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The Antioxidant Activity of Various Cultivars of Grape Skin Extract

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Abstract The aim of this study was to analyze the antioxidant properties of different cultivars of grape skin extract in an *in vitro* system. The extracts were prepared from eight grape cultivars: 'Campbell Early' (CE), 'Kyoho' (K), 'New Kyoho' (NK), 'Muscat of Alexandria' (MOA), 'Seibel' (S), 'Morgen Schow' (MS), 'Gold Finger' (GF), and 'Meru' (M). The total phenolic acid contents were highest in MS and K. Resveratrol content was high in NK (50.88 mg/1 g of coat), and quercetin content was significantly higher in K (0.68 mg/1 g of coat) than in the other grape species (0.21-0.44 mg/1 g of coat). The K and MS grape species, in which total phenol content was comparatively high (K: 24.15 μg/mL, MS: 25.52 μg/mL), also showed a high level of electron donating activity (K, 53%; MS, 59%). The hydrogen radical scavenging activity of M (50.36%) was significantly higher than the other grape species, including the S (50.21%), MS (49.43%), and K (49.06%) cultivars. Antioxidant activity varied depending on grape species, but overall it was highest in the MS and K cultivars.

Keywords: grape skin extract, antioxidant activity, resveratrol, quercetin

Introduction

Grapes are among the most widely consumed fruits in the world. They are rich in polyphenols, with approximately 75% of grape polyphenols existing in the seeds and skin. The major polyphenols in grape skin are the procyanidins, and particularly the proanthocyanidins, which include the condensed cyanidine-3-glucosides, malvidin-3-glucosides, and peonidine-3-glucosides (1, 2). Grape skin also contains another type of polyphenol known as anthocyanin, which usually has a purple color and amounts to 30% of the total polyphenols in grapes. All of these glycoside polyphenolic compounds are abundant in grape skin.

Yet unfortunately, useful materials such as grape seeds and skins have been underutilized. The annual production of grape waste in Korea is near 50,000 MT, which creates a disposal problem. The biotransformation products are humic, mineral substances. Grape waste has been subjected to composting studies in terms of its physicochemical and microbiological characteristics. These studies focused on its influence on pathogenic organisms, as well as the extraction of metal ions (3).

Extensive research has demonstrated that many types of biodegradable organic waste can be composted in a convenient and economical way. Composting may be considered as a means for treating different types of organic waste, as their emission into the environment would otherwise represent a risk. Some epidemiological studies have shown that red wine may reduce the mortality rate of coronary heart disease (4, 5). Anthocyanidins and catechins are known to play a defense role in the human body (6). The potential roles, including free radical scavenging, of antioxidant agents such as proanthocyanidin and resveratrol have been extensively studied for the prevention of numerous degenerative diseases (7-9). Thus, grape polyphenols may play an important role in preventing cardiovascular disease.

In order to effectively utilize grape skin waste, this study aimed to elucidate the antioxidant properties of different cultivars of grape skin extract through an *in vitro* model system.

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Materials and Methods

Materials Eight grape cultivars, 'Campbell Early' (CE), 'Kyoho' (K), 'New Kyoho' (NK), 'Muscat of Alexandria' (MOA), 'Seibel' (S), 'Morgen Schow' (MS), 'Gold Finger' (GF), and 'Meru' (M), a Korean wild type, were purchased from a local market in Okcheon in the autumn (October, 2004). All the solvents and chemicals used in the study were of analytical grade. 1, 1-Diphenyl-β-picrylhydrazyl (DPPH), folin-ciocalteu reagent, catechin, resveratrol, quercetin, chlorogenic acid, pyrogallol, egg yolk lecithin, thiobarbituric acid (TBA), $FeSO_4 \cdot 7H_2O$, 2-deoxy-ribose, trichloroacetic acid (TCA), and peroxidase were obtained from Sigma Chemical Co. (St. Louis, MO, USA.)

Preparation of grape skin extracts (GSE) The grape skin extracts were prepared according to the different varieties, where the skins were extracted from 8 types of berries harvested at commercial maturity. The grapes were separated into skins, pulp, and seeds. The skins were extracted separately with 1% trifluoroacetic acid (TFA) (Sigma Chemical Co.) aqueous ethanol for 3 hr, and concentrated using a vacuum rotary evaporator (Eyela, Tokyo, Japan) to remove the ethanol. Next, the skins were lyophilized. This was repeated 3 times.

Determination of total phenolic acid The total phenol acid contents were determined by the AOAC method (10).

Briefly, a 0.25 mL aliquot of extract solution was mixed with 0.25 mL of Folin-Ciocalteu reagent and 0.5 mL of saturated sodium carbonate (Na_2CO_3) solution, in addition to 4 mL of water. The mixture was allowed to stand at room temperature for 25 min and was then centrifuged. The supernatant absorbance was measured at 725 nm using a UV-visible spectrophotometer (Pharmacia Biotech, Cambridge, England). The results are expressed as chlorogenic acid (Sigma Chemical Co.) equivalents

Determination of resveratrol and quercetin The resveratrol and quercetin contents contained in the coats of the eight grape varieties were analyzed by HPLC (Younglin Associates, Anyang, Korea). The grape extracts were filtered onto Whatman (0.45 μm) filters and then analyzed on a C18 Bondapak (4.6×150 mm, Waters, Milford, MA, USA) column. Resveratrol was eluted with 40% methanol at 0.8 mL/min and detected at 320 nm. Quercetin was eluted with 65% methanol at a 0.8 mL/min and detected at 370 nm.

Scavenging activity of the DPPH free radical The electron donating ability of the extracts was assayed by the modified method of Kang *et al.* (11). Here 0.1 mL of grape extract and DPPH solution were vigorously mixed, and then placed at room temperature 30 min. Next, the mixed samples were measured using a spectrophotometer (Pharmacia Biotech) at 517 nm. The electron donating ability was calculated as follows:

EDA (%)=[1−(absorbance value of testing solution /absorbance value of control solution)]×100

Assay for superoxide dismutase (SOD)-like activity SOD-like activity was assayed using the method of Marklund and Marklund (12). The reaction solution was prepared by mixing 0.2 mL of sample solution, 3 mL of tris-HCl buffer (pH 8.5), and 0.2 mL of 7.2 mM pyrogallol, which was then placed at 25°C for 10 min. The oxidized pyrogallol was measured at 420 nm using a spectrophotometer (Pharmacia Biotech) after the reaction was stopped by adding 0.1 mL of 1.0 N HCl. The SOD-like activity is expressed as the reduction rate of absorbance.

SOD-like activity (%)=[1−(absorbance value of testing solution /absorbance value of control solution)]×100

Lipid peroxidation of egg yolk lecithin (13) To test for antioxidant activity, egg yolk lecithin dissolved in chloroform along with extract were added to a test tube and mixed well. After removing the solvent in a stream of nitrogen gas followed by vacuuming, the residue was dissolved in Tris-HCl (0.7 mL, 10 mM/L, pH 7.4)-KCl (0.175 M) buffer, with $FeSO_4$ (2 mM) and ascorbic acid (2 mM) added. This was mixed vigorously and exposed to ultrasonic waves (5210; Bransonic Ultrasonics Corp., Danbury, CT, USA) for 30 sec. The reaction mixture was incubated at 37°C for 30 min in order to induce peroxidation, and then cooled in an ice bath. The degree of lecithin peroxidation was measured by the TBA method, where 5 mM ethylene-diamine-tetra-acetic acid (EDTA), 1% phosphoric acid, and 0.7% TBA were added to the reaction mixture and then heated in water bath at 100°C for 45 min. After adding 4 mL of *n*-butanol, the mixture was centrifuged. The absorbance of the resulting solution was measured using a spectrophotometer (Pharmacia Biotech) at 532 nm. As a control, methanol was used as a blank. RAE_{TBARS} was calculated by the following equation:

RAE_{TBARS} (%)=(TBARS of the control−TBARS with extract /TBARS of the control)×100

Hydroxyl radical scavenging activity Hydroxyl radical scavenging activity was determined by the 2-deoxy-ribose oxidation method (14). The hydroxyl radical was generated by a Fenton reaction in the presence of $FeSO_4 \cdot 7H_2O$. The reaction mixture consisted of 200 μL of 10 mM $FeSO_4 \cdot 7H_2O$, 10 mM EDTA, and 10 mM 2-deoxy-ribose. The sample solution and a 0.1 M phosphate buffer (pH 7.4) were then added to generate a total volume of 1.8 mL. Finally, 200 μL of 10 mM H_2O_2 was added to the reaction mixture and then incubated at 37°C for 2 hr. After incubation, 1 mL of 2.8% TCA and 1.0% TBA were added to the reaction mixture. After boiling for 10 min, its absorbance was measured at 532 nm.

Hydrogen radical scavenging activity Hydrogen peroxide scavenging activity was determined according to the method of Muller (15). The test sample, dissolved in methanol, was mixed with 100 μL of 0.1 M PBS buffer (pH 5.0) and 20 μL of hydrogen peroxide in a tube. This was then incubated at 37°C for 5 min. Thirty μL of 1.25 mM 2,2'-azino-bis (3-ethylbenzthizaoline-6-sulfonic acid) (ABTS) and 30 μL of peroxidase (1 unit/mL) were added to the mixture, which was incubated at 37°C for 10 min. The absorbance was read with microplate reader (DigiScan 340; Asys Hitech, Eugendorf, Austria) at 405 nm.

Statistical processing The results of this study were averaged and a comparison between experimental groups was drawn through an ANOVA analysis based on the SAS system. After the ANOVA analysis, the significance level was computed using Duncan's multiple range test at $\alpha=0.05$.

Results and Discussion

Total phenolic compound content The total phenolic compound contents obtained from the 8 different cultivars are shown Table 1. Both MS (25.52 ug/mL) and K (24.15 ug/mL) had high levels of phenolic acid. There are many well known effects of phenolic compounds, including procyanidin, anthocyanin, and resveratrol, on human health (16-19). These components inhibit the oxidization of low density proteins and decrease the risk of heart disease (20, 21). Sun *et al.* (22) reported the isolation and purification of procyanidin (dimeric B_1, B_2, B_3, B_4, B_1-3-O-gallate, B_2-3-O-gallate, B_2-3'-O-gallate, trimer C_1, trimer T_2) from grape seeds by HPLC. Miceli *et al.* (23) measured the total phenolic substances in grape waste. The quantity of total phenolic substances in the grape seed extract (2.86±0.01 g/L) was higher than that obtained from the peel (1.11±0.01 g/L) and the marc (1.40±0.02 g/L).

Table 1. Total phenolic compound contents of extracts prepared from 8 different cultivars by (+)-catechin[1)]

	Total phenolic compounds[2)] (μg/1 g of coat)
K	24.15±0. 417[a]
CE	14.71±0.973[c]
MOA	8.32±1.112[e]
NK	10.19±0.139[d]
GF	5.47±0.139[f]
M	16.58±0.278[b]
S	9.90±0.556[d]
MS	25.53±0.417[a]

[1)]K, 'Kyoho'; CE, 'Campbell Early'; MOA, 'Muscat of Alexandria'; NK, 'New Kyoho'; GF, 'Gold Finger'; M, 'Meru'; S, 'Seibel'; MS, 'Morgen Schown'.
[2)]All values are means±SD, n=3. Different letters are significantly different by Duncan's multiple range test after an ANOVA analysis at α=0.05 (a>b>c>d>e>f).

Table 2. Resveratrol and quercetin contents of extracts prepared from different grape cultivars[1)]

	Resveratrol (mg/1 g of coat)	Quercetin (mg/1 g of coat)
K	5.80	0.68
CE	12.57	0.44
MOA	30.36	0.23
NK	50.88	0.21
GF	17.92	0.23
M	22.22	0.22
S	13.39	0.32
MS	20.87	0.31

[1)]K, 'Kyoho'; CE, 'Campbell Early'; MOA, 'Muscat of Alexandria'; NK, 'New Kyoho'; GF, 'Gold Finger'; M, 'Meru'; S, 'Seibel'; MS, 'Morgen Schown'.

Contents of resveratrol and quercetin In this study, phenolic components such as resveratrol and quercetin were found in great quantities in the grapes and grape seeds according to grape species (Table 2). Resveratrol content varied depending on the grape species. NK was the highest at 50.88 mg per 1 g of coat and MOA also contained a high level at 30.36 mg; while for K, the resveratrol content was very low at 5.80 mg per 1 g of coat. Before this research, measurable resveratrol content had been found in 'Campbell Early' grape coat extracts through the use of different solvents (24). It was found that the highest amount of resveratrol was acquired by an ethanol solvent, where HCl was added at 0.1%. After using the ethanol solvent it was determined that citric acid, added at 0.1%, resulted in the lowest level. Kim *et al.* (25) measured the trans-resveratrol contents of 32 grape varieties. 'Rehealvescol' of 32 grape varieties was found to contain the highest level at 207.14 μg/100 g and 'Blackolympia' was found to be lowest at 0.76 μg/100 g. In this study, quercetin content also varied depending on the grape species, and the quercetin content of K was significantly higher than in the other grape species.

Scavenging activity of the DPPH free radical The

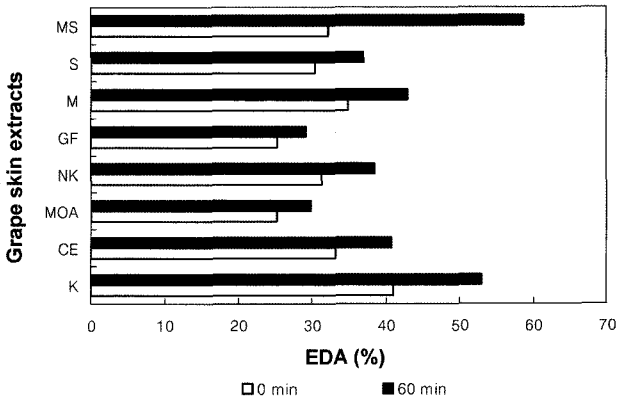

Fig. 1. Electron donating abilities of extracts prepared from 8 different grape cultivars. K, 'Kyoho'; CE, 'Campbell Early'; MOA, 'Muscat of Alexandria'; NK, 'New Kyoho'; GF, 'Gold Finger'; M, 'Meru'; S, 'Seibel'; MS, 'Morgen Schown'.

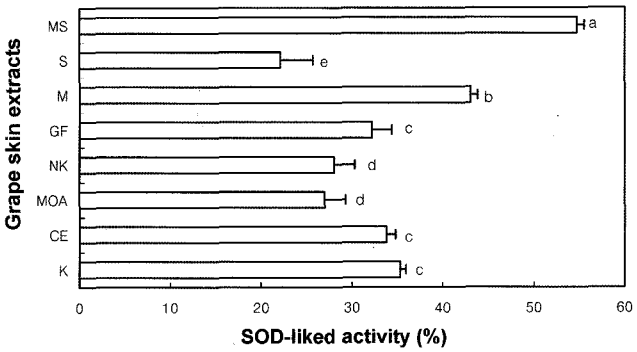

Fig. 2. SOD-like activity of extracts prepared from 8 different cultivars. All values are means±SD, n=3. Different letters are significantly different by Duncan's multiple range test after an ANOVA analysis at α=0.05 (a>b>c>d>e). K, 'Kyoho'; CE, 'Campbell Early'; MOA, 'Muscat of Alexandria'; NK, 'New Kyoho'; GF, 'Gold Finger'; M, 'Meru'; S, 'Seibel'; MS, 'Morgen Schown'.

electron donating ability of each extract at 60 min was measured (Fig. 1). The K and MS grape species, in which total phenol contents were comparatively high, showed electron donating activity levels that were also high. Chung *et al.* (26) reported that DPPH free radical scavenging ability was higher in grape seed extracts than BHT. Cho *et al.* (27) measured the electron donating activities of 'Kyoho' and 'Campbell Early' grape stem and seed extracts, and reported 70-80% in 500 μg/mL. In particular, the 'Kyoho' seed extract was reported to have an antioxidant effect equivalent to vitamin C.

SOD-like activity In the present study, of the 8 different grape cultivar extracts, MS had a SOD-like activity of 54.74%, whereas S showed 22.11% (Fig. 2). Halliwell *et al.* (28) emphasized the important role of SOD, and recommended the intake of food with high SOD for the prevention of diseases. Physicochemicals, especially plant phenolics and flavonoids, react with active oxygen radicals such as hydroxyl radicals and superoxide anion radicals, and inhibit lipid oxidation at early stages (29).

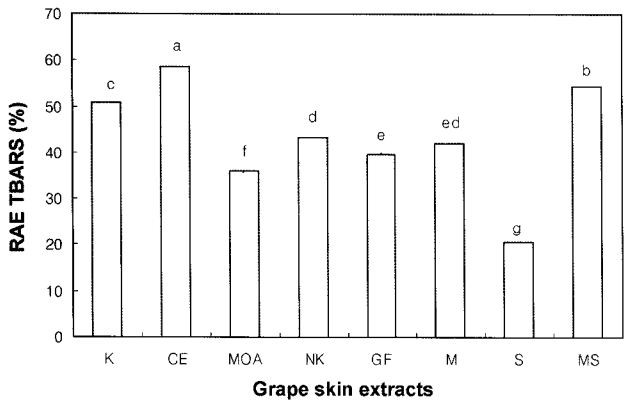

Fig. 3. Relative antioxidative effects of extracts prepared from 8 different grape cultivars. All values are means±SD, n=3. Different letters are significantly different by Duncan's multiple range test after an ANOVA analysis at α=0.05 (a>b>c>d>ed>e>f>g). K, 'Kyoho'; CE, 'Campbell Early'; MOA, 'Muscat of Alexandria'; NK, 'New Kyoho'; GF, 'Gold Finger'; M, 'Meru'; S, 'Seibel'; MS, 'Morgen Schown'.

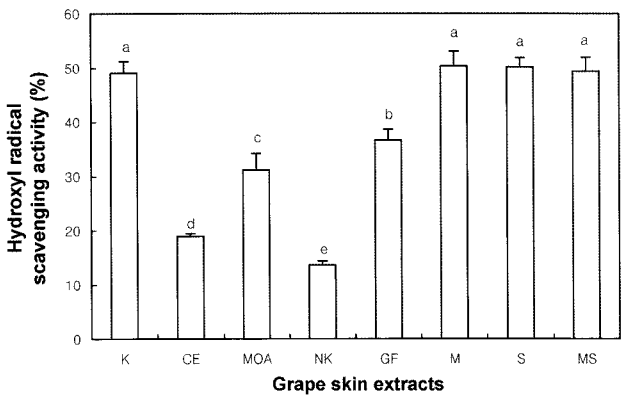

Fig. 5. Hydroxyl radical scavenging activity of extracts prepared from 8 different cultivars. All values are means±SD, n=3. Different letters are significantly different by Duncan's multiple range test after an ANOVA analysis at α=0.05 (a>b>c>d>e). K, 'Kyoho'; CE, 'Campbell Early'; MOA, 'Muscat of Alexandria'; NK, 'New Kyoho'; GF, 'Gold Finger'; M, 'Meru'; S, 'Seibel'; MS, 'Morgen Schown'.

Relative antioxidative effects When we assayed for the effects of the extracts on the lipid peroxidation activity of egg yolk lecithin induced by $FeSO_4$ (2 mM)/ascorbic acid, we found that K, CE, and MS showed stronger antioxidative effects than S (Fig. 3). Jang and Han (30) measured the oxidation inhibition of grape seed extract using a linoleic acid methyl ester system. Their measurements determined the following: grape stone extract (53%), ascorbic acid (52%), and α-tocopherol and BHT (37%). There was a difference between grape species in this research, and the grape coat's antioxidant activity was confirmed. Specifically, antioxidant activity was high in grape species in which total phenolic compound content was high. A strong correlation between DPPH radical and hydroxyl radical scavenging effects have been reported in red wine (31).

Hydrogen radical scavenging activity The hydrogen radical scavenging ability of the grape coat extracts by species (Fig. 4) was measured for M, K, MS, and C. The results show significantly high levels for each. Yuan *et al.* (32) reported that the proanthocyanidins in grape seeds inhibited oxidant injury when exposed to hydrogen peroxide in the cardiomyocyte.

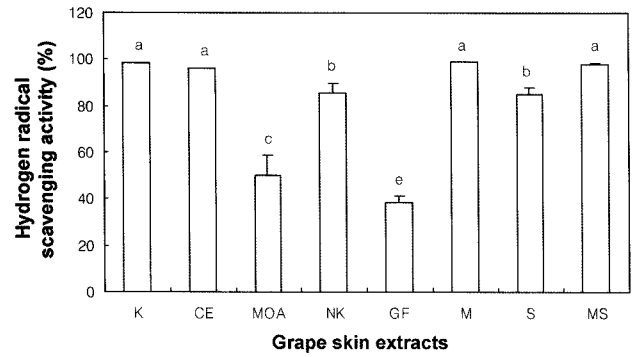

Fig. 4. Hydrogen radical scavenging activity of extracts prepared from eight different cultivars. All values are means± SD, n=3. Different letters are significantly different by Duncan's multiple range test after an ANOVA analysis at α=0.05 (a>b>c>d>e). K, 'Kyoho'; CE, 'Campbell Early'; MOA, 'Muscat of Alexandria'; NK, 'New Kyoho'; GF, 'Gold Finger'; M, 'Meru'; S, 'Seibel'; MS, 'Morgen Schown'.

Hydroxyl radical scavenging activity The results indicate there were high oxidation inhibiting effects (%) for M (50.36%), S (50.21%), MS (49.43%), and K (49.06%), which were significantly higher than the other grape species (Fig.5). Dipak *et al.* (33) stated that the proanthocyanidins in grape seeds have potent peroxyl radical and hydroxyl radical scavenging abilities, and can prevent coronary heart disease. Bagchi *et al.* (18) reported that grape seed proanthocyanidin extracts are safe, novel, highly potent, and bioavailable free radical scavengers and antioxidants, possessing a broad spectrum of health benefits.

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