



Bioactivity Screening and Identification of Secondary Metabolites from Fungal Endophytes of *Carica papaya* L. Leaves

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Abstract

Objectives: This work describes the chemical and *in-vitro* bioactivity studies on endophytic fungi derived from the leaves of *Carica papaya* L. **Methods:** Column Chromatography was employed for isolation of fungal metabolites. Nuclear Magnetic Resonance (NMR) spectral data was utilized to elucidate the structure of the isolated compounds and physicochemical properties of them were also examined. As a part of *in-vitro* bioactivity screening disc diffusion method, 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging assay and brine shrimp lethality bioassay were conducted to evaluate antimicrobial, antioxidant and cytotoxic activities, respectively. **Results:** A total of four fungal isolates were identified as endophytic fungi and purified from the young leaves of *Carica papaya*. These fungi, encoded as CPLE-1, CPLE-2, CPLE-3 and CPLE-4, were identified up to the genus level on the basis of their macroscopic and microscopic characteristics as *Colletotrichum* sp., *Carvularia* sp., and next two as *Alternaria* sp., respectively. Chemical profiling and preliminary bioactivity screening of the fungal endophytes signified strain CPLE-3 with highest potentiality to produce bioactive compounds which directs its large scale cultivation. Ergosterol and Alternariol monomethyl ether were isolated from the ethyl acetate extract of the fungus, CPLE-3. **Conclusion:** Bioactivity and chemical screening of the endophytic fungal extracts of *Carica papaya* leaf along with the isolation of fungal secondary metabolites suggested these endophytic fungi to be possible source of bioactive leads for developing new and improved medicines.

Keywords: Alternaria, Alternariol Monomethyl Ether, Carvularia, Colletotrichum, Ergosterol

1. Introduction

Plants are known to biosynthesize diverse range of chemical compounds with potent pharmacological activities. Endophytes isolated from plants are now gaining much attention for their ability to produce metabolites with therapeutic value. These metabolites

are prospective source of leads that can be developed as new drugs for treating both existing and newly invading diseases¹.

Bangladesh is a repository of medicinal plants belonging to various families, including *Caricaceae*. *Carica papaya* (Papaya, Bengali name: pepe), belonging to this family, is an economically important

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fruit crop cultivated worldwide including Bangladesh. The chemistry and pharmacology of different papaya plant parts such as, leaves, stems, roots, seeds, fruits and latex have already been evaluated². Several pharmacological activities including antibacterial, antitumor, antifungal and immunomodulatory activity have also been reported for papaya plant extract^{3,4}. Fruit and seed of papaya are rich source of vitamins, pectins and carotenoids which also have contraceptive efficacy^{5,6}. Overall, these findings indicate that this plant has tremendous medicinal value. Medicinal plants are host to endophytic fungi which may produce secondary metabolites or their derivatives alike host plant due to metabolic interactions. There are some reports on isolation of endophytic fungi of *Colletotrichum*, *Fusarium*, *Epicoccum*, *Aspergillus* and *Alternaria* genera from papaya collected in Nigeria and India with different pharmacological prospectus such as moderate antibacterial and cytotoxic activities, anti-quorum sensing activity against *Pseudomonas aeruginosa* etc⁷⁻¹⁰. So, this investigation was carried out to isolate endophytic fungi from papaya grown in Bangladesh with their chemical and *in-vitro* bioactivity screening which is therefore first report on the endophytic fungal distribution of *C. papaya* grown in Bangladesh.

2. Materials and Methods

2.1 Experimentation

Analytik Jena Specord 250 plus ultraviolet-visible (UV-VIS) spectrophotometer was used to measure absorbance at 517 nm. Column chromatography (CC) was conducted for separation of secondary metabolites on silica gel (70-230 and 230-400 mesh, Merck, Germany). Thin layer chromatography (TLC) was carried out on precoated silica gel (kieselgel 60 PF₂₅₄). The TLC plates were visualized under 254 nm and 365 nm followed by treating with spray reagent (1% vanillin-sulphuric acid) and heating at 110°C. Bruker 400 MHz NMR spectrometer (Bruker, Switzerland) was used to record 1D- and 2D-NMR spectra using Trimethylsilane (TMS) as an internal standard.

2.2 Plant Material Collection

The leaf samples from approximately 12 months old and fruiting *C. papaya* plants were collected from Jamalpur district (89°40' to 90°10' East and 24°40' to 25°20' North), Bangladesh in December 2016. The collected plant was identified from Bangladesh National Herbarium (BNH), Dhaka, Bangladesh. A voucher specimen of this collection was deposited under the accession number DACB- 3891.

2.3 Isolation and Identification of Fungi

After proper washing selected disease free and young leaves were cut into small pieces (1.5 cm x 1.5 cm). Leaf parts were then surface sterilized by treating with 70% ethanol (1 min), 1.3 mol/L sodium hypochlorite (approximately 5% available chlorine, 3 min), 70% ethanol (30 sec) and finally with distilled water under aseptic condition following a standard protocol¹¹. Surface sterilized leaf parts were placed on water agar (WA) media in petri dishes (3 leaf parts placed individually on each of 30 petri dishes) infused with streptomycin (200 µg/mL) along with negative control where unsterilized leaf parts were used. The petri dishes were incubated in a cooled incubator (Froilabo BRE 120, France) at 28 ± 2°C for 21 days until any fungal hypha becomes visible. Hyphal tips, upon visualization, were transferred onto potato dextrose agar (PDA) media and serial dilution technique was used on PDA to obtain pure fungal colonies. Fungal isolates were compared with control fungal isolates to separate the endophytic fungal strains. These isolated and purified endophytic fungal strains were preserved in 10% glycerol for long term storage.

For microscopic identification, slides of the matured fungal isolates were prepared by staining with lactophenol cotton blue¹². Microscopic observation of fungal isolates was carried out on transmitted light microscope (Kruss, Germany) with objective lens of 40 times magnification and 0.65 numerical apertures. For morphological identification different taxonomic key features such as growth pattern, hyphae, the color of the colony and medium, margin character, aerial mycelium, surface texture, sporulation and production of acervuli were checked until full growth of the fungi with intermittent observations^{13,14}. The morphological

characters were compared with those described in standard protocols^{14,15}.

2.4 Small Scale Cultivation in PDA Medium

Each fungal isolate was cultured on approximately 500 mL of PDA media in petri dishes for 21 days at $28 \pm 2^\circ\text{C}$. The culture media was extracted two times with ethyl acetate to obtain the crude extract according to the published literature and finally these extracts were concentrated by evaporation with rotary evaporator^{16,17}.

2.5 Antimicrobial Screening

The antibacterial activities were assayed by the disc diffusion method^{18,19}. Five pathogenic bacterial strains *Escherichia coli* (ATCC 28739TM), *Bacillus megaterium* (ATCC 25118TM), *Pseudomonas aeruginosa* (ATCC 27833TM), *Streptococcus pneumonia* (ATCC 49619TM) and *Staphylococcus aureus* (ATCC 25923TM), collected from American Type Culture Collection (ATCC), USA, were used for this investigation. The bacterial suspension of approximately 10^8 CFU/mL prepared from freshly subcultured specimens were used for inoculation. The zones of growth inhibition of the discs were measured after 24 hours of incubation at 37°C and the sensitivities of the bacterial species were compared to Azithromycin ($30\mu\text{g}/\text{disc}$) as positive control. Solvent discs were used as negative control.

2.6 Antioxidant Activity

The antioxidant activity of the isolates was measured by the DPPH radical scavenging method using the following equation²⁰:

$$\text{Scavenging ability (\%)} = [(A_{517} \text{ of control} - A_{517} \text{ of sample}) / A_{517} \text{ of control}] \times 100$$

Concentration of the isolates exhibiting 50% scavenging of the DPPH radical (IC_{50}) were calculated. Ascorbic acid (ASA) and Butylated hydroxyanisole (BHA) were used as positive controls.

2.7 Brine Shrimp Lethality Bioassay

The cytotoxic activity was performed by slight modification of the established Brine shrimp lethality bioassay method²¹. Eggs of *Artemia salina* shrimp

nauplii were hatched for 24 hours in simulated sea water with continuous supply of oxygen. After 24 hours, 10 of the hatched nauplii were placed in each test tube and final volume of these test tubes was made up to 5 mL with simulated sea water. The sample solutions (30 μL) of fungal extracts and column fractions in Dimethylsulphoxide (DMSO) at different concentration levels, obtained by serial dilution method, were added to these test tubes containing 10 shrimp nauplii in 5 mL of simulated sea water. This resulted in the final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562 $\mu\text{g}/\text{mL}$ for each sample. Test solutions were then incubated at room temperature for 24 hours. The concentration for 50% shrimp mortality (LC_{50}) of the isolates was measured by plotting percentage of mortality against the logarithm of the sample concentrations.

2.8 Isolation of Secondary Metabolites from Fungal Extract

Alternaria species (CPL-3) was cultured for 21 days at $28 \pm 2^\circ\text{C}$ on approximately 6 L of PDA media. The cultured media were extracted two times with ethyl acetate to obtain approximately 520 mg of the crude extract^{16,17}. The crude extract was fractionated by CC on silica gel, using solvents *n*-hexane, dichloromethane (DCM) and methanol (MeOH) in gradients of increasing polarity which resulted in a total of 70 fractions. After solvent evaporation of fractions eluted with gradient mixture of *n*-hexane/45-50% DCM, slightly impure crystals were formed which were further purified through solvent treatment to give compound 1. The crude extract was again subjected to CC for fractionation on silica gel, using solvents *n*-hexane, ethyl acetate (EtOAc) and MeOH in gradients of increasing polarity which resulted in a total of 40 fractions. These fractions were screened for the presence of similar components by TLC observation using solvent system of Toluene/10-50% EtOAc and Chloroform/2-20% MeOH depending on the polarity of each eluted fraction. Fractions eluted with gradient mixture of *n*-hexane/50-80% EtOAc were mixed after observing their similarity in TLC screening and termed as F-3. F-3 was subjected again to CC using solvents *n*-hexane, EtOAc and MeOH in gradients of increasing polarity which resulted in a total of 34 fractions.

Solvent evaporation of the sub CC fractions eluted with *n*-hexane/40-65% EtOAc [F-6] resulted in slightly impure crystal which was further purified through solvent treatment to give compound 2. NMR spectral analysis was performed to fix the structures of the pure compounds. Different column fractions, selected for *in-vitro* bioactivity studies, were shown in Table 1.

2.9 Statistical Analysis

All the experiments were conducted in triplicate. For antimicrobial screening results were presented as the mean \pm standard deviation of the mean. For antioxidant and brine shrimp lethality bioassay, 5% standard error values of the mean IC₅₀ and LC₅₀ data of each sample were presented, respectively.

3. Results

3.1 Identification of the Fungal Strains

A total of four fungal strains, namely CPLE-1, CPLE-2, CPLE-3 and CPLE-4 were isolated (Figure 1) from the papaya plant.

For strain CPLE-1 acervuli was found as disc-shaped or cushion-shaped, waxy, subepidermal, with dark spines or setae at the edge or among the conidiophores. Conidiophores were observed as simple, elongated. Conidia were hyaline, one-celled, both ovoid and oblong. The cultures on PDA had sparse, cottony, pale grey mycelium containing bright orange conidial masses produced in concentric rings in many mycelia.

Table 1. Sample code for Bioactivity screening from column fractions

Fraction no.	Bioactivity Sample
CPE 1 (<i>n</i> -hexane/15% EtOAc)	F-1
CPE 2 (<i>n</i> -hexane/20% EtOAc)	F-2
CPE 10 – CPE 21 (<i>n</i> -hexane/50-80% EtOAc)	F-3
CPE 24 – CPE 32 (<i>n</i> -hexane/85-100% EtOAc and EtOAc/0-2%MeOH)	F-4
CPE 37- CPE 40 (EtOAc/20-100% MeOH)	F-5
Subcolumn fractions of F-3	
CPF 8 – CPF 13 (<i>n</i> -hexane/40-65% EtOAc)	F-6
CPF 14 – CPF 15 (<i>n</i> -hexane/70-80% EtOAc)	F-7
CPF 16 – CPF 18 (<i>n</i> -hexane/85-95% EtOAc)	F-8
CPF 19 – CPF 20 (100% EtOAc)	F-9
CPF 21– CPF 22 (EtOAc/0-1% MeOH)	F-10
CPF 23 – CPF 24 (EtOAc/1.5-2% MeOH)	F-11
CPF 25 – CPF 28 (EtOAc/3-10% MeOH)	F-12
Subcolumn fractions of F-6	
CPf 1 –CPf 2 (<i>n</i> -hexane/30-35% DCM)	F-13
CPf 7 –CPf 8 (<i>n</i> -hexane/60-65% DCM)	F-14
CPf 11 –CPf 12 (<i>n</i> -hexane/80-85% DCM)	F-15
CPf 13 (<i>n</i> -hexane/90% DCM)	F-16
CPf 14 –CPf 15 (<i>n</i> -hexane/95-100% DCM)	F-17
CPf 21 (DCM/0-2% MeOH)	F-18

*CPE: Column fractions of fungal strain CPLE-3 ethyl acetate crude extract; CPF: Subcolumn fractions of F-3; CPf: Subcolumn fractions of F-6

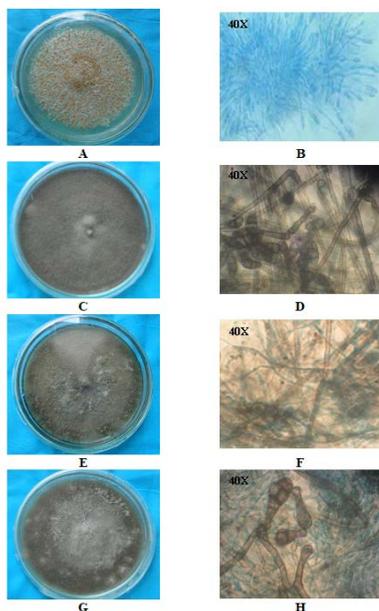


Figure 1. Macroscopic and microscopic view (magnification of 40x) of the endophytic fungi from *Carica papaya*. A. CPLE-1 (Macroscopic view), B. CPLE-1 (Microscopic view), C. CPLE-2 (Macroscopic view), D. CPLE-2 (Microscopic view), E. CPLE-3 (Macroscopic view) and F. CPLE-3 (Microscopic view), G. CPLE-4 (Macroscopic view) H. CPLE-4 (Microscopic view).

Based on all morphological characteristics this strain CPLE-1 was confirmed as *Colletotrichum* sp^{14,15}.

In case of strain CPLE-2, conidiophores were found to be brown, simple and sometimes observed as branched, bearing spores. Conidia was found to be brown, and cells lighter, 3 to 5 celled, rounded at the ends or sometimes fusiform, typically bent or curved, with one or two of the median cells enlarged. Blackish-gray coloration of colonies was observed in the PDA media. These morphological characteristics suggested strain CPLE-2 as *Curvularia* sp^{14,15}.

In case of strain CPLE-3 and CPLE-4, conidiophores were observed as dark, simple, bearing simple or branched conidia. Conidia was dark with cross and longitudinal septa; different shaped, obclavate to elliptical, frequently borne acropetally in long chains, less often borne singly with branched appendage at apex. The colony in the PDA media was flat to woolly and was covered by grayish, short, aerial hyphae. The

surface was observed as grayish black whereas the reverse side as of black color. Finally depending on these morphological characteristics, strain CPLE-3 and CPLE-4 were identified as *Alternaria* sp. by comparison with published standard taxonomic key^{14,15}.

3.2 Spectroscopic Characterization of Isolated Compounds

(1) Ergosterol: Compound 1 [Figure 2] was a white needle shaped crystal. It was soluble in DCM and chloroform. It exhibited a dark quenching spot on TLC ($R_f = 0.39$, Toluene/20% EtOAc) at 254 nm. Treatment of the developed plate with spray reagent gave a purple color.

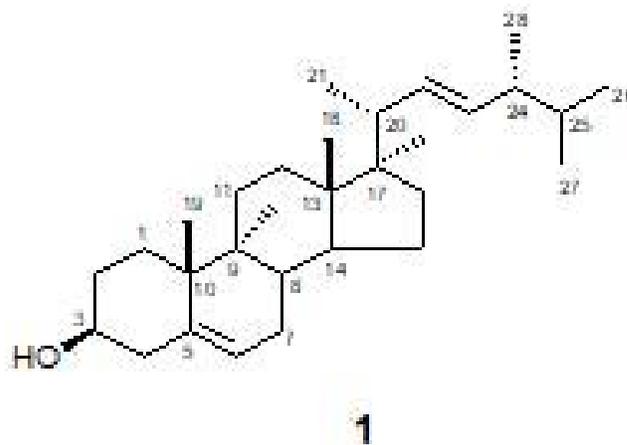


Figure 2. Compound 1 Ergosterol.

¹H-NMR (400 MHz, CDCl₃): $\delta = 3.63$ (1H, m, 3-H), 5.56 (1H, m, 6-H), 5.19 (3H, m, 7-H), 5.38 (2H, m, 22-H), 1.03 (3H, m, 21-H), 0.94 (3H, m, H-28), 0.83 (3H, s, 26-H), 0.62 (3H, m, 18-H). ¹³C-NMR (100 MHz, CDCl₃): $\delta = 39.2$ (C-1), 32.0 (C-2), 70.5 (C-3), 42.9 (C-4), 139.8 (C-5), 119.6 (C-6), 116.3 (C-7), 141.4 (C-8), 46.3 (C-9), 37.0 (C-10), 21.1 (C-11), 40.5 (C-12), 42.9 (C-13), 54.6 (C-14), 23.0 (C-15), 28.3 (C-16), 55.7 (C-17), 12.1 (C-18), 16.3 (C-19), 40.8 (C-20), 131.9 (C-22), 135.6 (C-23), 33.1 (C-25), 19.9 (C-26), 19.7 (C-27), 17.6 (C-28).

(2) Alternariol Monomethyl Ether: Compound 2 [Figure 3] was a white fine needle shaped crystal and soluble in Dimethyl sulphoxide. It exhibited a blue quenching spot on TLC ($R_f = 0.48$, Toluene/10% EtOAc) at 254 nm and a blue fluorescence at 365 nm.

Treating the developed plate with spray reagent gave purple spot.

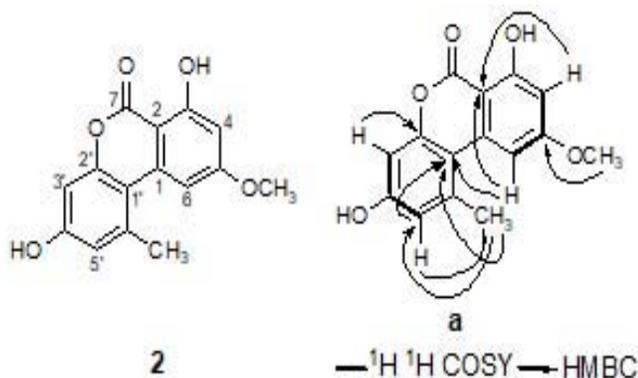


Figure 3. Connectivities of compound 2 Alternariol monomethyl ether.

$^1\text{H-NMR}$ (400 MHz, DMSO-D_6): $\delta = 6.61$ (1H, brs, 4-H), 7.21 (1H, brs, 6-H), 6.64 (1H, brs, H-3'), 6.72 (1H, brs, H-5'), 11.82 (1H, s, 3-OH), 3.90 (3H, s, 5-OCH₃), 10.36 (1H, brs, 4'-OH), 2.72 (3H, s, 6'-CH₃). $^{13}\text{C-NMR}$ (100 MHz, DMSO-D_6): $\delta = 137.8$ (C-1), 108.8 (C-1'), 98.5 (C-2), 152.6 (C-2'), 164.1 (C-3), 101.6 (C-3'), 99.2 (C-4), 158.6 (C-4'), 164.7 (C-5), 117.6 (C-5'), 55.8 (5-OCH₃), 103.4 (C-6), 138.4 (C-6'), 25.0 (6'-CH₃), 116.2 (C-7).

3.3 Antimicrobial Screening

The results of antimicrobial screening are shown in Table 2. Four crude fungal extracts showed good to moderate activity against *P. aeruginosa* whereas CPLE-1 and CPLE-3 exhibited prominent activity against *S. aureus* as compared to the standard Azithromycin. Column chromatographic separation of crude fungal extracts of CPLE-3 yielded 18 fractions containing different groups of metabolites based on polarity. Among these fractions, F-16 showed good antibacterial activity against *B. megaterium*, *S. aureus* and *P. aeruginosa*; whereas fraction F-3 showed moderate activity against *B. megaterium*, *S. aureus*, *E. coli* and *P. aeruginosa*.

3.4 Antioxidant Activity

Different fungal extracts and column fractions of fungal extract CPLE-3 were assayed for their free radical scavenging ability. All the fungal extracts exhibited good antioxidant activity with IC_{50} values of 2.41 to 4.80 $\mu\text{g/mL}$ as compared to the standard.

Column fraction F-3 and F-4 of CPLE-3 fungal extract showed most prominent antioxidant activity as shown in Figure 4.

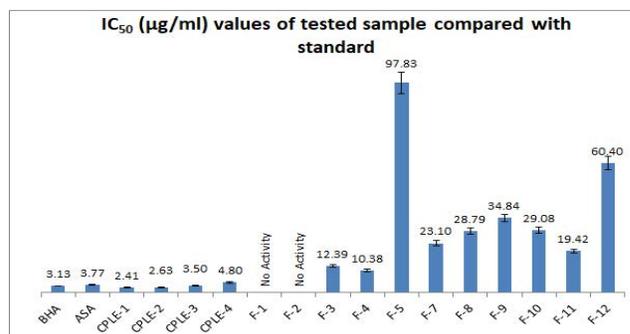


Figure 4. Free radical scavenging activity of fungal extracts and different column fractions of Endophytic fungal extract CPLE-3 with 5% error bar.

3.5 Brine Shrimp Lethality Bioassay

The crude fungal extracts were found to be toxic to the shrimp naupli (LC₅₀ values of 4.92 to 9.14 $\mu\text{g/mL}$) as compared to the standard (Figure 5). The main column fractions of the CPLE-3 fungal extracts also showed significant activity as compared to the standard. Fraction F-2 was highly cytotoxic as shrimp death occurred within 04 hours of application.

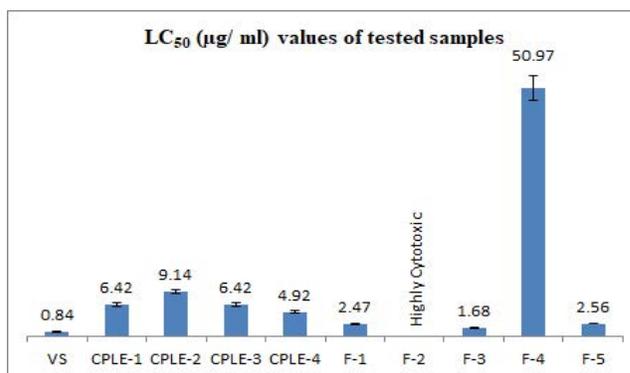


Figure 5. Cytotoxic effect of fungal extracts and different column fractions of Endophytic fungal extract CPLE-3 with 5% error bar.

4. Discussion

Endophytic fungi produced different pharmacologically important compounds of which some were found to be structurally analogous with the isolated host

Table 2. Antibacterial activities of fungal extracts of *Carica papaya* and column fractions of endophytic fungal extract, CPLE-3

Sample (100 µg/disc)	Zone of Inhibition (mm±SD)				
	Bacterial Strain				
	Gram positive			Gram negative	
	<i>Bacillus megaterium</i>	<i>Staphylococcus aureus</i>	<i>Streptococcus pneumonia</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
CPLE-1	ND	18.11±0.29	9.66±0.1	-	15.67±0.5
CPLE-2	ND	-	-	8.1±0.29	16.11±0.29
CPLE-3	ND	18.33±0.5	7.1±0.5	12.33±0.67	18.67±0.3
CPLE-4	ND	11.33±0.29	9.3±0.67	14.67±0.5	12.1±0.67
F-3	11±0.67	11.3±0.5	ND	11±0.5	15.3±0.1
F-4	10.33±0.67	9.33±1.1	ND	7.33±0.1	10.29±0.67
F-5	-	7.1±0.67	ND	-	7.33±0.1
F-6	11.1±0.29	9.7±0.29	ND	10.1±0.29	12.1±0.29
F-7	-	-	ND	-	-
F-8	-	7.5±0.5	ND	-	-
F-9	-	-	ND	-	-
F-10	-	-	ND	-	-
F-11	11.67±0.1	7.1±0.1	ND	7.0±0.5	7.1±0.5
F-12	-	7.33±0.1	ND	-	-
F-15	9.33±0.1	9.3±0.29	ND	8.1±0.29	10.1±0.29
F-16	15.1±0.29	15.67±0.1	ND	12.67±0.5	20.33±0.5
F-17	-	11.33±0.5	ND	12.1±0.67	9.1±0.29
F-18	-	7.1±0.29	ND	10.1±0.29	10.29±0.5
Positive control, AZM (30 µg/disc)	38.1±0.5	20.29±0.1	ND	21.33±0.1	22.1±0.1

“SD”-Standard deviation, “AZM”-Azithromycin; “-”-no sensitivity; “ND”-not done

plant compound²². Taking account of the medicinal importance of papaya plant, endophytic fungal diversity of them available in Bangladesh was investigated in this study. The four isolated fungi, CPLE-1 (*Colletotrichum* sp.), CPLE-2 (*Curvularia* sp), CPLE-3 (*Alternaria* sp.) and CPLE-4 (*Alternaria* sp), have important pharmacological value as several bioactive secondary metabolites have already been reported from these genera worldwide^{8,9,23-30}. Endophytic *Colletotrichum* species isolated from different medicinal plants in the Asian region had been reported for producing compounds with antimicrobial, antioxidant and anticancer properties such as Colletotric acid, 6-Isoprenylindole-3-carboxylic acid, Asparaginase,

Monocerin etc²³. Acropyrone, 4-hydroxyphenylacetic acid and indole-3-acetic acid were isolated from *Curvularia* sp. of Nigerian plant *Picralima nitida*²⁴. 2'-deoxyribolactone and hexylitaconic acid isolated from *Curvularia* sp. of *Rauwolfia macrophylla* collected in Cameroon exhibited antibacterial and cytotoxic activities²⁵. 11- α -methoxycurvularin isolated from *Curvularia* sp. of Indian *Oryza sativa* showed potential antibacterial, antifungal and larvicidal activities²⁶. Several compounds such as Alternariol, Altenusin and their derivatives isolated from endophytic *Alternaria* sp. collected from the Egyptian medicinal plant *Polygonum senegalense* exhibited significant cytotoxic activity by inhibiting proliferation of mouse

L5178Y lymphoma cells and also by inhibiting protein kinases²⁷. Another compound Alterperyleneol isolated from *A. tenuissima* of the Chinese medicinal plant *Erythrophleum fordii* exhibited cytotoxic activity on human colon cancer cell HCT-8²⁸. Because of the significant medicinal importance of these fungi, the bioactivity potentials of the four isolated endophytic fungi were examined. During antimicrobial screening, the two isolated *Alternaria* species, CPLE-3 and CPLE-4 exhibited good and moderate activity, respectively against the human pathogenic bacteria tested (Table 2). There is report on the quorum sensing inhibition activity of *A. alternata*, an endophyte isolated from *C. papaya* collected in India⁹. The isolated *Alternaria* species may produce this antibacterial activity by the similar mechanism. Moreover, Altersetin isolated from endophytic *Alternaria* sp. in Germany exhibited significant antibacterial activity against several human pathogenic bacteria²⁹. Additionally, strain CPLE-1 (*Colletotrichum* sp.) exhibited moderate to good activity against three of the four tested bacteria whereas CPLE-2 (*Curvularia* sp) exhibited moderate activity against the two Gram (-)ve bacteria tested. There is report on isolation of antimicrobial compounds from endophytic *Colletotrichum* sp. and *Curvularia* sp. of several medicinal plants^{23,25,26}. So, the isolated fungi have potentiality to produce biologically active compounds for antimicrobial agent development as these previous reports. The prominent antioxidant activity as shown by the isolated fungal extracts is suggestive of the presence of antioxidant compounds in these extracts which may play an important role to prevent diseases caused by reactive oxygen species like cancer, pathophysiological disorders. Furthermore, based on the LC₅₀ values (4.92 to 9.14 µg/mL) the order of cytotoxicity of the fungal strains was CPLE-4 < CPLE-3 < CPLE-1 < CPLE-2 (Figure 5). This result is in coherence with the previous reports on the isolation of cytotoxic compounds from endophytic *Colletotrichum* sp., *Curvularia* sp. and *Alternaria* sp. of several medicinal plants^{23,25,27,28,30}. However, *Colletotrichum gloeosporioides* isolated from leaves of *C. papaya* exhibited moderate antioxidant activity in the DPPH assay and moderate cytotoxic activity against cisplatin-sensitive ovarian cancer cell line 2780 (sens) and cisplatin-resistant ovarian cancer cell line 2780

(CisR)⁸. In this study the isolated *Colletotrichum* sp. exhibited good antioxidant activity in DPPH assay and also showed good cytotoxicity against *A. salina* naupli. These findings suggest that endophytic fungal colony differ with terrestrial region. For better antibacterial, antioxidant and cytotoxic property, strain CPLE-3 had been cultivated in large scale for bulk isolation of its bioactive compounds. Column and subcolumn fractions of strain CPLE-3 crude extract were screened for their bioactivity property for possible replication of the data in additional research on this fungus. F-3, F-4, F-6, F-11, F-15 and F-16 fractions showed moderate antibacterial property (Table 2) whereas F-3 and F-4 fractions showed most prominent antioxidant property (Figure 4). Fractions F-1, F-3 and F-5 exhibited significant cytotoxicity against *A. salina* naupli (Figure 5) along with the crude CPLE-3 extract (LC₅₀ value of 6.42 µg/mL) which indicates that these crude fungal extracts and fractions may contain metabolites with various pharmacological activities, thus necessitate further investigations with them. From the column chromatography of crude ethyl acetate extract of strain CPLE-3 eluted with *n*-hexane/45-50% DCM yielded compound **1** whereas subcolumn chromatography fraction of F-3 eluted with *n*-hexane/40-65% EtOAc (F-6) yielded compound **2**.

The ¹³C NMR spectrum (100 MHz, CDCl₃) of compound **1** displayed 28 carbons resonances. The 6 methyl signals at δ = 12.1, 16.3, 17.6, 19.7, 19.9 and 21.1 with an oxygenated methylene signal at δ = 70.5 and 6 olefinic carbon signals at δ = 116.3, 119.6, 131.9, 135.6, 139.8, and 141.4 observed in the ¹³C NMR spectrum suggested compound **1** as a sterol. The ¹H NMR spectrum (400 MHz, CDCl₃) of compound **1** isolated from the endophytic fungal extract showed two multiplets at δ = 3.63 and δ = 5.56 with one proton integration, typical for the signals of 3-H and 6-H of a steroidal nucleus. The multiplets at δ = 5.19 and δ = 5.38 could be attributed to three olefinic protons at C-7 and in the side chain of steroidal moiety, as would be expected for ergosterol. Comparing the spectroscopic data with those reported in literature^{31,32} compound **1** was identified as Ergosterol.

The ¹³C NMR spectrum (100 MHz, DMSO-D₆) of compound **2** from the endophytic fungal extract displayed 15 carbon resonances. The ¹H NMR spectrum

(400 MHz, DMSO- D_6) exhibited four broad singlets at δ 6.61, δ 6.64, δ 6.72 and δ 7.21, integrating one proton each, suggest the presence of two tetra substituted aromatic ring. One sharp singlet integrating one proton at δ 11.82 could be attributed to a phenolic chelated hydroxyl group. The relatively deshielded nature of its hydroxyl group may be caused by the intramolecular hydrogen bond with any lone pair of electrons of a functional group. The resonance at δ 2.72 (integrating for 3H) indicated presence of a methyl group linked to an aromatic ring. The resonances at δ 3.90 ppm in the 1H NMR spectrum and at δ 55.8 ppm in the ^{13}C NMR spectrum proved the presence of *O*-methyl group attached to one of the aromatic rings. Analysis of 1D- and 2D-NMR spectra including COSY, HSQC and HMBC guided assignment of the structure 'a' as shown in Figure 3. In this structure, C4-C5-C6 and C3'-C4'-C5' proton were assigned by tracing on cross peak in the COSY spectrum. The skeleton of alternariol monomethyl ether was disclosed by HMBC correlations (H-4/C-2; H-6/C-2; H-6/C-1'; H-3'/C-2'; H-5'/C-1'; H-5'/6'-CH₃; 5-OCH₃/C-5; 6'-CH₃/C-1' and 6'-CH₃/C-5'). Based on these data and comparing it with the report in literature compound **2** was identified as alternariol monomethyl ether³³. Compound **2** is a mycotoxin of dibenzopyranone group produced by some species of the genus *Alternaria* which may have agricultural application as herbicide or pesticide³⁴. Moreover, recent studies also reported antibacterial and cytotoxic potential of alternariol monomethyl ether^{27,30}. So, prominent cytotoxic activity of F-3 fraction was justified with isolation of the compound alternariol monomethyl ether (**2**) from this fraction.

Now-a-days the use of chemicals to prevent postharvest diseases is discouraged whereas more emphasis is given on biological control of infections through both field and post-harvest application of antagonistic microorganisms^{35,36}. Endophytic fungi can trigger pathogen-antagonist interactions through site exclusion, nutrient and space competition or by antibiotic production and thus can prevent postharvest diseases³⁵. Further study is necessary to ascertain the agricultural application of the isolated endophytic fungi in biological control of the infections occurred with papaya plant.

5. Conclusion

Now-a-days major concern of researchers is focused on the development of novel drugs that could be useful for treating various diseases. By this work, we were able to isolate two compounds from *Alternaria* sp namely, ergosterol and alternariol monomethyl ether. Antimicrobial, antioxidant and cytotoxic activities of the different fungal extracts of papaya plant have been investigated systematically in this research work. The results showed that some extracts had potent antimicrobial, antioxidant and cytotoxic activities. This discovery signifies the scientific and industrial potentials endophytic fungi isolated from papaya plant. Overall, these findings indicate that endophytic fungi of medicinal plants of Bangladesh could be an ideal target for discovery of potential bioactive lead structures for new drugs.

6. References

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