

Antibacterial activity of *Leptogium cochleatum* and *Leptogium moluccanum*

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Abstract

Currently there is an increase in the emergence and re-emergence of bacterial infections, and lichens are potential sources of pharmaceutically important secondary metabolites to halt this problem. Lichens are unique plant-like organisms which are relatively less explored in the Philippines. Here, lichen extracts of *Leptogium cochleatum* and *Leptogium moluccanum* were tested against *Escherichia coli*, *Enterobacter agglomerans*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* using paper disk diffusion assay. Hexane extract of *L. moluccanum* led to a 17.14 mm zone of inhibition (ZOI) against *K. pneumoniae*, while hexane extract of *L. cochleatum* led to a 15.28 mm ZOI against *E. agglomerans*. Dichloromethane extracts of *L. cochleatum* exhibited a ZOI of 15.86 mm against *E. agglomerans* and 14.84 mm ZOI against *E. coli*. The bioactivities exhibited by the lichen extracts may be due to the secondary metabolites detected using thin layer chromatography. Indeed, lichen-derived bioactive compounds hold great promise for pharmaceutical applications for the benefit of human life.

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INTRODUCTION

The worldwide public healthcare is challenged by various bacterial pathogens that cause infectious diseases leading to a large number of deaths^[1]. The emergence and re-emergence of infectious diseases pose a formidable threat to public health and welfare. Because of this, researchers and scientists are focused on the discovery and development of new and effective drugs^[2], and plants have been a valuable source of natural products with antibacterial properties^[3]. Bioactive molecules derived from plants inhibit the growth of pathogenic bacteria^[4], thus were developed into most medicines. However, some bacteria are resistant to antibiotics, thus alternative sources of pharmaceutically important secondary metabolites are being explored.

Lichens which are symbiotic organisms composed of fungi and algae and/or cyanobacteria in an intimate biological union^[5] has been one of the many alternative sources of biologically active metabolites. To date, 17,000 species worldwide^[6] and 789 species in the Philippines^[7] have been described and some are being used as traditional medicine despite the emergence of different modern medicines. Lichens have been used in the treatment of diverse diseases like arthritis, alopecia, constipation, kidney diseases, leprosy, pharyngitis, rabies, infection, worm infestation^[8] and other gastro-intestinal parasites in humans and livestock in Nordic countries such as Scandinavia^[9]. Lichen secondary metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects^[8].

One of the most common growth forms of lichen is the foliose lichen. Foliose lichens are characterized by flattened leafy thalli, and an upper and lower cortex. It has a highly structured growth form with a distinct lower cortex attached to the substratum usually by thread-like rhizinae^[10]. *Leptogium* is a well-known lichen genus under the family Collemataceae and is distributed throughout the tropical regions of the world^[11]. It is characterized by a foliose to subsquamulose thallus, which can be gelatinous, blue-grey to brown or blackish, adnate to loosely adnate, with isidia or lobule. There was initially no secondary metabolites reported in most of its species including *L. phyllocarpum*^[12] and *L. burnetiae*^[12,13]. However, a recent report noted two unknown chemical constituents in *L. azurem* and the presence of atranorin and norstictic acid in *L. burnetiae*, *L. burnetiae* var. *hirsutum*, and *L. cyanescens*^[11]. Thus, to determine if this report is true for other species of *Leptogium*, both species of *Leptogium cochleatum* and *Leptogium moluccanum* collected in Mt. Isarog, Camarines Sur were characterized, and the crude extracts were analyzed and tested for antibacterial activity against bacterial pathogens.

MATERIALS AND METHODS

Study site

Standing 2,000 meters above sea level, Mount Isarog is an inactive stratovolcano located in Luzon Island, Philippines. It has the coordinates 13°39'33" N 123°22'24" E, specifically located in the province of Camarines Sur. A dry period occurs from the month of January to May and the wet season starts in August but rainfall varies. In the lowlands it has a temperature

ranging from 20.8 to 33.6 °C. The temperature decreases by 0.6 °C for every 100 m elevation.

Collection of lichen samples

Foliose lichens were collected along the trails in Mount Isarog, Camarines Sur with purposive sampling technique which is a non-probability sampling technique. The lichens were obtained by detaching them from the substrate using a knife and scalpel. The collected samples were manually cleaned of any debris using syringe needles and were initially stored in paper bags. The samples were air-dried for 24 h and stored in a moist, cool, dry place until further use^[14]. Voucher specimens were stored at the UST Central Laboratories, University of Santo Tomas in Manila, Philippines.

Characterization and identification of foliose lichen samples

The lichen samples were characterized through observation of their morphological features and thalline spot tests K (potassium hydroxide), C (sodium hypochlorite), and IKI (iodine potassium iodide). Razor blades were used to cut the thallus open, followed by thalline spot test. A 10% aqueous solution of potassium hydroxide (KOH) was placed dropwise on the thallus for the K test. The same procedure was carried out for C test and I test, with a drop of 5.25% solution of sodium hypochlorite (NaOCl) and a drop of iodine potassium iodide (IKI) respectively. Resulting color change of the thallus, along with morphological characteristics, was the basis for identification in reference to the previously published dichotomous keys^[15–18].

Extraction and thin-layer chromatography of the lichen extracts

After air-drying, the samples were weighed, cut into small pieces, and crushed using a mortar and pestle. The samples were transferred to Erlenmeyer flasks and 100 mg of lichen thalli were soaked in 100 ml of the extracting solvents for 24 h, hexane being the first solvent used, followed by dichloromethane (DCM), then methanol. After 24 h, extracts were filtered and placed in pre-weighted vials, and the samples were soaked in the solvent again. The process was carried out every after 24 h until the extracts showed a clear and transparent appearance, and the solvent was evaporated using a rotary evaporator. The final amount of the crude lichen extracts of each species per solvent were then weighed^[19].

Thin-layer chromatography (TLC) was used to identify the metabolites or compounds present within the foliose lichen crude extracts. The extracts were spotted on TLC plates, and depending on the extracting solvent used, were run on solvent systems (A) hexane-dichloromethane, (B) dichloromethane, and (C) methanol-dichloromethane. Spots were observed under a UV light at a wavelength of 365 nm^[20]. Furthermore, the TLC plates were sprayed with the following spray reagents: potassium ferricyanide-ferric chloride, Dragendorff's reagent, Kedde reagent, Bornträger reagent, magnesium acetate in methanol, Van-Urk-Salkowski test, vanillin-sulfuric acid, and Naphthol-sulfuric acid. The visual appearance was used as the basis in identifying the compounds present^[21].

Test bacteria and paper disk diffusion assay

The test bacteria used include *Enterobacter agglomerans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The bacteria were initially grown in Mueller-Hinton agar (MHA) slant tubes. After incubation, each 24-h old culture was inoculated in a Normal

Saline Solution (NSS) and the turbidity was adjusted to that of 0.5 McFarland standard^[20].

In the paper disk diffusion assay, the standardized test bacteria were swabbed on MHA plates with an agar depth of 4 mm, and the bacteria were allowed to dry for at least 20 min. Sterile filter paper discs, with a diameter of 6 mm and thickness of 390 µm, were impregnated with 40 µL of lichen crude extracts. The plates were sealed and incubated at 37 °C for 1–2 d with constant observation. After 24 h, the diameter of the growth in the inhibition zone was measured using the Vernier caliper and were classified based on the zone of inhibition (ZOI) standards, < 10 mm as inactive, 10–13 mm as partially active, 14–19 mm as active, > 19 mm as very active^[21]. Chloramphenicol and tetracycline were used as positive controls.

Statistical analysis

Antibacterial activity was evaluated using the mean diameter of the inhibition zones formed. The data was subjected to one-way analysis of variance (ANOVA) to determine the errors and significance of ZOI.

RESULTS

Identification of the *Leptogium* samples

Leptogium is a well-known lichen genus found in the tropics. Morphologically, the thallus of both species was black, but *L. cochleatum* thallus is approximately 90–150 µm thick, while *L. moluccanum* is 30–60 µm thick. The thalline of *L. cochleatum* is wrinkled with flat apothecial disc while *L. moluccanum* has smooth thalline with convex apothecial disc. The morphology of *Leptogium* species used in the study is presented in Fig. 1.

The lichen *Leptogium* secondary metabolites

To test the presence of secondary metabolites in the lichen samples, the lichen thalli were crushed, extracted with different extracting solvents, and subjected to TLC analysis. The spray reagents used in TLC includes potassium ferricyanide-ferric chloride, Dragendorff's reagent, Kedde reagent, Bornträger reagent, magnesium acetate in methanol, Van-Urk-Salkowski test, vanillin-sulfuric acid, and α -naphthol-sulfuric acid. The results suggest that the *Leptogium* species contain secondary metabolites including phenols, tannins, flavonoids, alkaloids, anthrones, triterpenes, and sterols. However, TLC analysis did not detect cardenolides, antraquinones, coumarins, indoles, and sugars in both *L. cochleatum* and *L. moluccanum*. It is also worth noting that similar results were obtained using polar



Fig. 1 The morpho-anatomical structures of *Leptogium cochleatum* (left) and *Leptogium moluccanum* (right).

Table 1. Thin-layer chromatography analysis of *L. cochleatum* and *L. moluccanum* in solvent system.

Spray reagent	Positive result	<i>L. cochleatum</i>			<i>L. moluccanum</i>			Compounds present
		Hexane	DCM	Methanol	Hexane	DCM	Methanol	
A	Blue	+	+	+	+	+	+	Phenols, tannins, flavonoids
B	Brown-orange	-	+	+	+	+	+	Alkaloids
C	Blur to red-violet	-	-	-	-	-	-	Cardenolides
D	Orange, Yellow, Blue	-	-	-	-	-	+(2)	Antraquinones (1), anthrones (2), coumarines (3)
E	Orange-violet	-	-	-	-	-	-	Anthraquinones
F	Blue violet	-	-	-	-	-	-	Indoles
G	Blue violet	+	+	+	+	-	-	Triterpenes, sterols
H	Blue	-	-	-	-	-	-	Sugars

The spray reagents used include A, potassium ferricyanide-ferric chloride; B, Dragendorff's reagent; C, Kedde reagent; D, Bornträger reagent; E, magnesium acetate in methanol; F, Van-Urk-Salkowski test; G, vanillin-sulfuric acid; and H, α -Naphthol-sulfuric acid.

solvents DCM and methanol, as opposed to hexane, a non-polar solvent (Table 1).

Antibacterial assay

For the antibacterial activity assay of the lichen extracts of *L. cochleatum* and *L. moluccanum*, hexane extract of *L. moluccanum* showed the highest ZOI of 17.14 mm against *K. pneumoniae*, although the methanol extract of *L. cochleatum* showed activity. Both hexane and dichloromethane extract of *L. cochleatum* showed the highest ZOI against *E. agglomerans*, while the dichloromethane extract of *L. cochleatum* showed a ZOI of 14.84 mm against *E. coli*. The mean ZOI of the lichen extracts against each bacterium are shown in Table 2. Overall, four extracts showed activity against three bacterial pathogens. Hexane extract of *L. moluccanum* against *K. pneumoniae*, hexane and DCM extracts of *L. cochleatum* against *E. agglomerans*, and DCM extract of *L. cochleatum* against *E. coli* have very active inhibitory potential against the said organisms. Based on the ZOI standards by Guevarra^[21], five samples that showed activity against the pathogenic bacteria. One extract exhibited partial activity against *K. pneumoniae*, while the other showed higher activity against *K. pneumoniae* (1), *E. coli* (1) and *E. agglomerans* (2) (Fig. 2).

Statistical analysis using one-way analysis of variance (ANOVA) showed that there is significant difference on the potential of the different lichen extracts and positive controls,

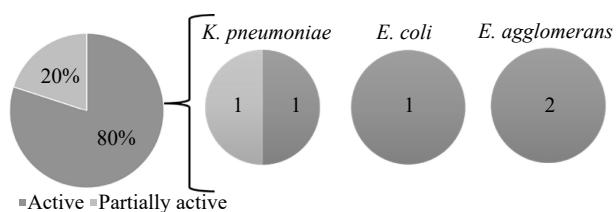


Fig. 2 Antibacterial activity of the lichen crude extracts against the test organisms based on the zone of inhibition (ZOI) standards by Guevara^[21].

Table 2. Mean of the ZOI (mm) of the *Leptogium* extracts against the test pathogenic bacteria.

	CP	TC	<i>L. cochleatum</i>			<i>L. moluccanum</i>		
			Hexane	DCM	Methanol	Hexane	DCM	Methanol
<i>K. pneumoniae</i>	32.06	24.46	7.96	8.94	10	17.14	7.72	6.89
<i>S. aureus</i>	35.7	30.6	7.68	6.79	8.83	8.18	6.34	6
<i>P. aeruginosa</i>	41.54	28.13	6.33	8.14	8.33	8	9.15	7.56
<i>E. agglomerans</i>	25.32	24.67	15.28	15.86	6.83	7.96	7.46	6
<i>E. coli</i>	25.62	20.4	6.72	14.84	6	8.81	6	6

CP, chloramphenicol; TC, tetracycline.

tetracycline, and chloramphenicol as antibiotic agents. In the *post hoc* test, the mean ZOI of hexane extract of *L. cochleatum* showed no significant difference with the mean ZOI of both the tetracycline and chloramphenicol with p-value of 0.275721 and 0.270317, respectively. This means that the hexane extract of *L. cochleatum* can also inhibit the growth of *E. agglomerans*. This suggests that the extracts can inhibit the growth of the bacterial pathogens and the lichen compounds detected in the extracts are responsible for the antibacterial activity of lichens.

DISCUSSION

Lichens are comprised of a fungal and an algal partner which are stable, ecologically obligate, self-supporting composite organisms^[22]. Lichens are a diverse group of organisms known to be bio-indicators of pollution and produce numbers of pharmaceutically important secondary metabolites used in traditional medicines for centuries^[23]. Thus, lichens and their products hold considerable interest as alternative treatments in various parts of the world^[24]. Lichen acids or secondary metabolites are either in crystal forms or liquid that are scattered in different parts of the thallus and are exported outside of the fungal hyphae^[25]. They are used in the regulation of primary metabolic pathways and help in the maintenance of balance in the environment, adapting to match the environmental needs. Secondary metabolites exhibit many biological activities such as anti-inflammatory, antifungal, anticancer and antimicrobial^[26]. Despite these, some species of *Endocarpon* and *Leptogium* showed no presence of secondary metabolites in their thalli^[12,13], yet the TLC results of the of *L. cochleatum* and *L. moluccanum* indicated that they contain phenols, tannins and flavonoids similar to other *Leptogium* species^[11].

The antibacterial activity indicates the effectivity of the secondary metabolites with antibacterial activity as extracted using hexane and dichloromethane. A study by Kamar et al.^[22] have not detected any secondary metabolite from *Leptogium*

burnetiae using methanol as the extracting solvent. However, this study detected phenol, tannins and flavonoids in both *Leptogium* species, which acts as growth inhibitors (bacteriostasis) on microbial organism^[27–29]. Alkaloids, triterpenes and sterols were also detected in *L. cochleatum* which are a large and structurally diverse group of compounds that have served a scaffold for important antibacterial drugs^[23].

CONCLUSIONS

This study shows the potential of *L. cochleatum* and *L. moluccanum* as sources of biologically active secondary metabolites with hexane and dichloromethane as effective extracting solvents. Further studies on the identification of these secondary metabolites should be performed.

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Conflict of interest

The authors declare that they have no conflict of interest.

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