

Antibacterial and Antioxidant Capacity of Polar Microorganisms Isolated from Arctic Lichen *Ochrolechia* sp.

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Abstract

Although many scientists have been studying lichens, the bacterial communities among lichens have not been fully explored. In this study, the biological activities of bacterial isolates from Arctic lichen *Ochrolechia* sp. were screened. Cell cultures were extracted using ethyl acetate, and these cultures were tested for antibacterial and antioxidant activities. To evaluate the antibacterial activities, we carried out a disk diffusion test and minimum inhibitory concentration assay. The inhibition zone diameter (mm) was 8 to 12, and its MIC value ranged from 222.0 to > 1000 µg/ml (control antibiotic ampicillin ranged from 0.31 to 0.58 µg/ml). PAMC26625 (*Sphingomonas* sp., sequence similarity: 99.108%) showed a high amount of total phenolic and total flavonoid contents within 5 extracts and also exhibited 77.23% and 80.02% in the 1,1-diphenyl-2-picryl-hydrazyl assay and the 2,2'-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid) assay, respectively. In the ferric reducing antioxidant power assay, it was determined that PAMC26625 contained a higher amount of ferrous ion [8.72 mM Fe(II)/mg extract] than control ascorbic acid [6.21 mM Fe(II)/mg extract]. Our data indicated that they had potential to be novel sources of antibacterials and antioxidants.

Key words: Arctic, antibacterial/antioxidant activity, bacterial association, lichen, *Ochrolechia* sp.

Introduction

Lichens are symbiotic organisms that are composed of a fungus and an alga, and they can survive in extreme environments such as tropical, desert, and polar regions. Lichens and their natural products have been used as cosmetics, decorations, dyes, foods, and medicines (Oksanen, 2006). They have been attracting the attention of many researchers because of their diverse pharmaceutical potentials as shown in their antiviral, anti-proliferative, anti-inflammatory, anti-tumor, and antimycobacterial activities (Ingólfssdóttir *et al.*, 1998; Lauterwein *et al.*, 1995; Lawrey, 1989; Molnár and Farkas, 2010; Morita *et al.*, 2009). Also, lichens are considered to be a source of natural antioxidants because while synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ) are commercially available, they have proven to be highly carcinogenic (Grice, 1996). Because of the lichens' inhibitory effects against microorganisms, their antimicrobial activities have also been widely investigated. In general, the natu-

ral products from lichens are their secondary metabolites, called "lichen substance", and most of these come from the fungal symbionts in lichens (Luo *et al.*, 2011; Stocker-Wörgötter, 2008).

Recent molecular studies have suggested a variety of bacterial communities among lichens, including their structural and ecological contributions. For examples, Gonzales *et al.* (2005) described the diversity of actinomycetes among lichens by DNA fingerprinting; they also evaluated the genes involved in the secondary metabolite production of 337 isolates. Cardinale *et al.* (2006 and 2008) showed that diverse bacteria are present in lichens by the analysis of their ribosomal internal transcribed spacer polymorphism and by fluorescence *in situ* hybridization analysis. Grube *et al.* (2009) also analyzed and compared the structure and composition of associated bacterial communities of some lichen species using combined microscopic and molecular techniques; however, their biological activities have not been studied in detail. The biological activities and bacterial diversity of Arctic lichen *Ochrolechia* sp. are still unknown. In previous studies, we screened

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nine bacterial isolates from Arctic lichen *Cladonia* sp., *Stereocaulon* sp., *Stereocaulon* sp., and *Umbilicaria* sp. and evaluated their antibacterial and antioxidant activities. We found that bacterial isolates from lichen exhibited some antibacterial activities against Gram-positive and Gram-negative clinically isolated microorganisms. They also showed better antioxidant activity than the control ascorbic acid (vitamin C). Therefore, the aim of this study was to screen the microorganisms from lichen *Ochrolechia* sp., and evaluate their antibacterial potential against six test microorganisms and discern their antioxidant potential.

Experimental

Materials and Methods

Collection and identification of lichen samples.

Lichen *Ochrolechia* sp. was collected in Ny-Ålesund, Svalbard, Dasan Korean Arctic Station (78, 91.140' N/ 011, 94.878' E) by the Korean Polar Research Institute (KOPRI). It was transferred at room temperature and stored at -20°C until further use. Bacterial isolates were deposited in polar and alpine microbial collection (PAMC).

Screening of microorganisms associates with lichen. The isolation of microorganisms was performed by KOPRI. Fragments from lichen thalluses were separated by sterilized scissors or knife. Sterilized 0.85% NaCl solution was added and this was followed by vortexing for 10 min. The solution was then discarded, and the steps above were repeated three times. The tissue was subsequently broken with a mortar in sterilized 0.85% NaCl solution. After spreading the tissue on malt extract-yeast extract (MY) agar media and Reasoner's 2A (R2A) agar, it was incubated at 10°C for 15 to 21 days. To obtain pure single colonies, subculturing was repeated three times and it was preserved at -80°C in 20% glycerol. The obtained bacterial isolates were identified by 16S rRNA gene sequences analysis. The 16S rRNA gene was amplified from a single colony of pure culture with two universal primers: 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') as described by Lane (1991). Polymerase chain reaction (PCR) was carried out with 25 μl reaction mixtures containing 1X PCR reaction buffer, 200 μM of dNTPs, 0.2 μM of each primer, a single colony as a template, and 1 unit of *Taq* DNA polymerase (In-Sung Science, Suwon, South Korea). The PCR procedure included an initial denaturing step at 95°C for 5 min and 30 cycles of amplification (95°C for 30 s, 56°C for 30 s, and 72°C for 30 s) and a final extension step at 72°C for 5 min. PCR products were purified with an AccuPrep PCR Purification Kit

(Bioneer, Daejeon, South Korea) and sequenced with the same primer used for PCR amplification. The sequence of the 16S rRNA gene was compared with that of type strains available in the database to find closely related species. All sample numbers were given by PAMC in KOPRI.

Culture and extraction of bacterial isolates. A total of five bacterial isolates were cultured in 50 ml of MY (4.0 g malt extract, 4.0 g yeast extract, 10.0 g glucose, and 1.0 L, pH 7.2 distilled water) and R2A (0.5 g proteose peptone, 0.5 g casamino acids, 0.5 g yeast extract, 0.5 g dextrose, 0.5 g soluble starch, 0.3 g dipotassium phosphate, 0.05 g magnesium sulfate-7H₂O, 0.3 g sodium pyruvate, and 1.0 L, pH 7.2 distilled water) liquid media at 15°C for 10 to 15 days. The culture broth was added to a double volume of ethyl acetate which was of analytical grade (Daejung, Shiheung, South Korea). Extraction was performed individually on each solvent at room temperature and then the layer of culture media was discarded after 2 hours. The solvent layer was concentrated using a rotary evaporator, and the obtained dried crude extract was dissolved in 500 μl of ethyl acetate. The concentrations of extract ranged from 1.2 g/ml to 1/7 g/ml. Each extract was diluted to 1 mg/ml for further experiments.

Evaluation of the antimicrobial activities of test microorganisms. Ethyl acetate extracts were tested against the 3 Gram-positive bacteria *Staphylococcus aureus* (KCTC1927), *Bacillus subtilis* (KCTC1028), and *Micrococcus luteus* (KCTC9341), and 3 Gram-negative bacteria *Escherichia coli* (KCTC1682), *Pseudomonas aeruginosa* (KCTC1637), and *Enterobacter cloacae* (KCTC1685) used in this study. All of the six test strains are known as clinical isolates. They were purchased from the Korean Collection for Type Cultures (KCTC) and Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, South Korea). All bacterial isolates were kept on Luria-Bertani (LB; 10.0 g trypton, 5.0 g yeast extract, 10.0 g NaCl, and 1.0 L, pH 7.2 distilled water) at 4°C .

Paper disk diffusion test. The paper disk diffusion test was performed according to Bauer *et al.* (1966) with some modifications. All reagents were purchases from Difco (Franklin Lakes, USA). Bacterial cells were standardized to 0.5 McFarland and then mixed with soft agar (0.04 g/ml). 9 ml of this mixture was inoculated onto Mueller-Hinton agar plates. Afterwards, each extract was loaded into paper disks (6 mm in diameter, Advantec, Osaka, Japan) and transferred onto the plates inoculated with the bacterial strains. Disks loaded with ethyl acetate were used as a control. All inoculated culture plates were incubated at 37°C , and the inhibition zones of bacterial growth were measured after 12 to 18 h.

Minimum inhibitory concentration (MIC) test. MIC was determined by the broth dilution method

(Swenson *et al.*, 1982) with some modifications. Dilutions of culture extract ranged from 0 to 1000 µg/ml in 5 ml of Mueller-Hinton broth. Final concentrations of test organisms were adjusted to 10⁶ CFU/ml. The inoculated tubes were incubated in a shaker (250 rpm) at 37°C for 24 h. MIC was measured by a UV-Vis spectrophotometer at 600 nm to determine growth.

Evaluation of antioxidant activities: total phenolic contents (TPC) and total flavonoid contents (TFC) tests. TPC was evaluated by the Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) with some modifications. All of the chemical reagents were purchased from Sigma-Aldrich (St. Louis, USA). The reaction mixture was incubated for 30 min at room temperature and the absorbance was determined at 760 nm using a UV/Vis spectrophotometer (Biochrom, Holliston, USA). Gallic acid used as the positive control and the reaction mixture without the extract was used as the negative control. The concentration of TPC was expressed in milligrams of gallic acid equivalent (GAE) per g of extract. TFC was evaluated by the colorimetric method described previously (Zhishen and Mengcheng, 1999). The absorbance of reaction mixture was measured at 510 nm using a UV/vis spectrophotometer. Catechin was used as a standard compound for the quantification of total flavonoids. All the values were expressed as milligrams of catechin equivalent (CE) per gram of extract. Each extract was measured three times.

Free radical scavenging activity using DPPH and ABTS. The free radical scavenging activity of the extract was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis[3-ethyl benzothiazoline-6-sulphonic acid] (ABTS) reagents and they were purchased from Sigma-Aldrich (St. Louis, USA). The DPPH free-radical scavenging activity of the five extracts was determined by the method of Blois (1958) with some modifications. The reaction mixture was incubated for 30 min at room temperature, and the absorbance was measured at 517 nm using a UV-visible spectrophotometer. 1 mM of ascorbic acid (vitamin C) was used as a positive control and pure ethyl acetate without extract was used as a negative control. The ABTS assay is also frequently used to measure antioxidant activities. The procedure that was used followed the method of Arnao (2010) with some modifications. The absorbance of reaction mixture was measured at 734 nm using a UV visible spectrophotometer. 1 mM of ascorbic acid was used as a positive control and pure solvents without the test sample were taken as a negative control. Free radical scavenging activity was described as the inhibitory percentage of DPPH and ABTS was calculated according to the following equation, with each extract measured three times: scavenging activity (%) = [1 - (Abs sample/Abs control)] × 100.

Ferric reducing antioxidant power (FRAP) assay.

The FRAP assay was carried out according to the modified Benzie and Strain method (1996) while all of chemical reagents were purchased from Sigma-Aldrich (St. Louis, USA). The absorbance of reaction mixture was measured at 593 nm using a UV/Vis spectrophotometer. Temperature was maintained at 37°C. The readings at 30 min were selected for the calculation of FRAP values.

Statistical analysis. All analyses were carried out in triplicates. The experimental values are the mean ± standard deviation (SD). Statistical comparisons using one-way analysis of variance (ANOVA) with $p < 0.05$ were regarded as significant, with $p < 0.01$ being highly significant.

Results and Discussion

Evaluation of antibacterial properties. A total of five bacteria were isolated from the Arctic lichen *Ochrolechia* sp (Table I). To evaluate the antibacterial potential of extracts from our bacterial isolates, a paper disk diffusion test and an MIC test were carried out. In the paper disk diffusion test, all of the five bacterial isolates showed antibacterial activities against both Gram-positive (*S. aureus*, *B. subtilis*, and *M. luteus*) and Gram-negative (*E. cloacae*, *P. aeruginosa*, and *E. coli*) bacteria. No antibacterial activity was observed in the control (disk without extract, only ethyl acetate), while the antibacterial strengths of our extract were similar with the zone of inhibition diameter ranging from 8 to 12 mm (Table II). Also, while the MIC test was carried out, we used ampicillin as control antibiotics because all of the Gram-positive and Gram-negative tested organisms had sensitive resistance to ampicillin. The MIC for the extracts in the tested bacteria was 222.0 to > 1000 µg/ml and all of the extracts inhibited the tested organism at higher concentrations compared to control ampicillin (from 0.31 to 0.58 µg/ml) (Table III). According to the 16S rRNA gene sequencing results, their sequence

Table I
Microorganisms isolated from the Arctic lichen *Ochrolechia* sp.

PAMC No.	Isolation media	Bacterial species (Closest strain)	Similarity* (%)
26605	MY	<i>Sphingomonas</i> sp.	98.888
26606	R2A	<i>Burkholderia</i> sp.	98.401
26607	R2A	<i>Burkholderia</i> sp.	99.296
26608	R2A	<i>Burkholderia</i> sp.	99.763
26625	MY	<i>Sphingomonas</i> sp.	99.763

* The value are expressed as sequence similarity with the closely related organisms and generated by Basic Local Alignment Search Tool (BLAST) search program (<http://blast.ncbi.nlm.gov/Blast>).

Table II
Inhibition zone diameter of microorganisms isolated from the Arctic lichen *Ochrolechia* sp.

Sample No.	Gram-positive			Gram-negative		
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>M. luteus</i>	<i>E. cloacae</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
26605	++	+	-	-	+	++
26606	-	+	++	-	++	-
26607	++	-	-	-	-	++
26608	+	+++	-	-	+	++
26625	++	-	-	-	++	+++

Each value was expressed in mm against test clinical isolates (-, no activity; +, 8 mm; ++, 9-11 mm; +++, 12 mm).

Table III
MIC of microorganisms isolated from Arctic lichen *Ochrolechia* sp.

Sample No.	Gram-positive			Gram-negative		
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>M. luteus</i>	<i>E. cloacae</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
26605	> 1000	571.4 ± 0.19	> 1000	> 1000	> 1000	518.3 ± 0.13
26606	303.1 ± 0.35	> 1000	> 1000	> 1000	523.7 ± 0.21	> 1000
26607	324.7 ± 0.24	602.1 ± 0.26	> 1000	> 1000	491.5 ± 0.66	531.7 ± 0.32
26608	409.8 ± 0.27	428.6 ± 0.23	> 1000	> 1000	408.9 ± 0.13	433.0 ± 0.56
26625	233.1 ± 0.16	> 1000	> 1000	> 1000	222.0 ± 0.95	417.2 ± 0.44
Ampicillin	0.58 ± 0.09	0.31 ± 0.05	0.49 ± 0.07	0.4 ± 0.08	0.48 ± 0.09	0.35 ± 0.02

Each value is expressed as mean ± SD (µg/ml). Ampicillin used as a positive control.

similarities are almost the same and the species name of some of these are also the same. However, the morphological, chemical, and biological characteristics of the bacterial associates are somewhat different (data not shown). There are even differences in antibacterial activities among them. It is possible that media composition such as carbon source and/or nitrogen source may have affected the active compound of each extract. To search for new antibacterial sources from nature, a variety of lichen has been screened (Bhattarai *et al.*, 2013; Celenza *et al.*, 2013; Paudel *et al.*, 2008 and 2010; Sultana and Afolayan, 2011). As for the lichen *Ochrolechia* species, however, there are few studies about their biological activities and active components (Millot *et al.*, 2007; Ranković *et al.*, 2010). Ranković *et al.* (2010) studied the antimicrobial activity of some lichens, including *Ochrolechia androgyna*, but not the bacterial associates of *O. androgyna*. Although antibacterial activities were lower than those of natural lichen *O. androgyna*, the obtained results indicated that the bacterial isolates described in this study had the potential to be a source of treatment for various diseases caused by these clinical isolates or related microorganisms.

Evaluation of antioxidant properties. Many publications concerning the antioxidant activities of lichen have been reported (Behera *et al.*, 2008; Gul-luce *et al.*, 2006; Kosanic *et al.*, 2011; Luo *et al.*, 2011; Stocker-Wörgötter, 2008). However, most of them used lichen or their fungal symbionts as their study

resource. Although bacterial symbionts are also present in lichens and they contribute to lichens structurally and ecologically (Cardinale *et al.*, 2006 and 2008; Gonzales *et al.*, 2005; Grube *et al.*, 2009), the biological activity of bacterial symbionts is still unexplored. Thus, the antioxidant potential of bacterial symbionts was evaluated. In general, antioxidant activity is dependent on phenolic contents and/or flavonoids contents (Halvorsen *et al.*, 2002; Gardner *et al.*, 2000; Pietta, 2000). Thus, TPC and TFC assays were carried out (Table IV). TPC values ranged from 1.07 (PAMC26607, *B. sordidicola*) to 10.44 (PAMC26625, *Sphingomonas* sp.) milligrams of GAE per gram of extract, and the TFC value ranged from 0.99 (PAMC26606, *B. sordidicola*) to 8.95 (PAMC26625, *Sphingomonas* sp.) milligrams of CE per gram of extract. PAMC26625 had the highest value of TPC and TFC among these extracts while *Sphingomonas* species had a higher amount of TPC and TFC than *Burkholderia* species in terms of bacterial species. Through these results, it was expected that PAMC26625 would have the strongest antioxidant activities. PAMC26625 showed 77.23% and 80.02% high, free-radical scavenging activity in the DPPH and ABTS assays (Table IV), respectively (control vitamin C was 29.31%). Extracts that have high amounts of phenolic and/or flavonoid content showed high antioxidant activities as expected. In the case of PAMC26607 and PAMC26605, however, the free radical scavenging activity of PAMC26605 showed stronger activity

Table IV
Evaluation of antioxidant activities of microorganisms isolated from the Arctic lichen *Ochrolechia* sp.

Sample No.	Evaluation of antioxidant activities				
	TPC*	TFC**	DPPH***	ABTS****	FRAP*****
26605	1.09 ± 0.11	5.46 ± 0.05	24.18 ± 0.66	22.73 ± 0.87	3.07 ± 0.16
26606	1.32 ± 0.18	0.99 ± 0.07	5.31 ± 0.17	6.55 ± 0.12	1.14 ± 0.11
26607	1.07 ± 0.11	1.65 ± 0.10	7.82 ± 0.13	5.91 ± 0.09	1.09 ± 0.09
26608	2.02 ± 0.08	5.27 ± 0.13	26.66 ± 0.15	28.97 ± 0.21	4.16 ± 0.08
26625	10.44 ± 0.04	8.95 ± 0.05	77.23 ± 0.96	80.02 ± 0.92	8.72 ± 0.22

Each value is expressed as mean ± SD.

* TPC (total phenolic contents) are expressed as gallic acid equivalents (mg GAE/g extract)

** TFC (total flavonoid contents) are expressed as catechin equivalents (mg CE/g extract)

*** DPPH (1,1-diphenyl-2-picryl-hydrazyl) and ABTS [2,2'-amino-bis(3-ethyl benzothiazoline-6-sulphoric acid)]; free radical scavenging activity was described as the inhibitory percentage of DPPH and ABTS

**** FRAP (ferric reducing antioxidant power) are expressed as mM Fe(II)/ mg extract

than that of PAMC26607-about 3.1 fold and 3.8 fold in both the DPPH and ABTS assays-while they have comparable values of TPC. It may be that all phenolics do not have same antioxidant activity and some of them may possess strong activity whereas others have weak activity. It is possible that there are synergistic or antagonistic interactions between phenolic compounds or different types of components such as carbohydrates and proteins (Rice-Evans *et al.*, 1995). The FRAP assay was also carried out due to its simplicity and reproducibility (Table IV). The reducing activity of the extract can evaluate based on the theory that antioxidants act as reductants by performing the reduction of ferric ions to ferrous ions (Benzie and Strain, 1996). Therefore, the determination of the ferrous ion formation can be used to predict the reducing power of the samples. The FRAP value of the extract varied from 1.09 to 8.72 mM of ferrous ion per mg of extract, and the significantly higher ($p < 0.05$) amount of ferrous ion was detected in PAMC26625. Other extracts had a lower amount of ferrous ions than the ascorbic acid that was used as a control (6.81 ± 0.44 mM Fe(II)/mg extract). Finally, the results of this research suggest that the extract of PAMC26625 showed high antioxidant activity compared with control ascorbic acid. Moreover, our obtained results indicate that bacteria from lichen possess antioxidant activity and could be considered as potential sources of natural antioxidants.

Conclusions. In this paper, the antibacterial and antioxidant potentials of five bacterial symbionts from the lichen *Ochrolechia* sp. were evaluated, with PAMC26625 finally showing the strongest antioxidant activities among these five bacterial isolates. Although the functional compounds were not confirmed, they

have the potential to be a novel source of antibacterials and antioxidants.

Paudel *et al.* (2008) found that the antioxidant activity of crude extract from polar lichen was more effective than other lichen species from tropical and temperate regions. It is expected that bacterial symbionts will follow a similar pattern. Thus, the biological activities of the bacterial symbionts in lichen that can be found in extreme environments from polar to desert can be compared.

It appears that this is the first study investigating the biological activity of bacterial associates isolated from the Arctic lichen *Ochrolechia* sp. Since most identified lichen substances come from the fungal symbionts of lichen, it is a novel approach to search for new antibacterial and antioxidant compounds from nature. In addition to this, this kind of study may help illuminate the unique survival mechanisms of lichen in extreme conditions. Therefore, it is important to screen and isolate microorganisms from lichens and investigate their biological activities in detail.

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