Asian Journal of Mycology 6(1): 51–57 (2023) ISSN 2651-1339

www.asianjournalofmycology.org **Article**

Doi 10.5943/ajom/6/1/5

Antibacterial potential of endolichenic fungi from lichen *Usnea*

Dumo ED[1], Espino AJT[1], Lina FD[1], Pua KD[1] and Paguirigan JAG[1,2*]

[1] Department of Biological Sciences, College of Science, University of Santo Tomas, Manila 1008 Philippines
[2] Korean Lichen Research Institute, Sunchon National University, 255 Jungang-ro, Suncheon-si, Jeollanam-do 57922 South Korea

Dumo ED, Espino AJT, Lina FD, Pua KD, Paguirigan JAG 2023 – Antibacterial potential of endolichenic fungi from lichen *Usnea*. Asian Journal of Mycology 6(1), 51–57, Doi 10.5943/ajom/6/1/5

Abstract

Lichens are unique organisms with medicinal benefits. However, lichens and endolichenic fungi in the Philippines are relatively less explored. In this study, the host lichen *Usnea* samples were collected from Mt. Talinis, Valencia, Negros Oriental and were surface sterilized for the isolation of endolichenic fungi. The endolichenic fungi were grown in culture, and secondary metabolites were extracted using ethyl acetate. The antibacterial activities of the crude culture extracts were analyzed against *Acinetobacter baumannii*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pantoea agglomerans*, and *Staphylococcus aureus* through disk diffusion assay. Following morphological characterization, the host lichens were identified as *Usnea baileyi, U. barbata, U. bismolliuscula, U. chaetophora, U. cornuta,* and *U. longissima*, from which six endolichenic fungi were chosen. Results of the paper disk diffusion assay showed that *Oidiodendron* sp. inhibited the growth of *Klebsiella pneumoniae* (30 mm ZOI), and *Geotrichum* sp. inhibited the growth of *Pantoea agglomerans* (16 mm ZOI). Our study confirms that endolichenic fungi isolated from the fruticose lichen *Usnea* can be a promising source of secondary metabolites with antibacterial activity.

Keywords – antimicrobial – antibacterial – bioactivity – lichen – secondary metabolites – endolichenic fungi – zone of inhibition – Usnea, crude extract

Introduction

The development and discovery of drugs have been successful in controlling many infectious diseases (Drews 2000, Muthuirulan 2018). However, the increasing emergence of multidrug-resistant bacteria poses a formidable threat to the public health and welfare of humans worldwide. Thus, the challenge lies in finding a new source of pharmacologically active molecules (Behera et al. 2005) to combat these emerging and re-emerging infectious diseases (Mercorelli et al. 2018, Zupin et al. 2022).

Alternative sources of biologically active secondary metabolites include lichens and endolichenic fungi (Agrawal et al. 2020). Endolichenic fungi are predominantly filamentous fungi that reside asymptomatically in the lichen thalli (Kellogg & Raja 2017). An estimated 13,500 filamentous fungal species are residing within the lichen thalli (Kellogg & Raja 2017, Singh et al. 2017). Most of these endolichenic fungi belong to orders *Pleosporales*, *Xylariales*, and *Hypocreales* (U'Ren 2012). Natural products from endolichenic fungi have been attracting increased attention for their ability to biosynthesize novel secondary metabolites (Agrawal et al.

Submitted 8 October 2022, Accepted 3 May 2023, Published 25 May 2023
Corresponding Author: Jaycee Augusto Paguirigan – e-mail – jgpaguirigan@ust.edu.ph

2020, Kellogg & Raja 2017). This is evident from the increase in publications devoted to endolichenic fungal metabolites over the past decade (Kellogg & Raja 2017). The bioactive metabolites produce by endolichenic fungi include alkaloids, furanones, peptides, acyclic compounds, quinones, steroids, sulfur-containing chromanone, terpenoids, and xanthone (Agrawal et al. 2020, Kellogg & Raja 2017). Analysis of metabolites extracted from endolichenic fungi showed diverse bioactivities, such as anticancer, antiviral, antibacterial, antifungal, and anti-Alzheimer's properties (Cimmino et al. 2019, Kellogg & Raja 2017, Singh et al. 2017).

Given the potential of endolichenic fungi as sources of novel compounds with pharmaceutical importance, this study aims to assess the antibacterial potential of the crude culture extracts of endolichenic fungi isolated from species of *Usnea* from the Philippines.

Materials and methods

Collection and identification of host lichens

Samples of lichen *Usnea* were collected from Mt. Talinis, Valencia, Negros Oriental. The lichen thalli were collected by detaching them from the substrates, placed inside separate paper bags, and stored in a cool, dry place. Identification of the lichen samples was accomplished through morphological characterizations, including the presence of reproductive structures (apothecia), types of branches, color, presence of soralia, and growth form. Identification through thalline spot test was also performed using potassium hydroxide (K), sodium hypochlorite (C), and combined (KC). The reagents were dropped directly on the exposed medulla, cortex, and central axis of the lichen, and an immediate color change indicates a positive result (Santiago et al. 2010).

Isolation of endolichenic fungi.

The lichen thalli were initially rinsed with distilled water to remove excess dirt. Sterile surgical scissors were used to cut the thalli into manageable portions, followed by surface sterilization by successively treating the thalli with 75% ethyl alcohol for 30 seconds, distilled water for 30 seconds, and 10% NaOCl for 30 seconds (Li et al. 2007). Following surface sterilization, the lichen explants were inoculated in malt extract agar (MEA) plates (five explants per plate, in triplicates) and incubated at room temperature for two weeks. To check for the effectiveness of the surface sterilization method, the treated thalli explants were tissue printed on MEA, where the absence of fungal growth ensures successful surface sterilization. The fungal hypha that grew from the lichen explants were transferred onto freshly prepared MEA plates for the isolation of the endolichenic fungi and incubated at room temperature (Padhi & Tayung 2015).

Mass production and extraction of secondary metabolites

The endolichenic fungal isolates were sub-cultured on MEA plates for one week. After incubation, the fungal colonies were cut with a sterile scalpel into squares (25 mm^2), and ten of these agar blocks were inoculated into 150 ml malt extract broth (MEB) in triplicates. The culture broths were then incubated at room temperature for 28 days, after which the fungal mycelia were macerated, and the culture was mixed with an equal volume of ethyl acetate at room temperature for 24 hours. The solvent layer was separated and evaporated under reduced pressure through rotary evaporation (45 ºC) at 121 rotations per minute (rpm). The crude culture extracts were air dried after transferring it into pre-weighed vials and re-dissolved with methanol to arrive at a final concentration of 30 µg/µl (Torres & Dela Cruz 2015) and 100 µg/µl.

Antibacterial assay using paper disk diffusion method

The following test bacteria were used in this study: *Acinetobacter baumannii* (TBRC 6950), *Enterococcus faecium* (TBRC 2163), *Klebsiella pneumoniae* (ATCC 13583), *Pseudomonas aeruginosa* (ATCC 27853), *Pantoea agglomerans*, and *Staphylococcus aureus* (ATCC 25923). The test bacteria were grown on Mueller Hilton Agar (MHA) at 35ºC for 24 hours. Following culture,

the test bacteria were suspended into 10 ml MHB tubes and standardized to 0.5 McFarland (De Jesus et al. 2016).

The standardized test bacteria were swabbed on MHA plates. Sterile discs were each impregnated with 10 µl of endolichenic fungal crude extracts with a concentration of 30 µg/µl and 100 µg/µl and placed on the swabbed MHA plates (Torres & Dela Cruz 2015). Methanol was used as the negative control, and tetracycline was used as the positive control. The culture plates were incubated at 35ºC for 24 hours. Following incubation, the diameter of the zone of inhibition was measured using a vernier caliper. The zone of inhibition (ZOI) was categorized as inactive (<10 mm ZOI), partially active (10-12 mm ZOI), active (13-19 mm ZOI), and very active (>19 mm ZOI) following the protocol of Quinto & Santos (2005).

Results

The lichen genus Usnea

The presence of a moist climate and minimal pollution in Mt. Talinis, Valencia, Negros Occidental allowed an extensive growth of the host lichens. Six lichen specimens collected in the area were described morphologically and identified as *Usnea baileyi*, *Usnea barbata*, *Usnea bismolliuscula*, *Usnea chaetophora*, *Usnea cornuta*, and *Usnea longissima*. (Figure 1). For the morphological characteristics of the lichens, *U. baileyi* exhibits grayish to green thalli, sub-pendulous growth form with a length of about 20.07 cm. It has fistulose branches with soralia but not apothecia. It is K+ and C+ for the thalline spot test. *Usnea barbata* has a yellowish to green thalli, pendulous growth form, and a length of about 12.95 cm. It has foveolate and ridged branches with soralia, but no apothecia observed. It is K+ and KC+ positive for the spot test. *Usnea bismolliuscula* has a grayish to green thalli, pendulous growth form, and a length of 24.38 cm. Its branches are perforated with attached soralia. It also shows K+ and KC+ spot test results. *Usnea chaetophora* has a yellowish-green thallus with a pendulous growth form and a length of 7.87 cm. Its branches are annulated with attached soralia. It exhibited C+ for the spot test. *Usnea cornuta* also has a yellowish-green thallus but has a shrubby growth form with a 19.30 cm length. The branches are shrubby and have soralia attached to them. It is also C+ and KC+ in the spot test. *Usnea longissima* has a pale green thallus, pendulous growth form, and about 26.16 cm long. Its branches are perpendicular with no soralia and apothecia. It also showed K+ and KC+ results for the spot test.

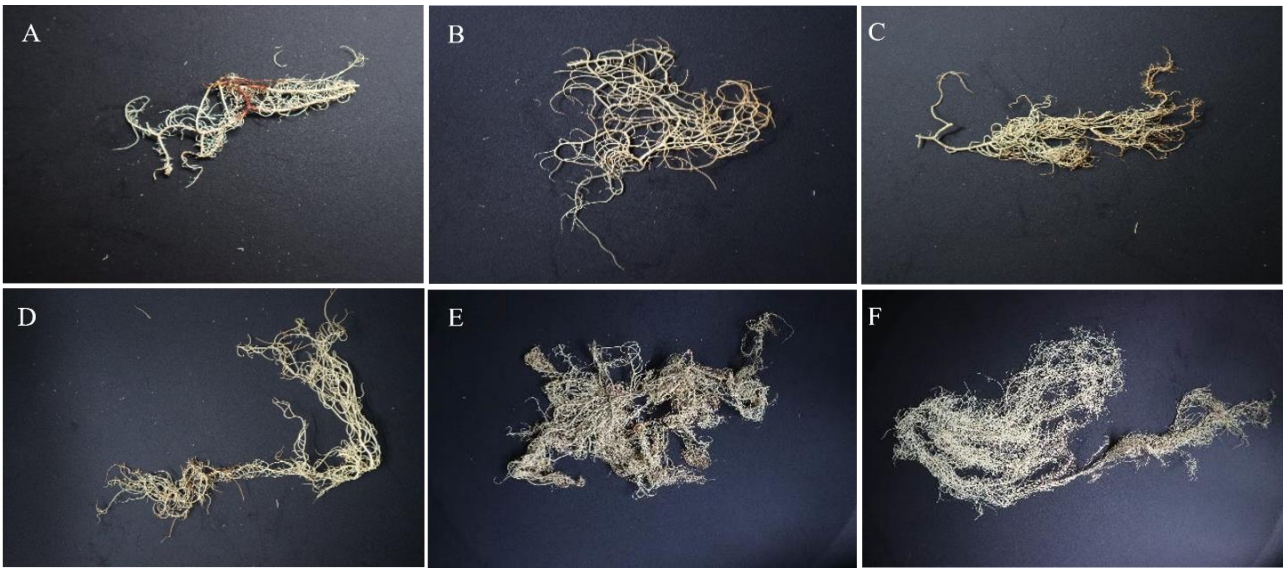

Fig. 1 – The genus *Usnea* in Mt Talinis, Valencia, Negros Oriental. A *Usnea baileyi*. B *Usnea barbata*. C *Usnea bismolliuscula*. D *Usnea chaetophora*. E *Usnea cornuta*. F *Usnea longissima*.

The isolated endolichenic fungi

A total of 26 endolichenic fungi were isolated from *Usnea* thalli. However, it is worth noting that majority of the fungal isolates were mycelia sterilia, and consequently, only ten morphologically identified endolichenic fungi were considered in the study. The ELFs included in the study were isolated from *U. barbata*, *U. cornuta*, and *U. longissima* and were identified under six genera, including *Botryotrichum*, *Coniothyrium*, *Cylindrocarpon*, *Geotrichum*, *Oidiodendron*, and *Papulospora* (Fig. 2).

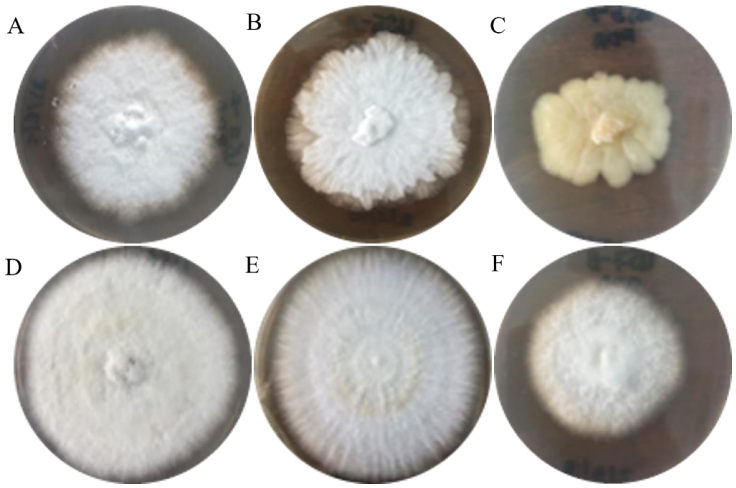

Fig. 2 – The endolichenic fungi isolated from *Usnea*. A *Botryotrichum* sp. B *Coniothyrium* sp. C *Cylindrocarpon* sp. D *Geotrichum* sp. E *Oidiodendron* sp. F *Papulospora* sp.

Botryotrichum sp. has white cottony mycelia with the reverse showing white with yellow tinges at the center. *Coniothyrium* sp. has a filamentous, white, dark brown discolored mycelium and a white reverse. *Cylindrocarpon* sp. has a filamentous, light-yellow mycelium and a yellow reverse color which fades as it reaches the edge. *Geotrichum* sp. has a white cottony mycelium with a white reverse, having tiny black spots. *Oidiodendron* sp. has white, filamentous mycelia with white reverse having tiny brown spots. *Papulospora* sp. has a white, cottony mycelium and a white reverse having yellow tinges in the center.

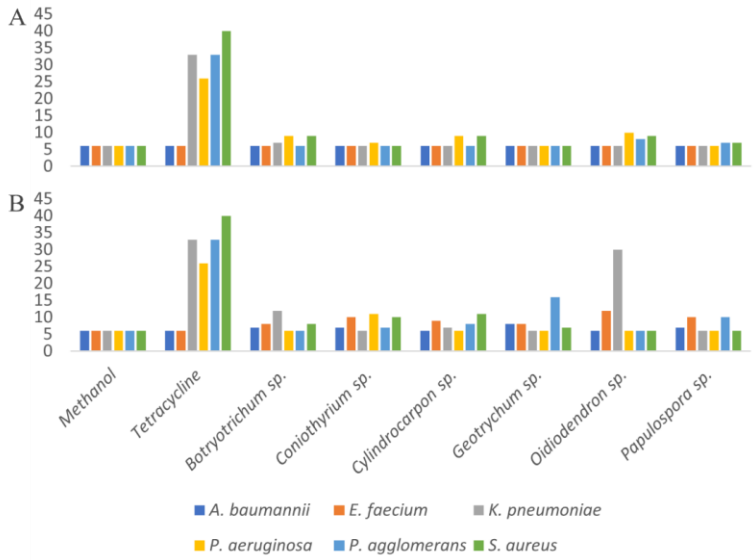

Fig. 3 – Antibacterial activity of the different endolichenic fungi against the bacterial pathogens. A 30 µg/µl. B 100 µg/µl crude extract concentration.

Antibacterial Activities of the endolichenic fungi

For the 30 µg/µl extract concentration, one extract exhibited a zone of inhibition (ZOI) against *K. pneumoniae*, four against *P. aeruginosa*, two against *P. agglomerans* and four against *S. aureus*. However, since most extracts exhibited a ZOI less than 10 mm and were considered inactive. For the 100 µg/µl extract concentrations, *Oidiodendron* sp. showed activity against *A. baumannii* (12 mm). *Botryotrichum* sp. and *Oidiodendron* sp. showed a ZOI of 12 mm and 30 mm against *K. pneumoniae*, respectively. *Coniothyrium* sp. showed a ZOI (11 mm) against *P. aeruginosa*; *Geotrichum* sp. (16 mm) against *P. agglomerans*, and *Cylindrocarpon* sp. (11 mm) against *S. aureus.* Methanol showed no ZOI to any pathogenic bacteria, while tetracycline showed a huge activity against *K. pneumoniae*, *P. aeruginosa*, *P. agglomerans*, and *S. aureus.*

To determine if there is a significant difference between the ZOI of the endolichenic fungi and the control, one-way ANOVA was performed. However, a significant difference was observed (Table 1). Given the observed absence of significant difference between the positive control and the endolichenic fungi (100 µg/µl) against *E. faecium*, this finding was ignored due to the overall inactivity exhibited by the samples (Figure 3, Table 1). Subsequently, we performed the Tukey post hoc test and discovered that there is no significant difference between the efficacy of 100 µg/µl extract of *Oidiodendron* sp. and tetracycline against *K. pneumoniae* ($p=0.285>0.05$).

Table 1. Statistical analysis of the zone of inhibition exhibited by the endolichenic fungi against the bacterial pathogens.

Bacteria	**30 µg/µl**		**100 µg/µl**	
	F value	F-crit value	F value	F-crit value
A. baumannii	-	-	296.07	2.22
E. faecium	-	-	1.10	2.22
K. pneumoniae	1050.92	2.72	780.27	2.72
P. aeruginosa	88.52	2.72	7844.05	2.72
P. agglomerans	572.64	2.72	15.76	2.72
S. aureus	545.43	2.72	21.30	2.72

Discussion

Lichens are self-sufficient organisms formed by a symbiotic relationship between a fungus and a photobiont. The photobiont, usually an alga or cyanobacteria, provides the carbohydrates needed by the fungus while the mycobiont (fungi) provides mineral nutrition and moisture to the photobiont (Komaty et al. 2016), but in addition to the fungal mycobiont, endolichenic fungi also resides within the lichen thalli (Kellogg & Raja 2017, Singh et al. 2017). Endolichenic fungi are believed to have evolved with lichen (Suryanarayanan & Thirunavukkarasu 2017) and is related to or similar to the fungal endophytes of vascular plants (Arnold et al. 2003). In this study, the *Usnea* species confirmed the presence of endolichenic fungi in the lichen's thalli which is similar to the previous report by Santiago et al. (2021). However, mycelia sterilia were not identified and used in the study. The endolichenic fungi from *U. barbata, U. cornuta*, and *Usnea longissima*, which can be identified morphologically, were the only ones chosen for the study. It has been previously mentioned that *U. baileyi*, *U. barbata*, *U. bismolliuscula*, *U. cornuta*, *U. longissima*, and *U. pectinata* harbor endolichenic fungi with antimicrobial activity (He & Zhang 2012, Rajapaksha et al. 2009, Santiago et al. 2021, U'Ren 2012). The isolated endolichenic fungi were classified under the classes Sordariomycetes and Eurotiomycetes (Santiago et al. 2021). These endolichenic fungi produce bioactive molecules, including alkaloids, quinones, furanones, as well as terpenes, steroids, and some allylic compounds (Kellogg & Raja 2017, Paranagama et al. 2007). These metabolites have many biological activities, which include antiviral, antibacterial, and antitumor (Aslan et al. 2001, Dülger et al. 1998, Huneck 1999, Kellogg & Raja 2017, Singh et al. 2017). The method of action of these metabolites on bacteria includes inhibition of biosynthesis of protein and cell wall of bacteria by blocking the action of transpeptidases (Adegboye & Babalola 2013, Padhi & Tayung 2015).

Although it is known that endolichenic fungi are good sources of bioactive metabolites, their identity and chemistry have not been studied extensively (Samanthi et al. 2015). In this study, a high concentration of endolichenic fungal extracts could inhibit the growth of some pathogenic bacteria. This result is similar to *Curvularia trifolii* isolated from *Usnea* sp., whose extracts have "bactericidal" activity against three test bacteria. Previous reports on endolichenic fungi isolated from *Usnea* also revealed that the endolichenic fungal crude extracts have bioactivity against *S. aureus* (Santiago et al. 2021). Also, this study confirms that differences in concentration of the crude culture extracts have an impact on the activity of the endolichenic fungal extract. A higher extract concentration exhibits a significantly higher activity as what is observed between the 30 µg/µl and 100 µg/µl *Oidiodendron* sp. extract against *K. pneumoniae,* although the same result was also observed with the other extracts. Finally, the bioactivity of molecules is indeed a concentration-dependent activity, where secondary metabolites are more potent and effective in high concentrations (Srivastava et al. 2013).

Conclusions

All lichens harbor endolichenic fungi that may exhibit antibacterial potential. The antibacterial activity of the crude extracts from endolichenic fungi exhibits a dose-dependent behavior, with higher concentrations leading to larger zones of inhibition against the pathogenic bacteria. Among the six endolichenic fungi, *Geotrichum* sp. and *Oidiodendron* sp. display promising bioactivity against *P. agglomerans* and *K. pneumoniae,* respectively. These findings indicate that endolichenic fungi are valuable sources of bioactive secondary metabolites.

Acknowledgments

We would like to thank the technical assistance of the members of the Fungal Biodiversity, Ergonomics, and Systematics (FBES) group at the RCNAS: Mr. Lawrence Timbreza, Mr. Paul Yap, Mr. Mark Gabriel Galinato, Mr. Arfel Tayona, and Prof. Kin Israel Notarte for their unwavering support in the fulfillment of this research endeavor. In addition, we would like to express our sincere gratitude to the technicians of the Biological Sciences Research Lab (BSRL), Mr. Ruperto Simbul and Mr. Gregorio Estioko, for their assistance.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Adegboye M, Babalola O. 2013 – Actinomycetes: a yet inexhaustive source of bioactive secondary metabolites. In: Medez-Vills A (ed.), Microbial pathogens and strategies for combating them: science, technology and education. Formatex pp. 786–795.

Agrawal S, Deshmukh SK, Reddy MS, Prasad R, Goel M. 2020 – Endolichenic fungi: a hidden source of bioactive metabolites. South African Journal of Botany 134, 163–186.

Arnold AE, Mejía LC, Kyllo D, Rojas EI et al. 2003 – Fungal endophytes limit pathogen damage in a tropical tree. In National Academy Sciences pp. 15649–15654.

Aslan A, Güllüce M, Atalan E. 2001 – A study of antimicrobial activity of some lichens. Bulletin of Pure and Applied Science 20, 23–26.

Behera BC, Verma N, Sonone A, Makhija U. 2005 – Antioxidant and antibacterial activities of lichen *Usnea ghattensis* in vitro. Biotechnology Letters 27, 991–995.

Cimmino A, Nimis PL, Masi M, De Gara L. et al. 2019 – Have lichenized fungi delivered promising anticancer small molecules? Phytochemistry Reviews 18, 1–36.

De Jesus E, Hur J-S, Notarte K, Santiago KA, Dela Cruz TE. 2016 – Antibacterial, antioxidant, and cytotoxic activities of the corticolous lichens *Canoparmelia aptata*, *Pannaria* sp., and *Parmotrema gardneri* collected from Mt. Banahaw, Quezon, Philippines. Current Research in Environmental and Applied Mycology 6, 173–183.

Drews J. 2000 – Drug discovery: a historical perspective. Science 287 (5460), 1960–1964.
Dülger B, GüciN F, Aslan A. 1998 – *Cetraria islandica* (L.) Ach. Likeninin Antimikrobiyal Aktivitesi. Journal of Biology 22, 111–118.
He Y, Zhang Z. 2012 – Diversity of organism in the *Usnea longissima* lichen. African Journal of Microbiology Research 6(22), 4797–4804.
Huneck S. 1999 – The significance of lichens and their metabolites. Naturwissenschaften 86, 559–570.
Kellogg JJ, Raja HA. 2017 – Endolichenic fungi: a new source of rich bioactive secondary metabolites on the horizon. Phytochemistry Reviews 16, 271–293.
Komaty S, Letertre M, Dang HD, Jungnickel H, et al. 2016 – Sample preparation for an optimized extraction of localized metabolites in lichens: application to *Pseudevernia furfuracea*. Talanta 150, 525–530.
Li W-C, Zhou J, Guo S-Y, Guo L-D. 2007 – Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. Fungal Diversity 25, 69–80.
Mercorelli B, Palù G, Loregian A. 2018 – Drug repurposing for viral infectious diseases: how far are we? Trends in Microbiology 26, 865–876.
Muthuirulan P. 2018 – Omics-informed drug target discovery in combating emerging infectious diseases. Drug Target Review 4, 52–54.
Padhi S, Tayung K. 2015 – *In vitro* antimicrobial potentials of endolichenic fungi isolated from thalli of *Parmelia* lichen against some human pathogens. Beni-Suef Univ. Journal of Basic and Applied Science 4, 299–306.
Paranagama PA, Wijeratne EMK, Burns AM, Marron MT, et al. 2007 – Heptaketides from *Corynespora* sp. inhabiting the cavern beard lichen, *Usnea cavernosa*: first report of metabolites of an endolichenic fungus. Journal of Natural Products 70, 1700–1705.
Quinto EA, Santos MAG. 2005 – Microbiology. In: B. Q. Guevara (ed.), A guidebook to plant screening: phytochemical and biological. UST Publishing House, España, Manila. pp 63–99.
Rajapaksha C, Paranagama PA, Kannangara S. 2009 – Bioactivities of endolichenic fungi of the lichen *Usnea barbata* at Hakgala montane forest. Proceeding of the International Forestry and Environment Symposium 2009, 59.
Samanthi U, Wickramarachchi S, Wijeratne K, Paranagama P. 2015 – Two new bioactive polyketides from *Curvularia trifolii*, an endolichenic fungus isolated from *Usnea* sp., in Sri Lanka. Journal of the National Science Foundation of Sri Lanka 43, 217–224.
Santiago KAA, Borricano JNC, Canal JN, Marcelo DMA, Perez MCP. 2010 – Antibacterial activities of fruticose lichens collected from selected sites in Luzon Island, Philippines. Philippine Science Letters, 18–29.
Santiago KAA, dela Cruz TEE, Ting ASY. 2021 – Diversity and bioactivity of endolichenic fungi in *Usnea* lichens of the Philippines. Czech Mycology 73, 1–19.
Singh BN, Upreti DK, Gupta VK, Dai X-F, Jiang Y. 2017 – Endolichenic fungi: a hidden reservoir of next generation biopharmaceuticals. Trends in Biotechnology 35, 808–813.
Srivastava P, Upreti DK, Dhole TN, Srivastava AK, Nayak MT. 2013 – Antimicrobial property of extracts of Indian lichen against human pathogenic bacteria. Interdisciplinary Perspectives in Infectious Diseases 709348.
Suryanarayanan T, Thirunavukkarasu N. 2017 – Endolichenic fungi: the lesser known fungal associates of lichens. Mycology 8, 189–196.
Torres J, dela Cruz TEE. 2015 – Antibacterial activities of fungal endophytes associated with the Philippine endemic tree, *Canarium ovatum*. Mycosphere 6, 266–273.
U'Ren JM, Lutzoni F, Miadlikowska J, Laetsch AD, Arnold AE. 2012 – Host and geographic structure of endophytic and endolichenic fungi at a continental scale. American Journal of Botany 99(5), 898–914.
Zupin L, Santos-Silva CA, Al Mughrbi ARH, Vilela LMB, et al. 2022 – Bioactive antimicrobial peptides: a new weapon to counteract zoonosis. Microorganisms 10, 1591.