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Effects of *Xylopia aromatica* (Lam.) Mart. fruit on metabolic and inflammatory dysfunction induced by high refined carbohydrate-containing-diet in mice

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ABSTRACT

Obesity is associated with chronic low-grade inflammation, which has been shown to be involved in the development of various comorbidities such as insulin resistance, glucose intolerance and liver damage. Phytochemicals found in some spices could modulate inflammation and improve health problems associated with the excess of adiposity. The extract of the Brazilian pepper *Xylopia aromatica* (Lam.) Mart. (CEXA) showed positive effects in the lower dose on oral glucose tolerance, hepatic steatosis and liver inflammation. The higher dose of CEXA also showed improvement on insulin sensitivity and release of anti-inflammatory cytokines IL-13 and IL-4 on adipose tissue and liver. Chemical analysis revealed the presence of phenolic acids and flavonoids in CEXA. This study suggests the potential of the Brazilian pepper to attenuate mice glucose resistance and liver inflammation, health problems commonly associated with obesity.

1. Introduction

Obesity is characterized by accumulation of excess fat in adipose tissues to the extent of inducing adverse effects on health, which can lead to a reduction in life expectancy and/or an increase in health hazards (Kopelman, 2000). The incidence of obesity is recently increasing at an enormous rate and becoming a worldwide health burden, which could be described as the pandemic of the 21st century (Speakman, 2003). Moreover, the excess of central adiposity is a major risk factor for the development of type 2 diabetes, atherosclerosis, cardiovascular disease and metabolic syndrome (Hossain, Kawar, & Nahas, 2007). The typical Western diet, which is rich in refined carbohydrates and saturated lipids, is associated with visceral fat accumulation and the development of obesity and its metabolic dysfunction, including insulin resistance and hepatic steatosis (Ferreira, Mario, Porto, Andrade, & Botion, 2011; Gregersen, Samocha-Bonet, Heilbronn, & Campbell, 2012; Rahimi & Landaverde, 2013).

Conventional drug treatments of metabolic dysfunction induced by obesity are often associated with undesirable and harmful side effects, rebound weight gain after discontinuation of drug intake, and the incidence of drug abuse. This necessitates a need of combined therapy that is better tolerated as well as more efficient (Bray & Tartaglia, 2000). The potential of natural products might provide an excellent strategy for the scientifically based development of safe and effective treatments. A variety of natural products, including crude extracts and isolated compounds, have been shown to induce body weight reduction and prevent diet-induced obesity and related diseases. Examples of these constituents are polyphenols, triterpenoidal and steroidal saponins, alkaloids, and carotenoids (Devalaraja, Jain, & Yadav, 2011; Sahib et al., 2012; Yuliana et al., 2011).

Several studies have confirmed that obesity is a state of chronic inflammation, as indicated by increased plasma concentration of proinflammatory cytokines (Gregor & Hotamisligil, 2011; Hotamisligil, 2006). The expansion of visceral fat tissue produces a wide range of pro- and anti-inflammatory mediators, both of which have been linked to the development of insulin resistance and glucose intolerance (Weisberg et al., 2003; Xu et al., 2003). Therefore, targeting obesity-related inflammatory components may be a useful strategy to either prevent or ameliorate the development of these obesity-related diseases (Hirai et al., 2010).

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Previous studies have shown that phytochemicals, such as phenolic compounds and alkaloids commonly present in spices, can modulate inflammation and improve health problems associated with obesity (Lampe, 2003; Woo et al., 2007). Black pepper, red chili, curcuma, cinnamon, and fenugreek are some of the spices that have been shown to modulate many targets linked to obesity and insulin resistance (Diwan, Poudyal, & Brown, 2011; Kang et al., 2010; Vijayakumar, Pandey, Mishra, & Bhat, 2010). Brazil is one of the world's most biodiverse countries, with over 40,000 different plant species representing 20% of the world flora. Among these are the fruits of *Xylopia aromatica* (Lam.) Mart. (Desmarchelier, 2010), a species known as "pimenta-de-macaco" (monkey-pepper) and found in Cerrado (Brazilian savannah). It is used as a condiment in food, and a carminative and aphrodisiac in traditional medicine (Corrêa, 1984; Oliveira, Yamada, Fagg, & Brandão, 2012; Silva & Rocha, 1981).

Several chemical constituents, including essential oils (Andrade, Silva, Maia, Carreira, & Oliveira, 2004; Staschenko, Jaramillo, & Martínez, 2004), alkaloids (Martins, Alvarenga, Roque, & Felicio, 1995; Moreira, Lago, & Roque, 2003) and phenolic compounds (Bouba, Njintang, Scher, & Mbofung, 2010), have been reported in the genus *Xylopia*. Additionally, the antioxidant, diuretic, hypoglycemic and hypolipidemic properties of these plants have been shown in some animal models (Nwozo, Orojobi, & Adaramoye, 2011; Ogbonnia, Adekunle, Bosa, & Enwuru, 2008; Ogbonnia et al., 2010; Somova, Shode, Moodley, & Govender, 2001). However, to the best of our knowledge, none of these studies evaluated the fruits of *X. aromatica* in laboratory studies. Here, we evaluated the potential of this species in modulating metabolic and inflammatory dysfunction induced by HC diet in mice.

2. Materials and methods

2.1. Plant material

Dried fruits from *X. aromatica* (Lam.) Mart. were obtained in Curvelo, Minas Gerais, in April and May of 2010. The samples were identified by J. Paula-Souza, and a voucher has been deposited in DATAPLAMT at the Federal University of Minas Gerais (number DAT-110/2010).

2.2. Total phenol and flavonoid content in X. aromatica fruits

Total phenol content was determined using the Folin–Ciocalteu assay as described by Zielinski and Kozlowska (2000) with some modifications. *Total phenol sample solution* (*TPSS*): Exactly 1 g of dried powdered fruit was refluxed with 50 ml of 60% methanol containing 0.1% HCl for 30 min and placed in water bath for 2 h at 85 °C to inactivate the vitamin C (Georgé, Brat, Alter, & Amiot, 2005). After cooling, the solution was filtered and supplemented with 80% methanol to a final volume of 100 ml. *Total phenol assay*: Exactly 5 ml of TPSS was transferred to a 25 ml volumetric flask and completed with distilled water. In a 25 ml volumetric flask, 2 ml of the TPSS/water solution, 1 ml of Folin–Ciocalteu reagent and 10 ml of distilled water were combined, and a 10.6% solution of anhydrous sodium carbonate was added to bring the volume to 25 ml. After 30 min in the dark, the absorbance at 760 nm was measured in a glass cuvette. Aqueous solutions of gallic acid (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 μg/ml) were used to obtain a standard curve. The mean of three readings was used, and the results are expressed as mg of gallic acid equivalents (GAE) per 100 mg of dry weight.

The total flavonoid content was measured by the aluminum chloride assay as described by Amorim et al. (2008), with some modifications. *Sample preparation*: Exactly 5 g of dried powdered fruit was refluxed with 50 ml of methanol for 45 min. After cooling, the solution was filtered, and methanol was added to a final volume of 100 ml. A 5 ml aliquot of this solution, 0.6 ml of glacial acetic acid and 2.5 ml of 8% aluminum chloride in methanol were added to a 25 ml volumetric flask, and methanol was added to bring the total volume to 25 ml. After 30 min in the dark, the absorbance at 420 nm was measured in a glass cuvette. Methanol solutions of rutin (5, 10, 20, 30 and 40 μg/ml) were used to obtain a standard curve. The mean of three readings was used, and the results are expressed as mg of rutin equivalents (RE) per 100 mg of dry weight.

2.3. Extract preparation

Dried and pulverized fruits were extracted by percolation with 95% ethanol, and the solution was evaporated in a vacuum until dry resulting in the crude extract (CEXA). The extracts were kept in a freezer at −18 °C until further analysis.

2.3.1. Identification of phenolics by TLC

The phytochemical screening of phenolics in the CEXA was performed by thin layer chromatography. Approximately 10 mg of each sample was dissolved in 1 ml of methanol. A small aliquot (10–20 μl) was applied to an aluminum plate covered with silica gel. It was used as an eluent, a mixture of ethyl acetate:acetic acid:formic acid:water (100:11:11:26). The plate was revealed with 1% methanol diphenylboric acid 2-amino-ethyl ester (NP) followed by 1% polyethylene glycol (PEG) 4000 in ethanol. The plate was heated for 5 min at 105 °C and then viewed under ultraviolet (UV) light at 365 nm. Samples of rutin references, quercetin and chlorogenic acid were used for each class of substance (Wagner & Bladt, 1996).

2.4. Quantification of phenolics in CEXA by HPLC

The CEXA was analyzed in a Shimadzu HPLC with a DAD UV detector. The column used was an NST C_{18} (250 mm × 4.6 mm, 5 μm) at 40 °C, and the mobile phases comprised of acetonitrile, phosphoric acid and water (7:0.4:92.6) (A) and acetonitrile and phosphoric acid (99.6:0.4) (B). The gradient elutions occurred as follows: 100:0 (A:B) for 0–17 min, 100:0 to 80:20 for 17–50 min, 80:20 to 0:100 for 50–51 min, 0:100 for 51–61 min, 0:100 to 100:0 for 61–62 min and 100:0 for 62–72 min, with a flow rate of 1 ml/min. The standards were prepared in methanol at concentrations of 0.025 mg/ml for chlorogenic and caffeic acids (Sigma-Aldrich®, São Paulo, SP, Brazil) and 0.07 mg/ml for rutin (Sigma-Aldrich®, São Paulo, SP, Brazil) and quercetin (Sigma-Aldrich®, São Paulo, SP, Brazil). The CEXA was prepared at 10 mg/ml, and the injection volume was 10 μl. The detection was performed at 330 nm (Finzelberg, 2000).

2.5. Animals, diet, tissue and blood collection

Male BALB/c mice aged 4–5 weeks were obtained from the animal care center at the Federal University of Minas Gerais (CEBIO/UFMG) and were kept in an environmentally controlled room under a 14/10 h light–dark cycle. Animals had free access to tap water and food and were maintained according to the ethical guidelines of our institution. The experimental protocol was approved by the Animal Ethics Committee at the university (CETEA/UFMG, number 211/2010). During the first 8 weeks of the experiment, mice were divided in two groups: the first group (n = 8) received a control diet (LABINA), and the second group (n = 32) received the HC diet. At the end of 8 weeks, the HC group had glucose and lipid metabolic alterations and increased levels of inflammatory parameters, just as described by Oliveira, Menezes-Garcia, et al. (2012). The HC diet was comprised of 45% condensed milk, 10% refined sugar and 45% of the control diet. The macronutrient composition of the control diet (4.0 kcal/g) was 65.8% by weight as carbohydrate, 3.1% as fat, and 31.1% as protein; and the HC diet (4.4 kcal/g) was 74.2% by weight as carbohydrate, 5.8% as fat, and 20% as protein. The HC diet contained at least 30% refined sugar (mostly sucrose) (Oliveira, Menezes-Garcia, et al., 2012).

After 8 weeks, the animals that were fed the HC diet were redistributed equally into four groups. One group continued to receive the HC diet alone, whereas the other three groups had their HC diet supplemented with three different concentrations of CEXA (50, 100 and 200 mg/kg). This treatment was performed for four weeks.

Mice were collectively housed, and food intake was measured twice a week for the first eight weeks and daily during the last four weeks of the experimental period. The body weight was measured once a week. At the end of the dietary treatment, animals were anesthetized with ketamine (130 mg/kg BW) and xylazine (0.3 mg/kg BW) and euthanized by exsanguination. Liver, epididymal, retroperitoneal, and mesenteric white adipose tissues were collected, weighed and stored at −20 °C for further analysis. The adiposity index was calculated as percentage of body fat (the sum of the epididymal, mesenteric and retroperitoneal adipose tissue weight) divided by the total body weight. Blood was collected, added to tubes and centrifuged (3000 rpm) for 10 min at 4 °C to obtain serum. The serum was aliquoted into individual tubes and frozen (−20 °C) to determine the glucose, triglyceride, cholesterol and insulin levels.

2.6. Histology

Epididymal adipose tissue and liver were excised and fixed in a phosphate-buffered formaldehyde solution for 48 h and were then incubated in 70% ethanol. Sections of the tissues were stained with hematoxylin–eosin and analyzed in original magnification of 200×. In the adipose tissue, the area encompassed by 100 adipocyte cells was measured in each animal using Image Pro-Plus software (Media Cybernetics, USA). ImageJ (National Institutes of Health, Bethesda, Maryland, USA) was used to calculate the mean adipocyte area (μm^2) and frequency distribution of adipocytes' cell surface area. Hepatic histological features were investigated, and lesions were evaluated by a scoring system of necroinflammation adapted from Brunt, Janney, Di Bisceglie, Neuschwander-Tetri, and Bacon (1999). Inflammatory infiltrate, perivascular inflammation and microsteatosis were classified by a scoring system (0–3) consisting of the following grades: 0 = absent, 1 = mild, 2 = moderate, and 3 = severe.

2.7. Oral glucose tolerance and insulin sensitivity tests

For the oral glucose tolerance test, mice that were fasted overnight orally received D-glucose (2 mg/kg BW). Tail blood samples were taken at 0, 15, 30, 60 and 90 min after glucose administration, and the blood glucose levels were measured using an Accu-Check glucometer (Roche Diagnostics, Indianapolis, IN). The sensitivity test was performed in fed mice after an intraperitoneal injection of insulin (0.75 units/kg BW; Sigma, St. Louis, MO). Tail blood samples were taken at 0, 15, 30 and 60 min after insulin injection to measure the blood glucose levels.

2.8. Determination of serum parameters

Fasting glucose, triglycerides and total cholesterol levels were measured using enzymatic kits (Katal, Belo Horizonte, MG). The fasting serum levels of insulin, adiponectin and resistin were assayed using ELISA kits (Millipore, Missouri, USA). The homeostasis model of the assessment for insulin resistance (HOMA-IR) and β cell function (HOMA-β) was calculated as described by Matthews et al. (1985) and updated by Bonora et al. (2000).

2.9. Total leukocyte, mononuclear cell and neutrophil counts

Tail blood samples were taken and diluted in 1:10 in Türk's solution. Total leukocyte counts were performed using a Neubauer chamber. Peripheral blood smears were stained with May-Grünwald–Giemsa, and differential leukocyte counts were determined under oil immersion (1000×) using standard morphology criteria.

2.10. ELISA assay

TNF-α, IL-6, IL-10, IL-4 and IL-13 levels were measured in the epididymal adipose tissue and liver. These analyses were performed using DuoSet ELISA development kits (R&D System, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

2.11. Statistical analysis

The data were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. Data and statistical analyses were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego California, USA). The results were reported as the mean ± SD, and differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Total phenol and flavonoid content in X. aromatica fruits

The total phenol content and the total flavonoid content were 2.01 ± 0.06 mg GAE/100 mg and 0.07 ± 0.01 mg RE/100 mg, respectively. Considering that the CEXA yield was 39.1%, the doses of 50 mg, 100 mg and 200 mg of CEXA represent 2.57, 5.14 and 10.28 mg of GAE and 0.9, 1.8 and 3.6 mg of RE, respectively.

3.2. Chromatographic analysis

Phytochemical screening by TLC allowed for the identification of the flavonoid rutin and caffeic and chlorogenic acids (data not shown). HPLC analysis of CEXA (Fig. 1) indicated and quantified the presence of 3 phenolic substances. Chlorogenic (0.22%) and caffeic (0.02%) acids at Rt 9.64 and 11.78 min, respectively, and rutin (0.11%) at Rt 34.86 were identified.

3.3. Mice feeding CEXA had smaller adipocytes

The body weight gain, visceral adiposity index and adipocyte morphology of the mice were evaluated. The body weight was similar between groups before and after the experimental period (data not shown). There were no differences in food intake and body weight gain (data not shown). However, the HC mice had a higher adiposity index and increased adipocyte area compared to the control mice (Fig. 2A). Although the mice that were supplemented with the three different doses of the CEXA showed no change in their adiposity index in relation to animals that were fed the HC diet alone, they presented with a smaller cell area (Fig. 2B). The animals that were fed the highest concentration of the CEXA showed the smallest adipocyte cell size (Fig. 2C, D).

3.4. CEXA improves glucose homeostasis but not serum lipid profile

We examined if the addition of CEXA to the HC diet was sufficient to improve the glucose intolerance and dyslipidemia induced by the HC diet. We used the performance oral glucose tolerance and insulin sensitivity tests and analyzed fasting glucose, insulin levels and the lipid profile as well as the HOMA indices. After 12 weeks on the HC diet, the animals showed an elevated glycemic response to glucose overload and a reduced response to insulin compared to animals fed the control chow diet (Fig. 3). The fasting glucose levels, total cholesterol levels, triglycerides and HOMA-IR increased, and decreased values of HOMA-β were observed in mice fed the HC diet (Table 1). Mice that were fed the HC diet supplemented with any of the CEXA doses evaluated

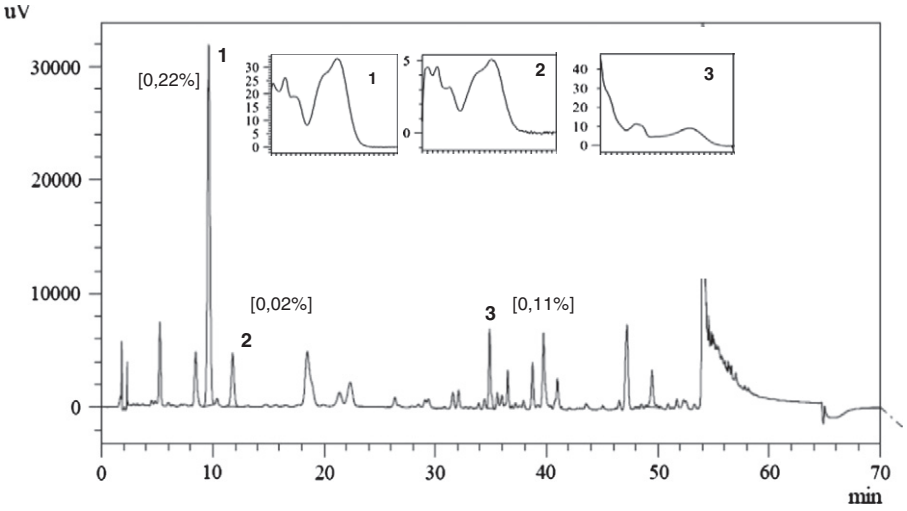

Fig. 1. HPLC/DAD chromatogram of the crude extract of *X. aromatica* fruit. Retention times: (1) Rt: 9.64 min, (2) Rt: 11.78 min and (3) Rt: 35.86 min, correspond, respectively, to chlorogenic acid, caffeic acid and rutin. Concentrations of each class of substances are expressed as mg/100 mg between [ ]: 1 = 0.22 mg/100 mg; 2 = 0.02 mg/100 mg, 3 = 0.11 mg/100 mg.

showed an improvement in the oral glucose tolerance test (Fig. 3A, B). The highest dose of CEXA also improved the insulin sensitivity (Fig. 3C, D) However, there were no differences in fasting glucose, lipid profile and HOMA indices in the mice fed the HC diet supplemented with CEXA (Table 1).

3.5. CEXA decreases the number of circulating immune cells

Mice that were fed the HC diet showed higher numbers of total leukocytes and circulating mononuclear cells over mice that were fed the control diet. The animals fed the HC diet supplemented with different

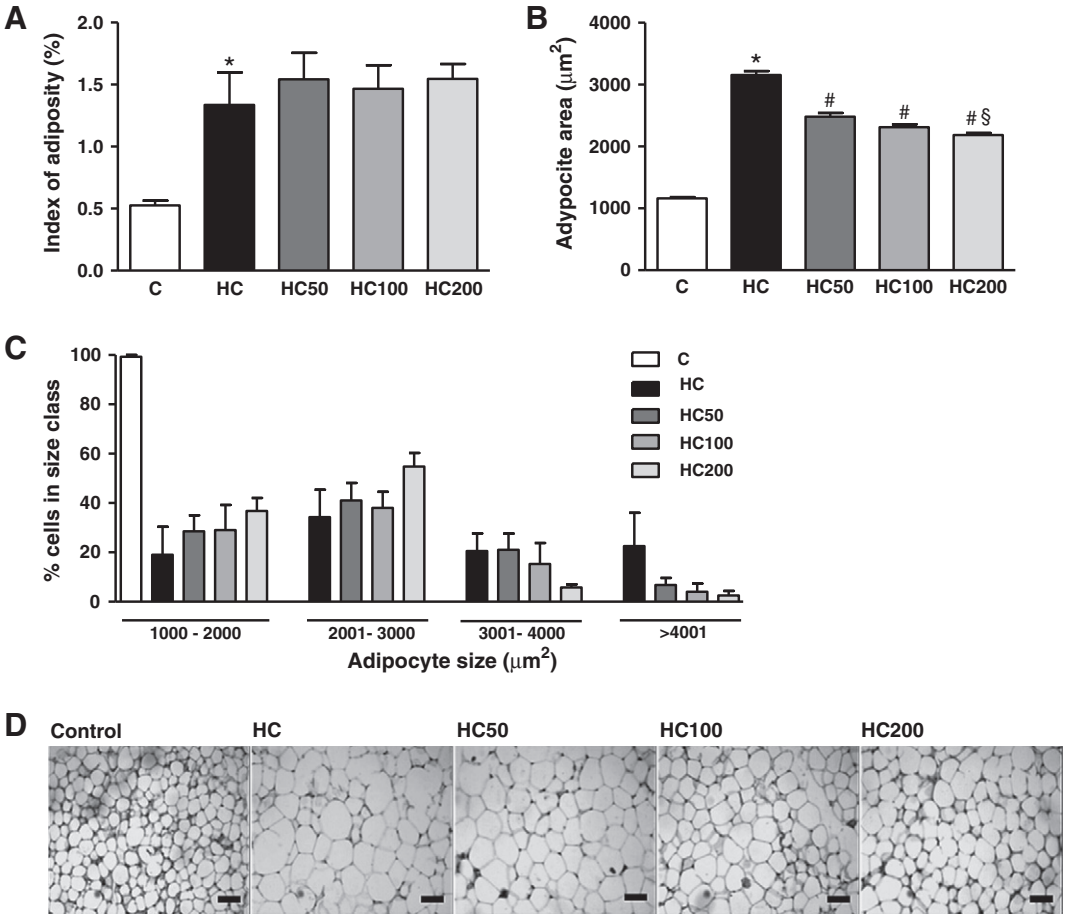

Fig. 2. Effect of CEXA intake on the visceral index of adiposity (A), morphology of epididymal adipose tissue (B), adipocyte size (C) and percentage of adipocyte cells in different size classes (D) in mice fed with the C diet, HC diet alone and HC diet supplemented with 50, 100 or 200 mg/kg BW of CEXA. The data are presented as the mean ± SD (n = 8). *Significant difference from the C group; #significant difference from the HC group; §significant difference from the H50 and HC100 groups ($P < 0.05$). C, animals fed with the control diet; HC, animals fed with the high carbohydrate diet; HC50/HC100/HC200, animals fed with the HC diet plus 50, 100 or 200 mg/kg BW of CEXA, respectively; CEXA, crude extract of *Xylopia aromatica*.

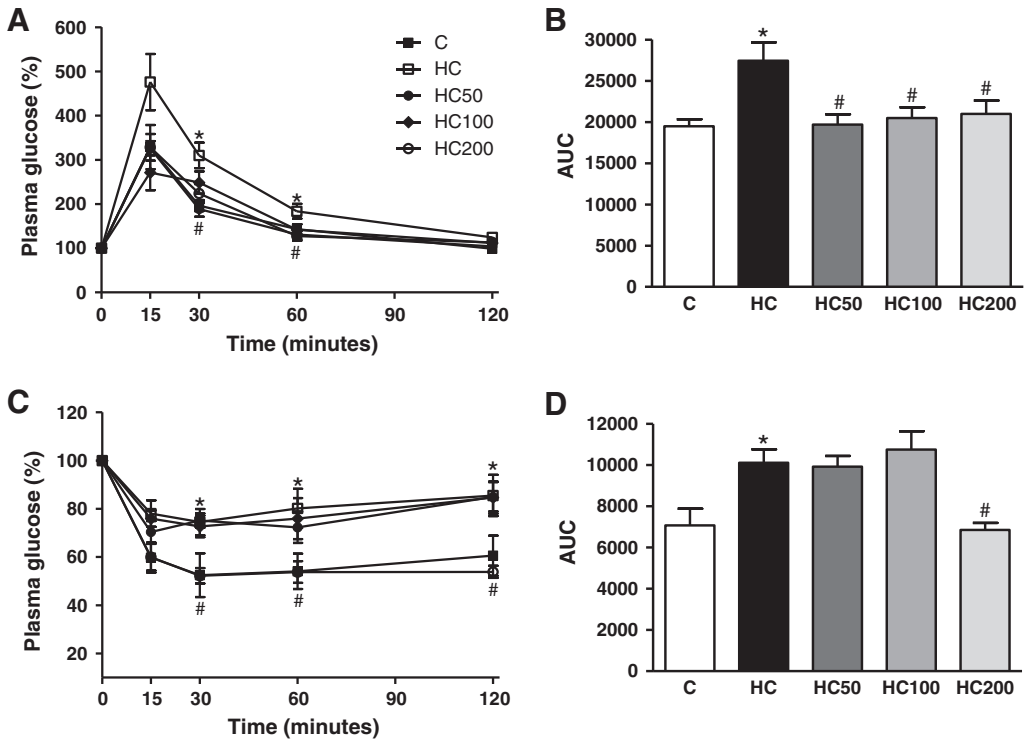

Fig. 3. Oral glucose tolerance test (A) and insulin sensitivity test (B) performed in mice fed with the C diet, HC diet alone and HC diet supplemented with 50, 100 or 200 mg/kg BW of CEXA. The data are presented as the mean ± SD (n = 8). *Significant difference from the C group; #significant difference from the HC group ($P < 0.05$). The groups are described in the legend of Fig. 2.

doses of CEXA showed lower counts of leukocytes and mononuclear cells (Fig. 4A, B). No differences in the neutrophil counts were observed between all of the groups (Fig. 4C).

3.6. CEXA improved cytokine levels in liver and epididymal adipose tissue

To test if the CEXA modulates the inflammation in metabolic tissues, we analyzed pro- and anti-inflammatory cytokines in adipose tissue and liver. The HC group had higher values of TNF-α, IL-6, IL-4, IL-10 and IL-13 in the adipose tissue (Fig. 5) and the liver (Fig. 6) compared to the control group. Supplementation of the HC diet with any of the tested concentrations of CEXA reduced the levels of TNF-α and IL-6 in the liver (Fig. 6A, B) but not in the adipose tissue (Fig. 5A, B). However, CEXA at the highest dose increased the levels of the anti-inflammatory cytokines IL-13 and IL-4 in both tissues (Figs. 5C, D; 6C, D). No changes were observed in IL-10 levels in adipose tissue and liver after CEXA supplementation (Figs. 5E, 6E).

3.7. Effect of CEXA on hepatic tissue

We evaluated if CEXA improves hepatic steatosis and inflammation induced by an HC diet. Mice that were fed the HC diet showed higher inflammatory infiltrate, perivascular inflammation and microsteatosis compared to mice that were fed the control diet (Fig. 7A, B). Animals that received CEXA at all doses presented with a lower total histopathological score compared to mice fed the HC diet alone (Fig. 7A).

4. Discussion

We first had to determine the doses of CEXA that would be suitable for this study. It is known that species within the same genus have similar chemical substances. Laboratory studies with *Xylopia aethiopica* fruits in animal models have shown positive effects on glucose and lipid metabolism, as well as on metabolic dysfunction, which is commonly associated with obesity (Ezekwesili, Nwodo, Eneh, & Ogbunugafor, 2010; Nwozo et al., 2011; Ogbonnia et al., 2008, 2010). An *in vivo* study showed an association of *X. aethiopica* fruits with other plant extracts in reducing serum glucose and lipid parameters at all of the four doses tested (50, 100, 250 and 500 mg/kg BW). An oral gavage of *X. aethiopica* (250 mg/kg/ BW) in a hypercholesterolemic mouse improved the lipid profile and endogenous antioxidant enzymes of the animals. Both reports primarily attributed the observed changes to the phenolic substances and sterols present in the fruit. CEXA was added to the animal feed in an attempt to mimic the ingestion of the

Table 1
Effect of CEXA intake on fasting glucose, insulin, HOMA indices and serum lipids in mice fed with the C diet, HC diet alone and HC diet supplemented with 50, 100 or 200 mg/kg BW of CEXA. The data are presented as the mean ± SD (n = 8). *Significant difference from the C group ($P < 0.05$). C, animals fed with the control diet; HC, animals fed with the high carbohydrate diet; HC50/HC100/HC200, animals fed with the HC diet plus 50, 100 or 200 mg/kg BW of CEXA, respectively; CEXA, crude extract of *Xylopia aromatica*.

Serum profile	Groups				
	Control	HC	HC50	HC100	HC200
Fasting glucose (mg/dl)	167.8 ± 30.7	259.6 ± 93.9*	220.8 ± 52.4	279.2 ± 41.0	264.0 ± 62.7
Insulin (ng/ml)	5.6 ± 0.8	5.9 ± 0.9	6.4 ± 0.6	5.7 ± 0.6	5.6 ± 0.4
HOMA-IR	2.3 ± 0.3	3.9 ± 0.8*	3.9 ± 1.7	3.8 ± 0.55	3.1 ± 0.5
HOMA-β	26.2 ± 9.9	14.1 ± 6.1*	17.1 ± 4.0	12.2 ± 1.9	12.1 ± 1.9
Total cholesterol (mg/dl)	160.9 ± 23.3	326.6 ± 48.2*	338.8 ± 77.4	351.5 ± 81.6	339.9 ± 53.0
Triacylglycerol (mg/dl)	199.7 ± 43.1	319.5 ± 46.7*	327 ± 59.6	349.8 ± 75.2	338.75 ± 72.4

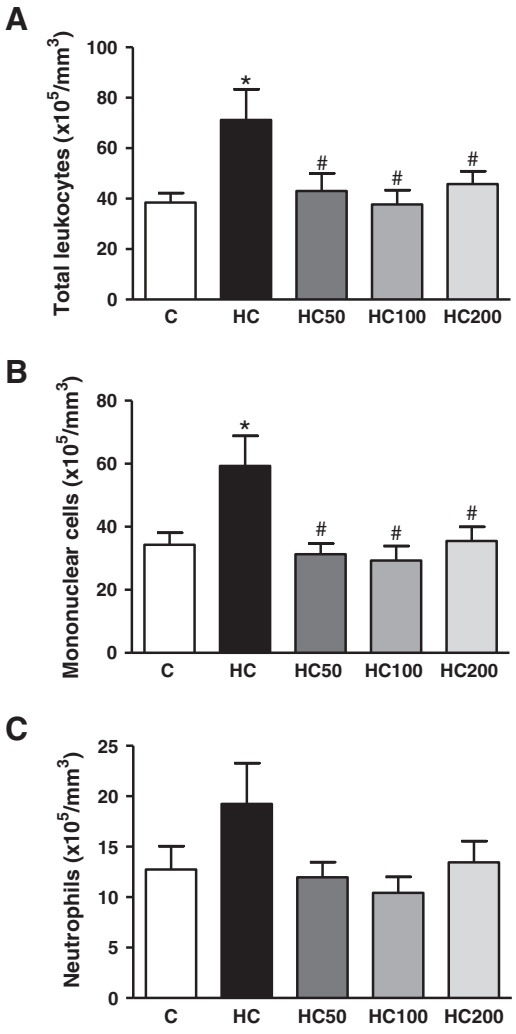

Fig. 4. Total leukocyte count and differential count of mononuclear cells and neutrophils. Total leukocyte count (A), mononuclear cell count (B) and neutrophil count (C) in mice fed with the C diet, HC diet alone and HC diet supplemented with 50, 100 or 200 mg/kg BW of CEXA. The data are presented as the mean ± SD (n = 8). *Significant difference from the C group; #significant difference from the HC group ($P < 0.05$). The groups are described in the legend of Fig. 2.

fruit and its role as a functional food. The development and maintenance of obesity and related metabolic disorders involve many complex molecular mechanisms and interconnected cell signaling pathways. In this context, the multi-component nature of medicinal herbs appears to be particularly suitable for treating complex diseases and offers great potential for exerting synergistic actions (Yang et al., 2014). Various bioactive substances in low concentrations are present in the crude extract, which can act synergistically. Some teas as well as fruit and vegetable extracts are a source of a complex mixture of bioactive substances, and many studies have shown that they can provide health benefits through the combination of additive and synergistic effects (Chantre & Lairon, 2002; Moreno, Ilic, Poulev, & Raskin, 2006; Seeram, Adams, Hardy, & Heber, 2014).

There is significant evidence of potential anti-obesity properties of polyphenols, which include simple phenolic acids such as chlorogenic and caffeic acids and flavonols such as quercetin and rutin (Williams et al., 2013). In this study, we quantified the total phenolic acid and total flavonoid levels by spectrophotometric analysis. CEXA showed 5.14% of GAE and 1.8% of RE. These values are similar to those of other plant extracts that have been shown to positively affect *in vivo* metabolic dysfunction models (Kang et al., 2012; Ortsäter, Grankvist, Wolfram, Kuehn, & Sjöholm, 2012; Williams et al., 2013). A Yerba Mate aqueous solution showed 5.6% of total phenolic acids and 0.3% of flavonoids (Bojić, Haas, & Maleš, 2013). The total phenolic levels in green and black teas normally range from 3.2 to 14.7% (Prior & Cao, 1999). HPLC analysis showed that the major substances found in the CEXA were chlorogenic acid (0.22%) followed by other two unidentified flavonoids and rutin (0.11%), a quercetin glycoside. These polyphenols are able to inhibit growth of adipose tissue by modulating adipocyte metabolism (Badimon, Vilahur, & Padro, 2010; Morikawa, Ikeda, Nonaka, & Suzuki, 2007). Hsu and Yen (2006) showed that chlorogenic acid can suppress cell growth as well as enhance cell apoptosis. Quercetin is a flavonoid that has shown the most promise as a potential anti-obesity treatment. Several reports have revealed that quercetin improved the effects against obesity-related inflammation, including attenuation of inflammation markers, insulin resistance and macrophages (Al-Fayez, Cai, Tunstall, Steward, & Gescher, 2006; Chuang et al., 2010). The presence of these substances in CEXA probably contributed to the positive results found in metabolic dysfunction mice.

In the present study, CEXA was shown to improve oral glucose tolerance, insulin sensitivity, hepatic steatosis, and inflammatory responses in adipose tissue and the liver. Although there were no observed changes in body weight and adiposity, the animals that were fed with any of the three doses of CEXA showed smaller adipocytes. The replacement of the hypertrophic cells by young adipocytes could create a more favorable metabolic environment because smaller adipocytes are more sensitive to insulin (Okuno, Tamemoto, & Tobe, 1998). Some plant extracts can reduce the size of adipocytes in obese and diabetic animals and promote improvements in glucose metabolism (Chung, Cho, Bhuiyan, Kim, & Lee, 2010; Kim et al., 2011; Krisanapun, Lee, Peungvicha, Temsiririrkkul, & Baek, 2011). Although we observed positive results on glucose metabolism, the addition of the three doses of CEXA in the HC diet did not change HOMA-IR and HOMA-β, which are indices that predict efficient insulin function and insulin production by the pancreatic beta cells, respectively, in an indirect manner (Song et al., 2007). It must be considered that these indices correlate to fasting glucose and insulin, which are parameters that did not change between the HC group and the HC mice supplemented with CEXA. Corroborating with our results, other studies also showed that some plant extracts improved the oral glucose tolerance but did not reduce fasting plasma glucose (Akase et al., 2011; Mang et al., 2006).

CEXA improved glucose metabolism in our study, but we did not observe any alterations in lipid metabolism after CEXA consumption. Contrary to our findings, Nwozo et al. (2011) showed a reduction of cholesterol levels and triglycerides in rats treated with *X. aethiopica*. The authors attributed these results to the presence β-sitosterol in *X. aethiopica* fruits, a compound that was neither identified nor quantified in this report. Moreover, the dosage of the *X. aethiopica* extract used was higher, and the length of the experiment was longer compared to those in our study.

Mice receiving CEXA showed a decrease in the number of total leukocytes and circulating mononuclear cells as well as reduced levels of TNF-α and IL-6 in the liver, suggesting an improvement in the inflammatory environment induced by the HC diet. Several reports have shown that obesity is a chronic low-grade inflammatory condition (Hotamisligil, Shargill, & Spiegelman, 1993; Wellen & Hotamisligil, 2005). The expansion of visceral fat tissue produces a wide range of pro- and anti-inflammatory mediators that have been linked to the development of insulin resistance, glucose intolerance and hepatic steatosis (Ferreira et al., 2011; Hotamisligil et al., 1993). Circulating immune cells and cytokines are released by macrophages, lymphocytes, and adipocytes to interact and regulate the inflammatory cascade and metabolism (Nishimura et al., 2009; Pearson et al., 2003). It is well documented that TNF-α and IL-6 stimulate increased transcription of proinflammatory cytokines, which impairs the action of intracellular tyrosine kinases and insulin activity in the liver and adipose tissue (Schenk, Saberi, & Olefsky, 2008; Wellen & Hotamisligil, 2005). Interestingly, there was no observed reduction in the levels of these

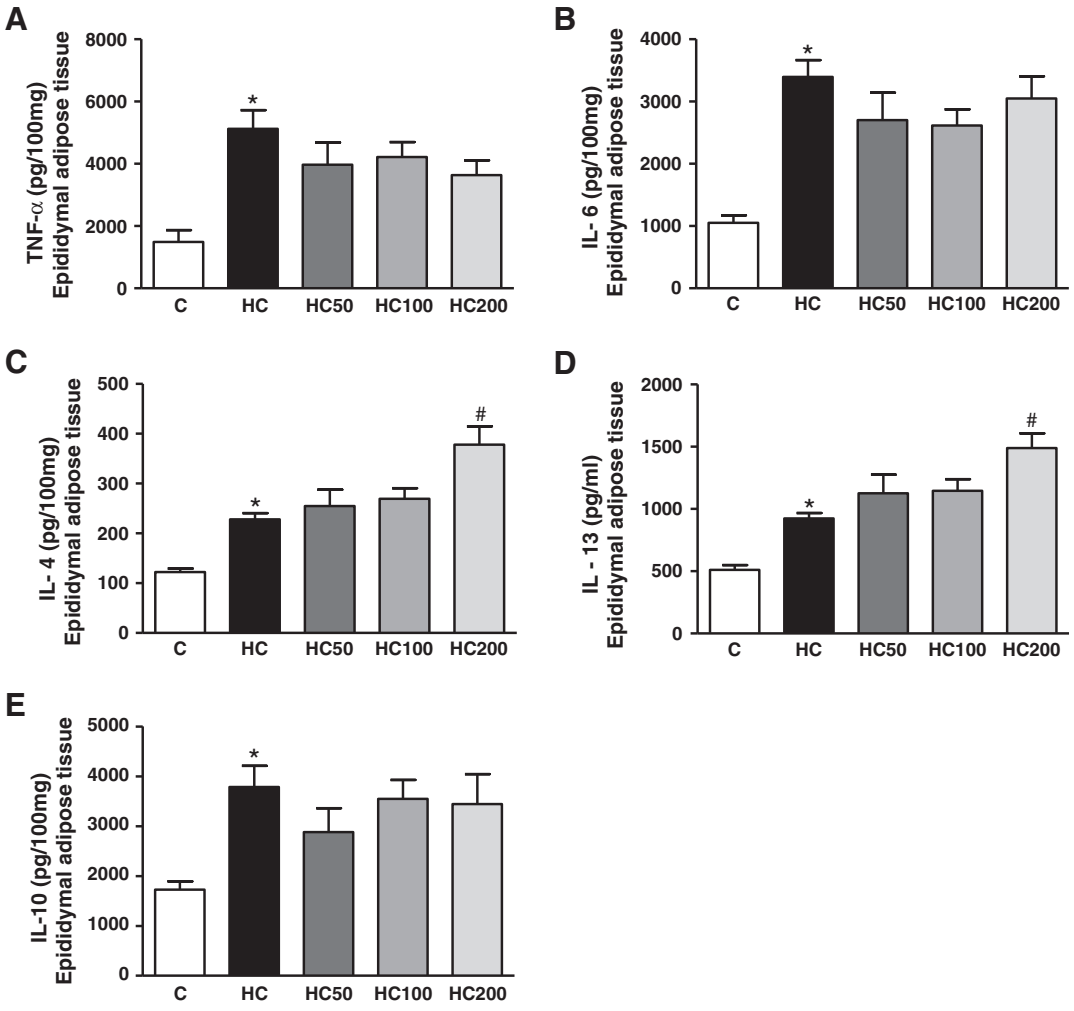

Fig. 5. Concentration of inflammatory mediators in visceral adipose tissue. Levels of the cytokines TNF-α (A), IL-6 (B), IL-4 (C), IL-13 (D) and IL-10 (E) in the epididymal adipose tissue of mice fed with the C diet, HC diet alone and HC diet supplemented with 50, 100 or 200 mg/kg BW of CEXA. The data are presented as the mean ± SD (n = 8). *Significant difference from the C group; #significant difference from the HC group ($P < 0.05$). The groups are described in the legend of Fig. 2.

pro-inflammatory cytokines in adipose tissue, suggesting that CEXA could modulate inflammation in mice in a cytokine and tissue-specific manner.

Concomitant with the reduced levels of pro-inflammatory cytokines in the liver, CEXA at the higher dose increased the levels of IL-4 and IL-13 in adipose tissue and liver and improved liver microsteatosis, inflammatory infiltrate and necrosis. Hepatic steatosis is a condition that is commonly found in patients with obesity and type 2 diabetes and is thought to be a major cause of hepatic insulin resistance (Lockman & Nyirenda, 2010). Recent studies have shown that IL-4 and IL-13 ameliorate liver inflammation and fibrosis (Das & Balakrishnan, 2011; Shimamura et al., 2008). IL-4 also regulates glucose metabolism by promoting insulin sensitivity and glucose tolerance (Chang, Ho, Lu, Huang, & Shiau, 2012; Winer & Winer, 2012). In accordance with our findings, oral administration of *Ilex paraguariensis* extract rich in phenolics (including chlorogenic acid and flavonoids) showed significant ameliorative effects on liver histopathology (Hussein et al., 2011).

The primary compounds in plant extracts possessing anti-inflammatory and antioxidant activities are polyphenols, specifically flavonoids (Talhouk, Karam, Fostok, El-Jouni, & Barbour, 2007; Ueda, Yamazaki, & Yamazaki, 2004). Flavonoids have been considered to be a potential antidiabetic agent with hypoglycemic and anti-hyperglycemic actions by mimicking and stimulating insulin secretion. They also have actions similar to drugs such as metformin, which increases insulin sensitivity but has no effect on its release (Chuang et al., 2010; Jung, Lee, Park, Kang, & Choi, 2006; Wu, Wu, Huang, Jao, & Yen, 2009).

Previous studies have reported the presence of alkaloids and essential oils in *X. aromatica* fruits (Moraes & Roque, 1988; Silva, Miranda, & Conceição, 2010). Alkaloids commonly found in spices have also shown positive effects in metabolic complications associated with obesity (Diwan et al., 2011; Kang et al., 2010), as well as essential oils (Talpur, Echard, Ingram, Bagchi, & Preuss, 2005). However, it must be considered that some alkaloids have hepatotoxic effects, and studies of the global effect of higher intake of this fruit should be performed (Li, Xia, Ruan, Fu, & Lin, 2011).

The higher concentration of anti-inflammatory interleukins in the adipose tissue and liver of mice that received the highest dose of CEXA likely contributed to the improvement on the insulin sensitivity test, a reduced adipocyte size and lower histopathological score. These results can be explained by the increased amount of bioactive substances present in the diet of the HC200 mice, and the biological activities observed in this study are likely due to the set of all substances present in CEXA.

Our findings showed that *X. aromatica* fruits can modulate metabolic changes associated with obesity, especially those related to glucose metabolism. The beneficial effects observed can be associated with a lower release of inflammatory mediators in adipose tissue and the liver. This study suggests the potential of *X. aromatica* in attenuating health problems associated with metabolic dysfunction induced by poor diet.

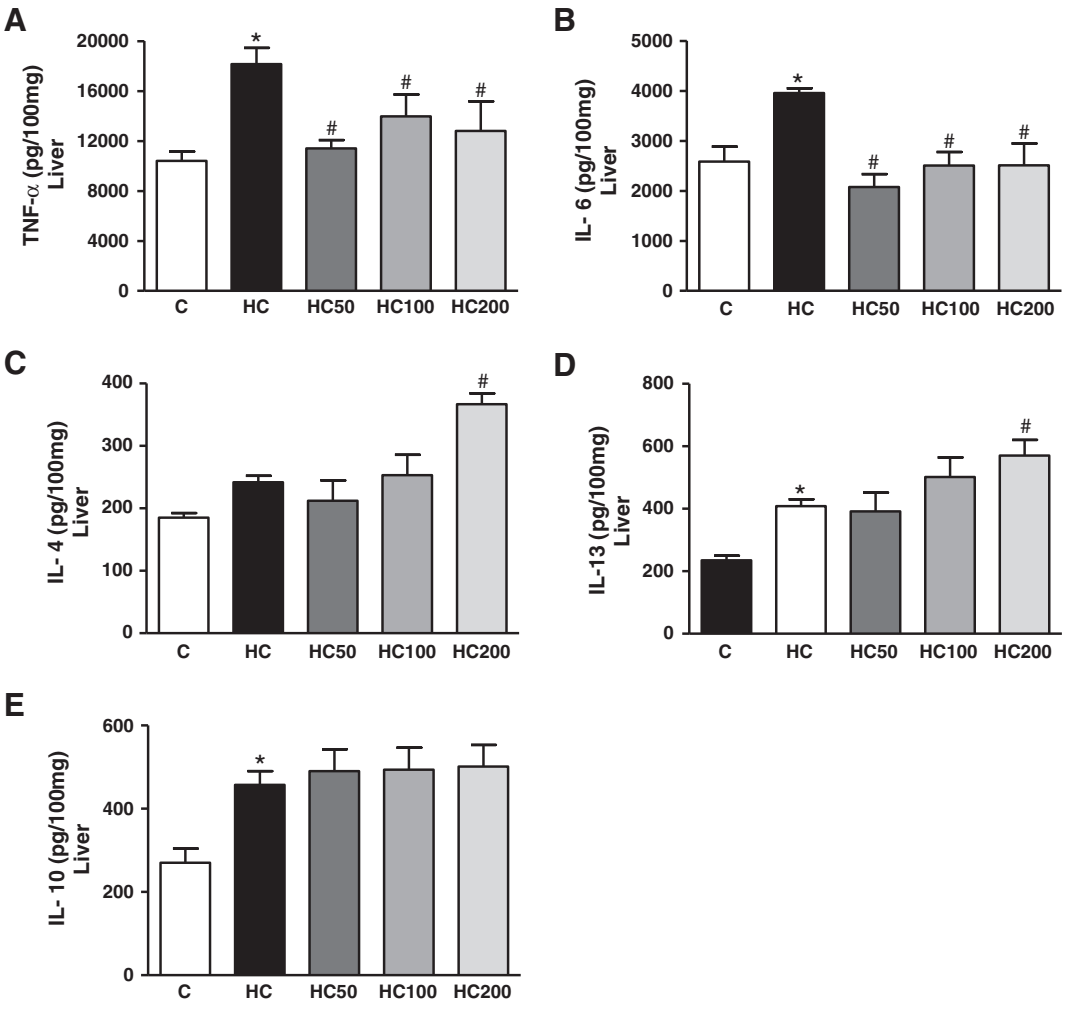

Fig. 6. Concentration of inflammatory mediators in the liver. Levels of the cytokines TNF-α (A), IL-6 (B), IL-4 (C), IL-13 (D) and IL-10 (E) in the livers of mice fed with the C diet, HC diet alone and HC diet supplemented with 50, 100 or 200 mg/kg BW of CEXA. The data are presented as the mean ± SD (n = 8). *Significant difference from the C group; #significant difference from the HC group ($P < 0.05$). §significant difference from the HC200 group. The groups are described in the legend of Fig. 2.

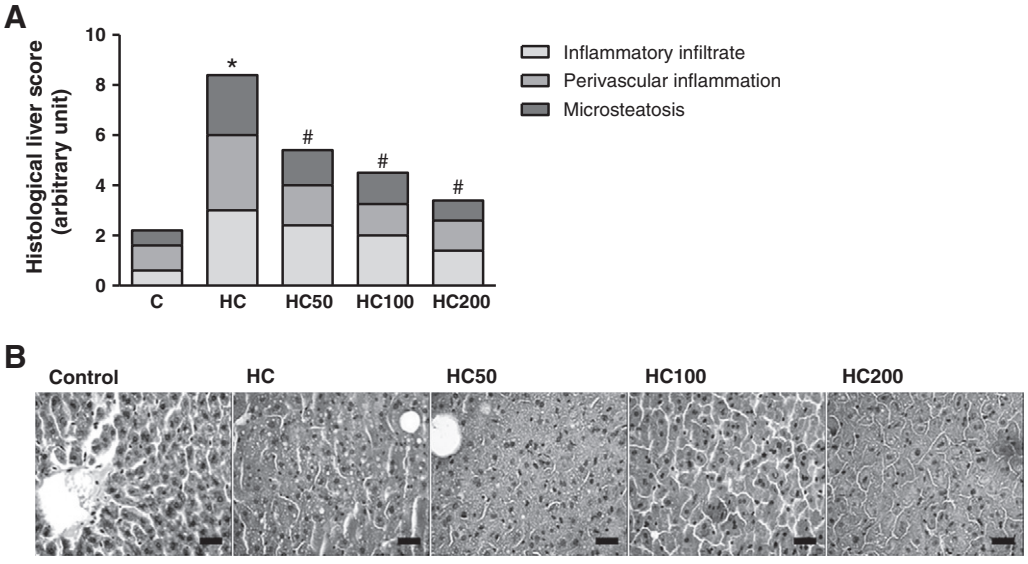

Fig. 7. Effect of CEXA on liver damage. (A) Total score of necroinflammation: inflammatory infiltrate score (0-3), perivascular inflammation score (0-3) and microesteatosis score (0-3). (B) Liver tissue morphology in mice fed with the C diet, HC diet alone and HC diet supplemented with 50, 100 or 200 mg/kg BW of CEXA. The data are presented as the mean ± SD (n = 8). *Significant difference from the C group; #significant difference from the HC group; §significant difference from the H50, HC100 and HC200 groups ($P < 0.05$). The groups are described in the legend of Fig. 2.

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