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Diversity and bioactivity of endolichenic fungi in *Usnea* lichens of the Philippines

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Endolichenic fungi (ELF; asymptomatic microorganisms living inside healthy lichen thalli) were isolated from three *Usnea* species, namely *U. baileyi*, *U. bismolliuscula* and *U. pectinata*, collected near the town of Sagada, Philippines. A total of 101 ELF were recovered representing 12 genera (classes *Sordariomycetes* and *Eurotiomycetes*), with the genera *Nemania* (50.5%, 51 isolates) and *Xylaria* (22.8%, 23 isolates) being the most abundant.

Comparative analyses on the antimicrobial activities of lichens and ELF revealed that lichen crude extracts were effective against the Gram-positive bacterium *Staphylococcus aureus* and the yeast *Candida albicans*, while ELF crude extracts were effective against *S. aureus*, *C. albicans* and the Gram-negative bacterium *Escherichia coli*. The broad-spectrum nature of ELF has provided medicinal and industrial advantages over the slow-growing lichens as shown on their respective bioactivities. Extracts from ELF also had a higher total flavonoid content (TFC; 6.29–85.69 mg QE/g of extract) and stronger antioxidant activities (IC_{50}: 0.57–19.63 mg/ml) than lichen-derived extracts. Although only culturable ELF were identified, this study provides the first evaluation of the diversity and bioactivities of culturable ELF from fruticose lichens of the genus *Usnea* in the Philippines.

Key words: antibacterial, anticandidal, antioxidant, lichen-associated, Sagada.

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Endolichenické houby (ELF; asymptomatické mikroorganismy rostoucí ve zdravých stélkách lišejníků) byly izolovány ze tří druhů rodu *Usnea*, konkrétně *U. baileyi*, *U. bismolliuscula* a *U. pectinata*,

sebraných v oblasti Sagady na Filipínách. Bylo zjištěno celkem 101 ELF z 11 rodů (zástupci tříd *Sordariomycetes* a *Eurotiomycetes*), přičemž nejhojnějšími rody jsou *Nemania* (50,5 %, 51 izolátů) a *Xylaria* (22,8 %, 23 izolátů).

Srovnávací analýzou antimikrobiálních aktivit lišejníků a ELF bylo zjištěno, že extrakty ze stélek lišejníků jsou účinné proti grampozitivní bakterii *Staphylococcus aureus* a kvasince *Candida albicans*, zatímco extrakty z ELF jsou účinné proti *S. aureus*, *C. albicans* i gramnegativní bakterii *Escherichia coli*. Z lékařského a průmyslového hlediska se ukazuje výhoda širokého záběru ELF oproti pomalu rostoucím lišejníkům. Extrakty z ELF mají take vyšší obsah flavonoidů (TFC; 6,29–85,69 mg QE na gram extraktu) a silnější antioxidační účinky (IC_{50}: 0,57–19,63 mg/ml) než extrakty získané z lišejníků. Ačkoli byly identifikovány jen kultivovatelné houby, studie poskytuje první zhodnocení diverzity a bioaktivity kultivovatelných ELF z keříčkovitých lišejníků rodu *Usnea* na Filipínách.

INTRODUCTION

Endolichenic fungi (ELF) are microorganisms which inhabit the interiors of healthy lichen thalli, resembling plant endophytes. These asymptomatic organisms are typically members of the phylum *Ascomycota*, although few are *Basidiomycota* (U'Ren et al. 2012). ELF have been reported from various ecosystems in the tropics as well as temperate and polar regions of the world. For example, foliose and fruticose lichens in Sri Lanka (Maduranga et al. 2018), India (Suryanarayanan et al. 2017) and China (He et Zhang 2012) harbour a wide variety of ELF. Similar observations of ELF have been reported from different parts of Europe (Petrini et al. 1990, Girlanda et al. 1997), the USA (Arnold et al. 2009) and the polar regions (Park et al. 2015, Zhang et al. 2016). These studies have also revealed that biotic and abiotic factors (e.g. climate, geographical location, host type, host lineage and elevation) may affect the taxonomic composition, occurrence and diversity of ELF communities in a lichen host.

In recent years, ELF have been studied to detect potential bioactive secondary metabolites, which were hypothesised to be distinct from those produced by their lichen hosts (Singh et al. 2017). These metabolites exhibit antibacterial (Ding et al. 2008), antifungal (Li et al. 2015), antioxidant (Samanthi et al. 2015), cytotoxic (Paranagama et al. 2007) and antiviral (He et al. 2012) activities. As such, ELF are increasingly popular as potential candidates for drug discovery. The occurrence of ELF as producers of bioactive compounds is highly advantageous as they serve as sources of bioactive compounds alternative to lichens, which are well-known slow-growing organisms.

The Philippines is home to a wide variety of lichens, among them the very abundant and extensively studied genus *Usnea*. Studies are mostly focused on the diversity (Galinato et al. 2017, Galinato et al. 2018) and bioactivities (Santiago et al. 2010, Santiago et al. 2013, Timbreza et al. 2017) of lichens, while the occurrence/distribution and diversity of their ELF is poorly understood.

This study aims at establishing the diversity of ELF in the lichen *Usnea* from Sagada, Mountain Province, one of the high-elevated mountain ranges in the country (mean temperature 29 °C, humidity 79%). ELF were identified using molecular methods, as the morphological approach alone may not necessarily be accurate enough (Raja et al. 2017). In addition, the bioactivities of culturable ELF were also evaluated using agar well assays, and compared to the bioactivities of their lichen hosts.

MATERIAL AND METHODS

Collection of lichens and isolation of ELF. *Usnea* species, namely *U. baileyi*, *U. bismolliuscula* and *U. pectinata*, were collected from pine trees (*Pinus merkusii*) at different accessible locations ranging from 1,500 to 1,550 m a.s.l. south of the town of Sagada, Mountain Province. GPS data and elevation of each collection site were recorded (Tab. 1). Each lichen specimen was identified according to standard methods (Ohmura 2012, Truong et Clerc 2012) using a stereomicroscope (100× magnification; Nikon Instruments Inc., New York, USA).

ELF were isolated from the lichen samples according to Samanthi et al. (2015) with modifications of the ethanol concentrations. Lichens were cleaned with tap water to remove all debris. The lichen thalli were then surface-sterilised by successively dipping them in different ethanol concentrations (70%, 80%, 85% and 90% v/v) for 1 min, followed by rinsing with sterile distilled water for 30 s inbetween each ethanol concentration. The lichen thalli were then dipped in 10% sodium hypochlorite (NaOCl) for 30 s followed by immersion in 95% ethanol for 30 s. Finally, the lichen thalli were rinsed with water for 30 s and then dried using filter paper under sterile conditions. The final rinsing water used in the surface sterilisation was plated onto potato dextrose agar (PDA; Merck, Darmstadt, Germany) to validate the efficacy of surface sterilisation. The lichen thalli were cut into small fragments (approx. 2 mm) and plated onto 2% malt yeast extract agar (MYE; Lab M Ltd., Heywood, UK). From each *Usnea* sample, 10 lichen fragments were carefully placed on each MYE agar (in triplicates) and were incubated at room temperature (26 ± 2 °C, under light for 12 h) for 14 days with daily observations. All growing fungi were immediately transferred to fresh MYE agar and stock cultures established using MYE agar slants.

Extraction of fungal genomic DNA. Pure isolates of each ELF were initially grown on potato dextrose broth (PDB; Merck, Darmstadt, Germany) for 7 days at room temperature. The mycelium was harvested and dried using filter paper under sterile conditions. The mycelium was ground until powdery with the addition of liquid nitrogen. Thirty milligrammes of dry fine powder of each ELF

Tab. 1. Description of collection sites at Sagada, Mountain Province, Philippines and number of isolated endolichenic fungal species.

Site	Elevation (m a.s.l.)	GPS data	Lichen host	Number of isolated ELF
1	1,516	17°04'33" N 120°53'59" E	*U. pectinata*	18
			U. bismolliuscula	8
2	1,499	17°04'25" N 120°53'46" E	*U. bismolliuscula*	9
3	1,550	17°04'22" N 120°53'48" E	*U. bismolliuscula*	13
			U. baileyi	10
4	1,539	17°05'11" N 120°54'07" E	*U. baileyi*	7
			U. bismolliuscula	9
			U. pectinata	8
5	1,542	17°05'11" N 120°54'06" E	*U. baileyi*	12
			U. bismolliuscula	7

was used for the DNA extraction using the commercially available Plant DNA Extraction Kit (Vivantis Technologies, Subang Jaya, Malaysia) following the manufacturer's instructions. The genomic DNA of each ELF isolate was kept at 4 °C until further use.

Molecular identification and phylogenetic analysis of selected ELF. The genomic DNA of ELF were subjected to PCR using the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3'; White et al. 1990) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; Gardes et Bruns 1993). The PCR reaction was performed following the protocol described by He et Zhang (2012). Briefly, the samples were subjected to initial denaturation at 98 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s. This was then followed by annealing at 55 °C for 30 s, extension at 55 °C for 30 s and final extension at 72 °C for 1 min. The PCR products (25 µl mixture reaction) were subjected to electrophoresis on 1% agarose gel and sent to Apical Scientific Sequencing (Seri Kembangan, Selangor, Malaysia). The fungal ITS rDNA fragments were manually edited using BIOEDIT 7.0 (Hall 1999) and their nucleotide sequences were compared with those present in GenBank using Basic Local Alignment Search Tool (BLAST) analysis (http://www.ncbi.nlm.nih.gov/) to determine their taxonomic identities. The sequences were then deposited in GenBank (accession numbers, see Electronic supplement). The phylogenetic analysis of the identified ELF was performed using the MEGA 7.0 software (Kumar et al. 2016). DNA sequences were initially aligned using the ClustalW option incorporated in the software and the phylogenetic relationship was inferred using the Maximum likelihood method based on the Tamura-Nei model. All positions containing gaps and missing data

were eliminated. One thousand bootstrap replications were used as statistical support for the nodes in the phylogenetic trees.

Assessment of ELF diversity. ELF with 99–100% sequence similarity were included in the biodiversity assessment (Tab. 2). Colonisation rate (CR) was calculated as the total number of lichen segments infected by fungi divided by the total number of segments incubated and expressed as percentages (Li et al. 2007). Species richness, abundance and evenness were also calculated for each lichen host using the Simpson Index of Diversity (D; Hunter et Gaston 1988), Shannon-Wiener Biodiversity Index (H'; Li et al. 2007) and Equitability (J'; Kricher 1972), respectively. The Diversity t test of the Shannon-Wiener diversity indices between the three lichen hosts was calculated using the PAST 3.24 software (Hammer et al. 2001).

Antimicrobial activities of the lichen hosts and selected ELF. Secondary metabolites were extracted from three lichen hosts and five ELF isolates. The extraction of lichen metabolites was performed following the protocol by Santiago et al. (2010), whereas the extraction of ELF metabolites and solid-state fermentation were conducted as described by Wu et al. (2011). Paper disk and agar well diffusion assays were used to evaluate the antimicrobial activities of lichen and ELF crude extracts, respectively, following the CLSI guidelines (CLSI 2002, Balouiri et al. 2016). The test organisms were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231. (+)-usnic acid (98% purity, Sigma-Aldrich, Merck, Darmstadt, Germany) was used as the reference standard, and chloramphenicol, polymyxin B and fluconazole (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK) as the positive controls. Acetone and ethyl acetate were the negative controls for lichen and ELF crude extracts, respectively. A concentration of 10 mg/ml was used for each crude extract. The minimum inhibitory concentration (MIC) was determined as recommended by CLSI (CLSI 2002, Balouiri et al. 2016).

Determination of total phenolic content, total flavonoid content and antioxidant properties of the lichen hosts and selected ELF. The total phenolic content (TPC) of the lichen and ELF crude extracts was evaluated spectrophotometrically following the Folin-Ciocalteu method (Lai et Lim 2011). The TPC for each crude extract was calculated from the gallic acid (GA; Friendemann Schmidt Chemical, Washington, USA) calibration curve. Data were expressed as gallic acid equivalents (GAE) per g of extract, which were calculated using the formula $y = 0.0991x + 0.0526$ ($R^2 = 0.9969$), where y is the absorbance value at 765 nm and x is the amount of GAE. The total flavonoid content (TFC), on the other hand, was evaluated using the aluminum chloride colorimetric method (Gunasekaran et al. 2017). The TFC for each crude

extract was calculated from the quercetin (Q; ChemFaces Biochemical Co., Wuhan, China) calibration curve. Data were expressed as quercetin equivalents (QE) per mg of extract, which were calculated using the formula $y = 0.1304x + 0.0442$ ($R^2 = 0.9963$), where y is the absorbance value at 510 nm and x is the amount of QE. Determinations were carried out in triplicates. Then, the DPPH radical scavenging activities of the crude extracts were determined (Lai et Lim 2011). Results were presented as IC_{50}, i.e. the concentration of crude extract required to scavenge 50% of the DPPH radical. In addition, ascorbic acid equivalent antioxidant capacity (AAEAC) was also calculated as previously described (Lai et Lim 2011). The IC_{50} of ascorbic acid (AA) was determined to be 0.021 mg/ml.

Statistical analyses. All bioassays, including determination of TPC and TFC, were performed in triplicates. One-way ANOVA was used to analyse all obtained data. The analyses were carried out using SPSS 23.0 (International Business Machines Corp., New York, USA) and means were compared using Tukey's HSD post hoc test ($P < 0.05$).

RESULTS

Colonisation rate and diversity assessment of ELF in the three *Usnea* species

A total of 101 ELF were recovered from the mentioned lichen hosts, with colonisation rate (i.e. the number of lichen segments with fungal growth divided by the total number of lichen segments plated) ranging between 30–43%. Among the three *Usnea* spp., *U. bismolliuscula* appeared to harbour the highest number of ELF (46 isolates), followed by *U. baileyi* (29 isolates) and *U. pectinata* (26 isolates) (Tab. 2).

Community analysis of the 101 isolates revealed 12 genera, representing five families (*Xylariaceae*, *Hypoxylaceae*, *Diaporthaceae*, *Nectriaceae* and *Aspergillaceae*) of the classes *Sordariomycetes* and *Eurotiomycetes* (Tab. 2, Fig. 1). The lichen *U. bismolliuscula* had the highest number of ELF isolates, but also the highest Simpson Index value (D = 0.8821) of all the lichen hosts. This indicates that the mycobiota of *U. bismolliuscula* is dominated by a few ELF species represented by several isolates. For example, *Nemania bipapillata* (12 isolates) and *N. diffusa* (10 isolates) dominated the ELF community in *U. bismolliuscula*, which was inhabited by 46 ELF isolates, while the remaining ELF species were represented by just a few isolates (< 4). Diversity was the highest in *U. bismolliuscula* (H' = 2.4046), followed by *U. baileyi* (H' = 2.0850) and *U. pectinata* (H' = 2.0303). These findings suggest that *U. bismolliuscula* had the highest number of isolated ELF species. The low H' value of *U. pectinata*, on the other hand, was expected

Tab. 2. Number of endolichenic fungal isolates from the three *Usnea* species.

ELF taxon	*U. baileyi*	*U. bismolliuscula*	*U. pectinata*
Aspergillaceae			
Penicillium simplicissimum (Oudem.) Thom	1	0	0
Penicillium sp.	0	1	0
Diaporthaceae			
Diaporthe longicolla (Hobbs) J.M. Santos, Vrandečić et A.J.L. Phillips	0	1	0
Hypoxylaceae			
Annulohypoxylon albidiscum J.F. Zhang, J.K. Liu, K.D. Hyde et Z.Y. Liu	0	1	1
Annulohypoxylon stygium (Lév.) Y.M. Ju, J.D. Rogers et H.M. Hsieh	0	0	2
Daldinia eschscholtzii (Ehrenb.) Rehm	0	1	1
Daldinia sp.	1	0	0
Nectriaceae			
Fusarium proliferatum (Matsush.) Nirenberg ex Gerlach et Nirenberg	0	1	0
Fusarium sp.	1	0	0
Xylariaceae			
Amphirosellinia fushanensis Y.M. Ju, J.D. Rogers & H.M. Hsieh	0	0	1
Astrocystis bambusae (Henn.) Læssøe et Spooner	0	1	0
Digitodochium cf. *rhodoleucum* Tubaki et Kubono	0	0	1
Kretzschmaria pavimentosa (Ces.) P.M.D. Martin	2	1	2
Nemania bipapillata (Berk. et M.A. Curtis) Pouzar	9	12	2
Nemania diffusa (Sowerby) Gray	5	10	11
Nemania primolutea Y.M. Ju, H.M. Hsieh et J.D. Rogers	0	0	2
Nodulisporium sp.	3	3	1
Xylaria apiculata Cooke	1	1	0
Xylaria atrosphaerica (Cooke et Massee) Callan et J.D. Rogers	1	0	0
Xylaria cubensis (Mont.) Fr.	0	2	0
Xylaria curta Fr.	0	0	1
Xylaria feejeensis (Berk.) Fr.	0	2	0
Xylaria cf. *heliscus* (Mont.) J.D. Rogers et Y.M. Ju	2	3	0
Xylaria intracolorata (J.D. Rogers, Callan et Samuels) J.D. Rogers et Y.M. Ju	0	1	0
Xylaria laevis Lloyd	0	1	0
Xylaria venustula Sacc.	3	3	1
Xylaria sp.	0	1	0
Total number of isolates	**29**	**46**	**26**
Number of lichen segments plated	**90**	**150**	**60**
Colonisation rate (%)	**32.2**	**30.7**	**43.3**
Number of species	**11**	**18**	**12**
Number of genera	**7**	**10**	**8**
Simpson Index of Diversity (D)	**0.8670**	**0.8821**	**0.8185**
Shannon-Wiener Biodiversity Index (H')	**2.0850**	**2.4046**	**2.0303**
Equitability (J')	**0.8695**	**0.8319**	**0.8171**
***P* value**	**0.170** (*U. baileyi* × *U. bismolliuscula*)	**0.179** (*U. bismolliuscula* × *U. pectinata*)	**0.844** (*U. baileyi* × *U. pectinata*)

* Diversity t test showed no significant differences between the Shannon-Wiener indices between the three lichen hosts at $P = 0.05$.

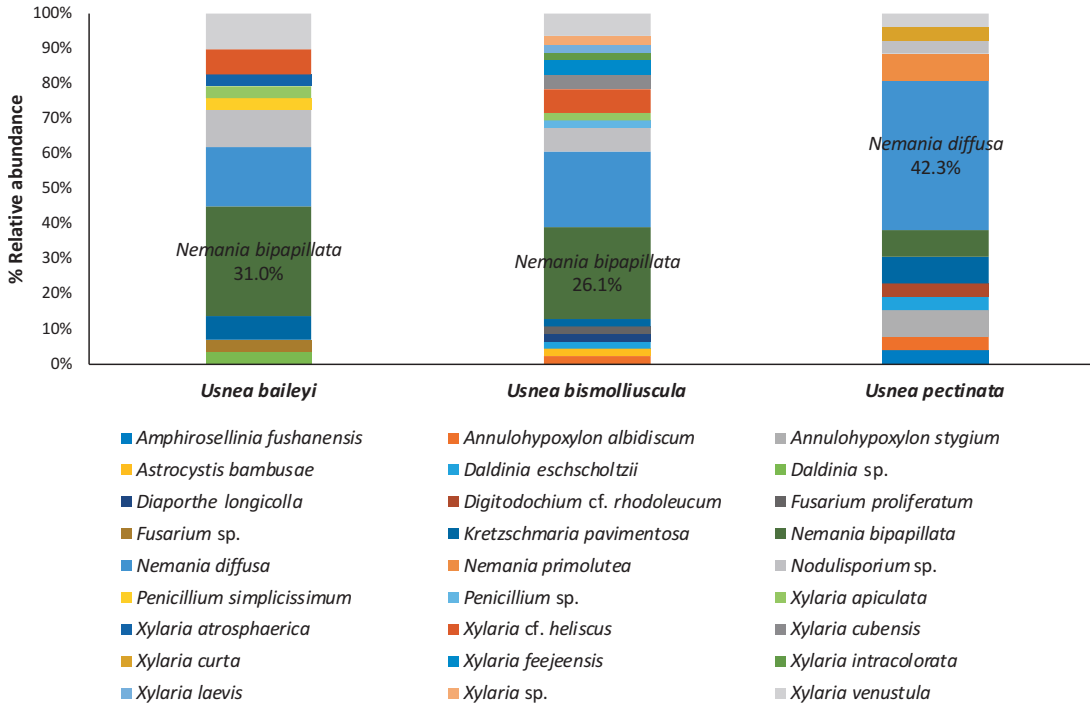
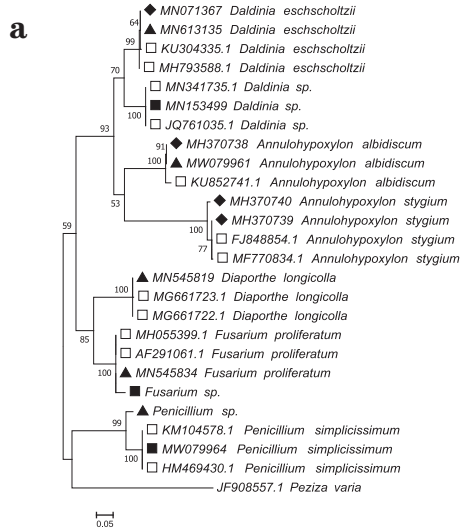
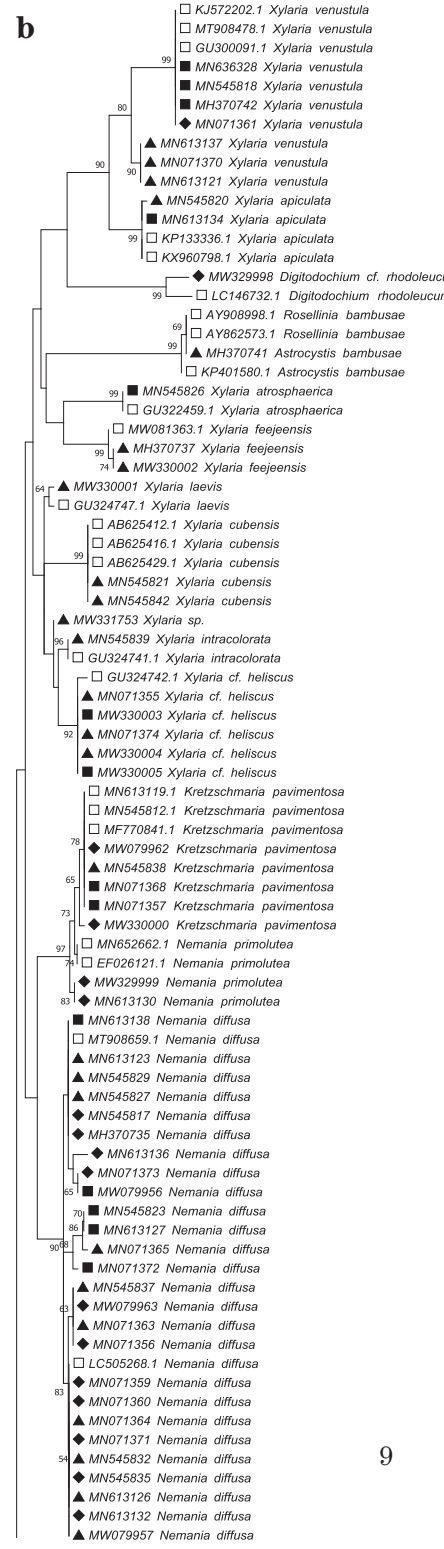


Fig. 1. Endolichenic fungal community structure at the species level inhabiting three *Usnea* lichens at Sagada, Mountain Province, Philippines. Different colours represent different species.

since this lichen host had the smallest D value and an uneven distribution of ELF (J' = 0.8171). The Diversity t test (*U. baileyi* × *U. bismolliuscula*, $P = 0.170$; *U. bismolliuscula* × *U. pectinata*, $P = 0.179$; *U. baileyi* × *U. pectinata*, $P = 0.844$; alpha = 0.05) showed no significant differences between the H' values of the three lichen hosts (Tab. 2).

Fig. 2. Maximum likelihood tree of the 101 ELF isolated from three *Usnea* species collected at Sagada, Mountain Province, Philippines: **a** – ELF species belonging to the families *Aspergillaceae*, *Diaporthaceae*, *Hypoxylaceae* and *Nectriaceae*; **b** – ELF species belonging to *Xylariaceae*.
The trees are drawn to scale based on 1000 repetitions, with branch lengths measured according to the number of substitutions per site. Numbers at each node represent the bootstrap support obtained from the maximum likelihood method. The lichen host from which the specific ELF was isolated is indicated using different symbols: *U. baileyi* (■), *U. bismolliuscula* (▲), *U. pectinata* (◆). Fungal isolates without accession numbers were not deposited in GenBank due to low similarity (< 90%). Reference sequences (□) retrieved from GenBank were included to validate the identification of isolated ELF. *Peziza varia* (JF908557.1) was used as the outgroup. ▶


a
MN071367 Daldinia eschscholtzii
MN613135 Daldinia eschscholtzii
KU304335.1 Daldinia eschscholtzii
MH793588.1 Daldinia eschscholtzii
MN341735.1 Daldinia sp.
MN153499 Daldinia sp.
JQ761035.1 Daldinia sp.
MH370738 Annulohypoxylon albidiscum
MW079961 Annulohypoxylon albidiscum
KU852741.1 Annulohypoxylon albidiscum
MH370740 Annulohypoxylon stygium
MH370739 Annulohypoxylon stygium
FJ848854.1 Annulohypoxylon stygium
MF770834.1 Annulohypoxylon stygium
MN545819 Diaporthe longicolla
MG661723.1 Diaporthe longicolla
MG661722.1 Diaporthe longicolla
MH055399.1 Fusarium proliferatum
AF291061.1 Fusarium proliferatum
MN545834 Fusarium proliferatum
Fusarium sp.
Penicillium sp.
KM104578.1 Penicillium simplicissimum
MW079964 Penicillium simplicissimum
HM469430.1 Penicillium simplicissimum
JF908557.1 Peziza varia
0.05


b
KJ572202.1 Xylaria venustula
MT908478.1 Xylaria venustula
GU300091.1 Xylaria venustula
MN636328 Xylaria venustula
MN545818 Xylaria venustula
MH370742 Xylaria venustula
MN071361 Xylaria venustula
MN613137 Xylaria venustula
MN071370 Xylaria venustula
MN613121 Xylaria venustula
MN545820 Xylaria apiculata
MN613134 Xylaria apiculata
KP133336.1 Xylaria apiculata
KX960798.1 Xylaria apiculata
MW329998 Digitodochium cf. rhodoleucum
LC146732.1 Digitodochium rhodoleucum
AY908998.1 Rosellinia bambusae
AY862573.1 Rosellinia bambusae
MH370741 Astrocystis bambusae
KP401580.1 Astrocystis bambusae
MN545826 Xylaria atrosphaerica
GU322459.1 Xylaria atrosphaerica
MW081363.1 Xylaria feejeensis
MH370737 Xylaria feejeensis
MW330002 Xylaria feejeensis
MW330001 Xylaria laevis
GU324747.1 Xylaria laevis
AB625412.1 Xylaria cubensis
AB625416.1 Xylaria cubensis
AB625429.1 Xylaria cubensis
MN545821 Xylaria cubensis
MN545842 Xylaria cubensis
MW331753 Xylaria sp.
MN545839 Xylaria intracolorata
GU324741.1 Xylaria intracolorata
GU324742.1 Xylaria cf. heliscus
MN071355 Xylaria cf. heliscus
MW330003 Xylaria cf. heliscus
MN071374 Xylaria cf. heliscus
MW330004 Xylaria cf. heliscus
MW330005 Xylaria cf. heliscus
MN613119.1 Kretzschmaria pavimentosa
MN545812.1 Kretzschmaria pavimentosa
MF770841.1 Kretzschmaria pavimentosa
MW079962 Kretzschmaria pavimentosa
MN545838 Kretzschmaria pavimentosa
MN071368 Kretzschmaria pavimentosa
MN071357 Kretzschmaria pavimentosa
MW330000 Kretzschmaria pavimentosa
MN652662.1 Nemania primolutea
EF026121.1 Nemania primolutea
MW329999 Nemania primolutea
MN613130 Nemania primolutea
MN613138 Nemania diffusa
MT908659.1 Nemania diffusa
MN613123 Nemania diffusa
MN545829 Nemania diffusa
MN545827 Nemania diffusa
MN545817 Nemania diffusa
MH370735 Nemania diffusa
MN613136 Nemania diffusa
MN071373 Nemania diffusa
MW079956 Nemania diffusa
MN545823 Nemania diffusa
MN613127 Nemania diffusa
MN071365 Nemania diffusa
MN071372 Nemania diffusa
MN545837 Nemania diffusa
MW079963 Nemania diffusa
MN071363 Nemania diffusa
MN071356 Nemania diffusa
LC505268.1 Nemania diffusa
MN071359 Nemania diffusa
MN071360 Nemania diffusa
MN071364 Nemania diffusa
MN071371 Nemania diffusa
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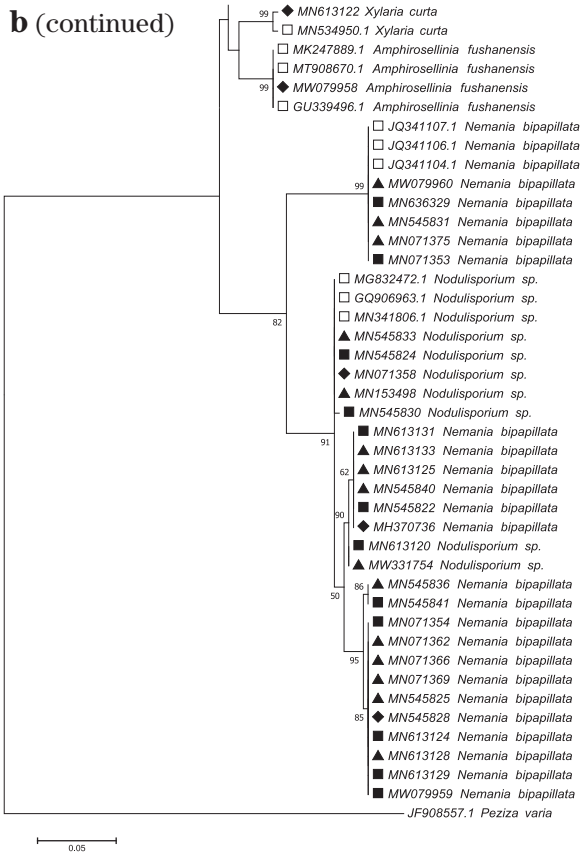
MN613132 Nemania diffusa
MW079957 Nemania diffusa


b (continued)
MN613122 Xylaria curta
MN534950.1 Xylaria curta
MK247889.1 Amphirosellinia fushanensis
MT908670.1 Amphirosellinia fushanensis
MW079958 Amphirosellinia fushanensis
GU339496.1 Amphirosellinia fushanensis
JQ341107.1 Nemania bipapillata
JQ341106.1 Nemania bipapillata
JQ341104.1 Nemania bipapillata
MW079960 Nemania bipapillata
MN636329 Nemania bipapillata
MN545831 Nemania bipapillata
MN071375 Nemania bipapillata
MN071353 Nemania bipapillata
MG832472.1 Nodulisporium sp.
GQ906963.1 Nodulisporium sp.
MN341806.1 Nodulisporium sp.
MN545833 Nodulisporium sp.
MN545824 Nodulisporium sp.
MN071358 Nodulisporium sp.
MN153498 Nodulisporium sp.
MN545830 Nodulisporium sp.
MN613131 Nemania bipapillata
MN613133 Nemania bipapillata
MN613125 Nemania bipapillata
MN545840 Nemania bipapillata
MN545822 Nemania bipapillata
MH370736 Nemania bipapillata
MN613120 Nodulisporium sp.
MW331754 Nodulisporium sp.
MN545836 Nemania bipapillata
MN545841 Nemania bipapillata
MN071354 Nemania bipapillata
MN071362 Nemania bipapillata
MN071366 Nemania bipapillata
MN071369 Nemania bipapillata
MN545825 Nemania bipapillata
MN545828 Nemania bipapillata
MN613124 Nemania bipapillata
MN613128 Nemania bipapillata
MN613129 Nemania bipapillata
MW079959 Nemania bipapillata
JF908557.1 Peziza varia
0.05

The genus *Nemania* (50.5%, 51 isolates) is the most abundant ELF detected, followed by the genus *Xylaria* (22.8%, 23 isolates). Rare genera were also isolated, which included *Amphirosellinia*, *Astrocystis*, *Diaporthe* and *Digitodochium* (1.0%, 1 isolate each). Interestingly, *Fusarium* and *Penicillium* (2.0%, 2 isolates each), known for their endophytic and soil-borne nature, respectively, were also isolated. All isolated ELF in this study had previously been reported as plant endophytes.

Only four (*Kretzschmaria pavimentosa*, *Nemania bipapillata*, *N. diffusa* and *Nodulisporium* sp.[1]) of the 27 ELF species were found in all three lichen hosts, while some ELF species were found in one lichen host only (Tab. 2, Fig. 1). For example, *Astrocystis bambusae*, *Diaporthe longicolla*, *Fusarium proliferatum* and *Xylaria intracolorata* were recovered only from *U. bismolliuscula*. These findings, however, do not confirm their host-specificity, as other factors may have likely influenced their occurrence. The phylogenetic analysis (Fig. 2) also revealed that most ELF species, despite differences in their lichen hosts, do not select a specific *Usnea* species for their occurrence and survival.

Antimicrobial activities of the lichen and ELF crude extracts

Three lichen (*Usnea baileyi*, *U. bismolliuscula* and *U. pectinata*) and five ELF (*Astrocystis bambusae*, *Annulohypoxylon albidiscum*, *Daldinia eschscholtzii*, *Nemania bipapillata* and *Xylaria venustula*) crude extracts were evaluated for their antimicrobial activities (Fig. 3). These five ELF isolates were selected based on their abundance (*N. bipapillata*), unique morphologies (*A. bambusae* and *X. venustula*), and relatively short incubation period (< 7 days) (*A. albidiscum* and *D. eschscholtzii*). The *U. bismolliuscula* extract had the strongest antibacterial activities against *Staphylococcus aureus* (MIC: 0.0625 mg/ml) of all lichen crude extracts. None of the three lichen crude extracts, however, inhibited *Escherichia coli*. All three lichen crude extracts had stronger antibacterial activities than the reference usnic acid. All five ELF crude extracts showed inhibition against both *S. aureus* and *E. coli*, suggesting a broad-spectrum nature. The fungal extract of *A. albidiscum* (MIC: 5 mg/ml) had the strongest activity against *S. aureus*, while *X. venustula* (MIC: 5 mg/ml) had the strongest activity against *E. coli*. In general, ELF crude extracts inhibited both test bacteria but were less effective than the positive controls. In addition, the antibacterial activities of ELF crude extracts against *S. aureus* were relatively weaker compared to those of the lichen crude extracts, as indicated by the lichen's lower MIC values.

The anticandidal activities of the lichen and ELF crude extracts were also evaluated (Fig. 3). Among the lichen crude extracts, *U. bismolliuscula* (MIC:

1 The isolates were identified as *Nodulisporium* sp. according to best BLAST hit with reference sequences of this taxon. However, according to the phylogenetic tree (Fig. 2) they might in fact belong to *Nemania bipapillata*.

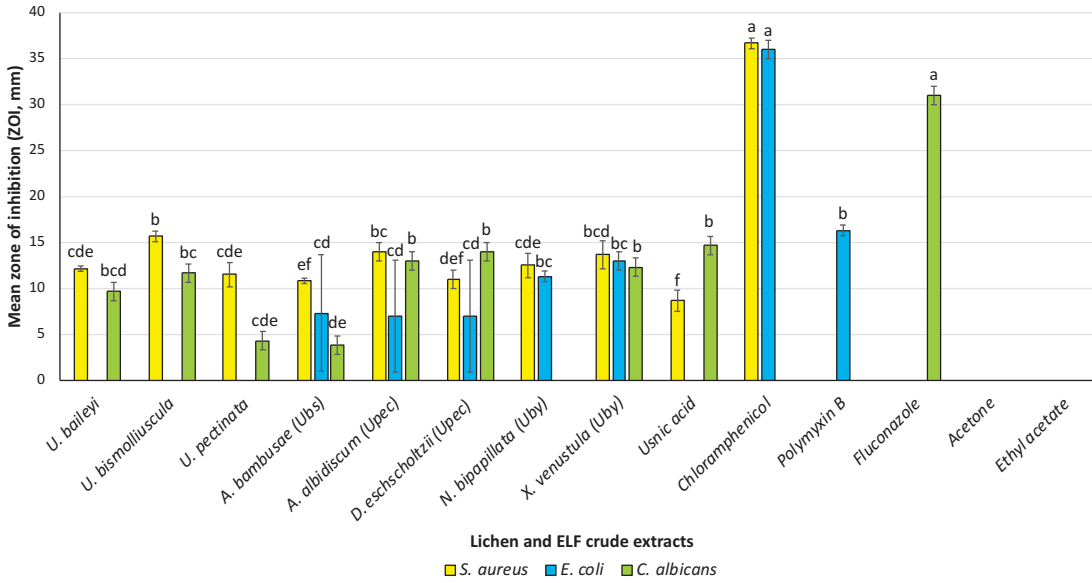


Fig. 3. Antimicrobial activities of the lichen and ELF crude extracts against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231, compared with the reference standard, positive and negative controls (for details, see Material and methods). The lichen hosts from which the ELF was isolated are indicated as Uby (*U. baileyi*), Ubs (*U. bismolliuscula*) and Upec (*U. pectinata*). Standard deviation values are indicated by error bars ($P < 0.05$). Letters above error bars indicate the statistical significance within groups using univariate analysis of variance (one-way ANOVA) and Tukey HSD.

0.0625 mg/ml) was found to be most active against *Candida albicans*. Usnic acid and fluconazole had stronger anticandidal activities than the lichen crude extracts. For the ELF crude extracts, four of the five extracts exhibited anticandidal activities, with *D. eschscholtzii* (MIC: 10 mg/ml) having the strongest inhibition against *C. albicans*. In general, the lichen crude extracts had more potent anticandidal activities than ELF crude extracts, with the lichen extract having significantly lower MIC values.

Total phenolic content, total flavonoid content and DPPH radical scavenging activities of lichen and ELF crude extracts

Three lichen (*Usnea baileyi*, *U. bismolliuscula* and *U. pectinata*) and five ELF (*Astrocystis bambusae*, *Annulohypoxylon albidiscum*, *Daldinia eschscholtzii*, *Nemania bipapillata* and *Xylaria venustula*) crude extracts were evaluated for their TPC, TFC and antioxidant activities (Tab. 3, Fig. 4). For the lichen crude extracts, *U. bismolliuscula* (24.18 mg GAE/g of extract) had the highest TPC,

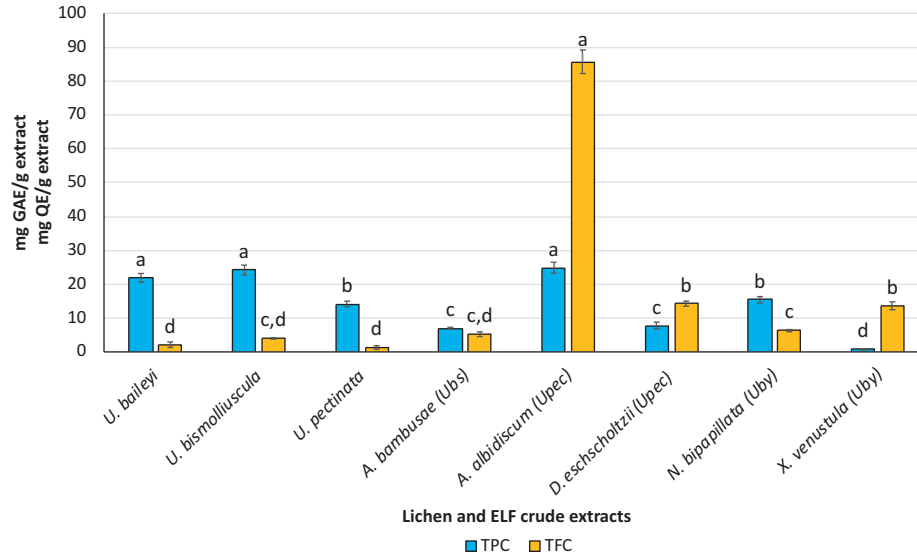


Fig. 4. Total phenolic content (TPC) and total flavonoid content (TFC) of the lichen and ELF crude extracts expressed as mg GAE/g of extract and mg QE/g of extract, respectively. The lichen hosts from which the ELF was isolated are indicated as Uby (*U. baileyi*), Ubs (*U. bismolliuscula*) and Upec (*U. pectinata*). Standard deviation values are indicated by error bars ($P < 0.05$). Letters above error bars indicate the statistical significance within groups using univariate analysis of variance (one-way ANOVA) and Tukey HSD.

Tab. 3. Antioxidant activities of the lichen and ELF crude extracts expressed as IC_{50} values of DPPH scavenging activities.

	Crude extract[a]	**IC_{50}** (mg/ml)	**AAEAC**[b] (mg AA/g of extract)
Lichen host	*Usnea baileyi*	13.24	158.6
	Usnea bismolliuscula	19.20	109.4
	Usnea pectinata	8.543	245.8
ELF	*Astrocystis bambusae* MH370741 (Ubs)	5.220	402.3
	Annulohypoxylon albidiscum MH370738 (Upec)	0.566	3710
	Daldinia eschscholtzii MN071367 (Upec)	3.991	526.2
	Nemania bipapillata MN071354 (Uby)	5.061	414.9
	Xylaria venustula MH370742 (Uby)	19.63	107.0

[a] The lichen hosts from which the ELF was isolated are indicated as Uby (*Usnea baileyi*), Ubs (*U. bismolliuscula*) and Upec (*U. pectinata*).

[b] AAEAC: ascorbic acid equivalent antioxidant capacity; IC_{50AA}: 0.021 mg/ml.

while *A. albidiscum* (24.85 mg GAE/g of extract) had the highest TPC of all ELF crude extracts. In general, the lichen crude extracts (14.13–24.18 mg GAE/g of extract) had a higher TPC than ELF crude extracts (0.703–24.85 mg GAE/g of extract). Furthermore, the TFC of lichen and ELF crude extracts was also determined. Out of the three lichen crude extracts, *U. bismolliuscula* (3.957 mg QE/g of extract) had the highest TFC, while *A. albidiscum* (85.69 mg QE/g of extract) had the highest TFC of all ELF crude extracts. In general, the TFC of ELF crude extracts (5.235–85.69 mg QE/g of extract) was significantly higher than that of the lichen crude extracts (1.288–3.957 mg QE/g of extract) (Fig. 4).

The antioxidant activities and scavenging abilities of the crude extracts were also evaluated (Tab. 3). The lichen *U. pectinata* had the strongest antioxidant and scavenging activities (IC_{50}: 8.543 mg/ml, AAEAC: 245.8 mg AA/g of extract) of all lichen crude extracts. The *A. albidiscum* extract, on the other hand, had the strongest antioxidant and scavenging activities (IC_{50}: 0.566 mg/ml, AAEAC: 3710 mg AA/g of extract) of all ELF extracts. Overall, ELF crude extracts (IC_{50}: 0.566–19.63 mg/ml, AAEAC: 107–3710 mg AA/g of extract) were more potent antioxidants than lichen crude extracts (IC_{50}: 8.543–19.20 mg/ml, AAEAC: 109.4–245.8 mg AA/g of extract).

DISCUSSION

Diversity of endolichenic fungi

The three species of *Usnea* (*U. baileyi*, *U. bismolliuscula* and *U. pectinata*) evaluated in this study harboured diverse ELF communities, belonging to the classes *Sordariomycetes* and *Eurotiomycetes*. The ELF isolates are primarily members of the family *Xylariaceae*, while a few of them belong to other families such as *Aspergillaceae*, *Diaporthaceae*, *Hypoxylaceae* and *Nectriaceae*. Our findings are consistent with other studies reporting that most ELF species isolated from the lichen *Usnea* belong to the class *Sordariomycetes* (particularly the family *Xylariaceae*) and a few to the class *Eurotiomycetes* (He et Zhang 2012, U'Ren et al. 2016, Suryanarayanan et al. 2017). Since culture-dependent techniques were employed during isolation, other non-sporulating and fastidious fungal species were presumed to have been completely excluded.

In this study, different ELF communities were observed in the three lichen hosts. Although the setup of the study and the sample size of this study do not allow for accurate identification of factors affecting the occurrence and diversity of ELF, the lichen host and elevation where the lichen was collected may have played significant roles. Specifically, the lichen host provides shelter as well as food for the growth of ELF. It was hypothesised that ELF, similar to other lichen

associates, depend on the lichen photobiont for its nutrition (Suryanarayanan et Thirunavukkarasu 2017). Elevation influences the amount of fog and dew in the air, which in turn, benefits the lichen as this organism depends on the atmosphere for its nutrition (Nash 2008). The higher the elevation, the more fog and dew are present in the environment, thus resulting in more food made available to lichens and at the same time, to ELF. The effects of these two factors are, therefore, interconnected. However, further studies are required to validate these assumptions. Similar results were observed in other studies which included the assessment of more biotic and abiotic factors affecting the occurrence and diversity of ELF (U'Ren et al. 2012, 2016).

All lichen hosts evaluated in this study belong to the fruticose lichen *Usnea*, thus not allowing to assess any host preference of ELF. Some ELF were isolated from a single *Usnea* species, but this finding does not suggest a host-specific behaviour as these ELF species were previously also reported from other lichen genera (U'Ren et al. 2016, Maduranga et al. 2018). It could be possible that other ELF species are more dominant, depriving yet other ELF of food. Similarly, the isolation technique and the surface sterilisation protocol employed in this study could also have favoured those ELF with simple growth requirements and those capable of withstanding the sterilising effects, making it impossible for the fastidious and weaker or more sensitive species to survive.

This study also showed the genera *Nemania* and *Xylaria*, both belonging to the family *Xylariaceae*, to be the most prevalent among all the isolated ELF. Similar observations were reported previously (U'Ren et al. 2016, Suryanarayanan et al. 2017). The prevalence of the genus *Nemania* is not new, as members of this genus were found abundantly in the lichens *Parmotrema*, *Flavoparmelia* and other *Usnea* species (U'Ren et al. 2016). Similarly, the abundance of the genus *Xylaria* in lichens was also expected, as these species were previously reported inhabiting the lichens *Cladonia*, *Heterodermia*, *Leptogium*, *Parmotrema* and other *Usnea* species (U'Ren et al. 2016, Suryanarayanan et al. 2017, Maduranga et al. 2018). These fungi were previously also reported as plant endophytes (Petrini et Petrini 1985). *Fusarium* and *Penicillium* were previously reported as ELF of other lichen genera (Petrini et al. 1990, Grishkan et Termina 2019), although these fungi are better known for their plant-endophytic or soil-borne nature (Ting et al. 2012, Chen et Ting 2015).

Muggia et al. (2017) reported that the lichen growth form influences the diversity of ELF community. For instance, members of *Eurotiomycetes*, *Leotiomycetes* and *Sordariomycetes* were mainly isolated from foliose and fruticose lichens, while *Chaetothyriomycetes* and *Dothideomycetes* were mainly recovered from saxicolous crustose lichens.

Antimicrobial activities of lichens and ELF

The crude extracts of ELF were found to have a greater range of bioactivities than lichen crude extracts. Similar findings on the broad-spectrum nature of ELF were previously reported (Padhi et Tayung 2015). The antimicrobial properties of ELF could be due to the presence of fatty acids, polyketides, terpenoids and anthraquinones (Yuan et al. 2015, Singh et al. 2017), which were probably present in the ELF crude extracts evaluated in this study. These findings indicate that ELF secondary metabolites are potential antimicrobial agents.

Furthermore, bioactivity of the lichen crude extracts against Gram-positive bacteria and yeasts were also common, which had also been reported previously (Santiago et al. 2010, 2013, Dandapat et Paul 2019). Selective inhibition of lichens against Gram-positive and Gram-negative bacteria was also reported earlier and could be ascribed to the differences in their cell wall composition (Guo et al. 2008). The antimicrobial activities of lichen crude extracts evaluated in this study could be due to the presence of usnic acid, which is commonly present in *Usnea* lichens. However, in this study, most of the lichen crude extracts exhibited stronger antibacterial activities than usnic acid. This indicates that other lichen secondary metabolites, other than usnic acid, could also have been responsible for the bioactivities. It is further possible that synergistic interactions between all lichen secondary metabolites present in the crude extracts resulted in strong antimicrobial activities.

Antioxidant activities of lichens and ELF

This study also discovered a strong antioxidant potential of ELF crude extracts. Similar findings on the antioxidant activities of ELF were reported by Samanthi et al. (2015). Both lichens and ELF were reported to possess high amounts of phenolic groups (i.e. phenols and flavonoids) (Wang et al. 2012, Dandapat et Paul 2019). However, in this study, huge differences in the amounts of TPC and TFC and antioxidant activities between lichen and ELF crude extracts were observed, with the latter showing stronger antioxidant potential. These findings could be due to exposure of the organism to its natural environment. Naturally, ELF are intact and kept 'hidden' inside the lichen thalli, while the lichen itself is strongly exposed to the environment.

It has been reported that light stimulates the synthesis of flavonoids and the natural presence of phenolic compounds in these organisms protect them against UV-B damage and cell death (Lai et Lim 2011). Therefore, organisms which are exposed to numerous stressors have increased their antioxidant activities. However, prolonged exposure to these stressors may lead to cell damage and are hence considered detrimental to the organism. In this study, more antioxidants

were produced by ELF, as indicated by their strong antioxidant and scavenging activities, since these microorganisms are exposed less to stressors than lichens.

Furthermore, this study also revealed that other non-phenolic compounds present in lichens and ELF may also be responsible for their antioxidant activities. Such a negative correlation between phenolic composition and antioxidant activities of both lichens and ELF had already been reported previously (Samanthi et al. 2015, Dandapat et Paul 2019). As such, other secondary metabolites present in these crude extracts could be effective antioxidants and should not be neglected. Overall, the strong antimicrobial and antioxidant activities demonstrated by ELF crude extracts suggest that ELF are excellent sources of bioactive compounds.

CONCLUSION

This study serves as a starting point in exploring the diverse ELF communities inhabiting the fruticose lichens of the genus *Usnea* in the Philippines and provides further impetus for studying ELF to acquire a more comprehensive understanding of their ecological roles in lichens and in nature. Our findings have shown the advantages of ELF over the slow-growing lichens as sources of metabolically active compounds, making them valuable alternative candidates for drug discovery.

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