ANTIMICROBIAL AND HERBICIDAL PROPERTIES OF THE FRUTICOSE LICHEN *Ramalina* FROM GUIMARAS ISLAND, PHILIPPINES

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ABSTRACT

Lichens, a unique symbiosis between a mycobiont and a photobiont organism, are known to produce metabolites that can be tapped as biopesticides for agriculture. Such property of the fruticose lichen *Ramalina* collected within Guimaras Island, Philippines was investigated in this study. A total of 195 specimens were collected and characterized using conventional morphological and chemical analyses. These lichens were identified as *Ramalina farinacea, R. roesleri,* and *R. nervulosa.* To test their potential application in agriculture, nine lichen specimens were extracted with acetone and assayed for its inhibitory activities against test bacteria, fungi, and weedy plants. All nine lichen extracts inhibited *Pseudomonas aeruginosa* (>19 mm ZOI) while only seven lichen extracts inhibited *Staphylococcus aureus* (13–19 mm ZOI). No inhibitory activity was observed among the fungal plant pathogens *Fusarium oxysporum, F. solani, F. verticillioides, Colletotrichum capsici,* and *C. gleosporioides*, and on the Gram-negative bacteria *Escherichia coli* and *Pectobacterium carotovorum* var. *carotovorum.* A decrease in the root (up to 27% reduction) and shoot (up to 39% reduction) lengths, and leaf chlorophyll content (up to 44% reduction) of rice weeds *Fimbristylis miliacea*, *Leptochloa chinensis* and weedy rice (*Oryza* sp.) were also observed. These results, therefore, suggested that the lichen crude extract from *Ramalina* is a potential biological control for weed management.

Keywords: antimicrobial, herbicide, lichens, tropics, weeds

INTRODUCTION

Lichens are an exceptional group of organisms having a mycobiont and a photobiont living in symbiosis. The photobiont component of lichen thalli can be algae, cyanobacteria or both (Nash 2008). Of the approximately 17,000 species of mycobionts, only about 40 species of photobionts are reported (Rikkinen *et al.* 2002). Lichens are widespread in many forest ecosystems (Dettki & Esseen 2003) and their number has increased to 25% since 1931. In spite of their huge diversity, lichens still remained poorly studied in many habitats. Little is known of the other missing species of lichens in the tropics (Sipman & Aptroot 2001). In the Philippines alone, many areas remain understudied in terms of lichen diversity. To complicate the matter, growing population and their activities also destroy pristine forests, thereby limiting the search for other lichen species.

Lichens produce several secondary metabolites including anthraquinones, xanthones, chromones, and secondary aliphatic acids and esters (Stojanović *et al.* 2011). Some of the most widely produced acids by lichens include usnic acid, stictic acid, and vulpinic acid (Stocker-Wörgötter & Elix 2004). These lichen acids are reported to have antimicrobial activities (Candan *et al.* 2007; Kosanić & Ranković 2010; De Jesus *et al.* 2016). Interestingly, one of the less studied lichen species in the Philippines is *Ramalina* of the

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family *Ramalinacea*. It is the richest in terms of regional endemism considering that some of the identified species are found only in a particular island (Krog & Østhagen 1980; Krog 1990; Aptroot & Schumm 2008; Kirk *et al.* 2008). These lichens are growing on a wide variety of substrates found in the rainforests (Nash 2008) and due to their renowned morphological plasticity (Pérez-Vargas & Pérez-Ortega 2014), had brought some difficulties to researches. The species is characterized as having shiny and bushy-like thallus and valued as food in Nepal and in Central and South Eastern Asian countries (Hanus *et al.* 2007). *Ramalina calicaris* (L.) Röhl is used as cosmetics in Europe and India, while *Ramalina farinacea* (L.) Ach. is valued for its antibacterial activity against *Staphylococcus aureus* (Hanus *et al.* 2007). Other species such as *Ramalina chilensis* Bertero ex Nyl., *R. farinacea, Ramalina arabum* (Dill. ex Ach.) Meyen & Flot., *Ramalina glaucescens* Kremp., *Ramalina unilateralis* F.Wilson, *Ramalina pacifica* Asah., and *Ramalina geniculata* Hook. f. & Taylor were studied for their lichen secondary metabolites (Tay *et al.* 2004; Hanus *et al.* 2007; Manojlovic *et al.* 2012). Studies show that among *Ramalina* species, similarities of lichen substances occur. For example, sekikaic acid was isolated from *R. geniculata, R. glaucescens, Ramalina tayloriana* Zahlbr., *Ramalina peruviana* Ach., and *R. farinacea.* Another common metabolite, norstictic acid, was found in *Ramalina subfraxinea* Nyl., *R. arabum, Ramalina lacera* (With.) J.R. Laundon and *R. farinacea.* Certain lichen substances may only occur on a specific *Ramalina* species and these include ramalinolic, lecanoric, divaricatic, homosekikaic, orsellic, and protocetraric acids. However, other lichen substances like, the orcinol depsides olivetoric acid, which are very common in several other large genera of lichens, are entirely unknown in *Ramalina* (Culberson 1965).

The Philippines is one of the mega diverse countries, yet little is known of their lichen flora. Researches on *Ramalina* in the Philippines were primarily based on the studies conducted by Vainio (1909) describing *Ramalina vittata* Nyl., *Ramalina pollinaria, Ramalina subfraxinea, Ramalina linearis* (Sw.) Ach., and *Ramalina gracilenta* Ach. Sevilla-Santos (1979) also studied *R. farinacea* while Gruezo (1979) focused on *R. pacifica* and *Ramalina nervulosa* (Müll. Arg.) Abbayes. Recently, the study of Santiago *et al.* (2010) identified four lichen genera including *Ramalina dendriscoides* Nyl. With this research gap, this study aimed to collect, characterize and identify different species of *Ramalina* from Guimaras Island based on morphological and chemical characteristics which were then tested for their antibacterial and antifungal activities against plant pathogens and for their herbicidal activity against weedy rice and rice weeds.

MATERIALS AND METHODS

Collection and Morphological Characterization of *Ramalina*

Located in the Western Visayas, Guimaras Island (10°34'N 122°35'E) is one of the smallest islands in the Philippines that has a total land area of 60,465 hectares and a land elevation ranging from 0 to ~300 meters above sea level. For this study, the island of Guimaras and its associated islets were divided into 12 equal quadrants. From these 12, the 10 accessible quadrants were chosen as the collection sites: Hoskyn (HO), Calingao (CA), Milan (MI), Piña (PI), Bulungawan (BU), Zaldivar (ZA), Morubuan (MO), Balacbacan (BA), Salvacion (SA), and Atgang (AT). Purposive sampling was used for the collection of lichen specimens within the collection site. Morphological characters were observed under dissecting and compound light microscopes and were used for the identification of each lichen specimen based on the ID keys of Stevens (1987), Goward (1999), Brodo *et al.* (2001) and Aptroot and Bungartz (2007).

Extraction and Identification of Secondary Metabolites of *Ramalina*

Of the 195 specimens collected, nine *Ramalina* specimens were air-dried and subjected to lichen acid extraction. Ten grams of the thallus were ground using a mortar and pestle until these became powdery. The powdered lichen was then placed in a 120 mL capped bottles, which were then added with 100 milliliter (mL) acetone and allowed to stand for 24 hours. The suspension was then filtered using Whatmann #6 filter paper, with the filtrate placed in a pre-weighed vial. Each filtrate was

left to dry until crystallized for 3-5 days and was reconstituted with acetone to make a final concentration of 10 mg/mL. The lichen crude extracts were stored in a 10°C refrigerator. The percent yield was calculated as the weight of crude extract over the weight of the thalli for extraction multiplied by 100. The chemical data were obtained following the standardized thin-layer chromatography (TLC) method. Acetone extracts of lichens were initially spotted on TLC plates (Merck Silica gel 60 F254) and developed using three solvent systems, namely, solvent system A [benzene:dioxane:acetic acid (180:45:5)], solvent system C [toluene:acetic acid (170:30)] and solvent system G [toluene:ethyl acetate:formic acid (139:83:8)] (Nash 2008; Ly *et al.* 2015). The Rf values of the lichen crude extracts were then calculated and compared with standard lichen acids.

Assay for Antimicrobial Activities

Antibacterial assay

The test bacteria, *Staphylococcus aureus* ATCC 25923, *Eschericia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Pectobacterium carotovorum* var. *carotovorum* were acquired from the UST Collection of Microbial Strains (USTCMS), University of Santo Tomas, Manila, Philippines and initially cultured on Nutrient Agar (NA, Hi-media) slants at 37°C for 24 hours. Following incubation, the bacterial suspensions were prepared and standardized by comparing with 0.5 McFarland standards (equivalent to $1.5x10^8$ cfu/mL). Using the Kirby-Bauer method, the bacterial inoculum was swabbed in a Petri dish pre-filled with 20 mL Mueller Hinton agar (MHA, Hi-Media). Twenty microliters (µL) of each lichen crude extract, with a concentration of 10 mg/mL, were poured into the paper discs (Whatmann, 6 mm diam.). The discs were then set to dry for 60 minutes in a separate empty sterile Petri dish before placing onto the inoculated culture plates (in triplicates). For positive control, Streptomycin (10 µg, BBL), Ampicillin (10 µg, BBL) and Gentamicin (10 µg, BBL) were used. The solvent acetone was used as the negative control. The plates were incubated at 37 °C for 18-24 hours, and then, the zones of inhibition were observed and measured in millimeters (mm) using a ruler. The bioactivity was assessed as follows: (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 & Santos 2005).

Antifungal Assay

Fusarium oxysporum BIO165255, *F. solani* BIO168270, *F. verticillioides* BIO169273, *Colletotrichum gleosporioides* BIO73202, and *C. capsici* BIO72199 were cultured in Potato Dextrose Agar (PDA, Hi-Media). All the test fungi were acquired from the Phytopathology Laboratory, SEAMEO-BIOTROP, Bogor, Indonesia. Initially, the test fungal pathogens were standardized to 10^6 spores/mL. These were then spread plated in triplicates on PDA plates following the protocol of Tiwari *et al.* (2011). For the treatment, Whatmann discs (6mm diam.) were loaded with 20 µL lichen extracts resulting in a final concentration of 200 µg, 1,000 µg and 2,000 µg per disc. The treated discs were air-dried for 60 minutes at room temperature and placed inside the plant pathogenic fungal culture plates. Commercially available Ketoconazole (10 mg) was used as the positive control. All plates were incubated for 5 days at room temperature. Antifungal activity was then evaluated by measuring the diameter of the zone of inhibition with the bioactivity assessed as follows: (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 & Santos 2005).

Assay for Herbicidal Activities

The rice weeds *Fimbristylis miliacea* (L.) Vahl and *Leptochloa chinensis* (L.) Nees and weedy rice (*Oryza* sp.) were used to assess the herbicidal activity of the lichen extracts. *F. miliacea* and *L. chinensis* seeds were provided by SEAMEO-BIOTROP, Indonesia while *Oryza* sp. (weedy rice) was obtained from the Philippine Rice Research Institute, Philippines. Seeds of weedy rice *Oryza* sp. and of the rice weeds *F. miliacea* and *L. sinensis* were initially allowed to germinate in a plastic tray up to 2-4 days in a greenhouse at temperatures of ± 34 °C. Five seedlings per pot in four replicates were placed in either a 500 gram- or 2 kg-capacity plastic pots containing

air-dried, well-sieved soil. The spray herbicide consisted of the lichen extracts dissolved in acetone (0.5 mL) and distilled water (49.5 mL) with a final concentration of 0.2 mg/mL. The formulated herbicide was then sprayed on the leaves of the 10-day old (four-leaf stage) test weeds. Distilled water with acetone was used as the control. On the 14th day of weed development, the length of the roots and shoots were measured. The chlorophyll content was also evaluated using a UV-VIS spectrophotometer at absorbances of 663 nm for chlorophyll a and 645 nm for chlorophyll b. The total chlorophyll content was computed and used to determine the percent reduction of chlorophyll.

RESULTS AND DISCUSSION

Ramalina of Guimaras Island

A total of 195 specimens belonging to three identified species were collected during two sampling times from five of the 10 sites in Guimaras Island: Hoskyn, Calingao, Milan, Piña, and Bulungawan (Table 1). Hoskyn has the highest number of species (all 3 species) identified from 54 samples, Calingao and Piña, has two species, *R. farinacea* and *R. nervulosa*, identified from 42 and 36 samples, respectively. Milan and Bulungawan, has only 1 species, *R. farinacea* identified from the 25 and 38 collected specimens respectively from these two sites. Past records of *Ramalina* lichen collections in the Philippines were mainly from the Luzon area (Vainio 1909; Sevilla-Santos 1979; Gruezo 1979; Santiago *et al.* 2010) which included the following species: *R. dendriscoides, R. farinacea, R. gracilenta, R. linearis R. nervulosa, R. pacifica, R. pollinaria, R. subfraxinea,* and *R. vittata*. The three collected specimens were differentiated using distinctive thallus characteristics (Table 1). This study reports for the first time, *R. roesleri* (Hochst. ex Schaer. Hue.) in the Philippines, as well as *R. farinacea* and *R. nervulosa* in Guimaras Island, thereby increasing the total number of *Ramalina* reported in the country to 10.

Lichen acids are known to possess herbicidal and antimicrobial properties (Halama & van-Haluwin 2004; Elix & Stocker-Wörgötter 2008; Tigre *et al.* 2012; Gazzano *et al.* 2013). Hence, the lichen acids present in *Ramalina* were also evaluated. The thin layer chromatography (TLC) profiles of the lichen acids from nine species in this study were determined using three solvent systems. Solvent system A detected seven lichen acids, solvent system C detected four, and solvent system G identified six lichen acids. Solvent system A detects dioxane bound with phenolic hydroxyl groups (Elix & Stocker-Wörgötter 2008). Solvent system C is a universal solvent used to differentiate lichen acids while solvent system G is useful in separating compounds with relatively low Rf values in solvents A and C (Nash 2008). A total of 14 lichen acids were identified, most of which were detected using solvent system A (Table 2). Among these, usnic acid, barbatic, stictic, norstictic, and salazinic are the most common. Remarkably, the sekikaic acid, which is a chemotaxonomic marker for the genus *Ramalina*, was also detected (Culberson 1965).

Table 1 The three *Ramalina* species collected from Guimaras Island

Taxa	Distinctive Morphological Traits	Number of Collected Specimens per Site[a]					Total
		HO	CA	PI	MI	BU	
 *R. farinacea*	Presence of dichotomous-anistomic branching and soralia on the margins and laminae of the thallus	39	40	32	25	38	174

Table 1 Continued

Taxa	Distinctive Morphological Traits	Number of Collected Specimens per Site[a]					Total
		HO	CA	PI	MI	BU	
 *R. nervulosa*	Presence of twisting and slit-like soralia	6	2	4	-	-	12
 *R. roesleri*	Presence of a granular apical soralia	9	-	-	-	-	9
Total		54	42	36	25	38	195

Note: [a]Hoskyn (HO), Calingao (CA), Milan (MI), Piña (PI), Bulungawan (BU). No lichen specimens were collected from Zaldivar (ZA), Morubuan (MO), Balacbacan (BA), Salvacion (SA), and Atgang (AT).

Table 2 The identified secondary metabolites from the collected lichen *Ramalina*

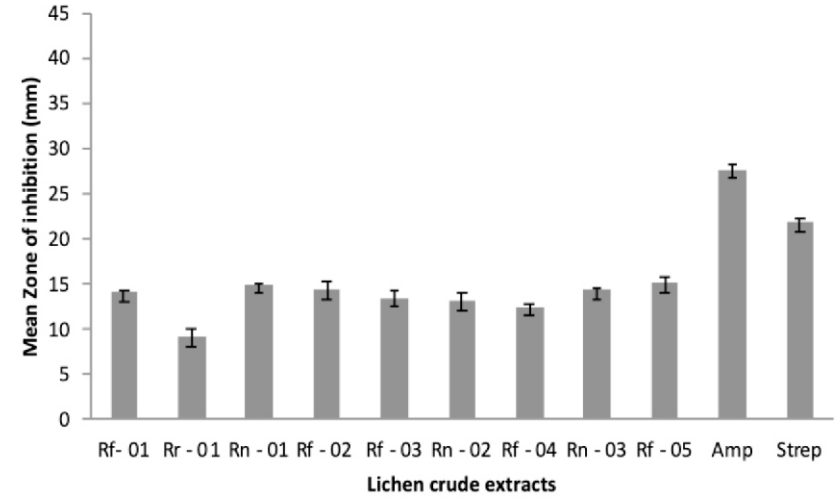
Solvent System	Detected Lichen Acids	Lichen Species								
		Rf01[a]	Rr01	Rn01	Rf02	Rf03	Rn02	Rf04	Rn03	Rf05
A	Barbatic	+[b]	-	+	+	+	+	+	+	+
	Constictic	-	+	-	-	-	-	-	-	-
	Hypoprotocetraric	+	+	+	+	+	+	+	+	+
	Nortstictic	+	+	+	+	+	+	+	+	+
	Perlatolic	-	+	-	-	-	-	-	-	-
	Stictic	-	-	-	-	-	-	-	-	+
	Usnic	-	+	+	-	+	-	-	-	-
C	Divaricatic	-	+	-	-	-	+	+	-	-
	Salazinic	-	+	+	+	+	+	+	+	+
	Sekikaic	+	+	+	+	+	+	+	+	+
	Usnic	+	+	+	+	+	+	+	+	+
G	Confumaprotocetraric	-	+	-	-	-	-	-	-	-
	Consalazinic	-	+	-	-	-	-	-	-	-
	Isonotatic	-	+	-	-	-	-	-	-	-
	Norstictic	+	-	+	+	+	+	+	+	+
	Protocetraric	-	-	+	-	-	-	-	-	-
	Usnic	-	+	-	+	+	-	+	-	-

Note: [a]Rf = *Ramalina farinacea*, Rr = *Ramalina roesleri*, Rn = *Ramalina nervulosa;*
[b](+) = Lichen acid detected; (-) = lichen acid not detected

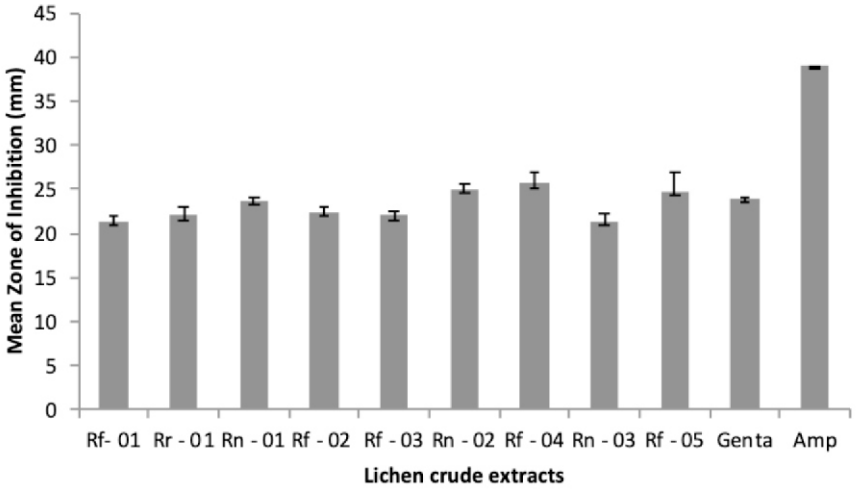
Inhibitory Activities of *Ramalina*

Antimicrobial Activities. Eight of the lichen crude extracts exhibited partially active (10-12 mm ZOI) to active (13-19 mm ZOI) inhibitory activities against *Staphylococcus aureus* while all nine extracts were very active (>19 mm ZOI) against *Pseudomonas aeruginosa* (Fig. 1). The observed bioactivities may be attributed to the impairment of DNA replication and RNA

synthesis of the test bacteria (Maciąg-Dorszyńska *et al.* 2014). The major substance of lichens, i.e. usnic acid, also possesses active centers that target the bacterial cells (Shrestha & St. Clair 2013). Specifically, 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 2008). However, no inhibition was observed against *Escherichia coli* and tomato rot-causing *Pectobacterium carotovorum* var *carotovorum*. In similar studies of Santiago *et al.* (2010; 2013), the lichen crude extracts against Gram-negative and/or Gram-positive bacteria were also inactive to partially active. Furthermore, the absence of inhibitory activity was observed against the five fungal plant pathogens, i.e. *Fusarium oxysporum*, *F. solani*, *F. verticillioides*, *Colletotrichum gleosporioides,* and *C. capsici*. The lichen crude extracts of *Ramalina* failed to suppress the mycelial growth of these test fungi. Although previous studies showed that other lichen species could inhibit the tested plant fungal pathogens, results in this study were similar to those obtained by Candan *et al.* (2006) and Tiwari *et al.* (2011). This selective activity may be attributed to the differences in the secondary metabolites produced by different lichen species (Halama & van Haluwin 2004; Goel *et al.* 2011).

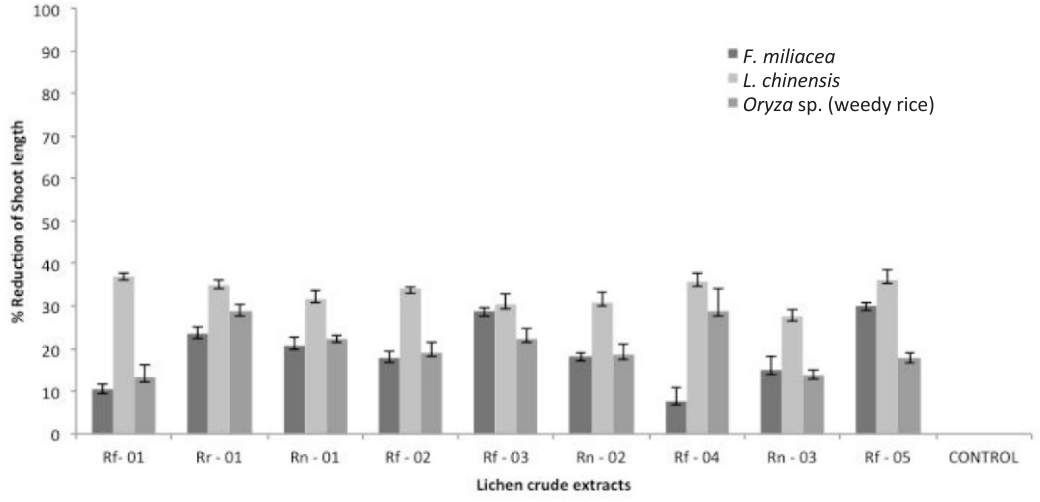


A

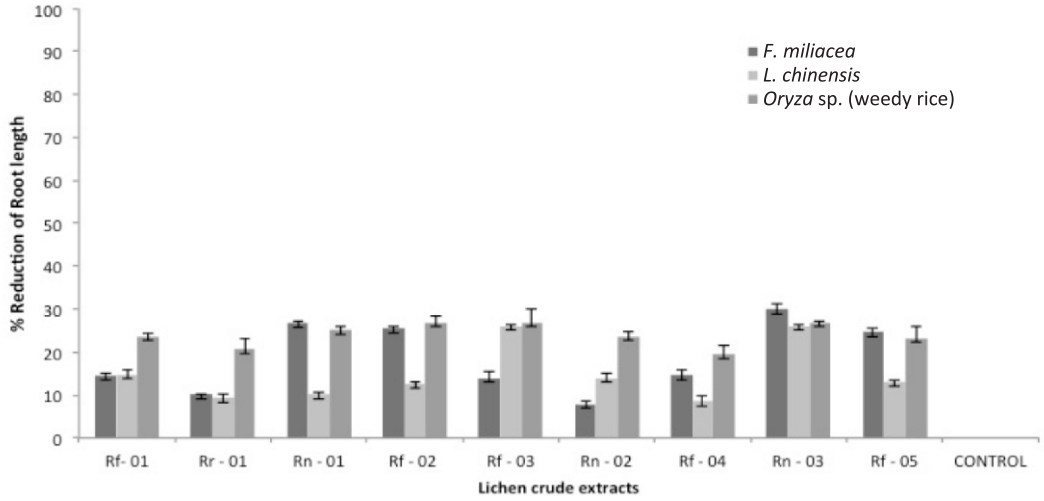


B

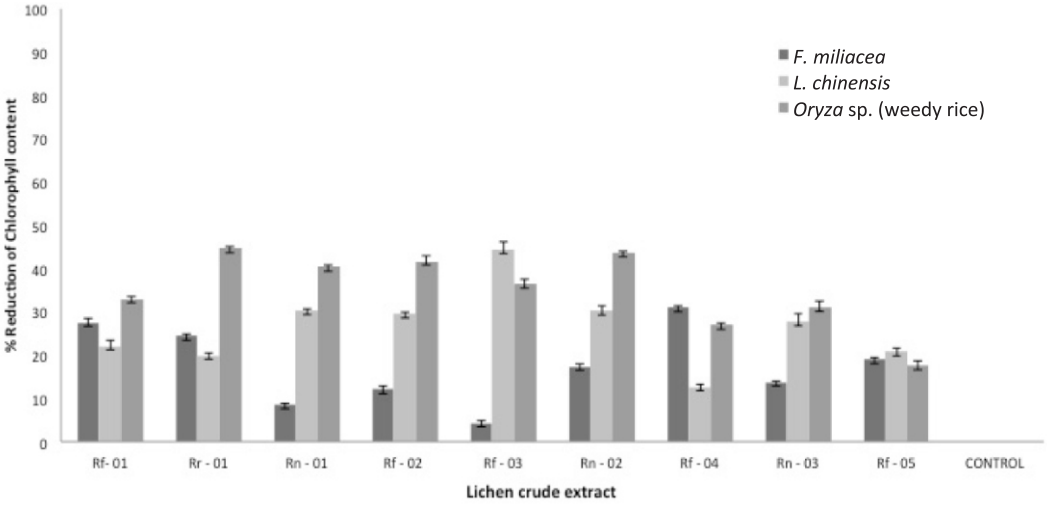
Figure 1 Zones of inhibition exhibited by lichen crude extracts against the *S. aureus* (A) and *P. aeruginosa* (B). Standard errors are indicated on bars



A



B



C

Figure 2 Percent reduction in shoot (A) and root (B) lengths and chlorophyll content (C) of the three test weeds. Standard error was indicated on bars

Herbicidal Activities. Results of this study showed a decrease in shoot (up to 39%) and root lengths (up to 27%) of the rice weeds *Fimbristylis miliacea*, *Leptochloa chinensis* and weedy rice *Oryza* sp. (Fig. 2). This indicates the potential growth-reducing activity of the *Ramalina* lichen crude extracts. Results also showed a reduction in the total chlorophyll content of up to 44%, a condition which would eventually result in chlorosis and necrosis in the affected leaf area as a decrease in chlorophyll level would affect the photosynthetic mechanism of plants (Radosevich *et al.* 2007).

The occurrence of weeds is a concern among farmers. Damage and stunted growth of crops due to these weeds has caused millions of losses in crop production (Quayyum *et al.* 2000; Marambe & Amarasinghe 2002). Unfortunately, the use of chemical herbicides to eradicate these weed pests has accompanying public health, environment, and safety concerns. Hence, there is a need to look for less toxic and eco-friendly but equally effective herbicidal agents. Lichens can be possibly tapped for this endeavor as the herbicidal potential of lichens was previously observed in *Cladonia verticillaris* (Raddi) Mont. (Tigre *et al.* 2012). The lichen extracts diminished the hypocotyl growth of the model plant, *Lactuca sativa.* By increasing the concentration of the lichen extracts, an abnormal growth was also observed on *L. sativa* (Tigre *et al.* 2012). Goel *et al.* (2013) also reported the allelopathic potential of the lichen *Parmelia reticulata* Tayl. against *Phalaris minor* Retz. by reducing shoot and root lengths and affecting seed germination.

The Philippines is as an agricultural country that produces rice in greater volume. An infestation by the rice weeds *F. miliacea* and *L. sinensis* and weedy rice *Oryza* sp. can have a disastrous effect in the county's food security and economy.

Although the mode of action of the lichen extracts as an herbicide was not determined in this study, changes in the entire morphology of the test weeds can happen due to the resulting alteration in the mitochondrial respiration processes, if a potential herbicidal compound is applied (Gniazdowska & Bogatek 2005). This will impede the ATP yield of the plant for biochemical processes that is eventually detrimental to the growth of the plant. The presence of usnic acid in the lichen extracts may also contribute to the herbicidal properties of *Ramalina*. Usnic acid has a blocking action against a specific key plant enzyme, 4-hydroxyphenylpyruvate dioxygenase (Conchietto *et al.* 2002). However, usnic acid has two enantiomers: (+) usnic and (-) usnic acid. Therefore, test is needed to determine if both or one of the enantiomers exhibits the herbicidal properties. Results of this study further substantiated the potential of lichens as biocontrol agents.

CONCLUSION

Three species of *Ramalina* were found mainly from the elevated areas in Guimaras Island, Philippines and were identified as *R. farinacea, R. nervulosa* and *R. roesleri*. This is the first record of *Ramalina* in Guimaras and in the Visayas. The species, *R. roesleri*, is also reported for the first time in the Philippines. A total of 14 lichen acids were detected. Changes attributed to the presence of lichen acids were also observed in the reductions in shoot and root lengths and in the total chlorophyll content in the rice weeds *Fimbristylis miliacea* and *Leptochloa chinensis,* and the weedy rice *Oryza* sp. This is supported by the fact that usnic acid, which is present in all collected samples, is known for its herbicidal activity. Moreover, the lichen crude extracts were active against the Gram-positive bacteria *S. aureus* and the Gram-negative *P. aeruginosa* as reported in similar studies. However, the *Ramalina* lichen extracts failed to inhibit any fungal plant pathogens. Nevertheless, fruticose lichens such as *Ramalina,* can still be tapped by industrial companies for the production of biocides.

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