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Research Paper

Anti-inflammatory potential of *Phaseolus calcaratus* Roxburgh, a oriental medicine, on LPS-stimulated RAW 264.7 macrophages

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Abstract

Objectives The seed of *Phaseolus calcaratus* Roxburgh (PHCR) has traditionally been used as a herbal medicine, considered to have anti-inflammatory potential. Here we examined the ability of PHCR seed extract to inhibit inflammatory responses of macrophages to bacterial toxin and the mechanism involved.

Methods In the present study, we prepared four fractions from an ethanol extract of PHCR seed and investigated their effects on the production of nitric oxide and cytokines, and the expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells.

Key findings The fractions inhibited LPS-induced nitric oxide production and cyclooxygenase-2 (COX-2) expression in the cells. The ethyl acetate fraction at 100 μg/ml almost completely suppressed NO production, iNOS and COX-2 expression, and TNF-α and IL-6 secretion in cells stimulated with LPS. The fraction also inhibited phosphorylation of extracellular signal-regulated kinase (ERK) and p38 in LPS-stimulated cells with the attendant suppression of IκBα nuclear translocation and nuclear factor (NF)-κB activation. Furthermore, PHCR seed extracts contained a large number of phenolic compounds having antioxidant potentials against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and hydroxyl radicals. We identified catechin-7-O-β-D-glucopyranoside as one of the active compounds responsible for the biological activity of PHCR seed extract.

Conclusions These results suggest for the first time that ethanol extracts from PHCR seed have anti-inflammatory potential on LPS-stimulated macrophages through the down-regulation of ERK/p38- and NF-κB-mediated signalling pathways.

Keywords anti-inflammation; lipopolysaccharide; mitogen-activated protein kinase; nuclear factor-κB; *Phaseolus calcaratus* R

Introduction

Nitric oxide (NO) is produced in macrophages after enzymatic reaction of inducible nitric oxide synthase (iNOS).[1] The expression of iNOS is increased in response to various pro-inflammatory cytokines including interferon-γ (IFN-γ), interleukin-6 (IL-6), IL-1β and tumour necrosis factor-α (TNF-α).[2,3] Over-production of NO by iNOS is closely involved in the pathogenesis of numerous diseases such as carcinogenesis, septic shock, rheumatoid arthritis and autoimmune diabetes.[1,4,5] Therefore, a direct and/or indirect modulation of macrophage-mediated NO production may reduce these inflammatory diseases.

Lipopolysaccharides (LPS) and macrophages are widely used as a model system for investigation of inflammatory responses, where LPS signals macrophages to produce a variety of inflammatory mediators through activation of mitogen-activated protein kinases (MAPKs).[6] Among MAPKs, extracellular signal-regulated kinase (ERK) and p38 are well known to be related to the up-regulation of LPS-stimulated iNOS induction and NO pro-

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duction in macrophages.[7] MAPKs also induce the activation of nuclear factor-κB (NF-κB), which is involved in the expression of pro-inflammatory genes.[8]

As preventive and therapeutic measures to deal with inflammatory diseases become more important, many investigators have been focusing their efforts on searching for anti-inflammatory materials from natural resources. Resveratrol and various polyphenolic compounds have been reported to possess anti-inflammatory potential.[9,10]

Adzuki beans such as *Phaseolus angularis* Wight and *Phaseolus calcaratus* Roxburgh (PHCR) are common legumes used in the daily diet in China and Korea.[11] The seed of PHCR is also used as a traditional medicine to treat inflammatory diseases in Korea. To date, however, there is no scientific basis to demonstrate the anti-inflammatory activity of PHCR seed or to indicate the mechanism by which it exerts this activity.

In this study, we examined the effects of ethanol extracts of PHCR seed on NO production, iNOS and cyclooxygenase (COX) expression, and cytokine secretion in LPS-stimulated RAW 264.7 macrophage cells. We also investigated the mechanism involved in the anti-inflammatory action of the extracts. Our present findings demonstrate that PHCR seed exerts an anti-inflammatory action on LPS-stimulated macrophages and has catechin-7-O-β-D-glucopyranoside as one of its major active compounds.

Materials and Methods

Chemicals and laboratory wares

Unless otherwise specified, chemicals and laboratory wares were purchased from Sigma Chemical Co. (St Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively.

Preparation of PHCR seed extracts

The dried PHCR seed was obtained from a traditional herbal market, located in Jeonju (South Korea) and identified by Dr H.K. Cho, director of the Center for Healthcare and Technology Development, HanPoong Pharmaceutical Co. Ltd (South Korea). A voucher specimen (HP-PHCRS) was deposited at the centre. Briefly, the dried seed (5.5 kg) was extracted with 50% ethanol by shaking at 20°C for 2 h, three times. The extract was concentrated using a rotary vacuum evaporator (Rotavapor R110, Büchi, Switzerland) and was then lyophilized to give a crude power (756 g). The ethanol extract was partitioned with 2000 ml distilled water and extracted stepwise with the same volume of the following solvents: *n*-hexane, ethyl acetate, *n*-butanol and distilled water. Each fraction was obtained by twice shaking the mixture of samples dissolved in distilled water and solvent at 20°C for 1 h. The fraction samples were concentrated, lyophilized and stored at –20°C until use. The samples were dissolved in ethanol, and the final concentration of ethanol did not exceed 0.5% in the culture medium in any of the experiments. The dose at 0.5% ethanol did not cause any side effects on RAW 264.7 cells and the control cells in the study included 0.3% ethanol.

Cell culture and treatment

RAW 264.7 macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM L-glutamine and antibiotics. When the cells had reached 70–80% confluence in 6- or 96-well flat-bottomed plates, they were treated with PHCR samples (0–100 μg/ml) just before exposure to 1 μg/ml LPS. They were then further cultured in the presence and absence of MAPK inhibitors. Human T lymphoma Jurkat cells were also cultured in DMEM containing 10% FBS. These were used in the assay for antioxidant activity of PHCR samples on hydroxyl-radical-mediated cytotoxicity.

MTT assay

3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) was used to evaluate the viability of the cells. In brief, 10 μl of MTT solution (5 mg/ml in PBS as stock solution) were added to the cultures incubated with PHCR samples and/or LPS in 96-multiwell plates. Four hours later the culture medium was changed to acidic isopropanol (70 μl per well), and the absorbance was then read at 570 nm using a SpectraCount™ ELISA reader (Packard Instrument Co., Downers Grove, IL, USA).

Measurement of nitric oxide production

Nitrite concentration in the culture medium was used as an indicator of the NO produced by LPS-stimulated macrophages. In brief, cells were treated with PHCR samples and/or MAPK inhibitors before exposure to 1 μg/ml LPS. After incubation for 48 h, the conditioned media (100 μl) were mixed with the same volume of Griess reagent, and absorbance of the mixtures was measured at 540 nm using an ELISA reader (Packard Instrument Co.).

Measurement of cytokines

The quantity of cytokines was determined by ELISA at the Bank for Cytokine Research (Chonbuk National University). Cells cultured in 24-well culture plates were pretreated with different concentrations of PHCR samples 30 min before stimulating them with 1 μg/ml LPS. Forty-eight hours later, the culture supernatants were collected and assessed by ELISA using TNF-α- or IL-6-specific OptEIA™ kit. The quantity of cytokines produced was calculated from standard curves generated using known concentrations of recombinant cytokine proteins.

Preparation of cell fractions

Whole-protein lysate[12] and nuclear protein fractions[13] were made in a lysis buffer as described elsewhere and the protein content in each sample was quantified according to the Bradford method.[14] For preparation of the cytosolic fraction, cells were incubated in 200 μl lysis buffer (250 mM sucrose, 20 mM N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml each of leupeptin, aprotinin and pepstatin A) on ice for 30 min. The lysates were

centrifuged at 750*g* for 10 min and then the supernatants were used as cytosolic proteins after additional centrifugation at 10 000*g* for 25 min.

Western blot analysis

Equal protein amounts of each sample were separated by 12% SDS-PAGE and blotted onto PVDF membranes. The blots were probed with primary and secondary antibodies and then developed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK) prior to exposure to X-ray film (Eastman-Kodak, Rochester, NY, USA). All mono- and polyclonal antibodies (COX-2, iNOS, ERK, p-ERK, p38, p-p38, β-actin, p-IκBα, IκBα) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Electrophoretic mobility shift assay

For the electrophoretic mobility shift assay (EMSA), the DNA-protein-binding reactions were performed for 30 min at room temperature with 10–15 μg protein in 20-μl buffer containing 1 μg/ml BSA, 0.5 μg/μl poly (dI-dC), 5% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 30 000 cpm of [α-^{32}P] dCTP-labelled oligonucleotides and the Klenow fragment of DNA polymerase. The samples were separated on 6% polyacrylamide gels and the dried gels were exposed to X-ray films (Eastman Kodak Co.) for 12–24 h at −70°C. The oligonucleotide primer sequences specific for NF-κB were: 5′-AAG GCC TGT GCT CCG GGA CTT TCC CTG GCC TGG A-3′ and 3′-GGA CAC GAG GCC CTG AAA GGG ACC GGA CCT GGA A-5′.

Determination of total phenolics

Total phenolic content was determined by a Folin-Ciocalteu reaction,[15] using gallic acid as a standard. Briefly, each PHCR fraction (2 mg) was dissolved in 2 ml of methanol/water mixture (50 : 50, v/v) and the fraction solution (300 μl) was mixed with 300 μl of 50% Folin–Ciocalteu reagent before the addition of 0.5 ml of 20% Na_2CO_3. Finally, the mixtures were centrifuged at 1000*g* for 5 min, and absorbance of the supernatants was measured at 730 nm. The total phenolic contents were expressed as gallic acid equivalents in milligrams per gram of sample.

Assays for antioxidant activity

For the cell-free assay of antioxidative activity, the scavenging activity of PHCR fractions on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was assessed according to the method reported by Gyamfi *et al*.[16] Briefly, 100 μl of each fraction (dissolved in 1 mg/ml) was mixed with 1 ml of 0.1 mM DPPH–ethanol solution and 450 μl of 50 mM Tris-HCl buffer (pH 7.4). After 30 min of incubation at room temperature, reduction of DPPH free radicals was measured by reading the absorbance at 517 nm.

For the cellular assay of antioxidative potential, Jurkat cells grown in 96-multiwell plates were treated with 100 μg/ml of PHCR samples 30 min before addition of 10 mU/ml glucose oxidase. After 24 h of incubation, the viability of the cells was determined by an MTT assay as described above.

Spectral identification of purified compound

Based on the inhibitory activities on NO production and reactive oxygen species (ROS) generation, PHCR seed extract (65 g) was applied to Amberlite XAD-4 resin (mesh size 20–60; Sigma Chemical Co.) and diluted successively with distilled water, 30% methanol, 60% methanol and 99% methanol to yield four fractions: distilled water (23.4 g), 30% methanol (13.6 g), 60% methanol (11.6 g) and 99% methanol (4.3 g). The 30% methanol fraction was further separated into eight sub-fractions (F_1 to F_8) by chromatography using a Sephadex LH-20 column (83 cm × 2 cm, Amersham Pharmacia Biotech, Freiburg, Germany) eluted with 30% methanol. After determining biological activities, F_6 was again applied to the Sephadex LH-20 column and eluted with 60% methanol to obtain four fractions: FF_1 (0.028 g), FF_2 (0.029 g), FF_3 (0.2 g) and FF_4 (1.3 g). Finally, the 30% methanol fraction and FF_4 were applied to a reverse-phase HPLC [Model 2695 with a 2487 detector (Waters Co.) and Shiseido Capcellpak C18 5 μm, 4.6 × 250-mm column] using a gradient of acetonitrile in 0.1% acetic acid. The FF_4 was further elucidated using ^{1}H-NMR and ^{13}C-NMR spectroscopic methods.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA; SPSS version 16.0 software) followed by Scheffe's test was applied to determine differences between the groups. A value of $P < 0.05$ was considered significant.

Results

PHCR extracts markedly inhibit nitric oxide production and COX-2 induction in LPS-stimulated macrophage cells

The ethyl acetate and butanol fraction did not cause cytotoxicity at a dose of 100 μg/ml, whereas the hexane and water fractions decreased the viability of the cells at a significant level, as shown by an MTT assay performed 48 h after sample treatment (Figure 1, upper left panel). We next examined the effect of PHCR samples on NO production in LPS-stimulated macrophage cells (Figure 1, lower left panel). When the cells were exposed to 1 μg/ml LPS for 48 h, the nitrite concentration in the culture medium increased approximately six-fold (55 ± 4.5 μM), compared to the control value (9.5 ± 2.9 μM). Both ethyl acetate and butanol fractions markedly attenuated LPS-stimulated NO production. In addition, the PHCR samples inhibited COX-2 expression in LPS-stimulated cells, with the ethyl acetate fraction being the most efficient (Figure 1, right panel).

Ethyl acetate fraction inhibits nitric oxide production and iNOS and COX-2 expression in a dose-dependent manner

We determined the suppressive ability of the ethyl acetate fraction on LPS-mediated inflammatory responses and a possible mechanism involved. Figure 2 shows that the fraction suppressed LPS-stimulated NO production in a dose-dependent manner; a significant decrease in nitrite concentration was observed at a treatment concentration of 20 μg/ml of

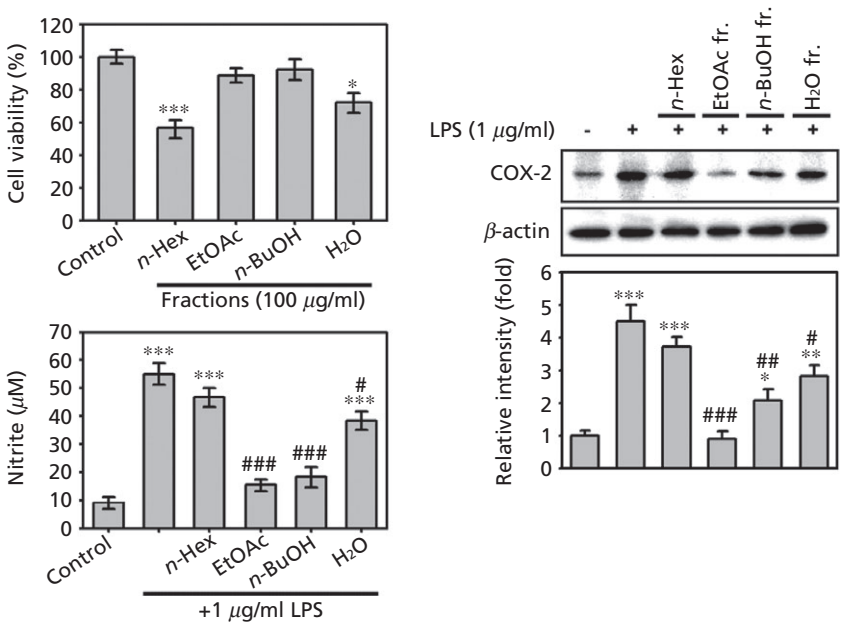

Figure 1 Effects of PHCR seed samples on viability, NO production and COX-2 expression in LPS-stimulated RAW 264.7 cells. Cells were treated with 100 μg/ml of each fraction and after 48 h of treatment the viability of the cells was determined by MTT assay (upper-left panel). Cells were also incubated for 48 h with 100 μg/ml of each sample in the presence of 1 μg/ml LPS and the level of NO in the conditioned media was measured (lower-left panel) In addition, cells were cultured for 24 h with 100 μg/ml of each sample in the presence of 1 μg/ml LPS and whole protein lysates from the cells were analyzed by Western blot analysis (right panel). The level of COX-2 expression was measured by densitometric analysis after normalising to that of β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs the non-treated control cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs the LPS treatment alone.

the fraction, while treatment at a concentration of more than 30 μg/ml reduced it to the base level. The fraction also showed a dose-sensitive inhibition of LPS-stimulated expression of iNOS protein in the cells. In addition, LPS-stimulated COX-2 expression in the macrophage cells was decreased to the base level by treating with 50 μg/ml of the fraction.

Ethyl acetate fraction inhibits phosphorylation of ERK1/2, p38 and IκBα and NF-κB-DNA binding in LPS-stimulated RAW 264.7 cells

Since ERK- and/or p38 MAPK-mediated pathways are closely involved in LPS-induced NO production, we determined the effect of ethyl acetate fraction on LPS-induced phosphorylation of these kinases. LPS treatment dramatically increased both ERK1/2 and p38 phosphorylation, whereas the ethyl acetate fraction suppressed their activation in a dose-dependent manner (Figure 3a). Treatment with 50 μg/ml of the sample reversed almost completely the increase in levels of p-ERK1/2 and p-p38 stimulated by LPS. To verify the role of MAPK in NO production, RAW 264.7 cells were treated with 10 μM of PD98059 or SB203580 together with the ethyl acetate fraction (100 μg/ml) 30 min before stimulation with LPS. LPS-induced NO production was clearly inhibited by treatment with the MAPK inhibitors or the sample. LPS treatment increased the DNA-NF-κB binding in these cells, but this was almost completely inhibited by addition of the fraction at dose of 50 μg/ml (Figure 3b). Furthermore, the ethyl acetate fraction significantly suppressed LPS-induced phosphorylation of IκBα and its subsequent degradation.

Ethyl acetate fraction inhibits LPS-induced production of TNF-α and IL-6 in RAW 264.7 cells

We assessed the effects of the ethyl acetate fraction on the production of the pro-inflammatory cytokines, TNF-α and IL-6, in LPS-exposed cells. Secretion of these cytokines was measured using the culture media of cells stimulated with 1 μg/ml LPS, alone or in combination with different concentrations (0–100 μg/ml) of the ethyl acetate fraction, for 48 h. Addition of LPS into the cells apparently increased the production of TNF-α (34.2 $\pm$ 3.8 ng/ml), whereas treatment with the fraction at 20 and 50 μg/ml significantly diminished its levels to 23.7 $\pm$ 1.9 ng/ml ($P < 0.05$) and 11.2 $\pm$ 2.6 ng/ml ($P < 0.001$), respectively (Figure 4). Similarly, treatment with the fraction produced a concentration-dependent suppression of LPS-stimulated IL-6 production in the cells. The fraction itself did not stimulate the production of these cytokines in the cells.

Ethyl acetate fraction contains high levels of phenolic compounds and antioxidant potential

Numerous studies have shown that the beneficial effects of medicinal plants are closely associated with the presence of phenolic compounds.[17,18] It is particularly likely that compounds with antioxidant properties may exert anti-inflammatory effects.[19] We therefore measured the content of phenolic compounds in the samples and determined their antioxidant potential using cell-free and cell-mediated systems. Figure 5a shows that the ethyl acetate and butanol fractions contained higher levels of phenolic compounds than the other

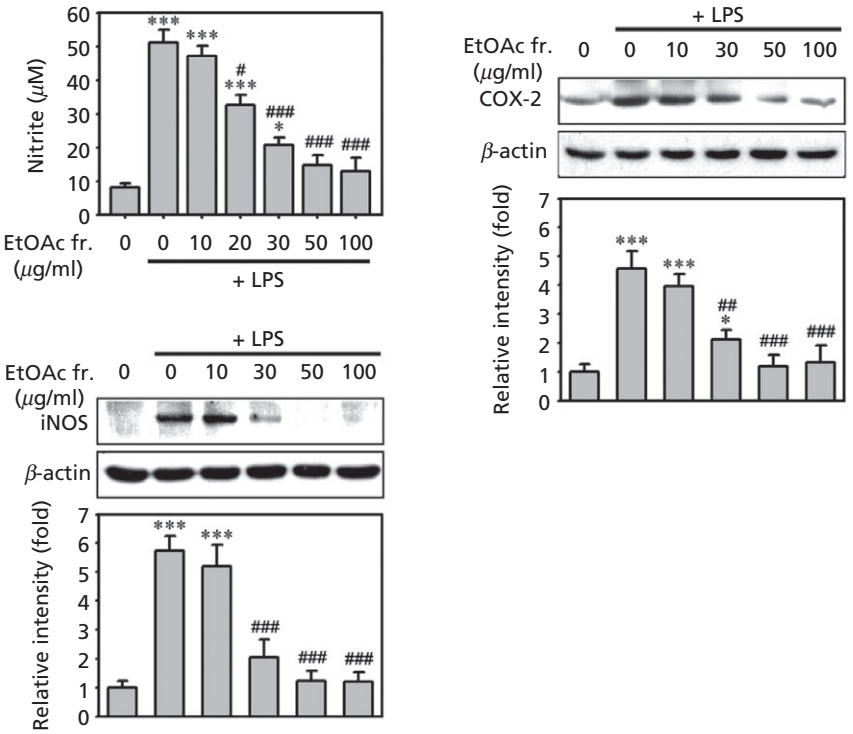

Figure 2 A dose-dependent suppression by ethyl acetate fraction of NO production, and iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells. Cells were exposed to the indicated concentrations (0–100 μg/ml) of the ethyl acetate fraction in the presence of 1 μg/ml LPS. After 48 h of incubation, the level of NO in the conditioned media was measured by determining the nitrite concentrations (upper-left panel). Whole protein lysates were also obtained from the cells exposed to the fraction and/or LPS and adjusted for the analyses of iNOS and COX-2 expression by immunoblotting at 24 h after the exposure. Quantification of iNOS and COX-2 proteins was measured by densitometric analysis after normalising the bands to that of β-actin. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs the non-treated control cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs the LPS treatment alone.

fractions. The samples also revealed the potential to scavenge DPPH free radicals in the following order: ethyl acetate = butanol > water > n-hexane fractions. In addition, the ethyl acetate and butanol fractions significantly inhibited glucose oxidase-mediated cytotoxicity in Jurkat cells (Figure 5b, upper panel). Similarly, the two fractions prevented LPS-mediated cytotoxicity in the cells (Figure 5b, below panel).

Identification of active compound

Among the numerous sub-fractions, FF_4 showed the most efficient potential for anti-inflammation and antioxidation (data not shown). Thus we initially applied FF_4 and its crude, parent, 30% methanol fraction, to HPLC (Figure 6). In the ^{1}H-NMR spectrum of FF_4, the typical ABX system signals in the aromatic proton at δ 6.75 (1H, d, J = 0.9 Hz, H-2′), δ 6.67 (1H, d, J = 7.8 Hz, H-5′), δ 6.58 (1H, dd, J = 0.9, 7.8 Hz, H-6′) were from the 3, 4-hydroxyphenyl moiety of the B-ring and the two hydroxyl-bearing carbon signals of C-3′, 4′ were observed downfield (δ 145.32, 145.31) compared with the peak of C-2′, 5′, 6′ (δ 114.9, 115.6, 118.8) in the ^{13}C-NMR spectrum. These observations indicate that the B-ring is a pyrocatechol moiety, which is substituted with a hydroxyl group in C-3′, 4′. Signals of two more aromatic protons, which were meta-coupled, and at δ 6.07 (1H, d, J = 1.2 Hz, H-8) and δ 5.97 (1H, d, J = 1.2 Hz, H-6), were observed in the ^{1}H-NMR spectrum, and the three carbon signals of C-5, 7 and 9 (δ 156.5, 157.2, 155.7) were deshielded and downfield-shifted, rather than C-6, 8 and 10 (δ 96.6, 95.0, 102.3) in the ^{13}C-NMR spectrum. These observations imply that the A-ring is related to a phlorglucinol moiety, which is substituted with a hydroxyl group at C-5, 7 and 9. Moreover, the proton signals at δ 4.54 (1H, d, J = 7.2 Hz, H-2), 3.85 (1H, m, H-3), 2.65 (1H, dd, J = 2.4, 8.1 Hz, H-4a) and 2.39 (1H, dd, J = 2.4, 8.1 Hz, H-4b) are observed in the ^{1}H-NMR and the carbon signals in ^{13}C-NMR spectrum at C-2,3 and 4 (δ 81.5, 66.5, 28.0) indicate a flavan-3ol moiety. In particular, the large coupling constant H-2 (J = 7.2 Hz) in the ^{1}H-NMR spectrum and the carbon signals of C-2 at δ 81.1 in the ^{13}C-NMR spectrum suggest a 2,3-*trans*-configuration (the *cis*-orientation between H-2 and 3 is associated with a broad singlet of H-2 in the ^{1}H-NMR spectrum and carbon signals of C-2 at about δ 75.0 in ^{13}C-NMR spectrum).

In the monosaccharide moiety, an anomeric proton signal at δ 4.69 (d, J = 7.8 Hz) and a carbon signal at δ 100.9 (glc-1), including oxygenated methine and methylene carbons such as δ 77.3 (glc-3), δ 77.0 (glc-5), δ 73.6 (glc-2), δ 70.0 (glc-4) and δ 61.1 (glc-6) suggest the presence of a β-glucosylpyranoside.[20,21] Thus the compound is presumed to be a catechin glycoside. To confirm the structure, we used the 2D-NMR technique. In the HMBC spectrum, owing to the correlation of the methylene proton signals at δ 4.69 (glc-1) with the carbons at δ 157.2, which were assigned to C-7, the glc-H should be linked to C-7 of the catechin skeleton. In

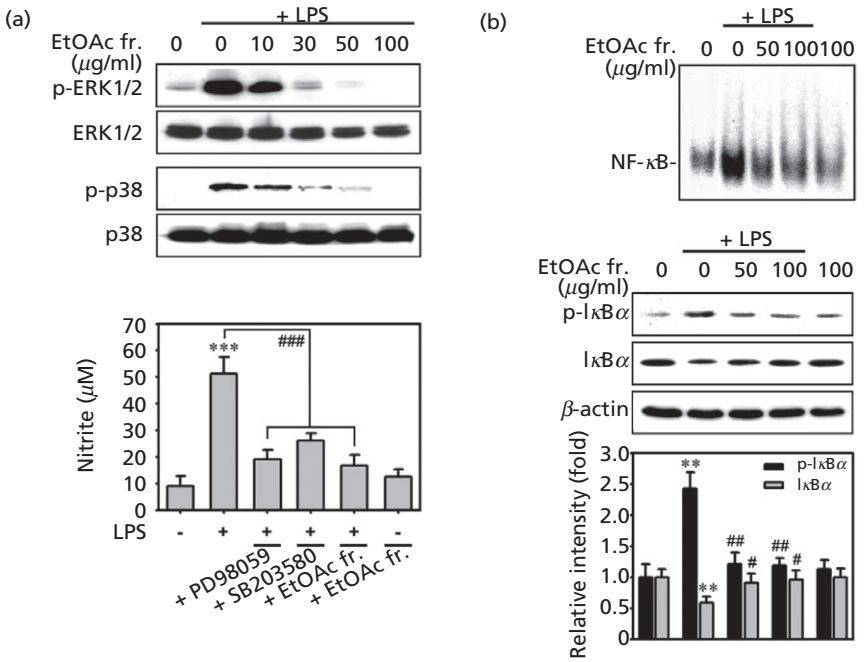

Figure 3 Effects of ethyl acetate fraction on MAPK activation and NF-κB DNA binding in LPS-stimulated RAW 264.7 cells. (a) Cells were exposed to various concentrations (0–100 μg/ml) of the fraction in the presence of LPS (1 μg/ml) for 30 min, and the levels of p-ERK and p-p38 MAPK in whole protein lysates were detected using their specific antibodies (upper panel). Representative data from triplicate experiments are shown. Cells were also exposed to 1 μg/ml LPS in the presence of PD98059 (10 μM), SB203580 (10 μM) or the ethyl acetate fraction (100 μg/ml) and at 48 h after the co-induction, nitrite concentrations in the conditioned media were determined (lower panel). ***$P < 0.001$ vs the untreated control cells. ###$P < 0.001$ vs LPS treatment alone. (b) Cells were incubated with the ethyl acetate fraction in the presence of 1 μg/ml LPS. After 1 h of incubation, nuclear and cytosolic fractions were prepared from the cells and processed for analysis of NF-κB binding activity by EMSA (upper panel) and IκB degradation by immunoblot analysis using p-IκBα and IκBα specific antibodies (lower panel), respectively. **$P < 0.01$ vs the non-treated control cells. #$P < 0.05$ and ##$P < 0.01$ vs the LPS treatment alone.

combination with previous data,[20–22] all these spectral data demonstrate that the compound is catechin-7-O-β-D-glucopyranoside. Finally, this compound showed anti-oxidative activity against DPPH and hydroxyl radicals and inhibited LPS-stimulated MAPK phosphorylation (data not shown).

Discussion

Inflammation is a host response to endotoxins and is mediated by complex processes with several classic signs. The most prominent phenomenon in the process of inflammation is the increase of NO and pro-inflammatory cytokines.[23] Although NO is also known to act as an intracellular messenger that mediates vascular relaxation and eliminates pathogens and tumour cells, it is primary involved in promoting inflammatory responses in many cases.[24–26] Thus over-production of NO by macrophages may lead to various pathological disorders such as inflammation, carcinogenicity, cytotoxicity and autoimmune diseases. In addition, COX-2 is involved in inflammatory responses, mediating the production of prostaglandins.[27] Therefore a selective inhibition of iNOS and COX-2 is regarded as the most efficient approach to alleviating a variety of disorders triggered by inflammatory mediators.

In the present study, we showed that PHCR seed extract inhibited NO production and COX-2 expression in LPS-stimulated macrophage cells. In particular, the ethyl acetate and butanol fractions revealed a potential for inhibiting LPS-mediated production of NO and cytokines such as TNF-α and IL-6, without any toxic effect on cells. These findings demonstrate for the first time the scientific basis supporting the anti-inflammatory potential of PHCR seed. Collectively, our present data suggest that PHCR seed extract can be useful as an active medicine to inhibit over-activation of macrophages followed by inflammatory mediators.

MAPKs regulate a broad range of cellular events, including proliferation, differentiation and apoptosis.[28] The kinases also mediate cellular signaling involved in activation of macrophages in responses to microbial pathogens.[29] Our current data show that PHCR seed extracts blocked LPS-induced NO production by inhibiting the phosphorylation of both ERK1/2 and p38 MAPK in LPS-stimulated RAW 264.7 macrophage cells. This is consistent with the previous report that luteolin suppressed LPS-induced NO production via inhibition of both ERK1/2 and p38 activation.[30] Sung *et al*.[7] also reported that vitisin A, a resveratrol tetramer, prevented LPS-induced NO production by inhibiting ERK and p38 phosphorylation in macrophages. However, there was an opposite mechanism showing that either ERK1/2 or p38 MAPK is related to NO production caused by LPS.[31–33] Although it is unclear whether the activation of both or either of ERK1/2 or p38 MAPK is required for LPS-induced NO production in macrophages, our data suggested that LPS activates macrophages through both ERK1/2 and p38 phosphorylation. It is also possible that

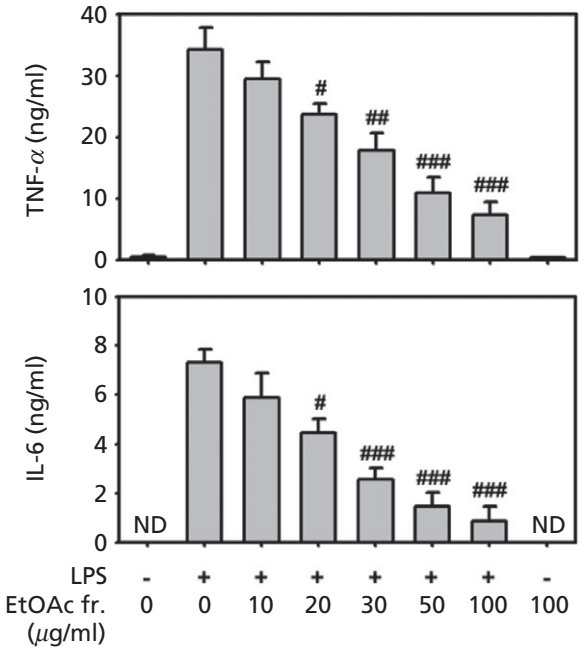

Figure 4 Effects of ethyl acetate fraction on the production of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells. Cells were stimulated with 1 μg/ml LPS in the presence of various concentrations (0–100 μg/ml) of the ethyl acetate fraction. After 48 h of co-incubation, the levels of TNF-α and IL-6 in the culture supernatants were measured by ELISA. Data represent the mean values of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs the LPS treatment alone. ND, not detected.

PHCR seed extract contains a variety of active compounds that are specific for the inhibition of ERK1/2 and/or p38 MAPK.

MAPKs affect the activity of NF-κB transcription factor, which is essential for iNOS gene transcription and subsequent NO production. The present findings reveal that the ethyl acetate fraction blocks LPS-induced NO production through the inhibition of ERK/p38-mediated NF-κB activation. The fraction-mediated suppression of LPS-stimulated cytokine production is also considered to be related to its inhibitory potential on NF-κB activation.[34,35] This observation is in accord with previous reports showing that bioactive substances such as bis-(3-hydroxyphenyl) diselenide and eutigoside C express their anti-inflammatory effect by inhibiting NF-κB-mediated pathways in LPS-stimulated RAW 264.7 cells.[36,37]

Many investigators have demonstrated the beneficial effects of phenolic compounds in antioxidative, antitumour and anti-inflammatory effects. In particular, phenolic compounds may exert their biological activity through inhibition of protein kinase.[38–40] Our results reveal that PHCR seed extracts contain a large quantity of phenolic compounds and protected cells from oxidative damage as well as from LPS itself. This indicates that phenolic substances contained in the PHCR seed extract are responsible for its anti-inflammatory and antioxidative effects.[41,42] More importantly, polyphenolic compounds, including catechin, quercetin, myricetin and myricetin-rutinosides, have been identified as the main components of the ethanol extract from *Phaseolus angularis*

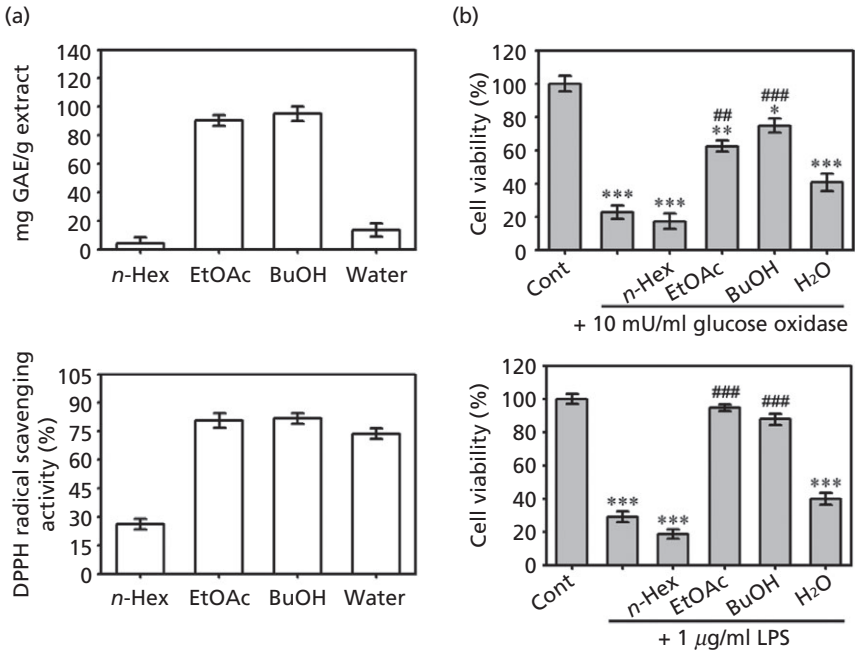

Figure 5 Total phenolic contents of PHCR samples and their anti-oxidant potential. (a) Contents of total phenolic compounds were measured according to the Folin–Ciocalteu method and the results reported are the mean ± SD from triplicate experiments (upper panel). The scavenging activity of PHCR fractions against DPPH free radicals was determined as described in the Materials and Methods section (lower panel). (b) Jurkat cells were incubated with 100 μg/ml of each fraction in the presence of 10 mU/ml glucose oxidase (upper panel) or 1 μg/ml LPS (lower panel), and viability of the cells was determined by MTT assay at 24 h after the incubation. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs the non-treated control cells. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs the LPS treatment alone.

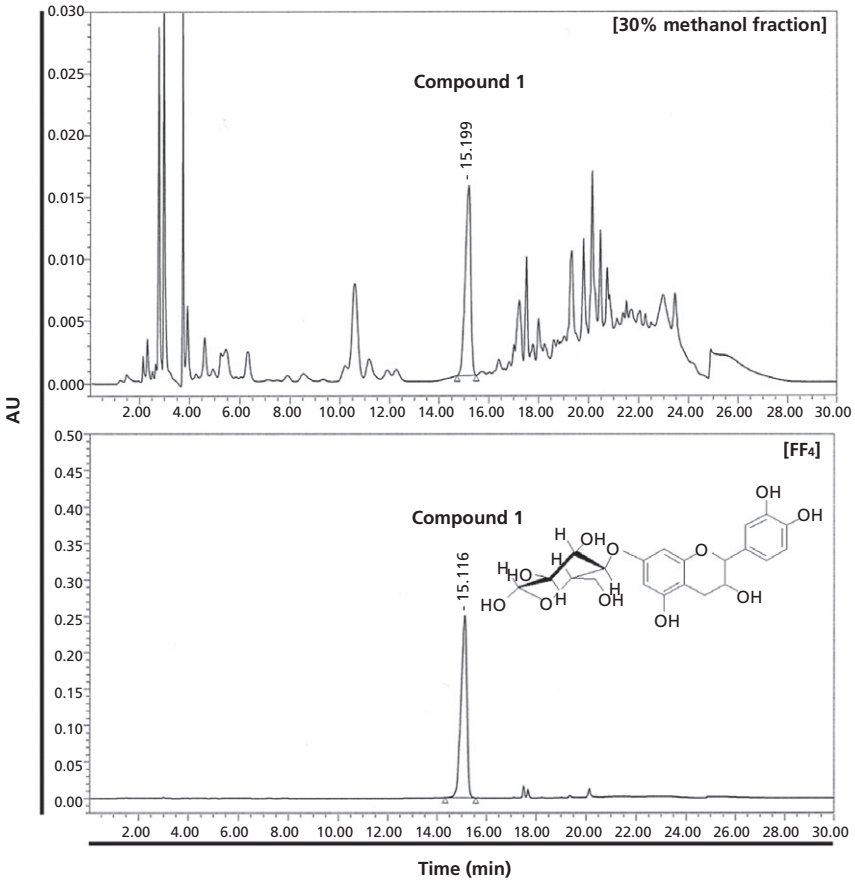

Figure 6 HPLC chromatogram of the 30% methanol fraction and its sub-fraction, FF_4. HPLC was performed with a reverse-phase column (Shiseido Capcellpak C18 5 μm, 4.6 × 250 mm) eluted with a linear gradient of acetonitrile in 0.1% acetic acid. The column temperature was 30°C, injection volume 10 μl each time, and flow rate 1 ml/min. The active compound 1 was catechin-7-O-β-D-glucopyranoside.

Wight, a kind of Adzuki bean.[11] Here we demonstrate for the first time the presence of catechin-7-O-β-D-glucopyranoside in the seed of PHCR, although this compound has previously been identified in other plants such as *Polygonum macrophyllum* D. Don,[20] *Ulmus davidiana* var. japonica,[21] and *Jatropha macrantha*.[22] Quantitative analysis by HPLC showed that 8.458 mg of the catechin derivative was recovered from 1 g of the ethanol extract from PHCR. Consequently, we suggest that this catechin compound and its derivatives have beneficial roles in decreasing inflammatory responses to LPS and preventing oxidative damage to living cells.

Conclusions

This study demonstrate that PHCR seed extracts have anti-inflammatory effects on LPS-activated macrophages, which is in part related to their antioxidative activity. This can be supported by the fact that ROS, which are produced in endotoxin-activated macrophages, play important roles in mediating signal transduction pathways involved in the inflammatory responses.[43,44] Overall we show that a catechin derivative is one of the bioactive phenolic compounds contained in PHCR seed, suggesting that the seed may be useful in a therapeutic approach for the treatment of inflammatory diseases.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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