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Article

Permitted daily exposure from preclinical studies of *Ginkgo biloba* L. dry extract

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Resolution 658/2022 of the Brazilian Regulatory Agency requires the determination of the permitted daily exposure (PDE) of pharmaceutical agents. *Ginkgo biloba* L. is used therapeutically to treat memory deficits and other brain diseases. However, published results indicate that more studies are needed to confirm the safety of *Ginkgo biloba*. This study aimed to evaluate the dry extract of *Ginkgo biloba* L. leaves PDE as an ingredient in an oral pharmaceutical product in preclinical studies using mice. Acute oral toxicity and repeated dose experiments were performed based on OECD guidelines, as well as genotoxicity tests. The results indicate that *Ginkgo biloba* L. has low acute toxicity, no liver toxicity, and does not alter blood glucose levels. No changes in weight gain were observed, but food intake decreased in males during the first week of treatment at the highest dose. Hematological parameters were not altered in males, whereas females presented lower leukocyte and lymphocyte counts and higher neutrophil counts at the highest dose. The lipid profile was not altered in males, whereas total cholesterol was increased in females. The estimated PDE was 0.1 mg/day and, when related to the maximum residual concentration, indicates that the cleaning process used is safe and does not require reassessment.

Keywords: Permitted daily exposure. *Ginkgo biloba* L. Oral pharmaceutical product. Toxicity. Preclinical studies. Genotoxicity.

INTRODUCTION

In Brazil, RDC 658/2022 establishes general guidelines for good drug manufacturing practices. Based on this regulation, pharmaceutical companies are required by ANVISA to carry out quality risk management that includes a toxicological assessment of the risk of cross-contamination between products (ANVISA, 2022).

A hazard that indicates a potential risk in the case of exposure is a prerequisite for conducting a risk assessment. Consequently, risk is associated with exposure to a hazard (Wiesner, Prause, Barle, 2018). In the context of cross-

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contamination, a hazard may be a source, situation, or event that has the potential to compromise patient health due to the lack of control measures to mitigate the risk of cross-contamination between products when the acceptable limit according to the permitted daily exposure (PDE) is exceeded (Sehner *et al*., 2019).

The determination of the PDE is mandatory for all active substances present on a production line, even if there is no emerging risk, as part of the quality risk management of each company (Brazil, 2022). The evaluation indicates the need to re-analyze the maximum permitted residual limits between products, with a view to validating the procedures for cleaning the surfaces of equipment. Thus, after determining the PDE, the value obtained should be compared with the result of the cleaning validation and other risk factors determined by the risk management.

In recent decades, a revaluation of phytotherapeutic preparations has been observed, with an advance and increase in their production by the pharmaceutical industry. Phytotherapy products are obtained from liquid intermediate raw materials (liquid extracts), solid intermediate raw materials (dry extracts), or semi-solid intermediate raw materials (soft extracts). Among the widely used raw materials for the development of phytotherapy products and considered one of the oldest species in the world, the therapeutic use of *Ginkgo biloba* L. dates back to 1436 for the treatment of memory deficits, dementia and other central nervous system diseases (Filho, Fakoury, Ferry, 2010; Russo *et al*., 2013; Yang *et al*., 2018; Burnett, 2018; Bonassi *et al*., 2018).

The main chemical components of *Ginkgo biloba* L. leaves are flavonoid glycosides, phenolic acids, polyprenols, terpene lactones, biflavones, alkylphenols and proanthocyanidins. It is important to highlight that terpene lactones represent a group of chemicals exclusive to *Ginkgo biloba* L. (Filho, Fakoury, Ferry, 2010; Burnett, 2018; Bonassi *et al*., 2018; National Toxicology Program, 2003). Usually, the positive biological effects described for *Ginkgo biloba* L. are related to the terpene lactones and flavonoid glycosides, whereas the ginkgolic acids are traditionally considered responsible for the negative biological effects (e.g. mutagenicity and cytotoxicity) (National Toxicology Program, 2003; van Beek, Montoro, 2009).

Two main mechanisms of action are associated with the health benefits of the *Ginkgo biloba* L. extract: antagonism of the platelet-activating factor (PAF) by ginkgolides and antioxidant activity of flavonoids by the elimination of reactive oxygen species. However, many other health-promoting mechanisms have been identified in *in vitro* or *in vivo* studies: increased superoxide dismutase concentrations, chelation of metal ions and glutathione S-transferases, antagonism of the main inhibitory receptors, glycine and GABAergic receptors of the central nervous system, inhibition of mitochondrial dysfunction, modulation of neurotransmitter concentrations or receptor densities, and reduced nitric oxide release (National Toxicology Program, 2003; Zhu *et al*., 2013).

The effect of *Ginkgo biloba* L. in humans is quite variable in the literature. While some studies indicate safe use with no reports of adverse effects, one review concluded that *Ginkgo biloba* L. had adverse effects in patients who were taking medications or had comorbidities (Diamond *et al*., 2000). In addition, a technical report published in the USA by the National Toxicology Program (NTP) showed carcinogenic activity of *Ginkgo biloba* L. extract in male and female rodents (National Toxicology Program, 2013). Furthermore, a recent meta-analysis suggests that more research is needed to confirm its safety (Yang *et al*., 2016). Thus, the design of studies evaluating the safety of *Ginkgo biloba* L. treatment is a priority.

Currently, there are many differences in the design of pre-clinical and clinical studies of *Ginkgo biloba* L. dry extract, making it difficult to select data to calculate the PDE. Therefore, this study followed the guidelines of the Organization for Economic Cooperation and Development (OECD). Within this context, it is essential to evaluate the efficacy of the methods for cleaning the equipment used to manufacture a solid pharmaceutical product containing *Ginkgo biloba* L. dry extract. Therefore, this study assessed the permitted daily exposure of *Ginkgo biloba* L. dry extract used as a raw material in an orally administered pharmaceutical product in pre-clinical studies to determine its cross-contamination potential on a multi-purpose production line.

MATERIAL AND METHODS

Material

Ginkgo biloba L. dry extract (Finzelberg GmbH & Co. KG, Germany) was obtained from the leaves of the tree and is characterized as a light brown powder containing 6.78% terpene lactones, 4.12% ginkgolides A, B, C, 2.66% bilobalide, 25.9% ginkgo flavonoids, 0.218% quercetin, and 0.25 ppm ginkgolic acids. The active ingredient was kindly donated by a pharmaceutical company, and the reagents ketamine, xylazine, trichloroacetic acid, zinc sulfate, glycerol, silver nitrate, pararosaniline (basic fuchsin), hydrochloric acid, distilled water, sodium metabisulfite and potassium, active charcoal, fast green, lysis solution, sodium hydroxide, sodium hypochlorite/sodium hydroxide and DMSO were purchased from Sigma Aldrich.

Methods

Pre-Clinical Trials

Ethics

Animals were used according to the ethical principles of the Brazilian legislation (Brazil, 2008; Conselho Nacional de Controle da Experimentação Animal, 2018), in accordance with the European Parliament and Council Directive of September 22nd, 2010 (2010/63/EU). Biosafety measures followed the recommendations of Feevale University.

This study was submitted to the Ethics Committee on the Use of Animals of Feevale University (CEUA) and was approved according to report nº 04/2020 – CEUA Feevale (project number 02.20.082).

Animals

Isogenic male and female BALB/c mice were used in this study. Animals aged 45 to 60 days and weighing 20 to 30 grams were provided by the Feevale University Vivarium. The animals were kept in plastic boxes (17 x 28 x 13 cm), grouped in a maximum of 5 animals per box, at 22°± 2°C in a 12-hour light/dark cycle (lights on from 7 a.m. to 7 p.m.), with an exhaust system and controlled humidity, with food and water *ad libitum*. The experiments were carried out from 10 a.m. to 4 p.m. All mice were given a 1-hour adaptation period in the experimentation room and were handled carefully to avoid stress and suffering.

***In Vivo* Toxicity**

Acute Oral Toxicity

Toxicity was evaluated in a single-dose exposure study according to NI 423 of the Organization for Economic Cooperation and Development (2001). Initially, *Ginkgo biloba* L. dry extract was solubilized in 5% DMSO, and a dose of 2000 mg/kg was administered (by gavage) to 3 mice. The animals were continuously observed for 14 days. Body weight and food intake were recorded. Frequency of deaths and toxicity symptoms (piloerection, eyelid ptosis, abdominal contortions, locomotion, hypothermia, muscle tone alterations, tremors, hind limb paralysis, salivation, bronchial secretion, and convulsions) were observed after 1, 2, 6, and 24 hours of treatment. The mice were euthanized on day 15 with a lethal dose of ketamine (90 mg/kg) and xylazine (10 mg/kg).

Repeated Dose Toxicity

This assay was carried out to assess the toxicity of repeated oral doses, based on NI 407 of the OECD (Organization for Economic Cooperation and Development, 2008) and the ICH (Harmonized Tripartite Guideline), with adjustments (Betti *et al.*, 2012; da Silva *et al.*, 2022). The mice were treated daily for 28 days with a vehicle (saline solution and 5% DMSO) and three different doses of *Ginkgo biloba* L. dry extract: an acceptable daily dose (120 mg/kg), twice (240 mg/kg), and four times (480 mg/kg) the dose. Twice the maximum daily dose, according to the therapeutic range described in the medication package insert, was considered for dose selection (240 mg/day or a single dose of 120-240 mg) (Gauthier, Schlaefke, 2014; Isah, 2015).

The chosen doses were also based on other studies that used similar or even higher doses than those

recommended for human use. Burnett (2018) tested up to 2000 mg/kg of *Ginkgo biloba* L. extract in animals. Wang *et al.* (2015) assessed polyprenols (natural active lipids present in *Ginkgo biloba* L. dry leaves) after administration of 500, 1000, and 2000 mg/kg to rats. The oral chronic toxicity of the extract was verified by EMA (2014) in rats at daily doses of 20, 300, 400 and 500 mg/kg for six months. In addition, ICH M3 (R2) (European Medicines Agency, 2009) sets the appropriate limit dose for subchronic and chronic toxicological studies at 1000 mg/kg/day for rodents and non-rodents. If the 1000 mg/kg/day dose is not 10 times the clinical exposure, the toxicity study should limit the exposure to the lowest value of the maximum feasible dose (MFD), 10 times the clinical dose, or the 2000 mg/kg/day dose.

Each group consisted of 10 animals. The mice were euthanized on day 29 with a lethal dose of ketamine and xylazine. Blood samples were obtained from the femur. Approximately 1 mL of blood was collected from the inferior vena cava using 20 µL heparin.

Relative Weight Gain

The weight of the mice was measured on a digital scale every two days during the 28-day treatment period. Relative weight gain was calculated considering the body mass index of the mice on day 1: relative mouse weight (%) = (mouse weight/mouse weight on day 1) x 100.

Food Intake

To monitor food consumption, the initial amount of food provided was weighed. Every two days, the remaining amount was removed and reweighed. The difference in food consumed per day was divided by the number of animals in each box. This procedure was repeated for 28 days.

Hematological Parameters

The hematological analysis was based on whole blood samples analyzed in KX 21M® equipment (Roche). The parameters evaluated were red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb), hematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW) and platelets. In addition to the blood count, leukocyte differentiation was performed by blood smear, counting segmented neutrophils, lymphocytes, and monocytes.

Biochemical Parameters

Following the hematological analysis, plasma was obtained through centrifugation (3,500 rpm, 10 minutes) for biochemical analysis using Mindray BS200E equipment and Bioclin® kits. AST and ALT levels were determined to assess hepatic toxicity. Creatinine, urea and uric acid were measured to evaluate renal toxicity. Glycemic profile was evaluated based on glucose levels and lipid profile was evaluated based on the levels of triglycerides, total cholesterol (COL) and its fractions, high density lipoproteins (HDL) and low-density lipoproteins (LDL).

Genotoxicity

The genotoxicity assays were performed on bone marrow, femur and blood samples from mice treated for 28 days.

Micronucleus Assay

Femoral bone marrow samples were prepared as described by Schmid (1975). After dissection, the proximal and distal ends of the bone were cut and the bone marrow cells were collected using fetal bovine serum. The suspension was centrifuged and the supernatant was discarded. The remaining cell pellet was resuspended in acetic acid:ethanol (3:1) and smeared on a clean glass slide. The slides were allowed to dry naturally for 12 hours and then stained with 10% Giemsa. All slides were coded and the cells were analyzed under an Olympus BX41 optical microscope.

Comet Assay

5 µl of blood was added to a glass slide and covered with a glass coverslip. The slides were kept in a

refrigerator until solidification. The coverslip was then removed and the slides were placed in a vertical glass tank containing refrigerated and light-protected lysis solution (100 mM ethylenediaminetetraacetic acid (EDTA), 2.5 M NaCl, 10 mM Tris, pH = 10, 1% Triton X-100 and 10% dimethylsulfoxide) for 7 hours. The slides were placed in a horizontal electrophoresis tank containing electrophoresis buffer (300 mM sodium hydroxide, 1 mM EDTA, pH > 13) for 20 minutes. The slides were then neutralized with 0.4 M Tris buffer (pH 7.5) for 24 hours. Finally, the slides were fixed with 15% trichloroacetic acid, 5% zinc sulfate, and 5% glycerol solution and stained with silver nitrate (Nadin, Vargas-Roig, Ciocca, 2001; Organization for Economic Cooperation and Development, 2016).

The samples were microscopically analyzed and categorized into five classes according to the size of the comet tail: zero (no genetic damage) to IV (maximum genetic damage). The DNA damage index was calculated by multiplying the total number of nucleoids observed in each damage class by the corresponding class number (Pitarque *et al*., 1999; Bücker, Carvalho, Alves-Gomes, 2006; Lima *et al*., 2019).

PDE Determination

The determination of the PDE of *Ginkgo biloba* L. dry extract administered orally was calculated using equation 1 based on the EMA ICH Q3C guideline (European Medicines Agency, 2022). For this purpose, the parameters described in Table I were used.

Equation 1:

$$PDE = \frac{NO(A)EL \times Body\ Weight}{F1 \times F2 \times F3 \times F4 \times F5}$$

TABLE I - Application of adjustment/uncertainty factors in the PDE calculation

Factors	Assessment *Based on ICH Q3C	Value considered (orally)
F1: interspecies extrapolation factor (2-12)	Extrapolation of data from mice to humans	12
F2: correction factor to account for variability between individuals (fixed at 10)	Interindividual variability	10
F3: safety factor that considers the duration of the studies (from 1 to 10)	Short study duration (less than 3 months in mice)	10
F4: safety factor related to severity of toxicity (from 1 to 10) (non-genotoxic carcinogenicity, neurotoxicity or teratogenicity)	Fetal toxicity in the absence of maternal toxicity	5
F5: variable factor to be applied if the NOEL is not established (up to 10) (NOAEL, LOEL or LOAEL)	The starting point is the NOAEL	1

The maximum residual concentration (MRC) determined in the cleaning validation study was then calculated using equations 2, 3 and 4. To do this, it was necessary to simulate information considering the batch size of the "worst case" product of the cleaning validation and its subsequent product on the production line, and the shared area in the equipment, among other data described in Table II.

Equation 2:

$$A = \frac{PDE \times FC}{MmaxTDsubs}$$

Equation 3 (Determination of the acceptance limit by area):

$$B = \frac{A \times MBSsubs}{SRSA}$$

Equation 4 (Determination of the acceptance limit in the analyzed sample):

$$C = \frac{B \times AREA}{VOLUME}$$

TABLE II - Fictitious data of the worst-case product, with active pharmaceutical ingredient *Ginkgo biloba* dry extract and subsequent product

Information	Values
Minimum batch size of the subsequent product in mL or g (MBSsubs)	100000 g
Minimum daily dose of contaminant in mg (MTDcont)	24 mg
Maximum daily dose of the subsequent product in g/day or ml/day (MmaxTDsubs)	2 g/day
Conversion factor from mg to µg (FC)	1000 µg/mg
Area shared by the products in cm^2 (SRSA)	100000 cm^2
Sampled area in cm^2 (AREA)	25 cm^2
Volume used for rinsing or swab recovery in mL (Volume)	5 mL
Permitted daily exposure (PDE)	1 mg/day

Statistical Analysis

Statistical analysis was carried out using Sigma Stat 2.03 (Jandel Scientific Corporation, San Rafael, CA, USA). Relative body weight gain and food intake were analyzed by two-way repeated measures (RM) analysis of variance (ANOVA), with treatment as the first factor and time interval as the second factor in the RM. One-way ANOVA was applied to the variables evaluated at a single moment (genotoxicity, biochemical, and hematological parameters). Post hoc Student Newman-Keuls was used to indicate where the differences were. One-way or two-way RM ANOVA was used after evaluating the normal distribution of the data. The differences were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

After analyzing the potential candidates for evaluation on a multi-purpose production line, *Ginkgo biloba* L. was selected for risk assessment because it is one of the best-selling medicinal plants and ranks as the fourth most reported plant with adverse effects (Micromedex®, 2023; Mei, 2017; Hauser, Gayowski,Singh, 2002; Benjamin *et al.*, 2001; Fessenden, Wittenborn, Clarke, 2001; Garcia, 1998; Vale, 1998; Blumenthal, 1987). In addition to the side effects already described for this species, there are some reports of drug interactions such as risk of bleeding when associated with anticoagulants, antiplatelet agents, non-steroidal anti-inflammatory drugs and/or thrombolytic agents; hypertension when concomitantly used with thiazide diuretics; decreased effectiveness of anticonvulsants and alteration of the effects of insulin, increasing its clearance; potentiation of the effects of monoamine oxidase inhibitors; and mental status changes when associated with buspirone or *Hypericum perforatum* (Bulario, 2023).

Acute Toxicity

The animals (n=3) were treated with a single oral dose of 2000 mg/kg *Ginkgo biloba* L.. None of the toxicity symptoms described in OECD 423 (Organization for Economic Cooperation and Development, 2001) was

observed in the first 24 hours and after 14 days, such as piloerection, eyelid ptosis, abdominal contortions, locomotion, hypothermia, muscle tone alterations, tremors, hind limb paralysis, salivation, bronchial secretion and seizures. Since the mice did not show any signs of toxicity, the limit test was performed in three additional animals. Also, female and male mice (Table III) did not show significant weight loss compared to the vehicle control group on the same day. The food intake of females and males (Table IV) was also unaffected by the treatment.

TABLE III - Body weight over 14 days of female and male BALB/c mice treated with vehicle (n=6) or 2000 mg/kg extract (n=6)

	Females		Males	
TREATMENT DAY	**Vehicle**	**Extract 2000 mg/kg**	**Vehicle**	**Extract 2000 mg/kg**
Day 1	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Day 4	101.4 ± 2.5	98.4 ± 2.7	97.2 ± 2.4	100.0 ± 2.6
Day 8	100.0 ± 0.0	96.8 ± 2.7	98.6 ± 2.4	103.5 ± 4.2
Day 12	100.0 ± 0.0	96.8 ± 2.7	97.3 ± 2.3	104.9 ± 4.2
Day 14	100.0 ± 0.0	96.8 ± 2.7	97.3 ± 2.3	104.9 ± 4.2

Note: Results expressed as mean ± standard deviation, considering day 1 as 100%. Two-way RM ANOVA. Females: F treatment (1,41) = 3.742, P = 0.124; F day (7,41) = 1.324, P = 0.286; F treatment x day (7,41) = 1.010, P = 0.451. Males: F treatment (1,41) = 5.678, P = 0.072; F day (7,41) = 1.266, P = 0.311; F treatment x day (7,41) = 0.891, P = 0.530.

TABLE IV - Food intake over 14 days of female and male BALB/c mice treated with vehicle (n=6) or 2000 mg/kg extract (n=6)

	Females		Males	
TREATMENT / **DAY**	**Vehicle**	**Extract 2000 mg/kg**	**Vehicle**	**Extract 2000 mg/kg**
Day 4	3.2 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	4.1 ± 0.1
Day 8	3.4 ± 0.2	3.7 ± 0.3	3.3 ± 0.2	4.6 ± 0.9
Day 12	3.3 ± 0.3	3.8 ± 0.3	3.7 ± 0.6	4.0 ± 0.4
Day 14	3.4 ± 0.1	3.7 ± 0.1	3.7 ± 0.3	3.8 ± 0.3

Note: Results expressed as mean ± standard deviation. Two-way RM ANOVA. Females: F treatment (1,15) = 6.025, P = 0.134; F day (3,15) = 1.494, P = 0.309; F treatment x day (3,15) = 0.243; P = 0.864. Males: F treatment (1,15) = 2.867, P = 0.232; F day (3,15) = 0.651, P = 0.611; F treatment x day (3,15) = 3.028; P = 0.115.

Based on the results presented above, it can be concluded that the *Ginkgo biloba* L. dry extract caused low acute toxicity (category 5), as there were no clinically relevant symptoms in the toxicological screening and no deaths during the observation period (2000 mg/kg dose). No changes in body weight were observed in the animals in the present study. However, Wang *et al.* (2015) reported significant weight gain in female and male mice treated with two oral doses of 21,500 mg/kg administered 4 hours apart. This weight change could be related to an increase in food intake, as the authors' results did not indicate clinical alterations

or death (Wang *et al*., 2015; Mei, 2017). It is suggested that the difference in the results of the present study may be related to the dose selected for the study, which followed the OECD recommendation and was also much lower than 43,000 mg/kg.

According to the EMA (European Medicines Agency, 2014), the seeds and leaves of *Ginkgo biloba* L. contain ginkgotoxin, a substance with reported cases of intoxication. At sublethal doses, reported symptoms of poisoning include epileptiform seizures, unconsciousness, and leg paralysis. An 11 mg/kg dose of ginkgotoxin alone triggered seizures in test animals. In addition, intraperitoneal administration of 30 to 50 mg/kg caused atrioventricular block and animal death. Doses higher than 400 to 600 mg/kg via the intraperitoneal route were required to cause convulsions in rats. In the present study, the amount of ginkgotoxin in the doses tested is unknown. Leistner and Drewke (2010) reported that the highest concentration of ginkgotoxin in *Ginkgo biloba* L. leaves was 5 µg, corresponding to 7 µg/g in leaves collected in early August. In addition, ginkgotoxins are also present in *Ginkgo biloba* L. seeds (Leistner, Drewke, 2010).

Repeated Dose Toxicity

The recommended therapeutic dose for *Ginkgo biloba* L. based oral formulations for adults and elderly patients is 240 mg/day or 120-240 mg (single dose). According to medical recommendations, treatment should last at least eight weeks to achieve efficacy (European Medicines Agency, 2015; Bulario, 2023).

Within this context, the doses selected for subchronic treatment in mice were 120, 240, and 480 mg/kg for 28 days, corresponding to 1x, 2x, and 4x the maximum daily dose according to the therapeutic range indicated in the package insert (Organization for Economic Cooperation and Development, 2008).

Control of Body Mass Gain

Following subchronic treatment with the dry extract of *Ginkgo biloba* L., alterations in the weight gain of female and male mice (Table V) were observed compared to the control group.

In female mice, the two-way RM ANOVA indicated a significant effect of treatment ($F_{(3,188)} = 4.143$; $P = 0.017$) and day of treatment ($F_{(6,188)} = 12.328$; $P < 0.001$), as well as a significant interaction between these two variables ($F_{(18,188)} = 1.887$; $P = 0.022$). The post hoc test indicated differences on days 4, 8, 18 and 22, with different doses causing significant weight loss (Table V). In male mice, the two-way RM ANOVA indicated a significant effect only on day of treatment ($F_{(3,230)} = 2.951$; $P = 0.049$; $F_{(6,230)} = 4.628$; $P < 0.001$; $F_{(18,230)} = 1.438$; $P = 0.119$) (Table V).

TABLE V - Control of body mass gain in female and male BALB/c mice

	Females				Males			
	Control	**Extract 120 mg/kg**	**Extract 240 mg/kg**	**Extract 480 mg/kg**	**Control**	**Extract 120 mg/kg**	**Extract 240 mg/kg**	**Extract 480 mg/kg**
Day 1	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Day 4	98.3 ± 4.2	96.1 ± 3.6	95.9 ± 1.8	92.9 ± 4.0*	99.9 ± 5.6	99.0 ± 2.9	101.3 ± 3.1	97.6 ± 3.1
Day 8	99.5 ± 4.0	97.6 ± 2.6	93.9 ± 3.6*	94.4 ± 3.4*	102.8 ± 5.6	99.0 ± 3.8	103.3 ± 3.2	98.9 ± 4.7
Day 13	98.0 ± 3.7	97.7 ± 4.8	93.1 ± 5.6	95.1 ± 2.8	101.4 ± 6.2	98.0 ± 3.8	102.9 ± 3.4	99.0 ± 3.6
Day 18	98.1 ± 5.0	92.9 ± 2.5*	91.1 ± 5.3**	93.0 ± 2.5*	98.8 ± 5.6	96.5 ± 2.5	101.4 ± 4.4	97.2 ± 3.0

TABLE V - Control of body mass gain in female and male BALB/c mice

	Females				Males			
	Control	**Extract 120 mg/kg**	**Extract 240 mg/kg**	**Extract 480 mg/kg**	**Control**	**Extract 120 mg/kg**	**Extract 240 mg/kg**	**Extract 480 mg/kg**
Day 22	99.5 ± 4.0	95.3 ± 2.9*	93.1 ± 5.1**	92.3 ± 6.3**	100.4 ± 5.6	95.3 ± 2.8	100.8 ± 3.6	97.2 ± 2.1
Day 28	98.9 ± 4.8	94.5 ± 1.7	95.9 ± 3.2	93.7 ± 3.6	99.0 ± 5.5	98.1 ± 3.2	102.0 ± 1.8	97.2 ± 2.1

Note: Percentage values in relation to day 1. Values expressed as mean ± standard deviation. Two-way RM ANOVA post hoc Student Newman-Keuls. Difference in relation to control on the same day *P < 0.05; **P < 0.01.

Food Intake

Based on the two-way RM ANOVA, in female mice (Figure 1A) there was no significant interaction between treatment and week (F treatment (4,51) = 0.673; P = 0.617; F week (3,51) = 1.095; P = 0.400; F treatment x week interaction (12,51) = 1.313; P = 0.277). In male mice (Figure 1B), there was a significant difference only for the highest dose during the first week of treatment (F treatment (4,51) = 3.624; P = 0.020; F week (3,51) = 2.030; P = 0.180; F treatment x week interaction (12,51) = 1.900; P = 0.090).

The results of the subchronic treatment indicate that *Ginkgo biloba* L. dry extract caused weight gain alterations only in female mice, especially on days 18 and 22 for all doses tested. However, females did not show differences in food intake, whereas males showed a decrease in food intake during the first week of treatment when treated with the highest dose (480 mg/kg). A study carried out by the National Toxicology Program (NTP) to evaluate the toxicity of *Ginkgo biloba* L. dry extract in male and female mice treated with 0, 125, 250, 500, 1000, and 2000 mg/kg for 3 months showed a significant decrease in body weight only in females treated with 2000 mg/kg. The other doses tested did not alter body weight (Burnett, 2018).

In a subchronic study to assess the toxicity of polyprenols (natural active lipids present in *Ginkgo biloba* L. dry leaves), female mice were treated with 500, 1000, and 2000 mg/kg for 91 days. The results obtained showed no significant variations in body weight and food intake (Wang *et al*., 2015).

Another study conducted by the NTP (National Toxicology Program, 2003) with rats treated (by gavage) with 0, 62.5, 125, 250, 500, and 1000 mg/kg of *Ginkgo biloba* L. dry extract for 14 weeks showed that the mean body weight of all experimental groups was similar to the control. These results differ from the present study, which indicated a significant decrease in body weight of female mice on days 4, 8, 18 and 22. Although the doses tested in both studies are close, the treatment duration was different, which may account for the difference in results.

On the other hand, the NTP (National Toxicology Program, 2003) reported a study of mice treated (by gavage) with 0, 125, 250, 500, 1000, and 2000 mg/kg of *Ginkgo biloba* L. dry extract for 14 weeks. The results indicated that the mean body weight of females treated with 2000 mg/kg was significantly lower than the control. These results corroborate the observation of the present study, although the duration and doses involved are different.

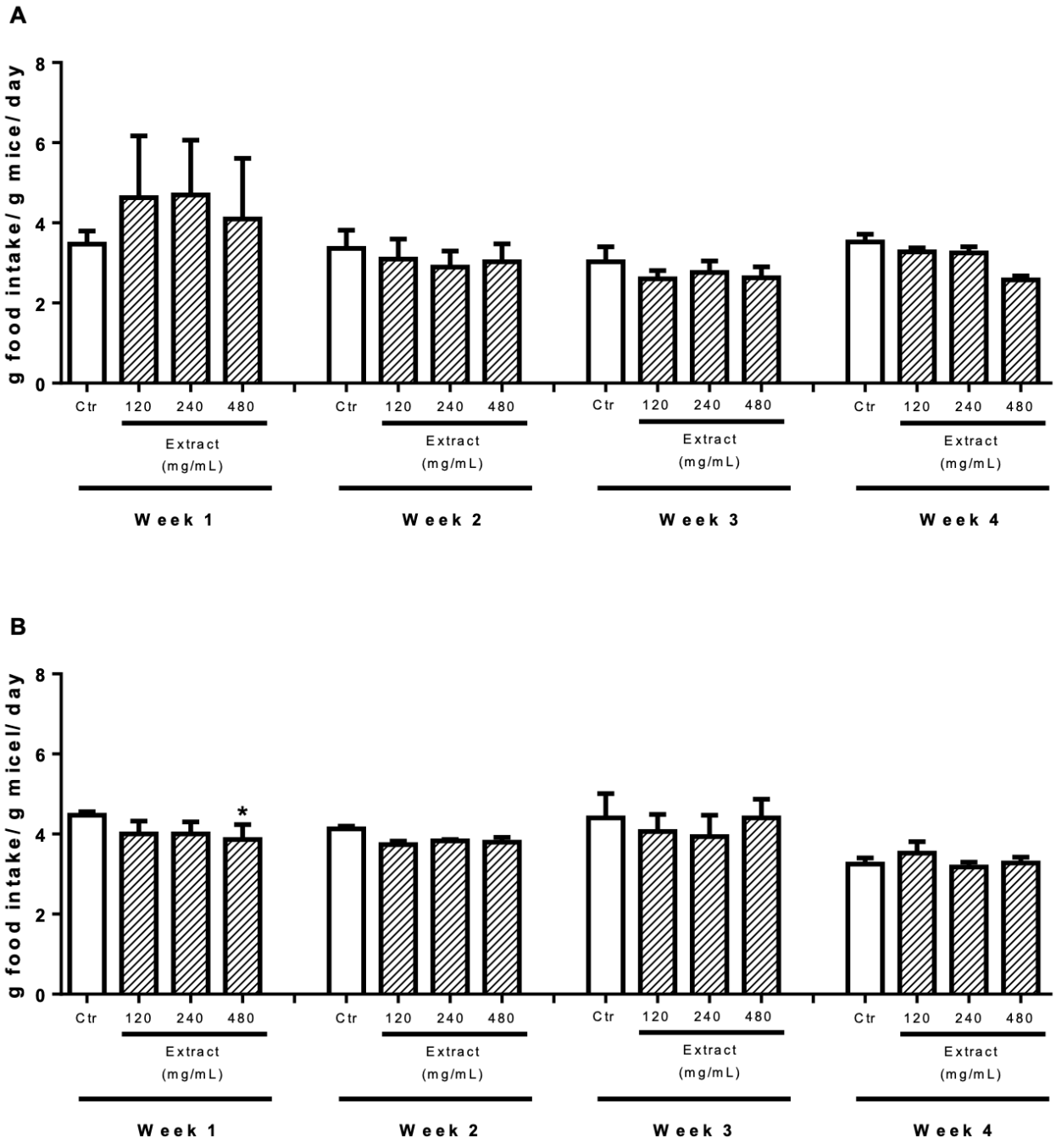

FIGURE 1 - Food intake of females (A) and males (B). Daily food intake in grams of chow per animal. Control and extract. Values expressed as mean ± error. Two-way RM ANOVA post hoc Student Newman-Keuls. Difference in relation to the vehicle in the same week *P<0.05.

Hematological Parameters

In female mice, the potential of the extract to cause anemia was analyzed based on the following parameters: hematocrit (HCT), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). These parameters were not altered by repeated administration of the extract at different doses compared to the control (HCT F (3,26) = 0.429; P = 0.734; Hb F (3,26) = 0.606; P = 0.618; MCH F (3,26) = 0.270; P =

0.846; MCHC F (3,26) = 0.336; P = 0.799), except for MCV (F (3,26) = 5.199; P = 0.007). In addition, the extract did not cause anisocytosis (RDW F (3,26) = 0.978; P = 0.420), alteration in platelet counts (platelets F (3,25) = 2.405; P = 0.095), or alteration in the concentration of red blood cells (erythrocytes F (3,26) = 0.375; P = 0.772). However, it did alter leukocyte counts (WBC F (3,26) = 5.751; P = 0.004) (Table VI). In the evaluation of blood distensions as a complementary parameter to the equipment, *Ginkgo biloba* L. dry extract increased neutrophil counts (F (3,14) = 10.66; P = 0.0007) at the highest dose, decreased lymphocyte counts (F (3,14) = 8.517; P = 0.0018) at the lowest and highest doses, but it did not alter the number of monocytes (F (3,15) = 0.8020; P = 0.5120) (Table VI).

Male mice showed no alterations in hematocrit (HCT F (3,29) = 0.1967; P = 0.8978), hemoglobin (Hb F (3,29) = 0.7377; P = 0.5382), mean corpuscular volume (MCV F (3,29) = 0.6510; P = 0.5888), mean corpuscular hemoglobin (MCH F (3,29) = 0.1219; P = 0.9465), mean corpuscular hemoglobin concentration (MCHC F (3,29) = 0.3907; P = 0.7606) and anisocytosis (RDW F (3,29) = 2.042; P = 0.1299). Alterations in platelet counts (platelets F (3,29) = 0.05592; P = 0.9823), total leukocyte concentration (WBC F (3,29) = 0.41506; P = 0.7435) and red blood cell concentration (erythrocytes F (3,29) = 0.2336; P = 0.8722) were not observed either (Table VI). The analysis of blood distensions indicated that the *Ginkgo biloba* L. dry extract did not cause any alterations in the number of neutrophils (F (3,29) = 0.6469; P = 0.5913), lymphocytes (F (3,29) = 0.3883; P = 0.7622) and monocytes (F (3,29) = 0.3053; P = 0.8213) at any of the doses tested (Table VI).

The evaluation of hematological parameters in blood distensions revealed alterations in leukocyte, neutrophil and lymphocyte counts in female mice, whereas no hematological parameter was altered in males. A 3-month study carried out by the NTP with doses up to 2000 mg/kg showed that female mice treated with ≥ 500 mg/kg had a significant decrease in total leukocyte counts, while reticulocyte counts decreased in both groups and platelet counts increased in male mice (National Toxicology Program, 2013). Conversely, the EMA (European Medicines Agency, 2014) reported no evidence of hematological damage in the chronic toxicity assessment of the extract in a 6-month study administering daily doses of 20, 300, 400, and 500 mg/kg to rats and 100, 300, and 400 mg/kg to dogs.

TABLE VI - Hematological parameters of female and male BALB/c mice

	Females				Males			
	Control	**Extract 120 mg/kg**	**Extract 240 mg/kg**	**Extract 480 mg/kg**	**Control**	**Extract 120 mg/kg**	**Extract 240 mg/kg**	**Extract 480 mg/kg**
Hb (g/dL)	13.6 ± 0.3	13.8 ± 0.4	13.7 ± 0.5	12.3 ± 4.7	13.9 ± 0.5	13.9 ± 0.4	13.9 ± 0.4	14.2 ± 0.5
HCT (%)	44.6 ± 1.3	45.6 ± 1.8	45.1 ± 3.0	40.9 ± 16.2	46.2 ± 3.1	45.5 ± 2,5	45.7 ± 2.2	46.4 ± 2.6
MCV (μm^3)	53.8 ± 0.7	53.4 ± 0.4	52.8 ± 0.4*	52.4 ± 0.9**	53.1 ± 0.5	52.6 ± 1.2	52.9 ± 0.7	52.6 ± 0.6
MCH (pg)	16.5 ± 0.3	16.2 ± 0.3	16.1 ± 0.7	16.5 ± 1.8	16.0 ± 0.5	16.2 ± 0.7	16.1 ± 0.3	16.1 ± 0.6
MCHC (g/dL)	30.6 ± 0.6	30.3 ± 0.7	30.5 ± 1.4	31.4 ± 4.0	30.2 ± 1.1	30.7 ± 0.9	30.5 ± 0.9	30.7 ± 1.3
RDW (%)	12.7 ± 0.4	12.3 ± 0.4	13.0 ± 0.5	12.9 ± 1.3	12.4 ± 0.7	12.5 ± 0.5	12.2 ± 0.6	12.9 ± 0.5
Platelets (x $10^3/mm^3$)	569.4 ± 104.1	463.7 ± 71.6	499.7 ± 108.2	376.1 ± 94.4	632.3 ± 40.5	646.4 ±93.1	658.9 ± 74.7	641.4 ± 143.0
RBC (x $10^6/mm^3$)	8.3 ± 0.2	8.5 ± 0.3	8.5 ± 0.5	7.7 ± 3.0	8.7 ± 0.5	8.6 ± 0.5	8.6 ± 0.3	8.8 ± 0.5
WBC (x 10^6)	10.9 ± 4.3	8.1 ± 1.4	6.2 ± 1.7**	5.5 ± 2.2**	7.9 ± 2.9	8.4 ± 2.4	7.6 ± 2.5	7.1 ± 1.6

TABLE VI - Hematological parameters of female and male BALB/c mice

	Females				Males			
	Control	**Extract 120 mg/kg**	**Extract 240 mg/kg**	**Extract 480 mg/kg**	**Control**	**Extract 120 mg/kg**	**Extract 240 mg/kg**	**Extract 480 mg/kg**
Neutrophils	17.0 ± 4.6	25.2 ± 2.7	21.6 ± 2.9	30.4 ± 4.0***	21.9 ± 5.8	26.9 ± 6.8	23.1 ± 7.3	26.3 ± 12.4
Lymphocytes	73.7 ± 4.7	61.8 ± 3.9*	68.0 ± 5.5	56.6 ± 5.8**	70.4 ± 5.0	66.6 ± 7.2	69.9 ± 8.3	66.4 ± 14.4
Monocytes	7.33 ± 1.2	9.2 ± 1.8	8.4 ± 1.5	8.7 ± 1.9	6.9 ± 1.1	6.0 ± 2.3	6.5 ± 1.9	6.3 ± 1.9

Note: Values expressed as mean ± standard deviation. ANOVA post hoc Student Newman-Keuls. Difference from control *P < 0.05; **P < 0.01; ***P < 0.0001.

Abbreviations: hemoglobin (Hb), red blood cell count (RBC), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular cell hemoglobin concentration (MCHC), red cell distribution width (RDW), neutrophils, lymphocytes, monocytes, eosinophils, leukocyte counts (WBC).

Biochemical Parameters

The subchronic administration of *Ginkgo biloba* L. dry extract to female mice did not alter glycemia (glucoses F (3,26) = 2.092; P = 0.129), renal parameters (creatinine F (3,20) = 0.9121; P = 0.4528; urea F (3,13) = 1.147; P = 0.3671 and uric acid F (3,23) = 3.135; P = 0.0450), or hepatic parameters (AST F (3,20) = 2.592; P = 0.0812; ALT F (3,19) = 0.8050; P = 0.5065). In the lipid profile, however, there was a significant increase in total cholesterol (cholesterol F (3,23) = 4.017; P = 0.0195) in animals treated with 480 mg/kg (132.9 ± 19.17 mg/dL) compared to the control (92.57 ± 14.72 mg/dL). Triglyceride levels were not altered (triglycerides F (3,23) = 0.2702; P = 0.8462; HDL F (3,23) = 0.2031; P = 0.8932 and LDL F (3,21) = 2.802; P = 0.0649) (Table VII).

For male mice, serum glucose (F (3,31) = 0.146; P = 0.932), creatinine (F (3,22) = 1.325; P = 0.2916), HDL (F (3,23) = 1.192; P = 0.338), LDL (F (3,23) = 0.682; P = 0.573; P = 0.6278), cholesterol (F (3,23) = 0.207; P = 0.891), AST (F (3,23) = 0.7987; P = 0.5073), ALT (F (3,26) = 0.2053; P = 0.8918), and uric acid (F (3,25) = 1.161; P = 0.3442) did not show any alterations. Triglyceride levels were significantly higher (F (3,23) = 3.458; P = 0.036) at the treatment dose of 120 mg/kg (189.0 ± 46.1 mg/mL), whereas urea levels were significantly lower in animals treated with 480 mg/kg (33.67 ± 7.062) (F (3,20) = 4.697; P = 0.0122) compared to the control (46.00 ± 6.841) (Table VII).

In the present study, the glycemic profile of males and females was not altered at the three doses tested (120, 240 and 480 mg/kg). On the other hand, Wang *et al.* (2015) administered *Ginkgo biloba* L. dry extract orally at doses of 500, 1000 and 2000 mg/kg for 91 days and observed a reduction in glucose levels in animals treated with 1000 and 2000 mg/kg. Wang *et al.* (2015) could not confirm that the extract caused an adverse effect, although the weight of the kidney and liver increased more in males than in females. With approximately three times longer treatment duration, no change in glucose levels was observed in animals treated with 500 mg/kg, as in the present study with lower test doses.

Hepatic toxicity was not observed in the present study, which is consistent with the EMA (European Medicines Agency, 2014), in which two other studies reported no evidence of organ damage or hepatic impairment. One study evaluated the chronic toxicity of the dry extract over six months, through oral administration of daily doses of 20, 300, 400, and 500 mg/kg in rats. In another study, rats and mice were treated for 27 weeks with oral doses of dry extract ranging from 100 to 1600 mg/kg.

TABLE VII - Biochemical parameters of female and male BALB/c mice

	Females				Males			
	Control	**Extract 120 mg/kg**	**Extract 240 mg/kg**	**Extract 480 mg/kg**	**Control**	**Extract 120 mg/kg**	**Extract 240 mg/kg**	**Extract 480 mg/kg**
GLU (mg/dL)	259.3 ± 60.0	283.0 ± 37.0	226.8 ± 34.6	262.3 ± 23.7	302.4 ± 81.4	286.6 ± 96.7	276.7 ± 57.8	284.2 ± 67.3
COL (mg/dL)	92.57 ± 14.7	120.3 ±14.9	106.9 ± 34.9	132.9 ± 19.2*	155.5 ± 39.1	152.3 ± 30.4	164.7 ± 29.2	161.5 ± 18.2
TRI (mg/dL)	116.6 ± 52.2	122.8 ± 31.2	106.7 ± 32.9	120.7 ± 16.4	135.8 ± 47.2	189.0 ± 46.1*	133.3 ± 14.7	169.0 ± 21.5
HDL (mg/dL)	64.4 ± 9.7	63.7 ± 18.8	64.1 ± 27.9	70.3 ± 9.3	76.3 ± 27.5	80.3 ±38.4	99.3 ± 16.2	97.0 ±14.7
LDL (mg/dL)	12.6 ± 8.8	30.3 ± 10.5	21.3 ± 11.4	38.3 ± 25.5	52.2 ± 40.4	34.3 ± 19.9	38.8 ± 31.9	30.8 ± 5.3
AST (U/L)	208.1 ± 77.5	270.2 ± 51.9	164.6 ± 57.9	241.7 ± 61.2	182.6 ± 66.6	206.7 ± 40.9	170.5 ± 39.3	176.3 ± 28.5
ALT (U/L)	29.5 ± 7.9	39.7 ± 14.1	38.8 ± 28.1	49.9 ± 30.9	33.0 ± 17.8	31.6 ± 15.5	27.6 ± 15.3	34.1 ± 20.7
CRE (mg/dL)	0.4 ±0.2	0.5 ±0.2	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.3	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.03
Urea (mg/dL)	34.7 ± 18.9	38.7 ± 4.3	52.7 ± 15.4	45.4 ± 15.9	46.0 ± 6.8	40.6 ± 3.1	38.6 ± 4.9	33.7 ± 7.0*
Uric acid (mg/dL)	4.5 ± 1.6	3.1 ± 1.2	3.5 ± 0.8	4.8 ± 0.9	4.3 ± 1.2	4.5 ± 0.8	5.5 ± 1.4	4.9 ± 1.4

Note: Values expressed as mean ± standard deviation. ANOVA post hoc Student Newman-Keuls. Difference from vehicle *P<0.05.

Abbreviations: blood glucose levels (GLU), total cholesterol (COL), high-density lipoproteins (HDL), low-density lipoproteins (LDL), triglycerides (TRI), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CRE).

In this study, female mice did not show any alterations in renal functions. However, male mice treated with 480 mg/kg showed a significant decrease in urea levels. Other studies evaluated the chronic toxicity of *Ginkgo biloba* L. dry extract in rats (27 weeks) and dogs (26 weeks), initially at doses of 20 and 100 mg/kg/day, respectively. During these studies, the doses were gradually increased to 500 mg/kg/day in rats and 400 mg/kg/day in dogs; the results showed no evidence of organ damage or liver or kidney impairment (European Medicines Agency, 2014).

The lipid profile of males did not show any changes at the doses evaluated, unlike females treated with 480 mg/kg, which showed an increase in total cholesterol without changes in triglycerides. In the study by Wang *et al.* (2015), triglyceride levels decreased in males treated with 1000 and 2000 mg/kg. Thus, the authors concluded that *Ginkgo biloba* L. reduces blood lipids. On the other hand, the results obtained in females at the dose of 480 mg/kg were different from those reported by Wang *et al.* (2015), who administered a similar dose (500 mg/kg) for more than three times the duration of the present study.

Genotoxicity

The genotoxicity of *Ginkgo biloba* L. dry extract was evaluated by micronucleus and comet assays.

Figure 2 shows the results of the damage index (DI) obtained in the comet assay of female (A) and male (B) mice after subchronic treatment with doses of 120, 240, and 480 mg/kg. The extract did not increase the damage index in lymphocytes at any of the doses tested in females ($F(3,20) = 0.8127$, $P = 0.5043$) and males ($F(3,32) = 0.1518$, $P = 0.9277$).

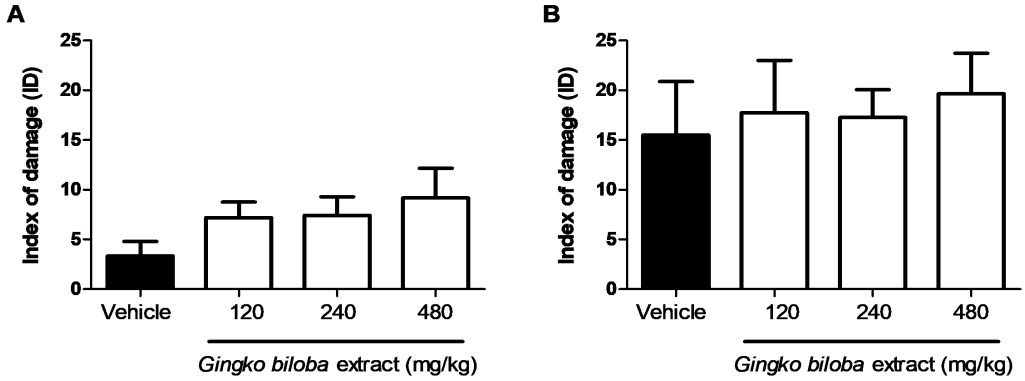

FIGURE 2 - Comet assay, damage index in lymphocytes of (A) female and (B) male mice after subchronic oral administration of the extract. Values expressed as mean ± error. Females F (3,20) = 0.8127, P = 0.5043. Males F (3,32) = 0.1518, P = 0.9277.

The comet assay showed that *Ginkgo biloba* L. dry extract did not significantly alter the damage index and the percentage of DNA in the tail. This result suggests that *Ginkgo biloba* L. has no potential to induce DNA damage in the range of doses tested. This evidence corroborates the results of a study by Maeda *et al.* (2014), in which no difference in the percentage of DNA in the tail was observed in male and female mice treated with *Ginkgo biloba* L. at doses of 500, 1000 and 2000 mg/kg/day. Furthermore, the EMA (European Medicines Agency, 2014) confirmed, through genotoxicity studies, the negative results for chromosomal mutations in two *in vivo* trials distinguishing peripheral erythrocytes and bone marrow cells in mice.

Figure 3 shows the frequency of micronuclei (MN) in 2000 polychromatic lymphocytes (PCD) (immature) from the bone marrow of female (3A) and male (3B) mice. After subchronic administration of the *Ginkgo biloba* L. dry extract, the frequency of MN was not significantly altered in females (F (3,26) = 0.1141, P = 0.9509) and males (F (3,31) = 0.9921, P = 0.4109) at the doses tested.

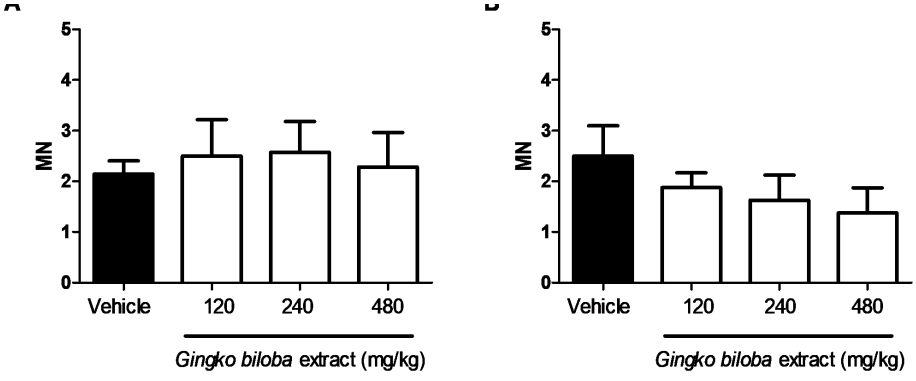

FIGURE 3 - Micronucleus test, micronuclei in 2000 bone marrow cells of (A) female and (B) male mice after subchronic oral administration of the extract. Values expressed as mean ± error. Females F (3,26) = 0.1141, P = 0.9509. Males F (3,31) = 0.9921, P = 0.4109.

A total of 1000 cells were counted per treatment, distinguishing between polychromatic (PCE) and normochromatic (NCE) erythrocytes. After subchronic administration, the results of the doses tested did not show a significant difference compared to the vehicle in male mice (Figure 4); however, a dose-dependent

relationship was observed in female mice (Figure 5). This significant increase in the PCE/NCE rate at the highest dose tested in females is due to the increase in immature cells, but has no clinical relevance.

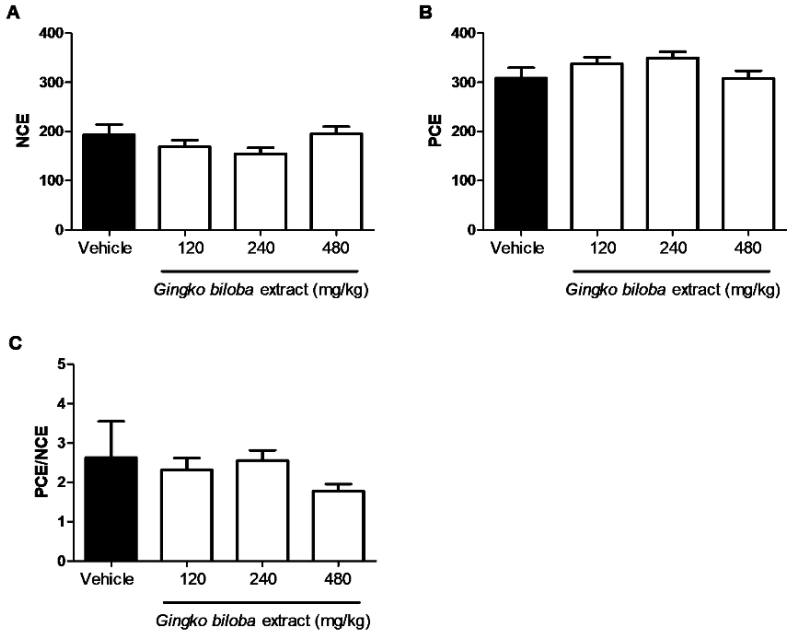

FIGURE 4 - Micronucleus test in 1000 bone marrow cells of male mice after subchronic oral administration of the extract. (A) Normochromatic lymphocytes (NCE) F (3,63) = 1.605, P = 0.1977. (B) Polychromatic lymphocytes (PCE) F (3,63) = 1,837, P = 0.1502. (C) PCE/NCE ratio F (3,63) = 0.5772, P = 0.6322. Values expressed as mean ± error.

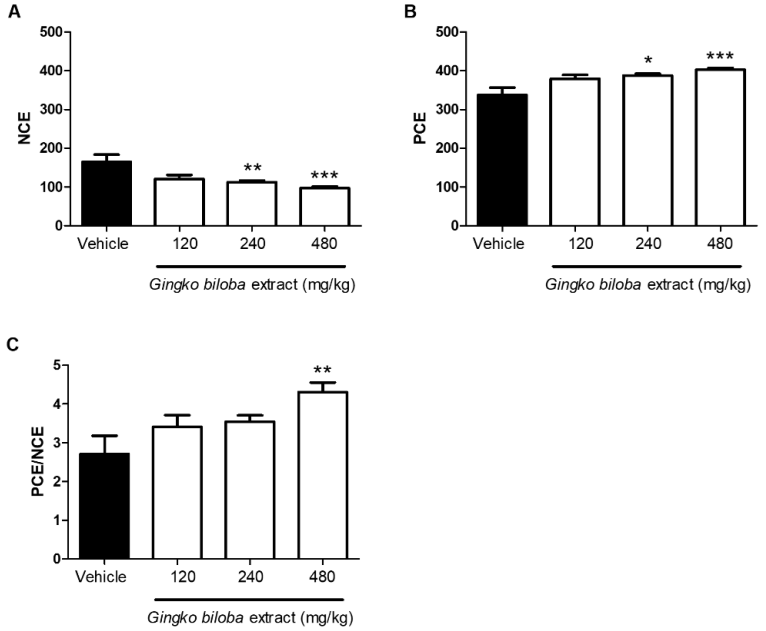

FIGURE 5 - Micronucleus test in 1000 bone marrow cells of female mice after subchronic oral administration of the extract. (A) Normochromatic lymphocytes (NCE) F (3,53) = 6,681, P = 0.0007. (B) Polychromatic lymphocytes (PCE) F (3,53) = 6,489, P = 0.0009. (C) PCE/NCE ratio F (3,53) = 4.310, P = 0.0088. Values expressed as mean ± error. ANOVA post hoc Tukey. Different from vehicle **P<0.01; ***P<0.001.

Based on the data presented above, it is possible to conclude that *Ginkgo biloba* L. did not induce potential chromosome breaks or total loss of chromosomal DNA. These outcomes suggest that genomic mutations were not induced, corroborating the results obtained by Maeda *et al.* (2014), who observed the absence of significant alterations in polychromatic erythrocyte percentages after oral treatment of mice for 3 days at doses of 500, 1000, and 2000 mg/kg/day. Caria *et al.* (1995) demonstrated that there is no induction of micronuclei by quercetin in mice treated with doses up to 558 mg/kg. Quercetin is one of the most common flavonoids present in plants. It was also present in the dry extract used in this study at levels ranging from 22 to 27%.

PDE Determination

The determination of the PDE of *Ginkgo biloba* L. dry extract administered orally, based on the results of the present study, was 0.1 mg/day. No correction factor was applied for the route of administration, as it is the same in humans. The NOAEL was considered to be 120 mg/kg/day, as this dose did not cause any side effects in the present study.

The PDE value of *Ginkgo biloba* L. dry extract was used to calculate the MRC, resulting in a concentration of 250 µg/mL. This value corresponds to the reception limit in the residual sample. Considering that the maximum residual limits of products between PDE and 10 ppm or 1/1000 must be compared and from this the lowest value obtained must be adopted as an acceptable residual limit, the health risk to patients using medicines based on this extract is mitigated.

Based on the evaluation of the results presented, it is possible to affirm that the procedures implemented to remove residues of *Ginkgo biloba* L. from the production line are safe and do not need to be re-evaluated based on the MRC result, since the PDE was 25 times higher than the value initially determined (10 ppm; µg/mL).

CONCLUSION

The present study shows that *Ginkgo biloba* L. has low acute toxicity and no genotoxicity based on micronucleus and comet assays. However, some alterations (such as renal and hepatic toxicity, lipid and glycemic parameters, body weight gain, and food intake) were observed when toxicity was evaluated at repeated doses, mainly in female mice treated with 480 mg/kg of *Ginkgo biloba* L. dry extract.

Initially, the PDE was determined to be 0.1 mg/day; however, considering the MRC, a value of 250 µg/mL was obtained. In other words, it was higher than 10 µg/mL, which is considered the permitted MRL after cleaning validation of the equipment. Therefore, it is possible to affirm that the current cleaning validation is safe for patients treated with medications containing *Ginkgo biloba* L. dry extract and does not need to be re-evaluated. However, for a detailed evaluation of any plant, data from other pre-clinical studies, including carcinogenicity and reproductive toxicity, as well as clinical data reporting on the use of the plant, must be considered.

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