International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 05-01-2022, Accepted: 20-01-2022, Published: 05-02-2022
Volume 7, Issue 2, 2022, Page No. 190-192

Secondary metabolites present in novel endolichenic fungi isolated from *Dirinaria applanata*

Jake B Canapim, Jennifer L Luyun
College of Allied Health Sciences, Cagayan State University Andrews Campus, Tuguegarao, Cagayan, Philippines

Abstract
The conduct of this study generally aimed to identify the secondary metabolites present in the endolichenic fungi isolated from *Dirinaria Applanata*. Specifically, it sought to identify the secondary metabolites present in the novel endolichenic fungi isolated from Dirinaria applanata through thin layer chromatography. The host lichen was collected from Luquilu, Cabagan, Isabela. The isolated endolichenic fungi were morphologically characterized. Mass production of endolichenic fungi was carried out to increase the amount of fungal crude extracts. Presence of secondary metabolites in the fungal crude extracts was determined through Thin-Layer Chromatography. These extracts were spotted on TLC plates through the use of DCM-methanol extracting solvent. Spots were observed under a UV light at the wavelength of 254/356 nm. The TLC plates were sprayed with several spray reagents. In reference to Dragendorff's reagent, brown orange spots indicate presence of alkaloids; triterpenes and sterols was determined through the presence of blue-violet spots after Vanillin-sulfuric acid reagent was sprayed on the TLC plate. Using ferricyanide-ferric chloride reagent, blue spots indicate presence of phenols, tannins and flavonoids. Also, brown spots produced after sprayingα-Naphthol-sulfuric shows positive results for glycosides. Lastly, presence of indoles can be detected using Van-Urk Salkowski test wherein positive result is indicated by blue-violet spots. Furthermore, the TLC plates were sprayed with several spray reagents and the visual appearance became the basis in identifying the compounds present. The isolated endolichenic fungi showed positive results to secondary metabolites including phenols, tannins, flavonoids, alkaloids, glycosides, indoles, triterpenes, and sterols.

Keywords: secondary metabolites, endolichenic fungi, host lichen, thin layer chromatography

Introduction
Lichens are appealing source of novel secondary metabolites, according to Kumar *et al*. (2014), (as cited by Suryanarayanan & Thirunavukkaru 2017) [14]. It is worthy to note that the lichen's unique symbiotic structure allows it to generate an amazing array of nutrients as well as remarkable phytochemical compounds. Lichens have long been used in traditional foods and medicines in Japan, China, Iceland, India, European countries, and other parts of the world. In Austria, Finland, Switzerland, and other countries, many dosage forms of usnic acid salt were used to treat wounds, ulcers, and burns (Huang *et al*., 2018, Kosanić *et al*., 2014) [11]. Despite the fact that the health-promoting potential of most lichens has been demonstrated, the majority of them have not been documented or reported for food consumption (Yingshu Zhao, Mingfu Wang, Baojun Xu, 2021). Emphasizing its scientific impact on health, the secondary metabolites produced by lichens include anti-inflammatory, antibiotic, antimycobacterial, antiviral, analgesic, antipyretic, antiproliferative, cytotoxic, and plant growth inhibitory compounds (Boustie & Grube, 2005 as cited by Zhao *et al*., 2021) [7]. In this sense, endolichenic fungi must be understood in order to realize and acknowledge the importance of lichens as determinants of ecological processes, which are frequently underappreciated and overlooked (Asplund and Wardle 2016). The goal of this study was to find essential metabolites biosynthesized by endolichenic fungi that have promising biologically active compounds and structural diversity.

Materials and Methods
Collection and Identification of Host Lichens
The *Dirinaria applanata* was collected at Luquilu, Cabagan, Isabela. The lichen samples collected were identified through characteristics and thalline spot tests using published identification keys and online keys and online catalogues. The lichen thalli were collected by detaching it from the substrata, placed inside a separate paper bag, and stored in a cool dry place (Stone *et al*., 2004). Identification of host lichen was accomplished through morphological characterizations including (1) presence or absence of reproductive structures (apothecia), (2) types of branch, (3) color, (4) presence of soralia, and (5) growth form. Identification through thalline spot test (K, K+C, C test) was done with the use of chemical reagents potassium hydroxide (K), sodium

hypochlorite (C), and a combination of both (KC). The reagents were dropped directly on the exposed medulla and cortex of the lichen, and an immediate color change indicates a positive result (Santiago *et al*., 2010).

Isolation of Endolichenic Fungi

The thalli from said lichens were initially rinsed with distilled water to remove excess dirt. Sterile surgical scalpel was used in order to cut the thalli into manageable portions. Surface sterilization was performed using the protocol of Li et. al (2007) with modifications to isolate the endolichenic fungi. The lichen thalli were successively treated four times with 75% ethyl alcohol for15 seconds, and distilled water for 15 seconds, and finally with 10% NaClO for 15 seconds. Following surface sterilization, the lichen explants were placed on Malt Extract Agar (MEA) plates (five explants per plate, in triplicates) and incubated at room temperature for 2 weeks. To check for the effectiveness of the surface sterilization method, the treated thalli explants were tissue printed on MEA. The absence of fungal growth on the tissue printed plates confirmed that the surface sterilization technique was efficient. The fungal hypha that grew from the lichen explants were then sub-cultured using sterile syringe needles, with the aid of a dissecting microscope to ensure that single hyphae from the fungal colony was transferred onto freshly prepared MEA plates for the isolation of the endolichenic fungi. All fungal isolates were maintained at room temperature (Padhi & Tayung, 2015) [15].

Morphological Characterization of Endolichenic Fungi

The isolated endolichenic fungi that were grown in the plates were morphologically characterized through their visible appearance under a dissecting microscope. The result of the characterization was validated by a registered microbiologist. The endolichenic fungi were characterized in terms of form, elevation, surface, margin and underside.

Mass Production and Preparation of Crude Extracts from Endolichenic Fungi

The endolichenic fungal isolates were sub-cultured on MEA plates for one week. After incubation, agar blocks were made with a cork borer into 8-mm diameter and inoculated in 300 mL Malt Extract Broth (MEB). The cultured broths were then incubated at room temperature for 1 month. Following inoculation, the fungal mycelia were filtered, and the cultured filtrates were mixed with equal volume of ethyl acetate at room temperature for 24 hours. The solvent layer was separated and evaporated under reduced pressure through rotary evaporation (45ºC) at 121 rotations per minute (rpm). The crude culture extracts were transferred into pre-weighed vials and air dried. The crude extracts were dissolved with ethyl acetate to arrive at final concentrations (Torres &dela Cruz, 2015) of 100, 200, 300 and 400 mg/ml. These extracts were used both for antibacterial assay and Thin Layer Chromatography.

Thin Layer Chromatography

Thin-layer chromatography was used to identify the secondary metabolites or compounds present within the lichen crude extracts. These extracts were spotted on TLC plates through the use of DCM-methanol extracting solvent. Spots were observed under a UV light at the wavelength of 254/356 nm (Timbreza, *et al*. 2017). The TLC plates were sprayed with several spray reagents. In reference to Dragendorff's reagent (spray reagent A), brown orange spots indicate presence of alkaloids; triterpenes and sterols was determined through the presence of blue-violet spots after Vanillin-sulfuric acid reagent (spray reagent B) was sprayed on the TLC plate. Using ferricyanide-ferric chloride reagent (spray reagent C), blue spots indicate presence of phenols, tannins and flavonoids. Also, brown spots produced after sprayingα-Naphthol-sulfuric (reagent D) shows positive results for glycosides. Lastly, presence of indoles can be detected using Van-UrkSalkowski test wherein positive result is indicated by blue-violet spots. Furthermore, the TLC plates were sprayed with several spray reagents and the visual appearance became the basis in identifying the compounds present, in reference to Guevara (2005).

Conclusion

Phenols, tannins and flavonoids are present across all isolated endolichenic fungi as manifested by color reaction of blue spots with spray reagent C which is potassium ferricyanide-ferric chloride reagent. The isolated endolichenic fungi also contained indoles as indicated by the presence of blue-violet spots with Van-Urk-Salkowski tests.

The TLC results of the endolichenic fungal extracts, in reference to spray reagent A (Dragendorff's reagent), were brown orange spots in all extracts except ELF 4 indicated that they contain alkaloids. Triterpenes and sterols were determined based on spray reagent B (Vanillin-sulfuric acid). The endolichenic fungi showed a positive result in TLC plates of all extracts except ELF 4. Also, spray reagent D (α-Naphthol-sulfuric) showed positive results in all extracts except for ELF 4.

Table 1: Secondary metabolites present in the endolichenic fungi through Thin Layer Chromatography.

Spray Reagent	Positive Result	ELF 1	ELF 2	ELF 3	ELF 4	ELF 5	ELF 6	ELF 7	Compounds Present
A	Brownorange spots	+	+	+	-	+	+	+	Alkaloids
B	Blue-violet spots	+	+	+	-	+	+	+	Triterpenes, Sterols
C	Blue spots	+	+	+	+	+	+	+	Phenols, tannins, flavonoids

D	Brown spots	+	+	+	-	+	+	+	Glycosides
E	Blue-violet spots	+	+	+	+	+	+	+	Indoles

The TLC results of the endolichenic fungal extracts, in reference to spray reagent A (Dragendorff's reagent), were brown orange spots in all extracts except ELF 4 indicated that they contain alkaloids. Alkaloids are a large and structurally diverse group of compunds that have served a scaffold for important antibacterial drugs (Cushnie *et al*, 2014) [2].

Triterpenes and sterols were determined based on spray reagent B (Vanillin-sulfuric acid). The endolichenic fungi showed a positive result in TLC plates of all extracts except ELF 4. Triterpene is a secondary metabolite that can be commonly found in the genus Maytenus that has medicinal properties (Mokoka *et al*., 2013) [8], while sterol belongs to the family of steroid that is often linked with beneficial effects on health and medicinal properties that researchers think have potential for drug discovery (Sultan and Raza, 2015) [13].

Spray reagent C was potassium ferricyanide-ferric chloride which determines the presence of phenols, tannins and flavonoids. All endolichenic fungi showed a positive result to these secondary metabolites. Phenols are common in nature and are probably the most abundant secondary metabolite in plant (Dai & Mumper, 2010) [3], and it acts as growth inhibition (bacteriostasis) on microbial organism (Sabbineni, 2016) [12]. Tannins are phenolic compounds that are found in leaves, bark, and wood of plants and are bound to proteins that form insoluble and soluble tannin-protein complexes. They mainly function as a defense mechanism towards mammals, herbivores and insects (Hassanpour *et al*., 2011) [5]. Flavonoid has biological functions in plants where it acts against pathogen infection (Falcone Ferreyra *et al*., 2012) [4].

Also, spray reagent D (α-Naphthol-sulfuric) showed positive results in all extracts except for ELF 4. All endolichenic fungal extracts showed positive result to Van-Urk-Salkowski test for the presence of indole.

References

1. Claustra AL, Madulid RS, Aguinaldo AM, Espeso EI, Guevara BQ, Nonato MG. *et al*. A guidebook to plant screening: phytochemical and biological. University of Santo Tomas Publishing House, Espana, Manila, 2005.
2. Cushnie TT, Cushnie B, Lamb AJ. Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. International journal of antimicrobial agents,2014:44(5):377-386.
3. Dai J, Mumper RJ. Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. Molecules,2010:15(10):7313-7352. https://doi.org/10.3390/molecules15107313
4. Falcone Ferreyra ML, Rius SP, Casati P. Flavonoids: biosynthesis, biological functions, and biotechnological applications. Frontiers in Plant Science, 2012, 3. https://doi.org/10.3389/fpls.2012.00222
5. Hassanpour S, MaheriSis N, Eshratkhah B. Plants and secondary metabolites (Tannins): A Review, 2011.
6. Kannangara B, Rajapaksha R, Paranagama P. Nature and bioactivities of endolichenic fungi inPseudocyphellariasp., Parmotremasp. andUsneasp. at Hakgala montane forest in Sri Lanka. Letters in Applied Microbiology,2009:48(2):203-209. https://doi.org/10.1111/j.1472-765x.2008.02512.x
7. Li YL, Zhu RX, Li G, Wang NN, Liu CY, Zhao ZT,. Secondary metabolites from the endolichenic fungus Ophiosphaerella korrae. RSC Advances,2019:9(8):4140-4149. https://doi.org/10.1039/c8ra10329a
8. Mokoka TA, McGaw LJ, Mdee LK, Bagla VP, Iwalewa EO, Eloff JN. Antimicrobial activity and cytotoxicity of triterpenes isolated from leaves of Maytenus undata (Celastraceae). BMC complementary and alternative medicine,2013:13(1):1-9.
9. Padhi S, Tayung K. *In vitro* antimicrobial potentials of endolichenic fungi isolated from thalli of Parmelia lichen against some human pathogens. Beni-Suef University Journal Of Basic And Applied Sciences,2015:4(4):299-306. doi: 10.1016/j.bjbas.2015.11.006
10. Padhi S, Masi M, Panda SK, Luyten W, Cimmino A, Tayung K. Antimicrobial secondary metabolites of an endolichenic Aspergillus niger isolated from lichen thallus of Parmotrema ravum. Natural Product Research,2019:34(18):2573-2580. https://doi.org/ 10.1080/14786419.2018.1544982
11. Ranković B, Kosanić M. Lichens as a potential source of bioactive secondary metabolites. In Lichen secondary metabolites. Springer, Cham, 2019, 1-29.
12. Sabbineni J. Phenol-an effective antibacterial agent. J Medic Organic Chem,2016:3(2):182-191.
13. Sultan A, Raza AR. Steroids: A diverse class of secondary metabolites. Med chem,2015:5(7):310-317.
14. Suryanarayanan TS, Thirunavukkarasu N. Endolichenic fungi: the lesser-known fungal associates of lichens. Mycology,2017:8(3):189-196. https://doi.org/10.1080/21501203.2017.1352048
15. Timbreza LP, De los Reyes JL, Flores CH, Perez RJLA, Stockel MA, Santiago KA. Antibacterial activities of the lichen Ramalina and Usnea collected from Mt. Banoi, Batangas and Dahilayan, Bukidnon, against multi-drug resistant (MDR) bacteria. Aust J Mycol,2017:26:27-42.
16. Torres J. Antibacterial activities of fungal endophytes associated with the Philippine endemic tree, Canarium ovatum. Mycosphere,2015:6(3):266-273. https://doi.org/10.5943/mycosphere/6/3/4
17. Zhao Y, Wang M, Xu B. A comprehensive review on secondary metabolites and health-promoting effects of edible lichen. Journal of Functional Foods,2021:80:104283. https://doi.org/10.1016/j.jff.2020.104283