Antioxidant and Anti-inflammatory Activities of Ethanol Extract from Leaves of *Cirsium japonicum*

Je-Hyuk Lee[1], Soo-Im Choi[1], Yong-Soo Lee[1,2], and Gun-Hee Kim[1,3]*

[1]*Plant Resources Research Institute, Duksung Women's University, Seoul 132-714, Korea*
[2]*College of Pharmacy, Duksung Women's University, Seoul 132-714, Korea*
[3]*Department of Food and Nutrition, Duksung Women's University, Seoul 132-714, Korea*

Abstract Antioxidant and anti-rheumatoid activities of *Cirsium japonicum* leaf extract (CJLE) were investigated in this study. CJLE had similar DPPH radical scavenging activity and reducing power to ascorbic acid and several flavonoids. Rheumatoid arthritis (RA) is a chronic inflammatory tissue-destructive disease, partly related with functions of hyaluronidases (HAases) and collgenases. CJLE (1,000 µg/mL) had approximately 60.7 and 31.9% inhibition of HAase and collagenase activity, respectively. Also, CJLE inhibited lipopolysaccharide (LPS)-induced nitrite production in a dose-dependent manner, and CJLE (1,000 µg/mL) suppressed approximately 70% of LPS-induced nitrite production effectively in RAW 264.7 macrophage cells. CJLE had inhibitory effects on the adherence of monocytic THP-1 to human umbilical vein endothelial cell (HUVEC) monolayers to the basal level. Inhibitory effect of CJLE on the adhesion was caused by suppression of tumor necrosis factor-a-upregulated expression of vascular cellular adhesion molecule-1 (VCAM-1) and E-selectin. We expect that CJLE may alleviate the inflammatory process in rheumatoid synovium, and these findings will raise the possibility of the usage of *C. japonicum* as a traditional pharmaceutical of anti-rheumatoid arthritis.

Keywords: *Cirsium japonicum*, antioxidant, anti-rheumatoid, nitric oxide (NO), HUVEC, monocyte, adhesion

Introduction

Cirsium japonicum which belongs to the Compositae family, is a wild perennial herb found in many areas of Korea, Japan, and China. *C. japonicum* has been used as a hypertensive, uretic, and a hepatitis agent in medicine. Also, it has been used in the treatment of tumors including liver cancer, uterine cancer, and leukemia (1). An anti-anxiety function and antimitotic activity of *Cirsium* sp. were reported by Grzycka *et al.* (2).

Flavonoid compounds; isokaempferide 7-*O*-β-D-(6''-methylglucuronide), apigenin 7-(6''-methylglucuronide), hispidulin 7-glucoside, isokaempferide 7-glucuronide, kaempferol 3-galactoside, pectolinarin, linarin, and apigenin 7-glucuronide were isolated from ethanol-extract of *Cirsium* sp., and had antimicrobial activity against *Micrococcus luteus, Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* ESBL, *Pseudomonas aeruginosa,* and *Candida albicans* (3). Antimicrobial activities of flower- and leaf-extracts of *Cirsium rivulare* were superior to other part extracts, and these bactericidal activities were good for Gram-positive bacteria (3). Antioxidant activities of *C. japonicum* var. *ussurense* Kitamura-extracts from different parts were reported (4). Leaf-extracts in *n*-butanol fraction and root-extracts in ethylacetate and *n*-butanol fractions had showed superior antioxidant activity. Also, the ethanol-extracts from the leaves of *Cirsium setidens* Nakai had higher antioxidant activities than those of stem- and root-part extracts (5).

Over-controlled reactive oxygen species (ROS) cause the oxidative stresses to normal cellular system. ROS leads to stimulate the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) related to rheumatoid arthritis (RA) as well as primary oxidative stresses (6,7). Also, it was reported ROS cause the adhesion of monocytes to human umbilical vein endothelial cells (HUVECs) (8,9) and the expression of cellular adhesion molecules (CAMs) in immunological inflammation (10-12), occurred in early stage of immunological inflammation. CAMs are stimulated by inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukins. Up-regulated expression of CAMs increases adherence of monocytes and neutrophils to HUVEC monolayer, and cause RA and atherosclerosis.

In this study, we investigated the biofunctions of *C. japonicum* leaf extract (CJLE) for antioxidant activity, the inhibitory influence on the nitrite production, and cell adhesion related to the progress of RA. From these results, we expect it will be supporting the evidence for the potential anti-rheumatoid activity of Korean domestic *C. japonicum*.

Materials and Methods

Reagents Dimethyl sulfoxide (DMSO), flavonoids, calcein *O,O'*-diacetate tetrakis (acetoxymethyl) ester (Calcein-AM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), collagenase (EC 3.4.24.3), hyaluronidase (HAase, EC 3.2.1.35), lipopolysaccharide (LPS), and N^G-monomethyl-L-arginine monoacetate (L-NMMA) were purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO, USA). Hyaluronic acid and PZ-peptide were from Fluka (Sigma-Aldrich Inc.). Cell culture medium and reagents, such as F-12K, RPMI-1640, fetal bovine serum (FBS), penicillin/

streptomycin, endothelial cell growth supplement (ECGS), and trypsin-EDTA were obtained from Gibco (Invitrogen Inc., Grand Island, NY, USA). TNF-α was purchased from BD Science (San Jose, CA, USA). Ethanol and other reagents were used as first grade reagents.

Preparation of CJLE   Leaves of *C. japonicum* were washed, dried by freeze drier, and crushed. Crushed leaves (30 g) were extracted with 100 mL of 70% ethanol (in water) for 24 hr at room temperature. Extracts were filtered through Whatman No. 1 filter paper. Filtered extracts were concentrated by evaporator under the reduced pressure. CJLE was redissolved in DMSO to 100 mg/mL of concentration as a stock.

Radical scavenging activity   Scavenging effect of CJLE on DPPH radical was monitored according to the method described by Lee *et al.* (13). A 0.2 mL of methanolic solution containing extracts was mixed with 4 mL of methanol, and a methanolic solution of DPPH (1 mmol/L, 0.5 mL) was added. The mixture was vortexed for 15 sec, left to stand at room temperature for 30 min, and the absorbance was read at 517 nm.

Reducing power   The reducing power of CJLE was determined by Fe^{3+} reduction (14-16). CJLE (10-1,000 μg/mL) in distilled water was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min. After that, 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 2,090×g for 10 min. A 2.5 mL of supernatant layer was added to 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance of the mixture was measured at 700 nm using UV-spectrophotometry (Agilent Technologies Inc., Santa Clara, CA, USA).

HAase inhibition assay   HAase inhibition assay was performed by the Morgan-Elson method (17,18). HAase solution (7,900 units/mL in 0.1 M acetate buffer, pH 3.5) was added to 100 μL of sample, and incubated at 37°C water bath incubator for 20 min. Then, 0.1 mL of 12.5 mM $CaCl_2$, as HAase activator, was added and further incubated for 20 min. For HAase reaction, 250 μL of hyaluronic acid (1.2 mg/mL in 0.1 M acetate buffer, pH 3.5) was added and incubated at 37°C water bath incubator for 40 min. For termination of HAase reaction, 0.1 mL of 0.4 N NaOH and 0.1 mL of 0.4 M potassium tetraborate were added, and warmed in boiling water for 3 min. After cooled completely, 3 mL of DMAB reagent (4 g of p-demethylaminobenaldehyde, 350 mL of 100% glacial acetic acid, and 50 mL of 10 N HCl) was added to the mixture, and was incubated at 37°C water bath incubator for 20 min. The HAase reaction product, 4-acetylglucosamine, was measured by UV-spectrophotometry (Agilent Technologies Inc.) at 585 nm wavelength. HAase inhibition activity was expressed by decreased percentage as compared to the control. Inhibitory activity was calculated by following equation;

% HAase inhibition=$[(A_c-A_s)/A_c] \times 100$

where A_c and A_s are the absorbance of control and sample, respectively. Control (1% DMSO solution) was used instead of CJLE samples.

Collagenase inhibition assay   Collagenase inhibition assay was performed by Sawabe's procedure (19,20) with a little modification. Collagenase (5.0 μg) was added to PZ-peptide (0.5 mg), a substrate of collagenase, in 0.1 M Tris buffer (pH 7.4) containing with or without CJLE. Total volume was 1.7 mL. The mixture was incubated at 37°C water bath for 30 min, and 1 mL of 25 mM citric acid solution was added for termination of enzyme reactions. After mixing with 5 mL of ethylacetate, the absorbance of organic layer was measured by UV-spectrophotometry (Agilent Technologies Inc.) at 320 nm. Percentage collagenase inhibition was calculated by following equation;

% Collagenase inhibition=$[(A_c-A_s)/A_c] \times 100$

where A_c is (the absorbance of control with collagenase - the absorbance of control without collagenase), and A_s was (the absorbance of CJLE with collagenase – the absorbance of CJLE without collagenase).

Cell culture   RAW 264.7 murine macrophage cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). RAW 264.7 cells were cultured in DMEM containing 10% FBS, and 100 units/mL of penicillin/streptomycin in 5% CO_2 humidified incubator at 37°C. RAW 264.7 cells were used at passage numbers 10-20 for LPS-induced nitrite production assay. Monocytic cell line, THP-1, was obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). Monocytes were cultured in RPMI-1640 medium containing 10% FBS and 100 units/mL of penicillin/streptomycin at 37°C in 5% CO_2 incubator under controlled moisture. Monocytes were subcultured after being collected by centrifugation at 2,090×g for 2 min due to the floating cell line. Monocytes were used for cell adhesion assay at passage numbers 20-30. HUVECs (CRL-2480; ATCC) were cultured with F-12K nutrient mixture (Kaighn's modification, Gibco) containing 10% FBS, 100 units/mL of penicillin/streptomycin, 0.1 mg/mL of heparin, and 0.03 mg/mL of ECGS. HUVECs were cultured at 37°C in 5% CO_2 humidified incubator. For subculture, RAW 264.7 macrophage cells and HUVEC monolayer were rinsed twice with PBS (pH 7.4) to remove all traces of serum which contains trypsin inhibitor, and was subdivided using 0.05% trypsin with 0.53 mM EDTA. HUVECs were used at passage numbers 20-30 for this study.

Cytotoxicity test   Cytotoxicity was examined by MTT assay. RAW 264.7 cells and HUVECs were plated at a density of 1×10^5 cells/well in 96-well tissue culture plate (Corning Inc., Corning, NY, USA). Plated cells were treated with indicated concentrations of CJLE. After 24 hr incubation, MTT was added to all well at 0.5 mg/mL of concentration, and incubated for 4 hr at 37°C. After discarding all medium from the plates, 100 μL of DMSO was added to the all well. The plates were placed for 5 min at room temperature with a shaking, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by UV-spectrophotometric plate reader (Emax, Molecular Devices Inc., Sunnyvale, CA, USA). Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

Nitrite assay RAW 264.7 cells were plated at 2×10^5 cells/well in a 96-well culture plate and incubated for 3-4 hr in 5% CO_2 humidified incubator at 37°C. Plated cells were treated with LPS (1 μg/mL) for stimulation of nitrite-production and an indicated concentrations of CJLE for 24 hr. LPS-stimulated nitrite-production from RAW 264.7 cells was measured by the Griess reaction (21,22). Briefly, 100 μL of each supernatant was mixed with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride in distilled water), and the absorbance of the mixture was determined by a microplate reader (Emax, Molecular Devices Inc.) at 540 nm. In this experiment, 10 μM of L-NMMA, an iNOS inhibitor, was used as a positive control (23,24).

Calcein-AM labeling of cells Prior to cell adhesion assay, monocytes were fluorescent-labeled by the incubation with 5 mM of calcein-AM in PBS (pH 7.4) for 30 min at 37°C. After loading of calcein-AM, cells were washed 3 times with PBS to remove the excess calcein-AM. Then cells were resuspended in RPMI 1640 medium at appropriate concentration.

Cell adhesion assay HUVECs were seeded at 1×10^5 cells/well in 96 well tissue culture plate (Corning 3603; Corning Inc.). After 24 hr incubation at 37°C, HUVEC monolayer was treated with CJLE for 24 hr, and then was stimulated with 5 ng/mL of TNF-α for 24 hr. The HUVEC monolayers were washed 3 times with PBS before cell adhesion assay (25). Calcein-AM labeled monocytes, THP-1, were cocultured at 5×10^5 cells/well with monolayer for 1 hr in 5% CO_2 humidified incubator at 37°C. Non-adherent monocytes were removed from monolayer by washing 4 times with PBS. Adherence of calcein-AM labeled monocytes was determined by fluorescent intensity, measured using a fluorescence plate reader (FL600; Bio-Tek Instruments, Inc., Winooski, VT, USA). The excitation and emission wavelengths for calcein-AM molecule are 485 and 530 nm, respectively.

For photographs, HUVECs were seeded on 24-well culture plates. Calcein-AM labeled THP-1 cells attached on HUVEC monolayer were photographed at a magnification of 200× using an inverted fluorescence microscope (IX 71; Olympus Inc., Tokyo, Japan) connected to an Olympus DP50 camera with Imaging software (ViewfinderLite, Ver. 1.0.134, Pixera Corporation, Los Gatos, CA, USA and OLYSIA BioAutoCell Ver. 3.2, Soft Imaging System, Tokyo, Japan).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for CAMs transcription Total RNA was isolated from HUVECs using RNeasy kit (Qiagen Inc., Valencia, CA, USA) after treated with CJLE. RT-PCR was performed using One-step RT-PCR kit (Qiagen and Bioneer Corp., Seoul, Korea) and primers at a final concentration of 1 μM. For PCR, human primers for CAMs were used as followed; vascular cellular adhesion molecule-1 (VCAM-1) forward primer: 5'-ATGCCTGGG-AAGATGGTCGTGA-3', VCAM-1 reverse primer: 5'-TG-GAGCTGGTAGACCCTCGCTG-3', intracellular adhesion molecule-1 (ICAM-1) forward primer: 5'-GGTGACGCT-

GAATGGGGTTCC-3', ICAM-1 reverse primer: 5'-GTCC-TCATGGTGGGGCTATGACTC-3', E-selectin forward primer: 5'-ATCATCCTGCAACTTCACC-3', E-selectin reverse primer: 5'-ACACCTCACCAAACCCTTC-3'. Total RNA (1 μg/μL) of basal HUVECs was used as a control, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, the house-keeping gene (26) were used to determine PCR efficiency; GAPDH forward primer: 5'-ATGACAA-CAGCCTCAAGATCATCAG-3', GAPDH reverse primer: 5'-CTGGTGGTCCAGGGGTCTTACTCCT-3'. Thermal cycling was performed according to modified method (16,17). For cDNA synthesis and predenaturation, 1 cycle of 50°C for 30 min and 95°C for 15 min was performed on total RNA. For PCR amplification, 30 cycles of 95°C for a 1 min denaturation, 55°C for 2 min annealing, 72°C for a 3 min extension, and 1 cycle of 72°C for a 10 min final extension were performed using Bio-Rad thermal cycler (MJ Mini; Bio-Rad Inc., Hercules, CA, USA). And RT-PCR product was stored at 4°C until agarose gel separation.

Protein assay For standardization of cell adhesion assay data, the amount of plated monolayer was measured by BCA protein assay (Pierce Inc., Rockford, IL, USA) using bovine serum albumin as a standard, following solubilizing cells using 0.1 N NaOH and 1% of 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate.

Statistical analysis The Student's *t*-test was used to determine the statistical significance of differences between values for a variety of experimental and control groups. Data was expressed as means±standard errors (SEM) of at least triplicate.

Results and Discussion

DPPH radical scavenging activity of CJLE Antioxidant activities of CJLE (500-2,000 μg/mL) for DPPH radical scavenge were shown in Fig. 1. Ascorbic acid (1,000 μM), an antioxidant, was used as a positive control. CJLE had the hydrogen-donating activity to DPPH radical in concentration-dependent manner. DPPH radical scavenging effect of CJLE (1,000 μg/mL) was approximately 64%, similar to ascorbic acid, used as an antioxidant *in vivo* and *in vitro*. Also, 2,000 μg/mL of CJLE had approximately 82% radical scavenging activity, which is similar to authentic 1 mM of flavonoids: kaempferol, qucertin, and rutin. When tested with 0-2,000 μg/mL of CJLE, EC_{50} value was 490 μg/mL. DPPH-scavenging results were consistent with flavonoids analysis data in *C. japonicum* extracts (27,28). Apigenin, quercetin, kaempferol, luteolin, and hispidulin were identified as major flavonoids in CJLE by HPLC and LC/MS. Also, kaempferol, quercetin, and rutin showed great DPPH-radical scavenging activity, but apigenin had little antioxidant activity, as reported by several researchers (29-31).

Reducing power of CJLE Reducing power of CJLE determined by Fe^{3+} reduction assay. The reducing power of Fe^{3+} was increased in concentration-dependent manner (Table 1). These results were consistent with DPPH radical scavenging results. CJLE had showed low reducing power of Fe^{3+}, where as pyrogallol (125 μg/mL) had high reducing
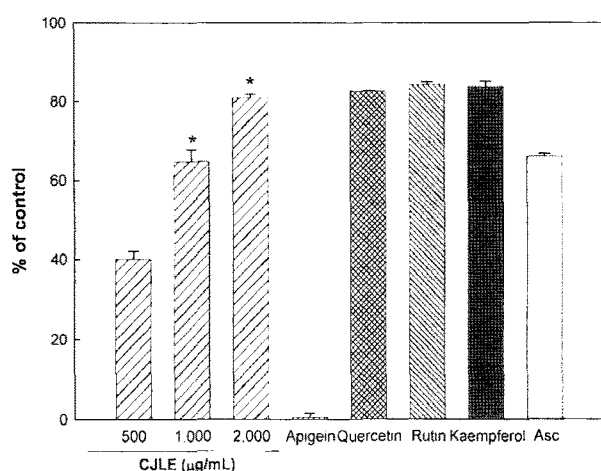
Fig. 1. DPPH radical scavenging activity of CJLE. DPPH radical scavenging activity was expressed by the percentage for control. Error bar has been omitted when the standard deviation was significantly small. *$p<0.05$, significantly different as compared to the control.

Table 1. Reducing power of CJLE

Samples	(μg/mL)	Reducing power at 700 nm[1]
	12.5	ND
	25	0.01±0.00
Circium japonicum	50	0.13±0.00
leaf extracts	100	0.15±0.00*
	500	0.19±0.01*
	1,000	0.40±0.01*
Pyrogallol	125	1.75±0.04*
Apigenin		ND
Kaempferol		0.12±0.00
Quercetin	50 μM	0.15±0.01*
Rutin		0.21±0.00*

[1]ND, not detected; *significant difference compared to the control at $p<0.05$.

Table 2. Inhibitory effect of CJLE on enzymes related to RA

	Inhibition (%)	
	Hyaluronidase	Collagenase
Cirsium japonicum leaf extracts		
500 μg/mL	62.00±4.30[1]	0.79±19.9
1,000 μg/mL	60.70±9.87[1]	31.86±7.48[1]

[1]Significant difference; $p<0.05$, as compared to the control.

power as a control (32). However, the reducing power of CJLE was similar to those of kaempferol, quercetin, and rutin (50 μM), like a case of DPPH radical scavenge.

The cell and tissue injury associated with acute and chronic inflammation is due to the toxicity of ROS generated and released by activated phagocytes (33,34). NADPH oxidase in phagocytes is responsible for the production of superoxide anion and represents a major host defense mechanism of phagocytes against invading microorganisms. Superoxide initiates the formation of other ROS, such as hydroxyl radical and oxygen singlet, which are known as strong oxidant products, and hydrogen peroxide and hypochlorous acid (35-38). Free radical scavenging activity and reducing power play the critical role for quenching superoxide anions and maintaining the cellular redox homeostasis against harmful oxidants and free radicals in cells. Also, the reducing power is generally associated with the presence of reductones, which have been shown to exert an antioxidant effect by donating a hydrogen atom and breaking the free radical chain (39,40). Reductones are reported to react with certain peroxide precursors, which preventing peroxide formation (41). In healthy blood, several enzymes, such as glutaredoxin (GRX) and glutathione reductase (GRD), and chemical compounds, such as ascorbic acid, vitamin E, and glutathione, play as antioxidant and reducing enzyme system for scavenging harmful superoxides and free radical compounds (42). In RA, the cellular reducing power may support to quench superoxide anions secreted from cells inside synovial membrane and prevent the inflammation responses in joint-cells. From this result, the reducing power and free radical scavenging activity of CJLE will help the relief on inflammatory symptoms, caused by harmful radical compounds.

Inhibition of CJLE on HAase and collagenase Inhibitory effects of CJLE (500 and 1,000 μg/mL) on HAase and collagenase were shown in Table 2. A 1,000 μg/mL of CJLE had approximately 60.7 and 31.9% inhibition on HAase and collagenase activity at a significant difference ($p<0.001$), respectively.

HAases are known as enzymes involved in inflammatory responses (43-45). Also, collagenase, a matrix metalloproteinase (MMP)-1, mainly breaks down collagen type I and type III (46). Conventionally collagenase-1 or MMP-1 has been considered responsible for degradation of collagen and proteoglycan cartilaginous matrix in joints (47). Abnormal breakdown of connective tissue components contributes to a large number of pathological conditions including RA, atherosclerosis, tumor invasion, and metastasis (48). More recently, collagenase-3 or MMP-13 has been found to be particularly effective in degradation of type II collagen in RA (50,51). CJLE consists of several flavonoids: apigenin, kaempferol, hispidulin 7-glucoside, pectolinarin, and linarin (3). These flavonoids are expected to play important roles for inhibition of HAase and collagenase. Also, the inhibitory effects of apigenin, quercetin, and kaempferol on HAase and collagenase were reported by several researchers (52-55).

Inhibition of CJLE on LPS-stimulated nitrite production from RAW 264.7 cells Prior to the inhibition assay of nitrite production, the cytotoxicity of CJLE (0-1,000 μg/ mL) on RAW 264.7 macrophages was determined by proliferation test (Fig. 2). CJLE did not affect cell viability in all-tested concentrations. CJLE (25-1,000 μg/mL) were used for the inhibition of nitrite production from RAW 264.7 macrophage cells.

CJLE inhibited LPS-induced nitrite production in a dose-dependent manner, as shown in Fig. 3. We observed LPS-stimulated nitrite production from RAW 264.7 cells, as compared to the basal. Also, L-NMMA (100 μM), which is one of selective NOS blockers (23), inhibited approximately 95% of LPS-induced nitrite production
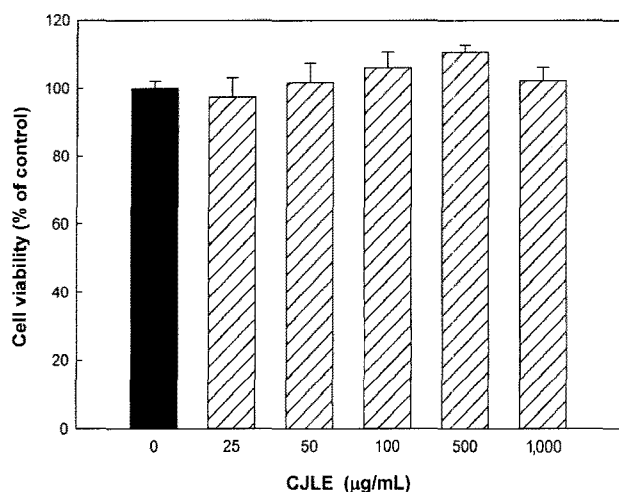
Fig. 2. Effect of CJLE on cell viability of RAW 264.7 macrophage cells. CJLE (0-1,000 mg/mL) were added to RAW 264.7 macrophage cells and incubated in humidified 5% CO_2 incubator for 24 hr in 37°C. Each group was no different to the control.
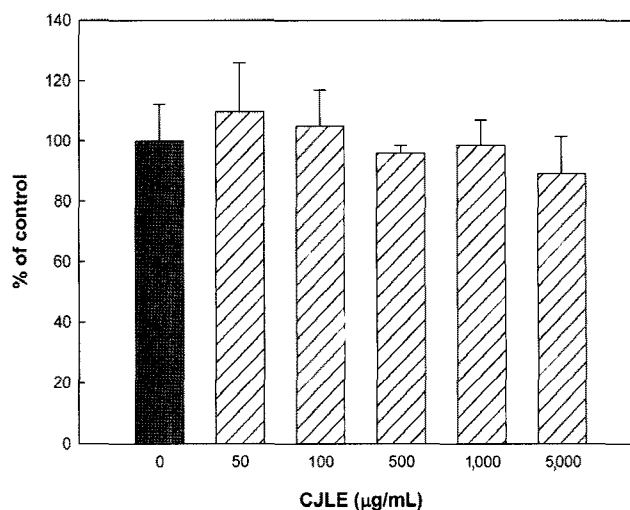

Fig. 4. Effect of CJLE on cell viability of HUVEC monolayers. CJLE (0-5,000 mg/mL) were added to HUVEC monolayers and incubated in humidified 5% CO_2 incubator for 24 hr in 37°C. Each group was no difference to the control.
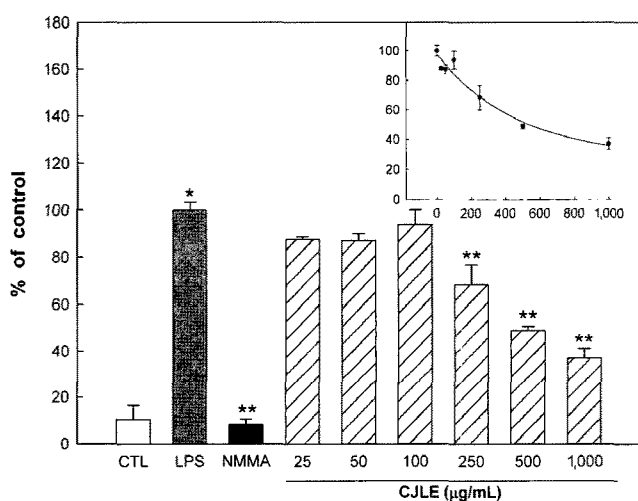

Fig. 3. Effect of CJLE on LPS-induced nitrite production from RAW 264.7 macrophage cells. IC_{50} was 277 μg/mL of CJLE for inhibition of LPS-induced NO production from RAW 264.7 macrophage cells. *$p<0.05$, as compared to control, **$p<0.001$, as compared to LPS-induced group.

significantly as a positive control. CJLE showed partly suppression of LPS-induced nitrite production from RAW 264.7 cells in a concentration-dependent manner (250-1,000 μg/mL). A 1,000 μg/mL of CJLE showed approximately 70% decrease in nitrite production, as compared to the LPS-induced nitrite production at a significant difference ($p<0.001$).

Nitric oxide (NO) is also related to the pathophysiology of inflammatory joint disease and plays a key role in cartilage catabolism mediated by inflammation (56). NO may represent key regulatory molecules in the inflammatory process in RA (57). Also, exogenously generated NO induces COX-2 expression in synovial cells. Apigenin, one of flavonoids in *Cirsium* sp. extracts, have minimal antioxidant activity in comparison to structurally related flavonoids: quercetin, mortin, and myricetin (30,31).

However, apigenin, kaempferol, and quercetin have inhibitory activity of iNOS reaction (58-60). As a whole, these findings showed that CJLE containing several flavonoids effectively inhibit LPS-induced nitrite production from RAW 264.7 macrophage cells.

Inhibition of CJLE on the adherence of monocytes to HUVEC monolayers CJLE (0-5,000 mg/mL) had a little cell toxicity (over 80% of cell proliferation) on HUVEC monolayers in all-tested concentration range (Fig. 4). CJLE had no harmful effects on proliferation of HUVEC monolayers related with cell adhesion for the initial steps of immunological inflammation. CJLE (500, 1,000, and 5,000 mg/mL) were used for cell adhesion assay without damages to HUVEC monolayers.

To investigate the anti-rheumatoid activity of CJLE, the inhibition on adhesion of THP-1 to HUVEC monolayers was examined. HUVEC monolayer was stimulated by TNF-α for adhesion assay. In response to TNF-α, the adherence of THP-1 to HUVEC monolayers had been stimulated significantly. CJLE reduced TNF-α-stimulated adhesion of THP-1 to HUVEC monolayers, as shown in Fig. 5. Figure 5A showed representative images of the stimulation of adhesion and the reduction of TNF-α-stimulated adhesion of THP-1 to HUVEC monolayers by CJLE. Fluorescent images showed that CJLE reduced the adhesion of calcein-AM labelled THP-1 to HUVEC monolayer to basal level. HUVEC monolayer background was shown in half-fluorescent images. A 1,000 μg/mL of CJLE had approximately 82% inhibition of adherence of THP-1 to HUVEC monolayers, and even 5,000 mg/mL of extract inhibited the adhesion to basal level (Fig. 5B). Otherwise, at 500 μg/mL of CJLE, no inhibitory effect on adhesion of THP-1 to HUVEC monolayers was shown.

When HUVECs in vascular system are exposed to the inflammation mediators, such as interleukin-1 and TNF-α, CAMs are expressed for the attachment with monocytes (61,62), which acts as one of the initial steps for RA and atherosclerosis. CJLE inhibited the adherence of THP-1 to
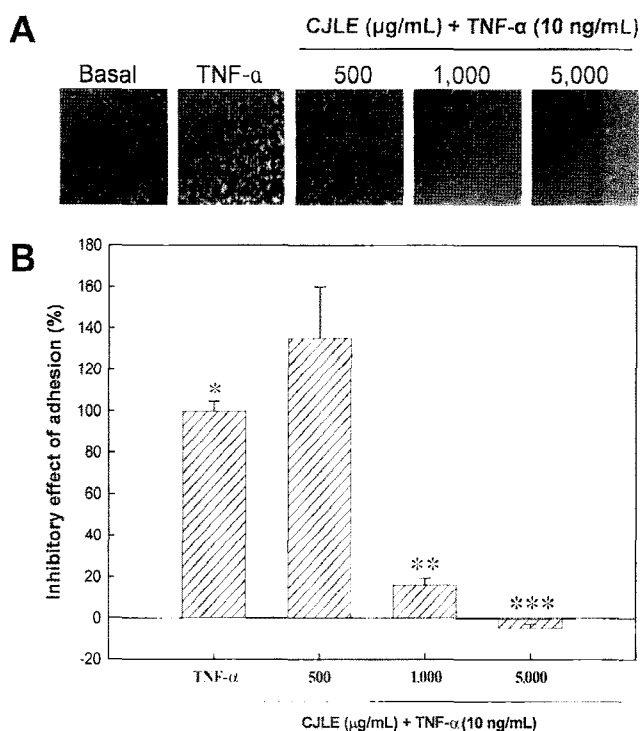
Fig. 5. **Inhibitory effect of CJLE on cell adhesion of THP-1 to HUVEC monolayers.** A, Adhesion of fluorescent-labeled THP-1 cells was photographed by fluorescence microscope with WB filter at 200× of magnification. B, Inhibition of cell adhesion. Significant difference; $*p<0.05$, as compared to the basal, $**p<0.05$, as compared to TNF-α treatment group as a positive control, $***p<0.05$, as compared to TNF-α treatment and 1,000 μg/mL extracts-addition group.

HUVEC monolayers *in vitro*. It is considered due to flavonoids components in CJLE, because apigenin, quercetin, and kaempferol have inhibitory activities for adherence of THP-1 to HUVEC monolayers (63,64). Therefore, we expected CJLE might decrease the adherence through the inhibitory effects on the transcription of TNF-α-upregulated CAMs in HUVECs.

Effect of CJLE on transcription of CAMs We observed no signal of the basal mRNA transcription of CAM proteins in HUVECs at gel migration pictures of RT-PCR products (Fig. 6). Addition of TNF-α stimulated significantly transcription of CAM proteins, such as VCAM-1, ICAM-1, and E-selectin. Also, we observed the suppression of mRNA transcriptional level for CAM proteins by CJLE in a dose-dependent manner. CJLE reduced TNF-α-stimulated upregulation of VCAM-1 effectively at 500 μg/mL concentration. Otherwise in case of ICAM-1 and E-selectin mRNA transcription, RT-PCR bands became fainter as concentration of CJLE increased and were reduced to basal level at 5,000 μg/mL of extracts.

Profiles for reduction of gene transcription by CJLE were different for each CAM. TNF-α-stimulated-VCAM-1 transcription was shown the most effective suppression among three CAMs (Fig. 6A). Transcription of VCAM-1 was suppressed significantly by 500 μg/mL extracts. Transcriptional level of VCAM-1 for each CJLE treated group is no difference to the basal and each other. Otherwise, the transcription of ICAM-1 and E-selectin were reduced as CJLE increased, progressively (Fig. 6B and 6C). In case of ICAM-1, the reduction of transcription was not suppressed in 500 μg/mL extracts-treated group. The suppression of transcription was appeared from 1,000 μg/mL of extracts-treated group, and 5,000 μg/mL extracts reduced approximately 86% of ICAM-1 transcription. Addition of 500 and 1,000 μg/mL of extract reduced approximately 26 and 35% of E-selectin transcription, and 5,000 μg/mL of extracts reduced E-selectin transcription to a basal level. RT-PCR bands for ICAM-1 and E-selectin became fainter as concentration of extract increased. These results were consistent with inhibitory effect data of cell-cell adhesion in Fig. 5.

In immunological inflammatory responses such as RA, the activation of monocytic THP-1 and neutrophils were performed by TNF-α and interleukin family (65). Also, TNF-α and interleukin family play a role as a stimulator for up-regulation of CAMs expression in HUVEC monolayers. Stimulation of CAMs protein expressions causes an increase in adherence of monocytes and neutrophils to
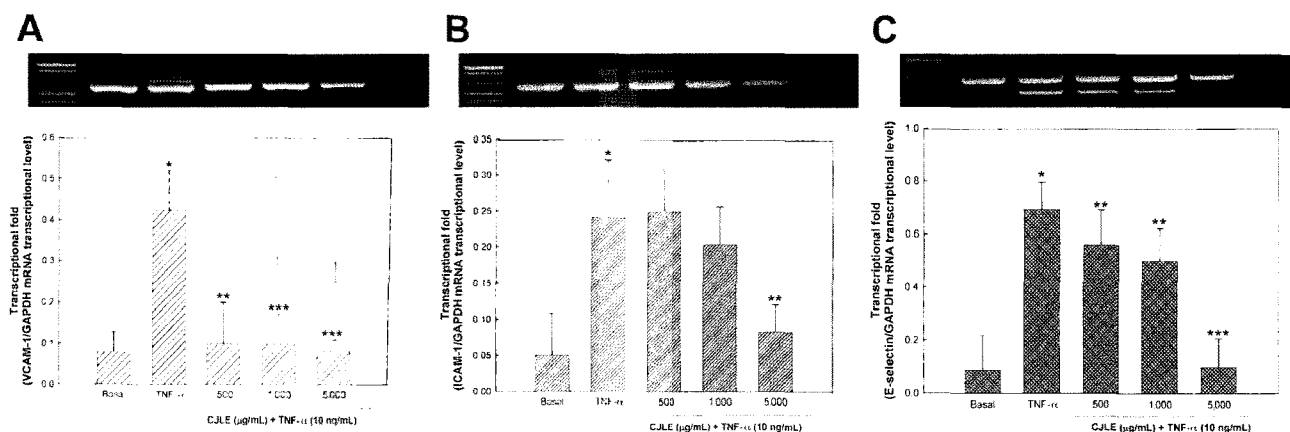

Fig. 6. **Influence of CJLE on TNF-α-induced mRNA transcription of CAMs.** A, Transcriptional change of VCAM-1. $*p<0.05$, as compared to basal, $**p<0.01$, $***p<0.05$, as compared to TNF-α treatment group; $**$ $***$ are no difference to basal. B, Transcriptional change of ICAM-1. $*p<0.05$, as compared to basal, $**p<0.05$, as compared to TNF-α treatment group, and $**p<0.5$, as compared to basal. C, Transcriptional change of E-selectin. $*p<0.05$, as compared to basal, $**p<0.5$, as compared to TNF-α treatment group, $***p<0.05$, as compared to TNF-α treatment group and to 1,000 μg/mL group and no difference with basal.

blood vessel cells, especially HUVEC monolayers. These responses are closely related to RA (25) and atherosclerosis (66). VCAM-1, ICAM-1, and E-selectin are constitutive, and were expressed by inflammatory agent-modulation among several CAMs (67). Also, mitogen-activated protein kinases (MAPKs) and nuclear factor-kB (NF-kB) pathways regulate the expressions of CAMs protein (68). In the future, it will be studied the effects of CJLE on the activation factors, such as p42/p44 MAPK, p38, and JNK for MAPKs pathways, which regulate the expression of CAMs.

From this study, we investigated CJLE suppressed LPS-induced NO production from RAW 264.7 cells and prevented the adhesion of monocyte to HUVEC monolayers, and may have anti-inflammatory function for rheumatoid arthritis. CJLE may be used as the functional food materials and an alternative medicine to help for the relief and prevention of RA symptoms.

Acknowledgments

References

1. Lui S, Luo X, Li D, Zhang J, Qui D, Lui W, She L, Yang Z. Tumor inhibition and improve immunity in mice treated with flavone from *Cirsium japonicum* DC. Int. Immunopharmacol. 6: 1387-1393 (2006)

2. Grzycka K, Krzaczek T, Milkowska J. Research on the biological activity if selected species of flower plant. Ann. Univ. Mariae Curie Sklodowska 33: 275-283 (1978)

3. Nazaruk J, Jakoniuk P. Flavonoid composition and antimicrobial activity of *Cirsium rivulare* (Jacq.) All. flowers. J. Ethnopharmacol. 102: 208-212 (2005)

4. Lee HK, Kim JS, Kim NY, Kim MJ, Park SU, Yu CY. Antioxidant, antimutagenicity, and anticancer activities of extracts from *Cirsium japonicum* var. *ussurense* KITAMURA. Korean J. Med. Crop Sci. 11: 53-61 (2003)

5. Lee SH, Jin YS, Heo SI, Shim TH, Sa JH, Choi DS, Wang MH. Composition analysis and antioxidant activity from different organs of *Cirsium setidens* Nakai. Korean J. Food Sci. Technol. 38: 571-576 (2006)

6. Ayasolla K, Khan M, Singh AK, Singh I. Inflammatory mediator and ß-amyloid (25-35)-induced ceramide generation and iNOS expression are inhibited by vitamin E. Free Radical Bio. Med. 37: 325-338 (2004)

7. Ayasolla KR, Giri S, Singh AK, Singh I. 5-Aminoimidazole-4-carboxamide-I-beta-4-ribofuranoside (AICAR) attenuates the expression of LPS- and Aß peptide-induced inflammatory mediators in astroglia. J. Neuroinflam. 2: 21-42 (2005)

8. Sorescu GP, Song H, Tressel SL, Hwang J, Dikalov S, Smith DA, Boyd NL, Platt MO, Lassèque B, Griendling KK, Jo H. Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress induces monocyte adhesion by stimulating reactive oxygen species production from a Nox-1-based NADPH oxidase. Circ. Res. 95: 773-779 (2004)

9. Simoncini S, Sapet C, Camoin-Jau L, Bardin N, Harlé JR, Sampol J, Dignat-George F, Anfosso F. Role of reactive oxygen species and p38 MAPK in the induction of the pro-adhesive endothelial state mediated by IgG from patients with anti-phospholipid syndrome. Int. Immunol. 17: 489-500 (2005)

10. Shimozawa M, Naito Y, Manabe H, Uchiyama K, Kuroda M, Katada K, Yoshida N, Yoshikawa Y. 7-Ketocholesterol enhances the expression of adhesion molecules on human aortic endothelial cells by increasing the production of reactive oxygen species. Redox

Rep. 9: 370-375 (2004)

11. Traore K, Sharma RB, Burek CL, Trush MA. Role of ROS and MAPK in TPA-induced ICAM-1 expression in the myeloid ML-1 cell line. J. Cell. Biochem. 100: 1010-1021 (2007)

12. Deem TL, Cook-Mills JM. Vascular cell adhesion molecule-1 (VCAM-1) activation of endothelial cell matrix metalloproteinases: Role of reactive oxygen species. Blood 104: 2385-2393 (2004)

13. Lee EJ, Kim KS, Jung HY, Kim DH, Jang HD. Antioxidant activities of garlic (*Allium sativum* L.) with growing districts. Food Sci. Biotechnol. 14: 123-130 (2005)

14. Oyaizu M. Studies on product of browning reaction: Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn. J. Nutr. 44: 307-315 (1986)

15. Amarowicz R, Troszyñska A, Shahidi F. Antioxidant activity of almond seed extract and its fractions. J. Food Lipids 12: 344-358 (2005)

16. Song HS, Moon KY. *In vitro* antioxidant activity profiles of β-glucans isolated from yeast *Saccharomyces cerevisiae* and mutant *Saccharomyces cerevisiae* IS2. Food Sci. Biotechnol. 15: 437-440 (2006)

17. Lee J, Lee SH, Min KR, Lee KS, Ro JS, Ryu JC, Kim Y. Inhibitory effects of hydrolysable tannins on Ca^{2+}-activated HAase. Planta Med. 59: 381-382 (1993)

18. Lee YM, Choi SI, Lee JW, Jung SM, Park SM, Heo TR. Isolation of hyaluronidase inhibitory component from the roots of *Astraglus membranaceus* Bunge (Astragali Radix). Food Sci. Biotechnol. 14: 263-267 (2005)

19. Sawabe Y, Yamasaki K, Iwagami S, Kazimura K, Nakagommi K. Inhibitory effects of natural medicines on the enzymes related to the skin. Yakuga. Zasshi 118: 423-429 (1998)

20. Park H, Sin BY, Kim HP. Inhibition of collagenase by anti-inflammatory synthetic flavones. J. Appl. Pharmacol. 14: 36-39 (2006)

21. Chattopadhyay S, Bhaumik S, Purkayastha M, Basu S, Chaudhuri AN, Gupta SD. Apoptosis and necrosis in developing brain cells due to arsenic toxicity and protection with antioxidants. Toxicol. Lett. 136: 65-76 (2002)

22. Dawson TM, Dawson VL. Nitric oxide: Actions and pathological roles. Neuroscientist 1: 7-18 (1995)

23. Sutherland H, Khundkar R, Zolle O, McArdle A, Simpson AWM, Jarvis JC, Salmons S. A fluorescence-based method for measuring nitric oxide in extracts of skeletal muscle. Nitric Oxide 5: 475-481 (2001)

24. Scheller M, Blobner M, Von Loewenich C, Schneck H, Stadler J, Franke C, Kochs E. The NO synthase inhibitors L-Name and L-NMMA, but not L-arginine, block the mammalian nicotinic acetylcholine receptor channel. Toxicol. Lett. 100-101: 109-113 (1998)

25. Ludwig A, Lorenz M, Grimbo N, Steinle F, Meiners S, Bartsch C, Stangl K, Baumann G, Stangl V. The tea flavonoid epigallocatechin-3-gallate reduces cytokine-induced VCAM-1 expression and monocyte adhesion to endothelia cells. Biochem. Bioph. Res. Co. 316: 659-665 (2004)

26. Corpe CP, Lee JH, Kwon O, Eck P, Narayanan J, Kirk KL, Levine M. 6-Bromo-6-deoxy- L-ascorbic acid: An ascorbate analog specific for Na^+-dependent vitamin C transporter but not glucose transporter pathways. J. Biol. Chem. 280: 5211-5220 (2005)

27. Kim SJ. Development of analytical methods for flavones in *Cirsium japonicum*. PhD thesis, Duksung Women's University, Seoul, Korea (2004)

28. Loizzo MR, Statti GA, Tundis R, Conforti F, Ando S, Menichini F. Antimicrobial activity and cytotoxicity of *Cirsium tenoreanum*. Fitoterapia 75: 577-580 (1994)

29. Nuutila AM, Puupponen-Pimiä R, Aarni M, Oksman-Caldentey K-M. Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. Food Chem. 81: 485-493 (2003)

30. Safari MR, Sheikh N. Effects of flavonoids on the susceptibility of low-density lipoprotein to oxidative modification. Prostag. Leukot. Ess. 69: 73-77 (2003)

31. McPhail DB, Hartley RC, Gardner PT, Duthie GG. Kinetic and stoichiometric assessment of the antioxidant activity of flavonoids

by electron spin resonance spectroscopy. J. Agr. Food Chem. 51: 1684-1690 (2003)

32. Tiwari AK. Imbalance in antioxidant defense and human diseases: Multiple approach of natural antioxidant therapy. Curr. Sci. India 81: 1179-1187 (2001)

33. Leirisalo-Repo M, Paimela L, Koskimies S, Repo H. Functions of polymorphonuclear leukocytes in early rheumatoid arthritis. Inflammation 17: 427-442 (1993)

34. Farber JL. Mechanisms of cell injury by activated oxygen species. Environ. Health Persp. 102: 17-24 (1994)

35. Babior BM. The respiratory burst oxidase. Adv. Enzymol. RAMB 65: 49-95 (1992)

36. Leusen J, Verhoeven A, Roos D. Interactions between the components of the human NADHP oxidase: Intrigues in the phox family. J. Lab. Clin. Med. 128: 461-476 (1996)

37. Shatwell K, Segal A. NADHP oxidase. Int. J. Biochem. Cell B. 28: 1191-1195 (1996)

38. Valentine JS, Wertz DL, Lyons TJ, Liou LL, Goto JJ, Gralla EB. The dark side of dioxygen biochemistry. Curr. Opin. Chem. Biol. 2: 253-262 (1998)

39. Duh PD. Antioxidant activity of burdock (*Arctium lappa* Linne): Its scavenging effect on free radical and active oxygen. J. Am. Oil Chem. Soc. 75: 455-461 (1998)

40. Gordon MH. The mechanism of antioxidant action *in vitro*. pp. 1-18. In: Food Antioxidants. Hudson BJF (ed). Elsevier, London, UK (1990)

41. Guo J, Wang MH. Antioxidant and antidiabetic activities of *Ulmus davidiana* extracts. Food Sci. Biotechnol. 16: 55-61 (2007)

42. Padayatty SJ, Levine M. New insights into the physiology and pharmacology of vitamin C. Can. Med. Assoc. J. 164: 353-355 (2001)

43. Sawabe Y, Nakagomi K, Iwagami S, Suzuki S, Nakazawa H. Inhibitory effects of pectic substances on activated hyaluronidase and histamine release from mast cells. Biochim. Biophys. Acta 1137: 274-278 (1992)

44. Edelstam GA, Laurent UB, Lundkvist OE, Fraser JR, Laurent TC. Concentration and turnover of intraperitoneal hyaluronan during inflammation. Inflammation 16: 459-469 (1992)

45. Noble PW, Mackee CM, Coweman M, Shin HS. Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha autoregulatory loop in murine macrophages. J. Exp. Med. 183: 2373-2378 (1996)

46. Park H, Sin BY, Kim HP. Inhibition of collagenase by anti-inflammatory synthetic flavones. J. Appl. Pharmacol. 14: 36-39 (2006)

47. Stenman M, Ainola M, Valmu L, Bjartell A, Ma G, Stenman UH, Sorsa T, Luukkainen R, Konttinen YT. Trypsin-2 degrades human type II collagen and is expressed and activated in mesenchymally transformed rheumatoid arthritis synovitis tissue. Am. J. Pathol. 167: 1119-1124 (2005)

48. Nagase H, Wossener JF. Matrix metalloproteinases. J. Biol. Chem. 274: 21491-21494 (1999)

49. Hanemaaijer R, Sorsa T, Konttinen YT, Ding Y, Suitinen M, Visser H, van Hinsbergh VW, Helaakoski T, Kainulainen T, Ronka H, Tschesche H, Salo T. Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblast and endothelial cells. Regulation by tumor necrosis factor-alpha and doxycycline. J. Biol. Chem. 272: 31504-31509 (1997)

50. Knäuper V, Lopez-Otin C, Smith B, Knight G, Murphy G. Biochemical characterization of human collagenase-3. J. Biol. Chem. 271: 1544-1550 (1996)

51. Koivunen E, Arap W, Valtanen H, Rainisalo A, Medina OP, Heikkila P, Kantor C, Gahmberg CG, Salo T, Konittinen YT, Sorsa T, Ruoslahti E, Pasqualini R. Tumor targeting with a selective gelatinase inhibitor. Nat. Biotechnol. 17: 768-774 (1999)

52. Tatamoto H, Muto N, Yim SD, Nakada T. Anti-hyaluronidase oligosaccharide derived from chondroitin sulfate A effectively reduces polyspermy *in vitro* fertilization of porcine oocytes. Biol. Reprod. 72: 127-134 (2005)

53. Seaton GL, Hall L, Jones R. Rat sperm 2B1 glycoprotein (PH20) contains a C-terminal sequence motif for attachment of a glycosyl phosphatidylinositol anchor. Effects of endoproteolytic cleavage on hyaluronidase activity. Biol. Reprod. 62: 1167-1676 (2000)

54. Sin BY, Kim HP. Inhibition of collagenase by naturally-occurring flavonoids. Arch. Pharm. Res. 28: 1152-1155 (2005)

55. Li M-W, Yudin AI, Vande Voort CA, Sabeur K, Primakoff P, Overstreet JW. Inhibition of monkey sperm hyaluronidase activity and heterologous cumulus penetration by flavonoids. Biol. Reprod. 56: 1383-1389 (1997)

56. Belmont HM, Levartovsky D, Goel A, Amin A, Giorno R, Rediske J, Skovron ML, Abramson SB. Increased nitric oxide production accompanied by the up-regulation of inducible nitric oxide synthase in vascular endothelium from patients with systemic lupus erythematosus. Arthritis Rheum. 40: 1810-1816 (1997)

57. Amin AR, Attur M, Abramson SB. Nitric oxide synthase and cyclooxygenases: Distribution, regulation, and intervention in arthritis. Curr. Opin. Rheumatol. 11: 202-209 (1999)

58. Scuro LS, Simioni PU, Grabriel DL, Saviani EE, Modolo LV, Tamashiro WMSC, Salgado I. Suppression of nitric oxide production in mouse macrophages by soybean flavonoids accumulated in response to nitroprusside and fungal elicitation. BMC Biochem. 5: 5-12 (2004)

59. Kang JS, Jeon YJ, Kim HM, Han SH, Yang KH. Inhibition of inducible nitric-oxide synthase expression by silymarin in lipopolysaccharide-stimulated macrophages. J. Pharmacol. Exp. Ther. 302: 138-144 (2002)

60. Lin HY, Juan SH, Shen SC, Hsu FL, Chen YC. Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264.7 macrophages involves heme oxygenase-1. Biochem. Pharmacol. 66: 1821-1832 (2003)

61. Amrani Y, Chen H, Panettieri R. Activation of tumor necrosis factor receptor 1 in airway smooth muscle: A potential pathway that modulates bronchial hyper-responsiveness in asthma? Respir. Res. 1: 49-53 (2000)

62. Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airway. J. Allergy Clin. Immun. 89: 958-967 (1992)

63. Choi JS, Choi YJ, Park SH, Kang JS, Kang YH. Flavones mitigate tumor necrosis factor-alpha-induced adhesion molecule upregulation in cultured human endothelial cells: Role of nuclear factor-kappa B. J. Nutr. 134: 1013-1019 (2004)

64. Sluss HK, Barrett T, Dérijard B, Davis RJ. Signal transduction by tumor necrosis factor mediated by JNK protein kinases. Mol. Cell. Biol. 14: 8376-8384 (1994)

65. Maksimowicz-McKinnon K, Bhatt DL, Calabrese LH. Recent advances in vascular inflammation: C-reactive protein and other inflammatory biomarkers. Curr. Opin. Rheumatol. 16: 18-24 (2004)

66. Koskinen PK, Lemstrom KB. Adhesion molecule P-selectin and vascular cell adhesion molecule-1 in enhanced heart allograft arteriosclerosis in the rat. Circulation 95: 191-196 (1997)

67. Silverman MD, Zamora DO, Pan Y, Texeira PV, Planck SR, Rosenbaum JT. Cell adhesion molecule expression in cultured human iris endothelial cells. Invest. Ophthalmol. Vis. Sci. 42: 2861-2866 (2001)

68. Lee CW, Lin WN, Lin CC, Luo SF, Wang JS, Pouyssegur J, Yang CM. Transcriptional regulation of VCAM-1 expression by tumor necrosis factor-α in human tracheal smooth muscle cells: Involvement of MAPKs, NF-kB, p300, and histone acetylation. J. Cell. Physiol. 207: 174-186 (2006)