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Inhibitory Effect of the Leaves of *Rumex crispus* L. on LPS-induced Nitric Oxide Production and the Expression of iNOS and COX-2 in Macrophages

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Abstract – In this study, the anti-inflammatory effects of ethanol extract of *Rumex crispus* L. and its fractions were investigated in RAW 264.7 macrophages. To evaluate the anti-inflammatory effects of extract, we studied nitric oxide (NO), prostaglandin E_2 (PGE_2), and tumor necrosis factor-alpha (TNF-α) levels in RAW 264.7 cells. The ethanol extract of *R. crispus* L. significantly decreased NO production and the levels of other inflammatory factors, such as PGE_2 and TNF-α, in lipopolysaccharide (LPS)-stimulated macrophages in a dose-dependent manner. We also assessed the inducible nitric oxide synthase (iNOS) and the cyclooxygenase 2 (COX-2) protein expression by western blot. Ethyl acetate fraction of *R. crispus* L. had the strongest anti-inflammatory effect. These results suggest that ethyl acetate extract of *R. crispus* L. might be beneficial in the treatment of chronic inflammatory diseases.
Keywords – *Rumex crispus* L., Nitric Oxide, PGE_2, TNF-α, iNOS, COX-2

Introduction

Rumex crispus L. is a perennial plant belonging to the Polygonaceae family. The height of the plant is about 50 - 100 cm. The stems of this plant can grow straight up, and the roots of this plant are larger. The fruits are heart-shaped and contain tannin component and are surrounded by an inner perianth. This plant grows naturally in many parts of Korea and in a humid area of fields or brook. This plant contains various bioactive ingredients.[1,2] It has been reported that *R. crispus* L. is effective when it is applied over festering wounds or wheals. Also, its fruit is sweet in taste and its texture is slippery, cool and harmless according to the botanical list. This plant has been reported to be effective in treating constipation, indigestion and so on.[3,4] Effective constituents of *R. crispus* L. include a variety of antioxidant substances such as emodin, nepodin, flavonoids and polyphenols.[3-6] *R. crispus* L. is a well-known oriental medicinal herb. Many studies have reported that the analysis of only the *R. crispus* L. leaves is not sufficient to understand the mechanism of action of this plant in inflammatory diseases.

Inflammation is a biological defense response to eliminate stimulation of the areas due to infection, exposure to toxins or cell damage and damaging factors. Usually, inflammation is self-limiting and is important for maintaining homeostasis in the body. However, chronic inflammation due to persistent stimulation causes tissue damage and various diseases.[7] When the inflammatory response occurs, NO an inflammatory mediator is synthesized from L-arginine and NO synthase (NOS). Typically, NO plays a role in the immune reaction to kill the bacteria and to eliminate the anti-tumor immune response.[8] However, when NO is produced excessively by lipopolysaccharide (LPS) or inflammatory cytokine-induced expression of inducible NOS (iNOS), it can aggravate the inflammatory response and can cause tissue damage, genetic mutations and nerve damage.[9,10]

Prostaglandin E_2 (PGE_2) is another inflammatory mediator. It is derived from arachidonic acid in the cell membrane by cyclooxygenase-2 (COX-2) and it inhibits

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tumor cell death. The pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α), which is expressed in inflammatory reactions is secreted by macrophages; and it induces the production of NO and PGE_2 that mediate various inflammatory responses.[11-13]

Recently, the concept of 'metainflammation' has been linked to nutrients or inflammatory response that is caused due to excessive supply of metabolites. Also, a study on insulin resistance, type 2 diabetes, association of cardiovascular disease with obesity and correlation of immune response in patients with metabolic syndrome is in progress.[14,15] Therefore, inhibiting the inflammatory response is helpful in preventing and treating various metabolic syndromes. However, the effects of the extract of *R. crispus* L. and its fractions have not been adequately studied in inflammatory diseases till date**.** Therefore, the purpose of this study was designed to determine the anti-inflammatory effect of the extract of *R. crispus* L. and its fractions in LPS-stimulated RAW 264.7 macrophages.

Experimental

Chemicals and reagents – Dulbecco's modified Eagle's medium (DMEM), antibiotics, trypsin-EDTA, fetal bovine serum (FBS) and other culture reagents were purchased from Gibco BRL CO (Grand Island, NY, USA). LPS, Trizma base, ethylenedinitrilo-tetraacetic acid disodium salt (EDTA-2NA), triton X-100, N,N,N',N'-tetra-methylethylenediamine (TEMED), acrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), tween 20, bicinchoninic acid kit (BCA kit) were obtained from Sigma Chemical Co. (St. Louis. Mo., USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon Ind., Ohio, USA). Primary antibodies, iNOS, COX-2, and secondary antibodies were purchased from Cell Signaling (MA., USA). PGE_2 and TNF-α ELISA kits were purchased from Cayman Chemical Company, Inc. (Ann Arbor, MI, USA). ECL Detection kit was obtained from Amersham Biosciences (Buckinghamshire, England). Immobilon-P transfer membrane was purchased from Millipore (Billerica Massachusetts, USA).

Plant material – The leaves of *Rumex crispus* L. were collected from Mt. Geumo in Gyeongsangbuk-DO province in March 2013. A voucher specimen (KMU-2013-02) of the plant was prepared at the College of Pharmacy at Keimyung University, Daegu, Korea.

Extraction and fractionation – Dried leaves of *R. crispus* L. (1.2 kg) were extracted 3 times with 70% ethanol for 24 h under room temperature. The resultant extract was condensed to 1 L using a rotary evaporator (EYELA, Tokyo, Japan). Finally, 70% extract (RC-Et, 276 g) was suspended in water and then partitioned successively with organic solvents of different polarities to obtain n-hexane (RC-H, 5.4 g), chloroform (RC-Ch, 3.2 g), ethyl acetate (RC-EA, 32.5 g), n-butanol (RC-Bu, 30.6 g), and aqueous (RC-W, 150.9 g) fractions.

Cell culture and cell viability – Mouse macrophage cell line was purchased from the Korean Cell Line Bank (KCLB). The cells were cultured in DMEM containing 10% FBS, 1% antibiotics. Cells were maintained at 37 ºC in a humidified atmosphere of 5% CO_2 and 95% air. Cells were subcultured once a day or every 2 days. Cell viability was determined by the MTT assay. The cells were plated on a 96-well plate at a density of 1×10^5 cells/well. RC extracts and fractions were added at various concentrations and incubated at 37 ºC under humidified atmosphere, 5% CO_2, and 95% air. After 24 h of culture 20 μL of MTT reagent (2.5 mg/ml) was added to each well and incubated for 4 hours, the supernatants were discarded, and the cells were dissolved in dimethyl-sulfoxide (DMSO). The absorbance was measured at 550 nm using a microplate reader (Molecular Devices, California, USA).

Nitrite assay – NO production was evaluated by measuring nitrite concentrations in cell-free culture supernatants using a colorimetric assay. Cells were seeded at a density of 1×10^5 cells/ml in a 96-well plate. After treatment with the RC extract, RC fractions and LPS (100 ng/ml) for 24 h, 100 ml aliquots of the culture supernatants were incubated with 100 ml of a modified Griess reagent (1 : 1 mixture of 1% sulfanilamide in 30% acetic acid and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 60% acetic acid) at room temperature for 10 min; at this time, the absorbance was measured at 540 nm using a spectrophotometer (Molecular Devices, California, USA). The concentration of NO was determined using a linear standard curve established with serial dilutions of sodium nitrite.

Western blot analysis – RAW 264.7 cells were seeded at a density of 1×10^6 cells/ml in a 6-well plate containing 2 ml of culture medium. The cells were incubated for 24 h and then treated with various concentrations of extracts and 100 ng/ml LPS. After 24 h of incubation, cells were lysed with RIPA buffer and centrifuged at 13,000 rpm at 4 ºC for 15 min. Protein concentration was determined with the Bradford method. Proteins were separated by SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% skim milk for 1 h, and incubated with primary antibodies (iNOS, COX-2)

Table 1. The primer sequences used for real-time PCR

Target gene	Primer sequences
GAPDH	GTATGATCCACTCACGGCA
	GGTCTCGCTCCTGGAAGAGG
iNOS	CAAGCTGAACTTGAGCGAGG
	TTTACTCAGTGCCCAGAAGCT
COX-2	TGCCAGGCTGAACTTCGAAA
	GCTCACGAGGCCACTGATAC

overnight at 4 °C. The membranes were washed and continuously incubated with secondary antibodies for 2 h, and detected by the ECL system.

PGE_2 and TNF-α assay – The levels of PGE_2 and TNF-α were determined by using commercially available kits from Cayman Chemical Company, Inc. (Ann Arbor, MI, USA). The assay was performed according to the manufacturer's protocol. RAW 264.7 cells were seeded at a density of 1×10^6 cells/ml in a 6-well plate containing 2 ml of culture medium. The cells were incubated for 24 h and then treated with various concentrations of LPS. Cell culture supernatants were collected immediately after treatment and centrifuged at 13,000 rpm for 2 min to remove any particulates. The medium was added to a 96-well plate that was pre-coated with affinity-purified polyclonal antibodies specific for PGE_2 and TNF-α. An enzyme linked polyclonal antibody specific for PGE_2 and TNF-α was added to the wells and left to react, and then the wells were washed 3 times to remove any unbound antibody-enzyme reagent. The intensity of the color detected at 420 nm and 450 nm was measured after the addition of a substrate solution, and it was proportional to the amount of PGE_2 and TNF-α produced.

Real Time PCR – Total RNA was isolated by using TRI reagent according to the manufacturer's protocol. The yield and the purity of the total RNA were determined by spectrophotometry. The primer sequences are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene for data normalization.

Statistical analysis – The results are expressed as means ± S.D., and the differences between means of the two groups were determined by unpaired Student's t test. The minimum significance level was set at a $*P$ value of < 0.05, $**P$ value of < 0.01 for all analysis. All experiments were performed at least three times.

Results and Discussion

R. crispus L. (Polygonaceae) is a well-known oriental medicinal herb which has been used for treating various diseases and has diverse pharmacological effects. Recently, Lee *et al.*[16] reported the analgesic and hepatoprotective effects of *R. crispus* L. Park *et al.*[17] reported the antimicrobacterial effect and antioxidant activity of *R. crispus* L. Most studies that assessed the effects of *R. crispus* L. used the roots and stem of this plant. However, a study assessing the effect of *R. crispus* L. leaves has rarely been reported. Also, the present study evaluated the anti-inflammatory activity of *R. crispus* L. extract and its fractions using in vitro assays. Our study offers great value since we used the leaves of *R. crispus* L.

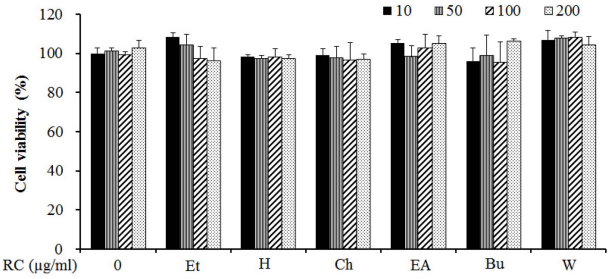

Fig. 1. Effect of *R. crispus* L. extract on cell viability of RAW 264.7 macrophages. RAW 264.7 cells were treated with the extract and fractions from *R. crispus* L. (50 μg/ml). Cell viability was calculated by using MTT assay. Results are expressed as the mean ± S.D. of three independent experiments.

MTT assay is widely used in the study of cells and it is very useful for the *in vitro* analysis of cell proliferation and viability. LPS-stimulated RAW 264.7 cells were treated with various concentrations of *R. crispus* L. extract and its fraction, and then cytotoxicity was measured using the MTT assay (Fig. 1). A more than 90% survival rate was observed in cells treated with *R. crispus* L. extract and its fractions. We also examined the range of non-toxic concentration.

Next, we measured LPS-induced NO production. LPS is a component of the cell wall of gram-negative bacteria. LPS increases the levels of pro-inflammatory cytokines such as NO, tumor necrosis factor-alpha (TNF-α), interleukin-5 (IL-6), interleukin-1β (IL-1β) in macrophages or monocytes. It also induces diverse disease-related inflammatory responses.[18] LPS-stimulated RAW 264.7 cells were treated with *R. crispus* L. extract and its fractions, and then we measured NO production in LPS-stimulated RAW 264.7 macrophages. LPS-induced NO production was 54.5 ± 2.1 μM in the LPS-treated group compared to LPS-non-treated group. All fractions of *R. crispus* L. inhibited NO production except for the water fraction. Especially, the ethyl acetate fraction had the most significant inhibitory effect of 86.9% (Fig. 2).

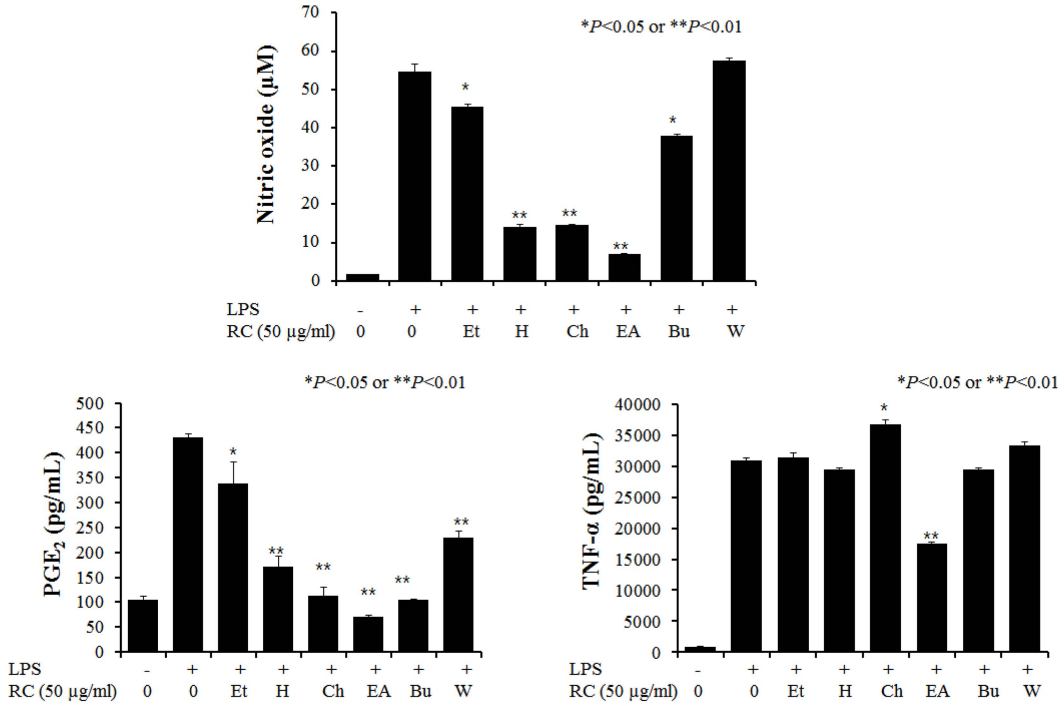

Fig. 2. Effect of *R. crispus* L. extract on LPS-induced NO, PGE_2 and TNF-α levels in RAW 264.7 macrophages. RAW 264.7 cells were treated with 50 μg/ml of *R. crispus* L. ethanol extract and its fractions and LPS (100 ng/ml) for 24 h. (A) NO production was determined in the culture supernatant by using the Griess reagent. The concentrations of (B) PGE_2 and (C) TNF-α were determined by using the ELISA kit. Results are expressed as the mean ± S.D. of three independent experiments. *P value of < 0.05, **P value of < 0.01.

PGE_2 is an inflammatory mediator whose synthesis is dependent on COX-2, which is a prostaglandin-endoperoxide synthase enzyme.[19] It is involved in inflammatory diseases. In other words, the production of PGE_2 occurs in the pre-inflammatory stage, and it causes pain, fever and vasodilatation.[20] TNF-α is secreted by macrophages, leukocytes following stimulation by lipopolysaccharides. It has cytotoxicity against tumor cells and it is also involved in chronic inflammation.[21] PGE_2 and TNF-α production was measured by the ELISA kit (Fig. 2). The PGE_2 level was 105 pg/ml in the LPS-non-treated group; however, it was markedly increased up to 429 pg/ml in the LPS-treated group. Also, when cells were treated with *R. crispus* L. extract and its fractions at a concentration of 50 μg/ml, we observed that the production of PGE_2 was suppressed. Especially, the PGE_2 level after treatment with the chloroform, ethyl acetate, and butanol fractions was 112 pg/ml, 71 pg/ml, 105 pg/ml, respectively; and they had an excellent inhibitory effect on the PGE_2 production.

We treated LPS-stimulated RAW 264.7 cells with *R. crispus* L. extract and its fractions and observed their inhibitory activity on TNF-α production. When LPS-stimulated RAW 264.7 cells were treated with *R. crispus* L. ethyl acetate fraction at a concentration of 50 μg/ml, LPS-induced TNF-α production was suppressed by up to 40%. Chae *et al.*[22] examined the inhibitory effects of *Terminalia chebula* Retz, *Lavandula spica* L., *Dalbergia odorifera* T. extracts on LPS-induced TNF-α production in RAW 264.7 cells. *Lavandula spica* L. and *Dalbergia odorifera* T. extracts showed slight inhibitory activity. However, when RAW 264.7 cells were treated with *Terminalia chebula* Retz extract at a concentration of 100 μg/ml, TNF-α production was decreased by about 50%. This study showed that the ethyl acetate fraction of *R. crispus* L. at a low concentration of 50 μg/ml effectively suppressed TNF-α production, and we observed an excellent anti-inflammatory activity of ethyl acetate fraction *of R. crispus* L..

There are 3 types of NOS, namely eNOS, nNOS and iNOS. eNOS and nNOS are the enzymes which are expressed inside the cells and maintain a low concentration of NO.[23] However, iNOS does not remain inside the cells, and it is the enzyme produced by external stimulation. It can produce more amount of NO over a long time period. NO produced by iNOS in inflammatory conditions is known to aggravate the inflammation by stimulating inflammatory responses and inflammatory-

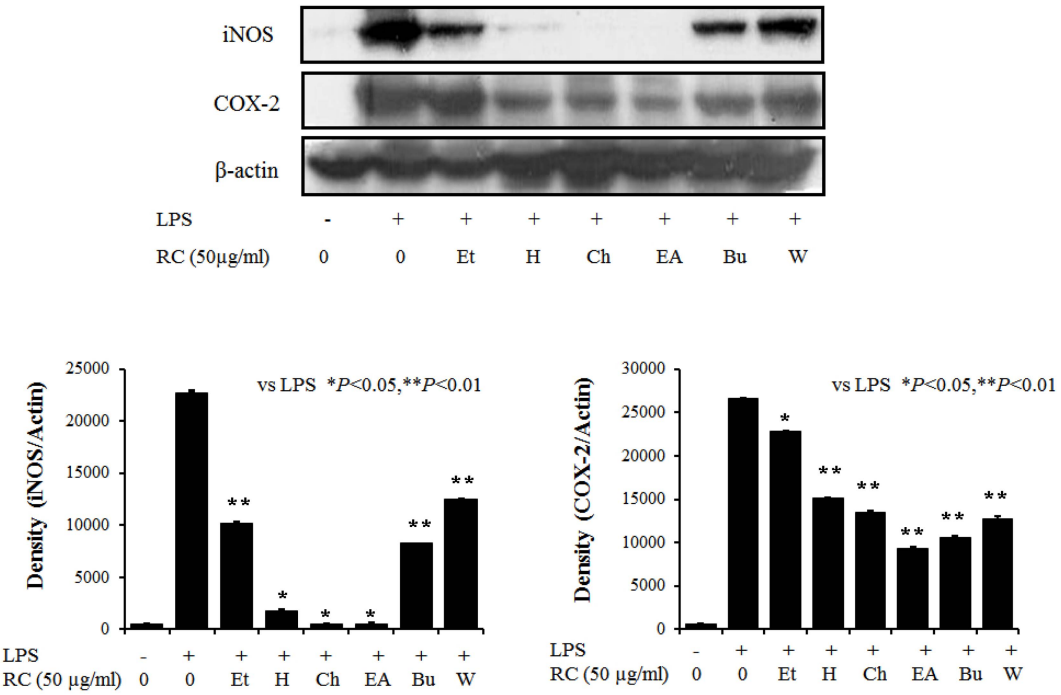

Fig. 3. Effect of *R. crispus* L. extract and its fractions on LPS-induced iNOS and COX-2 expression in RAW 264.7 macrophages. RAW 264.7 cells (1×10^6 cells/ml) were incubated for 24 h and then pretreated with *R. crispus* L. ethyl acetate fraction in the presence or absence of LPS (100 ng/ml) for 24 h. Equal amounts of total protein were resolved by SDS-PAGE.

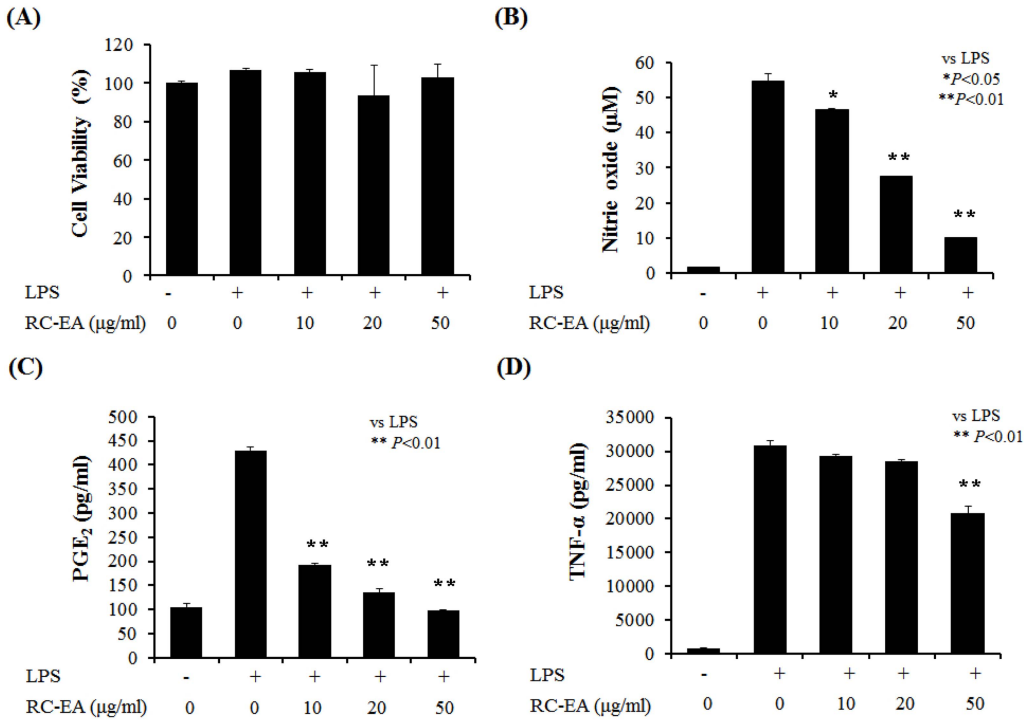

Fig. 4. Effect of *R. crispus* L. ethyl acetate fraction on LPS-induced NO, PGE_2 and TNF-α levels in RAW 264.7 macrophages. **P* value of < 0.05, ***P* value of < 0.01. RAW 264.7 cells were treated with 50 μg/ml of *R. crispus* L. ethanol extract and its fractions and LPS (100 ng/ml) for 24 h. (A) Cell viability was assessed by using MTT assay. (B) NO production was determined in the culture supernatant by using the Griess reagent. The concentrations of (C) PGE_2 and (D) TNF-α were determined by using the ELISA kit. Results are expressed as the mean ± S.D. of three independent experiments. **P* value of < 0.05, ***P* value of < 0.01.

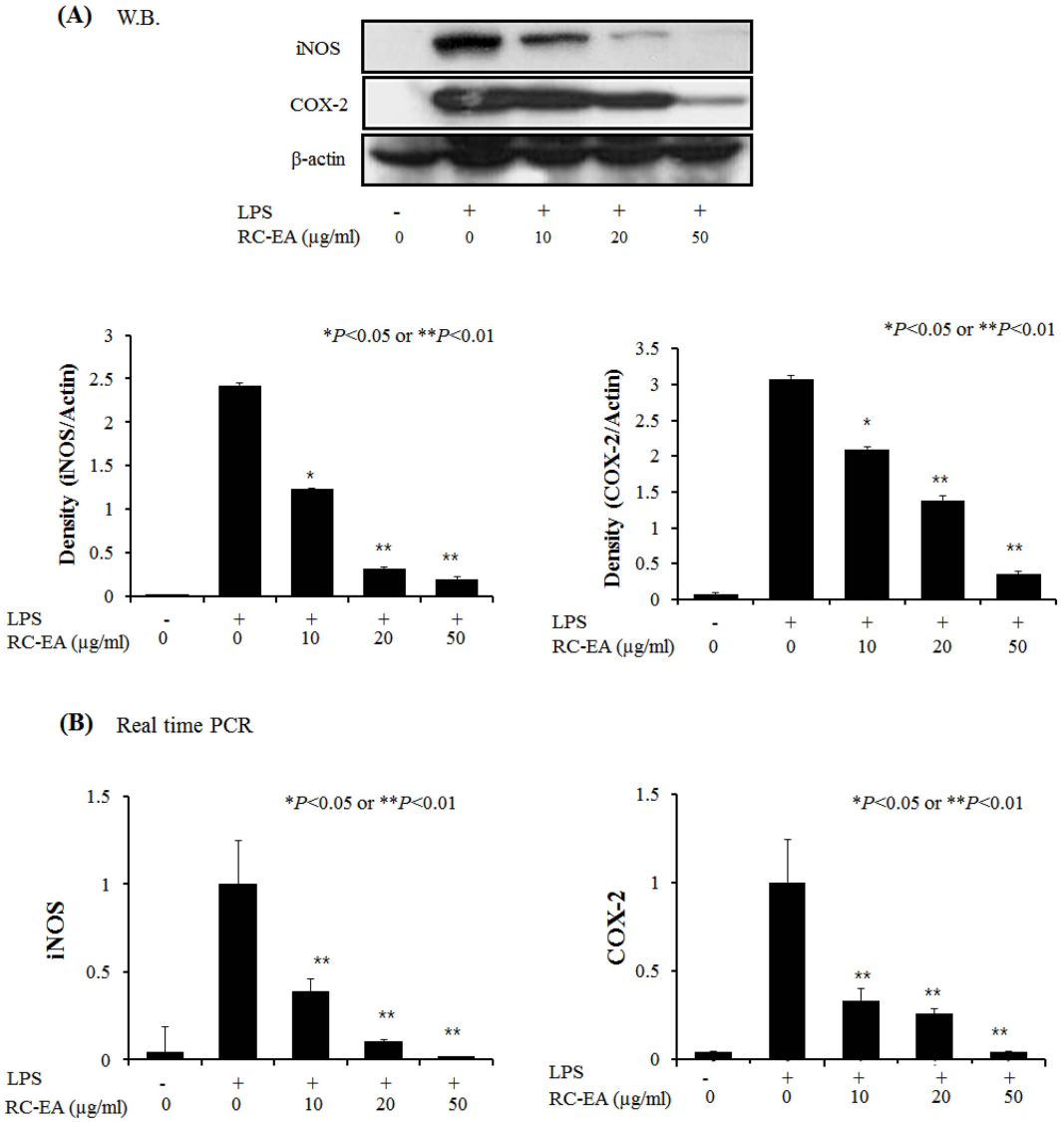

Fig. 5. Effect of *R. crispus* L. ethyl acetate fraction on LPS-induced iNOS and COX-2 expression and mRNA levels in RAW 264.7 macrophages. RAW 264.7 cells (1×10^6 cells/ml) were incubated for 24 h and then pretreated with *R. crispus* L. ethyl acetate fraction in the presence or absence of LPS (100 ng/ml) for 24 h. Equal amounts of total protein were resolved by SDS-PAGE. **P* value of < 0.05, ***P* value of < 0.01.

mediated biosynthesis of COX is the enzyme which converts arachidonic acid to PGE_2, and it is classified into COX-1 and COX-2. COX-2 is induced by inflammatory stimulating factor in inflammatory cells such as macrophages and fibroblasts. Prostaglandins are mostly produced by COX-2 during the inflammatory response.[24,25]

Therefore, we examined the expression ratio of iNOS and COX-2 protein inside the cells by western blot to investigate the effect of *R. crispus* L. extract and its fractions on iNOS and COX-2 protein expression (Fig. 3). When RAW 264.7 cells were treated with *R. crispus* L. extract and its fractions, the expression of iNOS protein, which was increased by treatment with LPS, was decreased. Especially, *R. crispus* L. hexane, chloroform, ethyl acetate fractions at a concentration of 50 μg/ml markedly decreased the expression of iNOS protein. Also, the expression of COX-2 protein, which plays an important role in the inflammatory response was increased by LPS treatment; and we finally observed the suppression of COX-2 protein expression after treatment with *R. crispus* L. hexane, chloroform, ethyl acetate fractions.

R. crispus L. ethyl acetate fraction had excellent anti-inflammatory effects in RAW 264.7 cells, and these cells were treated with a concentration of 10, 20, and 50 μg/ml

of *R. crispus* L. ethyl acetate fraction; then we measured cytotoxicity and suppression activity on NO production. The survival rate was up to 90% in cells treated with a high concentration of 50 μg/ml, and we could confirm significant inhibition of NO production. Also, we assessed the secretion rate of PGE_2 and TNF-α, which are inflammatory cytokines, by treating LPS-induced RAW 264.7 cells with several concentrations of *R. crispus* L. ethyl acetate fraction and the results were as follows (Fig. 4): when the *R. crispus* L. were treated, the secretion of PGE_2 and TNF-α, which was increased by treatment with LPS, was decreased significantly.

It was confirmed that the expression of decreased in a dose-dependent manner by treating RAW 264.7 cells with *R. crispus* L. ethyl acetate fraction. Kwak *et al*.[26] reported that the well-known mechanism of action of many anti-inflammatory drugs involves the suppression of prostaglandin synthesis and this suppression is closely related to the production of COX-2 inhibitors, such as nimesulide, celecoxib, and activity inhibition.[27] This study showed that *R. crispus* L. ethyl acetate fraction inhibited the production of PGE_2 and COX-2 protein expression. Therefore, we suggest that the mechanism of action of *R. crispus* L. ethyl acetate fractions is similar to the above mentioned mechanism. We could confirm the effects of *R. crispus* L. ethyl acetate fraction on iNOS and COX-2 gene expression by using real-time PCR (Fig. 5). We found that the mRNA expression of iNOS and COX-2 in LPS-stimulated control was increased; however, the expression of iNOS and COX-2 was significantly decreased in RAW 264.7 cells treated with *R. crispus* L. ethyl acetate fraction at a concentration of 10 μg/ml, 20 μg/ml and 50 μg/ml.

Kim *et al*.[28] reported that the polyphenol content of *R. crispus* L. ethyl acetate fraction was 703.9 ± 75.6 mg/g. Among all the contents, the polyphenol content was the highest. The RC_{50} value of *R. crispus* L. ethyl acetate fraction for the α-α-Diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity was 2.7 ± 0.2 μg/ml, and thus the *R. crispus* L. ethyl acetate fraction had the highest antioxidant activity. Therefore, a study to determine active constituents of the *R. crispus* L. ethyl acetate fraction with a focus on the polyphenol compound is needed.

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