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Antioxidant Profile and in Vitro Cardiac Radical-Scavenging versus Pro-oxidant Effects of Commercial Red Grape Juices (*Vitis vinifera* L. cv. Aglianico N.)

Gian Carlo Tenore,*[,†] Michele Manfra,[§] Paola Stiuso,[#] Luigi Coppola,[⊗] Mariateresa Russo,[⊥] Isabel Maria Gomez Monterrey,[†] and Pietro Campiglia[▽]

[†]Department of Chimica Farmaceutica e Tossicologica, Università di Napoli Federico II, Via D. Montesano 49, 80131 Napoli, Italy

[§]Department of Chimica, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy

[#]Department of Biochemistry and Biophysics, Second University of Naples, Napoli, Italy

[⊗]Coppola S.p.a., via S. Maria la Carità, Vicinale De Risi, Scafati (SA), Italy

[⊥]Department of Scienze e Tecnologie Agro-Forestali ed Ambientali, Università Mediterranea di Reggio Calabria, Reggio Calabria, Italy

[▽]Department of Pharmaceutical and Biomedical Sciences, University of Salerno, Salerno, Italy

ABSTRACT: Several works have reported on the pharmaceutical usefulness of grape phytochemicals. Nevertheless, the scientific literature needs further studies to consider grape extracts as useful dietary supplements. The aim of the present work was to hypothesize for the first time the use of whole commercial red grape juices as food supplements potentially useful against both physiological and induced cardiac oxidative stress. First of all, the results indicated a good antioxidant stability of the juice sample to lyophilization that may be reasonably regarded as a suitable process for the formulation of food supplements. Then, the processed sample (lioRGJ) was tested on cardiac-derived H9C2 myocytes to ascertain its effects on reactive oxygen species (ROS) generation and caspase-3 activity incubating cardiomyocytes with lioRGJ at increasing doses (0.01−1 μg). Experiments showed an appreciable direct radical-scavenging activity at a maximum sample dose of 0.01 μg that made the caspase-3 activity decrease by about 47% ($P < 0.001$). Cardiac cells were exposed to 1 μM doxorubicin and its combination with different doses of lioRGJ. A maximum sample aliquot of 0.01 μg seemed to effectively contrast the induced oxidant injury, decreasing the ROS levels by about 31% and depressing the caspase-3 activity by about 60% ($P < 0.001$). In both assays, pro-oxidant effects at higher sample concentrations were detected as indicated by the increase in both ROS generation and apoptotic activity. The data suggested the possible employment of the juice sample as a food supplement with prospective cardioprotective benefits, although further studies are needed to optimize its dosages to avoid harmful pro-oxidant effects.

KEYWORDS: *red grape juice, lyophilization, cardiomyocytes, radical-scavenging activity, pro-oxidant effects*

INTRODUCTION

Grape (*Vitis vinifera* fruit) is characterized by a high concentration and great variety of phenolic compounds, mainly flavonoids (catechin, epicatechin, quercetin, anthocyanins, and procyanidins) and resveratrol (3,5,4′-trihydroxystilbene), that are especially abundant in red grape products.[1] Particularly, positive health benefits of the consumption of red grape juice, such as improvement of the endothelial function, increase in the serum antioxidant capacity, protection of low-density lipoprotein (LDL) against oxidation, decrease in the native plasma protein oxidation, and reduction of platelet aggregation, have also been reported.[2] It must be emphasized that thermal treatments employed before (hot break process, mainly used in the United States) and/or after (pasteurization, concentration) grape extraction may lead to even deep changes in the polyphenolic profile of commercial grape juices from that of fresh grapes.[3] Some authors have affirmed that such processing may increase flavonol levels in juices by promoting extraction processes from all of the grape portions. In addition, hydrolysis mechanisms would enhance the accumulation in juices of individual phenolic compounds, mainly glucosides, galactosides, and aglycones along with some rutinosides and other glycosidic combinations linked to the flavonol nucleus, that could be better absorbed in the small intestine and reach the bloodstream.[4] Several works have reported the pharmaceutical usefulness of grape and wine phytochemicals.[5−7] Nevertheless, further studies are needed to legitimize grape extracts as useful dietary supplements. Therefore, investigation of their radical-scavenging properties is of interest, primarily to discover promising new sources of natural antioxidants, functional foods, food supplements, and nutraceuticals.

Myocardial mitochondria are an important source of oxidative stress. The mitochondrial electron transport chain (ETC), under conditions of reductive stress, is capable of generating reactive oxygen species (ROS) and reactive nitrogen species (RNS) that play a crucial role in the pathophysiology of a large variety of cardiovascular diseases including congestive

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heart failure, valvular heart disease, cardiomyopathy, hypertrophy, atherosclerosis, and ischemic heart disease.[8−10] Recently, grape seed proanthocyanidins, a group of polyphenolic bioflavonoids ubiquitously found in the lignified portions of grape clusters, were found to possess cardioprotective abilities by functioning as in vivo antioxidants and by virtue of their ability to directly scavenge ROS including hydroxyl and peroxyl radicals.[11−13] However, their pro-oxidant toxicity at higher doses (100−500 μg/mL) was also reported, particularly their ability to cause apoptosis in cardiomyocytes induced by ROS generation.[14−16] The effects on cardiomyocytes by directly testing the whole grape juice are unknown.

It is well-known that the clinical use of anthracyclines, especially doxorubicin, in the treatment of many neoplastic diseases is limited by acute and chronic dose related, cumulative, and essentially irreversible cardiotoxicities. Available laboratory evidence shows that doxorubicin and its metabolites induce generation of ROS that could interfere with iron metabolism and trigger the intrinsic mitochondria-dependent apoptotic pathway in cardiomyocytes.[17,18] Nevertheless, to date, only a few single chemicals or raw extracts have proven to be able to reduce the deleterious action of doxorubicin,[19,20] but no experiments with whole grape juices have been conducted.

Thus, the aims of this work were mainly two: (1) to study the antioxidant profile of a commercial red grape juice before (RGJ) and after lyophilization (lioRGJ) to evaluate its stability to processing for the formulation of food supplements; (2) to test the processed sample on cardiomyocytes to ascertain its effects on the physiological and doxorubicin-induced oxidative stress.

MATERIALS AND METHODS

Reagents and Standards. All chemicals and reagents were of analytical reagent or HPLC grade. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris-2,4,6-tripiridyl-2-triazine (TPTZ), iron(III) chloride (dry), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid monohydrate, aluminum chloride (dry), malvin (malvidin-3-*O*-glucoside) chloride, peonidin-3-*O*-glucoside chloride, delphinidin-3-*O*-glucoside chloride, cyanidin-3-*O*-glucoside chloride, petunidin-3-*O*-glucoside chloride, myricetin, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, isorhamnetin, Folin−Ciocalteu's phenol reagent, potassium phosphate buffer, 2′,7′-dichlorodihydrofluorescein diacetate, ethylenediaminetetraacetic acid (EDTA), Tris-HCl buffer, sodium dodecyl sulfate, diethylenetriaminepentaacetic acid, catalase, nitroblue tetrazolium, xanthine, bathocuproinedisulfonic acid, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Syringetin-3-*O*-galactoside was purchased from Extrasynthese (Lyon, France). Methyl alcohol (RPE) was purchased from Carlo Erba (Milano, Italy). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal bovine serum, and Dulbecco's phosphate-buffered saline (PBS) were purchased from Gibco-Invitrogen (Carlsbad, CA, USA). Tissue culture flasks and 24-well tissue culture plates were purchased from Corning (Corning, NY, USA).

Grapes and Grape Juice. *V. vinifera* L. cv. Aglianico N. red grape-based juices (four different production batches) were obtained from Coppola Spa (Luigi Coppola, Scafati, SA, Italy). Vines were cultivated in Scafati (40° 45′ N, 14° 32′ W, Italy), and grapes were harvested in 2010; all juices were manufactured during the same year. Purple juices were heat-extracted (approximately 50 °C) using pulp, seeds, and skin, with a subsequent pressing, and then submitted to pasteurization (at 85 °C). The juice was packaged and stored at 4 °C until the beginning of the analyses.

Lyophilization. A 10 mL aliquot of each RGJ of the four batches was lyophilized for 24 h (Edwards High Vacuum, West Sussex, UK). The residue (2.5 g) was stored in anhydrous atmosphere, at 4 °C in the dark, until the beginning of the analyses. Then, the sample was diluted to 10 mL with ultrapure water.

Spectroscopic Apparatus. Spectrophotometric analyses were performed using a Jasco V-530 UV−vis spectrophotometer (Tokyo, Japan) set at appropriate wavelengths for each assay.

Total Phenolic Content. The concentration of total phenolics was measured according to the method described by Singleton and Rossi,[21] with some modifications. Briefly, an aliquot (20 μL) of RGJ, lioRGJ, and calibration solutions of gallic acid (20, 40, 60, 80, and 100 mg/L) was added to a 25 volumetric flask containing 9 mL of ultrapure water (ddH_2O). A reagent blank using ddH_2O was prepared. One milliliter of Folin−Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 mL of Na_2CO_3 aqueous solution (7 g/100 mL) was added with mixing. The solution was then immediately diluted to volume with ddH_2O and mixed thoroughly. After incubation for 90 min at 23 °C, the absorbance versus prepared blank was read at 765 nm. Total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per 100 mL.

Total Flavonol Content. The total flavonol content was measured by a colorimetric assay developed by Zhishen et al.[22] A 50 μL aliquot of RGJ, lioRGJ, and calibration solutions of quercetin-3-*O*-glucoside (20, 40, 60, 80, and 100 mg/L) was added to a 5 mL volumetric flask containing 2 mL of ddH_2O. At zero time, 0.15 mL of $NaNO_2$ aqueous solution (5 g/100 mL) was added to the flask. After 5 min, 0.15 mL of $AlCl_3$ aqueous solution (10 g/100 mL) was added. At 6 min, 1 mL of 1 M NaOH was added to the mixture. Immediately, the reaction flask was diluted to volume with the addition of 1.2 mL of ddH_2O and thoroughly mixed. Absorbance of the mixture, pink in color, was determined at 510 nm versus prepared water blank. Total flavonol content was expressed as milligrams of quercetin-3-*O*-glucoside equivalents (QE)/ per 100 mL.

Total Monomeric Anthocyanin Content. The total monomeric anthocyanin content of the samples was evaluated by applying a pH-differential method.[23] An aliquot of 1 mL of RGJ, lioRGJ, and calibration solutions of malvin (0.1−10 mg/100 mL) was added to two vials containing 10 mL of acetate buffer (pH 3.6) and 1 N HCl, respectively. The difference between the absorbances read at 530 nm was calculated. Total anthocyanin content was expressed as milligrams of malvin equivalents (ME) per 100 mL.

Antioxidant Activity. The antioxidant activity of the juice samples was compared with that of antioxidant standard solutions (1 mg/mL). For each antioxidant assay, a Trolox aliquot was used to develop a 50−500 μmol/L standard curve. All data were then expressed as Trolox equivalents (micromoles of TE per 100 mL).

DPPH• Radical-Scavenging Assay. The ability of the samples to scavenge the DPPH radical was measured using the method of Brand-Williams et al.[24] Aliquots (20 μL) of RGJ, lioRGJ, and antioxidant standard solutions were added to 3 mL of DPPH solution (6×10^{-5} mol/L), and the absorbance was determined at 515 nm every 5 min until the steady state.

Reducing Potential Assay. The antioxidant potential of the samples was determined using the ferric reducing antioxidant power (FRAP) assay of Benzie and Strain.[25] A solution of 10 mmol/L TPTZ in 40 mmol/L HCl and 12 mmol/L ferric chloride was diluted in 300 mmol/L sodium acetate buffer (pH 3.6) at a ratio of 1:1:10. Aliquots (20 μL) of RGJ, lioRGJ, and antioxidant standard solutions were added to 3 mL of the FRAP solution, and the absorbance was determined at 593 nm every 5 min until the steady state.

HPLC Quantification of Anthocyanins and Flavonols. HPLC separation of anthocyanins and flavonols from samples was performed according to earlier studies.[26] Identification was possible by monitoring anthocyanins and flavonols at 520 and 353 nm, respectively, and by comparing their spectra and retention times with those of commercial standards and with those reported in previous works.[23] Aliquots (20 μL) of RGJ and lioRGJ were each directly injected after filtration through a 0.45 μm membrane filter. Elution conditions consisted of 10% formic acid in water (solvent A) and 10% formic acid in methanol (solvent B) gradients at a flow rate of 1.0 mL/min. The column selected was a C-18 Zorbax (150 mm × 4.6

mm, 5 μm packing; Agilent, USA) protected by an Agilent C-18 guard column. Analyses were run on a Finnigan HPLC system (Thermo Electron Corp., San Jose, CA, USA) provided with a photodiode array detector (DAD). The gradient conditions were as follows: 0 min, 18% B; 14 min, 29% B; 16 min, 32% B; 18 min, 41% B; 18.1 min, 30% B; 29 min, 41% B; 32 min, 50% B; 34.5 min, 100% B; 35−38 min, 18% B. Calibration curves consisted of 0.001−1 mg/mL quercetin-3-*O*-glucoside and 0.05−1 mg/mL malvidin-3-*O*-glucoside standard solutions.

The identity of anthocyanins and flavonols was confirmed with LC-ESI/MS/MS experiments, and data were compared with previous works.[26] The same chromatographic conditions were applied to a HP1100 HPLC system (Agilent, USA) coupled to a PE-Sciex API-2000 triple-quadrupole mass spectrometer (Warrington, Cheshire, UK) equipped with a Turbospray (TSI) source. MS detection was carried out in positive ion mode for anthocyanins and in negative ion mode for flavonols at unit resolution using a mass range of m/z 150−1500 and a mass peak width of 0.7 ± 0.1. Selected ion monitoring (SIM) experiments were carried out using the following operational parameters: vaporiszer, 350 °C; heated capillary, 150−200 °C; carrier gas, nitrogen, at a sheath pressure of 70 psi; auxiliary gas, nitrogen, to assist in nebulization, at a pressure of 30 psi; declustering potential, 44.0 eV; focusing potential, 340.0 eV; entrance potential, 10.0 eV; collision enegy, 33.0 eV for ion decomposition in the collision cell at 0.8 mTorr.

Cell Culture and Viability Test. Rat cardiac H9C2 cells (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin in 150 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2. The cells were fed every 2−3 days and subcultured once they reached 70−80% confluence. Cell viability and proliferation were assessed by incubating the culture with lioRGJ (0.01−1 μg) and 1 μM doxorubicin for 72 h.

Preparation of Cell Extract. Cardiac H9C2 cells were collected by centrifugation and then resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 2 mM EDTA. The cells were sonicated, followed by centrifugation at 13000*g* for 10 min at 4 °C. The resulting supernatants were collected and kept on ice for immediate measurements, as described below.

Measurement of Intracellular ROS Accumulation. 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA, 5 μM) was used to detect intracellular ROS levels in H9C2 cells.[27] DCF-DA is cell membrane permeable. Once inside the cells, DCF-DA is hydrolyzed by cellular esterases to form DCF, which is trapped intracellularly due to its membrane impermeability. DCF then reacts with intracellular ROS to form the fluorescent product, 2′,7′-dichlorofluoroscein. Then, the cells were washed once with PBS and lysed in 3 mL of ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.2% sodium dodecyl sulfate. The cell lysates were collected and centrifuged at 2000*g* for 5 min at 4 °C. The fluorescence of the supernatants was measured using a Perkin-Elmer luminescence spectrometer (LS50B) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

Measurement of Cellular Superoxide Dismutase (SOD) Activity. Total cellular SOD activity was measured as follows.[28] Briefly, a reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 1.33 mM diethylenetriaminepentaacetic acid, 1.0 U/mL catalase, 70 μM nitroblue tetrazolium, 0.2 mM xanthine, 50 μM bathocuproinedisulfonic acid, and 0.13 mg/mL BSA. A 0.8 mL aliquot of the reaction mixture was added to each cuvette, followed by the addition of 100 μL of lysate. The cuvettes were prewarmed at 37 °C for 3 min. The formation of formazan blue was monitored at 560 nm and 37 °C for 5 min. The sample total SOD activity was calculated using a concurrently run SOD (Sigma) standard curve and expressed as units per milligram of cellular protein. Cellular protein content was quantified with Bio-Rad protein assay dye (Hercules, CA, USA) on the basis of the method that makes use of BSA as the standard.

Measurement of Caspase-3 Activity. Caspase-3 activity was measured using the BD ApoAlert Caspase-3 Fluorogenic Assay (BD Biosciences Clontech, Palo Alto, CA, USA). Briefly, protein lysates were collected from cells that had been incubated with lioRGJ (0.01−1 μg) for 8 h, as per protocol. Activity was measured using a fluorescent microplate reader (PerSeptive Biosystems, Farmington, MA, USA).

Statistics. Unless otherwise stated, all of the experimental results were expressed as the mean ± standard deviation (SD) of three determinations of four batches. A one-way ANOVA was performed on the means to determine whether they differed significantly. P values of <0.05 were regarded as significant. The degree of linear relationship between two variables was measured using the Pearson product moment correlation coefficient (R). Correlation coefficients (R) were calculated by using Microsoft Office Excel application.

RESULTS AND DISCUSSION

Polyphenolic Composition and Antioxidant Capacity. The results obtained for RGJ polyphenolic composition (Figure.1) were generally higher than those reported elsewhere for other commercial samples.[1−3,29,30] Southern Italy is characterized by very high levels of exposure to sunlight, which is known to exert a marked influence on the

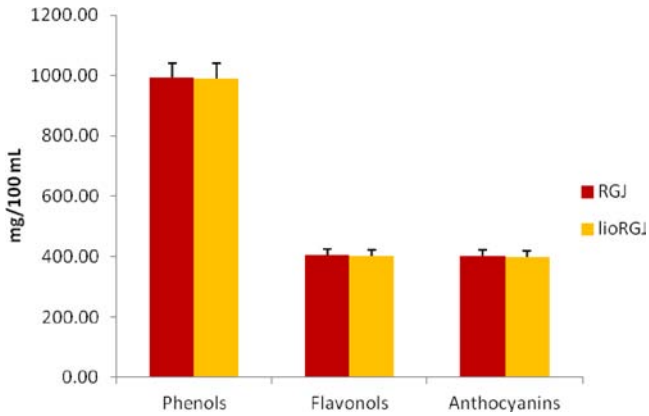

Figure 1. Polyphenolic contents in *Vitis vinifera* L. cv. Aglianico N. red grape juice before (RGJ) and after lyophilization (lioRGJ). Phenol contents are expressed as mg GAE/100 mL ± SD; flavonol contents are expressed as mg QE/100 mL ± SD; anthocyanin contents are expressed as mg ME/100 mL ± SD (P < 0.001).

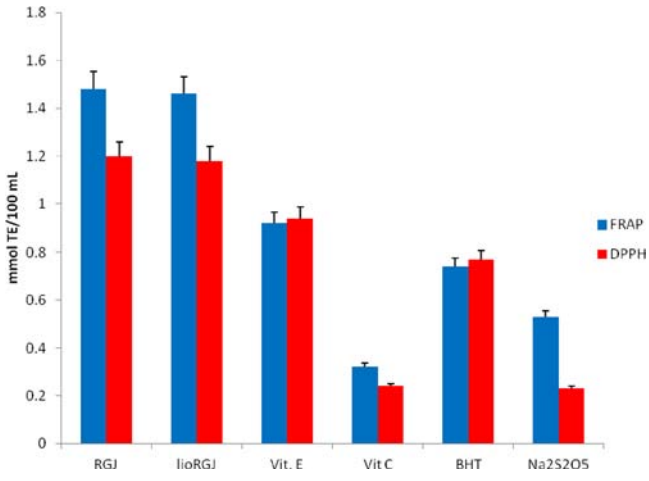

Figure 2. Reducing capacity (FRAP test) and radical-scavenging activity (DPPH test) of *Vitis vinifera* L. cv. Aglianico N. red grape juice before and after lyophilization versus antioxidant standards. Values are expressed ± SD (P < 0.001). RGJ, red grape juice; lioRGJ, lyophilized red grape juice; Vit. E, vitamin E; Vit. C, vitamin C; BHT, butylhydroxytoluene; $Na_2S_2O_5$, sodium metabisulfite. Standard solutions were of 1 mg/mL concentration.

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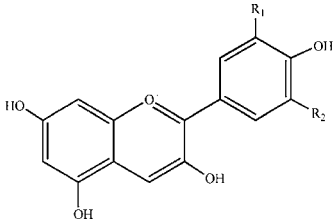
Table 1. LC-MS Data of Identified Anthocyanins in *Vitis vinifera* L. cv. Aglianico N. Red Grape Juice (RGJ) before and after Lyophilization (lioRGJ) and Their Quantitative Analysis Using DAD at 520 nm



Name	R_1	R_2
Cyanidin	OH	H
Peonidin	OCH_3	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

		mg ME/100 mL[a]						
peak	compound	RGJ	lioRGJ	retention time[b] (min)	m/z $[M+H]^+$	MS^{2c}	MS^{3c}	MS^{4c}
1	delphinidin-3-*O*-glucoside	34.78 ± 1.1	34.87 ± 1.3	5.14 ± 0.2	465	303	229, 257, 303	229, 257
2	cyanidin-3-*O*-glucoside	21.63 ± 1.2	21.63 ± 1.1	6.62 ± 0.3	449	287	213, 231, 241, 259, 287	213, 231, 241, 259, 287
3	petunidin-3-*O*-glucoside	25.63 ± 1.4	25.68 ± 1.0	8.12 ± 0.4	479	317	257, 274, 302, 317	218, 228, 246, 274
4	peonidin-3-*O*-glucoside	31.24 ± 1.0	31.15 ± 0.9	10.07 ± 0.5	463	301	286	230, 258, 268
5	malvidin-3-*O*-glucoside	114.63 ± 1.9	114.50 ± 1.7	11.50 ± 0.6	493	331	179, 242, 270, 287, 299, 315/316	213, 257, 285, 287, 313, 315
6	delphinidin-3-*O*-acetylglucoside	10.38 ± 1.5	10.40 ± 0.3	15.44 ± 0.7	507	303	229, 257, 303	229, 257, 303
7	cyanidin-3-*O*-acetylglucoside	5.08 ± 0.9	5.01 ± 0.4	16.86 ± 0.7	491	287	213, 231, 259, 287	213, 231, 259, 287
8	malvidin-3-(6-*O*-coumaroyl) glucoside (*cis* isomer)	12.38 ± 0.8	12.21 ± 0.4	22.35 ± 1.02	639	331	179, 242, 270, 287, 299, 315/316, 331	257, 285, 287, 313, 315
9	malvidin-(6-*O*-caffeoyl)glucoside	52.99 ± 1.2	53.00 ± 0.6	24.00 ± 1.09	655	331	179, 242, 270, 287, 299, 315/316, 331	257, 285, 287, 313, 315
10	peonidin-3-(6-*O*-coumaroyl) glucoside (*trans* isomer)	4.32 ± 1.0	4.32 ± 0.2	32.15 ± 0.7	609	301	286	230, 258, 268
11	malvidin-3-(6-*O*-coumaroyl) glucoside (*trans* isomer)	14.66 ± 0.8	14.35 ± 0.1	32.73 ± 0.6	639	331	179, 242, 270, 287, 299, 315/316, 331	225, 253, 281, 299

[a]ME, malvidin-3-*O*-glucoside equivalents, expressed as value ± SD ($P < 0.05$). [b]Expressed as mean value ± SD. [c]Base peak (100%) is underlined.

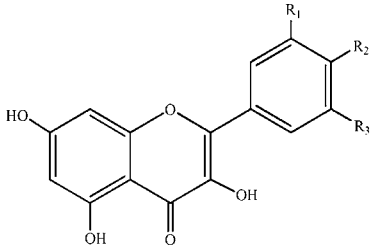
polyphenolic content of grapes. Thus, it has been shown that sun-exposed grapes can contain up to 10 times more total phenolics than grapes cultivated in the shade.[31] Moreover, thermal treatments employed during grape processing for grape juice production may be responsible for a more exhaustive extraction of polyphenols.[3] Particularly, RGJ high flavonol levels may be ascribed to hydrolysis processes, which would increase the monomeric compound content in the final product.[4] The risk of an overestimation, which is possible at 360 nm for the presence of other phenolic compounds (e.g., cinnamic acids, anthocyanins) that also have some absorbance, was avoided in this study by using a more specific spectral method of flavonol estimation. This method, based on complex formation with aluminum chloride,[22] is rather specific to flavonols, because the aluminum complexation requires a 4-keto group and at least one neighboring (3- or 5-) hydroxyl group, which are common features of flavonols. Similar features are also present in flavones and flavanones, but these compounds are not common in red grape. Interestingly, the almost identical RGJ and lioRGJ polyphenolic contents indicated a good stability of the juice to the lyophilization process.[32] The averages of total phenol, flavonoid, and anthocyanin contents of juice samples did not significantly differ at a level of $P < 0.001$.

Owing to the complex reactivity of phytochemicals, the antioxidant activities of food and food extracts cannot be evaluated by only a single method, but at least two test systems have been recommended for the determination of antioxidant activity to establish authenticity.[33] For this reason, the antioxidant activity of samples was determined by two spectrophotometric methods, DPPH and FRAP tests, and expressed as Trolox equivalents (TEs). The reduction of DPPH absorption is indicative of the capacity of the samples to scavenge free radicals, whereas the FRAP method is used to determine the capacity of reductants in a sample.

RGJ and lioRGJ showed quite comparable antioxidant activities when tested by both assays. The averages of total antioxidant activities of juice samples did not differ at a significance level of $P < 0.001$. These results confirmed that the quali-quantitative polyphenolic composition of the freeze-dried sample remained almost unchanged. Results revealed for the juice samples a good antioxidant activity when compared with that of authentic standards chosen as widely employed food preservatives and strong hydrophilic or lipophilic antioxidants (Figure 2). It is accepted that flavonoids and their metabolites, thanks to their both hydrophilic and relatively lipophilic properties, may interact with plasma proteins as well as the polar surface region of phospholipid bilayers in lipoproteins and cell membranes.[34] Because of the nature of these interactions, flavonoids may have the ability to protect against free radical attack in both aqueous and lipid environments, thus providing an effective antioxidant defense in biological systems. Grape juice is a rich source of antioxidant flavonoids, mainly catechin, epicatechin, quercetin, and anthocyanins. In vitro studies showed that grape juice has significant antioxidant activity and can inhibit the oxidation of LDL.[35,36] Human studies showed promising results but were limited by either the short duration of supplementation, the confounding effects of medications or other antioxidants, or the measurement of only a few indices of antioxidant status.[35]

Anthocyanin and Flavonol Profiles. RGJ and lioRGJ samples showed almost identical quali-quantitative anthocyanin and flavonol patterns (Tables 1 and 2). Although more than 11

Table 2. LC-MS Data of Identified Flavonols in *Vitis vinifera* L. cv. Aglianico N. Red Grape Juice before (RGJ) and after Lyophilization (lioRGJ) and Their Quantitative Analysis Using DAD at 353 nm



Name	R_1	R_2	R_3
Kaempferol	H	OH	H
Quercetin	OH	OH	H
Isorhamnetin	OCH_3	OH	H
Myricetin	OH	OH	OH
Laricitrin	OCH_3	OH	OH
Syringetin	OCH_3	OH	OCH_3

		mg QE/100 mL[a]						
peak	compound	RGJ	lioRGJ	retention time[b] (min)	m/z $[M-H]^-$	MS^{2c}	MS^{3c}	MS^{4c}
1	myricetin-3-*O*-glucoside	93.1 ± 0.3	91.7 ± 0.2	9.32 ± 0.3	479	316/317	242, 270/271, 287	171, 199, 227
2	quercetin-3-*O*-glucuronide	75.6 ± 0.2	73.5 ± 0.3	13.01 ± 0.4	477	301	151, 179,193, 257, 273	151
3	quercetin-3-*O*-glucoside	79.8 ± 0.07	78.4 ± 0.08	13.55 ± 0.4	463	301	151, 179,193, 257, 273	151
4	laricitrin-3-*O*-galactoside	13.3 ± 0.08	11.9 ± 0.06	14.96 ± 0.5	493	330, 331	151, 179,193, 316, 317	151, 164, 179, 219, 244, 270/271, 287/288
5	kaempferol-3-*O*-glucoside	5.6 ± 0.02	5.6 ± 0.01	15.68 ± 0.4	447	255, 284/285, 327, 401, 419, 429	227, 239, 255/256	212, 227
6	laricitrin-3-*O*-rhamnose-7- *O*-trihydroxycinnamic acid	18.2 ± 0.1	16.8 ± 0.2	16.86 ± 0.3	655	303, 314, 329, 347, 475, 501, 509	314	299
7	kaempferol-3-*O*-caffeoylate	16.8 ± 0.02	16.8 ± 0.02	17.77 ± 0.4	447	284/285	227, 239, 255/256	212, 227
8	isorhamnetin-3-*O*-glucoside	24.5 ± 0.03	23.1 ± 0.04	19.35 ± 0.4	477	271, 285, 314/315, 357	243, 257, 271, 285/286, 299/300	241/270
9	syringetin-3-*O*-galactoside	19.6 ± 0.05	20.3 ± 0.03	19.95 ± 0.4	507	344/345, 387, 479, 489	330	

[a]QE, quercetin-3-*O*-glucoside equivalents, expressed as value ± SD ($P < 0.05$). [b]Expressed as mean value ± SD. [c]Base peak (100%) is underlined.

Table 3. Effect of Lyophilized *Vitis vinifera* L. cv. Aglianico N. Red Grape Juice (lioRGJ) on Free Radical and Manganese Superoxide Dismutase Levels in Lysate of H9C2 Cardiomyocytes[a]

	control	0.01 μg of lioRGJ	0.05 μg of lioRGJ
TBARS, μM/μg protein	0.0043 ± 0.05	0.0025 ± 0.01	0.0047 ± 0.04
NO_2^-, nmol/μg protein	0.0010 ± 0.02	0.0039 ± 0.05	0.0080 ± 0.09
MnSOD, U/μg protein	0.0100 ± 0.12	0.0100 ± 0.15	0.0180 ± 0.28

[a]Values are expressed as means ± SD of at least three experiments ($P < 0.001$ compared to the control). lioRGJ, lyophilized red grape juice; TBARS, thiobarbituric acid reactive substances; MnSOD, manganese superoxide dismutase; control, untreated cell lines.

and 9 distinct chromatographic peaks, as regards anthocyanins and flavonols, respectively, were detected for each sample, some were present only in trace amounts, thus making their identification and quantification difficult. Their identification was based on MS experiments, UV−vis absorption spectra, and chromatographic retention times, which were compared with reference compounds and data from other studies.[26]

Single anthocyanin contents (Table 1) were generally higher than those reported elsewhere for other commercial samples.[1−3,29,30] As reported in the literature on *V. vinifera* fruit products,[37,38] the compound malvidin-3-*O*-glucoside was found as the main monomeric anthocyanin in the sample tested. Among the fruits and vegetables commonly consumed, grapes and their associated products are regarded as the most important source of our dietary anthocyanins. These compounds have been shown to contribute to the strong protection of red grape juice and wine against low-density lipoprotein oxidation.[3] Recent studies have demonstrated that the long-term intake of anthocyanins, which were administered as food matrix or enriched fractions, changed the markers for the oxidative status in some tissues and affected antioxidant enzyme expression levels and activities when compared with animals that did not receive polyphenols in the diet.[39] Thus, considering the dietary intake of anthocyanins (approximately 100 mg/diet)[40] and their potential health benefits, the juice sample could be regarded as a valuable anthocyanin source suitable for use as dietary supplement.

Our sample showed a higher concentration of flavonols than other more widely consumed red grape juices and wines,[3,41] as reported in Table 2. It has long been known that the increased biosynthesis of polyphenols, especially flavonols, is greatly influenced by sunlight exposure and temperature, so it is expected that the grapes which are grown in warmer, sunnier areas have a higher level of flavonols. In addition, industrial processing in which the juice is submitted to a heat treatment to obtain a product characterized by much better conditions for storage, transport, and preservation can increase the flavonol content due to more exhaustive extraction processes and digestion mechanisms.[4] Our study confirmed that the main flavonol glycosides in red grape juices and wines are derivatives

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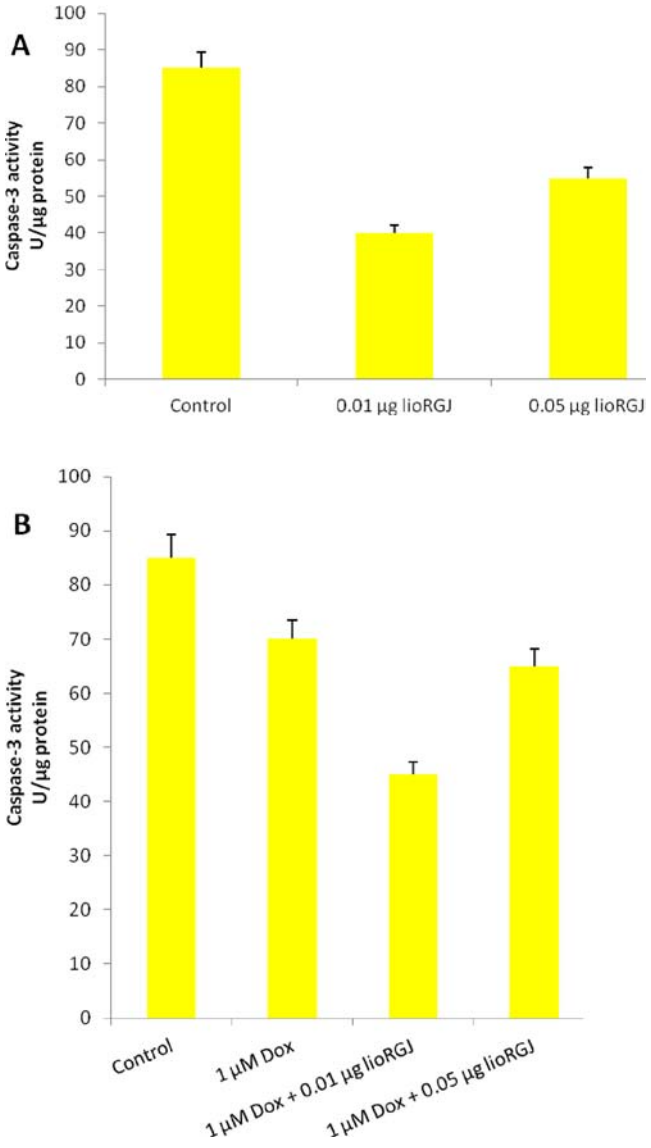

Figure 3. Effect of (A) lyophilized *Vitis vinifera* L. cv. Aglianico N. red grape juice (lioRGJ) and (B) doxorubicin (Dox) in association with lyophilized *V. vinifera* L. cv. Aglianico N. red grape juice (lioRGJ) on caspase-3 activity in lysate of H9C2 cardiomyocytes. Values are expressed as means ± SD of at least three experiments ($P < 0.001$). Control: untreated cell lines.

of myricetin and quercetin,[42,43] namely, myricetin-3-*O*-glucoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-glucuronide (Table 2). In contrast to the literature, low levels of kaempferol derivatives were found. Results suggested that the rates of hydrolysis of the flavonol glycosides in grape juice and wine were different according to the type of flavonol aglycone and also with respect to the nature of the glycoside moiety.[44] The differences between the means of polyphenolic compounds did not significantly differ at a level of $P < 0.05$.

Effects of lioRGJ on Cardiomyocytes. We examined the effect on free radical and manganese superoxide dismutase levels in cardiac-derived H9C2 myocytes exposed to increasing doses (0.01−1 μg) of lioRGJ (Table 3). Recent studies reported that grape seed proanthocyanidin extract (GSPE) would possess potent antioxidant activity against exogenous H_2O_2, hydroxyl radical, and superoxide and may chelate iron, when tested on cardiomyocyte culture.[11−13] Similarly, data reported in Table 3 demonstrated that antioxidants in the juice sample at a maximum sample dose of 0.01 μg were able to directly scavenge free radicals (with the exception of RNS) without interfering with cell antioxidant defensive system involving enzymes and proteins for cardioprotection. Nevertheless, exposure to increasing concentrations of lioRGJ resulted in pro-oxidant effects as demonstrated by the increase in ROS, RNS, and antioxidant enzyme levels at a sample dose of 0.05 μg (Table 3). These results would suggest what has already been stated in the literature for GSPE that higher doses of antioxidants occurring in the juice sample may cause apoptotic cell injury via effector caspase-3 activation and subsequent induction of ROS and RNS generation.[14−16] To confirm such a hypothesis, the influence of lioRGJ on caspase-3 activity in cardiac-derived H9C2 myocytes was tested (Figure 3). Among the many known regulators and effectors of apoptosis, caspases are a family of cytoplasmic proteases that play an important role in the execution phase of apoptosis. Two groups of caspases can be identified: upstream initiator caspases, which cleave and activate other caspases, and downstream effector caspases, including caspase-3, caspase-6, and caspase-7, which cleave a variety of cellular substrates or inactivating enzymes. Caspase-3 is a central executioner in apoptosis.[45] Cells were incubated with lioRGJ (0.01−1 μg) in medium for 8 h and then lysed to measure caspase-3 activity using a fluorogenic assay. The best result was achieved with a dose of 0.01 μg that made the caspase-3 activity decrease by about 47% (Figure 3). An increase in lioRGJ dose (from 0.01 to 0.05 μg) exposure to cardiomyocytes seemed to be less effective in reducing caspase-3 activity. Collectively, these data suggested that higher doses of lioRGJ caused cell death via the caspase-3-mediated apoptotic pathway. Other work suggests that flavonoid compounds can affect protein kinase C (PKC) activities. It is thus plausible that lioRGJ, at higher doses, may activate PKC isoforms that induce apoptosis (e.g., PKC-δ) or inhibit isoforms that protect against apoptosis (e.g., PKC-ε).[46]

To ascertain the potential effects of lioRGJ on the doxorubicin-induced oxidative stress in cardiac cells, H9C2 cardiomyocytes were exposed to 1 μM doxorubicin and a combination of doxorubicin and different doses of lioRGJ for 72 h (Table 4). A sample aliquot of 0.01 μg provided an

Table 4. Effect of Lyophilized *Vitis vinifera* L. cv. Aglianico N. Red Grape Juice (lioRGJ) on Doxorubicin-Induced Oxidative Stress in Lysate of H9C2 Cardiomyocytes[a]

	control	1 μM Dox	1 μM Dox + 0.01 μg of lioRGJ	1 μM Dox + 0.05 μg of lioRGJ
TBARS, μM/μg protein	0.0043 ± 0.05	0.0068 ± 0.09	0.0021 ± 0.02	0.0050 ± 0.05
NO_2^-, nmol/μg protein	0.0010 ± 0.02	0.0065 ± 0.08	0.0052 ± 0.07	0.0240 ± 0.21
MnSOD, U/μg protein	0.0100 ± 0.12	0.0080 ± 0.05	0.0080 ± 0.10	0.0350 ± 0.35

[a]Values are expressed as means ± SD of at least three experiments ($P < 0.001$ compared to the control). lioRGJ, lyophilized red grape juice; Dox, doxorubicin; TBARS, thiobarbituric acid reactive substances; MnSOD, manganese superoxide dismutase; control, untreated cell lines.

appreciable radical-scavenging activity as indicated by the decrease in the free radical levels (especially ROS species, about 31%) and the unchanged antioxidant defense system activity (Table 4). Interestingly, the association of doxorubicin with higher lioRGJ doses (from 0.01 to 0.05 μg) led to the enhancement of cardiac cell oxidative stress, probably due to sample pro-oxidant effects, as indicated mainly by the increase in RNS and antioxidant enzyme levels (Table 4). To confirm such a hypothesis, the influence of 1 μM doxorubicin and a combination of doxorubicin with different doses of lioRGJ on caspase-3 activity in cardiomyocytes was assayed (Figure 3). Our results showed that doxorubicin significantly up-regulated caspase-3 activity, whereas its combination with maximum sample aliquot of 0.01 μg seemed to effectively depress (by about 60%) the activity of this apoptotic factor (Figure 3). The means of the results from all of the above experiments were different at a significance level of $P < 0.001$.

In conclusion, our results showed a good antioxidant stability of the juice sample to lyophilization that may be reasonably regarded as a suitable process for the formulation of food supplements. In vitro experiments on cardiomyocyte cell culture indicated that low doses of lioRGJ were able to confer protection against both physiological reactive oxygen species (ROS) and doxorubicin-induced oxidant injury. It would be difficult to draw direct comparisons of our in vitro study, in which cardiomyocytes were directly exposed to lioRGJ, with animal models. The blood levels of antioxidants from lioRGJ were not measured in these experiments, and it would be difficult to extrapolate what sort of oral dosage would be required to achieve the equivalent levels of such antioxidants that cells in our experiments were exposed to. Our data suggest for the juice sample the possibility to be employed as a food supplement with prospective cardioprotective benefits, although further studies are needed to optimize its dosages to avoid harmful pro-oxidant effects.

AUTHOR INFORMATION

Corresponding Author

*Phone/fax: +39-081-678610. E-mail: giancarlo.tenore@unina.it.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ETC, electron transport chain; GSPE, grape seed proanthocyanidin extract; lioRGJ, lyophilized red grape juice; RGJ, red grape juice; ROS, reactive oxygen species.

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