Antibacterial activities of the lichen *Ramalina* and *Usnea* collected from Mt. Banoi, Batangas and Dahilayan, Bukidnon, against multi-drug resistant (MDR) bacteria

LAWRENCE P. TIMBREZA
JHORELLE L. DELOS REYES
CHARLES HENRY C. FLORES
ROBERT JOHN LOUIE A. PEREZ
MARIA AGNES S. STOCKEL
KRYSTLE ANGELIQUE A. SANTIAGO
Department of Biological Sciences
Institute of Arts and Sciences
Far Eastern University
Sampaloc Manila, Philippines
Email: lawrence.trimbreza@gmail.com

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Key words: bioactive secondary metabolites, lichen acids, paper disk diffusion assay, thin layer chromatography, TLC-bioautography.

Abstract: The present generation is at the start of the "Post-Antibiotic Era", wherein even the most common bacteria are resistant to a broad spectrum of antibiotics. Because of the rapid mutations of bacterial strains against antibiotics, scientists are immensely looking for novel bioactive compounds that can inhibit the growth of multi-drug resistant (MDR) strains. We aimed to determine the efficacy of the extracts from the fruticose lichen genera *Ramalina* and *Usnea* from the Philippines in inhibiting the growth of selected MDR bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, Methicillin-resistant *S. aureus*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa.* Results show that both MRSA and *S. pneumoniae* were inhibited as shown in their measured zone of inhibitions (ZOI) of 18mm and 18mm, respectively. The lichen crude extracts exhibited promising in vitro activities against the selected bacteria, since the ZOI are significantly higher than that of the positive control Vancomycin (30mg/ml, mean difference=7 mm, sig. α=.05). Interestingly, potential bioactive metabolites such as usnic acid and related compounds, depsides, depsidones, depsones, dibenzofurans, chromanones, monocyclic phenols, xanthones, naphtaquinones, anthraquinones and pulvic acid derivatives were detected by TLC-bioautography. Lichens, therefore, provide wide spectrum of opportunities for the discovery of potential antimicrobial agents.

Zusammenfassung: Die heutige Generation ist am Beginn der "post-antibiotischen Ära", wobei auch die häufigsten Bakterien gegen ein breites Spektrum an Antibiotika resistent sind. Aufgrund der schnellen Mutationen von Bakterienstämmen gegen Antibiotika suchen Wissenschaftler verstärkt nach neuartigen bioaktiven Verbindungen, die das Wachstum von multiresistenten (MDR)-Stämmen hemmen können. Wir wollten die Wirksamkeit der Extrakte aus den fruticosen Flechtengattungen Ramalina und Usnea von den Philippinen bei der Hemmung des Wachstums ausgewählter MDR-Bakterien bestimmen: *Bacillus subtilis, Staphylococcus aureus,* Methicillin-resistente *S. aureus* (MRSA), *Streptococcus pneumoniae* und *Pseudomonas aeruginosa*. Wie aus den gemessenen Hemmzonen (ZOI) von

18 mm bzw. 18 mm hervorgeht, wurden sowohl MRSA als auch *S. pneumoniae* gehemmt. Die Flechten-Rohextrakte zeigten vielversprechende in vitro-Aktivitäten gegen die ausgewählten Bakterien, da die ZOI signifikant höher sind als die der positiven Kontrolle Vancomycin (30 mg/ml, mittlere Differenz = 7 mm, sig. α = 0,05). Interessanterweise wurden potenzielle bioaktive Metabolite wie Usninsäure und verwandte Verbindungen, Depside, Depsidone, Depsone, Dibenzofurane, Chromanone, monocyclische Phenole, Xanthone, Naphtaquinone, Anthrachinone und Pulvinsäurederivate durch TLC-Bioautographie nach-gewiesen. Flechten bieten daher ein breites Spektrum an Möglichkeiten für die Entdeckung potentieller antimikrobieller Agenzien.

The antibiotic resistance of bacterial pathogens imposes an increasing serious threat to global public health that requires urgent action (KOSANIĆ & RANKOVIĆ 2015). As the relentless mutational mechanisms of bacterial resistance continue to transcend, other bacteria re-emerge as one of the propagator of resistance to multiple antimicrobial agents (NEONAKIS & al. 2011, WHITNEY & al. 2004). Among the most prevalent ones are the methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, β-lactamase-producing *Enterobacteriaceae* and multidrug-resistant Gram-negative bacteria (i.e. *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Klebsiella pneumoniae* and *Burkholderia cepacia*) (HAWKEY & JONES 2009). Thus, harmonized solutions are compulsory in order to keep up with the pace of bacterial resistance (HAWKEY & JONES 2009). As of today, the problem of increasing antimicrobial resistance is even more alarming, considering the very limited number of new developing antimicrobial agents (BOUCHER & al. 2009, ECDC 2009).

In the Philippines, the Antimicrobial Resistance Surveillance Program 2012 Report recommended that increasing rates of multidrug-resistant (MDR) and possible extensively drug resistant (XDR) organisms in the hospital should signal a review of infection control procedures and its implementation. The said program is composed of an Antimicrobial Resistance Surveillance Reference Laboratory (ARSRL) at the Research Institute for Tropical Medicine (RITM) and 22 active sentinel sites in tertiary or regional hospitals in the country (GLOOR 2014). Interestingly, a high percentage of hospital-acquired infections in the Philippines are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or multidrug-resistant Gram-negative bacteria. For example, people infected with MRSA, an etiologic agent of severe infections in the community and in hospitals, are likely to die by 64 % higher than those of non-resistant (GLOOR 2014). With the advent of such multidrug-resistant bacteria, the Infectious Disease Society of America reported that there are over 80000 MRSA related infections, 2.6 million cases of *Streptococcus* illnesses, and 51000 *Pseudomonas* infections annually (CDC 2013).

To find a possible solution to the rising of antibiotic-resistant bacteria, researchers have focused into the discovery of new classes of antimicrobial agents found in plants (RECIO & RIOS 1989, SILVER & BOSTIAN 1993, BUTLER & BUSS 2006). Plants contain a wide array of organic compounds that can be differentiated into primary and secondary metabolites; these metabolites can be discerned from one another but can also set boundaries in distinguishing the two (DEWICK 2002). However, despite the enormous therapeutic potentials of plants, other organisms are also tapped for drug discovery. For example, lichens also contain bioactive molecules with known therapeutic properties (SHUKLA & al. 2010, VALLE & al. 2015). The biological activities exhibited by lichens can be attributed to their secondary metabolites, which are exclusively produced by the mycobiont and are secreted onto the lichen thallus either in liquid or crystal forms (LAWREY 1986, BINGOL & al. 2009).

Previous studies showed that the fruticose lichen *Ramalina* and *Usnea* are potential agents against Gram-positive bacteria; thus, making these lichens a prolific source of bioactive secondary metabolites (ESIMONE & ADIKWN 1999, PERRY & al. 1999, WECKESSER 2007, KARAGÖZ & al. 2009, PRATEEKSHA & al. 2016). Additionally, lichens in the Philippines have also shown promising antibacterial results (QUISUMBING 1951, SANTIAGO & al. 2010, 2013). These studies are valuable resources for local medical scientists who seek to explore and substantiate the antibacterial activities of the two fruticose lichens (*Ramalina* and *Usnea*) commonly found in the Philippines, particularly against MDR bacteria (NASCIMENTO & al. 2000). However, despite of their abundance and unique potential as sources of novel drugs, the biological activities of the Philippine lichens remain less studied (SANTIAGO & al. 2010). Hence, this study assessed the presence of the lichen *Ramalina* and *Usnea* from Mt. Banoi, Batangas and Dahilayan, Bukidnon and the efficacy of the lichen extracts against the increasing prevalence of selected MDR strains.

Materials and methods

Study sites and the collected *Ramalina* and *Usnea*: The fruticose lichens *Ramalina* and *Usnea* were collected from Mt. Banoi in Batangas City, Luzon Island (13° 41’ 49.2” N, 121° 09’ 53.9” E) and Barangay Dahilayan, Manolo Fortich in Bukidnon, Mindanao Island (8° 12’ 32” N, 124° 51’ 34” E), respectively. The collected specimens were manually cleaned by removing the debris, and placed separately in sterile brown paper bags. These were set aside in cabinets for future use. Furthermore, herbarium materials of the collected specimens were prepared and kept at the Department of Biological Sciences Herbarium at Far Eastern University. These lichen specimens were then identified based on SWINSCOW & KROG (1975), CLERC (1998), HERRERA-CAMPOS & al. (1998), MCCUNE (2005), APTROOT & SCHUMM (2008), RANDLANE & al. (2009), OHMURA & al. (2010), OHMURA (2012), TRUONG & CLERC (2012), TRUONG & al. (2013), SHUKLA & al. (2014).

Thalline spot test: Thalline spot test is considered as a standard procedure in lichen taxonomy, although such biochemical test has yet to be proven accurate in lichen identification (Swinscow & Krog 1975). However, its utilization remains useful to identify species based on their medullary reactions (TRUONG & CLERC 2012). Thalline spot test was done following the protocol of Santiago & al. (2010). Three tests, namely K test, C test and KC test were performed. Initially, a portion of the thallus was scraped off using a razor blade, exposing its cortex, medulla and/or central cord. Using the two chemical reagents, potassium hydroxide (KOH) and sodium hypochlorite (NaOCl), additional information regarding the lichen identity were supplemented. All tests were done on different parts of the lichen thalli. For K test, a drop of KOH was spotted to the exposed portion of the lichen specimen. Similarly, a drop of NaOCl was spotted for C test. Finally, a drop of KOH and NaOCl were spotted simultaneously to another exposed portion of the specimen. Any change in color signifies a positive result. The color change in the cortex, medulla and/or central cord was recorded (NASH 2008).

Extraction of the obtained lichen specimens: Following the protocol of SANTIAGO & al. (2010), representatives of the lichen specimens were initially air-dried for 24 hours. After air drying, specimens were cut into small pieces and were pulverized using mortar and pestle to yield approximately one gram (g) of the powdered lichen specimen. Then, the powdered specimens were transferred into a screw-capped test tube (Pyrex, 150 × 16 mm) and were soaked into 10 ml laboratory grade acetone for 24 h. After soaking, extracts were filtered using a funnel and filter paper and were stored into pre-weighed amber stock bottles. The lichen crude extracts were concentrated by air-drying until the solvent has evaporated. Moreover, crystal-like substances (which are considered as the lichen substances) were observed and weighed. Furthermore, percentage yield were calculated using the formula:

$$\%\text{yield extract} = \text{WCE}/\text{WLT}$$

where WCE is the weight of the crude extract and WLT is the weight of the lichen thalli.

Finally, acetone was added onto each bottle containing the lichen substances until it reaches a final concentration of 10 mg/ml. All bottles were covered with cheesecloth and stored in the refrigerator until further use.

Paper disk diffusion assay: Test bacteria. The strains of *B. subtilis*, *S. aureus*, Methicillin-resistant *S. aureus* (MRSA), multidrug-resistant *S. pneumoniae* (MDRSP), and multidrug-resistant *P. aeruginosa* (MDRPA) were purchased from the Research Institute for Tropical Medicine (RITM), Alabang, Muntinlupa. All test bacteria were cultured on blood agar (Becton Dickinson, BA).

Preparation of Inocula. Each bacterium was initially streaked on a BA plate, and incubated at 37° C for 18–24 h. Following incubation, each 24-h-old bacterium was inoculated in Normal Saline Solution (NSS) and was adjusted into 0.5 McFarland standard. Mueller Hinton Agar (Becton Dickinson, MHA) was used to promote the growth of MRSA and MDR *P. aeruginosa*. Meanwhile, MHA with 5 % sheep blood was used to promote the growth of MDR *S. pneumoniae*.

Antimicrobial agents. Antibacterial drugs such as Tetracycline (BBL, 30 mg/ml), Vancomycin (Oxoid, 30 mg/ml), and Gentamicin (Oxoid, 30 mg/ml) were purchased from RITM, Alabang, Muntinlupa. All antibiotics served as positive control in paper disk diffusion assay and tube dilution assay.

Paper disk diffusion assay against Gram-positive bacteria. Selected lichen crude extracts were tested against the standardized *B. subtilis* and *S. aureus*. The standardized test organisms were swabbed on solidified MHA plates. Then, sterile blank paper disks were dipped into the lichen crude extracts using an incinerated forceps. Following incineration, the disks were air-dried for approximately 10–15 s or until the solvent has evaporated. These disks were carefully impregnated onto the agar. Tetracycline and acetone served as positive and negative controls, respectively. Triplicates were made for each extract. On the other hand, media without bacteria (called as check plates) were also made to ensure the purity of the cultures that are being maintained over a long period of time. All plates were incubated at 37 °C for 18–24 h. After the incubation period, ZOI were measured using Vernier caliper (mm). Statistical analysis was conducted using one-way analysis of variance (ANOVA) to determine the significant difference between variables using post host test via Duncan multiple range test (DMRT) at α=0.05 level of confidence.

Paper disk diffusion assay against MDR bacteria. All extracts showing big zones of inhibition were then chosen and tested against Methicillin-resistant *S. aureus* (MRSA), *S. pneumoniae* and *P. aeruginosa*. The standardized MRSA and *P. aeruginosa* were initially swabbed on solidified MHA plates. On the other hand, the standardized *S. pneumoniae* was swabbed on another set of MHA plates containing 5 % sheep's blood to promote the growth of the said bacterium. Then, sterile blank paper disk was dipped into the lichen crude extract using an incinerated forceps. The disk was then air-dried for approximately 10–15 s or until the solvent has evaporated. As positive control, vancomycin and gentamicin disks were impregnated onto the inoculated MHA plates of MRSA and *P. aeruginosa* respectively. Similarly, vancomycin disk was also impregnated onto the MHA plates with 5 % sheep's blood. For the negative control, acetone was used. All plates were done in triplicates. Also, check plates were made to ensure the purity of the cultures that are being maintained over a long period of time. MHA plates were incubated at 37 °C for 18–24 h, while MHA plates supplemented with 5% sheep's blood were incubated at 35 °C with 4.9 % carbon dioxide concentration for 18–24 h. Following the incubation period, ZOI were measured using Vernier caliper (mm). Statistical analysis was conducted using one-way analysis of variance (ANOVA) to determine the significant difference between variables using post host test via Duncan multiple range test (DMRT) at α=0.05 level of confidence.

Tube dilution assay: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the lichen crude extracts were analyzed via tube dilution assay with two-fold dilution (QUINTO & SANTOS 2005). All extracts exhibiting extensive ZOI against MRSA and *S. pneumoniae* were used and tested. Initially, the first tube contained 1 ml (10 mg/ml) of pure lichen extract. The second up to the eighth tube were utilized to perform a serial two-fold dilution, each containing Brain-Heart Infusion broth (Fluka, BHI) as diluent. The ninth tube contained uninoculated BHI broth and served as the negative control. Moreover, the 10th tube contained 1 ml (10 mg/ml) of vancomycin and served as the positive control. Then, tubes of MRSA were incubated at 37 °C for 18–24 h. Tubes of *S. pneumoniae*, on the other hand, were incubated at 35 °C with 4.9 % carbon dioxide

concentration for 18–24 h. Computation of MIC was based on the BHI broth assay tube with the least concentration of lichen extracts that showed absence of growth or turbidity. As positive control, MIC test strip evaluation for Vancomycin (256 µg/ml, Thermoscientific) was used. To determine the MBC, the tube showing the least MIC was streaked on MHA plates and were incubated at 37 °C for 18–24 h, while MHA plates with 5 % sheep's blood were incubated at 35 °C with 4.9 % carbon dioxide concentration for 18–24 h. After the incubation period, the presence (with growth) or absence (without growth) of the representative bacterium on these plates were recorded. The computation of MBC was based on the least concentration of crude extracts in the BHI broth assay tube that exhibited no growth or colony formation on the MHA plates.

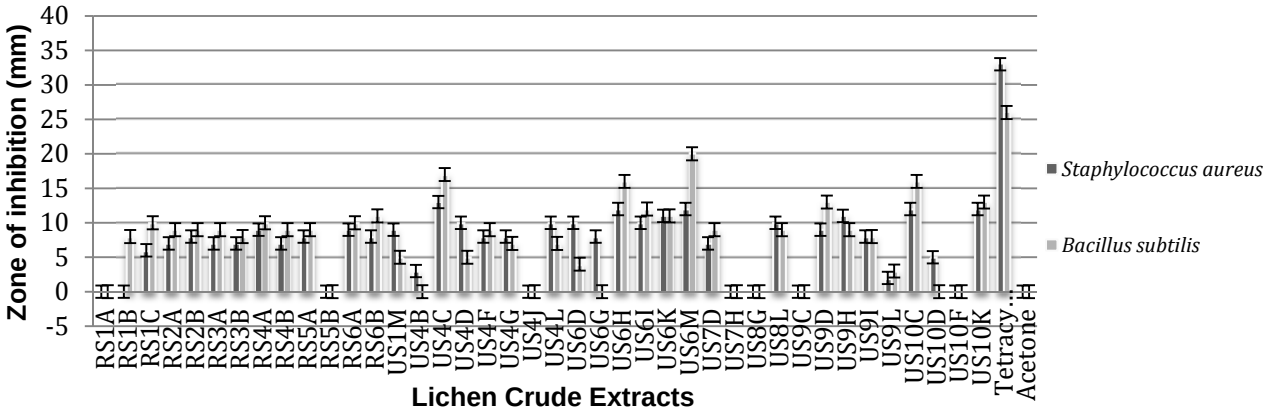


Fig.1. Bioactivities of the lichen crude extracts of *Ramalina* spp. and *Usnea* spp. against Gram-positive bacteria. Bioactivities: very active (>19 mm zone of inhibition), active (13-19 mm zone of inhibition), partially active (10-12 mm zone of inhibition); and inactive (<10 mm zone of inhibition).

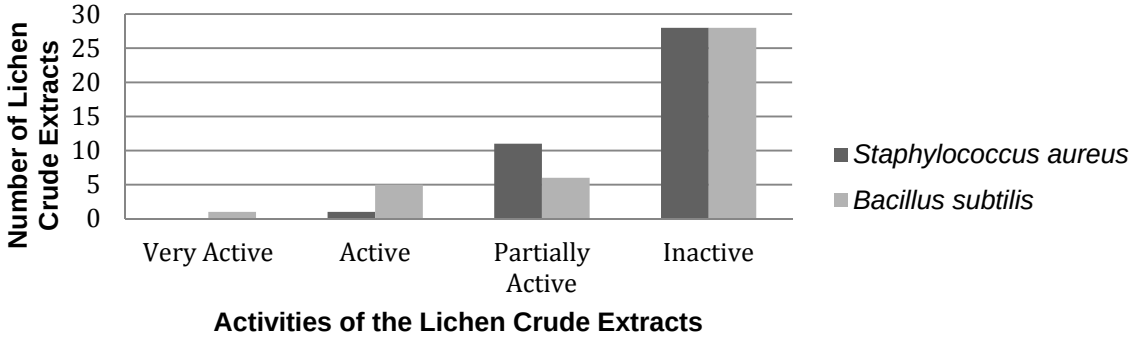


Fig. 2. Zones of inhibition of the 40 lichen extracts tested against Gram-positive bacteria *S. aureus* and *B. subtilis*. Positive control: Tetracycline (30mg/ml). Determinations determined in triplicate. Standard deviations are indicated above each bar ($p<0.05$).

Identification of bioactive lichen acids using TLC and TLC-bioautography: Thin layer chromatography (TLC). Crude extracts of the lichen specimens that showed extensive ZOI from the MDR test were spotted on TLC plates (Silica Gel Matrix, Sigma-aldrich). The plates were initiated in a series of solvent systems: solvent system A (36:9:1 toluene/dioxane/glacial acetic acid), solvent system C (20:30 toluene/glacial acetic acid) and solvent system G (139:83:8 toluene/ethyl acetate/formic acid) (CULBERSON 1972). Then, each TLC plate was sprayed with 97 % sulphuric acid and heated at 110 °C for 10 min following the protocol of SANTOS & MONDRAGON (1969). Similarly, spots were observed by exposing under ultraviolet light (~254 mm). Finally, the retention flow (Rf) values for each spot was determined and compared with the Rf values of known lichen acid standards for its identification (CULBERSON 1972, CULBERSON & JOHNSON 1982).

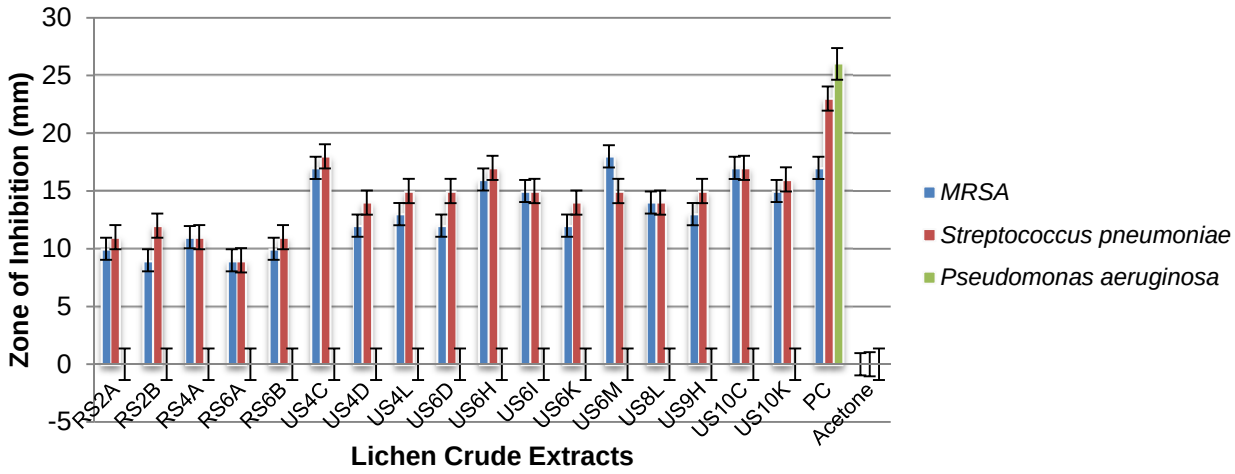


Fig. 3. Zones of inhibition exhibited by the 17 lichen crude extracts against selected multidrug-resistant bacteria. Positive control: Vancomycin (30mg/ml) and Gentamicin (30mg/ml). Determinations determined in triplicates. Standard deviations are indicated above each bar ($p<0.05$).

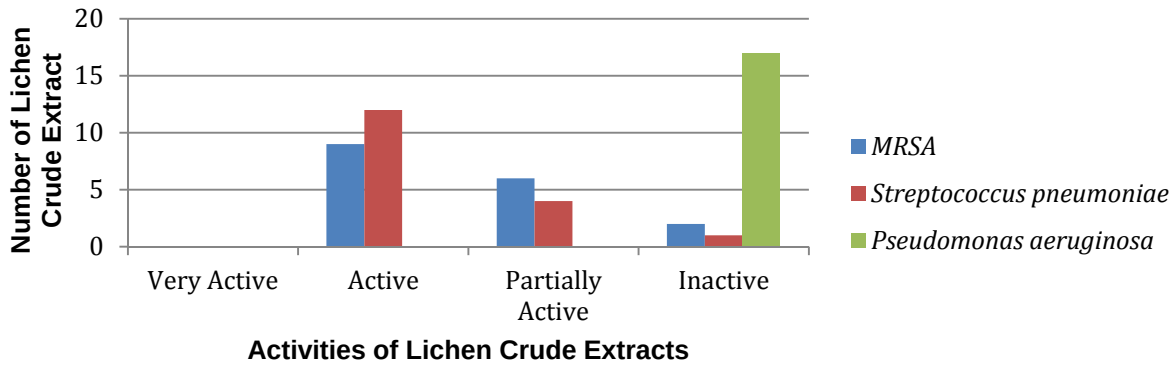
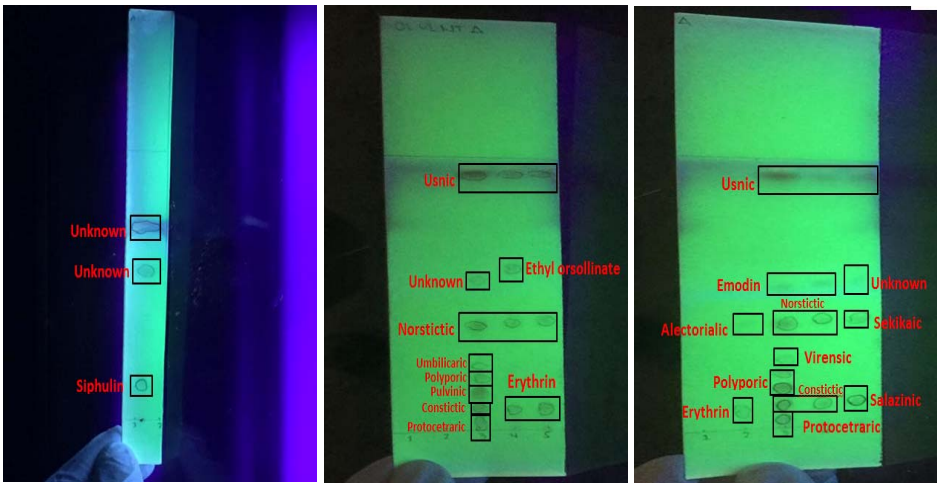
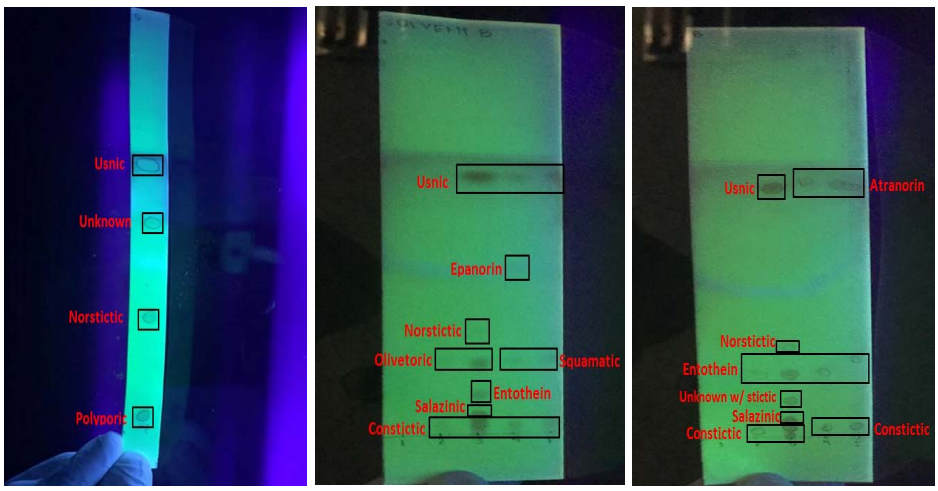


Fig. 4. Bioactivities of the lichen crude extracts of *Ramalina spp.* and *Usnea spp.* against the selected multidrug-resistant bacteria. Bioactivities: very active (>19 mm zone of inhibition), active (13-19 mm zone of inhibition), partially active (10-12 mm zone of inhibition); and inactive (<10 mm zone of inhibition).

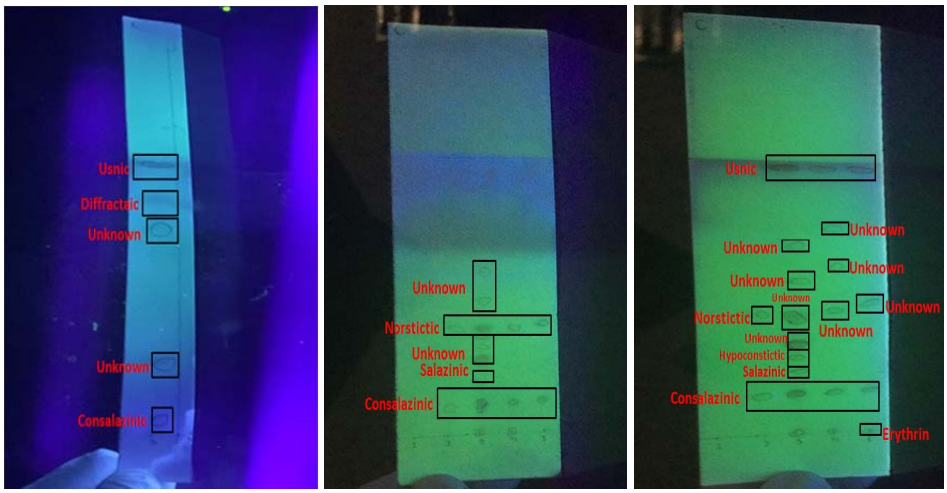
TLC bioautography. Bacterial suspensions were prepared using 24-h-old cultures of MRSA and *S. pneumoniae* and were adjusted to 0.5 McFarland standard. Then, 40 µl of the bacterial suspension was mixed with 40 ml cooled and semi-solid MHA (with and without 5 % sheep's blood depending on the bacterium used) and was designated as the seeded layer. A base medium, approximately 60 ml solidified MHA (with and without 5 % sheep's blood), was also prepared and poured on sterile, disposable petri plates. Once the base medium has solidified, the TLC plate was placed on top (with the silica side of the TLC plate facing upwards) using a sterile forceps. Then, the seeded MHA was poured simultaneously. All plates were refrigerated for two hours allowing the direct diffusion of metabolites to take place without allowing the test organism to grow. Culture plates were then incubated at 37 °C for 18–24 h. On the other hand, plates containing *S. pneumoniae* were incubated at 35 °C with 4.9% carbon dioxide concentration for 18–24 h. After the incubation period, spots of ZOI were observed. The identity of the bioactive lichen compounds was determined by comparing with the visualized TLC plates previously run.



A). Solvent System A: 36:9:1 toluene/dioxane/glacial acetic acid.



(B). Solvent System C: 20:3 toluene/glacial acetic acid.



(C). Solvent System G: 139:83:8 toluene/ ethyl acetate/formic acid.

Fig. 5. TLC plates showing the lichen acids detected in *Usnea* species.

Results

The collected fruticose lichens:

A total of 672 fruticose lichen specimens were collected from Mt. Banoi, Batangas and Dahilayan, Bukidnon. All specimens were subjected to morphological analysis and thalline spot test following published identification keys. Out of 672 collected lichen specimens, 464 specimens from Mt. Banoi, Batangas were identified as follows: *Ramalina chondrina*, R. *implectens*, *R. farinacea*, and *R. thrausta*. Two hundred eight (208) specimens from Dahilayan, Bukidnon were identified as: *Usnea aciculifera, U. amabilis, U. baileyi, U. barbata, U. bismolliuscula, U. ceratina, U. chaetophora, U. cornuta, U. diplotypus, U. esperantiana, U. filipendula, U. flammea, U. fragilescens, U. fragilescens var. mollis, U. glabrata, U. grandisora, U. hakonensis, U. himalayana, U. nidifica, U. nipparensis, U. orientalis, U. pangiana, U. pectinata, U. poliotrix, U. praetervisa, U. pygmoidea, U. rubicunda, U. rubrotincta, U. scabrata, U. subfloridana, U. substerilis, U. sphacelata, U. subdaseae, U. subrubricornuta, U. transitoria, U. wasmuthii*, and three unidentified specimens.

Antibacterial properties of *Ramalina* and *Usnea* species:

Against Gram-positive bacteria: Twelve out of the 40 lichen crude extracts of *Ramalina* spp. and *Usnea* spp. exhibited inhibitory activities against the Gram-positive bacteria, *B. subtilis* and *S. aureus* (Fig. 1). Most crude extracts of *Ramalina* spp. and *Usnea* spp. were partially active (10–12 mm ZOI) and inactive (<10mm ZOI) against *B. subtilis* (Fig. 1). Among the crude extracts tested, *U. filipendula* (US6M) had the highest mean ZOI (20 mm) against *B. subtilis* (Fig. 2). Interestingly, most of the specimens were either active (13–19 mm ZOI), partially active or inactive against *S. aureus* (Fig. 1). Among the 40 lichen crude extracts, *U. fragile*scens (US4C) had the highest mean ZOI (13 mm) against *S. aureus* (Fig. 2).

Against multidrug-resistant bacteria: Seventeen extracts were subjected to the MRSA test (Fig. 3). Nine extracts of *Usnea* spp. were active, six extracts (3 *Ramalina* spp. and 3 *Usnea* spp.) were partially active, and two extracts of *Ramalina* spp. had no antibacterial activity against MRSA (Fig. 4). Among the crude extracts tested, *R. farinacea* (RS4A) showed an average ZOI of 11 mm (Fig. 3) while the extract of *U. filipendula* (US6M) exhibited the largest ZOI (18 mm) in comparison with the positive control vancomycin (30 mg/ml) with an average ZOI of 17 mm (Fig. 3). In the multidrug-resistant *S. pneumoniae*, 12 extracts of *Usnea* spp. were active, four extracts of *Ramalina* spp. were partially active, and one extract of *R. farinacea* showed no antibacterial activity (Fig. 4). Furthermore, the extract of *R. farinacea* (RS4A) had an average ZOI of 11 mm (Fig. 3) while the extract *U. fragilescens* (US4C) showed the highest ZOI of 18 mm against MDR *S. pneumoniae* (Fig. 3).

Identification of bioactive lichen acids using TLC:

Five species of *Usnea* were ran in TLC. The extracted lichen acids had a percent yield ranging from 13–16 % per one gram air-dried lichen thalli (data not shown). Among the obtained lichen specimens, *U. rubicunda* had a maximum percentage yield extract of 16 % of the total dried weight while *U. fragilescens* and *U. bismolliuscula* both had the lowest yield of 13 %. Solvent system A detected 16 lichen metabolites. Interestingly, alectorialic acid, emodin acid, ethyl orsollinate acid, protocetraric acid, pulvinic

acid, sekikaic acid, siphulin acid, umbilicaric acid and virensic acid were specifically detected by this solvent system (Fig. 5, Tab. 2). The TLC profiles ran on solvent system C detected 11 lichen metabolites. Epanorin acid, entothein acid, olivetoric acid and S-2 with stictic acid were found only using this solvent system (Fig. 5, Tab. 2). Lastly, solvent system G detected 10 lichen metabolites. Consalazinic acid, diffractaic acid, fumarprotocetraric acid, hypoconstictic acid and stictic acid were unique to this solvent system (Fig. 5, Tab. 2). Additionally, atranorin, constictic acid, erythrin acid, norstictic acid, polyporic acid, salazinic acid, squamatic acid and usnic acid were all detected by the three solvent systems used (Fig. 5, Tab. 2). A total of 45 lichen acids were detected by solvent systems A, C and G. Twenty six of 45 detected lichen acids were identified using their Rf values. In addition, the remaining 19 were unidentified lichen acids that could be potentially new lichen acids.

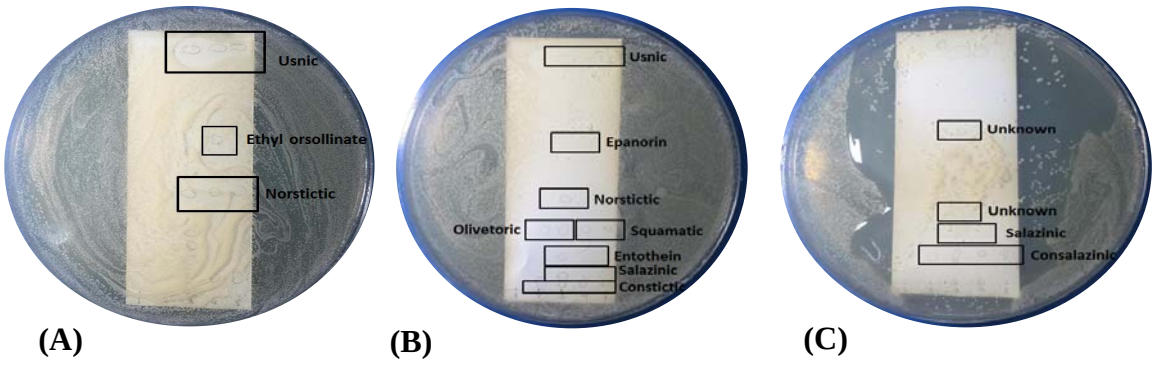


Fig. 6. Bioactive lichen metabolites of *Usnea* species tested against *S. pneumoniae*. **A.** Solvent system A. **B.** Solvent system C. **C.** Solvent system G).

Identification of bioactive lichen acids using TLC-bioautography:
Extracts of *Usnea bismolliuscula, U. chaetophora, U. filipendula, U. fragilescens* and *U. rubicunda* were tested against MRSA and *S. pneumoniae* (Fig. 6). Twenty-two bioactive lichen acids were detected, however, only 14 were identified. These include atranorin, consalazinic acid, constictic acid, entothein acid, epanorin acid, erythrin acid, ethyl orsollinate acid, norstictic acid, olivetoric acid, polyporic acid, salazinic acid, squamatic acid, S-2 with stictic acid, usnic acid and eight unknown lichen acids as shown in the clearing zone around these spots on the TLC plates (Fig. 6).

Discussion

Since distribution is an important part in determining the growth form lichens, the lichen communities inhabiting the forests provide information regarding the physicochemical factors of a specific habitat such as climate condition, and atmospheric condition (JOVAN 2008). Imposing both high elevations, Mt. Banoi, Batangas (608 m a.s.l.) and Dahilayan, Bukidnon (1376 m a.s.l.) shelter a lichen flora of fruticose forms. These sites exhibit low temperature and relatively high humidity. Furthermore, the province of Bukidnon also poses a rich diversity of other plants such as Pteridophytes and Bryophytes (AZUELO 2009). A total of 464 specimens belonging to the genus *Ramalina* were collected from Mt. Banoi, Batangas and 208 *Usnea* specimens were collected from Dahilayan, Bukidnon. Among the six sites in Mt. Banoi, the highest occur-

rence of *Ramalina* specimens was found in site 3 having four species. Meanwhile, sites 4 and 6 (among the 10 sites in Dahilayan), had the highest occurrence of lichen *Usnea* specimens, yielding 24 and 14 species, respectively.

Tab. 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) by the 5 lichen crude extracts against selected multidrug-resistant bacteria.

Code	Species	MIC				MBC (# of colonies)	
		MRSA		*Streptococcus pneumoniae*		MRSA	*Streptococcus pneumoniae*
		%	mg/ml	%	mg/ml		
US4C	*U. fragilescens*	50	0.50	12.5	0.0625	[a]0	0
US6H	*U. rubicunda*	6.3	0.03125	6.3	0.03125	0	0
US6M	*U. filipendula*	12.5	0.0625	25	0.125	0	0
US10C	*U. chaetophora*	50	0.25	3.13	0.015625	0	0
US10K	*U. bismolliuscula*	25	0.125	1.56	0.0078125	0	0

[a]0: No colony growth

The secondary metabolites of lichens, also known as the lichen acids, are exported outside the fungal hyphae and are deposited either as liquid or as crystal forms onto the different part of the thallus, often in the upper cortex or in specialized structure such as fruiting bodies (FAHSELT 1994, HUNNECK 1999, YAMAMOTO 2005). Usually, these substances contain many characteristic phenols such as depsides, depsidones, dibenzofurans and pulvinates (FAHSELT 1994, YAMAMOTO 2005), and aliphatic acids, pulvinic derivatives, hydroxybenzoic acid derivatives, anthraquinones, naphthoquinones and related compounds, and epidithiopiperzinediones (MÜLLER 2001).

Since lichens produce a broad spectrum of substances with inhibitory activities, it is expected that further screening programs will be conducted for the discovery of candidate compounds for the development of new antibiotics (KOSANIĆ & RANKOVIĆ 2015). Of our 40 extracts tested, 12 extracts exhibited inhibitory activities against the Gram-positive bacteria *B. subtilis* and *S. aureus* (Fig. 1). In fact, similar studies have shown such impressive activities of lichens against Gram-positive bacteria. For instance, SANTOS & al. (1964) and SANTIAGO & al. (2010) examined the inhibitory activities of fruticose lichens collected from the Philippines against *B. subtilis* and *S. aureus*. Most of their lichen crude extracts were active against these two test organisms. Also, lichens from temperate countries were reported active against Gram-positive bacteria (SAENZ & al. 2006, PAUDEL & al. 2008). Such inhibition, though not performed in our study, is due to the probable mechanisms of the secondary metabolites such as the inhibition of cell wall synthesis, inhibition of protein synthesis, alteration of the cell membrane, inhibition of nucleic acid synthesis, and anti-metabolite activity (KOSANIĆ & RANKOVIĆ 2015). Furthermore, our crude extracts were far by more active against *B. subtilis* than *S. aureus* (Fig. 2). The main defense of *S. aureus* is the production of an antiphagocytic capsule and zwitteronic capsule (both positively and negatively charged), which decreases the totipotency of the mechanisms of action of lichens (GORDON & LOWY 2008, SANTIAGO & al. 2013, MACIĄG-DORSZYŃSKA & AL 2014).

Multidrug-resistant microorganisms are major problems worldwide, which can cause a catastrophic event to people in every country in the world (CDC 2013). Multidrug-resistant is defined as the ability of microorganism to be insensitive in administered antimicrobial medicine (RITM & DOH 2014). To find a possible solution to the rising of multidrug-resistant bacteria, 17 lichen crude extracts showing extensive zones of inhibition from the antibacterial assay previously done were chosen and tested against the following MDR bacteria: Methicillin-resistant *S. aureus* (MRSA), *S. pneumoniae*, and *P. aeruginosa* (Fig. 3). These bacteria were used as a test microorganisms due to their clinical significance which imposes serious threats against individuals. Differences in the bioactivity of the extracts showing big zones of inhibition were reported (Figs. 3, 4). Results have proven that the fruticose lichen *Usnea* has promising activities against Gram-positive MDR bacteria. Involvement of the different lichen substances contributes to the excellent activities shown by the majority of the lichen crude extracts (YAMAMOTO 2005; CANSARAN & al. 2006). The lichen *Usnea* has exhibited greater antibacterial activities than the lichen *Ramalina.* This can be due to the presence of usnic acid as a constant substance (although it varies in amount depending on the location on the thallus) in the cortex of species of *Usnea* (Ohmura 2012). Moreover, all extracts did not exhibit antibacterial activity against the multidrug-resistant *Pseudomonas aeruginosa*. This can be ascribed to the sensitivity and differences in the permeability of the cell wall (NOSTRO & al. 2000). The cell wall composition of Gram-negative bacteria is made up of peptidoglycans, lipopolysaccharides and lipoproteins compared to the single cell membrane of Gram-positive bacteria which offers less protection against antibiotic substances; thus, Gram-negative bacteria were more resistant than Gram-positive ones (NOSTRO & al. 2000, MORTON & al. 2010). *Pseudomonas aeruginosa* shows all known enzymatic and mutational mechanisms of bacterial resistance (PECHERE & KOHLER 1999). For instance, BURKHOLDER & al. (1944) reported that no inhibitory activities were observed against Gram-negative bacteria such as *Escherichia coli* and *P. aeruginosa*. The different *Usnea* species were found active in inhibiting the growth of Gram-positive multidrug-resistant bacteria, particularly MRSA and *S. pneumoniae.* We further confirmed what is known of the activity of the lichen acid against Gram-positive multidrug-resistant bacteria (WECKESSER & al. 2007, IVANOVIC & al. 2013, SANTIAGO & al. 2013). Moreover, differences in the concentration of MIC/MBC of the lichen crude extracts were also observed (Tab. 1). Based on the results, *U. rubicunda* had the lowest MIC among the crude extracts tested (Tab. 1). This could be correlated with the medullary chemistry of *U. rubicunda* which is composed of stictic acid as the main susbstance, and norstictic and psoromic acids as accessories (RANDLANE & al. 2009) and is supported by our TLC results (Fig. 5). As stated earlier, stictic acid has been detected in TLC (Tab. 2). It is, therefore, not anymore surprising to learn that such species could have a low MIC value. Furthermore, the intensity of the antimicrobial effect depends upon the type of extract, its concentration, and the tested microorganisms (SRIVASTAVA & al. 2013, PRATEEKSHA & al. 2016). The inhibitory effect of the lichen crude extracts is more effective against Gram-positive bacteria such as *Micrococcus pyogenes* var. *aureus,* Penicillin-resistant *Micrococcus pyogenes*, *Bacillus subtilis*, and the acid-fast bacilli, *Mycobacterium tuberculosis* than the Gram-negative bacteria (SANTIAGO & al. 2013).

Tab. 2. Lichen metabolites detected from the five *Usnea* species using the three solvent systems on TLC.

Lichen Metabolite	[a]Solvent Systems		
	A	**C**	**G**
Alectorialic acid	[b]+	[b]-	-
Atranorin	-	+	+
Consalazanic acid	-	-	+
Constictic acid	+	+	-
Diffractaic acid	-	–	+
Emodin acid	+	-	-
Epanorin acid	-	+	-
Erythrin acid	+	-	+
Ethyl Orsollinate acid	+	-	-
Entothein acid	-	+	-
Fumarprotocetraric acid	-	-	+
Hypoconstictic acid	-	-	+
Norstictic acid	+	+	+
Olivetoric acid	-	+	-
Polyporic acid	+	+	-
Protocetraric acid	+	-	-
Pulvinic acid	+	-	-
Salazinic acid	+	+	+
Sekikaic acid	+	-	-
Siphulin acid	+	-	-
Squamatic acid	+	+	-
Stictic acid	-	-	+
Unknown with stictic acid	-	+	-
Umbilicaric acid	+	-	-
Usnic acid	+	+	+
Virensic acid	+	-	-
Total:	**16**	**11**	**10**

[a]Solvent System A: 36:9:1 tolouene/dioxane/glacial acetic acid
Solvent System C: 20:3 tolouene/glacial acetic acid
Solvent System G: 139:83:8 tolouene/ethyl acetate/ formic acid
[b]+=detected; -=not detected

The production and/or concentration of secondary metabolites in lichens varied with changing elevations (KOSANIĆ & RANKOVIĆ 2015) as supported in our metabolic profiles (Fig. 5). Identification of the lichen metabolites involved the standardized method of using three solvent systems, namely A, C, and G (CULBERSON 1972). This is an applied technique for the identification of secondary products of lichens and also for the confirmation of structures from certain types of new compounds that are found and extracted from the lichen specimens (CULBERSON 1972). The three solvent systems differ in terms of its chemical composition. The most effective, substantial and stable of all is solvent system C. It is also the best way to determine the dissemination of the lichen substances. In our study, several lichen acids (atranorin, constictic, epanorin, entothein, norstictic, olivetoric, salazinic, squamatic, unknown with stictic and usnic) were detected (Fig. 5, Tab. 2). Furthermore, various types of metabolites with phenolic hydroxy were detected by solvent system A such as alectorialic, constictic, emodin, erythrin, ethyl orsollinate, norstictic, polyporic, protocetraric, pulvinic, salazinic, sekikaic, siphulin, squamatic, umbilicaric, usnic and virensic acids (Fig. 5, Tab. 2). Interestingly, solvent system A is reputed to owe its distinctive characteristics to the ability of dioxane to associate with phenolic hydroxy groups (NASH 2008). On the other hand, solvent system G is convenient in separating compounds with relatively low Rf values in solvent systems A (b-orcinol depsidones) and C (hopane triterpenoids) (CULBERSON & JOHNSON 1982). Distinctive metabolites were present and detected by solvent system G such as atranorin, consalazinic, diffractaic, erythrin, fumarprotocetraric, hypoconstictic, norstictic, salazinic, stictic and usnic (Fig. 5, Tab. 2). To determine which among the detected lichen acids are bioactive, TLC-bioautography was performed. Interestingly, five lichen acids (diffractaic acid, norstictic acid, salazinic acid, stictic acid and usnic acid) previously reported as bioactive against *B. subtilis*, *S. aureus* (SANTIAGO & al. 2010) and *Nocardia asteroides* (SANTIAGO & al. 2013) were once again reported as biologically active against MRSA and *S. pneumoniae* (Figs. 6). The antibiotic activity could be correlated to the mechanisms of action of various lichen acids, particularly the inhibition of oxidative phosphorylation. As such event happens, oxygen consumption, electron transport chain and other mitochondrial functions are also inhibited, leading to cell death (FRANKOS 2005, HAUCK & JURGENS 2008).

Despite the fact that lichens are considered as one of the most promising reservoirs of low-molecular weight secondary compounds demonstrating some level of biological activity, a very limited number of compounds has been studied (BOUSTIE & GRUBE 2005). Our study, therefore, has opened opportunities for upcoming studies regarding the un- and under-explored lichens in the Philippines and its biological responses in resolving the emergence of multidrug-resistant bacteria.

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