

Isolation and Bioactivity Screening of Endophytic Fungi from *Commelina diffusa*

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ABSTRACT

Background: Endophytic fungi are able to produce many secondary metabolites and thus their availability and biological activity created new horizons for different pharmaceutical and agricultural approaches. **Objective:** For the ethnobotanical antiquity of *Commelina diffusa* in Bangladesh, this study is aimed at evaluating the endophytic fungal collection of *Commelina diffusa* and their bioactive potential. **Methods:** Endophytic fungi were isolated by the surface sterilization technique and identified using the microscopic procedure. Isolated fungal strains were solvent extracted. Disc diffusion method, DPPH free radical scavenging assay, brine shrimp lethality bioassay had been used for the determination of antibacterial, antioxidant, cytotoxic activities respectively. **Results:** Eight fungal strains were isolated and six of them were identified up to the genus level as *Fusarium* sp. (CDLE 1, CDRE 1, CDRE 2, CDRE 3, CDBE 1) and *Alternaria* sp. (CDBE 3) according to morphological and microscopical characters. Three of the eight isolated endophytes i.e., CDBE 2, CDRE 1 and CDRE 2 showed potential activity against *A. niger* in comparison with ketoconazole (30 µg/disc). Ethyl acetate extract CDBE 3 fungal extract demonstrated significant antioxidant and cytotoxic activities. **Conclusion:** The present study has proven that *Commelina diffusa* may be a rich source of medicinally important endophytic fungi and our findings may form a basis for further studies on endophytic fungi from medicinal plants for use in medicine, industry and agriculture. **Key words:** Endophytic fungi, *Fusarium* sp., *Alternaria* sp., Antibacterial, Antioxidant, Cytotoxic activity.

Submission Date: 21-07-2020;

Revision Date: 15-12-2021;

Accepted Date: 12-04-2021

DOI: 10.5530/ijper.55.3.156

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INTRODUCTION

Endophytes are micro-organisms (bacteria or fungi) that migrate to healthy plant tissue intercellularly and intracellularly without causing any noticeable symptoms of illness to the host species.¹ Until now they are ubiquitous, colonizing in all plants and produce no harm to the host plants. Endophytes isolated from medicinal plants showed bioactivity for broad spectrum of pathogenic micro-organisms to control microbial pathogens. Hundreds of natural products including alkaloids, terpenoids, flavonoids and steroids have already been isolated from endophytes.² Endophytes are capable of synthesizing bioactive secondary

metabolites that are known to have antibiotics, immunosuppressants, anticancer agents, biological control agents and other functionality. This potentially could result in improved host growth and the diverse ecological niches of these organisms may be helpful in agriculture. So far, more than ten thousand endophytic strains had already been isolated and characterized, including bacterium, fungus and actinomycete from the medicinal plants. As a part of our continuous screening for antimicrobial fungi from plant, *Commelina diffusa* Burm. F. has been investigated in this study.³ *Commelina diffusa* Burm. F. (Day flower, Bengali name:



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manaina), a medicinal herb is used to treat various diseases traditionally. Biological activity evaluation revealed its potential in anti-inflammatory,⁴ antioxidant,^{3,4} antibacterial,^{3,5} anti-fungal,⁵ nephroprotective,⁶ hepatoprotective,⁶ CNS depressant⁷ activities of this plant. The main objective of the present study was to isolate and characterize the endophytic fungi of *C. diffusa* available in Bangladesh and evaluation of their antibacterial, antioxidant and cytotoxicity activities.

MATERIALS AND METHODS

Collection and identification of plant material

Fresh and healthy (no visual infection) parts of the plant (leaves, bark and root) of *Commelina diffusa* were collected from Gazipur district, Bangladesh in June, 2017. The plants was identified a voucher specimen (ACC. No. 38390) and authenticated at the Bangladesh National Herbarium, Dhaka, Bangladesh. All the part (cutting length= 1 cm) of the plant were processed within 6 hr of collection and screened for the occurrence of endophytic fungi.

Surface sterilization and isolation of endophytic fungi

At first plant materials were washed several times under running tap water, followed by washing in distilled water. Surface sterilization was then done by the suitably modified method of Petrini *et al.* 1986.⁸ The plant materials were further cut aseptically at their perimeters to reveal the interior surface to the water agar media when sterilization process completed. For each plant part, three segments were placed in petri dishes containing water agar media adjusted with streptomycin at 200 mg/L concentration. The dishes were closed with parafilm and incubated at 28°C. After incubation period, the visual growth of fungus was observed from the plating date and isolated fungal tips were transferred to fresh potato dextrose agar (PDA) media without the addition of antibiotics to obtain pure fungal cultures. Nonsterilized culture used as negative control. The purified endophytic isolates were then transferred separately to PDA slants and stored at 4°C for further use.

Chemical screening

Chemical screening was performed on the fungal extracts of *C. diffusa* by thin layer chromatography (TLC) method as described by Wagner and Bladt, 1996 to ascertain the presence groups of starch, tannins, glycosides, terpenoids, flavonoids, steroids, alkaloids and so on.⁹ The presence of chemical compound in the extracts

were detected using TLC method with mobile phase of Toluene/10% EtOAc under UV light at 254 nm, 365 nm and by spraying with 1% vanillin in concentrated H₂SO₄ solution followed by heating at 105°C, respectively.^{10,11}

Macroscopic and microscopic identification

Morphological characterization was done on PDA following standard manuals^{12,13} and observing different morphological patterns such as growth rate, hyphae, color of the colony and medium, surface texture, margin character, aerial mycelium, the size and coloration of the conidia etc. The growth facade was then noted by observing both the back and front views of the plates. Fungal slides were prepared with lactophenol cotton blue reagent and observed at ×40 and ×100 magnifications following standard protocols.¹⁴

Small scale cultivation to obtain fungal extracts

All the isolated fungal strains were cultured on PDA with approximately 500 mL of media. After the completion of 21 days incubation period at 28°C, the culture media (PDA) were extracted with ethyl acetate to obtain the crude extracts. After each 05 days, media were filtrated through fresh cotton bed and finally with Whatman No.1 filter paper. The eight fungal extract were concentrated into solid residue by evaporation under rotary evaporator.¹⁵

Screening the antimicrobial activity of endophytic fungal extracts

The fungal extracts were subjected to screening for antimicrobial activities against four bacterial strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 28739, *Bacillus megaterium* ATCC 28318 and *Pseudomonas aeruginosa* ATCC 27833, two fungal strains *Aspergillus niger* and *A. flavus*. Antimicrobial activity was determined by the established disc diffusion method¹⁶ and compared with the standard antibiotics Kanamycin and Ketoconazole for antibacterial and antifungal activities respectively at concentration of 30µg/disc. Solvent was used as negative control. The antimicrobial activity of the fungal extracts (100 µg/disc) were determined by measuring the diameter of inhibition zone around each treated disc and expressed in millimeter ± standard deviation.

Screening the antioxidant activity of endophytic fungal extracts

A free radical scavenging assay was executed for the endophytic cultures as described by Braca *et al.* 2001¹⁷ using 2.0 µg/mL of DPPH in methanol. The free radical scavenging activities of the fungal extracts were measured at 517 nm and calculated according to the

equation: percentage scavenging (%) = $1 - (\text{absorbance of sample at } 517 \text{ nm} / \text{absorbance of control at } 517 \text{ nm}) \times 100\%$. The percent radical scavenging activity was determined by a comparison with the positive control, ascorbic acid and trolox. The experiment was repeated three times.

Screening the cytotoxic activity of endophytic fungal extracts

For the evaluation of cytotoxic activity, brine shrimp lethality bioassay, designed by Meyer *et al.* 1982¹⁸ was performed. Dimethylsulphoxide (DMSO) was used as a negative control, whereas anticancer drug vincristine sulphate used as positive control for this investigation. *Artemia salina* (brine shrimp) was hatched and incubated in sea water for 24 hr. Samples of different concentrations from 400 to 0.781 $\mu\text{g/mL}$ prepared by serial dilution in test tubes containing ten live nauplii in 5 ml of sea water. The median lethal concentrations (LC_{50}) of the endophytes were determined after 24 hr of observation by a plot of percentage of the shrimp mortality against the logarithm of the sample concentrations.

Statistical analysis

Mean IC_{50} values between groups were compared for antioxidant assay using independent student *t*-test for

equality of variances. For the scrutiny of the antimicrobial results, standard deviation data was calculated.

RESULTS AND DISCUSSION

A total of eight endophytic fungi such as CDLE 1 (leaf endophyte, Figure 1), CDLE 2 (leaf endophyte, Figure 2), CDRE 1 (root endophyte, Figure 3), CDRE 2 (root endophyte, Figure 4), CDRE 3 (root endophyte, Figure 5), CDBE 1 (bark endophyte, Figure 6), CDBE 2 (bark endophyte, Figure 7) and CDBE 3 (bark endophyte, Figure 8) were isolated and purified on PDA medium from *Commelina diffusa*. Among them five (CDLE 1, CDRE 1, CDRE 2, CDRE 3, CDBE 1) endophytes were taxonomically identified as *Fusarium* sp., CDBE 3 was distinguished as *Alternaria* sp. on the basis of their macroscopic and microscopic morphological characters and remaining two fungi could not be identified from these macroscopic and microscopic morphological characters.

Chemical screening

Results of preliminary mycochemical screening are summarized in Table 1. This investigation reveals the presence of various important chemical groups in different fungal extracts of this plant. These bioactive secondary metabolites produced by the isolated

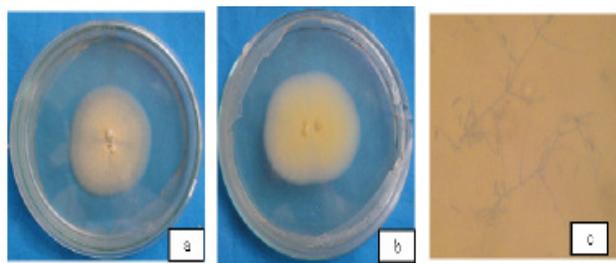


Figure 1: Macroscopic front (a) Macroscopic back (b) and Microscopic (c) view of leaves of *Fusarium* sp. (CDLE 1).

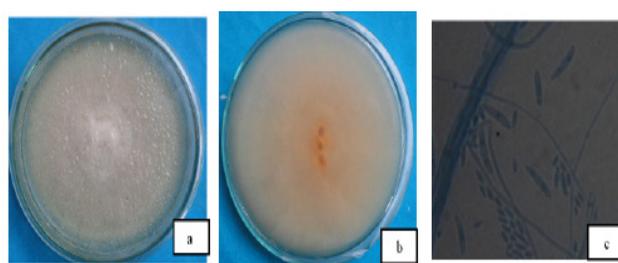


Figure 3: Macroscopic front (a) Macroscopic back (b) and Microscopic (c) view of root of *Fusarium* sp. (CDRE 1).

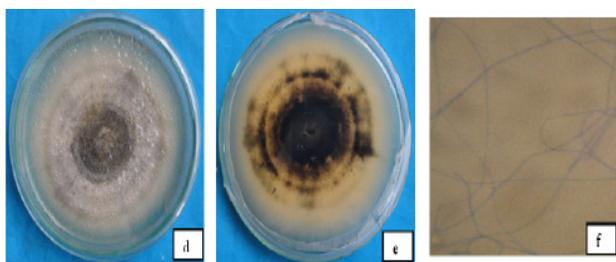


Figure 2: Macroscopic front (d) Macroscopic back (e) and Microscopic (f) view of leaves of *unidentified* sp. (CDLE 2).

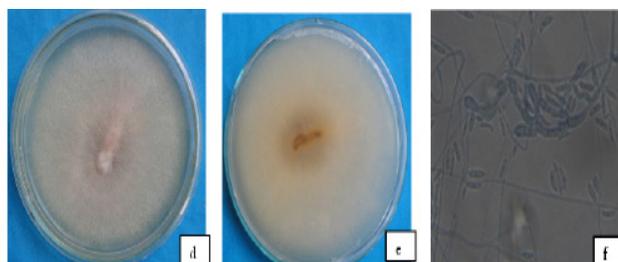


Figure 4: Macroscopic front (d) Macroscopic back (e) and Microscopic (f) view of root of *Fusarium* sp. (CDRE 2).

endophytes are known to generate characteristics physiological action beneficial to plant (Figure 9).

Macroscopic and microscopic identification

Strain CDLE 1, CDRE 1, CDRE 2, CDRE 3, CDBE 1

After 3 days of culture in PDA at 28°C, colony diameters of *Fusarium* sp. under light were found to be 3 to 4.4 cm (Figure 1, Figure 3-6). Aerial mycelia were abundant, densely floccose to fluffy and off white to pinkish in color and colony undersides on PDA were of pale orange and off white colored. Orange sporodochia were present in most isolates. No odor was detected. Microconidia were produced in false heads on monophialides or polyphialides, mostly nonseptate, oval, obovoid with a truncate base, elliptical in shape. Macroconidia were born in sporodochia, typically three-septate, fusiform, with slightly curved apical cell. Chlamydo spores were smooth, intercalary or terminal and produced in single or in pairs. Phialides of the conidiophores were cylindrical, consisting of short monophialides up to 10 µm long and 4.0 µm wide, or longer and more slender monophialides up to 41 µm long and 2.5 µm wide. Depending on these morphological characters these species were identified as *Fusarium* sp.

Strain CDBE 3

Strain CDBE 3 showed hyaline mycelium that turned to grey-brownish, multicelled, septate and irregularly branched (Figure 8). In early growing stage, hyphae were thin (2.84 µm in diameter), narrow, hyaline but

became slightly thick (4.42 µm in diameter) as they grew old. Conidiophores a rised singly or in clusters, pale olivaceous to olivaceous-brown, straight or curved, geniculate, slightly swollen at apex having terminal scars indicating the point of attachment of conidia. From these characteristics CDBE 3 was identified as *Alternaria* sp.

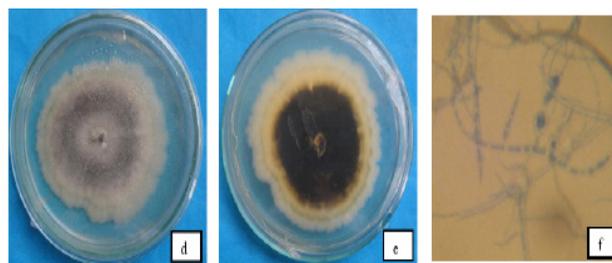


Figure 7: Macroscopic front (d) Macroscopic back (e) and Microscopic (f) view of bark of unidentified sp. (CDBE 2).

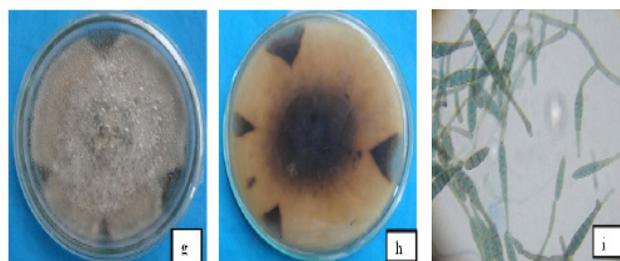


Figure 8: Macroscopic front (g) Macroscopic back (h) and Microscopic (i) view of bark of *Alternaria* sp. (CDBE 3).

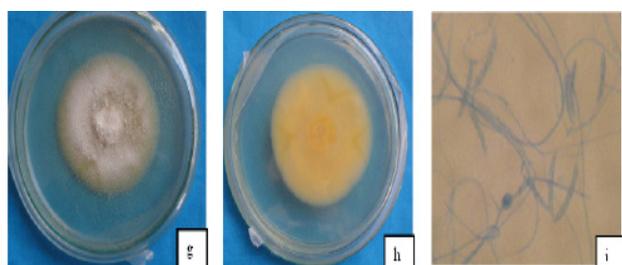


Figure 5: Macroscopic front (g) Macroscopic back (h) and Microscopic (i) view of root of *Fusarium* sp. (CDRE 3).

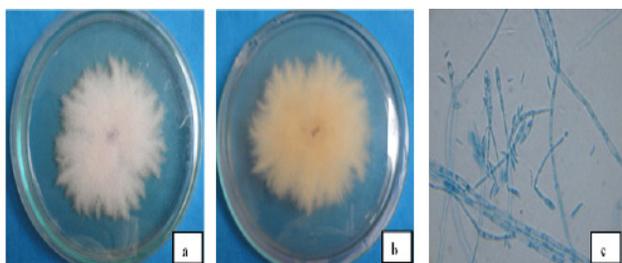


Figure 6: Macroscopic front (a) Macroscopic back (b) and Microscopic (c) view of bark of *Fusarium* sp. (CDBE 1).

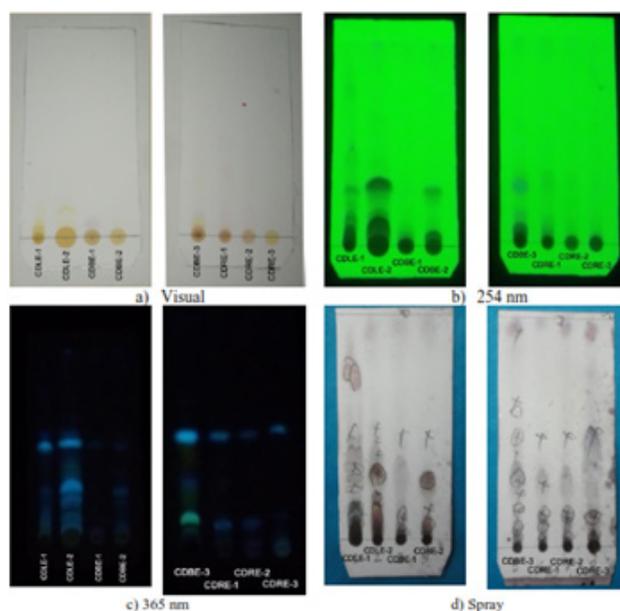


Figure 9: TLC screening of the fungal extracts (CDLE-1, CDLE-2, CDBE-1, CDBE-2, CDBE-3, CDRE-1, CDRE-2, CDRE-3) by visual observation (a), under UV at 254 nm (b), at 365 nm (c) and finally after spraying with spray reagent (d).

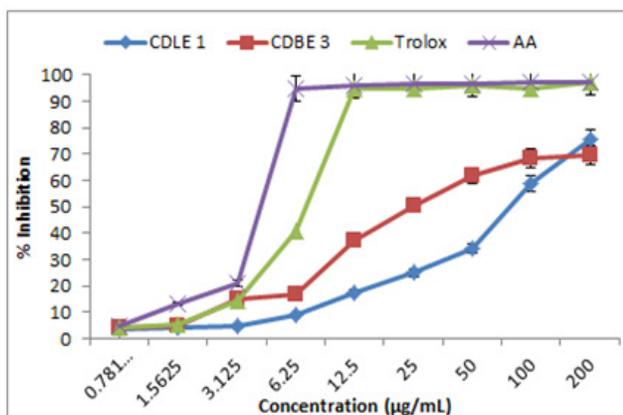


Figure 10: Free radical scavenging activity of different concentrations of endophytic fungi, *Fusarium* sp. (CDLE 1), *Alternaria* sp. (CDBE 3) and two standards.

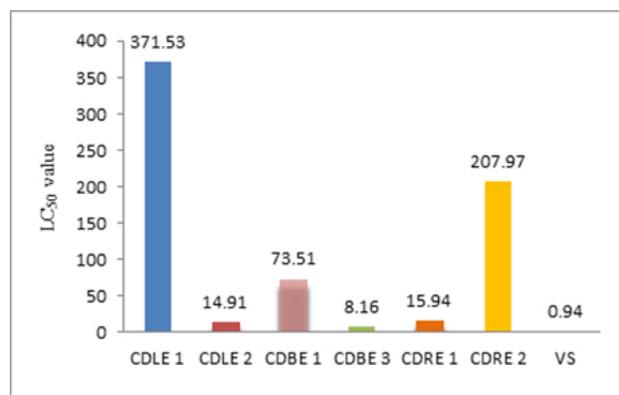


Figure 11: LC₅₀ values of the standard (VS) and different endophytic fungi of *Commelina diffusa*.

Table 1: Preliminary chemical screening of fungal ethyl acetate extracts.

| Fungal Extract | Visual color | UV light at 254 nm | UV light at 365 nm | After spray | Remarks |
|----------------|--------------|--------------------|--------------------|---------------|--------------------------|
| CDLE 1 | Yellow | Dark | Orangish | Purple | Anthraquinone/Flavonoid |
| | Orange Red | Dark | Blue | Purple | Anthocyanin |
| | - | Dark | Blue | Purple | Coumarin |
| | | Dark | - | Purple | Terpenoid |
| CDLE 2 | Yellow | Dark | Blue | - | Flavonoid |
| | - | Dark | Blue | Green | Coumarin/Flavonoid |
| | | Dark | Blue | Red/Purple | Coumarin |
| CDBE 1 | Light purple | Dark | - | Purple | Anthocyanin |
| | - | Dark | Blue | - | Coumarin |
| CDBE 2 | - | Dark | Blue | - | Coumarin |
| | - | Dark | - | Red/Brick red | Terpenoids |
| | - | Dark | Blue | Purple | Flavonoid/ Coumarin |
| CDBE 3 | Yellow | Dark | Orange | Purple | Flavonoid/Anthraquinone |
| | Orange red | Dark | Greenish | - | Coumarins |
| | | Dark | Blue | Purple | Flavonoid/Anthraquinone |
| CDRE 1 | Purple | Dark | - | Purple | Anthocyanin |
| | Orange red | Green | Red | Purple | Flavonoid |
| | - | Black/Blue | Blue | - | Coumarin |
| | | Dark | - | Purple/Brown | Terpenoids |
| CDRE 2 | Purple | Dark | - | Purple | Terpenoids/Anthraquinone |
| | Orange red | Green | Red | Purple | Flavonoid |
| | - | Blue | Blue | - | Coumarin |
| CDRE 3 | - | Dark | Blue | Red/Brick red | Flavonoid/ Coumarins |

Table 2: Antimicrobial activity of endophytic fungal strains isolated from *Commelina diffusa*.

| Sample | Diameter of Zone of Inhibition (mm) | | | | | | |
|-------------------------------|-------------------------------------|----------------------|----------------------|------------------|-----------------|------------------|----------|
| | Bacteria Strain (Mean±SD) | | | | Fungal Strain | | |
| | Gram Negative | | Gram Positive | | | | |
| | <i>E. coli</i> | <i>P. aeruginosa</i> | <i>B. megaterium</i> | <i>S. aureus</i> | <i>A. niger</i> | <i>A. flavus</i> | |
| CDLE 1 | 14.2±0.763 | 11.8±0.763 | 9.6±0.360 | 11.6±0.513 | - | - | |
| CDLE 2 | 11.5±0.5 | 9.6±0.513 | - | 9.6±0.793 | - | - | |
| CDBE 1 | - | - | - | - | - | - | |
| CDBE 2 | 10.4±0.351 | 10.5±0.5 | - | 10±0.5 | 27.5±0.5 | - | |
| CDBE 3 | 9.2±0.2 | 9.8±0.680 | - | 8.7±0.707 | - | - | |
| CDRE 1 | 11.8±1.755 | 10.4±0.513 | 9.5±0.5 | 10.7±1.101 | 29.7±0.642 | - | |
| CDRE 2 | 21.9±1.101 | 9.5±0.5 | 10.6±0.655 | 9.3±1.040 | 30.3±0.351 | 20.4±0.360 | |
| CDRE 3 | 9.9±0.360 | - | - | 11.4±1.216 | - | - | |
| Positive control (30 µg/disc) | KM | 33±1.0 | 34.5±0.5 | 49.6±0.513 | 36.5±0.5 | - | - |
| | KC | - | - | - | - | 39.8±0.763 | 42.5±0.5 |

(-) No Activity

CDLE 2 and CDBE 2 were not identified as their spore was absent (Figure 2, Figure 7). But their bioactivity was checked for potential response.

Antimicrobial activity screening

There were eight endophytic fungi isolated and screened for antimicrobial activity against the tested pathogenic micro-organism (Table 2). Three fungi (CDBE 2, CDRE 1, and CDRE 2) showed potential activity against *A. niger* in comparison with ketoconazole (30 µg/disc). Results showed that, two of the eight fungi (CDLE 1 and CDRE 2) showed moderate inhibitory effect towards *E. coli* with inhibition zone of more than 12 mm, while others fungi produced inhibition less than 10 mm on *P. aeruginosa*, *B. megaterium*, *S. aureus*.

Antioxidant activity screening

The endophytic DPPH radical scavenging potential was assessed in contrast to the positive control (ascorbic acid). To explore the antioxidant potential of the eight isolated endophytes, two were analyzed for their capacity to scavenge oxidative radicals. The results demonstrated that CDLE 1 and CDBE 3 had higher radical scavenging abilities with IC₅₀ value of 105.48 µg/mL and 32.36 µg/mL compared to the ascorbic acid (4.37 µg/mL) and trolox (4.79 µg/mL) respectively (Figure 10). CDLE-1 showed significant variation in mean IC₅₀ value as compared to standards ($p < 0.01$) whereas CDBE 3

did not exhibited significant variation in mean IC₅₀ value from the standards ($p > 0.05$). So CDBE 3 has the capacity to release secondary metabolites which may have high antioxidant activity.

Cytotoxic activity screening

In cytotoxic activity evaluation, the standard vincristine sulphate (VS) that was used as positive control for which LC₅₀ was found to be 0.94 µg/mL. The LC₅₀ values of CDLE 1, CDLE 2, CDBE 1, CDBE 3, CDRE 1 and CDRE 2 were found to be 371.53 µg/mL, 14.91 µg/mL, 73.51 µg/mL, 8.16 µg/mL, 15.94 µg/mL and 207.97 µg/mL respectively (Figure 11). Comparing these values with the value obtained from standard, it was observed that these endophyte CDBE 3 (*Alternaria* sp.) and CDRE 1 (*Fusarium* sp.) were quite lethal for the brine shrimp nauplii.

CONCLUSION

Successive sterilization, isolation and purification yielded a total of eight endophytic fungi. From the biological investigations, it is proved that, the endophytic fungi of *C. diffusa* exhibited moderate antimicrobial activity and strong antioxidant, cytotoxic activities. However, further, studies are required to isolate more bioactive compounds responsible for the activities of these endophytic fungi of *Commelina diffusa*.

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Cite this article: Nasrin M, Afroz F, Begum N, Rony SR, Sharmin S, Moni F, et al. Isolation and Bioactivity Screening of Endophytic Fungi from *Commelina diffusa*. Indian J of Pharmaceutical Education and Research. 2021;55(3):829-36.