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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

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

Original article http://dx.doi.org/10.1016/j.apjtb.2016.09.006

# Effects of bixin in high-fat diet-fed-induced fatty liver in C57BL/6J mice

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ARTICLE INFO

*Article history:*
Received 28 Mar 2016
Received in revised form 15 Jun, 2nd revised form 16 Jun 2016
Accepted 24 Aug 2016
Available online 6 Oct 2016

*Keywords:*
*Bixa orellana*
Bixin
Obesity
Enzymes
Metabolic disturbances
Diabetic

ABSTRACT

**Objective:** To evaluate the anti-obesity activity of bixin (BIX) on C57BL/6J mice which were fed a high-fat diet (HFD) and to determine the mechanism of this effect.
**Methods:** C57BL/6J mice were separately fed a high-calorie diet or a normal diet for 8 weeks, then they were treated with BIX for another 13 weeks. After administration for 13 weeks, the animals were sacrificed. Body adiposity, serum lipid level, and insulin resistance were evaluated. In addition, a histological assay of pancreas and liver, an evaluation of the inhibitory properties on pancreatic lipase, and α-amylase were conducted.
**Results:** Administration of BIX significantly decreased the body weight gain, adipocyte size, fat pad weights, hepatic lipid levels in HFD-induced obese mice. In addition, reduced liver weight exhibited decreased serum leptin levels, malic enzyme, glucose-6-phosphate dehydrogenase, hepatic fatty acid synthase, aspartate aminotransferase, alanine aminotransferase and hepatic phosphatidate phosphohydrolase activity. However, superoxide dismutase, catalase, glutathione peroxidase, and glutathione levels were increased in hepatic tissue. BIX also decreased lipid and carbohydrates absorption due to inhibition of pancreatic lipase and α-amylase. Long term supplementation of BIX significantly decreased hyperlipidemia, insulin resistance and glucose level. Decreased levels of hepatic steatosis and the islets of Langerhans appeared less shrunken in HFD-fed mice.
**Conclusions:** The antiobesity effect of BIX appears to be associated at least in part, to its inhibitory effect on lipids and carbohydrate digestion enzymes such as pancreatic lipase, α-glucosidase, and α-amylase. The results suggested that BIX also act as an antioxidant and may treat visceral obesity normalizing glucose levels, improving insulin resistance and increasing energy expenditure. Therefore, achiote which has a main component, the carotenoid BIX, could be a viable food for the treatment of obesity and diabetes.

## 1. Introduction

Obesity is a disorder of the metabolism of carbohydrates and fats resulting in an excessive fat deposition in adipose tissue and other organs [1]. Imbalance in energy causes obesity and visceral adiposity increasing production of reactive oxygen species (ROS) that predisposes individuals to complications such as oxidative stress, metabolic disturbances, insulin resistance, and lipogenesis. The adipose tissue is an endocrine organ that secretes some adipokines such as tumor necrosis factor-α (TNF-α), leptin, interleukin-6 (IL-6), chemoattractant proteins like chemokine (C–C motif) ligand-2, proteins and C–C chemokine receptor 2, plasminogen activator inhibitor-I [2].

Numerous enzymes such as pancreatic lipase, α-amylase and α-glucosidase are involved in lipid and carbohydrate metabolic pathways, representing therapeutic targets for obesity [3]. Currently, available anti-obesity agents have a modest efficacy and undesirable side effects. This is why new antiobesity drugs have been looked for in natural sources that may have minimal side effects and reduce body weight.

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All experimental procedures involving animals were conducted in accordance to Mexican Official Standard Technical Specifications for the Production, Care and Use of Laboratory Animals (NOM-062-ZOO-1999) and the Care and Use of Laboratory Animals (756/lab/ENCB), and approved by Ethics Committee Research (CEI-ENCB).

Foundation Project: Supported by Instituto Politecnico Nacional (Grant No. 20150541).

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

*Bixa orellana* seeds, also known as achiote or annatto is used for cooking in South America and Mexico. *Bixa orellana* seed coat contains apocarotenoids [4]. Bixin (BIX) reduces inflammatory response [5], improves lipid profiles, has antihyperglycemic activity, and protects from oxidative stress in diabetic rats [6]. Treatment with BIX enhances anti-adipogenic effect in 3T3-L1 adipocytes by regulating peroxisome proliferator-activated receptor alpha [7,8]. This study examined the effect of oral administration of BIX on obesity related biochemical parameters, weight loss, adipogenesis and metabolism changes in liver dysfunction, hepatic steatosis and oxidative stress in C57BL/6J mice.

## 2. Materials and methods

### 2.1. Mouse model of diet-induced obesity

Adult male C57BL/6J mice, (60 days, weighing 20–25 g) were provided by the bioterio of the National School of Biological Sciences of the National Polytechnic Institute. Mice were adapted under controlled conditions [12 h light/dark cycle and temperature (23 ± 2) °C] for a week. Subsequently, 105 mice were randomly divided into fifteen groups with seven mice per group. The experimental protocol used throughout the research followed the statutes for animal experimentation indicated by the Laboratory Animal Care (National Institute of Health publication 85-23), and Mexican Official Normativity (NOM-062-Z00-1999). Finally, all experiments were approved by committee of the use and handling of laboratory animals (permission number 756/lab/ENCB-IPN).

### 2.2. Supplementation of BIX

The C57BL/6J mice were individually housed in standard cages. The high-fat-diet (HFD) consisted of 24% corn oil, 6% sugar, 20% full-cream milk powder in 50% normal rat chow pellet [9]. All mice were fed experimental diet (HFD) *ad libitum* and with free access to water during the 8 weeks of the experimental period. During the feeding period, food intake and body weight were monitored daily and every 2 days respectively [9]. After 8 weeks of feeding HFD, the resultant obese mice were treated with BIX (Sigma–Aldrich, St. Louis, MO, USA) at doses of 5 (BIX-5), and 10 mg/kg (BIX-10), which were prepared dissolving the BIX in 1% Tween 80. Then, this carotenoid was orally administered at a volume of 0.1 mL daily for 14 weeks. The normal mice and obese control (HFD) groups were administered only 1% Tween 80, using orlistat (Roche, DF, Mexico) as standard (5 mg/kg). The administration of 5 mg/kg and 10 mg/kg of BIX was calculated on basis of the amount of commercial orlistat.

### 2.3. Biochemical analysis

At Week 14 after starting supplementations period, mice were sacrificed with diethyl ether to collect the retroperitoneal, epididymal, subcutaneous fat livers. Blood samples were collected from vena cava for biochemical parameters determination. Blood was centrifuged at 2000 r/min for 20 min at 4 °C, and the plasma was used for subsequent analyses. High density lipoprotein cholesterol (HDL-C), total cholesterol, triglycerides (TG), plasma activities of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were measured with commercial diagnostic kit from Cayman Chemical Company (Ann Arbor, Michigan, MI, USA). Low density lipoprotein cholesterol (LDL-C) was measured using Friedewald equation [10]. The non-esterified fatty acid levels were measured with free fatty acid (FFA) assay kit (Cat No: 700310, Cayman Chemical). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were calculated by enzymatic assay kits (Wako Pure Chemical, Osaka, Japan). Adiponectin and leptin were calculated using mouse ELISA kits (R&D Systems, Minneapolis, MN, USA), performed with a microplate spectrophotometer (Bio-Rad, Hercules, CA, USA). The insulin level was estimated using an ELISA kit (INSMSU-E01, ALPCO Diagnostics, Salem, NH, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated based on the following equation: HOMA-IR = Glucose (nmol/L) × Insulin (μIU/mL)/22.5.

Hepatic TNF-α was determinated using a mouse TNF-α ELISA kit (Millipore, Temecula, CA, USA). IL-1β and IL-6 were estimated using enzymatic kits (eBioscience, Inc., San Diego, CA). All samples were assayed in triplicate and procedures were performed according to the manufacturer instructions.

### 2.4. Faecal lipids excretion

Faeces were collected in the last days of experiment. The total amount of faeces was dried at 100 °C during 4 days, weighed and homogenized with twice the volume of water, and then faecal lipids were extracted using the Folch extraction method [11].

### 2.5. Lipid peroxidation (LPO) and xanthine oxidase (XO) inhibition assay

The ability of BIX to inhibit LPO in mice liver homogenate was determined by the formation of malondialdehyde (MDA) equivalents calculated by the thiobarbituric acid reactive substances (TBARS) assay [12]. Liver homogenate was mixed with 20% (w/v) acetic acid (pH 3.5), 8.1% sodium dodecyl sulfate and 0.8% (w/v) thiobarbituric acid. The reaction was heated for 60 min at 95 °C. Then a pyridine *n*-butano (1:15, v/v) mixture was added and centrifuged for 15 min at 3000 r/min then measured at 535 nm. Ascorbic acid was used as positive control.

Measurement of scavenging ROS was assayed by the XO assay kit (BioVision, USA) in liver homogenate.

### 2.6. Antioxidant effect of BIX on enzyme activity

Heme oxygenase 1 (HO-1) activity in liver and plasma was estimated with the HO-1 Mouse SimpleStep ELISA kit (Empire Genomics, Buffalo, NY, USA). Superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) reductase, and glutathione peroxidase (GSH-Px) activities were estimated using commercial kits. All the assay kits were purchased from Cayman Chemical (MI, USA), and all procedures were performed with the manufacturer instructions.

### 2.7. Effect of BIX on hepatic lipid regulating enzymes

Mitochondrial pellet was obtained from the liver [13]. Protein concentration was estimated base on Bradford method [14] using bovine serum albumin as standard. The fatty acid synthase (FAS) activity was determined with an assay in which the

activity of FAS was indicated as the amount of oxidized nicotinamide adenine dinucleotide phosphate in mmol/L/min/mg of protein [15]. Then enzymatic activity of glucose-6-phosphate dehydrogenase (G6PD) and malic enzyme (ME) was calculated with a commercial kit (Sigma–Aldrich, St. Louis, MO, USA). The phosphatidate phosphohydrolase (PAP) activity was determined using the method developed by Khokha *et al.* [16]. The α-glucosidase, pancreatic lipase and α-amylase inhibitory activities were evaluated with a specific assay kit (BioVision, CA, USA).

### 2.8. Histological analysis of liver and pancreas

Liver and pancreas were removed from the mice and fixed in a solution of formalin (10%). Then, tissues were embedded in paraffin and immediately sectioned to obtain 4 μm thick sections. The steatosis was classified according to the method described by Zelber-Sagi *et al.* [17]. Sections of the organs were stained with hematoxylin and eosin dyes and examined with an optical microscope (Nikon, Tokyo, Japan).

### 2.9. Statistical analysis

Each value was presented as the mean ± SD. One-way ANOVA was used for multiple comparisons, followed by the Tukey test with XLSTAT7.1 software, and a $P$ value of less than 0.05 was considered statistical significance.

## 3. Results

In this study, after 14 weeks of feeding a HFD to male C57BL/6J mice, the body weight was significantly increased (133%) compared with that of the normal diet mice, indicating that HFD produced obesity (Table 1). BIX was orally administered at 5 and 10 mg/kg over a period of 14 weeks; in the end BIX decreased the body weight of mice by 17% and 47% respectively compared with HFD control. During the last 14 weeks of experimentation, food intake among the groups HFD and HFD-BIX did not differ. Also, the weight of the retroperitoneal, subcutaneous, and epididymal adipose tissues at the end of the experiment was significantly ($P < 0.001$) higher in the HFD group than in the NC group. Body weight gain (g/day) produced by BIX-5 and BIX-10 mg/kg was significantly different when compared to HFD group. The weight of kidney and liver was significantly lower in the 5 and 10 mg/kg BIX groups than those of the HFD group.

Fecal lipid excretion in the HFD group was significantly ($P < 0.001$) higher compared with the NC diet group after 14 weeks of treatment. BIX 5 and 10 mg/kg supplementation increased fecal lipid excretion (50% and 56%, respectively) in comparison with the HFD group. This may be partially due to the decreased lipid absorption produced by inhibition of pancreatic lipase enzyme. In addition, there were no significant differences among the faecal lipid levels in the experimental groups (Table 1).

The weight of liver was significantly higher in the HFD supplemented mice compared to NC diet mice group. HFD mice had elevated FFA ($P < 0.001$), TG ($P < 0.001$) and cholesterol ($P < 0.01$) levels. BIX supplementation at dose of BIX-5 and BIX-10 significantly reduced liver weight (7% and 9%, respectively), ALT (31% and 54% decrease, respectively) and AST (21% and 31% decrease, respectively), FAS (50% and 57% inhibition, respectively), cholesterol (30% and 46% inhibition, respectively), TG (52% and 60% inhibition, respectively), LDL-C (29.59% and 43.33% inhibition, respectively) levels. However, HDL-C level was diminished in hyperlipidemic mice (HFD mice) and a significant rise of HDL-C was observed in BIX treatment mice. Data are presented in Table 2.

**Table 1**
Effect of BIX on body weight, food intake, adipose tissue weight, fecal total lipids and weight of kidney in HFD-fed obese mice.

| Groups | | NC | HFD | HFD + BIX-5 | HFD + BIX-10 | HFD + Orlistat |
|---|---|---|---|---|---|---|
| Body weight (g) | Initial body weight | 21.00 ± 1.76 | 21.12 ± 2.13 | 20.92 ± 2.65 | 21.76 ± 2.65 | 21.24 ± 2.67 |
| | Final body weight | 31.23 ± 1.26 | 49.38 ± 3.26 | 40.17 ± 5.73[a] | 35.31 ± 4.86[a] | 39.77 ± 3.19[c] |
| | Body weight gain (g/day) | 10.23 ± 2.71 | 28.26 ± 1.71 | 17.25 ± 1.34[b] | 11.55 ± 0.34[b] | 18.53 ± 2.04[b] |
| Food intake (g/mice/day) | | 2.65 ± 0.78 | 2.59 ± 1.59 | 2.53 ± 1.48 | 2.51 ± 1.32 | 2.50 ± 1.58 |
| Adipose tissue (g/100 g body weight) | Epididymal fat | 1.19 ± 0.26 | 1.91 ± 0.34 | 1.62 ± 0.54 | 1.34 ± 0.68 | 1.58 ± 0.48 |
| | Retroperitoneal fat | 0.90 ± 0.39 | 1.60 ± 0.65 | 1.37 ± 0.92[c] | 1.01 ± 0.46[c] | 1.33 ± 0.44[c] |
| | Subcutaneous fat | 0.38 ± 0.15 | 1.82 ± 0.28 | 1.69 ± 0.92[c] | 1.19 ± 0.17[c] | 1.75 ± 0.50[c] |
| | Total fat | 2.47 ± 0.75 | 5.33 ± 0.75 | 4.68 ± 0.69[a] | 3.54 ± 0.69[a] | 4.66 ± 0.69[b] |
| | Weight ratios kidney | 1.69 ± 0.03 | 1.44 ± 0.07 | 1.50 ± 0.04 | 1.55 ± 0.06 | 1.49 ± 0.05 |
| Fecal total lipids (mg/g wet weight) | | 39.45 ± 5.72 | 77.36 ± 7.48 | 116.18 ± 8.96[a] | 120.46 ± 8.52[c] | 118.54 ± 9.03[b] |

NC: Normal diet control. Values are expressed as mean ± SD ($n = 6$). [a]: $P < 0.05$, [b]: $P < 0.01$, [c]: $P < 0.001$, when compared to HFD group.

**Table 2**
Effect of BIX on hepatic lipid content, liver weight, hepatic TG, hepatic cholesterol, hepatic antioxidant capacity in C57BL/6J obese mice.

| Groups | NC | HFD | HFD + BIX-5 | HFD + BIX-10 | HFD + Orlistat | Ascorbic acid |
|---|---|---|---|---|---|---|
| Weight ratios liver (g/100 g body weight) | 5.51 ± 0.31 | 5.56 ± 0.35 | 5.67 ± 0.39[c] | 5.86 ± 0.39[c] | 3.57 ± 0.62[c] | |
| FFA (μmol/g liver) | 5.73 ± 1.45 | 23.84 ± 3.76 | 8.19 ± 1.53[c] | 6.32 ± 1.94[b] | 9.31 ± 3.21[c] | |
| Hepatic TG (mg/g) | 13.26 ± 3.19 | 48.65 ± 6.27 | 23.52 ± 5.40[b] | 19.21 ± 3.87[a] | 28.39 ± 4.58[b] | |
| Hepatic cholesterol (mg/g) | 5.72 ± 1.07 | 11.90 ± 3.45 | 8.34 ± 1.98[a] | 6.43 ± 1.21[b] | 8.78 ± 2.76[a] | |
| AST (IU/L) | 0.49 ± 0.03 | 0.77 ± 0.01 | 0.61 ± 0.02[c] | 0.53 ± 0.04[c] | 0.69 ± 0.02 | |
| ALT (IU/L) | 0.35 ± 0.04 | 0.84 ± 0.08 | 0.58 ± 0.08[a] | 0.46 ± 0.02[c] | 0.80 ± 0.02 | |
| LPO (MDA nmol/mg protein) | 0.86 ± 0.01 | 1.42 ± 0.57 | 0.88 ± 0.05[b] | 0.85 ± 0.03[a] | 1.49 ± 0.08 | 0.89 ± 0.07[a] |

Values are expressed as mean ± SD ($n = 6$). [a]: $P < 0.05$, [b]: $P < 0.01$, [c]: $P < 0.001$, when compared to HFD group.

The adipose specific hormone, leptin was elevated in obesity and it correlated with insulin resistance and adipose tissue mass. Serum leptin level increased in group compared to NC-fed mice (Table 3). BIX treated groups (HFD plus BIX-5 and BIX-10) exhibited decreased serum leptin levels compared with the HFD group.

**Table 3**

Effect of BIX on blood parameters after feeding for 14 weeks.

| Groups | NC | HFD | HFD + BIX-5 | HFD + BIX-10 | HFD + Orlistat | Ascorbic acid |
|---|---|---|---|---|---|---|
| Glucose (mg/dL) | 102.40 ± 5.89 | 178.10 ± 7.51[b] | 112.40 ± 7.14[a] | 102.30 ± 6.29[c] | 177.10 ± 5.38 | |
| Insulin (ng/mL) | 0.85 ± 0.04 | 100.34 ± 0.02[b] | 0.57 ± 0.08[a] | 0.52 ± 0.02[c] | 0.94 ± 0.02[c] | |
| HOMA-IR | 6.16 ± 0.64 | 9.75 ± 0.70 | 4.12 ± 0.28[c] | 4.02 ± 0.39[c] | 9.47 ± 0.32 [b] | |
| Cholesterol (mg/dL) | 89.21 ± 5.71 | 210.38 ± 9.32[a] | 138.21 ± 6.43[a] | 123.25 ± 7.62[b] | 123.11 ± 6.22[a] | |
| TG (mg/dL) | 131.45 ± 5.86 | 195.39 ± 8.20[b] | 150.48 ± 3.98[a] | 135.29 ± 4.18[a] | 138.34 ± 3.64[c] | |
| HDL-C (mg/dL) | 41.72 ± 2.80 | 34.01 ± 4.39[a] | 38.35 ± 5.06[c] | 40.87 ± 4.88[c] | 39.37 ± 2.45[c] | |
| LDL-C (mg/dL) | 22.76 ± 3.74 | 109.90 ± 5.64[a] | 77.37 ± 4.19[c] | 69.27 ± 6.36[b] | 79.27 ± 6.36[a] | |
| Atherogenic index | 0.49 ± 0.06 | 0.75 ± 0.03 | 0.59 ± 0.09[c] | 0.51 ± 0.07[a] | 0.54 ± 0.05 [b] | |
| GOT (Karmen/mL) | 165.43 ± 8.39 | 205.21 ± 7.87 | 173.56 ± 6.38[a] | 167.29 ± 7.87[a] | 194.53 ± 9.39 | |
| GPT (Karmen/mL) | 25.61 ± 4.73 | 128.19 ± 6.43 | 58.25 ± 5.28[b] | 49.87 ± 5.29[b] | 125.48 ± 7.17 | |
| AST (IU/L) | 67.60 ± 8.29 | 77.60 ± 6.94 | 72.20 ± 7.16[c] | 69.50 ± 9.38[c] | 77.10 ± 7.19 | |
| ALT (IU/L) | 35.60 ± 2.44 | 43.90 ± 1.76 | 28.40 ± 3.80[a] | 25.70 ± 5.23[c] | 23.10 ± 5.17 | |
| LPO (MDA μmol/L) | 19.86 ± 4.61 | 31.28 ± 6.52 | 24.32 ± 5.73[b] | 20.51 ± 4.73[a] | 32.29 ± 3.88 | 21.34 ± 5.06[a] |
| Leptin (ng/mL) | 4.16 ± 1.28 | 20.01 ± 5.74 | 10.92 ± 2.87[a] | 8.23 ± 4.19[a] | 20.62 ± 4.36 | |
| Adiponectin (μg/mL) | 7.56 ± 3.21 | 6.12 ± 4.77 | 6.90 ± 4.19 | 7.24 ± 3.61[a] | 6.16 ± 2.43 | |

[a]: $P < 0.05$, [b]: $P < 0.01$, [c]: $P < 0.001$, when compared to HFD group.

Metabolic parameters of the obese mice, including insulin levels, glucose, cholesterol, TG, and LDL were significantly elevated by HFD (Table 3). However, HDL cholesterol level was diminished in HFD group.

In this study, long term BIX supplementation significantly decreased hiperlipidemia, insulin resistance and glucose level in HFD fed mice (Table 3).

To assess the effect of BIX-supplemented mice on hepatic function, we measured the activity of enzymes GPT, GOT, AST and ALT. These activities in BIX-5 and BIX-10 treated groups were GOT (15% and 18% decrease, respectively), GPT (55% and 61% decrease, respectively), AST (21% and 31% decrease, respectively) and ALT (35% and 53% decrease, respectively). BIX supplementation resulted in a significant reduction in activity of enzymes GPT, GOT, AST and ALT (Table 3).

The hepatic TBARS was a marker of lipid peroxide production. Oxidative stress in the liver was calculated by measuring the levels of LPO, and the activities of related enzymes with the oxidative stress, including SOD, CAT, GSH-Px, GSH, HO-1 and XO in liver homogenates. The concentration of LPO was determined by the measuring of MDA, which was a product of lipid breakdown caused by peroxidation damage. Furthermore, liver MDA levels were elevated in the HFD-fed group compared with the NC group. MDA levels were significantly decreased in the liver of the BIX-supplemented mice (HFD + BIX-5 and HFD + BIX-10) and ascorbic acid treatment (HFD + ascorbic acid) compared with the NC mice (Table 3). Moreover, the result indicated a slight difference of inhibition activity between BIX and ascorbic acid.

Table 3 shows the effects of BIX on leptin and adiponectin. Significant increase in the levels of plasma of adiponectin was observed in the HFD fed groups for 14 weeks. After treatment with BIX, the plasma leptin was significantly decreased compared to leptin level in HFD-fed control groups. It also showed a significant reduction of leptin in the BIX-5 and BIX-10 treatment groups (45.4% and 58.9%, respectively).

HFD induced oxidative stress and inflammation in the liver, indicating the decline in the concentration of the antioxidant enzymes GSH, GSH-Px, SOD, CAT, HO-1 and XO (Table 4). BIX supplementation elevated activities of these enzymes. The assessment of antioxidant potential showed that the BIX possessed highly inhibitory effects on the XO ($IC_{50}$ = 15.6 μg/mL). Pro-inflammatory cytokine levels of TNF-α, IL-1β and IL-6 were elevated in the HFD group. However, cytokines IL-1β and IL-6 were not significantly different between the HFD control group after 4 weeks and BIX supplemented groups. While in contrast, TNF-α was increased by 29.0% ($P < 0.05$).

**Table 4**

Effect of treatment with BIX on liver antioxidant enzyme activity in C57BL/6J obese mice.

| Groups | SOD (IU/min) | CAT (IU/s) | GSH-Px (IU/mL) | GSH (IU/mL) | HO-1 (pg/mL) |
|---|---|---|---|---|---|
| NC | 6.60 ± 0.18[a] | 0.730 ± 0.002[b] | 99.35 ± 7.19[a] | 47.32 ± 3.91[b] | $2.27 \pm 10^2$ |
| HFD | 5.00 ± 0.23 | 0.620 ± 0.007 | 40.28 ± 4.38 | 25.13 ± 5.22 | $1.82 \pm 10^3$ |
| BIX-5 | 5.60 ± 0.43 | 0.660 ± 0.009 | 76.54 ± 5.21[b] | 36.78 ± 4.33[a] | $1.53 \pm 10^3$ |
| BIX-10 | 6.10 ± 0.42[a] | 0.700 ± 0.006[b] | 66.92 ± 5.89[a] | 31.09 ± 4.80[b] | $1.12 \pm 10^3$ |

Each value represents the mean ± SEM from 6 rats. [a]: $P < 0.05$ and [b]: $P < 0.01$ when compared to HFD group.

Table 5 shows the effects of BIX-supplementation on the liver lipogenic enzymes (G6PD, FAS, ME and PAP activities). The consumption of BIX at a dose of 10 mg/kg led to an important ($P < 0.05$) reduction of G6PD, FAS, ME, and PAP activities (19.28%, 57.05%, 31.46% and 35.17% respectively) in comparison with control group.

**Table 5**
Effect of supplementation with BIX on hepatic lipid regulating enzyme activities in C57BL/6J mice.

| Groups (nmol/mg protein/min) | Control HFD | HFD + BIX-5 | HFD + BIX-10 | HFD + Orlistat |
|---|---|---|---|---|
| FAS | 3.120 ± 0.039 | 1.560 ± 0.087[a] | 1.340 ± 0.067[a] | 3.010 ± 0.078 |
| G6PD | 1.970 ± 0.043 | 1.780 ± 0.071[b] | 1.590 ± 0.043[b] | 4.230 ± 0.540[a] |
| ME | 69.340 ± 4.230 | 52.230 ± 3.770[a] | 47.520 ± 4.840[a] | 72.270 ± 5.190 |
| PAP | 870.130 ± 40.360 | 615.360 ± 43.820[a] | 564.090 ± 29.730[b] | 903.170 ± 52.650 |

Values are expressed as mean ± SEM. [a]: $P < 0.05$, [b]: $P < 0.01$, when compared to HFD group.

As shown in Table 6, BIX inhibited pancreatic lipase activity in a concentration-related manner ($IC_{50}$ value of 1.06 mg/mL) which was higher than that achieved with orlistat ($IC_{50}$ value of 0.02 mg/mL). The $IC_{50}$ values of the α-amylase (34.24 μg/mL) and α-glucosidase (1.48 μg/mL) inhibitory effects of BIX were also higher than those presented by acarbose. BIX inhibited not only pancreatic lipase but also α-amylase and α-glucosidase activities.

**Table 6**
Inhibitory effect of BIX on pancreatic lipase, α-amylase, α-glucosidase, XO and LPO.

| Group | $IC_{50}$ (μg/mL) | | |
|---|---|---|---|
| | BIX | Orlistat | Acarbose |
| Lipase | 1.06 ± 0.09 | 0.02 ± 0.06 | – |
| α-Amylase | 34.24 ± 5.17 | – | 0.48 ± 0.01 |
| α-Glucosidase | 1.48 ± 0.06 | – | 0.67 ± 0.00 |
| XO | 15.6 ± 2.45 | – | – |

All values are expressed as mean ± SD ($n = 3$).

Normal histological architecture was seen in the pancreatic islet of the control group (Figure 1A). Hydropic degeneration, shrunken islets of Langerhans and lymphocyte infiltration were found in untreated obese animals. The nucleus of the necrotic cells indicated pyknosis or marginal hyperchromasia (Figure 1B). In the BIX treatment groups, the cells of the islets of Langerhans appeared less damaged, indicating that the cells were protected and suggesting the strong anti-obesity potential of BIX (Figure 1C,D).

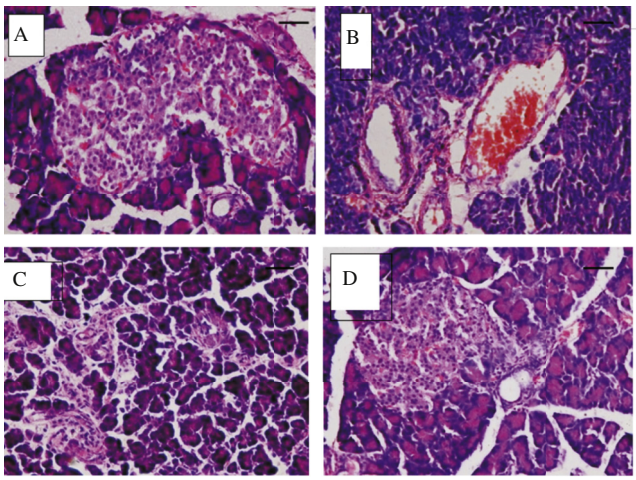


**Figure 1.** Histological pictures of cellular population in the islets of Langerhans in pancreas of mice.
A: Normal cellular population in the islets of Langerhans in pancreas of vehicle-treated mice; B: Extensive damage to the islets of Langerhans reduced dimensions of islets, and increased vacuolation; C and D: Restoration of normal cellular population size of islets with hyperplasia by BIX. Scale bar = 50 μm.

Figure 2 shows the histological findings of liver sections. No obese mice livers are presented in Figure 2A. Obese mice without any treatment showed a cumulation of spherical vacuoles of fat droplets, variability in nuclear size, inflammatory cell invasion, karyolysis and pyknosis (Figure 2B). These pathological alterations were dramatically ameliorated in liver sections of obese mice treated with BIX where the sections showed prominent nucleus and well-preserved cytoplasm, as well as decreased hepatic steatosis more like normal hepatic structure (Figure 2C,D). In addition, hyperlipidemic mice treated with BIX showed hepatic lobules appearing in radiating plates of strands of hepatocytes indicated a notable decrease of hepatocyte fat droplets.

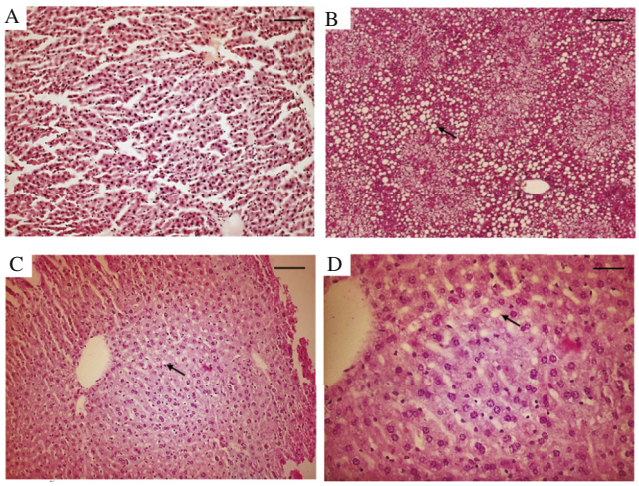


**Figure 2.** Histological findings of liver sections.
A: Normal cellular population in the liver tissue of vehicle-treated mice; B: HFD induced hepatic steatosis in mice; C and D: BIX supplementation decreased accumulation of hepatic lipid droplets in HFD mice. Scale bar = 50 μm.

## 4. Discussion

During the study period, BIX supplementation of obese mice decreased adipose tissue mass with a reduction of body weight without altering food intake. Measurements of the epididymal, retroperitoneal and subcutaneous adipose tissue mass indicated that BIX significantly reverted adipose tissue masses accumulation and adipocyte size increase compared to the HFD control group. Also, BIX supplementation produces remarkable reduction of the body fat percentage, visceral fat weight and total fat.

ALT, AST GOT and GPT levels are biomarker enzymes used to evaluate hepatic disturbance. The increase in the activities of these enzymes in obese mice indicated hepatic damage. BIX supplementation to hyperlipidemic mice produced remarkable reduction in the activities of these enzymes indicating a

hepatoprotective effect; it also improved lipid profiles and inhibited fat accumulation in the liver. Treatment with BIX produced different degrees of protection to the liver and pancreas which was confirmed by the histologic and microscopic study.

The reduction of serum total cholesterol, TG, LDL-C and rise in HDL-C were observed in BIX treated obese mice, which may be directly caused by an amelioration of insulin during treatment or indirectly by the effect of BIX on lipid regulation systems [18].

In obese mice, we found increased levels of TBARS, due to LPO activation in tissues producing cellular infiltration and cell damage [19]. In addition, we also found a decrease in the concentration of antioxidant enzymes GSH, GSH-Px, SOD, CAT, and HO-1 which are essential to prevent the effect of ROS to cells.

The consumption of BIX at 10 mg/kg led to an important ($P < 0.05$) reduction of TBARS levels and increased the concentrations of antioxidant enzymes compared to hyperlipidemic untreated mice group. These results suggested that BIX prevented oxidative stress. Given the importance of oxidative stress in the development of obesity, XO inhibitors could be of significant therapeutic benefit in obese patients.

Adipocytokine, leptin and adiponectin are secreted by adipose tissue and are important in the regulation of metabolic and cardiovascular homeostasis [20]. Leptin enhances fatty acid oxidation, stimulates thermogenesis, decreases glucose level, inhibits appetite, and food intake acting directly on the hypothalamus, thus reducing body weight and fat. Levels of plasma leptin are significantly elevated in obese mice. Adiponectin reduces levels of tissue TG, fatty acids content and increases tissue fat oxidation, which lead to an improvement in the insulin sensitivity. Levels of plasma adiponectin are significantly diminished in obese mice. Treatment with BIX produces a decrease of the level of plasmatic leptin in obese mice. BIX supplementation produces a significant weight loss which elevates plasmatic adiponectin level and reduces leptin.

An additional mechanism for the hypolipidemic effect of BIX is by reduction of G6PD, FAS, ME, and PAP activities. However, BIX improves hypoglycemic effect due to a decreased activity of the G6Pase probably by reducing homogentisate 1,2-dioxygenase.

To further investigate the properties of BIX on reduction of obesity risk, we examinated pancreatic lipase, α-glucosidase and α-amylase inhibitory activities, because these enzymes are the key enzymes in the digestion and absorption of lipids and carbohydrates [21]. α-Glucosidase and α-amylase reduce starch hydrolysis as a mechanism to lower glucose. Inhibition of pancreatic lipase is an important target for the treatment of obesity. A marked inhibition of these enzymes was observed after BIX administration.

In conclusion, the treatment with BIX in obese mice at two different doses (5 mg/kg and 10 mg/kg) for 14 weeks reduced visceral fat, suppressed body weight gain and plasma levels of leptin, TG and total cholesterol and ALT, GPT, GOT, AST and ALT activities compared with the high-fat-fed control mice. BIX also elevated fecal lipid excretion, adiponectin level and antioxidant enzymes GSH, GSH-Px, SOD, CAT, HO-1 and XO. In addition, BIX decreased hepatic FAS, G6PD and PAP activities. These results indicate that BIX has antioxidant and anti-visceral obesity effects mediated by antioxidant metabolism and regulation of lipid in obese mice. In addition, BIX shows hypoglycemic effect which normalizes the glucose levels and improves insulin resistance. Therefore, achiote which has a main component, the carotenoid BIX could be a viable food for the treatment of obesity and diabetes.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

This work was supported by Instituto Politecnico Nacional (Grant No. 20150541).

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