

# Isolation, Identification and Pharmacological Activities of Endophytic Fungi from *Aponogeton undulatus* Roxb.

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**How to cite this paper:** Chowdhury, N.S., Farjana, F. and Sohrab, Md.H. (2020) Isolation, Identification and Pharmacological Activities of Endophytic Fungi from *Aponogeton undulatus* Roxb. *Pharmacology & Pharmacy*, 11, 350-361.  
<https://doi.org/10.4236/pp.2020.1112028>

**Received:** November 5, 2020

**Accepted:** December 13, 2020

**Published:** December 16, 2020

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## Abstract

The objective of this study is to isolate, identify and investigate the pharmacological activities of the endophytic fungi from an aquatic plant *Aponogeton undulatus* Roxb. (*A. undulatus*). Endophytic fungi were isolated and identified based on morphological characters. The molecular identification of the fungal isolates was performed using by analyzing the DNA sequence based on mega BLAST program. Spectrums of antibacterial and antifungal activities were studied by Agar diffusion methods. Extracts of the endophytic fungal strains isolated from the plant *A. undulatus* were screened for probable cytotoxic activities using brine shrimp lethality bioassay. Six endophytic fungi, namely AULE-1, AULE-2, AULE-3, AURE-1, AURE-3 and AURE-4 were isolated and purified from the leaves and roots of the plant such as strains AULE-1 as *Trichoderma* sp., AULE-2 as *Carvularia* sp. AULE-3 as *Penicillium* sp. strains AURE-1 and AURE-4 as *Fusarium* sp. and AURE-3 as *Mucor* sp. Strain AURE-4 was further identified as *Fusarium solani*. At 300 µg/disc concentration, six ethyl acetate extracts of endophytic fungi of the plant *A. undulates* showed moderate to significant activities against most of the test bacteria and pathogenic fungi. The strain AURE-4 exhibited strong cytotoxic activities (10.18 µg/mL) compared to the standard antitumor agent vincristine sulphate and this positive result suggests that fungal extracts may contain antitumor or pesticidal compounds. This is the first study to report the isolation, identification, antimicrobial and cytotoxic properties of endophytic fungi of *Aponogeton undulatus* Roxb. in Bangladesh.

## Keywords

Endophytic Fungi, *Aponogeton undulatus*, *Fusarium solani*, Antibacterial Activity, Cytotoxic Activity

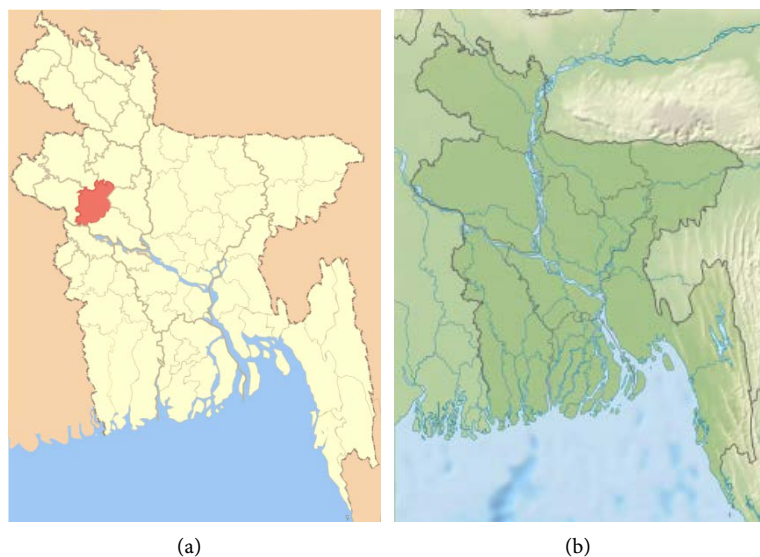
## 1. Introduction

Bangladesh possesses a rich flora of aquatic plants. Aquatic plants in wetlands carry out photosynthesis as primary producers in the ecosystem, with blooms at the surfaces of freshwater. Therefore, aquatic plants are considered useful resources because of their ability to purify water [1] [2] [3]. Aquatic plants or hydrophytes are highly adapted to their environment with unique morphological and physiological features [4] and these features are likely to make the aquatic plants unique habitats for the growth of potentially distinctive endophytic fungi. Endophytic fungi are ubiquitously found in the internal tissues of all plant species [5]. Despite the cryptic nature of their symbiotic existence in host plants, they have gained recognition as prolific producers of secondary metabolites that have potential in medical and agricultural applications useful to mankind [6]. However, much of the Earth's endophytic fungal population, estimated to be in the millions, remains uninvestigated [7]. Compared to their terrestrial counterparts endophytic fungi of aquatic plants are far less investigated with respect to secondary metabolite production and their biological activities [8] [9]. The plant growth promoting activity and the induction of systemic resistance (ISR) by these fungi in their host plants has been widely researched [10] [11] [12] [13] [14]. There are so many plants of Bangladesh which are untouched for investigation of their biological activity [15]. Aquatic genus *Aponogeton* is represented by approximately 57 species all over the world [16]. *Aponogeton undulatus* Roxb. (*A. undulatus*), commonly called Green bulb tree belongs to the family *Aponogetonaceae* and occurs in India, Sri Lanka, Myanmar, Bangladesh and China [17]. The plant is claimed to be effective against cough, tuberculosis, acne, cancer, diarrhoea, dysentery, jaundice etc. [18]. *A. undulatus* also possess remarkable antitumor activity [19]. The endophytic fungi of *Aponogeton undulatus* or their bioactive metabolites have not been investigated earlier. So the purpose of this present study is to provide the information about the isolation, identification and bioactive potential of endophytic fungi from leaves and roots of that plant *Aponogeton undulatus*.

## 2. Materials and Methods

The aquatic plants *Aponogeton undulatus* Roxb. were collected from Chalan Beel area (24.52N, 89.01E), Natore, Bangladesh during the rainy season (July) of 2017 (Figure 1). They were identified by Bangladesh National Herbarium, Dhaka, Bangladesh. A voucher specimen (DACB: 32072) has been deposited in the Herbarium for further reference. Healthy (showing no visual disease symptom) and mature plants were carefully chosen for sampling. Leaves and roots from different sites of the plant were randomly collected for the study. The plant material was brought to the laboratory in sterile bags and processed within a few hours after sampling. Fresh plant materials were used for isolation work to reduce the chance of contamination [20].

About 300 grams of fresh and healthy parts of the plant (leaves and roots)



**Figure 1.** Location of Natore in Bangladesh (a); Location of ChalanBeel at Natore in Bangladesh (b).

were cut with a sterile scalpel and stored at 4°C in a sterile polythene bag prior to use. Endophytic fungi were isolated from the fresh plant parts following the procedure, suitably modified [21].

### 2.1. Morphological Identification of Fungal Cultures

For the identification of endophytic fungal isolates, slides prepared from cultures were stained with lactophenol cotton blue reagent and examined with a bright-field and phase contrast microscope [22]. Identification was based on morphological characteristics such as growth pattern, hyphae, the color of the colony and medium, surface texture, margin character, aerial mycelium, sporulation and production of acervuli, coloration of the medium, and the size and coloration of the conidia using standard identification manuals [23]. The fungi were identified using relevant keys and taxonomic notes from various standard manuals [24] [25].

### 2.2. DNA Extraction, PCR Amplification and Sequencing

The identification of the fungal isolates was mainly performed using molecular characterization by analyzing the DNA sequence of the ITS region of the ribosomal RNA gene. Each fungus sample was cultured in potato dextrose agar (PDA) at room temperature. Mycelium of the fungus was filtered and the isolation of genomic DNA from the mycelia of each fungal isolate was carried out using DNA isolation kit for genomic DNA (Maxwell 16, Promega, USA) according to the published protocol [26] [27]. The ITS region of the isolated genomic DNA was amplified by polymerase chain reaction (PCR) using the forward primer ITS 1 (5'TCCGTAGGTGAACCTGCGG3') and the reverse primer ITS 4 (5'TCCTCCGCTTATTGATATGC3') under the conditions, initial denaturation of 5 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 1 min at 55°C and 2 min

at 72°C, with a final extension of 7 min at 72°C [28]. The amplified DNA was sequenced commercially and was BLAST analysed [National Center for Biotechnology Information (NCBI)]. The acquired gene sequences were submitted to the NCBI GenBank database and accession number (KY511422) was obtained. The fungal voucher specimens were preserved on PDA slants at 4°C at the Pharmaceutical Sciences Research Division, BCSIR Laboratories, Dhaka, Bangladesh.

### 2.3. Extraction of the Culture Medium

The six endophytic fungal strains were cultivated on a small scale at 28°C for 28 days in potato dextrose agar (PDA) medium. At the end of stationary phase of culture, fermented medium was filtered using three layers of muslin cloth to remove the mycelia. The resulting culture filtrate was extracted thrice with ethyl acetate. Then, the solvent phase was reduced under pressure using rotary vacuum evaporator (Heidolph, Germany) [20].

### 2.4. Antimicrobial Assay

Spectrums of antibacterial and antifungal activities were studied by using the technique described by Bauer [29]. Kanamycin and Ketoconazole sensitivity disc (30 µg/disc) were used as a positive control for bacteria and fungi, respectively. Solvents are used as negative control. The zones of growth inhibition around the discs were measured after 18 to 24 hrs of incubation at 37°C for bacteria and 48 to 96 hrs 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 millimeter. The test microorganisms used in the antimicrobial study included four pathogenic bacterial strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 28739, *Bacillus megaterium* ATCC 18 and *Pseudomonas aeruginosa* ATCC 27833, two fungal strains *Aspergillus niger* and *A. flavus*. All the cultures were obtained from ICDDR,B. The bacterial cultures were sub-cultured every two weeks on fresh nutrient agar (NA) slants and incubated at 37°C whereas the fungal cultures were sub-cultured every four weeks on the fresh potato dextrose agar (PDA) slants and incubated at 28°C.

### 2.5. Brine Shrimp Lethality Bioassay

Brine shrimp lethality bioassay was conducted to evaluate cytotoxicity of the fungal extracts. For the experiment, 4 mg of each of the endophytic fungal extracts was dissolved in dimethylsulfoxide (DMSO) and solutions of varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 µg/ml) were obtained by the serial dilution technique using simulated seawater. The solutions were then added to the pre-marked vials containing 10 live brine shrimp nauplii in 5 ml simulated seawater. After 24 h, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted [30]. The mortality endpoint of this bioassay was defined as the absence of controlled for-

ward motion during 30 s of observation. From this data, the percent of lethality of the brine shrimp nauplii for each concentration and control was calculated. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph paper and the values of LC<sub>50</sub> were calculated using Microsoft Excel 2003. Vincristine sulphate was used as the reference cytotoxic drug [31].

## 2.6. Statistical Analysis

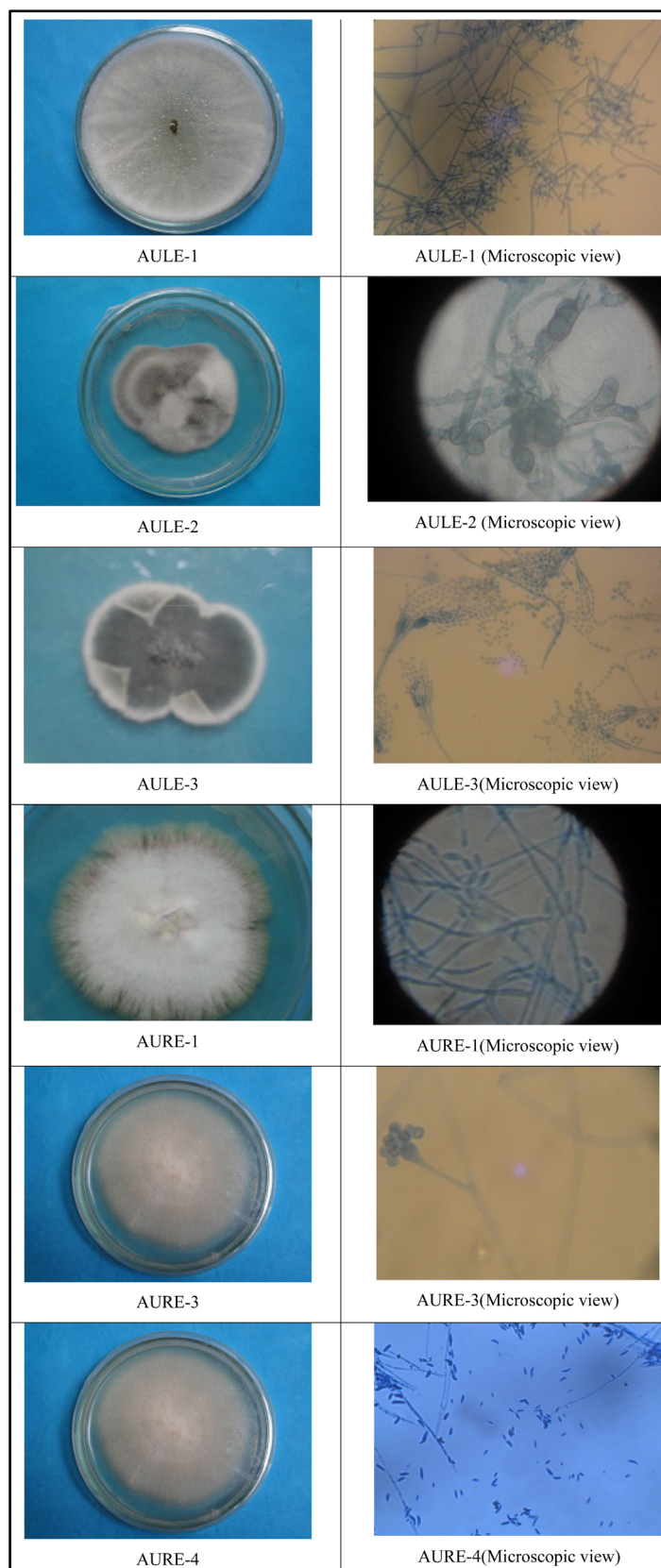
A statistical analysis was used to interpret the antimicrobial and cytotoxic results. The experiment was conducted in completely randomized design with 3 replicates. The results are presented as means  $\pm$  standard error of means using MS excel [32].

## 3. Result and Discussion

### 3.1. Morphological Identification of Fungal Cultures

Isolation, purification and cultivation of endophytic fungi have been done following the published method [33], suitably modified. A total of six endophytic fungi, namely AULE-1, AULE-2, AULE-3, AURE-1, AURE-3 and AURE-4 were isolated and purified from the leaves and roots of the plant *Aponogeton undulatus* Roxb. They exhibited characteristic colony and microscopic morphology that could be used to differentiate them. Fungi were identified taxonomically to the genus level on the basis of macroscopic and microscopic morphological characters in culture medium (Figure 2). Six (6) endophytic fungi, are identified such as strains AULE-1 as *Trichoderma* sp., strains AULE-2 as *Curvularia* sp., strains AULE-3 as *Penicillium* sp., strains AURE-1 and AURE-4 as *Fusarium* sp. and AURE-3 as *Mucor* sp.

The microscopic characters of strain AULE-I showed mycellium hyaline, pale or brightly colored branched and septate, conidiohores are arising from the mycellium singly or less often in synnemata, branched near the apex, ending in phialides, spores brightly colored mass, one celled, ovoid, in dry basipetal chain. These characteristics indicated that the fungus AULE-I belongs to *Trichoderma* sp. Strain AULE-2 showed profusely branched, septate and hyaline mycellium, conidophores are grey and simple, bearing spore apically, conidia dark, end cell lighter, typically bent, with one of the central cell enlarged. These morphological characteristics indicated the fungus AULE-2 belongs to *Curvularia* sp. Strain AULE-3 exhibited mycellium hyaline, pale or brightly colored branched and septate, conidiohores are arising from the mycellium singly or less often in synnemata, branched near the apex, ending in phialides, spores brightly colored mass, one celled, ovoid, in dry basipetal chain. These morphological characteristics indicated the fungus AULE-3 belongs to *Penicillium* sp. Strain AURE-1 was identified as *Fusarium* sp. It exhibited cottony mycellium, conidia or phialospores hyaline, two kinds of spores are observed. Macroconidia were several celled, slightly curved or bent at the pointed end. Microconidia were one celled,



**Figure 2.** Macroscopic and microscopic (100×) colony morphologies of the endophytic fungi from *Aponogeton undulatus* Roxb.

oblong, borne singly or in chain, colony was fluffy, spores cylindrical, septate and aseptate. In case of AURE-3, the mycelium consists of white grey cottony much-branched mycelium, rapid growth. The hyphae are prostrate, conidiospore producing globose sporangia on sporangiophores those are either solitary or branched. Rhizoids and stolons are absent. These characteristics indicated that the fungus AURE-3 belongs to *Mucor* species. Strain AURE-4 was identified as *Fusarium* sp. The mycelia profusely branched and septate, colony was fluffy white colored. Conidiospores produced macroconidial septation from 3 to 5, sparse to abundant cottony mycelium with white-creamy to white-reddish colour of aerial mycelium and the pigmentations were reddish brown with a dark brown zonation. The feature of conidiogenous cell with branched and long monophialides were commonly observed.

### **3.2. Molecular Identification of the Endophytic Fungus AURE-4 as *Fusarium solani***

One of the endophytic fungus (AURE-4) from the plant *Aponogeton undulatus* was grown on PDA for 28 days at 28°C and morphologically characterized. The mycelium was scraped directly from the surface of the agar culture (5 days old) and weighed. Nucleic acid was extracted and purified using the DNA isolation kit for genomic DNA (Maxwell 16, Promega, USA) using the manufacturer protocol. For identification and differentiation, the Internal Transcript Spacer regions (ITS4 and ITS5) and the intervening 5.8S rRNA region was amplified and sequenced using electrophoretic sequencing on an ABI 3730 x l DNA analyzer (Applied Biosystems, USA) using Big Dye Terminator v 3.1 cycle sequencing kit.

The PCR product was purified using 2% Agarose-Gel-Electrophoresis at 75 V for 60 minutes in TBE buffer. The agarose gel was then stained using 1% ethidium bromide. A 100 bp stained DNA fragment was then excised from the agarose gel. The next step of PCR product purification was performed using Gel Cleanup Kit. The binding buffer was mixed to the PCR product and incubated at 50°C for 10 minutes at 1000 rpm. The mixture was mixed with a volume of isopropanol and then centrifuged. The filtrate was discarded and the column was washed with wash buffer twice followed by centrifugation. Amplified fungal DNA (PCR product), which was incorporated into the column, was eluted by addition of elution buffer or molecular biology grade water to the center of the column. The column was then centrifuged to collect the filtrate, which was the fungal DNA dissolved in elution buffer. The amplified fungal DNA was then submitted for sequencing by a commercial service and the base sequence was compared with publicly available databases such as GenBank with the help of Blast-Algorithmus. Finally, the sequence data (ESI) was deposited to Genbank database (accession number KY511422). The sequence data revealed 99% similarity to another fungal isolate of accession number KC764913.1 that has shown 99% similarity with *Fusarium solani* (accession numbers KM235740.1, KJ207395.1, KJ719812.1, FJ719812.1, EU625403.1, KU382502.1 etc.) deposited in the US Na-

tional Center for Biotechnology Information (NCBI).

### 3.3. Antimicrobial Screening of Endophytic Fungi from *A. undulatus*

Six ethyl acetate extracts of endophytic fungi (Strains AULE-1, AULE-2, AULE-3, AURE-1, AURE-3 and AURE-4) of the plant *A. undulatus* showed moderate to significant activities against most of the test bacteria and pathogenic fungi (Table 1). The zone of inhibition were produced by the fungal extracts were found to be 11 - 36 mm at a concentration of 300 µg/disc. Extracts of the strains AURE-4 and AULE-1 were found to be more active compare to the other extracts. Extract of the strain AURE-4 was found to be exhibiting promising activities against *Escherichia coli* ( $28.55 \pm 0.35$  mm), *Staphylococcus aureus* ( $25.54 \pm 0.75$  mm), *Pseudomonas aeruginosa* ( $25.51 \pm 0.65$  mm) and *Bacillus megaterium* ( $24.50 \pm 0.45$  mm). Extract of the strain AULE-1 was found to show prominent activities against *Escherichia coli* ( $30.52 \pm 0.65$  mm), *Pseudomonas aeruginosa* ( $24.55 \pm 0.25$  mm), *Staphylococcus aureus* ( $25.54 \pm 0.75$  mm) and moderate activities against *Bacillus megaterium* ( $22.50 \pm 0.45$  mm). Interestingly the crude extract of the strain AURE-4 exhibited potent antifungal activities against *Aspergillus niger* ( $36.45 \pm 0.55$  mm) and *Aspergillus flavus* ( $33.65 \pm 0.35$  mm). The extracts of the strains AURE-4 and AULE-1 are very promising. This positive result suggests that all of the fungal extracts may contain metabolites with antimicrobial potency. This is the first report of antimicrobial activities of endophytic fungi from an aquatic plant *Aponogeton undulatus* Roxb.

### 3.4. Brine Shrimp Lethality Bioassay of Endophytic Fungi of *A. undulatus* Roxb.

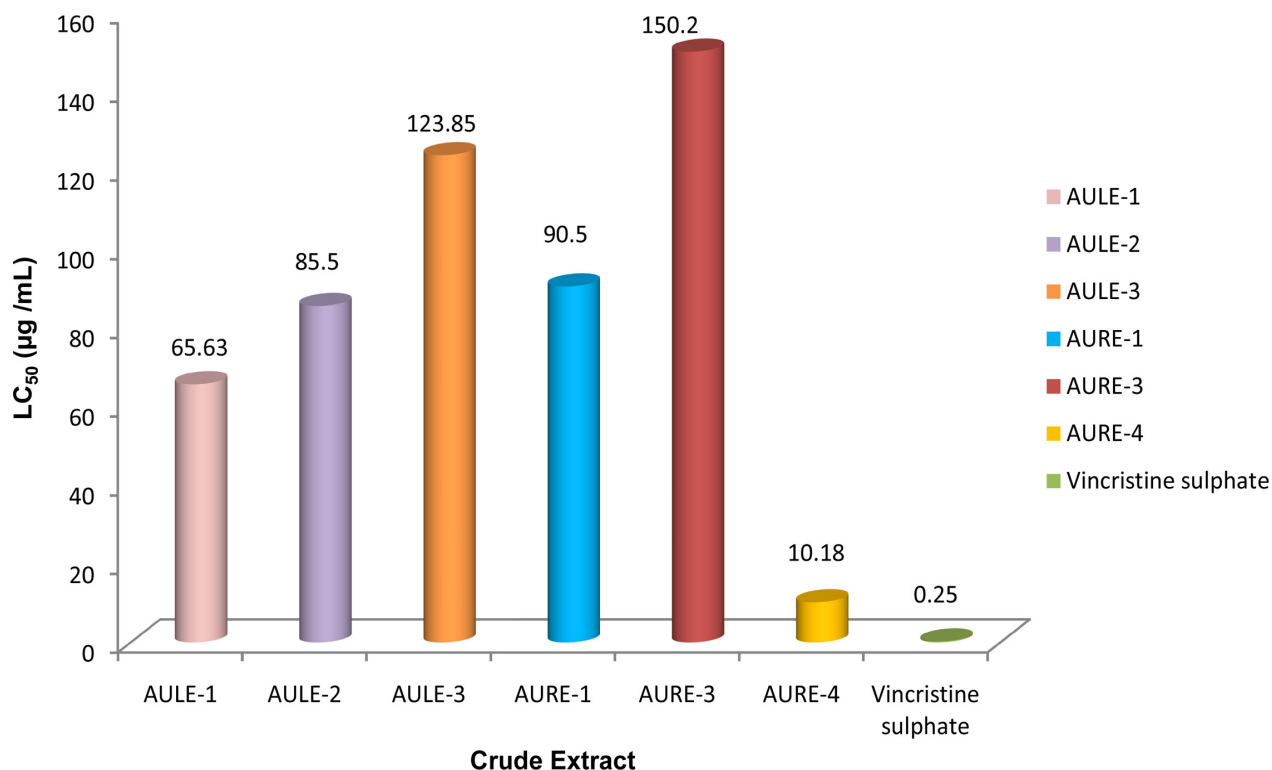
Extracts of the endophytic fungal strains isolated from the plant *A. undulatus* were screened for probable cytotoxic activities using brine shrimp lethality bioassay. The LC<sub>50</sub> value of the extracts of the strains AULE-1, AULE-2, AULE-3,

**Table 1.** Antimicrobial activity of the endophytic fungal extracts isolated from *Aponogeton undulatus* Roxb.

Test Microorganism	Diameter of the inhibition zone (mm) Crude extracts (300 µg/disc)						Standard Antibiotic/ Antifungal
	AURE-1	AURE-3	AURE-4	AULE-1	AULE-2	AULE-3	Kanamycin/ Ketoconazole
<i>B. megaterium</i>	14.50 ± 0.45	14.50 ± 0.55	24.50 ± 0.45	22.50 ± 0.45	14.52 ± 0.65	10.54 ± 0.75	31.35 ± 0.95
<i>S. aureus</i>	15.54 ± 0.75	---	25.54 ± 0.75	23.54 ± 0.75	15.52 ± 0.65	13.55 ± 0.25	32.52 ± 0.65
<i>P. aeruginosa</i>	25.51 ± 0.55	12.52 ± 0.65	25.51 ± 0.65	24.55 ± 0.25	17.55 ± 0.25	12.50 ± 0.45	31.35 ± 0.35
<i>E. coli</i>	20.55 ± 0.25	15.55 ± 0.35	28.55 ± 0.35	30.52 ± 0.65	17.50 ± 0.45	13.54 ± 0.75	30.37 ± 0.73
<i>A. niger</i>	---	25.45 ± 0.55	36.45 ± 0.55	---	---	---	41.52 ± 0.94
<i>A. flavus</i>	---	---	33.65 ± 0.35	---	---	---	48.56 ± 0.45

Note: The diameter of zone of inhibition are expressed as mean ± SD. (n = 3); a diameter less than 8 mm was considered inactive. Zone of inhibition (mm) determined after 24 h of incubation at 37°C.





**Figure 3.** Effect of plant and different fungal extracts of *A. undulatus* on brine shrimp nauplii.

AURE-1, AURE-3 and AURE-4 were found to be 65.63 µg/mL, 85.50 µg/mL, 123.85 µg/mL, 90.50 µg/mL, 150.20 µg/mL and 10.18 µg/mL, respectively (**Figure 3**) compared to the standard antitumor agent vincristine sulphate (LC<sub>50</sub> value 0.25 µg/mL). It is evident that most of the test samples were lethal to brine shrimp nauplii. All the fungal extracts showed low LC<sub>50</sub> values and thus demonstrated their quite potent cytotoxic activities. However, extract of the fungal strain AURE-4 was comparatively more active with minimum LC<sub>50</sub> values and the strain AURE-3 was less active with maximum LC<sub>50</sub> value. The strain AURE-4 exhibited strong cytotoxic activities and this positive result suggests that fungal extracts may contain antitumor or pesticidal compounds. This is also the first report of cytotoxic activities of endophytic fungi from an aquatic plant *Aponogeton undulatus* Roxb. However, this cannot be confirmed without further higher and specific tests.

#### 4. Conclusion

This is the first study to isolate the endophytic fungi of the native aquatic plant *Aponogeton undulatus* Roxb. and investigation of their biological activities. This study showed that the aquatic plant *Aponogeton undulatus* Roxb. harbours many endophytic fungi that are capable of producing antimicrobial as well as cytotoxic substances. The results presented here support the notion of an ecology based-approach to selection of fungal species for chemical studies, as this demonstrates that such an approach can be effective in the discovery of new bioactive fungal

metabolites. Thus it can be concluded that the endophytic fungi of *Aponogeton undulatus* Roxb. may be a potential source for the isolation of bioactive metabolites and more investigations should be performed to realize its true potential.

### Acknowledgements

The authors are thankful to the authority of BCSIR for supporting the work in this paper through a fellowship to NSC and also for providing the lab facilities related to the work on endophytic fungi.

### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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