



NRCP
RESEARCH JOURNAL

Full Paper

Effectiveness of Different Surface Sterilization Protocols for the Isolation of Endolichenic Fungi from *Ramalina*

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Endolichenic fungi (ELF) thrives within the thallus of lichens. Its isolation remains challenging given the indeterminate number of microorganisms cohabiting within the lichen thallus. This study determines the most effective surface sterilization method from the different protocols and their modifications to isolate ELF from the fruticose lichen *Ramalina*. Our findings showed that the protocol as described by Maduranga et al. (2018) with the treatment of the lichen thalli with 70% ethanol for 10 secs followed by 0.5% commercial bleach solution for 3 mins, and 1 min washing of sterile distilled water for 3 consecutive times remained the most ideal for isolation. All lichen thallus explants treated with this surface-sterilization method yielded 100% isolation rate while the culture plate for the tissue prints exhibited no fungal growth (100% effectivity rate). Modifications of this method resulted in 95–100% isolation rates, but lower effectivity rates of 80–86%. In comparison with the other tested surface-sterilization protocols and their modifications, their effectivity rates vary from 57-95%, albeit with isolation rates between 95-100%. All nine surface-sterilization protocols tested in this study resulted in the isolation of 156 ELF from a single lichen host. Our study highlighted Philippine lichens as ideal hosts for a diverse assemblage of endolichenic fungi.

Keywords; filamentous fungi, fruticose lichens, isolation protocol, laboratory method



Article history

Received : September 18, 2024

Revised : October 10, 2024

Accepted: December 18, 2024

Introduction

Lichens are described for centuries as a composite organism formed by two symbionts – a photobiont, typically a green alga, a cyanobacterium, or both, and a mycobiont, a filamentous fungus mainly from Ascomycota but also occasionally from Basidiomycota (Lutzoni & Miadlikowska, 2009). Lately, this symbiotic notion of two organisms in lichens has been challenged by the discovery of basidiomycetous yeasts living within the lichen thalli (Spribille et al., 2016). In their study, the basidiomycetous yeasts are embedded in the cortex of the lichen thallus and with varying abundance. The abundance appears to be correlated with the phenotypic variation observed between the two lichen hosts, although Mark et al. (2020) stated that the Cyphobasidiales yeast might not be as intimately associated with the symbiosis as the photobiont. With this new information, Hawksworth and Grube (2020) recently re-defined lichen as “a self-sustaining ecosystem formed by the interaction of an exhabitant fungus and an extracellular arrangement of one or more photosynthetic partners and an indeterminate number of other microscopic organisms”.

Interestingly, deep inside lichen thalli lies another fungal component different from the mycobiont, and these are known as ELF. They are said to be like fungal endophytes in vascular plants as ELF do not cause any harm to their lichen hosts (Arnold et al., 2009). ELF also produce different secondary metabolites such as alkaloids (Li et al., 2015), quinones (Xie et al., 2016), furanones (Zhang et al., 2014), pyrones (Kim et al., 2018), xanthonones (Wang et al., 2010), terpenes (Yuan et al., 2017), and peptides (Wu et al., 2011), making them ideal microorganisms for bioprospecting. The secondary metabolites produced by these ELF also had antibacterial (Santiago et al., 2021a; 2021b; 2022; Tan et al., 2020; 2024), antioxidant (Galinato et al., 2021), cytotoxic (Santhirasegaram et al., 2020), and antiviral (He et al., 2012) properties, and had anti-cancer activities, including a potential application against Alzheimer’s disease (Kellogg & Raja, 2017). ELF may help their lichen host from the stress of biotic and abiotic factors as well with the detoxification of substances that may cause harm inside lichen thalli, maintaining the balance

and stability needed by their lichen host to survive (Grube et al., 2014; Galinato et al., 2021).

In the Philippines, recent pioneering efforts have shown the potential of ELF for natural product research and drug discovery. In the study of Santiago et al. (2021a), three *Usnea* species collected in Sagada, Mountain Province became hosts to 101 ELF, which were identified as belonging to 12 genera with *Nemania* and *Xylaria* as the most abundant taxa, representing 73% of the isolated fungi. The ELF crude extracts which were tested showed broad-spectrum effectiveness as these target Gram-positive and Gram-negative bacteria and yeasts. In addition, the antioxidant activity and flavonoid content of these ELF crude culture extracts were higher than their lichen hosts. Interestingly, similar findings were observed with the same lichen hosts collected from Malaysia and their associated ELF (Santiago et al., 2022). Of the 62 ELF isolated from that study, the genera *Nemania* and *Xylaria* remained the most abundant taxa, also representing 73% of all isolated fungi. The ELF isolates could be host-generalist or host-specific to their lichen hosts and showed relatively strong antimicrobial and antioxidant activities. Interestingly, the *Usnea*-associated ELF from the Philippines had better antioxidant activities than the *Usnea*-associated ELF isolated from Malaysia, albeit their inhibitory activities were comparable. The promising nature of ELF as new sources of bioactive secondary metabolites was also highlighted by dela Cruz and Santiago (2021). They pointed out the steady increase in the number of publications related to ELF and the number of new metabolites from ELF as reported in published literature since 2007, yet the number of countries where these studies were reported remained dismal. Nevertheless, the discovery of novel metabolites from ELF remained a promising strategy (Santiago et al., 2021b). They used metabolomics-based approach to identify metabolites in both the lichen hosts and the culture extracts of their associated ELF. ELF isolates clearly produced distinct compounds than that of their lichen hosts.

Among the commonly reported fruticose lichens in the Philippines are species belonging to the genus *Ramalina*. This fruticose lichen is diverse and widespread with over 200 species reported worldwide (Oh et al., 2014), of which 11 species were reported in the Philippines (Paguirigan et al., 2020). *Ramalina* can also be seen from coastal areas (Gazo et al., 2019) to mountain and alpine habitats (Bannister et al., 2004; Oh et al., 2014). They are described as erect, sub-pendent to pendent

fruticose thallus that are usually attached by a basal holdfast to its substrate of barks, woods or rocks (Nash et al., 2004). Its thallus has also a strap-like appearance earning its nickname “strap lichen”. In the country, the first ever recorded *Ramalina* species dates back in 1909 when Finnish lichenologist Edvard August Vainio published a list of Philippine lichens and these included *R. pollinaria* (Westr.) Ach., *R. gracilentia* Ach., *R. linearis* (Sw.) Mull Arg., *R. subfraxinea* Nyl., and *R. vittata* Nyl. (Vainio, 1909). Of these, all except *R. linearis* are still considered valid names (Paguirigan et al., 2020). They are typically seen at lower elevations and capable of living in a wider temperature gradient as compared to another fruticose lichen, like *Usnea* species, which are typically seen at higher elevations (Galinato et al., 2018). The lichen *Ramalina* also contains secondary metabolites with antibacterial and herbicidal properties (Gazo et al., 2019), but due to general slow growth of fruticose lichens of about 2 cm per year, extraction of bioactive secondary metabolites from lichen thalli for biopharmaceutical use is not economical nor practical. Thus, metabolites from ELF associated with lichens are seen as preferable candidates for bioprospecting due to its faster growth and metabolite production *in vitro*.

While a growing number of research has shown the potential of ELF and the lichen host *Ramalina* collected in the Philippines (Gazo et al., 2019, Santiago et al., 2021a; Galinato et al., 2021), much are still needed to understand these symbiotic organisms. Research on ELF begins with its isolation from freshly collected lichen thalli. As such,

it is important to determine the best method for the surface-sterilization of lichen thalli to isolate ELF and to exclude other coinhabiting microorganisms such as epiphytic and transient bacteria and fungi. Much of the published research on the isolation of ELF follows the protocols of Li et al. (2007), Maduranga et al. (2018), and Yang et al. (2021). These protocols have been used in all major types of lichens – crustose, foliose and fruticose, and in different lichen genera. But which of these surface-sterilization methods is the most efficient in isolating ELF from the lichen thalli of *Ramalina* collected from the Philippines? Thus, this study investigated the different surface sterilization protocols and their modification on the isolation of ELF from a fruticose lichen *Ramalina* cf. *subfarinacea* collected in Cavite Province, Philippines.

Methods

Collection of the Lichen *Ramalina*

One specimen of the fruticose lichen *Ramalina* cf. *subfarinacea* was carefully collected from trunks of a pine tree within Tagaytay Picnic Grove (14°07'23.1"N, 120°59'56.7"E) located in Tagaytay City, Cavite Province, Philippines (Figure 1). In this study, identification of the collected lichen specimen was done following comparison of its morphometric features (i.e., thallus gross morphologies, presence of reproductive structures) with published literature, for example Oh et al. (2014). The collected lichen thalli were placed inside brown paper bags and immediately brought to the laboratory for surface sterilization and isolation of ELF.

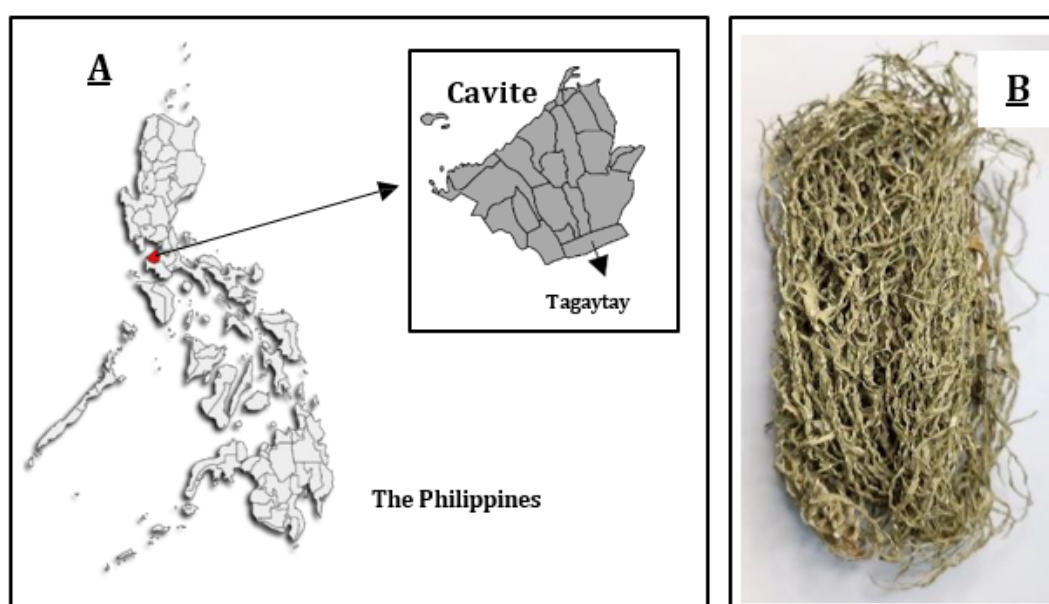


Figure 1.
The sampling locality (A) and thallus (B) of the fruticose lichen *Ramalina* cf. *subfarinacea*.

Evaluation of Surface Sterilization Protocols

Three surface sterilization protocols based on the studies of Li et al. (2007), Maduranga et al. (2018), and Yang et al. (2021) were used in this study. For the surface sterilization protocol developed by Li et al. (2007), the healthy lichen thalli were successively immersed in 75% ethanol for 1 minute followed by 2% sodium hypochlorite for 30 seconds, and another 75% ethanol for 30 seconds. This protocol was earlier used for three major types of lichens – crustose, foliose, and fruticose lichens. The protocol of Maduranga et al. (2018) used successive treatment of 70% ethanol for 10 seconds, followed by 0.5% sodium hypochlorite for 3 minutes, and then washed with sterile distilled water three times. This protocol was also used for

crustose, foliose, and fruticose lichens. Lastly, the surface sterilization protocol of Yang et al. (2021) was done with a foliose lichen which submerged the thalli into 70% ethanol for 90 seconds, followed by 0.4% sodium hypochlorite for 90 seconds, and then was rinsed with sterile distilled water for 120 seconds. This protocol was used only for the foliose lichen *Parmotrema tinctorum* (Despr. Ex Nyl.) Hale. Two modifications were made for each of the three surface-sterilization protocols, either with varying immersion time or varying bleach concentrations (Table 1). Furthermore, rinsing with sterile distilled water for 1 minute was done in between chemical treatments.

Table 1.

Surface sterilization protocols used in this study

Surface sterilization treatment no.	Main protocol and its modification ^a	Treatment # 1	Treatment # 2	Treatment # 3	Final rinsing with sterile distilled water
		Ethanol	Commercial Bleach	Ethanol	
1	Li et al. (2007)	75%, 1 min	2%, 3 min	75%, 30 sec	-
2	Modified Li #1	75%, 1 min	2%, 1 min	75%, 30 sec	-
3	Modified Li #2	75%, 1 min	2%, 5 min	75%, 30 sec	-
4	Maduranga et al. (2018)	70%, 10 sec	0.5%, 3 min	-	1 min, 3x
5	Modified Maduranga #1	70%, 10 sec	0.5%, 1 min	-	1 min, 3x
6	Modified Maduranga #2	70%, 10 sec	0.5%, 5 min	-	1 min, 3x
7	Yang et al. (2021)	70%, 90 sec	0.4%, 90 sec	-	2 min, 1x
8	Modified Yang #1	70%, 90 sec	1%, 90 sec	-	2 min, 1x
9	Modified Yang #2	70%, 90 sec	2%, 90 sec	-	2 min, 1x

^aRinsing with sterile distilled water for 1 minute was also done in between chemical treatments.

Isolation of Endolichenic Fungi

To isolate ELF, the collected healthy-looking lichen thalli (i.e., free of any visible signs of thallus damage) of *Ramalina* cf. *subfarinacea* were cut into 5-mm explant fragments, which were initially washed with tap water for 1 minute prior to surface sterilization. The washed lichen fragments were then subjected to the different surface-sterilization treatments described above and then plated on potato dextrose agar (PDA, TM Media, India) – five lichen fragments were placed equidistant on a 90-mm petri plate filled with the culture medium to a total of 20 explants plated per treatment. Five surface-sterilized thallus fragments were tissue-

printed on one PDA plate to test the effectivity of the surface sterilization protocols. This was done by placing thallus fragments on PDA plates for 1 minute, and then were removed afterwards. All culture plates, including the tissue print plates, were sealed and incubated at room temperature for up to 14 days. The growth of ELF on the lichen explants was observed and counted. Any fungi that have similar colonial growth to those present in the tissue print plates were designated as non-ELF and thus were not included in the fungal count. The data gathered was used to compute the isolation rate per treatment as follows:

$$\text{Isolation Rate (\%)}: \frac{\text{No. of explants with fungi}}{\text{No. of total explants}} \times 100$$

The effectivity rate per treatment was expressed with the formula:

$$\text{Effectivity rate (\%)}: \frac{\text{No. of ELF}}{\text{No. of isolates}} \times 100$$

Results

We collected a specimen of the fruticose lichen *Ramalina* growing on a pine tree. This lichen has shrub-like, greyish-green thallus, about 5 – 6 inches in length with numerous flattened branches arising from a single base which is attached to the substrate. Apothecia and soredia were also observed. The lichen host was then identified as *Ramalina* cf. *subfarinacea*. We plated a total of 180 thallus fragments from one specimen of this lichen host. From these surface-sterilized fragments, we were able to record 189 fungal isolates, 33 of which had colonial morphologies like those fungal isolates obtained from the tissue prints and thus were not

included (Table 2). Hence, in this study, from a single specimen of *R. cf. subfarinacea*, we isolated 156 ELF. As the study focused on the effectiveness of surface-sterilization protocols, we did not identify the isolated ELF, albeit the recorded counts were based on the differences in colonial morphologies (= morphospecies).

Among the three surface-sterilization protocols, the methods of Li et al. (2007) and Maduranga et al. (2018) yielded almost the same number of ELF, i.e., 55 – 56 ELF isolates. In contrast, the method of Yang et al. (2021) yielded only 45 ELF isolates. Comparing the surface-sterilization modifications with the original protocols, the number of ELF remained higher for two of the original protocols, except for the protocol of Yang et al. (2021). However, the study showed higher isolation rates, i.e., 95 % to 100% for all tested surface-sterilization treatments. But when the effectivity rate is considered, the different surface-sterilization protocols vary, with the described protocol of Maduranga et al. (2018) as the most effective due to the absence of any fungi from its tissue prints. Figure 2 shows representative ELF that were isolated from the fruticose lichen *Ramalina* cf. *subfarinacea* using the protocol of Maduranga et al. (2018).

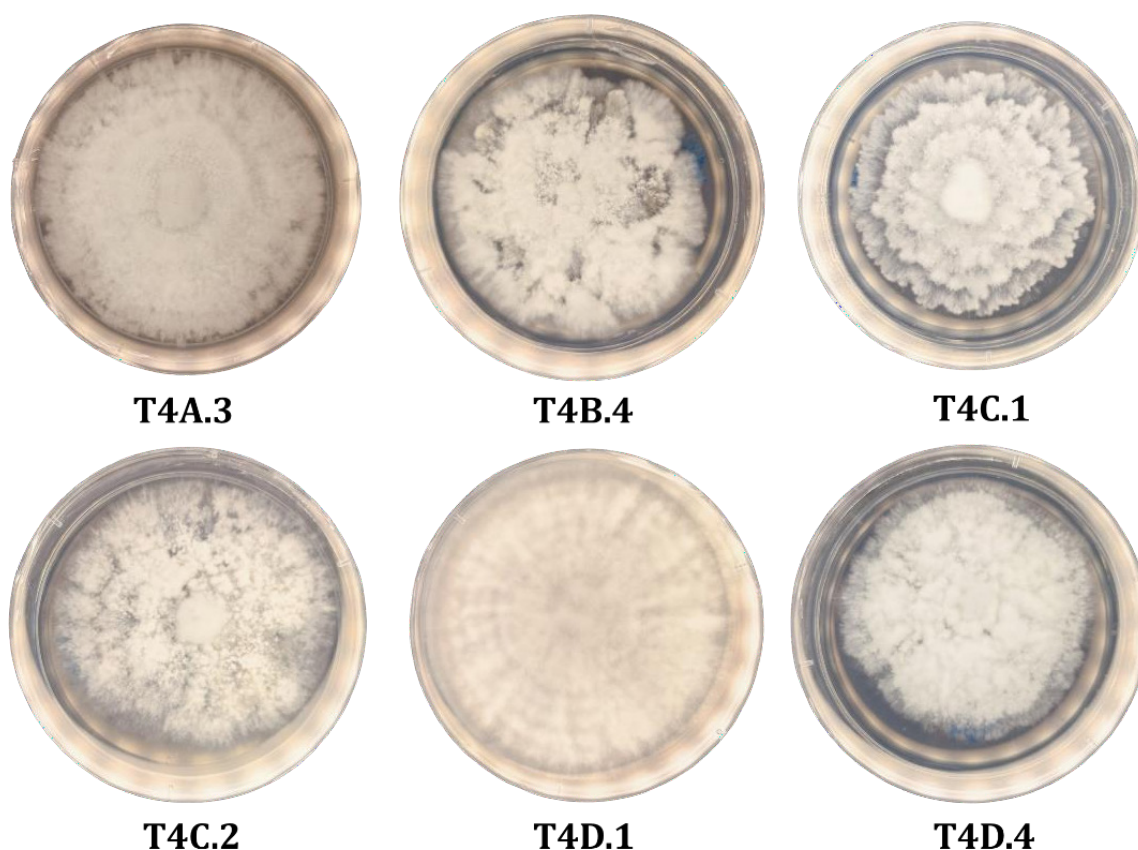


Figure 2.

Representative ELF isolated from *Ramalina* cf. *subfarinacea*. T4 denotes the lichen host while A-D denotes ELF isolate number

Table 2.

Number of fungal isolates and ELF following different surface sterilization protocols.

Treatment	Surface Sterilization Protocols	No. of explants w/ fungi (n=20)	No. of fungal isolates	No. of fungal isolates (as TP) ^a	No. of fungal isolates with similar morphology to TP cultures	No. of ELF	Isolation Rate (%)	Effectivity Rate (%)
1	Li et al. (2007)	19	24	2	4	20	95%	83%
2	Modified Li #1	20	21	4	3	18	100%	86%
3	Modified Li #2	19	19	3	1	18	95%	95%
Total		58	64	9	8	56		
4	Maduranga et al. (2018)	20	20	0	0	20	100%	100%
5	Modified Maduranga #1	20	20	3	4	16	100%	80%
6	Modified Maduranga #2	19	22	5	3	19	95%	86%
Total		59	62	8	7	55		
7	Yang et al. (2021)	20	21	5	8	13	100%	62%
8	Modified Yang #1	20	21	3	9	12	100%	57%
9	Modified Yang #2	20	21	4	1	20	100%	95%
Total		60	63	12	18	45		

^aTP = fungi isolated from tissue print culture plates

Discussion

The Philippines as one of the megadiverse countries is home to a diverse assemblage of lichens. In a recent bibliographic survey conducted by Paguirigan et al. (2020) on Philippine lichens, the country had 1,262 published taxa with 1,234 validated species names. About 25% of these lichen taxa, equivalent to 307 species, were described as new to science from specimens that were collected in the country. Among the most reported lichen species in the Philippines are the crustose lichen *Graphis* and the fruticose lichen *Usnea* (Paguirigan et al., 2020). There are also 11 reported species of *Ramalina* for the country. More recent studies also added new records and even new species to Philippine lichens (Gerlach et al., 2023; Taer et al., 2023; 2024). In addition to taxonomical and biodiversity studies, Philippine lichens were also explored for various biopharmaceutical applications, e.g., as antibacterial that targets antibiotic-sensitive and multi-drug-resistant Gram- positive bacteria (dela Cruz et al., 2023), as herbicidal that reduce root and shoot lengths and leaf chlorophyll content of *Fimbristylis miliacea* (L.) Vahl, *Leptochloa chinensis* (L.) Nees and the weedy rice, *Oryza* sp. (Gazo et al., 2019), and as cytotoxic to the human gastric adenocarcinoma and the human lung carcinoma (De Jesus et al., 2016). With the promising biological

activities exhibited by the lichen crude extracts but with a greater concern about its conservation given the slow growth of lichens (Sancho et al., 2007), it is not surprising that most recent studies have shifted its attention to the fungal components of lichens (Kumari et al., 2023; Rosabal & Pino-Bodas, 2024) and other thallus- associated microorganisms such as ELF (Kellog & Raja, 2017; Santiago & Ting, 2019; Wethalawe et al., 2021 Pawar et al., 2024). In the Philippines, the studies of Tan et al. (2020, 2024), Santiago et al. (2021a, 2021b) and Galinato et al. (2021) exemplified pioneering efforts to explore ELF associated with Philippine lichens.

The diversity of ELF has been studied through culture- dependent (Petrini et al. 1990., Muggia et al. 2017., Oh et al. 2020., Si et al. 2023) and culture- independent methods (Muggia and Grube, 2018, Park et al. 2014, Mendoza et al. 2017), which both affirms the enormous diversity of this fungal group within the lichen thalli. For the culture- dependent approach, ELF diversity varies and is dependent on different isolation conditions including the type of culture media used to grow the fungi. In the study of Muggia et al. (2017), the presence of different nutrients and minerals in the culture media improved the isolation of ELF which was affirmed by the study of Yang et al. (2021), wherein ELF from the foliose lichen *Parmotrema tinctorum* that were

cultured in potato dextrose agar (PDA) and malt and yeast extract agar (MY) had higher isolate density than Lysogeny broth (LB) and Bold's basal medium (BBM). The lichen age and the different parts of the lichen thallus also had different ELF isolates (Yang et al. 2021). More importantly, the severity of surface sterilization is one of the main factors that influence the number of ELF that can be isolated from a lichen thallus. It was observed that mild surface sterilization does not effectively remove microbial contaminants in the lichen surface while expectedly, potent surface sterilization procedures can kill culturable ELF (Yang et al. 2021). Thus, it is important to determine the best surface sterilization protocols for any given lichen host.

In this study, the three published protocols of Li et al. (2007), Maduranga et al. (2018) and Yang et al. (2021) were used and modified and then compared for the isolation of ELF from the fruticose lichen *Ramalina cf. subfaranicea*. These protocols have been used to isolate ELF from all lichen types – crustose, foliose and fruticose, and from various lichen species, e.g., *Cladonia coniocraea* (Flörke) Spreng, *Pyxine cocoes* (Sw.) Nyl., *Opegrapha medusulina* Nyl., *Xanthoria mandschurica* (Zahlbr.) Asahina, *Parmotrema tinctorum*, and including *Ramalina sinensis* Jatta. These lichen species also came from different habitats – as terricolous, saxicolous and corticolous (Li et al. 2007) and from different plant hosts, e.g., mangroves and mangrove-associated plants (Maduranga et al. 2018). Our modifications included varying immersion time (1 – 5 minutes for the commercial bleach treatment) or varying commercial bleach concentrations (0.4 – 2%) (Table 2). We isolated a total of 189 fungal specimens, although 33 fungal isolates came from the tissue prints. When the isolated fungi exhibiting similar colonial morphologies with those recorded from the tissue prints were excluded in the count, we recorded a total of 156 ELF. The presence of this enormous number of fungal isolates from a single specimen of lichen certainly supports the idea that lichens host a very diverse assemblage of associated ELF. Based on our results, we also observed a high isolation rate with all our tested surface sterilization protocols, i.e., 95 – 100%. However, if we consider the absence of any fungal growth on the tissue print cultures as an indication of the effectivity of the surface sterilization, only the original protocol of Maduranga et al. (2018) yielded 100% effectivity rate. Perhaps the effective concentration of 0.5% NaOCl solution and 70% ethanol combined with the effective exposure time of 3 minutes and 10 seconds for the chemical treatments, respectively, facilitate better removal of spores or hyphae of epiphytic

or transient fungi on the lichen thallus surface. Interestingly, the modifications in the protocols of Li et al. (2007) and Yang et al. (2021) improved the effectivity rate up to 95%, which indicates that these modifications better isolated ELF from our lichen host. This could be attributed to a longer exposure time, i.e., 5 minutes, to 2% NaOCl as opposed to the 3-minute exposure time to the same chemical sterilant in the original protocol of Li et al. (2007) and to a higher concentration of bleach treatment (2%) as opposed to 0.4% bleach solution in the original protocol of Yang et al. (2021). In conclusion, we proposed the use of the original protocol of Maduranga et al. (2018) – successive immersion of lichen thallus fragments in 70% ethanol for 10 seconds, followed by 0.5% commercial bleach for 3 minutes, and with rinsing of sterile distilled water in between chemical treatments and three times after the final chemical treatment for 1 minute, as the best surface-sterilization protocol to isolate ELF from the fruticose lichen *Ramalina cf. subfaranicea*. Our study further confirms that Philippine lichens are ideal hosts for diverse assemblages of ELF and offers opportunities for bioprospecting of fungal natural products.

Ethics Statement

Ethics approval is not required in this study.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Ghimel P. Espinosa: conceptualization, methodology, investigation, data curation, formal analysis, writing- original draft, writing- reviewing and editing. John Joshua T. Bellen: methodology, investigation, writing- reviewing and editing. Sittie Aisha B. Macabago: validation, supervision, writing- reviewing and editing. Melfei E. Bungihan: validation, supervision, writing- reviewing and editing. Thomas Edison E. dela Cruz: conceptualization, validation, formal analysis, supervision, funding acquisition, writing- reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Funding

This research was made possible through a research grant provided by the Department of Science and Technology (DOST) – National Research Council of

the Philippines (NRCP) – Project No. E-261 Project ELFHA: Biodiscovery of ELF to Mitigate Antimicrobial Resistance in Health and Agriculture.

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