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ORIGINAL PAPER

Anthocyanin-Rich Extracts of Calafate (*Berberis microphylla* G. Forst.) Fruits Decrease In Vitro Viability and Migration of Human Gastric and Gallbladder Cancer Cell Lines

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Abstract

Currently, gastric cancer (GC) and gallbladder cancer (GBC) constitute important causes of human deaths related to cancer worldwide. In the last years, several researches are focused on the role of dietary compounds in preventing cancers. The consumption of fruits with high antioxidants, mainly anthocyanins, represents a good option to reduce the risk of chronic human diseases. Calafate (*Berberis microphylla* G. Forst.) berries, recognized by their remarkable antioxidant properties and high content of anthocyanins, appear as a new alternative to treat degenerative diseases of public interest. The present work was aimed to evaluate the impact of crude and anthocyanin-rich extracts from Calafate fruits on in vitro cell viability and migration capacity of gastric (AGC) and gallbladder (G415) human cancer cell lines, as related with their antioxidant properties. Crude and anthocyanin-rich extracts were obtained from fruits of Calafate grown under field conditions in the south of Chile. Antioxidants, phenols, anthocyanins, and anthocyanidins were determined. In vitro cell viability and migration of AGS and G415 human cancer cell lines at different concentrations of extracts (25–800 μg mL^{-1}) were determined. Anthocyanin-rich extracts of Calafate berries showed comparable antioxidant activity (up to 1200 μg Trolox eq. g^{-1}DW), slightly lower total phenolic content (12%), but higher total anthocyanin content (25%) compared to the crude extract. The major anthocyanidin molecule detected in both extracts was delphinidin, followed by malvidin, and low concentrations of petunidin, cyanidin, and peonidin. As expected, all of these compounds were detected in higher levels in anthocyanin-rich extracts (up to 2-fold). Noteworthy, our study revealed that Calafate fruit extracts strongly decrease in vitro viability and migration capacity of gastric carcinoma (AGC model) and gallbladder carcinoma (G415 model) human cell lines; however, the anthocyanin-rich extract displayed higher inhibitory effects (up to 70%) compared to crude extracts. These findings allow suggesting that the in vitro antiproliferative potential of Calafate fruit extract is strongly related to the anthocyanin concentration, especially delphinidin.

Keywords Antioxidants · Antiproliferative effect · Calafate · Delphinidin · Gastric cancer · Gallbladder cancer

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1 Introduction

The World Health Organization (WHO) reported that cancers are currently the first cause of human deaths in the world (Wild et al. 2020). Indeed, gastrointestinal cancers, including gastric cancer (GC) and gallbladder cancer (GBC), have become an important contribution to the cancer burden worldwide (Wild et al. 2020). The GC is the fifth most frequently diagnosed cancer and the third most lethal malignancy worldwide, constituting an important public health problem (Wild et al. 2020). Each year, almost one million new GC cases are diagnosed, and ~700,000 people die of this disease, thus representing ~10% of the cancer-related deaths worldwide (Rawla and Barsouk 2019; Venerito et al. 2018). Although recent diagnostic and therapeutic advances have provided excellent survival for patients with early GC, the prognosis of patients with advanced cancer is still poor (Csendes and Figueroa 2017; Shah and Kelsen 2010). On the other hand, GBC is recognized as the most common malignancy of the biliary tract and the fifth most common malignant tumor of the digestive tract (Letelier et al. 2016). The evolution of this disease is usually asymptomatic, resulting in a late diagnosis of this malignancy and poor survival (Lazcano-Ponce et al. 2001; Letelier et al. 2014; Nemunaitis et al. 2018). Particularly, Chile has one of the highest mortality rates of GC with a mean of 19.2 deaths/100,000 people but reaching a mortality rate of 27.3/100,000 people in some regions (Bellolio et al. 2019; Csendes and Figueroa 2017). GBC is also a public health problem in Chile because the La Araucanía region has the highest mortality rate in the world, with 35 deaths/10,000 people (Lazcano-Ponce et al. 2001); therefore, it is key to be able to present measures that help reduce these malignancies in this country.

In the last decade, researchers have focused on the study of the role of different dietary compounds to prevent or control certain gastrointestinal cancers (Leung and Sung 2006). In this context, the consumption of fruits with a high level of antioxidants, especially anthocyanins, has been proposed as a good option to reduce the risk of chronic diseases development (Battino et al. 2019; Muceniece et al. 2019). Therefore, several studies have recently focused on chemoprotective properties of Chilean native berries, particularly rich in anthocyanins, which have gained quickly the interest of researchers around the world (Cespedes-Acuña et al. 2018; Fuentes et al. 2019; Mariangel et al. 2013; Ramirez et al. 2015; Ruiz et al. 2013a).

Berberis microphylla G Forst. (Calafate) is a native berry from southern Patagonia in Chile and Argentina (Ruiz et al. 2010), belonging to the Berberidaceae family (Mariangel et al. 2013; Ramirez et al. 2015). It is an evergreen shrub 2–3 m high with trifid thorns and little single yellow flowers, which in Chile is grown under wild conditions from Maule to Magallanes regions (Ulloa-Inostroza et al. 2017). Calafate fruits are dark blue-purple berries (7–11 mm caliber) (Mariangel et al. 2013) that generally are consumed fresh or as jellies, marmalades, and wines (Ruiz et al. 2010; Ulloa-Inostroza et al. 2017), being recognized by their remarkable antioxidant properties and high content of anthocyanins (Chamorro et al. 2019; Ruiz et al. 2013a). Thus, Calafate fruits appear as a new alternative of nutraceutical products potentially useful for the treatment of different degenerative diseases of public interest (Ruiz et al. 2013a), which has been poorly explored for this purpose. In the present study, we evaluate the effect of crude and anthocyanin-rich extracts of Calafate fruits on in vitro cell viability and migration capacity of AGS and G415 human cell lines, as related to their antioxidant properties. Thus, we have postulated that the anthocyanin-rich extract of Calafate fruits decreases in vitro cell viability and migration capacity of AGS and G415 human cell lines, which is related to its high antioxidant properties compared to the crude extract.

2 Materials and Methods

2.1 Crude Extracts Preparation of Calafate Fruits

Total crude extracts were obtained by macerating 10 g of calafate fruits (Fig. 1a) with ethanol 80% v/v in a chilled (4 °C) mortar. The extracts were centrifuged at 5000 rpm for 5 min, and the supernatants were recovered. The pellet was subjected to 3 sequential extractions, every time using ethanol 80% v/v until to obtain total extract (Fig. 1b). Then, the total extract was filtered, and the solvent was rotary-evaporated using Heidolph VV2020 to obtain the total dried extract. Prior use, the dried extracts were stored at 4 °C in the dark.

2.2 Obtaining of Anthocyanin-Rich Extracts from Calafate Fruits

Anthocyanin-rich extracts of Calafate fruits were obtained by chromatography fractionation of dried crude extracts using a glass column of 2.5 cm diameter (Fig. 1c), as described by Ribera-Fonseca et al. (2020). Briefly, the cotton pad was placed at the end of the column, which was packed with silica gel previously dissolved in dichloromethane to reach 12 cm of height. Then, dried Calafate fruits crude extracts were dissolved in dichloromethane and mixed with 1.5 g of silica gel to obtain a smooth paste, which was placed at the top of the column with a filter paper. The column was eluted with 1 L of ethyl acetate and then, with 1 L of acidified ethanol pH 1.0 [0.82 mL of hydrochloric acid (HCl, 37%) to 100 mL of absolute ethanol]. Finally, the solvent was rotary-evaporated to dryness (Heidolph VV2020).

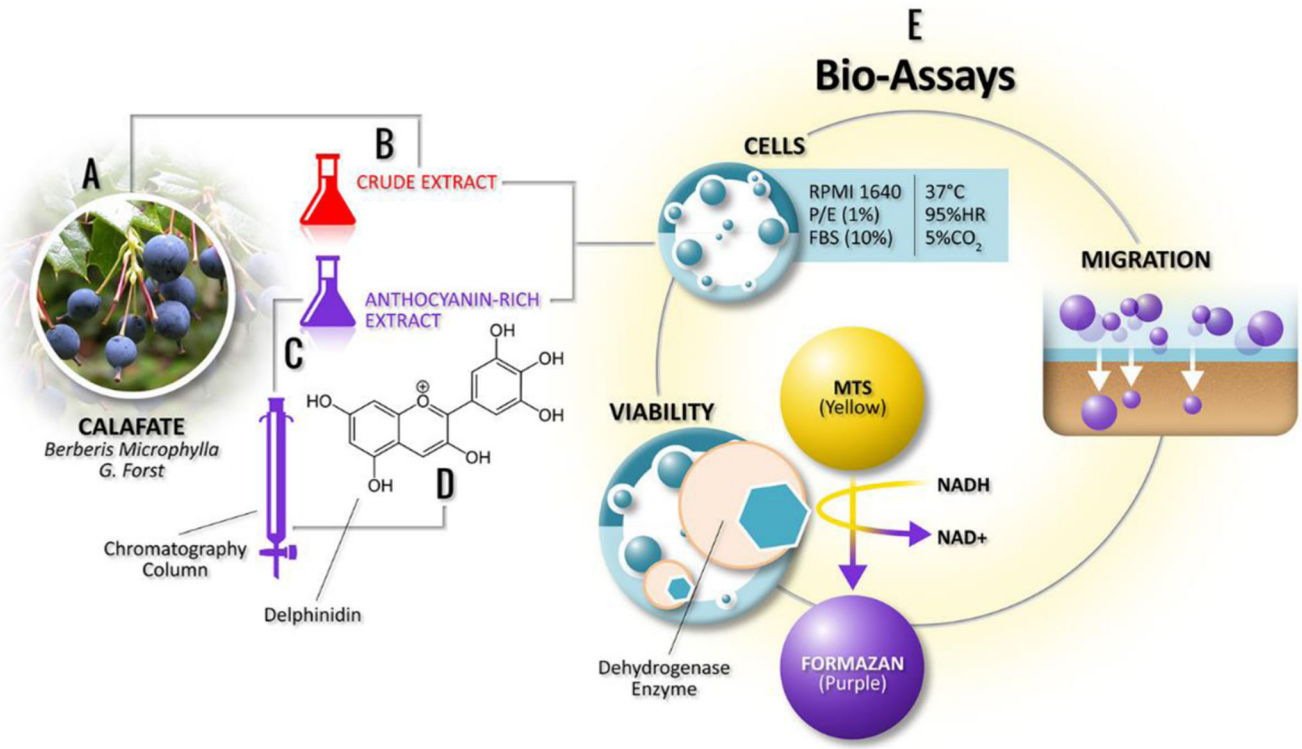

Fig. 1 Calafate fruits used (**a**), crude extract obtention (**b**), Anthocyanin-rich extract obtention (**c**), the main anthocyanidin structure (delphinidin) found (**d**) and the bioassays performed (**e**)

2.3 Determination of Antioxidant Activity in Calafate Extracts

The antioxidant activity (AC) by DPPH (2,2-diphenyl-2-picrylhydrazyl) method was determined, according to Chinnici et al. (2004). The absorbance of total and rich-anthocyanin extracts was determined at 517 nm using a multimodal detector (SynergyTM HT, Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Temuco, Chile). Results are expressed as microgramTrolox equivalents per gram of dry weight (μg TE g^{-1} DW).

2.4 Quantification of Total Phenolic Contents in Calafate Extracts

Total phenol concentrations were determined by the Folin-Ciocalteu method (Slinkard and Singleton 1977). The absorbance of total and rich-anthocyanin extracts was determined at 765 nm in a multimodal detector (SynergyTM HT, Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Temuco, Chile). Chlorogenic acid was used as a standard. Total phenol values were expressed as microgram of chlorogenic acid equivalent per gram of dry weight (μg CAE g^{-1} DW).

2.5 Assessment of Total Anthocyanin Content in Calafate Extracts

Total anthocyanin content in the extracts was determined by the differential pH method (Ribera et al. 2010). Briefly, dry samples were macerated with acidified ethanol. It was stirred in the dark overnight at 4 °C. Later, it was centrifuged (4 °C) at 13,000 rpm for 10 min. Supernatants were collected, and absorbance was measured in a multimodal detector (SynergyTM HT, Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Temuco, Chile) at 530 and 657 nm. Anthocyanin contents were expressed as mg of cyanidin-3-glucoside per g of dry weight (mg c3g g^{-1} DW).

2.6 Analysis of Anthocyanidin Composition in Calafate Extracts

The method used was based on anthocyanidin (anthocyanin aglycones) determination, according to Nyman and Kumpulainen (2001) with minor modifications (Ribera et al. 2010). Delphinidin, malvidin, petunidin, cyanidin, and peonidin provided by Sigma Chemical Co. (St. Louis, MO) were used as anthocyanidin standards. Signals were detected at 530 nm in a high-performance liquid chromatography (HPLC) system Jasco (LC-Net II/ADC) using a Kromasil reversed-phase (RP)-18 column (250 × 4.6 mm i.d.) equipped

with a photodiode array detector (DAD) (Jasco MD 2015 Plus). It is important to mention that according to Brito et al. (2014) and Ruiz et al. (2010, 2013a), the main anthocyanin molecules detected in Calafate fruits are delphinidin, cyanidin, petunidin, peonidin, and malvidin glycosides. Based on these antecedents, this study did not include pelargonidin as standard in the HPLC analysis of calafate extracts.

2.7 Culture of AGS and G415 Cell Lines

AGS cells (GC model) and G415 cells (GBC model) were used for the cell assays in this study. AGS cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), while G415 cells (GBC model) were purchased from Riken BioResource Center (Ibaraki, Japan). Both cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Biological Industries, USA) and 1% penicillin/streptomycin (Thermo Scientific Hyclone, Logan, UT, USA). Both cell lines were incubated at 37 °C, 5% CO_2, and 95% humidity.

2.8 Assessment of Cell Viability in Cell Lines Incubated with Calafate Extracts

Dried Calafate fruit extracts were used and dissolved in 100 μL of DPBS buffer 1× (Thermo Scientific Hyclone, Logan, Utah, USA) and then diluted in RPMI-1640 medium to reach final concentrations of 0, 25, 50, 100, 200, 400, and 800 μg mL^{-1}. On the other hand, AGS and G415 cell lines were plated into 96-well plates in triplicate at a final density of 3.5 × 103 cells/100 μL of culture medium. After an overnight attachment period, cells were treated with calafate extracts the different final concentrations mentioned above. At the end of 24 or 48 h of exposure to extract dilutions, cell viability was determined by the AQueousCellTiter 96® Non-Radioactive Cell Proliferation Test (Promega Corp., Madison, WI, USA). Briefly, 20 μL of 3-(4,5,dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazoliuminner salt (MTS) solution was added to each well and after incubation for 1 h at 37 °C, and the absorbance was determined at 490 nm (Fig. 1e) using a plate reader PHOmo (AutobioLabtec Instruments Co. Ltd., Zhengzhou Henan, China). This procedure was carried out similarly to that performed by Ribera-Fonseca et al. (2020). The percentage of cell viability was calculated using the formula: Cell viability (%) = (X × 100%)/Y, where "X" is the absorbance of treated cells and "Y" the absorbance of untreated cells.

2.9 Determination of In Vitro Cell Migration Capacity

Cell migration was measured in AGS and G415 cells, also in a similar manner to that performed by Ribera-Fonseca et al. (2020). Assays were performed using 24-well plates containing polycarbonate filters with an 8-μm pore size (BD Biosciences, Bedford, MA, USA). AGS and G15 cells were cultured in RPMI-1640 supplemented with 10% FBS until 70% of confluence. Before seeding, cells were exposed to the different concentrations mentioned above of calafate fruit extract for 1 h at 37 °C. Then, cells were harvested, and 2 × 104 cells were suspended in 500 μL of RMPI-1640 without FBS and seeded into the upper chamber. The complete RPMI-1640 medium (750 μL) was placed in the lower chamber. After 24 h, cells were fixed in methanol for 15 min and then stained with 0.05% crystal violet in 25% methanol/PBS solution for 15 min (Fig. 1e). Cells on top of the membrane were removed using a cotton swab, and the filters were washed with PBS. Cells on the underside of the filters were observed and counted under a microscope in 10 randomly selected fields.

2.10 Statistical Analysis

Statistical analysis was performed using Sigma Stat 2.0 (SPSS, Chicago). For the characterization of extracts, differences among groups were analyzed by two-way ANOVA, where the factors were extracts and concentrations of the extract. Significant differences among mean values were established by using Tukey's multiple comparisons test ($P \leq 0.05$). For in vitro assays (viability and migration), pair-wise comparisons between extracts (crude and anthocyanin-rich extracts) were evaluated by non-parametric Wilcoxon's test ($P \leq 0.05$) using GraphPad Prism software version 6.01 (Graph-Pad Software, CA). All data presented in the figures correspond to at least three independent analytical replicates (mean ± standard deviation).

3 Results

3.1 Antioxidant Properties of Crude and Anthocyanin-Rich Extracts of Calafate Fruits

The antioxidant properties of Calafate fruit extracts are shown in Fig. 2. Our results revealed that the antioxidant activity (AA) was constantly increased at increasing extract concentrations in dose-dependent manner (Fig. 2a). In fact, the AA of extracts at the highest doses (800 μg mL^{-1}) was 70% ($P < 0.001$) superior to those detected at the lowest concentration (10 μg mL^{-1}). In general, crude extract and anthocyanin-rich extract did not show significantly different AA ($P = 0.09$), except for the doses of 100 μg mL^{-1}, at which crude extract showed higher AA ($P < 0.001$) compared to the anthocyanin-rich extract (Fig. 2a). Similar observations were observed for total phenolic contents (Fig. 2b), which increased in response to the increment of extract concentration (in about 50% from the concentrations of 10 to 800 μg mL^{-1}), tending to be higher

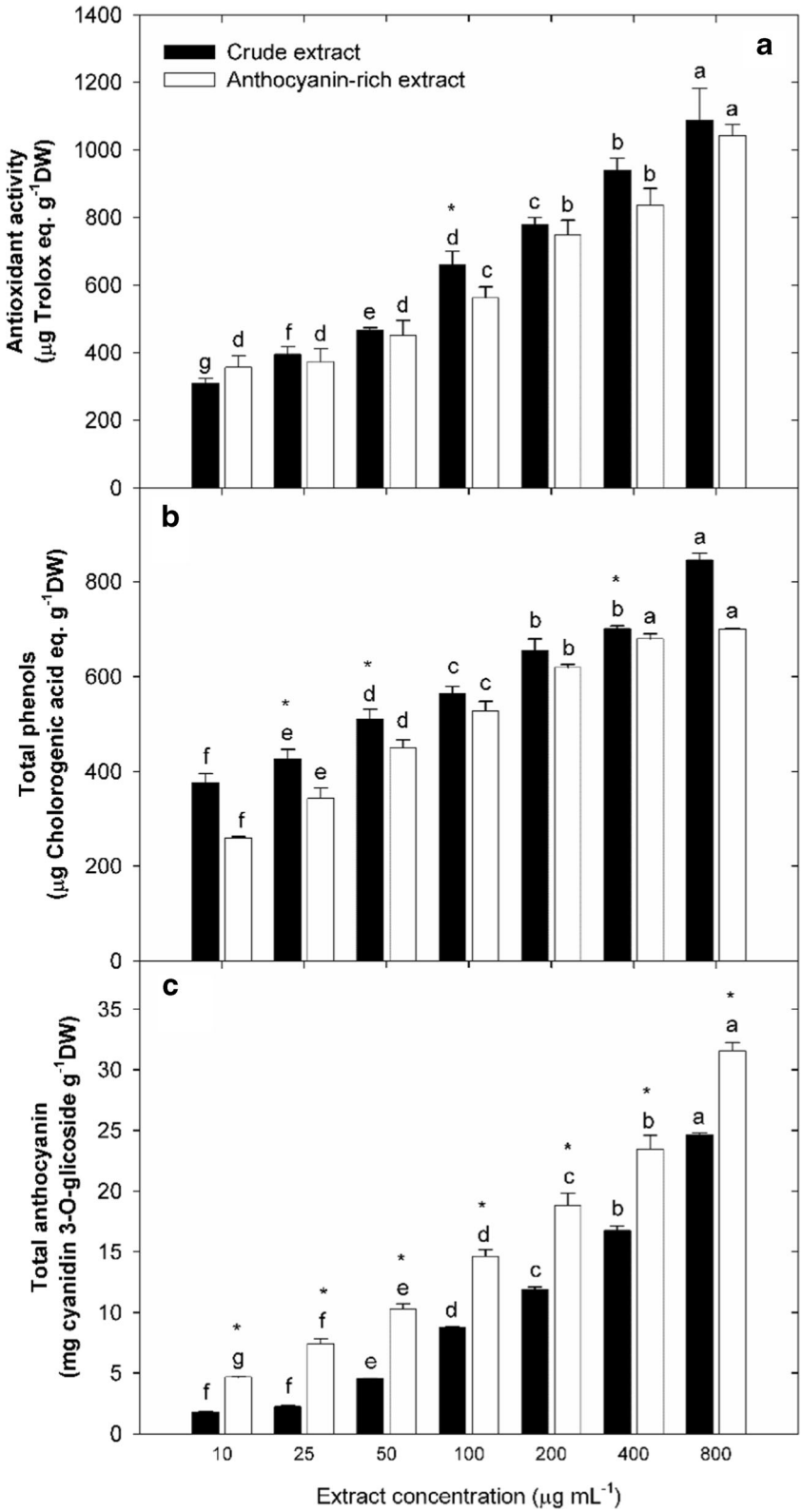

Fig. 2 Antioxidant activity (**a**), total phenolic (**b**), and total anthocyanin (**c**) of crude extracts and anthocyanin-rich extracts of calafate fruits. Different letters above bars denote statistic differences among extract concentrations, whereas asterisk (*) indicated statistic variations between crude extract and anthocyanin-rich extract, according to the Tukey test ($P \leq 0.05$)

in crude extract than in anthocyanin-rich extract (Fig. 2b). Contrarily and as expected, anthocyanin-rich extracts showed a significantly higher concentration of total anthocyanin in relation to crude extract (around 20%; $P < 0.001$) at all extract doses. This parameter also rose (~ 15%) across increasing extract concentrations (Fig. 2c).

Regarding anthocyanin composition (HPLC-DAD results), we observed that the concentration of all

anthocyanidin compounds detected linearly increased (more than 10-fold; $P < 0.001$) at increasing calafate extract concentration. In general, anthocyanin-rich extracts showed significantly higher levels of anthocyanidin molecules (up to 25% at the highest extract concentration; $P < 0.001$) compared to the crude extracts, with these differences more evident at the highest extract doses (Fig. 3). Moreover, we found that delphinidin was the major anthocyanidin in both calafate fruit extracts (until 1700 mg g^{-1} DW at the highest concentration of anthocyanin-rich extracts, Figs. 1 and 3a), which was more than 90% higher ($P < 0.001$) than malvidin (until around 130 mg g^{-1} DW at the highest concentration of anthocyanin-rich extracts, Fig. 3b), the second more abundant anthocyanidin (Fig. 3a, b). The HPLC analyses also revealed the presence of petunidin (until 60 mg g^{-1} DW at the highest concentration of anthocyanin-rich extracts), cyanidin (until around 20 mg g^{-1} DW at the highest concentration of anthocyanin-rich extracts), and peonidin (until around 1.8 mg g^{-1} DW at the highest concentration of anthocyanin-rich extract) in Calafate extracts. Interestingly, petunidin, cyanidin, and peonidin were detected in the anthocyanin-rich extract at all extract doses, while in the crude extract at low extract concentrations, sometimes are undetected (Fig. 3 c, d, and e).

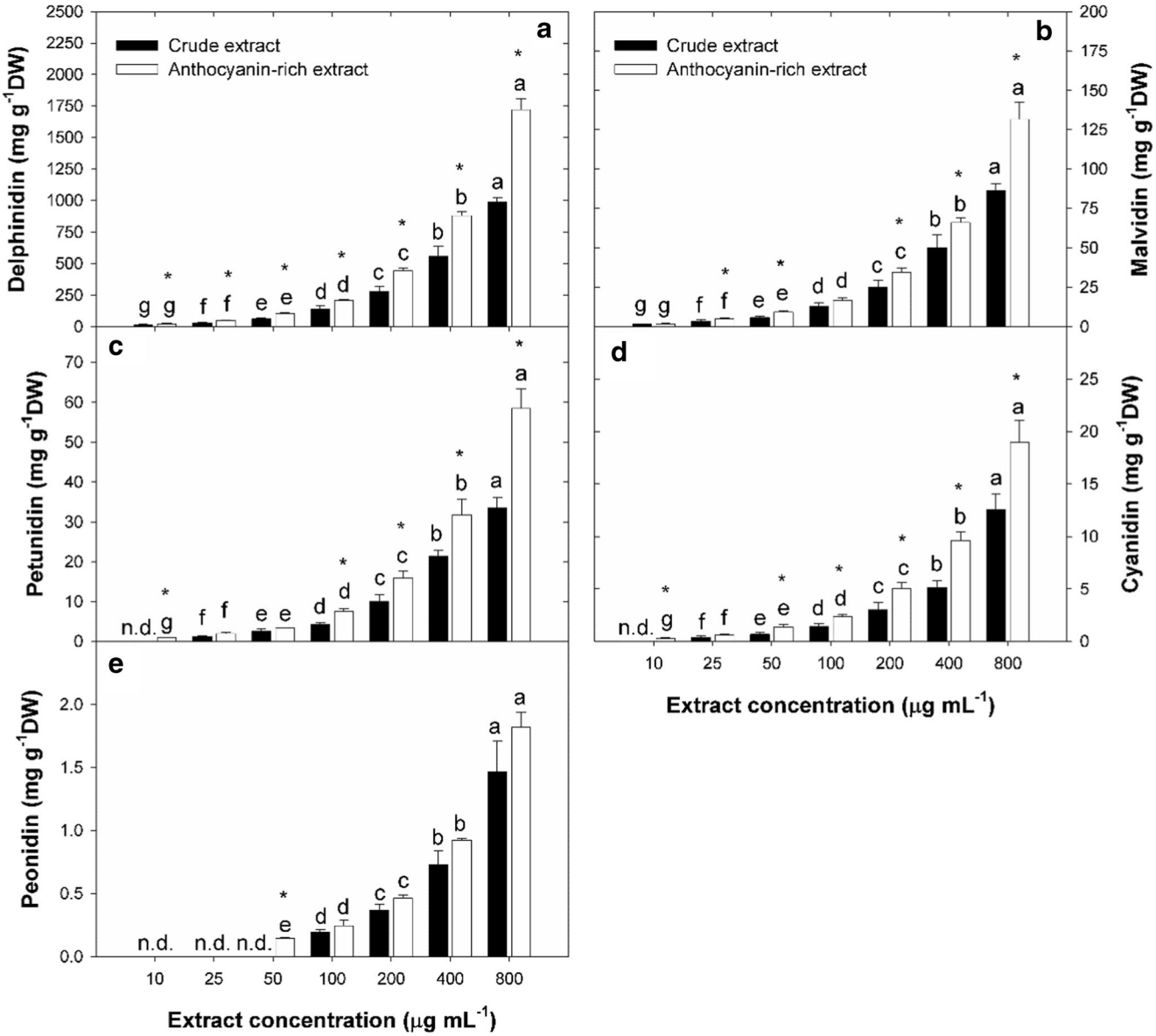

Fig. 3 Anthocyanidin composition (HPLC-DAD analysis) of crude extracts and anthocyanin-rich extracts of Calafate fruits. The graph showed the concentration of delphinidin (**a**), malvidin (**b**), petunidin (**c**), cyanidin (**d**), and peonidin (**e**) in both extracts. Abbreviation: n.d.: non-detected. Different letters above bars denote statistic differences among extract concentrations, whereas asterisk (*) indicated statistic variations between crude extract and anthocyanin-rich extract, according to the Tukey test ($P \leq 0.05$)

Indeed, peonidin, the anthocyanidin found at the lowest levels in both Calafate fruit extracts, was detected from 50 μg mL^{-1} in the anthocyanin-rich extract, but from 100 μg mL^{-1} in the crude extract (Fig. 3e).

3.2 Effect of Calafate Extracts on In Vitro Viability and Migration Capacity of Cancer Cells

In order to evaluate the antiproliferative activity of Calafate fruit extracts on cancer cells, AGS (GC model) and G415 (GBC model) cell lines were used. The effect of extracts on cell viability (after 24 h and 48 h of extract exposure) and migration capacity (after 48 h of extract exposure) were assessed at different concentrations of crude extracts or anthocyanin-rich extract (Figs. 4 and 5; Supplementary Figure). The results showed that, in general, Calafate extracts induced a reduction in the viability of both cells at different concentrations without differences between times (Supplementary Figure). For instance, the viability of AGS cells significantly decreased in ~ 50% with a concentration from 400 μg mL^{-1} of Calafate extracts at both extract exposure times (24 h and 48 h) (from $P < 0.001$). The highest dose of crude extract (800 μg mL^{-1}) significantly reduced AGS cell viability up to 70% after 48 h of extract exposure (Fig. 4a and b) ($P < 0.0001$). Noteworthy, the inhibitory effect on AGS cell viability was higher with anthocyanin-rich extract at both incubation times (~ 50% of cell viability at extract concentrations from 200 μg mL^{-1} onwards) (Fig. 4a and b; $P < 0.0001$). The inhibitory effect reached only 30% of cell viability at the highest extract concentration of 800 μg mL^{-1}. For both Calafate extracts, but mainly for the anthocyanin-rich extract, the inhibitory effects on cell viability were higher at 48 h compared to 24 h of extract exposure. Similar but more notorious inhibitory effects were observed for G415 cell viability, where the lowest viability levels were detected in response to the anthocyanin-rich extract at the higher doses ($P < 0.0001$) and 48 h of extract exposure (25 or 10% of cell viability at 800 μg mL^{-1} after 24 or 48 h of extract exposure, respectively). In comparison, the use of crude extract displayed lower inhibitory impacts on this parameter less evident (~ 25 o 15% of cell viability at 800 μg mL^{-1} after 24 and 48 h of extract exposure, respectively) (Fig. 4c and d; $P < 0.0001$).

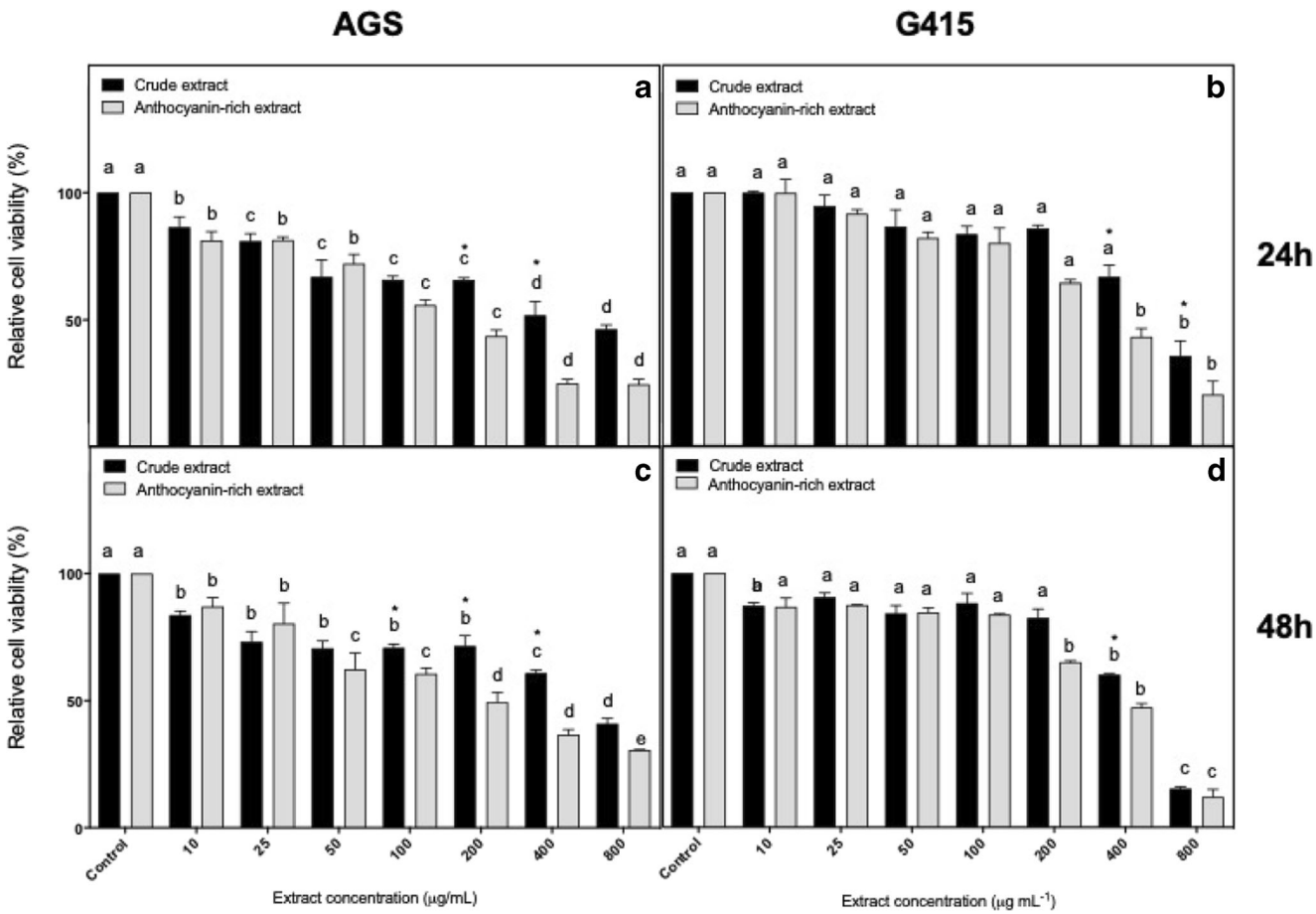

Fig. 4 In vitro viability of AGS (gastric model) and G415 (gallbladder model) cell lines in response to the incubation with Calafate fruit extracts. **a** AGS cell line after 24 h of incubation. **b** AGS cell line after 48 h of incubation. **c** G415 cell line after 24 h of incubation. **d** G415 cell line after 48 h of incubation. Different letters above bars denote statistical differences among extract concentrations, whereas asterisk (*) indicated statistical variations between crude extract and anthocyanin-rich extract, according to the non-parametric Wilcoxon's test ($P \leq 0.05$)

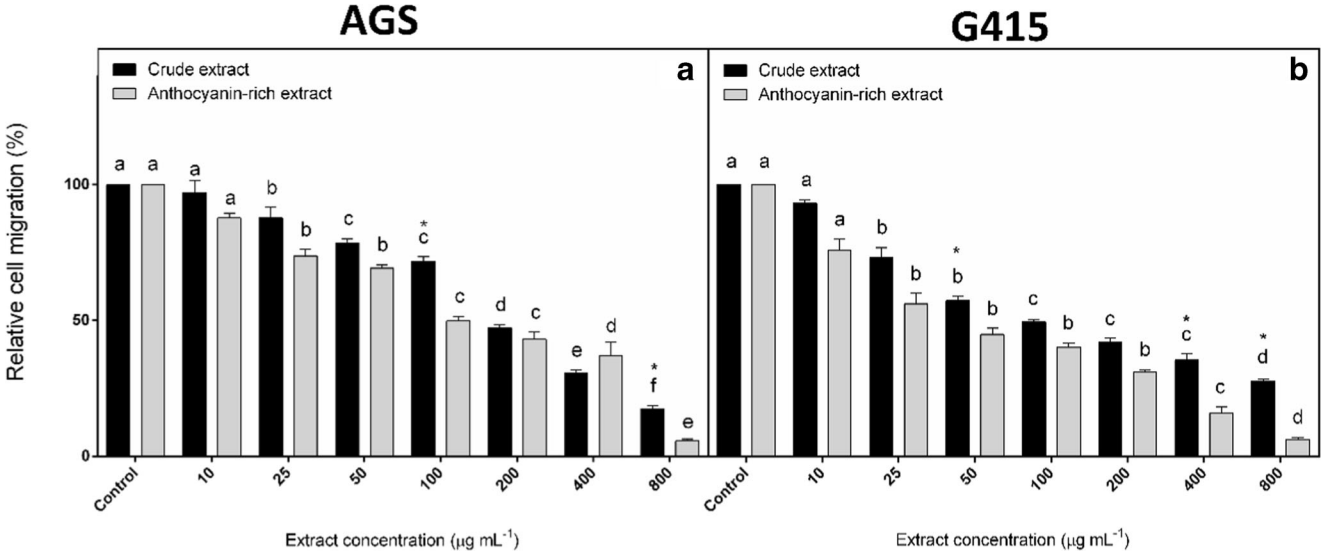

Fig. 5 In vitro migration capacity of AGS (gastric model) and G415 (gallbladder model) cell lines in response to the incubation with Calafate fruit extracts. **a** AGS cell line after 48 h of incubation. **b** G415 cell line after 48 h of incubation. Different letters above bars denote statistical differences among extract concentrations, whereas asterisk (*) indicated statistical variations between crude extract and anthocyanin-rich extract, according to the non-parametric Wilcoxon's test ($P \leq 0.05$)

On the other hand, the migratory capacity of both AGS and G415 cells was inversely proportional to the increase of the concentration in both extracts, reaching at least a ~ 80% decrease of migration by using Calafate extract at the highest doses of 800 μg mL^{-1} (Fig. 5, $P < 0.005$). Our results also showed that the impact of Calafate extracts was similar to the reduction of migration capacity between both cell lines, and G415 cells showed a decreased migration at lower concentrations of extract compared to AGS cells (Fig. 5a and b). Moreover, likewise to those observed for cell viability, the anthocyanin-rich extract was more effective to decrease cell migration in both cancer cell lines (up to ~ 5% of migration capacity at the highest concentration) compared to crude extracts (up to ~ 10–15% of migration at the highest concentration) (Fig. 5).

4 Discussion

Berries from Berberis species are recognized by their remarkable content of minerals, vitamins, alkaloids, and polyphenols (Belwal et al. 2017; Bhatt et al. 2017), having interesting nutritional and antioxidant properties (Chamorro et al. 2019; Negi and Subramani 2015). In fact, berberis plants have been used in various traditional medicine treatments worldwide (Adamus et al. 2019). In the present study, we have assessed the antioxidant and anti-cancerous properties of fruit extracts from *Berberis microphylla* (Calafate) growing in the south of Chile, one of the less-studied Berberis species, but a promising candidate for preventing and controlling different human diseases. Interestingly, our findings showed that both crude and anthocyanin-rich extracts of Calafate fruits were able to reduce in vitro viability and migration of AGS (gastric cancer) and G415 (gallbladder cancer) human cell lines. Several studies have reported that many species of Berberis have cardiovascular and hepatoprotective effects as well as antimicrobial and anti-cancerous activities (Adamus et al. 2019; Alamgeer et al. 2017; Behravan et al. 2019; Boudjlida et al. 2019; Khan et al. 2019; Srivastava et al. 2015). These effects have been explained mainly for their high contents of alkaloids such as berberine, which are the main secondary metabolite in berberis species (Hussain et al. 1981) with proved therapeutic effects in many fields of medicine (Srivastava et al. 2015). In fact, the anti-cancerous effect of berberine was recently reviewed by Xu et al. (2019), who reported that this alkaloid exerted antitumor effects in a variety of tumors in vivo, including breast cancer and lung cancer; nonetheless, the evidence is still insufficient in colorectal cancer and gastric cancer (Xu et al. 2019). It is important to highlight that, in fruits of calafate, berberine is present at low concentrations (0.001%), mainly in seeds (Ruiz et al. 2014). Interestingly and as was mentioned above, Berberis species have also been well-documented by their high content of polyphenols and antioxidant activity (Brito et al. 2014; Chamorro et al. 2019; Ruiz et al. 2010, 2013a, b, 2014); nevertheless, the relationship between anticancerous activity and polyphenols contents in Calafate fruits has been poorly explored.

In this regard, Khan et al. (2019) pointed out that, among plant secondary metabolites, phenolic compounds have received considerable attention, being the most studied natural product class for pharmaceutical and dietary concerns owing to their significant antiallergic, antimicrobial, anti-inflammatory, and antioxidant activities. Plant phenolics have radical scavenging and metal chelating potential, being useful to the

treatment of worldwide diseases like cardiovascular disorders and cancer (Mocan et al. 2016). In Chilean Patagonia, edible fruits of the woody or shrub forest species belonging to the Berberidaceae family, such as Calafate, are rich in antioxidant compounds (Brito et al. 2014; Fuentes et al. 2016; Ruiz et al. 2010; Schmeda-Hirschmann et al. 2019; Simirgiotis et al. 2013; Ulloa-Inostroza et al. 2017). Our results showed that the antioxidant activity, as well as total phenols and total anthocyanin content of Calafate extracts, increased concomitant with the increase of extract concentrations. Also, we found that despite the antioxidant activity of crude and the anthocyanin-rich extract was very similar, total phenols were higher in crude extract (around 850 μg CAE g^{-1}DW) compared with anthocyanin-rich extract (around 700 μg CAE g^{-1}DW). The high total phenols were due to in the crude extracts of Calafate fruits are present not only anthocyanins compounds but also another type of phenolic compounds like phenolic acids and flavonoids (Brito et al. 2014; Chamorro et al. 2019; Ruiz et al. 2013b; Ruiz et al. 2014). Flavonols and flavan-3-ols, mainly quercetin derivates and epicatechin, were detected in Calafate fruits by Ruiz et al. (2010). In the same way, Ruiz et al. (2013b) reported 20 hydroxycinnamic acids from Calafate fruits, being 5-caffeoylquinic acid the main compound found. According to the authors, other 13 hydroxycinnamoyl quinic acids and 6 caffeic acid esters with aldaric acid derivatives were also identified, with the glucaric-based hydroxycinnamic acid derivatives accounted for almost the half of total content of this kind of phenolic compounds in Calafate berries. Also, 3 phenolic acids (feruloyl quinic acid, chlorogenic acid, and neochlorogenic acid) and 5 flavonols (hyperoside, isoquercitrin, quercetin, rutin, myricetin, and isorhamnetin) were also detected by Brito et al. (2014) in Calafate berries. Likewise, Chamorro et al. (2019) evaluated the polyphenol composition of Argentinean Patagonia berries, including *B. microphylla*, *Berberis darwinii*, and *Fragaria chiloensis ssp. chiloensis f. patagonica* and found that the most complex polyphenol profile was found in the *Berberis* samples, with 10 anthocyanins, 27 hydroxycinnamic acids, 3 proanthocyanidins, 2 flavan-3-ol, and 22 flavonols. Brito et al. (2014) and Ruiz et al. (2010) demonstrated that Maqui (*Aristotelia chilensis*) and Calafate berries showed higher total polyphenol contents compared to other Patagonian and commercial berries. Thus, a significantly higher total phenol content [expressed as μmol of gallic acid equivalents (GAE) per gram of FW] for Maqui (97 μmol GAE g^{-1} FW), Calafate (87 μmol GAE g^{-1} FW), and Murta (*Ugni molinae*; 32 μmol GAE g^{-1} FW) compared to Blueberry (*Vaccicium corymbosum*; 17 μmol GAE g^{-1} FW) was reported by Ruiz et al. (2010). Additionally, some reports showed similar values of the total polyphenol content (as mg GAE per gram of DW) for Calafate (33.9 mg GAE g^{-1} DW) (Speisky et al. 2012), while other works revealed higher content of phenolic compounds for Calafate (65.5 mg GAE g^{-1} DW), followed by Arrayán (*Luma apiculata*; 27.6 mg GAE g^{-1} DW) and lower values for Murta (9.2 mg GAE g^{-1} DW) (Brito et al. 2014). Moreover, according to Ruiz et al. (2010), Calafate fruits showed a comparable flavonoid content (0.16 μmol g^{-1} FW) to those detected in Maqui fruits (0.12 μmol g^{-1} FW).

Regarding the polyphenol composition of the Patagonian berries, Maqui and Calafate have anthocyanins as the main molecule (Brito et al. 2014; Fuentes et al. 2016; Ruiz et al. 2010, 2013a; Schmeda-Hirschmann et al. 2019; Simirgiotis et al. 2013; Ulloa-Inostroza et al. 2017). As expected, our results showed that anthocyanin-rich extracts of Calafate fruits presented higher total anthocyanin concentration (32 mg cyanidin 3-O-glucoside g^{-1}DW) compared to crude extracts (25 mg cyanidin 3-O-glucoside g^{-1}DW), which were increased concomitant at increasing extract concentration. Several works suggest that the highest total anthocyanin content of berries can be found in Calafate and Maqui fruits, especially those harvested in the Chilean Patagonia (Ruiz et al. 2010, 2013a; Ulloa-Inostroza et al. 2017). Also, it was reported that the total anthocyanin contents were higher in Calafate fruit extract (between 23 and 36 μmol g^{-1} FW) (Ruiz et al. 2013a), followed by Maqui berries (between 16 and 20 μmol g^{-1} FW), being all high values compared to those detected in blueberries (2.0 μmol g^{-1} FW) (Ruiz et al. 2010). Similar results were reported by Brito et al. (2014), who found higher anthocyanin content (as mg cyanidin 3-O-glucoside g^{-1} DW) in fruits of Calafate (51.6), followed by Arrayán (15.2) and Murta (6.9). Regarding the anthocyanin profile of Calafate fruits, Ruiz et al. (2010) reported the identification of 18 anthocyanins in Calafate fruit, with total anthocyanin concentrations surpassing the polyphenol levels of several widely consumed berries, while 31 anthocyanins, mostly branched 3-O-glycosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin, were identified in Calafate fruits by Brito et al. (2014). Our findings also showed that the most abundant anthocyanidin in both crude extract and anthocyanin-rich extract of Calafate fruits was delphinidin, followed by malvidin, petunidin, cyanidin, and very low concentrations of peonidin. It is reported that the highest antioxidant activity among anthocyanin is delphinidin due to a chemical structure (Lazzè et al. 2004). In fact, Ruiz et al. (2010) found that the presence of delphinidin-3-glucoside, malvidin-3-glucoside, and petunidin-3-glucoside in Calafate fruits arouse the attention of this berry consumption. Indeed, according to Bustamante et al. (2018), the high antioxidant capacity detected in Calafate berries can be explained to their high anthocyanin diversity.

About 70–80% population of developing countries depends on plant-derived products for their health treatment due to that they are easily available, cheap, and without significant side effects. The use of herbal medicine is functional for alleviating many chronic and severe infections, providing therapeutic relief (Battino et al. 2019; Bhardwaj et al. 2018; Mollica et al. 2017). Thus, the epidemiological evidence

explaining the beneficial effect of consuming a diet rich in foods rich in polyphenols is strong (Amiot and Riva 2016; D'Archivio et al. 2010; Hussain et al. 2016). Noteworthy, our results revealed that both crude extract and anthocyanin-rich extract of Calafate fruits significantly reduced in vitro viability and migration of AGS and G415 cells and that the inhibitory effects were dose-dependent. Previous reports have used different cancer cell lines in order to demonstrate that anthocyanins have a role against several cancer-inducing processes (Wang et al. 2017). According to Adamus et al. (2019), the Pakistani plant extract of *Berberis orthobotrys* (BORM), derived from the root bark of the plant, exhibited antitumor effects on different types of breast and bone cancer cells in vitro. Also, BORM treatment has been shown to be effective against RMA cells with low side effects on healthy cells. Similarly, Reyes-Farias et al. (2015) observed that the extracts of maqui and Calafate fruits displayed a reduction of nitric oxide (NO) production, inhibition of the induction of nitric oxide synthase (NOS) and TNF-alpha, and induction of the interleukin 10 (IL-10) gene expression. On this basis, it has been suggested that they could be potential therapeutic tools against the co-morbidity associated with the development of obesity. Lately, Ribera-Fonseca et al. (2020) found that leaf extracts of highbush blueberry at a concentration equal or higher than 400 μg mL^{-1} caused more than 50% of AGS cell viability inhibition, reaching 60% of inhibition at the highest doses studied (3200 μg mL^{-1}). In contrast, fruit extract provoked only about 10% of inhibition of AGS cell viability at extract exposure during 48 h at the highest concentrations (1600 μg mL^{-1}). These findings showed that Calafate fruits displayed significantly higher inhibitory effects on AGS viability compared to blueberry fruits and that Calafate leaves could exhibit interesting antiproliferative activity, which has not been explored yet.

On the other hand, we also found that the anthocyanin-rich extract was more effective than crude extract in reducing the cell viability in the AGS cells compared to G415 cells. Similarly, the anthocyanin-rich extract was more effective than crude extract in reducing the cell migration in the G415 cells compared to AGS cells. In concordance with our results, Yi et al. (2005) evaluated the antiproliferative and proapoptotic effect of two colon cancer (HT-29 y Caco-2) cell lines with different fractions of phenolic compounds in three blueberry cultivars (Briteblue, Tifblue, and Powderblue). These authors indicated that the greatest inhibitory activity was found in the anthocyanin fraction, with a 50% inhibition of cellular proliferation, concluding that the blueberry intake could reduce the colon cancer risk. Accordingly with our results, Ribera-Fonseca et al. (2020) reported that anthocyanin-rich extracts of highbush blueberry significantly decreased AGS cell viability by around 20–30% at 100 μg mL^{-1}compared to the negative and positive control (quercetin 4 μM). Peiffer (2018) pointed out that the preparation of fractions possessing enriched amounts of the active constituents of Black Raspberry, including anthocyanins, protocatechuic acid, quercetin, ellagic acid (all of which have exhibited anti-cancer properties), may improve patients' access to the anti-cancer benefits afforded by this species. Likewise, Alarcon-Barrera et al. (2018) reported that the crude extract of Andean blueberries showed a higher protective effect against cytotoxic oxidative damage of human dermal fibroblasts. It was due to higher values of phenolic compounds such as flavonoids and anthocyanins of blueberries; however, the authors highlighted that both fruits represent a relevant source of bioactive compounds with promising benefits to human health. Faria et al. (2010) indicated that Blueberry anthocyanins and anthocyanin-pyruvic acid adduct extracts (250 μgmL^{-1}) had anti-invasive potential and inhibited proliferation of MDA-MB-231 and MCF7 breast cancer cell lines, acting as chemo-inhibitors. In a recent study, Giampieri et al. (2018) have shown that the overexpression of anthocyanidin synthase (ANS) gene in strawberry increased antioxidant capacity and cytotoxic effects on human hepatic cancer cells (human liver cancer cells HepG), demonstrating the high positive relationship among anthocyanin content and anti-cancer action in this fruit species. Our results showed that both extracts presented, as a major component, high concentration of delphinidin, showing 2-fold in the anthocyanin-rich extract compared to crude extract. Therefore, studies with commercial anthocyanin-rich extracts from Maqui (Delphinol®), mainly delphinidin and cyanidin, have been used to treat several diseases (Hidalgo et al. 2014). On the other hand, the anti-cancer effect of delphinidin and cyanidin on the induction of apoptosis of colon and human uterine cells (Lazzè et al. 2004). These authors confirmed that delphinidin, more than cyanidin, was able to induce apoptosis on these human cells, suggesting that the hydroxyl group in the position 5′ of B ring in the delphinidin, which is absent in cyanidin, would be very important in the anti-cancer activity of this molecule. Our findings also found that delphinidin was the most anthocyanidin compound present in our anthocyanin-rich extract of calafate, in which this compound could be responsible for reducing viability and migration of both AGS (gastric cancer) and G415 (gallbladder cancer) cell lines. Many reports showed that anthocyanin-enriched extracts reduce the actions of oxidative stress, proliferation, inflammation, angiogenesis, and invasion of cancer cells (Tsakiroglou et al. 2019; Wang and Stoner 2008). As mentioned above, our results showed that Calafate extract is rich in anthocyanin, and the major anthocyanidin molecule detected was delphinidin, followed by malvidin, petunidin, cyanidin, and peonidin. As a major anthocyanidin, delphinidin has a variety of biological functions that include an antioxidant, anti-inflammatory, anti-angiogenic, anti-invasiveness, and anti-carcinogenic effect (Watson and Schönlau 2015). Lamy et al. (2007) showed that delphinidin strongly suppresses the

transformation and migration of cancer cells during tumorigenesis compared with other anthocyanidin compounds tested. In other studies, it is reported that delphinidin suppresses proliferation and stimulates apoptosis of diverse cancer cells including breast (Ozbay and Nahta 2011), colon (Yun et al. 2009), liver (Feng et al. 2010), lung (Pal et al. 2013), prostate (Hafeez et al. 2008), and skin cancers (Kwon et al. 2009) through inhibition of various signal transduction cascades and cell cycle regulatory proteins. Lim et al. (2016) have been reported that delphinidin inhibited migration and induced apoptosis of human ovarian carcinoma cells through blocking AKT and ERK1/2 MAPK signaling pathways in a dose-dependent manner. AKT/PKB plays a significant role in endothelial cell migration (Michaelis 2014). In a recently published paper, we have shown that leaf extracts of highbush blueberries significantly decreased the viability and migration capacity of AGS cells (Ribera-Fonseca et al. 2020). In this work, we also evaluated the effect of leaf extracts over the expression of proteins related to gastric cancer (AKT/mTOR and mitogen-activating protein kinase (MAPK) signaling pathways). These results showed that the total protein of mTOR and AKT were not affected. However, it is observed almost a complete inhibition of ERK1/2 (p-ERK1/2) phosphorylated portion at a concentration higher than 100 $\mu g\ mL^{-1}$. Moreover, in this work, we found that blueberry extracts at the same concentrations slightly decreased the expression of the phospho-P70S6K protein. Based on these supports and according to our results, we suggest that a possible regulatory mechanism could be through the AKT7mTOR and MAPK signaling pathway modulation.

5 Conclusions

In spite of crude and anthocyanin-rich extracts of Calafate fruits reduced in vitro viability, and migration capacity of gastric (AGS) and gallbladder (G415) cancer cell lines, the extract rich on anthocyanins were significantly more effective. This outcome could mainly be due to the higher concentration of delphinidin present in this extract, the molecule recognized by its high antioxidant potential compared to other phenolic and anthocyanidin compound. Therefore, this research offers meaningful findings for further investigations, indicating that Calafate fruits are a promising material to discover phytopharmaceuticals compounds to manage human health. A complete secondary metabolite characterization of the crude and anthocyanin-rich extract of Calafate fruits could be a further step of our study, which certainly helps us to understand how the healthy properties of Calafate fruits have related a pool of natural products, probably another phenolic or alkaloid acting in a synergic way.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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