried fungal crude extracts were kept at 4 °C until further analysis.

2.5 Antimicrobial assay

The antimicrobial activity of the crude extracts was assessed by the disc diffusion method described by Bauer et al. (1966) with some modifications. The crude fungal extracts (2 mg) were dissolved in 200 µl of dichloromethane (DCM), and each disc in the agar plate received 10 µl (i.e., 100 µg $disc^{-1}$) of the prepared extract solution. Two Gram-positive bacterial strains, *Staphylococcus aureus* (ATCC 9144), *Bacillus megaterium* (ATCC 13578) and three Gram-negative bacterial strains, *Escherichia coli* (ATCC 11303), *Salmonella typhi* (ATCC 13311), *Pseudomonas aeruginosa* (ATCC 27833) were applied for the antimicrobial assay as pure cultures obtained from the Institute of Food Science and Technology (IFST), BCSIR Dhaka Laboratories, Bangladesh. Two pure fungal strains, *Aspergillus niger* (ATCC 1004) and *Aspergillus flavus* (UCFT 02), were collected from International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B). The experiment was carried out in triplicate. The mean zone of inhibition (in mm) of each extract was compared with that of two standards, antibacterial agent kanamycin (30 µg $disc^{-1}$) and antifungal agent ketoconazole (30 µg $disc^{-1}$). Here, DCM (10 µl $disc^{-1}$) was used to observe if there was any solvent effect on microorganisms.

2.6 Antioxidant activity

Using a slightly modified version of the approach published by Brand-Williams et al. (1995), the antioxidant capacity of the fungal crude extracts was assessed through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The antioxidant capacity of each extract was compared with that of two standards, the potent antioxidants ascorbic acid (vitamin C) and butylated hydroxyanisole (BHA). The crude extracts (1.6 mg) were dissolved in methanol (MeOH, 400 µl) and serially diluted to obtain 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.56 and 0.78 µg ml^{-1} concentrations. The extract solutions (2 ml) were mixed with 2 ml of a solution of DPPH in MeOH (20 µg ml^{-1}). Before measuring the absorbance at 517 nm, the mixture was kept in the dark at room temperature for 30 min. Scavenging activity (%) was measured using the equation below. Eventually, inhibitory concentration 50 (IC_{50} values, µg ml^{-1}), i.e., the concentration of each extract that reduced the DPPH absorbance by 50 % was estimated as a measure of their capacity to scavenge the radical:

$$\text{Scavenging activity (\%)} = \left[(A_{\text{blank}} - A_{\text{Sample}})/A_{\text{blank}} \right] \times 100$$

2.7 Brine shrimp lethality bioassay

The cytotoxicity of the fungal crude extracts was evaluated on brine shrimp nauplii following the technique reported by Meyer et al. (1982). After dissolving in 200 µl DMSO, the extracts (4 mg) were serially diluted to concentrations of 400, 200, 100, 50, 25, 12.50, 6.25, 3.125, and 1.56 µg ml^{-1}. Each test solution was added to 5 ml of simulated brine water containing 10 shrimp nauplii, and the test tubes were then maintained at room temperature for 24 h. Lethal concentration 50 (LC_{50} values, µg ml^{-1}), i.e., the concentration of each extract that caused a 50 % lethality of the shrimp nauplii, were compared with that of vincristine sulphate. The brine shrimp lethality bioassay is considered a convenient tool for preliminary toxicity assessment.

2.8 Preliminary chemical screening

The crude extracts of seaweed and its associated endophytic fungi were screened through TLC and NMR spectroscopic analysis for the preliminary visualization of chemical constituents. TLC separations were performed on pre-coated silica gel 60, PF_{254}, 0.2 mm aluminium foil (Macherey-Nagel, Germany), at 20 % ethyl acetate in toluene. Spots were detected using UV Lamp (Analytik Jena US, USA) at 254 and 365 nm and then 1 % vanillin-sulphuric acid as the spray reagent followed by heating for 5 min at 110 °C (Chowdhury et al. 2016). NMR spectra were recorded using a Bruker Ascend™ 600 spectrometer at room temperature. The chemical shifts are reported in ppm relative to residual solvent peaks.

2.9 Data and statistical analysis

All the bioactivity tests were repeated three times, and data were documented in triplicate. The IC_{50} and LC_{50} values were calculated using logistic regression. To identify significant changes relative to the standards, the data from this study were homoscedasticity-analysed, and then a one-way analysis of variance (ANOVA) was performed. Differences between means were accepted as significant at $p < 0.05$, and were then subjected to pairwise comparisons of group means using Tukey's *post hoc* test. The values are shown as mean ± standard deviation. Calculations and graphs were prepared using Microsoft Excel software.

3 Results

3.1 Identification of *Ulva* sp.

The seaweed was identified as *Ulva* sp. based on morphological characterization (Kipp et al. 2022; Peasura et al. 2015). The macroscopic characteristics of *Ulva* sp. revealed a vivid grass-green tubular frond and unbranched thalli throughout.

3.2 Endophytic fungi associated with *Ulva* sp.

The isolation and identification of fungal endophytes from *Ulva* sp. revealed six different fungi that were most closely related to *Chaetomium globosum, Nigrospora magnoliae, Curvularia* sp., *Curvularia moringae, Aspergillus terreus* and *Collariella* sp. (Table 1).

The isolate UE-1 (Figure 1) was a rapidly growing endophytic fungus. The surface of the colony was cottony and white initially, becoming greyish-olive with age. The reverse of the colony was yellowish to reddish brown. The colony diameter on PDA was approximately 7.8–8.0 cm at 28 ± 2 °C after 6 days in culture. Hyphae were septate with large, oval-shaped, brown-coloured ascomata with wavy filamentous hairs. Asci were stalked and club-shaped, containing ascospores. Ascospores were limoniform, single-celled and yellowish-brown. The colony morphology and microscopic observations suggested the identity of the isolate UE-1 to be a species of *Chaetomium* (Wang et al. 2016), which was confirmed by the nucleotide BLAST report generated by NCBI database (76.4 % similarity to BLAST best hit with 100 % query coverage). *Chaetomium globosum* (Accession no. NR 144851.1, connected with the marine environment) was discovered in the phylogenetic tree to be the closest to the isolate UE-1, with a bootstrap support of 92 %. Thus, the isolate UE-1 was recognized as *Chaetomium globosum*, and its morphology matched that of the relevant species.

The endophytic fungus UE-2 was initially white, becoming grey with abundant aerial mycelia (Figure 2). Black areas of conidiation appeared with age. Initially, the reverse of the colony was white and gradually became black. Colonies on PDA reached 7.5–7.8 cm diameter after 6 days at 28 ± 2 °C. Mycelia were superficial and immersed, composed of septate, branched, hyaline and brown hyphae with smooth and thick walls. Conidiophores were short, swelling and tapering at the point of conidium formation. The conidia were dark brown and almost round, slightly flattened. Morphological characteristics indicated that UE-2 was a

Table 1: Morphological and molecular identification of endophytic fungi associated with *Ulva* sp.

Isolate	**UE-1**	**UE-2**	**UE-3**	**UE-4**	**UE-5**	**UE-6**
Feature	**Proposed fungal taxon**					
	Chaetomium globosum	***Nigrospora magnoliae***	***Curvularia* sp.**	***Curvularia moringae***	***Aspergillus terreus***	***Collariella gracilis* or *Collariella virescens***
Growth rate	Rapid	Rapid	Moderate	Moderate	Moderate	Moderate
Diameter after 6 days (approx.)	7.8–8.0 cm	7.5–7.8 cm	4.5–5.0 cm	2.5–3.5 cm	3.0–3.5 cm	3.0–3.5 cm
Hyphae	Aerial, surficial, submerged	Aerial, surficial, submerged	Surficial, submerged	Surficial, submerged	Surficial, submerged	Surficial, submerged
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow	Shallow	Shallow
Form of colony	Filamentous	Irregular	Circular	Irregular	Filamentous	Circular or sometimes irregular
Colour of surface	White initially, becoming greyish olive with maturity	White initially, becoming grey with age	Light pink initially, gradually turned into black	Grey to black	White initially, then yellow, finally cinnamon brown	Translucent to off white
Colour of reverse	Yellowish to reddish brown	White to black	Same as the surface	Dark grey	Yellow to brown	Same as the surface
Texture of colony surface	Cottony	Woolly	Woolly	Woolly	Granular	Moist
Elevation of colony	Crateriform	Umbonate	Umbonate	Raised	Flat	Flat
Margin of colony	Filiform	Undulate	Entire	Undulate	Filiform	Entire or slightly undulate
Microscopic observation (spore view at)	28 days	21 days	7 days	7 days	5 days	45 days
BLAST (ITS sequence) Total score	211	889		1016	1110	795 or 789
BLAST (ITS sequence) Query cover/ identity (%)	100/76.4	90/98.2		95/97.8	96/100	88/94.3 or 88/94.1
Bootstrap support value (%)	92	85		88	66	61
GenBank accession no.	OR296827	OR335098		OR335205	OR335206	OR335210

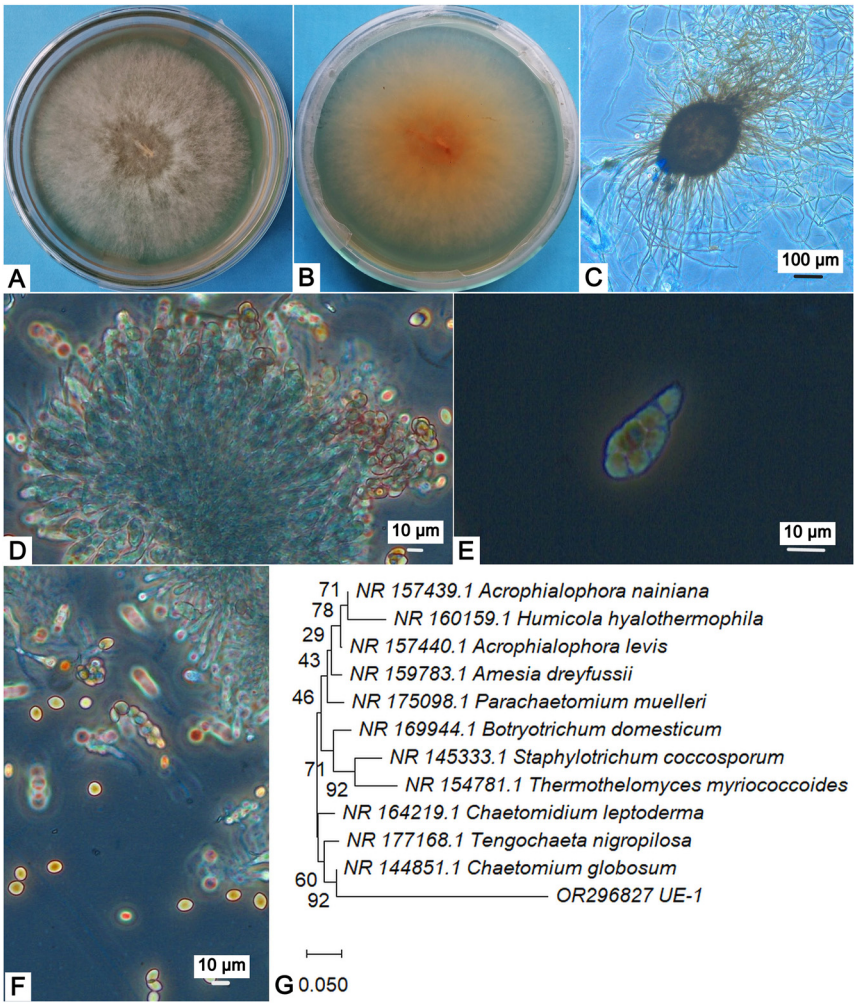

Figure 1: Isolate UE-1 (*Chaetomium globosum*). (A) Surface of colony, on potato dextrose agar after 6 days culture at 28 °C. (B) Reverse of colony. (C) Ascomata after 28 days culture. (D) Asci. (E) Ascus with ascospores. (F) Ascospores. (G) Phylogenetic tree inferred from internal transcribed spacer sequences using maximum likelihood method.

species of *Nigrospora* genus (de Silva et al. 2021). The nucleotide BLAST result showed 98.2 % similarity to its best hit with 90 % query coverage. Phylogenetic analysis revealed that this isolate was most closely related to *Nigrospora magnoliae* (Accession no. NR_172443.1, associated with terrestrial plants), sharing a monophyletic clade with a reasonably good bootstrap support of 85 % (Hillis and Bull 1993). As a result of phylogenetic analysis and morphology, isolate UE-2 was identified as *Nigrospora magnoliae* when compared to other *Nigrospora* species.

The isolate UE-3 initially appeared as a pinkish colony, growing moderately and gradually turning black; the reverse was the same as the surface (Figure 3A–C). The colony diameter on PDA was approximately 4.5–5.0 cm at 28 ± 2 °C after 6 days of culture. The colony was circular with an entire margin and a woolly texture. Hyphae were long, branched, septate and dark. At the site of conidium development, conidiophores were either simple and straight, branching and curved, or knobby. Conidia were big, typically had four cells, and ultimately took on a curved appearance from the enlargement of a central cell after 5–7 days of ageing. Conidia vary from those of *Bipolaris* sp. in that they have a darker central cell than the peripheral cells, a finer cell wall, thinner septa between cells, and a distinctive curve developed with maturity. These morphological characteristics indicated UE-3 as *Curvularia* sp. (Walsh et al. 2018).

The surface colour of isolate UE-4 was grey or black with a woolly surface (Figure 3D–F). The reverse of the colony was dark. The growth rate was moderate; the colony diameter on PDA was approximately 2.5–3.5 cm at 28 ± 2 °C after 6 days of culture. Microscopic examination revealed mycelia and conidiophores with conidia, similar to those of isolate UE-3. Moreover, the nucleotide BLAST report generated from the ITS sequence of isolate UE-4 supported this assumption and showed 97.8 % similarity to BLAST best hit with 95 % query coverage. The phylogenetic tree clearly showed that UE-4 was closely related to *Curvularia moringae* (Accession no. NR_171998.1, associated with terrestrial plant) with a bootstrap value of 88 %, indicating a well

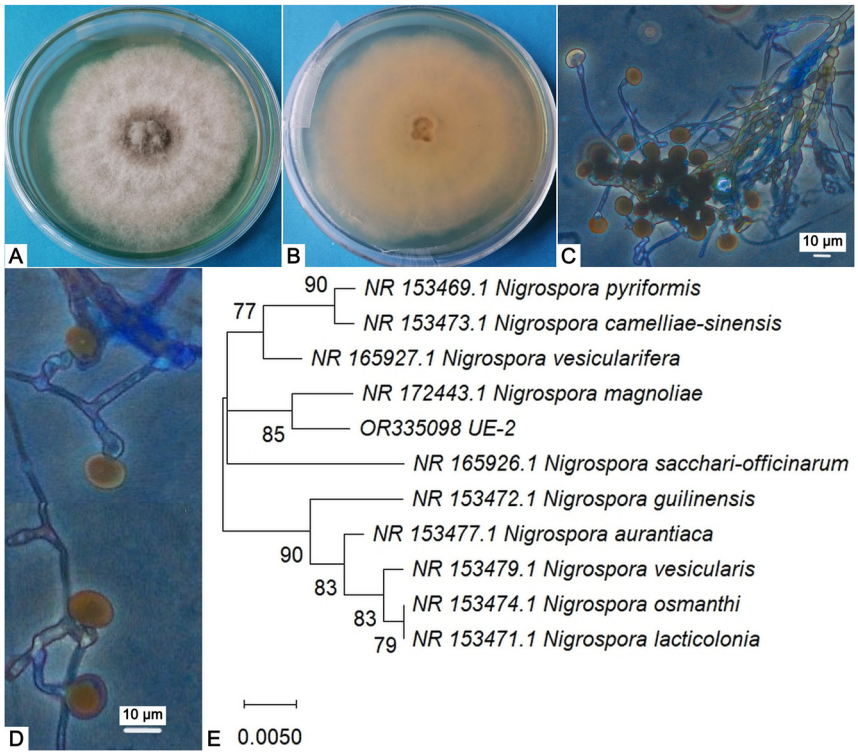

Figure 2: Isolate UE-2 (*Nigrospora magnoliae*). (A) Surface of colony, on potato dextrose agar after 6 days culture at 28 °C. (B) Reverse of colony. (C) Mycelia and conidia after 21 days culture. (D) Conidiogenus cells with conidia. (E) Phylogenetic tree inferred from internal transcribed spacer sequences using maximum likelihood method.

supported monophyletic clade. Given these observations, UE-4 was specified as *Curvularia moringae* among the other genetically similar *Curvularia* species.

The isolate UE-5 was a filamentous endophytic fungus with a powdery texture (Figure 4). Its growth rate was moderate, reaching 3.0–3.5 cm diameter on PDA at 28 ± 2 °C after 6 days of culture. The surface of the colony was at first white, and then a shade of yellow spreading from the centre and finally turned into cinnamon brown. The reverse of the colony was yellow to brown. Hyphae were septate along with short and smooth conidiophores. Phialides and metulae with chains of conidia were arranged solely on the top half of the vesicle, compact and column-like. Conidia were smooth and rounded. Microscopic and colony morphology indicated that the isolate was a species of *Aspergillus* (Walsh et al. 2018). The nucleotide BLAST result showed 100 % similarity to its best hit with 96 % query coverage. In the phylogenetic tree, UE-5 had the closest evolutionary relationship with *Aspergillus terreus* (Accession no. NR_131276.1, associated with indoor dust), forming a monophyletic clade with a bootstrap support of 66 %. Thus, the molecular verification distinguished UE-5 as *Aspergillus terreus* among other *Aspergillus* species.

Colonies of the isolate UE-6 on PDA (Figure 5A–C) were translucent to off-white, with an entire or slightly undulate edge, about 3.0–3.5 cm diameter at 28 ± 2 °C after 6 days of culture, and irregularly produced white aerial hyphae and dark ascospores later; the reverse was the same as the surface view. Ascomatal hairs were brown, septate, relatively seta-like or flexuous. Ellipsoidal ascospores were brown. In phylogenetic analysis, UE-6 belonged to a monophyletic clade along with two highly similar species, *Collariella gracilis* and *Collariella virescens*, supported by a bootstrap value of 61 % and the best hits in the nucleotide BLAST with 88 % query coverage showed 94.3 % and 94.1 % similarity, respectively, to these species. The two *Collariella* species shared a 98 % bootstrap support value, as shown in Figure 5D. According to von Arx et al. (1986), these *Collariella* species are very similar, and differ only in ascomata and ascospores. Due to morphological and molecular similarities, these species were previously known as *Chaetomium*. Isolate UE-6 was phylogenetically identified as either *Collariella gracilis* or *Collariella virescens* (Accession no. NR_147670.1 and NR_147671.1, respectively, associated with soil and rock), whose morphology was also found to be similar to the isolate (Wang et al. 2016).

3.3 Biological activities of fungal crude extracts

In antimicrobial, antioxidant and brine shrimp lethality bioassays, screening secondary metabolites generated by several marine endophytic fungi under the same fermentation

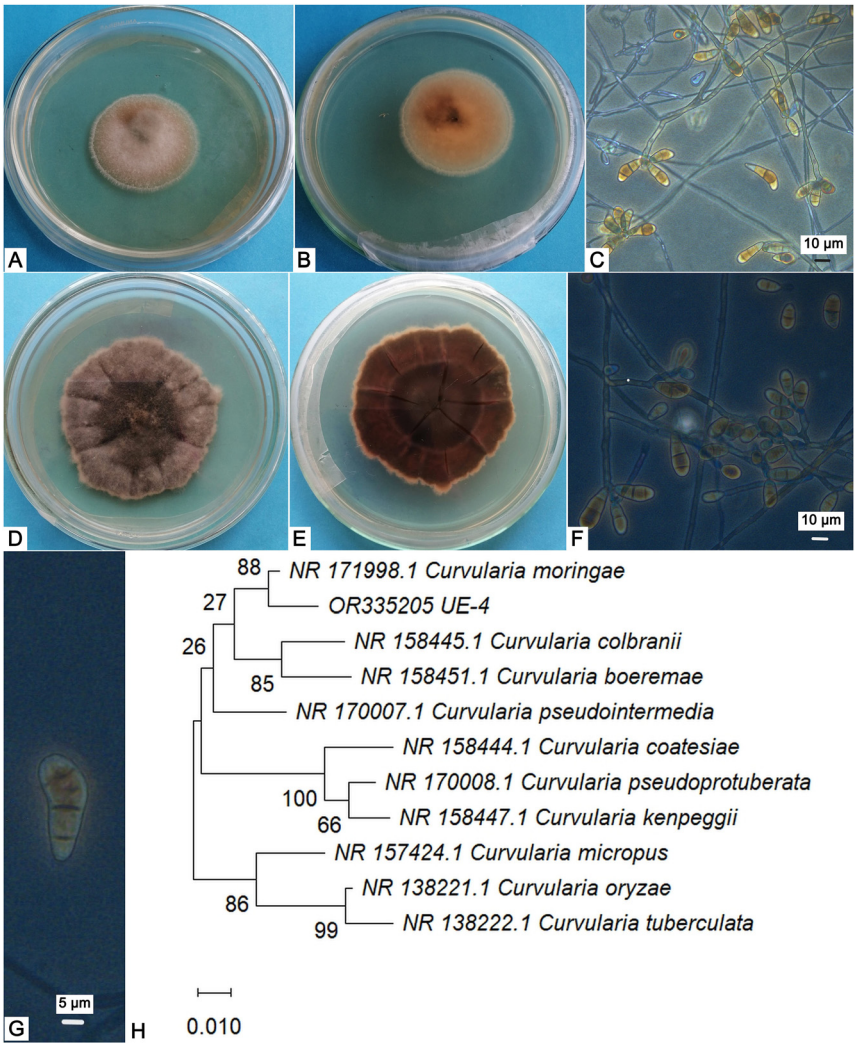

Figure 3: Isolates UE-3 (*Curvularia* sp., A–C) and UE-4 (*Curvularia moringae*, D–H). (A) Surface of colony, on potato dextrose agar (PDA) after 6 days culture at 28 °C. (B) Reverse of colony. (C) Mycelia and conidia after 7 days culture. (D) Surface of colony, on PDA after 12 days culture at 28 °C. (E) Reverse of colony. (F) Mycelia and conidia after 7 days culture. (G) Conidium. (H) Phylogenetic tree inferred from internal transcribed spacer sequences using maximum likelihood method.

conditions revealed that each fungal crude extract demonstrated one or more pertinent biological activities.

3.3.1 Antimicrobial assay

The study of the fungal crude extracts' capabilities against pathogenic microbes revealed that most extracts showed some inhibition of single or multiple microbial growth, although kanamycin and ketoconazole exhibited the most potent inhibitory activity against bacteria and fungi, respectively (Figure 6). However, most extracts were more potent against bacterial strains than fungal ones, and UE-2 was the only extract to inhibit the growth of both bacteria and fungi. The solvent (dichloromethane) exhibited no zone of inhibition, as expected. From a broad perspective, the extracts of isolates UE-1, -4, and -6 inhibited the growth of *Bacillus megaterium* and *Escherichia coli* more effectively. UE-6 showed the highest antibacterial activity (14.33 mm) against *Salmonella typhi* growth. UE-1, classified as *Chaetomium globosum*, provided the only extract that inhibited the growth of *Pseudomonas aeruginosa*.

3.3.2 Antioxidant activity

The DPPH radical scavenging capacity of the fungal crude extracts of marine endophytes derived from *Ulva* sp. was compared with the potent antioxidants ascorbic acid and BHA, which exhibited IC_{50} values of 10.67 ± 0.04 and 7.16 ± 0.07 µg ml^{-1}, respectively (Figure 7A). Among all the extracts examined, those of UE-1 and UE-5 also had low IC_{50} values (21.58 ± 0.06 and 17.80 ± 0.06 µg ml^{-1}, respectively) for scavenging DPPH radical, but the extract of UE-6 had only mild activity as a DPPH radical scavenger at the experimental concentration, while the other extracts had almost no scavenging activity (IC_{50} > 500 µg ml^{-1}).

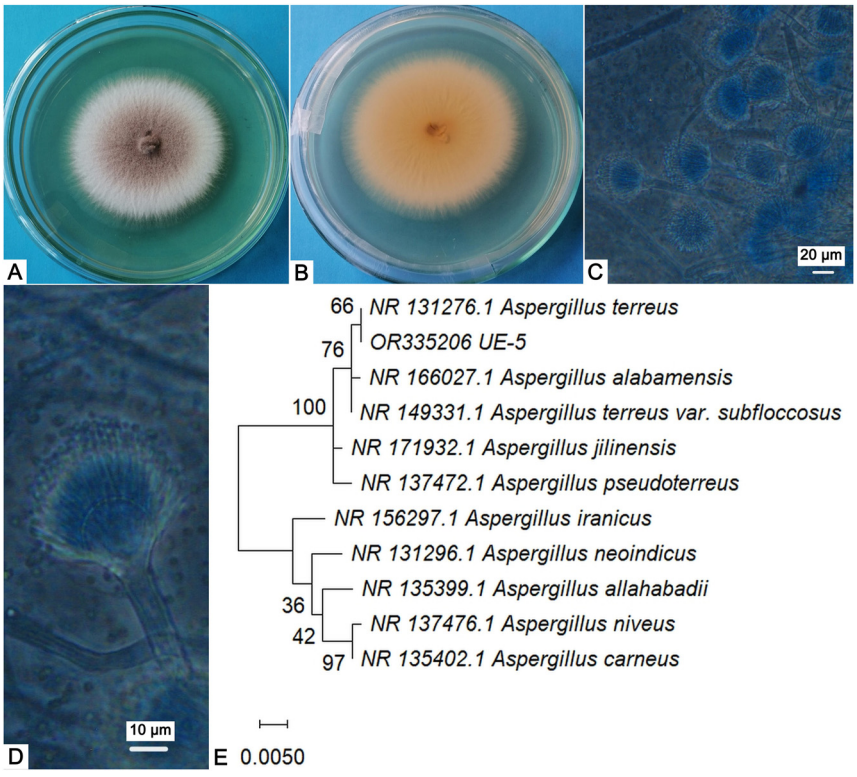

Figure 4: Isolate UE-5 (*Aspergillus terreus*). (A) Surface of colony, on potato dextrose agar after 6 days culture at 28 °C. (B) Reverse of colony. (C) Mycelia, conidiophores and conidia after 5 days culture. (D) Conidiophore with conidia. (E) Phylogenetic tree inferred from internal transcribed spacer sequences using maximum likelihood method.

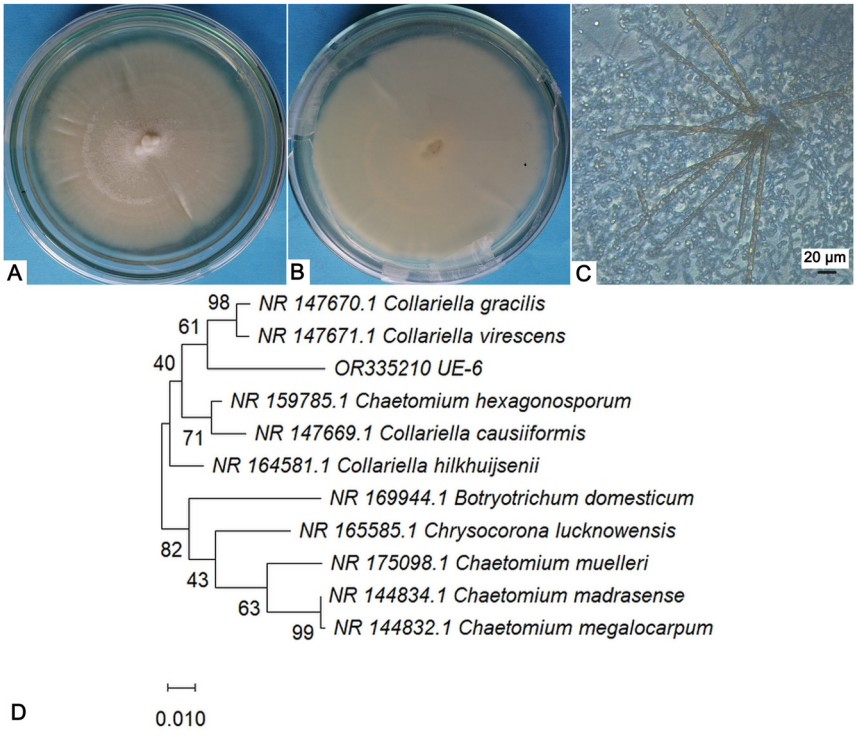

Figure 5: Isolate UE-6 (*Collariella* sp.). (A) Surface of colony, on potato dextrose agar after 12 days culture at 28 °C. (B) Reverse of colony. (C) Terminal ascomatal hairs with ascospores after 45 days culture. (D) Phylogenetic tree inferred from internal transcribed spacer sequences using maximum likelihood method.

3.3.3 Brine shrimp lethality bioassay

All fungal crude extracts associated with *Ulva* sp. demonstrated cytotoxic properties, according to the brine shrimp lethality bioassay relative to the potent cytotoxic agent vincristine sulphate (LC_{50}, 5.64 ± 0.65 µg ml^{-1}; Figure 7B). Four of the six extracts showed moderate lethality on brine shrimp nauplii (LC_{50}, range from 16 to 20 µg ml^{-1}). However,

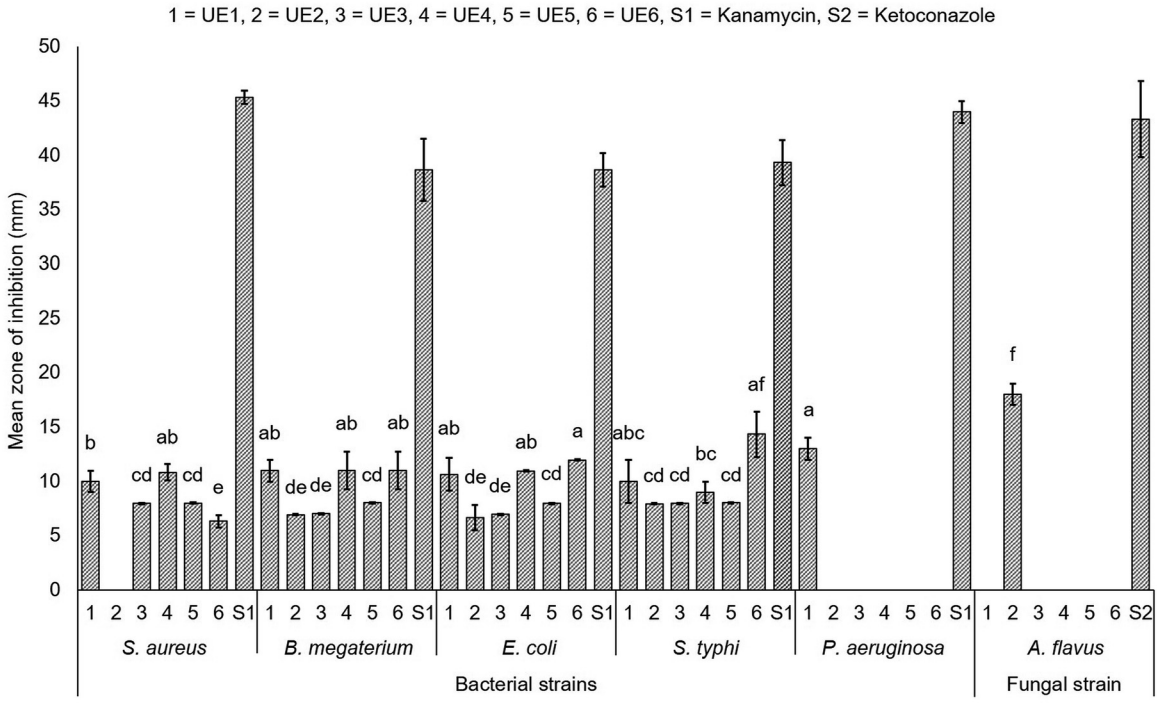

Figure 6: Antimicrobial activity of the crude extracts obtained from marine endophytic fungi associated with *Ulva* sp. against five bacteria (*Staphylococcus aureus*, *Bacillus megaterium*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*) and one fungus (*Aspergillus flavus*). Values are mean ± standard deviation, $n = 3$. Bars with different letters are significantly different according to Tukey's *post hoc* test at $p = 0.05$. Note: The solvent control (dichloromethane) showed no inhibition (0 mm). The strongest inhibitory effects were observed with the positive controls kanamycin (S1) and ketoconazole (S2).

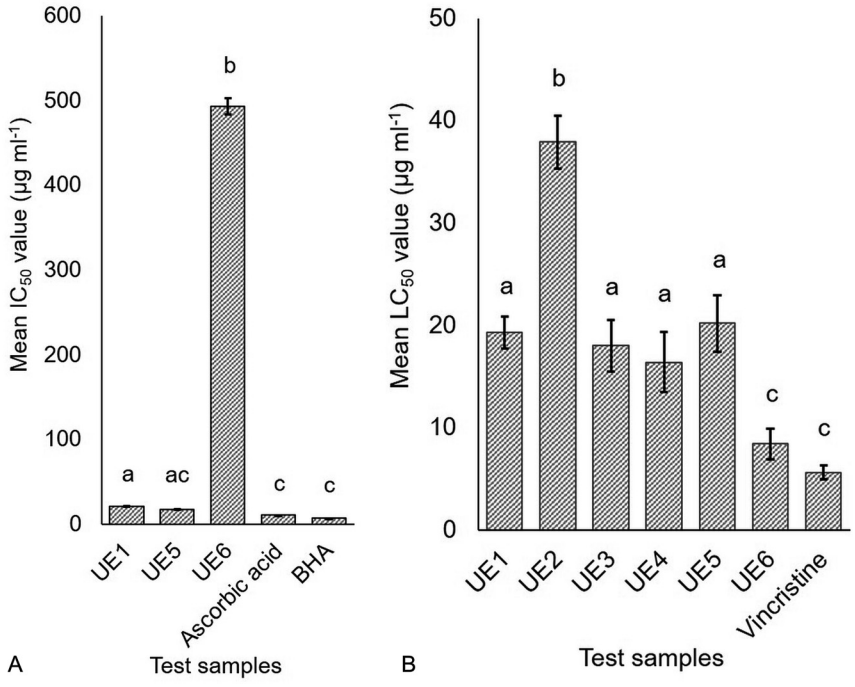

Figure 7: Antioxidant and cytotoxic potential of the crude extracts of fungi associated with *Ulva* sp. (A) DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, and (B) brine shrimp lethality bioassay. Values are mean ± standard deviation, $n = 3$. Bars with different letters are significantly different according to Tukey's *post hoc* test at $p = 0.05$.

the extract of UE-6 exhibited strong cytotoxicity with a LC_{50} value of 8.43 μg ml^{-1}, whereas the extract of UE-2 showed much milder cytotoxicity (LC_{50}, 37.91 μg ml^{-1}).

3.4 Preliminary chemical screening of crude extracts

Crude extracts were subjected to TLC and NMR spectroscopy for preliminary screening of secondary metabolites (Figures S1–S12 and Table S1). Analysis of the TLC spots and the chemical shifts in the spectra (^{1}H NMR and ^{13}C NMR) of the fungal crude extracts revealed the possible presence of diverse secondary metabolites such as sterols, terpenoids, flavonoids, isocoumarins, anthocyanins, anthraquinones and naphthoquinones or their derivatives.

4 Discussion and conclusion

4.1 Endophytic fungi associated with *Ulva* sp.

Ulva sp., the green seaweed found abundantly on the coastline, is also populated by endophytic fungal taxa, just like other seaweeds. The identification of *Ulva* sp. was carried out using morphological analysis. The present investigation revealed six different endophytic fungal isolates associated with *Ulva* sp. The identity of the isolates of *Ulva* sp. was established by definitive microscopic characteristics followed by macroscopic (phenotypic) observations. The most closely related species was selected more definitively using molecular (genotypic) analysis, such as by comparing the ITS sequence of fungal DNA (Schoch et al. 2012). Table S2 briefly describes the BLAST best hits of the isolates. Many fungi were misidentified previously due to phenotypic variations (Sarwar et al. 2019; Weiß and Göker 2011). Thus, the phylogenetic analysis of DNA sequence data along with morphological characterization has been proved to be an appropriate way to identify fungi correctly (Horiike 2016; Wang et al. 2022). The identification of six isolates derived from *Ulva* sp. revealed six different fungal species from five genera (*Chaetomium*, *Nigrospora*, *Curvularia*, *Aspergillus* and *Collariella*; Table 1) belonging to four classes, namely, Euascomycetes, Sordariomycetes, Dothideomycetes and Eurotiomycetes.

This is the first report of fungal endophytes in *Ulva* sp. collected from Saint Martin's Island in the Bay of Bengal, Bangladesh. However, very few reports are available on the diversity of endophytic fungi in green seaweeds. *Chaetomium* sp., *Phomopsis* sp., *Acremonium* sp., *Aspergillus niger* and *Cladosporium* sp. were isolated from *Ulva lactuca* collected from Kovalam (covelong), Chennai (Ahamed and Murugan 2019), whereas *Fusarium semitectum*, *Paecilomyces lilacinus*, *Aspergillus flavus*, *Penicillium expansum*, *P. roqueforti*, *Rhizopus* sp. and *Pythium* sp. were isolated from the same seaweed species from Johor, Malaysia (Zainee et al. 2021). Other studies have also reported the fungal endophytes *Acremonium fuci*, *Chaetomium globosum*, *Emericellopsis enteromorphae*, *E. phycophila*, *Monodictys putredinis*, *Parasarocladium alavariense*, *P. fusiforme* and *Penicillium* sp. from marine green macroalgae (Gonçalves et al. 2019; Singh et al. 2018). The fungal species isolated in this study have been reported previously from a diverse array of macroalgal hosts (Flewelling et al. 2015), except that *Nigrospora magnoliae* was first isolated from *Magnolia* plants collected in China and Thailand (de Silva et al. 2021), and this is the second report of an endophytic isolate characterised as *N. magnoliae*.

4.2 Biological activities of fungal crude extracts

The present work reflects the first evaluation of the crude extracts of six endophytic fungi associated with *Ulva* sp. from the Bay of Bengal, Bangladesh. Overall biological activities are summarized in Table 2. The extract of isolate UE-1, identified as *Chaetomium globosum*, was the most promising amongst all the evaluated extracts, demonstrating moderate antioxidant and brine shrimp lethality activity, and also inhibited the growth of all five pathogenic bacterial strains. The extract of isolate UE-2 appeared to have moderate antifungal and weak antibacterial and cytotoxic activities but had no antioxidant effect. The inhibition zones of the crude extracts may appear low in comparison with those of standards (Figure 6), but these zones may be increased if the specific compounds responsible for antimicrobial potential are later identified and analysed. Thus, isolate UE-2, recognized as *N. magnoliae*, yielded a noteworthy extract and requires further investigation as this species is only recently known to researchers and there is no prior information on its bioactivity. However, antifungal activity of *N. magnoliae* has been revealed for the first time in the present study. Both extracts of *Curvularia* sp. (isolates UE-3 and -4) exhibited moderate brine shrimp lethality and mild to moderate inhibition of bacterial growth but showed neither antifungal nor antioxidant activities. Additionally, isolate UE-5, identified as *Aspergillus terreus*, revealed prospective antioxidant (IC_{50}, 17.80 μg ml^{-1}) and cytotoxic (LC_{50}, 20.20 μg ml^{-1}) activity. Nevertheless, this extract had a weak inhibitory effect on bacterial growth (approx. 8 mm inhibition zone). Lini et al. (2020) reported the antioxidant (IC_{50},

Table 2: Overview of antimicrobial activity against five bacteria (*Staphylococcus aureus*, *Bacillus megaterium*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*) and one fungus (*Aspergillus flavus*), antioxidant activity and brine shrimp lethality bioassay of the crude extracts obtained from the marine endophytic fungi associated with *Ulva* sp.

Biological activity	Fungal crude extract[a]					
	UE-1	UE-2	UE-3	UE-4	UE-5	UE-6
	Chaetomium globosum	*Nigrospora magnoliae*	*Curvularia* sp.	*Curvularia moringae*	*Aspergillus terreus*	*Collariella* sp.
Staphylococcus aureus	++	–	+	++	+	+
Bacillus megaterium	++	+	+	++	+	++
Escherichia coli	++	+	+	++	+	++
Salmonella typhi	++	+	+	+	+	++
Pseudomonas aeruginosa	++	–	–	–	–	–
Aspergillus flavus	–	++	–	–	–	–
Antioxidant activity	++	–	–	–	++	+
Brine shrimp Lethality bioassay	++	+	++	++	++	+++

[a]'+++', '++' and '+' indicate strong, moderate and mild bioactivity, respectively; '–' no activity detected.

20.46 µg ml^{-1}), cytotoxic (LC_{50}, 2.85 µg ml^{-1}), and antibacterial (11–12 mm inhibition zone) activity of an ethyl acetate extract of *Aspergillus* sp. isolated from the green seaweed *Caulerpa peltata*. Isolate UE-6, identified as *Collariella* sp., produced an effective extract that might benefit from continued pharmaceutical development, since it exhibited powerful cytotoxicity (LC_{50}, 8.43 µg ml^{-1}), with slight antioxidant capacity and mild to moderate antibacterial effects. The IC_{50} and LC_{50} plots are presented in Figures S13 and S14, respectively.

Bioactivity screening of endophytic fungal extracts derived from macroalgal sources opens the door to future possibilities. Antibacterial, antifungal and antioxidant activities have been revealed in several endophytic fungi (e.g., *Aspergillus* sp., *Candida* sp., *Chaetomium* sp., *Curvularia* sp., *Nigrospora* sp., *Penicillium* sp., *Pichia* sp., *Pythium* sp. and *Rigidoporus* sp.) associated with seaweeds (e.g., *Caulerpa scalpelliformis*, *Chaetomorpha* sp., *Dictyota dichotoma*, *Euchema* sp., *Gracilaria edulis*, *Halymenia* sp., *Sargassum* sp., *Stoechospermum marignatum* and *Ulva* sp.) (Ahamed and Murugan 2019; Ravindran and Naveenan 2011; Suryanarayanan et al. 2010; Vega-Portalatino et al. 2023). Teixeira et al. (2019) revealed antitumour compounds in endophytic fungi derived from green macroalgae, such as, *Aspergillus versicolor*, *Chaetomium globosum*, *Coniothyrium cereal*, *Gibberella zeae* and *Penicillium* sp.

Even though there has been a lot of bioprospecting research on endophytic fungi associated with seaweeds, we still know very little about the diversity of fungi in marine ecosystems, the bioactive potential of fungal species, and the broader taxonomic groups that can be found in the Bay of Bengal, Bangladesh (Lini et al. 2020). The biological activities screened in this study have not been tested before with endophytic fungi associated with *Ulva* sp. The crude extracts from these isolates, cultured under identical circumstances, showed some notable variations in the bioactivity. Moreover, based on the variety in the bioactivity of endophytic fungi described here, exploring additional potential bioactivities and assessing how the fungal growth environment affects the generation of its secondary metabolites is crucial.

4.3 Preliminary chemical screening of crude extracts

The evaluation of TLC profiles and NMR spectroscopic data of crude extracts has revealed the existence of diverse metabolites. Visualization of multiple spots on the TLC plate (Figure S1) and ^{1}H NMR and ^{13}C NMR experiments (Table S1, Figures S1–S12) has indicated substance classes such as anthraquinones, naphthoquinones, anthocyanins (Chowdhury et al. 2017; Khan et al. 2018), terpenoids, steroids (Cohen et al. 2011; Ericsson and Ivonne 2009), flavonoids (Sohrab et al. 2004), isocoumarins (Krohn et al. 2004) and their derivatives (Harborne 1998; Liu 2021; Mahmud et al. 2020). The further isolation of prospective metabolites could be guided by the TLC profile or NMR resonance peaks of the crude extracts. Different crude extracts in this experiment developed different patterns on the TLC plate and NMR spectra, which

might be promising contributors to bioactive metabolites on further analysis.

Our study focused on the isolation of the endophytic fungal inhabitants of *Ulva* sp., a marine green macroalga found abundantly in the Bay of Bengal, Bangladesh, and the recognition of the most bioactive fungal species by performing preliminary analyses of their crude extracts. Six distinctive fungal endophytes were isolated from *Ulva* sp. for the first time in Bangladesh. In addition, the isolates were identified by comparing their ITS sequences with the most closely related species. Most of these isolates (except UE-5) also showed greater or smaller dissimilarities from their BLAST best hits to the nearest species. It would be worth investigating their taxonomy further in case the species isolated have not been sequenced or described before because the taxonomy of marine fungi is still understudied (Calabon et al. 2023; Pang et al. 2023; Pham et al. 2021; Weigand et al. 2019).

Although the seaweed *Ulva* sp. was identified based on morphology, a molecular analysis to determine the exact species is needed. Moreover, the preliminary analyses suggest that fungal endophytes from *Ulva* sp. produce secondary metabolites with a wide range of biological properties. The variety and potential of the bioactivities and chemical profiles displayed by the crude extracts of isolates related to *Nigrospora magnoliae, Chaetomium globosum* and *Curvularia moringae* have demonstrated the most attractive candidates for further analysis. Subsequent investigations should be conducted to unveil other potential pharmacological activities of these fungal crude extracts of marine origin. Future research should also focus on the determination of bioactive compounds from the host seaweed and its associated fungi.

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Bionotes

Sadia Noor
Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka, Bangladesh
Department of Pharmacy, University of Asia Pacific, Dhaka, Bangladesh
noorsadia.du@uap-bd.edu

Sadia Noor is a PhD student at the Department of Pharmaceutical Chemistry of University of Dhaka and an assistant professor at the Department of Pharmacy of University of Asia Pacific, Dhaka, Bangladesh. Her doctoral research focuses on the investigation of bioactive secondary metabolites from endophytic fungi associated with seaweeds located in the Bay of Bengal of Bangladesh. Currently she is doing her research work at Pharmaceutical Sciences Research Division, BCSIR Dhaka Laboratories, Bangladesh.

Mst. Nadira Begum
Biological Research Division, BCSIR Dhaka Laboratories, Dhaka, Bangladesh
nbegum470@gmail.com

Mst. Nadira Begum is a Senior Scientific Officer at Biological Research Division of BCSIR Dhaka Laboratories. She has experience in fungal biodiversity. Her current research interests are phytochemistry and prospective endophytic fungi.

Satyajit Roy Rony
Pharmaceutical Sciences Research Division, BCSIR Dhaka Laboratories, Dhaka, Bangladesh
satyajit_pharm@yahoo.com
https://orcid.org/0000-0002-6884-2904

Satyajit Roy Rony is working as a senior scientific officer at Pharmaceutical Sciences Research Division of BCSIR Dhaka Laboratories. His research is mainly focused on pharmaceutical science, new drug development,

analytical and natural product chemistry. He is also working as an assistant project director (APD) (additional charge) in the Annual Development Programme (ADP) of Government of Bangladesh named 'Institute of Bioequivalence Studies and Pharmaceutical Sciences'. He is a member of Bangladesh Pharmaceutical Society, Bangladesh Chemical Society and Bangladesh Botanical Society.

Md. Hossain Sohrab
Pharmaceutical Sciences Research Division, BCSIR Dhaka Laboratories, Dhaka, Bangladesh
mhsohrab@bcsir.gov.bd

Dr. Md. Hossain Sohrab is a chief scientific officer and scientist-in-charge at Pharmaceutical Sciences Research Division of BCSIR Dhaka Laboratories. He has been involved with research on pharmaceutical and natural product chemistry for over 25 years. He has an international patent. Presently he is working as a project director of the "Establishment of Institute of Bioequivalence Studies and Pharmaceutical Sciences" project, BCSIR, Dhaka.

Md. Abdul Mazid
Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka, Bangladesh
ma.mazid@du.ac.bd
https://orcid.org/0000-0003-2190-2591

Dr. Md. Abdul Mazid is a professor of the Department of Pharmaceutical Chemistry of University of Dhaka, Bangladesh. He is currently working and supervising research in the fields of pharmaceutical biotechnology and natural products chemistry. His research works are mainly targeting development of antibacterial and anticancer drugs. He is also studying molecular mechanisms of antibiotic resistance and genetic polymorphism in cancer.