

Lecanoric acid, a secondary lichen substance with antioxidant properties from *Umbilicaria antarctica* in maritime Antarctica (King George Island)

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Abstract Eight lichen species, *Cetraria aculeata*, *Cladonia furcata*, *Pseudephebe pubescens*, *Sphaerophorus globosus*, *Stereocaulon alpinum*, *Umbilicaria antarctica*, *Usnea antarctica* and *Usnea aurantiacoatra*, were collected from King George Island, maritime Antarctica, for the evaluation of antioxidant activities. Anti-linoleic acid peroxidation activity, free radical scavenging activity, reducing power and superoxide anion scavenging activity were assessed of methanol and acetone extract of the lichens in vitro. Extract of *Umbilicaria antarctica*, *Cladonia furcata*, *Sphaerophorus globosus* and *Usnea antarctica* were found to have strong in vitro antioxidant properties. In general, acetone extract exhibited stronger activities than methanol extract. The activity-guided bioautographic TLC and HPLC analysis demonstrated that lecanoric acid was the main antioxidant compound in the acetone extract of *Umbilicaria antarctica*, the most potent antioxidant lichen species among the test species. The results suggested that several Antarctic lichens and their substances can be used as novel bioresources of natural antioxidants.

Keywords Antarctic lichens · Antioxidant activity · Bioresources · Lecanoric acid · *Umbilicaria antarctica*

Introduction

Evidence is accumulating that oxidative damage mediated by reactive oxygen species (ROS) plays an important role in the etiology of several human diseases and ageing processes, and is also involved in food deterioration. Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions. Currently, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) are widely used by the food industry. However, a restriction on synthetic antioxidants is being imposed because of their toxicity to the liver and their carcinogenicity (Grice 1986; Wichi 1988). Therefore, the development and utilization of non-toxic and efficient antioxidants from natural origin is desirable. In the past years, the antioxidant properties of numerous crude extracts such as the primary and secondary metabolites of many plants have been widely reported (Hidalgo et al. 1994; Pietta et al. 1998).

Lichens, the dominant life-form throughout most of continental Antarctica, might be a good candidate for natural antioxidants on account of their adaptability in extreme conditions. Antarctica are extreme habitats, in terms of low temperature, prolonged drought, rapidly fluctuating thermal and hydric regimes, high solar irradiance and ultraviolet-B radiation levels, and prolonged winter darkness (Øvstedal and Lewis Smith 2001). Such extreme conditions increase the formation of ROS in organisms. Thus Antarctic lichens may contain considerable antioxidants to protect them from the damage of oxidative stress caused by the extreme condition. Like many other organisms, lichens are rich in cytoplasmic and membrane-bound antioxidants, such as glutathione, tocopherol and carotenoids. These intracellular

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antioxidants are essentially required for normal metabolism and stress response (Kranner et al. 2008). Meanwhile, lichens possess numerous extracellular deposits, which may potentially contribute to the overall antioxidant protection. Lichens synthesize a great variety of secondary metabolites which are often structurally unique, with only a small number of them being found in other fungi and higher plants (Stocker-Wörgötter 2008). Recently, much attention has been paid on the biological roles of lichen secondary substances; many lichen substances have been found to have a lot of bioactivities, such as antitumour, antibacterial, antifungal, antiviral, antiinflammatory and also antioxidant activities (Oksanen 2006). Some phenolic lichen secondary products are proved to have potent antioxidant activities (Hidalgo et al. 1994). Therefore the secondary metabolites of Antarctic lichens might be a potential resource of natural antioxidants and deserve to be tested for their antioxidant activities.

A previous study demonstrated the antioxidant activity of five species of Antarctic lichens and the results indicated that polar lichens exhibited antioxidant activities at a relatively low-dose level when compared to tropical lichens (Paudel et al. 2008). Nevertheless, these data of only five species is still scarce compared with the abundant lichen resources in Antarctica. Furthermore, the compounds responsible for the observed antioxidant activities are still unclear. Therefore, we collected other eight macrolichen species, which are widely distributed on maritime Antarctica, aiming to assess their antioxidant activities in vitro, and to isolate and characterize the effective compounds from the crude lichen extracts of high antioxidant lichen species.

Materials and methods

Lichen collections and identification of lichen samples

Eight species of Antarctic lichen specimens: *Cetraria aculeata*, *Cladonia furcata*, *Pseudephebe pubescens*, *Sphaerophorus globosus*, *Stereocaulon alpinum*, *Umbilicaria antarctica*, *Usnea antarctica*, and *Usnea aurantiacoatra* were collected from Barton Peninsula, the southwestern part of King George Island which is the largest island in the South Shetland Islands belonging to maritime Antarctic zone. Identification of all the species was previously reported (Kim et al. 2006). Voucher specimens were deposited in the lichen herbarium of Korean Lichen Research Institute (KoLRI) at Sunchon National University, Korea.

Preparation of the lichen extract

Air-dried lichen thalli (5 g) were cut into pieces (1 mm × 1 mm), shaken in 200 ml acetone twice (each

time 24 h, 150 rpm) at room temperature, and subsequently in methanol at the same condition. The combined resultant extracts of acetone and methanol fractions were respectively filtered using Whatman filter paper (No. 1) and then concentrated in vacuo at 40°C using a Rotary Evaporator. The residuals obtained were separately dissolved by acetone and methanol to different concentrations and stored in a freezer at –20°C until further study.

Ascorbic acid-linoleic acid assay

The inhibitory activity on linoleic acid peroxidation of lichen extracts was determined according to the thiocyanate method (Mitsuda et al. 1996) with a few modifications. Lichen extracts (0.1 ml, 2 mg/ml) were added to a 0.5 ml linoleic acid emulsion (0.02 M, pH 7.0) with a 0.5 ml phosphate buffer (0.2 M, pH 7.0). The linoleic acid emulsion was prepared by mixing linoleic acid with the same weight of tween 20 and a 100 ml phosphate buffer (0.2 M, pH 7.0), and then it was homogenized. The mixed solution was incubated at 37°C for 24 h. Then 1 ml of ammonium thiocyanate (3%, w/v) and 0.1 ml FeCl₂ (0.02 M, in 1 M HCl) were added into the 0.1 ml mixed solution. The antioxidant activity was determined by

Table 1 Antioxidant activities of Antarctic lichens and comparison with those of tropical or temperate lichens

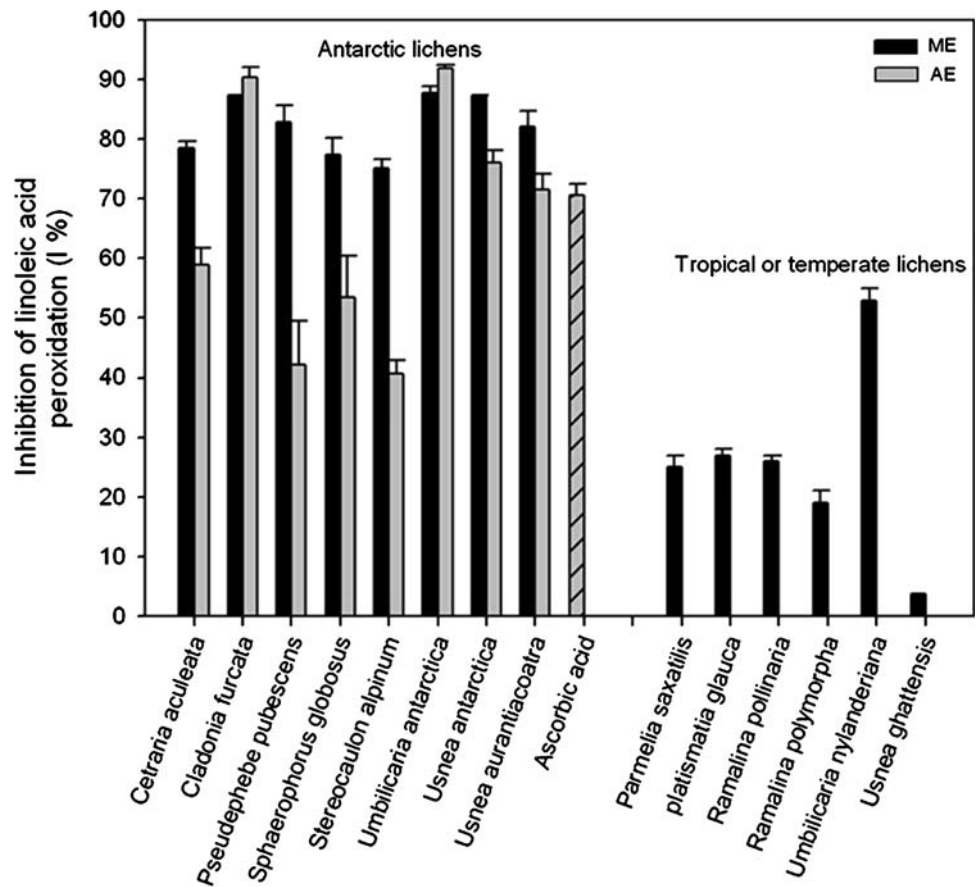
Lichen species	Free radical scavenging activity IC ₅₀ (µg/ml)	
	Methanol extract	Acetone extract
Antarctic lichens		
<i>Cetraria aculeata</i>	>1,000	>1,000
<i>Cladonia furcata</i>	>1000	471.3 ± 35.4
<i>Pseudephebe pubescens</i>	>1,000	>1,000
<i>Sphaerophorus globosus</i>	>1,000	>1,000
<i>Stereocaulon alpinum</i>	>1,000	>1,000
<i>Umbilicaria antarctica</i>	>1,000	121.3 ± 8.4
<i>Usnea antarctica</i>	>1,000	445.7 ± 26.7
<i>Usnea aurantiacoatra</i>	791.3 ± 103.6	642.6 ± 48.7
BHA ^a	4.2 ± 0.3	
Tropical or temperate lichens		
<i>Parmelia saxatilis</i>	NA	–
<i>Platismatia glauca</i>	NA	–
<i>Ramalina pollinaria</i>	NA	–
<i>Ramalina polymorpha</i>	NA	–
<i>Umbilicaria nylanderiana</i>	400.2 ± 0.5	–
<i>Usnea ghattensis</i>	>10,000	–

Data of tropical or temperate lichens were cited from Behera et al. (2006) and Gulluce et al. (2006). All the data presented are mean ± SD, n = 5

IC₅₀ 50% inhibition concentration, NA no activity observed

^a Positive control

Fig. 1 Anti-linoleic acid peroxidation activity of Antarctic lichens and tropical or temperate lichens are shown here. The data of tropical or temperate lichens are cited from Behera et al. (2006) and Gulluce et al. (2006). ME methanol extract, AE acetone extract, I% inhibition percentage



reading the absorbance at 500 nm. The solution without the extract was used as a negative control and one with the same concentration of ascorbic acid was used as a positive control. The activity was described as inhibition percentage (I%), which was calculated by the following equation:

$$I\% = (1 - A_{\text{sample}}/A_{\text{blank}}) \times 100,$$

where A_{blank} is the absorbance of the negative control, and A_{sample} is the absorbance of the tested extracts.

Reducing power determination

The reducing power of the lichen extract was determined following Oyaizu's method (Oyaizu 1986). Lichen extracts (0.1 ml, 2 mg/ml) were mixed with 0.25 ml phosphate buffer (0.2 M, pH 6.6) and 0.25 ml of potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$, 10 g/l], then the mixture was incubated at 50°C for 20 min. Afterwards, 0.15 ml of trichloroacetic acid (100 g/l) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The supernatant solution (0.3 ml) was mixed with 0.3 ml of FeCl_3 (1 g/l). The reducing power of lichen extracts was described as the A_{700} (absorbance at 700 nm) of the reaction mixture. The reaction mixture without extract was used as a negative

control and one with the same concentration of BHA was used as a positive control.

Free radical scavenging activity determination

The free radical scavenging activity of the lichen extract was determined by Blois' method (Blois 1958). Lichen extracts (0.1 ml, gradient concentration from 40 to 330 $\mu\text{g/ml}$) were added into a 0.1 mM of 1, 1-diphenyl-2-picryl-hydrazil (DPPH) methanol solution. After 30 min of incubation at room temperature, the absorbance was measured at 517 nm. A reaction mixture without the extract was used as a negative control and one with the same concentration of BHA was used as a positive control. The free radical scavenging activity was described as IC_{50} (50% inhibition concentration), calculated by SPSS 15.0 software (regression analyses).

Superoxide anion scavenging activity determination

The measurement of superoxide anion scavenging activity of lichen extracts was performed according to the protocol of Nishimiki's method (Nishimiki et al. 1972), with slight modifications. One ml of nitroblue

tetrazolium (NBT) solution (100 μ M NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468 μ M in 100 mM phosphate buffer, pH 7.4) and 1 ml of lichen extracts (2 mg/ml) were mixed. The reaction started by adding 100 μ l of phenazine methosulphate (PMS) solution (60 μ M PMS in 100 mM phosphate buffer, pH 7.4) to the mixture, which was then incubated at 30°C for 15 min. The absorbance was measured at 560 nm. A reaction mixture without the extract was used as a negative control and one with the same concentration of BHA was used as positive control. The calculation method of *I*% was the same with the one in the ascorbic acid–linoleic acid assay.

Bioautographic TLC assay for free radical scavenging activities of *Umbilicaria antarctica* acetone extract

Bioautographic TLC assay was based on Chaabib's method (Chaabib et al. 2003). Lichen extract (40 μ l, 2 mg/ml) was spotted on the TLC plate (silica gel 60, Merck) and then developed in the solvent of toluene: acetic acid (85:15, v/v). After developing the TLC plate, DPPH (0.4 mg/ml in methanol) was sprayed on the TLC plate as the spray

reagent. The active compounds were seen as the yellow white spots against a purple background. To identify the active spot, another TLC plate was also developed in the same solvent system, and then visualized by spraying 10% sulfuric acid.

HPLC analysis of the active compounds

Bioautographic TLC plate was used as the reference to locate of the antioxidant compounds on the prep-TLC plates developed in the same solvent system. The silica gel located at the active spot area were collected, and then dissolved by acetone. The solution was filtered prior to the HPLC analysis. HPLC (LC-10AT, Shimadzu, Japan) under the following conditions were: column, YMC-Pack ODS-A S-5 μ m 150 \times 4.6 mm I.D.; solvent system, MeOH:H₂O:H₃PO₄ (80:20:1, v/v); flow rate, 1 ml/min; photodiode array detector (range 180–700 nm); detecting wavelength, 254 nm for HPLC and 180–400 nm for UV-spectra analysis; temperature, 40°C. The lichen substances were identified by comparing their retention time and UV-spectra data with authentic substances (Yoshimura et al. 1994).

Fig. 2 Superoxide anion scavenging activity of Antarctic lichens and tropical or temperate lichens are shown here. The data of tropical or temperate lichens are cited from Behera et al. (2006). *ME* methanol extract, *AE* acetone extract, *I*% inhibition percentage

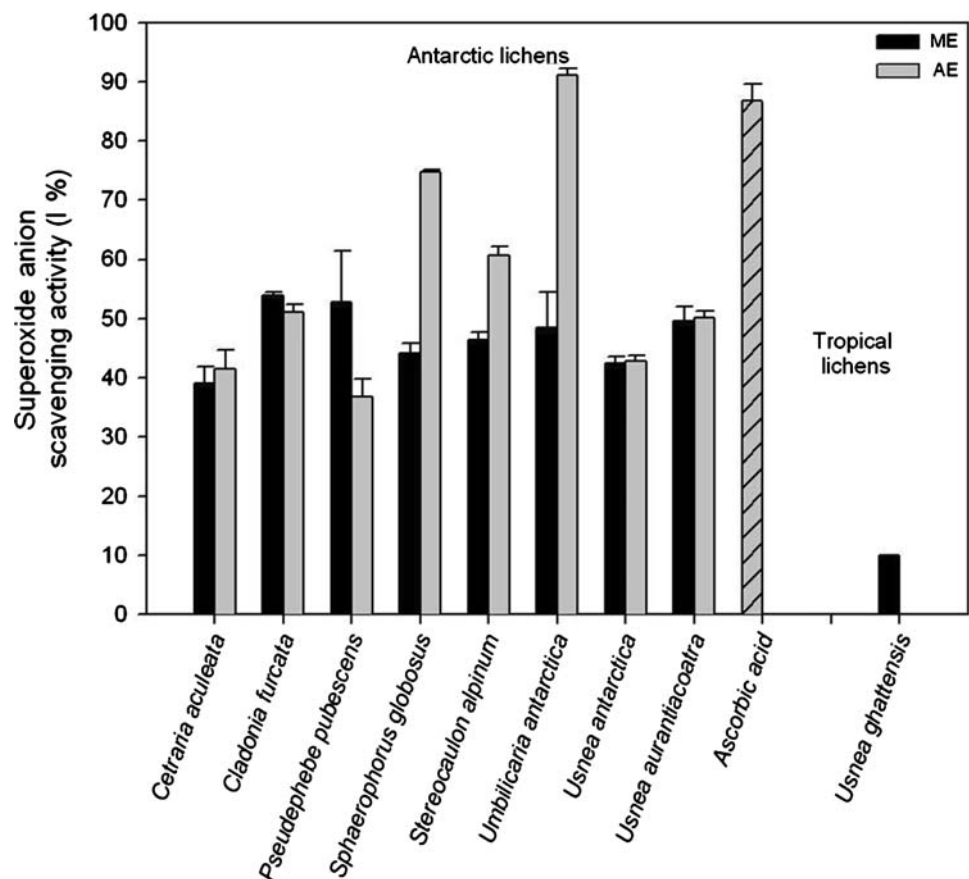
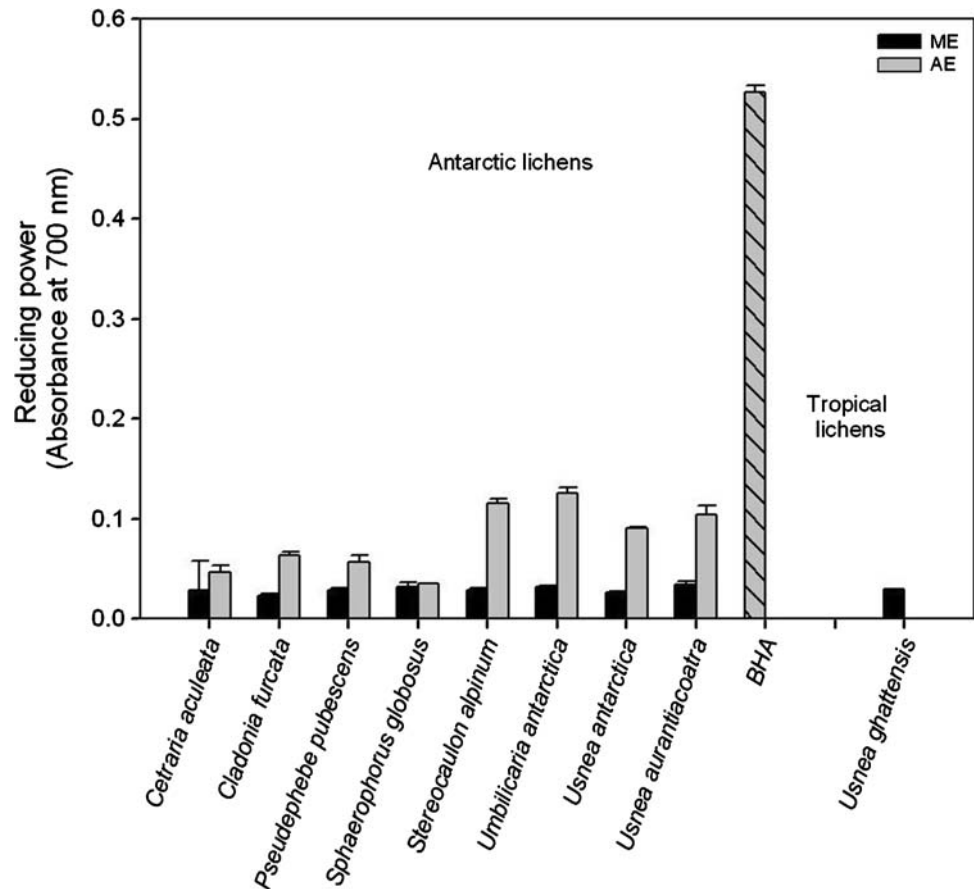


Fig. 3 Reducing power of Antarctic lichens and tropical or temperate lichens are shown here. The data of tropical or temperate lichens are cited from Behera et al. (2006). *ME* methanol extract, *AE* acetone extract, *I%* inhibition percentage



Results and discussion

DPPH assay has been extensively used for screening antioxidant activity because it can accommodate many samples in a short period and is sensitive enough to detect the active ingredients at low concentrations (Sanchez-Moreno 2002). As summarized in Table 1, the strongest free radical scavenging activity was observed in the acetone extract of *Umbilicaria antarctica*, which showed scavenging activity at much lower dose level ($IC_{50} = 121.3 \pm 8.4 \mu\text{g/ml}$) than that of the lichen extract from tropical and temperate origin (Behera et al. 2006; Gulluce et al. 2006). The result, which coincided with the conclusion of previous study (Paudel et al. 2008), suggested that Antarctic lichens contain more potent or larger amount of antioxidants to scavenge the free radicals caused by the extreme habitat such as severe dessication and strong solar radiation proposed by Kranner et al. (2005).

Moreover, Antarctic lichens extracts showed very significant inhibitory activity on linoleic peroxidation, compared with tropical and temperate lichens. As is shown in Fig. 1, methanol extract of all Antarctic species showed even stronger activity than that of ascorbic acid ($70.7 \pm 1.8\%$) as positive control. But the inhibitory

activity of tropical and temperate lichens extract are quite moderate at the same concentration, ranged from 3.8 to 53% and no extract showed higher activity than positive control. Several Antarctic lichen extracts also showed high activity on scavenging of superoxide anion (Fig. 2). The highest scavenging percentage were presented by the acetone extract of *Umbilicaria antarctica* ($91.1 \pm 1.1\%$) and followed by *Sphaerophorus globosus* ($74.7 \pm 0.5\%$). These activities were found to be stronger or close to that of the positive control BHA ($86.8 \pm 2.8\%$), and they are also much stronger than that of Indian lichen *Usnea ghattensis* (Behera et al. 2006). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al. 1995), however, unlike the activity in other three assays, all the tested lichen species were not effective in reducing power, compared to BHA as a positive control (Fig. 3). Although *Umbilicaria antarctica* showed the highest value of reducing power, it exhibited only 24% of the reducing power of the positive control at the same concentration.

Except for the ascorbic acid–linoleic acid assay, the acetone extracts generally showed stronger activity than the methanol extracts in free radical, superoxide anion scavenging and reducing power assay. The result indicated

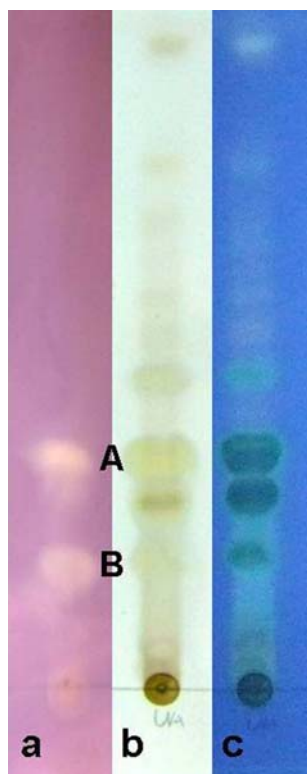
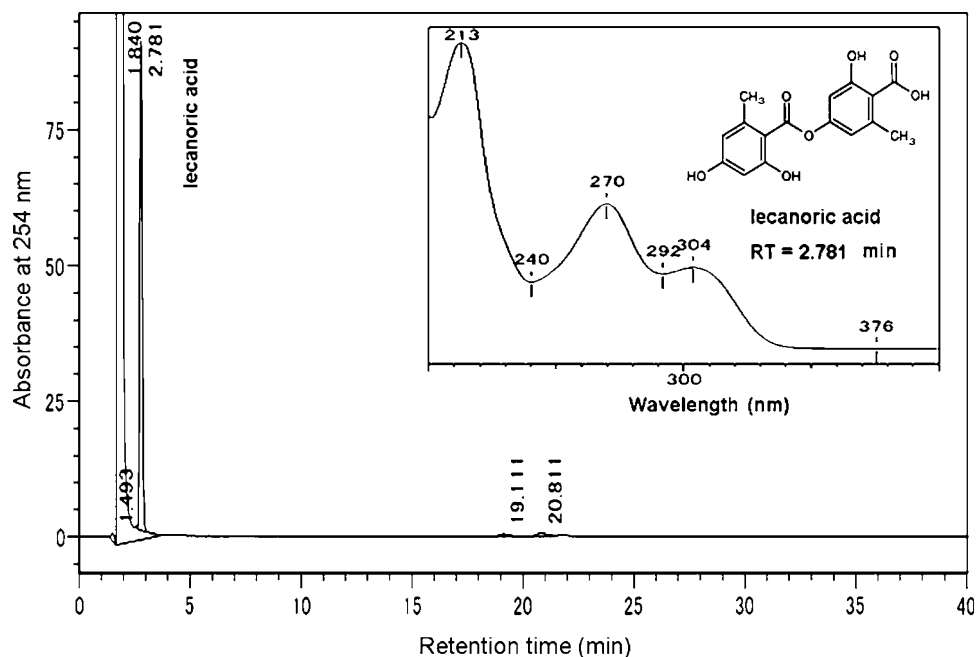


Fig. 4 Bioautographic TLC assay for acetone extract of *Umbilicaria antarctica*. **a** TLC plate sprayed by DPPH methanol solution; **b** TLC plate sprayed by 10% H₂SO₄, under visible light; **c** TLC plate sprayed by 10% H₂SO₄, under UV light; spot A lecanoric acid, spot B unknown

that the compounds with strong antioxidant activity in these species of lichens are of medium polarity. Furthermore, among the extracts assayed through all the four

methods, the acetone extract of *Umbilicaria antarctica* exhibited the strongest antioxidant activity. As an abundant foliose lichen species in maritime Antarctic regions, many systematical, physiological and ecological studies on *Umbilicaria antarctica* had been carried out. However, we could not find any reports dealing with the chemical composition of this lichen species. In order to obtain more information on the compound responsible to the strong free radical scavenging activity, bioautographic TLC assay was used, because this method gives a quick access for detection and localization of the active compounds in a complicated lichen extract. In this method DPPH scavenging activity was observed visually as the white yellow spots on a purple background. As shown in Fig. 4a, there were two active spots; spot A is relative strong while spot B is much weaker, demonstrating that spot A might be one of the main antioxidant compounds in *Umbilicaria antarctica*. To identify spot A, TLC developed in the same solvent system was also visualized by 10% sulfuric acid. Based on the R_f value and the color development of the spot under both visible light and UV light (Fig. 4b, c), spot A was identified as lecanoric acid (Huneck and Yoshimura 1996). The identification of lecanoric acid was further confirmed by HPLC analysis (Fig. 5). There are five detected peaks and the second peak (RT = 2.781 min) is the main component of the tested sample (the first peak with retention time of 1.840 min is acetone; the rest three peaks are pretty small, so we do not consider them to be the main components). The UV-spectra result suggested that the maximum absorption wavelengths (λ_{\max}) of the main compound are 213, 270 and 304 nm. By comparing both the retention time and the λ_{\max} with standard, the main

Fig. 5 HPLC chromatogram and UV-spectra of the antioxidant compounds isolated from acetone extract of *Umbilicaria Antarctica*. RT retention time. There are five detected peaks, the second peak (RT = 2.781 min) is the main component of this sample. The UV-spectra of the tested sample suggested that the maximum absorption wavelengths (λ_{\max}) of the sample are 213, 270 and 304 nm. By comparing both the retention time and the λ_{\max} with standard, the main compound of this sample was identified to be lecanoric acid. The molecular structure of lecanoric acid is presented in the insets



compound of the tested sample was identified to be lecanoric acid (Yoshimura et al. 1994).

Lecanoric acid is a common medullary lichen depside. Lecanoric acid and its derivatives had been found to have a lot of bioactivities in previous studies. Umezawa et al. (1974) demonstrated that lecanoriac acid is an inhibitor of histidine decarboxylase with extremely low toxicity; and another research indicated that 3',5'-dichloro-2,4'-dihydroxybenzanilide, a lecanoric acid analogue, exhibited the inhibitory activity on skin tumor promotion (Umezawa et al. 1984). Gomes et al. (2002) reported the potent fungitoxic activity of lecanoric derivatives. More recently, the free radical scavenging activity of lecanoric acid and its derivatives had also been evaluated in *Parmotrema tinctorum* (Lopes et al. 2008). In their study, orsellinic acid produced from lecanoric acid by alcohol treatment showed more than 8.5 times higher antioxidant activity than that of lecanoric acid (IC_{50} value is $42.87 \text{ mM} = 13.64 \text{ mg/ml}$). The value was much higher than that of the crude acetone extract of *Umbilicaria antarctica* ($121.3 \text{ }\mu\text{g/ml}$) found in our study. This is probably a consequence of synergistic interactions of lecanoric acid with other compounds such as orsellinic acid in the crude extract (Rice-Evans et al. 1997). In addition, some other unknown components may also importantly contribute to the overall activity of the extract, but in present study, they could not be separated and identified by bioautographic TLC assay. In addition to free radical scavenging activity, lecanoric acid may have photoprotection function of lichen symbionts in polar region as demonstrated by McEvoy et al. (2006). Lichens inhabited in maritime Antarctica are heavily exposed to strong solar radiation which causes oxidative stress to the symbiont. In the symbiotic state, photobionts are more tolerant to high irradiance, suggesting that substantial photoprotection is provided by the mycobiont, which forms a cortical layer above the photobiont. Most lichen mycobionts produce some type of cortical or medullar lichen compound which is an extracellular, crystallized compound deposited on the surface of the fungal hyphae (Huneck and Yoshimura 1996). Lecanoric acid has a strong absorption in the UV range, but only in a slight absorption in the violet region of the photosynthetic photo fluence rate band (Fig. 5). Thus, it can be speculated that lecanoric acid plays important role in protecting the lichen symbiont from damaging solar radiation, especially UV radiation in maritime Antarctica.

Based on all the tested antioxidant activities of each Antarctic lichen species compared with the positive control, *Umbilicaria antarctica*, *Cladonia furcata*, *Sphaerophorus globosus* and *Usnea antarctica* can be classified into high antioxidant lichen species. In addition,

when compared our data with those of the available previous study, we concluded that Antarctic lichens showed extraordinary stronger antioxidant activity than tropical or temperate lichens, and they contain more potent or larger amount of antioxidant substances, which could be used as potential bioresource for natural antioxidant in food and medicine industry. However, this study does not suggest exploiting the Antarctic lichen resources in an industrial scale, because of their slow growth. To apply these natural antioxidant resources in a sustainable way, the effective antioxidants in Antarctic lichens may be produced either by fermentation of lichen mycobionts, or by biosynthesis such as heterologous expression of PKS genes in other filamentous fungi (Stocker-Wörgötter 2008). These two applications are now undergoing for mass production of the lichen substances.

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