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Nitric oxide-scavenging compounds in *Agrimonia pilosa* Ledeb on LPS-induced RAW264.7 macrophages

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ABSTRACT

The extract of *Agrimonia pilosa* Ledeb, with a high polyphenol content, inhibited nitrite accumulation as an indicator of nitric oxide (NO) in LPS-induced RAW264.7 macrophages. The NO inhibitory compounds in the extract were isolated using open column chromatography and HPLC, and five phenolic compounds, namely aromadendrin (AD), dihydrokaempferol 3-*O*-*β*-*D*-glucoside (DK3-*O*-glc), quercitrin (QC), aglimonolide-6-*O*-*β*-*D*-glucoside (AG6-*O*-glc) and loliolide (LL), were determined by ^{1}H, ^{13}C NMR and LC/MS analyses. This is the first time that these compounds have been isolated from this plant. 4-Ethyl-2-hydroxyamino-5-nitro-3-hexenamide (NOR3), as a NO donor, was used in the presence of these compounds and then the nitrite-level, as an index of NO, decreased, indicating that these compounds would potentially have a NO-scavenging activity. An ESR study of the system containing NOR3 and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), as a NO detection reagent, with or without the compound, provided the evidence that these compounds directly scavenged NO and the scavenging activities of three flavonoids (AD, DK3-*O*-glc and QC) were remarkably high in comparison to those of the other phenolic compounds. These results indicated that one of the suppression mechanisms in cells would be the NO-scavenge of phenolic compounds.

1. Introduction

The function of nitric oxide (NO) has been elucidated in a variety of pharmacological conditions, including inflammation, carcinogenesis and atherosclerosis, and excess NO production or the peroxynitrite radical ($ONOO^{-}$) causes oxidative damage to membrane lipids, DNA, proteins and lipoproteins (Buney, Caufield, Niles, Wishnok, & Tannbaum, 1999; Stefen et al., 2007). These reactions have functional consequences, which may be deleterious to cells and tissues (Beckman & Koppenol, 1996; Yu et al., 1994). The development of substances to prevent the overproduction of NO has become a new research target to treat chronic inflammatory diseases (Hobbs, Higgs, & Moncada, 1999; Pacher, Joseph, Beckman, & Liaudet, 2007).

The production of NO is regulated by nitric oxide synthases, and three types of nitric oxide synthases, endothelium NO synthase (eNOS), neural NO synthase (nNOS) and inducible NO synthase (iNOS) have been identified. Lipopolysaccharide (LPS), from Gram negative bacteria, can be quantitatively iNOS-induced in various cells, such as macrophages, smooth muscle cells, and hepatocytes, to trigger several disadvantageous cellular responses and cause responses similar to inflammation, sepsis and stroke (Nathan, 1992; Shen et al., 2002). The large amount of NO production by iNOS induction has been closely correlated with pathophysiology in a variety of diseases and inflammations. Thus, NO production, through iNOS induction by LPS, may reflect the degree of inflammation and may provide a measure to assess the effect of drugs on the inflammatory process.

Polyphenols are widely distributed in dietary fruits, vegetables and medicinal herbs, and they generally potentially scavenge oxygen or nitrogen radicals (Cho et al., 2007; Nakagawa & Yokozawa, 2002; Tsai, Tsai, Yu, & Ho, 2007). Previous studies have demonstrated that a significant correlation exists between the total polyphenol content of a spice and its DPPH-scavenging ability (Katsube et al., 2004; Tsai et al., 2007). In our early study, we found that naturally or synthetic biphenyl compounds had a strong oxygen radical-scavenging effect and some of these have been developed as cosmetic materials to protect from skin damage causing excess free radical production due to sunlight-involved inflammation (Taira, Ikemoto, Mimura, Hagi, & Murakami, 1993; Taira, Ikemoto et al., 1992; Taira, Mimura et al., 1992). Based on such background studies, we initially examined the polyphenol contents of several hundred Asian plants. As a result, *Agrimonia pilosa* Ledeb, having antitumor activity, was found to contain a high polyphenol content (Koshiura, Miyamoto, Ikeya, & Taguchi, 1985). This study showed that the *A. pilosa* Ledeb extract had an inhibitory action on NO

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production in LPS-induced RAW 264.7 macrophages and the NO-scavenging compounds in the extract were identified.

2. Materials and methods

2.1. Reagents

Sodium nitrite, L-arginine and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from the Wako Chemical Pure Co. (Osaka, Japan). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) and 4-ethyl-2-hydroxyamino-5-nitro-3-hexenamide (NOR3) were purchased from Dojindo (Kumamoto, Japan). Interferon-γ (IFN-γ) and lipopolysaccharide (LPS) were purchased from Sigma (MO, USA). Dulbecco's modified eagle's medium (DMEM) and foetal bovine serum (FBS) were obtained from Gibco BRL (NY, USA).

2.2. Sample preparation

A. pilosa Ledeb (leaf-dry weight, 1 g) was extracted with ethanol (10 ml) for 20 h, using a shaker (RECIPRO SHAKER SR-2S, Taipec) at room temperature. The extract was filtered and concentrated using a centrifugal thickener (CEI Centrifugal Evaporator, Hitachi). It was dissolved in ethanol (1 ml) and the test sample was filtered using a disk filter (0.45 μM, Millipore) for the assay.

2.3. Cells

RAW264.7 cells (mouse macrophages, American Type Culture Collection) were cultured in DMEM medium (including 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a 5% CO_2 atmosphere. The cells (5×10^4 cells/well) with or without the test sample, were cultured in a 96-well microplate. Untreated cells were used as the control.

2.4. MTT assay

The MTT assay was used to examine the cell viability due to treatment with a test sample, as previously reported (Ferrari, Fomasiero, & Isetta., 1990). Briefly, the cells were seeded at a density of 5.0×10^5 cells/ml and cultured for 16 h, with or without the test sample. After the culture, MTT (0.5 mg/ml) was added to each well and incubated for 3 h and then the suspension was removed. Extraction with DMSO (100 μl) was measured at 570 nm with the reference at 630 nm, using a microplate reader (BIO-RAD Model 550, BIO-RAD, USA).

2.5. Nitrite assay on RAW264.7 macrophages

RAW264.7 macrophages in a 96-well microplate were treated with the various concentrations (0.025–0.25%) of the *A. pilosa* Ledeb extract or isolated compounds (25, 50 and 100 μM) with LPS (100 ng/ml), L-arginine (2 mM), and IFN-γ (100 U/ml). Cells, with or without LPS, IFN-γ and L-arginine, were used as the positive control and negative control, respectively. After culturing for 16 h, the nitrite-levels, as an NO indicator in the medium, were determined by the Griess method (Taira, Mišśík, & Riesz, 1997). Briefly, aliquots of 80 μl of medium and 20 μl of PBS were mixed in a 96-well plate and then 50 μl of a 1% sulfanilamide solution, containing 5% phosphoric acid, were added. The reaction mixture was incubated for 5 min at room temperature and then 50 μl of a 0.1% *N*-(1-naphthyl) ethylenediamine solution were added. After 10 min of incubation, the absorbance at 540 nm was measured on a microplate reader. $NaNO_2$ was used as the standard to calculate the nitrite concentration.

2.6. Cell viability

The inhibition (IH) of each assay was calculated using the following equation: IH (%) = {1 − [(test sample data) − (negative control data)]/[(positive control data) − (negative control data)]} × 100. The data are expressed as means ± SD of four test samples.

2.7. NO inhibitory action

NOR3 releases NO due to its spontaneous degradation at neutral pH, and the NO donor was used in the presence of phenolic compounds for confirming the NO-scavenge effect. Nitrite production, as an NO indicator, was monitored. The reaction mixture containing NOR3 (200 μM), with or without test compounds (50, 100 and 200 μM) in PBS solution, was incubated at room temperature for 60 min, and the nitrite accumulation in the reaction mixture was measured using previously described procedures (Taira et al., 1997).

2.8. Extraction and isolation

A. pilosa Ledeb (dry weight 951 g) was extracted with MeOH. After filtration, the extract was concentrated in vacuo and the MeOH extract was partitioned between EtOAc (1 l) and H_2O (1 l). The EtOAc extract (158.3 g) was suspended in 50% aqueous MeOH. The suspension was partitioned between hexane (500 ml), CH_2Cl_2 (500 mL) and *n*-BuOH (300 ml), respectively, to give the non-polar hexane extract (16.3 g), lipophilic $CHCl_3$ extract (38.3 g) and polar *n*-BuOH extract (36.1 g). The BuOH extract was partitioned between 50% aqueous MeOH (500 ml) and EtOAc (200 ml). The EtOAc phase was concentrated and the extract was partitioned between the hexane (200 ml) and 50% aqueous MeOH, and the 50% MeOH phase was further partitioned using CH_2Cl_2 (200 ml). The CH_2Cl_2 extract (1.4 g) was separated on an open column (Merck silica gel 60, particle size 0.040–0.063 mm, 230–400 mesh, ASTM), using hexane with an increasing proportion of EtOAc to give 17 fractions. Compound 1 (8.6 mg) was obtained from the 8th fraction (28 mg) using HPLC (Hitachi L-7110, HITACHI) equipped with an RI detector (R401, Waters) on a silica-column (COSMOSIL Si60 5SL-II, 10 i.d.×250 mm, Nacalai Tesque) with hexane/EtOAc (1/1 v/v). The 9th fraction (28 mg) was separated using an ODS-column (COAMOSIL 5C18 AR-II, 10 i.d. × 250 mm, Nacalai Tesque) with MeOH/H2O (5/1 v/v), and the 4th fraction in these fractions was purified with MeOH/H2O (1/1 v/v) to give compound 2 (0.9 mg). The 17th fraction (186.1 mg) was separated using a Sephadex column (LH-20, 20–100 mm particle size, Pharmacia Fine Chemical), with 10% aqueous MeOH as the eluent, to give 16 fractions and compound 3 (11.0 mg), compound 4 (8.4 mg) and compound 5 (13.5 mg) from each fraction were isolated, respectively, using HPLC with a YMC packed Ph HPLC column (A-423 PH12S05-2510WT, 10 i.d. × 250 mm, YMC) with MeOH/H2O (3/1 v/v).

2.9. NMR analysis

The ^1H, ^{13}C and 2D NMR spectra were recorded by a JEOL a-500 spectrometer (JEOL) and the ^1H and ^{13}C chemical shifts were referenced to the solvent peaks (dH 3.30 and dC 49.0 in CD3OD and dH 7.24 and dC 77.0 in CDCl3).

2.10. LC/MS analysis

LC/MS was used to determine the molecular weight of the isolated compounds and/or to monitor the reaction between NO and the compound. The chromatographic separations were carried out using a photodiode array detector and monitored in the

operating wavelength range from 210 to 500 nm at the flow rate of 0.34 ml/min on a reversed-phase chromatographic column, YMC-Pack *Pro* C18 (100 × 3.0 mm I.D., 5 μm particle size, YMC Co. Ltd., Japan) at 35.0 °C (Waters 2695 Separations Module, Waters Corporation, USA). The mobile phase consisting of a formic acid aqueous solution (0.1%) and acetonitrile, was used with a linear gradient. An initial isocratic step, with 0.1% formic acid, was held for 0.5 min and then a linear gradient to 10% acetonitrile for 1.5 min, 30% acetonitrile for 4 min and then held for 1 min. The final linear gradient to 100% acetonitrile was carried out for 1 min. The mass spectra were measured under the following conditions: cone voltage, 17 V for ESI positive ion mode; cone voltage, 21 V for ESI negative ion mode; capillary voltage, 3.00 kV; source temperature, 110 °C: desolvation temperature, 350 °C; cone gas flow, 50 l h^{-1}; and desolvation gas flow, 700 l h^{-1} (Micromass Quattro *micro* API Mass spectrometer, Waters Corporation., USA).

2.11. ESR measurement

NO-scavenging action of the compounds was confirmed by the ESR study, as previously reported (Pacelli, Taira, Cook, Wink, & Krishna, 1996). The reaction mixture of the compound (100 μM), NOR3 (200 μM) and NO detection reagent, carboxy-PTIO (25 μM) was prepared in PBS and incubated at room temperature for 30 min. An ESR measurement was performed by ESR spectroscopy (JES-FR30, JEOL) operating in the X-band with a modulation frequency of 100 kHz. The reaction mixture was transferred to the capillary (100 × 1.1 mm I.D., Drumnond Scientific. Co., USA) which was placed in a quartz cell (270 mm long, 5 mm I.D., JEOL DATUM LTD., Japan). The ESR signal was measured at 9.4 GHz resonant frequency under the following conditions: microwave power, 4 mW; modulation width, 0.1 mT; gain, 320; scan time, 1 min; time constant, 0.3 s. Manganese oxide was used as the internal standard.

3. Results

3.1. NO Inhibition of A. pilosa Ledeb in LPS-induced RAW264.7 macrophages

A. pilosa Ledeb was extracted with ethanol and the cytotoxicity of the extract was evaluated by an MTT assay. As shown in Fig. 1a, no cytotoxicity was detected in the selected concentration ranges. The various concentrations (0.025%, 0.50%, 0.10% and 0.25%) of the extract were evaluated for NO production in the LPS-induced RAW264.7 cells. The extract significantly inhibited the nitrite accumulation in a dose-dependent manner, suggesting that the NO inhibitory compounds would be contained in the extract (Fig. 1b).

3.2. Extraction and isolation of compounds

The extract of *A. pilosa* Ledeb was separated using open column chromatography and HPLC, and five compounds isolated from the extract were analysed using ^{1}H, ^{13}C and 2D NMR, and LC/MS. The resulting analytical data were as follows:

Compound 1 (Aromadendrin) *m/z* 286.92 $(M-H)^-$, ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 4.92 (1H, d, 12.0 Hz, H-2), 4.46 (1H, d, 12.0 Hz, H-3), 5.94 (1H, d, 2.5 Hz, H-6), 5.88 (1H, d, 2.5 Hz, H-8), 7.38 (1H, d, 8.5 Hz, H-2′), 6.82 (1H, d, 8.5 Hz, H-3′), 6.82 (1H, d, 8.5 Hz, H-5′), 7.38 (1H, d, 8.5 Hz, H-6′).

^{13}C NMR (125 MHz, $CDCl_3$) δ_C 83.2 (2), 72.1 (3) 195.9 (4) 100.3 (4a) 163.4 (5) 96.7 (6), 167.3 (7), 95.8 (8), 163.0 (8a) 127.3 (1′), 128.9 (2′), 115.5 (3′), 157.5 (4′), 115.5 (5′), 29.0 (6′).

Compound 2 (Loliolide) *m/z* 197.06 $(M+H)^+$, ^{1}H NMR (500 MHz, CD_3OD) δ_H 5.74 (1H, s, H-2), 1.52 (2H, dd, 14.0, 3.5 Hz H-4), 1.98 (2H, ddd, 14.0, 2.7, 2.7 Hz, H-4), 4.21 (2H, m, H-5), 1.72 (2H, m, H-6), 2.42 (2H, ddd, 14.0, 2.7, 2.7 Hz H-6), 1.75 (3H, s, 7-H), 1.27, (3H, s, 8-H), 1.46 (3H, s, 9-H).

^{13}C NMR (125 MHz, CD_3OD) δ_C 174.5 (1), 113.3 (2), 185.7 (2a), 37.2 (3), 48.0 (4), 67.3 (5), 46.4(6), 89.0 (6a), 27.4 (7), 31.0 (8), 27.0 (9).

Compound 3 (Aglimonolide-6-*O*-glucoside) *m/z* 474.94 $(M-H)^-$, ^{1}H NMR (500 MHz, CD_3OD) δ_H 2.94 (2H, m, H-1), 4.92 (1H, m, H-2), 6.52 (1H, d, 2.0 Hz, H-6), 6.50 (1H, d, 2.0 Hz, H-8), 2.03 (2H, m, H-9), 2.79 (2H, m, H-10), 7.14 (1H, d, 8.5 Hz, H-2′), 6.84 (1H, d, 8.0 Hz, H-3′), 6.84 (1H, d, 8.0 Hz, H-5′), 7.14 (2H, d, 8.5 Hz, H-6′), 3.74 (s, H-7′), 4.97 (d, 7.5 Hz, H-1″), 3.45 (1H, m, H-2″), 3.46 (1H, m, H-3″), 3.37 (t, 9.0, H-4″), 3.45 (1H, m, H-5″), 3.68 (1H, dd, 6.0, 2.0 Hz, H-5″), 3.89 (1H, dd, 2.5, 12.0 Hz, H-6″).

^{13}C NMR (125 MHz, CD_3OD) δ_C 33.10 (1), 80.09 (2), 171.35 (4), 104.00 (4a), 165.09 (5), 103.51 (6), 165.15 (7), 108.29, (8), 143.39 (8a), 37.79 (9), 31.10 (10), 134.33 (1′), 130.41 (2′), 114.96 (3′), 159.56 (4′), 114.96 (5′), 30.41 (6′), 55.65 (7′), 101.35 (1″), 74.69 (2″), 78.35 (3″), 71.23 (4″), 77.86 (5″), 62.42 (6″).

Compound 4 (Dihydrokaempherol-3-*O*-glucoside) *m/z* 448.83 $(M-H)^-$, ^{1}H NMR (500 MHz, CD_3OD) δ_H 5.22 (1H, d, 9.0 Hz, H-2), 4.91 (1H, d, 9.0 Hz, H-3), 5.89 (1H, d, 2.5 Hz, H-6), 5.84 (1H, d, 2.5 Hz, H-8), 7.23 (1H, d, 8.5 Hz, H-2′), 6.74 (1H, d, 8.5 Hz, H-3′), 6.74 (1H, d, 8.5 Hz, H-5′), 7.23 (1H, d, 8.5 Hz, H-6′), 4.64 (1H, d, 8.0 Hz, H-1″) 3.05 (1H, dd, 8.0, 9.5 Hz, H-2″), 3.3 (1H, t, 9.5, H-3″), 3.11 (1H, m, H-4″), 3.15 (1H, m, H-5″), 3.49 (1H, dd, 5, 1.5 Hz, H-5), 3.71 (2H, dd, 1.5, 11.5 Hz, H-6″).

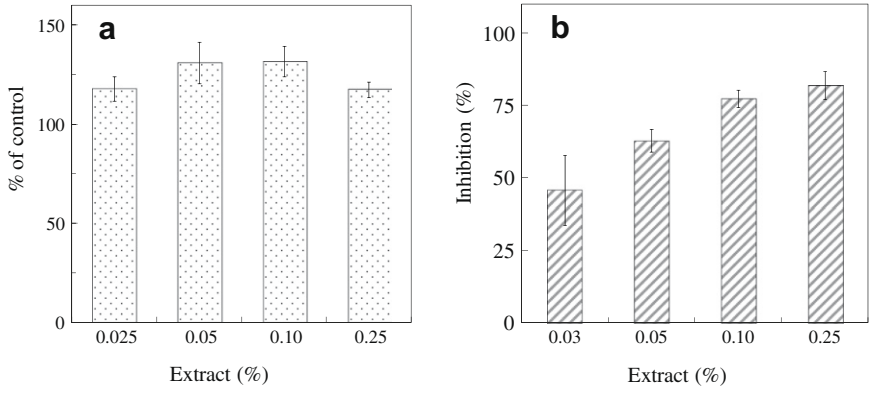

Fig. 1. Inhibitory action of *A. pilosa* Ledeb on NO production in LPS-induced RAW264.7 macrophages. (a) The effect of the various concentrations (0.025%, 0.50%, 0.10% and 0.25%) of the EtOH extract of *A. pilosa* Ledeb on the NO production in LPS-induced RAW264.7 cells was evaluated. The inhibitory action was indicated as the inhibition (%) of the sample-treated cells for the LPS treated cells. (b) Cell viability with treated samples at the test concentrations was examined by an MTT assay. The cell viability was expressed as % of control cells without sample.

^{13}C NMR (125 MHz, CD_3OD) δ_C 83.41 (2), 77.52 (3), 196.52 (4), 102.33 (4a), 165.55 (5), 97.30 (6), 169.00 (7), 96.31 (8), 164.13 (8a), 128.47 (1′), 130.53 (2′), 115.92 (3′), 159.03 (4′), 115.92 (5′), 130.53 (6′), 104.50 (1″), 75.47 (2″), 77.93 (3″), 71.47 (4″), 77.83 (5″), 62.86 (6″).

Compound 5 (Quercitrin) *m/z* 446.81 $(M-H)^-$. ^{1}H NMR (500 MHz, CD_3OD) δ_H 6.19 (1H, br s, H-6), 6.39 (1H, br s, H-8), 7.30 (1H, d, 8.0 Hz, H-2′), 6.74 (1H, d, 8.0 Hz, H-3′), 7.32 (1H, s, H-6′), 5.34 (1H, br s, H-1″), 4.21 (1H, br s, H-2″), 3.74 (1H, dd, 3.5, 9.5, Hz, H-3″), 3.39 (1H, dd, 9.5, 9.5 Hz, H-4″), 3.41 (1H, dd, 8.0, 5 Hz, H-5″) 0.93 (3H, d, 6.0, Hz, H-6″).

^{13}C NMR (125 MHz, CD_3OD) δ_C 158.52 (2), 136.23 (3), 179.64 (4), 105.88 (4a), 159.30 (5), 99.82 (6), 165.93 (7), 94.72 (8), 163.22 (8a), 122.86 (1′), 122.96 (2′), 116.36 (3′), 150.00 (4′), 146.41 (5), 116.93 (6′), 103.54 (1″), 71.90 (2″), 72.03 (3″), 73.25 (4″), 72.11 (5″), 17.65 (6″).

Compounds 1, 2, 3, 4 and 5 were assigned on the basis of these analytical data which were identical to aromadendrin (Lee et al., 2004; Rawat, Pant, Prasad, Joshi, & Pande, 1998), loliolide (Vardes, 1986), aglimonolide-6-*O*-glucopyranoside (Park, Oh, Kang, Sohn, & Kim, 2004), dihydrokaempferol-3-*O*-glucopyranoside (Lee et al., 2004) and quercitrin (Zheng, Liang, & Hu, 2005), respectively. Their chemical structures are depicted in Fig. 2.

3.3. NO inhibitory action in cells

Five phenolic compounds from the *A. pilosa Ledeb extract* were evaluated for NO production in the LPS-induced RAW264.7 cells. The nitrite accumulation in the cells increased due to the LPS treatment. When all the compounds were placed in the NO production system, the nitrite accumulation was significantly inhibited in a dose-dependent manner (25, 50 and 100 μM) (Fig. 3). The cytotoxicity of these compounds was not detected in the range of the test concentrations (data not shown). The NO-scavenging abilities of all compounds were similar, indicating that all of these compounds would act together in the extract against nitrosive stress. Flavonoids, as the major polyphenols in plants, are known to be nitrogen radical-scavengers, e.g. for NO and peroxynitrite, suggesting that the suppression of these compounds would also be due to a similar mechanism (Haenen, Paqary, Korthouwer, & Bast, 1997; Van et al., 1995).

3.4. NO-scavenge effect

NOR3 was used in the presence of phenolic compounds. The nitrite accumulation, with or without compounds, was examined. Nitrite accumulation from NOR3 was reduced in the presence of these compounds in a dose-dependent manner. These results suggested that these compounds would have a potentially NO or nitrogen radical-scavenging activity (Fig. 4). This result also suggested that the compounds would act together against excess NO production in the LPS-induced RAW264.7 macrophages (Fig. 3).

3.5. ESR measurement

To clarify the NO-scavenging action of the phenolic compounds, an ESR study was performed on the system containing carboxy-PTIO as NO detection reagent, in the presence of NOR3 and the

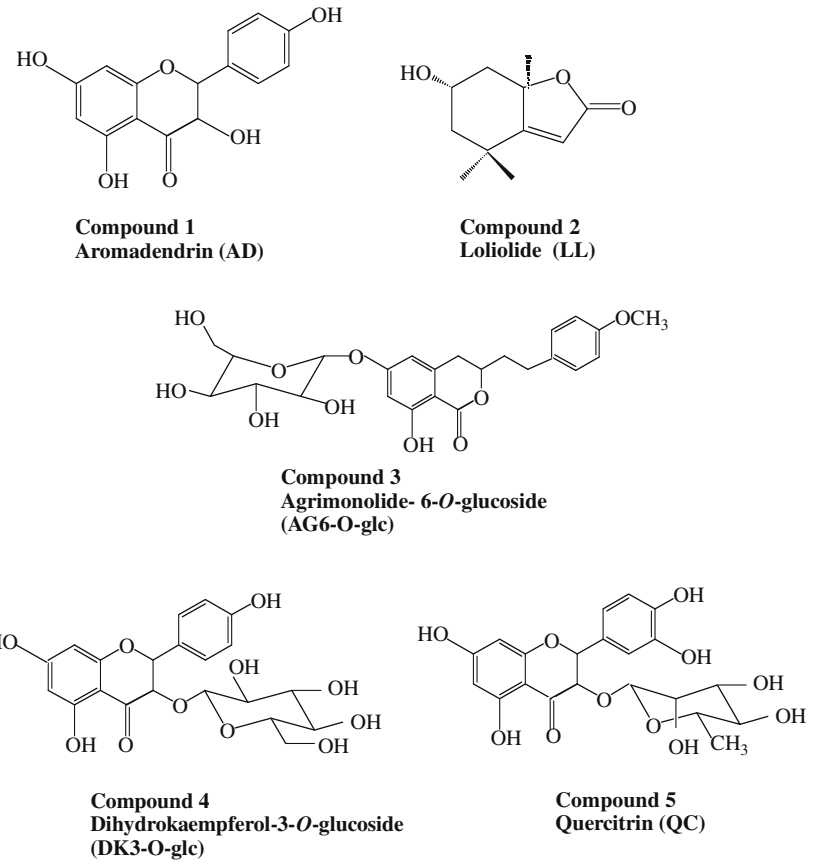

Fig. 2. Compounds isolated from *A. pilosa* Ledeb. Five phenolic compounds, compound 1 (aromadendrin, AD), compound 2 (loliolide, LL), compound 3 (aglimonolide-6-*O*-glucoside, AG6-*O*-glc), compound 4 (dihydrokaempferol-3-*O*-glucoside, DK3-*O*-glc) and compound 5 (quercitrin, QC), were isolated and determined.

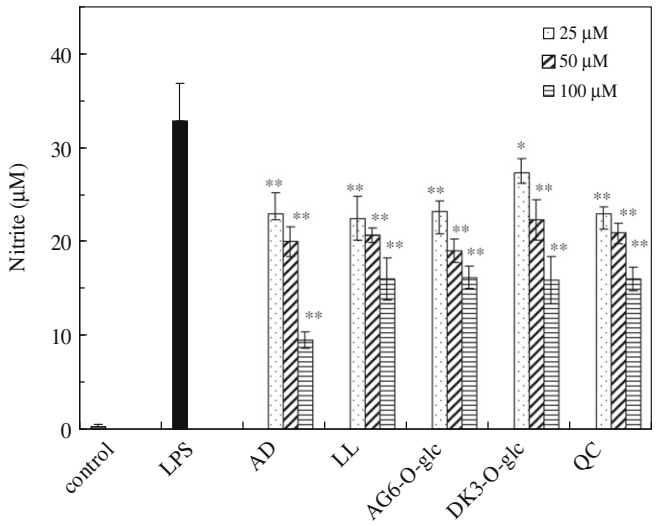

Fig. 3. Inhibition of phenolic compounds for NO production in LPS-induced RAW264.7 macrophages. The various concentrations (25, 50 and 100 μM) of aromadendrin (AD), loliolide (LL), aglimonolide-6-*O*-glucoside (AG6-*O*-glc), dihydrokaempferol-3-*O*-glucoside (DK3-*O*-glc) and quercitrin (QC) isolated from *A. pilosa* Ledeb, were evaluated for NO production in LPS-induced RAW264.7 macrophages. Data were expressed as means ± SD and the significant difference was analysed by Student's *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicated significant differences from LPS-treated cells as positive control.

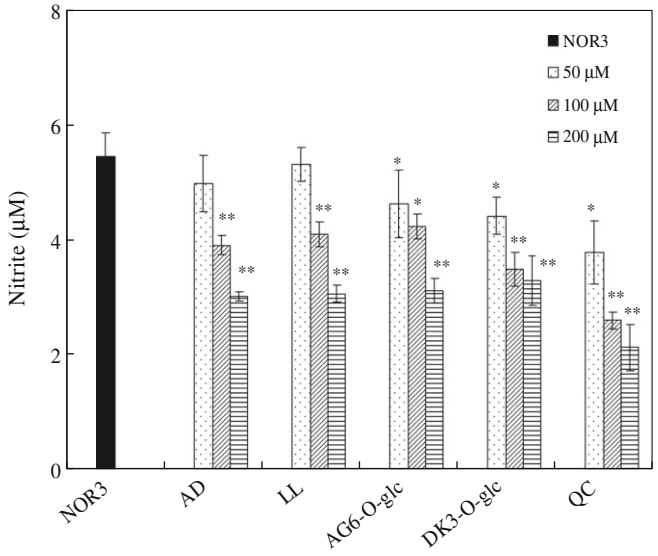

Fig. 4. NO reduction of phenolic compounds from *A. pilosa* Ledeb. The reaction mixture containing NOR3 (200 μM), with or without the test compounds aromadendrin (AD), loliolide (LL), aglimonolide-6-*O*-glucoside (AG6-*O*-glc), dihydrokaempferol-3-*O*-glucoside (DK3-*O*-glc) and quercitrin (QC) (50, 100 and 200 μM) in PBS solution, was incubated at room temperature for 60 min. The nitrite-level served as the NO indicator. Data were expressed as means ± SD and significant difference was analysed by Student's *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicated significant differences from NOR3 without test compounds.

compounds. As shown in Fig. 5, NO released from NOR3 was detected by carboxy-PTIO and it then produced carboxy-PTI radical, as indicated by the arrows in Fig. 5a and b. When a compound was present in the system, the carboxy-PTI radical was not detected, particularly AD and DK3-*O*-glc, and the QC clearly indicated NO-scavenging ability whereas the scavenging abilities of LL and AG6-*O*-glc were weak (Fig. 5c–g).

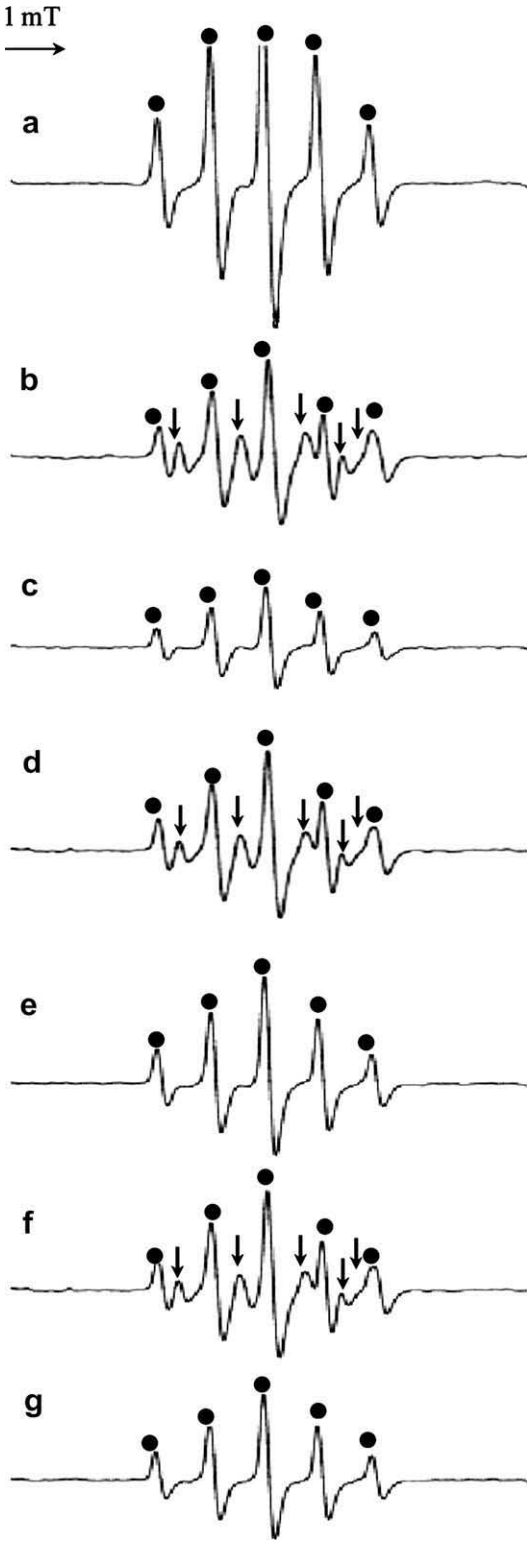

Fig. 5. NO-scavenging of phenolic compounds from *A. pilosa* Ledeb. The reaction mixture of compound (100 μM), NOR3 (200 μM) and NO detection reagent, carboxy-PTIO (25 μM) was prepared in PBS and incubated at room temperature for 30 min. (a) carboxy-PTIO (25 μM), (b) carboxy-PTIO (25 μM) and NOR3 (200 μM), and (b) with (c) aromadendrin (AD, 100 μM), (d), loliolide (LL, 100 μM), (e) dihydrokaempherol-3-*O*-glc (DK3-*O*-glc, 100 μM), (f) aglimonolide 6-*O*-glc (AG6-*O*-glc, 100 μM), and (g) quercitrin (QC, 100 μM). The carboxy-PTIO signal is indicated by the solid circle (●) and its detection with the NO-produced carboxy-PTI radical is indicated by arrows (↓). The ESR measurement was operated in the X-band with modulation frequency of 100 kHz, a 9.4 GHz resonant frequency and the following microwave powers: 4 mW; modulation width, 0.1 mT; gain, 320; scan time, 1 min; time constant, 0.3 s. Manganese oxide was used as the internal standard.

4. Discussion

The excess production of NO or peroxynitrite ($ONOO^-$), due to the reaction with superoxide in biological systems, gives rise to various diseases, such as inflammation, carcinogenesis, and atherosclerosis (Beckman & Koppenol, 1996; Pacher et al., 2007; Yu et al., 1994). The development of substances to prevent the overproduction of NO has become a new research target for treating chronic inflammatory diseases (Hobbs et al., 1999; Nakagawa & Yokozawa, 2002; Nathan, 1992; Shen et al., 2002). Recently, dietary fruits, vegetables and medicinal herbs, potentially containing various antioxidants and antinitrosive compounds, have been examined for protection against diseases causing oxidative stress (Cho et al., 2007; Katsube et al., 2004; Tsai et al., 2007). The total phenolic content in the plants has usually been found to correlate highly with the free radical-scavenging activity (Cho et al., 2007); therefore, the total amount of polyphenol is initially assessed for the plant extract, which will be a convenient way to find NO inhibitory compounds. We also initially examined the polyphenol contents or antioxidant activities of various Asian plants, showing that *A. pilosa* Ledeb, with a high polyphenol content (126 μmol-equivalents of gallic acid/g-dry weight), was a good target plant for examining the NO suppression activity in LPS-induced RAW264.7 macrophages. The extract showed NO suppression in cells, indicating that polyphenols would be responsible for the inhibitory action (Fig. 1). The compounds responsible for the NO suppression in the extract were isolated, and five phenolic compounds, including three flavonoids were determined (Fig. 2). The NO suppression abilities of these compounds were similar, suggesting that all of these compounds take part in the suppression of excess NO production in cells. Phenolic compounds are known to be free radical-scavengers and flavonoids, particularly, are effective for suppressing NO and peroxynitrite (Haenen et al., 1997; Van et al., 1995). In this study, the NO or nitrogen radical-scavenging effect was evaluated using NOR3 as a NO donor in the presence of each compound. All compounds inhibited the nitrite production in a dose-dependent manner; thus the NO-scavenging effects of these compounds can reduce excess NO production in cells (Figs. 3 and 4). The reactions between the phenolic compounds in the presence of NOR3 were monitored by LC/MS, but reaction products, such as the NO-scavenging products, or degradation products due to the NO, were not detected in the reaction (data not shown), suggesting that the redox of the NO inner molecule would occur in the reaction mixture. The specific structural feature of the phenolic compounds needed for scavenging the NO or peroxynitrite has been previously investigated. The OH-substituted functional group in the structure would be an important factor when considering the NO-scavenging mechanisms (Haenen et al., 1997; Van et al., 1995). Phenolic compounds from *A. pilosa* Ledeb would have a similar function (due to the OH substituted functional group in the molecule for NO suppression). An ESR study of the NO generation-detection system, using NOR3 and carboxy-PTIO, with or without compounds, provided evidence that phenolic compounds scavenged NO (Fig. 5). Particularly, the three flavonoids, AD, DK3-*O*-glc and QC, clearly showed more scavenging of NO than of the phenolic compounds. The NOR3/NO in the presence of carboxy-PTIO led to the formation of NO_2 which can oxidize GSH (Pfeiffer et al., 1996). A weak scavenging activity of the LL and AG6-*O*-glc in the presence of carboxy-PTIO suggested that they could scavenge NO_2 or the intermediate radicals, N_2O_3 and N_2O_4 during NO oxidation. Some flavonoids have been found to be excellent free radical-scavengers (of superoxide, NO, peroxynitrite and active oxygen species) during lipid peroxidation (Cho et al., 2007; Nakagawa & Yokozawa, 2002). Specific structural features of phenolic compounds, such as the presence of a catechol group of the B ring in a flavonoid, are definitely required for the excellent NO- or peroxynitrite-scavenging ability, and gallic acid linked to the flavan-3-ol also plays an important role in catechins (Nakagawa & Yokozawa, 2002). The hydroxyl groups of A–C in the molecule are significant functional groups for scavenging peroxynitrite. The results obtained from the ESR study were also similar to those of previous studies. The catechol group (C ring) of QC, the hydroxyl groups of the B and C rings of AD and the hydroxyl group of the B ring of DK3-*O*-glc would be responsible for the NO-scavenging. We demonstrated here the NO suppression mechanism against the overproduction of NO in LPS-induced RAW264.7 macrophages. Previous studies have demonstrated that flavonoids, such as oroxyrin A, quercetin and wogonin, showed an inhibition of NO production and PGE_2 induction through suppression of the iNOS and COX-2 protein expression in LPS-induced RAW264.7 cells (Chen, Shen, Chen, Lee, & Yang, 2001; Chen, Yang, & Lee, 2000; Číž et al., 2007). In these experiments that phenolic compounds decrease the level of NO which could involve both a decrease in NO production and NO-scavenging.

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