**N-trans-β-caffeoyl tyramine isolated from Tribulus terrestris exerts anti-inflammatory effects in lipopolysaccharide-stimulated RAW 264.7 cells**

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**Abstract.** Inflammation is induced by the expression of cyclooxygenase-2 (COX-2), which is an important mediator of chronic inflammatory diseases, such as rheumatoid arthritis, asthma and inflammatory bowel disease. *Tribulus terrestris* (*T. terrestris*) is known to have a beneficial effect on inflammatory diseases. In this study, we investigated the effects of *N*-trans-β-caffeoyl tyramine (CT) isolated from *T. terrestris* on the production of nitric oxide (NO), and the expression of pro-inflammatory cytokines and COX-2 in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. We also aimed to elucidate the molecular mechanisms involved. We found that the ethanolic extract of *T. terrestris* (EETT) and CT inhibited the production of NO, tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-10 in the LPS-stimulated RAW 264.7 cells in a dose-dependent manner. They were determined by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). In addition, CT markedly suppressed the expression of COX-2 and the production of prostaglandin E₂ (PGE₂) in response to LPS stimulation. Furthermore, CT markedly decreased p-c-Jun N-terminal kinase (p-JNK) protein expression in LPS-stimulated RAW 264.7 cells. COX-2 and p-JNK were measured by western blot analysis. Taken together, these findings indicate that CT isolated from *T. terrestris* is a novel and potent modulator of inflammatory responses. Thus, it may prove beneficial to further evaluate CT as a possible treatment for chronic inflammatory diseases.

**Introduction**

*Tribulus terrestris* (*T. terrestris*) is a herbal remedy that has a variety of uses in folk medicine. In traditional medicine, the extract from *T. terrestris* has been used to treat various diseases including hypertension, coronary heart disease (1), fungal diseases and infertility in both genders (2,3). It has also been described as a highly valuable drug that can help to restore decreased liver function, and it is used in the treatment of diabetes and hyperlipidemia (4,5). In traditional Chinese medicine, the fruit of *T. terrestris* has been used to treat pruritus, edema, tracheitis and inflammation (6). *N*-trans-β-caffeoyl tyramine (CT) is one of the compounds isolated from *T. terrestris* (7). A previous study reported that CT acts as an antioxidant and moderately inhibits acetylcholinesterase *in vitro* and *in vivo* (8). However, the anti-inflammatory effects of CT have not yet been completely elucidated.

Inflammation is a complex pathological process mediated by diverse molecules involving a variety of immune cells, such as leukocytes, macrophages and mast cells (9). Nitric oxide (NO) and prostaglandin E₂ (PGE₂) are involved in various pathophysiological processes, including inflammation, and inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) are mainly responsible for the production of large quantities of these mediators (10,11). NO produced by the constitutive isofom of NO synthase (NOS) is a key regulator of homeostasis; however, the generation of NO by iNOS plays a significant role in inflammation (12). Activated macrophages play a pivotal role in inflammatory diseases, as they excessively produce pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and inflammatory mediators, such as NO and PGE₂ (13,14). PGE₂ is another important inflammatory mediator and is produced from arachidonic acid metabolites by the catalysis of COX-2 (15). PGE₂ is related to the pathogenesis of acute and chronic inflammatory states (16), and specific COX-2 inhibitors decrease the symptoms of inflammation (17).

In the present study, we examined the anti-inflammatory effects of CT isolated from *T. terrestris* on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Our findings demonstrated that CT inhibited NO production and suppressed the expression COX-2 and cytokines related to inflammation in LPS-stimulated RAW 264.7 cells.
Materials and methods

Preparation of *T. terrestris* extract. The dried fruit of *T. terrestris* (Fructus Tribuli) was purchased from the Gyeongdong oriental Herbal Store, Seoul, Korea, in March 2012 and was formally identified by Professor Joa Sub Oh (College of Pharmacy, Dankook University, Cheonan, Korea). A voucher specimen (G46) was deposited at the Natural Products Research Laboratory, Gyeonggi Institute of Science and Technology Promotion, Suwon, Korea. The air-dried, crushed fruits of *T. terrestris* (10 kg) were pulverized and the extract was removed with 80% ethanol (EtOH; 3x18 liters) at room temperature (twice each day for 2 days).

Extraction and isolation of CT. The 80% EtOH extract was filtered and concentrated in vacuo at 40°C to yield 673.5 g of residue, and the residue was then suspended in water and partitioned with hexane (3x1.5 liters) to produce a hexane-soluble layer (40 g). The aqueous layer was partitioned with CHCl3 to provide a CHCl3-soluble residue (8.1 g). The CHCl3 layer was subjected to liquid chromatography [glass column (7x20 cm) packed with silica gel (230-400 mesh)] using CHCl3:MeOH (100:0, 99:1, 98:2, 97:3, 96:4, 92:8, 90:10, 80:20, 70:30, 60:40, 50:50, v/v) gradient mixtures as eluents. The eluent fractions G46-51-(1-13) were obtained from this initial liquid chromatographic separation. The fractions F001-F011 were subjected to an *in vitro* bioassay to evaluate their NO inhibitory activity. The fraction G46-51-7 exhibited promising inhibitory activity against NO production and was thus selected for further analysis. Column chromatography of the CHCl3-soluble layer (8.1 g) on a silica gel using MeOH, with increasing polarity, yielded 13 fractions, G46-51-(1-13). Fraction G46-51-7 (2.71 g) was further applied to flash column chromatography on a sephadex LH-20 column using CHCl3:MeOH (1:1), and 21 fractions were noted: G46-52-(1-21). Of these 21 fractions, CT (97.5 mg) was isolated from fraction G46-52-12, which was precipitated with CHCl3. 1H- and 13C-NMR spectra were recorded on a Bruker Ascend 700 MHz spectrometer (Bruker, Billerica, MA, USA) using CDCl3 as a solvent. Electrospray ionization (ESI) mass spectra were obtained on an LTQ Orbitrap XL (Thermo Scientific, Bremen, Germany) mass spectrometer.

*N-trans-α*-caffeoyl tyramine (CT). Amorphous powder; 1H-NMR (CD3OD, 700 MHz) δ: 7.40 (1H, d, J=15.4 Hz, H-7), 7.07 (2H, d, J=8.4 Hz, H-2, 6), 7.01 (1H, d, J=1.4 Hz, H-2'), 6.92 (1H, dd, J=8.4, 2.1 Hz, H-6'), 6.78 (1H, d, J=8.4 Hz, H-5'), 6.74 (2H, d, J=8.4 Hz, H-3, 5), 6.35 (1H, d, J=15.4 Hz, H-8), 3.47 (1H, t, J=7.0 Hz, H-7), 2.77 (1H, t, J=7.0 Hz, H-8); 13C-NMR (CD3OD, 175 MHz) δ 167.9 (C-9'), 155.5 (C-4), 147.3 (C-4'), 145.3 (C-3'), 140.8 (C-7), 129.9 (C-1'), 129.3 (C-2, 6), 126.9 (C-1), 120.7 (C-6'), 117.0 (C-8'), 115.0 (C-5'), 114.8 (C-3, 5), 113.6 (C-2'), 41.1 (C-8), 34.4 (C-7); ESI mass spectrometry (ESIMS; negative) m/z 298 [M-H]+ (18).

Reagents. The following pharmacological agents and antibodies were purchased from commercial sources: LPS from *Escherichia coli* serotype 0111:B4; celecoxib, Nα-nomonomethyl l-arginine (L-NNAME) and dexamethasone (all from Sigma-Aldrich, St. Louis, MO, USA); anti-COX-2 (M-19; sc-1747), anti-β-actin (13E5) and anti-GAPDH antibodies, and goat and mouse IgG-horseradish peroxidase conjugates (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-c-Jun N-terminal protein kinase (JNK; #9251) and anti-phospho-JNK (Thr183/Tyr185) antibodies (both from Cell Signaling Technology, Beverly, MA, USA).

Cell culture and NO assay. RAW 264.7 murine macrophages (TIB-71) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; both from Gibco® Life Technologies, Inc., Grand Island, NY, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin (both from Gibco® Life Technologies, Inc.) in a humidified atmosphere of 95% air with 5% CO2 at 37°C. On day 0, the cells were seeded in 96-well plates. After 24 h, the cells were stimulated with medium (0.5 µg/ml LPS in 10% FBS-DMEM) for 2 h, and then this medium was replaced with maintenance medium (10% FBS-DMEM). The cells were treated with various concentrations of CT (0-50 µM) for 24 h. We then measured the levels of nitrite, a stable metabolite of NO, using Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid; Sigma-Aldrich). Subsequently, the mixture was incubated at room temperature for 10 min, and the absorbance was measured at 540 nm. The quantity of nitrite was determined from a standard curve for sodium nitrite (Sigma-Aldrich).

Cell cytotoxicity assay. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay was used for the determination of cell viability *in vitro* in the RAW 264.7 cells. The cells were plated at a density of 4x10⁴ cells/well in 100 µl culture medium. One day after plating, a time zero control plate was made. Following stimulation of the cells with LPS for 2 h, CT was applied directly, and the cells were incubated for 24 h in a humidified atmosphere with 5% CO2 at 37°C. Cell culture was then performed. MTT (5 mg/ml in PBS) was added to each well, followed by incubation for 90 min. The medium was removed from the wells by aspiration; subsequently, 0.1 ml of buffered dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well, and the plates were shaken. The absorbance was measured on a microtiter plate reader at 540 nm.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed for the determination of the levels of cytokines *in vitro* in the RAW 264.7 cells. The cells were plated at a density of 4x10⁶ cells/well in 100 µl culture medium. One day after plating, a time zero control plate was made. Following stimulation of the cells with LPS for 2 h, CT was applied directly and the cells were incubated for 24 h in a humidified atmosphere with 5% CO2 at 37°C. Cell culture was then performed. The supernatants were harvested and assayed for cytokines by ELISA. The concentrations of interleukin (IL)-6, IL-10 and TNF-α in the culture medium were quantified using a platinum ELISA kit (eBioscience, San Diego, CA, USA), and the concentration of PGE2 in the culture medium was quantified using a competitive enzyme ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, respectively.
RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using a total RNA extraction kit (Ambion, Carlsbad, CA, USA). Five micrograms of RNA were used as a template for each RT-PCR reaction using the SuperScript™ III One-Step RT-PCR system (Invitrogen, Carlsbad, CA, USA). Newly synthesized cDNA from the RAW 264.7 control cells and CT-treated cells was amplified using specific primers and the Accupower® Pfu PCR PreMix (Bioneer, Daejeon, Korea). The sequences of the primers used for RT-PCR are shown in Table I.

Western blot analysis. The cells were harvested and washed with PBS and then collected by centrifugation at 13,000 rpm for 1 min at 4°C. To obtain the cell lysate, the cells were lysed on ice for 30 min in RIPA buffer [50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)], which contained protease inhibitors (Roche, Mannheim, Germany). Insoluble materials were removed by centrifugation at 13,000 rpm for 10 min at 4°C. A total of 50 mg of the supernatants was separated using a 10% polyacrylamide gel containing 10% SDS, 1.5 M Tris-HCl, 0.035% N,N,N′,N′-tetramethylethylenediamine and 7 mg ammonium persulfate. The separated proteins were electrically transferred onto a nitrocellulose membrane (Whatman, Dassel, Germany) at 36 mA in a transfer buffer containing 39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% MeOH. All western blot analyses were performed at least in triplicate, and representative blots are shown.

Statistical analysis. Data are expressed as the means ± SD. The statistical significance of the experimental results was analyzed (Student’s t-test and one-way ANOVA with a subsequent Dunnett’s multiple-range test). P-values <0.05 were considered to indicate statistically significant differences.

Results

Effects of CT on NO production and cytotoxicity in LPS-stimulated RAW 264.7 cells. The chemical structure of CT is illustrated in Fig. 1A. To examine the effects of CT on the inflammatory response, we measured the levels of NO production following treatment of the LPS (0.5 μg/ml)-stimulated RAW 264.7 cells with CT (0, 5, 25 or 50 μM) for 24 h. Treatment with CT induced a marked decrease in NO levels in the LPS-stimulated cells in a dose-dependent manner. Treatment with 50 μM CT induced an 84.07% decrease in NO production. We also confirmed that this result was similar to that achieved by treatment with 100 μM L-NMMA (Fig. 1B), as also previously demonstrated (19). To evaluate the cytotoxicity of CT, we conducted an MTT assay. Treatment with 5, 25 or 50 μM CT did not have a marked cytotoxic effect on the LPS-stimulated RAW 264.7 cells (Fig. 1C).

Effects of CT on the expression and production of cytokines in LPS-stimulated RAW 264.7 cells. We investigated the effects of CT on the expression of TNF-α, IL-6 and IL-10, which are pro-inflammatory cytokines, in the LPS-stimulated RAW 264.7 cells. Firstly, we measured the mRNA expression levels of TNF-α, IL-6 and IL-10 by RT-PCR following treatment with 5, 25 or 50 μM CT. We observed that treatment with CT suppressed the mRNA levels of TNF-α, IL-6 and IL-10 in a dose-dependent manner (Fig. 2A). Treatment with dexamethasone (25 μM), which is a potent synthetic member of the glucocorticoid class of steroid drugs, also inhibited the mRNA expression of TNF-α, IL-6 and IL-10 (Fig. 2A). We then confirmed the effects of CT on TNF-α, IL-6 and IL-10 at the protein level by ELISA. The protein levels of TNF-α, IL-6 and IL-10 in the conditioned medium were decreased following treatment with 5, 25 or 50 μM CT. In particular, treatment with 50 μM CT significantly inhibited the release of TNF-α, IL-6 and IL-10 by up to 44.13, 18.38 and 84.99%, respectively (Fig. 2B-D).

Effects of CT on COX-2 expression and phosphorylation of mitogen-activated protein kinase (MAPK) in LPS-stimulated RAW 264.7 cells. To determine the effects of CT on COX-2 expression, we examined whether the expression of COX-2 is reduced at both the mRNA and protein level in LPS-stimulated RAW 264.7 cells following treatment with 5, 25 or 50 μM of CT. As shown in Fig. 3A, CT significantly inhibited COX-2

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IL, interleukin; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α.
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Figure 1. Effects of N-trans-ρ-caffeoyl tyramine (CT) on nitric oxide (NO) production and cytotoxicity in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. (A) Structure of CT. (B) Following stimulation with LPS, the RAW 264.7 cells were treated with 0, 5, 25 or 50 µM of CT for 24 h. NO assay was performed using Griess reagent. (C) Cell viability was determined by MTT assay. Results of the experiments are the mean values of 3 independent experiments and are shown as a percentage cell viability compared with the viability of the untreated cells. *P<0.05 and **P<0.01, compared with the LPS-treated cells.

Figure 2. Effects of N-trans-ρ-caffeoyl tyramine (CT) on the expression of inflammatory cytokines in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Following stimulation, the RAW 264.7 cells were treated with 0, 5, 25 or 50 µM of CT for 24 h. (A) The representative mRNA levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-10 were assessed by RT-PCR. Inhibitory effects of CT on the production of (B) TNF-α, (C) IL-6 and (D) IL-10 were assessed by enzyme-linked immunosorbent assay (ELISA). Values represent the means ± SD of 3 independent experiments. *P<0.05 and **P<0.01, compared with the LPS-treated cells.
mRNA expression in a dose-dependent manner. Treatment with 5 µM of celecoxib, a well-known COX-2 inhibitor, significantly inhibited COX-2 expression at the mRNA level. In addition, treatment with 5, 25 or 50 µM CT also resulted in the suppression of COX-2 expression at the protein level in a dose-dependent manner, as evidenced by western blot analysis. Treatment with celecoxib also significantly inhibited COX-2 protein expression (Fig. 3B). Studies have demonstrated that the LPS-induced phosphorylation of MAPKs leads to the production of inflammatory cytokines (20,21). Thus, to determine whether the activation of the MAPK pathway is regulated by CT, we measured the phosphorylation levels of JNK. Treatment with CT (particularly with 50 µM CT) significantly inhibited the LPS-induced phosphorylation of JNK, but did not affect the expression of JNK (Fig. 3C).

Effects of CT on the PGE$_2$ level in LPS-stimulated RAW 264.7 cells. To confirm the effects of CT on PGE$_2$, one of the mediators produced by COX-2, we measured the secretion levels of PGE$_2$ following treatment of the LPS-stimulated RAW 264.7 cells with CT (5, 25 or 50 µM) and celecoxib (5 µM). The conditioned media were collected and the PGE$_2$ content was measured by ELISA. As shown in Fig. 4, the levels of PGE$_2$ in the conditioned media were significantly decreased following treatment with CT (50 µM) and celecoxib (5 µM).

Discussion

In this study, we demonstrated that CT isolated from T. terrestris has a marked effect on the inflammatory response and on the levels of related pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. We first examined the effects of an 80% ethanol extract of T. terrestris (EETT) on the inflammatory response using an NO assay, and we observed the dose-dependent suppression of NO production in the LPS-stimulated RAW 264.7 cells (data
Cox-2 is expressed in the host defense mechanisms; they are activated by exposure to interferon-γ, pro-inflammatory cytokines and bacterial LPS (10). NO is endogenously generated from L-NMMA by NOS, and it plays an important role in the regulation of a number of physiological processes (23). TNF-α, IL-6 and IL-10 are the most important pro-inflammatory cytokines. The cytokines, TNF-α, IL-6 and IL-10, are produced mainly by activated monocytes or macrophages (24). In the present study, we observed that treatment with 100 µM L-NMMA, a well-known NOS inhibitor, decreased NO production in the LPS-stimulated macrophages (Fig. 1B).

Macrophages are known to play a key role in the host defense mechanism; they are activated by exposure to interferon-γ, pro-inflammatory cytokines and bacterial LPS (10). NO is endogenously generated from L-NMMA by NOS, and it plays an important role in the regulation of a number of physiological processes (23). TNF-α, IL-6 and IL-10 are the most important pro-inflammatory cytokines. The cytokines, TNF-α, IL-6 and IL-10, are produced mainly by activated monocytes or macrophages (24). In the present study, we observed that treatment with 100 µM L-NMMA, a well-known NOS inhibitor, decreased NO production in the LPS-stimulated macrophages (Fig. 1B).

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