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Antibacterial and antioxidant potential of polar microorganisms isolated from Antarctic lichen *Psoroma* sp.

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Recent studies suggest that bacterial communities in lichens contribute structurally and ecologically, but their biological activities are not fully investigated. In this study, we explored biological potential of microorganisms that are isolated from Antarctic lichen *Psoroma* sp. Using their bacterial cell culture extracts, we evaluated antibacterial and antioxidant properties. Among 20 bacterial species that were isolated from Antarctic lichen *Psoroma* sp., PAMC 26508 (*Streptomyces* sp., similarity: 100%) showed antibacterial activities against all target bacteria with inhibition zone diameter of 7 to 9. PAMC 26537 (*Burkholderia sordidicola*, similarity: 98.828%) exhibited higher antioxidant potential with an inhibition rate of 60.12% in 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 58.69% in 2,2'-azino-bis[3-ethyl benzothiazoline-6-sulphonic acid] (ABTS) assay than control ascorbic acid (29.31%). Our results indicated that they have potential to be used as novel source of antibacterial and antioxidant agents.

Key words: Antarctica, antibacterial/antioxidant, lichen associated bacteria, lichen, *Psoroma* sp.

INTRODUCTION

Lichens are symbiotic organisms that are composed of a fungus and photosynthetic algae and/or Cyanobacteria. They can survive in extreme environments from desert to polar region because many lichens have a tolerance to extreme heat and coldness. In Antarctic ecosystems, lichens are the dominant vegetation as they are involved in soil processes, nutrient cycling and initial colonization (Lindsay, 1978). Lichens and their natural product, also called lichen substance, have been used for cosmetics, decorations, dyes, foods and medicine (Oksanen, 2006). Especially, they have been attracting attention from many researchers due to their diverse pharmaceutical potentials for antiviral, anti-proliferative, anti-inflammatory, anti-tumor and antimycobacterial activities (Lawrey, 1989; Lauterwein et al., 1995; Ingolfsdottir et al., 1998; Morita et al., 2009; Molnár and Farkas, 2010). Since they have inhibitory effect against microorganisms and high antioxidant activity, their antimicrobial and antioxidant activities are also being widely investigated (Richardson, 1998; Huneck, 1999; Muller, 2001). A few studies reported that

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lichen from polar region also have antibacterial and antioxidant activities (Paudel et al., 2008; Paudel et al., 2010; Bhattarai et al., 2013). In general, lichen substances come from the mycobiont (Stocker-Wörgötter, 2008; Luo et al., 2011).

Recent molecular studies showed that diverse bacteria are present in lichens, by the ribosomal internal transcribed spacer polymorphism and *in situ* hybridization analysis (Cardinale et al., 2006, 2008). Another research group also analyzed and compared their structure and compositions of associated bacterial communities of some lichen species, by using combined microscopic and molecular techniques (Grube et al., 2008). Despite those studies, biological activities of lichen, especially the Antarctic *Psoroma* sp. have not been studied yet, and biological activities of bacterial communities in Antarctic lichen *Psoroma* sp. are still unknown. In our previous studies, we screened 14 bacterial isolates from Arctic lichens such as *Cladonia* sp., *Umbilicaria* sp. and *Stereocaulon* sp., and antibacterial activities of these 14 bacterial isolates were determined against Gram-positive and negative clinical microorganisms (Kim et al., 2012a, 2013a). They also showed much better antioxidant activity than control ascorbic acid (Kim et al., 2012b, 2013b). The aim of this study, therefore, was to screen the antibacterial properties and antioxidant properties of 20 bacterial isolates from Antarctic lichen *Psoroma* sp. and to study this lichen as a novel source for the lichen substance.

MATERIALS AND METHODS

Collection and identification of lichen samples

The samples of lichen *Psoroma* sp. were collected in Barton Penninsula, King Geroge Island, South Shetland Island, and Antarctica (62.22 ' S/ 58.78 ' N) by Korean Polar Research Institute (KOPRI). They were transferred at room temperature and stored at -20°C until further use. The bacterial isolates were deposited in polar and alpine microbial collection (PAMC).

Screening of microorganisms associates with lichen

A fragment from a lichen thallus was separated by sterilized scissors or knife. Sterilized 0.85% NaCl solution was added, followed by vortexing for 10 min. After discarding the solution, the above steps were repeated. The tissue was subsequently broken with mortar in sterilized 0.85% NaCl solution. After spreading the tissue on malt extract-yeast extract (MY) agar media, international *Streptomyces* project (ISP) media, and Reasoner's 2A (R2A) agar, it was incubated at 10°C for 15 to 21 days. To obtain pure single colony, subculturing was repeated three times, and it was preserved at -80°C in 20% glycerol. The obtained bacterial isolates were identified by their 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). 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, Korea). The PCR procedure included an initial denaturing step at 95°C for 5 min, 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. The PCR products were purified using the AccuPrep PCR purification Kit (Bioneer, 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 culture in 50 ml of MY, ISP and R2A liquid media at 15°C for 10 to 15 days. The culture broth was added to double volume of ethyl acetate which was analytical grade (Daejeong, Korea). Extraction was performed individually to each solvent at room temperature, and then, the layer of culture media was discarded after 2 h. The solvent layer was concentrated using rotary evaporator, and the obtained dried crude extract was dissolved in 500 µl of ethyl acetate.

Evaluation of antimicrobial activities

Test microorganisms

Ethyl acetate extracts were tested against 3 Gram-positive bacteria: *Staphylococcus aureus* (KCTC 1928), *Bacillus subtilis* (KCTC 1918) and *Micrococcus luteus* (KCTC 1915), and 3 Gram-negative bacteria: *Escherichia coli* (KCTC 2441), *Pseudomonas aeruginosa* (KCTC 1637), and *Enterobacter cloacae* (KCTC 1685). They were purchased from the Korean Collection for Type Cultures (KCTC) and Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). All bacterial isolates were kept on Luria-Bertani (LB; trypton 10.0 g, yeast extract 5.0 g, NaCl 10.0 g, distilled water 1.0 L, pH 7.2) at 4°C.

Paper disk diffusion test

Paper disk diffusion test was performed according to Bauer et al. (1966) with some modifications, and all of the reagents were purchases from Difco (USA). Bacterial cells were standardized to 0.5 McFarland, and mixed with soft agar (0.04 g/ml), and 9 ml of this mixture was inoculated onto Mueller-Hinton agar plate. Then, each extract was loaded into paper disks (6 mm in diameter, ADVANTEC, Japan) and transferred onto the plates inoculated with the bacterial strains. Disks loaded with the 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. All experiments were done twice and were compared with the control.

Evaluation of antioxidant activities

Total phenolic contents (TPC) and total flavonoid contents (TFC) test

The total phenolic content was evaluated by the Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) with some modifications, and all the chemical reagents were purchased from Sigma-Aldrich (USA). After the final reaction mixture was incubated for 30 min at room temperature, the absorbance was determined at 760 nm. Gallic acid was 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 micrograms of gallic acid equivalent (GAE) per milligram

milligram of bacteria culture extract. Total flavonoid contents were evaluated by colorimetric method as described previously (Zhishen and Mengcheng, 1999). The 500 µl of lichen-bacterial culture extracts was taken, and 1.5 ml of distilled water was added to it, which was subsequently mixed with 0.15 ml of 5% $NaNO_2$ solution. After 6 min of incubation, 10% $AlCl_3$ solution was added and incubated for 6 min. Then, 2 ml of 4% NaOH solution was added to the mixture, and distilled water was added until the set final volume was reached at 5 ml. After 15 min at room temperature, the absorbance was measured at 510 nm using a spectrophotometer (Biochrome, USA). Catechin was used as a standard compound for the quantification of total flavonoids. All the values were expressed as g catechin equivalent (CE) per 100 g of extract. A single 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 (USA). The DPPH free-radical scavenging activity of the 5 extract was determined by the method of Blois (1958) with some modifications. The reaction mixture was incubated for 30 min at room temperature. The absorbance was measured at 517 nm using a UV-Visible spectrophotometer. The reason why we used ascorbic acid instead of synthetic antioxidant (butylated hydroxyanisole, BHA; butylated hydroxytoluene, BHT; and tertiary butylhydroquinone, TBHQ) is because our extract comes from bacteria in natural lichen (Grice, 1996; Thadhani et al., 2011). The ABTS assay is also frequently used to measure antioxidant activities. The procedure followed the method of Arnao et al. (2010) with some modifications. The reaction mixture was incubated for 30 min at room temperature, after which, the absorbance was measured at 734 nm using a UV Visible spectrophotometer. Ascorbic acid of 1 mM was used as positive control, and pure solvents without the test sample were taken as a negative control in both DPPH and ABTS assay. A single extract were measured three times. Free radical scavenging activity was described as the inhibitory percentage of DPPH. ABTS was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = [1 - (\text{Abs sample}/\text{Abs control})] \times 100$$

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the modified Benzie and Strain (1996) method and all of chemical reagents were purchased from Sigma-Aldrich (USA). The 900 µl of FRAP reagent, freshly prepared and warmed at 37°C, was mixed with 90 µl of distilled water and 10 µl of extract in different concentrations. The FRAP reagent contained 2.5 ml of a 10 mM 2,4,6-tripyridyl-striazine (TPTZ) solution in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$, and 25 ml of 0.3 mM acetate buffer pH 3.6. Absorbance was measured at the 593 nm using a UV/Vis spectrophotometer. Temperature was maintained at 37°C. The readings at 30 min were selected for calculation of FRAP values.

RESULTS AND DISCUSSION

Screening for antibacterial potential of 20 lichen-associated microorganisms

A total of 20 bacteria were isolated from Antarctic lichen *Psoroma* sp., and they were identified by their 16S rRNA gene sequence analysis as shown in Table 1. It was found that the morphological and biological characteristics of the 20 bacteria were somewhat different (data not shown). Although Hidalgo group (1993) reported that pannarin, isolated from *Psoroma pallidum*, had antioxidant activity, there is no study on microbial communities and biological activity from lichen *Psoroma*. To evaluate the antibacterial potential, the paper disk diffusion test was carried out, and some of the 20 bacterial symbionts showed antibacterial activities against Gram-positive/-negative bacteria containing *S. aureus*, *B. subtilis*, *M. luteus*, *E. cloacae*, *P. aeruginosa* and *E. coli* (Table 2). The zone of inhibition diameter ranged from 7 to 11 mm, and PAMC 26508 (*Streptomyces* sp., 100% similarity) especially had antibacterial activity against all of the 6 target bacteria. PAMC 26554 (*Hymenobacter* sp., 94.661% similarity) and PAMC 26517 (*Paenibacillus* sp., 93.523% similarity) showed strongest activity against *E. coli* (11 mm) and *E. cloacae* (11 mm), respectively. All these tested strains are known as clinical isolates related to human diseases. Although there are several strong antibiotics, our results indicated that bacterial isolates from *Psoroma* sp. have the potential to be a source or a starting point for the studies on their biological compounds and treatment of related diseases.

Screening for antioxidant potential of 20 lichen-associated microorganisms

Many publications on the antioxidant activities of lichen have been reported (Behera et al., 2005; Gulluce et al., 2006; Stocker-Wörgötter, 2008; Kosanic et al., 2011; Luo et al., 2011). However, most of them used lichen or their fungal symbionts as study resource. The fact that bacterial symbionts are also present in lichen and that they contribute to lichen structurally and ecologically are previously demonstrated (Gonzalez et al., 2005; Cardinale et al., 2006, 2008; Grube et al., 2008), but biological activity of bacterial symbionts are still unexplored. Thus, we evaluated antioxidant potential of bacterial symbionts. In general, antioxidant activities are dependent on their phenolic contents and/or flavonoids contents (Gardner et al., 2000; Pietta, 2000; Halvorsen et al., 2002). Thus, we carried out TPC and TFC assay (Table 3).

TPC value ranged from 1.93 (PAMC 26557, *Rhodanobacter* sp., 97.648% similarity) to 21.01 (PAMC 26505, *Rhodanobacter* sp., 98.371% similarity) microgram of GAE per milligram of extract, and TFC value ranged from 0.73 (PAMC 26508, *Streptomyces* sp., 100% similarity) to 26.79 (PAMC 26505, *Rhodanobacter* sp., 98.371% similarity) microgram of CE per milligram of extract. Because PAMC 26505 (*Rhodanobacter* sp.) had high value of TPC and TFC among our extracts, we expected it to have the strongest antioxidant activities.

PAMC 26537 (*Burkholderia sordidicola*, 98.828% similarity) showed 60.12 and 58.69% which are the highest

Table 1. Microorganisms isolated from the Antarctic lichen *Psoroma* sp.

PAMC no.	Bacterial species (Closest strain)	16S rRNA sequence Similarity (%)	Isolation media
26505	*Rhodanobacter* sp.	98.371	R2A
26506	*Burkholderia sordidicola*	98.904	R2A
26507	*Burkholderia sordidicola*	98.921	R2A
26508	*Streptomyces* sp.	100	R2A
26509	*Burkholderia* sp.	98.534	MY
26510	*Burkholderia sordidicola*	98.928	MY
26515	*Rhodanobacter* sp.	97.771	R2A
26517	*Paenibacillus* sp.	93.523	R2A
26518	*Rhodanobacter* sp.	98.081	R2A
26519	*Rhodanobacter* sp.	98.076	R2A
26537	*Burkholderia sordidicola*	98.828	R2A
26538	*Rhodanobacter* sp.	97.648	MY
26551	*Rhodanobacter* sp.	97.771	R2A
26552	*Rhodanobacter* sp.	97.751	R2A
26554	*Hymenobacter* sp.	94.661	R2A
26555	*Frigoribacterium* sp.	97.545	R2A
26556	*Sphingomonas* sp.	97.175	R2A
26557	*Rhodanobacter* sp.	97.648	MY
26561	*Sphingomonas* sp.	97.118	R2A
26633	*Burkholderia* sp.	98.491	ISP

Table 2. Antibacterial properties of microorganisms isolated from Antarctic lichen *Psoroma* sp.

Sample no.	Gram positive			Gram negative		
	S. aureus	*B. subtilis*	*M. luteus*	*E. cloacae*	*P. aeruginosa*	*E. coli*
26505	-	-	-	-	-	-
26506	-	-	-	-	-	-
26507	-	-	7	-	-	-
26508	7	8	7	7	9	9
26509	-	-	-	-	-	-
26510	-	-	-	-	-	-
26515	-	-	7	-	-	-
26517	9	8	-	11	-	-
26518	-	-	-	7	-	-
26519	-	-	-	-	-	-
26537	-	-	-	7	-	-
26538	7	9	-	7	8	8
26551	7	8	-	-	8	9
26552	-	-	7	-	-	-
26554	9	8	-	-	-	11
26555	8	8	10	-	-	-
26556	-	-	-	-	7	-
26557	-	-	7	-	-	7
26561	-	-	-	-	7	7
26633	7	8	-	-	8	9

The property was expressed as inhibition zone diameter in mm and '-' indicates no sensitivity against target bacteria. All values are expressed as mean (n=3).

Table 3. Antioxidant properties of microorganisms isolated from Antarctic lichen *Psoroma* sp.

Sample no.	TPC*	TFC**	DPPH***	ABTS***	FRAP****
26505	25.43	26.79	26.48	18.04	2.39
26506	15.61	13.24	14.25	16.89	2.19
26507	9.84	10.02	19.64	22.31	3.27
26508	2.21	0.73	40.55	44.32	7.50
26509	16.53	10.44	17.02	19.24	3.17
26510	8.90	13.05	18.33	16.54	2.26
26515	14.26	15.04	25.00	19.27	2.35
26517	5.31	5.32	4.23	6.41	1.24
26518	10.08	11.39	23.71	24.56	4.30
26519	19.08	20.08	45.35	30.24	6.94
26537	20.21	22.36	60.12	58.69	7.59
26538	21.01	24.09	44.16	35.17	6.43
26551	19.54	14.33	25.61	22.21	4.36
26552	17.61	15.30	22.17	19.08	3.29
26554	4.77	1.59	4.97	3.85	1.31
26555	4.92	1.64	11.20	10.64	2.22
26556	2.37	6.87	50.61	55.23	8.56
26557	1.93	1.45	35.84	43.27	7.44
26561	2.04	0.98	10.98	13.28	3.07
26633	12.22	13.38	21.03	18.00	2.34

All values are expressed as mean (n=3).*TPC are expressed as gallic acid equivalents (µg GAE/mg extract); **TFC are expressed as catechin equivalents (µg CE/mg extract); ***Vitamin C uses as control (29.31%); ****FRAP are expressed as mM Fe(II)/mg extract. Ascorbic acid was used as control (6.21 mM Fe(II)/mg extract).

free-radical scavenging activity in DPPH and ABTS assay, respectively. Most of the extracts with high amount of phenolic and/or flavonoid content showed high antioxidant activities, as we have expected and as shown in the case of PAMC 26519 (*Rhodanobacter* sp., 98.076% similarity) and PAMC 26538 (*Rhodanobacter* sp., 97.648% similarity).

However, in the cases of PAMC 26508, PAMC 26556 (*Sphingomonas* sp., 97.175% similarity), and PAMC 26557 (*Rhodanobacter* sp., 97.648% similarity), their free radical scavenging activities in both DPPH and ABTS assays showed stronger activity, although they have comparably low value of TPC and TFC. It may be due to the fact that all phenolics may not have the same antioxidant activity and some of them may possess stronger activity than others. It is possible that there are synergistic or antagonistic interactions between phenolic compounds or different type of components such as carbohydrates and proteins (Rice-Evans et al., 1995).

We also carried out a FRAP assay to evaluate the reducing activity of our extract, based on the theory that antioxidant acts as reductant by performing reduction of ferric ion to ferrous ion (Benzie and Strain, 1996). So, determination of the ferrous ion formation can be used to expect the reducing power of our samples.

FRAP value of our extract varied from 1.24 to 8.56 mM in ferrous ion/mg of extract, and high amount of ferrous ion was detected in PAMC 26556 within our extract. Other extracts have lower amount of ferrous ion than ascorbic acid that was used as control (6.21 mM Fe(II)/mg extract).

Antioxidants are important in the prevention of human disease, and in generally, living organisms have a natural defense mechanism of antioxidant (Halliwell, 1997). Sometime because low level of antioxidant molecules cause damage or kill cells (Devasagayam et al., 2004), finding new antioxidant from natural sources is highly desirable. Therefore, our results revealed the possibility that the bacteria from lichen possess antioxidant activity are able to be used as new sources of natural antioxidant.

Conclusions

Antarctica is harsh environment with low temperature and nutrient restriction. Vegetation in Antarctica is limited because these conditions are not typically ideal for their growth.

Paudel et al. (2008) found that antioxidant activity of crude extract from polar lichen was more effective than other lichen species from tropical and temperate regions.

This result may relate to unique mechanisms of lichen to avoid stress from high radiation, drought and very low temperature. We expected that bacterial isolates from Antarctic lichen will follow a similar pattern because of diverse bacterial communities affecting the lichen structure and ecology. In this paper, we evaluated antibacterial and antioxidant potential of 20 bacterial symbionts isolated from Antarctic lichen *Psoroma* sp. Although we did not confirm the actual active compound, they have potential to be used as novel source of antibacterial and antioxidants. To our knowledge, this is the first study investigating the biological activity of bacterial associates isolated from the Antarctic lichen *Psoroma* sp.

Since most of identified lichen substance comes from fungal symbionts of lichen, it is a novel approach to search for new antibacterial and antioxidant compound from the nature. In addition to this, this kind of study may help to understand the unique survival mechanisms of Antarctic lichen in extreme conditions.

Conflict of interest

The author(s) have not declared any conflict of interests.

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