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Comparative Study of Antioxidant Activity of Grape (*Vitis vinifera*) Seed Powder Assessed by Different Methods

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ABSTRACT

The phenolic content and antioxidant activity of grape (*Vitis vinifera*) seed powder extracted by *in vitro* physiological procedure and chemical procedure were investigated. The antioxidant potential of the extract were assessed by employing different *in vitro* assays such as CUPRAC, DPPH, ABTS, OH radical scavenging capacity, and peroxidation inhibiting activity. The *in vitro* physiological procedure yielded a higher phenolic content and antioxidant capacity than the chemical procedure. As for digestive enzymatic extracts, phenolic content and antioxidant capacity of the dialysates of grape seed powder were lower than those of the retentates. As for solvents extracts, extraction with acetone:water (70:30) led to the maximum phenolic content and antioxidant capacity, while water gave the lowest phenolic content and antioxidant capacity. Our results suggest that the biological properties of natural antioxidants determined by *in vitro* physiological procedure may be more useful for nutritional purposes than the values determined in solvent extracts.

Key words: grape seed powder, antioxidant activity, *in vitro* physiological procedure, chemical extraction

INTRODUCTION

Reactive oxygen species (ROS) cover a wide range of chemical components, including superoxide anion, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite. These free radicals have been implicated in over a hundred diseases in humans⁽¹⁻³⁾. However, the innate defense in human body may not be enough for severe or continued oxidative stress. Hence, exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the ROS.

Grape skins and seeds produced in large quantities by the winemaking industry are increasingly used to obtain functional food ingredients⁽⁴⁻⁵⁾. Grape seed is a better source of antioxidative constituents than skins of grape/wine byproducts. Functional ingredients of grape seed include several flavonoids with a phenolic nature such as monomeric flavanols, dimeric, trimeric and polymeric procyanidins, and phenolic acids⁽⁶⁻⁷⁾. The antioxidant activity of grape seed phenolic compounds is closely associated with activity against various cancer types, cardiovascular diseases and several dermal disorders⁽⁸⁾.

Water, aqueous mixtures of ethanol, methanol and acetone are commonly used to extract plant materials. The extracting solvents significantly affect extraction

yield, phenolic content and biological activities of plant materials⁽⁹⁻¹²⁾. It is not clear which solvent system is more effective in extracting phenolic content of different materials and evaluating the antioxidant activity. From a physiological point of view, the results using solvent extraction may differ quantitatively and qualitatively from extracts in the human gastrointestinal tract. However, feeding trials in human or animal subjects or model studies using intestinal sections are time consuming and expensive and often give variable results. On the other hand, the *in vitro* digestion method is simple, cheap, reproducible and widely applicable

The objective of this study was to conduct an assessment of the phenolic content and antioxidant activity of grape seed powder by *in vitro* digestive enzymatic extraction and chemical extraction.

MATERIALS AND METHODS

I. Chemicals

Ferric chloride, Folin-Ciocalteu's phenolic reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH), thiobarbituric acid (TBA), 2,9-dimethyl-1,10-phenanthroline (Neocuproine), piperazine-N,N-bis[2-ethane-sulfonic

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acid] disodium salt (PIPES), gallic acid and hydrogen peroxide were obtained from Sigma. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), (+)-catechin and (-)-epicatechin were obtained from Aldrich. Potassium persulfate was obtained from Merck. Enzymes pepsin (P-7000, porcine), pancreatin (P-1750, porcine) and bile extract (B-6831, porcine) were from Sigma. All other reagents were of analytical grade. The dialysis sac, with a molecular weight cut-off of 3600 (Spectrum Laboratories, Rancho Dominguez, CA, USA), was cut into 20 cm lengths to use.

II. Production of Grape Seed Powder

Cabernet Sauvignon grape (*Vitis vinifera*) seeds were obtained from wine technology laboratory of the Northwest A & F University (YangLin, China). Seeds were separated from the skin by rubbing the mixture over a coarse screen. Processing of grape seed powder included washing, drying, sterilizing, freezing and superfine grinding. Grape seed powders with a diameter of 2.5-22.5 μm were used.

III. Solvent Extraction

The extraction was carried out using four different solvents, i.e. ethanol:water (70:30, v/v), acetone:water (70:30, v/v), methanol and water. Grape seed powder (0.4 g) was extracted with 20 mL of solvents in a shaking incubator at 45°C for 2 h. The mixture was centrifuged at 5000 g for 10 min and subsequently decanted. The residue was re-extracted for 2 h and supernatants were combined and stored at -20°C until analyzed.

IV. In vitro Digestion

The digestion process used was that described by Argyri *et al.* (2006)⁽¹³⁾. Briefly, 0.2 g of grape seed powders were dissolved in 20 mL of phosphate buffer solution (pH 7.0). The homogenized sample extract was adjusted to pH 2.3 with HCl and pepsin was added and incubated at 37°C in a heated water bath for 2 h with shaking at 100 rpm. At the end of this incubation, pH of the samples was gradually adjusted from 2.3 to 6 with the aid of a dialysis sac, filled with 20 mL of PIPES buffer, pH 6.3. After 30 min, 5 mL of a pancreatin–bile salt mixture was added to the samples and the incubation continued for another 2 h. At the end of this incubation period, the dialysis sac was removed. The dialysates (fraction containing soluble compounds of molecular weight less than 3600) and the retentates (fraction containing soluble compounds of molecular weight above 3600) were collected. Then, samples were centrifugated (10 min, 5000 \times g) and supernatants were removed.

V. Determination of Total Phenolics (TP)

Amount of TP was assessed using Folin–Ciocalteu reagent procedure as described by Chaovanalikit and

Wrolstad (2004)⁽¹⁴⁾. Zero-point-five miniliter of Folin–Ciocalteu reagent and 7.9 mL deionized water were added to a test tube 0.1 mL of grape seed powder extract. The mixture was kept at room temperature for 10 min, and then 1.5 mL of 20 g/100 mL sodium carbonate was added. The mixture was heated in a water bath at 40°C for 20 min and then cooled in an ice bath before absorbance at 755 nm was measured. The results were expressed as gallic acid equivalents (GAE) per g of dry matter.

VI. Determination of Individual Phenolic by HPLC

A Shimadzu high performance liquid chromatograph consisting of a LC-10ATVP pump, a Rheodyne model 7725 injection valve with a 20 μL loop and a UV–Vis SPD-10AV detector was used. The separation of catechin, epicatechin and gallic acid was performed on a Shim-Pack VP-ODS C18 column (column 250mm \times 4.6mm). The elution conditions were as follows: flow rate 1 mL/min, column temperature 30°C, injection volume 20 μL , solvent A: water/ acetic acid (98:2, v/v), solvent B: acetonitrile/solvent A (80:20, v/v). A gradient elution was applied as follows: 0-35% B from 0 to 30 min, 35-50% of B in 5 min, 50-100% B from 35 to 50 min and 100% B isocratic for 5 min. Detection was at 280 nm. Peak identification was based on retention times and spiking with standard solutions. Quantification was performed using the calibration curves of each standard compounds.

VII. Hydroxyl Radical Scavenging

Hydroxyl radical scavenging was determined according to the method described by Halliwell, Gutteridge, and Aruoma (1987)⁽¹⁵⁾. Hydroxyl radicals were generated by hydrogen peroxide, ascorbate and FeCl_3 , in the presence or the absence of the test compound. The ability of the compound to compete with deoxyribose for scavenging hydroxyl radicals gives the rate constant of the reaction between hydroxyl radicals and the scavenger. The hydroxyl radical scavenging activity was expressed as EC_{50} values (mg grape seed powder per ml) for comparison.

VIII. Determination of Antioxidant Activity by DPPH Method

The ability for grape seed powder to scavenge DPPH free radicals was determined. Scavenging activity was based on the slightly modified method of Brandwilliams, Cuvelier and Berset (1995)⁽¹⁶⁾. Briefly, 0.1 mL of different extracts were added to 4 mL of a 6×10^{-5} M solution of DPPH in methanol. A control sample containing the same volume of solvent in place of extract was used to measure the maximum DPPH absorbance. After the reaction was allowed to take place in the dark for 30 min, the absorbance at 515 nm was recorded to determine the concentration of remaining DPPH. The DPPH radical scavenging activity was expressed as EC_{50} values for comparison.

IX. Antioxidant Activity by Radical Cation (ABTS⁺)

ABTS assay was based on the slightly modified method of Re *et al.* (1999)⁽¹⁷⁾. ABTS radical cation (ABTS⁺) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS⁺ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 732 nm. After addition of 100 μ L of sample or trolox standard to 3.9 mL of diluted ABTS⁺ solution, absorbance was measured at exactly 6 min. Results were expressed as trolox equivalent antioxidant capacity (TEAC).

X. Determination of Reducing Power

The cupric reducing antioxidant capacity (CUPRAC) of the extracts of grape seed powders was determined according to the method of Apak *et al.* (2004)⁽¹⁸⁾. Seven-point-five minimolar neocuprine, and NH₄Ac buffer (1 M, pH 7.0) solutions were added to a test tube 1 mL each of 10 mM Cu(II). Extracts were added to the initial mixture so as to make the final volume of 4.1 mL. The tubes were stoppered and the absorbance at 450 nm was recorded against a reagent blank after 30 min. Results were expressed as trolox equivalent antioxidant capacity.

XI. Antioxidant Activity by Thiobarbituric Acidreactive Substances Assay (TBARS)

The quantitative evaluation of the antioxidant capacity of the compounds against lipid peroxidation was determined through TBARS assay. Small unilamellar vesicles were prepared as described⁽¹⁹⁾ by sonication of multilamellar vesicles of egg yolk phosphatidylcholine. One miniliter of small unilamellar vesicles dispersion was incubated for 10 min at 37°C with extract and after

that the free radical generator AAPH was added (10 mM) to the mixture. The samples were incubated at 37°C for 2 h. The colorimetric reaction with thiobarbituric acid was then carried out by adding 250 μ L of sodium dodecyl sulfate (3 g/100 mL), 500 μ L of TBA (1 g/100 mL) and 500 μ L of HCl 7 mM to the samples. The mixture was heated at 95°C for 15 min and rapidly cooled on ice. The chromogen was extracted into 3 mL of butanol. Formation of TBARS was measured at 532 nm.

XII. Statistical Analysis

Experimental results were means \pm SD of three parallel measurements. Analysis of variance was performed by ANOVA procedures (DPS 7.55 for Windows). Significant differences between means were determined by Duncan's Multiple Range tests. Two significant levels (0.05 and 0.01) were employed.

RESULTS AND DISCUSSION

I. Phenolic Content of Extracts

Plant-derived phenolic compounds are well known to exhibit antioxidant activity through a variety of mechanisms, including free radical scavenging, lipid peroxidation and chelating of metal ions⁽²⁰⁾. Total phenolic contents of the grape seed powder extracted with different solvents were examined (Table 1). The amount of total phenolics varied among the different extracts and ranged from 0.75 to 4.04 g GAE/100 g dry matter. The amount of total phenolics in the digestive enzymatic extracts including the dialysates (fraction inside the dialysis sac) and the retentates (fraction outside the dialysis sac) were significantly higher than that in common solvent extracts. Total phenolics of the dialysates were

Table 1. Phenolic content in grape seed powder extracts

Sample	Total phenolic (GAE g/100 g powder)	Gallic acid mg/100 g powder)	Catechin (mg/100 g powder)	Epicatechin (mg/100 g powder)
methanol	2.02 \pm 0.07 D	23.4 \pm 3.7 BC	115 \pm 10.6 D	123 \pm 13.5 BC
ethanol:water (70:30)	2.53 \pm 0.06 C	14.2 \pm 1.1 D	162 \pm 19.3 B	121 \pm 10.2 BC
water	0.75 \pm 0.02 F	6.7 \pm 0.8 E	88 \pm 6.8 E	69 \pm 5.2 E
acetone:water (70:30)	2.98 \pm 0.04 B	21.4 \pm 1.8 C	149 \pm 12.9 C	117 \pm 15.0 C
dialysate	1.35 \pm 0.06 E	10.0 \pm 0.7 E	103 \pm 9.7 D	89 \pm 5.8 D
retentate	2.69 \pm 0.15 C	25.1 \pm 2.2 B	170 \pm 20.1 B	130 \pm 7.3 B
total digestive extracts	4.04 \pm 0.20 A	35.1 \pm 2.7 A	273 \pm 29.5 A	219 \pm 12.0 A

Value is expressed as mean \pm standard deviation (n = 3). GAE is gallic acid equivalent. Values that are followed by different letters are significantly different (P < 0.01). Dialysate is fraction inside the dialysis sac (fraction containing soluble compounds of molecular weight less than 3600) by *in vitro* physiological procedure. Retentate is fraction outside the dialysis sac (fraction containing soluble compounds of molecular weight above 3600) by *in vitro* physiological procedure.

significantly lower than those of the retentates. As for solvents extracts, extraction with acetone:water (70:30) led to the maximum phenolic content, while water gave the lowest phenolic content. This result indicated that aqueous solution of acetone was better than a single-compound solvent system for extraction of total phenolic from plant materials. We can establish the order of all the extracts with highest value of polyphenol content as follows: digestive enzymatic extracts > acetone:water > ethanol:water > retentate > methanol > dialysate > water.

Individual phenolic content (CT, EC, GA) determined by HPLC are presented in Table 1. Catechin, epicatechin and gallic acid content ranged from 88.7 to 274, 69.2 to 219.4, and 6.7 to 35.1 mg/100 g grape seed powder, respectively. Among individual phenolic content, the amounts of catechin and epicatechin were higher than gallic acid content. Three individual phenolics in the digestive enzymatic extracts including the dialysates and the retentates were significantly higher than that in common solvents extracts. Grape seeds contained higher amounts of monomeric, oligomeric, and polymeric flavan-3-ols than the different parts of the grape. Polymeric proanthocyanidins represented the largest proportion of the total flavan-3-ol content in the grape seeds. Polymeric proanthocyanidins possessed the property of liberating monomeric units under heated acidic conditions as result of the interflavanic bond cleavage. The flavan-3-ol monomeric units found in *Vitis vinifera* grapes were (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and (-)-epigallocatechin. Thus, the amounts of catechin and epicatechin of grape seed powder extracts by *in vitro* physiological procedure were higher because of acid and enzymatic depolymerization.

Grape seed is a complex matrix containing approximately 40% fiber, 16% oil, 11% proteins, and 7% complex phenols including tannins, in addition to sugars, and mineral salts, etc. The enzymatic treatments hydrolyze starch and protein, which may favor the release of phenolic compounds. Hydrolyzable phenolics and condensed tannins may be hydrolysed partially by the enzymatic and acid treatments. Janisch *et al.* (2006) investigated the flavonoids of grape seed powder under condition of simulated digestion by HPLC analysis⁽²¹⁾. Similarly, Nakamura and Tonogai (2003) reported the metabolism of grape seed phenolic compounds in rats by HPLC⁽²²⁾. All these results indicate that the release of phenolic compounds in the gastrointestinal tract is not only quantitatively but also qualitatively different from that in the chemical extraction.

II. Hydroxyl Radical Scavenging Activity

Hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology⁽³⁾. The hydroxyl radical scavenging activity of different extracts of grape seed powder is shown in Figure 1 and

the results are expressed as EC₅₀ values for comparison. Effectiveness of antioxidant properties inversely correlated with their EC₅₀ values. In our study, all the grape seed powder extracts exhibited appreciable hydroxyl radical scavenging activity ranging from 0.12 to 1.92 mg/mL. Acetone:water (70:30) extract of grape seed powder was more effective than other extracts as evidenced by lower EC₅₀ values. Hydroxyl radical scavenging activity of the extracts can be ordered as follows: acetone:water > ethanol:water > methanol > retentate > dialysate > water. Ahn *et al.* (2002) also reported that the radical scavenging activity evaluated by Chemiluminescence assay is 94.87% at 0.5 mg/mL concentration of grape seed⁽²³⁾.

III. DPPH Radical Scavenging Activity

The free radical scavenging activity of different solvent extracts of grape seed powders were determined by the DPPH method and the results are shown in Figure 2. Antioxidant molecules can quench DPPH free radicals and convert them to a colourless product, resulting in a decrease in absorbance at 517 nm. In our study, all the grape seed powder extracts exhibited appreciable scavenging activity ranging from 3.35 to 11.8 mg/mL (EC₅₀). The highest DPPH scavenging activities were shown by acetone–water extract of grape seed powder and the lowest DPPH scavenging activities were shown by water extract of grape seed powder. There was no significant difference between the scavenging activity of metha-

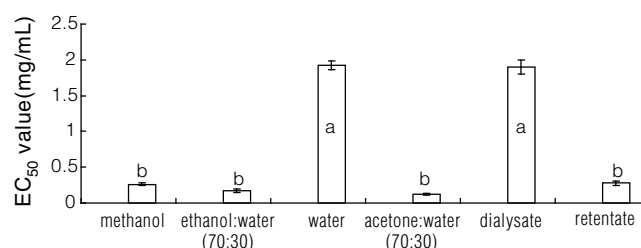


Figure 1. Hydroxyl radical scavenging activity of grape seed powder extracts. Values are means of triplicate determinations ($n = 3$) \pm standard deviation ($P < 0.05$). Dialysate is fraction inside the dialysis sac. Retentate is fraction outside the dialysis sac.

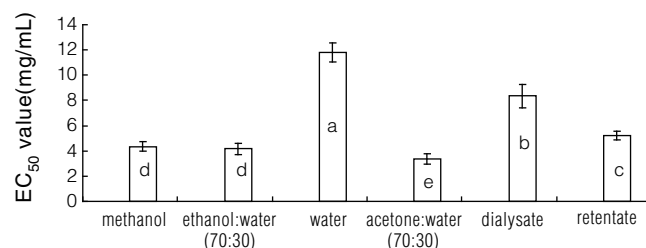


Figure 2. Free radical scavenging activity of grape seed powder extracts analyzed by DPPH method. Values are means of triplicate determinations ($n = 3$) \pm standard deviation ($P < 0.05$). Dialysate is fraction inside the dialysis sac. Retentate is fraction outside the dialysis sac.

nol and ethanol:water (70:30). For digestive enzymatic extracts, the retentates were superior over the dialysates in scavenging DPPH radicals. This result indicates that the amount of DPPH scavenging activity appeared to depend on the phenolic concentration of the extracts of grape seed powder.

Guendez *et al.* (2005) found that there is a significant correlation between DPPH scavenging activities of grape seed extracts and total phenolic content ($r = 0.82$, $P < 0.01$)⁽⁷⁾. In our study, there was a higher correlation ($r = -0.93$, $P < 0.01$). In addition, grape seed extracts exhibited the higher antioxidant activity compared to synthetic food antioxidants BHA, BHT, ascorbyl palmitate and to the natural food antioxidant, vitamin E^(10,12).

IV. ABTS Radical Cation Scavenging Activity

The effect of different solvent extracts of grape seed powder on ABTS radical cation scavenging activity is presented in Table 2. The activity of the tested sample extracts was expressed as Trolox equivalent (TE). High TEAC value indicates that the mechanism of antioxidant action of extracts was as a hydrogen donor and it could terminate the oxidation process by converting free radicals to the stable forms. In present study, all extracts possessed free radical-scavenging activity but at different levels. The highest activity was obtained from the digestive enzymatic extracts, with the TEAC value of $962.3 \pm 15.3 \mu\text{M TE/g dry matter}$. The digestive enzymatic extracts had the greatest ABTS scavenging activity, which was from 1.4 to 10.8-fold higher than the chemical extracts. The TEAC value of the dialysates was lower than that of the retentates. The antioxidant activity of plant foods daily consumed in the Spanish diet was determined by ABTS, and the result also revealed the *in vitro* physiological procedure yielded a higher anti-

oxidant capacity than the chemical procedure⁽²⁴⁾. These results indicate that determination of antioxidant capacity in food chemical extracts may underestimate the real antioxidant capacity that may be in close contact with the intestinal lumen. Therefore, the biological properties of antioxidants possibly depend on their release from the food matrix during the digestion process and may be more useful for nutritional purposes than the values determined in solvent extracts. In addition, we found that there is a moderate correlation between ABTS scavenging activities of grape seed extracts and total phenolic content ($r = 0.65$, $P < 0.05$). The antioxidant capacity of the extracts can be ordered as follows according to the results of ABTS⁺ radical bleaching: total digestive extracts > ethanol:water > retentate > methanol > acetone:water > dialysate > water. There was no significant difference among the scavenging activity of methanol, ethanol:water (70:30) and acetone:water (70:30).

V. Reducing Power Assay

The reducing power property indicates that the antioxidant compounds are electron donors and can reduce the oxidized intermediates of the lipid peroxidation process⁽²⁵⁾. FRAP assay is the most widely used method to determine the reducing power of antioxidants. However, FRAP has two major flaws: (1) FRAP assay is conducted at acidic pH 3.6 to maintain iron solubility; (2) FRAP assay does not measure thiol antioxidants, such as glutathione. Thus, FRAP may not give comparable relative values in physiological conditions. In the present study, we used CUPRAC assay which was based on reduction of Cu(II) to Cu(I) by antioxidants. The data for the reducing potential of different grape seed powder extracts are presented in Table 2. The result clearly indicated that the digestive enzymatic extracts of grape seed powder had the highest reducing power with the TEAC value of $3068 \pm 105 \mu\text{M TE/g dry matters}$. The digestive enzymatic extracts had the greatest antioxidant activity, which was from 1.8 to 6.5-fold higher than the chemical extracts. Other studies have revealed that the *in vitro* physiological procedure yielded a higher reducing power than the chemical procedure⁽²⁶⁾. Those results indicated that more antioxidants are liberated from solid grape seed powder because of acid and enzymatic depolymerization under *in vitro* physiological condition. Similar to the result of ABTS assay, the retentates appeared to have a higher reducing power. In addition, there is a moderate correlation between reducing power of grape seed extracts and total phenolic content ($r = 0.5$, $P < 0.05$). Reducing power of different solvent extracts of grape seed powder exhibited the following order: digestive enzymatic extracts > retentate > acetone:water > ethanol:water > methanol > dialysate > water. The reducing properties are generally associated with the presence of reductones. It is presumed that the grape seed powders phenolic compounds may act in a similar fashion as

Table 2. Antioxidant capacity of grape seed powder determined by ABTS and CUPRAC

Sample	ABTS assay ($\mu\text{M TE/g dry matter}$)	CUPRAC ($\mu\text{M TE/g dry matter}$)
methanol	664.2 ± 12.1 B	1328.3 ± 28.4 C
ethanol:water (70:30)	673.5 ± 10.2 B	1380.8 ± 23.1 C
water	88.6 ± 3.0 D	470.7 ± 8.9 D
acetone:water (70:30)	659.6 ± 10.5 B	1668.3 ± 45.0 B
Dialysate	291.3 ± 5.8 C	1321.5 ± 35.3 C
Retentate	671.0 ± 8.6 B	1746.5 ± 29.1 B
total digestive extracts	962.3 ± 10.0 A	3068.0 ± 61.1 A

Value is expressed as mean \pm standard deviation ($n = 3$). TE is Trolox equivalent. Values that are followed by different letters are significantly different ($P < 0.01$). Dialysate is fraction inside the dialysis sac. Retentate is fraction outside the dialysis sac.

reductones by donating electrons to terminate the free radical chain reaction.

VI. Antioxidant Activity by TBARS

Lipid peroxidation may cause peroxidative tissue damage in inflammation, cancer, toxicity of xenobiotics and aging⁽²⁷⁾. Some authors reported inhibition of peroxidation by extracts of grape seed in different model systems including linoleic acid peroxidation, rat liver peroxidation, copper-induced LDL oxidation and algae oil-in-water oxidation^(21,23,28).

We measured the potential of different extracts of grape seed powder to inhibit lipid peroxidation in egg yolk phosphatidylcholine, induced by AAPH peroxy radicals. In the present investigation, all sample extracts exhibited 55.1% to 81.8% inhibition of peroxidation at the present concentration in the reaction mixture (Figure 3). Effectiveness of dialysates and retentates towards inhibition of peroxidation was found to be greater than that of solvents extracts except the methanol extract. Interestingly, inhibition of peroxidation of water extract approached that of the ethanol and acetone extracts. In addition, there was no significant difference of the inhibition of peroxidation between dialysate and retentate. This result was different from the results of other methods and there was not a significant correlation between inhibition of peroxidation of grape seed extracts and total phenolic content. Inhibition of peroxidation of all extracts of grape seed powder exhibited the following order: methanol > dialysate > retentate > acetone:water > ethanol:water > water.

CONCLUSIONS

The results obtained in the present work denote that grape seed may constitute a good source of healthy compounds, therefore useful in the prevention of diseases in which free radicals are implicated. In addition, the biological properties of grape seed powder determined

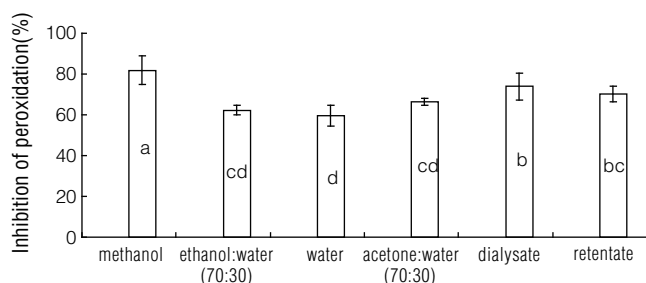


Figure 3. Inhibition of peroxidation of extracts of grape seed powder (10 mg/mL) as measured by the TBARS method. Values are means of triplicate determinations ($n = 3$) \pm standard deviation ($P < 0.05$). Dialysate is fraction inside the dialysis sac. Retentate is fraction outside the dialysis sac.

by *in vitro* physiological procedure may be more useful for nutritional purposes than the values determined in aqueous-organic extracts. However, further research is needed to identify the relation between the *in vitro* digestion method and the *in vivo* feeding trials.

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