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Original Article

Anti-oxidant and Anti-hypercholesterolemic Activities of *Wasabia japonica*

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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-picryhydrazyl (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 companied with modulations of cholesterol metabolism and decrease in liver XO activity.

Keywords: anti-oxidant – cholesterol – hypercholesterolemic – *Wasabia japonica*

Introduction

Wasabia japonica (WJ), also known as 'wasabi', is one of the popular spices in many Asian countries, especially in Korea and Japan. It has been used to treat rheumatic arthralgia, through promoting blood circulation and alleviation of pain (1). WJ contains several isothiocyanate (2), which are known for having anti-microbial, fungicidal, pesticidal activities as well as anti-carcinogenic effect (2–4). However, there are few reports on the anti-oxidant activities and anti-hypercholesterolemic effects of WJ, although it is well known that many plants have anti-oxidant and free radical scavenging activities (5–7).

Free radical oxidative stress, usually resulting from deficient natural anti-oxidant defenses (8), has been implicated in the pathogenesis of a wide variety of clinical disorders, such as the degenerative diseases (9), aging (10) and the progressive decline in the immune functions (11). Nitric oxide (NO) is one of the reactive oxygen species (ROS), and plays an important role in diverse physiological processes, including vasodilatation, neurotransmission and immune responses (12). The pathological roles of NO have been implicated in a wide range of inflammatory diseases,

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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-picryhydrazyl (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 leave ethanol extract (WJLE, 7.71 g), WJ root aqueous extract (WJR, 6.56 g) and WJ root ethanol extract (WJRE, 7.40 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 $\mu g\,ml^{-1}$). 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 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 $\mu g\,ml^{-1}$) 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 ethylenediamine dihyrochloride) 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 Dulbeco'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 $\mu g\,ml^{-1}$ of amphotericin D and 100 $\mu g\,ml^{-1}$ of streptomycin) and 1.5% sodium bicarbonate at 37°C in a humidified 5% CO_2 atmosphere. Cells were plated at a density of 1×10^6 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 $\mu g\,ml^{-1}$ 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 $U\,\mu l^{-1}$, 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 $U\,\mu l^{-1}$, 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 TGG ATG GC-3′, 5′-CAT GTA GGC CAT GAG GTC 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 1×10^6 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 $67.5\pm2.4\%$ and NO generation by $50.1\pm1.3\%$. On the other hand, WJLE, at 800 µg ml^{-1}, inhibited the DPPH by $59.3\pm1.8\%$ and NO by $45.8\pm3.1\%$. 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

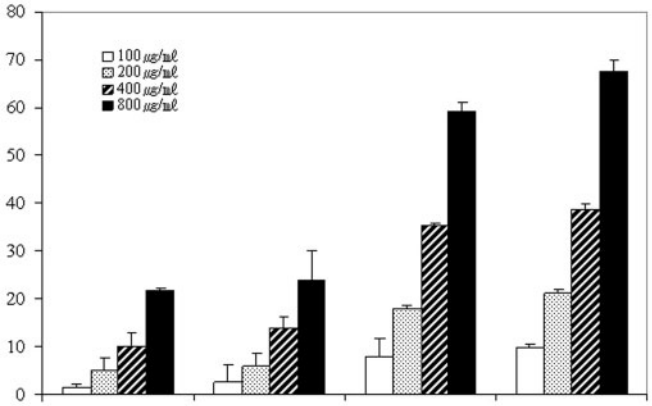

Figure 1. DPPH scavenging activity of WJ extracts *in vitro*. Results were expressed as percentage of inhibition, compared with the vehicle control (means ± SEM, $n = 3$). WJRE: root ethanol extract, WJR: root aqueous extract, WJLE: leaf ethanol extract, WJL: leaf aqueous extract.

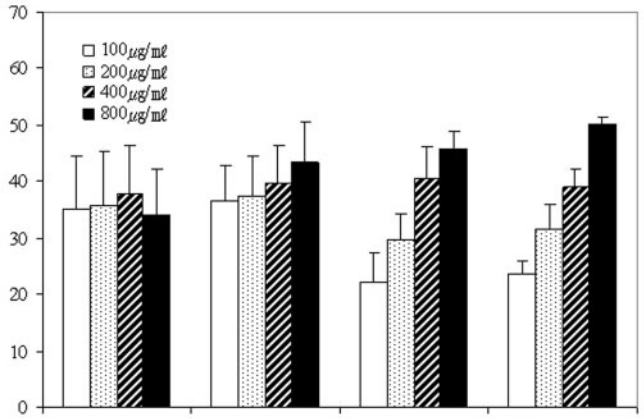

Figure 2. NO scavenging activity of WJ extracts *in vitro*. NO was produced by sodium nitroprusside. Results were expressed as percentage of inhibition, compared with the vehicle control (means ± SEM, $n = 3$). WJRE: root ethanol extract, WJR: root aqueous extract, WJLE: leaf ethanol extract, WJL: leaf aqueous extract.

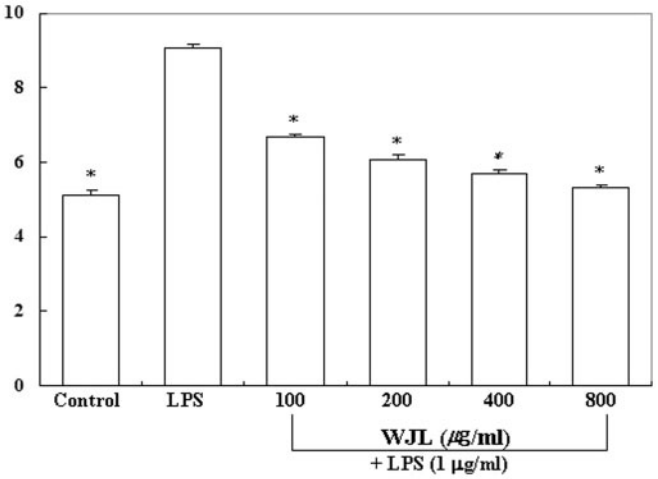

Figure 3. Inhibition of NO production in lipopolysaccharide (LPS; 1 ug ml^{-1})-stimulated RAW 264.7 cells by WJL extract (WJL, 100–800 ug ml^{-1}). RAW 264.7 cells were incubated with different concentrations of WJL in the absence of presence LPS for 24 h. The amount of NO generated was measured by Griess reaction. The data are expressed as mean ± SD of three separate experiments. *$P < 0.05$ significantly different from samples treat with LPS alone, analyzed by ANOVA test.

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.

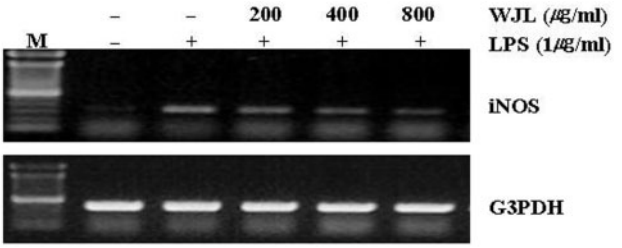

Figure 4. Effects of WJL extract (WJL, 200–800 ug ml^{-1}) on iNOS mRNA expression in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with 1 ug ml^{-1} LPS in the presence or absence of different concentrations of WJL for 24 h. iNOS mRNA was analyzed by RT-PCR. G3PDH was used as control genes.

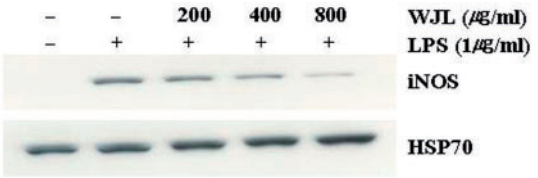

Figure 5. Effects of WJL (200–800 ug ml^{-1}) on the expression of iNOS protein in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with 1 ug ml^{-1} LPS in the presence or absence of different concentrations of WJL for 24 h. iNOS protein expression was analyzed by Western blotting as described in the method. HSP70 was used as a control genes.

Table 1. Effects of WJ diets on the total blood cholesterol level (mg dl^{-1}) in hypercholesterolemia rats

Treatment	TC (mg dl^{-1})	HDL (mg dl^{-1})	LDL (mg dl^{-1})
Group 1	58.8 ± 3.9*	36.5 ± 3.1	9.2 ± 2.4*
Group 2	69.2 ± 4.0	30.2 ± 2.5	25.8 ± 6.3
Group 3	66.9 ± 3.0	38.1 ± 1.6*	14.0 ± 1.3
Group 4	64.9 ± 2.9	41.7 ± 1.6*	11.0 ± 4.1*

Group 1: normal diet; Group 2: hypercholesterolemia rats + normal diet; Group 3: hypercholesterolemia rats + normal diet containing 5% WJ leaf; Group 4: hypercholesterolemia rats + normal diet containing 5% WJ root. Results are expressed as mean ± SEM, ($n = 5$). *$P < 0.05$ represent significantly different from hypercholesterolemia rats treated with normal diet (Group 2), analyzed by ANOVA test.

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.

Table 2. Effect of WJ diets on xanthine oxidase activity in hypercholesterolemia rats

Treatment	Xanthine oxidase (nmol mg^{-1} protein/min)
Group 1	1.41 ± 0.11*
Group 2	1.91 ± 0.12
Group 3	1.46 ± 0.04*
Group 4	1.34 ± 0.02*

Group 1: normal diet; Group 2: hypercholesterolemia rats + normal diet; Group 3: hypercholesterolemia rats + normal diet containing 5% WJ leaf; Group 4: hypercholesterolemia rats + normal diet containing 5% WJ root. Results are expressed as mean ± SE, ($n = 5$). *$P < 0.05$ represent significantly different from hypercholesterolemia rats treated with normal diet (Group 2), analyzed by ANOVA test.

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-methlysulfinyhexyl isothiocyanate isolated from wasabi was a potential inhibitor of human platelet aggregation *in vitro* (19). In additional, 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 DHPP. Furthermore, in hypercholesterolemia 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

1. Hur JM, Lee JH, Choi JW, Hwang GW, Chung SK, Kim MS, et al. Effects of Methanol Extract and Kaempferol Glycosides from Armoracia rusticana on the Formation of Lipid Peroxide in

Bromobenzene-treated Rats In Vitro. *Korean J. Pharmacog* 1998;29:231–6.
2. Yano T, Yajima S, Virgona N, Yano Y, Otani S, Kumagai H, et al. The effect of 6-methylthiohexyl isothiocyanate isolated from Wasabia japonica (wasabi) on 4-(methylnitrosamino)-1-(3-pyridyl)-1-buatnone-induced lung tumorigenesis in mice. *Cancer Lett* 2000;155:115–20.
3. Soledade M, Pedras C, Sorensen JL. Phyoalexin accumulation and antifungal compounds from the Crucifer wasabi. *Phytochemistry* 1998;49:1959–65.
4. Tajima H, Kimoto H, Taketo Y, Taketo A. Effects of synthetic hydroxy isothiocyanates on microbial systems. *Biosci Biotechnol Biochem* 1998;62:491–5.
5. Liao H, Banbury LK, Leach DN. Elucidation of Danzhixiaoyao Wan and its constituent herbs on antioxidant activity and inhibition of nitric oxide production. *Evid Based Complement Altern Med* 2007;4:425–30.
6. Ljubuncic P, Dakwar S, Portnaya I, Cogan U, Azaizeh H, Bomzon A. Aqueous extracts of *Teucrium polium* possess remarkable antioxidant activity *In Vitro*. *Evid Based Complement Altern Med* 2006;3:329–38.
7. Nishant P. Visavadiya, Narasimhacharya AVRL. *Asparagus* root regulates cholesterol metabolism and improves antioxidant status in hypercholesteremic rats. *Evid Based Complement Altern Med* [Epub ahead of print, August 27, 2007;1–8].
8. Halliwell B, Gutteridge JMC. Lipid peroxidation, oxygen radicals; cell damage and antioxidant therapy. *Lancet* 1984;23:1396–7.
9. Cross CE. Oxygen radicals and human disease. *Ann Intern Med* 1987;107:526–45.
10. Beckman KB, Ames BN. The free radical theory of aging matures. *Physiol Rev* 1998;78:547–81.
11. Pike J, Chandra RK. Effect of vitamin and trace element supplementation on immune indices in healthy elderly. *Int J Vitam Nutr Res* 1995;65:117–21.
12. Lee BG, Kim SH, Zee OP, Lee KR, Lee HY, Han JW, et al. Suppression of inducible nitric oxide synthase expression in RAW 264.7 macrophages by two b-carboline alkaloids extracted from Melia azedarach. *Eur J Pharmacol* 2000;406:301–9.
13. Kroncke KD, Fehsel K, Kolb-Bachofen V. Inducible nitric oxide synthase in human disease. *Clin Exp Immunol* 1998;113:147–56.
14. Shukla R, Gupta S, Gambhir JK, Prabhu KM, Murthy PS. Antioxidant effect of aqueous extract of the bark of Ficus bengalensis in hypercholesterolaemic rabbits. *J Ethnopharmacol* 2004;92:47–51.
15. Blois MS. Antioxidant determinations by the use of stable free radical. *Nature* 1958;26:1199–200.
16. Marcocci PL, Sckaki A, Albert GM. Antioxidant action of Ginkgo biloba extract EGP761. *Methods Enzymol* 1994;234:462–75.
17. Ryu SY, Oak MH, Kim KM. Yomogin inhibits the degranulation of mast cells and the production of the nitric oxide in activated RAW 264.7 cells. *Planta Med* 2000;66:171–3.
18. Della Corte E, Stirpe F. The regulation of rat-liver xanthine oxidase: activation by proteolytic enzymes. *FEBS Lett* 1968;2:83–4.
19. Morimitsu Y, Hayashi K, Nakagawa Y, Fujii H, Horio F, Uchida K, et al. Antiplatelet and anticancer isothiocyanates in Japanese domestic horseradish, Wasabi. *Mech Ageing Dev* 2000;116:125–34.
20. Watanabe M, Ohata M, Hayakawa S, Isemura M, Kumazawa S, Nakayama T, et al. Identification of 6-methylsulfinylhexyl isothiocyanate as an apoptosis-inducing component in wasabi. *Phytochemistry* 2003;62:733–9.
21. Morimitsu Y, Nakagawa Y, Hayashi K, Fujii H, Kumagai T, Nakamura Y, et al. A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway. *J Biol Chem* 2002;277:3456–63.
22. Wang CN, Shiao YJ, Kuo YH, Chen CC, Lin YL. Inducible nitric oxide synthase inhibitors from Saposhnikovia divaricata and Panax quinquefolium. *Planta Med* 2000;66:644–7.
23. Seo WG, Pae HO, Oh GS, Chai KY, Kwon TO, Yun YG, et al. Inhibitory effects of methanol extract of Cyperus rotundus rhizomes on nitric oxide and superoxide productions by murine macrophage cell line, RAW 264.7 cells. *J Ethnopharmacol* 2001;76:59–64.
24. Suzuki YJ, Forman HJ, Sevanian A. Oxidants as stimulators of signal transduction. *Free Radic Biol Med* 1997;22:269–85.
25. Kim NY, Kang TH, Song EK, Pae HO, Chung HT, Kim YC. Inhibitory effects of butanol fraction of the aqueous extract of Forsythia koreana on the nitric oxide production by murine macrophage-like RAW 264.7 cells. *J Ethnopharmacol* 2000;73:323–7.
26. Dhuley JN. Anti-oxidant effects of cinnamon (Cinnamomum verum) bark and greater cardamom (Amomum subulatum) seeds in rats fed high fat diet. *Indian J Exp Biol* 1999;37:238–42.
27. Prasad K, Kalra J. Oxygen free radicals and hypercholesterolaemic atherosclerosis: effect of vitamine E. *Am Heart J* 1993;125:958–73.
28. Kurosawa T, Itoh F, Nozaki A, Nakano Y, Katsuda S, Osakabe N, et al. Suppressive effect of cocoa powder on atherosclerosis in Kurosawa and Kusanagi-hypercholesterolemic rabbits. *J Atheroscler Thromb* 2005;12:20–8.
29. Tao L, Liu HR, Gao E, Teng ZP, Lopez BL, Christopher TA, et al. Antioxidative, antinitrative, and vasculoprotective effects of a peroxisome proliferator-activated receptor-gamma agonist in hypercholesterolemia. *Circulation* 2003;108:2805–11.
30. Jia L, Fu M, Tian Y, Xu Y, Gou L, Tian H, et al. Alterations of high-density lipoprotein subclasses in hypercholesterolemia and combined hyperlipidemia. *Int J Cardiol* 2007;120:331–37.
31. Knopp RH, Tsunehara C, Retzlaff BM, Fish B, Nguyen H, Anderson S, et al. Lipoprotein effects of combined ezetimibe and colesevelam hydrochloride versus ezetimibe alone in hypercholesterolemic subjects: a pilot study. *Metabolism* 2006;55:1697–703.
32. Das S, Snehlata XX, Das N, Srivastava LM. Role of ascorbic acid on in vitro oxidation of low-density lipoprotein derived from hypercholesterolemic patients. *Clinica Chimica Acta* 2006;372:202–5.
33. Chiang HC, Lo YJ, Lu FJ. Xanthine oxidase inhibitors from the leaves of Alsophila spinulosa (Hook) Tryon. *J Enzyme Inhib* 1994;8:61–71.
34. Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Van Poel B, et al. Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J Nat Prod* 1998;61:71–6.
35. Thomson MJ, Puntmann V, Kaski JC. Atherosclerosis and oxidant stress: the end of the road for antioxidant vitamin treatment? *Cardiovasc Drugs Ther* 2007;21:195–210.

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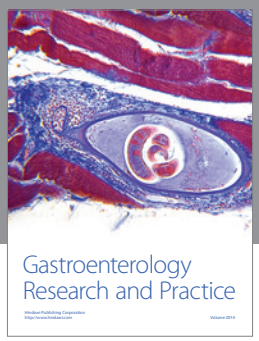

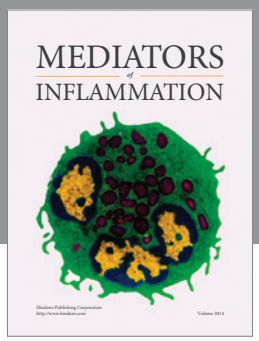

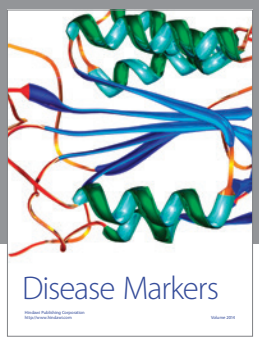

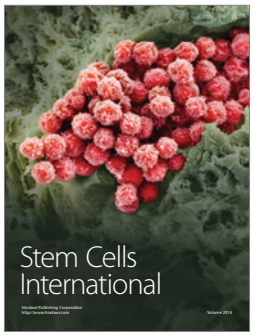

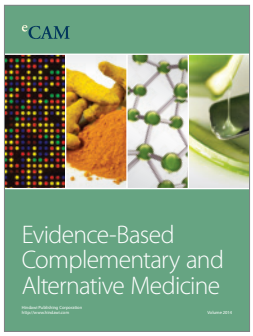

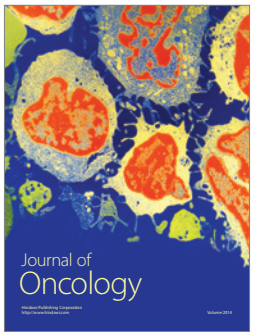

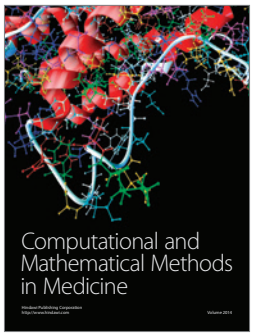

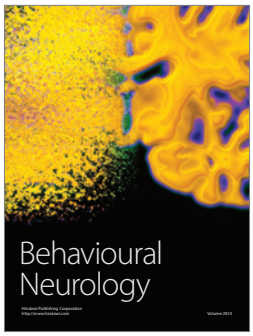

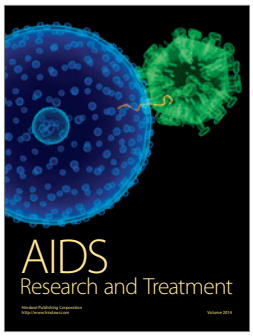

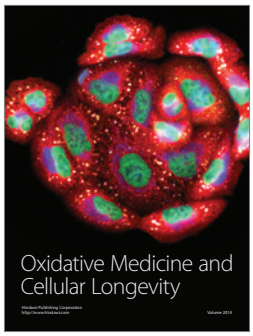

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