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Antioxidant effect of red wine polyphenols on red blood cells

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The protective effect of red wine polyphenols against hydrogen peroxide (H_2O_2)-induced oxidation was investigated in normal human erythrocytes (RBCs). RBCs, preincubated with micromolar amounts of wine extract and challenged with H_2O_2, were analyzed for reactive oxygen species (ROS), hemolysis, methemoglobin production, and lipid peroxidation. All these oxidative modifications were prevented by incubating the RBCs with oak barrel aged red wine extract (SD95) containing 3.5 mM gallic acid equivalent (GAE) of phenolic compounds. The protective effect was less apparent when RBCs were incubated with wines containing lower levels of polyphenols. Furthermore, resveratrol and quercetin, well known red wine antioxidants, showed lower antioxidant properties compared with SD95, indicating that interaction between constituents may bring about effects that are not necessarily properties of the singular components. Our findings demonstrate that the nonalcoholic components of red wine, mainly polyphenols, have potent antioxidant properties, supporting the hypothesis of a beneficial effect of red wine in oxidative stress in human system. © Elsevier Science Inc. 2000
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Introduction

The uncontrolled production of oxygen-derived free radicals is involved in the onset of many diseases such as cancer, rheumatoid arthritis, and atherosclerosis, as well as in degenerative processes associated with aging.[1–4] Moreover, reactive oxygen species (ROS) have been implicated in the mechanism of the damage to red blood cells (RBCs) in β-thalassemia, sickle cell anemia, and other hemoglobinopathies.[5–7] ROS are generated in biological systems through metabolic processes and exogenous sources such as food components, drugs, ultraviolet light, ionizing radiation, and pollution.[8] According to generally accepted mechanisms, major deleterious effects are caused by the hydroxyl radical ($^{\cdot}OH$), generated from hydrogen peroxide (H_2O_2), and by the superoxide ($O_2^{\cdot-}$) species in the presence of redox active transition metals.[9,10]

Many defense mechanisms have developed in living organisms to limit the levels of ROS and the damage they inflict. Included among them are endogenous enzymes such as superoxide dismutase, catalase, and glutathione peroxidase.[11] In addition to these endogenous mechanisms, much attention has been paid to the antioxidant role of some dietary compounds such as polyphenols, a class of molecules found in abundance in vegetables and red wine. Polyphenolic compounds have been shown to possess different biological properties, such as anti-inflammatory responses, prevention of low density lipoprotein oxidation, antihypertensive and antithrombic effects, and antiviral and carcinostatic properties.[12,13] Although there is a great deal of evidence from human epidemiologic and animal studies that suggests beneficial effects related to consumption of polyphenols,[14,15] only in the last few years have studies been published that show the presence of polyphenols, such as flavonoids and their glycosides, in human plasma at concentrations ranging between 0.5 and 1.6 μM.[16]

Among the dietary antioxidants with potential therapeutic effects, we focused our attention on wine polyphenols. The presence of these natural wine compounds depends principally on three sets of parameters: (1) the phenolic

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Table 1 Content of phenolic compounds in wines

Phenolic compounds	Red wine	White wine
	Content (mg/L)	
Catechin*	191.3	34.9
Myricetin*	8.5	0.0
Epicatechin*	82.0	21.2
Rutin*	9.1	0.0
Gallic acid*	95.0	6.8
Quercetin*	7.7	0.0
Resveratrol†	1.8	0.0

* Data from Teissedre et al. [19]
† Data from Goldberg et al.[20]

compounds in the grapes from which the wine is made; (2) the juice extraction and winemaking techniques; and (3) the numerous reactions that take place during aging and that are related to the wine's immediate environment and its physicochemical and biological characteristics.[17] A large number of different phenolic substances have been identified in wine: gallic, cinnamic, caffeic, gentisic, ferulic, and vanillic acids, trihydroxystilbenes (resveratrol), and flavonoids that consist mainly of anthocyanidins, flavonols, flavones, catechins, and flavanones.[18] *Table 1* lists the different content of phenolic compounds in red and white wines.[19,20] The polyphenol content of red wine is naturally higher than that of white wine. In red wine the breakdown of grape solids following the crush of grapes facilitates the liberation of phenolic compounds.

Data regarding human plasma levels of polyphenols after drinking wine are scarce. Recently, it has been shown that the plasma level of (+)− catechin, a major constituent of red wine, increased from less than 2 nmol/L to 91 ± 14 nmol/L in nine healthy volunteers after consuming 120 mL of red wine.[21]

The scope of the present study is to evaluate the protective role of red wine polyphenols against oxidative H_2O_2-induced damage in normal human RBCs. In fact, human RBCs, because they are oxygen carriers with high polyunsaturated fatty acid content on their membranes and high cellular concentration of hemoglobin are particularly exposed to oxidative damage. The hemoglobin released from erythrocytes is potentially dangerous because in reacting with H_2O_2 it is converted into oxidized forms: methemoglobin (met-Hb) and ferrylhemoglobin, which are powerful promoters of oxidative processes.[22] Moreover, the free hemoglobin exposed to H_2O_2 causes heme degradation with the release of iron ions catalytically active in initiating free radical reaction and lipid peroxidation.[23] These processes are involved in normal aging and in pathologic conditions.[5–7,24]

In this study, we provide evidence that an extract of oak barrel aged red wine with a high level of polyphenols is effective in reducing oxidative damage in normal human RBCs in vitro compared with red or white wine with a lower level of polyphenols. Moreover, the properties of the well known antioxidants resveratrol and quercetin are not completely able to justify the protective effect of red wine.

Methods and materials

Chemicals

Phosphate buffer saline (PBS) tablets were purchased from ICN Flow (Costa Mesa, CA USA); dichlorofluorescein-diacetate (DCFDA) from Molecular Probes (Eugene, OR USA); H_2O_2, quercetin, resveratrol, 2-thiobarbituric acid (TBA), butyl-hydroxy-toluene (BHT), and Folin-Ciocalteu's phenol reagent from Sigma Chemical Co. (St. Louis, MO USA). All other chemicals used were of research highest purity grade.

Preparation of erythrocytes

Human blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-treated tubes. Only samples with normal hematologic indices were used throughout the experiments. Consecutive centrifugation and washings in PBS removed plasma, platelets, and buffy coat. Aliquots of blood containing 1×10^5 cells/μL were used in each experiment.

Preparation of wine samples and determination of polyphenolic content

We tested two experimental red wines made from the same grapes but produced by different technological methods and a commercial white wine. Aglianico is a red wine obtained by 21 day maceration period at 25°C followed by oak barrel aging for 18 months (SD95). Novello is the same wine obtained by carbonic maceration of grapes (NW), and the white wine tested is obtained from grapes lacking a maceration process (WW).

To avoid ethanolic components, aliquots of each wine (1 mL) were lyophilized and resuspended in 0.4 mL PBS. The phenol content of each wine was determined using the Folin-Ciocalteu's phenol reagent according to the method of Singleton and Rossi.[25] The results were expressed in gallic acid equivalent (GAE), a naturally occurring polyphenol.[19]

Determination of hemolysis and met-Hb content

The extent of hemolysis was determined spectrophotometrically according to the method of Grinberg et al.[26] with some modifications. To evaluate the hemolysis induced by wine extracts, RBCs were preincubated with 20 μL of wine extracts for 1 hour. Cells, centrifuged and washed twice, were resuspended in PBS and the percent of hemolysis determined spectrophotometrically at 540 nm. The same test was also performed to detect the protective effect of wine extracts against exogenous oxidative stress. After 5 hours of incubation with 100 μM H_2O_2 at 37°C, the percent of RBC lysis was evaluated as above.

The met-Hb content was determined as a percentage of total hemoglobin (Hb), from the ratio of Hb released from the cells to the total Hb in samples incubated for 3 hours with 100 μM H_2O_2, according to the method of Winterbourn.[27]

Fluorescent measurement of intracellular ROS

RBCs were preincubated for 1 hour with 20 μL of wine extracts, corresponding to different GAE of polyphenols (*Table 2*), or with different concentrations of quercetin or resveratrol dissolved in absolute ethanol. Cells were then centrifuged and washed twice in PBS, and then gently resuspended in the same buffer and incubated 30 minutes in the presence of 10 μM DCFDA, a nonfluorescent compound that freely permeates cells. DCFDA is hydrolyzed to dichlorofluorescein (DCF), whose interaction with peroxides gives rise to 2′,7′-dichlorofluorescin detected spectrofluorimetrically. Cells were then centrifuged, washed, and incubated for 15 minutes in the presence of 100 μM H_2O_2 at 37°C. The determination of

Table 2 Effect of wine extracts

	mM (GAE)*	% Hemolysis
Control	—	2.6
SD95	3.5	2.7
NW	1.65	3.2
WW	0.3	3.1

* Molar concentrations of total polyphenols in gallic acid molecular weight equivalents.
SD95–oak barrel aged wine. NW–wine obtained by carbonic maceration of grapes. WW–white wine.

ROS production was performed by using a Perkin-Elmer LS 50B fluorimeter (Norwalk, CT USA) with excitation and emission settings at 495 and 530 nm, respectively.[28]

Evaluation of lipid peroxidation

The level of malondialdehyde (MDA), a secondary product of polyunsaturated fatty acid oxidation, was measured as MDA-2-thiobarbituric acid (TBA) complex by high performance liquid chromatography (HPLC) with fluorescence detector according to the method of Fukunaga et al.[29] RBCs were preincubated with 20 μL of SD95 for 1 hour at 37°C. Cells were centrifuged, washed twice, and resuspended in PBS. Samples were divided into two groups: One received treatment with 200 μM H_2O_2 for 2 hours at 37°C, the other represented the untreated control. All samples were then centrifuged, resuspended in 20 μL of PBS, and mixed with 2 mL of TBA (0.2% in 2 M sodium acetate pH 3.5) and 20 μL BHT (5% in absolute ethanol). The sample mixtures were heated 95°C for 1 hour and, after cooling, the MDA-TBA complex was extracted with 2 mL of *n*-butanol. The *n*-butanol extract was then passed through a 0.2 μm Millipore filter (Bedford, MA USA). The analysis was performed using a Beckman Model 126 HPLC (System Gold, Fullerton, CA USA). The MDA-TBA complex was separated with a Kromasil C18 (Azko Nobel, Bohus, Sweden; 250 × 4.6 mm inner diameter) column. The injection volume was 20 μL and the mobile phase water-acetonitrile was 4:1 v/v. The MDA-TBA complex was identified by fluorescence detection (Shimadzu model RF-551, Kyoto, Japan), with excitation at 515 nm and emission at 553 nm.

The data are reported as mean ± SD of duplicate determinations and are representative of at least three experiments.

Results

Protective effect of wine polyphenols against cellular damage

In our experiments, we used three different wines: oak barrel aged "Aglianico" from the 1995 vintage (SD95), which contained a large amount of polyphenols; "Novello" Aglianico (NW) with shorter maceration time, which contained a lower concentration of polyphenols; and a white wine (WW). The two red wines are produced from the same mixture of grapes in the same geographical area (Irpinia, Italy). Total polyphenol concentration of wine extracts is reported in *Table 2*.

We first demonstrated that the tested wine extracts did not have a harmful effect on erythrocytes. In fact, RBCs treated with different wine extracts showed the same hemolysis of control samples (*Table 2*).

The protective effect of wine extracts on RBC hemolysis was evaluated by oxidative stress induced experimentally using H_2O_2. Under the given conditions, H_2O_2 caused considerable RBC lysis that was significantly inhibited by wine extracts (*Figure 1*). It is worthy to note that SD95, the wine richest in polyphenols, exerted the strongest protective effect, providing 25% inhibition of RBC hemolysis.

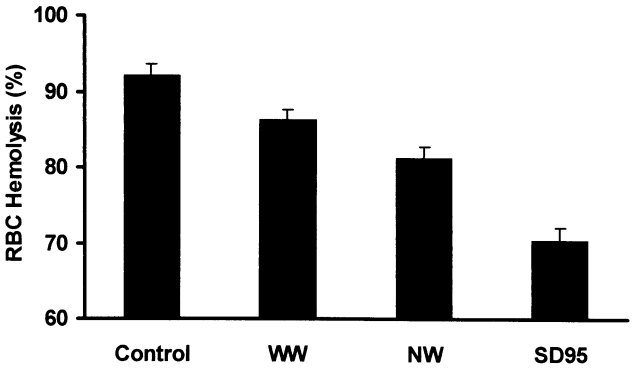

Figure 1 Hemolysis of red blood cells (RBCs) incubated for 1 hour with 20 μL wine extracts: oak barrel aged wine (SD95), wine obtained by carbonic maceration of grapes (NW), and white wine (WW) containing 3.5 mM [gallic acid equivalents (GAE)], 1.65 mM (GAE), and 0.3 mM (GAE) phenolic compounds, respectively. The oxidative stress was induced by adding 100 μM hydrogen peroxide for 5 hours. Hemolysis was evaluated as described in Materials and methods. Values are means ± SD.

Antioxidant activity of red wine

To confirm that SD95 exerts its protective activity by acting as general antioxidant, we measured three different parameters of oxidative damage: lipid peroxidation, ROS concentration, and met-Hb production. The intracellular level of MDA, which resulted directly from membrane lipid peroxidation, was measured as a marker of H_2O_2-induced cellular injury of RBCs.[30] As shown in *Figure 2*, the level of MDA increased when erythrocytes were treated with H_2O_2, indicating relevant oxidative damage of cellular membranes. Preincubation of RBCs with SD95 exerted significant protective effect against oxidative stress, maintaining the intracellular MDA at level comparable to cells without the oxidative stress. Treatment of RBCs with WW reduced the MDA production almost 10% compared with control.

Subsequent to oxidative stress, the major damage to RBC membrane lipids is due to ROS generation.[31] The level of ROS produced during oxidative stress indirectly indicates plasma membrane damage. We demonstrated that RBCs pretreated with 20 μL of different wine extracts showed a reduced production of ROS, mainly in presence of SD95, whose concentration of total polyphenols was the highest (3.5 mM GAE; *Figure 3*).

Finally, it is well known that free radicals cause formation of met-Hb and Heinz bodies.[27] The addition of H_2O_2 to erythrocytes pretreated with 20 μL of SD95 prevented oxidation of the oxyhemoglobin, as shown in *Figure 4*. The met-Hb content was comparable in both untreated and SD95-treated RBCs. The same level of met-Hb was detected in the control experiment and WW-treated RBCs.

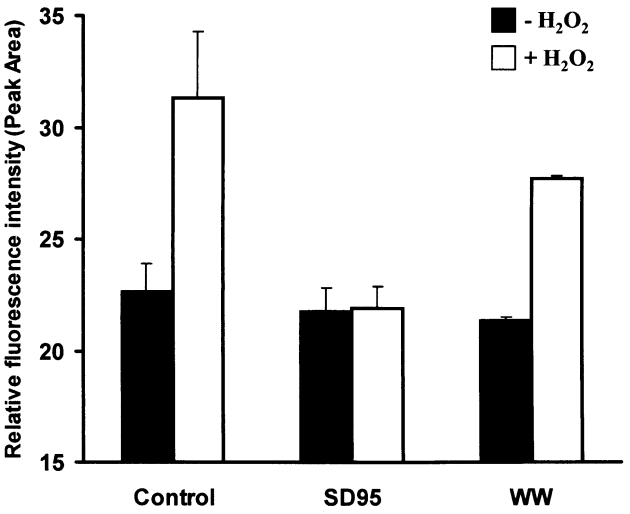

Figure 2 Effect of oak barrel aged wine (SD95) and white wine (WW) on MDA levels of hydrogen peroxide (H_2O_2)-treated red blood cells. Cells preincubated for 1 hour with 20 μL of wine extracts were treated with 200 μM H_2O_2 for 2 hours. The levels of MDA-TBA complex were indicated as relative fluorescence intensity (peak area). MDA assay was performed as described in Materials and methods. The data presented are means ± SD.

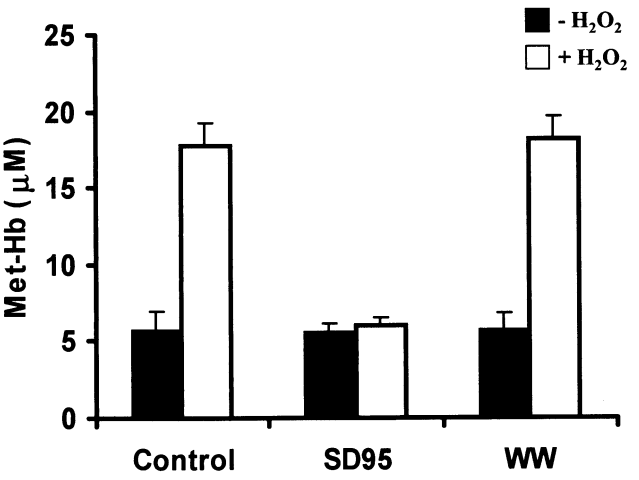

Figure 4 Methemoglobin (met-Hb) content in red blood cells treated with oak barrel aged wine (SD95) and white wine (WW). At the end of incubation with wine extracts, parallel sets of samples were treated with or without 100 μM hydrogen peroxide (H_2O_2) for 3 hours. Met-Hb assay was performed as described in Materials and methods. The data presented are means ± SD.

Involvement of quercetin and resveratrol in the antioxidant activity of red wine

To evaluate which component of SD95 was responsible for its antioxidant effects, we tested the activity of quercetin and resveratrol naturally present in red wines (*Table 1*). A protective effect of quercetin against oxidative damage has been previously reported.[32] At 2.5 μM concentration, which corresponded to the amount naturally present in SD95, quercetin showed a less pronounced protective activity (*Figure 5*). To reach an effect comparable to SD95 (*Figure 3*), we increased quercetin concentrations up to 20 μM (*Figure 5*). On the contrary, resveratrol at a concentration up to 20 μM did not show any protective effect on ROS production. The insert of *Figure 5* indicates that quercetin and resveratrol did not induce hemolysis at concentrations used in our experiment.

Discussion

In recent years, an increasing body of evidence has supported the hypothesis that a number of nutrients or nonnutrient dietary components—labeled as "antioxidants"—might have a beneficial role in retarding or reversing the course of chronic degenerative diseases.[8] In particular, it has been claimed that the polyphenolic components of the nonalcoholic fraction of red wine may be responsible for the protective effect of red wine against oxidative damage.[33,34]

The data presented in this study show that red wine extracts behave as potent scavengers of ROS. In fact, when intact human RBCs were preincubated with a red wine from Aglianico grapes aged in oak barrel and rich in polyphenols, a strong protective effect against H_2O_2-generated hemolysis and ROS production was observed. We demonstrated a clear correlation between the content of polyphenols in wines and antioxidant effects. In fact, in all experiments presented the strongest antioxidant effect was always associated with SD95. The most relevant difference among these

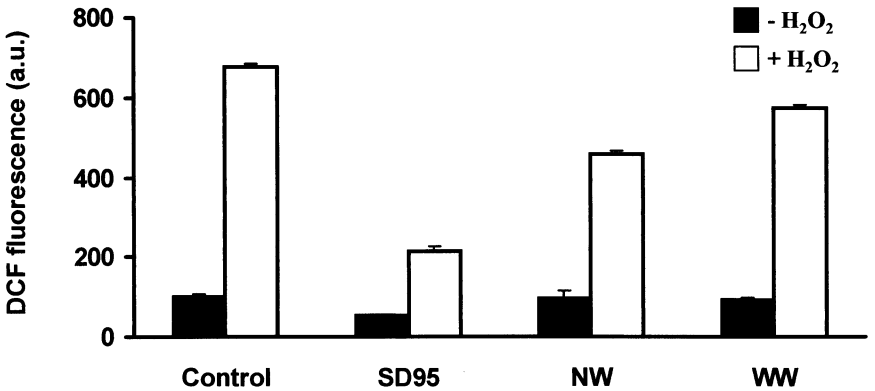

Figure 3 Reactive oxygen species (ROS) production in red blood cells pretreated with 20 μL of different wine extracts for 1 hour at 37°C and incubated for 15 minutes with 100 μM hydrogen peroxide (H_2O_2). At the end of incubation, ROS production was measured as dichlorofluorescein (DCF) fluorescence. Values are means ± SD. SD95, oak barrel aged wine; NW, wine obtained by carbonic maceration of grapes; WW, white wine.

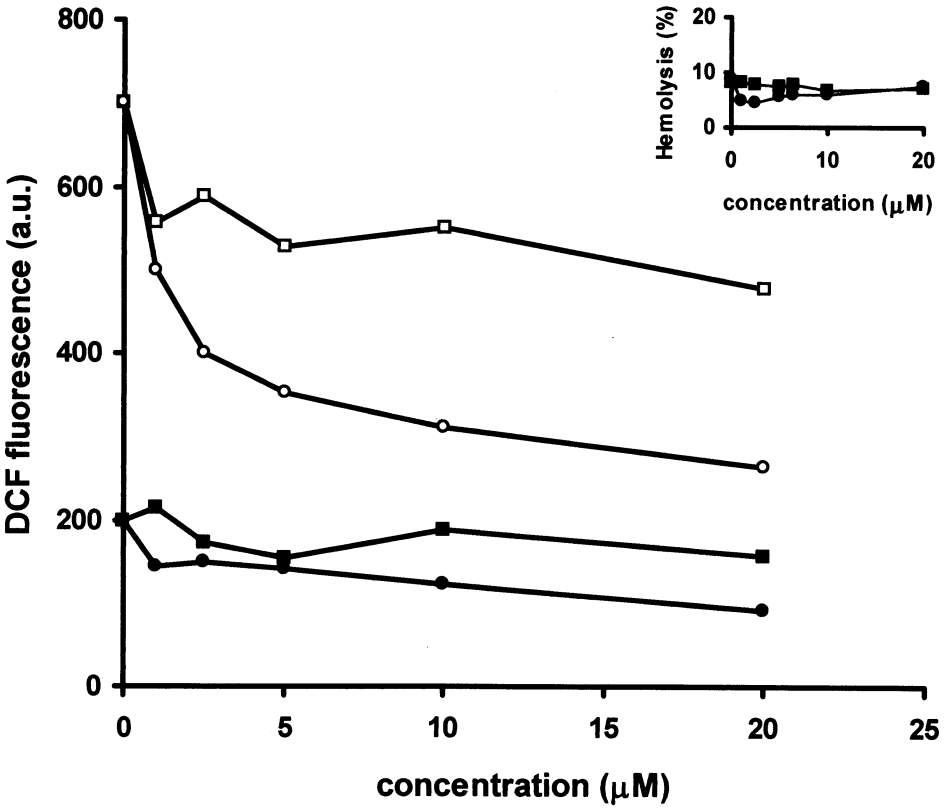

Figure 5 Dose-dependence effect of quercetin or resveratrol on reactive oxygen species (ROS) production in red blood cells (RBCs). Cells were pretreated for 1 hour with increasing amount of quercetin (-●-) or resveratrol (-■-). Parallel sets of samples received 100 μM hydrogen peroxide (H_2O_2) for 15 minutes in addition to quercetin (-○-) or resveratrol (-□-). ROS production was measured as described in Materials and methods. The insert shows the hemolysis of RBCs incubated with different concentrations of quercetin or resveratrol. DCF, dichlorofluorescein.

extracts was the relative abundance of polyphenols, which were highest in SD95 and lowest in WW.

Among the possible candidates that might explain the antioxidant activity of red wines, at a molecular level, there is quercetin because its concentration is significantly high in red wines but absent in white wines (*Table 1*). Several studies have demonstrated the protective effects of quercetin against cellular damage induced by ROS in different systems.[12,32] Accordingly, in our experimental conditions, pure quercetin showed a clear antioxidant activity on RBCs (*Figure 5*), but at a concentration of 20 μM, 10-fold higher than that naturally present in SD95 (2.5 μM). In addition, resveratrol, which is usually present in wines with prolonged contact to must and skins,[35] in our system fails to protect RBCs from the oxidative stress. This result allowed us to hypothesize that other polyphenolic compounds present in SD95 might account for the protection from oxidative injury. At the moment, we cannot exclude that the beneficial effect of red wine might be due to other unidentified components present in the nonalcoholic fraction of red wine and unrelated to polyphenols, a possibility currently under investigation. According to Halder and Bhaduri,[36] similar effects were observed on intact erythrocytes for black tea extracts versus free catechin, the most abundant polyphenol in tea. In fact, the antioxidant activity of catechin was lower than black tea extract in toto.

Red wine intake might contribute to the dietary prevention of those pathologies, whose etiology is related to the ROS-mediated cellular damage. The concentration of polyphenols in a regular serving of red wine during meals (two glasses of 150 cc) is approximately 4.8 g/L GAE, a value not far from the amount tested in our experiments. In addition, SD95 does not induce hemolysis in erythrocytes at the concentrations required for the antioxidant activity. Moreover, it is noteworthy that the wine extracts used in our study did not show any cytotoxic effect on human lymphocytes (data not shown).

Because both physical and chemical properties of individual phenolic compounds of red wine strongly affect their antioxidant activity, a more comprehensive and systematic structural analysis will be required to elucidate the contribution of each factor of the nonalcoholic fraction of red wine.

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