J Nat Med (2008) 62:481–484
DOI 10.1007/s11418-008-0257-9

NATURAL RESOURCE LETTER

Thin layer chromatography analysis of antioxidant constituents of lichens from Antarctica

Hari Datta Bhattarai · Babita Paudel · Soon Gyu Hong · Hong Kum Lee · Joung Han Yim

Received: 22 October 2007 / Accepted: 21 April 2008 / Published online: 17 June 2008

Abstract Antioxidant agents against reactive oxygen species can be used for several cosmetic and medicinal applications. Methanol–water (90:10 *v*/*v*) extracts of five polar lichen species—namely *Stereocaulon alpinum* Laurer (Stereocaulaceae); *Ramalina terebrata* Hook and Taylor (Ramalinaceae); *Caloplaca* sp. (Teloschistaceae); *Lecanora* sp. (Lecanoraceae); and *Caloplaca regalis* (Vain.)Zahlbr (Teloschistaceae) from King George Island (Antarctica)—were analyzed using thin layer chromatography (TLC) followed by a DPPH (2,2-diphenyl-1-picrylhydrazyl) spray technique. The experimental data showed that 33–50% of the major constituents of the test extracts were active antioxidants. *Stereocaulon alpinum* and *R. terebrata* showed a higher number (50%) of antioxidant constituents, although their activities were comparatively weak. The strength of antioxidant activity in terms of discoloration of DPPH was shown to be stronger by the constituents of *S. alpinum*, *C. regalis* and *C.* sp. In addition, phenolic content in these Antarctic lichen extracts was in the range of 17–47 mg/g, supporting the antioxidant data of TLC analysis. Thus, these results suggest that Antarctic lichen contains a variety of strong antioxidant constituents. Therefore, further study of the laboratory culture of lichen is warranted to investigate possible commercial production, followed by isolation and characterization of the active antioxidant agents, which can be used against various oxidative stress-related diseases.

Keywords Antioxidant activity · 2,2-Diphenyl-1-picrylhydrazyl (DPPH) · Polar lichens · Reactive oxygen species (ROS)

H. D. Bhattarai · B. Paudel · S. G. Hong · H. K. Lee · J. H. Yim (✉)
Polar BioCenter, Korea Polar Research Institute, KORDI Songdo Technopark, Songdo-dong 7-50, Yeonsu-gu, Incheon 406-840, South Korea
e-mail: jhyim@kopri.re.kr

B. Paudel
Department of Marine Biotechnology, Soonchunhyang University, Shinchang myun, Eupnae-ri 646, Asan City, Chunchannam-do 336-745, South Korea

Introduction

Oxidation reactions transfer electrons from a substance to an oxidizing agent producing free radicals. Free radicals start chain reactions and damage different cellular components, including nucleic acids and several extracellular components, and finally enhance a number of degenerative diseases such as premature aging, deoxygenation of ischemic tissues, atherosclerosis and cancer [1]. Free radicals have been reported to attack the unsaturated fatty acids of the cell membrane, resulting in lipid peroxidation, decrease in membrane fluidity, loss of enzyme and receptor activities and damage to membrane proteins [2]. These phenomena commonly occur when the human body comes in contact with negative environmental factors or ages. Such oxidative pathologies can be treated by the application of antioxidant agents [3]. Such agents terminate these chain reactions by removing free radical intermediates and inhibiting other oxidation reactions by being oxidized themselves. Several reports of the synthesis of compounds with strong antioxidant properties have been published recently [4, 5]. Because of the high carcinogenicity of synthetic antioxidants [6], there is preference to develop effective antioxidants of natural origin [7].

Lichens are non-flowering plants, which consist of two components, an alga (phycobiont) and a fungus (mycobiont),

living symbiotically. Because no easy method of lichen culture exists at an industrial level and it is extremely difficult to collect enough wild samples to study, extensive research of lichen metabolites had been overlooked for a long time. Since the last decade of the 20th century, a number of studies related to lichen culture, production, biochemical analysis and application of metabolites have been started [8]. Several bioactive secondary metabolites especially to antimicrobial, antioxidant have been continuously reported from lichens of both wild and culture specimens [9, 10] of tropical and temperate origins. On the basis of current information available, no report has been published describing the antioxidant constituents from the lichen of Antarctic origin. In this paper, for the first time, we report the various strong and weak DPPH free-radical-scavenging constituents present in five Antarctic lichen species.

Results and discussion

Lichens have been used as a biological source of several metabolites with various bioactivities [11]. For example, compounds with antimycobacterial activities such as usnic acid from *Cladonia arbuscula*, atranorin and lobaric acid from *Sterocaulon alpinum*, salazinic acid from *Parmelia saxatilis* and protolichesterinic acid from *Certaria islandica* [12] have already been isolated. Similarly, antibacterial compounds—methyl β-orsellinate and mixtures of methyl and ethyl orsellinates—have been isolated from *S. alpinum* and *Peltigera aphthosa*, respectively [13]. Longissiminone A and longissimione B were isolated from *Usnea longissima* [14] as anti-inflammatory agents. Similarly, mycosporine was isolated from *Collema cristatum* as a UV-B absorbing agent [15], and two naphthopyrones namely euplectin and coneuplectin were isolated from *Flavoparmelia euplacta* as cytotoxic agents [16].

Lichen extracts of temperate and tropical origins have been shown to have various antioxidant activities, such as DPPH-, superoxide- and hydroxyl-radical-scavenging capacity at a higher dose level than these Antarctic lichen species [17]. Thus, the experimental data of these previous reports showed that Antarctic lichen species must have contained strong active antioxidant constituents when compared with tropical and temperate lichen species. Therefore, we studied these five Antarctic lichen species. The data from our experiment showed that 33–50% of major Antarctic lichens resolved by means of TLC were active antioxidants (Fig. 1; Table 1) in terms of DPPH free-radical-scavenging capacity, supporting the previous report of strong antioxidant capacities of Antarctic lichen metabolites. The extracts of *Caloplaca regalis*, *Caloplaca* sp. and *S. alpinum* showed several resolved TLC bands with strong and few spots with weak anti-DPPH free-radical activities. However, *Ramalina terebrata* and *Lecanora* sp. showed faint spots, indicating relatively weak antioxidant activity of the resolved bands. The natures of the active antioxidant TLC bands of the crude extracts of all five lichens at two different solvent systems are presented in Fig. 1 and Table 1.

In general, phenolic compounds are well-known, high-level antioxidant constituents because of their high ability to scavenge toxic free radicals and reactive oxygen species such as singlet oxygen, superoxide free radicals and hydroxyl radicals [18]. In the present experiment, the phenolic content in the test lichen extracts was found to be higher (Fig. 2) than in other reports of tropical and temperate lichens species [8]. Interestingly, the phenolic contents in these five Antarctic lichens were found to be much higher than in black and white pepper [19]. Such experimental data after comparison with previously published reports clearly indicated that lichens from extreme environments, such as Antarctica, must contain various types of phenolic compounds with higher antioxidant activities. Therefore, further studies such as laboratory culture for mass production are warranted, as are purification and characterization of responsible antioxidant

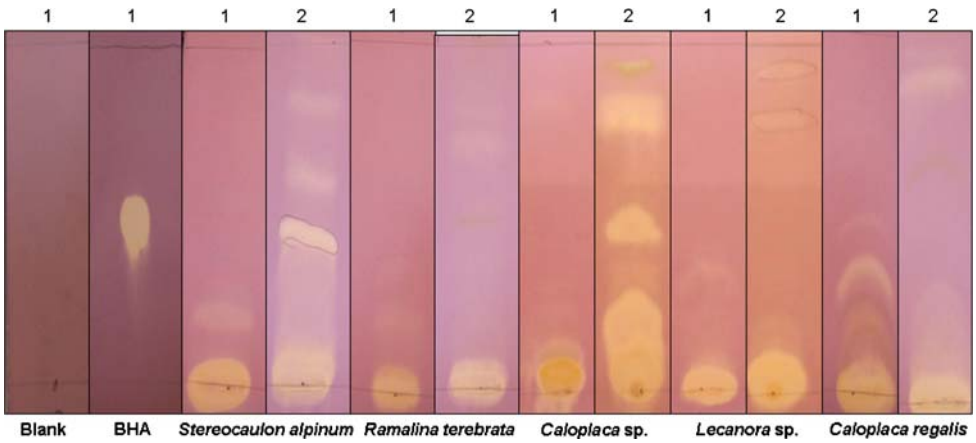

Fig. 1 Thin layer chromatography analysis of DPPH free-radical active agents from polar lichen species. *BHA* butylated hydroxyanisole, *1* chloroform, *2* 10% methanol in chloroform

Table 1 Thin layer chromatography (TLC) profile of DPPH free-radical active constituents of lichen crude extracts in two different solvent systems

Extract	Total number of TLC spots[a]	Antioxidant active TLC spots	Characteristics of antioxidant active spots
Mobile-phase chloroform			
Stereocaulon alpinum	6	0.28	*
		0.22	**
Ramalina terebrata	5	0.35	*
		0.22	**
Caloplaca sp.	4	0.87	***
		0.17	****
Lecanora sp.	5	0.42	***
Caloplaca regalis	7	0.45	***
		0.37	**
Mobile-phase 10% methanol in chloroform			
Stereocaulon alpinum	8	0.98	*
		0.85	**
		0.65	***
		0.47	**
Ramalina terebrata	6	0.98	*
		0.80	*
		0.72	***
Caloplaca sp.	9	0.99	***
		0.93	****
		0.53	**
Lecanora sp.	6	0.97	***
		0.83	***
Caloplaca regalis	8	0.95	***
		0.53	**
		0.30	***

*Comparatively weak in activity, observed as black band in UV at 254 nm; **Comparatively strong in activity, the band was not observed under UV light; ***Comparatively weak in activity and the band was not observed under UV light; ****Comparatively strong in activity, the band was observed as black at 254 nm

[a] Indicates a sum of UV detected and no detected TLC bands before DPPH spray

compounds and the evaluation of their pharmaceutical activities and mode of actions.

Materials and methods

Lichen specimens and collection site

Five lichen species—namely *S. alpinum* Laurer (Stereocaulaceae), *R. terebrata* Hook and Taylor (Ramalinaceae), *Caloplaca* sp. (Teloschistaceae), *Lecanora* sp. (Lecanoraceae) and *C. regalis* (Vain.) Zahlbr (Teloschistaceae)—were collected from the Korean Antarctic Research Station site on King George Island (60°13′S, 58°47′W), Antarctica. All species were identified with the help of previous descriptions [17].

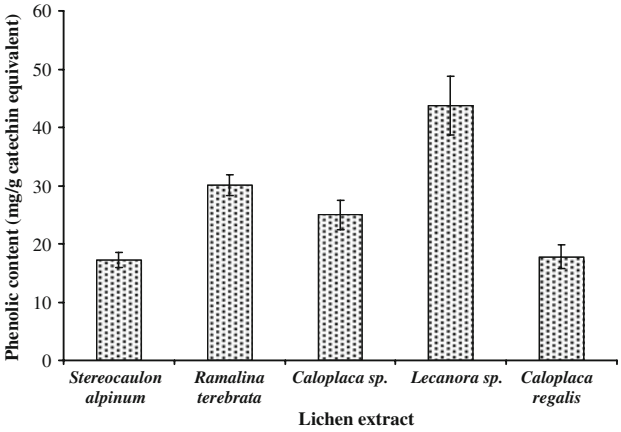

Fig. 2 Phenolic contents in the lichen extracts

Lichen extraction

About 10–130 g (dry weight) of each specimen was extracted separately in methanol–water (90:10 *v*/*v*), as described previously [17]. The solvent was evaporated in vacuum; samples were lyophilized and stored at −20°C until further use.

Thin layer chromatography analysis of antioxidant constituents

About 100 μg of extract of each lichen species was loaded on TLC plates (Merck, 10 × 10 cm^2). The plates were developed in four different solvent systems—chloroform, 5% methanol in chloroform, 10% methanol in chloroform and 20% methanol in chloroform to separate the various constitutes of the extracts. The developed plates were air dried and observed under visible and UV light. Various separated spots were noted as their Rf values. After this examination, 0.05% of DPPH solution in methanol was sprayed on the surface of developed TLC plates and incubated for 10 min at room temperature. The active antioxidant lichen constituents were detected as yellowish white spots produced by bleaching of DPPH by resolved bands on the TLC plates. After visual comparison with the intensity of bleached color of the TLC band of positive standard, the antioxidant strengths of lichen constituents were tentatively categorized as strong and weak activities. All detected active antioxidant constituents were noted according to their Rf values. Butylated hydroxyanisole (BHA) was used as positive control, and blank TLC plate was taken as negative control.

Estimation of phenolic compounds

Total soluble phenolic compounds in test extract were determined using pyrocatechol as a standard, according to the method of Slinkard and Singleton (1997) [20]. In short, 1 ml of test extract at various concentrations (0–1,000 μg/ml) was mixed with 1 ml of Folin–Ciocalteu reagent and mixed thoroughly. The reaction mixture was incubated for 5 min at room temperature, and 1 ml of Na_2CO_3 (20 g/l) was added and allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The pyrocatechol equivalent phenolics content was calculated using an obtained standard curve of commercially available pyrocatechol: $y = 0.03x - 0.04$, where y is the absorbance at 760 nm and x the concentration of the test sample.

Acknowledgments This work was supported by a grant to the Korea Polar Research Institute, KOPRI, under a project PE08050.

References

1. Halliwell B, Gutteridge JM (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 186:1–88
2. Dean RT, Davies MJ (1993) Reactive species and their accumulation on radical damaged proteins. Trends Biochem Sci 18:437–441
3. Tutour BL (1990) Antioxidative activities of algal extracts. Synergistic effect with vitamin E. Phytochemistry 29:3759–3765
4. Funasaka Y, Chakraborty AK, Komoto M, Ohashi A, Ichihashi M (1999) The depigmenting effect of α-tocopheryl ferulate on human melanoma cells. Br J Dermatol 141:20–29
5. Shimizu K, Kondo R, Sakai K, Takeda N, Nagahata T, Oniki T (2001) Novel vitamin E derivative with 4-substituted resorcinol moiety has both antioxidant and tyrosinase inhibitory properties. Lipids 36:1321–1326
6. Wichi HP (1988) Enhanced tumor development of butylated hydroxyanisol (BHA) from the prospective of effect on fore stomach and oesophageal squamous epithelium. Food Chem Toxicol 26:717–723
7. Anandjiwala S, Srinavasa H, Kalola J, Rajani M (2007) Free-radical scavenging activity of Bergia suffruticosa (Delile) Fenzl. J Natl Med 61:59–62
8. Behera BC, Verma N, Sonone A, Makhija U (2006) Determination of antioxidative potential of lichen *Usnea ghattensis* in vitro. Lebenson Wiss Technol 39:80–85
9. Boustie J, Grube M (2005) Lichens- a promising source of bioactive secondary metabolites. Plant Genet Resour 3:273–287
10. Behera BC, Verma N, Sonone A, Hakhija U (2007) Tissue culture of some lichens and screening of their antioxidant, antityrosinase and antibacterial properties. Phytother Res 21:1159–1170
11. Ingolfsdottir K (2002) Molecules of interest usnic acid. Phytochemistry 61:729–736
12. Ingolfsdottir K, Hjalmarsdottir MA, Sigurdsson A, Gudjonsdottir GA, Brynjolfsdottir A, Steingrimsson O (1997) In vitro susceptibility of *Helicobacter pylori* to protolichesterinic acid form the lichen *Cetraria islandica*. Antimicrob Agents Chemother 41:215–217
13. Ingolfsdottir K, Bloomfield SF, Hylands PJ (1985) In vitro evaluation of the antimicrobial activity of lichen metabolites as potential preservatives. Antimicrob Agents Chemother 28:289–292
14. Choudhary MI, Azizuddin SJ, ur-Rahman A (2005) Bioactive phenolic compounds from a medicinal lichen *Usnea longissima*. Phytochemistry 66:2346–2350
15. Torres A, Hochberg M, Pergament I, Smoum R, Niddam V, Dembitsky VM, Temina M, Dor I, Lev O, Srebnik M, Enk CD (2004) A new UV-B absorbing mycosporine with photo protective activity from the lichenized ascomycete *Collema cristatum*. Eur J Biochem 271:780–784
16. Ernst-Russell M, Chai CLL, Wardlaw JH, Elix JA (2000) Euplectin and coneuplectin, new naphthopyrones from the lichen *Flavoparmelia euplecta*. J Nat Prod 63:129–131
17. Paudel B, Bhattarai HD, Lee JS, Hong SG, Shin HW, Yim JH (2008) Antioxidant activity of polar lichens from King Geroge Island (Antarctica). Polar Biol 31:605–608. doi:10.1007/s00300-007-0394-8
18. Hall CA, Cuppett SL (1997) Structure-active of natural antioxidant. In: Auroma OI, Cuppett SL (eds) Antioxidant methodology in vivo and in vitro concepts. AOCS Press, Champaign, pp 141–170
19. Abbor GA, Vinson JA, Oben JE, Ngogang JY (2006) Comparative analysis of the in vitro antioxidant activity of white and black pepper. Nutr Res 26:659–663
20. Slinkard K, Singletion VL (1997) Total phenol analyses: automation and comparison with manual methods. Am J Enol Vitic 28:49–55