Anti-proliferative bioactivity against HT-29 colon cancer cells of a withanolides-rich extract from golden berry (*Physalis peruviana* L.) calyx investigated by Foodomics

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ABSTRACT

A comprehensive Foodomics study was carried out in this work to investigate the changes induced at gene and metabolite expression levels on HT29 colon cancer cell lines upon treatment with a bioactives-enriched extract from goldenberry calyx. As a result of the proposed multi-omics approach, the tested extract induced transcriptional activation of pro-apoptotic genes, and altered the expression of several genes related to the oxidative stress response in treated cancer cells. Metabolomics data confirm altered cellular redox homeostasis, providing additional evidence to transcriptomic results. Foodomics data integration also revealed alteration on relevant metabolic processes, suggesting inactivation of aminoacyl tRNA charging pathway, dysfunction on carnitine shuttle and beta-oxidation of fatty acids, and pyrimidine ribonucleotide interconversion impairment. These observations are in line with functional analysis and anti-proliferative activity results, where the viability of HT-29 colon cancer cells was notably reduced after 48h treatment without affecting the viability of normal human colon fibroblast cells.

1. INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer diagnosed and the second leading cause of cancer death, affecting both men and women in the world. In 2018, 1,849,518 people were diagnosed with CRC and 880,792 died by its cause (Cancer, 2019). CRC is the result of a progressive accumulation of genetic and epigenetic alterations related to mutational inactivation of tumor-suppressor genes (P53, APC and TGF- β) and activation of oncogene pathways (RAS, BRAF, and PI3K) leading to a marked genomic instability (Benarba & Pandiella, 2018). CRC can be caused by microbial infections, radiations, smoking habits, excessive alcohol consumption, high-level intake of red meat and obesity (Gothai et al., 2018). Despite of the advances in the CRC research field, which has indeed resulted in higher cure rates, this disease still remains a leading health challenge, consequently it is necessary to devote bigger efforts upon knowing the exact mechanism of CRC carcinogenesis, as well as to establish more significant strategies that contribute to their prevention and treatment (Narayanan et al., 2019).

In this context, the possibility of using naturally occurring materials to manage CRC has been an appealing alternative to overcome the expectations of this disease. CRC chemoprevention and management with dietary natural products and phytochemicals from medicinal plants have been widely studied, giving rise to innovative therapeutic approaches (Benarba & Pandiella, 2018; Gothai et al., 2018; Martínez Leo, Altamirano, & Segura Campos, 2018). The search of anti-cancer phytochemicals has also expanded to agro-food by-products, allowing their valorisation. However, the variety of starting natural materials, the chemical diversity of the potential bioactive compounds, the molecular complexities of the CRC and the recapitulation of preclinical tumour models make it necessary to ensure that the tested natural whole extract and/or their isolated compounds are fully and scientifically vetted to facilitate their eventual use for CRC management (Hussain, Kumar, & Ghosh, 2016). In this sense, cell culture studies can

80 reveal mechanistic aspects of the effects of the extracts/compounds and in this way establish
81 their anti-cancer potential.

82 Foodomics discipline can contribute to resolve several aspects mentioned above. Firstly, the
83 sustainability criteria of Green Foodomics can guide the selective recovery of bioactive
84 compounds based on theoretical modelling of solubility in green solvents, along with emergent
85 extraction strategies such as compressed fluids based techniques (Gilbert-López, Mendiola, &
86 Ibáñez, 2017). On the other hand, Foodomics can provide valuable information about changes
87 induced by bioactive compounds on a biological system (i.e., cell culture, tissue, organ or
88 organism) at different expression levels (Garcia-Canas, Simo, Herrero, Ibanez, & Cifuentes,
89 2012). For this, Foodomics involves the development, use and integration of omics approaches,
90 mainly, transcriptomics, proteomics, and metabolomics (A. Valdés, Cifuentes, & León, 2017).

91 Goldenberry (*Physalis peruviana* L.) is a yellow-orange fleshed berry of great commercial
92 interest due to its organoleptic quality, nutritional value and bioactive compounds content
93 (Puente, Pinto-Muñoz, Castro, & Cortés, 2011). This fruit is mainly consumed as fresh product,
94 although its processed products (sauces, juice, snacks and marmalades) are also widely
95 commercialized. The goldenberry grows on the calyx (or husk), which contributes to fruit
96 development (carbohydrates translocation) and with its protection from extreme climatic
97 conditions, mechanical damage, aerial distributed diseases, insects and birds (Lim, 2013). The
98 protection functions of calyx have been associated to the biosynthesis of repellent and,
99 antioxidant ~~and antifeeding~~ substances (Fischer & Herrera, 2011). Calyx is one of the
100 postharvest by-products and represents an expansive agro-food waste, since 1 to 2 tons of
101 biomass are generated per ha of goldenberry crop (Yıldız, İzli, Ünal, & Uylaşer, 2015).

102 Different research works have described a goldenberry calyx as source of bioactive compounds
103 (Calderón, Ruiz, & Castellanos, 2012; Lan et al., 2009). In previous studies (Diego Ballesteros-
104 Vivas et al., 2019; Ballesteros-Vivas, Alvarez-Rivera, Ibanez, Parada-Alfonso, & Cifuentes,

2019), we developed a sustainable strategy for goldenberry calyx valorisation, which allowed us to obtain a PLE extract enriched in high-added value compounds including withanolides, phenolic acids, flavonoids, sucrose esters, terpenoids, phytosterols and phytol derivatives with reported bioactive properties. These preliminary findings suggest a promising bioactive extract with high potential as health promoting agent. In this work we focused our efforts on: i) testing the anti-proliferative activity of the PLE-goldenberry calyx extract against a cell model of human colon adenocarcinoma (HT-29 cells) compared to normal colon cells, and ii) investigating the molecular changes upon treatment of HT-29 cells with the PLE extract, under a Foodomics strategy involving transcriptomic and metabolomics data analysis.

2. MATERIAL AND METHODS

2.1 Reagents and materials

D-Glucose-L-Cysteine, leucine, L-phenylalanine, pantothenic acid, S-Carboxymethyl-L-cysteine, N-Benzoyl-L-tyrosine ethyl ester, glutaric acid, benzoic acid, adenosine, nicotinic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640 cell culture medium, gentamicin (10 mg/mL), dimethyl sulfoxide (DMSO), Triton X-100, 5-fluorouracil and formic acid were purchased from Sigma-Aldrich (Madrid, Spain). Becton Dickinson (BD Pharmingen™, NJ, USA) provided the Annexin V, 7-amino-actinomycin D (7-AAD) and Annexin V Binding Buffer 10X. Fetal bovine serum (Gibco), 0.05% and trypsin-EDTA (Gibco) and TRIzol™ Plus RNA Purification Kit (Invitrogen) were purchased from Thermo Fisher Scientific (Rockford, IL).

Solvents employed were HPLC-grade. Acetonitrile and ethanol were acquired from VWR Chemicals (Barcelona, Spain), whereas ethyl acetate by Scharlau (Barcelona, Spain). Ultrapure water was obtained from a Millipore system (Billerica, MA, USA). For the UHPLC-q-TOF-MS analyses, MS grade ACN and water from LabScan (Dublin, Ireland) were employed.

2.2 Calyx extracts

The goldenberry calyx extract was obtained by a PLE process using an ~~commercial Accelerated~~ solvent extractor ASE 200 device (Dionex™ ASE™ 200) as described in our previous work (Diego Ballesteros-Vivas et al., 2019), where the extraction conditions (temperature and solvent composition) were optimized to maximize withanolide content, extraction yield, total phenolic content, total flavonoid content and antioxidant activity. Briefly, dried samples of goldenberry calyx were mixed with sea sand (1:2 w/w) and loaded into the extraction cell (11 mL stainless steel). PLE extraction was performed in static mode using an ethanol:ethyl acetate (75:25 v/v) mixture as solvent at 125 °C and 100 bar for 20 min. For comparison purposes, a dynamic maceration (DM) procedure was carried out using the same solvent composition. For this, samples of goldenberry calyx (~1 g) were agitated at 750 rpm (Thermomixer R Eppendorf AG, Hamburg, Germany) at 20 °C for 24 h. The extract was separated by centrifugation (Eppendorf Centrifuge 5810 R, Hamburg, Germany) at 5000 rpm for 10 min. After the extractions, the solvent was removed by evaporation with continuous stream of gaseous nitrogen.

2.3 Cells lines and cell culture

HT-29 (human colon adenocarcinoma) and CCD-18Co (normal human colon fibroblast) cells were purchased from the American Type Culture Collection. Cell lines were cultured in a growth medium (RPMI 1640 medium supplemented with Hepes 25 mM, L-glutamine 2.05 mM, fetal bovine serum 10% and gentamicin 50 µg/mL) and were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C.

2.4 Anti-proliferative activity assay

The anti-proliferative effect of goldenberry calyx extracts on the cell lines was determined by the MTT colorimetric assay. For this, the PLE- and DM-extract samples were diluted in DMSO

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at a concentration of 10 mg/mL and successively diluted in growth medium to a final concentration of 100, 50, 25, 12.5, and 6.25 µg/mL. Triton X was used at concentrations from 1.0×10^{-4} to 1.0% as a positive reference standard to determine the sensitivity of cell lines. Cells in exponential growth phase (80-90% confluence) were trypsinized, counted and seeded in 96-well microtiter plates at a density of 1.0×10^4 (HT-29) and 4.5×10^3 (CCD-18Co) cells/well, and allowed 24 h for attachment. Then, cells were treated with different concentrations of extracts, Triton X and the vehicle (DMSO 0.1% v/v) regarded as untreated controls and incubated at three different time points 24, 48, and 72 h. After the treatment time, the culture medium was replaced by 100 µL of MTT solution (0.25 mg/mL RPMI 1640 medium) and the plates were incubated for 4 h. After this time, medium was discarded and 100 µL of DMSO were added and the absorbance was measured at 570 nm (Tecan, Infinite® 200 PRO, Austria GmbH). Each experiment was performed as three independent tests with at least three replicates. The cell viability was expressed as percentage of live cells relative to controls. The IC₅₀ values (concentration of extract that causes 50% inhibition or cell death) were determined based on the dose-dependent response curves of extract using GraphPad Prism 7.0 software (GraphPAD Corp., San Diego, CA, USA). The IC₅₀ values from untreated controls and treatment groups were compared among themselves using Student's t-test. Differences were considered significant when *p*-value was < 0.05.

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2.5 Flow cytometry for cell cycle and apoptosis analysis

HT-29 cells (1.8×10^6 cells) were cultured in T25 cm² flasks and incubated with the calyx PLE-extract at IC₅₀ value according to the anti-proliferative activity results for 24, 48 and 72 h. In parallel, cells were treated with 50 µM of 5-FU regarded as positive control of apoptosis. After treatment, attached cells were collected by trypsinization and washed twice with PBS, cell pellet obtained was resuspended in 2 mL of PBS and split into two tubes, one for cell cycle analysis

180 and the other for apoptosis analysis. For cell cycle analysis, the cell pellets were fixed with 70%
181 ethanol for at least 1 h at 4°C, and stored at –20°C until use. The fixed cells were then washed
182 with PBS and stained with a 7-AAD solution for at least 15 min at room temperature.
183 Fluorescence intensity of the cells was analyzed using a BD Accuri™ C6 flow cytometer (BD
184 Biosciences, San Jose, USA). The percentages of cells in G₀/G₁ phase, S phase, and G₂/M phase
185 were analyzed using a trial version of the ModFit LT software, version 5.0.
186 The induction of apoptosis was examined using annexin V-PE/7-AAD apoptosis detection kit
187 (BD Pharmingen™, NJ, USA) following the manufacturer's instructions. Briefly, cells were
188 incubated for 15 minutes at room temperature in the presence of 5 µL annexin V-PE and 5 µL
189 7-AAD in 100 µL of 1x binding buffer. After incubation, 400 µL of 1x binding buffer was
190 added, and the percentage of apoptotic cells were analyzed using a BD Accuri™ C6 flow
191 cytometer (BD Biosciences, San Jose, USA) and the FlowJo software (TreeStar, Inc). Viable
192 cells were designated as AV-/7-AAD-, early apoptosis as AV+/7-AAD-, late apoptosis as
193 AV+/7-AAD+ and necrosis as AV-/7AAD+.

194

195 2.6 Morphological analysis

196 The morphological changes induced by exposure to the calyx PLE-extract (10 µg/mL) and 5-
197 FU (50 µM) in HT-29 cells were examined by light microscopy at x20 magnification
198 (Axiovert 40 CFL inverted microscope; Carl Zeiss, Germany) photographed with an AxioCam
199 HRm and processed by Zen 2.1, Blue edition, Zeiss, software. A sample representative of three
200 independent experiments was selected for the analysis.

201

202 2.7 Transcriptomics

203 HT-29 cells were seeded at a density of 1.0×10^4 cells/cm² in T25 cm² culture flasks and
204 incubated for 24 h at 37 °C with 5% CO₂ for cell attachment. Thereafter, cell cultures were

205 treated for 48 h with the calyx PLE-extract at IC₅₀ concentration according to the results of the
206 anti-proliferative activity assay (three replicates). In parallel, cell cultures were treated with
207 extract vehicle (DMSO 0.1% in RPMI 1640) regarded as untreated controls (three replicates).
208 Total RNA was isolated from pellets of $\sim 1 \times 10^6$ cells of treated and untreated cell cultures
209 using a TRIzol™ Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA) according to the
210 manufacturer's instructions. The quantity of purified RNA was determined by measuring the
211 absorbance at 260 and 280 nm using an NanoDrop (ND1000, Thermo, Spain). The RNA size
212 chromatography was carried out with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.,
213 Palo Alto, CA, USA). Only those samples with A260/A280 ratio between 1.8 and 2.1 and a
214 28S/18S ratio > 2 were further processed. 300 ng of total RNA were used and double-stranded
215 cDNA synthesis and generation of biotin-labelled cRNA was obtained following the
216 manufacturer protocol (Agilent Technologies). Each calyx PLE-extract treated sample (labelled
217 with Cy3) was randomly mixed with a control sample (labelled with Cy5) prior to hybridization
218 onto Agilent SurePrint G3 Human GE 8x60k Microarray (Agilent Technologies). Raw images
219 were obtained with the Scanner G2505C (Agilent Technologies) and processed with the Agilent
220 Feature Extraction software (Agilent Technologies). Generated gene-expression data files (.txt)
221 were processed using the read.maimages function included in the BioConductor package limma
222 (Linear Models for Microarray Data; (Smyth, 2005)). Background signal correction using the
223 normexp method with an offset of 50, within array normalization using the loess method, and
224 between array scaling using the quantile method were applied. Thereafter, gene signals were
225 submitted to a principal component analysis (PCA) using Statistica software Version 7
226 (Statsoft, Tulsa, OK), and limma package was used to carry out a significance analysis.
227 Moderated t statistics were used for significance analysis of each gene, and the Benjamini and
228 Hochberg's (Benjamini & Hochberg, 1995) method was applied for the control or estimation
229 of the false discovery rate (FDR). To identify the most significant differentially expressed genes

(DEG), a 1.5-fold change cut-off filter (equivalent to 0.585 and -0.585 in log₂ scale) between the two experimental conditions (treatment vs. control), and a statistical filter established at 5 % FDR (adjusted p value < 0.05) were applied. Raw and normalized data of microarray analysis were submitted to the Gene Expression Omnibus database and are accessible through GEO Series accession number GSE132855 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132855>). The lists of DEGs identified in microarray analyses were uploaded into the bioinformatics tool Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA, USA) to i) identify overrepresented canonical pathways and ii) to predict the activation state of different transcription factors. For the canonical pathway analysis, p-values were calculated using the Fisher's exact test based on the number of genes that participate in the pathways (from information stored in the Ingenuity® Knowledge Base) and the number of genes belonging to those pathways uploaded in the dataset, and considered significant when $p < 0.05$, and $1.5 < \text{z-score} < -1.5$ (Krämer, Green, Pollard, & Tugendreich, 2013). The transcription factor analysis was performed using the Upstream Regulator function of IPA, which uses the global direction of gene changes in the dataset and compares it with previously published targets of this regulator, predicting its activation/deactivation. P-values were calculated as for the canonical pathway analysis, and transcription factors were considered activated or "increased" if $\text{z-score} > 2$, and inactivated or "decreased" when $\text{z-score} < -2$.

249

250 *2.8 Metabolomics*

251 For metabolomic study, HT-29 cells were plated at a density of 1.0×10^4 cells/cm² in different
252 T175 cm² culture flasks and allowed 24 h for attachment. After the adhesion time, cell cultures
253 were treated with the calyx PLE-extract at IC₅₀ concentration according to the results of anti-
254 proliferative activity assay and with extract vehicle (regarded as untreated controls). After

255 treatments, cells were washed with PBS solution, trypsinized and centrifuged ($300 \times g$ for 5
256 min at 25°C). Supernatants were removed and cells were resuspended in PBS and counted
257 using trypan blue exclusion method. The estimated volume to have 10×10^6 cells from the cell
258 suspensions were centrifuged and the cell pellets were subjected to metabolite recovery using
259 an extraction method previously optimized in our laboratory (Ibáñez, Simó, Palazoglu, &
260 Cifuentes, 2017; Ibanez et al., 2015). In brief, cell pellets were mixed with 300 μL of ultrapure
261 water and 0.3 g glass beads (212–300 μm). The mixtures were vortexed and snap-frozen in
262 liquid nitrogen for 1 min for metabolite quenching. Subsequently, glass beads were removed
263 by centrifugation ($24,000 \times g$ for 10 min at 4°C) and the supernatants were collected. Protein
264 content was removed from supernatants by centrifugation ($14,000 \times g$ for 10 min at 4°C) using
265 3 kDa centrifugal filters (Amicon Ultra 0.5 mL, Millipore, Billerica, MA, USA).
266 Aqueous fractions were analyzed by liquid chromatography coupled to high resolution mass
267 spectrometry (LC-HRMS) in order to obtain metabolic profiles from treated and untreated HT-
268 29 cells. For this purpose, an Agilent 1290 UHPLC system (Agilent Technologies, Santa Clara,
269 CA, USA), coupled to an Agilent 6540 quadrupole-time-of-flight mass spectrometer (q-TOF
270 MS) equipped with an orthogonal electrospray source (ESI Agilent Jet Stream, AJS, Santa
271 Clara, CA, USA) was employed. In order to widen the metabolic coverage, the LC-q-TOF-MS
272 system was operated in both positive and negative ionization mode, using a Zorbax Eclipse Plus
273 C18 column (2.1×100 mm, 1.8 μm particle diameter, Agilent Technologies, Santa Clara, CA)
274 at 40°C . Elution was performed using the following mobile phases: H_2O + formic acid 0.1%
275 v/v (A) and acetonitrile + formic acid 0.1% v/v (B) for acquisition in positive ionization mode
276 (ESI+), and H_2O + formic acid 0.01% v/v (A) and acetonitrile + formic acid 0.01% v/v (B) for
277 acquisition in negative ionization mode (ESI–). The gradient elution was as follows: 0-30% B
278 in 0-10 min, 30-80% B in 10-10.5 min, 80-100% B in 10.5-11 min, 100% B in 11-13 min and
279 0% B in 13-14 min. The flow rate was 0.5 mL/min and the sample injection volume was 2 μL .

280 The mass spectrometer was operated in MS and MS/MS modes using the following parameters:
281 capillary voltage, 3,000 V; nebulizer pressure, 40 psi; drying gas flow rate, 11 L/min; gas
282 temperature, 300 °C; skimmer voltage, 45 V; fragmentor voltage, 110 V. The MS and Auto
283 MS/MS modes were set to acquire m/z values ranging between 50-1,100 and 50-800,
284 respectively, at a scan rate of 5 spectra per second. Auto MS/MS mode was operated selecting
285 4 precursor ions per cycle at a threshold of 200 counts. The reference compound solution for
286 internal mass calibration of the Q/TOF mass spectrometer containing 5 µM of purine
287 ([C₅H₅N₄]⁺ at 121.050873 m/z) and 2.5 µM HP-0921, hex-S-5akis(1H,1H,3H-
288 tetrafluoropropoxy) phosphazine ([C₁₈H₁₉O₆N₃P₃F₂₄]⁺ at 922.009798 m/z) in acetonitrile-water
289 (95:5, v/v) and was also from Agilent.

290 A randomized sequence of samples, including three replicates of control and treated cell
291 experiments, were injected in triplicate. An additional pool of all samples, combining equal
292 aliquots from each sample, was injected regularly throughout the samples sequence to monitor
293 the stability of the analysis. A mix solution of ten standards, were injected regularly throughout
294 the samples sequence, as quality control, to validate the LC-HRMS method in terms of retention
295 time and signal variability, and mass accuracy. Appropriate quality control assessment was
296 carried out to validate the robustness of the metabolomics data analysis. Retention time for the
297 target QC standards showed a relative standard deviation (RSD) below 0.4 %, whereas the
298 average RSD values for peak areas was 6.6%. Areas for each target standard were obtained
299 from the extracted ion chromatograms (EICs) reconstructed with mass accuracy below 10 ppm
300 (mass window). Furthermore, the average number of extracted MS features (cut-off threshold
301 > 200 counts) for all the analysed samples was 67, and no shift in features number was observed
302 through the sample sequence (RSD = 5.2%).

303 ~~A randomized sequence of samples, including three replicates of control and treated cell~~
304 ~~experiments, along with a pool of control and a pool of treated experiments was analysed. An~~

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~~additional pool of all samples, combining equal aliquots from each sample, was injected~~
~~regularly throughout the samples sequence as quality control to monitor the stability of the~~
~~analysis. Each sample was injected in triplicate.~~ Post-acquisition HRMS data treatment was
carried out using the Mass Hunter Qualitative (MHQ) and Profiler Professional (MPP) software
from Agilent, following a recursive analysis strategy. Thus, a list of candidates was obtained
for each sample in MHQ through an untargeted MS features detection (cut-off threshold > 200
counts), subsequently exported to an MS exchange format (.cef files). After convenient peak
alignment (RT window: 0.2 min and Mass window: 20ppm), a defined list of metabolites was
generated in MPP and exported for targeted data processing and metabolites detection in MHQ
(.cef files). After recursive analysis, a table of metabolite ion intensities filtered by frequency
(retaining entities that appear in at least 20% of the samples) is generated in MPP and exported
as .txt file for further filtering. Duplicated entities and peaks showing low signal to noise ratio
were removed (average of samples/average of blanks < 3.0). Gap filling (minimum value/2)
was applied before normalization. The resulting output.csv data tables of high-quality time-
aligned detected peaks with their corresponding retention time, m/z, and peak intensity obtained
for each sample was submitted to statistical analysis using the on-line Metaboanalyst program
(www.metaboanalyst.ca).
Univariate analysis based on T-test and Fold change (FC) analysis was applied to detect
significant molecular features (p-value (FDR) cutoff: 0.05) with a defined absolute value of
change (FC > 1.5) between control and treated cell samples. MS entities showing high VIP
score in the partial least squares-discriminant (PLS-DA) analysis were also considered as
relevant metabolites, as they allow explaining the differences between both groups of samples.
Selected metabolites from the resulting volcano plot and the multivariate PLS-DA analysis
were subjected to tentative identification on the basis of the information provided by MS data
(accurate mass, isotopic distribution), positive match of the MS/MS fragmentation pattern with

on-line MS databases (e.g. HMDB, Metlin), and information reported in literature. The Molecular Formula Generator algorithm within the Agilent Mass Hunter software was also to enhance the metabolite database search and mass accuracy calculation (<10 ppm). It is important to notice that the proposed metabolites identification is tentative, and for fully validation of metabolomic results the co-injection of authentic standards would be required or purification followed by NMR analysis. However, the robust data analysis and the reliability of the proposed identification was demonstrated to be a successful strategy to come up with new hypothesis, as discussed below.

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3. RESULTS AND DISCUSSION

3.1 Anti-proliferative activity

Colon cancer is among the most frequent cancer types. Cultured cancer cells are valuable resources for rapid screening of potential anti-cancer agents as well as for elucidation of mechanism of their activities. Therefore, in the present study, goldenberry calyx extracts were evaluated for their abilities to inhibit the growth of human colon adenocarcinoma HT-29 cells by the MTT assay. For this, HT-29 cells were treated with different concentrations of the PLE-extract (6.25–100 µg/mL) during 24, 48 and 72 h. Comparatively, anti-proliferative activity of the dynamic maceration DM-extract was studied under the same conditions. In addition, the PLE-extract was also tested on CCD-18Co cell line to determine its potential toxicity in non-cancer colon cells. Data obtained were expressed as the percentage of viability and the IC₅₀ values were calculated to compare the obtained results (see Fig. 1). As can be seen, after 24 h of treatment, DM-and PLE-extracts (Fig. 1A) exerted an equivalent ($p > 0.05$) inhibitory effect (IC₅₀ = 35.82 ± 2.77 µg/mL and 33.06 ± 2.57 µg/mL, respectively) on HT-29 cells, while PLE-extract did not affect the viability of CCD-18Co cells. In contrast, PLE-extract showed the

355 strongest inhibitory effect ($IC_{50} = 6.17 \pm 4.50 \mu\text{g/mL}$) on HT-29 cells and a slight effect on
356 CCD-18Co cells viability ($IC_{50} = 73.45 \pm 2.13 \mu\text{g/mL}$) at 48 h of exposition (Fig. 1B).
357 Meanwhile, the DM-extract exhibited a cytostatic effect on HT-29 cells at 48 and 72 h, since
358 the IC_{50} values did not change ($p > 0.05$) at these treatment periods (24.57 ± 3.45 and $20.55 \pm$
359 $1.79 \mu\text{g/mL}$, respectively). At 72 h, a partial recovery of HT-29 cell proliferation was observed
360 with PLE-extract treatment, since both the percentage of growth and IC_{50} value increased.
361 However, at the same incubation time, the PLE-extract produced a severe decrease of cell
362 viability on CCD-18Co cells (Fig. 1C). According to these results, the selectivity index (SI) of
363 PLE-extract on HT-29 respect to CCD-18Co cells was calculated, revealing values of 11.90
364 and 0.82 at 48 and 72 h, respectively (see Fig. 1). The SI approach allows to establish if a
365 compound or an extract exhibits selective toxicity toward cancer cells (SI value > 2) with a
366 minimal toxicity or no harm to normal cells, or whether, on the contrary, it is toxic even to
367 normal cells (SI value < 2) (Asif et al., 2017; Asif et al., 2016; Badisa et al., 2009). Following
368 these criteria, it is possible to assert that PLE-extract had selectivity toward HT-29 cells at 48
369 h, however, it was non-selective at 72 h of treatment. Furthermore, the observed anti-
370 proliferative behaviour of DM- and PLE-extracts toward HT-29 cells could be explained by
371 virtue of their differences in regard to withanolide content (For instance, the 4β -
372 hydroxywithanolide and withanolide E contents) as well as the phenolic content and
373 antioxidant capacity, which were characterized previously (Diego Ballesteros-Vivas et al.,
374 2019).

375 The anti-cancer properties of extracts from *P. peruviana* whole plant and its individual organs
376 have been studied on in vitro models, revealing a high anti-proliferative potential on several
377 human cancer cell lines including colon (Demir, Özen, & Hameş-Kocabaş, 2014; Wu et al.,
378 2009; Wu, Ng, Chen, et al., 2004; Wu, Ng, Lin, et al., 2004). In a previous work (Areiza-Mazo,
379 Maldonado, & Rojano, 2013), the anti-proliferative and apoptogenic activities of an aqueous

380 extract from *P. peruviana* fruit were determined against SW480 ($IC_{50} = 44.20 \mu\text{g/mL}$) and
381 SW620 ($IC_{50} = 85.10 \mu\text{g/mL}$). Furthermore, the anti-cancer effect of a methanolic extract from
382 *P. peruviana* twigs was also evaluated against HCT116 cells showing an important activity
383 ($IC_{50} = 12.26 \mu\text{g/mL}$) (Mbaveng et al., 2018). Quispe-Mauricio, Callacondo, Rojas, Zavala, and
384 Posso (2009), established the cytotoxic activity of the extracts of leaves ($IC_{50} = 0.35 \mu\text{g/mL}$)
385 and stems ($IC_{50} = 0.37 \mu\text{g/mL}$) from *P. peruviana* on HT-29 cells, which were more active
386 than antineoplastic compounds cisplatin and 5 FU used as positive controls. The anti-
387 proliferative effect observed toward HT-29 cells in the present work ($IC_{50} = 6.17 \mu\text{g/mL}$ at 48
388 h) is ranged into the previously reported values and to the best of our knowledge this is the first
389 contribution to the PLE-extract obtention from the *P. peruviana* calyx with selective anti-
390 proliferative activity against this cell line type. Despite the promising results obtained in
391 colorectal tumor cells growing in monolayer, further research work (e.g., with *in vivo* models
392 and other assays) should be carried out to estimate basic pharmacokinetic parameter in order to
393 establish the effective concentration under more realistic physiological conditions.

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394
395
396 The phytochemical profiling of the calyx PLE-extract was tentatively characterized by GC- and
397 LC-HRMS in our previous work (D. Ballesteros-Vivas et al., 2019). Fifty-six metabolites
398 belonging to different families, including phenolic acids, flavonoids, glycosylated flavonoids,
399 withasteroids and sucrose esters were identified by LC-HRMS. 4 β -Hydroxywhitanolide E
400 (4 β HWE), withanolide E (WE) and several of their isomers and derivatives were some of the
401 withasteroids found. The anti-cancer properties of 4 β HWE and WE have been described in
402 scientific literature on different cell lines showing a high cytotoxic activity and the 5,6-epoxy-
403 2-en-1-one moiety from their chemical structures has been related to biological activity (Lan et
404 al., 2009; Yen et al., 2010). In addition, the mechanism action of 4 β HWE on inhibition of HT-

29 cell growth has been described (Park, Sang-Ngern, Chang, & Pezzuto, 2016). In this sense, it allows thinking that withanolide type-compounds contribute to the anti-proliferative activity of PLE-extract observed on HT-29 cells.

3.2 Cell cycle study

The distribution of cell cycle phases in HT-29 cells at 24, 48 and 72 h post-treatment with the calyx PLE-extract was analysed by flow cytometry. As shown in Fig. 2, the incubation for 24 h did not change the distribution cell cycle phases, but at 48 h, it significantly increased ($p<0.05$) the percentage of cells in the S phase (48%) compared with the control (16.5%). Additionally, the treatment reduced the proportion of G₀/G₁ cells with respect to untreated control. At 72 h the profile also changed, and the percentage of cells in G₀/G₁ increased and the G₂/M population decreased.

The effects on cell cycle progression in cells treated with 5-FU were also evaluated at 24, 48 and 72h. As evidenced in Fig. 2, there were no significant variations in cell cycle phases at 48 h. However, an increase in the percentage of cells in S phase and a decrease in the G₀/G₁ phase were observed at 24 and 72 h, respectively.

3.3 Apoptosis induction

The effects on the phosphatidylserine (PS) externalization (typical marker of early phase apoptosis) in HT-29 cell after treatment with the calyx PLE-extract, was analysed using the Annexin V-PE/-7-AAD double staining. As evidenced in Fig. 3, the extract increased the percentage of attached cells in early apoptosis (AV+/7-AAD-) only at 24 h, while the distribution of cells in late apoptosis (AV+/7-AAD+) and necrosis (AV-/7-AAD+) was similar to control at all-time points. However, the analysis of the detached cells at 72 h clearly indicates an accumulation of cells in late apoptosis (50%), but also in necrosis (15%) (Fig. S1).

430

431 3.4 *Morphological analysis*

432 Light microscopy observation of HT-29 cells upon calyx PLE-extract (10 µg/mL) treatment
433 revealed a reduction of the cell population and morphological changes compared to control
434 cells, particularly at 48 and 72 h. In the control, cells grown as tight colonies of polygonal
435 shape with sharp boundaries between them. However, cells treated with the extract changed its
436 polygonal array to scattered spindly shape, shown alterations in nuclear morphology (shape and
437 definition nuclear), as well as cytoplasmic vacuolization. The cell treated with 5-FU (50 µM)
438 exhibited morphological features suggestive of apoptosis (apoptotic bodies, cell shrinkage and
439 rounding of cells) (Fig. 4).

440

441 3.5 *Transcriptomics*

442 A gene expression microarray experiment was performed to assess the effects of the calyx PLE-
443 extract on the transcriptome of HT-29 cells using the IC₅₀ at 48 h of treatment. After background
444 correction and signal normalization, the intensity of 62,975 represented genes in the microchip
445 were analysed by PCA, which indicates that the combination of the two principal components
446 captured more than 75% of the variance of the data (Fig. S24). In addition to PCA, the univariate
447 analysis indicates that 12,060 genes had a p-value < 0.05. To increase the confidence of the
448 results, a 1.5-fold change cut-off filter (in log2 scale) was applied and duplicate genes (with the
449 lowest p-value) were removed, and 3,221 genes were identified as differentially expressed
450 (1,541 down-regulated and 1,680 up-regulated, [Figure S24B and Table S1](#)). To explore and
451 characterize the molecular mechanisms underlying the effects of the extract on proliferation
452 and metabolism, a canonical pathway analysis was performed with the list of DEG using IPA
453 software. The top ten significantly overrepresented signalling and metabolic pathways are

shown in Table 1 (the whole list can be found as Supplementary Table S2), and the most interesting pathways and the genes involved are discussed below.

The pathway with the highest number of altered genes and predicted as inactive was the EIF2 signalling pathway (75 genes). EIF2 is crucial for translation initiation and it is primarily regulated through reversible phosphorylation of the eIF2 α subunit. The phosphorylation of eIF2 α inactivates EIF2, reducing the translation and, therefore, the protein synthesis. This phosphorylation occurs as a response to certain stresses such as oxidizing agents, nutrient deprivation, heat shock, heavy metals, high salt levels, and hypoxia (Clemens, 2001). However, eIF2 and phosphorylated eIF2 protein levels have been observed as increased in colon carcinomas, as well as their subcellular distribution (Lobo et al., 2000). On the other hand, a recent study has demonstrated that curcumin inhibits the proliferation of the colon cancer cell line HCT-116 via the inactivation of the EIF2 signalling pathway and the reduction of the protein synthesis (J. Wang et al., 2016). One of the most important genes that controls eIF2 activity is EIF2S2 (which encodes for the eIF2B protein) (Jennings & Pavitt, 2014; Kok-Sin et al., 2015). This gene was observed as down-regulated in our dataset (-0.90 in Log2 scale), and it might explain the anti-proliferative effect of the PLE-goldenberry calyx extract treatment.

Apart from decreasing protein synthesis, eIF2 α facilitates the translation of specific transcripts to restore the cellular homeostasis, such as ATF4 (B'Chir et al., 2013), which results in the activation of pro-survival or pro-apoptotic pathways (Baird & Wek, 2012; Bogorad, Lin, & Marintchev, 2017). In our study, ATF4 mRNA was highly down-regulated (-1.88), and as it will be later commented, the activity of this transcription factor is predicted to be inactive. In addition to the regulation of the cellular response to stress, ATF4 also controls genes involved in amino acid transport and metabolism, such as the cytoplasmic-localized aminoacyl-tRNA synthetases through the C/cbp-Atf-Response-Element (Shan et al., 2016). Among these genes, NARS (-0.72), LARS (-0.73), WARS (-1.05), GARS (-0.79), SARS (-1.43), MARS (-1.43) or

479 IARS (-0.86) were down-regulated, as well as others involved in the tRNA charging metabolic
480 pathway (YARS (-0.60), TARS (-1.53) and EPRS (-0.91)), and therefore this pathway was
481 overrepresented and predicted as inactive (Table 1) (an example is given in Fig. 5A). These
482 results suggest that even though the protein synthesis seems to be reduced, the cellular stress
483 response to restore the cellular homeostasis is not fully active. Complementary, other crucial
484 endoplasmic reticulum (ER) stress sensors such as ERN1 or PERK were not found altered, and
485 the XBP1 mRNA was found down-regulated (-1.00).

486 The metabolic pathway observed with the highest positive z-score value was the Glutathione
487 Redox Reactions I. Its overrepresentation is based on the up-regulation of several genes: GSTZ1
488 (0.62), GSR (0.67), GSTT2 (0.72), GPX3 (1.26), GPX2 (1.69), GPX4 (1.01), GSTP1 (0.67),
489 MGST3 (0.73), GSTT1 (0.81) and GSTK1 (0.72). Glutathione is the main responsible for the
490 antioxidant capacity of cells against reactive oxygen species (ROS), but it can only acts in their
491 reduced form, as glutathione disulfide. The genes presented above are involved in the
492 regeneration of this reduced form, which suggests that the cell is trying to increase its reducing
493 potential to increase its antioxidant capacity (Fig. 5B).

494 Finally, the Mitotic Roles of Polo-Like Kinase signalling pathway was also predicted as
495 inactive. The Polo-like protein kinases are a conserved family of enzymes that play important
496 role in G2/M transition, bipolar spindle formation and centrosome maturation (Glover, Hagan,
497 & Tavares, 1998). Among the different proteins involved in this pathway, the PLK1 has been
498 observed as overexpressed in several human cancers, such as colon, where it has been suggested
499 as a diagnostic/prognostic biomarker (Han et al., 2012; Rödel et al., 2010). The inactivation of
500 this pathway in our dataset is based on the expression of 25 genes, being the down-regulation
501 of PLK1 (-0.64), PLK2 (-1.76), CDK1 (-0.72), CCNB1 (-1.00), CCNB2 (-1.99), CDC20 (-
502 0.62), CDC25B (-0.61) and CDC25C (-1.04) some of the most relevant. The down-regulation
503 of these genes agree well with previous studies, where PLK1 has been demonstrated to be a

504 target of the DNA damage checkpoint, that blocks the activation of Cdk1/cyclin B by CDC25C,
 505 and triggers the cell-cycle arrests at multiple points in G2 and mitosis (Smits et al., 2000).
 506 Moreover, PLK1 is necessary during the S phase, where it facilitates the DNA replication under
 507 stress (Mandal & Strebhardt, 2013; Song, Liu, & Liu, 2012). These results may explain the cell
 508 cycle arrest observed in the S phase at 48 h. In accordance with the activation of the Mitotic
 509 Roles of Polo-Like Kinase signalling pathway, the Cell Cycle: G2/M DNA Damage Checkpoint
 510 Regulation pathway was predicted as activated by IPA software.

511 To complement the canonical pathway information, we explore the upstream transcriptional
 512 regulators that might be altered after the calyx PLE-extract treatment. For this aim, the list of
 513 altered genes was analysed using the Upstream Regulator function of IPA (the whole list of
 514 transcription factors is shown in Supplemental Table S3). The top ten transcription factors
 515 (based on the lowest p-value) and their activation z-scores are shown in Table 2. As previously
 516 commented, the inactivation of the EIF2 signaling pathway usually involves the transcriptional
 517 activation of ATF4. However, ATF4 mRNA was down-regulated and its transcriptional activity
 518 is predicted as inactive (assumption based on more than 60 genes) (Table 2). The main activity
 519 of ATF4 is the stress adaptation, and some of the most relevant genes controlled by ATF4 were
 520 observed as down-regulated: ATF3 (-0.61), DDIT4 (-2.34), DDIT3 (-1.00) and TRIB3 (-3.07).
 521 These genes are involved in resistance to cancer therapy (Tirado-Hurtado, Fajardo, & Pinto,
 522 2018), or in the initiation of apoptotic cell death after long-term activation of the ER stress
 523 responses (Rozpędek et al., 2016). Furthermore, TRIB3 has been suggested as a novel marker
 524 for prognosis in colorectal cancer (Ohoka, Yoshii, Hattori, Onozaki, & Hayashi, 2005), and its
 525 down-regulation may explain the anti-proliferative activity of the extract.

526 Another interesting transcription factor predicted as activated is NFE2L2 (3.00) (also known as
 527 NRF2). NRF2 regulates the expression of several genes involved in the cellular antioxidant
 528 capacity of cells (Mitsuishi, Motohashi, & Yamamoto, 2012), and some of them have been

described in the Glutathione Redox Reactions I pathway. This transcription factor has been demonstrated to be crucial in the antitumor effect of 4b-hydroxywithanolide E in breast cancer cells (Peng et al., 2016). In this work, 4b-hydroxywithanolide E induces the accumulation of ROS, the upregulation of Nrf2 and Nrf2-downstream gene, and the promotion of cell death. Moreover, pre-treatment of cells with reduced glutathione (GSH) or N-acetylcysteine (NAC), reduced the expression of ROS detoxifying enzymes (SOD and CAT), but reversed the expression of proteins involved in the G1 phase cell cycle arrest (CDK1, CDK2, CDK4, cyclin A and CHK2). Our data agree well with these results as the treatment with the calyx PLE-extract increases the mRNA levels of CAT (0.73), SOD1 (0.59) and SOD2 (0.99), decreases the levels of CDK1 (-0.72), and suggests elevated ROS accumulation. However, in contrast to the accumulation of cells in the G1 phase, we observed an arrest in the S phase, which might be explained by the different cell line used (Fig. 2).

Several transcription factors involved in different aspects of the cell cycle progression were predicted as altered, such as MYC, RB1, TP53, E2F3 and CDKN2A (Table 2). MYC transcription factor was predicted as the most inactive, prediction based on more than 250 genes, such as CCNA2 (-0.68), CCNB1 (-1.00) and CCNB2 (-1.99). The microarray results also reveals a down-regulation of its mRNA levels (-1.36). Apart from the cell cycle regulation, MYC activates or represses transcription of a large group of genes involved in metabolism, apoptosis, and protein synthesis; and its down-regulation has been related with the anti-proliferative activity of phenolic compounds or phenolic extracts in different cancer cell models (Manna, Mukherjee, Roy, Das, & Panda, 2009; Moon, Kim, Lee, Choi, & Kim, 2009; A. Valdés et al., 2012; Alberto Valdés, Sullini, Ibáñez, Cifuentes, & García-Cañas, 2015). In the case of RB1, it is a well-established tumour-suppressor protein which limits the transcription of cell cycle genes, primarily via regulation of the E2F transcription factor. This transcription factor also activates pro-apoptotic genes in response to genotoxic stress (Ianari et al., 2009).

554 However, RB1 expression in colorectal cancer is controversial (Yamamoto et al., 1999). In our
555 study, the mRNA levels of RB1 was closed to the cut-off threshold (-0.57), but the activity of
556 RB1 was predicted as activated based on more than 100 genes (Table 2). The activation of RB1
557 has been also predicted in the same colon cancer cell line (HT-29) and in leukaemia cells (K562)
558 after the treatment with a polyphenol enriched rosemary extract (A. Valdés et al., 2012; Alberto
559 Valdés et al., 2015). Associated with these transcription factors, the TP53 transcription factor
560 was predicted as active. The mutation of p53 tumour suppressor gene is one of the most frequent
561 events contributing to the transformation of colorectal cancer (Li, Zhou, Chen, & Chng, 2015).
562 However, polyphenols can exert anti-cancer effects up-regulating this signalling pathway, as
563 recently review (Khan et al., 2019). Some genes controlled by TP53 are well known pro-
564 apoptotic genes such as BAD, BAK1, and BCL2L11 (BIM), which mRNA levels were found
565 up-regulated (0.99, 0.70, and 0.59, respectively). These genes are involved in the mitochondrial
566 (or intrinsic) apoptotic pathway in response to cellular stress.

567 Other important genes found altered in our dataset belong to the chaperone family: HSP90AB1
568 (-0.67), HSPH1 (-0.67), HSPA8 (-1.45), HSPA9 (-0.75), HSPD1 (-1.63) and HSP70 (1.10), and
569 just below the established expression threshold, two other subunits of the HSP90 family,
570 HSP90AA1 (-0.57) and HSP90B1 (-0.57). Most of these genes are down-regulated, except
571 HSP70. These data correlate well with previous studies that demonstrate that Withaferin A and
572 4b-hydroxywithanolide E induce cell death in breast cancer cells due to their ability to inhibit
573 the heat shock protein 90 (H. C. Wang et al., 2012). The authors also observed an increase of
574 HSP70 protein, and how HSP70 induction attenuates HSP90 inhibitors cytotoxic effects. These
575 effects were attributed to the thiol oxidation induction and HSP90 aggregation based on the cell
576 death prevention by NAC. In addition, these effects have also been observed in pancreatic
577 cancer cells treated with Withaferin A (Yu et al., 2010). As previously discussed, several genes
578 involved in the glutathione disulfide regeneration were up-regulated in our dataset.

579 Furthermore, we also observed the down-regulation of the anti-apoptotic factors BIRC5 (-1.75)
580 and XIAP (-0.70), and the up-regulation of the pro-apoptotic genes BAK1 (0.62), BAD (0.99)
581 and CASP9 (1.61). These results agree well with the literature where using breast cancer cells,
582 the group of Wang et al. demonstrated that 4b-hydroxywithanolide E and Withaferin A decrease
583 the levels of the anti-apoptotic factors surviving (BIRC5), BCL-2, BCL-XL and XIAP (H. C.
584 Wang et al., 2019); and the group of Wu, Ng, Chen, et al. (2004) observed the down-regulation
585 of XIAP and the up-regulation of BAX and BAD in human hepatoma HepG2 cells treated with
586 a different goldenberry extract.

587

588 3.6 *Metabolomics*

589 The metabolic profiles of untreated controls and treated HT-29 cells with the calyx PLE-extract
590 were obtained by RP/LC-q-TOF-MS/MS, as described in section 2.7. Although methods based
591 on LC-ESI-MS are frequently run in positive ionization mode for metabolomics studies, the
592 metabolome coverage was improved in this work by operating in both positive and negative
593 ESI modes. The combination of analytical strategies is a critical issue to broaden the range of
594 metabolites, which is particularly relevant in metabolomics of cellular lysates, where the
595 number of detected metabolites is comparatively lower with regards to more complex biological
596 samples (e.g. blood, urine). After MS data treatment applying the parameters and data
597 processing workflow described in section 2.8, the resulting MS features obtained in positive
598 (99 entities) and negative (88 entities) ionization modes were submitted to unsupervised
599 multivariate analysis to evaluate the compositional variability of the data. According to the PCA
600 results obtained for both ESI modes, two principal components allow explaining most of the
601 data variability (PC1 + PC2 variance > 90%). The 2D-PCA scores plot (Fig. S3) illustrates an
602 evident distribution of the samples in two groups, suggesting a clear difference between treated
603 and untreated HT-29 cell samples in terms of expressed metabolites. Univariate analysis based

on t-test revealed a total of 38 differentially expressed metabolites (p value FDR adjusted < 0.05) detected in ESI + mode, whereas operating in ESI (-), a total of 25 compounds showed significant (p < 0.05) variation upon treatment with the calyx PLE-extract. It is worthy to highlight that only 4 compounds out of 24 target metabolites were detected by both ESI + and ESI + modes, evidencing the advantage of applying both complementary acquisition modes.

A list of 24 differentially expressed metabolites, most of them exhibiting FC values > 1.5 (-0.585 > Log2 FC > 0.585), is summarize in Table 3. Despite showing lower FC ratios, a group of 3 metabolites (xanthine, L-leucine, L-valine) were also included in the target list as they were shown to be significantly altered compounds with large weight in the discriminant analysis, according to the high variable importance in projection value (VIP > 1) in the first component of the PLS-DA analysis. It is important to notice that small changes in the levels of some metabolites might have a remarkable effect in the metabolism as discussed below for these metabolites. Quantitative pathway and enrichment analysis were carried in Metaboanalyst platform, integrating high-quality KEGG and SMPDB databases as the backend knowledge bases, in an attempt to tentatively identify the most relevant metabolic pathways in which the differentially expressed metabolites are involved. According to this analysis, beta-oxidation of fatty acids, aminoacyl-tRNA biosynthesis, branched-chain (Val, Leu) and aromatic (Phe, Tyr) amino acids, glutathione, pyrimidine and purine metabolic pathways are the most affected routes, as discussed below.

From the information provided in Table 3, a remarkable increase in the oxidized form of glutathione (GSSG) levels can be observed in treated cells. GSSG exhibits not only one of the highest FC values (2.0 in Log2 scale), but also shows the highest VIP score (5.59), indicating the large weight of this compound in the discriminant analysis. In this regard, glutathione redox pathway is reported to play an important role in cellular redox control of programmed cell death (e.g. apoptotic processes). Thus, shift in the cellular GSH-to-GSSG redox balance in favour of

the oxidized species, GSSG, constitutes an important signal of cell impairment (Circu & Aw, 2008). Reactive oxygen and nitrogen species (ROS/RNS) can oxidize cellular GSH or induce its extracellular export leading to the loss of intracellular redox homeostasis and activation of the apoptotic signaling cascade (Circu & Aw, 2012), and might explain the results observed in the anti-proliferative activity assay. The up-regulated levels of GSSG observed in treated HT-29 cells with the PLE-extract, in contrast to the non-significant changes detected for the reduced form (GSH), are in line with transcriptomic data showing altered levels of genes involved in the regeneration of the reduced form, in response to the high levels of GSSG as a result of an oxidative stress (Fig. 5A).

The altered levels of carnitine derivatives such as acetyl-, propionyl- (iso)valeryl-, (iso)butyryl- and hydroxybutyryl-L-carnitine indicates deregulation in the catabolic pathway of beta-oxidation of fatty acid in cancer cells upon treatment (Fig. 5C). As reported in literature, carnitine-dependent fatty acid uptake into mitochondria and beta-oxidation seem to be impaired in HT-29 cancer cells (Wenzel, Nickel, & Daniel, 2005). The observed down-regulated levels of short-medium chain acyl-carnitines and the up-regulation of carnitine palmitoyl transferase 2 (CPT2) might indicate a higher consumption of short-medium chain fatty acids in tumor cells, triggered by the impairment of the carnitine-dependent long chain fatty acid uptake into mitochondria in tumor cells (the mRNA levels of very long-chain acyl-CoA synthetase (SLC27A5) and long-chain acyl-CoA ligase 5 (ACSL5) were down-regulated). However, the mitochondrial very long-chain specific acyl-CoA dehydrogenase (ACADVL) and the short chain 3-hydroxyacyl-CoA dehydrogenase (HADH) mRNA were observed as up- and down-regulated, respectively.

In addition, during the fatty acid activation step, ATP is converted to AMP as a result of the CoA acylation process with a fatty acid molecule to produce acyl-CoA (Longo, Frigeni, & Pasquali, 2016). The observed down-regulated levels of adenosine monophosphate, might also

654 indicate an enhanced impairment in the activation step of fatty acid oxidation process, reducing
655 the uptake ability of long chain fatty acids into the beta-oxidation pathway. On the other hand,
656 increased fatty acid availability in mitochondria is reported to increase the generation of
657 superoxide anion (O_2^-) in colon cancer cells, leading to apoptosis-induced cell death (Wenzel
658 et al., 2005).

659 L-phenylalanine and L-tyrosine were shown to be the most altered amino acids, followed by L-
660 valine and L-leucine with large weight in the discriminant analysis (VIP score >1). The
661 observed down-regulation of genes involved in the aminoacyl- tRNA biosynthesis pathway
662 from the transcriptomic data might explain, to some extent, the altered levels of
663 abovementioned amino acids (Fig. 5B). This small group of essential branched-chain (Val, Leu)
664 and aromatic (Phe, Tyr) amino acids (BCAA and AAA, respectively) play important roles in
665 protein degradation and turnover, glycogen synthesis and energy metabolism, being reported
666 as early indicators of several diseases (Chen et al., 2016). Interestingly, the metabolic
667 connection between the previously discussed C3 and C5 acyl-carnitines with these essential
668 BCAA was reported by Newgard (2012). C5 acylcarnitines are intermediates in mitochondrial
669 Ile and Leu catabolism, respectively, whereas C3-acylcarnitine is a by-product of both Ile and
670 Val catabolism. Thus, the observed deregulated levels of these metabolites in treated cells are
671 expected to be associated to dysfunction in BCAA degradation pathway, and the mitochondrial
672 isovaleryl-CoA dehydrogenase (IVD) mRNA, involved in leucine catabolism, was observed as
673 down-regulated. Impairment in phenylalanine and tyrosine pathways (phenylalanine is
674 converted to tyrosine) might explain the up-regulated levels of phenylalanine in contrast to the
675 down-regulation observed for Tyrosine. These AAAs are described as altered metabolites in
676 patients with gastroesophageal cancer due to reduced action of phenylalanine hydrolase
677 (Wiggins, Kumar, Markar, Antonowicz, & Hanna, 2015).

Metabolomics data also indicated decrease in purine nucleosides such as inosine, xanthine and guanosine monophosphate, suggesting that purine metabolic pathway might be affected. Xanthine is an intermediate in the degradation of adenosine monophosphate to uric acid, being formed by oxidation of hypoxanthine. Lower levels of xanthine metabolite are in accordance with down-regulated levels of AMP, as discussed above. Inosine is another intermediate in the degradation of purines and purine nucleosides to uric acid that also occurs in the anticodon of certain transfer RNA molecules. Therefore, the observed decrease of inosine levels might affect aminoacyl-tRNA biosynthesis pathway. Elevated levels of the modified nucleosides 1-methyladenosine, generated by the processing of tRNA by methyltransferases (Chujo & Suzuki, 2012), reinforce this observation from transcriptomic results.

Deregulation in pyrimidine nucleosides metabolism is evidenced from the lower levels of uridine and uridine 5'-monophosphate, and the mRNA levels of genes involved in the pyrimidine ribonucleotide interconversion were mainly down-regulated (Fig. 5D). In turns, up-regulation of uridine diphosphate-N-acetylgalactosamine (UDP-GlcNAc) might also indicate dysfunction on amino-sugar metabolism. UDP-GlcNAc plays an important role in glycosaminoglycans, proteoglycans, and glycolipids biosynthesis. Impairment on this route may led to detrimental effects on intracellular signalling, thermal change and proteolytic attack in a number of diverse protein (Milewski, Gabriel, & Olchowy, 2006), which might contribute to explain the damage observed in HT-29 cells after treatment with the goldenberry calyx PLE-extract.

698

699 **4. CONCLUSIONS**

700 A multi-omics approach was applied, in this study, to evaluate the changes induced by a
701 goldenberry calyx PLE-extract on HT-29 colon cancer cells at different expressions levels (i.e.,
702 transcriptomic and metabolomics), in combination with cell viability, cell cycle and cell

morphology assays. The obtained results revealed that goldenberry calyx PLE-extract, and in a lesser extent the DM-extract, markedly affected the viability of HT-29 colon cancer cells at 48 h without affecting the viability of normal human colon cells. The PLE-extract was shown to block the cells in the S phase of the cell cycle, which might be PLK1-mediated, and alter the expression of several genes and metabolites related with the oxidative stress response. These findings are consistent with the reported NRF2-activating effect of 4b-hydroxywithanolide E, one of the most relevant phytoconstituents in the goldenberry extract, which also inhibits the expression of heat shock protein 90 and induces the expression of heat shock protein 70. Transcriptomics data also showed that the anti-apoptotic genes BIRC5 and XIAP are down-regulated, and the pro-apoptotic genes BAK1, BAD and CASP9 are up-regulated; however, the apoptosis experiment only showed the accumulation of apoptotic cells in the supernatants, but not on the attached cells, therefore multiple signalling pathways may also contribute to the cell death. Metabolomics data indicate altered cellular redox homeostasis, as deduced from the shift in the cellular GSH-to-GSSG redox balance in favour of the oxidized species. The dramatic up-regulated levels of GSSG in treated cells constitutes an important signal of cell impairment by oxidative stress. The deregulated levels of essential branched-chain (Val, Leu) and aromatic (Phe, Tyr) amino acids, along with the down-regulated levels of short-chain acyl-carnitines were identified as early indicators of dysfunctions in metabolic pathways involved in protein degradation and turnover, glycogen synthesis and energy metabolism. Additionally, the proposed Foodomics strategy, integrating both transcriptomics and metabolomics data, allowed the identification of significant changes on genes and metabolites involved in common metabolic processes such as inactivation of the tRNA charging signalling pathway, the carnitine shuttle and β -oxidation of fatty acids, and the pyrimidine ribonucleotide interconversion; which are key biochemical processes to sustain cell function.

727 The results and methodologies reported herein represent a valuable contribution to a better
728 understanding of the molecular mechanisms of action underlying the bioactivity of goldenberry
729 calyx extract against human colon cancer cells, corroborating the huge potential of Foodomics
730 to carry out this type of studies. Further research ~~work~~ is required to ~~explore more in depth the~~
731 ~~hypothesis generated in this work (e.g., such as validation using with orthogonal techniques, in~~
732 ~~vivo assays)~~ and to determine the main phytochemicals responsible for the observed bioactivity.

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1004 **Figure captions**

1005 **Figure 1.** IC₅₀ values and percentage of growth of HT-29 and CCD-18Co cells incubated for
1006 (A) 24, (B) 48 and (C) 72 h, with different concentrations of goldenberry calyx DM- and PLE-
1007 extracts. SI of PLE-extract was expressed as $\frac{IC_{50} \text{ CCD-18Co cells}}{IC_{50} \text{ HT-29 cells}}$ ratio. (*) indicates significant
1008 differences between the treated and control samples as determined by Student's t-test, $p < 0.05$.
1009 Error bars represent standard error of the mean.

1010 **Figure 2.** Goldenberry calyx PLE-extract induces changes on the cell cycle distribution in HT-
1011 29 cell. HT-29 cells were treated with PLE-extract (10 µg/mL), in the course of treatment, cell
1012 cycle distribution was analyzed by flow cytometer at 24, 48 and 72 h. Each bar represents
1013 mean ± SEM of the data obtained from three independent experiments. (*) indicate significant
1014 difference respect to control ($P < 0.05$) (Student's t-test).

1015 **Figure 3.** Flow cytometric analysis of apoptosis induction by goldenberry calyx PLE-extract in
1016 HT-29 cell. A) Flow cytometric dot plots from representative experiment showing percentage
1017 of viable (Q4-AV-/7-AAD+), early apoptotic (Q3-AV+/7-AAD-), late apoptotic (Q2-AV+/7-
1018 AAD+) and necrotic (Q1-AV-/7-AAD+) cells, after treatment with 5- FU (50 µM) and PLE-
1019 Extract (10 µg/mL). B) Bar chart indicates the proportion of early apoptosis, late apoptosis and

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1020 necrosis compared to untreated cells. Values are expressed as mean $\pm$ SD from at least three
1021 independent experiments. (*) indicate a significant increase from the untreated control ($P <$
1022 0.05) (Student's t-test).

1023 **Figure 4.** Light microscopy image (x20) illustrating the morphological changes induced by the
1024 calyx PLE-extract (10 μ g/mL) compared to positive (5-FU, 50 μ M) and negative Control in
1025 HT-29 cells. The morphological changes were also evaluated at 24, 48 and 72h.

1026 **Figure 5.** Representative pathways integrating transcriptomics and metabolomics results
1027 corresponding to A) t-RNA charging, B) Glutathione Redox Reactions ~~A) Glutathione Redox~~
1028 ~~Reactions I, B) t-RNA charging~~, C) Carnitine shuttle and fatty acid β -oxidation and D)
1029 Pyrimidine ribonucleotides interconversion. Rhombus-shape nodes represent the expression of
1030 genes and rectangular nodes represent metabolites. Down- and up-regulated components are
1031 shown on a green or red background, respectively.