



Antioxidant activities of fungi inhabiting *Ramalina peruviana*: insights on the role of endolichenic fungi in the lichen symbiosis

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Abstract

Apart from the fungal component (the mycobiont), other fungi reside inside lichens. Referred to as “lichen-associated fungi” or “endolichenic fungi” (ELF), these microorganisms have a poorly understood role in the lichen symbiosis. In this study, 11 morphoculturally-distinct ELF were isolated from the fruticose lichen *Ramalina peruviana* and identified as belonging to the genera *Colletotrichum* (1), *Daldinia* (3), *Hypoxylon* (1), *Nemania* (1), *Nigrospora* (1), and *Xylaria* (4). Each ELF was grown in two separate setups – submerged and solid-state fermentation – and were extracted with ethyl acetate for their secondary metabolites. Similarly, metabolites from the lichen host were also extracted. Among the 11 isolates, crude culture extracts of *Nemania primolutea* grown via the solid-state fermentation setup exhibited the highest radical scavenging activity (RSA = 89.7%), followed by *Colletotrichum eschscholtzii* grown using similar fermentation type (RSA = 80%). In contrast, extracts of the lichen host exhibited a slightly lower RSA (= 45.89%). Results showed that endolichenic fungi exhibited antioxidant activities greater than the lichen host, and possibly contributes to the protection of the lichen symbiosis through the synthesis of antioxidant compounds.

Key words – free radical scavengers – fruticose lichen – lichen-associated fungi – Philippine lichens – secondary metabolites

Introduction

For centuries, the dual nature of lichens has been the focus of many studies. Such partnership is considered as one of the most successful symbiotic relationships known in nature. However, studies over the past decade have uncovered the existence of endolichenic fungi or “ELF” (also known as lichen-associated fungi) within the lichen thalli which resemble the characteristics of

known plant fungal endophytes. The discovery of ELF gave lichens another reputation as a self-contained miniature ecosystem and a recognized microbial community (Nash 2008). ELF reside inside healthy lichen thalli and were observed to persistently occur near the photobionts (Arnold et al. 2009, U'Ren et al. 2010). ELF colonize the healthy tissues of lichens for shelter and to gain access to photosynthetic products for nourishment (Kellogg & Raja 2017, Yu et al. 2018). Moreover, other studies also suggested that ELF inside lichens strengthen their host's tolerance against environmental stress and assist in the formation and growth of the lichen thalli (Suryanarayanan & Thirunavukkarasu 2017, Yu et al. 2018, Oh et al. 2020). ELF also produced a set of metabolites distinct from that of their host lichen (Santiago et al. 2021b). In their paper, Santiago and co-authors hypothesized that ELF produced metabolites that target other "competitor" microorganisms, which in turn may have protected the lichen hosts from other possible invading microbes. Thus, the mycobiont is apparently not the only fungus benefiting from the lichen symbiosis. Interestingly, these ELF also represent lineages of Ascomycota that are distinct from the mycobiont, the lichenicolous fungi, and any incidental fungi that occur on lichen surfaces. It is therefore important to note that ELF, just like endophytic fungi in plants, are a distinct ecological group and are not simply "accidental colonizers" of lichens (U'Ren et al. 2010).

Associated fungi had been existing inside lichens for millions of years. A recent study found fossils of lichens from the Lower Devonian Era that contained traces of endolichenic microorganisms, i.e., actinobacteria, fungi (Honegger et al. 2013). While ELF are recognized as important members of the lichen symbiosis, little is known of their physiological role within the association. The partnership between a fungus, which provides physical protection, and free-living photosynthetic cells, which synthesize organic nutrients from CO₂ for the entire symbiosis, embody an array of complex processes that enable other microorganisms with limited means of sustenance to inhabit lichens. Though initially thought only to be "free-loaders", ELF are hypothesized to confer a multitude of benefits to their lichen host as they produce unique bioactive secondary metabolites that can add up to or compliment those that are being produced by the lichen. To date, only climate, host lineage, and geographic isolation are known to influence the diversity of endolichenic fungi (U'Ren et al. 2012). A previous study observed the "generalist" behaviour of ELF, or as not strictly host-specific (Chagnon et al. 2016). Perhaps, a better question now is: do ELF play significant roles in the survival of their hosts, especially under extreme conditions? After all, lichens are home to these fungi, hence the home must be protected from harsh environment at all costs.

The genus *Ramalina* is a fruticose lichen that occur in relatively lower elevated areas and under a wider temperature gradient as compared to other genera of the same type, e.g., *Usnea*, *Cladonia* (Galinato et al. 2017, 2018). While fruticose lichens are generally sensitive to air pollution (Nash 2008), samples of *Ramalina* were consistently observed during our fieldworks in areas where human activities are present, e.g., near urban roads or electricity infrastructure. As such, the level of air pollution and oxidative stress in the environment can force these organisms to upregulate their natural defence mechanism against external stressors. Fungi, including lichens, have primary antioxidant defences. When these defences are exhausted, fungal secondary metabolites act as adaptive response to stress conditions by scavenging reactive oxygen species (Le Devehat et al. 2014). This study therefore aimed to test for the antioxidant activities of the ELF associated with *Ramalina peruviana*. It is hypothesized that ELF contribute to resistance to external stress, if not the overall protection, of the lichen structure by synthesizing antioxidants potentially more active than those produced by the host.

Materials & Methods

Collection and identification of the lichen host

Samples of *Ramalina peruviana* were collected from the barks of *Shorea* trees in Tagaytay Tropical Greens, Mendez, Tagaytay City, Cavite Province, Southern Luzon, Philippines (14°6'N 120°54'E, ~600 masl). Collection of lichen samples included the attachment organ and a portion of

the substrate. Specimens were placed in brown, acid-free paper bags and were immediately transported back to the laboratory for initial identification. The specimen with the most numerous and healthy-looking thalli was then selected for the study and identified by morphological characterization following published literature (Oh et al. 2014). Identity of lichen sample was verified at the Lichen Herbarium, Biology Department, Ramkhamhaeng University, Thailand.

Isolation of endolichenic fungi

Isolation of ELF was done following the method described by Li et al. (2007) with slight modification on the agar medium used. It is important to process the lichen thalli within 48 hours of collection to preserve the viability of the ELF (Stone et al. 2004). The lichen thalli were initially washed in running tap water for 30 min. From healthy-looking thalli, i.e., those without any discoloration or were not dried, 15-20 pieces of lichen fragments (~1-2 cm in length) were obtained using flame-sterilized blade and placed in a sterile Petri plate. Surface sterilization was performed by consecutively immersing the thalli in various ethanol (EtOH) concentration (75%, 80% and 85%) for 1 min. Then, the thalli were immersed in 2% sodium hypochlorite (NaOCl) for 1 min, and in 90% EtOH for 30 sec. Final washing was done by immersing the thalli in sterile distilled water. Following surface sterilization, the thallus was dried with sterile paper towel which was aseptically placed inside a sterile Petri plate. The thalli were cut into small segments, ca. 0.5 cm², under aseptic conditions. Four lichen segments were evenly placed in a 90 mm Petri plate containing 2% malt extract agar (MEA, Merck, Germany) in five replicates. The plates were then sealed and incubated for 2 weeks under room temperature (22-25°C) with 12 hr/day exposure to fluorescence light. On a separate setup, to ensure that the growing fungi are indeed endolichenic fungi or originated inside the thalli, ~100 µl of the water used in the final washing was spread-plated on 10 plates with 2% MEA as control. Any filamentous fungi that grew in these plates and resembled those that grew in the isolation plates were treated as incidental fungi and excluded in the study. As soon as fungal growth was visible on any of the lichen fragments, each colony was continuously inoculated to a fresh 2% MEA plates until pure cultures were obtained.

Molecular identification of endolichenic fungi

Two-week old cultures of the isolated ELF were sent to Macrogen (South Korea) for DNA extraction, gene amplification, and sequencing. The fungal strains were identified through analysis of the nuclear internal transcribed spacer (ITS) region of the extracted DNA which were amplified by polymerase chain reaction (PCR) using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') (Gardes & Bruns 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer pair (White et al. 1990, Arnold & Lutzoni 2007). The PCR reaction mixture was composed of 5 µl of 10X buffer, 5 µl 2mM dNTPS, 1 µg Template DNA, 50 µl dH₂O, 10-25 pmole DNA sample and 5 µl of each of the primer pair for the sequenced ELF strains. Phylogenetic analysis of the gene sequences was done at the Mycology Laboratory, Research Center for Natural and Applied Sciences, University of Santo Tomas in Manila, Philippines. Sequences were initially edited using the BioEdit Sequence Assembly Software to check for sequence quality. Consensus sequences were then uploaded to the BLAST search engine (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to get related sequences. Only published sequences were used in the analysis. The related taxa, together with the fungal sequences, were aligned and edited using MEGA ver. 7.0 (Molecular Evolutionary Genetic Analysis) via the accessory application ClustalW multiple alignment. Phylogenetic trees were constructed based on a maximum likelihood (ML) analysis.

Extraction of lichen metabolites

Lichen acids were extracted from the thalli following the method used by Santiago et al. (2013). Air-dried lichen thalli of a single sample of *R. peruviana* (24 g) were ground using mortar and pestle until powdery. The powdered thalli were then placed in a clean 100 ml beaker and methanol was added until the sample was soaked. The mixture was allowed to stand overnight. The lichen extract was filtered using a funnel and filter paper and was then concentrated under reduced

pressure in a rotary evaporator. The crude extract was placed in small amber bottles for storage until further use.

Extraction of ELF metabolites

Two fermentation types were initially done prior to the extraction of ELF metabolites: (A) submerged and (B) solid-state fermentation.

Submerged fermentation setup – This setup was done following the protocols of Padhi & Tayung (2015). Pure cultures of each ELF were prepared on Malt Extract Glucose Yeast Extract Peptone Broth (MGYPB, Merck, Germany) by placing at least four small agar blocks from the fungal culture into two 250 ml sterile glass bottles per isolate containing 100 ml of the medium. The bottles were incubated under room temperature (22-25°C) for 50 days to ensure maximum production of secondary metabolites. During incubation, the liquid cultures were constantly disturbed by carefully shaking the bottles to redistribute the nutrients and the fungal growth. Then, the liquid broth was collected and extracted with equal volume of analytical-grade ethyl acetate (EtOAc) in a separating funnel done by vigorous shaking for 15 min.

Solid-state fermentation setup – This setup was done following the protocols of Gowthaman et al. (2001). Initially, four small agar blocks from each fungal culture were inoculated to 2% Malt Extract Broth (MEB, Merck, Germany) and incubated for one week with subsequent shaking under room temperature. Then, 10 ml of each broth culture was inoculated to sterile rice media in duplicates. Long grain white rice was washed thoroughly under running tap water and was soaked in distilled water overnight. Afterwards, the rice was dried on cheesecloth and was steamed for 30 minutes using an electric steamer. Fifty grams of steamed rice was added on each bottle. Then, 4 ml of distilled water was added to each bottle to adjust the moisture content. The fermentation bottles were sterilized at 121°C for 30 minutes. Each solid culture medium was inoculated with 10 ml of corresponding fungal culture and were incubated for 50 days to ensure maximum solid-state fermentation. Following incubation, the rice media were individually soaked with 200 ml EtOAc overnight. All culture extracts from both setups were filtered and concentrated under reduced pressure using a rotary evaporator.

Screening of antioxidant activities

Antioxidant activities of the *Ramalina peruviana* lichen thalli extract and the ELF culture extracts were tested following the protocol of Kosanić & Ranković (2010). Prior to the assay, all extracts were dissolved in methanol at a concentration of 1 mg/ml.

DPPH Radical Scavenging assay

1,1-diphenyl-2-picryl-hydrazil or DPPH (Sigma-Aldrich, India) was used to measure the free radical scavenging activity of the methanolic crude extracts. Briefly, 4 ml of the 0.1 mM DPPH solution was added to 1 ml of each crude extract (with a concentration of 1 mg/ml) dissolved in methanol. The commercially available Potentilla ascorbic acid (Pascual Laboratories, Philippines) with a concentration of 1 mg/ml was used as the positive control. The mixture was vortexed and allowed to stand for 30 minutes under room temperature. Following this, the absorbance was recorded at 517 nm (Spectronic-200, Thermo Fisher Scientific, USA) with pure methanol as the control. Lower optical density of the reaction mixture indicates higher DPPH free radical scavenging activity (Brand-Williams et al. 1995). All tests were performed in triplicates. Percentage of radical scavenging activity (% RSA) of the compounds on DPPH was calculated using the following formula:

$$\% \text{ RSA} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{control} is the absorbance of the control (DPPH solution without the crude extract) and A_{sample} is the absorbance of the test sample containing the mixture of DPPH and the crude extract.

Assay for total phenolic content

Total phenolic content of the extracts was determined using the Folin-Ciocalteu method (Slinkard & Singleton 1997). One ml of the crude extract was mixed with 1 ml of Folin-Ciocalteu reagent (Sigma-Aldrich, India) (diluted three-fold with distilled water). The mixture was allowed to react for 3 minutes before adding 3 ml of 2% sodium carbonate (Na_2CO_3) solution and 5 ml of distilled water. The mixture was again allowed to stand for 2 hours. Then, the absorbance was measured at 760 nm using a spectrophotometer. Ascorbic acid was used as the standard for the calibration curve. The total phenolic content was expressed in ascorbic acid equivalent per milligram sample (AAE/mg sample). All tests were performed in triplicates. One-way ANOVA was used to analyze all obtained data and the means were compared using post hoc test Tukey HSD ($P < 0.05$). The statistical analyses were done using the SPSS 23.0 (International Business Machines Corp., USA).

Detection of lichen and ELF metabolites

Thin-Layer Chromatography

The methanolic crude extracts were initially spotted on a silica gel TLC plates (Silica gel 60 F254 aluminum plates, Merck, Germany) using capillary tubes. For the crude lichen extracts, TLC plates were run on two solvent systems: (1) solvent system A: 36:9:1 toluene/dioxane/glacial acetic acid, and (2) solvent system G: 139:83:8 toluene/ethyl acetate/formic acid (Stocker-Wörgötter 2008, Santiago et al. 2013). Each plate was then brushed with 10% sulphuric acid and heated at 110°C for 5-10 minutes using TLC Plate Heater III (CAMAG, Switzerland). Identification of the lichen acid spots was done by comparison with the lichen acid standards (Culbertson 1972). For the endolichenic fungal crude culture extract, the TLC plate containing the extracts was run on a modified solvent system (100:4 dichloromethane/methanol). The retention factor (R_f) values were calculated by dividing the distance travelled of the spot (mm) with the distance travelled by the solvent front (mm). All TLC plates were also observed under shortwave (254 nm) and longwave (320 nm) ultraviolet light.

TLC Bioautography for antioxidant compounds

To detect the antioxidant-active compounds, TLC Bioautography method was done using the DPPH spray technique. Initially, 10% DPPH solution was prepared in methanol. Then, DPPH was sprayed generously onto the TLC plates containing the compounds until it is entirely covered with the solution. After ten minutes, the plate was observed for any white or light yellowish bands, indicating that these compounds are antioxidants (Bhattarai et al. 2008).

Results

The lichen host

Only one specimen of *Ramalina* was used to isolate ELF in this study to remove any bias in the results, i.e., physiological variation among the fungi as influenced by the mycobiont. The selected lichen specimen was identified as *Ramalina peruviana* Ach. Lich. Univ.: 1599 (1810) due to its narrowly elongated thalli that bear punctiform soralia in both lateral and laminal parts and the presence of chondroid strands (Fig. 1).

Identified endolichenic fungi

A total of 11 morphospecies of endolichenic fungi (ELF) were isolated (Table 1, Fig. 2). Of these, only five representative isolates were sent for sequencing (Figs 3-7).

Antioxidant activities of *Ramalina peruviana* and associated endolichenic fungi

Results showed that the 11 ELF crude extracts were more active than those of the lichen host (Table 2). Among all tested ELF crude culture extracts, both extracts of *Daldinia eschscholtzii*

showed consistently high radical scavenging activities (RSA) (Table 2). The highest RSA recorded was from the crude extracts of *Nemania primolutea* grown in solid rice medium (89.70%). All fungal extracts of *Xylaria* spp. exhibited good RSA but better when grown in solid rice medium. Notably, all genera belonging to the family Xylariaceae (*Daldinia*, *Xylaria*, *Nemania*) showed high RSA in this study except for the *Hypoxylon* sp., which did not manifest any activity (0%) despite several trials when grown in liquid medium. When grown in solid rice medium, however, the ELF crude extracts showed minimal RSA (23.80%). In general, the radical scavenging activity has no direct relationship to the total phenolic content of the extracts.



Fig. 1 – Lichen host *Ramalina peruviana* collected from Tagaytay City, Cavite with prominent [A] soralia (←), [B] chondroid strands (←). Scale bars: A = 3 mm, B = 1 mm.

Table 1 Identities of the endolichenic fungi (ELF) isolated from *Ramalina peruviana*.

ELF Code	Taxa	Family	GenBank Accession Number
ELF05	<i>Colletotrichum gloeosporioides</i>	Glomerellaceae	-
ELF03*	<i>Nigrospora sphaerica</i>	Trichosphaeriaceae	MW261780
ELF01	<i>Daldinia</i> sp. 2	Xylariaceae	-
ELF02	<i>Daldinia</i> sp. 3		-
ELF07*	<i>Daldinia eschscholtzii</i>		MW261783
ELF04*	<i>Xylaria</i> sp. 1		MW261781
ELF06*	<i>Hypoxylon</i> sp.		MW261782
ELF08	<i>Xylaria</i> sp. 2		-
ELF09*	<i>Nemania primolutea</i>		MW261784
ELF10	<i>Xylaria</i> sp. 3		-
ELF11	<i>Xylaria</i> sp. 4		-

*Isolates that were identified via gene sequence analysis

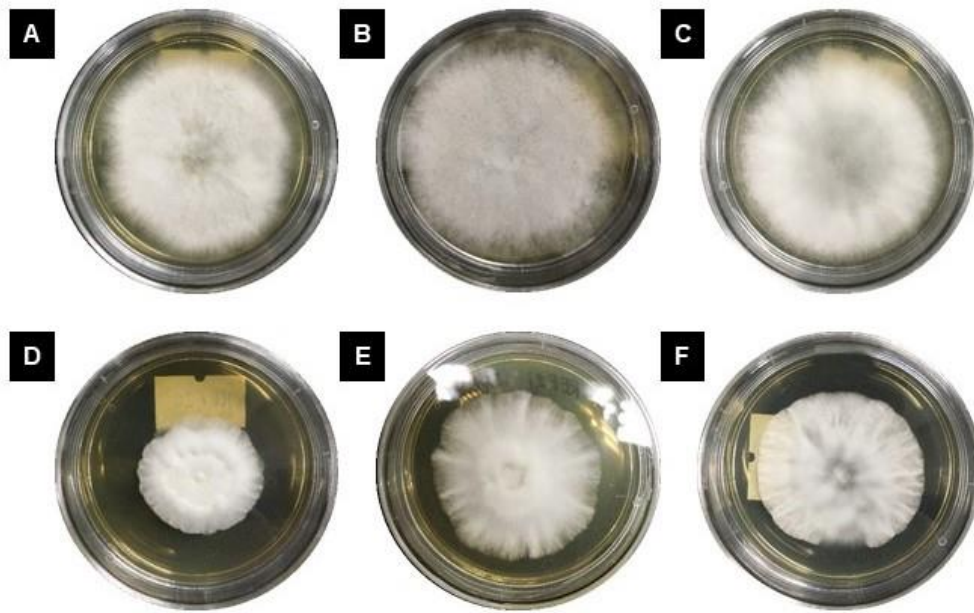


Fig. 2 – Cultural appearances of ELF grown on 2% MEA. [A] ELF01, [B] ELF02, [C] ELF05, [D] ELF08, [E] ELF10, [F] ELF11.

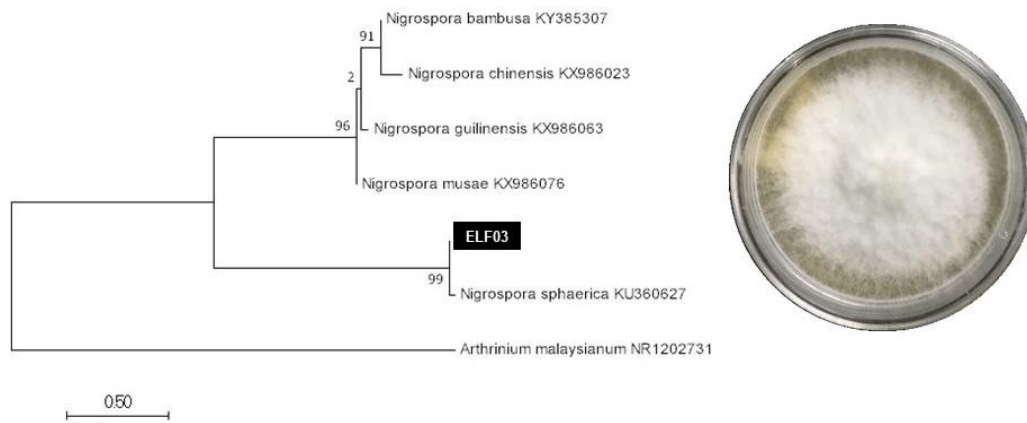


Fig. 3 – ML phylogenetic tree and colony morphology of the endolichenic fungal isolate ELF03. Related sequences were obtained from Wang et al. (2017).

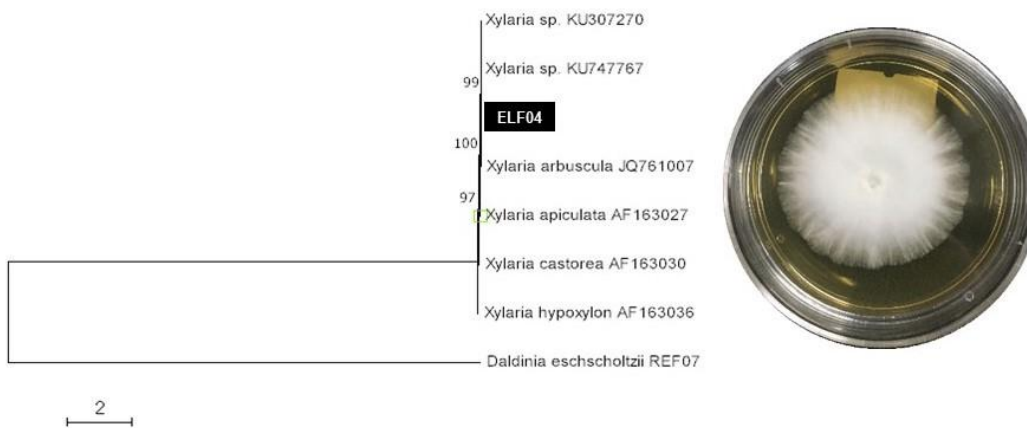


Fig. 4 – ML phylogenetic tree and colony morphology of the endolichenic fungal isolate ELF04. Related sequences were obtained from Lee et al. (2000).

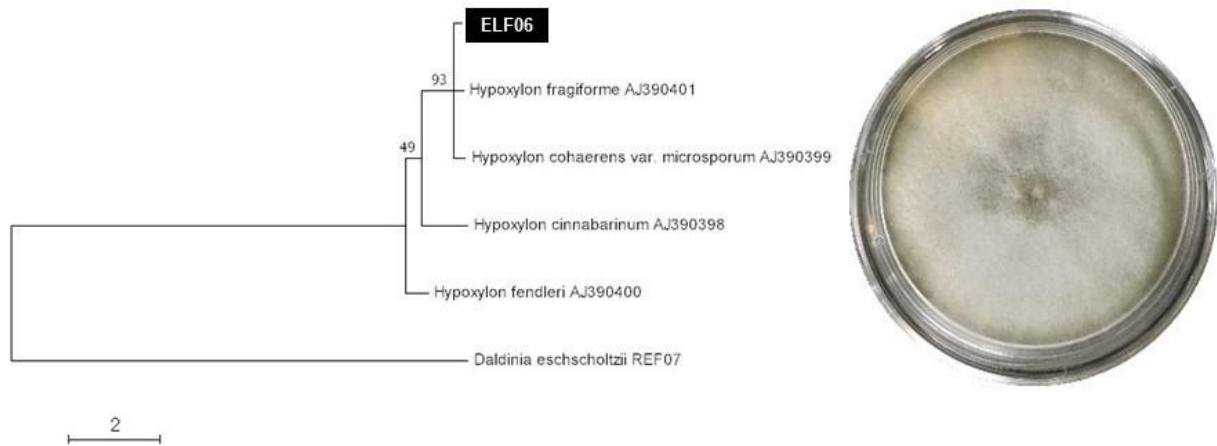


Fig. 5 – Phylogenetic tree and colony morphology of the endolichenic fungal isolate ELF06. Related sequences were obtained from Sánchez-Ballesteros (2000).

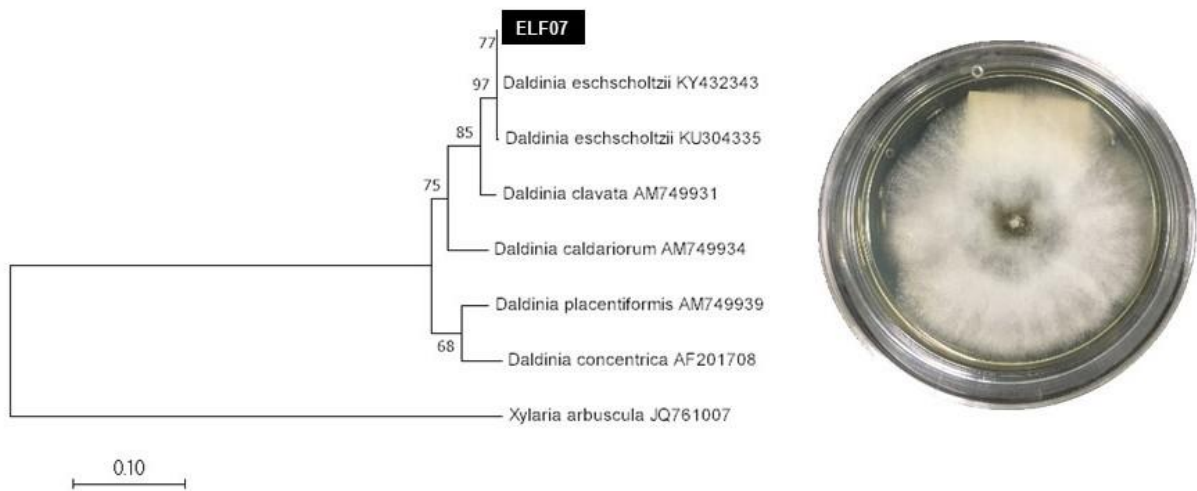


Fig. 6 – ML phylogenetic tree and colony morphology of the endolichenic fungal isolate ELF07. Related sequences were obtained from Stadler et al. (2014).

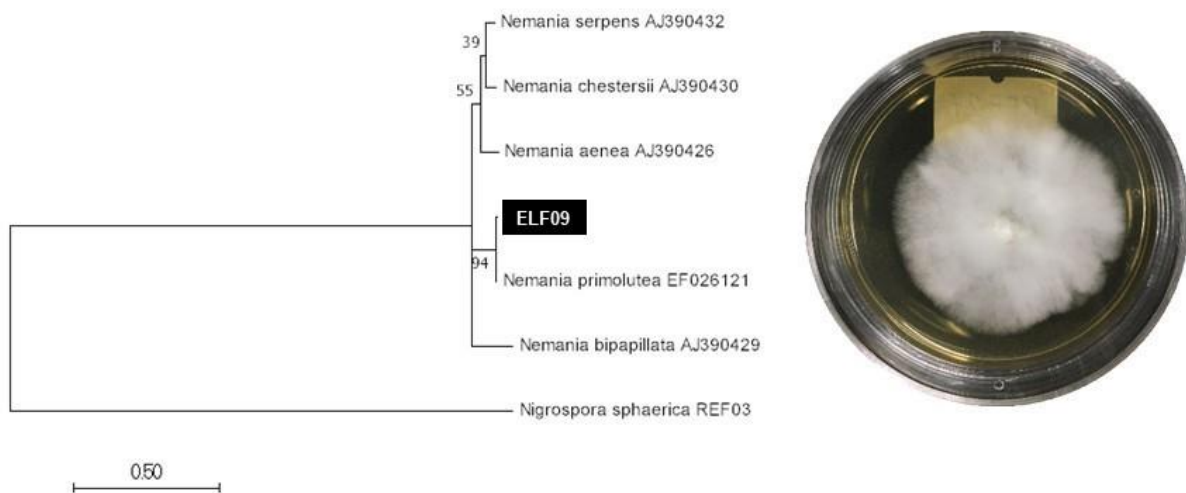


Fig. 7 – ML phylogenetic tree and colony morphology of the endolichenic fungal isolate ELF09. Related sequences were obtained from U'Ren et al. (2016).

Table 2 Radical Scavenging Activities (RSA) and Total Phenolic Content (AAE/mg sample) of the lichen host and endolichenic fungi (ELF).

Lichen Host		RSA (%)	AAE/mg sample		
<i>Ramalina peruviana</i> (Lichen host)		45.89	15.89		
Ascorbic Acid (Positive Control)		53.07	N/A		
ELF Code	Endolichenic Fungi	RSA (%)	AAE/mg		
		ELF grown in liquid medium	sample	ELF grown in solid rice medium	sample

ELF01	<i>Daldinia</i> sp. 2	33.33	5.60	42.80	25.58
ELF02	<i>Daldinia</i> sp. 3	22.03	8.96	30.00	29.41
ELF03	<i>Nigrospora sphaerica</i>	25.00	0.12	26.60	10.57
ELF04	<i>Xylaria</i> sp. 1	19.23	1.78	55.20	18.74
ELF05	<i>Colletotrichum gloeosporioides</i>	18.43	1.23	80.00	34.34
ELF06	<i>Hypoxyylon</i> sp.	0.00	4.88	23.80	31.07
ELF07	<i>Daldinia eschscholtzii</i>	78.26	11.76	65.20	17.41
ELF08	<i>Xylaria</i> sp. 2	33.33	1.73	54.50	10.82
ELF09	<i>Nemania primolutea</i>	56.67	5.32	89.70	4.69
ELF10	<i>Xylaria</i> sp. 3	31.63	1.49	38.20	9.54
ELF11	<i>Xylaria</i> sp. 4	54.74	2.64	69.00	16.66

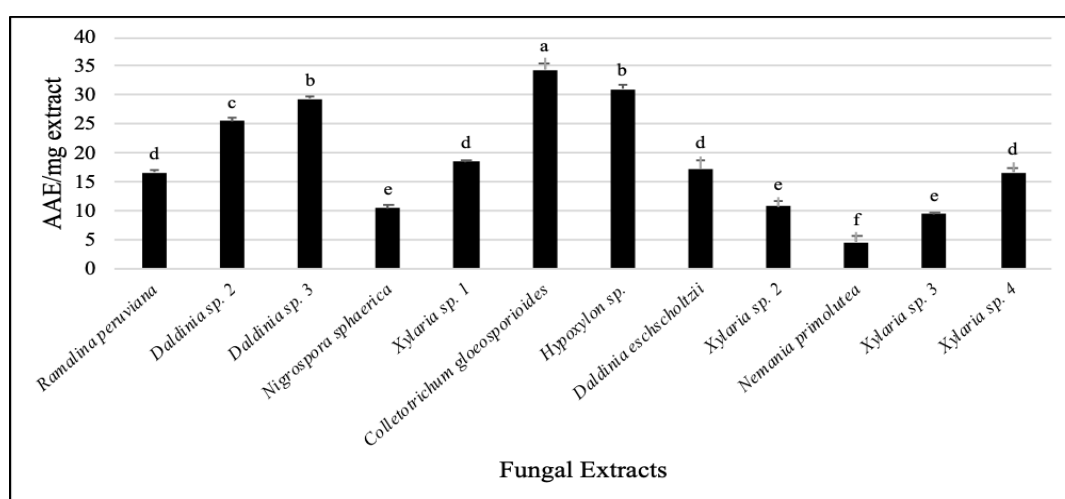


Fig. 8 – Total phenolic content (TPC) of lichen and endolichenic fungal extracts. [a] Very high TPC. [b] High TPC. [c] Moderately high TPC. [d] Moderately low TPC. [e] Low TPC. [f] Very low TPC. Data are mean \pm std of triplicate measurements conducted in each assay.

Secondary metabolites of the lichen host and endolichenic fungi

A total of six lichen acids were detected in the methanolic crude extracts of the lichen host *Ramalina peruviana*, namely: 1) homosekikaic acid, 2) hypostictic acid, 3) sekikaic acid, 4) usnic acid, and 5-6) 2 unconfirmed substances using TLC (Fig. 9). Unlike the secondary metabolites of the lichen host, the metabolite profile of ELF is not yet well-established. Hence, a general solvent system (100:4 dichloromethane:methanol) was used in this study to separate various metabolites of ELF to demonstrate the presence of different compounds in each extract (Fig. 10, Table 3). Most compounds are polar while majority of the visible spots are non-polar.

TLC Bioautography of secondary metabolites

Among the six lichen compounds found in the crude extract of *Ramalina peruviana*, usnic acid, homosekikaic acid, sekikaic acid, and one of the 2 unconfirmed lichen acids were the metabolites discovered to have antioxidant activities (Fig. 11).

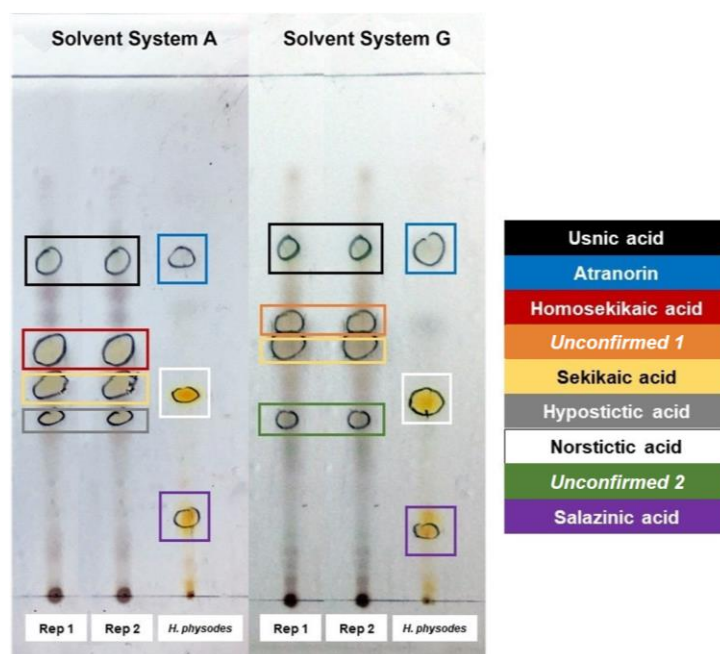


Fig. 9 – TLC profiles of *Ramalina peruviana* extracts (duplicate). Rep 1 = replicate 1, Rep 2 = replicate 2, *H. physodes* = standard. Solvent system A: 36:9:1 toluene/dioxane/glacial acetic acid, Solvent system G: 139:83:8 toluene/ethyl acetate/formic acid.

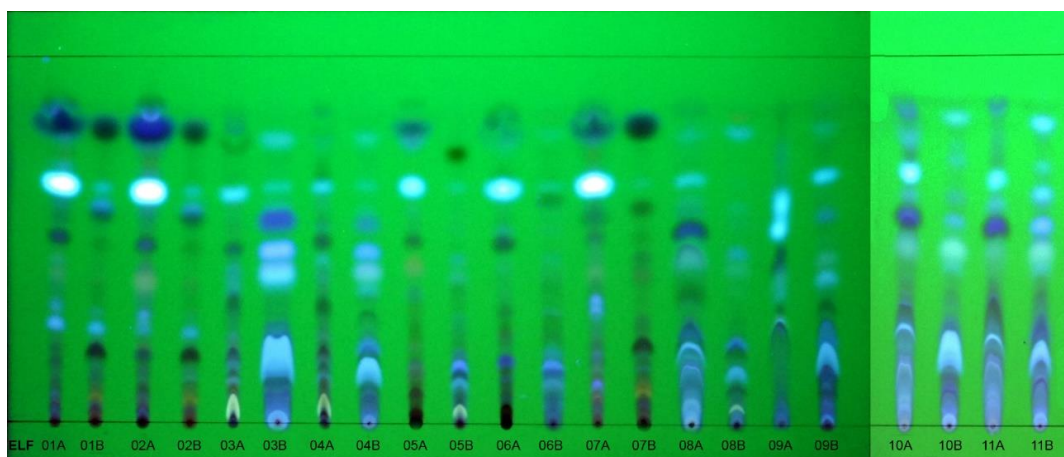


Fig. 10 – Bands of secondary metabolites of ELF crude extracts detected in TLC under mixed short- and long-wave UV light. [A] ELF grown in rice medium, [B] ELF grown in broth medium. *Daldinia* sp. 2 (01A, 01B), *Daldinia* sp. 3 (02A, 02B), *Nigrospora sphaerica* (03A, 03B), *Xylaria* sp. 1 (04A, 04B), *Colletotrichum gloeosporioides* (05A, 05B), *Hypoxylon* sp. (06A, 06B), *Daldinia eschscholtzii* (07A, 07B), *Xylaria* sp. 2 (08A, 08B), *Nemania primolutea* (09A, 09B), *Xylaria* sp. 3 (10A, 10B), *Xylaria* sp. 4 (11A, 11B).

Table 3 R_f values of the metabolites detected in the ethyl acetate crude extracts of ELF grown in both fermentation media using TLC.

Taxa	Extract codes	ELF grown in long grain rice medium* (solid-state fermentation)	Extract codes	ELF grown in liquid medium* (submerged fermentation)
<i>Daldinia</i> sp. 2	01A	0.20, 0.54, 0.61, 0.84	01B	0.20, 0.54, 0.60, 0.61, 0.63, 0.84
<i>Daldinia</i> sp. 3	02A	0.19, 0.45, 0.46, 0.61, 0.645, 0.84	02B	0.20, 0.46, 0.54, 0.61, 0.84

Table 3 Continued.

Taxa	Extract codes	ELF grown in long grain rice medium* (solid-state fermentation)	Extract codes	ELF grown in liquid medium* (submerged fermentation)
<i>Nigrospora sphaerica</i>	03A	0.30, 0.60, 0.80, 0.84	03B	0.30, 0.36 , 0.61, 0.80, 0.84
<i>Xylaria</i> sp. 1	04A	0.19, 0.45, 0.46, 0.61	04B	0.20, 0.36, 0.4 , 0.43 , 0.63
<i>Colletotrichum gloeosporioides</i>	05A	0.55, 0.76, 0.83	05B	0.75, 0.83
<i>Hypoxyylon</i> sp.	06A	0.41, 0.64, 0.8	06B	0.63, 0.75
<i>Daldinia eschscholtzii</i>	07A	0.45, 0.61, 0.64, 0.84	07B	0.45, 0.65, 0.84
<i>Xylaria</i> sp. 2	08A	0.3625, 0.425, 0.63	08B	0.3375, 0.625
<i>Nemania primolutea</i>	09A	0.38, 0.56, 0.6	09B	0.36, 0.38, 0.40, 0.56, 0.65
<i>Xylaria</i> sp. 3	10A	0.63 , 0.9	10B	0.49, 0.56, 0.7 , 0.81
<i>Xylaria</i> sp. 4	11A	0.6, 0.75 , 0.85	11B	0.41, 0.44, 0.49, 0.75 , 0.81

*Compounds with Rf values in bold are detected antioxidants in the TLC bioautography

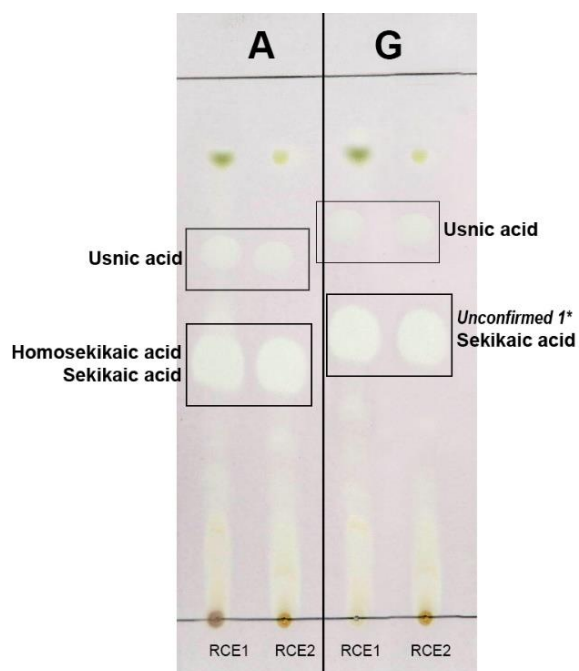


Fig. 11 – TLC Bioautography of *Ramalina peruviana* crude extracts (duplicate) using solvent systems A and G.

A modified TLC-Bioautography was also conducted on the ELF crude extracts to detect natural antioxidants. Twenty-one bands, each representing a metabolite, were revealed using the DPPH assay (Fig. 12). Nine and 12 bands were recorded from the solid-state and submerged fermentation set-up, respectively.

Discussion

The symbiosis of lichens produces unique secondary metabolites that are almost exclusive to them. These metabolites are often studied because of their great pharmacological application (de Jesus et al. 2016, Goga et al. 2020, Solárová et al. 2020, Ulus 2021). Recently, researchers have looked at the metabolites of lichens as compounds fashioned to interact with the symbionts and

other associated microorganisms. However, more work is needed to better understand the interaction between ELF and lichens, including their molecular and physiological facets (Tripathi & Joshi 2019).

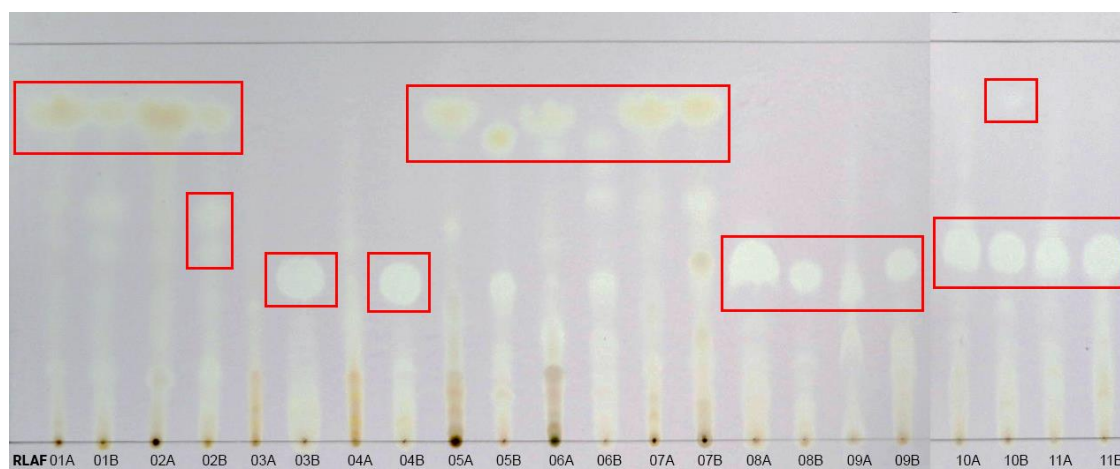


Fig. 12 – TLC Bioautography of ELF crude extracts. Antioxidant compounds (red boxes) were detected by DPPH reagent. See also Figure 10 for species names.

The fruticose lichen *Ramalina peruviana* is distributed throughout the Pacific region (Oh et al. 2014). Its abundance is reported across Indonesia (Kashiwadani & Moon 2007), Japan (Harada et al. 2004), China (Kashiwadani et al. 2006), Australia (Stevens 1987), East Africa (Krog & Swinscow 1976), and North America (Brodo et al. 2001). Based on local field observations, *Ramalina* is generally tougher and more resilient than other fruticose lichens, i.e., *Usnea*, *Cladonia*, as this genus can grow in wider gradients of elevation and temperature. Several species of *Ramalina* have been previously reported in the Philippines, with some taxa exhibiting antimicrobial and herbicidal activities (Santiago et al. 2010, Gazo et al. 2019, Paguirigan et al. 2020).

ELF or “lichen-associated fungi” are also abundant within the lichen symbiosis. Identities of ELF were determined primarily by molecular means since majority of the structural characteristics used to identify microfungi, e.g., presence of spores and other reproductive structures, were usually absent among the isolated and cultured ELF. Raja et al. (2017) recognized the benefit of molecular methods in identifying fungi in an event that morphological data render inconclusive. This study also discovered our ELF as belonging to the genera *Colletotrichum*, *Daldinia*, *Hypoxyylon*, *Nemania*, *Nigrospora*, and *Xylaria*. To the best of our knowledge, this is one of the very few papers to report *Nemania primolutea* as an endolichenic fungus. *Nemania primolutea* and *Daldinia eschsholtzii* were previously reported as endolichenic fungi in the foliose lichen *Parmotrema rampoddense* (Tan et al. 2020). The addition of this species brings the total number of reported endolichenic *Nemania* to 6. Interestingly, most isolated ELF in this study are members of the family Xylariaceae, while few belong to Glomerellaceae and Trichosphaeriaceae. Similar results were reported in previous studies (U’Ren et al. 2016, Maduranga et al. 2018, Becker & Stadler 2021). Only very few papers report the endolichenic nature of the genus *Colletotrichum* (Pettrini et al. 1990, Santiago & Ting 2019). However, the genus is often discovered in plants as fungal endophyte with high genetic diversity and antimicrobial activities (Manamgoda et al. 2013, Gonzaga et al. 2014, Moron et al. 2018, Apurillo et al. 2019, Ramirez et al. 2020). In contrast, *Daldinia*, *Hypoxyylon*, *Nigrospora*, and *Xylaria* are among the common genera of endolichenic fungi that are being reported in literature and targeted for bioprospecting purposes (Maduranga et al. 2018, Oh et al. 2020).

Lichens are generally considered as good sources of bioactive metabolites which include natural antioxidants. ELF are now being tapped for antioxidant compounds as they are novel sources and quite easy to isolate. In this study, the lichen host *R. peruviana* exhibited a relatively high radical scavenging activity expressed as %RSA. This level of activity has even been surpassed

by several number of the isolated ELF. Such results could be due to the exposure of ELF and the lichen host to its environment. Since the antioxidant mechanism of ELF has yet to be described, it is likely that their mechanism resembles with those of the plant endophytes. Interestingly, the antioxidant properties of ELF as evident by its %RSA are comparable to those recorded in plants, e.g., *Canarium ovatum* (Aril-dela Cruz et al. 2018), which are often considered rich in natural antioxidants. However, the RSA and TPC of the samples do not show any direct relationship in this study. For example, *N. primolutea* exhibited the highest RSA among all tested organisms but showed the lowest TPC (Table 2, Fig. 8). The same can be observed with *D. eschscholtzii* which exhibited consistent high RSA and low TPC in both culture media. These results entail that phenolic compounds are not the only active antioxidants present in the extracts and other non-phenolic compounds could also have antioxidant activities. Similar results were observed in previous literature (Hatami et al. 2014). Phenolic compounds are synthesized as a response to environmental stress (Lai & Lim 2011). Following this idea, the results of TPC can further support the idea in which ELF are protected from environmental stress, and thus might not need to synthesize much phenolic compounds.

The endolichenic strain of *Hypoxylon* in this study showed no RSA when grown in broth medium and showed only little activity when grown in rice. Such results are unexpected. *Hypoxylon* spp. usually occur as macrofungi in nature whose fruiting bodies are known sources of bioactive pigments with good antioxidant activities (Wu et al. 2008). However, in this study, the fungal strain was cultivated in the laboratory and occurred in its filamentous state, possibly deterring the production of antioxidant compounds, which, as mentioned, are only detected after the formation of fruiting bodies. Therefore, it is safe to assume that this species can only synthesize its most bioactive metabolites in its natural state than when it is cultured under laboratory conditions. Similar to the secondary metabolites of higher plants and other organisms, lichen metabolites are unessential to the growth and development of the organism (Bentley 1999). However, these compounds appear to contribute to the survival of the lichens. In this study, six lichen acids were detected in the methanolic crude extracts of *R. peruviana* (Fig. 9). Homosekikaic acid, sekikaic acid, and usnic acid were among the lichen metabolites extracted in the European specimens of *R. peruviana* (Oh et al. 2014). Furthermore, this study also identified the metabolites hypostictic acid in solvent system A, and two unconfirmed lichen acids in solvent system G. It should, however, be noted that hypostictic acid is uncommon in *Ramalina* and hence, further studies are needed to verify its identity. These acids were first isolated from foliose lichens (i.e., *Collema* and *Lobaria*) (Culbertson 1967), but are yet to be reported in *Ramalina*. Interestingly, these lichen species can be seen co-existing with *Ramalina* on barks of trees during fieldwork. As such, observation of these lichen acids in *Ramalina* lichens is feasible, but further studies must be done to confirm such assumption.

Unlike the secondary metabolites found in *Ramalina*, the metabolomics of endolichenic fungi, or even plant fungal endophytes, is not yet well established. In this study, a general solvent system (100:4 dichloromethane:methanol) was used to separate the different metabolites (Table 3, Fig. 10). These metabolites were detected under short- and long-wave UV lights. No detailed chemistry work was done to identify the detected metabolites, though some published literature provided hints to identify these metabolites. For example, some of the secondary metabolites detected from species under the family Xylariaceae included cytochalasin, daldinal, daldinol, and mellein (Stadler et al. 2001). However, these metabolites were only found in the stroma (fruiting bodies) of these fungi and not from mycelial cultures. Therefore, these metabolites may be absent in this study. Additionally, quinones of different structural classes have already been reported in *Nigrospora* spp. (Huang et al. 2016). It is possible that these types of metabolites were produced by the ELF, here identified as *Xylaria* and *Nigrospora* species.

Currently, there are limited studies that test whether some of the secondary metabolites of ELF are of lichen host origin. There may be a possibility that the secondary metabolic production of ELF is influenced by its occurrence within lichens and/or its interaction with the symbionts, but this idea needs further experimental support. In fact, a previous study reported the presence of the

known lichen acid scrobulin in the lichenicolous fungi inhabiting the lichen *Lobaria scrobiculata* (Spribille et al. 2010). In contrast, the study of Santiago et al. (2021b) stated that the ELF and the lichen host produced a different set of secondary metabolites. Therefore, as earlier stated, a more extensive study is required to support or contradict this proposed idea. The different lichen acids and fungal metabolites produced by ELF in this study proves that lichens and their endolichenic fungi are independent producers of natural antioxidants.

Our hypothetical concept and insights on the role of endolichenic fungi in the lichen symbiosis

The endolichenic fungi presented in this study are potential free radical scavengers. We hypothesize that ELF may serve as independent antioxidant producers within the lichen thalli, but this idea requires further testing with many specimens of same species coming from different ecoregions or geographical origins. Similar to the role of endophytes to plants (Fadiji & Babalola 2020), the interaction of lichens and ELF can also facilitate the protection of the lichen host against harmful conditions, perhaps through the formation of reactive oxygen species (ROS). To date, no study describes or demonstrates this possible specific role of ELF in the lichen symbiosis. However, literature on bioactivities of ELF provides a good perspective on how these microorganisms can contribute to rid “external stressors” of lichens. This study isolated six morphospecies of ELF, all of which showed antioxidant activities, with some even surpassing the level of activity of their host. This could be due to the organism’s exposure to environmental stresses, with ELF being less exposed to such stresses than their lichen hosts (Santiago et al. 2021a). As such, one of the many possible layers of lichen protection may include the enhancement of the host’s tolerance against ROS. Hypothetically, this tolerance is aided by the antioxidants produced by ELF, which are especially helpful during metabolic fluctuations and the lichen’s need to immediately photosynthesize after long periods of desiccation as described in the study of Kranner et al. (2009). The photobiont provides nutrition to the mycobiont and now, to the endolichenic fungi as well (Suryanarayanan & Thirunavukkarasu 2017). During harsh conditions, free radicals are produced as by-products of the photosynthesis of the photobionts (Álvarez et al. 2015).

As highlighted in this study, the antioxidant compounds found in ELF were more bioactive than the lichen acids in terms of radical scavenging activity. There is clearly a possibility that ELF may take over the role as the main producers of antioxidants inside the lichen symbiosis. This could also explain why samples of *Ramalina* in Tagaytay City are healthy and thriving despite the presence of air pollution brought about by the high influx of cars as the city is known as a local tourist destination. Perhaps lichens are indeed self-sustained miniature ecosystems that have developed the mechanism to entertain and house other ecological groups of fungi for their unique secondary metabolites. Therefore, the ELF in this study are looked upon as “free radical scavengers” residing inside the thalli of the lichen host *Ramalina peruviana*. However, our concept requires additional scientific validation through isolation, culture and testing of ELF isolated from other specimens of *Ramalina peruviana* collected in other localities or ecoregions.

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