Biomedicine & Pharmacotherapy 106 (2018) 363–372

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Evidence of hypoglycemic, lipid-lowering and hepatoprotective effects of the Bixin and Bixin: β-CD inclusion compound in high-fat-fed obese mice

Ana Delia Pinzón-García[a], Laura Alejandra Ariza Orellano[b], Marcela Guimarães Takahashi de Lazari[b], Paula Peixoto Campos[b], Maria Esperanza Cortes[c], Ruben Dario Sinisterra[a,*]

[a] Chemistry Department, Institute of Exact Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. Av. Antônio Carlos, 6627, Pampulha, 31270-901, Belo Horizonte, MG, Brazil

[b] Department of Pathology, Faculty of Medicine, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. Av. Antônio Carlos, 6627, Pampulha, 31270-901, Belo Horizonte, MG, Brazil

[c] Restorative Dentistry Department, Faculty of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. Av. Antônio Carlos, 6627, Pampulha, 31270-901, Belo Horizonte, MG, Brazil

ARTICLE INFO

Keywords:
Bixin
Inclusion compound
Diabetes type II
Obesity
Steatosis

ABSTRACT

Associations between obesity, diabetes type II, and steatosis have long been recognized. However, a pharmacotherapy that acts in a multifactorial manner controlling the interactions between these conditions is not available. A variety of natural plants, functional fatty acids, and other natural dietary compounds have been used in various anti-obesity products. We investigated the effects of oral administration of an antioxidant carotenoid pigment Bixin and Bixin: β-Cyclodextrin in an obese murine model. C57BL/6 male mice (4–5 weeks) received standard diet (2.18 kcal per 1 g) (CT) and high-fat diet (4.38 kcal per 1 g) (CT/OB, BIX and BIX/βCD) (n = 10 per group). After 16 weeks, the BIX and BIX/βCD were treated by gavage (100 μL day-1) for six weeks, with water (CT and CT/OB groups) and (50 mg kg-1 day-1), Bixin (BIX group) or Bix: β-CD (BIX/βCD). Body weight, Lee's Index, adiposity, CHT, TG, CHT/HDL-c, glucose levels (metabolic markers) and, liver markers (AST and ALT) were determined. All metabolic and liver parameters exhibited down-regulation after oral administration of BIX and BIX/βCD. Particularly relevant was Lee's Index and adiposity in BIX- and BIX/βCD-treated groups (339.18 g/cm -BIX and 327.58 g/cm -BIX/βCD vs. 360.68 g/cm -CT/OB animals), this finds associated with the insulin sensitivity test, showed a clear association between reduction of adipose tissue and decrease of peripherical insulin resistant. In conclusion, our study suggested that the oral administration of the Bixin and Bix: β-CD inclusion compound improved the metabolic parameters evaluate in obese mice, being more palatable and hepatoprotective.

1. Introduction

Obesity and overweight are major contributors to the global burden of chronic diseases and their complications, including diabetes, cardiovascular diseases, hypertension, osteoarthritis, some cancers and inflammation-based pathologies, which suggests that the obese are likely to have a disproportionate use of the health care system [1,2]. The World Health Organization (WHO) reported that in 2014, more than 1.9 billion adults were overweight worldwide, and of these adults, over 600 million were obese [3]. The high prevalence of overweight and obesity, combined with their concomitant risks, poses a particularly relevant worldwide public health challenge. At the same time, it is well established that obesity and metabolic syndrome are commonly associated with nonalcoholic fatty liver disease (NAFLD), one of the most common causes of chronic liver disease worldwide. In fact, it is so closely associated that hepatic steatosis has been proposed as a diagnostic criterion of metabolic syndrome. Hepatic steatosis is present in greater than 60% of obese and 90% of morbidly obese adults, and the prevalence of elevated alanine aminotransferase (ALT) in obese youth is a biological marker of this disease [4].

Abbreviations: BIX, Bixin (*BixaOrellana*); BIX:β-CD, Bixin and β cyclodextrins (inclusion complex); CT, control; CT/OB, obese control; HFD, high fat diet; NAFLD, nonalcoholic fatty liver disease; BCRJ, Banco de Células de Rio de Janeiro; DEX, dexamethasone; IST, insulin sensitivity test; CHT, cholesterol; TG, triglycerides; GLU, glucose; ALT/GTP, alanine aminotransferase; AST/GOT, aspartate amino transferase; CEUA, Ethics Committee of Animal Experimentation

* Corresponding author.

E-mail addresses: apfarmaceutica@gmail.com (A.D. Pinzón-García), lariza551@gmail.com (L.A.A. Orellano), takahashi.marcela@gmail.com (M.G.T. de Lazari), paulapet2003@yahoo.com.br (P.P. Campos), mecortes@yahoo.com (M.E. Cortes), sinisterra@ufmg.br (R.D. Sinisterra).

https://doi.org/10.1016/j.biopha.2018.06.144
Received 9 February 2018; Received in revised form 18 June 2018; Accepted 25 June 2018

A variety of natural plants (e.g., herbs, fruits, and vegetables), functional fatty acids (e.g., polyunsaturated fatty acids and conjugated fatty acids), and other natural dietary compounds have been used in various anti-obesity products [5]. There are also alternative strategies to treat obesity and diabetes using natural products, such as the polysaccharide portion of wolfberry, garlic-derived monomers, red grape-derived resveratrol, and milk thistle–derived substances [6,7], as well as peptides or proteins isolated from many animal sources [8,9] that have different action mechanisms in specific signaling pathways related to glucose and lipid metabolism. Among these natural products, antioxidant carotenoid pigments extracted from *Bixa Orellana L.* seeds, a shrub native to tropical America, and especially Bixin, its main constituent, are shown to have several bioactive properties including antioxidant effects [10] [11], activity in cancer cells [12,13], and anti-inflammatory activity [14,15]. The antioxidant effect of Bixin is important in the prevention of hyperlipidemia and arteriosclerosis [16] and in vitro and in vivo reports have shown that the partially purified extract of *Bixa Orellana L.* and purified Bixin showed hypoglycemic effects [17–20]. These results suggest that Bixin should potentially replace the actions of hypoglycemic drugs as thiazolidinediones and statins in the treatment of diabetes type 2 or hyperlipidemia. Unfortunately, the clinical relevance of Bixin is limited by its low solubility and stability against light and oxygen during processing and storage, owing to its largely hydrophobic structure [21,22].

In the pharmaceutical arsenal to increase water solubility and physicochemical stability, cyclodextrins are used in drug: cyclodextrin inclusion compound. Cyclodextrins have also been considered to be used in delivery systems based on their ability to form inclusion compounds and improve the solubility and bioavailability of drugs [23–29]. Some reports described the interaction between Bixin and cyclodextrins (α and β-CD) as a strategy to stabilize food formulations against air, ozone, light, and high temperatures [30,31]. Thus, we hypothesize that an inclusion compound of Bixin and cyclodextrin can be useful for treating obesity, hypoglycemia, and lipid-lowering or nonalcoholic fatty liver steatosis; thus, the present report aims to investigate in vitro or in vivo tests using Bix: β-CD inclusion complex oral formulations.

2. Materials and methods

2.1. Materials

Bixin (Bix) powder was extracted from seeds according to the methodology established by Barbosa et al. [32]. Beta-cyclodextrin (β-CD) was purchased from Cerestar Co, IN, USA. All other reagents (Acetone, $NaHCO_3$) were of analytical grade. For in vitro assays, 3T3-L1 mouse pre-adipocyte fibroblasts (BCRJ Code: 0019) were purchased from Banco de Células de Rio de Janeiro (BCRJ), and Dulbecco's Modified Eagle's Medium High Glucose (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution, 1-methyl-3-isobutyl xanthine (IBMX), dexamethasone (DEX) and insulin (INS) were all purchased from Gibco-Invitrogen, Brazil.

2.2. Inclusion compound preparation

The Bix: β-CD inclusion compound was prepared in a 1:1 molar ratio. Briefly, the first solution of 39.5 mg of Bix in 5 mL acetone was mixed in 50 mL $NaHCO_3$ solution (0.05%) to complete solubilization, and a mixture of a solution of 113.5 mg β-CD in 30 mL of distilled water was prepared using magnetic stirring for 12 h. The final solution was freeze-dried. For comparison, a 1:1 molar ratio physical mixture (PM) of Bix (3.9 mg) and β-CD (11.3 mg) was also prepared.

2.3. Physicochemical characterization of an inclusion compound

Physicochemical characterization was performed using Fourier transformed-infrared (FTIR) spectra collected with a Perkin Elmer Spectrum GX spectrophotometer, KBr pellets, and scanning between 4000 and 400 cm^{-1}. The X-ray diffraction patterns (XRD) were obtained on a Shimadzu XRD-7000 X-Diffractometer with Cu Kα = 1.54051 radiation. The scanning speed was 2θ/min. The thermal stability of the Bixin, Bixin-β-CD inclusion compound and physical mixture were assessed using an SDT Q600 TA analyzer (TG/DTG-DTA) over a temperature range of 25 °C–700 °C at a heating rate of 10 °C/min under a nitrogen purge. The one-dimensional NMR spectra (^1H) and two-dimensional spectra (2D ROESY) were obtained using a Bruker Avance DPX-400 (400 MHz). Samples were prepared in NMR tubes 8.00 inches in length with 5-mm external diameter.

2.4. In vitro assays

2.4.1. Culture and induce insulin resistance in 3T3-L1 cells

The 3T3-L1 pre-adipocytes was cultivated at a density of 10^5 cells/cm^2 in complete DMEM with 10% FBS and 37 °C and 5% CO_2 conditions until confluence. On the second day after confluence, the complete medium was replaced with differentiation medium (DMEM supplemented with IBMX 0.5 mM, DEX 0.1 μM and INS 10 mg mL^{-1} (1.72 mM), and cells were maintained for h. After this treatment, the cells were washed with PBS (pH 7.4), and the culture medium was replaced and changed every two days until completion after eight days [33]. Differentiated adipocytes were exposed for eight days by DMEM supplemented with DEX at a concentration of 1000 nM for 48 h. After exposure to DEX, the medium was replaced with DMEM with low glucose content. After one hour, the medium was supplemented with insulin (2500 nM) for glucose uptake stimulation for 24 h, and the residual glucose in the medium was quantified [34]. Differentiated adipocytes were exposed for 48 h to different concentrations of the Bix and Bix: β-CD inclusion compound. After this treatment, the cells were washed twice with PBS, and the medium was replaced with complete culture medium supplemented with DEX (1000 nM) and maintained for 48 h. The medium was replaced by DMEM with low glucose content and, after one hour, was supplemented with insulin (2500 nM). After 24 h of exposure to insulin, the residual glucose in the medium was quantified using the glucose liquiform enzyme-colorimetric endpoint assay (Labtest, Brazil) according to the instructions and standards of the manufacturer, and reading was performed with a spectrophotometric microplate format (96 well plates) in triplicate. The evaluation included the quantification of residual glucose after treatment in normal cells and the insulin resistance cell phenotype was induced by DEX [35,36].

2.5. In vivo studies

Male C57BL/6 mice aged 4–5 weeks were obtained from the animal care center at the Universidade Federal de Minas Gerais (CEBIO/UFMG) and kept under control conditions at room temperature (24 ± 2 °C), with alternating 12 h light and dark cycles with free access to food and water. They were maintained according to the ethical guidelines of our institution (experimental protocol approved by the Animal Ethics Committee at the university, CETEA/CEUA – UFMG, number 318/2015).

First, the mice were divided into two groups and treated for 16 weeks: the control group (n = 10) was fed with the Nuvilab CR-1® Quimtia mouse diet (Colombo, Brazil) with regular maintenance composed of 56.1% carbohydrate, 29.4% protein and 14.8% fat (3.03 kcal per 1 g of diet); the second group (n = 30, 10 animals per group) received the high-fat diet (HFD) to induce metabolic changes. The HFD diet was composed of 45% fat (42.1% lipid, 21.9% protein and 35.9% carbohydrates). The high-fat diet was prepared following the protocols described previously [37–39]. Lee index (calculated as one-third of body weight (grams)/Nasal-anal length (centimeters) x 1000) were used to determine obesity. Lee index > 344.32 after administration of the HF diet for 16 weeks were considered obese [37,40–42]. After 16 weeks, the mice from the HF diet group were redistributed equally into

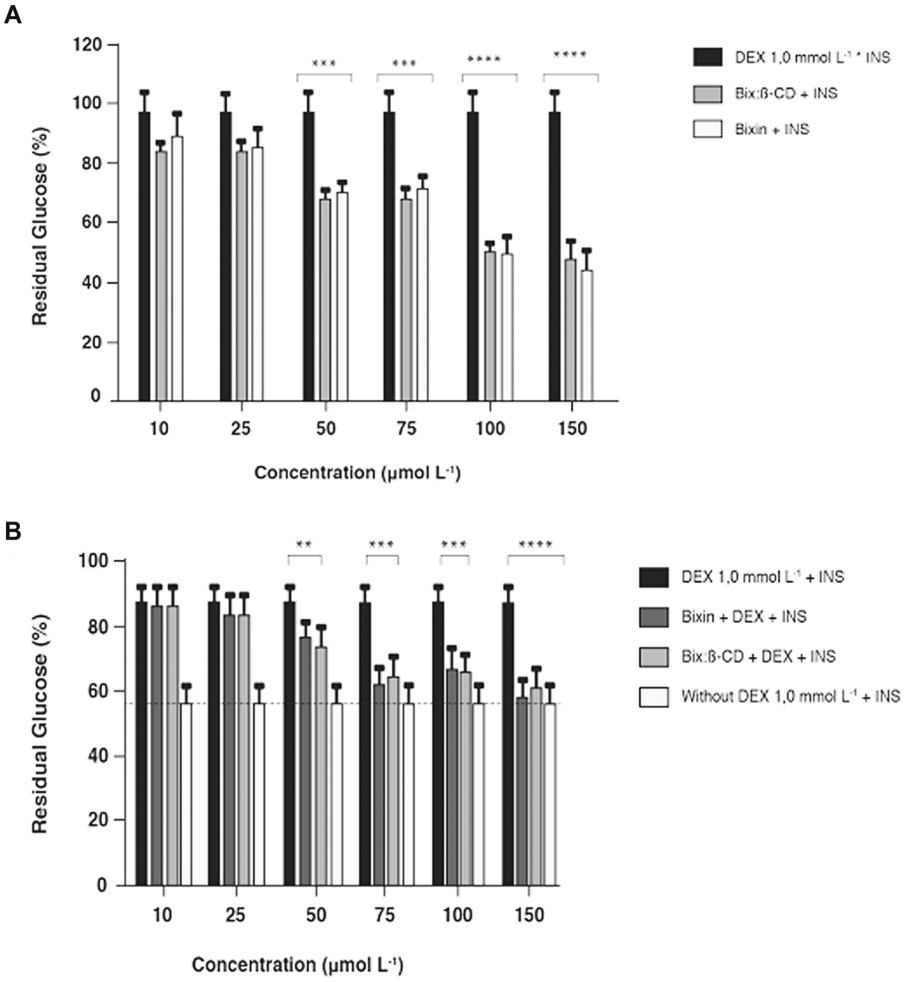

Fig. 1. Effect treatment with BIX and BIX/βCD on insulin-stimulated glucose uptake in a cellular model. A) Insulin-stimulated glucose uptake over normal 3T3-L1 cells of Bixin and Bix: β-CD inclusion compound in concentrations between 10 and 150 mmol L-1 after 48 h. B) Insulin-stimulated glucose uptake over insulin resistance phenotype differentiates adipocytes of Bixin and Bix:β-CD inclusion compound in concentrations between 10 and 150 mmol L-1 for 48 h. Was used the Glucose Liquiform enzyme-colorimetric assay. Data are expressed as mean ± SD, n = 4 replicates/group. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (two-way ANOVA).

four groups and subjected to treatment by gavage 100 µLday^{-1} for five weeks with the following protocols: water (CT and CT/OB groups), Bixin water suspension of Bixin (BIX) and Bix: β-CD inclusion compound (BIX/βCD). The doses administered for Bixin and inclusion compound Bix: β-CD were 50 mg kg^{-1}day^{-1}.

Food intake and water consumption were measured twice per week during treatment to calculate food efficiency (food intake/body weight). Overnight-fasted mice were killed by decapitation, and samples of blood, white adipose tissue (epididymal, mesenteric and retroperitoneal) and liver were collected, weighed and immediately frozen on dry ice and stored at −80 °C for subsequent analysis. The weight of fatty tissue was adjusted to body weight.

Metabolic parameters were assessed using the insulin sensitivity test (IST), which was performed for two time periods. Initially, at week 16, the initial condition of obese animals was assessed before administration of the respective treatments; IST was performed on overnight-fed mice after intraperitoneal injection of insulin (0.28 units kg^{-1} body weight [3,49 mg/mL] equivalent 100UI). Tail blood samples were taken at time 0 (before insulin administration), 30, 60, 90 and 120 min after injection.

Total serum cholesterol (CHT), triglycerides (TG), glucose (GLU) and the enzymes alanine aminotransferase (GTP/ALT) and aspartate aminotransferase (GOT/AST) were performed using enzymatic-colorimetric kits (Wiener Lab) according to the instructions and standards of the manufacturer, and measurements were taken using a spectrophotometric microplate format (96 well plate) in triplicate.

Adipose and liver tissue were excised and fixed in 10% buffered formalin, pH 7.4, and processed for paraffin embedding for histological studies. Sections with a thickness of 5 mm were stained with hematoxylin and eosin (H&E) for light microscopic studies and analyzed under 200× magnification. The hepatic histological features were investigated, and the lesions were evaluated using a scoring system for necroinflammation adapted from Brunt et al. [43].

2.6. Theory/calculation

The results were presented as the mean ± SEM. The assumptions of normality and homoscedasticity were determined for subsequent statistical analysis. Statistical analysis was performed using the GraphPad Prism 6.0 software for Windows (GraphPad Software, San Diego California, USA), by One-way ANOVA (interaction between independent time and diet variables) and two-way ANOVA, followed by post-test Newman-Keuls for one-way ANOVA, and post-test Bonferroni for two-way ANOVA multiple comparison. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Physico-chemical characterization

The Bixin, β-CD, and Bix: β-CD inclusion compounds and physical mixture (PM) were characterized using FTIR spectra and XRD pattern diffraction (Figs. 1S and 2S Supplemental material respectively). Thermal analysis TG, DTG, and DTA curves for Bixin, β-CD, Bix: β-CD and PM were performed and shown (Fig. 3A, B, and C Supplemental material). To obtain more insights on the intermolecular interactions between Bix and β-CD, two-dimensional 2D-ROESY experiments were performed because this technique is one of the most effective

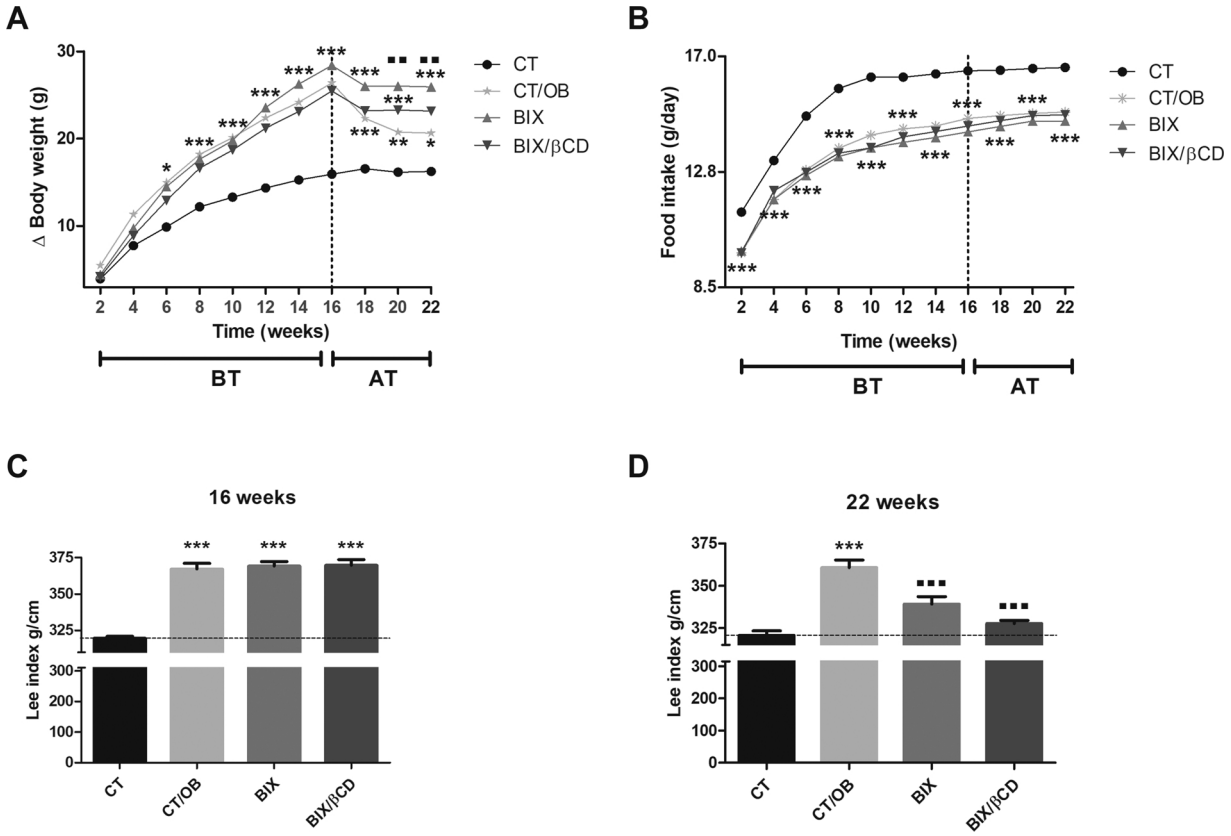

Fig. 2. Effect of a high-fat diet with and without treatment BIX and BIX/βCD compared with the normal diet. A) Time course evaluation of Δ in body weight (g) gain of high-fat fed mice compared to animals receiving standard diet. B) food intake throughout the experiment (16 weeks – before treatment (BT) – and 6 weeks with treatment (AT – after treatment), totaling 22 weeks). Effect of a high-fat diet in Lee's index C) before (16 weeks) and D) after treatment (22 weeks) with BIX and BIX/βCD compared with normal diet in C57BL/6 mice. Data are expressed as means SEM, n = 9 per group; *Significant difference between high-fat fed groups and CT group, ■Significant difference between treated groups and CT/OB group. *p < 0.05, **p < 0.01; ***p < 0.001; ■■ p < 0.01; ■■■p < 0.001. Two-way ANOVA; One-way ANOVA.

techniques for studying cyclodextrin host:guest interactions [24,44] and analyzing cross-peaks involving protons (H3 and H5) inside the β-CD cavity, Fig. 4S. These combined physico-chemical results were sufficient to demonstrate Bixin inclusion in the β-CD cavity. Afterward, this compound was used for in vitro and in vivo assays.

3.2. In vitro assays

The evaluation included the quantification of residual glucose after treatment with the Bixin and Bix:β-CD inclusion compound on insulin-stimulated glucose uptake in normal cells and cells with the insulin resistance phenotype induced by DEX [35,36]. Bixin and Bix:β-CD inclusion compound treatment in higher concentrations (100 and 150 μmolL^{-1}) enhanced insulin-stimulated glucose uptake by approximately 60%, whereas treatment with Bixin 10 and 25 μmol L^{-1} had no effect compared to untreated control cells, as shown in Fig. 1A.

As it may be observed, the addition of Bixin and Bix:βCD inclusion compound show that these substances behave as assisting agents in the glucose uptake process when compared to dexamethasone (insulin resistance-inducing agent) thereby assuring that differentiated cells have the ability to capture glucose under normal conditions.

When insulin-dependent glucose uptake was analyzed for the insulin resistance phenotype 3T3-L1 cells can be observed that the insulin stimulation exerts no effect on glucose uptake due to the presence of dexamethasone, and the treatment with the Bixin and Bix:β-CD inclusion compound at concentrations between 10 and 150 μmol L^{-1} for 48 h, higher reversed insulin resistance in cells was observed in a dose-dependent manner as seen in Fig. 1B. Additionally, the presence of pure β-cyclodextrin did not show changes in glucose levels in the medium.

3.3. In vivo assays

3.3.1. High-fat diet and effect of treatments on weight gain, food intake, and adiposity

The variation in body weight and food intake in C57BL/6 mice in a diet-induced obese model with a high-fat diet (HFD) feeding is shown in Fig. 2A–B. Starting from baseline, the HFD mouse groups (Obese control (CT/OB), BIX and Bix:β-CD inclusion compounds significantly increased body weight over time (16 weeks), after the begging of the treatments a reduction in weight gain was observed in BIX and Bix:β-CD groups. Body weight loss of the CT/OB group could be explained by a metabolic decompensation related with the peripheral resistance to insulin in obese animals without treatment [45]. There was no significant difference until the 22nd week of treatment in the BIX and BIX/βCD groups. As shown in Fig. 2B, the mean food consumption was significantly different between the groups that received high-fat diet (control group of obese mice (CT/OB), BIX and BIX/βCD) when compared to the animals that received standard diet (control group CT). Interestingly, there was no association between food intake and weight gain with the animals treated with HFD diet. The group with higher weight gain exhibited lower food intake compared to the control group (CT). These results demonstrate the caloric potential of HFD as reported previously by several authors in the same animal model of obesity and insulin resistance [46,47].

Lee index measure of the effect of the HFD before (confirm obesity) and after treatment (evaluated effect on adiposity) with BIX and BIX / βCD at the 16th and 22nd weeks, respectively. Adiposity was significantly increased by feeding the mice with the HFD compared to the standard diet (16 weeks) (Fig. 2C), and it was significantly reduced in

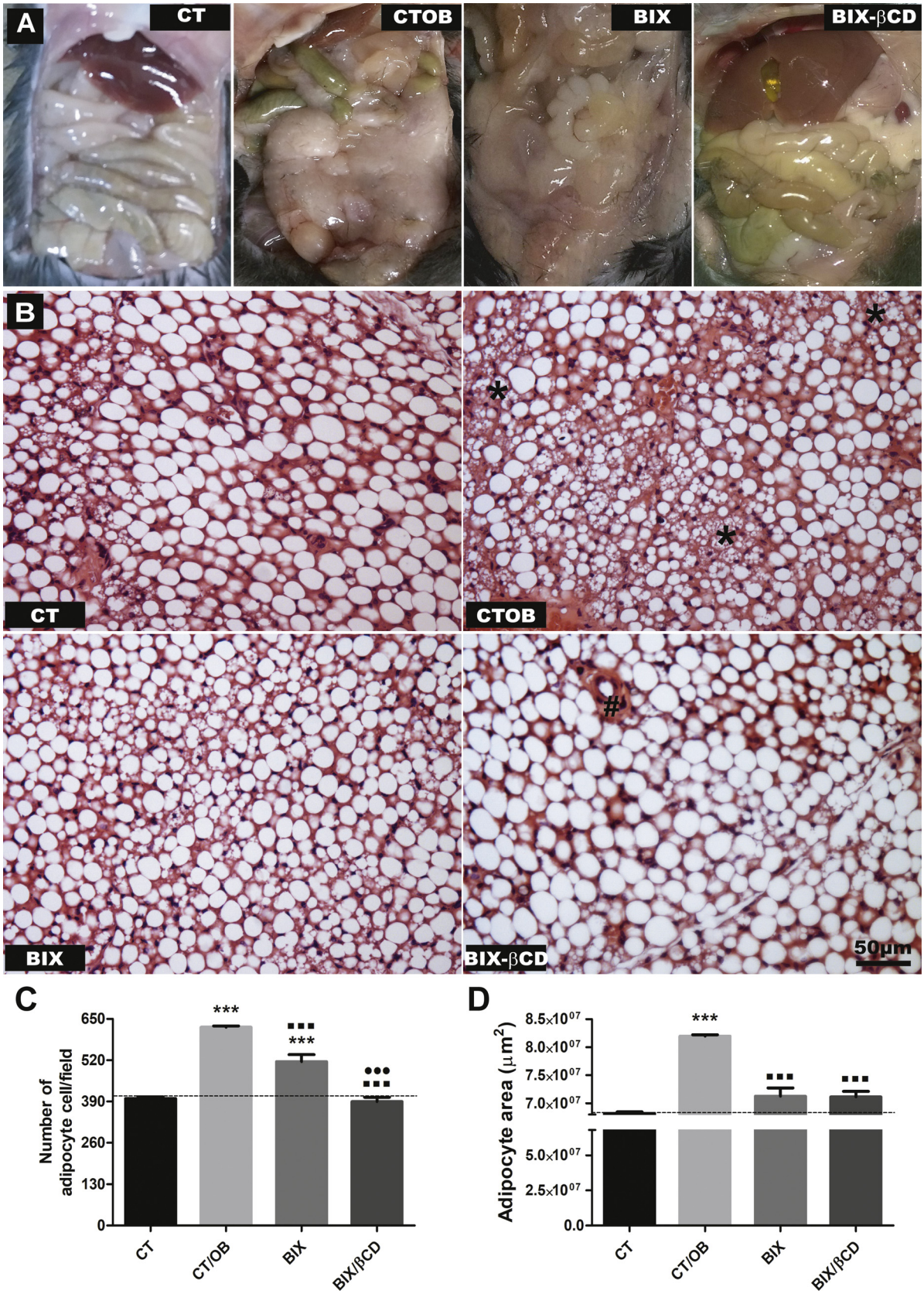

Fig. 3. Effect treatment with BIX and BIX/βCD on Adiposity in High-fat fed C57BL/6 obese mice. A), In situ images of visceral fat accumulation in all groups: CT; CT/OB; BIX and BIX/βCD after 22 weeks of experiments. B), histological findings of epididymal adipose tissue of CT; CT/OB; BIX and BIX/βCD groups, the treatment decreased the accumulation of adipose droplets. C) Effect of a high-fat diet in a number of adipocyte cells and D) adipocyte area, showed a decreased in these parameters in animals with treatment (BIX and BIX/βCD) compared with normal CT/OB C57BL/6 mice. Asterisk and hash indicate an increase in adipocyte proliferate and blood vessels, respectively. Data are expressed as mean ± SEM, n = 9 per group. *Significant difference between HFD groups and CT group, #Significant difference between treated groups and CT/OB group; &Significant difference between treated groups and BIX group; p < 0.001; ■■■p < 0.001; •••p < 0.001 (two-way ANOVA).

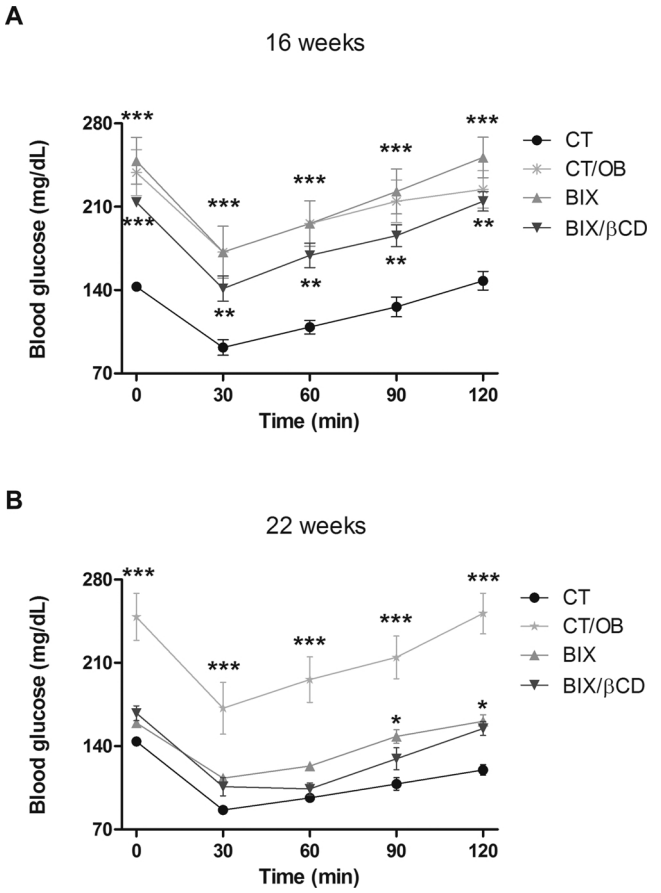

Fig. 4. Effect treatment with BIX and BIX/βCD on insulin resistance in High-fat fed C57BL/6 obese mice. Insulin Sensibility Test was performed in A) 16 weeks (before treatment) and B) 22 weeks (after 6 weeks of treatment). Treatment with BIX and BIX/βCD reversed peripheral resistance to insulin in high-fat fed C57BL/6 obese mice. Glucose levels were measured in 30, 60, 90 and 120 min after insulin administration (27.77uLkg-1 insulin [3,49 mg/mL] equivalent 100UI). Data are expressed as mean ± SEM, n = 9 per group. *p < 0.05 ***p < 0.001 (two- way ANOVA).

high-fat fed obese mice treated with BIX and BIX/βCD compared with equivalent obese mice non-treated (22 weeks), as observed, through Lee's index in the BIX and BIX/βCD treated groups (■■■p < 0.0001) compared to the CT/OB group after 22 weeks (Fig. 2D).

Parameters for visceral adipose tissue, number of adipocytes, adipocyte area and percentage of fat or adiposity index were also assessed (Fig. 3). Higher adiposity and fat mass for the mice fed with HFD than the control group. We observed a lower adiposity for groups treated with BIX and BIX/βCD compared to the CT/OB group, animals treated with BIX/βCD showed a significant decrease (•••p < 0.0001), followed by treatment with BIX (■■■p < 0.0001).

3.3.2. Insulin sensitivity test (IST)

To evaluate insulin resistance (IR) the insulin sensitivity test (IST) was performed, which evaluates plasma glucose levels after administration of a dose of insulin (INS) (27.77 μL kg-1 [3,49 mg/mL] equivalent 100UI) [47,48]. This test was performed after 16 weeks feeding mice with an HFD45% diet compared to the normal diet mice (CT group) and at 22 weeks, after 6 weeks of oral treatments with BIX and BIX/βCD, Fig. 4. The IST profile obtained at week 16 for groups with an HFD45% diet (CT/OB, BIX, and BIX/βCD) show high initial glucose levels (time 0) above 200 mg dL-1, indicating a hyperglycemic state, Fig. 4A.

Lower postprandial blood glucose values for the BIX and BIX/βCD treated groups was observed from 245 mg dL^{-1} to 150 mg dL^{-1} compared to the CT/OB group without treatments, Fig. 4B. In addition, the total recovery of blood glucose levels at 120 min after insulin administration was also observed, and these values are closer to the CT group values. The study also showed that after insulin administration, there was a decrease in glucose levels in plasma from all animal groups. This decrease was accompanied by recovery to near basal values glucose levels at 60 min for the BIX group and 90 min for the BIX/βCD and CT/OB group, compared to the CT group; recovery of basal glucose level after 120 min after insulin administration was as expected in the physiological response to insulin. These results suggest that the intake of the HFD diet induces an insulin-resistant state in all groups compared to the CT group (standard diet).

3.3.3. Metabolic parameters and hepatic function

Metabolic parameters were measured for obese mice, including fasting glucose, triglycerides, total cholesterol, HDL-c and hepatic enzymes (GOT and GPT), Fig. 5. According to results presented in Fig. 5A, the BIX and BIX/βCD-treated groups exhibited a reduction in fasting glucose levels compared to the CT/OB group.

The HFD diet caused a significant increase in the levels of triglycerides (TG), cholesterol (CHT) and non-HDL-c levels and a decrease in HDL-C compared to the normal diet, as seen in Fig. 5B-C-D.

Liver morphological aspect, tissue histology at the light microscopy, the quantification of lucent lesions areas and enzymes GOT and GPT activity of different groups are shown in Fig. 6. The Enlarged liver size was observed when animals were fed with HFD it. The macroscopic appearance pointed out changes to a more yellowish tone color and a brighter surface in the livers of untreated obese animals when compared to their counterparts that were treated, Fig. 6A. At the end of the treatment, we can see hepatocytes with a polygonal shape and a central core held and few lipid vacuoles, Fig. 6B. Increase in liver size was corroborated by the upsurge in organ weight, Fig. 6C and a minimum percentage of lucent lesions area in hepatocytes, significantly lower in both treatment groups, Fig. 6D. The results of enzymes GOT and GPT activity is depicted in Fig. 6E and F.

4. Discussion

Our study investigates the anti-obesity, hypoglycemic, lipid-lowering and reduction of nonalcoholic fatty liver steatosis effect of the oral administration of Bixin and Bix: β-CD in vivo using a C57BL/6 mice model with a high-fat diet. The in vitro results showed that, in a dose-dependent response, higher insulin resistance was reversed in the cells in a dose-dependent manner, as which was, observed when the differentiated adipocytes were treated with Bixin and Bix:β-CD inclusion compound at various concentrations. Our results demonstrated that Bixin treatment stimulates glucose uptake of differentiated adipocytes, similar to those reported in the literature by Goto et al. [18] and Takahashi et al. [19], who showed that Bixin and Norbixin increase glucose uptake in the presence of insulin through PPAR gamma receptor signaling that controls nuclear receptors for glucose transporters. Furthermore, the high-fat diet (HFD) rendered the obese mice, however the effect of treatments on weight gain, food intake and adiposity in vivo assays showed that the oral administration of BIX and BIX/βCD exhibited better control of weight than the CT/OB mice group, which exhibited a significant decrease in weight between the 18th to 22nd weeks. These results demonstrated that the treatments prevent metabolic decompensation in obese mice. These results are in accordance with findings by Lei et al. [49], Santos et al. [46] and Yang et al. [50] for this type of diet. However, the continuous HFD intake without Bix and BIX/βCD treatment produces metabolic decompensation, and these animals showed weight loss, as observed in chemically induced diabetes models described elsewhere [51,52]. In the present study, the administration of BIX/βCD significantly decreased body fat levels in obese mice and suggested that the administration of inclusion compounds can represent a significant advance in the treatment of

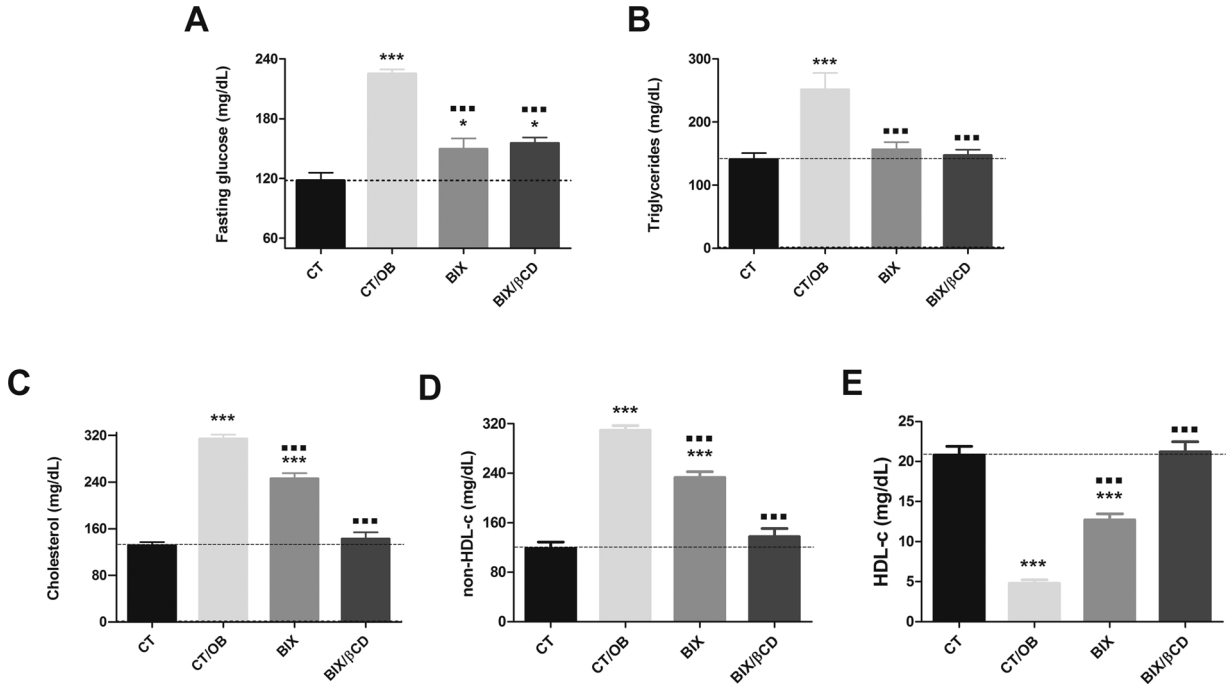

Fig. 5. Effect treatment with BIX and BIX/βCD on metabolic parameters in High-fat fed C57BL/6 obese mice. A) glycemic profile; B) triglycerides; C) total cholesterol; D) non-HDL cholesterol and E) HDL cholesterol in C57BL/6 mice. Data are expressed as mean ± SEM, n = 9 per group. *Significant difference between HFD groups and CT group, #Significant difference between treated groups and CT/OB group; ***$p < 0.001$, ■■■$p < 0.001$, (two-way ANOVA).

metabolic diseases such as diabetes, obesity, and metabolic syndrome. This decrease in adipocytes is directly related to the decrease in the adipose tissue area of these animals, and these values are comparable to animals fed the normal diet (CT group).

Literature reports have described that the administration of natural products such as pomegranate [49] or curcumin decreased adiposity in mice fed a high-fat diet, and in the case of curcumin, the effect was more pronounced when curcumin was used as a prophylactic [53]. In addition, these natural compounds exhibit activity on visceral adipose tissue and can inhibit angiogenesis in an adipose tissue. At the same time, our results agree with those established by Takahashi et al. [19], which demonstrate that Bixin decreases the differentiation of pre-adipocytes, reduces lipid accumulation, decrease body weight gain and decreases the chance of developing obesity. Based on the insulin uptake results, we suggest that the BIX and especially BIX/βCD oral administration showed a significant hypoglycemic effect and can be an alternative treatment for insulin resistance.

Previously, a study of the Bixin hypoglycemic effect in vitro studies with 3T3-L1 cells demonstrated that Bixin improves the absorption of glucose [19]. Likewise, studies conducted in obese mice demonstrated that the addition of 1.0% Bixin to the diet promotes the reduction of both glucose levels and insulin (32%) in serum and improves carbohydrate metabolism conditions [18]. The present study is the first report on both the weight loss effect and the hypoglycemic effect of the Bix: βCD inclusion compound. Thus, these new data indicate that the Bix: βCD formulation could potentially be used as a hypoglycemic in clinical medicine. Furthermore, lower concentrations of CHT, TG, and non-HDL-c and higher HDL-C was observed for the group treated with BIX/βCD compared to the CT/OB group. Our results are in agreement with the findings of Goto et al. [18], which indicated that Bixin acts as a ligand of PPARα and positively regulates lipid metabolism in the liver. At the same time, previous studies by de Paula et al. [54] established that annatto extract reduced the serum levels of total and LDL-cholesterol and increased HDL-cholesterol in animals fed a high lipid diet [54].

Due to the importance of hepatic function and its relationship to obesity, metabolic syndrome, insulin resistance and hyperlipidemia, were analyzed for the influence of BIX and BIX/βCD treatment on hepatic morphology and function. Hematoxylin and eosin staining of the liver tissue sections revealed that the CT/OB group without any treatment showed accumulation of spherical vacuoles of fat droplets, variability in nucleus position of hepatocytes and inflammatory cell invasion in some areas. These pathological alterations were dramatically ameliorated in the liver sections of obese mice treated with BIX and BIX/βCD, where the sections showed a prominent nucleus and well-preserved cytoplasm, as well as decreased hepatic steatosis that was more similar to normal hepatic structure.

In addition, obese mice treated with BIX/βCD showed hepatic lobules appearing in radiating plates of strands of hepatocytes, which indicated a notable decrease of lipid accumulation in hepatocytes. Increase in the activities of these enzymes in obese mice (CT/OB) indicates hepatic damage. According to the results obtained for the two hepatic enzymes, there was no change in GOT/AST levels, while the GTP/ALT levels showed an increase in the animals in the CT/OB group compared to animals in the CT group. Lower GTP/ALT levels for the mouse group treated with BIX and BIX/βCD were observed compared to the CT/OB group. These results suggest that the hepatoprotective effect improved the lipid profile and inhibited fat accumulation in the liver of the BIX/βCD formulations. Our results are in agreement with the findings of Lima et al. [16], and hepatoprotection was described when it was administered Bixa Orellana methanolic extract and quercetin [55].

In conclusion, the present work evidenced that the oral administration of the Bix: β-CD inclusion compound not only reduced body weight, Lee's Index, and various adipose pad weight percent but also efficiently decreased serum CHT, TG, glucose levels, and the CHT/HDL-c ratio as dyslipidemia risk factors and had a hepatoprotective effect in vivo.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors

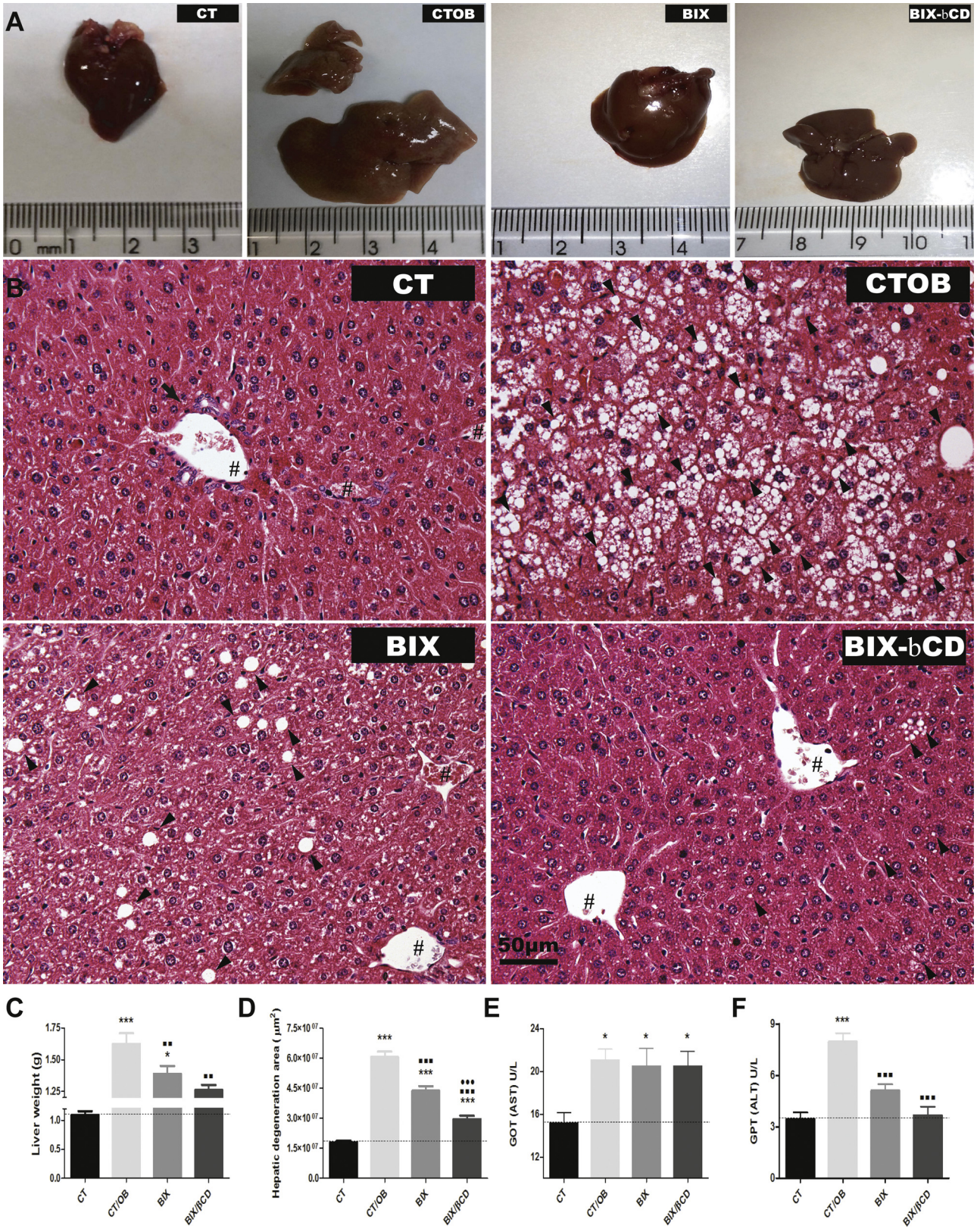

Fig. 6. Effect treatment with BIX and BIX/βCD on liver in High-fat fed C57BL/6 obese mice. A) Representative images of liver in CT group; CT/OB group; BIX and BIX/βCD treatment after 22 weeks; B) Histological findings of liver tissue of CT group; CT/OB group, BIX treatment, and BIX/βCD treatment, showed hepatocytes with decreased accumulation of lipid droplets after the treatment with BIX and BIX-βCD. **C)** Liver weight, D) hepatic degradation area (the quantification of lucent lesions areas), E) Hepatic enzymes GOT / AST and, F) GPT / ALT levels in serum. Samples collected on the last day of the experiment (week 22). Arrows, arrowheads, and hash indicate portal space, lipid vacuoles (steatosis) and blood vessels, respectively. Data are expressed as mean ± SEM, n = 9 per group; * Significant difference between HFD groups and CT group, # Significant difference between treated groups and CT/OB group; & Significant difference between treated groups and BIX group; *p < 0.05; ***p < 0.001; ■■p < 0.01; ■■■p < 0.001; •••p < 0.001 (two-way ANOVA).

Declarations of interest

None.

Acknowledgments

The authors would like to thank Adjunct Professor C. E. M. Jensen Pharmacy Department, UFSJ, MG, Brazil for the Bixin donation. Also, we would like to acknowledgment the Brazilian Research Support from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.06.144.

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