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Original Article

An Endophytic *Colletotrichum* sp. Closely Related to *C. siamense* from *Enhydra fluctuans*: Molecular Characterization, LC-MS-based Metabolite Profiling, and Bioactivity Evaluation

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Abstract

The study aimed to isolate and identify endophytic fungi from *Enhydra fluctuans* leaves and evaluate their secondary metabolites and biological activities. A fungal isolate EF-1 was cultivated on rice medium and extracted separately with ethyl acetate (EA), methanol, and n-hexane. All extracts were subjected to phytochemical screening and biological activity assays. The EA extract demonstrated the strongest antioxidant activity, with the lowest IC_{50} value (52.34 µg/mL). The EA and n-hexane extracts also exhibited strong antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, producing inhibition zones of up to 19.067 ± 0.076 mm and 19.883 ± 0.388 mm, respectively, with minimum inhibitory concentration values of 0.5 mg/mL against both pathogens. Molecular identification based on internal transcribed spacer sequencing indicated that isolate EF-1 was *Colletotrichum* sp., closely related to *Colletotrichum siamense*, and its sequence was deposited in GenBank with accession number PQ409585. A diverse range of bioactive compounds was putatively identified in the EA extract by liquid chromatography-mass spectrometry, comprising flavonoids, steroids, phenols, and terpenoids. The isolation, chemical profiling, and bioactivities of the *Colletotrichum* sp. closely related to *C. siamense* from *E. fluctuans* are reported here for the first time. These findings support further investigation of fungal-derived bioactive compounds for potential use in pharmaceutical, nutraceutical, and food industries.

Keywords

Antibacterial, antioxidant, *Colletotrichum* sp., *Enhydra fluctuans*, LC-MS

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Introduction

Natural sources, including plants and microorganisms, are widely explored as producers of bioactive compounds with potential antioxidant, anticancer, and antimicrobial activities.[1,2] *Enhydra fluctuans* Lour., family Asteraceae, is a tropical and subtropical plant traditionally used as a medicine for inflammation, skin diseases, nervous system disorders, smallpox, antidiabetic, and kidney stones.[3,4] Apart from that, *E. fluctuans*, an edible aquatic weed, is considered a healthy food in several countries such as India, Bangladesh, Sri Lanka, Burma, and other Southeast Asian countries. Its nutritional value is attributed to abundant bioactive compounds, minerals, essential and non-essential amino acids, protein, lipids, vitamins, and β-carotenoids.[5] Phytochemical investigations have shown that *E. fluctuans*

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contains diverse phytochemical classes, such as phenols, flavonoids, tannins, terpenes, and saponins. Most of the E. fluctuans extracts have bioactivities such as antioxidant and antimicrobial activity.[6,7]

Given the diverse bioactivities reported from *E. fluctuans*, its endophytic fungi may constitute an additional reservoir of chemically diverse metabolites. Recent studies have increasingly highlighted endophytic fungi as valuable sources of natural products, yielding compounds from various chemical classes, including alkaloids, flavonoids, phenolics, steroids, and terpenoids, with antioxidant, antimicrobial, antiproliferative, and anticancer properties.[8,9] Furthermore, fungal endophytes possess remarkable adaptive capabilities that enable them to survive under diverse environmental conditions, often accompanied by the production of structurally diverse secondary metabolites.[10] In addition, the cultivation of fungal endophytes offers a practical and environmentally sustainable alternative for metabolite production, reducing pressure on plant resources while facilitating the discovery of novel natural products.[8]

Recently, endophytic bacteria associated with *E. fluctuans* have been isolated and reported to possess antioxidant and antibacterial activities,[11] highlighting the potential of endophytic microorganisms as sources of bioactive compounds in this medicinal plant. However, no study has reported the isolation and characterization of endophytic fungi associated with *E. fluctuans*, leaving their biological activities and metabolite profiles largely unknown. Considering the remarkable capacity of endophytic fungi to synthesize structurally diverse secondary metabolites, the fungal community associated with *E. fluctuans* may represent a promising source of compounds with pharmaceutical potential. Therefore, this study aimed to isolate and molecularly characterize endophytic fungi from *E. fluctuans* leaves, followed by the evaluation of their antioxidant and antibacterial activities and characterization of their metabolite profiles using liquid chromatography-mass spectrometry (LC-MS) analysis.

Materials and Methods

Sampling and Isolation of Endophytic Fungi from E. Fluctuans Leaves

Leaves of *E. fluctuans* were identified and collected from Nagari Rangkiang Luluih, Solok Regency, West Sumatera, Indonesia (0.9496° S, 100.8777° E; approximately 800 m above sea level) in July 2024, according to the method described by Reis et al.[12] Leaves were washed with running tap water and surface sterilization was then carried out through sequential treatment with 70% ethanol (1 min), 3.5% sodium hypochlorite (NaOCl) (2 min), autoclaved distilled water (1 min), followed by a final rinse in 70% ethanol (30 s). Finally, the treated leaves were rinsed three times with sterile distilled water (30 s each rinse) and air-dried under aseptic conditions. Then, they were sliced and inoculated onto Potato Dextrose Agar (PDA). To confirm the effectiveness of sterilization, aliquots from the final wash water were also plated onto PDA. All plates were sealed and incubated at 28 °C. Growing fungi were sub-cultured based on colony shape and color onto fresh PDA to obtain pure cultures.

Molecular Identification of Endophytic Fungi

Molecular identification of endophytic fungi was carried out based on the internal transcribed spacer (ITS) rDNA sequence homology, following a modified protocol from a previous study.[13] Fungus EF-1 was grown on potato dextrose broth for 72 h. Genomic DNA was extracted using the i-genomic BYF DNA Extraction Mini Kit (iNtRON Biotechnology). The isolated genomic DNA was used as a template to amplify the ITS region using polymerase chain reaction (PCR) with ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers. Initial denaturation was performed at 95 °C for 7 min, followed by 30 cycles consisting of denaturation (95 °C, 30 s), annealing (58 °C, 45 s), and elongation (72 °C, 60 s). Final extension was carried out at 72 °C for 10 min. Electrophoresis on a 1% agarose gel was used to confirm the amplified products, and the Sanger method with ITS5 and ITS4 primers was used for sequencing. The BioEdit software was used to trim and assemble the sequenced data. The EF-1 ITS sequence was then analyzed using BLAST at the National Center for Biotechnology Information (NCBI) database.

Phylogenetic Characterization of Endophytic Fungal Isolate EF-1

The ITS region of EF-1, together with 14 highly homologous sequences retrieved from GenBank based on BLAST analysis and one outgroup sequence (*Xylaria curta*), was aligned using ClustalW. The aligned sequences were then subjected to construction of a phylogenetic tree based on the neighbor-joining method with default parameters. Branch support was assessed using 1,000 bootstrap replicates in MEGA 11.[14]

Fungal Cultivation and Extraction of Secondary Metabolites

Endophytic fungus EF-1 isolated from *E. fluctuans* was cultivated and extracted following a previous method.[13] The fungus was grown on 25 g of sterilized white rice and incubated at 25 °C for 3 weeks. After the incubation, the fungal cultures were harvested and extracted separately with methanol, ethyl acetate (EA), and n-hexane. Each extract was concentrated to obtain the corresponding crude extract. The crude extracts were then stored at 4 °C until further use.

Screening of Phytochemical Constituents

The crude extracts of EF-1 were subjected to qualitative phytochemical screening following previously described methods.[15] The formation of a characteristic precipitate or color change indicated the presence of the corresponding phytochemical constituents. Steroid and terpenoid tests were carried out by dissolving extracts in chloroform-ammonia and 2N H_2SO_4 to form two layers. The lower layer was evaporated and mixed with anhydrous acetic acid and H_2SO_4 p.a. Alkaloids were screened separately using Wagner's, Dragendorff's, and Mayer's reagents. Phenolic compounds were detected by adding 1 mL of 1% $FeCl_3$ solution to the crude extract. The flavonoids were identified by adding three drops of NaOH solution to the crude extracts of EF-1.

Antioxidant Activity by Using the Free Radical Scavenging Assay

The antioxidant potential of the EF-1 extracts was evaluated through their ability to quench 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals according to a modified procedure described previously.[16] Serial concentrations of each extract (7.81–500 µg/mL) were prepared and allowed to react with a methanolic 0.2 mM DPPH solution. The reaction mixtures were incubated for 30 min, after which the absorbance was measured at 517 nm using a UV–Vis spectrophotometer. Ascorbic acid, at concentrations ranging from 100 to 3.125 µg/mL, was used as the positive control. The percentage of radical scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity}\,(\%) = \frac{Control\ OD - Sample\ OD}{Control\ OD} \times 100$$

where control OD represents the absorbance of the DPPH solution without extract, while sample OD represents the absorbance of the DPPH solution in the presence of extract. IC_{50} values were calculated by nonlinear regression using GraphPad Prism software.

Antibacterial Activity

The paper disc diffusion method was employed to assess the antibacterial activity of the extracts following a previously reported method.[17] The extracts were prepared in dimethyl sulfoxide (DMSO) at concentrations of 1%, 5%, and 10% w/v, and the antibacterial activity was determined against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). The test bacterial cultures were spread on Mueller–Hinton agar plates. Sterile 6-mm paper discs were placed on the inoculated agar surface and loaded with 20 µL of each treatment. Amoxicillin (0.1%) and DMSO were used as positive and negative controls, respectively. After incubation at 37 °C for 24 h, the diameters of the inhibition zones were measured in millimeters using a digital caliper. Each treatment was conducted in triplicate. Data were analyzed by one-way ANOVA followed by Fisher's LSD post-hoc test using RStudio; significance was set at $p < 0.05$.

The minimum inhibitory concentration (MIC) of all crude extracts was determined following the method described previously by Suryelita et al.[15] with minor modifications. The assay was performed in sterile 96-well microtiter plates using double-strength Mueller–Hinton broth. Two-fold serial dilutions of each extract were prepared to obtain final concentrations ranging from 0.00195 to 1 mg/mL. The bacterial inoculum was standardized to approximately 7.5×10^5 CFU/mL. Each well contained 100 µL of the bacterial suspension and 100 µL of the extract. Growth control (medium and inoculum without extract), solvent control (medium with DMSO), and sterility control (medium only) were included, and all tests were conducted in triplicate. The plates were incubated at 37 °C for 24 h, and the MIC was determined as the lowest concentration of extract that completely inhibited visible bacterial growth

LC-MS Analysis

The metabolite contents in the EA extract of EF-1 were evaluated using a Shimadzu LC-MS-8040 system equipped with an FC-ODS column (2 × 150 mm, 3 µm). The column temperature was maintained at 35 °C, and the mobile phase consisted of 90% methanol in isocratic mode at a flow rate of 0.5 mL/min. The injection volume was 1 µL, and the total run time was 60 min. Peaks of various compounds eluted from the LC column were recorded along with their retention times (RT). Electrospray ionization in positive mode was employed for mass spectrum acquisition, covering a mass range of 10–1000 m/z. The compounds were putatively identified based on the observed m/z values, RT, and similarity indices reported in the analytical report generated using a Shimadzu LC-MS-8040 instrument.

Results and Discussion

Endophytes Fungal Isolates Associated with E. fluctuans

Two endophytic fungi, designated EF-1 and EF-2, were successfully isolated from *E. fluctuans* after surface sterilization using 70% ethanol and 3.5% NaOCl as the main sterilizing agent.[12] Morphological identification of EF-1 illustrated moderate growth frequency with finely granular conidial formation on PDA medium at 26 °C ± 2 °C, reaching a colony diameter of 35–60 mm in 7 days. In contrast, EF-2 showed slower growth under the same conditions. Colonies' surfaces were white and yellow for EF-1 (Figure 1A) and EF-2, respectively. Preliminary observations, including growth

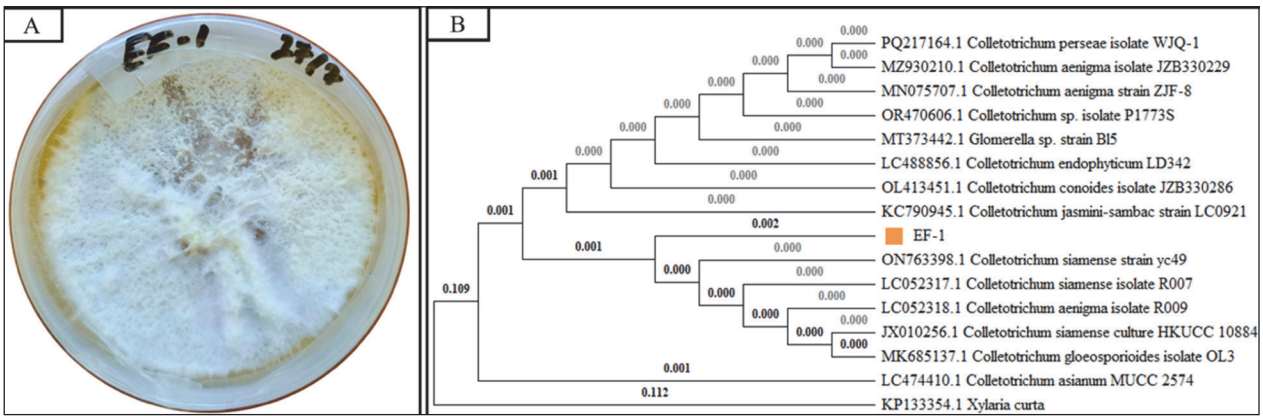

Figure 1. Fungal Endophytic EF-1Isolated from *E. Fluctuans* and Molecular Identification: (A) Surface of Culture on PDA, (B) Phylogenetic Tree.

characteristics and initial antioxidant screening, suggested that EF-1 was more promising for further investigation. Therefore, EF-1 was selected for molecular identification and bioactivity evaluation.

Molecular characterization of the isolate EF-1 involved aligning its ITS rRNA gene sequence with existing records in the NCBI GenBank database. A nucleotide sequence of the EF-1 ITS measuring 572 bp was obtained from PCR amplification using ITS4 and ITS5 primers. The sequence alignment of EF-1's ITS using the Nucleotide BLAST program produced a list of fungi in the GenBank database with a similarity of 99.65%–99.83%. The highest sequence similarity (99.83%) was obtained with *Colletotrichum siamense.* Based on the ITS sequence, the endophytic fungus EF-1 isolated from *E. fluctuans* was identified as *Colletotrichum* sp., closely related to *C. siamense*. The corresponding sequence has been deposited in the NCBI GenBank database under the assigned accession number PQ409585. Based on 1,000 bootstrap replicates, a phylogenetic reconstruction was performed using the ITS sequences of EF-1 and 15 selected sequences (including the outgroup). *Xylaria curta* served as the outgroup, and the Kimura 2-parameter model was applied within a neighbor-joining framework to determine the final topology[18,19] (Figures 1B). EF-1 clustered closely with *C. siamense* strain yc49 (ON763398) associated with *Camellia oleifera* in China. Genetic distance data obtained from 15 species ranged from 0.0000 (0.0%) to 0.0035 (0.3%) (Table S1). The closer the genetic distance of a species to another species, the closer the gene similarity. A minimal genetic difference of 0.18% was observed between EF-1 and *C. siamense* strain yc49 (ON763398). Therefore, the EF-1 isolate is hereinafter referred to as *Colletotrichum* sp., closely related to *C. siamense*.

Cultivation, Extraction, and Phytochemical Analyses

Fungal endophytes are well-known as potential producers of bioactive specialized metabolites, which underpin their documented antioxidant and antimicrobial properties.[20,21] Following previously reported cultivation protocols, *Colletotrichum* sp., closely related to C. siamense, was cultivated for 21 days prior to extraction.[12,13] To maximize metabolite recovery, fungal biomass was extracted using methanol, EA, and n-hexane, representing polar, semi-polar, and nonpolar solvents, respectively. The use of solvents with different polarities enables the extraction of diverse classes of secondary metabolites.[22]

Table 1. Phytochemical Screening of Isolate EF-1.

Phytochemical	EA Extract	MeOH Extract	n-Hexane Extract
Alkaloids	−	+	−
Flavonoids	+	−	−
Phenols	+	+	−
Steroids	+	+	+
Terpenoids	+	+	++

Note: (−): Absence, (+): Presence, (++): Abundance.

Preliminary phytochemical screening (Table 1) revealed that methanol and EA extracts contained phenols, steroids, and terpenoids. Alkaloids were exclusively detected in the methanol extract, whereas flavonoids were found only in the EA extract. In contrast, the n-hexane extract contained only steroids and terpenoids. These findings indicate that *Colletotrichum* sp. closely related to *C. siamense* produces a diverse range of secondary metabolites, supporting previous reports that endophytic fungi represent rich reservoirs of bioactive compounds.[20,21,23,24]

Antibacterial Activity of Fungal Extracts

In this study, *Colletotrichum* sp. closely related to *C. siamense* crude extracts obtained using three solvents at varying concentrations (1%, 5%, and 10%), were challenged against *S. aureus* and *E. coli*. All assays were performed in triplicate, and the results are presented as mean ± standard deviation ($n = 3$) in Table 2. A negative control was prepared

Table 2. Zones of Inhibition of Fungal Extracts (n = 3).

Extract	Concentration (%)	Zones of Inhibition (mm)	
		S. Aureus	*E. Coli*
MeOH	1	11.113 ± 0.076^{g}	10.917 ± 0.633^{f}
	5	14.433 ± 0.256^{f}	14.200 ± 0.218^{e}
	10	16.100 ± 0.087^{d}	15.333 ± 0.176^{d}
Amoxicillin		14.250 ± 0.050	13.167 ± 0.076
DMSO		00.00 ± 00.00	00.00 ± 00.00
EtOAc	1	15.067 ± 0.076^{e}	14.217 ± 0.153^{e}
	5	17.083 ± 0.175^{c}	16.117 ± 0.076^{c}
	10	19.067 ± 0.076^{b}	17.275 ± 0.126^{b}
Amoxicillin		14.725 ± 0.153	13.300 ± 0.050
DMSO		00.00 ± 00.00	0.00 ± 0.00
n-Hexane	1	15.217 ± 0.076^{e}	14.217 ± 0.076^{e}
	5	17.300 ± 0.150^{c}	17.067 ± 0.076^{b}
	10	19.317 ± 0.104^{a}	19.883 ± 0.388^{a}
Amoxicillin		14.282 ± 0.076	13.217 ± 0.104
DMSO		00.00 ± 00.00	00.00 ± 00.00

Note: Values are the mean ± standard deviation of three independent experiments. Statistical analysis was performed using one-way ANOVA + Fisher's LSD post-hoc test in RStudio, with significance set at $p < 0.05$.

by applying DMSO (20 µL/disc), which produced no observable inhibition zones against any of the test organisms. Conversely, amoxicillin 0.1% (20 µg/disc) was employed as a reference antibiotic, displaying the widest zone of inhibition against *S. aureus* (14.725 ± 0.153 mm) and the narrowest against *E. coli* (13.300 ± 0.05 mm).

All extracts inhibited bacterial growth, with inhibition zones ranging from 10.9 to 19.9 mm. The EA and n-hexane extracts exhibited greater antibacterial activity than the methanol extract. Based on the inhibition zone data, the EA extract was consistently more effective against Gram-positive *S. aureus* than Gram-negative *E. coli* across all concentrations tested. However, this trend was less pronounced for the n-hexane extract, particularly at 10%, where the activity against *E. coli* (19.883 mm) slightly exceeded that against *S. aureus* (19.317 mm). A positive correlation was observed between extract concentration and inhibition zone diameter, consistent with previous reports that higher concentrations enhance antibacterial effects. Increasing the extract concentration increases the concentration of the bioactive metabolites already present in the extract.[13,25]

To further evaluate the antibacterial potency of the extracts, the MICs were determined against both Gram-positive and Gram-negative pathogenic bacterial strains. The EA and n-hexane extracts exhibited MIC values of 0.5 mg/mL against both *S. aureus* and *E. coli*, whereas the methanol extract showed a higher MIC value of 1 mg/mL against both bacterial strains (Table 3). These findings are consistent with the results of the disc diffusion assay, in which the EA and *n*-hexane extracts exhibited comparable antibacterial activity, while the methanol extract showed lower activity. Although all extracts were less potent than the reference antibiotic amoxicillin (MIC = 0.0078 mg/mL), the EA and *n*-hexane extracts demonstrated promising antibacterial activity against both bacterial strains.

Table 3. MIC Values of Fungal Extracts.

MIC (mg/mL)				
Bacteria	n-Hexane Extract	EA Extract	MeOH Extract	Amoxicillin
S. aureus	0.5	0.5	1	0.0078
E. coli	0.5	0.5	1	0.0078

The higher activity of the EA extract may be associated with the presence of flavonoids, phenolics, and terpenoids detected during phytochemical screening. These compounds have been widely reported to possess antibacterial properties through multiple mechanisms, including disruption of cell wall and membrane integrity, inhibition of nucleic acid and protein synthesis, interference with enzymatic activity, and induction of oxidative stress.[26–28] Interestingly, the n-hexane extract, despite lacking flavonoids, exhibited comparable activity to the EA extract. This activity may be attributed to its terpenoid-rich composition, as terpenoids and steroids have been reported to suppress bacterial growth.[27,29]

Given the limited reports on the biological properties of *C. siamense*, the present results support and extend previous studies demonstrating its antimicrobial potential. Endophytic *C. siamense* isolated from *Stevia rebaudiana* exhibited high phenolic and flavonoid contents, which contributed to its inhibitory effects against both *Cladosporium herbarum* and *Bacillus subtilis*.[30] More recently, Daud et al.[31] reported that the EA extract of *C. siamense* from neem leaves exhibited significant antibacterial activity against *S. aureus*, with an inhibition zone of 18.0 ± 0.6 mm. Interestingly, our findings showed comparable activity, particularly with the n-hexane extract (19.317 ± 0.104 mm) and EA extract (19.067 ± 0.076 mm) against *S. aureus*. SEM analysis in their study further confirmed that the extract caused cell lysis and structural collapse of *S. aureus* cells, supporting the antibacterial potential of *C. siamense* as a promising source of bioactive compounds. Similarly, EA extracts of *C. siamense* colonizing *Coffea arabica* showed inhibitory effects against several pathogenic microorganisms, further supporting the potential of this species as a source of bioactive antibacterial metabolites.[32]

Antioxidant Activity of Fungal Extracts

The antioxidant activity of *Colletotrichum* sp. closely related to *C. siamense* extracts isolated from *E. fluctuans* was quantitatively assessed via DPPH radical scavenging assay (Figure 2). The extracts exhibited varying levels of antioxidant activity. Among the tested extracts, the EA extract displayed the strongest antioxidant activity, achieving more than 50% inhibition at concentrations ranging from 62.5 to 500 µg/mL. In comparison, the reference standard, ascorbic acid,

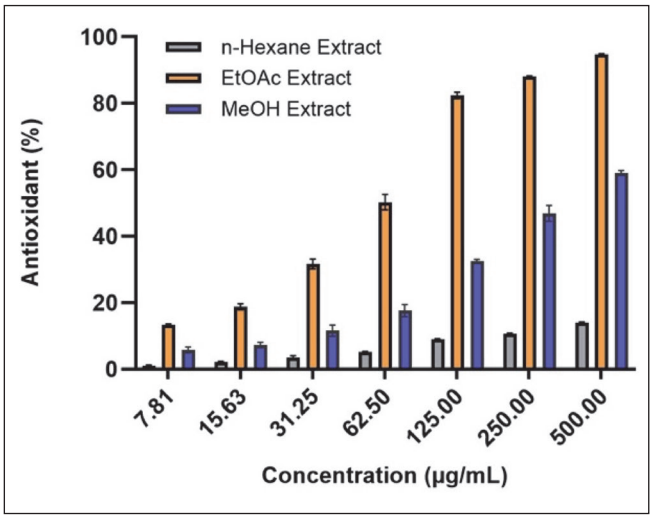

Figure 2. Antioxidant Activity of Fungal Extracts.

achieved more than 50% inhibition at lower concentrations of 25–100 µg/mL, indicating its higher antioxidant potency. In contrast, the methanol extract reached only 46.93% inhibition at 250 µg/mL, while the n-hexane extract demonstrated the weakest activity, with just 10.61% inhibition at the same concentration. The calculated IC_{50} values further confirmed the trend: EA extract had the lowest IC_{50} at 52.34 µg/mL, followed by the methanol (309.6 µg/mL) and n-hexane (>500 µg/mL) extracts. The ascorbic acid exhibited an IC_{50} value of 19.45 µg/mL, confirming its greater antioxidant potency than the tested extracts. According to standard classifications, an IC_{50} below 100 µg/mL suggests strong antioxidant activity, indicating that the EA extract possesses potent radical scavenging properties.

This superior antioxidant activity observed in the EA extract can be attributed to its phytochemical composition. While phenolic constituents were identified in both methanol and EA extracts, flavonoids were exclusively detected in the EA extract. These phytochemical classes are well-documented for their potent antioxidant capacity, primarily through the hydrogen atom donation mechanism. The absence of flavonoids in methanol and n-hexane extracts may explain their weaker activity compared to the EA extract.

Our results substantiate prior reports highlighting the EA fraction as a prime reservoir for bioactive metabolites in *Colletotrichum* species, particularly those isolated from *Andrographis paniculata*, which consistently demonstrated strong antioxidant activity.[33] Similarly, Gurgel et al.[34] reported that *Colletotrichum sp.* from *Arrabidaea chica* exhibited notable antioxidant activity with an EC_{50} value of 11 µg/mL. According to the latest investigation by Riga et al.[35] an IC_{50} of 87.6 µg/mL was achieved for the DPPH scavenging capacity of the EA extract from *Colletotrichum* sp.

The pronounced antioxidant activity of *Colletotrichum* sp. closely related to *C. siamense* EA extract suggests that this endophytic fungus represents a promising source of bioactive constituents. These findings further support the underexplored potential of endophytic *Colletotrichum* species as sources of antioxidant metabolites, while expanding current knowledge through the evaluation of *Colletotrichum* sp. closely related to *C. siamense* isolated from *E. fluctuans*.[36] Consequently, further studies involving advanced isolation techniques and structural characterization are warranted to identify the specific molecules responsible for this activity.

Chemical Profiling of Colletotrichum *sp. Closely Related to C. Siamense EA Extract Via LC-MS Analysis*

Our findings demonstrated that *Colletotrichum* sp. closely related to *C. siamense* isolated from *E. fluctuans* possessed both antibacterial and antioxidant activities. Since the EA extract exhibited the strongest antioxidant activity and antibacterial activity comparable to that of the n-hexane extract based on MIC values, it was selected for LC-MS-based metabolomic profiling.

The analysis putatively identified eleven non-essential and nine essential amino acids, as well as vitamins, carbohydrates, and a diverse array of bioactive compounds. Since the present study focused on bioactive secondary metabolites associated with the antibacterial and antioxidant activities of the EF-1 isolate, only the putatively identified secondary metabolites are presented and discussed in detail. Among the putatively detected secondary metabolites, flavonoids were predominant, followed by steroids, terpenoids, and phenolic compounds, whereas no alkaloids were detected. These results support the preliminary phytochemical screening (Table 1). Tables 4–6 and S2 present detailed data on the putatively identified secondary metabolites, including their RT, curve areas, observed m/z values, molecular formulas, similarity indices, and compound classes. The total ion chromatogram generated from LC-MS analysis of the EA extract in positive ionization mode is presented in Supplementary Figure S1. It should be noted that some metabolites have identical molecular formulas and molecular weights; therefore, their identities should be regarded as putative.

LC-MS analysis revealed that the EA extracts putatively contained 27 distinct flavonoid compounds (Table 4 and S2). Relatively high peak areas corresponding to metabolites putatively identified as quercetin, rutin, and kaempferol were detected, suggesting their possible contribution to antioxidant activities. These flavonoids have been reported to possess antioxidant and antiosteoporosis-related activities.[37] The antioxidants hesperetin and formononetin have been reported to exert neuroprotective effects and are considered promising candidates for Parkinson's disease.[38,39] A reduction in tumor growth and impaired viability of cancer cells were observed following biochanin A, naringenin, and fisetin treatment, suggesting that these compounds could serve as a foundation for developing novel antineoplastic therapies.[40–42] The antitumor, anticancer, and

Table 4. List of Major Flavonoids Putatively Identified in the EA Extract of Isolate EF-1.

Putative Compound	Rt (min)	Curve Area	Observed m/z	Molecular Formula	Similarity Index (%)
Formononetin	9.11200	350.56226	268.0736	$C_{16}H_{12}O_4$	92
Apigenin	9.36500	946.63625	270.0528	$C_{15}H_{10}O_5$	92
Naringenin	9.73200	2109.10272	272.0685	$C_{15}H_{12}O_5$	92
Biochanin A	10.01100	2395.39633	284.0685	$C_{16}H_{12}O_5$	92
Kaempferol	10.32200	2037.02724	286.0477	$C_{15}H_{10}O_6$	92
Luteolin	10.26500	1223.69855	286.0477	$C_{15}H_{10}O_6$	92
Fisetin	10.32500	1053.66225	286.0477	$C_{15}H_{10}O_6$	92
Rutin	35.51700	3471.10861	610.1534	$C_{27}H_{30}O_{16}$	92
Hesperetin 5-O-glucoside	24.04900	2106.44282	464.1319	$C_{22}H_{24}O_{11}$	92
Quercetin	11.42700	2433.10286	302.0427	$C_{15}H_{10}O_7$	92

Table 5. List of Putatively Identified Phenols in the EA Extract of Isolate EF-1.

Putative Compound	Rt (Min)	Curve Area	Observed m/z	Molecular Formula	Similarity Index (%)
Vanillic acid	2.79900	969.26248	168.0423	$C_8H_8O_4$	92
Gallic acid	3.04200	1785.62632	170.0215	$C_7H_6O_5$	92
Caffeic acid	4.64300	973.03584	180.0423	$C_9H_8O_4$	92
Ferulic acid	5.04300	1610.62508	194.0579	$C_{10}H_{10}O_4$	92
Syringic acid	5.17700	1031.76492	198.0528	$C_9H_{10}O_5$	92

Table 6. List of Putatively Identified Steroids and Terpenoids in the EA Extract of Isolate EF-1.

Putative Compound	Class	Rt (Min)	Curve Area	Observed m/z	Molecular Formula	Similarity Index (%)
Ergosterol	Steroid	13.2780	596.34155	396.3392	$C_{28}H_{44}O$	92
Eburicol	Steroid	19.7990	216.84636	440.4018	$C_{31}H_{52}O$	92
Fungisterol	Steroid	13.4990	436.32156	400.3705	$C_{28}H_{48}O$	92
Stigmasterol	Steroid	15.6380	271.10926	412.3704	$C_{29}H_{48}O$	92
Cycloartenol	Steroid	19.6180	431.10720	426.3862	$C_{30}H_{50}O$	92
β-sitosterol	Steroid	17.1630	223.22650	414.3862	$C_{29}H_{50}O$	92
Lanosterol	Steroid	19.1890	394.94632	426.3862	$C_{30}H_{50}O$	92
α-amyrin	Terpenoid	19.4380	544.20424	426.3862	$C_{30}H_{50}O$	92
β-amyrin	Terpenoid	19.3190	638.65523	426.3862	$C_{30}H_{50}O$	92

neuroprotective activities of these compounds are strongly associated with their antioxidant capacity, particularly through the attenuation of oxidative stress.

In parallel, several phenolic compounds such as vanillic acid, gallic acid, caffeic acid, ferulic acid, and syringic acid were also tentatively detected (Table 5). Syringic acid and caffeic acid have been well-characterized in previous studies as contributors to antioxidant and antibacterial activity.[43,44] However, ferulic acid faces significant challenges for practical applications due to its poor aqueous solubility and bioavailability. Therefore, these compounds continue to be investigated to increase their biopharmaceutical properties.[45]

LC-MS analysis also putatively indicated the presence of 26 steroids and four terpenoids (Table 6 and S2). Steroids and terpenoids are widely distributed fungal metabolites associated with antibacterial and antioxidant activities.[35,46] Their occurrence may contribute to the high bioactivity of the EA extract compared with the methanol and n-hexane extracts, although their individual contributions cannot be confirmed without bioactivity-guided fractionation**.** Notably, β-amyrin and α-amyrin, which were among the detected terpenoids, have been reported to exhibit antimicrobial and antioxidant activities.[47,48] Several putatively identified steroids, including ergosterol, eburicol, lanosterol, cycloartenol, stigmasterol, and β-sitosterol, have also been associated with pharmacological activities.[49–54] In contrast, the biological properties of other putatively detected steroids (Table S2) remain poorly characterized. Although their full pharmacological potential remains to be elucidated, their unique structures suggest the possibility of novel biological activities, warranting further investigation. These findings unravel the diversity of sterols in endophytic fungi and their possible applications.

These observations align with broader trends reported in endophytic fungi research. Between 2019 and 2022, endophytes isolated from various plants have demonstrated a broad spectrum of therapeutic potentials. Among the identified

genera, *Aspergillus* was the most dominant isolate, followed by *Alternari*a, *Penicillium*, *Trichoderma*, *Chaetomium*, *Nigrospora*, *Colletotrichum*, *Diaporthe*, *Epicoccum*, *Curvularia*, *Phoma*, *Emericella*, and several others.[55.] Specifically, *Colletotrichum* species have been reported to exhibit a diverse spectrum of bioactivities, including antioxidant, antibacterial, cytotoxic, and anticancer properties.[46,56] *C. siamense* is one of the *Colletotrichum* species that has been scarcely explored as a source of bioactive compounds. This present study contributes to this emerging field by identifying bioactive constituents in *Colletotrichum* sp. closely related to *C. siamense* from *E. fluctuans*, thereby providing a foundation for further investigations into the bioactive potential of *C. siamense*.

Conclusion

This study presents the first report on the isolation and characterization of *Colletotrichum* sp. isolate EF-1, closely related to C. *siamense* as an endophytic fungus associated with the leaves of *E. fluctuans*. The ITS sequence of isolate EF-1 showed the highest similarity to *C. siamense*, and the sequence has been deposited in GenBank (Accession No. PQ409585). Among the extracts tested, the EA extract exhibited the highest antioxidant activity (IC_{50} = 52.34 µg/mL). For antibacterial activity, both n-hexane and EA extracts showed comparable antibacterial activity against *S. aureus* and *E. coli*, with inhibition zones reaching up to 19.067 ± 0.076 mm and 19.883 ± 0.388 mm, respectively, and MIC values of 0.5 mg/mL against both bacterial strains. LC-MS analysis putatively identified a diverse chemical profile in the EA extract, including 27 flavonoids, 26 steroids, five phenolic compounds, and four terpenoids. These metabolites may contribute to the observed antioxidant and antibacterial activities. However, these identifications are tentative and require confirmation through MS/MS analysis. These findings highlight the potential of isolate EF-1 as a valuable source of natural compounds for pharmaceutical, nutraceutical, and biomedical applications, and provide a scientific foundation for further exploration of its bioactive metabolites.

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Authors' Contribution

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content. All authors have read and approved the final version of the manuscript and agreed to submit to the current journal.

Consent to Participate

This research did not involve human or animal biological material. Therefore, informed consent to participate was not required.

Consent for Publication

This research did not involve human or animal biological material. Therefore, consent to publish was not required.

Data Availability Statement

The ITS sequence of the endophytic fungus Colletotrichum sp. isolate EF-1, closely related to C. siamense, has been deposited in GenBank under accession number PQ409585. All other data generated or analyzed during this study are included in this published article. Additional datasets are available from the corresponding author upon reasonable request.

Declaration of Conflicting Interests

The authors declare that they have no competing financial or non-financial interests related to this work.

Ethical Approval

The authors confirm that this research did not involve human or animal biological material. Therefore, ethical approval was not required.

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Informed Consent

Not applicable.

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Use of Artificial Intelligence-assisted Tools

During the preparation of this manuscript, the authors used ChatGPT (OpenAI, free version) and DeepSeek solely to assist with English language editing, including grammar correction, wording improvement, and language polishing. Artificial intelligence (AI)-assisted text was used only for language editing, while no AI-generated research data, experimental results, scientific interpretations, or conclusions were used in this study. All AI-assisted suggestions were carefully reviewed, edited, and verified by the authors before being incorporated into the manuscript. The authors take full responsibility for the accuracy, originality, integrity, and validity of the content presented in this manuscript.

Supplemental Material

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