Antiproliferative and Antibacterial Potentials of Endophytic Fungi Associated with Bangladeshi Medicinal Plant *Tinospora Cordifolia*

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ABSTRACT: Endophytes provide mutual benefits and produce various biologically active secondary metabolites in plants. Our study was designed to isolate and identify fungal endophytes from fresh leaf and bark of *Tinospora cordifolia*, coupled with their bioactivity and preliminary chemical screening. Six endophytic fungi were isolated using surface sterilization technique. The fungi were identified by microscopic and molecular characterization and confirmed TCLE-1 as *Fusarium perseae*, TCLE-2 as *Fusarium keratoplasticum*, TCLE-3 as *fusarium macrosporum*, TCBE-1 as *Fusarium falciforme* and TCBE-2 as well as TCBE-3 leaving unidentified. Isolated strains were cultured in large scale on potato dextrose agar (PDA) medium and were extracted using ethyl acetate. Antibacterial activity of fungal extracts was performed by disc diffusion method and cytotoxicity using HeLa cell line by MTT assay. In antimicrobial test, all the fungal extracts except TCBE-2 showed moderate to potent activity in comparison to the standard kanamycin. All, except TCBE-2, demonstrated significant cytotoxic activity on HeLa cell. Preliminary chemical profiling using thin liquid chromatography (TLC) and analytical high performance liquid chromatography techniques indicated the presence of secondary metabolites in fungal extracts. In conclusion, endophytic fungi reside in the *T. cordifolia*, a native medicinal plant in Bangladesh, could be a potent source of putative antibacterial and anticancer compounds.

Key words: *Tinospora cordifolia*, endophytic fungi, secondary metabolites, bioassay.

INTRODUCTION

Endophyte is one of the potential sources of bioactive compounds. “Endophyte”, at first the term coined by *de Bary* at 1866, refers to any organism resides within plant tissues, by nature different from the organisms that live on plant surfaces. Endopytes, including bacteria and fungi, ensure mutual benefits for themselves as well as for plants without causing any appreciable symptom of disease or illness. They result in production of diverse bioactive secondary metabolites and have found active against a wide range of pathogenic microorganisms. A significant number of compounds from endophytes were obtained among which major groups include flavonoids, steroids, alkaloids, terpenoids etc.[1] Fungal endophytes produce more secondary metabolites than other endophytic microorganism classes (e.g., bacterium and actinomycetes).[2]

Few endophytic fungi produce pigments having bacteriostatic ability against *Staphylococcus aureus*, *Klebsiella pneumonia*, *Salmonella typhi* and *Vibrio cholera;* even few pigments were found more effective than streptomycin.[3] Since endophytes have

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the properties like phyto-stimulation, pigment and enzyme production, antimicrobial protection and so on, it is therefore evident that they can synthesize biologically active compounds and these compounds can be exploited by plants to defend pathogenic microbes. Researchers have found numerous bioactive compounds possessing capability to be utilized as lead for novel drug discovery. Schulz *et al.*[4] evaluated the biological potential of 6500 endophytic fungi; they obtained 135 secondary metabolites from these endophytes among which 51% was found new natural bioactive products. The research group pointed out that endophytic fungus has the great potentiality to explore numerous novel compounds.[4]

T. cordifolia (lacal name: Gulancha or Guduchi) is a spreading and mounting shrub with a lot twisting branches. The plant is from the family of *Menispermaceae*. Bangladesh, Vietnam, Myanmar, Thailand, South Africa, Philippines, India, Indonesia, Sri Lanka and Malaysia are the countries where they found in abundant as a native plant.[5,6] The whole plant of *T. cordifolia* has the traditional uses for treating jaundice, skin diseases, gout, ulcer and rheumatism by local healers. Ayurveda believes *T. cordifolia* stem could be used as rejuvenator and the healers advise different forms of the plant to treat jaundice, diarrhea, diabetes, dyspepsia, chronic urinary diseases and fever.[7] Use of *T. cordifolia* as antioxidant and immunomodulatory agent[8] and the use of stem juice as an anthelmintic in Kalenga forest (Chunarughat, Habiganj) of Bangladesh has previously been reported.[9]

Depending on the evidences that different plant parts of *T. cordifolia* is being used by traditional healers in different pathological conditions, it is assumed that the endophytic fungi isolated from the plant parts of *T. cordifolia* could be enriched with potential secondary metabolites which could exert diverse biological activities. However, present study was chosen to justify the antibacterial and cytotoxic effects of the fungal strains isolated from *T. cordifolia* planted in Bangladesh since significant antimicrobial[10-12] and cytotoxic[13, 14] effects from plant parts of this plant has previously been reported. In our investigation, we found some useful fungi producing antibacterial and cytotoxic metabolites.

MATERIALS AND METHODS

Isolation of endophytic fungi. *T. cordifolia* was collected from BCSIR Laboratory premises, Dhanmondi, Dhaka, Bangladesh. The plant was recognized and validated by a taxonomist of Bangladesh National Herbarium (BNH), Dhaka, Bangladesh and a receipt specimen under the accession number DACB-39195 for *T. cordifolia* was deposited at the BNH for future reference. Endophytic fungi were isolated following suitably modified surface sterilization technique.[15] In brief, healthy and mature plants were picked at an appropriate time and chosen plant parts were cleansed in running tap water followed by distilled water to remove dirt. After being cleaned, leaves and barks were chopped into 2-3 cm pieces and were transferred into aseptic condition for further processing. Fresh plant components (bark and leaf) were treated with 70% ethanol (1 to 1.5 min), 1.3 mol/L sodium hypochlorite (3 min), 70% ethanol (30 sec), and distilled water to extract endophytic fungi. Surface-sterilized plant pieces were placed on water agar (WA) media in petri dishes infused with streptomycin (200 µg/ml). The petri dishes were incubated in an incubator (Froilabo BRE 120, France) at 28 ± 2°C for 21 days until any fungal hypha becomes visible. Hyphal tips, as soon as visualized, were shifted to potato dextrose agar (PDA) media. A serial dilution technique was utilized to obtain pure fungal colony. Unsterilized plant parts were chosen as negative control. Isolated and purified endophytic fungal strains were preserved in 10% glycerol for long term storage.

Morphological identification. For morpho-logical identification, developed fungal cultures were stained with lactophenol blue and studied using a bright-field phase contrast microscope (Kruss, Germany) with 40x magnification and 0.65 numerical apertures. Color of colony and medium, texture, growth pattern, margin character, size and coloring

of conidia, surface character etc. were examined sporadically (after 3, 6, 9, 12 and 15 days) until complete growth of fungus and compared to the standard taxonomic key.[16, 17]

Molecular characterization. TCLE-1, TCLE-2, TCLE-3 from leaf and TCBE-1, TCBE-2, TCBE-3 from bark were subjected to molecular characterization. DNA isolation, amplification and sequencing of internal transcribed spacer (ITS) region and 5.8s region sequences were performed. Forward and reverse primers were ITS4 (5-TCCGTAGGTGAACCTGCGG-3) and ITS5 (5-TCCTCCGCTTATTGATATGC-3), respectively.[18] Fungal DNA was isolated using DNeasy Plant Minikit per manufacturer's instructions (QIAgen, USA). Using HotStarTaq Master Mix Kit, DNA was amplified by PCR (QIAgen, USA). Each PCR reaction mixture comprised 5-10 ng of genomic DNA, 1U of Hot StarTaq Polymerase, and 1M of ITS4 and ITS5 primers. The mixture was put on a thermal cycler (BioRad, USA) using the PCR cycle. Initial denaturation at 95°C for 5 minutes was followed by 35 cycles at 94°C for 30s, 48°C for 30s, and 95°C for 1 min, with a final extension at 72°C for 8 min.

Electrophoresis in Tris/borate/EDTA at 75V for 60 minutes was utilized. The agarose gel was stained with 1% ethidium bromide and a 550 bp fragment was extracted. Purification of PCR products was done using Eppendorf's ideal PrepGel Cleanup Kit (USA). The amplified pure fungal DNA (PCR product) was analyzed by electrophoretic sequencing on an ABI370X1 DNA analyzer (Applied Biosystems, USA). Using BLAST sequence match methods, the base sequences were compared to Genbank. Phylogenetic trees were constructed using the 'Neighbor-Joining' (NJ) technique with bootstrap support based on 1,000 replicates and evolutionary distances were computed using the 'Maximum Composite Likelihood' method based on 5.8S-rRNA-ITS sequences.

Extraction of crude fungal extract. All fungal strains were grown on PDA (Merck, Germany). After 28 days of incubation at 28^{o}C, fungi were extracted. Three times, the culture was steeped in ethyl acetate at room temperature on 5 days interval. After 5 days, media were filtered through fresh cotton and Whatman No.1 filter paper. The fungus extracts were concentrated using a rotary evaporator. Yield percentage was calculated; extract yield % = R/Sx100, where R is plant residue weight and S is plant raw sample weight.[19] On evaporation, we got crude mixture of fungal extracts that were transferred into weighted vial.

Antimicrobial activity screening. The antibacterial activity was screened by the disc diffusion method.[20-22] The test microorganisms used in the antibacterial study included two gram positive pathogenic bacterial strains *Bacillus megaterium* (ATCC 25918) and *Staphylococcus aureus* (ATCC 25923), two gram negative pathogenic bacterial strains *Escherichia coli* (ATCC 28739) and *Salmonella typhi* (ATCC 19430). The sample treated discs were employed within nutrient agar (NA) media inoculated with bacteria. The plates were incubated for 24 hrs at 37°C. Extent of sensitivity of particular microbial species to fungal extracts (100 µg/disc) were determined by measuring the diameter of zone of inhibition (in millimeter) around the sample treated disc and compared to kanamycin (30 µg/disc) as standard antibiotic. Triplicate screenings were performed.

Cytotoxicity assay. Cytotoxic assay was accomplished using the commercial services of Centre for Advanced Research in Sciences (CARS), University of Dhaka. Micro-culture tetrazolium test (MTT)[23] was executed for screening of cell antiproliferation. In brief, HeLa, a human cervical carcinoma cell line was maintained in DMEM (Dulbecco's Modified Eagles' Medium) containing 1% penicillin-streptomycin (1:1), 0.2% gentamycin and 10% fetal bovine serum (FBS). HeLa cells ($2.0x10^4$/100 µl) were seeded onto 96-well plate and incubated at 37^0C with 5% CO_2. After 24 hours, 25 µl of filtered sample was added on each well. Duplicate wells were used for each sample. Survival of cells was counted against each sample. Later quantitative cytotoxicity assay was performed after 48 hr of

incubation using cell counting kit-8 (CCK-8), a non-radioactive colorimetric cell proliferation & cytotoxic assay kit (Sigma-Aldrich, USA). Survival of cell in percentage was measured and recorded. Cytotoxicity of the fungal samples was examined in 4 concentrations: 0.3125, 0.625, 1.25 and 2.5 mg/ml.

Initial chemical screening by analytical HPLC and TLC. Detection and quantification of selected polyphenolic compounds in fungal extracts were determined by HPLC-DAD analysis[24], with slight modifications. Analytical HPLC was performed using a Shimadzu (LC-20A, Japan) equipped with a binary solvent delivery pump (SIL-20A HT), auto sampler (SIL-20A HT), column oven (CTO-20A), and photodiode array detector (SPD-M20A) and controlled by LC solution software. First, working standards were prepared. 16 phenolic compounds (value of 13 shown in table 3) were dissolved in methanol to make standard solutions. Stock solutions ranged from 4.0 to 50 g/ml. The appropriate volumes of each stock solution were mixed together and then diluted serially to prepare the working standard solutions. All solutions were stored under refrigeration. The mobile phase composed of A (1% acetic acid in acetonitrile) and B (1% acetic acid in water) with gradient elution: 0.01-20 min (5-25% A), 21-30 min (25-40% A), 31-35 min (40-60% A), 36-40 min (60-30% A), 41-45 min (30-5% A) and 46-50 min (5% A) was used in this study. The sample injection volume was 20 µl, and the flow-rate was set at 0.5 ml/min. Photodiode-array (PDA) detector set at 270 nm was applied for validation of method and analysis. The mobile phase was filtered and degassed via 0.45 m Nylon 6, 6 membrane-filters (India). For the calibration curve, a standard stock solution was prepared in methanol containing gallic acid (20 µg/ml), 3,4-dihydroxy benzoic acid (15 µg/ml), catechin hydrate (50 µg/ml), catechol, (-) epicatechin, rosmarinic acid (30 µg/ml each); caffeic acid, vanillic acid, syringic acid, rutin hydrate, p-coumaric acid, trans-ferulic acid, quercetin (10 µg/ml each); myricetin, kaempferol (8 µg/ml each); trans-cinnamic acid (4 µg/ml).

The endophytic fungal crude extracts were also subjected to thin layer chromatographic technique for initial identification of chemical constituents. Commercially available precoated silica gel (Kiesele gel 60 PF 254, Merck, Germany) plates were used with solvent system of tolune: 10% ethyl acetate. The screening of the TLC plates was performed by visual detection, under UV light at both 254 nm and 365 nm.

Statistical analysis. Independent Student t-test for equality of variances was used to compare mean IC_{50} values between groups. For cytotoxicity data, one-way ANOVA with Bonferroni's multiple comparison tests were used. Statistical analysis in antibacterial study was performed with GraphPad Prism 9.

RESULTS AND DISCUSSION

Identification of endophytic fungi by morphological and microscopic characteristics. Fungal endophytes were isolated and purified by standard protocol. Using morphological and microscopic observations (Figure 1), the isolates were identified to the genus level. Three leaf endophytes (TCLE-1, TCLE-2, and TCLE-3) and three bark endophytes (TCBE-1, TCBE-2, and TCBE-3) were isolated from *T. cordifolia*. Colony shape, medium properties and hyphal morphology were used to identify strains. Among six, four of the identified genera was *fusarium.* Two fungal isolates (TCBE-2 and TCBE-3) were unidentified.

After 3 days of incubation on PDA media under light at 25°C, colony diameter was found 3 to 4.5 cm. Aerial mycelia were plentiful, thickly floccose to fluffy, and pink to light violet. White and cottony colony morphology in the medium changed to a yellow, orange, or reddish core with a lighter periphery. Septet hyphae were seen. Long or short simple conidiophores with phialides were observed. Conidiophores observed were found with three or more septa branched or unbranched. Oval-shaped microconidia (conidiophores) with one or two cells were also observed. Microphores outnumbered macrophores and conidiophore phialides were

cylindrical. Observed morphological traits were matched with published data.[25,26] Finally the morphological characteristics TCLE-1, TCLE-2, TCLE-3 and TCBE-1 were confirmed as the genus of *Fusarium*.

Molecular identification of isolated fungal strains. Molecular analysis of fungal endophytes suggests that visually similar fungus may be genetically distinct. Based on BLAST analysis, sequence likeness (percent identity) was considered within 95 to 100%. Four species share a genus, *Fusarium*. All fungal strains were analyzed on the basis of 5.8S-rRNA-ITS regions. In few situations, non-identified species showed close % identity or vicinity, but their probability was excluded owing to morphological differences. In our work, traditional morphological identification was used to validate molecular data, which has been supported by earlier studies.[27] The accession numbers of the corresponding fungal strains are deposited in the 'U.S. National Center for Biotechnology Information' (NCBI). On table 1 the characteristic features of identified species (percent identity, accession number etc.) have been summarized.

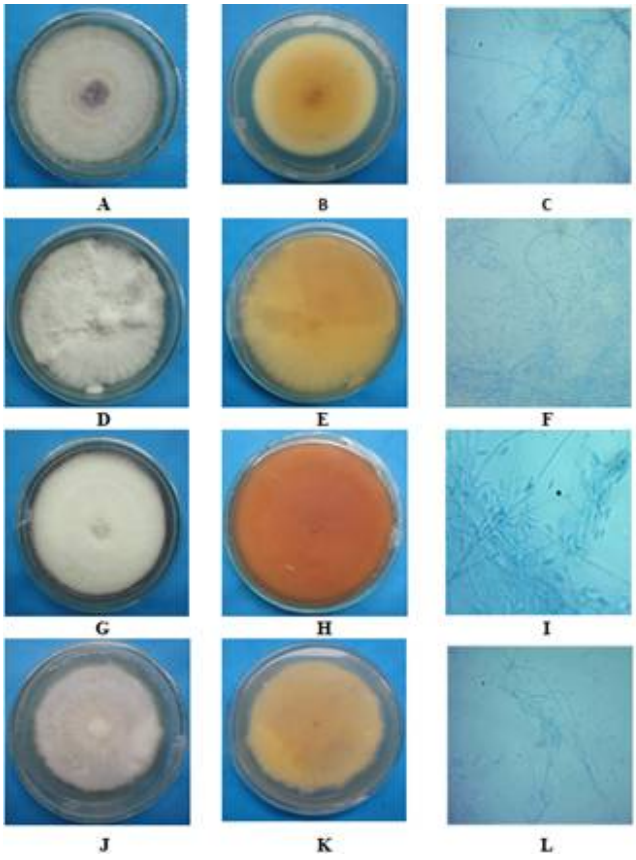

Figure 1. Macroscopic and microscopic view of isolated endophytic fungi from *T.cordifolia*. The picture represents macroscopic (A, B, D, E, G, H, J, K) and microscopic (40X) view (C, F, I and L) of genera *Fusarium* cultured on PDA media.

Table 1. **Molecular identification using ITS regions of endophytic fungi isolated from *T. cordifolia*.**

Fungal species	Internal strain no.	Query Cover (%)	E-value	% Identity	Accession no.
Fusarium perseae	TCLE-1	90	0.0	97.86	NR164415.1
Fusarium keratoplasticum	TCLE-2	95	0.0	99.63	NR130690.1
Fusarium macrosporum	TCLE-3	83	0.0	99.58	NR163291.1
Fusarium falciforme	TCBE-1	95	0.0	97.83	NR164424.1

Based on 5.8S-rRNA-ITS sections, phylogenetic relationship of fungal strain TCLE-1 was studied. Figure 2 shows that TCLE-1 is 98% identical to *Fusarium perseae* (accession no. NR164415.1). TCLE-1 was, therefore, confirmed as *Fusarium perseae* (Figure 2). TCLE-2 revealed 99.63% similarity to *Fusarium keratoplasticum* based on 5.8S-rRNA-ITS regions (accession no. NR130690.1). TCLE-2 was confirmed as *Fusarium keratoplasticum* after being verified with 5.8S-rRNA regions and published data (Figure 3). TCLE-3 resembles *Fusarium macrosporum* (NR163291.1) 99.58% morphologically. Other species (NR164415.1, NR169889.1) were close to the sample in the phylogenetic tree, although they didn't resemble it morphologically. Morphological evidence verified TCLE-3, therefore, as *Fusarium macrosporum* (Figure 4). The fungal strain TCBE-1 exhibited close phylogenetic relationship with both *Fusarium keratoplasticum* (accession no. NR130690.1) and *Fusarium falciforme* (accession no. NR164424.1); nevertheless, morphological likeness was closer to *Fusarium falciforme* and we designated TCBE-1as *Fusarium falciforme* (Figure 5). The fungal strain TCBE-2 and TCBE-3 were unidentified.

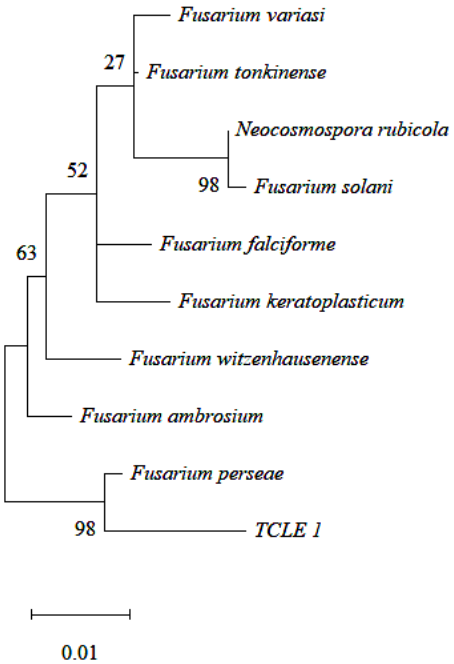

Figure 2. Phylogenetic relationship for 5.8S-rRNA-ITS regions between the endophytic fungus TCLE-1 and published data.

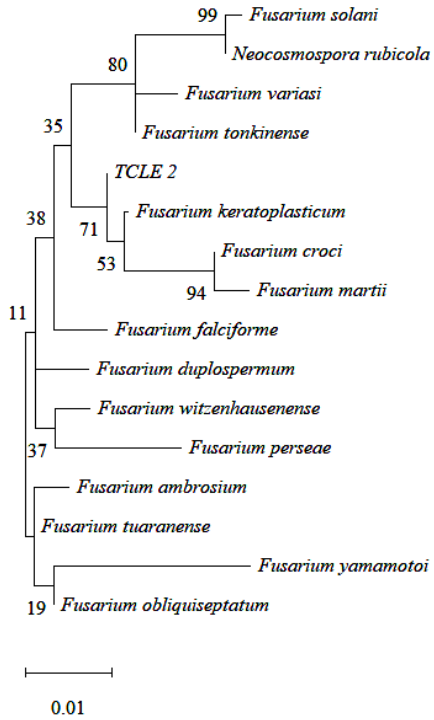

Figure 3. Phylogenetic relationship for 5.8S-rRNA-ITS regions between the endophytic fungus TCLE-2 and published data.

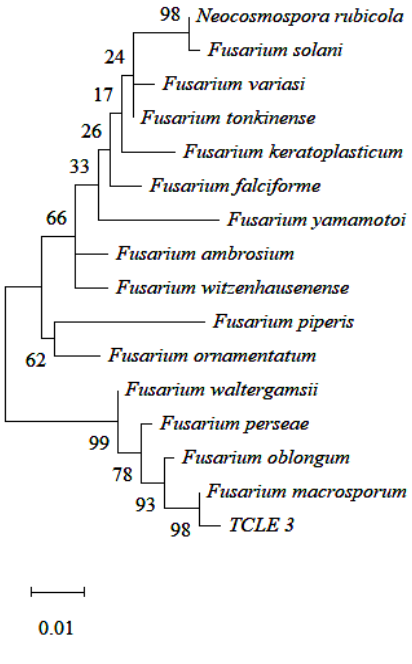

Figure 4. Phylogenetic relationship for 5.8S-rRNA-ITS regions between the endophytic fungus TCLE-3 and published data.

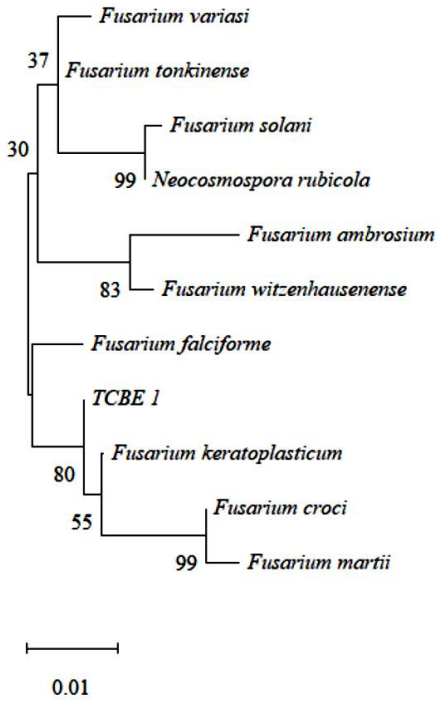

Figure 5. Phylogenetic relationship for 5.8S-rRNA-ITS regions between the endophytic fungus TCBE-1 and published data.

Chemical screening. TLC was employed in toluene-10% ethyl acetate to discover secondary metabolites. Toluene-10% ethyl acetate was used as the mobile phase; ethyl acetate having higher polarity (around 4) usually enhances compound separation. Visually and under UV light, shorter and longer wavelengths, extracts were examined (254 nm and 365 nm respectively). Under UV light, colorful dots showed the presence of a group of compounds (Figure 6). Some flavonoids, anthraquinones, alkaloids and coumarines are known to exhibit yellow, green, red and blue luminous zones in chromatogram.[28]

Analytical HPLC technique [Shimadzu; LC-20A, Japan] was also used for a quantitative phytochemical screening to determine the presence of phenolics (mg per 100 g dry fungal extract), and the results are reported in table 3. According to data, phenolic chemicals (particularly Myricetin, Quercetin and Kaempferol) are abundant in the three leaf-isolated strains (TCLE-1, TCLE-2, and TCLE-3) compared to those found in bark-isolated fungi. TLC qualitative analysis has previously shown that the leaf fungal strains contained more chemicals than their counterpart bark strains (Figure 6 and Table 2).

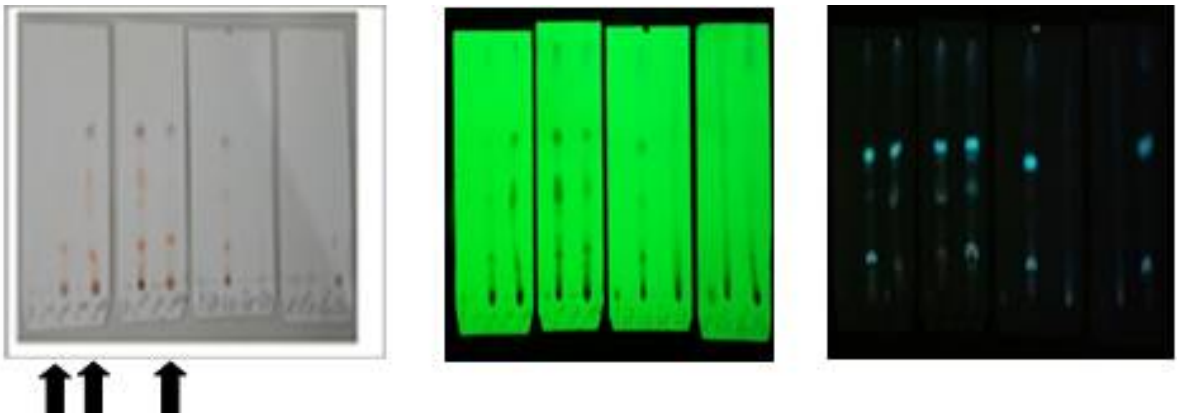

Figure 6. TLC screening of secondary metabolites of the ethyl acetate extract of fungal strains: (a) visual observation, (b) under UV lamp at 254 nm and (c) under UV lamp at 365 nm. Organic fungal extracts isolated from leaf, i.e., TCLE-1 TCLE-2 & TCLE-3 showed presence of more compounds (arrow marked) than polar counterparts as well as fungal strains isolated from bark.

Antibacterial test. Antibacterial test was conducted by disc diffusion method against both gram-positive (*Staphylococcus aureus* and *Bacillus megaterium*) and gram-negative (*Escherichia coli* and *Salmonella typhi*) human pathogenic bacteria. In this experiment, kanamycin (30 µg/disc) served as the standard. Measurements of the zone of inhibition, in millimeters, were used to evaluate the level of

antibacterial efficacy. *T. cordifolia* fungal extracts showed moderate activity against *E. coli*, *S. typhi*, and *S. aureus*, but significant activity against *B. megaterium*. In compared to the standard kanamycin (30 µg/disc), TCLE-2 and TCLE-3 demonstrated significantly higher action against all gram-positive and gram-negative bacteria. There were significant zones of inhibition displayed by TCLE-2 and TCLE-3 against all bacterial pathogens. The activity of TCBE-1 and TCBE-3 against all pathogenic microorganisms was low to moderate. We observed TCBE-2 extract had no effect against test pathogens. Also no antibacterial activity was found for the crude leaf and bark extracts of *T. cordifolia*. Figure 7 shows the graphical presentation of the zone of inhibition of fungal extracts.

Table 2. Chemical profiling of the crude extracts of *T. cordifolia* plant parts and its associated fungi on TLC technique.

Strains	Visual observation	On 254 nm	On 365 nm
TC leaf	Green, green, green	Light blue spot, light green, green	Red, red, red
TCLE-1 (aqueous part)	No color	Dark blue	Blue
TCLE-1 (organic part)	Light pink, light pink, brown	Orange red, light green, green	Light blue, red, light blue
TCLE-2 (aqueous part)	No color	No color	Purple
TCLE-2 (organic part)	Light pink, pink	Orange, dark spot	Light blue, red, light blue
TCLE-3 (aqueous part)	Green, green	Light green, light green	Red, light yellow, red, bluish
TCLE-3 (organic part)	Purple, orange, light pink, pink	Light green, dark orange, blue, orange	Sky blue, orange, light sky blue, yellow, light orange
TC bark	Green, light green	Light orange, light dark, light dark	Red, light blue, light red
TCBE-1 (aqueous part)	No color	No color	Violet
TCBE-1 (organic part)	Orange, light pink, pink	Light orange, dark black spot, light yellow, obscure	Sky blue, orange, light sky blue, sky blue, violet
TCBE-2 (aqueous part)	No color	No color	No color
TCBE-2 (organic part)	No color	Dark black, light blue, light dark, light dark	Sky blue, light sky blue, light yellow
TCBE-3 (aqueous part)	No color	No color	No color
TCBE-3 (organic part)	No color	Green	Blue, sky blue, light sky blue

Table 3. Chemical profiling of the crude extracts of *T. cordifolia* plant parts and its associated fungi on analytical HPLC technique (Values on table represent mg of the respective compound in per 100 g of dry fungal extract).

Compound	Leaf	TCLE-1	TCLE-2	TCLE-3	Bark	TCBE-1	TCBE-2	TCBE-3*
Catechin hydrate	0	0	0	0	66.8	0	0	0
Catechol	340.1	53.7	214.6	61.7	126.5	0	277.0	142.3
(-) Epicatechin	80.1	0	0	0	0	0	0	0
Caffeic acid	0	0	24.7	0	13.4	0	0	0
Syringic acid	234.6	0	0	0	44.6	0	0	0
Rutin hydrate	17.8	0	0	0	0	0	0	0
p-Coumaric acid	11.4	9.5	0	10.9	47.8	0	0	0
trans-Ferulic acid	140.7	0	0	0	192.3	0	0	0
Rosmarinic acid	319.1	0	36.3	14.9	464.7	0	5.2	11.2
Myricetin	22.1	37.9	106.0	31.3	20.6	0	0	24.9
Quercetin	0	21.1	25.1	7.3	0	0	0	0
trans-Cinnamic acid	6.4	6.9	7.5	4.4	0	6.4	5.1	16.0
Kaempferol	16.5	31.2	184.2	53.8	13.9	95.1	0	0

Natural alkaloids are crucial to producing antibacterial drugs. Several investigations found that plant-extracts containing phenols and alkaloids are effective against pathogenic bacteria.[29] Alkaloid, flavonoid and phenolic compounds were detected in our examined fungal strains (Tables 2 and 3), which could explain their logical background of antibacterial activity. Antibiotic resistance is increasing the interest in using natural resources to create antibacterial lead. Limited availability of new synthetic antibacterial[30] is another key factor. Our ultimate goal is to identify pure lead compounds from *T. cordifolia* leaf fungal extracts, which demonstrated promising action on present study.

Cytotoxicity assay. To assess cell viability and proliferation, micro-culture tetrazolium test (MTT) was performed. Extracts of endophytic *T. cordifolia* fungi were tested for cytotoxic activity using HeLa, a human cervical cancer cell. On qualitative cytotoxicity assay, cell rupture was observed (Figure 8). Cell survival was monitored and documented quantitatively. Standard MTT assay was used to estimate IC_{50} values (mg/mL) for cytotoxic fungal strains. 100% DMSO was used as a control. IC_{50} values were calculated by graphing various concentrations on the Y axis against the percentage of dead cells on the X axis.; subsequently calculating values from the equation of slop Y= mX+C.

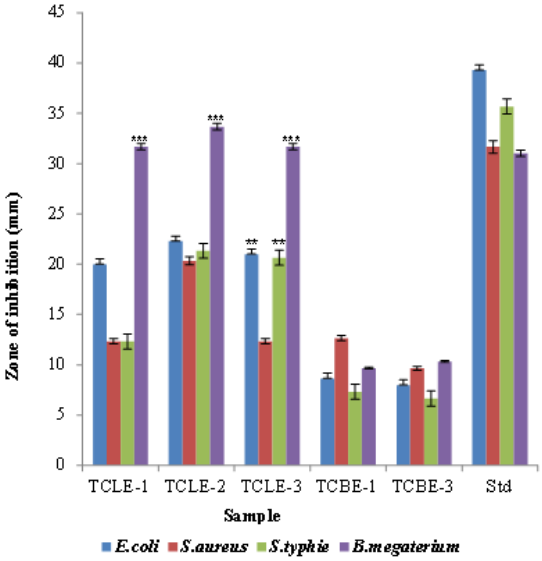

Figure 7. Zone of inhibition (mm) of fungal strains in comparison to the standard kanamycin. Values were found statistically significant for fungal leaf isolates (TCLE-1, TCLE-2 and TCLE-3) while compared by using two-tailed t-test with unequal variances. [Annotation: ***-significant at $p<.001$; **-significant at $p<.01$].

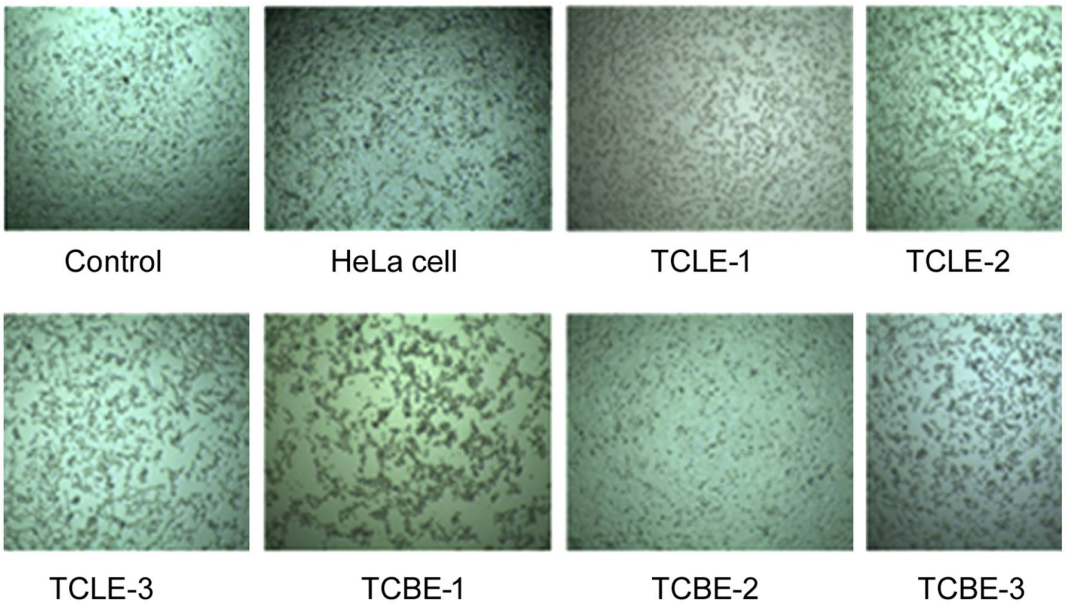

Figure 8. Observed cell cytotoxicity on qualitative assay. Except TCBE-2, appreciable cell ruptures were observed in all fungal strains compared to the control on human HeLa cell lines.

It is evident from the present study that all the test samples showed significant anti-proliferative effect. IC_{50} values, obtained from percent dead cells, were found 1.15, 2.28, 2.29, 2.34 and 1.70 mg/ml for fungal strains TCLE-1, TCLE-2, TCLE-3, TCBE-1 and TCBE-3 respectively. Percent dead cells were measured in four concentrations: 0.3125 mg/mL, 0.625 mg/mL, 1.25 mg/mL and 2.5 mg/mL. Higher percent dead cells were found in TCLE-1 and TCBE-1 fungal variants in respect of control dimethyl sulfoxide (DMSO) [Figure 9]. TCBE-2 was excluded from study because they showed no cytotoxic effect on preliminary qualitative screening. In a nut shell, except TCBE-2 all fungal strains showed moderate to higher cell cytotoxicity on HeLa cell line. Cytotoxic effect observed suggests that pure cytotoxic lead compound(s) could be obtained from the column/PTLC fractions of these fungal isolates.

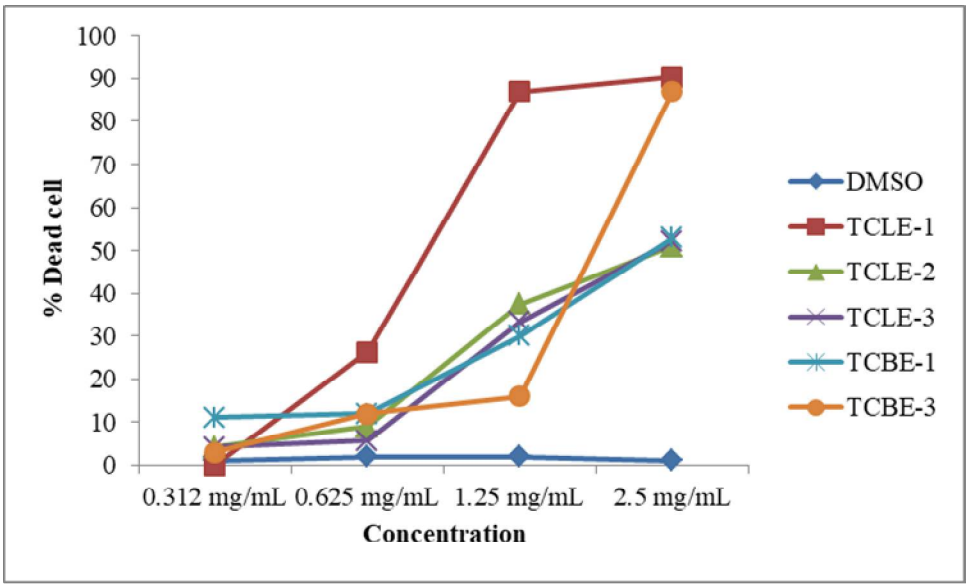

Figure 9. Percent dead cells were found higher at a higher concentration (2.5 mg/mL) for TCLE-1and TCBE-3 in comparison to control DMSO.

Anti-cancer effects on HeLa cervical cancer cells are mediated by apoptosis and reactive oxygen species (ROS).[31] Natural compounds that interfere with cell cycle arrest are also anticancer agents.[32, 33] Few alkaloids also decrease angiogenesis, which reduces cancer cell migration.[34] Few natural alkaloids, anthraquinones, and flavonoids demonstrate cytotoxic activity with less side effects and resistance than conventional chemo-therapeutics.[35] Chemical profiling of fungal endophytes demonstrated that isolated endophytes are rich in natural alkaloids, anthraquinone, flavonoids, coumarine, catechole (TCLE-3 & TCBE-2), caffeic acid (TCLE-2), myricetin (TCLE-, TCLE-2, TCLE-3 & TCBE-3) and campferol (TCLE-1, TCLE-2, TCLE-3 & TCBE-1), as shown in table 3. These substances may contribute to the cytotoxicity of the strains we identified; however, we propose advanced research. The specific underlying mechanism needs to be studied extensively.

Antitumor and antibacterial properties of *T. cordifolia* fungal endophytes were previously assessed.[36] Seven fungal isolates were identified as *Penicillium* genera. Significant antibacterial action, but no anti-proliferative efficacy observed with fungus isolates. Finding is different in our study; that could be due to a number of factors like soil quality-composition and climate, etc. Hypothetically, it's not unusual, thus we required our investigation to test fungal isolates from *T. cordifolia* planted in

Bangladesh. Fungal species and bioactivities differed as we assumed. We found potential anti-proliferative activity in all fungal strains except one (TCBE-2). Our result is persistent with the previous finding that *Furarium* genera are cytotoxic to HeLa cell lines.[37, 38] This is the first report on bioactivity of the fungal strains isolated from *T. cordifolia* planted in Bangladesh. Our study result shows that the bioactive potential of fungal endophytes of *T. cordifolia* planted in the soil of Bangladesh might be utilized to isolate pure bioactive compounds.

Conflict of interests

The authors declare no conflict of interest.

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