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Article

Anticholinesterase and Pro-dopaminergic Effects of Instant Coffee

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Abstract: Epidemiologic studies suggest an inverse correlation between coffee consumption and the occurrence of neurodegenerative diseases, but the role of caffeine and roasting degree are still matter of debate. The objective of this work was to evaluate the effects of caffeinated (light, medium, and dark roast) and decaffeinated instant coffee samples in acetylcholinesterase inhibition and antioxidant assays, as well as in animal models of Parkinson's disease. Caffeinated coffees inhibited the acetylcholinesterase in much smaller concentrations than decaffeinated coffee. All coffee samples showed antioxidant capacity without relation with the caffeine content. Dopaminergic-like activity in the haloperidol-induced catalepsy test was observed with caffeinated coffee, but not in the decaffeinated sample. The medium roast coffee reduced the number of rotations of rats after methamphetamine administration on the 6-hydroxydopamine unilateral lesion of the medial forebrain bundle. However, the coffee treatment did not avoid the loss of dopaminergic neurons on substantia nigra pars compact and only the smallest dose of coffee was able to avoid the decrease of dopamine levels in the lesioned side of the striatum. Altogether, these results suggest that coffee exerts moderate pro-cholinergic and pro-dopaminergic effects and caffeine seems to be the main responsible for these effects.

Keywords: *Coffea arabica*; antioxidant; acetylcholinesterase inhibition; catalepsy; unilateral 6-hydroxydopamine lesion; caffeine

1. Introduction

Coffee is one of the most popular beverages worldwide, exhibiting a complex source of multiple bioactive constituents, including alkaloids, diterpenes, chlorogenic acids, among others [1-2]. Epidemiologic studies suggest an inverse correlation between regular coffee consumption and neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD) [3-6]. PD is characterized mostly by the loss of dopaminergic neurons from the substantia nigra pars compacta and by α-synuclein deposition, in the form of Lewis bodies [7]. Clinical symptoms include akinesia or bradykinesia, impaired balance, postural changes, and in many cases, cognitive impairment. AD is mainly characterized by accumulation of amyloid-beta peptide (neuritic plaques) and neurofibrillary tangles with progressive degeneration of cholinergic neurons in neocortex, limbic system, and several other brain areas [8] leading to progressive loss of cognitive functions. Oxidative stress and the accumulation of reactive oxygen species are considered important factors associated with AD and PD development [9], which makes the use of antioxidants a potential strategy to reduce the risk of such diseases.

The supposed neuroprotective activity of coffee is attributed mainly to caffeine, a competitive adenosine receptor antagonist. There is a close structural and functional

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association between adenosine and dopamine receptors in some areas, especially in the basal ganglia, where adenosine negatively modulates the dopaminergic system [10], making the use of adenosine antagonists a relevant strategy for PD. Besides acting as an adenosinergic inhibitor, caffeine has also shown acetylcholinesterase inhibition [11], which makes this compound relevant to AD therapy, since cholinesterase inhibitors are the main class of drugs used in the treatment of AD. Furthermore, the possible neuroprotective effect of other coffee constituents cannot be ignored.

The current medicines used in PD and AD are able to improve the quality of life, but cannot avoid the progressive neuron degeneration, making it important to identify potential protective factors such as diet or life style, that could reduce the risk of developing such neurodegenerative diseases, or drugs which could avoid the disease progression. The present study aimed to evaluate the prophylactic/therapeutic potential of decaffeinated and caffeinated instant coffee samples submitted to different roasting degrees, assessing their antioxidant and anticholinesterase activities *in vitro* and their effects on experimental models of PD.

2. Materials and Methods

2.1. Drugs

Coffee bean water-soluble extracts were purchased from Nestlé®, in four different presentations (light, medium and dark roast coffee and decaffeinated coffee). Caffeine, rutin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), acetylcholinesterase, acetylthiocholine iodide, thiobarbituric acid, trichloroacetic acid, and 6-hydroxydopamine (6-OHDA) were purchased from Sigma (St. Louis, USA). Apomorphine was obtained from Tocris (St. Louis, USA) and methamphetamine was donated by the Brazilian Federal Police (the purity grade of 99% was confirmed through the HPLC-DAD). Haloperidol (Haldol®, Janssen-Cilag), rivastigmine (Exelon®, Novartis), anesthetic, antibiotic and anti-inflammatory drugs were acquired in local pharmacies.

2.2. Phytochemical profile and caffeine quantification

Quantitative analysis of caffeine contents in coffee samples was performed by means of HPLC analysis using a photodiode array detector (HPLC- PDA). The linearity test was performed by injecting the caffeine standard in ethanol with concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 0.9, and 1.0 mg/mL, respectively, into the HPLC system. Evaluation of each point was conducted in triplicates and the calibration curve was fitted by linear regression, performed by plotting the peak height (mAU) against concentration (mg/mL). The HPLC-PDA analyses were conducted on a Hewlett Packard 1090 II equipped with a degasser, an autosampler, and a photodiode array detector. The caffeine standard solutions were filtered with a 0.45 μm polytetrafluoroethylene (PTFE) filter, prior to injection of 31.2 μL into the HPLC system, using a reverse-phase, C18, Spherisorb ODS II (Hewlett Packard) column (4.6 × 250 mm, 5 μm), connected to a guard column. The mobile phases consisted of eluent A (0.1% aq. formic acid) and eluent B (methanol), and the gradient profile was: 0 min –20% B in A; 10 min – 30% B in A, 20 min – 50% B in A; 30 min – 70% B in A; 40 min– 90% B in A; 45 min – 40% B in A and finally returned to the initial conditions (20%B) to re-equilibrate the column prior to another run. The flow rate was kept constant at 1.0 mL/min, and the temperature of the column was maintained at 30°C. The chromatograms were recorded at 270 nm. Caffeine content was calculated with the linear regression equation y = bx + a from the standard calibration curve. The quantification of caffeine in each soluble coffee (1 mg/mL) was conducted in triplicates using the same conditions used for the construction of the calibration curve for caffeine, which was positively identified comparing the retention time and UV-spectrum [12].

The coffee samples were also analyzed by HPLC-ESI-MS/MS in order to detect the main coffee compounds. Spectral UV data from all peaks were collected in the spectral range 240 – 400 nm, and chromatograms were recorded at 270 nm (caffeine) and 330 (caffeoylquinic acid derivatives and feruloylquinic acids derivatives) [13].

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2.3. Animals

Male Swiss mice 3-4 months old (30-50 g) and male Wistar rats 2 months old (250-400 g) were provided by Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME) from Universidade Federal de São Paulo (UNIFESP). Animals were kept in rooms with controlled temperature (23 ± 2 ºC) and 12 hours light/dark cycle (light set on 7 am), with water and food *ad libitum*, and the procedures were performed during the morning period. All procedures were made under the approval of the University's ethical committee (#1637/09) in accordance with the Brazilian Law for Procedures for Animal Scientific Use.

2.4. Antioxidant assays

2.4.1. DPPH radical scavenging activity

The radical scavenging activity was measured using the DPPH radical according to the method described by Duarte-Almeida et al. [14]. Forty microliters of each coffee extract, at initial concentrations of 25, 50, 100, 200, and 400 µg/mL were incubated in microplate with 250 µL of DPPH methanolic solution at room temperature for 25 minutes. The DPPH solution without extract was used as control and rutin (6.25 - 100 µg/mL) as a standard antioxidant. All experiments were carried out in triplicates. The absorbance (A) was measured in a spectrophotometer (SpectraMax M2, Molecular Devices, San Jose, CA, USA) at 517 nm and the scavenging activity was calculated using the formula: $(\%_{inhibition}) = [(A_{control} - A_{sample})/ A_{control}] \times 100$. The concentration that scavenges 50% of DPPH (EC_{50}) was obtained by linear regression using the mean percentage of scavenging for each concentration from three independent assays.

2.4.2. Lipid peroxidation assay

The lipid peroxidation was measured by malondialdehyde concentration in health rat brain homogenate [15]. Rat brain was mixed with phosphate buffer pH 7.4 (1:4 weight/volume) in an Ultra-turrax (Ika) homogenizer and centrifuged at 3000 rpm for 15 minutes. The supernatant was diluted 1:3 with phosphate buffer and 50 µL of each coffee extract at initial concentrations of 125, 250, 500, 750, and 1000 µg/mL or 50 µL of water (control) were added to 3 mL of homogenate. Butylhydroxytoluene (6.25 - 100 µg/mL) was used as a standard antioxidant. A sample of 1 mL of each mixture was incubated at 37 °C for 60 min, while another sample was kept at room temperature. After 60 minutes, 1 mL of 5% trichloroacetic acid was added to all samples, which were centrifuged at 3000 rpm for 15 minutes. Then, 1 mL of thiobarbituric acid (6.7 mg/mL) was added to 1 mL of the supernatant for each sample and the mixture boiled for 20 minutes. After cooling, the absorbance was read in a spectrophotometer Beckmam DU 70 (Beckmam Instruments, Pasadena, USA) at 535 nm. The lipid peroxidation inhibition was determined between the incubated and non-incubated samples, according to the formula: $(\%_{inhibition}) = [1-(\Delta A_{sample}/\Delta A_{control})] \times 100$, where ΔA is the difference between the absorbance of incubated and non-incubated samples. The concentration that inhibits 50% of lipid peroxidation (IC_{50}) was obtained by linear regression using the mean percentage of inhibition for each concentration from three independent assays.

2.5. Acetylcholinesterase inhibition assay

Acetylcholinesterase (AchE) inhibition was evaluated *in vitro* according to Ellman's method with minor modifications, as described in Martins et al. [16]. Twenty microliters of coffee extracts at initial concentrations of 0.25, 0.5, 1, 2, 4, and 8 mg/mL or caffeine (0.1, 0.25, 0.35, 0.5, and 1 mg/mL) were pipetted in triplicate to a microplate containing 160 µL of DTNB (3.3 mM) in phosphate buffer pH 7.0. Then, 10 µL of AchE 1 U/mL was added to each well and the mixture was incubated at 37 °C for 5 minutes, when the basal absorbance was measured at 412 nm in a spectrophotometer (BioTek Instruments, Winooski, USA). Finally, 10 µL of the substrate acetylthiocholine iodide (20 mM) was added to the mixture and the absorbance was monitored at 412 nm for 20 minutes.

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Phosphate buffer, used to solubilize the samples, was used as control, and rivastigmine (0.03 - 1 mg/mL) was used as a standard drug. The inhibition percentage was calculated according to the formula: $(\%_{inhibition}) = [1-(\Delta A_{control} - \Delta A_{sample}/\Delta A_{control})]$ x100, where ΔA is the difference between final absorbance and basal absorbance. The concentration that inhibits 50% of acetylcholinesterase activity (IC_{50}) was obtained by linear regression using the mean percentage of inhibition for each concentration from four independent assays.

2.6. Haloperidol-induced catalepsy

Groups of 11 mice received haloperidol (5 mg/kg, ip), 30 minutes after the oral administration of water (control group) or coffee extracts (dark roast, medium roast, or decaffeinated coffee at doses of 50, 150, and 500 mg/kg). The negative control group was pretreated orally with water and then received saline (ip) instead of haloperidol. Cataleptic status was evaluated after 45 minutes of the haloperidol administration. Each mouse was placed with both front paws over bars suspended 5 cm from the surface and the time to remove the paws was counted, up to 10 consecutive trials or up to 10 minutes [17]. The catalepsy score was obtained dividing catalepsy total time (seconds) by the number of times the mice left the bar.

2.7. Unilateral 6-hydroxydopamine lesion

2.7.1. Surgery

Rats deeply anesthetized with ketamine (90 mg/kg, ip) and xylazine (13 mg/kg, ip) were placed in the stereotaxic apparatus and 12 µg of 6-OHDA (6 mg/mL, solubilized in saline with 0.2% ascorbic acid) were injected at a rate of 0.4 µL/min in the right medial forebrain bundle at the following coordinates: -1.9mm AP; -1.9mm ML; -7.2mm DV, calculated from the bregma according to the Atlas of Paxinos and Watson [18]. Saline was injected in negative control animals. After surgery, dental wax was applied on the skull to close the access hole, the head skin was sutured, and the animals were allowed to recover in individual boxes with controlled temperature and with food and water available.

2.7.2. Rotational behavior

Groups of 14-15 rats received oral administration of water (controls), medium roast, and decaffeinated coffee (50, 150, and 500 mg/kg) for 45 consecutive days, initiating 30 days before the 6-OHDA infusion. The medium roast coffee was selected for this experiment because the profile of action was similar for both caffeinated coffees and it was observed that this sample possesses more balanced amounts of caffeine and caffeoylquinic acids. Rotational behavior was accessed on the 14th day after surgery with the administration of apomorphine (0.1 mg/kg, sc) and on the 15th day, after administration of methamphetamine (2.5 mg/kg, ip). The number of complete (360°) contralateral and ipsilateral rotations was counted by a blind observer for one hour after the administration of each drug [17]. The treatment with coffee on the test days was made after the behavioral observation and the animals were euthanized approximately 24h after the last treatment.

2.7.3. Immunohistochemistry

Four rats from each group were randomly selected for the immunohistochemistry study. Approximately 24h after the behavioral test the animals were anesthetized with urethane (3000 mg/kg, ip) and then euthanized by intra-cardiac perfusion with 1% heparin solution in saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were dissected and stored at –80 °C until the moment they were sectioned. Sets of sections equally distributed throughout the anteroposterior brain regions were processed on a cryostat (Leica Biosystems, Buffalo Grove, USA) at 40 µm and collected into 24 well-microplates containing anti-freezing solution and stored at –20 ºC.

Free-floating immunohistochemistry was performed employing mouse monoclonal anti-tyrosine hydroxylase antibody diluted 5000X in 0.005% bovine serum albumin. The sections were then incubated with the secondary antibody and streptavidin-conjugated

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peroxidase (Dako K0690) and stained with 3-3'-diaminobenzidine (Dako K3468) according to the manufacturer's protocol. After that, the slices were transferred to gelatin-coated slides and kept at room temperature for 24h, when they were dehydrated with ethanol gradient and xylol and finally mounted with Entellan and coverslips.

The slides were screened to identify the sections where the substantia nigra pars compact (SNpc) was clearly separated from the ventral tegmental area by the medial terminal nucleus of the accessory optic tract. The selected sections were photographed using a Nikon DS-Ri1 digital camera coupled to a Nikon 80i microscope and analyzed using the software Nikon Niss-Elements F 3.0 and Carl Zeiss Axio Vision 4.8. Later, the images were independently analyzed by two blind investigators using ImageJ software (National Institute of Health, USA). The number of tyrosine hydroxylase immunoreactive (TH-positive) cells in each side was counted and the result was expressed as a relative percent in the lesioned side compared to the intact side for each section [19].

2.7.4. Determination of monoamine levels by HPLC

Eight to ten rats from each group were randomly selected to the dosage of central monoamines by HPLC. Animals were anesthetized as described in the previous session, intracardiacally perfused with PBS and the fresh brains were frozen at -80°C. Later, the right (lesioned) and left (control) striatum were dissected on a cold surface, weighed, and homogenized by ultrasonic probe using 500 µL of extraction solution (0.1 M perchloric acid containing 0.4 mM sodium metabisulfite and 0.2 mM ethylenediaminetetraacetic acid). The homogenates were centrifuged at 20,000 g for 15 min and filtered in a nitrocellulose membrane. Precipitates were processed and the protein concentration determined by colorimetric assay according to the manufacturer's kit (Pierce Chemical, Rockford, USA).

The quantification of monoamines and their metabolites was performed by HPLC according to the method described by Machado et al. [20]. Each sample was analyzed in duplicate for concentrations of dopamine (DA), noradrenaline (NE), serotonin (5-HT), and their metabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindolacetic acid (5-HIAA). The recovery rate of the analytes was determined by adding a predetermined amount of internal standard dihydroxybenzylamine for the homogenization of tissue. The peak areas of samples were compared with peak areas of standards of each neurotransmitter or metabolite, which were injected at known concentrations, at intervals between runs (see representative chromatograms in the supplementary figure S1).

2.8. Statistical analysis

Data was analyzed using the GraphPad Prism software. The EC_{50} and IC_{50} from the *in vitro* tests were calculated by linear regression using the mean percentage of inhibition for each concentration tested. Data from the behavioral tests, immunohistochemistry, and monoamine quantification were initially submitted to Shapiro-Wilk's normality test and Kolmogorov-Smirnov normality test. Behavioral (catalepsy, rotation) and immunohistochemistry data were found to be non-parametric and were analyzed by Kruskal-Wallis, followed by Mann-Whitney test. The monoamine levels were first analyzed by Kruskal-Wallis (inter group comparison) while the intragroup comparison (left vs right side) was made by Unpaired t test with Welch's correction for standard deviation, when necessary. Statistical significance was set at $p<0.05$.

3. Results

3.1. Phytochemical profile and caffeine quantification

The calibration curve for caffeine ($y = 837.8x + 45.0$) was found to be linear with $R^2 = 0.99$. The content of caffeine detected in the four samples of soluble coffee was the following: decaffeinated coffee: 0.1%; light roast: 9%; medium roast: 5.4%, and dark roast: 8.4%. The chromatographic profiles for each coffee sample are available at the supplementary

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figure S2. We observed a similar qualitative profile for the three caffeinated samples with small differences in the proportion of the constituents detected. Figure 1 show the chromatographic profile of medium roast coffee using HPLC-PDA-ESI-MS/MS in positive and negative ionization mode (the chromatographic profile of the light roast, dark roast and decaffeinated coffee samples are available at the supplementary figure S3). The main compounds identified were caffeic acid, 3-*O*-caffeoylquinic acid, caffeine, 5-*O*-caffeoylquinic acid, 3-*O*-feruloylquinic acid, 4-*O*-caffeoylquinic acid and 5-*O*-feruloylquinic acid.

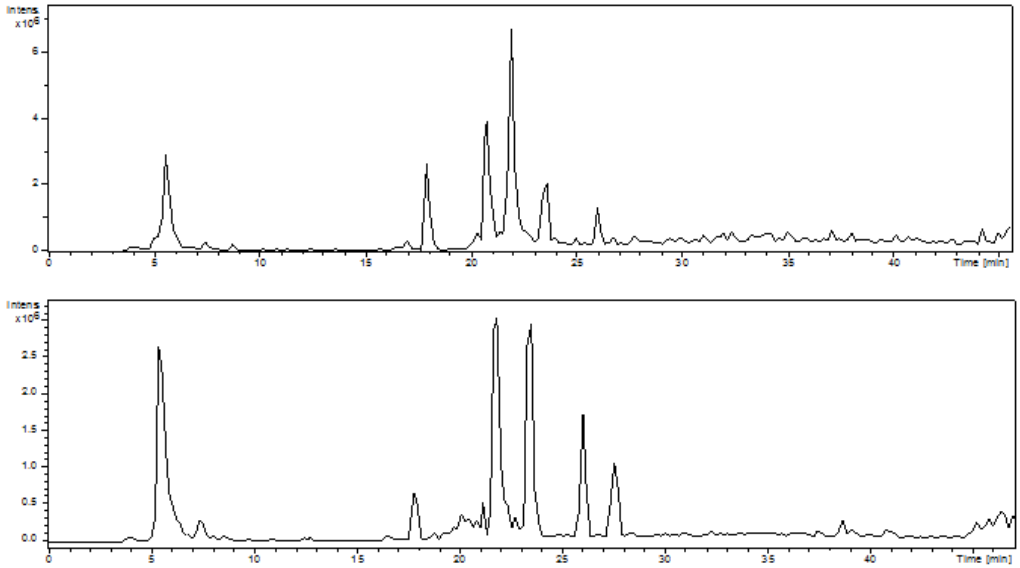

Figure 1. Chromatographic profile obtained by HPLC-ESI-MS/MS in positive ionization mode (upper panel) and negative ionization mode (lower panel) for the medium roast coffee. The main compounds detected were caffeic acid (5.5 min), 3-O-caffeoylquinic acid (17.5 min), caffeine (20.5 min), 5-O-caffeoylquinic acid (chlorogenic acid) (21.8 min), 3-O-feruloylquinic acid (23.5 min), 4-O-caffeoylquinic acid (26.0 min) and 5-O-feruloylquinic acid (27.5 min). Caffeine was not detected in negative ionization mode and 5-O-feruloylquinic acid was not detected in positive ionization mode.

3.2. Antioxidant assays

All coffee samples showed reducing power towards the free radical DPPH and inhibited the lipid peroxidation (Table 1). Dark roast coffee presented the strongest scavenging activity on DPPH assay (smallest IC_{50}), while light roast, medium roast, and decaffeinated coffee had similar values. On lipoperoxidation assay, the medium roast coffee presented the strongest antioxidant activity, followed by light roast, dark roast, and decaffeinated coffee (Table 1). Rutin and BHT, used as positive controls, presented typical antioxidant activity in both tests.

3.3. Acetylcholinesterase inhibition assay

The light roast coffee had the highest inhibitory effect on AchE, followed by dark roast, medium roast coffee, and decaffeinated coffee (Table 1). Caffeine presented IC_{50} = 20.54 μg/mL and rivastigmine, used as positive control, IC_{50} = 22.2 μg/mL.

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Table 1. Effective (EC_{50}) or inhibitory (IC_{50}) concentration 50% found for coffee extracts and positive controls in two antioxidant assays and in acetylcholinesterase inhibition assay, obtained by linear regression. Caffeine was not evaluated in the antioxidant assays. The values were obtained from triplicates and are expressed in μg/mL.

Sample	DPPH radical scavenging (EC_{50}, μg/mL)	Lipid peroxidation (IC_{50}, μg/mL)	Acetylcholinesterase inhibition (IC_{50}, μg/mL)
Light roast coffee	29,42	6.01	366
Medium roast coffee	29.81	5.86	402
Dark roast coffee	25.95	6.92	381
Decaffeinated coffee	29.81	7.92	578
Caffeine	-	-	20.5
Standard drug	11.3 (rutin)	0.53 (BHT)	22.2 (rivastigmine)

BHT = butylhydroxytoluene

3.4. Haloperidol-induced catalepsy

Haloperidol-induced catalepsy was evaluated in mice pre-treated with coffee or water (controls). Kruskal-Wallis test revealed that there was significant difference among the groups [H(10,119)=36.59; p<0.0001]. Medium roast and dark roast coffee (150 and 500 mg/kg) were able to block the cataleptic status induced by haloperidol (p<0.05), while decaffeinated coffee and lower doses of caffeinated coffees did not change the catalepsy score significantly (Figure 2).

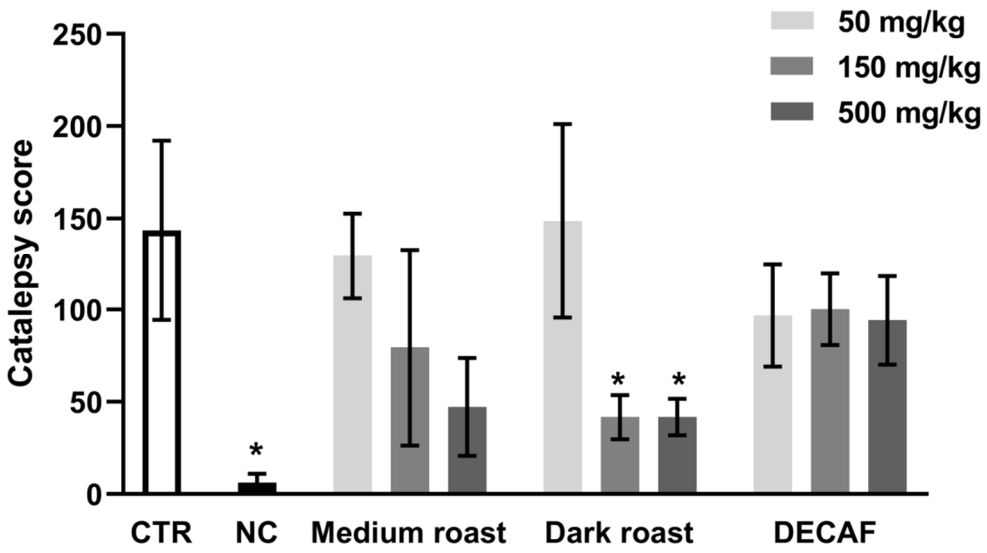

Figure 2. Catalepsy score in mice treated orally with water (CTR, control), 50, 150, and 500 mg/kg of medium roast, dark roast or decaffeinated (DECAF) coffee extracts. All animals received haloperidol (5 mg/kg, ip) 30 min after the pre-treatment, except the negative control group (NC), injected with saline. Results presented as mean ± standard error of mean. *p<0.05 in comparison to control (Kruskall-Wallis / Mann-Whitney, n=11).

3.5. Unilateral 6-hydroxydopamine lesion

3.5.1. Rotational behavior

Rats treated with medium roast and decaffeinated coffee were compared with lesioned control and negative control animals. Kruskal-Wallis test revealed significant difference among the groups on contralateral [H(7,105)=15.20; p=0.0335] and ipsilateral [H(7,105)=30.33; p<0.0001] rotations. As expected, lesioned control rats presented contralateral rotations induced by apomorphine and ipsilateral rotations induced by

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methamphetamine, while the negative control group did not show the rotational behavior (Figure 3). None of the treatments altered the rotational behavior induced by apomorphine when compared with the control group (Figure 3A). On the other hand, the highest dose of medium roast coffee (500 mg/kg) decreased significantly (p<0.05) the number of rotations induced by methamphetamine when compared with the control group (Figure 3B).

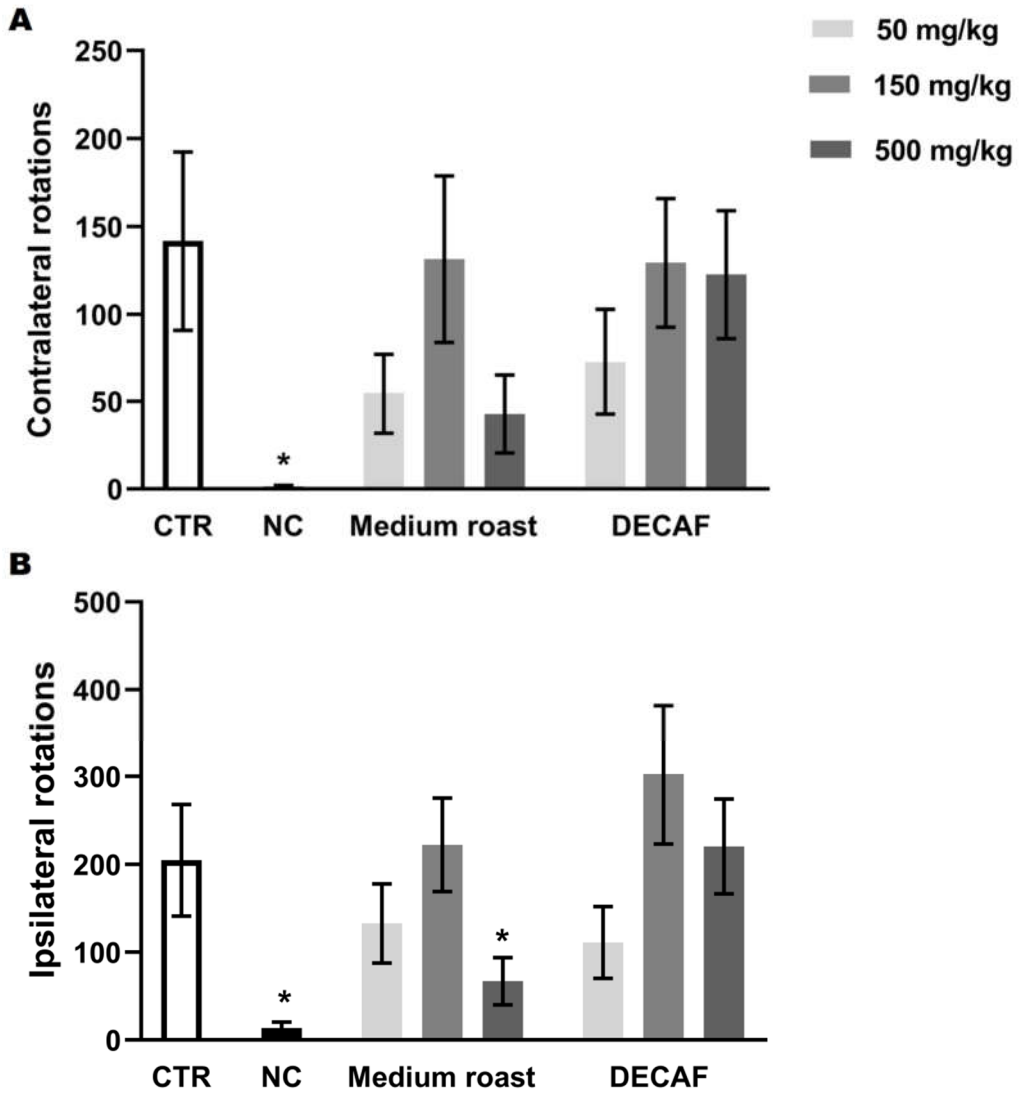

Figure 3. Number of rotations induced by (A) apomorphine (14th day after surgery) and (B) methamphetamine (15th day after surgery) in rats treated orally with water (CTR, control), 50, 150, and 500 mg/kg of medium roast or decaffeinated (DECAF) coffee extracts. The neurotoxin 6-OHDA was injected in the right nigrostriatal pathway of the animals, except that of negative control group (NC), injected with saline. Results presented as mean ± standard error of mean. *p<0.05 in comparison to control (Kruskall-Wallis / Mann-Whitney, n=12-15).

3.5.2. Immunohistochemistry

The effect of 6-OHDA infusion into the medial forebrain bundle was evaluated by the number of TH-positive neurons in the right (ipsilateral, lesioned side) in relation to the left (contralateral, intact side) substantia nigra. The 6-OHDA administration induced a reduction of 50% in the number of TH-positive neurons in the ipsilateral SNpc on the control group (Figure 4). The same pattern was observed for the treated groups, which presented a reduction of TH-positive neurons ranging from 40 to 67%. Rats that received

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saline infusion instead of 6-OHDA (negative control group) presented a small reduction (20%) of TH-positive cells in the ipsilateral side compared to the contralateral side. However, Kruskal-Wallis did not reveal significant difference among the groups [H(7,25)=9.751; p=0.2031].

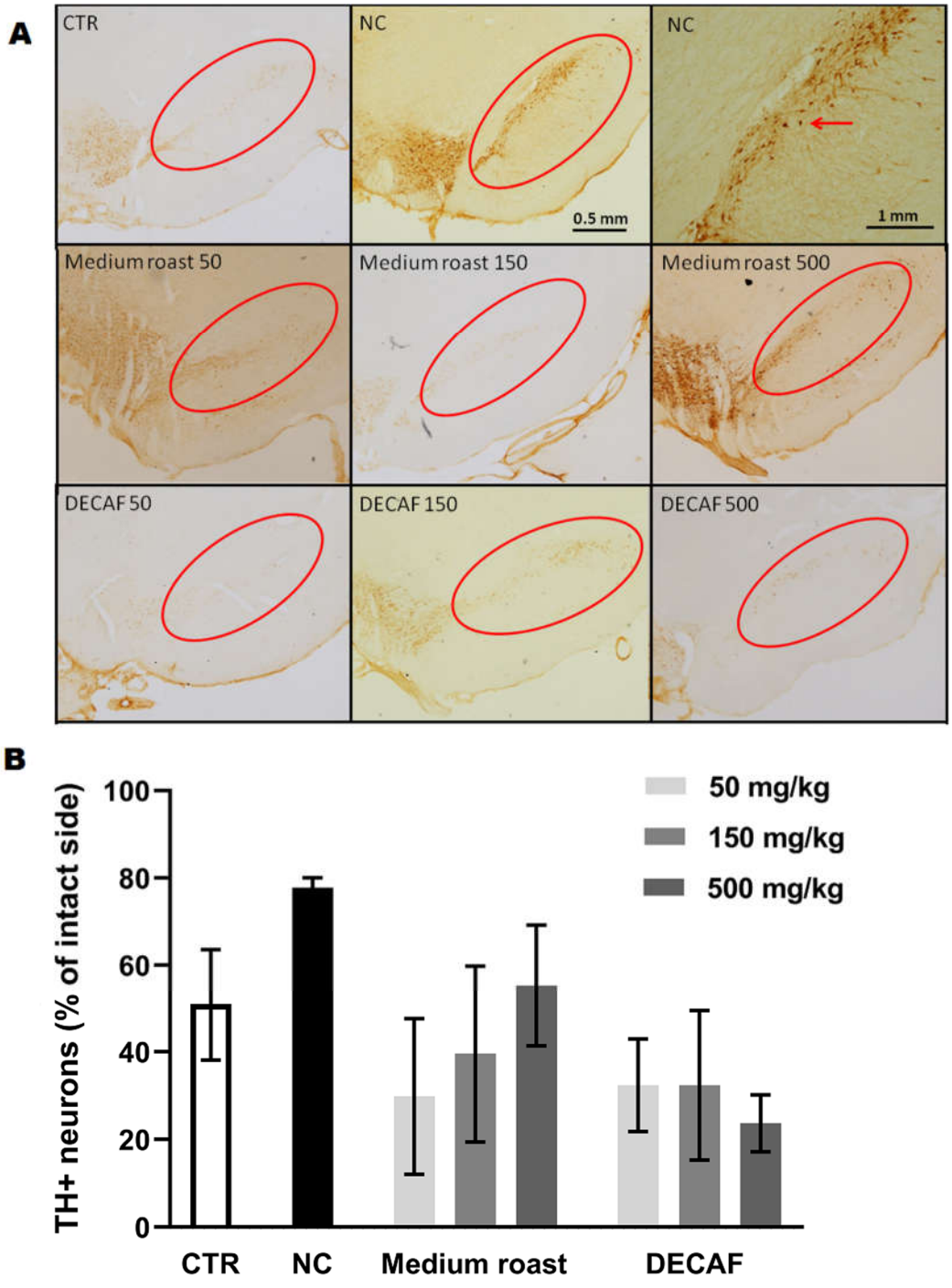

Figure 4. Tyrosine-hydroxylase (TH) positive cells marked in immunohistochemistry. (A) Representative photomicrographs of the right substantia nigra showing the areas analyzed (circles) and a TH-positive cell (arrow). (B) Percentage of TH-positive cells on the right (lesioned) substantia nigra compared to the left (intact) side in rats treated orally with water (CTR, control), 50, 150, and 500 mg/kg of medium roast or decaffeinated (DECAF) coffee extracts. The neurotoxin 6-OHDA was injected in the right nigrostriatal pathway of the animals, except that of negative control group (NC), injected with saline. Results presented as mean ± standard error of mean. (Kruskal-Wallis, n.s., n=4).

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3.5.3. Determination of monoamine levels by HPLC

The levels of monoamines and the main metabolites were determined on the right and left striatum of rats submitted to 6-OHDA lesion and in negative control animals. There was no difference among the groups as the content of the monoamines and metabolites analyzed in the left, intact striatum (Figure 5). Kruskal-Wallis test revealed significant difference among the groups in the content of DA [H(7,65)=14.79; p=0.0388] and NE [H(7,65)=14.66; p=0.0406] on the right striatum (lesioned side), but no differences were found on post-test comparisons. On the other hand, the infusion of 6-OHDA induced reduction of dopamine level on the right side compared to the left side (intra-group comparison) on groups control [t(16)=2.830; p=0.0121], medium roast coffee 150 mg/kg [t(14.27)=5.126; p=0.0001] and 500 mg/kg [t(17)=3.571; p=0.0024], decaffeinated coffee 50 mg/kg [t(15.82)=3.704; p=0.0020], 150 mg/kg [t(10.91)=5.949; p=0.0001], and 500 mg/kg [t(10.99)=2.849; p=0.0158] (Figure 5A). The levels of dopamine metabolites (DOPAC and HVA) were also diminished in several groups (Figures 5C and 5D). There was no difference on noradrenaline (Figure 5B), serotonin (Figure 5E), and 5-HIAA (Figure 5F) levels between left and right striatum in any group.

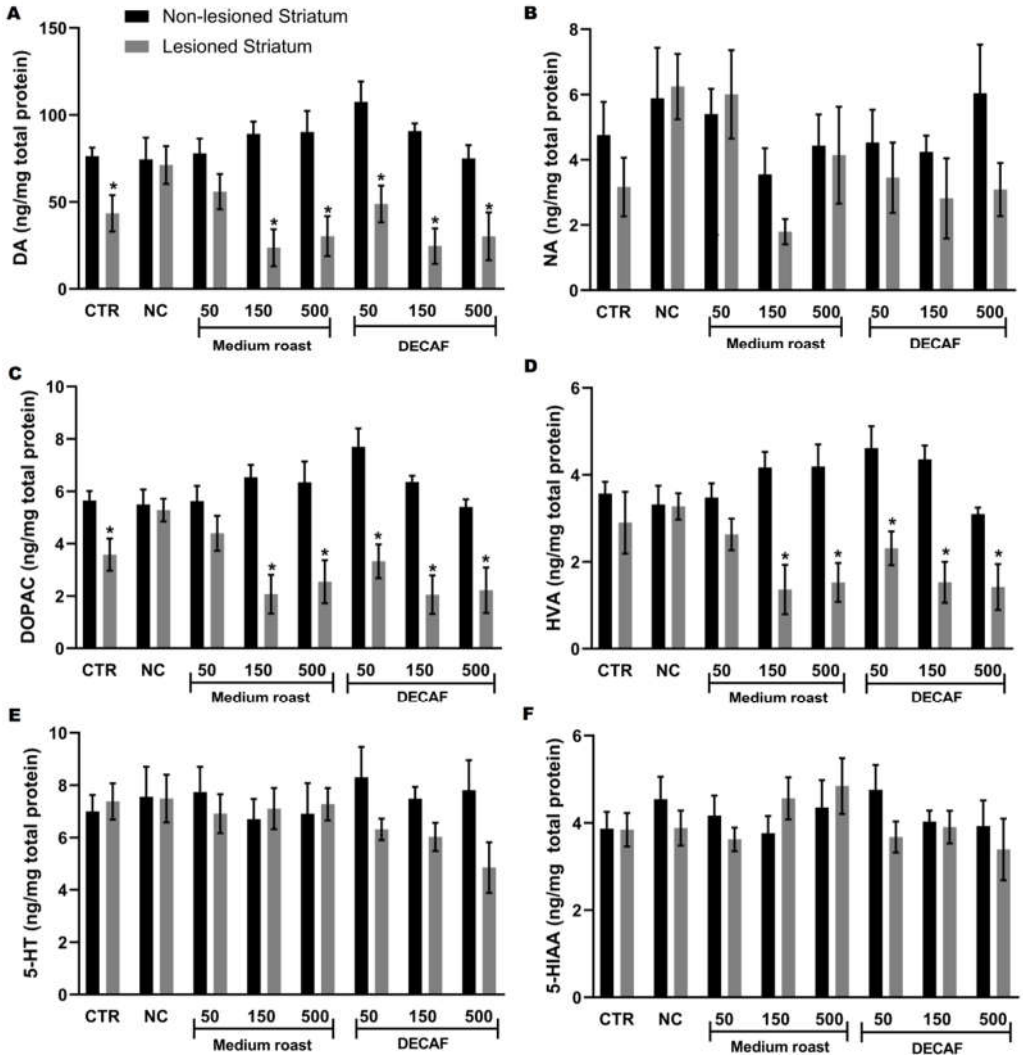

Figure 5. Levels of monoamines and their metabolites (ng/mg of total protein) in the left (non-lesioned) and right (lesioned) striatum of rats with unilateral lesion of the nigrostriatal pathway treated orally with water (CTR, control), 50, 150, and 500 mg/kg of medium roast or decaffeinated (DECAF) coffee extracts. The neurotoxin 6-OHDA was injected in the right MFB of the animals, except on negative control group (NC), injected with saline. (A) dopamine (DA), (B) noradrenaline (NE), (C) dihydroxyphenylacetic acid (DOPAC), (D) homovanillic acid (HVA), (E) serotonin (5-HT), (F) 5-hydroxyindolacetic acid (5-HIAA). Results presented as mean ± standard error of mean.

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*p<0,05, in comparison to the non-lesioned side (Unpaired t test with Welch's correction, when necessary, n=8-10).

4. Discussion

Observational studies and meta-analysis suggest a positive effect of coffee and other caffeinated beverages in neurological conditions, such as PD and AD [3-6,21-22]. Experimental studies support the neuroprotective role of caffeine and other coffee constituents in biochemical and animal models [23-28]. Our study assessed the potential prophylactic/therapeutic effects of instant coffee extracts on PD by different approaches, including antioxidant activity, pro-dopaminergic activity, and neuroprotection, as well as their inhibitory effect on acetylcholinesterase, which may be relevant for cognitive functions both in PD and AD.

First, we considered the influence of the roasting process and caffeine content in two *in vitro* antioxidant assays. The four coffee samples used in our study presented slight differences in the phytochemical profile, with the light roast and dark roast coffees having higher concentrations of caffeine, while decaffeinated coffee had only trace amounts. All tested coffee extracts presented quite similar antioxidant capacity, which could not be associated with the content of caffeine. Dark roast coffee presented the strongest DPPH scavenging activity, while the medium roast coffee presented the strongest antioxidant activity in the lipid peroxidation assay. The present data corroborates previous studies that show that the antioxidant activity in coffee is not related to the roasting degree, but mainly with the phenolic content. The origin, harvesting, processing, and preparation of the coffee extracts influence the content of caffeine, chlorogenic acids, melanoidins, and many other constituents and as result in the total antioxidant capacity [29].

The three samples of caffeinated coffee inhibited the AchE *in vitro*, which the order of potency (IC_{50}) related to the caffeine concentration: the light roast coffee (9% of caffeine) presented the strongest activity, followed by dark roast (8.4%) and medium roast coffee (5.4%). This result suggests that caffeine is important for the AchE inhibition, although other compounds in decaffeinated coffee also exert an inhibitory effect on AchE, because decaffeinated coffee also showed AchE inhibition in higher concentrations. A recent study observed a moderate correlation between the total phenolic content and AchE inhibition in extracts of roasted coffee, confirming that caffeine is the most active compound, but other components may contribute to the final effect [30]. Acetylcholinesterase inhibitors are used to improve the cognition and quality of life of AD patients and have also shown beneficial effects on the cognitive symptoms of PD [31].

It was suggested that coffee intake attenuates the risk of AD or related cognitive decline by reducing pathological cerebral amyloid deposition [32]. Caffeine was shown to reduce the production of beta-amyloid (Aβ) in experimental models [33] and it also protected mice against α-synucleinopathy [34] suggesting that this alkaloid may exert prophylactic effect against PD and AD. On the other hand, Mancini et al. [27] observed that phenylindanes formed in the roasting process showed a potent inhibitory effect of Aβ and tau fibrillization and Aβ oligomerization, while caffeine alone had no effect. It is important to highlight that these mentioned studies employed different methods and in general the literature supports that caffeine and other coffee components have synergistic effects.

The possible therapeutic effect of caffeine on PD is mainly attributed to its antagonism on the adenosinergic system, which in turn modulates the dopaminergic system [9]. Our results on the catalepsy test suggest that the presence of caffeine is essential to produce behavioral changes. The two caffeinated coffee samples tested were able to dose-dependent inhibit the cataleptic status induced by haloperidol, while the decaffeinated coffee had no effect. Drugs that can inhibit or reverse the cataleptic status induced by haloperidol are considered as possible treatments against the motor impairment in PD. Several adenosinergic antagonists, including caffeine, have shown effect at this animal model [35-36]. In a previous study, we demonstrated that a green coffee extract, rich in chlorogenic acid, was able to inhibit the haloperidol-induced catalepsy [17].

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To better investigate the coffee potential as neuroprotective agent in PD, we used the unilateral 6-OHDA lesion model. Coffee treatment began 30 days before the infusion of 6-OHDA and it continued for another 15 days. Thus, we could assess whether the previous coffee administration would exert some neuroprotection (its prophylactic effect), as it is supposed to occur in humans. The highest dose of medium roast coffee was able to decrease significantly the number of rotations induced by methamphetamine, which promotes ipsilateral rotation due to greater release of dopamine and reuptake blockade in the intact side. This dose of coffee also reduced the apomorphine-induced contralateral rotations in 69%, although it was not statistically significant. None of the other treatments changed the rotational behavior of rats after methamphetamine or apomorphine challenge.

Our results on the rotational model suggest that the highest dose of medium roast coffee reduced the unbalance between the lesioned and intact side. The turning behavior is influenced by the lesion extension and density of dopaminergic receptors in the intact and lesioned sides [37]. A minor change in the density of neurons is more easily accessed by methamphetamine, which can induce rotations with 50% of neuronal loss while apomorphine rotation requires 90% [38]. In fact, our protocol induced 50% of neuronal loss in the damaged SNpc of control animals compared to the intact side. The treatment with medium roast and decaffeinated coffee did not avoid the loss of dopaminergic neurons, but medium roast coffee at 500 mg/kg (the dose which reduced the metamphetamine-induced ipsilateral rotations) showed 22% more TH-positive neurons on the damaged side than the control group.

The 6-OHDA lesion also induced diminished levels of DA and its metabolite DOPAC in the lesioned side compared with the intact side in control animals and in the groups medium roast (150 and 500 mg/kg) and decaffeinated coffee (50-500 mg/kg). Interestingly, rats treated with the smallest dose of medium roast coffee (50 mg/kg) presented similar levels of DA and their metabolites in the striatum of both sides. Despite the fact that we did not use a tricyclic drug to inhibit the noradrenaline and serotonin transporter, we did not observe statistical differences on NE and 5-HT levels when comparing left and right striatum for all groups.

Previous studies have observed a neuroprotective effect of caffeine in the 6-OHDA experimental model [24,39]. It is important to notice that these two studies employed intraperitoneal injection of caffeine and induced striatal lesion. Instead, our protocol tried to mimic the nigrostriatal degeneration as it occurs in the early phase of PD and we administered coffee by oral route starting before the lesion in order to simulate the regular coffee consumption. Machado-Filho et al. [40] observed that caffeine (10-20 mg/kg) given orally for two weeks moderately reduced the contralateral rotations induced by apomorphine in 6-OHDA lesioned rats, but the authors did not evaluate the rotational behavior after amphetamine treatment.

Other studies using different neurotoxins and protocols also suggested the neuroprotective effect of caffeine in PD models [23,26,41-42]. In addition, many other coffee components have shown neuroprotective effects in *in vitro* assays and experimental models of PD. Among them, it is worth mentioning the chlorogenic acid [25], caffeic acid [43], and eicosanoyl-5-hydroxytryptamide [26,28]. Taking together, these data support that regular use of coffee is beneficial for brain health, as demonstrated by experimental and observational studies.

5. Conclusions

Our study brings additional evidence of the dopaminergic effect of coffee and its potential to the treatment or prevention of PD. The observed blockade of haloperidol-induced catalepsy and the decrease in the number of rotations in 6-OHDA lesion model suggest that coffee may indeed modulate the dopaminergic system. The antioxidant capacity observed suggests that coffee compounds are important to prevent the accumulation of reactive oxygen species and to inhibit cell damages, while the acetylcholinesterase

inhibitory activity can contribute to attenuate cognitive deterioration in patients suffering of PD and AD. Taking together, the data obtained in this study support the assumption that coffee exerts pro-cholinergic and pro-dopaminergic effects and caffeine seems to be the main responsible for these effects, although the contribution of other constituents cannot be rejected.

Supplementary Materials: Figure S1: Chromatograms obtained in the HPLC analysis for the standard monoamines/metabolites (A) and typical chromatograms of striatum from intact side (B) and lesioned side (C) of rats with 6-OHDA unilateral lesion of right medial forebrain bundle; Figure S2: Chromatographic profile obtained by PDA multi – 270 nm for caffeine (standard, panel A – at 20.5 min), light roast (B), medium roast (C), dark roast (D), and decaffeinated coffee (E); Figure S3: Chromatographic profile obtained by HPLC-ESI-MS/MS in positive ionization mode (left) or negative ionization mode (right) for light roast (panel A), dark roast (B), and decaffeinated coffee (C). The main compounds detected were caffeic acid (5.5 min), 3-*O*-caffeoylquinic acid (17.5 min), caffeine (20.5 min), 5-*O*-caffeoylquinic acid (21.8 min), 3-*O*-feruloylquinic acid (23.5 min) and 4-*O*-caffeoylquinic acid (26.0 min). The 5-*O*-feruloylquinic acid was not detected in positive ionization mode and caffeine was not detected in negative ionization mode.

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