Food & Function

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2013, **4**, 618

Hibiscus sabdariffa L. aqueous extract attenuates hepatic steatosis through down-regulation of PPAR-γ and SREBP-1c in diet-induced obese mice

Edgar Vinicio Villalpando-Arteaga,[a] Edgar Mendieta-Condado,†[b] Hugo Esquivel-Solís,[a] Arturo Alejandro Canales-Aguirre,[a] Francisco Javier Gálvez-Gastélum,[c] Juan Carlos Mateos-Díaz,[a] Jorge Alberto Rodríguez-González[a] and Ana Laura Márquez-Aguirre*[a]

Received 2nd October 2012
Accepted 22nd January 2013

DOI: 10.1039/c3fo30270a

www.rsc.org/foodfunction

The growing incidence of obesity is a worldwide public health problem leading to a risk factor for non-alcoholic fatty liver disease, which extends from steatosis to steatohepatitis and cirrhosis. We investigated whether the aqueous extract of *Hibiscus sabdariffa* L. (Hs) reduces body weight gain and protects the liver by improving lipid metabolism in high fat diet-induced obese C57BL/6NHsd mice. We found that oral administration of the Hs extract reduced fat tissue accumulation, diminished body weight gain and normalized the glycemic index as well as reduced dyslipidemia compared to the obese mice group that did not receive Hs treatment. In addition, Hs treatment attenuated liver steatosis, down-regulated SREBP-1c and PPAR-γ, blocked the increase of IL-1, TNF-α mRNA and lipoperoxidation and increased catalase mRNA. Our results suggest that the anti-obesity, anti-lipidemic and hepatoprotective effects of the Hs extract are related to the regulation of PPAR-γ and SREBP-1c in the liver.

1 Introduction

The overall increased incidence of obesity is rapidly becoming a serious public health problem. Obesity is a known risk factor for type 2 diabetes, dyslipidemia, metabolic syndrome, obstructive sleep apnea, hypertension, coronary heart disease, stroke, gall bladder disease, renal and hepatic diseases and osteoarthritis as well some forms of cancer (hepatocellular, breast, colorectal, endometrial and kidney).[1,2] A high-fat diet (HFD), along with reduced physical activity, induces excessive storage of triglycerides in adipocytes that leads to hypertrophy of the adipose tissue (AT). Hypertrophied AT reduces blood flow, resulting in hypoxia and secretion of pro-inflammatory adipokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6.[3,4] Low-grade chronic inflammation related to obesity causes insulin resistance and hyperglycemia, which often lead to type 2 diabetes and metabolic syndrome.[2] Obesity-related dyslipidemias are also related to heart diseases and liver pathologies.

The liver plays an essential role in regulating lipid homeostasis through low-density lipoproteins (LDL) clearance and recruitment of high-density lipoproteins (HDL). Hypertrophied AT in obese people manifests several altered metabolic properties of HDL cholesterol. These properties play a key role in obesity-related dyslipidemia as well as in the increase of free fatty acid (FFA) flux to the liver derived from lipolysis. The continuous FFA liver influx can result in liver steatosis. This is normally inhibited by insulin, but is over-stimulated in insulin-resistant states and associated with hypoxia of the adipose tissue. Non-alcoholic fatty liver disease (NAFLD) is regarded as a hepatic manifestation of metabolic syndrome.[5] NAFLD represents a broad spectrum of liver damage ranging from simple fatty infiltration of the liver parenchyma (steatosis), through the more severe fat accumulation and inflammation, to nonalcoholic steatohepatitis (NASH) and cryptogenic cirrhosis.[2,6] The widely accepted "two-hit" hypothesis for NAFLD progression sets the hepatic steatosis as the "first-hit" followed by the "second-hit" commonly associated with oxidative stress and inflammation.[2,7] A recent hypothesis of NAFLD pathogenesis includes the combined effects of (1) direct hepatocyte lipotoxicity, (2) hepatocellular oxidative stress secondary to free radicals produced during FFA oxidation, (3) endotoxin/TLR4 induced Kupffer cell (liver macrophage) cytokine release, (4) cytokine release (a proinflammatory profile of IL-6 and TNF-α) and (5) endoplasmic reticulum stress.[8] In both hypotheses, liver

[a]*Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ), Av. Normalistas 800, Guadalajara, Jalisco, 44270, Mexico. E-mail: amarquez@ciatej.net.mx; Tel: +55 33-3345-5200*

[b]*Departamento de Biología Celular y del Desarrollo, Instituto de Fisiología Celular Universidad Nacional Autónoma de Mexico (UNAM), Circuito Exterior S/N, Coyoacán, 04520, Mexico City, Mexico*

[c]*Unidad de Investigación Cardiovascular, Departamento de Fisiología, CUCS, Universidad de Guadalajara (UdeG), Sierra Mojada 950, Guadalajara, Jalisco, 44340, Mexico*

† Current address: Posgrado en Ciencias Genómicas, Universidad Autónoma de la Ciudad de México (UACM), Av. San Lorenzo 290, Ciudad de México, 03100, México.

steatosis is marked by the beginning of more severe liver pathologies like hepatitis, fibrosis, cirrhosis or hepatocarcinoma. Therefore, novel approaches to prevent obesity-related liver steatosis are urgently needed because the treatment for NAFLD is yet to be defined. Currently, research studies on effective therapeutic strategies for treating patients with NAFLD/NASH are underway and multi-modal approaches are being proposed as the standard of care.[9,10]

Mitochondrial dysfunction, increased reactive oxygen species, and the imbalance of the physiologic ratio between antioxidant and oxidant systems lead the progression of fatty liver disease to NASH. Therefore, natural and synthetic antioxidant agents have been tested for their beneficial roles in NASH patients as well as in animal models. Antioxidants have also shown a positive impact in liver homeostasis and attenuate liver pathology in obesity. These include vitamin E, glutathione precursors, and polyphenols.[11–15] *Hibiscus sabdariffa* L., a tropical herb rich in polyphenols, has proven antioxidant properties and has been effective in lowering lipidemia, hypercholesterolemia and triglyceridemia as well as in inducing weight loss in animal models of obesity and in obese patients.[16–19] Despite its protective effect against oxidative stress related to hepatic steatosis,[20–23] the role of *Hibiscus sabdariffa* L. has not been yet analyzed in obesity-induced liver steatosis. The objective of this study is to determine whether administration of the aqueous extract of *Hibiscus sabdariffa* L. (Hs) provides protection against hepatic steatosis in the mouse model of obesity induced by a high fat diet. We also evaluated the beneficial value of Hs on weight loss and molecular mechanisms related to liver homeostasis of lipid metabolism.

2 Materials and methods

2.1 Collection of *Hibiscus sabdariffa* L. (Hs) and obtaining the aqueous extract

We used a native variety of *Hibiscus sabdariffa* L. endemic to Xalisco, Nayarit, Mexico, which was initially selected and cultured due to its darker color. Fresh calyces were harvested during November and December 2010 (tropical winter; wet weather with a median temperature of 15.4 °C and 75% relative humidity). The material was collected 155 days after planting, then dried in an oven (Lumistell, model HTP-42) at 60 °C for 24 h. The dried calyces were frozen with liquid nitrogen and ground with a mortar until a fine powder was obtained. The extraction was carried out at ambient temperature by dissolving 10 g of powder in 100 mL of drinking water on an orbital shaker for 3 h at 250 rpm, then filtered through gauze and concentrated ten times in a rotovap. The extract was always protected from light.

2.2 Partial characterization of the aqueous extract of *Hibiscus sabdariffa*: HPLC analysis, quantification of total anthocyanins and antioxidant capacity assessment

The chemical composition of *H. sabdariffa* has been known for a long time. However, anthocyanins such as delphinidin-3-sambubioside and cyanidin-3-sambubioside have been previously detected as the main components in the aqueous extract of *H. sabdariffa*.[24,25] In order to partially characterize our aqueous extract of Hs, we performed an HPLC analysis. All chemicals were of analytical HPLC reagent grade. The aqueous extract of *Hibiscus sabdariffa* was freshly prepared and pre-filtered using Filters Millex type HN (Millipore, Alphaville-Barueri, Sao Paulo, BR) (0.45 μm, 13 mm) and directly injected into a Varian ProStar 335 (Varian, Walnut Creek, CA, USA) HPLC system and visualized by a diode array detector at 360 and 520 nm. The compounds of the Hs were separated by a Zorbax SB-C18 column (250 mm, 4.6 mm, 5.0 μm, Agilent Technologies, Palo Alto, CA, USA) at 30 °C and a flow rate of 1.0 mL min^{-1}. The injection volume was 20 μL. Mobile phases used for elution were: water with 1% formic acid (Sigma-Aldrich St. Louis, MO, USA) and acetonitrile (Acn) (M TEDIA® Company, INC. Fairfield, OH, USA). The linear gradient used for analysis and identification of anthocyanins was as follows: 0 min, 0% (Acn); 13 min, 20% (Acn); 30 min, 40% (Acn); 40 min, 20% (Acn); 50 min, isocratic of 0% (Acn). The data acquisition system included Galaxie chromatographic workstation v.1.9.3.2 software from Varian. The identification and peak assignment of anthocyanins was based on inclusion of chlorogenic acid (Sigma-Aldrich, St. Louis, MO, USA) as the internal standard. The chlorogenic acid was injected alone and also with the Hs. The retention times were similar in both cases (with and without the extract). Compounds of interest were detected between 23 and 33 minutes at 360 and 520 nm.

The total content of monomeric anthocyanins in this extract (Hs) was determined by the pH-differential method.[26] This method is based on the structural change of the anthocyanin chromophore between pH 1.0 and 4.5. In brief, aliquots of 50 μL of extract were taken and mixed with 450 μL of buffer pH 1 and 4.5, respectively. In both solutions absorbance at two wavelengths, 510 and 700 nm, was measured in a UV-Vis xMark Microplate of Bio-Rad spectrophotometer, using $A = (A_{510} - A_{700})_{pH\ 1} - (A_{510} - A_{700})_{pH\ 4.5}$. The content of pigment was calculated as monomeric anthocyanins (mg L^{-1}) = $(A \times M_W \times DF)/(\varepsilon \times \ell)$, where M_W is the molecular weight of cianidine-3-glucoside (g mol^{-1}), ε is the molar absorptivity (L mol^{-1} cm^{-1}), ℓ is the light path length in the cell (cm) and DF is the dilution factor. Finally, to verify the viability of the aqueous extract, antioxidant capacity of Hs was measured by the ABTS method.[27] The synthetic antioxidant Trolox was used as a reference at a concentration of 800 μM.

2.3 Diet-induced obese mice

Male C57BL/6NHsd mice, weighing 20–25 g, were purchased from Harlan Laboratories (Mexico City). A group of five mice were fed with a standard diet 2018S (SD) consisting of 24% protein, 58% carbohydrates and 18% fat (healthy control group) while two groups of 10 mice each were fed with a high fat diet TD.06414 (HFD) consisting of 18% protein, 22% carbohydrates and 60% fat. Diets were obtained from Teckland Research Diets, Harlan Laboratories. One of the high-fat diet groups was supplemented with Hs (33 mg of total anthocyanins per kilogram body weight) three times a week for 8 weeks by orogastric-way using a metallic cannula. Body weight, water and food intake were monitored

weekly. Animals were housed in a temperature-controlled room (20–25 °C) with a relative humidity of 55% and a photoperiod cycle of 12 h light × 12 h dark. All mice were fasted overnight before being euthanized with a lethal injection of sodium pentobarbital (200 mg kg^{-1} BW). Blood samples were collected from each animal by cardiac puncture immediately after death, and plasma or serum was separated and stored at −20 °C for subsequent measurements of glucose in plasma and cholesterol (TC), triglycerides (TG), high-density lipoproteins (HDL), low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) in serum. Glucose was quantified with the Onetouch-Ultra® system using reactive strips (Johnson & Johnson Medical). Serum parameters were measured on a Sincron-7 analyzer. Livers were removed and portions of liver were snap frozen in liquid nitrogen and stored frozen for subsequent analysis by lipoperoxidation assay, Western blotting and by reverse transcription real time-polymerase chain reaction (RT-PCR). The right lobe of the liver was fixed in 4% buffered paraformaldehyde for histological studies. Animal care and handling adhered to national and institutional regulatory rules.[28]

2.4 Valoration of liver histological changes

Liver histological changes were assessed over 5 μm liver sections using conventional staining techniques to analyze hepatocellular lipid accumulation (steatosis) with hematoxylin and eosin (H & E) collagen deposition with Masson's trichrome and glycogen accumulation with Periodic-Acid Schiff (PAS). Biopsies were classified into three grades depending on fat accumulation, using the proposed classification,[29] whereby a sample is classified as grade 1 when fat vacuoles (microvesicular) are seen in 5–33% of hepatocytes, grade 2 when 34–66% of hepatocytes are affected by fat vacuoles, and grade 3 when fat vacuoles are found in >66% of hepatocytes. The scale ranged from 0 to 3, where 0, 1, 2 and 3 signified absent, mild, moderate and severe, respectively.

2.5 Measurement of oxidative stress in liver

Liver lipoperoxidation, a consequence of oxidative stress, was determined by measuring thiobarbituric acid reactive substances (TBARs) in the liver. The amount of aldehydic products generated by lipoperoxidation was quantified by the thiobarbituric acid reaction, using 500 mg of total homogenate protein, as described.[30] Results are referred to TBARs and the concentrations were expressed as equivalents of malondialdehyde (MDA) that was used as the standard.

2.6 Measurement of antioxidant enzymes CAT, SOD1, GPX1, cytokines IL-1, IL-6, IL-10, TNF-α and transcription factors SREBP-1c and PPAR-γ expression in the liver

Total RNA was isolated from liver tissues using a TRIzol reagent (Invitrogen, Life Technologies, CA, USA) and retrotranscription was performed using 2 μg of total RNA and M-MLV reverse transcriptase (Invitrogen, Life Technologies, CA, USA). Two microliters of the cDNA reaction mixture were subjected to real-time PCR under the following conditions: pre-incubation 10 min/95 °C, and 45 cycles of 10 s/95 °C, 10 s/60 °C and 10 s/72 °C using TaqMan probes (Applied Biosystems, Life Technologies, CA, USA) to amplify catalase (CAT), superoxide dismutase1 (SOD1), glutathione peroxidase 1 (GPX1), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor (TNF-α), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, low-density lipoprotein receptor (LDL-R), Sterol Regulatory Element-Binding Protein-1c (SREBP-1c), Proliferator Peroxisome Activated Receptor-γ (PPAR-γ) and ribosomal 18 S housekeeping gene as the normalization control. Relative quantification of gene expression was performed with the comparative CT method (ΔΔCT method). The expression profile for each gene was reported as fold change $= 2^{-\Delta\Delta CT}$ plus standard deviation calculated as $\sigma = \sqrt{(\sigma_{(18S\ gene)}{}^2 + \sigma_{(Target\ gene)}{}^2)}$, according to the Applied Biosystem User Bulletin #2.

2.7 Statistical analysis

The results are expressed as mean ± SD. Assuming a non-normal distribution (nonparametric data), comparisons between groups were done using the Kruskal–Wallis test for one time analysis and Friedman test for paired longitudinal data followed by Dunn's multiple comparison test in both cases.

Differences were considered statistically significant at $p \leq 0.05$.

3 Results

3.1 Analysis of *Hibiscus sabdariffa* aqueous extract

The HPLC analysis showed typical profiles for *Hibiscus sabdariffa*[24,25] (Fig. 1). After identifying anthocyanins in the extract (Hs) by HPLC, we quantified the content of monomeric anthocyanins by the pH-differential method described before. We obtained 2.31 mg mL^{-1} of total anthocyanins in the extract and the Trolox equivalent antioxidant capacity (TEAC) was 2028 units, measured by the ABTS method.[27]

3.2 *Hibiscus sabdariffa* prevents body weight gain, hyperglycemia, white fat tissue accumulation and improves lipid metabolism in obese mice

As expected, during 8 weeks of study, diet-induced obese mice fed with a HFD gained more weight than animals fed with SD. Food intake in each group of mice was approximately the same, having no significant differences in food consumption along the study. However, water consumption increased ($p < 0.05$) in the obese mice group supplemented with the Hs extract (HFD-Hs) (Fig. 2A).

A difference of ~8 grams (62% overweight, $p < 0.05$) was found, which directly correlated with visceral white fat accumulation ($r^2 = 0.92$, Fig. 2B). Moreover, such obese mice developed moderate hyperglycemia and hypertriglyceridemia while significantly increasing the levels of LDL-cholesterol ($p < 0.05$) and moderately increasing levels of VLDL-c (Fig. 2C).

Also, dietary supplementation of mice with *Hibiscus sabdariffa* extract (HFD-Hs) significantly decreased the body weight gain ranking intermediate values among non-obese mice (SD) and obese mice (HFD) (Fig. 2B). It is well known that the main feature of obesity is the accumulation of fat in the adipose tissue and as a consequence, the imbalance of lipid metabolism

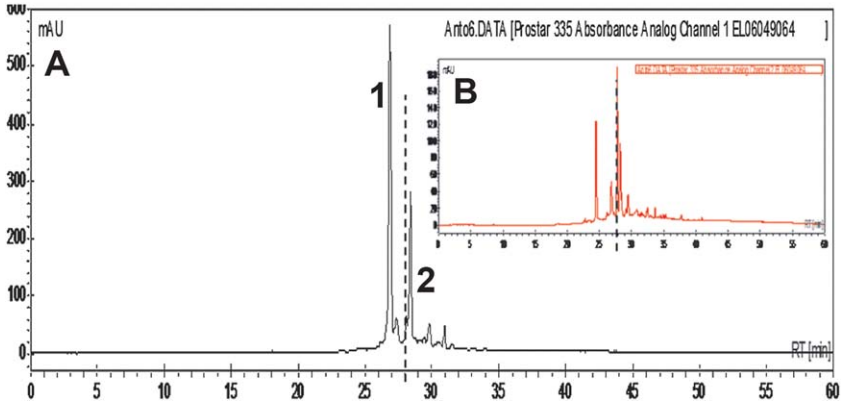

Fig. 1 HPLC analysis of *Hibiscus sabdariffa* aqueous extract. (A) *Hibiscus sabdariffa* aqueous extract was co-injected with a known concentration of chlorogenic acid as an internal standard at 360 nm. (B) The characteristic peaks 1 and 2, at a retention time of 27 and 28.5 minutes, correspond to previously reported delphinidin-3-sambubioside and cyanidine-3-sambubioside, respectively, at 520 nm. The dotted line in both chromatograms shows the retention time of the chlorogenic acid internal standard (28 minutes).

due to excessive intake of dietary fat. In our study, treatment with Hs had a significant benefic impact in this regard. The amount of visceral fat accumulated was significantly lower than that of obese non-treated mice ($p < 0.05$) (Fig. 2B). Serum levels of glucose and triglycerides after overnight fasting were also below ($p < 0.05$) those found in obese mice (Fig. 2C). Moreover, whereas total cholesterol remained unchanged, LDL-c was remarkably lower than obese mice and even lower than in normal mice ($p < 0.01$) with increase of HDL-c ($p < 0.05$), while VLDL-c remained normal (Fig. 2C).

3.3 *Hibiscus sabdariffa* L. extract attenuated steatosis progression and PPAR-γ and SREBP-1c expression in the liver of obese mice

Lipid accumulation in hepatocytes (steatosis) due to FFA flux in obesity is the precursor for development of more severe pathologies such as NAFLD or NASH.[31] In this study, we found that supplementation of the Hs extract in obese mice greatly diminished the accumulation of fat in the cytoplasm of hepatocytes. This attenuated steatosis by interrupting the progression of severe forms and reducing the moderate ones. This took place while increasing the appearance of mild forms and even blocking steatosis in about 40% of cases in comparison with the obese non-treated group (steatosis in 100%) (Fig. 3A and B). Moreover, livers of obese mice presented a high concentration of collagen surrounding the hepatocytes (intercellular) and were filled with glycogen droplets. These events were almost absent in livers of obese mice treated with the Hs extract (Fig. 3A). On the other hand, the transcription factors PPAR-γ and SREBP-1c, together with LDL-R and HMG-CoA, are master regulators of lipid homeostasis and are present in the liver. We found that the mRNA expression of PPAR-γ, SREBP-1c, LDL-R and HMG-CoA did not significantly increase in obese mice compared to non-obese mice. Interestingly, gene expression of both transcription factors PPAR-γ and SREBP-1c was significantly reduced in obese mice supplemented with Hs compared to obese mice ($p < 0.05$) (Fig. 3C).

3.4 *Hibiscus sabdariffa* L. has protective activities against oxidative stress and inflammatory gene expression in the liver of obese mice

Antioxidant properties of the Hs extract are attributed mainly to the peculiar combination of polyphenols that function as potent free radical scavengers, whereas oxidative stress and inflammatory signals in the liver are representative of liver injury induced by obesity. Thus, the effect of Hs extract on hepatocyte lipid peroxidation and on the liver oxidative defense system of obese mice was studied simultaneously to determine the effect on the inflammatory profile. We observed a doubling effect on lipoperoxidation of hepatocytes and on glutathione peroxidase (GPX1) gene expression in obese mice livers ($p < 0.05$). At the same time, we observed the induction of pro-inflammatory cytokine genes IL-1 and TNF-α in obese mice *versus* non-obese mice ($p < 0.05$). Conversely, in obese mice, the Hs extract exerts a protective effect by means of reducing lipid peroxidation, regardless of the non-significant difference, and inducing the CAT gene overexpression ($p < 0.05$), together with the reduction of IL-1 and TNF-α gene expression ($p < 0.05$). On the other hand, IL-6 and IL-10 mRNA levels were similar among the groups (Fig. 4).

4 Discussion

Considerable attention has been focused on food constituents that may be beneficial to the prevention and treatment of obesity and obesity-related pathologies. The chemical composition of *H. sabdariffa* has revealed the presence of flavonoids (quercetin, luteolin and its glycoside); chlorogenic acid, gossypetin, hibiscetin, phenols and some phenolic acids have also been reported. However, anthocyanins such as delphinidin-3-sambubioside and cyanidin-3-sambubioside have been detected as the main components in the aqueous extract of *H. sabdariffa*.[32,33] These molecules together or alone have been shown to possess powerful anti-obesity, antioxidant and anti-inflammatory activities and some anti-carcinogenic effects, and they also may help prevent cardiovascular disease and control

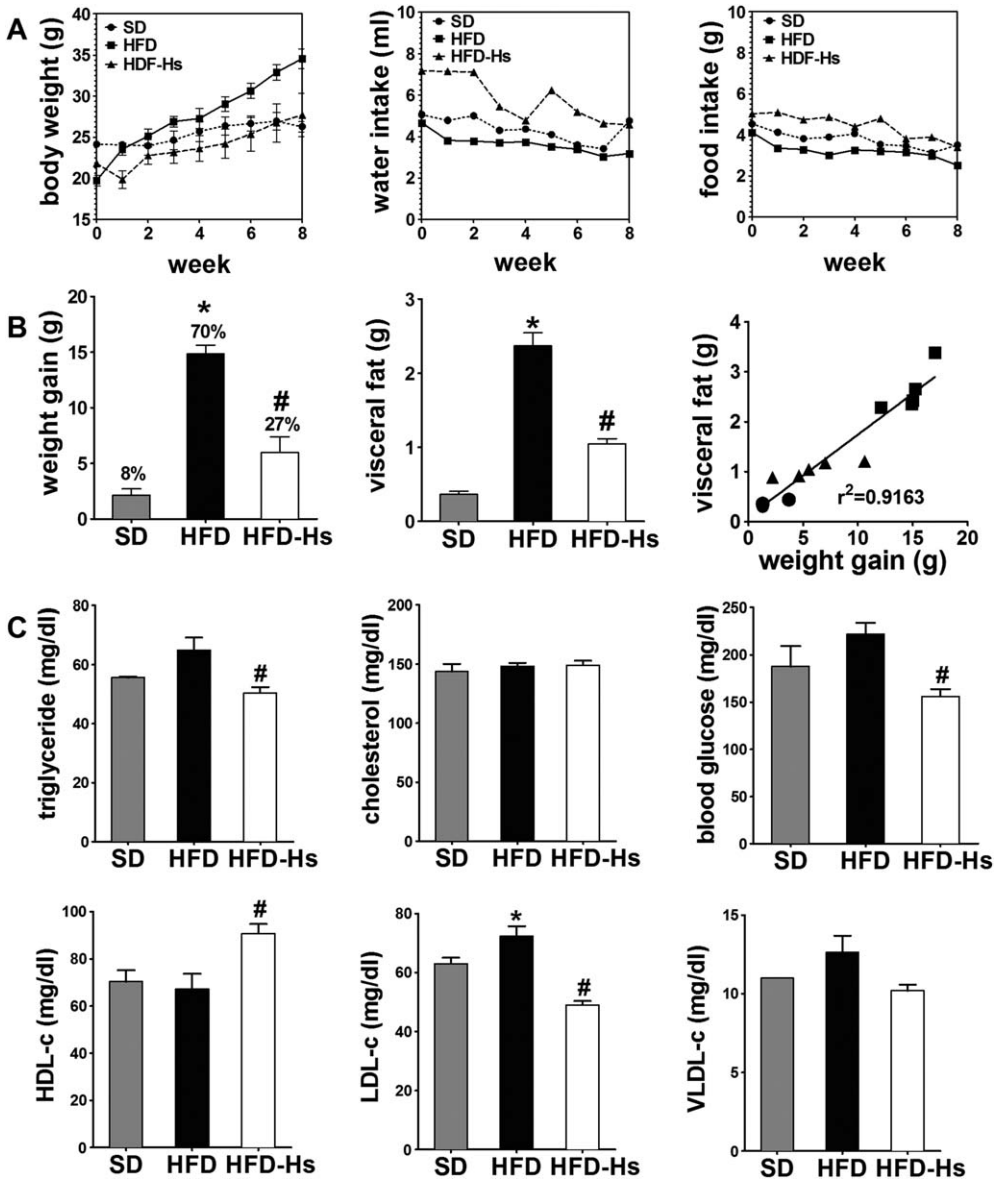

Fig. 2 *Hibiscus sabdariffa* reduces body weight gain, hyperglycemia, and white fat tissue accumulation and improves lipid metabolism in obese mice. (A) Obese mice supplemented with *Hibiscus sabdariffa* extract (HFD-Hs) significantly decreased their body weight and showed a significant increase ($p < 0.05$) in water consumption without change in the food intake. (B) Hs supplementation also significantly decreased the body weight gain and the amount of visceral fat accumulated ($p < 0.05$). (C) Serum levels of glucose and triglycerides after overnight fasting were also below those of obese mice due to the treatment of Hs ($p < 0.05$). LDL-c was remarkably lower compared to obese mice and even normal mice ($p < 0.01$) with increase of HDL-c ($p < 0.05$).

diabetes.[34,35] Previous research indicated that cyanidin-3-O-β-glucoside (C3G) significantly suppresses the development of HFD-induced obesity in mice[36] and that purified dietary C3G reduces blood glucose levels and improves insulin sensitivity in type 2 diabetic mice,[37] while black soybean anthocyanins improve systemic and hepatic lipid metabolism in HFD-induced obese mice.[38,39]

Herein, we show the effect of Hs aqueous extract at a dose of 33 mg of total anthocyanins per kg of body weight, in regulating HDL-cholesterol and glucose, triglyceride, LDL-c and VLDL-c, blocking their elevation in the blood of mice fed with a HFD compared to the obese group that did not received Hs. We found an increase in water consumption by animals that were supplemented with the extract of *Hibiscus sabdariffa*. It has been proposed that compounds present in *Hibiscus sabdariffa*, such as quercetin, effect the vascular endothelium, causing oxide nitric release and increasing renal vasorelaxation by increasing kidney filtration. Other compounds, such as anthocyanins and chlorogenic acid, are potentially responsible for the modulation of aldosterone, which results in a diuretic effect.[40,41] We also showed that Hs suppresses the body weight gain related to inhibition of fat accumulation. A previous study also showed body weight gain reduction at the same dose of Hs anthocyanins as our extract.[16] Even at lower doses (1.42 mg per kg of body weight), anthocyanins reduce the triglyceride concentration and increase HDL-c in humans with metabolic

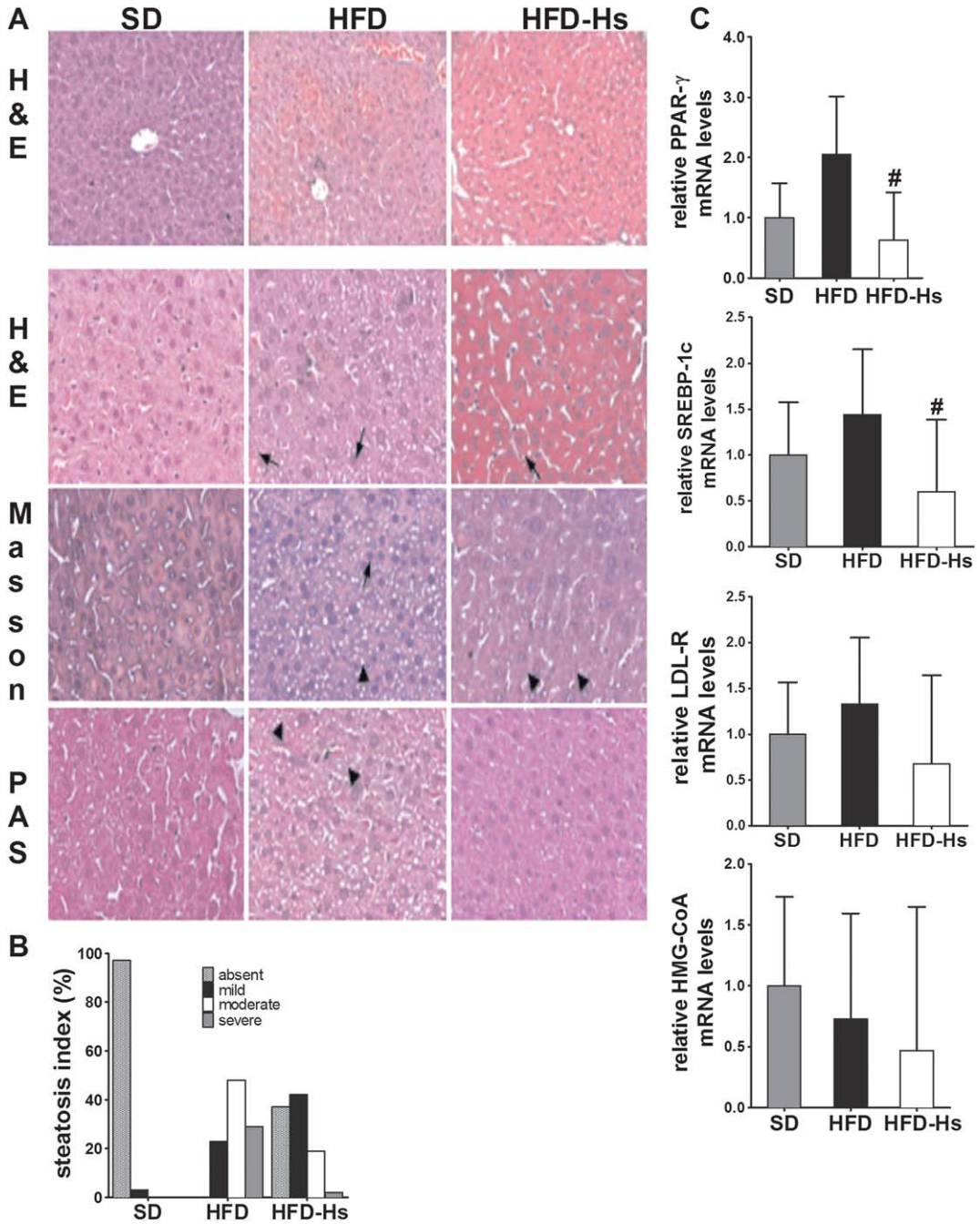

Fig. 3 *Hibiscus sabdariffa* L. extract attenuated steatosis progression and PPAR-γ and SREBP-1c expression in the liver of obese mice. The photomicrographs show the changes due to the effect of diet and treatment (H&E 20X and 40X, Masson's trichrome and PAS 40X). (A) Liver of obese mice presented abundant collagen surrounding the hepatocytes (intercellular) while there were plenty of glycogen droplets inside them, events that were almost absent in livers of obese mice treated with Hs extract. Also, the supplementation of the Hs extract in obese mice greatly diminished the accumulation of fat in the cytoplasm of hepatocytes, resulting in a high attenuation of steatosis. (B) Hs increased the appearance of mild forms and even blocked steatosis in about 40% of cases compared to 100% steatosis in the obese non-treated group. (C) Gene expression of both transcription factors PPAR-γ and SREBP-1c was significantly reduced in obese mice due to the supplementation with the Hs extract ($p < 0.05$).

syndrome.[18] Although our current data suggest that anthocyanins could be the major contributor of the Hs effects seen in the HFD-induced obesity in mice, the current study does not demonstrate this definitively since we used a crude extract and therefore other compounds in Hs could also be participating. Studies focusing on the particular combination of polyphenols in *H. sabdariffa* may be necessary because their interaction and absorption in the gastrointestinal tract may have particular determinants.[42] Moreover, although Hs has antioxidant properties,[43] the inhibition of lipid peroxidation induced by the Hs extract in our study might be attributed to the cellular antioxidant system evidenced by the over-expression of catalase, preventing the oxidative damage in the liver.

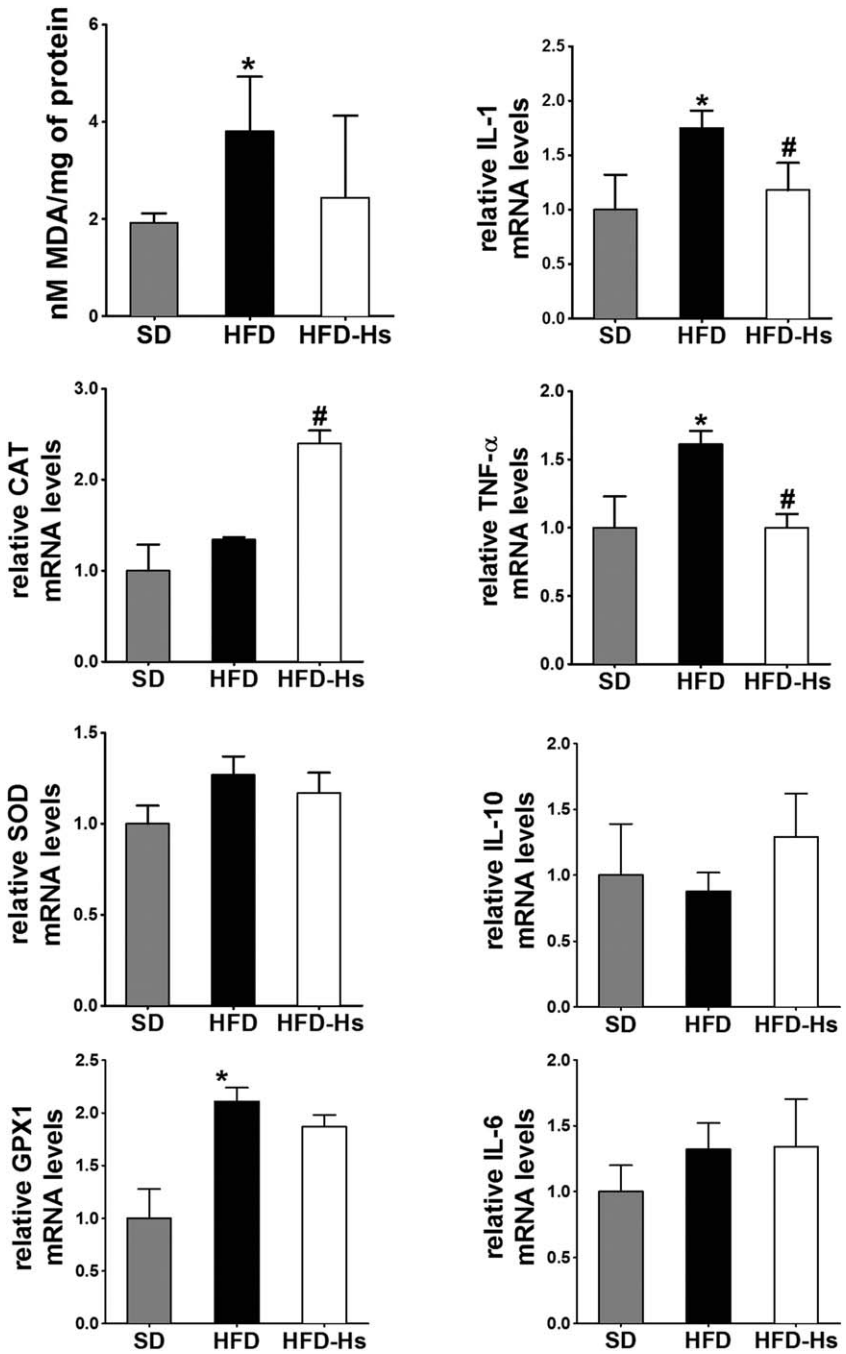

Fig. 4 *Hibiscus sabdariffa* L. has protective activities against oxidative stress and inflammatory gene expression in the liver of obese mice. We found an effect on lipoperoxidation of hepatocytes and on GPX1 gene expression in the liver of obese mice, parallel to the induction of pro-inflammatory cytokine genes IL-1 and TNF-α *versus* non-obese mice. In contrast, in obese mice the Hs extract exerts a protective effect inducing the CAT gene overexpression ($p < 0.05$), together with the reduction of IL-1 and TNF-α gene expression ($p < 0.05$). Ribosomal 18 S housekeeping gene was used as the normalization control. Relative quantitation of gene expression was performed with the comparative CT method and the expression was reported as fold change $= 2^{-\Delta\Delta CT}$ plus standard deviation calculated as $\sigma = \sqrt{(\sigma_{(18S\ gene)})^2 + \sigma_{(target\ gene)}^2)}$.

Accumulating evidence indicates that SREBP-1c and PPAR-γ are critical regulators of hepatic lipid metabolism. These transcription factors stimulate expression of several enzymes involved in liver fatty-acid synthesis and glucose transport, gluconeogenesis, and lipolysis.[44,45] Recent studies of obese patients have reported that increased SREBP-1c and PPAR-γ expression is strongly associated with fatty liver disease.[46] As we have shown, due to repression of SREBP-1c and PPAR-γ gene expression in mice fed with HFD and Hs, we propose that attenuation of liver steatosis seen in these mice is related to this effect, and could be also linked to the other signals of liver protection elicited by Hs, including the reduction of lipid peroxidation, and pro-inflammatory cytokine expression. Thus, fatty mass accumulation and body weight gain are inhibited. It

has been reported that adenosine monophosphate-activated protein kinase (AMPK) inactivates SREBP-1c and PPAR-γ transcription and inhibits hepatic steatosis in HFD–induced animal models.[14,47,48] Previous studies have shown that anthocyanins abundant in food significantly activate hepatic AMPK and reduce the SREBP-1c protein expression, agreeing with the hypolipidemic effect and the attenuation of steatosis seen in mouse liver and HepG2 hepatocytes.[49,50] In addition, Hs has shown potential antidiabetic effects.[51] All these data suggest that the effects of Hs over adipogenesis are mediated by SREBP-1c and PPAR-γ regulation through AMPK activation. It is supported by the fact that the *Hibiscus sabdariffa* extract inhibits the adipocyte differentiation through the AMPK-PPAR-γ pathway[52] and inhibits lipogenesis and promotes hepatic lipid clearance *via* AMPK-SREBP-1.[22] Since AMPK is considered a potential therapeutic target in the prevention and treatment of type 2 diabetes and obesity,[53,54] as supported by the basic drug for the treatment of type 2 diabetes (metformin) that activates AMPK,[55] studies evaluating the antidiabetic, antiobesic and anti-NALFD effects of Hs through AMPK activation are ongoing in our lab.

5 Conclusions

The present study has clearly shown that the Hs extract possesses antisteatogenic effects in the liver besides the antilipidemic and antiobesic effects in the HFD-induced obese mouse model. More importantly, we demonstrated for the first time that the Hs extract could regulate the lipid homeostasis through SREBP-1c and PPAR-γ inhibition, counteracting liver damage in an agonist-dependent manner.

Conflict of interest

The authors have declared no conflict of interest.

Abbreviations

Hs	*Hibiscus sabdariffa*
HFD	High-fat diet
SD	Standard-diet
TG	Triglycerides
VLDL-c	Very low-density lipoprotein cholesterol
LDL-c	Low-density lipoprotein cholesterol
HDL-c	High-density lipoprotein-cholesterol
SREBP-1c	Sterol regulatory element-binding protein-1
PPAR-γ	Proliferator peroxisome activated receptor-γ
CAT	Catalase
SOD	Superoxide dismutase
GPX	Glutathione peroxidase
ROS	Reactive oxygen species
NAFLD	Nonalcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
TBARS	Thiobarbituric acid reactive substances
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A reductase
AMPK	Adenosine monophosphate-activated protein kinase

Acknowledgements

This work was financially supported by Strategic Alliance for Process Innovation and Biotech Products Derived from Jamaica (87069). We thank Dr Ericka Flores, Dr Melchor Arellano Plaza and M.Sc. Maria Luisa Machuca for their assistance in this study. We also thank Kirk Allen and Matt Pickler from Peace Corps for reviewing the English grammar of this document.

Notes and references

1 U. Hoffler, K. Hobbie, R. Wilson, R. Bai, A. Rahman, D. Malarkey, G. Travlos and B. I. Ghanayem, *Endocrine*, 2009, **36**, 311.
2 G. Tarantino and A. Caputi, *World J. Gastroenterol.*, 2011, **17**, 3785.
3 G. H. Goossens, *Physiol. Behav.*, 2008, **94**, 206.
4 R. W. O'Rourke, A. E. White, M. D. Metcalf, A. S. Olivas, P. Mitra, W. G. Larison, E. C. Cheang, O. Varlamov, C. L. Corless, C. T. Roberts and D. L. Marks, *Diabetologia*, 2011, **54**, 1480.
5 E. J. Park, J. H. Lee, G. Y. Yu, G. He, S. R. Ali, R. G. Holzer, C. H. Osterreicher, H. Takahashi and M. Karin, *Cell*, 2010, **140**, 197.
6 H. Wang and D. Q. Peng, *Lipids Health Dis.*, 2011, **12**, 10.
7 M. Trauner, M. Arrease and M. Wangner, *Biochim. Biophys. Acta*, 2010, **1801**, 299.
8 Q. M. Anstee, A. K. Daly and C. P. Day, *Biochim. Biophys. Acta*, 2011, **1812**, 1557.
9 A. Alisi, A. E. Feldstein, A. Villani, M. Raponi and V. Nobili, *Nat. Rev. Gastroenterol. Hepatol.*, 2012, **9**, 152.
10 D. M. Torres, C. D. Williams and S. A. Harrison, *Clin. Gastroenterol. Hepatol.*, 2012, **8**, 837.
11 N. P. Chalasani, A. J. Sanyal, K. V. Kowdley, P. R. Robuck, J. Hoofnagle, D. E. Kleiner, A. Unalp, J. Tonascia and J. NASH CRN Research Group, *Contemp. Clin. Trials*, 2009, **30**, 88.
12 S. A. Harrison, S. Torgerson, P. Hayashi, J. Ward and S. Schenker, *Am. J. Gastroenterol.*, 2003, **98**, 2485.
13 J. E. Lavine, J. B. Schwimmer, M. L. Van Natta, J. P. Molleston, K. F. Murray, P. Rosenthal, S. H. Abrams, A. O. Scheimann, A. J. Sanyal, N. Chalasani, J. Tonascia, A. Ünalp, J. M. Clark, E. M. Brunt, D. E. Kleiner, J. H. Hoofnagle and P. R. Robuck, *J. Am. Med. Assoc.*, 2011, **305**, 1659.
14 Y. Li, S. Xu, M. M. Mihaylova, B. Zheng, X. Hou, B. Jiang, O. Park, Z. Luo, E. Lefai, J. Y. Shyy, B. Gao, M. Wierzbicki, T. J. Verbeuren, R. J. Shaw, R. A. Cohen and M. Zang, *Cell Metab.*, 2011, **13**, 376.

15 I. Sinha-Hikim, A. P. Sinha-Hikim, R. Shen, H. Kim, S. W. French, N. D. Vaziri, A. Crum, T. B. Rajavashisth and K. C. Norris, *Exp. Mol. Pathol.*, 2011, **91**(suppl. 1), 419.
16 F. J. Alarcón-Aguilar, A. Zamilpa, M. D. Perez-Garcia, J. C. Almanza-Perez, E. Romero-Nuñez, E. A. Campos-Sepulveda, L. I. Vazquez-Carrillo and R. Roman-Ramos, *J. Ethnopharmacol.*, 2007, **114**, 66.
17 O. Carvajal-Zarrabal, S. M. Waliszewski, D. M. Barradas-Dermitz, Z. Orta-Flores, P. M. Hayward-Jones, C. Nolasco-Hipólito, O. Angulo-Guerrero, R. Sánchez-Ricaño, R. M. Infanzón and P. R. Trujillo, *Plant Foods Hum. Nutr.*, 2005, **60**, 153.
18 C. M. Gurrola-Díaz, P. M. García-López, S. Sánchez-Enríquez, R. Troyo-Sanromán, I. Andrade-González and J. F. Gómez-Leyva, *Phytomedicine*, 2010, **17**, 500.
19 E. S. Kao, J. D. Hsu, C. J. Wang, S. H. Yang, S. Y. Cheng and H. J. Lee, *Biosci., Biotechnol., Biochem.*, 2009, **73**, 385.
20 C. H. Lee, C. Y. Kuo, C. J. Wang, C. P. Wang, Y. R. Lee, C. N. Hung and H. J. Lee, *Biosci., Biotechnol., Biochem.*, 2012, **76**, 646.
21 L. C. Liu, C. J. Wang, C. C. Lee, S. C. Su, H. L. Chen, J. D. Hsu and H. J. Lee, *J. Sci. Food Agric.*, 2010, **90**, 329.
22 M. Y. Yang, C. H. Peng, K. C. Chan, Y. S. Yang, C. N. Huang and C. J. Wang, *J. Agric. Food Chem.*, 2010, **58**, 850.
23 G. Yin, L. Cao, P. Xu, G. Jeney and M. Nakao, In Vitro *Cell. Dev. Biol.: Anim.*, 2011, **47**, 10.
24 L. A. Galicia-Flores, Y. Salinas-Moreno, B. M. Espinoza-García and C. Sánchez-Feria, *Revista Chapingo Serie Horticultura*, 2008, **14**(suppl 2), 121–129.
25 I. C. Rodríguez-Medina, R. Beltrán-Debón, V. Micol-Molina, C. Alonso-Villaverde, J. Joven, J. A. Menéndez, A. Segura-Carretero and A. Fernández-Gutiérrez, *J. Sep. Sci.*, 2009, **32**, 3441.
26 M. M. Giusti and R. E. Wrolstad, in *Proanthocyanidins: Extraction, Purification, and Determination of Subunit Composition by HPLC*, Jonh Wiley & Sons, Inc, 2001, I1.1.
27 R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, *Free Radical Biol. Med.*, 1999, **26**, 1231.
28 NORMA Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio.
29 D. E. Kleiner, E. M. Brunt, M. Van Natta, C. Behling, M. J. Contos, O. W. Cummings, L. D. Ferrell, Y. C. Liu, M. S. Torbenson, A. Unalp-Arida, M. Yeh, A. J. McCullough and A. J. Sanyal, *Hepatology*, 2005, **41**, 1313.
30 I. Aguilar-Delfín, F. López-Barrera and R. Hernández-Muñoz, *Hepatology*, 1996, **24**, 657.
31 Y. Takahashi, Y. Soejima and T. Fukusato, *World J. Gastroenterol.*, 2012, **18**, 2300.
32 N. Gaet, *Hibiscus sabdariffa* L. in *Medicinal Plants of the World*, ed. A. Iva, Human Press, New York, 1999, pp. 165–170.
33 A. Segura-Carretero, M. A. Puertas-Mejía, S. Cortacero-Ramírez, R. Beltrán, C. Alonso-Villaverde, J. Joven, G. Dinelli and A. Fernández-Gutiérrez, *Electrophoresis*, 2008, **29**, 2852.
34 J. He and M. M. Giusti, *Annu. Rev. Food Sci. Technol.*, 2010, **1**, 163.
35 L. S. Wang and G. D. Stoner, *Cancer Lett.*, 2008, **269**, 281.
36 T. Tsuda, *J. Agric. Food Chem.*, 2008, **56**, 642.
37 R. Sasaki, N. Nishimura, H. Hoshino, Y. Isa, M. Kadowaki, T. Ichi, A. Tanaka, S. Nishiumi, I. Fukuda, H. Ashida, F. Horio and T. Tsuda, *Biochem. Pharmacol.*, 2007, **74**, 1619.
38 S. H. Kwon, I. S. Ahn, S. O. Kim, C. S. Kong, H. Y. Chung, M. S. Do and K. Y. Park, *J. Med. Food*, 2007, **10**, 552.
39 I. T. Nizamutdinova, Y. C. Jin, J. I. Chung, S. C. Shin, S. J. Lee, H. G. Seo, J. H. Lee, K. C. Chang and H. J. Kim, *Mol. Nutr. Food Res.*, 2009, **53**, 1419.
40 J. Alarcon-Alonso, A. Zamilpa, F. A. Aguilar, M. Herrera-Ruiz, J. Tortoriello and E. Jimenez-Ferrer, *J. Ethnopharmacol.*, 2012, **139**, 751.
41 E. Jiménez-Ferrer, J. Alarcón-Alonso, A. Aguilar-Rojas, A. Zamilpa, I. Jiménez-Ferrer, J. Tortoriello and M. Herrera-Ruiz, *Planta Med.*, 2012, **18**, 1893.
42 S. Fernández-Arroyo, M. Herranz-López, R. Beltrán-Debón, I. Borras-Linares, E. Barrajón-Catalán, J. Joven, J. A. Fernández-Gutiérrez, A. Segura-Carretero and V. Micol, *Mol. Nutr. Food Res.*, 2012, **56**, 1590.
43 V. Hirunpanich, A. Utaipat, N. P. Morales, N. Bunyapraphatsara, H. Sato, A. Herunsale and C. Suthisisang, *J. Ethnopharmacol.*, 2006, **103**, 252.
44 A. Tailleux, K. Wouters and B. Staels, *Biochim. Biophys. Acta*, 2012, **1821**, 809.
45 F. Yap, L. Craddock and J. Yang, *Int. J. Biol. Sci.*, 2011, **7**, 645.
46 P. Pettinelli and L. A. Videla, *J. Clin. Endocrinol. Metab.*, 2011, **96**, 1424.
47 M. J. Frederico, M. F. Vitto, P. A. Cesconetto, J. Engelmann, D. R. De Souza, G. Luz, R. A. Pinho, E. R. Ropelle, D. E. Cintra and C. T. De Souza, *Scand. J. Gastroenterol.*, 2011, **46**, 381.
48 M. S. Sozio, C. Lu, Y. Zeng, S. Liangpunsakul and D. W. Crabb, *Am. J. Physiol.: Gastrointest. Liver Physiol.*, 2011, **301**, G739.
49 Y. P. Hwang, J. H. Choi, E. H. Han, H. G. Kim, J. H. Wee, K. O. Jung, K. H. Jung, K. I. Kwon, T. C. Jeong, Y. C. Chung and H. G. Jeong, *Nutr. Res.*, 2011, **31**, 896.
50 M. Takikawa, S. Inoue, F. Horio and T. Tsuda, *J. Nutr.*, 2010, **140**, 527.
51 C. H. Peng, C. C. Chyau, K. C. Chan, T. H. Chan, C. J. Wang and C. N. Huang, *J. Agric. Food Chem.*, 2011, **59**, 9901.
52 J. K. Kim, H. So, M. J. Youn, H. J. Kim, Y. Kim, C. Park, S. J. Kim, Y. A. Ha, K. Y. Chai, S. M. Kim, K. Y. Kim and R. Park, *J. Ethnopharmacol.*, 2007, **114**, 260.
53 B. Kola, A. B. Grossman and M. Korbonits, *Front. Horm. Res.*, 2008, **36**, 198.
54 L. F. Yu, B. Y. Qiu, F. J. Nan and J. Li, *Curr. Top. Med. Chem.*, 2010, **10**, 397.
55 S. A. Hawley, A. E. Gadalla, G. S. Olsen and D. G. Hardie, *Diabetes*, 2002, **51**, 2420.

