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Antibacterial Properties Associated with Microorganisms Isolated from Arctic Lichens

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A total of 5 different polar microorganisms were isolated from Arctic lichens and their bioactive compounds were extracted from cell culture using different solvents including acetone, water, chloroform, diethylether, ethanol, ethyl acetate, methanol, and petroleum ether. The antibacterial properties of the extracts were evaluated by disk diffusion tests and minimal inhibitory concentration tests against 6 bacterial pathogens; *Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Enterobacter cloacae, Pseudomonas aeruginosa* and *Escherichia coli*. Among the extraction samples, ethyl acetate extracts of *Burkholderia sordidicola* S5-B^T (KOPRI 26644) showed the highest activity (inhibition zone, 7-10 mm; MIC value, 57.5-1000 ug/ml) against targeted bacteria. Among the various solvents used for extraction, chloroform extract exhibited the weakest, but still obvious, activity.

Key words: Antibacterial activity, lichen, minimal inhibitory concentration test, paper disk diffusion test, Polar microorganisms

Introduction

Lichens are symbiotic organisms between fungi, algae, or cyanobacteria. They are ubiquitous and can grow in extreme environment such as tropical region and polar area. Lichens produce a wide range of bioactive metabolites and they have been used as medicines, cosmetics, dyes, foods, and decorations [3, 5, 13].

About 350 lichen metabolites have been found and the chemical structures of approximately 200 of them have been established. Usnic acid is one of the most common and widely investigated lichen compounds [7]. Its antimicrobial, antiviral, anti-proliferative, anti-inflammatory, anti-tumor, and analgesic activities have been reported [8-12]. Lichen compounds are also known to show some biological activities against microorganisms. Most of these are come from fungi of lichen symbiosis. Recent study showed that bacteria communities also contribute to lichen structurally and ecologically [1, 4, 6], however, their biological activities have not been studied in detail. Especially, bacterial communities of these lichen species *Cladonia* sp., *Sterocaulon* sp., *Umbilicaria* sp., *Cetraria* sp. are not well-known. Some study reported antibacterial and antioxidant activities of these lichens, but not bacterial symbiont [14].

In the recent work, we isolated 5 microorganisms from the Arctic lichens *Cladonia* sp., *Sterocaulon* sp., *Umbilicaria* sp., *Cetraria* sp.. To evaluate their antibacterial activities, cell culture extraction was carried out using different solvents including acetone, water, chloroform, diethylether, ethanol, ethyl acetate, methanol, and petroleum ether. Subsequently, antibacterial activities of these polar microorganisms against 6 bacteria such as *Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Enterobacter cloacae, Pseudomonas aeruginosa* and *Escherichia coli* were investigated.

Materials and Methods

Lichens and lichen-associated with microorganisms

Lichen samples were collected by Korean Polar Research Institute (KOPRI). All lichen-associated with bacterial species were identified by KOPRI and their 16S rRNA

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sequences were analyzed. All sample numbers were given by KOPRI.

Screening of lichen-associated microorganisms

A segment of lichen thallus was separated by scissors and then added the sterilized 0.85% saline solution. Vortex in ten minute and then discard the solution and repeat above steps about two more times. Break the tissue with mortar with sterilized 0.85% saline solution and spread on selective media. Cultures were incubated at 28°C for 15-21 days. Selective media is Humic acid vitamin agar (Humic acid 10.0 g, Na_2HPO_4 0.5 g, KCl 1.71 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $CaCO_3$ 0.02 g, vitamin 1.0 ml, distilled water 1.0 L, agar 16.0 g), Bennett's vitamin agar (D-glucose 10.0 g, yeast extract 1.0 g, peptone 2.0 g, beef extract 1.0 g, vitamin 1.0 ml, distilled water 1.0 L, agar 16.0 g), ISP4 (Difco soluble starch 10.0 g, K_2HPO_4 anhydrous 1.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, NaCl 1.0 g, $(NH_4)_2SO_4$ 2.0 g, $CaCO_3$ 2.0 g, ISP trace salt solution 1.0 ml, distilled water 1.0 L, agar 16.0 g) and water agar (distilled water 1.0 L, agar 16.0 g). The obtained single colony was taken from the selective media and they have kept on fresh media that mentioned above, respectively.

Microorganisms

Gram-positive pathogenic bacteria *Staphylococcus aureus* (KTCC1928), *Bacillus bacillus* (KTCC1918), *Micrococcus luteus* (KTCC1915) and Gram-negative pathogenic bacteria *Escherichia coli* (KTCC2441), *Pseudomonas aeruginosa* (KTCC1637), *Enterobacter cloacae* (KTCC1685), were used. Bacteria were kept on LB at 4°C. These strains were cultured at 37°C in Mueller-Hinton for 6 hrs.

Extraction

For extraction, 50 ml of total 5 microorganisms were cultured on Bennett's vitamin liquid media (D-glucose 10.0 g, yeast extract 1.0 g, peptone 2.0 g, beef extract 1.0 g, vitamin 1.0 ml and distilled water 1.0 L) and ISP4 liquid media (soluble starch 10.0 g, NaCl 1.0 g, K_2HPO_4 (anhydrous) 1.0 g, $CaCO_3$ 2.0 g $MgSO_4 \cdot 7H_2O$ 1.0 g, $(NH_4)_2SO_4$ 2.0 g, ISP trace salt solution 1.0 ml and distilled water 1.0 L) at 15°C for 10-15 days. Culture broth centrifuged and the supernatant were taken and added to double volume of non-polar solvents of diethyl ether, ethyl acetate, petroleum ether, chloroform, ethyl acetate. The mixtures were shaken at room temperature for 2 hrs, and then discard the layer of media. In case of polar solvent extractions such as acetone, ethanol, methanol, and aqueous extract, cell free supernatants were mixed with chloroform and metabolites were converted into chloroform layer. Then, chloroform layer was collected and re-converted into polar solvent. Solvent layer was concentrated using rotary evaporator. The obtained dried crude extract was dissolved in 500 ul of solvent that was same as above.

Evaluation of antibacterial activity

We carried out the disk diffusion test with some modifications [2]. Bacteria strains were inoculated onto LB agar plate. The each extract was loaded into paper disks (6 mm in diameter, ADVANTEC, Japan). These disks were transferred into the inoculated microorganism media. Pure aqueous, acetone, chloroform diethyl ether, ethanol, methanol, ethyl acetate and petroleum ether loaded and dried disks used as negative control. The bacterial plates were incubated for 9 hrs at 37°C. Growth was evaluated visually by comparing the extract-containing disks with the control disks in a particular plate. The inhibition zones for bacteria were measured after 12-18 hrs. All experiments were done twice and checked with the control plates.

Determination of minimum inhibitory concentration (MIC)

MIC was determined by a broth dilution method with some modifications [15]. Dilutions of lichen extract ranging from 0 to 1,000 ug/ml in 5 ml of Mueller-Hinton broth in duplicate were prepared in 15 ml sterile falcon tubes. The range of test concentration for the extracts was determined in an initial range finding experiment. An overnight broth culture where target strain seeded was used to inoculate fresh broth culture to make final cell density of 10^6 CFU/ml. The inoculated tubes were incubated at optimum temperature for each target strain and shaken at 250 rpm for 24 hrs. Microbe growth was measured by UV/Vis spectrophotometer at 530 nm.

Fatty acid analysis

For the fatty acid analysis, microorganisms isolated from Arctic lichens were grown on R2A at 28°C for 3 days. Cellular fatty acid was methylated and analyzed using gas chromatography followed MIDI standard protocol. Identification of fatty acid was confirmed using a standard protocol of HP MIS.

Results and Discussion

We isolated total 5 microorganisms from the Arctic lichen samples. The results of 16S rRNA sequence analysis are summarized in Table 1. Colonies have pigments and their shapes were either circlar or irregular. The detailed

Table 1. Detailed information of samples and 16S rRNA sequence analysis results.

Sample No.	Lichen source	16SrRNA sequence analysis (Closest strain)	Similarity (%)	Culture media	Culture conditions
KOPRI 26643	*Cladonia* sp.	*Burkholderia sordidicola* S5-B^T	98.9	ISP 4	15°C, 10~15 days
KOPRI 26644	*Sterocaulon* sp.	*Burkholderia sordidicola* S5-B^T	98.9	Bennett's vitamin agar	15°C, 10~15 days
KOPRI 26645	*Cetraria* sp.	*Sphingomonas faeni* MA-olki^T	99.8	ISP 4	15°C, 10~15 days
KOPRI 26646	*Umbilicaria* sp.	*Burkholderia sordidicola* S5-B^T	98.9	Bennett's vitamin agar	15°C, 10~15 days
KOPRI 26647	*Umbilicaria* sp.	*Burkholderia sordidicola* S5-B^T	98.9	ISP 4	15°C, 10~15 days

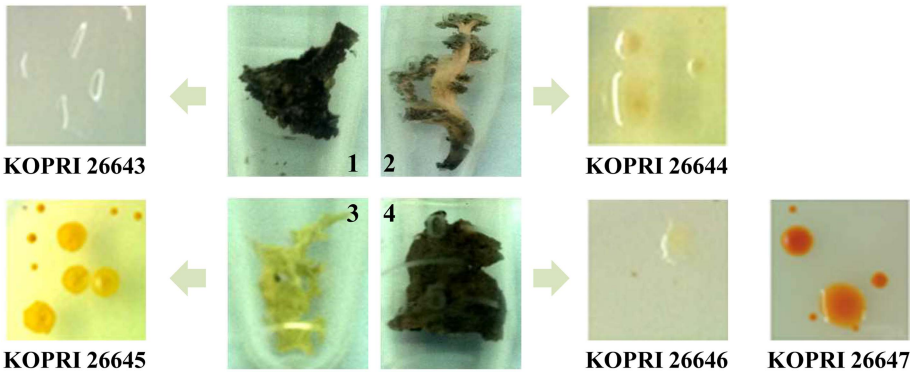

Fig. 1. Colony characteristics of isolated polar microoroganisms. 1 (*Cladonia* sp.), KOPRI 26643 have white pigment and irregular shape; 2 (*Sterocaulon* sp.) KOPRI 26644 have ivory pigment and irregular shape; 3 (*Cetraria* sp.), KOPRI 26645 have yellow pigment and circle shape; 4 (*Umbilicaria* sp.), both KOPRI 26646 have irregular shape and their pigment is white and orange, respectively.

Table 2. Physiological and biochemical test results.

Characteristics	26643	26644	26645	26646	26647
Pigment	White	Beige	Yellow	White	Orange
Growth range of					
Temparature*	15-28	15-28	15-28	15-28	15-28
pH	6.0-8.0	6.0-8.0	6.0-8.0	6.0-8.0	6.0-8.0
Oxidaseactivity	+	+	+	+	+
Nitrate reduction	−	−	−	−	−
H2S production	−	−	−	−	−
Acid production from :					
Arabinose	−	−	+	−	−
Glucose	+	+	+	+	+
Assimilation of :					
N-Acetyl-D-glucosamine	+	+	−	+	+
D-Arabinose	−	−	+	−	−
L-Arabinose	−	−	+	−	−
Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
Xylose	+	+	+	+	+
Major Quinone(s)**	UQ-7, UQ-9	UQ-7, UQ-9	UQ-10	UQ-7, UQ-9	UQ-7, UQ-9
DNA G + C contents***	61.3	60.0	63.1	61.0	61.1

Strain 26643, 26644, 26646 and 26647 are similar with *Bulkholderia sordidicola* S5-B^T. Strain 26645 is similar with *Sphingomonas faeni* MA-olki^T.
*Temperature was expressed as °C
**UQ: ubi-quinone.
***DNA G+C contents was expressed as mol%.

Table 3. Antibacterial activities of polar microorganisms culture extracts.

Target bacteria		Solvents*	Polar microorganisms**					
			26643	26644	26645	26646	26647	Standard***
Gram positive	*Staphylococcus aureus*	Aq	NS	NS	NS	NS	NS	NS
		Ace	NS	NS	NS	7	NS	NS
		Chl	NS	NS	NS	NS	NS	NS
		Di	NS	NS	NS	NS	NS	NS
		Et	NS	NS	NS	NS	NS	NS
		EtoAC	7	9	NS	NS	NS	NS
		Met	NS	NS	NS	NS	NS	NS
		Pe	NS	NS	NS	NS	NS	7
	Bacillussubtilis	Aq	NS	NS	NS	NS	NS	NS
		Ace	NS	NS	NS	NS	NS	7
		Chl	9	8	NS	9	7	NS
		Di	8	7	NS	NS	NS	NS
		Et	NS	NS	NS	NS	NS	NS
		EtoAC	9	9	9	8	8	NS
		Met	NS	NS	NS	NS	NS	NS
		Pe	NS	NS	NS	NS	NS	7
	Micrococcus luteus	Aq	NS	NS	NS	NS	NS	NS
		Ace	NS	NS	NS	NS	NS	NS
		Chl	NS	7	NS	7	NS	7
		Di	NS	NS	NS	NS	NS	NS
		Et	NS	NS	NS	NS	NS	NS
		EtoAC	9	10	8	10	9	NS
		Met	NS	NS	NS	NS	NS	NS
		Pe	NS	NS	NS	NS	NS	7
Gram negative	*Enterobacter cloacae*	Aq	NS	NS	NS	NS	NS	NS
		Ace	NS	NS	NS	7	NS	7
		Chl	NS	8	NS	NS	NS	NS
		Di	NS	9	NS	8	NS	NS
		Et	NS	NS	NS	NS	NS	NS
		EtoAC	9	10	9	NS	9	NS
		Met	NS	NS	NS	NS	NS	NS
		Pe	NS	NS	NS	NS	NS	7
	Pseudomonas aeruginosa	Aq	NS	NS	NS	NS	NS	NS
		Ace	NS	NS	8	NS	NS	7
		Chl	7	8	8	7	NS	NS
		Di	NS	NS	NS	NS	NS	NS
		Et	NS	NS	NS	NS	NS	7
		EtoAC	7	10	9	NS	NS	NS
		Met	NS	NS	NS	NS	NS	NS
		Pe	NS	NS	NS	NS	NS	7
	Escherichia coli	Aq	NS	NS	NS	NS	NS	NS
		Ace	NS	NS	7	NS	NS	NS
		Chl	NS	8	8	NS	NS	7
		Di	8	9	9	NS	NS	NS
		Et	NS	NS	NS	NS	NS	7
		EtoAC	8	10	8	8	NS	NS
		Met	NS	NS	NS	NS	NS	NS
		Pe	NS	NS	NS	NS	NS	7

The activity was expressed as inhibition zone diameter in mm and NS means no sensitive against test pathogenic strains.
*Aq, aqueous; Ace, acetone; Chl, chloroform; Di, diethyl ether; Et, ethanol; EtoAC, ethyl acetate; Met, methanol; Pe, petroleum ether
** The number was given from KOPRI
***standard : only solvents without extract.

Table 4. Minimum inhibitory concentration (MIC) of extract against the test organisms.

Target bacteria		Solvents*	Polar microorganisms**					
			26643	26644	26645	26646	26647	Standard***
Gram positive	*Staphylococcus aureus*	Aq	NA	NA	NA	NA	NA	0.58
		Ace	> 1000	> 1000	NA	NA	NA	
		Chl	295.6	497.3	> 1000	663.6	571.4	
		Di	NA	NA	NA	NA	NA	
		Et	NA	NA	NA	NA	NA	
		EtoAC	287.6	283.3	492.1	533.6	752.1	
		Met	NA	NA	NA	NA	NA	
		Pe	NA	NA	NA	NA	NA	
	Bacillussubtilis	Aq	NA	NA	NA	NA	NA	0.31
		Ace	842.2	> 1000	> 1000	> 1000	> 1000	
		Chl	> 1000	852.4	645.7	495.2	> 1000	
		Di	NA	NA	NA	NA	NA	
		Et	NA	NA	NA	NA	NA	
		EtoAC	154.1	145.2	378.0	842.2	559.7	
		Met	> 1000	> 1000	> 1000	> 1000	> 1000	
		Pe	NA	NA	NA	NA	NA	
	Micrococcus luteus	Aq	NA	NA	NA	NA	NA	0.49
		Ace	133.2	117.4	122.8	324.0	541.0	
		Chl	80.5	78.6	100.7	301.7	> 1000	
		Di	NA	NA	NA	NA	NA	
		Et	> 1000	> 1000	> 1000	> 1000	> 1000	
		EtoAC	133.6	57.5	267.9	752.4	> 1000	
		Met	> 1000	> 1000	NA	NA	NA	
		Pe	NA	NA	NA	NA	NA	
Gram negative	*Enterobacter cloacae*	Aq	NA	NA	NA	NA	NA	0.4
		Ace	> 1000	> 1000	> 1000	> 1000	> 1000	
		Chl	871.6	765.2	998.4	954.2	> 1000	
		Di	NA	NA	NA	NA	NA	
		Et	> 1000	> 1000	> 1000	> 1000	> 1000	
		EtoAC	> 1000	704.4	954.1	977.3	924.5	
		Met	> 1000	> 1000	> 1000	> 1000	> 1000	
		Pe	NA	NA	NA	NA	NA	
	Pseudomonas aeruginosa	Aq	NA	NA	NA	NA	NA	0.48
		Ace	> 1000	> 1000	> 1000	> 1000	> 1000	
		Chl	> 1000	> 1000	> 1000	> 1000	> 1000	
		Di	NA	NA	NA	NA	NA	
		Et	NA	NA	NA	NA	NA	
		EtoAC	423.5	267.2	598.4	709.3	> 1000	
		Met	936.4	987.1	> 1000	> 1000	> 1000	
		Pe	NA	NA	NA	NA	NA	
	Escherichia coli	Aq	NA	NA	NA	NA	NA	0.35
		Ace	> 1000	> 1000	> 1000	> 1000	> 1000	
		Chl	> 1000	> 1000	> 1000	> 1000	> 1000	
		Di	NA	NA	NA	NA	NA	
		Et	NA	NA	NA	NA	NA	
		EtoAC	139.4	98.2	291.2	532.1	> 1000	
		Met	423.7	389.3	> 1000	> 1000	> 1000	
		Pe	NA	NA	NA	NA	NA	

Values given as μg/ml for lichen associated with microorganism extracts and as μg/ml for antibiotics. NA means no active.
*Aq, aqueous; Ace, acetone; Chl, chloroform; Di, diethyl ether; Et, ethanol; EtoAC, ethyl acetate; Met, methanol; Pe, petroleum ether
**The number was given from KOPRI
***standard : only solvents without extract and ampicillin is used as control.

morphological, physiological and biochemical characteristic results are summarized in Fig. 1 and Table 2. To evaluate their antibacterial activity, disk diffusion test and MIC test were carried out using various solvents extraction (acetone, water, chloroform, diethyl ether, ethanol, methanol and petroleum ether) of total 5 microorganisms isolated from Arctic lichens (*Cladonia* sp., *Sterocaulon* sp., *Umbilicaria* sp. and *Cetraria* sp.).

Disk diffusion test summarized in Table 3 showed antibacterial activity against the 6 pathogenic bacteria. All

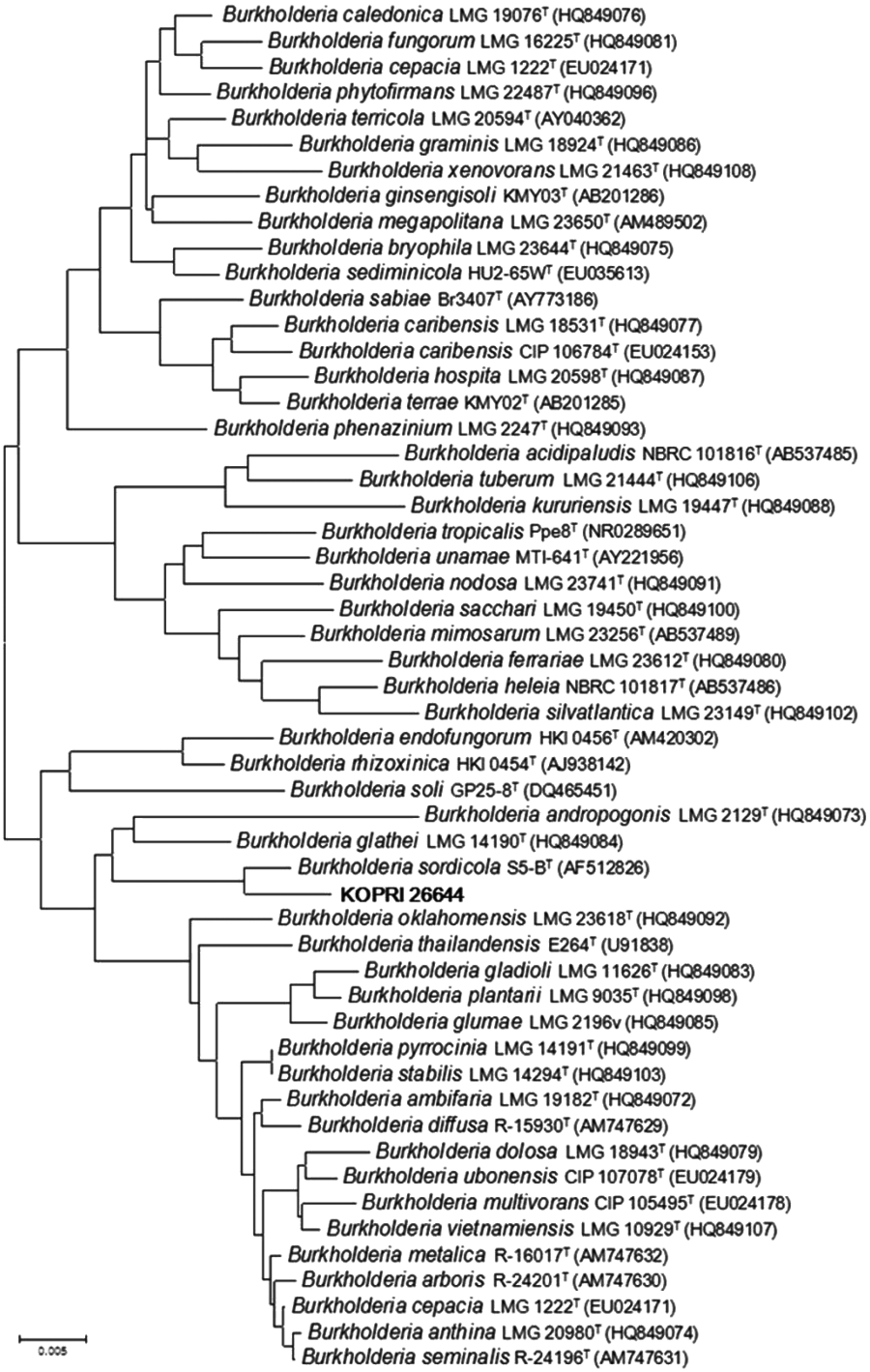

Fig. 2. Phylogenetic relationship of KOPRI 26644 and *Burkholderia* sp. The tree was constructed based on 16S rRNA sequence analysis and according to neighbor-joining method.

the aqueous extracts of 5 microorganisms showed no activity against all 6 target strains containing Gram positive pathogenic bacteria (*Staphylococcus aureus, Bacillus subtilis* and *Micrococcus luteus*) and Gram negative pathogenic bacteria (*Enterobacter cloacae, Pseudomonas aeruginosa* and *Escherichia coli*). Extracts of 26643 has weak activity against all target strain *S.aureus, B. subtilis, M. leutus, E. cloacae, P. aeruginosa, E. coli* when compared with 6 mm diameter of paper disk. Among the extracts, ethyl acetate (EtoAC) extract showed good activity, especially ethyl acetate extract of SM-1 showed best activity (10 mm) against *B. subtilis*. 26644 extracts showed better activity than other isolated strains. Chloroform (Chl), diethyl ether (Di), ethyl acetate extracts exhibit activity against all target strains and ethyl acetate extract of 26644 showed best activity (10 mm) against *M. luteus, E. cloacae, P. aeruginosa and E. coli.* 26645 extracts have no activity against *S. aureus* but showed weak activity against other pathogenic bacteria, especially gram negative pathogenic bacteria strains. Ethyl acetate extract of 26645 showed relatively higher activity (9 mm) against *B. subtilis, E. cloacae*, and *P. aeruginosa* than other extracts of 26645. 26646 extracts have weak activity against all pathogenic bacteria. Although acetone, chloroform and diethyl ether extract showed activity, ethyl acetate extraction is more effective than other solvents. Ethyl acetate extract of 26646 showed activity (10 mm) against *M. luteus*. In case of 26647, they have no activity against *S. aureus, P. aeruginosa and E. coli*. Ethyl acetate extract exhibits weak activity against *B. subtilis* (8 mm), *M. luteus* (9 mm) and *E. cloacae* (9 mm). Also, chloroform extract has weak activity (7 mm) against *B. subtilis*.

MIC test results are also similar with disk diffusion test results as summarized in Table 4. Ampicillin, used as a positive control, showed MICs against target stain in the range 0.31-0.58 μg/ml but five tested samples showed inhibition activity at very high concentration against all 6 pathogenic bacteria.

In both disk diffusion test and MIC test, 26644 showed good activities in all solvents. Therefore, we carried out fatty acid analysis which is used to characterize strain, to investigate this strain. Phylogenetic tree and fatty acid profiles of 26644 are summarized in Figs. 2 and 3. The results suggest that 26644 is similar with strain of *Burkholderia sordidicola* S5-B^T. There is no report about active compound from *B. sordidicola* S5-B^T. So, this strain might have antibacterial compound from this strain.

The results indicate only few microorganisms showed antibacterial activity and against both Gram-positive bacteria and Gram-negative bacteria. Zone diameters were roughly same for extract of same bacterial samples. In case of solvents, ethyl acetate extraction was more effective against 6 target bacteria. Solvents with high polarity, like ethanol, and with very low polarity, like petroleum ether, did not extract efficiently. All the aqueous extracts have no antibacterial activity against target bacteria. It is probable that they have no water soluble active compounds.

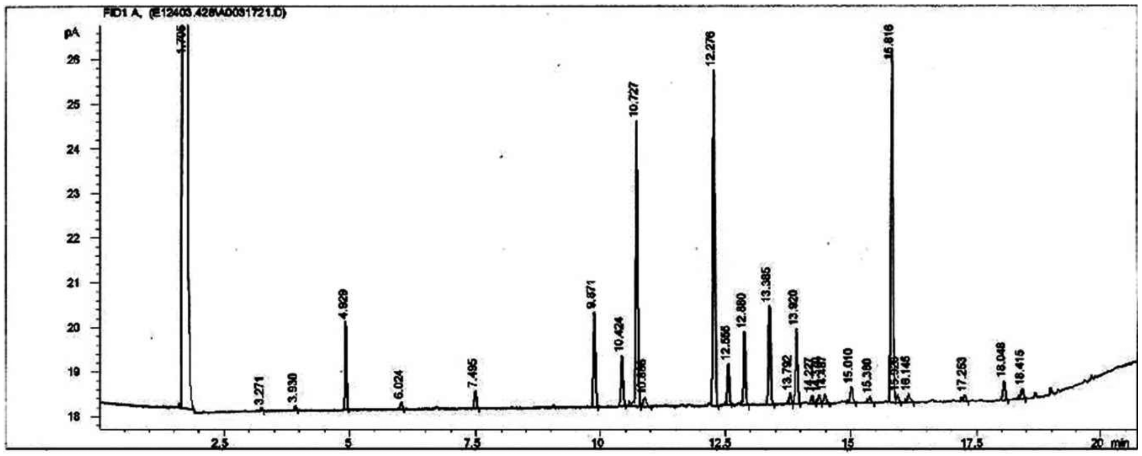

Fig. 3. Fatty acid profile of KOPRI 26644. Numerical data in figure indicated retention time of cellular fatty acids and expressed in follow form: fatty acid (retention time). Solvent peak (1.705); $C_{10:0}$ (3.271); $C_{12:0}$ aldehyde (3.930); $C_{12:0}$ (4.929); $C_{13:1}$ (6.024); $C_{14:0}$ (7.495); $C_{12:0}$ aldehyde (9.871); summed in $C_{16:1}$ w7c/$C_{16:1}$ w6c (10.422); $C_{12:0}$ (10.727); $C_{17:0}$ cyclo (12.276); $C_{16:1}$ 2OH (12.555); $C_{16:0}$ 2OH (12.880); $C_{16:0}$ 3OH (13.385); $C_{16:0}$ (10.726); summed in $C_{18:1}$ w7c/$C_{18:1}$ w6c (13.920); $C_{18:0}$ (14.227); $C_{18:1}$ w7c 11 methyl (14.370); $C_{17:0}$ iso 3OH (14.487); $C_{19:0}$ cyclo w8c (15.816); $C_{18:1}$ 2OH (16.145); $C_{20:2}$ w6,9c (17.253).

Up to date, little is known about antibacterial active compound from *Stereocaulon* sp. and there are many reports that crude extract of lichen samples, not lichen-associated bacteria, showed good antibacterial activity. This is the first study to evaluate antibacterial activity of microorganisms isolated from the Arctic lichen *Stereocaulon* sp. In this study, the obtained results showed that the tested extracts of 5 polar microorganisms isolated from Arctic lichen samples have some antibacterial effect which suggests a possibility of their use in treatment of various diseases caused by these and similar microorganisms. Further, we need to investigate for screening and isolation of the microorganisms of lichen symbiosis and detail investigation about their activities is essential.

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국문초록

북극 지의류 유래 미생물의 항균성

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북극 지의류로부터 서로 다른 5종의 극지 미생물을 분리하였고, 그들의 생리활성 물질들은 아세톤, 물, 클로로포름, 다이에틸에테르, 에탄올, 에틸아세테이트, 메탄올 및 페트롤륨 에테르 등 다양한 용매를 이용하여 균 배양액으로부터 추출되었다. 이러한 추출물들의 항균성은 *Staphylcoccus aureus, Bacillus subtilis, Micrococcus luteus, Enterobacter cloacae, Pseudomonas aeruginosa* 및 *Escherichia coli* 등 6종의 병원체에 대한 디스크 확산법과 최소억제농도 측정법에 의해 조사되었다. 여러 추출 샘플 중, *Burkholderia sordidicola* S5-B^T (KOPRI 26644) 유사미생물 종의 에틸아세테이트 추출물이 표적박테리아에 대한 높은 항균활성을 나타내었다(억제대, 7-10 mm; 최소억제농도, 57.5 → 1000 μg/ml). 또한, 다양한 추출 용매 중, 클로로포름 추출물에서 약하지만 분명한 활성을 나타내었다.