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# Acute and subacute oral toxicity assessment of dry encapsulated and non-encapsulated green coffee fruit extracts

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## Abstract

The coffee fruit is a high source of bioactive compounds such as phenolic acids and methylxanthines, comprising chlorogenic acids and caffeine, respectively. Extract from this matrix may be used as supplement or active ingredient of functional foods, energy drinks, cosmetics or drugs. Safety of caffeine- and chlorogenic acid-rich encapsulated and non-encapsulated hydroethanolic extracts from green coffee fruit (GCFE) was assessed by acute and subacute toxicity tests. In the acute test, oral single dosage until 1000 mg/kg per body weight (bw) did not show any adverse effect on both female and male mice according to the Hippocratic screening and clinical parameters for a period of 14 days. While the oral median lethal dose of non-encapsulated GCFE was 5000 mg/kg bw/day, that of encapsulated GCFE was not detectable likely due to the delayed release of caffeine and other compounds from GCFE. Non-encapsulated GCFE displayed a stimulating effect at a dose of 1000 mg/kg bw/day after 30 min of oral administration, but not after 60 min. Daily consumption of encapsulated GCFE for 30 days showed no adverse effect in male rats even at the highest dose. Extrapolating this value of no-observed-adverse-effect level (1000 mg/kg bw/day) to human consumption, a human equivalent dose of 189 mg/kg bw/day or 11.34 g/day could be estimated for encapsulated GCFE considering a 60 kg adult body weight.

**Keywords:** Mice, No-observed-adverse-effect level, Rats, Safety, 5-O-Caffeoylquinic acid

## 1. Introduction

Not only the efficacy but also the safety of any medicine, food component or additive, dietary supplement, chemical, or substance is of paramount importance. So, before using a

substance in humans, including encapsulated products, its safety must be assessed by *in vitro* tests, *in silico* studies and studies in animals, especially rodents and other species [1,2]. A lot of information is in fact required by the regulatory agencies for a petition for use of a new food

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ingredient, among which are the estimated daily intake (EDI), acceptable daily intake (ADI), no-observed-adverse-effect level (NOAEL), etc. [3].

Microencapsulation is a technique applied to protect bioactive from external environment, to prevent the interaction between core and food matrix nutrients, and/or to provide a controlled release of compounds from core [4,5]. The development of nano/microencapsulation systems that can carry, protect and deliver food ingredients has been a key breakthrough in the food industry [6]. However, the development of oral controlled release systems is still a challenge, due to the inability to restrain and localize the system at targeted areas of gastrointestinal tract [4].

Several researches have been developed aiming to improve safety of substances using different microencapsulation techniques [7–9]. With specific reference to systems for bioactive compounds release, safety issues concern also the features of carrier materials [10,11]. Because of their excellent biocompatibility, non-toxicity, biodegradability and ability to provide controlled-drug release, natural polymers such as maltodextrin (MD) and gum Arabic (GA) are often used to prepare microspheres [11]. For this purpose, the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) provided online documents about the acceptable daily intake of substances applied in encapsulation technologies, based on animal toxicology or human studies and expressed in mg per kg of body weight per day (mg/kg bw/day). Whereas, the ADI values of GA and MD are not specified, likely because these substances are considered to display very low or no toxicity, limited daily intakes are reported for other polymers such as polypropylene glycol alginate (ADI = 70 mg/kg bw/day) and beta cyclodextrin (ADI = 5 mg/kg bw/day) [1,12].

Due to their safety and cost-effectiveness, GA and MD (DE < 20) were chosen in this study as wall material to encapsulate hydroethanolic extracts from the whole fruit of *Coffea canephora*. The resulting dry extracts, which are rich in caffeine and chlorogenic acids, are claimed to be used as food additive and/or nutritional supplement in foodstuff. The health benefits of green coffee extract are already well documented [13–15]; among them, antidiabetic [16], antiobesogenic [13,17], antioxidant [18] and antihypertensive [19] effects stood out in clinical studies, but safety of isolated compounds such as chlorogenic acid [20,21] and, especially, caffeine is still a matter of debate [22–24].

Heimbach et al. [25] assessing the safety of ethanolic extracts from whole cherry coffee fruit through daily ingestion for 90 days by rats (approximately 3446 and 4087 mg/kg bw/day) did not observe any adverse effects. However, this type of extract (Coffeeberry® Energy Brand) was used in non-encapsulated form and had remarkably lower average contents of chlorogenic acids (0.3–14%) and caffeine (0.0–4.46%) [26] than that used in the present study.

The main objective of this study was to test the safety of encapsulated and non-encapsulated extracts from the whole green coffee fruit (GCFE). With this aim in mind, we determined the acute safety, acute effect on behavior and locomotor activity, and the oral median lethal dose of GCFEs in mice. In addition, since preclinical studies are a vital preliminary step to assess safety before human consumption, the NOAEL of encapsulated GCFE was also assessed by subacute 30-day study in rats.

## 2. Materials and methods

### 2.1. Plant material

Powder extracts tested by acute and subacute toxicity methods were obtained from the green fruits (GCFE) of an organic culture of *C. canephora* grown in the Experimental Farm of Leopoldina (Minas Gerais, Brazil) belonging to the Company EPAMIG.

### 2.2. Extraction and microencapsulation process

Powders were submitted to percolation extraction (PE) at a flow rate of 1.0 mL/min. A mass of 900 g batch of dried whole coffee fruit powder (particle size between 250 and 710  $\mu\text{m}$ ) was uniformly moistened with thrice its weight (2.7 kg) of a 68% (w/w) ethanol solution in water for 12 h (pre-swelling phase). After carefully transferring and evenly loading this material into a 10-L stainless steel percolator (Revitec Ltda, São Paulo, SP, Brazil), more solvent was added to achieve a solid-to-solvent ratio of 0.9:10 (w/w) and allowed to remain in contact with the powder for more 24 h (intermediate maceration phase). The flow rate was then adjusted to  $0.20 \pm 0.05$  mL/min at room temperature ( $25 \pm 1$  °C) (percolation phase). The residual solvent was removed at  $40 \pm 2$  °C under vacuum in a rotary evaporator, model R-220 SE (Buchi, Flawil, Switzerland), taking care to maintain 15% of solids in the extract before spray drying.

For microencapsulation, a carrier dispersion containing 30% of solids comprised of maltodextrin

(MD) and gum Arabic (GA) (1:1, w/w) was added to the concentrated extract (1:1, w/w). The resulting dispersions were placed overnight in refrigerator ( $5 \pm 1$  °C) to complete the hydration according to Faria et al. [27] and then dried with a laboratory scale spray dryer under the following conditions: feed flow rate  $500 \text{ mL} \cdot \text{h}^{-1}$ , drying air flow rate  $45,000 \text{ OU} \cdot \text{min}^{-1}$ , inlet temperature  $160 \pm 2$  °C and outlet temperature  $125 \pm 2$  °C.

### 2.3. Physicochemical characterization of extracts

Free and microencapsulated spray dried extracts were analyzed by HPLC-DAD according to Rodrigues and Bragagnolo [28], whereby 5 mg of each extract were suspended in  $1250 \mu\text{L}$  of a methanol: water (80:20 v/v) solution, and mixtures vortexed for 30 s and centrifuged at  $25,000 g$  for 10 min at 10 °C. Supernatants were filtered through membranes with  $0.45 \mu\text{m}$  pore diameter (Millipore, São Paulo, SP, Brazil) and injected into the HPLC-DAD, model 10 (Shimadzu, Kyoto, Japan).

The quantitative analysis was carried out with a HPLC equipped with a LC10 binary pump (Shimadzu), degasser with helium (DGU-2A), automatic injection system (SIL-10A) and a SPD-M-10A diode array detector (DAD-UV-Vis) (Shimadzu). Bioactive compounds were separated out using a Shim-pack ODS-C18 column ( $5 \mu\text{m}$ ,  $250 \times 4.6 \text{ mm}$ , Shimadzu, Kyoto, Japan) coupled to an ODS-C18 pre-column ( $5 \mu\text{m}$ ,  $4 \times 3 \text{ mm}$ , Phenomenex, Torrance, CA, USA). Separation was carried out according to a gradient elution mode with a mobile phase made up of solvent A consisting of 80% (v/v) 10 mM citric acid (pH 2.5) and 20% (v/v) methanol and solvent B consisting of methanol [28]. The flow rate was  $1.0 \text{ mL/min}$  and the column oven temperature 30 °C. To detect 5-caffeoylquinic acid (5-CQA), trigonelline and caffeine, chromatograms were processed at wavelengths of 262, 272 and 325 nm, respectively, and external calibration curves of the same compounds at six concentration levels were used for quantification.

The moisture content was measured gravimetrically in a circulation oven at 105 °C. The water activity ( $A_w$ ) of samples was measured with a Testo 650 thermo-hygrometer (Testo AG, Lenzkirch, Germany) and a hermetic chamber, while the particle size by a laser diffractometer, model Mastersizer X (Malvern Instruments, Malvern, UK), fitted with 45-mm lens. Particles were dispersed in 99.9% ethanol.

The ability of encapsulated (En-GCFE) and non-encapsulated (Non-En-GCFE) extracts to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was

investigated according to the method reported previously by Brand-Williams et al. [29] with slight modification. Briefly, 5.0 mg of Non-En-GCFE or 15 mg of En-GCFE were dispersed in 2.0 mL of a (50:42:8 v/v/v) methanol: water: acetic acid solution agitated for 1 min with a vortex mixer. Afterward, the sample was kept in an ultrasonic bath, model USC 800 (Unique, São Paulo, SP, Brazil), for 10 min, centrifuged at  $15,000 g$  for 15 min at 20 °C and then filtered through a nylon filter ( $45 \mu\text{m}$ ). The resulting GCFE solution was diluted five folds to determine the concentration required to obtain a 50% antioxidant effect ( $\text{EC}_{50}$ ), and then 0.1 mL of each dilution were added to 3.9 mL of a  $6 \times 10^{-5} \text{ mol/L}$  DPPH solution. The mixture was then mixed and left for 30 min at room temperature in the dark. The absorbance of samples was measured at 515 nm using a UV-VIS Spectrophotometer, model UV-1800 (Shimadzu, Chiyoda-ku, Tokyo, Japan). The analysis was done in triplicate either for standards or extracts. The antioxidant capacity of each extract was determined as percentage absorbance reduction. Physicochemical characteristics of powders are listed in Table 1.

### 2.4. Toxicity study methods

For toxicity study, dosage of En-GCFE and Non-En-GCFE was equated by using 5-CQA as the chemical marker. Since its mass in Non-En-GCFE was about 3.5 folds that in En-GCFE, a 3.5 correction factor was applied for adjustment of the test dosage.

Acute oral toxicity and subacute oral toxicity studies were carried out in strict accordance with the recommendations in the Test Guidelines 423 and 407 of the Organization for Economic Cooperation and Development (OECD) [30,31] respectively. The assays were conducted at Federal University of Mato Grosso (UFMT) Biochemistry Laboratory belonging to the Department of Chemistry (Cuiabá, MT, Brazil), and the protocols were approved by the Committee on the Ethics of Animal Experiments of the UFMT under number 23108101038/2015-91. Experimental procedures were conducted in compliance with the Brazilian College of Animal Experimentation (COBEA) and with the principles set forth in the Principles of Good Laboratory Practice and Compliance Monitoring [32].

#### 2.4.1. Animals

Female and male Swiss–Webster albino mice *Mus musculus* (20–25 g) and male albino rats *Rattus norvegicus* (180–220 g) were used. Animals, provided by the Central Animal House of the UFMT, were

acclimated for 6 days in polypropylene cages before treatments. Throughout the trials, animals were kept in the Animal Care Unity of the Biochemistry Laboratory under controlled temperature ( $20 \pm 1$  °C), relative humidity ( $50 \pm 5\%$ ), 12 h light/dark cycle, and provided with water and feed (Nuvilab<sup>®</sup> autoclavable CR1 Sogorb, São Paulo, Brazil) *ad libitum* except when they were fasted overnight prior to blood sampling or during behavioral testing.

#### 2.4.2. Acute toxicity tests

Acute oral assays of En- and Non-En-GCFEs were performed on male ( $n = 36$ ) and female ( $n = 36$ ) mice in order to determine the oral median lethal doses ( $LD_{50}$ ), i.e., the dose which kills 50% of a group of test animals, as well as the tolerable dosages to conduct the subacute toxicity tests in rats. Groups of 6 mice/sex/dose received single dose of 100, 500, 1000, 2500, 5000 mg/kg of body weight (bw) or vehicle (distilled water, 1 mL/kg bw) by orogastric gavage, after a 12 h-overnight fast. Animals were monitored individually for clinical signs of toxicity or mortality by using the Hippocratic screen according to Malone and Robichaud [33] in the open 5, 10, 15, 30, 60, 120, and 240 min following administration of powders, and daily for a period of 14 days. Body weight, food and water intake were measured daily. On the 15th day, animals were euthanized by carbon dioxide overdose and subjected to gross necropsy (i.e., examination of external surface of the body, all orifices, and the thoracic and abdominal cavities and their contents). Organs were removed to determine the relative weight, which was expressed as percentage of organ weight to body weight.

Table 1. Physicochemical characteristics of the encapsulated and non-encapsulated green coffee fruit extracts (GCFEs).

	Encapsulated GCFE	Non-encapsulated GCFE
Appearance	Pale yellow powder	Greenish/brown powder
Moisture content (%)	$1.65 \pm 0.06$	$2.04 \pm 0.06$
Water activity	$0.10 \pm 0.08$	$0.70 \pm 0.01$
Particle size distribution ( $\mu\text{m}$ )	1.19–12.65	0.88–4.60
Total chlorogenic acids (%) <sup>a</sup>	$6.08 \pm 0.07$	$21.70 \pm 0.40$
Caffeine (%)	$3.08 \pm 0.01$	$8.75 \pm 0.20$
Trigonelline (%)	$1.02 \pm 0.08$	$3.55 \pm 0.12$
Antioxidant capacity (%) <sup>b</sup>	$9.30 \pm 0.8$	$2.23 \pm 0.08$

<sup>a</sup> 3-O-caffeoylquinic acid, 4-O-caffeoylquinic and 5-O-caffeoylquinic acid.

<sup>b</sup> Expressed as  $IC_{50}$  of DPPH scavenging activity ( $\mu\text{g}\cdot\text{mL}^{-1}$ ). The extraction solvent used in the extraction process was ethanol:water (68%, w/w) for both encapsulated and non-encapsulated GCFE. All values are given as means of triplicates  $\pm$  standard deviations.

#### 2.4.3. Subacute toxicity tests

Male Wistar rats ( $n = 40$ ) were placed in individual metabolic cages and randomly distributed into one of the following groups ( $n = 10/\text{group}$ ): control group (C), rats treated with 100 mg/kg bw/day of En-GCFE (En-GCFE<sub>100</sub>), rats treated with 500 mg/kg bw/day of En-GCFE (En-GCFE<sub>500</sub>), and rats treated with 1000 mg/kg bw/day of En-GCFE (En-GCFE<sub>1000</sub>). The control group received the vehicle (distilled water, 1 mL/100 g bw/day), and the En-GCFE dose was adjusted from the Non-En-GCFE one using the 3.5 correction factor. To dissolve microparticles in the vehicle, the powder was weighed daily into a conical tube and solubilized in distilled water using a vortex mixer up to the achievement of 1 mL solution/100 g bw.

Treatment was administered orally once a day for 30 days. Body weight of animals, food intake and feces mass were determined once a week, while water consumption every 3 days. Rats were also observed every two days for behavior alteration or for presence of any signs and symptom of toxicity. Near the end of the study, they were evaluated by functional observational battery (FOB) that assessed excitability, autonomic function, gait, sensorimotor coordination, reactivity, sensitivity, and other abnormal clinical signs in an open field. At the end of the study, after fasting for 12 h, animals were euthanized. Blood samples collected in ethylenediaminetetraacetic acid (EDTA) in Vacutainer<sup>®</sup> tubes were used to determine complete blood count (CBC) using a veterinary hematology analyzer, model Poch-100iV Diff (Sysmex do Brazil Indústria e Comércio, São José dos Pinhais, PR, Brazil). Other blood samples collected in anticoagulant-free Vacutainer<sup>®</sup> tubes were analyzed for biochemical parameters [glucose (GLU), urea (Ur), uric acid (UA), creatinine (Cre), alanine aminotransferase (ALT), aspartate aminotransferase (AST),  $\gamma$ -glutamyl transferase (GGT), amylase, total bilirubin (TBILI), direct bilirubin (DBILI), indirect bilirubin (IBILI), total cholesterol (CHO), HDL cholesterol, LDL cholesterol, triglycerides, alkaline phosphatase (ALP), total protein (TP), albumin and globulin] in an automatic biochemical analyzer, model Labmax Plenno (Labtest, Belo Horizonte, MG, Brazil), and for hormonal cortisol as stress biomarker by chemiluminescence using an immunoassay system, model Immulite<sup>®</sup> 1000 (Siemens Healthineers, Belo Horizonte, Brazil).

Liver, kidney, stomach, spleen, heart, lungs, and brain were removed and their relative weights, expressed as percentages of organ weight to body weight, were determined before placing fragments in 10% formalin for histological analysis.

#### 2.4.4. Histopathological study

Histological sections of liver, brain, testicles, stomach, lungs, spleen, heart, kidneys, and small and large intestine were processed by a conventional histopathological method, followed by staining with hematoxylin and eosin and visualization by optical microscopy with a 40X objective. Animal tissues were examined blindly, and the criteria for assessing the occurrence of lesions in organs were done according to the methodology proposed by Giordani et al. [34]. Briefly, the criteria for liver injury were vacuolization of hepatocytes and pyknotic hepatocyte nuclei, number of Kupffer cells and enlargement of sinusoids. Moreover, the histopathological change of lungs was based on congestion, edema, inflammation and hemorrhage. Microscopic features of the organs of En-GCFE-fed rats were compared with those of the control group.

#### 2.4.5. Estimation of human dose

The human equivalent dose (HED) was estimated, from the no-observed-adverse-effect level (NOAEL) of En-GCFE determined in male rats by subacute toxicity tests, by means of the empirical equation [35]:

$$\text{HED (mg/kg)} = \text{NOAEL (mg/kg bw/day)} \times K_m \text{ ratio} \quad (1)$$

where the NOAEL corresponds to the effective animal dose, and the  $K_m$  ratio to the ratio of human  $K_m$  factor to that of the animal. The  $K_m$  factors were estimated by dividing the average body weight (kg) of human or rats by its body surface area ( $\text{m}^2$ ). The average human body weight and body surface area were considered to be 60 kg and 1.62  $\text{m}^2$ , respectively, while the average animal body weight was that determined at the end of the toxicological study (around 0.3 kg) and the body surface 0.043  $\text{m}^2$  [35].

#### 2.5. Behavioral test

Male mice ( $n = 60$ ) were also treated with Non-En-GCFE and analyzed for locomotor activity by the open-field test as described by Zomkowski et al. [36]. The experimental conditions included four doses of Non-En-GCFE (100, 500, 1000 or 2000 mg/kg bw,  $n = 6$  per group), and a control group fed with vehicle (distilled water 1 mL/kg bw,  $n = 6$  per group). Animals were pretreated with extract or vehicle twice, after 30 min and 1 h prior to the open-field test. The duration of this study was around 10 (ten) days with around six days of acclimatization of

animals; at the end of the test, they were euthanized by carbon dioxide overdose.

The apparatus consisted of a wooden box ( $40 \times 60 \times 50$  cm) with visible lines dividing the floor into twelve equal squares drawn using a black sticker. The number of squares crossed with all paws (crossings) or rearing was counted in a 6-min session. The light was maintained at minimum and any interference such as noise was avoided during the test. Mice were individually placed in the center of the apparatus and allowed to explore freely, while a blinded observer scored the number of times a mouse crossed one of the grid lines with all four paws or rearing occurrence (event).

#### 2.6. Data analysis

The GraphPad Prism (GraphPad Software 5.00, San Diego, CA, USA) was used for statistical analysis and plotting. The results of parametric tests were expressed as mean  $\pm$  standard error of the mean (SEM) or standard deviation, according to circumstances. To compare the means of more than two samples from different treatments we used one-way analysis of variance (ANOVA), observing the significance by *F-test* and, when significant, Tukey–Kramer test at 5% and 1% probability levels.

### 3. Results and discussion

#### 3.1. Acute toxicity

##### 3.1.1. Hippocratic screening

The Hippocratic screening is often used to provide a general estimate of pharmacological and toxicological nature of a given compound [37]. For animal welfare reasons, starting doses of Non-En-GCFE and En-GCFE used in this study ranged from 100 to 1000 mg/kg of body weight (bw). No behavioral alterations or death were observed in both female and male mice submitted to these dosages. A mild analgesic effect was noticed at doses from 500 to 1000 mg/kg bw both in Non-En-GCFE and En-GCFE treatment groups, but this effect was reversed around 4 h after a single dose administration both in male and female mice.

In accordance with OECD guideline 423 [30], which recommends performing a limit test when available information suggests that mortality is unlikely at the highest starting dose level, dosages of 2500 and 5000 mg/kg bw were also assessed in a second step. Although the additional upper dose level is usually tested for substances with relatively low acute toxicity hazard, under certain circumstances it may pose a danger to the vulnerable population [31].

Non-En-GCFE at a dosage of 5000 mg/kg bw caused tonic convulsion followed by death of four male mice and three female mice. Two male mice died right after the administration of the maximum dose, and two more did so after a period of ataxia of about 4 h. Among females, two showed an acute tonic convulsion episode and subsequent death, while the third remained ataxic for about 8 h and then died. No human endpoints criteria were adopted to minimize the suffering before the death of these animals because the deaths were very fast in half part of the animals, and between animals that showed symptoms of ataxia before die, the survival was expected. On the other hand, to determine the median lethal dose (LD<sub>50</sub>) in toxicity studies, no euthanasia intervention should be performed before the end of the assay in order not to alter the dose–response results.

Even though doses of 2500 mg/kg bw of Non-En-GCFE and 5000 mg/kg bw of En-GCFE did not cause any death, other effects on the autonomic system were noticed among both female and male mice such as the Straub tail phenomenon accompanied by piloerection after about 30 min as well as initial excitation with increased motor activity and respiratory rate in the first 15 min. Then, an increased fugue reaction and aggressiveness were observed especially at 2500 mg/kg bw of Non-En-GCFE in mice of both sexes. These side effects are characteristic of caffeine intoxication present with hyperventilation, dizziness, anxiety, tinnitus, tremor, and agitation [38].

Enophthalmos after 60 min along with palpebral ptosis (more sedation than a true blepharospasm) were observed among great part of the animals that survived the treatment with 5000 mg/kg bw of En-GCFE and with 2500–5000 mg/kg bw of Non-En-GCFE. These effects were reversed after 8 h of administration, and no other deaths occurred after this period among the other treatment groups for 14-day observation.

### 3.1.2. Median lethal dose (LD<sub>50</sub>)

In accordance with the criteria established by the OECD guideline 423 [30], Non-En-GCFE belongs to the hazard category 5, since the oral LD<sub>50</sub> for both male and female mice was achieved at the dose of 5000 mg/kg bw. In this category includes substances characterized by relatively low acute toxicity hazard, with oral or dermal LD<sub>50</sub> in the range of 2000–5000 mg/kg or equivalent doses by other routes.

As reported by Schafer et al. [39] oral LD<sub>50</sub> of chlorogenic and caffeic acids in red wing blackbird

>100 mg/kg bw, corresponding to 0.282 and 0.555 mmol/kg, respectively, but sex and strain of model animals were not provided. Despite the lack of information in the scientific literature about LD<sub>50</sub> values of these substances administered orally to rats in studies performed under good laboratory practice or equivalent, a Non-Commercial Scientific and Production Partnership reported acute oral toxicity of chlorogenic acid >2000 mg/kg bw in rats and >1580 mg/kg bw in mice (NSPP, 2006). In a systematic review, Adamson [2] reported 367 mg/kg bw as the most accurate acute median lethal dose of caffeine administered orally in male albino rats. Based on these findings, the caffeine content in Non-En-GCFE may have been responsible for the death of more than 50% of mice given that the 5000 mg/kg bw dose provided around 400 mg/kg bw of caffeine. The fatal blood concentration of caffeine in humans is 80–100 mg/L, which means that, in order to achieve this concentration, a person would need to ingest as much as 5000–10,000 mg of pure caffeine [40]. In addition, it has been speculated that caffeine's mechanism of death is usually associated with ventricular arrhythmia provoked by a block of the adenosine receptor, due to similarity of its molecular structure to that of adenosine. The increased intracellular calcium concentration, causing noradrenaline release and sensitizing dopamine receptors, can trigger arrhythmia [41]. Although the dosages of En-GCFE and Non-En-GCFE were equivalent, it was not possible to determine the oral LD<sub>50</sub> of the former, probably due to the delayed release of caffeine and other compounds from GCFE.

### 3.1.3. Body weight, food intake and water consumption

As shown in Table 2, in the male group treated with 2500 mg/kg bw of En-GCFE the mean weekly body weight gains were significantly lower than those in the controls after 7 and 14 days ( $p < 0.05$ ). However, this effect was not dose-dependent since no difference was observed with the highest dosage in the control group. On the other hand, the female body weight values were always close to those of the control group.

The food intake (Fig. 1) and food efficiency (data not shown) were also similar among females in both treatments. On the other hand, among males, the food efficiency at dosage of 2500 mg/kg of Non-En-GCFE was 31% lower compared to En-GCFE. Nonetheless, among males treated with Non-En-GCFE, the food intake and food efficiency were similar to the control group. It is well known that

food efficiency, which is the ratio of body weight gained per gram of food eaten, is subject to sex-specific responses to the effects of voluntarily increased food intake by animals. In general, female animals exhibit a higher percent increment of body fat than males [42], which corroborates with the higher food efficiency observed in this study for females compared to males at all dosages.

As far as water consumption is concerned, despite the variations in both sex and treatment, groups did not show any significant difference among them (Fig. 2). Based on these results, we can conclude that single doses of En-GCFE and Non-En-GCFE did not interfere with food intake or water consumption.

3.1.4. Relative organ weight

Changes in body and organ weights are a clear indication of damage caused by the ingestion of a toxic substance, the latter being considered as the most sensitive indicator of its toxic effect [43]. As it can be seen in Table 3, no significant differences in relative organ weight were observed between

female groups treated with Non-En- and En-GCFE ( $p > 0.05$ ), with the exception of the liver one at dosage of 1000 mg/kg bw of the latter extract that showed higher value compared to the other groups. However, this effect was not dosage-dependent since the groups treated with 2500 and 5000 mg/kg bw did not show any significant difference between them. Relative liver weight, which is considered a more sensitive toxicity indicator than absolute liver weight [44], can be enlarged by several conditions that may be basically divided into two categories: those without histopathological alterations and injuries caused by diseases such as tumors [45,46]. Conditions of the former category, which can be brought about by a variety of compounds metabolized by the liver such as drugs, food additives, insecticides, carcinogens and normal body constituents such as cortisone, thyroxine, estradiol, testosterone and androsterone [46], were likely to occur in the present study as a result of oral acute toxicity tests, since liver necropsy compared to the control group did not show

Table 2. Results of the oral acute toxicity study using female and male mice.

Group	Dose (mg/kg bw)	Sex	Body weight (g) <sup>a</sup>			Clinical Signs	Macroscopical lesions findings	Mortality (%) (Dead/total)
			Day 0	Day 7	Day 14			
<sup>b</sup> Non-En-GCFE								
G1	0	M	23.1 ± 0.7	32.0 ± 1.1	35.1 ± 0.6	N	0	0% (0/6)
		F	25.4 ± 0.8	29.8 ± 0.7	30.6 ± 0.9	N	0	0% (0/6)
G2	100	M	22.1 ± 0.9	23.0 ± 0.8	37.5 ± 0.8	N	0	0% (0/6)
		F	22.7 ± 1.2	26.7 ± 1.0	28.5 ± 1.2	N	0	0% (0/6)
G3	500	M	22.4 ± 0.6	31.0 ± 0.4	34.8 ± 0.8	N	0	0% (0/6)
		F	24.6 ± 0.9	29.0 ± 0.8	30.2 ± 0.9	N	0	0% (0/6)
G4	1000	M	22.1 ± 0.8	27.9 ± 1.5	32.4 ± 1.5	N	0	0% (0/6)
		F	24.2 ± 0.8	27.3 ± 1.0	29.2 ± 0.7	N	0	0% (0/6)
G5	2500	M	22.7 ± 0.6	33.3 ± 1.1	37.0 ± 1.3	Ab	0	0% (0/6)
		F	24.3 ± 0.6	26.2 ± 1.0	28.5 ± 0.7	Ab	0	0% (0/6)
G6	5000	M	22.6 ± 0.8	27.7 ± 1.3	32.4 ± 1.7	Ab	0	66.6% (4/6)
		F	26.0 ± 0.5	28.8 ± 0.7	29.7 ± 0.6	Ab	0	50% (3/6)
<sup>c</sup> En-GCFE								
G1	0	M	23.2 ± 0.5	30.7 ± 1.1	34.2 ± 1.0	N	0	0% (0/6)
		F	23.2 ± 0.6	28.0 ± 0.9	30.0 ± 0.8	N	0	0% (0/6)
G2	100	M	24.5 ± 0.7	33.2 ± 0.2	34.0 ± 0.3	N	0	0% (0/6)
		F	22.0 ± 0.5	28.0 ± 0.5	31.1 ± 0.4	N	0	0% (0/6)
G3	500	M	22.8 ± 0.9	30.7 ± 1.1	34.8 ± 0.9	N	0	0% (0/6)
		F	23.7 ± 0.7	28.5 ± 0.8	30.6 ± 0.6	N	0	0% (0/6)
G4	1000	M	22.9 ± 0.5	28.8 ± 0.7	34.1 ± 1.1	N	0	0% (0/6)
		F	23.1 ± 0.7	26.9 ± 0.9	30.2 ± 1.2	N	0	0% (0/6)
G5	2500	M	23.7 ± 0.3	24.6 ± 0.3**	30.7 ± 0.3**	N	0	0% (0/6)
		F	24.9 ± 0.4	26.1 ± 0.6	28.4 ± 0.5	N	0	0% (0/6)
G6	5000	M	22.4 ± 0.6	27.0 ± 1.2	32.0 ± 0.8	Ab	0	0% (0/6)
		F	24.2 ± 0.6	27.6 ± 0.3	29.3 ± 0.3	Ab	0	0% (0/6)

\* $p \leq 0.05$  and \*\* $p \leq 0.01$  represent significant differences from the control male or female group. G1 = Control; G2 = Group 2 (100 mg/kg bw); G3 = Group 3 (500 mg/kg bw); G4 = Group 4 (1000 mg/kg bw); G5 = Group 5 (2500 mg/kg bw); G6 = Group 6 (5000 mg/kg bw); M = male; F = female; N = Normal; Ab = Abnormal.

<sup>a</sup> Results were expressed as mean ± standard error of mean (SEM) of 6 rats.

<sup>b</sup> Encapsulated (En-) and.

<sup>c</sup> Non-encapsulated (Non-Enc-) green coffee fruit extract (GCFE). Analysis by ANOVA followed by Tukey–Kramer test.

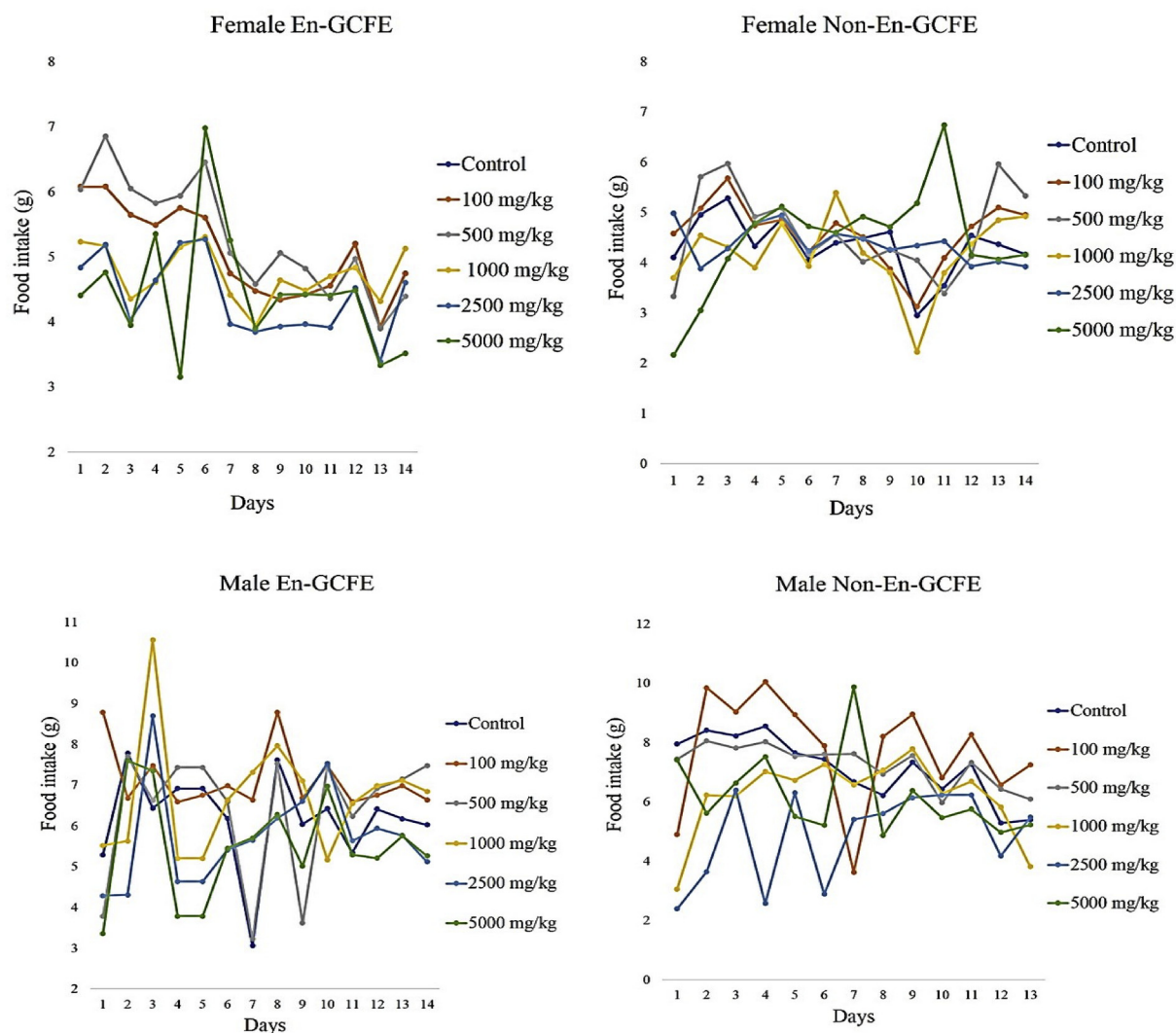


Fig. 1. Female and male mice food intake during 14-day after single dose of En-GCFE and Non-En-GCFE. \*encapsulated (En-) and non-encapsulated (Non-En-) green coffee fruit extract (GCFE).

any macroscopic alteration, both in females and males.

In male groups, statistically significant decreases ( $p < 0.05$ ) were observed for relative kidney weight at dosages of 500 and 5000 mg/kg bw of Non-En-GCFE when compared to the control group, but not at 100, 1000 and 2500 mg/kg bw. Although such an effect did not appear to be dose-dependent, it could be so in a longer subacute test. A decrease in kidney weight can happen due to malnutrition or even to atrophy induced by reduced water consumption, but both situations must be excluded in the present study for both male and female groups. Moreover, gross examination of the main organs including liver, spleen, stomach, kidneys, lungs, heart, and brain did not show any abnormal findings (data not shown). Therefore, these results taken together

indicate that oral administration of a single dose until 2500 mg/kg bw did not show toxic effects in mice. However, there were mild reversible reactions which may be suggestive of caffeine action on the central nervous system.

Even among dead mice, neither macroscopic alteration in selected organs nor relative organ weight alteration were observed. Despite being clear the role of caffeine in these fatal intoxications, specific organ alterations, i.e. brain edema and mild erosion with hemorrhage in the stomach caused by caffeine overdose [41], were not observed in this toxicity study.

Acute toxicity not only provides initial information on the mode of toxic action of a substance, but also allows establishing a safe dose of a new compound and is helpful in determining doses in animal

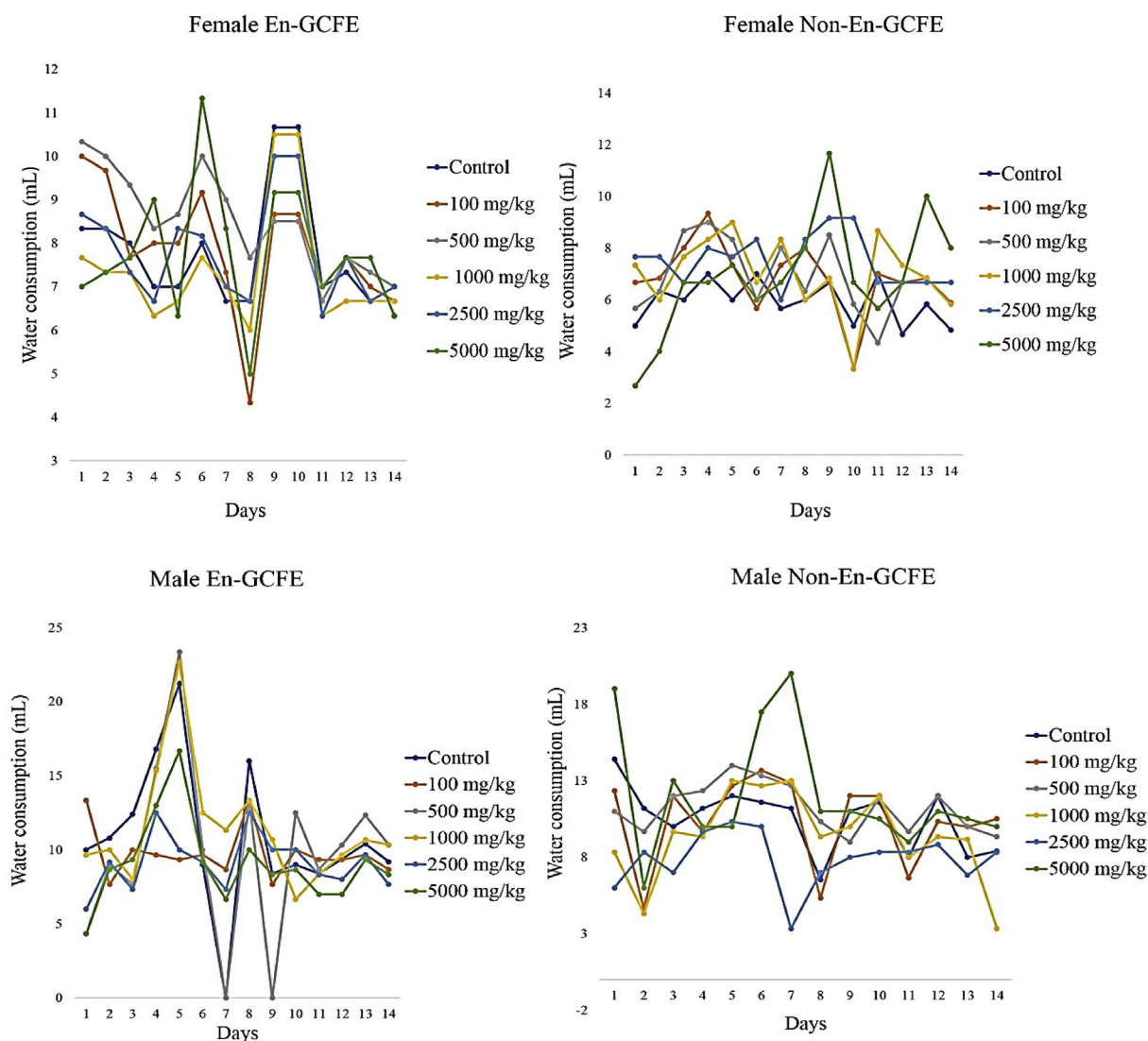


Fig. 2. Female and male mice water consumption during 14-day after single dose of En-GCFE and Non-En-GCFE. \*encapsulated (En-) and non-encapsulated (Non-En-) green coffee fruit extract (GCFE).

studies [34]. Based on this, doses until 1000 mg/kg bw of En-GCFE, which were well tolerated in the acute test, were selected to conduct a subacute study in male Wistar rats in order to calculate the no-observed-adverse-effect level (NOAEL) and to help in clinical studies.

### 3.2. Subacute toxicity

Since the toxicological profile of GCFE may have been influenced by a synergistic effect of compounds, *in vivo* data are essential to be sure of its safety. For this

purpose, repeated dose toxicity testing is usually carried out for no less than 28 days, with the test substance administered daily preferentially through the oral route [31], and a rodent of any gender and age of 5–6 weeks selected as a model [47]. In this study, the subacute test was conducted orally administering to male rats only En-GCFE via gastrogavage for 30 days, because this extract was proven to exert less adverse effect than Non-En-GCFE in Hippocratic screening. During this period, no treatment-related clinical symptoms or death were recorded, and autonomic and central nervous system, somatomotor activity and

Table 3. Relative body weights (%)<sup>a</sup> of female and male mice after 14-days toxicity study.

Organ/Groups	Males (dose, mg/kg bw)					Females (dose, mg/kg bw)						
	Control	100	500	1000	2500	5000	Control	100	500	1000	2500	5000
<b>b</b> Non-En-GCFE												
Spleen (%)	0.60 ± 0.04	0.49 ± 0.01	0.54 ± 0.08	0.48 ± 0.02	0.51 ± 0.05	0.52 ± 0.13	0.69 ± 0.08	0.61 ± 0.01	0.59 ± 0.02	0.61 ± 0.03	0.44 ± 0.02	0.53 ± 0.05
Heart (%)	0.62 ± 0.03	0.62 ± 0.03	0.58 ± 0.05	0.58 ± 0.04	0.46 ± 0.01	0.57 ± 0.10	0.53 ± 0.03	0.52 ± 0.01	0.55 ± 0.02	0.54 ± 0.05	0.51 ± 0.04	0.66 ± 0.06
Stomach (%)	0.97 ± 0.04	1.09 ± 0.07	1.05 ± 0.05	0.96 ± 0.03	1.01 ± 0.03	0.90 ± 0.05	1.03 ± 0.04	0.99 ± 0.03	1.11 ± 0.07	1.05 ± 0.04	1.02 ± 0.12	1.19 ± 0.06
Liver (%)	5.14 ± 0.19	5.27 ± 0.18	5.09 ± 0.20	5.01 ± 0.16	4.85 ± 0.19	4.70 ± 0.17	5.46 ± 0.23	4.92 ± 0.11	4.56 ± 0.09	4.51 ± 0.21	4.71 ± 0.21	4.82 ± 0.25
Lungs (%)	0.66 ± 0.04	0.63 ± 0.02	0.64 ± 0.04	0.76 ± 0.04	0.63 ± 0.02	0.65 ± 0.02	0.82 ± 0.04	0.67 ± 0.02	0.75 ± 0.03	0.74 ± 0.03	0.72 ± 0.02	0.91 ± 0.03
Kidneys (%)	1.48 ± 0.01	1.45 ± 0.06	1.26 ± 0.03*	1.38 ± 0.04	1.30 ± 0.05	1.25 ± 0.04*	1.19 ± 0.02	1.18 ± 0.02	1.14 ± 0.03	1.16 ± 0.06	0.99 ± 0.24	1.21 ± 0.05
<b>c</b> En-GCFE												
Spleen (%)	0.41 ± 0.04	0.51 ± 0.06	0.42 ± 0.01	0.46 ± 0.03	0.51 ± 0.03	0.46 ± 0.03	0.49 ± 0.05	0.53 ± 0.06	0.52 ± 0.02	0.55 ± 0.03	0.57 ± 0.03	0.45 ± 0.01
Heart (%)	0.46 ± 0.04	0.54 ± 0.02	0.51 ± 0.02	0.54 ± 0.01	0.57 ± 0.02*	0.53 ± 0.01	0.49 ± 0.03	0.45 ± 0.02	0.49 ± 0.04	0.44 ± 0.03	0.47 ± 0.01	0.47 ± 0.02
Stomach (%)	0.83 ± 0.05	0.90 ± 0.05	0.87 ± 0.08	0.98 ± 0.02	1.03 ± 0.06	0.95 ± 0.03	1.02 ± 0.05	0.95 ± 0.05	1.09 ± 0.07	1.01 ± 0.07	1.03 ± 0.05	1.04 ± 0.08
Liver (%)	6.23 ± 0.69	6.47 ± 0.21	5.94 ± 0.05	5.48 ± 0.24	6.31 ± 0.68	5.09 ± 0.12	5.14 ± 0.14	4.98 ± 0.12	5.19 ± 0.15	5.45 ± 0.14*	4.55 ± 0.18	4.64 ± 0.22
Lungs (%)	0.62 ± 0.05	0.75 ± 0.03	0.70 ± 0.04	0.79 ± 0.04	0.68 ± 0.02	0.77 ± 0.06	0.70 ± 0.04	0.63 ± 0.02	0.67 ± 0.01	0.67 ± 0.01	0.66 ± 0.02	0.72 ± 0.02
Kidneys (%)	1.33 ± 0.01	1.39 ± 0.06	1.36 ± 0.05	1.37 ± 0.01	1.49 ± 0.03	1.32 ± 0.05	1.19 ± 0.03	1.10 ± 0.04	0.90 ± 0.18	1.16 ± 0.03	1.10 ± 0.01	0.96 ± 0.12

\*p ≤ 0.05 and \*\*p ≤ 0.01 represent significant differences from the control group.

<sup>a</sup> Relative body weight (%) = [(g/g body weight) × 100].<sup>b</sup> Encapsulated (En-) and.<sup>c</sup> Non-encapsulated (Non-Enc-) green coffee fruit extract (GCFE). Results are expressed as mean ± standard error of mean (SEM) of 6 mice. Analysis by ANOVA followed by Tukey–Kramer test.

behavior pattern were found to be normal in all the experimental groups.

### 3.2.1. Body weight, food intake and water consumption

There were no significant differences in body weight, water consumption, feces weight, food intake (Fig. 3), or feeding efficiency (data not shown) in treated animals compared to the control group ( $p > 0.05$ ). En-GCFE did not affect gastrointestinal transit since symptoms like diarrhea or intestinal constipation were not observed, feces weight increased at the last time-point in accordance with animal growth (Fig. 3D), and food intake, body weight, and water consumption varied only a little throughout the study (Fig. 3A–C). In addition, the moderate, but non-significant decrease in body weight gain occurred among animals of the control group after 7 weeks (data not shown) and in the last week indicates food avoidance, which may have happened due to some discomfort caused by gastrogavage. Differently, in their 90-day dietary sub-chronic study, Heimbach et al. [25] observed significant body weight gain, food consumption and food efficiency at dosages of 3446 and 4087 mg/kg bw/day, which however were not considered adverse or toxicologically significant.

### 3.2.2. Relative and absolute organ weights and histopathological findings

Systemic toxicity manifests itself through decreases in ponderal development as well as food and water consumption, behavioral alteration, apathy, bad condition of the coat such as hair loss, biochemical and hematological alterations, and relative organ weight alterations [48–50]. In the present study, the terminal body weight of rats did not show any significant difference ( $p > 0.05$ ) when comparing the groups treated with En-GCFE and the control group (Table 4 and Fig. 3).

No significant difference was even found in relative or absolute rat organ weights (Table 4). Contrariwise, Heimbach et al. [25] observed some statistically significant changes in these parameters for kidneys, heart and liver using either mid (~2000 mg/kg bw/day) or high dose (~4000 mg/kg bw/day) of ethanolic extract from whole coffee fruit incorporated in rat diet.

Figs. 4–6 shows photomicrographs of some of main organs analyzed histologically. The section of heart, liver and kidney tissues stained with hematoxylin and eosin revealed normal morphology without inflammatory cell infiltration. In all groups, including the control, some alterations were observed in the superficial stomach tissue, which

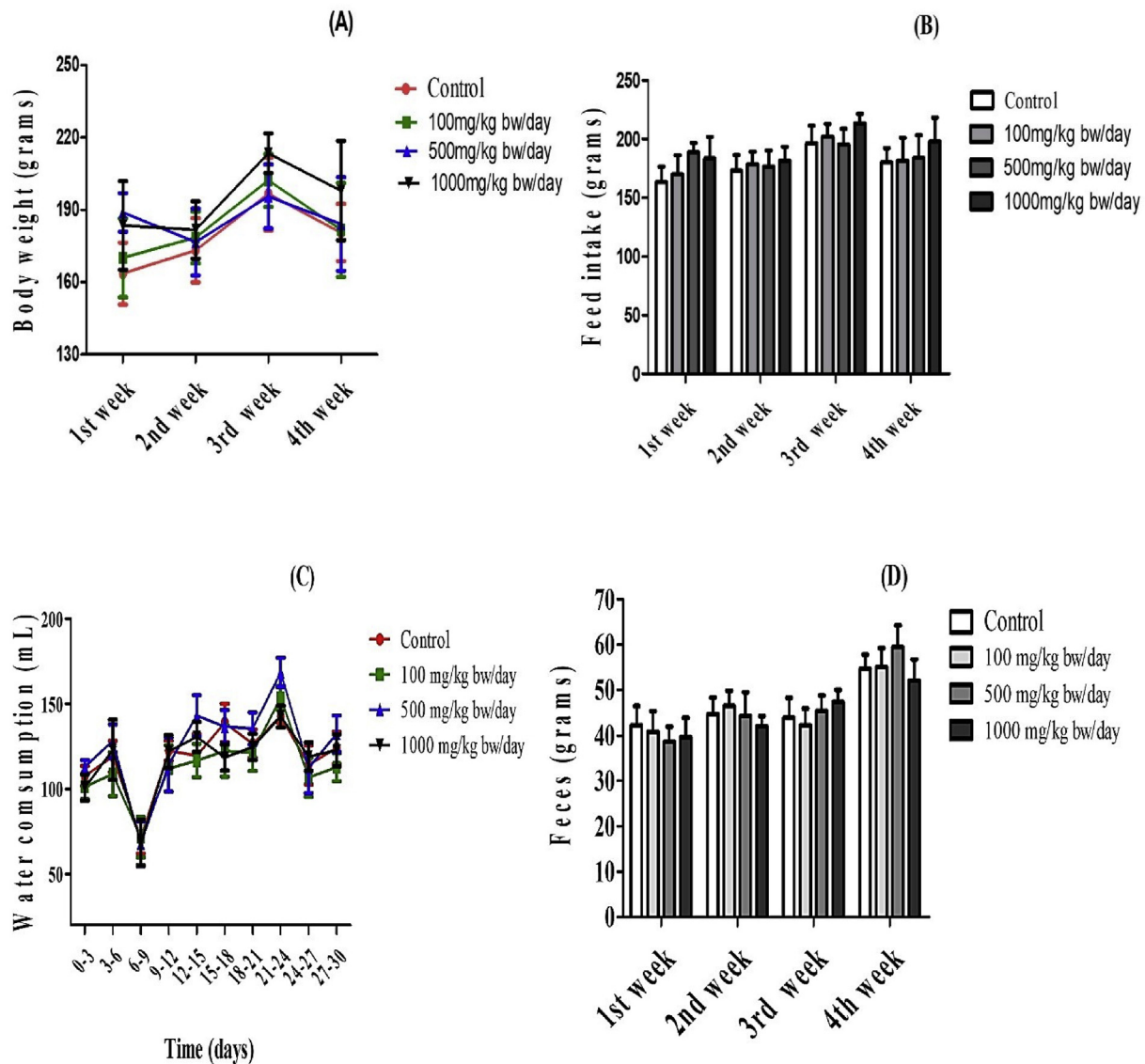


Fig. 3. Subacute toxicity study using male Wistar rats treated with encapsulated green coffee whole fruit extract. (A) body weight gain, (B) feed intake, (C) water consumption and (D) feces weight.

suggest colonization by *Candida* sp., whereas no alteration was evident in other stomach regions.

Some studies reported effects of coffee and its compounds such as caffeine and chlorogenic acid on the gastrointestinal system [51–53]. Both coffee and caffeine can stimulate gastrin hypersecretion and gastric acid secretion causing mild erosive and hemorrhagic points in stomach [41,51,52]. Decaffeinated coffee, which is commonly produced from the same Robusta beans used in this study, was found to be more acidic than the regular one due to higher concentrations of organic acids such as chlorogenic acid compared with the Arabica ones [54]. Nevertheless, En-GCFE did not cause any macro or microscopic damage in the architecture of

the stomach mucosa, likely due to protection of superficial tissue of this organ from CGFE offered by the Arabic gum (GA)/maltodextrin (MD) wall material. The role of GA as prebiotic and antioxidant agent has been studied widely [55–57], and its effectiveness in targeted delivery has been ascribed to resistance in stomach and small intestine [58]. Moreover, combinations of MD and GA like that used in this study have been proposed to protect phenolic compounds [59,60].

At dosage of 1000 mg/kg bw/day, no alteration in the architecture or inflammatory infiltration was observed in the large or small intestine structures likely because microencapsulation protected intestine endothelium from chlorogenic acids and

Table 4. Terminal body weight, absolute and relative main organs weights<sup>a</sup> from subacute toxicity study using male rats.

	Treatment with encapsulated GCFE (mg/kg bw/day)			
	Control	100	500	1000
Terminal body weight (g)	296.3 ± 10.2	298.2 ± 8.8	300.9 ± 12.2	296.9 ± 7.8
Brain weight (g)	1.91 ± 0.04	1.84 ± 0.05	1.86 ± 0.05	1.84 ± 0.04
Relative brain weight (%) <sup>a</sup>	0.65 ± 0.02	0.62 ± 0.02	0.62 ± 0.03	0.62 ± 0.02
Liver weight (g)	12.27 ± 0.60	12.96 ± 0.55	13.04 ± 0.66	12.16 ± 0.70
Relative liver weight (%)	4.12 ± 0.08	4.34 ± 0.13	4.33 ± 0.14	4.07 ± 0.12
Heart weight (g)	1.21 ± 0.05	1.24 ± 0.05	1.16 ± 0.05	1.18 ± 0.05
Relative heart weight (%)	0.40 ± 0.01	0.41 ± 0.02	0.38 ± 0.01	0.39 ± 0.01
Lungs weight (g)	2.16 ± 0.16	2.37 ± 0.12	2.14 ± 0.08	2.08 ± 0.13
Relative lungs weight (%)	0.72 ± 0.04	0.80 ± 0.04	0.72 ± 0.03	0.70 ± 0.05
Stomach weight (g)	1.97 ± 0.07	1.88 ± 0.08	1.93 ± 0.08	1.88 ± 0.12
Relative stomach weight (%)	0.67 ± 0.02	0.63 ± 0.03	0.65 ± 0.04	0.63 ± 0.04
Spleen weight (g)	0.81 ± 0.04	0.81 ± 0.04	0.80 ± 0.04	0.80 ± 0.03
Relative spleen weight (%)	0.27 ± 0.01	0.27 ± 0.01	0.26 ± 0.06	0.27 ± 0.01
Kidney weight (g)	2.84 ± 0.11	2.91 ± 0.10	3.01 ± 0.10	2.87 ± 1.13
Relative kidney weight (%)	0.96 ± 0.03	0.98 ± 0.02	1.00 ± 0.02	0.96 ± 0.02
Testis weight (g)	3.75 ± 0.17	3.37 ± 0.01	3.36 ± 0.15	3.31 ± 0.15
Relative testis weight (%)	1.27 ± 0.06	1.13 ± 0.03	1.13 ± 0.07	1.12 ± 0.07

\* $p \leq 0.05$  and \*\* $p \leq 0.001$  represent significant differences from the control group.

<sup>a</sup> Relative body weight (%) = [(g/g body weight) × 100]. Results were expressed as mean ± standard error of mean (SEM) of 6 rats. Analysis by ANOVA followed by Tukey–Kramer test.

caffeine present in GCFE. Contrariwise, Du et al. [61] reported that a high dose of chlorogenic acid (7 mg/kg bw) administered via parenteral route caused severe architecture damage, manifested by loss of ileum villi, villus congestion and massive

infiltration of inflammatory cells, as the likely result of oxidative stress due to an increase in reactive oxygen species and, consequently, to enhancement of inflammatory mediators in intestinal endothelium. A dose as high as 4000 mg/kg bw/day of

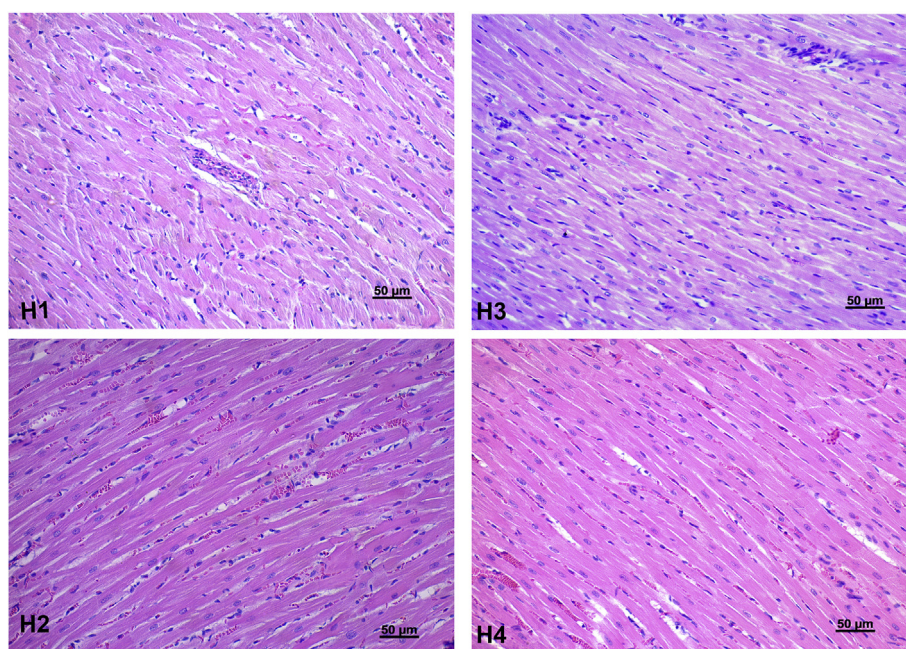


Fig. 4. Photomicrographs of heart (50  $\mu\text{m}$ ) analyzed histologically. H1 = control group; H2 = 100 mg/kg bw/day; H3 = 500 mg/kg bw/day; H4 = 1000 mg/kg bw/day.

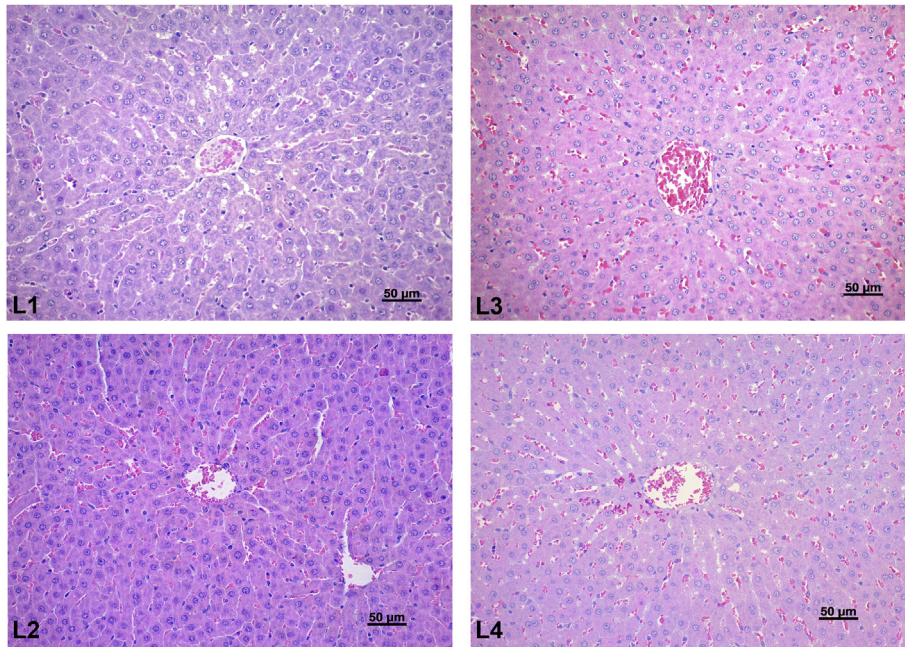


Fig. 5. Photomicrographs of liver (50 µm) analyzed histologically. L1 = control group; L2 = 100 mg/kg bw/day; L3 = 500 mg/kg bw/day; L4 = 1000 mg/kg bw/day.

ethanolic extract from whole coffee fruit, containing 40% phenolic acids and 0.6–9.0% caffeine and administered orally for 14 days, caused colon/intestinal distention in 3 of 10 female rats, but it was

not considered a toxic effect since no histopathological alteration was seen [25]. A possible reason of conflicting results is the different administration route employed in these studies.

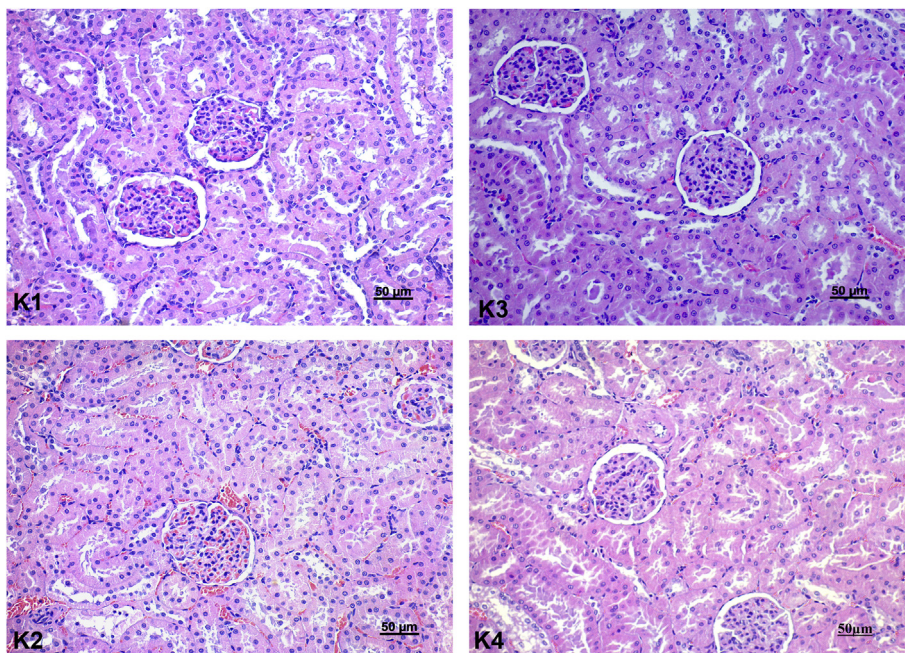


Fig. 6. Photomicrographs of kidney (50 µm) analyzed histologically. K1 = control group; K2 = 100 mg/kg bw/day; K3 = 500 mg/kg bw/day; K4 = 1000 mg/kg bw/day.

In a chronic study conducted by Chan et al. [62], where 1000 mg/kg bw/day green tea extract were administered by gavage for 3 months, liver alterations were found in 3 of 10 female rats likely due to its content of caffeine (4.99%, ~15 mg/kg bw), which is known to act as a potent CYP1A2 inducer [63]. However, despite its higher caffeine content (24 mg/kg bw/day), En-GCFE did not lead, in the present study, to any macroscopic or histological liver alteration among treated or control male rats; neither did it cause structural alteration in testis, corroborating findings reported by Heimbach et al. [25].

Despite no organ weight changes, some alterations in brain and lungs were seen in both treated and control animals. Particularly, midbrain-diencephalic junction and rostrocaudal brain region showed mild neutrophil vacuolization and mild acute neuronal necrosis, which, however, were presumably due to the increased brain glutamate postmortem levels caused by the CO<sub>2</sub> euthanasia protocol [64]. In addition, CO<sub>2</sub> anesthetic levels may have been responsible for hemorrhage and acidosis observed in the brain [65]. Moderate alveolar wall thickening with the presence of mononuclear and polymorphonuclear cell infiltrates and the absence of any bacterial agent in lung tissue were also observed among all animal groups, including the control one, but these findings are commonly observed in enclosed individuals [66]. No histological alterations in treated groups were significant compared to the control animals, thereby excluding any role of En-GCFE in tissue toxic effect.

### 3.2.3. Biochemical and hematological parameters

Consumption of En-GCFE caused no alteration in hematological parameters and only small changes in biochemical parameters of the treated animals compared to the control ones. Specifically, mean albumin concentration was found to decrease at the highest En-GCFE dose, while glucose concentration to increase at 500 and 1000 mg/kg bw/day compared to the control group ( $p < 0.05$ ). Likewise, Heimbach et al. [25] found only mild significant alterations in mean platelets concentration when using mid (2030 mg/kg bw/day) and high (4087 mg/kg bw/day) doses of whole coffee fruit extract as well as an increased glucose level at low dose (965 mg/kg bw/day) after a 90-day dietary study.

Albumin is a well-known body's predominant serum-binding protein responsible for the transport of various compounds, including bilirubin, fatty acids, metals, ions, hormones and exogenous drugs [67], whose level can be decreased by various

adverse conditions including malnutrition, nephrotic syndrome, hepatic cirrhosis, heart failure and, more commonly, acute and chronic inflammatory responses [68]. However, although all groups exhibited a serum albumin level below the reference range indicated by Giknis and Clifford [69], no other hematological or biochemical alterations ascribable to possible causes of hypoalbuminemia were found in comparison to the control group (Table 5), such as increased levels of blood cells indicating inflammatory process, or of biomarkers of liver and heart function (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase), or even of more specific markers of liver ( $\gamma$ -glutamyl transpeptidase, total, direct and indirect bilirubin) and kidney (urea, creatinine and uric acid) functions. In addition, even though lab markers of malnutrition such as globulin and total protein decreased, reducing total cholesterol level and lymphocytes count [70], such variations were not statistically significant when compared to the control group ( $p > 0.05$ ).

The dose-dependent raise in serum glucose level in healthy rats disagrees with the *in vitro* antidiabetic effect of green coffee extract reported by Henry-Vitrac et al. [71], as well as the capability of trigonelline and chlorogenic acids taken through regular coffee consumption to lower the risk of type 2 diabetes mellitus in humans [72–75], and to exert an anti-hyperglycemic effect in diabetic rats [76]. Finally, En-GCFE ingestion did not lead to any statistically significant increase in cortisol level compared to the control group ( $p > 0.05$ ), like that induced by caffeine and coffee on this and other stress hormones [77,78].

In summary, these results taken as whole demonstrate that oral administration of En-GCFE did not cause any deterioration of hematological and biochemistry parameters, thereby confirming the findings of histopathological analyses that showed a normal tissue architecture in all selected organs. Besides, no alterations of clinical signs and behavior were observed among the treated and control groups even at the highest dose.

These findings allowed establishing an En-GCFE NOAEL of 1000 mg/kg bw/day for male rats. Considering the human equivalent dose defined by Equation (1), the safe dose established for humans is 189 mg/kg bw/day or 11.34 g/day considering an adult with 60 kg of body weight. Nonetheless, it is worth mentioning that the above equation assumes body surface area as the unique important characteristic, not considering physiological, biochemical and pharmacokinetic aspects.

Table 5. Effects on haematological and biochemical parameters in male rats after 30-day of ingestion of encapsulated GCFE.

Hematological parameters	Treatment with encapsulated GCFE (mg/kg bw/day)				Reference <sup>A</sup>
	Control	100	500	1000	
Red blood cell (10 <sup>6</sup> /mL)	8.81 ± 0.21	8.91 ± 0.27	8.76 ± 0.19	8.18 ± 0.30	7.27–9.65
Hemoglobin (g/dL)	16.09 ± 0.40	16.31 ± 0.57	15.95 ± 0.21	14.92 ± 0.44	13.7–17.6
Hematocrit (%)	51.44 ± 1.22	51.67 ± 1.89	50.88 ± 0.87	46.62 ± 1.73	39.6–52.5
MCV (m <sup>3</sup> )	58.51 ± 1.27	58.01 ± 1.34	58.21 ± 1.21	57.08 ± 1.11	48.9–57.9
MCH (pg)	18.28 ± 0.34	18.33 ± 0.44	18.25 ± 0.37	18.30 ± 0.37	17.1–20.4
MCHC (%)	31.28 ± 0.28	31.60 ± 0.35	31.37 ± 0.24	32.07 ± 0.32	32.9–37.5
Leukocytes (10 <sup>3</sup> /μ <sup>3</sup> )	6.67 ± 1.58	9.61 ± 0.83	6.77 ± 0.86	7.7 ± 1.01	1.96–8.25
Segmented (%)	21.56 ± 6.04	14.44 ± 1.11	16.88 ± 1.34	15.5 ± 1.10	6.2–27.6
Eosinophils (%)	6.33 ± 0.92	7.67 ± 0.78	7.33 ± 0.37	8.00 ± 0.73	0.2–3.5
Basophils (%)	0	0	0	0	0
Lymphocytes (%)	70.11 ± 6.63	76.33 ± 1.43	70.55 ± 1.27	75.25 ± 1.08	66.6–90.3
Monocytes (%)	1.33 ± 0.16	1.56 ± 0.17	1.88 ± 0.77	1.25 ± 0.16	0.8–3.8
Platelets (10 <sup>3</sup> /μL)	748.89 ± 76.12	814.22 ± 60.48	871.22 ± 58.09	792.04 ± 79.04	638–1177
Biochemical parameters					
Glucose (mg/dL)	72.32 ± 7.65 <sup>a</sup>	77.77 ± 4.60 <sup>a</sup>	88.31 ± 9.50 <sup>b*</sup>	90.95 ± 12.67 <sup>b*</sup>	70–208
Ur (mg/dL)	51.54 ± 3.46	53.22 ± 3.49	45.28 ± 2.11	43.28 ± 3.04	10.7–20.0
Cre (mg/dL)	0.77 ± 0.04	0.80 ± 0.03	0.71 ± 0.01	0.68 ± 0.01	0.3–0.5
CHO (mg/dL)	100.11 ± 4.19	110.65 ± 7.30	99.55 ± 6.10	97.01 ± 7.98	37–95
HDL (mg/dL)	43.98 ± 2.40	46.73 ± 1.79	43.24 ± 2.33	40.46 ± 3.05	–
LDL (mg/dL)	46.32 ± 2.68	49.58 ± 7.40	43.20 ± 6.01	50.16 ± 7.79	–
VLDL (mg/dL)	9.80 ± 0.75	11.96 ± 1.60	10.56 ± 1.41	10.80 ± 1.11	–
TG (mg/dL)	53.10 ± 2.88	66.44 ± 6.93	65.55 ± 8.43	54.30 ± 3.92	27–140
UA (mg/dL)	1.03 ± 0.08	1.41 ± 0.23	1.50 ± 0.36	1.17 ± 0.22	–
TBILI (mg/dL)	0.10 ± 0.02	0.09 ± 0.03	0.10 ± 0.03	0.13 ± 0.05	0.05–0.15
DBILI (mg/dL)	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03–0.05
IBILI (mg/dL)	0.07 ± 0.01	0.07 ± 0.02	0.07 ± 0.03	0.10 ± 0.05	0.01–0.12
TP (g/dL)	7.65 ± 0.22	7.60 ± 0.18	7.25 ± 0.20	7.07 ± 0.18	5.2–7.1
Alb (g/dL)	3.11 ± 0.07 <sup>a</sup>	3.24 ± 0.07 <sup>a</sup>	2.96 ± 0.07 <sup>a</sup>	2.73 ± 0.11 <sup>b*</sup>	3.4–4.8
Globulins (g/dL)	4.53 ± 0.17	4.34 ± 0.12	4.28 ± 0.15	4.33 ± 0.18	1.58–2.67
ALT (UI/L)	57.76 ± 6.43	66.28 ± 3.73	60.24 ± 6.47	58.27 ± 8.01	18–45
AST (UI/L)	130.20 ± 26.58	137.84 ± 23.80	125.36 ± 23.13	129.38 ± 26.13	74–143
AMI (UI/L)	468.48 ± 45.73	480.01 ± 38.46	493.64 ± 37.57	442.18 ± 25.37	–
GGT (UI/L)	6.73 ± 0.85	5.74 ± 0.75	7.90 ± 0.97	7.07 ± 0.58	–
ALP (UI/L)	121.15 ± 10.45	103.18 ± 9.04	102.91 ± 11.14	103.08 ± 6.54	62–230
COR (mg/dL)	0.81 ± 0.02	0.82 ± 0.02	0.84 ± 0.02	0.84 ± 0.02	–

Results are expressed as mean ± standard error of mean (SEM) of 10 trials. One-way ANOVA followed by Tukey–Kramer test. \* $p \leq 0.05$  and \*\* $p \leq 0.01$  represent significant differences from the control group. Difference ( $p \leq 0.05$ ) between groups of the same treatment represented by the letters <sup>a</sup>, <sup>b</sup> and <sup>c</sup>. Glu, glucose; AMI, amylase; Ur, urea; LDL, low density lipoprotein; VLDL, very low-density lipoprotein; HDL, high density lipoprotein; TG, triglycerides; CHO, total cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT,  $\gamma$ -glutamyl transferase; Alb, albumin; TP, total protein; UA, uric acid; Cre, creatinine; TBILI, total bilirubin; DBILI, direct bilirubin; IBILI, indirect bilirubin, FAL, alkaline phosphatase. COR, cortisol.

<sup>A</sup> Giknis & Clifford [69].

### 3.3. Behavioral test

The autonomic effects observed among mice after an oral single dose of 2500 and 5000 mg/kg bw of Non-En-GCFE allowed selecting a maximum limit dose of 2000 mg/kg bw by the open-field test.

In the study proposed by Nehlig et al. [79], caffeine supplied in gavage exerted muscle relaxant activities and sedative effects or psychostimulant effects. These possible nonspecific effects of Non-En-GCFE neither changed the number of rearings and crossings when compared to the control group after 60 min of administration until doses of 1000

and 2000 mg/kg bw, respectively (Fig. 7). On the other hand, statistically significant increase in the number of crossings ( $p < 0.01$ ) and decrease in that of rearings ( $p < 0.01$ ) were observed after 30 min among animals that received a dose of 1000 mg/kg bw compared to the control ones. The group treated with 2000 mg/kg bw decreased significantly the number of rearings after 30 and 60 min of administration of Non-En-GCFE (Fig. 7).

It was reported by Czok and Lang [80] that chlorogenic acid alone had a weak neurostimulating effect in rats, but chlorogenic acid co-administered with caffeine showed an enhanced, dose-dependent

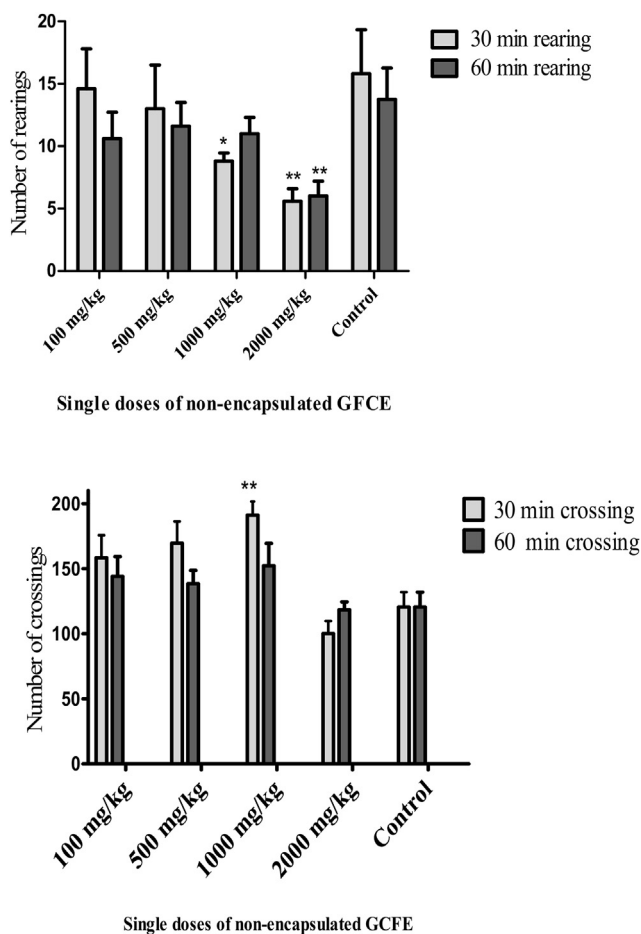


Fig. 7. Number of crossings and rearings after 30 and 60 min of the GCFE single dose. Results are expressed as mean  $\pm$  standard error (SEM) of 6 samples. One-way ANOVA followed by Tukey–Kramer test. \* $p \leq 0.05$  and \*\* $p \leq 0.01$  represent significant differences from the control group.

central stimulant action. In a more recent study assessing the effects of chlorogenic, caffeic and *m*-coumaric acids on spontaneous locomotor activity in mice, Ohnishi et al. [81] observed that chlorogenic acid has a weak caffeine-like psychostimulant effect, but at a dose of 2.8 mmol/kg it significantly increased locomotor activity, although more weakly than the other two acids. In a clinical random trial, adult males who received 200 mg of caffeine from green coffee extract showed an increase in epinephrine level similar to the control, but no significant change in blood pressure or heart rate from baseline levels after 60 and 120 min post-GCFE dose, thereby suggesting that natural caffeine sources may have different impacts on excitatory neurotransmitters, particularly epinephrine [78].

In the present study, the stimulant effect of GCFE was shown by the increased number of crossings

30 min after receiving a dose of 1000 mg/kg bw, but not by the number of rearings. On the other hand, at a dose of 2000 mg/kg bw a significant neurodepressive effect referred to the number of crossings took place compared to other treated groups ( $p < 0.05$ ) (data not shown), but not compared to the control ( $p > 0.05$ ). These results may be ascribed to some discomfort caused by the extract like that observed at 2500 mg/kg dosage in the Hippocratic screening, given that high doses of caffeine can cause hypotension, dizziness, nausea, among other symptoms [41].

#### 4. Conclusion

Acute toxicity tests provided important information on the safety of encapsulated (En-) and non-encapsulated (Non-En-) green coffee fruit extract (GCFE) and allowed determining for the former a median lethal dose ( $LD_{50}$ ) in mice of 5000 mg/kg bw/day. Despite En-GCFE contained theoretically the same amount of 5-CQA taken as a chemical marker as Non-En-GCFE, it was not possible to determine its  $LD_{50}$  likely due to a delay in the release of the caffeine. Doses of 100, 500 and 1000 mg/kg bw/day of En-GCFE, prepared to furnish the same amounts of chemicals as in Non-En-GCFE, were selected to assess the subacute safety in male Wistar rats, since they showed fewer side effects in the Hippocratic screening. In the subacute toxicity assay, oral administration of the highest dose (1000 mg/kg bw/day) of En-GCFE for 30 days did not lead to any adverse effects; therefore, it was assumed as no-observed-adverse-effect level, which allowed estimating a human equivalent dose for an adult with 60 kg of body weight of 189 mg/kg bw/day or 11.34 g/day. These results indicate that En-GCFE could be safe for daily consumption.

#### Author contributions

**Conceptualization** – Wanessa, Nair Honda, Neura, Edemilson, Wander, Waldenia.

**Data curation** – Wanessa, Waldenia.

**Formal Analysis** – Wanessa

**Funding Acquisition** – Wander, Neura, Wanessa

**Investigation** – Wanessa, Waldenia.

**Methodology** – Alessandra, Natalie, Suelem, Wanessa

**Project administration** – Wanessa

**Supervision** – Neura, Edemilson.

**Writing - original draft** – Wanessa

**Writing – review & editing** – Attilio, Neura, Nair.

**Visualization** – Wanessa, Neura.

## Conflict of interest

There is no conflict of interest to declare.

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