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# Evaluation of Antioxidant Activity and Inhibitory Effect on Nitric Oxide Production of Some Common Vegetables

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The objectives of this study were to study the antioxidant activities and nitric oxide (NO) scavenging effects of vegetables in vitro systems and to study the inhibitory effects of vegetables on the NO production and NO-induced DNA damage in RAW 264.7 macrophage. The results indicated that water extracts from Indian lotus, Jew's ear, shiitake, eggplant, and winter mushroom showed stronger antioxidant activity and free-radical-scavenging ability than that of other vegetable extracts. The scavenging effects of vegetable extracts on NO derived from sodium nitroprusside (SNP) were in decreasing order of water spinach > Indian lotus > eggplant and garland chrysanthemum. In the macrophage model system, the water extracts from fresh daylily flower, sponge gourd, pea sprout, and eggplant exhibited over 80% inhibition on NO generation stimulated by lipopolysaccharide. The extract from fresh daylily flower that expressed the strongest inhibition on NO production was attributed to the ability to reduce the inducible nitric oxide synthase (iNOS) induction. However, the extracts from pea sprout and eggplant suppressed the NO production by scavenging on NO and inactivating toward iNOS enzyme. In addition, the water extracts from fresh daylily flower, sponge gourd, pea sprout, and eggplant also showed over 40% inhibitory effect on DNA damage induced by SNP in RAW 264.7 macrophage. The data also indicated that eggplant and pea sprout extracts contained higher total phenolic compounds, anthocyanins, and ascorbic acids and appeared to be responsible for their antioxidant activities and scavenging effects on NO derived from SNP.

**KEYWORDS: Vegetables; antioxidant activity; nitric oxide; inducible nitric oxide synthase (iNOS); DNA damage; macrophage**

## INTRODUCTION

Recently medical researches showed that reactive oxygen species (ROS), reactive nitrogen species (RNS), and free radicals have been implicated in mediating various pathological processes including cancer, aging, and atherosclerosis (*1*). Oxidative stress is thought to result from an imbalance between the generation of ROS and free radicals and the antioxidants that scavenge them. Therefore, much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit lipid peroxidation or to protect the damage of free radicals (*2*, *3*). The protection that fruits, vegetables, medicinal plants, and herbal plants provide against diseases has been attributed to various antioxidant phytonutrients, such as flavonoids, phenylpropanoids, phenolic acid, ascorbic acid, tocopherol, and carotenoids contained in these plants. Several studies have shown that increased dietary intake of polyphenol and flavonoids correlates with reduced oxidative stress, inflammation, tumor, and coronary heart disease (*4*−*6*). Thus, the study on the protection against oxidative damage of crude extracts from fruits, vegetables, and other plant materials has been gaining attention. Antioxidants may act by preventing first-chain initiation or breaking chain promotion by scavenging free radicals. In this study, linoleic acid oxidation and α,α-diphenyl-β-picrylhydrazyl (DPPH) radical-scavenging methods have been used to measure the antioxidants property of the vegetable samples.

With the exception of ROS, the overproduction of RNS has also been associated with oxidative stress and with chronic inflammation, which were involved in the pathophysiology of various diseases such as arthritis, diabetes, atherosclerosis, and carcinogenesis (*7*−*10*). Nitric oxide (NO) and its derivative peroxynitrite ($ONOO^-$), produced from NO and superoxide, were the main source of RNS in vivo. NO is an important intra- and intercellular regulatory molecule of multiple physiological functions and is enzymatically synthesized via the oxidation of the terminal guanidine nitrogen atom of L-arginine by a family of nitric oxide synthase (NOS), which are either constitutive (cNOS) or inducible (iNOS) (*11*). iNOS is not detectable in healthy tissues but is expressed after an immunological challenge or injury. iNOS is expressed in various cell types including smooth muscle cells, macrophages, hepatocytes, and astrocytes

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only after exposure to specific stimulants such as cytokines (*12*, *13*). Overexpressed iNOS has been detected in several human tumors (*14*, *15*). The damage from NO during the inflammation or carcinogenic process might be decreased by the NO scavengers and NOS enzyme inhibitors. A number of pytochemicals, such as quercetin, tocopherol, and catechins have been found to inhibit the damage of NO (*16*−*18*). Therefore, how to utilize the natural antioxidants in dietary plants to prevent or improve RNS-mediated injury becomes very important.

Phagocytic cells, especially macrophages, have been implicated in immunopathological disorders related to oxidative stress, including inflammation and diseases. Macrophages are sensitive to changes in the oxidant−antioxidant balance because of the production of ROS and RNS as part of their normal function. Therefore, macrophages offer an excellent model system to study the antioxidant and NO inhibitory activities of natural materials (*19*, *20*). In this study, we investigate the scavenging effects of water extracts from vegetables on NO derived from sodium nitroprusside (SNP) in vitro systems and study the inhibitory effects of vegetables on NO production, iNOS enzyme activity, and its protein expression in RAW 264.7 macrophage activated by lipopolysaccharide (LPS).

## MATERIALS AND METHODS

**Chemicals.** SNP, LPS; *Escherichia coli*, serotype O55:B5), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and DPPH were purchased from the Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco/BRL Life Technologies (Eggenstein, Germany). All other reagents were of analytical grade.

**Vegetables and Sample Extraction.** A total of 25 various vegetables used in this study were purchased from a local market in Taichung, Taiwan. Vegetables included (a) plant: asparagus (*Asparagus officinalis* L.), bitter gourd (*Momordica charantia* L.), bottle gourd [*Lagenaria siceraria* (Molina) Standl.], cauliflower (*Brassica oleracea* L. var. *botrytis* L.), celery [*Apium graveolens* L. var. *dulce* (Mill.) Pers], coriander (*Coriandrum sativum* L.), dried daylily (*Hemerocallis fulva* L.), eggplant (*Solanum melongena* L.), fresh daylily (*Hemerocallis fulva* L.), garland chrysanthemum (*Chrysanthemum coronarium* L.), Indian lotus (*Nelumbo nucifera* Gaertn.), mung bean seedling [*Vigna radiata* (L.) Wilczek], leek (*Allium tuberosum*), onion (*Allium cepa* L.), pea sprout (*Pisum sativum* L.), potato (*Solanum tuberosum* L.), sponge gourd [*Luffa cylindrica* (L.) Roem.], sweet potato [*Ipomoea batatas* (L.) Lam.], tomato (*Lycopersicon esculeutum* Mill.), water spinach (*Ipomoea aquatica* Forsk.), and wild rice stem (*Zizania latifolia* Turcz.); and (b) mushroom: Jew's ear (*Auricularia auricula*), shiitake (*Lentinula edodes*), straw mushroom (*Volvariella volvacea*), and winter mushroom (*Flammulina velutipes*). Edible parts of vegetables were weighed and finely minced. Each vegetable except dried daylily and Jew's ear was homogenized with 4 volumes of deionized water at room temperature using a blender, and the mixture was then centrifuged at 4 °C at 9000*g* for 30 min. Dried daylily and Jew's ear were homogenized with 10 and 15 volumes of deionized water, respectively. The supernatants of vegetable extracts were freeze-dried and then storaged at −20 °C.

**Antioxidant Activity in a Linoleic Acid System.** The antioxidant activities of water extracts from vegetables were determined by the thiocyanate method (*21*). Each sample in 0.5 mL of distilled water was mixed with linoleic acid emulsion (2.5 mL, 0.02 M at pH 7.0) and sodium phosphate buffer (2 mL, 0.2 M at pH 7.0) and incubated at 37 °C. The antioxidant activity was evaluated from the peroxide value determined by measuring the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate at various intervals during incubation. All samples were assayed, in triplicate, in three independent experiments.

**Measure of the Antioxidative Ability by the TEAC Method.** The measure of the antioxidative ability of water extracts from vegetables was carried out as describd by Arnao et al. (*22*). Briefly, ABTS radical cation ($ABTS^{\cdot+}$) solution was diluted in 5 mM phosphate buffered saline (PBS) to obtain an optical density at 734 nm of about 0.80 unit of absorbance. The solution was placed in a plastic cuvette; 50 $\mu$L of the antioxidant solutions was added; and the absorbance was read after exactly 1 min. A dose−response curve was plotted for trolox, and the antioxidant ability was expressed as TEAC.

**Scavenging Effects on DPPH Radicals.** The scavenging effects of water extracts from vegetables on DPPH radicals were estimated according to the method of Shimada et al. (*23*). Vegetable extracts in 4 mL water were added to a 1 mL solution of DPPH in methanol. The final concentrations of vegetable extracts and DPPH were 200 $\mu$g/mL and 0.2 mM, respectively. The mixture was shaken vigorously and was allowed to stand for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer.

**Scavenging Effects on Nitrite Oxide.** The scavenging effects of water extracts from vegetables on NO were measured according to the method of Marcocci et al. (*24*). A total of 4 mL of vegetable extract solution was added in the test tubes to 1 mL of SNP solution (25 mM), and the tubes were incubated at 37 °C for 150 min. An aliquot (0.5 mL) of the incubation solution was removed and diluted with 0.3 mL of Griess reagent (1% sulfanilamide in 5% $H_3PO_4$ and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 570 nm and referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent. Scavenging effect (%) = [1 − (nitrite concentration of the sample with 5 mM SNP/nitrite concentration of the control)] × 100.

**Cell Culture.** The murine macrophage cell line RAW 264.7 was obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). RAW 264.7 cells were cultured in 25 or 75 $cm^2$ plastic flasks with DMEM supplemented with 10% heat-inactivated FCS and antibiotics (Gibco/BRL Life Technologies, Eggenstein, Germany). These cells were activated with 1 $\mu$g/mL LPS (*E. coli*, Serotype 0.55:B5) and cultured for 20 h at 37 °C in an atmosphere of 5% $CO_2$.

**Inhibitory Effects on NO Production Activated by LPS.** The inhibitory effects of water extracts from vegetables on NO were estimated according to the method of Dirsch et al. (*25*). Cells were seeded in 96-well plates ($8 \times 10^4$/200 $\mu$L), cultured for 2 days, and then incubated with or without LPS (1 $\mu$g/mL) in the absence or presence of vegetable extracts (200 $\mu$g/mL) at 37 °C for 20 h. As a parameter of NO synthesis, the nitrite concentration was assessed in the supernatant of macrophages RAW 264.7 by the Griess reaction. Briefly, 100 $\mu$L of cell culture supernatant was removed and combined with 60 $\mu$L of 1% sulfanilamide in 5% $H_3PO_4$ and 60 $\mu$L of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in $H_2O$ in a 96-well plate, followed by spectrophotometric measurement at 570 nm using a SPECTRA microplate reader (SLT-Labinstruments). The nitrite concentration was determined through a comparison with a sodium nitrite standard curve.

To determine that the observed NO inhibition was not false-positive because of cytotoxic effects, cell respiration, an indicator of cell viability, was determined through the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. After the supernatants were removed from the plate for nitrite determination, the cells were incubated at 37 °C with MTT (0.5 mg/mL) for 45 min. The medium was aspirated, and the cells were solubilized in DMSO (250 $\mu$L) for at least 2 h in the dark. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm.

**iNOS Assay.** iNOS activity was assayed in a macrophage by the method of Dirsch et al. (*26*). The cells were seeded in 25 $cm^2$ flask ($8 \times 10^4$/200 $\mu$L), cultured for 2 days, and then incubated with or without LPS (1 $\mu$g/mL) in the absence or presence of vegetable extracts (200 $\mu$g/mL) for 20 h. The cells were collected and washed 3 times with cold PBS, frozen immediately, and stored at −80 °C until iNOS activity was measured. iNOS activity was determined by measuring the conversion of L-[$^3$H]arginine to L-[$^3$H]citrulline. Briefly, the cells were homogenized in 25 mM Tris (pH 7.4) containing EDTA (1 mM), EGTA (1 mM), and the protease inhibitor phenylmethyisulfonyl fluoride

(PMSF, 1 mM). The reaction mixture (40 $\mu$L) consisted of NADPH (10 mM), L-[$^3$H]arginine (1 $\mu$Ci/$\mu$L), 50 mM Tris-HCl (pH 7.4), 6 $\mu$M $H_4B$, and 2 $\mu$M FAD. After incubation for 30 min at 37 °C, the reaction was stopped by adding stop buffer (0.5 M $NH_4Cl$). The reaction mixture was separated by means of ion exchange on a Dowex 50 W ($Na^+$ form) column, and the activity of the eluted L-[$^3$H]citrulline was quantified by means of scintillation counting (Packard liquid scintillation analyzer 1500). Bio-Rad protein assay solution was used for protein determination, with BSA employed as a standard.

**Western Blotting of the iNOS Enzyme.** RAW 264.7 cells were incubated in a 25 cm$^2$ flask with or without LPS in the absence or presence of water extracts from vegetables (200 $\mu$g/mL) for 20 h. Cells were washed, harvested, and homogenized. The lysate was centrifuged at 15000*g* for 20 min and standardized to contain 1.0 mg/mL total protein. Using Tris-glycine gel (7−12.5%), electrophoresis was carried out using a Mini-PROTEAN II apparatus (Bio-Rad, Hercules, CA). Bands were transferred into a Hybond-C nitrocellulose membrane (Amersham, Braunschweig, Germany) using a Bio-Ray Trans-Blot semi-dry transfer cell. iNOS antibody (PA3-030A, ABR, CO) was incubated, and iNOS bands were visualized through the treatment with secondary antibody (31340, Pierce, IL) and a NBT/BCIP detection system (34042, Pierce, IL). Signal intensities were evaluated through densitometric analysis.

**Inhibitory Effects on DNA Damage Induced by SNP.** Cells were incubated with or without SNP (1 mM) in the absence or presence of 200 $\mu$g/mL vegetable extracts for 1 h at 37 °C in a dark incubator, together with untreated control samples. Samples were then centrifuged at 800 rpm, and the RAW 264.7 cells were resuspended in low melting agarose (LMA). SNP-induced DNA damage was estimated using single-cell gel electrophoresis (comet assay) (*27*). Briefly, fully frosted slides were covered with 0.5% normal melting agarose (NMA) as the first layer, with a mixture of the cell suspension and 0.5% of LMA as the second layer and finally with 0.5% of LMA (without the cell suspension) as the third layer. After solidification at 4 °C, all slides were immersed in the lysing buffer (2.5 M NaCl and 100 mM EDTA at pH 10, with freshly added 1% Triton X-100 and 10% DMSO) at 4 °C for 1 h, and the slides were then placed in a horizontal electrophoresis tank. The tank was filled with freshly prepared electrophoresis solution (300 mM NaOH and 1 mM EDTA at pH 10 and 4 °C), and the slides were left in the solution for 20 min to allow for DNA unwinding and expression of alkali-labile damage before electrophoresis. Electrophoresis was then conducted at 4 °C for 20 min using 25 V and 300 mA. After electrophoresis, the slides were neutralized in neutralization buffer, stained with ethidium bromide, kept in a humidified airtight container, and examined using a fluorescence microscope. Images of 100 randomly selected cells from each slide were analyzed. The degree of DNA damage is expressed as the tail moment value. Tail moment = (tail length × tail DNA%)/100.

**Determination of Total Phenolic Compounds.** The content of total phenolic compounds in vegetable extracts was measured according to the method of Taga et al. (*28*) and calculated using gallic acid as a standard. The vegetable extracts (0.1 g) was dissolved in 5 mL of 0.3% HCl in methanol/water (60:40, v/v). The resulting solution (100 $\mu$L) was added to 2.0 mL of 2% $Na_2CO_3$. After 2 min, 50% Folin−Ciocalteu reagent (100 $\mu$L) was added to the mixture, which was then left for 30 min. Absorbance was measured at 750 nm using a spectrophotometer.

**Determination of Flavonoid Content.** The spectrophotometer assay for the quantitative determination of flavonoid content was carried out as described by Jia et al. (*29*). A total of 1 mL of water vegetable extracts (200 $\mu$g/mL) was added to 5.7 mL of distilled water and 0.3 mL of 5% $NaNO_2$. After 5 min, 3 mL of 10% $AlCl_3$ was added 5 min later. After another 6 min, 2 mL of the mixture solution was added to 2 mL of 1 N NaOH. Absorbance was measured at 510 nm using a spectrophotometer. Quercetin was used as the standard for a calibration curve.

**Determination of Ascorbic Acid Content.** Determination of ascorbic acid content was performed according to the method of Klein and Perry (*30*). Vegetable extracts (0.1 g) were extracted with 10 mL of 1% metaphosphoric acid. After the vegetable extracts were filtered, the filtrate (1 mL) was added to 9 mL of 50 $\mu$M 2,6-dichloroindophenol (DIP), and the absorbance at 515 nm was read with a Hitach model 2000 spectrophotometer.

**Determination of Total Anthocyanins.** The content of total anthocyanins of vegetable extracts was determined according to the method of Cheng and Breen (*31*). Absorbance of 200 $\mu$g/mL water extracts from vegetable solutions (pH 1.0 and 4.5) was measured at 510 and 700 nm using a spectrophotometer. The total anthocyanin absorbance was calculated using the following formula: $A = [(A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}]$. Delphidin was used as the standard for a calibration curve.

**Statistical Analysis.** Each experiment was performed in triplicate from separate experiments. Statistical analyses were performed according to the SAS User's Guide. Analyses of variance were performed using the ANOVA procedure. Significant differences ($p < 0.05$) between the means were determined using Duncan's multiple range test.

## RESULTS AND DISCUSSION

**Antioxidant Activity and Free-Radical-Scavenging Effect.** The antioxidant activities and DPPH-radical-scavenging effects of water extracts from vegetables are summarized in **Table 1**. Among 25 various vegetables, 6 vegetable extracts showed over 50% inhibition at 200 $\mu$g/mL in linoleic acid peroxidation system and decreased in the order of Indian lotus > coriander > Jew's ear > shiitake > eggplant > winter mushroom. Among 6 vegetables, Indian lotus and eggplant extracts exhibited more scavenging effects on DPPH radicals, followed by shiitake and Jew's ear extracts, and coriander showed the weakest scavenging effects. Indian lotus, winter mushroom, eggplant, Jew's ear, and mung bean seedling showed stronger antioxidant activity in the TEAC method. The relational coefficients between the linoleic acid and TAEC methods, the linoleic acid and DPPH-scavenging methods, the TEAC and DPPH-scavenging methods were 0.578 ($p = 0.0025$), 0.612 ($p = 0.0011$), and 0.682 ($p = 0.0002$), respectively. Therefore, the antioxidant activity of vegetable extracts may be partly due to their scavenging effects on radicals and blocking the chain reaction in the peroxidation of linoleic acids. Data in **Table 1** indicate that the extracts from rhizome of Indian lotus exhibit the strongest antioxidant activity and radical-scavenging effect. Hu and Skibsted (*32*) reported that extracts from rhizome and the rhizome knot of edible lotus displayed good antioxidant activity and high total phenol content. Results above also showed that mushrooms could reduce the oxidative damage of free radicals. Cheung et al. (*33*) compared the antioxidant activities of methanol and water crude extracts from shiitake and straw mushroom. The results showed that the water extract from shiitake exhibited the most potent radical-scavenging activity in all mushroom extracts, and positive correlations were found between the total phenolic content in mushroom extracts and their antioxidant activities. In this study, three of four mushroom extracts presented good antioxidant activities. Therefore, mushrooms could be a source of phenolic compounds that provide beneficial effects against oxidative damage.

**NO-Scavenging Effect.** It is well-known that NO has an important role in the various types of pathophysiological processes. In the present study, the vegetable extracts were evaluated for their scavenging effects on NO derived from SNP. The compound SNP is known to decompose in aqueous solution at physiological pH, producing NO. Under aerobic conditions, NO reacts with oxygen to produce the stable products nitrate and nitrite, which can be determined using Griess reagent (*24*). Data in **Figure 1** show that the scavenging effects of vegetable extracts on NO are in decreasing order of water spinach > Indian lotus > eggplant and garland chrysanthemum. Although the

**Table 1.** Antioxidant Activity and Free-Radical-Scavenging Effects of Water Extracts from Vegetables

| sample[a] | edible part | percent yield of extracts (%) | inhibition of peroxidation (%) | trolox quivalent antioxidant capacity (TEAC, mM) | scavenging effect on DPPH (%) |
|---|---|---|---|---|---|
| plant | | | | | |
| asparagus | stem | 3.81 | 20.6 ± 0.6 | 0.09 ± 0.03 | 10.0 ± 1.5 |
| bitter gourd | fruit | 2.61 | 5.9 ± 0.9 | 0.06 ± 0.01 | 10.4 ± 0.4 |
| bottle gourd | fruit | 3.12 | 14.1 ± 0.2 | 0.06 ± 0.00 | 6.2 ± 1.3 |
| cauliflower | flower/stem | 4.83 | 7.3 ± 0.6 | 0.09 ± 0.01 | 13.0 ± 0.9 |
| celery | leaf/stem | 2.43 | 43.6 ± 0.2 | 0.08 ± 0.01 | 11.1 ± 0.4 |
| coriander | leaf/stem | 5.02 | 78.5 ± 0.2 | 0.07 ± 0.02 | 11.7 ± 1.0 |
| daylily (dried) | flower | 3.65 | 2.8 ± 0.1 | 0.06 ± 0.01 | 17.1 ± 0.8 |
| daylily (fresh) | flower | 3.70 | 12.7 ± 0.3 | 0.06 ± 0.02 | 22.5 ± 0.5 |
| eggplant | fruit | 2.17 | 61.0 ± 0.3 | 0.10 ± 0.00 | 46.1 ± 0.7 |
| garland chrysanthemum | leaf | 5.22 | 26.3 ± 0.3 | 0.06 ± 0.02 | 8.4 ± 1.1 |
| Indian lotus | rhizome | 5.31 | 83.5 ± 0.2 | 0.13 ± 0.02 | 48.4 ± 0.2 |
| mung bean seedling | seedling | 2.20 | 17.6 ± 0.1 | 0.10 ± 0.01 | 16.1 ± 2.1 |
| leek | leaf | 3.05 | 16.3 ± 0.2 | 0.08 ± 0.01 | 14.4 ± 0.8 |
| onion | bulb | 5.90 | 13.4 ± 0.2 | 0.06 ± 0.02 | 4.0 ± 0.1 |
| pea sprout | sprout | 3.35 | 25.6 ± 0.2 | 0.09 ± 0.02 | 11.7 ± 1.4 |
| potato | tuber | 2.38 | 32.7 ± 0.3 | 0.07 ± 0.01 | 1.2 ± 0.4 |
| sponge gourd | fruit | 2.79 | 13.9 ± 0.3 | 0.06 ± 0.02 | 17.9 ± 0.4 |
| sweet potato | root | 6.26 | 4.7 ± 0.3 | 0.03 ± 0.01 | 5.9 ± 2.7 |
| tomato | fruit | 3.41 | 2.8 ± 0.4 | 0.07 ± 0.02 | 18.1 ± 0.9 |
| water spinach | leaf/stem | 3.62 | 29.3 ± 0.1 | 0.06 ± 0.02 | 10.9 ± 0.8 |
| wild rice stem | stem | 2.69 | 10.5 ± 0.2 | 0.06 ± 0.02 | 5.7 ± 0.1 |
| mushroom | | | | | |
| Jew's ear | fruit body | 0.42 | 72.7 ± 0.4 | 0.10 ± 0.00 | 22.4 ± 2.7 |
| shiitake | fruit body | 3.86 | 68.4 ± 0.2 | 0.06 ± 0.02 | 24.0 ± 0.1 |
| straw mushroom | fruit body | 2.43 | 8.6 ± 0.2 | 0.06 ± 0.00 | 8.3 ± 1.6 |
| winter mushroom | fruit body | 5.67 | 58.9 ± 0.1 | 0.12 ± 0.02 | 32.4 ± 0.7 |
| standards | | | | | |
| trolox | | | 97.9 ± 0.0 | | 95.9 ± 0.6 |
| BHA | | | 90.8 ± 0.1 | 2.20 ± 0.01 | 94.8 ± 0.2 |
| α-tocopherol | | | 78.8 ± 0.0 | 1.30 ± 0.02 | 95.5 ± 0.9 |

[a] The final concentration of the sample in the reaction mixture was 200 μg/mL.

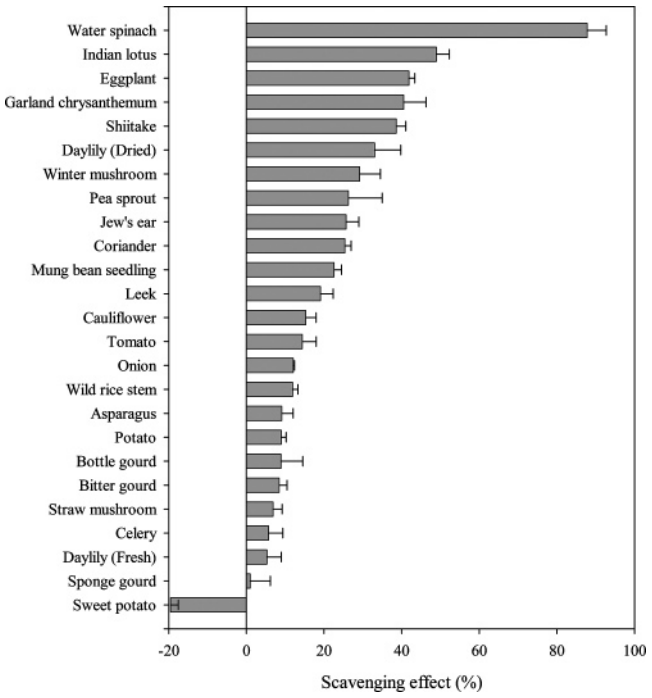


**Figure 1.** Scavenging effect of water extracts from vegetables on NO derived from SNP during incubation. The concentration of vegetable extracts used for treatment was 200 μg/mL.

relational coefficient were not very good, the positive correlations were observed in the scavenging effect on NO and the antioxidant activity ($R = 0.463$, $p = 0.0199$) and scavenging effect on DPPH radicals ($R = 0.415$, $p = 0.0391$). The extract from sweet potato promoted the NO generation; however, the reason is still unknown and requires more study.

**Effects on NO Generation and iNOS Expression in LPS-Stimulated Macrophages.** The effects of vegetable extracts on the NO generation were assayed in LPS-stimulated RAW 264.7 macrophages. The viability of RAW 264.7 cells were over 90% by the addition of all vegetable extracts at the concentration of 200 μg/mL, as determined by the MTT assay. As shown in **Figure 2**, the water extracts from fresh daylily flower, sponge gourd, pea sprout, and eggplant displayed over 80% inhibition on the NO generation stimulated by LPS. However, the dried daylily flower exhibited a slight promotion on the NO generation. The edible part of fresh daylily is the immature flower (green); however, the dried daylily is processed from a mature flower (yellow) by drying and blenching with sulfite. Therefore, the components that provided an inhibitory effect on the NO generation may be altered in the flower maturation process or may be affected by sulfite and remains as a matter to be studied further.

The results of inhibitory effects on the NO generation in LPS-stimulated RAW 264.7 macrophages were different to that of scavenging effects on NO derived from SNP. The fresh daylily and sponge gourd exhibited little effects on SNP-derived NO (**Figure 1**); however, these two plant extracts possessed very significantly activities on suppressing the NO production in LPS-stimulated RAW 264.7 macrophages (**Figure 2**). When macrophages were activated by LPS, macrophages generate NO from L-arginine via the catalysis of iNOS. Therefore, the mechanism of inhibition on the NO generation in LPS-activated macrophages may be not only attributed to the scavenging effects on NO but also effect the action of iNOS. For realized

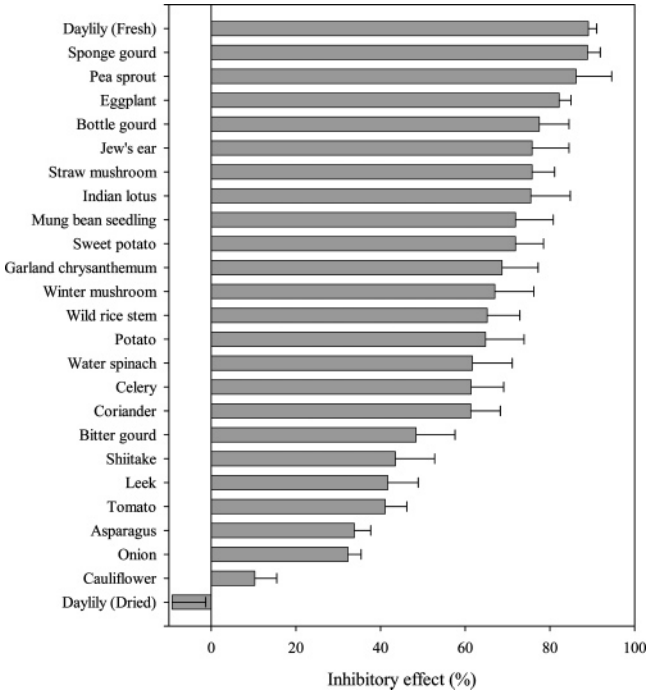


**Figure 2.** Effects of water extracts from vegetables on the production of NO induced by LPS in RAW 264.7 macrophages. The concentration of vegetable extracts used for treatment was 200 $\mu$g/mL.

effects of vegetable extracts on iNOS, the enzyme activity and protein expression of iNOS in LPS-activated macrophages were further evaluated. LPS increased iNOS activity as measured by the conversion of L-[$^3$H]arginine to L-[$^3$H]citrulline. The water extracts from pea sprout, fresh daylily flower, eggplant, and sponge gourd inhibited 25−35% iNOS activity, which was significantly lower compared with the control (data not shown). The effects of vegetable extracts on the production of the iNOS protein were also measured by Western blotting analysis (**Figure 3**). Among four various vegetable extracts, fresh daylily flower exhibited markedly inhibition on protein induction in LPS-activated macrophages. However, the extracts from pea sprout and eggplant showed no effects on the production of iNOS protein. As shown above, the extract from fresh daylily flower that expressed the strongest inhibition on the NO production was contributed to the ability to reduce the iNOS induction. The extracts from pea sprout and eggplant, on the other hand, suppressed the NO production by scavenging on NO and inactivating toward the iNOS enzyme. The mechanism responsible for NO inhibition of several plant extracts had been reported. Wadsworth and Koop (*34*) indicated that the *Ginkgo biloba* extract (EGb 761) directly scavenged NO and inhibited LPS-induced levels of iNOS protein. Moreover, the *Ginkgo biloba* extract suppressed the inflammation of a rat by blocking the iNOS protein expression and its anti-inflammatory effect on the eye (*35*). The inhibition of many medicinal plant extracts on NO production was also found to be due to direct scavenging of NO, suppression of iNOS protein, or reduction on iNOS mRNA expression (*36*). In the present study, the vegetable extracts expressed NO inhibition via one or more mechanisms.

**Effect on SNP-Mediated Macrophages DNA Damage.** There are increasing lines of evidence that NO and its donors induced mutations in cultured mammalian cells (*37*). Several reports also showed that NO or NO donors induced DNA strand breaks in vitro and in vivo (*38*, *39*). The effects of vegetable extracts on SNP-mediated DNA damage were measured by the comet assay, a simple and sensitive technique used to detect

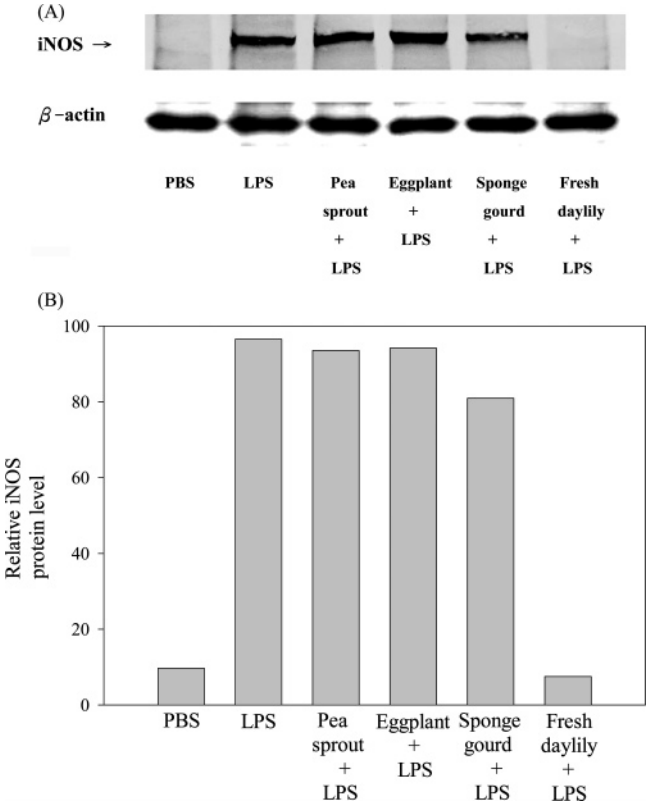


**Figure 3.** Western blotting analysis of the iNOS protein in LPS-activated RAW 264.7 macrophages. Cells were stimulated with LPS (1 $\mu$g/mL) and incubated in the absence or presence of the vegetable sample (200 $\mu$g/mL) for 20 h at 37 °C. (A) Bands of iNOS were identified by Western blotting with anti-iNOS antibodies. (B) Immunoblots were analyzed by densitometry, and the data were generated as integrated density units.

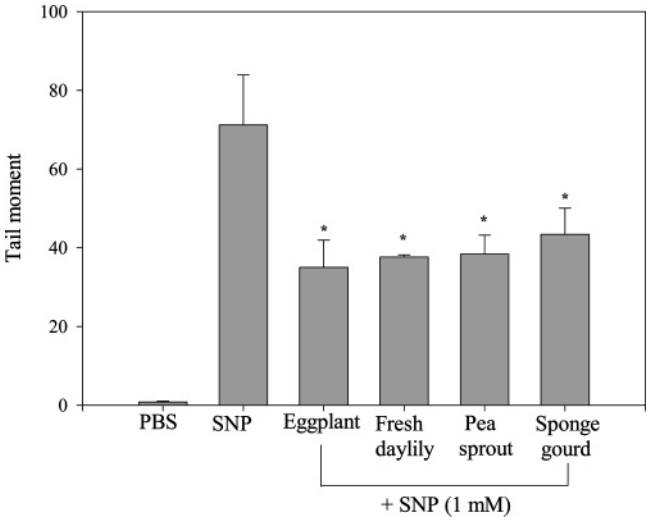


**Figure 4.** Effect of water extracts from vegetables on DNA damage induced by SNP in RAW 264.7 macrophages. The concentration of the vegetable sample used for treatment was 200 $\mu$g/mL. (*) Significiantly different from SNP treated only ($p < 0.05$).

DNA damage in individual cells. A positive increase in the tail moment value was seen following 1 mM SNP treatment of macrophages in comparison with the untreated control (**Figure 4**). However, 42−48% genotoxicity of SNP was inhibited by fresh daylily flower, sponge gourd, pea sprout, or eggplant, and this decreasing effect may be partly attributed to the antioxidant activities and NO-scavenging abilities of vegetable extracts.

**Table 2.** Analysis of Total Phenolics, Flavonoids, Total Anthocyanins, and Ascorbic Acid Contents of Water Extracts from Vegetables

| sample | total phenolics[a] (mg/g) | flavonoids (mg/g) | total anthocyanins (mg/g) | ascorbic acid (mg/g) |
|---|---|---|---|---|
| pea sprout | 48.20 a[b] | 17.16 a | 1.33 a | 2.20 a |
| eggplant | 33.70 b | 17.33 a | 1.67 b | 2.25 a |
| fresh daylily | 22.68 c | 22.44 b | 0.50 c | 0.59 b |
| sponge gourd | 20.74 d | 17.94 a | 0.50 c | 1.20 c |

[a] The contents of constituents in vegetable extracts were expressed by constituent (in milligrams)/ vegetable water extracts (in grams). Values are means of three replicate analyses. [b] Values in a column with the different superscripts are significantly different ($p < 0.05$).

**Analysis of Main Antioxidant Compounds.** The antioxidant activity and radical-scavenging ability of plant extracts most attributed to the antioxidant compounds including phenolics, ascorbic acid, tocopherols, and carotenoids, etc. However, the contributions of antioxidant compounds in the inhibitory effects on NO generation and iNOS action in LPS-stimulated RAW 264.7 macrophages should be studied further. Therefore, the contents of total phenolics, flavonoid, total anthocyanins, and ascorbic acid in four vegetable extracts that exhibited good inhibition on NO generation in LPS-stimulated RAW 264.7 macrophages were assayed. From the results shown in **Table 2**, the content of total phenolics of the pea sprout is the highest among all of the samples, followed by eggplant, fresh daylily, and sponge gourd. The flavonoid content of fresh daylily extracts was higher than that of the other three vegetable extracts. The contents of total anthocyanins and ascorbic acid in the eggplant and pea sprout extracts were higher than that of fresh daylily and sponge gourd extracts. It is well-known that plant phenolic compounds, such as phenolic acids, flavonoids, and anthocyanins act as both free-radical scavengers and antioxidants (*40*, *41*). Yokozawa et al. (*42*) examined the direct NO-scavenging effects of 31 traditional crude drugs and several pure compounds and found that tannins and alkaloids were the active principles responsible for scavenging NO. Nakagawa and Yokozawa (*43*) also indicated that tea catechins with the galloly group showed stronger scavenging effects on NO. In our results, the positive correlation ($R = 0.990$, $p = 0.0099$) was only observed between the content of anthocyanins and the scavenging effects on NO. The extracts from eggplant and pea sprout that showed stronger antioxidant activity and scavenging effects on NO derived from SNP than that of fresh daylily and sponge gourd could be correlated with their contents of total phenolic compounds, anthocyanins and ascorbic acids. With the exception as a free-radical scavenger, the effect of phenolic compounds on the action of iNOS has been interested and intensively studied. Blonska et al. (*44*) indicated that flavonoids in propolis, including chrysin, galangin, kaempferol, and quercetin, significantly decreased the iNOS mRNA level and NO production in LPS-induced macrophages. Packer et al. (*45*) also reported that a mixture of flavonoids, mainly procyandins and phenolic acids extracted from *Pinus maritime*, modulated the NO generation in activated macrophages by quenching the NO radical and inhibiting both iNOS mRNA expression and iNOS activity. Matsuda et al. (*46*) and Kim et al. (*47*) evaluated the inhibitory effects of flavonoids on NO production in LPS-activated macrophages and indicated that the activities of flavones were stronger than those of corresponding flavonols and flavanones. In addition, their inhibitory activity might be due to the reduction of iNOS enzyme expression. In the present study, the extracts from fresh daylily exhibited more inhibition on the NO generation in LPS-stimulated RAW 264.7 macrophages. However, further studies on the effective components in fresh daylily extracts that contributed to the ability to reduce the iNOS induction are required.

In conclusion, the present study showed that the extracts from several vegetables were found to possess strong antioxidant activity and scavenging effects on free radicals and NO. Some kinds of vegetable extracts not only effectively scavenged NO but also potently inhibited the generation of NO in macrophages. The mechanism responsible for NO inhibition in macrophages seems to involve direct scavenging of NO, inactivation in iNOS activity, and reduction in the induction of iNOS. Because ROS and RNS are thought to be associated with chronic infection and inflammation diseases, the inhibitory effects on the NO generation by vegetable extracts determined in this study may provide the assessment of the health effects of vegetables.

## LITERATURE CITED


(1) Darley-Usmar, V.; Wiseman, H.; Halliwell, B. Nitric oxide and oxygen radicals: A question of balance. *FEBS Lett.* **1995**, *369*, 131−135.

(2) Halliwell, B. Antioxidants and human disease: A general introduction. *Nutr. Rev.* **1997**, *55*, 44S−49S.

(3) Vendemiale, G.; Grattagliano, I.; Altomare, E. An update on the role of free radicals and antioxidant defense in human disease. *Inter. J. Clin. Lab. Res.* **1999**, *29*, 49−55.

(4) Laranjinha, J.; Alemeida, L.; Madeira, V. Reactivity of dietary phenolic acids with peroxyl radicals: Antioxidant activity upon low-density lipoprotein peroxidation. *Biochem. Pharm.* **1994**, *48*, 487−494.

(5) Ness, A. R., Powles, J. W. Fruit and vegetables, and cardiovascular disease. *Inter. J. Epidemiol.* **1997**, *26*, 1−13.

(6) Meng, Q. H.; Lewis, P.; Wahala, K.; Adlercreutz, H.; Tikkanen, M. J. Incorporation of esterified soybean isoflavones with antioxidant activity into low-density lipoprotein. *Biochem. Biophys. Acta* **1999**, *1438*, 369−376.

(7) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharm. Rev.* **1991**, *43*, 109−142.

(8) Schmidt, H. H. H. W.; Walter, U. NO at work. *Cell* **1994**, *78*, 919−925.

(9) Kroncke, K.; Fehsel, K.; Kolb-Bachofen, V. Nitric oxide: Cytotoxicity versus cytoprotection-how, why, when, and where? *Nitric Oxide* **1997**, *1*, 107−120.

(10) Murphy, M. P. Nitric oxide and cell death. *Biochim. Biophy. Acta* **1999**, *1411*, 401−414.

(11) Moncada, S.; Higgs, A. The L-arginine−nitric oxide pathway. *N. Eng. J. Med.* **1993**, *329*, 2002−2012.

(12) Busse, R.; Mulch, A. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.* **1990**, *275*, 87−90.

(13) Zhang, X.; Morrison, D. C. Lipopolysacchride-induced selective priming effects on tumor necrosis factor α and nitric oxide production in mouse peritoneal macrophages. *J. Exp. Med.* **1993**, *177*, 511−516.

(14) Thomsen, L. L.; Miles, D. W.; Happerfield, L.; Bobrow, L. G.; Knowledges, R. G.; Moncada, S. Nitric oxide synthase activity in human breast cancer. *Br. J. Cancer* **1995**, *72*, 41−45.

(15) Gallo, O.; Masini, E.; Morbidelli, L.; Franchi, A.; Fini-Storchi, I.; Vergari, W. A.; Ziche, M. Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. *J. Natl. Cancer Inst.* **1998**, *90*, 587−596.

(16) Arroyo, P. L.; Hatch-Pigott, V.; Mower, H. F.; Cooney, R. V. Mutagenicity of nitric oxide and its inhibition by antioxidants. *Mutat. Res.* **1992**, *281*, 193−202.

(17) Chan, M. M.; Fong, D.; Ho, C.; Huang, H. Inhibition of inducible nitric oxide synthase gene expression and enzyme activity by epigallocatechin gallate, a natural product from green tea. *Biochem. Pharm.* **1997**, *54*, 1281−1286.

(18) Kawada, N.; Seki, S.; Kuroki, T. Effect of antioxidants resveratrol, quercetin and *N*-acetylcystein, on the functions of cultured rat hepatic stellate cells and kupfer cells. *Hepatology* **1998**, *27*, 1265−1274.

(19) Saha K.; Lajis, N. H.; Israf, D. A.; Hamzah, A. S.; Khozirah, S.; Khamis, S.; Syahida, A. Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. *J. Ethnopharmacol*. **2004**, *92*, 263−267.

(20) So, H. S.; Park, R. K.; Oh, H. M.; Pae, H. O.; Lee, J. H.; Chai, K. Y.; Chung, S. Y.; Chung, H. T. The methanol extract of *Spiraea prunifolia* var. *simpliciflora* root inhibits the generation of nitric oxide and superoxide in RAW 264.7 cells. *J. Ethnopharmacol*. **1999**, *68* 209−217.

(21) Mitsuda, H.; Yasumodo, K.; Iwami, K. Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Eiyoto Shokuryo* **1966**, *19*, 210−214.

(22) Arnao, M. B.; Cano, A.; Hernandez-Ruiz, J.; Garcia-Canovas, F. Acosta, M. Inhibition by L-ascorbic acid and other antioxidants of the 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) oxidation catalyzed by peroxidase: A new approach for determining total anti-oxidant status of foods. *Anal. Biochem.* **1996**, *236*, 255−261.

(23) Shimada, K.; Fujikawa, K.; Yahara, K.; Nakamura, T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agric. Food Chem.* **1992**, *40*, 945−948.

(24) Marcocci, L.; Maguire, J. J.; Droy-Lefaix, M. T.; Packer, L. The nitric oxide-scavenging properties of *Ginkgo biloba* extract EGB 761. *Biochem. Biophys. Res. Comm.* **1994**, *201*, 748−755.

(25) Dirsch, V. M.; Stuppner, H.; Vollmar, A. M. The Griess assay: Suitable for a bio-guided fractionation of anti-inflammatory plant extracts? *Planta Med.* **1998**, *64*, 423−426.

(26) Dirsch, V. M.; Kiemer, A. K.; Wagner, H.; Vollmar, A. M. Effect of allicin and ajoene, two compounds of garlic, on inducible nitric oxide synthase. *Atherosclerosis* **1998**, *139*, 333−339.

(27) Green, M. H. L.; Lowe, J. E.; Delaney, C. A.; Green, I. C. comet assay to detect nitric oxide-dependent DNA damage in mammalian cells. *Methods Enzymol.* **1996**, *269*, 243−266.

(28) Taga, M. S.; Miller, E. E.; Pratt, D. E. Chia seeds as a source of natural lipid antioxidants. *J. Am. Oil Chem. Soc.* **1984**, *61*, 928−931.

(29) Jia, Z.; Tang, M.; Wu, J. The determination of flavonoids contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* **1999**, *64*, 555−559.

(30) Klein, B. P.; Perry, A. K. Ascorbic acid and vitamin A activity in selected vegetables from different geographical areas of the United States. *J. Food Sci*. **1982**, *47*, 941−945.

(31) Cheng, J. W.; Breen, P. J. Activity of phenylalanine ammonia-lyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. *J. Am. Soc. Hortic. Sci.* **1991**, *116*, 865−869.

(32) Hu, M.; Skibsted, L. H. Antioxidative capacity of rhizome extract and rhizome knot extract of edible lotus (*Nelumbo nuficera*). *Food Chem.* **2002**, *76*, 327−333.

(33) Cheung, L. M.; Cheung, P. C. K.; Ooi, V. E. C. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chem.* **2003**, *81*, 249−255.

(34) Wadsworth, T. L.; Koop, D. R. Effects of *Ginkao biloba* extract (EGb 761) and quercetin on lipopolysaccharide-induced release of nitric oxide. *Chem.-Biol. Interact.* **2001**, *137*, 43−58.

(35) Ilieva, I.; Ohgami, K.; Shiratori, K.; Koyama, Y.; Yoshida, K.; Kase, S.; Kitamei, H.; Takemoto, Y.; Yazawa, K.; Ohno, S. The effects of *Ginkgo biloba* extract on lipopolysaccharide-induced inflammation in vitro and in vivo. *Exp. Eye Res.* **2004**, *79*, 181−187.

(36) Tezuka, Y.; Irikawa, S.; Kaneko, T.; Banskota, A. H.; Nagaoka, T.; Xiong, Q.; Hase, K.; Kadota, S. Screening of Chinese herbal drug extracts for inhibitory activity on nitric oxide production and identification of an active compound of *Zanthoxylum bungeanum*. *J. Ethnopharm.* **2001**, *77*, 209−217.

(37) Lin, W.; Xue, H.; Liu, S.; He, Y.; Fu, J.; Zhou, Z. Genotoxicity of nitric oxide produced from sodium nitroprusside. *Mutat. Res.* **1998**, *413*, 121−127.

(38) Delaney, C. A.; Green, L. C.; Lowe, J. E.; Cunningham, J. M.; Butler, A. R.; Renton, L.; Costa, L. D.; Green, M. H. L. Use of the comet assay to investigate possible interactions of nitric oxide and reactive oxygen species in the induction of DNA damage and inhibition of function in an insulin-secreting cell line. *Mutat. Res.* **1997**, *375*, 137−146.

(39) Lin, W.; Wei, X.; Xue, H.; Kelimu, M.; Tao, R.; Song, Y.; Zhou, Z. Study on DNA strand breaks induced by sodium nitroprusside, a nitric oxide donor, in vivo and in vitro. *Mutat. Res.* **2000**, *466*, 187−195.

(40) Sato, M.; Ramarathnam, N.; Suzuki, Y.; Ohkubo, T.; Takeuchi, M.; Ochi, H. Variental difference in the phenolic content and superoxide radical scavenging potential of wines from different sources. *J. Agric. Food Chem*. **1996**, *47*, 37−41.

(41) Rice-Evans, C.; Miller, N. J.; Paganga, G. Structure−antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(42) Yokozawa, T.; Chen, C. P.; Tanaka, T. Direct scavenging of nitric oxide by traditional crude drugs. *Phytomedicine* **1999**, *6*, 453−463.

(43) Nakagawa, T.; Yokozawa T. Direct scavenging of nitric oxide and superoxide by green tea. *Food Chem. Toxicol.* **2002**, *40*, 1745−1750.

(44) Blonska, M.; Bronikowska, J.; Pietsz, G.; Czuba, Z. P.; Scheller, S.; Krol, W. Effects of ethanol extract of propolis (EEP) and its flavones on inducible gene expression in J774A.1 macrophages. *J. Ethnopharmacol*. **2004**, *91*, 25−30.

(45) Packer, L.; Rimbach, G.; Virgili, F. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free Radical Biol. Med.* **1999**, *27*, 704−724.

(46) Matsuda, H.; Morikawa, T.; Ando, S.; Toguchida, I.; Yoshikawa, M. Structural requirements of flavonoids for nitric oxide production inhibitory activity and mechanism of action. *Bioorg. Med. Chem.* **2003**, *11*, 1995−2000.

(47) Kim, H. K.; Cheon, B. S.; Kim, Y. H.; Kim, S. Y.; Kim, H. P. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure−activity relationships. *Biochemical. Pharmacol.* **1999**, *58*, 759−765.

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