

# ***N-trans- $\alpha$ -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- $\alpha$ -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- $\alpha$  (TNF- $\alpha$ ), 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 E<sub>2</sub> (PGE<sub>2</sub>) 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- $\alpha$ -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 E<sub>2</sub> (PGE<sub>2</sub>) 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 isoform 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- $\alpha$  (TNF- $\alpha$ ) and inflammatory mediators, such as NO and PGE<sub>2</sub> (13,14). PGE<sub>2</sub> is another important inflammatory mediator and is produced from arachidonic acid metabolites by the catalysis of COX-2 (15). PGE<sub>2</sub> 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 of COX-2 and cytokines related to inflammation in LPS-stimulated RAW 264.7 cells.

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## 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 CHCl<sub>3</sub> to provide a CHCl<sub>3</sub>-soluble residue (8.1 g). The CHCl<sub>3</sub> layer was subjected to liquid chromatography [glass column (7x20 cm) packed with silica gel (230–400 mesh)] using CHCl<sub>3</sub>:MeOH (100:0, 99:1, 98:2, 97:3, 96:4, 94:6, 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 CHCl<sub>3</sub>-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 CHCl<sub>3</sub>: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 CHCl<sub>3</sub>. <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were recorded on a Bruker Ascend 700 MHz spectrometer (Bruker, Billerica, MA, USA) using CDCl<sub>3</sub> as a solvent. Electrospray ionization (ESI) mass spectra were obtained on an LTQ Orbitrap XL (Thermo Scientific, Bremen, Germany) mass spectrometer.

***N-trans-*o**-caffeoyl tyramine (CT).** Amorphous powder; <sup>1</sup>H-NMR (CD<sub>3</sub>OD, 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); <sup>13</sup>C-NMR (CD<sub>3</sub>OD, 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]<sup>-</sup> (18). The structure of CT is presented in Fig. 1A.

**Reagents.** The following pharmacological agents and antibodies were purchased from commercial sources: LPS from *Escherichia coli* serotype 0111:B4, celecoxib, N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) 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% CO<sub>2</sub> 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 4x10<sup>4</sup> 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% CO<sub>2</sub> 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 4x10<sup>4</sup> 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% CO<sub>2</sub> 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 PGE<sub>2</sub> 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.

Table I. The primer sequence used for RT-PCR.

| Target        | Primer sequence  | Accession no. |
|---------------|--|---------------|
| GAPDH         | Sense: 5'-GTATGACTCCACTCACGGCAAA-3'<br>Antisense: 5'-GGTCTCGCTCCTGGAGAGATG-3'        | NM_008084     |
| IL-6          | Sense: 5'-CACTTCACAAGTCGGAGGCTT-3'<br>Antisense: 5'-GCAAGTGCATCATCGTTGTTTC-3'        | NM_031168     |
| IL-10         | Sense: 5'-CCTGGTAGAAGTGATGCCCCAGGCA-3'<br>Antisense: 5'-CTATGCAGTTGATGAAGATGTCAAA-3' | NM_010548     |
| COX-2         | Sense: 5'-GGAGAGACTATCAAGATAGTGATC-3'<br>Antisense: 5'-ATGGTCAGTAGACTTTTACAGCTC-3'   | NM_011198     |
| TNF- $\alpha$ | Sense: 5'-AGCCTGTAGCCACGTCGTA-3'<br>Antisense: 5'-TCTTTGAGATCCATGCCGTTG-3'           | NM_013693     |

IL, interleukin; COX-2, cyclooxygenase-2; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

**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 phenylmethanesulfonyl 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  $\pm$  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  $\mu$ g/ml)-stimulated RAW 264.7 cells with CT (0, 5, 25 or 50  $\mu$ M) 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  $\mu$ M CT induced an 84.07% decrease in NO production. We also confirmed that this result was similar to that achieved by treatment with 100  $\mu$ M 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  $\mu$ M 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- $\alpha$ , 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- $\alpha$ , IL-6 and IL-10 by RT-PCR following treatment with 5, 25 or 50  $\mu$ M CT. We observed that treatment with CT suppressed the mRNA levels of TNF- $\alpha$ , IL-6 and IL-10 in a dose-dependent manner (Fig. 2A). Treatment with dexamethasone (25  $\mu$ M), which is a potent synthetic member of the glucocorticoid class of steroid drugs, also inhibited the mRNA expression of TNF- $\alpha$ , IL-6 and IL-10 (Fig. 2A). We then confirmed the effects of CT on TNF- $\alpha$ , IL-6 and IL-10 at the protein level by ELISA. The protein levels of TNF- $\alpha$ , IL-6 and IL-10 in the conditioned medium were decreased following treatment with 5, 25 or 50  $\mu$ M CT. In particular, treatment with 50  $\mu$ M CT significantly inhibited the release of TNF- $\alpha$ , 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  $\mu$ M of CT. As shown in Fig. 3A, CT significantly inhibited COX-2

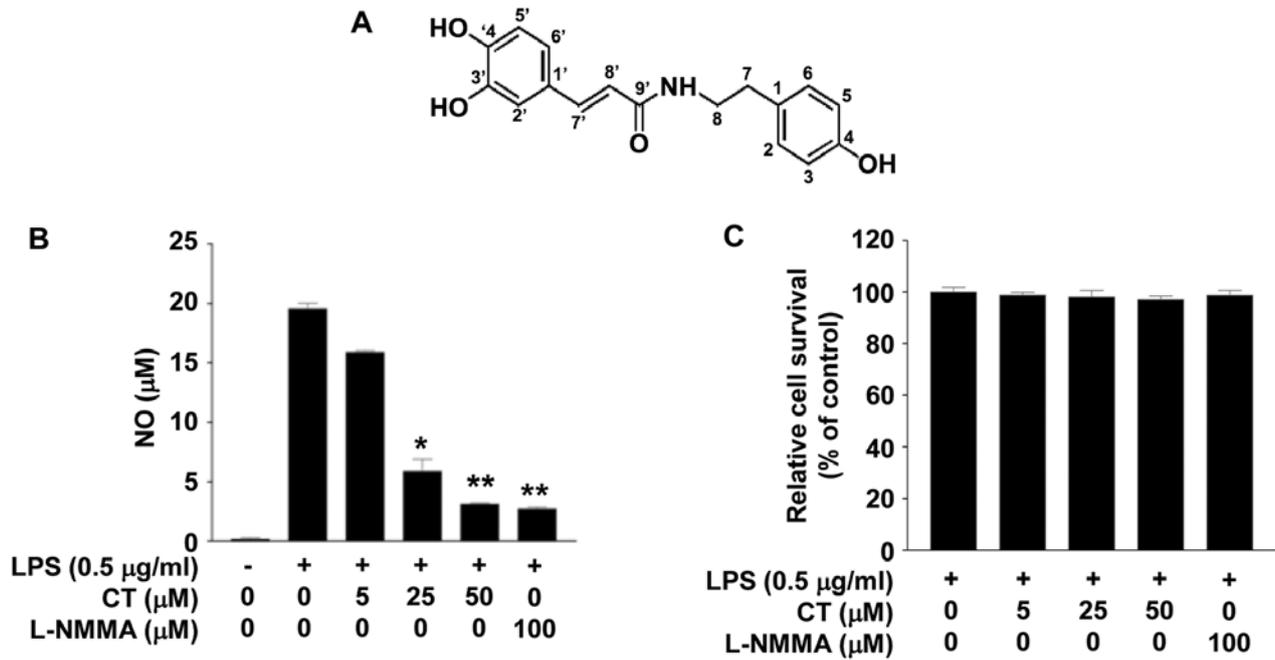


Figure 1. Effects of *N-trans*-*o*-caffeoyl tyramine (CT) on nitric oxide (NO) production and cytotoxicity in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. (A) Structure of CT. (B) Following stimulation with LPS, the RAW 264.7 cells were treated with 0, 5, 25 or 50 µM of CT for 24 h. NO assay 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.

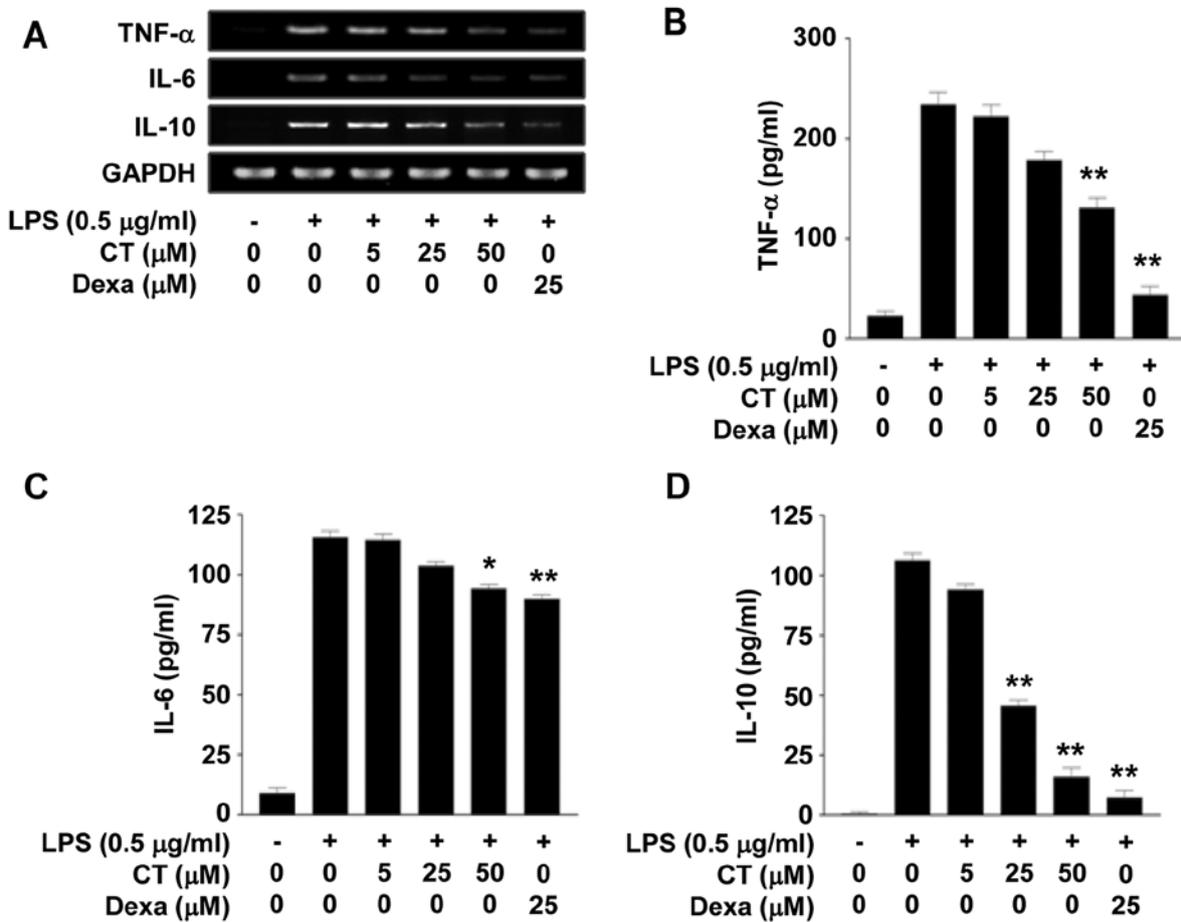


Figure 2. Effects of *N-trans*-*o*-caffeoyl tyramine (CT) on the expression of inflammatory cytokines in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Following stimulation, the RAW 264.7 cells were treated with 0, 5, 25 or 50 µM of CT for 24 h. (A) The representative mRNA levels of tumor necrosis 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.

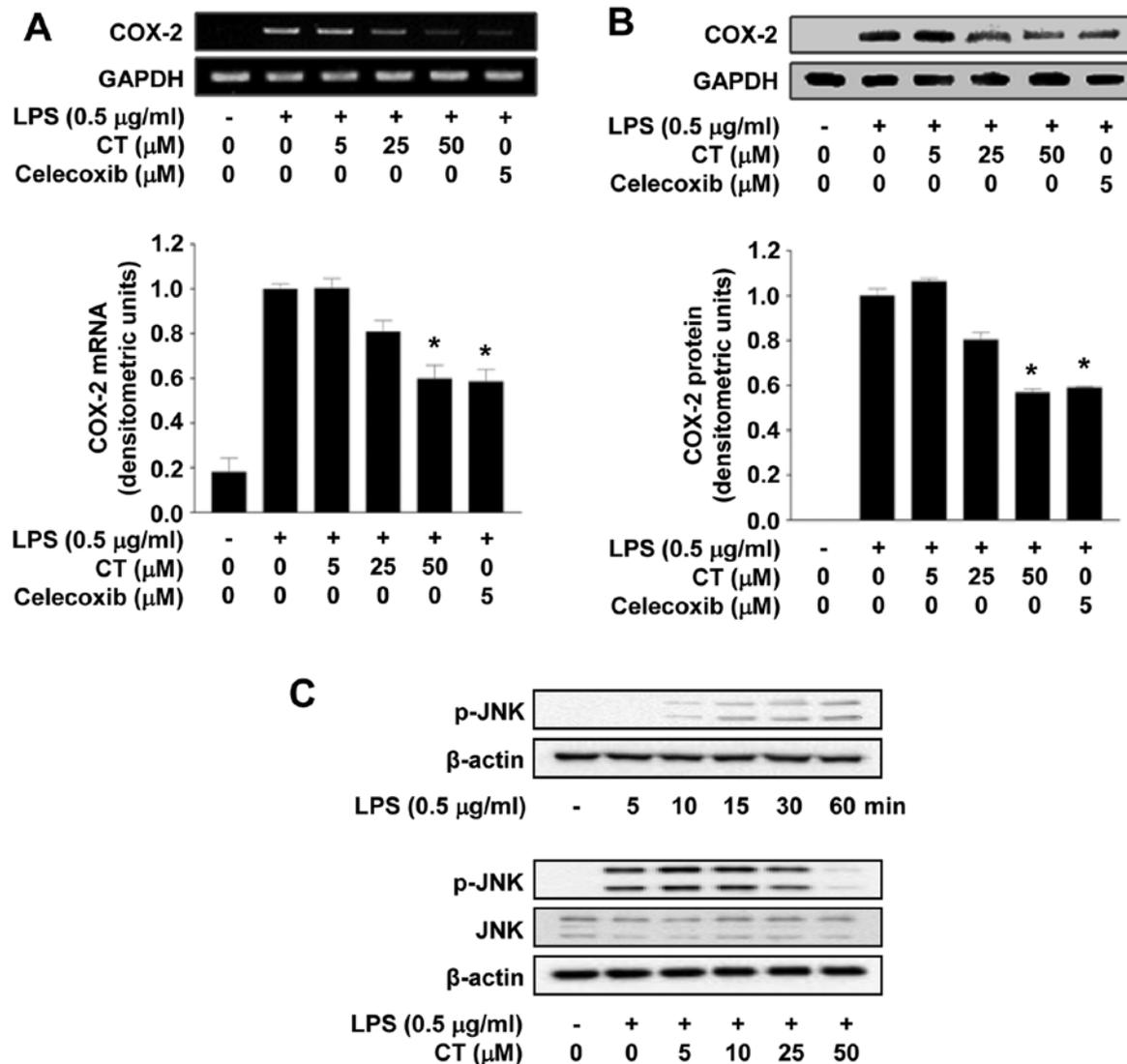


Figure 3. Effect of *N-trans-α*-caffeoyl tyramine (CT) on the expression of the cyclooxygenase-2 (COX)-2 and the phosphorylation of c-Jun N-terminal protein kinase (JNK) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Following stimulation, the RAW 264.7 cells were treated with 0, 5, 25 or 50 μM of CT for 24 h. The CT-treated cells exhibited a decrease in (A) COX-2 mRNA expression and (B) COX-2 protein expression and (C) phosphorylation of JNK when compared with the untreated cells. We confirmed this effect by using quantitative densitometric analysis. Values represent the means ± SD of 3 independent experiments. \*P<0.05, 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<sub>2</sub> level in LPS-stimulated RAW 264.7 cells.* To confirm the effects of CT on PGE<sub>2</sub>, one of the media-

tors produced by COX-2, we measured the secretion levels of PGE<sub>2</sub> 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<sub>2</sub> content was measured by ELISA. As shown in Fig. 4, the levels of PGE<sub>2</sub> 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

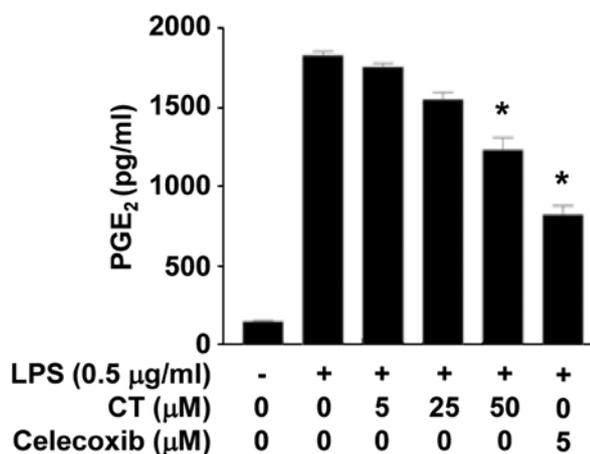


Figure 4. Effect of *N-trans*-*q*-caffeoyl tyramine (CT) on the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Following stimulation, the RAW 264.7 cells were treated with 0, 5, 25 or 50  $\mu$ M CT for 24 h. PGE<sub>2</sub> secretion from the RAW 264.7 cells was determined using an enzyme-linked immunosorbent assay (ELISA). Values represent the means  $\pm$  SD of 3 independent experiments. \* $P$ <0.05, compared with the LPS-treated cells.

not shown). A previous study demonstrated that *T. terrestris* inhibited COX-2 expression using the promoter assay (22). In the present study, we isolated CT from the EETT, and we examined its anti-inflammatory effects on RAW 264.7 murine macrophages. We demonstrated that treatment with CT resulted in a decrease in NO production in the LPS-stimulated macrophages and that it did not cause cytotoxicity under our experimental conditions. We also observed that treatment with 100  $\mu$ M 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- $\gamma$ , 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- $\alpha$ , IL-6 and IL-10 are the most important pro-inflammatory cytokines. The cytokines, TNF- $\alpha$ , IL-6 and IL-10, are produced mainly by activated monocytes or macrophages (24). In the present study, we noted that the LPS-stimulated cells exhibited increased levels of expression and production of pro-inflammatory cytokines compared to the unstimulated cells. Our data indicated that treatment with CT reduced the expression of TNF- $\alpha$ , IL-6 and IL-10 at the mRNA level (Fig. 2A), and it suppressed the secretion of TNF- $\alpha$ , IL-6 and IL-10 at the protein level in the LPS-treated macrophages (Fig. 2B).

Glucocorticoids are a class of steroid hormones with pleiotropic effects. At pharmacological concentrations, glucocorticoids are used to prevent and suppress inflammation and the activation of the immune system. Steroids exert their anti-inflammatory effects mainly by modulating the transcription of a variety of genes involved in controlling inflammatory processes (25). Our results indicated that treatment with dexamethasone, which is one of the glucocorticoids, induced a decrease in the levels of TNF- $\alpha$ , IL-6 and IL-10 by up to 81.39, 22.19 and 93.13%, respectively (Fig. 2B-D). However, glucocor-

ticoids are known to have serious side-effects (26), and hence it was our aim to obtain a drug from natural sources.

Prostaglandins (PGs) are key inflammatory mediators; they are produced from the conversion of arachidonic acid by COX. There are two isoforms of COX: COX-1 and COX-2 (27). COX-1 is the constitutively expressed isoform under normal physiological conditions, whereas COX-2 is expressed in response to inflammatory signals, such as cytokines and the bacteria endotoxin LPS. Celecoxib, which is a COX-2 selective inhibitor, is a useful drug for the treatment of acute pain and chronic inflammatory diseases, particularly arthritis (28); however, it is known to cause various side-effects. In this study, we demonstrated that treatment of the cells with 25 or 50  $\mu$ M of CT, or 5  $\mu$ M celecoxib, inhibited the expression of COX-2 at the mRNA and protein level (Fig. 3A and B). These findings suggest that CT isolated from *T. terrestris* exerts a therapeutic effect and prevents inflammatory responses by acting as a COX-2 selective inhibitor, and may thus be a potentially safe naturally-derived drug which may be used in the treatment of inflammatory diseases. Salvemini *et al* reported that NO modulates the activity of COX-2 and plays a role in the release of PGE<sub>2</sub> by activating COX-2 (29). COX-2 produces large amounts of PGE<sub>2</sub> that induce an inflammatory response (17). Therefore, the release of the inflammatory mediator PGE<sub>2</sub> is promoted by COX-2 activation. Our results demonstrated that treatment with CT (50  $\mu$ M) induced a 32.70% decrease in PGE<sub>2</sub> levels (Fig. 4). These results suggest that CT exerts an anti-inflammatory effect by suppressing COX-2 expression, which results in the inhibition of PGE<sub>2</sub> synthesis.

In conclusion, in this study, we demonstrated that CT can markedly inhibited macrophage-mediated inflammatory responses through the suppression of the production of NO and pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-6 and IL-10. Moreover, CT inhibited the expression of COX-2, the phosphorylation of JNK and PGE<sub>2</sub> synthesis. These findings suggest that CT has a therapeutic effect and may be used to prevent inflammatory diseases. Thus, it can be considered as a potential drug candidate for the treatment of arthritis and other inflammatory diseases, functioning as a COX-2-specific inhibitor.

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