J Food Sci Nutr
Vol 14, p 277～282 (2009)
DOI: 10.3746/jfn.2009.14.4.277

J Food Science
and Nutrition

Free Radical Scavenging Activity and Protective Ability of Methanolic Extract from *Duchesnea indica* Against Protein Oxidation and DNA Damage

Weicheng Hu[1], Wei Shen[2], and Myeong-Hyeon Wang[1†]

[1]*School of Biotechnology, Kangwon National University, Gangwon-Do 200-701, Korea*
[2]*College of Guangling, Yangzhou University, Yangzhou, 225127, China*

Abstract

The antioxidant potency of methanolic extract of *Duchesnea indica* (MDI; Indian strawberry) was investigated by employing various established *in vitro* systems, such as total phenolic content, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, reducing power assay, metal chelating assay, superoxide radical scavenging activity and protective ability of DNA damage and protein oxidation. MDI inhibited metal chelating by 75.57% at 2 mg/mL, scavenged 50% DPPH free radical at 29.13 μg/mL, and eliminated approximately 46.21% superoxide radical at the concentration of 1 mg/mL. In addition, MDI showed strong ability on reducing power, DNA damage protection and protein oxidation protection. Overall, results suggested that MDI might be beneficial as a potent antioxidant and effectively employed as an ingredient in food applications.

Key words: *Duchesnea indica*, reactive oxygen species, antioxidant, DNA damage, protein oxidation

INTRODUCTION

Oxidative stress, induced by oxygen radicals, plays a critical role in various degenerative diseases, such as cancer, atherosclerosis, and gastric ulcers (1). The human body has a complex antioxidant system to protect the cellular molecules against the oxygen radical-induced damage, including antioxidative enzymes, such as superoxide dismutase, catalase and glutathione peroxidase (2). This innate defense is not enough for severe oxidative stress, and therefore overproduction of oxidative radicals may cause tissue damages (3). Consequently, certain amounts of exogenous antioxidants are constantly required to balance the reactive oxygen species (ROS) in the human body (4). Several synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ), are commercially available, but their applications are restricted due to potential risks related to health (5). Therefore, much attention has been focused on natural antioxidants. Plants contain a variety of free radical scavenging molecules such as phenolic compounds, nitrogen compounds, vitamins, terpenoids, and other endogenous metabolites that are rich in antioxidant activity. Some plants have been extensively investigated for antioxidant in the last few decades (6,7).

Duchesnea indica, commonly known as Indian strawberry, belongs to the Rosaceae family, is widely distributed in China, and also possesses a variety of biological activities, including cytotoxic, antibiotic, antioxidative, and anti-inflammatory activities (8-10). A number of chemical compounds like triterpenes, triterpene glycodides, flavonoid glycodides and sterols have been identified from *D. indica* (11-13). Previously, Kim et al. (9) reported the antioxidant activity of a polysaccharide-enriched fraction isolated from *Duchesnea chrysantha*. However, to the best of our knowledge, there is limited literature on free radical scavenging activity and protective ability of methanolic extract from *Duchesnea indica* (MDI) against protein oxidation and DNA damage. To investigate the potential antioxidant properties of *D. indica*, we employed a number of *in vitro* assay systems, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, reducing power assay, metal chelating assay, superoxide radical scavenging assay and protective ability on DNA damage and protein oxidation.

MATERIALS AND METHODS

Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2-deoxy-D-ribose, α-tocopherol, ethylenediaminetetraacetic acid (EDTA), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), trichloroacetic acid (TCA), BHT, nitro blue tetrazolium (NBT), ferric chloride, phe-

†Corresponding author. E-mail: mhwang@kangwon.ac.kr
Phone: +82-33-250-6486, Fax: +82-33-241-6480

nazine methosulphate (PMS), and dinucleotide-reduced (NADH) were purchased from Sigma (St. Louis, MO, USA). 2-Thiobarbituric acid (TBA) was purchased from Alfa Aesar (Karlsruhe, Germany). All other reagents were of analytical grade.

Preparation of the extract

The whole plant of *Duchesnea indica*, cultivated in Anhui, China, was dried in the shade at room temperature and then powdered. Two hundred grams of the whole plant powder were extracted with absolute methanol at 60°C for 3 hr. The extract was filtered and evaporated under reduced pressure using a vacuum rotary evaporator to produce a crude extract of 17.83 g. The dried samples were weighed and kept in a refrigerator until further analysis. The positive controls and MDI were dissolved in methanol to the concentration of 10 mg/mL as stocks.

Determination of the total phenolic content

One milliliter of MDI (200 μg/mL) was mixed with 1.8 mL of Folin-Ciocalteu reagent, and the solution was allowed to stand at 25°C for 5 min before the addition of 1.2 mL of a 15% sodium carbonate solution. Tannic acid (Tan) was used as the standard to create a calibration curve. The total phenolic content is expressed as mg Tan/g MDI.

DPPH radical-scavenging activity

The free radical scavenging activity of MDI was determined by the DPPH test (14). Briefly, 0.5 mL of 0.1 mM DPPH solution (in methanol) was added to a test tube containing 0.5 mL of the MDI at various concentrations ranging from 1 μg/mL to 100 μg/mL. The mixture was shaken vigorously for 1 min and kept at room temperature for 30 min in the dark. The absorbance of each solution was subsequently measured at 517 nm. α-Tocopherol and BHT were used as positive controls. The capability to scavenge the DPPH radical was calculated using the following equation:

$$I\ (\%) = [1 - (A_i - A_j)/A_c\ ] \times 100$$

A_c is the absorbance of the DPPH solution without sample (0.5 mL DPPH solution + 0.5 mL of methanol); A_i is the absorbance of the test sample mixed with DPPH solution (0.5 mL sample + 0.5 mL DPPH solution) and A_j is the absorbance of the sample without DPPH solution (0.5 mL sample + 0.5 mL methanol).

Reducing power assay

The reducing power assay was determined according to the method of Hu et al. (15). Various concentrations (100, 200, 300, 400, and 500 μg/mL sample [1 mL]) were mixed with 2.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 0.1 % potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After adding 2.5 mL of 10% TCA, the mixture was centrifuged at 3000 × *g* for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% of ferric chloride, and the absorbance was measured at 700 nm. α-Tocopherol and BHT were used as positive controls.

Metal-chelating activity

The chelation of ferrous ions by MDI was estimated as described previously (16). In brief, various concentrations (0.5, 1, and 2 mg/mL MDI [1 mL]) were mixed with 3.7 mL of absolute methanol and 0.1 mL of 1 mM $FeCl_2$. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine followed by vigorous shaking; the mixture was then left to react at room temperature for 10 min. The absorbance was measured at 562 nm. EDTA at the concentration of 0.1 mg/mL was used as a positive control.

Superoxide radical scavenging assay

The superoxide radical scavenging activity was determined by the PMS-NADH generating system which was described by Singh and Rajini (17) with minor modifications. Briefly, 100 μL of the extract solution at various concentrations (0.0625, 0.125, 0.25, 0.5 and 1 mg/mL) was mixed with 150 μL of NADH (166 μM), 450 μL of NBT (86 μM) and 150 μL of PMS (16.2 μM). After 5 min of incubation at room temperature, the absorbance at 560 nm was measured. Gallic acid at the concentration of 0.125 mg/mL was used as a positive control.

DNA damage protection assay

The method used to test for hydroxyl radical-induced DNA damage in the plasmid pBR 322 was modified from Qian et al. (18). Briefly, 3 μL of MDI at various concentrations (0.1, 0.5, and 1 mg/mL) and 0.5 μg of pBR 322 were mixed and incubated for 10 min at room temperature followed by the addition of 7 μL of Fenton's reagent (3 μL of 30% H_2O_2, 2 μL of 5 mM $FeSO_4$, and 2 μL of 50 mM PB). The resulting mixture (final volume 20 μL) was incubated for 30 min at 37°C. The DNA was electrophoresed on 1% agarose gels and visualized using ethidium bromide and a Mini BIS image analysis system (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel). Densitometric analysis was done using image analysis software (Quantity One, Bio-Rad, Hercules, CA). The extent of DNA damage protection (%) was calculated by comparing the proportion of supercoiled DNA with Fenton's reagent to that without Fenton's reagent.

Protein damage protection assay

The effects of MDI on protein oxidation were carried out according the method of Kızıl et al. (19). BSA was oxidized by a H_2O_2/Fe^{3+}/ascorbic acid system. The reaction mixture (1.0 mL), containing sample (100 μg/mL or 200 μg/mL), phosphate buffer (20 mM pH 7.4) BSA (1 mg/mL), $FeCl_3$ (50 μM), H_2O_2 (1 mM) and ascorbic acid (100 μM) were incubated for 3 hr at 37°C. After incubation, the reaction mixture was analyzed by electrophoresis in 10% SDS polyacrylamide gel. The gel was stained with brilliant blue R staining solution for 2 hr and then destained and digitally photographed. To determine the amount of protein damage, band intensity was estimated using the image analysis software (Quantity One, Bio-Rad, Hercules, CA).

Statistical analysis

All tests were carried out independently in triplicate ($n=3$). The data are expressed as the mean ± standard derivation (SD). All analyses were performed using SPSS 7.5 (SPSS Institute, Cary, NC, USA); individual comparisons were made using Tukey's multiple-range test, which was used to determine the differences between the means. $p<0.05$ was considered significantly.

RESULTS AND DISCUSSION

Total phenolic content

Solvents used for extraction affected the yield (20). The yield of the MDI was 8.92% to dry material. Shoemaker et al. (21) had previously reported that the water extract of *D. indica* had a 100 mg/g dry herb. Phenolic compounds widely exist in plants are bioactive substances. It is well known that phenolic compounds in plants are viewed as powerful *in vitro* antioxidants due to their ability to donate hydrogen or electrons and to form stable radical intermediates (22). In our work, the total phenolic content of the samples was 161.0 mg/g dry weight, expressed as tannic acid equivalent (Fig. 1).

DPPH radical-scavenging activity

Free radicals are known as the major cause of oxidative damage of biological molecules in human body, including coronary heart disease, aging, cancer and dementia. DPPH has been used to evaluate the free-radical scavenging capacity of antioxidants (23,24). The method is based on the reduction of the absorbance of DPPH solution at 517 nm in the present of a proton-donating substance, owing to the formation of the diamagnetic molecule by accepting an electron or hydrogen radical (25). As shown in Table 1, the MDI showed the IC_{50} (The effective concentration at which DPPH radicals were scavenged by 50%) at the concentration of 29.13 μg/mL. However, scavenging activity of α-tocopherol and BHT, known antioxidants, used as positive controls, were relatively more pronounced than that of MDI.

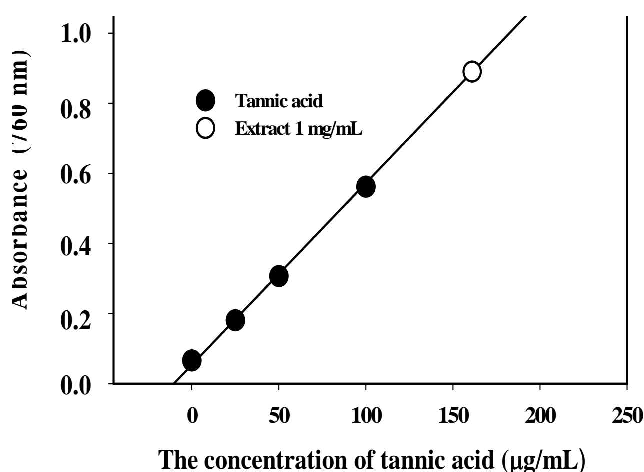

Fig. 1. Total phenolic content of methanolic extract from *D. indica*. Tannic acid was used as a standard for measuring the total phenolic content. Each value is expressed as the mean ± SD ($n=3$).

Table 1. DPPH free radical scavenging activity of methanolic extract from *D. indica*.

Samples		DPPH radical activity (IC_{50}: μg/mL)
MDI		29.13 ± 0.45^c
Positive control	α-Tocopherol	8.34 ± 1.23^b
	BHT	5.07 ± 0.47^a

α-Tocopherol and BHT were used as positive control.
[a-c]Values in the same column are significantly different by Tukey's multiple range test ($p<0.05$).

Reducing power assay

The reducing power of a compound is associated with antioxidant activity and provides a strong indication of its antioxidant capability. Some researchers have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts (26,27). For the measurement of the reductive activity, we investigated the Fe^{3+}-Fe^{2+} transformation in presence of MDI. As shown in Fig. 2, reducing power of MDI, α-tocopherol and BHT increased with increasing amount, which revealed EC_{50} (the effective concentration at which the absorbance was 0.5) of 0.23 mg/mL, 0.44 mg/mL and 0.21 mg/mL, respectively. Results showed that the reducing power of MDI was relatively more pronounced than that of α-tocopherol, but a bit weaker than that of BHT.

Metal-chelating activity

It is reported that transition metals are involved in both

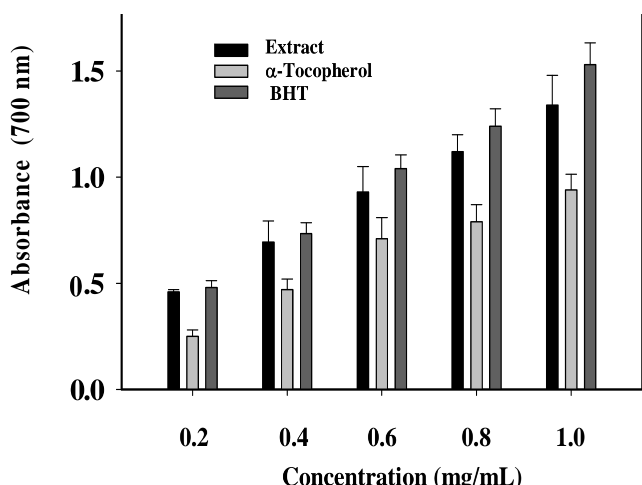

Fig. 2. Reducing power ability of methanolic extract from *D. indica*. α-Tocopherol and BHT were used as positive controls. Each value is expressed as the mean ± SD ($n = 3$).

initiation and propagation of oxygen free radicals in the organisms. Transition elements, such as iron and copper, are powerful catalysts of oxidation reactions because they contain one or more unpaired electrons that can participate in electron transfer reactions (22). In this assay, both MDI and EDTA interfered with the formation of ferrous and ferrozine complex, revealing that they have chelating activity and can capture ferrous ion before ferrozine. The Fe^{2+} chelating ability of MDI at various concentrations is shown in Fig. 3. The ferrous ion chelating effect of MDI was increased at the varying concentration, exhibiting a highest metal chelating activity of 75.57%, which was lower than EDTA at the concentration of 100 μg/mL.

Superoxide radical scavenging assay

Superoxide radical is very harmful to cellular components as a precursor of ROS, such as single oxygen and hydroxyl radicals. Scavenging of superoxide radicals is one of the most important ways of clarifying the mechanism of antioxidant activity (28). Hence, a NBT assay was carried out to test studying the scavenging effects of MDI on superoxide radicals. As shown in Fig. 4, MDI scavenged 46.21% superoxide radical at the concentration of 1 mg/mL. Gallic acid was used as the positive control showed 55.39% superoxide scavenging activity at the concentration of 125 μg/mL. These results indicated that MDI had a notable effect on scavenging of superoxide radicals.

DNA damage protection assay

Oxidative DNA damage has been implicated in various degenerative diseases (29). DNA damage by ROS can initiate carcinogenesis or beget the pathogenesis of neurodegenerative diseases such as Parkinson's and Alzheimer's disease. Among the types of ROS, hydroxyl radical is regarded as a DNA damaging agent with physiological significance (30). The antioxidant effect of MDI was evaluated based on the ability to protect against hydroxyl radical-induced DNA damage in the plasmid pBR 322. The plasmid exists in three forms: supercoiled, open circular, and linear. When the plasmid was subjected to the Fenton reaction for 30 min, the intact supercoiled DNA was broken into an open circular form compared to the untreated plasmid. As shown in Fig. 5, MDI dose-dependently protected supercoiled DNA by 9.23%, 71.4%, and 92.3% at the concentrations of 100, 500, and 1000 μg/mL, respectively.

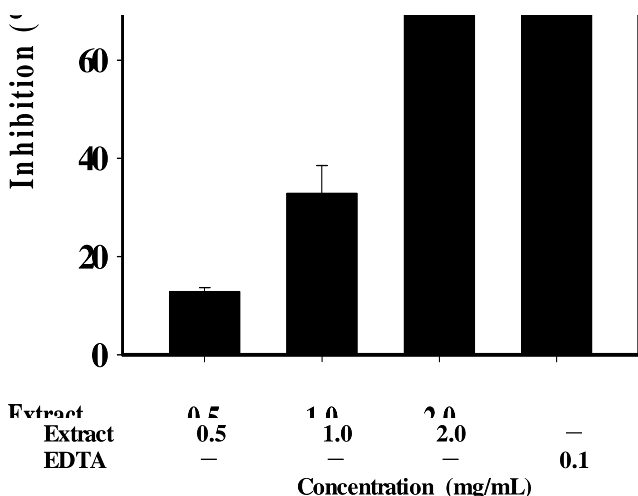

Fig. 3. Metal chelating activity of methanolic extract from *D. indica*. EDTA was used as a positive control. Each value is expressed as the mean ± SD ($n = 3$). $p < 0.05$, when compared to the control.

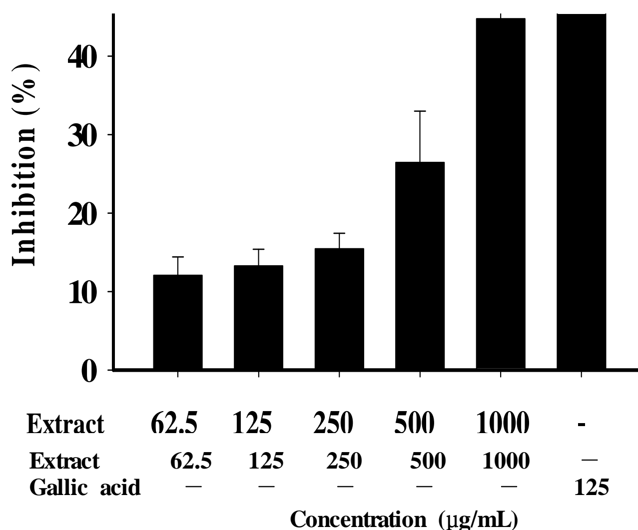

Fig. 4. Superoxide radical scavenging activity of methanolic extract from *D. indica*. Gallic acid was used as a positive control. Each value is expressed as the mean ± SD ($n = 3$). $p < 0.05$, when compared to the control.

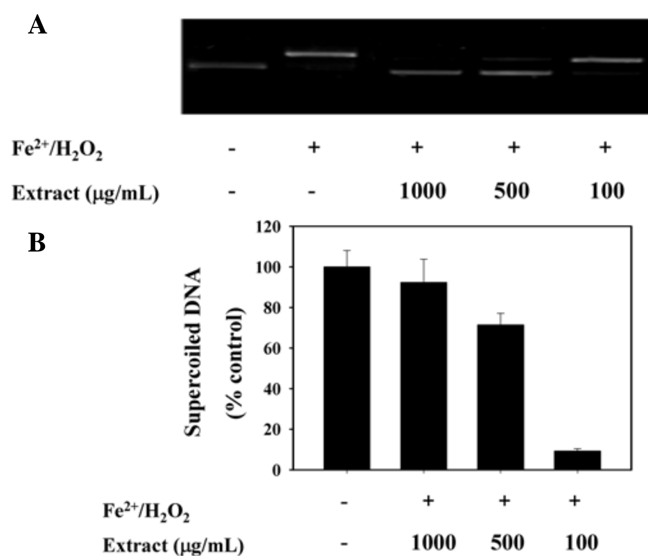

Fig. 5. (A) Visualization of the damage induced by hydroxyl radicals on plasmid DNA in the presence of methanolic extract from *D. indica* by agarose gel electrophoresis. (B) Histogram showing the protective effect of different concentration of extract against DNA damage based on densitometric measurements. Line 1, DNA incubated without Fenton's reagent; Line 2, DNA incubated with Fenton's reagent; Lines 3~5, DNA incubated with Fenton's reagent in the presence of 1000 μg/mL, 500 μg/mL, and 100 μg/mL of MDI, respectively.

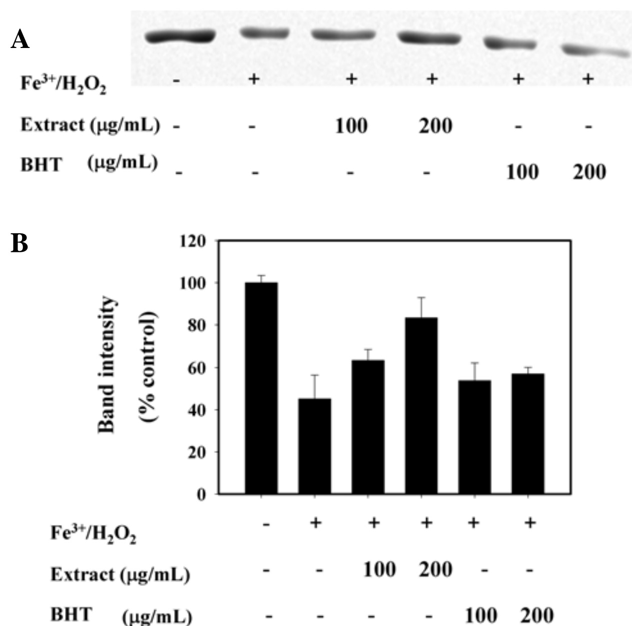

Fig. 6. (A) Protection of BSA oxidative damage by methanolic extract from *D. indica* and BHT. (B) The densitometric analysis of corresponding band intensity.

Protein damage protection assay

Because of their abundance in biological systems, proteins are a major target for oxidants. The protein damages induced by free radicals have been demonstrated to play a significant role in aging and other pathological events (31). Radical-mediated damages to proteins might be initiated by electron leakage, metal-ion dependent reactions, and autoxidation of lipids and sugars (32). Electrophoretic patterns of BSA after incubation with Fe^{3+}/H_2O_2/ascorbic acid system in the presence of the MDI and BHT was shown in Fig. 6. The density of BSA band decreased to 45.2% of control after incubated with Fe^{3+}/H_2O_2/ascorbic acid. A 200 μg/mL of MDI and BHT restored BSA band intensity to 83.36% and 57% of the control levels, respectively. These results suggest MDI is more potent than BHA in Fe^{3+}/H_2O_2/ascorbic acid induced protein damage.

The methanolic extract of *D. indica* exhibited different level of antioxidant activity in all the systems studied. The above findings showed that *D. indica* possesses significant free radical scavenging activity. Further investigation into *D. indica* for its lead active compounds and *in vivo* antioxidant mechanisms is warranted.

REFERENCES

1. Bandyopadhyay U, Das D, Banerjee RK. 1999. Reactive oxygen species: oxidative damage and pathogenesis. *Cur Sci* 77: 658-666.
2. Clarkson PM, Thompson HS. 2000. Antioxidants: what role do they play in physical activity and health. *Am J Clin Nutr* 72: 637-646.
3. Wang G, Alamuri P, Maier RJ. 2006. The diverse antioxidant systems of *Helicobacter pylori*. *Mol Microbiol* 61: 847-860.
4. Shukla S, Mehta A, John J, Singh S, Mehta P, Vyas SP. 2009. Antioxidant activity and total phenolic content of ethanolic extract of *Caesalpinia bonducella* seeds. *Food Chem Toxicol* 47: 1848-1851.
5. Valentao P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. 2002. Antioxidative properties of cardoon (*Cynara cardunculus* L.) infusion against superoxide radical, hydroxyl radical and hypochlorous acid. *J Agric Food Chem* 50: 4989-4993.
6. Cai Y, Luo Q, Sun M, Corke H. 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci* 74: 2157-2184.
7. Samarth RM, Panwar M, Kumar M, Soni A, Kumar M, Kumar A. 2008. Evaluation of antioxidant and radical-scavenging activities of certain radioprotective plant extracts. *Food Chem* 106: 868-873.
8. Lee IR, Kim YH. 1986. Studies on the antitumor activity of *Duchesnea Indicae* herba. *Arch Pharm Res* 9: 1-4.
9. Kim IG, Jung IL, Oh TJ, Kim KC, Shim HW. 2002. Polysaccharide-enriched fraction isolated from *Duchesnea chrysantha* protects against oxidative damage. *Biotech Lett* 24: 1299-1305.
10. Kim KC, Kim JS, Son JK, Kim IG. 2007. Enhanced induction of mitochondrial damage and apoptosis in human leukemia HL-60 cells by the *Ganoderma lucidum* and *Duchesnea chrysantha* extracts. *Cancer Lett* 246: 210-217.
11. Lee IR, Yang MY. 1994. Phenolic compounds from *Duchesnea chrysantha* and their cytotoxic activities in hu-

man cancer cell. *Arch Pharm Res* 17: 476-479.
12. Qiao W, Bao Z, Zhang W, Duan HQ. 2009. Two new triterpens from *Duchesnea indica*. *Chin Chem Lett* 20: 572-575.
13. Peng JN, Lu YR, Chen DH. 1995. Studies on the chemical constituents of Indian mock strawberry. *Chin Tradit Herb Drugs* 26: 338-341.
14. Ghasemi K, Ghasemi Y, Ebrahimzadeh MA. 2009. Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tisssues. *Pak J Pharm Sci* 22: 277-281.
15. Hu W, Heo SI, Wang MH. 2009. Antioxidant and anti-inflammatory activity of *Kalopanax pictus* leaf. *J Korean Soc Appl Biol Chem* 52: 360-366.
16. Que F, Mao L, Zhu C, Xie G. 2006. Antioxidant properties of Chinese yellow wine, its concentrate and volatiles. *LWT-Food Sci Technol* 39: 111-117.
17. Singh N, Rajini PS. 2004. Free radical scavenging activity of an aqueous extract of potato peel. *Food Chem* 85: 611-616.
18. Qian ZJ, Jung WK, Byun HG, Kim SK. 2008. Protective effect of an antioxidative peptide purified from gastrointestinal digests of oyster, *Crassostrea gigas* against free radical induced DNA damage. *Bioresour Technol* 99: 3365-3371.
19. Kızıl G, Kızıl M, Ceken B. 2009. Protective ability of ethanol extracts of *Hypericum scabroides* Robson & Poulter and *Hypericum tiquetrifolium* Turra against protein oxidation and DNA damage. *Food Sci Biotechnol* 18: 130-136.
20. Jung CH, Seong HM, Choi IW, Park MW, Cho HY. 2006. Antioxidant properties of various solvent extracts from wild ginseng leaves. *LWT-Food Sci Technol* 39: 266-274.
21. Shoemaker M, Hamilton B, Dairkee SH, Cohen I, Campbell MJ. 2005. *In vitro* anticancer activity of twelve Chinese medicinal herbs. *Phytother Res* 19: 649-651.
22. Wang J, Yuan X, Jin Z, Tian Y, Song H. 2007. Free radical and reactive oxygen species scavenging activities of peanut skins extract. *Food Chem* 104: 242-250.
23. Ferrari CKB, Torres EAFS. 2003. Biochemical pharmacology of functional foods and prevention of chronic diseases of aging. *Biomed Pharmacother* 57: 251-260.
24. Siriwardhana N, Lee KW, Jeon YJ, Kim SH, Haw JW. 2003. Antioxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. *Food Sci Technol Int* 9: 339-346.
25. Milardović S, Iveković D, Grabarić BS. 2006. A novel amperometric method for antioxidant activity determination using DPPH free radical. *Bioelectrochemistry* 68: 175-180.
26. Pin-Der-Duh X. 1998. Antioxidant activity of burdock (*Arctium lappa* Linne): its scavenging effect on free-radical and active oxygen. *J Am Oil Chem Soc* 75: 455-461.
27. Tanaka M, Kuie CW, Nagashima Y, Taguchi T. 1988. Applications of antioxidative Maillard reaction products from histidine and glucose to sadine products. *Nippon Suisan Gakkaishi* 54: 1409-1414.
28. Je JY, Kim SK. 2006. Reactive oxygen species scavenging activity of aminoderivatized chitosan with different degree of deacetylation. *Bioorg Med Chem* 14: 5989-5994.
29. Loft S, Fischer-Nielsen A, Jeding IB, Vistisen K, Poulsen HE. 1993. 8-Hydroxydeoxyguanosine as a urinary biomarker of oxidative DNA damage. *J Toxicol Environ Health* 40: 391-404.
30. Je JY, Ahn CB, Oh MJ, Kang SY. 2009. Antioxidant activity of a red seaweed *Polysiphonia morrowii* extract. *Food Sci Biotechnol* 18: 124-129.
31. Du J, Gebicki JM. 2004. Proteins are major initial cell targets of hydroxyl free radicals. *Int J Biochem Cell Biol* 36: 2334-2343.
32. Bahramikia S, Ardestani A, Yazdanparast R. 2009. Protective effects of four Iranian medicinal plants against free radical-mediated protein oxidation. *Food Chem* 115: 37-42.

(Received September 10, 2009; Accepted November 12, 2009)