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ARTICLE

Betulinic Acid, a Natural Pentacyclic Triterpenoid, Prevents Abdominal Fat Accumulation in Mice Fed a High-Fat Diet

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In the search for potential antiobese agents from natural sources, this study investigated the effects of betulinic acid (BA), a pentacyclic triterpene from *Clusia nemorosa* L. (Clusiaceae), in mice on a high-fat diet (HFD). Adult male Swiss mice ($n = 8$) treated or not with BA (50 mg/L, in drinking water) were fed a HFD during 15 weeks. Mice treated with BA and fed a HFD showed significantly ($P < 0.05$) decreased body weights, abdominal fat accumulation, blood glucose, plasma triglycerides, and total cholesterol relative to their respective controls fed no BA. Additionally, BA treatment, while significantly elevating the plasma hormone levels of insulin and leptin, decreased the level of ghrelin. However, it caused a greater decrease in plasma amylase activity than the lipase. These findings suggest that BA has an antiobese potential through modulation of fat and carbohydrate metabolism, and it may be a suitable lead compound in the treatment of obesity.

KEYWORDS: Betulinic acid; pentacyclic triterpene; high-fat diet; antiobesity effect; hypoglycemic effect

INTRODUCTION

The prevalence of obesity has been increasing worldwide, which has a great impact on lifestyle-related disorders such as coronary heart disease, atherosclerosis, and diabetes (*1*). Excess visceral abdominal fat accumulation appears to be a key feature of abdominal obesity contributing to the development of the metabolic syndrome (*2*). Therefore, preventing abdominal fat accumulation is an ideal option for the treatment of obesity and related diseases. Although most of the available drugs, such as orlistat, sibutramine, and rimonabant, have modest clinical efficacy, their use is often associated with gastrointestinal or cardiovascular and central nervous system side effects (*3*).

The rich potential of nature to combat obesity has not been fully explored yet, and many newer leads can be obtained from natural sources. In this context, much attention has been focused on phytoconstituents present in fruits, vegetables, and medicinal herbs that may be helpful in preventing diet-induced body fat accumulation and the possible risk of diabetes and heart disease. For example, Cornelian cherries (*Cornus mas*) are used in the preparation of beverages in Europe and also to treat diabetes-related disorders in Asia. Investigations on the most abundant bioactive compounds in *C. mas* fruits, the anthocyanins and ursolic acid, revealed their ability to ameliorate obesity and insulin resistance in C57BL/6 mice fed a high-fat diet (*4*). Likewise, the plant-derived flavonoids, anthocyanins, and saponins have been shown to effectively suppress abdominal fat accumulation in experimental animals (*5*−*8*). Experimental studies on pentacyclic triterpenoid compounds evidence inhibition of several different enzyme systems closely related to carbohydrate and lipid absorption/metabolism, such as lipase and α-amylase (*9*, *10*), protein tyrosine phosphatase 1B (*11*), inhibition of glycogen phosphorylase (*12*), diacylglycerol acetyltransferase (DGAT) (*13*), and α-glucosidase (*14*).

Betulinic acid (BA) is a naturally occurring plant-derived pentacyclic triterpenoid present in many fruits and vegetables (*15*, *16*) that exhibits a wide variety of pharmacological and biochemical effects including anti-inflammatory and anticancer activities (*16*, *17*), promotes vascular function by up-regulation of eNOS expression and down-regulation of NADPH oxidase (*18*), and inhibits DGAT, thereby reducing triglyceride formation (*13*). The consumption of fat-rich foods can activate an inflammatory response in the hypothalamus, which disturbs the anorexigenic and thermogenic signals generated by the hormones leptin and insulin, leading to anomalous body mass control (*19*). Obesity is associated with an increase in adipose tissue macrophages, which also participate in the inflammatory process through the elaboration of cytokines. It is also characterized by the accumulation of triacylglycerol in adipocytes. DGAT catalyzes the final reaction

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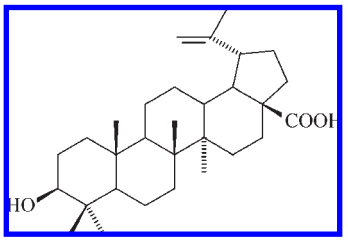

Figure 1. Chemical structure of betulinic acid.

of triacylgycerol synthesis, and several DGAT inhibitors of natural and synthetic origin have been reported (*20*). Given that inflammation and DGAT play crucial roles in adiposity and because BA exhibits both anti-inflammatory and DGAT inhibitory properties, it is hoped that it might be a potential lead compound for the treatment of obesity and associated disorders. In the present study, we therefore examined the possible inhibitory effect of BA on adipogenesis in mice fed a high-fat diet.

MATERIALS AND METHODS

Animals. Male Swiss mice, weighing 25−30 g, obtained from the Central Animal House of this university, were used. They were kept in propylene cages, at a room temperature of 24 ± 2 °C on a 12 h light/dark cycle with food (chow) and water provided ad libitum unless otherwise noted. Experimental protocols (31/08) were approved by the Federal University of Ceara Institutional Committee on Care and Use of Animals for Experimentation, in accordance with the guidelines of the National Institutes of Health, Bethseda, MD.

Plant Material, Extraction, and Isolation of Betulinic Acid. *Clusia nemorosa* L. (Clusiaceae) was collected in August 2005 from Pico Alto, Guaramiranga County, State of Ceara, Brazil, and identified by Professor Edson Paula Nunes. A voucher specimen (34495) has been deposited at the Herbario Prisco Bezerra of the Federal University of Ceara. Air-dried and powdered roots (1.2 kg) were extracted exhaustively with *n*-hexane and then with EtOH at room temperature. Upon concentration of the EtOH extract a precipitate was obtained (16.5 g), which was filtered and recrystallized successively with EtOH to yield a pure compound (6.1 g, 0.5% relative to plant material weight). Melting points were determined using a digital Mettler Toledo FP90 apparatus. The optical rotations were measured on a Perkin-Elmer 341 digital polarimeter. IR spectra (KBr pellets) were recorded using a Perkin-Elmer FT-IR 1000 spectrometer. NMR spectra were recorded on a Bruker Avance DRX-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer using pyridine-d_5 as solvents. The compound was identified as betulinic acid (**Figure 1**) [pf 288−290 °C; $[\alpha]_D^{25} = +34$ (*c* 0.14, pyridine)] upon spectroscopic analysis including IR, NMR, and comparison with literature data (*21*,*22*). TLC was performed on precoated silica gel polyester sheets (kieselgel 60 F_{254}, 0.20 mm, Merck), and the purity of the compound was detected by spraying with vanillin/perchloric acid/EtOH solution followed by heating at 120 °C (molecular weight 456; $C_{30}H_{48}O_3$). The betulinic acid was submitted to HPLC (Shimadzu UFLC system consisting of three pumps LC-20AT) using a semipreparative C_{18} reversed-phase column (250 mm × 10 mm i.d. × 5 μm) from Phenomenex, in the isocratic mode, using acetonitrile/water (86:14, v/v) at a flow rate of 2.0 mL min^{-1}, with an injection volume ("loop") of 100 μL and UV detection at 254 nm (*23*). The purity of BA was 96.5%.

Experimental Diets. The standardized high-fat diet used for the study (*24*) comprised the following hypercaloric constituents: 15 g of laboratory animal chow, 10 g of roasted ground nut, 10 g of milk chocolate, and 5 g of maizen bisquets. These ingredients were ground and prepared in the form of pellets that actually contain, by weight, 20% protein, 48% carbohydrate, 20% lipids, 4% cellulose, and 5% vitamins and minerals. The net energy content of this diet was 21.40 kJ/g. To avoid autoxidation of the fat components, food was stored at ~3 °C. Laboratory pellet chow was used as a control diet.

Animal Treatment and Experimental Protocol. Swiss male mice (6 weeks old) were randomly divided into four groups ($n = 8$) matched for body weight after 1 week of being fed laboratory pellet chow. The control group continued to be fed laboratory pellet chow ad libitum and was designated ND (normal diet fed group). The remaining mice consumed high-fat diet (HFD, control) or HFD + betulinic acid (BA, 50 mg/L in drinking water) or HFD plus sibutramine (SIB, 50 mg/L) for 15 weeks. The choice for the drug concentrations adopted for BA and SIB was based on preliminary studies that showed their safety and efficacy. Betulinic acid was suspended initially in 2% (v/v) Tween 80 and then further in water. HFD-fed controls received the same vehicle. SIB being water-soluble, no vehicle was used. BA- or vehicle-water was changed twice a week and weekly consumption of water (mL/week) noted.

The body weight of each mouse was measured once a week; the total amount of food consumption was recorded every day for 15 weeks and weekly consumption of food (g/week) noted. At the end of this period, animals were starved for 6 h, blood was taken by venous puncture under light anesthesia with diethyl ether, and then they were sacrificed by cervical dislocation. The plasma or serum was prepared and either used within a few hours or frozen at −70 °C until analysis. The liver and abdominal adipose tissues (epididymal and parametrial) were dissected, weighed, and expressed in milligrams per 10 g of body weight.

Biochemical Measures. Plasma amylase and lipase were determinated by a kinetic method using the commercial kits for amylase (Labtest, Minas Gerais, Brazil) and lipase (Bioclin, Minas Gerais, Brazil). The assays were performed according to the manufacturer's instructions, and their levels expressed in units per liter. Plasma glucose, triglycerides, and total cholesterol were analyzed using commercial kits (Labtest) and the levels expressed as milligrams per deciliter. Serum alanine amino transferase (ALT), aspartate amino transferase (AST), and alkaline phosphatase (ALP) activities expressed in units per liter were analyzed by a kinetic method using commercial kits (Labtest). Plasma insulin, leptin, and ghrelin (Crystal Chem, Downer's Grove, IL) were measured by enzyme-linked immunosorbent assay (ELISA) performed in duplicate and were expressed in nanograms per milliliter.

Histology. Tissue samples of hepatic and epididymal fat pads were fixed with 4% buffered formalin and embedded in paraffin. Standard sections of 5 mm were cut and stained with hematoxylin and eosin (H&E), viewed with an optical microscope, and photographed at a final magnification of 100× or 400×.

Statistical Analysis. The results were presented as mean ± standard error of the mean (SEM). The data were analyzed by analysis of variance (ANOVA) and Tukey's test, using the GraphPad Prism program (version 3.0). $p < 0.05$ was considered to be significant for all comparisons.

RESULTS

Effects of BA and SIB Treatments on Initial and Final Body Weights, Net Food and Water Consumption, Relative Liver and Abdominal Adipose Tissue Weights, and Levels of Serum Enzymes ALT, AST, and ALP in Mice Fed Experimental Diets for 15 Weeks. There were no significant differences in the initial body weights among the four groups. At the end of 15 weeks, significant differences were observed in the final body weights and the net food consumption between the control ND group (39.88 and 36.95 g, respectively) and the HFD group (49.38 and 29.25 g, respectively) (**Table 1**). When compared to the ND group, HFD-fed mice showed a significant increase in body weight (23.8%, $p < 0.05$), The observed final body weights were significantly lower in animal groups that received HFD + BA (13.2%, $p < 0.05$) or HFD + SIB (10.9%, $p < 0.05$) when compared to vehicle-treated HFD fed mice. However, there was no obvious difference in food consumption among the HFD group and the groups treated with BA or SIB. In a similar way, HFD-fed animals consumed less water than the ND-fed mice or groups fed HFD that received BA or SIB. The relative weight of the accumulated abdominal fat was significantly higher by feeding HFD than the value for the ND mice (3.4-fold greater, $p < 0.05$), which was decreased to 1.6- and 2.6-fold, respectively, with BA and SIB treatments. The relative hepatic weights in HFD group were found to be significantly higher than those of ND-fed mice. However, this increase was not seen in animal groups

Table 1. Effects of Betulinic Acid Treatment on Initial and Final Body Weights, Net Food and Water Intake, Relative Weights of Abdominal Fat and Liver, and Serum ALT, AST, and ALP Levels in Mice Fed Experimental Diets for 15 Weeks[a]

group	ND	HFD	HFD + BA	HFD + SIB
initial body wt (g)	25.20 ± 0.38	25.27 ± 0.69	25.67 ± 0.66	25.67 ± 0.81
final body wt (g)	39.88 ± 0.51	49.38 ± 1.07*	42.88 ± 1.52**	44.00 ± 0.68**
net food intake (g/week)	36.95 ± 1.89	29.25 ± 1.24*	30.61 ± 1.21*	32.82 ± 1.40
net water intake (mL/week)	42.50 ± 1.65	38.06 ± 2.35	40.77 ± 1.95	38.55 ± 1.24
abdominal fat (mg/10 g of body wt)	201.80 ± 29.22	887.60 ± 121.10*	332.60 ± 0.57**	540.40 ± 93.93**
liver wt (mg/10 g of body wt)	341.50 ± 16.78	419.00 ± 24.23*	365.00 ± 7.19**	349.00 ± 11.87**
ALT (U/L)	41.88 ± 1.68	42.17 ± 2.51	40.00 ± 3.89	41.00 ± 2.92
AST (U/L)	94.83 ± 6,89	122.80 ± 9.13	122.40 ± 11.63	142.80 ± 9.14*
ALP (U/L)	81.17 ± 18.19	107.30 ± 19.10	92.17 ± 19.52	119.70 ± 5.76*

[a] Values are means ± SEM (n = 8). ND, normal diet; HFD, high fat diet; SIB, sibutramine.*, $p < 0.05$ vs mice fed ND; **, $p < 0.05$ vs mice fed HFD (ANOVA followed by Tukey's test).

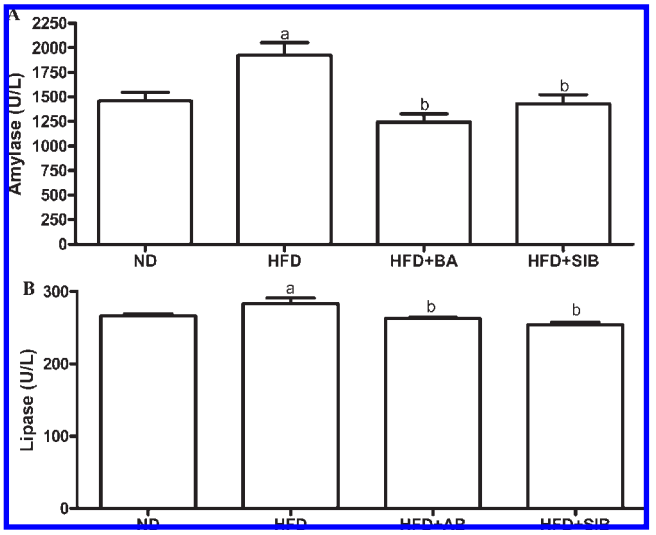

Figure 2. Effects of betulinic acid and sibutramine treatments on plasma levels of (**A**) amylase and (**B**) lipase in mice fed experimental diets for 15 weeks. ND, normal diet; HFD, high-fat diet; BA, betulinic acid; SIB, sibutramine. Each value is the mean ± SEM (n = 8). a, $p < 0.05$ vs ND; b, $p < 0.05$ vs HFD (ANOVA and Tukey's test).

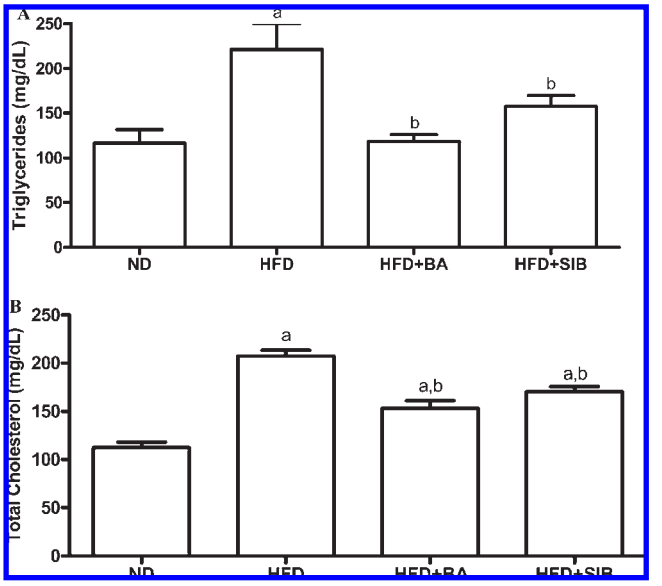

Figure 3. Effects of betulinic acid and sibutramine treatments on plasma levels of (**A**) triglycerides (TG) and (**B**) total cholesterol (TC) in mice fed experimental diets for 15 weeks. ND, normal diet; HFD, high-fat diet; BA, betulinic acid; SIB, sibutramine. Each value is the mean ± SEM (n = 8). a, $p < 0.05$ vs ND; b, $p < 0.05$ vs HFD (ANOVA and Tukey's test).

that received BA or SIB treatment. In general, the serum levels of ALT, AST, and ALP were not significantly different between the various groups, with the exception of one group receiving HFT + SIB that demonstrated a statistically significant increase in AST and ALP when compared to the ND group (**Table 1**).

Plasma Levels of Lipase, Amylase, Total Cholesterol, and Triglycerides. Figure 2 shows the effects of BA and SIB treatments on plasma amylase and lipase activities. The activities of these enzymes were significantly elevated in HFD-fed mice compared to the ND group. Both BA and SIB treatments caused significant reductions in these enzyme activities (amylase by 32 and 25.8%; lipase by 6.6 and 8.8%, respectively). The HFD-induced increase in plasma levels of total cholesterol and triglycerides were also significantly ($p < 0.05$) lowered by treatments with BA or SIB to the extent of 24 or 25%, respectively (**Figure 3**).

Plasma Levels of Glucose, Insulin, Leptin, and Ghrelin. The plasma levels of glucose and insulin were significantly elevated in mice fed the HFD compared to mice on ND. Whereas SIB treatment showed no significant influence on the HFD-induced increases in these parameters, BA treatment showed a significantly decreased level of plasma glucose and an elevated insulin level (**Figure 4**). Mice on the HFD showed a lower level of ghrelin but a higher leptin level compared to the ND group. SIB treatment had no significant influence on HFD-induced changes in the levels of ghrelin and leptin. In contrast, the BA-treated group showed a further decrease in ghrelin and an increase of leptin (**Figure 5**).

Histology of Liver and Adipose Tissues. At histology (**Figure 6**), the HFD resulted in diffuse cytoplasmic vacuolization, indicating hepatocellular steatosis with no signs of necro-inflammation. BA and SIB treatments reduced the intensity of microvacuolization. An increase in cell size of adipocytes was, however, apparent in HFD-fed animals compared to ND-fed mice (**Figure 7**). The adipose cell size in mice treated with BA or SIB did not apparently differ from that observed in ND-fed animals.

DISCUSSION

The results obtained clearly show that a HFD for 15 weeks promotes abdominal adiposity and weight gain in Swiss mice, and treatments with the plant triterpenoid, BA, or a known anorectic agent, SIB, in drinking water (50 mg/L) prevent this adipogenesity and weight gain. In the evaluation of test drugs, besides the adipose tissue weights, body weights, and net food intake measurements, plasma levels of amylase, lipase, glucose, TC, and TG, and the food regulatory peptide hormones ghrelin and leptin, and insulin were quantified. Among the various enzymes involved in lipid metabolism, amylase and lipase provide interesting targets in

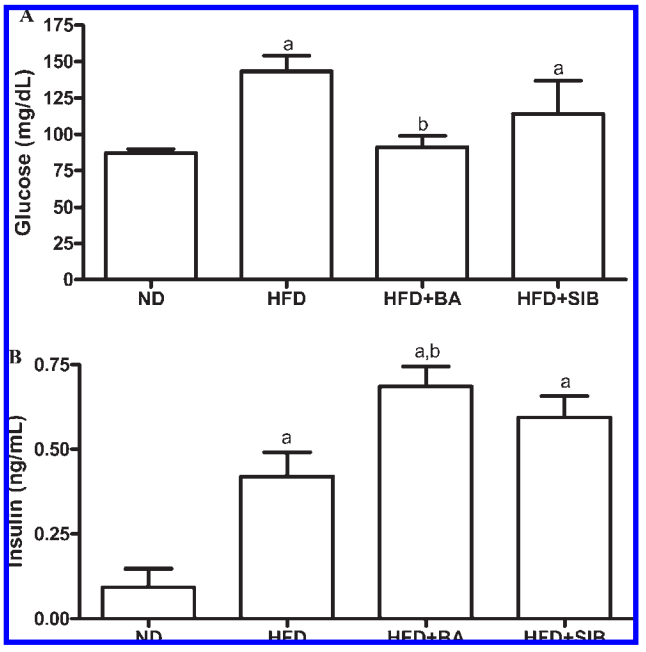

Figure 4. Effects of betulinic acid and sibutramine treatments on plasma levels of glucose (**A**) and insulin hormone (**B**) in mice fed experimental diets for 15 weeks. ND, normal diet; HFD, high-fat diet; BA, betulinic acid; SIB, sibutramine. Each value is the mean ± SEM ($n = 8$). a, $p < 0.05$ vs ND; b, $p < 0.05$ vs HFD (ANOVA and Tukey's test).

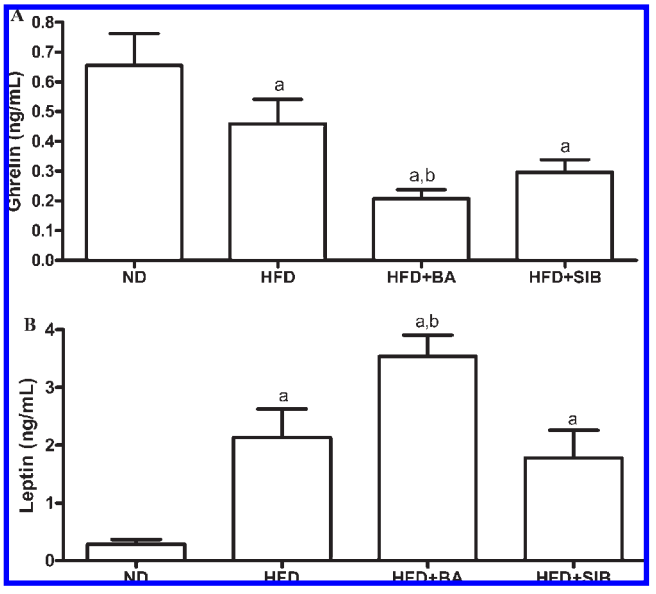

Figure 5. Effects of betulinic acid and sibutramine treatments on plasma levels of ghrelin (**A**) and leptin (**B**) in mice fed experimental diets for 15 weeks. ND, normal diet; HFD, high-fat diet; BA, betulinic acid; SIB, sibutramine. Each value is the mean ± SEM ($n = 8$). a, $p < 0.05$ vs ND; b, $p < 0.05$ vs HFD (ANOVA and Tukey's test).

the development of antiobesity compounds. Activating lipase or inhibiting pancreatic lipase would have an antiobesity effect. Pacreatic lipase, the principal lipolytic enzyme, hydrolyzes dietary fats, the first step of lipid metabolism. Orlistat is a potent, specific, and irreversible inhibitor of lipase clinically employed to prevent obesity and hyperlipidemia. Studies of Jang et al. (*25*) have shown that BA inhibits pancreatic lipase activity in vitro. However, our study demonstrates only a weak inhibitory effect of BA and SIB under in vivo conditions. In contrast, both BA and SIB displayed

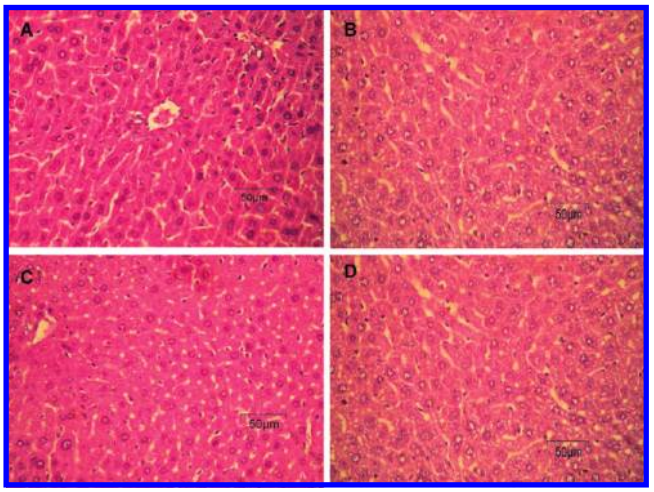

Figure 6. Histology of liver tissue of mice fed the experimental diets for 15 weeks. Representative microphotographs of mouse liver fed (**A**) normal diet, showing normal arquitexture and hepatocytes, (**B**) high-fat diet, showing diffuse cytoplasmic vacuolization indicating steatosis, (**C**) high-fat diet + betulinic acid, having comparatively less steatosis, and (**D**) high-fat diet + sibutramine, which presents moderate steatosis (H&E, 400×).

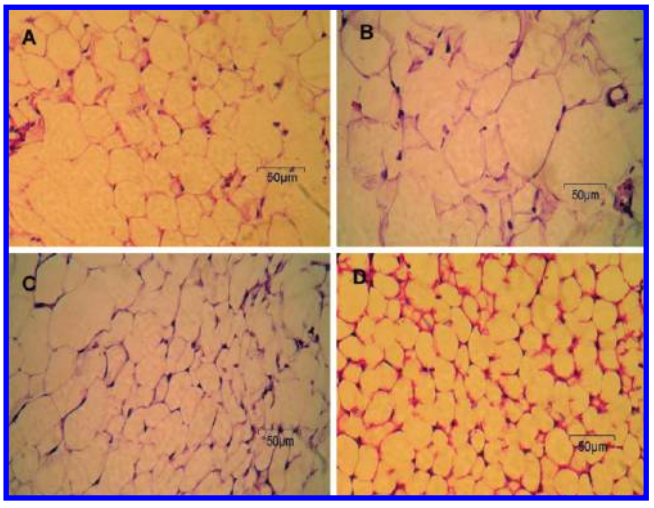

Figure 7. Histology of adipose tissue of mice fed the experimental diets for 15 weeks. Representative microphotographs of mouse epididymal fat pad (**A**) fed normal diet showing normal arquitexture of adipocytes, (**B**) fed high-fat diet showing increased size of adipocyte, (**C**) high-fat diet + betulinic acid, and (**D**) high-fat diet + sibutramine, which presents smaller-sized adipocytes comparable to normal diet (H&E, 100×).

a higher inhibition of amylase activity. The inhibition of α-amylase activity may hinder the digestion of carbohydrates, thereby accounting for prevention of body weight increase. The α-amylase inhibitory activity of pentacyclic triterpenoids such as oleanolic acid, ursolic acid, and lupeol has been previously described (*10*). Betulinic acid is a pentacyclic triterpenoid that belongs to the lupane series, and from the results obtained we assume that it impairs lipid and carbohydrate metabolism through the inhibition of lipase and amylase and thus may, in part, account for the observed antiobese and weight loss effects. In support of this was our finding with BA that showed a favorable influence on blood glucose level in the glucose tolerance test.

The diet-regulating hormones ghrelin and leptin show a major influence on energy balance (*26*), and therefore measuring their plasma levels may indicate the sensitivity of an animal to weight

gain when exposed to a HFD. Leptin, the adipocyte hormone, is a mediator of long-term regulation of energy balance, suppressing food intake and thereby accounting for weight loss. It is believed to tonically act as an afferent signal from adipose tissue to the hypothalamus, as a part of a negative feedback loop. On the other hand, ghrelin secreted from the stomach is a fast-acting brain−gut peptide with growth hormone-releasing and appetite-stimulating activities, acting as an afferent signal to the hypothalamus as a part of a positive feedback loop. In the present study, mice fed a HFD for 15 weeks showed increased body weight and fat mass, despite an affective decrease in the circulating level of the orexigenic hormone ghrelin and an increase of the anorexigenic hormone leptin, a finding consistent with earlier reports (*27*, *28*). Possibly, the ghrelin and leptin secretions are dysregulated with high-fat diets, impairing the homeostasis and eventually promoting obesity. Interestingly, BA treatment further decreased ghrelin and enhanced leptin secretions, without an apparent change in net food and water consumption, implying participation of other mechanisms in its antiobese action.

Adipose tissue is a potential source of another bioactive substance, adiponectin. Although this investigation did not analyze the levels of adiponectin, earlier studies have shown its inverse relationship with body weight, especially abdominal visceral fat accumulation. Adiponectin has an insulin-sensitizing property, and low plasma adiponectin levels are associated with insulin resistance as found in obesity and the peroxisome proliferator-activated receptor gamma (PPARλ), a nuclear factor that regulates the expression of key genes involved in lipid and glucose metabolism and adipocyte differentiation. Agonists such as rosiglitazone used widely in the treatment of diabetes mellitus seem to promote adiponectin secretion, accounting for its insulin-sensitizing property (*29*). Mice fed a HFD for 15 weeks presented hyperglycemia, dyllipidemia, and insulin resistance as evidenced by increased levels of plasma glucose, insulin, and total cholesterol and triglycerides. BA treatment ameliorated hyperglycemia and dyslipidemia but did not modify the plasma insulin. In this context, a recent study has shown that insulin stimulates adiponectin secretion in 3T3-L1 adipocytes (*30*). By judging its performance in the present work, we assume that BA possibly influences adiponectin secretion or the pancreatic β-cell secretion, which needs to be addressed in a future study.

Sibutramine, a weight control drug included in this study as a positive control, demonstrated decreased accumulation of abdominal fat and significant reductions in triglycerides and total cholesterol. The decreased body weight and fat mass observed in the BA- and SIB-treated mice are most likely due to increased β-oxidation in the liver and energy expenditure. Besides, both treatments not only decreased total cholesterol and triglycerides but also reduced the steatosis in liver and the sizes of adipocytes, indicating that they decrease lipid accumulation in the visceral adipocytes of mice fed a HFD. In conclusion, the results of this investigation show that BA, when administered daily (50 mg/L in drinking water) to HFD-fed mice, produced significant decreases in body weight gain, abdominal fat accumulation, and enhanced insulin sensitivity. It is unlikely that these effects of BA are a result of its cytotoxicity. Several studies have shown that betulinic acid is a very promising candidate for the clinical treatment of various forms of cancer, but in contrast to the potent cytotoxicity of BA against a variety of cancer types, nonmalignant cells and normal tissue seem to remain relatively resistant to BA, indicating a therapeutic window (*17*). Because it functioned as both a hypolipidemic and a hypoglycemic agent in the present investigation, BA may have a therapeutic potential in combating type 2 diabetes mellitus and obesity, modulating effectively the various enzymes and hormones involved in the absorption and metabolism of carbohydrates and lipids. Also, BA appears to be a safe and effective agent to promote weight loss, because it did not effect adversely the hepatic enzymes ALT, AST, and ALP.

ABBREVIATIONS USED

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BA, betulinic acid; DGAT, diacylglycerol acetyltransferase; HFD, high-fat diet; ND, normal diet; PPARγ, peroxisome proliferator-activated receptor gamma; TC, total cholesterol; TG, triglycerides; SIB, sibutramine.

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