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Radical scavenging and anti-inflammatory activity of extracts from *Opuntia humifusa* Raf.

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Abstract

Opuntia humifusa Raf. (*O. humifusa* Raf.) is a member of the Cactaceae family. To determine the antioxidative and anti-inflammatory effects of this herb, various solvent fractions (methanol, hexane, chloroform, ethyl acetate, butanol, and water) prepared from the leaves of cacti were tested using DPPH (2,2-diphenyl-1-picrylhydrazyl radical) and xanthine oxidase assays, and nitric oxide (NO)-producing macrophage cells. We found that *O. humifusa* Raf. displayed potent antioxidative and anti-inflammatory activity. Thus, all solvent fractions, except for the water layer, showed potent scavenging effects. The scavenging effect of the ethyl acetate fraction was higher than that of the other fractions, with IC50 values of 3.6 and 48.2 $\mu\text{g mL}^{-1}$. According to activity-guided fractionation, one of the active radical scavenging principles in the ethyl acetate fraction was found to be quercetin. In contrast, only two fractions (chloroform and ethyl acetate) significantly suppressed nitric oxide production from the lipopolysaccharide (LPS)-activated RAW264.7 cells. In addition, chloroform and ethyl acetate fractions significantly blocked the expression of inducible nitric oxide synthetase (iNOS) and interleukin-6 (IL-6) from the RAW264.7 cells stimulated by LPS. Moreover, ethyl acetate fractions significantly blocked the expression of IL-1 β from the RAW264.7 cells stimulated by LPS. Therefore, the results suggested that *O. humifusa* Raf. may modulate radical-induced toxicity via both direct scavenging activity and the inhibition of reactive species generation, and the modulation of the expression of inflammatory cytokines. Finally, *O. humifusa* Raf. may be useful as a functional food or drug against reactive species-mediated disease.

Introduction

The prickly pear (*Opuntia humifusa* Raf.) is a member of the Cactaceae family. It is widely distributed in semi-arid countries throughout the world, especially in the Mediterranean area and Central America (Goldstein & Nobel 1994; Acuna et al 2002; Lee et al 2002). In Korea, *O. humifusa* Raf. has been cultivated for a long time. It, however, is different from *O. ficus-indica* var. *saboten* in several ways. Firstly, *O. humifusa* Raf. can be cultivated during a Korean winter, even with temperatures reaching below -20°C (Goldstein & Nobel 1994). Secondly, *O. ficus-indica* var. *saboten* can grow to be more than 1 m, whereas *O. humifusa* Raf. usually grows near the surface of ground.

Concerning the pharmacological profile in *Opuntia* spp., the total phenols in an ethanolic extract from South Korean *O. ficus-indica* var. *saboten* were responsible for the radical scavenging activity towards superoxide and hydroxyl anions (Lee et al 2002; Dok-Go et al 2003). In addition, an ethanol extract of *O. ficus-indica* var. *saboten* showed analgesic effects in the abdominal constriction test induced by acetic acid, and an anti-inflammatory effect against gastric lesions (Park et al 1998). Bwititi et al (2000) demonstrated that administration of leaf extracts of *O. megacantha* not only reduced blood glucose levels, but resulted in a weight decrease in diabetic animals.

Whereas *O. ficus-indica* var. *saboten* and other *Opuntia* spp. have been extensively studied for their biological effects, such as their therapeutic properties against arthritis and cancer (Park & Chun 2001; Butera et al 2002; Galati et al 2002; Lee et al 2002; Dok-Go et al 2003;

de La Barrera & Nobel 2004; Wiese et al 2004), nothing is known about the pharmacological efficacy of *O. humifusa* Raf.

The role of reactive oxygen species (ROS) has been determined in many human degenerative diseases, including ageing, cancer, arthritis, and Parkinson's disease (Hogg 1998; Droge 2002; Inoue et al 2003; Carreras et al 2004). For example, hydrogen peroxide (H_2O_2), a prominent ROS, caused lipid peroxidation and DNA damage in cells (Halliwell & Aruoma 1991). The antioxidant action of some natural compounds, such as vitamins and minerals, polyphenols and other non-nutrient compounds of plants, inhibiting the generation of reactive oxygen species or the scavenging of free radicals, was believed to be beneficial for human health (Braca et al 2002; Badami et al 2003).

Indeed, natural antioxidants have displayed a wide range of pharmacological activity, such as anticancer, anti-inflammatory and anti-ageing actions (Mates et al 1999; Noguchi & Niki 2000; Mayne 2003; Pinnell 2003). In this study, we have examined whether *O. humifusa* Raf was able to modulate free radical generation or pro-inflammatory responses.

Materials and Methods

Reagents

Ascorbate, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), lipopolysaccharide (LPS), Griess reagent, and xanthine oxidase were obtained from the Sigma Co (St Louis, MO). Xanthine was from the Merck Co. (Milwaukee, WI, USA). All other reagents were of the first grade.

Solvent extract and isolation of active ingredients

O. humifusa Raf. was collected in October from the province of Asan (Korea). The powder (100 g) from the leaf of *O. humifusa* Raf. was extracted with 0.7 L methanol overnight. The methanol extract was subsequently filtered through filter paper (Whatman No. 3) and centrifuged at 5000 g for 10 min. The filtrate was evaporated to dryness in a Rotavapor (yield 13.64 g (19.5%)). The methanol extract was successively extracted except for 0.62 g of extract, which was suitable for a methanol fraction, by using hexane, chloroform, ethyl acetate, n-butanol and water. The yield of extraction by hexane, chloroform, ethyl acetate, n-butanol, and water was 9.2%, 2.6%, 1.2%, 3.9%, and 83.1%, respectively. The crude extracts were stored at -20°C until use.

For identification of the ingredient having the strongest antioxidant activity, we purified the ingredients. Air-dried whole plants of *O. humifusa* Raf. (3 kg) were percolated with MeOH at 25°C for three weeks. The residue obtained after removal of the solvent (48 g) was diluted with H_2O (1 L) and extracted with EtOAc (1 L \times 3). The EtOAc extract (6 g) was chromatographed on a column of Si gel with $CHCl_3$ and MeOH mixtures of increasing polarity. The active fractions were further purified by Sephadex LH-20 column chromatography using MeOH- H_2O (8:2) as the solvent system. Final purification was effected using HPLC (C18 column) with an

acetonitrile- H_2O gradient solvent system. Active fractions were then pooled, evaporated, and re-dissolved in water for LC/MS. Quercetin and its derivatives were structurally identified by ESI-LC-MS/MS (Finnigan LCQ Advantage MAX ion trap mass spectrometer (San Jose, CA, USA)) and 1H NMR (CD_3OD , 300 MHz), and ^{13}C NMR (CD_3OD , 75 MHz) analysis.

Cell culture

RAW264.7 cells were maintained in DMEM supplemented with 100 U mL^{-1} penicillin and $100\ \mu\text{g mL}^{-1}$ streptomycin, and 5% fetal bovine serum. Cells were grown at 37°C and 5% CO_2 in humidified air.

DPPH radical scavenging activity

The DPPH assay measured hydrogen atom (or one electron) donating activity and hence provided an evaluation of antioxidant activity due to free radical scavenging. DPPH, a purple-coloured stable free radical, was reduced into the yellow-coloured diphenylpicryl hydrazine. The Blios method was used with slight modifications in this experiment (Blois 1958). Each solvent fraction of *O. humifusa* Raf. was tested at a concentration of $50\ \mu\text{g mL}^{-1}$. For the ethyl acetate and chloroform fractions, dose-dependency (ethyl acetate fraction; between 1 and $500\ \mu\text{g mL}^{-1}$, chloroform fraction; between 1 and $1000\ \mu\text{g mL}^{-1}$) was evaluated.

The fresh batch of a radical stock solution was prepared daily. The electron-donating activity (EDA) described the difference of absorbance between the mixture and the control solution as a percentage: $EDA (\%) = (\text{the absorbance of the control} - \text{the absorbance of the mixture}) / \text{the absorbance of the control} \times 100$.

Assay for inhibition of xanthine oxidase activity

The activity of xanthine oxidase with xanthine, as a substrate, was measured spectrophotometrically by using the procedures of Noro et al (1983), with the following modifications. The final concentration of xanthine oxidase was $250\ \mu\text{U mL}^{-1}$ in 0.1 mM phosphate buffer (pH 7.4). Each solvent fraction of *O. humifusa* Raf. was tested at a concentration of $50\ \mu\text{g mL}^{-1}$, and in the case of an ethyl acetate or chloroform fraction, dose-dependency (ethyl acetate fraction; between 1 and $40\ \mu\text{g mL}^{-1}$, chloroform fraction; between 1 and $500\ \mu\text{g mL}^{-1}$) was evaluated. Xanthine and xanthine oxidase were mixed in cuvette with either compounds tested or vehicles. The difference of absorbance was measured at 295 nm for 3 min and the enzyme activity was calculated with references: $(\text{the activity of control} - \text{the activity of the mixture}) / (\text{the activity of control}) \times 100$.

Measurement of nitrite

To determine the concentration of nitric oxide, nitrite (NO_2^-) was measured using the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid), as described by Hong et al (2003). After pre-incubation of the RAW 264.7 cells (1×10^6 cells mL^{-1}) for 18 h, the cells were incubated with 50 or $100\ \mu\text{g mL}^{-1}$ of

various extracts of *O. humifusa* Raf. with LPS ($1 \mu\text{g mL}^{-1}$) for 24 h. Supernatant ($100 \mu\text{L}$) from each well of the cell culture plates was transferred into 96-well microplates. The supernatant was then mixed with an equal volume of Griess reagent at room temperature. The absorbance at 550 nm was determined in a Spectramax 250 microplate reader. The concentration of nitrite was calculated from regression analysis using serial dilutions of sodium nitrite as a standard. The percentage inhibition was calculated based on the ability of extracts to inhibit nitric oxide formation by cells compared with the control (cells in media without extracts containing triggering agents and DMSO), which was considered as 0% inhibition.

MTT assay for the measurement of cell proliferation

A cell proliferation assay was performed to exclude the possibility that the observed nitric oxide inhibition was falsely positive due to cytotoxic effects. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Hong et al (2003). All experiments for the measurement of nitric oxide inhibition were conducted three times, each time with three independent observations, and the results were averaged.

Extraction of total RNA

Total RNA from the LPS-treated RAW264.7 cells were prepared by adding Easy blue Reagent (iNtRON Biotechnology Co., Korea), according to the manufacturer's protocol. The total RNA solution was stored at -70°C until used.

Semi-quantitative RT-PCR amplification

Semi-quantitative RT reactions were carried out using RT premix (Bioneer Co., Korea). Briefly, total RNA ($2 \mu\text{g}$) were incubated with oligo-dT₁₈ at 70°C for 5 min and cooled on ice for 3 min, and for 90 min after the addition of RT premix at 42.5°C . The reactions were terminated at 95°C for 5 min for the inactivation of reverse transcriptase. The PCR reaction was further conducted using the PCR premix (Bioneer Co., Korea) with the appropriate sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense primer, 5'-CAC TCA CGG CAA ATT CAA CGG C-3'; antisense primer, 5'-CCT TGG CAG CAC CAG TGG ATG CAG G-3'), inducible nitric oxide synthetase (iNOS; sense primer, 5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3'; antisense primer, 5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3'), tumour necrosis factor- α (TNF- α ; sense primer, 5'-TTG ACC TCA GCG CTG AGT TG -3'; antisense primer, 5'-CCT GTA GCC CAC GTC GTA GC-3'), interleukin-1 β (IL-1 β ; sense primer, 5'-CAG GAT GAG GAC ATG AGC ACC-3'; antisense primer, 5'-CTC TGC AGA CTC AAA CTC CAC-3'), and IL-6 (sense primer, 5'-GTA CTC CAG AAG ACC AGA GG-3'; antisense primer, 5'-TGC TGG TGA CAA CCA CGG CC-3') under incubation conditions (a 45-s denaturation time at 94°C , an annealing time of 45 s at $55\text{--}60^\circ\text{C}$, an extension time of 45 s at 72°C , and final extension of 10 min at 72°C at the end of the cycles). The PCR products were separated in 1% agarose

using electrophoresis of BioRad Co. The relative intensity was calculated using Eagle eyes image analysis software (Stratagene Co., La Jolla). The resulting densities of the iNOS, TNF- α , IL-1 β , and IL-6 bands were expressed relative to the corresponding densities of the GAPDH bands from the same RNA sample. GAPDH, a housekeeping gene, was used as RNA internal standard.

Statistical analysis

Student's *t*-test and two-way analysis of variance were used to determine the statistical significance of differences between values for the experimental and control groups. Data represent the means \pm s.e.m. of three experiments conducted in triplicate. *P* values of 0.05 or less were considered as statistically significant.

Results and Discussion

Antioxidant activity of various extracts of *O. humifusa* Raf.

To compare the antioxidant capacity of leaf, root, and fruit of *O. humifusa* Raf., the antioxidant activity of water extracts of leaf, root and fruit was measured using a xanthine oxidase assay. One-hundred $\mu\text{g mL}^{-1}$ of each extract showed antioxidant activity without any significant differences between the samples tested (Figure 1). Therefore, we used the leaf of *O. humifusa* Raf. for the following experiments.

Of the various solvent fractions tested, the ethyl acetate fraction from *O. humifusa* Raf. showed the highest antioxidative effect on xanthine oxidase assay with an IC₅₀ value of $3.6 \pm 0.1 \mu\text{g mL}^{-1}$ (Table 1). The chloroform fraction from *O. humifusa* Raf. showed the lowest antioxidative effect with

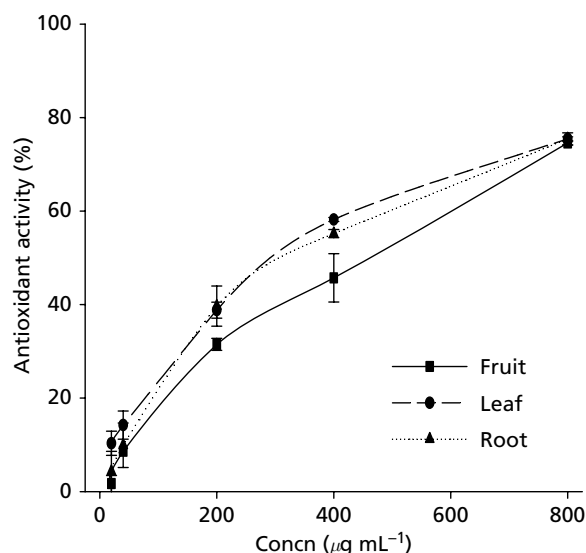


Figure 1 Antioxidant activity of water extracts of root, fruit, and leaf of *O. humifusa* Raf. in the xanthine oxidase assay.

Table 1 Antioxidant activity of various solvent extracts from *O. humifusa* Raf. in the xanthine oxidase (XO) assay and DPPH assay. Phosphate buffer (0.1 mM, pH 7.4, for XO assay) or acetate buffer (10 mM, pH 5.5, for DPPH assay) was mixed with the various solvent extracts (50 $\mu\text{g mL}^{-1}$), and assay was carried out as described in Materials and Methods

Solvent extract	Activity at 50 $\mu\text{g mL}^{-1}$		IC50 ($\mu\text{g mL}^{-1}$)	
	XO assay	DPPH assay	XO assay	DPPH assay
Methanol	61.9 \pm 3.3	11.3 \pm 0.5	—	—
Chloroform	64.5 \pm 3.1	25.1 \pm 0.6	32.0 \pm 3.2	247.5 \pm 1.8
Hexane	56.8 \pm 1.1	8.2 \pm 0.3	—	—
Ethyl acetate	88.3 \pm 2.1	74.5 \pm 1.4	3.6 \pm 0.1	48.0 \pm 1.1
Butanol	67.4 \pm 2.6	4.8 \pm 0.3	—	—
Water	9.2 \pm 5.8	0.1 \pm 0.4	—	—
Quercetin			97.0 \pm 5.1 nM	23.5 \pm 8.1 μM

Each value is the mean \pm s.e. of three determinations, performed in duplicate.

an IC50 value of 32.0 \pm 3.2 $\mu\text{g mL}^{-1}$. Standard compounds (ascorbic acid and caffeic acid) displayed scavenging activity with IC50 values of 180 and 152 μM , respectively, similar to a previous report (Kweon et al 2001).

The DPPH assay was used to confirm the scavenging effect of *O. humifusa* Raf. (Aruoma et al 1997). As shown in Table 1, the ethyl acetate extract from *O. humifusa* Raf. highly scavenged the radical generation with an IC50 of 48 \pm 1.1 $\mu\text{g mL}^{-1}$ comparable with that of ascorbic acid (42 $\mu\text{g mL}^{-1}$). The scavenging activity (e.g. IC50 of 247.5 \pm 1.8 $\mu\text{g mL}^{-1}$) of the chloroform fraction from *O. humifusa* Raf. in the DPPH assay was less than with the ethyl acetate fraction, as in the case of the xanthine oxidase assay.

According to activity-guided fractionation, we found that quercetin was one of the powerful antioxidative ingredients in the ethyl acetate fraction, with IC50 values of 97.5 nM (xanthine-xanthine oxidase assay) and 23.5 μM (DPPH assay), respectively (Table 1). Due to this compound, it may be suggested that the radical scavenging effect of the ethyl acetate fraction was more potent in the xanthine-xanthine oxidase assay (IC50 = 3.6 $\mu\text{g mL}^{-1}$) than in the DPPH assay (IC50 = 48.2 $\mu\text{g mL}^{-1}$). In addition, we found that a minor quantity of taxifolin (e.g. dihydroquercetin) was contained in the chloroform extract of the cacti, using LC-ESI-MS analysis (data not shown). Furthermore, we examined the total phenolic content of each fraction. We found that the phenolic content of the ethyl acetate fraction was approximately four times greater than that of the chloroform fraction (Lee & Rhee, unpublished results). This suggested that quercetin and its derivatives played a major role in the antioxidative effects of the cacti. Some flavonoids (quercetin, (+)-dihydroquercetin, quercetin 3-methyl ether, rutin, and isorhamnetin) have been found in the ethyl acetate fraction from the prickly pears of *O. ficus-indica* var. *saboten* (Butera et al 2002; Dok-Go et al 2003; Galati et al 2003). Judged by the inhibition of lipid peroxidation, the antioxidative effects may be mediated by these flavonoid compounds.

The other possibility is that the betalain pigments (e.g. betanin and indicaxanthin) take a role in scavenging activity of the cactus, since it has been reported that these pigments from the methanol extract of the prickly pear fruit of *O. ficus-*

indica var. *saboten* have a marked antioxidant activity (Butera et al 2002).

Effects of *O. humifusa* Raf. on the production of nitric oxide

The solvent fractions from *O. humifusa* Raf. exhibited potent antioxidant effects, and so we investigated whether *O. humifusa* Raf. was capable of modulating a ROS-mediated pathological inflammatory response, such as LPS-mediated pro-inflammatory activity by macrophages. Indeed, most antioxidants such as quercetin and *N*-acetyl-L-cysteine showed modulatory effects on the LPS-mediated cytokine production and release of inflammatory mediators such as NO and prostaglandin E₂ (PGE₂) (Gerhauser et al 2003; Saha et al 2004). We found that quercetin isolated from the ethyl acetate fraction of *O. humifusa* Raf. also showed an IC50 of 12.5 μM in LPS-stimulated NO production of RAW 264.7 cells, suggesting that it may be one of the active NO inhibitory principles of the ethyl acetate fraction.

To exclude the possibility that the inhibitory effect of extract of *O. humifusa* Raf. in NO production was due to the cytotoxic effect of the extract in itself, we carried out a cell viability test using the MTT assay. As shown in Figure 2A, methanol, hexane, ethyl acetate, butanol, and water fractions did not show cytotoxicity, even at 100 $\mu\text{g mL}^{-1}$. The chloroform fraction, however, was slightly cytotoxic (25%) at 100 $\mu\text{g mL}^{-1}$. Figure 2a shows that the methanol, hexane, chloroform, and ethyl acetate extracts of *O. humifusa* Raf. inhibited NO production. Moreover, 100 $\mu\text{g mL}^{-1}$ of the chloroform extract almost completely inhibited LPS-induced NO production and cytokine release. On the other hand, 1 $\mu\text{g mL}^{-1}$ LPS induced cell toxicity to macrophages via the NO generation and similarly the NO inhibitor, *N*-nitro-L-arginine methyl ester, strongly blocked LPS-induced cytotoxicity (data not shown). It seemed that co-exposure to ethyl acetate or chloroform fraction and 1 $\mu\text{g mL}^{-1}$ LPS blocked LPS-induced cytotoxicity. The ethyl acetate fraction of *O. humifusa* Raf. may contain cell-protective components. It is well known that NO rapidly and spontaneously reacts with a superoxide anion (O₂⁻) to form a peroxynitrite anion (ONOO⁻), which is more toxic to biological systems than O₂⁻ or NO, by causing a

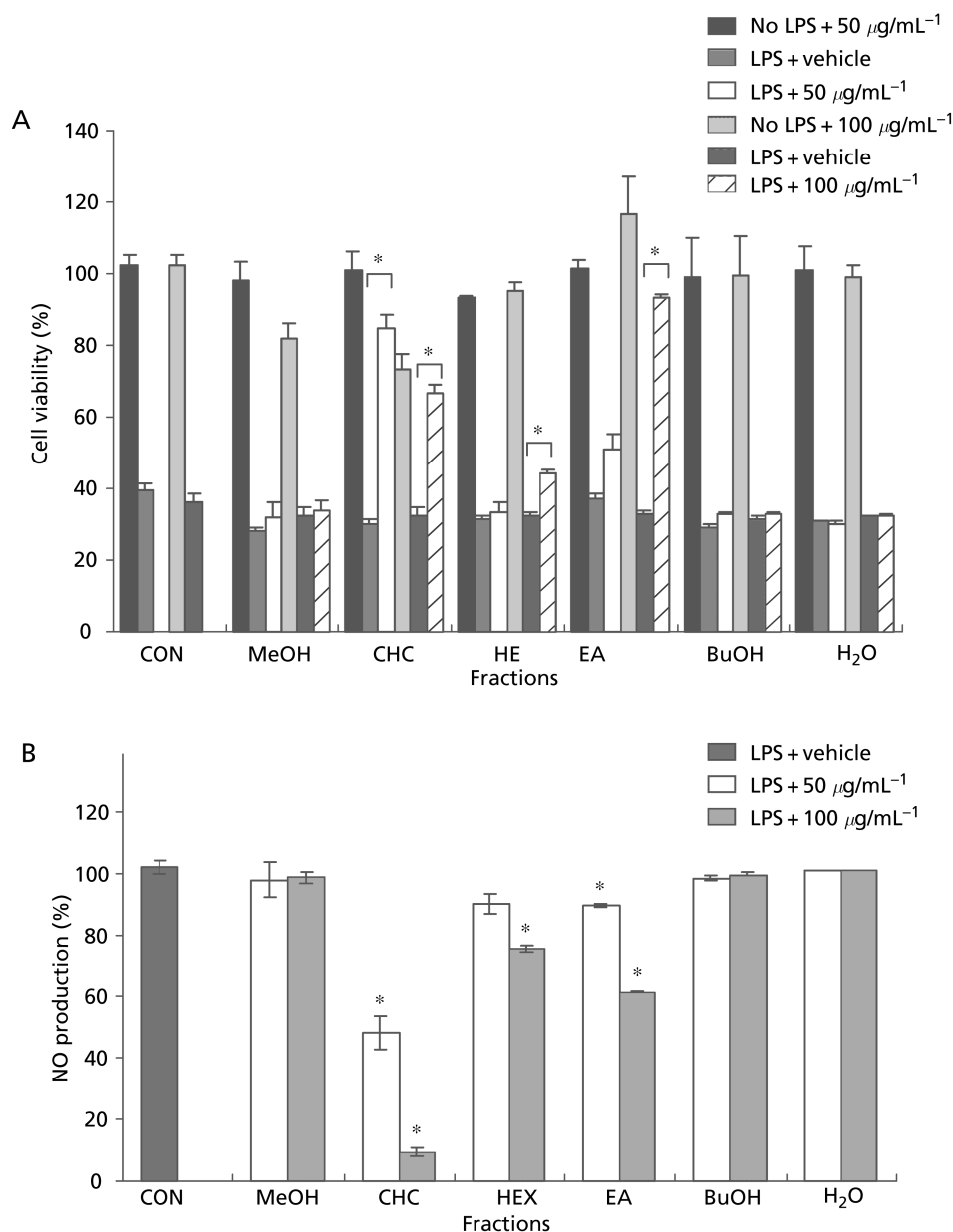


Figure 2 Effect of various extracts of *O. humifusa* Raf. on the cytotoxicity (A) and NO production (B) of RAW 264.7 cells (1×10^6 cells mL^{-1}). A. RAW 264.7 cells (1×10^6 cells mL^{-1}) were incubated with either various extracts of *O. humifusa* Raf., LPS ($1 \mu\text{g mL}^{-1}$), or *O. humifusa* Raf. and LPS ($1 \mu\text{g mL}^{-1}$). B. RAW 264.7 cells (1×10^6 cells mL^{-1}) were stimulated by LPS ($1 \mu\text{g mL}^{-1}$) and incubated with methanol (MeOH), hexane (HEX), chloroform (CHC), ethyl acetate (EA), n-butanol (BuOH), and water extracts (H₂O) of *O. humifusa* Raf. Each value is the mean \pm s.e.m. of three independent experiments performed in triplicate. * $P < 0.05$.

modification of proteins or DNA damage (Kim et al 2000). We assumed that some components, having radical scavenging activity, played an important role in protecting the cell from NO-induced cell toxicity by directly scavenging the peroxynitrite anion (ONOO⁻). In addition, flavonoids (e.g. quercetin and rutin) and nonflavonoids (e.g. caffeic and its derivatives), which are distributed in many medicinal plants (e.g. *Opuntia ficus-indica*), are known to inhibit the expression of iNOS through modulation of NF κ B signalling (Shen et al 2002; Song et al 2002; Dok-Go et al 2003; Shin et al

2004). They probably have a role in the cytoprotective effects against LPS-induced cytotoxicity. We assumed that extracts of *O. humifusa* Raf. might have contained a cell-protective component through the inhibition of NO production in immune cells such as RAW 264.7 cells, which would be either radical scavenger or direct inhibitor of the NOS gene.

The production of NO may not only be a marker of immune activation, but may actually contribute to the pathogenesis of a disease as a modulator of cell physiology. In this case, mechanisms by which NO may exert such functions

likely include modulation of apoptotic cell death and the regulation of gene expressions. Among others, it has been demonstrated that NO has the capability to upregulate factors such as the tumour necrosis factor- α (TNF- α), IL-8 and iNOS itself. We, therefore, intended to determine whether the chloroform and ethyl acetate extracts from *O. humifusa* Raf. regulated the expression of inflammatory-related genes, such as iNOS, TNF- α , IL-1 β and IL-6.

Effects of *O. humifusa* Raf. on the expression of inflammatory cytokines produced by LPS-stimulation

The chloroform and ethyl acetate fractions of *O. humifusa* Raf. inhibited the production of NO stimulated with LPS ($1 \mu\text{g mL}^{-1}$); therefore, we determined whether both fractions were capable of negatively regulating the iNOS gene in murine macrophages (RAW264.7). As expected, both fractions potently abrogated the LPS-induced iNOS upregulation with a concentration of 50 and $100 \mu\text{g mL}^{-1}$ (Figure 3A, B). We next examined whether both fractions could modulate the

expressions of the TNF- α genes. Neither the chloroform nor ethyl acetate fraction inhibited the expression of TNF- α genes even at a concentration of $100 \mu\text{g mL}^{-1}$ (Figure 3A, B). It was interesting to note that the ethyl acetate extract potently inhibited the LPS-induced upregulation of the IL-1 β genes but the chloroform fraction did not (Figure 3A, B). On the other hand, both fractions modulated the LPS-stimulated upregulation of IL-6 genes at the different potencies (Figure 3A, B). The extent of the inhibition of chloroform extracts against the LPS-induced IL-6 gene upregulation was higher than that of the ethyl acetate extracts. Different fractions of *O. humifusa* Raf. differentially regulated the expression of pro-inflammatory cytokines such as IL-1 β and IL-6, suggesting that different active ingredients of each extract played an important role in the development of anti-inflammation.

Conclusions

We tested whether various extracts of *O. humifusa* Raf. showed antioxidant activity by using the xanthine oxidase assay and DPPH assay. In both assays, the ethyl acetate extract showed potential antioxidant activity. In addition, some fractions of *O. humifusa* Raf. showed nitric oxide inhibitory activity as well as cell protective activity against LPS-induced cytotoxicity. Moreover, the chloroform and ethyl acetate fractions of *O. humifusa* Raf. differentially modulated the expression of the iNOS, IL-1 β and IL-6 genes. According to activity-guided fractionation by radical scavenging activity assays, quercetin and its derivatives (e.g. taxifolin) seemed to be active principles in the ethyl acetate and chloroform fractions.

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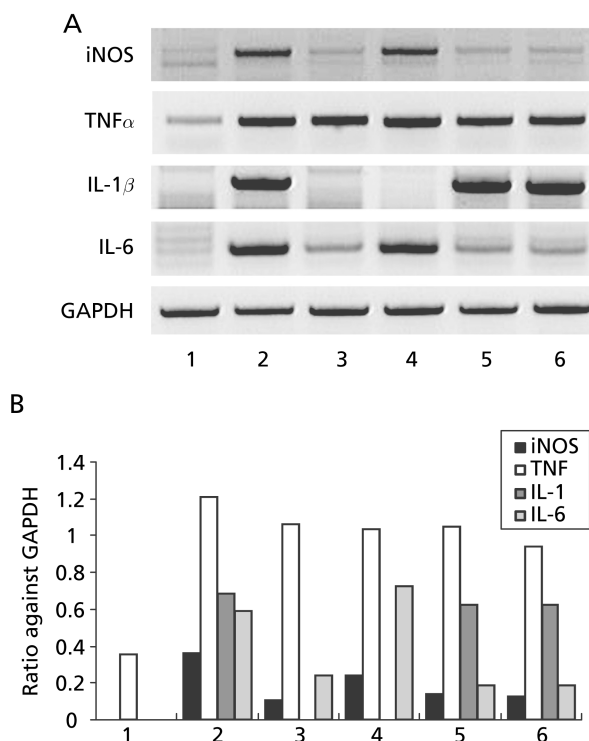


Figure 3 Effects of various extracts of *O. humifusa* Raf. on the mRNA expression of pro-inflammatory cytokines in LPS-activated RAW264.7 cells. The mRNA levels of iNOS, TNF- α , IL-1 β , IL-6 and GAPDH from the RAW264.7 cells were determined by semi-quantitative RT-PCR as described in Materials and Methods. The figures present the representative results from three separate experiments, which gave similar results. Lane 1, none; 2, LPS; 3, LPS+ $100 \mu\text{g mL}^{-1}$ ethyl acetate; 4, LPS+ $50 \mu\text{g mL}^{-1}$ ethyl acetate; 5, LPS+ $100 \mu\text{g mL}^{-1}$ chloroform; 6, LPS+ $50 \mu\text{g mL}^{-1}$ chloroform. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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