Antibacterial activities of fruticose lichens collected from selected sites in Luzon Island, Philippines

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Lichens are known prolific sources of biologically active natural products. Thus, our research study explores the antibacterial activities of lichen acids extracted from fruticose lichens and aims to identify their bioactive metabolites. Sixty-three lichens were collected from different sites in Luzon Island, Philippines: Bataan, Batangas, Benguet, Cavite, Laguna, and Quezon. Morphological characterization and biochemical tests were used to identify the collected fruticose lichens as *Usnea baileyi* (6), *Ramalina dendriscoides* (55), *Stereocaulon massartianum* (1), and *Cladonia gracilis* (1). The lichen thalli were air-dried and their secondary metabolites extracted with acetone. Lichen crude extracts were then tested against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus)* and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa)* using the paper disk diffusion assay. Our results showed that all 45 tested extracts inhibited at least one of the test bacteria. However, only 38 extracts were found to be very active (> 19 mm zone of inhibition) against Gram-positive bacteria. Extracts from *R. dendriscoides* were observed to be the most active. Selected lichen extracts also showed activities against *S. aureus* even at a volume of 30 µl and MIC/MBC values of 156 µg/ml and 2500 µg/ml in the tube dilution assay. Eight lichen acids were detected in the crude extracts by thin layer chromatography. TLC-bioautography showed barbatic acid, stictic acid, diffractaic acid, galbinic acid, norstictic acid, salazinic acid, and usnic acid to be the bioactive lichen acids.

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lichens, fructicose type, lichen acids, bioactive secondary metabolites, antibacterial activity

INTRODUCTION

The Philippines, though known as a megahotspot of biodiversity, has limited studies on lichens, particularly on fruticose lichens. Earlier study by Herre (1957) reported only 68 lichen species from 26 provinces in Luzon (12), Visayas (6), and Mindanao (8). Majority of the species were foliose (45 species) and crustose (36 species) type of lichen growth. Eleven species were recorded as fruticose, while only one species was noted as squamulose. Among the fruticose type of lichens reported were species of *Cladonia* (2), *Stereocaulon* (2), and *Usnea* (7). Herre (1963) also reported from Bataan, Ilocos Sur, Misamis, Negros Oriental, and Rizal five species of *Usnea*: *U. hossei, U. longissima, U. marivelensis, U. misamisensis*, and *U. squarrosa*.

Recently, Dulnuan (2006) reported 3 species of lichens with fruticose type of growth from a total of 52 lichen genera collected in Ifugao, Mountain Province. The total number of lichen species credited now for the Philippines is 790 (DENR 1999; Tacio 2004).

Lichens are good sources of biologically active secondary metabolites. They have been used as medicine in treating wounds, stomach diseases, and whooping cough in America and in Europe (Crockett et al. 2003; Rankovic et al. 2007). They are also reported to produce secondary metabolites with antimicrobial and anticancer activities (Ingólfsdóttir et al. 1985; Manojlovic et al. 2000; Gulluce et al. 2006; Rankovic et al. 2007). However, in spite of their potential as sources of drugs, the biological activities of Philippine lichens remain less studied. Quisumbing (1951) earlier reported the medicinal properties of fruticose lichen *Usnea philippina*. Santos et al. (1964) tested the biological activities of these lichens and other fruticose lichens, e.g., *Usnea* sp., *Ramalina* sp., and *Stereocaulon* sp., and reported their inhibitory activities against Gram-positive bacteria such as *Micrococcus pyogenes* var *aureus* 209 P (syn=*Streptococcus pyogenes*), penicillin-resistant *Micrococcus pyogenes* var *aureus, Bacillus subtilis*, and the acid-fast bacilli, *Mycobacterium tuberculosis* 607. Interestingly, the latter is known to have acquired resistance against major anti-TB drugs due to incomplete or partial treatment and necessitates treatment with new antibiotics (WHO 2009). Santos and Mondragon (1969) also conducted thin layer chromatographic analysis of these lichens and detected the following lichen acids: salazinic acid, stictic acid, usnic acid, barbatic acid, protocetraric acid, zeorin, atranorin, lecanorin, and homosekikaic acid. However, it was not reported whether any of these metabolites is responsible for its antibacterial activities.

The search for novel bioactive secondary metabolites is of primary concern since infectious diseases are continuously emerging and re-emerging. For example, *Mycobacterium tuberculosis* infects approximately 9 million new individuals every year with 1.7 million deaths annually (WHO 2009). Since lichens offer alternative sources of bioactive metabolites, our study explores the antibacterial activities of fruticose lichens belonging to the genera *Cladonia*, *Ramalina*, *Stereocaulon*, and *Usnea* collected from selected provinces of Luzon. It is hoped that the lichen acids extracted from these species may be potentially novel and biologically active against emerging and re-emerging diseases.

MATERIALS AND METHODS

Collection sites

The Luzon Island (14° 35' N, 121º 00 E) is located on the northern part of the Philippine archipelago. It is bounded by the Philippine Sea on the east and the South China Sea on the west. The Luzon Island, the largest in the Philippines, has a typical tropical climate with two distinguishable seasons: the wet and dry. Fruticose lichens were collected from six provinces in Luzon Island, Philippines: (1) Laguna Province (200 meters above sealevel, masl): Los Baños and San Pablo; (2) Benguet Province (1600 masl): Camp John Hay and Baguio City; (3) Cavite Province (640 masl): Silang and Tagaytay City; (4) Batangas Province (600 masl): Lipa City, Lemery, Batulao, Laurel, Alaminos, and Caleruega; (5) Quezon Province (141 masl): Dolores; and (6) Bataan Province (600 masl): Orani and Mt. Natib. Fresh specimens were collected and placed in brown paper bags, air-dried and then stored in dry wooden cabinets until used in the study.

Characterization and identification of fruticose lichens

All collected fruticose lichens were initially characterized based on morphology and biochemical tests, e.g., (1) growth form: length of thallus and description of branches and branchlets; (2) color and texture of thallus; (3) presence or absence of reproductible structures (apothecium); and (4) thalline spot test (K, KC and C test). For the thalline spot test, the upper cortex of each specimen was initially scraped off with a razor blade to expose the medulla. Then, different chemical reagents, e.g., potassium hydroxide (K) and sodium hypochlorite (C), were spotted directly onto the exposed medulla. Any immediate change in color of the thallus indicates a positive reaction (Hale 1979). Identification was done following comparison of the morphometric and biochemical test results with those in published literature and identification keys, e.g., Herre (1963), Hale (1979), Nash III (1996), and Sipman (2005). Representative specimens were also sent to Mr. Virgilio C. Linis, Botany Division, National Museum of the Philippines, Manila, for the confirmation of the identities of the collected lichens.

Extraction and identification of lichen acids

One gram, air-dried thalli of 45 representative lichen specimens from the six provinces were initially cut into small pieces, ground using mortar and pestle until powdery and then soaked overnight in 10 ml acetone. Acetone was used for the microscale extraction of the lichen acids as recommended (Huneck and Yoshimura 1996), as most lichen substances are soluble in this solvent. After 24 hours, the extracts were filtered, concentrated by air-drying for 4 – 5 days or until the extracts crystallized, and the weight/yield of the crude extracts were determined.

To identify the lichen acids present, the extracted lichen crude extracts were dissolved in acetone to a final concentration of 10 mg/ml. The crude extracts were then spotted on silica gel thin layer chromatography (TLC) plates (silica gel 60 F_{254} aluminum plates, Merck) and run in three different solvent systems: (1) Solvent System A: 36:9:1 toluene/dioxane/glacial acetic acid, (2) Solvent System B: 24:18:4 hexane/diethyl ether/formic acid, and (3) Solvent C: 20:3 toluene/glacial acetic acid (Culberson et al. 1972). Each TLC plate was then sprayed with 0.5 ml glacial acetic acid and 1 ml 97 % sulfuric acid and heated at 105°C for 5 minutes to visualize the lichen acids (Santos and Mondragon 1969). The R_f values for each spot were

determined and compared with selected lichen acid standards: (1) norstictic acid, (2) usnic acid, (3) salazinic acid, (4) caperatic : usnic acid, 25:1, (5) protocetraric acid, (6) stictic : constictic acid, 2:1, (7) diffractaic acid, (8) barbatic acid, and (9) galbinic acid. The lichen acids standards were generously provided by Prof. Dr. Jack A. Elix, Australian National University, Canberra, Australia.

Assay for antibacterial activities of fruticose lichens

Paper disk diffusion assay. The lichen crude extracts (10 mg/ml) from 45 lichen specimens were then tested for their inhibitory activities against representative test bacteria: (1) Gram-positive bacteria: *Bacillus subtilis* and *Staphylococcus aureus*, and (2) Gram-negative bacteria: *Escherichia coli* and *Pseudomonas aeruginosa*. Bacterial cell suspension was prepared from a 24-hour old culture, adjusted to 0.5 McFarland standard, and swabbed on petri plates pre-filled with 25 ml Nutrient Agar (NA, Hi-Media). Antibiotic disks (Whatman) measuring 6 mm in diameter were then placed onto the inoculated NA plates (three disks per plate). Each disk was impregnated with 50 µl lichen crude extracts (10 mg/ml). The positive and negative controls were 50 µl of 10 mg/ml tetracycline (Sigma)/streptomycin (Sigma) and the solvent acetone, respectively. The assay was done in triplicates. Plates were then incubated at 37°C for 18-24 hrs. Following incubation, zones of inhibition including paper disks were then measured with a ruler and recorded. Inhibition zones of the control (acetone), if present, were deducted from those of the lichen extracts. Bioactivity was assessed using the following rating system: (1) very active, > 19 mm zone of inhibition; (2) active, 13-19 mm zone of inhibition; (3) partially active, 10-12 mm zone of inhibition; and (4) inactive, < 10 mm zone of inhibition (Quinto and Santos 2005).

To determine the effectivity of the lichen crude extracts at different volumes, four selected lichen extracts (Rd19, Rd26, Rd39 and Ub04) exhibiting antibacterial activities were tested against *S. aureus*. Bacterial suspensions for the test bacterium were prepared as described above. To each paper disk, varying volumes (40 µl, 30 µl, 20 µl and 10 µl) of each lichen crude extract (10 mg/ml) were added. The culture plates (in triplicates) were then incubated at 37°C for 18-24 hours. The zones of inhibition were then measured with a ruler, recorded and assessed as described above.

Tube dilution assay. To determine the Minimum Inhibitory Concentration (MIC) and/or Minimum Bactericidal Concentration (MBC) of the bioactive lichen crude extracts, tube dilution assay with a two-fold dilution was done (Quinto and Santos 2005). Extracts of Rd06 and Rd42, which exhibited relatively larger zones of inhibition, were tested against the Gram-positive *S. aureus*. Initially, the first tube contained only pure lichen extracts (10 mg/ml). A serial two-fold dilution was done from the second tube up to the 9th tube with Nutrient Broth (Hi-Media) as diluent. The 10th tube which contained only Nutrient Broth (NB) was the negative control. The 11th tube which contained 10 mg/ml streptomycin was the positive control. The 12th tube contained only uninoculated Nutrient Broth and served as the point of comparison for turbidity. All assay tubes were incubated at 37°C for 18-24 hours. The MIC was computed based on the NB assay tube with the least concentration of lichen extract that showed absence of growth or turbidity. For the determination of the MBC, the three assay tubes exhibiting the least MIC were streaked on NA plates to confirm the presence or absence of growth of the test organism. NA plates were then incubated at 37°C for 18-24 hours. Following incubation, the MBC was computed based on the least concentration of crude extract in the NB assay tube that exhibited no growth or colony formation on NA plate.

Identification of the bioactive lichen acids using TLC-bioautography

To determine the bioactive lichen acid/s, eight lichen extracts from representative lichen species (Rd40, Rd38, Rd44, Ub31, Sm13, Cg32, Ub39 and Rd42) were initially spotted on TLC plates. The TLC plates were then run in Solvent System A (36:9:1 toluene/dioxane/glacial acetic acid) (Culberson et al. 1972) and the spots were visualized under ultraviolet (UV) light (254 nm). Identification of the lichen spots was done following comparison with the lichen acid standards. Prior to the TLC-bioautography, the TLC plates were allowed to air-dry for at least 24 hours to remove any traces of the solvent system. A bacterial suspension was prepared from a 24-hour old *S. aureus* culture, adjusted to 0.5 McFarland Standard. About 100 µl of the bacterial suspension were mixed with 100 ml cooled, melted NA. The seeded NA was poured on top of the base medium (approximately 15 ml solidified NA) and allowed to solidify. The TLC plates were placed on top of the seeded layer and stored for two hours inside a refrigerator. This is to allow the metabolites to diffuse directly into the seeded layer without allowing the growth of the test organism. After two hours, the TLC plates were carefully removed and the culture plates were then incubated at 37°C for 18-24 hours. Following incubation, the spots having zones of inhibition were identified and noted. The identity of the bioactive lichen acid was determined following comparison with the visualized TLC plates.

RESULTS

The collected fruticose lichens

A total of 63 specimens of fruticose lichens were collected from six provinces in Luzon Island: Benguet, Laguna, Batangas, Cavite, Bataan, and Quezon (Table 1). Based on published descriptions of the morphological and biochemical characteristics of lichens, 55 specimens were identified and confirmed as *Ramalina dendriscoides*, 6 as *Usnea baileyi*, 1 as *Cladonia gracilis*, and 1 as *Stereocaulon massartianum*. Among the collection sites, the Batangas province has the most number of collections, totaling 23 specimens, which belonged to the genera *Ramalina* and *Usnea*. On the other hand, the Benguet province has the least number of collections (4 specimens) and with only the genus *Usnea*. *Ramalina* species were also

Table 1. Distribution and thalline spot test results of the four lichen species collected.

Collection Sites	Species[b]	thalline spot test[a]		
		K Test (KOH)	C Test (5.25% NaOCl)	KC Test (K+C)
Bataan	*Usnea baileyi* (1)	Y	NR	Y
	Cladonia gracilis (1)	P	NR	R
	Stereocaulon massartianum (1)	Y	NR	Y
Benguet	*Usnea baileyi* (4)	R/Y	R	Y/R
Batangas	*Ramalina dendriscoides* (22)	R/Y	R/Y	Y/R
	Usnea baileyi (1)	R	R	R
Cavite	*Ramalina dendriscoides* (14)	R/Y	R/NR	Y/R/NR
Laguna	*Ramalina dendriscoides* (15)	Y/NR	Y/ NR	Y/NR
Quezon	*Ramalina dendriscoides* (4)	R	NR	R

[a] Color observed in the thalline spot test and their corresponding class of secondary metabolites
K test: Y (yellow) = atranorin, R (red) to P (purple) = anthraquinones
C test: R (red) = m-dihydroxy phenols
KC test: Y (yellow) = usnic acid, R (red) = depsides and depsidones
NR = no observable color change in the thalline spot test

[b] Number in parenthesis are the number of specimens tested for each species.

collected from Cavite (14 specimens), Laguna (15 specimens), and Quezon (4 specimens). The two species, *Stereocaulon* and *Cladonia*, were collected only in Bataan.

Though similar species were collected, our thalline spot test results showed variations in their thallus color, and thus, also in the metabolites detected (Table 1). The variation was observed among different specimens of the similar species collected from different sites.

The lichen acids of *R. dendriscoides*, *U. baileyi*, *C. gracilis* and *S. massartianum*

Extraction of lichen acids of *R. dendriscoides* with acetone yielded the highest crude extract of up to 8.21% of the total dried weight. On the other hand, *C. gracilis* gave the lowest crude extract yield, only 0.51%. The other two fruticose lichens, *S. massartianum* and *U. baileyi*, gave 3.07% and 6.14% of the total dried weight, respectively.

TLC detected 4 to 6 lichen acids using the three solvent systems used (Figure 1, Table 2). However, specific lichen acids were detected by each of the solvent systems. For example, solvent system A (36:9:1 toluene/dioxane/glacial acetic acid) detected barbatic and salazinic acids while solvent system B (24:18:4 hexane/diethyl ether/formic acid) detected only galbinic and protocetraric acids. Solvent system C (20:3 toluene/glacial acetic acid) detected stictic acid. Furthermore, differences in lichen acids were detected in the two lichen species, *Usnea baileyi* and *Ramalina dendriscoides*. Barbatic acid was observed only in *Ramalina* species. Interestingly, several unidentified spots were also detected: 8 spots for solvent system A and C, and 13 spots for solvent system B.

Antibacterial activities of lichen crude extracts and their bioactive metabolites

Of the 45 fruticose lichen extracts tested, 38 exhibited inhibitory activities against the Gram-positive bacteria, *S. aureus* and *B. subtilis* (Figure 2, Table 3).

Only ten lichen extracts were found partially active while 7 were recorded as inactive. Two lichen extracts of *R. dendriscoides* (Rd14 and Rd16) from two sites in Batangas

Table 2. TLC profile of lichen species as detected using Solvent Systems A, B, and C[a].

Lichen Acids	*Ramalina dendriscoides*			*Usnea baileyi*		
	A	B	C	A	B	C
Barbatic Acid	+	-	-	-	-	-
Caperatic Acid	-	-	-	-	-	-
Diffractaic Acid	+	-	+	-	+	-
Galbinic Acid	-	+	-	-	-	-
Norstictic Acid	+	+	+	+	+	-
Protocetraric	-	+	-	-	-	-
Salazinic Acid	+	-	-	+	-	-
Stictic Acid	+	-	+	-	-	-
Usnic Acid	+	+	+	+	+	+

[a] Solvent System A: 36:9:1 toluene/dioxane/glacial acetic acid
Solvent System B: 24:18:4 hexane/diethyl ether/formic acid
Solvent System C: 20:3 toluene/glacial acetic acid

Table 3. Bioactivity of lichen extracts against *Staphylococcus aureus* and *Bacillus subtilis*.

Species	Number of Extracts	Bioactivity against *S. aureus*[a]			
		Very Active	Active	Partially Active	Inactive
Cladonia gracilis	1	1	0	0	0
Ramalina dendriscoides	37	3	24	8	2
Stereocaulon massartianum	1	0	0	0	1
Usnea baileyi	6	0	1	1	4
Species	Number of Extracts	Bioactivity against *B. subtilis*			
		Very Active	Active	Partially Active	Inactive
Cladonia gracilis	1	1	0	0	0
Ramalina dendriscoides	37	29	8	0	0
Stereocaulon massartianum	1	0	1	0	0
Usnea baileyi	6	4	1	1	0

[a] Bioactivity was assessed using the following rating system:
very active = >19 mm zone of inhibition
active = 13-19 mm zone of inhibition
partially active = 10-12 mm zone of inhibition
inactive = < 10 mm zone of inhibition

showed the highest mean zone of inhibition against *B. subtilis* (29 mm) and *S. aureus* (23 mm), respectively. Analysis of the percentage of lichen specimens with bioactivities showed that majority (76%) were very active against *B. subtilis* (Figure 3). More than 50% of the lichen specimens were active against *S. aureus*.

Against Gram-negative bacteria, only two specimens of *R. dendriscoides* (Rd44 and Rd45, also from Batangas) and one specimen of *U. baileyi* (Ub04, from Benguet) were partially active against *E. coli* (10 – 12 mm zone of inhibition, data not shown). Seven lichen extracts from *R. dendriscoides* (collected from four provinces) were found partially active against *P. aeruginosa* (10 – 12 mm zone of inhibition, data not shown). So, generally, lichen extracts were not very effective against Gram-negative bacteria *E. coli* and *P. aeruginosa*, but observed to be very effective against Gram-positive bacteria *B. subtilis* and *S. aureus*.

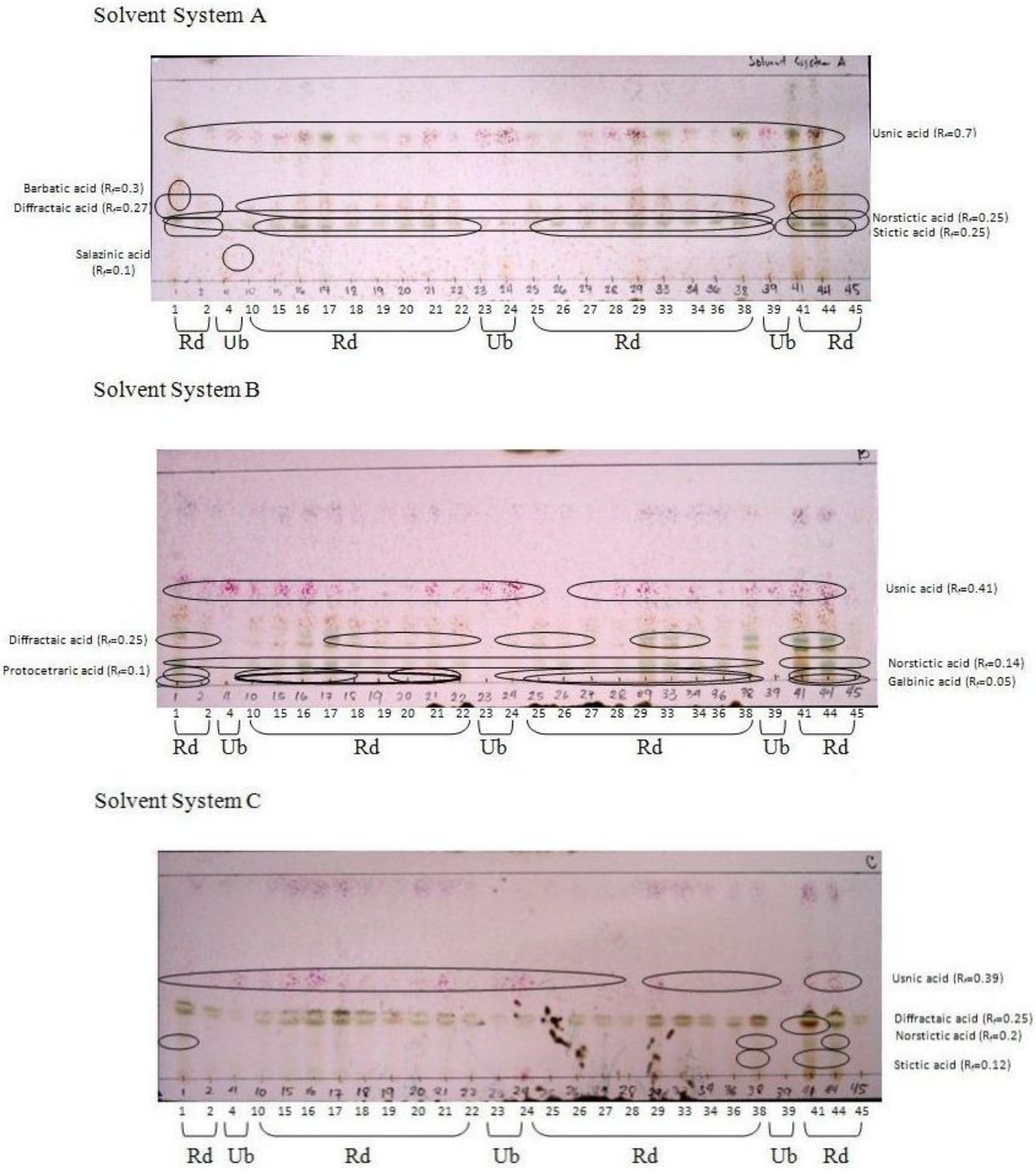

Figure 1. TLC profiles of the lichen extracts run in Solvent Systems A, B and C.

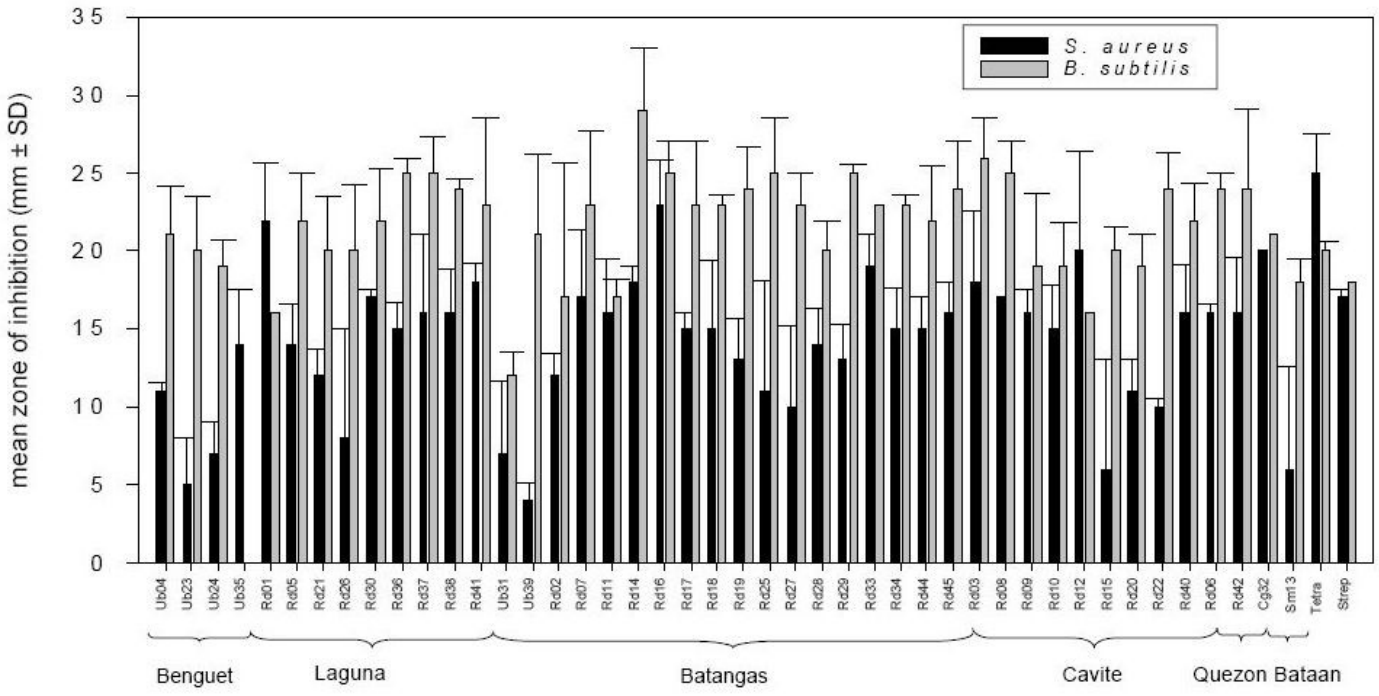

Figure 2. Zones of inhibition of 45 lichen extracts (*Ramalina dendriscoides*, Rd; *Usnea baileyi*, Ub; *Cladonia gracilis,* Cg; *Stereocaulon massartianum,* Sm) tested against gram-positive bacteria *S. aureus* and *B. subtilis*. Positive control: tetracycline (10mg/ml) and streptomycin (10mg/ml). Determinations determined in triplicate. Standard deviations are indicated above each bar.

Among the four species of fruticose lichens, *R. dendriscoides* yielded extracts that showed relatively significant activity against Gram-positive test organisms. Twenty-four *R. dendriscoides* extracts were found active against *S. aureus* (Table 3). Moreover, 29 of the *R. dendriscoides* extracts were very active against *B. subtilis* (Table 3). Interestingly, many lichen crude extracts showed zones of inhibition against *B. subtilis* that were of similar size as observed with the antibiotics tetracycline and streptomycin (Figure 2).

Different volumes of four selected lichen crude extracts (Rd19, Rd26, Ub39 and Ub04) also showed inhibitory activities against the tested Gram-positive bacterium *S. aureus* (Figure 4). Rd19, Ub39 and Ub04 extracts were still active even with an extract volume of 30 µl, whereas Rd26 was active only at 40 µl.

The Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the lichen crude extracts from Rd06 and Rd42 were also determined against *S. aureus*. Rd06 and Rd42 extracts gave MIC/MBC of 156 µg/ml and 2500 µg/ml, respectively.

TLC–bioautography was also done to identify the bioactive lichen acids of 8 lichen extracts of *R. dendriscoides* and *U. baileyi*. Our results showed usnic acid, norstictic acid, salazinic acid, stictic acid, diffractaic acid, barbatic acid, and galbinic acid to be the bioactive lichen acids (Figure 5). Several bioactive spots could not be identified based on the lichen acid standards.

DISCUSSION

Fruticose lichens remain less explored in the Philippines. Herre (1957) reported only 68 fruticose lichens from different sites within our country. Furthermore, Quisumbing (1951) and Santos et al. (1964) reported the biological activities of these lichens. Since then, very few studies were done on fruticose lichens in our country. In our study, we collected fruticose lichens from 16 sites in 6 provinces: Laguna, Benguet, Cavite, Batangas, Quezon and Bataan. Morphological characterization identified these specimens as *Ramalina dendriscoides, Usnea baileyi, Stereocaulon massartianum*, and *Cladonia gracilis* (Table 1). All of our specimens were found mainly in areas at high elevation (between 141 to 1600 masl) and at a cooler annual temperature of 25°C. Interestingly, *U. baileyi* was found more frequently at high elevations (1600 masl) and mostly on pine trees while *R. dendriscoides* was found at lower elevations and mostly on coconut trees. In our study, we also observed variations in the thalline spot test of all collected fruticose lichens, i.e., differences in the color produced were observed

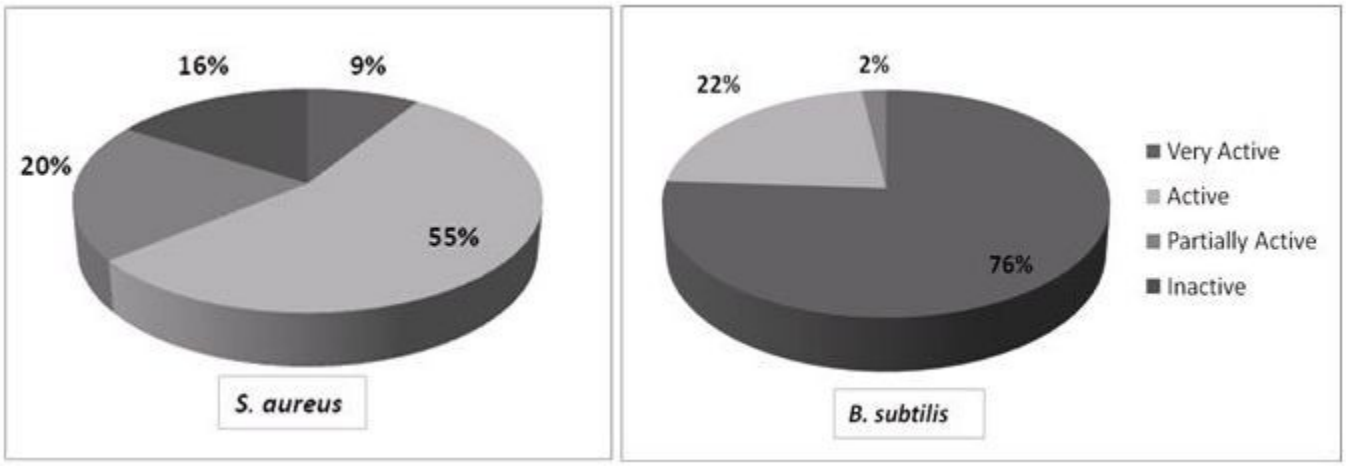

Figure 3. Percentage of lichen crude extracts showing bioactivities against *S. aureus* and *B. subtilis.* Assessment of bioactivities: very active (>19 mm zone of inhibition), active (13 – 19 mm zone of inhibition), partially active (10 – 12 mm zone of inhibition); and inactive (<10 mm zone of inhibition).

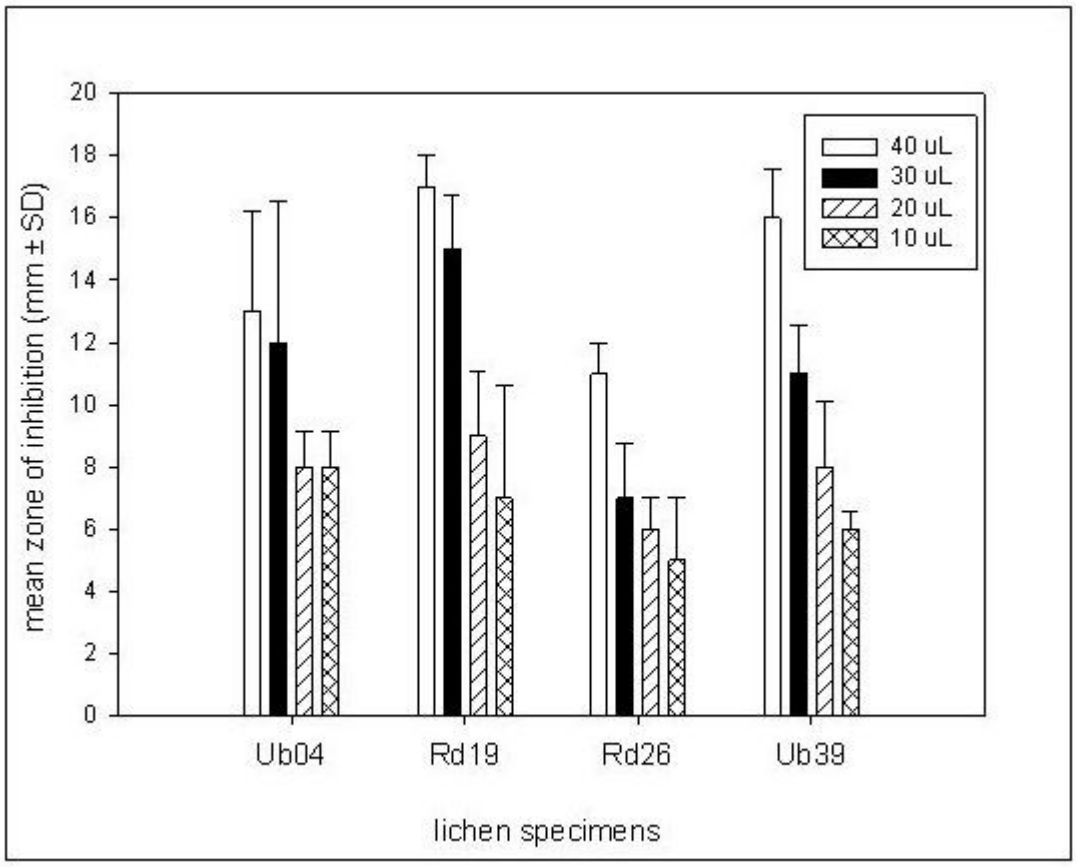

Figure 4. Rd19, Rd26, Ub39, and Ub04 extracts exhibiting zones of inhibitions against *S. aureus* at different volumes (40, 30, 20, and 10 µl) of lichen crude extracts (*Ramalina dendriscoides*, Rd; *Usnea baileyi*, Ub).

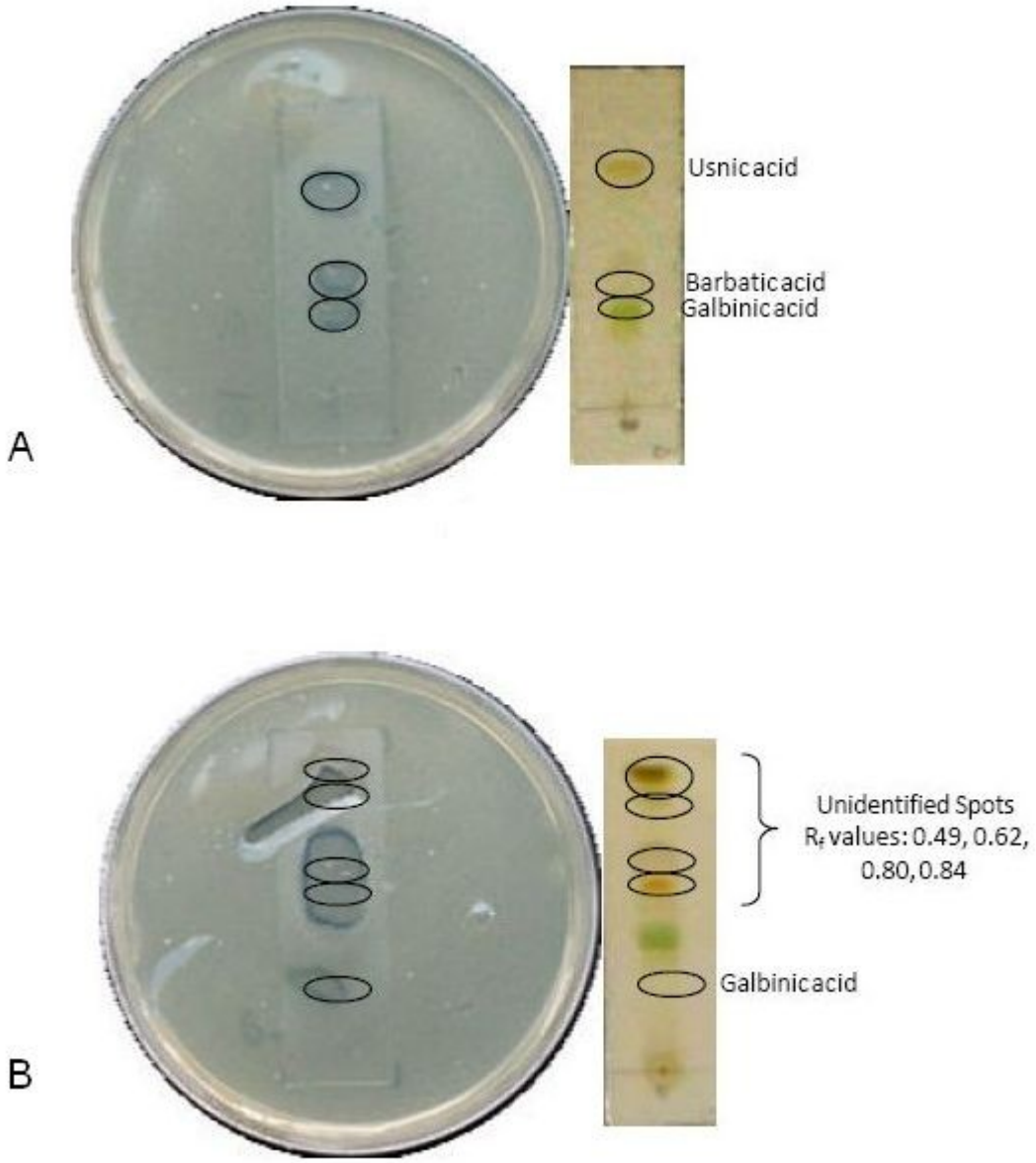

Figure 5. Bioactive lichen acids, galbinic, barbatic, and usnic acids, detected from Rd40 crude extract showed inhibition of *S. aureus* (A). Bioactive galbinic acid, together with four unidentified bioactive metabolites from Rd38 crude extract, also showed inhibition of *S. aureus* (B).

among the different species and among the different specimens of similar species (Table 1). Nash III (1996) indicated that synthesis of secondary metabolites within lichen populations of same species could be due to many factors, e.g., differences in the mycobiont component or total loss of major compounds typically present in thalli, or differences in the secondary metabolites or their biosynthetic origins. Thus, variability is probably innate and not correlated with their ecological preferences (Nash III 1996). In our study, only 17 *R. dendriscoides* specimens, four *U. baileyi*, and one *S. massartianum* showed the presence of yellow and red colors in the K test. Nash III (1996) and Sipman (2005) reported that

these results confirmed the presence of atranorin and other related compounds (yellow color in the thalline spot test), as well as anthraquinone pigments (red color in the thalline spot test). Atranorin was reported as a moderate enzyme inhibitor, while anthraquinones have antiviral activity (Müller 2001). For the C test, only two *R. dendriscoides* and two *U. baileyi* specimens showed red color, indicating the presence of *m*-dihydroxyphenols (Nash III 1996). As similarly observed with the K test, the majority of *R. dendriscoides, U. baileyi, S. massartianum*, and *C. gracilis* showed yellow color which indicates the presence of usnic acid (Nash 1996; Sipman 2005).

The wide variety of biological activities of lichens is generally correlated with their special ecological niche and with the production of metabolites that are involved in their antimicrobial actions (Boustie and Grube 2005). However, the choice of solvent system also greatly influences the lichen acids that will be detected. In our study, solvent systems A, B, and C were used because these solvent systems can easily detect lichen acids. To visualize the lichen acids, a spray reagent containing 0.5 ml anisaldehyde in 50 ml glacial acetic acid and 1 ml 97 % sulfuric acid was used following charring at 105°C. Charring detects the broadest range of compound types, including virtually all terpenes and phenolic derivatives in lichens (Nash III 1996). Our results showed that all the lichen crude extracts were positive for usnic acid (Figure 1, Table 2). Most specimens have norsticstic acid, stictic acid, diffractaic acid, galbinic acid and barbatic acid. Interestingly, stictic acid was also found in the genera *Pertusaria* and *Roccella* (Saenz et al. 2006). On the other hand, diffractaic and barbatic acids were found in *Hypogymnia hengduanensis* (Jiang-Chun and Wang-Fu 1998), while norstictic and barbatic acids were detected in the genus *Xanthoparmelia* (Giordani et al. 2002; Nash III 1996). In comparison, Santos and Mondragon (1969) detected salazinic, stictic, usnic, protocetraric, barbatic, atranorin, homosekikaic, and zeorin acids in the lichen specimens of *Usnea, Ramalina, Stereocaulon, Parmelia*, and *Crocynia* collected from the Philippines. In our study, three lichen acids, norstictic, diffractaic, and galbinic acids, were detected from our specimens but were absent from the same specimens in the previous study (Santos and Mondragon 1969). Caperatic and constictic acids were also not visualized in the three solvent systems we used. Fourteen lichen metabolites were detected with TLC. Twenty-one lichen spots could not be identified.

The lichen specimens were then tested for their biological activities, specifically for their antibacterial activities. Species of *Ramalina, Stereocaulon*, and *Cladonia* were previously reported to possess bioactive metabolites and thus, were used in medicinal, perfumery, and cosmetics industries (Ingólfsdóttir 2002). Müller (2001) likewise reported lobaric acid from species of *Stereocaulon*, which showed anti-inflammatory activities. Lichen extracts and their components also have antimicrobial activities (Rankovic et al. 2007). In our study, we tested the antibacterial activities of the lichen crude extracts. Of the 45 extracts tested, 34 and 4 lichen extracts were found very active against *B. subtilis* (16 – 29 mm zone of inhibition) and *S. aureus* (4 – 23 mm zone of inhibition), respectively (Figure 2, Table 3). Similarly, Santos et al. (1964) observed that 30 of the 38 lichen extracts tested inhibited at least one of the 12 test microorganisms, particularly the Gram-positive test bacteria. Some of the lichen crude extracts gave big zones of inhibition against *B. subtilis* and *S. aureus* as similarly observed with the antibiotics tetracycline and streptomycin (Figure 2). The lichen crude extracts were also active even at lower volumes (Figure 4). More than 71% of the lichen extracts tested were reported as active and very active (Figure 3). Furthermore, the MIC/MBC of Rd06 and Rd42 lichen extracts against *S. aureus* was determined to be 156µg/ml and 2,500 µg/ml, respectively. *S. aureus* was used as test organism due to its clinical importance. Of note, the lichen crude extracts can be later tested against methicillin-resistant *S. aureus*. However, the MIC values of these two lichen extracts were greater than the expected MIC value of the control antibiotic tetracycline (Andrews 2001). Other studies also confirmed several lichen metabolites to be active against Gram-positive microorganisms. For example, Saenz et al. (2006) found that 4 species of lichens, namely, *Ramalina canariensis, R. subfarinacea, Cladonia firma*, and *Leconara moralis*, were most active against Gram-positive bacteria. The bactericidal action was attributed to the presence of usnic acid and other bioactive acids such as lichesterinic, stictic, evernic, and ursolic acids. These metabolites may be involved in a synergistic manner. Interestingly, the active centers of usnic acid molecule seem to be the benzofuran or dihydrodibenzofuran nucleus, the phenolic hydroxyl groups, and the 4,4a-double bond in the dihydro-aromatic ring. The antibiotic action of usnic acid is due to the inhibition of oxidative phosphorylation (Asahina and Shibata 1954; Nash III 1996). However, very few of our lichen crude extracts showed inhibitory activities against Gram-negative bacteria. Burkholder et al. (1944) reported that no inhibitory activities were observed against *E. coli* by the 42 lichens tested. Due to a limited number of specimens, we could not pursue our observation of partial or limited activity against *E. coli* and *P. aeruginosa*.

TLC-bioautography was also conducted to detect the bioactive lichen acids. Our results showed galbinic acid, salazinic acid, usnic acid, norstictic acid, stictic acid, diffractaic acid, and barbatic acid to be the bioactive lichen acids (Figure 5). Stictic acid was detected in *Cladonia* species, while species of *Usnea* contained norstictic, usnic and diffractaic acids. *Ramalina* species, on the other hand, have usnic and barbatic acids as their bioactive metabolites. Interestingly, galbinic acid was the most commonly detected bioactive lichen acid among the representative lichen extracts. Nash III (1996) and Müller (2001) classified galbinic acid and three other lichen acids, salazinic acid, norstictic acid, and stictic acid, as depsidone. This metabolite was reported to have an anti-inflammatory effect and as an anti-constrictor in smooth muscles of humans (Müller 2001). Usnic acid was classified as dibenzofuran (Nash III 1996) and exhibits antiviral, antiprotozoal, antiproliferative, anti-inflammatory, and analgesic activities (Ingólfsdóttir 2002). Its

antibiotic action is due to the inhibition of oxidative phosphorylation, thereby, inhibiting oxygen consumption, electron transport chain, and other key mitochondrial functions in cells (Nash III 1996; Frankos 2005). In our study, several spots exhibiting bioactivity could not be identified (Figure 5). Two spots from *U. baileyi* (Rf values: 0.54, 0.13), 7 spots from *R. dendriscoides* (Rf values: 0.41, 0.42, 0.48, 0.49, 0.62, 0.80, 0.84), 3 spots from *C. gracilis* (Rf values: 0.47, 0.51, 0.60), and 1 spot from *S. massartianum* (Rf value: 0.42) could not be identified and, perhaps, are new bioactive metabolites.

CONCLUSION

Our study showed the potential of lichens from the Philippines and their crude extracts as new sources of biologically active secondary metabolites. Crude lichen extracts from 4 lichen species were found active against *S. aureus* and *B. subtilis*. The good inhibitory activities exhibited against these Gram-positive bacteria show the potential application against emerging multi-drug resistant bacteria, e.g., methicillin-resistant *S. aureus* and Vancomycin-resistant *Enterococcus faecalis*. The presence of unidentified bioactive lichen metabolites indicates the possibility of isolating novel antimicrobial compounds from the lichens in the Philippines.

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CONTRIBUTION OF THE INDIVIDUAL AUTHORS

All the authors contributed to the framing of the hypotheses and experimental design, to laboratory or field work, and to data analysis and interpretation. Manuscript preparation was done by Krystle Angelique A. Santiago and Thomas Edison E. dela Cruz.

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