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Polyphenolic pattern and *in vitro* cardioprotective properties of typical red wines from vineyards cultivated in Scafati (Salerno, Italy)

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ABSTRACT

Wines are the subject of increasing numbers of investigations owing to the pharmaceutical usefulness of grape phytochemicals. The aim of the present work was to hypothesize the use of lyophilised red wines for the formulation of food supplements potentially useful against both physiological and induced cardiac oxidative stress. Cardiac derived H9C2 myocytes were incubated with increasing doses (0.01–1 μg) of lyophilised Aglianico wine (lioAW). Experiments showed an appreciable direct radical scavenging activity at a maximum lioAW dose of 0.03 μg that made the caspase-3 activity decrease by about 41%. Cardiac cells were exposed to 1 μM doxorubicin and its combination with different doses of lioAW. Maximum lioAW aliquot of 0.03 μg seemed to effectively contrast the induced oxidant injury decreasing the reactive oxygen species (ROSs) levels by about 38%and depressing the caspase-3 activity by about 63%. In both assays, pro-oxidant effects at higher lioAW concentrations were detected.

1. Introduction

The health-protective properties of wines are attributed to their antioxidant activities, i.e., their capability to eliminate free radicals. Consequently, numerous papers have been focused on the determination of the antioxidant capacity of wines (Fernandéz-Pachón, Villaño, Troncoso, & García-Parrilla, 2006) as well as on their polyphenolic content, probably responsible for the antioxidant action. 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 (Saija et al., 1995). 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 defence in biological systems. There is a plethora of scientific evidence demonstrating the pharmaceutical usefulness of grape and wine phytochemicals, but to prove their legitimacy as useful dietary supplements grape extracts deserve a much better documentation than what is offered today (Milos Sovak, 2001). 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 (ROSs) and reactive nitrogen species (RNSs) that play a crucial role in the pathophysiology of a large variety of cardiovascular diseases including congestive heart failure, valvular heart disease, cardiomyopathy, hypertrophy, atherosclerosis, and ischaemic heart disease (Murphy, 2009; Vanden Hoek, Shao, Li, Schumacker, & Becker, 1997; Venditti, Masullo, & Di Meo, 2001). 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 reactive oxygen species including hydroxyl and peroxyl radicals (Baydar, Özkan, & Yaşar, 2007; Shao, Becker, et al., 2003; Shao et al., 2009). However, it is also reported on their pro-oxidant toxicity at higher doses (100–500 μg/mL), particularly their ability to cause apoptosis in cardiomyocytes induced by ROS generation (Lluís et al., 2011; Shao, Vanden Hoek, et al., 2003; Shao et al., 2006).

Doxorubicin has long been one of the most effective chemotherapeutic agents for the treatment of various types of cancer. However, it is well-known that the clinical use of anthracyclines in the treatment of many neoplastic diseases is limited by acute

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and chronic dose related, cumulative, and essentially irreversible cardiotoxicities (Reeve et al., 2007). 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 (Reeve et al., 2007). Since cellular apoptosis is at least partially responsible for the pathogenesis of doxorubicin cardiac toxicity, *in vitro* and in vivo studies have been conducted employing anti-apoptotic remedies to manage this devastating complication. Nevertheless, to date, only few single chemicals or raw extracts have proven to be able to reduce the deleterious action of doxorubicin. Liu et al. (2008) studied the capacity of *Ginkgo biloba* extracts to counteract the deleterious effects deriving from doxorubicin activation of the p53-mediated, mitochondrion-dependent cell apoptosis. Other authors (Chang et al., 2011) have recently shown that the flavone Baicalein, isolated from the roots of *Scutellaria baicalensis*, protects against cellular adverse effects of doxorubicin, confirming that flavonoids, the most abundant antioxidant naturally occurring in food, possess excellent radical-scavenging properties. Actually, very few reports regarding the protective effects of grape and grape product antioxidants against doxorubicin induced cardiotoxicity are available (Du & Lou, 2008). Therefore, the search for an effective and safe antagonist of doxorubicin cardiac toxicity remains a critical issue in both cardiology and oncology.

Based on this accepted knowledge, the aims of this research were (1) to study the antioxidant profile of Aglianico red wine (a high-quality and late-ripening grape autochthonous cultivar of Campania, Italy) before (AW) and after lyophilisation (lioAW) in order to evaluate its stability to processing for the formulation of food supplements; (2) to test the processed sample on cardiomyocytes in order to ascertain its effects on the physiological and doxorubicin-induced oxidative stress.

2. Materials and methods

2.1. Reagents and standards

All chemicals and reagents were analytical-reagent or HPLC grade. DPPH (1,1-diphenyl-2-picrilhydrazyl), 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, aluminium 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 dodecylsulfate, diethylenetriaminepentaacetic acid, catalase, nitroblue tetrazolium, xanthine, bathocuproinedisulfonic acid, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). 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, foetal bovine serum, and Dulbecco's phosphate-buffered saline (PBS) were purchased from Gibco-Invitrogen (Carlsbad, CA). Tissue culture flasks and 24-well tissue culture plates were purchased from Corning (Corning, NY).

2.2. Wine samples

The wine samples (*Vitis vinifera* L. cv. Aglianico N.; vintage: 2010; four lots) were obtained from Coppola S.p.a. (Luigi Coppola, Scafati, SA, Italy). Vines were cultivated in Scafati (40°45′ north, 14°32′ west, Italy), grapes were harvested in 2010 and wines were manufactured during the same year. Wines were stored in the dark at 15 °C until analysis, and after they were opened, the bottles were purged with nitrogen and closed with the original cork.

2.3. Lyophilisation

A 10 mL aliquot of wine was lyophilised 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.

2.4. Spectroscopic apparatus

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

2.5. Total phenolic content

The concentration of total phenolics was measured by the method described by Singleton and Rossi (1965), with some modifications. Briefly, an aliquot (20 μL) of AW, lioAW 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 double distilled water (ddH_2O). A reagent blank using ddH_2O was prepared. One mL 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 mg gallic acid equivalents (GAE)/100 mL wine.

2.6. Total flavonol content

The total flavonol content was measured by a colorimetric assay developed by Zhishen, Mengcheng, and Jianming (1999). A 50 μL aliquot of of AW, lioAW and calibration solutions of quercetin-3-O-glucoside (20, 40, 60, 80 and 100 mg/L) were added to a five volumetric flask containing 2 mL ddH_2O. At zero time, 0.15 mL $NaNO_2$ aqueous solution (5 g/100 mL) was added to the flask. After 5 min, 0.15 mL $AlCl_3$ aqueous solution (10 g/100 mL) was added. At 6 min, 1 mL 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 colour, was determined at 510 nm versus prepared water blank. Total flavonol content was expressed as mg quercetin-3-O-glucoside equivalents (QEs)/100 mL of wine.

2.7. Total monomeric anthocyanin content

The total monomeric anthocyanin content of of AW and lioAW was evaluated applying a pH-differential method. An aliquot of 1 mL of wine and of calibration solutions of malvin (0.1–10 mg/100 mL) have been added to two vials containing 10 mL of acetate buffer (pH 3.6) and HCl 1 N, respectively. The difference between the absorbances read at 530 nm has been calculated. Total anthocyanin content was expressed as mg malvin equivalents (MEs)/100 mL of wine.

2.8. HPLC quantification of anthocyanins and flavonols

HPLC separation of anthocyanins and flavonols from wine was performed according to earlier studies (Downey & Rochfort, 2008). 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 (Downey et al., 2008). Wine samples (20 μL) were directly injected after filtration through a 0.45 μm membrane filter. Elution conditions consisted in 10% formic acid in water (Solvent A) and 10% formic acid in methanol (Solvent B) gradient 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 Corporation, San Jose, California) provided with photodiode array detector (DAD). The gradient conditions were: 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 in 0.001–1 mg/mL quercetin-3-O-glucoside and 0.05–1 mg/mL malvidin-3-glucoside standard solutions.

The identity of anthocyanins and flavonols was confirmed with LC-ESI/MS/MS experiments and data were compared with previous works (Downey et al., 2008). 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, U. K.) equipped with a Turbospray (TSI) source. MS detection was carried out in positive ion mode for anthocyanins and negative ion mode for flavonols at unit resolution using a mass range of 150–1500 m/z and a mass peak width of 0.7 ± 0.1. Selected ion monitoring (SIM) experiments were carried out using the following operational parameters: vaporizer, 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.

2.9. Cell culture and viability test

Rat cardiac H9C2 cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% foetal 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 lioAW (0.01–1 μg) and doxorubicin 1 μM for 72 h.

2.10. 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 13,000*g* for 10 min at 4 °C. The resulting supernatants were collected and kept on ice for immediate measurements, as described below.

2.11. Measurement of intracellular ROS accumulation

2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA, 5 μM) was used to detect intracellular ROS levels in H9C2 cells. 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′-dichlorofluorescein. Then, the cells were washed once with PBS and lysed in 3 mL ice-cold 10 mM Tris–HCl buffer (pH 7.4) containing 0.2% sodium dodecylsulfate. 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.

2.12. Measurement of cellular superoxide dismutase activity

Total cellular superoxide dismutase (SOD) activity was measured as follows. 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 bovine serum albumin (BSA). A 0.8 mL aliquot of the reaction mixture was added to each cuvette, followed by addition of 100 μL of lysate. The cuvettes were pre-warmed at 37 °C for 3 min. The formation of formazan blue was monitored at 560 nm, 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 mg of cellular protein. Cellular protein content was quantified with Bio-Rad protein assay dye (Hercules, CA) based on the method which makes use of BSA as the standard.

2.13. Measurement of caspase-3 activity

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

2.14. Statistics

Unless otherwise stated, all of the experimental results were expressed as mean ± standard deviation (SD) of three determinations. 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.

3. Results and discussion

3.1. Polyphenolic profile

The results obtained for the wine polyphenolic composition (Fig. 1) were generally higher than those reported elsewhere for other valuable red wine varieties from Italy and abroad (Ginjom, D'Arcy, Caffin & Gidley, 2010; Landrault et al., 2001; Minussi et al., 2003; Monagas, Gomez-Cordoves, & Bartolome, 2006). Southern Italy is characterized by very high levels of exposure to sunlight, which is known to exert a marked influence on the 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. (Spayd, Tarara, Mee, & Ferguson, 2002). The content of flavonols in the selected wines in this study was higher than that reported in previous works (Cliff, King, & Schlosser, 2007). 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

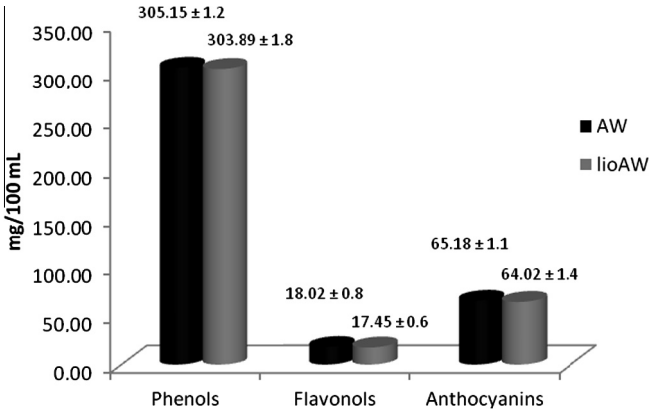
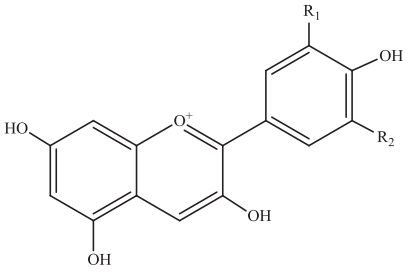


Fig. 1. Polyphenolic contents in *Vitis vinifera* L. cv. Aglianico N. wine before (AW) and after lyophilisation (lioAW). Phenol contents are expressed as mg GAE/100 mL ± SD; Flavonol contents are expressed as mg QE/100 mL ± SD; Anthocyanin contents are expressed as mg ME/100 mL ± SD ($P < 0.001$).

absorbance, was avoided in this study by using a more specific spectral method of flavonol estimation. This method, based on a complex formation with aluminium chloride (Zhishen et al., 1999), is rather specific to flavonols, because the aluminium complexation requires a 4-keto group and at least one neighbouring (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 wines. Interestingly, the almost identical AW and lioAW polyphenolic contents indicated a good stability of the wine to the lyophilisation process. The averages of total phenol, flavonoid and anthocyanin contents of wine samples were significantly different at a level of $P < 0.001$.

Although more than 11 and 9 chromatographic distinct peaks, as regards anthocyanins and flavonols, respectively, were detected for each wine 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 (Downey et al., 2008). Single anthocyanin contents (Table 1) resulted generally higher than those reported elsewhere for wines from other countries (Landrault et al., 2001; Minussi et al., 2003; Ginjom, D'Arcy, Caffin, & Gidley, 2010; Monagas et al., 2006). As reported in literature on *Vitis vinifera* wines (Monagas, Nunez, Bartolome, & Gomez-Cordoves, 2003), the compound malvidin-3-*O*-glucoside was found as the main monomeric anthocyanin in all of the samples tested. The extractability of the anthocyanins from grapes during fermentation depends on their variety, geographical origin, vintage and wine-making techniques. The anthocyanin composition or profile has been used in several studies to fingerprint grape varieties. It was found that the anthocyanin profile of Spanish red wines could be used to distinguish grape cultivar, wine origin, and also the wine-making method (Gonzalez-San Jose, Santa-Maria, & Diez, 1990). Among the fruits and vegetables commonly consumed, grapes and their associated products are regarded as the most important source of our dietary

Table 1
LC/MS data of identified anthocyanins in *Vitis vinifera* L. cv. Aglianico N. wine before (AW) and after lyophilisation (lioAW) and their quantitative analysis using DAD at 520 nm.

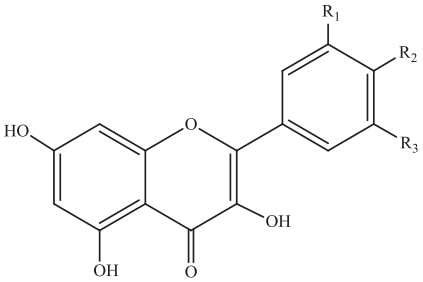


Name	R_1	R_2
Cyanidin	OH	H
Peonidin	OCH_3	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Peak	Compound	mg ME/100 mL*		Retention time** (min)	*m/z* $[M + H]^+$	MS^{2a}	MS^{3a}	MS^{4a}
		AW	lioAW					
1	Delphinidin-3-*O*-glucoside	4.29 ± 0.1	4.01 ± 1.3	5.07 ± 0.2	465	303	229, 257, 303	229, 257
2	Cyanidin-3-*O*-glucoside	2.11 ± 0.1	1.98 ± 1.1	6.45 ± 0.3	449	287	213, 231, 241, 259, 287	213, 231, 241, 259, 287
3	Petunidin-3-*O*-glucoside	7.11 ± 0.3	6.89 ± 1.0	8.01 ± 0.4	479	317	257, 274, 302, 317	218, 228, 246, 274
4	Peonidin-3-*O*-glucoside	3.19 ± 0.4	3.02 ± 0.9	10.07 ± 0.5	463	301	286	230, 258, 268
5	Malvidin-3-*O*-glucoside	24.21 ± 1.2	24.02 ± 1.7	11.32 ± 0.6	493	331	179, 242, 270, 287, 299, 315/316	213, 257, 285, 287, 313, 315
6	Delphinidin-3-*O*-acetylglucoside	6.27 ± 0.7	6.03 ± 0.3	15.41 ± 0.7	507	303	229, 257, 303	229, 257, 303
7	Cyanidin-3-*O*-acetylglucoside	3.12 ± 0.4	2.98 ± 0.4	16.67 ± 0.7	491	287	213, 231, 259, 287	213, 231, 259, 287
8	Malvidin-3-(6-*O*-coumaroyl)glucoside (*cis* isomer)	2.01 ± 0.3	1.89 ± 0.4	22.14 ± 1.02	639	331	179, 242, 270, 287, 299, 315/316, 331	257, 285, 287, 313, 315
9	Malvidin-(6-*O*-caffeoyl)glucoside	10.01 ± 1.0	9.87 ± 0.6	23.89 ± 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)	2.18 ± 0.6	2.03 ± 0.2	32.01 ± 0.7	609	301	286	230, 258, 268
11	Malvidin-3-(6-*O*-coumaroyl)glucoside (*trans* isomer)	3.00 ± 0.6	2.76 ± 0.1	32.65 ± 0.6	639	331	179, 242, 270, 287, 299, 315/316, 331	225, 253, 281, 299

AW: Aglianico wine; lioAW: lyophilised Aglianico wine.
* ME: Malvidin-3-*O*-glucoside Equivalents, expressed as value ± SD ($P < 0.05$).
** Expressed as mean value ± SD.
[a] Base peak (100%) is underlined.

Table 2
LC/MS data of identified flavonols in *Vitis vinifera* L. cv. Aglianico N. wine before (AW) and after lyophilisation (lioAW) 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

Peak	Compound	mg QE/100 mL*		Retention time** (min)	m/z [M-H]⁻	MS[2a]	MS[3a]	MS[4a]
		AW	lioAW					
1	Myricetin-3-*O*-glucoside	5.12 ± 0.4	5.02 ± 0.2	9.24 ± 0.3	479	316/317	242, 270/271, 287	171, 199, 227
2	Quercetin-3-*O*-glucuronide	3.32 ± 0.3	3.28 ± 0.3	12.87 ± 0.4	477	301	151, 179,193, 257, 273	151
3	Quercetin-3-*O*-glucoside	1.26 ± 0.08	1.21 ± 0.08	13.37 ± 0.4	463	301	151, 179,193, 257, 273	151
4	Laricitrin-3-*O*-galactoside	2.01 ± 0.07	1.89 ± 0.06	14.78 ± 0.5	493	330, 331	151, 179,193, 316, 317	151, 164, 179, 219, 244, 270/271,287/288
5	Kaempferol-3-*O*-glucoside	0.50 ± 0.02	0.43 ± 0.01	15.48 ± 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	1.68 ± 0.04	1.51 ± 0.2	16.66 ± 0.3	655	303, 314, 329, 347, 475, 501, 509	314	299
7	Kaempferol-3-*O*-caffeoylate	0.40 ± 0.02	0.35 ± 0.02	17.59 ± 0.4	447	284/285	227, 239, 255/256	212, 227
8	Isorhamnetin-3-*O*-glucoside	1.15 ± 0.05	1.08 ± 0.04	19.24 ± 0.4	477	271, 285, 314/315, 357	243, 257, 271, 285/286, 299/300	241/270
9	Syringetin-3-*O*-galactoside	2.00 ± 0.06	1.87 ± 0.03	19.79 ± 0.4	507	344/345, 387, 479, 489	330	

AW: Aglianico wine; lioAW: lyophilised Aglianico wine.
* QE: Quercetin-3-*O*-glucoside equivalents, expressed as value ± SD ($P < 0.05$).
** Expressed as mean value ± SD.
[a] Base peak (100%) is underlined.

anthocyanins. These compounds have been shown to contribute to the strong protection of the red grape juice and wine against low-density lipoprotein oxidation (Frankel, Bosanek, Meyer, Silliman, & Kirk, 1998). 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 (Hassimotto & Lajolo, 2011). Thus, considering the dietary intake of anthocyanins (approximately 100 mg/die) and their potential health benefits, the wine sample could be regarded as a valuable anthocyanin source suitable for use as dietary supplement. All of the wine samples showed a higher concentration of flavonols in comparison to conventional wines (Rastija, Srečnik, & Medić-Šarić, 2009), 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 wines made from grapes which are grown in warmer, sunnier areas, have a higher level of flavonols. Our study confirmed that the main flavonol glycosides in red wines are derivatives of quercetin and myricetin (Gawel, 1998), namely quercetin-3-*O*-glucuronide and myricetin-3-*O*-glucoside (Table 2). In contrast to literature (Gawel, 1998), low levels of kaempferol-3-*O*-glucoside, kaempferol-3-*O*-caffeoylate and quercetin-3-*O*-glucoside were found. The results suggested that the rates of hydrolysis of the flavonol glycosides in wine were different according to the type of flavonol aglycone and also with respect to the nature of the glycoside moiety (Castillo-Muñoz, Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez, 2007). The differences between the means of polyphenolic compounds were considered significantly different at a level of $P < 0.05$.

3.2. Effects of lioAW on cardiomyocytes

Cardiac derived H9C2 myocytes were exposed to increasing doses (0.01–1 μg) of lioAW and the sample influence on cellular free radical and manganese superoxide dismutase levels was evaluated (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 (Baydar et al., 2007; Shao, Becker, et al., 2003; Shao et al., 2009). Similarly,

Table 3
Effect of lyophilised *Vitis vinifera* L. cv. Aglianico N. wine (lioAW) on free radical and manganese superoxide dismutase levels in lysate of H9C2 cardiomyocytes.*

	Control	0.03 μg lioRGJ	0.08 μg lioRGJ
TBARS μM/μg protein	0.0043	0.0022	0.0051
NO_2^- nmol/μg protein	0.0010	0.0037	0.0086
MnSOD U/μg protein	0.0100	0.0100	0.0175

lioAW: lyophilised Aglianico wine.
TBARS: Thiobarbituric Acid Reactive Substances.
MnSOD: manganese superoxide dismutase.
* Values are expressed as means ± SD of three independent experiments ($P < 0.001$ compared to the control).

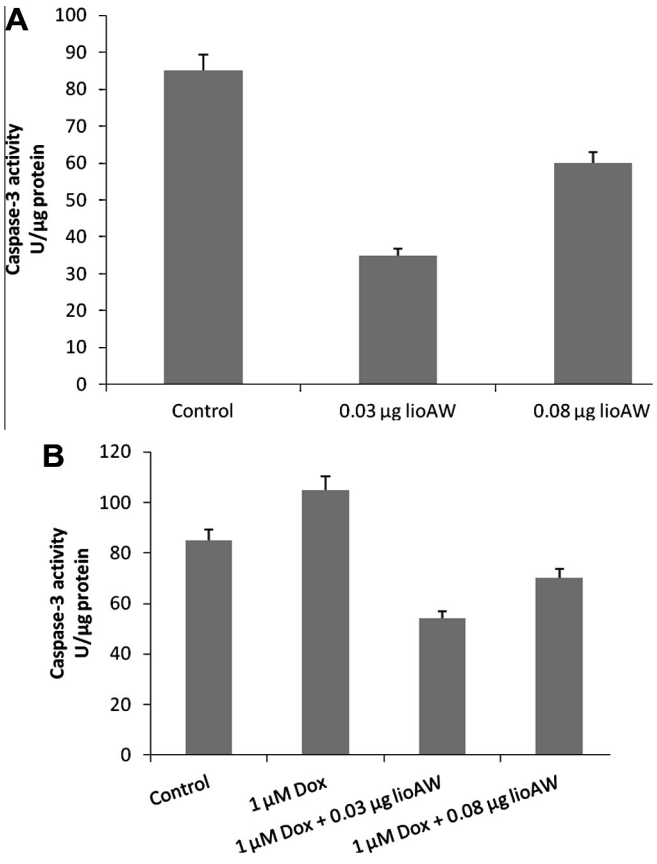


Fig. 2. (A) Effect of lyophilised *Vitis vinifera* L. cv. Aglianico N. wine (lioAW) on caspase-3 activity in lysate of H9C2 cardiomyocytes. (B) Effect of doxorubicin (Dox) in association with lyophilised *Vitis vinifera* L. cv. Aglianico N. wine (lioAW) on caspase-3 activity in lysate of H9C2 cardiomyocytes. Values are expressed as means ± SD of three independent experiments ($P < 0.001$).

data reported in Table 3 demonstrated that antioxidants in the wine sample at a maximum sample dose of 0.03 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 lioAW resulted in pro-oxidant effects as demonstrated by the increase in ROS, RNS and antioxidant enzyme levels at a sample dose of 0.08 μg (Table 3). These results would suggest what already stated in literature for GSPE that higher doses of antioxidants occurring in the wine sample may cause apoptotic cell injury via effector caspase-3 activation and subsequent induction of ROS and RNS generation (Lluís et al., 2011; Shao, Vanden Hoek, et al., 2003; Shao et al., 2006). To confirm such hypothesis, the influence of lioAW on caspase-3 activity in cardiac derived H9C2 myocytes was tested (Fig. 2A). Among the many known regulators and effectors of apoptosis, caspases are a family of cytoplasmic proteases that plays an important role in the execution phase of apoptosis. Two groups of caspases can be identified: upstream initiator caspases, that cleave and activate other caspases, and downstream effector caspases, including caspase-3, caspase-6, and caspase-7, that cleave a variety of cellular substrates or inactivating enzymes. Caspase-3 is a central executioner in apoptosis (Thornberry & Lazebnik, 1998). Cells were incubated with lioAW (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 0.03 μg that made the caspase-3 activity decrease by about a 41% (Fig. 2A). An increase in lioAW dose (from 0.03 to 0.08 μg) exposure to cardiomyocytes seemed to be less effective in reducing caspase-3 activity. Collectively, these data suggested that higher doses of lioAW 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 lioAW, 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) (Picq, Dubois, Munari-Silem, Prigent, & Pacheco, 1989).

In order to ascertain the potential effects of lioAW 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 lioAW for 72 h (Table 4). Sample aliquot of 0.03 μg provided an appreciable radical-scavenging activity as indicated by the decrease in the free-radical levels (especially ROS species, about 38%) and the unchanged antioxidant defence system activity (Table 4). Interestingly, the association of doxorubicin with higher lioAW doses (from 0.03 to 0.08 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 hypothesis, the influence of 1 μM doxorubicin and a combination of doxorubicin with different doses of lioAW on caspase-3 activity in cardiomyocytes was assayed (Fig. 2B). Our results showed that doxorubicin significantly up-regulated caspase-3 activity while its combination with maximum sample aliquot of 0.03 μg seemed to effectively depress (by about 63%) the activity of this apoptotic factor (Fig. 2B). The means of the results from all of the above experiments were different at a significant level of $P < 0.001$.

4. Conclusions

In conclusion, our results showed a good antioxidant stability of the wine sample to lyophilisation 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 lioAW were able to confer protection both against physiological reactive oxygen species (ROSs) 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 lioAW, with animal models. The blood levels of antioxidants from lioAW were not measured in these experiments, and

Table 4
Effect of lyophilised *Vitis vinifera* L. cv. Aglianico N. wine (lioAW) on doxorubicin-induced oxidative stress in lysate of H9C2 cardiomyocytes.*

	Control	Dox 1 μM	Dox 1 μM + 0.03 μg lioAW	Dox 1 μM + 0.08 μg lioAW
TBARS μM/μg protein	0.0043	0.0068	0.0026	0.0065
NO_2^- nmol/μg protein	0.0010	0.0065	0.0048	0.0211
MnSOD U/μg protein	0.0100	0.0150	0.0100	0.0308

lioAW: lyophilised Aglianico wine.
Dox: doxorubicin.
TBARS: Thiobarbituric Acid Reactive Substances.
MnSOD: manganese superoxide dismutase.
* Values are expressed as means ± SD of three independent experiments ($P < 0.001$ compared to the control).

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 wine sample the possibility to be employed as a food supplement with prospective cardioprotective benefits although further studies are needed to optimize its dosages in order to avoid harmful pro-oxidant effects.

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