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The Chilean wild raspberry (*Rubus geoides* Sm.) increases intracellular GSH content and protects against H_2O_2 and methylglyoxal-induced damage in AGS cells

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ABSTRACT

The Chilean raspberry *Rubus geoides* Sm. (Rosaceae) is a native species occurring in the Patagonia. Five *R. geoides* samples were assessed for phenolic content and composition, antioxidant activity, effect on total reduced glutathione (GSH) synthesis and protective effect against H_2O_2 and methylglyoxal (MGO)-induced stress in epithelial gastric AGS cells. The HPLC–DAD/ESI-MS profiles allowed the tentative identification of 39 phenolics including flavonol glycosides and tannins. *R. geoides* presented higher total phenolic and flavonoid content than *Rubus idaeus*. Two out of the five phenolic enriched *R. geoides* extracts (PEEs) exhibited better antioxidant activity than *R. idaeus* in the DPPH, FRAP and TEAC assays. A significant cytoprotective activity was observed when AGS cells were pre-incubated with extracts and subsequently challenged with H_2O_2 or MGO. Treatment with the PEEs increased the intracellular GSH content. *R. geoides* fruit extracts may induce the activation of intracellular protection mechanisms against oxidative and dicarbonyl-induced stress.

1. Introduction

The Chilean wild raspberry *Rubus geoides* Sm. (Rosaceae) is a native plant occurring in humus-rich soils of the western Andean slopes from southern Chile to Patagonia and Tierra del Fuego. Its fruits have a pleasant scent, sweet taste and intense red color (Fig. 1). *R. geoides* fruits have been consumed since pre-hispanic times and are still gathered by the Pehuenche Indians (Rapoport, Ladio, & Sanz, 2003). Cyanidin-3-sambubioside (Ruiz et al., 2013), galloylquinic acid, caffeoyl hexoside, quercetin hexoside-pentoside and quercetin-3-galactoside were reported from *R. geoides* (Ruiz et al., 2015).

Berries are considered to be a source of biologically active compounds and are main ingredients of a healthy diet (Szajdek & Borowska, 2008). The *Rubus* species, including *Rubus spectabilis* and *Rubus chamaemorus*, are relevant as food and medicine for indigenous people from Alaska (Kellogg et al., 2010). These species contain cyanidin-3-glucoside and cyanidin-3-arabinoside, as well as A-type proanthocyanidin polymers (dimers, trimers and tetramers). Proanthocyanidin-enriched fractions of these berries reduced lipid accumulation in adipocytes and glycaemia in mice (Kellogg et al., 2010). *Rubus glaucus* Benth is a South American species occurring in the northern part of the Andes. It was recently included in a review of Andean berries from Ecuador (Carrillo-Perdomo, Aller, Cruz-Quintana, Giampieri, & Alvarez-Suarez, 2015) and according to the authors it is the only native *Rubus* species commercially cultivated in Central and South America.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt; AGS, human gastric epithelial adenocarcinoma cells; CE, cyanidin equivalents; DAD, diode array detection; DPPH, 2,2-diphenyl-1-picryl hydrazyl; ESI, electrospray ionization; FBS, fetal bovine serum; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; GSH, reduced glutathione; HPLC, high performance liquid chromatography; IRh, isorhamnetin; K, kaempferol; MGO, methylglyoxal; MeOH, methanol; MS, mass spectrometry; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenytetrazolium bromide; NAC, *N*-acetyl-L-cysteine; PEE, phenolic enriched extract; Q, quercetin; QE, quercetin equivalents; ROS, reactive oxygen species; TE, trolox equivalent; TEAC, trolox equivalent antioxidant capacity; TF, total flavonoids; TP, total phenolics; TPTZ, 2,4,6-tri(2-pyridyl)1,3,5-triazine; HHDP, hexahydroxydiphenoyl.

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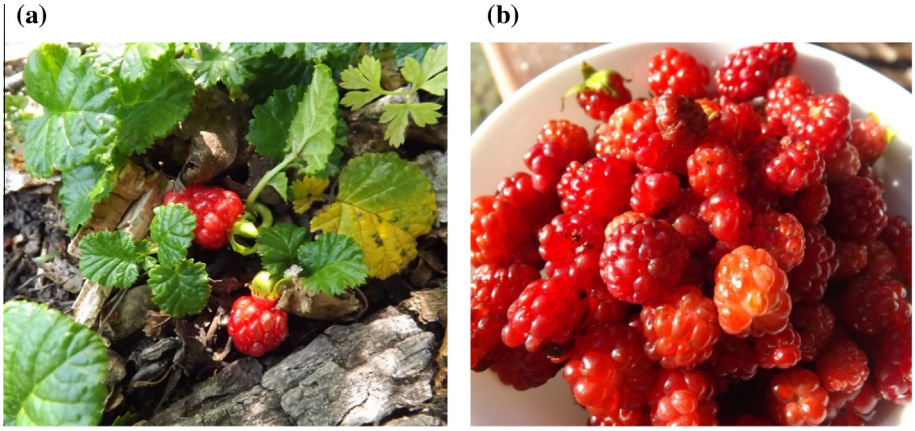


Fig. 1. (a) Wild growing *Rubus geoides* with ripe fruits; (b) ripe fruits collected for consume.

Recent studies on the effects of strawberry consumption add relevant evidence on the potential of berries as health beneficial food. The daily consumption of 0.5 kg fresh strawberries for one month improved the biochemical markers of antioxidant activity, lipid profile, increased plasma antioxidant activity and platelet function in healthy human volunteers (Alvarez-Suarez et al., 2014). Strawberry phenolics block free radical products, modulate gene expression and protect and repair DNA damage (Giampieri et al., 2015). Soft fruits extracts including strawberries, raspberries and blueberries inhibit α-glucosidase and α-amylase, suggesting hypoglycaemic potential (McDougall et al., 2005). In addition, there are several native berries used by the Cree Nation in Canada to treat diabetes (Leduc, Coonishish, Haddad, & Cuerrier, 2006). The hyperglycaemia associated with diabetes is responsible for the generation of endogenous reactive carbonyl species such as glyoxal and methylglyoxal (MGO) (Lo, Hsiao, & Chen, 2011). Methylglyoxal is produced by cells during glycolysis as a side-product of the spontaneous degradation of glyceraldehyde 3-phosphate. It has been shown that plasma from diabetic patients contains higher levels of MGO compared with normal subjects (Kong et al., 2014). The high reactivity of MGO has been associated with deleterious effects such as induction of cellular senescence and post-translational modifications in proteins, like advanced glycation end products. For these reasons, it is interesting to determine the effect of polyphenols from native fruits as a strategy to prevent deleterious effects mediated by MGO. The intracellular content of glutathione (GSH) has been widely used as an indicator of the redox-state in cells. Moreover, GSH is the main intracellular antioxidant and plays a major role as a co-factor in the intracellular detoxification of MGO and reactive oxygen species (ROS). Therefore, the modulation of the levels of total reduced GSH by polyphenols may be considered as an strategy to prevent oxidative damage.

Food analysis comprises the study of bioactivity and functional properties and it is important to count with chemical assays and with cell-based models. The use of cultured cells implies a further step in complexity considering that they are living systems (Laguerre, Decker, Lecomte, & Villeneuve, 2010). Moreover, the need of reliable *in vitro* cellular models as an alternative complementary to animal studies has become an important issue. For antioxidant studies, the use of immortalized cell lines with no altered functional responses to oxidative stress is critical (Cheli & Baldi, 2011). In this context, a human gastric adenocarcinoma cell line (AGS) has been successfully used to evaluate the antioxidant activity of several lentil cultivars (Xu & Chang, 2010).

There is an increasing interest in South American native berries, mainly because of their potential as nutraceuticals (Schreckinger, Lotton, Lila, & Gonzalez de Mejia, 2010). A recent review on the native Chilean berry *Aristotelia chilensis* summarizes the information on the composition and health benefits of this fruit (Fredes & Robert, 2014). However, the information available on South American berries covers only a small part of the existing biodiversity. This scenario encouraged us to investigate the bioactive potential, chemical composition and antioxidant activity of several samples of Chilean *R. geoides* fruits. Following with our studies on South American food plants, we now report the cytoprotective effect of *R. geoides* extracts against H_2O_2 and MGO induced-damage and the effect on intracellular reduced glutathione (GSH) content using AGS cells. Total phenolic, flavonoid and anthocyanin content, antioxidant activity and polyphenol profiling in *R. geoides* is presented and discussed.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents were of analytical grade. Folin–Ciocalteu phenol reagent, TPTZ (2,4,6-tri(2-pyridyl)1,3,5-triazine), KCl, sodium acetate, DPPH (2,2-diphenyl-1-picrylhydrazyl radical), quercetin, gallic acid, $AlCl_3$, L-glutamine, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), rutin, methylglyoxal (MGO), *N*-acetyl-L-cysteine (NAC), Amberlite XAD-7 resin and sodium bicarbonate were purchased from Sigma–Aldrich (St. Louis, MO, USA). ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, sodium carbonate, acetic acid, $FeCl_3{\cdot}6H_2O$, hydrogen peroxide, MeOH and formic acid were purchased from Merck (Darmstadt, Germany). Culture media, antibiotics and fetal bovine serum (FBS) were from Invitrogen Corp. (Grand Island, NY, USA). Ultrapure water was obtained using a Barnstead EasyPure water filter system (Thermo Scientific, Dubuque, IA, USA).

2.2. Plant material and sample preparation

Ripe fruits from *R. geoides* were collected during the summer season at the following locations: (1) Araucania region, Las Raíces (38°25′30″S; 71°32′38″W); (2) Aysen region, Cerro Castillo (45°58′47″S; 71° 58′50″W); (3) Magallanes region, Cerro Negro (52°02′16″S; 71°56′58″W); (4) Magallanes region, San Juan (53°38′55″S; 70°56′58″W) and (5) Magallanes region, Lago Blanco (54°04′08″S; 69°02′29″W) (Fig. 2). The raspberry *Rubus idaeus* purchased at the Talca market was used as comparative control.

Fruits were homogenized in a Waring blender and extracted four times in the dark with MeOH:formic acid (99:1 v/v) in a 1:3

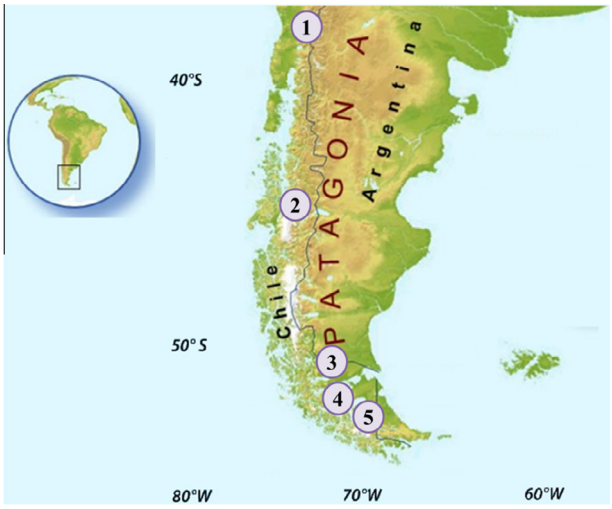


Fig. 2. Map of Chile showing the collection places of *Rubus geoides*. (1) Las Raíces, Araucania region; (2) Cerro Castillo, Aysen region; (3) Cerro Negro, Magallanes region; (4) San Juan, Magallanes region; and (5) Lago Blanco, Magallanes region (source: http://www.elciudadano.cl, with slight modifications).

v/v homogenate:solvent ratio (15 min each) under sonication. The combined extracts were taken to dryness under reduced pressure. An aliquot was taken and freeze dried for analysis and percent extraction yield determination. A pre-conditioned Amberlite XAD-7 resin was used to obtain phenolic-enriched extracts (PEE). The MeOH extract was dissolved in water, filtered and mixed with Amberlite XAD-7 in a ratio of about 1:5 (extract:Amberlite) and stirred for 40 min. The resin was filtered, washed with water and phenolics were desorbed with MeOH. The PEE was evaporated under reduced pressure at 40 °C and freeze-dried. All antioxidant assays (DPPH, FRAP and TEAC) as well as the experiments with cells were carried out using the PEE.

2.3. Total phenolic (TP), total flavonoid (TF) and total anthocyanin (TA) content

The total phenolic content (TP) was determined by the Folin–Ciocalteu method with slight modifications (Simirgiotis, Bórquez, & Schmeda-Hirschmann, 2013). Stock solutions of MeOH extracts (5 mg/mL) were prepared in H_2O. For TP, results are expressed as g gallic acid equivalent (GAE)/100 g MeOH extract. The total flavonoid content (TF) was determined as described with slight modifications (Simirgiotis et al., 2013). The TF is expressed as g quercetin equivalent (QE)/100 g of MeOH extract. Total anthocyanins (TA) were evaluated by the pH differential method (Giusti & Wrolstad, 2001). Methanolic extracts were dissolved in 0.025 M potassium chloride solution (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5). Absorbances of each solution were measured at 510 and 700 nm after 15 min of incubation at room temperature. The content of TA is expressed as mg cyanidin-3-glucoside equivalents (CE)/100 g of MeOH extract.

2.4. DPPH radical scavenging effect

The free radical scavenging activity of the samples was determined by the discoloration of the free radical DPPH (20 mg/L) as previously described (Simirgiotis et al., 2013). Briefly, the PEEs were dissolved in H_2O to a final concentration of 300 μg/mL. Serial dilutions of the stock solutions of each sample were prepared in 96-well plates in order to calculate SC_{50} values. Methanol was used as negative control and quercetin as the reference compound. Absorbance was measured at 517 nm in a universal microplate reader (Biotek Instruments Inc., ELx 800, Winooski, VT, USA). Scavenging of DPPH radical was calculated as percent of discoloration using the following equation: % discoloration = $[1 - (A_{sample} - A_{background})/A_{blank}] \times 100$. The SC_{50} values are expressed as μg/mL.

2.5. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed as previously described (Simirgiotis et al., 2013). Absorbance was read at 593 nm using a Thermo Spectronic Helios Alfa spectrometer (Cambridge, U.K.). Quantification was performed using a standard curve of the antioxidant Trolox. Results are expressed as millimol Trolox equivalents (TE)/g of PEE.

2.6. Trolox equivalent antioxidant activity (TEAC) assay

ABTS radical-scavenging activity was determined according to Nenadis, Wang, Tsimidou, and Zhang (2004). The ABTS· was generated by mixing ABTS solution with potassium persulfate. The solution was stored in the dark for 16 h, and diluted with MeOH to a final absorbance of 0.700 ± 0.050 at 734 nm. Determinations were carried out by mixing ABTS· with fresh standard (1 mM Trolox) or extract (100, 150, 200, 250 and 300 μg/mL). Absorbances were read at 734 nm after 6 min. Results are expressed as μmol/L TE/g of PEE.

2.7. AGS cell culture

Human epithelial gastric cells AGS (ATCC CRL-1739) were grown as monolayers in Ham F-12 medium containing 1 mM L-glutamine and 1.5 g/L sodium bicarbonate, supplemented with 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were grown in a humidified incubator with 5% CO_2 in air at 37 °C. For the subsequent experiments, cells were plated at a density of 2.5×10^4 cells/mL.

2.8. Cytotoxicity assay

Cytotoxicity values (IC_{50}, μg/mL) were required as a reference to determine the working concentrations of the extracts in the experiments described below. Confluent cultures of AGS cells were treated during 24 h with medium containing the PEEs at concentrations ranging from 0 up to 1000 μg/mL. The samples were dissolved in medium supplemented with 2% FBS. Cells treated with medium only were used as controls. Cell viability was determined at the end of the incubation by means of the MTT reduction assay (Cheli & Baldi, 2011). Each concentration was tested in quadruplicate and experiments were repeated 2 times using different cell preparations.

2.9. Cytoprotection against H_2O_2 or MGO stress

Confluent cultures of AGS cells were treated overnight with different concentrations of PEEs (0, 62.5, 125, 250 and 500 μg/mL). Cells treated with quercetin (100 μg/mL) were used as positive controls (Li, Zheng, Sang, & Lv, 2014). Samples were dissolved in medium supplemented with 2% FBS and antibiotics. At the end of the incubation, culture medium was completely removed via vacuum aspiration. Then, the stress was induced with 13 mM H_2O_2 or 15 mM MGO during 2 h. The H_2O_2 and MGO solutions were freshly prepared using medium only. Untreated cells were used as 100% viability controls. Cells treated with H_2O_2 or MGO served as damage controls. Cell viability was determined by means of the MTT reduction assay (Cheli & Baldi, 2011). Each concentration was

tested in quintuplicate and experiments were repeated 2 times using different cell preparations. Results are expressed as percentage of the 100% viability control.

2.10. Cellular reduced glutathione (GSH) content

One day after confluence, AGS cells were incubated overnight with different concentrations of PEEs (0, 125, 250 and 500 μg/mL). Samples were dissolved in medium supplemented with 2% FBS and antibiotics. At the end of the incubation, culture medium was completely removed via vacuum aspiration and replaced with culture medium only. Cells were removed with a cell scraper, re-suspended with cold MES buffer (50 mM, pH 6–7) and lysed by sonication. Test tubes containing the cell preparations were kept on ice. The GSH content was determined using a colorimetric kit (BioAssays Systems, Hayward, CA, USA). The GSH synthesis stimulant *N*-acetyl-L-cysteine (750 μM) was included as positive control. Each concentration was tested in triplicate and experiments were repeated 2 times using different cell preparations. Results are expressed as nmol of soluble reduced sulfhydryls/10^6 cells.

2.11. HPLC–DAD analysis

The HPLC–DAD analysis was performed using a Shimadzu system (Shimadzu Corporation, Kyoto, Japan) consisting of a LC-20AT pump, a SPD-M20A UV diode array detector, CTO-20AC column oven and a LabSolution software. A MultoHigh 100 RP 18–5 μ (250 × 4.6 mm) column (CS-Chromatographie Service GmbH, Langerwehe, Germany) maintained at 25 °C was used. The HPLC analyses were performed using a linear gradient solvent system consisting of 1% formic acid in water (A) and acetonitrile (B) as follows: 90% A to 75% A over 30 min, followed by 75% A to 40% A from 30 to 55 min, and maintained in 40% A from 55 to 75 min, finalizing with 90% A for 5 min. The flow rate was 1 mL/min and the volume injected was 20 μL. The compounds were monitored at 254, 330 and 510 nm and UV spectra from 200 to 600 nm were recorded for peak characterization. The main flavonoids were quantified using a calibration curve prepared with rutin as standard ($r > 0.999$) considering the areas of the peaks at 254 nm. All determinations were carried out in duplicate in two separate experiments and results are expressed as mean values ± SD.

2.12. HPLC–DAD/ESI-MS^n characterization of extracts

Mass spectra were recorded using an HPLC HP1100 (Agilent Technologies Inc., Santa Clara, CA, USA) connected through a split to an Esquire 4000 Ion Trap LC/MS System (Bruker Daltonik, Bremen, Germany). Full scan mass spectra were measured between m/z 150 and 2000 u in positive and negative ion mode. Nitrogen was used as nebulizer gas at 27.5 psi, 350 °C and at a flow rate of 8 L/min. The mass spectrometric conditions were: electrospray needle, 4000 V; end plate offset, −500 V; skimmer 1: 56.0 V; skimmer 2: 6.0 V; capillary exit offset, 84. V; and capillary exit, 140.6 V. Collision induced dissociation spectra were obtained with a fragmentation amplitude of 1.00 V (MS/MS) using helium as the collision gas. Mass spectrometry data were acquired in the negative mode for all phenolic compounds except anthocyanins, which were acquired in the positive ion mode. The compounds were tentatively identified comparing the Rt, UV and MS fragmentations patterns with literature.

2.13. Statistical analyses

Determinations of TP, TF, TA, DPPH, FRAP and TEAC were performed in triplicate and results are expressed as mean values ± SD. For the TEAC assay, a curve was plotted for each sample and a correlation coefficient (r) with 95% confidence limit was established. Statistical differences between different treatments and their respective control were determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The level of significance was set at $p < 0.05$. All statistical analyses were carried out using the software SPSS 14.0 for Windows (IBM, Armonk, NY, USA).

3. Results

3.1. General analysis

Larger *R. geoides* fruits were found in the Araucania ($n = 392$; 0.77 ± 0.33 g/fruit) and Aysen regions ($n = 222$; 0.67 ± 0.28 g/fruit) and smaller fruits were found in the Magallanes region ($n = 21$; 0.40 ± 0.25 g/fruit). The commercial raspberry used for comparative purposes, had an average weight of 2.04 ± 0.44 g/fruit. The moisture content in *R. geoides* fruits ranged between 65.58% and 82.58%. The percent of MeOH extraction yields was between 4.18% and 8.34%. The highest XAD-7 yield was found in the Cerro Negro, Magallanes region sample (2.58%). Results are summarized in Table 1.

3.2. Total phenolic (TP), flavonoid (TF) and total anthocyanin (TA) content

The TP content of the ripe fruits ranged between 2.89 and 3.78 GAE/100 g MeOH extract. The TF values were in the range of 0.11 to 0.25 QE/100 g MeOH extract (Table 2). Overall, the TP and TF content of *R. geoides* were higher compared with *R. idaeus*. However, when considering TA, the commercial *R. idaeus* had a higher content (290.56 mg CE/100 g MeOH extract), compared with the 48.31–142.61 mg CE/100 g MeOH extract for *R. geoides*.

Table 1
Mean *Rubus geoides* fruit weight, moisture and yields of extractions on the basis of fresh fruit weight.

Sample	Mean weight (g)	Moisture (%)	MeOH extraction (%)	XAD-7 retention (%)
Araucania region				
Las Raíces	0.77 ± 0.33	82.05	4.18	0.19
Aysen region				
Cerro Castillo	0.67 ± 0.28	82.58	8.34	1.23
Magallanes region				
Lago Blanco	0.43 ± 0.18	74.72	5.43	0.95
San Juan	0.34 ± 0.20	[a]	6.70	[a]
Cerro Negro	0.46 ± 0.31	65.58	8.00	2.58
Rubus idaeus (Talca market)	2.04 ± 0.44	84.27	8.21	0.16

[a] Not determined.

Table 2
Total phenolic (TP), flavonoid (TF) and anthocyanin (TA) content in methanolic extracts of *Rubus geoides* fruits.

Sample	TP (g GAE/100 g MeOH extract)	TF (g QE/100 g MeOH extract)	TA (mg CE/100 g MeOH extract)
Araucania region			
Las Raíces	3.78 ± 0.11	0.25 ± 0.01	85.28 ± 6.46
Aysen region			
Cerro Castillo	2.89 ± 0.09	0.16 ± 0.00	48.31 ± 4.36
Magallanes region			
Lago Blanco	3.61 ± 0.07	0.12 ± 0.04	142.61 ± 13.16
San Juan	3.54 ± 0.05	0.25 ± 0.00	130.58 ± 6.59
Cerro Negro	3.54 ± 0.06	0.11 ± 0.02	104.98 ± 8.54
Rubus idaeus (Talca market)	1.50 ± 0.03	0.03 ± 0.00	290.56 ± 9.84

3.3. Antioxidant activity

In the DPPH assay, the PEEs of the most active samples showed SC_{50} values of 7.20 ± 0.61 and 9.10 ± 0.97 μg/mL for the Magallanes (San Juan) and Araucania, respectively.

The most active sample in the FRAP assay was from Araucania (2591.85 ± 99.99 μmol TE/g PEE), followed by San Juan, Magallanes, (2357.85 ± 160.16 μmol TE/g PEE). Both samples were about two fold more active than *R. idaeus* (1235.38 ± 74.97 μmol TE/g PEE). The highest TEAC value was found for the Araucania sample, with 2416.57 ± 17.65 μM TE/g PEE, followed closely by San Juan, with 2324.75 ± 15.79 μM TE/g PEE. Like the results in the FRAP assay, *R. geoides* presented higher antioxidant activity than *R. idaeus* (1149.66 ± 16.14 μM TE/g PEE). Results are summarized in Table 3.

3.4. Cytotoxicity

The *Rubus* PEEs were devoid of toxicity towards AGS cells, with IC_{50} values >1000 μg/mL, except the samples from Araucania (898.1 ± 2.6 μg/mL) and San Juan (811.0 ± 1.4 μg/mL). A concentration of 500 μg/mL has been described to be close to the normal consumption and absorption of phenolics from berries (Schantz, Mohn, Baum, & Richling, 2010). Therefore, a maximum concentration of 500 μg/mL was selected for the subsequent experiments.

3.5. Cytoprotection against H_2O_2 or MGO stress

AGS cells were exposed to H_2O_2 or MGO to induce stress in separate experiments. Hydrogen peroxide (13 mM) and MGO (15 mM) significantly diminished cell viability to 42.0 ± 3.3% and 62.8 ± 2.3%, respectively, compared to untreated cells. Quercetin (100 μg/mL) was used as the positive control. Interestingly, under our experimental conditions quercetin did not protect cells from oxidative damage induced by H_2O_2 (46.6 ± 2.7%, $p > 0.05$) but protected cells significantly against the injury caused by MGO (83.7 ± 1.7%, $p < 0.05$). *Rubus* PEEs significantly protected cells in a dose-dependent manner. All samples at 500, 250 and 125 μg/mL protected AGS cells from H_2O_2 oxidative stress. The best activity was found for the Lago Blanco sample at 500 μg/mL (63.6 ± 1.8%, $p < 0.05$) (Fig. 3). The *R. idaeus* extract presented significant activity only at 500 and 250 μg/mL.

When MGO was used as the stressor agent, only the samples from San Juan (67.8 ± 1.3%) and Lago Blanco (64.8 ± 1.6%) showed a significant cytoprotective effect at 500 μg/mL ($p < 0.05$) (Fig. 4). At lower concentrations (250, 125 and 62.5 μg/mL) only the sample from Las Raíces showed significant protection against MGO.

In co-incubation experiments, no significant protection against both stresses was observed. In this experimental model, quercetin (100 μg/mL) effectively protected AGS cells against H_2O_2 (81.4 ± 2.7%, $p < 0.05$) and MGO (84.8 ± 2.1%, $p < 0.05$) (data not shown).

3.6. Determination of cellular reduced GSH content

In general, *Rubus* PEEs induced an increase in the intracellular GSH content. Results are expressed as percent compared to untreated controls. At the highest concentration (500 μg/mL) a significant increase in GSH levels was found for the samples from San Juan (75.90%), Lago Blanco (48.63%), Araucania (23.86%) and *R. idaeus* (39.90%) (Fig. 5). A dose–response effect was only observed in the samples from Las Raíces and San Juan. The reference compound NAC, a known stimulant of GSH synthesis, increased intracellular GSH content by 34.71% ($p < 0.05$).

3.7. HPLC–DAD comparison of the PEEs

The phenolic constituent profiles from the four main *R. geoides* collections were compared by HPLC–DAD at 254 nm. The *R. idaeus* was included as a reference. The PEEs showed patterns with three main groups of constituents, namely two anthocyanins (A and B), four tannins (C–F) and four flavonoids (G–K) (Fig. 6). The

Table 3
Antioxidant activity of PEEs from *Rubus geoides* fruits.

Samples	DPPH SC_{50} (μg/mL)	FRAP (μmol TE/g PEE)	TEAC (μM TE/g PEE)
Araucania region			
Las Raíces	9.10 ± 0.97	2591.85 ± 99.99	2416.57 ± 17.65
Aysen region			
Cerro Castillo	46.71 ± 3.28	881.91 ± 48.53	904.54 ± 5.96
Magallanes region			
Lago Blanco	36.51 ± 2.10	1037.11 ± 5.30	939.39 ± 5.15
San Juan	7.20 ± 0.61	2357.85 ± 160.16	2324.75 ± 15.79
Cerro Negro	64.75 ± 1.32	467.89 ± 15.20	282.93 ± 2.02
Rubus idaeus (Talca market)	30.99 ± 2.55	1235.38 ± 74.97	1149.66 ± 16.14

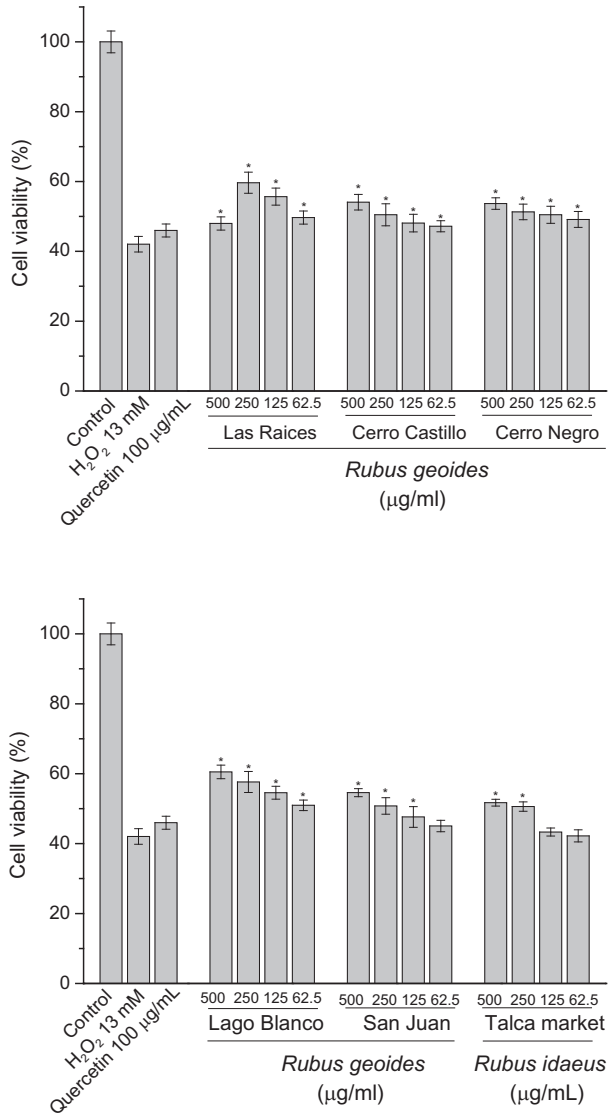


Fig. 3. Effect on the viability of AGS cells pre-treated with *Rubus geoides* and *Rubus idaeus* PEEs and subsequently challenged with H_2O_2. The cell viability was determined by MTT reduction assay. Results are expressed as means ± SD ($n = 5$). $^{*}p < 0.05$ compared to H_2O_2 controls.

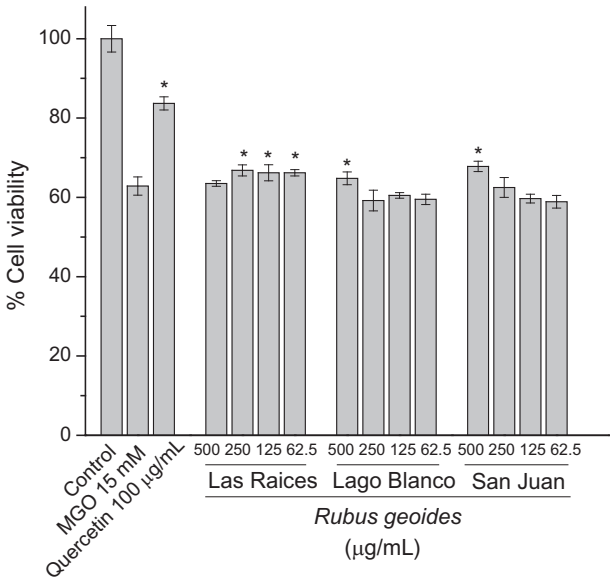


Fig. 4. Effect on the viability of AGS cells pre-treated with *Rubus geoides* PEEs and subsequently challenged with MGO. The cell viability was determined by MTT reduction assay. Results are expressed as means ± SD ($n = 5$). $^{*}p < 0.05$ compared to MGO controls.

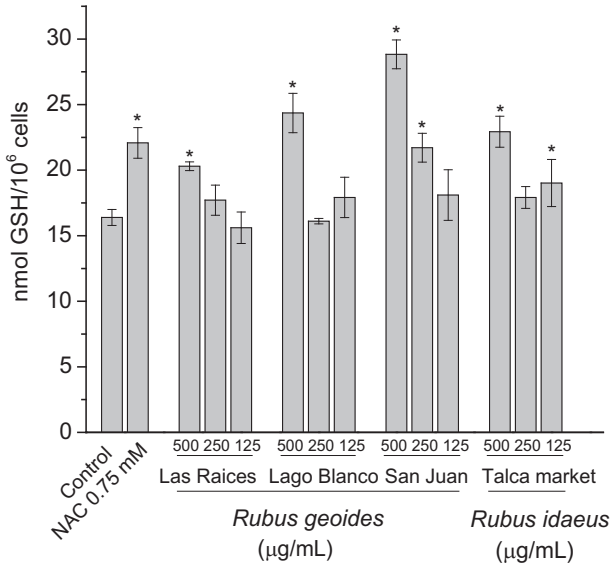


Fig. 5. Effect of *Rubus geoides* and *Rubus idaeus* PEEs on GSH content in AGS cells. $^{*}p < 0.05$ compared to untreated controls.

anthocyanins A and B eluting at Rt 14.15–16.47 min are more abundant in *R. idaeus*, where two major peaks easily differentiate the commercial raspberry from *R. geoides*. The second signal set (C–F) is centered between 18.41 and 23.84 min and present UV spectra compatible with tannins, with common compounds in both species. The flavonoid glycoside (G) elutes at Rt 25.58 min and occurs in all *R. geoides* accessions but not in *R. idaeus*. Four additional minor constituents (H–K) shows UV spectra compatible with flavonol glycosides. The main flavonoid (G) is Q-hexoside pentoside (compound **22**, Tables 4 and 5) while the minor compounds H, I and J were identified as Q hexoside **22a**, isorhamnetin glucuronide **23** and Q hexoside **25**, respectively (Tables 4 and 5).

In the antioxidant assays one the most active samples was that from San Juan. This sample showed the best effect increasing intracellular GSH content (at 500 and 250 µg/mL), a significant cytoprotective effect in the challenge with MGO (at 500 µg/mL) and was effective to protect AGS cells against H_2O_2-induced damage in a dose-dependent manner. The HPLC pattern shows that the flavonoid **22** content (Q-hexoside pentoside) differentiates this accession from other *R. geoides* samples and suggests a relationship among the flavonoid identity, content and biological activity (Fig. 6). The *R. idaeus* PEE was active only in the H_2O_2 and GSH assay. Quantification of the main flavonoid by HPLC using a calibration curve of rutin shows that the concentration of compound **22** in *R. geoides* ranged between 0.49 and 1.09 g rutin equivalent/100 g PEE (Table 4).

3.8. Identification of phenolic compounds

Phenolics in the PEEs were tentative identification by HPLC–DAD-MS/MS. A representative HPLC–MS chromatogram is presented in Fig. 7. The results from HPLC–MS analyses are summarized in Table 5 and the structures of some phenolics tentatively identified are shown in Fig. 8.

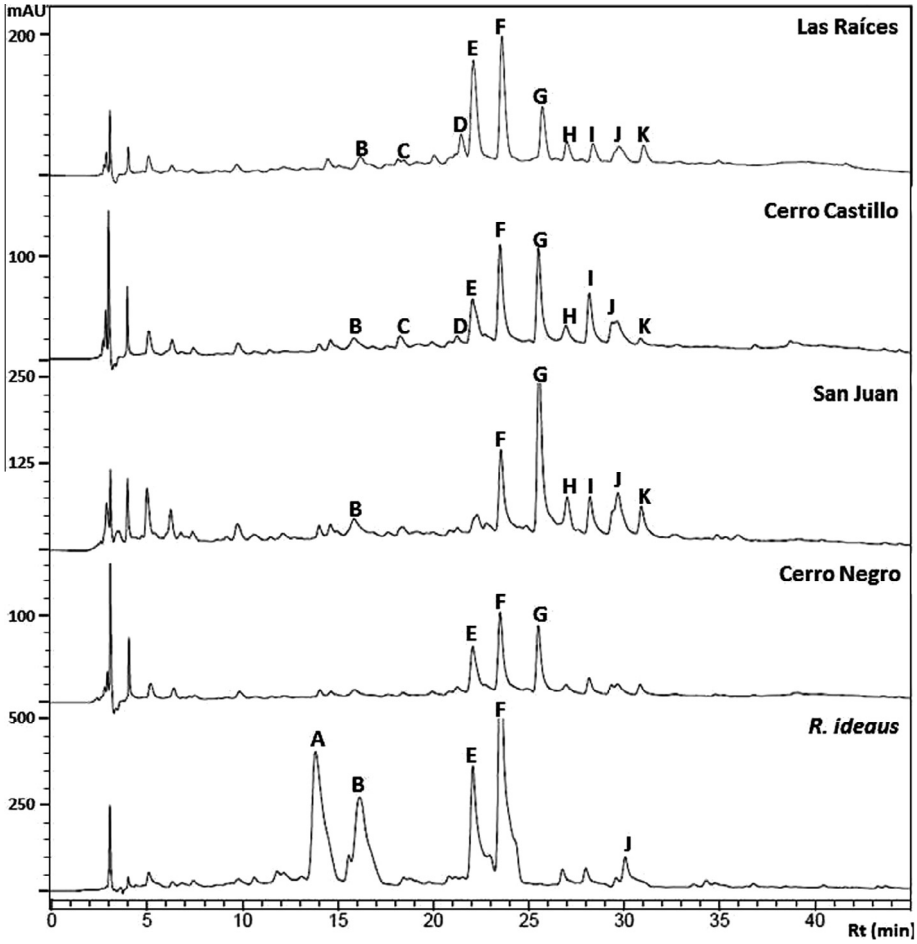


Fig. 6. Comparative HPLC chromatograms at 254 nm of wild growing *Rubus geoides* (Las Raíces, Cerro Castillo, San Juan and Cerro Negro) collections and commercial *R. idaeus* fruits. Compounds (A and B) anthocyanidins; (A) cyanidin derivative (Rt 14.15 min, UV/Vis. max: 517, 280 nm); (B) cyanidin hexoside pentoside 2 (Rt: 16.47 min, UV/Vis max: 516, 280 nm). (C–F) tannins; (C) (Rt: 18.41 min, UV max: 279, 239 nm); (D) (Rt: 21.32 min, UV max 280sh, 244 nm); (E) E-5 HHDP galloyl glucose derivative 16 (Rt: 22.48 min, UV max: 285sh, 240 nm); (F) E-7 Galloyl-bis-HHDP-glucose 19 (Rt: 23.84 min; UV max: 280sh, 239 nm). (G–K) flavonol glycosides; (G) Q hexoside pentoside 22 (Rt: 25.58 min, UV max: 354, 266sh, 255 nm); (H) Q hexoside 22a (Rt: 27.13 min, UV max: 352, 266sh, 236 nm); (I) IRh glucuronide 23 (Rt: 28.30 min, UV max: 364, 285sh, 236 nm); (J) Q hexoside 25 (Rt: 30.24 min, UV max: 353, 266sh, 236 nm); (K) K hexoside 26 (Rt: 31.01 min, UV max: 350, 280sh, 266sh nm). The compound numbers are referred to Table 5.

Table 4
Quantification of the main flavonoid glycosides **22**, **22**a, **23** and **25** [a]occurring in Amberlite XAD-7-retained methanolic extracts of *Rubus geoides* expressed as g rutin equivalent/100 g phenolic-enriched extract.

Compound	Rubus geoides			
	Las Raíces	Cerro Castillo	San Juan	Cerro Negro
Q hexoside pentoside **22**	0.49 ± 0.03	0.62 ± 0.00	1.09 ± 0.01	0.51 ± 0.01
Q hexoside **22**a	0.18 ± 0.00	0.10 ± 0.00	0.11 ± 0.01	0.06 ± 0.00
Isorhamnetin glucuronide **23**	0.17 ± 0.00	0.29 ± 0.00	0.29 ± 0.00	0.12 ± 0.01
Q hexoside **25**	0.26 ± 0.01	0.28 ± 0.00	0.48 ± 0.01	0.16 ± 0.00

All determinations were carried out in duplicate in two separate experiments and results are expressed as mean values ± SD.
[a] Compound numbers referred to Table 5.

3.8.1. Anthocyanins

MS/MS experiments of compounds **1**–**5** lead to a *m*/*z* ion at 287 u, indicating the presence of cyanidin glycosides. While the MS/MS spectra of compounds **3** and **6** show the loss of 162 u (hexose), **2** and **4** presented losses of a pentose (132 u) and a hexose (162 u) while **1** losses two hexoses units, leading to cyanidin. Compound **1** was assigned as cyanidin dihexoside, **2** and **4** as cyanidin hexoside pentoside, **3** and **5** as cyanidin hexosides, respectively. The compounds, with different Rt might differ in the identity of the sugars and/or the placement of the sugars in the molecules. Compound **2** was the main anthocyanin in the fruit.

3.8.2. Flavonoids

The UV spectra of the main flavonoids show maxima of 354 nm for compound **22** and 352 nm for **25**, respectively. MS/MS experiments led to a base peak at *m*/*z* 301, in agreement with quercetin

Table 5
HPLC–MS analysis of *Rubus geoides* fruits PEEs.

Compound	Rt (min)	MS	MS/MS	Tentative identification
1	13.5	611	287	Cyanidin-3-dihexoside
2	14.9–15.3	581	287 (100)	Cyanidin 3-hexoside pentoside
3	15.4	449	287 (100)	Cyanidin 3-hexoside
4	15.7–15.9	581	287 (100)	Cyanidin-3-hexoside pentoside
5	15.8	449	287 (100)	Cyanidin-3-hexoside
6	15.5	579	447, 285	K-pentoside hexoside
7	18.7	289		Catechin/epicatechin
8	19.0–20.4	561	289 (100)	(epi)Afzelechin/(epi)Catechin
9	19.7	289	245 (100), 205 (35)	Catechin
10	20.9–21.1	477	301 (100)	Q-glucuronide
11	21.2	935	765 (32), 633 (100), 301 (91)	E-1 Potentillin/casuarictin isomer Galloyl-bis-HHDP-glucose
12	21.4	915 (28)	765 (51), 633 (100), 301 (91)	E-2 HHDP galloyl glucose derivative
13	22.1	1250 (82)	934, 633 (51), 301 (100)	E-3 Lambertianin C isomer without ellagic acid
14	22.3	1261 (100)	1110 (29), 905 (27), 633 (26), 301 (77)	E-4 HHDP galloyl glucose derivative
15	22.5	625	505 (16), 463 (30), 445 (33), 301 (100)	Q dihexoside
16	22.6	1912	1272 (54), 1121.7 (16), 948, 633 (33), 301 (100)	E-5 HHDP galloyl glucose derivative
17	22.7	477	315 (100)	Isorhamnetin hexoside
18	23.3	1251 (29)	935, 633 (100), 301 (74)	E-6 Lambertianin C related isomer without ellagic acid
19	23.4–23.6	935	783 (26), 633 (83), 301 (100)	E-7 Galloyl-bis-HHDP-glucose
20	24.2	935 (10)	633 (83), 617 (100), 467 (86), 301 (71)	E-8 Galloyl-bis-HHDP-glucose
21	24.2, 27.2	935 (2)	633 (100), 463 (5), 301 (53)	E-9 Galloyl-bis-HHDP-glucose
22	25.6	595	473 (16), 301 (100)	Q hexoside pentoside
22a	26.7	473	301 (100)	Q hexoside
23	29.0	491	315 (100)	Isorhamnetin glucuronide
24	29.0	1249 (38)	933, 924 (69), 783 (10), 633 (100), 301 (86)	E-10 Lambertianin C isomer without ellagic acid
25	29.1	463	301 (100)	Q-hexoside
26	29.4	447	285 (100)	K-hexoside
27	29.6	579	447 (21), 285 (100)	K-pentoside hexoside
28	29.8–30.0	863	573 (100)	Procyanidin trimer
29	29.8	301	257 (26)	Ellagic acid
30	30.0	863	573 (100)	Procyanidin trimer
31	31.8	577	289 (100)	(epi)Catechin-(epi)catechin Procyanidin B
32	32.8	505	463 (83), 301 (100)	Q hexoside acetate
33	33.5	863	573	Procyanidin trimer
34	34.3	631	341 (100), 289 (10)	Procyanidin derivative
35	36.4	1249 (13)	924 (100), 633 (84), 301 (46)	E-11 Lambertianin C isomer without ellagic acid
36	36.7	933	633, 463, 301	Castalagin/vescalagin
37	36.8	1247 (17)	953 (58), 944.6, 655 (100), 301 (88)	E-12 Ellagitannin
38	36.9	1250 (5)	933, 924 (100), 633 (12), 301 (19)	E-13 Lambertianin C isomer without ellagic acid
39	43.4	301	178 (100), 150 (90)	Q

(Q). Both compounds differ in the number and identity of the sugars. Compound **22** losses a hexose and a pentose, while one hexose is lost in **25**. The compounds were identified as Q-hexoside pentoside and Q-hexoside, respectively. Compounds **10**, **15** and **32** showed the loss of a glucuronide, two hexoses, a hexose and an acetate unit, respectively, leading to quercetin (*m*/*z* 301). The compounds were assigned as Q-glucuronide **10**, Q-dihexoside **15** and Q-hexoside acetate **32**.

The MS spectrum of compound **23** showed the loss of glucuronic acid, leading to an aglycone with a *m*/*z* of 315 and MS^2 in agreement with isorhamnetin (IRh) and was assigned as IRh glucuronide. The compound **17** with a $[M-H]^-$ of *m*/*z* 477, loses a hexose (162 u), leading to the *m*/*z* ion at 315, being identified as IRh hexoside. The glycosides **6**, **26** and **27** showed a base peak at *m*/*z* 285 u, in agreement with kaempferol (K) and differ in the number and identity of the sugars. The diglycosides **6** and **27** consecutively losses a pentose and a hexose while **26** was identified as K-hexoside.

3.8.3. Proanthocyanidins

The $[M-H]^-$ ion at *m*/*z* 289 and MS/MS fragmentation of the compounds **7** and **9** are in agreement with catechin/epicatechin. The MS of compounds **28**, **30** and **33** with $[M-H]^-$ at *m*/*z* 863 are in agreement with a procyanidin trimer. Compound **31** shows a $[M-H]^-$ ion at *m*/*z* 577 and MS/MS fragmentation of 289. This can be associated with (epi)catechin-(epi)catechin dimer, compatible with procyanidin B or procyanidin C. The MS of compound **34** shows the loss of 290 u (catechin or epicatechin) and a base peak at *m*/*z* 341 which losses 52 u leading to 289 u (epicatechin/catechin). The compound was assigned as a procyanidin derivative.

3.8.4. Ellagitannins

Ellagic acid (compound **29**) was identified in *R. geoides* by its *m*/*z* ion at 301 and MS^2 at *m*/*z* 257. In ellagitannins, the neutral loss of 302 u pointed up to a hexahydroxydiphenoyl (HHDP) group and a 170 u loss to a gallate unit. The compounds **11**, **19**, **20** and **21**, with a $[M-H]^-$ ion at *m*/*z* 935, showed consecutive losses of 302, 170 and 162 u, indicating the presence of ellagitannins bearing two HHDP units and a gallate unit linked to the hexose core (galloyl-bis-HHDP-glucose). The compounds were tentatively assigned as casuarictin/potentillin isomers. The compounds **13**, **18**, **24**, **35** and **38** showed a $[M-H]^-$ ion at *m*/*z* 1249.0, 1249.5, 1250.0 and 1250.7 u, respectively. Compounds were tentatively identified as lambertianin C derivatives. The mass spectrum of compound **36** with a $[M-H]^-$ ion at *m*/*z* 933 shows the consecutive loss of ellagic acid, gallic acid and a hexose, leading to ellagic acid (*m*/*z* 301). The structure of the tannin is in agreement with a galloyl-bis-HHDP hexoside with a molecular formula of $C_{41}H_{26}O_{26}$. The compounds **12**, **14** and **16**, with $[M-H]^-$ ion at *m*/*z* 915, 1261 and 1912 u, respectively, show a common core of HHDP galloyl glucose (*m*/*z* 633 and 301) and differ in the identity of the additional substituents. The compounds were assigned as HHDP-galloyl glucose derivatives.

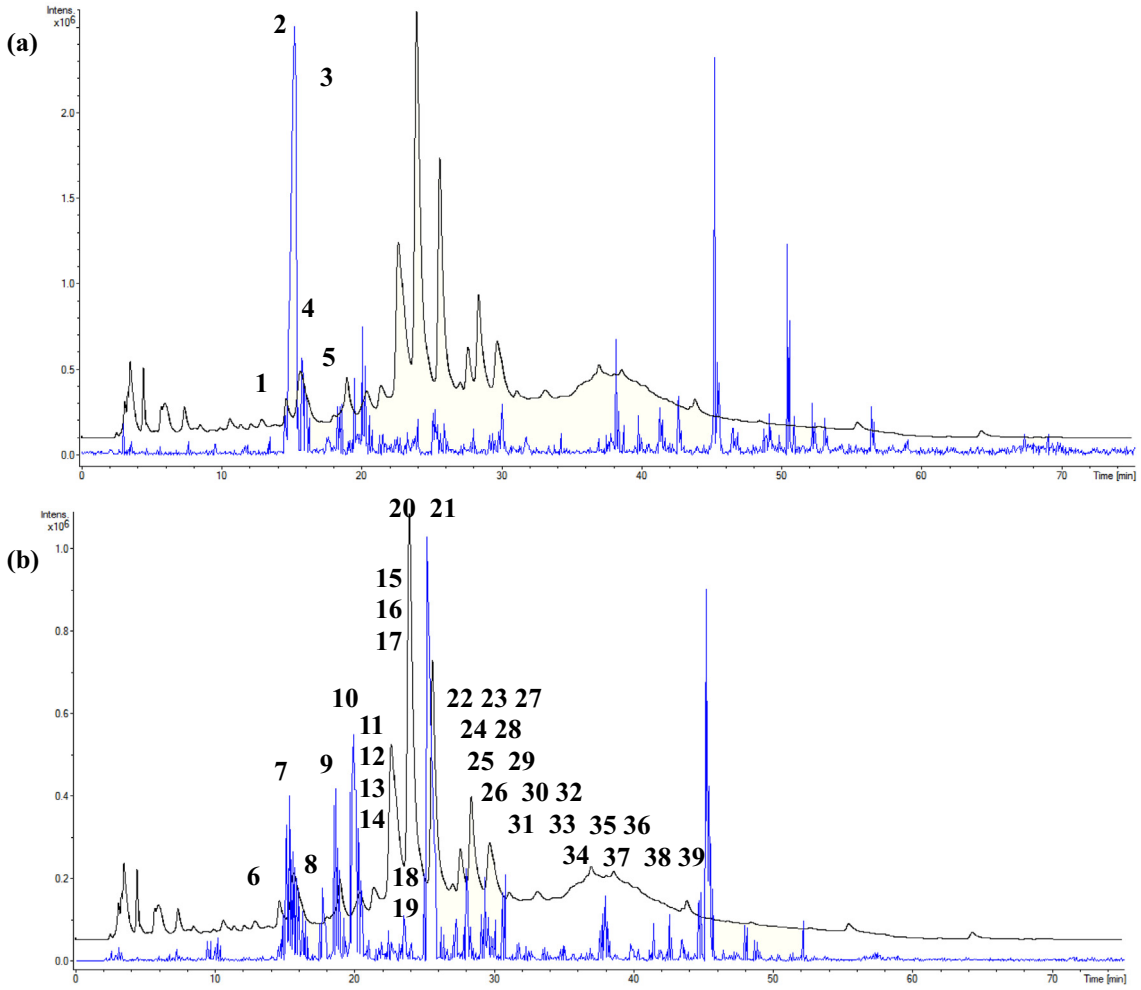


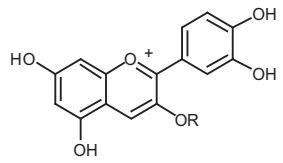
Fig. 7. HPLC–DAD chromatogram (black trail) and TIC (blue trail) of the *R. geoides* PEE from San Juan, Magallanes region. (a) Positive ion mode; (b) negative ion mode. Detection: UV, 254 nm.

4. Discussion

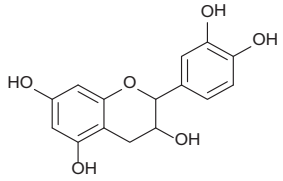
Raspberries are worldwide consumed due to their good taste and health benefits (Rao & Snyder, 2010; Szajdek & Borowska, 2008). Several *Rubus* species have been investigated for phenolic composition and antioxidant activity, including the red raspberry (*R. idaeus*) (Rao & Snyder, 2010) and wild species such as *Rubus arcticus*, (Maatta-Riihinen, Kamal-Eldin, & Torronen, 2004), *R. chamaemorus* and *R. coreanus* (Im et al., 2013), among others. The major phenolic acids, anthocyanins, vitamin C, TEAC and CUPRAC of *R. geoides* have been recently reported (Ruiz et al., 2013, 2015) but there are no reports on the fruit bioactivity. Hence, our aim was to disclose some of the bioactivities of the fruit using AGS cell-based experimental models.

Anthocyanins and other flavonoids may exert their protective antioxidant effects in humans before their absorption into the systemic bloodstream, protecting cells of the gastrointestinal tract (Halliwell, Rafter, & Jenner, 2005). The endpoint to determine the antioxidant protection was the reduction of MTT which indicates mitochondrial metabolic rates (Cheli & Baldi, 2011). Our results show that AGS cells were significantly protected by *R. geoides* PEEs against H_2O_2 oxidative stress. In a study on the protective effect of *R. coreanus* fruits on PC-12 cells against H_2O_2 injury, the cyanidin glycosides effectively diminished intracellular oxidative stress, while the non-anthocyanin fraction of the extract had no significant effect (Im et al., 2013). A protective potential of anthocyanins isolated from strawberries against the radical AAPH [2,2′-azino-bis (2-amidinopropane)] in human dermal fibroblasts was described (Giampieri et al., 2014). The antioxidant effect of an anthocyanin extract from bilberries was also reported in Caco-2 and HT-29 cell lines (Schantz et al., 2010). The anthocyanins present in *R. geoides* are cyanidin-3-glycosides, mainly the 3-sambubioside and 3-sophoroside (Ruiz et al., 2013). Our HPLC–DAD-MS/MS analyses allowed the identification of cyanidin hexosides, dihexoside and hexoside-pentoside derivatives (compounds **1**–**5**). *R. idaeus* showed a more complex anthocyanin pattern than the Chilean raspberry (Lee, Dossett, & Finn, 2012). As the Chilean raspberry contains higher TP and TF than *R. idaeus*, the protective activity might be attributed to procyanidins, tannins and flavonoids. Among the flavonoid compounds, the main constituent in *R. geoides* was Q hexoside pentoside, which was not detected in *R. idaeus*.

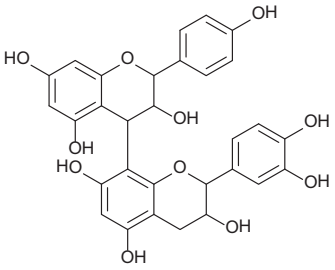
Dietary phenolics are known to exert direct antioxidant effects by ROS scavenging, but also indirect effects through the induction of endogenous protective mechanisms. This include the regulation of intracellular GSH concentration (Moskaug, Carlsen, Myhrstad, & Blomhoff, 2005) and the expression levels of GSH-related detoxifying enzymes, such as glutathione S-transferase (Feng et al., 2005) and glutathione peroxidase (Fernandez-Pachon et al., 2009), among others. After pre-incubation with the extracts, AGS cells significantly increased their intracellular level of total reduced GSH. However, when cells were co-incubated with the PEEs and the



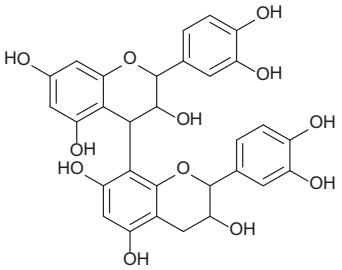
2 R: Hexoside pentoside
3 R: Hexose



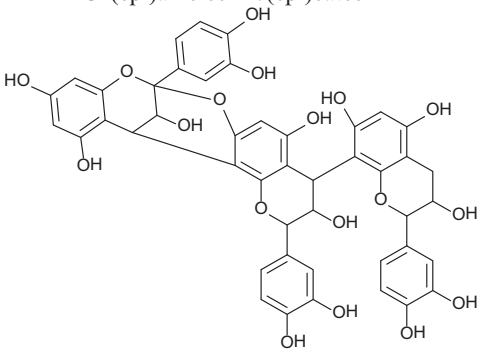
9 (epi)catechin



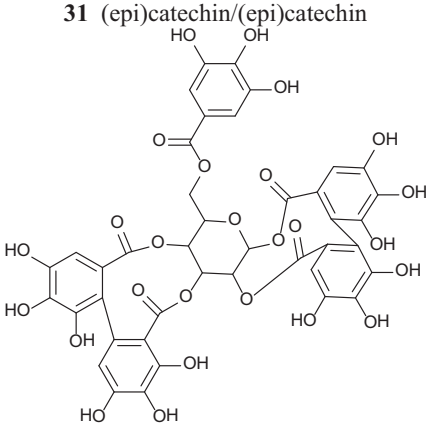
8 (epi)afzelechin/(epi)catechin



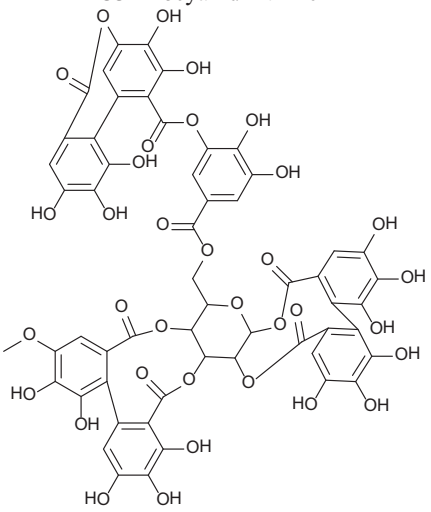
31 (epi)catechin/(epi)catechin



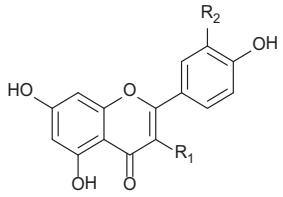
33 Procyanidin trimer



11 Potentillin/Casuarictin



Lambertianin C-related tannin



	R_1	R_2
26	Hexoside	H
27	Pentoside-hexoside	H
10	Glucuronide	OH
15	Dihexoside	OH
22	Hexoside pentoside	OH
25	Hexoside	OH
32	Hexoside acetate	OH
39	OH	OH
17	Hexoside	OCH_3
23	Glucuronide	OCH_3

Flavonol derivatives

Fig. 8. Structures of some phenolics tentatively identified in *Rubus geoides* fruits.

stressor agents, no protective effect was observed. These results indicate that the cytoprotection elicited by *R. geoides* PEEs might be associated to an increase in the intracellular concentrations of GSH, rather than a direct scavenging activity of H_2O_2 or MGO. It is important to consider that GSH is needed as a substrate and co-factor in the detoxifying activity of numerous enzymes such as glutathione peroxidase and glyoxalase, involved in the inactivation of H_2O_2 and MGO, respectively (Battin & Brumaghim, 2009; Sousa Silva, Gomes, Ferreira, Ponces Freire, & Cordeiro, 2013). The flavonol quercetin significantly protected cells in the co-incubation, but not in the pre-incubation experiments. These results suggest that direct scavenger activity and cellular signaling could be involved in the protective effect of polyphenols against damage induced by ROS and dicarbonyl compounds. *R. geoides* PEEs contain a complex mixture of anthocyanins, flavonoids, ellagitannins and proanthocyanidins. The main compounds exhibiting

antioxidant activity in a *R. idaeus* variety were ellagic acids, flavonoids and vitamin C (Mullen et al., 2002). Eleven flavonol derivatives were tentatively identified in our samples of *R. geoides*. The compounds are mainly monoglycosides or diglycosides of quercetin (Q) (compounds **10**, **15**, **22**, **25**, **32**), kaempferol (K) (**6**, **26** and **27**) or isorhamnetin (IRh) (**17** and **23**). The flavonol glycosides quercetin hexoside-pentoside and quercetin-3-galactoside were found in *R. geoides* fruits (Ruiz et al., 2015). In raspberries, glycosides of Q have been reported, but K and IRh derivatives were not detected (Mullen, Yokota, Lean, & Crozier, 2003).

Proanthocyanidins or condensed tannins have been reported in wild and cultivated *Rubus* species from Mexico (Cuevas-Rodriguez et al., 2010). In our analyses, compound **31** can be associated with (epi)catechin-(epi)catechin dimer, compatible with procyanidin B or procyanidin C, previously reported from *R. chamaemorus* (Maatta-Riihinen et al., 2004). The proanthocyanidin **8** showed in the MS/MS spectrum, the neutral loss of 272 u and a base peak at 289 u, pointing out to an (epi)afzelechin/(epi)catechin isomer. The procyanidins from *R. geoides*, with a molecular weight of 864 are related to pavetannin and cinnamtannin ($C_{45}H_{36}O_{18}$) isolated from *Pavetta owariensis* (Baldé et al., 1991) and to the trimers from *Arachis hypogaea* skins (Lou et al., 2004). In the north American species *R. chamaemorus* and *R. spectabilis*, the berries contained A-type proanthocyanidins dimers and trimers, some of them species-specific (Kellogg et al., 2010). The fragmentation patterns of compounds **28**, **30** and **33** with $[M-H]^-$ at *m*/*z* 863 are in agreement with three (epi)catechin units linked through a C4–C8 or C4–C6 bonds (B type) with an additional C2–O–C7 link (A-type) (Tarascou et al., 2011).

Ellagitannins, ellagic acid and gallic acid conjugates have been described for *Rubus* fruits (Gasperotti, Masuero, Vrhovsek, Guella, & Mattivi, 2010). For *R. idaeus*, *R. glaucus*, *Rubus adenotrichus* and *Rubus fruticosus*, sanguiin H-6 and lambertianin C were reported as the main ellagitannins (Lee et al., 2012). Casuarictin, with a *m*/*z* 936, was reported from *R. coreanus* and *R. idaeus* (Heo et al., 2011). The tannin 2,3-hexahydroxydiphenoyl-4,6-sanguisorboyl glucose was reported from *R. sanctus* (Hussein, Ayoub, & Nawwar, 2003). Our findings with *R. geoides* confirm the trend observed for this genus, but also provides evidence of strong differences in the identity of the main fruit tannins. Zoom mass spectra showed intervals between the isotope peaks of 0.5 u, suggesting double charge of the ions corresponding to MW 2500 for **13**, **18**, **24** and **35** and MW 2502 for **38**. MS/MS shows a fragmentation pattern in agreement with lambertianin C without an ellagic acid unit, similar to the tannins described for *R. idaeus* and *R. fruticosus* (Gasperotti et al., 2010). The fragmentation pattern of compound **36** was compatible with castalagin/vescalagin (Vivas, Laguerre, de Boissel, de Gaulejac, & Nonier, 2004), terflavin C (Tanaka et al., 1991) or its isomers. Ellagitannins with the same fragmentation pattern were described for *Luma apiculata* and *Luma chequen* berries (Simirgiotis et al., 2013).

5. Conclusions

Our results show that PEEs of *R. geoides* present higher antioxidant capacity than the commercial raspberry and shows protective effect on AGS cells against oxidative and dicarbonyl stress-induced damages. This antioxidant activity may be related with the capacity of these extracts to increase intracellular levels of reduced GSH. Further work could confirm up- or down-regulation of critical enzymes involved in H_2O_2 and MGO detoxification. Five anthocyanidin glycosides were identified, being the main compound cyanidin 3-hexoside pentoside. Ellagitannins included 15 compounds, some of them related with casuarictin/potentillin, while condensed tannins comprised catechin/epicatechin dimer, trimers and tetramers. The ellagitannins and proanthocyanidins found in *R. geoides* fruits are reported for the first time. The cytoprotective effects in AGS cells and phenolic content support the use of this native berry as a potential functional food.

Acknowledgments

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