

HEPATOTOXIC AND NEPHROTOXIC EFFECTS OF ORAL ADMINISTRATION OF HERBALIFE® PRODUCTS IN MICE

EFEITOS HEPATOTÓXICOS E NEFROTÓXICOS DA ADMINISTRAÇÃO ORAL DE PRODUTOS HERBALIFE® EM CAMUNDONGOS

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Abstract

The increase in the consumption of dietary supplements has been growing and the absence of pre-clinical studies that demonstrate the impact of the consequences of their chronic consumption is the biggest gap. Therefore, the objective of the present study was to evaluate the effects of supplements of chronic use of Herbalife® products (Shake and Tea). For this, the caffeine in the products was first quantified. Mice were separated into four groups: Control, Tea, Shake and Shake + Tea and treated for 28 days by gavage. The weight of the animals, the open field test, biochemical tests, morphometric analysis and the oxidative stress of the kidney and liver were evaluated. Our results showed the detection of caffeine in the Shake a 10-fold higher dose in tea samples. Animals exposed to the “Shake+Tea” association show changes in their behavior pattern. In addition, we observed an AST increase (+175%) in Shake + Tea for the control group, as well as a ~10-fold increase in AOPP in kidney tissue for all groups. Histopathological of liver and kidney and biochemical analysis tissue showed no changes in the experimental groups. Our results indicate that the chronic use of Herbalife® products contributes to changes in the pattern of motor behavior, increased AST and protein oxidation in renal tissue. In this sense, it is suggested that more experimental studies are needed to support the safety of these products that are widely consumed by the general population.

Keywords: herbal products; non-herbal products; protein oxidation; hepatotoxicity, nephrotoxicity.

1 INTRODUCTION

Since ancient times until today, herbs have been widely used for medicinal purposes in most of the countries due to its human health benefits (DZOBO, 2022; VERMA AND THULUVATH, 2007; LEE, BAE, 2017) and/or as a complementary nutritional supplement (ELINAV *et al.*, 2007). However, has been observed an excessive increase in the use of dietary supplements from natural and/or industrial origin, due to the strong appeal to the healthy lifestyle (ZHANG *et al.*, 2020). This herbs product is associated with the misconception that herbal products have being considered “natural and free of adverse reactions” (SCHOEPFER *et al.*, 2007), raise unfounded hopes and expectations for the application of these herbs in the treatment of different diseases (STICKEL, 2007). Furthermore, there is no relevant evidence of the beneficial clinical effects of these types of products in short, medium and/or long-term application (ELINAV *et al.*, 2007; SCHOEPFER *et al.*, 2007). In addition, the exposed dose can cause organ toxicity due to plants that produce metabolites that can be toxic to health (LÓPEZ-GIL *et al.*, 2017; GULDIKEN *et al.*, 2018), causing risks to human health

due to unnecessary supplementation (STICKEL *et al.*, 2010). In addition, herbal-drug interaction should be evaluated as they may reduce the drug's effectiveness or cause toxicity (BORSE, SINGH AND NIVSARKAR, 2019).

These products are based on plants or even herbs fortified with different nutrients (eg vitamins and oligosaccharides), however, their exact composition is unknown (SCHOEPFER *et al.*, 2007). In this sense, the absence of experimental studies showing the impact of the toxicity of these products can be considered the greatest gap in understanding the potential consequences of their chronic consumption. Although this, the consumption of herbal products whit “benefits to human health” has steadily increased (ELINAV *et al.*, 2007; SCHOEPFER *et al.*, 2007; STICKEL *et al.*, 2011; MENGUAL-MORENO *et al.*, 2015).

A classic example is Herbalife® products, that are related to harmful effects (TESCHKE *et al.*, 2013; JURČIĆ *et al.*, 2019). Herbalife® is an enriched over-the-counter dietary supplement enriched with herbs that promise to lose weight and have a “healthy life” has people who consume (JURČIĆ *et al.*, 2019), however, you consume has associated a

hepatotoxicity (ELINAV *et al.*, 2007; JURČIĆ *et al.*, 2019). In addition, it should be noted that individuals consume more than one product at the same time, which increases the chance of toxicity (JURČIĆ *et al.*, 2019).

Due to these factors mentioned above, there is a need to evaluate the intoxication mechanism of herb-based products that are highly consumed in the world. Therefore, the aim of the present study was to evaluate the effects of the chronic use of two products widely used as dietary supplements, Shake (non-herbal based) and Tea (herbal based), on the behavioral and motor biochemical effects, Histological parameters and protein oxidation effects in Balb/C mice, mitigating potential clinical confounders.

3 MATERIAL AND METHODS

Identification and quantification of caffeine

The thin layer chromatography was performed on silica gel GF254 precoated plate as stationary phase (adsorbent) and solvent system of ethyl acetate: methanol and water (25;3,3;1,25) as mobile phase. Samples of the shake (5 µL of a solution in 1% methanol), Tea (5 µL of a solution in 1% methanol) and standard of caffeine (solution in 1% methanol) were applied in bands of 1 cm. After end of the elution, the plate was dried at room temperature and derivatization was done by ultraviolet light at 254nm. The R_f value of 0,5 corresponded the standard caffeine and then was compared with samples (SIRIWARDANE, DHARMADASA, SAMARASINGHE, 2013).

The high-performance liquid chromatography (HPLC) analysis was performed using a WATER HPLC system with manual injector, UV detector of 273nm and column Nucleosil RP 18 (250 mm X 4,4 mm and 3 µm). The mobile phase was a mixtures 1:1 of methanol:water (TFA 0,3%) in isocratic

elution condition and flow of 0,50ml/min. Standard solution of the caffeine in 50, 25, 12,5, 6,25, 3,125 and 1,562 µg/mL concentration were used to obtain the calibration curve. The equation was used to determine the caffeine concentration in the samples of shake and tea previously prepared by extraction, in ultrasonic apparatus, 1g of samples in 25mL of methanol (Shehata AB, Rizk MS, Rend EA 2016).

Animals

The experiments were conducted using male Balb/C mice weighing between 25 and 40 g which were obtained, bred and maintained from the animal care facility at the Experimental Monitoring Laboratory of Vila Velha University (UVV). The mice were housed in individual plastic cages with air exhaustion, controlled temperature (~22°C) and humidity (60%) and were exposed to a 12/12-h light–dark cycle. The availability of feed and water *ad libitum* occurred for 15 hours/day and remaining time was replaced by oral supplementation with herbal (Tea) and non-herbal (Shake) as described below. The data collection was done always during the same period of the day and the analysis was always blind to minimize the effects of subjective. All experimental procedures were performed in accordance with the guidelines for the care and handling of laboratory animals as recommended by the National Institutes of Health (NIH Publication N 85-23, 1996), and the study protocols were approved by the Institutional Animal Care Committee of the UVV (CEUA-UVV: # 299/2013).

Experimental group and oral Supplementation

Mice were randomized into four groups: 1) Control (semi-skimmed milk, 0.5mL/day, n= 10); 2) Tea (0.5mL/day, n= 12); 3) Shake (0.5mL/day, n= 12); 4) Shake+Tea (0.25mL/day each one, n=

12). All the treatment were for 28 days and made by gavage. While Tea was solubilized in water (0.35 g of Tea in 6.5 mL), the Shake was diluted in semi-skimmed milk (1.01 g of Shake in 6.5 mL). According to the manufacturer's instructions (Herbalife International, Inc.), the chemical composition of Tea is: Maltodextrin, Fructose, Orange Pekoe Extract, Green Tea Extract, Lemon Peel Extract (Lemon Peel, Modified Food Starch, Ascorbic Acid, Citric Acid), Cardamom Seed Extract (Extract of Cardamom, Soybean Oil, Silicon Dioxide), *Malva sylvestris* Extract and Hibiscus Flower Powder. The composition of Shake of the same trademark is: Fructose, lecithin powder, soy protein isolate, maltodextrin, calcium caseinate, sodium caseinate, corn bran, flavours and artificial flavours, guar gum, lecithin liquid, magnesium oxide, carrageenan, disodium phosphate, citrus pectin, honey powder, silicon dioxide, ascorbic acid, ferrous fumarate, potassium iodide, DL alpha tocopheryl acetate, sodium selenite, niacinamide, copper gluconate, zinc oxide, manganese sulfate, biotin, papain, bromelain, vitamin A palmitate, D-calcium, pantothenate, pyridoxine hydrochloride, thiamine mononitrate, riboflavin, folic acid, chromium chloride and sodium molybdate.

Body weight, food intake, water intake was daily measured during all the treatment. Cumulative food intake was calculated from difference in the weight from that before feeding. The average caloric food consumption was determined under the following provisions: carbohydrate (4 kcal/g), protein (4 kcal/g) and fat (9 kcal/g) proportionally with food intake.

Open Field Test

The open field test apparatus consists of a square Plexiglas cage (30 × 30 × 16 cm) subdivided in 16 (7,5 cm x 7,5 cm)

squares with walls to minimize outside light and noise. The animals, 10 per group, were individually placed in the center of box and were left to move freely during a 5 min period. The measured behaviors were: locomotor activity (number of segments crossed by the animal with all four legs), rearing (number of times that the animal stood on its hind legs), self-grooming (number of times that an animal preened its fur or tail with its mouth or forepaws), defecation (number of fecal boli) and urination (number of times a mouse urinated). The experiments were conducted between 13:00 and 17:00 h. To avoid possible bias due to odor trails left by previous animals, after behavioral monitoring the floor was cleaned with 70% ethanol solution and left to dry before testing the next animal. The observation was done blinded to minimize the effects of subjective bias.

Metabolic and Biochemical Parameters

Glycemia determinations were carried out with a glucometer system (Roche, Mannheim, Germany), using blood samples obtained from the tail vein after 28 days of treatment (DAMIÁN *et al.*, 2014). At the same day, the animals were euthanized an overdose of sodium thiopental overdose (100 mg/kg, i.p.) and the venous blood samples were obtained by puncturing the right ventricles of the animals and the liver and kidneys were removed.

The Serum was separated immediately (1200g for 10 minutes) and kept at -20°C until biochemical serum analysis (Total cholesterol, Triglycerides, Urea, Creatinine, Uric acid, ALT (alanine aminotransferase), AST (aspartate aminotransferase) and C reactive protein CRP). All these measurements were realized in an automatic spectrophotometric analyzer (AU 400 or 680 (Olympus/Beckman Coulter, Munich, Germany) from a clinical analysis

laboratory (Tommasi Laboratory, Vitoria, ES, Brazil).

Liver and Kidney morphometric parameters

A portion of the liver and the kidneys samples were embedded and fixed in Bouin's solution (1:4) for 48 hours at 4 °C and histologic paraffin sections (5 µm) were made and stained with hematoxylin and eosin. Images of individual tissues were captured with a color video camera AxioCam ERC 5s (Carl Zeiss, Germany) connected to a microscope (AX70, Olympus, Center Valley, PA, USA). All histological analyses were done by a blind examiner to the experimental groups (CHISTÉ *et al.*, 2019; DE SOUZA SANTOS *et al.*, 2019).

AOPP determination in liver and kidney

AOPP were measured according to the method described by BARBOZA *et al.* (2018) using spectrophotometry by the formation of triiodide ion through the oxidation of KI with chloramine-T. A total 200 µL of homogenate of liver and kidney (200 mg diluted 1:5 in PBS, w/v), was added to 10µL of KI (1.16 mol/L) and 20µL glacial acetic acid were placed in each well of a 96-well microtiter plate (Becton Dickinson Labware, Lincoln Park, NJ, USA). After, the sample were mixed on a plate shaker and the absorbance of the reaction were read at 340 nm in a microplate reader (Spectra-MAX-190, Molecular Devices, Sunnyvale, CA, USA). The AOPP levels were recorded as µmol/L of chloramine-T per mg/protein by Bradford methods (BRADFORD, 1976).

Statistical analysis

The normality of the variables was evaluated using the Kolmogorov–Smirnov test. The statistical analysis was performed by one-way analysis of

variance (ANOVA) using Prism software (Prism 6.0, GraphPad Software, Inc., San Diego, CA, USA). When ANOVA showed significant differences, the Tukey's test was used as a post hoc analysis. The differences were considered significant when $p < 0.05$. All data are expressed as mean \pm standard error mean (SEM).

4 RESULTS AND DISCUSSION

Years ago, clinical cases of liver injury were first reported in association with the use of oral supplementation with some non-herbal products (ELINAV *et al.*, 2007; BALLOTIN *et al.*, 2021). Despite this evidence, there are still no preclinical studies demonstrating the impact of chronic consumption of these supplements on risks and/or toxicity reactions. So, this is the first study to investigate the effect of the chronic use of two dietary products (Shake and Tea), widely marketed as food supplements, on locomotor and anxiety behaviors, biochemical and histological parameters. The first reports were mentioned in 2005 in Switzerland and Spain (STICKEL *et al.*, 2011), however in fact there are more than 50 cases described worldwide with the majority of patients involved using this type of supplementation aimed at loss weight (ELINAV *et al.*, 2007; SCHOEPFER *et al.*, 2007; STICKEL *et al.*, 2011; GARCÍA-CORTÉS *et al.*, 2016; MENGUAL-MORENO *et al.*, 2015; BALLOTIN *et al.*, 2021).

Analysis of caffeine in the Tea and the Shake of Herbalife® products

The caffeine in tea samples (Figure 1A) was detected by observing a typical spot similar to Rf pure caffeine, without changing the resolution even with the presence of the other components of the formulation. Interestingly, by HPLC analysis it was possible to quantify the

caffeine in tea (Figure 1B) and Shake (Figure 1C) in two samples used in the experiment. After obtaining the equation of pure caffeine with the calibration curve ($y = 168306.9918x - 638538.1667$ with $r^2 = 0.9938$) was observed in the product "Tea" means the presence of 25 mg caffeine/g while the shake has been detected the presence of 2.5 mg caffeine/g sample.

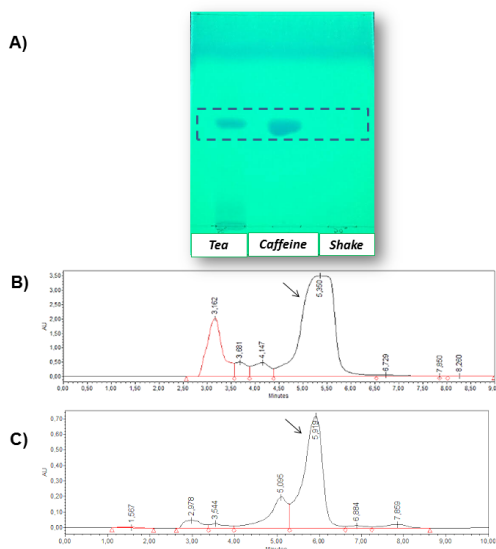


Figure 1. Detection of caffeine. Using the Chromatography on silica gel GF254 technique, caffeine band in the Tea was identified by Rf value similar to that of the standard evidenced in box with dotted lines (Figure A). In the figures "B" and "C" are shown the typical chromatograms of samples of Tea and Shake (respectively) obtained by high-performance liquid chromatography (HPLC). Arrows indicate the presence of caffeine in both samples, (clearly superior in the Tea sample).

The chemical characteristics of herbal and non-herbal products showed that the amount of caffeine in Tea (25 mg of caffeine per gram of sample) was the half than the expected (50 mg/g, product label). Although there is no mention in the label product, it was observed the presence of caffeine in Shake sample (2.5 mg/g). It must be emphasized that herbal

and non-herbal products are considered food supplements and, thereat, are exempt of the approval routinely imposed on synthetic drugs or medicinal products (ELINAV *et al.*, 2007; STICKEL, 2011; RASCHI AND DE PONTI, 2015). Nevertheless, there are advertising in websites and papers attesting that these products present quality control (IGNARRO *et al.* 2008). It is noteworthy that in plant-derivate products the chemical constitution may vary according to season, location and altitude of cultivation, so the same product presents different composition from batch to batch (RASCHI AND DE PONTI, 2015), which could in part explain the outliers in our quantification.

Biometric parameters of experimental group

The analysis of table 1 shows increased body weight in Control animals (+16%, $p < 0.05$). Simultaneously, in the Tea and Shake+Tea groups, the food intake was decreased 20% and 35%, respectively ($p < 0.05$). Similarly, the average caloric consumption was diminished in the same groups (Tea: -19%; Shake+Tea: -33%, $p < 0.05$), compared with Control. Water intake was not different between groups.

Table 1. Biometric parameters, chow and water intake in Control, Shake, Tea and Shake +Tea groups.

Parameters	Control (n=6)	Tea (n=10)	Shake (n=10)	Shake + Tea (n=10)
Initial body weight (g)	33.7 ± 1.7	34.3 ± 1.0	30.7 ± 1.5	33.2 ± 1.0
Final body weight (g)	39.1 ± 2.5 (16%) [§]	35.2 ± 1.5 (1%)	30.4 ± 2.2 (1%)	33.0 ± 2.1 (1%)
Medium food intake (g)	6.9 ± 0.4	5.6 ± 0.3*	6.3 ± 0.3	4.6 ± 0.2**
Average caloric consumption** (Kcal)	24.1 ± 1.4	19.5 ± 1.1*	22.2 ± 1.1	16.2 ± 0.7**
Medium water intake (mL)	12 ± 1.0	11.7 ± 0.6	12 ± 0.9	13 ± 1.3

Table 1: Results expressed as mean ± standard error of the mean. [§] $p < 0.05$ vs. Initial body weight, * $p < 0.05$ vs. Control, # $p < 0.05$ vs. Shake, **supplement + food. ANOVA-1 one way, followed by Tuckey *post hoc*).

The advertisements of food supplements highlight the commitment to provide

healthy solutions for the worldwide epidemic of obesity, by nutritional supplements distribution geared mainly for weight control and promotion of well-being (STICKEL, 2011; IGNARRO *et al.*, 2008; GARCÍA-CORTÉS *et al.*, 2016;). However, ESTEGHAMATI *et al.* (2015) emphasize the shortage of data showing that long-term treatment with a mix of herbal products for weight loss is safe and effective, claiming insignificant clinical benefits, such as the use of placebo. Thereby, our data shows that the herbal and non-herbal products evaluated in the present study may contribute to weight control, as indicated in the label, since caffeine can stimulate lipolysis accompanied by thermogenesis (VAN SCHAİK *et al.*, 2021), suggesting that part of this effect could be attributed to this methylxanthine. Based on our HPLC results, the animals treated with tea and the association of tea and shake were exposed to 0.9 mg and 0.45 mg of caffeine/day, respectively. GURLEY *et al.*, (2015) demonstrated similar results in regular consumers of teas with low caffeine content, such as green tea. Nevertheless, our data are not sufficient to validate these supplements for weight control, because other possible risks inherent to the continuous or chronic consumption of these caffeine rich products can trigger toxic effects, which are widely described in the literature (GURLEY *et al.*, 2015; GARCÍA-CORTÉS *et al.*, 2016). Considering that the manufacturer suggests replacing two daily meals for shake and several times a day the tea consumption for weight loss, we could deduce that the consumers are exposed to a supranormal dose of caffeine (AHLUWALIA *et al.*, 2015) daily (130 mg in Shake + 85 mg in Tea), considering that the typical dose of caffeine is around 100 mg per drink (WILLSON, 2018).

Table 2 shows the effects of supplementation on locomotor and

anxiety-like behavior parameters obtained by the open-field test. The Tea and Shake+Tea groups showed diminished locomotor activity (40% and 42%, respectively), rearing (63% and 44%, respectively) and self-grooming (43% and 51%, respectively), compared with Control group ($p < 0.05$). The number of fecal pellets and the frequency of urination was not different between groups.

Table 2. The effects of supplementation on behavioral responses observed in Control, Shake, Tea e Shake + Tea groups.

Parameters	Control (n=6)	Tea (n=5)	Shake (n=5)	Shake + Tea (n=5)
Ambulation (number)	34 ± 5	21 ± 7*#	36 ± 5	20 ± 4*#
Rearing (number)	9.2 ± 2.0	3.4 ± 1.1*#	8.4 ± 2.0	5.2 ± 1.0*#
Self-Grooming (number)	3.3 ± 0.8	1.9 ± 0.4*	2.1 ± 0.5	1.6 ± 0.2*
Fecal Pellets (number)	1.7 ± 0.5	1.6 ± 0.3	1.7 ± 0.3	1.8 ± 0.4
Urination (number)	0.13 ± 0.08	0.18 ± 0.1	0.25 ± 0.09	0.08 ± 0.05

Table 2: Results expressed as mean ± standard error of the mean. * $p < 0.05$ vs. Control # $p < 0.05$ vs. Shake. ANOVA-1 one way, followed by Tuckey *post hoc*.

Interestingly, the motor behavior was diminished in animals from Tea and Shake+Tea groups, determined by a decrease in locomotion, self-grooming and rearing, which could be attributed to a psych stimulant, as caffeine (ALMOSAWI *et al.*, 2018). ALMOSAWI *et al.*, (2018) showed diminished motor activity after administration of high doses of caffeine combined with stress, in mice. Furthermore, according to HASSIEN *et al.*, (2020) wild animals tend to escape, whereas laboratory animals tend to be paralyzed (freezing stress) when exposed to a stressful situation. Accordingly, the supplementation with tea could have intensified the low exploratory behavior of the groups Tea and Shake+Tea.

Biochemical parameters of experimental group

Biochemical evaluation (table 3) showed no significant difference in glycaemia, total cholesterol, and triglycerides profile due to oral supplementation with herbal and non-herbal products between groups. In addition, no difference was observed in renal function biomarkers (urea and creatinine). However, we observed an increase in liver function biomarkers. Tea and Shake isolated groups showed augmented levels of AST (77% and 72%, respectively) compared with Control ($p < 0.05$). Shake+Tea group presented an augmentation in ALT (88%, not significant) and in AST (175%, $p < 0.05$) compared with Control.

Table 3. Comparison of biochemical serum parameters between Control, Shake, Tea and Shake + Tea groups.

Parameters	Control (n=6-10)	Tea (n=8-12)	Shake (n=8-12)	Shake + Tea (n=8-12)
Glucose (mg/dL)	113 ± 6	98 ± 3	96 ± 8	114 ± 6
Glucose variation (%)	12.6 ± 9.4	-6 ± 7	-5.6 ± 11	-4.0 ± 8
Total cholesterol (mg/dL)	85 ± 5	90 ± 4	77 ± 9	80 ± 5
Triglycerides (mg/dL)	117 ± 28	154 ± 14	168 ± 27	146 ± 36
Urea (mg/dL)	76 ± 3	66 ± 5	78 ± 5	65 ± 5
Creatinine (mg/dL)	0.15 ± 0.02	0.10 ± 0.01	0.11 ± 0.01	0.15 ± 0.02
Uric acid (mg/dL)	3.4 ± 0.7	3.3 ± 0.3	3.6 ± 0.6	4.3 ± 1.1
ALT (U/L)	58 ± 7	73 ± 18	71 ± 12	109 ± 39
AST (U/L)	140 ± 12	248 ± 82*	241 ± 39*	386 ± 78*
CRP (mg/dL)	1.02 ± 0.35	0.91 ± 0.19	0.95 ± 0.44	1.6 ± 0.95

Table 3: Results expressed as mean ± standard error of the mean. * $p < 0.05$ vs. Control. ANOVA-1 one way, followed by Tuckey *post hoc*. ALT: alanine aminotransferase; AST: aspartate aminotransferase; CRP: C reactive protein.

The supposed correlation between liver injury and the supplementation with herbal and non-herbal products aroused the interest of clinicians due to increasing case-reports (ELINAV *et al.*, 2007; SCHOEPFER *et al.*, 2007; STICKEL, 20011; MENGUAL-MORENO *et al.*,

2015; GARCÍA-CORTÉS *et al.*, 2016; BALLOTIN *et al.*, 2021).) showing the elevation of liver transaminases, signaling hepatocellular toxicity (ROTUNDO, PYRSOPOULOS, 2020).

JURČIĆ *et al.*, (2019) reported in a case report that a patient using ten different Herbalife® products at the "recommended dose" had liver toxicity due to elevated liver markers and jaundice. And the products were suspended, all clinical changes regressed. Our data corroborate this clinical finding, above all in Shake+Tea group, showing a synergistic effect, since the dose of the respective products were halved compared with Shake and Tea groups alone. Many herbal supplements present hepatotoxic properties, especially in patients with comorbidities that require multiple long-term therapies (TESCHKE AND EICKHOFF, 2015; RASCHI AND DE PONTI, 2015). Interestingly, 30-40% of hospitalized patients do not describe to their doctors the consumption of these products (VERMA AND THULUVATH, 2007), often because of self-medication practice (STICKEL, 2011) or because these supplements are from natural origin and, therefore, safe (SCHOEPFER *et al.*, 2007). Thereby, our data support that during the clinical investigation of possible etiologic agents involved in liver injury, the use of herbal and non-herbal supplements must be considered as a possible variant in patients with augmented hepatic enzymes levels. **The** Although blood glucose, triglycerides and cholesterol data from this study were not different between groups.

Oxidative damage in the renal tissue

Oxidative protein damage was indirectly determined by AOPP assay. As demonstrated in figure 2, no difference was observed in liver protein oxidation between groups (Tea: 4.4 ± 0.7 ; Shake: 4.8 ± 1 ; Shake+Tea: 3.9 ± 0.3 $\mu\text{mol/mg}$ of protein) compared with Control (4.7 ± 0.4

$\mu\text{mol}/\text{mg}$ of protein). On the other hand, AOPP concentration measured in kidney was significantly increased in the groups supplemented with herbal and non-herbal products (Tea: 2.7 ± 0.7 ; Shake: 3.7 ± 1 ; Shake+Tea: $3.2 \pm 1 \mu\text{mol}/\text{mg}$ of protein) compared with Control ($0.3 \pm 0.05 \mu\text{mol}/\text{mg}$ of protein).

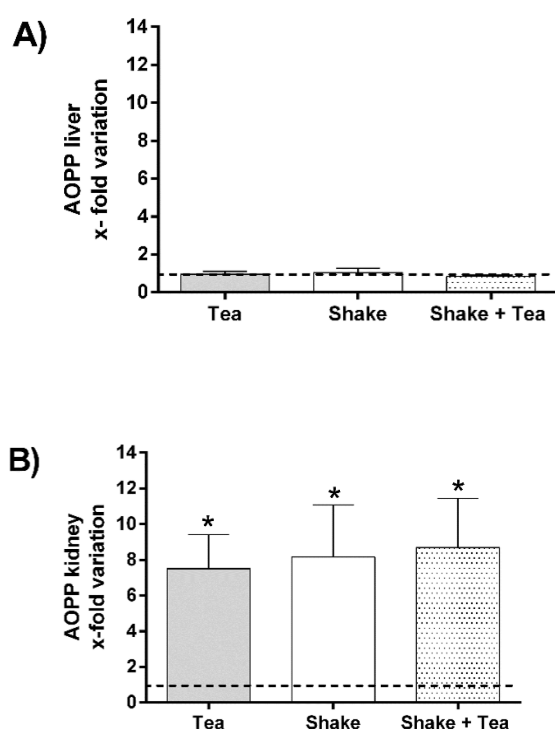


Figure 2. AOPP quantification. Levels of advanced oxidation protein products (AOPP) in liver and kidneys from mice treated with Tea (n=4), Shake (n=5), and Shake+Tea (n=7). The bar graph shows the AOPP in liver (A) and kidney (B) expressed in x-fold variation relative to the Control group. Note the remarkable increase in the AOPP in kidneys of all treatments. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The values are presented as the mean \pm SEM.

The imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense characterizes the oxidative stress (PEREIRA *et al.*, 2021). Despite the media appeal suggesting that herbal and non-herbal products supplementation could antagonize oxidative stress, we find augmented renal protein oxidation in animals treated with

Tea, Shake and Shake+Tea. A recent study demonstrated that high doses of green tea can disrupt the oxidative balance by the inhibition of antioxidant enzymes, probably due to augmented catechin levels (BÁRTÍKOVÁ *et al.*, 2015), leading to colorectal cancer and hepatic and renal damage (MURAKAMI, 2014). On the other hand, others demonstrated that low and medium doses of green tea presented beneficial effects in mice tissue, especially in the large intestine, liver and kidneys, in contrast with the use of high doses (MURAKAMI, 2014). In the present study, we found no difference in liver AOPP levels between groups, which could be explained by the greater antioxidant activity in liver compared with renal tissue (ATLI *et al.*, 2020), maintaining a balance in ROS levels. Still, we do not exclude the possibility of risk of oxidative damage induced by Herbalife® products in tissues with low redox status, especially after chronic exposure (as observed in the kidneys), or with high doses, or in patients with impaired antioxidant system (NOCELLA *et al.*, 2019; PINHEIRO, OLIVEIRA, 2020).

Liver and kidney morphometric parameters

Figure 3 showed typical photomicrographs of the histology of liver of the experimental groups.

The treatment of Tea and Shake showed a normal tissue architecture in all supplemented groups. Similarly, showed figure 4, the kidneys do not appear injure between the treatments.

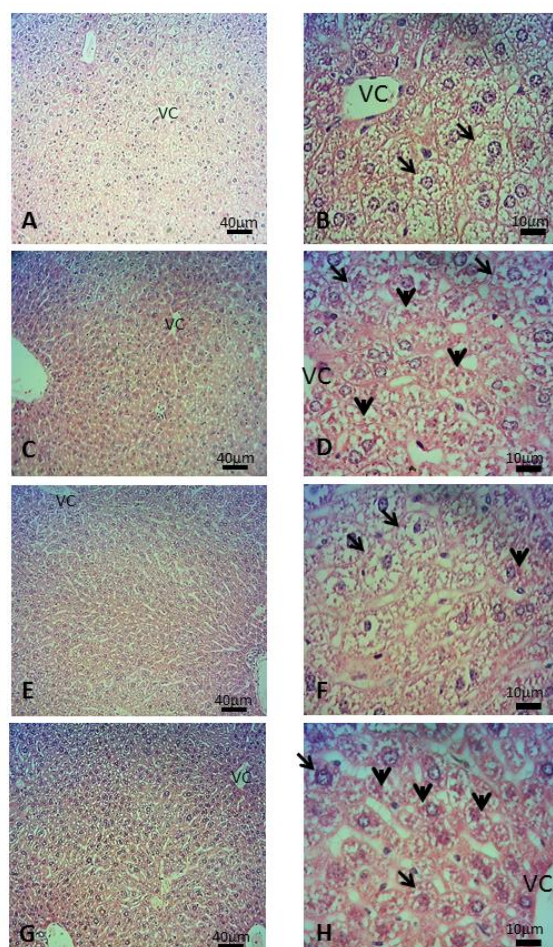


Figure 3. Liver histology. Representative micrographs showing liver histology in all groups studied after 28 days of supplementation: Control (A and B); Tea (C and D), Shake (E and F), and Shake+Tea (G and H). All groups showed generalized cytoplasmic vacuolization of hepatocytes (black arrows) distributed throughout the hepatic lobule. In Tea, Shake and Shake + Tea groups was observed small hyaline deposits (arrowheads). Staining with Hematoxylin and eosin (HE) reagents. Magnification: 40x (first column) and 100x (second column).

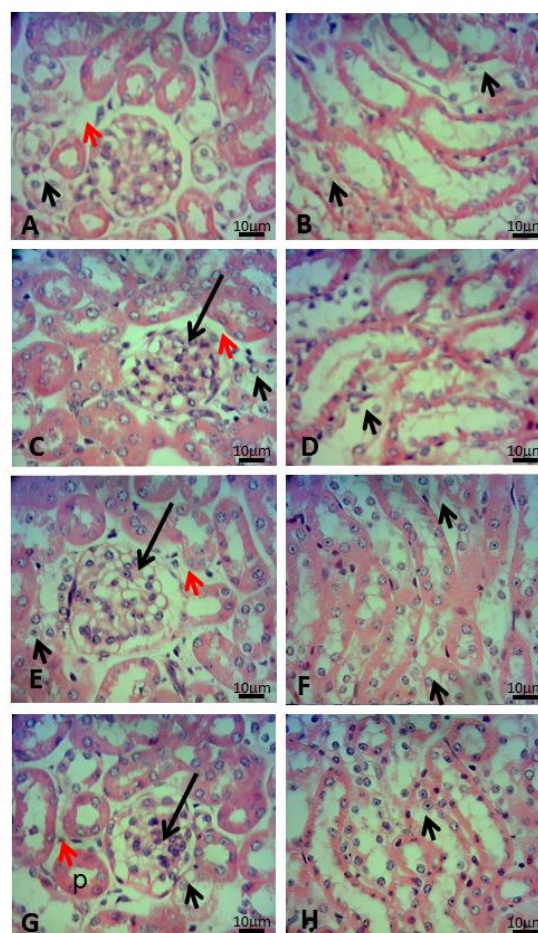


Figure 4. Kidney histology. Representative micrographs showing kidney histology in all groups studied after 28 days of supplementation. Control (A and B); Tea (C and D), Shake (E and F), and Shake+Tea (G and H). The glomeruli did not show any obvious changes in glomerular structure. In the tubular interstitium, slight edema was observed (red arrow) highlighted in A, C, E and G. There is some slight vacuolization of tubular epithelial cells in the medulla (black arrows) in B, D, F and H. These sections were stained with hematoxylin and eosin (HE) reagents. Magnification: 100x.

In the present study, the dosing interval stipulated for each group was based on the product label suggestions, therefore we replaced 2 meals per day by the supplementation and maintained one free meal, so during one day the animals were exposed only to supplements for 9 hours. As mentioned above, there are no previous studies determining the ideal dose of these products for mice, thus we established Tea and Shake doses based on an appropriate gastric volume supported

by mice (BARBOZA *et al.*, 2018) but maintaining the same dilution indicated on the label of the products. In parallel, we converted the human doses for mice based on weight adjusted to body surface of the specie using a formula in accordance with NAIR *et al.*, (2018). We observed that the daily doses of Shake should be 47% lower compared to conventionally dose suitable for humans (26 g/portion). Using the same conversion formula, we found that the ideal dose of Tea calculated was 4.8 g of Tea/day, which is 3-fold higher than indicated in the product label (1.7 g/portion). Interestingly, in the group Shake+Tea in which Herbalife® products was administrated in association (and which we observed better results), the dose of Shake must be 73% lower, while the dose of tea was similar to the conventionally used by humans (2.4 g/serving/day).

5 CONCLUSION

In conclusion, our results indicate that chronic use of Herbalife® products could be contribute to weight control. However, the group Shake+Tea presented changes in motor behavior pattern. Despite the negative histological results, we observed an increase of AST and of protein oxidation in renal tissue of these animals, suggesting the need for further experimental studies to support the safety of those products widely consumed by general population.

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