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Morpho-anatomical Characterization and Biochemical Profiling of Two Lichen Species, *Physmabyrsaeum* (Ach.) Tuck and *Leptogium azureum* (Sw. ex Ach.) Mont., collected from Mount Banahaw de Lucban, Quezon Province, Philippines

John Dave A. Dicuangco[1]* and Glen S. Nolasco[2]
[1]Department of Biology, College of Arts and Sciences, Don Honorio Ventura State University, Bacolor, Pampanga 2001, Philippines
[2]Department of Biology, Institute of Arts and Sciences, Mabalacat City College, Mabalacat City, Pampanga2010, Philippines
jdadicuangco@dhvsu.edu.ph

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Abstract

Lichens are renowned for their diverse secondary metabolites with diverse bioactivities and uses. This study generally aimed to characterize and determine the phytochemical constituents of two lichen species purposively collected from Mount Banahaw de Lucban. Morpho-anatomical and chemical characterization identified the specimens as Physmabyrsaeum and Leptogium azureum. The lichens were then subsequently extracted with two different extracts of acetone and dichloromethane to obtain polar and non-polar phytochemical constituents. The phytochemical screening by test tube reaction showed presence of potent alkaloids, cardiac glycosides, anti-oxidant rich flavonoids, foaming saponins, condensed tannins, and aromatic terpenoids. Thin-layer Chromatography (TLC) also revealed the presence of various lichen products that could possibly belong to substance class anthraquinones, xanthones, pulvinic and usnic acid derivatives, β-Orcinol depsides and terpenoids and infrequent substances napthaquinone, bi- and diphenyl. In conclusion, collected lichens of P. byrsaeum and L. azureum from Mount Banahaw de Lucban showed substantial presence of phytochemicals that may contribute valuable insights for their chemical diversity.

Keywords: Lichens, morpho-anatomical, spot tests, thin-layer chromatography, Mount Banahaw de Lucban.

Introduction

Lichenized fungi, commonly known as ―lichens, are ecologically essential, stable and self-supporting organism formed from a symbiotic relationship between the inhabitant photobiont and the exhabitant mycobiont. Photobionts are organisms that are capable to photosynthesize (the autotrophic partners) that can either be an alga, cyanobacteria or both. Meanwhile, mycobiont are organisms that act as host partner of the photobiont which are the fungi[1]. Lichen species were believed to range from 13,500-17,000 species[2]. However, updated estimate revealed that the lichen species worldwide reach approximately 28,000 species[3].

The diversity and unique various compounds possessed by these lichen species caught the interest of scientific community[4]. Lichens can be found almost in all habitats ranging from poles to tropics, terrestrial to marine and fresh water aquatic habitats and also to xeric environments. It was estimated that the dominant life forms of lichens cover 8% of the Earth's land surface. This high diversity can be accounted to their capacity to colonize wide range of substrates[1].

Lichens produce secondary metabolites[5] and many of them are known to possess biological and/or therapeutic properties in the field of medicine[6,7]. Secondary metabolites are produced commonly through biosynthetic polyketide pathway while some are from shikimate and mevalonate pathways[8]. These physiological processes consumed high cost of energy; however, carbon plays a defense mechanism from either biotic or abiotic factors[9]. Lichens are also well-known for their sources of great diversity of biologically active natural substances that shows various biological activities including antimicrobial, anti-inflammatory, analgesic, antipyretic, anti-proliferative, cytotoxic activities and other important activities.

These biologically active natural substances are also referred as phytochemicals[10]. Important phytoconstituents include potent alkaloids, astringent tannins, antioxidant-rich flavonoids, aromatic terpenoids, and other phenolic compounds such as cardiac glycosides and foaming saponins[11].

Meanwhile, Mount Banahaw is one of the places worth studying because of its biodiversity. It is bordered by Laguna Lake (N), Tayabas Bay (S), Bicol Peninsula (SE) and tail end of Sierra Madre Mountains (E)[12]. The area has rough terrain, intermediate slopes, 1875 m.a.s.l. elevation[13], tall canopies and small bushes. Biodiversity studies, including plant[14], small mammals[15], and herpetofaunal[16], have been conducted.

However, studies about lichen biodiversity and their biochemical constituents are still limited.

This research generally aimed to characterize the purposively collected lichens and determine the lichen substances and phytochemicals possessed by it. Specifically, it aimed to identify the collected lichen samples based on the morpho-anatomical and chemical characterization. Additionally, it aimed to profile the polar and non-polar lichen metabolites present in collected samples.

This study is beneficial primarily on scientific community in providing insights about the characteristics of lichens from Mount Banahaw de Lucban and their lichen substances and phytochemical composition. The study mainly focuses on the morpho-anatomical and chemical characterization and limited in determining selected phytochemicals.

Materials and Methods

Research Locale and Design: The collection of lichen samples was conducted at Mount Banahaw de Lucban in Quezon Province, Luzon, Philippines. The lichen samples were collected from 600-1000 meter above sea level (m.a.s.l.) of the area. The study used descriptive type of research design. Purposive sampling was used in lichen collection collecting only two species that can provide substantial amounts of extracts. Morpho-anatomical and chemical characterizations were used in lichen characterization and identification. Test-tube tests and Thin-layer Chromatography (TLC) were used in determining lichen metabolites.

Research Procedure: Characterization of Collected Lichen Samples: The collected lichen samples were observed and characterized macroscopically and microscopically to determine its external and internal morphology including its growth form, lobes, margin, description of its upper and lower surface, describing the presence of apothecia and perithecia and the features of its ascospores using digital dissecting microscope. The observed parts of the lichens were captured using iPad 4. Histological aspect was performed by free-hand sectioning and was examined in a compound light microscope to check for the detailed parts of the lichens.

The thallines pot test, also known as the color test, is a rapid and convenient method usually used during field collection. It quickly indicates the chemically-related group by giving specific color reaction. The lichens were subjected into K, C, and KC test in order to initially determine some of the presence of secondary metabolites by subjecting the lichen thallus in different respective reagents[17]. Identification of lichen samples was conducted using a dichotomous key[18] in accordance with the morpho-anatomical and chemical characteristics of the collected lichens.

Extraction of Lichen Metabolites: Two hundred grams (200g) of air-dried lichen thalli were grounded using blender until pulverized. The pulverized lichen samples were soaked in 2000mL each of acetone and dichloromethane separately in a 1 gram:10mL ratio to acquire the polar and non-polar compounds present respectively. Lichen samples were left for three consecutive days at room temperature in a 1000mL beaker tightly sealed with paper and aluminum foil to avoid vaporization for its boiling points are both low. The samples were filtered using Whatman filter paper no. 1. The extracting reagents used were allowed to evaporate using rotary evaporator set in 56◦C and 39◦C boiling temperature for acetone and dichloromethane extracts respectively to acquire the crude extracts[18].

Biochemical Profiling: Test-tube Test: Different extracts obtained were tested qualitatively for the presence of various phytochemical compounds[20]. Ferric chloride test was conducted to test the presence of tannins. Two milliliters of lichen crude extract was mixed with about 3-5 drops of 5% ferric chloride solution in a test tube until color reaction occurs. Blue color indicates that there could be presence of hydrolysable tannins and brown for the condensed tannins. Dragendorff's test was used to test the presence of alkaloids. Two milliliters of crude lichen extract was added with about 3-5 drops of 1% HCl and set aside for 10 minutes. Thereafter, 6 drops of Dragendorff's reagent were added to the mixture. Reddish brown precipitate indicates that there could be presence of alkaloids. Froth's test was used to determine the presence of saponins. Two milliliters of crude lichen extract was mixed with 5mL of distilled water in a test tube and was agitated vigorously. The formation of stable foam indicates the presence of saponins. Sodium hydroxide test was performed to detect the presence of flavonoids. Two milliliters of crude lichen extract was added to 2mL of 10% NaOH solution. Formation of yellow to orange colour indicates that there could be presence of flavonoids. Salkowski's test was conducted to test the presence of terpenoids in which two 2mL of crude lichen extract was added with 1mL of chloroform and was agitated.

Biochemical Profiling: Thin-layer Chromatography*:* Qualitative analysis for the presence of lichen substances and phytochemicals were determined using thin-layer chromatography (TLC). Using a capillary tube, for all the samples, the extracts loaded on the tube was spotted into the TLC plate strips for 20 times. The spots should be dried in every interval of every spotting.

After 15-20 spots, the TLC plate strips were then placed into a developing jar containing solvent system C having 170:30 ratio of toluene:glacial acetic acid. The solvents were run until it reached the solvent front through capillary action. Thereafter, it was allowed to dry. For the identification of lichen acids, 10% sulfuric acid (H_2SO_4) was sprayed onto the TLC plate strips. The Rf values were computed following the formula below.

$$R_f = \frac{\text{distance spot traveled}}{\text{distance solvent traveled}}$$

Lichen substances were presumed using the computed Rf values based on the ―Catalogue of Standardized Chromatographic Data and Biosynthetic Relationships for Lichen Substances[20].

Results and Discussion

Characterization of Collected Lichen Samples: The morpho-anatomical characterization was conducted through observation of macro- and microscopic view of the lichen samples. It includes the examining and recording of the name and type of substrate, collection site, growth forms, shape of lobes and margin, description of upper and lower surface, presence of isidia and rhizine on surface, apothecia or perithecia type of fruiting body, and its ascopore features.

Morpho-anatomical Characterization: Figure-3 shows the morpho-anatomical of the Mount Banahaw de Lucban Lichen 1 (MBDL-L01) and Mount Banahaw de Lucban Lichen 2 (MBDL-L02). Based on the observations, the MBDL-L01 has foliose-growth form, radiating and oblong lobes, and entire margin. The upper surface was observed having ridged and wrinkled texture, and showed absence of isidia and apothecial-type of reproductive structure. The apothecia were also described to be laminal. The lower surface has a pale rough texture and showed presence of rhizines. The ascospore, within the ascus, was observed with ellipsoidal shape.

Meanwhile, the MBDL-L02 were observed having also foliose-growth form, rotund lobes and orbicular margin. The upper surface was described as smooth and gelatinous in texture. It also possessed apothecial-type of reproductive structure and absence of isidia. The apothecia were also described as laminal. The lower surface was observed as pale smooth and contains rhizines presence. The ascospores were ellipsoidal in shape enclosed within the ascus. The results were summarized in Table-1 and furtherly discussed along with the collection information about the lichen specimens of MBDL-L01 and MBDL-L02.

Both samples have its corresponding host plant. During the collection, MBDL-L01 was found on Mahogany trees while MBDL-L02 was usually found on pandan plants and narra trees. Based on the table, it depicts that MBDL-L02 were mainly found around 800-1000 m.a.s.l. while MBDL-L01 is around 600-700 m.a.s.l.

For the morpho-anatomical characteristics of the collected lichen samples, both samples exhibit foliose type of growth form, entire margin, absence of perithecia and isidia structure on the upper surface, and the presence of apothecia, and rhizine on the lower surface. *MBDL-L01* and *MBDL-L02* differ in shape of the lobe, ascospore, and the characteristics of their lower and upper surface. This set of features pinpoints that it is *Physmabyrsaeum*. *P. byrsaeum* has wholly-developed cortical layer and the thalline apothecial exciple are wrinkled. Moreover, features of the thalli are rosulate, adnate which are 6-8cm across, 200–350μm in thickness having radiating lobes and 1-5mm wide. *P. byraseum* also has thickened entire margin and the upper surface is ridged, brownish black and lacks isidia; thin cortex, pale brown color of the lower surface having *Nostoc* photobiont; cushion-like indumentums was formed by rhizines of interwoven hyphae having whitish to black coloration; abundance of apothecial ascomomata, laminal and sessile having 1-4mm wide. Furthermore, the study confirms that the disc is concave to plane having reddish brown coloration constituting exciple thick thalline (wrinkled, lobed, concolorous with thallus). *P. byrsaeum* comprises simple ascospores, ellipsoidal (12-15X10–12μm), 2–3μm thick episporium, laminal pycnidia, 2-3μm long conidia[21].

The second lichen sample collected at Mt. Banahaw de Lucban, based on the observed morpho-anatomical features was determined to be *Leptogium azureum. L. azureum* exhibits foliose type of thallus having blue to blue gray coloration and 3-8 cm in diameter[22] similar to what was observed in the *MBDL-L02*. Moreover, L. *azureum* bears orbicular to elongated lobes which are 5mm broad having orbicular margin. *Leptogium* species also has distinctly gelatinous thallus having cyanobacterium *Nostoc* as photobiont possessing common, laminal, sessile to short stipitate, 0.2-2.5mm wide apothecia composing of flattened discs[23]. Lastly, *L. azureum* also has 8-spore cylindrico-clavate asci[24].

Chemical Profiling: The Table-2 presents the result of the thalline spot testing conducted with the collected lichens. It consists of the three different tests of K, C and KC tests. It also shows the color reaction and its indication from the different color spot tests conducted on the lichen thallus sample. It shows that *Physmabyrsaeum* and *Leptogium azureum* were positive from all of the tests conducted indicating that both lichens have presence of *o*-hydroxyl aromatic aldehydes, dihydroxy dibenzofuran strepsilin and usnic acid as indicated by their color reaction.

A study[25] supported that the *Leptogium azureum* were positive to the K, C and KC test. However, in *Physmabyrsaeum*, it is argued that the K, C and KC were positive which was indicated as negative by another study conducted[21].

The lichen specimens were pre-identified up to its genus level in which it describes that the lichen specimen belongs to the family Collemataceae (*Leptogium*, *Collema* and *Physma*) by exhibiting the thallus a shade of brown, olive, black or bluish gray; lacking cyphellae; lower side without veins; large lobes; lower side not tementose; and gelatinous, jelly-like when wet, non-stratified thallus[18].

The accumulated results coincide with the study conducted[21] stating that *P. byrsaeum* has wholly-developed cortical layer and the thalline apothecial exciple are wrinkled and the features of the thalli are rosulate and adnate. Moreover, *P. byraseum* has thickened entire margin and the upper surface is ridged,

brownish black and lacks isidia; thin cortex, pale brown color of the lower surface having cushion-like indumentum were formed by rhizines of interwoven hyphae having whitish to black coloration; abundance of apothecial ascomomata, laminal and sessile[26]. The second lichen sample which is claimed to be *L. azureum* was supported by the study published confirming that *L. azureum* exhibits foliose type of thallus having blue to blue gray coloration and bears[28].

Biochemical Profiling: Test-tube Reaction: Table-3 shows that the results of the test tube reaction from the acetone and dichorome thane extracts of the *Physmabyrsaeum* and *Leptogium azureum*. It tested the possible presence of the alkaloids, cardiac glycosides, flavonoids, saponins, tannins and terpenoids.

The acetone extracts of *P. byrsaeum* were positive for the presence of tannins, alkaloids, flavonoids and terpenoids but negative for the presence of saponins and cardiac glycosides. For its dichloromethane extracts, it was positive for the presence of tannins, alkaloids, flavonoids, saponins and terpenoids but negative for the cardiac glycosides.

Conversely, it also shows that the acetone extracts of *L. azureum* were positive for the presence of tannins, alkaloids, flavonoids, and terpenoids but negative for cardiac glycosides and saponins. Its dichloromethane extracts were positive for all of the phytochemicals.

In a study conducted[10], the acetone lichen extracts were mostly positive for the presence of the tannins, flavonoids and terpenoids. Minimally, it was only positive for the presence of the alkaloids in the acetone lichen extract of *Telos chistes flavicans*. Also, all the acetone lichen extracts were negative for the presence of the cardiac glycosides.

Additionally, another study[28] showed that the tannins, alkaloids, flavonoids, and saponins can also be found in nonpolar extracts like chloroform and dichloromethane. The primary terpenoids are normally extracted from non-polar solvents since it is exclusively composed of hydrocarbons. Generally, the alkaloids can be extracted by non-polar solvents.

It was explained that quaternary alkaloids are slightly soluble to non-polar solvents. However, polar solvents are much preferred when other classified alkaloids (primary, secondary and tertiary) are also present which can also be polar in structure[29]. Meanwhile, the cardiac glycosides were explained that can be polar or nonpolar depending on the quantity of the ketone and alcohol groups in glycone structure[30].

Thin-layer Chromatography: The Tables 3-6 show the results of the thin-layer chromatography (TLC) in identifying the lichen substances of acetone and dichloromethane extracts of *P. byrsaeum* and *L. azureum*. It presents the possible identified lichen substances and its class by determination and comparison of its color, distance traveled, Rf class and Rf value with the standard values[20].

Acetone extract of *P. byrsaeum* showed lichen products of pulvinic acid dilactone, allorhizin, contortin, xanthorin, fallacinal, pigmentosin, barbatic acid, and 15α-Acetoxyhopane-22-ol [Dolichorrizin]. Acetone extract of *L. azureum* showed lichen products of pulvinic acid dilatone, calycin, 8-O-Methylthiomelin Leprolomin, barbatic acid, obstusatic acid, 7-Chloroemodin, Anhydrofusaburin lactol methyl ketal, and rhein.

For the dichloromethane extract of *P. byrsaeum*, lichen products of pulvinic acid dilactone, atranorin, usnic acid, vulpinic acid and xanthorin were determined. Meanwhile, dichloromethane extracts of *L. azureum* showed lichen products of pulvinic acid dilactone, parietin, atranaorin, usnic acid, dichloropannarin, methyl psoromate, Fern-9(11)-ene-3,19-dione, 29-Nor-21α-hopane-3,22-dione and xanthorin.

Based on further observation, it can be inferred that the dichloromethane extracts of *L. azureum* contains the greatest number of lichen substances. In contrast, the dichloromethane extracts of *P. byrsaeum* shows the least. It can also be implied that there was more polar lichen substances extracted from the acetone and more non-polar lichen substances from the dichloromethane indicated by the Rf values. The possible lichen substances such as pulvinic acid dilactone, usnic acid, atranorin, barbatic acid and xanthorin were frequently extracted from the lichen specimen. Based on the lichen substances, the two lichen species were commonly observed with the presence of the lichen classes of pulvinic acid derivatives, B-orcinol depsides and anthraquinones.

A study[31] claimed that the secondary metabolites in *Leptogium azureum* and *Physmabyrsaeum* could not be detected by TLC analysis. However, in this study, the lichens of *Leptogium azureum* and *Physmabyrsaeum* possessed various secondary metabolites. Presence of unknown substances in *Leptogium* which was visible in crystallization and TLC visualization was seen by one study[25].

Moreover, the author also stated that their *Leptogium* collected possessed substances rich in aromatic compounds. In a similar study[21], it was showed that the *Leptogium azureum* contains various unknown substances as revealed by High Performance Liquid Chromatography (HPLC). Other species of genus *Leptogium*, the *L. quercicola*, was also showed by HPLC containing carotenoids[27].

Genus *Collema*, under family Collemataceae which is also family where *Physmabyrsaeum* and *Leptogium azureum* belongs, showed various secondary lichen metabolites in which most abundant are fatty acids and great variation of ester composition which was characterized by GC-MS, HPLC and HR-TLC. Moreover, indole alkaloids and other nitrogen–containing metabolites were also isolated[32].

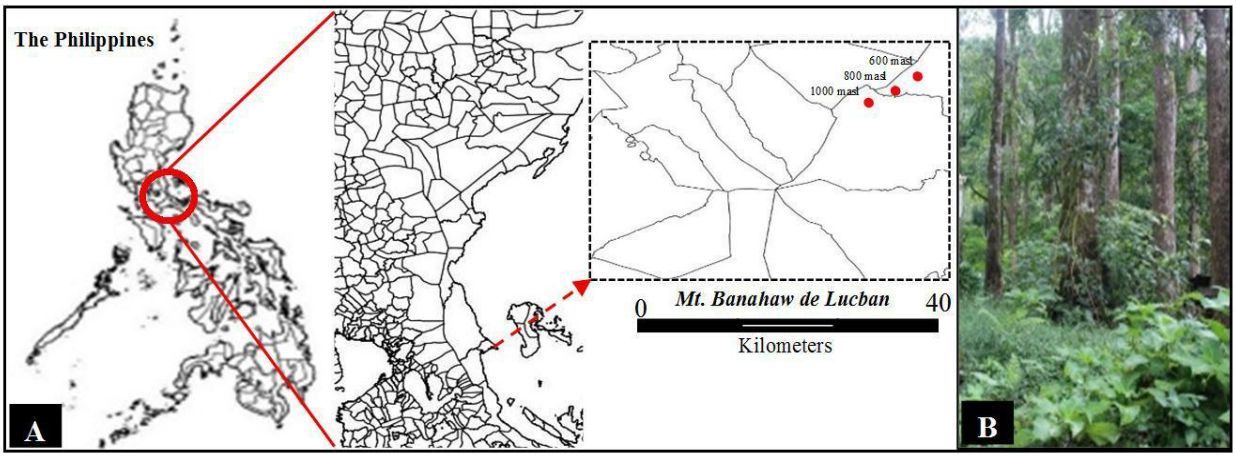


Figure-1: The study area. (A) Map of the Mt. Banahaw in Quezon Province, showing the plotted collecting points (generated using the Quantum GIS, v1.8 from 600 to 1000 m.a.s.l. (B) Actual photo of collection site.

Figure-2 (A-J): Morpho-anatomical Characteristics of MBDL-L01 (A-E) and MBDL-L02 (F-J) (A & F) External Morphology. (B & G) Upper Surface. (C & H) Lower Surface. (D & I) Dissected Thallus. (E & J) Ascus.

Table-1: Collection, Characterization, and Identification Information Table of Collected Lichen Samples.

Lichen	Identified	Collection Information			Lichen Morphoanatomical Characters											
Code	Species Name	Substrate		Elevation (masl)	Growth Form	Lobes	Margin	Upper Surface		Lower Surface		Reproductive Structure				Ascopore
		Type	Name					Isidia (+/-)	Description	Rhizine (+/-)	Description	Apothecia (+/-)	Description	Perithecia (+/-)	Description	
MBDL-	*Physma*	Plant	Mahogany	600-700	Foliose	Oblong,	Entire	-	Ridged,	+	Pale, brown	+	Wrinkled	-	-	Simple,
L01	*Byrsaeum*		(*Swietenia macrophylla*)			radiating			wrinkled		rough		exiple laminal			Ellipsoidal
MBDL-	*Leptogium*	Plant	Pandan	800-1000	Foliose	Rotund	Orbicular	-	Smooth	+	Pale, smooth	+	Laminal	-	-	Ellipsoidal
L02	*azureum*		(*Pandanus* sp.)													
			Narra (*Pterocarpus* sp.)													

(+) – Present, (-) – absent.

Table-2: Thalline Spot Test Result of the Collected Lichens and their Indication.

Lichen Code	Lichen Name	Thalline Spot Testing			Indication
		Test	Color Reaction	+/-	
MBDL – L01	*Physma byrsaeum*	K test	Light yellow	+	Presence of *o*-hydroxyl aromatic aldehyde
		C test	Yellow green	+	Presence of dihydroxy dibenzofuran strepsilin
		KC test	Dark yellow	+	Presence of usnic acid
MBDL – L02	*Leptogium azurem*	K test	Light yellow	+	Presence of *o*-hydroxyl aromatic aldehyde
		C test	Yellow green	+	Presence of dihydroxy dibenzofuran strepsilin
		KC test	Yellow green	+	Presence of usnic acid

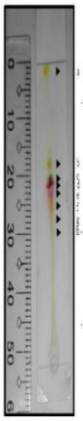
(+) – Positive, (-) – negative.

Table-3: Phytochemical Screening Results of Collected Lichen Samples through Test-tube Reaction.

Lichen Code	Lichen Species Name	Phytochemicals	Crude Extract	
			Acetone	Dichloromethane
MBDL – LO1	*Physma byrsaeum*	Alkaloids *(Dragendorff's test)*	+	+
		Cardiac Glycosides *(Keller-Killiani's test)*	-	-
		Flavonoids *(Sodium hydroxide test)*	+	+
		Saponins *(Froth's test)*	-	+
		Tannins *(Ferric chloride test)*	+	+
		Triterpenoids *(Salkowski's test)*	+	+
MBDL – LO1	*Leptogium azureum*	Alkaloids *(Dragendorff's test)*	+	+
		Cardiac Glycosides *(Keller-Killiani's test)*	-	+
		Flavonoids *(Sodium hydroxide test)*	+	+
		Saponins *(Froth's test)*	-	+
		Tannins *(Ferric chloride test)*	+	+
		Triterpenoids *(Salkowski's test)*	+	+

(+) – Present, (-) – absent.

Table-4: Thin-layer Chromatography (TLC) of the Lichen Substances of Acetone Extract of Physma byrsaeum.

Crude Extract	Image		Color	d	R_f value	R_f class	Standard			Possible Lichen Product	Lichen Class
							R_f	Color	Remarks		
MBDL – L01		a	Yellow	52	0.98	8	0.9	Yellow	-	Pulvinic acid dilactone	Pulvinic acid derivatives
Physma byrsaeum		b	Yellow	35	0.66	6	0.65	Yellow	-	Allorhizin	β-orcinol depsidones
Acetone Extract		c	Green	31	0.58	6	0.58	Green	-	Contortin	Biphenyl
		d	Dark Red	30	0.57	6	0.57	Red	K+	Xanthorin	Anthraquinones
		e	Pink	29	0.55	6	0.55	Pink	-	Fallacinal	Anthraquinones
		f	Brown	28	0.53	6	0.53	Brown	K+	Pigmentosin	Napthpyrones
		g	Light Yellow	27	0.51	5	0.51	Yellow	KC+	Barbatic Acid	β-orcinol depsidones
		h	Light Brown	23	0.43	5	0.43	Brown	-	Dolichorrhizin	Terpenoids

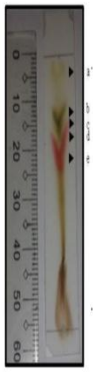
+ = K/C/KC test positive; - = no remarks; Rf =retention factor; d = distance travelled.

Table-5: Thin-layer Chromatography (TLC) of the Lichen Substances of Dichloro-methane extract of Physma byrsaeum.

Crude Extract	Image		Color	d	R_f value	R_f class	Standard			Possible Lichen Product	Lichen Class
							R_f	Color	Remarks		
MBDL – L01		a	Dark Yellow	54	0.98	8	0.9	Yellow	-	Pulvinic acid dilactone	Pulvinic acid derivatives
Physma byrsaeum		b	Light Yellow	49	0.89	8	0.88	Yellow	-	Calycin	Pulvinic acid derivatives
Dichloro-methane Extract		c	Orange	31	0.56	6	0.56	Orange	-	8-*o*-methylthiomelin	Xanthones
		d	Dark Green	30	0.55	5	0.54	Green	-	Leprolomin	Diphenyl ethers
		e	Yellow	28	0.52	5	0.52	Yellow	KC+	Barbatic acid	β-orcinol depsidones
		f	Gray	27	0.49	5	0.47	Gray	KC+	Obtusatic acid	β-orcinol depsidones
		g	Yellow	26	0.47	5	0.47	Yellow	K+	7-chloroemodin	Anthraquinones
		h	Gray	24	0.44	5	0.45	Gray	K+	Anhydrofusaburin lactol methyl ketal	Napthaquinones
		i	Red orange	23	0.43	5	0.47	Red orange	K+	Rhein	Anthraquinones

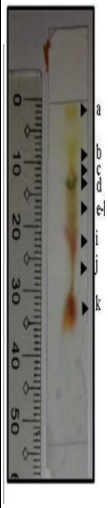
+ = K/C/KC test positive; - = no remarks; Rf =retention factor; d = distance travelled.

Table-6: Thin-layer Chromatography (TLC) of the Lichen Substances of Acetone Extract of Leptogium azureum.

Crude Extract	Image		Color	d	R_f value	R_f class	Standard			Possible Lichen Product	Lichen Class
							R_f	Color	Remarks		
MBDL – L02		a	Light Yellow	54	0.96	8	0.9	Yellow	-	Pulvinic acid dilactone	Pulvinic acid derivatives
Leptogium azureum		b	Orange	44	0.79	7	0.79	Orange	K+	Atranorin	β-orcinol depsidones
Acetone Extract		c	Green	42	0.75	6	0.75	Green	K+	Vulpinic acid	Usnic acid derivatives
		d	Yellow Green	40	0.71	6	0.7	Green	KC+	Usnic acid	Pulvinic acid derivatives
		e	Red Orange	33	0.59	6	0.6	Red orange	K+	Xanthorin	Pulvinic acid derivatives

+ = K/C/KC test positive; - = no remarks; Rf =retention factor; d = distance travelled.

Table-7: Thin-layer Chromatography (TLC) of the Lichen Substances of Dichloromethane Extract of Leptogium azureum.

Crude Extract	Image		Color	d	R_f value	R_f class	Standard			Possible Lichen Product	Lichen Class
							R_f	Color	Remarks		
MBDL – L02		a	Yellow	52	0.96	8	0.9	Yellow	-	Pulvinic acid dilactone	Pulvinic acid derivatives
Leptogium azureum		b	Yellow	45	0.83	8	0.82	Yellow	K+	Parietin	Anthraquinones
Dichloro-methane Extract		c	Orange	42	0.78	7	0.79	Orange	K+	Atranorin	β-orcinol depsidones
		d	Green	41	0.76	6	0.7	Green	KC+	Usnic acid	Usnic acid derivatives
		e	Brown	39	0.72	6	0.75	Brown	-	Dechloropannarin	β-orcinol depsidones
		f	Brown	37	0.69	6	0.67	Brown	-	Methyl psoromate	β-orcinol depsidones
		g	Brown	35	0.65	6	0.65	Brown	-	Fern-9(11)-ene-3, 19-dione	Terpenoids
		h	Brown	34	0.63	6	0.59	Brown	-	29-Nor-21α-hopane-3,22-dione	Terpenoids
		i	Red orange	31	0.57	6	0.6	Red orange	K+	Xanthorin	Anthraquinones
		j	Brown	27	0.5	5	-	-	-	-	-
		k	Red-brown	21	0.39	5	-	-	-	-	-

+ = K/C/KC test positive; - = no remarks; Rf =retention factor; d = distance travelled.

Conclusion

Two common lichen samples, *Physmabyrsaeum* and *Leptogium azureum*, were characterized and identified. Test-tube tests and Thin-layer chromatography (TLC) revealed that the lichen samples possess various phytochemicals and lichen substances. The phytochemicals of alkaloids, cardiac glycosides, flavonoids, saponins, tannins, and terpenoids were present except for cardiac glycoside in *Physmabyrsaeum*. Thin-layer Chromatography (TLC) also revealed the presence of lichen substances that could possibly belong to substance class anthraquinones, xanthones, pulvinic and usnic acid derivatives, β-Orcinol depsides and terpenoids and infrequent substances napthaquinone, bi- and diphenyl based on the R_f value.

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