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Further Isolation of Peroxynitrite and 1,1-Diphenyl-2-picrylhydrazyl Radical Scavenging Isorhamnetin 7-*O*-glucoside from the Leaves of *Brassica juncea* L.

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From the leaves of *Brassica juncea*, an radical scavenging isorhamnetin 7-*O*-glucoside on peroxynitrite and 1,1-diphenyl-2-picrylhydrazyl (DPPH) was isolated and characterized based on the spectroscopic evidence. The compound showed the peroxynitrite and DPPH scavenging activities with IC_{50} values of 2.07 ± 0.17 and 13.3 μM, respectively. Penicillamine and L-ascorbic acid as positive control exhibited radical scavenging activities with IC_{50} values of 3.17 ± 0.39 and 12.78 μM, respectively.

Key words : *Brassica juncea*, Isorhamnetin 7-*O*-glucoside, Peroxynitrite, 1,1-Diphenyl-2-picrylhydrazyl

INTRODUCTION

Brassica juncea L., a cormophyte vegetable that belongs to cruciferous family, originated from China and has been widely distributed in Korea and Japan (Lee, 1985). The seeds are consumed for mustard (a spice) and the leaves are used as food spices or folkloric uses such as stimulant, diuretic and expectorant (Farrell, 1985). Previously we reported that the EtOAc fraction of the MeOH extract of *B. juncea* exerts *in vitro* scavenging activities using spin trapping and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Kim *et al.*, 2002), and authentic peroxynitrite (Zou *et al.*, 2002). From this EtOAc fraction, sinapic acid was isolated as one of the active principles. Sinapic acid efficiently scavenged authentic peroxynitrite as well as peroxynitrite derived from the peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1) (Zou *et al.*, 2002). In the course of continuous study on the active principles of this plant, we isolated isorhamnetin 7-*O*-β-D-glucopyranoside (**1**). The flavonoid was evaluated for scavenging on DPPH radicals, and authentic peroxynitrite.

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MATERIALS AND METHODS

General experimental procedures

^{1}H- and ^{13}C-NMR spectra were measured by Varian UNITY-Plus 400 spectrometer. Chemical shifts were referenced to the respective residual solvent peaks (δ_H 2.50 and δ_C 39.5 for DMSO-d_6). HMQC, and HMBC spectra were recorded on a Varian UNITY-Plus 400 using pulsed field gradients. Column chromatography was carried out using Si gel (Merck, 70-230 mesh). TLC was performed on the precoated Merck Kieselgel 60 F_{254} plates (0.25 mm), and 50% H_2SO_4 was used as spray reagent. L-ascorbic acid, DPPH, 3-Morpholinosydnonimine (SIN-1), and DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dihydrorhodamine 123 (DHR 123) and $ONOO^-$ were from Molecular Probes (Eugene, Oregon, USA) and Cayman (Ann Arbor, MI, USA), respectively. All other chemicals were of the highest purity available from either Sigma Chemical Co. (St. Louis, MO, USA) or Junsei Chemical Co. (Tokyo, Japan).

Plant materal

The leaves of *Brassica juncea* L. were collected in August 1998, Yosu in Chonnam Province, Korea. A voucher speciemn (No. 980802) was deposited in the

Herbarium of the Department of Food and Nutrition, Pusan National University.

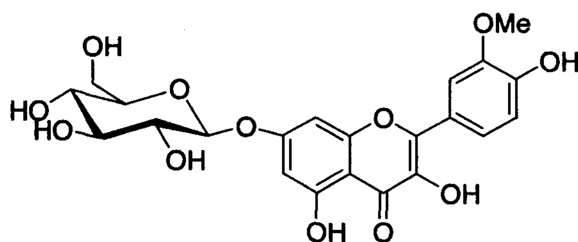
Extraction and isolation

The powdered dried leaves (3.67 kg) of *B. juncea* were refluxed with MeOH for three hours (9 L × 3). The total filtrate was concentrated to dryness *in vacuo* at 40°C to render the MeOH extract (400 g), and this extract was suspended in distilled water and partitioned with CH_2Cl_2 (76 g), EtOAc (2.5 g), *n*-BuOH (31 g), and H_2O (286 g) in sequence. The EtOAc fraction (2.50 g) was applied to a Si gel column which was eluted with EtOAc-MeOH (10 : 1) to yield 10 subfractions. Subfraction 10 (400 mg) was further chromatographed on a Sephadex LH-20 column eluting with MeOH to give compound **1** (65 mg).

Compound 1 (Isorhamnetin 7-*O*-glucoside) : ^{1}H-NMR (400 MHz, DMSO-d_6) : δ 3.85 (3H, s, OMe), 5.05 (1H, d, *J*=7.32Hz, H-1"), 6.43 (1H, *J*= 2.1 Hz, H-6), 6.85 (1H, d, *J*= 2.1 Hz, H-8), 6.95 (1H, d, *J*= 8.9 Hz, H-5'), 7.73 (1H, dd, J=2.1 & 8.9Hz, H-6'), 7.77 (1H, d, *J*=2.1Hz, H-2'), 9.58 (1H, brs, 3-OH), 9.79 (1H, brs, 4'-OH), 12.46 (1H, brs, 5-OH) ; ^{13}C-NMR (100.0 MHz, DMSO-d_6) : δ 176.03 (C-4), 162.71 (C-7), 160.29 (C-5), 155.66 (C-9), 148.97 (C--3'), 147.38 (C-4'), 147.28 (C-2), 136.15 (C-3), 121.88 (C-6'), 121.78 (C-1'), 115.53 (C-5'), 111.74 (C-2'), 104.65 (C-10), 100.02 (C-1"), 98.74 (C-6), 94.62 (C-8), 77.26 (C-5"), 76.45 (C-3"), 73.11 (C-2"), 69.60 (C-4"), 60.61 (C-6"), 55.76 (OMe)

Acid Hydrolysis of Compound 1 : On acid hydrolysis with 5%-H_2SO_4 (3 hr), **1** gave glucose (co-TLC, *n*-BuOH-HOAc-H_2O, 4:1:5) and an aglycone **(1a),** identified as isorhamnetin.

Compound 1a (Isorhamnetin) : ^{1}H-NMR (400 MHz, DMSO-d_6) : δ 3.84 (3H, s, OMe), 6.19 (1H, *J*= 2.2 Hz, H-6), 6.48 (1H, d, *J*= 2.2 Hz, H-8), 6.93 (1H, d, *J*= 8.3 Hz, H-5'), 7.68 (1H, dd, J=2.0 & 8.3Hz, H-6'), 7.75 (1H, d, *J*=2.0, H-2'), 9.44 (1H, s, 3-OH), 9.74 (1H, s, 4'-OH), 10.77 (1H, s, 7-OH), 12.47 (1H, brs, 5-OH) ; ^{13}C-NMR (100.0 MHz, DMSO-d_6) : δ 175.85 (C-4), 163.88 (C-7), 160.65 (C-5), 156.12 (C-9), 148.78 (C-4'), 147.33 (C-3'), 146.60 (C-2), 135.79 (C-3), 121.94 (C-6'), 121.69 (C-1'), 111.68 (C-2'), 115.51 (C-5'), 103.00 (C-10), 98.17 (C-6), 93.56 (C-8)



Structure of compound **1**

Measurement of $ONOO^-$ scavenging activity

$ONOO^-$ scavenging was measured by monitoring the oxidation of dihydrorhodamine 123 by modifying the method of Kooy *et al.* (1994). A stock solution of DHR 123 (5mM) in dimethylformamide was purged with nitrogen and stored at -80°C. Working solution with DHR 123 (final concentration, 5 μM) diluted from the stock solution was placed on ice in the dark immediately prior to the study. The buffer of 90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride with 100 μM (f.c.) diethylenetriaminepentaacetic acid (DTPA) was purged with nitrogen and placed on ice before use. $ONOO^-$ scavenging by the oxidation of DHR 123 was measured on a microplate fluorescence spectrophotometer FL 500 (Bio-Tek Instruments, USA) with excitation and emission wavelengths of 485 nm and 530 nm respectively at room temperature. The background and final fluorescent intensities were measured 5 min after treatment with or without SIN-1 (f.c. 10 μM) or authentic $ONOO^-$ (f.c. 10 μM) in 0.3 M sodium hydroxide. Oxidation of DHR 123 by decomposition of SIN-1 gradually increased whereas authentic $ONOO^-$ rapidly oxidized DHR 123 with its final fluorescent intensity being stable over time.

DPPH radical scavenging effect

The DPPH radical scavenging effect was evaluated according to the method first employed by Blois (1958). Four milliliters of MeOH solution of varying sample concentrations (1.5-45 μM) was added to 1.0 mL DPPH methanol solution (1.5×10^{-1} M). After mixing gently and leaving for 30 min at room temperature, the optical density was measured at 520 nm using a spectrophotometer. The antioxidant activity of each sample was expressed in terms of IC_{50} (μg/mL or μM required to inhibit DPPH radical formation by 50%) and calculated from the log-dose inhibition curve.

RESULTS AND DISCUSSION

Column chromatography of the EtOAc-soluble part of the methanol extract of the plant yielded a known compound **1**. The structure of known **1** was identified by comparisons with published spectral data as isorhamnetin 7-O-glucoside (**1**) (Singh & Pandey, 1986; Wolbis, 1989; Agrawal, 1989). The structure was further identified by chemical reaction (acid hydrolysis) of **1** and detailed analysis of the ^{1}H- and ^{13}C-NMR spectra, aided by HMQC, and HMBC experiments. In the HMBC spectrum of **1** the signal of glucose anomeric proton at δ 5.05 was correlated with that of isorhamnetin C-7 at δ 162.71,

suggesting that the glucose was connected with isorhamnetin at C-7. On the basis of these results, the structure of **1** was established as isorhamnetin 7-*O*-β-D-glucopyranoside. This is the first report of its occurrence in *Brassica* species.

The compound **1** showed scavenging activities of DPPH radical and peroxynitrite, with IC_{50} values of 13.3, and 2.07 ± 0.17 μM, respectively. The radical scavenging activities were comparable to that of L-ascorbic acid (IC_{50} = 12.78 μM) and Penicillamine (IC_{50} = 3.17 ± 0.39 μM).

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