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Isolation of Specific Cranberry Flavonoids for Biological Activity Assessment

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1. Introduction

Plants are the primary source of dietary phenolic compounds that have been suggested to be responsible for many health benefits. Cranberry phenolics include simple phenolic acids, and flavonoids that include anthocyanins, proanthocyanidins (PACs), as well as flavonols (Gregoire, Singh, Vorsa, & Koo, 2007). These phenolics are differentiated according to the degree of unsaturation, oxidation of the three-carbon segment, and polymerization, which may influence their biological activity. Only a small percentage of the total flavonol content in a cranberry or cranberry juice (CBJ) exist as aglycones such as free myricetin, quercetin and kaempferol (Haekkinen, Kaerenlampi, Heinonen, Mykkaenen, & Toerroenen, 1999).

Cranberry phenolics have been associated with many possible beneficial effects. CBJ consumption is reputed to be protective against urinary tract infections (Avorn, Monane, Gurwitz, Glynn, Choodnovskiy, & Lipsitz, 1994), an effect associated with A-Type PAC trimers (Foo, Lu, Howell, & Vorsa, 2000). CBJ polyphenolics stimulate nitric oxide synthase mediated vasodilation in a rat model system (Maher, Matczynski, Stephaniak, & Wilson, 2000) and improve the human lipoprotein profile (Ruel, Pomerleau, Coutre, Lemieux, Lamarche, & Couillard, 2005). CBJ phenolics are also known to provide a rich antioxidant capacity that can protect LDL from oxidative injury (Wilson, Porcari & Harbin, 1998; Wilson, Porcari & Maher, 1999).

Polymerization has been observed to affect the antioxidant activity for PACs from non-cranberry sources (Plumb, De Pascual-Teresa, Santos-Buelga, Cheynier, Williamson, G. 1998; Lotito, et al., 2000) and the antioxidant activity for chocolate PACs increases with increasing degrees of polymerization (Counet & Collin, 2003). The antioxidant capacity of crude cranberry PAC fractions has also been reported (Porter, Krueger, Wiebe, Cunningham, & Reed, 2001; Yan, Murphy, Hammond, Vinson, & Neto, 2002), although the chemical purity of PAC fractions used in these studies was not always been well defined. Previous investigators

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have also reported on the content or biological activities of cranberry aglycones (Vinson, Bose, Proch, Al Kharrat & Samman, 2008; He & Liu, 2006; Chen, Zou & Deng, 2001;). Development of an improved isolation methodology will improve the elucidation of the medicinal chemistry of cranberry phenolics.

The isolation of cranberry monomeric flavonols, anthocyanins and PAC isolates using chromatography has been especially problematic because of their labor intensive nature, low yield, low isolate purity (Wang, Du, & Francis, 1978), and poor stability (Chen, Zuo, & Deng 2001; Fergusson, Kurowska, Freeman, Chambers, & Koropatnick, 2004). Methods for isolating and identifying larger MW flavonoids are further complicated due to the degree of polymerization, and overlapping of peaks/poor separation on HPLC column. Poor charge-mass separation characteristics of PACs make them especially problematic for isolation with HPLC-MS and MALDI-TOF (Howell, Reed, Kreuger, Winterbottom, Cunningham & Leahy, 2005).

HPLC column isolate separation is extremely dependent upon mobile phase gradient ratios and pH, as well as column flow rate, however, very few investigators have characterized these parameters in their published isolation methods. The lack of clarification makes it difficult to compare cranberry flavonoid isolate yields and biological activities between different manuscripts, even if a quantitative yield is reported. Semi-preparative and analytical HPLC has proven to be useful for effective isolation and identification of flavonoids, PACs and anthocyanins from cranberry fruit and cranberry juice (Wilson, Meyers, Singh, Limburg, & Vorsa 2008; Wilson, Singh, et al., 2008; Vvedenskaya, Rosen, Guido, Russell, Mill, & Vorsa, 2004.].

Determination of the biological activities of specific flavonol, PAC, and anthocyanins constituents is important for evidence-based selection of cranberry variants with favorable polyphenolic profiles and improved health benefits. This study describes an improved chromatographic method utilizing a HPLC-RP-18 column for quantitative isolation of cranberry flavonols, PACs, and anthocyanidins at 99% purity, as well as confirming the antioxidant activity of some of these constituents. Determination of quantitative yield is important for determining if these compounds are present at levels that are nutritionally or pharmaceutically relevant.

2. Materials and methods

2.1 Chemicals

Myricetin-3- β-galactoside, Myricetin-3-α-arabinofuranoside, quercetin-3- β-galactoside, quercetin-3- β-glucoside, quercetin-3- rhamnopyranoside, quercetin-3-O-(6″-p-benzoyl)-β-galactoside, epicatechins (monomer, dimer and trimer) used for antioxidant assays were isolated and characterized using LC-MS and compared with previously published data (Vvedenskaya, Rosen, Guido, Russel, Mills, & Vorsa, 2004; Gregoire, Singh, Vorsa, & Koo, 2007). For isolation and extraction HPLC grade methanol, ethyl acetate, acetone, acetonitrile, formic acid, acetic acid and water were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Polyvinylidene difluoride Company (Duluth, GA, USA). Deionized water (18Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corp., New Bedford, MA, USA).

2.2 Extraction and pre-purification of cranberry flavonoids

Flavonol, anthocyanin, and proanthocyanidin-rich fractions were prepared from cranberry fruits of the ‘Stevens’ variety harvested from a bog located in Burlington county New Jersey (October 2005) and stored at -20°C until use. Frozen cranberry fruits (5 kg frozen fruits equivalent to 500 g dry weight) were crushed, macerated with 7 L of aqueous acetone (80:20 acetone: water) and extracted at room temperature for 12 hours with agitation. The resulting

extract was filtered through Whatman filter paper, extraction was repeated on the remaining solids, and the two aqueous acetone extracts were combined. The acetone was removed by rotary evaporation at 35°C under high vacuum and frozen at -20°C.

The extracts were pre-purified using a method described below and modified as described previously (Vvedenskaya, Rosen, Guido, Russell, Mills & Vorsa 2004). After removal of acetone, the aqueous layer (one liter) was partitioned into 1.2 L hexane to remove carotenoids, fats and waxes, followed by additional partitioning into 1.8 L of ethyl acetate to selectively extract proanthocyanidins with anthocyanin glycosides and flavonols. The ethyl acetate extract was concentrated by vacuum evaporation. The ethyl acetate extract (41 g) was dissolved in a small amount of methanol for transfer to column chromatography system pre-packed with sephadex LH-20 (column size 100mm × 45 mm).The column was subsequently eluted with water (5 L), 20% methanol in water (3 L), 60% methanol in water (4L) 100% methanol (2.5 L) and 1.5 L of 80% acetone in water. The water (first liter) eluate yielded organic acids, with subsequent 20% methanol in water elution of anthocyanins, which were pooled together on the basis of color and TLC. The fraction eluted with 60% methanol in water, methanol and 80 % acetone in water yielded the flavonol glycoside fraction (2g), proanthocyanidin fraction (2.5g). Each fraction was further characterized using LC-MS, HPLC and TLC.

2.3 Preparative HPLC purification procedure

Flavonoids were isolated using an HPLC (Waters, Milford, MA, USA) equipped with Empower software, waters 600 pump controllers, 996 Photodiode Array detector (PDA), a fraction collector II, and a 10 ml sample loop size (Gregoire, Singhm Vorsa & Koo, 2007). Two solvents were used: A, 90% water and 10% methanol (pH 3.5 with formic acid); B, 60% acetonitrile,10% water and 20% methanol (pH 3.5 with formic acid). Using a 21 ml/min flow rate the elution profile was: 0-5 min, 100% A (isocratic); 5-10 min, 0-20% B in A (linear gradient); 10-20 min, 20-60% B in A (linear gradient); 20-30 min, 60-80% B in A (linear gradient); 30-35 min,80-100% B in A (linear gradient); 35 -40 min ,100% B (isocratic elution); 40 to 42 min, 0 to 100% A in B, 42 to 50 min,100% A (isocratic). The effluent absorbance was monitored at 280 nm for proanthocyanidins and at 366 nm for flavonols so that each peak fraction could be collected according to its specific elution profile. HPLC separation was performed using Luna column 5 μm C18 (2) 100 A of size 250 × 21 mm (Phenomenex Inc., Torrance, CA, USA) A relatively high mobile phase flow rate was important to prevent an excessively broad peak with long elution time. The partially purified fraction (95% purity) was then further purified by reversed-phase semi-preparative HPLC under the same conditions to yield flavonols and PACs (above 99% purity). The absorption spectra of isolated proanthocyanidin and flavonol peaks were checked at 280 and 366nm.. PDA software (Waters Empower) was used to examine each peak, a component with a peak purity angle lower than its purity threshold was considered to be a pure substance and the separation would be acceptable at 99% purity. The absorption spectra of both isolated proanthocyanidins and flavonols peaks were checked at 280 and 366 nm. The fractionated pure compounds were collected from the preparative HPLC and characterized using LC-MS and NMR spectra as described below. In addition,, mass fragmentation patterns for each isolate were also compared with published data (Gregoire, et al., 2007; Vvedenskaya et al., 2004).

2.4 Compound identification using LC-MS-MS and NMR spectroscopy

Following purification, flavonols and proanthocyanidins were identified by high performance liquid chromatography coupled with mass spectrometry (HPLC-MS-MS). The HPLC system (Shimadzu Co., 10VP Series, Columbia, MD, USA) employed a Hypersil Gold C_{18} (3 μm particle size; 150 mm length × 3.0 mm ID; Thermo Electron Co., Bellefonte, PA). Five μL was injected onto the column and a gradient elution was used for separations. Solvent A consisted of 10% MeOH in H_2O adjusted to pH 3.5 with formic acid. Solvent B consisted of 20% H_2O

(pH 3.5), 20% MeOH, and 60% acetonitrile. At a flow rate of 0.3 mL min^{-1}, the following gradient was used: 0 min, 100% A; 10 min 20% A; 20 min, 40% A; 40 min, 0 % A; held at 0% A for 15 min. Five minutes of equilibration at 100% A was performed before and after each injection. Effluent from the column was introduced into a triple-quadrupole mass spectrometer (Micromass, Inc., Beverly, MA, USA) equipped with a pneumatically-assisted electrospray ionization source (ESI). Mass spectra were acquired in the negative ion mode under the following parameters: capillary voltage, 3 kV; source block temperature, 120 °C; desolvation gas temperature, 400 °C. Nitrogen was used as the drying and nebulizing gas at flow rates of approximately 50 and 450 L/h. For full-scan HPLC-ESI-MS analysis, spectra were scanned in the range of 50 to 1200 *m/z*. Data acquisition and processing were performed using a Mass-Lynx NT 3.5 data system (Micromass Inc., Beverly, MA, USA).

^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AM 500 instrument at room temperature (RT) using 3 mm tubes. Samples (5 mg) were dissolved in MeOH-D4. Chemical shifts were expressed in parts per million (ppm) relative to tetramethyl silane (TMS) as an internal standard.

2.5 Antioxidant activity assessment

The ability of cranberry polyphenolics to prevent the oxidative modification of LDL by cupric ions was assessed using methods described previously (Wilson, et al., 2000). Following approval of the WSU Human Subjects committee and completion of an informed consent document, fasting venous blood was collected in EDTA from six volunteers and LDL fractions isolated by sequential ultracentrifugation. Following dialysis to remove EDTA, LDL isolates were merged and the LDL protein concentration determined with the BioRad DC Protein Assay (Hercules, CA).

Phenolic extracts were re-solubilized in molecular biology grade dimethylsulfoxide (Sigma-Aldrich Inc., St Loius, MO) prior to sequential dilution in deionized water and a final solution containing phosphate buffered saline (pH 7.40) containing freshly prepared and dialyzed LDL (100 μg LDL protein /ml). LDL oxidation was promoted at 37°C with 10 μM cupric sulfate (Sigma Aldrich Inc., St Loius, MO) in the presence/absence of CBJ constituents while the absorbance at 234 nm was recorded to determine their relative ability to delay the lag-time for the formation of lipid conjugated dienes. Trimeric and larger PACs were not evaluated because of presumed precipitation that was observed when these relatively pure isolates were solublized into PBS.

2.6 Statistical analysis

Data are expressed as mean ± standard deviation. The effect of cranberry constituent classes or compounds on antioxidant activity was analyzed using ANOVA, Least Squares Means and Dunnett's adjustment for multiple comparisons (SAS Institute Inc., Cary, NC). Differences between means were considered to be significant when the P value was < 0.05.

3. Results and discussion

3.1 Optimization of semi preparative-HPLC isolation method

To ascertain the purity of semi purified cranberry crude extract isolated from sephadex column was first analyzed by analytical HPLC. Conditions required to achieve analytical purity were converted to semi-preparative isolation using Waters Prep CalculatorTM (Waters Co., Milford, MA, USA). In our prep-HPLC method and development, different mobile phases containing different mixture of water, methanol, acetonitrile with different pH using formic acid, different flow rates were all tested. The result indicated that the mobile phase for solvent A was water and methanol mixture of pH 3.5 at volume ration of 90:10 (v/v) and for solvent B was

acetonitrile, water and methanol mixture of pH 3.5 at a volume ratio of 60:20:20 (v/v/v), and the flow rate, and detection wavelength were set at 21 ml/min, and 280 and 366 nm, which were most suitable for our analysis and isolation. The above conditions provided for optimal separation of the target compounds and the HPLC chromatograms of the pure compounds are shown in Fig-2

3.2 Cranberry flavonoid isolation, identification and yield

Cranberry flavonols, anthocyanins, and proanthocyanidins have been previously characterized with HPLC (Porter, Kreuger, Wiebe, Cunningham, & Reed, 2001; Prior, Lazarus, Cao, Muccitelli, & Hammerstone, 2001; Yan, Murphy, Hammond, Vinson, & Neto, 2002; Vvedenskaya, Rosen, Guido, Russell, Mills, & Vorsa, 2004; Wilson, Meyers, Singh, Limburg, & Vorsa, 2008;). Previous attempts at isolating constituent cranberry flavonol glycosides by semi-preparative HPLC have been reported though not quantitatively (Yan Murphy, Hammond, Vinson, & Neto, 2002), our previous reports are to our knowledge the only cranberry reports that provide quantitative yields (Wilson, Singh, et al., 2008; Wilson, Meyers, Singh, Limburg, & Vorsa, 2008). The use of methanol-water-acetonitrile-formic acid for the scale separation of these cranberry compounds by preparative-HPLC has advantages over these previously used mobile phase protocols. In contrast, the current solvent system and gradient procedure permitted isolation of flavonols and PACs. A relatively high mobile phase flow rate was used to prevent excessively broad peaks with long elution times and overlapping peaks.

Cranberry PAC and flavonol isolate structures, HPLC chromatograms and representative LC-MS spectra are described in Figures 1, 2 and 3, respectively, their quantitative yields (μg/gram based on dry weight of frozen fruits) are described in Table 1. Isolated PAC-A type included epicatechin (monomer), dimer (epicatechin-(4 β→8, 2 β→O→7)-epicatechin), and trimer (epicatechin-(4 β→8)-epicatechin)-(4 β→8, 2 β→O→7)-epicatechin). Isolated cranberry flavonols included myricetin-3- β-galactoside, myricetin-3-α-arabinofuranoside, quercetin-3-β-galactoside, quercetin-3- β-glucoside, quercetin-3- rhamnospyranoside, and quercetin-3-O-(6″-p-benzoyl)-β-galactoside (QBG). NMR was also used to confirm PAC and flavonol constituent identities. ^{1}H- and ^{13}C-NMR data matches for isolated flavonols and proanthocyanidins correlated with our previously published values (Foo, Lu, Howel, & Vorsa, 2000,Vvedenskaya, Rosen, Guido, Russell, Mills,& Vorsa, 2004;Gregoire, Singh, Vorsa, & Koo, 2007).

Anthocyanins were isolated as a crude extract, however further constituent separations were not performed for the following reasons. Cranberry anthocyanins have not been implicated in urinary tract or cardiovascular benefits, nor were the crude anthocyanin extract isolates associated with antioxidant activity, as will be discussed later in this report, although we have characterized specific anthocyanins including cyanidin and peonidin-3-glycosides from a 100% commercially available CBJ (Wilson, Meyers, Singh, Limburg,& Vorsa, 2008).

3.3 Cranberry flavonoid antioxidant activity verification

The relative antioxidant activity of crude flavonol, anthocyanin, and proanthocyanin extracts were compared by measuring their ability to delay the lag-time for LDL oxidation (Figure 4; Table 2). Flavonols proved best able to protect LDL from oxidative injury at a 0.10 μg/ml concentration and proanthocyanidins provided a similar but slightly reduced capacity to inhibit oxidative injury to LDL at 0.10 μg/ml, however the anthocyanin extract isolated from CBJ was not associated with a significant antioxidant activity at 0.10 μg/ml. None of these phenolic types provided significant antioxidant protection at the 0.05 μg/ml concentration, in spite of the relative 99% purity of each extract.

Vinson, Bose, Proch, Al Kharrat, & Samman (2008) compared crude polyphenol contents (mg/g fresh weight) of many commercially available cranberry products, but does not quantify the constituent flavonoids in their extracts. The study by Prior, Lazarus, Cao, Muccitelli, & Hammerstone (2001) discusses cranberry antioxidant activity in ORAC units, but again does not provide an accurate characterization of the individual anthocyanin constituents. While crude cranberry PAC extracts have been observed to inhibit Cu++-mediated LDL oxidation (Porter, Kreuer, Weibe, Cunningham, & Reed, 2001), neither their anthocyanin nor flavonol rich extracts were able to significantly inhibit LDL oxidation. The discordance between our flavonol antioxidant results and those of Porter's (2001) study results may reflect differences in experimental conditions in the two studies. Porter's study (2001) did not report the amount of flavonoids present in the crude extracts used in their antioxidant assays, so there may have been significantly less anthocyanin and flavonol in their extracts relative to those used in our study. Antioxidant activities of cranberry fruit flavonol aglycones (no sugar moiety) such as quercetin have been previously evaluated and demonstrated to possess antioxidant properties. However, the antioxidant properties of constituent flavonol glycosides and PACs, which make up the bulk of flavonoids present in cranberries, remain relatively unknown, presumable because of a lack of effective methods for constituent isolation and identification. In this regard, Q-3-O-(6″-p-benzoyl)-β-galactoside (QBG), Q-3-galactose, Q-3-glucose, Q-3-rhamnose, M-3-galactose and M-3-arabinofuranoside all significantly exhibited antioxidant activity at 75 μM when compared to LDL oxidized in the absence of constituent flavonols (Table 2). Our antioxidant activity assessments for these glycones were similar to that observed by Yan, Murphy, Hammond, Vinson, & Neto, 2002, and confirms the observations of many others using identical flavonols from non-cranberry sources (Peng et al., 2003; Montoro, Braca, Pizza, & De Tommasi, 2005; Burda & Oleszek 2001; Tsao, Yang, Xie, Sockovie & Khanizadeh, 2005).

Previous investigators have determined that proanthocyanidins with a high degree of purity extracted from grape seeds (Plumb, et al, 1998; Natella, Belelli, Gentili, Ursini, & Scaccini, 2002; Sano, Uchida, et al., 2007) and chocolate (Lolito, Actis-Goretta, et al, 2000; Counet & Collin 2003) have an antioxidant capacity that is increased with increasing polymer size. Cranberry proanthocyanidins have not been purified and examined with respect to their ability to inhibit LDL oxidation. We also confirmed that at 75 μM cranberry proanthocyanidin monomer and dimer both effectively inhibit LDL oxidation.

3.4 Conclusions

Our semi-preparative method used a methanol—water—acetonitrile—formic acid solvent system and gradient procedure for the successful separation of pure flavonol and proanthocyanidin compounds at 99% purity. This is a great improvement over previous methods used to study cranberry flavonoids. Cranberry fruits are rich in flavonoids, many of which are proanthocyanidins with A-type linkage featuring both 4ß→O8 and 2ß→O7 interflavanoid bonds. Crude cranberry flavonol and proanthocyanidin extracts were associated with a rich antioxidant activity. A large percentage of plant flavonols and proanthocyanidins are thought to form glycosides and polymers in the plant tissue. The glycosylated and polymerized forms may represent the bulk of polyphenolic mass in this regard. Future studies will need to characterize the effects of the larger cranberry PACs and attempt to characterize how all cranberry flavonoid constituent compounds identified here may be individually responsible so specific health beneficial biological activities and a determination of whether in vitro effects associated with anyone phenolic isolate have biological activity in vivo.

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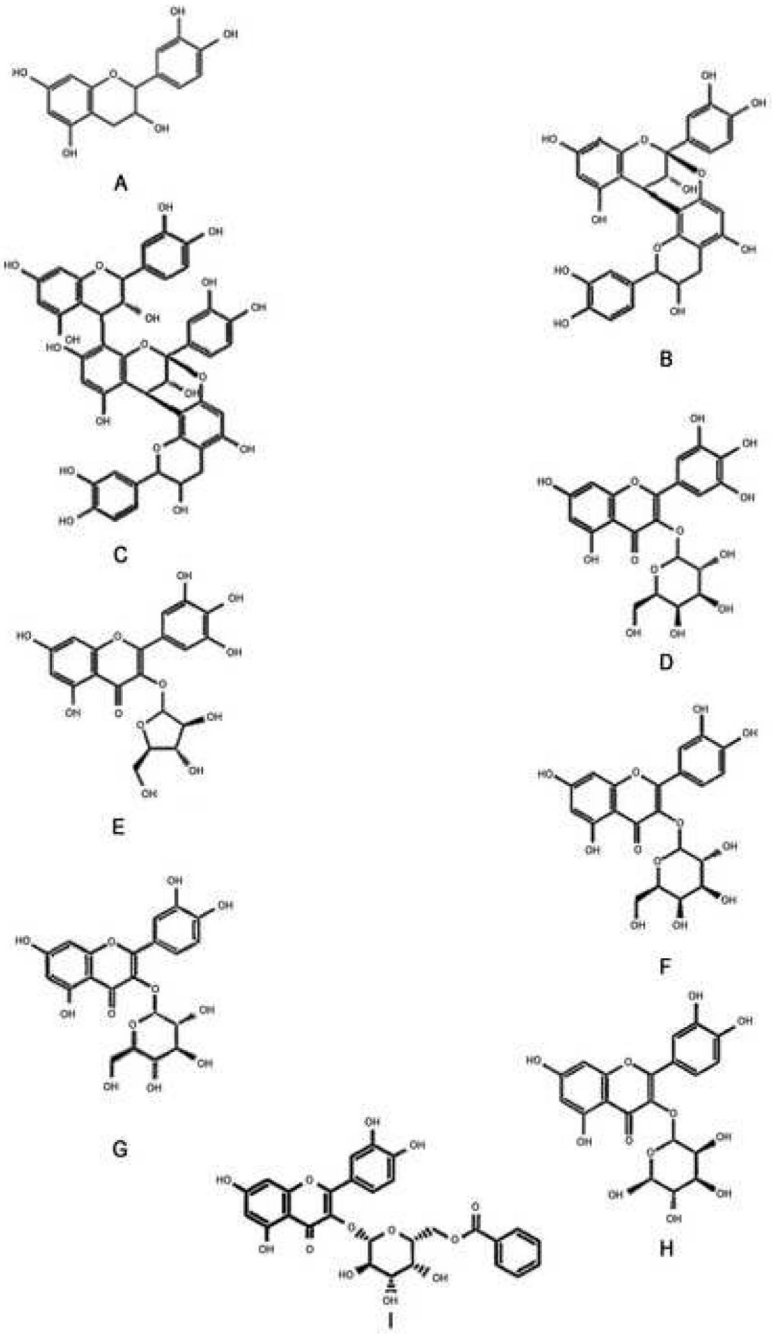

Fig.1.

Chemical structure of selected flavonols and proanthocyanidins isolated from cranberry fruits: A- monomers; B- dimers; C- trimers; D- myricetin-3-galactoside; E- myricetin-3-arabinofuranoside;F- quercetin-3-galactoside; G- quercetin-3-glucoside; H- quercetin-3-rhamnoside; and I- quercetin-3-benzoylgalactoside derivative.

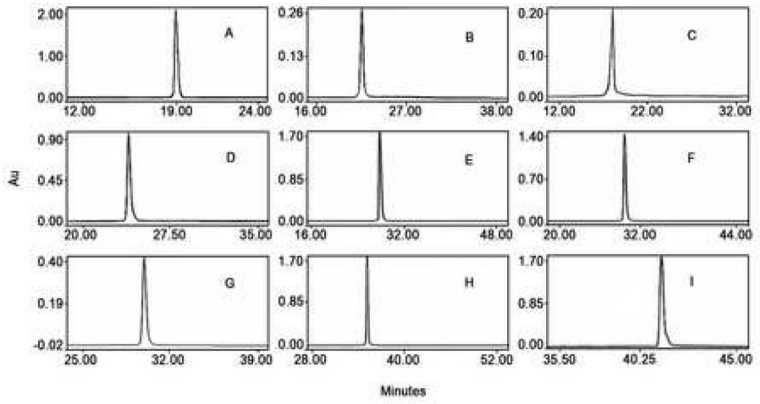

Fig. 2.

HPLC chromatogram of isolated pure fractions: A- Monomers; B- Dimers; C- trimers; D- myricetin-3-galactoside; E- myricetin-3-arabinofuranoside; F- quercetin-3-galactoside; G- quercetin-3-glucoside; H- quercetin-3-rhamnoside; and I- quercetin-3-benzoylgalactoside derivative.

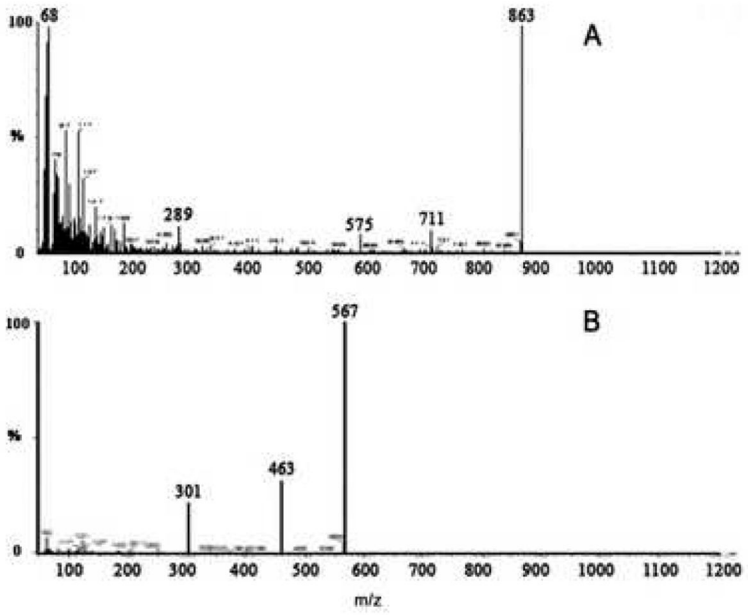

Fig. 3.
Mass fragmentation chromatogram of isolated pure fractions: A- trimers; and B-quercetin-3-benzoylgalactoside derivative.

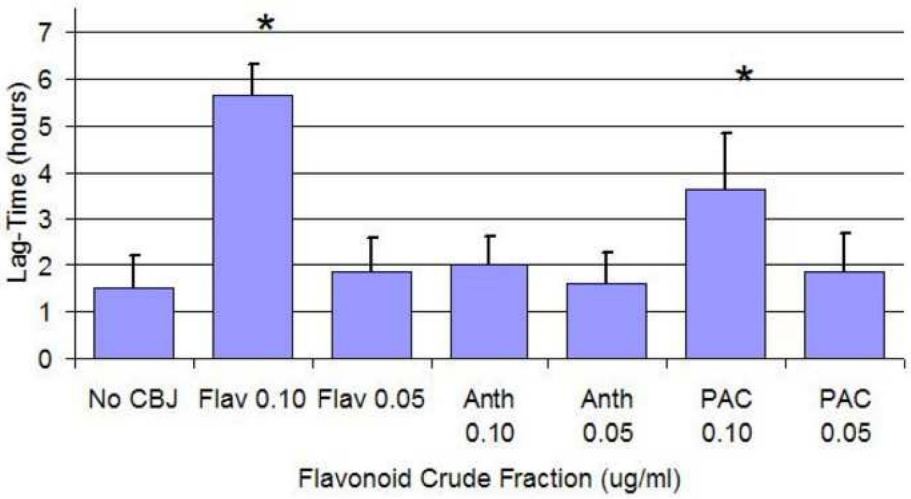

Fig. 4.

Crude cranberry flavonol (Flav) and proanthocyanidin (PAC) extracts were associate with a significant inhibition of cupric ion mediated oxidation of LDL lipids (lag-time at 234 nm) at 0.10 μg/ml but not at 0.05 μg/ml, while crude anthocyanidin (anth) extracts were not associated with a significant antioxidant activity. *Significantly different from control $P < 0.01$.

Table 1

Characterization of flavonoid isolate ESI-MS mass fragmentation pattern and yield.

Name	$[M-H]^-$; fragment(s) in ESI-MS	Yield (μg/ g dry wt)
Proanthanthocyanidins		
Epicatechin (monomers)[a,b]	289; 126, 117	18.4
Dimers (epicatechin-(4 β →8, 2 β →O→7)-epicatechin) [a,b,c]	575; 289	42.8
Trimers (epicatechin-(4 β →8)-epicatechin-(4 β →8, 2 β →O→7)-epicatechin) [a,b,c]	863; 575, 289	20.0
Flavonols		
Myricetin-3- β-galactoside [a,b,c,d]	479; 317	12.0
Myricetin-3-α-arabinofuranoside [a,b,c,d]	449; 317	6.0
Quercetin-3- β-galactoside [a,b,c,d]	463; 301	100
Quercetin-3-glucoside [a,b,c,d]	463; 301	1.0
Quercetin-3-rhamnoside [a,b,c,d]	447; 301	42.4
Q-3-O-(6″-p-benzoyl)-β-galactoside [a,b,c,d]	567; 463, 301	5.0

[a] Based on comparisons with authentic standards

[b] Based on LC-MS mass fragmentation pattern.

[c] In conjunction with Wilson, Singh, et al., (2008).

[d] In conjunction with Vvedenskaya, Rosen, Guido, Russell, Mills & Vorsa, (2004).

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Table 2

Lag-time (234 nm) for cupric ion mediated oxidation of LDL lipids in the presence of cranberry flavonols and proanthocyanidins. LDL oxidation lag-time in absence of cranberry constituents was 1.05 ± 0.12 hours.

Cranberry Constituent Compound and Concentration					
	75 μM	**7.5 μM**		**75 μM**	**7.5 μM**
PAC^ Monomers	10.12 ± 0.84*	1.28 ± 0.18	Quercetin-3-Glucoside	10.92 ± 0.17*	1.61 ± 0.79
PAC^ Dimers	7.68 ± 1.58*	1.13 ± 0.18	Quercetin-3-Rhamnoside	11.00 ± 0.00*	1.57 ± 0.75
PAC^ Trimers	Precipitated	1.56 ± 0.30	Myricetin-3-Galactoside	11.00 ± 0.00*	1.37 ± 0.65
Q-3-O-(6″-p-benzoyl)-β-galactoside	11.00 ± 0.00*	1.25 ± 0.29	Myricetin-3-Arabino furanoside	9.90 ± 0.29*	0.92 ± 0.07
Quercetin-3-galactoside	10.82 ± 0.36*	1.10 ± 0.36	Quercetin (Reference)	7.43 ± 0.19*	0.99 ± 0.04

Cranberry Constituent Compound					
	Proanthocyanidins			**Flavonols**	
Concentration	**Monomers**	**Dimers**	**Trimers**	**Q-3-O-(6″-p-benzoyl)-β-galactoside**	**Quercetin-3-galactoside**
75 μM	10.12 ± 0.84*	7.68 ± 1.58*	Precipitated	11.00 ± 0.00*	10.82 ± 0.36*
7.5 μM	1.28 ± 0.18	1.13 ± 0.18	1.56 ± 0.30	1.25 ± 0.29	1.10 ± 0.36

	Flavonols				**Reference**
	Quercetin-3-Glucoside	**Quercetin-3-Rhamnoside**	**Myricetin-3-Galactoside**	**Myricetin-3-Arabinofuranosid**	**Quercetin**
75 μM	10.92 ± 0.17*	11.00 ± 0.00*	11.00 ± 0.00*	9.90 ± 0.29*	7.43 ± 0.19*
7.5 μM	1.61 ± 0.79	1.57 ± 0.75	1.37 ± 0.65	0.92 ± 0.07	0.99 ± 0.04

^ Proanthocyanidin (PAC)

* Significantly different from control ($P > 0.01$)