

IDENTIFICATION AND BIOACTIVE POTENTIAL OF ENDOPHYTIC FUNGI FROM *GYNURA PROCUMBENS* (LOUR.) MERR.

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Abstract

Ten endophytic fungi were isolated from the leaves and stems of *Gynura procumbens* (Lour.) Merr. The identification of all the fungi was carried out up to the genus level based on colony morphology and microscopic characteristics. Seven of these fungi were further identified at the species level, namely: *Muyocopron laterale*, *Ectophoma multirostrata*, *Nemania abortiva*, *Diaporthe rosae*, *Colletotrichum aciculare*, *Fusarium perseae*, and *N. feicuiensis*, utilizing BLAST analysis of the amplified sequences of the internal transcribed spacer (ITS) region. This study marks the first report of these fungi as endophytes of *G. procumbens* in Bangladesh. This study also performed a biochemical assessment on ethyl acetate extracts from the isolated endophytic fungi, including antimicrobial, antioxidant, and thin-layer chromatography (TLC). Most of the crude extracts from the fungi exhibited one or more biological activities. TLC analysis revealed a variety of phytochemical components, such as flavonoids, terpenoids, anthraquinones, coumarins, isocoumarins, and steroids in all isolates. These results imply that the endophytic fungi associated with *G. procumbens* could serve as a valuable source for investigating potential bioactive compounds or as starting points for developing new drugs.

Introduction

Endophytes are essential for the development of their respective host (Strobel 2003). Recently, endophytic fungi have been regarded as an excellent source of bioactive natural products with unique characteristics. It has been predominantly documented that these substances harbor significant bioactive constituents, including coumarins, isocoumarins, alkaloids, anthraquinones, naphthoquinones, terpenoids, steroids, flavonoids, and lactones (Krohn *et al.* 2004, Khan *et al.* 2018). These compounds are crucial in biotechnology, pharmaceuticals, and agrochemical sectors. Endophytic fungi produce bioactive natural substances with promising potential for use in human health. *Taxomyces andreanae* has been reported to produce anti-cancer agent Paclitaxel (Stierle *et al.* 1993). The antidepressant agent hypericin is isolated from an endophytic fungus associated with *Hypericum perforatum* (Kusari *et al.* 2008).

Gynura procumbens is a medicinal plant belongs to Asteraceae native to Southeast Asia, China, and Africa. This plant contains various chemical constituents, including kaempferol, quercetin, terpenoids, tannins, alkaloids, saponins, and astragaloside, which exhibit excellent therapeutic effects (Abrika *et al.* 2013, Saeed *et al.* 2014). It has been shown to exhibit antihypertensive, antimicrobial, antioxidant, anticancer, antihyperglycemic, fertility-enhancing, hepatoprotective, anti-genotoxic, anti-plasmodial, cardioactive, and anti-inflammatory properties (Tan *et al.* 2016). Reports on the diversity of endophytic fungi in *G. procumbens* are very few (Jamal *et al.* 2022, Rahman *et al.* 2023).

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Although limited research has been conducted on fungal endophytes associated with *G. procumbens* in Bangladesh, the present study provides a detailed characterization and evaluation of the bioactive potential of endophytic fungi isolated from *G. procumbens* with an aim to discover novel therapeutic drugs.

Materials and Methods

Gynura procumbens samples were collected from the medicinal garden, BCSIR campus, Dhaka and it was identified in the Dhaka University Salar Khan Herbarium, Department of Botany, University of Dhaka, with the accession number DUSH:10821. Endophytic fungi were isolated from the stem and leaf following the procedure described by Chowdhury *et al.* (2016) with suitable modifications. In this investigation, 24 leaves and 24 stem segments of *G. procumbens* were inoculated on water agar media amended with streptomycin and incubated at 28 ± 2 °C. After 3-4 weeks, the emerging endophytic fungi were sub-cultured onto potato dextrose agar (PDA) medium for further isolation and growth. Isolated endophytes were identified morphologically based on standard taxonomic keys, like hyphae, growth pattern, colony morphology, coloration of media, margin character, surface texture, and spore characters, etc. Identification was confirmed based on relevant literature (Booth 1971, Ellis and Ellis 1976, Sutton 1980, Barnett and Hunter 2000, Chowdhury *et al.* 2016).

Molecular identification was done by comparing the sequences of the internal transcribed spacer (ITS) region (Adeniyi *et al.* 2018). For this purpose, a colony of 7-10 day old culture was used. This was followed by scraping with a sterile surgical blade and crushing with a pestle and mortar in liquid nitrogen. Then, DNA was isolated using the Maxwell® 16 LEV Plant DNA Kit (AS1420, Promega, USA) (Cappuccino and Sherman 1996). RNA contamination from the extracted DNA was eliminated using DNase-free RNase (30 min at 37 °C). PCR was conducted using the ITS1F and ITS4 primers for the ITS gene (White *et al.* 1990, Gardes and Bruns 1993). Sequencing was done using the Sanger dideoxy sequencing technique (White *et al.* 1990). After editing in BioEdit 7.2, the initial sequence was compared to those available in the National Center for Biotechnology Information's (NCBI) GenBank's rRNA/ITS database with the help of blast-search tools.

All the purified fungi were cultured on PDA medium. The culture medium was extracted with ethyl acetate after 21-28 days of incubation at 28 ± 2 °C. The culture media were filtered every seven days through a new cotton bed and then through Whatman No.1 filter paper. Evaporation in a rotary vacuum evaporator (Hei-VAP series, Heidolph Instruments) at 45-50°C and reduced pressure was used to concentrate the ten fungal solvent extracts into a solid residue (Chowdhury *et al.* 2017, Khan *et al.* 2018).

The antimicrobial abilities of the endophytic fungal crude extracts were evaluated by the disc diffusion method (Bauer *et al.* 1966) against four bacterial strains, i.e., *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 28739, *Bacillus megaterium* ATCC 28318, and *Salmonella typhi* ATCC 13311 and two fungi, i.e., *Aspergillus niger* and *A. flavus*. Kanamycin and Ketoconazole (30 µg/disc) were used as antibacterial and antifungal activity standards, respectively. The antimicrobial activity of the fungal extracts (100 µg/disc) was measured by measuring the inhibition zone around each treated disc following a 24 hrs incubation period at 37°C for bacteria and 48-96 hrs at 28°C for fungi and expressed it in millimeters \pm standard deviation.

A slightly modified Brand-Williams' DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging method was used to test the antioxidant activity of fungal extracts (Brand-Williams 1995). Ascorbic acid and butylated hydroxyanisole (BHA) were positive controls. Each fungal

crude extract (1.6 mg) was dissolved in MeOH (400 μ l) and serially diluted to obtain concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 μ g/ml. The extract solutions (2 ml) were mixed with 2 ml of DPPH solution in MeOH (20 μ g/ml), and the mixture was kept in the dark at room temperature for 30 min. The absorbance was taken at 517 nm using a UV-visible spectrophotometer. The following formula was used to determine the scavenging capacity.

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

Results were presented as an IC₅₀ (sample concentration that produced 50% scavenging of the DPPH radical)

Chemical constituents of endophytic fungal crude extracts were screened using TLC (Chowdhury *et al.* 2016). The initial chemical screening of the crude extracts was carried out using precoated silica gel DC Kieselgel 60 PF₂₅₄ 0.2 mm aluminium foil (Macherey-Nagel, Germany) with a mobile phase of 10% ethyl acetate in toluene. TLC plate was detected using a UV viewing cabinet (UVGL-58 handheld UV lamp, USA) at 254 and 365 nm, and 1% vanillin-sulphuric acid was then sprayed over and heated for 5 min at 110°C. (Chowdhury *et al.* 2016).

All the bioactivity tests were repeated three times, and data were documented in triplicate. The IC₅₀ value were calculated using logistic regression. The values are shown as mean \pm standard deviation. Calculations and graphs were prepared using Microsoft Excel software.

Results and Discussion

During this study, three fungi (GPLE 1- 3) from the leaves and seven fungi (GPSE 1-7) from the stem were isolated. All the ten fungi were found to show distinct morphological characteristics, and the features described below were used to identify the endophytic fungal isolates before molecular confirmation.

The isolate GPLE 1 produced branched, septate, and hyaline mycelia. The colony's surface is pale gray-brown, with irregular margins; the reverse view of the colony is brown in the centre and white to the periphery. Light brown to dark brown appressoria present (Fig. 1A-C). Based on these morphological features, it was identified as *Muyocopron* sp.

The culture morphology of the isolate GPLE 2 on PDA was initially white and then became olivaceous brown, with an irregular margin. Usually globose, single, dark brown or black pycnidia are distributed over the agar surface. Alpha conidia 5.5 - 6.8 \times 1.8 - 3.0 μ m enteroblastic, ovate to ellipsoidal, single-celled, smooth, and hyaline. Beta conidia not observed (Fig. 1D-F). These morphological observations were similar to those of *Diaporthe* sp. and was identified as *Diaporthe* sp.

Colonies of the isolate GPLE 3 were grey-green on the front, dark grey on the back, margin entire, cottony, and some grayish-black spots scattered over the colony surface. Colony diameter 48-50 mm in 5 days. Mycelia were profusely branched, septate, and hyaline. Conidia were cylindrical, single-celled, smooth, had rounded ends, and were hyaline (Fig. 1G-I). These morphological characteristics indicated that the fungus could be *Colletotrichum* sp.

The fungal colonies of the isolate GPSE 1 were dark-olivaceous, covered with grey mycelial growth, while the reverse of the colony was dark-black. Pycnidia were black, solitary or confluent, embedded in the culture media. The spores were ellipsoid, single-celled, hyaline, and abundant (Figs 1J-L). These morphological findings were similar to *Ectophoma* sp.

The isolate GPSE-2 grows slowly on the PDA with a diameter of 4 cm after 12 days at 28°C. The colony on the surface was white, thick, and flat in the middle, with shallow edges, irregular bands, and rosettes. Colony reverse was off-white. Not sporulating on PDA (Fig. 1M-O). These morphological features were similar to those of *Nemania* sp.

The isolate GPSE 3 initially appeared white; it gradually turns brownish when cultured on PDA. Colony margin irregular, black, globose pycnidia distributed over the agar surface. Two types of conidia were present. Alpha conidia were hyaline, smooth, one-celled, ovate to ellipsoidal, and straight. Beta conidia were $20\text{-}26.5 \times 0.9\text{-}1.4 \mu\text{m}$, fusiform, aseptate, and hyaline (Fig. 1P-R). These morphological findings were similar to those of *Diaporthe* sp.

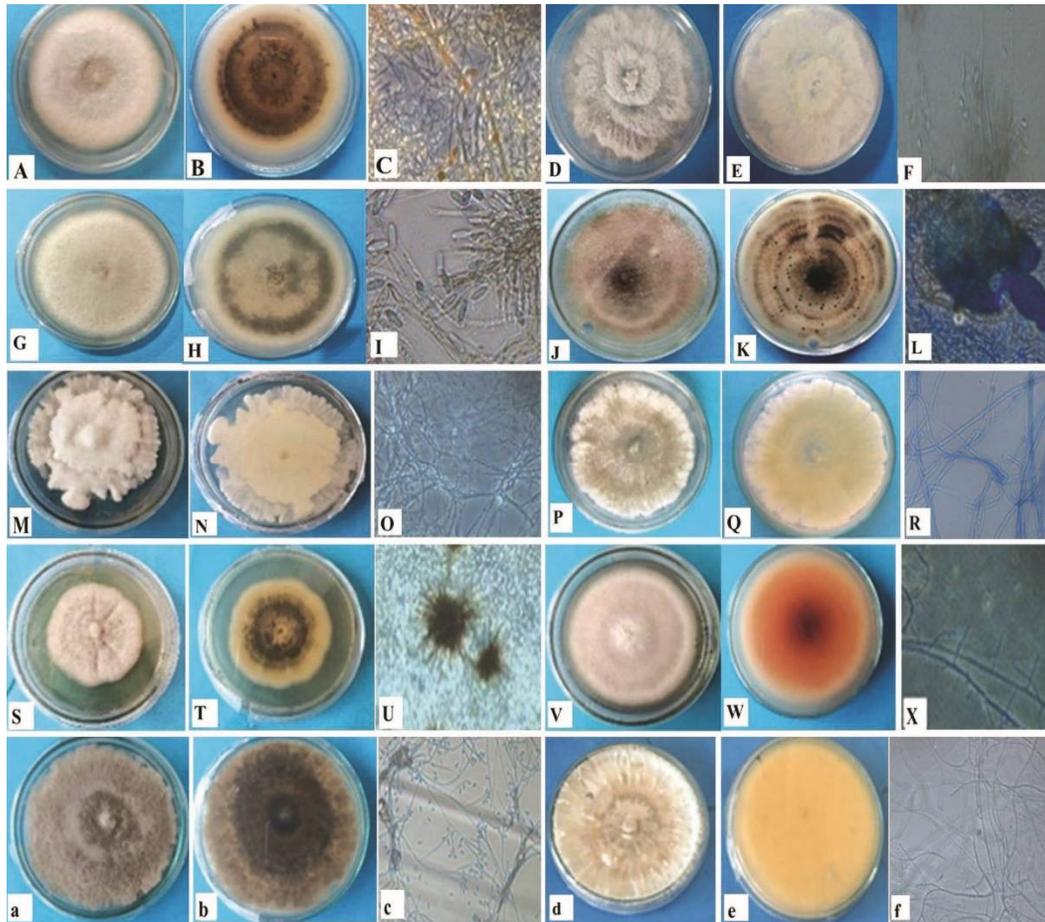


Fig. 1. Colony morphology (Top and bottom views) on PDA medium and microscopic view of isolated fungi. A-C: *Muyocopron laterale*, D-F: *Diaporthe* sp., G-I: *Colletotrichum* sp., J-L: *Ectophoma multistrostrata*, M-O: *Nemania abortiva*, P-R: *Diaporthe rosae*, S-U: *Colletotrichum aciculare*, V-X: *Fusarium perseae*, a-c: *Daldinia* sp., d-f: *Nemania feicuiensis*

The upper surface of the GPSE 4 fungus colony was pale grey to dark grey, the reverse was dark brown, and small black granules covered the entire surface. The conidia were single-celled, smooth, and falcate, gradually tapering towards each end. Brown colour setae were present (Figs. 1S-U). So the fungal isolate was morphologically identified as *Colletotrichum* sp.

The upper surface of the fungal colony of GPSE 5 was pinkish white, and the lower surface of the colony was brownish pink. Mycelia septated, hyaline, branched. Two kinds of hyaline conidia

were observed. Microconidia were one-celled, oblong, and macroconidia were 3 to 4-celled, slightly curved or bent at the pointed end (Fig. 1V-X). These morphological characteristics indicated that the fungus was *Fusarium* sp.

A fast-growing grey felty colony and black-gray coloration on the reverse side of the isolate GPSE 6 was observed. Septate hyphae, dichotomously branched conidiophores, and ovoid to ellipsoid single-celled conidia were observed under the microscope (Fig. 1a-c). These morphological findings were similar to those of *Daldinia* sp.

The slow growing mycelial mat on PDA was initially white but later turned brown to ash, and was noticed in isolate GPSE 7. Mycelia were thin-walled, septate, branched, and hyaline. Candle-shaped white to greyish stromata present on PDA (Fig. 1d-f). These morphological findings were similar to *Nemania* sp. Rahman *et al.* (2023) isolated three endophytic fungi from *G. procumbens*; however, they were not identified morphologically or molecularly, and only the antimicrobial assay was conducted.

ITS sequence-based molecular analysis was also conducted to identify the isolated fungi. Seven endophytic fungi isolated from the *G. procumbens* were identified at their species level through molecular identification (Table 1). Isolate GPLE 1 showed 99.21 % sequence similarity and 100% query coverage with *Muyocopron laterale* (NR_164055.1), clustering closely with this species in the phylogenetic tree. Isolate GPSE 1 exhibited 92.32 % similarity and 97 % query coverage with *Ectophoma multirostrata* (NR_158226.1), also clustering nearby in the phylogenetic analysis. Similarly, GPSE 2 showed 91.02 % similarity and 97 % query coverage with *Nemania abortiva* (NR_121350.1), while GPSE 3 had 99.65 % similarity and 96 % query coverage with *Diaporthe rosae* (NR_172401.1), both forming close clusters with their respective reference strains. Isolate GPSE 4 exhibited 99.81 % similarity and 86 % query coverage with *Colletotrichum aciculare* (NR_138009.1), and GPSE 5 showed 97.09 % similarity and 88 % query coverage with *Fusarium perseae* (NR_164415.1), both of which were supported by phylogenetic clustering. Lastly, GPSE 7, clustering closely in the phylogenetic tree, demonstrated 93.16 % similarity and 86 % query coverage with *Nemania feicuiensis* (NR_177560.1). These results collectively confirm the taxonomic placement of the isolates within their respective genera and species.

Table 1. Morphological and molecular identification of endophytic fungi from *Gynura procumbens*.

Fungal isolate	Morphological identification	Molecular identification			
		Identified fungi	Accession No.	Coverage (%)	Max identity (%)
GPLE 1	<i>Muyocopron</i> sp.	<i>Muyocopron laterale</i>	PP159174	100	99.21
GPSE 1	<i>Ectophoma</i> sp.	<i>Ectophoma multirostrata</i>	PP440235	97	92.32
GPSE 2	<i>Nemania</i> sp.	<i>Nemania abortiva</i>	PP440227	97	91.02
GPSE 3	<i>Diaporthe</i> sp.	<i>Diaporthe rosae</i>	PP438317	96	99.65
GPSE 4	<i>Colletotrichum</i> sp.	<i>Colletotrichum aciculare</i>	PP437249	86	99.81
GPSE 5	<i>Fusarium</i> sp.	<i>Fusarium perseae</i>	PP349788	88	97.09
GPSE 7	<i>Nemania</i> sp.	<i>Nemania feicuiensis</i>	PV628739	86	93.16

Antimicrobial activity of the isolated endophytic fungi was tested by the disc diffusion method. The crude extract of all the endophytic fungi showed mild to significant activity against most of the test bacteria (Table 2). The zone of inhibition produced by the fungal extracts was $7 \pm 0.09 - 27 \pm 0.26$ mm at a concentration of $100 \mu\text{g}/\text{disc}$. Extract of *Fusarium perseae* exhibited promising activity against *Escherichia coli* (19.9 ± 0.21 mm), *Staphylococcus aureus* (18.0 ± 0.12 mm), *Salmonella typhi* (27 ± 0.26 mm), and *Bacillus megaterium* (12.2 ± 0.18). These results suggest that most of the fungal extracts may contain antibacterial metabolites. Extracts of *Diaporthe rosae* showed prominent activities against *Aspergillus niger* (17 ± 0.26 mm) and *A. flavus* (24.1 ± 0.19 mm). The crude extracts of other endophytic fungi did not exhibit considerable antifungal activity (Table 2). This means the test fungi *Aspergillus niger* and *A. flavus* were found to be resistant to them. *Diaporthe rosae* can produce secondary metabolites with potentially high antifungal activity.

Table 2. Antimicrobial activity of isolated fungal extract against selected bacteria and fungi.

Endophytic fungi	Diameter of inhibition zone (mm)						
	Bacteria				Fungi		
	<i>E. coli</i>	<i>S. typhi</i>	<i>B. megaterium</i>	<i>S. aureus</i>	<i>A. niger</i>	<i>A. flavus</i>	
<i>Muyocopron laterale</i>	7.0 ± 0.09	-	8.1 ± 0.06	-	-	-	
<i>Diaporthe</i> sp.	7.2 ± 0.15	-	-	-	-	-	
<i>Colletotrichum</i> sp.	7.2 ± 0.18	-	7.9 ± 0.26	6.1 ± 0.05	-	-	
<i>Ectophoma multirostrata</i>	8.9 ± 0.26	9.0 ± 0.09	9.0 ± 0.15	7.1 ± 0.09	-	-	
<i>Nemania abortiva</i>	11.1 ± 0.21	11.0 ± 0.12	11.4 ± 0.15	7.1 ± 0.19	-	-	
<i>Diaporthe rosae</i>	7.2 ± 0.15	-	10.1 ± 0.23	10.6 ± 0.06	17 ± 0.26	24.1 ± 0.19	
<i>Colletotrichum aciculare</i>	8.1 ± 0.18	9.2 ± 0.12	7.0 ± 0.09	9.4 ± 0.6	-	-	
<i>Fusarium perseae</i>	19.9 ± 0.21	27.0 ± 0.26	12.2 ± 0.18	18.0 ± 0.12	-	-	
<i>Daldinia</i> sp.	10.0 ± 0.15	7.0 ± 0.09	8.1 ± 0.06	-	-	-	
<i>Nemania feicuiensis</i>	-	7.9 ± 0.09	6.1 ± 0.06	7.0 ± 0.15	-	-	
Positive control	Kanamycin	37.1 ± 0.21	46.1 ± 0.32	37.4 ± 0.26	32.1 ± 0.06	ND	ND
	Ketoconazole	ND	ND	ND	ND	30.2 ± 0.19	30.1 ± 0.18

‘-’ indicates no activity, and ND indicates not done.

The IC_{50} values of fungal extracts and reference standards are presented in Fig. 2. Among the endophytic fungi, *Nemania abortiva*, *Muyocopron laterale*, and *Daldinia* sp. exhibited potent antioxidant activity, with IC_{50} values of 9.83, 30, and 36.12 $\mu\text{g}/\text{mL}$, respectively. *Diaporthe* sp. showed moderate antioxidant activity ($\text{IC}_{50} = 72.28 \mu\text{g}/\text{ml}$), while *Nemania feicuiensis* displayed weak activity with an IC_{50} of 124.58 $\mu\text{g}/\text{ml}$. In comparison, the standard antioxidants showed significantly lower IC_{50} values: ascorbic acid (1.55 $\mu\text{g}/\text{ml}$) and BHA (2.47 $\mu\text{g}/\text{ml}$). The scavenging activity of the other extracts was negligible ($\text{IC}_{50} > 250 \mu\text{g}/\text{ml}$). It is noteworthy that *Muyocopron laterale*, *Nemania abortiva*, and *Daldinia* sp. represent promising sources of antioxidant compounds and may serve as valuable resources for future research. However, further studies are required to isolate more bioactive compounds responsible for the activities of these endophytic fungi. Antibacterial and antioxidant activities have been reported in several endophytic fungal species, including *Fusarium* spp., *Phomopsis liquidambaris*, *Colletotrichum* spp., *Aspergillus* spp.

Curvularia spp., *Penicillium* spp., *Pestalotiopsis* spp., and *Diaporthe perseae*. These fungi are associated with a variety of host plants, such as *Thysanolaena maxima* Roxb., *Commelina diffusa*, *Dracaena spicata* Roxb., *Dillenia indica* L., and *Aglaonema hookerianum* Schott (Chowdhury *et al.* 2016, Mahmud *et al.* 2020, Nasrin *et al.* 2021, Hoque *et al.* 2023, Zinnurine *et al.* 2024).

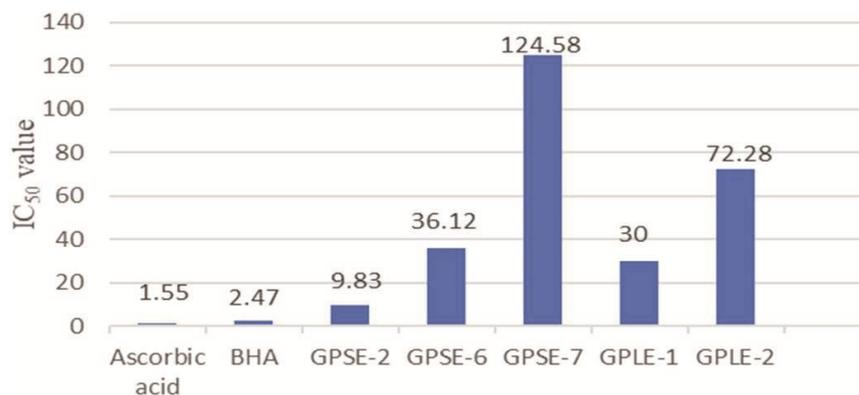


Fig. 2. IC₅₀ value of standard and crude extracts of isolated endophytic fungi. BHA: Butylated hydroxyanisole, GPSE 2: *Nemania abortiva*, GPSE 6: *Daldinia* sp., GPSE 7: *Nemania feicuiensis*, GPLE 1: *Muyocopron laterale* and GPLE 2: *Diaporthe* sp.

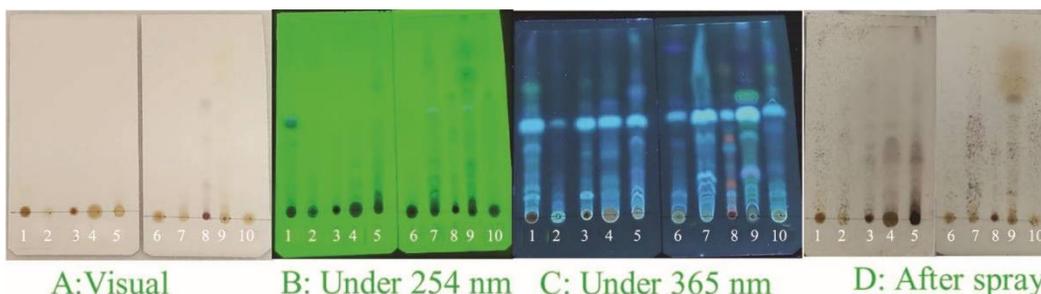


Fig. 3. TLC screening of the fungal extracts by visual observation, under UV at 254 nm, 365 nm, and after spray. 1: *Muyocopron laterale*, 2: *Diaporthe* sp., 3: *Colletotrichum* sp., 4: *Ectophoma multirostrata*, 5: *Nemania abortiva*, 6: *Diaporthe rosae*, 7: *Colletotrichum aciculare*, 8: *Fusarium perseae*, 9: *Daldinia* sp., and 10: *Nemania feicuiensis*.

Thin-layer chromatography was employed to determine the presence of secondary metabolites. Visual detection, UV light (at 254 and 365 nm), and the vanillin-H₂SO₄ spray reagent were used to screen all extracts (Fig. 3 and Table 3). TLC spot analysis of the extracts revealed several secondary metabolites, including terpenoids, flavonoids, steroids, quinones, coumarins, isocoumarins, and anthraquinones. This finding is consistent with previous reports of diverse bioactive compounds in extracts of endophytic fungi (Krohn *et al.* 2004, Khan *et al.* 2018, Mahmud *et al.* 2020).

In this study, ten prominent fungi were identified using both morphological and molecular methods from the leaf and stem of *Gynura procumbens*. Antioxidant and antimicrobial assays, as well as chemical screening, were conducted, yielding promising results that highlight their potential for next-generation drug research and development.

Table 3. Chemical screening of fungal extracts by thin-layer chromatography.

Endophytic fungi	Visual observation	Visibility under UV light		Visibility after spray	Compounds
		254 nm	365 nm		
<i>Muyocopron laterale</i>	Yellow	Blue & dark quenching,	Blue, yellow	Light purple	Flavonoids, coumarins, anthraquinones, terpenoids, isocoumarins.
<i>Diaporthe</i> sp.	-	Light quenching	Blue	Light purple	Steroids, isocoumarins, coumarins, or their derivatives.
<i>Colletotrichum</i> sp.	-	light quenching	Blue, sky blue	Light purple	Coumarins, isocoumarins, steroids, terpenoids.
<i>Ectophoma multirostrata</i>	-	Dark quenching	Blue, sky blue	Dark, brownish purple	Terpenoids, steroids, coumarins.
<i>Nemania abortiva</i>	Yellow	Dark quenching	Blue, sky blue	Dark purple	Coumarins, flavonoids, terpenoids, steroids, anthocyanins.
<i>Diaporthe rosae</i>	-	Light blue, blue quenching	Blue, sky blue	Light purple	Coumarins, anthocyanins, terpenoids, steroids.
<i>Colletotrichum aciculare</i>	Yellow	Dark quenching	Blue, sky blue, light green	Light purple	Terpenoids, steroids, coumarins, isocoumarins, anthocyanins.
<i>Fusarium perseae</i>	Light purple, redish	Dark quenching	Blue, sky blue, red	Light purple	Coumarins, anthocyanins, terpenoids, quinone, steroids.
<i>Daldinia</i> sp.	Yellow	Light blue, dark quenching	Blue, sky blue, light green	Brownish purple	Terpenoids, steroids, coumarins, isocoumarins, flavonoids.
<i>Nemania feicuiensis</i>	Light yellow	Dark quenching	Blue, sky blue, light green	Light purple	Steroids, flavonoids, anthocyanins, terpenoids, coumarins, isocoumarins.

'-' indicates absence of a distinguishable character

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