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Bioavailable phytoprostanes and phytofurans from *Gracilaria longissima* have anti-inflammatory effects in endothelial cells

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Background: An array of bioactive compounds with health-promoting effects has been described in several species of macroalgae. Among them, phytoprostanes (PhytoPs) and phytofurans (PhytoFs), both autoxidation products of α-linolenic acid, have been seen to exert immunomodulatory and antiinflammatory activities *in vitro*. The purpose of this study was to explore the bioaccesibility, bioavailability, and bioactivity of PhytoPs and PhytoFs obtained from the edible red algae *Gracilaria longissima*, and to gain insight into the anti-inflammatory activity of their bioavailable fraction in human endothelial cells. *Methods*: The PhytoPs and PhytoFs profile and concentration of *G. longissima* were determined by UHPLC-QqQ-MS/MS. Algal samples were processed following a standardised digestion method including gastric, intestinal, and gastrointestinal digestion. The bioavailability of the PhytoPs and PhytoFs in the characterized fractions was assessed in a Caco-2 cell monolayer model of the intestinal barrier. The inflammation response of these prostaglandin-like compounds in human endothelial cells, after intestinal absorption, was investigated *in vitro*. *Results*: Simulated digestions significantly reduced the concentration of PhytoPs and PhytoFs up to 1.17 and 0.42 μg per 100 g, respectively, on average, although permeability through the Caco-2 cell monolayer was high (up to 88.2 and 97.7%, on average, respectively). PhytoP and PhytoF-enriched extracts of raw algae impaired the expression of ICAM-1 and IL-6 inflammation markers. The inflammation markers progressed in contrast to the relative concentrations of bioactive oxylipins, suggesting pro- or anti-inflammatory activity on their part. In this aspect, the cross-reactivity of these compounds with diverse receptors, and their relative concentration could explain the diversity of the effects found in the current study. *Conclusions*: The results indicate that PhytoPs and PhytoFs display complex pharmacological profiles probably mediated through their different actions and affinities in the endothelium.

Introduction

The sea is a source of a largely unexplored diversity of organisms. In the case of macroalgae, almost ten thousand species have been described in the marine environment,[1] while less than 5% of this diversity is ingested by humans. The growing interest of the food industry in functional foods of natural origin is due to the health benefits associated with them.[2–4] In this regard, powerful bioactive compounds that are responsible for several health-promoting effects, including their anticancer and anti-inflammatory activities, have been described in several species of macroalgae.[2,5,6] *Gracilaria longissima* is a species of edible red seaweed that grows along the coast of Spain, which is widely sold and eaten.[7] Recently, the oxylipins present in higher plants, especially phytoprostanes (PhytoPs) and phytofurans (PhytoFs), have been suggested as (sometimes partially) responsible for a wide range of biological activities, inducing the biosynthesis of bioactive secondary metabolites, the expression of several genes involved in detoxification, and regulation of the oxidative stress-related mitogen-activated protein kinase pathway.[8,9]

Both PhytoPs and PhytoFs are autoxidation products of α-linolenic acid (ALA, C18:3 ω3), one of the most abundant polyunsaturated fatty acids in the membranes of higher

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plants.[9–11] Two regioisomeric series (16-G_1- and 9-G_1-PhytoPs) are generated, depending on the position oxidation the ALA structure.[12,13] In turn, G_1-PhytoPs are precursors of A_1, B_1, D_1, E_1, F_1, dJ_1, and L_1-PhytoPs (the regioisomer of B_1-PhytoP).[14,15] The cellular redox imbalance produced under pro-oxidation agents leads to the enhanced formation of the above plant constitutive prostaglandin-like compounds.[9–11] In higher plants, PhytoPs and PhytoFs can be found either in free or esterified forms.[16] In plant-based foods, these plant oxylipins are being detected in nuts, hazelnuts, almonds, vegetable oils, cereals, olives, wine, peas, rice, several tropical fruits, cocoa, macroalgae, and especially in legumes, where the concentration is the remarkedly highest of them (2.3–26.3 mg per 100 g fw and 20–500 mg per 100 g fw for PhytPs and PhytoFs, respectively).[10,16–33]

As regards the bioaccessibility and bioavailability of PhytoPs and PhytoFs, studies have revealed that F_1-PhytoPs are absorbed at intestinal level, are present in plasma in a conjugated form, and are excreted in urine as free compounds.[34] Moreover, an evaluation of their biological activity has suggested the involvement of E_1-PhytoPs in inhibiting the production of interleukin-12 (IL-12) by dendritic cells and, hence, polarization of the immune response towards a pro-allergenic T helper type 2 (Th2) response, at least *in vitro*.[35] This modulatory activity is developed *via* the peroxisome proliferator-activated receptor gamma (PPAR-γ) and the transcriptional nuclear factor-kappa B (NF-κB).[36,37] Similarly, Gutermuth *et al.* (2007) found that E_1 and F_1-PhytoPs partially inhibited Th1, as well as Th2 cytokine production *in vivo*,[38] while Karg *et al.* (2007) reported that A_1 and J_1-PhytoPs display anti-inflammatory activity in human HEK293 embryonic kidney cells and RAW264.7 murine macrophages, by down-regulating the NF-κB factor and inhibiting nitric oxide (NO) synthesis.[34] More recently, it has also been noted that B_1-PhytoP exhibits neuroprotective effects against oxidation and promotes myelination through PPAR-γ activation.[39] All these *in vivo* or *in vitro* actions could be associated with human oxylipins, – bioactive compounds with a wide range of biological activities – owing similar structure relative to these plant oxylipins.[9,40–43] These results have encouraged the reassessment of foodstuffs containing high concentrations of PhytoPs and PhytoFs in as regards their oxylipin dependent biological functions, which might provide theoretical support to the design of new health-promoting foods.

Even though these materials could be incorporated as functional ingredients, it is still mandatory to understand the impact of human gastrointestinal (GI) digestion on the suitability of administering plant oxylipins in the diet, in order to take full advantage of their biological power.[44]

The current study aimed to assess the bioaccessibility of PhytoPs and PhytoFs after their *in vitro* GI digestion in hydromethanolic extracts and raw red macroalgae, thus providing accurate and valuable information on changes in their stability and profile, as well as on their bioavailability *in vitro*. Closely connected with this first objective, the capacity of such extracts to modify gene expression was assessed in a model of human endothelial dysfunction. Using correlation analyses, the results concerning their bioavailability and bioactivity were further processed to identify the PhytoPs and PhytoFs responsible for any biological activities observed.

Materials and methods

Chemicals and reagents

The PhytoPs, 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP, and the PhytoFs *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (Table 1), were synthesized according to our published procedures,[45–50] and provided by the Institut des Biomolécules Max Mousseron (IBMM) (Montpellier, France). Hexane was obtained from Panreac (Castellar del Valles, Barcelona, Spain), and butylated hydroxyanisole (BHA), and bis–tris(bis-(2-hydroxyethyl)-amino-tris (hydroxymethyl)methane) were purchased from Sigma–Aldrich (St Louis, MO, USA). All LC-MS grade solvents, methanol and acetonitrile, were purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was treated in a Milli-Q water purification system from Millipore (Bedford, MA, USA). The solid phase extraction (SPE) cartridges used were Strata cartridge (Strata X-AW,

Table 1 Preparation of stock solutions of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

	Stock concentration		Simulated gastric fluid (SGF, pH 3)		Simulated Intestinal fluid (SIF, pH 7)	
Constituent	g L^{-1}	mol L^{-1}	Volume of stock solution (mL)	Concentration of SGF (mmol L^{-1})	Volume of stock solution (mL)	Concentration of SGF (mmol L^{-1})
KCl	37.3	0.50	6.90	6.90	6.80	6.80
KH_2PO_4	68.0	0.50	0.90	0.90	0.80	0.80
$NaHCO_3$	84.0	1.00	12.50	25.00	42.50	85.00
NaCl	117.0	2.00	11.80	47.20	9.60	38.40
$MgCl_2(H_2O)_6$	30.5	0.15	0.40	0.10	1.10	0.33
$(NH_4)_2CO_3$	48.0	0.50	0.50	0.50	—	—
For pH adjustment						
NaOH	1.0		—	—	—	—
HCl	6.0		1.30	15.60	0.60	8.40

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100 mg per 3 mL) and were acquired from Phenomenex (Torrance, CA, USA).

Collection of red macroalgae material and preparation of analytical extracts of phytoprostanes and phytofurans

The red seaweed *G. longissima* was acquired from the company "*La huerta marina*" (Huelva, Spain), and dried before extraction.

The PhytoPs and PhytoFs from *G. longissima* were extracted using the methodology described previously, with minor modifications.[24,51] Briefly, 1 g of powdered algae was mixed with 5 mL of methanolic butylated hydroxyanisole (BHA) (99.9 : 0.1, v/w) and the extracts were cleaned-up by SPE. The PhytoPs and PhytoFs extracts were prepared at room temperature (RT). After SPE, the extracts were then reconstituted in 200 μL of MeOH/MilliQ-water (50 : 50, v/v), filtered through a 0.45 μm filter (Millipore, MA USA), and immediately analysed by UHPLC-ESI-QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany).

In vitro gastrointestinal digestion

For the development of the gastric, intestinal, and GI digestions, the analytical (methanol/BHA (99.9 : 0.1, v/w)) extract or the raw algal material were processed according to the consensus *in vitro* digestion method reported by Minekus *et al.* (2014).[52] Briefly, 5 mL of the analytical extract or 5 g of raw *G. longissima* were mixed with 0.8 or 4.0 mL of simulated gastric fluid (SGF) stock electrolyte solution, respectively (Table 1), to obtain a final SGF ratio of 50 : 50 (v/v) after the addition of other recipients and water. Porcine pepsin (EC 3.4.23.1) was added to obtain a final concentration of 2000 U mL^{-1}, followed by $CaCl_2$ (0.075 mM). The final pH of 3.0 was achieved by adding 1 M HCl. The gastric digestion was developed for 2 h at 37 °C with continuous shaking. For the intestinal digestion, five parts of gastric chime were mixed with four parts of simulated intestinal fluid (SIF) electrolyte stock solution (Table 1) to obtain a final gastric chime/SIF ratio of 50 : 50 (v/v) after the addition of other recipients and water. The final pH required was achieved by adding 1 M NaOH. Then, porcine pancreatin and bile salts were added to the mixture to achieve the final concentration of 100 U mL^{-1} and 10 mM, respectively. The intestinal digestion was performed for 2 h at 37 °C. The resulting gastric, intestinal, and GI digestion samples were frozen immediately at −80 °C and lyophilized. The lyophilized extracts were then dissolved in 2 mL of MeOH/BHA (9.9 : 0.1, v/w), sonicated for 10 min, and immediately cleaned-up by SPE.

The SPE clean-up was developed using Strata X-Aw cartridges (100 mg per 3 mL), according to the procedure described previously.[51] The target compounds were eluted with 1.0 mL of MeOH and dried using a SpeedVac concentrator. The dry extracts were reconstituted with 200 μL of ultrapure-water/MeOH (50 : 50, v/v), sonicated for 10 min, and filtered through a 0.45 μm filter (Millipore, MA, USA).

UHPLC-ESI-QqQ-MS/MS analysis

Chromatographic separation of PhytoPs and PhytoFs was performed using a UHPLC coupled with a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany) and a BEH C_{18} analytical column (2.1 × 50 mm, 1.7 μm) (Waters, Milford, M.A.), applying the methodology described by Collado-González *et al.* (2015) and Domínguez-Perles *et al.* (2018).[24,51] The PhytoPs and PhytoFs were quantified using authentic standards according to standard curves freshly prepared each analysis day. The chemical names follow the Taber/Robert and Muller nomenclature system of Table 2.

Cell lines and culture conditions

Colorectal Caco-2 (ATCC®HTB37) and endothelial Ea.hy 926 (ATCC®CRL2922) human cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in DMEM containing 4.5 g L^{-1} glucose, supplemented with GlutaMAX™, 10% foetal bovine serum (FBS), and 1% non-essential amino acids, at 37 °C in a humidified atmosphere containing 5% CO_2. The passage number of the cells used in this study was between 30–35 and 3–8 for the Caco-2 and the Ea.hy926 cell lines, respectively.

Cell viability

Toxicity of the analytical extract (up to 50 ng mL^{-1} PhytoPs and 20 ng mL^{-1} PhytoFs) dissolved in complete medium was tested

Table 2 UHPLC/MS/MS parameters for the quantification and confirmation of phytoprostanes and phytofurans in *G. longissima* macroalgae (in negative ionization mode)

Compound	Retention time (min)	MRM transition (*m*/*z*)
Phytoprostanes		
ent-16-*epi*-16-F_{1t}-PhytoP[c]	1.583	327.1 > 283.2[a] 327.1 > 225.1[b]
9-F_{1t}-PhytoP	1.631	327.2 > 273.1 327.2 > 171.0
ent-16-F_{1t}-PhytoP[c]	1.712	327.2 > 283.2 327.2 > 225.1
9-*epi*-9-F_{1t}-PhytoP	1.785	327.2 > 272.8 327.2 > 171.0
9-D_{1t}-PhytoP	1.791	325.2 > 307.3 325.2 > 134.7
9-*epi*-9-D_{1t}-PhytoP	2.022	325.2 > 307.2 325.2 > 134.9
16-B_1-PhytoP	2.620	307.2 > 223.2 307.2 > 235.1
9-L_1-PhytoP	3.079	307.2 > 185.1 307.2 > 196.7
Phytofurans		
ent-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF	0.906	344.0 > 300.0 344.0 > 255.9
ent-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF	1.501	343.9 > 209.0 343.9 > 201.1
ent-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF	1.523	343.0 > 171.1 343.0 > 97.2

[a] Quantification transition. [b] Confirmation transition. [c] Coeluting diastereoisomers quantified together.

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in Caco-2 cells. For this, exponentially growing cells were seeded into a 96-well plate at a density of 10^4 cells per well. After 48 h of incubation with the diluted extract, the cells were incubated with tetrazolium (MTT) solution at a final concentration of 1 mg mL^{-1} for 4 h at 37 °C. The formazan crystals formed in the intact cells were dissolved in 200 μL dimethyl sulfoxide (DMSO) for 30 min and the absorbance was measured in a microplate reader (Bio-Tek Synergy HT, Winooski, VT, USA) at 570 nm and 620 nm of reference. Cell viability was calculated as average optic density (OD) of wells/average OD of control wells, and expressed as a percentage (%).

Transport experiments in the Caco-2 monolayer model of the intestinal barrier

To assess the bioavailability of PhytoPs and PhytoFs *in vitro*, Caco-2 cells were seeded at a density of 3×10^5 cells per well on high-throughput screening (HTS)-Transwell®-12 well permeable supports (0.4 μm pore polyester membrane) (Costar, Corning, Birmingham, UK). The Caco-2 cells were allowed to differentiate for at least 21 days before the experiments, replacing the culture media every 48–72 hours. Cell monolayer integrity was checked by reference to the transepithelial electrical resistance (TEER) of Caco-2 cells measured by a commercial apparatus (Millicell ERS; Millipore Co., Bedford, MA) using Ag–AgCl electrodes, according to the manufacturer's instructions. The final values are expressed as Ω cm^2 on the basis of the following equation: 'TEER = $(R - R_b) \times A$', where R is the resistance of the filter insert with cells, R_b is the resistance of the filter alone and A is the growth area of the filter in cm^2. Only the inserts with a TEER higher than 600 Ω cm^2 were used for the experiments. Seventy microlitres of six analytical extract samples and 300 μL of six digested extracts (2 gastric, 2 intestinal, and 2 GI) were added to the apical chamber, and the transport from the apical chamber to the basolateral compartment was evaluated after 60 min at 37 °C.

Endothelial dysfunction model and quantitative RT-PCR for inflammatory markers

In order to study the effects of PhytoPs and PhytoFs on the phenotype of the human endothelial cell line EA.hy926, cells were seeded in 24-well plates at 8×10^5 cells per well in DMEM supplemented with 5% FBS. Cells were treated with 70 μL of the analytical and bioavailable fraction extracts (in triplicate) for 16 hours. Then, an inflammatory stimulus (20 ng mL^{-1} TNF-α) was added and cells were maintained at 37 °C and 5% CO_2 for another six hours.

Total RNA was extracted from EA.hy926 cells treated with PhytoPs and PhytoFs and the inflammatory stimulus using 300 μL Trisure reagent (Bioline, Taunton, MA, USA), and Direct-zol RNA MiniPrep (Zymo Research Irvine, Irvine, CA, USA) according to the manufacturer's protocol. Total RNA was reverse-transcribed into complementary DNA by using Sensifast cDNA™ Synthesis kit (Bioline, Taunton, MA, USA). The mRNA level of the target genes was quantified by RT-PCR using SensiFAST SYBER Hi-ROX Kit (Bioline, Taunton, MA, USA) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Briefly, 5 μL of 1 : 5 diluted cDNA in water (v/v) was added to the qPCR reaction containing 10 μL 2× SensiFAST Mix and 400 nM of each primer in a total volume of 20 μL.

Specific and validated primers for the human genes actin, intercellular adhesion marker-1 (ICAM-1), vascular cell adhesion marker-1 (VCAM-1), endothelial nitric oxide synthase (eNOS), and interleukin 6 (IL-6) were used (all from Sigma-Aldrich Chemical Co., Saint Louis, MO, USA). The relative mRNA expression of the genes of interest was represented by: '2^(−DDCT) = [CT(gene of interest) − CT(Actin)]test − [CT (gene interest) − CT(Actin)]control'.

Relative quantification of the gene expression was carried out using the comparative fold change 2^DDCT method.[53] The average value of each target gene after actin normalization at the moment of highest expression was used as a calibrator to determine the relative levels in the rest of the experimental conditions. All experiments and qPCR reactions were performed in triplicate.

Statistical analysis

All the methanolic extractions, as well as the gastric, intestinal, and GI digestions were performed in triplicate (n = 3) and the data were expressed as the mean ± least significant differences (LSD) (n = 3). All statistical tests were performed at 5% of the significance level using the SPSS 24.0 software package (LEAD Technologies, Inc., Chicago, USA). All the data were subjected to one-way analysis of variance (ANOVA), confirming that the ANOVA requirements were met especially as regard the normal distribution of the residuals and the homogeneity of variance, by means of the Kolmogorov–Smirnov (with Lilliefors correction) and Levene tests, respectively. When statistical differences were identified, the variables were compared using the Tukey's multiple range test.

For the gene expression study, data were expressed as mean ± standard deviation (SD) (n = 3). A paired Student's t-test was used to compare the mean of two groups. Statistical analyses were performed with SPSS 24.0 and the significance of the differences found was set up at $p < 0.05$.

Results and discussion

Total and individual PhytoPs and PhytoFs composition in *Gracilaria longissima*

Assessment of the analytical extracts of *G. longissimi* identified eight PhytoPs (9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-PhytoP) and three PhytoFs (*ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF). These eleven plant oxylipins were identified according to their pseudomolecular ion and retention time based on previous descriptions by Collado-González *et al.* (2015) and Domínguez-Perles *et al.* (2018), and by comparison with authentic standards.[24,51]

Quantification of the individual and total PhytoPs and PhytoFs (Fig. 1 and 2, respectively) showed that *ent*-16-*epi*-16-

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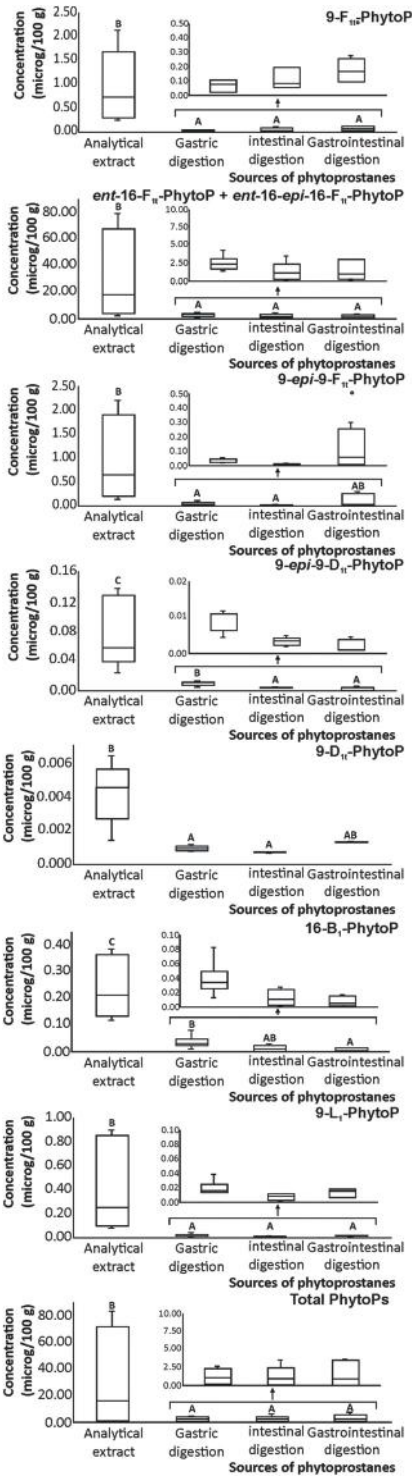

Fig. 1 Box plots with quartiles (upper values 75%, median 50%, and lower values 25%) of PhytoPs (9-F_{1t}-PhytoP, 9-*epi*-9-F_1t-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, 9-L_1-PhytoP, and total PhytoPs) bioaccessible upon *in vitro* gastric, intestinal, and GI digestions of *Gracilaria longissima* (μg per 100 g dry algae material). Boxes with a different letter within each plot indicate statistically significant differences at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range test. When necessary, box-plots insets were included showing the proper autoscale to observe differences between gastric, intestinal and gastrointestinal digestions.

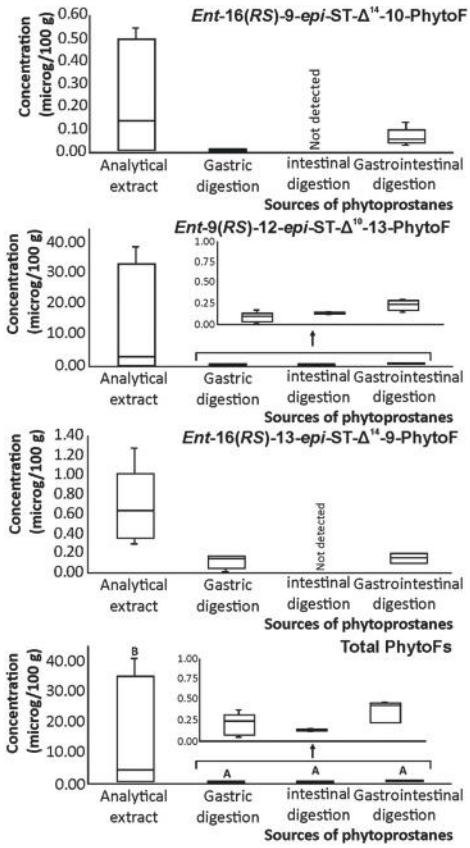

Fig. 2 Box plots with quartiles (upper values 75%, median 50%, and lower values 25%) of PhytoFs (*ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, and total PhytoFs) bioaccessible upon *in vitro* gastric, intestinal, and GI digestions of *Gracilaria longissima* (μg per 100 g dry algae material). Boxes with a different letter within each plot indicate statistically significant differences at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range test. When necessary, box-plots insets were included showing the proper autoscale to observe differences between gastric, intestinal and gastrointestinal digestions.

F_{1t}-PhytoP + *ent*-16-F_{1t}-PhytoP were the most abundant PhytoPs (the chromatographic conditions applied in the present work were not able to separate the two C-16 epimers of the 16 series of F_{1t} PhytoPs, as mentioned by Domínguez-Perles *et al.*, 2018).[24] They reached concentrations ranging between 2.638 and 78.697 μg per 100 g of raw material. The remaining PhytoPs presented average concentrations in the lower range 0.001–2.113 μg per 100 g of raw material (Fig. 1).

Bioactive PhytoPs are found in small quantities in plants (*e.g.*, olive oil, rice, almonds).[19,20,24] However, their content is strongly affected by agricultural conditions, as reveal their diverse concentration (4.0 to 23.8 μg per 100 g) in raw almonds found by Carrasco-del Amor *et al.* (2015), being in the same range as the content observed in *Gracilaria longissima* in the current study.[19]

As regards PhytoFs, these plant oxylipins were present at significantly lower concentrations than PhytoPs, the highest average concentration being the 13.213 μg per 100 g of raw

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material found for *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF (Fig. 2). Rice flours had average values of 1.030 μg per 100 g,[31] slightly lower than the PhytoFs content of the algae under study.

Thus, due to the increasing importance of *Gracilaria* genus in the food industry, this study provides new perspectives about the use of red algae as a source of bioactives molecules and nutraceutics.

Bioaccessibility: impact of gastrointestinal digestion on the PhytoPs and PhytoFs extracted from *Gracilaria longissima*

According to the preliminary results obtained, the *in vitro* GI digestion of analytical extracts of PhytoPs and PhytoFs from *G. longissima*, strongly broke-down these lipid compounds. However, PhytoFs were even more sensitive to the chemical and enzymatic conditions of the intestinal digestions, which did not permit their further detection.

As regards the stability of both oxylipins following GI digestion of the raw plant material, the concentrations recorded for the PhytoPs and PhytoFs were, as expected, below the concentrations recorded in the analytical extracts (Fig. 1 and 2). However, the total amount of PhytoPs and PhytoFs remaining after GI digestion of the raw material (1.17 and 0.42 μg per 100 g of raw material, respectively) was slightly higher than digestion of the analytical extract (0.5 ng mL^{-1} and undetected, respectively) (data not shown). This would probably be due to the protective effect of the cellular wall of the plant tissues, as well as to the contribution of additional structures that might hinder access of the enzymes responsible for the break-down of the oxylipins, thus protecting the insoluble compounds present in the raw material.[54] However, it should be noted that the low solubility of oxylipins in the gastric and intestinal digestive fluids did not protect their molecular integrity during intestinal digestion. In this aspect, many oxilipins have demonstrated low stability in the presence of other biomolecules.[55] Indeed, the data obtained in the present study suggest that the presence of bile salts contributes to emulsifying and solubilizing oxylipins, as previously mentioned in the case of other amphipathic bioactive compounds, such as kale polyphenols.[56] Anyways, the observed impact of the GI digestion process on the molecular stability of these lipidic compounds shows that their integrity at gastric and intestinal pH is low.

Cytotoxicity of analytical extract of PhytoPs and PhytoFs in intestinal epithelium cells

To investigate the impact of PhytoPs and PhytoFs on the viability of Caco-2 cells, the cytotoxicity of rising percentages of the analytical (non-digested) extract in the culture medium was assessed by the MTT method. The data obtained revealed no cytotoxicity on intestinal epithelial cells by PhytoPs and PhytoFs with concentrations of up to 50 and 20 ng mL^{-1}, respectively (Fig. 3). The following oxylipin concentrations had no effects on cell proliferation nor induced cell death.

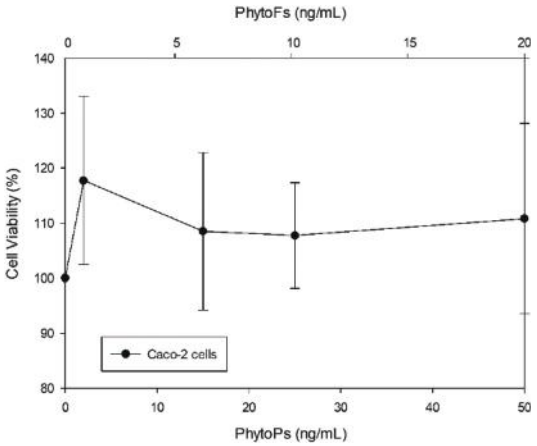

Fig. 3 MTT-based viability of colorectal Caco-2 cells exposed to rising concentrations of PhytoPs and PhytoFs for 48 hours.

Bioavailability of PhytoPs and PhytoFs in extracts from *Gracilaria longissima*

To better understand the biological activity of bioavailable PhytoPs and PhytoFs, testing their absorption by Caco-2 cells an *in vitro* intestinal barrier model was considered essential. Based on the hydrophobicity of PhytoPs and PhytoFs, somehow, the uptake of these oxylipins by cells was expected to be faster compared with the uptake of other bioactive hydrophilic molecules.[57,58] Therefore, the bioavailability of PhytoPs and PhytoFs in the analytical and digested extracts (after gastric, intestinal, and GI digestion) was evaluated *in vitro* following a previously described methodology.[57] The percentage of PhytoPs and PhytoFs added to the cell culture in the apical (AP) compartment that crossed the intestinal epithelial barrier of cells and those found in the culture medium in the basolateral (BL) compartment was calculated. These concentrations provide information on the bioavailability of PhytoPs and PhytoFs.

The results obtained in this respect suggested that PhytoPs and PhytoFs are absorbed by intestinal epithelial cells (Fig. 4 and 5, respectively).

Indeed, the efficiency of the absorption/transport of these compounds was found to be higher than the value described for other secondary metabolites of plants, such as phenolic compounds,[58] achieving values of up to 88.2 and 97.7%, on average, for PhytoPs and PhytoFs, respectively. Detailing the bioavailability for each individual oxylipin, the average percentage calculated for bioavailability in the analytical extract decreased as follows: *ent*-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (50%) > 9-D_{1t}-PhytoP (40%) > 9-*epi*-9-F1t-PhytoP (30%), while the rest of the PhytoPs reached less than 10% bioavailability. The result after gastric digestion pointed to an average bioavailability of 80% for PhytoPs. In the case of the PhytoFs, *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF (60%) showed maximum bioavailability followed by *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF (40%) and *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF (20%) in the analytical extract. Hence, a higher percentage of bioavailability was

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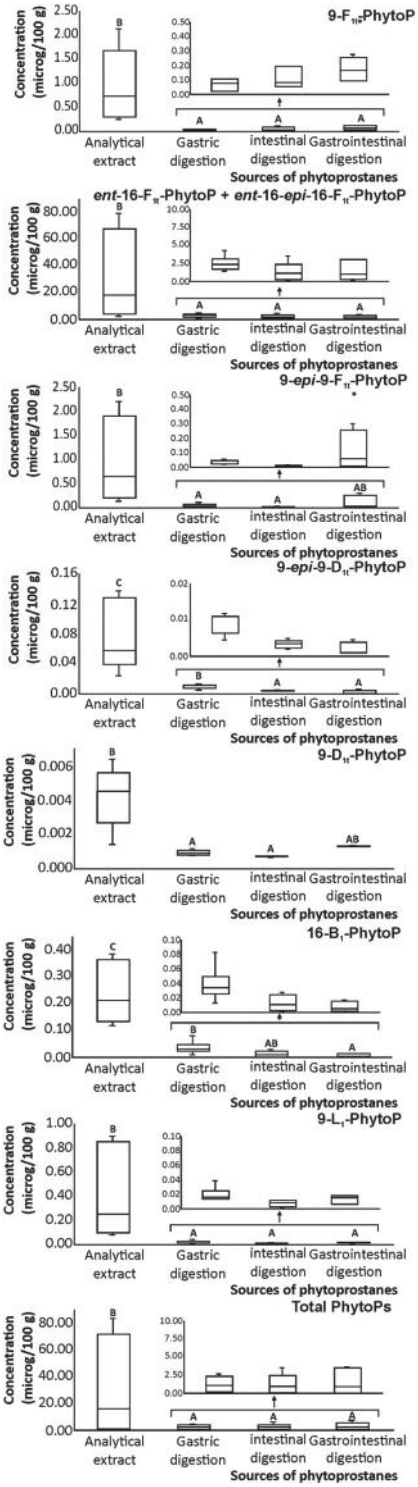

Fig. 4 Box plots with quartiles (upper values 75%, median 50%, and lower values 25%) of percentages of PhytoPs (9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 16-B_1-PhytoP, 9-L_1-PhytoP, and total PhytoPs) bioavailable after crossing the Caco-2 cell barrier. Boxes with a different letter within each plot indicate statistically significant differences at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's test. When necessary, box-plots insets were included showing the proper autoscale to observe differences between gastric, intestinal and gastrointestinal digestions.

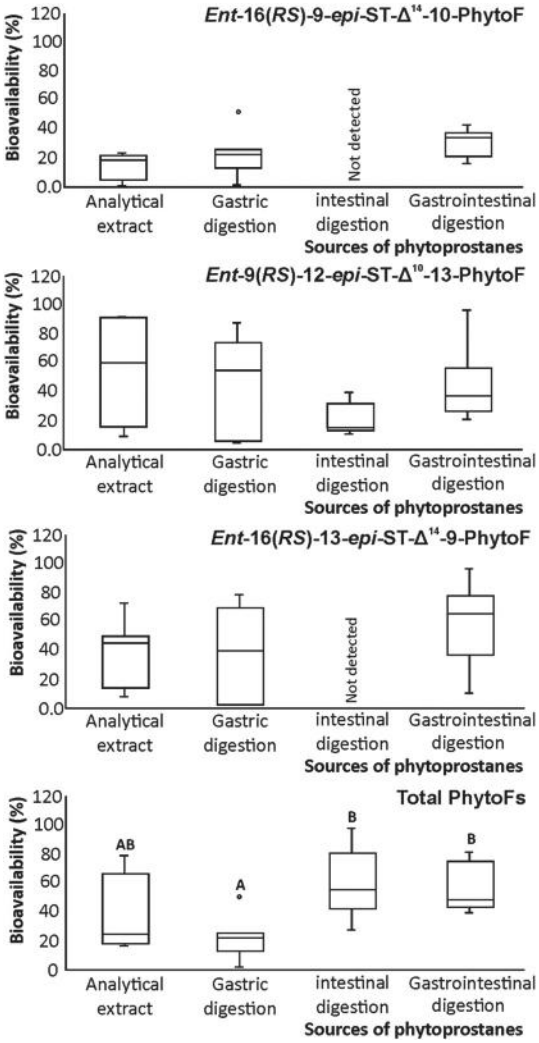

Fig. 5 Box plots with quartiles (upper values 75%, median 50%, and lower values 25%) of percentage of PhytoFs (*ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, and total PhytoFs) bioavailable after crossing the Caco-2 cell barrier. Boxes with a different letter within each plot indicate statistically significant differences at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's test.

observed for PhytoFs following GI digestion than in the case of PhytoPs. However, no significant differences in transport/absorption efficiency were observed for either oxylipins after the different digestions, probably due to the high dispersion of the results (Fig. 4 and 5).

Evaluation of these data together with those on the bioaccessibility of the raw algae material described in the present work (Fig. 1 and 2) underline the relative stability and bioavailability of PhytoPs and PhytoFs in GI conditions. These joint findings strongly suggest the potential of these oxylipins for developing biological activities in humans after the dietary ingestion of plant-based foods, the source of both compounds.

Data available on the *in vivo* bioavailability of oxylipins are scarce, and, to the best of our knowledge, only two animal and human studies exist.[34,59]

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Moreover, previous studies have suggested the presence in plasma of conjugative metabolites of these compounds,[34,59] and tentatively, in tissues (although this has not been demonstrated to date). Indeed, all the evidence in this respect suggests the interest to further evaluate the chemical derivatives of these compounds using untargeted metabolomic approaches and to establish the functionality of both free and conjugated metabolites through both *in vitro* and *in vivo* studies using efficient esterases described recently by our group.[16]

Nonetheless, it should be noted that the *in vitro* digestion models (specially the static models like that used in the present work) cannot faithfully simulate the complex physiochemical events occurring during *in vivo* physiological digestion processes, and so the results obtained from *in vitro* models will require to be verified by scaling such studies to animal or human models.

Bioactivity of the analytical extract in endothelial cells

Due to the structural analogy between PhytoPs and human prostanoids, for instance, between the E-series PhytoPs and prostaglandin E_2 (PGE_2) (an anti-inflammatory prostaglandin with vascular effects),[9] the present work explored whether the analytical extract of PhytoPs and PhytoFs from *G. longissima* could mediate inflammatory pathways in human endothelial cells. With this objective, analytical extracts or their bioavailable fractions obtained through the Caco-2 intestinal barrier model were applied directly to endothelial cells exposed to inflammatory stimulus.

When human endothelial cells were treated with 20 ng mL^{-1} TNF-α, the transcription of ICAM-1, VCAM-1, and IL-6 genes increased and eNOS expression decreased, both compared with the results obtained in control conditions (untreated cells) (Fig. 6). The exposure of cells to the analytical and bioavailable extracts of PhytoPs and PhytoFs, with no inflammatory stimulus did not significantly affect the basal expression of any of the inflammation markers monitored. However, when both PhytoPs and PhytoFs extracts (analytical and bioavailable) were added to stimulated cells, the level of ICAM-1 decreased significantly ($p < 0.005$) (Fig. 6A). Similar results were found for IL-6, although the high dispersion of the data recorded did not allow significant differences to be identified concerning the capacity of the PhytoPs and PhytoFs extracts to modulate the secretion of this cytokine. In this respect, and despite the lack of statistically significant differences, the results obtained suggest that the addition of the analytical extract strongly contributed to the recovery of basal IL-6 expression levels (Fig. 6B). As regards VCAM-1, during the incubation of endothelial cells with the analytical extract, it was seen that, in absence of an inflammatory stimulus, the level of this adhesion marker did not change relative to basal levels. However, when cells were stimulated with TNF-α, the exposure to the analytical extract induced a significant increase in its transcription (Fig. 6C). The analytical extract also significantly increased the expression of eNOS suggesting

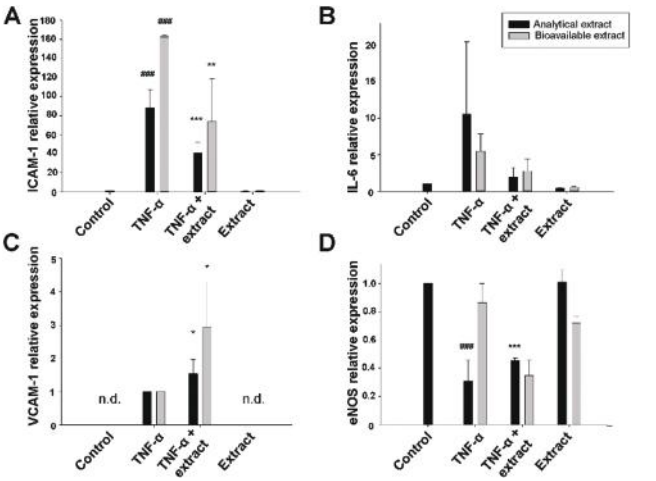

Fig. 6 Relative mRNA expression in Ea.hy926 cells (A) ICAM-1; (B) IL-6; (C) VCAM-1; (D) eNOS after treatment with the analytical extract and the bioavailable PhytoPs and PhytoFs. Data shown represent averaged values of five independent experiments. Statistic differences compared with 20 ng mL^{-1} TNF-α were found at *($p < 0.005$), **($p < 0.01$), and *** ($p < 0.05$). ### indicate statistically significant differences compared with unstimulated cells (CONTROL) ($p < 0.05$).

a potential vasorelaxing effect, although such an effect was not found in the presence of the bioavailable extract.

A comparison of the modulatory effects of both analytical and bioavailable PhytoPs and PhytoFs extracts pointed to a similar capacity to modify the inflammation and adhesion markers. More specifically, the relative expression of VCAM-1 was slightly higher when the endothelial cells were treated with very low concentrations of both bioavailable compounds. For their part, ICAM-1 and eNOS expression markers were higher when the compounds were more abundant in the analytical extract. Therefore, it seems that such effects are related to the concentrations of the compounds, decreasing after crossing the Caco2 barrier (Fig. 4). Based on the current data, a dose-dependent effect on the endothelium might be envisaged, although more additional research is needed to make sense of the situation. Besides, the abundance of the oxylipins should be mentioned due to this antagonist concentration–effect relationship. Other compounds of a different nature and structure also display antagonistics' effects, as seen from a multitarget model that is involved in pathologies such as endothelial dysfunction and inflammation.[60,61] However, the mechanisms underlying such actions remain underexplored, pointing to the need for further studies to identify the target molecules and receptors interacting with PhytoPs and PhytoFs.

Although to the present date the information available on this issue is scarce, given the structural similarity of PhytoPs and PhytoFs with human prostaglandins, the possible mechanisms of action of PhytoPs and PhytoFs could be envisaged in the base of the molecular pathways responsible for the pro- and anti-inflammatory activity of prsotaglandins. Hence, similarly to these human oxylipins, PhytoPs and PhytoFs could

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Table 3 Correlation between the concentration of individual phytoprostanes and phytofurans and the modulation of nRNA codifying for different concentration of intercelluar adhesion molecules 1 (ICAM-1), interleukine-6 (IL-6), vascular cell adhesion protein-1 (VCAM-1), and endothelial nitric-oxide synthase (eNOS)

	Inflammatory markers			
Plant oxylipin	ICAM-1 R^2(p-value)[a]	IL 6 R^2(p-value)	VCAM-1 R^2(p-value)	eNOS R^2(p-value)
9-*epi*-9-D_{1t}-PhytoP	−0.124(N.S.)	**−0.428($p < 0.05$)**	**−0.515($p < 0.01$)**	**−0.675($p < 0.001$)**
9-D_{1t}-PhytoP	−0.165(N.S.)	−0.339(N.S.)	**−0.537($p < 0.01$)**	**−0.559($p < 0.01$)**
9-F_{1t}-PhytoP	−0.008(N.S.)	−0.266(N.S.)	**−0.522($p < 0.05$)**	**−0.467($p < 0.05$)**
9-*epi*-9-F_{1t}-PhytoP	0.214(N.S.)	0.069(N.S.)	−0.020(N.S.)	0.052(N.S.)
9-L_1-PhytoP	0.032(N.S.)	−0.352(N.S.)	−0.512(N.S.)	**−0.546($p < 0.05$)**
16-B_1-PhytoP	−0.113(N.S.)	0.109(N.S.)	0.076(N.S.)	0.104(N.S.)
ent-16-*epi*-16-F_{1t}-PhytoP + *ent*-16-F_{1t}-PhytoP	−0.151(N.S.)	**−0.472($p < 0.05$)**	**−0.536($p < 0.01$)**	**−0.724($p < 0.001$)**
ent-9-(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF	0.016(N.S.)	0.267(N.S.)	−0.302(N.S.)	0.374(N.S.)
ent-16-(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF	−0.135(N.S.)	**−0.549($p < 0.05$)**	−0.001(N.S.)	**−0.798($p < 0.001$)**
ent-16-(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF	−0.241(N.S.)	0.269(N.S.)	0.278(N.S.)	0.278(N.S.)

[a] N.S: non-significant.

exert their inflammatory modulation by activating transmembrane spanning G protein-coupled receptors. In this aspect, the prostanoid receptor subfamily is comprised of the E prostanoid receptor (EP) 1, 2, 3, and 4, that interact with the prostaglandins belonging to class E, the D prostanoid receptor (DP) 1 that interact with the prostaglandins belonging to class D, and the F prostanoid receptor (FP) that interact with the prostaglandins belonging to class F. So, given the structural analogy between human oxylipins and those of higher plants, PhytoPs and PhytoFs could exert their biological actions by interacting with these receptors according to their chemical structure. The interaction with such receptorsn would activate a myriad of intracellular signaling pathways, responsible for the effects of the receptors activation on cell function.[62]

Correlation structure–activity

In order to shed some light on the relative contribution of the individual PhytoPs and PhytoFs to the modulation of ICAM-1, IL-6, VCAM-1 and eNOS levels, the correlation between the concentration of the individual compounds and the modulatory effect was assessed.

These determinations suggested that each oxylipin would possess multi-target affinity (Table 3); for instance, the 9-*epi*-9-D_{1t}-PhytoP and *ent*-16-F_{1t}-PhytoP showed a significantly negative correlation with IL-6 and VCAM-1 (both at $p < 0.05$), suggesting that these PhytoPs could contribute to the induction of anti-inflammatory responses. Moreover, these oxylipins also correlated in a significantly positive way with the expression of mRNA codifying eNOS and so may also be related with enhanced vasorelaxation (Fig. 6). Besides, according to the correlation analysis (Table 3), 9-D_{1t}-PhytoP, 9-F_{1t}-PhytoP, 9-L_1-PhytoP, and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF seem to induce NO dysfunction, although they did not mediate in the changes observed in the rest of the inflammation markers studied.

In light of the antecedents mentioned in the literature concerning the biological functions of PhytoPs and PhytoFs and the results obtained in the present study, it is clear that these oxylipins (PhytoPs and PhytoFs) can exert opposite biological functions. In this respect, as many as nine human G protein-coupled prostanoid receptors with different affinities have been described.[63] Hence, the existence of multiple affinity-receptors, the cross-reactivity that seem to characterize the individual PhytoPs and PhytoFs, and their concentration in different plant-based foods, as well as their differential bioaccessibility and bioavailability could contribute to our understanding of the seemingly contradictory effects observed in the inflammatory process. These pharmacological actions affect the endothelium in functionally opposing directions. Indeed, this phenomenon has also been described in studies of human prostanoids, such as thromboxane-A_2 (vasoconstrictor) and prostacyclin (PG-I_2, vasodilator and anti-inflammatory). Thus, this suggests that oxylipins (of plant and mammal origin) are responsible for complex pharmacological profiles, probably mediated by their reactivity with diverse receptor/signalling pathways and affinity over the endothelium. Whether or not oxylipins display endothelial protective effects has not been formally demonstrated, but the observations reported in the present work suggest they have a dose-dependent role in vascular cells.

Conclusions

The results retrieved upon the present study further support the biological interest of PhytoPs and PhytoFs by describing the stability of these plant oxylipins through gastro-intestinal digestion and their bioavailability *in vitro* that reached values equal or even higher than those previously described for paradigmatic secondary metabolites related to the benefits for health associated to plant-based foods (phenolic compounds). However, bioaccessibility and bioavailability is not the same of the diverse individual PhytoPS and PhytoFs, reaching the highest values for *ent*-16-*epi*-16-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, and *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF. Besides, when evaluat-

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ing the capacity to prevent inflammation, it was found that 9-*epi*-9-D_{1t}-PhytoP and *ent*-16-F_{1t}-PhytoP could contribute to prevent inflammation, while impaire vasorelaxation. On the other hand, 9-D_{1t}-PhytoP, 9-F_{1t}-PhytoP, 9-L_1-PhytoP, and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF seem to induce NO dysfunction. All these findings suggest that although these plant oxylipins seem to play a major role as chemical signals in humans, the putative reactivity of individual PhytoPs and PhytoFs regarding receptors eliciting diverse responses associated to the course of an array of pathophysiological situations, in addition to the dose-dependent activity, make difficult to attribute a final cellular response to any specific oxylipin. Hence, we understand that the data presented herein might be interpreted with cautions, requiring modelling approaches that contribute to complementing the *in vitro* studies the outcomes reported.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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