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Original Article

Inhibition of LPS-stimulated NO production in RAW264.7 macrophages through iNOS suppression and nitrogen radical scavenging by phenolic compounds from *Agrimonia pilosa* Ledeb

Junsei Taira[1], Wakana Ohminea[1], Hitoshi Nanbub[2], Katsuhiro Uedab[2]

[1]*Department of Bioresource Technology, Okinawa National College of Technology, Nago City;*
[2]*Department of Chemistry, Biology and Marine Sciences, University of the Ryukyus; Okinawa, Japan*

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Corresponding Author
Junsei Taira
Department of Bioresource Technology, Okinawa National College of Technology, 905 Henoko, Nago City, Okinawa 905-2192, Japan.
taira@okinawa-ct.ac.jp

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RAW264.7 macrophages

Abstract

In this study, the six phenolic compounds, such as kaempherol, catechin, glucodistylin, aglimonolide-6-*O*-glucoside and quercitrin from the extract of *Agrimonia pilosa* Ledeb inhibited nitrite accumulation as an indicator of nitric oxide (NO) in LPS-stimulated RAW264.7 macrophages. 4-ethyl-2-hydroxyamino-5-nitro-3-hexenamide (NOR3) as an NO donor was used in the presence of these compounds and the nitrite level then decreased, indicating that these compounds would potentially have nitrogen radical scavenging activity. An electron spin resonance (ESR) study of the NO generating system containing NOR3 and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) as an NO detection reagent with or without the compound provided evidence that these compounds, such as catechin, glucodistylin and hyperin, directly scavenged NO and that the presence of a catechol group on the B ring in the molecule is responsible for the NO scavenging activity. All of the tested phenolic compounds suppressed the iNOS induction in LPS-stimulated cells. Particularly, the suppression of iNOS protein expression by hyperin and quercitrin was distinct among in the examined compounds. This study demonstrated that the compounds can decrease the level of NO in the cells through involving both a decrease in NO production and a nitrogen radical-scavenging effect.

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INTRODUCTION

The production of nitric oxide (NO) is formed by NO synthases (NOSs), and three types of NOSs including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) have been identified. The function of NO has been elucidated in a variety of pharmacological conditions including inflammation, carcinogenesis, and atherosclerosis; however, the excess NO production or the peroxynitrite ($ONOO^-$) produced during reaction of NO with superoxide anion radical ($O_2{\bullet}^-$) can cause oxidative damage such as membrane lipid peroxidation, DNA fragmentation and lipoprotein oxidation [1, 2]. These reactions have functional consequences, which may be deleterious to cells and tissue [3-5]. The development of substances to prevent the overproduction of NO has become a new research target to treat chronic inflammatory diseases [6, 7].

Gram negative bacterial lipopolysaccharide (LPS) stimulates the production of pro-inflammatory mediators and proteins, such as iNOS, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and cyclooxygenase (COX-2) in various cells and causes to trigger several disadvantageous cellular damages, such as inflammation, sepsis and stroke [5, 8]. The large amount of NO production by iNOS induction has been closely correlated with the pathophysiology in a variety of diseases and inflammation. 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.

Agrimonia pilosa Ledeb is a medicinal herb which contains various polyphenols such as flavonoids, coumarins, phenolic glycosides, and these phenolic compounds have pharmacological properties of antitumor activity, antibacterial activity, acetylcholinesterase inhibitory and NO scavenging activity [9-14]. In a recent study, the *A.pilosa* Ledeb extract showed a potential anti-inflammatory activity through inhibition of NO production due to suppression of iNOS and reduction of a carrageenan-induced rat paw edema [15]. In this study, the six phenolic compounds from the extract of *A.pilosa* Ledeb inhibited NO production in LPS-stimulated inflammatory RAW264.7 cells, suggested that these compounds would contribute to anti-inflammatory effect.

MATERIALS AND METHODS

Materials

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). L-arginine and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), interferon-γ (IFN-γ) and LPS were obtained from Wako Pure Chemical Co (Osaka, Japan). Dulbecco's modified Eagle`s medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (NY, USA).

Cell culture

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.

Cell viability

The MTT assay was used to examine the cell viability due to treatment with a test sample as previously reported [14]. Briefly, the cells were seeded at a density of 5 x 10^5 cells/ml and cultured for 16 h with or without the test sample. After the culture, MTT (0.05%) was added to each well and incubated for 3 h and then the suspension was removed. Extraction with DMSO (100 µl) was obtained at 570 nm with the reference at 630 nm using a microplate reader (BIO-RAD Model 550, Philadelphia, PA, USA).

Nitrite assay

The nitrite concentration in the medium or reaction mixtures was measured as an NO indicator according to the Griess method as previously reported [16]. Briefly, the solution containing the test sample (80 µl), PBS (20 µl), 1% sulfanilamide containing 5% phosphoric acid (50 µl) and 0.1% N-(1-naphthyl) ethylenediamine (50 µl) was incubated on a 96-well plate and the absorbance at 540 nm was measured on a microplate reader.

Nitrite production on RAW264.7 macrophages

The cells (5 x 10^5 cells/well) with or without various concentrations of the compounds (25, 50 and 100 µM) in the presence of LPS (100 ng/ml), L-arginine (2 mM), and IFN- γ (100 U/ml) were cultured on a 96-well microplate. Cells with or without LPS, IFN-γ and L-arginine were used as the positive control and the control, respectively. After culturing for 16 h, the nitrite concentrations in the medium were determined by previously reported procedures [16].

Nitrogen radical scavenging activity

NOR3 as an NO donor was used to evaluate the NO or nitrogen radical scavenging effect of the compounds. The reaction mixture containing NOR3 (200 µM) with or without the test compounds (10, 50 and 100 µM) in PBS solution was incubated at room temperature for 60 min, and the nitrite accumulation in the reaction mixture was measured using the previously described procedures [16].

Extraction and isolation of compounds 1-4

A.pilosa Ledeb (dry weight 951 g) was extracted with MeOH (methanol). After filtration, the extract was concentrated in vacuum and the MeOH extract was partitioned between EtOAc (ethyl acetate, 1 l) and H_2O (1 l). The EtOAc extract (158.3 g) was suspended in 50% aqueous MeOH. The aqueous (50% MeOH) was successively extracted with hexanes and dichloromethane. The suspension was partitioned between hexane (500 ml), CH_2Cl_2 (dichloromethane, 500 ml) and n-BuOH (n-butanol, 300 ml), respectively, to give a non-polar hexane extract (16.3 g), a lipophilic CH_2Cl_2 extract (38.3 g) and a polar n-BuOH extract (36.1 g). The n-BuOH extract (36.1 g) was partitioned between 50% aqueous MeOH (500 ml) and EtOAc (200 ml). The EtOAc phase was concentrated and the extract was partitioned between 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 a silica gel-column (Merck silica gel 60, particle size 0.04-0.063 mm, 70-230 mesh, ASTM, Merck) using hexane with an increasing proportion of EtOAc to give 17 fractions. The 9th fraction (17.2 mg) was separated using an ODS-column (COAMOSIL 5C18 AR-II, 10 i.d. , 250 mm, Nacalai Tesque) with MeOH/H_2O (5/1, v/v) to give compound 1 (kaempferol, 1.2 mg). The 50% aqueous MeOH layer from the n-BuOH extract (36.1 g) was partitioned using EtOAc and extracted with n-BuOH.

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The n-BuOH extract (15.1 g) was separated by an open-column (COAMOSIL 75-C18 OPN, Nacalai Tesque) using H_2O/MeOH (3/1, v/v) with an increasing proportion of EtOAC to give 6 fractions. The first fraction (9.1 g) was separated on a Sephadex column (TOYOPEARL HW-40, TOYO SODA Mfg) with MeOH to give 14 fractions. The 5th fraction (2.1 g) among these fractions was separated by a Sephadex-column (Sephadex LH-20, Pharmacia Fine Chemicals, Uppsala, Sweden) using MeOH to give 12 fractions. The 8th fraction (1.6 g) was partitioned between EtOAC and H_2O. The compound 2 (63.9 mg, glucodistylin) was purified from the EtOAC extract (843.1 mg) with MeOH/H_2O (1/1, v/v) using HPLC on an ODS column. The 11th fraction (27 mg) was purified by HPLC on an ODS column using MeOH/H_2O (2/1, v/v) to give compound 3 (4.1 mg, catechin) and compound 4 (4 mg, hyperin).

Nuclear magnetic resonance (NMR) analysis

The ^{13}C spectra were recorded by a JEOL α-500 spectrometer (JEOL, Tokyo, Japan) and the ^{13}C chemical shifts were referenced to the solvent peaks (δ C 77.0 in $CDCl_3$).

Liquid chromatography and mass spectrometry (LC/MS) analysis

LC/MS (Agilent 1200, Agilent Technologies, Santa Clara, CA, USA), which was used to determine the molecular weight of the isolated compounds was carried out using a photodiode array detector and monitored in the operating wavelength range from 210 to 700 nm at flow rates of 0.8 ml/min on a reversed-phase chromatographic column, YMC-Pack Pro C18 (100 x 4.6 mm i.d., 5 μm particle size, YMC Co Ltd, Kyoto, Japan) at 40°C. The mobile phase, consisting of a formic acid aqueous solution (0.1%) and acetonitrile, was carried out using a linear gradient. An initial isocratic step with 0.1% formic acid/30% acetonitrile was held for 0.5 min followed by a linear gradient to 100% acetonitrile for 5.5 min and held for 2.5 min. The mass spectra were obtained under the following conditions: electrospray ionization (ESI) negative ion mode; desolvation temperature, 350°C; desolvation pressure, 35 psi; and desolvation gas flow, 12.01 ml/min (6120 Quadrupole LC/MS spectrometer, Agilent Technologies).

Electron spin resonance measurement

The NO scavenging action of the compounds was confirmed by an electron spin resonance (ESR) study as previously reported [14, 18]. The reaction mixture of the compound (25 and 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 Ltd, Tokyo, Japan) operating in the X-band with a modulation frequency of 100 kHz. The reaction mixture was transferred to a capillary (100 x 1.1 mm i.d., Drummond Scientific, Broomall, PA, USA) which was placed in a quartz cell (270 mm long, 5 mm i.o., JEOL Ltd, Tokyo, 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 sec. Manganese oxide was used as the internal standard.

Reverse transcriptase-polymerase chain reaction

LPS-stimulated RAW264.7 macrophages on a 12-well microplate (2.5 x 10^6 cells/ml) were treated with the compounds (25 μM), such as kaempherol, catechin, glucodistylin, hyperin, aglimonolide-6-*O*-glucoside (AG6-*O*-glc) and quercitrin (QC). Total RNA from cells was isolated from the cell lysate according to the manufacturer's instructions using the RNeasy Mini Kit (Invitrogen). The total of RNA concentration and quality were determined spectrophotometrically with a nanoVette microliter cell (Beckman Coulter DU800, Beckman). cDNA was synthesized using the SuperScript® Choice system as a manual procedure (Invitrogen). Amplification of the cDNA was performed by incubating in LA PCR buffer 0.1 M Tris-HCl buffer (pH 8.0, Takara Bio Inc, Shiga, Japan), containing 0.5 M KCl, 0.025 M $MgCl_2$, 250 μM dNTPs and 50 U/ml of Taq DNA polymerase with the iNOS primers: 5`-CCT TGT TCA GCT ACG CCT TC-3`and 5`-CTG AGG GCT CTG TTG AGG TC-3` using polymerase chain reaction (PCR, GeneAmp® PCR System 9700, Applied Biosystems). The following PCR condition were used; initial denaturation was performed at 94℃ for 2 min, followed by 30 thermal cycles at 94℃ for 30 sec, 60℃ for 30 sec and at 72℃ for 1 min. The PCR product of cDNA (100 ng/μl) was loaded on a DNA chip (Agilent DNA 1000 kit, Agilent Technologies) and the electrophoresis was performed by a micro DNA analyzer (Agilent 2100 Bioanalyzer, Agilent Technologies).

Western blot

After stimulation, the cells were washed with PBS, and then treated with lysis buffer. The cellular lysates were centrifuged at 13,800g at 4℃ for 5 min. The total cellular extracts were separated on SDS-polyacrylamide gels (4-12% SDS-polyacrylamide, Invitrogen) and transferred to a nitrocellulose membrane (Blot Gel Transfer Mini, Invitrogen) using an iBlot Gel Transfer Device (Invitrogen). The membrane treatment and detection of the protein expression were carried out by manual procedures using an immunodetection system (Invitrogen) while incubated with polyclonal anti-iNOS (ab3523, abcam) overnight at 4ºC.

RESULTS

Extraction and isolation of compounds

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

Compound 1 (kaempherol) *m/z* 285.1 (M-H), ^{13}C NMR (125 MHz, CD_3OD) δ: 159.3 (2), 136.2 (3) 179.6 (4) 105.9 (4a) 163.2 (5) 99.8 (6), 165.9 (7), 94.7 (8), 158.5 (8a), 122.9 (1'), 116.9 (2'), 146.4 (3'), 148.7 (4'), 116.3 (5'), 123 (6'), 103.5 (1''), 72.1 (2''), 72 (3''), 73.3 (4''), 71.9 (5''), (6'') 17.7.

Compound 2 (glucodistylin) *m/z* 465.0 (M-H), ^{13}C NMR (125 MHz, CD_3OD) δ: 83.3 (2), 77.7 (3), 196.1 (4), 102.3 (4a), 165.5 (5), 96.4 (6), 169.1 (7), 97.3 (8), 164 (8a), 128.9 (1'), 115.9 (2'), 146.9 (3'), 145.9 (4'), 116.3 (5'), 121.2 (6'), 104.6 (1''), 75.4 (2''), 77.8 (3''), 71.4 (4''), 77.8 (5''), 62.7 (6'').

Compound 3 (catechin) *m/z* 289.1 (M-H), ^{13}C NMR (125 MHz, CD_3OD) δ: 82.9 (2), 115.3 (2'), 68.8 (3), 146.2 (3'), 28.5 (4), 146.2 (4'), 100.8 (4a), 157.9 (5), 116.1 (5'), 95.5 (6), 120 (6'), 157.6 (7), 96.3 (8), 156.9 (8a), 132.2 (1')

Compound 4 (hyperin) *m/z* 463 (M-H), ^{13}C NMR (125 MHz, CD_3OD) δ: 158.7 (2), 135.8 (3), 179.5 (4), 105.4 (4a), 163 (5), 100 (6), 166.4 (7), 94.8 (8), 158.5 (8a), 122.9 (1'), 117.8 (2'), 145.8 (3'), 150 (4'), 116.1 (5'), 122.9 (6'), 105.6 (1''), 73.2 (2''), 75.1 (3''), 70 (4''), 77.2 (5''), 61.9 (6'').

Compounds 1, 2, 3 and 4 were assigned on the basis of these analytical data which were identical to kaempherol [18], glucodistylin [20], catechin [21] and hyperin [22], respectively. Their chemical structures are depicted in Fig.1.

NO inhibitory action in cells

Four flavonoids from the *A.pilosa* Ledeb extract were evaluated for their inhibitory effect on NO production in 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.2a). The cytotoxicity of these compounds was not detected in the range of the test concentrations indicating that the inhibition was due to the effect of the compounds (Fig.2b).

Nitrogen radical-scavenging effect

The nitrogen radical-scavenging activity of the compounds was examined using NOR3 as an NO donor. The nitrite accumulation from NOR3 in the presence of the compounds was reduced in a dose-dependent manner (Fig.3). These results suggested that these compounds would have a potentially NO or nitrogen radical scavenging activity. This result suggested that the effect of the radical scavenging of the compounds would contribute to inhibition of excess NO production in the LPS-induced RAW264.7 macrophages (Fig.2a).

Electron spin resonance measurement

To clarify the NO scavenging action of the compounds, an ESR study was performed on the system containing a carboxy-PTIO as an NO detection reagent in the presence of NOR3 and the compounds. As shown in Fig.4, NO released from NOR3 was detected by carboxy-PTIO and then produced a carboxy-PTI radical as indicated by the arrows in Fig.4. When a compound was present in the system, the carboxy-PTI radical was not detected for catechin and glucodistylin or was only slightly detected for hyperin, but kaempherol did not show the effect of NO-scavenging, even though a high concentration of 100 μM kaempherol was used in the system (data not shown). These results indicated that catechin, glucodistylin and hyperin can scavenge NO, resulting in a decrease in the overproduction of NO in cells.

Suppression of iNOS induction

Six phenolic compounds, including aglimonolide-6-*O*-glucoside (AG6-*O*-glc) and quercitrin from the *A.pilosa* Ledeb extract, were examined in the LPS-stimulated RAW264.7 cells. The iNOS mRNA gene expression in cells was induced due to the LPS stimulant (Fig.5a).

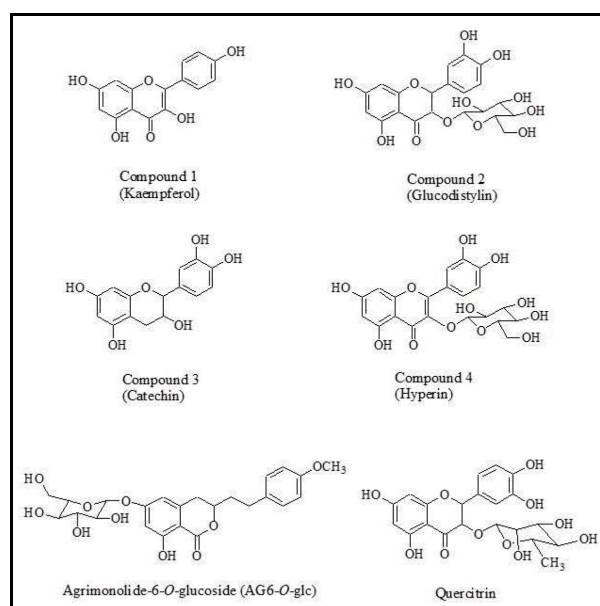

Figure 1. Phenolic compounds isolated from *A. pilosa* Ledeb; four phenolic compounds, such as compound 1 (kaempferol), compound 2 (glucodistylin), compound 3 (catechin) and compound 4 (hyperin), were isolated from the *A.pilosa* Ledeb extract in this study. Aglimonolide-6-*O*-glucoside (AG6-*O*-glc) and quercitrin were previously isolated from *A. pilosa* Ledeb [14].

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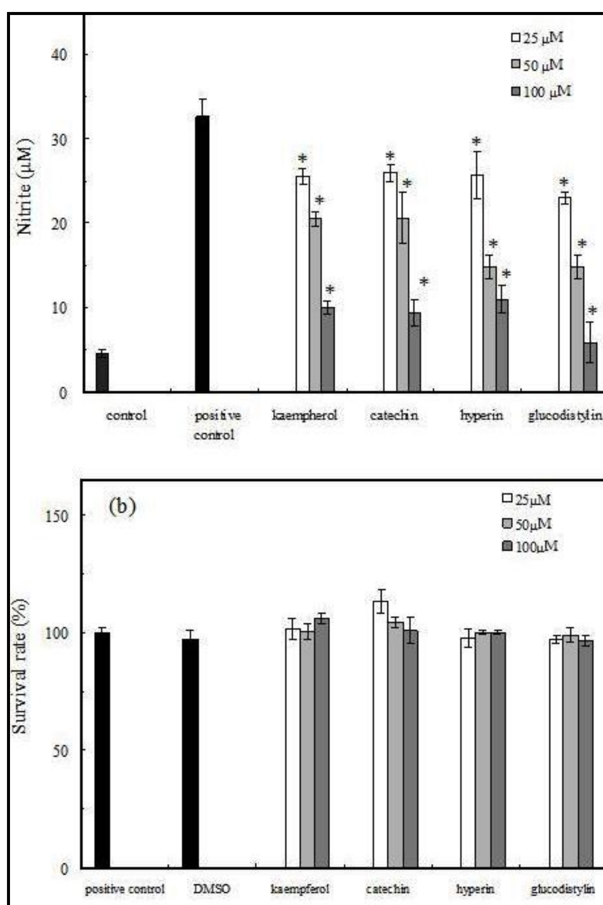

Figure 2. Inhibition of phenolic compounds for NO production in LPS-stimulated RAW264.7 macrophages; **(a)** the various concentrations (25, 50 and 100 μM) of kaempferol, catechin, hyperin and glucodistylin isolated from *A. pilosa* Ledeb were evaluated for NO production in LPS-stimulated RAW264.7 macrophages; **(b)** the cytotoxicity of the test compounds was determined by MTT assay and indicated as survival rate (%) for LPS-treated cells as the positive control. Data is expressed as mean ± SD and the significant difference was analyzed by the Student`s t-test. *P < 0.01 indicates significant difference from the positive control.

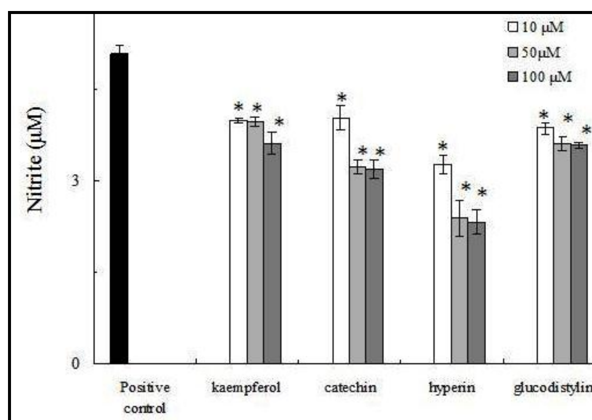

Figure 3. Nitrogen radical scavenging of flavonoids from *A.pilosa* Ledeb; the reaction mixture containing NOR3 (200 μM) with or without the test compounds of kaempferol, catechin, hyperin and glucodistylin (10, 50 and 100 μM) in PBS solution was incubated at room temperature for 60 min. The nitrite level was used as the NO indicator. Data were expressed as mean ± SD, and the significant difference was analyzed by the Student`s t-test. *P < 0.01 indicates significant difference from NOR3 without the test compounds.

When the compounds were placed in the cells, the compounds except for glucodistylin suppressed the iNOS gene expression; particularly, both hyperin and quercitrin indicated a strong inhibition of LPS-stimulated iNOS expression. The iNOS protein production was also examined in the same cell system. As shown in Fig.5b, the iNOS protein was produced in LPS-stimulated cells. When the compound was placed in the cells, the protein production was suppressed; particularly, the inhibitory effect of quercitrin was clear as well as the result of the gene expression. These results demonstrated that the phenolic compounds can decrease excess NO production in LPS stimulated cells through suppression of the iNOS expression.

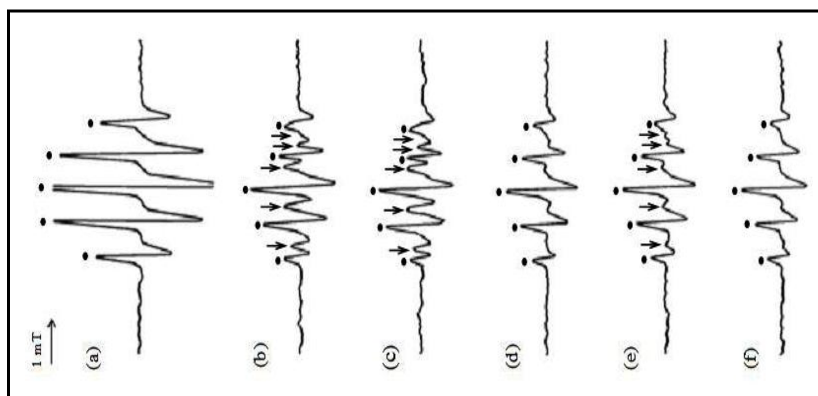

Figure 4. NO scavenging of flavonoids from *A.pilosa* Ledeb; the reaction mixture of the test compound (25 μM), NOR3 (200 μM) and NO detection reagent, carboxy-PTIO (25 μM) was prepared in PBS and incubated at room temperature for 30 min: **(a)** carboxy-PTIO, **(b)** carboxy-PTIO and NOR3, and with **(c)**, kaempferol, **(d)** catechin, **(e)** hyperin and **(f)** glucodistylin. 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 a modulation frequency of 100 kHz, a 9.4 GHz resonant frequency and the following microwave power, 4 mW; modulation width, 0.125 mT; gain, 320; scan time, 1 min; and time constant, 0.3 sec. Manganese oxide was used as the internal standard.

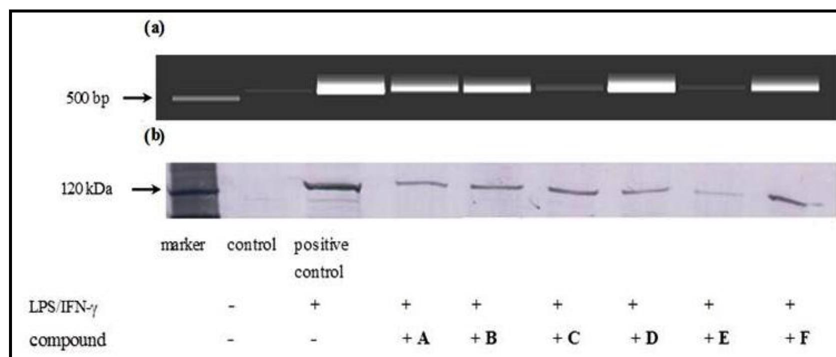

Figure 5. Inhibitory effect of the phenolic compounds from *A.pilosa* Ledeb on LPS-stimulated iNOS in RAW264.7 macrophages; the inhibitory effect of the iNOS induction due to the phenolic compounds (A, kaempferol; B, catechin; C, hyperin; D, glucodistylin; E, AG6-*O*-glc; and F, quercitrin from *A.pilosa* Ledeb extract was examined in cells. Cells were treated with (positive control) or without (control) LPS/IFN-γ; **(a)** effect of the phenolic compounds (25 μM) for the iNOS mRNA expression in LPS- stimulated RAW264.7 macrophages, and **(b)** suppression of the iNOS protein production by phenolic compounds (100 μM). A representative example of two experiments is shown.

DISCUSSION

The excess production of NO or $ONOO^-$ due to the reaction of NO with O_2^- in biological systems has given rise to various diseases, such as inflammation, carcinogenesis, and atherosclerosis [3-5]. Therefore, substances for preventing the overproduction of NO have become a new research target for preventing chronic inflammatory diseases [6, 7]. Phenolic compounds are known as free radical scavengers; particularly, flavonoids are effective for suppressing NO, $ONOO^-$ and reactive oxygen species during lipid peroxidation [22-25]. In our previous study, we reported that *A.pilosa* Ledeb, having high polyphenol content, inhibited NO production in LPS-stimulated RAW264.7 macrophages, and five phenolic compounds including flavonoids, coumarins and phenolic glycosides were identified as NO scavenging compounds [14]. In this study, the four flavonoids, kaempherol, glucodistylin, catechin and hyperin, were isolated from *A.pilosa* Ledeb, and they contributed to decreasing the NO production in cells (Fig.2). The NO or nitrogen radical scavenging effect of each flavonoid was evaluated in the NOR3/NO system (Fig.3). All tested compounds inhibited the nitrite production in a dose-dependent manner. The scavenging effects of these compounds would contribute to the reduction of NO production in cells. In the previous studies, the OH substituted functional group in the structure was reported to be an important factor in NO suppression [23-25]. An ESR study of the NO generation detection system using NOR3 and carboxy-PTIO with or without the compounds provided evidence that phenolic compounds scavenged NO (Fig.4). The specific structural feature of phenolic compounds such as the presence of a catechol group on the B ring in a molecule is definitely required for excellent NO or $ONOO^-$ scavenging ability [24]. In the present study, the five flavonoids, except for kaempherol, clearly showed a scavenging effect for NO (Fig.4). This study clearly demonstrated that the presence of a catechol group on the B ring in the structure would contribute to the NO scavenging. NOR3/NO in the presence of carboxy-PTIO led to the formation of NO_2 [26]. Thus, the kaempherol in the NOR3/NO system in the presence of carboxy-PTIO could scavenge NO_2 or the intermediate radicals, N_2O_3 and N_2O_4 during NO oxidation, resulting in decreasing the nitrite levels in cells (Fig.2).

Treatment of RAW264.7 macrophages with LPS/ IFN-γ has been shown to result in iNOS induction. The gene expression of iNOS and COX-2 is mainly regulated at the transcriptional level. Previous studies have demonstrated that flavonoids, such as oroxylin A, quercetin and wogonin, inhibited the NO production and PGE2 induction through suppression of the iNOS and COX-2 protein expression in LPS-induced RAW264.7 cells [27-28]. The phenolic compounds, such as kaempherol, glucodistylin, catechin, hyperin, AG6-*O*-glc and QC from the *A.pilosa* Ledeb extract suppressed the iNOS gene expression and the protein production in cells with LPS/IFN-γ treatment (Fig.5). The expression of iNOS in murine macrophages has been shown to be dependent on NF-κB activation and both iNOS and COX-2 gene promoters contain the transcription factors for NF-κB [29-30]. A previous study demonstrated that kaempherol was a potential inhibitor of iNOS and COX-2 induction at the level of gene transcription [31]. In the present study, the similar activity of kaempherol was indicated, and a potential nitrogen radical-scavenging activity of the compound also would contribute to the reduction of the nitrite level in LPS-stimulated cells (Figs.2&5). A previous study showed that a flavonoid, oroxylin A, inhibited the iNOS and COX-2 gene expression through blocking of NF-κB activation [27]. A recent study indicated that an electrophile, such as 1,2-naphthoquinone, suppresses the IKKb/NF-κB/NO signaling induced by LPS through covalent modification of IKKb thiols [32]. A

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possible explanation for the phenolic compounds, except for kaempherol, such the presence of a catechol group on the B ring in a molecule-mediated inhibition of such a signaling is that the phenolic compound undergoes oxidation to yield the ortho-quinoid species, such as 1,2-naphthoquinone, that is covalently bound to IKKb. Thus, the phenolic compounds having a catechol group may suppress the NO production by disrupting the NF-κB/NO signaling.

In conclusion, the phenolic compounds such as flavonoids, glycoside flavonoids and coumarins from *A.pilosa* Ledeb inhibited the overproduction of NO in LPS-stimulated inflammatory RAW264.7 macrophage cells. These compounds decreased the level of NO in cells through involving both the suppression of iNOS induction and a nitrogen radical-scavenging effect, which would contribute to the anti-inflammatory effect of *A.pilosa* Ledeb extract.

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