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Diversity, bioactivity, and phytochemistry of endophytic fungi in various organs of nipa palm (*Nypa fruticans*) mangrove

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Abstract. *Nasution SSA, Elfita, Widjajanti H, Ferlinahayati. 2024. Diversity, bioactivity, and phytochemistry of endophytic fungi in various organs of nipa palm (*Nypa fruticans*) mangrove. Biodiversitas 25: 3928-3942.* Nipa palm (*Nypa fruticans* Wurmb), a traditional medicinal mangrove species, grows along the coastal areas of South Sumatra. This study explores the diversity of endophytic fungi isolated from the stem, frond, and mesocarp of *N. fruticans*. A total of eighteen fungal isolates were identified and tested for antioxidant and antibacterial activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the disk diffusion method. Phytochemical profiles of these isolates were analyzed through thin layer chromatography (TLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Among the isolates, *Aspergillus niger* (NfBa5, NfP7, and NfBu4), present in all organs, showed consistent antioxidant and antibacterial activities with similar major compounds across these isolates. Other endophytic fungi demonstrated significantly higher antioxidant and antibacterial activities compared to the host plant, highlighting their potential as promising sources of bioactive compounds. Despite some shared compound patterns, the fungal profiles differed from those of *N. fruticans*, underscoring the unique bioactive potential of endophytes. This study emphasizes the diversity of endophytic fungi in *N. fruticans* and reveals significant differences in their biological activities and phytochemical profiles compared to the host. These findings provide foundational insights for developing medicinal compounds from *N. fruticans* endophytic fungi for future pharmaceutical applications.

Keywords: Antibacterial, antioxidant, *Aspergillus niger*, biodiversity, sustainability

INTRODUCTION

Indonesia, recognized as a megadiverse country, hosts a vast array of ecosystems that contribute significantly to its natural wealth and cultural heritage. With approximately 30,000 plant species out of the 40,000 species recorded worldwide, Indonesia ranks among the richest in biodiversity (Rahardjanto et al. 2021; Zeng et al. 2022). This biodiversity is not only vital for the environmental balance but also foundational to the development of traditional practices that have sustained local communities for generations. In coastal regions especially, biodiversity plays a vital role in the daily lives of local communities, providing resources such as food, clothing, shelter, and medicinal plants, including mangroves (Genilar et al. 2021; Tresnati et al. 2021).

Mangroves are integral to Indonesia's coastal ecosystems. Among the 202 identified species are climbers, herbs, and epiphytes (Kusmana and Sukristijiono 2016; Khairunnisa et al. 2020; McElwain et al. 2024). These plants not only protect shorelines from erosion and storms but also offer critical resources for various traditional uses. Among the most notable species is *N. fruticans*, a mangrove palm widely found along the coasts of Sumatra, which has been utilized by local communities for generations (Das et al. 2021; Herningtyas et al. 2023; Muhtadi et al. 2023; van Hespen et al. 2023). This species plays a significant role in the livelihood of coastal populations, as it provides materials for food, clothing, shelter, and traditional medicine.

Traditional communities in coastal Sumatra have long utilized *N. fruticans* for its medicinal properties. Through centuries of practice, they have used this mangrove species to treat a range of ailments, including asthma, gout, fever, diabetes, leprosy, and rheumatism. Recent studies confirm the bioactive properties of *N. fruticans*, which include antioxidant, antibacterial, antidiabetic, and antipyretic activities, attributed to compounds like polyphenols, phenolics, flavonoids, alkaloids, tannins, diterpenes, and steroids (Rahayu and Sunarto 2020; Nugroho et al. 2022; Fitri et al. 2023). This species is therefore a promising candidate for further research and development in the pharmaceutical sector.

However, with increasing interest in medicinal plants, the overexploitation of mangroves raises sustainability concerns. Mangroves serve multiple purposes for coastal communities, and overharvesting them for medicinal compounds could threaten their availability for other critical uses, such as food, construction materials, and ecosystem services. In light of these challenges, it is crucial to find sustainable alternatives to meet the medicinal demand without depleting the mangrove population. One such promising alternative is the exploration of bioactive

compounds from endophytic fungi. This study explores the bioactive potential of endophytic fungi from *N. fruticans* as a sustainable source of medicinal compounds.

Endophytic fungi, which reside within plant tissues, are recognized for producing diverse bioactive compounds-antioxidants, antimicrobials, and anti-inflammatory agents that often mirror or enhance their host plants' medicinal properties (Akram et al. 2023; Choudhary et al. 2023; Jha et al. 2023; Singh and Kumar 2023). Host specificity significantly influences the bioactive profiles of these fungi, making them ideal for sustainable pharmaceutical development (dos Reis et al. 2022; Wen et al. 2023; Wijesekara and Xu 2023; Rai et al. 2024). Cultivating endophytic fungi offers efficiency and sustainability benefits, as they grow rapidly and require minimal plant biomass, thus enabling large-scale production of medicinal compounds without depleting natural plant populations (Hashem et al. 2023; Shen et al. 2024). While mangroves like *N. fruticans* hold significant potential for the pharmaceutical industry, sustainable approaches are necessary to prevent the depletion of their multiple uses. Endophytic fungi offer a viable solution, enabling the medicinal properties of mangroves to be harnessed without compromising this invaluable resource. This approach not only supports environmental sustainability but also creates new pathways for developing natural medicines from Indonesia's rich biodiversity.

This study provides new insights into the bioactive potential of endophytic fungi from *N. fruticans*, focusing on the consistent production of bioactive compounds across different plant organs. Emphasizing the stable biosynthetic capabilities of these fungi, this research highlights their potential as sustainable alternatives to the host plant for bioactive compounds, supporting pharmaceutical development while preserving mangrove ecosystems. These findings underscore the value of endophytic fungi as reliable sources for natural medicine, aligning with biodiversity conservation and sustainable pharmacology.

MATERIALS AND METHODS

Preparation of samples and isolation of endophytic fungi

Fresh *N. fruticans* mangrove samples were collected from the waters of Sungsang IV, South Sumatra (-2.3494680, 104.9062378). The samples were then identified at Generasi Biologi Indonesia (BT-072478). The stem, frond, and mesocarp of the fruit were washed thoroughly with running water. Surface sterilization of the samples was conducted inside a Laminar Air Flow by immersing them in 70% ethanol and rinsing with aquadest for approximately 1 min each. The samples were then soaked in sodium hypochlorite (NaOCl) for 30 sec, rinsed again with 70% ethanol, and sterile distilled water. The sterilized samples were aseptically cut. The samples were cultured at room temperature for a period of 3 to 7 days after being inoculated on potato dextrose agar (PDA) medium in Petri plates. Every day until fungal growth was apparent, observations were taken. Colonies were transferred to new PDA media and incubated for 48 hours at room temperature in order to obtain pure cultures. The purified fungal colonies were then transferred to culture media for macroscopic and microscopic characterization observations (Oktiansyah et al. 2023).

Cultivation and extraction of endophytic fungi

The isolated endophytic fungi were then cultivated by placing 5 agar blocks of pure culture (5 mm in diameter) in 15 culture bottles, each containing 300 mL of potato dextrose broth (PDB). The cultures were kept in a static environment at room temperature for four weeks. Filter paper was used to separate the mycelium from the media following the incubation period. After that, a 1:1 ratio of ethyl acetate solvent was added to the medium, and it was extracted. The ethyl acetate extract was separated and evaporated from the liquid culture using a rotary evaporator. The concentrated extract was subsequently dried in an oven at 45°C and weighed (Oktiansyah et al. 2023).

Characterization and identification of endophytic fungi

Observation of endophytic fungal colony characteristics was carried out at 3-7 days of age, including color, texture (cottony, granular, powdery, slimy), the presence of radial lines, concentric rings, and exudate droplets. Microscopic characterization was performed by preparing microscopic slides using Henrici's slide culture method. Microscopic observations included spore shape and the presence or absence of septa in the hyphae (Walsh et al. 2018). Identification was based on macroscopic and microscopic characteristics observed and compared with the literature (Watanabe 2010; Walsh et al. 2018).

Extraction of host plants

Fresh samples of *N. fruticans* (stem, frond, and fruit mesocarp) were cleaned with clean water, air-dried, and then cut into small pieces. The samples were dried and ground into a fine powder. From each sample, 100 g of dried powder was macerated for three consecutive days using methanol solvent (3 repetitions). Each obtained macerate was concentrated using a rotary evaporator to produce concentrated methanol extracts (Muharni et al. 2020).

Antioxidant activity test

The 1-diphenyl-2-picrylhydrazyl (DPPH) technique was utilized to determine the antioxidant activity. Three times, methanol was used to dissolve the host plant's methanol extract as well as the endophytic fungi's ethyl acetate extract at concentrations of 500, 250, 125, 62.5, 31.25, 15.625, and 7.81 µg/mL. The 3.8 mL of a 0.05 mM DPPH solution was added at 0.2 mL of each concentration. After homogenising the mixture, it was placed in a dark tube for half an hour. Absorbance at λ max 517 nm was measured using a UV-Vis spectrophotometer (Elfita et al. 2012). Ascorbic acid served as the positive control, acting as a standard for DPPH radical scavenging activity, while the negative control consisted of the DPPH solution without any extract to confirm the specificity of the antioxidant response (Abbas et al. 2021). Antioxidant activity was quantified using the IC_{50} value (the concentration at which

50% inhibition occurs) and the percentage inhibition of DPPH absorbance at each concentration, allowing for a comparative analysis of antioxidant effectiveness between the plant host and endophytic fungal extracts.

$$\% \text{ Inhibition} = \frac{A_k - A_s}{A_s}$$

Where:
A_k : Absorbance of control
A_s : Absorbance of samples

Antibacterial activity test

Antibacterial activity was tested using the disk diffusion method (Witasari et al. 2022). Each methanol extract from the host plant and ethyl acetate extract from the endophytic fungi was prepared at a concentration of 4% using dimethyl sulfoxide (DMSO) as a solvent. The extracts were then tested on nutrient agar (NA) medium against four bacterial strains: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), and *Salmonella typhi* (IPCCCB.11.669), with three repetitions for each test. All bacterial suspensions were adjusted to a concentration of 1.5×10^8 CFU/mL, following the 0.5 McFarland standard. A 100 µL bacterial suspension with a density of 5×10^5 CFU/mL was inoculated onto petri dishes, and paper discs (6.0 mm) impregnated with 10 µL of the concentrated extract were placed on the inoculated agar plates. Tetracycline was used as the positive control to benchmark antibacterial activity, providing a standard for comparison against the test samples. DMSO served as the negative control to confirm that any observed antibacterial effects were due solely to the active compounds and not influenced by the solvent. Following a 24 h incubation at 37°C, the diameter of the inhibition zone around each extract was measured and compared to the standard antibiotic. Based on a comparison with the positive control, the extract antibacterial activity was categorized as strong (with an inhibition zone over 70%), moderate (with an inhibition zone between 50% and 70%), or weak (with an inhibition zone under 50%) (Elfita et al. 2014).

Thin layer chromatography (TLC) test

The endophytic fungal extract was spotted onto a TLC plate and eluted with a suitable solvent system. The spots were observed under a UV lamp at λ 254, providing further information on the compounds in the extract. After that the chromatogram was sprayed with 20% H_2SO_4 and heated on a hotplate at 100°C. The spot patterns and retention factors (Rf) were observed as indicators of the diversity of secondary metabolites present in the extracts (Aisyiyah et al. 2023).

LCMS/MS analysis

A mass spectrometer was connected to an ultra-performance liquid chromatography (UPLC) system to for high-resolution mass spectrometry analysis. The column, a C18, was kept at 50°C while the ambient temperature was set at 25°C. The mobile phase for the liquid chromatography consisted of acetonitrile with 0.05% formic acid (B) and water with 5 mM ammonium formate (A), flowing at 0.2 mL/min in a step gradient over 23 minutes. Prior to injection, the sample was filtered using a 0.2 µm syringe filter, and 5 µL was injected. Electrospray ionization (ESI) in positive mode was used for mass spectrometry (MS) analysis, covering a mass range of 50-1200 m/z. The desolvation temperature was set at 350°C, while the ion source temperature was set at 100°C. The cone gas flow rate was 0 L/hr, and the desolvation gas flow rate was 793 L/hr. Collision energy ranged between 4 and 60 eV, and data were acquired, analyzed, and controlled using MassLynx software version 4.1 (Ismed et al. 2022).

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

Eighteen endophytic fungal isolates were obtained from various parts of *N. fruticans*, including the stem, frond, and fruit mesocarp. These comprised five isolates from the stem (NfBa1-NfBa5), seven from the frond (NfP1-NfP7), and six from the fruit mesocarp (NfBu1-NfBu6). The macroscopic characteristics, including front and reverse views, along with microscopic results of the endophytic fungi isolated from the stem, frond, and mesocarp of *N. fruticans* are shown (Figures 1, 2, 3). The morphological characteristics of the fungal colonies isolated from these parts are detailed for each isolate (Table 1, Table 2).

Based on morphological characteristics, five endophytic fungal isolates from the stem of *N. fruticans* were identified (NfBa1-NfBa5), belonging to four fungal genera: *Diaporthe* (NfBa1), *Aspergillus* (NfBa2 and NfBa5), *Penicillium* (NfBa3), and *Apophysomyces* (NfBa4). From the frond, seven endophytic fungal isolates were identified (NfP1-NfP7), with six different genera: *Paecilomyces* (NfP1 and NfP3), *Humicola* (NfP2), *Nodulisporium* (NfP4), *Fusarium* (NfP5), *Mortirella* (NfP6), and *Aspergillus* (NfP7). In the mesocarp, six endophytic fungal isolates were found (NfBu1-NfBu6), belonging to four different genera: *Aspergillus* (NfBu1, NfBu2, and NfBu4), *Papulaspore* (NfBu3), *Pestalotiopsis* (NfBu5), and *Cladosporium* (NfBu6).

Biodiversity of endophytic fungi in *Nypa fruticans*

A total of 18 endophytic fungal isolates were successfully isolated from the stem, frond, and mesocarp of *N. fruticans*, representing 12 different genera. The genus mapping of the endophytic fungi found in each organ is provided. The diversity of endophytic fungi isolated from the stem, frond, and mesocarp of *N. fruticans* is presented (Table 3). The analysis of fungal diversity in different parts of *N. fruticans* reveals distinct fungal colonization patterns. A total of 18 isolates from 12 different genera were identified, with *Aspergillus* being the most dominant genus, found in all parts (stem, frond, and mesocarp), with six isolates.

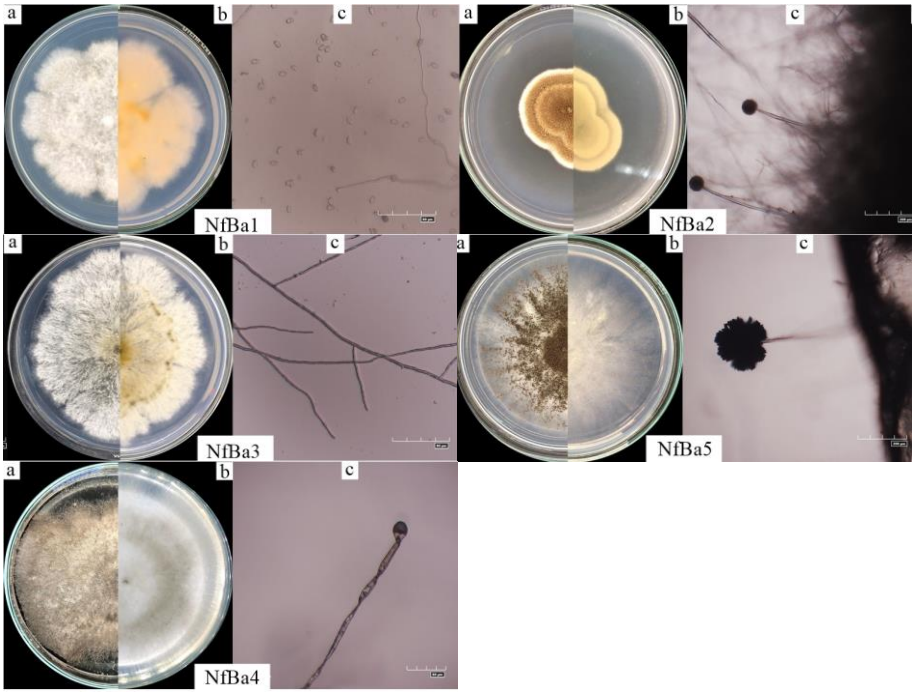

Figure 1. Macroscopic characteristics. (a: front view, b: reverse view) and microscopic characteristics (c) of endophytic fungi isolated from the stem of *Nypa fruticans*

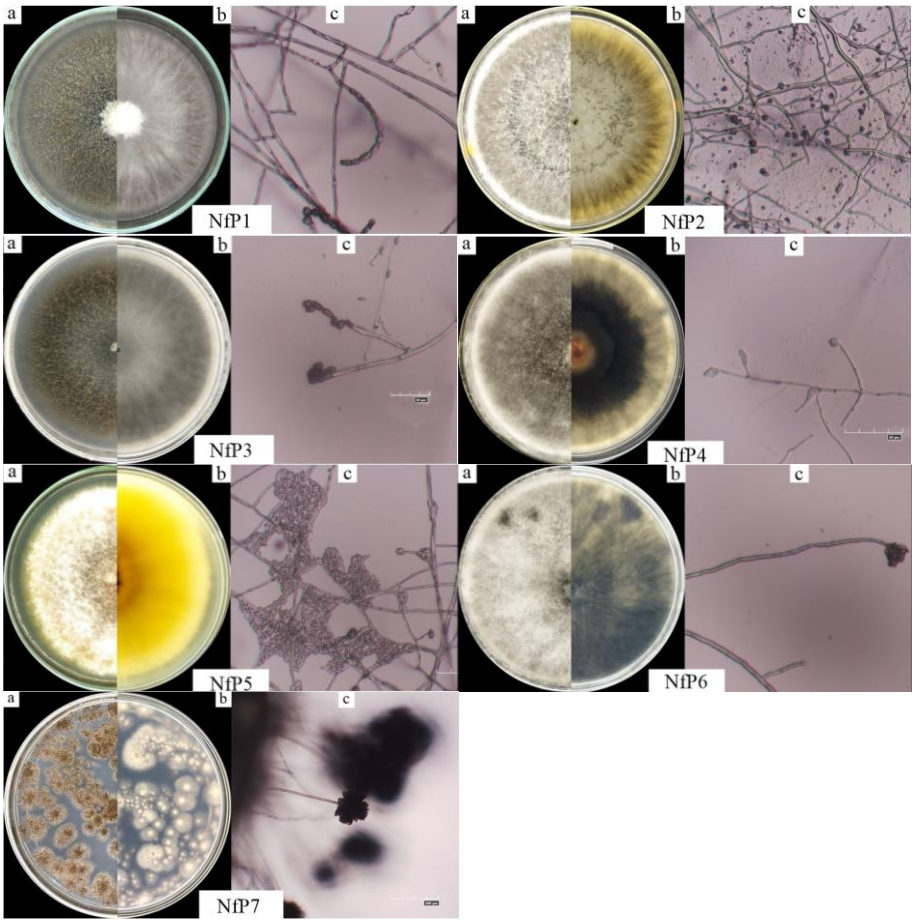

Figure 2. Macroscopic characteristics (a: front view, b: reverse view) and microscopic characteristics (c) of endophytic fungi isolated from the frond of *Nypa fruticans*

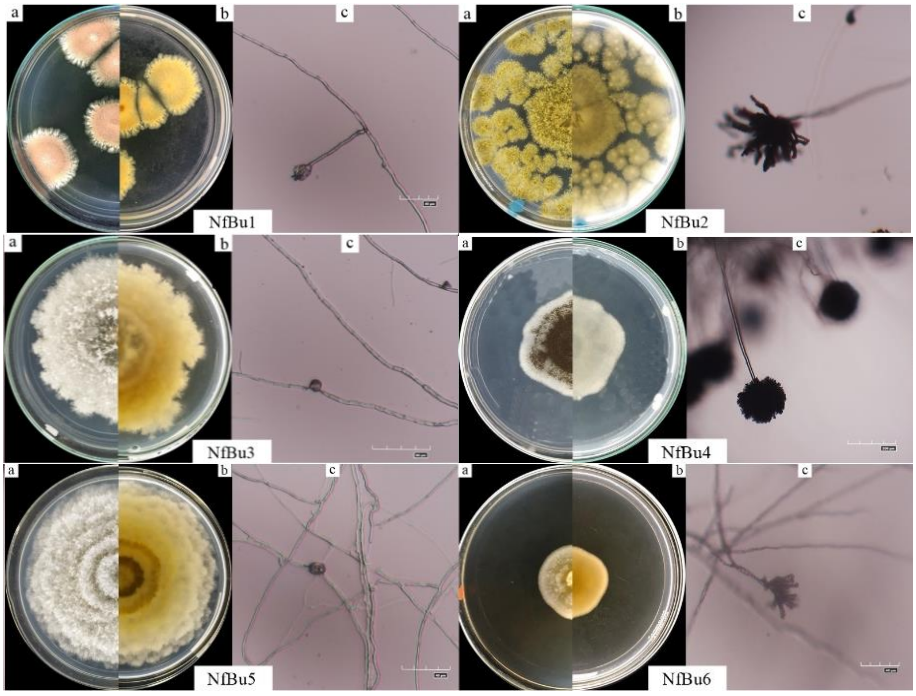

Figure 3. Macroscopic characteristics (a: front view, b: reverse view) and microscopic characteristics (c) of endophytic fungi isolated from the mesocarp of *Nypa fruticans*

Table 1. Colony characteristics of endophytic fungi from the stem, frond, and mesocarp of *Nypa fruticans*

Code	Surface colony	Reverse colony	Structure	Elevation	Pattern	Exudate drops	Radial line	Concentric circle
NfBa1	White	White to orange	Cottony	Raised	Flowery	-	-	-
NfBa2	Browny with white border	White goldish	Powdery	Flat	Zonate	-	-	√
NfBa3	White with yellowish centre	Pale yellow with brownish green	Velvety	Flat	Flowery	-	-	√
NfBa4	Dark brown	White to brown	Cottony	Flat	Irregular	-	-	-
NfBa5	Black with white border	White to cream	Powdery	Umbonate	Spread	-	-	-
NfP1	Brownish grey with white center	Grey with white	Cottony	Umbonate	Radiate	-	-	√
NfP2	Grey with white	Brownish grey	Cottony	Flat	Radiate	-	-	-
NfP3	Grey with brown	Grey to black	Cottony	Flat	Zonate	-	-	-
NfP4	Grey with white	Black with tan border and orange center	Cottony	Raised	Zonate	-	-	-
NfP5	White to light yellow	Pale yellowish brown	Cottony	Flat	Radiate	-	-	-
NfP6	White to grey	Grey to black	Cottony	Raised	Circular	-	-	-
NfP7	Black with white border	White to cream	Powdery	Umbonate	Spread			
NfBu1	Cinnamon Brown with white lobate	Yellowish brown	Powdery	Flat	Zonate	-	-	√
NfBu2	yellow green	Goldish to brown	Powdery	Umbonate	Spread	-	-	-
NfBu3	White to cream	Yellowish brown	Velvety	Raised	Zonate	-	-	√
NfBu4	Black with white border	White to cream	Powdery	Umbonate	Spread	-	-	-
NfBu5	White with concentric ring	Yellowish brown	Velvety	Raised	Zonate	-	-	√
NfBu6	White to yellowish	Pale brown	Cottony	Umbonate	Circular	-	-	√

Note: NfBa1-NfBa5: Endophytic fungal colonies isolated from the stem; NfP1-NfP7: Endophytic fungal colonies isolated from the frond; NfBu1-NfBu6: Endophytic fungal colonies isolated from the mesocarp

The other eleven genera appeared only in specific tissues. The diversity index supports this observation, with the Simpson index of diversity (1-D) showing that the frond had the highest fungal diversity with a value of 0.8163, followed by the stem with a value of 0.72, and the fruit mesocarp with a value of 0.6667. The Shannon diversity index (H') also reflects the same trend, with the highest diversity in the frond with a value of 1.748 and the lowest in the mesocarp with a value of 1.242, confirming that the frond provides a more complex habitat for fungal colonization. The diversity data is visually presented (Figure 4).

Table 2. Microscopic characteristics of the isolated endophytic fungi from the stem, frond, and mesocarp of *Nypa fruticans*

Isolate	Spore	Shape	Hyphae	Characteristic	Species of identification
NfBa1	Conidia	Globose	Septate	Aseptate conidia depending on the species	*Diaporthe* sp.
NfBa2	Conidia	Globose	Septate	Conidiophores are unbranched and terminate in a vesicle	*Aspergillus* sp.
NfBa3	Conidia	Globose	Septate	The conidiophores terminate in phialides, which are flask-shaped cells where conidia are produced	*Penicillium* sp.
NfBa4	Sporangia	globose	Nonseptate	Sporulation occurs exclusively on broad media Hyphae are often non-septate and branching	*Apophysomyces* sp.
NfBa5	Conidia	Subglubose	Septate	Phialides radiate around the vesicles and are arranged in a biseriate pattern	*Aspergillus niger*
NfP1	Conidia	Ovate	Septate	Hyaline, erect, tapering gradually from base to apex, conidiophores carry catenulate conidia apically	*Paecilomyces* sp.
NfP2	Conidia	Globose	Septate	Conidia globose or spindle-shaped aleurioconidia	*Humicola* sp.
NfP3	Conidia	Ovate	Septate	Catenulate conidia apically or branched in the middle	*Paecilomyces* sp.
NfP4	Conidia	Ellipsoidal	Septate	Conidiophores can be apically branched irregularly, erect, hyaline, or verticillate	*Nodulisporium* sp.
NfP5	Conidia	Fusiform	Septate	Hyaline, erect, lengthy conidiophores that contain masses of spores apically at each branch	*Fusarium* sp.
NfP6	Sporangia	Globose	Septate	Sporangiophores can be simple or branching at the lower half and bear sporangia at the terminal end	*Mortirella* sp.
NfP7	Conidia	Subglubose	Septate	The metulae are twice as long as the phialides, radiate around the vesicles and are arranged in a biseriate pattern	*Aspergillus niger*
NfBu1	Conidia	Elliptical	Septate	The conidiophores are relatively short and the phialides occur only on the upper half of the vesicle	*Aspergillus* sp.
NfBu2	Conidia	Globose	Septate	The conidiophores possess foot cells at the base and are swollen at the tip, forming globose vesicles	*Aspergillus* sp.
NfBu3	Conidia	Irregular	Septate	Papulaspores are formed on small conidiophores that are identical to the hyphae or intercalary	*Papulaspore* sp. 1
NfBu4	Conidia	Subglubose	Septate	The metulae are twice as long as the phialides, which radiate around vesicles in a biseriate pattern	*Aspergillus niger*
NfBu5	Conidia	Globose	Septate	Conidiophores are carried in one or two conidia cells as they mature	*Pestaloptiosis* sp.
NfBu6	Conidia	Subglobose	Septate	Conidia upright, branching at the apical portions, and bearing catenulate conidia in each branch	*Cladosporium* sp.

Notes: NfBa1-NfBa5: Endophytic fungal colonies isolated from the stem; NfP1-NfP7: Endophytic fungal colonies isolated from the frond; NfBu1-NfBu6: Endophytic fungal colonies isolated from the mesocarp

Table 3. Biodiversity of endophytic fungi from *Nypa fruticans*

Genera	Part of plat *Nypa fruticans*			Total
	Stem	Frond	Fruit mesocarp	
Aspergillus	2	1	3	6
Diaporthe	1	0	0	1
Penicillium	1	0	0	1
Apophysomyces	1	0	0	1
Paecilomyces	0	2	0	2
Humicola	0	1	0	1
Nodulisporium	0	1	0	1
Fusarium	0	1	0	1
Mortierella	0	1	0	1
Papulaspore	0	0	1	1
Pestaloptiosis	0	0	1	1
Cladosporium	0	0	1	1
Number of fungal isolates	5	7	6	18
Simpson index (D)	0.28	0.1837	0.3333	-
Simpson index of diversity (1-D)	0.72	0.8163	0.6667	-
Shannon index of diversity (H^l)	1.332	1.748	1.242	-

The PCA (Principal Component Analysis) plot illustrates the distribution and diversity of fungal genera in different parts of *N. fruticans*, including the stem, frond, and mesocarp (Figure 4). Each axis on the plot represents a principal component, with Component 1 explaining the largest variance in the fungal community, while Component 2 explains the second largest variance. The fungal genera are plotted based on their influence on the two components, showing how they are distributed across different plant parts. In the fruit mesocarp, *Aspergillus* is highly dominant, as it is located far to the right on Component 1, indicating its significant role in shaping the fungal community in this part. The frond shows a higher fungal diversity, with *Paecilomyces* being the most significant contributor, positioned in the upper left quadrant of the plot. Other genera such as *Nodulisporium*, *Mortierella*, *Fusarium*, and *Humicola* were also found in the frond, contributing to the complexity of the fungal community in this section. The stem of *N. fruticans* occupies a more central position on the plot, with genera such as *Diaporthe*, *Penicillium*, and *Apophysomyces* clustered around it. This indicates that while the stem has moderate fungal diversity, no genus is as dominant as *Aspergillus* in the fruit mesocarp.

Antibacterial and antioxidant activity of host plant extracts and endophytic fungal extracts isolated from *Nypa fruticans*

The endophytic fungi isolated from *N. fruticans*, extracted with ethyl acetate, demonstrate potential as antibacterial and antioxidant agents (Table 4). The antioxidant activity exhibits IC_{50} values classified as very strong and strong, while the antibacterial activity ranges from strong, moderate, to weak categories. The bioactivity of the host plant extract and the endophytic fungal extracts shows promising antibacterial and antioxidant activity, as shown (Figure 5, Figure 6).

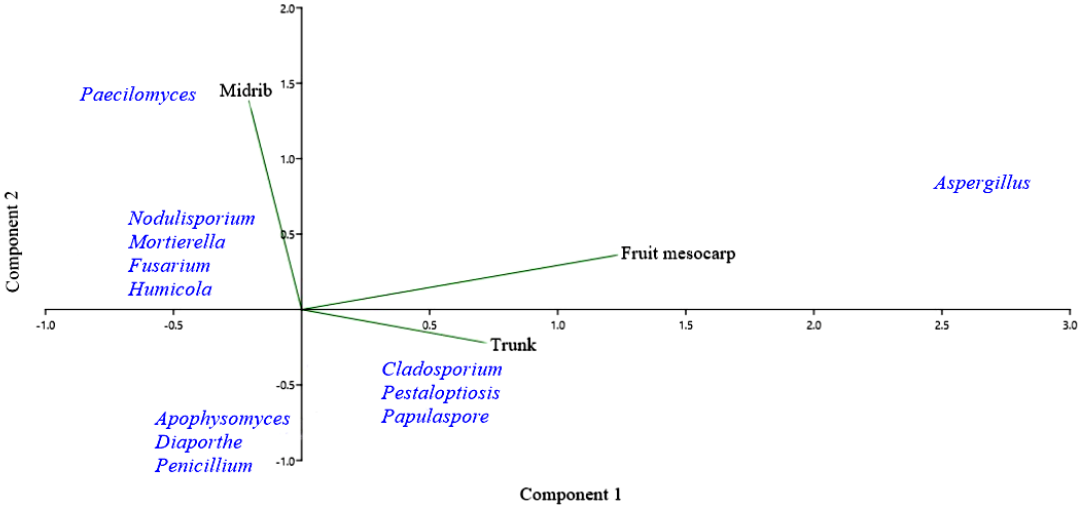

Figure 4. Principal Component Analysis (PCA) of endophytic fungi from *Nypa fruticans*

Table 4. Antibacterial and antioxidant activity of endophytic fungal extracts from the stem, frond, and mesocarp of *Nypa fruticans*

Sample/ extract	Weight of media extract (g)	Antibacterial activity				Antioxidant activity IC_{50} (µg/mL)
		E. coli	*S. aureus*	*S. thypi*	*B. subtilis*	
Host plant						
Methanol of nipah's stem	-	32.40±0.21*	39.85±1.41*	38.23±0.71*	39.21±0.14*	62.13***
Methanol of nipah's frond	-	46.77±1.41*	36.83±0.14*	60.96±0.03**	39.74 ±0.71*	34.56***
Methanol of nipah's mesocarp	-	26.44±0.21*	38.72±0.71*	50.90±0.14**	45.41±0.21*	17.21****
Endophytic fungi						
NfBa1	1.4	55.16±0.03**	50.99±0.21**	64.98±0.14**	53.04±0.71**	29.74***
NfBa2	1.6	41.94±0.71*	58.92±0.07**	57.45±0.14**	41.13±1.41*	18.36****
NfBa3	0.7	46.79±0.03*	45.09±1.41*	29.66±1.41*	36.16±0.21*	44.82***
NfBa4	1.2	49.65±1.41*	49.42±0.14*	51.28±0.03**	50.92±0.07**	17.21****
NfBa5	1.3	52.47±0.14**	60.17±0.71**	60.00±0.14**	46.17±0.21*	59.90***
NfP1	1.7	70.30±0.14***	71.90±0.07***	57.74±1.41**	62.70±0.07**	37.75***
NfP2	1.2	69.51±0.71**	56.87±0.14**	56.08±0.03**	61.75±0.21**	33.45***
NfP3	0.7	69.46±0.14**	63.43±0.14**	65.58±0.00**	56.16±0.03**	42.77***
NfP4	1.6	76.88±1.41***	95.07±0.00***	92.78±1.41***	76.22±0.14***	13.21****
NfP5	2.0	82.57±0.07***	98.64±0.03***	95.70±0.14***	87.08±0.71***	15.07****
NfP6	1.3	47.91±0.21*	53.18±1.41**	48.54±0.07*	70.29±0.14***	43.69***
NfP7	1.4	47.94±0.14*	42.98±0.71*	57.69±0.14**	48.48±0.14*	55.35***
NfBu1	1.9	61.69±0.14**	70.82±0.71***	70.85±1.41***	75.76±0.07***	19.33****
NfBu2	2.3	52.94±0.71**	72.15±0.21***	56.63±0.07**	57.40±0.14**	98.48***
NfBu3	0.9	60.11±0.21**	71.83±0.71***	57.21±0.21**	46.68±1.41*	75.39***
NfBu4	1.6	47.05±0.71*	56.13±0.14**	59.23±0.14**	34.24±1.41*	66.43***
NfBu5	0.7	68.03±1.41**	65.40±0.14**	74.03±0.71***	67.15±0.21**	42.25***
NfBu6	1.0	57.66±0.03**	52.73±0.21**	61.01±0.03**	45.50±0.71*	16.58****
Positive control		Tetracyclin 100***	Tetracyclin 100***	Tetracyclin 100***	Tetracyclin 100***	Ascorbic acid 10.47****

Note: NfBa1-NfBa5: endophytic fungal colonies isolated from the stem; NfP1-NfP7: endophytic fungal colonies isolated from the frond; NfBu1-NfBu6: endophytic fungal colonies isolated from the mesocarp. Antibacterial activity percentage: ***: >70% (strong), **: 50-70% (moderate), *: <50% (weak). Antioxidant activity IC50 (µg/mL): ****: <20 µg/mL (very strong), ***: 20-100 µg/mL (strong), **: 101-500 µg/mL (moderate); *: >500 µg/mL (weak)

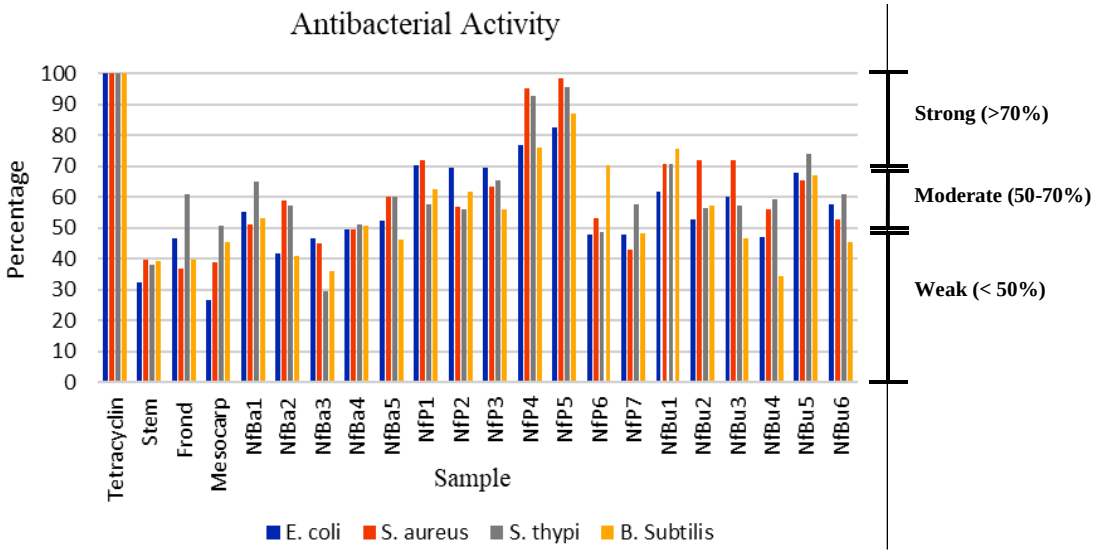

Figure 5. Antibacterial activity of host plant extracts and endophytic fungal extracts from *Nypa fruticans*. Note: NfBa1-NfBa5: endophytic fungal colonies isolated from the stem; NfP1-NfP7: endophytic fungal colonies isolated from the frond; NfBu1-NfBu6: endophytic fungal colonies isolated from the mesocarp

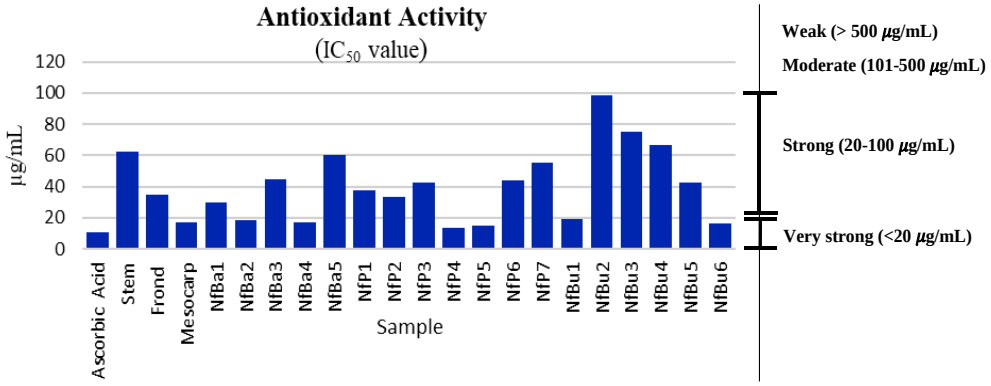

Figure 6. Antioxidant activity of host plant extracts and endophytic fungal extracts from *Nypa fruticans.* Notes: NfBa1-NfBa5: Endophytic fungal colonies isolated from the stem; NfP1-NfP7: Endophytic fungal colonies isolated from the frond; NfBu1-NfBu6: Endophytic fungal colonies isolated from the mesocarp

The antibacterial activity shows that isolate NfBa1 exhibited the best antibacterial activity among the other isolates, with moderate activity against all four test bacteria (*S. aureus*, *E. coli*, *S. typhi*, and *B. subtilis*). Compared to the extract from the nipa palm stem, the NfBa1 extract had better antibacterial activity. The antioxidant activity of isolates NfBa2 and NfBa4 was classified as very strong (IC_{50}<20 µg/mL), while isolates NfBa1, NfBa3, NfBa5, and the methanol extract from the stem of *N. fruticans* were categorized as strong (IC_{50}<100 µg/mL). Isolates NfP4 and NfP5 showed the best antibacterial activity compared to other isolates and the extract from the nipa frond, with strong activity against all four test bacteria. The antioxidant activity of isolates NfP4 and NfP5 was classified as very strong (IC_{50}<20 µg/mL), while isolates NfP1, NfP2, NfP3, NfP6, NfP7, and the methanol extract from the frond of *N. fruticans* were categorized as strong (IC_{50}<100 µg/mL). Isolate NfBu1 showed the best antibacterial activity among the other isolates and the mesocarp extract, with strong activity against three test bacteria (*S. aureus*, *S. typhi*, and *B. subtilis*) and moderate activity against one test bacterium (*E. coli*). The antioxidant activity of isolates NfBu1, NfBu6, and the methanol extract from the mesocarp of *N. fruticans* was categorized as very strong (IC_{50}<20 µg/mL), while isolates NfBu2, NfBu3, NfBu4, and NfBu5 were classified as strong (IC_{50}<100 µg/mL). This variation in bioactivity could be attributed to the content of secondary compounds or metabolites in each extract. The results of the TLC (Thin Layer Chromatography) tests from each endophytic fungal extract showed diverse spot patterns (Figure 7). Several endophytic fungal extracts that showed moderate and strong antibacterial activity, or those closest to the activity of the positive control, were isolates NfP4, NfP5.

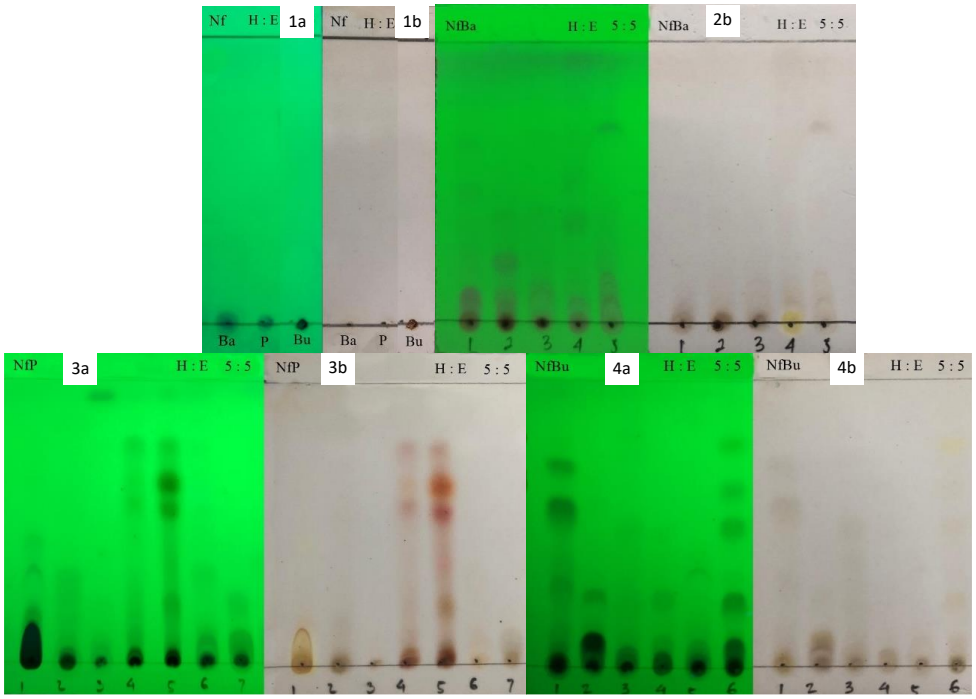

Figure 7. Thin Layer Chromatography (TLC) results of methanol extracts of *Nypa fruticans*and endophytic fungal extracts. Notes: (1: Host plant extract (Nf: Ba, P, Bu); 2: Endophytic fungal extract from the stem (NfBa1-NfBa5); 3: Endophytic fungal extract from the frond (NfP1-NfP7); 4: Endophytic fungal extract from the fruit mesocarp (NfBu1-NfBu6); a: TLC under UV light, b: TLC after being sprayed with 20% H_2SO_4)

The TLC results of the host plant and endophytic fungal extracts isolated from various parts of *N. fruticans* are shown (Figure 7). The spots for each endophytic fungal extract display different patterns, numbers of spots, and retention factor (Rf) values. This indicates that each endophytic fungal extract contains different compounds. After spraying with 20% H_2SO_4 as the reagent, the spot patterns suggest the presence of specific compounds in each endophytic fungal extract. These compounds, particularly phenolic groups, are known to have antioxidant and antibacterial properties (Bisso et al. 2022; El-Zahar et al. 2022; Mapfumari et al. 2022; Mehmood et al. 2022). No spots were observed for the host plant extract, possibly due to the polar nature of the compounds. The solvent used in this study was ethyl acetate, a semi-polar solvent. The secondary metabolites in the extract were more diverse because ethyl acetate may bind both polar and nonpolar molecules.

The bioactivity test results show that isolates NfP4, NfP5, and NfBu1 exhibit the most potent antibacterial and antioxidant activity. Furthermore, the TLC results indicate the presence of unique secondary metabolites in these three isolates. Additionally, the same fungal species, *A. niger*, was found in all three parts of the plant (stem, frond, and mesocarp), with equivalent antibacterial and antioxidant activity in each part (NfBa5, NfP7, and NfBu4). The spot patterns on the TLC plate also show similarities in the initial spots, indicating common metabolites during the early stages of separation.

Compound analysis in extracts using LCMS/MS

The three *A. niger* endophytic fungal extracts from different plant parts, namely NfBa5, NfP7, and NfBu4, were analyzed for compound content using LCMS/MS. Each chromatogram of the three *A. niger* extracts showed one major peak and one minor associated peak. The chromatograms of the three *A. niger* endophytic fungal extracts are displayed (Figure 8). The predicted compounds from a major peak and an attached peak in the LCMS/MS chromatogram of the endophytic fungus *A. niger*, which was isolated from various plant organs, are shown (Table 5). This study indicates that the compounds contained in the endophytic fungus *A. niger* isolated from different parts of the plant, specifically the stem, frond, and mesocarp, have the same compound composition.

LCMS/MS analysis identified key compounds such as (−)-Lycorine (1) and 5-Amino-1-(2-pyrimidinyl)-1H-pyrazole-4-carbonitrile (2) in all three parts (Figure 9). Interestingly, these tentatively identified compounds have not been found in the host plant, *N. fruticans*, which contains different compounds such as chlorogenic acid and kaempferol, according to literature. The biological activity of all three *A. niger* extracts is relatively equivalent, with antibacterial and antioxidant activity tests showing comparable results between the extracts from the stem, frond, and mesocarp (Table 6).

The stem extract of *A. niger* (NfBa5) exhibited higher antibacterial activity against all four test bacteria compared to the methanol extract from the host plant's stem. Additionally, while the NfBa5 extract demonstrated stronger antioxidant activity than the host plant's stem extract,

studies have shown that other parts of the host plant, such as the frond and mesocarp, may exhibit superior antioxidant activity compared to the endophytic fungus. Despite the higher antioxidant activity in certain parts of the host plant, the diversity and capability of *A. niger* to produce unique bioactive compounds make it a valuable potential source for the development of natural antibacterial and antioxidant agents. The significant difference in compound composition between the endophytic fungus and its host opens up opportunities for further exploration of novel compounds from endophytic fungi that could potentially be more effective than those found in their host plants.

Cichorin A (3), a compound traditionally associated with the plant *Cichorium intybus* L. (chicory), was notably isolated for the first time from an endophytic fungus *Pestalotiopsis* sp. residing in the mangrove palm *N. fruticans* (Alade et al. 2017). Comparative analysis of chemical compounds in the ethanol fraction of nipa fruit endosperm (Astuti et al. 2020) HPLC test results (Prasad et al. 2013), and LC-MS/MS analysis of compounds identified from the *A. niger* isolate on *N. fruticans* revealed significant differences between the host plant and fungal isolates. Prasad et al. (2013) identified several key phenolic compounds, including chlorogenic acid (4), protocatechuic acid (5), gallic acid (6), and hydroxybenzoic acid (7), as well as flavonoids such as kaempferol (8), rutin (9), cinnamic acid (10), and quercetin (11). These phenolic and flavonoid compounds were not identified in the LCMS/MS results of the *A. niger* isolate. Instead, compounds such as Lycorine were found, indicating a clear difference in the chemical profiles between the compounds produced by the host and those produced by the endophytic fungus. Prasad et al. (2013) demonstrated that flavonoids and phenolic acids dominate in raw nipa endosperm extract, compounds known for high antioxidant activity. In contrast, the *A. niger* isolates produced distinct secondary metabolites, such as 5-Amino-1-(2-pyrimidinyl)-1H-pyrazole-4-carbonitrile, typically associated with fungal metabolism.

Similarly, the *N. fruticans* endosperm, as characterized by Astuti et al. (2020), was found to contain predominantly furfural compounds, including furfural (12), 5-methylfurfural (13), and furfuryl alcohol (14). However, these compounds were absent in the LC-MS/MS results for the *A. niger* isolate, suggesting they are either not produced by the fungus or are not dominant in its secondary metabolites. These findings underscore the distinct secondary metabolite profiles of *N. fruticans* and its associated endophytic fungi, such as *Pestalotiopsis* sp., highlighting the unique biosynthetic pathways of endophytes compared to their host plants. Compounds isolated from endophytic fungi (1-3) and its host (4-14) can be seen (Figure 9). These distinct differences between the host and fungal isolates reveal the potential of fungal endophytes to serve as alternative sources for novel bioactive compounds, independent of their host plant's metabolic profile.

Table 5. Predicted compounds from major and associated peaks in the LCMS/MS Chromatograms of *A. niger* endophytic fungal extracts isolated from different organs

Extract of *A. niger* isolated from	Retention Time (RT)	% area	Measured mass (m/z) $[M + H]^+$	Formula	Tentative identification
Stem (NfBa5)	8.07	8.10	288.124	$C_{16}H_{17}NO_4$	(−)-Lycorine
Frond (NfP7)	7.85	3.65	288.125	$C_{16}H_{17}NO_4$	
Mesocarp (NfBu4)	8.14	10.17	288.125	$C_{16}H_{17}NO_4$	
Stem (NfBa5)	8.31	32.38	187.076	$C_8H_6N_6$	5-Amino-1-(2-pyrimidinyl)-1H-pyrazole-4-carbonitrile
Frond (NfP7)	8.14	44.29	187.076	$C_8H_6N_6$	
Mesocarp (NfBu4)	8.42	40.72	186.178	$C_8H_6N_6$	

Table 6. Antioxidant and antibacterial activity of *A. niger* endophytic fungal extracts isolated from different organs

Extract of *A. niger* isolated from	Antibacterial activity				Antioxidant activity
	E. coli	*S. aureus*	*S. thypi*	*B. subtilis*	IC_{50} (µg/mL)
Stem (NfBa5)	52.47±0.14**	60.17±0.71**	60.00±0.14**	46.17±0.21*	59.90***
Frond (NfP7)	47.94±0.14*	42.98±0.71*	57.69±0.14**	48.48±0.14*	55.35***
Mesocarp (NfBu4)	47.05±0.71*	56.13±0.14**	59.23±0.14**	34.24±1.41*	66.43***

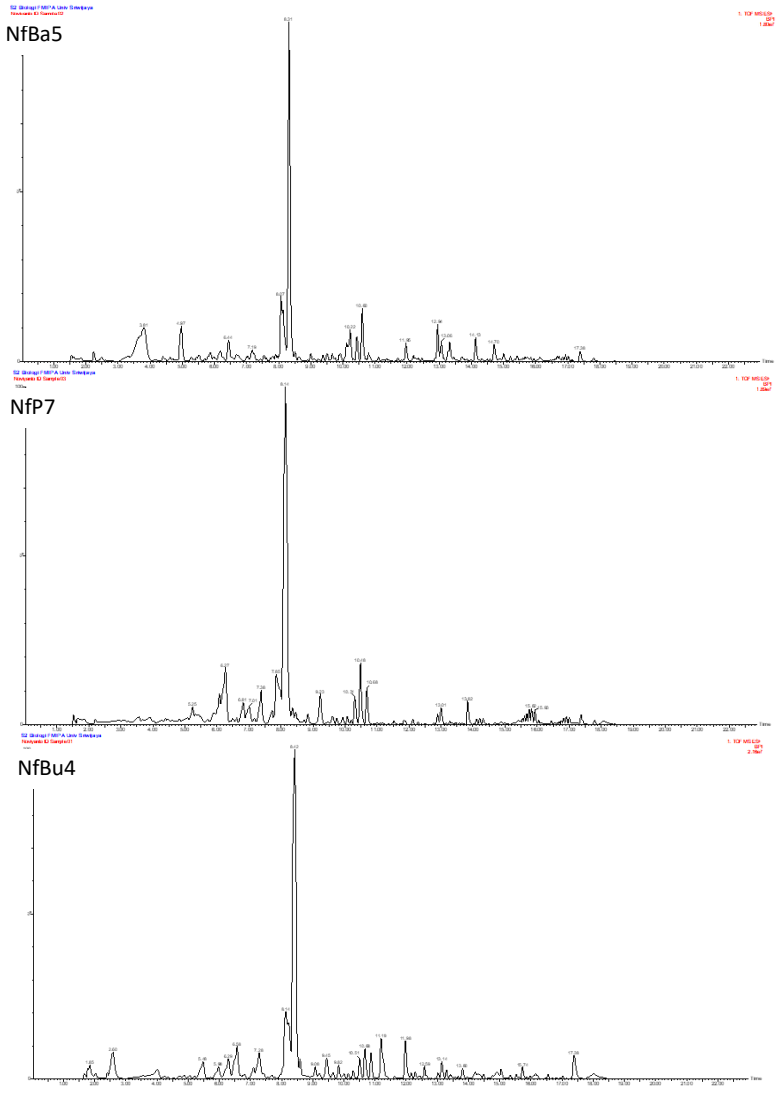

Figure 8. LCMS/MS chromatograms of NfBa5, NfP7, and NfBu4

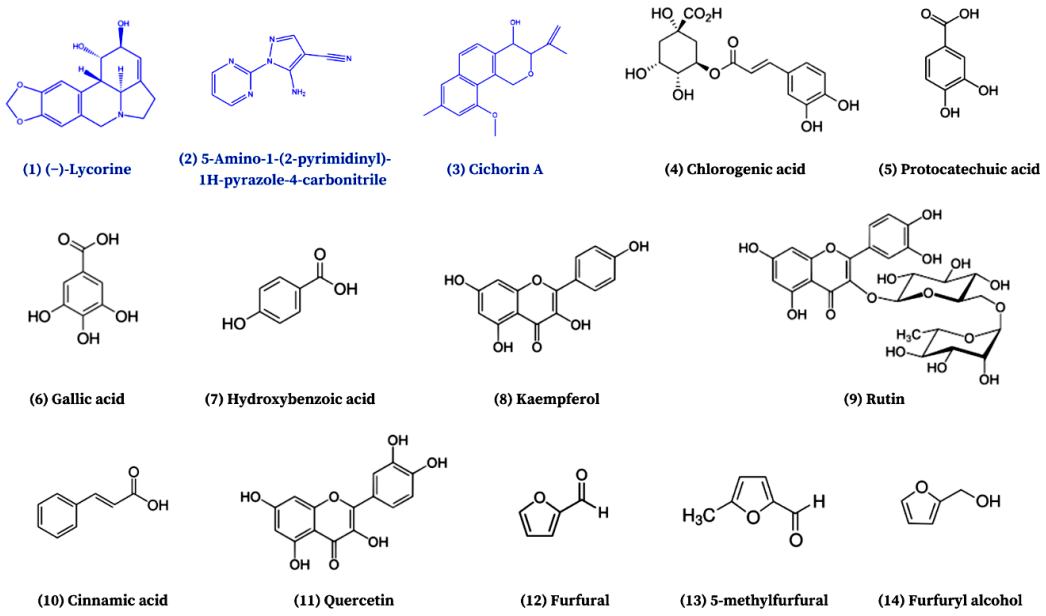

Figure 9. Compounds isolated from endophytic fungi (1-3) and its host (4-14)

Discussions

This study identified 18 endophytic fungal isolates from the stem, frond, and mesocarp of *N. fruticans*, belonging to 12 different genera, namely *Diaporthe*, *Aspergillus*, *Penicillium*, *Apophysomyces*, *Paecilomyces*, *Humicola*, *Nodulisporium*, *Fusarium*, *Mortierella*, *Papulaspora*, and *Cladosporium*. Among these, the genus *Aspergillus* was the only one present in all three plant parts of *N. fruticans*. This discovery suggests that endophytic fungus from the *Aspergillus* genus do not exhibit habitat specialization within plant tissues. However, some studies suggest that the genus *Aspergillus* is specifically found in certain organs of its host plant (Pescador et al. 2022; Taylor et al. 2022). Our results show that endophytic fungi from the *Aspergillus* genus can adapt to varying anatomical and physiological conditions within their host.

The Simpson Index of Diversity (1-D) and Shannon Diversity Index (H') showed that the frond had the highest fungal diversity, followed by the stem, and the mesocarp. This confirms that the frond provides a more complex habitat for fungal colonization. Overall, PCA revealed distinct fungal communities in each part of *N. fruticans*, with the mesocarp being dominated by *Aspergillus*, the frond hosting a diverse community centered around *Paecilomyces*, and the stem having a more balanced fungal composition (Figure 4). These findings align with diversity index analyses, supporting the unique fungal ecosystems present across plant parts. The higher fungal diversity in the frond is attributed to its more heterogeneous microenvironment, nutrient richness, and more intense interactions with the external environment. The stem, while supporting fungal communities, is more stable and protected, resulting in slightly lower diversity, while the mesocarp is a more closed and limited environment, supporting lower fungal diversity, often dominated by specific fungi such as *Aspergillus*.

The bioactivity of extracts from *N. fruticans* (stem, frond, and mesocarp) and endophytic fungal extracts (Table 4) showed varying levels of antibacterial activity, ranging from weak to strong, while their antioxidant activity ranged from strong to very strong. Isolates NfBa2, NfBa4, NfP4, NfP5, NfBu1, NfBu6, and the mesocarp fruit extract exhibited very strong antioxidant activity (IC_{50}<20 µg/mL) compared to host plant extracts and other endophytic fungi. Isolates NfP4, NfP5, and NfBu1 demonstrated strong antibacterial activity against several test bacteria. The extracts from these isolates showed IC_{50} values and antibacterial activity percentages close to the positive control. Generally, extracts from the stem, frond, and mesocarp alone exhibited weaker antibacterial activity than those derived from endophytic fungi, indicating that endophytic fungi significantly enhance antibacterial properties. Endophytic fungi thus hold considerable potential for pharmaceutical development as sources of bioactive compounds, especially in addressing challenges like antibiotic resistance. Their production of secondary metabolites with antibacterial and antioxidant activities positions them as promising candidates for novel drugs. Furthermore, producing these compounds via laboratory fermentation offers an efficient, sustainable alternative to traditional plant extraction methods, with potential applications as natural supplements or phytopharmaceutical ingredients.

N. fruticans is known to contain polyphenols, phenolics, flavonoids, alkaloids, tannins, diterpenes, and steroids (Nugroho et al. 2022). This indicates that the synergy between the compounds present in the extract leads to the varying antibacterial and antioxidant activities. The results of the TLC test show varying patterns and numbers of spots, supporting the argument that there is a synergistic effect from the compounds contained in the host plant extract (Figure 7). Research has shown that these secondary metabolites exhibit both antibacterial and antioxidant activities (Hayat et al. 2020; Kumar et al. 2021; Suleiman et al. 2021).

A. niger is an opportunistic pathogenic fungus that can grow in a variety of situations. Its spores can quickly move through the air (aerosols) and may be breathed by humans, possibly entering the respiratory system and contributing to the development of allergies (Mousavi et al. 2016; Latgé and Chamilos 2020; Yu et al. 2021). Despite being a known pathogen, research has shown that *A. niger* can rapidly spread in association with plants, and there is currently no information available about its host specificity (Lahlali et al. 2022; Mundim et al. 2022; Silva et al. 2022). Fungi isolated from medicinal plants are known to exhibit significant bioactivity (Chugh et al. 2022; Rehman et al. 2022; Vaou et al. 2022). This suggests that *A. niger*, which lives in part of the *N. fruticans* plant, is able to produce secondary metabolites that are present in their host plant as well. According to a number of studies, *A. niger* isolated from medicinal plants has antibacterial and antioxidant properties due to structural similarities with the compounds in its host plant (Rahimi et al. 2022; Wei et al. 2022). The ethyl acetate extracts of *A. niger* (NfBa5, NfP7, and NfBu4) found in all parts of the *N. fruticans* plant exhibited comparable antibacterial and antioxidant activities. This analogous biological activity is linked to the chemicals found in the *A. niger* extract. The fungus possesses a significant number of cryptic biosynthetic gene clusters (BGCs), which allow it to synthesise diverse biomolecules as secondary metabolites with a wide range of activities (Yu et al. 2021; Mózsik et al. 2022; Wang et al. 2022).

TLC test results showed that this fungus produces various secondary metabolites, as evidenced by the pattern and number of spots on the TLC plate (Figure 7). The TLC profiles of *A. niger* ethyl acetate extracts across all parts of *N. fruticans* showed consistent initial spots. LC-MS/MS analysis revealed key compounds, including (−)-Lycorine and 5-Amino-1-(2-pyrimidinyl)-1H-pyrazole-4-carbonitrile, both of which have not been identified in *N. fruticans*. Lycorine, an alkaloid from the Amaryllidaceae family, has significant potential as an anticancer agent (Roy et al. 2018). These tentatively identified compounds are absen in the host plant, *N. fruticans*, which contains different compounds. This can be seen from the results on the TLC plate using n-hexane: ethyl acetate (5:5) as the eluent, where, unlike the *A. niger* fungus, the host plant did not display any spots.

The chemical composition of the ethanol fraction from nipa fruit endosperm was dominated by furfural compounds (furfural, 5-methylfurfural, and furfuryl alcohol) (Astuti et al. 2020). HPLC analysis of raw endosperm extract identified chlorogenic acid, kaempferol, and protocatechuic acid as major components, alongside gallic acid, hydroxybenzoic acid, rutin, quercetin, and cinnamic acid (Prasad et al. 2013). The chemical compounds found in the *A. niger* isolate from the *N. fruticans* plant are different from those found in the nipa fruit endosperm. Common phenolic and flavonoid compounds in nipa endosperm (such as chlorogenic acid, quercetin, and rutin) were not identified in the *A. niger* fungal isolate, indicating differences in the secondary metabolites between the host plant and its endophytic fungus.

Differences in physiological functions, environmental adaptations, biochemical pathways, and organismal interactions result in the production of unique and overlapping metabolites across plant parts. Similar metabolites may serve distinct functions depending on their location, while unique metabolites cater to specific needs within each plant section. These adaptive responses allow plants to thrive in diverse environmental conditions (Chen et al. 2022; Priyashantha et al. 2023; Toppo et al. 2024). Each part of the plant produces specific metabolites that are most suited to its function; for example, the frond of *N. fruticans*, which is part of the leaf that supports the structure of the leaves and flowers, produces metabolites that benefit the plant, such as antimicrobial compounds that help protect the frond from pathogen infection. Endophytic fungi in the stem play a crucial role in the mangrove ecosystem, aiding in the decomposition of organic matter and returning nutrients to the soil. The nipa fruit mesocarp also serves as a habitat for various fungi that contribute to the nutrient cycle in the mangrove ecosystem. Plants must also adapt to various environmental stresses, such as pathogen attacks, herbivores, UV light, and varying soil conditions. Each part of the plant exists in different environmental conditions, leading them to produce different metabolites as an adaptive response. The synthesis of secondary metabolites is tightly regulated by the plant's biochemical pathways, which are influenced by internal factors such as enzyme activity, gene expression, and developmental stages. Different parts of the plant express distinct sets of genes and enzymes, resulting in the production of specific metabolites tailored to their unique roles and environmental challenges (Divekar et al. 2022; Al-Khayri et al. 2023; Manickam et al. 2023; Salam et al. 2023).

In conclusion, this study underscores the crucial role of endophytic fungi from *N. fruticans*, particularly *A. niger*, in producing bioactive compounds with strong antibacterial and antioxidant activities. The tentative identification of metabolites, such as (−)-Lycorine and 5-Amino-1-(2-pyrimidinyl)-1H-pyrazole-4-carbonitrile in fungi but not in the host plant emphasizes the unique bioactive potential of these fungi. The distinctive metabolite profiles of fungi and host suggest a synergistic relationship that enhances plant bioactivity. These findings underscore the pharmaceutical potential of endophytic fungi as contributors to antibacterial and antioxidant properties.

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