2008-1-287

PHYTOTHERAPY RESEARCH
Phytother. Res. **22**, 1635–1639 (2008)
Published online 19 September 2008 in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/ptr.2538

Antioxidant Activity of *Sanionia uncinata*, a Polar Moss Species from King George Island, Antarctica

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Antioxidant agents counter reactive oxygen species (ROS) and can be used in cosmetic and medicinal applications. The goal of this study was to evaluate the antioxidant activity of an Antarctic moss species from King George Island (Antarctica), tentatively designated as KSJ-M5. On the basis of morphological characteristics, KSJ-M5 was identified as *Sanionia uncinata* (Hedw.) Loeske (Amblystegiaceae). The identification was confirmed by comparing the partial sequence of the ITS (internal transcribed spacer) region with that in GenBank. The antioxidant activity of an ethanol extract of KSJ-M5 was evaluated by analyzing its reducing power, superoxide scavenging activity, ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] cation scavenging activity, and DPPH (1,1-diphenyl-2-picrylhydrazyl) free-radical scavenging activity. The reducing power of 1 mg of KSJ-M5 extract was equivalent to 31.9 ± 0.9 μg (Mean ± SD, n = 3) of the commercial standard, BHT (butylated hydroxytoluene). IC_{50} values of the KSJ-M5 extract for DPPH free-radical scavenging activity, superoxide scavenging activity, and ABTS cation scavenging activity were found as 356 ± 26.8 μg/mL, 466.2 ± 43.4 μg/mL, and 181.3 ± 12.2 μg/mL, respectively. The total phenolic content in 1 mg of KSJ-M5 extract was equivalent to 12.7 ± 2.7 μg of pyrocatechol. These results clearly showed that KSJ-M5 could be an important source of natural antioxidant agents for improved medicinal and cosmetic applications.

Keywords: free radicals; phenolic compounds; reducing power; superoxide scavenging activity; *Sanionia uncinata*.

INTRODUCTION

The production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radicals is a common physiological process in the human body (Nordberg and Arner, 2001). Exogenous chemicals and endogenous metabolic processes can produce high levels of ROS. ROS are directly linked to hypertension, atherosclerosis, and diabetes (Cai and Harrison, 2000). In addition, emphysema, cirrhosis, inflammation, genotoxicity and cancer have been correlated with ROS effects. ROS damage cells and enhance a number of degenerative diseases, including premature aging and deoxygenating of ischemic tissues (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 and cutaneous aging (Ames *et al.*, 1993). ROS may also attack unsaturated fatty acids of cell membranes, resulting in lipid peroxidation, a decrease in membrane fluidity, loss of enzyme and receptor activities, and membrane protein damage (Dean and Davies, 1993).

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Antioxidant agents can treat oxidative pathologies by neutralizing ROS, chelating catalytic metals and acting as oxygen scavengers (Gulcin *et al.*, 2003). The most commonly used antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl galate and *tert*-butylhydroquinone (Sherwin, 1990). In addition, several reports on the synthesis of compounds showing strong antioxidant properties have been published (Funasaka *et al.*, 1999; Shimizu *et al.*, 2001). Because of the high carcinogenicity of synthetic antioxidants (Grice, 1986), the development of effective antioxidants of natural origin is of great interest (Wang *et al.*, 1996; Bergman *et al.*, 2001; Behera *et al.*, 2006).

Sanionia uncinata (Hedw.) Loeske is a polar alpine species, which is distributed over a large area of Antarctica (Ochyra, 1998). Polar mosses have been reported to respond to ultraviolet light B (UV-B) and enhanced temperatures by producing some specific secondary metabolites (Huttunen *et al.*, 2005). Secondary metabolites that protect mosses from environmental stresses such as UV, drought, and high temperatures were described previously (Rozema *et al.*, 2001). For example, bryophyte flavonoids, which exhibit an important protective function, contain flavone, flavonol glycones and glycosides, anthocyanins and their derivatives, aurones, biflavonoids, dihydroflavonoids, isoflavons, and triflavons (Markham, 1990). We describe the antioxidant activity of an ethanol-water extract of *S. uncinata* in the contexts of free radical scavenging activity,

Received 4 November 2007
Revised 29 January 2008
Accepted 8 February 2008

reducing power, superoxide radical scavenging activity, and ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] cation scavenging activity.

MATERIALS AND METHODS

Chemicals and reagents. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ferric chloride, trichloroacetic acid, potassium ferricyanide, ferric chloride, nitro blue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), phenazine methosulfate, riboflavin, and an antioxidant assay kit (Product code CS0790) were purchased from Sigma-Aldrich (St Louis, MO, USA). All reagents and solvents used in this experiment were analytical grade.

Moss sampling and identification. A moss specimen (KSJ-M5) 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), the KSJ-M5 specimen was identified as *Sanionia uncinata* (Hedw.) Loeske. The identification was further confirmed by comparing partial sequencing data from the ITS (internal transcribed spacer) region with that on record at the National Center for Biotechnology Information (NCBI). The GenBank accession number of ITS region for KSJ-M5 is- (EU267027).

Extraction. A freeze-dried sample (20 g) was extracted in 70% ethanol (in water) at room temperature (RT). The solvent was evaporated under vacuum at 45 °C and lyophilized. A total of 2.99 g (yield, 14.6%) of dried extract was obtained. The KSJ-M5 extract was stored at –20 °C until further use.

DPPH free radical scavenging assay. Free radical scavenging activity for the KSJ-M5 extract was estimated using a previously described method (Blois, 1958). One mL of DPPH solution (0.1 mM of DPPH in methanol) was mixed with 3 mL of various concentrations of the KSJ-M5 extract. The mixture was incubated at RT for 30 min and the absorbance was measured at 517 nm in a UV-Visible spectrophotometer (SCINCO). Reaction mixtures without the KSJ-M5 extract and with BHA were used as negative and positive controls, respectively. The experiment was conducted in triplicate.

Reducing power determination. Reducing power was determined after minor modification in a previously described method (Oyaizu, 1986). Various concentrations of the KSJ-M5 extract (1 mL in methanol) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K_3Fe (CN) $_6$] solution (2.5 mL, 1%) in distilled water. Reaction mixtures were incubated at 50 °C for 20 min. A trichloroacetic acid solution (2.5 mL, 10% in water) was added and centrifuged (3,000 rpm) at RT for 10 min. The supernatant (0.5 mL) was mixed with 1 mL of distilled water and 0.5 mL of ferric chloride solution (0.1% in water). Absorbance was measured at 700 nm in a UV-Visible spectrophotometer. Reaction mixtures without the KSJ-M5 extract and with BHA were used as negative and positive controls, respectively. The experiment was conducted in triplicate.

Superoxide radical (O_2^-) scavenging activity. The inhibition of blue formazan formation by scavenging superoxide radicals in a riboflavin-light-NBT system (Beauchamp and Fridorich, 1971) was evaluated for various KSJ-M5 extract concentrations. A reaction mixture of 3 mL contained 50 mM phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA, and 0.1 mg NBT (added in that sequence). The reaction was started by illuminating (fluorescent lamp) the mixture for 3 min after the addition of various concentrations of KSJ-M5 extract. Immediately after illumination, absorbance was measured with a UV-Visible spectrophotometer at 590 nm. The reaction mixture without KSJ-M5 extract was used as a control. An identical set of reaction mixtures was kept in the dark (blanks). Triplicate experiments were conducted.

ABTS cation scavenging activity. The antioxidant activity of the KSJ-M5 extract was estimated by comparing with a water-soluble vitamin E analog, trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxilic acid), as a standard (Rice-Evans and Miller, 1994). A standard curve, $y = -0.972x + 0.41$ (y, represents absorbance at 405 nm and x, represents concentration of trolox), for antioxidant activity was obtained for 0 to 100 µg/mL of trolox. Various concentrations of KSJ-M5 extract were applied to the free radical (cation) generation system (280 µL reaction mixtures) following the antioxidant assay kit (Sigma, St Louis, MO, USA) protocol. In the reaction mixture, the chromogen cations of ABTS (2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) were produced by oxidizing ABTS with ferryl myoglobin radical. The ferryl myoglobin radical was produced by the reaction of metmyoglobin with hydrogen peroxide. The ABTS radical produces a green color and can be read spectrophotometrically at 405 nm.

Determination of total phenolic compounds. Total soluble phenolic compounds were determined as described previously (Slinkard and Singleton, 1997) using pyrocatechol as a standard. KSJ-M5 extract (1 mL) at various concentrations was added to 1 mL of Folin-Ciocaltue reagent and mixed thoroughly. After 5 min at RT, 1 mL of Na_2CO_3 (2%) was added and allowed to stand for 2 h with intermittent shaking, and absorbance at 760 nm was measured. The concentration of total phenolic compounds was determined as microgram of pyrocatechol equivalent by comparing with the pyrocatechol standard curve, $y = 0.03x - 0.04$ (y, represents absorbance at 760 nm and x, represents the concentration of pyrocatechol), obtained from 0–1000 µg/mL of pyrocatechol.

RESULTS AND DISCUSSION

DPPH free radical scavenging. The KSJ-M5 extract was able to reduce the stable DPPH radical diphenylpicrylhydrazine (visible, yellow) in a concentration-dependent manner in an assay based on the reduction of DPPH in the presence of a hydrogen donating

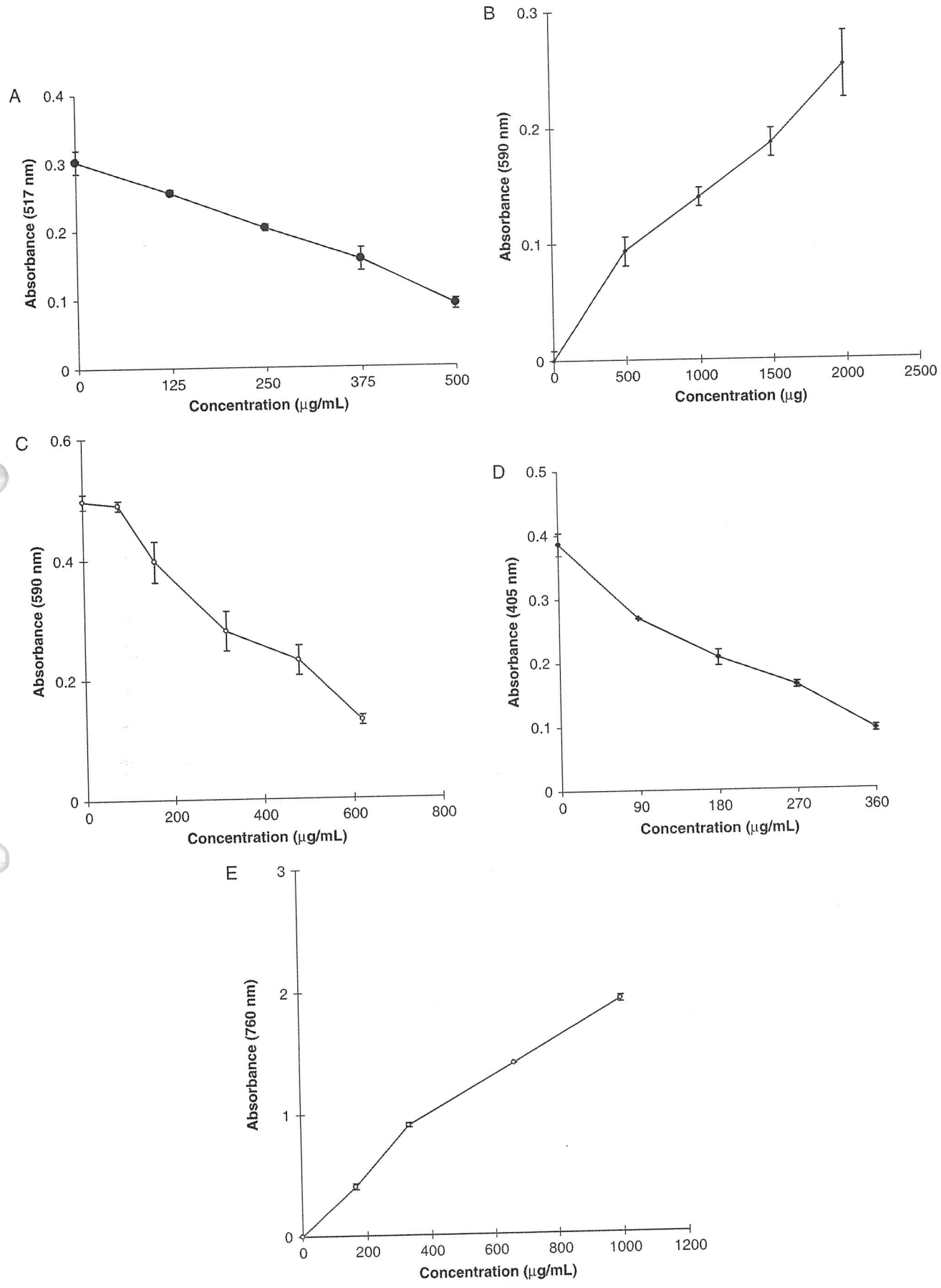

Figure 1. Various antioxidant activities of KSJ-M5 extracts. (A) DPPH free radical scavenging capacity; (B) Reducing power; (C) Superoxide radical (O_2^-) scavenging capacity; (D) ABTS cation scavenging capacity; (E) Total phenolic compounds content.

antioxidant. DPPH radical scavenging activities of various KSJ-M5 extract concentrations is shown in Fig. 1A. In the present experiment, the average IC_{50} concentrations for the commercial standard, BHA, and for the KSJ-M5 extract were found to be 3.5 ± 0.2 µg/mL and 356 ± 26.8 µg/mL, respectively. The KSJ-M5 extract concentration dependent DPPH free radical scavenging activity clearly indicated that KSJ-M5 must be a potent source of compounds that can donate hydrogen atoms to act as an antioxidant. Therefore, KSJ-M5 should be the subject of further work focused on the isolation, purification and characterization of the active antioxidant compounds.

Reducing power. The reducing strength of the KSJ-M5 extract is presented in Fig. 1B. The reducing power of plant extracts was reported to be directly correlated with their antioxidant activity (Pin-Der-Duh *et al.*, 1999). The reducing properties of plant extracts are based on the presence of reductones, which exert antioxidant activity by breaking the free radical chain and donating a hydrogen atom (Gordon, 1990). In this experiment, the reducing power of the KSJ-M5 extract was observed to be proportional to test concentration. The result showed that 1 mg of KSJ-M5 extract was equivalent to 31.9 ± 0.9 µg (i.e., 3.13 ± 0.09 %) of commercial standard, BHT. Clearly, KSJ-M5 must be a prominent source of natural compounds with reducing power, which may be used in therapeutic applications. The isolation and identification of responsible agents from KSJ-M5 is essential for the development of active therapeutics.

Superoxide radical (O_2^-) scavenging assay. The O_2^- radical scavenging activity of various concentrations of the KSJ-M5 extract is presented in Fig. 1C. O_2^- radicals, which function as precursors of ROS (Halliwell and Gutteridge, 1990), are very harmful to cells. In a riboflavin- NBT-light system *in vitro*, the photochemical reduction of flavin generates O_2^- radicals, which reduce NBT, causing blue formazan formation (Beauchamp and Fridovich, 1971). Crude KSJ-M5 extract inhibited blue formazan formation in a concentration dependent manner, with an IC_{50} of 466.2 ± 43.4 µg/mL. In the present experiment, the IC_{50} for a standard, ascorbic acid was obtained as 12.7 ± 1.2 µg/mL. Comparatively, these data showed that KSJ-M5 could be a source of compounds with potent superoxide scavenging activity.

$ABTS^+$ scavenging activity. After regression analysis of the data, it was observed that 0 µg/mL of trolox produced an absorbance of 0.41 at 405 nm after 3 min incubation, while 100 µg/mL of trolox showed no absorbance, indicating complete inhibition of $ABTS^+$ production in the reaction mixture (figure not shown). This curve was taken as a reference during the evaluation of the antioxidant activity of KSJ-M5.

The antioxidant activity of various concentrations of crude KSJ-M5 extract is presented in Fig. 1D. Like trolox, the KSJ-M5 extract reduced the reaction mixture absorbance at 405 nm in a concentration dependent manner. In the present experiment, the IC_{50}s for trolox and for the KSJ-M5 extract were observed at 46.35 ± 5.1 µg/mL and 181.3 ± 12.2 µg/mL, respectively. The observed strength of inhibition of $ABTS^+$ production activity by the KSJ-M5 extract (in terms of IC_{50}) clearly showed that KSJ-M5 may be a source of compounds with potent antioxidant activity.

Total phenolic compound content. Because of the ability to scavenge free radicals and reactive oxygen species (like singlet oxygen, superoxide free radicals, and hydroxyl radicals) phenolic compounds are called strong antioxidant agents (Hall and Cuppett, 1997). In this experiment, an equivalent of 12.7 ± 2.7 µg of pyrocatechol (a phenolic compound) was detected in the KSJ-M5 extract (Fig. 1E). Because of the presence of high solar UV-B radiation, moss species of the Polar Regions produce diverse flavonoid compounds to protect themselves from harmful radiation (Rozema *et al.*, 2001). Considering this point, the Antarctic moss could be a promising source of strong antioxidant agents for various therapeutic applications.

In this study we found that an ethanol-water extract of KSJ-M5 at crude level exhibited various antioxidant activities. Since polar mosses produce several phenolic secondary metabolites in response to high solar UV-B radiation (Rozema *et al.*, 2001), it is highly possible that KSJ-M5 could be a prominent natural source of strong antioxidant compounds. Such compounds may serve as alternatives to commercially available, synthetic antioxidant agents and may have better therapeutic applicability. Hence, this species should be further explored for the isolation and characterization of bioactive molecules.

Acknowledgement

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

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