Comparison of the bioactive potential of roselle calyx and its by-product: phenolic characterization by *UPLC-QTOF-MS^E* and their effect in an *in vivo* model

Diana Amaya-Cruz[a], Iza F. Peréz-Ramírez[a], Jara Pérez-Jiménez[b], Gerardo Nava-Morales[a], Rosalía Reynoso-Camacho[a]

[a] Research and Graduate Studies in Food Science, School of Chemistry, Universidad Autónoma de Querétaro, C.U., Cerro de las Campanas S/N, Queretaro 76010, Mexico

[b] Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), José Antonio Novais 10, 28040, Madrid, Spain

E-mail address:

Diana M. Amaya Cruz: dmamayacruz@hotmail.com

Iza F. Pérez Ramírez: iza.perez@hotmail.com

Jara Pérez-Jiménez: jara.perez@ictan.csic.es

Gerardo Nava Morales: gerardomnava@gmail.com

- *Rosalía Reynoso Camacho: rrcamachomx@yahoo.com.mx. Corresponding author. Tel: +52 (442) 1921200 ext. 5576.

Authors contributors:

Diana Amaya-Cruz: Quantifications and *in vivo* assays, the data analysis and wrote the draft.

Iza F. Peréz-Ramírez: Realized the identification of polyphenolic compounds and organic acids in roselle calyx and by-product by UPLC-QTOF-MS^E

Jara Pérez-Jiménez: Made a critical revision of the manuscript

Rosalía Reynoso-Camacho: Designed the work and revised the manuscript

Abstract

The comparison of roselle calyces and its by-product (BP) in terms of extractable (EPP) and non-extractable polyphenols (NEPP), and organic acids profile, likewise the effect of their consumption in a high fat high fructose diet was carried out. In the detailed profile,

141 EPP, 16 organic acids, and 35 hydrolysable polyphenols (HPP) were identified; this is the first detailed profile for EPP and organic acids for BP, and the HPP for both materials. The *in vivo* results showed that the supplementation with calyces and BP were effective for significantly reducing body weight gain (14 and 10%), adiposity (13 and 17%), insulin resistance (59 and 48%), hypertriglyceridemia (12 and 18%) and hepatic steatosis (36 and 29%), besides increased the excretion of lipids from the diet (26 and 14%, respectively). The *in vivo* effects observed for calyces supplementation could be due to their higher content of EPP and organic acids, while those for BP would be due to a synergistic effect between dietary fiber and NEPP. These results indicate the potential of roselle calyces and specially the BP as functional ingredient and as an alternative for the integral use of the BP.

Keywords: Roselle, hydrolysable polyphenols, extractable polyphenols, by-product, calyx, obesity.

Abbreviations:

EPP: extractable polyphenols

NEPP: non-extractable polyphenols

NEPA: non-extractable proanthocyanidins

HPP: hydrolysable polyphenols

BP: by-product

DP: decoction process

TDF: total dietary fiber

SDF: soluble dietary fiber

IDF: insoluble dietary fiber

HF/HFr: high fat high fructose

C3G: cyanidin-3-glucoside

1. Introduction

Obesity is a multifactorial disease due to an energy imbalance caused by high caloric intake or/and a low energy expenditure, causing the excess energy to be stored in the adipose tissue as fat, and producing an increase in the number (hyperplasia) and the size (hypertrophy) of adipocytes (Siriwardhana et al., 2013). This pathology increases the risk of complications such as non-alcoholic fatty liver, dyslipidemia and type 2 diabetes (Malnick & Knobler, 2006). Among the alternatives for obesity prevention are the functional foods that contain phenolic compounds and dietary fiber (Trigueros et al., 2013).

Roselle (*Hibiscus sabdariffa* L.) calyces are a source of these compounds, and these are used to prepare beverages, whose process generates a by-product. Calyces contain dietary fiber and polyphenolic compounds, including extractable polyphenols (EPP) and non-extractable polyphenols (NEPP) (Sáyago-Ayerdi, Velázquez-López, Montalvo-González, & Goñi, 2014). EPP are low and medium molecular weight polyphenols that MAY be extracted with aqueous-organic solvents and these are bioavailable in stomach and small intestine; intead, NEPP MAY be single polyphenols as hydrolysable polyphenols (HPP) associated to proteins and polysaccharides, or polymeric compounds as non-extractable proanthocyanidins (NEPA). NEPP are retained in the residue of extraction and partially metabolized by the colonic microbiota and absorbed in the colon (Pérez-Jiménez, Díaz-Rubio, & Saura-Calixto, 2013). Total EPP and NEPP contentos have been reported for calyces and roselle by-products (Amaya Cruz, Pérez Ramírez, Ortega, Rodríguez García, & Reynoso-Camacho, 2018; Sáyago-Ayerdi et al., 2014). EPP profile has been reported for extracts or roselle calyces (Morales-Luna et al., 2018) but a detailed profile of EPP and HPP for calyces and their by-products has not been reported.

In vitro and *in vivo* studies and clinical trials have demonstrate that roselle extracts rich in EPP have antihyperlipidemic, antiobesity, antihypertensive, anti-inflammatory and antimicrobial effects (Riaz & Chopra, 2018). However, there is not enough information about roselle calyces powder as source of EPP and NEPP and their effects in health, although some scarce studies have shown a beneficial effect for this supplementation. Rats fed with a hypercholesterolemic diet and supplemented with roselle power showed minor lipids contents compared to control group (El-Saadany, Sitohy, Labib, & El-Massry, 1991).

Recently Moyano et al. (2016) reported that roselle calyces decreased body weight, adiposity and plasma concentration of total cholesterol and glucose concentration in rats fed with a high fat diet. And regarding the by-product (BP) or decocción resides of roselle, to our best knowledge there are no reports about their *in vivo* effects. Nevertheless, the search for alternative utilization of the by-product are important due to beverages consumption of these calyces has increased in the last years (Solangi et al., 2017)

Therefore, the purpose of this study was to compare the profile of bioactive compounds (EPP, HPP, dietary fiber, organic acids) of Jamaica calyces powders and their by-products, and to assess their effects on obesity control and its complications, with the purpose of promoting an integral use of roselle.

2. Materials & methods

2.1. Sample preparation

Roselle calyces were obtained from producers of Guerrero state, Mexico. The calyces were disinfected and used to prepare a soft drink: 60 g of calyces were added to 1 L of boiling water and heated for 15 min, later the extract was filtered. The disinfected calyces and the by-products were dried in a forced circulation dryer during 24 h at 45 °C. The materials were ground and sieved to obtain particle size lower than 420 µm.

2.2. Quantification of bioactive compounds

2.2.1. Dietary fiber content (DF)

Total dietary fiber (TDF) and its fractions: soluble (SDF) and insoluble (IDF), were quantified in calyces and by-products according to the method of AOAC (2002) using a total dietary fiber assay kit (Sigma-Aldrich). One g of sample was incubated with α-amylase, protease and amyloglucosidase at pH, time and temperature established by the manufacturer.

2.2.2. Extractable polyphenols (EPP) content

For obtaining the EPP fraction, 0.25 g of each sample was extracted during 1 h with 10 mL of a mixture of methanol/water (50:50 v/v) acidified with HCl (pH 2), the supernatant was kept, and the residue was extracted for 1 h with 10 mL of a mixture of acetone/water

(70:30, v/v); later both supernatants were mixed (Hassan, Ismail, Abdulhamid, & Azlan, 2011).

In the extract, total phenolics compounds were quantified according to Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1998) and results were expressed as mg of gallic acid equivalents (GAE)/g of DB. Total flavonoids were quantified using a colorimetric assay method according to Heimler, Vignolini, Dini, Vincieri, & Romani, (2006), and reported as mg of catechin equivalents (CE)/g DB, respectively. The monomeric anthocyanins were quantified by the method described by Giusti & Wrolstad, (2001) with slight modifications. Briefly, 50 µL of each extract was individually mixed with 175 µL of potassium chloride solution (0.025 M, pH: 1) and 175 µL of sodium acetate solution (0.4 M, pH: 4.5) in a microplate. The absorbance was recorded at 510 and 700 nm, and results were calculated as cyanidin-3-glucoside (C3G) equivalents according to the next equation:

$$\text{Total anthocyanins}=[(Abs_{510nm}\text{-}Abs7_{00nm})_{pH1}\text{-}(Abs_{510nm}\text{-}Abs_{700nm})pH_{4.5}]x[(MWxDF)/\varepsilon xD]$$

MW is the molecular weight for C3G (448.8), DF is the dilution factor, Ɛ is the molar absorptivity coefficient (26900) and D is the height of the sample in the well.

2.2.3. Non-extractable polyphenol (NEPP) content

The residue of the extraction obtained in 2.2.2 section was used for the extraction of NEPP. For non-extractable proanthocyanidins (NEPA), 100 mg of the residue were hydrolyzed for 60 min at 100 °C with n-butanol/HCl (95:5 v/v) and iron reagent (2% w/v ferric ammonium sulphate in 2 mol/L HCl). Absorbances were registered at 450 and 555 nm, and results are expressed as mg of proanthocyanidins/g of DB (PA mg/g), using a calibration curve reported with carob pod as standard (Zurita, Díaz-Rubio, & Saura-Calixto, 2012). For hydrolysable polyphenols (HPP) the residue obtained in 2.2.2 was hydrolyzed during 20 h at 85 °C with methanol/H_2SO_4 (90:10, v/v) (Saura-Calixto, Serrano, & Goñi, 2007) and the compounds released were quantified with the Folin–Ciocalteu assay (Singleton et al., 1998).

2.2.4. Identification of polyphenolic compounds and organic acids in roselle calyx and BP by UPLC-QTOF-MS^E

For the EPP fraction the extraction process carried out in the section 2.2.2 was performed with mass grade solvents for the chromatographic analysis; organic acids were also identified in this extract. For hydrolysable polyphenols fraction obtained in 2.2.3, the pH was adjusted to 5.0 with NaOH 6M (Pérez-Jiménez & Saura-Calixto, 2015). The EPP and HPP extracts were speed-vacuum dried, reconstituted in the mobile phase (water with 0.1% formic acid) and filtered using a syringe filter (0.45 μm, PVDF) prior to inject 2 μL of sample in a column Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 μm). The identification was realized by UPLC QTOF MS^E with an electrospray ionization (ESI) interphase (Vion; Waters Co, Milford, USA). The chromatographic and ionization conditions were according to reported by Amaya-Cruz et al., (2019). The mobile phase consisted of A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) at a flow rate of 0.4 mL/min. The solvent gradient changed from 95% A over 2 min, linear gradient 95 to 5% A over 20 min, maintained isocratic condition for 2 min and a linear gradient 5 to 95% A over 3 min.

A high definition MS^E ionization was used, in positive mode for anthocyanins and organic acids and in negative mode for phenolic compounds. For data acquisition, the mass range was stablished between 50–1800 Da. The mass spectrometer settings were 2000 V for capillary voltage and 120 ºC as source temperature. The collision energy was set to 5 eV (low) and a ramp energy at 15–45 eV (high). Nitrogen was used as desolvation and cone gas at 800 L/h, 450 °C, and 50 L/h. Leucine-enkephalin solution (50 pg/mL,) was used for lock mass correction at 10 μL/min. For the identification, the exact mass (<10 ppm mass error), isotope distribution, and fragment pattern were analyzed; Phenol-Explorer, PubChem and FooDb databases were used for comparing mass spectra.

2.3. Effect of supplementation of roselle calyces and BPs in a high fat high fructose (HF/HFr) model

2.3.1. Animals and diets

Thirty-two male Wistar rats (180 ± 20 g) were purchased from the Institute of Neurobiology, UNAM (Querétaro, Mexico) and allowed to acclimate for one weak. The experimental procedure followed the Guiding Principles in the Care and Use of Animals and it was approved by the bioethics committee of the Universidad Autónoma de Querétaro (CBQ17/071). The animals were housed individual per cage under a 12 h light-dark cycle

at 25 ºC with unrestricted access to food and water. Four groups of eight animals were formed. Standard diet group was fed with commercial powdered Rodent Lab chow 5001, while HF/HFr group was fed with a diet added with 20% of fat and 18% of fructose. The two remaining groups corresponded to animals fed with HF/HFr diet supplemented with the 4% of dietary fiber either from calyces or from BP. The study was carried out for eighteen weeks, body weight and food intake were measured weekly, and in the last week feces were collected and kept at 70 °C. At the end of the study, the animals were sacrificed after a 12 h fasting period and blood, adipose tissue and liver were collected. In serum, glucose and triacylglycerol were measured with enzymatic kit (Spinreact, Spain) and insulin was measured by sandwich ELISA (Millipore). Glucose and insulin values were used to calculate the HOMA (Homeostatic Model Assessment) index.

2.3.2. Histological analysis

For the evaluation of histological changes, adipose tissue and liver were fixed in 10% neutral buffered formaldehyde. Then, the tissues were paraffin-embedded and sectioned in a microtome. The slides were stained with hematoxylin and eosin and analyzed by light microscope (300x). Adipocytes relative size were obtained with ZEISS ZEN microscope software and steatosis was evaluated according to Kleiner classification (Kleiner et al., 2005)

2.3.3 Quantification of triacylglycerol in liver and feces

The lipids of the frozen liver were extracted according to Norris et al., (2003). Triacylglycerols were quantified by an enzymatic kit (Spinreact, Spain). The results were normalized by the protein content by BCA protein assay (Bio-Rad).

For feces, the extraction method of Yetukuri et al., (2007) was used with modifications. 50 mg of dried and ground feces were homogenized for 1 min with 200 µL of 0.9% NaCl and 800 µL of chloroform:methanol (2: 1). The samples were centrifuged at 7000 x g for 10 min, the supernatant was incubated for 4 h at -20 °C. Then, the samples were centrifuged at 11 200 x g for 5 min at 4 °C and the lower phase was recovered and evaporated to dryness. The triacylglycerols were measured with a enzymatic kit (Spinreact, Spain).

2.4 Statistical analysis

Data were analyzed using JMP statistical discovery^{TM} v5.0, by applying a one-way analysis of variance (ANOVA) and t-student, and Tukey's test ($p < 0.05$).

3. Results & discussion

3.1 Dietary fiber and phenolic compounds content

Dietary fiber and phenolic compounds have been associated with benefits for obesity and their complications (Fuller, Beck, Salman, & Tapsell, 2016; Rodríguez-Pérez, Segura-Carretero, & del Mar Contreras, 2017); therefore, they were quantified. The decoction process (DP) affected the content of DF, EPP and NEPP showing statistical difference for all the values quantified (Table 1). In particular, the TDF, IDF and SDF content increased significantly in 62.3, 67.0 and 38.2%, respectively when the calyces were subject to a DP. This behavior is due to the fact that some compounds like carbohydrates, proteins, minerals, lipids, antioxidants and, perhaps, SDF were lixiviated to the beverage (Sáyago-Ayerdi et al., 2014); so the DP is a method to concentrate DF. The IDF was the most abundant fraction but in both samples SDF was about 30%, the percentage described as adéquat for achieving all DF health properties. It has been reported that the beneficial effect of DF consumption is shared by both fractions (Weickert & Pfeiffer, 2018).

After the DP, the extractable polyphenols decreased their concentration in 52.0, 45.7 and 57.1%, for phenolic compounds, flavonoids and anthocyanins, respectively. This decrease may be due to the lixiviation of these compounds to the water or tge degradation by the thermal process. This applies especially for anthocyanins, since these compounds are highly sensitive to thermal process, which causes oxidation and cleaving of covalent bonds (Patras, Brunton, O'Donnell, & Tiwari, 2010). The behavior of NEPP was opposite, with an I crease in their content after DP. HPP and NEPA of BP were 71.2 and 74.4% higher than in calyces, respectively, due to a concentration effect. Overall, the different profile of EPP, HPP, NEPA and DF in the two samples may give place to differential health effects.

3.2 Polyphenolic profile of calyces and by-products by UPLC-QTOF-MS^E

A detailed profile of the EPP (Table 2) and HPP (Table 3) fractions was realized by UPLC-QTOF-MS^E; besides, organics acids were identified since the antiobesogenic effect of roselle has also been attributed to these compounds (Morales-Luna et al., 2018). To our best

knowledge this is the first report of EPP, HPP and organic acids for roselle BP, and of HPP for calyces. For EPP, 35 hydroxycinnamic acids, 19 hydroxybenzoic acids, 2 hydroxyphenylpropanoic acids, 8 flavanals, 8 flavanones, 31 flavonols, 3 dihydroflavonols, 34 anthocyanins and 16 organic acids were identified based on their exact mass (<10 ppm mass error), fragment pattern and isotope distribution. Analyzing the total count for each family, the higher loss was found for anthocyanins (81.2%) and the individual compounds with the largest decreases after DP were pelargonidin 3-O-glucoside (95.9%), dihydroquercetin (93.3%), cyanidin 3-O-sambubioside (92.1%), pelargonidin 3-O-galactoside (91.9%) and dihydrocaffeic acid (91.4%). Among these compounds, the majority were glycosylated anthocyanins; indeed, it has been reported that monoglycosylated are less stable than their diglycosylated derivatives (Brauch, Kroner, Schweiggert, & Carle, 2015), the glycoside first presents a ring opening forming a chalcone glycoside, follow the deglycosilation producing a chalcone (Sadilova, Carle, & Stintzing, 2007). On the other hand, the lowest losses were for the clases of organic acids (36.2%) and flavanones (40.7%) and the following individual compounds: glutaric acid (0.3%), naringenin (3.3%), 4-hydroxybenzoic acid 4-O-glucoside (8.1%), eriodictyol (11.6%) and ascorbic acid (13.6%). However, some EPP compounds were concentrated in the roselle BP: eriocitrin (increase of 149.6%), 2,4-dihydroxybenzoic acid (81.9%), 3-hydroxybenzoic acid (78.0%), ellagic acid (65.6%) and gluconic acid (52.7%), therefore, these compounds were well retained in the food matrix.

Regarding HPP, 12 hydroxycinnamic acids, 11 hydroxybenzoic acids, 3 flavanals, 3 flavanones and 6 flavonols were identified, with 28 of them being present in BP, and 24 of them in calyces (Table 3). Pérez-Jiménez & Saura-Calixto (2015) reported phenolic acids, flavonols and flavanones as constituents of HPP fractions of fruits and vegetables, so in this study flavanals were additionally detected.

For hydroxycinnamic and hydroxybenzoic acids, there was an increase of 64.0 and 53.8%, respectively in BP as compared to calyces, with the following phenolic acids being just detected in the BP: 3-caffeoylquinic, p-coumaroyl malic, isoferulic, 3,5-dicaffeoylquinic, vanillic, 2,6-dihydroxybenzoic and protocatechuic. During DP, flavonoids as flavanals, flavanones and flavonols were increased 2.5, 2.0 and 0.7-fold, respectively. Besides, there

were flavonoids as prodelphinidin dimer B3, hesperidin, rhamnetin and kaempferol that were only identified in the BP. Compounds as (-)-epigallocatechin gallate, (-)-epicatechin 3-O-gallate and kaempferol 3-O-glucoside were not detected in the EPP fraction, but these compounds were found in HPP. Most of HPP were increased in the BP; this is due to their association with proteins and polysaccharides (Pérez-Jiménez & Saura-Calixto, 2015), which increases their retention in the food matrix and, consequently, their content after the decoccion process. So roselle BP are a good source of DF and NEPP such as HPP and NEPA, while the calyces showed a great content of organic acids and EPP, specially anthocyanins. Both materials were tested in a *in vivo* model in order to evaluate potential differential effects.

3.3 Effect of the consumption of roselle BP and calyces on a HF/HFr diet

3.3.1 Weight gain

After eighteen weeks, animals fed with a HF/HFr diet presented a significative increase ($p=0.05$) in body weight up to 25% when compared to the standard diet-fed group. This increase is caused by the excess energy ingested which is stored as triglycerides in the adipose tissue, which can be synthesized either from the fatty acids in the diet or through the metabolism of fructose- a lipogenic sugar that may lead to obesity, insulin resistance and hyperlipidemia (Lim, Mietus-Snyder, Valente, Schwarz, & Lustig, 2010).

Treatment with calyces and BP reduced the body weight in 14 and 10%, respectively, so calyces supplementation exerted the greatest anti-obesogenic effect. The reduction in weight gain was related to a lower hypertrophy of the adipocytes (Figure 2). Thus, the size of the adipocytes was statistically decreased in standard diet group (28.3%) and treatments with calyces (12.7%) and BP (17.3%) as compared to HF/HFr diet group, with no statistical difference observed between the treatments ($p=0.05$), so both supplementations could be promoting less triglycerides storage in adipose cells. The highest effect in the calyces group could be to a higher content of organic acids and EPP SUCH as anthocyanins. Moreover, organic acids as hibiscus, dimethyl hibiscus and hydroxy citric, identified in high quantity in calyces (Table 2), have been associated with the prevention of body weight gain and adipocytes hyperplasia (Morales-Luna et al., 2018).

3.3.2 Insulin resistance and hypertriglyceridemia

The consumption of a HF/HFr diet produced in the animals an increase in fasting glucose, insulin and triacylglycerol (TAG) content in serum in 55.7, 100.0, and 55.9% in comparison to the rats of the standard diet group (Table 4). The increase in blood glucose is related to the high consumption of both fat and fructose in the diet. In this way, it has been reported that a high-fat diet increases hepatic glucose production through glycogenolysis and gluconeogenesis (Jin, Beddow, Malloy, & Samuel, 2013). The supplementation with calyces and BP showed a hypoglycemic effect reducing the glucose concentration in 38 and 36.6% respectively compared to group fed with HF/HFr diet; and insulin in 41.7 and 23.4%, respectively. As regards to insulin resistance, HOMA-IR was calculated; it was reduced by calyx and BP in 59.1 and 47.8%, respectively, being statistically similar to the standard diet group (Table 4).

The above data show that supplementation with roselle calyces and BP was effective for decreased the insulin resistance caused by the consumption of HF/HFr diet. Moyano et al., (2016) attribute the hypoglycemic effect of the roselle calyces in powder to an increase in the concentration of GLP-1, which increases insulin secretion and pancreatic β cell mass, improves insulin sensitivity, increases satiety and decreases gastric emptying (Prasad-Reddy & Isaacs, 2015). In this study, no augments in insulin secretion nor in satiety (data not shown) were observed, while the HOMA values suggest an improvement in insulin secretion. Besides the hibiscus acid and hibiscus acid 6-O-methyl ester (Table 2) have been reported as alpha amylase inhibitors (Hansawasdi, Kawabata, & Kasai, 2000), slowing down the digestion of carbohydrates and therefore its absorption. With regard to BP supplementation, the effect was lower, however the diets with a high content of IDF have been reported to decrease the risk of diabetes in prospective cohort studies (Weickert & Pfeiffer, 2018).

Regarding TAG content in serum, the highest reduction occurred in the BP group (17.8%). The decrease in the TAG serum levels, by both treatments, could be due to a reduction in the absorption of the fat from the diet, since the supplementation with calyces and BP increased the TAG content in feces in 26.4 and 13.8%, respectively (Table 4), as compared to the HF/HFr diet. In addition, it has reported that NEPP, the main phenolic fraction in BP,

cause a reduction in lipid biosynthesis (Martín-Carrón, Saura-Calixto, & Goñi, 2000).Therefore, this could explain the major effect produced by BP.

3.3.3 Hepatic steatosis

Steatosis was determined histologically. The animals fed with the standard diet (Figure 2a) did not show lipid vacuoles within hepatocytes, showing score 0 for steatosis. However, for HF/HFr diet (Figure 2b) steatosis score was 2, decreasing to 1 for the animals supplemented with calyces (Figure 2c) and BP (Figure 2d). Therefore, supplementation reduced the fatty deposits in the liver. In addition, the hepatic triglycerides content was determined being 2.5-fold in animals fed with HF/HFr diet ascompatef to standard diet group, since fructose intake causes an increase in *de novo* hepatic fatty acid synthesis, producing a hepatic triglyceride accumulation (Huang et al., 2011). A significant effect ($p=0.05$) in hepatic content was found by the consumption of BP and calyces, with no difference between the treatments, decreasing the content in 15.5 and 24.7%, respectively.

The lower concentration of triglycerides in animal livers could be related to the content of polyphenols and DF. Thus, it has been reported that phenolic compounds can decrease and/or prevent damage to hepatocytes through various mechanisms of action: a) reducing *de novo* lipogenesis by decreasing SREBP-1c, as reported for roselle extracts (Kao, Yang, Hung, Huang, & Wang, 2016; Villalpando-Arteaga et al., 2013) (b) β- oxidation of fatty acids (c) improving insulin sensitivity, which was one of the effects proven in this study (d) reducing oxidative stress, and (e) attenuating inflammatory pathways (Rodríguez-Ramiro, Vauzour, & Minihane, 2016). The *in vivo* effects found for the BP group could be attributed a high content of DF and, specially, NEPP. It has been reported than an advantage of NEPP consumption is that their colonic metabolites remain in the body longer periods than those derived from EPP. Moreover, the higher DF content in BP than in calyces could increase the fermentation of NEPP, since synergistic processes take place between both constituents (Saura- Calixto et al., 2010).

4. Conclusions

Roselle calyces were characterized by a high content of EPP like phenolic acids and flavonoids (specially anthocyanins), as well as oforganic acids. Instead, roselle BP showed

a high level of DF and NEPP(present as NEPA and HPP) . Both materials showed promising effects -with no statistical difference between them- for the prevention of obesity, reducing body weight and adiposity and its complications like insulin resistance, hypertriglyceridemia and hepatic steatosis, likewise by reducing the absorption of lipids from the diet. Therefore, roselle calyces and its BP could be a functional ingredient with nutraceutical potential.

Declaration of interest

None.

Financial support

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Figure captions

Figure 1. Histological pictures (300x) of rats following different diets: (a) Standart diet, (b) HF/HFr diet, (c) HF/HFr diet plus calyces, (d) HF/HFr diet plus by-product (e) relative size of adipocytes in all dietd

Figure 2. Histological pictures of liver sections (300x) in rats following different diets: (a) Standard diet, (b) HF/HFr diet, (c) HF/HFr diet plus calyces, (d) HF/HFr diet plus by-product (e) hepatic triglycerides content (mg tg/g protein) in all diets

Table 1. Content of bioactive compounds in roselle calyces and its by-product

Compound	Calyces	By-product
Total dietary fiber (%)	41.37±2.40 [b]	67.16±0.85 [a]
Insoluble dietary fiber (%)	28.99±1.70 [b]	48.43±0.75 [a]
Soluble dietary fiber (%)	13.34±0.75 [b]	18.44±0.73 [a]
Extractable polyphenols		
Total phenolic compounds (GAE mg/g)	14.24±0.77 [a]	6.83±0.18 [b]
Flavonoids (CE mg/g)	10.37±0.67 [a]	5.63±0.46 [b]
Anthocyanins (mg C3G/g)	5.76±0.43 [a]	2.47±0.17 [b]
Macromolecular antioxidants		
Hydrolysable polyphenols (GAE mg/g)	2.85±0.01 [b]	6.18±0.08 [a]
Non-extractable proanthocyanidins (PAE mg/g)	3.82±0.31 [b]	6.67±0.03 [a]

Results are expressed on dry basis (DB) and are the average of three independent determinations ± SD. Means within a same line with different superscript letters indicate significant difference by t-student ($p < 0.05$). GAE: gallic acid equivalents, CE catechin equivalents, C3GE cyanidin-3-glucoside equivalents, PAE: proanthocyanidin equivalents

Table 2. Identification of extractable polyphenols and organic acids by UPLC-ESI-QTOF MS^E of roselle calyces and its by-product.

Component name	Retention time (min)	Molecular formula	Expected mass (Da)	Observed mass (Da)	Mass error (ppm)	Adducts	Roselle by-prod
Hydroxycinnamic acids							
Cinnamic acid	1.48	C9H8O2	148.0524	148.0513	-7.9027	$[M-H]^-$	225.04 ± 7.
3-Caffeoylquinic acid	2.56	C16H18O9	354.0951	354.0953	0.7208	$[M-H]^-$	501017.88 ± 231
m-Coumaric acid	2.80	C9H8O3	164.0473	164.0468	-3.0898	$[M-H]^-$	431.17 ± 39
Caffeoyl tartaric acid	3.37	C13H12O9	312.0481	312.0505	7.7697	$[M-H]^-$	109.97 ± 0.
Cinnamoyl glucose	3.40	C15H18O7	310.1053	310.1060	2.4070	$[M-H]^-$	217.35 ± 0.
o-Coumaric acid	3.69	C9H8O3	164.0473	164.0471	-1.4475	$[M-H]^-$	5216.63 ± 10
p-Coumaroylquinic acid	3.69	C16H18O8	338.1002	338.1006	1.3100	$[M-H]^-$	35678.22 ± 15
Rosmarinic acid	3.69	C18H16O8	360.0845	360.0828	-4.8642	$[M-H]^-$	7629.28 ± 52
Caffeic acid 4-O-glucoside	3.80	C15H18O9	342.0951	342.0954	0.7976	$[M-H]^-$	6206.90 ± 56
4-Caffeoylquinic acid	3.92	C16H18O9	354.0951	354.0952	0.4587	$[M-H]^-$	163913.16 ± 71
4-Sinapoylquinic acid	4.08	C18H22O10	398.1213	398.1230	4.2492	$[M-H]^-$	ND
p-Coumaroyl glucose	4.14	C15H18O8	326.1002	326.1001	-0.0716	$[M-H]^-$	1488.65 ± 20
p-Coumaric acid	4.15	C9H8O3	164.0473	164.0473	-0.4223	$[M-H]^-$	912.14 ± 12
5-Caffeoylquinic acid	4.18	C16H18O9	354.0951	354.0956	1.4804	$[M-H]^-$	211839.33 ± 76
Caffeic acid	4.19	C9H8O4	180.0423	180.0421	-0.7888	$[M-H]^-$	15058.83 ± 37
Hydroxycaffeic acid	4.19	C9H8O5	196.0372	196.0368	-1.9387	$[M-H]^-$	146.31 ± 4.
p-Coumaroyl malic acid	4.40	C13H12O7	280.0583	280.0570	-4.7846	$[M-H]^-$	174.51 ± 33
Caffeic acid ethyl ester	4.42	C11H12O4	208.0736	208.0725	-5.2651	$[M-H]^-$	151.04 ± 28
4-p-Coumaroylquinic acid	4.56	C16H18O8	338.1002	338.1004	0.7261	$[M-H]^-$	1330.32 ± 95
Ferulic acid 4-O-glucoside	4.56	C16H20O9	356.1107	356.1109	0.5237	$[M-H]^-$	1111.61 ± 6.
Isoferulic acid	4.61	C10H10O4	194.0579	194.0577	-1.0468	$[M-H]^-$	1709.44 ± 38
3-Feruloylquinic acid	4.63	C17H20O9	368.1107	368.1114	1.8853	$[M-H]^-$	13050.82 ± 63
5-p-Coumaroylquinic acid	4.82	C16H18O8	338.1002	338.1005	0.9370	$[M-H]^-$	18129.74 ± 41
Ellagic acid arabinoside	4.92	C19H14O12	434.0485	434.0452	-7.6913	$[M-H]^-$	210.54 ± 0.
p-Coumaroyl tartaric acid	4.94	C13H12O8	296.0532	296.0545	4.3476	$[M-H]^-$	356.78 ± 28
Ellagic acid acetyl-xyloside	4.95	C21H16O13	476.0591	476.0602	2.4268	$[M-H]^-$	ND
4-Feruloylquinic acid	5.13	C17H20O9	368.1107	368.1116	2.4956	$[M-H]^-$	6302.55 ± 28
3,4-Dicaffeoylquinic acid	5.47	C25H24O12	516.1268	516.1270	0.4257	$[M-H]^-$	1239.82 ± 36
5-Feruoylquinic acid	5.58	C17H20O9	368.1107	368.1115	2.0179	$[M-H]^-$	8955.46 ± 27
p-Coumaroyl glycolic	5.62	C11H10O5	222.0528	222.0525	-1.6387	$[M-H]^-$	1279.91 ± 81

acid								
Ellagic acid	5.65	C14H6O8	302.0063	302.0063	0.0888	$[M-H]^-$	2186.92	± 61
Ferulic acid	5.72	C10H10O4	194.0579	194.0576	-1.5485	$[M-H]^-$	554.03	± 2.5
Sinapic acid	5.79	C11H12O5	224.0685	224.0680	-1.9877	$[M-H]^-$	565.63	± 22
3,5-Dicaffeoylquinic acid	6.30	C25H24O12	516.1268	516.1270	0.5040	$[M-H]^-$	5121.86	± 10
4,5-Dicaffeoylquinic acid	6.74	C25H24O12	516.1268	516.1270	0.4415	$[M-H]^-$	7669.55	± 23
Total counts							1020191.39	
				Hydroxybenzoic acids				
Galloyl glucose	1.09	C13H16O10	332.0743	332.0757	4.1567	$[M-H]^-$	505.02	± 9.5
2,3-Dihydroxybenzoic acid	1.13	C7H6O4	154.0266	154.0266	-0.2324	$[M-H]^-$	879.60	± 24
2-Hydroxybenzoic acid	1.21	C7H6O3	138.0317	138.0318	0.5111	$[M-H]^-$	929.24	± 38
2-Hydroxybenzoic acid 4-O-glucoside	1.21	C13H16O8	300.0845	300.0846	0.3629	$[M-H]^-$	1901.99	± 42
Gallic acid 4-O-glucoside	1.46	C13H16O10	332.0743	332.0747	1.0631	$[M-H]^-$	8631.54	± 36
Protocatechuic acid 4-O-glucoside	1.63	C13H16O9	316.0794	316.0799	1.5491	$[M-H]^-$	82410.93	± 22
Vanillic acid	1.69	C8H8O4	168.0423	168.0421	-0.8113	$[M-H]^-$	1875.01	± 16
2,4-Dihydroxybenzoic acid	1.94	C7H6O4	154.0266	154.0267	0.4600	$[M-H]^-$	5078.73	± 17
3-Hydroxybenzoic acid	3.12	C7H6O3	138.0317	138.0316	-0.6198	$[M-H]^-$	8241.42	± 24
2,6-Dihydroxybenzoic acid	3.32	C7H6O4	154.0266	154.0265	-0.8566	$[M-H]^-$	909.53	± 9.6
3-Hydroxybenzoic acid 4-O-glucoside	3.71	C13H16O8	300.0845	300.0838	-2.3637	$[M-H]^-$	ND	
Gallic acid	4.22	C7H6O5	170.0215	170.0214	-0.6540	$[M-H]^-$	4987.29	± 14
Gallic acid 3-O-gallate	4.23	C14H10O9	322.0325	322.0335	3.1189	$[M-H]^-$	ND	
Benzoic acid	4.27	C7H6O2	122.0368	122.0367	-0.2766	$[M-H]^-$	1338.03	± 35
4-Hydroxybenzoic acid 4-O-glucoside	4.35	C13H16O8	300.0845	300.0843	-0.7473	$[M-H]^-$	2316.49	± 81
Gallic acid ethyl ester	4.50	C9H10O5	198.0528	198.0526	-0.9592	$[M-H]^-$	1605.62	± 68
Syringic acid	4.84	C9H10O5	198.0528	198.0520	-4.1343	$[M-H]^-$	153.69	± 8.3
Protocatechuic acid	4.93	C7H6O4	154.0266	154.0265	-0.4363	$[M-H]^-$	4444.19	± 10
4-Hydroxybenzoic acid	6.69	C7H6O3	138.0317	138.0316	-0.6430	$[M-H]^-$	1833.43	± 10
Total counts							128041.77	
				Hydroxyphenylpropanoic acids				
Dihydro-p-coumaric acid	3.75	C9H10O3	166.0630	166.0627	-1.9825	$[M-H]^-$	295.50	± 3.9
Dihydrocaffeic acid	7.61	C9H10O4	182.0579	182.0571	-4.2781	$[M-H]^-$	206.18	± 1.4
Total counts							501.68	
				Flavanals				
Procyanidin dimer B2	3.59	C30H26O12	578.1424	578.1439	2.6286	$[M-H]^-$	ND	
(+)-Catechin	3.86	C15H14O6	290.0790	290.0781	-3.1342	$[M-H]^-$	162.88	± 6.3
(-)-Epicatechin	4.72	C15H14O6	290.0790	290.0794	1.1163	$[M-H]^-$	ND	

(-)-Epigallocatechin gallate	4.78	C15H14O7	458.0849	458.0832	-3.6407	[M-H]-	ND	
(+)-Gallocatechin	4.79	C15H14O7	306.0740	306.0721	-5.9957	[M-H]-	307.03	± 7.9
(+)-Gallocatechin 3-O-gallate	5.13	C22H18O11	458.0849	458.0828	-4.6105	[M-H]-	277.53	± 0.0
(-)-Epicatechin 3-O-gallate	5.87	C22H18O11	442.0900	442.0937	8.4310	[M-H]-	ND	
Prodelphinidin dimer B3	7.43	C30H26O14	610.1323	610.1321	-0.3353	[M-H]-	37076.15	± 24
Total counts							37823.60	
				Flavanones				
Naringin	5.53	C27H32O14	580.1792	580.1799	1.1405	[M-H]-	ND	
Naringenin 7-O-glucoside	5.79	C21H22O10	434.1213	434.1210	-0.7202	[M-H]-	233.17	± 0.0
Naringin 4-O-glucoside	6.09	C33H42O19	742.2320	742.2282	-5.2126	[M-H]-	508.41	± 0.0
Eriodictyol	6.76	C15H12O6	288.0634	288.0658	8.3489	[M-H]-	179.90	± 98
Hesperidin	6.78	C28H34O15	610.1898	610.1890	-1.2157	[M-H]-	346.06	± 14
Narirutin	7.01	C27H32O14	580.1792	580.1813	3.5491	[M-H]-	ND	
Naringenin	8.72	C15H12O5	272.0685	272.0680	-1.9082	[M-H]-	1126.69	± 1.
Eriocitrin	9.82	C27H32O15	596.1741	596.1710	-5.2451	[M-H]-	971.49	± 15
Total counts							3365.72	
				Flavonols				
Quercetin 3-O-(6-malonyl-glucoside)	3.56	C24H22O15	550.0959	550.0916	-7.8563	[M-H]-	ND	
Kaempferol 3,7,4-O-triglucoside	3.67	C33H40O21	772.2062	772.2057	-0.6334	[M-H]-	ND	
Quercetin 3-O-glucoside	3.77	C21H20O12	464.0955	464.0965	2.2632	[M-H]-	12030.02	± 30
Kaempferol 3-O-sophoroside	4.00	C27H30O16	610.1534	610.1531	-0.4029	[M-H]-	1402.30	± 1
Quercetin 3-O-(6-acetyl-galactoside) 7-O-rhamnoside	4.02	C29H32O17	652.1639	652.1656	2.6023	[M-H]-	ND	
Kaempferol 3-O-xylosyl-glucoside	4.19	C26H28O15	580.1428	580.1432	0.6335	[M-H]-	347993.48	± 67
Kaempferol 3-O-glucuronide	4.91	C21H18O12	462.0798	462.0808	2.0525	[M-H]-	1213.97	± 10
Quercetin 3,4-O-diglucoside	4.97	C27H30O17	478.0747	478.0738	-1.9902	[M-H]-	832.69	± 81
6,8-Dihydroxykaempferol	4.98	C15H10O8	318.0376	318.0374	-0.6057	[M-H]-	2644.24	± 9.0
Kaempferol 2-O-(2''-rhamnosyl-6''-acetyl-galactoside) 7-O-rhamnoside	5.13	C34H40O21	784.2062	784.2042	-2.5278	[M-H]-	2325.22	± 14
Quercetin 3-O-sophoroside	5.23	C27H30O17	626.1483	626.1484	0.2092	[M-H]-	7874.96	± 30
Quercetin 3-O-xylosyl-rutinoside	5.23	C32H38O20	742.1956	742.1961	0.5775	[M-H]-	13776.96	± 17
Kaempferol 3-O-glucosyl-rhamnosyl-glucoside	5.25	C33H40O20	756.2113	756.2116	0.3447	[M-H]-	4955.60	± 13

Myricetin 3-O-glucoside	5.32	C21H20O13	480.0904	480.0911	1.4984	$[M-H]^-$	43391.09	± 18
Kaempferol 3-O-sophoroside 7-O-glucoside	5.33	C33H40O21	772.2062	772.2043	-2.4998	$[M-H]^-$	ND	
Quercetin 3-O-(6-malonyl-glucoside) 7-O-glucoside	5.35	C30H32O20	712.1487	712.1514	3.8424	$[M-H]^-$	ND	
Quercetin 3-O-glucosyl-xyloside	5.35	C26H28O16	478.0747	478.0760	2.6756	$[M-H]^-$	2402.47	± 10
Kaempferol 3-O-(6''-acetyl-galactoside) 7-O-rhamnoside	5.53	C29H32O16	636.1690	636.1699	1.4395	$[M-H]^-$	253.46	± 0.
Kaempferol 3-O-(2''-rhamnosyl-galactoside) 7-O-rhamnoside	5.60	C33H40O19	740.2164	740.2176	1.7060	$[M-H]^-$	2023.13	± 94
Quercetin 3-O-galactoside 7-O-rhamnoside	5.75	C27H30O16	610.1534	610.1539	0.7666	$[M-H]^-$	101829.40	± 36
Kaempferol 3-O-acetyl-glucoside	5.84	C23H22O12	490.1111	490.1112	0.1834	$[M-H]^-$	224.43	± 0.
Myricetin 3-O-rhamnoside	5.93	C21H20O12	464.0955	464.0962	1.6555	$[M-H]^-$	106177.96	± 37
Kaempferol 3-O-galactoside 7-O-rhamnoside	6.23	C27H30O15	594.1585	594.1586	0.2351	$[M-H]^-$	18310.62	± 81
Quercetin 3-O-xyloside	6.23	C20H18O11	434.0849	434.0865	3.5681	$[M-H]^-$	268.24	± 10
Quercetin 3-O-rhamnoside	6.42	C21H20O11	448.1006	448.1014	1.8057	$[M-H]^-$	15897.25	± 34
Quercetin 3-O-acetyl-rhamnoside	6.62	C23H22O12	490.1111	490.1114	0.5734	$[M-H]^-$	785.61	± 5.
Myricetin	6.77	C15H10O8	318.0376	318.0373	-0.9378	$[M-H]^-$	71000.42	± 11
Rhamnetin	7.42	C16H12O7	316.0583	316.0563	-6.2177	$[M-H]^-$	155.42	± 4.
Quercetin	7.89	C15H10O7	302.0427	302.0424	-0.8965	$[M-H]^-$	188623.30	± 49
Kaempferol	8.90	C15H10O6	286.0477	286.0474	-1.0541	$[M-H]^-$	16215.84	± 34
Isorhamnetin	9.12	C16H12O7	316.0583	316.0579	-1.3591	$[M-H]^-$	409.02	± 22
Total counts							963017.11	
				Dihydroflavonols				
Dihydroquercetin 3-O-rhamnoside	4.00	C21H22O11	450.1162	450.1182	4.3305	$[M-H]^-$	1726.10	± 36
Dihydromyricetin 3-O-rhamnoside	4.13	C21H22O12	466.1111	466.1118	1.3853	$[M-H]^-$	4236.58	± 19
Dihydroquercetin	5.22	C15H12O7	304.0583	304.0581	-0.5487	$[M-H]^-$	173.23	± 13
Total counts							6135.90	
				Anthocyanins				
Delphinidin 3-O-glucosyl-glucoside	3.61	C27H31O17	627.1561	627.1557	-0.7027	$[M-e]^+$	1268.68	± 27
Delphinidin 3-O-arabinoside	3.69	C20H19O11	435.0927	435.0919	-1.9893	$[M-e]^+$	167.93	± 0.
Delphinidin 3-O-glucoside	3.72	C21H21O12	465.1033	465.1034	0.1074	$[M-e]^+$	18669.91	± 30

Delphinidin 3-O-sambubioside	3.72	C26H29O16	597.1456	597.1457	0.2036	$[M-e]^+$	841271.66	± 521
Cyanidin 3-O-sophoroside	3.97	C27H31O16	611.1612	611.1609	-0.4783	$[M-e]^+$	1352.39	± 15
Cyanidin 3-O-glucoside	4.18	C21H21O11	449.1084	449.1087	0.6123	$[M-e]^+$	15328.38	± 24
Cyanidin 3-O-(6"-malonyl-glucoside)	4.58	C24H23O14	535.1088	535.1067	-3.9681	$[M-e]^+$	ND	
Pelargonidin 3-O-sambubioside	4.61	C26H29O14	565.1557	565.1567	1.7786	$[M-e]^+$	830.99	± 20
Cyanidin 3-O-(3",6"-O-dimalonyl-glucoside)	4.67	C27H25O17	621.1092	621.1138	7.4314	$[M-e]^+$	439.07	± 14
Cyanidin 3-O-xyloside	4.69	C20H19O10	419.0978	419.0956	-5.4000	$[M-e]^+$	161.83	± 25
Cyanidin 3-O-(6"-malonyl-3"-glucosyl-glucoside)	4.87	C30H33O19	697.1616	697.1654	5.4053	$[M-e]^+$	805.57	± 0.(
Cyanidin 3-O-sambubioside 5-O-glucoside	5.23	C32H39O20	743.2035	743.2044	1.3178	$[M-e]^+$	3206.79	± 26
Delphinidin 3,5-O-diglucoside	5.23	C27H31O17	627.1561	627.1567	0.9311	$[M-e]^+$	1344.62	± 8.(
Pelargonidin 3-O-(6"-malonyl-glucoside)	5.24	C24H23O13	519.1139	519.1131	-1.4416	$[M-e]^+$	ND	
Cyanidin 3-O-glucosyl-rutinoside	5.25	C33H41O20	757.2191	757.2232	5.3801	$[M-e]^+$	975.34	± 2.(
Cyanidin 3-O-sambubioside	5.44	C26H29ClO15	616.1195	616.1192	-0.5174	$[M-e]^+$	1290.65	± 7.3
Delphinidin 3-O-galactoside	5.44	C21H21O12	465.1033	465.1031	-0.3770	$[M-e]^+$	6652.42	± 16
Delphinidin 3-O-xyloside	5.44	C20H19O11	435.0927	435.0926	-0.2243	$[M-e]^+$	ND	
Cyanidin 3-O-arabinoside	5.58	C20H19O10	419.0978	419.1009	7.3172	$[M-e]^+$	ND	
Pelargonidin 3-O-glucosyl-rutinoside	5.60	C33H41O19	741.2242	741.2247	0.6549	$[M-e]^+$	288.89	± 0.(
Cyanidin 3-O-xylosyl-rutinoside	5.66	C32H39O19	727.2086	727.2134	6.7312	$[M-e]^+$	ND	
Cyanidin 3,5-O-diglucoside	5.75	C27H31O16	611.1612	611.1621	1.4957	$[M-e]^+$	22164.69	± 17
Pelargonidin 3,5-O-diglucoside	5.75	C27H31ClO15	630.1351	630.1351	-0.0337	$[M-e]^+$	705.83	± 65
Pelargonidin 3-O-glucoside	5.78	C21H21O10	433.1135	433.1139	0.9036	$[M-e]^+$	187.00	± 0.(
Cyanidin 3-O-rutinoside	5.80	C27H31O15	595.1663	595.1664	0.2514	$[M-e]^+$	362.10	± 14
Pelargonidin 3-O-sophoroside	6.23	C27H31O15	595.1663	595.1672	1.5912	$[M-e]^+$	2670.15	± 21
Delphinidin 3-O-(6"-acetyl-glucoside)	6.24	C23H23O13	507.1139	507.1171	6.2822	$[M-e]^+$	150.34	± 0.(
Cyanidin 3-O-galactoside	6.41	C21H21O11	449.1084	449.1088	0.9211	$[M-e]^+$	689.88	± 22
Cyanidin 3-O-(6"-caffeoyl-glucoside)	7.11	C30H27O14	611.1401	611.1407	1.0948	$[M-e]^+$	2915.26	± 57
Pelargonidin 3-O-arabinoside	7.14	C20H19O9	403.1029	403.1025	-0.9678	$[M-e]^+$	ND	
Pelargonidin 3-O-galactoside	7.41	C21H21O10	433.1135	433.1131	-0.8553	$[M-e]^+$	112.15	± 0.(

Delphinidin 3-O-(6"-p-coumaroyl-glucoside)	7.43	C30H27O14	611.1401	611.1406	0.8644	$[M-e]^+$	7040.75	± 10
Delphinidin 3-O-feruloyl-glucoside	7.54	C31H29O15	641.1506	641.1465	-6.4428	$[M-e]^+$	ND	
Cyanidin 3-O-(6"-p-coumaroyl-glucoside)	7.95	C30H27O13	595.1452	595.1451	-0.0297	$[M-e]^+$	4411.03	± 74
Total counts							935464.29	
				Organic acids				
Gluconic acid	0.57	C6H12O7	196.0583	196.0584	0.5986	$[M-H]^-$	1869.13	± 77
Fumaric acid	0.60	C4H4O4	116.0110	116.0111	0.8337	$[M-H]^-$	335.93	± 5.
Hydroxycitric acid	0.60	C6H8O8	208.0219	208.0219	-0.0001	$[M-H]^-$	122935.53	± 13
Malic acid	0.60	C4H6O5	134.0215	134.0222	5.3796	$[M-H]^-$	768.04	± 66
Hibiscus acid	0.63	C6H6O7	190.0114	190.0112	-0.6328	$[M-H]^-$	514694.86	± 70
Succinic acid	0.63	C4H6O4	118.0266	118.0266	-0.3947	$[M-H]^-$	826.29	± 23
Ascorbic acid	0.64	C6H8O6	176.0321	176.0315	-3.2016	$[M-H]^-$	105.64	± 0.
Glutaric acid	0.78	C5H8O4	132.0423	132.0415	-5.5200	$[M-H]^-$	269.81	± 44
Maleic acid	0.83	C4H4O4	116.0110	116.0111	0.9672	$[M-H]^-$	253.19	± 1.
Hibiscus acid 6-O-methyl ester	0.88	C7H8O7	204.0270	204.0270	-0.0503	$[M-H]^-$	43304.45	± 12
Citric acid	1.01	C6H8O7	192.0270	192.0259	-5.7809	$[M-H]^-$	ND	
Hibiscus acid hydroxyethyl dimethyl esther	1.45	C10H16O8	264.0845	264.0832	-4.8959	$[M-H]^-$	ND	
Hibiscus acid dimethyl ester	1.46	C8H10O7	218.0427	218.0425	-0.6725	$[M-H]^-$	595.61	± 13
Hibiscus acid hydroxyethyl dimethyl esther	1.63	C8H12O8	236.0532	236.0532	-0.1657	$[M-H]^-$	1170.55	± 29
Quinic acid	3.93	C7H10O5	192.0634	192.0631	-1.3690	$[M-H]^-$	74274.51	± 26
Shikimic acid	4.83	C7H10O5	174.0528	174.0526	-1.3078	$[M-H]^-$	2957.36	± 59
Total counts							764360.90	

Results are expressed as counts ± SD. ND: not detected.

Table 3. Identification of hydrolysable polyphenols by UPLC-ESI-QTOF MS^E of roselle calyces and its by-product

Component name	Molecular formula	Expected mass (Da)	Observed mass (Da)	Mass error (ppm)	Retention time (min)	Adducts	Roselle by-	
Hydroxycinnamic acids								
Cinnamic acid	C9H8O2	148,0524	148,0513	-7,9027	1,48	$[M-H]^-$	280,70	±
3-Caffeoylquinic acid	C16H18O9	354,0951	354,0953	0,7208	2,56	$[M-H]^-$	138,61	±
o-Coumaric acid	C9H8O3	164,0473	164,0471	-1,4475	3,69	$[M-H]^-$		ND
Caffeic acid	C9H8O4	180,0423	180,0421	-0,7888	4,19	$[M-H]^-$	1177,61	±
p-Coumaroyl malic acid	C13H12O7	280,0583	280,0570	-4,7846	4,40	$[M-H]^-$	267,93	±
Caffeic acid ethyl ester	C11H12O4	208,0736	208,0725	-5,2651	4,42	$[M-H]^-$		ND
Ferulic acid 4-O-glucoside	C16H20O9	356,1107	356,1109	0,5237	4,56	$[M-H]^-$		ND
Isoferulic acid	C10H10O4	194,0579	194,0577	-1,0468	4,61	$[M-H]^-$	216,98	±
3-Feruloylquinic acid	C17H20O9	368,1107	368,1114	1,8853	4,63	$[M-H]^-$		ND
Ellagic acid	C14H6O8	302,0063	302,0063	0,0888	5,65	$[M-H]^-$	530,02	±
Ferulic acid	C10H10O4	194,0579	194,0576	-1,5485	5,72	$[M-H]^-$	2161,45	±
3,5-Dicaffeoylquinic acid	C25H24O12	516,1268	516,1270	0,5040	6,30	$[M-H]^-$	228,87	±
Total counts								5002.
Hydroxybenzoic acids								
2-Hydroxybenzoic acid 4-O-glucoside	C13H16O8	300,0845	300,0846	0,3629	1,21	$[M-H]^-$		ND
Vanillic acid	C8H8O4	168,0423	168,0421	-0,8113	1,69	$[M-H]^-$	112,49	±
2,4-Dihydroxybenzoic acid	C7H6O4	154,0266	154,0267	0,4600	1,94	$[M-H]^-$	414,57	±
3-Hydroxybenzoic acid	C7H6O3	138,0317	138,0316	-0,6198	3,12	$[M-H]^-$	4746,12	±
2,6-Dihydroxybenzoic acid	C7H6O4	154,0266	154,0265	-0,8566	3,32	$[M-H]^-$	127,97	±
3-Hydroxybenzoic acid 4-O-glucoside	C13H16O8	300,0845	300,0838	-2,3637	3,71	$[M-H]^-$	268,38	±
Gallic acid	C7H6O5	170,0215	170,0214	-0,6540	4,22	$[M-H]^-$	266,17	±
Benzoic acid	C7H6O2	122,0368	122,0367	-0,2766	4,27	$[M-H]^-$	371,19	±
Gallic acid ethyl ester	C9H10O5	198,0528	198,0526	-0,9592	4,50	$[M-H]^-$	425,16	±
Protocatechuic acid	C7H6O4	154,0266	154,0265	-0,4363	4,93	$[M-H]^-$	167,47	±
4-Hydroxybenzoic acid	C7H6O3	138,0317	138,0316	-0,6430	6,69	$[M-H]^-$	150,47	±
Total counts								7049.
Flavanals								
(-)-Epigallocatechin gallate	C15H14O7	458,0849	458,0832	-3,6407	4,78	$[M-H]^-$		ND
(-)-Epicatechin 3-O-gallate	C22H18O11	442,0900	442,0937	8,4310	5,87	$[M-H]^-$	1036,88	±
Prodelphinidin dimer B3	C30H26O14	610,1323	610,1321	-0,3353	7,43	$[M-H]^-$	705,05	±
Total counts								1741.
Flavanones								

Naringenin 7-O-glucoside	C21H22O10	434,1213	434,1210	-0,7202	5,79	$[M-H]^-$	399,50	±
Hesperidin	C28H34O15	610,1898	610,1890	-1,2157	6,78	$[M-H]^-$	1250,31	±
Naringenin	C15H12O5	272,0685	272,0680	-1,9082	8,72	$[M-H]^-$	413,42	±
Total counts								2063.
			Flavonols					
Kaempferol 3,7,4-O-triglucoside	C33H40O21	772,2062	772,2057	-0,6334	3,67	$[M-H]^-$		ND
Myricetin 3-O-rhamnoside	C21H20O12	464,0955	464,0962	1,6555	5,93	$[M-H]^-$	433,43	±
Kaempferol 3-O-glucoside	C21H20O11	448,1006	448,1005	-0,0578	6,42	$[M-H]^-$	448,44	±
Rhamnetin	C16H12O7	316,0583	316,0563	-6,2177	7,42	$[M-H]^-$	375,10	±
Quercetin	C15H10O7	302,0427	302,0424	-0,8965	7,89	$[M-H]^-$	413,26	±
Kaempferol	C15H10O6	286,0477	286,0474	-1,0541	8,90	$[M-H]^-$	84,09	±
Total counts								1754.

Table 4. Body weight and biochemical parameters in rats fed with a HF/HFr diet and supplemented with roselle calyces and its by-product.

	Standard diet	HF/HFr	HF/HFr + calyx	HF/HFr + by-product
Body weight (g)	541.4 ± 9.3 [c]	678.5 ± 11.2 [a]	583.7 ± 15.3 [bc]	610.0 ± 18.3 [b]
Glucose (mg/dL)	140.3 ± 7.4 [b]	218.5 ± 25.7 [a]	134.8 ± 9.3 [b]	138.6 ± 6.9 [b]
Insulin (ng/mL)	1.0 ± 0.1 [b]	2.0 ± 0.1 [a]	1.2 ± 0.1 [b]	1.6 ± 0.2 [ab]
HOMA index	9.0 ± 3.1 [b]	25.2 ± 4.2 [a]	10.3 ± 2.4 [b]	13.1 ± 2.2 [b]
Plasmatriglyceride (mg/dL)	83.9 ± 5.9 [b]	130.8 ± 8.9 [a]	115.3 ± 8.1 [a]	107.5 ± 7.1 [ab]
Triglyceride in feces (µg/g)	8.1 ± 0.6 [b]	8.4 ± 0.6 [b]	10.6 ± 0.4 [a]	9.6 ± 0.3 [ab]

Results are expressed as mean ± SE. Means within a same line with different superscript letters differ significantly by Tukey's test ($p < 0.05$).

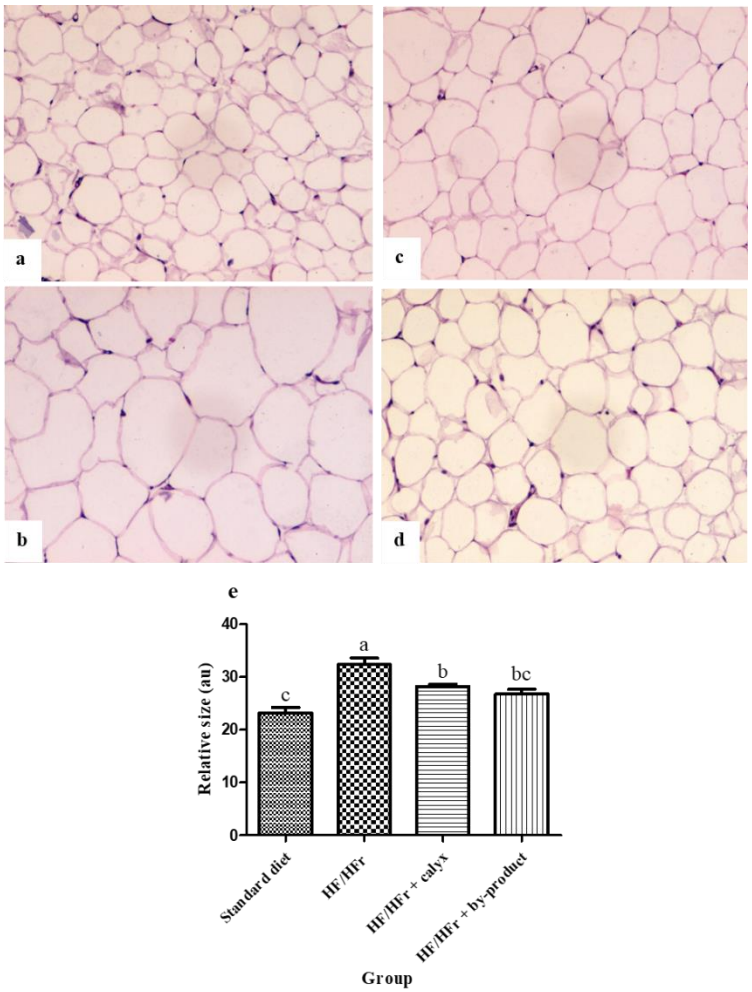

Figure 1. Histological pictures of adipocites (300x) of rats following different diets: (a) Standart diet, (b) HF/HFr diet, (c) HF/HFr diet plus calyces, (d) HF/HFr diet plus by-product (e) relative size of adipocytes in all diets

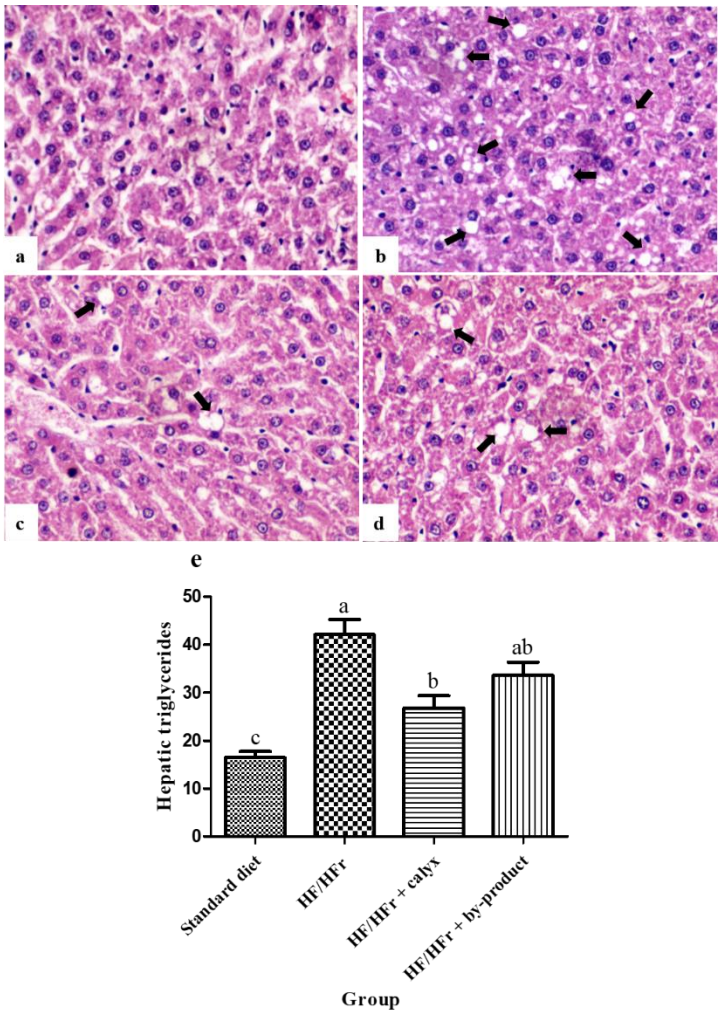

Figure 2. Histological pictures of liver sections (300x) in rats following different diets: (a) Standard diet, (b) HF/HFr diet, (c) HF/HFr diet plus calyces, (d) HF/HFr diet plus by-product (e) hepatic triglycerides content (mg tg/g protein) in all diets