Effects of *Corydalis Tuber* on Synthesis of NO and PGE_2 in Murine Macrophage RAW 264.7 Cells Stimulated by LPS

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Corydalis Tuber has traditionally been used for the treatment of water retention in the body. Administration of the aqueous extract of *Corydalis Tuber* has been known to be effective for the control of pain and treatment of arthritis. It was reported that *Corydalis Tuber* possesses anti-inflammatory activity and modulates the intestinal immune system. The effect of *Corydalis Tuber* against LPS-stimulated expressions of COX-2, iNOS, and IL-1β in cells of the murine RAW 264.7 macrophages was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), PGE_2 immunoassay, and NO detection. The aqueous extract of *Corydalis Tuber* was shown to suppress PGE_2 production by inhibition on the LPS-stimulated enhancement of COX-2 enzyme activity, IL-1β, and iNOS expression in the RAW 264.7 macrophages. Present results suggest that *Corydalis Tuber* exerts anti-inflammatory and analgesic effects probably by suppressing of COX-2, iNOS, and IL-1β expressions, resulting in inhibition of PGE_2 synthesis. *Corydalis Tuber* has anti-inflammatory and analgesic effects probably by suppressing of COX-2, iNOS, and IL-1β mRNA expressions, resulting in inhibition of PGE_2 and NO synthesis.

Key words : *Corydalis Tuber*, COX-2, Inflammation, iNOS, PGE_2

Introduction

Many of evidences suggest that prostaglandins (PGs) and nitric oxide (NO) are closely implicated in various pathophysiological processes including inflammation. Cyclooxygenase (COX-2), interleukin-1 beta (IL-1β), and nitric oxide synthase (iNOS) are mainly responsible for the production of these mediators[1,2].

Two isoforms of COX, designated as COX-1 and COX-2, have been known to catalyze the biosynthesis of PGs from arachidonic acid[3]. COX-1 is constitutively expressed in most tissues and seems to be responsible for housekeeping roles for normal physiological functions. In contrast, COX-2 is not detectable in normal tissues, but is induced by pro-inflammatory cytokines, growth factors, oncogenes, carcinogens, and tumor promoters, implicated in both inflammation and control of cell growth[4,5]. Thus, compounds that inhibit the activity or expression of COX-2 might be an important target for cancer chemoprevention or anti-inflammation.

Nitric oxide synthase (NOS) is also an important enzyme in regulation of inflammation. Nitric oxide (NO) is generated via oxidation of the terminal guanidine nitrogen atom of L-arginine by NOS. NO is released during a variety of pathophysiological responses including circulatory shock, carcinogenesis, and inflammation[6,7].

IL-1β is a pleiotropic proinflammatory cytokine, which is actively involved in the initiation and maintenance of both acute and chronic inflammation[8]. Tissue contents of IL-1β are up-regulated in stressful and pathological conditions[9,10]. IL-1β is expressed on many cell types such as neurons, astrocytes, microglia, and tissue macrophages[11-13]. Although IL-1β may exert its effects directly on responsive cells, it can also act indirectly as an autocrine or paracrine mediator to induce the synthesis and secretion of arachidonic acid, PGs, and NO that act on local cells[14-16].

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Lipopolysaccharide (LPS) initiates a number of major cellular responses which play a vital role in the pathogenesis of inflammatory responses including activation of inflammatory cells and production of cytokines and other mediators. While COX-1 is a constitutively expressed form in normal physiologic functions, COX-2 is expressed only in response to inflammatory signals such as bacterial endotoxin LPS. COX-2 produces large amounts of PGE_2 that induce inflammatory reaction[17,18].

Corydalis Tuber has traditionally been used for the treatment of water retention in the body. Administration of the aqueous extract of *Corydalis Tuber* has been known to be effective for the control of pain and treatment of arthritis. It was reported that *Corydalis Tuber* possesses anti-inflammatory activity and modulates the intestinal immune system[19-21].

However, the mechanism of anti-inflammatory action of *Corydalis Tuber* has not been clarified yet. In the present study, the effect of *Corydalis Tuber* against LPS-stimulated expressions of COX-2, iNOS, and IL-1β in cells of the murine RAW 264.7 macrophages was investigated using 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), PGE_2 immunoassay, and NO detection.

Materials and Methods

1. Cell Culture

Cells of the murine macrophage RAW 264.7 were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) at 37℃ in 5% CO_2-95% O_2 in a humidified cell incubator. To obtain the water extract of *Corydalis Tuber*, 200 g of *Corydalis Tuber* was added to distilled water, and extraction was performed by heating at 80℃, concentrated with a rotary evaporator and lyophilized. The resulting powder, weighing 30 g, was dissoved in saline.

2. MTT Cytotoxicity Assay

Cell viability was determined using the MTT assay kit (Boehringer Mannheim GmbH, Mannheim, Germany) as per the manufacurer's protocols. In order to determine the cytotoxicity of *Corydalis Tuber*, cells were treated with *Corydalis Tuber* at concentrations of 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, and 5 mg/ml for 24 h. Cultures of the control group were left untreated. Ten μℓ of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. Solubilization solution of 100 μℓ was then added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 195 nm and a reference wavelength of 690 nm. Optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) × 100.

3. RNA Isolation and RT-PCR

To identify expressions of iNOS and COX-2 mRNAs, RT-PCR was performed. Total RNA was isolated from RAW 264.7 cells using RNAZOLTMB (TEL-TEST, Friendswood, TX, USA). Two μg of RNA and 2 μℓ of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65℃ for 10 min. One μℓ of AMV reverse transcriptase (Promega), 5 μℓ of 10 mM dNTP (Promega), and 5 μℓ of 10× AMV RT buffer (Promega) were the added to the mixture, and the final volume was brought up to 50 μℓ with diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42℃ for 1 h.

PCR amplification was performed in a reaction volume of 40 μℓ containing 1 μℓ of the appropriate cDNA, 1 μℓ of each set of primers at a concentration of 10 pM, 4 μℓ of 10× RT buffer, 1 μℓ of 2.5 mM dNTP, and 2 units of Taq DNA polymerase (TaKaRa, Shiga, Japan). For mouse iNOS, the primer sequences were 5′-GTGTTCCACCAGGAGATGTTG-3′ (a 21-mer sense oligonucleotide) and 5′-CTCCTGCCCAC TGAGTTCGTC-3′ (a 21-mer anti-sense oligonucleotide). For mouse COX-2, the primer sequences were 5′-TGCATGTGGCT GTGGATGTCATCAA-3′ (a 25-mer sense oligonucleotide) and 5′-CACTAAGACAGACCCGTCATCTCCA-3′ (a 25-mer antisense oligonucleotide). For cyclophilin, the internal control used in the study, the primer sequences were 5′-ACCCCACC GTGTTCTTCGAC-3′ (a 20-mer sense oligonucleotide starting at position 52) and 5′-CATTTGCCATGGACAAGATG-3′ (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 583 bp (for COX-2), 500 bp (for iNOS), and 299 bp (for cyclophilin).

For iNOS and COX-2, the PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: initial denaturation at 94℃ for 5 min, followed by 40 amplification cycles, each consisting of denaturation at 94℃ for 30 sec, annealing at 58℃ for 30 sec, and extension at 72℃ for 30 sec, with an additional extension step at the end of the procedure at 72℃ for 5 min. For cyclophilin, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were

executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular AnalystTM version 1.4.1 (Bio-Rad, Hercules, CA, USA).

4. Determination of Nitric Oxide Synthesis

In order to determine the effect of Corydalis Tuber on NO synthesis, the amount of nitrite in the supernatant was measured using a commercially available NO detection kit (Intron Biotech., Seoul, Korea). After collection of 100 $\mu\ell$ of supernatant, 50 $\mu\ell$ of N1 buffer was added, and the plate was incubated at room temperature for 10 min. N2 buffer was then added, and the plate was incubated at room temperature for 10 min. The absorbance of the content of each well was measured at 450 nm. The nitrite concentration was calculated from a nitrite standard curve.

5. Measurement of Prostaglandin E_2 Synthesis

Assessment of PGE_2 synthesis was performed using a commercially available PGE_2 competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). Cells were lysed and cell lysates and standard were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE_2 antibody and peroxidase-conjugated PGE_2 were added to each well, and the plate was incubated at room temperature and shaken for 1 h. The wells were drained and washed, and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution was added. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30 min through the addition of H_2SO_4. The absorbance of the content of each well was then measured at 450 nm.

6. Statistical Analysis

Results are expressed as mean ± standard error mean (S.E.M.). Data were analyzed by one-way ANOVA followed by Duncan's post-hoc test using SPSS. Differences were considered statistically significant at $p < 0.05$.

Results

1. Effect of Corydalis Tuber on RAW 264.7 Cell Viability

The viabilities of cells incubated with *Corydalis Tuber* at 0.01 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, and 5 mg/ml for 24 h were 108.25 ± 11.28 %, 100.59 ± 10.42 %, 94.37 ± 16.21 %, 94.30 ± 11.28, 91.53 ± 9.59 %, and 90.23 ± 10.46 % of the control value, respectively. The MTT assay revealed that *Corydalis Tuber* exerted no significant cytotoxicity in the macrophage RAW 264.7 cells (Fig.1).

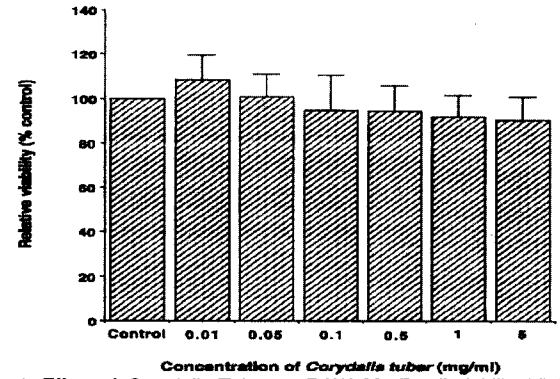

Fig. 1. Effect of *Corydalis Tuber* on RAW 264.7 cell viability Viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT) assay. Results are presented as mean ± standard error mean (S.E.M.).

2. Effect of *Corydalis Tuber* on mRNA Expressions of iNOS, COX-2, and IL-1β

RT-PCR analysis of the mRNA levels of iNOS, COX-2 and IL-1β was performed in order to provide an estimate of the relative levels of expressions of these genes. In the present study, the mRNA levels of iNOS, COX-2, and IL-1β in the control cells were used as a control value 1.00. The level of iNOS mRNA was markedly increased to 7.60 ± 0.58 following treatment with 5 μg/ml LPS for 24 h, while decreased to 0.93 ± 0.59 and 1.00 ± 0.68 in cells treated with *Corydalis Tuber* at 0.1 mg/ml and 1 mg/ml (Fig. 2). The level of COX-2 mRNA following treatment with 5 μg/ml LPS for 24 h was significantly increased to 12.09 ± 0.69, while decreased to 7.80 ± 0.97 and 0.99 ± 0.16 in cells treated with *Corydalis Tuber* at 0.1 mg/ml and 1 mg/ml (Fig. 3). The level of IL-1β mRNA following treatment with 5 μg/ml LPS for 24 h was significantly increased to 5.59 ± 0.64, while decreased to 6.27 ± 0.52 and 1.56 ± 0.61 in cells treated with *Corydalis Tuber* at 0.1 mg/ml and 1 mg/ml (Fig. 4).

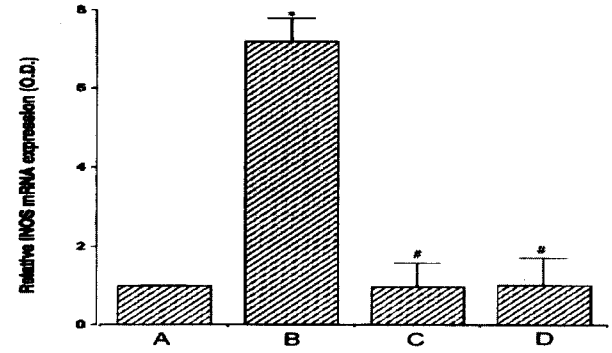

Fig. 2. Results of reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA level of inducible nitric oxide synthase (iNOS). A : Control B : LPS-treated group C : LPS- and 0.1 mg/ml Corydalis Tuber-treated group D : LPS- and 1 mg/ml Corydalis Tuber-treated group * represents $p < 0.05$ compared to the control. # represents $p < 0.05$ compared to the lipopolysaccharide (LPS)-treated group.

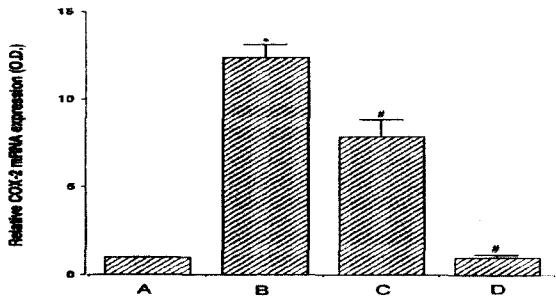

Fig. 3. Results of reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA level of cyclooxygenase-2 (COX-2). A : Control B : LPS-treated group C : LPS- and 0.1 mg/ml Corydalis Tuber-treated group D : LPS- and 1 mg/ml Corydalis Tuber-treated group * represents $p < 0.05$ compared to the control. # represents $p < 0.05$ compared to the lipopolysaccharide (LPS)-treated group.

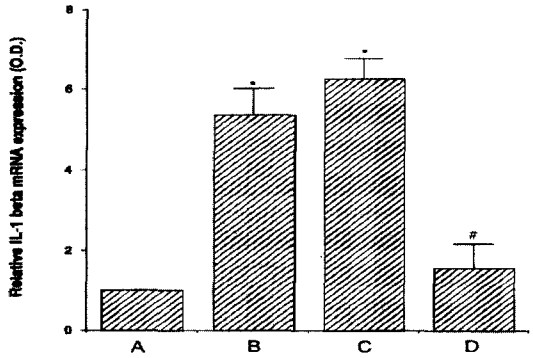

Fig. 4. Results of reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA level of interleukin-1beta (IL-1β). A : Control B : LPS-treated group C : LPS- and 0.1 mg/ml Corydalis Tuber-treated group D : LPS- and 1 mg/ml Corydalis Tuber-treated group * represents $p < 0.05$ compared to the control. # represents $p < 0.05$ compared to the lipopolysaccharide (LPS)-treated group.

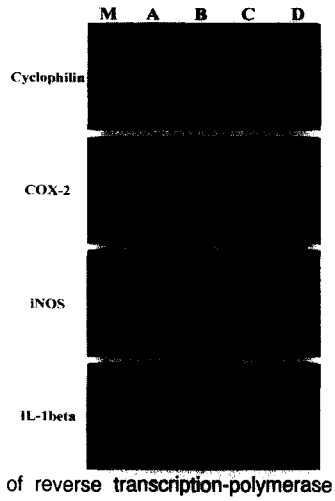

Fig. 5. Results of reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA expressions of COX-2, iNOS, and IL-1β). A : Control B : LPS-treated group C : LPS- and 0.1 mg/ml Corydalis Tuber-treated group D : LPS- and 1 mg/ml Corydalis Tuber-treated group * represents $p < 0.05$ compared to the control. # represents $p < 0.05$ compared to the lipopolysaccharide (LPS)-treated group.

3. Effect of Corydalis Tuber on NO Synthesis

From NO dectection assay, after 24 h of exposure to LPS, the amount of nitrite was increased from 0.98 ± 0.01 μM to 16.02 ± 3.04 μM, while decreased to 10.34 ± 2.51 μM and 7.34 ± 1.63 μM by treatment with *Corydalis Tuber* at 0.1 mg/ml and 1 mg/ml (Fig. 6).

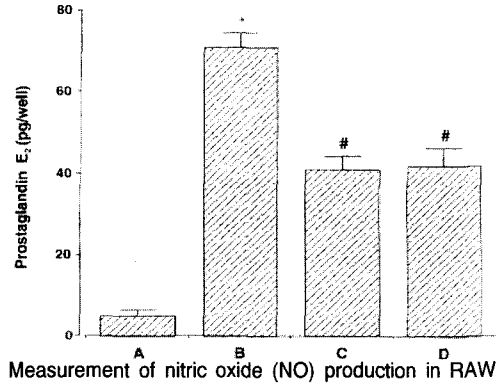

Fig. 6. Measurement of nitric oxide (NO) production in RAW 264.7 cells. A : Control B : LPS-treated group C : LPS- and 0.1 mg/ml Corydalis Tuber-treated group D : LPS- and 1 mg/ml Corydalis Tuber-treated group * represents $p < 0.05$ compared to the control. # represents $p < 0.05$ compared to the lipopolysaccharide (LPS)-treated group.

4. Effect of Corydalis Tuber on PGE_2 Synthesis

From PGE_2 immunoassay, after 24 h of exposure to LPS, the amount of PGE_2 was increased from 5.00 ± 1.24 pg/well to 71.00 ± 3.27 pg/well, while decreased to 41.00 ± 3.30 pg/well and 42.00 ± 4.15 pg/well by the treatment with *Corydalis Tuber* at 0.1 mg/ml and 1 mg/ml (Fig. 7).

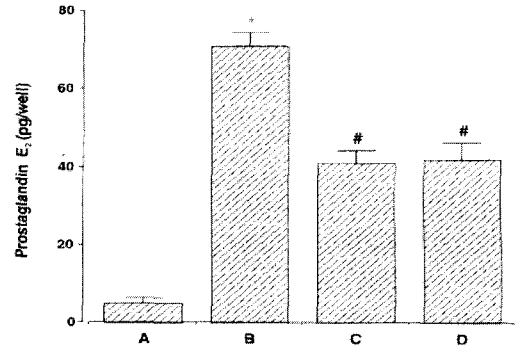

Fig. 7. Measurement of prostaglandin E_2 (PGE_2) in RAW 264.7 cells. A : Control B : LPS-treated group C : LPS- and 0.1 mg/ml Corydalis Tuber-treated group D : LPS- and 1 mg/ml Corydalis Tuber-treated group * represents $p < 0.05$ compared to the control. # represents $p < 0.05$ compared to the lipopolysaccharide (LPS)-treated group.

Discussion

The present study was undertaken to elucidate the pharmacological effects of *Corydalis Tuber* on the production of inflammatory mediators in macrophages. Macrophages play an important role in the regulation of inflammation and immune response. When activated, macrophages release growth factors,

cytokines, and lipid mediators such as prostaglandins and leukotrienes, which promote inflammation by directing cellular migration to the site of inflammation through the production and release of proinflammatory cytokines such as interleukins (IL-1, -6, and -8). Present results showed that *Corydalis Tuber* suppressed the production of NO and PGE_2 in LPS-stimulated RAW 264.7 cells. This suppression was correlated with down-regulation of gene expressions of iNOS, COX-2, and IL-1 β. These finding suggest that *Corydalis Tuber* may prevent tissue injury during acute endotoxemia by suppressing inflammatory genes.

NO and PGs, which are produced by iNOS and COX-2, respectively, have been implicated as important mediators in endotoxemia and inflammatory conditions[22]. It has been demonstrated that NO plays a pivotal role as neurotransmitter, vasodilator, and immune regulator in a variety of tissues at physiological concentration[23]. High levels of NO produced by iNOS, however, have been defined as a cytotoxic molecule in inflammation and endotoxemia. PGE_2 is a pleiotropic mediator produced at inflammatory sites by COX-2 and induces pain, swelling, and stiffness[24]. Thus, potential inhibitors of iNOS and COX-2 have been considered as anti-inflammatory drugs. We here demonstrated that *Corydalis Tuber* inhibits significantly the gene expressions of iNOS, COX-2, and IL-1β, resulting in suppression on NO and PGE_2 production in LPS-stimulated macrophages.

In the production of NO, PGE_2, IL-1β, iNOS and COX-2 play an important role in the immune response to many inflammatory stimuli. However, excessive production of these mediators is seen in many acute and chronic human diseases including septic shock, hemorrhagic shock, multiple sclerosis, rheumatoid arthritis, ulcerative colitis, and atherosclerosis[25,26]. Thus, suppression of these mediators may be an effective therapeutic strategy for preventing inflammatory reaction and diseases. Expressions of iNOS, COX-2, and IL-1β are an important mechanism for the overproduction of the inflammatory mediators in macrophages in response to LPS. Several natural products, such as curcumin, theaflavin, and sesquiterpene lactone, have been shown to directly inhibit the expressions of iNOS and COX-2 genes[27,28] and thus reduce inflammation in endotoxemia[29]. Present study demonstrated that *Corydalis Tuber* inhibits NOS and PGs activation in RAW 264.7 cells stimulated by LPS. This inhibitory effect of *Corydalis Tuber* is associated with down-regulation of iNOS, COX-2, IL-1 β expression.

Inflammatory stimuli such as LPS induce cytokines, such as IL-1β, in the process of macrophage activation, which mediates tissue responses in different phases of inflammation in a sequential and concerted manner[30]. Many evidences indicate that an abnormality in the production or function of IL-1β plays an essential role in many inflammatory lesions[31]. Inhibition of cytokine production or function serves as a key mechanism in the control of inflammation[32]. Natural phenolic antioxidants and flavonoids exhibit potent anti-inflammatory activity by inhibiting production of cytokines and prevent lethal shock induced by LPS. Based on the present results, it is possible that *Corydalis Tuber* inhibits IL-1β expression in RAW 264.7 cells stimulated by LPS. This finding provides the evidence that *Corydalis Tuber* possesses a potent anti-inflammatory activity.

In the present study, the aqueous extract of *Corydalis Tuber* was shown to suppress PGE_2 production by inhibition on the LPS-stimulated enhancement of COX-2 enzyme activity, IL-1β, and iNOS expression in the RAW 264.7 macrophages. Present results suggest that *Corydalis Tuber* exerts anti-inflammatory and analgesic effects probably by suppressing of COX-2, iNOS, and IL-1β expressions, resulting in inhibition of PGE_2 synthesis.

Conclusion

In the present study, the effect of *Corydalis Tuber* on synthesis of NO and PGE_2 in cells of the murine RAW 264.7 macrophages was elucidated using 3-(4,5-dimethylthiazol-2yl)-2.5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-CRT), PGE_2 immunoassay, and NO detection. The results are as follows The MTT assay revealed that Corydalis Tuber exerted no significant cytotoxicity. From the RT-PCR analysis, Corydalis Tuber suppressed the mRNA expressions of iNOS, COX-2, and IL-1β. From the NO and PGE_2 immunoassay, *Corydalis Tuber* inhibited the synthesis of NO and PGE_2. In conclusion, *Corydalis Tuber* has anti-inflammatory and analgesic effects probably by suppressing of COX-2, iNOS, and IL-1β mRNA expressions, resulting in inhibition of PGE_2 and NO synthesis.

Acknowledgement

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