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IDENTIFICATION AND BIOACTIVE POTENTIAL OF ENDOPHYTIC FUNGI FROM MARINE WEEDS AVAILABLE IN THE COASTAL AREA OF BANGLADESH

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ABSTRACT: Endophyte is a rich source of bioactive metabolites for medicinal exploitation as an increasing number of novel compounds with bioactive potential are now being isolated from this source. This study was conducted to characterize and explore endophytic fungi from marine weeds available in Bangladesh. The whole weed was used for isolation of endophytic fungi in the water agar media and the isolated fungi were identified through macroscopic and microscopic observation. Small scale cultivation was performed on liquid Wickersham media. Cytotoxicity, antibacterial, antioxidant activities were evaluated following brine shrimp lethality bioassay, disc diffusion method and DPPH free radical scavenging assay, respectively. A total of five endophytic fungi were isolated, purified and characterized from four seaweeds; *Hypnea musciformis* (HM), *Sargassum crassifolium* (SC), *Dictyota dichotoma* (DD) and *Caulerpa peltata* (CP). They were coded as HMWE-1, HMWE-2, SCWE-1, DDWE-1 and CPWE-1. The fungal strains HMWE-1 and SCWE-1 were identified morphologically as *Fusarium* sp., HMWE-2 and DDWE-1 as *Penicillium* sp. and CPWE-1 as *Aspergillus* sp. DDWE-1 fungal strain showed significant antioxidant activity in comparison to the standard, ascorbic acid. The fungal extracts CPWE-1 (LC_{50} value of 2.85 µg/mL) and DDWE-1 (LC_{50} value of 6.38 µg/mL) showed significant cytotoxic activity. The extracts exhibited weak to good activity against a wide range of human pathogenic microorganisms during anti-microbial activity screening. Preliminary chemical screening by thin-layer chromatography (TLC) indicated the presence of a number of secondary metabolites. Thus, this study explored the potential entophytes from marine weeds of the Bay of Bengal as a producer of leads for drugs.

INTRODUCTION: Marine weeds are broadly classified into red, brown and green macroalgae which have shown various therapeutic properties.

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Extract of *Hypnea musciformis* (red algae) belonging to the family Hypneaceae, exhibited antioxidant and antihypertensive activities; also showed antidepressant, psychotropic, and anxiolytic profile; whereas increased plasma insulin effects in diabetic animals [1, 2].

Sargassum crassifolium (brown algae), native to the family Sargassaceae, is reported to produce a great variety of secondary metabolites with antioxidant, anti-bacterial, anti-fungal, anti-

coagulant and anti-radiation UV-B, wound healing and cell structure regeneration potential. This weed is also rich in phytochemical constituents such as flavonoids, tannins, phenolics, sterols and terpenoids that activate defense mechanism against different infectious diseases [3]. *Dictyota dichotoma* (brown algae), belonging to the family Dictyotaceae, has medicinal implications for its secondary metabolite content with anti-thrombolytic, antifouling, anti-tumor, antibacterial and antifungal properties [4]. *Caulerpa peltata* (green algae), belonging to the family Caulerpaceae, is traditionally used for its antibacterial, anti-proliferative, and immuno-stimulatory properties [5].

During the last decade, numerous secondary metabolites isolated from the marine weeds have shown to possess significant pharmacological effects as compared to the current medicines which offer a great opportunity to develop new classes of medicinal agents [6]. On the other hand, marine micro-organisms including bacteria, cyanobacteria, microalgae and fungi have also become an important source of new pharmacologically active metabolites. Marine fungi have been reported as the potential source of new and bioactive natural products regarding the chemical diversity of their secondary metabolites [7].

The Bay of Bengal is a rich source of flora and fauna but endophytic fungi from the marine weeds are nearly untapped. Therefore, this study was conducted to explore endophytic fungi from the marine weeds and their potentiality as the producer of bioactive compounds from the coastal region of the Bay of Bengal.

MATERIALS AND METHODS:

Chemicals: Ethyl acetate, toluene, ethanol, methanol, chloroform and hydrochloric acid (37%, HCl) were purchased from Merck KGaA, Germany. 1, 1-diphenyl-2-picryl hydrazyl (DPPH) was brought from Sigma-Aldrich Chemicals Pvt. Ltd. (Germany). Magnesium chloride, calcium sulphate, sodium hydroxide, sodium carbonate, and sodium chloride were purchased from Merck Specialties Private Limited, Mumbai, India. Nutrient agar, malt agar and Potato Dextrose Agar (PDA) were from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India) and the rest of the chemicals and solvents used were of analytical grade.

Isolation of Endophytic Fungi from Marine Weeds: Marine weeds were collected from St. Martin's Island in the North-eastern part of the Bay of Bengal, about 9 km south of the tip of the Cox's Bazar-Teknaf peninsula in February 2017. Healthy and mature weeds were carefully chosen for sampling. Weeds were rinsed gently in running tap water to remove dust and debris. After proper washing weeds were cut into small pieces (1.5 cm) and further processed under aseptic condition. Endophytic fungi were isolated from fresh marine weeds parts following the suitably modified procedure of Kjer *et al.* 2010 [8].

Morphological Identification: For the identification of endophytic fungal strains, slides were prepared from 02 days cultures by staining with lactophenol cotton blue. The prepared slides were examined for morphological characteristics by checking under a bright-field and phase-contrast microscope concerning different standard taxonomic keys [9, 10].

Small Scale Fermentation in Liquid Medium: The isolated five fungi were cultivated on a small scale and extracted by a slight modification of the standard method [8]. 300 mL of liquid Wickerham's medium was poured in 1000 mL Erlenmeyer flasks and autoclaved at 121 °C for 20 min. The isolated endophytic fungal strains were inoculated aseptically into all flasks. The flasks were kept in a static condition at room temperature for 21 days for their proper growth. After 21 days, the content of the cultured flask was mixed with 250 mL of ethyl acetate and kept it for 24 h.

Then the mixture was filtered under negative pressure using a Buchner funnel and the mycelium residue was discarded. The filtrate was then transferred into a separating funnel and left it undisturbed for several minutes for separation of the organic layer from the aqueous phase.

The organic ethyl acetate layer was separated and the remaining aqueous layer was again extracted twice with 300 mL of ethyl acetate. All the ethyl acetate extract of the fungi were collected and concentrated into solid residue by evaporation under reduced pressure in rotary evaporator **Table 1**.

TABLE 1: AMOUNT OF CRUDE EXTRACTS OF ISOLATED FUNGI

Entophytic fungi	Ethyl acetate extract (gm)
HMWE-1	0.0327
HMWE-2	0.0227
SCWE-1	0.0926
DDWE-1	0.0428
CPWE-1	0.2029

Bioactivity Screening:

Antimicrobial Screening: The preliminary antibacterial and antifungal activities were screened by the disc diffusion method [11]. The test microorganisms used in the antimicrobial study included four pathogenic bacterial strains *Bacillus megaterium* (ATCC 13578), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 28739) and *Pseudomonas aeruginosa* (ATCC 27833), two fungal strain *Aspergillus niger* and *Aspergillus flavus*. The zones of growth inhibition around the discs were measured after 18 h of incubation at 37 °C for bacteria and 48 h of incubation at 28 °C for fungi. The sensitivities of the microorganism species to the fungal extract (100 µg/disc) were determined by measuring the diameter of inhibitory zones in millimeters compared to kanamycin (30 µg/disc) and ketoconazole (30 µg/disc) as standard antibiotics for antibacterial and antifungal screening, respectively.

Anti-oxidant Activity: The antioxidant activities of the entophytic fungal extracts were assayed by the discoloration of the methanolic solution of DPPH [12]. The scavenging activity of the fungal extract was measured spectroscopically (UV-Vis Spectrophotometer, Analytik Jena, Germany) at 517 nm. The measurement was carried out in triplicate and averaged. The scavenging ability was calculated by the following method:

Scavenging ability (%) = (Absorbance of control – Absorbance of sample) × 100 / Absorbance of control

For each sample, the result was presented as an IC_{50} (sample concentration that produced 50% scavenging of the DPPH radical). Ascorbic acid (ASA) was used as a positive control in this study.

Brine Shrimp Lethality Bioassay: The cytotoxic activity was performed by a slight modification of the brine shrimp lethality bioassay method [13]. The samples of fungal extract in Dimethyl sulphoxide (DMSO) at different concentration obtained by serial dilution method were added to test tubes containing 10 shrimps in 5 mL of simulated seawater to obtain final concentration of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562 µg/mL in test tubes. Each of these test solutions was incubated at room temperature for 24 h. The median lethal concentration (LC_{50}) of the test samples was determined by a plot of percentage of the shrimp mortality against the logarithm of the sample concentrations. DMSO was used as a negative control.

Statistical Analysis: IC_{50} values between groups were compared using independent student's t-tests. For the analysis of the cytotoxicity results, continuous variables between groups were compared to one-way analysis of variance (ANOVA). Mean values between groups were compared using independent student t-test for equality of variances.

RESULTS:

Identification of Marine Weeds: Collected marine weeds **Fig. 1** were identified as *Hypnea musciformis*, Sargassum crassifolium, *Dictyota dichotoma*, and *Caulerpa peltata* according to published literature [14, 15]. *Hypnea musciformis* **Fig. 1(a)** is a bushy marine weed with spreading slender branches, often with spineless branchlets and cozier tip. The height of this weed is nearly 45 cm long and looks off-white in color. *Sargassum crassifolium* **Fig. 1(b)** is a structurally large weed with a basal holdfast and receptacle branch. The upper portion of the weed is busy but leaves of the lower portions are larger, simple or divided.

Dictyota dichotoma **Fig. 1(c)** is a yellowish-brown marine weed in color and 15-20 cm in height with a thick basal holdfast. The upper portion has repeatedly dichotomously branched with equal angles between branches wide apices but rarely found slight unequal angles. *Caulerpa peltata* **Fig. 1(d)** is a marine weed with structures like fleshy umbrellas; with a thick circular portion (about 1-1.5 cm across) on a little stalk. These little umbrellas emerge along the length of a 'horizontal root' that creeps over the surface. Some form loose clusters. This weed is bright yellow-green to bluish-green in color.

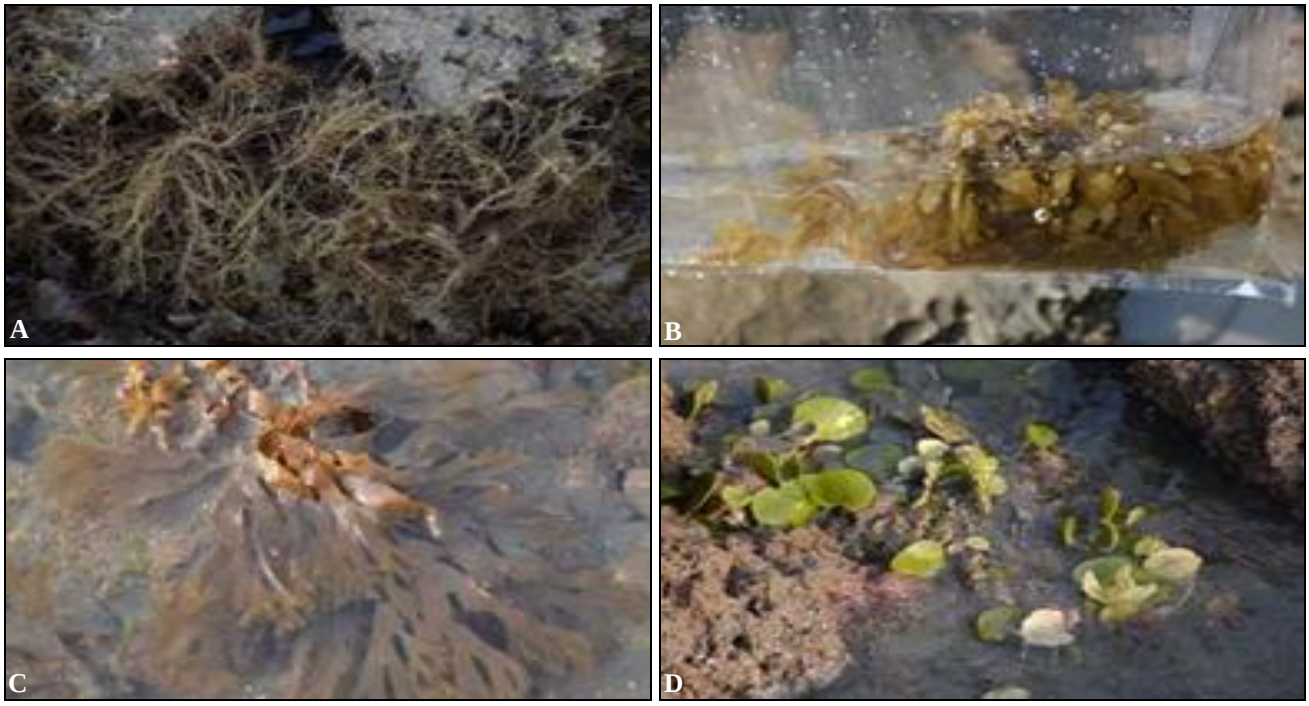
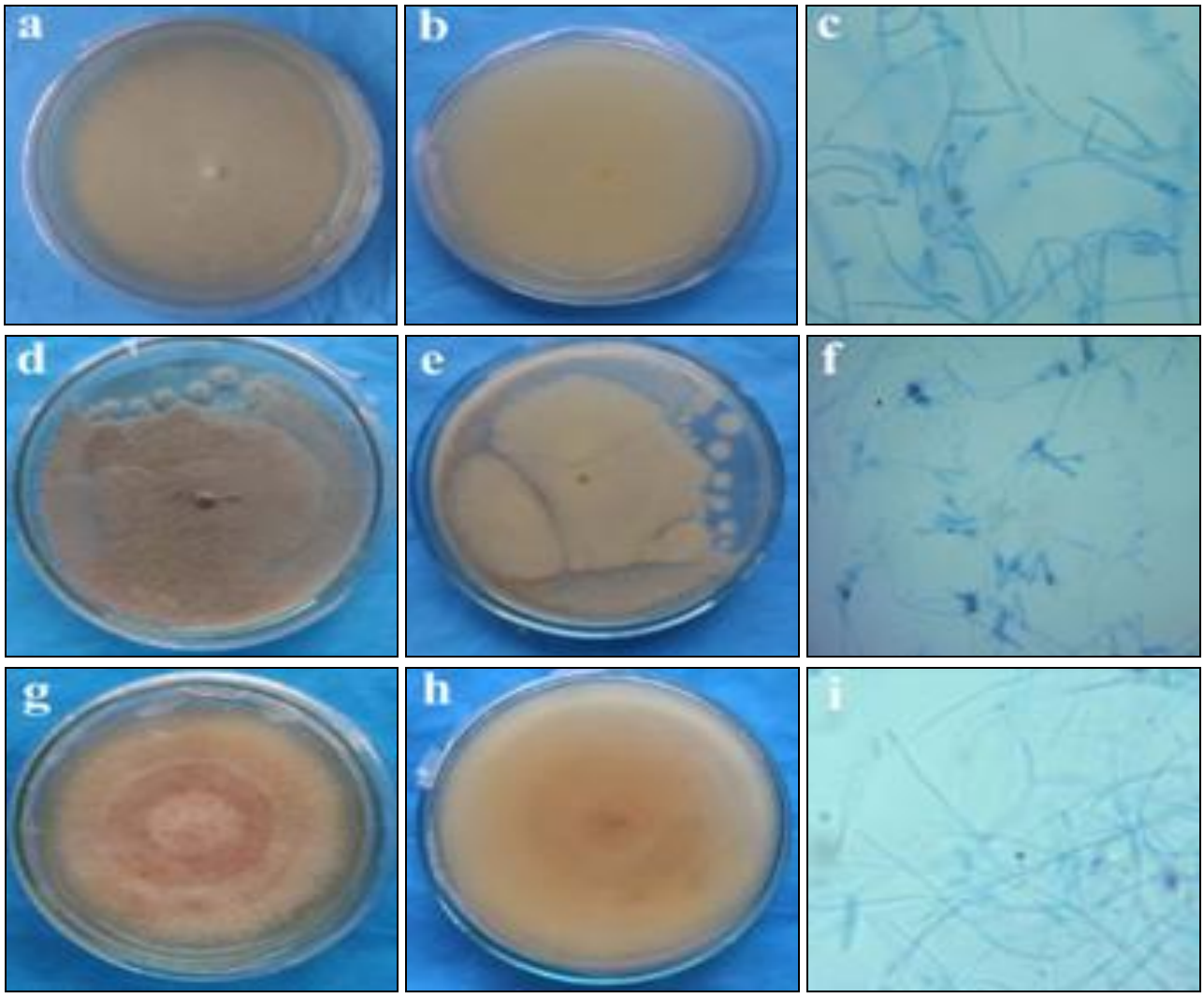


FIG. 1: DIFFERENT SEAWEEDS FROM SAINT MARTIN'S ISLAND. (A) *HYPNEA MUSCIFORMIS*, (B) *SARGASSUM CRASSIFOLIUM*, (C) *DICTYOTA DICHOTOMA*, (D) *CAULERPA PELTATA*



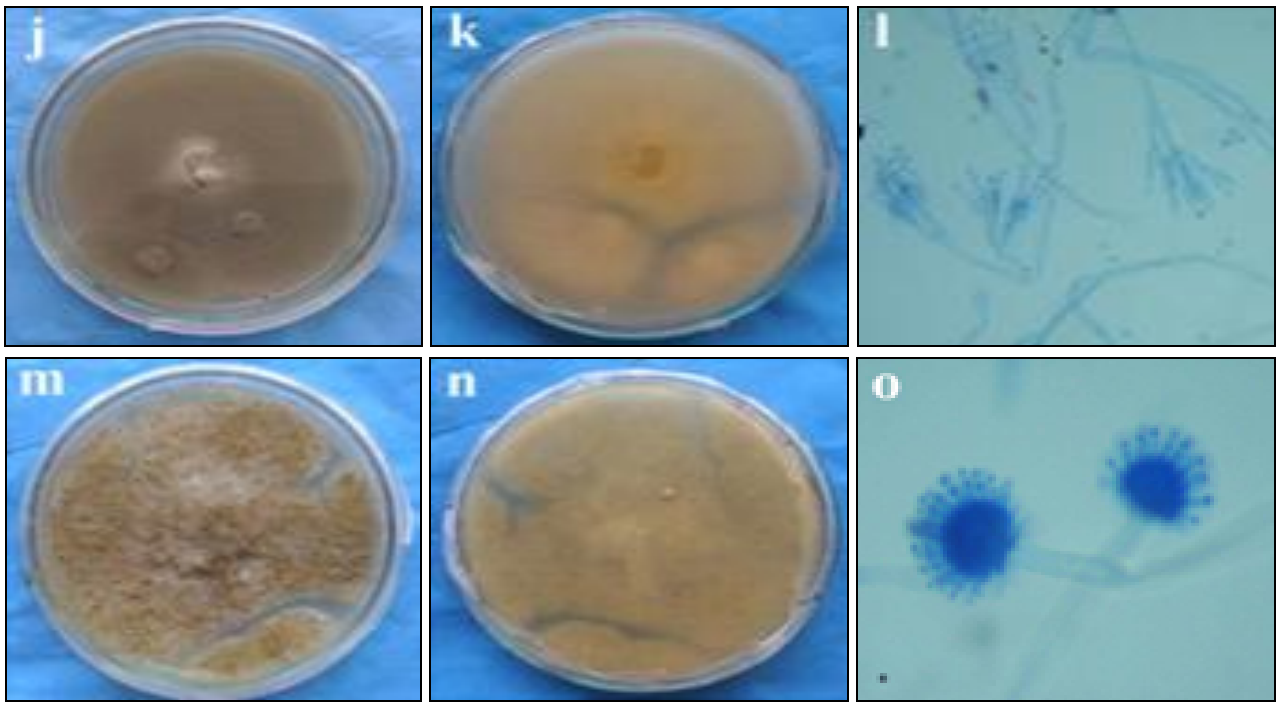


FIG. 2: MACROSCOPIC AND MICROSCOPIC COLONY MORPHOLOGIES OF THE ENDOPHYTIC FUNGI FROM DIFFERENT MARINE WEEDS. MACROSCOPIC (A, B) AND MICROSCOPIC (C) VIEWS OF HMWE-1; MACROSCOPIC (D, E) AND MICROSCOPIC (F) VIEWS OF HMWE-2; MACROSCOPIC (G, H) AND MICROSCOPIC (I) VIEWS OF SCWE-1; MACROSCOPIC (J, K) AND MICROSCOPIC (L) VIEWS OF DDWE-1; MACROSCOPIC (M, N) AND MICROSCOPIC (O) VIEWS OF CPWE-1

A total of five endophytic fungi were isolated, purified and characterized by the four marine weeds. Isolated endophytic fungi with the internal strain numbers HMWE-1 and HMWE-2 were from *Hypnea musciformis*, SCWE-1 was from *Sargassum crassifolium*, DDWE-1 was from *Dictyota dichotoma* and CPWE-1 was from *Caulerpa peltata*, where the fungal strains HMWE-1 **Fig. 2 a, b, c** and SCWE-1 **Fig. 2 g, h, i** were identified as *Fusarium* sp., HMWE-2 **Fig. 2 d, e, f** and DDWE-1 **Fig. 2 j, k, l** were identified as *Penicillium* sp. and CPWE-1 **Fig. 2 m, n, o** was identified as *Aspergillus* sp. after comparing their morphological character with those described by standard taxonomic identification protocols [10, 16, 17].

Strain HMWE-1 and Strain SCWE-1: In the colony morphology the colony looked as white and cottony at an early stage, but developed a pink or violet center with a lighter periphery within a very short time. Later species became tan or orangey.

In the microscopic observation, the hyphae were found to be septate. There were two types of conidiophores unbranched or branched conidiophores with phialides and long or short simple conidiophores. Unbranched or branched conidiophores produced large sickle or canoe-shaped macroconidia with three to five septa and 2-6 × 14-80 µm in size. The conidiophores (microconidia) were observed as long or short bearing small, oval, one or two-celled conidia of 2-4 × 4-8 µm size. Based on all morphological characteristics these two strains HMWE-1 and SCWE-1 were confirmed as *Fusarium* sp.

Strain HMWE-2 and Strain DDWE-1: In the morphological examination of the colony the fungal strains were found as soft, flat, powdery to velvety, white to tan, later became tan yellow with a yellow or white edge and gray-green coloration in the center. Microscopically the fungi were observed as smooth conidiophores with three to five metulae, each metula contains four to six phialides. Conidia were short, smooth or slightly rough, formed chains, round to oval in shape and 2-3 × 2.5-4 µm in size.

Finally, from the morphological characteristics strain HMWE-2 and DDWE-1 were identified as *Penicillium* sp which was usually thermally dimorphic.

Strain CPWE-1: In the colony, morphology surface was observed as suede-like, usually having radial groves, a colour often varied, commonly green or tan with spots of yellow or orange. In the microscopic morphology, the hyphae were found to be septate; conidiophores were smooth, medium length, nearly 200-500 × 4-7 µm; vesicles were 9-16 µm in diameter. Conidiophores were also biseriate with metulae nearly the same size as the phialides, roughly covered half to most of the vesicle. Conidia were round, slightly irregular and 2-3.5 µm in diameter. The morphological characteristics of strain CPWE-1 were confirmed as *Aspergillus* sp. by comparison with a published standard taxonomic key.

Bioactivity Screening:

Anti-microbial Activity: Antimicrobial study was conducted using the disc diffusion method against a wide range of Gram-positive and Gram-negative human pathogenic bacteria. All extracts obtained from fungal endophytes were tested at 100 µg/disc concentration for the screening of antibacterial activity against the standard at 30 µg/disc concentration. Most of the extracts showed moderate activity against tested human pathogenic bacteria; on the other hand, they did not show any antifungal activity against the pathogenic fungal strains **Table 2**.

TABLE 2: ANTIMICROBIAL ACTIVITY OF HMWE-1, HMWE-2, SCWE-1, DDWE-1 AND CPWE-1

Sample	Zone of Inhibition* (mm)					
	Escherichia coli	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Bacillus megaterium*	*Aspergillus niger*	*Aspergillus flavus*
HMWE-1	14.66 ± 0.1	11.1 ± 0.5	11.33 ± 0.3	13.33 ± 0.1	-	-
HMWE-2	11.33 ± 0.5	11.5 ± 0.1	-	12.66 ± 0.5	-	-
SCWE-1	12.1 ± 0.1	-	11.66 ± 0.1	12.1 ± 0.5	-	-
DDWE-1	13.33 ± 0.5	11.66 ± 0.3	-	12.33 ± 0.3	-	-
CPWE-1	11.1 ± 0.3	11.33 ± 0.1	12.1 ± 0.3	11.66 ± 0.1	-	-
KM	25.66 ± 0.1	30.1± 0.1	25.33 ± 0.3	22.33 ± 0.5	ND	ND
KC	ND	ND	ND	ND	23.33 ± 0.3	25.66 ± 0.1

*Values are expressed as mean ± SD (n = 3); "-" indicates no sensitivity, "ND" means not done, "KM" indicates Kanamycin, "KC" indicates Ketoconazole.

Evaluation of Antioxidant Activity: In this study, all extracts were subjected to the DPPH scavenging test in order to evaluate the free radical scavenging activity of the fungal extracts. Fungal strain DDWE-1 (12.16 µg/mL) and CPWE-1 (20.46 µg/mL) showed good antioxidant activity compared to standard, ascorbic acid (9.01 µg/mL). Besides, other strains HMWE-1 (55.94 µg/mL), HMWE-2 (87.68 µg/mL), SCWE-1 (64.36µg/mL) showed weak antioxidant activity as shown in **Fig. 3**.

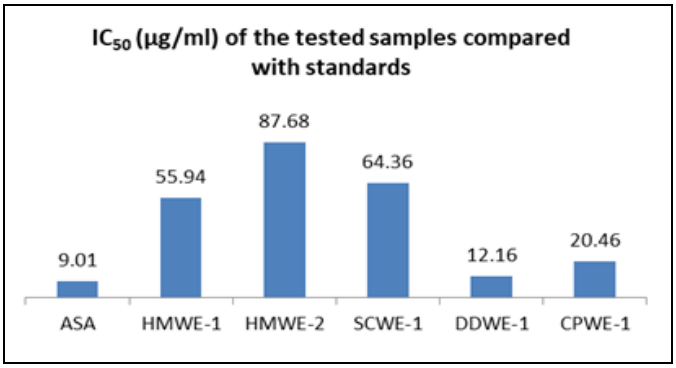


FIG. 3: FREE RADICAL SCAVENGING ACTIVITY OF DIFFERENT FUNGAL EXTRACTS

Brine Shrimp Lethality Bioassay: In the brine shrimp lethality bioassay, the result of cytotoxic potential of extracts in terms of percent (%) mortality of brine shrimps is considered for calculating LC_{50} value. The degree of lethality was directly proportional to the concentration of the extracts.

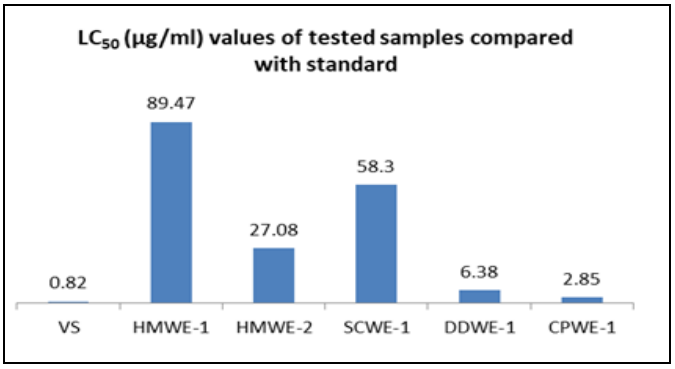


FIG. 4: CYTOTOXIC ACTIVITY OF DIFFERENT FUNGAL EXTRACTS

In the brine shrimp lethality assay, the LC_{50} value of the fungal extracts was assayed. DDWE-1 (LC_{50} value 6.38 µg/mL) and CPWE-1 (LC_{50} value 2.85

µg/mL) showed strong cytotoxic activity, whereas HMWE-2 (LC_{50} value 27.08 µg/mL) showed good cytotoxic activity as shown in **Fig. 4**.

DISCUSSION: The importance of fungi as a new source of bioactive compounds was first focused by the discovery of penicillin from *Penicillium notatum* in 1928 by Alexander Flemming. A molecular study reported that fungal communities differ between marine lives collected at the same site and also differ from those present in the surrounding marine water [18-20]. Up to 2016, nearly 600 new compounds were reported from marine-derived symbiotic microorganisms of which over 70% were obtained from epi/endophytes derived from marine weeds, invertebrates and woody substrates [21]. Thus, marine-derived endophytic fungi prolong to be a potential source of unique secondary metabolites with interesting structural features, a significant number of which exhibited promising biological activities [22, 23]. The disc diffusion method is extensively used to explore the preliminary anti-microbial activity of natural substances and plant extracts.

Endophytic *Penicillium* sp. is known to be an important source of antibiotics [24, 25]. There are reports on azaphilones isolated from mangrove rhizosphere soil-derived fungus of *Penicillum* sp. exhibiting significant antibacterial activity against *S. aureus* [26]. Moreover, azaphilonidal derivatives isolated from the marine fungus strain *P. sclerotiorum* exhibited strong antibacterial activity against *S. aureus*, *P. aeruginosa*, *Klebsiella pneumonia* and *E. coli* [27]. The investigated *Penicillium* sp. isolated from *Hypnea musiformis* and *Dictyota dichotoma* were also exhibited strong anti-microbial activity against *S. aureus*, *E. coli* and *B. megaterium*. Thus, these marine seaweeds can be a valuable source of potential antibacterial agents. Moreover, the isolated marine endophytic strains identified as *Fusarium* sp. also exhibited strong antibacterial activity against the virulent strain of *E. coli*, *S. aureus*, *P. aeruginosa* and *B. megaterium*. The previous report on marine endophytic *Fusarium* sp. suggested strong anti-microbial activity against different virulent strains such as *E. coli*, *P. aeruginosa*, *K. pneumonia*, *Proteus vulgaris*, *S. aureus*, *Bacillus cereus*, *Streptococcus pyogenes*, *B. subtilis*, *P. syringae* pv. lachrymans or acid ovorax avenae and anti-mycobacterial activity against *Mycobacterium tuberculosis* [28-32]. Terrestrial endophytic *Fusarium* sp. Like *Fusarium* solani, *F. oxysporum*, *F. acuminatum*, *F. tricinctum* were reported to release structurally diversified anti-microbial secondary metabolites [28, 33-36]. Some investigators also claimed that marine-derived *Fusarium* sp. is able to produce novel anti-bacterial compounds like cyclodepsipeptide enniatin B37, sesterterpenoid neomangicols [22, 37], bromomethylchlamydosporols A38 and antifungal compound, fusarielin E37. So, it can be assumed that the investigated marine fungi can produce antimicrobial secondary metabolites like pest alone due to bacterial competition against other micro-organisms in the marine environment developed through its chemical defense mechanisms [20]. Thus, the five isolates derived from four marine weeds are presumed to be good antibiotic-producer candidates.

DPPH free radical assay is a rapid and reliable method for evaluating the antioxidant activity of the extracts or pure compounds [39]. The extracts exhibited IC_{50} values in the order DDWE-1 < CPWE-1 < HMWE-1 < SCWE-1 < HMWE-2 with lowest for DDWE-1 (*Penicillium* sp.) of 12.16 µg/mL and highest for HMWE-2 (*Penicillium* sp.) of 87.68 µg/mL. So, DDWE-1 (*Penicillium* sp.) and CPWE-1 (*Aspergillus* sp.) fungal extracts exhibiting strong anti-oxidant activity with lowest IC_{50} values (within ~20 µg/mL) are a promising candidate for use as natural products based antioxidant agent for the health of the human being. In the same laboratory, another investigation was reported that the fungal isolates from fresh aquatic plant *Monochoria hastate* (L.) Solms except one showed very insignificant antioxidant activity whereas the one isolated *Trichoderma* sp. exhibited the only week antioxidant property [40]. It indicates that the metabolite profile of fungal endophytes is exclusively dependent on the biochemical environment of the host as well as ecological or environmental conditions [41].

The brine shrimp lethality test has been reported as a safe, practical and economic method for the determination of preliminary toxicity of synthetic and natural compounds. This method was conducted in order to evaluate the toxicity of isolated marine fungal isolates. LC_{50} value from regression analysis showed activity in the order

CPWE-1 < DDWE-1 < HMWE-2 < SCWE-1 < HMWE-1 with lowest for CPWE-1 (*Aspergillus* sp.) of 2.85 µg/mL and highest for HMWE-1 (*Fusarium* sp.) of 89.47 µg/mL. Comparison of this result with the standard Vincristine Sulfate (0.82 µg/mL) indicated the most lethality of the crude extracts CPWE-1 (*Aspergillus* sp.), DDWE-1 (*Penicillium* sp) and HMWE-2 (*Penicillium* sp.) with LC_{50} values of within ~30 µg/mL. This result indicates that the crude fungal extracts may contain metabolites with various pharmacological activities like anti-tumor, pesticidal or herbicidal activities which require further investigations to be proved.

CONCLUSION: This is the first report of isolation, identification and bioactivity screening of fungal endophytes from marine weeds of the northeastern part of the Bay of Bengal which is the most untapped territory for investigation of endophytic fungal diversity. This study indicates the potentiality of fungal endophytes as isolated endophytic fungi from the marine weeds possessed diverse metabolites that may have different pharmacological activity, warranting further investigations on the fungi with a selection of proper fermentation media for large scale cultivation and maximum production of secondary metabolites.

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CONFLICTS OF INTEREST: Authors have no conflicts of interest to declare.

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