The effects of *Wasabia japonica* (WJ) were investigated *in vitro* and *in vivo* for their anti-oxidant and anti-hypercholesterolemic activities. It was found that the aqueous extracts of WJ leaves (WJL) had strong scavenging activities towards 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) free radicals in cell free systems. WJL also inhibited NO production and the expressions of inducible NO synthase (iNOS) mRNA and enzyme protein, determined by Griess reactions, RT-PCR or Western blotting respectively in Lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages cells. The anti-hypercholesterolemic effects of WJ diet were investigated in hypercholesterolemia rats. Sprague-Dawley rats were divided into four groups and were fed with either normal diet (Group 1), or diet containing 1% (w/w) cholesterol (Groups 2, 3 and 4). After 4 weeks, Group 2 was changed to normal diet, Groups 3 and 4 were changed to the diet containing 5% WJ leaf and or 5% WJ root, respectively. 3 weeks after WJ diets, Serum HDL-cholesterol levels were significantly increased in WJ diet groups compared with the normal diet hypercholesterolemia rats. In contrast, the serum LDL-cholesterol levels and liver xanthine oxidase (XO) activity in WJ diet groups were significantly decreased. The results indicate that the WJ extracts have significant anti-oxidant activities, and the WJ diet exhibited anti-hypercholesterolemic action in high cholesterol diet rats, which was accompanied with modulations of cholesterol metabolism and decrease in liver XO activity.

**Keywords:** anti-oxidant – cholesterol – hypercholesterolemic – *Wasabia japonica*
such as sepsis, arthritis, multiple sclerosis and systemic lupus erythematosus (13). It has been also reported that hypercholesterolemia is increased free radical production and reduced free radical scavenging effect (14). Therefore, certain natural products with anti-oxidant activities may have potential anti-hypercholesterolemia actions.

In this study, we investigated the effects of WJ extracts on the inhibition of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and NO formation in cell free system, as well as the expressions of iNOS mRNA and enzyme protein in RAW 264.7 murine macrophage cells. Furthermore, we studied the anti-hypercholesterolemic effects of WJ diet in hypercholesterolemia rats in vivo.

**Methods**

**Preparation of WJ**

WJ used in this study was supplied and identified by Dr Man Jong Bae from the Efficacy and Safety Research Center for Traditional Oriental Medicines, Kyungbuk Technopark, South Korea. The voucher specimen (No. ESRC 020303) was deposited in the herbarium at the above mentioned research center. The root and leaf of *WJ* (400 g) were extracted three times with 1500 ml of ethanol and distilled water. The extracts were then evaporated in vacuo to obtain WJ leave aqueous extract (WJL, 8.34 g), WJ root ethanol extract (WJRE, 7.40 g), WJ root aqueous extract (WJR, 6.56 g) and WJ leave ethanol extract (WJLE, 7.71 g), respectively.

**DPPH Scavenging Assay**

The hydrogen-donating ability of each extract was examined according to the method previously described (6,15) in the presence of a DPPH stable radical. The extracts and positive control Vitamin C were diluted in methanol to prepare a sample solution (800, 400, 200 and 100 µg/ml). A total of 500 µl of the sample solution was then mixed with 500 µl of 5×10−4 M DPPH solution for 10 s. Absorbance of the methanolic DPPH tincture was measured with a spectrophotometer (DU530, Beckman Coulter, CA, USA) at 517 nm.

**Nitric Oxide Scavenging Assay**

Nitric oxide generated from sodium nitroprusside was measured as described by Marcocci *et al*. (16) using the Griess reagent. Various concentrations (800, 400, 200 and 100 µg/ml) of the extracts (2 ml) and 25 mM sodium nitroprusside (0.5 ml) were incubated at 25°C for 150 min. After incubation, samples (0.5 ml) and 0.5 ml of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylenedianine dihydrochloride) were incubated at room temperature for 10 min. The absorbance of the chromophore formed was measured with a spectrophotometer (DU530, Beckman Coulter, CA, USA) at 540 nm.

**Cell Culture**

Murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC; TIB71, Maryland, USA). The cells were maintained in complete Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 1% antibiotics/anti-mycotics (100 U ml−1 of penicillin, 2.5 µg ml−1 of amphotericin D and 100 µg ml−1 of streptomycin) and 1.5% sodium bicarbonate at 37°C in a humidified 5% CO2 atmosphere. Cells were plated at a density of 1×10⁶ cells/well in a 60 mm dish, and allowed to attach for 2 h. For stimulation, the medium was replaced with 1% FBS contained DMEM, and the cells were then stimulated with 1 µg ml−1 of LPS in the presence or absence of various concentrations of WJL for 48 h.

**Measurement of Nitrite**

NO synthesis in cell culture was measured by a Griess reagent according to the previous study (17). Briefly, a 100 µl/well sample was incubated with an equal volume of the Griess solution (1% sulfanilamide in 5% phosphoric acid + 1% naphthylamide in distilled aqueous) at room temperature for 10 min. The absorbance was measured with a micro Enzyme-Linked Immunosorbent Assay (ELISA) reader at 540 nm. Nitrite was determined by using sodium nitrite as the standard.

**Total RNA Isolation and RT-PCR**

Murine macrophage RAW 264.7 cells in the TRIzol reagent were well homogenized and vortexed after a 1/10 volume of chloroform was added. After incubating the mixture on ice for 15 min, the samples were centrifuged at 12 000 r.p.m., for 15 min at 4°C. The aqueous phase was transferred to a new 1.5 ml micro centrifuge tube. RNA from the aqueous phase was precipitated by mixing it with the same volume of isopropyl alcohol, and was centrifuged at 12 000 r.p.m. for 15 min at 4°C, following 30 min of incubation on ice. Precipitated RNA pellets were washed once with 70% ethyl alcohol, and were redissolved in DEPC-treated water (Quality Biological Inc., Gaithersburg, MD, USA). RT reaction of 4 µg of RNA was performed in a 20 µg RT reaction mixture containing 0.5 µl of MMLV reverse transcriptase (200 U µl−1, Promega), 4.0 µl of 5×MMLV RT buffer (Promega), 2.0 µl of dNTP mixture (10 mM, BM), 0.5 µl of RNasin (RNase inhibitor, 40 U µl−1, Promega) and 2.0 µl of oligo dT (50 µM) in DEPC-treated water. The reaction was performed under the conditions of 42°C for 1 h and 95°C for 5 min. The cDNAs were amplified in
a PCR with following primer sets: 5'-GAC AAG CTG CAT GTG ACA TC-3', 5'-GCT GGT AGG TTC CTG TTG TT -3' for iNOS. 5'-CCA CCC AGA AGA CTG CAC CAC-3' for G3PDH. PCR was carried out with the use of 1.0 μl of RT products as templates: 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, and elongation at 72°C for 45 s. The last cycle was followed by a 10 min extension step at 72°C. The amplified products were analyzed by ethidium bromide-stained agarose gel electrophoresis.

**Western Blotting**

Cellular lysate were prepared by suspending 1×10⁶ cells in 100 μl of lysis buffer (50 mM Tris–Cl, 25 mM EDTA, 650 mM NaCl, 5% Triton X-100, 100X PMSF, a 100X protease inhibitor cocktail, 5X lysis buffer). Proteins in the cell lysates were then separated by a 12% SDS-polyacrylamide gel electrophoresis and transferred to a Protran® nitrocellulose membrane (S&S, GmbH, Germany). The membrane was then blocked with 5% skim milk in PBS-Tween-20 for 1 h at room temperature and then incubated with an anti-iNOS antibody (Transduction lab, Lexington, KY, USA). After washing in PBS-Tween-20 three times, the blot was incubated with a secondary antibody. Detection of specific proteins was carried out with an ECL Western blotting kit (Amersham Pharmacia Biotech, USA) according to the manufacture’s instructions.

**In Vivo Experiments**

Male Sprague-Dawley rats (180–200 g) were supplied from Dae-Han Laboratory Animal Research Center Co. (Choongbook, Korea) and maintained on a 12 h light/dark cycle. Purina Rodent Chow (Bio Genomics, Inc., Korea) and tap water provided ad libitum. All procedures relating to animals and their care were in accordance with the institution guideline for animal research.

Animals were divided into four groups comprising six rats in each group. Group 1 served as the healthy control groups and was given the normal diet and the other Groups (2, 3 and 4) received a 1% cholesterol diet. After 4 weeks, the Groups 1 and 2 received a normal diet, Group 3 received a normal diet containing 5% leaf powder of WJ and Group 4 received a normal diet containing 5% root powder of WJ.

After 3 weeks treatment, all the animals were sacrificed and blood was collected by decapitation to determine the levels of total cholesterol, HDL-cholesterol and LDL-cholesterol. The liver was removed, weighed and homogenized with POLYTRON® PT-MR2100 (Kinematica, Swiss), in ice chilled in a 0.25 M sucrose solution. The suspension was centrifuged at 3000 r.p.m. at 4°C for 10 min, and the supernatant was used for the determination of XO activity. The enzyme activity of XO was determined according to the method of Della Corte and Strip (18). The unit of enzyme activity was expressed as nano moles of produced uric acid of 1 mg protein per min.

**Statistical Analysis**

One-way analysis of variance (ANOVA) was used to evaluate the statistical significance of changes in all indices as a function of dose and treatment, followed by Duncan’s new multiple range test using P<0.05 as the level of significance.

**Results**

**Scavenging activity on DPPH and NO radicals**

The scavenging activity of WJ extracts towards DPPH and NO radicals were shown in Figs 1 and 2. A concentration-dependent inhibition of DPPH and NO radicals were observed for all WJ extracts tested. At 800 μg ml⁻¹ WJL inhibited DPPH generation by 67.5 ± 2.4% and NO generation by 50.1 ± 1.3%. On the other hand, WJLE, at 800 μg ml⁻¹, inhibited the DPPH by 59.3 ± 1.8% and NO by 45.8 ± 3.1%. Among the tested extracts, WJL showed a strong scavenging activity towards DPPH and NO. Therefore, it was used for further experiments.

**NO Production in LPS-Stimulated Macrophages**

As previously reported, LPS markedly induced NO production in RAW 264.7 cells compared with unstimulated cells. WJL significantly inhibited NO production in LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Fig. 3). The observed effect was not due to a potential
cytotoxicity of WJL, since WJL showed no alteration of cell viability (data not shown).

iNOS mRNA and iNOS Protein Expression in RAW 264.7 Cells

WJL (200–800 μg ml⁻¹) markedly decreased iNOS mRNA expression in LPS-stimulated cells in a dose-dependent manner (Fig. 4). Similarly WJL extract caused a dose-dependent inhibition of expressions of iNOS enzyme protein in Western blot analysis (Fig. 5). On the other hand, a normal expression of HSP70 was observed with same WJL concentrations.

Cholesterol Levels and Hepatic XO Activity in vivo

Compared with the hypercholesterolemia rats with normal diet group (Group 2), HDL-cholesterol was significantly (P < 0.05, one-way ANOVA followed by the Duncan’s new multiple range test) increased in the hypercholesterolemia rats with 3 weeks 5% WJ leaf diet (Group 3) or 5% WJ root diet (Group 4) (Table 1). In contrast, LDL-cholesterol was significantly (P < 0.05, one-way ANOVA followed by the Duncan’s new multiple range test) decreased in the Groups 3 and 4. However, Group 4 has a significantly (P < 0.05, one-way ANOVA followed by the Duncan’s new multiple range test) decreased LDL-cholesterol level compared with the Group 2.
Hepatic XO activity was significantly ($P < 0.05$, one-way ANOVA followed by the Duncan’s new multiple range test) decreased in both Groups 3 and 4, compared with Group 2 (Table 2).

### Discussion

The major findings of the present study are the demonstration of anti-oxidant activities of WJ extracts in vitro and the discovery of an anti-hypercholesterolemic effect of WJ diet in hypercholesterolemia rats. WJ contains several kinds of isothiocyanate that have been found to inhibit lung tumorigenesis (2). The 6-methylsulfinylhexyl isothiocyanate isolated from wasabi was a potential inhibitor of human platelet aggregation in vitro (19). In addition, wasabi ethanol extract has been shown to inhibit cell growth of human monoblastic leukemia U937 cells (20). Furthermore, Morimitsu (21) reported that the anti-oxidant activities of WJ might contribute to its anti-cancer effects. However, to our best knowledge, no anti-hypercholesterolemic effects of WJ have been demonstrated. In the present study, the scavenging activities on DPPH and NO radicals by aqueous and ethanol extracts of WJ root and leaf were first demonstrated in cell-free assays. The finding of strong radical scavenging activity by WJL led us to investigate further the biological significance of anti-oxidant of WJL by measuring NO production and iNOS mRNA and protein expressions in RAW 264.7 cells. As shown in the present study, WJL significantly inhibited NO production, as well as iNOS mRNA and protein expressions in LPS-stimulated RAW 264.7 cells. Furthermore, there seems a dose related correlation between the inhibition of NO production and the inhibitions of iNOS mRNA and iNOS protein expressions by WJL in RAW 264.7 cells. The observed effect of WJL was unlikely due to any cytotoxicity of WJL, since it did not affect the cell viability or the normal expression of HSP70 under the experimental conditions. The results suggest that WJL reduces LPS-induced NO production through the inhibition of iNOS mRNA expression, which leads to a decreased iNOS protein expression. It is likely that this effect of WJL may relate to its anti-oxidant activities, since it is known that anti-oxidant molecules can decrease the gene expression of iNOS (22–24). A number of plant materials have been reported to inhibit NO production (12,16,23,25). Since high levels of NO can induce host cell death and inflammatory tissue damage and iNOS-mediated NO production has been involved in a variety of diseases or pathological conditions such as infection and rheumatic disorders (12,25), the observed effect of WJ may support the traditional use of WJ in the treatment of certain diseases such as rheumatic arthralgia, or anti-microbial activities (2–4). However, it still remains to be established whether the clinical used doses of WJ can achieve therapeutic efficacy in these conditions.

It has been reported that hypercholesterolemia increased production of oxygen-free radical and raise malondialdehyde level (26,27). Animals fed with high cholesterol diet were shown with a depressed anti-oxidant system and formation of free radical (14). Thus, it has been suggested that the anti-oxidant effects may potentially improve hypercholesterolemic status (28,29). The present study has demonstrated for the first time that WJ diet had a significant anti-hypercholesterolemic effect in hypercholesterolemia rats in vivo. Three weeks after WJ diet serum HDL-cholesterol level was increased while at the same time the serum LDL-cholesterol level was decreased. It is well know that the improvement of HDL and LDL ratio benefits hypercholesterolemic conditions (30–32). Furthermore, the liver XO activity in hypercholesterolemic rats was also inhibited by WJ diet. XO is the enzyme responsible for the formation of uric acid from the purines hypoxanthine and xanthine. It serves as an important biological source of oxygen-derived free radicals that contribute to oxidative damage to living tissues that are involved in many pathological processes such as inflammatory, atherosclerosis, cancer and aging (33–35). Therefore, WJ diet may improve the status of hypercholesterolemia through the modulation of HDL, LDL-cholesterol and anti-oxidant system.

In conclusion, the present study has clearly demonstrated that WJ extracts tested exhibits free radical scavenging activity towards NO and DHP. Furthermore, in hypercholesterolemic rats, WJ diet significantly increases blood HDL-cholesterol but decreases blood LDL-cholesterol levels. The liver XO activity was also decreased by WJ diet. These results may provide a rational for using WJ leaf extract or diet in certain conditions related with oxidative stress or hypercholesterolemia.

### References
