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Endolichenic fungi from common *Usnea* lichens found in a montane forest in Malaysia: a study on diversity and bioactivity profiling

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Abstract

This study evaluated the diversity of endolichenic fungi (ELF) isolated from three common lichen species (*Usnea baileyi, U. bismolliuscula* and *U. pectinata*) found in Bukit Larut, Peninsular Malaysia. Diversity indices and phylogenetic analysis were performed to identify the various species found in lichens, and their bioactivity profiles were established based on antimicrobial and antioxidant activities. Sixty-two (62) ELF isolates were identified, mostly belonging to the family Xylariaceae (88.7%, 55 isolates), followed by Hypoxylaceae (6.5%, four isolates), Nectriaceae (3.2%, two isolates) and Sporocadaceae (1.6%, one isolate). The common genera were *Nemania* (45.2%, 28 isolates) and *Xylaria* (27.4%, 17 isolates). In this study, ELF isolates have shown both host-generalist and host-specific behaviours towards their lichen hosts. The ELF also have relatively strong antimicrobial (minimum inhibitory concentration:0.0625-10.00mg/ml) and antioxidant (IC_{50}:5.72-17.8mg/ml) properties. This study highlights that a diverse ELF collection rich in bioactive compounds can be found in lichens in the montane forest in Malaysia.

Keywords – Bukit Larut – fruticose lichen – host specificity – tropical forest – Xylariaceae

Introduction

Lichens are symbiotic organisms that typically comprise a fungal partner (mycobiont) with an autotrophic partner (photobiont, i.e., green algae or cyanobacteria) (Asplund & Wardle 2017). The possibility of a third lichen symbiont has been hypothesized with the discovery of the yeast-like basidiomycete (*Cyphobasidium* sp.) from the cortex of the lichen *Bryoria tortuosa* (Spribille et al. 2016). In recent years, other bacterial and fungal communities have also been discovered in lichens (Honegger et al. 2013). Among these are a group of non-obligate microfungi called endolichenic fungi (ELF) (Agrawal et al. 2020). ELF are taxonomically and ecologically different from the lichen mycobiont (the primary fungal component of the lichen) and other lichenicolous fungi (fungi with obligatory association with lichens as saprotrophs or parasites) (Arnold et al. 2009, U'Ren et al. 2010). They do not cause symptoms in the lichen thalli, and this association is similar to endophytic fungi with their host plant (Santiago & Ting 2019, dela Cruz & Santiago 2022). Most species of ELF are members of the phylum Ascomycota (Arnold et al. 2009), with species from

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Basidiomycota and Zygomycota reported as well (Zhang et al. 2015).

The *Usnea* lichens are among the common lichens found in tropical forests, valued for their ecological and pharmaceutical significance. They are also known to host a diverse species of ELF. *Usnea cavernosa* reportedly harbours *Corynespora* sp., the first ELF explored for its bioactive metabolites (Paranagama et al. 2007). Other *Usnea* spp. are host to ELF from the genera *Curvularia, Fusarium, Nigrospora, Cladosporium* and *Chrysosporium* (Kannangara et al. 2009, Samanthi et al. 2015). The diversity of ELF from their lichen hosts has been reported by researchers from Europe (Petrini et al. 1990, Girlanda et al. 1997), North America (U'Ren et al. 2012) and Eastern Asia (Oh et al. 2020, Yang et al. 2021). However, profiling of ELF from their lichen hosts from Southeast Asia is rare (Santiago et al. 2021a). Existing studies documented the lichen flora (Din et al. 2010, Zulkifly et al. 2011, Rajan et al. 2016, Paguirigan et al. 2019, Paguirigan et al. 2020) but excluded details on their ELF. This work, therefore, explores the ELF diversity and occurrence of *Usnea* lichens in Malaysia to complement our initial report on the *Usnea* lichens from the Philippines (Santiago et al. 2021a). Additionally, the bioactivity profile of ELF is also explored due the limited information availability (Tan et al. 2020, Santiago et al. 2021a, 2021b). However, studies on the biological activities of the lichen host are available (Yusof et al. 2015, de Jesus et al. 2016, Rajan et al. 2016, Gazo et al. 2019).

This study, therefore, embarks to investigate the ELF assemblages in three common *Usnea* lichens found in Bukit Larut in Peninsular Malaysia using a culture-dependent technique. We aim to compare these assemblages with our recent study in the Philippines (Santiago et al. 2021a), with relatively similar environmental conditions (i.e., weather, rainfall, elevation and vegetation type) to determine if similar lichen hosts collected from another tropical country in the Southeast Asian region will harbour similar ELF assemblages. Our study will provide insights into the possible influence of lichen hosts on the diversity and occurrence of ELF. The profiling of antimicrobial and antioxidant activities in ELF further establishes the potential of ELF as an alternative source for bioactive compounds.

Materials & Methods

Collection and identification of lichens

Usnea lichens of the species *U. baileyi* (Stirt.) Zahlbr., *Usnea bismolliuscula* Zahlbr. and *U. pectinata* Stirt., (= *Eumitria pectinata*) were collected from Bukit Larut in Perak, Malaysia (4°51′44″ N, 100°47′36″ E). The lichens were randomly sampled from barks of pine trees (*Pinus* sp.) found at different accessible elevations (Fig. 1). All collected lichens were placed in sterile plastic bags. GPS data and elevation of the site were recorded. Annual weather data (annual temperature: 27 °C, humidity: 84%) were retrieved from http://www.customweather.com.

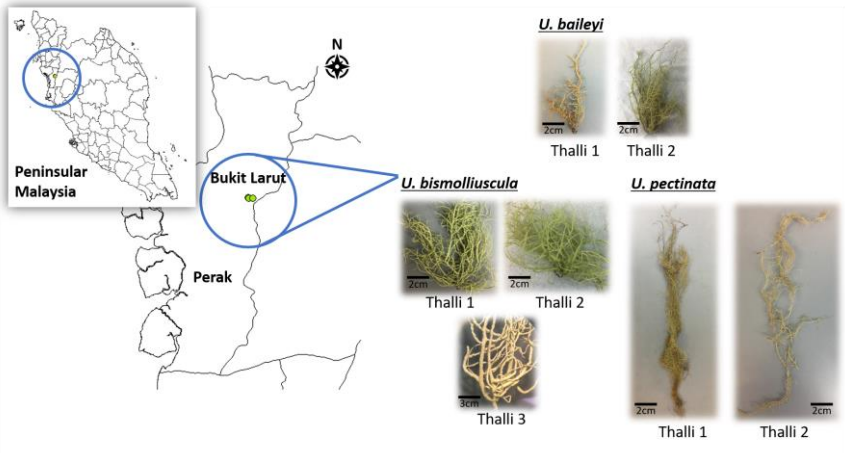

Fig. 1 – The collection site and the seven lichen hosts. The map is drawn using the software DIVA-GIS (Hijmans et al. 2012)

Each lichen specimen was viewed under a stereomicroscope (4x, Nikon Instruments, Inc.) to observe the morphological characteristics such as the length of thallus (e.g., shrubby, sub-pendent, pendent), the shape of lichen segments (e.g., terete (inflated), deformed or ridged), branch shape and texture, type of axis (e.g., thick, thin or hollow) and medulla (e.g., compact, dense, loose), presence of common reproductive structures and their shape (soredia) and presence and location of pigmentation, to subsequently categorize the *Usnea* lichens into three species groups (Truong et al. 2011, Ohmura 2012, Truong & Clerc 2012). Lichen specimens with shrubby or sub-pendent thalli, fistulose or hollow axis, red pigmentation in the medulla near the cortex and the presence of punctiform soralia were identified as *U. baileyi* (Ohmura 2012). Specimens characterized by shrubby to sub-pendent thalli, terete, perforated, smooth and glossy branches, loose medulla and irregular-shaped soralia were identified as *U. bimsolliuscula* (Ohmura 2012). *Usnea pectinata* lichens were characterized by pendent and ridged thalli, uninflated branches, thick axis, compact medulla and absence of soralia (Ohmura 2012).

The collected lichens were also subjected to thalline spot test to supplement the morphological data. Briefly, the upper cortex of each lichen specimen was initially scraped off to expose the medulla. Different chemical reagents (e.g., potassium hydroxide (K, KOH), sodium hypochlorite (C, NaOCl) and the combination of KOH and NaOCl (KC test)) were then spotted on the exposed medulla. Each test was done on different parts of the thallus (Santiago et al. 2010). An immediate change in the color of medulla was recorded, whereby each color represents the presence of a specific lichen metabolite or type of compound. For the K test, the presence of yellow turned red color indicates most *o*-hydroxyl aromatic compounds. For the C test, the red color indicates the presence of *m*-dihydroxy phenols, while green indicates dihydroxy dibenzofurans. For the KC test, a change in yellow, blue and red indicates the presence of usnic acid, dihydroxy dibenzofurans and depsides and depsidones, respectively (Elix & Stocker-Wörgötter 2008).

Molecular confirmation of the identity of the lichen hosts

Molecular typing for lichens was performed using protocols from Seymour et al. (2007). Thirty milligrams (30 mg) of dry fine powder of each lichen was used for DNA extraction using Plant DNA Extraction Kit (Vivantis Technologies, Subang Jaya, Malaysia) following the manufacturer's instructions. The genomic DNA was then amplified using the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) with PCR conditions based on the protocol by Seymour et al. (2007). Briefly, samples were subjected to initial denaturation at 95 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 1 min. This was followed by annealing at 55 ºC for 1 min, extension at 72 ºC for 1 min and final extension for 5 min at 72 ºC. The PCR products were subjected to electrophoresis on 1.6% agarose gel and were sequenced by Apical Scientific Sequencing (Malaysia). The fungal ITS rDNA fragments were manually edited using BioEdit 7.0 (Hall 1999), and their nucleotides were compared with those in GenBank using Basic Local Alignment Search Tool (BLAST) analysis (http://www.ncbi.nlm.nih.gov/) to determine their taxonomic identities. The sequences were deposited in GenBank, and their respective accession numbers were assigned and presented in the results (Fig. 2).

The phylogenetic analysis of the identified lichen hosts was performed using MEGA 7.0 (Kumar et al. 2016). The DNA sequence alignment was completed using the ClustalW option incorporated in the software. 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, and 1000 bootstrap replications were used as statistical support for the phylogenetic tree. The phylogenetic position of the lichen hosts is then discussed in relation to the phylogeny of the isolated ELF.

Isolation of ELF

The isolation of ELF from the collected lichen sample was conducted according to Samanthi et al. (2015) with modifications to the ethanol concentrations. Briefly, lichens were initially cleaned

with tap water to remove all debris. The lichen thalli were then surface sterilized by successively dipping in different ethanol concentrations (70%, 80%, 85% and 90% v/v) for 1 min, with water rinsing in between ethanol concentrations. The lichen thalli were then dipped in 10% NaOCl for 30 s followed by immersing in 95% ethanol for another 30 s. The lichen thalli were finally rinsed with water and dried using sterile filter paper. To validate the efficacy of the surface sterilization technique, the final rinsing water used in the surface sterilization procedure was spread-plated onto potato dextrose agar (PDA, Merck, Darmstadt, Germany). The lichen thalli were cut into small fragments (approx. 2 mm) and plated onto 2% (wt/wt) malt yeast extract agar (MYE, Lab M Limited, Heywood, UK). Ten lichen fragments from each *Usnea* sample were carefully placed on each MYE (in triplicates) and were incubated at room temperature (26 ± 2 ºC, under light for 12 h). Fungal colonies formed were transferred to fresh MYE agar plates and established as pure cultures, and subsequently transferred to agar slants to establish stock cultures.

Molecular identification of ELF

Isolates were first cultured in potato dextrose broth (PDB, Merck, Darmstadt, Germany) for seven days at room temperature (26 ± 2 ºC, under light for 12 h). The mycelium was harvested and dried using filter paper under sterile conditions. The mycelium was ground until powdery with the addition of liquid nitrogen. Dry fine powder of each ELF isolate was used for DNA extraction using Plant DNA Extraction Kit (Vivantis Technologies, Subang Jaya, Malaysia) following the manufacturer's instructions. The resulting genomic DNA was subjected to PCR using the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Honegger et al. 2013). The PCR reaction was performed following the protocol described by He & Zhang (2012). Briefly, the samples were subjected to initial denaturation at 98 ºC for 3 min, followed by 30 cycles of denaturation at 94 ºC for 30 s. These were followed by annealing at 55 ºC for 30 s, extension at 55 ºC for 30 s and final extension for 1 min at 72 ºC. The PCR products were subjected to electrophoresis on 1% agarose gel and sequenced by Apical Scientific Sequencing (Malaysia). The fungal ITS rDNA fragments were manually edited using BioEdit 7.0 (Hall 1999), and their nucleotides were compared with those in GenBank using Basic Local Alignment Search Tool (BLAST) analysis (http://www.ncbi.nlm.nih.gov/) to determine their taxonomic identities. The sequences were deposited in GenBank, and their respective accession numbers were assigned.

Phylogenetic analysis of ELF isolates

The phylogenetic analysis of the identified ELF was performed using MEGA 7.0 (Kumar et al. 2016). DNA sequence alignment was done using the ClustalW option incorporated in the software. 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, and 1000 bootstrap replications were used as statistical support for the phylogenetic tree. The fungus *Peziza varia* (JF908557.1) was used as the outgroup for a point of comparison for the ingroup and to allow the phylogeny to be rooted.

Diversity assessment and ecological analysis of ELF

The diversity assessment for ELF was assessed based on the presence and absence of a specific species in the study site. The colonization rate was calculated as the total number of lichen segments with fungi divided by the total number of plated lichen segments and expressed as percentages (Li et al. 2007). The Simpson Diversity Index (D′), Shannon-Weiner Diversity Index (H′), and Shannon's Equitability of the lichen hosts were calculated based on the number of isolated ELF using the software PAST 3.24 (Hammer et al. 2001). The similarity matrix was graphically represented via dendrogram using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm. The Venn Diagram was created manually to present similarities in species composition. The Cole Rarefaction of the lichen hosts was calculated using EstimateS (Colwell 2013) and graphically represented using Microsoft Excel.

Extraction of secondary metabolites produced by the lichen hosts and selected ELF

Three lichen hosts and four representative ELF isolates were extracted for their secondary metabolites and screened for their antimicrobial and antioxidant activities. The extraction of lichen acids was performed following the protocol of Santiago et al. (2010). Briefly, 1 g of each lichen specimen was ground into powder form, and then soaked with acetone overnight. The crude extract was filtered using filter paper (Whatman no. 1, Tisch Scientific, Ohio, USA) (under sterile conditions), and the supernatant was collected in a pre-weighed vial. Each vial was carefully covered with cheesecloth until the acetone evaporated to yield the crude extracts.

For ELF, 10 mycelial plugs (approx. 15 mm in diameter) were inoculated into glucose malt yeast extract (GMY, Lab M Limited, Heywood, UK) broth and incubated at room temperature (26 ± 2 ºC, under light for 12 h) (135 rpm) for 15 days to obtain the seed culture. The solid-state fermentation was then initiated using rice (160 g in 240 ml of distilled water) as the substrate. The rice media were autoclaved, and the seed culture was inoculated and incubated at room temperature for 50 days (Santiago et al. 2021b). Following incubation, each rice medium was transferred to a clean jar and soaked with ethyl acetate overnight. The rice medium was filtered using a cheesecloth (under sterile conditions), and the supernatant (i.e., solvent) was collected. The solvent was evaporated via reduced pressure to yield the crude extracts.

Antimicrobial activities of the lichen hosts and selected ELF

Paper disk diffusion and agar well assays were used to evaluate the antimicrobial properties of lichen and ELF crude extracts, respectively. These were carried out following the CLSI guidelines (Balouiri et al. 2016). The test organisms used were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231. Additionally, (+)-usnic acid (98% purity, Sigma-Aldrich, Merck, Darmstadt, Germany), chloramphenicol, polymyxin B and fluconazole (ThermoFisher Scientific Oxoid Ltd, Basingstoke, UK) were used as the reference standard and positive controls, respectively. A concentration of 10 mg/ml of crude extract was used throughout the study. In addition, the minimum inhibitory concentration (MIC) was determined for each crude extract as recommended by CLSI (Balouiri et al. 2016). The MIC data of the lichen and ELF crude extracts presented here, however, were also reported in our previous work (Santiago et al. 2021b). The inclusion of these data in the present study is necessary to emphasize the antimicrobial activities of the crude extracts.

Total phenol content (TPC), total flavonoid content (TFC) and antioxidant activities of lichen hosts and selected ELF

The TPC of the lichen and ELF crude extracts was evaluated spectrophotometrically, following the Folin-Ciocalteau method (Lai & Lim 2011). Briefly, the methanol extract (1 mg/ml) was mixed with 10% Folin-Ciocalteau's reagent (Sigma-Aldrich, Merck, Darmstadt, Germany) and 7.5% sodium carbonate (Sigma-Aldrich, Merck, Darmstadt, Germany). Each sample mixture was kept in the dark for 30 min before measuring the absorbance using the Spark™ multimode microplate reader (Tecan Trading AG, Switzerland) at 765 nm. A blank was prepared by replacing the crude extract with methanol. The TPC for each crude extract was calculated from the gallic acid (Friendemann Schmidt Chemical, Washington, USA) calibration curve and was expressed as gallic acid equivalents (GAE) per g of extract.

The TFC of lichen and ELF crude extracts was evaluated using the aluminium chloride colourimetric method (Gunasekaran et al. 2017). Briefly, the methanol extract (1 mg/ml) was mixed with distilled water and 5% sodium nitrate ($NaNO_2$) (Friendemann Schmidt Chemical, Washington, USA). The solution was incubated for 6 min, and 10% aluminium chloride (AlCl3) (Friendemann Schmidt Chemical, Washington, USA) was added and further incubated for another 6 min. Then, 1M sodium hydroxide (NaOH) (Friendemann Schmidt Chemical, Washington, USA) was added and made up to mark with distilled water and left at room temperature for 15 min.

Absorbance was measured using a Spark™ multimode microplate reader (Tecan Trading AG, Switzerland) at 510 nm. A blank was prepared by replacing the crude extract with methanol. The TFC for each crude extract was calculated from the quercetin (ChemFaces Biochemical, Hubei, China) calibration curve and was expressed as quercetin equivalents (QE) per g of extract. Determinations were carried out in triplicates.

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activities of the extracts were determined following Lai & Lim (2011). The DPPH solution (Friendemann Schmidt Chemical, Washington, USA) was added to three different concentrations of the crude extract. The solution was incubated in the dark for 30 mins and the absorbance was measured at 517 nm using a Spark™ multimode microplate reader (Tecan Trading AG, Switzerland). Results were presented as IC_{50}, the concentration of crude extract required to scavenge 50% of the DPPH radical. In addition, results were presented as ascorbic acid equivalent antioxidant capacity (AAEC) as described by Lai & Lim (2011). The IC_{50} of ascorbic acid (AA) was determined to be 0.021 mg/ml.

Statistical analysis

All microbiological laboratory tests (antimicrobial assays, determination of TPC and TFC and antioxidants) were done in triplicates. One-way ANOVA was used to analyze all obtained data using SPSS 23.0 (International Business Machines Corp., New York, USA), and the means were compared using Tukey's HSD post hoc test (P<0.05).

Results

The collected lichen hosts

Seven individual lichens, belonging to *U. baileyi* (2 specimens), *U. bismolliuscula* (3 specimens) and *U. pectinata* (2 specimens) were collected from accessible areas (1000 to 1100 meters above sea level, masl) within Bukit Larut (Figs 1,2). Colonization rates of ELF in these lichens were between 23-46% (per individual lichen host) and 18-45% (pooled data per lichen species) (Table 1), with *U. pectinata* having the highest ELF isolates. From these lichens, a total of 62 ELF was isolated. The species accumulation curve for both individual lichen host and pooled lichen species did not exhibit a plateau to indicate that the expected number of observed species is met, suggesting that there could be more ELF species present within the lichen thalli (Fig. 3).

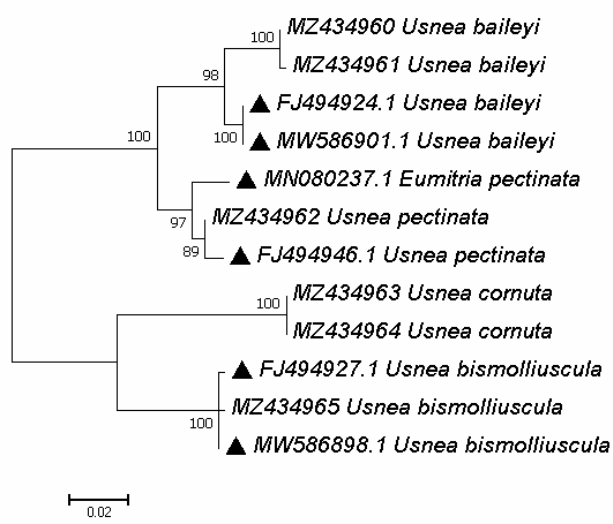

Fig. 2 – Maximum likelihood of the three *Usnea* spp., namely *U. baileyi*, *U. bismolliuscula* and *U. pectinata* (=*Eumitria pectinata*), collected from Bukit Larut, Malaysia. The tree is drawn to scale based on 1000 repetitions, with branch lengths measured in the number of substitutions per site. Numbers near each node represent the bootstrap support obtained from maximum likelihood

method, showing only those above 50%. The lichen *U. cornuta* were included to emphasize the relationship of the three *Usnea* lichens evaluated in this study. Reference sequences (▲) retrieved from GenBank were included to validate the identification of the lichen specimens.

Table 1 Taxonomic and diversity indices of the endolichenic fungi in relation to the lichen host.

Lichen Host[a]	No. of Individuals	No. of Species	No. of Genera	S/G Ratio	Colonization Rate (%)	Simpson Diversity Index (D′)	Shannon-Weiner Diversity Index (H′)[b]	Shannon's Equitability
Uby1	7	3	2	1.5	23.3	0.653	1.079	0.981
Uby2	4	4	2	2.0	13.3	0.750	1.386	1.00
Pooled (Uby)	11	4	2	2.0	18.3	0.711	1.295	0.912
Ubs1	8	6	4	1.5	26.7	0.813	1.733	0.943
Ubs2	6	6	5	1.2	20.0	0.833	1.792	1.00
Ubs3	10	3	2	1.5	33.3	0.460	0.802	0.743
Pooled (Ubs)	24	12	7	1.7	26.7	0.837	2.152	0.717
Up1	13	8	4	2.0	43.3	0.793	1.839	0.787
Up2	14	6	3	2.0	46.7	0.786	1.649	0.867
Pooled (Up)	27	10	5	2.0	45.0	0.829	1.973	0.719

[a] Lichen hosts: (Uby1, Uby2) – *U. baileyi* thalli 1 and 2, (Ubs1, Ubs2, Ubs3) – *U. bismolliuscula* thalli 1, 2 and 3, (Up1, Up2) – *U. pectinata* thalli 1 and 2.

[b] Diversity t test showed significant difference between pooled data from *U. baileyi* x *U. bismolliuscula* and *U. baileyi* x *U. pectinata* at $P < 0.05$, whereas no significant difference was observed between *U. bismolliuscula* x *U. pectinata*. Similarly, significant differences were observed between individual thalli of the three *Usnea* spp. at $P < 0.05$: Uby1 x Ubs1, Uby1 x Ubs2, Uby1 x Up1, Uby1 x Up2, Ubs1 x Ubs3, Ubs2 x Ubs3, Ubs3 x Up1 and Ubs3 x Up2

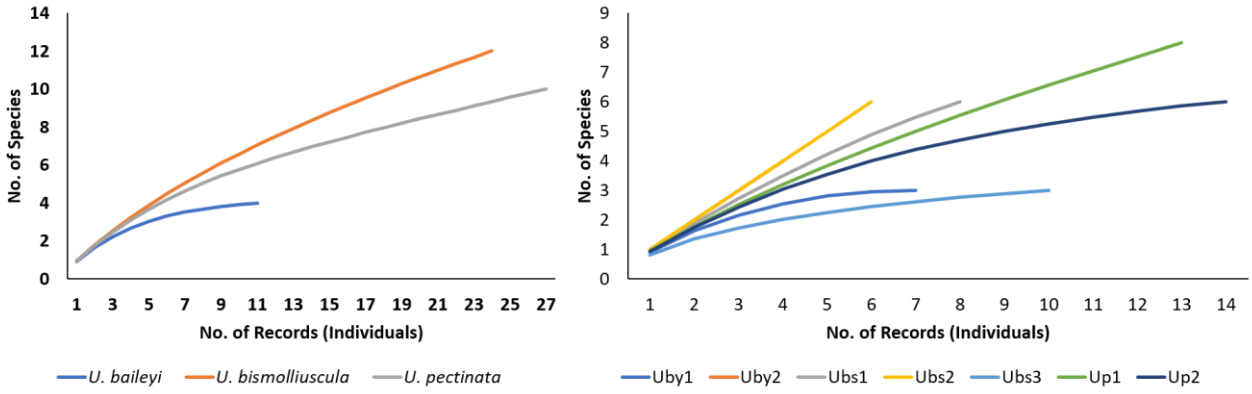

Fig. 3 – Species accumulation curve (Cole rarefaction) for ELF isolated from the (a) three *Usnea* spp. (pooled) and (b) seven individual lichen thalli. Data were randomized 100 times for plotting the graph.

Composition of endolichenic fungal assemblages

The ELF assemblages in *Usnea* lichens were assigned to four families (Hypoxylaceae, Nectriaceae, Sporocadaceae and Xylariaceae), encompassing nine genera and 18 species of the class Sordariomycetes (Fig. 4, Table 2). Of the four families, Xylariaceae was the most dominant (88.7% abundance, 55 isolates), followed by Hypoxylaceae (6.5%, four isolates), Nectriaceae (3.2%, two isolates) and Sporocadaceae (1.6%, one isolate) (Fig. 4a). At the genus level, *Nemania* was the most frequently isolated ELF (45.2%, 28 isolates), followed by *Xylaria* (27.4%, 17 isolates) and *Kretzschmaria* (12.9%, eight isolates) (Fig. 4b). The genera *Annulohypoxylon*, *Daldinia*, *Fusarium*, *Hypoxylon*, *Nodulisporium* and *Pseudopestalotiopsis* were the least isolated

ELF, represented by only 1–2 isolates (rare taxa). Among the species, *Xylaria* sp. (21.7%, 13 isolates) and *Nemania diffusa* (20%, 12 isolates) were the most abundant ELF among the *Usnea* lichens. Both these ELF species were isolated from all three lichen hosts, whereas rare taxa were only recovered from one lichen host (Fig. 4, Table 2). For example, *Annulohypoxylon*, *Daldinia*, *Hypoxylon* and *Nodulisporium* were only recovered from *U. bismolliuscula* and the ELF *Fusarium* and *Pseudopestalotiopsis* from *U. pectinata* (Table 2).

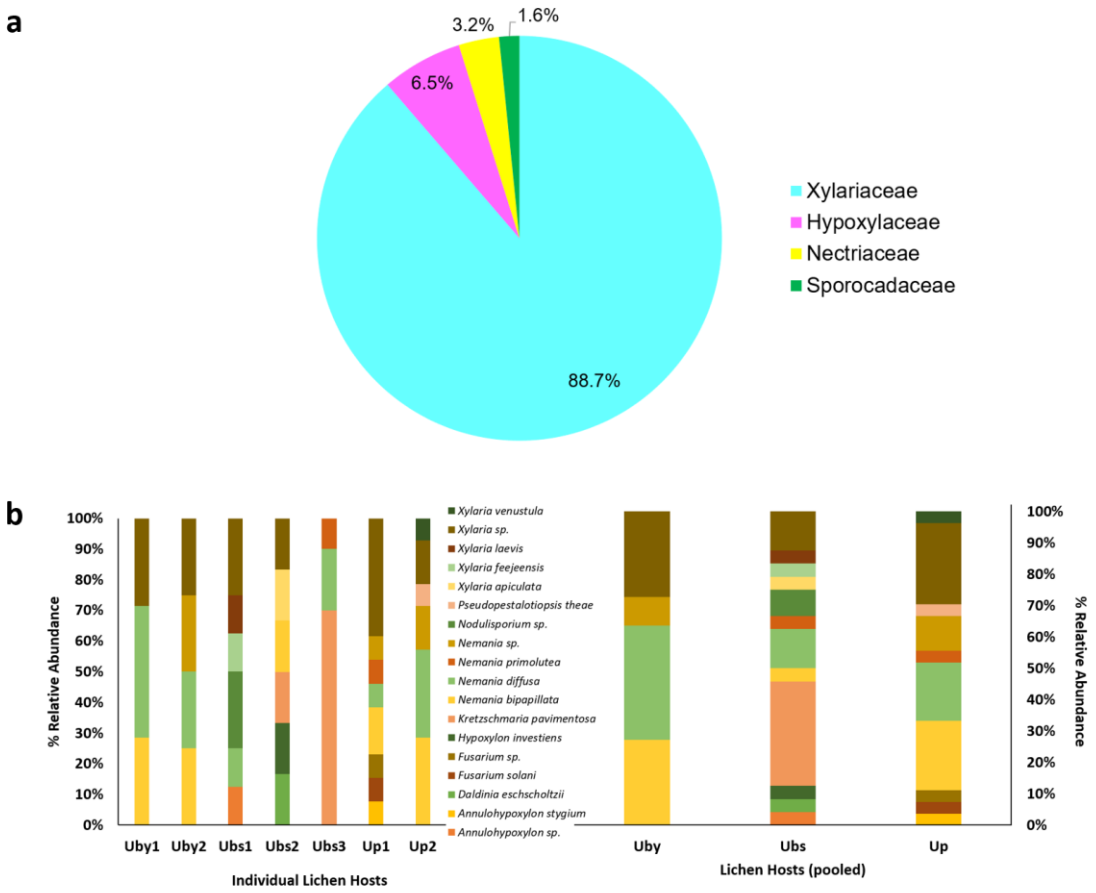

Fig 4 – The relative abundance of ELF species based on their families, (a) isolated from (b) the seven lichen specimens and (c) the three *Usnea* spp. (pooled). Lichen hosts: (Uby1, Uby2) – *U. baileyi* thalli 1 and 2, (Ubs1, Ubs2, Ubs3) – *U. bismolliuscula* thalli 1,2 and 3, and (Up1, Up2) – *U. pectinata* thalli 1 and 2. Different colors represent different species

Table 2 The 18 ELF species isolated from the lichen *Usnea*.

ELF Species	***U. baileyi***			***U. bismolliuscula***				***U. pectinata***			**Total**
	Uby1[a]	Uby2	Pooled	Ubs1	Ubs2	Ubs3	Pooled	Up1	Up2	Pooled	**No. of Isolates**
Annulohypoxylon stygium	0	0	0	0	0	0	0	1	0	1	**1**
Annulohypoxylon sp.	0	0	0	1	0	0	1	0	0	0	**1**
Daldinia eschscholtzii	0	0	0	0	1	0	1	0	0	0	**1**
Hypoxylon investiens	0	0	0	0	1	0	1	0	0	0	**1**

Table 2 Continued.

ELF Species	*U. baileyi*			*U. bismolliuscula*				*U. pectinata*			Total
	Uby1[a]	Uby2	Pooled	Ubs1	Ubs2	Ubs3	Pooled	Up1	Up2	Pooled	**No. of Isolates**
Fusarium solani	0	0	0	0	0	0	0	1	0	1	**1**
Fusarium sp.	0	0	0	0	0	0	0	1	0	1	**1**
Pseudopestalotiopsis theae	0	0	0	0	0	0	0	0	1	1	**1**
Kretzschmaria pavimentosa	0	0	0	0	1	7	8	0	0	0	**8**
Nemania bipapillata	2	1	3	0	1	0	1	2	4	6	**10**
N. diffusa	3	1	4	1	0	2	3	1	4	5	**12**
N. primolutea	0	0	0	0	0	1	1	1	0	1	**2**
Nemania sp.	0	1	0	0	0	0	0	1	2	3	**4**
Nodulisporium sp.	0	0	0	2	0	0	2	0	0	0	**2**
Xylaria apiculata	0	0	0	0	1	0	1	0	0	0	**1**
X. feejeensis	0	0	0	1	0	0	1	0	0	0	**1**
X. laevis	0	0	0	1	0	0	1	0	0	0	**1**
Xylaria sp.	2	1	3	2	1	0	3	5	2	7	**13**
X. venustula	0	0	0	0	0	0	0	0	1	1	**1**
Total No. of Isolates	**7**	**4**	**11**	**8**	**6**	**10**	**24**	**13**	**14**	**27**	**62**

[a] Number of isolated ELF per lichen thalli/host

The effects of lichen host on the ELF assemblage

In this study, the effects of the lichen host on the occurrence of ELF are rather inconclusive. There were ELF species that were confined in only one lichen host (i.e., host-specific), while other ELF were found widespread in all three lichen species (i.e., host-generalist). For example, the ELF *Kretzschmaria pavimentosa*, represented by eight lichen specimens, was only isolated from the lichen *U. bismolliuscula* (Table 2). The ELF *Nemania bipapillata*, *N. diffusa* and *Xylaria* sp., on the other hand, were isolated from all three *Usnea* species (Table 2). These findings suggest that lichen hosts may influence the composition of their ELF assemblages by, perhaps, providing their necessary growth requirements.

U. bismolliuscula had the most diverse ELF assemblage, followed by *U. pectinata* and *U. baileyi* (Table 1). Data suggest that *U. bismolliuscula* could have been a better or more "welcoming" host for most ELF species isolated in this study. Furthermore, a similar trend was observed when assessing ELF dominance among lichen hosts. *U. bismolliuscula* had the highest number of dominant ELF species, followed by *U. pectinata* and *U. baileyi* (Table 1). Among the individual lichen thalli evaluated, *U. bismolliuscula* specimens also gave the highest D′ values, which could be attributed to the presence of eight *K. pavimentosa* isolates inhabiting this lichen host (Table 1).

In addition to the diversity indices evaluated in this study, a phylogenetic tree was constructed to look for any trend or pattern in relation to the genetic diversity of the ELF and their lichen hosts (Fig. 5). Data revealed that different members of Xylariaceae were identified from all *Usnea* lichens. However, members of other remaining three families (i.e., Hypoxylaceae, Nectriaceae and Sporocadaceae) were found in two lichen hosts, but not in *U. baileyi* (Fig. 5). At the genus level, *Xylaria* and *Nemania*, the most abundant ELF in this study, were isolated from all three *Usnea* spp. (Fig. 5) suggesting that these ELF can be described as host-generalists as also noted previously. The eight isolates of *K. pavimentosa*, however, were consistently isolated from *U. bismolliuscula* and hence, may be described as host-specific. The diversity and phylogenetic data both suggested that some ELF species may have host preferences while others do not.

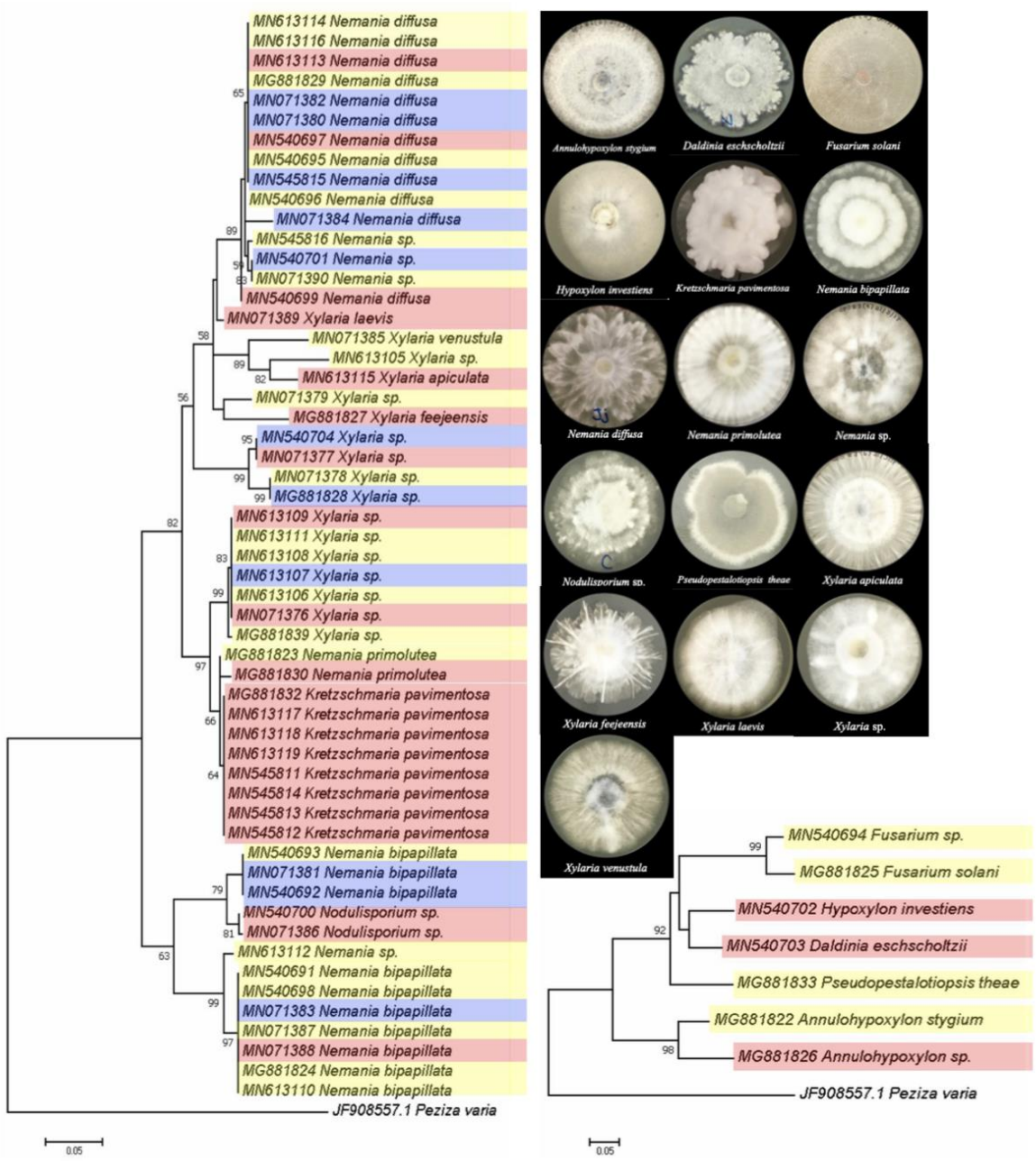

Fig 5 – Maximum likelihood tree of the 62 identified ELF isolated from the three *Usnea* spp. collected from Bukit Larut, Malaysia: (a) ELF isolates belonging to the family Xylariaceae and (b) the families, Hypoxylaceae, Nectriaceae and Sporocadaceae. The tree is drawn to scale based on 1000 repetitions, with branch lengths measured in the number of substitutions per site. Numbers near each node represent the bootstrap support obtained from maximum likelihood method, showing only those above 50%. Lichen host where the specific ELF was isolated is indicated using different colors: blue – *U. baileyi*, red – *U. bismolliuscula* and yellow – *U. pectinata.* The fungus *Peziza varia* JF908557.1 serves as the outgroup. Images of representative ELF species are shown on the left.

Five and seven ELF species were only found in *U. pectinata* and *U. bismolliuscula*, respectively, whereas none were unique or specific to *U. baileyi* (Fig. 6). The lichen hosts Up2 and Uby1 (per individual lichen host) and *U. baileyi* and *U. pectinata* (per *Usnea* species) had the most

similar ELF assemblages as reflected by their cluster analyses (Fig. 7). We then compared the ELF in relation to the phylogenetic position of our lichen hosts. It is interesting to note that the lichens *U. pectinata* and *U. baileyi* are more phylogenetically related (Fig. 2) and could be an important link to the similarity in their ELF assemblages. Such findings may also provide insights into the influence of lichen hosts on the occurrence of their ELF. However, we recognized the limitations of our study, and we suggest further analysis with more lichen hosts be done on this concept.

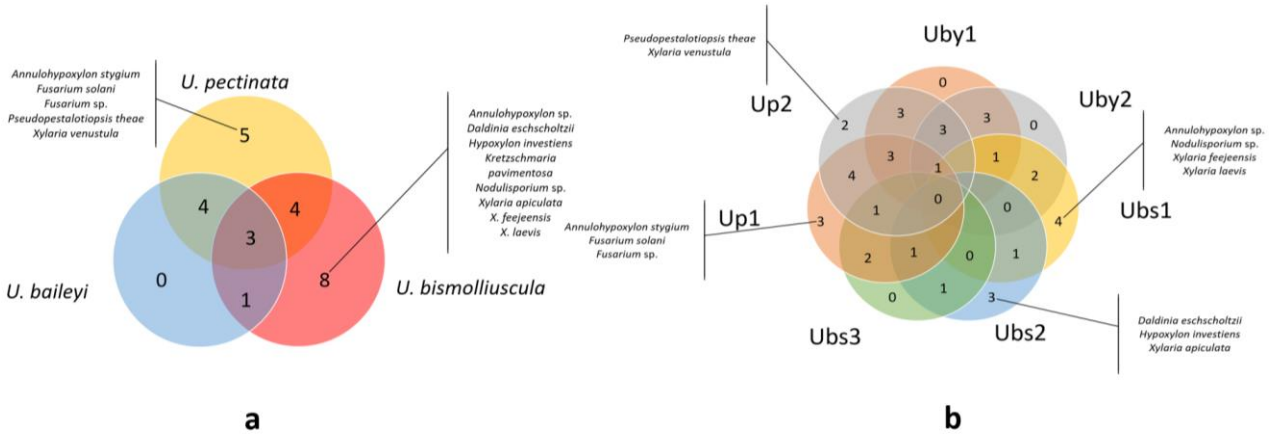

Fig 6 – ELF assemblages in (a) the three *Usnea* spp. (pooled) and (b) all seven lichen thalli. Unique ELF species found in a specific lichen host are listed. Lichen hosts: (Uby1, Uby2) – *U. baileyi* thalli 1 and 2, (Ubs1, Ubs2, Ubs3) – *U. bismolliuscula* thalli 1, 2 and 3, and (Up1, Up2) – *U. pectinata* thalli 1 and 2.

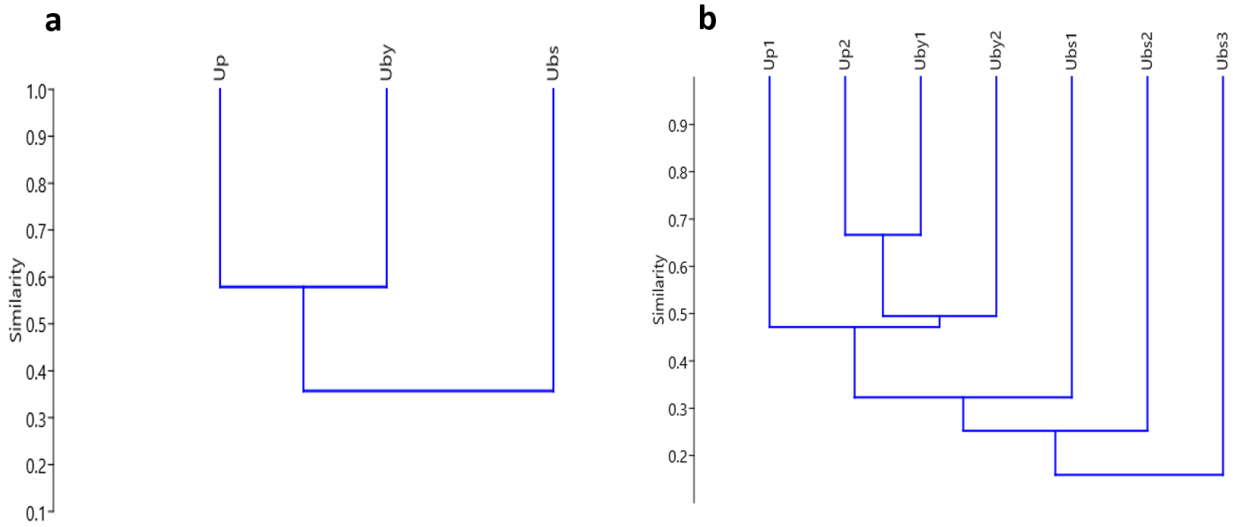

Fig 7 – The cluster analysis of (a) each *Usnea* species and (b) individual lichen thalli. Lichen hosts: (Uby1, Uby2) – *U. baileyi* thalli 1 and 2, (Ubs1, Ubs2, Ubs3) – *U. bismolliuscula* thalli 1,2 and 3, and (Up1, Up2) – *U. pectinata* thalli 1 and 2.

Antimicrobial and antioxidant activities of the three Usnea spp. and the selected ELF species

The bioactive compounds extracted from both lichen and ELF revealed antimicrobial properties. For lichen crude extracts, all were active against *Staphylococcus aureus*, with *U. bismolliuscula* (MIC: 0.0625 mg/ml) demonstrating the strongest activity, while none inhibited *Escherichia coli* (Fig. 8). For the ELF crude extracts, *Fusarium solani* (MIC: 10 mg/ml) and

Pseudopestalotiopsis theae (MIC: 10 mg/ml) demonstrated the strongest activities against *S. aureus* and *E. coli*, respectively (Fig. 8). Additionally, both lichen and ELF were active against *Candida albicans* (Fig. 8), with *U. bismolliuscula* (MIC: 0.0625 mg/ml) and *Annulohypoxylon* sp. (MIC: 0.0625 mg/ml) having the strongest activity among lichen and ELF crude extracts, respectively (Fig. 8). These findings revealed that the level of bioactivities of ELF does not reflect those of their lichen hosts. For example, the ELF *Annulohypoxylon* sp. had shown stronger activity against *C. albicans* than its lichen host *U. pectinata*. Similarly, *U. bismolliuscula* effectively inhibited *S. aureus*, while its associated ELF *Xylaria* sp. showed relatively weak antibacterial activity.

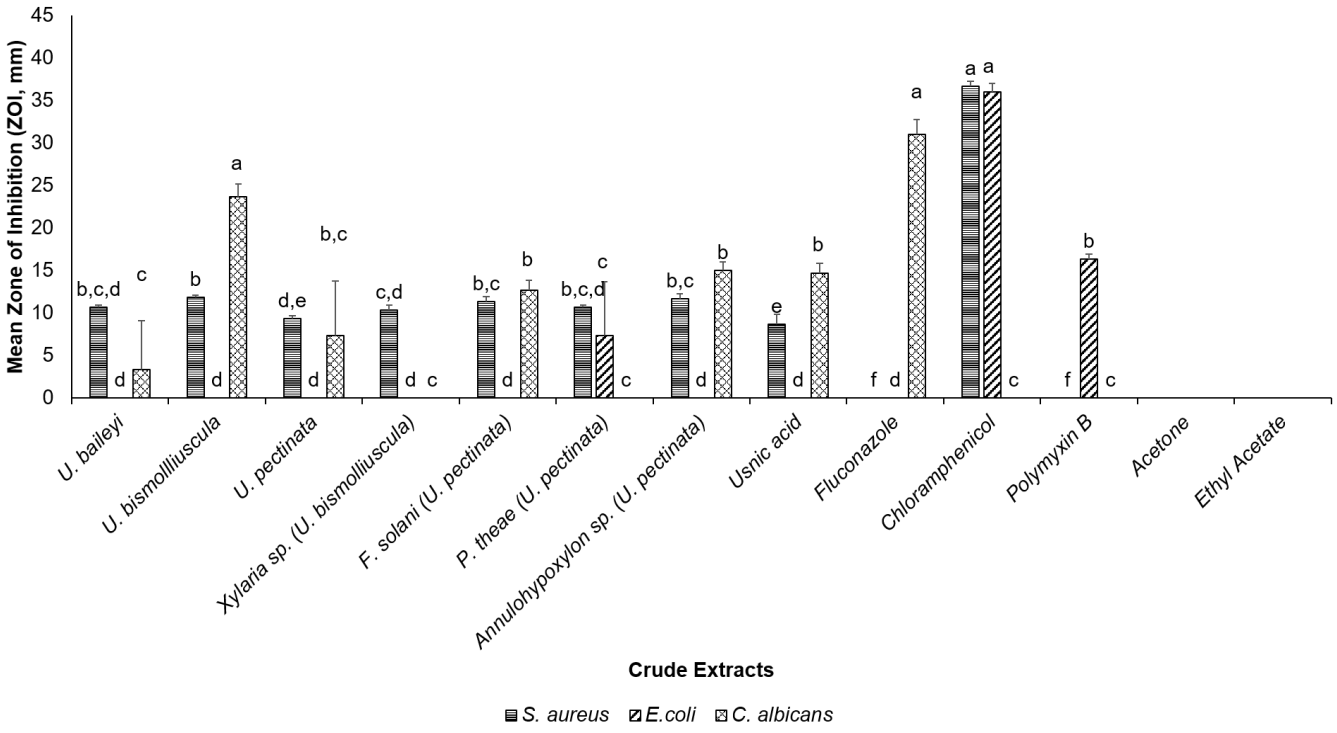

Fig 8 – Antimicrobial activities of the lichen and ELF crude extracts against *S. aureus* ATCC 25923, *E. coli* ATCC25922 and *C. albicans* ATCC 10231. The lichen host where the ELF was isolated is indicated in the parenthesis (). Standard deviation values are indicated by the error bars ($P < 0.05$) Letters above error bars indicated the statistical significance between respective lichen or ELF crude extracts using one-way ANOVA and Tukey HSD.

The lichen and ELF crude extracts also exhibited antioxidant activities. In this study, the amount of phenolic compounds present in the lichen crude extracts ranged from 11.09 to 33.01 mg GAE/g of extract, while ELF extracts had 5.52 to 22.4 mg GAE/g of extract (Fig. 9). *U. baileyi* (33.01 mg GAE/g of extract) and *P. theae* (22.4 mg GAE/g of extract) exhibited the highest TPC among the lichen and ELF crude extracts, respectively (Fig. 9). The amount of flavonoids in the lichen extracts ranged from 3.49 to 7.37 mg QE/g of extract, while ELF extracts had 5.53 to 19.9 mg QE/g of extract. *U. pectinata* (7.37 mg QE/g of extract) and *Xylaria* sp. (19.9 mg QE/g of extract) exhibited the highest TFC among the lichen and ELF crude extracts, respectively (Fig. 9). Furthermore, the lichen *U. pectinata* demonstrated an IC_{50} value of 11.9 mg/ml (FRS: 176.0 mg AA/mg of extract), while the ELF *F. solani* exhibited an IC_{50} value of 5.72 mg/ml (FRS: 367.0 mg AA/mg of extract) (Table 3). These findings suggest that both lichen and ELF crude extracts have high amounts of phenolics and flavonoids, however, ELF have shown better antioxidant properties than their lichen hosts. Additionally, these data did not reveal a correlation between TPC and antioxidant activities.

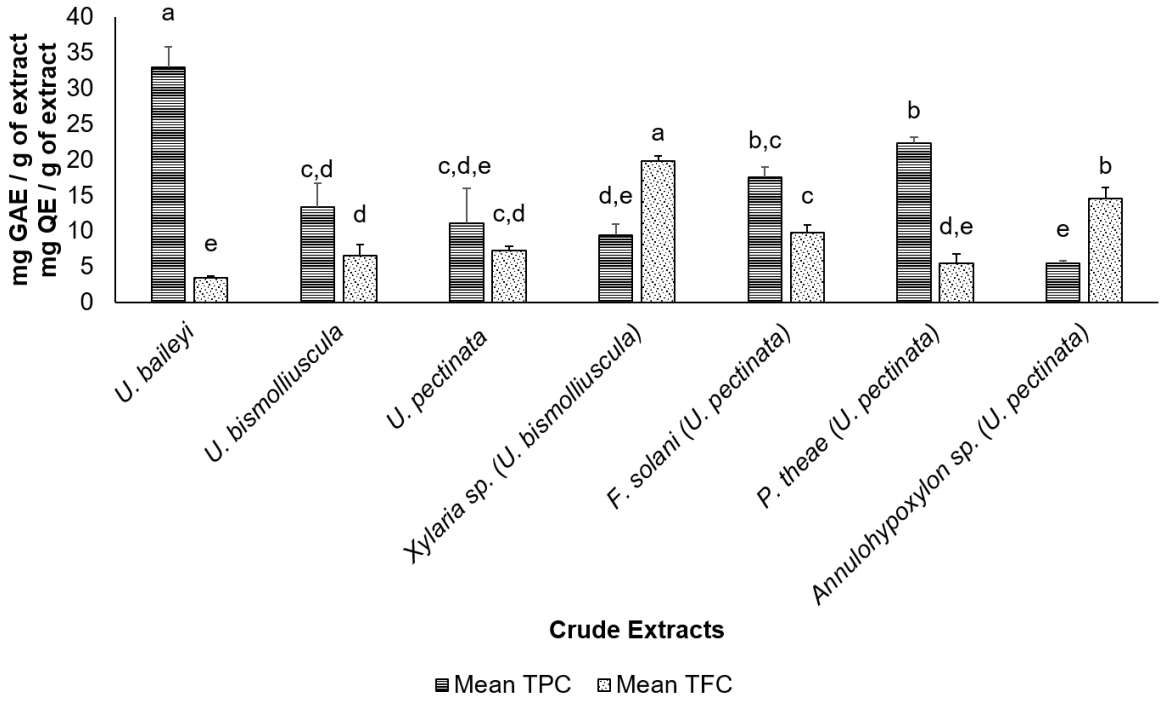

Fig 9 – Total phenol content (TPC) and total flavonoid content (TFC) of the lichen and ELF crude extracts expressed as mg GAE/g of extract and mg QE/g of extract, respectively. TPC was calculated using the formula y = 0.991x + 0.0526 (R2 = 0.9969). TFC was calculated using the formula y = 0.1304x + 0.0442 (R2 = 0.9963). The lichen host where the ELF was isolated is indicated in the parenthesis (). Standard deviation values are indicated by the error bars ($P < 0.05$) Letters above error bars indicated the statistical significance between respective lichen or ELF crude extracts using one-way ANOVA and Tukey HSD.

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

	Crude Extract	**IC_{50} (mg/ml)**	**AAEAC[b] (mg AA/g of extract)**
Lichen Host	*U. baileyi*	22.7	92.6
	U. bismolliuscula	43.2	48.7
	U. pectinata	11.9	176.0
ELF	*Xylaria* sp. (Ubs)[a]	14.3	147.0
	F. solani (Up)	5.72	367.0
	P. theae (Up)	9.09	231.0
	Annulohypoxylon sp. (Up)	17.8	118.0

[a]The lichen hosts where the ELF species was isolated are indicated as Uby (*U. baileyi*), Ubs (*U. bismolliuscula*) and Up (*U. pectinata*)
[b]AAEAC: ascorbic acid equivalent antioxidant capacity; IC_{50AA}: 0.021 mg/ml

Discussion

Among the three *Usnea* spp. investigated in this study, only *U. baileyi* was previously reported in Malaysian forests. For example, *U. baileyi* was found in Bukit Larut and was profiled for its bioactive compounds (Din et al. 2010). It was also found in Cameron Highlands and was reported to exhibit antibacterial and antioxidant activities (Saidi et al. 2018). Studies on the other two *Usnea* spp. (*U. bismolliuscula* and *U. pectinata*), however, remain scarce in the country. Similarly, *U. baileyi* is also among the common lichens found in the Philippine forests (Santiago et al. 2010, Galinato et al. 2017, Timbreza et al. 2017), albeit few studies on *U. bismolliuscula* and *U.*

pectinata were previously reported (Timbreza et al. 2017). The presence of the three *Usnea* lichens in these tropical forests suggests a resemblance in their environmental conditions, which led us to further explore if their ELF assemblages are also similar.

This study revealed that the three *Usnea* spp. (*U. baileyi*, *U. bismolliuscula* and *U. pectinata*) harbour diverse ELF assemblages. All the ELF species isolated belong to the class Sordariomycetes, most of these collections belonging to the family Xylariaceae. Our work agrees with the findings of several studies reporting Sordariomycete-dominant ELF assemblages in various lichens, particularly those belonging to Xylariaceae (U'Ren et al. 2016, Rajulu et al. 2020, Yang et al. 2021). In our previous work where we evaluated the three *Usnea* spp. from the Philippines (Santiago et al. 2021a), the classes Sordariomycetes and Eurotiomycetes were reported, with the former dominating the entire ELF assemblage. The difference in the class level of ELF assemblage composition of this study with that of our previous work could have been affected by altitude, where a higher number and more diverse ELF isolates were found in lichens inhabiting high elevations. Wang et al. (2016) emphasized the significant influence of altitude on the lichen-associated fungal composition within the lichen *Hypogymnia hypotrypa*, particularly at the operational taxonomic unit (OTU), class and phylum levels. Furthermore, other studies that evaluated *Usnea* spp. as the lichen host observed ELF species belonging to classes other than Sordariomycetes, such as Dothideomycetes, Eurotiomycetes and Leotiomycetes (U'Ren et al. 2010, U'Ren et al. 2012). Such diverse ELF assemblages were influenced by several factors including altitude, climate and biogeographic location.

While ecological factors such as altitude may have played an important role in the number and diversity of ELF, the isolation method can also influence the number and diversity of ELF species detected in a host lichen. Our study used a culture-dependent technique to uncover ELF assemblages in each lichen host, hence, underestimation of ELF species is possible. Previous studies reported the effects of culture-dependent and culture-independent techniques in estimating fungal community richness and distribution (U'Ren et al. 2014, Zhang et al. 2015, Zhang et al. 2016). Although the conventional culture-dependent technique is the most common method utilized in evaluating the diversity of lichen-inhabiting fungi, certain fungal species may be excluded such as those with obligate host associations, specialized growth requirements, and very slow growth rates or low competitive ability (U'Ren et al. 2014). Similarly, using a culture-independent technique (e.g., direct environmental sequencing) can also fail to recover species detected in a culture-dependent method (Arnold & Lutzoni 2007, U'Ren et al. 2014, Pecundo et al. 2021a), albeit will detect diverse species (Pecundo et al. 2021b). Hence, additional work utilizing both techniques is necessary to provide a more in-depth and comprehensive view of ELF diversity in a lichen specimen.

In this study, *U. bismolliuscula* revealed the most diverse ELF assemblage, which was also the lichen host that harboured the most diverse ELF assemblage in our previous work in the Philippines (Santiago et al. 2021a). Altitude appeared to have played a significant role that could have influenced the diversity of ELF in both the Philippine and Malaysian *Usnea* lichens as noted earlier. Both lichen hosts and their associated ELF benefited from the high altitude as more water and nutrients are made available. Studies on *U. bismolliuscula* as a host for ELF are, however, limited. Furthermore, the lichen *U. baileyi* had the least diverse ELF assemblage in this study, whereas *U. pectinata* revealed the least diverse ELF in the Philippine lichens (Santiago et al. 2021a). Such differences were due to the unequal number of specimens collected for each *Usnea* species, resulting in an incomparable number of isolated ELF.

The ELF species profiled in this study show that the two most dominant genera are *Xylaria* and *Nemania*. The dominance of these xylariaceous fungi is well known as these are often reported from lichens as ELF in other studies (U'Ren et al. 2016, Suryanarayanan et al. 2017, Masumoto & Degawa 2019). In our recent work with ELF from similar *Usnea* species in the Philippines, these two genera were also abundantly detected inhabiting *U. baileyi*, *U. bismolliuscula* and *U. pectinata* (Santiago et al. 2021a), albeit a few unique ELF was found. In addition, these genera are among the most common endophytic fungi found both in lichens and higher plants (Tang et al. 2009, U'Ren et

al. 2016, Suryanarayanan et al. 2017). As such, these ELF are described as hosts generalists as also observed among *Xylaria* and *Nemania* inhabiting Philippine *Usnea* specimens (Santiago et al. 2021a).

The other least common genera reported in this study, such as *Annulohypoxylon, Daldinia, Fusarium, Hypoxylon, Nodulisporium* and *Pseudopestalotiopsis*, were also previously reported as plant endophytes (Tang et al. 2009, Maharachchikumbura et al. 2016, Al-Fadhal et al. 2019) and hence, may also be described as host generalists. These ELF genera, excluding *Pseudopestalotiopsis*, were also found in the Philippine *Usnea* specimens suggesting their host-generalist behaviour. These observations also support the hypothesis that endolichenism may have served as an evolutionary source for fungal transitions (Arnold et al. 2009). We, however, do not consider these fungi as saprobes or as transient fungi growing on the lichen surface due to the stringent surface sterilization treatment performed in this study. Furthermore, only the ELF *Kretzschmaria pavimentosa* was consistently found in all collected specimens of *U. bismolliuscula* in Malaysia and was initially described as host-specific. However, when compared with the Philippine samples, *K. pavimentosa* was found in all three *Usnea* spp., which suggests a host-generalist behaviour. Such comparison suggested that *Usnea* in Malaysia and the Philippines may differ in their ELF assemblages, depending on the nutrients available for these ELF.

While the lichen hosts in this study harboured diverse ELF assemblages, we did not see any clear specific pattern in relation to the genetic diversity or phylogeny of the ELF. However, we showed from our similarity, diversity, and phylogenetic analyses that ELF species may have host preference, with some species described as host generalists and some as host-specific, such as those ELF only found in one *Usnea* species. When comparing the current results of this study with our previous work (Santiago et al. 2021a), the ELF assemblages were not identical. This suggests that ELF species may vary, despite investigating similar lichen hosts, between habitats in the tropical region. We, therefore, hypothesized that ELF species do not have a specific rule for their occurrence. Their nature, in relation to their host lichen, will depend on the availability of growth factors they require to survive. As such, our results agree with U'Ren et al. (2010) that the lichen hosts play an important role in building their ELF assemblages. Additionally, we observed that the rare ELF species identified in this study were previously reported as plant endophytes.

The extracts from lichen and ELF both showed antimicrobial activities that were effective against the pathogens tested. The antimicrobial nature of ELF has been reported previously. For example, the ELF *Ulocladium* sp. inhabiting the lichen *Everniastrum* sp. exhibited strong antimicrobial activities against *Bacillus subtilis,* methicillin-resistant *Staphylococcus aureus, Candida albicans* and *Aspergillus fumigatus* (Wang et al. 2012). Specifically, the metabolites 7-hydroxy-3, 5-dimethyl-isochromen-1-one and griseoxanthone C were identified as bioactive. Similarly, the ELF *Aspergillus quandricinctus* isolated from the lichen *Usnea longissima* inhibited the biofilm formation of *Pseudomonas aeruginosa*, albeit the specific compound responsible for the antibacterial activity was not specified (Prateeksha et al. 2020). *Usnea* lichens, particularly the species that we used, are known sources of bioactive compounds. For example, the metabolites eumitrin F and G isolated from *U. baileyi* revealed moderate antibacterial activity against *Escherichia coli* and *B. subtilis* (Nguyen et al. 2022). In their earlier work, the compound eumitrin D from *U. baileyi* exhibited cytotoxic activity against representative cell lines (Nguyen et al. 2020).

In this study, *U. bismolliuscula* showed the strongest bioactivities, followed by *U. pectinata* and *U. baileyi*. Their bioactivities could probably be due to the presence of usnic, salazinic and norstictic acids (Santiago et al. 2013, Sepahvand et al. 2021). For ELF, we found *Fusarium solani* and *Pseudopestalotiopsis theae* isolated from *U. pectinata* as the most active. Although no specific bioactive compounds were reported from these ELF, the presence of polyketides, terpenoids, steroids, alkaloids or cyclic peptides may be responsible for their bioactivities (Sepahvand et al. 2021). When compared with the Philippine lichen samples, *U. bismolliuscula* exhibited the strongest antimicrobial activities, followed by *U. baileyi* and *U. pectinata* (Santiago et al. 2021a). These findings suggest that the bioactivities of similar *Usnea* species may vary depending on their habitat as quantitative differences in the production of secondary metabolites are commonly

observed in lichen populations (Santiago et al. 2021b). For ELF, *Annulohypoxylon albidiscum* and *Xylaria venustula*, isolated from Philippine *U. pectinata* and *U. baileyi*, respectively, showed the strongest antimicrobial activities. Since different ELF species were evaluated in both studies, no comparison between bioactivities can be made. However, it can be observed that the bioactivities of ELF do not reflect the bioactivities of their respective lichen hosts. Additionally, ELF species appear to inhibit a wider group of microorganisms when compared with their respective lichen hosts.

While plants, including their fruits and fungal endophytes, are reported for antioxidant properties (Akinsanya et al. 2015, Eskandarighadikolaii et al. 2015, Aril-dela Cruz et al. 2018), our study also discovered the potential of ELF species as antioxidant producers. The level of antioxidant activity exhibited by ELF has surpassed the activity of lichens. A similar observation was noted in our previous work involving Philippine *Usnea* (Santiago et al. 2021a). Although limited studies have been done on the antioxidant mechanism of ELF, the host lichen likely protected the ELF against external stressors as earlier reported by Galinato et al. (2021) and Santiago et al. (2021a), thereby increasing the antioxidant potential of these fungi. While organisms exposed to various stressors tend to have increased antioxidants (Lai & Lim 2011, Santiago et al. 2021a), prolonged exposures may lead to detrimental effects on the organism. In our study, ELF had stronger antioxidant activities than lichens. Since these ELF were kept hidden (as they inhabit the interiors of the lichen thalli), they were protected by the lichen host against various environmental stressors. Similar observations were noted in our recent works (Galinato et al. 2021, Santiago et al. 2021a). However, additional analyses are required to validate these hypotheses. In general, ELF may be an effective source of antioxidants, thus reducing the need to collect (or over-collect) the slow-growing lichens.

Conclusion

This study revealed that *Usnea* lichens in Malaysia harbour ELF with a diverse profile that resembles, though not identical, the *Usnea* lichens in the Philippines. The main ELF species were *Xylaria* and *Nemania,* which are postulated to be host generalists. Similarly, rare ELF such as *Annulohypoxylon*, *Daldinia*, *Fusarium*, *Hypoxylon*, *Nodulisporium* and *Pseudopestalotiopsis* were isolated from a single *Usnea* species but were also reported in hosts other than *Usnea* lichens, thus described as host generalists. Both *Usnea* lichens and their associated ELF produce compounds that are antimicrobial and antioxidant, with the lichen *U. bismolliuscula* and the ELF *F. solani* and *P. theae* having good antimicrobial and antioxidant activities. The bioactivities of ELF and lichens are unique to themselves as geographical distribution does not influence their bioactivities. This study also shows that the ELF from a humble lichen in a tropical montane forest can be cultured and could be a prospect of upscaling for medical use.

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Accessibility of data

All data generated and analysed during the study are included in the manuscript.

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