Antioxidant and anti-inflammatory activity of red and white wine extracts

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**ABSTRACT**

Two commercially available Greek wines were treated with two different extraction methods in order to obtain either the total lipid fraction or several fractions containing different classes of phenolic compounds.

Chemical determinations were performed on each fraction and their capacity to inhibit lipoxygenase activity, lipid peroxidation, catalysed by Fe²⁺, and their antiradical activity, using the DPPH assay, were tested. Most of the fractions act as inhibitors against lipoxygenase and also possess scavenging capacity against DPPH radical, while, only a few of them inhibit non-enzymatic lipid peroxidation. These results indicate that a fraction's antiradical activity does not necessarily correlate to its inhibitory activity against lipid peroxidation. Additionally, a fraction's total phenolic and ortho-phenolic concentration does not necessarily determine its ability to inhibit lipoxygenase and its antiradical activity.

These results give information about the anti-inflammatory and antiradical effects of wine micro-constituents and support the idea that the molecular structure of phenolics rather than their amount is important for the biological activity of a wine.

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1. Introduction

Oxidative stress, the consequence of the imbalance between prooxidants and antioxidants in an organism, is considered to play a very important role in the pathogenesis of several degenerative diseases, such as diabetes, cancer and cardiovascular diseases, including atherosclerosis. Reactive oxygen species (ROS), including hydroxyl radicals, superoxide radicals and singlet oxygen, as well as reactive nitrogen species, are continuously generated in the cell, as a result of normal human metabolism, and can be harmful, as they can attack biological macromolecules, cause membrane and DNA damage, and enzyme inactivation. The mechanisms by which free radicals interfere with cellular functions are not yet fully understood, but one of the most important processes seems to be the formation of lipid hydroperoxides. Lipid hydroperoxides could be produced either through non-enzymatic ways, through the action of ROS on polyunsaturated fatty acids, or as specific products of lipoxygenase (LOX) and cyclooxygenase activities (Dröge, 2002). These enzymes also play an important role in inflammation, since they are involved in the biosynthesis of inflammatory lipid mediators, such as leukotrienes and prostaglandins, and their inhibition is considered as one of the targets for the prevention of diseases, whose development is linked to oxidative stress and inflammation (Rådmark & Samuelsson, 2007), such as cancer and atherosclerosis.

Data support the idea that diet could have beneficial effects against diseases and several patterns of diet or individual foods are highlighted. Amongst them, the consumption of foods rich in antioxidants, such as fruits, vegetables and wine, is considered to promote health. Thus, the beneficial role of dietary polyphenols under oxidative stress conditions is being investigated. The proposed mechanisms for their action include (i) direct radical scavenging, (ii) inhibition of enzymes, such as NO-synthase, xanthine oxidase, cyclooxygenase and lipoxygenase, (iii) iron chelation and (iv) direct inhibition of lipid peroxidation (Nijveldt et al., 2001).

During the last few years, the attempt to interpret the 'French paradox' (Renaud & de Lorgeril 1992), which is the low incidence of coronary heart disease in southern France, in spite of high fat intake, place wine in the centre of the scientific investigation (Fragopoulou, Demopoulos, & Antonopoulou, 2009). Its protective action against cardiovascular diseases is attributed not only to ethanol, but also to its micro-constituents. White and, especially, red wine are considered as rich sources of anti-oxidant polyphenolic compounds that exert both anti-inflammatory and antioxidant actions, such as (a) inhibition of platelet aggregation, (b) inhibition of LDL oxidation (antioxidant properties), (c) increase of HDL, (d) vasorelaxation and (e) modulation of endothelial action (Cordova, Jackson, Berke-Schlessel, & Sumpio, 2005).
2. Materials and methods

2.1. Reagents and chemicals

Sodium molybdate dihydrate, perchloric acid 70–72%, Folin–Ciocalteau reagent and organic solvents were purchased from Merck (Darmstadt, Germany). Gallic acid, quercetin, resveratrol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), soybean lipoxygenase (Type I-B), linoleic acid and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). All reagents and chemicals used were of analytical grade.

2.2. Samples

The two types of wine studied are commercially available in the Greek market and widely consumed. Domaine Hatzimichalis kindly offered the selected wines: Cabernet Sauvignon (red wine, main grape Cabernet Sauvignon, 2003) and Ambelon (white wine, main grape Robola, 2005).

2.3. Preparation of wine extracts

Two extraction techniques were used in order to obtain either the total lipid fraction or several fractions containing different classes of phenolic compounds.

2.3.1. Extraction No. 1 – Extraction of wine total lipids by the Bligh–Dyer method

Total lipids (TL) were extracted from 750 ml of wine according to the method of Bligh and Dyer (1959), taking into account the ethanol content of each wine. The aqueous phase (W) was stored at −20°C, while the chloroform phase (TL) was evaporated and dissolved in chloroform:methanol (1:1 v/v). One tenth of TL fraction was weighted and stored at −20°C, while the rest was further separated into polar (PL) and neutral (NL) lipid fractions by counter-current distribution (Galanos & Kapoulas, 1962). All fractions were sealed under nitrogen and stored at −20°C for further analysis.

2.3.2. Extraction No. 2 – Extraction of wine in order to obtain different classes of phenolic compounds

Liquid/liquid extraction methods were performed on 750 ml of wine, in order to obtain several fractions containing different classes of polyphenolic compounds (Ghiselli, Nardini, Baldi, & Scaccini, 1998). Alcohol removal was performed by vacuum distillation (at 30°C and 30 mbar). A 150 ml aliquot of the dealcoholised wine (pH 2.0) was first extracted with ethyl acetate (three times with 100 ml of ethyl acetate each), obtaining an aqueous residue (FI fraction) and an organic phase. The organic phase (ethyl acetate) was evaporated and redissolved in 100 ml of water at pH 7.0 and a further extraction with ethyl acetate (three times with 100 ml of ethyl acetate each) was performed. The ethyl acetate phase is the FII fraction. The aqueous residue from this extraction was adjusted to pH 2.0 and extracted again with ethyl acetate (three times with 100 ml of ethyl acetate each) to obtain the FIII fraction (ethyl acetate phase) and FIV fraction (water phase).

2.4. Chemical determinations

Total phenolic content was determined using the modified method of Singleton and Rossi (1965). Briefly, samples were dried under a stream of nitrogen and dissolved in 3.5 ml of water. A volume of 0.1 ml of Folin–Ciocalteau reagent was added followed after 3 min by 0.4 ml of Na2CO3 35% w/v. The reaction mixture was rested for 1 h and the intensity of blue colour was measured at 725 nm. Standards of gallic acid were prepared similarly.

Ortho-diphenolic content was determined following the method described by Arranz, Cert, Pérez-Jiménez, Cert, and Saura-Calixto (2008), with modifications. Briefly, samples were dried under a stream of nitrogen and dissolved in 0.1 ml ethanol. Then, 1.15 ml of a 1.087% w/v solution of sodium molybdate dihydrate in ethano/lwater (1:1, v/v) was added, and the mixture was shaken. After 15 min at room temperature, the absorbance at 370 nm was measured. Standards of quercetin were prepared similarly.

Carbohydrate determination was carried out according to the method of Fisher, Hansen, and Norton (1955) with some modifications. Briefly, samples were dried under a stream of nitrogen and dissolved in 1 ml of water. A volume of 2 ml of a solution of 5-methylresorcinol (2 mg/ml in 70% sulfuric acid, v/v) was added, followed by heating at 80°C for 20 min. On cooling, the absorbance of the solution was measured at 505 nm. Standards of glucose were prepared similarly.

Phosphorus was determined according to the method of Bartlett (1959). The amount of inorganic phosphorus that water fractions (FI, FIV and W) contained was determined by repeating the same procedure without incubating the samples with the perchloric acid at 180°C. Standards of KH2PO4 were prepared similarly.

2.5. Soybean lipoxygenase inhibition assay

The assay was performed according to a previously described procedure (Axelrod, Cheesbrough, & Laakso, 1981), with some modifications. The incubation mixture consisted of the appropriate amount of the sample solution in the chosen solvent (water or dimethyl sulfoxide) and 1.33 μl of the enzyme solution (100 units/μl in boric acid buffer) in 0.2 M boric acid buffer, pH 9.0. After incubation at room temperature for 10 min in the dark, the reaction was started by adding 0.53 μl of linoleic acid solution (450 mM in dimethyl sulfoxide). The total volume of the reaction solution was 800 μl. The conversion of linoleic acid to 13-hydroperoxylinoleic acid was recorded at 234 nm (room temperature) and compared to the appropriate standard solution, which did not contain the extracts. The effect of wine extracts upon lipoxygenase was also studied in the absence of substrate, in order to exclude the possibility that any substance may absorb at 234 nm or be produced.
simultaneously. Every sample was tested in triplicate at several concentrations. The results were expressed as IC₅₀ values (mg/ml of the reaction mixture), which are the extract concentration needed to achieve 50% inhibition of the lipoxygenase activity. Moreover, the amount (µl) of wine that corresponds to this mass of extract was calculated.

2.6. Linoleic acid peroxidation assay

This assay was performed according to the method of Choi et al. (2002), with modifications. Briefly, the reaction mixture contained 500 µl linoleic acid (20 mM), 30 µl Tris HCl (100 mM, pH 7.5), 10 µl of ascorbic acid (20 mM) and an amount of 500 mg of each wine extract in ethanol or water (up to 100 µl). Linoleic acid peroxidation was initiated by the addition of 10 µl FeSO₄·7H₂O (40 mM), incubated for 30 min at 37 °C and terminated by the addition of 103.5 µl trichloroacetic acid (40% v/v). An amount of 200 µl of thiobarbituric acid (1% w/v in 50 mM NaOH) was added to 0.5 ml of the reaction mixture, followed by heating at 95 °C for 10 min. The mixtures were centrifuged at 3500 g for 10 min and the absorbance of thiobarbituric acid-reacting substances (TBARS) in the supernatant was read at 532 nm and converted into the percentage of antioxidant activity using the following equation:

Linoleic acid peroxidation inhibition (%) = \( \frac{(A_{\text{control}} - A_{\text{blank}})}{A_{\text{control}}} \times 100 \)

where, A is the absorbance of control, blank and sample solutions, meaning without extract, without extract and FeSO₄·7H₂O, and containing the extracts, respectively. Every sample was tested in triplicate. The results are expressed as % inhibition when 500 mg of each fraction was used (corresponding to 0.77 mg/ml) and as IC₅₀ (mg/ml) for the standards.

2.7. Determination of DPPH radical-scavenging activity

The DPPH assay was used to measure the free radical-scavenging capacity of the wine extracts, according to a previously reported method (Brand-Williams, Cuvelier, & Berset, 1995), with modifications. The appropriate amount of each sample in ethanol was mixed with 0.035 ml of a freshly-prepared ethanolic solution of 0.4 mg/ml DPPH in microplate wells. The total volume of the assay was 0.2 ml. The solutions were incubated at 37 °C for 30 and 60 min and the absorbance were measured at 492 nm with a microplate reader (Sunrise, Tecan Group Ltd., Mannedorf, Switzerland). The percentage of inhibition was calculated by the formula:

% Inhibition = 100 × \( \frac{A_{\text{control}} - A_{\text{blank}}}{A_{\text{control}}} \)

where \( A_{\text{control}} \) is the absorbance of the control without the extract and \( A_{\text{extract}} \) is the absorbance with the extract. The EC₅₀ values, which reflect the equivalent concentration able to scavenge 50% of the DPPH radicals, were estimated by the plot of % scavenging against concentration and were expressed in terms of mg sample/ml of the reaction mixture and as µl of wine that corresponds to that mass of extract. Every sample was tested in triplicate.

2.8. Statistical analysis

All analyses of individual samples were performed in triplicate. The results presented are the mean ± standard deviations of the obtained values. Data manipulation was performed by means of Microsoft Excel (Microsoft Corp., Redmond, WA).

3. Results and discussion

3.1. Extraction yield and chemical composition of the extracts

Two different types of wine – a red (Cabernet Sauvignon, CS) and a white (Robola, R) – were extracted using two different methods of extraction. Extraction No. 1 was performed in order to obtain wine total lipid fractions. Since phenolics constitute the majority of bioactive compounds in wines, a second extraction method was performed, in order to obtain fractions with different polyphenolic content (Extraction No. 2). Table 1 shows the yield of each extraction method.

Extraction method No. 1 isolates the total lipid (TL) fraction from the water–soluble constituents (W), while the subsequent countercurrent distribution separates into polar (PL) and neutral (NL) lipids. TL fractions comprise less than 2.5% of wine extractable compounds, whereas the rest consists of water-soluble constituents. The majority of total lipids are distributed in the PL fraction, whereas the amount of NL is negligible in both cases, as previously reported (Fragopoulou et al., 2000; Fragopoulou et al., 2001). Moreover, as our previous data indicate, the biological activity (inhibition of platelet aggregation) of total lipids is mainly attributed to the polar lipids and for this reason, as well as their negligible amount, NL fractions were not further studied.

The majority of the extracted compounds from both wines by method No. 2 are mainly distributed in the first water fraction, FI, followed by FIJ, FIJV and FIJV fractions.

The chemical composition of wine extracts is essential for understanding their activity. Therefore, chemical determinations of total phenolics and carbohydrates were performed. Since previous data (Fragopoulou, Antonopoulou, & Demopoulos, 2002; Fragopoulou et al., 2000; Fragopoulou et al., 2001) revealed the existence of phospholipids in both tested wines, the determination of lipidic (organic) phosphorus, as well as inorganic phosphorus in water fractions was also performed. In addition, since literature data indicate that the ortho-phenolic compounds are stronger antioxidants than the rest of the phenolic constituents (Torres de Pinedo, Penalver, & Morales, 2007), the ortho-phenolic content of wine extracts was also determined. The results are presented in Table 2. In order to estimate the contribution of each fraction to wine total content, the results are expressed as mg of the corresponding standard (gallic acid, quercetin, glucose or phosphorus) per litre of wine, and % w/w, with respect to each fraction mass, in order to estimate the concentration in each fraction.

### Table 1

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Robola (R)</th>
<th>Cabernet Sauvignon (CS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1 W (water fraction)</td>
<td>13.3 x10⁻³ (98.1%)*</td>
<td>23.2 (97.5%)</td>
</tr>
<tr>
<td>TL (total lipid fraction)</td>
<td>0.340 (1.7%)</td>
<td>0.596 (2.5%)</td>
</tr>
<tr>
<td>PL (polar lipid fraction)</td>
<td>0.305 (87.3%)</td>
<td>0.558 (93.6%)</td>
</tr>
<tr>
<td>NL (neutral lipid fraction)</td>
<td>0.0006 (0.2%)</td>
<td>0.0010 (0.2%)</td>
</tr>
<tr>
<td>No. 2 FI (fraction I)</td>
<td>16.3 (94.1%)</td>
<td>23.5 (92.1%)</td>
</tr>
<tr>
<td>FI (fraction II)</td>
<td>0.540 (3.11%)</td>
<td>1.35 (3.30%)</td>
</tr>
<tr>
<td>FIJV (fraction III)</td>
<td>0.150 (0.80%)</td>
<td>0.270 (1.07%)</td>
</tr>
<tr>
<td>FIJV (fraction IV)</td>
<td>0.320 (1.85%)</td>
<td>0.380 (1.49%)</td>
</tr>
</tbody>
</table>

a Results are expressed in grams extracted from 750 ml of wine.
b Results are expressed as % w/w of each fraction with respect to each fraction mass.
c Expressed as % of the TL fraction.
As was expected, because of the different winemaking process of red wine, the amount of total phenolics and ortho-phenolics in CS is higher than in R, regardless of the extraction method used. The same observation is applied to the amount of carbohydrates, while the amount of total phosphorus is similar in both wines.

In both wines, W fraction mainly contributes to the amount of total phenolics, ortho-phenolics, carbohydrates and phosphorus. However, the distribution of phenolics and ortho-phenolics is different in the two wines' corresponding fractions. More specific, in R, the TL fraction and its subfraction PL are relatively rich in ortho-phenolics, while in CS, both W and TL fractions, as well as the PL subfraction of TL, have similar proportions of both. Concerning the carbohydrates % w/w concentration in fractions, it is quite similar in both wines. In contrast, R possesses a higher organic phosphorus amount than CS.

Concerning extraction method No. 2, the first water fraction, FL, is the one that mainly contributes to the amount of phenolics, ortho-phenolics, carbohydrates and total phosphorus (as the sum of organic and inorganic) in wines, followed by the first organic fraction, FII. Nevertheless, fraction FII possesses higher % w/w concentration in phenolics, including ortho-phenolics, in both wines. Moreover, FL of white and FI and FIV of red wine are the richest in carbohydrates.

Similar total phenolic content values for red (1000–4000 mg/l) and white (100–700 mg/l) wines and their extracts have also been reported by others (Ghiselli et al., 1998; Roussis et al., 2008). More specifically, the reported total phenolic content (as gallic acid equivalents) values of Rompola (258 and 185–192 mg/l) (Psarra, Makris, Kalithraka, & Kefalas, 2002; Roussis et al., 2008) and Cabernet Sauvignon wines (2000 mg gallic acid/l) (Landraut et al., 2001) are comparable to those reported here (210–306 mg/l and 2300–3400 mg gallic acid/l respectively). As far as we know, no data exist concerning the ortho-phenolic content of either Robola or Cabernet Sauvignon wines. The ortho-phenolic content of Rompola ranges between 100 and 119 mg quercetin/l, while Cabernet Sauvignon contains 500–600 mg quercetin/l. Their phosphorus content is 130 mg/l and 112 mg/l, respectively.

Although the separation and identification of the bioactive compounds is out of the scope of the present work, previous studies of our team have shown that several bioactive compounds identified by electrospray mass spectrometry for Extraction No. 1 fractions, belong to the classes of phenolic glycosides and/or phospho- or glyco-glycerolipids (Table 3) (Fragopoulou, Antonopoulou, Nomikos, & Demopoulos, 2003; Fragopoulou et al., 2000, 2001).

Moreover, concerning the selected method of phenolic fractionation, previous reports indicate the chemical composition of each fraction (Ghiselli et al., 1998; Roussis et al., 2008) (Table 3). More specifically, fraction FL contains all the anthocyanic compounds, FII, the subclass of procyanidins, catechins and several flavonols (mainly quercetin 3-O-glucoside), whereas FIII contains phenolic acids and quercetin 3-O-glucuronide. Fraction FIV contains the rest of the components that are not distributed between the previous fractions. Concerning Robola, the predominant phenolic acids reported are caftaric acid, coumaric acid, 2-S-glutathionyl-caftaric acid, ferraric acid, cinnamic acid and caffeic acid, while the amount of gallic acid is very small (Roussis et al., 2008). Additionally, the existence of trans-resveratrol in Robola wine has been reported (Douroglou, Makris, Bois-Dounas, & Zonas, 1999).

Concerning Cabernet Sauvignon wine, the main anthocyanins reported are malvidin, petunidin and delphinidin glycosides (Sán-
3.2. Determination of lipoxygenase inhibitory activity of wine extracts

The anti-LOX activity of the fractions was measured as inhibition of linoleic acid’s peroxidation to hydroperoxylinoleic acid, a reaction, which is catalyzed by soybean lipoxygenase. Soybean LOX was chosen as it constitutes a suitable model for mammalian lipoxygenases in several aspects, such as reaction mechanism and kinetic parameters. The results are demonstrated in Table 4. Moreover, quercetin, resveratrol and gallic acid were studied against lipoxygenase’s activity, for evaluation of the activity of the tested wine extracts, and the results are presented in the same table.

Red wine (CS) fractions derived from the first extraction method are in general more effective inhibitors of lipoxygenase than the corresponding fractions from white wine (R), with the exception of the water fractions (W), where the inhibitory activity is comparable in both wines. Water fractions (W) are more effective LOX inhibitors than TL fractions, as three to five times higher concentration of the latter does not affect at all the enzyme’s activity. Due to solubility problems, it was not possible to determine the IC₅₀ value of TL and PL fractions of white wine, but the highest tested concentrations gave negligible inhibition of the enzyme’s activity. On the other hand, TL fraction of Cabernet Sauvignon is almost as active as PL fraction, which indicates that the activity of TL fraction is probably attributed to the PL fraction.

Amongst the 2 fractions from method No. 2, the procyanidin fraction FII is the strongest inhibitor in both types of wine. Due to solubility problems, it was not possible to determine the IC₅₀ value of FI and FIII of white wine. However, the highest tested concentration did not affect at all the enzyme’s activity.

It is worth mentioning that the combination of the data from Tables 2 and 4 revealed that the % w/w of total phenolics and ortho-phenolics concentration is not necessarily correlated to the observed anti-LOX activity, and that the individual phenolics in each class should play an important role in their anti-LOX activity.

As far as we know, limited data report the effect of wine or wine extracts on eicosanoid metabolism (Soleas, Diamandis, & Goldberg, 1997), while there are no data on the effect of such wine extracts on lipoxygenase activity. However, a dose dependent in vitro activity of supplements containing red wine polyphenol extracts against 5-lipoxygenase has been reported (Leifert & Abeywardena, 2008).

The examined standards (Table 4), such as quercetin, resveratrol and gallic acid, exert more potent inhibitory activity against lipoxygenase than wine fractions, as expected, since wine fractions are mixtures of ingredients with different – often opposite – activities. The IC₅₀ values reported in the literature are 17 μM for quercetin (Kim, Kim, & Chung, 2005) and higher than 50 and 100 μM for resveratrol and gallic acid, respectively (Ngoc et al., 2008; Shibata, Nagayama, Tanaka, Yamaguchi, & Nakamura, 2003), values that are similar to those reported here.

3.3. Linoleic acid peroxidation assay

This assay was performed in order to investigate a possible correlation between the ability of wine extracts to inhibit lipoxygenase action and their ability to inhibit peroxidation of linoleic acid. Specifically, an amount of 500 mg of each fraction was tested in the assay (0.77 mg/ml of assay) and the results are shown in Table 5.

Generally, the water fractions, meaning W, FI and FIV, as well as FIII, had no effect at the tested concentrations. TL fractions inhibit peroxide formation by about 70% and 50% for white and red wine, respectively. Moreover, since the PL fraction is 97% of the TL fraction, the higher activity of TL over PL reveals that the NL fraction is probably active against non-enzymatic linoleic acid’s peroxidation. However, the amount of NL fraction present was insufficient to perform the assay. Fraction FII of Robola was the only effective inhibitor of lipid peroxidation amongst the fractions of extraction.

<table>
<thead>
<tr>
<th>Wine</th>
<th>Extraction No. 1</th>
<th></th>
<th>Extraction No. 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TL²</td>
<td>PL²</td>
<td>FI²</td>
<td>FIII²</td>
</tr>
<tr>
<td>Robola (R)</td>
<td>0.193 ± 0.003</td>
<td>0.203 ± 0.015</td>
<td>71.6 ± 7.4</td>
<td>36.5 ± 2.9</td>
</tr>
<tr>
<td>Cabernet Sauvignon (CS)</td>
<td>&gt;0.700</td>
<td>&gt;1200</td>
<td>43.5 ± 5.3</td>
<td>No effect</td>
</tr>
</tbody>
</table>

Standards mg/ml μM

- Quercetin² 0.099 ± 0.017 326 ± 57
- Resveratrol² 0.056 ± 0.016 244 ± 72

<table>
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<tbody>
<tr>
<td></td>
<td>TL²</td>
<td>PL²</td>
<td>FI²</td>
<td>FIII²</td>
</tr>
<tr>
<td></td>
<td>FIV²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Robola (R)</td>
<td>0.252 ± 0.017</td>
<td>0.297 ± 0.024</td>
<td>58.7</td>
<td>36.5 ± 2.9</td>
</tr>
<tr>
<td>Cabernet Sauvignon (CS)</td>
<td>&gt;0.400</td>
<td>&gt;1540</td>
<td>15.7 ± 1.6</td>
<td>No effect</td>
</tr>
</tbody>
</table>

Standards mg/ml μM

- Quercetin² 0.028 ± 0.002 93 ± 6
- Resveratrol² 0.026 ± 0.002 114 ± 1
- Gallic acid² 0.042 ± 0.008 247 ± 47

a Each value is expressed as mean % inhibition ±standard deviation, when 500 mg of each fraction was used (0.77 mg/ml).
b Each value is expressed as IC₅₀ (mg/ml of assay and as μM final concentration in assay) ±standard deviation.

Table 4

The IC₅₀ values of wine fractions and phenolic standards against soybean lipoxygenase.

<table>
<thead>
<tr>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>W²</td>
<td>TL²</td>
<td>PL²</td>
<td>FI²</td>
</tr>
<tr>
<td>Robola (R)</td>
<td>0.028 ± 0.002</td>
<td>0.193 ± 0.003</td>
<td>&gt;0.700</td>
<td>&gt;160</td>
</tr>
<tr>
<td>Cabernet Sauvignon (CS)</td>
<td>0.026 ± 0.002</td>
<td>&gt;0.400</td>
<td>&gt;1200</td>
<td>&gt;1540</td>
</tr>
</tbody>
</table>

Standards mg/ml μM

- Quercetin² 0.028 ± 0.002 93 ± 6
- Resveratrol² 0.026 ± 0.002 114 ± 1
- Gallic acid² 0.042 ± 0.008 247 ± 47

a Each value is expressed as mean IC₅₀ (mg/ml of assay and μM of wine that corresponds to this mass of extract) ±standard deviation.

b Each value is expressed as mean IC₅₀ (mg/ml of assay and as μM final concentration in assay) ±standard deviation.
method No. 2, in contrast to red wine’s FII fraction, whose effect was negligible at the same concentration.

Although no data exist for these specific wines, Kondo, Ohnishi, and Kawaguchi (1999) studied the inhibition of linoleic acid peroxidation by Japanese wine extracts using a chemiluminescence method. Their results indicated that the antioxidant activity of red wine extracts against linoleic acid peroxidation is higher than that of white wine extracts. Also, the anthocyanin and flavanol fraction’s antioxidant activity is higher than that of the phenolic acid fraction of the same wine. On the contrary, in the present study the fraction of procyonidine (FII) is the active one. However, it should be mentioned that different methods have been used in the fractionation and in the evaluation of fraction’s activity against linoleic acid peroxidation between Kondo et al. (1999) and the present study, which makes a direct comparison between them difficult.

Moreover, the same assay was performed using standard phenolic compounds. The tested standards, quercetin and resveratrol, are stronger inhibitors than wine fractions, as expected. Resveratrol is more effective, based on its lower IC$_{50}$ value. The reported IC$_{50}$ value for quercetin is 0.063 mg/ml (Choi et al., 2002), which is similar to the one reported here.

3.4. Antiradical capacity of wine extracts

The antiradical activities of extracts were determined using the DPPH free radical assay in order to evaluate their antioxidant capacity from a different point of view. The absorbance was measured after 30 and 60 min incubation of DPPH radical with the wine extracts, and their radical-scavenging activities, were expressed as mean EC$_{50}$ values (mg/ml) and as volume of wine that contains the effective amount of each fraction.

The results are shown in Table 6 and represent the absorbance values after 30 min incubation. The antiradical activities of standard phenolic compounds, resveratrol, quercetin and gallic acid, are also reported at Table 6. The results (data not shown) of the antiradical activity of these fractions and standards after 60 min of incubation with the DPPH radical showed a time-dependent inhibition and the corresponding EC$_{50}$ values were lower. The order of the antiradical activity of tested fractions remained the same after 60 min incubation.

Generally, the scavenging capacity of the tested wines is attributed to different fractions. In Robola (R), the water fraction (W) was a less potent scavenger, since its EC$_{50}$ value is about 3 times higher than the value of the TL fraction and 6 times higher than that of the PL fraction. Exactly the opposite was observed in Cabernet Sauvignon (CS) fractions, since the W fraction is about 5 times more effective as a scavenger than TL.

Amongst the Robola fractions from extraction method No. 2, FII fraction was the most potent quencher of DPPH radical, followed by FIII, while the rest showed similar activity. On the other hand, the most active scavenger of Cabernet Sauvignon was the F I fraction, followed by FIII and FIV, while FII was about 5 times less active than FI. Similar results have been reported (Ghiselli et al., 1998) for the antiradical activity of red wine (Sangiovese) fractions. In the aforementioned study, the anthocyanin (FI) fraction exerts an effective antiradical activity, while procyonidine (FII) and phenolic acids (FIII) fractions’ activity is weak.

The majority of the studies take for granted the positive correlation between the antiradical activity of extracts and their total phenolic content (Roussis et al., 2008), and usually express this activity as mg of phenolics/ml. Nevertheless, during recent years this correlation has been a subject of debate and investigation. In this study, although the antiradical activity of all fractions of white wine is associated with the phenolic % w/w concentration, this association is not observed in the case of red wine. For example the FII fraction of Robola is richer in total phenolics (mg/100 mg of extract) and a better quencher against DPPH radical, compared to the FII fraction of Cabernet Sauvignon, which is the least efficient scavenger but contains the highest total phenolic concentration.

The above data support the idea that the high phenolic amount of a fraction is not crucial for its antiradical activity. However, there are reports that suggest that the antioxidant properties of red wine are correlated to their content of flavan-3-ols (Teissedre, Frankel, Waterhouse, Peleg, & Bruce German, 1996) and anthocyanins (Ghiselli et al., 1998). The data from the present study support the idea that the antiradical activity should not only be attributed to the different classes of phenolic compounds, but probably to the molecular structures of the individual phenolic compounds that each fraction contains.

Although the antiradical activity of individual fractions of Robola and Cabernet Sauvignon has not been reported in other papers, the antiradical activity of total wine has been reported. Thus, Rompolà’s activity was found to be 0.55 mM (Psarra et al., 2002) and that of Cabernet Sauvignon was 20.0 mM (Landrault et al., 2001) of Trolox equivalents. The last data further support the opinion that total phenolic content, as well as wine’s colour, is not essential for the observed antioxidant activity.

In order to evaluate the antiradical activity of the wine extracts, four compounds that possess antiradical activity were also tested in the same assay (Table 6). Quercetin, resveratrol, gallic acid and

### Table 6
The EC$_{50}$ values of radical-scavenging activity of wine fractions and phenolic standards.

<table>
<thead>
<tr>
<th></th>
<th>Robola (R)$^a$</th>
<th>Cabernet Sauvignon (CS)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml of assay</td>
<td>µl of wine</td>
</tr>
<tr>
<td><strong>Extraction No. 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>1.71 ± 0.03</td>
<td>18.9 ± 0.3</td>
</tr>
<tr>
<td>TL</td>
<td>0.524 ± 0.022</td>
<td>225 ± 9</td>
</tr>
<tr>
<td>PL</td>
<td>0.331 ± 0.007</td>
<td>163 ± 3</td>
</tr>
<tr>
<td><strong>Extraction No. 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FI</td>
<td>2.58 ± 0.28</td>
<td>2.37 ± 0.26</td>
</tr>
<tr>
<td>FII</td>
<td>0.082 ± 0.005</td>
<td>2.25 ± 0.14</td>
</tr>
<tr>
<td>FIII</td>
<td>1.37 ± 0.11</td>
<td>132 ± 11</td>
</tr>
<tr>
<td>FIV</td>
<td>2.23 ± 0.20</td>
<td>103 ± 9</td>
</tr>
<tr>
<td><strong>Standards</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin$^b$</td>
<td>0.0086 ± 0.0013</td>
<td>29.0 ± 6.0</td>
</tr>
<tr>
<td>Resveratrol$^b$</td>
<td>0.0006 ± 0.0044</td>
<td>268 ± 27.0</td>
</tr>
<tr>
<td>Gallic acid$^b$</td>
<td>0.0011 ± 0.0002</td>
<td>6.7 ± 2.0</td>
</tr>
<tr>
<td>Ascorbic acid$^b$</td>
<td>0.0077 ± 0.0005</td>
<td>44.0 ± 4.0</td>
</tr>
</tbody>
</table>

$^a$ The results are expressed as mean EC$_{50}$ (mg/ml of assay and as µl of wine that corresponds to this mass of extract) ± standard deviation.

$^b$ Results are expressed as mean EC$_{50}$ (mg/ml of assay and as µM final concentration in assay) ± standard deviation.
Lipid peroxidation in vivo may occur either by enzymatic or non-enzymatic pathways. The possible mechanisms through which bioactive compounds act are the inhibition of enzyme activity, direct radical scavenging, iron chelation, and the direct antagonism with oxidation substrate. The three assays reported here provide information about the aforementioned ways. The first assay used indicates the interaction with lipoxygenase that catalyses peroxidation (Fe²⁺) and lipoxygenase activity amongst Robola fractions. In contrast, Robola FIV and W fraction’s anti-LOX and anti-radical activities do not correlate. These samples are also ineffective against Fe²⁺-induced lipid peroxidation. On the other hand, FII fraction is the most potent inhibitor of enzymatic lipid peroxidation (anti-LOX) amongst Cabernet fractions, but is inactive against non-enzymatic lipid peroxidation and a less potent quencher of free radicals. The Cabernet Sauvignon fractions of anthocyanins (Fi), phenolic acids (FIII), FIV and W fractions are effective radical quenchers. These conclusions are summarised in Fig. 1.

The standard phenolic compounds tested, quercetin and resveratrol, possess similar inhibitory activity against LOX, while resveratrol exerts the highest activity against non-enzymatic lipid peroxidation and quercetin possesses the highest scavenging capacity against DPPH radical.

4. Conclusions

The present study indicates the presence of micro-constituents in red and white wine extracts that inhibit lipid peroxidation, mainly through the inhibition of lipoxygenase activity. In general, the majority of Cabernet Sauvignon extracts are more potent scavengers and inhibitors of lipid peroxidation than Robola extracts. However, individual fractions of white wine exert similar or even higher activity than the corresponding fractions of red wine. Additionally, the procyanidin fraction seems to be a potent inhibitor of lipoxygenase-induced lipid peroxidation in both wines.

It is worth mentioning that the total phenolic or ortho-phenolic content of the fractions does not seem to determine their antiradical and anti-inflammatory activity. The molecular structure of each phenolic, regardless of the phenolic class to which it belongs, seems to contribute to the fraction’s activity in the studied bioassays.

References


