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Bioactivity Guided Fractionation and Characterization of Secondary Metabolites Isolated from the Endophytic Fungus, *Daldinia Eschscholtzii* and their Broad Spectrum Anti-Microbial Activities

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Abstract

Antibiotic resistance by pathogenic bacteria has become a global health threat. In this scenario, natural products with activity against pathogens could aid in countering the microbial resistance mechanisms. Fungal endophytes offer a rich source of untapped molecules with potential antimicrobial activity. This study aims to isolate the endophytic fungi from the medicinal plant, *Abutilon indicum* (L.) sweet (AbL). The extracts and compounds isolated from this endophyte were tested for their Ability for inhibiting biofilm formation against gram-negative as well as gram-positive bacteria. We describe two fungal endophytic isolates of of *Daldinia eschscholtzii* (DAE), AbLEF 001 and AbLEF 002 for the first time from the plant AbL. The crude extracts of DAE upon chromatographic separation, resulted in te isolation of five compounds. Spectroscopic characterization showed the compounds to be 2-phenylbenzo chromen 4-one (1), 4-Hydroxy-3,5-dimethoxy benzaldehyde (2), 3,4,5-trimethoxybenzoic acid (3), Furan 2 carboxylic acid (4) and Gossypetin-3′-O-glycoside (5).The isolated compounds showed promising anti-bacterial activities and exhibited anti-biofilm activity against *Staphylococcus aureus*, *Streptomyces griseus*, *Clostridium botulinum*, *Helicobacter pylori*, *Pseudomonas aeruginosa* and *Salmonella enterica*. DAE from AbL was identified as a new source of bioactive molecules including Gossypetin-3′-O-glycoside. All of the compounds from DAE showed anti-microbial and anti-biofilm activity with Gossypetin-3′-O-glycoside having the strongest anti-biofilm activity.

Keywords: Endophyte, *Daldinia eschscholtzii*, secondary metabolites, Gossypetin-3′-O-glycoside, Antioxidants

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1. Introduction

The evolution of new pathogenic strains and the spread of antibiotic resistance are becoming a great threat to the global health community [1]. The human pathogenic microorganisms have developed resistance in response to the arbitrary use of commercially available anti-microbial drugs. Hence, development of novel, effective and affordable medicines as the treatment for anti-microbial infections has become the need of the hour [2]. In this scenario, the use of natural products derived from various medicinal plants and microorganisms emerge as one of the possible strategies in the treatment of infectious pathogens. Currently, there is a growing interest in endophytic microorganisms, which in close association with the host produces a diverse number of biologically active metabolites [3-5]. Secondary metabolites that may be beneficial to the host plant, due its antifungal, antibacterial and other regulatory activities [6]. Owing to their rich biodiversity, these are considered as potential sources for new drug candidates [7]. Earlier research has highlighted the possible application of metabolites in

industries, agriculture as well as in modern medicine as anticancer, immunosuppressant, antimycotics, and antibiotics compounds [8].

Studies have also shown that endophytic fungi are rich source of phenols and other metabolites with broad spectrum anti-microbial activity [9]. Overall, endophytes are a more reliable source for meeting the growing need for biologically active drugs in an ecologically acceptable manner [10-11]. Plant has been shown to contain a number of bioactive secondary metabolites, including alkaloids, saponins, flavonoids, gallic acid, caffeic acid, and beta sitosterol were reported with potential antioxidant and anti-inflammatory activity [12]. Several strains of endophytic fungus, including *Fusarium* sp, *Cladosporium* sp, *Nigrospora* sp, *Aspergillus niger and Alternaria alternata*, were previously identified in this plant [13-14]. Considering the fact that the extracts and compounds of AbL exhibit effective antimicrobial activity, it is possible that the metabolites of endophytic fungus isolated from AbL have therapeutic relevance.

In this study, an attempt was made to isolate and identify novel endophytic fungus species from AbL pods and leaves, and the results showed that this was successful. Leaf tissues were used to identify a notable species of *Daldinia*, but pods did not exhibit any endophyte colonization signs. According to literature reports, there has been no reported case of DAE from any region of AbL until date. This research is supported by thorough phenotypic, molecular, as well as morphological characterization. In addition, efforts have been undertaken to describe the bioactive compounds as well as identify the main metabolites and forinvestigating the potential anti-microbial activity of extracts and compounds against bacteria *Staphylococcus aureus*, *Streptomyces griseus*, *Clostridium botulinum*, *Helicobacter pylori*, *Pseudomonas aeruginosa* and *Salmonella enteriac* that could be useful for further better applications in the field of medicine. In addition, the anti-biofilm activity was tested with the minimum inhibitory concentration (MIC) obtained.

2. Materials and Methods

2.1 Collection of AbL leaves

During the month of November, fresh leaves and pods from AbL were harvested from Potheri, TamilNadu, India. Phytopathologist Prof. P. Jayaraman of the Plant Anatomy Research Centre in Tambaram, India, recognized as well as authenticated the entire plant (Reg. No. PARC/2019/3951), as well as voucher specimen was already submitted.

2.2 Isolation of endophytic fungi from leaves and pods of AbL

Pedra et al. [15] proposed a technique for cultivating endophytic fungi from plant parts, which was followed with fewer modifications. Endophytic fungi have been cultivated from pods (2 nos) as well as leaves (2 nos) following adequate surface sterilization within a biosafety cabinet. Pods as well as fresh leaves have been carefully rinsed with running tap water before being surface sterilized for 2-3 minutes with 4 percent hypochlorite and washed three times with autoclaved distilled water, followed by washing with 70 percent ethanol. The leaves have been cut into small parts (0.5cm × 0.5cm) plated on potato dextrose agar plates (PDA: 20g/L agar, 300g/L diced potatoes, 20g/L dextrose,) under sterile conditions. Additionally, from the pods one single leaflet removed and placed on the agar plates in a similar manner. On every plate, four leaf as well as pod segments have arranged in a circle. It was necessary to add 50 µg/ml of chloramphenicol (Antibiotic) to the culture medium in order to inhibit bacterial growth. The samples have been incubated at 28°C for 5-7 days. For further investigation, fungal hyphae in PDA that emerged from leaves were sub cultured whereas pure cultures have been stored at 4°C as PDA slants. The presence of fungus in the leaf samples demonstrated again by placing them in Oatmeal agar (12.5g/L Agar and 60g/L oatmeal).

2.3 Identification of the endophyte

2.3.1 Morphological analysis

Morphological characteristics like topography, texture, and colour of isolates were studied on OA and PDA using light microscope as defined earlier [16]. Lactophenol cotton blue (LCB) staining was used to stain the isolates. It was crucial to analyze the fungal development on a daily basis during the incubation period at 30°C. The stained fungus has been viewed and photographed using a microscope - BF (Zeiss, Germany) with images being processed using ZEN 10 software (Magnification – 100X).

2.3.2 DNA extraction, amplification and sequencing

The fungal mycelia grown on agar plates were carefully collected using sterile forceps and added to sterilized PBS (pH 7.4), which was vortexed for 5 minutes with glass beads. A total of 0.2 mL of the suspension was used for extraction of DNA utilizing DNeasy Plant Mini kit (Qiagen, USA) as per the manufacturer's instructions. The purity of DNA samples were analyzed using nanodrop. The extracted DNA upon dilution with milliQ water preserved at 4˚C. PCR has been carried out with the help of "primersF:5′AATCAGTTATAGTTTATTTGATGGTGGT3′, R:3′TCTCAGGCTCCCTCTCCGGAACC5′."The reaction has been carried out in a 25µl final volume containing 1U Taq DNA polymerase, 0.2 mM dNTPs, 1.5 mM $Mgcl_2$, 1X Taq.pol buffer, 10 pM of each primer and 0.1µg of genomic DNA. For PCR, thermal parameters used are, 94˚C (3min) followed by 35 cycles which executed the following parameters for every cycle, 94˚C 30 s, 55˚C – 40 s, 72˚C – 35 s, and final extension at 72˚C for 7 min. The obtained PCR products were analyzed using 1.5% agarose gels in TAE buffer and purified with PCR clean-up kit. The Nextseq 500 DNA Analyzer has been utilized to sequence the purified PCR products. The sequences have been modified as well as compared manually with accessible data from databases (Genbank) utilizing BLASTN software, which served as the underlying foundation for identifying the fungus in this study.

2.3.3 Phylogenetic analysis

The phylogenetic analysis of obtained fungal ITS sequences along with closely related sequences (GenBank) using a BLAST search, was utilized to identify the endophytes [17]. It was possible to collect 67 sequences, of which 14 sequences have been chosen for phylogenetic reconstruction. The chromatogram of the original sequences modified with the chromas version 2.6.6 software (Technelysiumpty ltd) and aligned with Clustal W (version 1.8). The phylogenetic tree was built utilizing the NJ (neighbor-joining) technique with *Hypoxylon fragiforme*

serving as out-group, and the results were analyzed with MEGA 10.1.8 software.

2.3.4 Mass cultivation of endophytic fungi

Endophytic fungal isolates were cultured in Potato dextrose broth (20g/L dextrose, PDA; 300g/L diced potatoes). Fungal mycelium discs (8 mm in diameter) were punctured with a cork borer, inoculated aseptically into 500ml PDA broth erlenmeyer bottles (15 in total) that contained 200 ml of PDA broth each which were further grown at 28˚Celsius in an incubator shaker with for 21day period continuous shaking.

2.3.5 Preparation of crude extracts

The media from the fully-grown AbLEF cultures (AbLEF 001 along with AbLEF 002) was filtered using filter paper (whattman No.1) and the fungal mat was air dried and weighed. Fungal mycelia (AbLEF 001g–800 g, AbLEF 002g-715g) were then extracted consecutively 3 times (24 h each) at room temperature with equal volume of methanol (1000ml) by cold maceration method. The extracts were again filtered filtered and the filtrate was evaporated under vacuum. Using a separating funnel, dried AIEF extracts were partitioned further (thrice) with equal amounts of ethyl acetate (EA) along with water (300 ml) to acquire the crude EA extracts (EFEA 001 and EFEA 002). The total crude extract of EFEA 001 was 4.8 g and EFEA 002 was 3.7 g

2.4 Phytochemical screening of crude extracts

Preliminary phytochemical analysis for the obtained fungal extracts were done following the method of Rahman et al. [18]. The extracts were analyzed for the phytocompounds like flavonoids, glycosides, terpenoids, saponins, tannins and
Steroids

2.5 Anti-bacterial activity of crude extracts - resazurin assay

Chemicals

Nutrient agar, peptone, Yeast extract, Chloramphenicol, Resazurin, Potassium per sulfate, Dextrose, Acridine orange from Sigma-Aldrich. *Staphylococcus aureus* (SA) ATCC 25923 (Gram-positive) and *Salmonella enterica* (SE) ATCC 10708 (gram negative) were used for determining the MIC of the crude extracts. Individual bacterial colonies inoculated in the nutrient broth (LB) under sterile conditions. The bacterial cultures incubated and monitored for growth utilizing a UV-1800 spectrophotometer (Shimadzu, Japan). The experiment was carried out in an aseptic environment on a 96well plate following the method suggested by Monteiro et al [19] with minor changes. Briefly, 13.5 mg of reassuring was dissolved in 2.0 mL of sterile water and this solution was used as reassuring stock, a chromogenic indicator. Various dilutions (10 0.03mg/ml) of the extracts (EFEA 001 &EFEA 002) have been added to appropriate wells containing microbial cultures (10µl) along with control. 10µl of broad-spectrum antibiotic ciprofloxacin in serial dilution (5mg/ml stock) has been utilized as positive control. For 24h, at 37 º C, the plates were incubated and color change has been assessed at the end of the incubation. Bacterial growth will result in a color change from purple to pink because of resazurin transformation. Graph plotted with concentration of extracts against percentage inhibition to determine the minimum inhibitory concentration (MIC) value.

2.6 Fractionation of EFEA using column chromatography

Column chromatography using silica gel was done to purify the compounds from both EFEA. The crude extracts were weighed (4.8 g), admixture was prepared with equal amounts of silica, and the same was loaded on to the silica packed column. CC on silica gel (1x25 cm), 100-200mesh size, Hex/EA 1:9 – 8:2, 2 ml /min, followed by TLC detection of eluates (Iodine, VanillinSulphuric acid). TLC was used to monitor the fractions, and those with comparable compound profiles have been pooled, the solvent was evaporated under vacuum. While EFEA 002 had compound profile that was similar to EFEA 001, with a few differences, EFEA 002 was exposed to a comparable column chromatography separation and the compounds were separated in the same way. Standard spectroscopy methods were used to identify and characterize the isolated phytocompounds.

2.7 Spectroscopic characterization of isolated compounds

Compounds (1) – (5) obtained from EFEA 001 and compounds (1), (3), and (4) obtained from EFEA 002, were characterized utilizing UV, IR, NMR, along with mass spectrometry. For UV, the compounds were dissolved in methanol (20µg/ml) and maximum Absorbance was recorded using the spectrophotometer (UV1800, SHIMADZU, Kyoto, Japan). For FT-IR, KBr pellets were prepared and the analysis was done using "Bruker alphaE&T spectrophotometer (Lab India). ^{13}C, ^{1}H, HSQC and HMBC spectra were recorded using NMR (500 MHz - BRUKER), with TMS (tetramethylsilane) serving as an internal control. Compounds" (1) – (3) dissolved in $CDCl_3$ while compounds (4) and (5) have dissolved in D_6-DMSO solvent. The mass was determined using SHIMADZU mass spectrophotometer.

2.8 Quantification of compounds by HPTLC technique

In this study, the total amount of each of the five distinct compounds (1) – (5) in their corresponding EFEA extracts was determined using HPTLC. The experiment was done using an 8 x 10-cm2 TLC plate (Merck, Germany) and CAMAG HPTLC analyzer. For compounds (1) – (4), the appropriate mobile phase used was chloroform, methanol and glacial acetic acid; whereas for compound (5) the mobile phase used was n-butanol, acetic acid and water. The samples (EFEA: 10 mg/ml, compounds (1): 7.5 to125 µg/ml, (2) – (5): 60 to 1000µg/ml) were sprayed on the TLC plate using an automated TLC applicator (Lino 5). Which were then eluted using methanol: chloroform: acetic acid (0.2:9.6:0.2) for (1)–(4) and n-butanol: water: acetic acid (4:4:2) for (5) and the plate was further scanned and visualized using CAMAG TLC scanner and visualizer (254nm and 366nm). The linearity range fixed as 60-1000µg/band. The amount of each compound in EFEA was measured by plotting its concentration against the peak region gained from its HPTLC chromatogram (CATS software).

2.9 In vitro anti-bacterial screening of compounds (1)-(5) using resazurin assay

Compounds (1) – (5) at concentrations 5 – 0.015 mg/ml were individually tested for their anti-microbial activity using resazurin assay (Refer section: Anti-bacterial activity of crude extracts). Gram-positive bacteria

Staphylococcus aureus (SA) ATCC 25923, *Streptomyces griseus* (SG) ATCC 10137, *Clostridium botulinum* (CB) ATCC 3502 and gram negative bacteria *Helicobacter pylori* (HP) ATCC 26695, *Pseudomonas aeruginosa* (PA) ATCC 27853, *Salmonella enterica* (SE) ATCC 10708 were used for determining the MIC of the isolated compounds.

2.10 Biofilm inhibition assay – Crystal violet staining

Biofilm formation, a characteristic feature of microbes was visualized using crystal violet staining as described previously by Selvaraj et al [20]. Briefly, 1% of the culture was seeded in 24 well sterile microtitre plate followed by the addition of compounds (1) – (5) (MIC obtained from resazurin assay) and incubated at 37˚C for 24 h. Nutrient broth along with cultures served as vehicle control. The plate was then rinsed with distilled water in order to extract any remaining unbound cells [21-22]. After that, the biofilm cells have been stained using 0.4% crystal violet solution and again washed with distilled water to remove excess stain. The formation of biofilms was observed using Phase contrast microscope (Leica, Germany) at 40X magnification.

2.11 Statistical analysis

The results obtained were subjected to ANOVA analysis. $p < 0.05$ statistically significant.

3. Results and discussion

Endophytic fungi are found in nearly all plant tissues and are well recognised as source of novel compounds with a wide range of applications. Secondary metabolites generated from these fungi have diverse structural groups with potent bioactivities [11]. In the search for new therapeutics, endophytic fungus from traditional medicinal plants is becoming increasingly relevant. In this study, our findings report two fungal endophytic isolates (AbLEF001 and AbLEF002) from AbL leaves and were recognized as *Daldinia eschscholtzii* (DAE). Previous findings report the existence of the endophyte *Alternaria alternata* in AbL petiole and root [13]. However, only a few species of endophytic fungus have been identified from AbL so far (TN, India). Consequently, the current research focussing on "endophytic fungi along with their metabolites extracted from the leaves of AbL will be tremendously valuable.

3.1 Morphological study

After surface sterilization, AbL leaf samples were cultured on PDA (potato dextrose agar) as well as OA (oatmeal agar) agar plates, where fungus grew rapidly. In both OA and PDA, development of white cottony hyphae has been detected at inoculation site after 3 days, indicating the presence of a viable fungus (Fig 1A & 1B). From same AbL plant, the pod samples, treated in the same way as the leaves, did not display any growth of fungus after 5 days (Data not shown). These results show that endophytic fungus is likely to be restricted to the plant's leaf tissue [23]. The structural characteristics of the isolated fungus visualized with the aid of light microscopy (LCB staining). The fungus comprised thick and thin walled septate hyphae (Fig 2A) with hyaline and cylindrical conidiogenous cells (Fig 2B). Conidiophores have been branched in an irregular pattern, with conidiogenous cells sprouting from each end of the length of the conidiophore. (Fig 2C). A trichotomous and dichotomous branching pattern is formed by the septate conidiophores, with clusters of conidia at the end of each branch (Fig 2D). Thickwalled hyphae with brown exudates on their surfaces (Fig 2E). Ellipsoid and hyaline conidia with an attenuated base, ranging in length from 4.3 to 4.7 µm and diameter from 2.0 to 2.25 µm (Fig 2F). These distinct characteristics signify that the isolates belong to the family *Hypoxylaceae* and genus Daldinia. Both the AbLEF 001 and AbLEF 002 fungal isolates had comparable morphological and structural features, representing that they are both members of the Daldinia genus [24].

3.2 Molecular identification

To determine their relationships, DNA sequences were subjected to molecular phylogenetic analysis. The phylogenetic tree was generated using ITS1-5.8-ITS2 sequences from fungal DNA that were compared to almost identical sequences from the GENBANK database. An Ncbi-BLAST search of the DNA sequences of AbLEF 001 and AbLEF 002 revealed that they are both DAE. Furthermore, the BLAST results revealed that the isolates' DNA sequences contained a number of closely related sequences. The isolates (Table S1) were examined using the NJ method in combination with DAE reference isolates and other closely related DAE species from BLAST, resulting in a cluster within the DAE group (Fig 3). *Hypoxylon fragiforme* was used as an out-group. Both fungal isolates' DNA sequences had a maximum similarity of 100 percent to data available at the National Center for Biotechnology Information (NCBI) and were identified as *Daldinia*. The isolates AbLEF 001 and AbLEF 002 displayed high bootstrap values (100 %) when compared to the DAE strain FR852577 (Fig. 3). This indicates that the isolates are confident in their clade's position. Because of the phylogenetic analysis, the isolates were revealed DAE. The ITS sequences of the AbLEF 001 and AbLEF 002 were submitted to GenBank and given the accession codes MT712203 and MT712204, respectively. DAE strain, called A630, was identified from the herb *Pogostemon cablin* [25]. However, no DAE has ever been isolated from any component of the plant AbL. Hence, the identification of DAE in this study is a significantly important finding.

3.3 Phytochemical analysis of crude extracts

The crude extracts (EFEA 001, EFEA 002) obtained after further fractionation with ethyl acetate and water was exposed to phytochemical screening for the presence of terpenoids, glycosides, saponins, steroid hormones, tannins, and flavonoids. With the exception of Tannins, EFEA001 confirmed the occurrence of all phytochemicals tested. In contrast, only terpenoids, saponins, steroids, and flavonoids were found in high concentrations in EFEA002. (Table S2). Despite the fact that both endophytic fungi shared the same host, their phytochemical profiles were substantially different.

3.4 Anti-bacterial activity of crude extracts

Resazurin assay was done to measure the minimum inhibitory concentration of the extracts. This assay is an easy, reliable and feasible assay used to differentiate dead and live bacteria and also efficiently used as a screening technique for demonstrating the MIC values [26]. In the present study, EFEA 001 showed concentration dependent growth inhibition against both SA and SE. Against bacteria SA, the

extract showed inhibition from concentrations 10mg/ml to 0.3mg/ ml and from 10mg/ml to 0.07mg/ ml against SE (Fig S1). EFEA 002 showed inhibition from 10 to 0.6 mg/ml against SA and from 10 to 0.3 mg/ml against SE. Ciproflaxacin, which used as positive control showed inhibition against the bacteria SA and SE against all tested concentrations (Data not shown). EFEA 001 showed higher inhibitory activity against both bacteria SA and SE when compared to EFEA 002. The findings are in line with studies that show, extracts from endophytic fungi possess excellent antimicrobial activities. Also, compounds isolated from these endophytic fungi have the ability to inhibit a wide variety of infectious pathogens [27].

3.5 Purification of bioactive compounds from EFEA fractions

The positive bioactivity results in this investigation imply that DAE fungal extract contains one or more bioactive phytocompounds, which is consistent with prior findings. Previous efforts to describe secondary metabolites from various sources using Sepadex LH and HPLC led in the finding of polar metabolites, polyketides, and cytochalasins, according to the literature [21-23-28-29]. Such phytochemicals have been shown to have potent immunomodulatory properties [23]. The focus of this research is on the mid-polar EA extract (partitioned using H2O/EA). The purification of EFEA 001 and EFEA 002 was achieved using column chromatography (Silica gel) with different proportions of EA and Hexane. The EFEA 001 fraction was purified by column chromatography into seven main fractions, five of which were identified as pure substances (1) – (5).

Also, purification of the EFEA 002 fraction resulted in three main compounds that were structurally identical to the EFEA 001 fraction's compounds (1), (3), and (4). Compounds (2) and (5) were either not detected in EFEA 002 or were present in amounts insufficient for isolation using conventional column separation. TLC analysis indicated a distinct band with Rf values of 0.9, 0.75, 0.7, and 0.5 for compounds (1) – (4), and 0.3 for compound (5), when using chloroform:acetic acid:methanol (9.4:0.3:0.3) as mobile phase. For the isolated compounds (1) – (5), spectral analysis, including 13C NMR, 1H, Mass, FT-IR, and UV-VIS, performed, and the results were used to derive the molecular structure (Fig S2 – S6). Furthermore, the acquired spectroscopic values compared to known literature reports on the compounds, confirming their structure (Figure 4a-e) [30-34].

Compound (1): 2-phenylbenzo chromen 4-one (αNapthoflavone). “Isolated as white amorphous powder.” R_f : 0.9; mp : 153 - 157˚C ^{1}H NMR ($CDCl_3$) δ7.69 (1H, d)6.97 (1H,s)7.58 (3H,t)7.72 (2H,dd)7.79 (1H,d)7.94 (1H,d)8.02 (2H,dd)8.18 (1H,d)8.61 (1H,dt) ^{13}C NMR ($CDCl_3$) 178.3 (C=O), 162.7, 153.6, 136.07, 132.02, 131.59, 129.29, 129.23, 128.29, 127.20, 126.29, 125.40, 124.18, 122.39, 120.80, 120.31, 129.31, 129.20, 108.88. “UV λmax (MeOH) nm (log ε) 270; FTIR (KBr, cm^{-1}) 1609. (C=O),”660, 1026, 1160, 1361; MS m/z: 272 (M^+). Compound (2): 4-Hydroxy-3, 5-dimethoxy benzaldehyde (Syringaldehyde): “Isolated as colourless crystals.” R_f: 0.75; mp: 110 - 113˚C; ^{1}H NMR ($CDCl_3$) δ 3.9 (6H, s) 7.15 (2H, d) 9.81 (1H,s) ^{13}C NMR ($CDCl_3$)190 (C=O), 56.2 (O-Me), 140, 147,106,128. UV λmax (MeOH) nm (log ε) 300; FTIR (KBr, cm^{-1}) 1670 (C=O), 1038, 1172, 1423, “1514; MS m/z: 182 (M^+)”

Compound (3): 3, 4, 5 trimethoxy benzoic acid (Gallic acid trimethyl ether): Isolated as colorless crystals. R_f : 0.7; mp : 168 - 172˚C; “^{1}H NMR ($CDCl_3$) δ 3.81 (6H,s) 3.93 (3H,s) 7.28 (2H,d) ^{13}C NMR ($CDCl_3$) 179 (C=O), 56.2(O-Me), 60.9 (OMe), 142.9, 152.9,107.4,124.1; UV λmax (MeOH) nm (lo ε) 260; FTIR (KBr cm^{-1}) 1686 (C=O),”2840, 2950, 1320,1519,1620,3022,3400; MS m/z: 201(M^+). Compound (4): Furan 2 carboxylic acid (2 Furoic acid): Isolated as white crystalline solid; R_f : 0.5; mp : 128 132˚C; ^{1}H NMR (D6-DMSO) δ 6.65 (1H,dd) 7.22 (1H,dd) 7.9 (1H, d)^{13}C NMR (D6-DMSO) 112.5, 118.2, 145.3,147.4,159.8,; UV λmax (MeOH) nm (log ε) 265; FTIR (KBr cm^{-1}) 1694,1715,3150; MS m/z: 112.08 (M^+). Compound (5): 2-(3,4-dihydroxyphenyl)-5,7,8trihydroxy-3-[(2S,5S,6R)-3,4,5-trihydroxy6(hydroxymethyl)oxan-2-yl]oxychromen-4-one (Gossypetin3′ O glycoside): “Isolated as yellow amorphous powder;” R_f : 0.3; mp : 229 230˚C;. ^{1}H NMR (D6-DMSO)” δ12.34 (1H,s)3.24 (1H, s)3.32 (3H, dd)3.42 (2H, t)4.66 (1H, d)4.84 (1H, d)5.08 (1H, d)5.32 (1H, d)6.26 (1H, s)6.91 (1H, d)7.81 (1H, dd)7.88 (1H, d)9.31 (1H, s)9.46 (1H, s) 9.65 (1H, s). ^{13}C NMR (D6-DMSO) 61.1, 69.81, 74.7, 76.6, 77.7, 99, 103.6, 106.9, 116.1, 116.3, 121.6, 122.7, 125.7, 136.4, 145.5, 147.9, 148.49, 149.02, 157.02, 157.07, 176.5. UV λmax (MeOH) nm (log ε) “380; FTIR (KBr cm^{-1}) 1597, 1657 (C=O), 3175 (-OH); MS m/z: 480 (M^+).

Compound (1) has been identified as 2-phenylbenzo chromen 4-one, (2) as 4-Hydroxy-3, 5-dimethoxy benzaldehyde, (3) as 3, 4, 5-trimethoxybenzoic acid, (4) as Furan 2 carboxylic acid, and (5) as gossypetin-3'-O-glycoside based on spectroscopic literature reports and data. There no known occurrences of such five compounds being isolated and characterized from either DAE or AbL, according to a careful examination of previous literature examples. As a result, this the first time that all five of these DAE compounds have identified. Compounds (1), (2), and (4) have been discovered in a variety of plant species as well as endophytic fungi [30-31-34]. Similarly, while (3) has been found in another *Daldinia* sp. [35], it has yet to be discovered in DAE. Compound (5) was reported from a single plant, *Talipariti elatum* S [34]. The EA extraction techniques redone with a new batch of fungal cultures and the existence of substances effectively confirmed. Furthermore, the PD medium tested separately to exclude out it as a source of the compounds. The discovery of DAE in plant AbL is major step forward in the development of alternative sources for the manufacture of such phytocompounds.

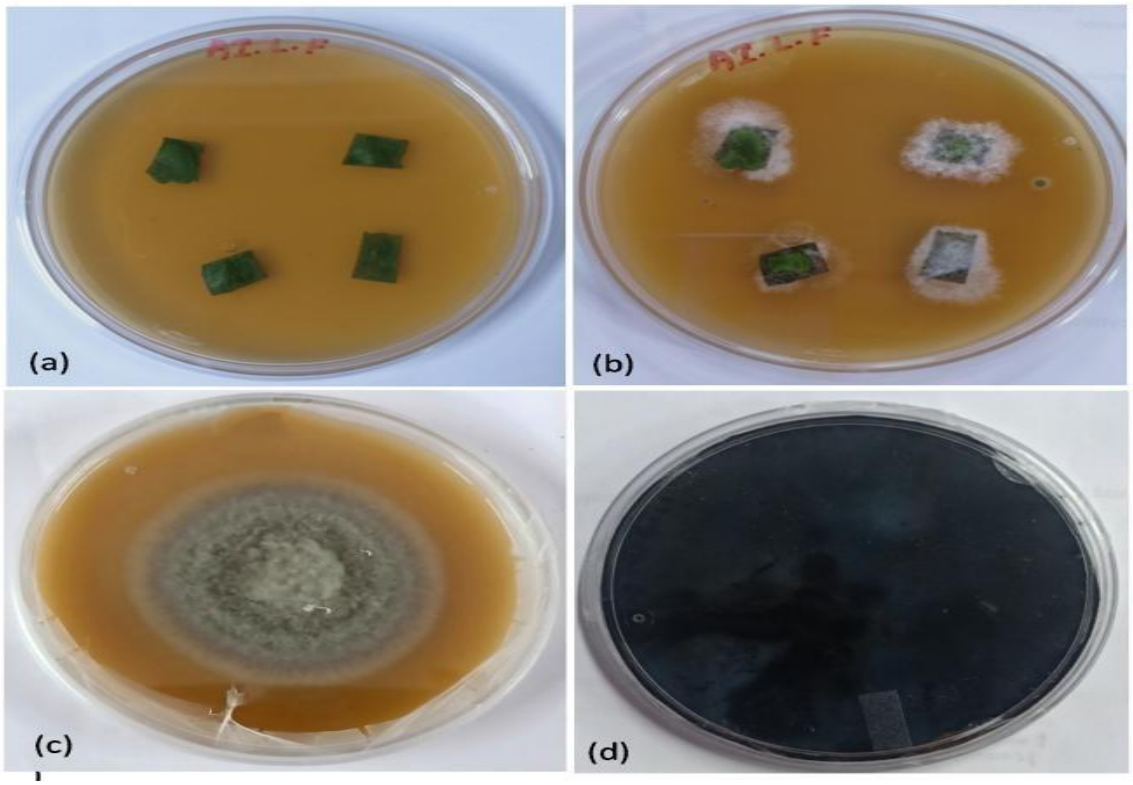

Figure 1A. PDA plates containing endophytic fungi DAE isolated from AbL Leaf samples. (a) Day 1- surface sterilized AI fresh leaves cut into 0.5x0.5 cm. (b) Day 3 – White cottony isolates grow on the surface of AbL leaves. (c) Day 12 – Sub cultured fungal isolate showing concentric circles – grey colored mass. (d) Black coloration on the reverse side of the completely grown DAE fungal culture.

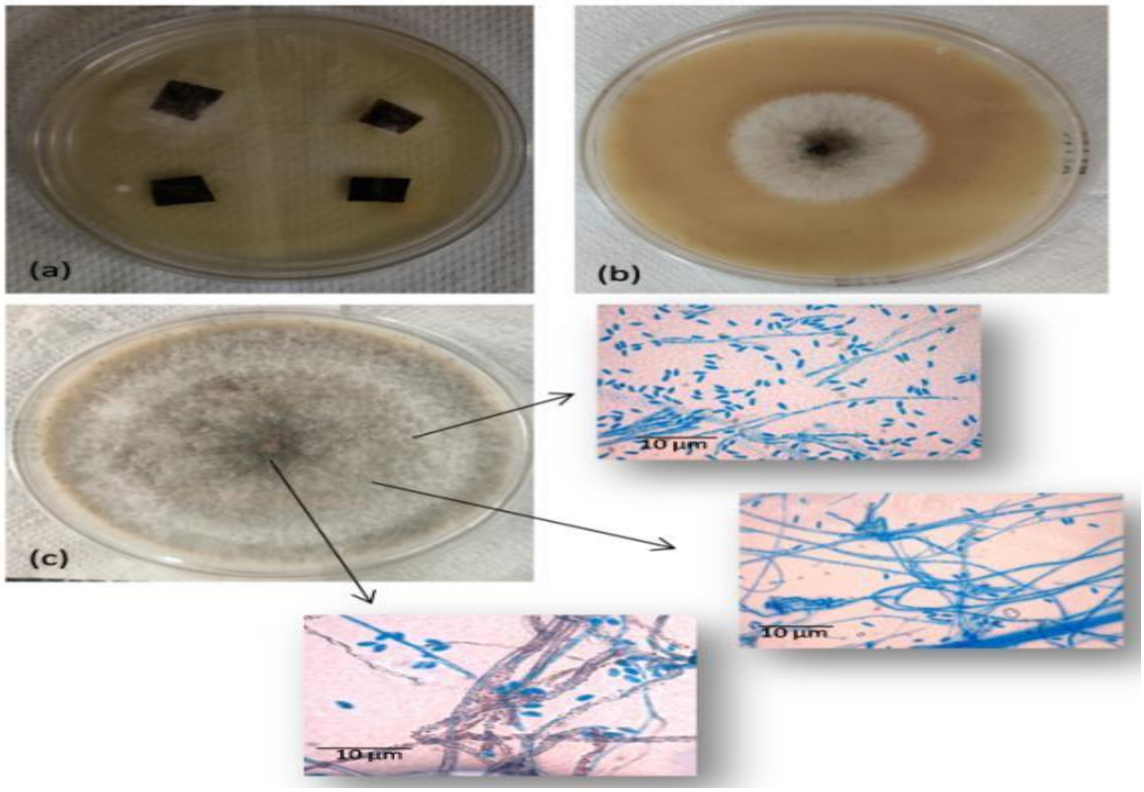

Figure 1B. Images of Oatmeal agar (OA) plates containing endophytic fungi DAE isolated from AbL Leaf samples (i) Day 3surface sterilized AI leaves with white cottony isolates grow on the surface (ii) Day 7 –Sub cultured fungal isolate showing concentric circles (iii) Fully grown DAE fungal culture and LCB stained culture showing conidia and coniophores with septate hyphae

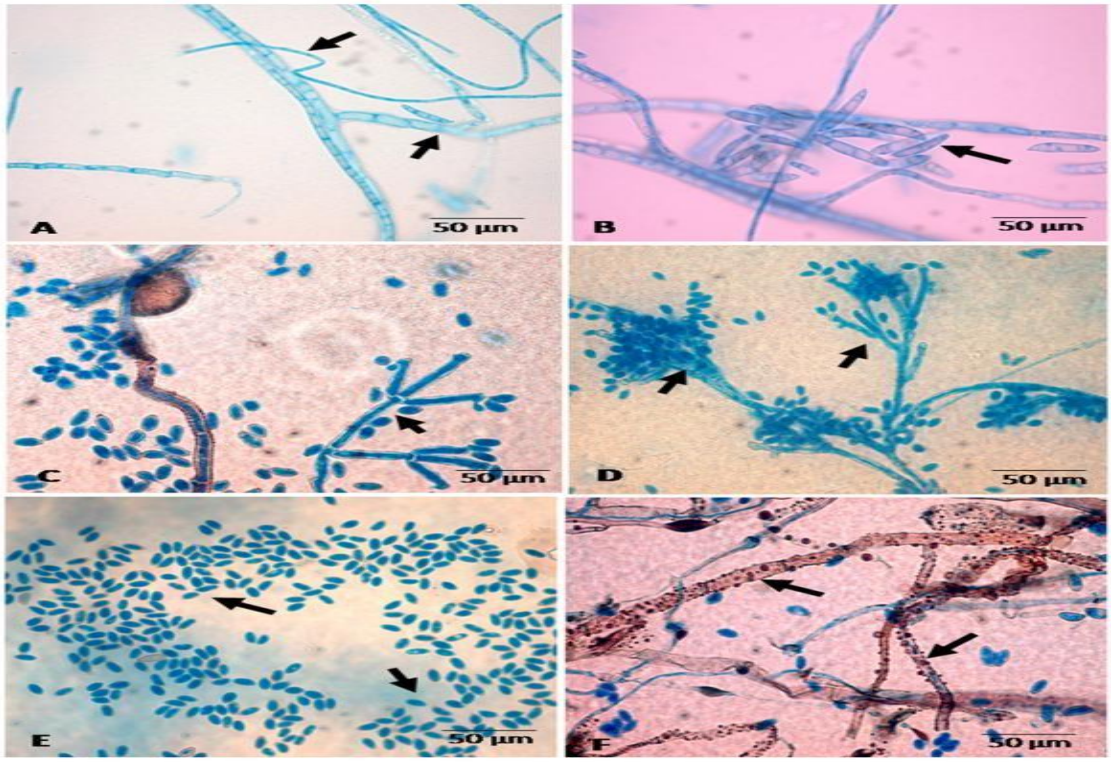

Figure 2. Bright field microscopic imaging and identification of DAE structures using Lactophenol cotton blue staining. (A) Thin and thick walled septate Hyphae. (B) Hyaline and cylindrical conidiogenous cells. (C) Intermittently divided conidiophores with conidiogenous cells originating from the each ends. (D)The septate conidiophores form dichotomous and trichotomous branches with clusters of conidia at the terminus. (E) Brownish black exudates on the surface of thick walled hyphae. (F) Hyaline conidia with ellipsoid and attenuated base - length 4.3 - 4.7 µm and diameter 2.0 – 2.25 µm. Magnification-100X (oil immersion) Scale bar represents 50µm

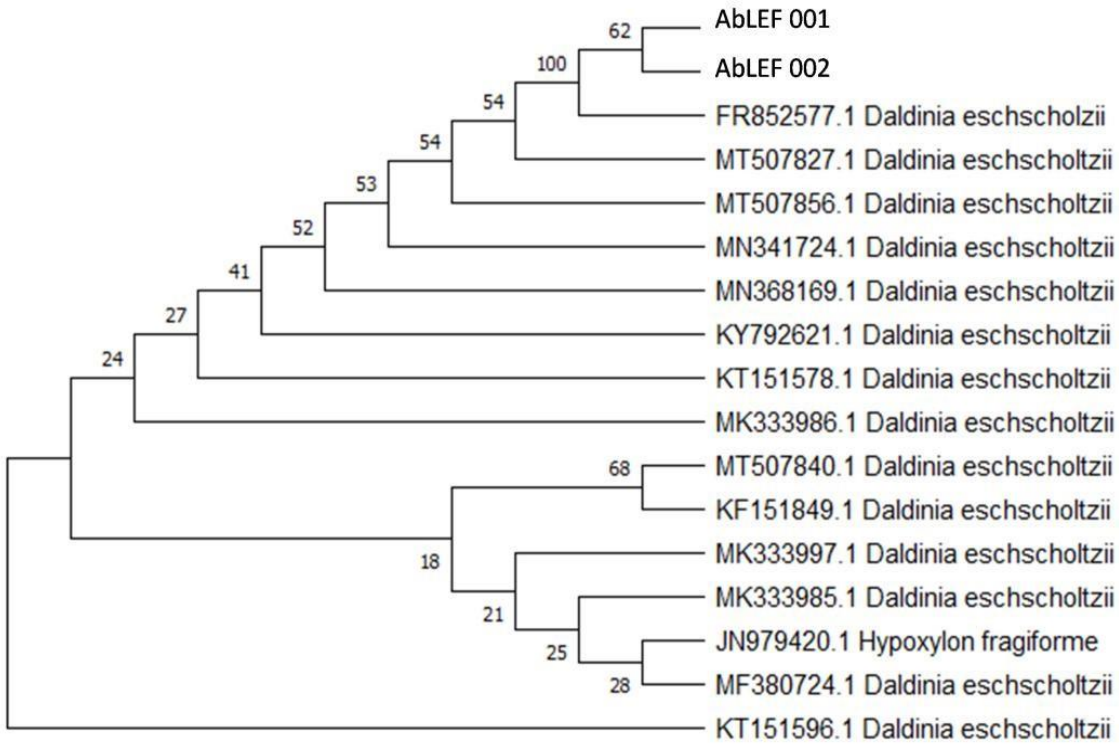

Figure 3. Phylogenetic consensus tree based on the Neighborhood joining (NJ) analysis inferred from the nearest neighbors of endophytic fungi, DAE isolated from AbL with maximum Bootstrap values indicated above each branch. Both AbLEF 001 and AbLEF 002 formed a cluster within the clade

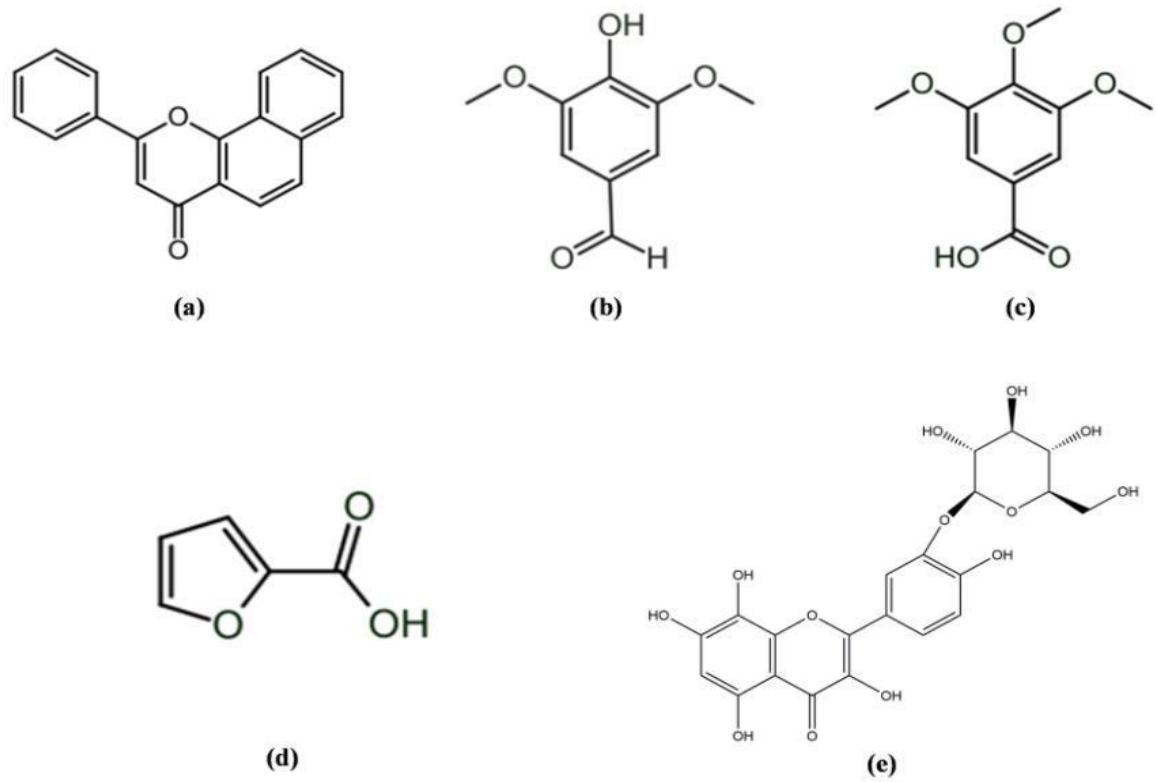

Figure 4. Structure of compounds: (a) 2-phenylbenzo chromen 4-one, (b) 4-Hydroxy-3, 5-dimethoxy benzaldehyde, (c) 3, 4, 5-trimethoxy benzoic acid, (d) Furan 2 carboxylic acid, (e) Gossypetin 3′O glycoside

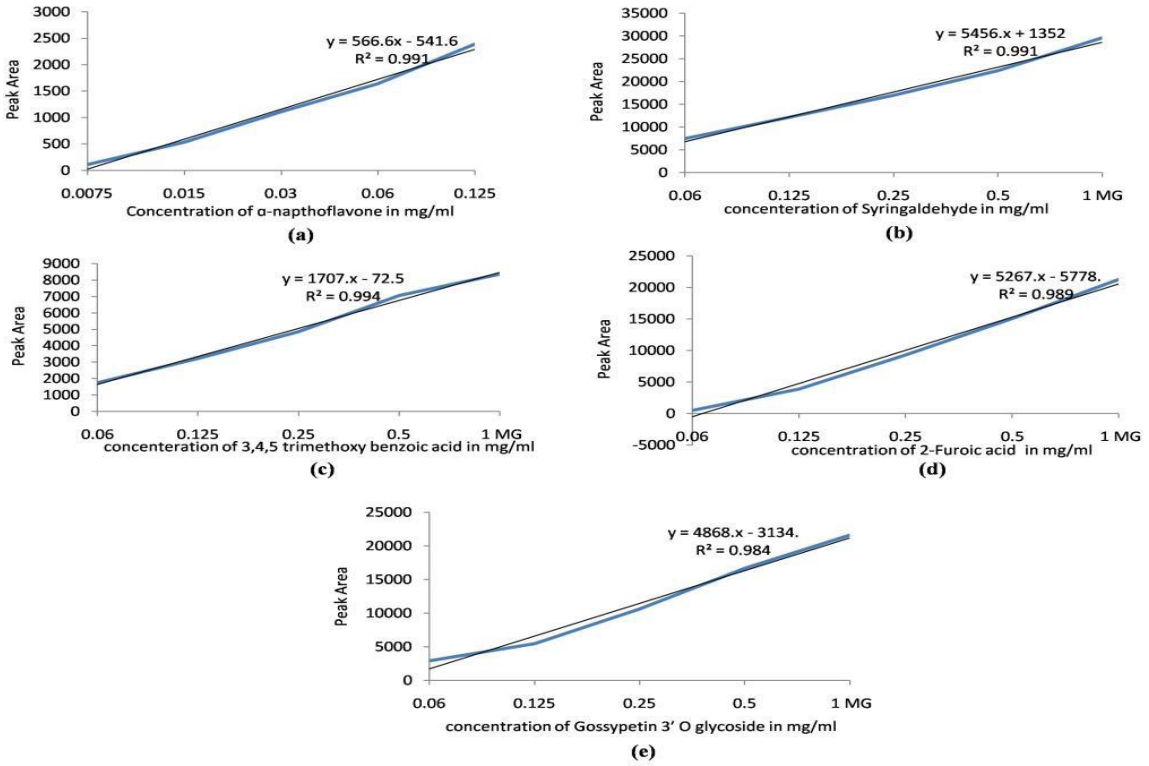

Figure 5. HPTLC Quantification -Calibration curve of compounds 1 – 5 in the EFEA fractions. (a) 2-phenylbenzo chromen 4-one (b)4-Hydroxy-3,5-dimethoxy benzaldehyde (c) 3,4,5 trimethoxy benzoic acid (d) Furan 2 carboxylic acid(e) Gossypetin 3′O glycoside

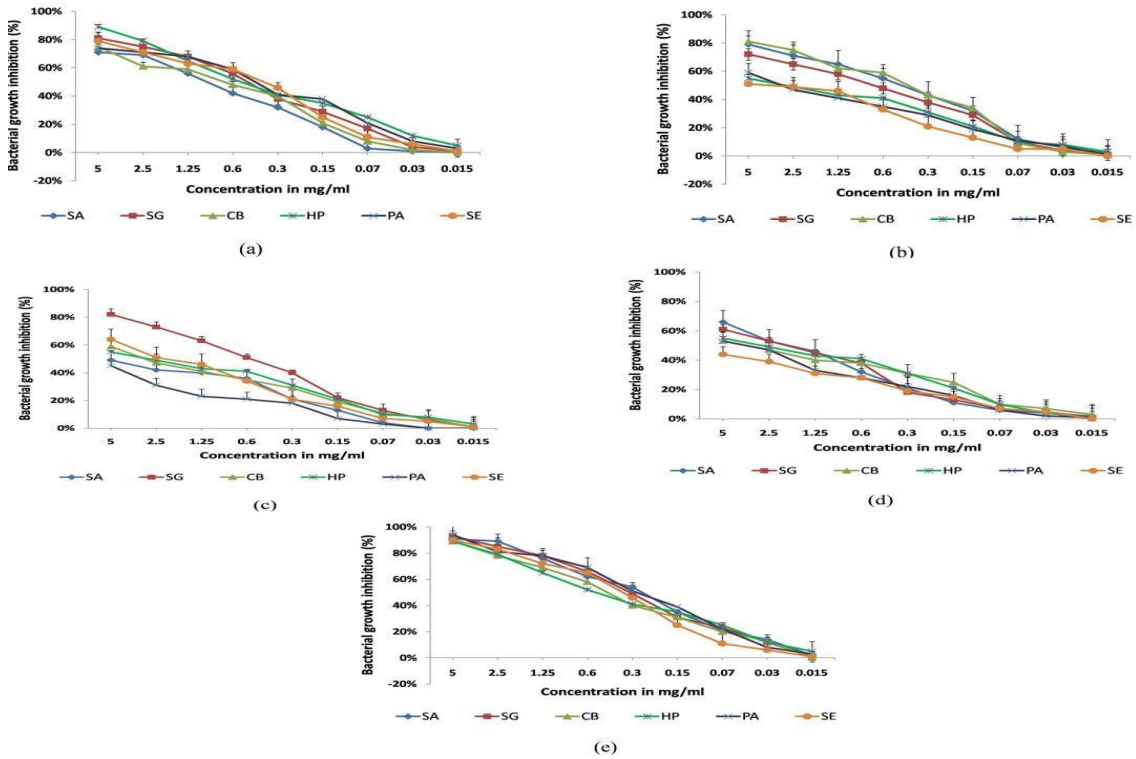

Figure 6. Anti-bacterial activity of (a) 2-phenylbenzo chromen 4-one. (b) 4-Hydroxy-3, 5-dimethoxy benzaldehyde. (c) 3, 4, 5 trimethoxy benzoic acid. (d) Furan 2 carboxylic acid. (e) Gossypetin 3′O glycosideby resazurin assay treated against SA, SG, CB (gram positive) and HP, PA, SE (gram-negative bacteria).

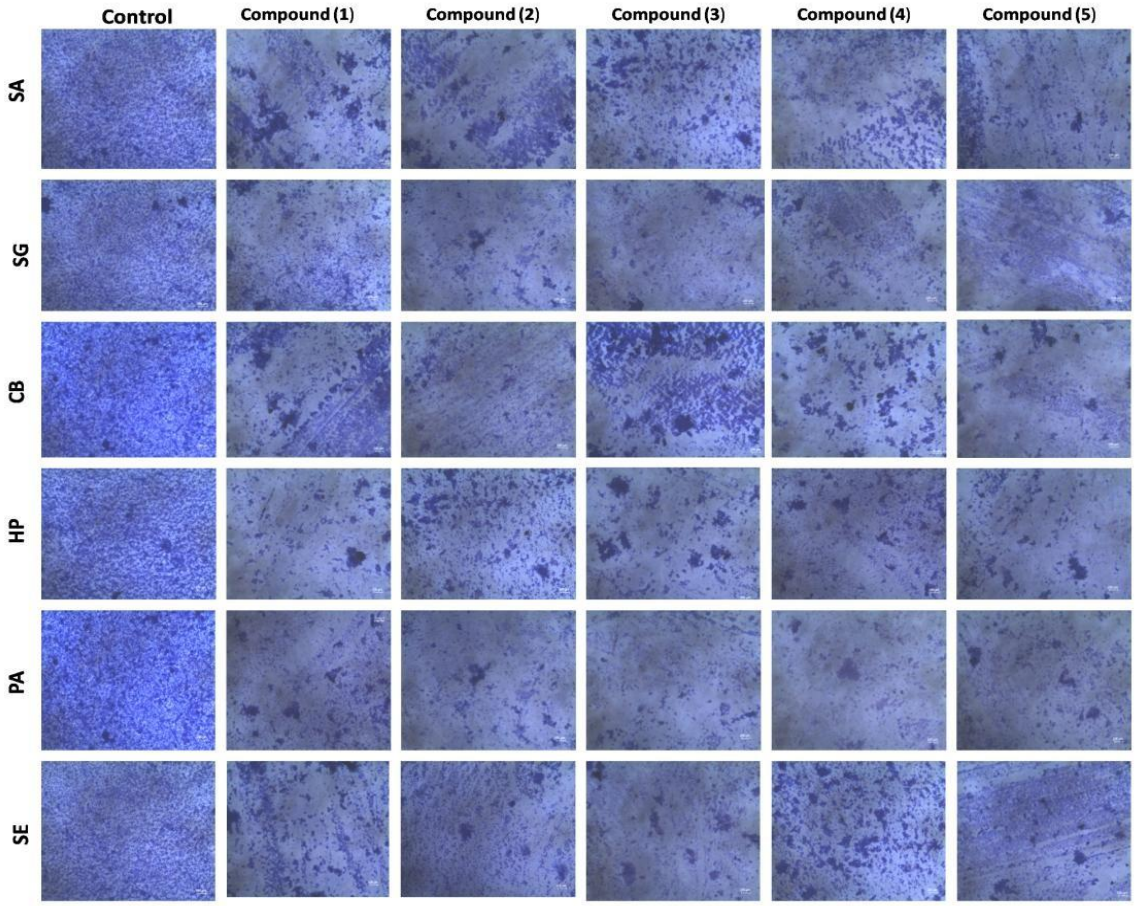

Figure 7. Anti-biofilm activity of compounds 1 – 5 against bacteria SA, SG, CB, HP, PA and SE by crystal violet staining.

Anti-microbial activity - MIC

S. No	Gram positive			Gram negative		
Compounds (mg/ml)	*Staphylococcus aureus*	*Streptomyces griseus*	*clostridium botulinum*	*Helicobacter pylori*	*Pseudomonas aeruginosa*	*Salmonella enterica*
(1)	0.15	0.07	0.15	0.03	0.07	0.15
(2)	0.07	0.15	0.15	0.15	0.07	0.3
(3)	0.15	0.07	0.15	0.15	0.3	0.3
(4)	0.15	0.15	0.15	0.15	0.15	0.15
(5)	0.03	0.03	0.03	0.03	0.07	0.07

3.6 Quantification of isolated compounds

Using HPTLC, we quantified the five compounds isolated from the endophytic fungus DAE. For verifying and quantifying phytoconstituents, HPTLC has proven to be a valuable and important approach [36]. The ease of usage and the flexibility of utilizing different solvent systems make it a suitable instrument for measuring numerous components concurrently in the EFEA. The linearity of every compound has been determined by plotting various concentrations (ranging from 7.5 to 1000 g/ml) against the peak area of the corresponding peak in the chromatogram. Individual compounds each had a distinct Rf value, which was as follows: 2-phenylbenzo chromen 4-one (0.93), 4-Hydroxy3, 5-dimethoxy benzaldehyde (0.70), 3, 4, 5 trimethoxy benzoic acid (0.68), Furan 2 carboxylic acid (0.39) and Gossypetin 3′ O glycoside (0.90) (Fig S7 & S8). The Rf values of the compounds were used to compare them to EFEA, while peak areas were used to calculate the amount of compounds in the extract (Table S3). The linearity plot used to generate correlation coefficients and regression equations for each individual compound, which were then used to calculate the quantity of compounds in EFEA (Fig 5). The compounds' (1) – (5) in crude fraction EFEA 001 have been determined to be 2phenylbenzo chromen 4-one – 3.29 mg; 4-Hydroxy-3,5dimethoxy benzaldehyde – 2.24 mg; 3,4,5 trimethoxy benzoic acid – 4.8 mg; Furan 2 carboxylic acid – 6.4 mg; and Gossypetin 3′ O glycoside – 4.6 mg of dried extract. These findings imply that, despite the fact that the compounds in EFEA 002 were identical to those in EFEA 001; the yield of the compounds in EFEA 002 was significantly lower than in EFEA 001.

3.7 Determination of MIC of isolated compounds

The antimicrobial activity of EFEA previously validated by resazurin assay results. This activity could shared by EFEA's five distinct compounds, which would be interesting to learn more about. The assay was performed using resazurin and the compounds (1) – (5) were tested against SA, SG, CB (gram positive) and HP, PA, SE (gram-negative) bacteria. The minimum inhibitory concentration values of compounds are listed in Table 1. All of the compounds demonstrated antibacterial action against gram negative and gram-positive microorganisms (Fig 6a-e). The maximum activity was observed in compound (5) which exhibited an MIC at lowest concentration of 0.03 mg/ml against the tested bacteria (Fig 6e). Literature reports the antibacterial activities of compounds of DAE from the medicinal plant *Dendrobium chrysotoxum* [37]. Chigozi et al [38] reported the antibacterial activity of DAE extracts against bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus subtilis".* Our results are in line with these previous reports confirming the compounds' antimicrobial activity isolated from DAE.

3.8 Anti-biofilm activity of isolated compounds

Antibiotic resistance can be reduced using an alternate therapeutic strategy called anti-biofilm activity. Biofilms considered as important virulence factor that causes

persistent chronic infections. As a result, bacteria can adhere to a surface and remain even in the existence of antimicrobial agents [39]. Studies report that species like *Staphylococcus; Pseudomonas; Salmonella* etc cause infections, which are not easy to treat because of their ability to form biofilms. Several natural compounds such as phenols, flavanoids, terpenes etc. have been tested for their efficacy to inhibit these biofilms [40]. In the present study, light microscopic analysis used qualitatively analyze the biofilms formed against gram negative and gram positive bacteria showed thick aggregated biofilm formation in the control samples. While the samples showed, disruption of biofilms when treated with MIC of compounds (Fig 7). These results strongly showed the compounds' ability for inhibiting the destruction or formation of biofilms by decreasing the adherence of microbes to the surface. Hence, these compounds isolated from DAE are of therapeutic importance especially as antimicrobial agent.

4. Conclusions

The current study validates the first report of endophytic fu *D.eschscholtzii* from AbL leaves. Furthermore, secondary metab such as 2-phenylbenzo chromen 4-one (1), 4-Hydroxy-3,5-dimet benzaldehyde (2), 3,4,5-trimethoxybenzoic acid (3), Fura carboxylic acid (4), and Gossypetin 3′ O glycoside (5) were iso and characterized using chromatographic and spectros investigations. This study is the first to demonstrate this compound from DAE. The isolation of Gossypetin 3' O glycoside from DA notable because, to our knowledge, only one study has revealed phytoconstituent from a plant, *Taliparitielatum S*. This study fu proved the antibacterial properties of the isolated compounds. Research also shows that drugs have the ability to suppress production of microbial biofilms. As a whole, the current conclusively reveals the therapeutic ability of the endophytic fu *D.eschscholtziii* in producing important phytochemicals, particu flavones that have the potential to use in innovative bioa applications.

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Conflicts Of Interest

The authors declare no conflict of interest.

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