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RESEARCH ARTICLE

Evaluation of the toxicology, anti-lipase, and antioxidant effects of *Callistemon citrinus* in rats fed with a high fat-fructose diet

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ABSTRACT

Context: *Callistemon citrinus* Skeels (Myrtaceae) exhibits many biological activities.

Objective: This study analyzes for the first time, the toxicity, obesogenic, and antioxidant effects of *C. citrinus* in rats fed with a high fat-fructose diet (HFFD).

Materials and methods: Four studies using male Wistar rats were conducted: (a) 7 groups ($n = 3$): control (corn oil) and ethanol extract of *C. citrinus* leaf (single oral dose at 100–4000 mg/kg) for acute toxicity; (b) 2 groups ($n = 8$): control (corn oil) and *C. citrinus* (1000 mg/kg/day) for 28 days for subacute toxicity; (c) 3 groups ($n = 4$) with single oral dose of lipid emulsion: control (lipid emulsion), *C. citrinus* and orlistat (250 and 50 mg/kg, respectively) for lipid absorption; (d) 4 groups ($n = 6$): control (normal diet) and 3 groups fed with HFFD: HFFD only, *C. citrinus* and simvastatin (oral dose 250 and 3 mg/kg, respectively) for 13 weeks. Antioxidant enzymes and biomarkers were evaluated and inhibition of pancreatic lipase was determined *in vitro*.

Results: Toxicological studies of *C. citrinus* showed no differences in biochemical parameters and lethal dose (LD_{50}) was higher than 4000 mg/kg. *C. citrinus* inhibited pancreatic lipase activity, with IC_{50} of 392.00 μg/mL, and decreased lipid absorption by 70%. Additionally, it reduced the body weight 22%, restored the activities of antioxidant enzymes, and reduced the biomarkers of oxidative stress.

Conclusions: *Callistemon citrinus* showed an effect against oxidative stress by reducing biomarkers and induced antioxidant system, without toxic effects.

Introduction

The use of natural products has increased due to their low toxicity and lack of side effects normally found in drugs. The medicinal properties of plants are found in substances of diverse chemical composition. Most plants present phenolic compounds, many of them having good biology effects. Some of them have been reported to be hematotoxic and hepatotoxic (Michalowicz and Duda 2007). As a consequence, it is important to determine their pharmacological effects and toxicity (Saidu et al. 2010). Studies in toxicity are widely used to identify undesirable effects and mortality.

Obesity is a chronic metabolic disorder produced by a disproportion between energy intake and expenditure (Spiegelman and Flier 2001). It is a disease that contributes to health deterioration via oxidative damage, which increases the possibility of morbidity and eventually mortality (Marseglia et al. 2014). High levels in the production of reactive oxygen species (ROS) are related to cardiovascular diseases (CVD) produced by atherosclerosis complications. One initial event in this process is the oxidised low-density lipoprotein. Consequently, the induction of the antioxidant enzyme system and the reduction of biomarkers of oxidative stress would be a possible way to control CVD (Pignatelli et al. 2018). Oxidative stress is a cellular state of elevated concentrations of ROS that generate molecular damage in cellular components, both in structure and function of vital molecules (Rani et al. 2016). Oxidative stress contributes to increase preadipocyte proliferation, adipocyte differentiation, and mature adipocyte size in obesity (Masschelin et al. 2020).

One of the strategies in the prevention of obesity is to change lipid metabolism by inhibiting fat absorption (Gholamhose et al. 2009). Orlistat is one of the few drugs used for obesity treatment; it is a strong inhibitor of lipases, mainly pancreatic lipase, responsible for the digestion of dietary triacylglycerol into free fatty acids, and monoacylglicerol and diacylglycerol that can be absorbed by the organism (Zeng et al. 2018). However, orlistat can cause many side effects, some of them include oily spotting, increased bowel movements, abdominal pain, and headache among others. Mechanisms of action of phytochemicals comprise the following: inhibition of dietary lipid digestion via inhibition of pancreatic lipase activity, appetite regulation via hormones associated with food intake, and inhibition of white adipose tissue development via attenuation of oxidative stress in obesity (Sun et al. 2016).

Callistemon citrinus Skeels (Myrtaceae) (syn. *Callistemon lanceolatus* (Sm.) Sweet), also called bottlebrush, is considered as an ornamental tree in Mexico (Ríos-Chávez et al. 2019). Aboriginal

Australians used it as traditional food (Radulović et al. 2015). Beneficial phytotherapeutic properties of *C. citrinus* have been reported such as anticancer activity in colon (López-Mejía et al. 2019) and in breast (Ahmed et al. 2019), anti-α-glucosidase activity (Fayemi et al. 2019), anti-inflammatory activity (Radulović et al. 2015), and positive effect against type-2 diabetes (Kumar et al. 2011). These medicinal properties have been attributed mainly to the synergistic effect of the phenolic, flavonoid, and terpenoid compounds (Ahmed et al. 2019; Ríos-Chávez et al. 2019). The phytochemical analysis of the terpenoid compounds has been characterised by GC-MS and two-dimensional gas chromatography by Petronilho et al. (2013), and the phenolic compounds using ID- and 2 D-NMR and ESI-MS by Khanh et al. (2016). Bhushan et al. (2014) reported that *C. citrinus* dry leaf extract administrated with a dose of 2 g/kg in mice was not toxic. No prior research has focussed on the effects of this plant in the oxidative stress produced in obese rats. The purpose of this study is to evaluate the toxic, antiobesogenic, and antioxidant effects of *C. citrinus* leaf extract in rats fed with a high fat-fructose diet.

Materials and methods

Plant collection and preparation of ethanolic extract

Leaves of *Callistemon citrinus* were collected in Morelia, Mexico (19° 41′ 11.3″ N latitude and 101° 12′ 18.4″ O longitude) in February 2019. A specimen of the plant was deposited at the Herbarium of the Faculty of Biology of the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH), and verified by a taxonomy expert MSc. Patricia Silva (voucher number EBUM23538). Fresh leaves were macerated in a 1:10 ratio (1 g of vegetal tissue in 10 mL of 95% ethanol). The extract was allowed to stand at room temperature in dark for 5 days. Subsequently, it was dried by evaporation with vacuum removal in a rotary evaporator at 45 °C and was stored at 4 °C until its later use.

Phytochemical analysis

Callistemon citrinus leaves were collected from 4-year-old plants. At this stage, leaves have high contents of limonene, α-terpineol, and 1,8-cineole (Petronilho et al. 2013). López-Mejía et al. (2021) standardised and characterised the antioxidant capacity and phenolic, flavonoid and terpenoid compounds in *C. citrinus* leaf extract. Figure 1 shows the stability of the *C. citrinus* leaf extract in a period of 6 months using the gas chromatography-mass spectrometry technique.

Animals and treatments

Male Wistar rats, 8-12 weeks old with an average weight of 200–300 g were kept at a room temperature of 24 °C with a 12 h light/dark cycle. They were fed with chow standard diet and water *ad libitum*. The animals came from the bioterium of the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH). The protocol for these experiments was approved by the Institutional Committee for Use of Animals of the UMSNH, and followed the recommendations of the regulatory standard for the use of animals issued by the Mexican Ministry of Agriculture in its Federal Regulations for the Use and Care of Animals (NOM062-ZOO-1999).

Acute toxicity assay

Twenty-one male Wistar rats were randomly divided into 7 groups with three animals each. Control group (A) received corn oil as vehicle, whereas groups B, C, D, E, F, G received a single orally dose by gavage of the extract at 100, 300, 1000, 2000, 3000, and 4000 mg/kg body weight, respectively. The animals were observed for signs of toxicity or death during the first 4, 24, and 48 h, and then 15 days after the treatment. At the end of the period, rats were euthanized with a pentobarbital sodium (50 mg/kg) injection to look for toxicity signs in their internal
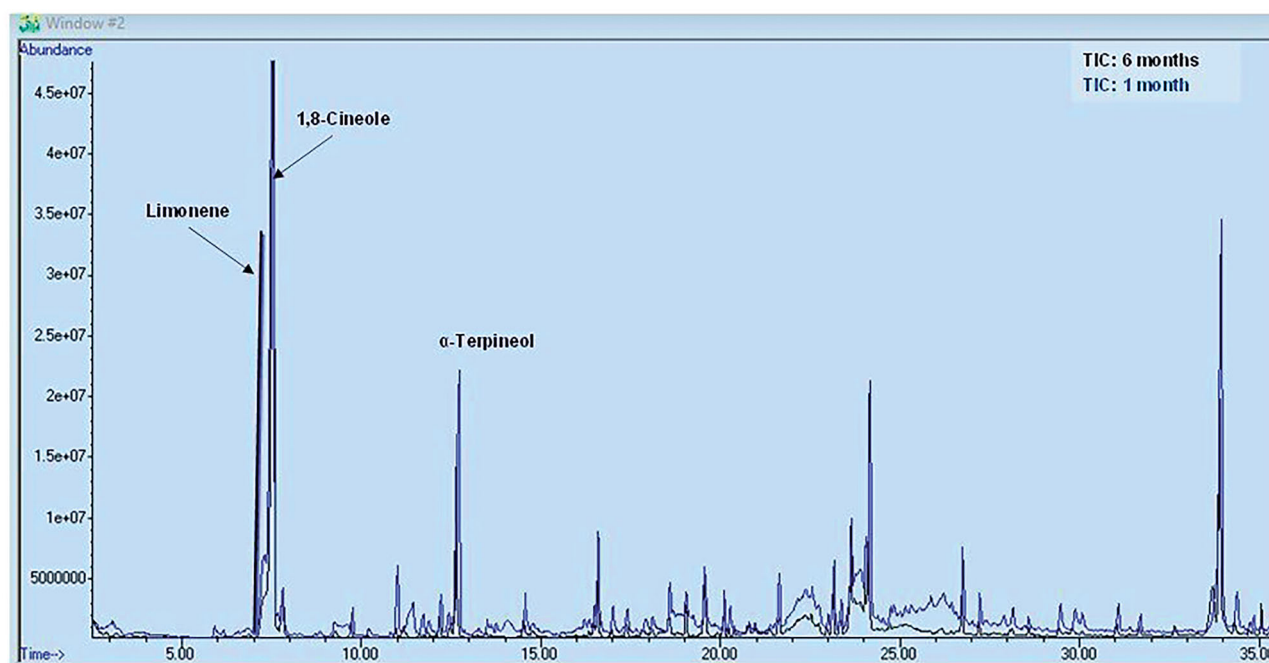

Figure 1. GC-MS total ion chromatogram of *C. citrinus* leaf extract (one month and six months old).

organs. The acute toxicity test was carried using the Organisation for Economic Cooperation and Development (OECD) guidelines 423.

Subacute toxicity assay

Sixteen male Wistar rats were randomly divided into 2 groups with eight animals each. The control group received corn oil as vehicle; the experimental group received an oral dose by gavage of *C. citrinus* leaf extract at 1000 mg/kg body weight once daily for 28 days. Any toxicity signs presented by the animals were evaluated during the test. Animals were weighted and blood was collected to assess biochemical parameters: aspartate amino transferase (AST), alanine amino transferase (ALT), and lactate dehydrogenase (DHL) were obtained using kits. Total serum protein and albumin were determined according to Bradford (1976) and Gendler (1984) assays, respectively. The subacute toxicity test was carried using the Organisation for Economic Cooperation and Development (OECD) guidelines 407 ([OECD] Organization for Economic Cooperation and Development 2008).

Sample preparation

Rats were euthanized with a pentobarbital sodium (50 mg/kg) injection. Subsequently, the internal organs were analysed to search for toxicity signs. Later, the liver was weighed and sliced for pathological assessment. These pieces were washed with physiological saline solution and fixed in 10% formaldehyde solution for histopathological examination. Tissues were embedded in paraffin, thinly sectioned using a microtome (4 μm), and stained with haematoxylin and eosin. Afterwards, the samples were observed under a light microscope.

Pancreatic lipase inhibition assay

Lipase inhibitory activity was performed according to Jaradat et al. (2017). *Callistemon citrinus* leaf extract and orlistat were prepared in dimethyl sulfoxide 1% (50, 100, 200, 300, and 400 μg/mL). Reaction mixture comprised 0.2 mL of the different concentrations, 0.1 mL of a solution of the pancreatic lipase (1 mg/mL), and 0.7 mL of 25 mM tris-HCl buffer pH 7.4; then, incubated for 15 min at 37 °C. 0.1 mL of a *p*-nitrophenyl butyrate solution was then added and again incubated for 30 min at 37 °C. Finally, the samples were measured at 405 nm. Orlistat was used as a positive control. The lipase inhibition (I) was calculated with Equation (1), where A is the activity without an inhibitor; a is the negative control without an inhibitor; B is the activity with inhibitor and b is the negative control with inhibitor.

$$I\% = \frac{(A - a) - (B - b)}{(A - a)} \times 100 \tag{1}$$

The concentration of 50% lipase inhibition (IC$_{50}$) was performed using a range of 50–400 μg/mL.

Determination of fat absorption in rats

Another group of 12 male Wistar rats, 10 weeks old with an average weight of 290 g, were randomly divided into 3 groups with four animals each. Plasma triacylglycerol was measured, with slight modification from a previous study (Kim et al. 2009).

Rats were fasted for 10 h before 3 mL of lipid emulsion was orally administrated. Lipid emulsion containing 10 mL of corn oil, cholic acid (80 mg), 10 mL saline solution, and 5 mL of egg yolk as cholesterol substitute to determine fat absorption. *Callistemon citrinus* leaf extract and orlistat were administered by oral gavage (250 and 50 mg/kg, respectively). Blood samples were taken from the tail at 1, 2, and 3 h after the administration of the lipid emulsion. The triacylglycerol (TAG) level was determined using an Accutrend® Plus system – Roche Diagnostics.

Model of obesity by feeding high-fat-fructose diet

Twenty-four male Wistar rats, 8 weeks old with an average weight 200–250 g were randomly divided into 4 groups with six animals each. Group I: (Control) rats fed *ad libitum* with pellet chow food for 13 weeks. Group II: (HFFD) represented a negative control; the animals were fed *ad libitum* for 13 weeks with slight modifications of the high fat-diet reported by García-Berumen et al. (2019). The modified diet that rats received consisted of: 45.4% pellet chow plus vegetable shortening 14.8% (*w/w*) (saturated mono: ∼1.79 g, unsaturated ∼3.44 g, poly-unsaturated ∼8.45 g); lard 14.8% (*w/w*) (saturated mono ∼5.28 g, unsaturated ∼6.12 g, poly-unsaturated ∼1.48 g) and fructose 25% (*w/w*). Group III: (HFFD + SIMV), the rats received HFFD *ad libitum* plus simvastatin (3 mg/kg) once a day by oral gavage for 13 weeks. The dose of simvastatin was selected based on Bais et al. (2014). Group IV: (HFFD + *C. citrinus*) rats received HFFD *ad libitum* and additionally *C. citrinus* leaf extract (250 mg/kg) once a day by oral gavage for 13 weeks. The dose of *C. citrinus* leaf extract was selected according to the study of López-Mejía et al. (2019).

Measurement of morphometric parameters

The body weight, food, and water intake were recorded daily for 13 weeks. At the end of this period the following morphometric parameters were measured: total body weight (BW) with an electronic scale, nose to anus length (NAL), and nose to tail length using a tape measure. The adiposity index (AI) (measurement of adiposity where the fat level increases) was calculated as AI = (total adipose tissue weight/final BW) × 100. In addition, the body mass index (BMI) (BMI = kg/m^2) was also determined. In rats, the Lee index (LI) is similar to BMI in humans, and it was calculated as LI = $(3\sqrt{BW})$/NAL) × 10. The weight gain (D) was calculated as D = [(Final BW – initial BW)/initial BW] × 100 (Novelli et al. 2007). At the end of these measurements, animals were euthanized with a sodium pentobarbital (50 mg/kg) injection, and the total adipose, liver, heart, stomach, and kidney tissue samples were removed, washed, weighed, and stored at −20 °C.

Biochemical parameters

Triacylglycerol, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very-low density lipoprotein (VLDL), glucose, AST and ALT were measured in a spectrophotometer using commercial kits of enzymatic colorimetric methods (SPINREACT®). The protein in serum and in each tissue homogenate was estimated by the method of Bradford using bovine serum albumin as the standard in spectrophotometer (590 nm).

Biochemical assays for antioxidant enzyme activity

Preparation of tissue homogenates

Liver, heart, stomach, and kidney tissues (0.25 g each) were homogenised in 1 mL of 50 mM phosphate buffer pH 7.4 with 0.1 M EDTA, and centrifuged at 13,000 rpm, for 20 min at 4 °C. The supernatant was recovered, and stored at −20 °C.

Superoxide dismutase (SOD)

Superoxide dismutase activity was based on the generation of superoxide anions that reduces nitroblue tetrazolium chloride (NTB) to a formazan product (Giannopolitis and Ries 1977). The enzyme specific activity of SOD was expressed in U/mg protein. One unit of SOD activity was the enzyme amount required for 50% inhibition of the rate of the NBT reduction.

Catalase (CAT)

Catalase activity was measured with the disappearance of hydrogen peroxide using the method reported by Aebi (1984). One unit of CAT activity was defined as the amount to decompose 1 μM of H_2O_2 per min, based on molar extinction coefficient of 43.6 m^2/mol.

Glutathione peroxidase (GPx). Glutathione peroxidase activity was measured as proposed by Prabhu et al. (2001). One unit of GPx activity was expressed as the amount of enzyme that catalyses the oxidation of 1 nmol of NADPH/min/mg protein, based on molar extinction coefficient of 6.22 m^2/mol.

Paraoxonase (PON1)

The arilesterase or PON1 activity was measured according to Dantoine et al. (1998). One unit of PON1 activity was defined as 1 μM of phenol formed per min/per mg protein, based on the molar extinction coefficient 14,000 m^2/mol.

Glutathione-S-transferase (GST)

Glutathione-*S*-transferase activity was determined as described in (López-Mejía et al. 2019). One unit of GST activity was presented as nmol of GSH-CDNB conjugate per min/mg protein, based on the molar extinction coefficient of 9.6 m^2/mol.

Biochemical assays for biomarker of oxidative stress

Glutathione (GSH)

Reduced glutathione was estimated as described by Sedlak and Lindsay (1968). The units are expressed in mM GSH/g tissue.

Malondialdehyde (MDA) and hydroxyalkenals (HNE)

Malondialdehyde and hydroxyalkenals (lipid oxidation products) were quantified as described by Johnston et al. (2007).

Protein carbonyls (PCO)

The determination of carbonyl proteins allows to confirm the high degree of oxidative stress by lipid oxidation products. PCO were measured using the method described by Levine et al. (1990). This method is based on the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the protein carbonyl groups, leading to create the stable 2,4-dinitrophenylhydrazone. The PCO content was expressed as μM using the molar extinction coefficient of 22,000 m^2/mol.

Statistical analysis

The test results were expressed as mean ± standard error (SEM) or standard deviation (SD). Data were analysed using GraphPad Prism (version 8) by one-way analysis of variance. To determine statistical differences to morphometric and biochemical parameters between groups, Tukey's multiple comparison test was conducted. Dunnett's *post hoc* test to multiple comparisons between the control and the treatment groups was used in fat absorption, body weight, enzyme activity, and biomarkers of oxidative stress. Values were considered to be indicative of statistical significance *$p \leq 0.05$.

Results

Acute and subacute toxicity

All the animals had normal behaviour, no toxicity signs, and no damage was observed in internal organs, neither death during the 15 days even at the highest dose of 4000 mg/kg. None of the treatments affected food and water intake. No signs of abnormal behaviour or death were observed during the subacute toxicity. There was a slight weight reduction in the group treated with *C. citrinus* leaf extract when compared to control group. Both groups had the same food and water intakes during the 28 days treatment period. There were no significant changes in the weight of the visceral organs after 28 days in both groups, and macroscopic observation did not show any change. The biochemical parameters did not have any changes (Table 1).

Histopathological analysis

The subacute administration of *Callistemon citrinus* leaf extract (1000 mg/kg body weight) during 28 days did not produce any histopathological alteration or necrosis in the liver (Figure 2).

Pancreatic lipase activity *in vitro*

Callistemon citrinus was able to inhibit 50.0% of the pancreatic lipase activity in a dose-dependent manner with IC$_{50}$ 392.00 μg/mL (Figure 3(A)). In addition, orlistat used as the reference compound had 83.0% inhibition with IC$_{50}$ 35.10 μg/mL. Therefore, the anti-lipase activity of *C. citrinus* could be considered as a moderate inhibitor.

Table 1. Body and organ weights and biochemical parameters of Wistar rats treated during 28 days with a single dose 1000 mg/kg of *Callistemon citrinus* leaf extract.

	Control	C. citrinus
Body weight (initial day)	293.30 ± 9.50	279.10 ± 35.80
Body weight (final day	262.20 ± 8.80	336.80 ± 21.30
Weight gain on day 28 (g)	62.00 ± 16.60	57.00 ± 14.20*
Liver	13.30 ± 2.81	12.00 ± 1.50
Heart	1.44 ± 0.04	1.39 ± 0.02
Kidney	3.21 ± 0.02	3.27 ± 0.03
Stomach	1.85 ± 0.07	1.69 ± 0.09
Biochemical parameters		
AST (U/L)	93.70 ± 9.90	91.10 ± 8.90
ALT (U/L)	58.57 ± 6.00	50.37 ± 6.60
LDH (U/L)	755.90 ± 80.20	668.3 ± 35.60
Total protein (mg/mL)	6.70 ± 0.80	6.60 ± 0.10
Albumin (mg/mL)	3.60 ± 0.30	3.70 ± 0.40

Values presented as mean ± SD ($n = 8$; Dunnett's *post hoc* test; *values are statistically significant at $p \leq 0.05$ when compared to control group).
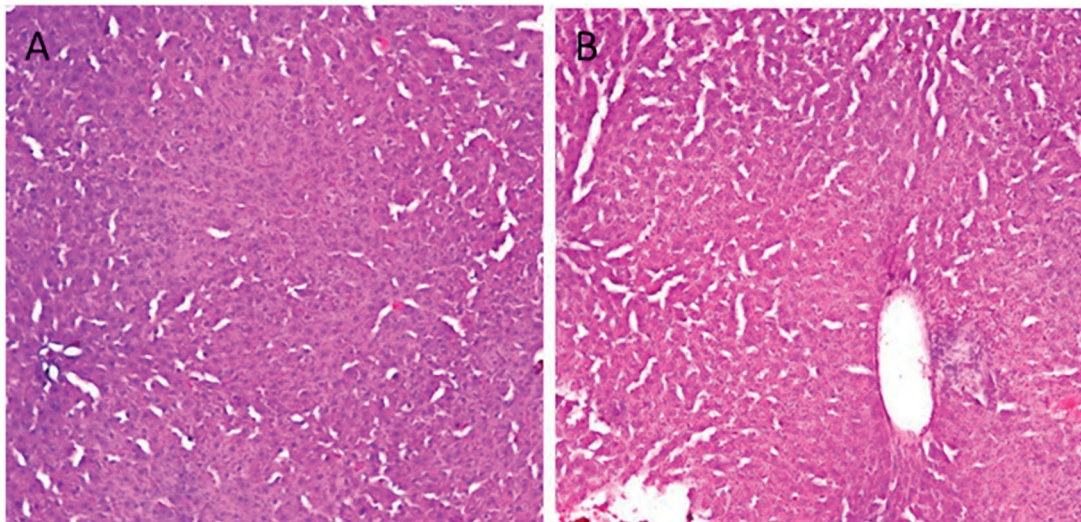
Figure 2. Histological sections (20×) of rat livers treated in the subacute toxicity study: (A) control, (B) *C. citrinus* leaf extract (1000 mg/kg). No significant damage was detected in the treatment.
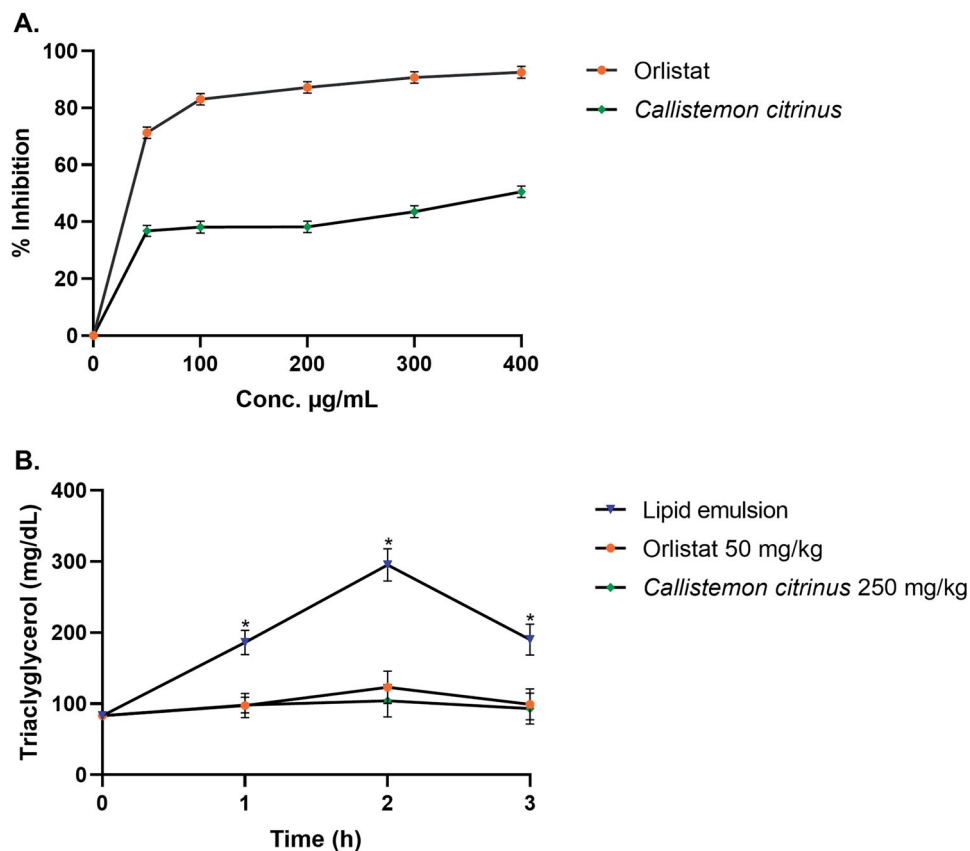

Figure 3. (A). Inhibitory effect of *Callistemon citrinus* extract and orlistat on the activity of porcine pancreatic lipase. Data represent values of three determinations per sample. (B). Effect of orlistat (50 mg/kg B.W) and *Callistemon citrinus* (250 mg/kg B.W) on rat plasma triacylglycerol levels after oral administration of a lipid emulsion. Values expressed as mean ± SEM ($n = 4$, Dunnett's *post-hoc* test, $*p \leq 0.05$ vs. lipid emulsion treatment).

Estimation of plasma triacylglycerol after oral administration of lipid emulsion in rats

Figure 3(B) displays the plasma triacylglycerol levels after oral administration of the lipid emulsion. Control group shows an increase after the 2nd h, followed by a gradual decrease. Meanwhile, the *C. citrinus* (250 mg/kg) and orlistat (50 mg/kg) groups kept low triacylglycerol levels. Despite the differences in the inhibitory percent of lipase activity *in vitro*, between the *C. citrinus* and orlistat groups, the triacylglycerol serum level remained similar.

Weight, morphometric parameters and biochemical plasma values

Rats fed with a high fat-fructose diet during 13 weeks, showed a significant increase of body weight between the 6th (15.5%) and

13th (18.0%) week as compared to the control group. All the animals consumed the same amount of food and water. The groups treated with simvastatin or *C. citrinus* maintained similar weight compared to the control group in all weeks. Table 2 shows that simvastatin and *C. citrinus* leaf extract significantly attenuated the weight gained in HFFD fed groups by 14.6 and 22.0%, respectively ($p \leq 0.05$). These results showed that *C. citrinus* (250 mg/b.w./daily) inhibited the weight gain of the rats fed with HFFD.

Table 2 also displays the morphometric parameters of the rats fed with a high fat-fructose diet at the end of 13 weeks. After the last week of the treatment period, the HFFD group presented significant increases in abdominal circumference (14.4%), body weight gain (almost 50.0%), BMI (26.4%), adiposity index (66.0%) and Lee index (12.1%) with respect to the control group. However, the *C. citrinus* extract restored the biometrical parameters as seen by significant reductions in abdominal circumference (12.2%), body weight gained (40.4%), BMI (24.2%), adiposity index (27.6%), and Lee index (9.1%), respectively ($p \leq 0.05$) as compared to the HFFD group. The HFFD plus simvastatin group presented similar reductions.

Finally, neither the heart, stomach, nor kidneys showed any differences in weight in all groups. Nevertheless, liver weight and retroperitoneal fat were significantly increased in the HFFD treated group, as compared with the control group. Conversely, the groups treated with simvastatin and *C. citrinus* extract showed 50.0% less fat than the HFFD group.

Table 3 shows biochemical parameters. The HFFD group presented high triacylglycerol levels, whereas oral supplementation of *C. citrinus* extract and simvastatin reduced triacylglycerol levels. The other parameters: total cholesterol, HDL, LDL, VLDL, glucose, total proteins, albumin, and the activity of the serum enzymes AST and ALT had the same values in all the groups.

Enzyme activities

This study included the stomach because this tissue is also exposed to harmful compounds from diets that could increase ROS levels and produce some gastric diseases (Camilleri et al. 2017).

SOD activity decreased in the liver, kidney, and stomach, and increased in the heart of the HFFD treated group. This result reflects an adaptation of this tissue to oxidative stress in the HFFD treated group. The kidney and heart of the HFFD treated group presented an increase in GPx and GST activities, whereas in the liver and stomach a decrease was observed. Low catalase activity found in the liver, heart, stomach, and kidney could be caused by a decreased in H_2O_2 levels due to the transformation of this reactive oxygen species into hydroxyl radical. Moreover, the PON activity in the liver, stomach, kidney, and heart of the HFFD group was higher than the activity in the other groups (Table 4). After 13 weeks of *C. citrinus* treatment, the activities of antioxidant enzymes in the liver, heart, stomach, and kidney were similar to those of the control group. Our results revealed that *C. citrinus* extract prevented the oxidative stress produced in the HFFD fed rats by increasing the antioxidant enzymes and caused a significant decrease of PON activity in the four tissues. This extract can be acting as scavenger by eliminating ROS due to the high content of terpenes, phenolic, and flavonoids compounds presented in this plant.

Table 2. Effect of *C. citrinus* on morphometric parameters, organ weights and obesity markers in rats.

Morphometric parameters	Control	HFFD	HFFD + SIMV	HFFD + *C. citrinus*
Initial weight (g)	261.00 ± 27.12^a	270 ± 16.87^a	246.00 ± 14.86^a	245.83 ± 19.09^a
Final weight (g)	439.16 ± 14.16^b	536.00 ± 19.69^a	457.50 ± 25.91^b	446.50 ± 19.87^b
Abdominal circumference (cm)	19.83 ± 1.60^b	23.16 ± 1.16^a	20.50 ± 1.37^b	20.33 ± 1.36^b
Nose-to-anus length (cm)	25.41 ± 1.20^a	24.33 ± 1.50^a	25.41 ± 0.66^a	24.41 ± 0.91^a
Nose-to-tail length (cm)	45.33 ± 1.86^a	45.16 ± 3.32^a	45.50 ± 0.89^a	44.66 ± 1.63^a
Markers of obesity				
BMI (Kg/m^2)	0.67 ± 0.02^b	0.91 ± 0.12^a	0.68 ± 0.03^b	0.69 ± 0.09^b
Body weight gain (%)	63.47 ± 30.37^b	117.53 ± 17.88^a	$90.75 \pm 19.40^{a,b}$	70.00 ± 17.64^b
Adiposity index	2.90 ± 0.60^c	8.54 ± 1.84^a	$4.57 \pm 1.15^{b,c}$	6.18 ± 0.39^b
Lee index	0.29 ± 0.01^b	0.33 ± 0.02^a	0.30 ± 0.01^b	0.30 ± 0.01^b
Organs weights				
Liver weight (g)	14.56 ± 3.33^b	25.89 ± 2.86^a	15.98 ± 1.72^b	12.04 ± 3.19^b
Stomach weight (g)	1.80 ± 0.07	1.79 ± 0.32	1.82 ± 0.12	1.81 ± 0.05
Heart weight (g)	1.56 ± 0.43^a	1.39 ± 0.18^a	1.34 ± 0.22^a	1.13 ± 0.20^a
Kidney weight (g)	2.91 ± 0.28^a	2.74 ± 0.35^a	2.75 ± 0.20^a	2.37 ± 0.20^a
Retroperitoneal fat (g)	9.17 ± 1.57^b	28.11 ± 1.57^a	14.54 ± 1.72^b	14.94 ± 1.72^b

All values expressed as mean ± SD ($n = 6$; values statistically different (^{a}, ^{b}, ^{c}) among groups ($p \leq 0.05$) according to Tukey test.

Table 3. Effect of *C. citrinus* on the biochemical parameters in rats fed with a high fat-fructose diet (HFFD).

Biochemical determinations	Control	HFFD	HFFD + SIMV	HFFD + *C. citrinus*
Triacilglycerol (mg/dL)	119.66 ± 59.83^a	246.16 ± 64.70^b	126.16 ± 45.40^a	136.33 ± 66.96^a
Total cholesterol (mg/dL)	60.83 ± 14.72^a	58.83 ± 12.28^a	53.00 ± 17.82^a	62.00 ± 18.92^a
HDL (mg/dL)	27.33 ± 6.37^a	25.83 ± 5.70^a	24.00 ± 7.58^a	33.00 ± 8.31^a
LDL (mg/dL)	9.50 ± 7.68^a	11.00 ± 7.18^a	5.00 ± 4.85^a	11.66 ± 7.58^a
VLDL (mg/dL)	24.00 ± 11.74^a	29.16 ± 13.02^a	25.33 ± 9.02^a	27.33 ± 13.60^a
Triacilglycerol in liver (mg/dL)	3.70 ± 1.24^a	8.33 ± 1.76^b	5.65 ± 1.44^{ab}	$5.66 \pm 1.44^{a,b}$
Blood glucose (mg/dL)	107.00 ± 9.89^a	106.50 ± 17.67^a	104.00 ± 14.14^a	97.00 ± 4.24^a
Total protein (mg/mL)	15.99 ± 2.3^a	12.77 ± 5.46^a	18.96 ± 0.86^a	18.50 ± 0.85^a
Albumin (mg/mL)	3.58 ± 0.52^a	2.99 ± 0.68^a	3.51 ± 0.17^a	3.10 ± 0.25^a
AST (U/L)	116.66 ± 11.55^a	75.16 ± 19.06^a	113.66 ± 64.41^a	105.00 ± 62.41^a
ALT (U/L)	52.00 ± 9.46^a	52.66 ± 6.53^a	74.83 ± 39.40^a	54.33 ± 13.63^a

All values expressed as mean ± SD ($n = 6$; values statistically different (^{a}, ^{b}, ^{c}) among groups ($p \leq 0.05$) according to Tukey test.

Table 4. Effect of *C. citrinus* on antioxidant system in liver, heart, stomach, and kidney in rats fed with high fat-fructose diet (HFFD).

Group	Organ	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)	GST (U/mg protein)	PON1 (U/mg protein)
Control	Liver	124.30 ± 15.31	127.44 ± 5.50	49.72 ± 3.60	73.07 ± 6.62	3009.01 ± 538.55
	Heart	508.09 ± 27.19	63.65 ± 5.00	23.19 ± 3.48	170.75 ± 25.28	1994.04 ± 225.96
	Kidney	29.37 ± 2.28	23.22 ± 1.46	0.008 ± 0.00	4.04 ± 0.06	224.33 ± 21.43
	Stomach	63.30 ± 8.40	13.79 ± 2.28	11.50 ± 0.46	696.11 ± 35.50	4765.00 ± 234.31
HFFD	Liver	71.47 ± 10.83*	100.88 ± 5.50*	22.01 ± 3.12*	36.72 ± 6.62*	8149.86 ± 439.72*
	Heart	659.70 ± 31.39*	41.34 ± 5.00*	39.83 ± 3.48*	271.54 ± 25.28*	3215.47 ± 225.96*
	Kidney	10.75 ± 0.36*	17.94 ± 0.50*	0.076 ± 0.02*	7.45 ± 0.33*	320.08 ± 21.43*
	Stomach	50.31 ± 2.16*	6.90 ± 1.43*	4.98 ± 0.32*	2260.14 ± 256.83*	10621.00 ± 472.45*
HFFD + SIMV	Liver	104.25 ± 15.31	144.62 ± 5.50	56.01 ± 3.60	60.82 ± 5.41	1963.25 ± 439.72
	Heart	501.68 ± 31.39	52.10 ± 5.78	23.19 ± 3.48	169.18 ± 21.89	1889.08 ± 195.69
	Kidney	31.45 ± 0.61	21.24 ± 1.39	0.007 ± 0.00	4.72 ± 0.11	256.23 ± 19.171
	Stomach	64.47 ± 5.85	16.41 ± 1.07	12.3 ± 0.97	866.00 ± 41.26	4556.02 ± 271.11
HFFD +*C. citrinus*	Liver	116.88 ± 13.26	146.12 ± 5.50	41.80 ± 3.60	49.63 ± 4.68	2866.34 ± 380.81
	Heart	419.91 ± 27.19	73.98 ± 5.00	26.30 ± 3.02	120.55 ± 25.28	1735.36 ± 225.96
	Kidney	30.69 ± 2.20	20.09 ± 0.99	0.007 ± 0.00	4.62 ± 0.36	238.75 ± 21.43
	Stomach	63.78 ± 4.02	14.62 ± 1.06	11.13 ± 0.53	756.06 ± 11.40	3966.08 ± 234.03

Values expressed as mean ± SEM ($n = 6$; Dunnett's *post hoc* test, value of *$p \leq 0.05$ vs. the control group is significant).

Table 5. Effect of *C. citrinus* over the biomarkers of oxidative stress in liver, heart, stomach, and kidney in rats fed with high fat-fructose diet (HFFD).

Biomarkers oxidative stress in treatments

Group	Organ	GSH (mM GSH/g tissue)	MDA (nmol/mg protein)	HNE (nmol/mg protein)	PCO (µM PCO/mg protein)
Control	Liver	2.90 ± 0.08	0.23 ± 0.02	0.14 ± 0.04	56.44 ± 3.61
	Heart	2.33 ± 0.05	0.12 ± 0.01	0.03 ± 0.01	9.44 ± 2.67
	Kidney	48.32 ± 0.09	0.21 ± 0.00	0.09 ± 0.01	0.27 ± 0.01
	Stomach	26.55 ± 1.74	0.24 ± 0.09	0.15 ± 0.05	0.49 ± 0.04
HFFD	Liver	1.90 ± 0.09*	0.36 ± 0.03*	0.48 ± 0.04*	103.52 ± 4.17*
	Heart	1.03 ± 0.04*	0.15 ± 0.01	0.17 ± 0.01*	22.79 ± 2.67*
	Kidney	54.00 ± 1.10*	0.26 ± 0.01*	0.22 ± 0.01*	1.32 ± 0.21*
	Stomach	19.69 ± 1.29*	0.27 ± 0.08	0.34 ± 0.80*	0.98 ± 0.05*
HFFD + SIMV	Liver	2.70 ± 0.08	0.31 ± 0.02	0.26 ± 0.04	55.88 ± 3.61
	Heart	2.25 ± 0.04	0.11 ± 0.01	0.06 ± 0.01	9.42 ± 2.31
	Kidney	50.83 ± 0.24	0.24 ± 0.01	0.08 ± 0.01	0.37 ± 0.01
	Stomach	23.81 ± 1.87	0.25 ± 0.03	0.14 ± 0.09	0.59 ± 0.03
HFFD + *C. citrinus*	Liver	3.00 ± 0.09	0.30 ± 0.02	0.31 ± 0.04	49.50 ± 4.17
	Heart	2.27 ± 0.04	0.12 ± 0.01	0.06 ± 0.01	9.66 ± 2.67
	Kidney	47.94 ± 1.37	0.22 ± 0.00	0.06 ± 0.00	0.39 ± 0.01
	Stomach	23.29 ± 2.60	0.23 ± 0.07	0.17 ± 0.03	0.53 ± 0.05

Values expressed as mean ± SEM ($n = 6$; Dunnett's *post hoc* test, value of *$p \leq 0.05$ vs. the control group is significant).

Biomarkers of oxidative stress

Table 5 shows biomarkers of oxidative stress. The HFFD group showed low GSH level in liver, stomach, and heart tissues. Meanwhile a high level was seen in the kidney. Conversely, HNE and PCO levels showed significant increases ($p > 0.05$) in liver, heart, stomach, and kidney tissues as compared to the others groups. The MDA level of the HFFD group was increased in liver and kidney tissues, but no change was found in stomach and heart tissues. Again, the biomarker values of oxidative stress (GSH, MDA, HNE and PCO) in the groups treated with simvastatin or C. citrinus were similar to the control group.

Discussion

This study shows for the first time the toxicological evaluation and the obesogenic and antioxidant effects of *Callistemon citrinus* leaf extract in rats fed with a high fat-fructose diet. The results obtained from the acute and sub-acute studies showed that the oral administration of C. citrinus extract was tolerated up to a dose of 4000 mg/kg body weight. LD$_{50}$ was not obtained because there was no lethality in the animals. Unlike our study, Bhushan et al. (2014), using dried leaves of *Callistemon citrinus*, found a LD$_{50}$ of 3200 mg/kg body weight in mice. In our study we used fresh leaves and the extract did not show signs of toxicity. Moreover, the rats had normal behaviour and no damage was observed in internal organs, nor was death during the 15 days even in the case of using a dose of 4000 mg/kg. According to the OECD ([OECD] Organization for Economic Cooperation and Development 2001), C. citrinus extract has the lowest toxicity class 5. In the subacute toxicity study, the macroscopic examinations of all the internal organs of the rats treated with C. citrinus (1000 mg/kg) did not show hypertrophy or any changes that could indicate toxicity. The unvarying activities of ALT, AST, and LDH suggest that C. citrinus is safe, as corroborated by the histopathological examinations of the liver which lacked any granular and vacuolar degeneration signs. Macedo et al. (2010) found similar results with no significant differences in biochemical analysis and body weight in rats in the acute and subacute toxicity study with essential oil of *Eucalyptus staigeriana* F.Muell. ex Bailey (Myrtaceae). This plant and C. citrinus contain high levels of terpenes. 1,8-Cineole, the major terpene in C. citrinus leaves (Petronilho et al. 2013) is found as either a major, or a minor constituent in many aromatic plants, and it is used in

many food and pharmaceutical products as well. However, the use of 1,8-cineole can produce severe changes in liver and kidney tissues, despite having a LD$_{50}$ 3849 mg/kg (Xu et al. 2014).

Herbal medicine is used to treat several diseases as an alternative to drugs because of the lack in the side effects and resistance. One advantage of using herbal medicines (phytomedicines) is that they are a mixture of compounds that can act synergistically to be more effective in certain diseases than the use of a single compound (Carmona and Soares 2013). Our results clearly indicated that *C. citrinus* can be safely used.

Oxidative stress is an event that occurs during obesity and is responsible for several complications in this condition (Novelli et al. 2007). Currently, there is a small number of products used commercially to reduce body weight with undesirable side effects. Despite having a lower inhibitory activity than orlistat on pancreatic lipase activity *in vitro*, *C. citrinus* leaf extract was able to suppress dietary fat absorption *in vivo*. The consumption of fats, as opposed to carbohydrates, favours the increase of fat deposition in the body. Therefore, the reduction of fat assimilation helps to avoid obesity (Kim et al. 2009). Thus, plants with a pancreatic lipase inhibitory capacity, without side effects, such as those reported for orlistat are suitable candidates to control weight gain. Moreover, the administration of *C. citrinus* leaf extract during a period of 13 weeks did not produce side effects in the rats.

In this study we observed a normal food consumption in all the groups receiving a high fat-fructose diet (HFFD). The HFFD group increased weight after six weeks, whereas the HFFD effect on the *Callistemon citrinus* group was negligible because the animals showed similar body weight to the control group. López-Mejía et al. (2019) reported that *C. citrinus* has a modulating effect on body weight. A positive correlation was observed between the Lee and adiposity indices in the HFFD group, which was not present in the other groups.

Despite the differences in morphometric parameters in HFFD treated rats, only TAG level increased in serum and liver tissue while the other biochemical parameters showed no changes. Similar results were reported by Muñoz et al. (2018). Additionally, there are many reports showing that different classes of fatty acids from diet can produce various hypercholesterolaemia states (de Angelis-Pereira et al. 2017). Magri-Tomaz et al. (2018) found variations in serum and liver triacylglycerol and cholesterol levels in Wistar rats fed with high fat diet (34.9%). Our results showed high levels of triacylglycerol in the HFFD group, whereas the *C. citrinus* and simvastatin treated groups had similar levels to the control group. Similar results were reported by Matos et al. (2005). Obesity is a medical condition that accumulates triacylglycerol in the cells of the liver (El-Anany and Ali 2018).

Callistemon citrinus leaves have α-glucosidase inhibitory activity (Fayemi et al. 2019). Quiroga et al. (2013) showed that the essential oil of *Lippia turbinata* Griseb (Verbenacea) has limonene and 1,8-cineole as the main terpenes, with an inhibitory capacity of pancreatic lipase. Petronilho et al. (2013) showed that 1,8-cineole, limonene, α-terpineol, and α-pinene are the main compounds of *C. citrinus* leaf extract. Hence, *C. citrinus* extract might modulate fat and carbohydrate metabolism, and as a result, could contribute to reduce body weight which was confirmed in our study by observing the morphometric parameters and the fat decrease in adipose tissue in the HFFD plus *C. citrinus* group.

Mitochondrial and peroxisomal β-oxidation of fatty acids increase with a hypercaloric diet consumption. This metabolic adaptation increases the production of ROS and free radicals, which induces oxidative stress (Begriche et al. 2013). Our results showed that the increase in oxidative stress parameters such as MDA, HEN, and PCO was related to the decrease of antioxidant enzyme activities in the liver of rats fed with a HFFD. Conversely, the rats treated with the extract of *C. citrinus* did not present variations in these parameters (Table 5). This suggests that the compounds present in *C. citrinus* leaves have a protective effect against the damage caused by oxidative stress, acting synergistically to improve the oxidant-antioxidant status with an increase in the activities of antioxidant enzymes (Table 4). This behaviour could be connected to the presence of terpenes and flavonoids found in this plant (Petronilho et al. 2013; Khanh et al. 2016). The inhibition of free radical formation, via modulation of radical scavenging activity, prevents oxidative stress. These phytochemicals play the role of antioxidants to prevent the oxidative stress, as previously reported in the evaluation of the antioxidant capacity of *C. citrinus* extract (Sampath et al. 2016). At the same time, *C. citrinus* showed antioxidant-detoxifying mechanisms by regulating the activities of PON1 and GST enzymes. This effect could be attributed to the inhibition of the formation of electrophilic molecules. Previous studies reported that *C. citrinus* has anticancer properties (López-Mejía et al. 2019), antioxidant capacity (López-Mejía et al. 2021), and anti-diabetic and anti-inflammatory activities (Kumar et al. 2011; Radulović et al. 2015), which could contribute to improved health in obesity.

The high-antioxidant capacity of *C. citrinus* (López-Mejía et al. 2019, 2021) maintained the normal concentration of ROS and decreased the risks of oxidative stress normally presented in colon cancer. Furthermore, differences in the antioxidant enzymes and biomarkers of oxidative stress in liver, heart, stomach, and kidney tissues could be a compensatory adaptation of the tissues against the oxidative stress in HFFD. This specific response depends not only on ROS levels but also on the tissue (Ribeiro et al. 2017). GSH is an endogenous defense against the oxidative stress that can act with GST and GPx enzymes or in the process of lipid peroxidation. Therefore, GSH level reveals the potential for detoxification (Deng et al. 2019). In our study, the HFFD group showed low GSH levels in the liver, stomach, and heart tissues, unlike *C. citrinus* or simvastatin treated groups.

Our results proved that statins not only play a role in cholesterol reduction but also contribute to the protection of oxidative stress due to their antioxidant capacity through the uptake of superoxide anions and hydroxyl radicals. However, statins present adverse effects in liver and muscle as hepatic dysfunction and rhabdomyolysis respectively (Stancu and Sima 2001).

This study demonstrated the efficiency of *Callistemon citrinus* to attenuate body weight gain and to reduce biometrical parameters of obesity such as: abdominal circumference, body weight gain, BMI, adiposity index, and Lee index. *Callistemon citrinus* had anti-pancreatic lipase activity and high antioxidant capacity on oxidative stress in animals with obesity, restoring enzymatic activities (SOD, CAT, GPx, GST and PON1) and glutathione levels. It also inhibited the formation of highly oxidative products such as MDA, HNE, and PCO. Limonene and 1,8-cineole, the major compounds in *C. citrinus*, exhibit pleiotropic effects on many of the antioxidant enzymes involved in counteracting ROS that occur in oxidative stress. Also, *C. citrinus* extract has an inhibitory effect on pancreatic lipase and α-glucosidase activities, key enzymes in the digestion of lipids and carbohydrate (Chaudhary et al. 2012; Greiner et al. 2013; Ryu et al. 2014; Kim

et al. 2015; Fayemi et al. 2019; López-Mejía et al. 2019). Therefore, *C. citrinus* leaves could be used as a therapeutic alternative for the treatment of obesity, preventing the oxidative stress presented in this condition.

Conclusions

Callistemon citrinus leaf extract exhibited anti-lipase activity and showed weight loss through a decrease in fat accumulation. Liver, kidney, heart and stomach presented different levels of oxidative stress in the obese rats; however, *C. citrinus* induced antioxidant enzymes and reduced the biomarkers of the oxidative stress in all tissues. Given the promising results of this study, further investigation about bioavailability and pharmacokinetics would be advised to ensure the safe and effective use of *C. citrinus*.

Ethical approval

All authors read and approved the final manuscript.

Author contributions

LG O-P, JS P-S, OR M-R, LA A-R, AJ G-C, P R-CH contributed to analysis, design, critically revised the manuscript, gave final approval and agreed to be accountable for all aspects of work ensuring integrity and accuracy. LA A-R carried out the toxicology assays. D G-H contributed to analysis and gave final approval and agreed to be accountable for all aspects of work ensuring integrity and accuracy.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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