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In Vitro and ex Vivo Antihydroxyl Radical Activity of Green and Roasted Coffee

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The specific antiradical activity against the hydroxyl radical of the water soluble components in green and dark roasted *Coffea arabica* and *Coffea robusta* coffee samples, both in vitro by the chemical deoxiribose assay and ex vivo in a biological cellular system (IMR32 cells), were determined. All the tested coffee solutions showed remarkable antiradical activity. In the deoxiribose assay, all the tested solutions showed similar inhibitory activity (IA%) against the sugar degradation (IA values ranged from 45.2 to 46.9%). In the cell cultures, the survival increase (SI%) ranged from 197.0 to 394.0% with *C. robusta* roasted coffee being significantly more active than the other samples. The coffee solutions underwent dialysis (3500 Da cutoff membrane) to fraction their components. In both systems, the dialysates (MW < 3500 Da) either from green or roasted coffee, showed antiradical activity, while the only retentates (MW > 3500 Da) from the roasted coffee samples were active. The preparative gel-filtration chromatography of roasted coffee *C. robusta* dialysate gave three fractions active in the biological system, all containing chlorogenic acid derivatives. The most active fraction was found to be that containing the 5-*O*-caffeoilquinic acid, which shows a linear relation dose−response ranging from 0.02 to 0.10 mM. The results show that both green and roasted coffee possess antiradical activity, that their more active component is 5-*O*-caffeoyl-quinic acid, and moreover that roasting process induces high MW components (later Maillard reaction products, i.e., melanoidins), also possessing antiradical activity in coffee. These results could explain the neuroprotective effects found for coffee consumption in recent epidemiological studies.

KEYWORDS: Coffee; antiradical activity; neurodegenerative disorders; 5-*O*-caffeoyl-quinic acid

INTRODUCTION

Epidemiological studies over the past decade showed very consistent beneficial effects of an increased consumption of vegetable and fruits only partially ascribable to the known nutrients and micro-nutrients such as vitamins A, E, C, and β-carotene. The protective effects on human health concern chronic diseases such as cancer, cardiovascular, inflammatory, and neurodegenerative pathologies, including aging, all being phenomena in which oxidative stress has been generally recognized as a cause of cell degeneration (*1*−*3*). Oxidative stress is caused by an excessive production of reactive oxygen and nitrogen species (ROS and RNS), which our defense mechanisms are no longer able to promptly remove (*4*). The strong protection that fruits and vegetables provide has been attributed to antioxidant compounds different than the known vitamins such as polyphenolic compounds ubiquitously present in plant material (*5*). The main class of polyphenols are defined according to the nature of their carbon skeleton as phenolic acids, flavonoids, and furthermore, stilbenes and lignans. Their antioxidant properties depend on the hydrogen-donating and ion chelating capability due to the presence of hydroxyl groups on their molecule.

Regarding aging and neurodegenerative diseases, it is common belief that the increase in oxidative stress may be one of the contributing factors in neuronal death occurring after any ischemic and hypoxic insults. In particular, in Alzheimer's disease (AD), a direct link seems to exist between the putative disease-causing agent, amyloid-β-peptide, and oxidative processes occurring within the brain and in the cerebrospinal fluid (*6*). In fact, it has been described that AD histopathological cortical lesions are caused by oxidizing stress and by a subsequent accumulation of free radicals that bring on a lipoperoxidation, thus causing lesions to neuronal membranes (*7*−*8*). ROS and RNS, such as the highly reactive hydroxyl radical, peroxinitrite or hypoclorite, are able to initiate the chain reaction of lipid peroxidation, and their action is inhibited by free radical antioxidants.

In addition to the clinical trials using antioxidant drugs such as vitamin E, Selegeline, Gingko extracts, and idebenone (*9*),

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dietary antioxidants represent an emerging aspect, as previously described. In fact, in humas, the antioxidant protection against toxic intermediates may be heavily influenced by nutrition (*4*), and it becomes important to underline that a healthy diet may reduce the risk of chronic conditions such as cancer and cardiovascular diseases as well as age-related degenerative brain disorders (*10*). Thus, a healthy diet should be considered the first line of defense against both the development and progression of AD, as all the other chronic diseases (*11*−*12*).

Regarding AD, there is strong evidence that the incidence and prevalence of this pathology is affected by diet, with high risk factors such as alcohol, fat, refined carbohydrates, salt, and total caloric consumption, and protective factors such as antioxidants, essential trace minerals, estrogens for post-menopausal women, fish, fish oil, wine, and coffee consumption (*1*). Again, recent epidemiological studies have established an association between the common consumption of coffee, or other caffeinated beverages, and a reduced risk of developing another neurodegenerative disease, such as Parkinson's disease (*13*). Furthermore, recently, two large prospective epidemiological studies (*14*−*15*) have greatly strengthened the concept of a link between higher coffee and caffeine intake and a lower incidence of Parkinson's disease.

Regarding coffee beverage, it has also been reported that, at low doses, coffee can suppress the in vitro mutagenicity of oxidants such as *tert*-butyl-hydroperoxide and can also inhibit lipid peroxidation and malondialdehye formation (*16*). Additionally, in a previous research, coffee (mainly roasted coffee) was found to possess strong antiperoxyl radical activity, both in a chemical (linoleic acid-βcarotene micellar system) and in a biological system (rat liver microsomes) (*17*).

The aim of this research is to verify both in a chemical (deoxyribose assay) and in a biological system (MTT assay), the antiradical activity of green and dark roasted coffee, either from *Coffea arabica* or *Coffea robusta* against the hydroxyl radical and to isolate and characterize the compounds responsible for such activity.

MATERIALS AND METHODS

Chemicals. 5-*O*-Caffeoyl-quinic acid (5-*O*-CQA), caffeine, trigonelline hydrochloride, nicotinic acid, blue dextran, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich S.r.l. (Italy). Fetal bovine serum, penicillin, streptomycin, and glutamine were purchased from Gibco (Paisley, UK). Lysozyme was purchased from Merck.

Coffee Solution Preparation. One sample of *Coffea arabica* and one sample of *Coffea robusta* beans, either green or dark roasted, were ground in a laboratory scale mill and sieved through a no. 30 sieve. The roasted samples were prepared in a pilot roaster apparatus. Coffee solutions were prepared using the brewed coffee procedure. A 6-g sample of green or dark roasted coffee was boiled for 10 min in 100 mL of Millipore grade water. The solution was then filtered on Millipore membranes of cellulose acetate/cellulose nitrate mixed esters (0.45 μm).

Standard Solution Preparation. 5-*O*-CQA, caffeine, nicotinic acid, trigonelline hydrochloride aqueous standard solutions were assayed at the concentrations of 1.30, 3.00, 1.50, 0.15 mM, respectively, to determine the antihydroxyl radical activity using the chemical and the biological assay.

Dialysis. Dialysis was performed using Spectra/Por Biotech cellulose ester membrane with molecular weight cutoff at 3500 Da. A 10-mL aliquot of the above-mentioned coffee solutions was fractionated with 3500 Da cutoff membrane in 1000 mL of distilled water for 6 h at 4 °C. The dialysates and the retentates were brought to the initial volume of the coffee solutions to be tested for deoxyribose assay and MTT colorimetric assay. The 5-*O*-CQA used as standard molecular weight marker, was recovered at a percentage that was higher than 95%.

Gel Filtration Chromatography (GFC). The GFC apparatus was a 655A-11 Merck-Hitachi liquid chromatograph with a variable wavelength UV monitor at 200, 270, and 324 nm. The GFC separation of the dialysate and the retentate obtained from the dark roasted *C. robusta* sample were performed using a Superformance Universal glass-cartridge system (300- × 10-mm) (Merck). The stationary phase was TSK gel Toyopearl HW-40F (exclusion limits 100−10 000 Da) (Tosoh Biosep GmbH), the mobile phase was Millipore grade water at a flow rate of 0.5 mL/min.

Spectrophotometric Analysis. The GFC fractions obtained from the dialysate were spectrophotometrically analyzed after dilution in the 200−340 nm range (DU 750 Beckman).

Deoxyribose Assay. The scavenger activity of the coffee solutions, based on the inhibition of the deoxyribose degradation caused by the attack of hydroxyl radicals, was evaluated using the Aruoma et al. (*18*) method and included some modifications.

In a final volume of 1.2 mL, the reaction mixture contained the following reagents at the final concentrations: $FeCl_3$ (25 μM) premixed with EDTA (100 μM) in KH_2PO_4/KOH buffer (pH 7.4), 2-deoxy-D-ribose (2.8 mM), H_2O_2 (2.8 mM), ascorbic acid (100 μM), and 12 μL coffee solutions (sample), or all the same volumes of KH_2PO_4/KOH buffer (control sample). Both samples were placed in a water bath at 37 °C for 1 h and then 1 mL of 1% thiobarbituric acid and 1 mL of 2.8% trichloroacetic acid were added. The reaction mixtures were heated in a water bath at 80 °C for 20 min, kept in ice for 5 min, and then centrifuged for 5 min at 3000 rpm to separate the particles. The absorbance of the samples' supernatant and of the control sample were read in a spectrophotometer at 532 nm against the relative solutions prepared as described but without ascorbic acid (ΔAbs) to correct for interference due to the juice color and thiobarbituric acid-reactive substances that might naturally occur in coffee solutions.

The scavenger activity was expressed as the percent of inhibitory activity (IA%) of deoxyribose degradation in the presence of the coffee solution (sample), relative to the control sample (without the coffee solution), using the equation

$$\mathrm{IA\%} = 100 - \frac{\Delta\mathrm{Abs\ sample}}{\Delta\mathrm{Abs\ control\ sample}} \times 100$$

The scavenger activity was also determined for an aqueous solution of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C), which was assayed at three final concentrations of 50, 100, and 200 μM.

Cell Cultures. IMR32 cell cultures (human neuroblastoma) were grown in an RPMI 1640 medium containing 10% (v/v) fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and 2 mM glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Treatment and Assessment of Cell Viability (MTT Colorimetric Assay). At day 0, IMR32 cells were plated at a density of 5×10^4 viable cells per well in 96-well plates coated with 0.5% gelatin. On the day of the experiment, cells were incubated for 1 h at 37 °C and in the presence of diluted samples (1/20). After this incubation period, cells were treated for 24 h with hydrogen peroxide 500 μM. The protective effect of the different samples were then evaluated using MTT colorimetric assay. MTT is an indicator of the mithocondrial activity in living cells. In the present study, cells were exposed to an MTT solution in a phosphate buffer solution (1 mg/mL at pH 7.4). Following 4 h incubation with MTT and treatment with sodium dodecyl sulfate for 24 h, the reduction of living cells was quantified using a microplate reader Bio-Rad model 550.

The protective activity was expressed as the survival increase percent (SI%) of IMR32 cells in the presence of the coffee solutions (sample) relative to that of the control sample (without the coffee solutions), using the following equation

$$\mathrm{SI\%} = \frac{\mathrm{ABSsample} \times 100}{\mathrm{ABScontrolsample}} - 100$$

The protective activity was also determined for a 250 μM Trolox C solution.

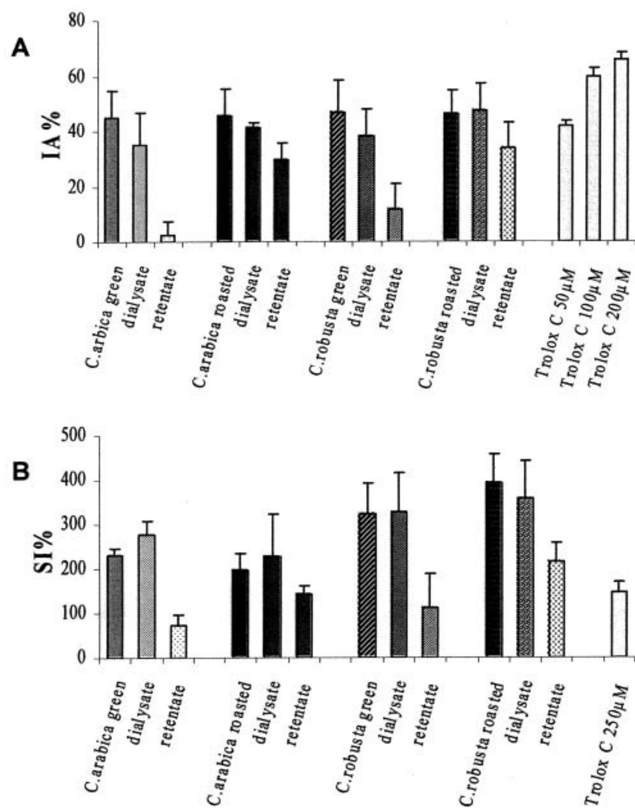

Figure 1. (**A**) In vitro antihydroxyl radical activity (IA%) of *C. arabica* and *C.* robusta green and dark roasted coffee solutions, dialysates and retentates. (*B*) Ex vivo antihydroxyl radical activity (SI%) of *C. arabica* and *C. robusta* green and dark roasted coffee solutions, dialysates, and retentates.

Statistical Analysis. The values represent the mean values of at least eight replications for the deoxyribose assay and five independent experiments on quadruplicate samples for the MTT assay. Data were analyzed by analysis of variance (ANOVA) with the statistical package Statgraphic Plus (1998). Means were separated with the LSD method at a confidence level of 95%.

RESULTS AND DISCUSSION

We determined, both in vitro and in a biological cellular system, the specific antiradical activity, against the hydroxyl radical, of the water soluble components occurring in green and dark roasted coffee beans belonging to the two most important commercial species (i.e., *C. arabica* and *C. canephora*) usually indicated as *C. robusta* according to the most commonly used variety.

To test the antiradical activity of the coffee solutions in a chemical system, we used the deoxyribose assay (*18*). The antiradical activity is expressed as the coffee extract inhibitory activity (IA%) against the degradation of deoxyribose, which acts as a target for the hydroxyl radical attack. To evaluate the activity of green and dark roasted coffee components against hydrogen peroxide-induced cell death, IMR32 cell line was chosen for the present study because this cellular model is sensitive to oxidative stress-induced cell death in a concentration dependent manner.

All the four tested coffee solutions showed remarkable antiradical activity both in the chemical and in the biological system. In the deoxiribose assay, all the tested coffee solutions showed similar inhibitory activity (**Figure 1A**); in fact, the sugar degradation products, induced by the hydroxyl radical attack and measured as TBA-RS, decreased in the presence of the coffee solutions in amounts ranging from 45.2 to 46.9%. No significant differences were found among either green or roasted coffees of the same species or among the samples belonging to the two different species when their IA values were compared statistically. In the cell cultures, the cell survival increase percentage (SI%) ranged from 197.0 to 394.0% when coffee solutions were present (**Figure 1B**). In this case, no significant differences were found among the green and roasted samples from the same species. Significant differences ($p < 0.05$) were found among SI values given by the Robusta dark roasted coffee and the samples belonging to the *C. arabica* species.

Table 1. In vitro (IA%) and ex vivo (SI%) antihydroxyl radical activity of standard compounds

compound	concn (mM)	IA%	SI%
caffeine	3.00	27.2 ± 6.4	25.2 ± 5.2
trigonelline hydrochloride	1.50	19.3 ± 12.6	15.7 ± 4.1
nicotinic acid	0.15	19.2 ± 6.7	23.2 ± 4.8
5-O-caffeoyl-quinic acid	1.30	34.6 ± 7.5	264.3 ± 56.2

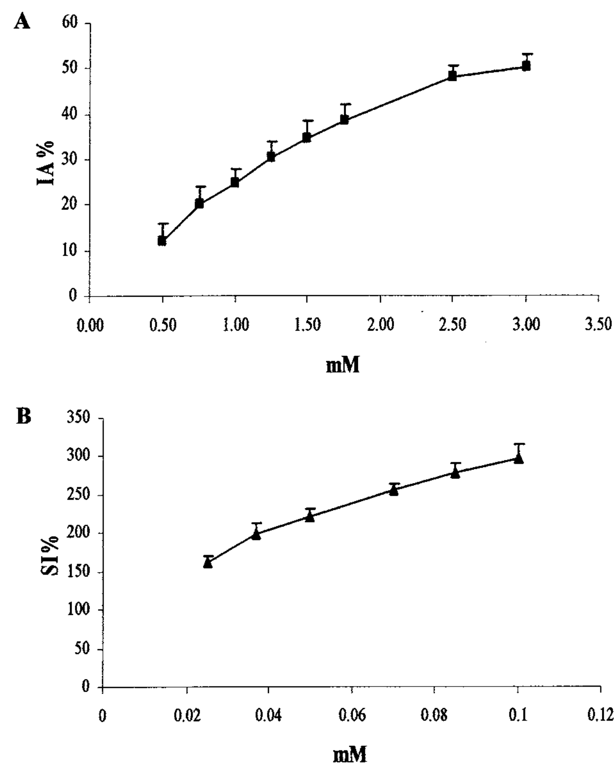

Figure 2. (**A**) In vitro antihydroxyl radical activity (IA%) versus concentration of 5-*O*-CQA. (**B**) Ex vivo antihydroxyl radical activity (SI%) versus concentration of 5-*O*-CQA.

To obtain preliminary information about the compounds responsible for the revealed activity, several standards of coffee components such as caffeine, trigonelline hydrochloride, nicotinic acid, and 5-*O*-CQA, were analyzed at concentrations commonly found in dark roasted *C. robusta* coffee samples (*19*). In the chemical system, all the tested compounds resulted active with the 5-*O*-CQA giving the highest IA value. On the other hand, in the biological system, only the 5-*O*-CQA resulted as active, with a strong antiradical activity (**Table 1**). In both the systems 5-*O*-CQA showed an effect that was concentration-dependent (**Figure 2A−B**).

Thereafter, the previously tested coffee solutions were submitted to dialysis to fraction their components on the basis of their molecular weight (MW) using a membrane with cutoff

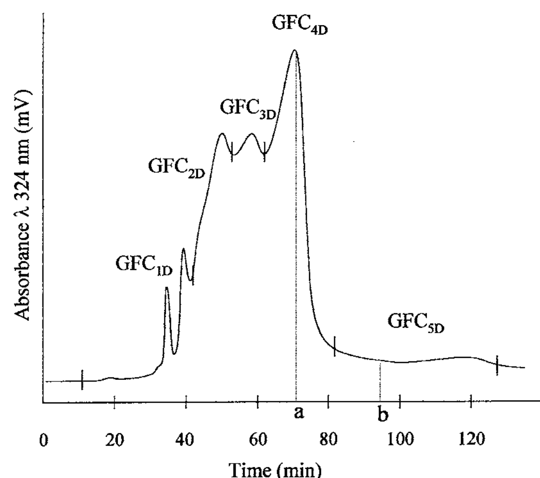

Figure 3. GFC of *C. robusta* coffee dialysate. Operative conditions were described in the text. (**a**) 5-*O*-CQA (Rt = 70 min), (**b**) caffeine (Rt = 95 min).

at 3500 Da separating lower MW components such as monomeric polyphenols from polymeric polyphenols and lower MW melanoidins from the higher MW polymers.

Both of the fractions obtained from each coffee solution (*C. arabica* and *C. robusta*), which are the dialysate (MW < 3500 Da) and the retentate (MW > 3500 Da) were reported at the initial volume of the fractioned beverages and were submitted to the chemical and biological tests (**Figure 1**). In both systems, the dialysates (MW < 3500 Da), either from green or roasted coffees, possessed antiradical activity, while the retentates were active, in both systems, only when derived from the roasted coffee samples. Both in the chemical and in the biological systems, no significant differences were found among the values given by the dialysates obtained from the tested coffee samples. The retentate obtained from *C. robusta* resulted significantly more active when compared to the one obtained from *C. arabica* and tested in the biological system. In any case, the results given by both assays indicate that the roasting process on one hand destroyed most of the chlorogenic acid derivatives but on the other hand induced the formation of new compounds, probably melanoidins, with MW > 3500 Da and with antiradical activity in both the chemical and in the biological systems. The mean values given by the dialysates in both the systems were close to those given by the corresponding coffee solutions. Consequently, the activity of the dialysate and retentate on the whole resulted higher than that of the corresponding coffee solutions for roasted samples.

Our studies continued on the dark roasted samples, which are commonly the only ones used to prepare coffee beverages, by choosing *C. robusta*, the species that was the most active in the biological system. The dark roasted coffee dialysate and retentate were submitted to preparative GFC to further separate their components using blue dextran to determine dead volume time and lysozyme, 5-*O*-CQA and caffeine standard compounds. The GFC eluate obtained from the dialysate, monitored at 324 nm (λ_{max} 5-*O*-CQA) and 275 nm (λ_{max} caffeine) was subdivided into five fractions (**Figure 3**). The fourth fraction (the widest peak) showed the same retention time of the 5-*O*-CQA (Rt = 69 min) and the fifth one of caffeine (Rt = 95 min). The GFC_{2D}, GFC_{3D}, and GFC_{4D} fractions, when spectrophotometrically analyzed, all showed, in the range λ = 220−340 nm, the spectrum characteristics of the chlorogenic acid derivatives. The five GFC fractions, reported of the initial volume of the coffee beverage submitted to dialysis, were tested in the cell culture.

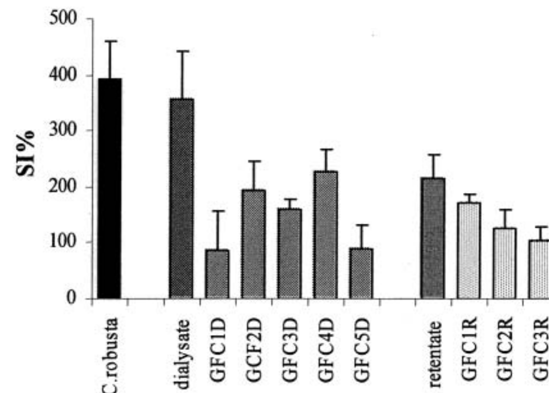

Figure 4. *Ex vivo* antihydroxyl radical activity (SI%) of dark roasted coffee solution, dialysate, retentate, and their GFC fractions.

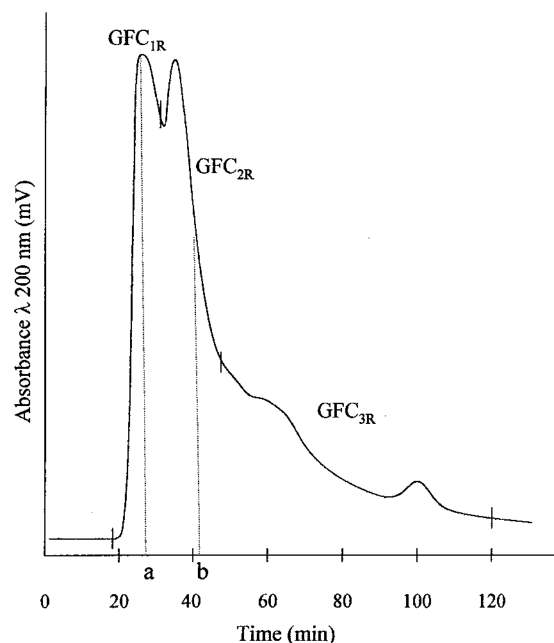

Figure 5. GFC of *C. robusta* coffee retentate. Operative conditions were described in the text. (**a**) Blue dextran (Rt = 27 min), (**b**) lysozyme (Rt = 42 min).

The data obtained showed the fractions GFC_{2D}, GFC_{3D}, and GFC_{4D} as active with the higher activity for the GFC_{4D} containing the 5-*O*-CQA (**Figure 4**). These results seem to indicate that in the dark roasted coffee there are at least three chlorogenic acid derivatives which seem to be active.

The GFC eluate obtained from the retentate (**Figure 5**) was subdivided into three fractions that were then assayed for the antiradical activity in the biological system. The first fraction shows the retention time of blue dextran and the second one a retention time close to the retention time of lysozyme. The results obtained from the biological assay show that GFC_{1R} fraction is active with SI value of 171.0% and that GFC_{2R} has weak activity which could be due to a poor resolution of the mixture (**Figure 4**).

Green and dark roasted coffee from *C. arabica* and *C. robusta* show a specific antihydroxyl radical activity both in the chemical and in the biological system, with *C. robusta* dark roasted coffee being significantly more active than the other samples. Roasted coffee and any beverages prepared from it are very complex mixtures of several hundred chemicals that occur both naturally or are induced by the roasting process. Green coffee contains large, but variable, amounts of phenolic acids (caffeic acid and

chlorogenic acid derivatives). On the other hand, coffee beverages are commonly prepared with roasted coffee, where a part of the polyphenolic compounds are destroyed, while Maillard reaction products with antioxidant properties are in turn generated.

The activity of the green coffee is only due to the low MW compounds, while in the dark roasted coffee, even the fractions with high MW show antiradical activity. These results demonstrate that the roasting process, which on one hand causes the partial degradation of the naturally occurring coffee polyphenols, on the other hand, induces the formation of new brown compounds, known as high MW coffee melanoidins, which show antiradical activity and have a MW higher than 3500 Da.

The low MW compound most responsible for the coffee-revealed activity seems to be the 5-*O*-CQA occurring in coffee. Nevertheless, in the deoxiribose assay, a number of other low MW coffee components can protect the deoxyribose against the hydroxyl radical attack. This seems to indicate that, in the chemical system, almost any compound can protect sugar from degradation, probably because it simply makes it more difficult for the radicals to reach the target. In the biological system, only the compounds that actually possess antioxidant properties, such as chlorogenic acid derivatives, show protective activity against the action of the oxidative stress.

Regarding the high molecular weight fraction obtained from dark roasted coffee, the results show that the roasting process induces in coffee at least one type of melanoidin compound able to protect the IMR32 cells from the oxidative damage.

Thus, the present study indicates that the water soluble components of green and dark roasted coffee from *C. arabica* and *C. robusta* display significant antioxidant activity and are able to rescue and protect IMR32 cell cultures against hydrogen peroxide-induced toxicity. This is consistent with, and supports, further earlier data reporting the protective effects of coffee against lipid peroxidation in a chemical model (i.e., the micellar system linoleic acid β-carotene) and in an ex vivo system of oxidative-stress-induced toxicity in the rat liver cell microsomes (*17*).

This study indicates a coffee component that actually could contribute to the neuroprotective effects established by epidemiological studies for coffee consumption. In particular, in AD where a direct link seems to exist between the putative disease-causing agent, amyloid-β-peptide, and oxidative stress, the antiradical compounds could help in restoring the normally occurring redox status and could be useful in reducing the risk of developing this neurodegenerative disorder as well as all the other chronic diseases.

This study awaits further research efforts to better isolate and characterize other components different than 5-*O*-CQA low MW and high MW coffee components active against oxidative stress.

ABBREVIATIONS USED

ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5-*O*-CQA, 5-O-caffeoylquinic acid; SDS, sodium dodecyl sulfate; GFC, gel filtration chromatography; IA, inhibitory activity; EDTA, ethylenediaminetetracetic acid; SI, survival increase; ANOVA, analysis of variance; LSD, least significance difference; MW, molecular weight

LITERATURE CITED

(1) Mattson, M. P. Emerging neuroprotective strategies for Alzheimer's disease: dietary restriction, telomerase activation, and stem cell therapy. *Exp. Gerontol.* **2000**, *35*, 489−502.

(2) Beal, M. F. Ageing, energy, and oxidative stress in neurodegenerative diseases. *Ann. Neurol.* **1995**, *38*, 357−366.

(3) Ratan, R. R.; Murphy, T. H.; Baraban, J. M. Oxidative stress induces apoptosis in embryonic cortical neurons *J. Neurochem.* **1994**, *62*, 376−379.

(4) Benzie, I. F. Evolution of antioxidant defence mechanisms. *Eur. J. Nutr.* **2000**, *39*, 53−61.

(5) Velioglu, Y. S.; Mazza, G.; Gao, L.; Oomah, B. D. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agric. Food Chem.* **1998**, *46*, 4113−4117.

(6) Arlt, S.; Beisiegel, U.; Kontush, A. Lipid peroxidation in neurodegeneration: new insights into Alzheimer's disease. *Curr. Opin. Lipidol.* **2002**, *13*, 289−294.

(7) Brewer, G. J. Neuronal plasticity and stressor toxicity during aging. *Exp. Gerontol.* **2000**, *35*, 1165−1183.

(8) Pratico, D.; Delanty, N. Oxidative injury in disease of the central nervous system: focus on Alzheimer's disease. *Am. J. Med.* **2000**, *109*, 577−585.

(9) Mayeux, R.; Sano, M. Drug Therapy: treatment of Alzheimer's disease. *N. Engl. J. Med.* **1999**, *341* (220), 1670−1679.

(10) Duthie, G.; Crozier, A. Plant-derived phenolic antioxidants. *Curr. Opin. Clin. Nutr. Metab. Care* **2000**, *3*, 447−451.

(11) Grant, W. B. Dietary links to Alzheimer's disease: 1999 Update. *J. Alzheimers Dis.* **1999**, *1*, 197−201.

(12) Grant, W. B.; Campbell, A.; Itzhaki, R. F.; Savory, J. The significance of environmental factors in the etiology of Alzheimer's disease. *J. Alzheimers Dis.* **2002**, *4*, 179−189.

(13) Chen, F.; Xu, K.; Petzer, J. P.; Staal, R.; Xu, Y. H.; Beilstein, M.; Sonsalla, P. K.; Castagnoli, K.; Castagnoli, N. Jr.; Schwarzschild, M. A. Neuroprotection by Caffeine and A2A Adenosine Receptor Inactivation in a Model of Parkinson's Disease. *J. Neurosci.* **2001**, *21*, 1−6.

(14) Ascherio, A.; Zhang, S. M.; Hernan, M. A.; Kawachi, I.; Colditz, G. A.; Speizer, F. E.; Willett, W. C. Prospective study of caffeine intake and risk of Parkinson's disease in men and women, *Ann. Neurol.* **2001**, *50*, 56−63.

(15) Ross, G. W.; Abbott, R. D.; Petrovitch, H.; Morens, D. M.; Grandinetti, A.; Tung, K. H.; Tanner, C. M.; Masaki, K. H.; Blanchette, P. L.; Curb, J. D.; Popper, J. S.; White, L. Association of coffee and caffeine intake with the risk of Parkinson's disease. *J. Am. Med. Assoc.* **2000**, *283*, 2674−2679.

(16) Stadler, R. H.; Turesky, R. J.; Muller, O.; Markovic, J.; Leong-Morgenthaler, P. M. The inhibitory effects of coffee on radical-mediated oxidation and mutagenicity. *Mutat. Res.* **1994**, *308* (2), 177−190.

(17) Daglia, M.; Papetti, A.; Gregotti, C.; Bertè, F.; Gazzani, G. In vitro antioxidant and ex vivo protective activity of green and roasted coffee. *J. Agric. Food Chem.* **2000**, *48*, 1449−1454.

(18) Aruoma, O. I. Deoxyribose Assay for Detecting Hydroxyl Radicals. *Methods Enzymol.* **1994**, *233*, 57−66.

(19) Belitz, H. D.; Grosh, W. Coffea, tea, cocoa. In *Food Chemistry*; New York: Springer-Verlag: Heidelberg, Berlin; 1999; 874−883.

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