Mycoscience (2010) 51:391–395
DOI 10.1007/s10267-010-0048-5

NOTE

Antioxidant activities of edible lichen *Ramalina conduplicans* and its free radical-scavenging constituents

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Received: 20 October 2009 / Accepted: 2 March 2010 / Published online: 8 April 2010

Abstract The aim of this study was to evaluate the antioxidant properties of an edible lichen *Ramalina conduplicans*. The extract exhibited potent anti-linoleic acid peroxidation activity, free radical-scavenging activity, and reducing power. The total phenolic contents were found to be high in the extract. Activity-guided bioautographic thin layer chromatography (TLC) and HPLC identified sekikaic acid and homosekikaic acid as the main free radical-scavenging compounds in *R. conduplicans* extract (IC_{50} [50% inhibition concentration] = 0.082 and 0.276 mg/ml, respectively). The results suggested that this edible lichen species have the potential to be utilized as food additives or as protective drugs.

Keywords Bioautographic TLC · Homosekikaic acid · In vitro · Sekikaic acid · Total phenolic contents

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Evidence is accumulating that oxidative damage mediated by reactive oxygen species (ROS) plays an important role in the etiology of several human diseases and aging processes (Scandalios 1997). Therefore, antioxidant defense systems, including antioxidant enzymes, food, and drugs are important in the prevention of many diseases. ROS are also involved in food deterioration, so synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ), all of which were synthesized many years ago, have been used in the food industry as preservatives. However, these compounds have been suspected to cause liver damage and to increase cancer risk (Grice 1986; Wichi 1988), leading to a need for the development and utilization of less dangerous antioxidants of natural origin.

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 to the biological roles of lichen secondary substances; many lichen substances have been found to have several bioactivities, such as antitumor, antibacterial, antifungal, antiviral, anti-inflammatory, and antioxidant activities (Oksanen 2006). Over the past few years, several lichen species from different regions of the world have been screened for their potential antioxidant properties, and some of them showed very strong antioxidant activities (Gülçin et al. 2002; Odabasoglu et al. 2005; Behera et al. 2006; Gulluce et al. 2006; Luo et al. 2009). However, most of these tested lichens are inedibles (or there are no records of them having been eaten), so the utilization of these lichens in food or the pharmaceutical industry is more risky than using edible lichens. Furthermore, most of these lichen extracts have not been fractioned, so the compounds responsible for the observed antioxidant activities are still unclear.

Ramalina conduplicans Vain. is an edible lichen species that is commonly used in central and southeastern Asian countries. In Yunnan province, southwestern China, people cook this lichen to prepare a traditional cold dish served at marriage banquets, or they use it in a stir-fried pork dish (Wang et al. 2001). *R. conduplicans* are also usually used as traditional food by the Rai and Limbu communities of East Nepal (Hanus et al. 2008) and they are used as a spice in many places in India (Upreti et al. 2005). A previous study determined the trace elements of this lichen, and the results suggested that the lichen is rich in necessary trace elements and has high nutritional value (Liu and Fu 2003). However, the bioactivity and therapeutic utilization of this lichen have never been evaluated before.

Therefore, to promote the utilization of this edible lichen for public health, and to search for new active free radical scavengers from a natural resource, the purposes of this study were to evaluate the antioxidant properties of *R. conduplicans* by comparing their antioxidant activities with those of synthetic antioxidants such as BHA and ascorbic acid, and to isolate and identify the free radical-scavenging constituents from *R. conduplicans* secondary metabolites through bioactivity-guided isolation.

Lichen specimens of *R. conduplicans* Vain. were collected from high-altitude regions of Yunnan Province (China) and identified at Kunming Institute of Botany, Chinese Academy of Science (CAS), China. The voucher specimen is deposited in CAS, along with their duplicates in the Lichen and Allied Bioreources Center, Korean Lichen Research Institute (KoLRI), Sunchon National University, Korea. Air-dried and fractionated lichen thalli (50 g) were extracted twice with 2000 ml methanol for 24 h at room temperature. The combined extracts were filtered using Whatman filter paper (No. 1) and then concentrated at 40°C using a rotary evaporator. The residue was then dissolved in methanol to 2 mg extract/ml and stored in a freezer at −20°C.

Firstly, the free radical-scavenging activity of the lichen extract was measured by DPPH (1,1-diphenyl-2-picrylhydrazyl), using the method of Blois (1958). Free radical-scavenging activity was described as IC_{50} (50% inhibition concentration) and calculated (regression analyses) with SPSS 15.0 software (SPSS Inc., Chicago, USA). The inhibitory effect of the lichen extract on linoleic acid peroxidation was determined according to the thiocyanate method (Mitsuda et al. 1996) with a few modifications (Luo et al. 2009). Solution without extract served as a blank (negative control) and a solution with ascorbic acid (same concentration as the lichen extract) was the positive control. Activity was described as the inhibition percentage ($I\%$) calculated by the following equation:

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

where A_{blank} is the absorbance of the negative control, and A_{sample} is the absorbance of the tested extracts. The reducing power of the lichen extract was determined according to the method of Oyaizu (1986). Absorbance at 700 nm indicated reducing power and BHA served as the positive control. The amount of total soluble phenolic contents in the *R. conduplicans* extract was determined with Folin–Ciocalteu reagent according to the method of Slinkard and Singleton (1977), using pyrocatechol as a standard. The concentration of total phenolic compounds was expressed as micrograms of pyrocatechol equivalents per milligram of lichen extract.

In order to identify the compounds responsible for the strong free radical-scavenging activity of this lichen extract, bioautographic TLC assays were performed. The bioautographic TLC assay was based on Chaaib et al.'s (2003) method. Lichen extract (40 µl, 2 mg/ml) was spotted on a TLC plate (silica gel 60; Merck KGaA, Darmstadt, Germany) and developed in a solvent system C (toluene:acetic acid = 85:15, v/v). After drying, the plates were sprayed with DPPH solution (0.4 mg/ml in methanol) and examined 30 min later. Antioxidant samples appeared as yellow-white spots against a purple background. To identify the active compounds responsible for the antioxidant properties, another TLC plate was developed in the same solvent system and visualized by spraying with 10% sulfuric acid. Bioautographic TLC plates were used as a reference to locate antioxidant compounds on preparative TLC plates developed in the same solvent. Silica gel at the active spot area was collected, dissolved in acetone, and filtered. The solution was then analyzed by HPLC (LC-10AT; Shimadzu, Kyoto, Japan) under the following conditions: YMC-Pack ODS-A S-5 µm 150 × 4.6 mm internal diameter [ID] column; solvent, methanol:H_2O:H_3PO_4 (80:20:1, v/v); 1 ml/min flow rate; photodiode array detector (range 180–700 nm); detecting wavelength, 254 nm for HPLC and 180–400 nm for UV-spectrum analysis; temperature, 40°C. Lichen substances were identified by comparing their retention times and UV spectra with the database of the authentic substances in the Laboratory of Advanced Bio-Production Science at Akita Prefecture University. The free radical-scavenging activities of the active compounds isolated by preparative TLC were also determined by the same method as that mentioned above; the activities were described as IC_{50} values.

In the present study, methanol extract of *R. conduplicans* showed strong DPPH free radical-scavenging activity (Fig. 1). The inhibition percentage reached 55.8% at the concentration of 330 µg/ml. The scavenging activity was found to be concentration-dependent for both BHA and

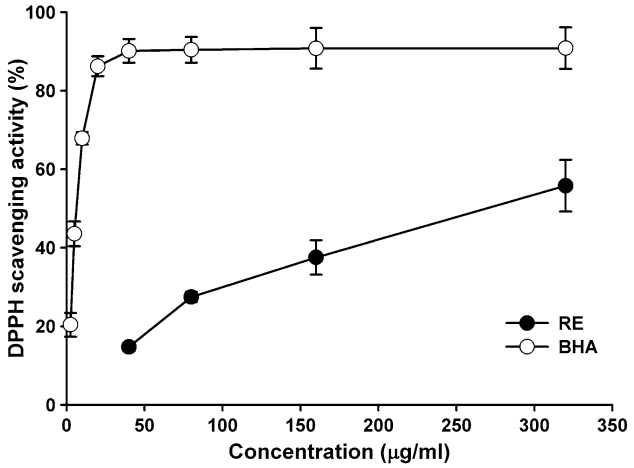

Fig. 1 *DPPH* (1,1-diphenyl-2-picrylhydrazyl) free radical-scavenging activity of different concentrations of methanol extract of *Ramalina conduplicans* (*filled circles*) and butylated hydroxyanisole (*BHA*; *open circles*). *RE*, *R. conduplicans* extract. Results are means ± standard deviation of three different experiments

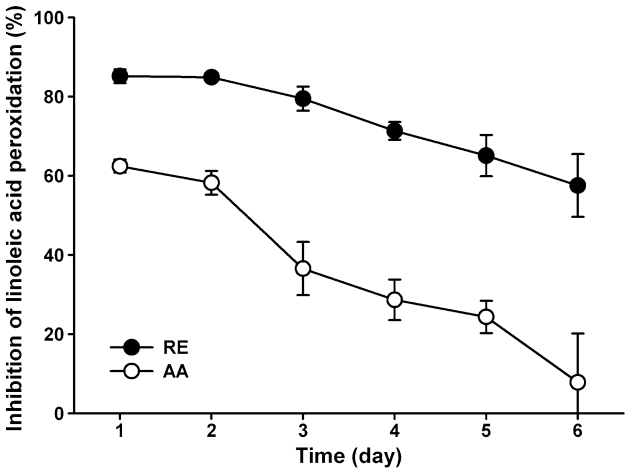

Fig. 2 Anti-linoleic acid peroxidation activity of methanol extract of *R. conduplicans*. *AA*, ascorbic acid; *RE*, *R. conduplicans* extract. *Lines* indicate time-dependent inhibition percentage (%) of AA (*open circles*) and the lichen extract (*filled circles*). Results are means ± standard deviation of three different experiments

R. conduplicans extract. The DPPH scavenging activity is also described as the IC_{50} value. When comparing the IC_{50} value with that in a previous study, we found that *R. conduplicans* showed DPPH scavenging activity at a relatively low concentration ($IC_{50} = 0.232$ mg/ml) compared with those of many other lichen species (Behera et al. 2006; Gulluce et al. 2006) and also many species of edible mushrooms (Lo and Cheung 2005; Tsai et al. 2009). The result indicated that *R. conduplicans* contains strong free radical-scavenging constituents or a large amount of these constituents and would be a very good health-improving food resource for human beings against oxidative damage.

The products of lipid peroxidation are reactive aldehydes, such as 4-hydroxyl nonenal and malondialdehyde, many of which can cause damage to proteins and DNA, and they are highly toxic to cells (Yu and Yang 1996). Therefore, the inhibition of lipid peroxidation is important for the survival of organisms in oxidative stress. Inhibition of lipid peroxidation by an external agent is often used to evaluate its antioxidant capacity. In the present study, *R. conduplicans* extracts exhibited very significant antioxidant activity and inhibited 85.2% of linoleic acid peroxidation at the concentration of 2 mg/ml, which was much stronger than the inhibition of ascorbic acid (positive control) at the same concentration. Peroxidation inhibition by *R. conduplicans* extract was also measured over a period of 6 days (Fig. 2). After 6 days' incubation, the inhibition percentage of ascorbic acid decreased to only 7.8%, while the *R. conduplicans* extract still exhibited 52.7% inhibition, which indicates that the *R. conduplicans* extract showed stronger and more stable inhibition of linoleic acid peroxidation than ascorbic acid. The result suggested that this lichen extract has strong antioxidant activity and has great potential as a food additive to prevent food deterioration caused by lipid peroxidation.

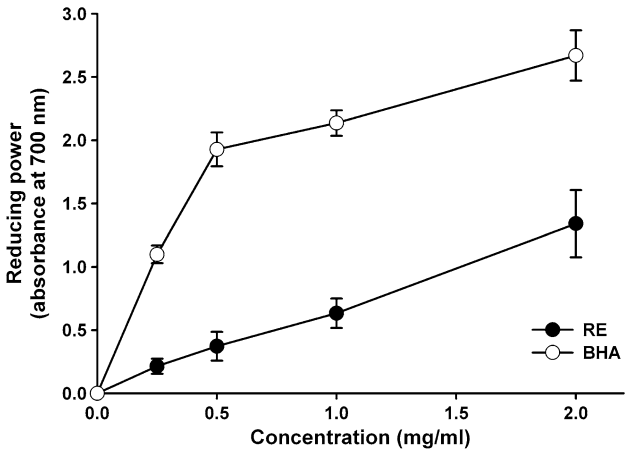

Fig. 3 Reducing power of methanol extract of *R. conduplicans*. *RE*, *R. conduplicans* extract. *Lines* indicate concentration-dependent reducing power of BHA (*open circles*) and the lichen extract (*filled circles*). Results are means ± standard deviation of three different experiments

Figure 3 illustrates a significant increase of Fe^{2+} concentration due to the reducing power of *R. conduplicans* with increases in the extract concentrations. This result indicates that methanol extract of *R. conduplicans* possesses strong electron donors which could react with free radicals to convert them into more stable products and terminate radical chain reactions.

Phenolic compounds are high-level antioxidants because they act as hydrogen donors and single oxygen quenchers, and therefore have high redox capacity (Rice-Evans et al. 1995). Lichens produce numerous phenolic compounds such as depsides, depsidones, and dibenzofurans. It has

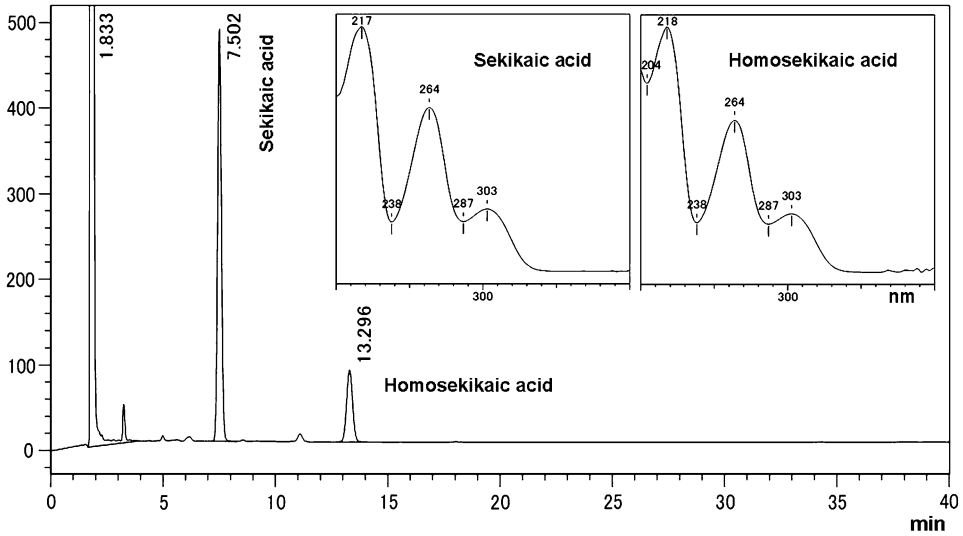

Fig. 4 HPLC chromatograms and UV spectra of antioxidant compounds isolated from methanol extract of *R. conduplicans*. The third (retention time, RT = 7.502 min) and the fourth peaks (RT = 13.296 min) are the main components (the peak with RT = 1.833 min is acetone). *Insets* UV spectra of sekikaic acid and homosekikaic acid

been demonstrated previously that the antioxidant capacities of lichen extracts are dependent on their phenolic constituents (Gulluce et al. 2006). Therefore, we measured the total phenolic contents of the methanol extract of *R. conduplicans*. In our investigation, 136.5 μg pyrocatechol equivalent phenolic contents were detected in 1 mg of *R. conduplicans* methanol extract. This value was significantly higher than that of *Usnea ghattensis* (13 μg pyrocatechol equivalent/mg extract) and *Cetraria islandica* (0.0387 μg pyrocatechol equivalent/mg extract), both of which have been proven to be potent antioxidant lichen species in previous studies (Gülçin et al. 2002; Behera et al. 2006). The result suggested that *R. conduplicans* contains a relatively large amount of phenolic contents, which might play an important role in the strong antioxidant activities of *R. conduplicans*.

Bioautographic TLC showed that *R. conduplicans* contains two major antioxidant compounds (Rf value: 0.68 and 0.64). HPLC and UV spectrometry identified these compounds as the depside compounds sekikaic acid (peak area percentage = 46.9%) and homosekikaic acid (peak area percentage = 18.3%) (Fig. 4). The IC_{50} values of the free radical-scavenging activity of sekikaic acid and homosekikaic acid were then measured to be 0.082 and 0.276 mg/ml, respectively. The results demonstrated that these two compounds are promising antioxidant agents and their contents in *R. conduplicans* methanol extract are abundant. The potent antioxidant activity of sekikaic acid had been reported in previous studies. Choudhary et al. (2009) reported strong superoxide anion-scavenging activity of this compound isolated from the lichen *Heterodermia obscurata*. Sekikaic acid and homosekikaic acid have also been isolated from other species of *Ramalina*, such as *R. intermedia* (Bowler and Rundel 1974). Dias and Urban (2009) reported the antitumor and the antibacterial properties of the mixture of sekikaic acid and 5-chlorosekikaic acid isolated from the Australian lichen *R. glaucescens.* In the present study, the potent free radical activity of homosekikaic acid was reported for the first time.

The results obtained in the present study are noteworthy, because this is the first study that aimed to evaluate the bioactivity and therapeutic utilization of the edible lichen *R. conduplicans*, which is commonly used in central and southeastern Asian countries. On the basis of the results it is suggested that the methanol extract of *R. conduplicans* has potent antioxidant activities. Furthermore, we identified sekikaic acid and homosekikaic acid as the compounds responsible for the potent free radical-scavenging activity of *R. conduplicans* methanol extract. Therefore, these lichen substances have great potential to be used as bioresources for novel natural antioxidants.

However, because lichens grow slowly, they are not suitable for industrial-scale exploitation. Fortunately, a method for the culture of lichens has been established (Yamamoto et al. 1985); both lichen mycobionts and photobionts could be cultured under artificial conditions. Therefore, *R. conduplicans* could be sustainably produced by artificial culture. As for the free radical-scavenging constituents sekikaic acid and homosekikaic acid, they could be produced either by the fermentation of lichen mycobionts or by biosynthesis such as the heterologous expression of *PKS* genes in other filamentous fungi (Stocker-Wörgötter 2008). We are currently investigating the feasibility of these two means of mass production of lichen antioxidant substances.

Acknowledgments This work was supported by a grant from the Korea National Research Resource Center Program (Grant 20090062634) and also by a Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2007-313-C00669).

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