ANTIMICROBIAL AND HERBICIDAL ACTIVITIES OF THE FRUTICOSE LICHEN RAMALINA FROM GUIMARAS ISLAND, PHILIPPINES

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ABSTRACT

Lichens, a unique symbiosis between two or three organisms, are known to produce metabolites that can be tapped as biopesticides for agriculture. In this research study, the fruticose lichen *Ramalina* was collected within Guimaras Island, Philippines. 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 for 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 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 against the fungal plant pathogens *Fusarium oxysporum, F. solani, F. verticillioides, Colletotrichum capsici,* and *C. gleosporioides*, and the Gram-negative bacteria *Escherichia coli* and *Pectobacterium carotovorum* var. *carotovorum*. Furthermore, there was also 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 *Fimbristylis miliacea*, *Leptochloa chinensis* and weedy rice (*Oryza* sp.). These results, therefore, suggested the potential of lichen extracts from *Ramalina* as a biological control for weed management.

Keywords: antimicrobial, herbicide, lichens, 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 III 2008). There are approximately 17,000 species of mycobionts whereas only about 40 species of photobionts were reported (Rikkinen *et al.* 2002). Lichens are widespread in many forest ecosystems (Dettki & Esseen 2003) and this number has increased to 25% since 1931. In spite of their huge diversity, many habitats still remained poorly studied for lichens. It was suggested that the other missing species of lichens could be found in the tropical parts of the world (Sipman & Aptroot 2001). In the Philippines alone, many areas remain understudied in terms of lichen diversity. To complicate the matter, anthropological activities as well as the growing population also destroy pristine forests, therefore 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

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include usnic acid, stictic acid, and vulpinic acid (Stocker-Wörgötter & Elix 2004). These lichen acids were 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* under the lichen family *Ramalinacea*. This 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*. 2008). *Ramalina calicaris* (L.) Röhl, in particular, is used as cosmetics in Europe and India, while *R. farinacea* (L.) Ach. is valued for its antibacterial activity against *S. aureus* (Hanus *et al.* 2008). Other species such as *R. chilensis* Bertero ex Nyl., *R. farinacea, R. arabum* (Dill. ex Ach.) Meyen & Flot*., R. glaucescens* Kremp*., R. unilateralis* F.Wilson*, R. pacifica* Asah*.,* and *R. geniculata* Hook. f. & Taylor were studied for their lichen secondary metabolites (Tay *et al.* 2004; Hanus *et al.* 2008; Manojlovic *et al.* 2012). It was shown that among *Ramalina* species, similarities of lichen substances might occur. For example, sekikaic acid was isolated from *R. geniculata, R. glaucescens, R. tayloriana* Zahlbr*., R. peruviana* Ach*.,* and *R. farinacea.* Another common metabolite, norstictic acid, was found in *R. subfraxinea* Nyl*., R. arabum, R. lacera* (With.) J.R. Laundon and *R. farinacea.* However, there are certain lichen substances that may only occur on a specific *Ramalina* species and these include ramalinolic, lecanoric, divaricatic, homosekikaic, orsellic, and protocetraric acids. Interestingly, there are also lichen substances such as the orcinol depsides olivetoric acid, which are very common in several other large genera of lichens, but are entirely unknown in *Ramalina* (Culberson 1965).

The Philippines is considered as one of the most diverse countries, yet a study on their lichen flora is limited. Researches on *Ramalina* in the Philippines were primarily based on the studies conducted by Vainio (1909) describing *R. vittata* Nyl*., R. pollinaria, R. subfraxinea, R. linearis* (Sw.) Ach*.,* and *R. gracilenta* Ach. Furthermore, Sevilla-Santos (1979) focused on *R. farinacea* while Gruezo (1979) studied *R. pacifica* and *R. nervulosa* (Müll. Arg.) Abbayes. A recent study of Santiago *et al.* (2010) identified four lichen genera in which *Ramalina dendriscoides* Nyl. was identified. With this huge gap, our research study aimed to collect, characterize and identify different species of *Ramalina* from Guimaras Island based on their morphological and chemical tests. In addition, extracted secondary metabolites from each lichen thalli were then tested for antibacterial and antifungal activities against plant pathogens. Finally, the herbicidal activity of the lichen crude extracts against weedy rice and rice weeds was assessed.

MATERIALS AND METHODS

Collection and Morphological Characterization of *Ramalina*

Guimaras Island (10°34'N 122°35'E) is one of the smallest islands in the Philippines located in Western Visayas. It has a total land area of 60,465 hectares and a land elevation ranging from 0 to ~300 meters above sea level. In this study, the island of Guimaras and its associated islets were divided into 12 equal quadrants. From these, 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 the strategy used for the collection of lichen specimens within the collection site. Morphological characters were observed for each lichen specimen under dissecting and compound light microscopes and used for their identification 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 until powdery using a mortar and pestle. The powdered lichen was then placed in a 120 ml capped bottles, which

was then added with 100 milliliter (ml) acetone and allowed to stand for 24 hours. Then, the suspension was 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. Also, percent yield was calculated as the weight of crude extract over the weight of the thalli for extraction multiplied by 100. Furthermore, the chemical data were obtained following the standardized 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 III 2008; Ly *et al.* 2015). The Rf values of the lichen extracts were 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, bacterial suspensions were prepared and standardized by comparing with 0.5 McFarland standard (equivalent to $1.5x10^8$ cfu/ml). Using the Kirby-Bauer method, the bacterial inoculum was swabbed in a Petri dish pre-filled with 20ml Mueller Hinton agar (MHA, Hi-Media). Twenty microliters (µl) of each lichen extract, with a concentration of 10 mg/ml, were added to 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 (10µg, BBL), Ampicillin (10µg, BBL) and Gentamicin (10µg, BBL) were used. The solvent acetone was used as negative control. Plates were incubated at 37°C for 18-24 hours, and then the zones of inhibition were observed and measured using a ruler (in millimeters, mm). 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 test fungi were acquired from the Phytopathology Laboratory, SEAMEO-BIOTROP, Bogor, Indonesia. Initially, 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 rendering 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 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 while *Oryza* sp. (weedy rice) was obtained from the Philippine Rice Research Institute, Philippines. Seeds of *Oryza* sp. (weedy rice) and 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 (Temperature: ± 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 constituted 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 test weeds after 10 days (four-leaf stage). 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

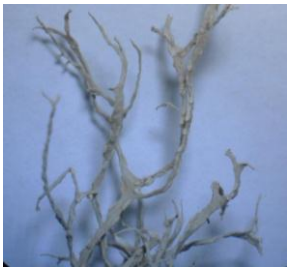
The *Ramalina* of Guimaras Island

The lichen *Ramalina* is one of the largest lichen genera (Kirk *et al.* 2008). It is the richest in terms of regional endemism (Aptroot & Schumm 2008) wherein some of the identified species were found only in a particular island (Krog & Østhagen 1980; Krog 1990). *Ramalina* are found in the rainforests from a wide variety of substrates (Nash III 2008). Moreover, studying this genus is also a difficult one due to its renowned morphological plasticity (Pérez-Vargas & Pérez-Ortega 2014).

In this study, a total of 195 specimens were collected during two sampling times from five of the 10 sites in Guimaras Island: Hoskyn, Calingao, Milan, Piña, and Bulungawan (Table 1). Among the collected *Ramalina* samples, Hoskyn had the highest number of species (all 3 species) identified from 54 samples. In Calingao and Piña, two species, *R. farinacea* and *R. nervulosa*, were identified from 42 and 36 samples, respectively. In Milan and Bulungawan, only 1 species, *R. farinacea*, was identified from the 25 and 38 collected specimens in these two sites. Past records of *Ramalina* lichens in the Philippines were mainly collected 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). In this study, we report for the first time *R. roesleri* (Hochst. ex Schaer.) Hue. in the Philippines, as well as *R. farinacea* and *R. nervulosa* in Guimaras Island. This brings the total number of *Ramalina* reported in the country to 10.

Lichen acids have shown various biological activities as an antimicrobial and herbicide (Halama & Van-Haluwin 2004; Elix & Stocker-Wörgötter 2008; Tigre *et al.* 2012; Gazzano *et al.* 2013). Hence, it is interesting to evaluate the lichen acids present in *Ramalina*. In our study, nine specimens were extracted with acetone as most lichen acids were soluble with this organic solvent. Thin layer chromatography (TLC) profiles 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 III 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. Interestingly, 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 marginal and laminal on the thallus	39	40	32	25	38	**174**
 *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**

[a] Hoskyn (HO), Calingao (CA), Milan (MI), Piña (PI), Bulungawan (BU)
[b] 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	-	+	-	+	+	-	+	-	-

[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 *S. aureus* while all nine extracts were very active (>19 mm ZOI) against *P. 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). In addition, the major substance of lichens, i.e. usnic acid, has active centers that targets 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 III 2008). However, no inhibition was observed against *E. coli* and tomato rot-causing *P. carotovorum* var *carotovorum*. In the studies of Santiago *et al.* (2010, 2013), the lichen 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).

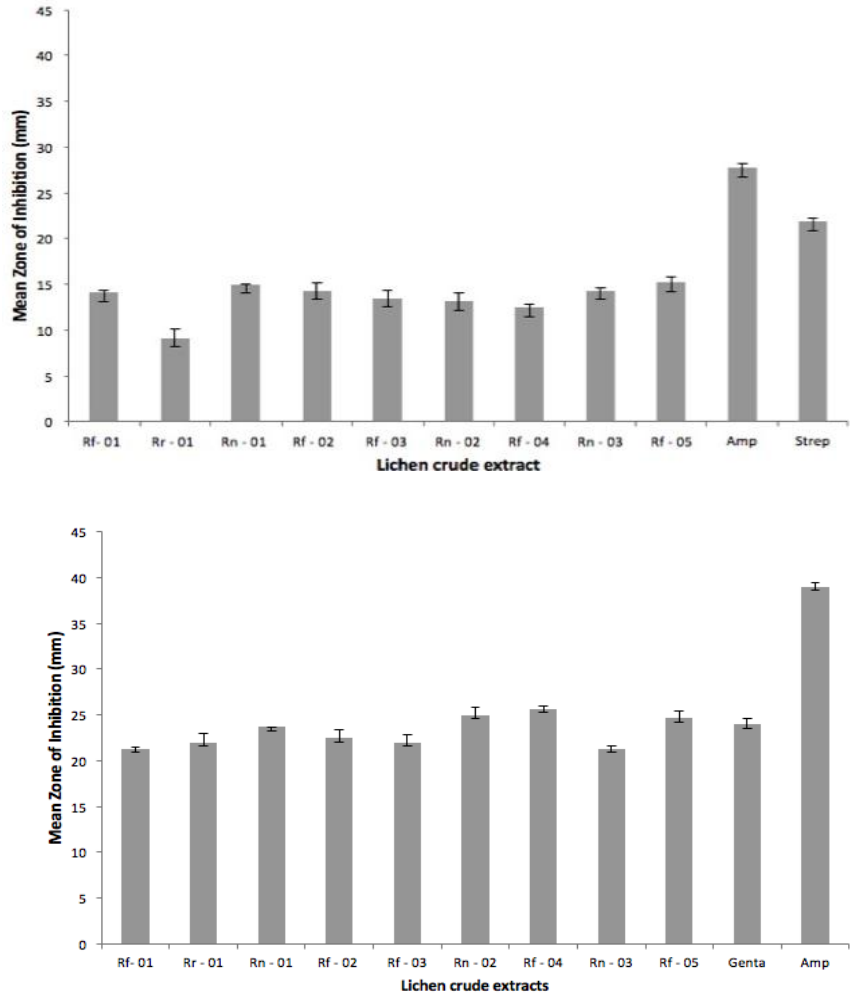
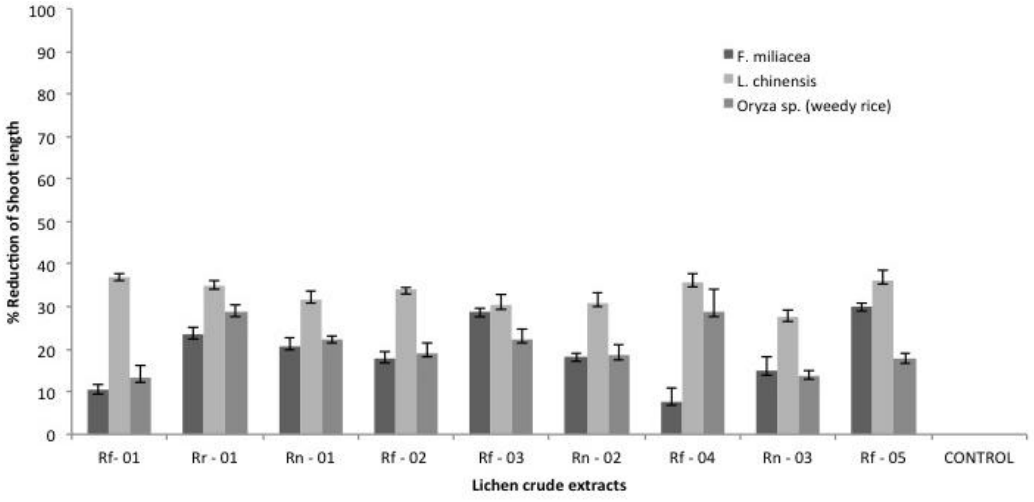


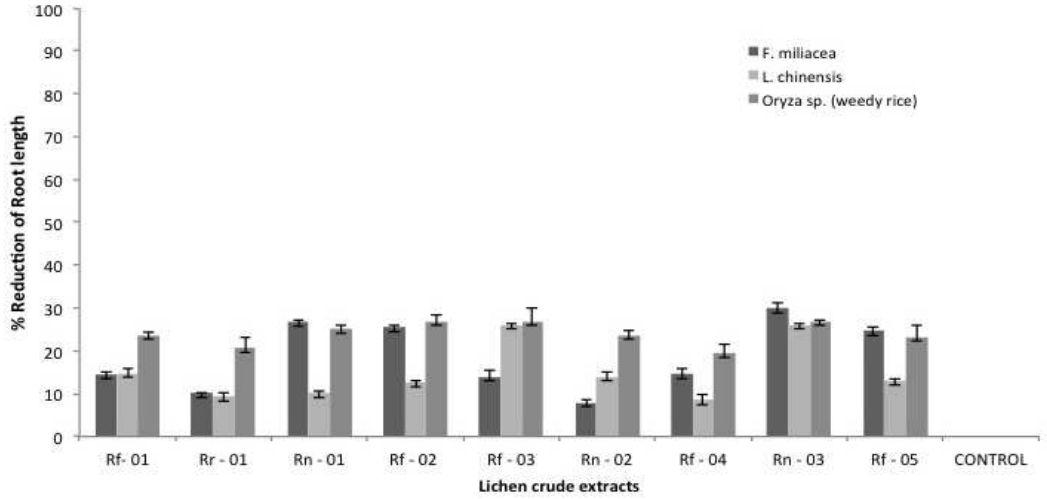
Figure 1 Zones of inhibition exhibited by lichen crude extracts against the *S. aureus* (A) and *P. aeruginosa* (B). Standard error were indicated on bars

Herbicidal Activities. There is a concern among farmers with regards to the occurrence of weeds. Damage and stunted growth of crops due to these weeds 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 also has 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, e.g. in *Cladonia verticillaris* (Raddi) Mont. (Tigre *et al*. 2012). In this study, 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. 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. In our study, we tested our lichen extracts against rice weeds and weedy rice. The Philippines is as an agricultural country that produces rice in greater volume. An infestation by these weeds can have a disastrous effect in our county's food security and economy. Results of our tests showed a decrease in shoot (up to 39%) and root lengths (up to 27%) of the weedy rice and rice weeds (Fig. 2). This indicates the potential growth-reducing activity of the *Ramalina* lichen extracts.

To further assess the herbicidal potential of our lichen extracts, we also measured the chlorophyll content of the test weeds. Our data showed a reduction of total chlorophyll content up to 44% (Fig. 2). This eventually resulted in chlorosis and necrosis in the affected leaf area. Radosevich *et al*. (2007) noted that a decrease in chlorophyll level would affect the photosynthetic mechanism of plants. The mode action of the lichen extracts as an herbicide was not determined in this study. However, based on the previous report of Gniazdowska & Bogatek (2005), changes in the entire morphology of the test weeds can be due to the alteration in the mitochondrial respiration processes if a potential herbicidal compound is applied. This will impede ATP yield of the plant for biochemical processes that will eventually lead to the detrimental growth of the plant. Interestingly, the presence of usnic acid in our lichen extracts may also contribute to the herbicidal properties of *Ramalina*. Conchietto *et al*. (2002) argued that usnic acid has a blocking action against a specific key plant enzyme, 4-hydroxyphenylpyruvate dioxygenase. The presence of usnic acid in our lichen extracts may also contribute to the herbicidal properties of *Ramalina*. However, usnic acid has two enantiomers: (+) usnic and (-) usnic acid. Therefore, it remains to be investigated if both or one of the enantiomers exhibited the herbicidal activities. Results of our study further substantiated the potential of lichens as biocontrol agents.



A



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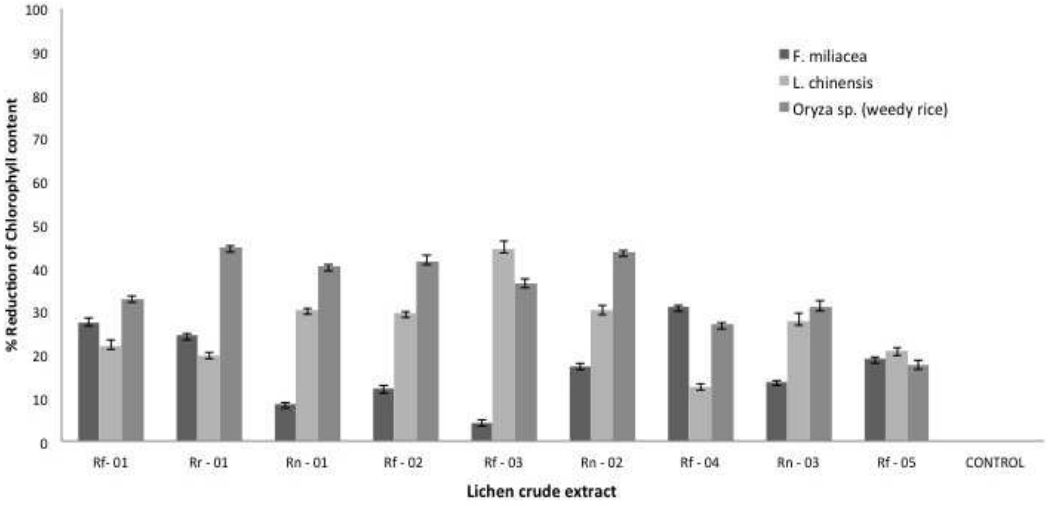

C

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.

CONCLUSION

Our study reported three species of *Ramalina* in the island of Guimaras: *R. farinacea, R. nervulosa* and *R. roesleri*. These were recorded mainly from elevated areas within the island. Interestingly, this is the first time *Ramalina* is reported in Guimaras and in the Visayas. One species, *R. roesleri*, is also reported for the first time in the Philippines. Furthermore, a total of 14 lichen acids were detected. Reductions in shoot and root lengths and in total chlorophyll content in the weedy rice and rice weeds were observed. These changes can be attributed to the presence of lichen acids. In fact, 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 similarly reported in other studies. However, the *Ramalina* lichen extracts failed to inhibit any fungal plant pathogens. Nevertheless, fruticose lichens such as *Ramalina,* can be tapped by industrial companies for the production of biocides.

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