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Exploring medicinal plant associated fungal endophytes

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Abstract Fungal endophytes play a vital ecological role in terms of plant defense from different environmental stresses. Fungal communities in the healthy leaves of kown Philippine medicinal plants, *Ocimum sanctum* and *Plectranthus amboinicu*s were isolated and identified morphologically through agar-block method. Active mycochemical properties were also determined qualitatively subjecting each isolate to mycelial mat production and ethanolic extraction. Presence of lapachol, a natural napthoquinone was also evaluated. Fungal isolates positive for lapachol were subjected to antibacterial assay using standard microbiological procedures. Eight endophytic fungal species belonging to four different families were isolated and identified from *O. sanctum* leaves namely *Lasiodiplodia* sp., *Cladosporium cladosporioides*, *Aspergillus terreus*, *Aspergillus ustus*, *Alternaria alternata*, *Fusarium vertillioides*, *Penicillium chrysogenum*, and Mycelia sterilia. On the other hand, five species and the sterile mycelia were isolated and identified from *P. amboinicus* leaves including *Aspergillus tamarii*, *Aspergillus terreus*, *Aspergillus niger*, *Penicllium oxalicum*, and Mycelia sterila. Qualitative mycochemical analyses exhibited that ethanolic extracts of each fungal isolates contain active biochemical compounds like anthraquinone, tannins, saponins, flavonoids, glycosides, alkaloids, terpenes, and sterols which can further be utilized for therapeutic and cosmeceutical development. It was recorded that the mycochemical properties exhibited by *A. terreus* differed from each plant species. Lapachol was detected positive among *A. alternata*, *A. niger* and A. *terreus* both from *O. sanctum* and *P. amboinicus*. Antibacterial assay showed potential inhibitory activities against *E. coli* and *S. aureus*. This current study shows the potential of these endophytic fungi for pharmacologic evaluations.

Keywords: Endophyte fungi, Lapachol, Medicinal plants, Secondary metabolites

Introduction

Endophytes are microbes residing inside plant tissues causing no harm or symptoms of pathogenicity. They live either internally or within the

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intracellular spaces of the host plants that include roots, stems, leaves, flowers, and fruits. Commonly, endophytic microbes are considered symbionts with the plant host. Frequently, most isolated fungi comprise separate subspecies having both endophytic and pathogenic properties (Schouten, 2019). While most pathogenic microorganisms produce toxins harmful to the host plant, the secondary metabolites produced by endophytic strains contain antimicrobial agents that protect their host plants by inhibiting pathogenic microorganisms (Schouten, 2019; Ludwig-Muller, 2019). Endophytes can also control the expansion and development of host plants (Neilson *et al.,* 2013; Martinez-Klimova *et al.,* 2017; Schouten, 2019; Ko Ko *et al.,* 2011; Rustamova *et al.,* 2020b).

Endophytic microorganisms are known over the years. However, reports on these organisms and their bioactive compounds received little to no attention until the past decade. Endophytes are considered as sources of bioactive natural products. As endophytes live together with a eukaryotic host, these microbes are less potent to form toxic products or harm host tissues. The bioactive compounds that they create to safeguard the host are proven helpful to humans, medicine, agriculture, and other industries (Campos and Jacob, 2021; Campos *et al.,* 2019). In the past years, the biochemistry of plant-associated endophytic fungi has materialized as focuses of progressively essential importance to our understanding of plant and microbial ecosystems and their multiple applications in pharmaceutical industries. In the Philippines, medicinal plants play a vital role on folkloric medicine. Medicinal plants including *Plectranthus amboinicus*, locally known as "oregano" and *Ocimum sanctum*, vernacularly known as "biday" are widely used plant in tropical countries such as India, the Philippines, and Cuba. Traditionally, these plants are used in various ailments such as colds, cough, diarrhea, nasal congestion, throat infections, constipation, and digestive problems (Rout and Panda, 2010; Cohen, 2014).

Understanding the mechanism of these fungi associated with medicinal plants leads to an improved knowledge of their phytochemistry, including isolation, analysis, synthesis, biotransformation, and various fungal components. Meanwhile, biological studies permit development in systems of strain isolation, microbiological characterization, and understanding of mechanisms of biological diversity and plant-fungal relationship (Caroll *et al.*, 2019; Rusatmova *et al*., 2020a). The study aimed to investigate the primary fungal endophytes presented from the two medicinal plants, determined their bioactive compounds, and evaluated its bioactivities. This study would further contribute to a role of fungi as natural products and new knowledge towards drug discovery.

Materials and methods

Collection of plant materials

Mature and healthy leaf samples of *Ocimum sanctum* (biday) and *Plectranthus amboinicus* (oregano) were collected inside Isabela State University, Echague, Isabela Philippines in early morning. An experienced botanist assisted in the authentication of plant materials using taxonomic keys. The researchers transported the leaf samples back to the laboratory, stored them in a sterile polypropylene bag, and immediately processed them 24 to 48 hours after collection.

Isolation of endophytic fungi

Leaves of each medicinal plant were initially washed with running tap water to remove dirt and attached debris. Leaf explants with a diameter of 6 mm were surface sterilized through sequential washing of 75% ethyl alcohol (EtOH) for one minute, 5% commercial bleach (NaClO) for 3 minutes, and 75% EtOH for another 30 seconds, then lastly washed with distilled water three times. For each host medicinal plant, five surface-sterilized leaf explants were plated on each of the six potato dextrose agar (PDA) plates supplemented with 500 mg/L streptomycin sulfate to inhibit the growth of leaf-associated bacteria. Tissue imprints were prepared by touching leaf fragments on agar plate for 10 seconds to test the efficacy of the surface sterilization method. The absence of any fungal or bacterial growth on the tissue imprints indicated an effective surface sterilization. The plates were then incubated at room temperature (30 ℃) and checked for fungal growth daily for five to seven days. Observation was extended up to a month to isolate fungi which grew even after one week. All fungi growing out of the leaf explants were subcultured for isolation on an agar slant.

Identification of endophytic fungi

Endophytic fungi associated with the leaves of “biday”, and “oregano” were identified based on their macroscopic and microscopic characteristics, such as structural and spore morphology. The colony morphology of the isolated endophytes was observed on Coconut Water Agar (CWA), Potato Dextrose Agar (PDA), and Malt Extract Agar (MEA) using a three-point inoculation method. Microscopic examination of morphological structures was conducted using the agar block method. Fungal identification was made using

the keys and descriptions provided by Quimio and Hanlin (1999) and Samson *et al*. (2014).

Production of fungal mats and ethanolic extraction

Mycelial mats of each isolated endophytic fungus were produced in a liquid culture using Coconut Water Broth (CWB). Approximately 150 microwavable containers with 100 ml coconut water broth were prepared to obtain at 150-200g of fresh mycelia. Each sterile container was inoculated with fungal blocks using a sterile inoculating needle. These were then incubated at 32 ℃ for seven to 14 days to allow mycelial proliferation. Mycelial mats were harvested, oven-dried at 40 ℃, and pulverized prior for subjecting to solvent extraction. The previously prepared fungal mats were pulverized using a sterile mechanical grinder. A total of 50g pulverized endophytic fungi was soaked in a 500-ml laboratory-grade 95% ethanol for 48 hours. It was then vacuum filtered and was subjected to a rotary evaporator at 400rpm at 40 ℃ for two hours or until a sticky residue was obtained.

Determination of active myco-chemical compounds

The ethanolic extracts of each fungal endophyte were analyzed for the presence of the active mycochemical compounds using the following standard methods by Sofowara (1993) modified by Jacob and David (2016).

Test for anthraquinones

Approximately 10 ml of benzene was added in 6g of the pulverized sample in an Erlenmeyer flask, soaked for 10 minutes, then filtered. Meanwhile, 10 ml of 10% ammonia solution was added to the filtrate while shaking vigorously for 30 seconds. A resulting pink, violet, or red color indicated the presence of anthraquinones in the ammonia phase.

Test fortannins

10 ml of bromine water was added to 0.5 g ethanolic extract of each sample. Clearing of bromine water confirmed the presence of tannins.

Test for saponins

5 ml of distilled water was mixed with ethanolic extract of the sample in a test tube while shaking vigorously. The resulting froth was then mixed with 2-3 drops of olive oil and while shaking vigorously. The appearance of stiff foam determined the presence of saponins.

Tests for flavonoids

Shinoda test was done using pieces of magnesium ribbons and concentrated HCl which were carefully mixed with the ethanolic extract of the sample. Pink coloration indicated the presence of flavonoids.

Alkaline reagent test was done using 2 ml of 2.0% NaOH mixture which was mixed with ethanolic extract of the sample, when a deep yellow color was produced, and added two drops of dilute HCl to the mixture. A colorless result showed the presence of flavonoids.

Tests for glycosides

Keller-Kiliani Test was done by 4-ml glacial acetic acid that added with one drop of 2.0% $FeCl^3$. The resulting mixture was then added with 10 ml ethanolic extract of the sample and 1ml concentrated H_2SO_4. A formation of brown rings between the layers indicated the presence of cardiac steroidal glycosides.

Test for alkaloids

Wagner's tes was done using a fraction of each ethanolic extract that treated with Wagner's reagent, which contained 1.27 grams of iodine and 2 grams of potassium iodide in 100 ml of distilled water. The formation of a reddish-brown colored precipitate confirmed the presence of alkaloids.

Test for terpenoids

Salkowki's test was done using one ml of chloroform which carefully added to 2 ml of each extract while adding 2-3 drops of concentrated H^2SO_4 to form a lower layer. The formation of a reddish-brown precipitate indicated the presence of terpenoids.

Test for phenols

$FeCl_3$ test was done by the ethanolic extract of each endophyte treated with 5% ferric chloride. Deep blue or black coloration confirmed the presence of phenols.

Lapachol detection assay

Methods on the identification of lapachol (Napthaquinone) was carried out using the modified protocols of Thomson (1987), as cited by Sadananda *et al.* (2011). Dried endophytic fungal mats were soaked and extracted with ethyl acetate. Subsequently, 1g of the resulting extract of endophytic fungi was then re-crystallized in petroleum ether and benzene using an 80:60 ratio. These were

then heated at 135-140 ℃ for 5 minutes. After which, 2-ml ferric chloride solution was added and observed for a yellow color that confirmed the presence of Napthaquinone.

Antibacterial activity assay

Only those fungal isolates tested positive for lapachol were further tested for their antibacterial activity via the agar well-diffusion method. Petri plates containing 15-20 ml Mueller-Hinton Agar (MHA) were inoculated with a 12-hour culture of test organisms, *E. coli* and *S. aureus* with three replicates. Then, a 5-mm cork borer was then used to cut and remove four equidistantly circular agar plugs, creating wells in each plate. Different cork borers were used for different test organisms. Then, 50 µl of each liquid extract was carefully pipetted in four wells on each plate. Plates were then incubated at 30 ℃ for 24 hours. Zones of inhibition were observed and recorded every eight hrs within the incubation period. Treatments were analyzed using one-way nalaysis of variance (ANOVA).

Results

Isolation and identification of endophytic fungi

The identified fungal endophytes from the leaf samples of *O. sanctum* and *P. amboinicus* is shown on Table 1. A total of 28 endophytic fungal isolates belonging to five genera and a mycelia sterila group were identified from the two medicinal plants. Genus *Aspergillus* recorded the highest number of isolated fungi including *A. terreus, A. ustus, A. tamarii*, and *A. niger* It was followed by Genus *Penicillium* with two species namely, *P. chrysogenum* and *P. oxalicum*. Other isolated fungal endophytes were *Lasiodiplodia* sp., *C. cladosporioides*, and *A. alternata*. Moreover, mycelia sterila was represented by two isolates.

Mycochemical analysis

Among the 10 isolates, anthraquinone was found to be present on all the isolates, except on *C. cladosporioides* and *F. verticillioides* (Table 2). Tanins were absent on the two isolates namely *F. verticillioides* and Mycelia sterila and presented on the other eight isolates. Saponins were also presented among the eight isolates but absent in *A. alternata* and *A. ustus*. Moreover, the presence of flavonoids was appeared, except on *A. terreus* and *A. ustus*. Moreover, glycosides did not persist on all the isolates, and was found to be

absent in *A. niger*, *A. terreus*, *A. ustus* and *P. oxalicum*. Alkaloids were absent within the four isolates namely, *A. niger*, *C. cladosporioides*, *Lasioplodia sp*. and *P. oxalicum*. Four from the isolates did not exhibit terpenes including *A. alternata*, *C. cladosporioides*, *F. verticillioides* and Mycelia sterilia. Further, sterols were presented on seven isolates but absent on *A. alternata, P. oxalicum,* and *M. sterila*. Interestingly, the active mycochemicals exhibited by *A. terreus* from *O. sanctum* differed from that of *P. amboinicus* as shown in the table.

Table 1. List of endophytic fungi isolated from the leaves of *O. sanctum* and *P. amboinicus*

	Ocimum sanctum		
Isolates	**Species count**	**Family**	**Fungal species**
OSL1	3	*Botryosphaeriaceae*	*Lasiodiplodia sp.*
OSL2	2	*Cladosporiaceae*	*Cladosporium cladosporioides*
OSL3	1	*Trichocomaceae*	*Aspergillus terreus*
OSL4	2	*Trichocomaceae*	*Aspergillus ustus*
OSL5	2	*Trichocomaceae*	*Alternaria alternata*
OSL6	3	*Nectriaceae*	*Fusarium verticillioides*
OSL7	4	*Trichocomaceae*	*Penicillum chrysogenum*
OSL8	1		Mycelia sterila
	Plectranthus amboinicus		
PA1	2	*Trichocomaceae*	*Aspergillus tamarii*
PA2	3	*Trichocomaceae*	*Aspergillus terreus*
PA3	2	*Trichocomaceae*	*Aspergillus niger*
PA4	2	*Trichocomaceae*	*Penicillum oxalicum*
PA5	1		Mycelia sterila

Table 2. Results of the mychochemical analysis of endophytic fungi ethanolic extracts

Fungal isolates	**Mycochemicals**							
	Anth	***Tan***	***Sap***	***Fla***	***Gly***	***Alk***	***Ter***	***Ste***
A. alternata	+	+	-	+	+	+	-	-
A. niger	+	+	+	+	-	-	+	+
***A. terreus* (OS)**	+	+	+	-	-	+	+	+
***A. terreus* (PA)**	+	+	+	+	-	-	-	+
A. ustus	+	+	-	-	-	+	+	+
C. cladosporioides	-	+	+	+	+	-	-	+
F. verticillioides	-	-	+	+	+	+	-	+
***Lasioplodia* sp.**	+	+	+	+	+	-	+	+
P. chrysogenum	+	+	+	+	+	+	-	+
P. oxalicum	+	+	+	+	-	-	+	-
Mycelia sterilia (OS)	-	-	-	-	-	-	-	-
Mycelia sterilia (PA)	-	-	-	-	-	-	-	-

Note: (+ present; - absent); Anth – anthroquinone, Tan – tannins, Sap – saponins, Fla – flavonoids, Gly – Glycosides, Alk – alkaloids, Ter – Terpenoids, Ste – sterols.

Lapachol detection assay

Results on the lapachol detection assay was shown in Table 3. Among the isolates, only four species were detected with lapachol including *A. alternata, A. niger,* and *A. terreus* isolated both from medicinal plants.

Table 3. Results of the lapachol identification assay on different fungal endophytic isolates

Fungal endophytes	Presence of Lapachol
A. alternata	+
A. niger	+
***A. terreus* (OS)**	+
***A. terreus* (PA)**	+
A. ustus	-
C. cladosporioides	-
F. verticillioides	-
***Lasioplodia* sp.**	-
P. chrysogenum	-
P. oxalicum	-
Mycelia sterile (OS)	-
Mycelia sterile (PA)	-

Note: (+) present; (-) absent

Antibacterial assay

The antibacterial activities of the four fungal endophytes tested positive for lapachol are presented in Figure 1. For the test on antibacterial efficacy against *E. coli*, *A. terreus* from *O. sanctum* exhibited the highest zone of inhibition with 16.78 mm after 24 hours of incubation followed by *A. niger* with 16.45mm, *A. terreus* from *P. amboinicus* with 15.88mm, and *A. alternata* with 14.65mm. The zones of inhibition against *S. aureus* indicated that among the four fungal isolates, *A. niger* had the most potent inhibitory activity on Gram-positive bacteria with 14.27 mm mean diameter. Moreover, *A. terreus* (OS), *A. terreus* (PA), and *A. alternata* exhibited a mean diameter of 13.66 mm, 12.84 mm, and 12.35 mm respectively as shown in Figure 1.

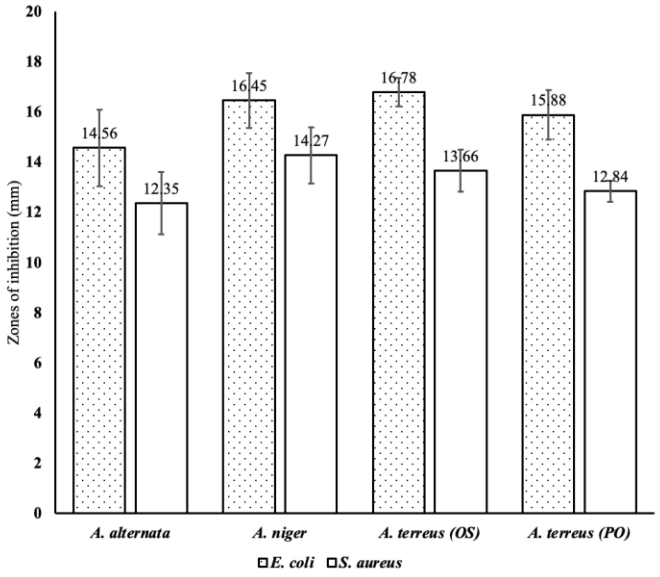

Figure 1. Zones of inhibition exhibited by the ethanolic extracts of fungal endophytes against *E. coli* and *S. aureus*

Discussion

In the present study, a total of 28 morphospecies of fungal endophytes were collected and morphologically identified. Prevalently, these endophytic fungi belong to a single Phylum, Ascomycota with four families, five genera, and 13 species. Most number of families were found under *Trichocomaceae* and the most prominent genera was found to be *Aspergillus.* Results of the present study corroborated with Shen *et al.* (2014), who reported that most endophyted identified from moso bamboo seeds belong to Ascomycota. Another study by Khan *et al*. (2007) showed that among the endophytic fungi isolated from *Calotropis procera* (Ait.) R. Br, the most distinguished genus was found to be Aspegillus which also conforms with the results of this study. It indicated that genus Aspergillus is prevalent and common among medicinal plants.

The experimental results showed that the identified fungal endophytes from the two Philippine medicinal plants contain bioactive compounds. Eze *et al.* (2019) reported that endophytes isolated from three medicinal plants of Nigeria contains bioactive compounds namely, ethyl 4-hydroxyphenyl acetate and ferulic acid, ruspolinone, protocatechuic acid, scytalone, and cladosporin, indole-3-acetic acid, and indole-3carbaldehyde. On the other hand, a wide-range bioactive compounds including steroids, xanthones, phenols,

isocoumarines, perylene derivatives, quinones, furandiones, terpenoids, depsipeptides, and cytochalasines have been found to be unique from endophytic fungi. These unique compounds are synthesized through polyketide pathway from mevalonate derived C5 units and using the non-ribosomal protein synthesis (Nisa *et al.,* 2015; Schulz and Boyle, 2005; Santos *et al.,* 2003). Moreover, naphthoquinone, such as lapachol was also found in present study. It is a naturally occurring compound that is mostly found in bacteria, fungi, plants, and animals. For instance, *Penicillium notatum* was reported by Otten and Rosazza (1983) to contain lapachol. However, in this study, only four fungal endophytes, A. *alternata*, *A. niger*, *A. terreus* from *O. sanctum* and *A terreus* from *P. amboinicus* posseses lapachol which indicated that it is not common in all filamentous fungi.

The study also showed antibacterial activities of the isolated endophytic fungi which is in congruence with the results obtained by Verma *et al.* (2007) and Ramasamy *et al. (*2010) in their studies. The antibacterial activity exhibited by the endophytic fungal extracts can be attributed to the compounds present in the extracts such as tanins, flavonoids, saponins, and terpenoids. According to Jacob and David (2016), the presence of tannins can cause an adverse effect on the productivity, nutrient availability, digestibility, impaired digestive physiology and may be mucosal perturbations to organisms while terpenoids could cause cytotoxic effects, growth hormones, and tumor promoters. On the same manner, alkaloids have high nitrogen organic content that can be poisonous and addictive.

Results obtained from this study highlighted the possibility of endophytic fungi from medicinal plants as an alternative and novel source of bioactive compounds which can be useful in the pharmaceutical field. Their presence could produce valuable chemical compounds that significantly contributed to plants' medicinal value as these fungi also played an essential role in the bioactivities, functionality, and ecology of their plant hosts. This study suggests that endophytic fungi can further be developed from a low spectrum antimicrobial into a wide-range antimicrobial agent through the determination of other compounds that might present in the fungal isolates using other forms of organic solvents such as ethylacetate, and methanol. Furthermore, it is suggested that the present bioactive compounds be characterized and elucidated.

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