Metabolic fate and cardiometabolic effects of phenolic compounds from red-fleshed apple in hypercholesterolemic rats: A comparative study with common white-fleshed apple. The AppleCOR Study.

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Abstract

The present study aimed to investigate the metabolic fate and the cardiometabolic effects of phenolic compounds (PC) provided by a red-fleshed apple variety biofortified in anthocyanins (ACN). Wistar rats were feed with high-fat diet (HFD) to induce hypercholesterolemia and supplemented with red-fleshed apple (HFD+R), white-fleshed apple (HFD+W) or an ACN-rich infusion from aronia fruit (HFD+A) providing matched content and profile of ACN. Plasma biochemical parameters, histological analysis and phenol biological metabolites were determined. Plasma, urine and faeces showed a significant increase of ACN metabolites after HFD+R and HFD+A, while flavan-3-ols were significantly increased after HFD+W and dihydrochalcones derivatives increased after both apples supplementation. A cardioprotective effect was observed after both apples and aronia infusion supplementation in the reduction of aortic thickness. The kidney function was improved after all supplementations but only in females, probably related to the higher phenol bioavailability reported in females. A decrease in insulin plasma concentration after both apples supplementation was also observed only in males. Our findings support that ACN without apple matrix can induce cardioprotective effects. ACN or flavan-3-ols, together with dihydrochalcones, compose a phenolic phytocomplex in red and white-fleshed apples, respectively, that could act synergistically in the attenuation of cardiovascular outcomes in hypercholesterolemic rats.

Keywords: anthocyanins, red-fleshed apple, phenolic compounds, aortic thickness, Bowman’s space, sustained intake, UPLC-MS/MS.

INTRODUCTION

Phenolic compounds (PC) are among the most abundant phytochemicals present in the human diet, and increasing evidence highlights their important health-promoting effects.[1,2] At present, PC garner much attention due to their protection against cardiovascular diseases (CVD),[3] which are the first cause of death globally (17.9 million people died from CVD in 2016).[4] Understanding the bioavailability of PC, their metabolism and tissue distribution is critical since their physiological impact depend on their delivery to target tissues.[5,6] Once the PC are absorbed in the upper part of the gastrointestinal tract, they are mainly metabolized in the small intestine and liver by phase-II enzymes to their sulphate, methyl, and glucuronide conjugates. However, it is estimated that only 5–10% of the total polyphenol intake is absorbed in the small intestine. The remaining 90–95% may accumulate in the large intestinal lumen where they are subjected to the enzymatic activities of the gut microbial community.[7] All these metabolites are those that reach circulation and target tissues and may be responsible for the health effects derived from the consumption of PC-rich foods.

Another fact to consider in the study of PC bioavailability and their health effects is the food matrix in which these compounds are found, since in most fruits and vegetables many of these compounds are linked to carbohydrates, fiber, proteins and cell walls as well as to other PC by covalent bonds, hydrogen bonding, and hydrophobic and hydrophilic interactions.[8] During gastrointestinal digestion, the binding of PC to these molecules affects their release in the gut as well as the efficacy by which they are transported across the mucosal epithelium and this may lead to a decrease in the PC bioaccessibility[9,10] and, in consequence in their bioactivity.

Anthocyanins (ACN) have been extensively reported to manifest therapeutic properties against hyperlipidaemia and CVD, among others.[11-13] Some authors reported that after oral intake short-term experiments in animals, intact ACN were mainly detected in tissues like heart, liver, kidney, lung[14] or brain.[15] In the last few years, there has been an increasing interest in potential crops for coloring food naturally, such as red-fleshed apples cultivars. Due to their enhanced content of ACN, different studies have shown that the total phenolic content and antioxidant capacity of red-fleshed apples were significantly higher compared to common white-fleshed apple cultivars, which indicates that they could have presumably added healthy properties.[16] Indeed, red-fleshed apple supplementation in rats has already shown protective effects against colon carcinogenesis retarding/diminishing the appearance of the precancerous markers and the expression of genes related to this cancer.[17]

The emerging potential of red-fleshed apples as a novel ACN-rich fruit along with the differences reported in the PC bioavailability and bioactivity depending on the food matrix, substantiates the present research focused on the posible health benefits of red-fleshed apple. The main objective of the present study was to investigate the cardiometabolic effects of the diet supplementations with red-fleshed apple and compared to common white-fleshed apple in hypercholesterolemic rats through the analysis of histological parameters in target tissues (liver, heart, kidney and aorta). To study the apple matrix effect, rats were also supplemented with an ACN-rich infusion from aronia fruit (*Aronia melanocarpa*). To fully understand the observed effects, we also performed a comprehensive analysis of the metabolic fate and metabolic pathways of ACN and other PC from apple in rat plasma, urine and faeces.

1. MATERIALS AND METHODS

1.1. Chemicals and reagents

Cyanidin-3-*O*-galactoside, eriodictyol, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, procyanidin dimer B_2, phloretin-2'-*O*-glucoside, *p*-coumaric acid, and caffeic acid were purchased from Extrasynthese (Genay, France). *p*-Hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (aka protocatechuic acid), hippuric acid, 3-(4'-hydroxyphenyl)acetic acid, 3-(3',4'-dihydroxyphenyl)acetic acid, 3-(3'-hydroxyphenyl)propionic acid, 3-(3',4'-dihydroxyphenyl)propionic acid (aka dihydrocaffeic acid), 3-(3'-hydroxy-4'-methoxyphenyl)propionic acid (aka dihydroferulic acid), epicatechin, and 5-O-caffeoylquinic acid (aka chlorogenic acid) were from Sigma-Aldrich (St. Louis, MO, USA). Vanillic acid and ferulic acid were from Fluka (Buchs, Switzerland). Vanillic acid-4-*O*-sulphate, catechol-4-*O*-sulphate, and 4-methyl catechol sulphate were synthesized according to [18], and were kindly supplied by Dr. Claudia N. Santos (University of Lisbon, Portugal).

Methanol (HPLC grade), acetonitrile (HPLC grade), acetic acid, and hydrochloric acid (HCl) were purchased from Scharlab Chemie (Sentmenat, Catalonia, Spain). The water used was Milli-Q quality (Millipore Corp, Bedford, MA, USA).

Stock solutions of standard compounds were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L, and stored in a dark flask at −30 ºC.

1.2. Red- and white-fleshed apples and aronia fruit infusion

To study and compare the cardioprotective effects, two different apple varieties were selected: i) the red-fleshed “Redlove” apple variety, a new genotype naturally biofortified in ACN, and ii) the common white-fleshed Granny Smith apple variety (both provided by NUFRI SAT, Mollerussa, Lleida, Spain) without ACN. Additionally, and to study the effect of ACN without the possible interaction of the apple matrix, an ACN-rich infusion from aronia fruit was selected.

To minimize changes in the bioactive compounds of the apples, a freeze-dried format was selected to prepare the supplemented diets. Briefly, the apple core was removed and the whole apple (with peel) was cut into 1 cm-sized cubes. The apple cubes were immediately frozen in liquid nitrogen and then lyophilized on a Lyobeta 15 TELSTAR Lyophilizer (Terrassa, Spain). The freeze-dried apple cubes were immediately transferred to airtight plastic containers and stored in refrigeration (2ºC) until the analysis of their phenolic composition, and the preparation of the supplemented diets. To obtain the ACN-rich extract, a cold water infusion of *Aronia melanocarpa* fruit powder (Aronia Pulver, BIOJOY, Nuremberg, Germany) was prepared, which was equivalent in dose and type of ACN to the red-fleshed apple. Briefly, distilled water was added to aronia fruit powder (in proportion 1:1), and the mixture was homogenized (Kinematica Polytron, Polytron Corporation, Montreal, Canada) for 60 seconds. The resulting mixture was centrifuged (5403 *g* for 5 min), and the supernatant was analyzed and added to the drinking water of the rats. The phenolic composition of the freeze-dried apples, and the aronia infusion is shown in **Table 1**.

1.3. Animals and experimental procedure

Thirty *Wistar* rats weighting between 300 and 350 g were purchased from Charles River Laboratories (Barcelona, Spain). They were divided into 5 groups of 6 animals each one (3 males and 3 females) as follows, Group 1: standard chow-diet (SCD) (Teklad 2014, rodent maintenance diet, Envigo, Huntingdon, Cambridgeshire, United Kingdom); Group 2: high-fat diet (HFD) (Atherogenic Rodent Diet TD. 02028, Envigo, Huntingdon, Cambridgeshire, United Kingdom) to induce hypercholesterolemia; Group 3: HFD supplemented with white-fleshed apple (HFD+W), Group 4: HFD supplemented with red-fleshed apple (HFD+R), and Group 5: HFD supplemented with anthocyanin extract from aronia (HFD+A).

The design of the study is shown in **Supplemental Figure 1.**

Group 1 was fed with chow diet for 9 weeks. The other groups were fed during 3 weeks with a HFD and the following 6 weeks with the HFD supplemented with the different products. For HFD+R and HFD+W (Groups 3 and 4), HFD pellets were crushed in a mill along with the freeze-dried apples. For HFD+A (Group 5), the Aronia extract was dissolved daily in the drinking water. Moreover, diets from Group 2 (HFD) and Group 5 (HFD+A) were modified by adding 25% of chow diet in the same proportion as apples, so that all groups except Group 1, would take the same proportion of HFD during the supplementation period.

To prepare the supplemented diets, HFD pellets and lyophilized apples (red- or white-fleshed) were crushed in a mill (MC300132, Moulinex, Alençon, France) until a homogeneous powder was obtained and mixed. Then distilled water (10%) was added, the mixture was homogenized and new apple-enriched pellets were prepared and dried in an oven (JA Selecta, Barcelona, Spain) at 25ºC for 3 days (at darkness).

The supplementation of ACN through red-fleshed apple or aronia was based on the human equivalent dose of 70 mg/day of ACN, according to [19]. So, the quantity of red-fleshed apple administered and the aronia fruit extract in water was adjusted to a dose of 1.8 and 1.9 mg/day/rat of ACN, respectively (**Table 1**). The quantity of white-fleshed apple to prepare HFD+W was equivalent to HFD+R.

During the 9 weeks of the experiment, the rats were housed in cages on a 12 h light-12 h dark schedule at controlled temperature (21 ± 1 ºC), and humidity (55 ± 10%). Food and water were available *ad libitum*. The body weight, food and water intakes were recorded every 3 days.

The day before sacrifice, the rats were caged in metabolic cages for 24 h to collect urine and faeces. The rats were sacrificed by an intracardiac puncture after isoflurane anesthesia (IsoFlo, Veterinarian Esteve, Bologna, Italy). Blood was collected in EDTA tubes, and plasma samples were obtained by centrifugation (3000 *g*, 10 min at 4 ºC) and stored at –80 ºC until chromatographic analysis of phenolic metabolites. After blood collection, the rats were perfused with an isotonic solution of sodium chloride (0.9%) to remove the remaining blood in tissues. The heart, aorta, liver and kidneys were excised and immediately frozen in liquid nitrogen. A part of the tissue samples were stored at –80 ºC until analysis and the other part were fixed in 10% (v/v) formalin for a minimum of 24 h.

The animal procedures were conducted in accordance with the guidelines of the European Communities Directive 2010/63/EU regulating animal research. The protocols were approved by the Animal Ethical Committee of the University of Lleida (CEEA 01-10/17), and performed under a Generalitat de Catalunya Project License (10038). The study complies with the ARRIVE guidelines developed by the NC3Rs.[20]

1.4. Plasma, urine and faeces analysis of anthocyanin and phenolic metabolites by Ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)

1.4.1. Pre-treatment of plasma samples

Before the chromatographic analyses, the rat plasma samples were pre-treated by micro-Elution solid-phase extraction (µSPE) using OASIS HLB (2 mg, Waters, Milford, MA) micro-cartridges. The methodology used is reported in our previous study.[21] Briefly, the micro-cartridges were conditioned sequentially with 250 µL of methanol and 250 µL of 0.2% acetic acid. Then, 350 µL of 4% phosphoric acid solution was added to 350 µL of the rat plasma sample, and then this solution was loaded into the micro-cartridges. The loaded micro-cartridges were cleaned-up with 200 µL of Milli-Q water, and 200 µL of 0.2% acetic acid. Afterwards, the retained phenolic compounds were eluted with 2 x 50 µL of methanol. Each sample was prepared in duplicate.

1.4.2. Pre-treatment of urine samples

The urine samples were also pre-treated by µSPE. The micro-cartridges and their conditioning and equilibration steps were the same as reported for plasma samples. In this case, 100 µL of phosphoric acid at 4% was added to 100 µL of the urine sample, and this solution was loaded into the micro-cartridge. The retained phenolic compounds were then eluted with 2 x 50 µL of methanol. Each sample was prepared in duplicate.

1.4.3. Pre-treatment of faeces samples

The faeces were pre-treated as it was reported in our previous study.[22] Briefly, 100 mg of lyophilized faeces were mixed in 1 ml of the solution methanol/HCl/Milli-Q water (79.9/0.1/20, v/v/v), and shaken in a vortex (Multi vortex, VWR, Franklin, MA, USA) for 15 min. After that, the sample was centrifuged at 8784 *g* for 10 min at 4 ºC, and the supernatant was collected, and centrifuged under the same conditions. Finally, the supernatant was filtered with a 0.22 µm syringe Nylon filter and transferred into chromatographic vials until the chromatographic analysis.

1.4.4. Chromatographic analysis (UPLC-MS/MS)

LC analyses were carried out on an AcQuity Ultra-Performance^{TM} liquid chromatography and tandem mass spectrometry equipment from Waters (Milford, MA, USA). Two chromatographic methods were used for the analysis of 1) anthocyanins and their metabolites, and 2) the rest of the phenolic compounds and their metabolites. In both methods, the flow rate was 0.3 mL/min, and the injection volume 2.5 µL. The UPLC-MS/MS conditions were the same used in our previous studies.[16, 21, 23] Tandem MS analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface.

Due to the lack of commercial phenolic standards and their generated metabolites, some of these compounds were tentatively quantified by using the calibration curve of their precursor or another phenolic compound with a similar structure. **Supplemental Table 1** shows the selected reaction monitoring (SRM) conditions as well as its cone voltage and collision energy used for the quantification of these phenolic compounds. This table also shows in which phenolic standard compound, these phenolics have been quantified.

1.5. Histological analysis

To investigate the possible protective effects against the high fat diet of red-fleshed apple, white-fleshed apple or aronia infusion, different histological stains of aorta, kidney, heart, and liver tissues were performed and different parameters were assessed.

1.5.1. Aorta and kidney haematoxylin-eosin staining

Aorta and kidney samples were fixed in 10% formaldehyde, dehydrated in a graded alcohol series and cleared in xylene. Later, the samples were embedded in paraffin (Panreac Quimica Slu, Castellar del Vallès, Spain) and the different sections were cut using a microtome (Microm HM 340E, Barcelona, Spain). Each paraffin block was cut to 4-µm thickness. The sections were stained with haematoxylin and eosin for light microscopic examination according to standard procedures. Microscopic tissue images were taken in an Olimpus BX50 microscopic system (Olympus Corporation, Shinjuku, Tokyo, Japan) at x10 magnification.

To evaluate possible deposition of lipids in aorta, which can lead to the formation of atherosclerotic plaques, its thickness was measured in the stained sections.

To assess possible protective effects in kidney, the renal structure was evaluated through the analysis of the Bowman's space, which can be altered after the chronic administration of dietary lipids.

The aortic thickness and the Bowman's space were determined using the program CellSens Entry (Microscopy Imaging Software by Olympus Life Science, Olympus Corporation, Shinjuku, Tokyo, Japan). To evaluate the thickness of the aorta and the Bowman's space 60 and 30 measurements were taken per animal, respectively.

1.5.2. Liver Oil-Red staining

A frozen preserved portion of the liver of each rat was subjected to cryostat (Microm HM 505N, Barcelona, Spain) section to a thickness of 8 µm. The stock solutions of oil red O were prepared by completely dissolving 0.5 g of oil red O (Merck, Darmstadt, Germany) with 100 ml 2-isopropanol 60% (Sigma Aldrich, St Louis, Missouri, United States) using a magnetic stirrer and later it was boiled and filtered using a Whatman No. 2 filter paper (GE Healthcare Life Sciences). The samples were stained following the procedure recommended by the manufacturer. Finally, the nuclei were lightly stained by dipping the slides into haematoxylin solution (Casa Álvarez, Madrid, Spain) for 5 min and rinsing with distilled water for examination using light microscopy (Olympus BX50 microscopic system).

1.5.3. Heart Masson's trichome staining

To assess possible protective effects on heart fibrosis, heart samples were stained with Masson's Trichrome stain to observe collagen deposition. Heart samples were embedded in paraffin and cut following the same procedure as kidney and aorta. The sections were stained with Masson's trichrome staining. Briefly, the paraffin samples were deparaffinized by submerging into xylol and rehydrated by submerging in ethanol 100%, 95% and 70% in this order. The slides were submerged in Bouin's solution, in haematoxilin by Weigert, in trichromic solution and in green light solution in this order (with distilled water washes between each step). After that, slides were washed with distillated water and put in ethanol 100%. Before observation, slides were dipped into xylol and xylol-eucalyptol and finally mounted with cover slip for examination using light microscopy (Olympus BX50 microscopic system).

1.6. Plasma biochemical parameters

Total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDLc), non-high-density lipoprotein cholesterol (non-HDLc), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glucose were measured in the Wistar rat plasma by standardized methods using the Cobas Mira Plus autoanalyzer (Roche Diagnostics, Spain). Insulin was measured by Mercodia Rat Insulin Enzyme-linked Immuno Sorbent Assay (ELISA; reference 10-1250-10) from AD Bioinstruments S.L. (Barcelona, Spain). TC, TG, HDLc, non-HDLc, and Glucose are expressed as mg/dL, insulin is expressed as µg/L, and ALT and AST are expressed as U/L. Non-HDLc was calculated by subtracting the HDLc value from the TC value, for each case.

1.7. Statistical analysis

The results are presented as mean values ± standard deviation (SD) for phenolics in SCD, HFD, HDF+W, HFD+R, and HFD+A. The concentration of the anthocyanins and phenolic metabolites in plasma, urine, and faeces samples are presented as mean values ± standard error of the mean (SEM). For the concentration of the phenolic metabolites, the values of males and females, and the sum of males and females were compared intra-groups and inter-day with one-way repeated measures analysis of variance General Linear Model and ANOVA one-way.

The results of the plasma biochemical parameters were presented as mean values ± standard deviation (SD), and were analyzed using Student t-test comparing each treatment versus HFD.

The results of the histological analysis were presented as mean values ± SEM. The mean of Bowman's space and the mean of the aorta thickness were compared intra-groups with Student t-test (between males and females) and compared between the males of the different groups and between the females of different groups with analysis of variance General Linear Model and ANOVA one-way.

Differences were considered significant at $p < 0.05$. All data were analyzed with Minitab Statistical Software, version 17.2.1 (Minitab Inc., State College, Pennsylvania, United States).

2. RESULTS

2.1. Daily dose of phenolics administered

A complete phenolic characterization and quantification of the administered products (red-fleshed apple, white-fleshed apple and aronia infusion) were performed in order to study their metabolic fate after their supplementation in rats and to evaluate their possible biological activities depending on the product administered. **Table 1** shows the daily dose of PC administered to the rats through the supplemented diets.

The daily dose of total PC administered through white-fleshed and red-fleshed apples was very similar, 9.60 and 11.6 mg/day/rat, respectively. Regarding the phenolic composition, both apples had a similar phenolic profile and content of phenolic acids, flavonols, flavanones, and dihydrochalcones, being the daily dose of ACN the main difference between them. Both apples were also different in the flavan-3-ols content, observing that white-fleshed apple contained around 8-fold higher amounts of flavan-3-ols than red-fleshed apple (**Table 1**).

Aronia fruit administered as an infusion was selected as a rich source of ACN without the components of the apple matrix, containing cyanidin-3-*O*-galactoside and cyanidin arabinoside, the main ACN present in red-fleshed apple. Although, the daily dose of total PC through aronia infusion (4.10 mg/day/rat) was lower than the red-fleshed apple (11.6 mg/day/rat), the ACN dose was nearly the same (around 2 mg/day/rat). Half of the phenolic dose in aronia was ACN (46.5%), and the other half were phenolic acids, being caffeoylquinic acid and protocatechuic acid the most abundant (**Table 1**).

2.2. Phenolic profile in plasma, urine and faeces, and their metabolic pathways

To study the metabolic fate of the PC administered through both apples and the aronia infusion, we analyzed the phenol biological metabolites in plasma, urine and faeces, and different metabolic pathways were proposed. **Supplemental Tables 2, 3 and 4** show the concentration of the individual phenolic metabolites that presented a concentration significantly higher than the control diets (SCD or HFD) in plasma, urine and faeces, respectively. These data is presented independently for males and females and also as the sum of both. For a better understanding of the results, data from plasma, urine and faeces have been summarized and represented by the sum of compounds of the main phenolic groups in **Figure 1.** The detected metabolites were derived from the phenolic families of anthocyanins, phenolic acids (benzoic, phenylacetic and phenylpropionic acids), flavan-3-ols, and dihydrochalcones. The phenolic metabolites were mainly phase-II sulphated, glucuronided and/or methylated conjugates, and also microbial catabolites from colonic degradation. In order to elucidate how each phenolic metabolite was generated from its precursor present in apples and aronia infusion, respectively, in **Figure 2** we propose the main metabolic pathways after the intake of the three administered products.

Additionally, in the present study, qualitative and quantitative differences on the metabolic fate of PC depending on the gender have been reported, which can be observed in **Figure 1** and in **Suplemental Tables 2, 3 and 4**. The most significant differences between males and females are commented in sections below and also reflected in the metabolic pathways represented in **Supplemental Figures 2, 3, and 4,** which refer to urinary metabolites detected after HFD+W, HFD+R, and HFD+A, respectively. In addition, the significant differences between males and females observed in faeces samples are shown in **Supplemental Figure 5**.

2.2.1. Anthocyanin metabolites

As expected, ACN and its metabolites were higher after HFD+R and HFD+A than HFD+W, specially reflected in urine samples (**Figure 1b**). Although the supplemented diets HFD+R and HFD+A provided the same daily amount and type of ACN (2 mg/day/rat), qualitative and quantitative differences were observed in the biological samples studied.

As observed in **Figures 1a** and **1b**, the concentration of ACN in plasma and urine was significantly higher after HFD+A compared with HFD+R. In plasma samples, cyanidin-3-*O*-galactoside was the main anthocyanin detected after red-fleshed apple and aronia supplementation. Remarkably, peonidin galactoside was only detected in HFD+A group (**Supplemental Table 2**).

In urine samples, cyanidin-3-*O*-galactoside was also the main anthocyanin detected in HFD+R, however, peonidin galactoside appeared to be the most abundant in HFD+A. Urinary peonidin arabinoside and peonidin galactoside in HFD+A appeared to be significantly higher than in HFD+R, whereas peonidin glucuronide was significantly higher in HFD+R (**Supplemental Table 3**).

It is noteworthy, that in faeces only the ACN parent compounds were detected, being cyanidin-3-*O*-galactoside the main ACN detected in HFD+R, and cyanidin arabinoside in HFD+A (**Supplemental Table 4**).

Figure 2, also shows some other differences observed in the ACN metabolic pathways between red-fleshed apple (red arrows) or aronia infusion (purple arrows). Protocatechuic acid and dihydroxyphenylpropionic acid are the phenolic acids generated after the fission of B-ring (colonic degradation) of cyanidin glycosides.[24] Our results show that after HFD+R and HFD+A, colonic catabolites from protocatechuic acid and dihydroxyphenylpropionic acid route were generated. Although similar metabolites were observed between HFD+R and HFD+A, a higher amount of metabolites were detected in urine and plasma after HFD+A, such as protocatechuic acid sulphate, vanillic acid glucuronide and catechol sulphate (**Supplemental Tables 2 and 3**). It must be noted that the metabolites derived from dihydroxyphenylpropionic acid could also be generated by colonic degradation *via* valerolactones of flavan-3-ols.

No notable differences in ACN metabolism were observed between males and females ($p < 0.05$).

2.2.2. Phenolic acid metabolites

The phenolic acid metabolites detected in plasma were protocatechuic acid sulphate and vanillic acid glucuronide, and together with caffeic acid sulphate, these were also the main excreted metabolites in urine after the three diet supplementations (**Supplemental Tables 2 and 3**). As seen in **Figure 1**, the total phenolic acids concentration only appeared to be significantly higher after HFD+A intake compared to other groups, in plasma and urine samples. Our results are in accordance with a previous study in rats that reported phase II metabolites from protocatechuic acid and vanillic acid in plasma and urine after the intake of an ACN-rich extract.[25]

In faeces, the metabolites detected were protocatechuic acid and hippuric acid. Although in plasma and urine phenolic acids were only detected in higher amounts after aronia supplementation, in faeces, these metabolites appeared in higher concentration after both apple supplementations compared to aronia (**Figure 1d, 1e, 1f**).

It is remarkable that hippuric acid was only detected in faeces samples. Regarding the possible metabolic routes of hippuric acid, we hypothesize three different pathways: 1) by protocatechuic acid derived from the colonic metabolism of cyanidin glycoside; 2) by hydroxyphenylpropionic acid, which has been reported to be a colonic metabolite of dihydrochalcones[26-28]; and 3) by dihydroxyphenylpropionic acid, which is a colonic metabolite of flavan-3-ols (**Supplemental Figure 5).**

Regarding the gender differences, we observed that in general terms total phenolic acid metabolites were detected in significantly higher amounts in females compared to males, mainly observed in plasma (**Figure 1**). Specifically, after HFD+W, females excreted in urine 6-fold more vanillic acid glucuronide than males (**Supplemental Table 3**), and in plasma this metabolite also appeared 7-fold higher in females than in males after HFD+A, and was only detected in females after HFD+R (**Supplemental Table 2**).

As observed in **Figure 1**, phenolic acid metabolites were also detected in significant amounts in SCD and HFD (control diets). Although protocatechuic and vanillic acid metabolites have been reported as colonic metabolites from cyanidin and peonidin glycosides (B-ring fission), respectively,[24] vanillic acid glucuronide could also be generated by β-oxidation from ferulic acid, which was present in significant amounts in SCD and HFD (control diets) (**Supplemental Table 5 and Supplemental Figure 6)**.

2.2.3. Phenylacetic and phenylpropionic acid metabolites

After the three supplemented diets (HFD+W, HFD+R and HFD+A), significant differences in phenylacetic acids (PAA) and phenylpropionic acids (PPA) were only observed in urine and faeces.

In urine samples, the total concentration of PPA and PAA was only significantly higher after HFD+A (**Figure 1j**), being dihydroxyphenylacetic acid sulphate the most abundant metabolite. PPA and PAA metabolites could be generated mainly from ACN by B-ring fission (colonic degradation) in HFD+A, but also from flavan-3-ols in diets supplemented with both apples (**Figure 2**).

Similarly to phenolic acids, PAA and PPA presented a different response in faeces compared to urine, being significantly higher in urine after HFD+A and higher in faeces after HFD+R (**Figure 1g, 1h**). Specifically, *m*-hydroxyphenylacetic was the predominant metabolite in faeces significantly incremented after HFD+R and was only detected in females (**Supplemental Table 4 and Supplemental Figure 5b**).

2.2.4. Flavan-3-ol metabolites

Flavan-3-ols were more abundant in the white-fleshed apple (**Table 1**), which was clearly reflected in biological samples, specially in plasma, observing significant higher levels of all the derived metabolites after HFD+W (**Figure 1i**). The sum of flavan-3-ol metabolites includes both phase II metabolites and colonic metabolites (valerolactones). These monomers (catechin and epicatechin) can also be generated from dimer by depolymerization (**Figure 2**).

In plasma, all the detected metabolites were significantly higher after HFD+W than in other groups, being catechin glucuronide the most abundant (**Supplemental Table 2**).

Similarly, in urine samples, the concentrations of all the individual metabolites were significantly higher in the HFD+W than other groups (**Supplemental Table 3**). In this case, hydroxyphenyl-γ-valerolactone sulphate was the most abundant metabolite. It is noteworthy that significant gender differences were reported in urine in some flavan-3-ols metabolites in the HFD+W group. For instance, we observed that after HFD+R females excreted almost 3-fold more methyl epicatechin suphate than males ($p < 0.05$). Also, after HDF+A intake, females presented almost 60-fold more hydroxyphenyl-ɣ-valerolactone sulphate compared to males ($p < 0.05$).

In faeces samples, (epi)catechin, dimer, trimer and methyl catechin sulphate were detected, being all metabolites also significantly higher after HFD+W compared to other groups (**Supplemental Table 4**).

2.2.5. Dihydrochalcone metabolites

Dihydrochalcones, and particularly phloretin, are a specific phenolic group from apples, and this was reflected in our results, observing that their derived metabolites were only detected after both apples supplementation. It was also noted that dihydrochalcones were the most abundant excreted in urine after the supplementation with both apples (HFD+R and HFD+W), representing around the 90% of total metabolites excreted in the HFD+R group. The concentration of dihydrochalcones in red-fleshed apple was around 4-fold higher than in white-fleshed apple (**Table 1**), and this fact was reflected in urine and in faeces observing that the total urine dihydrochalcone concentration in HFD+R was significantly higher than in HFD+W (**Figures 1l, 1m**). In urine samples, phloretin sulphate was the most abundant metabolite.

In faeces, only the parent compounds (hydroxyl) phloretin and (xylosyl) glucoside were detected after supplementation of both apples. The total concentration in HFD+R was similar to HFD+W and the concentrations of the three compounds followed the same trend (**Supplemental Table 4**).

2.3. Aorta, kidney, liver and heart histological analysis

2.3.1. Aorta samples: Differences in thickness

To evaluate possible deposition of lipids in the aorta, which can lead to the formation of atherosclerotic plaques, its thickness was measured in sections stained with Haematoxylin-eosin. Results showed significant differences between males and females in all diet groups, therefore, we analyzed the results independently (**Figures 3a** and **3b)**. Regarding rat males, the aortic thickness of the HFD group (136 ± 10.1 μm) was significantly higher than the other groups (**Figure 3a** and **Supplemental Figures 7a-e)**. Male aortic thickness of HFD+W (112 ± 8.38 μm), HFD+R (109 ± 8.17 μm) and HFD+A

(107 ± 8.00 µm) groups were similar to that of SCD control group (112 ± 8.32 µm).

Unlike the males, the females had different response depending on the administered diet (**Figure 3b** and **Supplemental Figures 7f-j**). The aortic thickness in HFD+W (106 ± 7.92 µm) was similar to those in SCD group (102 ± 7.62 µm) and lower compared to males in the same group. In the HFD+R group (115 ± 8.64 µm) a significant improvement was observed compared to the HFD group (127 ± 9.44 µm), although the effect was not as effective as HFD+W. By contrast, the HFD+A diet (128 ± 9.50 µm) did not show a significant reduction in aortic thickness compared with HFD control group.

2.3.2. Kidney samples: Bowman's space

To assess possible protective effects against a fatty diet on the kidneys, differences in Bowman's space were evaluated. Since significant differences were observed between males and females of the same group, results were presented separately (**Figures 3c and 3d**). It has been proven that the chronic administration of dietary lipids alters the renal structure and one of the parameters that is altered is the Bowman's space that increases and leads to glomerular atrophy and functional loss of glomeruli and tubules.[29,30]

In males, no significant differences were observed in the Bowman's space area among groups (**Figure 3c**). In contrast, female samples showed that after HFD+W (1675 ± 177 µm^2), HFD+R (1560 ± 164 µm^2) and HFD+A (1578 ± 166 µm^2), the Bowman's space area was significantly reduced compared to the HFD group (2388 ± 251 µm^2) ($p<0.05$), but significantly higher than SCD (889 ± 93.8 µm^2).

2.4. Fatty liver development by Oil-Red staining

To evaluate possible attenuation effects of PC on fatty liver development, samples were stained with oil-red staining to assess the lipid accumulation. As it can be observed in **Figure 4**, HFD led to an increase in cellular lipid accumulation compared to SCD. However, the diet supplementation with either apples or aronia did not reduce the lipid accumulation, inflammation or modified hepatocytes, in comparison to the HFD group.

2.5. Heart Masson's trichrome staining

To assess possible protective effects of PC on the development of heart fibrosis that occurs in a chronic inflammatory process, heart samples were stained with Masson's Trichrome stain to observe collagen deposition. As seen in **Figure 4**, this staining revealed that widths of cardiac myocytes and the collagen deposition (blue color) in the perivascular area that leads to cardiac fibrosis increased similarly after HFD, HFD+R, HFD+W and HFD+A compared to the SCD group. No differences were observed between the groups supplemented with apples or aronia infusion.

2.6. Plasma biochemical parameters

Data of various biochemical parameters including insulin, glucose, lipid profile and liver enzymes are presented as Mean ± SD values in **Table 2**. In general, high intra-individual variability was observed in all parameters**.** The effects of the HFD were reflected in a significant increase of TC, non-HDLc, ALT, AST, and insulin values ($p < 0.05$) compared to SCD. Results showed that the supplemented diets with apples or aronia did not attenuate these effects, observing no significant reductions compared to HFD in all parameters, except for insulin. Remarkably, this depletion of insulin levels was only observed in males in the three supplemented HFD diets, being statistically significant ($p < 0.05$) in HFD+R and HFD+W diets.

DISCUSSION

In the present study, we investigate the response to a sustained intake of red-fleshed apple biofortified in ACN on the phenolic metabolism and their cardiovascular effects associated. For this purpose, we supplemented rats that fed a HFD to induce hypercholesterolemia with red-fleshed apple and compared results to a common white-fleshed apple without ACN. To evaluate the impact of the components of the apple matrix, a group supplemented with ACN-rich infusion was included.

Regarding the results on absorption and metabolic fate of ACN, although the amount and profile of ACN in red-fleshed apple and aronia infusion were the same, the metabolic profile detected in biological samples differed quantitatively and qualitatively. The differences observed could be related to the effect of the apple matrix. In apple fruit, the ACN are bound to fiber and saccharides, wheareas in the aronia infusion the ACN are in their free forms more available, which favour their gastrointestinal absorption and metabolism. The metabolites observed in the present study were in agreement with previous studies describing the metabolic fate of ACN after the intake of red-fleshed apple in a postprandial study in humans,[21] and aronia in rats.[31,32] The apple matrix effect was also observed in phenolic acids, PPA and PAA metabolites, which appeared in higher concentration in urine after HFD+A, and in faeces after HFD+R. This fact indicates that ACN and phenolic compounds linked to apple fiber are absorbed more slowly compared to aronia infusion, and therefore, they reach the large intestine where they are catabolized by the colonic microbiota appearing in higger amounts in faeces. Regarding the other phenolic metabolites, our results from flavan-3-ols are in agreement with previous studies, reporting similar phase II metabolites and valerolactones after the intake of flavan-3-ol rich extracts, such as apple,[21] or wine.[33] The detected dihydrochalcone metabolites were also reported by other authors in biological samples after the intake of cider,[34] and red-fleshed apple snack in humans.[21,23] To our knowledge, the colonic metabolism of dihydrochalcones has been scarcely studied, and the obtained results were in agreement with previous studies.[26-28]

When studying the metabolic fate of PC after apple and aronia supplementation, a gender effect was also reported. In general terms, the differences between males and females detected in urine samples could be sumamarized as: i) the methylated, glucuronided, methylglucuronided and methylsulphate conjugates were found in higher concentrations or predominantly in females; and ii) the sulphate conjugates in males. These results were in agreement with those reported in previous studies in rat model[35,36] and in humans[37] showing that the sulphatation and glucuronidation were more intense in males than in females.[37] Contrary to our results, several studies have reported that some isoforms of catechol-*O*-methyltransferases (COMT) enzymes may be more active in males than in females.[36,38,39] In quantitative terms, the most significant differences between genders were observed in phenolic acid metabolites, observing that females absorbed and excreted significant higher amounts of these metabolites after all the supplemented diets. These differences could be explained by the sex-dependent expression of many isoforms of gastrointestinal enzymes or/and transporters that participate in the absorption of phenolics.[40] For example, in rats, β-glucosidase activity is greater in females than in males.[41]

Concerning the cardioprotective parameters studied, a significant reduction was reported in aortic thickness as a consequence of apple (red- and white-fleshed) and aronia intake compared with HFD, indicating a protective atherogenic effect of the three administered products.

Particularly, the effect observed in HFD+W group could be mainly due to flavan-3-ols metabolites, being catechin glucuronide and hydroxypheny-γ-valerolactone sulphate the most abundant metabolites detected in plasma and urine samples, respectively **(Supplemental Tables 2 and 3)**. The anti-atherosclerotic potential of flavan-3-ols from apples has already been demonstrated in mice, after the administration of cider apple extracts in apo-E deficient mice.[42] Similarly, the diet supplementation with an apple phenolic extract rich in flavan-3-ols for 12 weeks in hypercholesterolemic male mice[43] produced a reduction of the mean atherosclerotic lesion area in the aortic sinus. Similar effects were also observed in male hypercholesterolemic rabbits after the sustained intake of apple juices producing a significant reduction of the atherosclerotic lesions of coronary arteries.[1] Also, in agreement with our results, after the chronic administration in Sprague Dawley rats of a persimmon fruit extract rich in catechin derivatives, a significant decrease in aortic thickness was observed.[44]

Regading the HFD+R group, ACN and dihydrochalcones were the most abundant phenolic compounds and also their metabolites in plasma and urine, so we hypothesize that the reduction of the aortic thickness at levels of SCD group could be mainly related to ACN and dihydrochalcones, with a minor contribution of flavan-3-ols. Similar effects have been reported in rabbits and mice studies supplementing with apples in which dihydrochalcones were studied.[1,42,43] The potential anti-atherosclerotic role of ACN was also consistent with other studies in which lower fatty deposition in the arteries of hypercholesterolemic male rabbits was observed after the intake of sun-dried berries for 8 weeks,[11] or after the administration of red-fruit juices like raspberry in hypercholesterolemic hamsters.[12]

In the case of aronia supplementation, the attenuation effects observed in the aorta can be attributed to ACN, as these were the main PC administered through the aronia infusion and was also reflected in a higher amount of their derived metabolites in plasma and urine. These results are in agreement with a previous study[13] in which it was observed a decrease in epididymal fat accumulation in hypercholesterolemic Wistar rats after the administration of aronia fruit (at two different concentration, 3 or 6 mg daily) for 6 weeks.

Unlike the males, the females had different response on the aortic thickness depending on the administered diet, observing a lower attenuation effect and no effect after aronia intake in females. In relation to this, contradictory results are described in the literature regarding the anti-atherogenic effects of ACN. Some studies with males of different species such as rabbits,[11] hamsters[12] or Wistar rats[13] have reported the anti-atherogenic effects of ACN, whereas other studies[45,46] did not report histological differences in aorta after the sustained administration of aronia melanocarpa (106.8 mg anthocyanins per 100 ml water) in males Wistar rats. Regarding the HFD+W group, a reduction of aortic thickness was detected in both males and females, which corroborates the protective effect of flavan-3-ol in the development of atherosclerotic lesions.

The attenuation effects in the reduction of aorta thickness were not related with the plasma lipid profile, since no significant differences were observed in TC, TG, HDL, non-HDL after the three supplemented diets compared with HFD group. This fact could indicate that the absolute cholesterolemia is not an indicative parameter of the aortic fatty streak deposition.[47,48]

Regarding the studied effects on kidney, an attenuation effect in the renal structure was only observed in females after the three supplemented diets compared to HFD. This fact could probably be related to the gender-related differences in the phenol bioavailability, as we observed higher amounts of urinary phenolic metabolites in females compared to males. Although the differences were not significant, the total excretion of phenolic metabolites in urine was greater in females after the three supplementation diets, suggesting that an accumulation of phenolic metabolites in the kidney could lead to major effects in female kidneys. This higher excretion of phenolic metabolites observed in females is consistent with studies in which a greater excretion of isoflavone conjugates[49] or hydroxytyrosol derivatives[50] was observed in females after the administration of soy milk or high doses of hydroxytyrosol in humans and in Sprague-Dawley rats, respectively.

As it was discussed for the aorta, the attenuation effects reported in kidney after the supplemented diets could be mainly related to ACN, flavan-3-ols, and dihydrochalcones. The effect observed after HFD+W on the accumulation of fat in the kidney could be mainly attributed to the flavan-3-ol metabolites, being hydroxyplenyl-γ-valerolactone sulphate (urine) and catechin glucuronide (plasma and urine) the main metabolites detected.

The effect observed in kidney structure after both apple supplementation, especially after HDF+R, could be also attributed to the dihydrochalcone metabolites detected in high concentration in urine, being phloretin sulphate the main metabolite with a higher concentration in females. The effects observed in kidney after the apple consumption are consistent with the studies reported in the literature. After the intake of a dried apple rich in flavan-3-ols and dihydrochalcones, kidney function improved, and the concentration of uric acid, urea, and creatinine decreased in 8 Sprague Dawley hyperlipidemic rats.[2] In addition, after apple supplementation (approximately 20% of the daily intake) of obese Zucker rats, the glomerulopathy with the consequent proteinuria was reduced.[51]

In addition, ACN could be also responsible for the effects observed in the HFD+A group, and to a lesser extent in the HFD+R group as commented for aorta results, which could be in agreement with previous studies. After the administration of black soybean in hypercholesterolemic Sprague Dawley rats, ACN reversed the effects of HFD on body weight, serum lipids and decreased the weights of epididymal and perirenal fat pats.[52] In another study in Wistar rats, after the supplementation with aronia melanocarpa extract (with phenol doses approximately eight-fold higher than those administered in our study), the antioxidant status was improved, especially, the concentration of a lipid peroxidation indicator, the thiobarbituric acid reactive substances (TBARS) in the kidneys.[53]

It is noteworthy that, apart from the effects observed in aortas and kidneys, a significant decrease in insulin values was also observed in the male plasma from the supplemented groups with apples (3.49 ± 0.63 and 3.22 ± 0.34 µg/L in HFD+W and HFD+R, respectively) compared with HFD group (5.27 ± 0.34 µg/L). These results are in agreement with the results reported in the literature.[13,54] After the administration of cranberry extract rich in ACN and flavan-3-ols[54] or aronia extract[13] in obese mice, the insulin values decreased with respect to the control HFD. One of the effects of HFD diets is their induction of adipose tissue dysfunction. This can alter diverse factors, inducing to systemic insulin resistance, which is a major contributor to the development of type 2 diabetes.[55] The anti-insulin resistance effect of PC is partly due to their ability to reverse this dysregulation. It has also been reported that after chokeberry extract consumption rich in ACN, the Ppargamma mRNA expression increased, the Fabp4, Fas and Lpl mRNA levels were suppressed, a decrease of mRNA expression of TNF-α and Ilb and Il6 was induced and in consequence, the plasma levels of TNF-α and IL-6 were decreased.[13] In other studies in diet-induced insulin-resistant animals, after the administration of polyphenols from cinnamon,[56] and green tea,[57] Fas and Lpl mRNA expression and other genes related with lipogenesis were inhibited leading to improve systemic insulin sensitivity and dyslipidemia by enhancing insulin signaling.

The obtained results in liver and heart stainings are in agreement with plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values, since no significant differences were observed concerning HFD after diet supplementations. These results may be due to the fact that the HFD diet was so powerful that the attenuation effect of PC administered in relatively low doses could not be appreciated at the macromolecular level. These results contrast with another study[51] performed in another rat model (obese Zuckers rats) in which after the administration of a similar dose of lyophilized apple, a cardioprotective (decreasing TG in the heart) and hepatoprotective (limiting liver steatosis) effects were observed.

The main limitation of the study is the low number of animals per group, so future studies with higher *n* are necessary to corroborate the observed effects and also to determine the molecular mechanisms.

Conclusions

Our study showed the *in vivo* cardiometabolic protective effects of both red-fleshed and white-fleshed apples and aronia infusion supplementation in hypercholesterolemic rats, specifically in the reduction of the aorta thickness, the improvement of the renal function and the reduction of insulin levels. Our findings support that ACN without apple matrix through aronia infusion can induce cardioprotective effects. In the case of apples, ACN or flavan-3-ols, together with dihydrochalcones, compose a phenolic phytocomplex in red and white-fleshed apples, respectively, that could act synergistically in the attenuation of cardiovascular outcomes.

A gender effect was also reported probably related with the differences observed in the metabolic fate of PC. These differences in phenol metabolism were specially noted in the kidney function which was improved only in females, and may be related to the higher phenol bioavailability observed in females. So our results suggest that differences in the metabolic fate of PC underlie the possibility that PC can differently influence the health outcomes of males and females.

Moreover, an apple matrix effect was reported between red-fleshed apple and aronia infusion observing a higher absorption and excretion of ACN after aronia supplementation without the apple components.

ACKNOWLEDGEMENTS

This study was supported by the Spanish Ministry of Industry, Economy and Competitiveness through the AGL2016-76943-C2-1-R and AGL2016-76943-C2-2-R projects (co-funded by the European Social Fund, European Union). I.A.L. enjoys a post-doctoral contract (2017PMF-POST2-19) from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement and from the Universitat Rovira i Virgili (URV). S.Y. was supported by a grant from the University of Lleida. Ú.C. has a Pla estratègic de recerca i innovació en salut (PERIS) post-doctoral grant (SLT002/16/00239; Catalunya, Spain) from Generalitat de Catalunya. A.P. enjoys a post-doctoral grant (PTQ-15-08068; Spain). In addition, the authors are grateful to NUFRI SAT (Mollerussa, Lleida, Catalonia, Spain) for providing the red-fleshed apples. We are grateful to A. Martínez (Department of Medicine, University of Lleida) for helpful with the histological stains. Finally, we are grateful to SCT-Estabulari of the University of Lleida where the animal experiment was carried out.

CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Figure Captions

Figure 1. Anthocyanin, phenolic acids, phenylpropionic & phenylacetic, flavan-3-ols and dihydrochalcones derivatives content in plasma, urine and faeces of rats fed with SCD, HFD, HFD+W, HFD+R and HFD+A.

Data expressed as mean values ± standard error of mean. Different lowercase letters: indicates differences between males (M) in the different groups; different capital letters: indicates differences between females (F) in the different groups; and different Numbers indicates differences between males + females (M+F) in the different groups (General Linear Model, and One-way ANOVA, $P < 0.05$). * indicate differences between males & females of the same group (t-student test, $P < 0.05$).

Figure 2. Schematic representation of the main metabolic pathways of the generated phenolic metabolites after the HFD+W, HFD+R, and HFD+A intakes in M+F and in urine samples.

Figure 3. Aortic thickness (µm) in males (a) and in females (b); and Bowman's space (µm^2) in males (c) and in females (d) of the groups SCD, HFD, HFD+W, HFD+R and HFD+A.

Data expressed as mean values ± standard error of mean. Values not displaying the same letter are significantly different (one-way ANOVA, Tukey's test between all means, $P < 0.05$). * indicates differences between males and females of the same group (t-student test $P < 0.05$).

Figure 4. Representative images (at a magnification of 20x) of Oil-Red-stained livers of females and males from: a) SCD, b) HFD, c) HFD+W, d) HFD+R, and e) HFD+A.

Representative images (at a magnification of 20x) of Masson’s trichrome stained hearts of females and males from: f) SCD, g) HFD, h) HFD+W, i) HFD+R, and j) HFD+A.

Table 1. Daily dose (mean ± standard deviation) of the main phenolics (µg phenol / day / rat) ingested through the diet supplementation with white-fleshed apple, red-fleshed apple, and aronia infusion by LC-MS/MS.

Phenolic compounds	**White-fleshed apple (µg / 5 g / day)**	**Red-fleshed apple (µg / 5 g / day)**	**Aronia (µg / 20 ml infusion / day)**
Cyanidin arabinoside	6.88 ± 6.12	167 ± 6.00	480 ± 18.1
Cyanidin galactoside	21.8 ± 18.1	1690 ± 36.0	1426 ± 48.0
Total Anthocyanins	***28.6 ± 24.2***	***1857 ± 42.0***	***1906 ± 67.0***
Protocatechuic acid	n.d.	108 ± 67.3	462 ± 65.2
Coumaric acid hexoside	48.7 ± 7.03	48.7 ± 7.03	2.00 ± 0.03
Ferulic acid hexoside	67.3 ± 7.44	134 ± 16.4	5.00 ± 0.02
Vanillic acid	n.d.	n.d.	14.0 ± 1.01
Vanillic acid hexoside	58.2 ± 4.05	271 ± 7.24	138 ± 22.0
5-*O*-caffeoylquinic acid	1386 ± 28.08	5004 ± 174	814 ± 434
3-*O*-caffeoylquinic acid	n.d.	n.d.	362 ± 97.0
Gallic acid	n.d.	n.d.	36.0 ± 5.03
Gallic acid hexoside	n.d.	n.d.	25.0 ± 6.02
Caffeic acid	n.d.	n.d.	32.0 ± 1.04
Homogentisic acid	n.d.	n.d.	21.0 ± 7.12
Total Phenolic acids	***1837 ± 56.2***	***5566 ± 211***	***1911 ± 638***
Catechin	377 ± 25.3	n.d.	n.d.
Epicatechin	1867 ± 27.1	353 ± 49.3	12.0 ± 1.12
Dimer	3663 ± 121	438± 18.1	32.0 ± 7.15
Trimer	350 ± 51.6	82.0± 7.34	8.00 ± 1.06
Total Flavan-3-ols	***6259 ± 226***	***875 ± 74.9***	***52.0 ± 9.00***
Quercetin arabinoside	166 ± 24.3	232 ± 28.7	5.00 ± 1.07
Quercetin rhamnoside	230 ± 26.6	587 ± 59.7	n.d.
Quercetin glucoside	541 ± 81.0	279± 35.9	133 ± 11.2
Quercetin rutinoside	n.d.	n.d	87.0 ± 12.3
Total Flavonols	***938 ± 132***	***1098 ± 125***	***225 ± 24.0***
Eriodictyol hexoside	n.d.	26.4 ± 1.42	7.00 ± 2.05
Naringenin	n.d.	n.d.	n.d.
Total Flavanones	***n.d.***	***26.4 ± 1.42***	***7.00 ± 2.00***
Phloretin glucoside	247 ± 34.5	1371 ± 160	n.d.
Phloretin xylosyl glucoside	276 ± 17.6	739 ± 32.7	n.d.
Hydroxyphloretin xylosyl glucoside	14.2 ± 1.01	20.5 ± 1.52	n.d.
Total Dihydrochalcones	***536 ± 53.1***	***2130 ± 195***	***n.d.***
TOTAL PHENOLICS	***9598 ± 487***	***11552 ± 1420***	***4101 ± 740***

The number of replicates was three. (*n*=3). n.d.: not detected.

Table 2. Biochemical parameters of rat plasma from the different studied groups.

		TC (mg/dL)	**TG (mg/dL)**	**HDLc (mg/dL)**	**Non-HDLc (mg/dL)**	**ALT (U/L)**	**AST (U/L)**	**Insulin (µg/L)**	**Glucose (mg/L)**
HFD	F (n=3)	426 ± 147	130 ± 51.4	47.4 ± 12.4	379 ± 147	110 ± 50.8	447 ± 146	1.24 ± 0.66	225 ± 23.0
	M (n=3)	224 ± 62.0	151 ± 43.0	36.3 ± 11.9	188 ± 52.8	189 ± 70.0	314 ± 76.8	5.27 ± 0.34	211 ± 11.2
	M&F (n=6)	325 ± 150	141 ± 44.0	41.8 ± 12.5	283 ± 144	149 ± 70.0	381 ± 127	3.26 ± 2.25	218 ± 18.0
SCD	F (n=3)	69.2 ± 7.72 (ns)	56.4 ± 29.3 (ns)	52.5 ± 2.83 (ns)	16.8 ± 6.14 (0.013*)	43.3 ± 16.2 (ns)	170 ± 88.6 (0.048*)	0.46 ± 0.10 (ns)	222 ± 18.6 (ns)
	M (n=3)	73.5 ± 13.3 (0.046*)	141 ± 90.9 (ns)	54.6 ± 7.27 (ns)	18.9 ± 7.09 (0.029*)	44.3 ± 10.1 (0.024*)	179 ± 1.73 (ns)	0.84 ± 0.13 (<0.001*)	264 ± 39.9 (ns)
	M&F (n=6)	71.4 ± 10.0 (0.009)	96.4 ± 76.0 (ns)	53.5 ± 5.07 (ns)	17.8 ± 6.05 (0.006*)	43.8 ± 12.1 (0.013*)	175 ± 56.3 (0.005*)	0.61 ± 0.23 (0.035*)	243 ± 36.2 (ns)
HFD+ W	F (n=3)	477 ± 190 (ns)	115 ± 7.97 (ns)	33.5 ± 15.6 (ns)	443 ± 174 (ns)	107 ± 61.4 (ns)	410 ± 280 (ns)	1.07 ± 0.74 (ns)	228 ± 13.2 (ns)
	M (n=3)	227 ± 41.0 (ns)	91.5 ± 29.8 (ns)	55.3 ± 27.9 (ns)	172 ± 39.6 (ns)	241 ± 102 (ns)	494 ± 219 (ns)	3.49 ± 0.63 (0.013*)	228 ± 13.7 (ns)
	M&F (n=6)	352 ± 184 (ns)	103 ± 23.4 (ns)	44.4 ± 23.5 (ns)	307 ± 187 (ns)	174 ± 105 (ns)	452 ± 229 (ns)	2.52 ± 1.44 (ns)	228 ± 12.0 (ns)
HFD+ R	F (n=3)	548 ± 307 (ns)	216 ± 144 (ns)	44.8 ± 23.5 (ns)	503 ± 289 (ns)	101 ± 48.4 (ns)	380 ± 93.7 (ns)	1.33 ± 0.94 (ns)	278 ± 62.3 (ns)
	M (n=3)	233 ± 33.6 (ns)	88.3 ± 27.3 (ns)	40.6 ± 9.37 (ns)	192 ± 24.4 (ns)	210 ± 40.4 (ns)	318 ± 61.8 (ns)	3.22 ± 0.34 (0.002*)	236 ± 14.5 (ns)
	M&F (n=6)	390 ± 261 (ns)	152 ± 116 (ns)	42.7 ± 16.2 (ns)	347 ± 250 (ns)	156 ± 71.5 (ns)	349 ± 78.8 (ns)	2.28 ± 1.21 (ns)	257 ± 46.7 (ns)
HFD+ A	F (n=3)	536 ± 157 (ns)	212 ± 68.6 (ns)	40.3 ± 2.60 (ns)	495 ± 154 (ns)	88.7 ± 61.7 (ns)	358 ± 197 (ns)	1.39 ± 1.03 (ns)	260 ± 44.3 (ns)
	M (n=3)	236 ± 66.8 (ns)	137 ± 84.1 (ns)	43.4 ± 13.4 (ns)	193 ± 72.7 (ns)	246 ± 23.5 (ns)	450 ± 83.3 (ns)	2.08 ± 2.24 (ns)	209 ± 42.3 (ns)
	M&F (n=6)	386 ± 196 (ns)	174 ± 80.0 (ns)	41.8 ± 8.78 (ns)	344 ± 198 (ns)	167 ± 95.8 (ns)	404 ± 144 (ns)	1.73 ± 1.60 (ns)	235 ± 47.8 (ns)

Concentration is expressed as mean ± standard deviation. Data were analyzed using Student t-test comparing each treatment versus HFD. * $p < 0.05$ each treatment versus HFD.