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Research Article

Antibacterial Activities of Fungal Endophytes from Philippine Endemic Plant *Dillenia philippinensis*

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ABSTRACT

Fungal endophytes represent a group of microorganisms that establish symbiotic associations with plants and hold significant ecological importance. Their ability to produce a diverse array of biologically active secondary metabolites has garnered considerable interest in the search for novel drug leads. In this study a total of 33 fungal endophytes were isolated from leaf specimens of the Philippine endemic tree *Dillenia philippinensis* (Rolfe). The morphological characterization of the fungal isolates revealed their taxonomic affiliation with the following eight genera: *Alternaria* sp., *Aspergillus* sp., *Geotrichum* sp., *Guignardia* sp., *Nigrospora* sp., *Paecilomyces* sp., *Pestalotiopsis* sp., and *Phialophora* sp. A representative set of 22 fungal endophyte isolates was selected from the pool of isolates and subjected to large-scale cultivation, followed by extraction of their bioactive metabolites through liquid-submerged fermentation. The resulting crude extracts were evaluated for their inhibitory potential against two Gram-positive bacteria, namely *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA); and two Gram-negative bacteria, namely *Escherichia coli* and Multi-drug resistant *Pseudomonas aeruginosa* (MRPA), using the disc diffusion assay. The results indicate that the crude extracts obtained from endophytic fungi colonizing *D. philippinensis* represent a promising source of bioactive metabolites that exhibit noteworthy inhibitory activity against *S. aureus*, *E. coli*, and MRSA, with an effective concentration of 10 mg/mL. This study demonstrates that the fungal endophytes associated with *Dillenia philippinensis* foliage represent a rich source of bioactive metabolites with significant inhibitory activity against Gram-positive and Gram-negative bacteria. These lead to exploring the potential of these fungal endophytes as a viable source of novel therapeutics.

Keywords: Antimicrobial activities, Bioactive metabolite, Dillenia philippinensis, Endophytes

Introduction

Fungi are regarded as important groups of eukaryotic organisms due to their ability to synthesize metabolites that have medical and clinical applications [1]. Fungal endophytes, fungi that reside within plant tissues, are said to be emerging in their diversity because of their roles in the biome. Their roles are for plant growth and survival, plant responses to pathogens herbivores, environmental change, and interactions with other organisms. Given that fungal endophytes appear ubiquitous among plants in natural ecosystems [2], their presence has been reported in virtually every plant species studied to date, with their distribution variable among different plant parts. Taxonomically, fungal endophytes are commonly classified under the Division Ascomycota [3]. The presence of these fungi not only serves as an indication of potential diseases in plants, but they also act as prolific producers of diverse and numerous bioactive secondary metabolites [3, 4]. The secondary metabolites of fungal endophytes are often bioactive, usually low molecular weight, and produced as families of related compounds at restricted parts of the life cycle [5]. Over 8,600 bioactive metabolites of fungal origin have been described [6]. Isolating such natural products could offer a potential alter-

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native approach for developing resistance against pathogens [7]. Phenolics, coumarin, sterols, and terpenoids are among the common types of secondary metabolites reported to be highly effective against *Gordonia terrae*, *Eschericia coli*, and *Staphylococcus aureus* [6,7].

The Philippines is known as a rich biodiversity hotspot not only for animals but also for plants. It has been reported that some of these plants possess novel phytochemicals that can be used in different pharmacological activities [8-11]. Over 6,000 endemic plants are known in the Philippines [12], *Dillenia philippinensis* (Rolfe), commonly known as "katmon," has been classified as a near-threatened species as of 2020 [13]. *D. philippinensis* is one of those least studied species, especially concerning the possible endophytic fungal communities it hosts. Interestingly, the fungal endophytes are reported to be good sources of secondary metabolites that can be used to develop novel drugs. However, studies on this plant mainly focus on fruit extract and its medicinal uses, which has been found to have anti-leukemia and antioxidant properties that can remedy coughs and fevers [12, 14].

Considering the limited studies on *D. philippinensis* and its associated fungal endophytes, further studies on *D. phlippinensis* provide the opportunity to widen the current knowledge on this plant species, as well as, take part in the scientific effort of isolating and characterizing the associated fungal endophytes. The primary objective of this study was to establish a baseline understanding of *D. philippinensis* and the fungal endophytes that are associated with it. To this end, the research involved the isolation and identification of fungal endophytes from *D. philippinensis* using morphological and cultural characteristics, followed by an evaluation of the antibacterial activity of the extracted bioactive metabolites against *S. aureus* and *E. coli*, as well as methicillin-resistant *S. aureus* and multi-drug resistant *Pseudomonas aeruginosa*.

Material and Methods

Collection of D. philippinensis samples

Samples of *D. phlippinensis* were collected from the Pasonanca Natural Park in Zamboanga City (N 6°58'53.8" and E 122°04'02.2"), which is situated at an altitude of 70.0 meters above sea level. The plant's height and diameter at breast height were measured to be 20.0 meters and 56.6 meters, respectively. The leaf specimens of the plant were placed in 10 separate zipper plastic bags, each containing two leaf samples. The samples were processed within 24 hours, and only the leaves were used as a substrate for the isolation of fungal endophytes.

Isolation of Fungal Endophytes

As per protocol [15], two individual leaf samples (about 17 inches long) from different branches were taken and cut into approximately 5 × 5 centimeter pieces. A total of six explants were placed in each plate. Surface sterilization was done by immersing plant materials in 75% ethanol for a minute, followed by NaOCl for 5 minutes, then submerged in 75% ethanol for 30 seconds. Then, the samples were thoroughly rinsed with sterile distilled water. Next, the samples were imprinted onto a tissue plate agar and transferred into one-fourth strength Potato Dextrose Agar (PDA, DIFCO) supplemented with 500 mg/L of streptomycin (Research Biolab). To verify the effectiveness of the surface sterilization step, aliquots from the rinsed water were plated onto PDA plates. Five plates for each leaf sample containing six explants per plate were then incubated at room temperature for two weeks and examined daily for fungal growth. Any endophytic fungi growing from the leaf tissues were isolated and purified for further identification and bioassay studies. Fungal cultures were maintained on PDA slants at 4°C at De La Salle University Microbiology laboratory.

Characterization and identification of fungal Endophytes

The identification of fungal endophyte (FE) isolates was done based on morphocultural characteristics [16, 17]. Slide cultures were prepared, and microscopic examination was done using light compound microscope. Morphological characteristics considered were the structures of the mycelia, conidiophores, conidia, and hyphae; while cultural characteristics considered were colony growth, surface texture, and margin character. Observed characteristics were then compared with reported fungal taxonomic keys, such as "Illustrated Genera of Imperfect Fungi" by Barnett and Hunter [18], to aid in the identification of fungal isolates.

Production and extraction of bioactive metabolites from fungal Endophytes

The liquid submerged fermentation set-up described in previous literature [12] was used for the

extraction of bioactive metabolites. For the mass production of these FE, 22 FE representing all morphospecies were initially grown on Sabouraud Dextrose Agar (SDA) slants for two weeks at room temperature (21-23˚C. The culture protocol involved adding 5 mL of sterile distilled water to the culture slants and dislodging the fungal mycelia and spores using an aseptic technique. The resulting fungal inocula were then transferred to glass bottles containing 100 mL of Sabouraud Dextrose Broth (SDB, Titan Biotech) and incubated at room temperature (21-23°C) for a period of four weeks. The mycelia mats were macerated to release bioactive metabolites produced by the fungal endophytes and soaked with ethyl acetate (RCI Labscan) for 24 hours. The ethyl acetate extracts containing the metabolites were concentrated *in vacuo* through the IKA RV 10 rotary evaporator. Concentrated crude extracts were placed in pre-weighed vials and were air-dried for 24 hours. Crude extracts were resuspended in a 1:1 methanol acetone solution at a final concentration of 10 mg/mL.

Assay for biological activities of fungal Endophyte crude extracts

The antimicrobial assay was done through the disc diffusion method. The protocol described [19] was followed and the interpretation of results was based on the reported literature [20]. The test organisms used in this study included ATCC strains of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, as well as multi-drug resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*. These isolates were obtained from De La Salle University's existing microbial collection, and their identities were confirmed through phenotypic characterization, including Gram-staining and biological assays [21]. Bacterial cell suspensions were prepared from 24-hour old cultures of test bacteria. Each inoculum concentration was adjusted to 0.5 McFarland standard (1.5×10^8 CFU/mL), and aseptically swabbed on freshly prepared sterile Muller-Hilton Agar (MHA) plates following Ortez (2005) protocol in line with the CLSI standards [20]. Twenty-five (25) µL of each crude culture extract was added and air-dried to each paper disc containing 250 µg of crude extract. The paper discs were placed onto inoculated culture plates. Positive controls included streptomycin (250 µg) for Gram-positive bacteria and ampicillin (250 µg) (Westmont Pharmaceuticals) for Gram-negative bacteria. Sterile distilled water and methanol acetone served as negative controls. All inoculated plates in triplicates were incubated at 37°C for 24 hours and inhibitory activities were evaluated based on the measured average zones of inhibition (ZOI). Interpretation of results was as follows: >19mm ZOI (very active), 14-19 mm ZOI (active), 10-13mm ZOI (partially active), and <10mm ZOI (inactive) [12, 20, 22-24].

Results and Discussion

Fungal Endophytes from D. philippinensis

A total of 33 fungal endophytes (FE) were isolated from the leaves of *D. philippinensis*, comprising 22 distinct morphospecies. Among these morphospecies, representatives from eight genera, namely *Alternaria* sp., *Aspergillus* sp., *Geotrichum* sp., *Guignardia* sp., *Nigrospora* sp., *Paecilomyces* sp., *Pestalotiopsis* sp., and *Phialophora* sp., were selected for mass production of bioactive metabolites. Morphological and cultural characterization of these endophytes is provided in Figure 1 and Table 1. These eight genera of endophytes have been previously reported derived from different host plants, including *Echinochloa glabrescens* (barnyard grass), *Pandanus amaryllifolius* (Pandan), and *Oryza granulate* (wild rice roots) [3, 22, 25-27].

Guignardia (FE 11, 12, 17, 21, and 22 isolates) and *Nigrospora* (FE 1, 2, 7, 13, and 14 isolates) were the most commonly observed fungal endophytes. However, three isolates (FE 10, 19, and 20) could not be identified due to their inability to produce spores. It is possible that the growth of these organisms was influenced by the type of culture medium used, which might have impeded the induction of sporulation.

Antibacterial activities of fungal Endophytes

This study assessed the antibacterial activities of 22 representative fungal endophytes isolated from the leaves of *Dillenia phlippinensis*. It is interesting to note that a total of eight fungal genera were identified from leaf samples of the endemic plant, *D. philippinensis*. Leaf substrates have a high di versity of endophytic fungi [28-29] probably due to the large surface area exposed to the other environment and the presence of stomata that can act as a passageway for the mycelia [3, 4, 7]. The identified endophytic fungi in this study have also been reported in various scientific studies [3, 22,

Table 1. Morphological description of the selected fungal endophytes

Code	Endophyte	Characteristics of Conidia	Colony Pigment
FE 1 (DP-S1-1A I)	*Nigrospora* sp.	Black and shiny, borne singly, apically on a special flat hyaline cell	white
FE 2 (DP-S1-1A II)	*Nigrospora* sp.	Black and shiny, borne singly, apically on a special flat hyaline cell	white
FE 3 (DP-S1-1C)	*Aspergillus* sp.	In dry chains; conidiophores apex enlarged, rounded	Dark green
FE 4 (DP-S1-2A I)	*Pestalotiopsis* sp.	Dark brown, appendage bearing conidia	white
FE 5 (DP-S1-2A II)	*Geotrichum* sp.	Segmentation of hyphae, rod-shaped	white
FE 6 (DP-S1-3A)	*Paecilomyces* sp.	Fusiform to lemon-shaped	green
FE 7 (DP-S1-3B)	*Nigrospora* sp.	Black and shiny, borne singly, apically on a special flat hyaline cell	white
FE 8 (DP-S1-4)	*Geotrichum* sp.	Segmentation of hyphae, rod-shaped	white
FE 9 (DP-S1-5A)	*Alternaria* sp.	Sharply attenuated at apex	Dark green
FE 10 (DP-S1-5C)	Unidentified FE	Mycelia sterile	white
FE 11 (DP-S2-1A I)	*Guignardia* sp.	Elliptical spores, hyaline and aseptate	Dark green
FE 12 (DP-S2-1A II)	*Guignardia* sp.	Elliptical spores, hyaline and aseptate	Dark green
FE 13 (DP-S2-1B)	*Nigrospora* sp.	Black and shiny, borne singly, apically on a special flat hyaline cell	white
FE 14 (DP-S2-2A)	*Nigrospora* sp.	Black and shiny, borne singly, apically on a special flat hyaline cell	white
FE 15 (DP-S2-3A)	*Phialophora* sp.	Phialides with enlarged base with flaring collar; conidia hyaline	Dark green
FE 16 (DP-S2-3B I)	*Alternaria* sp.	Sharply attenuated at apex	white
FE 17 (DP-S2-3B II)	*Guignardia* sp.	Spores obovate to elliptical, hyaline and aseptate	Dark green
FE 18 (DP-S2-3C)	*Alternaria* sp.	Sharply attenuated at apex	Dark green
FE 19 (DP-S2-4A I)	Unidentified FE	Mycelia sterile	grey
FE 20 (DP-S2-4A I)	Unidentified FE	Mycelia sterile	grey
FE 21 (DP-S2-4A II)	*Guignardia* sp.	Spores obovate to elliptical, hyaline and aseptate	Dark green
FE 22 (DP-S2-4A II)	*Guignardia* sp.	Spores obovate to elliptical, hyaline and aseptate	Dark green to black

25-27, 30]. For the interpretation of results of the zones of inhibition for fungal endophyte extracts, extracts with zones of inhibition less than 10 mm were considered as inactive, 10-13 mm were partially active, 14-19 mm were active, and those greater than 19 mm were considered to be very active. This follows the classification presented in previous study [20] which uses the same classification based on fungal endophyte crude extract studies [12, 22, 23, 24].

Out of the 33 fungal endophyte isolates, 22 representative fungal endophyte (FE) crude extracts were tested against *E. coli* (EC), multi-drug resistant *P. aeruginosa* (MRPA), *S. aureus* (SA),

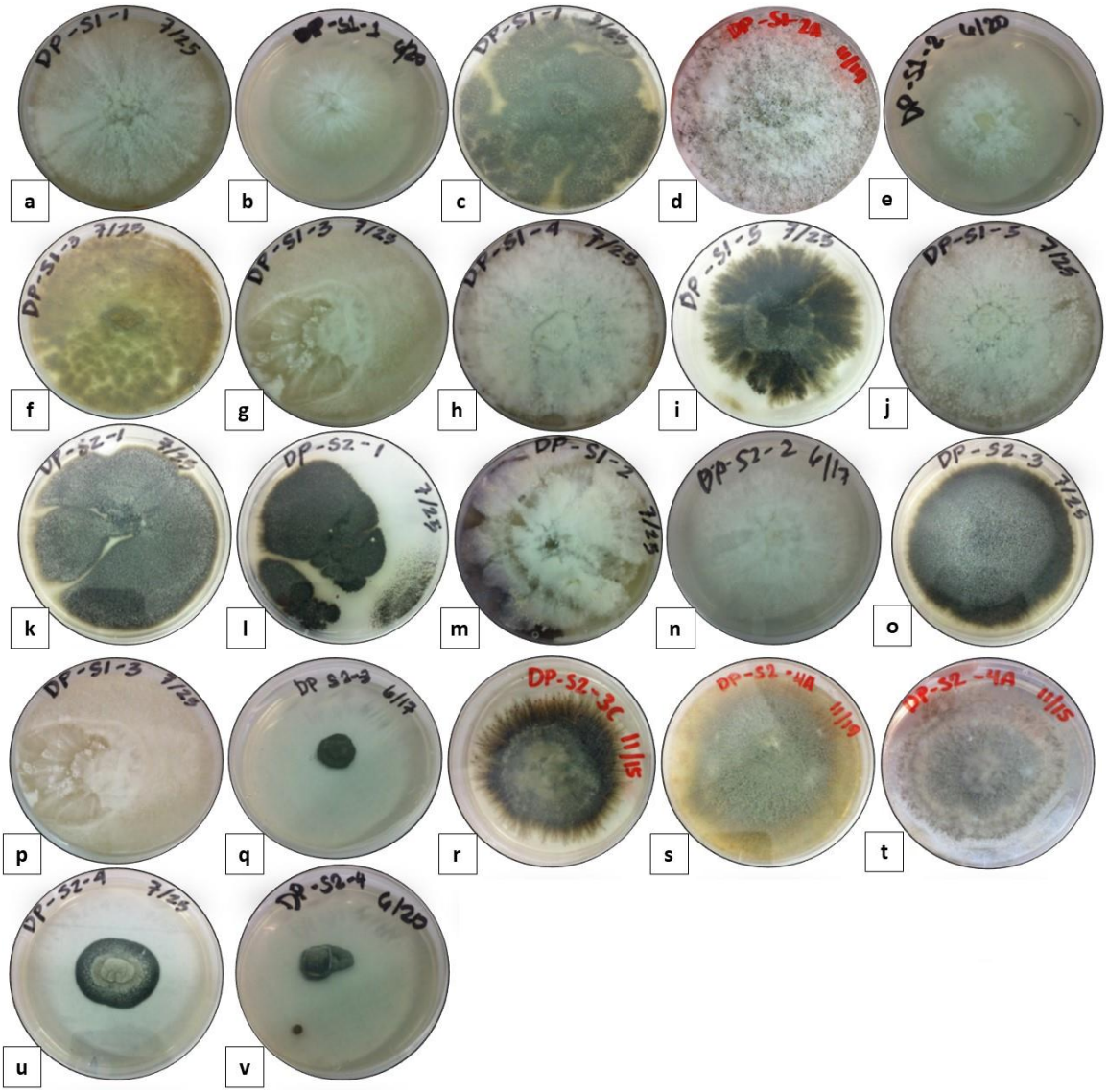

Figure 1. Colony morphology of the fundal endophytes (FE) grown on PDA. (a) FE 1_*Nigrospora* sp., (b) FE 2_*Nigrospora* sp., (c) FE 3_*Aspergillus* sp., (d) FE 4_*Pestalotiopsis* sp., (e) FE 5_*Geotrichum* sp., (f) FE 6_*Paecilomyces* sp., (g) FE 7_*Nigrospora* sp., (h) FE 8_*Geotrichum* sp., (i) FE 9_*Alternaria* sp., (j) FE 10_Unidentified, (k) FE 11_*Guignardia* sp., (l) FE 12_*Guignardia* sp., (m) FE 13_*Nigrospora* sp., (n) FE 14_*Nigrospora* sp., (o) FE 15_*Phialophora* sp., (p) FE 16_*Alternaria* sp., (q) FE 17_*Guignardia* sp., (r) FE 18_*Alternaria* sp., (s) FE 19_Unidentified, (t) FE 20_Unidentified, (u) FE 21_*Guignardia* sp., and (v) FE 22_*Guignardia* sp.

Table 2. Number of crude extracts with inhibitory activities

*Test Organism	Partially Active	Active	Very Active
EC	1	1	13
MRPA	0	0	0
SA	2	1	13
MRSA	1	4	7

*Test organism: EC = *Escherichia coli*, MRPA = Multi-Drug Resistant *Pseudomonas aeruginosa*, SA = *Staphylococcus aureus*, and MRSA = Methicillin-resistant *Staphylococcus aureus*.

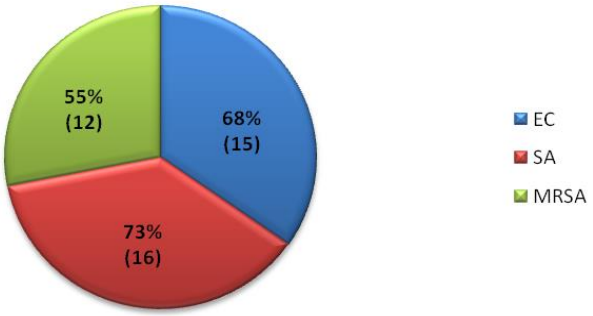

Figure 2. The percentage of inhibitory activities of fungal endophyte crude extracts against *E. coli* (EC), *S. aureus* (SA), and MRSA, with the number of fungal endophytes with effective extracts enclosed in parentheses.

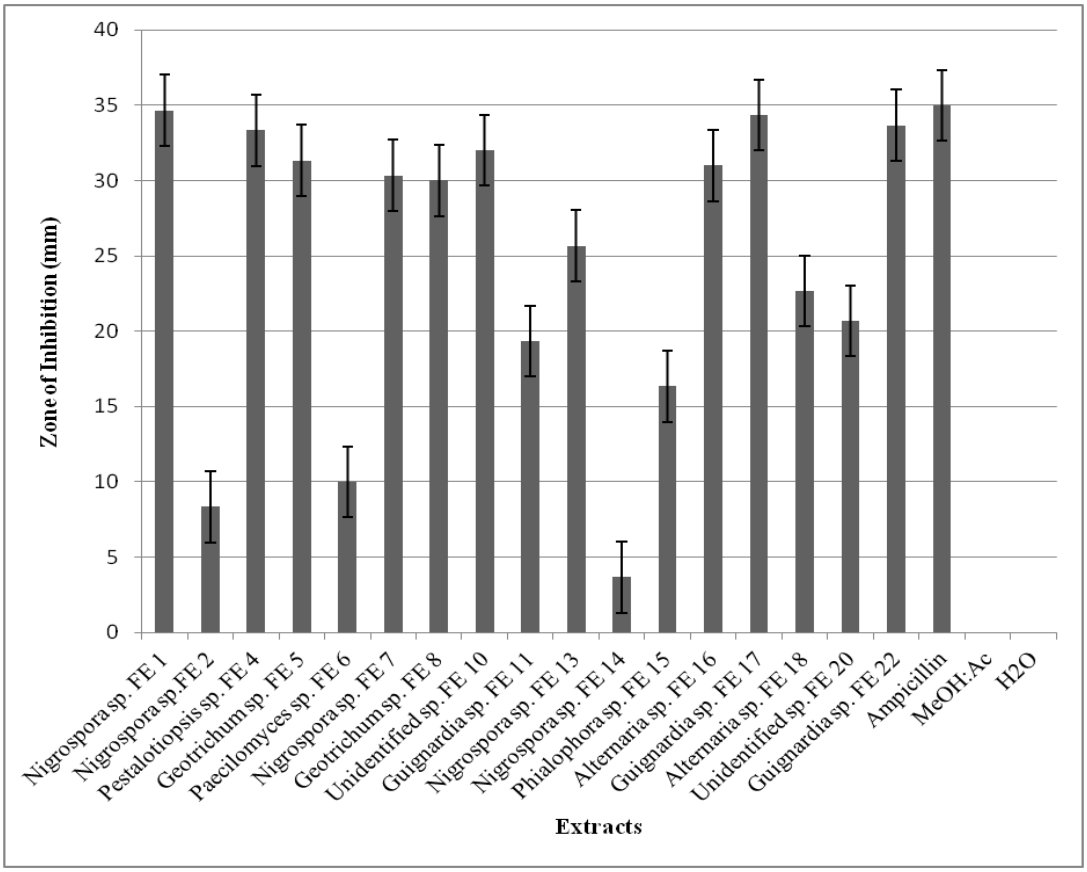

Figure 3. Antimicrobial activities of FE crude extracts against Gram-negative *E. coli*, with standard deviation shown as error bars (n=3).

and Methicillin-resistant *S. aureus* (MRSA). As shown in Figure 2, the FE extracts were most effective against SA, with 16 out of 22 extracts yielding zones of inhibition (ZOI) and an inhibition rate of 73%. The extracts also showed an inhibition rate of 65% against EC and 55% against MRSA. However, none of the extracts showed activity against MRPA. Table 2 shows that 13 crude extract samples were very active against *E. coli* and *S. aureus*, while seven extracts showed very active results against MRSA.

Antimicrobial Activities of FE against E. coli

Figure 3 shows 15 FE crude extracts that were effective against *E. coli*. Thirteen of these extracts were found to be very active against *E. coli*,

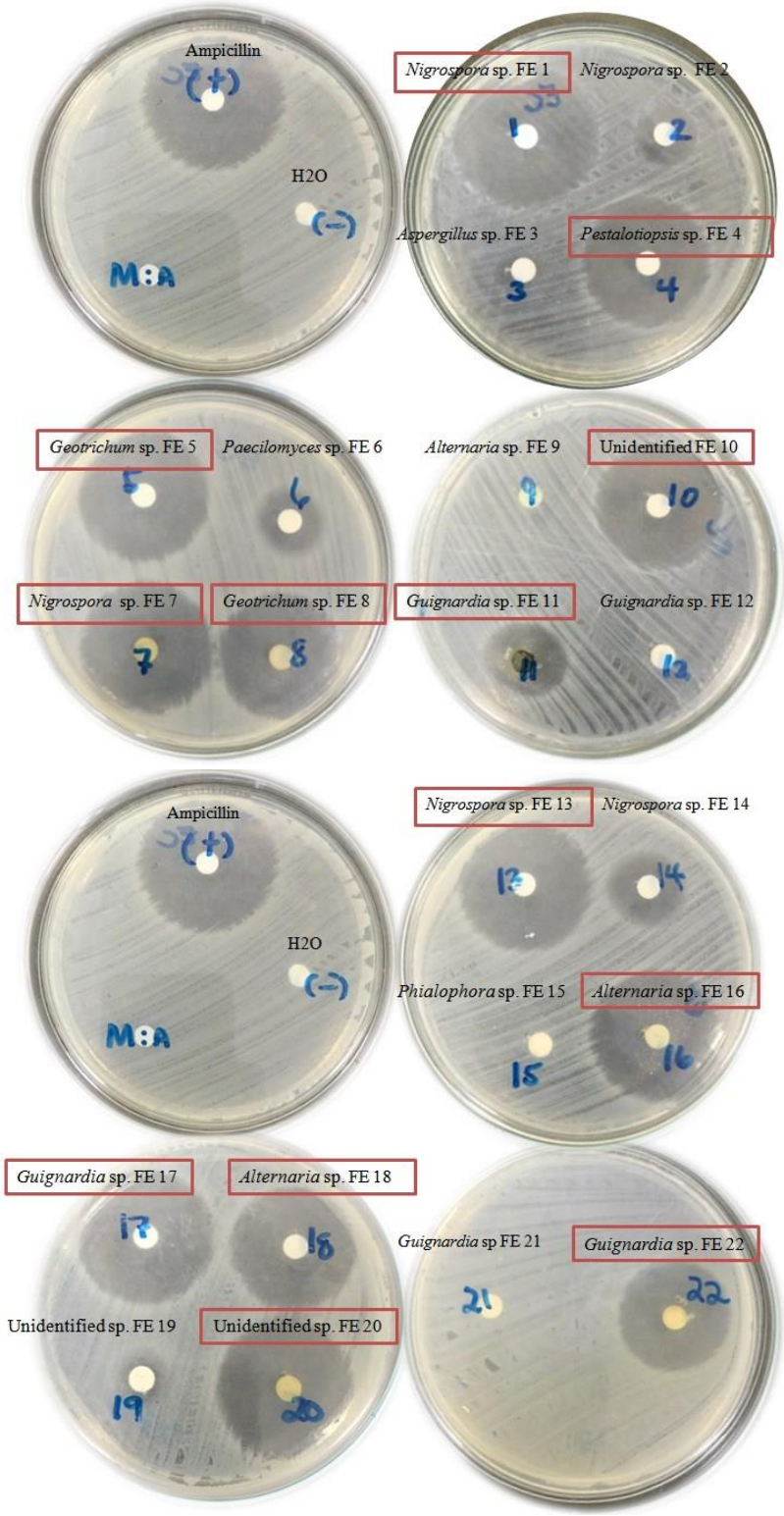

Figure 4. Zones of inhibition displayed by FE crude extracts against *E. coli*. Extracts with a high degree of activity are highlighted in red boxes

namely: *Nigrospora* sp. FE 1, *Nigrospora* sp. FE 7, *Nigrospora* sp. FE 13 (mean ZOI of 34.67 mm, 30.33 mm, and 25.67 mm respectively); *Pestalotiopsis* sp. FE 4 (mean ZOI of 33.33 mm), *Geotrichum* sp. FE 5 and *Geotrichum* sp. FE 8 (mean ZOI of 31.33 mm and 30.00 mm); *Guignardia* sp. FE 11, *Guignardia* sp. FE 17, *Guignardia* sp. FE 22 (mean ZOI 19.33 mm, 34.33mm, and 33.67

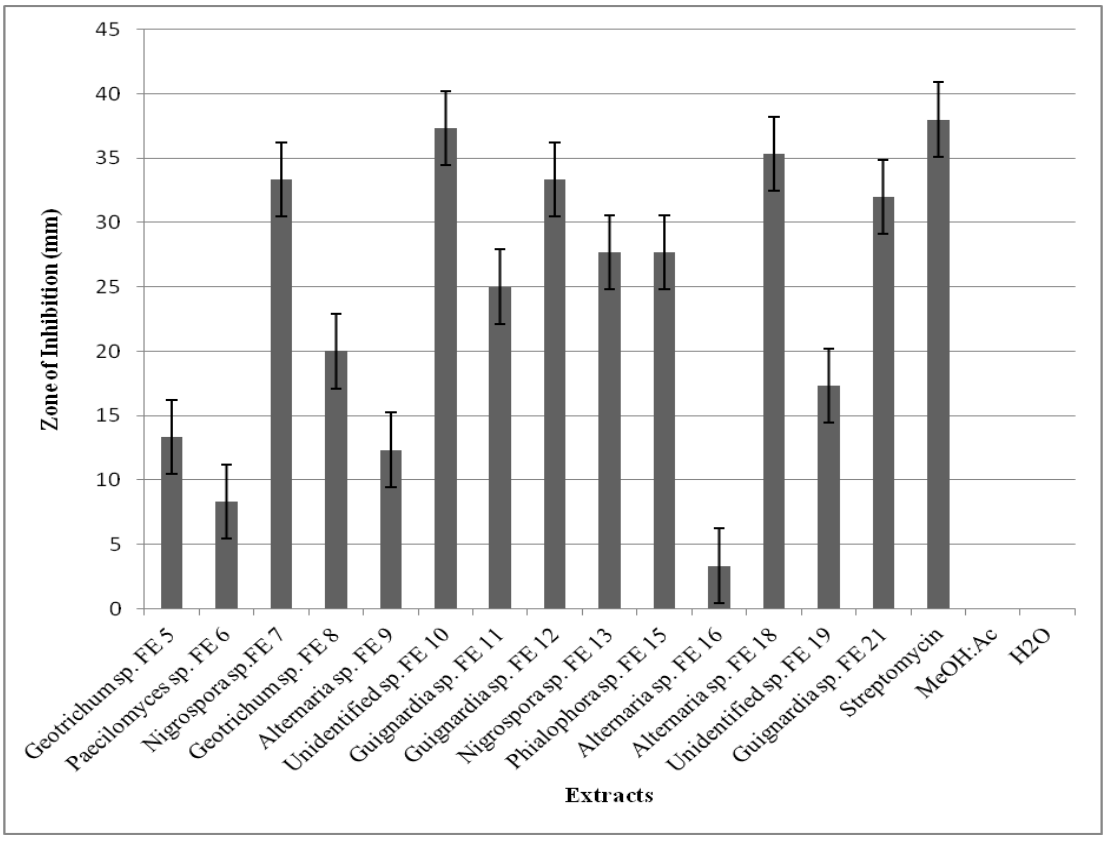

Figure 5. Antimicrobial activities of the FE crude extract against Gram-positive *Staphylococcus aureus*. Standard deviation can be observed as error bars (n=3)

mm); *Alternaria* sp. FE 16 and *Alternaria* sp. FE 18 (mean ZOI of 31.00 mm, and 22.67 mm) ; and two other Unidentified genera FE 10 and FE 20 with mean ZOI of 32.00 mm and 20.67 mm. *Phialophora* sp. FE 15 (mean ZOI of 16.33 mm) was classified as active, and *Paecilomyces* sp. FE 6 (mean ZOI of 10.00 mm) was classified as partially active (Figure 4).

Antimicrobial activities of FE against S. aureus

The results in Figure 5 demonstrates the antimicrobial activities against *S. aureus*, with partially active crude extracts from *Geotrichum* sp. FE 5 and *Alternaria* sp. FE9, an active crude extract from Unidentified FE 19, and highly active crude extracts from *Nigrospora* sp. FE 1, *Nigrospora* sp. FE 2, *Aspergillus* sp. FE 3, *Pestalotiopsis* sp. FE 4, *Nigrospora* sp. FE 7, *Geotrichum* sp. FE 8, Unidentified FE 10, *Guignardia* sp. FE 11, *Guignardia* sp. FE 12, *Nigrospora* sp. FE 13, *Phialophora* sp. FE 15, *Alternaria* sp. FE 18, and *Guignardia* sp. FE 21.

Interestingly, *Nigrospora* sp. FE 1, *Nigrospora* sp. FE 2, *Aspergillus* sp. FE 3, and *Pestalotiopsis* sp. FE 4 resulted in zones of inhibition (ZOI) that were too large to measure (Figure 6), suggesting the presence of potent antimicrobial compounds in these fungal endophyte isolates that are highly effective against *S. aureus*. It should be noted, however, that some plates exhibited pinpoint bacterial growth, which could be attributed to variations in swabbing techniques.

According to Figure 7, out of the 22 crude extracts that were tested against MRSA, seven were found to be highly active. These extracts included *Nigrospora* sp. FE 2 and *Nigrospora* sp. FE 7, which exhibited ZOI of 32.67 mm and 29.67 mm, respectively. *Pestalotiopsis* sp. FE 4 also showed high activity with a ZOI of 29.67 mm. Additionally, *Geotrichum* sp. FE 8, *Alternaria* sp. FE 9, *Alternaria* sp. FE 18, and *Guignardia* sp. FE 11 demonstrated significant activity with ZOI ranging from 20.00 mm to 31.00 mm. Overall, these seven crude extracts can be classified as “highly active” against MRSA.

Figure 8 illustrates that four of the tested extracts displayed antibacterial activity, with *Nigrospora* sp. FE 1, *Aspergillus* sp. FE 3, *Guignar-*

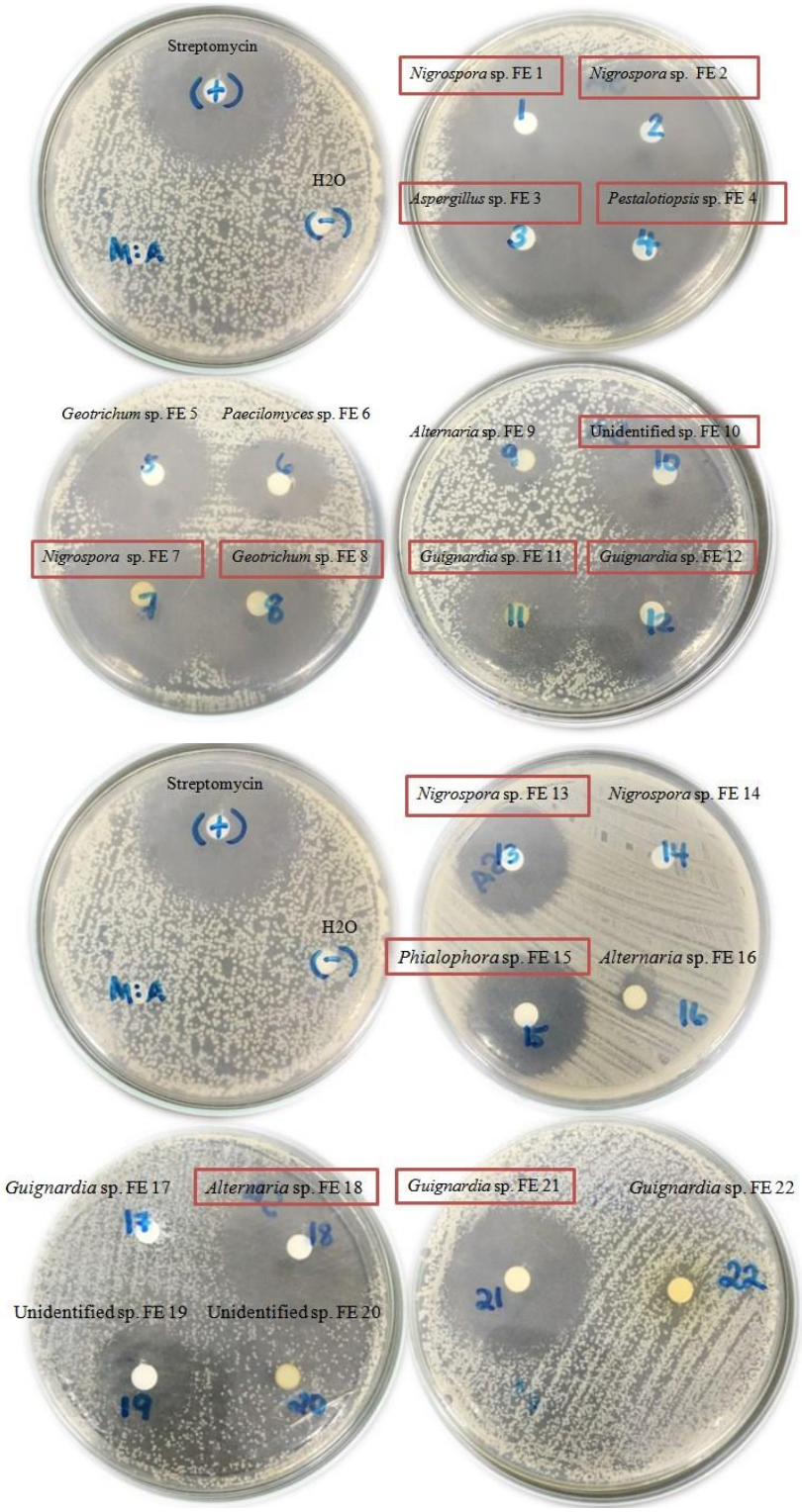

Figure 6. Zones of inhibition exhibited by FE crude extracts against *S. aureus*. Extracts classified as 'very active' are enclosed in red boxes.

dia sp. FE 12, and an unidentified species (FE 20) showing zones of inhibition (ZOI) measuring 17.00 mm, 16.00 mm, 15.33 mm, and 15.67 mm, respectively. Nonetheless, it is noteworthy that some plates in Figure 8 exhibited pinpoint bacterial growth, which may be attributed to inconsistencies in swabbing techniques.

Among the crude culture extracts, *Nigrospora*

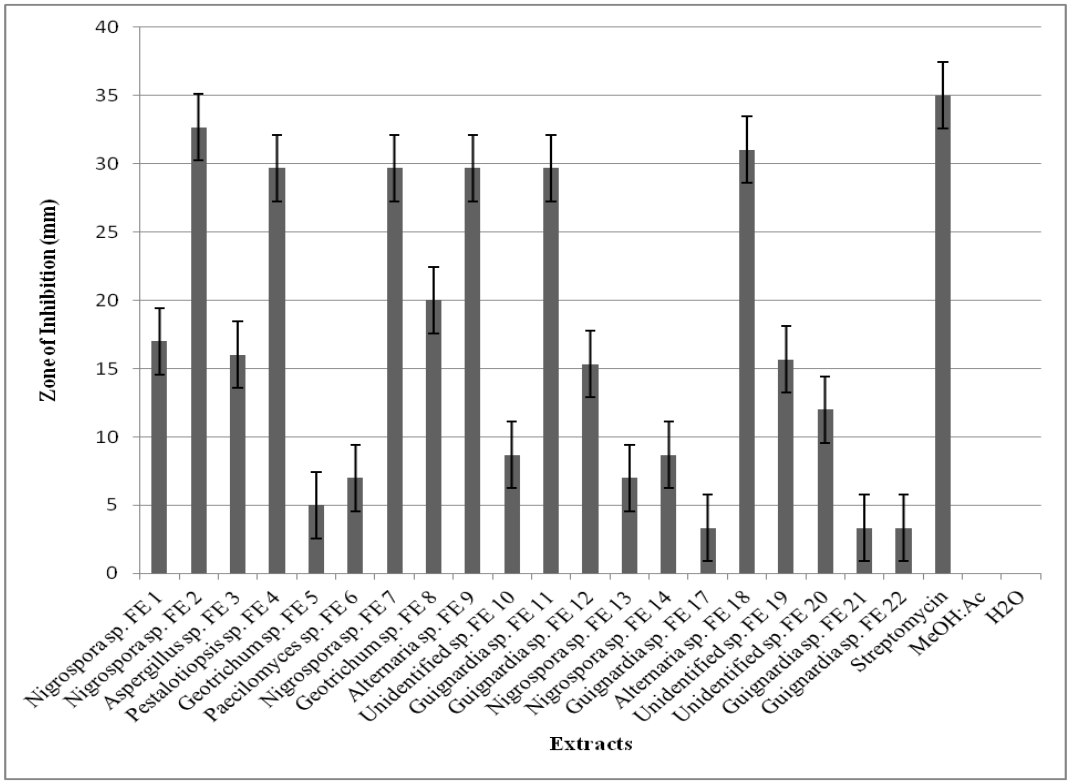

Figure 7. Antimicrobial activities of the FE crude extract against Gram-positive Methicillin-resistant *Staphylococcus aureus*. Standard deviation can be observed as error bars (n=3).

sp. FE 1, *Nigrospora* sp. FE 2, *Aspergillus* sp. FE 3, *Pestalotiopsis* sp. FE 4, *Nigrospora* sp FE 7., *Geotrichum* sp. FE 8, *Alternaria* sp. FE 9, *Guignardia* sp. FE 11, *Guignardia* sp. FE 12, *Alternaria* sp. FE 18, and Unidentified FE 19 were found to have broad-spectrum activity against Gram-positive and Gram-negative bacteria (mean ZOI ranging from 15 to 33 mm) with the exception of multi-drug resistant *P. aeruginosa*. In addition to the nature of the *P.* aeruginosa used in this study being a multi-drug resistant, it is possible that the metabolites were not active against the test microorganism. Hence, no activity was observed. These results suggest that *Dillenia philippinensis*-associated fungal endophytes do synthesize biologically active substances that can be considered as medically important metabolites.

According to a previous report [31], differences in the production of secondary metabolites exist among endophytes of the same species, and these differences are influenced by various factors, such as the host plant, season, and local environment. In another study [32], it was found that endophytic fungi from the genus *Guignardia* sp. exhibited antibacterial and antifungal activities. Similarly, *Pestalotiopsis* sp., a fungal endophyte isolated from *Eucalyptus exserta*, also displayed antimicrobial properties [33]. Variations in substrate location from which fungal endophytes were isolated may affect the amount and kinds of bioactive metabolites produced, leading to observed differences in the antimicrobial activity profiles of extracts from the same genus, as reported previously [12] for fungal endophytes isolated from *Canarium ovatu*m, another endemic plant in the Philippines.

Notably, previous reports on fungal endophytes from the Philippines [30, 34] also showed similar antibacterial profiles with similar endophytic fungi. Several studies that investigated associated fungal endophytes from mangroves and tested the inhibitory activity of their crude extracts also exhibited comparable antibacterial inhibitory patterns against the test microorganisms [30, 34].

These results suggest that similar species of endophytic fungi can be isolated from different substrate types such as leaves [30], roots [35], and stems [36] with the same bioactivity profiles. The fungal endophytes in this report can be further studied for bioactive compounds that are possible sources of novel drugs and antibiotic compounds for pharmaceutical use. In addition, this study also

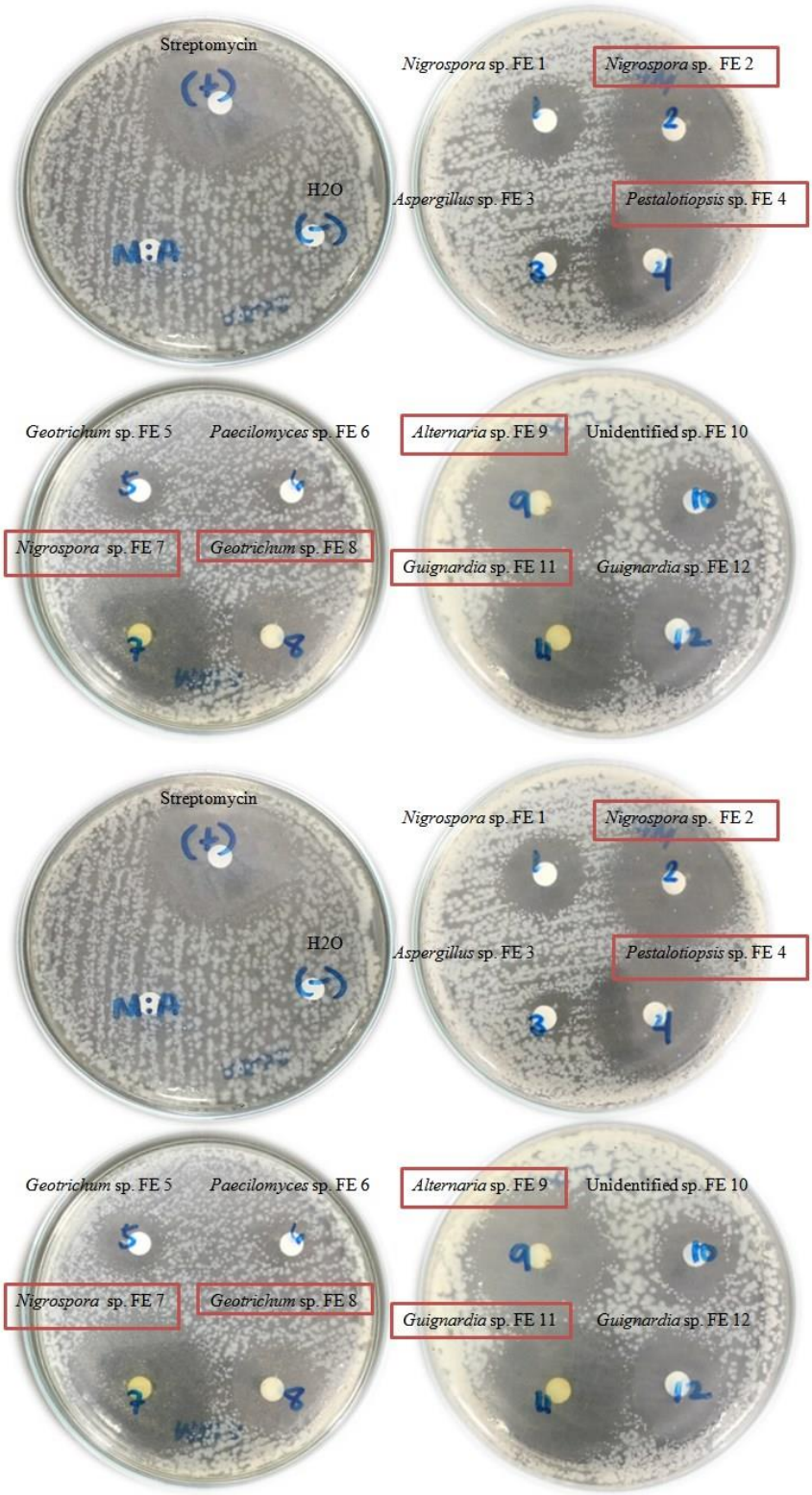

Figure 8. Zones of inhabitation exhibited by FE crude extracts against Methicillin-resistant *S. aureus*. Extracts classified as very active are enclosed in red boxes.

highlights the significance of the endemic plant, *D. philippinensis*, in harbouring fungal endophytes with various medicinal applications.

Conclusion

In conclusion, we successfully isolated fungal endophytes from the leaves of *D. philippinensis*,

and morphocultural characterization indicated that the fungal isolates belonged to eight different genera. Further testing against four different microorganisms revealed that several fungal endophytes, including *Nigrospora* sp. FE 1, *Nigrospora* sp. FE 2, *Aspergillus* sp. FE 3, *Pestalotiopsis* sp. FE 4, *Nigrospora* sp FE 7, *Geotrichum* sp. FE 8, *Alternaria* sp. FE 9, *Guignardia* sp. FE 11, *Guignardia* sp. FE 12, *Alternaria* sp. FE 18, and unidentified FE 19, demonstrated broad-spectrum activity against both Gram-positive and Gram-negative bacteria, with the exception of MRPA. These findings emphasize the potential of endophytic fungi from endemic plants as valuable sources of metabolites that can be further explored for the discovery of novel bioactive compounds.

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