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In vitro antioxidant activity of the Antarctic lichen *Caloplaca regalis* and its GC/MS based phytochemical analysis

Babita Paudel, Hari Datta Bhattarai, and Joung Han Yim*
Division of Life Sciences, Korea Polar Research Institute, KOPRI, Songdo Technopark, Songdo-dong 7-50, Yeonsu-gu, Incheon 406-840, South Korea

Abstract Antioxidant agents, which inhibit the destructive actions of free radicals or reactive oxygen species (ROS), are widely used as food additives as well as in medicines and cosmetics. Antioxidant agents derived from natural sources are believed to be safer than synthetic compounds. In order to identify a new potential source of natural antioxidant compounds, we evaluated various antiradical and antioxidant activities of an ethanol-water extract of *Caloplaca regalis* (Vain.) Zahlbr. (Teloschistaceae), a lichen collected from the Antarctica. The experimental data indicated that even the crude extract of *C. regalis* exhibited a significant potential to inhibit free radical production. The GC/MS analysis showed that *C. regalis* extract contained various derivatives of benzoic acids, furanone, anthraquinone and several fatty acids. In addition, the total phenolic content in the lichen extract was found to be more than 8% by weight. We conclude that *C. regalis* does indeed produce antioxidant active compounds, which would have much strong activities if tested in a purified form. Thus, further research should be conducted to purify and characterize the compounds responsible for the antioxidant activity of *C. regalis* extract, as these compounds may be an alternative source of medically relevant antioxidant.

Keywords: Antioxidant, *Caloplaca regalis*, free radicals, GC/MS, nitric oxide.

INTRODUCTION

Living organisms accumulate reactive oxygen species (ROS) and reactive nitrogen species through both normal metabolic processes and exogenous sources. ROS, such as superoxide anions (O2), hydroxyl radicals (OH), hydrogen peroxide (H2O2), and hypocholorous acid (HOCl), have been asscociated with inflammation, cardio vascular diseases, cancer, aging-related disorders, metabolic disorders, and atherosclerosis (Ames et al,. 1993). ROS are dangerous because they can attack unsaturated fatty acids and cause membrane lipid peroxidation, decreases in membrane fluidity, loss of enzyme receptor activities, and damage to membrane proteins, ultimately leading to cell inactivation (Dean and Davies, 1993).

Living organisms possess a natural defense mechanism that counters the deleterious effects of ROS. Despite the existence of such a mechanism, increasing ROS accumulation over the lifetime of a cell can cause irreversible oxidative damage (Tseng et al., 1997). Thus, antioxidant agents that can slow or prevent the oxidation process by removing free radical intermediates are desired. Several strong synthetic antioxidants have already been reported (Shimizu et al., 2001); however they have proven to be highly carcinogenic (Wichi, 1988). For this reason it has become necessary

to derive antioxidants from natural sources for use as supplements to human health. A wide range of natural compounds, including phenolic compounds, nitrogen compounds, and caretenoids (Velioglu et al., 1998), have antioxidant properties.

Lichens are the symbiotic association of a fungus (mycobiant) and an alga (photobiant)/and or cynobacteria, which resemble non-flowering plants. In lichens, the fungus forms a thallus or lichenized stroma that may contain characteristic secondary metabolites (Ahmadjin, 1993). Due to the difficulties in collecting enough amount of natural samples and little knowledge for the mass production, less research had been conducted than on higher plants. Advance methods designed for tissue culture, mass production and biochemical analysis of lichens have brought momentum in lichen research (Behera et al., 2006). Some of the lichen metabolites from several chemical classes, including the aliphatic acids, depsides and depsidones, dibenzofurans, diterpenes, anthraquinones, naphoquinones, usnic acids, pulvinic acids, xanthones, and epidithiopiperazinediones with various biological activities such as cytotoxic, fungicidal, antimicrobial, antioxidant and anti-inflammatory have already been reported (Müller, 2001). Most of the known antioxidant activities of lichens are derived from species of tropical and sub-tropical origin. Fewer studies have been conducted on the antioxidant activities of lichens from Antarctic regions (Paudel et al., 2008). In this paper, we describe various in vitro antioxidant activities and phytochemical analysis of Caloplaca regalis, from Antarctica.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and reagents used for *in vitro* antioxidant assays were of analytical grade and were purchased from Sigma-Aldrich, USA. The solvents used during extraction were HPLC grade and were purchased from DUKSAN pure chemicals Co. Limited, Korea.

Collection and identification of lichen species

The lichen species, *Caloplaca regalis* (Vain.) Zahlbr. (Teloschistaceae), was collected from the Korean Antarctic Research Station site on Barton peninsula, in King George Island (61°50–62°15S and 57°30–59°01W), Antarctica in January, 2010. The specimen was identified, as described previously (Paudel *et al.*, 2008).

Extraction

A completely freeze-dried grinded lichen sample (127.4 g) was extracted in a mixture of ethanol and water (70:30 v/v) at room temperature for 24 h. The extract was filtered and solvent was evaporated at 45 °C in vacuum. The extraction procedure was repeated three times to ensure the complete extraction of extractable compounds. The extract (20.65 g), after freeze-drying, was stored at -20 °C until further use.

Antioxidant assay

Various in vitro antioxidant activities such as DPPH and $ABTS^{\bullet+}$ radical scavenging capacity (Blois, 1958; Rice-Evans and Miller, 1994), Fe^{+++} reducing power (Oyaizu, 1986), superoxide anion

scavenging capacity in riboflavin-nitroblue tetrazolium (NBT)-light system (Beauchamp and Fridovich, 1971), ferrous ion chelating capacity (Dinis *et al.*, 1994), nitric oxide radical scavenging capacity (Sumanont *et al.*, 2004) of the *C. regalis* extract were determined by comparing to commercially available standard compounds (Table 1). In addition, the total phenol assay (TFA) by Fojin-Ciocalteu reagent (FCR) was also performed (Slinkard and Singleton, 1997) to measure the reduction capacity of the test extract. The TFA data are always in excellent linear correlations with the total phenolic profiles of the test samples (Huang et al. 2005). These experiments were modified at various degrees as described previously (Bhattarai *et al.*, 2008).

Gas chromatography mass spectrometry (GC/MS) analysis

Gas chromatography mass spectrometry (GC-MS) analysis of crude extract of *C. regalis* was performed in Clarus 500 gas chromatograph (Perkin Elemer) equipped with a 30 m x 0.32 mm PerkinElmer Elite-5MS low bleed capillary column with 0.25 μm film phase. The temperature of injector was 200 °C. Sample analysis was performed following a temperature program, 90 °C for 3 min, then 5 °C min^{-1} until 260 °C. The GC was coupled with a Clarus 500 mass spectrometer (Perkin Elemer) with a mass limit 1,185. The analysis was performed in EI mode (ionization energy 70 eV, source temperature 180 °C). The mass fragmentation pattern of GC separated compounds was analyzed and compared with NIST and WILEY libraries.

RESULTS AND DICUSSION

Four antioxidant assays based on electron transfer (ET) or hydrogen atom transfer (HAT) system- (DPPH free radical, $ABTS^{\bullet+}$ scavenging capacities, Fe^{+3} reducing power, total phenol assay by Folin-Ciocalteu Reagent) and three other antioxidant assays against biologically relevant oxidants were used to investigate the antioxidant activity of *C. regalis* extract. In addition, GC/MS analysis of the extract helped to make a tentative identification of the responsible antioxidant after comparison with the previously published reports (Müller, 2001). Antioxidant capacities of *C. regalis* as observed in various assays are presented here (Table 1). The experimental data clearly showed that the antioxidant capacities of the test lichen extract were more or less comparable with commercial standards even at crude form in different assays suggesting the presence of potential antioxidant constituents in the test extract. In the present experiments, the antioxidant activity data for *C. regalis* from the Antarctica was obtained in microgram level. The antioxidant activity for the lichen species of tropical and temperate origin were reported in milligram level (Behera et al. 2006).

DPPH (1, 1-diphenyl-2-picrylhydrazyl) is a commercially available organic nitrogen radical. The antioxidant compounds converted DPPH into DPPH-H by donating a hydrogen atom.

Table 1. Antioxidant activity of ethanol-water extract of *C. regalis*

	50% Inhibition concentration (IC_{50})						
Test samples	DPPH free radical (µg/mL)	ABTS free radical (µg/mL)	Nitric oxide scavenging (µg/mL)	Superoxide scavenging (µg/mL)	Metal chelating (µg/mL)	Reducing Power	Total phenol (Pyrocatechol equivalent) (mg/g)
C. regalis extract	42.3±3.6	70.3±1.7	60.3±2	55.8±4.5	120.8±3.1	10 µg of extract ≈ 1 µg of BHT	83.7±5.1
BHA	4.97±0.9	-	-	-	-	-	-
Trolox	-	46.35±5.1	-	-	-	-	-
Ascorbic acid	-	-	-	12.7±1.2	-	-	-
EDTA	-	-	-	-	71.8±0.8	-	-
Curcumin	-	-	8.4±0.3	-	-	-	-

BHA, Butylated hydroxyanisole; BHT, Butylated hydroxytoluene; EDTA, Ethylenediaminetetraacetic acid

This conversion by the extract of *C. regalis* could easily be read by a spectrophotometer by obtaining a decreased absorbance at 517 nm. Similarly, the inhibition of production of chromogen cations of ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) in a specially designed cations generation system (Rice-Evans and Miller 1994) by *C. regalis* extract could be measured at 405 nm by a spectrophotometer. Ferric ion reducing antioxidant power (or reducing power) assay measures the electron transferring capacity of an antioxidant to convert Fe^{+3} to Fe^{+2} inside a complex molecule. Total phenol assay by Folin-Ciocalteu Reagent (FCR) actually measured the test samples' reducing capacity (antioxidant activity) which was often found excellent linear correlation with total phenolic profiles of the test sample (Huang *et al.*, 2005). *C regalis* extract clearly showed its potent activity on these assays.

In addition, *C regalis* extracts showed dose dependent activity against the biologically active free radicals like superoxide ($O_2^{\bullet-}$) and nitric oxide (NO). Both radicals were well known for their oxidative damages such as inflammation and cancer in human body (Halliwell and Gutteridge, 1990, Moncada *et al.*, 1991). In the present experiment of riboflavin-NBT (Nitroblue tetrazolium) - light system, the photochemical reduction of flavin generated $O_2^{\bullet-}$ radicals, which induced NBT, causing the formation of blue formazan (Beauchamp and Fridovich, 1971). The inhibition of blue formazan formation by the *C. regalis* extract was indicated as decreased absorbance at 590 nm by a spectrophotometer in a concentration-dependent manner. Similarly, nitric oxide after reacting with superoxide forms peroxynitrite ($ONOO^-$). The protonated form of $ONOO^-$ (peroxynitrus acid, ONOOH) is a very strong oxidant which often causes nitration and hydroxylation of tyrosine. In the nitric oxide radical scavenging assay, the sodium nitroprusside in the reaction

mixture generated nitric oxide, which interacted with oxygen to produce nitrite ions that could be detected using Griess reagent. The nitric oxide scavenger constituents competed with oxygen resulting in the inhibition of production of nitrite ions, which could be read by a spectrophotometer at 540 nm (Sumanont *et al.*, 2004). The *C. regalis* extract showed concentration-dependent decrease in absorbance at 540 nm, which indicated its potent nitric oxide radical scavenging capacity.

Iron is capable of generating free radicals from peroxides by the Fenton reaction and is associated with many diseases (Halliwell and Gutteridge, 1990). Ferrous ions (Fe^{2+}) produce oxyradicals and lead to lipid peroxidation. Chelating agents decreased the effective concentration of Fe^{2+} available for the Fenton reaction and, thus, could protect against oxidative damage. Chelating agents are effective as secondary antioxidants due to their ability to form σ-bonds with metal and reduce its redox potential by stabilizing the oxidized form of the metal ion (Gordon, 1990). The Fe^{+2} chelating capacity of *C. regalis* extract was measured by using a spectrophotometer at 562 nm and found to be effective in a concentration-dependent manner. In this reaction system, ferrozine could form complexes with Fe^{2+}. But, in the presence of *C. regalis* extract, the complex formation was disrupted and absorbance was decreased.

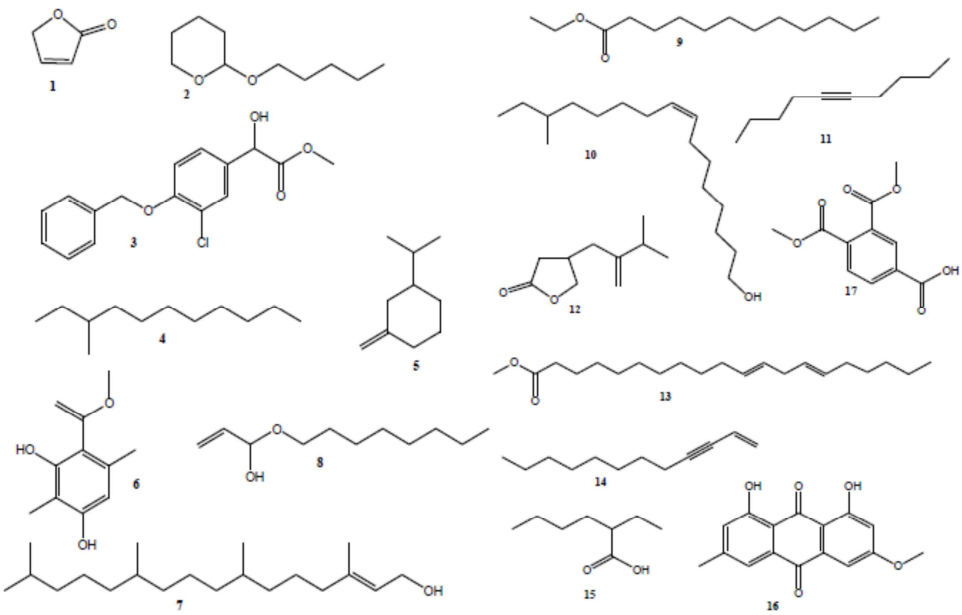

Figure 1. Compounds (**1-17**) identified from GC/MS analysis of *C. regalis* extract.

Table 2. Phytochemical constituents of *Caloplaca regalis* after GC/MS analysis.

Compounds	RT(min)	MW	Name of compounds
1	5.18	84	2(5H)-Furanone
2	7.59	72	2-Pentoxy-tetrahydropyran
3	7.91	306	Hydroxy(4-benzyloxy-3-chlorophenyl)acetic acid methyl ester
4	10.58	170	Undecane, 3-methyl
5	25.22	138	M-Menth-1(7)-ene, (R)-(-)-
6	25.98	196	Benzoic acid, 2,4-dihydroxy-3,6-dimethyl-,methyl ester
7	28.81	296	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
8	31.29	184	2-Propenoic acid, octyl ester
9	31.93	228	Dodecanoic acid, ethyl ester
10	34.03	254	14-methyl-8-hexadecen-1-ol
11	34.42	138	5-Decyne
12	34.54	168	2(3H)-Furanone,4,5-dihydro-4-(2-methyl-3-methylenebut-4-yl)-
13	34.93	322	11,14-Eicosadienoic acid, methyl ester
14	35.03	168	1-Dodecen-3-yne
15	35.58	144	Hexanoic acid, 2-ethyl
16	42.15	284	9,10-Anthracenedione,1,8-dihydroxy-3-methoxy-6-methyl
17	45.4	238	1,2,4-Benzenetricarboxylic acid, 1,2-dimethyl ester

RT, Retention time; MW, Molecular weight

Thus, the experimental data (Table 1, Fig. 1A-G) showed that even the crude extract of *C. regalis* showed strong potential to inhibit free radical production. The GC/MS data (Table 2) showed that *C. regalis* extract contained various derivatives of benzoic acids, furanone, anthraquinone, well known antioxidant compounds (Hieder *et al.*, 2006, Yen *et al.*, 2000). Phenolic compounds and their derivatives are the major secondary metabolites produced by lichens (Nash, 1996). Such compounds are referred to as strong antioxidant agents because they have the ability to scavenge free radicals and reactive oxygen species, such as singlet oxygen, superoxide free radicals, and hydroxyl radicals (Hall and Cupett, 1997). The antioxidant properties of polyphenols are due to the presence of their many phenolic hydroxyl groups, which have high potential for scavenging free radicals (Sawa *et al.*, 1999). The phenolic compounds can easily donate hydrogen to reactive radicals and break the reaction of lipid oxidation at the initiation step (Gülcin *et al.*, 2004). We conclude that *C. regalis* does indeed produce various antioxidant active compounds,

which would have much strong activities if tested in a purified form. We expect that it might be a source of novel antioxidant metabolites because this specimen was collected from an extreme environment (low temperature, high UV content). Therefore, further research should be conducted to purify and characterize the compounds responsible for the antioxidant activity of *C. regalis* extract, as these compounds may be an alternative source of medically relevant antioxidants.

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