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RESEARCH ARTICLE

Identification of D-Friedoolean-13-en-3-one (Taraxerone) as an Antioxidant Compound from Sedom (*Sedum sarmentosum*)

Eun Kyoung Mo, Byung Hee Han, Seung Mi Kim, Sun A Yang, Sung Kwon Kang, Chang Jin Oh, Ran Kim, Cheong Gyu Kim, Hyo Jin Kang, and Chang Keun Sung

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Abstract A pentacyclic triterpenoid compound was isolated from the ethyl acetate extract of sedum (*Sedum sarmentosum*) and identified as D-friedoolean-13-en-3-one (taraxerone) by GC-MS and crystallographic analysis. The extraction yield of taraxerone was 74.12±0.57 mg/kg sedum (dry weight). The IC_{50} values of taraxerone were 102.34±1.53 μM and 1,763.81±12.63 μM/mL (Trolox equivalent) by the DPPH and ferric reducing ability of plasma (FRAP) assays, respectively. Taraxerone exhibited comparable antioxidant capacities with butylated hydroxytoluene (BHT) by the DPPH (p=0.117) and FRAP (p=0.179) assays. The production of inducible nitric oxide in lipopolysaccharide-stimulated murine macrophage was inhibited by taraxerone (IC_{50}=38.49±3.77 μM) via downregulation of inducible nitric oxide synthase (iNOS) expression at the transcriptional level. The inhibitory effect of taraxerone on nitric oxide generation was significantly more effective than that of caffeic acid and/or gallic acid.

Keywords: sedom (*Sedum sarmentosum*), D-friedoolean-13-en-3-one (taraxerone), ferric reducing ability of plasma (FRAP), iNOS

Chang Jin Oh, Ran Kim, Cheong Gyu Kim, Hyo Jin Kang, Chang Keun Sung (✉)
Department of Food Science and Technology, Chungnam National University, Daejeon 305-764, Korea
Tel: +82-42-821-6722; Fax: +82-42-822-2287
E-mail: ekymo@hanmail.net

Eun Kyoung Mo, Byung Hee Han, Seung Mi Kim, Sun A Yang
Research and Development Center, DBIO Incorporation, Daejeon 305-764, Korea

Sung Kwon Kang
Department of Chemisty, Chungnam National University, Daejeon 305-764, Korea

Introduction

Reactive oxygen species (ROS) such as superoxide anion radical, hydroxyl radical, and nitric oxide cause oxidative damage to cellular molecules and severe human disease when generated in excess and/or antioxidant defense systems malfunction. The oxidative deterioration of foods can be prevented by synthetic antioxidants. Although efficient in the prevention of peroxidation, only a few synthetic compounds are currently approved for use in the food industry because of their potential toxicity and carcinogenicity (1). Therefore, due to consumers' preferences for natural food additives over synthetic ones, there is an increasing need to identify natural antioxidants from plant and botanical materials for use as less harmful alternatives to synthetic antioxidants (2).

Sedum (*Sedum sarmentosum* Bunge, stonecrop, *dolnamul*) is a type of perennial herb widely distributed in Asia, Europe, and North America. Generally, fresh sedum leaves are consumed as an ingredient in salads, and sedum juice has been used in the making of gelatin jelly (3,4). Furthermore, sedum has been used as a hepatoprotective medicinal plant for a long time in the Asian countries. Recently, a number of studies on the antioxidant capacities of the sedum extracts and/or fractions have been published (5-8). However, no antioxidant compound has been identified or reported from sedum. The aims of this study are to identify sedum's antioxidant compound for the first time and to determine its antioxidant capacities.

Materials and Methods

Chemicals and reagents Silica gel (Kieselgel 60, 70-230 mesh) and TLC plate (precoated Si gel 60 F254) were

purchased from Merck (Darmstadt, Germany). DPPH, butylated hydroxytoluene (BHT), MTT, lipopolysaccharide (LPS), N^{ω}-nitro-L-arginine methyl ester (L-NAME), caffeic acid, and gallic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trolox was purchased from Fluka Chemie (Buchs, SG, Switzerland). All other chemicals used were of analytical grade.

Plant materials and preparation Fresh sedum leaves (general edible portion) were harvested in Seoul, Korea in January 2009. A voucher specimen (No. DBP-090131) was deposited in the Herbarium of the Research and Development Center of the DBIO Incorporation (Daejeon, Korea). The sedum was washed and immediately freeze-dried. Fifty g of lyophilized sedum powder were extracted with 3 L of ethyl acetate (EA) for 12 h at 20±3ºC. To increase the extraction yield, this procedure was repeated twice. The solvent extracts (EAE) were concentrated with a rotary vacuum evaporator at 35±1ºC and stored at −20ºC until further use.

Separation and determination of molecular weight To separate the antioxidant compound, the EAE was chromatographed on column (25×60 cm) chromatography using silica gel and eluted with chloroform:EA:water= 5:2:1 (v/v). Each fraction from the column chromatography was collected and spotted on a TLC plate and developed with the same elution solvent. The fractions with similar retention factor (Rf) values on TLC were combined, and the solvent was evaporated to obtain subfractions. The combined subfractions were chromatographed on a silica gel column (2×80 cm) and eluted with *n*-hexane:EA=8:1 (v/v). Each fraction from the column chromatography was collected and spotted on a TLC plate and developed with the same elution solvent. All spots were detected by treating the TLC plates with a 10% H_2SO_4 solution followed by heating at 110ºC. Each fraction was monitored on the plates, and the fractions with the same Rf values on the TLC were combined.

The molecular weight of the isolate was estimated by GC-MS (6890N; Aglient, Santa Clara, CA, USA), and the operating conditions were consistent with those from an earlier report (9).

Determination and refinement of the X-ray structure X-ray intensity data were collected on a SMART APEX-II CCD diffractometer (Bruker, Madison, WI, USA) using graphite monochromated Mo Kα radiation (λ=0.71073 Å) at 173 K. The structure was solved by applying the direct method using a SHELXS-97 (Fayre; XXII IUCr Congress, Madrid, Spain) and refined by a full-matrix least-squares calculation on F^2 using SHELXL-97 (10). All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed in ideal positions riding on their respective carbon atoms (B_{iso}=1.2 B_{eq} for CH_2 and 1.5 B_{eq} for CH_3 groups).

Crystallographic data for the structure reported here have been deposited into the Cambridge Crystallographic Data Center (Deposition No. CCDC-823740), and the data can be obtained.

Antioxidant capacity Each fraction with same Rf factor on the TLC plate was added into the antioxidant capacity assay systems described before (8). The free radical scavenging capacity was assessed by the DPPH assay, and the metal reducing power was determined by a ferric reducing ability of plasma (FRAP) assay as previously reported (8). The free radical scavenging capacity was expressed as IC_{50} value (μM), and the reducing power was exhibited as Trolox equivalents (TE, μM/mL). Gallic acid, caffeic acid, BHT, and Trolox were used as positive controls.

Nitric oxide (NO) determination and iNOS expression For the determination of NO production, RAW264.7 cells were obtained from the Korean Cell Line Bank (#40071; Seoul, Korea). The cells were grown in RPMI1640 media (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) at 37ºC in a humidified 5% CO_2 incubator (NU-4750G; NuAire, Plymouth, MN, USA) under standard conditions. The cells were cultured in plates at 5×10^5 cells/mL for the measurement of viability and nitrite accumulation. Cell viability and nitrite concentration were determined by the MTT assay (11) and Griess assay (12), respectively. LPS (1 μg/mL), and L-NAME were used as an activator and an inhibitor of NO generation, respectively.

Total RNA was extracted with TRIzol^R reagent (Invitrogen, Carisbad, CA, USA) according to the manufacturer's protocol. iNOS primers (F: 5'-ATGCCCGATGGCACCA TCAGA-3', and R; TCTCCAGGCCCATCCTCCTGC-3'; GeneBank Accession No. D14051), and GAPDH primers (F: 5'-CAACTACATGGTTTACATGTTCC-3', and R: 5'-GGACTGTGGTCATGAGTCCT-3'; GeneBank Accession No. M17851) were used. After an initial denaturation of 5 min at 94ºC, 30 cycles of amplification (94ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s) were performed, followed by a 5 min extension at 72ºC. Five μL of RT-PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining (394-bp iNOS fragment and 416-bp GAPDH fragment).

Statistical analysis All data were expressed as the means ±standard deviation (SD) ($n\geq3$). One-way analysis of variance (ANOVA) and Scheffe's *post hoc* test were conducted to test for significant differences by SPSS (ver.

14; SPSS, Chicago, IL, USA). The *p*-values less than 0.05 were considered statistically significant.

Results and Discussion

Identification According to the earlier report (8), the EAE showed highest antioxidant capacity among some kinds of solvent extracts (methanol-, butanol-, and hexane-extracts). Therefore, the EAE was partitioned over a silica gel column using chloroform:EA:water=5:2:1, and several fractions were obtained (Fig. 1). A fraction of Rf factor 0.86 showed the highest antioxidant activity among them (data not shown), and this fraction (441.26±1.48 mg/kg sedum, d.w.) was chromatographed on silica gel column using *n*-hexane:EA=8:1. An important reaction was observed when the subfractions were developed on the TLC plate, spots of Rf factor 0.58 were collected, and the solvent evaporated at room temperature (20±2°C) to crystallize: the isolate showed yellowish-white crystals. GC-MS analysis demonstrated that the molecular weight of the isolate was 424, and the extraction yield was 74.12±0.57 mg/kg sedum (d.w.). The crystallographic data and structure refinement parameters for the isolate [$C_{30}H_{48}O$] are summarized in Table 1. The selected bond distances and bond angles are summarized in Table 2. An Oak Ridge Thermal-Ellipsoid Plot program (ORTEP; Oak Ridge National Laboratory, Oak Ridge, TN, USA) view including the atomic numbering scheme is shown in Fig. 2. The isolate was identified as taraxerone.

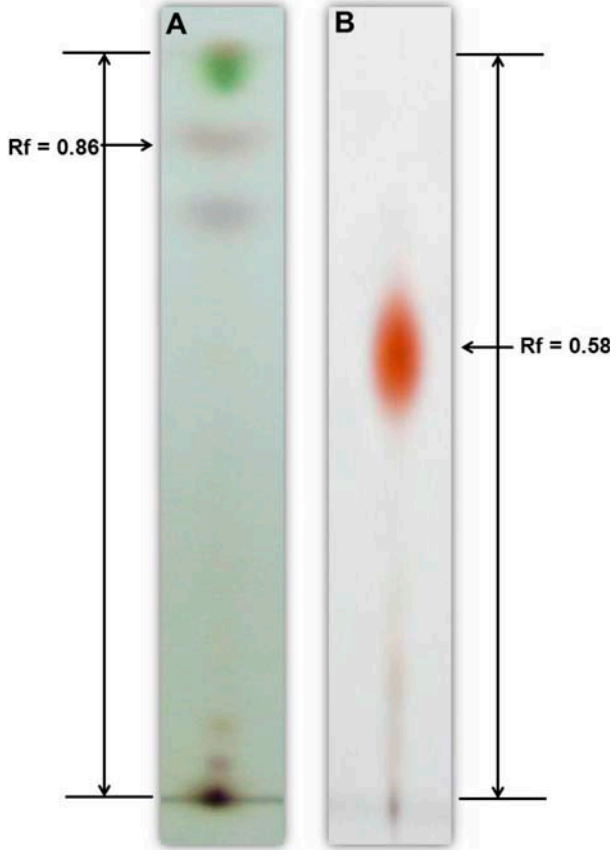

Fig. 1. TLC of the ethyl acetate extract of sedum. A; TLC plate was developed with chloroform:EA:water=5:2:1, B; TLC plate was developed with *n*-hexane:EA=8:1

Antioxidant capacity of taraxerone The organic radical scavenging capacity of taraxerone was determined by the DPPH assay (Table 3). Although taraxerone's radical scavenging ability was significantly lower than that of gallic acid or Trolox, no significant differences were detected between taraxerone and BHT (p=0.117) and caffeic acid (p=0.283). Based on the principle of DPPH assay and the structure of taraxerone, it was suggested that these results were due to hydrogen donation from taraxerone.

According to the FRAP assay, gallic acid exhibited the

Table 1. Crystal data and structure refinement for the isolate [$C_{30}H_{48}O$]

Chemical formula	$C_{30}H_{48}O$	
Formula weight	424.68	
Temperature	174(2) K	
Wavelength	0.71073 Å	
Crystal system, space group	Monoclinic, $P\,2_1$	
Unit cell dimensions	a=12.4896(9) Å	α=90°
	b=7.2379(5) Å	β=90.560(2)°
	c=28.093(2) Å	γ=90°
Volume	2,539.5(3) Å^3	
Z, Calculated density	4, 1.111 Mg/m^3	
F(000)	944	
Crystal size	0.22×0.14×0.14 mm	
Theta range for data collection	1.63 to 25.50°	
Reflections collected/unique	19,640/7,718 [R_{int}=0.0310]	
Goodness-of-fit of F^2	1.033	
Final R indices [$I>2\sigma(I)$]	R_1=0.0493, wR_2=0.1259	
R indices (all data)	R_1=0.0604, wR_2=0.1345	
Largest diff. peak and hole	0.468 and -0.212 e Å^{-3}	

Table 2. Selected bond distances (Å) and angles (°) for the isolate [$C_{30}H_{48}O$]

O(1)-C(2)	1.217(3)	C(2)-C(3)	1.533(4)
C(2)-C(23)	1.499(4)	C(3)-C(4)	1.565(3)
C(4)-C(5)	1.531(3)	C(4)-C(21)	1.544(3)
C(3)-C(24)	1.543(4)	C(16)-C(17)	1.344(3)
O(1)-C(2)-C(3)	121.6(3)	O(1)-C(2)-C(23)	122.2(3)
C(11)-C(16)-C(17)	123.2(2)	C(11)-C(16)-C(15)	112.7(2)

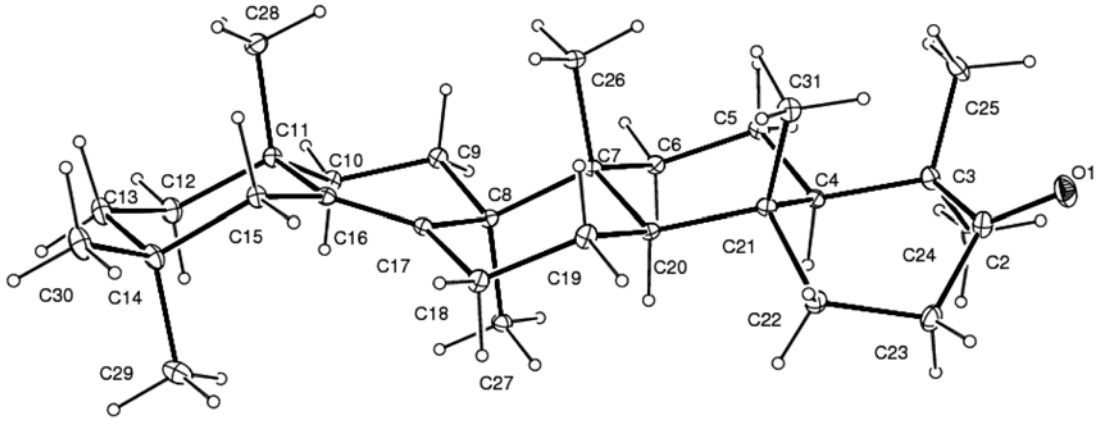

Fig. 2. Molecular structure of the isolate showing the atom numbering scheme and 30% probability ellipsoids.

Table 3. Antioxidant capacity of taraxerone

	Taraxeone	Caffeic acid	Gallic acid	BHT	Trolox	L-NAME
DPPH (IC_{50}[1)], μM)	102.34±1.53[a2)]	101.51±4.56[a]	16.06±0.79[b]	91.49±5.53[a]	67.16±2.56[c]	NA
FRAP (TE, μM/mL)	1,763.81±12.63[a]	1,935.18±60.19[b]	2,511.04±112.09[c]	1,794.84±9.12[a]	NA	NA
Nitrite (IC_{50}, μM)	38.49±3.77[a]	138.84±17.29[b]	> 40	NA	986.39±15.42[c]	21.74±1.09[d]

[1)]IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.
[2)]Different letters within a column denote values that were significantly different (p<0.05); NA, not applicable

highest antioxidant ability, and the reducing power of taraxerone was lower than that of caffeic acid (Table 3). Similar to the DPPH assay, there was no significant difference between taraxerone and BHT (p=0.179) in the FRAP assay. This result could be due to the basic concept that antioxidants are reducing agents of metal ions. Taraxerone could be capable of donating a single electron or hydrogen atom for reduction.

Over 90% of non-LPS-stimulated RAW264.7 cells in the presence of taraxeone, caffeic acid, and Trolox (1-1,000 μM) were healthy and viable, according to the MTT assay, and no nitrite was detected. However, only 75% of the cells survived in the 40 μM gallic acid-treated group, so IC_{50} of gallic acid on the NO production could not be acquired. The NO generation in LPS-activated macrophage was inhibited by the treatment of taraxerone. Despite the fact that the IC_{50} value of taraxerone on NO generation was significantly higher than that of L-NAME, taraxerone IC_{50} value was significantly more effective than that of caffeic acid and/or Trolox (Table 3). iNOS expression of the taraxerone-treated group was downregulated in a dose-dependent manner (Fig. 3). The inhibitory effect of taraxerone on NO production in LPS-activated macrophages was considered to result from the downregulation of the iNOS mRNA expression.

Antioxidant capacity can be assessed in multiple ways because there are many methods of measuring antioxidant activity available. Some spectrophotometric assays have problems with substances exhibiting UV wavelengths similar to those of the test materials, resulting in interference. If the

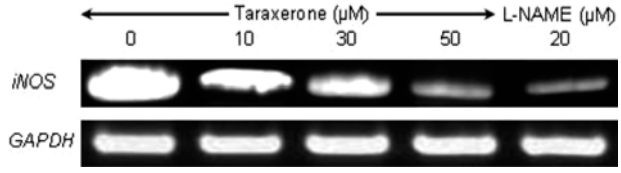

Fig. 3. Effects of taraxerone on LPS-stimulated iNOS expression in RAW264.7 cells. Cells were treated with different concentrations of taraxerone and LPS (1 μg/mL) for 12 h. Total RNA was subjected to RT-PCR, and the PCR products were resolved in 2% agarose gel. L-NAME (20 μM), a NO inhibitor, was used as positive control.

method changes, the hierarchy of antioxidant activity will also change (13). The DPPH, oxygen radical absorbance capacity (ORAC), and FRAP assays are the most common assays used to determine *in vitro* antioxidant activity. In general, at least 2 of these methods are used in combination to provide comprehensive information on the total antioxidant activity of the tested materials, taking into account the pros and cons as well as the applicability of each assay (14). FRAP determines a sample's ability to reduce a metal, and DPPH evaluates a sample's free radical scavenging activity. As shown in the results of the DPPH and FRAP assays, taraxerone isolated from the EAE of sedum exhibited comparable antioxidant capacities to BHT.

The activation of macrophages plays an important role in inflammatory reaction by releasing inducible nitric oxide, which can be produced by iNOS. In this study, the NO generation and iNOS expression of LPS-stimulated murine macrophage were inhibited by taraxerone. Trolox,

gallic acid, and/or caffeic acid showed better antioxidant ability than taraxerone in DPPH and/or FRAP assays, while they exhibited inferior NO inhibitory effects to taraxorone. The modulation of NO production by inhibiting iNOS expression is potentially therapeutic in relation to inflammation (15). This result suggests that taraxerone has anti-inflammatory ability via antioxidant power. Thus, taraxerone could be applied to the treatment of NO-mediated diseases as well as an antioxidant.

Taraxerone has been identified from higher plants, and it has cytotoxic activity against cancer cell lines (16), inhibitory activity on topoisomerase (16), antimicrobial activity (17), antiviral activity (18), antiparasitic activity (19), and insecticidal activity (20). However, antioxidant ability of taraxerone has not been reported. Recently, many studies on the antioxidant ability of sedum were reported (5-8), but no antioxidant compound has been identified or reported from sedum. In the present study, we are the first to identify an antioxidant compound, taraxerone, from sedum by GC-MS and crystallographic analysis. Taraxerone exhibited comparable radical scavenging capacity and reducing power to BHT, and showed better inhibitory effect on LPS-stimulated NO production than gallic acid and/or caffeic acid. These results indicated that sedum could be a natural antioxidant source due to antioxidant capacity of taraxerone. Moreover, these data will provide a scientific basis for the medicinal use of sedum and useful information on a new natural antioxidant source that can be used in the food and nutraceutical industries.

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