

Comparison of antioxidant activity between green and roasted coffee beans using molecular methods

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Abstract. Coffee is one of the most popular and widely consumed beverages worldwide due to its pleasant taste and aroma. A number of studies have been performed to elucidate the possible beneficial effects of coffee consumption on human health and have shown that coffee exhibits potent antioxidant activity, which may be attributed mainly to its polyphenolic content. However, there is also evidence to suggest that coffee roasting (the procedure which turns green coffee beans to the dark, roasted ones from which the beverage derives) may alter the polyphenolic profile of the beans (e.g., via the Maillard reaction) and, concomitantly, their antioxidant activity. In the present study, the antioxidant activity of 13 coffee varieties was examined in both green and roasted coffee bean extracts using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) radical scavenging assays. In addition, 5 selected varieties were also examined for their protective effects against peroxyl and hydroxyl radical-induced DNA strand cleavage. Finally, C2C12 murine myoblasts were treated with non-cytotoxic concentrations of the most potent extract in order to examine its effects on the cellular redox status by measuring the glutathione (GSH) and

reactive oxygen species (ROS) levels by flow cytometry. Our results revealed that, in 8 out of the 13 coffee varieties, roasting increased free radical scavenging activity as shown by DPPH and ABTS^{•+} assays. Moreover, we found that when one coffee variety was roasted for different amounts of time, the increase in the antioxidant activity depended on the roasting time. By contrast, in 5 varieties, roasting reduced the antioxidant activity. Similar differences between the roasted and green beans were also observed in the free radical-induced DNA strand cleavage assay. The observed differences in the antioxidant activity between the different coffee varieties may be attributed to their varying polyphenolic content and composition, as well as to the different molecules produced during roasting. In addition, in the cell culture assay, the tested coffee extract led to increased GSH levels in a dose-dependent manner, indicating the enhancement of cellular antioxidant mechanisms.

Introduction

It is well-known that aerobic organisms are exposed to oxygen-free radicals, including reactive oxygen species (ROS), under various conditions (1). Thus, free radicals are produced under physiological conditions and participate in a variety of normal cellular functions, such as the regulation of signaling pathways, gene expression and apoptosis (1-3). In addition, free radicals are produced under abnormal conditions, such as in the cases of poor diet, smoking and exposure to ionizing or ultra-violet (UV) radiation. Excess free radicals in cells may interact and cause damage to proteins, lipids and DNA (4). Living organisms possess a complex endogenous defensive mechanism against free radicals that consists of both enzymatic and non-enzymatic compounds (5-7). The overproduction of free radicals may lead to oxidative stress, a pathological condition in which an imbalance between the production of free radicals and the antioxidant mechanisms is observed. Oxidative stress has been shown to be associated with a variety of diseases and pathological conditions, such as cancer, diabetes, obesity and neurodegenerative and autoimmune diseases (8-12).

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Abbreviations: GSH, glutathione; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; H₂O₂, hydrogen peroxide; O₂^{•-}, superoxide radical; OH[•], hydroxyl radical; PBS, phosphate-buffer saline; ROO[•], peroxyl radical; ROS, reactive oxygen species; TPC, total polyphenolic content

Key words: coffee beans, roasting, polyphenols, melanoidins, antioxidant activity, glutathione

Aside from its endogenous mechanisms, an organism may also acquire antioxidant components through diet (13,14). Some of the most important antioxidants, which are found particularly in plant foods, are polyphenols (15). These constitute a category of products of the plant's secondary metabolism and play an important role in a number of cellular functions (16,17). When plant foods are consumed, the absorbed polyphenols may elicit a variety of important bioactivities which have beneficial effects on human health (17). Such polyphenols can also be found in coffee, which is one of the most popular beverages worldwide due to its pleasant taste and aroma; the annual production of coffee is approximately 8 Mt, and the average daily consumption is 2.3 billion cups a day (18). Traditionally, the beneficial effects of coffee on human health were mainly attributed to its most-investigated ingredient, caffeine; however, other components also contribute to its valuable properties, such as its antioxidant activity (17). The latter is attributable mainly to its polyphenolic content, with the most abundant polyphenols being chlorogenic acid (CGA) (19-21). Several studies have been performed to investigate the quantity, as well as the antioxidant and other disease-related properties, of CGA (19,21-24).

However, although we are aware that coffee beans undergo roasting prior to consumption, little data exist on the effects of roasting on coffee composition, or on the differences in antioxidant activity between green and roasted beans (25,26). For instance, it is known that the roasting procedure (which may be different for each variety of coffee) markedly affects CGA, leading to their hydrolysis (27). However, new compounds are formed from the products of this hydrolysis, which may alter the overall antioxidant capacity of the beans (25,28). Therefore, in the present study, we aimed to examine the free radical scavenging activity of 13 coffee varieties (both green and roasted coffee beans). Furthermore, 5 selected varieties were also examined for their protective activity against free radical-induced DNA damage. Finally, C2C12 murine myoblasts were treated with non-cytotoxic concentrations of the most potent extract in order to examine its effects on the cellular redox status by measuring the glutathione (GSH) and ROS levels.

Materials and methods

Coffee beans and roasting conditions. A total of 13 coffee bean varieties were used, specifically 12 from *Coffea arabica* (varieties 1-5 and 7-13) and one from the *Coffea canephora robusta* species (variety 6). The coffee beans were roasted to different degrees. The roasting degrees depended on the roasting time and temperature, with high values indicating less roasting and low values more roasting. The roasting degrees were as follows: 110 (12 min 30 sec; 210°C) for variety 1, 110 (12 min; 210°C) for variety 2, 100 (12 min 30 sec; 211°C) for variety 3, 105 (12 min; 211°C) for variety 4, 96 (11 min; 215°C) for variety 5, 154 (12 min 30 sec; 218°C) for variety 6, 95 (12 min; 208°C) for variety 7, 95 (12 min 30 sec; 215°C) for variety 8, 101 (12 min 30 sec; 215°C) for variety 9, 110 (12 min 30 sec; 209°C) for variety 10, 100 (12 min 30 sec; 215°C) for variety 11, and 144 (11 min 30 sec; 194°C) for variety 12. For variety 13, 4 different roasting times (R1: 7 min 15 sec; R2: 6 min 5 sec; R3: 5 min 32 sec; R4: 3 min 52 sec) at 215°C were used in order to examine the effects of roasting time on the antioxidant activity.

Preparation of extracts from coffee beans. For each variety, 2 g of either green or roasted beans were added to 20 ml distilled water and ground using a mortar and pestle. Each sample was sonicated for 20 min (70% amplitude, 0.7 sec cycle), and then stirred for a further 20 min under moderate heat (35°C). The extract was separated from solid residues by centrifuging each sample (7,000 x g, 10 min, 25°C). Finally, each extract was separated into aliquots that were kept at -80°C for future use.

Assessment of the total polyphenolic content (TPC) of the extracts. The TPC of the coffee extracts was determined using Folin-Ciocalteu reagent, as previously described (29). A 20- μ l sample of extract was added to a tube containing 1 ml deionized water. A total of 100 μ l Folin-Ciocalteu reagent was added to the reaction mixture, followed by incubation for 3 min at room temperature. Subsequently, 280 μ l 25% w/v sodium carbonate solution and 600 μ l deionized water were added to the mixture. Following 1 h of incubation at room temperature in the dark, the absorbance was measured at 765 nm vs. a blank containing Folin-Ciocalteu reagent and distilled water without the extract. The measurement of absorbance was conducted on a Hitachi U-1900 radio beam spectrophotometer (serial no. 2023-029; Hitachi, Tokyo, Japan). The optical density of the sample (20 μ l) in 25% w/v solution of sodium carbonate (280 μ l) and distilled water (1.7 ml) at 765 nm was also measured. The TPC was determined using a standard curve of absorbance values correlated with standard concentrations (50-1500 μ g/ml) of gallic acid. The TPC is presented as μ g of gallic acid equivalents per mg of extract in percentage form.

Assessment of CGA concentration of the coffee extracts. A liquid chromatography (LC)-mass spectrometry (MS; 2010) system was used for the analysis of CGA. CGA ($\geq 95\%$) was also purchased from Sigma-Adrich (St. Louis, MO, USA) for making stock solutions. Stock solutions of CGA at a concentration of 100 ppm were prepared in methanol. The working solutions of the analytes (0, 0.5, 1, 2.5 and 5 ppm) were prepared by further dilutions of the stock solutions. All solutions were stored at -20°C in the dark. To a volume of 50 μ l of each sample, 950 μ l of methanol were added, following by vortexing and centrifugation at 14,000 rpm for 5 min; 20 μ l of the supernatant was injected into the LC system for analysis. The system comprised of a binary LC pump (Shimadzu Prominence LC; Shimadzu, Kyoto, Japan), a vacuum degasser, an autosampler, a diode array detector (SPD-M20A Prominence; Shimadzu, Kyoto, Japan; serial no. L201545) and a column oven. A gradient of 0.1% formic acid in water (solvent A) and methanol (solvent B) was selected as the mobile phase, with a flow rate of 0.7 ml/min: starting at 20% solvent B (1 min), 95% solvent B (13 min linear ramp), and finally 20% solvent B (13.01 min). The separation of the analytes was achieved on a Discovery® C18 HPLC column (250x4.6 mm, 5 μ m) thermostated at 30°C. A diode array detector was used for the determination of the analytes. The maximum wavelength for CGA was 320 nm. The CGA retention time was 8.93 min.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The free-radical scavenging capacity (RSC) of the extracts was evaluated by DPPH radical assay, as previously described (29). Briefly, a 1.0 ml freshly prepared methanolic

solution of DPPH radical (100 μM) was mixed with the tested extract solution at various concentrations (0.5–100 $\mu\text{g/ml}$). The contents were vigorously mixed, incubated at room temperature in the dark for 20 min, and the absorbance was measured at 517 nm. The measurement was conducted on a Hitachi U-1900 radio beam spectrophotometer (Hitachi). In each experiment, the tested sample alone in methanol was used as a blank and DPPH alone in methanol was used as the control.

The percentage RSC of the tested extracts was calculated using the following equation: $\text{RSC} (\%) = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$, where A_{control} and A_{sample} are the absorbance values of the control and the test sample, respectively. Moreover, in order to compare the radical scavenging efficiency of the extracts, the IC_{50} value indicating the concentration that caused 50% scavenging of the DPPH radical was calculated from the graph-plotted RSC percentage against the extract concentration. All experiments were carried out in triplicate and on at least two separate occasions.

2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{++}) radical scavenging assay. The ABTS^{++} RSC of the extract was determined as previously described in the study by Cano *et al* (31) with minor modifications. Briefly, the reaction was carried out in 1 ml distilled water containing ABTS^{++} (1 mM), hydrogen peroxide (H_2O_2) (30 μM) and horseradish peroxidase (6 μM) in 50 mM phosphate-buffered saline (PBS; pH 7.5). The solution was vigorously mixed followed by incubation at room temperature in the dark for 45 min until ABTS^{++} radical formation occurred. Subsequently, 10 μl extracts, of various concentrations, were added to the reaction mixture and the absorbance at 730 nm was read. The measurement was conducted on a Hitachi U-1900 radio beam spectrophotometer (Hitachi). In each experiment, the tested sample in distilled water containing ABTS^{++} and H_2O_2 in 50 mM PBS-pH 7.5 was used as a blank, and the ABTS^{++} radical solution with H_2O was used as the control. The RSC percentage and the IC_{50} values were determined as described above for the DPPH method. All experiments were carried out in triplicate and on at least two separate occasions.

Hydroxyl radical-induced DNA plasmid strand cleavage. DNA strand breakage was measured by the conversion of supercoiled pBluescript (SK+) plasmid double-stranded DNA to the open circular form. Hydroxyl radical-induced DNA relaxation assay was performed according to the method described in the study by Keum *et al* (32) with some modifications. The reaction mixture (10 μl) consisted of 1 μg pBluescript (SK+) plasmid DNA, 10 mM Tris-HCl-1 mM EDTA, the tested extract at various concentrations (600, 1,000, 1,500, 2,000, 3,300 and 6,000 $\mu\text{g/ml}$) and 40 mM H_2O_2 . Hydroxyl radicals ($\text{OH}^\bullet$) were generated from UV photolysis of H_2O_2 following irradiation of the reaction mixture with a 300 W UV lamp (OSRAM GmbH, Munich, Germany) for 3 min at a distance of 50 cm. The reaction was terminated by the addition of 3 μl loading buffer (0.25% bromophenol blue and 30% glycerol) and analyzed by 0.8% agarose gel electrophoresis at 80 V for 1 h. The gels were stained with ethidium bromide (0.5 $\mu\text{g/ml}$), destained with water, photographed and analyzed by UV transillumination using the Alpha Innotech Multiimage (ProteinSimple, San Jose, CA, USA). In addition, pBluescript (SK+) plasmid DNA was

treated with each extract alone, at the highest concentration used in the assay, in order to examine the effects of the extracts on plasmid DNA conformation. It should be noted that isolated pBluescript (SK+) plasmid DNA contained approximately 10% open-circular DNA prior to treatment. Each experiment was repeated at least 3 times. The preventive activity of the tested extracts against hydroxyl radical-induced DNA strand breakage was assessed by measuring the inhibition of the conversion of supercoiled conformation to the open-circular form. The percentage inhibition of radical-induced DNA strand cleavage by the extracts was calculated using the following formula: $\% \text{ inhibition} = [(S - S_0)/(S_{\text{control}} - S_0)] \times 100$, where S_{control} is the percentage of supercoiled DNA in the negative control sample (plasmid DNA alone), S_0 is the percentage of supercoiled plasmid DNA in the positive control sample (without tested extracts but in the presence of the radical initiating factor), and S is the percentage of supercoiled plasmid DNA in the sample with the tested extracts and the radical initiating factor. Moreover, in order to compare the percentage inhibition of the extracts, the IC_{50} value indicating the concentration that caused 50% scavenging of the DPPH radical was calculated by comparing the graph-plotted percentage inhibition to the extract concentration. All experiments were carried out in triplicate and on at least two separate occasions.

Peroxyl radical-induced DNA plasmid strand cleavage.

The assay was performed using the procedure previously described in the study by Chang *et al* (33). Peroxyl radicals ($\text{ROO}^\bullet$) were generated from the thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). The reaction mixture (10 μl) containing 1 μg pBluescript (SK+) plasmid DNA, 2.5 mM AAPH in PBS and the tested extract at various concentrations (50, 60, 75, 100, 150 and 300 $\mu\text{g/ml}$) was incubated in the dark for 45 min at 37°C. Following incubation, the reaction was terminated by the addition of 3 μl loading buffer (0.25% bromophenol blue and 30% glycerol) and analyzed by gel electrophoresis. Each experiment was repeated 3 times. The preventive effects of the tested extracts against peroxyl radical-induced DNA strand breakage were assessed as described above for hydroxyl radical-induced DNA strand breakage.

Cell culture conditions. The C2C12 murine myoblasts were a gift from Professor Koutsilieris (National and Kapodistrian University of Athens, Athens, Greece). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin and 100 U/ml streptomycin (all from Gibco, Paisley, UK) in plastic disposable tissue culture flasks at 37°C in an atmosphere with 5% carbon dioxide.

XTT assay. Cell viability was assessed using an XTT assay kit (Roche, Mannheim, Germany). Briefly, the C2C12 cells were subcultured in a 96-well plate with 1×10^4 cells per well in DMEM. Following 24 h of incubation, the cells were treated with various concentrations of the coffee extract in serum-free DMEM for 24 h. Subsequently, 50 μl XTT test solution, which was prepared by mixing 50 μl XTT labeling reagent with 1 ml electron coupling reagent, were added to each well. Following 4 h of incubation, absorbance was measured

at 450 nm and also at 690 nm as a reference wavelength in a BioTek ELx800 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Serum-free DMEM was used as a negative control. In addition, the absorbance of the grape-extract concentration alone in serum-free DMEM and XTT test solution was tested at 450 nm. The absorbance values of the grape extracts alone were subtracted from those derived from cell treatment with coffee extract. Data were calculated as the percentage of inhibition using the following formula: inhibition (%) = $[(OD_{\text{control}} - OD_{\text{sample}})/OD_{\text{control}}] \times 100$, where OD_{control} and OD_{sample} indicate the optical density of the negative control and the tested compounds, respectively. All experiments were carried out in triplicate and on two separate occasions.

Assessment of GSH and ROS levels by flow cytometry. The intracellular GSH and ROS levels were assessed using mercury orange and 2,7-dichlorofluorescein diacetate (DCF-DA), respectively. The fluorescent mercury orange binds directly to GSH, while DCF-DA within cells is deacetylated by esterases and is further converted to fluorescent DCF by the oxidative action of ROS. A 400-mM stock solution of mercury orange was prepared in acetone and stored at 4°C, and a fresh 400-mM stock solution of DCF-DA was prepared in methanol. To measure the GSH and ROS levels, the cells were resuspended in PBS at 1×10^6 cells/ml and incubated in the presence of mercury orange (40 μ M) or DCF-DA (10 μ M) in the dark at 37°C for 30 min. The cells were then washed, resuspended in PBS, and subjected to flow cytometric analysis using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) with excitation and emission wavelengths at 488 and 530 nm for ROS and at 488 and 580 nm for GSH, respectively. In addition, forward-angle and right-angle light scattering showing the cell size and cell internal complexity, respectively, were measured. The cells were analyzed at a flow rate of 1,000 events/sec. Analyses were performed on 10,000 cells per sample, and the fluorescence intensities were measured on a logarithmic scale of 4 decades of log of fluorescence. Data were analyzed using BD Cell Quest software (Becton-Dickinson). Each experiment was repeated at least 3 times.

Statistical analysis. All results are expressed as the means $\pm$ standard deviation. A Spearman's correlation analysis for examining the results from the TPC, DPPH and ABTS^{•+} assays was performed. A P-value <0.05 was considered to indicate a statistically significant difference. In addition, one-way ANOVA was applied, followed by Tukey's test for multiple pair-wise comparisons using SPSS software (SPSS, Inc., Chicago, IL, USA).

Results and Discussion

The TPC was determined in each coffee variety before and after roasting (Fig. 1). The TPC percentage by mass varied from 2.7 to 4.7% for the roasted beans, with a mean value of 3.8%, whereas for the green beans, the values varied from 3.2 to 5.2%, with a mean value of 3.8%. In 7 of the 13 varieties, the green coffee beans had higher amounts of polyphenols, as was expected (Fig. 1A). However, in the remaining 6, the roasted beans had more polyphenols than their respective green beans (Fig. 1B and C). The polyphenolic percentages obtained

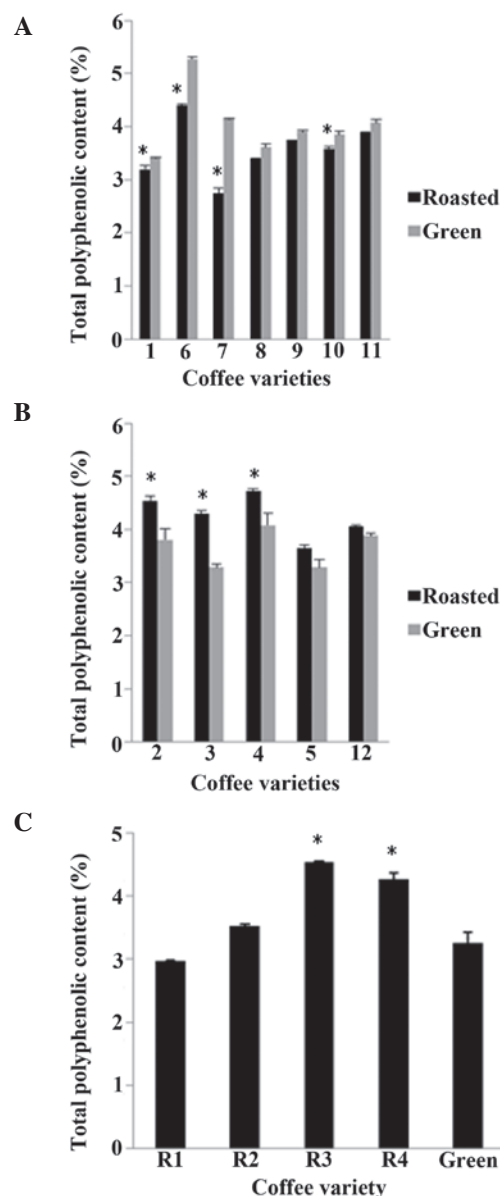


Figure 1. Total polyphenolic content (TPC) of each variety of coffee. TPC is expressed as a percentage by mass of each tested sample, as measured by Folin-Ciocalteu assay. (A) Varieties of green beans which had a higher TPC compared with their respective roasted beans. (B) Varieties of roasted seeds which had a higher TPC. (C) Effects of roasting time on the TPC of variety 13. The roasting times were as follows: R1, 7 min 15 sec; R2, 6 min 5 sec; R3, 5 min 32 sec; R4, 3 min 52 sec. All data are expressed as the means $\pm$ SD. *P<0.05 indicated a statistically significant difference between roasted beans and the respective green beans.

in the present study are in agreement with those presented in the relevant literature, despite the fact that, depending on the variety, large variations have been detected (27,34,35).

Following the determination of the polyphenolic content, the antioxidant activity of each sample was evaluated using DPPH and ABTS^{•+} assays (Figs. 2 and 3). In order to examine the antioxidant potency of the polyphenols contained in each coffee sample, the IC_{50} value obtained from the assays was divided by the amount of polyphenols contained in each mg of the respective coffee extract. According to both assays, in 8 of the 13 varieties, the roasted beans exhibited an increased antioxidant activity compared with their respective green

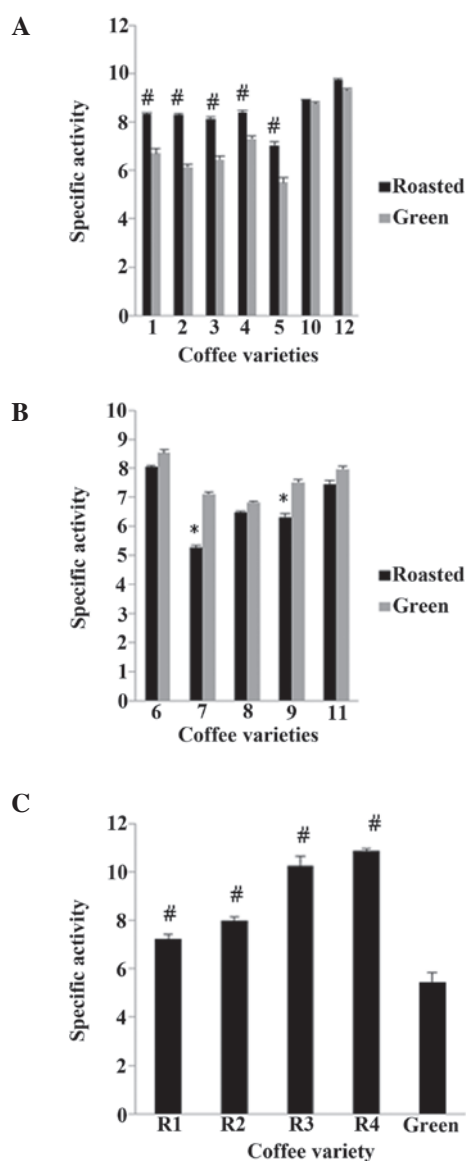


Figure 2. Antioxidant activity as assessed using DPPH assay. The specific activity of each variety is shown, as measured in units of activity per mg of coffee extract. A unit represents the amount of polyphenols required to scavenge 50% of the DPPH radical. (A) Varieties of roasted seeds which exhibited higher levels of activity compared with their respective green beans. (B) Varieties of green beans which exhibited higher levels of activity. (C) Effects of roasting time on DPPH radical scavenging by variety 13. The roasting times were as follows: R1, 7 min 15 sec; R2, 6 min 5 sec; R3, 5 min 32 sec; R4, 3 min 52 sec. All data are expressed as the means $\pm$ SD. * $P < 0.05$ and # $P < 0.01$ indicated a statistically significant difference between roasted beans and the respective green beans.

beans (Fig. 1A and C and Fig. 2A and C), whereas in the remaining 5 varieties, the opposite was observed (Figs. 2B and 3B). Specifically, in varieties 1, 2, 3, 4 and 5, the roasted beans exhibited a significant ($P < 0.01$) increase in antioxidant activity compared to the green beans by 24.1, 35.1, 26.0, 15.2 and 27.9%, respectively, in the DPPH assay, and in the ABTS⁺ assay, this increase was 30.4, 36.2, 42.0, 25.2 and 34.1%, respectively. By contrast, in varieties 7 and 9, the antioxidant activity decreased significantly ($P < 0.05$) after roasting, by 26.0 and 16.0%, respectively, for the DPPH assay, whereas in varieties 7 and 8, the decrease was 16.4 and 11.0%, respectively, in the ABTS⁺ assay. The differences between the DPPH and ABTS⁺ assays

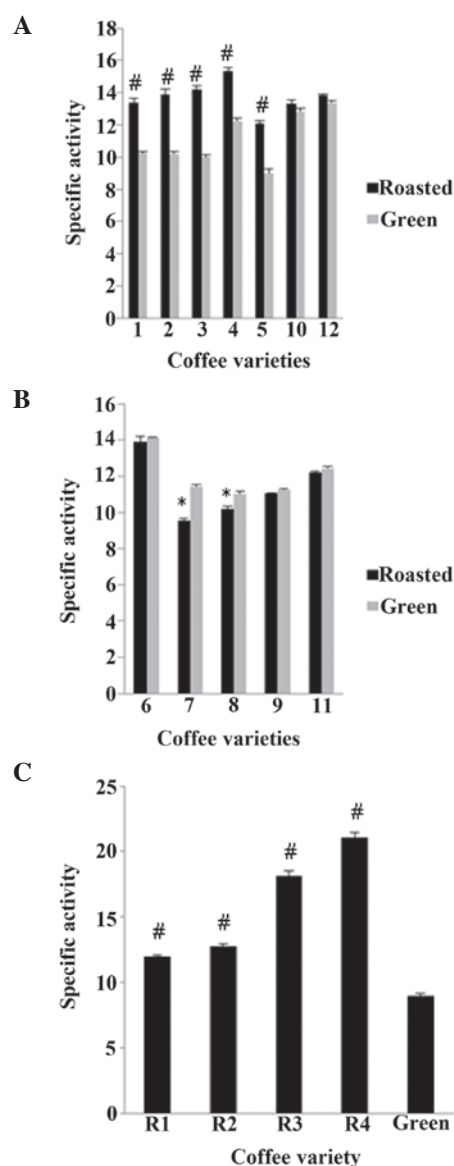


Figure 3. Antioxidant activity as assessed using ABTS⁺ assay. The specific activity of each variety is shown, measured in units of activity per mg of coffee extract. A unit represents the amount of polyphenols required to scavenge 50% of the ABTS⁺ radical. (A) Varieties of roasted seeds which exhibited higher levels of activity compared with the respective green beans. (B) Varieties of green beans which exhibited higher levels of activity. (C) Effects of roasting time on ABTS⁺ radical scavenging by variety 13. The roasting times were as follows: R1, 7 min 15 sec; R2, 6 min 5 sec; R3, 5 min 32 sec; R4, 3 min 52 sec. All data are expressed as the means $\pm$ SD. * $P < 0.05$ and # $P < 0.01$ indicated a statistically significant difference between roasted beans and the respective green beans.

may be ascribed to the varying reactivity of these 2 radicals to the components of each coffee sample. The fact that roasting increased the antioxidant activity in some samples and reduced it in others may be explained by the different phenolic composition of these varieties. It is well known that roasting greatly affects the chemical composition of the coffee beans due to the high temperatures used (28,36). For example, new compounds, such as melanoidins, are formed, which exhibit antioxidant activity, whereas other ingredients, such as CGAs are broken down (25,37).

Moreover, we noted that the results from both DPPH and ABTS⁺ assays significantly correlated with the TPC (Table I).

Table I. Correlation coefficient (r) between values of TPC, DPPH and ABTS^{•+} assays.

Assays	Correlation coefficient (r)
TPC-DPPH	0.647 ^a
TPC-ABTS ^{•+}	0.766 ^a
DPPH-ABTS ^{•+}	0.926 ^a

^aP<0.05 indicates a statistically significant difference. TPC, total polyphenolic content.

Specifically, the correlation coefficient (r) was 0.647 (P<0.01) between TPC and DPPH, 0.766 (P<0.01) between TPC and ABTS^{•+}, and 0.926 (P<0.01) between DPPH and ABTS^{•+} (Table I). The correlations between the TPC and both the free radical scavenging assays, DPPH and ABTS^{•+}, indicated that there was an association between the total amount of polyphenols and the antioxidant activity, that is, higher amounts of polyphenols led to enhanced potency. In addition, the correlation between DPPH and ABTS^{•+} suggests that the same compounds of the extracts are likely responsible for the scavenging of both free radicals.

For variety 13, there were 4 different roasted bean samples, and each one was roasted for a different amount of time at

215°C. The results revealed that the antioxidant activity of the beans was dependent on the roasting time (Figs. 2C and 3C). More specifically, for all 4 samples, the roasted beans exhibited significantly (P<0.01) higher levels of activity than the green ones, at roasting times of 7 min 15 sec (R1), 6 min 5 sec (R2), 5 min 32 sec (R3) and 3 min 52 sec (R4) by 32.8, 46.2, 87.9 and 99.6%, respectively for DPPH assay, and by 33.4, 42.4, 102.0 and 135.0%, respectively for ABTS^{•+} assay (Figs. 2C and 3C). The roasting conditions which were used for these beans are all within the range of those typically used when making coffee beverages. The considerable differences observed under varying roasting conditions are in accordance with those presented in other studies (36,38,39). In addition, an LC-MS analysis was performed to examine the effect of roasting on the levels of CGA. For this analysis, 2 samples from variety 13 were used: the green extract and a sample roasted for 3 min and 52 sec (R4) at 215°C. According to the results, CGA diminished from 2028.4 ppm in the green beans to 38.8 ppm in the roasted sample (Fig. 4). This difference denotes the importance of novel antioxidant substances that are formed during the roasting procedure (e.g., melanoidins). Specifically, although one of the most prominent antioxidant compounds in green beans is practically non-existent in the roasted ones, the latter exhibited higher levels of antioxidant activity. It has previously been reported that polyphenolic compounds can be incorporated into melanoidins during roasting, either as intact units or following their breakdown to simpler phenols (40).

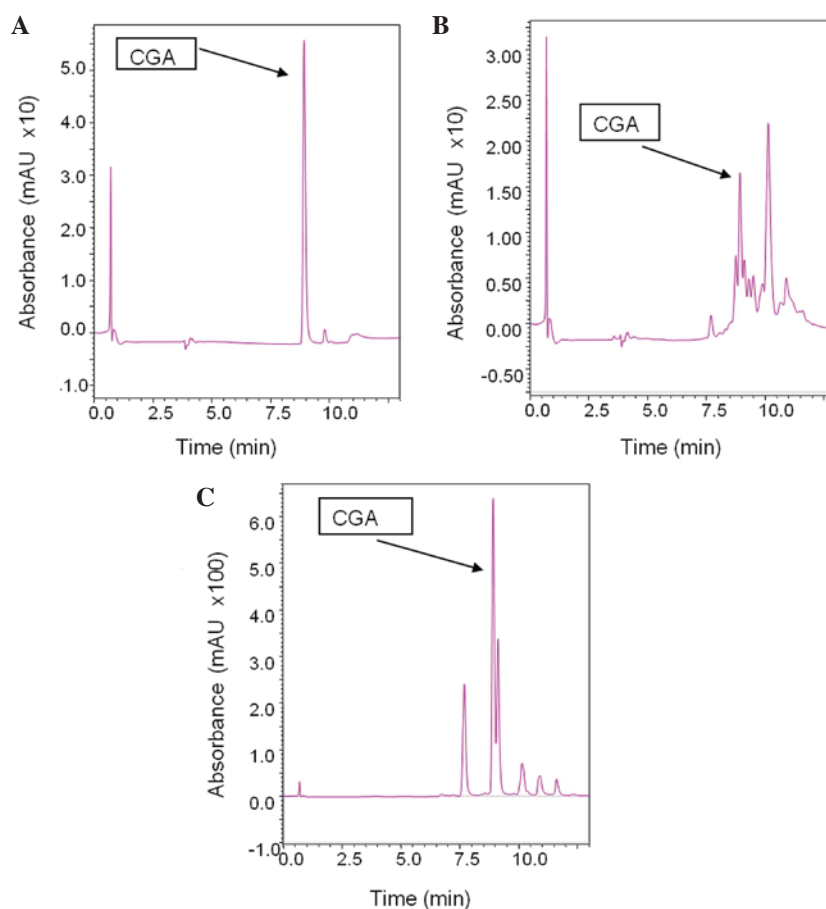


Figure 4. Liquid chromatography (LC)-mass spectrometry (MS) chromatograms of chlorogenic acid (CGA) of (A) standard solution at the concentration of 10 ppm, (B) roasted coffee at the found concentration of 38.8 ppm, and (C) green coffee at the found concentration of 2028.4 ppm.

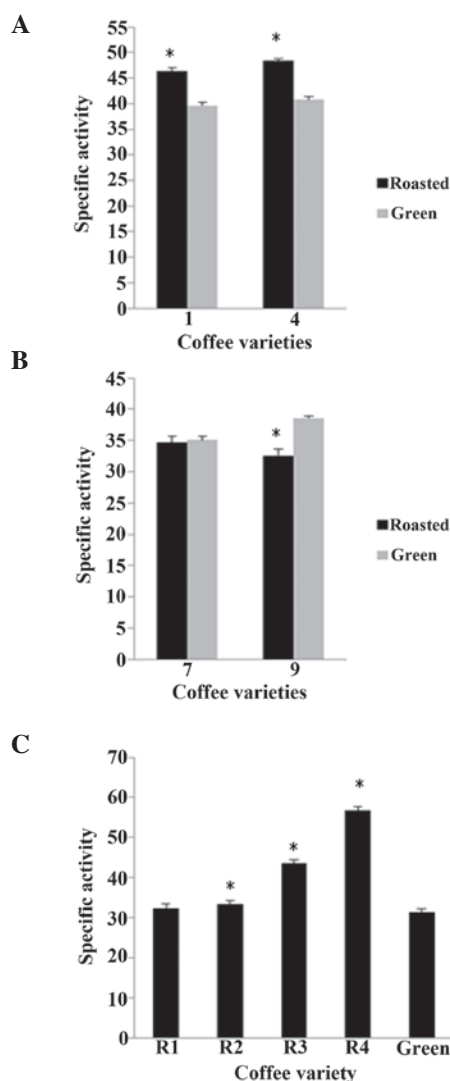


Figure 5. Protection from hydroxyl radical-induced DNA damage as assessed using a DNA plasmid strand cleavage assay. The specific activity of each variety is shown, measured in units of activity per mg of coffee extract. A unit represents the amount of polyphenols required to prevent plasmid relaxation by 50%. (A) Varieties in which roasted seeds which exhibited higher levels of activity compared with their respective green beans. (B) Varieties of green beans which exhibited higher levels of activity. (C) Effects of roasting time on protection from hydroxyl radical-induced DNA damage by variety 13. The roasting times were as follows: R1, 7 min 15 sec; R2, 6 min 5 sec; R3, 5 min 32 sec; R4, 3 min 52 sec. All data are expressed as the means $\pm$ SD. * P <0.01 indicated a statistically significant difference between roasted beans and the respective green beans.

To further investigate the antioxidant capacity of the coffee extracts, 2 assays assessing the protective effects of the extracts against ROS-induced DNA damage were carried out. For the ROO[•]- and OH[•]-induced DNA plasmid strand breakage assays, 5 varieties were selected, namely 1 and 4 (those varieties in which higher activity was observed for the roasted beans compared to the green beans in the DPPH and ABTS^{•+} assays), 7 and 9 (where the green beans were more potent than the roasted beans according to DPPH and ABTS^{•+} assays), and variety 13, in which the effect of the roasting time was tested (Figs. 5 and 6). These 2 methods yielded similar results with the DPPH and ABTS^{•+} assays. Specifically, in the case of OH[•], the roasted beans of varieties 1 and 4 exhibited higher

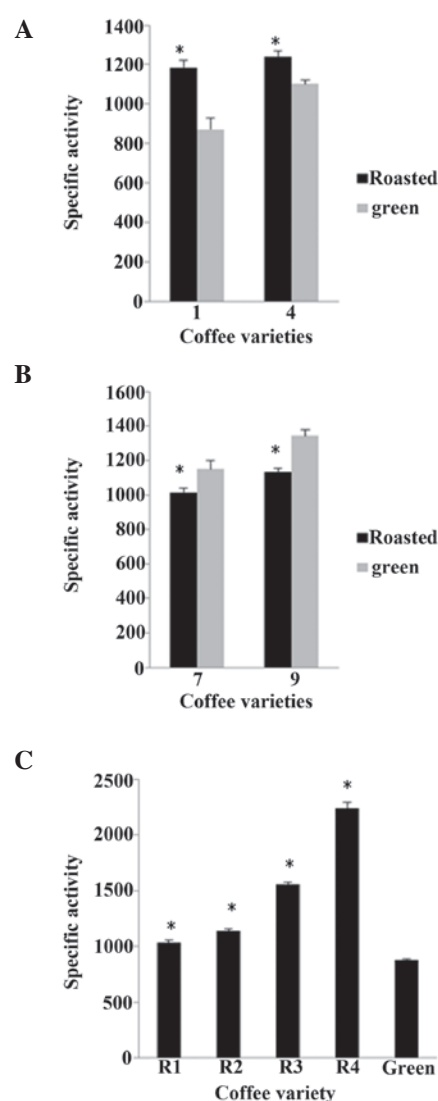


Figure 6. Protection from peroxyl radical-induced DNA damage as assessed using DNA plasmid strand cleavage assay. The specific activity of each variety is shown, measured in units of activity per mg of coffee extract. A unit represents the amount of polyphenols required to prevent plasmid relaxation by 50%. (A) Varieties of roasted seeds which exhibited higher levels of activity compared with their respective green beans. (B) Varieties of green beans which exhibited higher levels of activity. (C) Effects of roasting time on protection from peroxyl radical-induced DNA damage by variety 13. The roasting times were as follows: R1, 7 min 15 sec; R2, 6 min 5 sec; R3, 5 min 32 sec; R4, 3 min 52 sec. All data are expressed as the means $\pm$ SD. * P <0.01 indicated a statistically significant difference between roasted beans and the respective green beans.

specific activity by 17.0 and 19.0%, respectively, compared to the green beans, whereas in varieties 7 and 9 the green beans had a higher activity by 4.1 and 18.5%, respectively, than the roasted beans (Fig. 5A and B). Moreover, for variety 13, all 4 groups of roasted beans had significantly (P <0.05) higher activities than the green ones, by 3.2, 6.2, 39 and 81.1% for R1, R2, R3 and R4 time points of roasting, respectively (Fig. 5C). As for the assay using ROO[•], again in varieties 1 and 4 the roasted beans higher activities by 35.6 and 12.7%, respectively, compared to the green beans, whereas in varieties 7 and 9, the green beans exhibited higher levels of activity, by 13.2 and 18.4%, respectively, than the roasted beans (Fig. 6A and B). In addition, in variety 13 the roasted beans exhibited

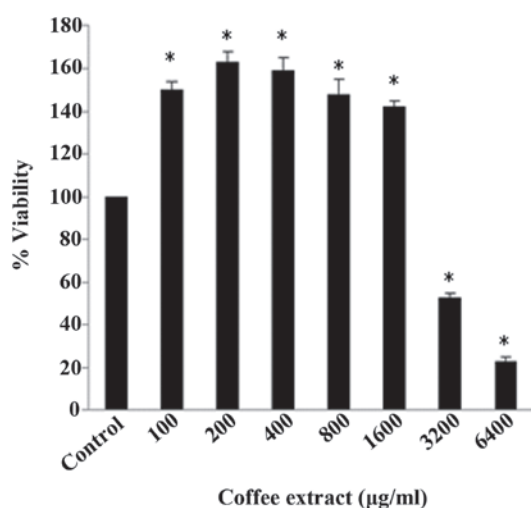


Figure 7. Assessment of effects of variety 13 roasted extract (roasting conditions: 3 min 52 sec; 215°C) on the viability of C2C12 murine myoblasts after 24 h treatment. Cytotoxicity was estimated via XTT assay. All data are expressed as the means $\pm$ SD. * $P < 0.01$ indicated a statistically significant difference compared to the control.

significantly higher levels of activity compared with the green ones, by 17.0, 29.1, 77.3 and 154.0% for R1, R2, R3 and R4 time points of roasting, respectively (Fig. 6C).

Thus, we noted that all the tested coffee extracts exhibited protective activity against free radical-induced DNA damage, with the most potent being the less roasted sample from variety 13 (R4). As shown in Figs. 5 and 6, the much lower specific activities observed against $\text{OH}^\bullet$ -induced DNA strand breaks may be due to the high reaction rate of $\text{OH}^\bullet$ with DNA, and thus it is more difficult for antioxidant molecules to exert their protective effects (41). To the best of our knowledge, this is the first study to report the protective effects of coffee extracts against DNA damage induced by $\text{OH}^\bullet$ and $\text{ROO}^\bullet$ radicals. However, other studies have been performed using different oxidants, and the oxidants in these coffee extracts also exerted a significant protective effect against mutagenesis (42). Specifically, coffee inhibited *tert*-butylhydroperoxide-induced mutagenicity in *Salmonella typhimurium* strains TA100 and TA102. This activity was partly attributed to cafestol and kahweol, two diterpenes commonly found in coffee (43,44).

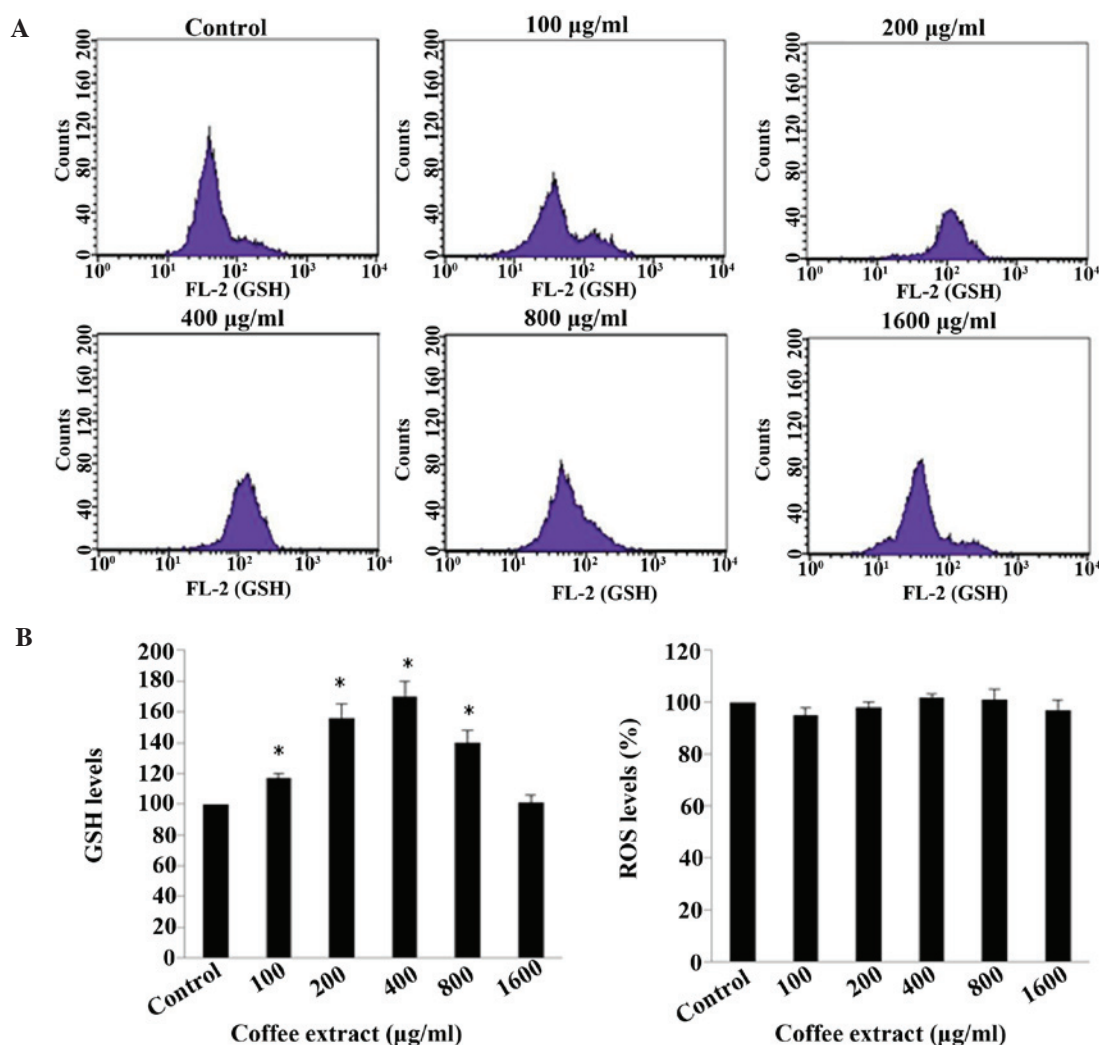


Figure 8. Effects of variety 13 roasted extract (roasting conditions: 3 min 52 sec; 215°C) after treatment for 24 h on glutathione (GSH) and reactive oxygen species (ROS) levels in C2C12 cells, as assessed by flow cytometry. (A) The histogram of cell counts versus fluorescence of 10,000 cells, as analyzed by the flow cytometer in order to detect GSH levels. FL-2 represents the detection of fluorescence using 488 and 580 nm as the excitation and emission wavelength, respectively. (B) Bar charts showing the GSH and ROS levels, as calculated by BD Cell Quest software. All results are expressed as the means $\pm$ SD. * $P < 0.05$ indicated a statistically significant difference between roasted beans and the control.

To summarize, all coffee extracts exhibited an antioxidant activity similar to that observed in previous studies by our research group on polyphenolic extracts; the antioxidant activity of the extracts was comparable to that of grapes (45) and pomegranates (unpublished data).

The most potent antioxidant extract [variety 13, roasted for 3 min and 52 sec at 215°C (R4)] was selected in order to examine its effects on the cellular redox status (specifically in C2C12 murine myoblasts) by assessing the GSH and ROS levels by flow cytometry. Non-cytotoxic concentrations were used (according to the results of the XTT assay) and, more specifically, the non-cytotoxic concentrations ranged from 100-1,600 µg/ml extract (Fig. 7). The results of flow cytometric analysis revealed that the GSH levels were increased significantly ($P<0.05$), by 17, 56, 70 and 40% at 100, 200, 400 and 800 µg/ml extract, respectively, compared to the control (Fig. 8). Although the extract increased the levels of the antioxidant molecule, GSH, the ROS levels were not significantly affected by the extract (Fig. 8). In previous studies of ours, we also found that the ROS levels are not always accompanied by changes in oxidative stress levels or antioxidant mechanisms (46,47). Moreover, the increase in the GSH levels was not linear; rather, the increase in the GSH levels peaked at 400 µg/ml and subsequently declined, and no increase at 1,600 µg/ml was noted, compared with the control. The observed decline in the GSH levels may be explained by the pro-oxidant activity of coffee extracts after reaching a certain concentration, as has been observed in relation to other plant polyphenolic extracts (48-51). Indeed, 1,600 µg/ml was a crucial concentration, since it was the highest concentration used which did not exert cytotoxic effects (Fig. 7). Thus, our results suggest that the tested coffee extract improved the cellular redox status by increasing the levels of GSH, one of the most important antioxidant molecules. Importantly, it has been previously reported that coffee extracts lead to protein localization of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein, a key transcription factor which is associated with antioxidant systems in HT-29 cells (52,53). Interestingly, one of the enzymes whose expression is regulated by Nrf2 is gamma-glutamylcysteine synthetase, the first enzyme in the biosynthetic pathway of GSH (54). However, when tested in humans, considerable inter-individual differences were observed in Nrf2 localization, suggesting that the effect of coffee extracts is genotype-dependent (55,56). However, as each coffee variety has a different chemical composition and thus performs a different activity, determining the potential of the coffee extracts used in our study to induce Nrf2 activity will be an intriguing task.

In conclusion, the findings of the present study indicated that coffee extracts from green or roasted beans exhibited potent free radical scavenging activity and also served to protect against the DNA damage induced by free radicals. Moreover, we noted differences in the levels of antioxidant activity between green and roasted bean extracts derived from the same variety. In some coffee varieties, bean roasting reduced antioxidant activity, whereas in others the opposite was noted. It appears that the final effect depends on the chemical composition of the beans of each coffee variety, but this hypothesis requires further investigation. In addition, roasting time was shown to affect the antioxidant activity of roasted coffee beans. This observation suggests that the roasting time should be

optimized in order to maintain the levels of antioxidant activity as high as possible. Finally, the coffee extract with the highest antioxidant activity (variety 13) was also shown to enhance the antioxidant mechanisms in myoblast cells by increasing GSH levels. Currently under way is a study in which cells treated with this extract are used in DNA microarray analysis, and this is being undertaken in order to examine its effects on whole genome expression, and thus investigate in depth the molecular mechanisms responsible for its antioxidant activity. Understanding the mechanisms through which coffee acts as an antioxidant will lead to improvements in the extraction and roasting processes and the ability to fully exploit its properties.

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