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Original Research Article

Secondary Metabolites Isolation from Endophytic Fungi *Diaporthe Arecae* and *Colletotrichum Gloeosporioides* Isolated from *Syzygium Cumini* Linn.

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ABSTRACT

This study reports some secondary metabolites produced from *Diaporthe arecae* and *Colletotrichum gloeosporioides*. These endophytic fungi were isolated from *Syzygium cumini* Linn plant. The fungal strains were identified based on morphological characteristics and DNA sequence analysis. They were isolated from the plant, followed by its small-scale cultivation and extraction with ethyl acetate. Secondary metabolites from the endophytic fungi were isolated through chromatographic separation and recrystallization. The structures of the isolated secondary metabolites were characterized by ^{1}H-NMR, ^{13}C-NMR, and DEPT-135 spectroscopic data. Column chromatographic treatment of the crude ethyl acetate extract of *Diaporthe arecae* with solvents of different polarity yielded the compound 1 (6.5 mg) and compound 2 (4.7 mg) respectively. In contrast, the crude extract of *Colletotrichum gloeosporioides* yielded the compound 3 (2 mg) after treatment with solvents of different polarity. Spectroscopic characterization revealed compound 1 as Ergosterol, compound 2 as 3-(3'-(3"-(3"'-acetoxypropanoyloxy) propanoyloxy) propanoyloxy) propanoic acid and compound 3 as a mixture of ergosterol and 4-methoxy phenol, respectively. The isolation of these compounds implies that endophytic fungi from *Syzygium cumini* may be a prominent source for the discovery of potential bioactive compounds or lead structures for new drug development.

Keywords: Characterization, Extraction, Potential, Discovery, Lead.

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Introduction

Almost every plant within the biosphere harbors one or more endophytic microorganisms. Certain medicinal plants, recognized for their therapeutic properties, may serve as significant reservoirs of endophytes that produce valuable secondary metabolites..[1] *Syzygium cumini*, a member of the Myrtaceae family, is a traditional medicinal plant with a long history of use. The seeds of this plant have been utilized in the treatment of diabetes mellitus and are also reported to exhibit a range of bioactivities, including antioxidant, anti-inflammatory, antimicrobial, anti-diarrheal, gastroprotective, and radioprotective effects.[1,2] Phytochemical analyses have elucidated the presence of various bioactive compounds, such as gallic acid, cyanidin glycoside, jamboline, triterpenoids, tannins, gallitanins, essential oils, myricetin, β-sitosterol, and myricyl alcohol. [3,4].

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In the context of our ongoing investigation into endophytic fungi, two fungal species, namely *Diaporthe arecae* and *Colletotrichum gloeosporioides*, were successfully isolated from the bark of *Syzygium cumini*. Notably, both *Colletotrichum gloeosporioides* and *Diaporthe* species are known to induce infections in plants, fruits, and vegetables. [5,6,7] But As an endophytic fungus, it acquires nutrients and benefits from the host's defense mechanisms. It is widely believed that the fungus' metabolites enhance the host's growth and competitiveness, thereby sustaining a mutualistic relationship. These endophyte-generated metabolites are regarded as a valuable resource for the development of new drugs, pesticides, and antibiotics, such as podophyllotoxin.,[9, 10] camptothecin,[11] hypericin, [12], emodin and azadirachtin.[13] In previous studies *Colletotrichum gloeosporioides* has been reported to produce 10-hydroxy camptothecine, aspergillomarasmine A and B, antimicrobial gloeosporone, anticancer taxol, piperine,rohitukine,[14-17] novel compound viz. 2-phenylethyl 1H-indol-3-yl-acetate,[18] gloeosporone,[19] ferricrocin,[20] azaphilones, colletotric acid and a ring B aromatic steroid.[21, 22] Also a new compound arecine[23], together with twenty known diketopiperazines including cordysinin A, bacillusamide B, Staphyloamide were isolated from *Diaporthe arecae.* [24] Since endophytic microbes serve as natural craftsmen for the synthesis of bioactive metabolites, they attract attention in the chemical library and in the pharmaceutical industry. [1, 25] Therefore, this study aimed to isolate and characterize the secondary metabolites from the endophytic fungus *Diaporthe arecae* and *Colletotrichum gloeosporioides* isolated from bark of *Syzygium cumini.* Linn. growing in Bangladesh.

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Materials and Methods

Isolation of fungal material

The fungi *Diaporthe arecae* and *Colletotrichum gloeosporioides* were isolated from the fresh, healthy bark of *Syzygium cumini* Linn. The plant sample was collected from the Jahangirnagar University Botanical Garden (23.8825°N 90.2482°E). The plant material was identified, and a voucher specimen was deposited at the Bangladesh National Herbarium (BNH) in Dhaka under the accession number DACB-47653. The isolation of endophytic fungi from fresh plant components was conducted following the established procedures at the Pharmaceutical Sciences Research Division, BCSIR Laboratories, Dhaka, Bangladesh.[26,27]

Taxonomical identification of fungal cultures

Morphological and Molecular identification

Fungal cultures were prepared to create slides stained with lactophenol cotton blue and examined using bright field and phase contrast microscopes to identify the strains. [28] A molecular biological protocol was followed for molecular identification of the fungal strains. [29, 30] The fungal DNA was amplified and submitted for sequencing. The resulting base sequences were then compared using the BLAST algorithm with publicly accessible databases, including GenBank.

2.2.2 Isolation and structure elucidation of secondary metabolites

After surface sterilization of the bark from the *Syzygium cumini* plant, *Diaporthe arecae and Colletotrichum gloeosporioides* (internal strain No. SCBE-1 and SCBE-2 respectively) were grown on medium potato dextrose agar on a small scale at 28°C for 21 days. The cultured media were then extracted twice with ethyl acetate to obtain crude extract.[31] The crude extract of *Diaporthe arecae* was subjected to column chromatography on silica gel (70–230 and 230–400 mesh, Merck, Germany) using n-hexane/dichloromethane gradients, followed by dichloromethane, then dichloromethane/methanol gradients, and finally methanol gradients. Different fractions were collected and subjected to TLC. Then the fractions were treated with different solvent and pure compounds were obtained. The structures of the pure compounds were determined by NMR data analysis using $CDCl_3$ on a Bruker 400 MHz NMR spectrometer (Bruker, Switzerland).

Results and Discussion

Identification of fungal strain

According to the protocol, morphological characteristics were used to identify endophytic fungal strains.[4, 32] Molecular analysis of the fungus (SCBE-1) revealed 99% similarity to another isolate of accession number KF918581 that was identified as *Diaporthe arecae*. They are similar to other related taxa (99%, accession numbers FJ 478124.1, MK673909.1, MH142457.1, KT821501.1, KT821501.1 etc.) deposited in NCBI.

>Seq1 [organism= *Diaporthe arecae*] 5.8 s rRNA

CGCCTTATTGATATGCTTAAGTTCAGCGGGTATTCCTACCTG
ATCCGAGGTCAAATTTTCAGAAGTTGGGGGTTTAACGGCAG
GGCACCGCCAGGGCCTTCCAGAGCGAGGGTTTAACTACTGC
GCTCGGGGTCCTGGCGAGCTCGCCACTAGATTTCAGGGCCT
GCTTCGTTAAAAGCAGTGCCCCAACACCAAGCAATGCTTGA
GGGTTGAAATGACGCTCGAACAGGCATGCCCTCCGGAATAC
CAGAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTG
AATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCT
TCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTT
GATTCATTTGTGTTTTTTCTCAGAGTTTCAGTGTAAAAACAA
GAGTTAACTTGGCCGCCGGCGTGCCTGCTCCCTGTCTCCAGG
GGGCCCCGAGGGGGCCAGCATGCGCCGAGGCAACAGTAAG
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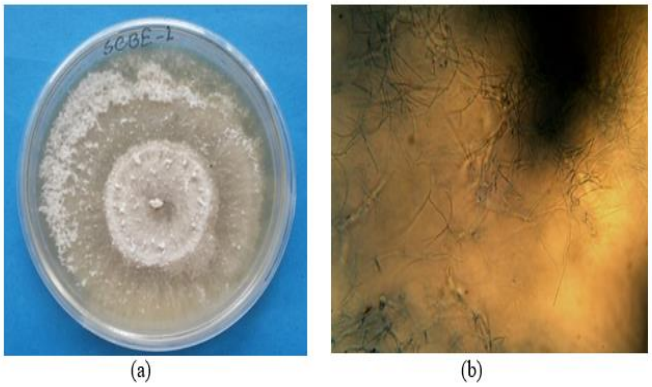

Figure 1: (a) Macroscopic and (b) Microscopic view of *Diaporthe arecae* sp.

The pictures represent macroscopic view of *Diaporthe arecae* from bark of *Syzygium cumini* after 12 days of cultivation on PDA media and also represent microscopic view (40X).

Another molecular analysis of another fungus (SCBE-2) based on 5.8 s rRNA gene revealed 99% similarity to another fungal isolate of accession number MH863850.1 that itself was identified as *Colletotrichum gloeosporioides*. The sequence of the fungus is:

GAAAAAATAANNNTAGAAGACAAATGACAANGANNGAAA
ATAAAAAAAAAAANNTTAAGAGANNAAGANNAAANAAATA
AGAAAGAATNATAAGATGATCCGAGGTCAACCTTTGGAAA
ATTGGGGGTTTTACGGCAAGAGTCCCTCCGGATCCCAGTGC
GAGACGTAAAGTTACTACGCAAAGGAGGCTCCGGGAGGGT
CCGCCACTACCTTATGAGGGCCTACATCAGCTGTAGGGCCC
CAACACCAAGCAGAGCTTGAGGGTTGAAATGACGCTCGAAC
AGGCATGCCCGCCAGAATGCTGGCGGGCGCAATGTGCGTTC
AAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTT
ATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGA
GATCCGTTGTTAAAAGTTTTGATTATTTGCTTGTACCACTCA
GAAGAAACGTCGTTAAATCAGAGTTTGGTTATCCTCCGGCG
GGCGCCGACCCGCCCGGAGGCGGGAGGCCGGGAGGGTCAC
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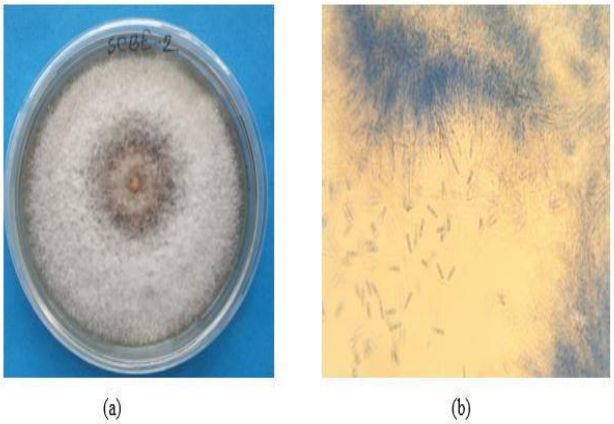

Figure 2: (a) Macroscopic and (b) Microscopic view of *Colletotrichum gloeosporioides* sp. The pictures represent macroscopic view of *Colletotrichum gloeosporioides* from bark of *Syzygium cumini* after 12 days of cultivation on PDA media and also represent microscopic view (40X).

Structure elucidation of isolated compounds

After performing column chromatography on the crude extract of *Diaporthe arecae*, a total of 59 fractions, each 100 ml in volume, were

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collected. The thin-layer chromatography (TLC) patterns of these fractions were observed for purification purposes. Treatment of fraction number 12 with n-hexane and dichloromethane (DCM) yielded Compound 1 (6.5 mg). Additionally, cultivating *Colletotrichum gloeosporioides* at a small scale on potato dextrose agar (PDA) medium led to the extraction of Compound 3 (2 mg). This represents the first reported isolation of 4-methoxyphenol from the endophyte *Colletotrichum gloeosporioides*, sourced from the Bangladeshi medicinal plant *Syzygium cumini*. The structures of the compounds were confirmed using 1H NMR and 13C NMR spectroscopic data. Since the compounds, except for Compound 2, are known metabolites, they were identified by comparing their spectroscopic data with existing literature.

Compound 1

After treatment with *n*-hexane and dichloromethane of column fraction from the crude extract of *Diaporthe Arecae*, compound 1 (6.5 mg) was obtained as white color solid . It appeared purple color on the TLC plate (R_f = 0.402, Toluene / 10% ethyl acetate) under UV light at 254 nm. It was invisible at 365 nm. It is soluble in $CHCl_3$ and CH_2Cl_2. A purple color was provided by spraying the formed plate with vanillin-sulfuric acid reagent, followed by heating.

Compound 1's 13C NMR spectrum (100 MHz, CDCl3) revealed 28 carbon resonances, while the DEPT 135 experiment reported these signals as 6 methyls, 7 methylenes, 11 methines and 4 quaternary carbons; and also, 24 no carbons, were bound to protons. Two one-proton multiplets at δ = 3.64 and δ = 5.59 were seen in the 1H NMR range (400MHz, CDCl3), signifying H-3, H-6 steroidal signals. The multiplets at δ=5.39 and δ= 5.59 could be accounted for three olefinic protons at C-7 which is expected for ergosterol. The ^{13}C NMR spectral data reported a sterol with 6 methyl signals at δ = 12.1,17.6,21.1,19.6,19.9,16.2; oxygenated methylene signals at δ= 70.5 and six olefinic carbon signals at δ=139.8,119.6,116.3,141.4,135.6,132.Finally, the structure of compound 1 was confirmed as ergosterol by comparison with the published data. [33, 34] The structure of compound 1 is given in Figure 3 and the data is given in the following.

Compound 1: (Ergosterol)

White crystalline solid; ^{1}H NMR ($CDCl_3$): δ 3.64 (1H, m,H-3), δ 5.59 (1H, m,H-6), δ5.39 (1H, m,H-7), δ 0.97 (3H, s,H-18), δ 0.65 (3H, s,H-19), δ1.06 (3H, d, *J* = 6.4 Hz,H-21), δ5.22 (1H, m,H-22,H-23), δ 0.86 (3H, d, *J* = 6.4 Hz,H-26, H-27), δ0.90 (3H, d, *J* = 6.8 Hz,H-28); ^{13}C NMR ($CDCl_3$): 38.4, 32.0, 70.5, 40.8, 139.8, 119.6, 116.3, 141.4, 46.3, 37.0, 21.1, 39.1, 42.9, 54.6, 22.7, 28.3, 55.7, 12.1, 17.6, 40.4, 21.1, 135.6, 132.0, 42.8, 33.1, 19.6, 19.9, 16.2.

Compound 2

Compound 2 (4.7 mg) was isolated from column fraction 30 of the crude extract of *Diaporthe arecae* by eluting with CH_2C_{l2}/0.3% Methanol. It was obtained as a colourless crystal.It was not observed visually or under UV light at 365 nm. It appeared purple on the TLC plate (R_f = 0.3571; Toluene / 10 % ethyl acetate) under UV light at 254 nm. The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of Compound 2 displayed 4 signals of carbon resonances, while the DEPT 135 experiment enlisted these signals into methyl, methylene and quarternary carbons. The ^{1}H NMR spectrum (400MHz, $CDCl_3$) of compound 2 showed singlet at δ=2.71 ppm (3H, acetyl-CH_3 group of aliphatic chain), two triplets centered at δ 3.07 ppm (8H, 4 CH_2 groups of the aliphatic chain) and δ 4.68 ppm (8H, 4-CH_2 groups adjacent to the oxygen group of the aliphatic chain). A carboxyl group was reported by a signal at δ 174.1 ppm. In the ^{1}H NMR spectrum, the coupling constant of the two triplets (*J*=6.0 Hz) indicated that they are mutually coupled. On this basis of these spectral data, Compound 2 was assumed as 3-(3′-(3″-(3‴-acetoxypropanoyloxy) propanoyloxy) propanoyloxy) propanoic acid and its structure was given in the Figure 3. After the literature review, it was expected that this would be a novel compound. For further confirmation the compound requires 2D (COSY, HSQC and HMBC) NMR and MS spectroscopic data analyses.

Compound 2: (3-(3′-(3″-(3‴-acetoxypropanoyloxy) propanoyloxy) propanoyloxy) propanoic acid)

Colourless crystal; ^{1}H NMR ($CDCl_3$): δ 4.68 (2H, t, *J* = 6.0 Hz,H-1,H-3′, H -3″, H -3‴), δ 3.07 (2H, t, *J* = 6.0 Hz, H -2, H -2′, H -2″, H -2‴), δ 2.71 (3H, s,H-1‴′);^{13}C NMR ($CDCl_3$): 69.3 (C-1, C -3′, C -3″, C -3‴, 40.2 (C -2, C -2′, C -2″, C -2‴), 174.1 (C-3, C-1′, C-1″, C-1‴),30.7(C-1‴′).

Compound 3

Mixture of ergosterol and 4-methoxyphenol

The ^{1}H NMR spectrum (400MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectra of Compound 3 showed signals representing a minor and a major compound in the ratio of approximately 1:3.94. It was identified as a mixture of ergosterol and 4-methoxyphenol compared with the published NMR data.[33]

Ergosterol

From the ^{13}C NMR spectrum (100 MHz, $CDCl_3$), 28 carbon resonances were observed as a major compound while 6 methyls,7 methylene, 11 methines and 4 quaternary carbons were sorted from the DEPT 135 experiment. Two one-proton multiplets at δ =3.66 and δ = 5.59, typical for the signals of H-3, H-6 of a steroidal nucleus were shown by the ^{1}H NMR spectrum (400MHz, $CDCl_3$). The multiplets at δ=5.4 could be attributed to three olefinic protons at C-7 and in the side chain, as would be expected for ergosterol. The ^{13}C NMR spectral data also suggested that this compound was a sterol with 6 methyl signals at δ = 12.1, 17.6, 40.4, 19.6, 19.9, 16.2; oxygenated methylene signals at δ= 70.5 and six olefinic carbon signals at δ=139.8, 119.6, 116.3, 141.4, 135.6, 132. The structure of the minor compound was confirmed as Ergosterol by comparison with the published NMR data.[33] The structure of the compound is given in Figure 3.

4-methoxyphenol

The minor compound of the mixture of Compound 3 displayed two symmetric pairs of coupled two proton signals at δ=6.27 (H-2 and H-6) and at δ= 6.53 (H-3 and H-5) assigned to a 1, 4-disubstituted benzene ring. The resonance at δ 3.51 ppm in the ^{1}H NMR and at δ 56.2 ppm in the ^{13}C NMR spectra, in conjunction with the DEPT-135 spectrum, proved the presence of a heteroatom bonded methyl group that is present, -OCH_3 in the case of Compound 3. These spectral data were compound reported as 4-methoxy phenol and found identical. Based on these spectroscopic data and literature review, the minor compound of the mixture was established as 4-methoxy phenol.[34] The structure of the compound is given in Figure 3.

Compound 3: (Mixture of Ergosterol and 4-methoxyphenol) Ergosterol

M.P. 149–150 °C; ^{1}H NMR ($CDCl_3$): δ 3.66 (1H, m,H-3), δ 5.59 (1H, m,H-6), δ5.40 (1H, m,H-7), δ 0.97 (3H, s,H-18), δ 0.65 (3H, s,H-19), δ1.06 (3H, d, *J* = 6.4 Hz,H-21), δ5.23 (1H, m,H-22,H-23), δ 0.85 (3H, d, *J* = 6.4 Hz,H-26, H-27), δ0.94 (3H, d, *J* = 6.8 Hz,H-28); ^{13}C NMR ($CDCl_3$): 38.4, 32.0, 70.5, 40.8, 139.8, 119.6, 116.3, 141.4, 46.3, 37.0, 21.1, 39.1, 42.8, 54.6, 23.0, 28.3, 55.8, 12.1, 17.6, 40.4, 21.1, 135.6, 132.0, 42.8, 33.1, 19.7, 20.0, 16.3.

4-methoxyphenol

^{1}H NMR ($CDCl_3$): δ 6.27 (1H, d, *J* = 8.4 Hz,H-2, H-6), δ 6.53 (1H, d, *J* = 8.4 Hz, H-3, H-5), δ 3.51 (3H, s, -OCH_3); ^{13}C NMR ($CDCl_3$): 130.8 (C-2), 135.2 (C-3), 135.6 (C-5), 132.0 (C-6), 56.2(-OCH_3)

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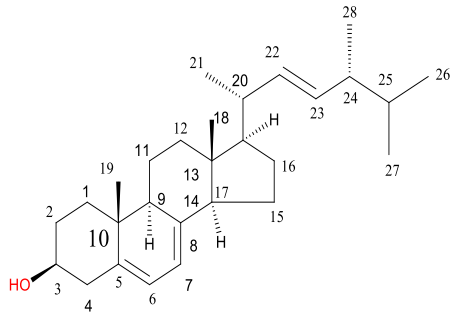
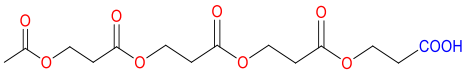
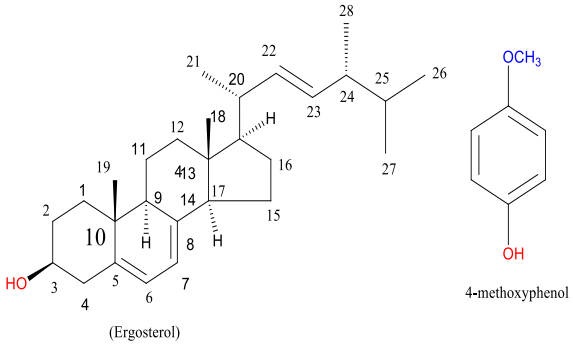


Compound 1(Ergosterol)



(3-(3'-(3"-(3'"-acetoxypropanoyloxy) propanoyloxy) propanoyloxy) propanoic acid)

Compound 2



(Ergosterol) 4-methoxyphenol

Compound 3

Figure 3: Secondary metabolites from *Diaporthe arecae* isolated from *S. cumini* –Ergosterol,[23] 3-(3′-(3′′-(3′′′-acetoxypropanoyloxy)propanoyloxy)propanoyloxy)propanoic acid[32] and metabolites from *Colletotrichum gloeosporioides* – Mixture of ergosterol and 4-methoxyphenol.[8]

Conclusion

In conclusion, this report has identified two secondary metabolites from the fungal strain *Diaporthe arecae*: ergosterol and 3-(3′-(3′′-(3′′′-acetoxypropanoyloxy) propanoyloxy) propanoyloxy) propanoic acid. This study is the first to document compounds 1 and 2 from the endophytic fungus *Diaporthe arecae*. Notably, compound 2 has not been previously recorded in the literature. Additionally, compound 3, a mixture of ergosterol and 4-methoxyphenol, was isolated from the endophyte *Colletotrichum gloeosporioides*. There is potential for discovering more secondary metabolites from these two endophytes, but further investigation is required. Hence, it can be concluded that the endophytic fungi of *Syzygium cumini* may serve as a valuable source for discovering secondary metabolites, which could lead to the development of new bioactive compounds or future drugs.

Conflict of Interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original. Any liability for claims relating to this article will be borne by us.

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