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Mango leaf tea promotes hepatoprotective effects in obese rats

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ABSTRACT

As mango leaf tea contains mangiferin and other bioactive compounds, this study investigated its anti-inflammatory, antioxidant and hepatoprotective effects on rats with high-fat induced obesity. Three groups were established: a control group (AIN93M diet), an obese group (high-fat diet HFD) and a treatment group (HFD with mango leaf tea for 8 weeks). Mango leaf tea increased antioxidant enzymes, total antioxidant capacity, AdipoR2 and PPAR-α mRNA and proteins expressions and, it also inhibited the NF-κB p65 and SREBP1c genes expressions in the liver. This beverage also leads to Cpt1 overexpression and a significant decrease in the accumulation of fat droplets, improving the hepatic steatosis. Molecular docking suggested a positive interaction between mangiferin, the main bioactive compound of mango leaf tea, and PPAR-α. Mango leaf tea exhibited a hepatoprotective effect through activating PPARα and decreasing the NF-κB p65 expressions, reducing oxidative stress and steatosis, and improving the lipid metabolism.

1. Introduction

Obesity is one of the most prevalent disorders worldwide and the main risk factor for the development of inflammatory process and oxidative stress to name a few. In 2016, an estimated of 41 million children (under the age of 5 years), 340 million children and adolescents (aged 5–19), and 650 million adults (aged 18 years and over) were overweight or obese. In accordance to the World Health Organization these global estimates are increasing (Fernández-Sánchez et al., 2011; World Health Organization, 2016). Fats excessive consumption results in a lipid metabolism disorder which can increase lipid delivery to the liver and reduce fatty acid oxidation. This can manifest in an accumulation of fatty acids (as triacylglycerols) in hepatocytes, causing hepatic steatosis (Fabbrini, Sullivan, & Klein, 2010; Koo, 2013). The body has a particular sensitivity to high-fat consumption, so it is more exposed to an imbalance of the redox homeostasis (between reactive oxygen species and antioxidants) and an increasing of proinflammatory mediators (Fernández-Sánchez et al., 2011; Vincent, Innes, & Vincent, 2007). Therefore, obesity by high fat diet intake is strongly related to oxidative stress and hepatic steatosis (Koo, 2013). During obesity, chronic inflammation occurs due to inflammatory molecules production (cytokines) and cells activation of the immune system (neutrophils and macrophages), as a result of the excessive fat cells accumulation, leading to the activation of different signalling pathways. The nuclear factor kappa B (NF-κB) and peroxisome proliferator-activated receptor (PPAR) pathways has been demonstrated to be involved in obesity inflammation process (Asghar & Sheikh, 2017; Lee, 2013; Pawlak, Lefebvre, & Staels, 2015; Tailleux, Wouters, & Staels, 2012).

Due to the serious effects of obesity on the human metabolism, many new treatments are being developed, including the use of natural and phytotherapeutic products. Teas and extracts containing multiple bioactive compounds have been widely studied and used. Teas are natural, inexpensive, and contain several bioactive compounds with functional properties, representing a great alternative for the treatment and prevention of obesity and its alterations (Chakrabarti, 2009; Jobu et al., 2013; Lee et al., 2011; Moreira et al., 2017).

Mango (*Mangifera indica*) is a tropical fruit rich in bioactive compounds with high therapeutic potential. The mango leaf is less used and it is considered a kind of crop waste. However, it is an important source of mangiferin, phenolic, flavonoids, benzophenones and antioxidants

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with free radical scavenging activity. These bioactive compounds has been linked to biological activities including anti-inflammatory, antioxidant, antidiabetic and others (Medina Ramírez et al., 2016; Pan et al., 2018; Ribeiro & Schieber, 2010; Ribeiro, Barbosa, Queiroz, Knödler, & Schieber, 2008; Zhang et al., 2011). Since tea is important to human life and mango leaves contain many bioactive compounds, we developed and studied a tea using processed mango leaves of Ubá variety. We have previously showed that the mango leaf tea contain 0.72 ± 0.08 mg ml^{-1} of mangiferin, 1.59 ± 0.11 mg GAE mL^{-1} of total phenolics and 80.33 ± 0.18% of radical scavenging activity (Medina Ramírez et al., 2016). Also we demonstrated that this beverage decreases the visceral fat accumulation, regulates glucose metabolism, stimulates the anti-inflammatory markers and improves adipocytes hypertrophy, confirming its anti-obesity effects (Medina Ramírez et al., 2017). In contrast, currently, there are not studies that report its effects on the liver of obese rats after treatment with mango leaf tea. Consequently, we hypothesized that mango leaf tea can modulate markers related to hepatic lipid accumulation, enhance antioxidation, and improve liver alterations caused by the high fat-diet intake. Thus, the current research aims to evaluate the hepatoprotective effects of mango leaf tea on liver damage in obese rats.

2. Materials and methods

2.1. Tea preparation

Young leaves from *M. indica*, Ubá variety, were collected in October (spring, 2015) from the Zona da Mata area (20°60′ S, 43°06′ W, 183 m), Minas Gerais State (Brazil). The plants were identified, and voucher specimens were deposited at the herbarium of the Federal University of Viçosa under the number No. VIC37611. The leaves were washed, sanitized, dried and crushed as described previously (Medina Ramírez et al., 2016). The fine powder obtained (50 g) was blended with one litre of water (5% of final concentration), boiled for 5 min and then filtered (Melitta filter paper N°4). The mango leaf tea was previously characterized by Medina Ramírez et al. (2016), where mangiferin was analysed via high-performance liquid chromatography (HPLC), total phenolic was estimated colorimetrically using the Folin-Ciocalteu reagent and the antioxidant activity was analysed by using the 2,2-diphenyl-1-picrilhidrazil-DPPH assay.

2.2. Assay biologic

A total of twenty-four, sixty-day old, male Wistar rats (200 ± 50 g) were placed under controlled conditions: 12/12-h light–dark cycle (AM 07:00-PM 07:00), room temperature 22 ± 3 °C and constant humidity (80%). The animals were supplied by the Animal Laboratory of the Biological Science and Health Centre, Federal University of Viçosa. All the experimental procedures were performed in accordance with the Ethic Committee for Animal Research of the Federal University of Viçosa, Brazil (approval registered under the number 29/2016). The experimental design and dietary intervention were previously described (Medina Ramírez et al., 2017). According to the method, the control non-obese group (CG) received AIN-93M diet and water, the obese group (OB) was fed a high-fat diet-HFD and water, and the treated group (TF) received a HFD and 50 mL/day of mango leaf tea, during eight weeks. OB and TF groups were both fed for 7 weeks with HFD before the intervention started. Water was administered to rats *ad libitum*. The tea was prepared daily and administered via oral using sipper bottles which were washed and changed daily, simultaneously with the tea. The experimental diets are presented in Table 1 (RESEARCH-DIETS®, 2006). The animals were anesthetized with Isoforine® 100% (Cristália, SP, Brazil) and euthanized by cardiac puncture. Blood and liver tissue were collected and stored at −80 °C, and a liver fragment was separated in order to perform histological analysis.

Table 1
Diets composition and caloric density.

Ingredients (g 100 g^{-1})	AIN-93M	HFD
Casein	14.00	19.50
Maltodextrin	15.50	10.00
Saccharose	10.00	34.10
Corn starch	46.57	5.32
Soybean oil (mL)	4.00	1.00
Lard	0.00	20.00
Cellulose	5.00	5.00
Mineral mix	3.50	3.50
Vitamin mix	1.00	1.00
Bitartarate choline	0.25	0.25
L-cystine	0.18	0.18
Cholesterol	0.00	0.15
Butylated hydroxytoluene	0.0008	0.004
Total	100.00	100.00
Calories (Kcal)		
Casein	56.00	78.00
Maltodextrin	62.00	40.00
Saccharose	40.00	136.40
Corn starch	186.28	21.28
Soybean oil (mL)	36.00	9.00
Lard	–	180.00
CD	3.80	4.70

CD: caloric density (Kcal g^{-1})

2.3. Food consumption, body weight and serum parameters

Tea and food consumption were measured daily and once per week respectively. For the tea intake control, drink spills and the surplus beverage in drinking bottles were collected and measured. The animals weight was monitored once per week at the same time. Food efficiency was calculated as follows: FE = [body-weight gain (g)/energy intake (kcal)] * 1000. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), and triacylglycerols (TG), were determined previously (Medina Ramírez et al., 2017), using commercial kits (Bioclin Quibasa, SP, Brazil).

2.4. Analyses of liver oxidative stress

All chemicals were of analytical grade and were purchased from Sigma-Aldrich (Saint Louis, USA). Liver homogenate was prepared homogenizing 200 mg of the liver tissue (one per animal of each group) in Tris-HCL buffer solution (10 mM pH 7.4). Afterwards it was centrifuged for 10 min at 4 °C and 12,000*g*. The supernatant was carefully collected and stored at −80 °C. Values obtained were normalized by the amount of total protein.

2.4.1. Bradford protein assay

Total protein was quantified by Bradford protein assay (Bradford, 1976). For the reaction, 5 µl of homogenate, 395 µl of distilled water and 100 µl of Bradford reagent (0.01% Coomassie Brilliant Blue [G250], 4.7% ethanol [95%], and 8.5% phosphoric acid [85%]), were combined. Samples were vortex for 30 s and allowed to stand for 15 min, without light illumination at room temperature (20 °C). The absorbance was read (spectrophotometer Thermo Scientific MultiSkan™ GO) at 595 nm. Values were expressed as milligrams of protein per millilitre (mg Ptn mL^{-1}), using a standard curve of Bovine serum albumin protein (2 mg mL^{-1}) with concentrations varying from 2 to 44 µg mL^{-1}.

2.4.2. Superoxide dismutase (SOD)

SOD activity was determined using the method based on the enzyme capacity to inhibit 50% of pyrogallol oxidation (Marklund & Marklund, 1974). For the reaction 20 µl of liver homogenate, 6 µl of MTT (bromide (3- [4,5-dimethylthiazol-2 M] -2,5-diphenyltetrazolium), 1.25 mM), 15 µl of pyrogallol (1 mM, HCL 10 mM) and 259 µl of Tris-EDTA buffer

(Tris 50 mM, EDTA 1 mM, pH 8.2), were mixed. The mixture was incubated for 5 min at 37 °C and the absorbance was read (spectrophotometer Thermo Scientific MultiSkan™ GO) at 570 nm. Measurements of blank (without sample and pyrogallol) and a standard (only reagents) were made. Values were expressed as a Unity of SOD per milligrams of protein (U SOD mg Ptn^{-1}).

2.4.3. Catalase activity (CAT)

CAT was determined by the spectrophotometric assay of hydrogen peroxide, where one unit (1 U) of catalase decomposed one micromole (1 μmol) of hydrogen peroxide, in one minute (1 min) of reaction (Aeib, 1884; Góth, 1991). According to the modifications, 10 μl of liver homogenate was added into 1 mL of phosphate-buffered saline (NaCl 136.9 mM, Na_2HPO_4 0.27 mM, KH_2PO_4 1.1 mM, pH 7.4) that contained 30% of hydrogen peroxide. Subsequently, the absorbance was measured (T70 + UV/VIS spectrometer, PG Instruments Ltd, Leicestershire, UK) at 240 nm on the times 0 and 60 s. The blank consisted of homogenate and phosphate buffer. Values were expressed as the unity of CAT per milligrams of protein (U CAT mg Ptn^{-1}), as follow: U CAT mg Ptn^{-1} = [(Abs_{t0} − Abs_{t60}) * 2.361]/(mg protein of sample * Abs_{t0}), where Abs was absorbance measured.

2.4.4. Total antioxidant capacity (TAC)

TAC was determined by ELISA using the Antioxidant Assay Kit (CS0790, SIGMA-ALDRICH, Saint Louis, USA), according to the manufacturer's instructions. The results were expressed as millimole of Trolox per liter of homogenate (mM Trolox).

2.4.5. Malondialdehyde (MDA)

MDA was measured by the TBARS test (thiobarbituric acid reactive substances) (Buege & Aust, 1978; Kohn & Liversedge, 1944). 100 μl of homogenate and 200 μl of TBARS solution (15% trichloroacetic acid, 0.375% thiobarbituric acid, HCL 0.25 M), were mixed. The mixture was incubated for 40 min at 90 °C and allowed to stand for 5 min. 300 μl of *n*-butanol was then added and mixed using a vortex for 2 min. Subsequently the mixture was centrifuged for 5 min at 3500*g* and the supernatant was collected. Absorbance was measured (spectrophotometer Thermo Scientific MultiSkan™ GO) at 535 nm. The results were expressed as nanomole of MDA per milligram of protein (nMol mg Ptn^{-1}) using a standard curve of TMPO (1,1,3,3 tetraethoxypropane) with concentrations varying from 2.5 to 20 μM.

2.5. Liver genes expression

RNA was extracted from the 100 mg of liver tissue, using Trizol reagent (Thermo Fisher Scientific, Carlsbad, USA). The cDNA was prepared from 2 μg of RNA using the M-MLV Reverse transcriptase kit (Thermo Fisher Scientific, Carlsbad, USA). Genes were analysed by Real-Time polymerase chain reaction (RT-qPCR) using 2 μl of cDNA and a SYBR Green Master Mix (Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions. RT-qPCR was also conducted under the following parameters: denaturation step at 95 °C for 10 min, 40 cycles of 95 °C for 15 s, annealing at 60 °C for 30 s and one extension steps at 72 °C for 30 s, followed by melting curve. The results were expressed as the fold change calculated according to the relative expression method, using a standard curve (Range of 1000–31.25 ng), and normalized to the OB group. cDNA samples were run in the AB SepOne Rt-PCR System equipment (Applied Biosystem). The primers sequences (Table 2) were obtained from the Primer3 plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

2.6. Proteins expressions by ELISA assay

Proteins were quantified by ELISA assay. Serum concentrations of adiponectin and resistin were determined by using the Rat Adiponectin (EZRADP-62 K, Merck Millipore KGaA, Darmstadt, Germany) and Rat Resistin (SEA847RA, Wuhan USCN Business Co., Ltd.-Life science, Houston, USA) kits, according to the manufacturer's instructions. The results were expressed as micrograms and nanograms of adiponectin and resitin respectively, per milliliter of serum.

For the PPAR-α and NF-κB p65 proteins analysis, nuclear protein fractions were extracted from the liver tissue using a Thermo Scientific™ NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Rockford, USA). Nuclear protein of PPAR-α and NF-κB p65 was quantified using the Rat PPAR-α (N° E-EL-R0725) and the Rat NF-κB p65 (N° E-EL-R0674) kits (Elabscience Biotechnology Co.,Ltd, USA), according to the manufacturer's instructions. Results were expressed as nanograms per milligram of nuclear protein (ng mg $Ptn^{-1)}$. Ratios of NF-κB p65/PPAR-α protein values were obtained.

2.7. Western blot analysis

Nuclear and cytoplasm protein fractions of liver tissue were prepared with Thermo Scientific™ NE-PER™ Nuclear and Cytoplasmic Extraction Kit (CA, USA). Total protein of NF-kB p65 was detected by Western blot assay using a Vertical Electrophoresis System (Loccus Verti-10, LPS 600 V, SP, Brazil). Nuclear and cytoplasm extracts (30 μl) containing 15 μg of total protein and 10 μl of buffer solution 4X, were mixed and applied in the gel. In the electrophoresis step, the homogenates were passed through SDS-polyacrylamide gel (Tris 1.5 M, pH 8.8; 30% acrylamide; 10% SDS; 10% ammonium persulfate; ultrapure water) in TAE 1x buffer, at a constant current of 100 V for 2 h 40 min. The proteins were transferred into polyvinylidene fluoride (PVDF) membranes (BioRad®, CA, California) using a transfer buffer (Tris 0.25 M, glycine 2.0 M and 20% methanol), at a constant current of 0.35A for 90 min. After transferred, membranes were washed with ultrapure water and stained with Ponceau 0.5% (red colorant). Then, unspecified proteins were blocked with 3% of bovine serum albumin (BSA) solution at 4 °C for 12 h and subsequently washed with TBS-Twin solution at room temperature. Membranes were incubated with a monoclonal primary antibody (anti-NF-kB p65, 65 kDA, ab13594, diluted 3:1000 in TBS-T and 3% BSA, Ab Cambridge, United Kingdom) at 4 °C for 12 h. Then, membranes were washed with TBS-Twin solution, incubated again in a conjugated secondary antibody (Horseradish Peroxidase - HRP, A9044, diluted 3:1000 in TBS-T and 3% of BSA, Sigma-Aldrich Ltda, St Louis, MO, USA) at room temperature for 1 h and washed as described previously. The NF-kB proteins were revealed using a mix solution (Tris-HCl 50 mM, pH 7.6; 3,3-Diaminobenzidine tetrahydrochloride; 30% H_2O_2) for 15 min at room temperature.

2.8. Molecular modelling

Molecular docking of the mangiferin with a PPAR-α receptor was carried out as previously described (Moreira et al., 2017). All computer applications were run on OpenSUS Tumbleweed. According to the modifications, mangiferin and Aleglitazar (ligand used in redocking analysis) were constructed using Maestro 10.2.010 (Schrödinger Release 2015-2: Maestro, version 10.2.010, LLC, New York, NY, USA). The software LigPrep 3.4 (Schrödinger Release 2015-2: LigPrep, version 3.4, LLC) with OPLS_3 force field and ionization state for pH 7.0 ± 2.0 was used for the preparation of the ligands involved in these studies. The crystallographic structure of PPAR-α (Protein Data Bank [PDB] ID: 3G8I) (Bénardeau et al., 2009) was obtained from the database PDB. The software Protein Preparation Wizard (Schrödinger Release 2015-2: Schrödinger Suite 2015-2 Protein Preparation Wizard; Epik version 3.2; Impact version 6.7; Prime version 4.0, Schrödinger, LLC) was used for the preparation of this receptor. The OPLS3 force field in the MacroModel 9.9 was used for optimization. Studies of molecular docking between PPAR-α and the ligand were performed using the Induced Fit Docking protocol (Small-Molecule Drug Discovery Suite 2015-2: Schrödinger Suite 2015-2 Induced Fit Docking protocol; Glide version 6.7; Prime version 4.0; Schrödinger, LLC), the grid box area was defined

Table 2
Oligonucleotides for qt-PCR analysis on the liver of experimental animals.

Target gene	Forward (5′-3′)	Reverse (5′-3′)
AdipoR2	CATGTTTGCCACCCCTCAGTA	ATGCAAGGTAGGGATGATTCCA
PPAR-α	CATTTCTGCTCCACACTATGAA	CGGGAAGGACTTTATGTATGAG
Cpt1	GTAAGGCCACTGATGAAGGAAGA	ATTTGGGTCCGAGGTTGACA
SREBP1c	CGCTACCGTTCCTCTATCAATGAC	AGTTTCTGGTTGCTGTGCTGTAAG
NF-κB p65	CTGCGCGCTGACGGC	TCGTCGTCTGCCATGTTGAA
TNF- α	ACGGCATGGATCTCAAAGAC	AGATAGCAAATCGGCTGACG
GAPDH	AGGTTGTCTCCTGTCACTTC	CTGTTGCTGTAGCCATATTC

ADIPOR2, Adiponectin receptor 2.
PPAR-α, peroxisome proliferator-activated receptor alpha.
CPT1, carnitine palmitoyltransferase I.
SREBP1c, Sterol regulatory element-binding proteins.
NF-κB p65, nuclear factor kappa B.
GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

as 20 × 20 × 20 Å. All computer programs belong to the Schrödinger suite.

2.9. Histology

Fragments of the liver were fixed in formalin (10%) and impregnated in the glycolmethacrylate resin (Leica, Historesin®). Sections of 5 μm were laminated and stained with hematoxylin-eosin (HE). The images were obtained using the Nikon-Elipse E600YF-L photomicroscope (Japan). Percentages of the nucleus, fat cells, inflammatory infiltrate, cytoplasm and blood vessels of liver tissue were analysed with ImageJ software (Fiji). The diameters of the nucleus and hepatocytes cytoplasm were measured by Image-Pro Plus 7.0 software. The nucleus/cytoplasm ratio (NCR) was calculated. The steatosis was measured semi-quantitatively according to the fat percentage as follows: grade 0, if < 5%; grade 1, if ≥5% and ≤33%; grade 2, if > 33% and ≤66%; and grade 3, if > 33% (Kleiner et al., 2005). The hepatosomatic index (HSI) was calculated as follows: HSI (%) = [(liver weight/final body weight of animal) × 100].

2.10. Statistical analysis

The study was designed with a power of 80%, the values obtained were expressed as a mean and standard error (SEM). Parametric one-way analysis of variance (ANOVA) followed by the *post hoc* Tukey test were used to compare the groups. A statistical analysis was performed using GraphPad Prism software version 5.0 (San Diego, CA). Results with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of the mango leaf tea in the body weight and serum parameters

No differences were observed in caloric intake between OB and TF groups until 8 week which showed an increase in the TF group. However, no differences were either observed in calorie intake per day. This similar calorie intake guaranteed that the effects observed in this study were not associated with the food consumption variation (Fig. 1A). After the third week, the mango leaf tea significantly suppressed weight gain. This effect was accompanied by a significantly lower values of food efficiency in the TF group (Fig. 1B and C). Tea consumption was 169 ± 1 mL/week (25 mL/day) and it did not change throughout the eight weeks of treatment (Fig. 1D).

Serum levels of Triacylglycerols and alanine aminotransferase (ALT) were significantly higher in the OB group when compared to the CG and TF groups. No differences in aspartate aminotransferase (AST) were observed between the groups. Total cholesterol was statistically lower when compared with OB group (Fig. 1E–H).

3.2. Effects of mango leaf tea on the oxidative stress

To investigate the effects of mango leaf tea on oxidative stress, SOD, CAT, MDA and TAC were evaluated. Lower concentrations of SOD were observed in the OB group, whereas CAT and TCA were not affected by the HFD. The mango leaf tea stimulated concentrations of CAT (32.4 ± 1.9 U CAT mg Ptn^{-1}) and TAC (0.27 ± 0.01 mM Trolox), nearly doubling the OB and CG values (Fig. 2A–C and E). The HFD increased the formation of lipid peroxidation products (MDA), in response to oxidative stress. Interestingly, lower values of MDA formation were observed in the animals treated (Fig. 2D).

3.3. Mango leaf tea on genes and proteins expression

After eight weeks of treatment, mango leaf tea significantly augmented 225 times mRNA levels expression of PPAR-α and attenuated proinflammatory genes NF-κB p65 and tumour necrosis factor alpha (TNF-α). Moreover, it leaded to upregulation (12 times) of adiponectin receptor II (AdipoR2) mRNA expression levels and overexpressed marker implicated in the β-oxidation regulation, carnitine palmitoyltransferase (Cpt1). The significantly increased expression of NF-κB p65, TNF-α and SREBP1c due to the HFD consumption, was inhibited by this beverage (Fig. 3).

Furthermore, to evaluate the effect of mango leaf tea on the excess adiposity and weight loss of the rats, adiponectin and resistin serum proteins were analysed elevating adiponectin serum levels and decreasing resistin values. However, the HFD had negative changes on these parameters. Correlation between adiponectin and resistin proteins confirmed it. In both serum protein concentrations, no differences were observed between the TF and CG groups (Fig. 4A–C).

In agreement with mRNA expression findings, the treatment with mango leaf tea stimulated the PPAR-α protein transcription (Fig. 4D). No changes were observed in the NF-κB p65 protein expression between the CG and the OB group. Though, correlation between NF-κB and PPAR-α proteins, indicated predominance of NF-κB in the OB group compared with the control group (Fig. 4E and F).

3.4. The PPAR-α and mangiferin interaction

Molecular docking of the mangiferin with PPAR-α receptor (Bénardeau et al., 2009) was performed and presented a docking score value (GScore) of −10.286 kcal mol^{-1}. This interaction occured mainly through 7 bond Hydrogen interactions with the following amino acid residues of the PPAR-α receptor site: Asn219 (two interactions), Ser280, Glu282, Thr283, Tyr314 and Hle440, in addition to 322 favourable hydrophobic interactions (van der Waals). Validated molecular redocking studies were performed on the align result with a RMSD value of 0.119 Å. (PDB code: 3G8I). The main interactions demonstrating that the affinity score could lead the agonist receptor activity

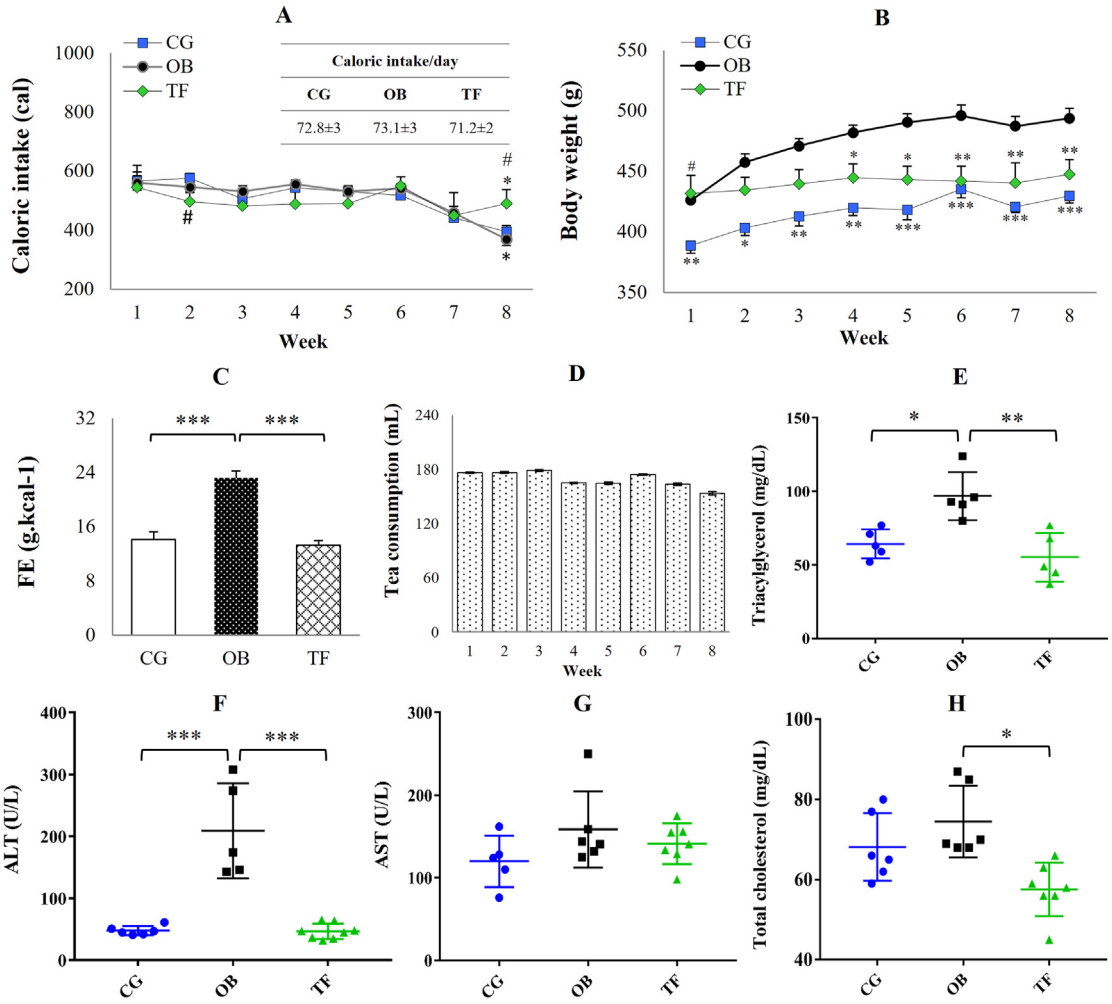
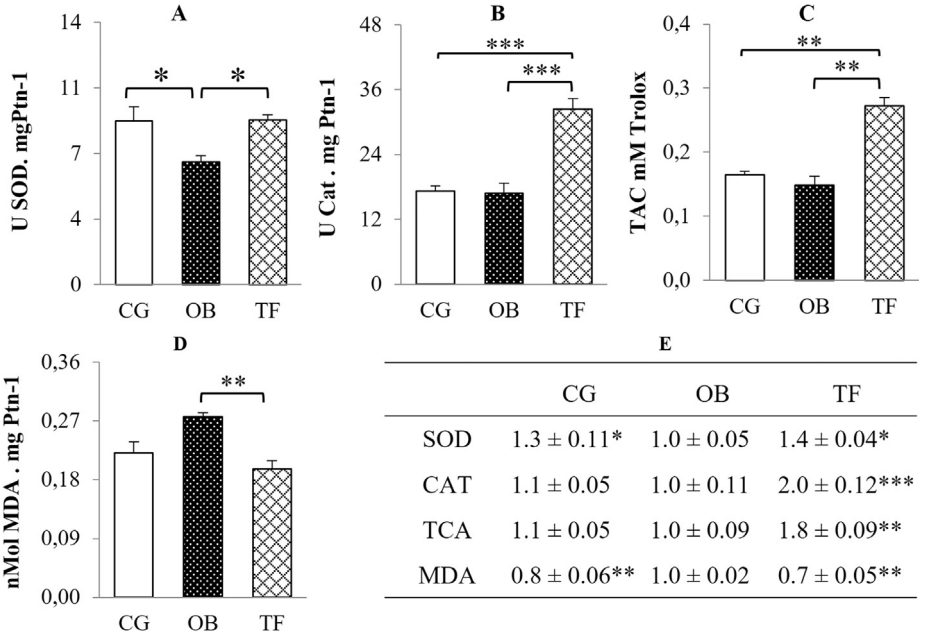


Fig. 1. (A) Caloric intake per week and per day, (B) body weight, (C) food efficiency (FE = [body-weight gain (g)/energy intake (kcal)] * 1000), and (D) tea consumption, of the experimental animals. Serum concentrations in animals of (E) Triacylglycerol, (F) alanine aminotransferase, (G) aspartate aminotransferase, and (H) Total cholesterol. The values represent averages and SEM (n = 8). Data were analyzed using ANOVA followed by the *post hoc* Tukey test. *P < 0.05, **P < 0.01, ***P < 0.001 indicated differences respect to the OB group; #P < 0.05 TF versus CG. Rats were fed an AIN93M diet (CG), HFD (OB) and HFD and treated with mango leaf tea (TF). No differences were observed in tea consumption between the eight weeks.



E

	CG	OB	TF
SOD	1.3 ± 0.11*	1.0 ± 0.05	1.4 ± 0.04*
CAT	1.1 ± 0.05	1.0 ± 0.11	2.0 ± 0.12***
TCA	1.1 ± 0.05	1.0 ± 0.09	1.8 ± 0.09**
MDA	0.8 ± 0.06**	1.0 ± 0.02	0.7 ± 0.05**

Fig. 2. Oxidative stress parameters on the liver of experimental rats after eight weeks treatment. (A) superoxide dismutase, (B) catalase, (C) total antioxidant capacity, (D) malondialdehyde, (E) results expressed as fold of values respect to the OB group. The values represent averages and SEM (n = 8). Data were analyzed using ANOVA followed by the *post hoc* Tukey test, *P < 0.05, **P < 0.01, ***P < 0.001. Rats were fed an AIN93M diet (CG), HFD (OB) and HFD and treated with the mango leaf tea (TF).

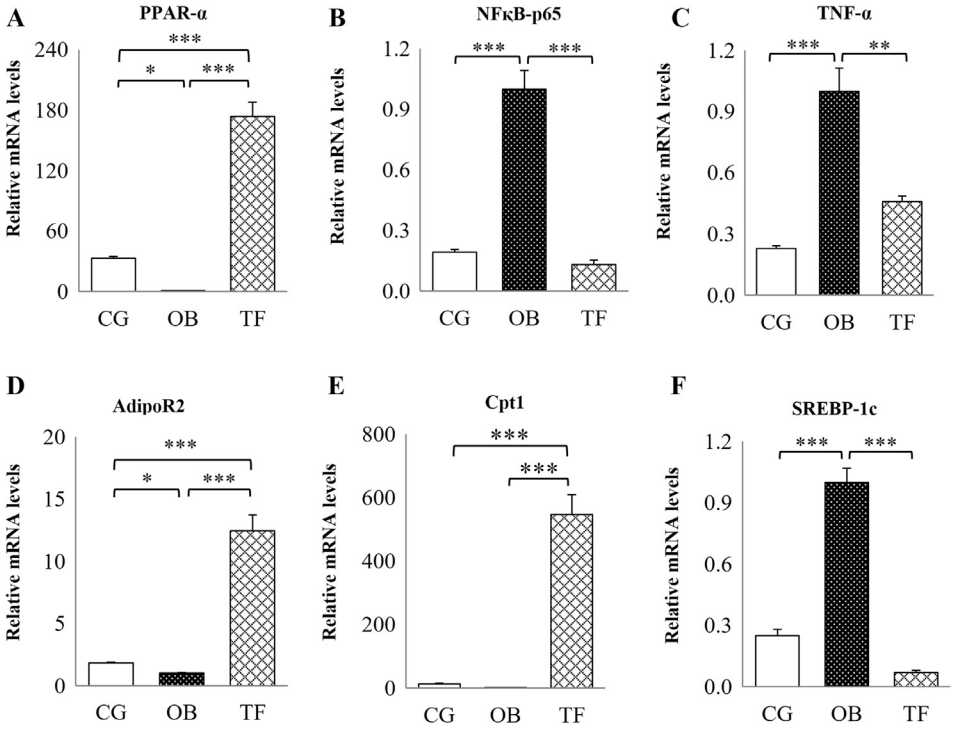


Fig. 3. Effects of the mango leaf tea on gene expression for the regulatory markers of inflammation and steatosis, on the liver of experimental rats after eight weeks treatment. mRNA levels of (A) peroxisome proliferator-activated receptor alpha, (B) nuclear factor kappa B p65, (C) tumour necrosis factor alpha, (D) adiponectin receptor II, (E) carnitine palmitoyltransferase I, (F) sterol regulatory element-binding proteins 1c. Data were quantified using the relative expression method (GAPDH as endogenous) and normalized to the OB group. All values represent averages and SEM (n = 5). Data were analysed using ANOVA followed by the *post hoc* Tukey test, *P < 0.05, **P < 0.01, ***P < 0.001. Rats were fed an AIN93M diet (CG), HFD (OB) and HFD and treated with mango leaf tea (TF).

(Fig. 5). Fig. 5A illustrates the results of molecular docking in a 2D format of the mangiferin with amino acids of PPAR-α, and Fig. 5B illustrates the best docking poses of the compound evaluated.

3.5. Changes in liver tissue morphometry

The OB group revealed normal hepatic architecture lost with inflammatory cell infiltration in vascular endothelium, whereas the TF group showed normal structure with blood vessels increased (Fig. 6A).

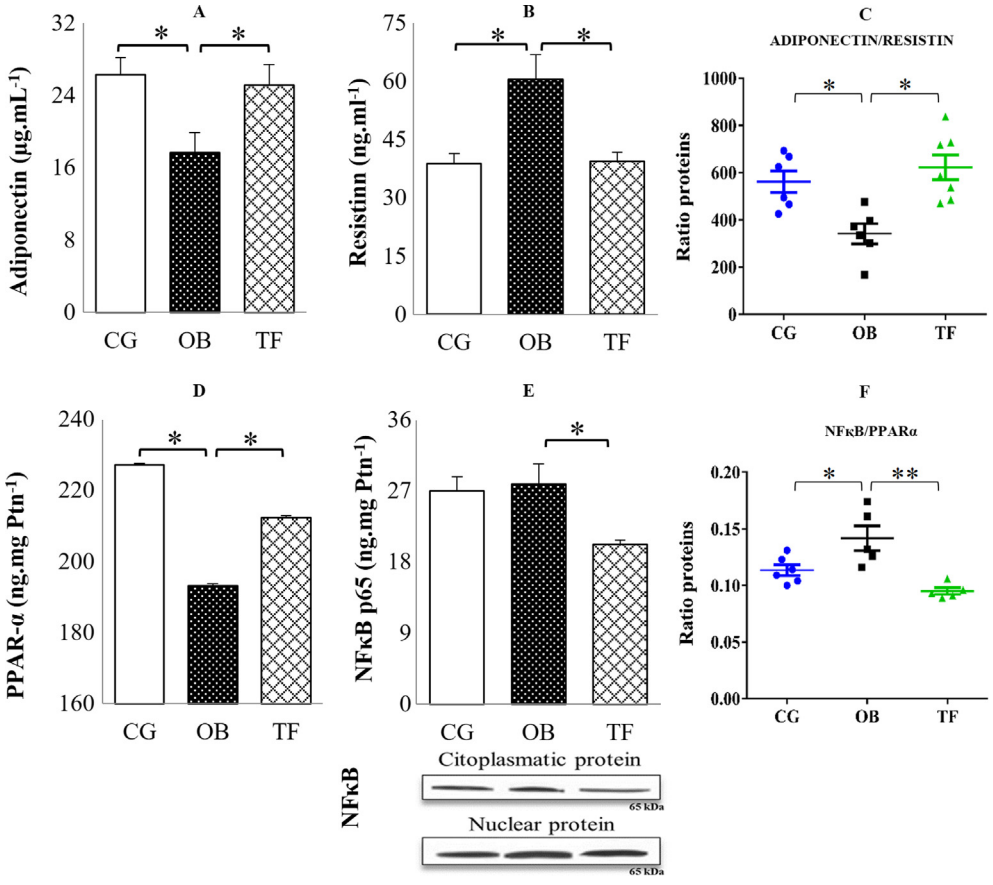


Fig. 4. Effects of the mango leaf tea on the protein concentrations of experimental rats after eight weeks treatment. Serum proteins of (A) adiponectin, (B) resistin, and (C) Ratios between the adiponectin and resistin values. Hepatic nuclear proteins of (D) PPAR-α, (E) NF-κB p65 and representative gel blots for levels of citoplasmatic and nuclear NF-κB p65 using western blot analysis, and (F) Ratios between the proteins values of NF-κB p65/PPAR-α. All values represent averages and SEM (n = 8). Data were analysed using ANOVA followed by the *post hoc* Tukey test, *P < 0.05, **P < 0.01, ***P < 0.001. Rats were fed an AIN93M diet (CG), HFD (OB) and HFD and treated with mango leaf tea (TF).

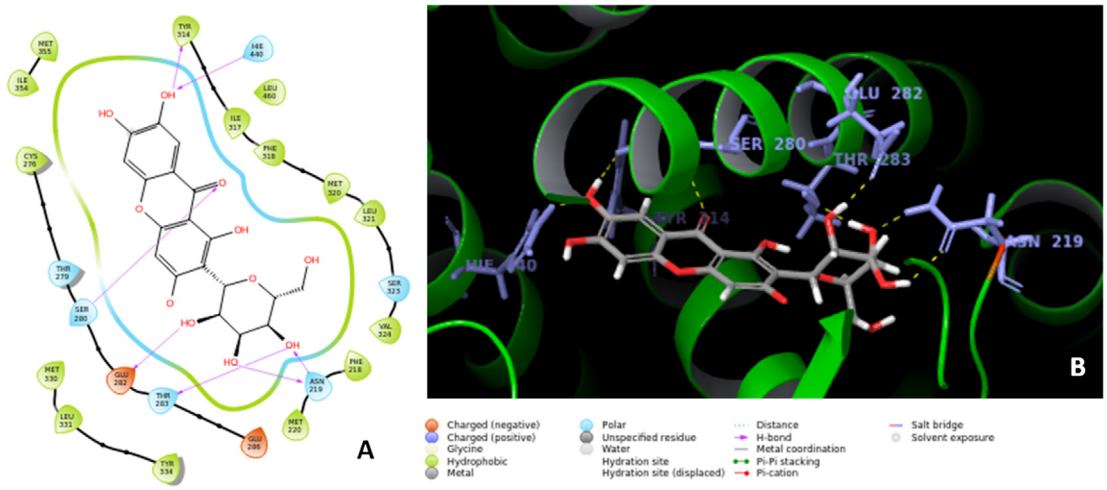


Fig. 5. (A) Interactions between amino acids of PPAR-α receptor site and mangiferin; (B) representation of the best pose of molecular docking result between mangiferin and PPAR-α.

However, inflammatory infiltrated cells did not differ between the three experimental groups (Fig. 6E). HFD triggers hepatic steatosis evidenced by the elevated percentage of accumulated cell fat (OB = 46%) which negatively altered the cytoplasm size and increased liver weigh in the OB group. According to the fat percentage, the steatosis grade obtained for each group was CG = 0, OB = 2 and TF = 1 (Fig. 6B). The hepatosomatic index was elevated in the OB group (3.8 ± 0.1%) when compared with the CG (2.9 ± 0.1%) and the TF (3.1 ± 0.1%) groups (Fig. 6D). After treatment with mango leaf tea, an increase in nucleus, cytoplasm and blood vessels percentages, were observed. This result agreed with lower percentage of fat droplets accumulated and, therefore, with the decrease of hepatic steatosis grade in the TF group (Fig. 6).

4. Discussion

In this study, *in vivo* effects of mango leaf tea were evaluated on stress oxidative, inflammatory processes and steatosis in the liver of obese-induced rats. We have shown that mango leaf tea treatment accompanied by a HFD can revert the diet alterations, by increasing antioxidant enzymes and anti-inflammatory biomarkers.

Based on the values previously reported (Medina Ramírez et al., 2016), we can establish that the treated animals consumed 121.2 ± 4.7 $mg.mL^{-1}$ of mangiferin and 269.5 ± 6.7 mg EGA mL^{-1} of total phenolics, per week. Some studies reported that mangiferin reduces lipid accumulation by activation of AMPK pathway, prevents liver steatosis by regulating hepatic proteins transcription, reduces HFD-induced liver toxicity and decreases inflammation by reducing JNK activation in plasma and liver of animal models of obesity (Benard & Chi, 2015; Lim et al., 2014; Liu et al., 2013; Niu et al., 2012; Wang et al., 2017; Xing et al., 2014). Phenolics also had positive effects on obesity and their alterations via signalling mechanisms, in animal and human models (Hsu & Yen, 2008).

As expected, HFD consumption increased the fat accumulated cells, affecting the body weight regulation and fatty acid metabolism. Although caloric intake was similar between the three groups, mango leaf tea supressed weight gain showing a preventive effect.

Higher levels of AST and ALT are an indicative of hepatocellular damage and these enzymes are important biomarkers of non-alcoholic fatty liver disease (NAFLD), and therefore, hepatic steatosis. Similarly, elevated triacylglycerols level is associated with increased delivery of fatty acids to the liver, leading to hepatic steatosis (Koo, 2013; Wedemeyer et al., 2010). High fat consumption alters cholesterol homeostasis and bile acid biosynthesis which plays an important role in the intestinal absorption of cholesterol and other fatty acids. Thereby, higher concentration of serum or plasma cholesterol reflects elevated intestinal absorption of cholesterol (A. E. Morgan, Mooney, Wilkinson, Pickles, & Mc Auley, 2016; Simonen, Gylling, Howard, & Miettinen, 2000). The lower values observed in the treated animals, suggested that mango leaf tea can reduce the intestinal absorption of cholesterol, decreasing the free cholesterol in the body. On the other hand, this beverage also decreased serum concentrations of triacylglycerols, ALT and total cholesterol, indicating that it could promotes positive changes in injury liver by HFD. A study demonstrated that mango tree leaf extract decreases triacylglycerols and cholesterol concentrations, and improve lipid metabolism in liver of animal model (Zhang et al., 2013).

In the oxidative stress model, ROS formation is increased, generating an imbalance between oxidant and antioxidant species, resulting in inflammation and cell degradation (Burton & Jauniaux, 2011; Radak, Zhao, Koltai, Ohno, & Atalay, 2013). Superoxide dismutase and catalase are enzymes with high antioxidant capacity which are used as markers of oxidative stress and reduce fat cell accumulation. As HFD stimulates ROS production, these enzymes increases its activity in response of an adaptive process to protect tissues. However, this adaptation is deficient when excessive formation of ROS overwhelms the antioxidant defence system and, therefore, the antioxidant enzymes quantities are not sufficient to balance oxidant and antioxidant species (Fernández-Sánchez et al., 2011; Matsuda & Shimomura, 2013). Curiously, we observed a decreasing in SOD but not for CAT in the obese group, and an overstimulation of CAT and the total antioxidant capacity in the treated group. The increased CAT in the obese group could be due to its activation as antioxidant defence system to control of redox homeostasis. This indicates the mango leaf tea could decrease in the ROS level by stimulation of superoxide dismutase and catalase activities, without forgetting lipid peroxidation decreased.

AdipoR2 functions as a mediator of adiponectin through the activation of several molecules, including PPAR-α pathway (Deepa & Dong, 2009; Kadowaki & Yamauchi, 2005; Smolinska et al., 2014). Some authors reported that up-regulation of PPAR-α improves lipid metabolism, inflammation and oxidative stress in obese mice (Nomaguchi et al., 2011; Park, Liu, Kim, & Shin, 2016; Pawlak et al., 2015). Negative modulation of AdipoR2 and PPAR-α is associated to hepatic steatosis (Peng et al., 2009). We found that a HFD decreases mRNA expression of AdipoR2 and PPAR-α, whereas mango leaf tea stimulates these genes expressions, attenuating the steatohepatitis. Furthermore, mango leaf tea overexpressed the Cpt1 marker, which is an enzyme strongly involved in fatty acid oxidation and mitochondrial β-oxidation (McGarry & Brown, 1997; Serra, Mera, Malandrino, Mir, & Herrero,

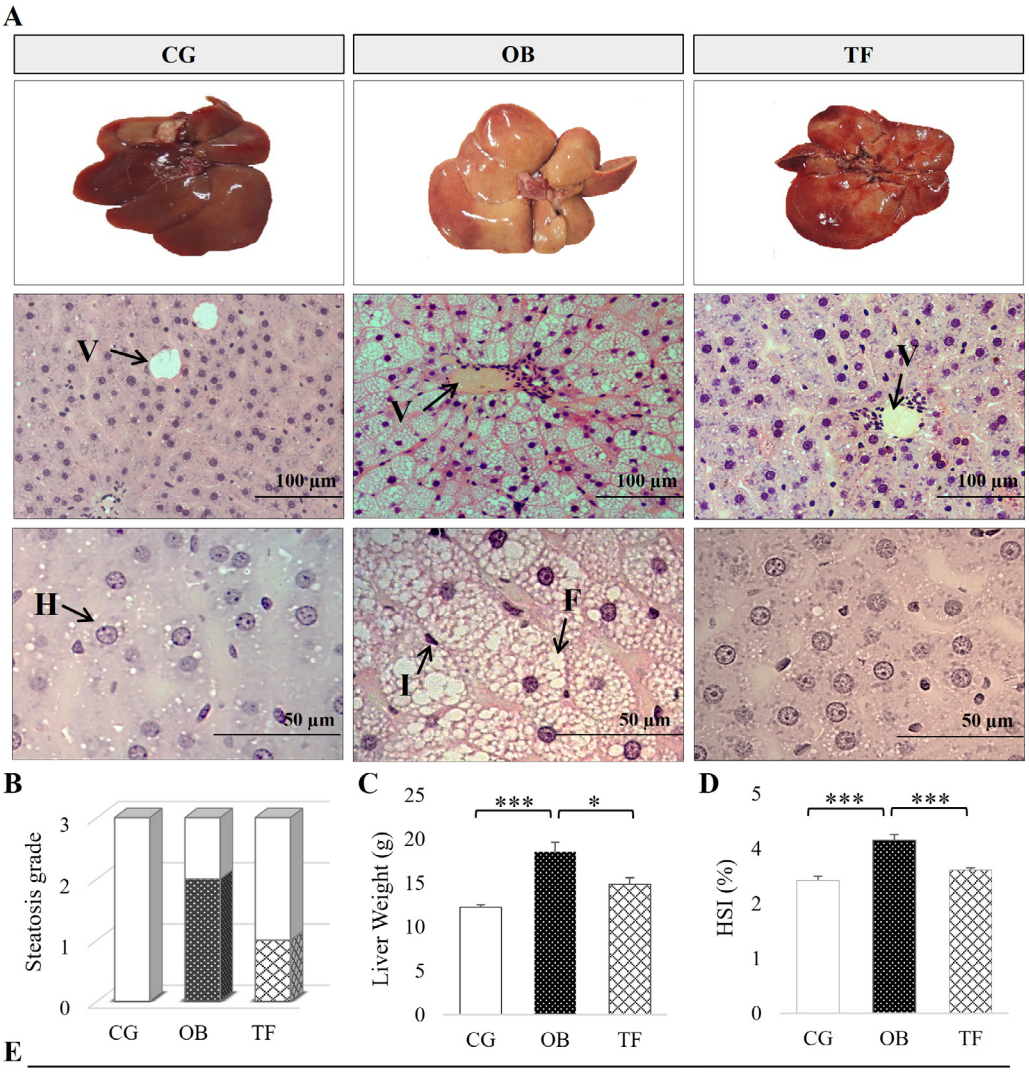


Fig. 6. (A) Liver tissue histology of experimental rats after eight weeks treatment (H, hepatocyte; V, hepatic vein; I, Inflammatory cell infiltration; F, Fat); (B) steatosis grade; (C) Liver weight; (D) hepatosomatic index percentage (HSI (%) = [(liver weight/final body weight of animal) × 100]), and (D) liver morphometric parameters (¥ percentage by total area of 52900 µm^2). Values are expressed as the means ± SEM (n = 8). Statistically significant differences were determinate using ANOVA followed by the *post hoc* Tukey test, *P < 0.05, **P < 0.01, ***P < 0.001. Rats were fed an AIN93M diet (CG), HFD (OB), and HFD and treated with mango leaf tea (TF).

E

	CG	OB	TF
Nucleus ¥	7.1 ± 0.8**	4.5 ± 0.9	5.3 ± 0.7**
Fat droplets ¥	1.4 ± 0.5***	46.3 ± 7.3	6.8 ± 3.3***
Inflammatory cell infiltration ¥	1.7 ± 0.3	1.7 ± 0.4	1.4 ± 0.3
Cytoplasm ¥	10.7 ± 2.3**	7.5 ± 1.1	10.2 ± 1.4**
Blood vessels ¥	78.8 ± 2.1***	39.9 ± 6.8	75.5 ± 3.1***
Diameter of cytoplasm (µm)	18.9 ± 0.9***	25.4 ± 0.9	20.7 ± 0.9***
Diameter of nucleus (µm)	8.2 ± 0.2	8.2 ± 0.2	8.3 ± 0.2
Ratios Nucleus/Cytoplasm	0.44 ± 0.0***	0.33 ± 0.0	0.42 ± 0.0***

2013). On the other hand, the SREBP1c, a major transcription factor that contributes to lipid synthesis, was overexpressed in the liver of obese rats (Eberlé, Hegarty, Bossard, Ferré, & Foufelle, 2004). We observed that Cpt1 mRNA expression could be responsible for improving the diet-induced lipid toxicity via β-oxidation. As well, it decreased the percentage of accumulated fat droplets and regulates the SREBP1c in the liver of the treated group.

TNF-α is an essential mediator of inflammatory responses that exerts pleiotropic effects on many types of cells by mediating a wide variety of biologic activities, including the NF-κB pathway activation (Mitoma, Horiuchi, Tsukamoto, & Ueda, 2018). NF-κB activity plays an important role in numerous biological processes; such as inflammation, stress responses, cell differentiation and programmed cell death. In this study, a HFD increased the levels of accumulated fat leading to ROS formation, generating oxidative stress and therefore activating the NF-κB pathway. Our data showed that lower values of MDA can be indicative of decreased ROS that could be associated to the down regulation of NF-κB p65 and upregulation of PPAR-α genes. This suggests the inhibitory role of mango leaf tea over lipid peroxidation and pro-inflammatory genes, via TNF-α and NF-κB modulation (Mitchell, Vargas, & Hoffmann, 2016; Morgan & Liu, 2011; Nakano et al., 2006).

Increased adiponectin positively participates in glucose metabolism, β-oxidation and weight loss, and also acts as anti-inflammatory. Some authors reported that this protein suppresses SREBP1c expression and prevents steatosis of the liver in mice. We suggest that the major adiponectin level observed in the treated group could be associated with the higher values of AdipoR2 and Cpt1 expressions, with the body weight gain suppressed and with SREBP1c inhibition (Awazawa et al., 2009; Fukushima et al., 2009). Resistin is expressed in adipose tissue, interferes in insulin activity, and has suggested to be directly proportional to lipid accumulation (Kusminski, Mcternan, & Kumar, 2005; McTernan, Kusminski, & Kumar, 2006; Steppan et al., 2001). Interestingly we found that mango leaf tea down-regulated serum resistin concentration to the point that it even matched the non-obese group. A

more recent study suggests that mango leaf tea improves glucose metabolism and β-cell function, by stimulating glucose uptake in the 30–120 min interval and, insulin secretion (Medina Ramírez et al., 2017).

The proteins expression levels suggested that mango leaf tea stimulates the PPAR-α transcription, which interacts with NF-κB p65 factor promoting its down regulation. This is due to PPAR-α acting as an antagonist of the transcriptional process of the pro-inflammatory markers related to obesity. This prevents liver fat cell accumulation, stimulating fatty acid oxidation and improving lipid homeostasis (Lefebvre, Chinetti, Fruchart, & Staels, 2006; Tailleux et al., 2012). Our findings suggest that mango leaf tea exerts anti-inflammatory functions and improves hepatic steatosis, via NF-κB modulation and PPAR-α activation.

Molecular docking studies were performed in order to verify the probable agonist profile of the compound mangiferin with the PPAR-α. The results presented good affinities values, corresponding to Gscore energy, for the compound analysed of $-10.286\ \text{kcal.mol}^{-1}$. The chemical structure of mangiferin presents various functional groups where hydrogen bond interactions are possible. The docking results indicated 7 interactions, as well as 322 Van der Waals contacts in hydrophobic regions of mangiferin, reinforcing the indication of possible agonist action of mangiferin. The results suggest an interaction that can lead to an agonist activity of the mango leaf tea in relation to the PPAR-α receptor, corroborating with the experimental results described in this study.

The morphometric analysis of liver tissue showed that a HFD increases the cytoplasm size of hepatocytes and leads to nucleus displacement. Evidently, this cytoplasm size was due to higher fat droplets accumulated and lipid toxicity caused by high fat consumption. This effect was also demonstrated by the nucleus/cytoplasm ratio. In OB group, the percentage of hepatocytes affected (46%) was indicative of hepatic steatosis grade 2 (Kleiner et al., 2005). This condition was reverted by the mango leaf tea, whose animals presented a hepatic steatosis grade 1 (6.8% of liver fat), and were not different to the control group. The hepatosomatic index is an indicator of energy reservoir in the liver and of hepatomegaly (Suchy, 2004). The rats supplemented with mango leaf tea showed a lower hepatosomatic index compared to the obese group, which can be associated to the higher reduction of fat droplets and lipids in the liver. PPAR-α was associated with lipid metabolism, increasing the fat acid oxidation and decreasing the fat droplets accumulation (Grygiel-Górniak, 2014). The absence of hepatomegaly in the treated group could be due to the high levels of PPAR-α (Ye et al., 2003). Since mango leaf tea treatment improves body weight, reduces biomarkers associated with oxidative stress and leads to lipid accumulation reduction, it is feasible to have positive effects on the histomorphometric and physiopathological parameters of liver tissue.

The animals treated with mango leaf tea consumed approximately $18\ \text{mg ml}^{-1}$ of mangiferin per day in order, to obtain these effects (25 mL of tea/day). Extrapolating to human dose per day and considering a human adult with a 60 kg body weight, this value corresponding to $2.9\ \text{mg Kg}^{-1}$ of mangiferin which are equivalent to 240 mL of mango leaf tea (Reagan-Shaw, Nihal, & Ahmad, 2008).

This study demonstrates that mango leaf tea has anti-inflammatory effects by modulating NF-κB p65 and increasing AdipoR2, PPAR-α and Cpt1 mRNA expressions in the liver. Moreover, this beverage is able to inhibit lipogenesis and lipid peroxidation, improving oxidative stress and liver steatosis in obese rats. In addition, the main bioactive compound of mango leaf tea, the mangiferin, could be responsible to induce anti-inflammatory markers. In conclusion, mango leaf tea has hepatoprotective potential against diet-induced obesity in rats.

Conflict of interest

The authors declare that they have no conflict of interest.

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