



Identification of foliose lichens and antibacterial screening of *Parmotrema neopustulatum* collected from communal forest in Shilan, La Trinidad, Benguet, Philippines

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Abstract

Lichens are symbiotic organisms composed of a mycobiont and a photobiont of either algae or cyanobacteria. Despite the reported 1,262 lichen species in the Philippines, the biodiversity and bioactivity of lichens in some areas of the Philippines remained understudied. In this study, we collected foliose lichens from a communal forest in Shilan, La Trinidad Benguet, Philippines, and subjected them to morphological and chemical analysis. A total of 19 lichen species were identified belonging to six genera, including *Bulbothrix*, *Crocodia*, *Heterodermia*, *Hypotrachyna*, *Parmotrema*, and *Relicina*. Specifically, *Parmotrema neopustulatum*, the most collected lichen thalli by mass, was extracted using ethyl acetate and was evaluated for its antibacterial properties against ESKAPE pathogens via paper-disk diffusion assay. The crude extract exhibited inhibitory activity against *Enterobacter aerogenes*, with a 13 mm zone of inhibition (ZOI). It also inhibited the growth of *Staphylococcus aureus* (18 mm ZOI), *Klebsiella pneumoniae* (21 mm ZOI), *Acinetobacter baumannii* (17 mm ZOI), and *Enterococcus faecalis* (16 mm ZOI). Based on Thin-Layer Chromatography (TLC), the extract contained aldehydes, alkaloids, cardenolides, essential oils, indoles, and sugars. This study addresses research gaps in Philippine lichenology, confirms the diversity of understudied foliose lichen species in some regions of the country such as La Trinidad, Benguet, and affirms that lichens are a potential source of bioactive secondary metabolites.

Keywords – disk-diffusion assay – ESKAPE pathogens – foliose – lichens – thin-layer chromatography

Introduction

Lichens are symbiotic organisms consisting of a mycobiont and a photobiont, a green algae, a cyanobacteria, or both. Lichens are perennial, resilient, and globally distributed organisms that primarily function as bioindicators of pollution, food for animals, traditional medicine, and treatment of diseases (Crawford 2015, Elkhateeb et al. 2022). About 18,500 different lichen species have been described worldwide, with the majority of mycobiont from the phylum Ascomycota (Kosanić & Ranković 2015). In the Philippines, a megadiverse archipelagic country, lichen flora is

relatively large and diversified, which consist of 1,262 taxa, distributed across 65 families and 229 genera, with *Graphis*, *Usnea*, *Porina*, *Leptogium*, and *Parmotrema* as the top five genera with the most species (Paguirigan et al. 2020). The occurrence of foliose lichens has been recorded at several collection sites in the Philippines, such as in Abra, Benguet, Ifugao, Ilocos Norte, Isabela, Mountain Province, Nueva Vizcaya, Quezon, and Calabarzon in Luzon; Negros Oriental in Visayas; and Agusan, Davao de Oro, Davao del Sur, and Bukidnon in Mindanao (Guzman & Fabito 2016, Bawingan et al. 2017, 2019, Azuelo & Puno 2018, Paguirigan et al. 2019, Cababan et al. 2020, Magday et al. 2020, Dela Tina-Picaza & Picaza 2023, Taer et al. 2023). Specifically, foliose lichens are reported in several natural parks and mountainous areas, including Mt. Kalatungan Range Natural Park in Bukidnon (Azuelo & Puno 2018). Meanwhile, 24 foliose lichens were reported in Mt. Apo Natural Park, Davao del Sur, with Lobariaceae as the most species-rich family (Magday et al. 2020). Despite this, lichens, in general, are still considered understudied in many regions of the country (Paguirigan et al. 2020). For example, the Shilan Communal forest in La Trinidad, Benguet, a landlocked, mountainous municipality in the Cordillera Administrative Region, offers a unique environment for biodiversity studies. However, only one ethnobotanical survey on edible wild fruits has been conducted (Chua-Barcelo 2014), and currently, no studies have been conducted on the diversity of foliose lichens in the area.

In addition to the limited studies on lichen biodiversity, there is a lack of understanding regarding the bioactivity of the various secondary metabolites they produce. Even though there are at least 1,000 secondary metabolites found in lichens such as secondary aliphatic acids, esters, mononuclear phenolic compounds, depsides, tri-depsides, benzyl esters, depsidones, diphenyl esters, depsones, dibenzofurans, usnic acids, anthraquinone, xanthones, triterpenes, and terphenylquinones (Ranković & Kosanić 2019), several lichens species have not been studied for their secondary metabolite profile, specifically of their bioactivity.

One notable type of lichen, based on their growth, is foliose lichen. Foliose lichens are known for their leaf-like thallus and distinct upper and lower cortices (Shukla et al. 2014). According to several studies, they have been studied for their antifungal (Karabulut & Ozturk 2015, Millot et al. 2017), anti-inflammatory (Mendili et al. 2022), antibacterial, and antioxidant potential (Dwarakanath et al. 2022, Tartouga et al. 2022). Specifically, some foliose lichens, such as *Leptogium moluccanum* and *L. cochleatum*, exhibited an inhibitory activity against *Klebsiella pneumoniae*, *Enterobacter agglomerans*, and *Escherichia coli* (Manlapaz et al. 2022). In addition, *Parmotrema reticulatum*, another foliose lichen, inhibited the growth of *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Jain et al. 2016). Several other foliose lichens with antibacterial activity include *Parmotrema andium* (Sahoo et al. 2021), *Lobaria* sp. (Zheng et al. 2018), *Peltigera*, *Umbilicaria*, and *Xanthoparmelia* (Shrestha et al. 2014) to name a few. This is why foliose lichens are considered promising candidates for alternative sources of biologically active compounds to combat the growing threat of antimicrobial resistance (MDR).

The increasing antimicrobial resistance has been a growing threat to global public health (Salam et al. 2023). Antimicrobial resistance is a natural occurrence in the natural population of pathogens driven by genetic mutations and selective pressures. However, extensive application, as well as inappropriate usage of antibiotics, such as inadequate dosing, poor adherence to treatment protocol, and abuse of over-the-counter availability of medicines, has significantly increased the incidence of microbial resistance (Santajit & Indrawattana 2016). Treatment of infections has become more difficult with fewer treatment options available (Chinemerem-Nwobodo et al. 2022). In 2017, the World Health Organization (WHO) compiled a list of priority pathogens to emphasize the urgent need for research and development of novel antimicrobials. Among them are the ESKAPE pathogens comprising *E. faecalis*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. aerogenes*, which are recognized multidrug-resistant pathogens of clinical relevance worldwide (Idris & Nadzir 2023).

The discovery of alternative sources of bioactive compounds, such as those in foliose lichens, would expand the range of available treatment options against human pathogens that have developed resistance to standard medications (Ren et al. 2023). For this reason, the current study

aims to document and identify foliose lichens in the Shilan Communal Forest in La Trinidad, Benguet, Philippines to address research gaps in Philippine lichenology. Additionally, it seeks to assess the antibacterial activity of *Parmotrema neopustulatum* to explore alternative solutions to the growing issue of multidrug-resistant pathogens that threaten public health.

Materials & Methods

Collection of Lichen Samples

The study was conducted in one of the communal forests of Shilan, La Trinidad, Benguet, Philippines. We submitted a formal request letter to the Barangay Captain to secure authorization to conduct research within the barangay's boundaries. Additionally, permits from the Department of Environment and Natural Resources (DENR) were also secured to ensure compliance with local laws and environmental regulations during the study.

We comprehensively surveyed the study area with the assistance of a local guide. Each collected lichen was photographed in its natural habitat, while initial identification was conducted based on their observed morphological characteristics. Coordinates of the collected specimens were recorded using a Garmin hand-held GPS.

The specimen collection followed the standard protocol for field collection outlined by Nayaka (2014). Lichens were carefully removed from their substrates with a knife, including the thallus' center and margin. When present, fruiting bodies, such as apothecia and perithecia, were also included in the collection (Ohmura 2014). At least two thalli per lichen samples were collected for the subsequent morpho-anatomical and chemical examination, antibacterial assay, and vouchering. The collected samples were eventually air-dried to prevent deterioration and maintain their quality for further examination and/or preservation.

Identification of Lichen Samples

We identified foliose lichens according to their morphological, anatomical, and chemical characteristics with the aid of previously reported lists and taxonomic keys (Sipman 1998, 2005, Bawingan et al. 2017, 2019, Paguirigan et al. 2020).

Specifically, morphological structures of foliose lichens, including thalli, cilia, rhizines, and pseudocyphellae, are noted. In addition, the anatomy of lichen thallus and its reproductive structures, known as fruiting bodies or ascocarps, were examined under a compound microscope. A color spot test was also performed to classify lichen species according to the reaction of lichen substances to certain chemicals (Paguirigan et al. 2019) in which a color change is observed for specific reagents applied. The reagents used for color spot tests include an aqueous solution of potassium hydroxide, bleaching powder or aqueous solution of calcium hypochlorite, and aqueous solution of paraphenylenediamine. Color changes produced from the specific spot tests were recorded, wherein a positive color change was recorded with a positive (+) sign along with the color produced. Meanwhile, no color change was recorded with a negative (-) sign (Rashmi & Rajkumar 2014).

Extraction of *Parmotrema neopustulatum*

The most predominant foliose lichen species, *P. neopustulatum*, was cleaned by removing the debris and the substrate from the lichen. The lichens were placed in a glass bottle and suspended in ethyl acetate at room temperature for 24 hours. The supernatant was dried using a rotary evaporator and reconstituted with 4 ml methanol. The extract was transferred to a pre-weighed vial using a 0.2 µm filter syringe. After drying, methanol was added to arrive at a final concentration of 100mg/ml.

Paper Disk Diffusion Assay

The paper disk diffusion assay was performed to determine the effectiveness of the specific foliose lichen extracts against the test bacteria. Pure cultures of *E. faecalis*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *E. aerogenes* were taken from the Mycology

Laboratory of the University of Santo Tomas. They were inoculated into 5 ml 0.85% Normal Saline Solution. The bacterial suspensions were adjusted to 0.5 McFarland Standard (equivalent to 1.5×10^8 CFU/ml) following the preparation of Aryal et al. (2021). The bacterial culture was swabbed aseptically onto culture plates pre-filled with 2 ml 4-mm deep Mueller Hinton Agar (MHA). Sterile paper discs (6 mm in diameter) in triplicates were impregnated with 40 μ l of 100 mg/ml crude extract. We dried the treated disks at room temperature for 10–15 minutes and placed them on each swabbed MHA. The positive controls used were tetracycline and gentamicin, while methanol was the negative control. All culture plates were incubated for 24 hours at 37 °C, after which the zone of inhibition (ZOI) was measured using a vernier caliper and assessed following Quinto & Santos (2005): (1) very active, > 19 mm ZOI, (2) active, 13–19 mm ZOI, (3) partially active, 10–12 mm ZOI, and (4) inactive, < 10 mm ZOI.

Thin-Layer Chromatography

The presence of different lichen metabolites was identified through thin-layer chromatography following the protocol suggested by Manlapaz et al. (2022). The lichen extract was first spotted on TLC plates and was run on (blank) as a solvent system. The spots were then observed under UV light at a wavelength of 365 nm and were sprayed with spraying reagents, such as antimony (III) chloride, potassium ferricyanide-ferric chloride, Dragendorff's reagent, Borntrager reagent, magnesium acetate in methanol, and Van-Urk-Salkowski test. The appearance of the TLC results was observed to characterize the lichen metabolites present in the extract.

Results

Nineteen foliose lichen species belonging to three families were collected in Shilan Communal Forest, La Trinidad, Benguet (Figure 1). The collected specimens were from the families Parmeliaceae, Peltigeraceae, and Physciaceae and were further classified under six genera, including *Bulbothrix*, *Crocodia*, *Heterodermia*, *Hypotrachyna*, *Relicina*, and *Parmotrema*. The three species not reported in the Philippines before are marked with asterisk (*). Lichen vouchers of the lichen samples were submitted to the UST Herbarium.

Parmeliaceae

Bulbothrix subdissecta (Nyl.) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'9"N, 120°37'6"E, 1670 masl; 16°27'3"N, 120°37'4" E, 1660 masl] on bark of *Pinus* sp.

Bulbothrix tabacina (Mont. & Bosch) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'3"N, 120°37'4"E, 1660 masl; 16°27'3"N, 120°37'4"E, 1660 masl; 16°27'6"N, 120°37'4"E, 1670 masl; 16°27'6"N, 120°37'5"E, 1670 masl; 16°27'7"N, 120°37'5"E, 1680 masl; 16°27'13"N, 120°37'4"E, 1660 masl.] on bark of *Pinus* sp.

Crocodia aurata (Ach.) Link – Shilan Communal Forest, La Trinidad, Benguet [16°27'14"N, 120°37'2"E, 1660 masl] on bark of *Pinus* sp.

Heterodermia diademata (Taylor) D.D. Awasthi – Shilan Communal Forest, La Trinidad, Benguet, [43° NE, 16°27'13"N, 120°37'4"E, 1660 masl] on bark of *Pinus* sp.

Heterodermia isidiophora (Nyl.) D.D. Awasthi – Shilan Communal Forest, La Trinidad, Benguet [16°27'6"N, 120°37'5"E, 1670 masl; 16°27'7"N, 120°37'5"E, 1680 masl] on the bark of *Pinus* sp.

**Hypotrachyna brevirhiza* (Kurok.) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'7"N, 120°37'5"E, 1670 masl; 16°27'13"N, 120°37'4"E, 1660 masl; 16°27'7"N, 120°37'5"E, 1670 masl] on bark of *Pinus* sp.

**Hypotrachyna microblasta* (Vain.) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'13"N 120°37'4"E, 1660 masl; 16°27'13"N, 120°37'4"E, 1660 masl] on bark of *Pinus* sp.

Hypotrachyna reducens (Nyl.) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'6"N, 120°37'4"E, 1670 masl; 16°27'6"N, 120°37'4"E, 1670 masl; 16°27'6"N, 120°37'5"E, 1680 masl; 16°27'7"N, 120°37'4"E, 1670 masl; 16°27'6"N, 120°37'5"E, 1680 masl; 16°27'7"N, 120°37'5"E, 1680 masl; 16°27'7"N, 120°37'5"E, 1670 masl; 16°27'13"N, 120°37'4"E, 1660 masl; 16°27'14"N, 120°37'2"E, 1650 masl] on bark of *Pinus* sp.

**Hypotrachyna pseudosinuosa* (Asahina) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'14"N, 120°37'2"E, 1660 masl; 16°27'9"N 120°37'6"E, 1670 masl] on bark of *Pinus* sp.

**Hypotrachyna rockii* (Zahlbr.) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'7"N, 120°37'5"E, 1670 masl] on bark of *Pinus* sp.

Parmotrema clavuliferum (Räsänen) Streimann – Shilan Communal Forest, La Trinidad, Benguet [16°27'7"N, 120°37'5"E, 1680 masl] on bark of *Pinus* sp.

Parmotrema cristiferum (Taylor) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'14"N, 120°37'0"E, 1610 masl] on bark of *Pinus* sp.

Parmotrema dilatatum (Vain.) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'3"N, 120°37'4"E, 1660 masl] on bark of *Pinus* sp.

Parmotrema neopustulatum Kurok. – Shilan Communal Forest, La Trinidad, Benguet [16°27'4 N, 120°37'3"E, 1660 masl; 16°27'7"N, 120°37'5"E, 1670 masl; 16°27'9"N, 120°37'6"E, 1670 masl; 16°27'9"N, 120°37'6"E, 1670 masl; 16°27'14"N, 120°37'1"E, 1650 masl; 16°27'14"N, 120°37'1"E, 1650 masl] on bark of *Pinus* sp.

Parmotrema reticulatum (Taylor) M. Choisy – Shilan Communal Forest, La Trinidad, Benguet [16°27'9"N, 120°37'5"E, 1680 masl; 16°27'13"N, 120°37'4"E, 1660 masl; 16°27'13"N, 120°37'4"E, 1660 masl; 16°27'14"N, 120°37'2"E, 1650 masl; 16°27'10"N 120°37'5"E, 1670 masl] on bark of *Pinus* sp.

Parmotrema saccatilobum (Taylor) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'7"N, 120°37'5"E, 1670 masl; 16°27'7"N, 120°37'5"E, 1670 masl; 16°27'8"N 120°37'5"E, 1680 masl; 16°27'13"N 120°37'4"E, 1660 masl] on bark of *Pinus* sp.

Parmotrema tinctorum (Despr. ex Nyl.) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'6N, 120°37'5"E, 1670 masl] on bark of *Pinus* sp.

Relicina planiuscula (Kurok.) Hale – Shilan Communal Forest, La Trinidad, Benguet [16°27'7"N, 120°37'5"E, 1660 masl] on bark of *Pinus* sp.

The lichen species collected in the communal forest of Shilan La Trinidad, Benguet, were identified as belonging to three families of lichens: Parmeliaceae, Peltigeraceae, and Physciaceae. In addition, the lichen samples were further classified under six different genera, including *Bulbothrix*, *Crocodia*, *Heterodermia*, *Hypotrachyna*, *Parmotrema*, and *Relicina* (Table 1).

Table 1. List of lichen genera with number of identified species (n = 19).

Genera	Species	Percentage	Genera	Species	Percentage
<i>Crocodia</i>	1	5%	<i>Bulbothrix</i>	2	11%
<i>Relicina</i>	1	5%	<i>Hypotrachyna</i>	5	26%
<i>Heterodermia</i>	2	11%	<i>Parmotrema</i>	8	42%

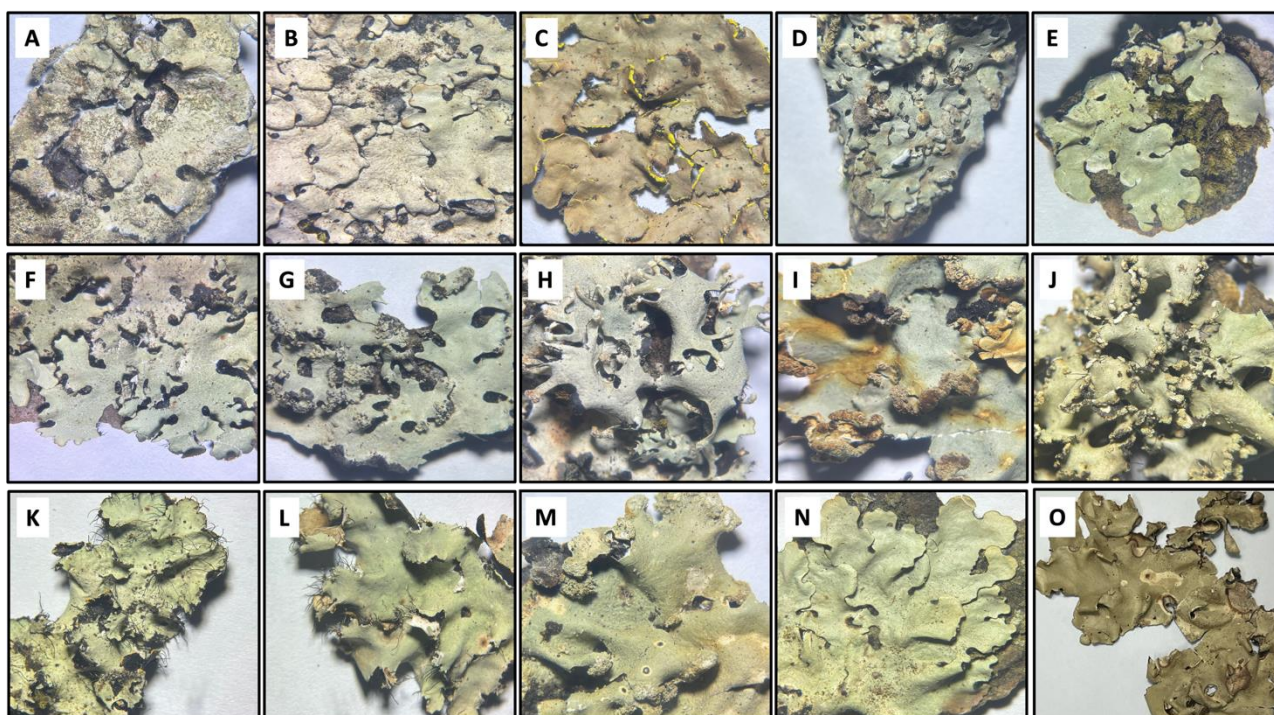


Fig. 1 – Lichens species found in Shilan forest community in La Trinidad, Benguet. A *Bulbothrix subdissecta*. B *Bulbothrix tabacina*. C *Crocodia aurata*. D *Hypotrachyna brevirhiza*. E *Hypotrachyna microblasta*, F *Hypotrachyna pseudosinuosa*. G *Hypotrachyna rockii* H. *Parmotrema clavuliferum*. I *Parmotrema cristiferum*. J *Parmotrema dilatatum*. K *Parmotrema neopustulatum*. L *Parmotrema parahypotropum*. M *Parmotrema reticulatum*. N *Parmotrema saccatilobum*. O *Parmotrema tinctorum*.

Paper Disk Diffusion Assay

The antibacterial activity of the crude extract of *Parmotrema neopustulatum* against ESKAPE was evaluated, and the zone of inhibition was noted. Specifically, *P. neopustulatum* crude extract exhibited very active inhibitory activity against *K. pneumoniae* with a ZOI of 20.80 mm. In addition, active inhibition was noted against *S. aureus* (18.27 mm ZOI), *A. baumannii* (16.87 mm ZOI), and *E. faecalis* (16.07 mm ZOI), while partial activity was observed against *E. aerogenes* (12.83 mm ZOI) (Table 2).

Table 2. Zones of inhibitions (mm) of *P. neopustulatum* crude extract and the controls - tetracycline, gentamicin, and methanol against the ESKAPE pathogens.

Bacterial Pathogens	Tetracycline (30 µg)	Gentamicin (10 µg)	<i>P. neopustulatum</i> (400 µg)	Methanol (20 µl)
<i>E. faecalis</i>	29.60 ± 0.85	22.00 ± 2.39	16.07 ± 1.87	0.00
<i>S. aureus</i>	21.63 ± 1.73	15.10 ± 5.11	18.27 ± 2.67	0.00
<i>K. pneumoniae</i>	23.90 ± 0.67	16.93 ± 2.51	20.80 ± 2.76	0.00
<i>A. baumannii</i>	28.00 ± 3.27	21.70 ± 3.97	16.87 ± 3.60	0.00
<i>P. aeruginosa</i>	16.03 ± 2.26	22.23 ± 1.91	0.00	0.00
<i>E. aerogenes</i>	22.93 ± 0.84	16.50 ± 1.43	12.83 ± 8.91	0.00

Thin-Layer Chromatography

To identify the metabolites present in *P. neopustulatum*, the crude extract was subjected to thin-layer chromatography analysis. The crude extract showed positive results for phenols, tannins, flavonoids, alkaloids, cardenolides, anthraquinones, sugars, and essential oils (Table 3).

Table 3. Thin Layer Chromatography of *Parmotrema neopustulatum*. The TLC plates display the distinct separation of compounds, with visible spots indicating different components based on their retention factors. Positive and negative results suggest the presence and absence of the other metabolites tested.

Spray reagent*	Positive result	<i>P. neopustulatum</i> crude extract	Compounds present
A	Yellow to orange; fluoresce under UV 365 nm	-	Flavonoids, steroids
B	Blue	+	Phenols, tannins, flavonoids
C	Brown-orange	+	Alkaloids
D	Blue to red-violet	+	Cardenolides
E	Orange-violet	+	Anthraquinones
F	Blue-violet	-	Indoles
G	Blue	+	Sugars
H	Wide range of colors	+	Essential oils

* A antimony(III) chloride. B potassium ferricyanide-ferric chloride. C Dragendorff's reagent. D Kedde reagent. E magnesium acetate in methanol. F Van-Urk Salkowski Test. G α -naphthol-sulfuric test, and H 5% sulfuric acid in ethanol.

Discussion

The collected foliose lichens in the communal forest of Shilan La Trinidad, Benguet, were identified as belonging to Parmeliaceae, Peltigeraceae, and Physciaceae. Foliose lichens generally have a leaf-like thallus typically adnate to the substratum, with laminal apothecia and pycnidia (González-Burgos et al. 2019).

Bulbothrix species generally are characterized by their bulbate cilia, which are either simple or branched. Their thallus is distinguished by their gray-green color, indicating the presence of atranorin. They bear rhizines as well as apothecia with discs. Morphologically, *B. subdissecta* differs from *B. tabacina* because it has more frequent isidia and more imbricate lobes (Bawingan et al. 2019). In Asia, *B. subdissecta* is only found in Malaysia and the Philippines. Records of *B. subdissecta* occurrences in the Philippines include Ilocos Norte and Quezon (Bawingan et al. 2019), while *B. tabacina* was reported in Cavite, Bukidnon, Negros Oriental, Baguio City, Ifugao, and Mt. Kabuyao in Benguet (Bawingan et al. 2019).

Another Parmeliaceae member with which *Bulbothrix* shares similar morpho-anatomic features is *Relicina*. Like *Bulbothrix*, *Relicina* is distinguished from other lichen species by its bulbate cilia, presence or absence of cortical maculae, isidia, soredia, and lobules. Both genera bear black, simple, sparsely, or densely branched rhizines and apothecia. However, the two differ in thalli color. The two genera also vary in chemical profile, ecological requirements, and geographic distribution (Bawingan et al. 2019). While both have centers of diversity in the tropics, unlike *Bulbothrix*, which thrives primarily in South America and Africa, as discussed above, *Relicina* species are predominantly found in Southeast Asia. *Relicina* is endemic in the Philippines. *R. planiuscula* that is collected in Shilan, La Trinidad, and Benguet, distinguished for their imbricate thallus and abundant isidia, which are either globose or cylindrical, also thrives in Mt. Ugo, Benguet, Ifugao, and Mountain Province (Bawingan et al. 2019). To date, there are about 17 species of *Relicina* reported for the Philippines (Paguirigan et al. 2020).

Hypotrachyna is morphologically characterized by the presence of pores on its epicortex, narrow, sublinear to linear elongate lobes with truncate apices, and dichotomously branched rhizines (Kirika et al. 2019). Diverse *Hypotrachyna* species are found chiefly in tropical montane forests, especially in moderate to high altitudes of tropical America (Divakar et al. 2006). However, there are about thirteen species already reported for the Philippines (Paguirigan et al. 2020). In this study, three species of *Hypotrachyna* were first reported in the Philippines, including *H. brevirhiza*, *H. microblasta*, and *H. rockii*. The identification of *H. microblasta* (Vain.) Hale was confirmed

through morphological features including small, globose to cylindrical isidia (20–40 µm diameter) densely covering the upper surface, lobe width of 2–4 mm with rounded apices, black simple to sparsely furcate rhizines, and spot test results (K⁺ yellow, P⁺ pale yellow) indicating atranorin and salazinic acid, consistent with descriptions by Hale (1975) and Sipman (1998). *Hypotrachyna rockii* was initially examined as *H. taylorensis* but subsequently re-identified as *H. rockii* (Zahlbr.) Hale following additional chemical testing with calcium hypochlorite, which yielded a diagnostic C⁺ rose reaction indicative of gyrophoric acid. This chemical distinction is taxonomically significant, as *H. rockii* produces gyrophoric acid whereas *H. taylorensis* does not (Hale 1975). The collected *H. breviphiza* species can be distinguished from the other *Hypotrachyna* species by its distinct soralia, often capitate, sometimes finely pustular, and generally terminal or subterminal on lobules (Sipman 1998). *Hypotrachyna rockii* also has distinct soralia, but unlike *H. breviphiza*, its clusters of soredia are pustulate and terminal on lobules, and its lobes are less imbricate (Sipman 1998). According to the Lichen Consortium, Herbaria, *H. breviphiza*, and *H. microblasta* are prominently occurring in tropical regions, particularly at the borders of South America and the continents Africa and Oceania. Notably, *H. microblasta* is also found in Asia, particularly in Brunei. The species have no occurrences recorded yet in the Philippines, but their evident distribution in tropical continents makes them more likely to thrive in the country (Jayala et al. 2013). In contrast to these new records of *Hypotrachyna* species of Philippines, *H. pseudosinuosa* is observed in Asian countries like Taiwan, Japan, South-East Asia and Papua New Guinea. *Hypotrachyna pseudosinuosa* (Asahina) Hale was initially tentatively identified as *H. sinuosa* but was subsequently corrected based on broader thalli (6–8 mm wide lobes vs. 3–5 mm in *H. sinuosa*), more densely reticulate maculae patterns, and chemical spot test results (K⁺ yellow, P⁺ orange-yellow) confirming the presence of atranorin and salazinic acid (Hale 1975, Sipman 1998).

Several *Parmotrema* species were also collected in Shilan La Trinidad, Benguet. Generally, this genus is characterized by its broad, rotund lobe apices, absence of pseudocyphellae, presence of isidia or soredia, marginal cilia, simple or dimorphous rhizines, and broad erhizinate or papillate marginal zone on their lower cortex. Its cortical spot test results and chemistry usually indicate the presence of atranorin, chloroatranorin, usnic acid, and lichexanthone (Louwhoff & Elix 1999, Bawingan et al. 2017). *Parmotrema* species have a wide geographic distribution, but its center of diversity is in the Neotropics—the tropical and subtropical regions. *Parmotrema clavuliferum* has multiple occurrences across Asia, especially in Japan, Taiwan, Thailand, Malaysia, Nepal, and the Philippines. It has been reported in Benguet, specifically in Tuba, Itogon, Atok, Mt. Santo Tomas near Baguio, and Puguis (Bawingan et al. 2017), which are close to the communal forest of Shilan La Trinidad, Benguet. *Parmotrema cristiferum*, *P. dilatatum*, *P. neopustulatum*, *P. parahypotropum*, *P. reticulatum*, and *P. saccatilobum* has also been previously reported in several provinces in the Philippines, including in Benguet and/or nearby provinces (Bawingan et al. 2017). Chemical spot tests of *P. tinctorum* specimens yielded K⁺ yellow→red and P⁺ orange-red reactions, confirming the presence of salazinic acid, consistent with the chemotype documented by Bawingan et al. (2017).

Meanwhile, *Crocodia* is part of the Peltigeraceae family that features large, irregularly spreading, lobed thalli. The upper surface can be smooth, wrinkled, or ridged, while the lower surface may be corticate or non-corticate with cyphellae or pseudocyphellae. Their photobionts are typically green algae or cyanobacteria, sometimes within cephalodia. Reproductive structures like soredia or isidia may also occur within this family (Cannon et al. 2021). The genus *Crocodia* is known for its distinct yellow pseudocyphellae, which are present as punctiform or effigurate breaks in the lower cortex. *Crocodia aurata* has a wide distribution observed in numerous countries on different continents, including the Asia-Pacific region, and has been reported specifically in Japan, South Korea, Papua New Guinea, and the Philippines (Paguirigan et al. 2020).

Lastly, the *Heterodermia* genus, which belongs to the family Physciaceae, has variable thallus morphology, ranging from foliose to crustose forms, and it has a consistent association with *Trebouxia* photobionts. Specifically, *H. diademata* is distinguished by the presence of marginal soredia, pseudocyphellae, and laminar rhizines. *Heterodermia* species are primarily found in

tropical regions, and *H. diademata* is significantly concentrated in Mexico, South Korea, and South America. According to the Consortium of Lichen Herbaria, in the Philippines, this species has reported occurrences in Benguet, Bontoc, Bukidnon, and Cotabato.

The identification of *P. neopustulatum* was confirmed through comprehensive morpho-chemical analysis. The specimens exhibit conspicuous, well-developed pustules (0.5-2 mm diameter) on the upper surface, broad lobes (8-15 mm wide), and chemical spot test results (K+ yellow→red, C+ rose, P+ orange-red) indicating salazinic acid with trace amounts of gyrophoric acid. These characteristics align with the chemotype reported for *P. neopustulatum* by Kurokawa (1991) and confirmed by Bawingan et al. (2017). The well-developed pustular morphology distinguishes this species from morphologically similar taxa and is consistent with specimens from the species' known Southeast Asian distribution range (Bawingan et al. 2017)

Parmotrema neopustulatum crude extract exhibited very active inhibitory activity against *K. pneumoniae*, an active inhibition against *S. aureus*, *A. baumannii*, and *E. faecalis*, and a partial activity against *E. aerogenes*. The susceptibility and resistance of the lichen extract to the bacterial specimens may be attributed to several factors, including the test bacteria's structural properties and virulence factors. For instance, *E. faecalis* and *S. aureus* are Gram-positive bacterial species characterized by the absence of their outer membrane, which could have allowed the antibacterial substance from the extract to penetrate through the bacteria, thereby inhibiting it. *K. pneumoniae*, on the other hand, although classified as a Gram-negative bacteria possessing an outer membrane barrier, exhibited the highest inhibition from the crude extract. This could be due to the higher affinity and potency of *Parmotrema*'s metabolites to *K. pneumoniae*'s virulence factors, such as its capsular polysaccharide, the primary determinant of its virulence. Additionally, although studies about *P. neopustulatum* are limited, several *Parmotrema* studies proved how foliose lichens exhibit antimicrobial efficacy through their unique secondary metabolites (Jain et al. 2016). The wide-ranging effectiveness of the crude extract, especially against multidrug-resistant organisms like *A. baumannii* and *K. pneumoniae*, highlights its promise as a candidate for the development of antimicrobial agents.

The metabolites present in *Parmotrema neopustulatum*, such as tannins, alkaloids, flavonoids, cardenolides, and sugars, are also present in other *Parmotrema* species (Rashmi & Rajkumar 2014). In addition, phenols, anthraquinones, and essential oils were also detected in *P. neopustulatum*. Specifically, the color spot tests revealed that the phenolic compounds in *P. neopustulatum* include fumarprotocetraric, protocetraric, or succinprotocetraric acid, which are categorized under depsidones. Some other depsidones reported for *Parmotrema* species, such as *P. cristiferum* include parmoferone, parmosidone K, albifolione, and 4-chloroorcinol (Duong et al. 2024). Naturally occurring depsidones have been reported to possess diverse structures and bioactivity, including antimicrobial activities (Khayat et al. 2023). In the study of Vivek et al. (2014), methanolic extract of *Parmotrema* species containing depsidones showed dose-dependent antibacterial activity and was observed to be more gram-positive bacteria than gram-negative bacteria. The occurrence of depsidones in *P. neopustulatum* reinforces the extensive potential of lichen species in biomedical research, as exhibited in the anti-tumor, anti-inflammatory, bactericidal, antiseptic, antioxidant, anti-aging, skin regenerating, diuretic, anti-allergic, and antimicrobial properties of the identified secondary metabolites (Goga et al. 2018, Manivannan & Johnson 2020, Elkhateeb et al. 2022, Martucciello et al. 2022, Adu-Amankwaah et al. 2023).

Several lichen genera from the phyla Ascomycota and Basidiomycota have been used in traditional cultures locally and worldwide (Crawford 2015, Azuelo & Puno 2018), generally attributed to the secondary metabolites that not only help defend themselves from external predators and extreme environmental conditions but also enable them to perform biological activities. Additionally, there are at least 1000 metabolites specific to lichens that have been shown to possess anticancer, antibacterial, anti-inflammatory, and antifungal properties (Goga et al. 2018, Sutar et al. 2021), supporting their long-standing application in traditional medicine; however, only 39% of lichen species have traditional uses with reported biological activity and 12% of traditionally important lichens have yet to be investigated for their biological activities (Sutar et al.

2021). Much of the lichen's secondary metabolites are deposited in the cortex or the medulla (Ranković & Kosanić 2019), often in a crystallized form. These crystals are confirmed to be very stable when formed, in addition to a no significant decrease in secondary metabolite concentration in voucher specimens. Furthermore, environmental and physiological factors also affect secondary metabolite production, including light, UV exposure, elevation, temperature, the age of lichens, and the location of secondary metabolites in the thallus (Goga et al. 2018).

Conclusions

Among the foliose lichens collected in Shilan Communal Forest, La Trinidad, Benguet, 19 species from six genera were identified, with three species from the genus *Hypotrachyna* being newly recorded in the Philippines. The most abundant species by weight, *Parmotrema neopustulatum*, was extracted using ethyl acetate, and its antibacterial activity was evaluated against ESKAPE pathogens using the paper disk diffusion assay. The crude extract demonstrated favorable susceptibility against *Enterococcus faecalis*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* but showed resistance to *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter aerogenes*. Thin-layer chromatography (TLC) and chemical spot tests confirmed the presence of secondary metabolites, specifically phenols, tannins, flavonoids, alkaloids, cardenolides, anthraquinones, sugars, and essential oils, to which the *P. neopustulatum* extract's antibacterial activity could be attributed. With the rise of antimicrobial resistance, these findings highlight the potential of lichen-derived secondary metabolites as promising candidates for future antibiotic research.

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Accessibility of data

Voucher specimens of the identified lichen species were deposited at the University of Santo Tomas Herbarium in Manila, Philippines.

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