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Radical Scavenging Activity of Grape-Seed Extracts Prepared from Different Solvents

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Abstract Antioxidant activities of grape seeds extracted with various solvents were evaluated by measuring total phenol and flavanol contents, thiobarbituric acid reactive substances (TBARS) following lipid peroxidation, 2-deoxyribose degradation, SOD-like activity, 2,2'-azino-bis(3-ethylbenzthizaoline-6-sulfonic acid (ABTS) radical-scavenging ability, and electron-donating ability using 1,1-diphenyl-2-pycryl hydrazil (DPPH) method. Total phenol and flavanol contents of mixted-solvent extracts were higher than those of single-solvent extracts, with the mixing ratio of 17:3 (ethyl acetate : water) (EW) showed the highest contents. Antioxidant activities (%) of TBARS following phosphatidylcholine peroxidation were 14, 45, 45, 7, 4, 25, 21, 23, and 20% for ascorbic acid (AA), butylated hyroxytoluene (BHT), quercetin (Q), acetone extract (AT), ethyl acetate (EA) extract, methanol (MeOH) extract, 4:1 (EA) extract, 9:1 (EW)-extract, and 17:3 EW extract, respectively. Antioxidant activities for 2-deoxyribose degradation were 5, 80, 87, 78, 56, 73, 64, 60, and 75% in AA, BHT, Q, AT, EA, MeOH extract, 4:1 EW extract, 9:1 EW extract, and 17:3 EW extract, respectively. MeOH grape seed extract showed distinctly stronger electron-donating activity than other solvent extracts.

Keywords: antioxidant, grape seed, thiobarbituric acid reactive substances, 2-deoxyribose, 1,1-diphenyl-2-pycryl hydrazil

Introduction

The grape seed is a rich source of monomeric flavan-3-ols (such as (+)-catechin, (-)-epicatechin, and their galloylated derivatives) as well as oligomeric and polymeric procyanidins, and only the procyanidin type of proanthocyanidins are detected from the seed (1, 2). Jayaprakasha, Singh, and Sahariah reported the antioxidant activity of grape seed extracts using â-carotene-linoleate and linoleic acid peroxidation methods (3). In terms of the antioxidant activities of procyanidins in grape seed, the grape seed extract was a better radical scavenger than buthylated hydroxyanisole (BHA) when measured by HPLC method using 1,1-diphenyl-2-picrylhydrazyl (DPPH) (4). Availability of extracts of red grape marc and its peels and seeds as natural antioxidants was evaluated by determining the antioxidant activities through the β-carotene bleaching test (5). The DPPH scavenging ability of the grape seed extract was 89% and the amount of TBARS in the liver was lower after oral administration of persimmon seed (6). Yamakoshi, Kataoka, Koga, and Ariga suggested that, as a major polyphenol in red wine, proanthocyanidin inhibits the oxidation of low density lipoprotein (LDL) by removing the reactive oxygen species in the plasma and interstitial fluid of the arterial wall, thus showing an anti-atherosceloric activity (7). Tebib *et al.* reported that dietary grape seed extract showed a pronounced anti-hypercholesterolenic effect of enhancing the reverse cholesterol transport (8). Koga *et al.* reported that the intake of proanthocyanidins increased the resistance of plasma against oxidative stress and contributed to physiological functions of plant food such as wine through their *in vivo* antioxidant activities (9). For these reasons, proanthocyanidin-rich extracts from grape seeds are marketed as main nutritional supplements in the United States, Australia, Japan, Korea, and many other countries. The grape seed extract is also used as an additive for various food applications, especially in Japan (10). In addition, there is a report that procyanidin serves to inhibit the growth of cariogenic *streptococcus mutans* and the aggregation of blood platelets, and acts as an antimutagenic and antiviral agent as well (11). Tebib *et al.* also reported that, when fed with monomeric and polymeric diets containing no vitamin E, rats fed with the polymeric diet showed higher increase in the bioactivation of redox enzymes (12). Therefore, in this study different solvents were used to optimize the extractions of the monomeric and polymeric flavonoids from grape seeds, and the monomeric and polymeric flavoniod contents, and antioxidant effect were measured.

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Materials and Methods

Materials Red grapes (*Vitis labrusca*, Korea) were purchased at commercial maturity at a local store. All solvents and chemicals used were of analytical grade. Catechin, epicatechin, quercetin, chlorogenic acid, and tannic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Polymeric grape seed extract Dried grape seeds were powdered and shaken with hexane for 6 hr in a shaker to remove the fatty matters. The defatted seed powder (50 g) was extracted in a Soxhlet extractor for 10 hr at 60-70°C with 150 mL extractants, such as acetone, ethyl acetate, and methanol. The extracts were concentrated in a vacuum

evaporator (Eyela, Tokyo, Japan) to obtain a viscous liquid. The polymeric grape seed extracts were precipitated by adding a double volume of hexane to the viscous liquid, and the precipitate was collected by filtration under vacuum. The liquid extract was freeze-dried to obtain the grape seed extracts, and the extraction yield was measured. The obtained extract was kept at -70°C in a deep freezer. In a separate extraction process, the defatted grape seed powder (50 g) was twice extracted for 5 hr at 60-70°C in a shaking water bath with 150 mL EtOAc-water mixture at different ratios of 9:1, 17:3, and 4:1. The extracts were concentrated in the vacuum evaporator to obtain a viscous liquid. The polymeric grape seed extracts were precipitated by adding the double volume of hexane to the viscous liquid, and the precipitate was collected by filtration under vacuum. The liquid extract was freeze-dried to obtain the grape seed extract. The obtained extract was stored at -70°C in a deep-freezer.

Total phenolic content One milligram of each extract from the dried grape seed was dissolved in 1 mL dimethyl sulfoxide (DMSO). Samples were mixed with 2% Na_2CO_3, added with Folin-Ciocalteu reagent, and kept for 30 min at room temperature. The absorbance was measured three times at 750 nm using UV-visible spectrophotometer (Pharmacia Biotech, Cambridge, England) to determine the contents of phenolic compounds in each extract.

Total flavanol content The flavanol content was obtained based on the weight (mg) of (+) catechin/100 g extract, with (+) catechin treated under the same conditions as a standard of comparison. One milliliter of the extract dissolved in dimethyl sulfoxide (DMSO) was added to 0.1 % (w/v) *p*-dimethylaminocinnamaldehyde in methanolic/ HCl (3/1; v/v) reagent (13), and after 10 min the absorbance was measured at 640 nm.

HPLC analysis Monomeric flavanols were quantified by HPLC with catechin and epicatechin as the external standards. Ten microliters of the extract dissolved in DMSO solution was filtered through Whatman (0.45 μm) and analyzed on a Develosil ODS-5 column using 0.5% each of formic acid (A) and acetonitrile (B). The elution program at 1.0 mL/min flow rate was as follow:

0-5 min, 0-12% B in A; 6-10 min, 12% B in A; 10-35 min, 12-21% B in A; 35-40 min, 21% B in A; and 40-50 min, 25% B in A.

Flavanols were detected at 280 nm, and the content of monomeric flavanols was estimated as the sum of monomeric flavanols, such as catechin and epicatechin.

Scavenging capacity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) The DPPH radical-scavenging capacity was evaluated by a slightly modified method of Okada and Okada (14). The assay mixture contained 0.3 mL of 1.0 mM DPPH radical solution, 2.4 mL of 99% ethyl alcohol, and 0.3 mL sample solution. The solution was rapidly mixed, and the scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. Ascorbic acid, BHT, and quercetin were used as positive controls. The scavenging ability of the sample was expressed as EC_{50}, that is, the effective concentration of the sample at which 50% DPPH radical-scavenging effect was obtained.

SOD-like activity The superoxide anion radical-scavenging capacity was determined by the method of Marklund *et al.* (15). A 50 μl sample solution and Tris-HCl buffer (pH 8.5) were mixed with 50 μl of 24 mM pyrogallol. The absorbance of the reaction mixture was measured for 10 min at 420 nm.

Lipid peroxidation induced by the Fe^{2+}/ascorbate system Phosphatidylcholin in a chloroform solution was dried under nitrogen gas, and the dried lipid film was dispersed in a 10 mM Tris-HCl buffer (pH 7.4) by vigorous shaking on a vortex mixer. Transition metal ion-dependent peroxidation was induced by the addition of 2 mM $FeSO_4$ and 2 mM ascorbic acid. Incubation was carried out for two hr in a 37°C water bath with constant shaking. Products of lipid peroxidation were analyzed by the thiobarbituric acid method, with their absorbances measured at 532 nm.

Deoxyribose degradation by iron-dependent hydroxyl radical Degradation of deoxyribose was carried out in a sodium phosphate buffer (0.1 mM, pH 7.0) containing deoxyribose (10 mM), H_2O_2 (10 mM), $FeSO_4$-EDTA (10 mM), and a sample at a final volume of 2 mL. The reaction mixture was incubated for 2 hr at 37°C, and thiobarbituric acid (TBA) reactivity was determined by adding 2 mL TBA reagent (0.67% w/v in 0.1 N NaOH) and 1 mL TCA (20% w/v), and heating the mixture for 15 min at 100°C. The mixture was then cooled and centrifuged with butanol and pyridine added at a 14:1 ratio. The absorbance was measured at 532 nm against appropriate blanks. The inhibition percent (%) of deoxyribose degradation was calculated in percent by the following equation:

$$Inhibition\ (\%) = [(A_0-A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control reaction (all reagents except the test compound), and A_1 is the absorbance of the test compound.

Inhibition of oxidation induced by AAPH Blood was collected from Sprague-Dawely (SD) male rats. The serum was used for experiment immediately after centrifugation. The antioxidant activity was determined as the inhibition of serum oxidation induced in an aqueous solution by the free radical initiator 2,2'-Azobis(2-amidino-propane) dihydrochloride (AAPH). The serum was oxidized by 10 mM AAPH (final concentration) and incubated for 2 hr at 37°C in a shaking water bath. The reaction mixture was mixed with 0.67% TBA and 20% TCA, and heated for 15 min in a boiling water bath. After cooling, the mixture was centrifuged with butanol and pyridine added at a 14:1 ratio. The absorbance was read at 532 nm against appropriate blanks.

ABTS free radical scavenging activity The ABTS radical-scavenging activity was determined by a slightly modified method of Kim *et al.* (16) In brief, 2.0 mM AAPH as a radical initiator was mixed with 150 μM

ABTS in acetate buffer (pH 5.4, 50 mM). The mixed solution was heated at 55°C for 1 hr in a water bath. A 25-μl sample was added to 3.125 mL ABTS radical solution, and the mixture was incubated at 25°C for 14 min in a water bath under restricted light conditions. The absorbance was measured at 734 nm against appropriate blanks. Total antioxidant capacity of grape seed extracts was defined as ability to scavenge blue-green ABTS radical anions. The radical stock solution was prepared freshly for each test. The tests were carried out in triplicates, and the results were averaged.

Statistical analysis Results are presented as mean values ± standard deviation. Analysis of variance was performed using ANOVA procedures. Significance in difference among means was determined at a level of $P<0.05$ using Duncan's multiple range test.

Results and Discussion

Yield of grape seed extracts and their total phenolic and flavanol contents Table 1 and Fig. 1 show the total phenolic and flavanol contents and the yields obtained using different extractants. Because the antioxidant activity of plant materials is correlated with the content of phenolic compounds (17), it is very important to consider the effect of total phenolic content on the antioxidant activity of grape seed extracts from various solvents. The total contents of phenolic acids extracted using acetone, ethyl acetate, methanol, ethyl acetate : water (9:1), ethyl acetate : water (17:3), and ethyl acetate : water (4:1) were 640, 30, 720, 790, 820, and 280 mg/g, respectively, among which the mixture of ethyl acetate and water (17:3, v/v) was found to be the most effective. Although the methanol extract showed the highest yield, its total phenolic content was lower than that of the ethyl acetate : water (17:3, v/v) extract. Jayaprakasha *et al.* (3) reported that the methanol extract showed the highest yield, and Baydar *et al.* (18) reported that grape seed extracts prepared from the acetone : water : acetic acid (90:9.5:0.5) mixture gave a higher extraction of phenolic acid compounds from grape seeds than the ethyl acetate : methanol : water (60:30:10) mixture. Kallithraka *et al.* (1) reported that methanol was the best solvent for the extraction of (+) catechin, (-) epicatechin, and epigallocatechin, and the total content of phenolic acids was highest when extracted with 70% acetone. In this study, the acetone extract showed a higher total phenolic acid content than the ethyl acetate extract. No significant differences were observed in the total flavanol content among grape extracts except ethyl acetate extract; however, the procyanidin content was higher in the mixture of ethyl acetate and water compared to single solvents such as acetone, ethyl acetate, and methanol (Table 2).

Table 1. Extract yield of grape seed extracts

Solvents used for extraction[1)]	Extract yield (% dry grape seeds)
AT	1.73±0.14[2)c]
EA	1.89±0.07[b]
MeOH	2.89±0.07[a]
EW(9 : 1)	0.96±0.01[d]
EW(17 : 3)	0.94±0.02[d]
EW(4 : 1)	0.76±0.01[e]

[1)]AT = Acetone, EA = Ethyl acetate, MeOH = Methanol, EW (9:1) = Ethyl acetate : Water (9:1), EW (17:3) = Ethyl acetate : Water (17:3), EW (4:1) = Ethyl acetate : Water (4:1)
[2)]Values expressed are Mean±S.D. of three experiments
[3)]Value within a column with different superscripts are significantly different at $p<0.05$ by Duncan's multiple range test (NS; not significant)

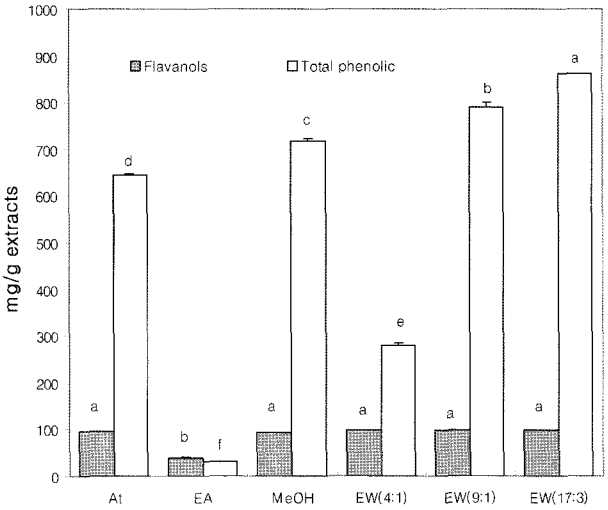

Fig. 1. Total flavanol and phenolic acid contents of grape seed extracts prepared with various solvents. Each bar is a mean standard deviation for triplication experiment. Alphabets on the bars show a statistical difference at $p<0.05$ by Duncan's multiple range test. *AT = Acetone, EA = Ethyl acetate, MeOH = Methanol, EW (9:1) = Ethyl acetate : Water (9:1), EW (17:3) = Ethyl acetate : Water (17:3), EW (4:1) = Ethyl acetate : Water (4:1)

DPPH radical-scavenging ability of grape seed extracts The DPPH scavenging-abilities of grape seed extracts obtained from different solvents are shown in Fig. 2. The radical-scavenging capacity of grape seed extracts from different solvents was determined through spectrophotometric assay as the level of DPPH radical inhibition.

Table 2. Composition of grape seed extracts (%) (in catechin equivalents /100 g extract)

Component	AT[1)]	EA	MeOH	EW(9:1)	EW(17:3)	EW(4:1)
Monomeric flavanols (%)	6.13±1.39[2)]	1.28±0.71	5.97±0.20	4.45±0.07	4.44±1.86	4.07±0.60
Polyneric flavanols (%)	3.54±1.40	2.63±0.51	3.45±0.19	5.35±0.08	5.43±1.07	5.77±0.61
Total flavanols (%)	9.66±0.01[a3)]	3.91±0.20[b]	9.42±0.01[a]	9.80±0.01[a]	9.87±0.01[a]	9.83±0.01[a]

[1)]AT = Acetone, EA = Ethyl acetate, MeOH = Methanol, EW (9:1) = Ethyl acetate : Water (9:1), EW (17:3) = Ethyl acetate : Water (17:3), EW (4:1) = Ethyl acetate : Water (4:1)
[2)]Values expressed are Mean±S.D. of three experiments
[3)]The value within a column with different superscripts are significantly different at $p<0.05$ by Duncan's multiple range test (NS; not significant)

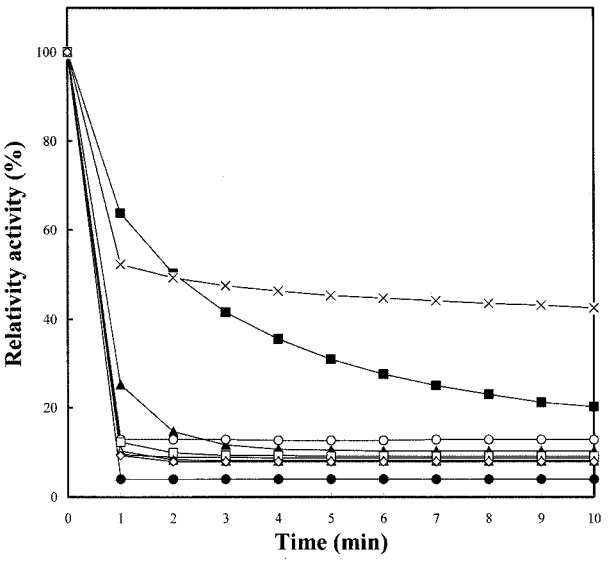

Fig. 2. DPPH radical scavenging capacities of grape seed extracts obtained from different solvents. Each bar is a mean standard deviation for triplication experiment. Alphabets on the bars show a statistical difference at $p<0.05$ by Duncan's multiple range test. (●) Ascorbic acid; (■) Butylated hydroxytoluene; (▲) Quercetin; (◆) Acetone extract; (×) Ethyl acetate extract; (○) Methanol extract; (□) Ethyl acetate : Water = 9:1 extract ; (△) Ethyl acetate : Water = 17:3 extract; (◇) Ethyl acetate : Water = 4:1 extract.

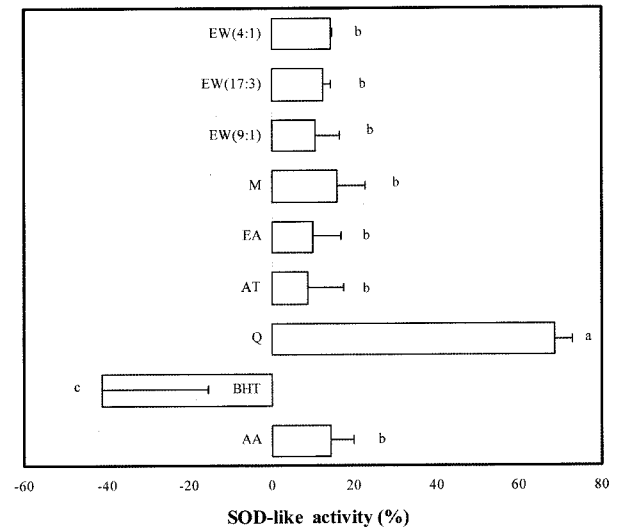

Fig. 3. SOD-like activity of grape seed extracts with different solvents. Each bar is a mean standard deviation for triplication experiment. Alphabets on the bars show a statistical difference at $p<0.05$ by Duncan's multiple range test. *AA = Ascorbic acid, BHT = Butylated hydroxytoluene, Q = Quercetin, AT = Acetone, EA = Ethyl acetate, MeOH = Methanol, EW (9:1) = Ethyl acetate : Water (9:1), EW (17:3) = Ethyl acetate : Water (17:3), EW (4:1) = Ethyl acetate : Water (4:1)

DPPH is a free-radical compound widely used to test the free radical-scavenging ability of various samples (19). It is also well known as a free radical playing an important role in antioxidation of unsaturated lipids in food stuffs (20). For example, the oxidation of muscle cholesterol may be initiated by free radicals generated during the oxidation of poly-unsaturated fatty acids. This is why DPPH is used as a free radical for evaluating the antioxidant activities of natural sources. Fig. 2 shows the radical-scavenging abilities of different grape seed extracts measured using the DPPH method. The extracts obtained using a 9:1, 17:3, and 4:1 mixture of water and ethyl acetate, acetone, ethyl acetate, and methanol showed relative activities of 90.8, 91.9, 92.1, 91.3, 57.5, and 87.2, each at 1 mg/mL concentration. The reduction in DPPH radicals was most remarkable in ascorbic acid used as a reference compound. The DPPH free radical-scavenging ability was also higher in grape seed extracts than the commercial antioxidant BHT. Mixtures of 17:3 EW, 9:1 EW, 4:1 EW, and AT showed similar levels of (about 90%) DPPH radical-scavenging ability. The methanol extract showed much lower EC_{50} value (0.089 mg/mL), which represents the amount of antioxidants required for decreasing the initial concentration of DPPH radicals up to 50%, compared to the other extracts. These results show most of the grape seed extracts except the EA extract have higher radical-scavenging activity and higher inhibition percentage compared to BHT (Table 2). A rapid decrease in the absorbance indicates the presence of many anti-oxidant-active compounds, which act as hydrogen donors (2). Jayaprakasha *et al.* (3) reported that the EW (17:3) extract had the highest reducing power among grape seeds extracted with different solvents, and the antioxidant action of reductone took place as the free radical chain was being destroyed during the donation of hydrogen atoms. In other words, the grape seed flavanol/procyanidin compounds are likely to act in a similar fashion to reductones in the aspect of donating electrons, terminating the chain reaction of free radicals, thereby converting the free radicals into more stable products.

SOD-like activity Superoxide dismutase-like activity of grape seed extracts was measured using the pyrogallol method. Pyrogallol induces O_2, which helps control the death of cells such as mesangial, human lymphoma, and human glioma cells (22). It undergoes rapid autoxidation in aqueous solutions, and SOD is known to take part in the process (23). Therefore, the autoxidation pyrogallol can be inhibited where SOD or SOD-like active substances exist. The logic is to measure the effect of decreasing pyrogallol autoxidation after the addition of a grape seed extract to an aqueous solution, which is expected to cause pyrogallol oxidation. quercetin showed a significantly higher SOD-like activity (68.4%) than other samples, while BHT accelerates the autoxidation of pyrogallol. No significant difference was observed among grape seed extracts, and their SOD-like activities were similar to that of ascorbic acid. Also in addition, the superoxide-anion-scavenging activity is considered to have been(was) mainly influenced by flavanols (proanthocyanidins) in the grape seed extracts.(tense-Is this part of the study results or stating reported finding?) These results suggest that the phenolic compounds of grape seed may show a superoxide-anion-scavenging effect or SOD-like activity, and that grape-seed extract may have an oxygen-scavenging effect and antioxidant property by inhibiting free radical reactions in chronic diseases.

Lipid peroxidation induced by the Fe^{2+}/ascorbate system Fig. 4 shows the antioxidant effects of various extracts of grape seeds in terms of peroxidation of phosphatidylcholine measured by the TBA method. Fe (II)- and ascorbic acid-induced lipid peroxidations are determined based on the formation of TBARS. In the oxidation process, peroxides gradually decompose into lower molecular weight compounds, and their relative concentrations are measured using the TBA method (24). Ferrous ion acts to promote the lipid peroxidation of phosphatidylcholine through various mechanisms such as decomposition of lipid peroxides, generation of hydroxyl radicals, and formation of perferryl (25). Reactions initiated by the oxidation and peroxidation processes are evaluated by measuring the malondialdehyde (MDA) formation based on the thiobarbituric-acid colormetric reaction (26). The production of TBARS is evaluated by measuring the lipid peroxidation of phosphatidylcholine first when antioxidants are in existence and assigning an oxidation rate of 100% to these samples. In terms of phosphatidylcholine oxidation, the amount of TBARS, which is a breakdown product of phosphatidylcholine that is produced during lipid peroxidation, can be used as an index of lipid peroxidation in phosphatidyl. Our results showed that BHT and quercetin have highest antioxidant activities, and the relative TBARS values (%) of MeOH and EW (9:1) were significantly higher than those of EA, AT, and ascorbic acid. However, although no significant differences were observed, MeOH and EW (9:1) showed an increasing tendency in the relative TBARS value (%) when compared to EW (4:1) and EW (17:3).

Deoxyribose degradation by iron-dependent hydroxyl radical The hydroxyl radical-scavenging activities of grape seed extracts prepared with different solvents were measured by investigating the competition between deoxyribose and grape seed extracts against hydroxyl radicals generated from the Fe^{2+}-H_2O_2 system. As the hydroxyl radicals attack deoxyribose and set off a series of reactions, TBARS is eventually formed. 2-Deoxyribose is oxidized by OH formed through the Fenton reaction and degraded into malondialdehyde (27). Fig. 5 shows the hydroxyl radical-scavenging abilities of grape seed extracts obtained from various solvents. The extents to which hydroxyl radicals generated by Fe^{2+}-H_2O_2 decompose deoxyribose were 5.49, 89.49, 87.8, 78.7, 56.7, 73.8, 64.6, 60.4, and 75.6% in ascorbic acid, BHT, quercetin, AT, EA, MeOH, EW (4:1), EW (9:1), and EW (17:3), respectively. In this study, MeOH, AT, and EW (17:3) exhibited stronger inhibition effects on deoxyribose oxidation than other grape seed extracts. Furthermore, all grape seed extracts showed higher inhibition levels (%) than ascorbic acid against deoxyribose degradation. Accordingly, grape seed extracts were able to protect deoxyribose from the damage associated with the direct binding of iron to deoxyribose and the subsequent attack of OH radicals generated via the Fenton reaction.

Inhibition of oxidation induced by AAPH Oxidation of serum exposed to AAPH was followed by the production of TBARS. The antioxidant abilities of grape seed extracts are shown in Fig. 6. 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) is a well-known water-soluble radical initiator, which has been also introduced as a successful initiator of lipid peroxidation. AAPH allows carbon-centered radicals to be formed and causes the transformation of lipids and DNA under aerobic conditions, where alkylperoxyl radicals and alkylperoxides are yielded (28). The antioxidant capacity of grape seed extracts was measured using peroxyl radicals generated

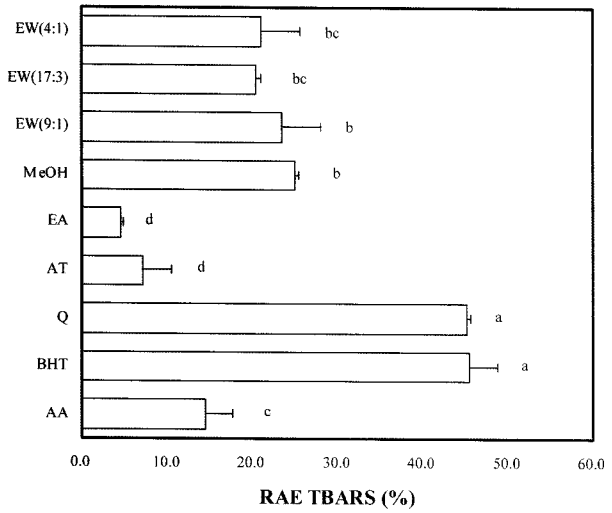

Fig. 4. Relative TBARS of grape seed extracts with different solvents by egg yolk peroxidation. Each bar is a mean standard deviation for triplication experiment. Alphabets on the bars show a statistical difference at $p<0.05$ by Duncan's multiple range test. *AA = Ascorbic acid, BHT = Butylated hydroxytoluene, Q = Quercetin, AT = Acetone, EA = Ethyl acetate, MeOH = Methanol, EW (9:1) = Ethyl acetate : Water (9:1), EW (17:3) = Ethyl acetate : Water (17:3), EW (4:1) = Ethyl acetate : Water (4:1).

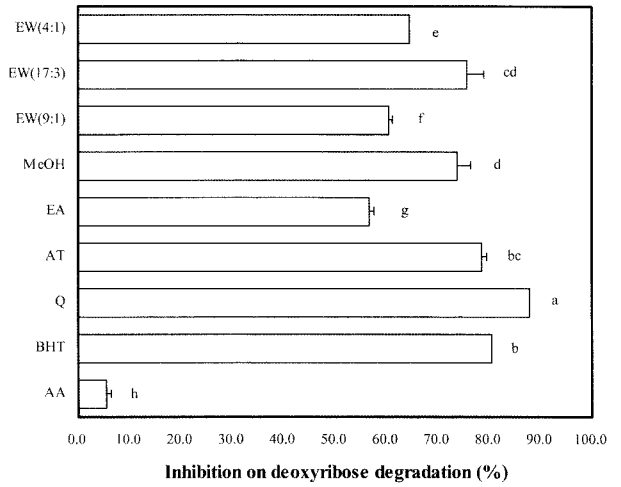

Fig. 5. Inhibition on deoxyribose degradation of grape seed extracts with different solvents by hydroxyl radicals induced by Fe^{2+}. Each bar is a mean standard deviation for triplication experiment. Alphabets on the bars show a statistical difference at $p<0.05$ by Duncan's multiple range test. *AA = Ascorbic acid, BHT = Butylated hydroxytoluene, Q = Quercetin, AT = Acetone, EA = Ethyl acetate, MeOH = Methanol, EW (9:1) = Ethyl acetate : Water (9:1), EW (17:3) = Ethyl acetate : Water (17:3), EW (4:1) = Ethyl acetate : Water (4:1).

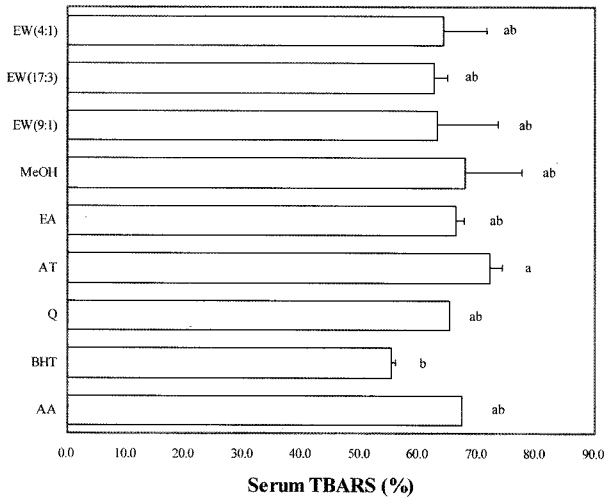

Fig. 6. Serum TBARS of grape seed extracts with different solvents on AAPH-induced peroxidation. Each bar is a mean standard deviation for triplication experiment. Alphabets on the bars show a statistical difference at $p<0.05$ by Duncan's multiple range test. *AA = Ascorbic acid, BHT = Butylated hydroxytoluene, Q = Quercetin, AT = Acetone, EA = Ethyl acetate, MeOH = Methanol, EW (9:1) = Ethyl acetate : Water (9:1), EW (17:3) = Ethyl acetate : Water (17:3), EW (4:1) = Ethyl acetate : Water (4:1).

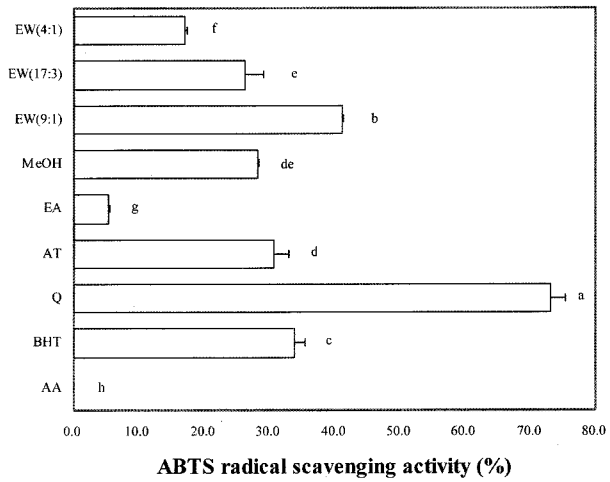

Fig. 7. ABTS radical scavenging activities of grape seed extracts with different solvents. Each bar is a mean standard deviation for triplication experiment. Alphabets on the bars show a statistical difference at $p<0.05$ by Duncan's multiple range test. *AA = Ascorbic acid, BHT = Butylated hydroxytoluene, Q = Quercetin, AT = Acetone, EA = Ethyl acetate, MeOH = Methanol, EW (9:1) = Ethyl acetate : Water (9:1), EW (17:3) = Ethyl acetate : Water (17:3), EW (4:1) = Ethyl acetate : Water (4:1)

from AAPH in the serum. After the addition of AAPH to the serum, the oxidation of oxidizable materials was measured by TBARS produced in the reaction process. During the induction period, this oxidation is inhibited by the antioxidants in the serum. AT, MeOH, EA, and EW mixtures of 9:1, 17:3, and 4:1 showed 72, 68, 66, 63, 63, and 64% antioxidant levels, respectively. The AT extract demonstrated the highest antioxidant ability with all samples achieving higher than 50% antioxidant level. Furthermore, the grape seed extracts showed higher levels of antioxidant ability than BHT used as a reference compound.

ABTS free-radical scavenging activity Presented in Fig. 7 are the total antioxidant abilities of grape seed extracts determined by scavenging ABTS radical anions. Radical $ABTS^+$ scavenging activity is widely used to measure the radical scavenging capacity. The $ABTS^+$ radicals can be generated through an enzymatic or chemical oxidation reaction. In this study, the grape seed extracts prepared using various solvents were evaluated by measuring their scavenging activities against $ABTS^+$ radicals produced by AAPH. The study used 0.1 mg/mL as the concentration of grape seed extracts and commercial antioxidants. The antioxidant activity of ascorbic acid was not found at 100 ppm concentration. Quercetin proved to be the most effective among all samples, and the scavenging effect was higher in the EW (9:1) extract than BHT and other grape seed extracts. There was no significant difference between AT and MeOH in the scavenging ability, but showed higher antioxidant activities than EW (17:3) and EW (4:1). The EA extract showed the lowest antioxidant activity, as in the case of DPPH testing. These results indicate antioxidants can be obtained most effectively from grape seed extracts prepared with a mixed solvent of ethyl acetate and water, which show fast and potent inhibition of ABTS radical cations.

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Table 3. 50% effective concentration (EC_{50}) in DPPH radical scavenging of different solvent extracts from grape seeds

	AA	BHT	Q	AT	EA	MeOH	EW (9:1)	EW (17:3)	EW (4:1)
EC_{50} (mg/ml)	0.092	0.307	0.042	0.102	0.523	0.089	0.214	0.141	0.177

*AA = Ascorbic acid, BHT = Butylated hydroxytoluene, Q = Quercetin, AT = Acetone, EA = Ethyl acetate, MeOH = Methanol, EW (9:1) = Ethyl acetate : Water (9:1), EW (17:3) = Ethyl acetate : Water (17:3), EW (4:1) = Ethyl acetate : Water (4:1)

Table 4. 50% Effective concentration (EC_{50}) in ABTS radical scavenging of different solvents from grape seeds

	AA	BHT	Q	AT	EA	MeOH	EW (9:1)	EW (17:3)	EW (4:1)
EC_{50} (mg/mL)	5.508	0.131	0.085	0.291	2.382	0.325	0.252	0.147	0.436

*AA = Ascorbic acid, BHT = Butylated hydroxytoluene, Q = Quercetin, AT = Acetone, EA = Ethyl acetate, MeOH = Methanol, EW (9:1) = Ethyl acetate : Water (9:1), EW (17:3) = Ethyl acetate : Water (17:3), EW (4:1) = Ethyl acetate : Water (4:1)

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