



Inhibitory effect of epigallocatechin from *Camellia sinensis* leaves against pro-inflammatory mediator release in macrophages

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Abstract To investigate the anti-inflammatory activity of natural products, we determined the anti-inflammatory activity of purified epigallocatechin (EGC) from *Camellia sinensis* leaves. In the present study, we found that EGC inhibited the production of pro-inflammatory mediators (IL-6, TNF- α , NO, and PGE₂) in lipopolysaccharide (LPS)-stimulated Raw 264.7 cells. Suppression of IL-6 seems to be at least partly attributable to the inhibitory effect of EGC. TNF- α is a major cytokine produced by LPS-induced macrophages, and they have a wide variety of biological functions including regulation of inflammation. The inhibition of IL-6 and TNF- α production by EGC may downregulate the acute-phase response to LPS, thereby reducing LPS-induced inflammation. In addition to IL-6 and TNF- α , EGC effectively reduced the production of other key inflammatory mediators, including NO and PGE₂. The inhibitory effect of EGC on NO and PGE₂ production was supported by the suppression of inducible nitric oxide synthase and COX-2 at protein levels. These results support the traditional use of EGC in the alleviation of various inflammation-associated diseases and suggest that EGC might be useful in the development of new functional foods for inflammatory diseases.

Keywords Anti-inflammation · *Camellia sinensis* · Cytokine · Epigallocatechin · Raw 264.7 cells

Introduction

Green tea has long been considered a beverage in North Eastern Asia. It contains polyphenols, caffeine, theophylline, amino acids, vitamins, minerals, and many other compounds. The polyphenols in green tea play an important role in imparting scent, color, and taste. It also has antioxidant, anti-aging, cancer cell growth inhibition, sterilization, and many other biological activities (Freudenberg 1920; Cowan 1999; Alessio et al. 2002; Chang et al. 2002). The catechins present in green tea are epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate, epicatechin (EC), and others. EGCG comprises 40% of the total polyphenol content and has been studied for its strong biological activity. Several researches on the compounds of green tea (Yosioka et al. 1970; Yosioka et al. 1971; Rah et al. 1992) and their anti-cancer activity (Liao et al. 1995; Stoner and Mukhtar 1995; Yamane et al. 1995) have reported that green tea can be used as a safe and economical source for the inhibition of prostate cancer and skin diseases (Liao and Hiipakka 1995).

The type of tannin in green tea leaf is widely known and categorized into condensed tannin and hydrolysable tannin depending on its structure (Haslam 1966; Ham et al. 1997; Cho et al. 2011). Condensed tannin is a flavan-3-ol derivative. Epicatechin and catechin type is widely known. Also, it produces anthocyanidin type compounds when treated with strong acid. Therefore, the compound follows the name of proanthocyanidin (Butler et al. 1982). Tannins are water-soluble phenolic compounds known for their adverse effect on viruses, bacteria, fungi, insects, reptiles, birds, and mammals (Swain et al. 1979), and they can be found widely in vascular plants (Bate 1973; Swain et al. 1979). They play an important role in protecting plant tissues from herbivores (Feeny 1970; Roades and Cates 1976). Recently, Bernays (1981) reported the differences in the effect of tannins on herbivores and insects. However, Bate and Metcalfe (1957) have cautioned against premature generalizations regarding their ecological and evolutionary significances.

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The researches on green tea tannins were mainly focused on epigallocatechin-3-gallate (Park et al. 2004). In addition, several studies have been conducted on the antimutagenic, antibacterial, cholesterol lowering, antioxidant, anti-tumor, and cancer preventive activities of EGCG (Kada et al. 1985; Muramatsu et al. 1986; Senji et al. 1989; Hayashi et al. 1990; Kazuko et al. 1991; Ikabe et al. 1991; Juwon and Hyosun 1993; Toyoshima et al. 1994). However, there have been no researches on EGC, owing to its low content. Although other tannin compounds are continually being identified (Liao and Hiipakka 1995; Valcic et al. 1996), researches on EGC, which has remarkable biological activity, is thought to be necessary. EGC is known to have higher content in unfertilized green tea than oolong tea and black tea (Lee et al. 1998).

In this study, we separated EGC and investigated its anti-inflammatory effect in LPS-stimulated Raw 264.7 cells.

Materials and Methods

Preparation of extracts from *Camellia sinensis*

The sample used in this study was purchased from a market at Daegu in Korea. Fresh leaves were dried in an oven at 45 °C for 24 h and ground to 40-mesh size. To separate compound with anti-inflammatory activity, water, ethanol, methanol, acetone and butanol as various solvents were used for extraction and then the extracts were used to determine NO production inhibitory effect at macrophage cell to identify anti-inflammatory effect. Producing extract for purification of compound with anti-inflammatory activity, 100 mL of ethanol was added to 1 g of the ground powder, homogenized for 1 min at 20,000 rpm using a homogenizer, and stirred for 24 h before extraction. The extract was filtered using Whatman No. 1 filter paper (Piscataway, NJ, USA) and concentrated using a rotary vacuum evaporator (Eyela NE, Tokyo, Japan), if necessary.

Determination of phenolic content in the extracts

The total phenolic content of each extract was determined using the Folin-Denis method (Folin and Denis 1912). Briefly, 0.5 mL of 1 N Folin-Ciocalteu reagent (Junsei Chemical Co., Tokyo, Japan) was added to 1 mL of the extract mixed with 5 mL of distilled water and 1 mL of 95% ethanol. Na₂CO₃ solution (5%, 1 mL) was added after 5 min. The optical density (OD) at 725 nm was determined within 1 h using an UV-visible spectrophotometer (Optitizen 3220UV, Mecasys Co., Ltd., Daejeon, Korea). The total phenolic content was determined using a standard curve of gallic acid (Sigma Co., St, Louis, MO, USA).

Conditions for column chromatography purification

Lyophilized ethanol extract of *C. sinensis* was solvent fractionated using H₂O/ethyl acetate/n-BuOH (200 mL each) in order and H₂O, ethyl acetate, and n-BuOH fractions were obtained. The

solvent from each *C. sinensis* fraction was removed and diluted with distilled water. The compound was purified by chromatography system. Sephadex LH-20 column (Pharmacia Co., Uppsala, Sweden) was used, and the compounds were separated based on their adsorption characteristics. Ethanol (60%) was used as the eluting solvent in the normal phase column. The extract was eluted with ethanol→water mixture (100→0%) in the order of polarity at a flow rate of 20 mL per minute. Compound with anti-inflammatory activity was concentrated and dried in the TLC phase after identifying the presence of a phenolic compound and type. Purification to single compound, Reverse phase chromatography was performed using a high-porosity polystyrene gel (MCI gel) (Supelco, Bellefonte, PA, USA) and eluted with H₂O→MeOH (0→100%) solvent. The phenol compound was separated on a TLC plate (Sigma Co.).

Identification of the chemical structure of the purified compound

The melting point of the sample (1 mg) was determined using a microelectrothermal system (Fisher-Jhons, Mexico City, Mexico). Purified alkali halides were used for Infrared spectrometry (IR) (Perkin-Elmer IG-1330, Perkin Elmer, Waltham, MA, USA). The separated sample (1 mg) and KBr (100 mg) powder were mixed, and the purified compound was obtained by applying pressure. In ¹H and ¹³C-Nuclear Magnetic Resonance (NMR) spectroscopy (ARX-250, Burker Co., Billerica, MA, USA), Pulse Fourier Transform method was performed by eluting pure refined sample (10 mg), and the determining solvent was CDCl₃+DMSO-D₆+D₂O at 5–20% (w/v) with [TMS (Tetramethylsilane), (CH₃)₄Si] as the standard. Then, the proton magnetic resonance (PMR) was measured at 300 MHz. The negative ion fast atom bombardment (FAB)-mass spectrum (Jeol JMX-PX 300, Tokyo, Japan) was determined by chemical ionization method using the solid sample (1 mg) at decompressed phase (10⁻⁴–10⁻⁶ mmHg). Thioglycerol was used as a determining solvent, and the emitter electric current at 22–28 eV was selected as the determining condition. Mass spectrometry analysis was performed with accelerated ion source pressure at 6–7 kV.

Cell culture for the determination of anti-inflammatory effect

The murine macrophage cell line Raw 264.7 was purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured at 37 °C and 5% CO₂ with Dulbecco's modified Eagle's medium (DMEM) (Hyclone, GE Healthcare Life Sciences, Chicago, IL, USA) containing 10% fetal bovine serum (FBS) (Hyclone, GE Healthcare Life Sciences) and 1% penicillin/streptomycin (Hyclone, GE Healthcare Life Sciences) 100 unit/mL. The cells were maintained at 5% CO₂ and subcultured to a density of 2–3×10⁶/mL in a cell culture 75T plate. During the experiment, 80% confluency and 20 passages were maintained. The cells were cultured for 12 h in FBS-free medium before the experiment (Cho and An 2008; Lee 2011).

Determination of cell toxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Raw 264.7 cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin (Sigma Co.) at 37 °C in a humidified atmosphere with 5% CO₂, unless specified otherwise. Cell toxicity was determined as described previously (Carmichael et al. 1987). The cells were seeded in a 96-well plate at a density of 5×10⁴ cells/mL. Then, 0.02 mL of the extract at different concentrations was added, and the cells were incubated at 37 °C for 24 h. The MTT solution (5 mg/mL, 0.02 mL/well) was added, and the culture medium was collected after 4 h. The culture media from each well were incubated with 0.15 mL of dimethyl sulfoxide (DMSO) at room temperature for 30 min and OD₅₄₀ was measured. Cell toxicity was determined by comparing the absorbance of the treated and untreated cells using the following formula: (1–absorbance of control/absorbance of sample)×100. The control cells were treated with distilled water.

Determination of NO levels

NO levels were determined by measuring the nitrite amount in the cell culture medium using the Griess reagent (Sigma Co.). Raw 264.7 cells (1×10⁶ cells/mL) were washed two times with phosphate buffered saline (PBS) and cultured in serum-free medium (Hyclone, GE Healthcare Life Sciences) for 12 h. Then, the cells were stimulated with 1 µg LPS for 2 h, followed by the addition of the extract at different concentrations. The control was stimulated with LPS for 2 h without adding the extracts. The supernatants were collected after 24 h, and the NO levels were determined by measuring OD₅₂₀ 10 min after the addition of Griess reagent (Sigma Co.). Data are expressed relative to NO production in LPS-treated cells, which was set to 100%. NO (%) was expressed as (1–absorbance of control/absorbance of sample)×100.

Determination of inducible nitric oxide synthase (iNOS) and COX-2 protein expression using the western blot analysis

To determine iNOS protein expression, Raw 264.7 cells were cultured for 24 h in a 6-well plate at 5×10⁵ cells/mL. Then, the cells were treated with different concentrations of extracts for 24 h. The medium was removed and the cells were washed two times with PBS. The cell suspension (100 µL) was added to the Mammalian protein extraction reagent (M-PER) buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitors (complete Mini 1 tablet added to 10 mL M-PER buffer) followed by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was collected, transferred to a new tube, and stored at 20 °C until further use or for the determination of protein concentration using the Bradford assay.

The proteins (20 µL) were separated on 10% sodium dodecyl sulfate polyacrylamide gels (Sigma Co.). The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA) by using a semi-dry transfer cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA) and

incubated for 1 h with blocking buffer [5% skim milk in Tris-buffered saline containing Tween-20 (TBST)] at room temperature. The membrane was washed with TBST three times (10 min each) and incubated overnight with primary antibodies against iNOS (1:1,000; BD Biosciences, San Jose, CA, USA), COX-2 (1:1,000; Cayman Chemicals, Ann Arbor, MI, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4 °C. The membrane was washed with TBST three times (10 min each) and incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG (1:1,000; Santa Cruz Biotechnology) and HRP-conjugated bovine anti-goat IgG (1:1,000; Santa Cruz Biotechnology). After washing 3 more times, the membranes were incubated with enhanced chemiluminescence reagent (Millipore, Bedford, MA, USA) in a darkroom and exposed to an X-ray film. The intensity of each band was determined by using a Molecular Imager (Bio-Rad Laboratories) (Syu-ichi et al. 2005; Cho and An 2008; Cho 2011).

Determination of PGE₂ levels

Raw 264.7 cells were cultured in 6-well culture plates at a density of 5×10⁵ cells/mL for 24 h. After 18–24 h, the cells were treated with LPS, and the supernatants were collected and stored at 70 °C. PGE₂ levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). Calibrator diluent RD5-56 was added to the wells as follows: 200 µL to the non-specific binding (NSB) well, 150 µL to the zero standard (B₀) well, and 150 µL to other cells. Then, the primary antibody solution (50 µL) was added to all wells, except the NSB well. The culture plate was covered and incubated for 1 h at room temperature with shaking. PGE₂ conjugate (50 µL) was added to all wells, followed by 2 h of incubation with shaking and rinsing four times with a washing buffer. The substrate solution (200 µL) was added after washing, and the plate was incubated at room temperature for 30 min in the dark. Then, 100 µL of the stop solution was added and OD₄₅₀ was measured.

Cytokine assay

The cultured macrophages were inoculated at a density of 5×10⁵/mL cells in a 6-well plate. They were then treated with different agonist (cell stimulator) concentrations every hour. After LPS treatment for 1 h, the cytokine levels were determined by treating the cells in cell culture medium hourly for 24 h. The collected medium was stored at –70 °C before determination of cytokine levels. Enzyme immunoassay kit was used for determining the levels of TNF-α, IL-1β, and IL-6. Each cytokine level was converted using the standard curve obtained from the reaction of the standard material (Anfernee et al. 2005; Syu-ichi et al. 2005; Iwona et al. 2006).

Statistical analysis

All tests were repeated three times and statistical analyses were

performed using the SAS program (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range tests were used to analyze the differences. $p < 0.05$ was considered to be statistically significant.

Results and Discussion

The phenolic content and NO production inhibitory effect at macrophage cell in various solvent extracts and optimal concentration for extraction

To extract phenolic compound from green tea leaf as bio-activity compound, water, ethanol, methanol, acetone, butanol as solvents were used for extraction. Eluted phenolic compound in solvent was determined as Fig. 1A. Also, No production amount was determined using LPS stimulated Raw 264.7 macrophage cell with treating extracts (Fig. 1B). As a result, ethanol and acetone extract showed the highest phenolic content. Also in anti-inflammatory effect determination, ethanol extract showed high NO inhibitory effect up to 40% compared to control group. The highest anti-inflammatory effect was shown with the ethanol extract and determined phenolic compound in concentration dependent manner shown as Fig 1C showing that 60% ethanol showed the highest phenolic compound of 16.41 ± 0.37 mg/g. Therefore, 60% ethanol extract was used for further experiment.

Purification and identification of the compound from *C. sinensis* leaves

Lyophilized 60% ethanol extract of *C. sinensis* (30 g) was solvent fractionated using H₂O/ethyl acetate/n-BuOH (200 mL each) in order and H₂O, ethyl acetate, and n-BuOH fractions were obtained. The solvent from each *C. sinensis* fraction was removed and diluted with distilled water. The compound was purified by chromatography system (Sephadex LH 20 and MCI gel CHP column). The purified compound was uncolored and in powder form, with a melting point of 217–219 °C. The chemical structure of EGC was identified by using ¹H NMR (CDCl₃, 300 MHz) spectrum. The result of ¹H NMR is as follows: PMR 2.56 (1 H, dd, $J=16.3$ Hz, 4-H), 2.70 (1 H, dd, $J=16.4$ Hz, 4-H), 4.12 (1 H, m, 3-H), 4.73 (1 H, brs, 2-H), 5.87, 5.98 (each 1 H, d, $J=2$ Hz, 6, 8-H), 6.51 (2 H, s, 2',6'-H). ¹³C NMR is as follows: 31.4, 66.3, 81.2, 95.7, 96.1, 100.7 ppm. The molecular weight of the purified compound was determined to be 305 using FAB-MS, and it was identified as a compound having an extra oxygen molecule added to (–)EC and (+)catechin. The result of the PMR spectrum was 4-H of 2.56 ppm (dd), 2.70 ppm (dd) and 2,3-H spectrum of 4.12 ppm (m), 4.73 ppm (brs), which was similar to the PMR spectrum of (–)EC but showed difference of 2',6'-H in singlet of 2 hydrogen doses at 6.51 ppm; therefore, the compound was identified as (–)EGC (Cho et al. 1993).

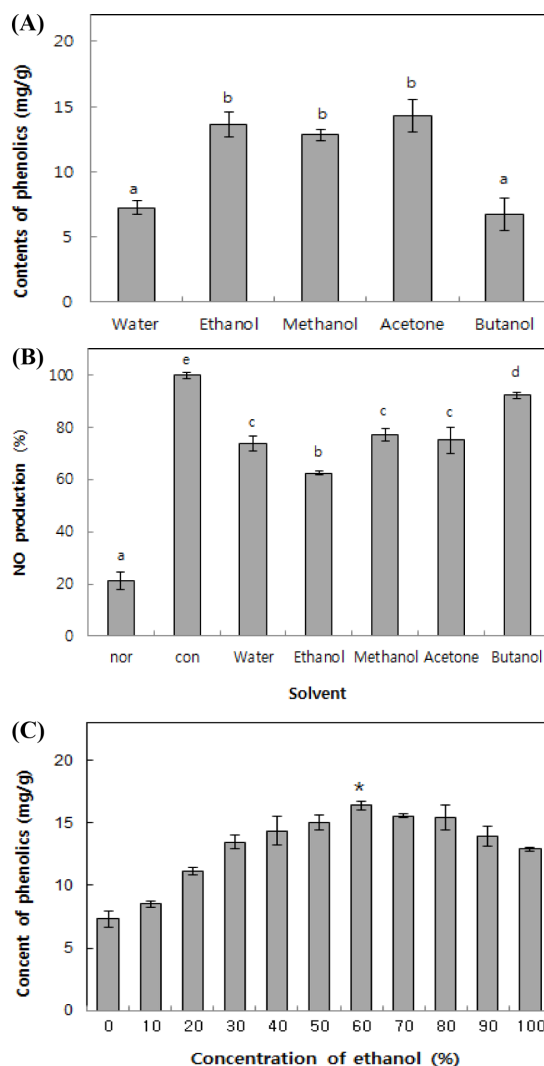


Fig. 1 The phenolic content in extracts by various solvent (A) and effect of NO production (B) by various solvent extracts on LPS-induced pro-inflammatory cytokine expression in Raw 264.7 cells and the change of phenolic content in extract by ethanol concentration (C). The cells were treated with the indicated concentrations of EGC or LPS or both (1 μ g/mL) for 24 h. The results were expressed as percentages relative to the untreated control. The results were expressed as percentages relative to the untreated control. ^{a-c}Means \pm error ($n=3$), the means with different superscript letters within the column were observed to be significantly different at $p < 0.05$ by Duncan's multiple range tests

EGC inhibits IL-6 and TNF- α production, but not IL-1 β in LPS-stimulated Raw 264.7 cells

TNF- α , IL-1 β , and IL-6 are representative pro-inflammatory cytokines produced by the macrophages. To determine the potential inhibitory effects of EGC on LPS-induced pro-inflammatory cytokine release, Raw 264.7 cells were incubated with EGC in the absence or presence of LPS for 24 h, and the cytokine levels were

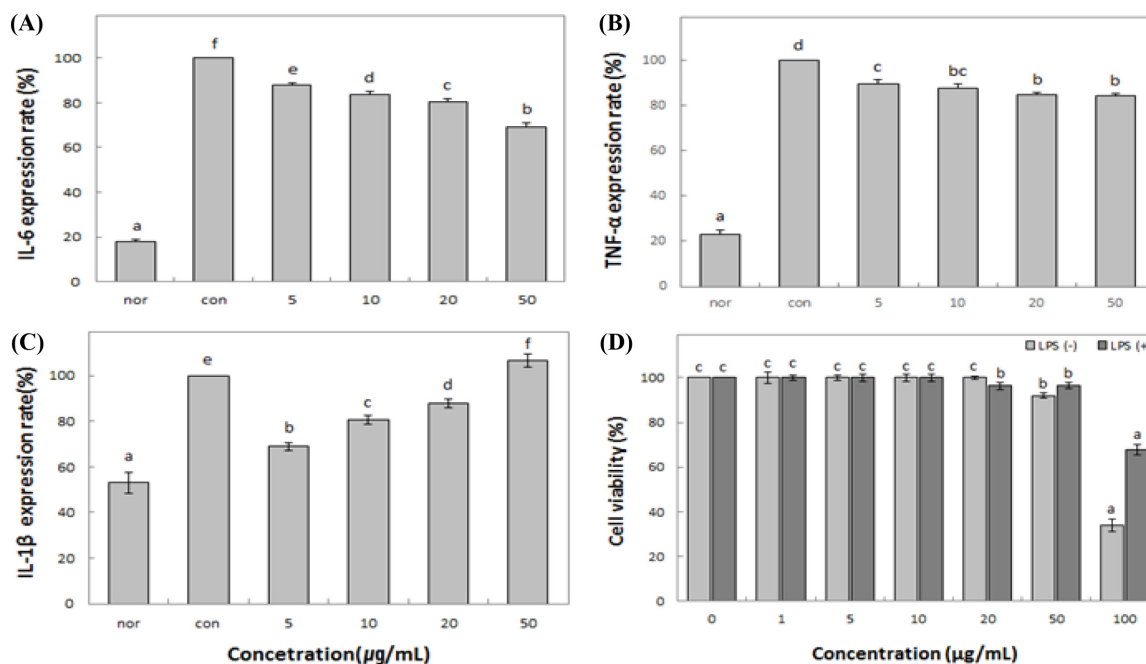


Fig. 2 Effects of EGC on LPS-induced pro-inflammatory cytokine expression in Raw 264.7 cells. The cells were treated with the indicated concentrations of EGC or LPS or both (1 µg/mL) for 24 h. The levels of IL-6 (A), TNF-α (B), and IL-1β (C) in the culture medium were measured using ELISA. The results were expressed as percentages relative to the untreated control. Cell viability (D) after treatment with EGC or LPS or both (1 µg/mL) for 24 h was examined using the MTT assay, and the results were expressed as percentages relative to the untreated control. ^{a-f}Means ± standard deviation ($n=3$), the means with different superscript letters within the column were observed to be significantly different at $p < 0.05$ by Duncan's multiple range tests

evaluated in the culture supernatant. LPS significantly induced IL-1β release following incubation (Figs. 2A, B, C). Co-treatment with EGC significantly and dose-dependently inhibited the LPS-induced release of IL-6 and TNF-α. EGC treatment did not inhibit IL-1β release, but increased its production instead. The inhibitory effect of EGC on the expression of pro-inflammatory cytokines might be owing to its toxicity in LPS-stimulated Raw 264.7 cells. To exclude this possibility, a cell toxicity assay was performed on Raw 264.7 cells treated with EGC alone or together with LPS for 24 h. EGC treatment with or without LPS did not affect cell viability (Fig. 2D); no obvious toxicity was observed for EGC even at 50 µg/mL. However, toxicity was observed at 100 µg/mL, showing approximately 35% cell viability. Notably, in our study, EGC strongly inhibited the expression of IL-6 and TNF-α, but not of IL-1β, in LPS-stimulated Raw 264.7 cells. This suggests that EGC may suppress LPS-induced inflammation by selectively inhibiting IL-6 and TNF-α expression. IL-6 has a potent role as a stimulus for acute-phase response in the earliest stages of inflammation. Similar anti-inflammatory effect was observed for chokeberry extract (Apple et al. 2015).

EGC inhibits NO and PGE₂ in LPS-stimulated Raw 264.7 cells

NO production is important in removing tumor or in terminating bacteria; however, excess NO formation causes inflammation,

organ damage, gene mutation, or neuronal damage (Harris et al. 2002; Lee et al. 2003; Liao et al. 2004). The two most important steps involved in inflammation are the production of NO and PGE₂ from iNOS and COX-2, respectively. To examine whether EGC could affect the production of NO and PGE₂ in LPS-stimulated Raw 264.7 cells, the levels of NO and PGE₂ in the cell culture medium were measured using the Griess reaction and ELISA assay, respectively. LPS markedly induced NO production in cells not treated with EGC (Fig. 3A). EGC significantly inhibited NO production in a dose-dependent manner as 28.6% at 50 µg/mL concentration. Additionally, 24 h of exposure to LPS resulted in a marked increase in PGE₂ release in cells not treated with EGC (Fig. 3B). However, EGC slightly inhibited LPS-mediated PGE₂ production in a dose-dependent manner. Purified EGC showed higher NO production inhibitory effect as 72.7% at 50 µg/mL concentration than extract.

EGC inhibits iNOS and COX-2 expression in LPS-stimulated Raw 264.7 cells

NO is produced from NOS. LPS-induced iNOS expression resulted in the production of excess amount of NO for a long period. NO resulted in the activation of guanylyl cyclase, which led to cell toxicity. Therefore, in Raw 264.7 cells, LPS-induced decrease in iNOS protein level was expected to produce an anti-inflammation effect. Previous studies have reported that in

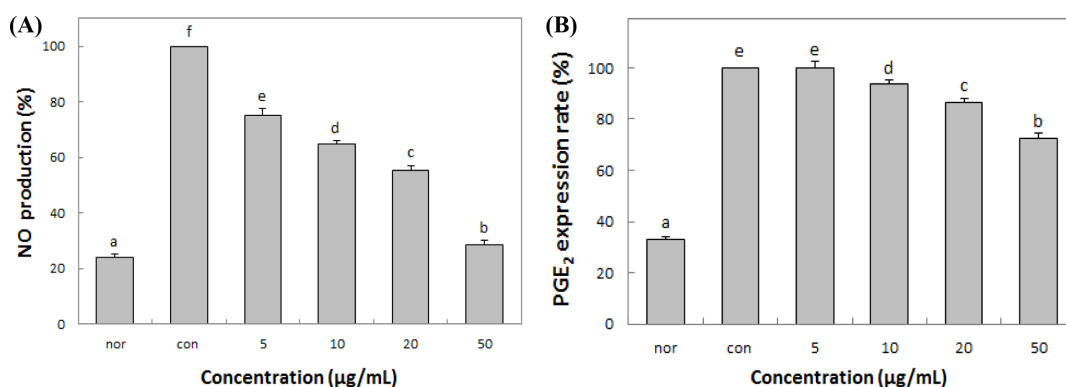


Fig. 3 Effects of EGC on LPS-induced NO and PGE₂ production in Raw 264.7 cells. Raw 264.7 cells were stimulated with the indicated concentrations of EGC in the presence or absence of LPS (1 µg/mL) for 18 h. The amount of NO (A) in the culture medium was determined using the Griess reaction. PGE₂ levels (B) in the cell culture medium were detected by ELISA assay. ^{a-f}Means ± standard deviation ($n=3$), the means with different superscript letters within the column were observed to be significantly different at $p<0.05$ by Duncan's multiple range tests

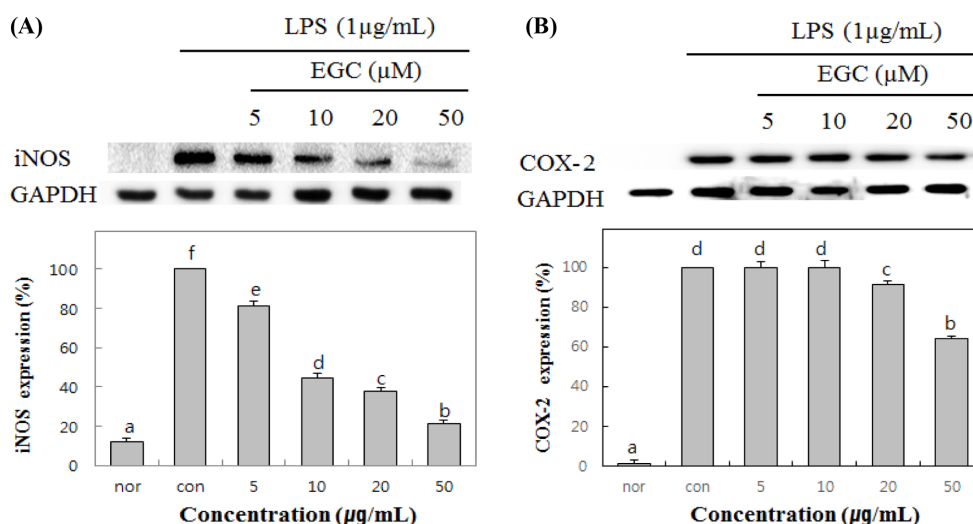


Fig. 4 Effects of EGC on the expression of iNOS and COX-2 in LPS-stimulated Raw 264.7 cells. Raw 264.7 cells were treated with the indicated concentrations of EGC or LPS or both (1 µg/mL) for 24 h. The protein levels of iNOS (A) and COX-2 (B) were determined using western blot analysis. The graph shows the quantification results normalized to GAPDH levels. ^{a-f}Means ± standard deviation ($n=3$), the means with different superscript letters within the column were observed to be significantly different at $p<0.05$ by Duncan's multiple range tests

macrophages, the anti-inflammatory effect was expected to be caused by a decrease in COX-2 protein level, which is a factor that increases the levels of proinflammatory cytokines such as TNF- α and IL-6 (Suh 2002; Kim et al. 2004). To determine whether inhibition of NO and PGE₂ release by EGC was associated with reduced levels of iNOS and COX-2 expression, we quantified the expression of iNOS and COX-2 protein by using western blot analysis. LPS treatment for 24 h markedly increased iNOS protein expression, and this increase in iNOS protein level was suppressed significantly as 21% at 50 µg/mL concentration and dose-dependently by EGC treatment (Fig. 4A). COX-2 protein expression was also induced by treatment with LPS, and the LPS-induced increase in COX-2 protein level was suppressed significantly in a dose-dependent manner by EGC treatment (Fig. 4B). EGC exerts potent anti-inflammatory effects

by inhibiting the LPS-induced expression of iNOS and COX-2 in Raw 264.7 cells. The inhibitory effect of EGC on NO and PGE₂ production were supported by the suppression of iNOS and COX-2 at protein levels. These inhibitory effects are caused by the suppression of NF- κ B activation and MAPK phosphorylation.

These results support the use of EGC in the alleviation of various inflammation-associated diseases and suggest that EGC might be useful in the development of new functional foods for inflammatory diseases.

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