Cytoprotection by Neutral Fraction of Tannat Red Wine against Oxidative Stress-Induced Cell Death

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Some of the beneficial effects of the Mediterranean diet on human pathologies have been attributed to red wine polyphenols. It has been postulated that the antioxidant activity of the latter would be also responsible for the cytoprotective capacity of red wine that has been reported in a few papers. Nevertheless, red wine shows a complex composition, and the active fraction is not known yet. In this context, the protective capacity of total lyophilized extracts of red wine and anthocyanin, neutral, or acidic fractions, was explored in PC12 cells in culture after a hydrogen peroxide insult. Although all fractions showed high antioxidant activity, only the neutral fraction was cytoprotective. The analysis of this active fraction showed that it was rich in the aglycons quercetin and myricetin as well as the glycosides of kaempferol, isorhamnetin, epicatechin, and catechin, some of which are known to be cytoprotective. This is the first paper to reveal the active fraction of total wine responsible of its cytoprotection.

KEYWORDS: Red wine; polyphenols; flavonoids; cell cultures; cytoprotection; PC12; H₂O₂

INTRODUCTION

During the past decade, many epidemiological studies have shown that the Mediterranean diet appears to have beneficial effects on cardiovascular diseases, a fact that has come to be known as the "French paradox" (1–3). Epidemiological studies have also indicated that dietary habits could influence the incidence or evolution of pathologies such as neurodegenerative disorders (4–6) in which oxidative stress has been identified as a main contributor (7, 8). The regular and moderate consumption of wine, especially red wine, appears to be one of the main reasons for these healthy effects (9, 10). For example, data from the so-called Personnes Agees Quid (PAQUID) study showed that people drinking three to four glasses of wine per day have an 80% less incidence of dementia and Alzheimer's disease than those who drank less or did not drink at all (11). There is evidence indicating that the antioxidant action of wine phenolic compounds is involved in the beneficial effects of the Mediterranean diet (12, 13).

In this sense, it has been demonstrated that the total antioxidant capacity (AC) of blood plasma increases after polyphenolic compounds ingestion (14, 15).

The ultimate healthy aim of antioxidant activity of natural or synthetic molecules is the prevention of cellular death in those tissues—the endothelium, the skin, or the brain—where cells are at risk because of oxidative stress. In contrast with the many papers dealing with the study of the antioxidant properties of wine and its relationship with its components, only one paper (De Ruvo et al., 2000) has shown the in vitro cytoprotective activity of red wine. In this case, the lyophilized whole wine showed neuroprotection on apoptotic neurons (5). This lack of protective results for total wine contrasts with the cytoprotective activity already demonstrated for phenolic compounds of wine such as quercetin, resveratrol, kaempferol, or epicatechin when tested individually (16, 17). Another related work published by Roig et al. showed that a mixture of flavonoids resembling those of wine has better cytoprotective effect than individual phenols against an oxidative stress cell damage (18). Accordingly, nothing is known about cytoprotection by the different fractions of wine and what relationship it could have—if any—with their antioxidant capacity. There is a growing awareness that functions other than antioxidants are important for flavonoids' complex actions in the organism.

In this context, this paper was aimed at assessing the cytoprotective capacity of lyophilized whole wine (LWW) or its acidic (AcF), anthocyanin (AntF), and neutral fractions (NF) against an oxidative stress stimulus in the pheochromocytoma

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cell line PC12. The study further assessed the AC and the phenolic composition of each fraction.

MATERIALS AND METHODS

Wine and Isolation of Polyphenolic Fraction. Three different commercial brands of red wine cv. Tannat were selected for the experiments among other Uruguayan red wine varieties because in preliminary experiments they have shown the highest AC and phenolic content.

Liquid/liquid extraction methods were performed to obtain the neutral (NF), acid (AcF), and anthocyanin fractions (AntF) (*19*). Alcohol was removed under vacuum (at 25 °C and 30 mbar). The dealcoholized wine was adjusted to pH 7, and 6 mL was first extracted with ethyl acetate (three times with 3 mL of EtOAc each), obtaining an aqueous residue and an organic phase. The EtOAC of the organic phase was evaporated to obtain the NF. The aqueous residue was adjusted to pH 2 and extracted again with EtOAc (three times with 3 mL of EtOAc each) to obtain the AcF by means of evaporating the EtOAC. The remaining aqueous residue is the AntF.

Lyophilized whole wine (LWW) was obtained by means of dealcoholized wine lyophilization.

Determination of Total Polyphenol. Total polyphenol (TP) contents of LWW and its different fractions were determined according to the Folin−Ciocalteu method (*20*). Distilled water (3950 μL) was combined and vortexed with 50 μL of sample appropriately diluted and 250 μL of Folin−Ciocalteu reagent. After that, 750 μL of sodium carbonate (20%) was added, and the mixture was vortexed again and allowed to stand at room temperature for 35 min. The absorbance was read at 750 nm, and the total polyphenol concentration was calculated from a calibration curve, using gallic acid as a standard. Results were expressed as micrograms per milliliter of gallic acid equivalents (GAE).

Analysis of Phenolics by HPLC. The concentration of individual polyphenols was determined using a Merck−Hitachi HPLC system equipped with an L-6200 pump and an L-7450 photodiode array detector controlled by D-7000 Chromatography Data Station software (*21*). Reversed phase separations were carried out using a Supelco LC-18 Supelcosil 250 × 4.6 mm i.d., 5 μm, column. Elution was carried out by using a gradient system that makes use of 2% formic acid (solvent A) and 2% formic acid/50% methanol (solvent B).The following proportions of solvent A were used: 0−2 min, 100%; 2−3 min, 98%; 3−4 min, 95%; 4−4.5 min, 90%; 4.5−5 min, 85%; 5−10 min, 75%; 10−15 min, 60%; 15−20 min, 20%; 20−25 min, 0%; 25−30 min, 50%; and finally 5 min of washing with 100%. The flow rate was 1 mL/min. Monitoring of the eluate was performed simultaneously at 280, 313, 360, and 518 nm for benzoic acids and flavanol, hydroxycinnamate, flavonol, and anthocyanin quantification, respectively. Free flavonols were detected in the nonhydrolyzed NF, whereas the total flavonol content was determined after acid hydrolytic cleavage of the flavonol conjugates, which released the aglycons (*22*). The conjugated flavonol level was estimated by subtracting the amount of flavonol found in the unhydrolyzed sample from the quantity present in the acid-hydrolyzed extract. With each analysis, the flavonol content of the hydrolyzed sample was corrected for sample handling/hydrolysis losses on the basis of the recovery of the morin internal standard.

Free and conjugated hydroxycinnamate concentrations were determined before and after AcF alkaline hydrolysis (*21*), with a sinapic acid as internal standard.

Measurement of Antioxidant Capacity. The AC of LWW and its fractions was evaluated by studying its ability to scavenge the ABTS$^{\bullet+}$ radical (*23*). ABTS$^{\bullet+}$ was produced by the reaction of ABTS solution (7 mM) with ammoniun persulfate (140 mM) (both solutions were prepared in bidistilled water) for 12−16 h, in the dark and at room temperature. Prior to use, the stock solution was filtered and diluted in PBS so as to achieve an absorbance at 734 nm of 0.70 ± 0.02. One milliliter of diluted ABTS solution was mixed with 10 μL of sample, and the percentage decrease of absorbance at 734 nm was calculated. The IC$_{50}$ (μg/mL of GAE) was determined for LWW or its fractions (*24*).

Cell Culture Conditions. PC12 cells were grown and maintained in a growth medium consisting of 85% RPMI, 10% horse serum (heat-treated for 30 min at 56 °C), 5% fetal bovine serum, 75 mg/L penicillin, and 50 mg/L streptomycin, in collagen-coated 7 mL culture flasks. Cultures were fed twice a week with complete growth medium. When they reached near-confluent state 1 week later, subculturing was carried out (the split ratio was 1:5). Maintenance was in a 37 °C incubator in a water-saturated atmosphere containing 5% CO$_2$ (*25*).

Cytotoxic and Cytoprotective Activity Assay. Cells were seeded in 96-well plates at a density of 20000 cells/well. For the LWW and fractions, the TP concentrations chosen were from 0 to 2000 μg/mL of GAE. After 24 h of subculture, LWW or the fractions were added. For the study of cytotoxicity, treated cells were incubated for 30 min at 37 °C. For the cytoprotective assessment 200 μM H$_2$O$_2$ was concomitantly added, and cells were incubated for 30 min at 37 °C. Two kinds of controls were used (without any treatment or vehicle treatment), and no significant differences were found between them. Finally, the treatment medium was removed, and viability was determined according to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (*26*). Cells were incubated for 2 h at 37 °C with MTT (0.5 mg/mL final concentration) and dissolved in fresh complete medium, in which metabolically active cells reduced the dye to purple formazan. Crystals were dissolved with dimethyl sulfoxide (DMSO), and the absorbance was measured at 570−630 nm.

Statistical Analysis. The results were expressed as means ± standard deviation (SD). Differences among different experimental groups were tested for significance using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons tests, taking $p < 0.05$ as significant.

RESULTS AND DISCUSSION

The cytoprotective activity of red wine and its fractions against oxidative stress insult in culture was explored in this work. The H$_2$O$_2$ oxidative insult has been widely utilized for the assessment of cytoprotection, especially in PC12 cells in culture (*27−29*), and it offers the possibility of a graded regulation of the severity of cell death in relationship with the concentration applied. In our model, exposure of PC12 cells to 200 μM H$_2$O$_2$ for 30 min resulted in 50% remaining cellular viability (*30*).

The LWW did not show cytoprotection at any of the concentrations tested, and the application of >500 μg/mL of GAE was cytotoxic per se.

Among the fractions used in this study, the NF was the only one that prevented H$_2$O$_2$-induced cell death, even at concentrations that were toxic when NF was applied alone (**Figure 1**). Concentrations ≤100 μg/mL GAE did not show cytoprotective effects (**Figure 1**). Neither AcF nor AntF showed any cytoprotective activity, and both were toxic at concentrations higher than 150 and 500 μg/mL of GAE, respectively. The cytotoxic effect of these two fractions could explain why the LWW was not cytoprotective. It is likely that in low concentrations of LWW, NF would not reach the concentration to offer cytoprotection to cells. At high concentrations of LWW applied to cell cultures, the cytotoxic effect of AcF and AntF would outweigh the cytoprotective effect of NF, because they represent together >70% of the whole wine.

Several mechanisms could be hypothesized to be involved in the cytoprotective effect of NF. Because cell death induced by H$_2$O$_2$ is originally mediated by oxidative stress, the AC of the fraction could be the main force counteracting the oxidative insult. For this reason the scavenger activities of LWW, NF, AcF, and AntF were evaluated by the ABTS assay (**Figure 2**). LWW and all of the fractions showed higher AC compared with Trolox, which has an IC$_{50}$ of 3.7 μg/mL.

However, although all of the fractions have a marked AC, only the NF protected PC12 cells against the oxidative insult, showing that there is no direct relationship between the

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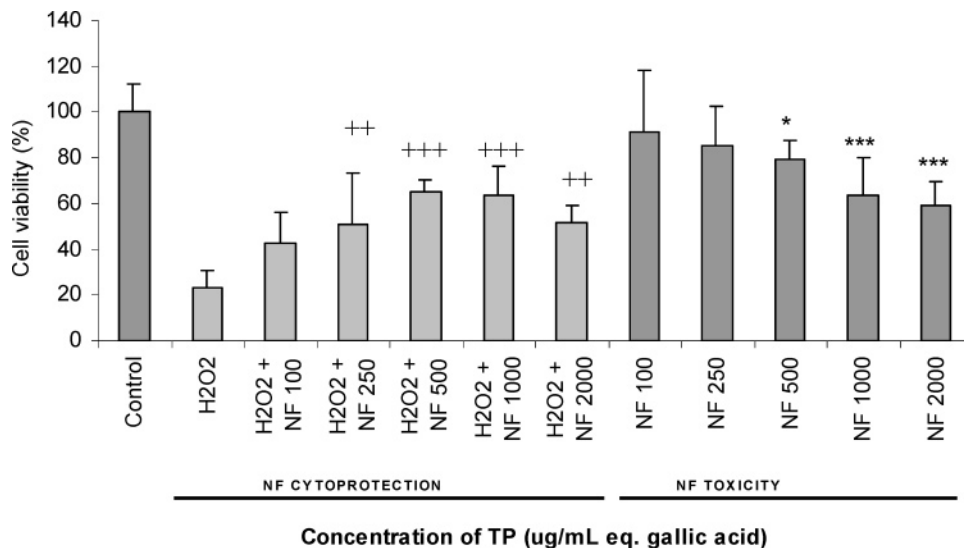
Figure 1. Evaluation of cell viability in PC12 cells after treatment with neutral fraction (NF) or NF plus H_2O_2 (200 μM during 30 min). Data are represented as mean $\pm$ SD of $n = 6$: ++, $p < 0.01$, and +++, $p < 0.001$, comparing treatment with H_2O_2; *, $p < 0.05$, and ***, $p < 0.001$, comparing treatments with control. Statistical analysis was done with Tukey's multiple-comparisons test.
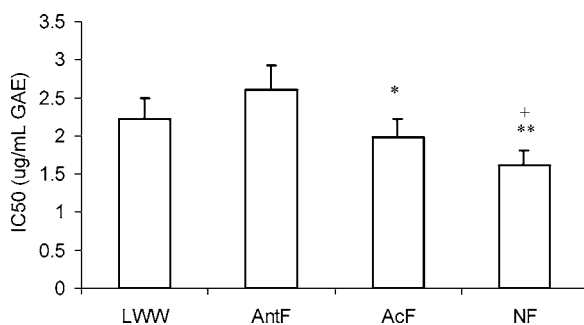
Figure 2. Analysis of antioxidant capacity of different red wine fractions evaluated by the ABTS assay. NF = neutral fraction, AntF = anthocyanic fraction, AcF = acidic fraction. Data are expressed as mean $\pm$ SD of IC_{50}: **, $p < 0.01$ (NF vs AntF); *, $p < 0.05$ (AcF vs AntF); and +, $p < 0.05$ (NF vs LWW). Tukey's multiple-comparisons test was utilized for statistical analyses.

Table 1. Phenolic Compounds Identified in Neutral, Acidic, and Aqueous Fractions

fraction	compound	μM/(mg/mL of GAE)
neutral	flavan-3-ols	
	catechin	478.8
	epicatechin	643.2
	other[a]	697.6
	total	1820.0
	free flavonols	
	myricetin	14.4
	quercetin	7.2
	conjugated flavonols	
	kaempferol	4.8
	isoramnetin	13.7
acidic	hydroxybenzoic acids	
	gallic	35.0
	protocatechuic	37.6
	hydroxycinnamic acids	
	caftaric[b]	1779.8
	cutaric[c]	238.4
	caffeic	92.5
anthocyanic	free anthocyanins[d]	
	delphinidin 3-glucoside	2.3
	cyanidin 3-glucoside	0.7
	petunidin 3-glucoside	4.6
	peonidin 3-glucoside	0.1
	malvidin 3-glucoside	28.4

[a] Other flavan-3-ols are expressed as catechin. [b] Caftaric acid is expressed as caffeic acid. [c] Cutaric acid is expressed as p-coumaric acid. [d] Free anthocyanins are expressed as cyanidin-3-glucoside. Data are based on HPLC analysis.

cytoprotective effect and the potency of AC. It must be taken into account that in the ABTS assay the compounds have total access to the ABTS radical, and in the cell culture the accessibility to the radicals depends on their ability to pass through the cell membrane. Furthermore, the scavenger capacity was tested using the carbon-centered synthetic free radical ABTS, which differs from the kind of free radicals generated in the H_2O_2 cell culture model. Finally, the ABTS assay cannot differentiate the antioxidant reactivity of the different species, an issue likely involved in cytoprotective activity.

Another clue for the understanding of the cytoprotective effect of NF is the analysis of its components. The HPLC analysis of the polyphenolic composition of each fraction is shown in **Table 1**. The NF fraction had a predominant flavan-3-ols composition (mainly catechin and epicatechin), free flavonols (myricetin and quercetin), and conjugated flavonols (kaempferol and isorhamnetin glycosides) (**Table 1**). The components determined in the AcF were gallic and protocatechuic acids from the hydroxybenzoic acid family and caftaric, cutaric, and caffeic acids from the hydroxycinnamic acid family (**Table 1**). In the AntF the five free anthocyanins present in the grapes were found (malvidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, and peonidin 3-glucoside) and malvidin 3-glucoside-coumarylade.

Although phenolic families contained in FN of the different Tannat bottles tested are unchanged, the combination of compounds from each phenolic family and its concentration vary depending on the bottles of wine used. Indeed, in NF the concentration of flavan-3-ols goes from 1265 to 2240 μM/(mg/mL of GAE), the free flavonols vary between 12.8 and 21.6 μM/(mg/mL of GAE), and conjugated flavonols vary between 37.2 and 136 μM/(mg/mL of GAE). However, for the same TP concentration, the NF of different bottles of wine showed similar results in cell culture and AC assays. Thus, the figures and table presented here illustrate the results obtained from an experimental series of one bottle of Tannat red wine.

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Many of the compounds found in the NF are known as cytoprotective molecules in different cell culture models, including PC12 cells exposed to oxidative injury (*31*−*33*). Hydrophobicity could be determinant for the cytoprotective activity of the compounds because hydrophobic molecules have a greater chance to get into the cell where the injury process is developed (*34*). In this sense, the decreased hydrophobicity of anthocyanin ionized molecules is a factor that should be taken into account to explain why AnF, with good antioxidant potency, did not have a cytoprotective effect on PC12 because anthocyanin ionized molecules decrease hydrophobicity.

An additional point is that oxidative stress induced by H_2O_2 also increases intracellular Ca^{2+} and activates intracellular signals involved in cell death in PC12 cells (*35*−*37*). In this sense, although free radical scavenging activity has been the action most widely attributed to flavonoids (*38*), they have also several other activities such as iron chelation, enzyme activation or inhibition, and calcium homeostasis (*39*) participating in intracellular signaling cascades and likely interfering with cell death signals. Moreover, it has been shown that individual flavonoids present in the NF inhibit xanthine oxidase, IP3 kinase, and PKC kinase (*39*). In this context, beyond their known scavenger action, the cytoprotective effects of flavonoids present in NF could be explained by their ability to interact with intracellular targets implicated in programmed cell death. It could be hypothesized that flavonoids such as flavonols and flavan-3-ol included in NF have cytoprotective effects affecting downstream intracellular signaling cascades. Thus, NF probably has a unique cytoprotective profile given by a marked AC, adequate hydrophobicity, and cytoprotective compounds acting in secondary messenger systems involved in the cell death process. These results are in agreement with the numerous studies pointing to natural polyphenols as potential neuroprotective compounds (*40*, *41*) and show the specific phenolic fraction that is involved in wine cytoprotective properties, perhaps acting synergistically as was previously suggested (*18*).

ABBREVIATIONS USED

AC, antioxidant capacity; LWW, lyophilized whole wine; AcF, acidic fraction; AntF, anthocyanin fraction; NF, neutral fractions; EtOAc, ethyl acetate; TP, total polyphenols; GAE, gallic acid equivalents; HPLC, high-performance liquid chromatography; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); ABTS$^{•+}$, ABTS radical; PBS, phosphate-buffered saline; RPMI, basal medium designed at Roswell Park Memorial Institute; H_2O_2, hydrogen peroxide; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

LITERATURE CITED

(1) St. Legere, A. S.; Cochrane, A. L.; Moore, F. Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. *Lancet* **1979**, *i*, 1017−1020.

(2) Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kornhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet* **1993**, *342*, 1007−1011.

(3) Renaud, S.; de Lorgeril, M. Wine, alcohol, platelet and the French Paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

(4) Commenges, D.; Scotet, V.; Renaud, S.; Jacqmin-Gadda, H.; Barberger-Gateau, P.; Dartigues, J. F. Intake of flavonoids and risk of dementia. *Eur. J. Epidemiol.* **2000**, *16*, 357−363.

(5) De Ruvo, C.; Amodio, R.; Algeri, S.; Martelli, N.; Intilangelo, A.; D'Ancora, G. M.; Esposito, E. Nutritional antioxidants as antidegenerative agents. *Int. J. Dev. Neurosci.* **2000**, *18*, 359−366.

(6) Youdim, K. A.; Spencer, J. P.; Schroeter, H.; Rice-Evans, C. Dietary flavonoids as potential neuroprotectants. *Biol. Chem.* **2002**, *383*, 503−519.

(7) Kehrer, J. P. Free radicals as mediators of tissue injury and disease. *Crit. Rev. Toxicol.* **1993**, *23*, 21−48.

(8) Deschamps, V.; Barberger-Gateau, P.; Peuchant, E.; Orgogozo, J. M.; Nutritional factors in cerebral aging and dementia: epidemiological arguments for a role of oxidative stress. *Neuroepidemiology* **2001**, *20*, 7−15.

(9) Renaud, S.; Ruf, J. C. The French paradox: vegetables or wine. *Circulation* **1994**, *90*, 3118−3119.

(10) Orgogozo, J. M.; Dartigues, J. F.; Lafont, S.; Letenneur, L.; Commenges, D.; Salomon, R.; Renaud, S.; Breteler, M. B. Wine consumption and dementia in the elderly: a prospective community study in the Bordeaux area. *Rev. Neurol.* **1997**, *3*, 185−192.

(11) Lemeshow, S.; Letenneur, L.; Dartigues, J. F.; Lafont, S.; Orgogozo, J. M.; Commenges, D. Illustration of analysis taking into account complex survey considerations: the association between wine consumption and dementia in the PAQUID study. *Am. J. Epidemiol.* **1998**, *148*, 298−306.

(12) Frankel, E. N.; Kanner, J.; German, J. B.; Parks, J. E. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **1993**, *341*, 454−457.

(13) Teissedre, P. L.; Frankel, E. N.; Waterhouse, A. L.; Peleg, H.; German, J. B. Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wine. *J. Sci. Food Agric.* **1996**, *70*, 55−61.

(14) Cao, G. H.; Russell, R. M.; Lischner, N.; Prior, R. L. Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. *J. Nutr.* **1998**, *44*, S126−33.

(15) Maxwell, S.; Cruickshank, A.; Thorpe, G. Red wine and antioxidant activity in serum. *Lancet* **1994**, *344*, 193−194.

(16) Schroeter, H.; Spencer, J. P.; Rice-Evans, C.; Williams, R. J. Flavonoids protect neurons from oxidised low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-jun and caspase-3. *Biochem. J.* **2001**, *358*, 547−557.

(17) Jang, J. H.; Surh, Y. J. Protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells. *Mutat. Res.* **2001**, *496*, 181−190.

(18) Roig, R.; Cascon, E.; Arola, L.; Blade, C.; Salvado, M. Procyanidins protect Fao cells against hydrogen peroxide-induced oxidative stress. *Biochim. Biophys Acta* **2002**, *1572*, 25.

(19) Ghiselli, A.; Nardini, M.; Baldi, A.; Scaccini, C. Antioxidant activity of different phenolic fractions separated from an Italian red wine. *J. Agric. Food Chem.* **1998**, *46*, 361−367.

(20) Singleton, V.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic−phosphotungstic reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.

(21) Burns, J.; Gardner, P. T.; O'Neil, J.; Crawford, S.; Morecroft, I.; McPhail, D. B.; Lister, C.; Matthews, D.; MacLean, M. R.; Lean, M. E. J.; Duthie, G. G.; Crozier, A. Relationship among antioxidant activity, vasodilation capacity, and phenolic content of red wines. *J. Agric. Food Chem.* **2000**, *48*, 220−230.

(22) McDonald, M. S.; Hughes, M.; Burns, J.; Lean, M. E. J.; Matthews, D.; Crozier, A. Survey of the free and conjugated myricetin and quercetin content of red wines of different geographical origins. *J. Agric. Food Chem.* **1998**, *46*, 368−375.

(23) Miller, N. J.; Rice-Evans, C.; Davies, M. J.; Gapinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in prematures neonates. *Clin. Sci.* **1993**, *84*, 407−412.

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(24) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(25) Greene, L. A.; Aletta, J. M.; Rukenstein, A.; Green, S. H. PC12 pheochromocytoma cells: culture, nerve growth factor treatment, and experimental exploitation. *Methods Enzymol.* **1987**, *147*, 207−216.

(26) Denizot, F.; Lang, R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **1986**, *89*, 271−277.

(27) Yamakawa, H.; Ito, Y.; Naganawa, T.; Banno, Y.; Nakashima, S.; Yoshimura, S.; Sawada, M.; Nishimura, Y.; Nozawa, Y.; Sakai, N. Activation of caspase-9 and -3 during H_2O_2-induced apoptosis of PC12 cells independent of ceramide formation. *Neurol. Res.* **2000**, *22*, 556−564.

(28) Jiang, D.; Jha, N.; Boonplueang, R.; Andersen, J. K. Caspase 3 inhibition attenuates hydrogen peroxide-induced DNA fragmentation but not cell death in neuronal PC12 cells. *J. Neurochem.* **2001**, *76*, 1745−1755.

(29) Wang, R.; Zhou, J.; Tang, X. C. Tacrine attenuates hydrogen peroxide-induced apoptosis by regulating expression of apoptosis-related genes in rat PC12 cells. *Brain Res. Mol. Brain Res.* **2002**, *107*, 1−8.

(30) Dajas, F.; Rivera, F.; Blasina, F.; Arredondo, F.; Echeverry, C.; Lafón, L.; Morquio, A. Cell culture protection and *in vivo* neuroprotective capacity of flavonoids. *Neurotoxicity Res.* **2003**, *5*, 377−384.

(31) Wang, H.; Joseph, J. A. Structure−activity relationships of quercetin in antagonizing hydrogen peroxide-induced calcium dysregulation in PC12 cells. *Free Radical Biol. Med.* **1999**, *27*, 683−694.

(32) Ishige, K.; Schubert, D.; Sagara, Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biol. Med.* **2001**, *30*, 433−446.

(33) Arredondo, F.; Blasina, F.; Echeverri, C.; Morquio, A.; Ferreira, M.; Abin-Carriquiry, J. A.; Lafon, L.; Dajas, F. Cytoprotection by *Achrocline satureioides* (Lam) D.C. and some of its main flavonoids against oxidative stress. *J. Ethnopharmacol.* **2003**, *3105*, 1−8.

(34) Sasaki, N.; Toda, T.; Kaneko, T.; Baba, N.; Matsuo, M. Protective effects of flavonoids on the cytotoxicity of linoleic acid hydroperoxide toward rat pheocromocytoma PC12 cells. *Chem.−Biol. Interact.* **2003**, *145*, 101−116.

(35) Satoh, T.; Sakai, N.; Enokido, Y.; Uchiyama, Y.; Hatanaka, H. Free radical-independent protection by nerve growth factor and Bcl-2 of PC12 cells from hydrogen peroxide-triggered apoptosis. *J. Biochem.* **1996**, *120*, 540−546.

(36) Wang, R.; Zhou, J.; Tang, X. C. Tacrine attenuates hydrogen peroxide-induced apoptosis by regulating expression of apoptosis-related genes in rat PC12 cells. *Brain Res. Mol. Brain Res.* **2002**, *30*, 107, 1−8.

(37) Suzaki, Y.; Yoshizumi, M.; Kagami, S.; Koyama, A. H.; Taketani, Y.; Houchi, H.; Tsuchiya, K.; Takeda, E.; Tamaki, T. Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 (BMK1) and the MEF2C signaling pathway in PC12 cells: potential role in cell survival following oxidative insults. *J. Biol. Chem.* **2002**, *15*, 9614−9621.

(38) Juurlink, B. H.; Paterson, P. G. Review of oxidative stress in brain and spinal cord injury: suggestions for pharmacological and nutritional management strategies. *J. Spinal Cord Med.* **1998**, *21*, 309−334.

(39) Middleton, E., Jr.; Kandaswami, C.; Theoharides, T. C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. *Pharmacol. Rev.* **2000**, *52*, 673−751.

(40) Bastianetto, S.; Quirion, R. Natural extracts as possible protective agents of brain aging. *Neurobiol. Aging* **2002**, *23*, 891−897.

(41) Sun, A. Y.; Simonyi, A.; Sun, G. Y. The "French Paradox" and beyond: neuroprotective effects of polyphenols. *Free Radical Biol. Med.* **2002**, *15*, 314−318.