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Effect of decaffeination of green and roasted coffees on the *in vivo* antioxidant activity and prevention of liver injury in rats

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Article

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Abstract: Decaffeination and roasting affects the composition of the chlorogenic acids in coffee, which have antioxidant potential. The aim of this study was to evaluate the effects of coffee decaffeination on the *in vivo* antioxidant activity and the prevention of liver damage. The Wistar rats received intraperitoneal doses of carbon tetrachloride and daily doses of Arabica coffee brews (whole and decaffeinated, both green and roasted) by gavage for fifteen days. The activity of liver marker enzymes aspartate aminotransferase, alanine aminotransferase and serum albumin were measured as well as the quantification of the thiobarbituric acid reactive species and the content of liver total lipids. Aspartate aminotransferase and alanine aminotransferase are good indicators of liver damage: the results showed that all studied coffee brews decreased the activity of aspartate aminotransferase and alanine aminotransferase, and liver levels of thiobarbituric acid reactive species and total lipids. The compounds presents in coffee brews are able to decrease the hepatic lipid peroxidation induced by carbon tetrachloride, making a significant hepatoprotective effect, in accordance with the liver function tests. The coffee brews are hepatoprotective regardless of the decaffeination process and our results suggest a better protection against liver damage for the roasted coffee brews compared with green coffee brews.

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Introduction

Many studies have demonstrated that coffee, due to its antioxidant compounds, has protective effects in the liver against diseases such as cirrhosis and reduces the risk of developing hepatocellular carcinoma (Ozercan et al., 2006; Bravi et al., 2007; Muriel & Arauz, 2010; Masterton & Hayes, 2010; Carvalho et al., 2010; Shi et al., 2010), which is the most common form of cancer in the liver. Among the bioactive compounds investigated, we focused on the chlorogenic acids, melanoidins and caffeine.

Liver diseases are a public health problem worldwide. The evolution of liver disease begins with steatosis, hepatitis, fibrosis, cirrhosis following to hepatocellular carcinoma. Research has shown that reactive oxygen species are related to the cascade of events that regulate the initiation and the progression of liver disease, regardless of the agent that originated (Loguercio & Frederico, 2003; Vitaglione et al., 2004). Thus, the use of antioxidant therapies, drugs that interfere in the metabolism (CYP-450) and hepatotoxic agents that increase the activity of enzyme systems of defense are the options used in the study of hepatotoxicity (Weber et al., 2003; Porchezhian & Ansari, 2005; Lima et al., 2007).

The confirmation of liver damage is carried out through specific serological diagnostic tests, as determination of the enzymatic activity of transaminases: alanine aminotransferase (ALT) and aspartate aminotransferase (AST), enzymes cellular function whose presence in the blood is a consequence of defective release into the circulation.

The use of carbon tetrachloride (CCl_4) in experimental protocols helps to elucidate the mechanisms of hepatotoxicity and its consequences: inflammation, steatosis, hepatitis, fibrosis, cirrhosis and carcinogenesis (Schatzki, 1963; Weber et al., 2003; Lima et al., 2007).

The quest for coffee without caffeine has mobilized researchers around the world to meet the

increasing demand of people wanting to eliminate the side effects, such as insomnia, caused by this stimulant substance (Lima et al., 2010). Many studies have been conducted on the physiological effects of this substance, but there is not yet a consensus on the positive and negative aspects of the results of these studies (Corrao et al., 2001; Ruhl & Everhart, 2005; Masterton & Hayes, 2010; Chu et al., 2012). Decaffeination and roasting processes caused changes in the levels of important bioactive compounds, such as chlorogenic acids (Toci et al., 2006; Lima et al., 2010).

This study may contribute to a deeper understanding of this issue, as research on the use of decaffeinated coffees as preventives of liver damage in rats is scarce and is the main objective of this work.

Materials and Methods

Plant material

Samples of whole and decaffeinated coffee (blends of *Coffea arabica* L. grown in several areas of Brazil) were given by COCAM Industry, located in Catanduva-SP, Brazil. Decaffeination was performed using water and dichloromethane (Toci et al., 2006; Antonio et al., 2010). Samples of coffee (500 g) were roasted in a laboratory roaster (Probatino, Leogap model, Brazil), with a capacity of 1 kg, at the average degree of roasting. The tone of the final color of the grains was determined visually and with instruments (Chomameter-2 Reflectance, Minolta, Osaka, Japan). Next, the roasted beans were ground (Pinhalense electric grinder, ML-1, Brazil) into fine grains (20 mesh), packed in polyethylene/aluminum, sealed and stored at -20 °C until use. The green beans were finely ground in a mill cooled to 4 °C (Tecnal, model TE 631/2, Brazil) using liquid nitrogen (Lima et al., 2010).

Analysis of chlorogenic acid and caffeine by HPLC

For the determination of caffeine and chlorogenic acid levels in the coffee brews, a hot water extraction method was employed (Vitorino et al., 2001); the extraction was followed by dilution with 100 mL/0.5 g of distilled water and, finally, HPLC analysis with a Shimadzu Brand chromatograph (model M10AVP, Japan) equipped with a C-18 reverse-phase column (Shimadzu 100 mm long x 0.3 mm ID, 4,6 µm particle size, Japan). The HPLC was coupled to a UV/visible spectrophotometric detector (Shimadzu SPD-10A model) connected by an interface (CBM-101) to a microcomputer for data processing. The conditions of analysis used were as follows: flow (1 mL/min); mobile phase (methanol, water and acetic acid in a ratio of 20:80:1); injection volume was 20 µL and wavelength detection at 272 nm. The concentrations of the compounds were determined with standard concentration curves. Caffeine and 3-*O*-caffeoylquinic acid (chlorogenic acid, 3-CQA) were purchased from Sigma-Aldrich Chemical. Curves obtained had R^2 greater than 0,999.

Preparation of brews

A filter coffee brews was prepared according to the methodology described by Lima et al. (2010), 10 g of coffee powder were added in commercial filter paper, and then, 100 mL of deionized water at 90 °C were poured into the coffee contained in the filter. The coffee brews were always prepared at the time of administration to animals.

Experimental animals

The study included 24 male adult Wistar rats (*Rattus norvegicus*), which were divided into six equal groups. The animals were maintained at 22±2 °C, 55±10% humidity, with a 12-h light-dark cycle. The rats were fed commercial Nuvilab CR-1® (Nuvital Nutriente S/A, Colombo, PR, Brazil) 1®. To induce liver injury, CCl_4 was injected through an intraperitoneal route at a dose of 1.5 $mL.kg^{-1}$ (suspended with olive oil at a rate of 1:1) on days 11, 13 and 15 during the 15-day treatment.

The animals were divided into six groups of four animals each: Group 1, the negative control group (NC), was given water; Group 2, the positive control group (PC), was administered CCl_4 to induce liver injury, along with water; Group 3 was administered whole green coffee and CCl_4 (WGC); Group 4, decaffeinated green coffee and CCl_4 (DGC); Group 5, whole roasted coffee and CCl_4 (WRC) and Group 6, decaffeinated roasted coffee (DRC) and CCl_4.

The coffee brews were administered to the animals by gavage once daily for fifteen days and the dose used was 5.7 $mL.kg^{-1}$ per day, which is equivalent to an adult human drinking eight 50 mL cups of coffee brew (10%) per day.

On the sixteenth day, the animals fasted for 12 h, were anesthetized for the removal of blood by cardiac puncture, and immediately after, the rats were euthanized in a CO_2 chamber for liver removal. The blood was centrifuged to separate the serum. The livers were washed with 0.9% saline solution, stored at -20 °C and submerged in phosphate buffer (pH 7.4). The University Ethical Committee for Animal Research (Unifal, MG, Brazil) approved the protocols used in this study (protocol 155/2007).

Biochemical analysis: biochemical parameters in the serum and oxidative stress in the liver

The serum obtained by centrifuging the blood from the rats was kept under -20 °C until analysis. Alanine

aminotransferase (ALT), aspartate aminotransferase (AST) and albumin levels were determined by the colorimetric method.

The protein concentration of liver homogenate was determined by Bradford (1976), using bovine serum albumin (BSA) as the standard. An aliquot of 10 µL containing liver homogenate was added to 790 mL of distilled water and 200 mL of Coomassie Brilliant Blue G-250 in ethanol 95% w/v. Then, the optical density was measured at 595 nm in a spectrophotometer. Protein concentration (µg/10 µL) was calculated by inserting a calibration curve previously made with BSA (5-25 mg/mL).

To determine whether drinking coffee was able to reduce oxidative stress, we analyzed the lipid peroxidation of isolated rat livers. The lipid peroxidation was assessed by the formation of thiobarbituric acid reactive substances (TBARS) (Winterbourn et al., 1981). The lipid peroxidation products react with thiobarbituric acid to produce a compound that has an absorbance at 532 nm.

The livers were weighed and homogenized in a tissue homogenizer in an ice bath after the addition of 0.1 M PBS, pH 7.4 (with a volume equal to four times the weight of fresh tissue). The homogenate was centrifuged at 3000 x *g* for 10 min at 4 °C, and the supernatant that was kept on ice was used in the tests. Aliquots of 500 mL of the supernatant were mixed with 500 mL of hydrochloric acid 25% (v/v), 500 mL of thiobarbituric acid 1% (w/v in 0.05 M NaOH) and 45 µL of BHT 2% (w/v in ethanol).

The mixture was heated at 100 °C in a water bath for 10 min. After cooling in an ice bath for 10 min, 1.5 mL of butanol as added, and the samples were shaken vigorously. The samples were then centrifuged at 900 x *g* for 5 min, and the fraction containing butanol was collected and used to determine the absorbance at 532 nm. The TBARS concentration was calculated from the standard curve of malonic dialdehyde (MDA; tetraetoxipropano 1,1,3,3). The results were expressed as nmol MDA/g protein.

Determination of total liver lipids

Total liver lipids were measured according to the methodology described by AOAC (1995) with slight modifications. The livers were lyophilized in a lyophilizer Liobrás (L101) and crushed in a mortar; 1 g of the resulting powder was placed into cartridges cellulose, and lipids were extracted with diethyl ether for 6 h in a Soxhlet apparatus. The process is based on the weight loss of the material submitted to extraction with ether or the amount of material solubilized by the solvent. The results were expressed as a percentage of the lipids in relation to the total matter.

Statistical analysis

The values obtained for the chemical composition of the coffee samples were analyzed by one way ANOVA followed by post hoc Tukey test. A global analysis of variance was made with all the *in vivo* treatments, with the aim to obtain the residue average square to test the factorial and to apply Dunnett's test at 5% to compare the control treatment (negative and positive) with the others. The F test was used to test treatments in a factorial way.

Results and Discussion

The results of the HPLC analysis are presented in Table 1. Bioactive compounds, such as chlorogenic acid (CGA) and caffeine were identified by comparison of the sample retention times with standards. The amount of each compound present was quantified using an external standard calibration. The retention times for the chlorogenic acid and caffeine standards, were 9 and 10 min, respectively.

Table 1. Concentrations of chlorogenic acid (CGA) and caffeine in the aqueous extracts of whole green coffee (WGC), decaffeinated green coffee (DGC), whole roasted coffee (WRC) and decaffeinated roasted coffee (DRC) (g/100 g of ground).

	CGA	Caffeine
WGC	4.95±0.043[a]	0.92±0.031[a]
DGC	1.95±0.041[b]	0.03±0.008[b]
WRC	0.40±0.017[c]	0.93±0.009[a]
DRC	0.38±0.008[c]	0.04±0.005[b]

*Values are expressed as mean±SD (n=3). Means within a column, followed by different letters (a,b,c) are significantly different by Tukey's test, $p<0.05$.

The CGA content was reduced with the decaffeination process, with losses of 60% in the decaffeinated green sample. The decrease must have occurred mostly due to isomerization of the CGA when exposed to high temperatures for solvent evaporation and drying of the beans during decaffeination with dichloromethane (Toci et al. 2006).

The roasting led to losses of 92% in levels of CGA, which is consistent with other studies where decaffeinated and whole commercial coffees showed a loss of up to 93% after roasting (Fujioka & Shibamoto, 2008). During the roasting of the beans, part of chlorogenic acids is transformed into other substances that are formed during this process, and are related to color and flavor of coffee (Farah et al., 2005).

The results showed the stability of caffeine after roasting, although small caffeine losses occur by sublimation at 178 °C (Toci et al., 2006). The caffeine content found in whole green coffee is in agreement with

values reported in the literature for green Arabica coffee beans -0.9 to 1.3 g% (Ramalakshmi & Raghavan, 1999; Toci et al., 2006; Lima et al. 2010). The caffeine content in the decaffeinated green sample (0.03%) is in accordance with Brazilian law, which sets a level of 0.1% caffeine in decaffeinated coffee (Anvisa, 1999).

Biochemical analyses

Acute administration of carbon tetrachloride causes centrilobular necrosis and steatosis, a fact confirmed in this work, because their intra-peritoneal injection caused liver injury in rats, which is evident from the significant difference in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) between the negative and positive control groups. The results of AST and ALT are respectively shown in Tables 2 and 3.

Table 2. Concentration of the enzyme aspartate aminotransferase, AST (U/L) in the groups treated with the whole or decaffeinated samples of coffee (green and roasted), and the control groups.

Type of coffee	Processing		Mean
	Green	Roasted	
Whole Coffee	248.05*†	259.22*†	253.63
Decaffeinated Coffee	243.47*†	244.88*†	244.18
Mean	245.76	252.05	
Negative control	108.19		
Positive control	327.49		
CV (%)#	5.75		

*differs from the positive control by Dunnett's test (p<0,05); †differs from the negative control by Dunnett's test (p<0,05); #coefficient of variation.

Table 3. Concentration of the enzyme alanine aminotransferase, ALT (U/L) in the groups treated with the whole or decaffeinated samples of coffee (green and roasted), and the control groups.

Type of coffee	Processing		Mean
	Green	Roasted	
Whole Coffee	141.24*†	141.91*†	141.57
Decaffeinated Coffee	144.57*†	145.39*†	144.98
Mean	142.91	143.65	
Negative control	39.68		
Positive control	233.0		
CV (%)#	9.11		

*differs from the positive control by Dunnett's test (p<0,05); †differs from the negative control by Dunnett's test (p<0,05); #coefficient of variation.

The group treated with carbon tetrachloride (PC) showed AST and ALT levels significantly higher than the group treated with water (NC). In all of the groups treated with coffee, there was a significant decrease in the levels of AST and ALT when compared with the PC group (treated with carbon tetrachloride). However, these rates did not reach their baseline values (the negative control group). Among the groups treated with the different coffee brews, there was no significant difference, which demonstrates that regardless of the process of decaffeination and roasting, the coffee brews protected the liver from injury caused by carbon tetrachloride.

A study from Ozercan and coworkers (2006) reports the effects of coffee on liver damage caused by carbon tetrachloride which are consistent with the results of this study. They found levels of AST and ALT in the group treated with carbon tetrachloride to be significantly higher than in the control group. In this same work, the observed levels of AST and ALT in the group treated with carbon tetrachloride over coffee were significantly lower than in the groups treated with carbon tetrachloride (p<0.05) (Ozercan et al., 2006).

Albumin is the most abundant protein in the blood plasma, constituting approximately 50 to 65% of the total protein. Albumin is synthesized in the liver, and the most decisive factor for its concentration in the blood is the liver's ability to synthesize it. The accumulation of fat causes a reduction in the hepatic synthesis capacity of this body, reducing the concentration of albumin (Nicoluzzi et al., 2000). The concentrations of albumin are presented in Table 4.

Table 4. Serum albumin (g/dL) in the groups treated with the whole or decaffeinated samples of coffee (green and roasted), and the control groups.

Type of coffee	Processing		Mean
	Green	Roasted	
Whole Coffee	4.77*†	5.22*†	5.00
Decaffeinated Coffee	4.90*†	5.24*†	5.07
Mean	4.84[b]	5.23[a]	
Negative control	5.73		
Positive control	4.27		
CV (%)#	2.09		

*differs from the positive control by Dunnett's test (p<0,05); †differs from the negative control by Dunnett's test (p<0,05); #coefficient of variation. Means within a line, followed by different letters ([a, b]) are significantly different by Tukey's test (p<0,05).

The positive control group showed significantly lower albumin content than the negative control group, indicating that the carbon tetrachloride decreased the levels of serum albumin in rats. Tetrachloride-treated groups that were given coffee drinks had significantly higher values of albumin than the positive control group and had lower values than the negative group control.

The serum albumin levels in the groups treated with roasted coffee (WRC and DRC) were significantly

higher than the groups treated with unroasted coffee (WGC and DGC). These results show that roasted coffee offered greater protection compared with the green coffee. The process of decaffeination did not influence the levels of serum albumin.

The presence of xenobiotics in the liver, such as carbon tetrachloride, caused injury and a consequent accumulation of lipids in the liver, which led to hepatic steatosis (Ramakrishna et al., 2011). The results were expressed as a percentage of fat in relation to the total matter and are presented in Table 5.

Table 5. Concentration of total lipids in the liver (%) in relation to the total matter in the groups treated with the different coffee samples and the control groups.

Type of coffee	Processing		Mean
	Green	Roasted	
Whole Coffee	4.06*†	4.08*†	4.07
Decaffeinated Coffee	4.77*†	3.91*†	4.34
Mean	4.42	4.00	
Negative control	2.53		
Positive control	7.34		
CV (%)#	12.69		

*differs from the positive control by Dunnett's test (p<0,05); †differs from the negative control by Dunnett's test (p<0,05); #coefficient of variation.

The total lipid content of the livers in the groups treated with carbon tetrachloride was significantly higher than that in the negative control group (treated with water), indicating that xenobiotics elevate liver lipid levels. The groups treated with carbon tetrachloride in conjunction with the coffee brews exhibited a decrease in lipid levels compared with the positive control group. There was no significant difference among the coffee drinks. The coffee drinks, regardless of the type and the processing, prevented the accumulation of fat in the liver but did not return to the baseline values found in the negative control group.

Substances capable of combating oxidative stress are able to protect the liver from improper fat accumulation (Ramakrishna et al., 2011), thus the antioxidants present in the coffee brews may have contributed to the decreased accumulation of hepatic fat.

Lipid peroxidation is a major consequence of liver injury caused by CCl_4 and is mediated by the production of free radicals derived from CCl_4. However, antioxidant activity and the inhibition of free radical generation are important in the protection against liver damage caused by carbon tetrachloride (Teselkin et al., 2000; Campo et al., 2001; Ramakrishna et al., 2011).

The inhibition of lipid peroxidation was calculated as the concentration of thiobarbituric acid reactive substances (TBARS). The levels of MDA in the different groups are presented in Table 6.

All samples demonstrated inhibition of lipid peroxidation, and roasting was a positive influence in this inhibition. The decaffeination process did not influence lipid peroxidation.

Table 6. Content of thiobarbituric acid reactive substances (nmol MDA/mg protein) in the groups treated with the different samples of coffee and the control groups.

Type of coffee	Processing		Mean
	Green	Roasted	
Whole Coffee	0.290*†	0.240*†	0.265
Decaffeinated Coffee	0.295*†	0.230*†	0.263
Mean	0.293[a]	0.235[b]	
Negative control	0.148		
Positive control	0.378		
CV (%)#	12.22		

*differs from the positive control by Dunnett's test (p<0,05); †differs from the negative control by Dunnett's test (p<0,05); #coefficient of variation. Means within a line, followed by different letters (a, b) are significantly different by Tukey's test (p<0,05).

The coffee brews that were analyzed have phenolic compounds, mostly chlorogenic acids, which may contribute, at least in part, to the antioxidant activity demonstrated in this study. Moreover, the complexation of Fe^{+2} to phenolic compounds can reduce the availability of the metal involved in the Fenton reaction in the initiation and propagation of lipid peroxidation (Lima et al., 2006).

Since the content of chlorogenic acid was reduced by decaffeination, other substances may have been formed during the roasting, that acted synergistically with the phenolic compounds for the inhibition of lipid peroxidation observed in the coffee brews (Lima et al., 2010). Several authors have suggested that the Maillard reaction products, which are formed during the roasting of the coffee, have antioxidant activity and may have contributed to the observed results (López-Galilea et al., 2006).

Furthermore, hydroxyl radical scavenging characteristics of highmolecular weight melanoidin-like compounds are stronger than those of low-molecular weight phenolic compounds (Delgado et al., 2005).

Conclusion and perspectives

Regardless of the decaffeination process, the decaffeinated coffee brews protected the rat livers, no significant differences were observed between whole and decaffeinated brews. Our results suggest a better protection against liver damage caused by carbon tetrachloride for the roasted coffee brews compared with green coffee brews.

More studies are needed to determine the mechanism(s) of effect of coffee and its bioactive

compounds on liver metabolism. Because of the mixture of bioactive components present in the coffee, it is possible that more than one mechanism underlying this effect is involved.

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Authors' contributions

ARL, SAA contributed to chromatographic analysis running the laboratory work. ARL, SAA, FBAP and SMSD contributed to biological studies analysis. MGZ undertook the statistical analysis and interpretation of data. ARL, SAA, RGFAP, FBAP and SMSD designed the study, analysis of the data and drafted the manuscript. RGFAP, FBAP and SMSD supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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