

Full Paper

The Water-Extracted *Ampelopsis brevipedunculata* Downregulates IL-1 β , CCL5, and COX-2 Expression via Inhibition of PKC-Mediated JNK/NF- κ B Signaling Pathways in Human Monocytic Cells

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Abstract. The anti-inflammatory and anti-hepatotoxic effects of *Ampelopsis brevipedunculata* (*A.bre*) have been well known in folk medicine. An ethanol-extract of *A.bre* has been reported to inhibit carbon tetrachloric acid induced hepatic injury, suggesting that extracted components from *A.bre* could potentially treat inflammatory disease. To test this hypothesis, in this study, we extracted polysaccharide components from leaves of *A.bre* and investigated the anti-inflammatory effects in PMA stimulated THP-1 cells. THP-1 cells activated by PMA in the presence or absence of *A.bre* demonstrated that a water-extract of *A.bre* inhibited the expression of pro-inflammatory cytokine IL-1 β and chemokine CCL-5 in a dose-dependent manner. In addition, *A.bre* suppressed production of cyclooxygenase (COX)-2 in THP-1 cells activated by PMA. Moreover, *A.bre* markedly down-regulated the expression of p-JNK1/3, whereas it did not inhibit production of the phosphorylated form of p38 and extracellular signal-regulated kinase in THP-1 cells treated by PMA. Particularly, *A.bre* inhibited the translocation of transcription factor NF- κ B from the cytosol into the nucleus in PMA-stimulated THP-1 cells. Collectively, our data showed that water-extracted *A.bre* inhibited the protein kinase C–JNKs/NF- κ B signaling pathways, resulting in the suppression of IL-1 β , CCL-5, and COX-2 expression. This study suggests that water extracted *A.bre* may be a therapeutic agent against inflammatory disease.

Keywords: IL-1 β , anti-inflammation, *Ampelopsis brevipedunculata*, PMA, THP-1

Introduction

Inflammation is a biological protective response to harmful stimulation. Inflammation is also associated with diseases such as rheumatic arthritis, diabetes, and even cancer (1 – 3). Monocytes and macrophages play an important role in induction of several inflammation responses (4). Coming from a human monocytic leukemia cell line, THP-1 cells can be differentiated by PMA into macrophages. Differentiated THP-1 macrophages have been used as an in vitro model to study human inflammatory diseases (5). PMA activates macrophages to produce pro-inflammatory mediators and pro-inflam-

matory cytokines, including cyclooxygenase-2 (COX-2), interleukin-1 β (IL-1 β), and chemokine C-C motif ligand 5 (CCL-5) (6, 7). Therefore, IL-1 β , COX-2, and CCL-5 play a pivotal role in the regulation of inflammatory process. *Ampelopsis brevipedunculata* (*A.bre*), commonly called porcelain vine and very popular in Northeastern Asia, has been used to cure inflammatory and liver diseases in folk medicine. Previous studies demonstrated that ethanol extracted *A.bre* decreases hepatic injury (8, 9) as well as exerts anti-hepatitis B virus activity (10). However, the anti-inflammation effects of the water extracted *A.bre* have not been investigated. In this study, the anti-inflammatory effects of water extracted *A.bre* in PMA-stimulated THP-1 macrophages were investigated by detecting the expression of pro-inflammatory mediators and pro-inflammatory cytokines such as IL-1 β , COX-2, and CCL-5. Moreover, we examined whether

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the inhibition of activated NF- κ B and MAPK is critical for the anti-inflammatory functions of *A.bre*.

Materials and Methods

Materials

We obtained the human monocyte cell line THP-1 from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in RPMI medium (Welgene Incorporation, Daegu, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA). The cells were incubated under 5% CO₂ and 37°C and were subjected to a maximum of 20 cell passages.

Preparation of *A.bre*

A.bre leaves were collected directly by one of us, Hye Nyoung Noh, in June 2010 in Korea. The water extracted *A.bre* was deposited in Prof. Se Chul Chun's Laboratory, Faculty of Konkuk University (Seoul, Korea). The authenticity of the plant was confirmed by Dr. Suk Keun Park, a plant botanist in College of Life and Environment Science, Konkuk University.

Organic solvent and water extraction of *A.bre*

Leaves (1 kg) of *Ampelopsis brevipedunculata* (Korean name: Gaemeoru) were ground with a blender and mixed with 1 liter of ethanol. The ground leaves in ethanol were sonicated for 1.5 h and precipitated for 1.5 h. The ground leaf suspension of ethanol was filtered with a vacuum pump. A 3-ml aliquot of the filtrate in ethanol was used to test as "ethanol extract materials" and the rest was evaporated, resulting in 1.667 g of the extract. The extract was then dissolved in 300 ml of milliQ water for further extraction by different organic solvents. After being mixed with 300 ml of *n*-hexane and placed into separating funnels with glass stopcock, the extract was again collected and evaporated, resulting in 0.07 g. With the same methods, the extracts of ethyl acetate, chloroform, and butanol were collected, resulting in 0.18, 0.15, and 0.19 g, respectively. The remaining materials were dissolved in water after all the organic solvent extracts were evaporated, and 0.95 g of the water extract was harvested.

Analysis of sugars

All organic solvent extracts did not have any anti-inflammatory activities except for water extract in preliminary tests, leading into analyzing sugars in water extract. Water extract was dissolved in 200 μ l of deionized water. This solution was filtered with 1 m syringe filter (Pall Syringe Filters with PVDF Membrane, 13 mm, 0.45 μ m) and analyzed for HPLC. Analysis of

Table 1. Analysis of sugars in the water extract of *A.bre*

Instrument	Waters 515 HPLC pump
	Waters 717 plus auto-sampler
	Waters pump control module
Column	Grace Prevail carbohydrate ES 5 μ m (250 mm $\times$ 4.6 mm)
Mobile phase	75% Acetonitrile
Detection	Waters 410 Differential Refractometer Detector
Flow rate	0.9 ml/min (isocratic mode)
Injection volume	10 μ l
Oven temperature	27°C
Software	Empower pro

the sugar was conducted with HPLC that is consisted of Waters 515 HPLC pump, Waters 717 Plus Auto-sampler, Waters 410 Differential Refractometer Detector, Waters Column Heater Module, Waters Pump Control Module, and Empower pro software. A solution of 75% acetonitrile was passed through a HPLC column, Grace Prevail Carbohydrate ES 5 μ m (250 $\times$ 4.6 mm), with 0.9 ml/min in isocratic mode as indicated in the Table 1. Injection volume was 10 μ l and sugars were detected with Waters 410 Differential Refractometer Detector (27°C). Reference materials of Monosaccharide kit, Disaccharide kit, Oligosaccharide kit, Sugar alcohol kit (Supelco; Sigma Aldrich Korea, Seoul, Korea) were used. The standards for HPLC were fructose, glucose, glycerol, lactose, mannitol, myo-inositol, ribose, sorbitol, trehalose, and xylose, which were used at the concentrations of 0.5%, 1%, 2% in deionized water for HPLC analysis.

MTS assay

Cell viability was quantified using an MTS reagent. The MTS assay is a colorimetric assay that measures the activity of mitochondrial reductases that reduce tetrazolium to formazan (11). The THP-1 (1×10^4 cells/well) cells were seeded in 100 μ l medium in 96-well plates and then treated with *A.bre* at the ratio of 1/800 – 1/100 for 24 h with 10 nM PMA. The effect of *A.bre* on cell proliferation was assessed by CellTiter 96 Aqueous One Solution Assay (Promega, Madison, WI, USA) containing MTS and phenazine methosulfate (PMS), an electron coupling reagent. An aliquot of 20 μ l Aqueous One Solution reagent was added to each well, and the cells were incubated for another 1 h. Absorbance at 492 nm was measured by a microplate reader (Apollo LB 9110; Berthold Technologies GmbH, Bad Wildbad, Germany). The percentages of viable cells were estimated by comparison with untreated controls. The cell proliferation assay was repeated three times.

RNA extraction and mRNA expression analysis

The THP-1 cells (3×10^6 cells/well) were seeded in 6-well plates. Cells were treated with water extracted *A.bre* (1/1600 – 1/400), JNK inhibitor SP600125 (10 μ M) followed by PMA (10 nM) for 24 h. The treated cells were collected and lysed using the Easy-BLUE Total RNA Extraction kit (iNtRon Biotechnology, South Korea) according to the manufacturer's instructions. The Oligo(dT)-primer RNA (5 μ g) was reverse transcribed using M-MuLV reverse transcriptase (New England Biolabs, Beverly, MA, USA). RNA was converted to cDNA by RT-PCR using PROSTARTM (Stratagene, La Jolla, CA, USA). After cDNA was synthesized using Oligo(dT), the cDNA was PCR-amplified with a PCR thermal cycler Dice instrument (TaKaRa, Otsu). The following sets of primers were used; hIL-1 β : 5'-ATG GCA GAA GTA CCT AAG CTC GC (forward), 5'-ACA CAA ATT GCA TGG TGA AGT CAG TT-3' (reverse); COX-2: 5'-GAA ACC CAC TCC AAA CAC AG (forward), 5'-CCC TCG CTT ATG ATC TGT CT (reverse); CCL-5: 5'-GCT GTC ATC CTC ATT GCT AC (forward), 5'-CAT TTC TTC TCT GGG TTG GC (reverse); and GAPDH: 5'-TGA TGA CAT CAA GAA GGT GGT-3' (forward), 5'-TCC TTG GAG GCC ATG TAG GCC-3' (reverse). GAPDH was used as an internal control. The PCR products were separated on a 1.5% agarose gel and detected by ethidium bromide (EtBr) staining.

IL-1 β and CCL-5 secretion assay

IL-1 β and CCL-5 levels in culture supernatant were measured by sandwich ELISA (R&D System, Minneapolis, MN, USA). To detect the concentrations of IL-1 β and CCL-5 in THP-1 cells, a flat-bottom 96-well plate was coated with mouse monoclonal anti-human IL-1 β or CCL-5 antibody (4.0 μ g/ml in PBS R&D Systems). The wells were incubated overnight at room temperature, washed three times with wash buffer [0.05% Tween-20 in PBS (PBST)], and blocked with 300 μ l 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. After washing the wells three times with PBST, 100 μ l of sample was added per well and incubated for 2 h at room temperature. Recombinant human IL-1 β and CCL-5 were used as standard. The wells were washed three times with PBST and incubated for 2 h at room temperature with 100 μ l biotinylated goat anti-human IL-1 β or CCL-5 at 200 ng/ml (1% BSA in PBS, R&D Systems). The wells were washed again with PBST and incubated with Streptavidin HRP in 0.1% BSA / 0.05% Tween-20 in PBS for 20 minute at room temperature, avoiding direct light. The wells were washed again and 100 μ l tetramethylbenzidine (TMB) was added to each well and incubated for 20 min at room temperature,

avoiding direct light. To stop the reaction, 50 ml 2 NH₂SO₄ was added to each well. Developed colors were detected on an Apollo LB 9110 microplate plate at 450 nm (corrected by absorption at 570 nm). The measurement was performed in triplicate.

Western blot analysis

The THP-1 cells (2×10^6 cells/well) were seeded and treated with water extracted *A.bre* (1/400) and/or protein kinase C (PKC)-delta inhibitor rottlerin (10 nM) and/or pan PKC inhibitor Go6850 (10 μ M) followed by 10 nM phorbol 12-myristate 13 acetate (PMA). The treated cells were collected by centrifugation at 5,500 rpm for 5 min at 4°C. The cells were then washed with ice cold PBS and re-centrifuged at 5,500 rpm for 5 min at 4°C. The cell pellets were re-suspended in a lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, 0.1% SDS, 0.25% SDC, 1mM EDTA, 1 mM EGTA, 1 mm orthovanadate, aprotinin (10 μ g/ml) and 0.4 mM PMSF (phenylmethylsulfonyl fluoride)] at 4°C for 2 h. The lysate was centrifuged at 13,000 rpm for 30 min at 4°C before the supernatant was collected. We performed the Bradford assay (Biorad, Hercules, CA, USA) and a UV spectrophotometer (Bio-wave II; Biochrom WPA, Cambridge, UK) to equalize loading protein. Equal amounts of protein (50 μ g) were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Milipore, Billerica, MA, USA). The membranes were incubated with blocking buffer [5% non-fat dry-milk in TBS containing 0.1% Tween-20 (TBST)] for 1 h at room temperature. Membranes were then incubated with primary antibodies to extracellular signal-regulated kinase (ERK) 1/2, p-ERK 1/2, JNK1/3, p-JNK1/3, and phosphor-(Ser) PKC Substrate (Cell Signaling Technology, Danver, MA, USA) overnight at 4°C and washed three times for 10 min per wash with TBST. Membranes were incubated with HRP-conjugated rabbit or mouse IgG secondary antibodies for 1 h at room temperature. After washing three times for 10 min per wash by TBST, proteins were detected by enhanced chemiluminescence (ECL) solution (Amersham Bioscience, Buckinghamshire, UK) and x-ray film.

Cell fractionation

The THP-1 cells (2×10^6 cells/well) were seeded and pretreated with water extracted *A.bre* (1/400) and/or SP600125 (10 μ M) followed by 10 nM PMA for 1 h prior to harvesting and fractionation with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc., Rockford, IL, USA) according to the manufacturer's instructions. Briefly, cells were collected by centrifugation at $1,980 \times g$ and 4°C for 5 min, washed with PBS, and re-centrifuged. The cell pellet was

suspended with buffer I, vortexed, and incubated on ice for 10 min. Buffer II was added and the cells were incubated for 1 min prior to centrifugation at $16,000 \times g$ and 4°C for 5 min, yielding a cytoplasmic extract as the supernatant. The insoluble pellet was then suspended in buffer III, incubated on ice for 40 min, and centrifuged at $16,000 \times g$ for 10 min, yielding the nuclear extract as the supernatant. Equal quantities of protein from these extracts ($50 \mu\text{g}$) were separated by SDS-PAGE and then transferred to PVDF membranes; the remainder of the procedure was as described for the western blots. Primer antibodies to NF- κB p65, p50, and c-Jun were utilized for both the cytosol and nucleus fractions. PARP and β -actin were used as markers for nucleus and cytosolic proteins, respectively.

Luciferase assay for IL-1 β promoter

The IL-1 β promoter region (–126 to +11) was PCR-amplified from THP-1 genomic DNA. The primer set was 5'-CCACCAATACTCTTTTCCCC-3' (sense) and 5'-CTGGCAGATACCAAACCTCT-3' (antisense). The PCR product was digested with *Kpn*I and *Bgl*II restriction enzymes. The digested fragment (–126 to +11) was ligated into the pGL3-Basic vector (12). THP-1 cells (2×10^6 cells/well) were transfected overnight with pGL3- IL-1 β promoter ($1 \mu\text{g}$) and pRL-null (Renilla, $0.1 \mu\text{g}$) using the NeonTM transfection system (Invitrogen, Carlsbad, CA, USA). Cells were treated with *A.bre* (1/400) and/or 10 nM rottlerin and/or $10 \mu\text{M}$ SP600125 followed by 10 nM PMA for 24 h prior to lysis. The promoter activity was measured by using the Luciferase Reporter Assay System (Promega).

Results

The analysis of sugars in the water extract of *A.bre*

The water extract ($10 \times$ dilution of original extract) of *A.bre* contained D-glucose (2.62) and D-fructose (2.14 g)/L most significantly, followed by D-ribose (1.04 g), myo-inositol (0.20 g), D-xylose (0.11 g), α -lactose (0.08 g), glycerol (0.08 g), and a sugar alcohol such as mannitol (0.03 g) (Table 2 and Fig. 1). These sugars are not specific as components of the polysaccharide. The biological activities may be attributed not to the types of sugar components but to the shape and chemical structure. To the best of our knowledge, the polysaccharides of *A.bre* never have been characterized and tested for their biological activities. The water extract of *Ampelopsis brevipedunculata* was designated as *A.bre* in the following experiments.

Effects of water extracted *A.bre* on cell viability in THP-1 macrophages

The effects of *A.bre* on the viability of THP-1 cells were determined by MTS assay after incubating the cells for 24 h in the absence or presence of PMA. The cell viability was not affected by *A.bre* at a concentration up to 1/400 dilution (Fig. 2). Therefore, for all subsequent experiments, concentrations of *A.bre* less than 1/400 were used.

Effects of water extracted *A.bre* on expression of IL-1 β , CCL-5, and COX-2 in THP-1 cells stimulated by PMA

We investigated the effects of *A.bre* on PMA-induced IL-1 β , CCL-5, and COX-2 mRNA levels. THP-1 cells were treated with 10 nM PMA in the presence or absence of *A.bre* (1/1600 – 1/400); then the mRNA expression levels of IL-1 β , CCL-5, and COX-2 were measured

Table 2. Volatile compounds identified in water extracted *A.bre*

Component	Equation	R ²	Standard error
Fructose	$Y = (3.64 \times 10^2 \times X) + (8.82 \times 10^4)$	0.9993	1.006583×10^4
Glucose	$Y = (3.04 \times 10^2 \times X) - (2.44 \times 10^5)$	0.9990	1.026878×10^4
Glycerol	$Y = (2.18 \times 10^2 \times X) + (3.87 \times 10^5)$	0.9979	1.077260×10^4
α -Lactose	$Y = (1.70 \times 10^2 \times X) - (3.11 \times 10^4)$	0.9969	1.022879×10^4
Mannitol	$Y = (3.41 \times 10^2 \times X) + (1.88 \times 10^4)$	0.9991	1.082126×10^4
myo-Inositol	$Y = (3.71 \times 10^2 \times X) - (6.37 \times 10^3)$	0.9404	1.009065×10^5
Ribose	$Y = (5.55 \times 10^1 \times X) - (3.96 \times 10^4)$	0.9999	1.003363×10^4
Sorbitol	$Y = (3.12 \times 10^2 \times X) + (1.94 \times 10^4)$	0.9999	1.091501×10^3
Trehalose	$Y = (1.56 \times 10^2 \times X) - (1.14 \times 10^5)$	0.9999	1.008643×10^4
Xylose	$Y = (9.47 \times 10 \times X) + (2.40 \times 10^4)$	0.9901	1.021213×10^4

Reference materials of the monosaccharide kit, disaccharide kit, oligosaccharide kit, and sugar alcohol kit were used. The standards for HPLC were fructose, glucose, glycerol, lactose, mannitol, myo-inositol, ribose, sorbitol, trehalose, xylose; and these standards were used at the concentration of 0.5%, 1%, and 2% in deionized water for HPLC analysis.

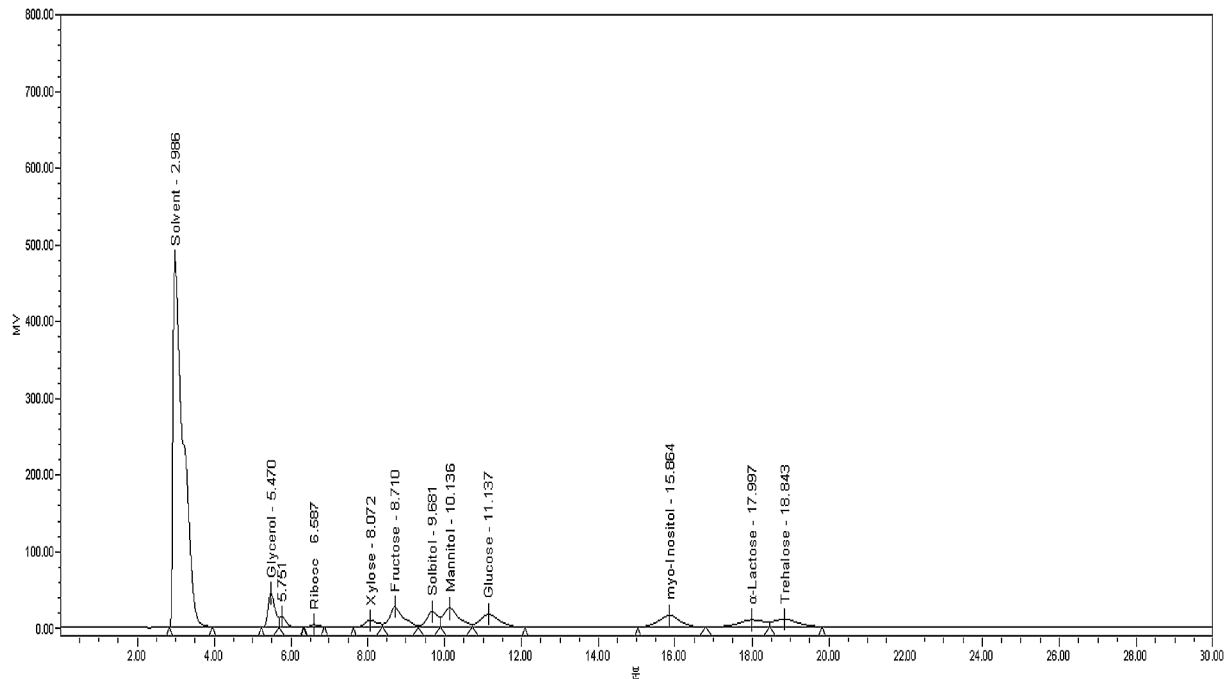


Fig. 1. The HPLC analysis of sugar composition in the water extract of *A.bre*. The 10 sugars in the sugar composition of the sample were analyzed by comparing the spectrum of the sample and that of each standard, for which a standard curve was obtained; the peak area for each component of the sample was used to determine the amount of the component from the corresponding standard curve.

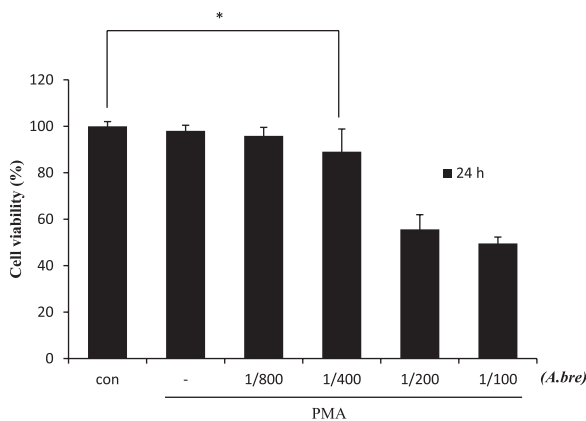


Fig. 2. The cytotoxic effect of water extracted *A.bre* on PMA-treated THP-1 cells. THP-1 cells were pre-treated with water extracted *A.bre* in a dose-dependent manner (1/800 – 1/100) and then activated by PMA (10 nM) for 24 h. Cell proliferation was determined by MTS assay. The percentage of viable cells was calculated using 100% for stimulation in the absence of treatment. All data represents that the mean values and are expressed as the mean $\pm$ S.D. of 3 experiments conducted in triplicate. The concentration of *A.bre* (1/400) was not cytotoxic compared to non-treated control cells (* $P < 0.05$).

(Fig. 3A). PMA treatment significantly induced IL-1 β , CCL-5, and COX-2 mRNA levels, while the pre-treatment of the THP-1 cell line with *A.bre* (1/1600 – 1/400) resulted in down-regulation of IL-1 β , CCL-5, and

COX-2 mRNA levels in a dose-dependent manner. In addition, western blot analysis showed that the expression of COX-2 was strongly increased by PMA, while the application of *A.bre* (1/1600 – 1/400) for 1 h prior to PMA treatment resulted in a marked reduction in the level of COX-2 protein in a dose-dependent manner in THP-1 cells (Fig. 3B). To determine whether *A.bre* affects the secretion of inflammatory cytokines in PMA-stimulated THP-1 cells, the secretion levels of IL-1 β and CCL-5 were detected using ELISA. As shown in Fig. 3C, the levels of IL-1 β and CCL-5 significantly increased after PMA challenge compared with those of the control group, while the pretreatment with *A.bre* (1/1600 – 1/400) reduced the production of IL-1 β and CCL-5 in a dose-dependent manner. Altogether, the results showed that *A.bre* attenuated the inflammatory response via suppressing productions of pro-inflammatory cytokine and chemokines such as IL-1 β , CCL-5, as well as COX-2, a mediator of inflammation, in PMA-stimulated THP-1 cells.

Effects of water extracted A.bre and PKC inhibitors on the phosphorylation of MAPKs in PMA-stimulated THP-1 macrophages

In order to determine whether the *A.bre* induced suppression of inflammation was mediated through a

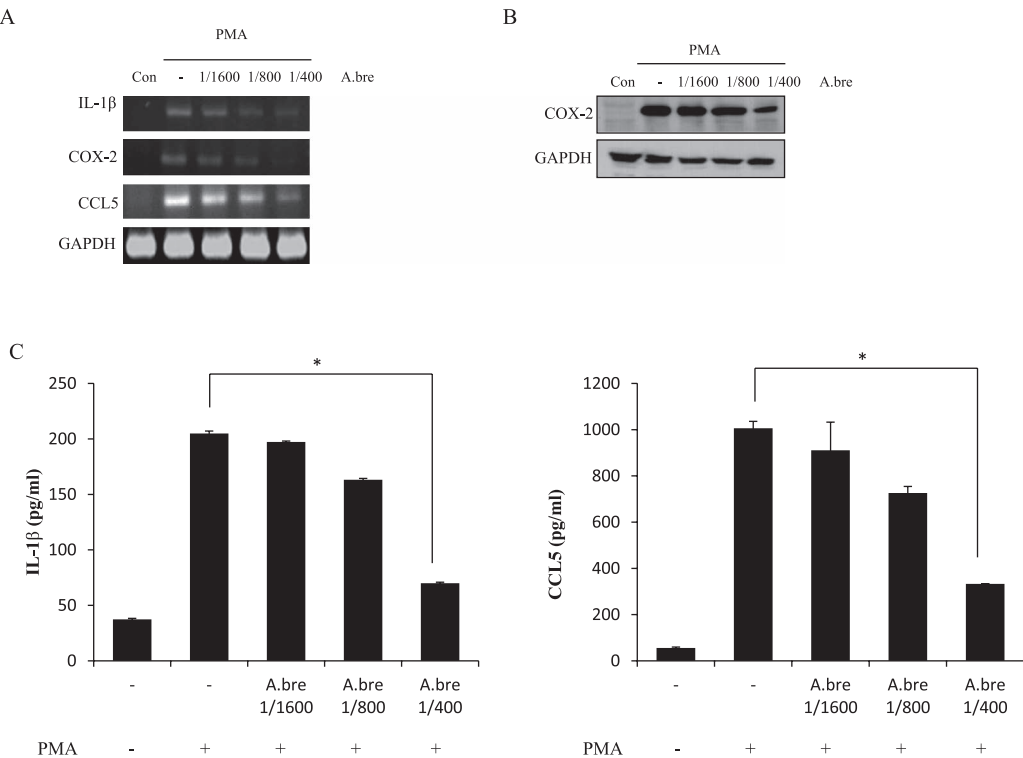


Fig. 3. Effect of water extracted *A.bre* on IL-1 β , CCL-5, and COX-2 expression. THP-1 cells were pre-treated with water extracted *A.bre* (1/1600 – 1/400) for 60 min then induced by PMA (10 nM) for 24 h. A) IL-1 β , CCL-5, and COX-2 transcript expression was measured by RT-PCR. B) COX-2 protein expression was measured by western blot analysis. C) The concentration of IL-1 β and CCL-5 in the supernatant was measured by ELISA. Data represent the mean $\pm$ S.D. of 3 experiments conducted in triplicates (* $P < 0.05$).

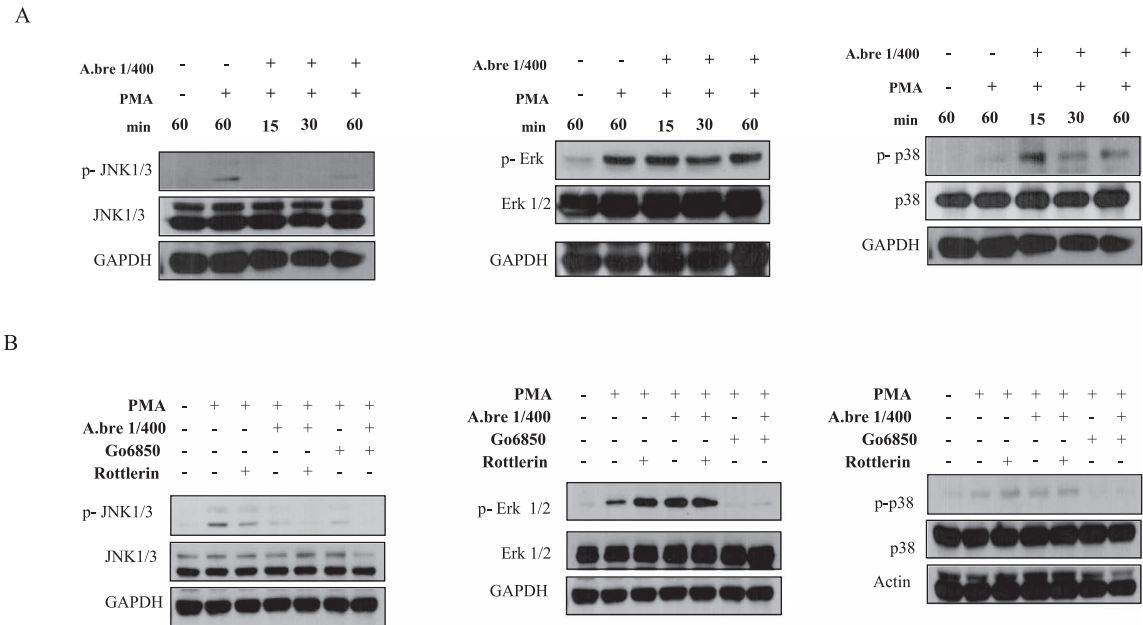


Fig. 4. Effects of water extracted *A.bre* and PKC inhibitors on phosphorylation of JNK1/3, ERK1/2 in PMA-treated THP-1 cells. A) THP-1 cells were treated with water extracted *A.bre* (1/400) and stimulated by PMA (10 nM) for 60 min. The protein levels of JNK1/3, ERK1/2 and their phosphorylated forms were measured by western blot analysis. B) THP-1 cells were treated with water extracted *A.bre* (1/400) and/or the PKC δ inhibitor rottlerin (10 μ M) and/or pan PKC inhibitor Go6850 (10 μ M) followed by PMA (10 nM) for 60 min. The protein levels of JNK1/3, ERK1/2, and their phosphorylated forms were measured by western blot analyses.

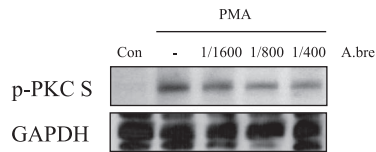


Fig. 5. Effect of water extracted *A.bre* on PKC activity. THP-1 cells were pre-treated with water extracted *A.bre* (1/1600–1/400) for 60 min then induced by PMA (10 nM) for 60 min. The protein levels of phosphor-(Ser) PKC Substrate were measured by western blot analysis.

MAPK pathway, we assessed the effects of *A.bre* on the PMA-induced phosphorylations of ERK, JNK MAPKs in THP-1 macrophages. As shown in Fig. 4A, pre-treatment with *A.bre* for 15, 30, and 60 min, at the concentration of 1/400 dilution, significantly inhibited the PMA-induced phosphorylation of JNK in a dose-dependent manner, but not those of ERK and p38. In contrast, the expression levels of ERK1/2 and JNK1/3 MAPKs were unaffected by PMA or *A.bre*. We next studied the effect of PKCs on MAPKs activation in THP-1 cells induced by PMA, using the PKC-delta inhibitor rottlerin and pan PKC inhibitor Go6850. The results showed that the PKC-delta inhibitor rottlerin partially suppressed p-JNK protein level after 60-min treatment of PMA, while it did not inhibit p-ERK and p-p38 protein level. Moreover, co-treatment PKC-delta inhibitor rottlerin and *A.bre* completely inhibited p-JNK. We also assessed pan PKC inhibitor to THP-1 treated with PMA. As we can see, the pan PKC inhibitor significantly inhibited 3 MAPKs (Fig. 4B). These results suggested that *A.bre* could inhibit the inflammatory effects through suppressing the PKC/JNKs MAPK pathway.

The inhibitory effect of water extracted A.bre on PKC activity in PMA-treated THP-1 cells

To investigate whether *A.bre* suppresses the PKC activity, THP-1 cells were treated with 10 nM PMA in the presence or absence of *A.bre* (1/1600–1/400). We assessed the effects of *A.bre* on the PMA-induced phosphor-(Ser) PKC Substrate in THP-1 macrophages. As shown in Fig. 5, phosphor-(Ser) PKC Substrate level was enhanced with PMA treatment, whereas it was inhibited in a dose dependent manner by *A.bre*. These results suggested that *A.bre* regulated the JNKs MAPK signaling pathway via inhibiting PKC activity.

A.bre suppresses NF-κB translocation from cytosol to nucleus in PMA-stimulated THP-1 cells

To investigate whether the anti-inflammatory effects of *A.bre* are mediated via the NF-κB or AP-1 pathways, the nuclear translocation of p65, p50, and c-jun was

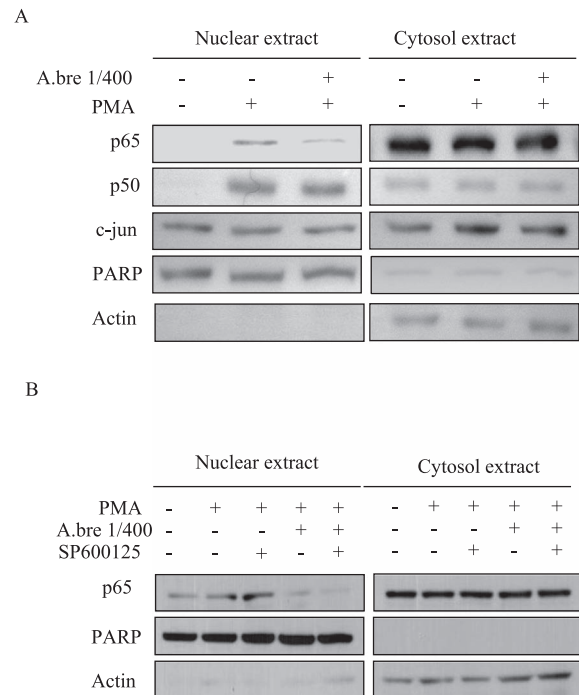


Fig. 6. Effects of water extracted *A.bre* and JNK inhibitor SP600125 on expression of transcription factors NF-κB and AP-1 in PMA-treated THP-1 cells. A) THP-1 cells were treated with water extracted *A.bre* (1/400) and stimulated by PMA (10 nM) in 60 min. The protein levels of p65, p50, and c-jun were measured by western blot analysis in the nucleus and cytosol extract. B) THP-1 cells were treated with *A.bre* (1/400) and/or JNK inhibitor SP600125 (10 μM), followed by PMA (10 nM) for 60 min. The protein level of p65 was measured by western blot analysis in the nucleus and cytosol extract.

detected by western blotting. As shown in Fig. 6A, the translocation of p65 into the nucleus was inhibited by *A.bre*, while translocations of p50 and c-jun were not affected by *A.bre* treatment. The data demonstrated that *A.bre* might exert the anti-inflammatory effects through the NF-κB pathway. We also elucidated the effect of JNK inhibitor on NF-κB p65 translocation. As shown in Fig. 6B, the JNK inhibitor SP600125 (10 μM) did not inhibit the NF-κB p65 translocation from cytosol to nucleus. These results indicated that the modulating effect of *A.bre* on NF-κB p65 translocation could not be mediated via the JNK MAPK pathway.

Effect of JNK inhibitor on IL-1β, CCL-5, and COX-2 expression

In order to investigate whether JNK MAPK regulates IL-1β, CCL-5, and COX-2 expression, we used the JNK inhibitor SP600125. THP-1 cells were seeded and treated with water extracted *A.bre* (1/400) and/or SP600125 (10 μM) followed by PMA (10 nM) for 24 h. Then mRNA levels of IL-1β and CCL-5 and COX-2 and

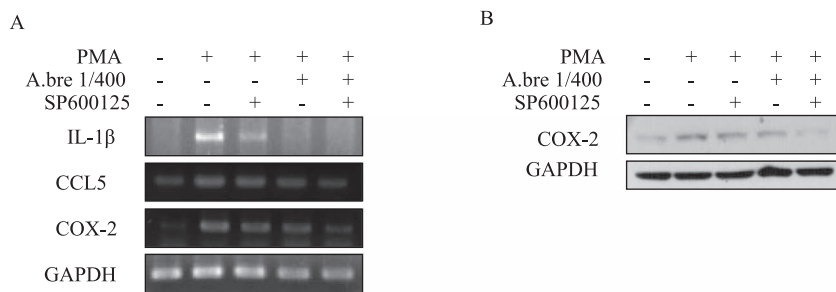


Fig. 7. Effect of water extracted *A.bre* on IL-1 β , CCL-5, and COX-2 expression. THP-1 cells were pre-treated with *A.bre* (1/1600 – 1/400) and/or JNK inhibitor SP600125 (10 μ M) for 60 min and then induced by PMA (10 nM) for 24 h. A) IL-1 β , CCL-5, and COX-2 transcript expression was measured by RT-PCR. B) COX-2 protein was expression measured by western blot analysis.

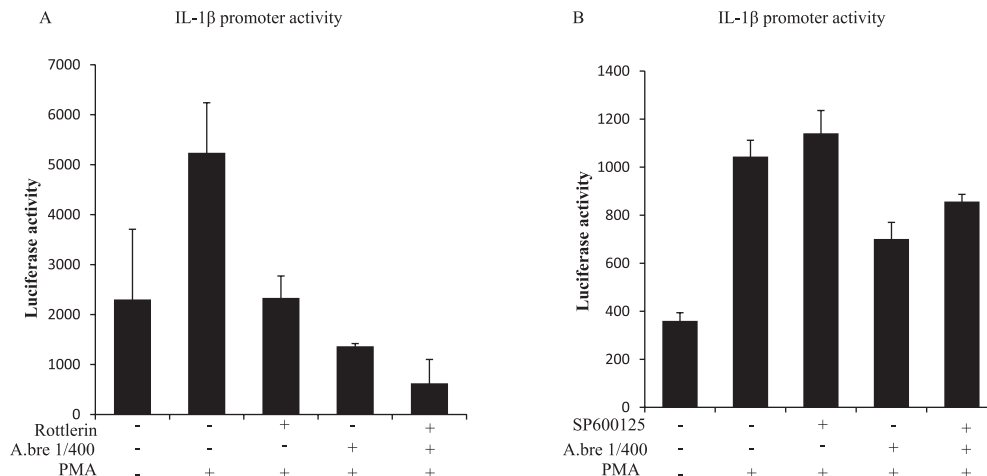


Fig. 8. The effect of water extracted *A.bre*, PKC δ inhibitor rottlerin, and JNK inhibitor SP600125 on IL-1 β promoter activity. THP-1 cells were transfected with IL-1 β promoter and incubated overnight. A) Cells were pre-treated with *A.bre* (1/400) or PKC δ inhibitor rottlerin (10 μ M) for 60 min and then induced by PMA for 24 h. IL-1 β promoter activity was measured using the Luciferase assay. B) Cells were pre-treated with *A.bre* (1/400) or JNK inhibitor SP600125 (10 μ M) for 60 min and then induced by PMA for 24 h. IL-1 β promoter activity was measured using the Luciferase assay.

COX-2 protein level were measured. The results showed that the JNK inhibitor SP600125 could partially inhibit IL-1 β , CCL-5, and COX-2 expression and additionally suppressed by co-treatment of the JNK inhibitor SP600125 and *A.bre*. The data demonstrated that *A.bre* inhibited IL-1 β , CCL-5, and COX-2 expression through JNK MAPK (Fig. 7: A and B).

A.bre inhibits IL-1 β promoter activity mediated via PKC activation in PMA-treated THP-1 cells

In order to inquire whether *A.bre* would inhibit IL-1 β promoter activity and which pattern this follows, the PKC delta inhibitor rottlerin and JNK inhibitor SP600125 were used as described. Luciferase activity of cells pretreated with *A.bre* or the PKC-delta inhibitor rottlerin was down-regulated compared to cells only treated with PMA. Moreover, *A.bre* and the PKC-delta inhibitor rottlerin have an additional inhibitory effect on IL-1 β promoter activity (Fig. 8A). However, JNK inhibitor could not affect the IL-1 β promoter activity (Fig. 8B).

This result showed that *A.bre* could regulate IL-1 β promoter activity via PKC activity in THP-1 stimulated by PMA.

Discussion

A.bre, a porcelain vine popular in Northeastern Asia, has been used to cure liver and inflammatory diseases in folk medicine. In the present study, we evaluated the anti-inflammatory effects and elucidated the mechanism of action of *A.bre* in PMA-stimulated THP-1 human monocytic leukemia cells. Inflammation is a complex process that is mediated by various stimuli, including the immune reaction. In the mammals, macrophages are the prime candidates for the innate immunity that defends against invading bacteria and their products. Without inflammation, wounds and infections would never heal. However, excessive inflammation can itself lead to a host of diseases, such as fever, asthma, rheumatoid arthritis, and even cancer. Cells from THP-1, a

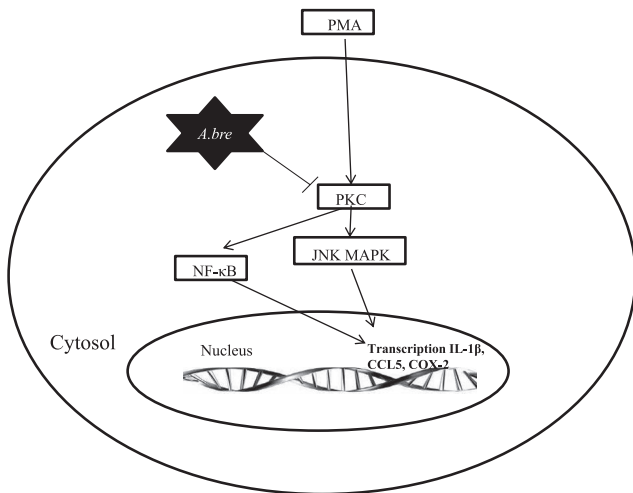


Fig. 9. Schematic illustration of water extracted *A.bre* on signaling pathways mediated by PMA in THP-1 cells. *A.bre* can inhibit the expression of IL-1 β , CCL-5, and COX-2 in PMA-stimulated macrophages via inhibition of the PKC-mediated JNK/NF- κ B signaling pathways.

human monocytic leukemia cell line, can be differentiated by PMA into macrophages (13). Macrophages are significantly activated during the inflammatory process by producing pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6. IL-1 β mediates the inflammatory effect as a major pro-inflammatory cytokine at the local and systemic levels (14 – 16). The CCL-5, also known as the regulated upon activation normal T cell-expressed and secreted RANTES protein, was first identified as a gene expressed during late T cell activation (17). Reportedly, CCL-5 plays pivotal roles in many diseases including asthma, rheumatoid arthritis, atherosclerosis, and cancer (18 – 22). To explore the potential anti-inflammatory effects of *A.bre*, the effects of *A.bre* on the production of pro-inflammatory cytokine IL-1 β and chemokine CCL-5 were examined. The results indicated that *A.bre* attenuated the mRNA levels as well as the secretion of IL-1 β and CCL-5 in PMA-stimulated THP-1 cells in a dose-dependent manner. COX-2 and iNOS are enzymes important to inflammation and injury repair. Over-expression of iNOS and COX-2 can induce tissues damage (23, 24). Our results showed that PMA-stimulated THP-1 macrophages decreased both mRNA level and protein expression of COX-2 by *A.bre*. However, iNOS expression was not affected by *A.bre* (data not shown). The activation of MAPK signaling has been confirmed in a variety of inflammatory diseases (25). The MAPKs are a family of protein serine/threonine kinases, including ERK, JNK, and p38 MAPK (26). The inhibition of any of these 3 MAPK pathways plays a necessary role in suppressing chronic inflammatory disorders (27).

Accordingly, we investigated the protein levels of phosphorylated JNK and ERK MAPKs in PMA-stimulated THP-1 cells. In our study, *A.bre* strongly decreased PMA-induced activation of p-JNK1/3, whereas it had no effect on ERK1/2 and p38 activation (Fig. 4A). PKC has been shown to be involved in the regulation of MAPKs by various stimuli in some cell types (28 – 30). There is a report demonstrating that depletion of PKC-delta with inhibitor rottlerin decreased neuropeptide substance P (SP)-induced ERK, JNK, NF- κ B, and AP-1 activation in pancreatic acinae cells (31). We addressed the effect of PKC-delta on MAPKs in PMA-induced THP-1 cells by using the PKC-delta inhibitor rottlerin and pan PKC inhibitor Go6850. Our findings suggested that PKC-delta could inhibit the JNK MAPK activity but could not inhibit ERK and p38 MAPKs in THP-1 cells induced by PMA. It also showed that other PKCs might regulate the ERK and p38 MAPK activation (Fig. 4B). Furthermore, our data indicated that *A.bre* could gradually inhibit PKC activity as phosphor-(Ser) PKC Substrate protein level was decreased by *A.bre* treatment in a dose-dependent manner in THP-1-induced by PMA (Fig. 5). Collectively, our results suggested that *A.bre* may trigger a vital anti-inflammatory role, possibly through inhibiting the PKC/JNK signaling pathway.

NF- κ B and AP-1 are the transcription factors that regulate the expression of genes involved in inflammation, differentiation, and proliferation. Therefore, NF- κ B and AP-1 are considered the major targets and key players in inflammatory diseases that regulate the inflammatory process (32). The blocking of NF- κ B translocation from cytosol into the nucleus suppresses the expression of IL-1 β and COX-2 and other inflammation cytokines (33 – 35). AP-1 is a ubiquitous protein that locates in the cytoplasm as a homo- or heterodimer protein with c-jun and c-fos families, while NF- κ B is composed of p65 and p50. As shown in Fig. 6A, *A.bre* suppressed p65 translocation from the cytosol into the nucleus, whereas it did not affect the translocation of p50 and c-jun. We also confirmed that the translocation of NF- κ B p65 was not affected by the JNK inhibitor SP600125 (Fig. 6B). The data demonstrated that *A.bre* pretreatment can block NF- κ B p65 activation and translocation of the NF- κ B p65 may not depend on the JNK MAPK signaling pathway. Many studies have shown that JNK MAPK regulates expression of IL-1 β , CCL-5, and COX-2 (36 – 38). Our study demonstrated that JNK MAPK participates in expression of IL-1 β , CCL-5, and COX-2; and *A.bre* inhibited these streams of pathways in THP-1 cells induced by PMA. We verified that PMA-induced IL-1 β , CCL-5, and COX-2 expressions in THP-1 cells were partially inhibited by the JNK inhibitor SP600125. Furthermore, when we co-treated the cells

with *A.bre* and the JNK inhibitor SP600125, the inhibition of CCL-5 and COX-2 expression was increased (Fig. 7). Interestingly, the JNK inhibitor SP600125 could not affect IL-1 β promoter activity, while the PKC inhibitor rottlerin could significantly diminish IL-1 β promoter activity (Fig. 8). These data indicated that *A.bre* could mediate the IL-1 β promoter activity via PKC activity. On the other hand, the down-regulation on IL-1 β expression of JNK MAPK may undergo post-transcriptional control (39–41). In summary, these studies demonstrated that *A.bre* can inhibit the expression of IL-1 β , CCL-5, and COX-2 in PMA-stimulated THP-1 cells by suppressing both PKC/JNK/NF- κ B signaling pathways (Fig. 9). Therefore, *A.bre* can be effective anti-inflammatory components that may be a potential agent for preventing inflammatory diseases.

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References

- Choy E, Ganeshalingam K, Semb AG, Szekanecz Z, Nurmohamed M. Cardiovascular risk in rheumatoid arthritis: recent advances in the understanding of the pivotal role of inflammation, risk predictors and the impact of treatment. *Rheumatology* (Oxford). 2014. In press.
- Duran-Salgado MB, Rubio-Guerra AF. Diabetic nephropathy and inflammation. *World J Diabetes*. 2014;5:393–398.
- Ling S, Feng T, Jia K, Tian Y, Li Y. Inflammation to cancer: the molecular biology in the pancreas (Review). *Oncol Lett*. 2014;7:1747–1754.
- Kahles F, Findeisen HM, Bruemmer D. Osteopontin: a novel regulator at the cross roads of inflammation, obesity and diabetes. *Mol Metab*. 2014;3:384–393.
- Michee S, Brignole-Baudouin F, Riancho L, Rostene W, Baudouin C, Labbe A. Effects of benzalkonium chloride on THP-1 differentiated macrophages in vitro. *PLoS One*. 2013;8:e72459.
- Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, et al. Interleukin-1 β -mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature*. 2001;410:471–475.
- Marques RE, Guabiraba R, Russo RC, Teixeira MM. Targeting CCL5 in inflammation. *Expert Opin Ther Targets*. 2013;17:1439–1460.
- Yabe N, Tanaka K, Matsui H. An ethanol-extract of *Ampelopsis brevipedunculata* (Vitaceae) berries decreases ferrous iron-stimulated hepatocyte injury in culture. *J Ethnopharmacol*. 1998;59:147–159.
- Yabe N, Matsui H. *Ampelopsis brevipedunculata* (Vitaceae) extract inhibits a progression of carbon tetrachloride-induced hepatic injury in the mice. *Phytomedicine*. 2000;7:493–498.
- Pang R, Tao JY, Zhang SL, Chen KL, Zhao L, Yue X, et al. Ethanol extract from *Ampelopsis sinica* root exerts anti-hepatitis B virus activity via inhibition of p53 pathway in vitro. *Evid Based Complement Alternat Med*. 2011;2011:939205.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65:55–63.
- Kim MS, Kang JW, Lee DH, Bak Y, Park YS, Song YS, et al. IL-32 θ negatively regulates IL-1 β production through its interaction with PKC δ and the inhibition of PU.1 phosphorylation. *FEBS Lett*. 2014;588:2822–2829.
- Park EK, Jung HS, Yang HI, Yoo MC, Kim C, Kim KS. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm Res*. 2007;56:45–50.
- Gao D, Madi M, Ding C, Fok M, Steele T, Ford C, et al. Interleukin-1 β mediates macrophage-induced impairment of insulin signaling in human primary adipocytes. *Am J Physiol Endocrinol Metab*. 2014;307:E289–E304.
- Dinarello CA. Proinflammatory cytokines. *Chest*. 2000;118:503–508.
- Wojdasiewicz P, Poniatowski LA, Szukiewicz D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators Inflamm*. 2014;2014:561459.
- Schall TJ, Jongstra J, Dyer BJ, Jorgensen J, Clayberger C, Davis MM, et al. A human T cell-specific molecule is a member of a new gene family. *J Immunol*. 1988;141:1018–1025.
- Alam R, York J, Boyars M, Stafford S, Grant JA, Lee J, et al. Increased MCP-1, RANTES, and MIP-1 α in bronchoalveolar lavage fluid of allergic asthmatic patients. *Am J Respir Crit Care Med*. 1996;153:1398–1404.
- Boiardi L, Macchioni P, Meliconi R, Pulsatelli L, Facchini A, Salvarani C. Relationship between serum RANTES levels and radiological progression in rheumatoid arthritis patients treated with methotrexate. *Clin Exp Rheumatol*. 1999;17:419–425.
- Braunersreuther V, Steffens S, Arnaud C, Pelli G, Burger F, Proudfoot A, et al. A novel RANTES antagonist prevents progression of established atherosclerotic lesions in mice. *Arterioscler Thromb Vasc Biol*. 2008;28:1090–1096.
- Robinson SC, Scott KA, Wilson JL, Thompson RG, Proudfoot AE, Balkwill FR. A chemokine receptor antagonist inhibits experimental breast tumor growth. *Cancer Res*. 2003;63:8360–8365.
- Soria G, Ben-Baruch A. The inflammatory chemokines CCL2 and CCL5 in breast cancer. *Cancer Lett*. 2008;267:271–285.
- Misko TP, Trotter JL, Cross AH. Mediation of inflammation by encephalitogenic cells: interferon gamma induction of nitric oxide synthase and cyclooxygenase 2. *J Neuroimmunol*. 1995;61:195–204.
- Alhouayek M, Muccioli GG. COX-2-derived endocannabinoid metabolites as novel inflammatory mediators. *Trends Pharmacol Sci*. 2014;35:284–292.
- Kyriakis JM, Avruch J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiol Rev*. 2012;92:689–737.
- Geest CR, Coffey PJ. MAPK signaling pathways in the regulation of hematopoiesis. *J Leukoc Biol*. 2009;86:237–250.
- Arthur JS, Ley SC. Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol*. 2013;13:679–692.
- Shigemoto-Mogami Y, Koizumi S, Tsuda M, Ohsawa K, Kohsaka S, Inoue K. Mechanisms underlying extracellular ATP-evoked interleukin-6 release in mouse microglial cell line, MG-5. *J Neurochem*. 2001;78:1339–1349.

- 29 Comalada M, Xaus J, Valledor AF, Lopez-Lopez C, Pennington DJ, Celada A. PKC epsilon is involved in JNK activation that mediates LPS-induced TNF-alpha, which induces apoptosis in macrophages. *Am J Physiol Cell Physiol*. 2003;285:C1235–C1245.
- 30 Shibukawa Y, Takahashi M, Laffont I, Honke K, Taniguchi N. Down-regulation of hydrogen peroxide-induced PKC delta activation in N-acetylglucosaminyltransferase III-transfected HeLaS3 cells. *J Biol Chem*. 2003;278:3197–3203.
- 31 Ramnath RD, Sun J, Adhikari S, Zhi L, Bhatia M. Role of PKC-delta on substance P-induced chemokine synthesis in pancreatic acinar cells. *Am J Physiol Cell Physiol*. 2008;294:C683–C692.
- 32 Roebuck KA, Carpenter LR, Lakshminarayanan V, Page SM, Moy JN, Thomas LL. Stimulus-specific regulation of chemokine expression involves differential activation of the redox-responsive transcription factors AP-1 and NF-kappaB. *J Leukoc Biol*. 1999;65:291–298.
- 33 Yang DJXue J, Dong JH, Huang GD, Qu XF, Wu G, Dong XR. NF-kappaB signaling modulates radiationinduced microglial activation. *Oncol Rep*. 2014;31:2555–2560.
- 34 Yang DJ, Chang YY, Lin HW, Chen YC, Hsu SH, Lin JT. Inhibitory effect of litchi (*Litchi chinensis* Sonn.) flower on lipopolysaccharide-induced expression of proinflammatory mediators in RAW264.7 cells through NF-kappaB, ERK, and JAK2/STAT3 inactivation. *J Agric Food Chem*. 2014; 62:3458–3465.
- 35 Bak MJ, Truong VL, Kang HS, Jun M, Jeong WS. Anti-inflammatory effect of procyanidins from wild grape (*Vitis amurensis*) seeds in LPS-induced RAW 264.7 cells. *Oxid Med Cell Longev*. 2013;2013:409321.
- 36 Choi EM, Lee YS. Luteolin suppresses IL-1beta-induced cytokines and MMPs production via p38 MAPK, JNK, NF-kappaB and AP-1 activation in human synovial sarcoma cell line, SW982. *Food Chem Toxicol*. 2010;48:2607–2611.
- 37 Tsai MH, Lin ZC, Liang CJ, Yen FL, Chiang YC, Lee CW. Eupafolin inhibits PGE2 production and COX2 expression in LPS-stimulated human dermal fibroblasts by blocking JNK/AP-1 and Nox2/p47(phox) pathway. *Toxicol Appl Pharmacol*. 2014;279:240–251.
- 38 Lam KP, Chu YT, Kuo CH, Wang WL, Tok TS, Chin YY, et al. Suppressive effects of procaterol on expression of IP-10/CXCL 10 and RANTES/CCL 5 by bronchial epithelial cells. *Inflammation*. 2011;34:238–246.
- 39 Mercer TR, Dinger ME, Bracken CP, Kolle G, Szubert JM, Korbie DJ, et al. Regulated post-transcriptional RNA cleavage diversifies the eukaryotic transcriptome. *Genome Res*. 2010;20:1639–1650.
- 40 Fadda A, Ryten M, Droll D, Rojas F, Farber V, Haanstra JR, et al. Transcriptome-wide analysis of trypanosome mRNA decay reveals complex degradation kinetics and suggests a role for co-transcriptional degradation in determining mRNA levels. *Mol Microbiol*. 2014;94:307–326.
- 41 Jiao X, Chang JH, Kilic T, Tong L, Kiledjian M. A mammalian pre-mRNA 5' end capping quality control mechanism and an unexpected link of capping to pre-mRNA processing. *Mol Cell*. 2013;50:104–115.