Anti-inflammatory and Antinociceptive Activities of Decaffeinated Coffee

Maria Eliza de Castro Moreira1, Rosemary Gualberto Fonseca Alvarenga Pereira2, Daniele Ferreira Dias3, Claudinei Alves da Silva4, Thiago Corrêa de Souza5, Éderson D’Martin Costa6, Bárbara Pereira da Silva1*, Stella Maris da Silveira Duarte6, Alexandre Giusti-Paiva7, Marcelo Henrique dos Santos8

1Faculdade Dinâmica do Vale do Piranga, Ponte Nova, Brazil
2Department of Food Science of Federal University of Lavras, Lavras, Brazil
3Institute of Chemistry of Federal University of Alfenas, Alfenas, Brazil
4Federal University of Jataí, CIEX, Jataí, Brazil
5Institute of Natural Sciences of Federal University of Alfenas, Alfenas, Brazil
6Faculty of Pharmaceutical Sciences of Federal University of Alfenas, Alfenas, Brazil
7Department of Biomedical Science of Federal University of Alfenas, Alfenas, Brazil
8Department of Chemistry of Federal University of Viçosa, Viçosa, Brazil

*Corresponding author: barbaraperreira2805@gmail.com

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Abstract Epidemiological and experimental studies have found that the consumption of regular coffee has various health benefits. In the present study, we investigated the effect of decaffeinated coffee extracts on the inflammation process and found that aqueous extracts of green and roasted coffee have anti-inflammatory activity. The anti-inflammatory and antioxidant effects of aqueous extract of decaffeinated coffee (AD) and ethanol extract of decaffeinated coffee (ED) were evaluated in animal models using a DPPH radical scavenging test. In the formalin test, the extracts reduced licking activity in both late phases. The inhibitory values of oedema 3 h post-carrageenan indicated an anti-inflammatory effect. In the paw pressure test, the animals treated with extracts exhibited a reduction in the withdrawal threshold. These results provide evidence for the anti-inflammatory and antioxidant properties of decaffeinated coffee extracts that can be attributed to the potential of compounds present in coffee.

Keywords: antioxidant activity, chlorogenic acid, coffea arabica, hypernociception, inflammation

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1. Introduction

Coffee consumption is used for social engagement, leisure, enhancement of work performance and well-being [1]. In recent years, there has been increasing interest in both coffee and coffee constituents for their potentially beneficial and adverse health effects [2]. The coffee beverage is rich in biologically active substances such as nicotinic acid, trigonelline, quinolinic acid, tannic acid, chlorogenic acids, cafestol, pyrogallic acid and caffeine [3,4]. Of the many bioactive molecules of coffee, clinical studies have focused almost exclusively on caffeine. However, coffee contains several other biologically active substances whose relative concentrations vary depending on the type of coffee as well as the brewing process used. Polyphenols and melanoids are present at relatively high concentrations and have been found to exhibit properties, such as antioxidant, anti-inflammatory, hepatoprotective and immunomodulator agent, that benefit human health [5,6,7,8,9]. Coffee is a rich source of antioxidants belonging to the hydroxycinnamic acid family (caffeic, chlorogenic, coumaric, ferrulic and sinapic acids) [10], which can markedly change the total polyphenol intake.

The relationship between decaffeinated coffee consumption and health is sometimes used to determine whether the apparent effects of coffee are due to caffeine or other coffee ingredients. A study of the traits of decaffeinated coffee consumers in the US found that decaffeinated coffee consumption could be related to a history of illness in some people but to a healthy lifestyle in others [11]. Another study found that concerns associated with regular coffee could be voided almost entirely with a transition from regular to decaffeinated coffee [12]. Studies have reported that decaffeinated coffee consumption reduces the risk of type 2 diabetes [13,14,15], improve the insulin sensitivity [16] and improve the intestinal barrier integrity [17]. Others studies utilizing decaffeinated coffee managed to reduce inflammatory markers [18,19].

Despite the worldwide consumption of coffee and evidence of its anti-inflammatory properties [12,20], little
is known about the anti-inflammatory and nociceptive activity of its bioactive compounds. Thus, the present study investigated decaffeinated coffee extracts and utilized inflammation assays, nociception animal models and a chemical analysis of the bioactive compounds in decaffeinated coffee.

2. Materials and Methods

2.1. Materials

Analytical grade reference standards of caffeine, chlorogenic acid (CGA), caffeic acid, gallic acid, trigonelline hydrochloride, Folin-Ciocalteu’s reagent, quercetin, carrageenan, morphine, indomethacin, carboxymethyl cellulose (CMC) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) were supplied by Sigma Chemical Co. (St. Louis, MO, USA). The high purity chromatography solvents were supplied by Merck (Darmstadt, Germany). Glacial acetic acid was obtained from J.T.Baker®. Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Decaffeinated coffee was obtained from a local trader in Alfenas, Minas Gerais, Brazil.

2.2. Preparation of the Extracts and Reference Drugs

For preparation of the aqueous extract (AD) sample, powdered decaffeinated coffee (100 g) was extracted with 1,000 ml of 90°C water followed by filtration. For the ethanol extract (ED) sample, powered roasted decaffeinated coffee beans (100 g) were extracted with 1,000 ml 90% ethanol three times followed by filtration. The solvent was removed under reduced pressure, and the water of the extracts (ED and AD) was removed using a freeze-dryer (Liobras L101, São Paulo, Brazil) to produce a powder for subsequent use in the experiments.

The extracts ED and AD were administered (p.o.) in 30, 100 and 300 mg/kg doses after being suspended in a vehicle (aqueous solution of 0.5% CMC). The control group animals received the same experimental treatment as the test groups, except that the drug treatment was replaced with corresponding volumes of the dosing vehicle. Indomethacin (10 mg/kg, p.o.) and morphine sulphate (1 mg/kg, i.p.) in the vehicle solution were used as reference drugs.

2.3. Compositional Analysis of Extracts by HPLC and Determination of Total Phenolic and Flavonoid Content

The liquid chromatography (HPLC) profile was obtained using Shimadzu LC-100 equipment with a diode array detector (DAD, monitoring 254 nm), an automatic injector and LC solution software. The HPLC apparatus was equipped with a C18 column (Shimadzu CLC-ODS; 4.6 x 250 mm; 5 μm). The mobile phase consisted of 0.5 mM aqueous acetic acid (A) and acetonitrile (B) with a flow rate of 0.7 ml/min. The following elution profile was used: 0-5 min 95:5 (A:B) (isocratic), 5-13 min 87:13 (isocratic) and 27-30 95:5 (linear). An equilibration period of 10 min was included between runs.

The total content of phenolic phytochemicals was measured using the Folin-Ciocalteu method [22]. The total phenolic content is expressed in mg of gallic acid equivalents (GAE) per g of extract. Samples were analysed in triplicate. The flavonoid content was measured using a previously developed colorimetric assay [23]. The flavonoid content was expressed as mg of quercetin equivalent (QE) per g of extract. Samples were analysed in triplicate.

2.4. Analytical Curves

The liquid chromatography (HPLC) profile was obtained using Shimadzu LC-100 equipment with a diode array detector (DAD, monitoring 254 nm), an automatic injector and LC solution software. The HPLC apparatus was equipped with a C18 column (Shimadzu CLC-ODS; 4.6 x 250 mm; 5 μm). The mobile phase consisted of 0.5 mM aqueous acetic acid (A) and acetonitrile (B) with a flow rate of 0.7 ml/min. The following elution profile was used: 0-5 min 95:5 (A:B) (isocratic), 5-13 min 87:13 (linear), 13-27 min 87:13 (isocratic) and 27-30 95:5 (linear). An equilibration period of 10 min was included between runs (24). The analytical curves consisted of six data points, and three replicate injections for each concentration level were performed [24].

2.5. DPPH Free Radical-scavenging Activity

The property to scavenge DPPH free radicals was measured [25]. The scavenging property was estimated based on the percentage of DPPH radicals scavenged using the following equation: scavenging effect (%) = ([control absorbance - sample absorbance]/control absorbance) x 100. The values are presented as the mean of triplicate analyses. The IC50 value is the effective concentration that could scavenge 50% of the DPPH radicals. Ascorbic acid and BHT standards were used as positive controls.

2.6. Pharmacological Procedures

2.6.1. Animals

Adult male Swiss mice (25-35 g) obtained from the Central Animal Facility of the Federal University of Alfenas were housed under controlled light (12:12 h light-dark cycle; lights on at 06:00 am) and temperature conditions (22 ± 1°C) with access to water and food ad libitum. The animals were allowed to habituate to the housing facilities for at least one week before the experiments. At the end of the experiment, mice were euthanized using an overdose of halothane anaesthetic. All experiments were conducted in accordance with the Declaration of Helsinki on the welfare of experimental animals and with the approval of the Ethics Committee of the Federal University of Alfenas (290/2010).

2.6.2. Carrageenan-induced Rat Paw Oedema

Acute inflammation was induced by subplantar injection of 1 mg carrageenan (100 μl) in the right hind paw. The vehicle, AD, ED or indomethacin was orally administered 1 h before carrageenan injection, and the effects were...
evaluated at 1 h intervals from 1 to 4 h after the inflammatory stimulus. Oedema was assessed by measuring the volume of the right hind paw (Plethysmometer 7140, Ugo Basile, Rome, Italy) before and after carrageenan injection [26]. The results are presented as change in paw volume (ml) relative to the basal values.

2.6.3. Formalin-induced Nociception

A formalin solution (5% in 0.9% saline; 20 µl/paw) was injected into the hind paw plantar surface (i. pl.), and the animals were individually placed in transparent observation chambers as previously described [27]. Oral treatments (p.o.) with vehicle, indomethacin, AD or ED were administered 1 h prior to formalin injection. Morphine was administered (i.p.) 30 min before the test. The time spent licking the injected paw was recorded and expressed as the total licking time in the early (0–5 min) and late (20–30 min) phases after formalin injection.

2.6.4. Paw Pressure Test

For the paw pressure test, a hind paw flexion was induced with a handheld force transducer (Electronic anesthesiometer Insight model EFF-302, Brazil) adapted with a 0.5 mm2 polypropylene tip. The investigator was trained to apply the polypropylene tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The stimulation of the paw was repeated until the animal presented two similar measurements [28]. Vehicle, AD or ED was orally administered 1 h before the i.pl. injection of carrageenan (20 µl/paw) or morphine (1 mg/kg, i.v.). The results are expressed as the delta (Δ) withdrawal threshold (in grams), which is calculated by subtracting the zero-time mean measurements from the mean measurements at the indicated times after carrageenan (hypernociceptive agent) injection.

2.7. Statistical Analysis

The results were expressed as the means ± S.E.M. The data obtained were subjected to one-way analysis of variance (ANOVA) and compared using the Newman-Keuls test, with p < 0.05 for significance. A nonlinear regression was performed on the antioxidant activity data to obtain the IC50 values (concentrations that inhibit the presence of free radicals by 50.0%), and the curves were compared statistically using an F test.

3. Results

3.1. Chemical Characterisation

Table 1 shows the results for the phenolic and flavonoid contents, compound quantification by HPLC and scavenging activity. As antioxidant activity is frequently coupled with phenolic content, the total phenolics expressed in gallic acid equivalents (GAE) were determined using the Folin-Ciocalteu C test. The values obtained and the flavonoid content were expressed as mg of quercetin equivalent (QE) per g of extract. The contents of trigonelline, CGA, caffeine and caffeic acid were analysed for HPLC, and each solute concentration was calculated using an analytical curve for the standards. The antioxidant activities of ED were greater than those of the reference antioxidant BHT and AA (3.2 and 3.8-fold, respectively), while AD exhibited less antioxidant activity than can be monitored spectrophotometrically at 517 nm.

The limits of detection (LOD) and quantitation (LOQ) were 0.94 and 2.84 µg/ml for CGA 0.63 and 1.9 µg/ml for caffeine, 0.89 and 2.7 µg/ml for caffeic acid, 2.2 and 6.8 µg/ml for trigonelline hydrochloride, 1.9 and 5.7 µg/ml for total flavonoids and 1.5 and 4.6 µg/ml for total phenolics, respectively. The criteria used to determine the LOD and LOQ were based on the determination of the slope (S) of the analytical curve and the standard deviation of responses (SD) in accordance with the formulas LOD = 3.3 SD/S and LOQ = 10 SD/S. The standard deviation of the response was determined from the standard error estimated by the regression line (Table 2).

Figure 1. Oral effect of decaffeinated coffee extracts (aqueous-AD (A) and ethanol extracts-ED (B)) in the carrageenan-induced mouse paw oedema assay. The values represent the mean ± SEM (n = 8). The asterisks indicate the following significance levels when compared to the vehicle control group: ***p < 0.001 and **p < 0.01 (Newman-Keuls)
Table 1. Quantification of chlorogenic acid (CGA), caffeine, caffeic acid, trigonelline, total phenolics and total flavonoids and scavenging activity of DPPH of extracts DPPH and the standard ascorbic acid (AA) and butylated hydroxytoluene (BHT) in ethanolic (ED) and aqueous (AD) decaffeinated coffee extract

| Samples | CGA (mg/g) | Caffeine (mg/g) | Caffeic acid (mg/g) | Trigonelline (mg/g) | Total flavonoids (mg/g) | Total phenolics (mg/g) | IC50 (μg/ml) |
|---------|------------|-----------------|---------------------|---------------------|------------------------|------------------------|-------------|
| ED      | 17.50 ± 0.04 | ND              | ND                  | 25.70 ± 1.50        | 16.20 ± 0.04           | 100.00 ± 1.00          | 2.40 ± 0.49a |
| AD      | 25.60 ± 1.60 | ND              | ND                  | 33.20 ± 0.56        | 11.30 ± 0.85           | 87.30 ± 2.20           | 19.21 ± 3.39c |
| AA      | -           | -               | -                   | -                   | -                      | -                      | 9.14 ± 0.75b |
| BHT     | -           | -               | -                   | -                   | -                      | -                      | 7.66 ± 0.88b |

ND: not detected; - : not performed. Each value is expressed as mean ± standard deviation (n=3). a, b, c. Means within a line, followed by different letters are significantly different by F test, p<0.05.

3.2. Pharmacological Procedures

3.2.1. Carrageenan-induced Rat Paw Oedema

The AD and ED extracts (Figure 1) significantly inhibited carrageenan-induced rat paw oedema (F4,34 = 5.95; p = 0.001 and F4,34 = 37.11; p = 0.0001, respectively). Maximal swelling of the carrageenan-injected paws was observed 3 h after carrageenan injection. The inhibitory values of oedema 3 h post-carrageenan injection were 61 and 53% for 100 and 300 mg/kg, respectively, for the AD extract (p < 0.01, Newman-Keuls). Indomethacin produced a percentage inhibition of 70%. The ED extract inhibited oedema by 42, 62 and 53% for 30, 100 and 300 mg/kg (p < 0.01, Newman-Keuls), respectively. This result is not only similar to that observed for the group treated with indomethacin, which inhibited oedema formation by 74%.

3.2.2. Formalin-induced Nociception

In the formalin test, the ED and AD extracts had significant antinociceptive activity compared to the control in both the early (F5,39 =7.29; p < 0.001 and F5,40 = 17.41; p < 0.001, respectively) and late phases (F5,41 = 11.09; p < 0.001 and F5,41 = 10.22; p < 0.001, respectively). The reference drug indomethacin suppressed only the second phase of the formalin test, whereas morphine inhibited both phases of the pain stimulus (p < 0.001; Figure 2).

Figure 2. Effects of orally administered aqueous (AD, panels A and B) and ethanol extracts (ED, panels C and D) of decaffeinated coffee on formalin-induced licking in mice. The animals were pretreated orally with vehicle, ED (30, 100 and 300 mg/kg), indomethacin (Indo; 10 mg/kg) or morphine (10 mg/kg) prior to formalin injection. The total time spent licking the hind paw was measured in the first (panels A and C) and second (panels B and D) phases after intraplantar injection of formalin. Each column represents the mean ± S.E.M. (n = 8). The asterisks indicate the following significance level when compared to the control group: ***p < 0.001 (Newman-Keuls)
Table 2. Linearity, detection and quantitation limits determined for the chlorogenic acid (CGA), caffeine, caffeic acid, trigonelline, total phenolics and total flavonoids in decaffeinated coffee extract

| Parameter                        | CGA                          | Caffeine         | Caffeic acid  | Trigonelline hydrochloride | Total flavonoids | Total phenolics |
|----------------------------------|------------------------------|------------------|---------------|-----------------------------|------------------|-----------------|
| Concentration range* (µg/mL)     | 1.00 – 30.00                 | 1.00 – 30.00     | 1.00 – 30.00  | 10.0 – 150.00               | 5.00 – 70.00     | 10.00 – 80.00   |
| Intercept                        | -11892                       | -7070            | -21472        | 21613                       | -0.00987         | 0.00632         |
| Slope                            | 25865                        | 77215            | 52506         | 28383                       | 0.01574          | 0.00813         |
| Coefficient of correlation(r)    | 0.9994                       | 0.9997           | 0.9995        | 0.9999                      | 0.9996           | 0.9997          |
| Standard error estimate          | 7359                         | 14829            | 14182         | 19297                       | 0.00892          | 0.00377         |
| Limit of detection (µg/mL)       | 0.94                         | 0.63             | 0.89          | 2.20                        | 1.90             | 1.50            |
| Limit of quantitation (µg/mL)    | 2.80                         | 1.90             | 2.70          | 6.80                        | 5.70             | 4.60            |

* six data points, three replicate injections at each concentration level.

3.2.3. Paw Pressure Test

In the paw pressure test, the animals treated with AD exhibited withdrawal threshold reductions of 48, 58 and 53% at 1 h and 48, 46 and 42% at 3 h (for 30, 100 and 300 mg/kg, respectively). For the animals treated with ED, the reductions were 45 and 58% in the first hour (for 100 and 300 mg/kg, respectively) and 43, 37 and 62% in the third hour (30, 100 and 300 mg/kg). The reductions for morphine were 98 and 82% at 1 and 3 hours, respectively (Figure 3).

4. Discussion

Although clinical studies on coffee have focused almost exclusively on caffeine, there is increasing evidence that other coffee components are responsible for the effects associated with coffee. Coffee brews are a remarkable source of antioxidants, with contents comparable to those of the bioactive compounds in tea and wine [29,30]. In the present study, we evaluated the anti-inflammatory, antinociceptive and antioxidant properties of decaffeinated coffee extracts.

Studies suggest that the consumption of polyphenol-rich foods reduces the incidence of cancer, coronary heart disease and inflammation [31,32,33]. Chlorogenic acid (CGA) is one of the most abundant polyphenol compounds in the human diet [34]. Decaffeination produced a relative 17% average increase in the levels of total CGA in green coffee, caused mainly by the lixiviation of other compounds during decaffeination and differences in CGA content of regular (caffeinated) and decaffeinated roasted coffees may appear to be relatively small [35]. That the antioxidant activities of the extracts in the present study could be monitored spectrophotometrically suggests that the scavenging activity of the extracts was likely due to the high concentration of phenolic compounds obtained through extraction.

The statistical analysis of the data revealed excellent linearity (r > 0.99) for the method from concentrations of 1.00 to 150 µg/ml. The contents of trigonelline, CGA, caffeine and caffeic acid in commercial coffee may be greatly influenced by the coffee species, variety, geographical origin and roasting conditions [36,37]. Decaffeination is performed prior to the roasting process. The most common and least costly caffeine extraction methods in the coffee industry utilise an organic solvent, such as dichloromethane or ethyl acetate, and the use of water/vapour prior to and after extraction [38]. This decaffeination process changes the chemical composition of coffee, with the caffeine content typically reduced to 0.02-0.3% by the end of the process. Losses of key flavour components of coffee also tend to occur in the process of decaffeination, especially when solvents that lack specificity, such as water, are used. Among the compounds lost may be CGA and related compounds [35]. In the compositional analysis of coffee extracts, we did not verify any caffeine and caffeic acid.

Prior oral treatment with the decaffeinated coffee extracts was effective in reducing the oedematogenic response evoked by carrageenan, and these effects were similar to those exhibited by the group of rats treated with indomethacin. The development of oedema induced by carrageenan is a biphasic event. The early phase (1-2 h) is mediated primarily by histamine, serotonin and bradykinin. The late phase is sustained by the release of
prostaglandins and nitric oxide, with a peak at 3 h, and is produced by inducible isoforms of cyclooxygenase (COX-2) and nitric oxide synthase [39]. The reduced response may be caused by inhibition of one or more intracellular signalling pathways that mediate the inflammatory response [40].

These results are in agreement with those of reported that aqueous extracts of C. arabica L. display considerable anti-inflammatory action by alleviating paw oedema, formalin-induced pain and reducing LPS-induced leukocyte migration in the peritonitis test [20]. The mechanism of effect may be due to the presence of anti-inflammatory substances like flavonoids and antioxidants, which are present in Coffea. Kim et al. [41] reported that kahweol, a coffee-specific diterpene, significantly reduces inducible nitric oxide synthase, carrageenan-induced paw oedema and the level of prostaglandin E2 production in the inflamed paw. Furthermore, our findings corroborate those of other authors [34] that chlorogenic acids began to inhibit carrageenan-induced paw oedema by the second hour of the experimental procedure. Coffee contains antioxidants called polyphenols and flavonoids that scavenge free radicals and protect cells, tissues and organs from damage. Kim et al. [42] demonstrated that certain flavonoids modulate the activities of arachidonic acid-metabolising enzymes, such as phospholipase A2 cyclooxygenase, lipoxygenase and nitric oxide synthase. Accordingly, the inhibition of these enzymes by flavonoids represents an important cellular mechanism underlying anti-inflammatory activity.

In the formalin test, which consists of two distinct phases, the extracts exhibited a significant antinociceptive activity compared to the control during both phases. The first phase, or neurogenic phase (immediately after formalin injection), appears to be a direct effect of formalin on sensory C-fibres. The second phase, or inflammatory phase (starting approximately 20 min after injection), is associated with the development of an inflammatory response and the release of nociceptive mediators [43]. It was previously reported that substance P and bradykinin participate in the onset of the early phase responses and that histamine, serotonin, prostaglandin and bradykinin are involved in the late phase responses [44]. Centrally acting drugs, such as opioids, inhibit both phases equally, while peripherally acting drugs, such as non-steroidal anti-inflammatory drugs and corticosteroids, inhibit only the late phase [43]. The finding of the present study that decaffeinated coffee extracts produce analgesic effects in the early and late phases of the formalin test indicates that the extracts act centrally and peripherally to reduce inflammatory pain.

These results for the formalin test are in agreement with those obtained by Marrassini et al. [45] and Yonathan et al. [46] who similarly concluded that chlorogenic acids contribute to anti-inflammatory and antinociceptive activities. However, the authors [20] reported that aqueous extracts of Coffea produced antinociception only against the inflammatory phase of formalin. Some authors [34] demonstrated that chlorogenic acids inhibit the number of flinches in the late phase of the formalin-induced pain test. Such activities may be derived from the inhibitory action of CGA in the peripheral synthesis/release of inflammatory mediators involved in these responses.

Authors [35] proposed that the pharmacological action of CGA is more evident in decaffeinated coffee because of the absence of caffeine.

Because inflammation is correlated with and influenced by various cytokines and chemokines, a reduction in these markers should indicate a decrease in overall inflammation. Coffee is a potentially beneficial substance because it contains compounds such as CGA, caffeine, cafestol, kahweol and trigonelline, which all have antioxidant or anti-inflammatory potential [47]. The present findings indicate analgesic effects of the ED and AD extracts at all three hours of the paw pressure test. In general, acute inflammatory pain is characterised by hypernociception (evaluated here by the Von Frey method) due to the sensitisation of primary sensory nociceptive neurons, also referred to as hyperalgesia or allodynia [48]. After the initial tissue injury, several specific primary mediators are synthesised and/or released. Eicosanoids and sympathetic amines are the most important primary mediators ultimately responsible for mechanical hypernociception induced by several stimuli in mice [49].

### 5. Conclusion

The decaffeinated coffee extracts had higher concentrations of chlorogenic acid and trigonelline, total phenolics and total flavonoids. AD and ED reduced oedema in treated animals, inhibited both phases of the painful stimulus in the formalin test and produced analgesic effects according to the paw pressure test. As evidenced by previous reports, the mechanism that produces these effects may involve anti-inflammatory and analgesic substances, such as flavonoids and antioxidants, present in Coffea. Thus, the extracts exhibited analgesic activity even in the absence of caffeine and other compounds such as polyphenols may be responsible for such activity. More broadly, the results obtained in the animal experiments demonstrated that decaffeinated coffee had beneficial health effects.

### List of Abbreviations

| AA | Ascorbic acid |
| AD | Aqueous extract decaffeinated coffee |
| ANOVA | Analysis of variance |
| BHT | Butylated hydroxytoluene |
| CGA | 5-cafeoylquinic acid; chlorogenic acid |
| CMC | Carboxymethyl cellulose |
| COX-2 | Cyclooxygenase 2 |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl radical |
| DPHRL | 1-diphenyl-2-picrylhydrazyl |
| IC50 | 50% inhibitory concentration |
| ED | Ethanol extract decaffeinated coffee |
| FC | Folin-Ciocalteu test |
| GAE | Gallic acid equivalent |
| HPLC | High performance liquid chromatography |
| i.p. | Intraperitoneal |
| i.pl. | Intraplantar |
| p.o. | Per oral |
| QE | Quercetin equivalent |
| UV/Vis | Ultraviolet/visible |
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Statement of Competing Interests

The authors have not competing interests.

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