J. Korean Soc. Appl. Biol. Chem. 52(5), 539-544 (2009)

Article

Antioxidant Properties of Proanthocyanidin Fraction Isolated from Wild Grape (*Vitis amurensis*) Seed

Hye-Ryun Lee[1], Min-Ji Bak[2], Woo-Sik Jeong[2], Young-Chan Kim[3], and Shin-Kyo Chung[1]*

[1]*Department of Food Science and Technology, Kyungpook National University, Daegu 702-701, Republic of Korea*
[2]*Food Science Institute, School of Food & Life Sciences, College of Biomedical Science & Engineering, Inje University, Gimhae 621-749, Republic of Korea*
[3]*Korea Food Research Institute, Sungnam 463-746, Republic of Korea*

Received May 18, 2009; Accepted August 12, 2009

In order to investigate the utilization of wild grape by-products, isolation of proanthocyanidin from the wild grape seed (*Vitis amurensis*) and its antioxidant capacities were examined. 70% acetone crude extract of the wild grape seed was fractionated. The ethyl acetate fraction was applied to Sephadex LH-20 column chromatography, which was eluted with 50%, 75% methanol, and 75% acetone, respectively, and 9 fractions were collected, finally. Their proanthocyanidin characteristics and contents were investigated by vanillin-H_2SO_4 and BuOH-HCl methods. The Fr. 5 has the highest proanthocyanidin content (86.00±6.74 g%) among all the fractions. Fr. 6-2 was identified as (+)-catechin by LC-MS analysis. The antioxidant activities of the proanthocyanidin were evaluated by total phenolic contents, DPPH radical scavenging, and FRAP assays. Fr. 8 shows the strongest antioxidant activities with high proanthocyanidin content. The wild grape seed containing fairly high amounts of proanthocyanidin could be utilized as a natural antioxidant material.

Key words: *antioxidant activity, proanthocyanidin, Sephadex LH-20 column chromatography, wild grape* (Vitis amurensis) *seed*

Wild grape (*Vitis amurensis)* is distributed in the far-east Asia, and is currently cultivated on the south foothill in Korea. Because of its strong acidity and astringency, most wild grapes are processed into wine, and juice products. The wild grape pomace remained after processing, which consists of seeds and peels, and are mainly discarded, although it have been partly reutilized as the ingredients of animal feeds. There have been few reports on the biological and physiological functions of the wild grape except winemaking [Kim 1996, Kim and Kim, 1997]. The proanthocyanidin is a kind of condensed tannin which is contained in grape seeds and skins abundantly, and has attracted a lot of attention for health-benefit functional material, due to their several biological and physiological functions [Haslam, 1996; Yamakoshi *et al.*, 1999; Bagchi *et al.*, 2000; Hughes-Formella *et al.*, 2007], such as anti-inflammatory [Torras *et al.*, 2005], anti-asthmatic [Lau et al., 2004], anticancer [Ray *et al.*, 2005] function. Especially, plant polyphenols, including proanthocyanidin compounds, have been widely studied on their antioxidant functions for preventing various metabolic diseases induced by reactive oxygen radical species (ROS). Kim and Chung [2002] and Ahn [2009] had also reported the ROS scavenging and antioxidant activities of some Korean medicinal herbs. Therefore, it might also be meaningful and interesting to isolate and identify the proanthocyanidin in the wild grape pomace for the development of health-benefit function materials. From this point of view, the proanthocyanidin fractions from the Korean wild grape seed have been studied, as well as their antioxidant capacity.

*Corresponding author
Phone: +82-53-950-5778; Fax: +82-53-950-6772
E-mail: kchung@knu.ac.kr

Abbreviations: DPPH, α,α-Diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant power; HPLC, high performance liquid chromatography; MS, mass spectrometry; ODS, Octadecyl silica gel; PA, proanthocyanidin; TLC, thin layer chromatography; TPTZ, 2,4,6-tripyridyl-s-triazine

doi:10.3839/jksabc.2009.091

Materials and Methods

Plant material. Wild grape (*Vitis amurensis*) seeds were collected from the wild grape pomace from Doorae,

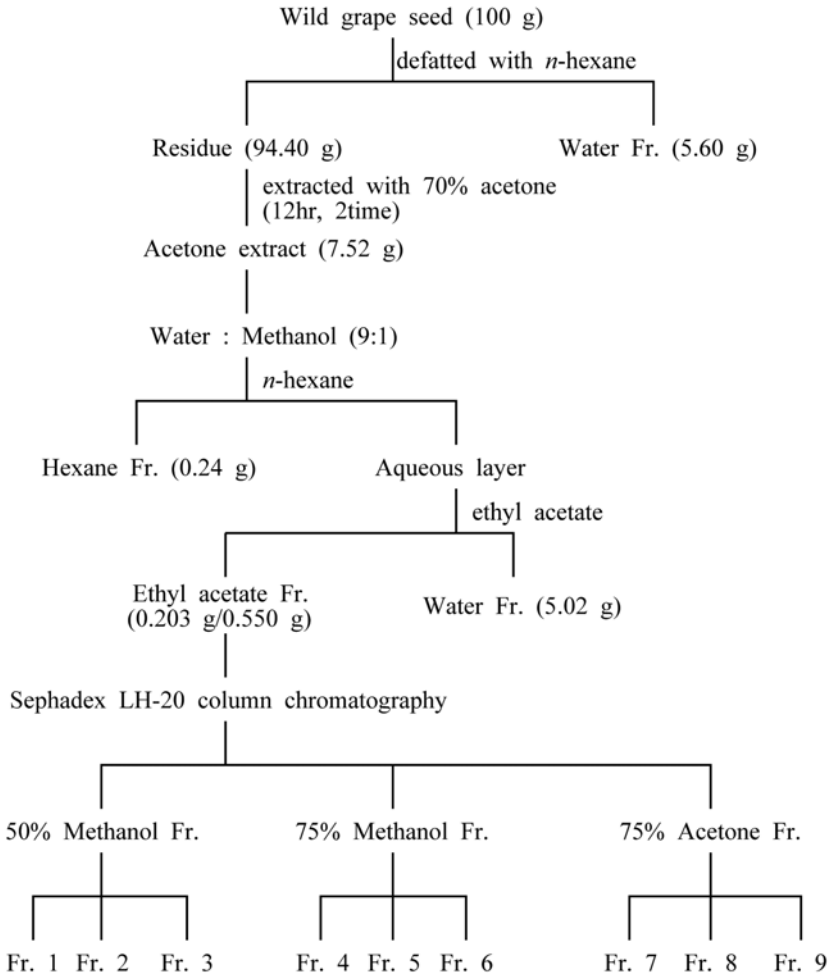

Fig. 1. The isolation scheme of proanthocyanidin from wild grape (*Vitis amurensis*) seed.

Inc. (Hamyang, Korea), November, 2006.

General apparatus and chemicals. Spectrophotometric analyses for the assays were performed by a UV-Vis spectrophotometer (UV 1601 PC, Shimadzu Co., Kyoto, Japan). Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) and thin layer chromatography (TLC) plate (Silica gel 60, E. Merck Co., Darmstadt, Germany) were used for the isolation and monitoring of proanthocyanidin fractions. LC-MS (API^{TM} 2,000 LC/MS/MS System, Applied biosystems Co., Foster City, CA, USA) was used for the mass spectra analyses. Vanillin, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), Folin-Ciocalteu reagent, and (+)-catechin were obtained from Sigma Co. (St. Louis, MO, USA). All organic solvents were the analytical grade of Merck (Darmstadt, Germany), except for HPLC (J. T. Baker, Phillpsburg, NJ).

Extraction and isolation. The isolation scheme of proanthocyanidin fraction from the wild grape seeds were shown in Fig. 1. The defatted wild grape seeds (100 g) by *n*-hexane were extracted two times at room temperature with 1 L of 70% (v/v) aqueous acetone for 12 h, and evaporated to dryness with a rotary evaporator. The crude acetone extract was further fractionated with the order of hexane, ethyl acetate, and water. The ethyl acetate fraction (203 mg)was applied on the Sephadex LH-20 column (25×500 mm) which was eluted with 300 mL of 50 %, 75% methanol, and 75% acetone, respectively. The eluted solution was collected by 100 mL to give nine fractions (Fr. 1~9). The fractions were monitored by the TLC with the mobile phase of toluene/acetone/formic acid (6:6:1, v/v/v), visualized by a 1% vanillin solution of 70% hydrochloric acid [Andriambeloson *et al*., 1998]. And (+)-catechin was used as a standard. Fr. 6 with 2 spots in TLC (Fig. 2.) was isolated and purified by preparative TLC. The mass spectrum of the Fr. 6-2 was measured by LC-MS in an ODS column (4.6×250 mm, 5 μm, Thermo Hypersil Gold, Waltham, MA, USA) at the 1 mL/min flow rate of the gradient mobile phase of 1% aqueous formic acid (solvent A) and methanol 100% (solvent B) with and the selected ion monitoring (SIM) method under a negative mode. The mobile phase was

programmed as follows; 0-30% B (0-30 min), 30-60% B (30-50 min). The fragmentor was set at 20 V for all compounds to observe the pseudomolecular ion. Spray chamber parameters were as follows: 5.0 l min^{-1} drying gas, 325°C drying gas temperature, 200°C vaporizer temperature, 60 psig. nebulizer pressure and 2000 V capillary voltage.

Proanthocyanidin analyses. The proanthocyanidin contents of the fractions were determined by the vanillin-H_2SO_4 method [Baoshan *et al*., 1998]. Briefly, a sample solution 200 μL was added to 500 μL of 1.2% vanillin solution and 500 μL of 20% H_2SO_4 solution. The reaction was carried out in the dark at room temperature for 20 min, and then absorbance was measured absorbance at 500 nm. Proanthocyanidin contents were expressed as a (+)-catechin equivalents in mg per 100 g. The proanthocyanidin profile of the fraction was investigated by the absorbance scanning measurement between 450 and 600 nm of the sample solution reacted by BuOH/HCl method [Vivas *et al*., 2006].

Ferric reducing antioxidant power (FRAP) assay. In the FRAP assay, antioxidants in the sample reduce Fe^{3+}/tripyridyltriazine complex, present in stoichiometric excess, to the blue colored ferrous form, with the increase of absorbance at 590 nm. The stock solutions was composed of 23 mM acetate buffer (pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_36H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_36H_2O$ solution and then warmed at 37°C before using. The working solution 175 μL was mixed with 25 μL of sample solutions. The mixture was shaken and incubated at 37°C for 30 min in the darkness, and then measured the absorbance at 590 nm. Results are expressed as mmol trolox equivalents (TE) per 1 L of sample solution [Benzie *et al*., 1996].

DPPH radical scavenging activity. The DPPH solution (100 μM) 900 μL was added to 100 μL of the sample soultion. The reaction for scavenging DPPH radicals was carried out at room temperature in the dark for 30 min, and measured the absorbance at 517 nm. The radical scavenging activity was calculated using the following equation:

$$\text{Activity (\%)} = [1 - (Ab_s/Ab_c)]\ 100\ (\%)$$

Where, Ab_s is the absorbance of the sample, and Ab_c is the absorbance of the control solution [Kang *et al*., 2001].

Total phenolic contents. Total phenolic contents was determined by the Folin-Ciocalteu reagent method [Sato *et al*., 1996]. Briefly, a sample soultion (100 μL) reacted with 50 μL of Folin-Ciocalteu reagent for 4 min at room temperature was added with 300 μL of saturated sodium carbonate (20%), and was stood for 15 min in the dark at room temperature. Finally, 1 mL of water was added, centrifuged at 1,250 rpm for 5 min, and the absorbance of the supernatant solution were measured at 760 nm. Total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per 100 mL.

Statistical treatments. All the experiments were executed in triplicate and the determination values were expressed as means±standard deviation. The regression equations for the standard curves and the correlation coefficients were obtained using Microsoft office Excel 2007 (Microsoft, Redmond, WA, USA) software.

Results and Discussion

Isolation of proanthocyanidin fractions and their characteristics. Proanthocyanidin is widely distributed in the plant, especially in almost all part of the grapevine. There has been no complete study on the perfect extraction method of proanthocyanidin, however the most popular solvents for the extraction were methanol, acetone, and their aqueous solution [Oszmianski and Lee, 1990]. The crude extract was obtained from the defatted wild grape seed with 70% aqueous acetone [Ricardo da Silva, 1990]. The crude 70% acetone extract was fractionated and isolated as shown in Fig. 1. Finally 9 fractions were obtained through the Sephadex LH-20 column chromatography. Fr. 1 and 2 eluted with 50% methanol had a little bit low yields, however, the yields were gradually increased from the fr. 3 and reached the highest at fr. 5 (22.01%), followed by fr. 8. (19.07%). The determination of proanthocyanidin contents was executed by the vanillin-H_2SO_4 method, which is widely used for rapid proanthocyanidin determination in plant because of the specific coloring reaction of several kinds of proanthocyanidins [Butler *et al*., 1982, Deshpande *et al*., 1986]. The proanthocyanidin contents of the fractions from wild grape seeds were shown in Table 1. The Fr. 5 had the highest proanthocyanidin contents of all the fractions, and Fr. 6~8 had also fairly higher contents than any other fractions. The fractions isolated were monitored on the proanthocyanidin characteristics by the TLC method [Andriambeloson *et al*., 1998]. The TLC chromatogram was shown in Fig. 2. The results were shown in a light red color in Fr. 5~8 spot. Fr. 5 was developed to 1 spot, showing the same Rf value, 0.55 with (+)-catechin as a standard. The 2 spots (Rf 0.55 and 0.23) were developed in Fr. 6, and one (Fr. 6-2) had also the same Rf value with (+)-catechin. Fr. 7, 8 were detected more than five spots. The Fr.6-1 and Fr. 6-2 were separated from Fr. 6 by the preparative TLC, and analyzed by LC-MS. The mass spectrum of Fr. 6-2 was

Table 1. The yields and proanthocyanidin contents of Sephadex LH-20 fractions from wild grape (*Vitis amurensis*) seed

Fractions	Yield (%)	Proanthocyanidin contents[1)] (g CE/100 g)
Fr. 1	2.41	0
Fr. 2	4.13	0
Fr. 3	2.31	0.33±0.16
Fr. 4	2.80	1.05±0.04
Fr. 5	22.01	86.00±6.74
Fr. 6	10.32	60.09±3.88
Fr. 7	11.11	50.82±1.44
Fr. 8	19.07	64.70±4.35
Fr. 9	1.47	3.58±1.12

Values reflect the means±SD (n=3)
[1)]Test solutions of 1,000 ppm, CE; catechin equivalents.

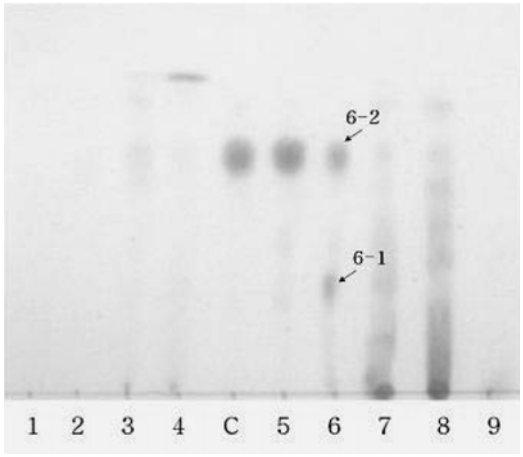

Fig. 2. TLC chromatogram of Sephadex LH-20 fractions from wild grape (*Vitis amurensis*) seed. The spots were visualized by spraying with a 1% vanillin solution in 70% hydrochloric acid. C, (+)-catechin; 1~9, Fr. 1~9.

shown in Fig. 3. The *m/z* of 289 $[M-H]^-$ indicates the compound with the molecular weight of 290. Therefore the Fr. 6-2 was identified as (+)-catechin. The Fr. 7, 8 showed the typical red coloring reaction of proanthocyanidin on the vanillin-HCl reagent spray, although the development was not fairly good in this mobile system. The visible ray absorption spectrum of isolated fractions were investigated from 450 nm to 600 nm after BuOH-HCl reaction (Fig. 4). The Fr. 5-8 showed the absorbance band around 550 nm, indicating the presence of oligomer proanthocyanidins.

Antioxidant activities of proanthocyanidin fractions. Numerous research studies propose that the pathogenesis of various diseases by the oxidative stress, such as atherosclerosis, diabetes mellitus, and carcinogenesis might be prevented by the consumption of plant-derived antioxidants. Up to now, the antioxidant evaluation on the plant and agricultural materials, and products was performed by several methods in diverse fields, however no method could absolutely fulfill the ideal requirements of the antioxidant efficacy measurement [Prior *et al*., 2005].

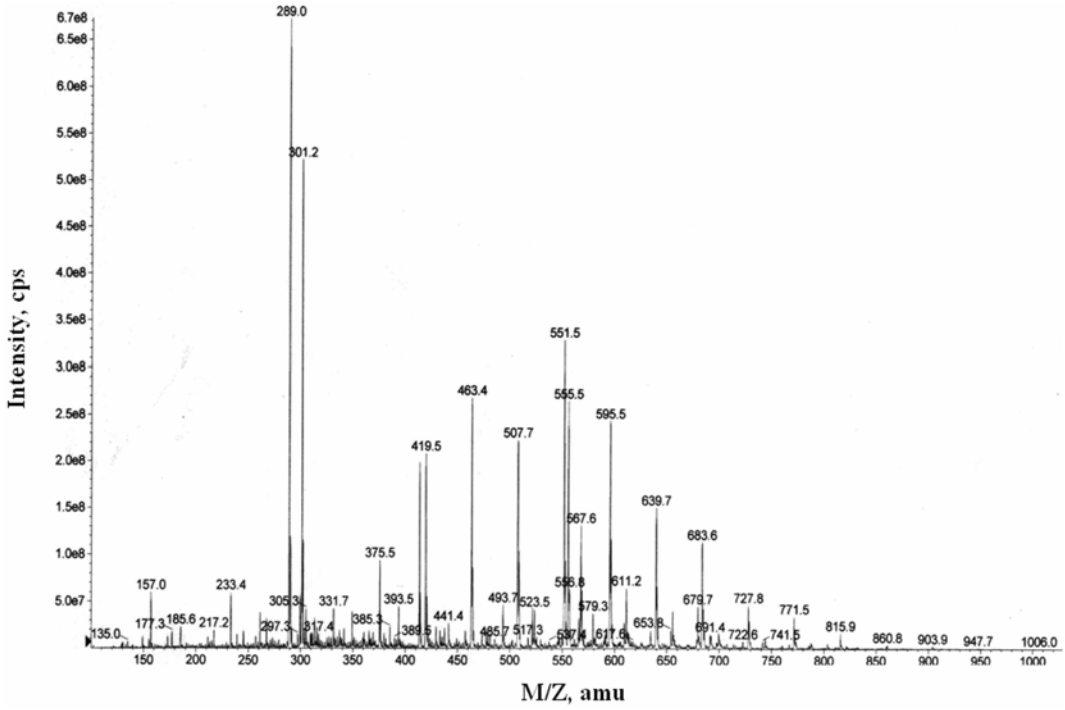

Fig. 3. LC-MS spectrum of Fr. 6-2 isolated from wild grape (*Vitis amurensis*).

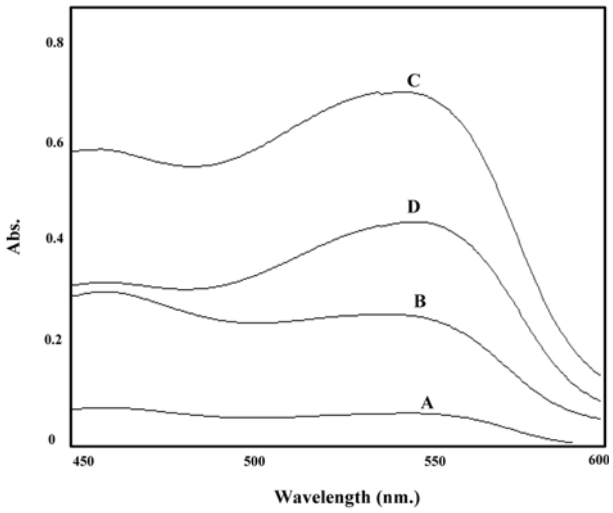

Fig. 4. UV-Vis absorption spectra for BuOH-HCl reaction of Sephadex LH-20 fractions from wild grape (*Vitis amurensis*) seed. A, Fr. 5; B, Fr. 6; C, Fr. 7; D, Fr. 8(2 times diluted).

The spectrophotometric measurements by the free radical scavenging and oxidation-reduction potential power were very simple, but robust and widely used antioxidant activity methods. The antioxidant activities of proanthocyanidin fractions isolated from wild grape seed were measured by DPPH radical scavenging and FRAP assays. The FRAP assay is widely used in the evaluation of the antioxidant activity, like as DPPH assay. The total phenolic contents of the proanthocyanidin fractions were also determined. Fr. 8 which was eluted with 75% acetone showed the strongest activities by both assays. The total phenolic content of fr. 8 was also the highest among all the fractions, almost 10 times of the reported value of the crude methanol extracts of grape seeds ranged from 4.66 to 5.12 g/100 g [Rababah *et al*., 2008]. Fr. 5, 6, and 7 showed a little weaker activities than fr. 8. This result might be somehow caused by the fact that the antioxidant activity of proanthocyanidin depends on the degree of polymerization [Jayaprakasha *et al*., 2001]. The strong antioxidant activity of the proanthocyanidin might be caused to the synergistic effects of its direct radical scavenging and chelating effects of transition metals, which might be due to the catechin structure with the *o*-dihydroxyl moiety at the B ring [Xiao *et al*., 2008]. The correlation coefficients between the proanthocyanidin contents and antioxidant activities and total phenolic contents were shown in Table 3. The correlation between proanthocyanidin contents and DPPH radical scavenging activities was fairly high (r=0.937), similar to the coefficient between proanthocyanidin contents and total phenolic contents (r=0.904). In contrast, the correlation between proanthocyanidin contents and FRAP values was silghtly low (r=0.709). The antioxidant activity by trolox equivalent antioxidant capacity (TEAC) and FRAP assays were also highly correlated with proanthocyanidins in several *Cassia fistula* L. organs [Luximon-Ramma *et al*. 2002].

Table 2. The DPPH radical scavenging activities, FRAP values and total phenolic contents of Sephadex LH-20 fractions from wild grape (*Vitis amurensis*) seed

Fractions	DPPH radical scavenging activities[1)] (%)	FRAP values[2)] (mmol TE/L)	Total phenolic contents[3)] (g GAE/100 mL)
Fr. 1	2.66±0.01	0	1.89±0.13
Fr. 2	7.11±1.63	3.57±0.55	6.60±0.26
Fr. 3	31.51±4.76	27.84±7.82	24.02±0.61
Fr. 4	39.17±1.20	24.67±3.49	24.98±0.32
Fr. 5	80.92±4.00	32.90±4.62	49.86±1.09
Fr. 6	66.15±0.95	31.05±1.39	41.66±0.32
Fr. 7	60.44±2.78	26.36±6.39	40.09±1.73
Fr. 8	90.15±1.64	37.06±4.28	53.02±0.35
Fr. 9	12.20±4.44	6.30±0.66	10.79±0.50

Values reflect the means±SD (n=3); Test solutions were [1)]100 ppm, [2)]100 ppm, [3)]1,000 ppm.
TE; Trolox equivalents, GAE; gallic acid equivalents.

Table 3. The correlation coefficients between proanthocyanidin contents and DPPH radical scavenging activities, FRAP values and total phenolic contents of the Sephadex LH-20 fractions

	DPPH radical scavenging activities (%)	FRAP values (mmol of TE/L)	Total phenolic contents (g GAE/100mL)
Proanthocyanidin contents (g CE/100 g)	0.937	0.709	0.904

*CE; catechin equivalents, GAE; gallic acid equivalents, TE; Trolox equivalents.

Conclusion. The proanthocyanidin fractions were isolated from the wild grape seed very efficiently using the solvent fractionation and Sephadex LH-20 column chromatography. These proanthocyanidin fractions with strong antioxidant capacities could be developed as a health-benefit material through further research, such as purification, structure identification, and efficacy *in vivo*, etc.

Acknowledgment. This study was supported by Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries, Republic of Korea.

References

Ahn BY (2009) Free radicals scavenging effects of ethanol extract from *Areca catechu*. *J Korean Soc Appl Biol Chem* **52**, 92-95.

Andriambeloson E, Magnier C, Haan-Archipoff G, Lobstein A, Anton R, Beretz A, Stodet JC, and Andriantsitohaina R (1998) Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. *J Nutr* **12**, 2324-2333.

Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, Joshi SS, and Pruess HG (2000) Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. *Toxicology* **148**, 187-197.

Baoshan S, Jorge MR, and Isabel S (1998) Critical factors of vanillin assay for catechins and proanthocyanidins. *J Agric Food Chem* **46**, 4267-4274.

Benzie IFF and Stranin JJ (1996) The ferric reducing ability of plasma as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* **239**, 70-76.

Butler LG, Price ML, and Brotherton IE (1982) Vanillin assay for proanthocyanidins (condensed tannins): Modification of the solvent for estimation of the degree of polymerization. *J Agric Food Chem* **30**, 1087-1089

Deshpande SS, Cheryan M, and Salunkhe DK (1986) Tannin analysis of food products. *Crit Rev Food Sci Nutr* **24**, 401-449.

Haslam E (1996) Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J Nat Prod* **59**, 205-215.

Hughes-Formella B, Wunderlich O, and Williams R (2007) Anti-inflammatory and skin-hydrating properties of a dietary supplement and topical formulations containing oligomeric proanthocyanidins. *Skin Pharmacol Physiol* **20**, 43-49.

Jayaprakasha GK, Singh RP, and Sakariah KK (2001) Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models in vitro. *Food Chem* **73**, 285-290.

Kang MH, Park CG, Cha MS, Seong NS, Chung HK, and Lee JB (2001) Component characteristics of each extract prepared by different extract methods from by-products of *Glycyrrhizia uralensis*. *J Korean Soc Food Sci Nutr* **30**, 138-142.

Kim SK (1996) Deacidification of new wild grape wine. *Korean J Food Nutr* **9**, 265-270.

Kim SY and Kim SK (1997) Winemaking from new wild grape. *Korean J Food Nutr* **10**, 254-262.

Kim YC and Chung SK (2002) Reactive oxygen radical species scavenging effects of Korean medicinal plant leaves. *Food Sci Biotechnol* **11**, 407-411.

Lau BHS, Riesen SK, Truong KP, Lau EW, Rohdewald P, and Barreta RA (2004) Pycnogenol® as an adjunct in the management of childhood asthma. *J Asthma* **41**, 825-832.

Luximon-Ramma A, Bahorun T, Soobrattee MA, and Aruoma OI (2002) Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of *Cassiafistula*. *J Agric Food Chem* **50**, 5042-5047.

Oszmianski J and Lee CY (1990) Isolation and HPLC determination of phenolic compounds in red grapes. *Am J Enol Vitic* **41**, 204-206.

Prior RL, Wu X, and Schaich K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* **53**, 4290-4302.

Rababah TM, Ereifej KI, Al-Mahasneh MA, Ismaeal K, Hidar A-G, and Yang W (2008) Total phenolics, antioxidant activities, and anthocyanins of different grape seed cultivars grown in Jordan. *Int J Food Prop* **11**, 472-479.

Ricardo da Silva JM (1990) Separation and quantitative determination of grape and wine procyanidins by high performance reversed phase liquid chromatography. *J Sci Food Agr* **53**, 85-92.

Ray SD, Parikh H, and Bagchi D (2005) Proanthocyanidin exposure to B6C3F1 mice significantly attenuates dimethylnitrosamine-induced liver tumor induction and mortality by differentially modulating programmed and unprogrammed cell deaths. *Mutat Res* **579**, 81-106

Sato M, Ramarathnam N, Suzuki Y, Ohkubo T, Takeuchi M, and Ochi H (1996) Varietal differences in the phenolic content and superoxide radical scavenging potential of wines from different sources. *J Agric Food Chem* **44**, 37-41.

Torras MAC, Faura CA, Schonlau F and Rohdewald P (2005) Antimicrobial activity of Pycnogenol®. *Phytother Res* **19**, 647-648.

Vivas V, Nonier MF, Pianet I, de Gaulejac NV, and Fouquet E (2006) Proanthocyanidins from *Quercus petraea* and *Q. robur* heartwood: quantification and structures. *C R Chimie* **9**, 120-126.

Xiao J, Suzuki M, Jlang X, Chen X, Yamamoto K, Ren F, Xu M (2008) Influence of B-ring hydroxylation on interactions of flavonols with bovine serum albumin. *J Agric Food Chem.* **56**, 2350-2356.

Yamakoshi J, Kataoka S, Koga T, and Ariga T (1999) Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* **142**, 139-149.