Serum Metabolites of Proanthocyanidin-administered rats decrease lipid synthesis in HepG2 cells

Guerrero L[†#], Margalef M[†], Pons Z[†], Quiñones M[†], Arola L[†§], Arola-Arnal A[†*], and Muguerza B[†§].

[†] Department of Biochemistry and Biotechnology, Rovira i Virgili University, Tarragona, 43007 Spain.

[#] Department of Research, Nutrition and Innovation, ALPINA S.A, Bogotá, Colombia.

[§] Centre Tecnològic de Nutrició i Salut (CTNS), Reus, 43204, Spain

Running title: Lipid-lowering effect proanthocyanidin metabolites.

*Corresponding author: Dr. Anna Arola-Arnal

Dpt. Bioquímica i Biotecnologia, Universitat Rovira i Virgili, C/ Marcel.lí Domingo s/n 43007 Tarragona, Spain.

Telephone: +34 977 55 86 30

Fax: +34 977 55 82 32

E-mail: anna.arola@urv.cat

Abstract

The regular consumption of flavonoids has been associated with reduced mortality and a decreased risk of cardiovascular diseases (CVD). The proanthocyanidins found in plasma are very different from the original flavonoids in food sources. Thus, the use of physiologically appropriate conjugates of proanthocyanidins is essential for the *in vitro* analysis of flavonoid bioactivity.

In this study, the effect of different proanthocyanidin-rich extracts, which were obtained from cocoa (CCX), French maritime pine bark (Pycnogenol extract, PYC) and grape seed (GSPE), on lipid homeostasis was evaluated. Hepatic human cells (HepG2 cells) were treated with 25 mg/L of CCX, PYC or GSPE. We also performed *in vitro* experiments to assess the effect on lipid synthesis that is induced by the bioactive GSPE proanthocyanidins using the physiological metabolites that are present in the serum of GSPE-administered rats. For this, Wistar rats were administered 1g/Kg of GSPE and serum was collected after 2h. The semi-purified serum of GSPE-administered rats was fully characterised by LC-QqQ/MS^2. The lipids studied in the analyses were free cholesterol (FC), cholesterol ester (CE) and triglycerides (TG).

All three proanthocyanidin-rich extracts induced a remarkable decrease in the *de novo* lipid synthesis in HepG2 cells. Moreover, GSPE rat serum metabolites reduced the total percentage of CE, FC and particularly TG; this reduction was significantly higher than that observed in the cells directly treated with GSPE. In conclusion, the bioactivity of the physiological metabolites that are present in the

serum of rats after their ingestion of a proanthocyanidin-rich extract was demonstrated in Hep G2 cells.

Key words: Grape seed proanthocyanidin extract; Serum metabolites; lipid synthesis; cell cultures; HepG2 cells.

1. Introduction

Cardiovascular disease (CVD) is the main cause of death worldwide. According to the World Health Organization, 17.3 million people died from CVD in 2008; this number represents 30% of the total deaths worldwide. In addition, starting from 2015, it is estimated that approximately 20 million people will die every year from this disease. Among the modifiable risk factors for CVD, dyslipidaemia, hypertension, smoking and diabetes mellitus are of particular relevance [1]. Specifically, hyperlipidaemia, which is the elevation of cholesterol and/or triglyceride (TG) levels, is a significant risk factor for the development of atherosclerosis and heart disease.

There is a body of evidence that indicates that a diet rich in vegetables and fruits decreases the risk of CVD [2-4]; in addition, this decrease has been attributed to the phenolic compounds that are present in plants. Flavonoids are phenolic compounds that are commonly found in fruits and vegetables at high concentrations, and their regular consumption has been associated with a reduced mortality and a decreased risk of CVD [5-8]. More specifically, grapes, wine, cocoa and pine are known to be significant sources of flavonoids, particularly flavan-3-ols and proanthocyanidins [9, 10].

It is well known that proanthocyanidins improve human health by affecting the cellular and physiological processes. Several studies have shown the beneficial effects of proanthocyanidins on lipid metabolism in different experimental models [11, 12] and humans [13, 14]. Our group has previously shown that the oral

administration of grape seed proanthocyanidins significantly reduces the postprandial levels of TG-rich and ApoB-containing lipoproteins and improves several atherosclerotic risk indexes in normolipidemic rats [15]. In addition, grape seed proanthocyanidins lead to a reduction in the production and secretion of TG in the human hepatocarcinoma cell line HepG2 [16]. However, the effect of other proanthocyanidin extracts on lipid production and secretion has not been studied in hepatic cells.

It is generally accepted that polyphenol bioavailability is relatively poor [17], although the monomeric flavan-3-ols (or flavanols) are among the polyphenols that exhibit higher bioavailability [18]. Moreover, the rapid conversion of flavonoids into their metabolites is well known [19]. Therefore, the large amount of scientific data that has been generated using non-physiologically relevant forms and/or concentrations of flavonoids is questionable [20, 21]. In fact, the limited description of flavonoid bioactivity that is based on studies that use non-physiologically relevant forms and/or concentrations of these compounds is considered the most important limitation in flavonoid research [20]. Thus, the development of *in vitro* models using physiologically appropriate conjugates, forms and concentrations of flavonoids that are more similar to those observed in tissues after the intake of these compounds is an important requirement for the analysis of flavonoid bioactivity [21]. Hence, we hypothesised that this question could be addressed through the treatment of cells with the sera of rats that were orally administered a proanthocyanidin-rich extract. In this study, the cells were incubated directly with the flavonoid metabolites in the rat sera, which would simulate the physiological

conditions that occur within the body. The use of the sera of rats that were previously administered the compound under study for the treatment of cell cultures has been recently described. In fact, a previous study confirmed the bioactivity of bezafibrate, which is a known PPAR-γ ligand, in HeLa cells using serum [22]. In a similar manner in this study rats were used as a tool to produce flavanoid metabolites. These physiological forms were utilized to treat HepG2 cells, allowing the evaluation of the functionality of the bioactive forms.

Therefore, the aim of this study was to first evaluate the effect of different proanthocyanidin-rich extracts on the lipid production and secretion in HepG2 cells. Moreover, the lipid-lowering effect of bioactive proanthocyanidins was also evaluated using an *in vitro* system; in this system, the cells were treated with the physiological metabolites that were present in the serum of rats that ingested a grape seed proanthocyanidin extract (GSPE).

2. Materials and Methods

Chemicals and reagents

Chromatographic analysis: Methanol (Scharlab S.L., Barcelona, Spain), acetone (Sigma-Aldrich, Madrid, Spain) and glacial acetic acid (Panreac, Barcelona, Spain) were of HPLC analytical grade. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). The 1000 mg/L standard stock solutions of (+)-catechin, (-)-epicatechin, gallic acid, epigallocatechin gallate, proanthocyanidin B2 and proanthocyanidin B1 (all from Fluka/Sigma -Aldrich,

Madrid, Spain) in methanol and the 2000 mg/L standard solution of pyrocatechol in methanol, which was used as an internal standard (Fluka/Sigma Aldrich, Madrid, Spain), were stored in a dark-glass flask at -20ºC.

Standard stock mixtures with a concentration of 200 mg/L of (+)-catechin, (-)-epicatechin, epigallocatechin gallate and gallic acid in methanol and 100 mg/L of proanthocyanidin B2 and proanthocyanidin B1 in methanol were prepared weekly and stored at -20ºC. These standard stock solutions were diluted daily to the desired concentration using an acetone/water/acetic acid (70:29.5:0.5, v:v:v) solution.

Cell culture: Dulbecco´s modified Eagle´s medium (DMEM), foetal bovine serum (FBS), L-glutamine, penicillin and streptomycin were purchased from Bio Whittaker Europe (Verviers, Belgium). The Bradford protein reagent was obtained from Bio-Rad Laboratories (Life Science Group, Hercules, CA, USA). ^{14}C-acetate was purchased from Amersham Biosciences (Buckinghamshire, England).

Proanthocyanidin-rich extracts

The grape seed proanthocyanidin extract (GSPE) was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this proanthocyanidin extract contains monomeric (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%) and oligomeric (>5) (35.7%) proanthocyanidins.

The cocoa proanthocyanidin extract (CCX) used for this study was provided by Natraceutical (Valencia, Spain). This extract was obtained from a polyphenol-

144 rich cocoa powder that was produced from unfermented, blanch-treated, non-
145 roasted cocoa beans, which preserve the degradation of polyphenols [23]. The
146 data obtained using normal-phase HPLC showed that the proanthocyanidin cocoa
147 extract contains monomeric (23.7%), dimeric (15.8%), trimeric (18.4%), tetrameric
148 (13.9%) and oligomeric proanthocyanidins (>5) (36.2%) [24].

149 The pycnogenol extract (PYG) was provided by Shirota Functional Foods
150 (Tarragona, Spain). The pycnogenol extract contains monomeric (38.0%), dimeric
151 (40.9%) and oligomeric (containing more than 3 monomeric units, 62.0%)
152 proanthocyanidins. [25].

153

154 **Experimental procedure in rats**

155 In this study, 17 to 20-week-old male Wistar rats that weighed 300-350 g were
156 used. The Animal Ethics Committee of our university approved all procedures
157 (reference number 6777 by Generalitat de Catalunya). The animals were obtained
158 from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters
159 at 22ºC with a 12/12-hour light/dark cycle (light from 9:00 a.m. to 21:00 p.m.). The
160 animals consumed tap water and a standard chow diet (Panlab A04, Barcelona,
161 Spain) ad libitum throughout the experiment. The rats were randomly divided into
162 two groups: control (n=4) and GSPE (n=3). The rats from the experimental group
163 were administered 1 mL of 1 g/kg of body weight of GSPE by oral gavage. The
164 control group was orally administered 1 mL of water. The corresponding treatments
165 were administered between 9 and 10 am after overnight fasting. Two hours after
166 the treatment, the rats were anaesthetised with sodium pentobarbital (80 mg/kg),

and their blood was collected by cardiac puncture (Figure 1). The blood was maintained at room temperature for 30 min. Once the blood coagulated, it was centrifuged at 2000 x g and 4ºC for 15 min to obtain the serum. The sera was inactivated at 56ºC for 30 min to avoid the risk of complement-mediated cell lysis and stored at -80ºC until analysis. All of the methods were in accordance with the guidelines for the care and use of laboratory animals of the University Rovira i Virgili (Tarragona, Spain).

Extraction of serum proanthocyanidins

Prior to the cell culture and chromatographic analysis, the rat serum samples were pretreated by off-line μSPE following the methodology that was previously described by Martí et al. (2010) [26] using 30-μm OASIS HLB μElution Plates (186001828BA, Waters, Barcelona, Spain). Briefly, the micro-cartridges were sequentially conditioned with 250 μL of methanol and 250 μL of 0.2% acetic acid. Prior to extraction, the serum was centrifuged at 2000 x g and 4ºC for 5 min. Two serum aliquots (each of 350 μL) were mixed, each of them, with 300 μL of 4% phosphoric acid and 50 μL of pyrocatechol (2000 ppb) and then loaded onto two different plates. The two loaded plates were washed with 200 μL of Milli-Q water and 200 μL of 0.2% acetic acid. The retained flavanols on each plate were eluted with 2 x 50 μL of an acetone/Milli-Q water/acetic acid (70:29.5:0.5, v:v:v) solution. The two elutions were mixed to obtain a final volume of 200 μL. Part of the solution (25 μL) was evaporated to dryness using a SpeedVac Concentrator SPD 2010 SAVANT (Thermo Scientific, USA) at room temperature and redissolved in 25 μL

of an acetone/Milli-Q water/acetic acid (70:29.5:0.5, v:v:v) solution. These samples were then directly injected in the LC-QqQ/MS^2 for chromatographic analysis; the sample volume used was 2.5 µL. The remaining 175 µL of the semi-purified serum was also evaporated to dryness at room temperature using the same procedure described above and then stored at -80ºC until its use in the cell culture experiments (Figure 1).

Chromatographic analysis

The chromatographic analysis was performed using a 1200 LC Series coupled to a 6410 QqQ/MS^2 (Agilent Technologies, Palo Alto, USA). The separations were achieved using a Zorbax C18 (100 mm x 2.1 mm i.d., 1.8-µm particle size) chromatographic column from Agilent Technologies. The mobile phase consisted of 0.2% acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 mL/min. The elution gradient was 0-10 min, 5-55% B, 10-12 min, 55-80% B, 12-15 min, 80% B isocratic, and 15-16 min, 80-5% B. A post run of 10 min was applied. The ESI conditions were the following: drying gas temperature and flow rate of 350ºC and 12 L/min, respectively, nebuliser gas pressure of 45 psi, and capillary voltage of 4,000 V. The QqQ was operated in the negative mode. The QqQ acquisition was performed in the MRM mode for the analysis of the proanthocyanidins and their metabolites.

Method validation and sample quantification

To validate the quantitative method, the calibration curves, linearity, extraction recovery, precision, sensitivity, and the method detection and

quantification limits were studied through an analysis of the standard solutions and blank serum samples that were spiked with standard flavanols. The calibration curves were obtained by plotting the analyte/IS peak abundance ratios and the corresponding analyte/IS concentration ratios. The extraction recovery was evaluated through a comparison of the responses of the spiked samples with the calibration curves of the standard solutions. The precision of the method was assessed from the relative standard deviation (RSD) in a triplicate analysis of a spiked sample. The sensitivity was evaluated by determining the limit of detection (LoD), which is defined as the concentration that corresponds to three times the signal-to-noise ratio, and the limit of quantification (LoQ), which is defined as the concentration that corresponds to 10 times the signal-to-noise ratio. The method detection and quantification limits (MDL and MQL, respectively) were calculated in the analysis of 350 µL of a sample. Table 1 shows the values that were obtained for each quality parameter.

To quantify the samples, eight spiked blank samples with different concentration levels were used to obtain the calibration curves. The standard compounds in the samples were then quantified through the interpolation of the analyte/IS peak abundance ratios in these curves. Due to the lack of appropriate standards, the catechin, epicatechin and epicatechin gallate metabolites were tentatively quantified using the calibration curves of the catechin, epicatechin and EGCG standards, respectively. Similarly, the proanthocyanidin B3 dimer and the proanthocyanidin trimer were quantified using the calibration curves of the proanthocyanidin B2 dimer.

Cell culture

The human hepatocellular carcinoma cell line HepG2 (ATCC code HB-8065, Manassas, VA, USA) was cultured in DMEM medium supplemented with 10% (v/v) foetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine in a cell culture flask at 37ºC in a humidified atmosphere with 5% CO_2. The cells were fed every 2-3 days.

Lipid analysis

Once they reached 80-90% confluence, the HepG2 cells were seeded at a cell density of 500 x 10^3 cell/ well in 12-well plates. The growth medium was replaced by supplemented culture media 12 hours before the addition of the treatments. In the first *in vitro* study, the HepG2 cells were treated with 25 mg/L of GSPE, CCX, or PYG, which was dissolved in 1% EtOH, or vehicle (1% EtOH), which was used as the negative control. In the second *in vitro* study, the HepG2 cells were treated with the semi-purified rat serum. The dried semi-purified serum was redissolved in supplemented culture medium and then added to the growth medium on the well (1:10, v/v). GSPE (25 mg/L) and EtOH (1%) were used as positive and negative controls, respectively. In both *in vitro* studies, ^{14}C-acetate (0.6 µCi/mL) was added to the cell culture medium at the same time as the treatment to assess the lipid synthesis.

After 6 hours of treatment with the different proanthocyanidin extracts or sera, the media and cells were collected. The lipid fractions were obtained through a hexane/isopropanol (3:2, v:v) extraction and separated through thin layer chromatography (TLC). The lipids analysed in both studies were cholesterol ester (CE), free cholesterol (FC) and triglycerides (TG). TLC was performed as previously described [27] with an additional separation using a hexane/MTBE/NH_3 (30:20:0.1, v:v:v) solvent to obtain the TG fraction [16]. The obtained lipid fractions were separated, and the radioactivity was measured by scintillation counting. The values were corrected per milligram of protein, which was determined using the Bradford methodology [28].

Statistical analysis

The results are expressed as the mean ± standard error (SEM) of the mean and were analysed by Student's t-test and one-way ANOVA using the SPSS software. The differences between the groups were assessed using the Bonferroni test (to correct for multiple comparisons). The differences between the means were considered significant when $p < 0.05$.

3. Results

Proanthocyanidin-rich extracts decrease lipid synthesis and excretion in HepG2 cells

All three tested proanthocyanidin-rich extracts (GSPE, CCX and PYC) induced a 50-60% decrease in the synthesis of CE in HepG2 cells; no differences were observed between the three extracts (Figure 2A). Furthermore, GSPE and PYC also induced a 40-50% decrease in the levels of both FC and TG. However, even though CCX also induced a decrease in the FC and TG levels, this decrease was not statistically significant (Figures 2B and 2C).

Moreover, no significant difference was measured in the amount of CE, FC and TG in the medium in response to any of the three proanthocyanidin-rich extracts. Therefore, the observed decrease in the synthesis of FC, CE and TG was due to a significant reduction of the lipids inside the cells (Figure 2).

Characterisation of the GSPE metabolites in the rat serum

To assess the bioactive compounds in GSPE that affect the *de novo* lipid synthesis and excretion, we characterised the proanthocyanidin metabolites that are present in the sera of GSPE-administered rats by LC-ESI-QqQ/MS. This analysis was performed 2 hours after the administration of 1 g/kg GSPE to the rats. Figure 3 illustrates the extracted ion chromatograms of the analysis of the flavonoids and their metabolites. The results of this analysis are shown in Table 2, which details the amount of each flavonoid and metabolite in the rat serum. Hence, 2 hours after GSPE ingestion, the main compounds detected in the rat sera were conjugated forms of the monomeric flavan-3-ols (catechin and epicatechin). Specifically, the flavan-3-ol metabolites (epi)catechin glucuronide, methyl-(epi)catechin-glucuronide, epicatechin-sulphate, methyl-epicatechin and methyl-

(epi)catechin-sulphate were detected at concentrations of at least 0.15 μM (Table 2). The glucuronidated forms were present in the sera at a substantially higher concentration than the concentrations of the methylated and sulphated conjugates. However, in contrast to the high amount of these compounds in the extract, free forms of unconjugated catechin, epicatechin and dimeric proanthocyanidins were detected in low amounts [29]. Moreover, other compounds that were also abundant in GSPE were not detected in the sera, such as monomeric gallate conjugates and trimeric proanthocyanidins. The retention times of each compound are shown in Figure 3.

The metabolites in the serum of GSPE- administered rats decrease lipid synthesis and excretion in HepG2 cells.

Two hours after the administration of GSPE, the rat serum was extracted, semi-purified and used to treat HepG2 cells as a source of bioactive GSPE metabolites. The results show that the metabolites in the serum extracted from GSPE-administered rats significantly reduced the total percentage of CE and FC in both the cells and the culture medium compared with the effect obtained with the sera of rats that were administered water (Figures 4A and 4B). The percentage of TG was also reduced inside the cell but not in the medium (Figure 4C). The GSPE metabolites induced 39%, 52% and 72% reductions in the cellular levels of CE, FC and TG.

4. Discussion

Dietary proantocyanidins are known to have numerous potential health benefits; these compounds present a protective role against different cardiovascular risk factors [30, 31], including high serum lipid levels. In fact, our research group has reported a reduction in postprandial TG as a result of the administration of 25 mg/kg of grape seed proanthocyanidin extract to normolipidemic rats [16], mice [32] and rats that receive a high-fat diet [33]. In addition, the GSPE-induced suppression of some lipogenic enzymes, which are induced by a high-fat diet, has been described [33, 34]. Moreover, a previous *in vitro* study reported the reduction of *de novo* TG synthesis and secretion in HepG2 cells that were treated with 50 mg/kg GSPE [16].

In our study, the GSPE-treated HepG2 cells exhibited hypolipidemic effects compared to the control cells. In particular, we found a significant reduction in the accumulation of intracellular ^{14}C-lipids but no significant differences in the lipid contents in the media. This result is likely because the incorporated radioactivity was measured after 6 and not after 12 hours of treatment, as previously described [16]. Interestingly, our results show that half of the dose of GSPE (25 mg/L) and half of the treatment duration was sufficient to reduce lipid synthesis and production. Similar results were observed with the other proanthocyanidin-rich extracts (CCX and PYC). However, CCX did not significantly decrease the syntheses and the amount (both the cell and medium contents) of FC and TG (Figures 4B and 4C, respectively); this difference is likely because of the different composition of the extracts.

346 The data obtained using non-bioactive molecules and extracts to treat
347 cultured cells have been questioned [22]. More specifically, the *in vitro* results
348 obtained using non-physiologically relevant forms and/or concentrations of
349 flavonoids is considered an important limitation of the studies that aim to determine
350 the *in vivo* bioactivity of these compounds [20, 21]. An important feature of
351 polyphenols is the changes that occur to these molecules during first-pass
352 metabolism. Thus, the molecular forms that reach the peripheral circulation and
353 tissues are different from those that are present in foods [20]. In addition, the
354 microflora extensively affects polyphenol hydrolysis and hence further contributes
355 to the variation in the molecular forms of the polyphenols that are found in the
356 blood and tissues [12, 35, 36]. These findings suggests that the bioactive forms of
357 polyphenols are different from those that are present in food and that *in vitro*
358 experiments with food polyphenols do not necessarily capture the *in vivo* situation
359 [37]. In fact, GSPE is rich in monomers, namely catechin and epicatechin, which
360 are found both free and conjugated to a gallate moiety, and dimers [29]. However,
361 these free forms are not found in a high concentration in the serum, which is
362 predominantly composed of conjugated metabolites, such as catechin and
363 epicatechin glucuronide. Moreover, only dimeric proanthocyanidins are detected in
364 serum, whereas trimeric proanthocyanidins are not detected despite their
365 abundance in GSPE [29]. The serum metabolites that were detected in this study
366 are similar to those that were previously reported by other authors that analysed
367 plasma after the administration of the same dose of GSPE [29]. Therefore, the
368 bioactive flavanols in rat serum differ considerably from the compounds that are
369 present in GSPE. Hence, it is important to perform flavan-3-ol functionality studies

using the metabolites in the serum of GSPE-administered rats for the treatment of cells. This approach is particularly important when extracts and not pure compounds are used to treat cells since the extracts consist of a complex mixture of different molecules. Moreover, the post-absorption metabolization of the extract mixture yielding numerous metabolites [29], turns into impossible to obtain them from other sources rather than from the rat.

In this study, we combined the *in vivo* and *in vitro* system described to establish the bioactivity of proanthocyanidins on *de novo* lipid synthesis and excretion. Thus, HepG2 cells were incubated with the physiological metabolites that are present in the serum of GSPE-administered rats. It is important to point out that the sera metabolites comes from the metabolization not only by the liver hepatocytes, but also by the intestinal cells and by the microbiota. Therefore, the metabolites used in our study contained all the possible bioactive forms of polyphenols that are present in the sera of the animals 2 hours after the administration of an extract rich in proantocyanidins. The rats were administered with an acute dose of 1g/Kg body weight of GSPE to obtain sufficient amount of the proanthocyanidins metabolites to observe a further functionality in HepG2 cells [29]. The rat serum was obtained 2 hours after the administration of the proanthocyanidin-rich extract because the highest plasma peak concentrations of flavanols are obtained 2 to 3 hours after the ingestion of the extract in a dose-dependent manner [38, 39]. Specifically, the maximum peaks were obtained in the plasma of rats that received 1 g/kg of GSPE 1 to 2 hours post-administration [29]. Moreover, before its use for the treatment of HepG2 cells and chromatographic

393 analysis, the sera were semi-purified using a micro-solid-phase extraction
394 procedure (μSPE), which is a standard method that has been used prior to the
395 chromatographic analysis of the polyphenols in biological samples, such as plasma
396 [40]. Through this method, the numerous interferences in the sera, which are
397 mainly proteins, are reduced. In addition to the pretreatment of the plasma, this
398 method pre-concentrates the phenolic compounds because these are present in
399 the plasma and serum in trace amounts [40]. Therefore, we consider this semi-
400 purification and pre-concentration step crucial to obtain mostly purified bioactive
401 flavonoids and metabolites for both chromatographic analysis and *in vitro* cell
402 treatment.

403 The results obtained using the metabolites in the sera of GSPE-
404 administered rats, which were compared to the results obtained with the sera of
405 rats that were orally administered water, showed a decrease in the *de novo* lipid
406 synthesis and excretion in HepG2 cells, especially of TG. Similarly, a marked
407 decrease in the TG level was also observed in previous *in vivo* studies that
408 administered GSPE to rats, which showed that GSPE induced a clear
409 hypotriglyceride effect in animals [15]. The lowering lipid effect of semi-purified rat
410 sera is indicative that the physiological forms of the GSPE flavonoids also exhibit
411 hypolipidemic properties. It was recently postulated that orally administered
412 quercetin and likely other flavonoids are conjugated in the intestine and liver prior
413 to its transport to the bloodstream and that the flavonoid released from its
414 glucuronidated metabolite is responsible for its activity [41, 42]. Although our study
415 has been performed with other cell types and using other flavonoids that are

different from quercetin, as well as with extracts instead of pure compounds, the obtained results also show the bioactivity of the conjugated metabolites.

Finally, although this study was realized with the phase II metabolites and some aglycones (i.e.; a time-point of 2h was selected), this methodology can be a particularly useful tool to test the bioactivity of microbial metabolites, through the use of serum from rats administered proanthocyadins after long time-points. These studies could be especially relevant taking into account that the microbial metabolites are thought to be the responsible forms of a great part of the health effects of proanthocyanidns [43].

In conclusion, this study demonstrates the high hypolipidemic *in vitro* effect of different proanthocyanidin-rich extracts. Furthermore, the bioactivity of the proanthocyanidin rat sera metabolites of GSPE was also demonstrated in HepG2 cells, which exhibited a decrease in lipid synthesis and excretion. All these results demonstrated that the proposed *in vivo-in vitro* combined system is useful to study the functionality of the bioactive forms of flavonoids.

Acknowledgements

This study was supported by grants from the Ministerio de Educación y Ciencia of the Spanish Government (AGL 2008-00387/ALI) and the Universitat Rovira i Virgili-Banco Santander (2011 LINE-12 and 2011 LINE-13).

437

438

References

1. Smith SC,Jr. Multiple risk factors for cardiovascular disease and diabetes mellitus. Am J Med. 2007 Mar;120(3 Suppl 1):S3-S11.

2. Hollman P, Hertog M, Katan M. Role of dietary flavonoids in protection against cancer and coronary heart disease. Biochem Soc Trans. 1996;24:785-9.

3. Joshipura KJ, Hu FB, Manson JAE, Stampfer MJ, Rimm EB, Speizer FE, et al. The effect of fruit and vegetable intake on risk for coronary heart disease. Ann Intern Med. 2001;134(12):1106-14.

4. Liu S, Lee IM, Ajani U, Cole SR, Buring JE, Manson JAE. Intake of vegetables rich in carotenoids and risk of coronary heart disease in men: The physicians' health study. Int J Epidemiol. 2001;30(1):130-5.

5. Hertog MGL, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, et al. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch Intern Med. 1995;155(4):381.

6. Ariga T. The antioxidative function, preventive action on disease and utilization of proanthocyanidins. Biofactors. 2004;21(1−4):197-201.

7. Rasmussen SE, Frederiksen H, Struntze Krogholm K, Poulsen L. Dietary proanthocyanidins: Occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. Mol. Nutr. Food Res. 2005;49(2):159-74.

8. Hooper L, Kroon PA, Rimm EB, Cohn JS, Harvey I, Le Cornu KA, et al. Flavonoids, flavonoid-rich foods, and cardiovascular risk: A meta-analysis of randomized controlled trials. Am J Clin Nutr. 2008;88(1):38-50.

9. Hammerstone JF, Lazarus SA, Schmitz HH. Procyanidin content and variation in some commonly consumed foods. J Nutr. 2000;130(8):2086S-92S.

10. Borriello A, Cucciolla V, Della Ragione F, Galletti P. Dietary polyphenols: Focus on resveratrol, a promising agent in the prevention of cardiovascular diseases and control of glucose homeostasis. Nutr Metab Cardiovas. 2010;20(8):618-25.

11. Bladé C, Arola L, Salvadó MJ. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. Mol. Nutr. Food Res. 2010;54(1):37-59.

12. Gonthier MP, Donovan JL, Texier O, Felgines C, Remesy C, Scalbert A. Metabolism of dietary procyanidins in rats. Free Radical Bio Med. 2003;35(8):837-44.

13. Baba S, Natsume M, Yasuda A, Nakamura Y, Tamura T, Osakabe N, et al. Plasma LDL and HDL cholesterol and oxidized LDL concentrations are altered in normo-and hypercholesterolemic humans after intake of different levels of cocoa powder. J Nutr. 2007;137(6):1436-41.

14. Sugiyama H, Akazome Y, Shoji T, Yamaguchi A, Yasue M, Kanda T, et al. Oligomeric procyanidins in apple polyphenol are main active components for inhibition of pancreatic lipase and triglyceride absorption. J Agric Food Chem. 2007;55(11):4604-9.

15. Del Bas JM, Fernández-Larrea J, Blay M, Ardèvol A, Salvadó MJ, Arola L, et al. Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. The FASEB journal. 2005;19(3):479-81.

16. Del Bas JM, Ricketts ML, Baiges I, Quesada H, Ardevol A, Salvadó MJ, et al. Dietary procyanidins lower triglyceride levels signaling through the nuclear receptor small heterodimer partner. Mol. Nutr. Food Res. 2008;52(10):1172-81.

17. Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. J Nutr. 2000;130(8):2073S-85S.

18. Tomas-Barberán FA, Cienfuegos-Jovellanos E, Marín A, Muguerza B, Gil-Izquierdo Á, Cerdá B, et al. A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. J Agric Food Chem. 2007;55(10):3926-35.

19. Walle T. Absorption and metabolism of flavonoids. Free Radical Bio Med. 2004;36(7):829-37.

20. Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C, et al. How should we assess the effects of exposure to dietary polyphenols in vitro? Am J Clin Nutr. 2004;80(1):15-21.

21. Kay CD. The future of flavonoid research. Br J Nutr. 2010;104:91.

22. del Bas JM, Laos S, Caimari A, Crescenti A, Arola L. Detection of bioavailable PPARγ modulators by a cell-based luciferase reporter system. Anal Biochem. 2012.

23. Quiñones M, Miguel M, Muguerza B, Aleixandre A. Effect of a cocoa polyphenol extract in spontaneously hypertensive rats. Food Funct. 2011;2(11):649-53.

24. Andújar I, Recio MC, Giner RM, Cienfuegos-Jovellanos E, Laghi S, Muguerza B, et al. Inhibition of ulcerative colitis in mice after oral administration of a

polyphenol-enriched cocoa extract is mediated by the inhibition of STAT1 and STAT3 phosphorylation in colon cells. J Agric Food Chem. 2011;59(12):6474-83.

25. D'Andrea G. Pycnogenol: A blend of procyanidins with multifaceted therapeutic applications? Fitoterapia. 2010;81(7):724-36.

26. Martí MP, Pantaleón A, Rozek A, Soler A, Valls J, Macià A, et al. Rapid analysis of procyanidins and anthocyanins in plasma by microelution SPE and ultra−HPLC. Journal of separation science. 2010;33(17−18):2841-53.

27. Pill J, Aufenanger J, Stegmeier K, Schmidt F, Müller D. Thin-layer chromatography of radioactively labelled cholesterol and precursors from biological material. Fresenius J Anal Chem. 1987;327(5):558-60.

28. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.

29. Serra A, Macià A, Romero MP, Valls J, Bladé C, Arola L, et al. Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. Br J Nutr. 2010;103(7).

30. Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, et al. (–)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proc Natl Acad Sci U S A. 2006;103(4):1024-9.

31. Heiss C, Keen CL, Kelm M. Flavanols and cardiovascular disease prevention. Eur Heart J. 2010;31(21):2583-92.

32. Del Bas JM, Ricketts M, Vaqué M, Sala Eea. Dietary procyanidins enhance transcriptional activity of bile acid-activated FXR in vitro and reduce triglyceridemia in vivo in a FXR-dependent manner. Mol. Nutr. Food Res. 2009;53:805–814.

33. Quesada H, Del Bas J, Pajuelo D, Diaz S, Fernandez-Larrea J, Pinent M, et al. Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. Int J Obes. 2009;33(9):1007-12.

34. Baiges I, Palmfeldt J, Blade C, Gregersen N, Arola L. Lipogenesis is decreased by grape seed proanthocyanidins according to liver proteomics of rats fed a high fat diet. Mol Cell Proteomics. 2010;9(7):1499-513.

35. Kühnau J. The flavonoids. A class of semi-essential food components: Their role in human nutrition. World Rev Nutr Diet. 1976;24:117.

36. Dall'Asta M, Calani L, Tedeschi M, Jechiu L, Brighenti F, Del Rio D. Identification of microbial metabolites derived from in vitro fecal fermentation of different polyphenolic food sources. Nutrition. 2012;28(2):197-203.

37. Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MRA, Williamson G. Human metabolism of dietary flavonoids: Identification of plasma metabolites of quercetin. Free Radic Res. 2001;35(6):941-52.

38. Serafini M, Bugianesi R, Maiani G, Valtuena S, De Santis S, Crozier A. Plasma antioxidants from chocolate. Nature. 2003;424(6952):1013.

39. Rein D, Lotito S, Holt RR, Keen CL, Schmitz HH, Fraga CG. Epicatechin in human plasma: In vivo determination and effect of chocolate consumption on plasma oxidation status. J Nutr. 2000;130(8):2109S-14S.

40. Suárez M, Romero MP, Ramo T, Macià A, Motilva MJ. Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants. J Agric Food Chem. 2009;57(4):1463-72.

41. Menendez C, Dueñas M, Galindo P, González-Manzano S, Jimenez R, Moreno L, et al. Vascular deconjugation of quercetin glucuronide: The flavonoid paradox revealed? Mol. Nutr. Food Res. 2011.

42. Galindo P, Rodriguez-Gómez I, González-Manzano S, Dueñas M, Jiménez R, Menéndez C, et al. Glucuronidated quercetin lowers blood pressure in spontaneously hypertensive rats via deconjugation. PloS one. 2012;7(3):e32673.

43. Del Rio D, Rodriguez-Mateos A, Spencer JP, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal. 2013; 18(14):1818-92.

Figure legends

Figure 1. Graphical representation of the *in vivo* and *in vitro* systems used in this study.

Figure 2. Effect of proanthocyanidin-rich extracts on lipid synthesis and secretion in HepG2 cells. Changes induced by GSPE, CCX and PYC on the *de novo* synthesis and secretion of CE (A), FC (B) and TG (C). The HepG2 cells were simultaneously incubated with ^{14}C-labelled acetate and 25 mg/L of GSPE, CCX, PYG or vehicle (control). After 6 hours of treatment, the radioactivity that was incorporated into the media and cellular lipids was measured. %CE, %TC and %TG were calculated considering the control as the 100%. All of the values are the mean ± SEM of triplicates of three independent experiments; the bars with different letters indicate statistically significant differences compared to the control ($p < 0.05$).

Figure 3. Extracted ion chromatograms of the compounds in Table 2. These chromatographs were used for the analysis of the serum of GSPE-administered rats. The serum was extracted 2 hours after the administration of 1 g/kg of GSPE. The following compounds were analysed: (1) gallic acid; (2) B1 dimer; (3) B1+B3 dimer; (4) B2 dimer; (5) methyl- catechin-glucuronide; (6) methyl-epicatechin-glucuronide; (7) catechin glucuronide; (8) epicatechin glucuronide; (9) catechin; (10) epicatechin; (11) epicatechin sulphate; (12) epicatechin gallate; (13) methyl-catechin-O-sulphate; (14) methyl-epicatechin-O-sulphate; (15) 3-methyl-epicatechin; and (16) 4-methyl-epicatechin.

Figure 4. Effect of semi-purified serum from GSPE-administered rats on lipid synthesis and secretion in HepG2 cells. Changes induced by the semi-purified serum of GSPE-administered rats on the *de novo* synthesis and secretion of CE (A), FC (B) and TG (C). The serum was extracted 2 hours after the administration of water (control group) or 1 g/kg of GSPE. The HepG2 cells were simultaneously incubated with ^{14}C-labelled acetate and semi-purified rat serum. After 6 hours of treatment, the radioactivity that was incorporated into the media and the cellular lipids was measured. %CE, %TC and %TG were calculated considering the control as the 100%. All of the values are the mean ± SEM of triplicates of three independent experiments.

Table 1. Quality parameters of the quantitative method used for the determination of proanthocyanidins by LC-ESI-QqQ/MS^2.

Compound	Calibration curve	Determination coefficient (R^2)	Linearity (µM)	Recovery (%)	Precision (%RSD, n=3)	LoD (nM)	LoQ (nM)	MDL[a] (nM)	MQL[a] (nM)
Catechin	y=0.1472x	0.99	0.19-39	99	0.29	20.70	68.90	5.90	19.70
Epicatechin	y=0.1127x	0.99	0.18-36	80	1.06	12.40	41.30	3.50	11.80
B2 dimer	y=0.2474x	0.99	0.04-9.0	92	0.27	6.90	23.00	2.00	6.60
B1 dimer	y=0.3335x	0.99	0.04-8.6	69	0.85	31.10	103.70	8.90	29.60
EGCG	y=2.0337x	0.99	0.20-12	73	8.77	1.20	4.00	0.30	1.10
Gallic acid	y=0.2719x	0.98	0.31-61	48	0.42	35.30	117.60	10.10	33.60

[a] Method detection and quantification limits are expressed in µmol/L of fresh sample, which was calculated for the analysis of a 350-µl serum sample.

Abbreviations: EGCG, Epigallocatechin Gallate; LOD, Limit of Detection; LOQ, Limit of Quantification; MDL, Method Detection Limit; MQL, Method Quantification Limit

610 **Table 2.** Flavonoids and metabolites in the serum of rats that ingested an acute dose of 1
611 g/kg of grape seed proanthocyanidin extract (GSPE). The quantification was performed 2
612 hours after the administration of GSPE. The data are presented as the mean (μM) ± SEM
613 (n=4).

Compound	**Total amount (μM)**
Catechin	0.08±0.02
Epicatechin	0.52±0.14
Procyanidin B1 dimer	0.10±0.06
Proanthocyanidin B2 dimer	0.19±0.50
Proanthocyanidin B3 dimer [(3)]	0.10±0.02
Gallic acid	0.89±0.13
Epicatechin gallate[(4)]	n.d.
Dimer gallate [(3)]	n.d.
Trimer[(3)]	n.d.
EGCG	n.d.
Metabolites	
Catechin-glucuronide[(1)]	>39±14.89
Epicatechin-glucuronide[(2)]	>36±14.51
Methyl-catechin-glucuronide[(1)]	14.89±1.96
Methyl-epicatechin-glucuronide[(2)]	12.35±1.16
Catechin-sulphate[(1)]	n.d.
Epicatechin-sulphate[(2)]	0.76±0.16
3-o-methyl-epicatechin[(2)]	0.15±0.03
4-o-methyl-epicatechin[(2)]	0.34±0.05
Methyl-catechin-o-sulphate[(1)]	1.50±0.30
Methyl-epicatechin-o-sulphate[(2)]	3.91±0.63

614 Abbreviations: n.d.= not detected; n.q.= not quantified; EGCG= Epigallocatechin
615 Gallate
616 [1] Quantified as Catechin
617 [2] Quantified as Epicatechin
618 [3] Quantified as Dimer B2
619 [4] Quantified as EGCG
620
621