Isolation and Characterization of Endothelium-Dependent Vasorelaxing Compounds from Grape Seeds

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Previous work has shown that red wines, grape juices, and other grape products cause endothelium-dependent relaxation (EDR) of blood vessels in vitro by increasing nitric oxide production. In this paper we describe the isolation and characterization of some of the compounds responsible for EDR activity. Concord grape seeds were extracted with methanol and the compounds were separated by Toyopearl TSK HW-40S chromatography. Resulting fractions (primarily phenolic acids, catechins, and proanthocyanidins) were further separated semipreparatively by reversed-phase HPLC, and peaks were collected and bioassayed for EDR activity using the rat aorta preparation. EDR-active compounds were subsequently characterized by HPLC retention times and electrospray−ion-trap mass spectrometry. The compounds exhibiting the most EDR activity were proanthocyanidin trimers, tetramers, pentamers, and polymers and their gallates, as well as a dimer gallate (EC_{50} values in the range of 0.6−2.5 μg catechin equivalents/mL). These compounds should be useful for in vitro and in vivo studies, particularly as they relate to improvement of cardiovascular function.

Keywords: *Grape seed; proanthocyanidin; endothelium; EDR; nitric oxide; mass spectrometry*

INTRODUCTION

Considerable attention is currently focused on the putative cardioprotective effects of red wine and other grape products and, indeed, increased consumption of fruits and vegetables in general. These beneficial effects are usually attributed to the well-known antioxidant effects of various flavonoid compounds found in grapes and other food plants (Frankel et al., 1993; Wang et al., 1996; Rice-Evans et al., 1997; Koga et al., 1999). We have shown (Fitzpatrick et al., 1993) that several red wines, grape juice, and grape skin extracts cause endothelium-dependent relaxation (EDR) of rat aortic rings, that this activity is accompanied by elevated levels of cyclic GMP in the aortic rings, and that these effects are blocked by nitric oxide synthase inhibitors such as N^{G}-nitro-L-arginine and its methyl ester. Subsequently, EDR activity was demonstrated in extracts of a variety of other commonly consumed fruits, vegetables, nuts, and spices (Fitzpatrick et al., 1995). Andriambeloson et al. (1997) and Cishek et al. (1997) have observed similar EDR effects induced by red wine and wine polyphenolic compounds. Thus, in addition to the well-recognized antioxidant effects of these types of compounds, they may also provide the additional benefits of enhanced nitric oxide production by endothelial cells.

Little information is available concerning the identity of the compounds responsible for EDR activity. Leucocyanidol (flavan-3,4-ol) produced EDR effects and increased NO production in rat aorta, whereas (+)-catechin, a closely related compound, had no such effect (Andriambeloson et al., 1997). Epicatechin 3-*O*-gallate isolated from grape seeds was shown to have some EDR activity (Fitzpatrick et al., 1997), but other unidentified catechin-type compounds, including the proanthocyanidins, appeared to be considerably more active.

The proanthocyanidins are a complex group of compounds made up of oligomers and polymers of polyhydroxyflavan-3-ol monomer units, which are (+)-catechin and (−)-epicatechin in the case of procyanidins (Ricardo da Silva et al., 1991). The units are most often linked by 4→8 interflavanoid bonds, and less often by 4→6 linkage. Some such compounds may also be galloylated at the C-3 position on one or more of the constituent units in the procyanidin molecule. Only in recent years have many of the constituent procyanidins of plants been isolated and identified (Ricardo da Silva et al., 1991; Escribano-Bailon et al., 1992; Prieur et al., 1994; De Freitas et al., 1998). Most of these studies have utilized grape seeds, a rich and easily extractable source of proanthocyanidins. The aims of this study were to isolate and characterize the compounds in grape seed extracts that are (1) EDR-active; (2) of relatively low molecular weight; and, (3) present in relatively high concentrations in the extracts.

MATERIALS AND METHODS

Preparation of Seed Extracts and Preliminary Fractionation by Toyopearl Chromatography. Frozen Concord grapes (kindly provided by Welch Foods Inc., Concord, MA) were thawed and seeds were removed by hand, rinsed, and dried. The seeds (25 g) were crushed, and were extracted by stirring with methanol (100 mL, 30 min at room-temperature × 3). Following filtration, the methanol was evaporated, and the residue was dissolved in 6 mL of methanol and centrifuged to remove undissolved material. Total recovery of phenolics in the extract was 204 ± 24 mg catechin equivalents from 25 g of seeds. Five ml of the extract was placed on a preparatory

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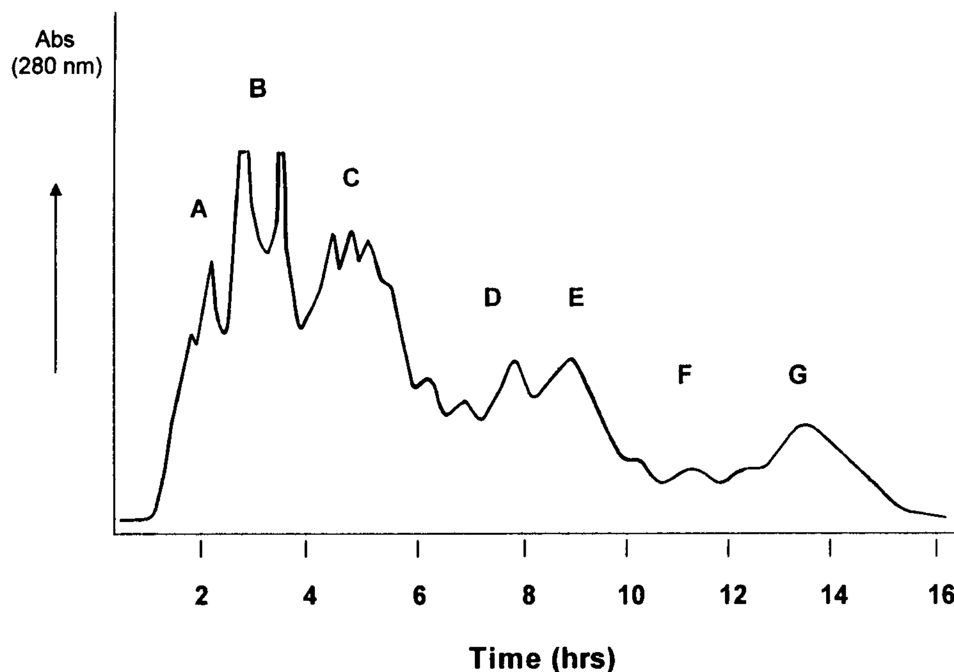

Figure 1. Toyopearl TSK HW(40) elution profile of grape seed extract eluted with methanol, absorbance measured at 280 nm.

column containing Toyopearl TSK HW-40 (F) (35 × 2.5 cm i.d.) preequilibrated in methanol and, using methanol as mobile phase, eluted at a rate of 0.8 mL/min. Seven fractions were collected, the methanol was evaporated, and the residues were dissolved in water (for bioassay) or methanol (for spectrometry and HPLC). The EDR-active compounds were found, in preliminary investigation, to be readily soluble in both methanol and water. The concentration of phenolics in each fraction was estimated by the general phenolic assay method of Hagerman and Butler (1994) using (+)-catechin as calibration standard, and expressing concentrations as catechin equivalents. Aliquots of each fraction were used for bioassay of vasorelaxing activity and for HPLC and mass spectrometry as described below.

Analytical HPLC. A Waters HPLC system was employed and consisted of a U6K injector, two 510 pumps, and a 481 UV/Vis detector, in conjunction with a Radial Pak reverse-phase NovaPak C18 column, protected by a guard column of the same material. The gradient was similar to the water/acetic acid gradient of Sun et al. (1998). Mobile phase A was water, mobile phase B was 10% acetic acid in water, and the gradient ran from 25% B up to 75% B over the first 47 min; from 85 to 100% B over 47 to 50 min; and 100% B isocratic over 50 to 55 min. Thereafter, the gradient was returned to mobile phase A to prepare for the next run. A 10 to 30 μL aliquot of original extract in methanol or Toyopearl fractions was injected. Flow rate was 1.0 mL/min and detection was made at 280 nm.

Semipreparative HPLC for Peak Collection. HPLC for peak collection involved the same HPLC procedure as described above except that larger sample volumes were used (100 μL) and peaks were collected manually. To obtain sufficient peak material for subsequent bioassay, mass spectrometry, and re-HPLC, several identical HPLC runs were made and peaks were collected. Corresponding peaks from the several runs were combined, frozen in a dry ice/acetone bath, then lyophilized. The resulting residue was dissolved in water (for bioassay) or methanol (for mass spectrometry and re-HPLC). In some cases, peak material was treated with tannase for detection of gallates by the method of Rigaud et al. (1993).

Electrospray–Ion-Trap Mass Spectrometry (ES–IT-MS). Fractions eluted from the Toyopearl column and individual peaks were examined by mass spectrometry using a Bruker-Esquire ITMS with an electrospray ionization source and run in the negative ion mode (Fries et al., 1998; Fleming et al., 1999). The electrospray matrix was 80% MeOH/20% H_2O. A syringe pump was used to deliver the samples to the needle with a flow rate of 25 μL/hr.

Aortic Ring Preparation and Bioassay of Fractions and Peaks. The procedure for preparation of rat aortic rings and general aspects of determining mechanical activity has been previously described (Fitzpatrick et al., 1993). Briefly, male Sprague–Dawley rats (200–250 g) were euthanized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and bled. The thoracic aorta was excised and cleaned, and rings 3–4 mm in length were cut, taking care not to disturb the endothelium. In some instances, the endothelium was deliberately removed by gently rubbing the lumen with a curved forceps. The rings were suspended in tissue baths containing a physiologic salt solution (in millimolar): 118 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 0.026 EDTA, 1.5 $CaCl_2$, and 11 glucose. The solution was bubbled continuously with O_2/CO_2 (95%/5%), and maintained at 37 °C. Activity was recorded on a Grass polygraph. After equilibration for at least 1 h under 1.5 g of tension, tissues were contracted submaximally (approximately 80% of Emax) with 1 μM phenylephrine. Then 3 μM acetylcholine, a known EDR-active compound, was added to the bath to test for intactness of the endothelium. This concentration of acetylcholine is sufficient to produce maximum endothelium-dependent relaxation in intact rings. Preliminary experiments produced a mean (± SEM) EC_{50} value of 0.14 ± 0.02 μM as an indication of the sensitivity of aortic rings to the contractile effects of phenylephrine, whereas the EC_{50} for EDR to acetylcholine was 0.12 ± 0.06 μM. Rings were washed with physiological salt solution three times over the next 45 min. prior to the next sequence.

Screening of extracts, Toyopearl fractions, and HPLC peaks was conducted as follows. Aortic rings were contracted by addition of phenylephrine, and cumulative additions of each sample were made, beginning with a concentration determined in preliminary experiments to be below the threshold for relaxation, and increased until a relaxation of approximately 15% (relative to the relaxation induced by 3 μM acetylcholine) was achieved. The concentration of sample required to produce this degree of relaxation (15%) was arbitrarily set as the “threshold” for demonstrating relaxation potency for the purpose of rapidly screening the many samples. Subsequently, full concentration–response curves were generated for peak compounds exhibiting the greatest relaxing activity. To test for endothelium-dependence, denuded aortic rings were used. Successful endothelium removal was established by a lack of relaxation response to 3 μM acetylcholine. Upon testing of the

fractions and peaks, none exhibited any relaxing activity using de-endothelialized rings.

Statistics. Tabular data are presented as either means ± SEM or 95% confidence intervals. Effective concentrations of extracts/Toyopearl fractions/HPLC peaks required for producing 50% relaxation (EC_{50}) were determined from concentration−relaxation response curves, utilizing nonlinear regression analysis software (GraphPad Prizm, San Diego, CA).

RESULTS AND DISCUSSION

Preliminary Fractionation using Toyopearl. Toyopearl TSK HW-40S fractionation of grape seed polyphenolics using methanol as eluent yielded 7 fractions, labeled A through G (Figure 1). The compounds present in the early fractions were easily identified by HPLC by their coelution with available standards, e.g., fraction A contained primarily gallic acid, fraction B contained (+)-catechin and (−)-epicatechin, and fraction C contained (−)-epicatechin gallate plus other compounds subsequently identified as flavan-3-ol dimers. These three fractions exhibited very little EDR activity when bioassayed, whereas the remaining Toyopearl fractions containing proanthocyanidins displayed varying degrees of vasorelaxing potencies (see below).

HPLC Separation of Grape Seed Polyphenolics. Figure 2 shows the HPLC pattern for the entire grape seed extract (Figure 2A), an EDR-active Toyopearl fraction (fraction G, Figure 2B), and one peak within this fraction (peak G6, Figure 2C). The fraction G and peak G6 chromatograms are considerably expanded compared to those of the entire extract. The entire extract contained 11 major peaks, many of which, from inspection, appeared to contain more than one compound. This became more apparent by the fact that more than 30 peaks were resolved by Toyopearl fractionation, followed by HPLC. Fraction G contains 7 major peaks, 6 of which exhibited EDR activity (Table 1). Peak G6 was of particular interest to us because (1) it exhibits relatively potent EDR activity (see below); (2) its molecular mass is relatively small; and, (3) it is present in relative abundance compared to other EDR-active compounds. Therefore this peak was studied in more detail by mass spectrometry, as described below.

EDR Activity of Grape Seed Proanthocyanidins. Relaxation responses of the original grape seed extract, one of the Toyopearl fractions (fraction G), and one of the peaks comprising fraction G (peak G6) are shown in Figure 3. All three samples relaxed phenylephrine-contracted, intact (endothelium present) rat aortic rings. None of the three relaxed de-endothelialized rings (not shown). N^G-Nitro-L-arginine, an inhibitor of nitric oxide synthase, reversed the relaxation. L-Arginine, the normal substrate for nitric oxide synthase, by competing with N^G-nitro-L-arginine, converted the contraction back to a relaxation. Finally, sodium nitroprusside (an endothelium-*in*dependent vasodilator) completely relaxed all aortic rings. These results indicate that the proanthocyanidin-containing samples cause relaxation by stimulating nitric oxide production and release by endothelial cells.

Mass Spectrometry of Grape Seed Compounds. Electrospray−ion-trap mass spectrometry was performed on all of the Toyopearl fractions and the major individual peaks in each fraction. In some cases MS/MS was also run on individual peaks in order to determine the proanthocyanidin structure of the compounds. Figure 4A shows the negative mode ES−ITMS

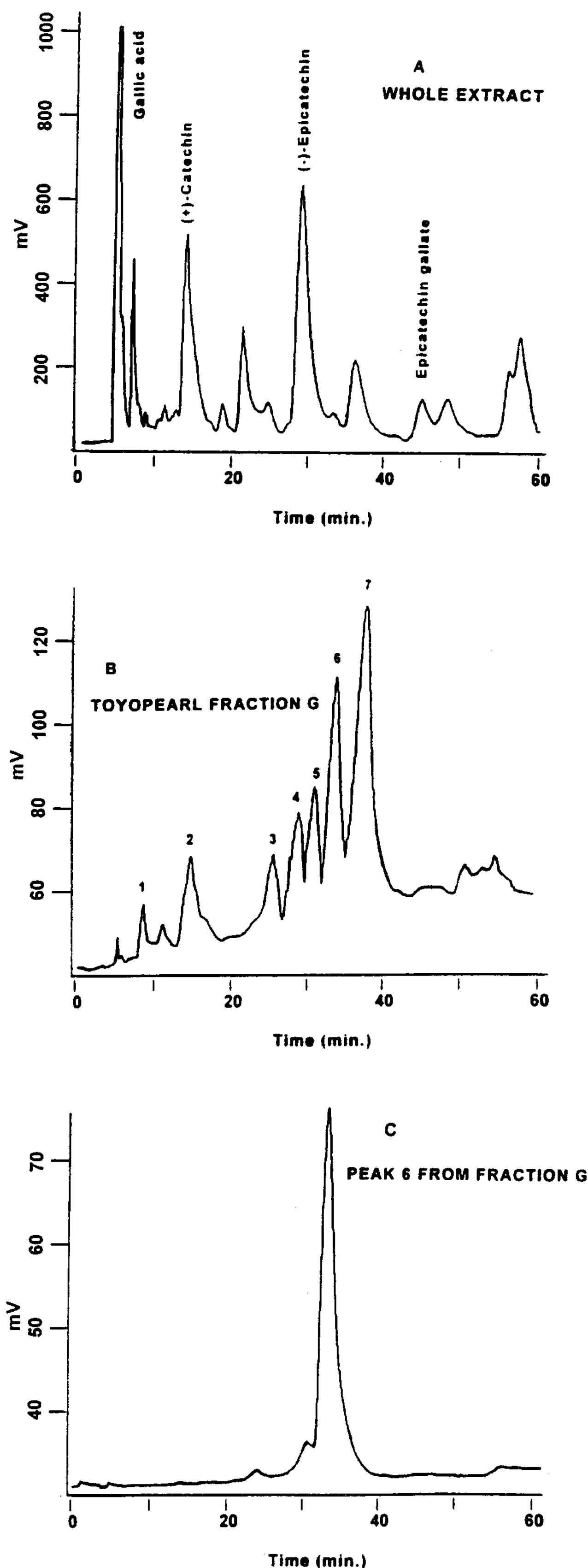

Figure 2. HPLC pattern, monitored at 280 nm, of A, the original grape seed extract; B, Toyopearl fraction G; and, C, peak G6 isolated from Toyopearl fraction G. All samples were dissolved in methanol prior to injection.

spectrum of the HPLC peak G6 after the material had been freeze-dried then redissolved in methanol and

Table 1. HPLC Retention Times, Relaxation Data, and ES−ITMS Information on Toyopearl Fractions and HPLC Peaks[a]

HPLC fraction	HPLC peak	HPLC peak retention times (min)	threshold[b] for EDR (μg/mL)	ES−ITMS info *m*/*z* [M−H]$^-$	ES−ITMS peak compounds
fraction A		6.2; 7.5	NR	169; n.d.	gallic acid, other phenolic acid
fraction B		13.1; 28.3	NR	289	flavanol monomers (catechin and epicatechin)
fraction C		-	> 4	577; 441	dimers; monomer-G[c] (epicatechin-gallate)
fraction D		-	> 4	729; 865	dimer-G; trimer
	D1	6.3	NR	865	trimer
	D3	5.1	> 4	865	trimer
	D4	17.1	NR	865	trimer
	D6	31.5	2−3	729	dimer-G
	D7	36.7	NR	865	trimer
fraction E		-	1−2	865; 1017; 1153	trimer; trimer-G; tetramer
	E1	13.3	1−2	1153	tetramer
	E2	18.6	NR	865	trimer
	E3	24.7	1	1017	trimer-G
	E4	36.2	1	1153	tetramer
fraction F		-	2−3	729; 865; 1153	dimer-G; trimer; tetramer
	F3	23.1	1−2	1153	tetramer
	F4	25.1	1	729	dimer-G
	F5	32.8	1−2	1153	tetramer
	F6	36.0	0.5−1	1153	tetramer
	F7	43.8	3	865	trimer
fraction G		-	1.0	1017; 1153; 1305; 1441	trimer-G; tetramer; tetramer-G; pentamer
	G2	14.7	1−2	1441	pentamer
	G3	24.6	2−4	1153	tetramer
	G4	27.6	< 0.5	1305	tetramer-G
	G5	29.8	< 0.5	1305	tetramer-G
	G6	31.9	< 0.5	1017	trimer-G
	G7	35.5	< 0.5	1441	pentamer

[a] Abbreviations used: EDR, endothelium-dependent relaxation; *m*/*z* [M−H]$^-$, mass/charge ratio of [molecular ion − H]$^-$; Rt, retention time on HPLC; n.d., not determined; NR, no relaxation. [b] Concentration (μg catechin equivalents/mL) of fraction or peak material required to produce at least 15% relaxation. [c] -G, suffix denoting galloylated compound.

water. This spectrum shows only a single [M−H]$^-$ ion peak at 1017 *m*/*z*, indicating the HPLC fraction contains only one component. The mass peaks at 407 and 729 *m*/*z* correspond to ions produced by fragmentation of the parent ion at 1017 *m*/*z*. The molecular weight of 1018 corresponds to a trimer unit containing one gallic acid ester. Because other compounds could have the same molecular weight a MS/MS experiment was performed on *m*/*z* 1017. Figure 4B shows the MS/MS results and clearly fingerprints a gallic acid ester of a procyanidin trimer. Major fragment ions are observed at *m*/*z* 999, 891, 865, 847, 771, and 729.

Figure 5 shows the most likely structure of the molecule based on the observed ion fragmentation reactions and overall steric considerations, although the precise monomeric catechin/epicatechin makeup and order of monomers are not known. The circled numbers indicate the major fragmentation points. The ion at *m*/*z* 999 corresponds to the loss of water occurring at certain sites. The ion at *m*/*z* 891 is due to the loss of the B ring of the monomer unit with the loss of a hydroxyl group (cleavage 1). The ion at *m*/*z* 865 corresponds to the retro-Diels−Alder opening of the 'C' ring (cleavage 2) with the fragment at *m*/*z* 847 arising from the subsequent loss of water (cleavage 3). This fragmentation has been observed by other research groups for similar compounds without gallic ester groups (Barofsky, 1989; Karchesy et al., 1986; Self, 1986). The 'C' ring is also cleaved at point 4 to produce the 771 *m*/*z* fragment. We have observed ring-opening fragments at points 2 and 4 in ES−ITMS of commercial catechin and epicatechin−gallate samples. The loss of one monomer unit produces the *m*/*z* 729 monomer−monomer−gallate fragment (cleavage 5). The loss of gallic acid (cleavage 6) also produces *m*/*z* 847. On the basis of this information we conclude that peak G6 is a trimeric procyanidin gallate. Treatment of G6 with tannase, followed by HPLC and MS of the products, yielded gallic acid plus a trimer (data not shown), thus confirming the identification. Because of the stereochemistry of the proanthocyanidin compounds we are not able to ascertain the difference between catechin and epicatechin units at this time using MS only. However, we have fingerprinted the major classes of lower molecular weight procyanidin compounds (through pentamers) from grape seed extracts using ES−ITMS/MS data. Definitive identification will await thiolytic degradation of the compounds in acid, followed by HPLC and mass spectrometry of resultant products (Rigaud et al., 1991).

Summary of EDR Activity, Mass Spectrometry, and HPLC Results. Table 1 summarizes the relaxation and ES−ITMS results for most of the Toyopearl fractions and for their constituent peaks. Some peaks were numbered initially but were present in concentrations too small to collect and test for EDR activity, therefore, they are not listed in the table. Little or no relaxing activity was observed in fractions A, B, or C, which contained only phenolic acids, monomeric, and dimeric flavanol compounds, respectively, although fraction C (which also contains epicatechin−gallate) showed some relaxing activity at concentrations > 4 μg/mL. Relatively more activity (indicated by the "threshold") was seen in subsequent fractions, with activity generally increasing from fraction D through fraction G. EC_{50} values (confidence interval) of active Toyopearl fractions (fractions D − G) were fraction D, 4.37 (1.30−14.60); fraction E, 2.97 (1.65−5.38); fraction F, 2.55 (1.75−3.72); and, fraction G, 1.20 (0.84−1.71). Fractions eluting later than

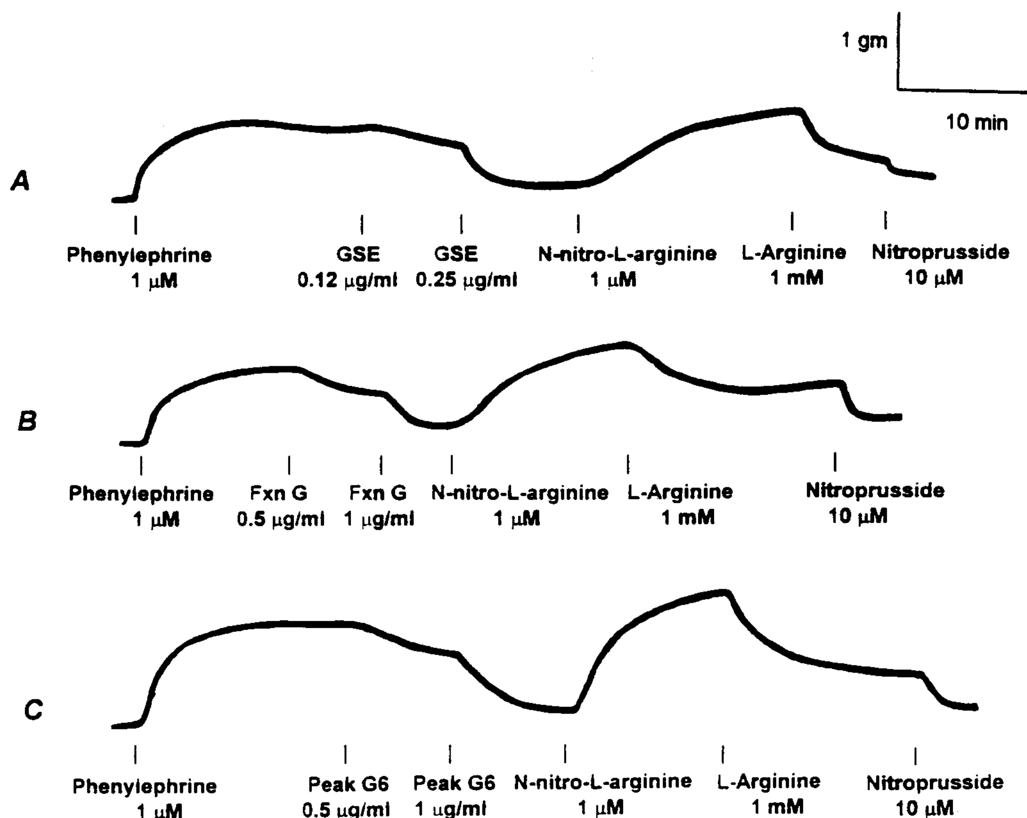

Figure 3. Endothelium-dependent relaxing (EDR) activity of A, the original grape seed extract (GSE); B, Toyopearl fraction G (Fxn G); and C, HPLC peak G6 isolated from Toyopearl fraction G. Vertical lines indicate where additions were made to the bath. Rat aortic rings were contracted with phenylephrine, then test substances (GSE, Fxn G, or peak G6) were added to relax the rings. The nitric oxide synthase inhibitor, N^G-nitro-L-arginine reversed the relaxations, and the substrate, L-arginine, caused tone to again be relaxed. Finally, nitroprusside was added to totally relax the tissues.

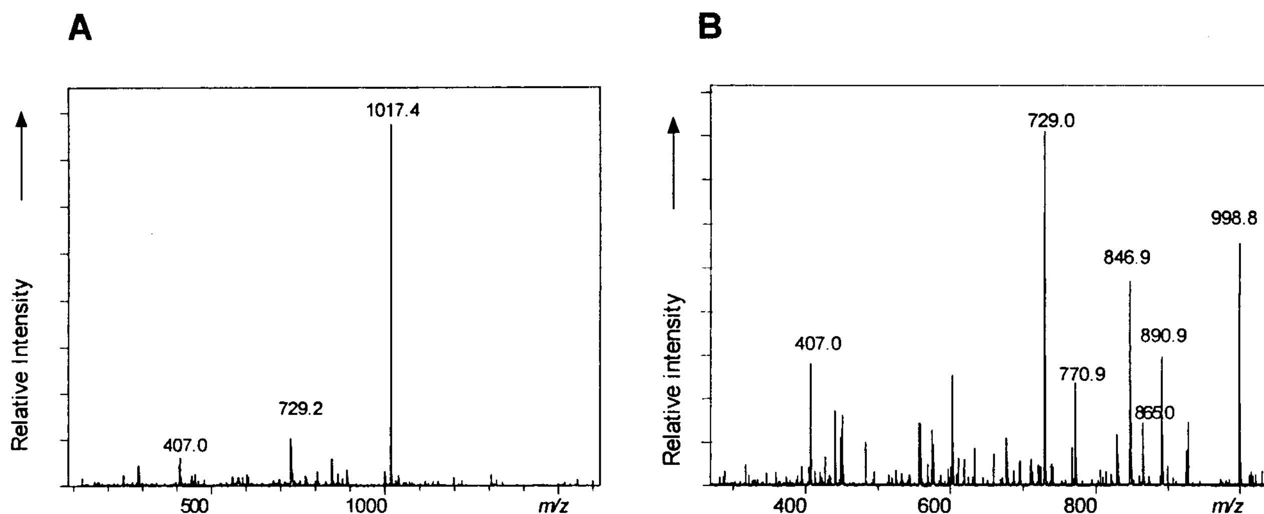

Figure 4. A, ES–ITMS in the negative mode $(M-H)^-$ of the HPLC peak G6. B, MS/MS spectrum of the $m/z = 1017$ ion in the negative ionization mode.

fraction G (using 70% acetone in water) were quite active but were not pursued because they were higher molecular weight compounds which would probably not be bioavailable and thus would be of less interest to us for future in vivo studies.

HPLC peaks derived from fractions E, F, and G that exhibited the greatest EDR activity are indicated in Table 2, along with their EC_{50} values. These values ranged from approximately 0.6 μg/mL to 2.6 μg/mL. The most active compounds include proanthocyanidins larger than dimers (i.e., trimers, tetramers, and pentamers) and their gallates. Galloylation appeared to increase activity at any given molecular size, e.g., the activity of trimer gallates was greater than that of most trimers, and dimer gallate activity was greater than that of the mostly inactive dimers. There also seem to be differences in EDR activity among the members of the isomeric families, e.g., all compounds identified as tetramers were not equally active, suggesting that the specific monomeric makeup of the compounds, and possibly the order of the monomeric components within the oligomer, are important for activity.

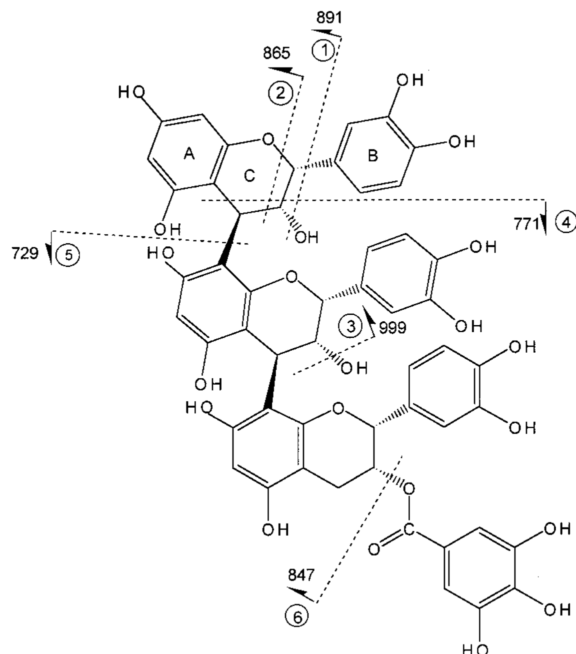

Figure 5. Model of a trimer−gallate (epicatechin-(4α-8)-epicatechin-(4α-8)-epicatechin-3-*O*-gallate) showing the ESI−ITMS fragmentation points, depicted by the circled numbers, and the observed fragment ion negative mode $(M-H)^-$ *m/z* values.

Table 2. EC_{50} Values of the Most Active Peaks

peak no.	compound	EC_{50} (C. I.)[a]	n[b]
E1	tetramer	2.59 (2.49−2.69)	4
E3	trimer-G	1.55 (1.28−1.89)	4
E4	tetramer	2.25 (2.14−2.37)	3
F3	tetramer	1.54 (1.27−1.87)	4
F4	dimer-G	1.25 (0.90−1.73)	4
F6	trimer	1.17 (0.96−1.43)	6
G4	tetramer-G	0.93 (0.83−1.04)	3
G5	tetramer-G	0.57 (0.49−0.67)	6
G6	trimer-G	1.00 (0.92−1.09)	6
G7	pentamer	1.05 (0.85−1.29)	6

[a] Mean concentration of peak material (μg catechin equivalents/mL) required to produce 50% relaxation (C. I., 95% confidence interval). [b] Number of concentration−response curves determined on different aortic rings, and peak material derived from at least two different extracts.

CONCLUSIONS

The results of this study demonstrate that vasoactive plant-derived compounds can be isolated and identified by the methods described herein: solvent extraction, separation of categories of proanthocyanidins by Toyopearl column pre-fractionation, and semipreparative and analytical HPLC combined with electrospray−ion-trap mass spectrometry and MS/MS. The in vitro results of this study cannot be extrapolated to the in vivo situation, because little is known about the absorption and metabolism of these compounds (Koga et al., 1999; Yamakoshi et al., 1999). However, recent reports provide indirect evidence that there are compounds present in grape juice and red wines that cause two well-known in vivo effects of nitric oxide: vasodilation and inhibition of platelet aggregation. In one of these studies, Stein et al. (1999) showed that short-term ingestion of purple grape juice improves endothelium-dependent, flow-mediated vasodilation in humans. Red wine consumption by rats resulted in a marked prolongation of bleeding time and a nitric oxide-dependent reduction in ex vivo thrombus formation (Wollny et al., 1999). Importantly, these effects of red wine were prevented by the nitric oxide synthesis inhibitor, N^G-nitro-L-arginine methyl ester, while L-arginine, the normal nitric oxide synthase substrate, reversed the latter effect. Further in vivo work remains to be done with purified EDR-active proanthocyanidins to confirm or deny a role for the potentially beneficial effects of these compounds.

ABBREVIATIONS USED

EDR, endothelium-dependent relaxation; ES−ITMS, electrospray−ion-trap mass spectrometry.

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