Food Sci. Biotechnol. Vol. 15, No. 2, pp. 312 ~ 316 (2006)

Food Science and Biotechnology

© The Korean Society of Food Science and Technology

Antioxidant Activity of Extracts from *Dangyuja* (*Citrus grandis* Osbeck) Fruits Produced in Jeju Island

Hee-Kyoung Lim, Eun-Sook Yoo, Jeong-Yong Moon[1], You-Jin Jeon[2], and Somi K. Cho[1,3]*

Department of Medicine, Cheju National University, Jeju, Jeju 690-756, Korea

[1]Faculty of Biotechnology, College of Applied Life Sciences, Cheju National University, Jeju, Jeju 690-756, Korea

[2]Faculty of Applied Marine Science, Cheju National University, Jeju, Jeju 690-756, Korea

[3]The Research Institute for Subtropical Agriculture and Biotechnology, College of Applied Life Sciences, Cheju National University, Jeju, Jeju 690-756, Korea

Abstract Crude extract of young *dangyuja* (*Citrus grandis* Osbeck) fruit was investigated for its antioxidant activity as measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity. Among the samples, including 4 *Citrus* species and various solvent-extracted-fractions of young *dangyuja* fruit, the water-extracted fraction (WF) and butanol-extracted fraction (BF) showed the greatest DPPH free radical scavenging activity. WF and BF were further examined for their antioxidant activities by three different *in vitro* assays. The cell viability tests using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay showed that both fractions significantly reduced H_2O_2–induced cytotoxicity in HepG2 cells dose-dependently. Generation of the reactive oxygen species (ROS) was also reduced in cells pretreated with both fractions. In addition, BF showed a higher level of lipid peroxidation inhibitory capacity than WF in H_2O_2-treated HepG2 cells. Taken together, these results indicate that young *dangyuja* fruit can be used as an easily accessible source of natural antioxidants.

Keywords: *Citrus grandis* Osbeck, antioxidant activity, radical scavenging, reactive oxygen species, HepG2 cells

Introduction

Oxidative stress is widely recognized as one of the causes for the development of chronic disease including alcohol-mediated organ damage, various forms of neuro-degenerative diseases, many types of cancers, cardiovascular diseases, lung diseases, and UV-mediated skin diseases (1-3). It is mediated by reactive oxygen species (ROS) that are generated as byproducts of normal and irregular metabolic processes that utilize molecular oxygen. ROS are known to attack cellular biomolecules and are responsible for increased oxidation of proteins, DNA, polyunsaturated fatty acids, and lipids. This oxidative stress-induced damage disrupts cellular function and membrane integrity, thereby leading to cell death through apoptosis (4, 5). Being a major component of ROS, H_2O_2 has been extensively used as an inducer of oxidative stress (6).

Antioxidant bioactive compounds have been shown to play a major role in blocking oxidative stress induced by free radicals (7-11). They have been reported to reduce the incidence of chronic degenerative disease including heart diseases and cancer (12-14). The genus *Citrus*, with several species cultivated worldwide, produces a large amount of antioxidant bioactive compounds. Many researchers have found antioxidants in juice and edible parts of oranges of different origin and from different varieties (15-17). As far as the peel is concerned, extracts from this part of the fruit were found to have a good total radical antioxidative potential (16). *Dangyuja* (*Citrus grandis* Osbeck) is one of native *Citrus* species and the fruits have been utilized in Jeju Island for a long time as a folk remedy for hangover and protection against liver damage. However, previous studies have focused mainly on the changes of limonoid contents in different tissues during fruit growth and on the evaluation of antioxidant activity of the mature *dangyuja* fruit tissues (flavedo, albedo, and segment membrane) by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity and β-carotene bleach assay (18, 19). Few studies have been conducted to evaluate the antioxidant activities of young *dangyuja* whole fruit extract in comparison with those of mature *dangyuja* whole fruit extract and other *Citrus* cultivars extracts.

In the present work, the DPPH radical scavenging activity and the antioxidant activity of young *dangyuja* whole fruit extract were evaluated by a well differentiated, transformed cell line: the human hepatoma HepG2 line. The samples tested for DPPH free radical scavenging activity included *Citrus unshiu* Marc., [(*C. unshiu* Marc. × *C. sinensis* Osb.) × *C. reticulata* Bla.], *C. sinensis*, mature *dangyuja*, and young *dangyuja*. Young *dangyuja* whole fruit extracts and residues were prepared for examination using successive solvents of varying polarity and by partitioning the methanol fraction with hexane, chloroform, ethyl acetate, and *n*-butanol. Furthermore, the effect of water- and butanol-extracted fractions (WF and BF, respectively) of young *dangyuja* fruit on antioxidant defense at the cellular level was investigated by a human hepatoma HepG2 cell line, for the first time.

We report here that WF of young fruit extracts from *dangyuja* protects the HepG2 cells from oxidative stress and acts as a direct scavenging agent of ROS.

*Corresponding author: Tel: 82-64-754-3348; Fax: 82-64-756-3351
E-mail: somikim@cheju.ac.kr
Received February 7, 2006; accepted March 17, 2006

Materials and Methods

Materials *Dangyuja* fruits were obtained from the National Institute of Subtropical Agriculture Jeju Province, Korea. Young fruits were collected on June 28, 2005 from *dangyuja* trees about 30 days after flowering and the mature fruits were collected in January 2005. *C. unshiu* Marc., [(*C. unshiu* Marc. × *C. sinensis* Osb.) × *C. reticulata* Bla.], and *C. sinensis* fruits were purchased from a market in Jeju city. All the chemicals and reagents were purchased from Sigma Chemicals (St. Louis, MO, USA) and Invitrogen Gibco (Grand Island, NY, USA).

Preparation of fruit extracts Air-dried *Citrus* whole fruits were pulverized using a milling machine and extracted with 80% methanol by stirring for 3 days at room temperature (RT). The extract was filtered, concentrated with a vacuum rotary evaporator under reduced pressure, and lyophilized. The resulting methanol extract (ME) was suspended in water (1 L) and further fractionated by additional extraction with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol in a stepwise manner. Each solvent was extracted three times at RT and solvent fractions from each step were removed by rotary evaporation and freeze-dried (Fig. 1). The extract powder(s) was dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS, pH 7.4) to give the final concentrations.

Determination of antioxidant activity using DPPH The free radical scavenging activity of the fruit extracts was measured by the DPPH radical assay described by Blois (21). Total extracts of citrus fruit at a final concentration of 0.5 mg/mL and solvent fractions obtained from young *dangyuja* fruit extracts at final concentrations of 1, 0.5, 0.25, and 0.125 mg/mL were tested individually by addition to 150 μM solution of DPPH in ethanol. The mixtures were vigorously mixed and allowed to stand in the dark at RT for 30 min. The absorbance of the remaining DPPH was measured at 520 nm using a spectrophotometer. These experiments were run in triplicate. The radical scavenging activity was calculated according to following equation:

$$\text{Radical scavenging activity (\%)} = [(A_o - A)/A_o] \times 100,$$

where, A_o is the A_{520} of DPPH without sample (control), A is the A_{520} of DPPH with sample.

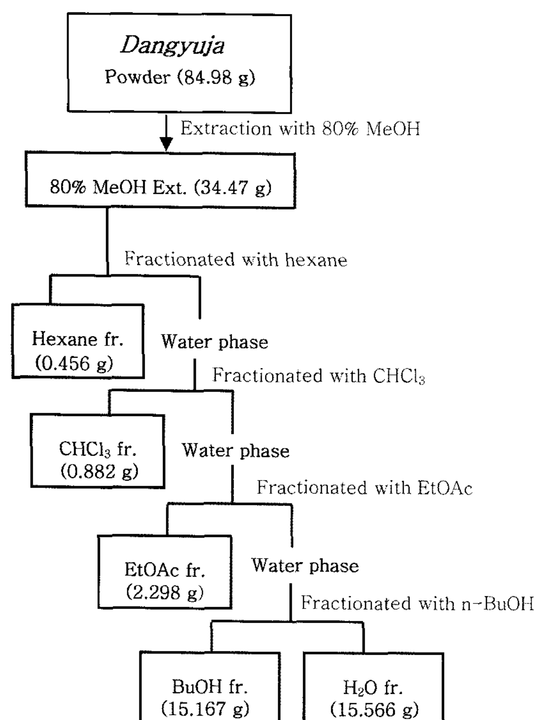

Fig. 1. Flow diagram of fractionation of *dangyuja* extract.

Cell culture The human hepatoma HepG2 (KCLB No. 58065) cells were purchased from the Korea Cell Line Bank (Seoul, Korea). They were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10%(v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Cells were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere. The culture medium was changed twice a week, and the cells were subcultured at a 1: 4 ratio once a week.

Determination of cell viability The cell viability was estimated by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, which is based on cleavage of a tetrazolium salt by mitochondria dehydrogenases in viable cells (22). HepG2 cells were cultured at a density of 1×10^5 cells/mL on 96-well microplates for 16 hr, washed twice using PBS, and pretreated with different concentrations of *dangyuja* fruit extracts. After 30 min incubation, H_2O_2 solution was added to the wells, and the cells were re-incubated for 24 hr. MTT reagent (5 mg/mL) was added to each well, and the plate was incubated at 37°C for an additional 4 hr. The media were then removed, and the intracellular formazan product was dissolved in DMSO. The absorbance of each well was then measured at 570 nm, and the cell viability was expressed as a percentage of counts relative to the untreated control cells.

Determination of reactive oxygen species Cellular ROS were quantified by dichlorofluorescein (DCFH) assay (23), in which the esterified from of 2',7'-dichorofluoresceindiacetate (DCFH-DA) diffuses through the cell membrane and is enzymatically deacetylated by intracellular esterase. The resulting compound, dichlorofluorescein (DCFH), is reactive with ROS to give an oxidized fluorescent compound, dichlorofluorescein (DCF). HepG2 cultures were pretreated with either WF or BF of young *dangyuja* fruit extracts for 30 min, then the cultures were washed twice and incubated with 1 mM H_2O_2 for 10, 30, 60, and 90 min. DCFH-diacetate was added to the culture plates at a final concentration of 25 μM and DCF fluorescence was detected over a period of 90 min at 37°C at an excitation wavelength of 485 nm and an mission wavelength of 530 nm using Genios multiwell, fluorescence plate reader (Genios, Tecan, Salzburg, Austria).

Lipid peroxidation inhibitory capacity Lipid peroxidation was assayed by measuring malondialdehyde (MDA) according to the method of Ohkawa *et al.* (24). Lipid peroxides react with thiobarbituric acid (TBA) and develop a pink color due to the formation of TBARS (TBA reactive substances). In this method cells were exposed to WF or BF of young *dangyuja* fruit extract at various concentrations in the incubation medium for 60 min, followed by 1 mM H_2O_2 for 60 min. Oxidation of phospholipids and evaluation of TBARS formation was achieved in a single 96-well microplate and the amount of MDA formed as a breakdown product was measured spectrophotometrically at 530 nm.

Statistical analysis Experimental data were analyzed by Student's *t*-test and expressed as mean ±SD. A probability value of $p<0.05$ was considered statistically significant.

Results and Discussion

Antioxidant activity The use of DPPH free radicals is a common method to evaluate antioxidant activities in a relatively short time compared with other methods. The reduction capability of DPPH radicals was determined by the antioxidant-induced decrease in their absorbance at 520 nm. The DPPH free radical scavenging potentials of the 5 citrus samples at concentration of 0.5 mg/mL are present in Fig. 2. The DPPH radical scavenging activities of ME samples were found to be in order of young *dangyuja* (*C. grandis* Osbeck) > *C. unshiu* Marc. > Navel Orange (*C. sinensis*) > Hallabong [(*C. unshiu* Marc. × *C. sinensis* Osb.) × *C. reticulata* Bla.] > mature *dangyuja* (*C. grandis* Osbeck) (Fig. 2A). Since the crude extracts of young *dangyuja* fruit exhibited significant antioxidant activity, various solvents extracts of young *dangyuja* fruit were then prepared and the antioxidant activities of these fractions were determined. The various solvent fractions of young *dangyuja* fruit at a concentration of 0.5 mg/mL showed the DPPH radical scavenging activity in the decreasing order of WF (70%) > BF (41%) > ethyl acetate fraction (34%) > chloroform fraction (14%) > hexane fraction (8%) (Fig. 2B). The results strongly showed that the radical scavenging activity of WF approached the activity of the standard, α-tocopherol, and exhibited a higher antioxidant activity than the standard, butylated hydroxytoluene, did, at a concentration of 0.5 mg/mL. In the previous study, the antioxidant activities of ethyl acetate extracts of *C.* Osbeck crude extracts exhibited strong antioxidant activities in flavedo and albedo with slightly less antioxidant activities than the standards, α-tocopherol, and butylated hydroxyanisole, as measured by DPPH scavenging activity (19). However, the DPPH radical scavenging activities of hexane and chloroform fractions were not significantly different. These results suggest that the antioxidant activity of ME of young *dangyuja* fruit is mainly attributable to both WF and BF. The results in Fig. 3 show that at concentrations ranging from 0.125 to 0.5 mg/mL, these two fractions exhibited dose-dependent, free radical scavenging activity. This concentration range of 0.125 to 0.5 mg/mL was consequently used for all subsequent experiments.

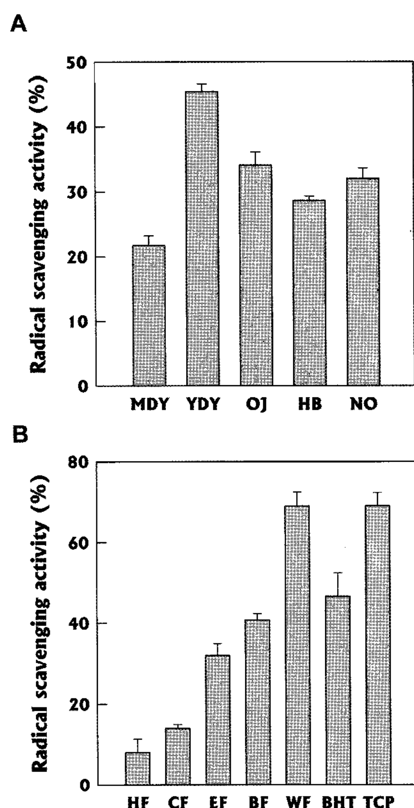

Fig. 2. The effects of total extracts from *Citrus* fruits (A) and various solvent-extracted fractions from YDY (B) on scavenging DPPH free radicals. All the extracts, butylated hydroxytoluene, and α-tocopherol were added at 0.5 mg/mL. Values are means ± SD (n =3). (A) MDY mature *C. grandis* Osbeck; YDY young *C. grandis* Osbeck; OJ *C.* unshiu Marc., HB;[(*C. unshiu* Marc. × *C. sinensis* Osb.) × *C. reticulata* Bla.], and NO, *C.* sinensis. (B) HF, hexane; CF, chloroform-; EF, ethyl acetate-; BF: butanol-; WF, water extracted fractions; BHT, butylated hydroxytoluene; and TCP, α-tocopherol.

Effect of water and butanol fractions on H_2O_2-induced cell growth suppression The role of young fruit extract in the protection of the HepG2 cells from H_2O_2-induced oxidative stress was evaluated by MTT assay. The HepG2 cell line is a reliable model, well characterized, and widely

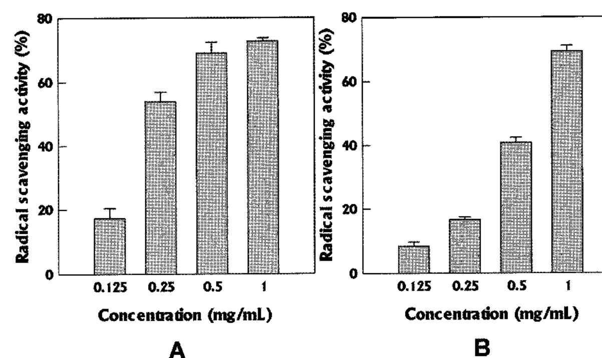

Fig. 3. Antioxidant activity of water fraction (A) and butanol fraction (B) at various concentrations as measured by free radical scavenging effects. Values are means ± SD (n=3).

used for biochemical and nutritional studies where many antioxidants and conditions can be assayed (20). When human hepatoma HepG2 cells were pretreated with either WF or BF for 30 min prior to being submitted to an oxidative, the cell toxicity was greatly inhibited in a dose-dependent manner (Fig. 4). The cells exposed 1 mM H_2O_2 for 24 hr without pretreatment of fruit extracts exhibited about 49% of cell viability, whereas cell viability was restored back to 80% by pretreatment of 0.5 mg/mL of WF, indicating that WF-treated cells were protected against the oxidative insult. A similar protective effect was observed in a dose-dependent manner when the HepG2 cells were pretreated with BF. At a dose of 0.5 mg/mL, BF increased cell viability by 64% compared with the control.

Effect of water and butanol fraction on reactive oxygen species (ROS) generation in HepG2 cells To investigate whether or not the difference in viability of the HepG2 cells upon exposure to H_2O_2 was associated with ROS formation, the level of intracellular ROS was measured by quantification of DCF fluorescence (23). Cells were treated for 30 min with either WF or BF and then the fraction was removed from the culture medium and 1 mM H_2O_2 was added for the 90 min of the ROS assay.

A significant increase in ROS production was observed over time in the presence of 1 mM H_2O_2 as compared to the unstressed control (Fig. 5). Pretreatment of HepG2 cells with either 0.5 mg/mL or 0.25 mg/mL WF exhibited very similar DCF fluorescence, implying the effect of WF on ROS generation is dose-dependent only at lower concentrations under 0.5 mg/mL (Fig. 5A). However, the DCF fluorescence of cells pretreated with BF evoked a greater decrease in ROS generation than WF in a dose-dependent manner. At a concentration of 0.5 mg/mL, DCF fluorescence dropped significantly from 10,343 to 4,578 units after 90 min reaction in cells to which BF of young *dangyuja* was added (Fig. 5B).

Effect of water and butanol fraction on lipid peroxidation inhibitory activity in HepG2 Lipid peroxidation is a free radical-mediated propagation of oxidative insult to polyunsaturated fatty acids, involving several types of free radicals, and termination occurs through enzymatic means or free radical scavenging activities of antioxidants (25). To evaluate the antioxidant activity of *dangyuja*, the effects of WF and BF of young *dangyuja* on lipid peroxidation in H_2O_2-treated HepG2 cells were also assessed. Pretreatment of HepG2 cultures with WF for 60 min increased the lipid peroxidation inhibitory activity, although its effect was a lot less than that on DPPH radical scavenging activity (Fig. 6). The inhibition of lipid peroxidation was much more pronounced when cells were pretreated with BF. Lipid peroxidation inhibitory activity increased by 30, 60, 75, and 83% with pretreatment of BF at a concentration of 62.5, 125, 250, and 500 μg/mL, respectively. The results suggest that BF of young *dangyuja* fruit is capable of amelioration of hepatocyte lipid peroxidation caused by H_2O_2.

In conclusion, the results of our study show that both WF and BF of young *dangyuja* fruit have strong anti-

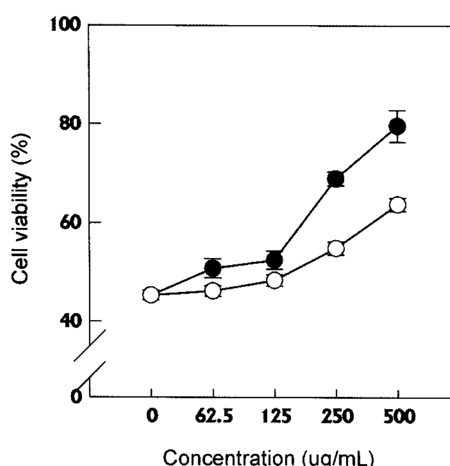

Fig. 4. Effect of young *dangyuja* extracts on H_2O_2-induced cytotoxicity in HepG2 cells. Values are mean ± SD (n=4). ● -water fractions, ○ -butanol fractions.

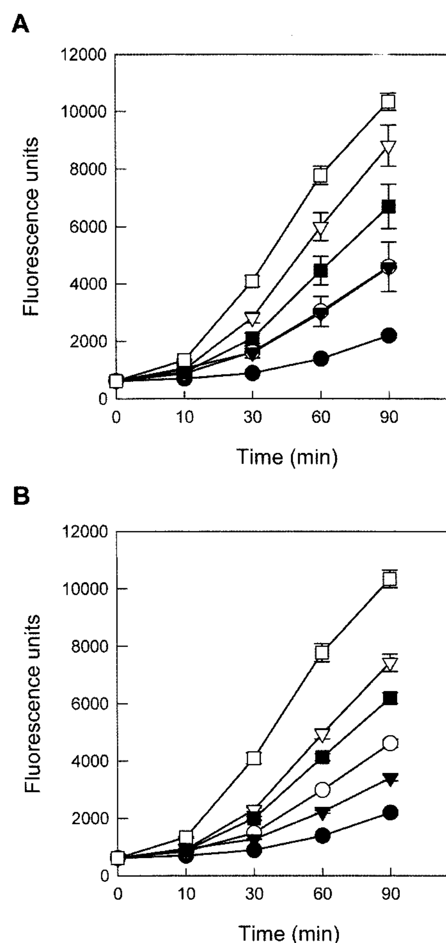

Fig. 5. Effect of young *dangyuja* fruit extracts on H_2O_2-induced intracellular reactive oxygen species (ROS) generation in HepG2 cells. (A) and butanol fractions (B) and then incubated with 1 mM H_2O_2 for the indicated period of time. Values are presented as means ± SD (n=4). □ , 1 mM H_2O_2 only; ▽ , 62.5 μg/mL; ■ , 125 μg/mL; ○ , 250 μg/mL, ▼ , 500 μg/mL; ● , unstressed control.

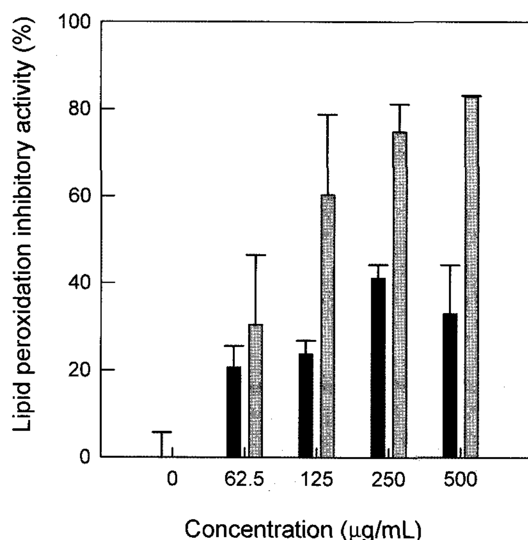

Fig. 6. Lipid peroxidation inhibitory activity of young *dangyuja* fruit extracts against H_2O_2-induced oxidative damage in HepG2 cells. Black (■), concentrations of water fractions; gray (▒), concentrations of butanol fractions.

oxidant activity as measured by DPPH radical scavenging activity, cell viability, ROS generation, and inhibition of lipid peroxidation. Moreover, among the five fractions (hexane-, chloroform-, ethyl acetate-, butanol-, and water-extracted fractions) of young *dangyuja* fruit, WF showed the highest DPPH radical scavenging activity, whereas BF showed a greater effect on ROS generation and higher lipid inhibitory peroxidation than WF. Therefore, the antioxidant activity found in the complete extracts of young *dangyuja* fruit is attributable to both BF and WF. These two fractions have promising potential as oxidative preventatives in fruit juices and food products, and also as health supplements. Full identification of the responsible components is currently underway.

Acknowledgments

This study was supported by Technology Development Program of the Ministry of Agriculture and Forestry, Repulic of Korea.

References

1. Kehrer JP. Free radicals as mediators of tissue injury and disease. Crit. Rev. Toxicol. 23: 21-48 (1993)
2. Willcox JK, Ash SL, Catignani GL. Antioxidants and prevention of chronic disease. Crit. Rev. Food Sci. Nutr. 44: 275-295 (2004)
3. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. P. Natl. Acad. Sci. USA 90: 7915-7922 (1993)
4. Fiers W, Beyaert R, Declercq W, Vandenabeele P. More than one way to die: apoptosis and necrosis and reactive oxygen damage. Oncogene 18: 7719-7730 (1999)
5. Deng G, Su JH, Ivins KJ, Houten BV, Cotman CW. Bcl-2 facilitates recovery from DNA damage after oxidative stress. Exp. Neurol. 159: 309-318 (1999)
6. Satoh I, Sakai N, Enokido Y, Uchiyama Y, Hatanaka H. Free radical independent protection by nerve growth factor and Bcl-2 of PC12 cells from hydrogen peroxide triggered-apoptosis. J. Biochem. 120: 540-546 (1996)
7. Oliva MR, Iradi A, Garrido F, Ramos M, Oltra AM, Muniz P, Saez GT. Oxidative stress induces the expression of the major histocompatibility complex in murine tumor cells. Free Radical Res. 35: 119-128 (2001)
8. Ng TB, Liu F, Wang ZT. Antioxidative activity of natural products from plants. Life Sci. 66: 709-723 (2000)
9. Park YK, Lee WY, Park SY, Ahn JK, Han MS. Antioxidant activity and total phenolic content of *Callistemon citrinus* extracts. Food Sci. Biotechnol. 14: 212-215 (2005)
10. Choi YM, Ku JB, Chang HB, Lee JS. Antioxidant activities and total phenolics of ethanol extracts from several edible mushrooms produced in Korea. Food Sci. Biotechnol. 14: 700-703 (2005)
11. Heo SJ, Park PJ, Park EJ, Cho SM, Kim SK, Jeon, YJ. Antioxidative effect of proteolytic hydrolysates from *Ecklonia cava* on radical scavenging using ESR and H_2O_2-induced DNA damage. Food Sci. Biotechnol. 14: 614-620 (2005)
12. Ross JA, Kasum CM. Dietary flavonoids; bioavailability, metabolic effects, and safety. Annu. Rev. Nutr. 22: 19-34 (2002)
13. Huang HY, Alberg AJ, Norkus EP, Hoffman SC, Comstock GW, Helzlsouer KJ. Prospective study of antioxidant micronutrients in the blood and the risk of developing prostate cancer. Am. J. Epidemiol. 157: 335-344 (2003)
14. Knekt P, Jarvinen R, Seppanen R, Hellovaara M, Teppo L, Pukkala E, Aromaa A. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am. J. Epidemiol. 146: 223-230 (1997)
15. Miller NJ, Rice-Evans CA. The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. Food Chem. 60: 331-337 (1997)
16. Rapisarda P, Tomaino A, Lo Cascio R, Bonina F, De Pasquale A, Saija A. Antioxidant effectiveness as influenced by phenolic content of fresh orange juices. J. Agric. Food Chem. 47: 4718-4723 (1999)
17. Roberts, QG, Gordon, MH. Determination of the total antioxidant activity of fruits and vegetables by a liposome assay. J. Agric. Food Chem. 51: 1486-1493. (2003)
18. Mokbel MS, Hashinaga F. Evaluation of the antioxidant activity of extracts from butan (*Citrus grandis* Osbeck) fruit tissues. Food Chem. 94: 529-534 (2006)
19. Sun, CD, Chen, KS, Chen, Y, Chen. QJ. Contents and antioxidant capacity of limonin and nomilin in different tissues of citrus fruit of four cultivars during fruit growth and maturation. Food Chem. 93: 599-605 (2005)
20. Alia M, Ramos S, Mateos R, Bravo L, Goya L. Response of the antioxidant defense system to t-Butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). J. Biochem. Mol. Toxic. 19: 119-128 (2005)
21. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 181: 1199-1200 (1958)
22. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Method 119: 203-210 (1989)
23. Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radical. Bio. Med. 27: 612-616 (1999)
24. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95: 351-358 (1979)
25. Heim KE, Tagliaferro AR, Bobilya, DJ. Flavonoid antioxidants: chemistry and structure-activity relationships. J. Nutr. Biochem. 13: 572-584 (2002)