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Anti-inflammatory effect of polyphenols from Chilean currants (*Ribes magellanicum* and *R. punctatum)* after *in vitro* gastrointestinal digestion on Caco-2 cells
Anti-inflammatory activity of *in vitro* digested Chilean currants

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ABSTRACT

The polyphenol-enriched extracts (PEEs) of Chilean currants *Ribes magellanicum* and *R. punctatum* were submitted to *in vitro* gastro-intestinal digestion and their anti-inflammatory activities were assessed using differentiated human Caco-2 (clone C2BBe1) cells stimulated with interleukin 1β (IL-1 β). The inhibitory effect of non-digested and digested PEEs towards human cyclooxygenase 1 (COX-1) and COX-2, and the gene expression of COX-2 and inducible nitric oxide synthase (iNOS) was also evaluated. The digested PEE from *R. punctatum* decreased the secretion of IL-8, IL-6, and TNF-α; whereas *R. magellanicum* reduced IL-6 and TNF-α in the Caco-2 cells ($p < 0.05$). Both digested extracts significantly down-regulated the mRNA expression of COX-2 and iNOS ($p < 0.05$). PEEs showed 60% of inhibition towards COXs, with higher inhibition against COX-2. The PEEs from *R. punctatum* displayed better anti-inflammatory activity in all the experiments. Our results suggest that *R. magellanicum* and *R. punctatum* might be useful against intestine inflammatory conditions.

1. Introduction

Inflammatory bowel diseases (IBD) are chronic conditions including Crohn's disease (CD) and ulcerative colitis (UC). These illnesses are associated with a dysregulation of the immune response against the resident microbiota and are characterized by epithelial injuries and immune cell infiltration (Sartor, 2006). The population from North American and European countries are the most affected by IBD; however, the prevalence and incidence in industrialized countries from Asia, the Middle East, Africa, and South America is increasing. This raise is associated with environmental and genetic factors, as well as to the diet (Kaplan & Ng, 2017).

The IBD treatment is focused on the control of the inflammatory and immune responses, and often involves long-term administration of non-steroidal anti-inflammatory drugs (NSAIDs) – and corticosteroids. The long-term use of these drugs results in a latent risk of side effects, lack of response to the treatment and recurrence of the disease (Biasi, Astegiano, Maina, Leonarduzzi, & Poli, 2011). Pro-inflammatory cytokines such as interleukin 1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) are overexpressed in the intestinal mucosa from patients with IBD and seem to play an important role in its etiology and pathogenesis. In addition, the enzymes cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are overexpressed in the injured tissues, releasing prostaglandin E_2 (PGE_2) and nitric oxide

Abbreviations: CD, Crohn's disease; COX, cyclooxygenase; GAE, gallic acid equivalents; GID, gastrointestinal digestion; IBD, intestinal bowel disease; IL, interleukin; iNOS, inducible nitric oxide synthase; GD-PEE, gastric digested polyphenol-enriched extract; ID-PEE, intestinal digested polyphenol-enriched extract; NO, nitric oxide; NSAIDs, non-steroidal anti-inflammatory drugs; PEE, polyphenol-enriched extract; PG, prostaglandin; UC, ulcerative colitis; TNF-α, tumor necrosis factor alpha

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(NO), respectively, which are directly linked to cancer development (Surh et al., 2001). The regulation of cytokines levels through diet-derived compounds might be helpful in the prevention and/or as a co-adjuvant treatment. In this sense, polyphenols are good candidates due to the increasing evidence of their modulating capacity of the acute and chronic inflammatory status in humans (Leyva-López, Gutiérrez-Grijalva, Ambriz-Pérez, & Heredia, 2016).

Fruits from *Ribes* species (currants), largely cultivated and commercialized, are important sources of polyphenols and antioxidants, thus, there is a growing interest in these berries as a potential source of functional ingredients (Gopalan et al., 2012). Studies focused on the anti-inflammatory activity of currant extracts have been mainly performed with *Ribes nigrum* (blackcurrant). The juice of blackcurrant, white currant (*Ribes sativum*), and gooseberry (*Ribes hirtellum*) modulate COX-2 expression levels in prostatic adenocarcinoma cells (PC-3) through the NF-kB pathway (Boivin, Blanchette, Barrette, Moghrabi, & Béliveau, 2007). Lyall et al. (2009) reported a decrease of TNF-α and IL-6 in human monocytes (THP-1). Huebbe et al. (2012) showed a reduction of mRNA levels of TNF-α, IL1β, and iNOS in murine macrophages (RAW264.7) as a consequence of the pre-treatment with blackcurrant extract. Similar results were obtained in a Caco-2/RAW264.7 co-cultured cell model, in which a reduction of IL-1α and IL-1β, COX-2 and iNOS was observed (Olejnik et al., 2016). In addition, a decreased COX-2 expression was observed in rats fed with anthocyanin-rich black currant skin extracts (Bishayee et al., 2013).

The Chilean currants *Ribes magellanicum* and *R. punctatum* are also rich in polyphenols with some reported health-beneficial bioactivities. The polyphenolic-enriched extracts (PEEs) contain high amounts of hydroxycinnamic acids, dihydroflanovols, flavonols, and anthocyanins, with high antioxidant capacity and cytoprotective activity against oxidative stress (Jiménez-Aspee, Thomas-Valdés et al., 2016; Jiménez-Aspee, Theoduloz et al., 2016). The cytoprotection occurs by means of the induction of the antioxidant enzymes catalase, superoxide dismutase, glutathione peroxidase and reductase (Theoduloz, Burgos-Edwards, Schmeda-Hirschmann, & Jiménez-Aspee, 2018). In addition, the PEE from *R. magellanicum* and *R. punctatum,* submitted to *in vitro* gastrointestinal digestion, showed inhibitory activity against α-glucosidase, thus indicating a potential role in the prevention of postprandial hyperglycemia (Burgos-Edwards, Jiménez-Aspee, Thomas-Valdés, Schmeda-Hirschmann, & Theoduloz, 2017). Among Chilean berries, extracts from maqui (*Aristotelia chilensis*), calafate (*Beberis microphylla*) (Reyes-Farias et al., 2015) and white strawberry (Molinett, Nuñez, Moya-León, & Zúñiga-Hernández, 2015) have been assayed as anti-inflammatory agents with promising results. However, no studies on Chilean currants could be found in literature so far.

Most *in vitro* studies have been conducted with crude extracts. However, the gastrointestinal environment affects the stability of polyphenols, generating qualitative and quantitative changes in the phenolic profile (Burgos-Edwards et al., 2017; Olejnik et al., 2016). In a previous study we isolated the main anthocyanins in both Chilean currants, which were unequivocally identified as cyanidin-3-glucoside and cyanidin-3-rutinoside. In addition, 3-caffeoylquinic acid and caffeoyl hexoside were the main hydroxycinnamic acid derivatives in the PEEs (Burgos-Edwards et al., 2017; Jiménez-Aspee, Thomas-Valdés et al., 2016). Other compounds occurring in *R. magellanicum* and *R. punctatum* PEE include flavonoid glycosides based on the flavonols myricetin, quercetin and kaempferol. Proanthocyanidin monomers and oligomers were identified in both Chilean currant species. The content of the main compounds after intestinal digestion decreased roughly by 90% for cyanidin-3-glucoside and 80% for the corresponding rutinoside. This effect was more pronounced for the hydroxycinnamic acid derivatives, with losses of nearly 98% for 3-caffeoylquinic acid and about 50–65% for caffeoyl hexoside (Burgos-Edwards et al., 2017). The *in vitro* digestion models became a useful alternative to simulate the influence of the human digestion process on food constituents. The absence of invasive methods and/or experimental animals, the low cost, and the fast results turned them into a widely employed procedure for these purposes (Minekus et al., 2014). In addition, polyphenol extracts submitted to *in vitro* gastrointestinal digestion (GID) show comparable bioactivity to that observed in *in vivo* studies (Brown et al., 2014). After GID, the intestinal tissue is often exposed to higher concentrations of polyphenols, unlike other tissues which require previous absorption of these metabolites (Biasi et al., 2011). The Caco-2 cell model has been widely used to emulate the epithelial barrier functions, and the C2BBe1 clone presents a homogeneous morphology forming a polarized monolayer with the apical brush border comparable to that of the human colon cells. In addition, this clone is responsive to inflammatory stimuli (Leonard, Collnot, & Lehr, 2010).

The aim of our study was to assess the anti-inflammatory activity of the PEEs from *R. magellanicum* and *R. punctatum*, after *in vitro* gastrointestinal digestion in a Caco-2 cell inflammation model. The levels of pro-inflammatory cytokines (IL-6, TNF-α) and the pro-angiogenic chemokine IL-8, the expression of iNOS and COX-2, as well as the inhibitory activity against COX-1 and COX-2 were determined.

2. Materials and methods

2.1. Chemicals and standards

Methanol and formic acid were from Merck (Darmstadt, Germany). Potassium phosphate monobasic, sodium chloride and sodium hydroxide were from Scharlau (Barcelona, Spain). Pepsin A was from Biomol GmbH (Hamburg, Germany). Amberlite XAD-7 HP resin, dimethylthiazol diphenyl tetrazolium bromide (MTT), pancreatin, lipase and α-amylase from porcine pancreas, porcine bile extract, amyloglucosidase from *Aspergillus niger*, trizma® maleate buffer, penicillin (10,000 U/mL) and streptomycin (10 mg/mL) solutions, L-glutamine, human transferrin, sodium pyruvate, Dulbecco's Modified Eagle's Medium – high glucose (DMEM) were from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained from a Barnsted EasyPure water system (Thermo Scientific, Marietta, OH, USA). 0.25% Trypsin-EDTA was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Fetal bovine serum was acquired from Corning Mediatech, Inc. (Manassas, VA, USA). IL-1β was from PeproTech, Inc. (Pepro Tech EC, Ltd., London, UK). Phosphate buffered saline without Ca and Mg (10x) was acquired from BioWhittaker® (Lonza, Verviers, Belgium).

2.2. Sample collection and polyphenol-enriched extracts (PEEs) preparation

The collection of the ripe fruits from Chilean currants was carried out during summer (January-February) 2015 and 2016. The *R. magellanicum* fruits were from Laguna Verde, Región de la Araucanía, Parque Nacional Conguillío (39°29′S; 71°41′W), while *R. punctatum* fruits were from Las Trancas, Región del Bío-Bío (36°54′S; 71°25′W). Both samples were stored at −20 °C until extraction and analyses within one week. The fruits were washed, homogenized in a blender (Thomas TH-501V, Thomas Elektrogeräte, China) and lyophilized. The freeze-dried samples were extracted five times using MeOH/formic acid (99:1 v/v) under sonication for 15 min each. Then, the solvent was evaporated at 37 °C in a rotary evaporator and enriched in polyphenols with the Amberlite XAD-7-HP® resin to obtain the polyphenol enriched-extracts (PEEs), according to the methodology described by Jiménez-Aspee, Thomas-Valdés et al. (2016).

2.3. Simulated gastrointestinal digestion of polyphenol-enriched extracts (GID)

The PEEs were submitted to a two-step *in vitro* digestion procedure following a previously described method (Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010). The simulated gastric fluid contained NaCl (0.2% w/v), pepsin (0.32% w/v), HCl (0.7% v/v) and ultrapure water (pH 1.2) and the simulated intestinal fluid was composed

of K_2HPO_4, NaOH, pancreatin (1% w/v) and ultrapure water (pH 7.5). Trizma-maleate buffer (0.2 M, pH 6.8) was used to dissolve amyloglucosidase and α-amylase, while PBS buffer (pH 7.5) was employed for bile extracts (0.058 g) and lipase (0.023 g) solutions.

Briefly, 0.5 g of each sample was incubated at 37 °C in the dark with simulated gastric fluid (pH 1.2) for 30 min under constant shaking (180 rpm). Then, the pH was adjusted to 4.5 ± 0.2 with NaOH (0.5 M) before the addition of amyloglucosidase solution (120 mg/mL). After 30 min of incubation, the pH was adjusted to 6.9 ± 0.2 and the α-amylase solution (120 mg/mL) was added, incubating for further 45 min to finish the gastric phase. At the end of the process, a fraction was taken, centrifuged (10 min, 3000 rpm, 4 °C), and stored at −80 °C for further analyses. The remaining solution was further incubated for 30 min with simulated intestinal fluid (pH 7.5). Lipase and bile extract solutions were then added, and the mixture was incubated for additional 30 min. Finally, the digested samples were centrifuged (10 min, 3000 rpm, 4 °C) and the supernatant was stored at −80 °C for further analyses. The experiments were carried out in triplicate.

Polyphenols were recovered from the gastrointestinal solution by means of solid-phase extraction using HF Bond Elut C18 cartridges (Agilent Technologies, Santa Clara, CA, USA), yielding the gastric (GD-PEE) and intestinal digested PEE (ID-PEE) (Burgos-Edwards et al., 2017).

2.4. Cell culture

Caco-2 cells clone C2BBe1 (ATCC CRL-2102) were grown as monolayers in low bicarbonate (1.5 g/L) DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 µg/mL transferrin, 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in a humidified incubator at 37 °C in a 5% CO_2. The medium was renewed every two days and the cells were sub-cultured every week at a split ratio of 1 to 20 by trypsinization (0.25% trypsin-EDTA). Cells were plated at a density of $6.0x10^4$ cells/cm^2 for the subsequent experiments.

2.5. Cytotoxicity assay

The cytotoxicity of the ID-PEEs was determined employing the dimethylthiazol diphenyl tetrazolium bromide (MTT) reduction assay (Van Meerloo, Kaspers, & Cloos, 2011). After 21 days of cell differentiation in 96-well plates, cells were treated with the ID-PEE samples previously dissolved in dimethyl sulfoxide (DMSO), at concentrations ranging from 0 to 500 µg/mL. The concentration of DMSO in cell medium did not exceeded 0.5%, v/v. Medium with 0.5% of DMSO was used for controls. After 24 h incubation, the treatments were removed and each well was washed twice with sterile phosphate saline buffer (PBS). Then, MTT solution (1 mg/mL) was added to each well and incubated for 1 h. The MTT solution was removed and DMSO was added to dissolve the formazan salt. The absorbance at 570 and 690 nm (for sample and background, respectively) were measured with a microplate reader (SPECTROstar® Nano, Offenburg, Germany). The results were expressed as a percentage of viability compared to the untreated controls. Each treatment was performed in quintuplicate with two independent experiments.

2.6. Inflammatory stimulation and treatment conditions

Caco-2 cells were grown on 24-well plates, forming a fully differentiated monolayer after 21 days in culture. First, cells were incubated during 4 h with the ID-PEE from *R. magellanicum* and *R. punctatum* at final concentrations of 500, 250, 125 and 62.5 µg/mL. At the end of the incubation, cells were stimulated with IL-1β (10 ng/mL) for 24 h. Stimulated cells without the extracts were employed as inflammation controls (positive), while those without any treatment were employed as negative controls. After incubation, the culture media were transferred to sterile microtubes and the cells were lysed with 200 µL of TRI Reagent® solution (Thermo Fischer Scientific, MA, USA). Culture media, as well as cell lysates, were stored at − 80 °C until analysis for no more than 3 weeks. Three biological replicates were performed.

2.7. Quantification of pro-inflammatory cytokines

The release of IL-8, IL-6, and TNF-α into the cell medium was measured, using ABTS ELISA Development Kits (Pepro Tech EC, Ltd., London, UK) following the manufacturer instructions. Samples were diluted 1:2 for IL-8 determinations. Three independent experiments were performed, assessing each sample in duplicate.

2.8. Pro-inflammatory gene expression by quantitative PCR

Total RNA from Caco-2 cells was isolated with TRI reagent® solution (ThermoFisher Scientific, MA, USA) according to the manufacturer instructions. The purity and concentrations of isolated RNAs were verified with a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). The cDNAs were synthesized from 1 µg of RNA with the PrimeScript RT Master Mix Kit (Takara Bio, Otsu, Japan). The qPCR was performed with 25 ng of cDNA in a 10 µL reaction mixture, with Perfecta SYBR Green SuperMix (Quantabio, Beverly, MA). Sequence of primer pairs are detailed in Table 1. The quantitative PCRs thermal conditions were at 94 °C during 10 min for initial denaturation, followed by 15 s at 94 °C, and 60 s at 62 °C to complete 40 PCR cycles. Each cDNA sample was amplified in triplicate in a BioRad CFX96 System (BioRad, Hercules, CA, USA). The relative quantification of mRNA level was determined by means of the $2^{-\Delta\Delta Ct}$ method, using the constitutive glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene expression levels.

2.9. COX-1 and COX-2 inhibition assay

The inhibitory capacity of undigested and digested PEEs towards COX-1 and COX-2 was performed using a Human COX Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer protocol. The PEE, GD-PEEs, and ID-PEE from both *Ribes* species were assessed at 250 and 25 µg/mL, while indomethacin was employed as a positive control. Briefly, the extracts were incubated with COX-1 or COX-2 for 10 min, followed by the addition of arachidonic acid. After 2 min, HCl 1 M was added to stop the reaction. Finally, PGE_2 was determined by means of ELISA. The absorbance was measured at 405 nm on a plate reader (Biotek ELx801). The experiments were carried out by duplicate.

Table 1
Primers employed for quantitative PCR analysis.

Primer	Forward	Reverse
iNOS	5′-CGG TGC TGT ATT TCC TTA CGA GGC GAA-3′	5′-GGT GCT GTC TGT TAG GAG GTC AAG TAA-3′
GADPH	5′-GAA GGT GAA GGT CGG AGT-3′	5′-GAA GAT GGT GATGGG ATT TC-3′
COX-2	5′-TCC TTG CTG TTC CCA CCC ATG-3′	5′-CAT CAT CAG ACC AGG CAC CAG-3′

iNOS, inducible NO synthase; GADPH, glyceraldehyde 3-phosphate dehydrogenase; COX-2, cyclooxygenase 2.

2.10. Statistical analysis

The statistical analyses were carried out using the Statistical Package for Social Sciences for Windows software, version 22.0 (SPSS Inc., Chicago, IL, USA) Statistically significant differences between different cell treatments were analyzed using the Student's *t*-test. Statistical significance was established at $p \leq 0.05$. All data were expressed as the mean ± SD.

3. Results and discussion

In the present work, we investigated the effect of digested *Ribes* PEEs on intestinal inflammatory parameters using a Caco-2 cell model. For this purpose, gene expression of the inflammatory enzymes COX-2 and iNOS, the secretion of cytokines and chemokines (TNF-α, IL-6 and IL-8), and the inhibitory effects towards COX-1 and COX-2 enzymes was measured.

3.1. Cytotoxicity of the intestinal digested polyphenol-enriched extract (ID-PEE) from Chilean currants on intestinal Caco-2 cells

The cytotoxicity of the ID-PEEs was assessed to avoid death interference and to establish the concentrations to be used during the subsequent experiments. Cell proliferation was assessed by means of the MTT reduction assay after the incubation with each ID-PEE for 24 h. The extracts were devoid of toxicity towards Caco-2 cells (data not shown). In agreement with our results, the PEEs from *R. magellanicum* and *R. punctatum* were not toxic towards human gastric epithelial cells (AGS) at concentrations up to 500 μg/mL (Jiménez-Aspee, Theoduloz et al., 2016). Taking into account our results, the maximum concentration was set at 500 μg/mL for the subsequent experiments. The employed concentrations correspond to 93.5 and 101.5 μg gallic acid equivalents (GAE)/mL for *R. magellanicum* and *R. punctatum*, respectively (Burgos-Edwards et al., 2017). The concentration used are in agreement with physiologically relevant doses, since after the consumption of a polyphenol rich source, these compounds reach the intestinal level at concentrations of several hundreds of micromole per liter (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004).

3.2. Effect of ID-PEE from Chilean Ribes species on IL-8, IL-6, and TNF-α release

The clone C2BBe1 from Caco-2 cell line was chosen to emulate the human intestinal epithelium due to its capability to develop a more homogeneous brush border than the parental cells (Peterson & Mooseker, 1992). Moreover, they can reproduce the physiopathology of the inflamed intestinal mucosa (Leonard et al., 2010).

Under our experimental conditions, the IL-1β (10 ng/mL) induced the secretion of IL-8, IL-6 and TNF-α by the Caco-2 cell monolayer, similar to the observations of Leonard et al. (2010). Without the IL-1β stimulus, the cells secreted background levels of IL-8, TNF-α, and IL-6 of about 20, 30, and 35 pg/mL, respectively. The treatment for 24 h with IL-1β significantly raised ($p < 0.05$) the concentration of TNF-α and IL-6 by almost three-fold, while IL-8 levels were increased by almost 30 times, compared to the untreated cells (Fig. 1).

The pre-treatment of Caco-2 cells with the ID-PEE from *R. magellanicum* did not significantly reduce the secretion of IL-8 in response to the IL-1β stimulation, compared to the inflamed controls (Fig. 1A). Interestingly, the pre-treatment of Caco-2 cells with the ID-PEE from *R. punctatum* significantly decreased by 32% and 23% the IL-8 levels at 500 and 250 μg/mL, respectively. The observed differences between both species might be related to their polyphenol content and composition. Several flavonoids can inhibit IL-8 over-secretion in IL-1β-stimulated Caco-2 cells (Romier, Van De Walle, During, Larondelle, & Schneider, 2008). In this sense, the total flavonoid content was higher in the ID-PEE of *R. punctatum*, whereas *R. magellanicum* ID-PEE presented a higher anthocyanin content (Burgos-Edwards et al., 2017). In addition, the ID-PEE of *R. punctatum* showed the presence of several dihydroquercetin (taxifolin) derivatives that were not found in the *R. magellanicum* samples (Burgos-Edwards et al., 2017). In murine bone marrow and LPS-stimulated dendritic cells, dihydroquercetin-3-glucoside inhibited the production of cytokines and NO (Kim, Choi, Lee, & Lee, 2008). Moreover, the structurally related flavanone dihydrokaempferol decreased the IL-8 production in a keratinocyte cell line (Venditti et al., 2013). Hence, dihydroquercetin derivatives from *R. punctatum* might contribute to the observed IL-8 reduction in cells treated with the ID-PEE.

It is noteworthy that not all polyphenol extracts show the same inhibitory effects towards the IL-8 secretion in Caco-2 cells. For example, Romier-Crouzet et al. (2009) evaluated polyphenol-enriched extracts from pomegranate, oak, grape seed, sugar cane, cocoa and mangosteen on the over-secretion of IL-8 levels. The authors reported that the best effect was found in pomegranate and oak, a moderate effect was found for sugar cane and grape seed, while cocoa and mangosteen were devoid of activity. In the same intestinal model, the polyphenol-rich extracts of cranberry bean (*Phaseolus vulgaris*) reduced IL-8 levels at all tested doses, reaching complete inhibition at the highest concentration (Chen et al., 2017). A few *in vitro* studies on the effect of berries on the IL-8 secretion at the intestinal level could be found. Most of the studies were assessed at the gastric level using human gastric epithelial adenocarcinoma (AGS) cells. Blackberry (*Rubus fruticosus*), raspberry (*Rubus idaeus*), wild strawberry (*Fragaria vesca*), and strawberry (*Fragaria* × *ananassa*) decreased the high levels of IL-8 in AGS cells (Sangiovanni, Fumagalli, & Dell'Agli, 2017). The pre-treatment of a Caco-2/Raw264.7 model with a *Ribes nigrum* extract, submitted to simulated gastrointestinal digestion, reduced the IL-8 mRNA expression by 54% (Olejnik et al., 2016). In comparison, the ID-PEE of *R. punctatum* showed slightly less inhibition on the secretion of this chemokine, probably due to the lower doses employed in our experiments. Similarly, phenolic compounds from *Aronia melanocarpa* submitted to GID decreased the secretion of IL-8 in a Caco-2/endothelial co-culture model (Wu et al., 2018). Our work is the first report addressing the IL-8 modulation by polyphenols from Chilean currants.

The expression of the IL-8 chemokine is found to be up-regulated in the colonic mucosa of patients with active UC and CD (Sartor, 2006). The rise of IL-8 during inflammation plays an important role in the chemotaxis and activation of immune cells, such as neutrophils, monocytes, and eosinophils. These cells produce free radicals in response to the stimulus, triggering oxidative stress and even cancer if the inflammation persists (Ribeiro, Freitas, Lima, & Fernandes, 2015). In addition, it has been reported that the overexpression of IL-8 increases the rate of colon cancer cell migration and invasion. This chemokine is also involved in angiogenesis, which is associated with tumorigenesis and growth, as well as resistance to chemotherapeutics drugs (Ning et al., 2011). Thus, naturally-occurring compounds from fruits might serve as IL-8 regulators, helping to control active inflammatory-related diseases. In this sense, our results suggest that *R. punctatum* fruits might be a good source of bioactive polyphenols potentially useful to prevent IL-8 over-secretion.

Cytokines are considered as biomarkers of chronic inflammation-related diseases, including IBD. TNF-α and IL-6 are the most well-studied cytokines and constitute drug targets in the treatment of chronic inflammation. These cytokines are over-secreted by inflamed tissues and are found in higher levels in patients suffering this type of illness (Leyva-López et al., 2016).

We observed that the treatments with digested polyphenols from the two Chilean currants were able to significantly decrease the secretion of IL-6 and TNF-α in Caco-2 cells. The levels of TNF-α in medium from cells incubated with ID-PEEs were lower than that of the positive inflammation control (Fig. 1B). The inhibition percentages ranged from 51 to 85% for *R. magellanicum* and 79 to 89% for *R. punctatum* at the different concentrations assayed. Similarly, the IL-6 concentrations

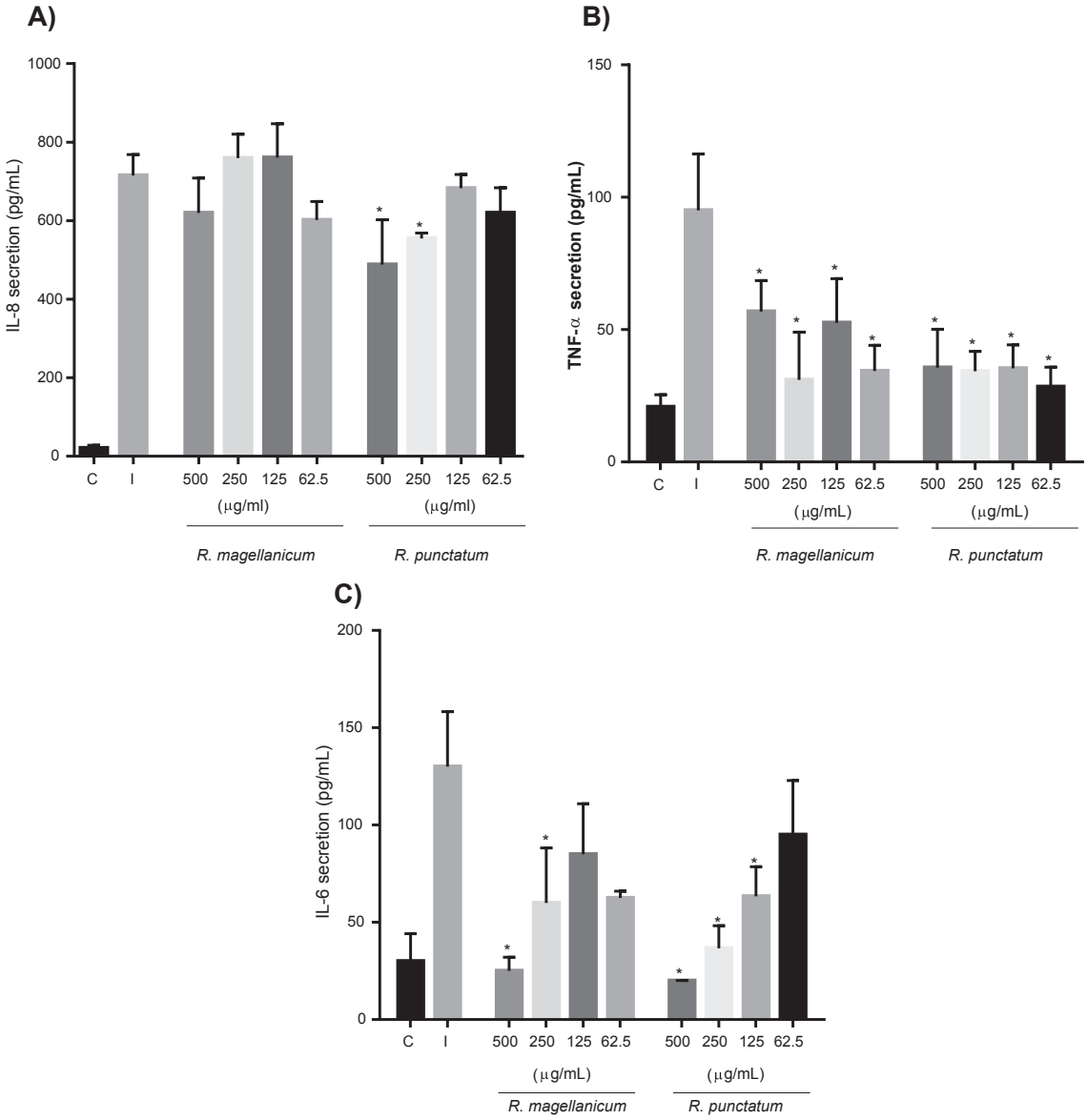

Fig. 1. Effect of intestinal-digested polyphenol-enriched extracts (ID-PEE) from *R. magellanicum* and *R. punctatum* on the secretion of cytokines and chemokines A) IL-8; B) TNF-α; and C) IL-6 in intestinal Caco-2 cells stimulated with interleukin 1β (IL-1β). Letters C and I, means negative and inflammation controls, respectively. The results were expressed as means ± SD (n = 3). The symbol (*) on the bars show significant differences of each treatment compared to the inflammation control (Student's *t* test; $p < 0.05$).

were significantly lower in cells treated with ID-PEE of *R. magellanicum*. At 250 μg/mL, a reduction of 70% was observed, whereas at 500 μg/mL, the IL-6 levels were similar to the negative control (Fig. 1C). In a similar way, the digested extract of *R. punctatum* significantly prevented, in a dose-dependent manner, the production of IL-6 at three of the four tested concentrations. At 500 and 250 μg/mL, the concentration of IL-6 in the cell media was maintained close to the negative control, while at 125 μg/mL, it was reduced by 67% compared to the inflammation control (Fig. 1C). These results are in agreement with those observed for the European relative *R. nigrum* in several inflammation models. Both, the undigested and the *in vitro* gastrointestinal digested extracts of *R. nigrum,* decreased the IL-6 and TNF-α mRNA levels, in an inflamed Caco-2/RAW264.7 co-cultured cell model (Olejnik et al., 2016). Huebbe et al. (2012) observed the same reduction in the mRNA levels of TNF-α employing only RAW264.7 cells, after the pre-incubation with a *R. nigrum* polyphenol-rich juice (Huebbe et al., 2012). Furthermore, the anthocyanin-rich extract from *R. nigrum* fruits reduced the secretion of TNF-α and IL-6 in LPS-stimulated acute monocytic leukemia (THP-1) cells, suggesting that anthocyanins are mainly responsible for this activity (Lyall et al., 2009).

Although both Chilean currants decreased IL-6 and TNF-α secretion, *R. punctatum* was the most active. Thus, as we pointed out above, the regulation of these cytokines might also be associated with the high content and variety of flavonoids occurring in *R. punctatum* (Burgos-Edwards et al., 2017). In agreement with our results, Olejnik et al. (2016) suggested that anthocyanins might have only a partial contribution to the inflammation-preventive effect of *R. nigrum* extracts. Flavonoids in general, and particularly quercetin and its derivatives, have been well characterized as TNF-α and IL-6 suppressors (Ribeiro et al., 2015). In addition, dihydroquercetin-3-glucoside showed inhibition of TNF-α production in bone marrow and splenic dendritic cells (Kim et al., 2008).

The regulation of the pro-inflammatory cytokine TNF-α by Chilean native fruit extracts have been previously reported. In LPS-stimulated RAW264.7 cells pre-treated with maqui and calafate extracts a reduction in the gene expression of TNF-α was observed (Reyes-Farias et al.,

2015). In addition, rats fed with white strawberries (*Fragaria chiloensis*) showed a decreased gene expression of TNF-α, IL-6, and IL-1β in the liver, as well as lower serum concentrations of these cytokines compared to the controls (Molinett et al., 2015).

Several studies propose the implication of the inflammatory mediators TNF-α and IL-6 in the pathogenesis of IBD. During inflammation, the intestinal resident cells release cytokines. Both TNF-α, and IL-6 stimulate the production of adhesion molecules and endothelial activation, as well as binding and recruitment of polymorphonuclear cells and monocytes to the inflammatory focus (Sartor, 2006). The constant infiltration of immune cells and the release of oxidant species generate lesions to the tissue (Ribeiro et al., 2015). TNF-α induces the synthesis of IL-6, other cytokines and eicosanoids. IL-6 mediates the change of the inflammatory status, from acute to a chronic condition. In addition, it has been reported that IL-6 is produced by several types of cancer cells (Leyva-López et al., 2016). Therefore, the selective inhibitors of IL-6 and TNF-α are effective in the control of active IBD (Biasi et al., 2011; Leyva-López et al., 2016). The polyphenols from Chilean currants *R. magellanicum* and especially *R. punctatum* seem to down-regulate cytokine and chemokine secretion in an intestinal cell model of inflammation, at physiologically relevant doses even after simulated intestinal digestion.

3.3. Effect of the digested PEEs on COX-2 and iNOS expression in Caco-2 cells

The enzymes COX-2 and iNOS are over-expressed in inflammatory disorders, and IBDs are not an exception. The characteristic cytokine over-production occurring in active IBDs induces the activation of signaling pathways with transcription of enzymes, including iNOS and COX-2 (Leyva-López et al., 2016). These enzymes are responsible for the synthesis of prostaglandins (PGs) and nitric oxide (NO), respectively. Both are considered to be potent pro-inflammatory mediators with involvement in the pathogenesis of several types of cancer (Surh et al., 2001).

The results of the treatments with ID-PEE from each Chilean currant on the gene expression of COX-2 and iNOS in Caco-2 C2BBe1 cells are shown in Fig. 2. The ID-PEE of *R. magellanicum* decreased by 63% and 88% the mRNA expression of COX-2 at 250 and 500 μg/mL, respectively ($p < 0.05$). Similarly, the iNOS mRNA expression was down-regulated by 43% and 58% at 250 and 500 μg/mL, respectively, compared to the inflammation control (Fig. 2B). The ID-PEE of *R. punctatum* showed higher reductions in the mRNA expressions of the mentioned enzymes compared to the ID-PEE from *R. magellanicum*. The ID-PEE from *R. punctatum* significantly decreased the COX-2 gene expression at all tested concentrations, reaching a complete inhibition at 500 μg/mL. The iNOS mRNA levels were also significantly reduced after the treatments at 500, 250, and 125 μg/mL (Fig. 2B).

The anti-inflammatory activity of *R. nigrum* (black currant) is the most well characterized among *Ribes* species, including the COX-2 and iNOS gene expression. The gastro-intestinally digested extract from *R. nigrum* decreased the mRNA levels of COX-2 and iNOS by 44% and 15%, respectively, in a Caco-2/RAW264.7 co-cultured model (Olejnik et al., 2016). The treatment with black currants also down-regulated the mRNA levels of iNOS in LPS-induced RAW 264.7 murine macrophages (Huebbe et al., 2012). Boivin et al. (2007) observed that *R. nigrum*, *R. sativum* and *R. hirtellum* inhibited the expression of COX-2 by 43%, 49%, and 83%, respectively, in prostatic adenocarcinoma cells (PC-3), while *R. rubrum* did not show any anti-inflammatory activity. In addition, the anthocyanin-rich extract of *R. nigrum* also decreased the up-regulated levels of COX-2 expression at hepatic level in rats (Bishayee et al., 2013). It has been reported that cyanidin-3-glucoside and cyanidin-3-rutinoside down-regulate iNOS and COX-2 gene expression (Jung, Kwak, & Hwang, 2014). Cyanidin-3-glucoside and cyanidin-3-rutinoside are the main anthocyanins occurring in the studied *R. magellanicum* and *R. punctatum* fruits. However, the digested extract

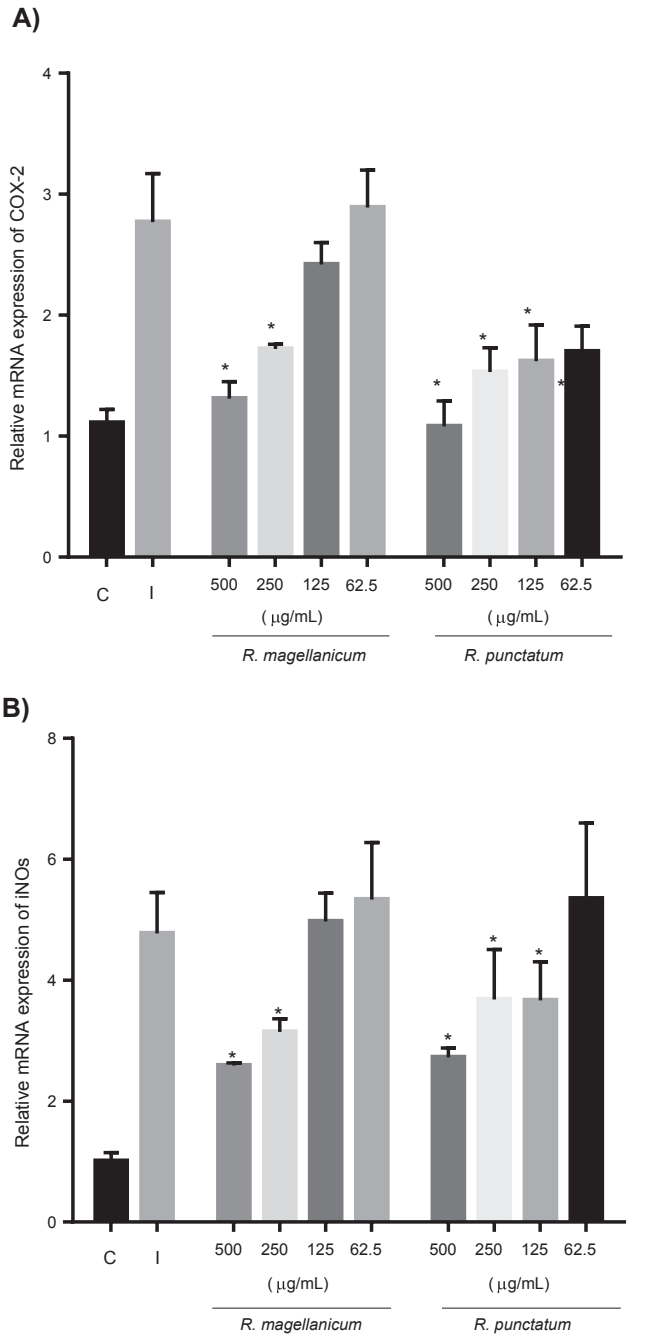

Fig. 2. Effect of the intestinal-digested polyphenol-enriched extracts (ID-PEE) from *R. magellanicum* and *R. punctatum* on the relative mRNA expression of A) COX-2; and B) iNOS in intestinal Caco-2 cells stimulated with interleukin 1β (IL-1β). Letters C and I, means negative and inflammation controls, respectively. The results were expressed as means ± SD (n = 3). The symbol (*) on the bars show significant differences of each treatment compared to the inflammation control (Student's *t* test; $p < 0.05$).

of *R. punctatum* showed more activity than *R. magellanicum*, despite having a slight less anthocyanin content (Burgos-Edwards et al., 2017). This differential activity could be associated to the higher flavonoid content of *R. punctatum*, contributing to the anti-inflammatory effect.

Regarding other Chilean berries, few *in vitro* studies have been focused on the expression of these pro-inflammatory enzymes. It was reported that maqui polyphenolic extracts reduced the expression of COX-2 and iNOS, as well as PGE_2 and NO release in RAW264.7 macrophages (Schreckinger, Wang, Yousef, Lila, & Gonzalez de Mejia, 2010). These results were supported by Céspedes-Acuña et al. (2018),

who observed that different varieties of maqui berry inhibit COX-2 and iNOS gene expression in the same cell line. It has been reported that calafate berry also decreases iNOS gene expression in RAW264.7 cells (Reyes-Farias et al., 2015). However, none of these reports considered the influence of the GID on the content and composition of the polyphenols.

COX-2 is over-expressed in inflamed tissues, where this enzyme is responsible for the synthesis of PGE_2. This eicosanoid enhances metastatic potential of cancer cells by decreasing apoptosis, promoting cell proliferation and angiogenesis (Desai, Prickril, & Rasooly, 2018). As previously mentioned, iNOS is also expressed as a response to inflammation, producing NO. Although low levels of NO have important physiological functions, its over-production may become harmful. NO is involved in the generation of powerful oxidant reactive nitrogen species with cytotoxic effects as well as tumor progression through angiogenesis (Soufli, Toumi, Rafa, & Touil-Boukoffa, 2016; Surh et al., 2001). High levels of nitrite/nitrate have been found in the lumen of the colon of IBD patients and in those within the active phase of UC and CD (Soufli et al., 2016). Therefore, the excessive COX-2 and iNOS expression has been associated with the pathogenesis of IBD and may lead to tumor development. Our results suggest that the polyphenols from the studied Chilean currants, after GID, are able to reduce the expression of these pro-inflammatory genes in an *in vitro* intestinal inflammation cell model.

3.4. Inhibitory activity of Chilean currants digested extracts towards COX-1 and COX-2

Desai et al. (2018) proposed that polyphenols might prevent inflammation and cancer by means of the inhibition of cyclooxygenases (COX). COX-1 is a constitutive enzyme expressed along the gastrointestinal tract (GI); while COX-2 is and inducible enzyme expressed only in inflammatory conditions in the GI (Desai et al., 2018). In the present work, the inhibitory capacity of the non-digested and digested PEEs from *R. magellanicum* and *R. punctatum* towards COX-1 and COX-2 was assessed.

The inhibition percentages, induced by *R. magellanicum* and *R. punctatum* PEEs, towards COX-1 and COX-2 are shown in Table 2. The PEEs from both species inhibited COX-1 in the range of 80–89%, while the inhibition of COX-2 was in the range of 89–99%. After the gastric step, the inhibition of COX-1 was in the range of 78–89%, and for COX-2 64–97%. The PEE obtained after the intestinal digestion inhibited COX-1 by 77–89%, while the inhibition of COX-2 was in the range of 59–99%. These results point out that the inhibitory effect of the polyphenols from *R. magellanicum* and *R. punctatum* withstand the simulated digestion process. In line with the results described above, the PEE of *R. puntatum* was the most active. The inhibitory activity was slightly higher than that reported for a *R. nigrum* extract at 40 μg/mL, which inhibited by 78 and 71% COX-1 and COX-2, respectively (Strugała, Gładkowski, Kucharska, Soko ł-Łętowska, & Gabrielska, 2016). The inhibition towards COX-2 decreased after the simulated digestion in both PEEs at 25 μg/mL, mainly after the intestinal step. The highest reduction was observed for *R. magellanicum*, possibly due to the flavonoid loss resulting from the simulated digestion. The PEE from *R. magellanicum* shows a higher content of anthocyanins than the PEE from *R. punctatum*. These compounds are more sensitive to the pH changes occurring in the digestion, being more affected than other polyphenols (Burgos-Edwards et al., 2017).

The non-steroidal anti-inflammatory drugs (NSAIDs) are COX inhibitors usually prescribed to ameliorate pain and inflammation in patients. Under our experimental conditions, the positive control indomethacin showed IC_{50} values of 0.08 and 0.01 μg/mL, for COX-1 and COX-2, respectively. However, indomethacin is not devoid of adverse effects such as dyspepsia, esophagitis, among others (Leyva-López et al., 2016). The PEEs from both Chilean currants show *in vitro* anti-inflammatory effect, with sustained activity despite the simulated GID process. Our results suggest that polyphenols from *R. magellanicum* and *R. punctatum* might be helpful in ameliorating IBDs. However, further *in vivo* studies are needed to corroborate these effects.

4. Conclusions

The *in vitro* digested polyphenol-enriched extracts from *R. magellanicum* and *R. punctatum* showed anti-inflammatory properties on an intestinal inflammation model (IL-1β-stimulated Caco-2 C2BBe1 cells). The digested extracts decreased the secretion of the cytokines IL-6 and TNF-α and the chemokine IL-8. A difference was observed between both species, in terms of their capacity to inhibit the IL-8 secretion. This might be associated to the high content of total flavonoids in the ID-PEE from *R. punctatum,* in particular with the presence of dihydroquercetin derivatives. The digested extracts from both species downregulated the gene expression of the pro-inflammatory enzymes COX-2 and iNOS. In addition, the non-digested and digested PEEs from both *Ribes* species inhibited human COX-1 and COX-2 *in vitro*. To the best of our knowledge, this is the first report on the anti-inflammatory properties of gastrointestinal digested Chilean currants at physiologically relevant doses. However, further *in vivo* studies are needed to confirm these observations.

5. Ethics statement

The authors did not include any human subjects and animal experiments.

Table 2
Percentage of inhibition of the PEEs from *Ribes magellanicum* and *R. punctatum* towards human COX-1 and COX-2.

Samples	Inhibition of COX-1 (%)		Inhibition of COX-2 (%)	
	250 μg/mL	25 μg/mL	250 μg/mL	25 μg/mL
R. magellanicum				
PEE	80.33 ± 0.45	88.45 ± 0.00	97.90 ± 0.20	89.12 ± 0.35
GD-PEE	80.89 ± 0.00	77.89 ± 2.32	97.14 ± 0.28	64.31 ± 0.58
ID-PEE	80.44 ± 0.95	88.69 ± 1.10	88.63 ± 1.56	59.16 ± 6.71
R. punctatum				
PEE	80.22 ± 3.03	81.21 ± 0.46	98.9.3 ± 0.00	94.22 ± 0.42
GD-PEE	79.12 ± 2.19	88.80 ± 1.27	96.41 ± 0.17	77.37 ± 3.82
ID-PEE	78.90 ± 1.88	76.50 ± 1.52	98.86 ± 1.07	75.33 ± 2.19
Indomethacin*	IC_{50} = 0.08 ± 0.01 μg/mL		IC_{50} = 0.01 ± 0.00 μg/mL	

PEE: phenolic-enriched extract; PEE-GD: gastric digested phenolic-enriched extract; ID-PEE: intestinal digested phenolic-enriched extract; COX-1: cyclooxygenase 1; COX-2: cyclooxygenase 2.
* Reference compound.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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