

Mycopharmacological Properties of Endophytic Fungi Isolated from Cuban Oregano (*Plectranthus amboinicus* Lour.) Leaves

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ABSTRACT

Endophytic fungi reside internally and asymptotically in plant tissues and play significant role in the ecosystem. Medicinal plants, such as oregano (*Plectranthus amboinicus* Lour.), are valuable sources of important endophytic fungi which are known to confer several benefits to their hosts. In this study, endophytic fungi were isolated from the leaves of *P. amboinicus* and assessed for their mycopharmacological properties. The fungal endophytes were isolated then identified based on their morphological characteristics. They were subjected to qualitative mycochemical analysis and antibacterial screening. Three fungal endophytes were isolated from the leaves of Mexican oregano, all of which belong to the genus *Aspergillus*. The fungal endophytes were morphologically identified as *Aspergillus niger*, *Aspergillus tamarii* and *Aspergillus terreus*. Each endophytic fungus was determined to contain valuable mycochemical compounds such as alkaloids, flavonoids, saponins, tannins, glycosides, sterols, terpenoids, quinones and phenols which can be exploited for therapeutic development. In the antibacterial assay, preliminary screening using agar plug diffusion method revealed that the endophytes were able to impressively suppress the growth of both *Escherichia coli* and *Staphylococcus aureus*. The results were further affirmed by subjecting the ethanol extracts of the endophytes against the same test bacteria using the agar well diffusion method. This study shows the potential of these endophytic fungi for pharmaceutical exploitations.

Key words: Antibacterial, Endophytes, Indian borage, Mycochemical, Oregano.

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INTRODUCTION

Endophytic fungi are microbes that inhabit internal plant tissues without causing any apparent symptoms.^[1] These mutualistic microorganisms are believed to render beneficial metabolic interactions with their hosts such as pathogen resistance, increased nutrient uptake and resilience during extreme conditions.^[2-6] Plants used in traditional medicine have a very significant role in the search for new bioactive strains of endophytic fungi, as it is possible that the beneficial characteristics and properties of these medicinal plants

can be related with the metabolites produced by their endophytic community.^[7,8] *Plectranthus amboinicus*, known as Indian borage or Cuban thyme, is a widely utilized medicinal plant found commonly found in tropical countries such as India, Philippines and Cuba.^[9] Traditionally, this plant is used in variety of medicinal applications such as treating fever, cough and indigestion, nasal congestion and throat infection and constipation and digestive problems.^[10-12]

Resistance of microorganisms to drugs caused by the overuse of antibiotics is becoming a serious global concern. It demands the urgent search for new sources of antibiotics that are effective, cheap and have minimal environmental impact.^[13] Endophytes are relatively unstudied and offer potential origin of novel natural products for exploitation in medicine, agriculture and the pharmaceutical industry.^[14] Despite their huge biological potential, endophytes of many Philippine medicinal plants have remained unexplored, necessitating investigation in this field. Thus, the present study was

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carried out to evaluate the mycopharmacological properties endophytic fungi from the leaves of *P. amboinicus*.

MATERIALS AND METHODS

Collection of Plant Material

Mature and healthy leaf samples of *P. amboinicus* were collected during May 2018 from Echague, Isabela (16.6701° N, 121.7171° E). The authentication of plant materials was done on the basis of taxonomic characters through the assistance of an experienced botanist. Samples were transported in sterile polypropylene bags and processed within 6 hrs of collection.

Isolation of Endophytic Fungi

Surface sterilization of plant samples and isolation of endophytic fungi was done using the standard method with minor modifications.^[15] Leaves of *P. amboinicus* were washed and rinsed with running tap water and cut into 10 mm (length) by 5 mm (width) segments. Then, each segment was surface sterilized by sequential immersion in 75% ethanol for 2 min, 1% Sodium hypochlorite (NaOCl) for 3 min and then once again in 75% ethanol for 1 min. The leaf segments were finally rinsed three times in sterile distilled water to remove excess sterilant and blot dried in sterile filter paper. Afterwards, the leaf segments of *P. amboinicus* were inoculated onto Potato Dextrose Agar (PDA) plates supplemented with streptomycin (1 ml/L) to suppress bacterial growth. Four (4) leaf segments were equidistantly placed on each amended PDA plate. The plates were then sealed with parafilm and incubated at 28°C until the growth of endophytic fungi was detected. The hyphal tip of each endophytic fungi growing out from the leaf segments were separately transferred into new amended PDA plates and routinely maintained.

Identification and Characterization of Endophytic Fungi

Endophytic fungi were identified according to their macroscopic and microscopic characteristics such as the morphology of fruiting structures and spore morphology. Colony morphology of the endophytes was observed on Coconut Water Agar (CWA), Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA). To ascertain identification of species, microscopic examination of morphological structures was conducted using the agar block technique. The identification of fungi was done using the keys and descriptions of various literatures.^[16,17]

Mycelial Mat Production

Mycelial culture mats of each endophytic fungus were produced in a liquid culture using Coconut Water Broth (CWB) as medium. One hundred and fifty (150) containers with CWB were prepared to obtain at least 200 g of mycelial fresh weight for each endophytic fungus. The containers were inoculated with mycelial blocks using sterile inoculating needle. After which, they were incubated at 32°C for seven (7) to 14 days to allow mycelial growth. The resulting mycelial mats were harvested, oven-dried and pulverized for ethanolic extraction.

Preparation of Ethanolic Extract

Thirty (30) g of powdered mycelial sample of each endophytic fungus was soaked in 500 ml laboratory grade 95% Ethanol for 48 hr. The mixture was then filtered using Whatman filter paper No. 1 in an Erlenmeyer flask. The filtrate was refluxed using a rotary evaporator until a sticky residue is obtained. The resulting extract was used for the mycochemical analysis and assessment of antibacterial properties.

Mycochemical Analysis

The ethanol extracts of the fungal endophytes were screened qualitatively for the presence of mycochemicals in accordance with the prescribed method for mycochemical detection.^[18,19] Each test was qualitatively expressed as negative (–) or positive (+) and the intensity of the characteristic color or precipitate was expressed as +, ++ or +++. The different mycochemical tests undertaken were as follows;

a. Test for Alkaloids

Wagner's test: A fraction each extract was treated with Wagner's test reagent (1.27g of iodine and 2g of potassium iodide in 100 ml of distilled water) and observed for the formation of reddish-brown colored precipitate.

b. Test for Saponins

Foam test: A small amount of each extract was vigorously shaken with distilled water and observed for the formation of persistent foam.

c. Test for Tannins

Braymer's test: Each extract was treated with 10% alcoholic ferric chloride solution and observed for the formation of dark green, blue or brown color which indicates the presence of tannins.

d. Test for Glycosides

Keller-Kelliani test: Five (5) ml of the ethanolic extracts were mixed with two (2) ml of glacial acetic acid (CH₃CO₂H) containing one (1) drop of FeCl₃. The mixture was carefully added to a prepared one (1) ml of

concentrated sulfuric acid H_2SO_4 to form a lower layer. The presence of a brown ring at the interphase indicates presence of glycosides.

e. *Test for Flavonoids*

NaOH test: A small amount of each extract was treated with aqueous NaOH and HCl and observed for the formation of yellow orange color.

f. *Test for Terpenoids*

Salkowski's test: One (1) ml of chloroform was added to 2 ml of each extract and carefully added with a few drops of concentrated H_2SO_4 to form a lower layer. The formation of a reddish-brown precipitate indicates presence of terpenoids.

g. *Test for Sterols*

Liebermann–Burchard test: Extracts (1ml) were treated with chloroform, acetic anhydride and carefully added with drops of H_2SO_4 . Change of color to dark green indicates presence of sterols.

h. *Test for Quinones*

HCl test: A small amount of extract was treated with concentrated HCl and observed for the formation of yellow color precipitate.

i. *Test for Phenols*

FeCl₃ test: The extract was treated with 5% ferric chloride and observed for formation of deep blue or black color.

Antibacterial Assay

Source of Test Bacteria

Bacterial cultures of *E. coli* and *S. aureus* were used for the antibacterial assay. The bacteria were obtained from the culture collections of the Microbiology and Bio-Industry Laboratory, College of Arts and Sciences, ISU-Echague and were maintained in Nutrient Broth (NB) medium.

Agar Plug Diffusion Method

The endophytic fungi were subjected to preliminary screening through agar plug diffusion method.^[20] This method involved the use of mycelial agar discs as carrier of fungal exudates with antibacterial activities.

Fungal isolates of the endophytic fungi were grown separately on PDA plates without antibiotics and maintained for 14 days to allow the diffusion of fungal exudates to the agar medium. After incubation, the fungal isolates were immobilized using ultraviolet (UV) light inside a laminar flow hood for 15 mins. A 10-mm cork borer was then used to aseptically bore and obtain immobilized mycelial agar discs.

Meanwhile, 15 mL sterile Mueller-Hinton Agar (MHA) was aseptically dispensed into sterile Petri plates then

cooled prior to inoculation of bacterial samples. Bacterial cultures of *E. coli* and *S. aureus* were then spread thoroughly onto the MHA plates using sterile cotton swab. Afterwards, the mycelial agar discs were aseptically transferred onto the surface of MHA with inoculum. The assay was conducted in triplicates for each fungus. The zone of inhibition of each endophyte was observed and recorded every eight (8) hr within a 24hr incubation period using a calibrated digital Vernier caliper.

Agar Well Diffusion Method

The ethanolic extracts of the endophytes were further tested for their antibacterial activity using the agar well diffusion method. Petri plates containing 20 ml MHA were seeded with 24hr culture of the bacterial strains. A 10-mm cork borer was then used to equidistantly cut and remove four (4) agar plugs to create agar wells in each plate. Different cork borers were used for different test organisms. Afterwards, 50 μ l of each liquid treatment was carefully pipetted in each individual well. All plates were incubated at 37°C for 24 hrs and resulting zones of inhibition were observed and recorded every eight (8) hrs within the incubation period. Zones of inhibition were measured using a calibrated digital Vernier caliper. The assay was conducted in triplicates with the treatments as distilled water (T1-negative control), streptomycin sulfate (T2-positive control), laboratory grade 95% ethanol (T3) and ethanol extracts (T4).

Data Analysis

Each of the tests were carried out using Completely Randomized Design (CRD) with three replicates for each treatment. All the recorded data were treated statistically using one-way Analysis of Variance (ANOVA). The means were compared by Tukey's Honest Significant Difference test at $p < 0.05$ using IBM™ SPSS v25.

RESULTS

All plants in natural ecosystems appear to be symbiotic with fungal endophytes. Three fungal endophytes belonging to genus *Aspergillus* were isolated and identified from the leaves of *P. amboinicus*, namely *A. tamarii*, *A. niger* and *A. terreus*. The obtained microscopic descriptions of the *Aspergillus* endophytes coincide with the existing descriptions. The morphology and growth characteristics of the *Aspergillus* endophytes on CWA, PDA and MEA were summarized in Table 1 and Figures 1,2.

The colony color of the *A. tamarii* endophyte ranges from yellow to olive green turning dark green to brown with age. Formation of conidial heads are more

Table 1: Macroscopic and microscopic characteristics of the *Aspergillus endophytes*.

Endophytic Fungi	Macroscopic Characteristics				Microscopic Characteristics		
	Culture Media	Colony Color	Reverse Color	Colony Density	Shape of Vesicle	Texture of Conidia	Seriation
<i>A. tamarii</i>	CWA	Brown-green	White	Abundant	Sub-globose	Smooth/Finely roughened	Biseriate
	PDA	Parrot green	White	Luxuriant			
	MEA	Yellow	Light yellow	Abundant			
<i>A. terreus</i>	CWA	Beige	Tan	Sparse	Pyriform	Smooth	Biseriate
	PDA	Cinnamon	Brown	Sparse			
	MEA	Cream yellow	Yellow orange	Abundant			
<i>A. niger</i>	CWA	Grey	White	Luxuriant	Globose	Echinulate	Biseriate
	PDA	Black	Cream	Abundant			
	MEA	Brown-black	Black	Luxuriant			

Legend: CWA = Coconut Water Agar; PDA = Potato Dextrose Agar; MEA = Malt Extract Agar

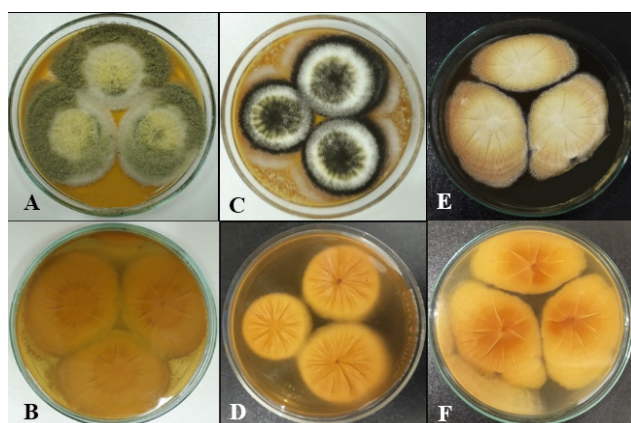


Figure 1: Colony morphology of (A, B) *A. tamarii*, (C, D) *A. niger* and (E, F) *A. terreus* on PDA after 1 week of incubation.

prominent towards the center of the mycelia. The margin and form are both filamentous and elevation is slightly raised. Production of colorless exudates and brown-black sclerotia was observed in various plates after extended incubation. Conidial head is radiate to loosely columnar with long and hyaline stipe. Vesicle is globose to sub-globose. Conidia are smooth to finely roughened and occurs in chains with yellowish green color.

Colony morphology of *A. niger* has a distinct black-brown colony. The colonies initially grow with felt-like yellow to white hyphae, turning black with the formation of conidia. It shows both filamentous on margin and form and umbonate elevation. Formation of black sclerotia and black exudate beads was observed. Conidial head is globose, splitting with age. Stipe is long, smooth and hyaline. Vesicles is globose and thick-walled. Conidia are globose to elliptical, echinulate and in chains. Colonies of *A. terreus* has a distinct beige to cinnamon color. Mycelia are initially floccose white which eventu-

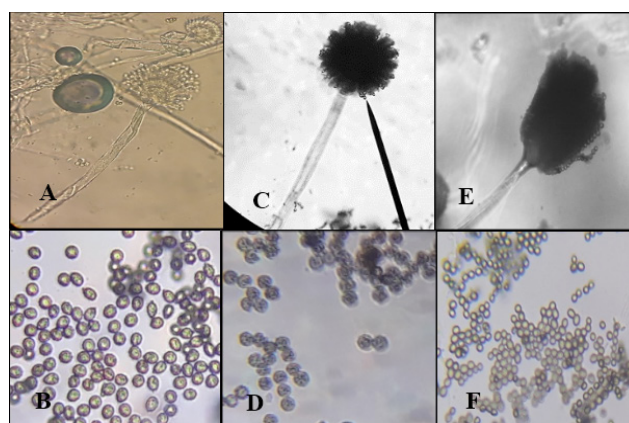


Figure 2: Conidiophore and conidia of (A, B) *A. tamarii*, (C, D) *A. niger* and (E, F) *A. terreus*.

ally turns brown to yellow-brown consisting of a dense felt of conidiophores. Reverse morphology is brownish to orange in color, indicating the secretion of metabolites into the medium. Sclerotia is not present. Conidial heads are highly columnar with short and smooth stipe. Vesicle is pyriform to hemispherical. Conidia are globose and smooth, occurring in long chains which makes a compact column.

Mycochemical Analysis

The results of the mycochemical analyses for the detection of alkaloids, saponins, tannins, glycosides, flavonoids, terpenoids, steroids, quinones and phenols are presented in Table 2.

The mycochemical analysis for the ethanolic extracts of *A. tamarii* revealed that tannins and phenols were abundantly present while saponins were moderately present in the extracts. Flavonoids, terpenoids, sterols and quinones were present in traceable amounts but glycosides and alkaloids were determined to be absent. Mycochem-

ical screening of *A. niger* ethanolic extracts showed that tannins and terpenoids were found to be abundantly present while phenols were moderately present and alkaloids, sterols and quinones were traceably present. Glycosides, saponins and flavonoids were absent from the extracts. The ethanolic extracts of *A. terreus* showed the abundance of alkaloids, tannins and phenols. Terpenoids and sterols were moderately present while saponins and quinones were detected in traces. Glycosides and flavonoids were not detected.

Antibacterial Assay

The antibacterial activities of the endophytic fungi against *E. coli* and *S. aureus* are presented in Table 3. For the test on antibacterial efficacy against *E. coli*, *A. flavus* exhibited the largest zone of inhibition after 24 hrs with 24.91 ± 0.53 mm, followed by *A. terreus* with 24.05 ± 1.782 mm of inhibition and *A. niger* with 21.87 ± 0.49 mm. The zones of inhibition produced by the endophytes against *E. coli* followed an upslope pattern during the 24hr

incubation period it gradually increased with the incubation period. Statistical analysis of results after 24 hrs indicated that *A. flavus* and *A. niger* were significantly different while *A. terreus* is statistically comparable with the results of *A. flavus* and *A. niger* Figure 3.

The zones of inhibition against *S. aureus* indicate that *A. niger* has the most potent inhibitory property against the Gram-positive bacteria with 34.90 ± 0.71 mm of mean diameter. Moreover, *A. terreus* and *A. flavus* also inhibited bacterial growth by 32.72 ± 1.19 mm and 32.22 ± 1.11 mm respectively.

Although the agar plug diffusion assay had produced results which are indicative of antibacterial properties of these endophytic fungi, further confirmatory test was done through agar well diffusion method using ethanolic extracts from cultured mycelial mats of the three fungal isolates. The zones of inhibition generated by the endophytes are shown in Table 4.

Table 2: Mychochemical constituents of the ethanol extracts of the endophytes.

Mycochemical	Endophyte Extracts		
	<i>A. tamarii</i>	<i>A. niger</i>	<i>A. terreus</i>
Alkaloids	-	+	+++
Saponins	++	-	+
Tannins	+++	+++	+++
Glycosides	-	-	-
Flavonoids	+	-	-
Terpenoids	+	+++	++
Sterols	+	-	++
Quinones	+	-	+
Phenols	+++	++	+++

Legend: (-) absent, (+) traceably present, (++) moderately present, (+++) abundantly present

Table 3: Zones of inhibition of the mycelial agar plugs against *E. coli* and *S. aureus*.

Mycelial Agar Plug	Zone of Inhibition (mm)		
	8 hrs	16 hrs	24 hrs
<i>E. coli</i>			
<i>A. tamarii</i>	22.41 ± 2.00^a	24.15 ± 0.46^b	24.91 ± 0.53^b
<i>A. terreus</i>	21.38 ± 1.31^a	24.02 ± 1.57^b	24.05 ± 1.78^{ab}
<i>A. niger</i>	21.17 ± 1.07^a	21.64 ± 0.49^a	21.87 ± 0.49^a
<i>S. aureus</i>			
<i>A. tamarii</i>	21.67 ± 0.93^a	31.96 ± 0.85^a	22.22 ± 1.11^a
<i>A. terreus</i>	24.38 ± 1.00^{ab}	34.00 ± 1.19^b	34.05 ± 1.15^{ab}
<i>A. niger</i>	25.91 ± 1.55^b	34.81 ± 0.74^b	34.90 ± 0.71^b

Note: Values are means of three replications. Means in the same column not sharing the same superscript are significantly different at 5% significance level.

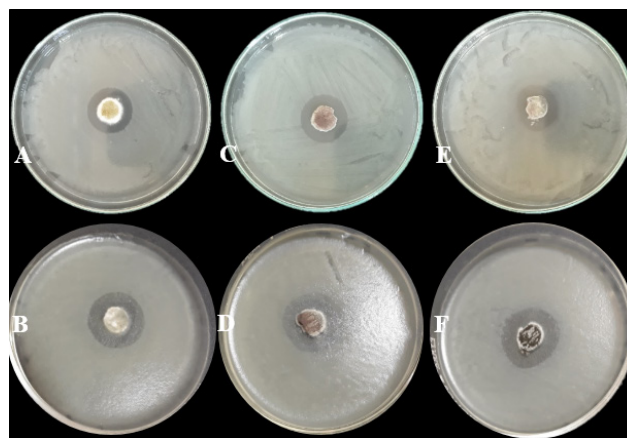


Figure 3: Antibacterial activities of (A, B) *A. tamarii*, (C, D) *A. terreus* and (E, F) *A. niger* mycelial agar plugs against (A, C, D) *E. coli* and (B, D, F) *S. aureus*.

Table 4: Zones of inhibition of the ethanol extracts against *E. coli* and *S. aureus*.

Endophytic Fungi	Treatment	Zone of Inhibition	
		<i>E. coli</i> (mm)	<i>S. aureus</i> (mm)
<i>A. tamarii</i>	Distilled water	11.00±0.00 ^a	11.00±0.00 ^a
	Streptomycin	28.19±3.77 ^b	34.39±0.66 ^b
	Ethanol	11.00±0.00 ^a	11.00±0.00 ^a
	Ethanol Extract	27.80±2.52 ^b	29.83±0.97 ^c
<i>A. terreus</i>	Distilled water	11.00±0.00 ^a	11.00±0.00 ^a
	Streptomycin	32.18±1.57 ^b	34.94±1.75 ^b
	Ethanol	15.53±7.85 ^a	11.00±0.00 ^a
	Ethanol Extract	18.45±1.06 ^a	18.81±0.65 ^c
<i>A. niger</i>	Distilled water	11.00±0.00 ^a	11.00±0.00 ^a
	Streptomycin	29.12±2.21 ^b	34.20±0.09 ^b
	Ethanol	11.00±0.00 ^a	11.00±0.00 ^a
	Ethanol Extract	12.79±0.57 ^a	12.21±0.31 ^a

Note: Values are means of three replications. Means in the same column not sharing the same superscript are significantly different at 5% significance level.

Among the three endophytes, *A. flavus* exhibited the greatest antibacterial activities followed by *A. terreus* and *A. niger* against both *E. coli* and *S. aureus*. The ethanolic extracts of *A. flavus* produced mean inhibition of 27.80±2.52 and 29.83±0.97 for *E. coli* and *S. aureus* respectively. Meanwhile, the ethanolic extracts of *A. terreus* exhibited zones of inhibition of 18.45±1.06 for *E. coli* and 18.81±0.65 for *S. aureus*. On the other hand, *A. niger* showed relatively smaller inhibitory zones of 12.79±0.57 and 12.21±0.31 against *E. coli* and *S. aureus* respectively.

Statistical analysis revealed that the ethanolic extracts of *A. flavus* against *E. coli* were statistically comparable with that of streptomycin, suggesting that the efficacy of the extracts against the bacteria was relatively similar with the antibiotic. Meanwhile, the extracts of *A. flavus* against *S. aureus* were significantly different with other treatments.

DISCUSSION

The study yielded positive results on the mycochemical screening and antibacterial assay for the three endophytes. Fungi of the genus *Aspergillus* produce important metabolites that are of considerable interest to the scientific research community.^[21,22] Cultures of *A. niger* have indicated the presence of alkaloids, steroids, terpenoids, phenols, glycosides.^[23] Gluconic and fumaric acids have also been produced with *A. niger* and it has become a source of a variety of enzymes that are well established as technical aids in several industries.^[24] Furthermore, a metabolite called asterriquinone (ASQ), an antitumor

agent, has been isolated from the mycelium of *A. terreus*.^[25] An endophytic strain of *A. terreus* from mangroves which contains various bioactive secondary metabolites has also been elucidated.^[26]

The antimicrobial properties of secondary metabolites derived from various groups of fungi are widely reported and have been playing a promising role in the search for novel drugs.^[27] The results acquired from this study are in congruence with other studies from various authors. Fungal endophytes have been recognized as a repository of novel compounds of immense value in agriculture, industry and medicine. Some endophytic fungi have also been found to produce similar medicinal compounds to that of the host.^[14] Isolates of *A. terreus* and *A. niger* showed great zones of inhibition against *S. aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *E. coli*.^[13,28] Similarly, *A. niger* also possessed antimicrobial properties as resolved by different studies.^[23,29]

The production of antibacterial substances by strains of *Aspergillus* has been observed by a number of workers. Several studies of the bactericidal and bacteriostatic activities of *A. niger* have supported the potential of the fungus as source of antibiotic compounds.^[30] It was also determined that compounds isolated from broth extract of *A. niger* have high degree of antibacterial activity against bacterial organisms.^[31] A handful of congruent studies on the antibiotic activities of *A. terreus* have also been conducted. An active sesquiterpene named terrecyclic acid A was isolated from *A. terreus* in 1986, which exhibits cytotoxic activity against human cancer

lines and are also active antimicrobial compounds.^[32] A terrestrial species of *A. terreus* have shown antibacterial activities against *S. aureus*, *E. faecalis*, *B. subtilis*, *P. aeruginosa* and *E. coli* with promising results.^[33] This was further affirmed wherein *A. terreus* var. *africanus* was identified as a potent isolate which possess both cytotoxic and antibacterial activities.^[34]

CONCLUSION

The results obtained in this study indicates the potential of endophytic fungi isolated from *P. amboinicus* as sources of novel natural products with possible applications in medicine, agriculture and the pharmaceutical industry. Furthermore, it is possible that endophytic strains of *Aspergillus* species play a crucial role in the production of beneficial chemical compounds by *P. amboinicus* and contribute in the medicinal attributes of the plant, as endophytic fungi play important physiological and ecological roles in the life of their hosts.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in the subject matter or materials discussed in this manuscript.

ABBREVIATIONS

CWA: Coconut Water Agar; **PDA:** Potato Dextrose Agar; **MEA:** Malt Extract Agar; **ANOVA:** Analysis of Variance; **ASQ:** Asterriquinone.

SUMMARY

This study aimed to determine the mycopharmacological properties of endophytic fungi from Cuban Oregano. Three fungal endophytes namely: *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus tamarii* were determined to inhabit the leaves of the plant. The endophytic fungi showed varying presence of mycochemicals. Furthermore, ethanolic extracts of each respective fungi exhibited positive results on the antibacterial assay against *E. coli* and *S. aureus*.

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