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Antioxidant Activities of Bacterial Culture Extracts Isolated from Arctic Lichens

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Lichens are a symbiosis between fungi, algae and cyanobacteria. Our group recently studied the antioxidant properties of some bacterial species isolated from Arctic lichens and we confirmed that they possess high antioxidant activities. In this paper, we investigated the antioxidant capacity of 5 microorganisms newly isolated from 4 Arctic lichen species, *Cladonia* sp., *Sterocaulon* sp., *Umbilicaria* sp. and *Cetraria* sp., using various solvent extractions. We carried out 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid) (ABTS) free radical scavenging activity test and ferric reducing antioxidant power (FRAP) assay. Also total phenolic and flavonoid content assays were performed. Among the bacterial culture extracts of the tested lichen-microorganisms, ethyl acetate extracts of *Burkholderia sordidicola* S5-B(T) had not only a high antioxidant activity (72.9%) when compared with the ascorbic acid used as the control (51.3%) in the DPPH assay, but also a high amount of phenolic content as well as flavonoid content. As a result, these lichen-microorganisms may be potentially useful sources of natural antioxidants.

Key words: ABTS, antioxidant activity, Arctic lichen, DPPH, TPC

Introduction

Reactive oxygen species such as hydroxyl radicals, peroxides and superoxide anions are important because they are major causes of oxidative damage causing carcinogenesis, mutagenesis, aging and cardiovascular diseases [6, 9]. Antioxidants inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions and hence protect the human body from oxidative stress-related diseases [19]. They are also used to protect foods from rancidity, discoloration or deterioration due to auto-oxidation [12]. Although several strong synthetic antioxidants have already been reported such as butylated hydroxyanisole, butylated hydroxytoluene and tertiary butylhydroquinone, demand to develop less harmful antioxidants from natural sources is increasing because synthetic antioxidants have proven to be highly carcinogenic compound [9, 19].

Lichens are composed of fungi, algae, or cyanobacteria which are able to survive in extreme environmental conditions from desert to polar area. Lichens and their secondary metabolites have been widely used as food, dyes, perfumes, natural medicines and decoration [12]. Especially, their secondary metabolites have been investigated for a long time. Usnic acid, for example, is representative secondary metabolite found in several lichen species and has potential as antioxidant, antibiotic and antitumor drug [4, 11, 15]. Little is known about biological activities of lichen-associated bacterial symbionts [5], while lichen-forming fungi have been extensively described although study of lichen-forming fungi is difficult due to their very slow growth rates [18]. Diversity of bacterial community in lichen symbiosis has been reported and recent research suggested that these bacterial symbionts have antibacterial and antioxidant activity [8]. On this background, the aim of this study is to evaluate antioxidant activities of bacterial culture extracts isolated from the Arctic lichens.

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Materials and Methods

Collection and Identification of Lichen Samples

Lichen species are provided by the Korean Polar Research Institute (KOPRI). All species were identified by KOPRI and their 16S rRNA analysis results were summarized in Table 1. All sample numbers were also given from the KOPRI.

Culture Condition and Preparation of the Lichen-Bacterial Culture Extracts

The 5 microbial species were isolated by selective appropriate media [10, 16]. KOPRI 26643, 26645 and 26647 strains were isolated from ISP4 agar media [16] while 26644 and 26646 strains were isolated from Bennett's vitamin agar media [10] at 28°C. In addition, these 5 microbial species were inoculated on the corresponding media as shown above at 28°C for 10~13 days. All the bacterial cultures were treated with various solvents (water, acetone, methanol, ethyl acetate, chloroform, ethanol, diethyl ether, and petroleum ether) at room temperature. And then the solvent was evaporated in vacuum at 40°C. The residuals were then dissolved with the same solvents and stored in a freezer at -20°C until further study.

Total Phenolic Contents (TPC) and Total Flavonoid Contents (TFC) assay

The total phenolic content in the lichen associated bacterial culture extract was evaluated by the Folin-Ciocalteu reagent according to the method of Slinkard and Singleton with some modifications [17]. 30 μL of the test extracts were mixed with 30 μL of 1 N Folin-Ciocalteu reagent (Sigma-Aldrich, USA) and the reaction mixture was mixed thoroughly. These mixtures are incubated for 3 min at room temperature and 600 μL of 2% Na_2CO_3 was added to the reaction mixture. The mixture was incubated for 30 min at room temperature and the absorbance was measured at 760 nm. Gallic acid used as the positive control and the reaction mixture without the extraction sample was used as the negative control. The concentration of TPC was expressed in micrograms of gallic acid equivalent per milligram of lichen-bacterial culture extract. Total flavonoid contents were evaluated by using modified colorimetric method [20]. The 500 μL of lichen-bacterial culture extracts was taken. The 1.5 mL of distilled water was added to it and subsequently mixed with 0.15 mL of 5% $NaNO_2$ solution. After 6 min incubation, 10% $AlCl_3$ solution was added and incubated for 6 min. And then 2 mL of 4% NaOH solution was added to the mixture, and distilled water was added to set the final volume to be 5 mL. After 15 min at room temperature, the absorbance was measured at 510 nm using a spectrophotometer (Biochrome, U.S.A.). 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 lichen-bacterial culture extract was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azino-bis[3-ethyl benzothiazoline-6-sulphonic acid] (ABTS) reagents. These assays are colorimetric method and their result can be read visually. The DPPH radical scavenging assay is an easy rapid and sensitive method for the antioxidant screening of plant extracts. The DPPH free-radical scavenging activity of the 5 lichen-bacterial culture extract was determined by the method of Blois with some modifications [3]. 0.1 mM of DPPH was prepared in methanol. Then, 950 μL of DPPH solution was mixed with 50 μL of lichen-bacterial extraction samples with various solvents. The mixture was incubated for 30 min at room temperature, and the absorbance was measured at 517 nm using a UV-Visible spectrophotometer to determine the content of DPPH free radical. 1 mM of ascorbic acid (vitamin C) was used as positive controls and pure solvents without lichen-bacterial culture extract were used as a negative control. The ABTS assay is also frequently

Table 1. Microorganisms isolated from the Arctic lichen samples.

KOPRI No.	Source	Bacterial species (Closest strain)	Similarity (%)
26643	*Cladonia* sp.	*Burkholderia sordidicola* S5-B(T)	98.932
26644	*Sterocaulon* sp.	*Burkholderia sordidicola* S5-B(T)	98.932
26645	*Cetraria* sp.	*Sphingomonas faeni* MA-olki(T)	99.851
26646	*Umbilicaria*sp.	*Burkholderia sordidicola* S5-B(T)	98.932
26647	*Umbilicaria* sp.	*Burkholderia sordidicola* S5-B(T)	98.932

used to measure antioxidant activities. For ABTS assay, the procedure followed the method of Arnao with some modifications [1]. 7.4 mM of ABTS was prepared in methanol. ABTS was kept in the dark for 12 h to generate free radicals from the ABTS salt, and then 950 μL of ABTS solution was mixed with 50 μL of lichen-bacterial extraction samples. The mixture was incubated for 30 min at room temperature and the absorbance was measured at 734 nm using a UV-Visible spectrophotometer. 1 mM of ascorbic acid was used as 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. A single extract were measured three times.

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

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was carried out according to the modified Benzie and Strain method [2]. 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 samples 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 spectrophotometer. Temperature was maintained at 37°C. The readings at 30 min were selected for calculation of FRAP values.

Results and Discussion

Total Phenolic and Flavonoid Contents of Microorganisms

The fact that antioxidant activities of lichen-bacterial culture extracts are dependent on their phenolic constituents was previously demonstrated [7]. Thus, we evaluated the total phenolic contents of lichen-bacterial culture extracts (Table 2). Total 5 bacterial cultures were extracted in various solvents such as aqueous, acetone, methanol, ethyl acetate, chloroform, ethanol, diethyl ether, and petroleum ether. Some lichen-bacterial species showed high-level of TPC, range of 3.42 (aqueous extract of *Bulkholeria sordidicola* S5-B(T), sample No. 26647) to 72.26 (ethyl acetate extract of *Bulkholeria sordidicola* S5-B(T), sample No. 26644). Especially, extract of *Bulkholeria sordidicola* S5-B(T) had the highest TPC among the other bacterial species. Ethyl acetate and chloroform extracts showed high TPC than other solvents used. We also carried out TFC assay and results are summerized in Table 2. Total flavonoid content was in the order of 26644>26645> 26646>26647>26643, regardless of extraction solvents. Similar pattern was observed for total phenolic content although amount of phenolic is lower than TPC. Furthermore, it was found that both TPC and TFC were dependent on concentration of extracts (data not shown). Taken together, the high levels of TPC and TFC indicate that extracts of microorganisms, isolated from the Arctic lichen samples, might have antioxidant activity.

DPPH and ABTS Free Radical Scavenging Activities of Microorganisms

DPPH and ABTS assays using various solvent extracts

Table 2. Total phenolic and lavonoid contents of lichen-bacterial cell extracts.

Sample No.		Solvents							
		Water	Acetone	Chloroform	Diethyl ether	Ethanol	Ethyl acetate	Methanol	Petroleum ether
TPC[a]	26643	10.04 ± 3.31	27.02 ± 1.82	45.12 ± 1.42	51.22 ± 1.37	12.30 ± 0.51	39.06 ± 1.26	30.14 ± 1.82	24.14 ± 1.30
	26644	6.44 ± 1.20	27.52 ± 0.42	47.06 ± 0.23	71.21 ± 0.26	15.29 ± 0.62	72.26 ± 2.11	48.87 ± 3.42	14.16 ± 0.33
	26645	13.72 ± 0.26	13.25 ± 0.63	31.13 ± 0.57	60.51 ± 1.82	19.21 ± 0.32	61.24 ± 0.24	11.27 ± 1.72	10.21 ± 0.28
	26646	8.81 ± 0.47	10.24 ± 0.23	29.08 ± 0.71	72.01 ± 0.21	20.03 ± 0.33	49.45 ± 2.68	19.33 ± 4.23	4.13 ± 1.40
	26647	3.42 ± 0.35	8.22 ± 0.93	29.12 ± 3.21	36.21 ± 0.11	33.43 ± 0.54	46.36 ± 3.50	52.92 ± 5.23	14.06 ± 0.16
TFC[b]	26643	3.12 ± 0.22	6.48 ± 1.01	12.01 ± 0.76	15.32 ± 0.87	5.05 ± 0.09	11.95 ± 1.03	11.55 ± 0.71	18.09 ± 0.84
	26644	0.21 ± 0.01	6.05 ± 0.48	12.04 ± 1.02	34.25 ± 0.95	6.11 ± 0.12	35.24 ± 1.71	26.07 ± 0.49	6.01 ± 0.67
	26645	3.84 ± 0.03	5.19 ± 0.32	11.09 ± 1.01	30.07 ± 0.45	6.89 ± 0.34	32.10 ± 1.09	6.14 ± 0.34	3.95 ± 0.59
	26646	2.98 ± 0.20	5.02 ± 0.64	10.46 ± 1.45	33.16 ± 0.26	10.02 ± 0.28	29.81 ± 1.55	8.06 ± 0.65	0.72 ± 0.09
	26647	0.55 ± 0.18	4.12 ± 0.14	10.52 ± 2.01	20.11 ± 0.41	9.14 ± 0.71	25.33 ± 1.49	30.02 ± 0.41	0.43 ± 0.07

[a]Total phenolic contents are expressed as gallic acid equivalents (mg GAE/g extract)
[b]Total flavonoid contents are expressed as catechin equivalents (mg CE/g extract)

Table 3. Evaluation of antioxidant properties using various solvents.

Sample No.		Solvents							
		Water	Acetone	Chloroform	Diethyl ether	Ethanol	Ethyl acetate	Methanol	Petroleum ether
DPPH	26643	3.1 ± 1.29	7.7 ± 1.02	54.3 ± 1.42	45.2 ± 1.35	11.5 ± 0.45	13.1 ± 0.73	38.6 ± 0.84	20.2 ± 2.18
	26644	7.2 ± 0.30	37.5 ± 1.19	22.4 ± 1.17	29.8 ± 1.04	12.3 ± 0.33	72.9 ± 0.41	8.1 ± 0.52	23.1 ± 1.93
	26645	14.9 ± 0.41	41.1 ± 2.12	22.1 ± 0.33	9.3 ± 0.35	19.9 ± 1.41	55.9 ± 0.73	8.9 ± 1.16	41.3 ± 0.62
	26646	5.1 ± 1.01	9.8 ± 0.75	6.1 ± 1.72	42.1 ± 2.26	48.3 ± 0.82	21.9 ± 0.32	11.2 ± 0.94	32.7 ± 0.97
	26647	9.6 ± 1.58	15.7 ± 0.92	10.4 ± 0.84	36.1 ± 0.71	21.5 ± 1.44	20.5 ± 0.51	31.9 ± 0.72	34.7 ± 3.57
Control		Ascorbic acid (1 mM) : 51.3 ± 0.32							
ABTS	26643	5.4 ± 0.73	7.0 ± 0.42	22.3 ± 0.65	10.1 ± 0.37	6.09 ± 0.33	9.0 ± 0.37	13.8 ± 0.86	10.4 ± 0.68
	26644	8.7 ± 0.81	12.5 ± 1.02	19.9 ± 0.57	12.8 ± 1.12	7.7 ± 0.28	34.9 ± 0.65	18.6 ± 0.42	11.7 ± 1.72
	26645	3.2 ± 0.90	22.0 ± 1.34	21.9 ± 0.38	20.3 ± 0.18	9.0 ± 0.43	15.7 ± 1.12	5.9 ± 0.89	15.6 ± 0.37
	26646	5.3 ± 1.25	17.2 ± 2.38	24.3 ± 1.22	14.1 ± 0.96	11.0 ± 1.12	5.4 ± 0.51	6.7 ± 0.99	22.3 ± 0.97
	26647	4.7 ± 0.52	22.9 ± 0.42	22.1 ± 0.94	6.1 ± 3.20	13.8 ± 0.92	9.6 ± 0.71	12.8 ± 1.42	9.1 ± 2.14
Control		Ascorbic acid (1 mM) : 25.8 ± 0.41							

Free radical scavenging activity was described as the inhibitory percentage of DPPH and ABTS.

of 5 lichen-bacterial species are performed to evaluate the antioxidant activity (Table 3). The DPPH free radical scavenging activity of each lichen-bacterial culture extract was compared with that of the natural antioxidant, ascorbic acid (vitamin C). All the tested extracts and the control (ascorbic acid) exhibited DPPH and ABTS free radical scavenging activity was depend on concentration of extracts (data not shown). Also, the rate of scavenging activity was variable for each extract and each solvent. In case of DPPH assay, radical scavenging activities range from 3.1 (aqueous extract of *Bulkholeria sordidicola* S5-B(T), KOPRI 26643) to 72.9 (ethyl acetate extract of *Bulkholeria sordidicola* S5-B(T), KOPRI 26644). Similarly, radical scavenging activities in ABTS assay range from 3.2 (aqueous extract of KOPRI 26645) to 34.9 (ethyl acetate extract of KOPRI 26644). Among 5 bacterial species, *Bulkholeria sordidicola* S5-B(T) showed stronger activity than ascorbic acid about 1.4 fold and 1.3 fold in DPPH and ABTS assay, respectively. In almost all cases, extracts of bacterial species that have high amount of TPC showed high antioxidant activity. However, such as chloroform extract of *Burkholderia sordidicola* S5-B(T) (KOPRI 26643) showed high antioxidant activities than chloroform extract of *Burkholderia sordidicola* S5-B(T) (KOPRI 26644) in spite of that total phenol contents of KOPRI 26643 is lower than KOPRI 26644 (Table 3). This indicates that all phenolics may not necessarily have same antioxidant activity. Some of them may have more potent activity and others have a moderate or even weak antioxidant activity. It is possible there are synergistic or antagonistic interactions of phenolic compounds with other phenolics, or differing types of components such as carbohydrates and proteins [14].

Ferric Reducing Antioxidant Power (FRAP) of Microorganisms

We carried out ferric reducing antioxidant power to evaluate the reducing activity of microorganism extract. In this assay, antioxidant acts as reductant by performing reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). So, determination of the ferrous ion formation can be used to

Table 4. Ferric reducing antioxidant power activity of extracts.

Sample No.	Solvents							
	Water	Acetone	Chloroform	Diethyl ether	Ethanol	Ethyl acetate	Methanol	Petroleum ether
26643	0.07 ± 0.01	0.10 ± 0.01	0.14 ± 0.02	0.12 ± 0.01	0.11 ± 0.02	0.21 ± 0.06	0.10 ± 0.01	0.13 ± 0.01
26644	0.19 ± 0.02	0.21 ± 0.03	0.51 ± 0.02	0.15 ± 0.01	0.19 ± 0.02	0.72 ± 0.14	0.18 ± 0.23	0.18 ± 0.02
26645	0.15 ± 0.01	0.18 ± 0.01	0.31 ± 0.01	0.20 ± 0.02	0.15 ± 0.02	0.56 ± 0.11	0.13 ± 0.03	0.16 ± 0.02
26646	0.13 ± 0.01	0.19 ± 0.03	0.27 ± 0.03	0.21 ± 0.02	0.17 ± 0.02	0.43 ± 0.05	0.11 ± 0.06	0.13 ± 0.01
26647	0.10 ± 0.01	0.20 ± 0.04	0.25 ± 0.01	0.21 ± 0.02	0.18 ± 0.01	0.33 ± 0.05	0.10 ± 0.01	0.15 ± 0.03
Ascorbic acid	6.81 ± 0.44							

Free reducing antioxidant power activities are expressed as mM Fe(II)/mg extract and ascorbic acid was used as control.

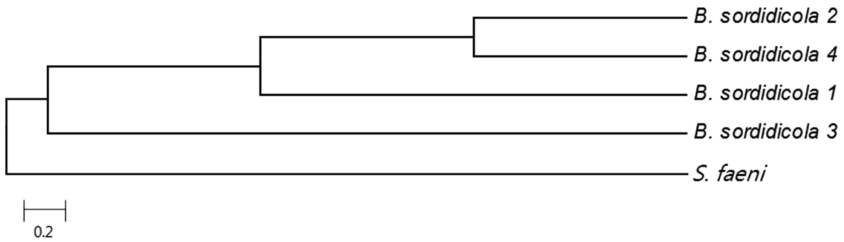

Fig. 1. Phylogenetic relationship of 5 bacterial strains. *B. sordidicola* 1, KOPRI 26643 (from *Cladonia* sp.); *B. sordidicola* 2, KOPRI 26644 (*Sterocaulon* sp.); *S. faeni*, KOPRI 26645 (*Cetraria* sp.); *B. sordidicola* 3, KOPRI 26646 (*Umbilicaria* sp.); and *B. sordidicola* 4, KOPRI 26647 (*Umbilicaria* sp.).

expect the reducing power of samples, and results are summarized in Table 4. The FRAP value for the ethyl acetate extract of KOPRI 26644 was high. This sample has high level of phenolic and flavonoid contents, and also it's free radical scavenging activity was good in both DPPH and ABTS assay. Although some value is lower than standard compound, all our microorganisms, isolated from the Arctic lichen samples, has antioxidant activity.

In this paper, we isolated various microorganisms from the Arctic lichens and evaluated their antioxidant activities using various solvent extractions. The results showed that ethyl acetate extract of *Bulkholeria sordidicola* S5-B(T) exhibits a stronger activity than ascorbic acid about 1.4 fold and 1.3 fold in DPPH and ABTS assay, respectively. Also, amount of their total phenolic component and total flavonoid contents was higher than other extraction samples. Therefore, these strains might be potentially useful natural antioxidants which might be able to replace several synthetic antioxidant compounds proven to be highly carcinogenic. Interestingly, *Bulkholeria sordidicola* S5-B(T) was isolated from several lichen species, but their antioxidant activity showed some variations (Table 2). Even their 16S rRNA sequence similarity was almost same in phylogenetic analysis (Fig. 1), media compositions such carbon source and/or nitrogen source may effect on their active compound. Therefore, further investigation is needed about their correlation between media composition and active compounds. These active compounds are may control UV protection system on lichen. In respect of the cosmetics/pharmaceutical industry, these properties are very significant because these active compounds might be able to replace several synthetic antioxidant compounds which known to be carcinogenic. Lichens are well adapted to the polar area where they have limited nutrients. In previously study, some group found that antioxidant activity of crude extract from polar lichen was more effective than other lichen species from tropical and temperate region [13]. Although it's different with us because we investigated antioxidant activity of lichen-associated bacteria, our results showed similar pattern with previous study. In addition to, this kind of study that investigation of lichen-associated bacteria symbiont will help understand unique mechanisms of lichen ecology in extreme condition.

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국문초록

북극 지의류로부터 분리한 미생물 배양 추출액의 항산화 활성

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지의류는 곰팡이, 조류 및 남조균류의 공생체이다. 본 연구팀은 최근 북극 지의류로부터 분리한 몇몇 미생물 종의 항산화 활성에 대하여 연구하였고, 그들의 높은 항산화 활성을 확인하였다. 본 연구에서는 *Cladonia* sp., *Sterocaulon* sp., *Umbilicaria* sp. 및 *Cetraria* sp. 총 4종류의 지의류로부터 5종의 미생물을 새롭게 분리하였고 배양 후, 다양한 용매 추출법으로 그들의 항산화능을 조사하였다. DPPH와 ABTS 자유 라디컬 소거능 측정법 및 FRAP 분석 등을 수행하였다. 또한 총 폴리페놀함량과 총플라보노이드 함량 분석 역시 수행하였다. 지의류 유래 미생물 배양 추출액 중, *Burkholderia sordidicola* S5-B(T) 유사 미생물 종의 에틸아세테이트 추출액은 DPPH 분석에서 대조군인 아스코르빈산 (51.3%)에 비해 72.9%로 높은 항산화 활성을 보였을 뿐만 아니라, 높은 플라보노이드와 폴리페놀 함량을 나타내었다. 결과적으로, 이러한 지의류 유래 미생물 종들은 잠재적으로 천연 항산화제의 원천소재로서 이용가능할 것이다.