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In vitro Antioxidant Capacities of Two Benzonaphthoxanthenones: Ohioensins F and G, Isolated from the Antarctic Moss *Polytrichastrum alpinum*

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Antioxidant agents against reactive oxygen species can be used for several cosmetic and medicinal applications. This study's objective was to evaluate the antioxidant activities of *Polytrichastrum alpinum* (Hedw.) G. L. Sm. (Polytrichaceae), an Antarctic moss species collected from King George Island (Antarctica). The identification of the moss species was performed on the basis of morphological characteristics and molecular sequencing of the 18S rRNA gene. Two benzonaphthoxanthenones: ohioensins F and G, were isolated from the extract after several chromatographic procedures. The various *in vitro* antioxidant capacities of a methanolic extract of *P. alpinum* and the isolated compounds were evaluated by analyzing the scavenging capacities of free radicals of 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), the total phenol assay with Folin-Ciocalteu reagent, the ferric ion (Fe^{3+}) reducing power and the nitric oxide (NO) scavenging activity and compared to those of commercial standards for each assay. The experimental data showed that even the crude extract of *P. alpinum* exhibited potent antioxidant activity. The antioxidant activity was increased two- to seven-fold for the purified compounds. The antioxidant activities of both purified compounds were found to be more or less the same in all experiments. However, the obtained data showed that the Fe^{3+} reducing power of the purified compounds and crude methanolic extract was almost the same suggesting the presence of other stronger reducing agents in the methanolic extract which could not be isolated in the present experiment. Therefore, further work on the isolation of these stronger antioxidant agents from this moss specimen of the extreme environment is warranted. Developments of laboratory mass culture techniques are anticipated to achieve bulk production of the active constituents for commercial application.

Key words: ABTS, DPPH, *Polytrichastrum alpinum*, Nitric Oxide

Introduction

Oxidation reactions transfer electrons from a substance to an oxidizing agent, producing free radicals which start chain reactions, damage different cellular components, including nucleic acids, and enhance a number of degenerative diseases, such as premature aging, deoxygenating of ischemic tissues, atherosclerosis, and cancer (Halliwell and Gutteridge, 1990), cardiovascular diseases (Kris-Etherton *et al.*, 2002), neurodegenerative diseases including Parkinson's and Alzheimer's diseases (Di Matteo and Esposito, 2003), as well as inflammation caused by cells and cutaneous aging (Ames *et al.*, 1993). Free radicals have been reported to attack unsaturated fatty acids of cell membranes resulting in lipid peroxidation, a decrease in membrane fluidity, loss of enzyme and receptor activities, and damage to membrane proteins (Dean and Davies, 1993). 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 antioxidants (Totour, 1990), which terminate these chain reactions by removing free radical intermediates and inhibiting other oxidation reactions by being oxidized themselves. Several reports on the synthesis of compounds showing strong antioxidant properties have been published in the past years (Shimizu *et al.*, 2001). Because of the high carcinogenic activities of synthetic antioxidants (Grice, 1986), the develop-

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ment of effective antioxidants of natural origin is widely preferred (Bergman *et al.*, 2001; Li *et al.*, 2008).

Polytrichastrum alpinum (Hedw.) G. L. Sm., the mountain hair moss, is an alpine species which is distributed over a large area of Antarctica. *P. alpinum* responds to UV-B and enhanced temperatures by producing some specific secondary metabolites (Huttunen *et al.*, 2005). Several secondary metabolites that protect mosses against environmentalstresses such as UV light, drought, and high temperatures have been well described previously (Rozema *et al.*, 1997). For example, bryophyte flavonoids, which have shown an important protective function, contain flavone and flavonol glycones and glycosides, anthocyanins and their derivatives, aurones, biflavonoids, dihydroflavonoids, isoflavones, and triflavones (Markham, 1990). In the present paper we describe the various *in vitro* antioxidant capacities of the methanolic extract of Antarctic *P. alpinum* and two benzonaphthoxanthenones, ohioensin F and ohioensin G, isolated very recently from the species.

Material and Methods

Chemicals and reagents

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), curcumin, ferric chloride, trichloroacetic acid, potassium ferricyanide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), pyrocatechol and the antioxidant assay kit (product code CS0790) were purchased from Sigma-Aldrich (St. Louis, USA). All reagents and solvents used in the present study were of analytical grade.

Moss sampling and identification

A moss specimen designated as KOPRI-M1 was collected from the Korean Antarctic Research Station site on King George Island (60°13' S, 58°47' W) in January 2006. On the basis of morphological characteristics described previously (Ochyra, 1998), KOPRI-M1 was identified by Dr. Y. K. Lee, Korea Polar Research Institute, KOPRI, Incheon, South Korea as *Polytrichastrum alpinum* (Hedw.) G. L. Sm. The identification was further confirmed by comparing the sequence data of the 18S rRNA gene with those present in the gene bank. The gene bank accession number of the 18S rRNA gene of *P. alpinum* is EU272035.

Extraction and isolation of antioxidant compounds

A freeze-dried sample of *P. alpinum* (100 g) was extracted with methanol (1000 mL × 3) at room temperature for 24 h. A fraction (5 g) of the resulting crude methanolic extract (10.5 g) was fractionated by automated mild pressure liquid chromatography (MPLC) using a C_{18} functionalized silica gel column (3 × 15 cm). Two very recently known metabolites, ohioensin F (**1**) and ohioensin G (**2**), were isolated by various chromatographic techniques. The compounds were identified by comparing the HPLC (retention time), EI-MS and spectroscopic data with those described in our previous report (Seo *et al.*, 2008).

In vitro antioxidant assays

Various *in vitro* antioxidant activities such as DPPH and $ABTS^{\bullet+}$ radical scavenging capacity (Blois, 1958; Rice-Evans and Miller, 1994), Fe^{3+} reducing power (Oyaizu, 1986), and nitric oxide radical scavenging capacity (Sumanont *et al.*, 2004) of the *P. alpinum* extract and isolated compounds were determined by comparing to commercially available standard compounds (Table I). In addition, the total phenol assay (TFA) with Folin-Ciocalteu reagent was also performed (Slinkard and Singleton, 1997) to measure the reduction capacity of the test extract and isolated compounds. These experiments were modified at various degrees as described previously (Bhattarai *et al.*, 2008).

Results and Discussion

In order to identify a new potential source of natural antioxidants, four antioxidant assays based on the electron transfer (ET) system (DPPH free radical and $ABTS^{\bullet+}$ scavenging capacities, Fe^{3+} reducing power, total phenol assay with Folin-Ciocalteu reagent) and one more antioxidant assay against biologically relevant oxidants (nitric oxide) were used to investigate the antioxidant capacities of the methanolic extract of *Polytrichastrum alpinum* (Hedw.) G. L. Sm. (Polytrichaceae). Similar assays were also performed for the purified compounds. The obtained experimental data (Table I) showed that even the crude extract of

P. alpinum exhibited potential antiradical activities against the free radicals of ABTS and DPPH. Similarly, the extract also showed potential nitric oxide scavenging capacity.

Two benzonaphthoxanthenones: ohioensin F (**1**) and ohioensin G (**2**) (Fig. 1), were isolated from the methanolic extract of *P. alpinum* by several chromatographic procedures and identified using spectroscopic data as described previously (Seo *et al.*, 2008). Both compounds showed potent antiradical activities against $ABTS^{\bullet+}$ and DPPH free radicals. The test compounds and the crude extract converted DPPH into DPPH-H by donating a hydrogen atom. This conversion could easily be noticed by a spectrophotometer with which a decreased absorbance at 517 nm in a dose-dependent manner could be observed. Similarly, the extract and the isolated compounds inhibited the production of the chromogen cation of ABTS in a specially designed cation generation system (Rice-Evans and Miller, 1994) in a dose-dependent manner which could be measured at 405 nm by a spectrophotometer. The ferric ion reducing antioxidant (or reducing power) assay measures the electron transfer capacity of the test samples convertinf Fe^{3+} to Fe^{2+} inside a complex molecule. In the present experiment, the crude extract and the isolated compounds showed almost equal Fe^{3+} reducing capacity. Similarly, the total phenol assay with Folin-Ciocalteu reagent where the reducing capacity of the test sample is measured showed only a two-fold increment in the reducing power of pure compounds compared to the crude extract. Such data suggested that the crude extract must have contained some other stronger reducing agents than the isolated compounds. In addition, the crude extract and purified compounds were moderately active against nitric oxide (NO) in a dose-dependent manner. NO is a well known free radical causing oxidative damage such as inflammation and cancer in the human body (Halliwell and Gutteridge, 1990). The overall experimental data showed that both purified compounds have almost equal activities in each antioxidant assay conducted here.

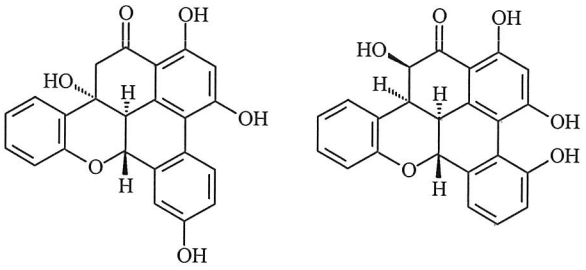

Fig. 1. Chemical structure of the isolated compounds ohioensin F (**1**) and ohioensin G (**2**).

Ohioensins A, B, C, D and E containing a polycyclic skeleton were isolated for the first time from the moss *Polytrichum ohioense* (Polytrichaceae) and showed potent cytotoxic activities against 9PS murine leukemia and the human tumour cell lines A-549 lung carcinoma, MFC-7 breast adenocarcinoma and HT-29 colon adenocarcinoma (Zheng and Chang, 1993). Similarly, ohioensin F and ohioensin G isolated from *P. alpinum* in our previous study (Seo *et al.*, 2008) showed tyrosine phosphatase 1B inhibitory activity. In this report

Table I. *In vitro* antioxidant capacities of the methanolic extract of *P. alpinum* and the isolated compounds.

Sample	Test assays				
	50% inhibition concentration (IC_{50})				
	DPPH [μg/mL]	ABTS [μg/mL]	Nitric oxide [μg/mL]	Fe^{3+} reducing power[a] [μg]	Total phenol[b] [μg]
P. alpinum extract	56.8 ± 0.8	103.98 ± 9.8	145.6 ± 8.2	10 ± 1.2	12.5 ± 1.2
Ohioensin F (**1**)	10 ± 0.16	14.3 ± 1.2	63 ± 5.1	9.8 ± 0.07	6.76 ± 0.5
Ohioensin G (**2**)	10.1 ± 1.5	14.8 ± 1.5	62.1 ± 5.0	9.6 ± 1.2	7.4 ± 0.8
Trolox	–	46.35 ± 5.1	–	–	–
BHA	4.97 ± 0.9	–	–	–	–
Ascorbic acid	–	–	–	–	–
Curcumin	–	–	8.4 ± 0.3	–	–

[a] Reducing power is expressed in terms of equivalents to 1 μg of BHT.
[b] Total phenol is expressed in terms of equivalents to 1 μg of pyrocatechol.

we presented *in vitro* antiradical and antioxidant activities of ohioensin F and G and the crude extract of *P. alpinum*.

In conclusion, the methanolic extract of *P. alpinum* and the isolated compounds did show potent antiradical and antioxidant capacities *in vitro*. The quantitative LC/MS analysis of the crude methanol-soluble extract after removing hexane-soluble pigments showed the presence of 1.1% of ohioensin G and 3.3% of ohioensin F (data not shown). Based on the obtained data on antioxidant activities of the crude extract and the purified compounds as well as on the content of the purified compounds in the crude extract, it is obvious that there must be more stronger antioxidant constituents which could not be obtained in this purification system. Therefore, further work is necessary to obtain the various active antioxidant constituents for diverse therapeutic applications.

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