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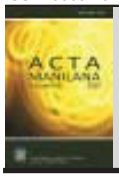

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In pursuit of promising microbes for drug discovery: tapping endolichenic fungi (ELF) from lichens

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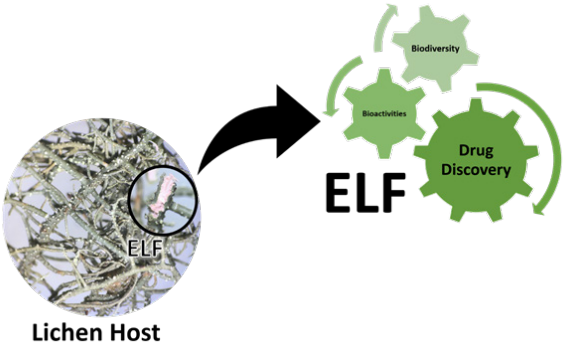
Graphical Abstract



Abstract

The emergence of novel and re-emergence of latent infectious diseases coupled with rising cases of antimicrobial resistance necessitates the continuous search for new antibiotics. The Kingdom Fungi has been at the centerpiece of any drug discovery program ever since the beginning of the antibiotic era. The key strategy is to find novel taxa and/or ecologically defined fungal groups for the screening of bioactive secondary metabolites. In this paper, we presented the endolichenic fungi as promising fungi for bioprospecting. We searched for published papers on these fungi and presented reports of their biological activities. Through this paper, we hope to increase interest on these microorganisms among natural product researchers.

Keywords: bioactivities; bioprospecting; endolichenic fungi; fungal natural products; lichen

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Introduction

The current COVID-19 pandemic has made it known the enormous danger of communicable diseases brought about by infectious entities. According to the World Health Organization (WHO), of the ten leading causes of death in 2019, three are communicable diseases [1]. This number increased to four among the lower-middle income countries and to six among low-income countries. Among these communicable diseases, bacterial infection such as tuberculosis tops the list. This is further aggravated by the rise of antimicrobial resistance (AMR). In 2019, an estimated 10 M were infected with TB worldwide, with more than 200,000 cases reported with multi-drug resistant TB [2]. The Philippines is among the eight countries with 30 high TB burden, which accounted for 87% of new cases. WHO has thus identified AMR as among the top 10 global public health threats. This coupled with the declining effectivity of anti-infective drugs available in the market necessitates the continuous exploration of bioactive metabolites for drug discovery. In this mini-review paper, we described lichen-associated microorganisms as promising sources of potentially novel and bioactive metabolites for drug discovery development.

Lichens: a partnership of more than two organisms. Lichens are mutualistic association between a filamentous fungus, also referred to as the mycobiont, and at least one photosynthetic partner, known as the photobiont, which could either be a green alga, a cyanobacterium or both [3]. Lichenization is described as a mechanism of acquiring fixed carbon by fungi from a population of minute, living algal and/or cyanobacterial cells [4]. In this lichenization process, the mycobiont, or the lichen-forming fungus which often belongs to the Phylum Ascomycota, overgrow the photobiont on or within the substratum to form the lichen thallus [5], a unique vegetative structure. While the photobiont provides nutritional benefits (e.g., sugars) to the association, the mycobiont provides a "shelter" and facilitates absorption of water from the environment. As a result of this symbiotic relationship, both the mycobionts and the photobionts can exist in various habitats, where separately they would be rare or non-existent [6]. In this context, lichens are regarded as the most successful symbiosis in the natural world. They comprise more than 20% of the global fungal biodiversity [7], and exist in almost all terrestrial habitats, from the tropics to polar regions including extreme environments such as extremely dry deserts [6]. In the Philippines, a total of 1,234 lichen species has so far been recorded, of which 307 species or equivalent to 24% of the recorded taxa were first described from specimens collected in the country [8]. This represents an enormous diversity from which bioactive metabolites for drug discovery can be explored. Our studies on Philippine lichens at the UST Research Center for the Natural and Applied Sciences have shown their antimicrobial, cytotoxic, and herbicidal potentials [9-12]. However, despite their promising bioactivities, lichens grow very slowly which raises concerns of over-collection, leading to species population decline and possible species extinction. The *in vitro* culture of its mycobionts may not always be a good option as the lichen-forming fungi may not necessarily produce the bioactive metabolites extracted from the lichen thalli. Interestingly, the lichen-forming fungi are not the only organisms within the lichen association that can be explored for natural products.

Lichens are described for its intricate architecture, the lichen thalli, which involves the fungal component sheltering the photosynthetic partners [13]. Although the dual nature of lichens is widely recognized for centuries, the unexpected discovery of a basidiomycetous yeast, identified as *Cyphobasidium* sp., from the cortex of the lichen *Bryoria* [14] led to the recognition of the "third" symbiont. The yeast cells are embedded in the cortex of the lichen thallus and may contribute significantly to its morphology. The yeasts are also ubiquitous, having been reported over large geographical distances and across the six continents, and therefore, are believed to be essential partners for most lichens and are not casual colonizers or parasites. Interestingly, recent studies also showed the presence of bacteria which provides vitamins and cofactors in the lichen thalli [15-17]. These led Hawksworth & Grube [18] to re-define the lichen symbiosis 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." These also showed the enormous diversity of microorganisms from different kingdoms and domains of life entering the lichen symbiosis.

In the study of Arnold et al. [19], they earlier concluded that lichens could be the 'cradle' of fungal diversification. True enough, the discovery of Endolichenic Fungi (ELF) as another microbiota living inside healthy lichen thalli without causing any infections to the host is recently reported. Described as similar in functions or roles to plant endophytes but represented a lineage of Ascomycota distinct from the lichen mycobiont, lichenicolous fungi, and other incidental fungi found on the surface of the thalli, endolichenic fungi represent another ecologically defined group of microorganisms within lichens [19-21]. ELF have been reported to occur in taxonomically diverse lichens encompassing various lichen growth forms (e.g., crustose, foliose, fruticose), substrates (e.g., epiphytic, saxicolous, terricolous), and ecosystems (e.g., boreal, temperate, tropical, and Antarctic) [22-24]. In the next section, we describe the possible role/s of endolichenic fungi in the lichen association.

Endolichenic fungi (ELF) and their role in the lichen symbiosis. Investigations on the nature of endolichenic fungi have progressed in the recent years – from learning the similarities of these microorganisms with plant endophytic fungi to the production of unique and bioactive secondary metabolites. There are, however, other biological aspects of these group of asymptomatic fungi that require further studies. Among these is the identification of their role/s in the lichen symbiosis to gain significant insights for the better understanding of their diversity and occurrence as well as their physiological, functional, and ecological nature. Previous studies have attempted to identify these roles of ELF, albeit most were only based on comparison with the lifestyle of plant endophytic fungi, also known as fungal endophytes. Fungal endophytes reside within tissues of plants and have been shown to exhibit antimicrobial and cytotoxic activities. For example, we have reported bioactivities of fungal endophytes associated with terrestrial plants [25], mangroves [26-28], and even macroalgae [29-31]. Suryanarayanan and Thirunavukkarasu [32] further hypothesized that ELF, like plant endophytes, aid in the abiotic and biotic stress tolerance of their lichen hosts, influence the gene regulation and alteration of the fitness of the host lichen, and perhaps be involved in the degradation of the dead parts of the lichen thalli.

Similarly, Zhang and colleagues [24] reported the possible functional role of ELF in litter degradation, since their isolated ELF species were previously reported as plant endophytes that exhibited such role in leaf decay. Now, just like fungal endophytes from plants, the bioactive secondary metabolites produced by ELF were also explored. Kellogg and Raja [33] reported that ELF species produce a suite of biologically active and functional secondary metabolites, in exchange for the shelter provided by the host lichen. These metabolites were hypothesized to provide chemical protection for the host [34]. Galinato et al. [35] recently reported that ELF could be leading producers of antioxidants within the lichen thalli, since ELF crude culture extracts exhibited stronger antioxidant activities than the crude lichen extracts. Similar results were reported by Santiago et al. [36]. It is, thus, possible that ELF protects the host against harmful conditions through the production of reactive oxygen species (ROS). Furthermore, Santiago and colleagues [37] recently reported that ELF metabolites may have protected the lichen from other possible harmful or invading microorganisms. Such assumption resulted from the metabolomics analyses of lichen hosts and their associated ELF, where a distinction between lichen and ELF metabolic profiles was observed.

While these studies may have provided ample information on the role of ELF in the lichen symbiosis, additional analyses involving various lichens and ELF are still required to understand the complexity of endolichenism. Furthermore, there are other significant observations in these studies that also require attention. For example, ELF is considered analogous to plant endophytic fungi. While this observation was proven accurate by several studies [19, 38-41], the nature of the secondary metabolites of ELF could have been different from those produced by plant endophytes. It is worth noting the ability of fungal endophytes to produce metabolites similar to their plant hosts [42]. However, in a recent metabolomics analyses conducted [37], different set of metabolites was observed between ELF and their lichen hosts, thereby indicating possibly a different role for ELF, and a role perhaps distinct from that of the fungal endophytes. Proving this observation by conducting more extensive analyses will aid in identifying the role of ELF in the lichen symbiosis, particularly from the perspective of ELF.

ELF and drug discovery. Since the first isolation of bioactive secondary metabolites produced by ELF in 2007 [43], a continuous natural product exploration on the potential of ELF in drug discovery followed. In a relatively short period of time, there has been a plethora of research where ELF metabolites and their biological activities are reported. To show this growing trend, we conducted a simple journal search using Google Scholar on June 23, 2021, using only one keyword, endolichenic fungi. We read through more than 800 hits and selected only journal articles that reported endolichenic fungi and their bioactivities, and that were published in reputable journals. Our simple search strategy is illustrated in Figure 1. Our study resulted in the identification of varied bioactivities of ELF, and included but not limited to antibacterial [37, 44-46], antifungal [47-50], antioxidant [35, 51-54], cytotoxic [43, 55-59], anti-inflammatory [60-62], anti-Alzheimer's disease [63, 64], antiviral [65], and anti-biofilm [66-68] (Figure 2, Table 1). From these studies, a total of 592 compounds was reported, with half of these metabolites identified as novel (Figure 3). This observation alone is sufficient to prioritize endolichenic fungi for chemical diversity studies.

Interestingly, in addition to the biopharmaceutical applications mentioned above, some of the ELF metabolites were also tested for their agricultural potential such as nematicidal [69], biocontrol [70], and phytotoxic activities [71, 72]. In the Philippines, the first reports of ELF were that of Galinato et al. [35], Santiago et al. [36], and Tan et al. [44]. Galinato and colleagues isolated 11 morphologically distinct ELF from the fruticose lichen, *Ramalina peruviana*, collected in Tagaytay City, Cavite Province. Their ELF showed antioxidant activities, with three species reporting higher % RSA than the lichen host and the positive control, ascorbic acid [35]. On the other hand, Santiago et al. [36] isolated ELF from another fruticose lichen, *Usnea*, specifically, from *U. baileyi*, *U. bismolliuscula*, and *U. pectinata*, all collected from Sagada, Mountain Province. Their studies also reported the antimicrobial and antioxidant activities of ELF, with many isolates exhibiting better activities than the lichen hosts. Tan et al. reported the biodiscovery of antibacterial constituents from three endolichenic fungi isolated from the foliose lichen *Parmotrema rampoddense*, with the isolation of three compounds: bis(2-ethylhexyl)terephthalate, acetyl tributyl citrate, and fusarubin [44]. Acetyl tributyl citrate also exhibited moderate antibacterial activity against *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* With the recent listing of more than 1,200 Philippine lichens and with 307 species described as novel from specimens collected in the country [8], it is now easy to imagine the high diversity of our endolichenic fungi and their enormous potential for bioprospecting.

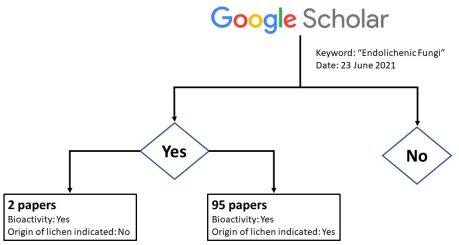

Figure 1. A simple search strategy for the screening of journal articles on endolichenic fungi.

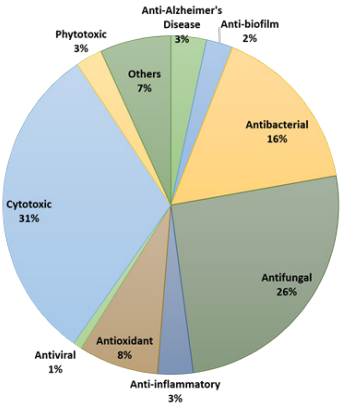

Figure 2. The reported biological activities exhibited by the metabolites produced by ELF. Data are based from 97 publications searched through Google Scholar on June 23, 2021, with the keyword "endolichenic fungi". Papers were published from 2007 until May 2021. The category "others" refers to uncommon biological activities such as heat shock activation, allelopathic, anti-algal, biocontrol, anti-proliferative, α-amylase inhibition, anti-quorum sensing, anti-migratory (wound healing), and glucose-uptake.

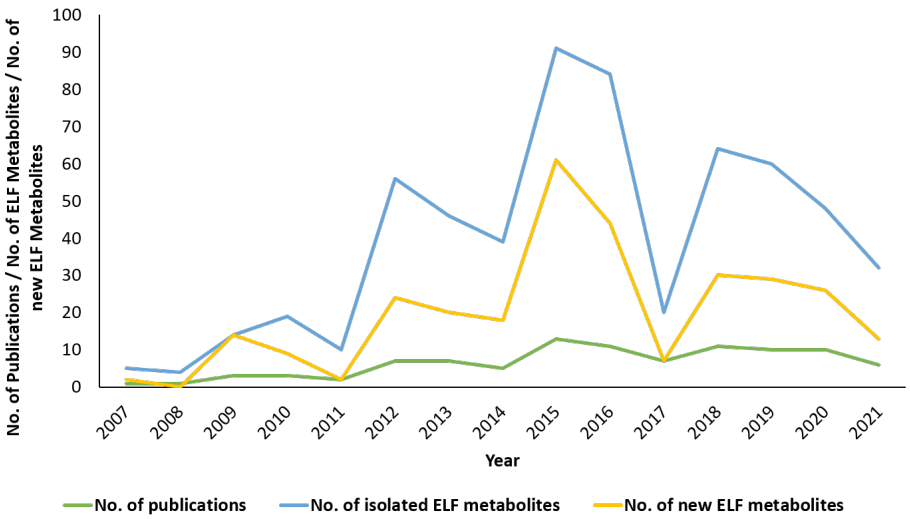

Figure 3. Number of published scientific articles on the bioactivities of endolichenic fungi. Included here are the number of isolated ELF metabolites and those identified as novel compounds from 2007 until May 2021 based on 97 publications obtained from our journal search through Google Scholar on June 23, 2021, with the keyword "endolichenic fungi".

Strategies for the biodiscovery of novel bioactive metabolites from ELF. While several bioactive and novel secondary metabolites were discovered from different ELF species, none have so far reached the next stage in the drug discovery pipeline. Numerous *in vitro* investigations have been conducted confirming the efficacy of these secondary metabolites, but very few studies have continued to the development of these bioactive compounds. For example, Lagarde et al. [73] reported the strong cytotoxic activities of ELF crude extracts against human cancer cell lines. Such results are promising, but the isolation and characterization of these bioactive compounds are yet to be determined. Though, there are several reports on the isolation, characterization, and structure elucidation of ELF metabolites [60, 74-76], most of which also demonstrated interesting bioactivities. As such, it is strongly encouraged to continuously explore these microorganisms, gathering more information beyond the first few steps of laboratory routines (i.e., preliminary screening) and continue throughout the drug discovery process.

One of the main concerns in the drug discovery research is the re-isolation of known compounds. While a higher chance of getting novel metabolites is expected from novel species, endolichenic fungi, many of which are taxonomically similar with fungal endophytes, may have produced less chemically diverse compounds. Therefore, strategies to maximize the chemical diversity and biological activities of ELF are needed, which in turn are also very useful in the whole drug discovery pipeline. Among these strategies is the OSMAC or "one strain, many compounds" approach. It is a simple yet effective strategy that aims to increase the number of secondary metabolites produced by a target microorganism by altering the fermentation conditions, such as media composition, temperature, pH, aeration, and addition of enzymes [77].

Wang and colleagues [78] applied such strategy to the ELF *Ulocladium* sp. inhabiting *Everniastrum* lichen. This then led to the isolation and characterization of more ELF metabolites exhibiting strong cytotoxic activities. Likewise, Padhi and Tayung [79] observed more ELF metabolites with varying bioactivities by altering the culture media and incubation parameters.

Another strategy to further maximize the chemical and biological diversity of ELF metabolites is the exploration of structure-activity relationship, albeit still uncommon in the field of endolichenism. Wijeratne and colleagues [80] applied such strategy to two strains of the ELF *Geopyxis* sp. that yielded analogues of the novel metabolites geopyxins. Preliminary structure-activity relationship screening of these analogues, however, showed low cytotoxic and heat-shock-inducing activities, suggesting that these substances may be weak anticancer drugs.

Table 1. Listing of bioactive secondary metabolites produced by endolichenic fungi. The host lichens and the types of bioactivities are also indicated with the reference journals.

Lichen Host	Endolichenic Fungi	Bioactive Secondary Metabolites	Bioactivities	References
Amandinea medusulina	*Xylaria psidii*	(*Z*)-3-{(3-acetyl-2-hydroxyphenyl) diazenyl} -2,4-dihydroxybenzaldehyde	Cytotoxic	Santhirasegaram et al. [73]
Cetrelia sp.	*Aspergillus* sp.	Isocoumarindole A	Antifungal, Cytotoxic	Chen et al. [74]
Everniastrum nepalense	*Chaetomium globosum*	Chaetoglobosin Y, E, G, B, C, Isochaetoglobosin D	Cytotoxic	Zheng et al. [59]
Everniastrum sp.	*Ulocladium* sp.	Tricycloalternarenes 9b, 6-O-methylnorlichexanthone, norlichexanthone, griseoxanthone, alterlactone, altenusin	Antibacterial, Antioxidant, Cytotoxic	Wang et al. [75], Wang et al. [76]
Lepraria incana	*Aspergillus chevalieri*	Asperglaucin A, Asperglaucin B	Antibacterial	Lin et al. [77]
Lethariella zahlbruckneri	*Tolypocladium cylindrosporum*	Pyridoxatin	Cytotoxic	Li et al. [78]
Lobaria retigera	*Aspergillus versicolor*	8-*O*-methylversicolorin A, 8-*O*-methylversicolorin B, 8-*O*-methylaverythin, 1'-*O*-ethyl-6,8-di-*O*-methylaverantin	Cytotoxic	Dou et al. [79]
Parmelia sp.	*Eupenicillium javanicum*	Javanicol E, (+)-terrein	Anti-inflammatory	Xu et al. [61]
Parmotrema rampoddense	*Fusarium proliferatum*	Acetyl tributyl citrate	Antibacterial	Tan et al. [44]
Parmotrema sp.	*Daldinia eschscholtzii*	8-methoxynaphthalen-1-ol	Radical scavenging activity	Manthrirathna et al. [54]
Parmotrema sp.	*Penicillium citrinum*	10-ethylidene-2,4,9-trimethoxy-10,10a-dihydro-7,11-dioxa-benzo[b]heptalene-6,12-dione	Antioxidant	Wickramarachchi et al. [53]
Parmotrema tinctorum	*Lecythophora* sp.	Oxaspirol B	Cytotoxic	Wijeratne et al. [80]
Physciaceae physcia	*Ophiosphaerella korrae*	Ophiosphaerellin C	Anti-Alzheimer's Disease	Li et al. [81]
Ramalina sp.	*Myrothecium inundatum*	Myrotheols A and B, Myrothesides C and D, Sphaeropsidin A, Hymatoxin L	Cytotoxic	Basnet et al. [82]
Stereocaulon tomentosum	*Dothideomycetes* sp.	Dothideopyrone F	Cytotoxic	Kim et al. [83]
Umbilicaria sp.	*Ulospora bilgramii*	Ulosporin G	Cytotoxic	Xie et al. [84]
Usnea cavernosa	*Corynespora* sp.	Corynesporol, 1-hydroxydehydroherbarin, herbarin, dehydroherbarin, naphthoquinone	Cytotoxic	Paranagama et al. [43]
Xanthoparmelia angustiphylla	*Talaromyces* sp.	Talaromycin A	Cytotoxic, Antioxidant	Yuan et al. [85]
Xanthoria sp.	*Aspergillus* sp.	Asperunguisin C	Cytotoxic	Li et al. [58]

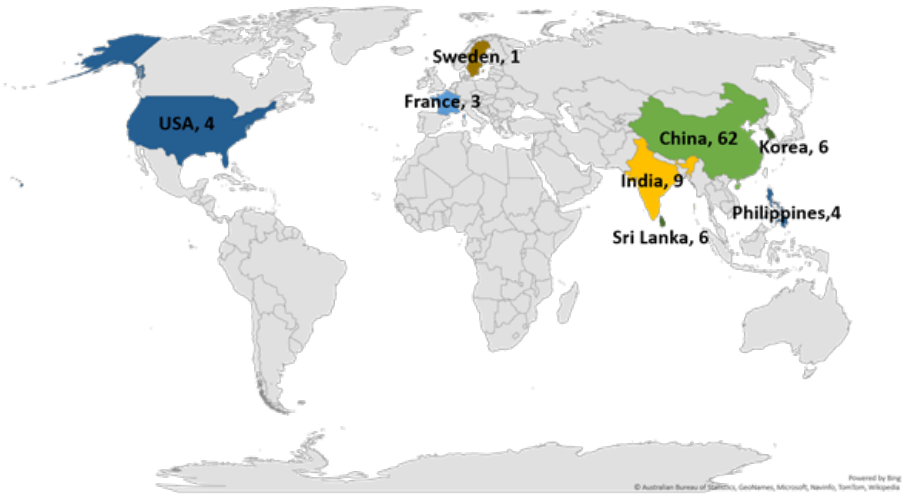

Figure 4. Geographic locations of the lichen hosts where the metabolite-producing ELF were isolated. Numbers indicate the number of published papers for each country where the host lichen was collected. Data are based on 95 publications obtained from our Google Scholar journal search on June 23, 2021, with the keyword "endolichenic fungi". Papers were published from 2007 until May 2021. Two articles did not indicate the origin of the lichen hosts, and thus, were excluded from the graph.

Despite that, such observation gave an insight regarding the target selectivity of the compounds and may instead provide broader anticancer drugs with different modes of action. It therefore serves as an opportunity to further explore the structure-activity relationship for the development of better drug leads. The success of ELF in drug discovery research lies with the high probability of discovering new compounds. This is also expected to increase, since more countries have started exploring this ecologically important group of microorganisms (Figure 4). Furthermore, the use of asymptomatic endolichenic fungi can serve as alternative target organisms, especially when conservation of the host lichens owing to their very slow growth is considered.

Conclusions

Fourteen years into the study of endolichenic fungi, only about 97 publications were reported. However, despite this meager number as compared to other fungal groups, a diverse list of bioactivities was known from these microorganisms. These have shown the enormous potential of this unique, ecological fungal group for bioprospecting. In the Philippines, only a handful of studies reported endolichenic fungi, mainly from the lichen genus *Usnea* and *Ramalina*.

With more than a thousand species of lichens reported in the country, a quarter of which are described first in the Philippines, these represented many potential hosts for endolichenic fungi, and expectedly may harbor unique, if not, novel species of fungi awaiting to be discovered and screened for fungal natural products, and thereby are very good candidates for drug development programs. We only need to explore our own backyard for these promising microbes.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, T.E.E.DC.; original draft preparation, T.E.E.DC. and K.A.A.S.; review and editing of the draft, T.E.E.DC. and K.A.A.S. All authors have read and agreed to the final version of the manuscript.

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