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Chemical characterization of polyphenol extracts from Andean and industrial Solanum tuberosum tubers and their cytotoxic activity on human hepatocarcinoma cells

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Research

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CONFLICTS OF INTEREST
There are no conflicts of interest for any of the authors.

ABSTRACT

Background: Polyphenols are plant metabolites that have been largely studied for their beneficial effect on human health. Potato is one of the most important crops worldwide and is a relevant source of human dietary nutrients and antioxidants. Particularly, pigmented potatoes contain the highest levels of polyphenolic compounds. Hepatocellular carcinoma is one of the most frequent types of cancers worldwide and despite the existence of treatments; it is yet associated with a high mortality rate. Thus, new drugs are needed, and polyphenols are a potential source of anti-hepatocellular carcinoma compounds. The objectives of this study were to determine the content of different groups of polyphenols (phenolic acids, anthocyanins and flavan-3-ols) in five potato polyphenolic extracts, and to study their antioxidant and cytotoxic activities on a human hepatocellular carcinoma cell line.

Methods: four Andean varieties and one industrial potato variety were selected for this study. Polyphenolic quantification and composition were determined by spectrophotometric assays and HPLC-DAD analysis. The antioxidant and cytotoxic activities were evaluated through DPPH and MTS assays respectively.

Results showed that pigmented varieties possessed higher levels of the analyzed phenolic compounds. HPLC analysis showed that chlorogenic acid was the main phenolic acid in all the potato polyphenolic extracts. Also, pigmented potatoes presented higher levels of antioxidant activity compared to non-pigmented ones, showing a positive correlation with the total phenolic content. Finally, treatment with three of the studied potato polyphenolic extracts reduced the viability of Hep3B. Furthermore, one extract from a non-pigmented variety affected cell viability to a similar extent as extracts from pigmented potatoes, suggesting that other compounds, besides anthocyanins, may be responsible of the cytotoxic effect of this polyphenolic extracts.

Conclusion: These results suggested that polyphenolic compounds present in the Andean potato varieties could be used as a potential source of anti-

hepatocarcinoma drugs.

Keywords: potato, phenolic acids, anthocyanins, flavan-3-ols, antioxidant activity, cytotoxicity.

INTRODUCTION

The potential health benefits of antioxidant compounds have been broadly reported. Furthermore, several epidemiological studies showed that consuming foods with high levels of antioxidants, like polyphenols, might correlate with lower risk of developing some diseases such as cardiovascular diseases, diabetes or cancer [1-5]. Polyphenols are a group of plant metabolites whose main role is to protect the plant from different types of abiotic and biotic stresses, such as: UV radiation, wounds, ROS, and herbivores [6, 7]. In addition to their relevance in plants, the effects of these compounds on human health have been extensively studied. Different *in vitro* and *in vivo* assays have described polyphenols as antioxidant, anti-inflammatory, anti-microbial and anti-cancer compounds [8-10]. Particularly, *in vitro* results showed that phenolic acids, like chlorogenic acid (CGA) or anthocyanins, like pelargonidin, exerted different biological activities in various cellular models [11-14].

Potato (*Solanum tuberosum*) is one of the most important crops worldwide, and is a significant source of carbohydrates, minerals and vitamins for the human diet [15, 16]. Also, due to its high intake levels, potato represents one of the main sources of dietary antioxidants such as, carotenoids and polyphenols [17]. Compared to non-pigmented potatoes, pigmented varieties contain higher levels of polyphenols, mainly because of the presence of both anthocyanins and phenolic acids [18-20]. Several studies reported CGA as the main phenolic acid in both pigmented and non-pigmented potato varieties [21-23]. Moreover, pigmented and non-pigmented potatoes present a similar profile of phenolic acids but, pigmented potato varieties also present antochyanins like pelargonidin and malvidin [24] and, compared to non-pigmented, pigmented potato varieties exhibit higher levels of antioxidant capacity [23, 25-28].

Hepatocellular carcinoma (HCC) is one of the most frequently occurring tumors worldwide and is associated with high mortality [29, 30]. Different etiological factors are related to the development of HCC, including chronic hepatitis B or C virus infections, mycotoxin consumption and alcoholic cirrhosis [29]. Despite the existence of treatments, patients diagnosed with late HCC still have a poor prognosis [31, 32]. Thus, the development of new anti-HCC drugs is essential, and natural compounds like polyphenols, are a promising source of potential molecules for cancer treatment. Particularly, anti-HCC activity has been described for polyphenolic extracts (PEs) from various commonly consumed vegetables or herbs [33-35]. In the case of potato polyphenolic extracts (PPEs) their cytotoxic activity has been demonstrated *in vitro* in different tumoral cell lines, including HCC [21, 36-40]. The objectives of this work were to characterize and quantify the composition of polyphenolic extracts from potatoes with different pigments (*S. tuberosum L ssp. tuberosum and S. tuberosum L ssp. andigena*), and to evaluate their antioxidant and cytotoxic activities against human HCC cells *in vitro*.

MATERIALS AND METHODS

Plant material

Four *S. tuberosum L ssp. andigena* potatoes varieties (CCS1283, CCS1307, CS1418 and CCS1385), here after referred to as Andean, were grown in a field located in Yavi Department (22° 6' 4" S, 65° 35' 44" O, 3377 MAMSL), Jujuy, Argentina, during the 2012/2013 season. One *S. tuberosum* L ssp. *tuberosum* variety (52.1-10) here after referred to as the industrial variety was grown in an experimental field located in Balcarce (37° 49' 9.65" S, 58° 13' 11" W, 130 MAMSL), Buenos Aires, Argentina, during the 2012/2013 season of McCain Argentina S.A. All potato varieties and their pigmentation and morphological characteristics are presented in Figure 1. All varieties were planted in random plots and harvested at the end of their respective cycles. The tubers were transported to the laboratory where they were washed, peeled and potato flesh from the different varieties were frozen in liquid nitrogen and stored at -80 °C for further analysis.

Preparation of potato polyphenolic extracts

Two grams of potato tuber flesh were homogenized with liquid nitrogen, and extracted with 40 mL 100 % methanol (HPLC grade, Pharmco-aaper) at 4 °C overnight, in darkness with constant agitation. Only for the purple variety, CCS1385, the extracts were prepared from the whole tubers due to their small size. Then, extracts were centrifuged at 6000 rpm for 20 min at 4 ° C, concentrated using a rotary evaporator (Senco) and resuspended in 1 mL of methanol 30 % (v/v). After centrifugation at 13000 rpm for 15 min at 4 °C the supernatant was filtered through a 0.22 µm filter. Potato polyphenolic extracts (PPEs) were stored at -20 °C until analysis.

Determination of total phenolic content

The total phenolic content was determined using the Folin - Ciocalteu colorimetric method, as previously described [41]. Briefly, 20 µL of PPE were diluted to a final volume of 0.5 mL with methanol (HPLC-grade). Then 7.5 mL of water was added, mixed with 0.5 mL of Folin - Ciocalteu reagent (Merck), diluted in water (1:7), and after 3 min of reaction, 1 mL of 0.5 M Na_2CO_3 was added and allowed to react for 10 min. Finally, absorbance at 725 nm was measured in a visi-

ble light spectrophotometer (Hitachi 156 U-1900). Distilled water was used as a blank. Chlorogenic acid (CGA, Sigma-Aldrich) was used as a standard, and total phenolic content was expressed as µg of CGA equivalents per 1 g of potato tuber fresh weight (µg of CGA equiv. / gfw). Each sample was measured in triplicate in four independent experiments.

Determination of total flavan-3-ols

Total flavan-3-ols were calculated as previously described [42]. Briefly, 100 µL of PPE were diluted to a final volume of 200 µL and mixed with 1 mL of 4-(Dimethylamino) - cinnamaldehyde (DMCA, Sigma), 30/70 (v/v). Finally, absorbance at 640 nm was measured. Catechin (Sigma) was used as standard, and the total flavan-3-ols quantity was expressed as µg of catechin equivalents per 1 g of potato tuber fresh weight (µg of catechin equiv. / gfw). Each sample was measured in triplicate in four independent experiments.

Determination of total monomeric anthocyanin content

Total anthocyanin content was calculated using the pH-differential method [43]. Two dilutions of the sample were prepared: 100 µL of PPE were diluted with 2000 µL of 0.025 M KCl buffer (pH 1) and another 100 µL of PPE were diluted 2000 µL of 0.4 M $CH_3CO_2Na_3$ (pH 4.5). After 15 min of reaction, absorbance at 500 nm and 700 nm, respectively was measured in a visible light spectrophotometer. The difference in absorbance (A) at different pH values and wavelengths was calculated according the equation below:

$A = (A_{500}\text{-}A_{700})\ pH1 - (A_{500}\text{-}A_{700})\ pH4.5$

Anthocyanin concentration (AC) of the PPE was calculated in terms of cyaniding – 3 - glucoside, using the following formula:

$AC\ (mg/L) = (A \times MW \times DF \times 1000) / (e \times 1)$

Where, MW is cyanidin-3-glucoside (C-3-G) molecular weight of 449.2 g / mol; *e,* is the extinction coefficient of 26900 L / cm / mol; and DF, is the dilution factor. Anthocyanin content was reported as µg of C-3-G per 1 g of potato tuber fresh weight (µg C-3-G equiv. / gfw). Each sample was measured in triplicate in four independent experiments.

Determination of antioxidant activity

The total hydrophilic antioxidant activity was measured using the DPPH (2, 2 – diphenyl - 1 -picrylhydrazyl, Sigma) assay, as previously described [44]. Briefly, 10 µL of PPE were diluted to a final volume 150 µL with methanol (HPLC-grade). Then, 4 mg of DPPH were diluted in 100 mL of methanol to obtain a working solution with an absorbance at 515 nm of 1-1.1. Diluted PPE was mixed with 2.85 mL of DPPH and incubated 24 h at room temperature in the dark. Finally, absorbance at 515 nm was measured in a visible light spectrophotometer. Methanol (HPLC-grade) was used as a blank, and trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid, Sigma) was used for the standard curve. Antioxidant activity was expressed as µg of trolox equivalents per 1 g of potato tuber fresh weight (µg trolox equiv. / gfw). Each sample was measured in triplicate in four independent experiments.

Characterization and quantification of phenolic acids, flavan-3-ols and anthocyanidins by HPLC-DAD

Quantification of phenolic acids, flavan-3-ols and anthocyanidins was carried out with a high performance liquid chromatography (HPLC) system (Shimadzu, Prominence) equipped with a diode array detector (DAD). To analyze phenolic acids and flavan-3-ols, 20 µL of the sample (previously filtered through a 0.45 µm PVDF membrane, Millipore), were injected using a flow rate of 1 mL/min, onto a C-18 Phenomenex Luna column 153 (250 x 4.6 mm i.d.; 5 µm particle size). The mobile phase was: (A) acidified distilled water (pH 2.3) and (B): CH_3CN. The gradient used was: 0-20 min, linear gradient of B 20% to 100%; 20-25 min, B was decreased back to 20% and 25-30 min conditions were kept constant.

For characterizing anthocyanidins, only PPEs from the pigmented varieties (CS1418, CCS1385) were used. The samples were hydrolyzed with a final concentration of 2 M HCl at 100° C for 1 h. Anthocyanin detection was also carried out with an octadecylsilane C - 18 column (250 L x 4.6, 5 µm particle size). A flow rate of 0.8 mL/min was used and sample injection volume was 20 µL. The mobile phase was: (A) 4% H_3PO_4 buffer, (B) CH_3CN. The gradient used was: 0-25 min, B was increased from 15% to 25%; 25-30 min, B was increased to 27%; 30.5-33 min B was returned to 15%.

The phenolic acids [CGA, caffeic acid (CA), ferulic acid (FA) and coumaric acid (CoA), Sigma], anthocyanins (pelargonidin, peonidin, petunidin, malvidin and delphinidin, Sigma) and flavan-3-oles [catechin and epicatechin, Sigma] were charazterized and quantified by comparing retention times and spectra of the different standards.

Human hepatocarcinoma cell line

Hep3B cells (American Type Tissue Collection, ATCC), derived from human hepatocellular carcinoma, were grown in minimal Eagle's medium (MEM, Gibco), supplemented with 100 mL / L fetal bovine serum (FBS, Natocor), 2 mmol / L glutamine (Gibco), 1.5 g/L sodium bicarbonate, 1 mmol / L nonessential amino acids (Gibco), and 1 mmol / L sodium pyruvate (Gibco). For experiments, FBS was reduced to 10 mL / L. The cells were cultured at 37 °C in a humidified atmosphere, containing 5 % CO_2.

Determination of cell cytotoxicity

To analyze the cytotoxic activity of the PPEs, Hep3B cells were seeded in 96 multiwell plates and incubated at 37° C, 5% CO_2 for 24 h. Cells were treated with different concentrations (25, 50, 100, 200 and 400 µg / mL) of the PPEs for 24 h. Controls with 30 % methanol and non-treated cells were also included. After incubation, cytotoxicity was measured by an MTT assay (3 - (4, 5 -dimethiylthiazol – 2 – yl) 5 - (3 – carboxymethoxyphenyl) – 2 - tetrazolium, Sigma) and absorbance was read at 570 nm. The viability percentage was calculated as % = (Absorbance of treated cells/ Absorbance of control (30 % methanol) cells) x 100. The 50% cytotoxic concentration (CC_{50}) was calculated for each potato variety.

Statistical analysis

All experiments were carried out four times, and expressed as the mean ± standard error of the mean (SEM). Phenolic acids, flavan-3-ols, and anthocyanins quantification, HPLC analysis and cytotoxic activity experiments of the PPEs were analyzed using parametric one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. Correlations among TPc, total anthocyanin content, flavan-3-ols, CGA, CA, FA, CoA, and antioxidant activity (DPPH) were calculated following Pearson's correlation method. All statistical analyses were performed using GraphPad Prism 6.

RESULTS

Quantification of total phenolic acids, monomeric anthocyanins, and flavan-3-ols contents

First, the total amount of three groups of polyphenols (phenolic acids, flavan-3-ols and anthocyanins) in the PPEs of the different varieties was quantified. Total phenolic compounds from four Andean varieties (CCS1283, CCS1307, CS1418 and CCS1385) and the industrial variety (52.1-10) were quantified using the Folin - Ciocalteu method. As shown in Figure 1, only CS1418 and CCS1385 varieties exhibit pigmented flesh, being white with red spots or purple, respectively. The quantification resulted in a total phenolic acid content ranging from 427.22 ± 68.04 to 1118.15 ± 90.33 µg of CGA equiv. / gfw. CS1418 and all white fleshed varieties showed similar values of this group of compounds, with no significant differences between Andean varieties (CS1418, CCS1283 and CCS1307) and the industrial variety (52.1-10). The purple variety CCS1385 contained the highest levels of phenolic acids, 1118.15 ± 90.33 µg of CGA equiv. / gfw (Figure 2A).

Figure 1. Potato tubers from studied Andean and industrial varieties. (A) Morphology of entire tubers as well as cross-sections is shown. The arrow indicates the cross-section of the 1385 variety. Scale bar = 10 cm. **(B)** Table shows subspecies, common name, accession numbers used for their identification at the Germoplasm bank (INTA-EEA-Balcarce), and the description color of flesh and skin from varieties.

A

B

Specie	Number bank design*	Common name	Skin color	Flesh color
S. tuberosum L. ssp. *tu-*	52.1-10	Summerside	White	White
S. tuberosum L. ssp. *Andíge-*	CCS1283	Waicha	Reddish	White
S. tuberosum L. ssp. *Andíge-*	CCS1307	Moradita	Purple	Yellow
S. tuberosum L. ssp. *Andíge-*	CS1418	Chaqueña	White	White with Red Spots
S. tuberosum L. ssp. *Andíge-*	CCS1385	Moradita	Purple	Purple

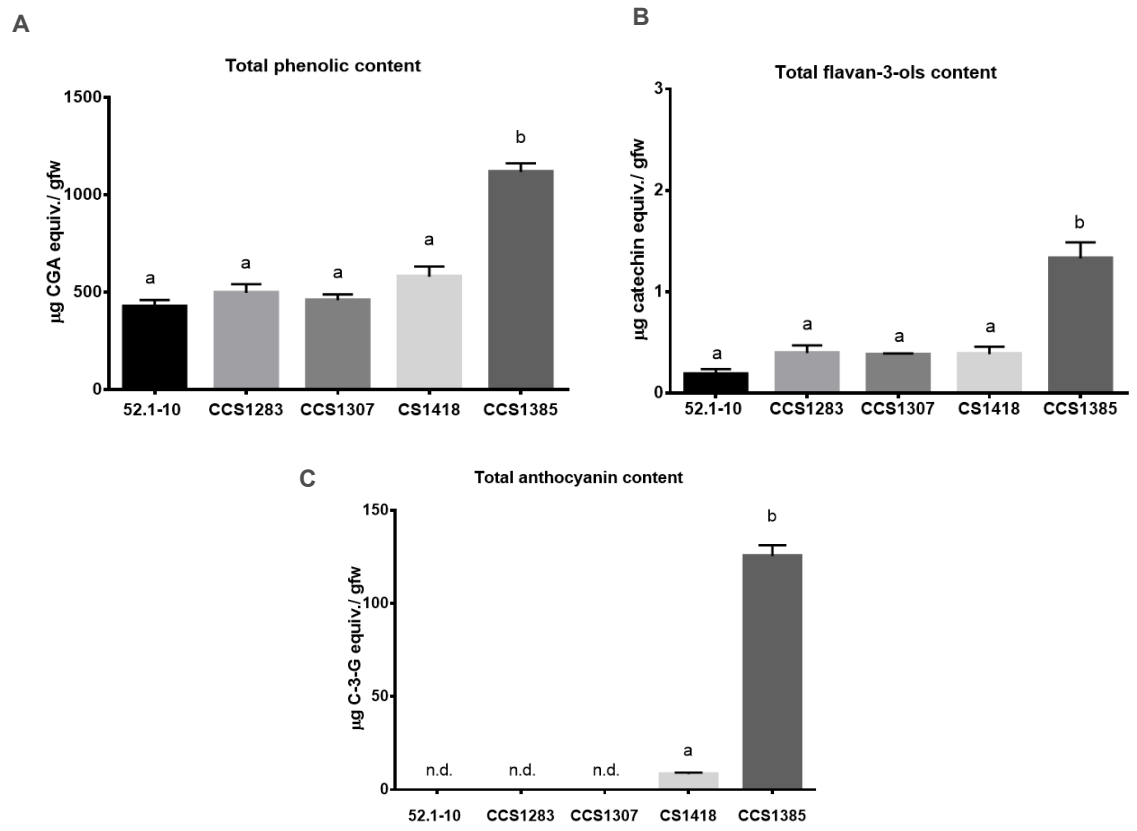

Figure 2. Phenolic acids, anthocyanins and flavan-3-ols content in PPEs of Andean and industrial varieties. (A). Total phenolic content was determinated by the Folin-Ciocalteu reagent. Values were expressed as µg CGA equiv/g fw. **(B)** Total flavan-3-ol content was calculated and values were expressed as µg catechin equiv/g fw**. (C)** Total monomeric anthocyanin content was determined by the pH differential method. Values were expressed as µg C-3-G equiv/g fw. Different letters indicate significant differences between varieties by one-way ANOVA followed by Bonferroni's multiple comparison test ($p< 0.05$). n.d. indicated not detected. Results are expressed as the mean ± SEM of four independent experiments.

Then, the total flavan-3-ol levels were quantified in all the PPEs. Coinciding with phenolic acid content, the purple variety CCS1385 presented the highest amounts of flavan-3-ols: 1.33 µg catechin equiv./gfw. Again, none of the other PPEs from the other four varieties (Andean or industrial) presented significant differences in flavan-3-ol levels (Figure 2B).

Finally, total anthocyanin content was measured in all PPEs by the differential pH method. No anthocyanins were detected in white or yellow fleshed varieties (52.1-10, CCS 1283, and CCS1307). The purple variety CCS 1385 showed the highest levels of total anthocyanin content: 125.43 ± 10.16 µg C-3-G equiv. / gfw (Figure 2C).

Characterization and quantification of phenolic acids, flavan-3-ols and anthocyanidins content by HPLC-DAD

To determine the content of the phenolic acids: chlorogenic acid (CGA), caffeic acid (CA), ferulic acid (FA), and coumaric acid (CoA), the five PPEs were analyzed by HPLC-DAD. Flavan-3-ols catechin (CT) and epicatechin (ECT) were also quantified. Table 1 shows the phenolic acid profiles found in the different PPEs. CGA represented the main phenolic acid in all the PPEs, showing significantly higher levels in the pigmented varieties. CA (only absent in CCS1307), CoA (absent in 52.1-10 and CC1418) and FA appeared in lower proportions, than CGA, and in similar quantities in all the PPEs. CT and ECT were not detected in any of the PPEs by this method.

Based on the results of total anthocyanins content obtained by the differential pH method, only the two pigmented varieties, CS1418 and CCS1385, were analyzed by HPLC. Table 2 shows the anthocyanidin profiles found for both cultivars. In agreement with the monomeric anthocyanin quantification, PPE from CCS1385 showed the highest levels of anthocyanidins. Also, the anthocyanidin profile CCS1385 was more diverse than that of CS1418. Particularly, pelargonidin was the most abundant anthocyanin found in CS1418, and in a lower proportion peonidin and cyanidin. Instead, CCS1385 presented a completely different profile, with malvidin as the main anthocyanin, followed by peonidin and in similar quantities pelargonidin, delphinidin, and cyanidin.

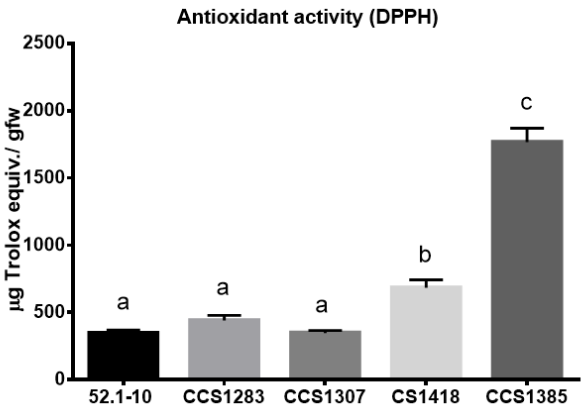

Figure 3. Antioxidant activity of PPEs of Andean and industrial varieties. Total antioxidant activity was determinated by DPPH assay. Values were expressed as µg trolox equiv/g fw. Different letters indicate significant differences between varieties by one-way ANOVA followed by Bonferroni's multiple comparison test ($p< 0.05$). Results are expressed as the mean ± SEM of four independent experiments.

Determination of antioxidant activity by the DPPH assay

The antioxidant activity of the five PPEs was measured by the DPPH assay. This activity ranged from 344.71 ± 47.09 to 1765.73 ± 207.17 µg Trolox equiv. / gfw. Similarly to what was observed in the polyphenol quantification, both PPEs from pigmented varieties (CCS1385 and CS1418) showed high antioxidant activity, exhibiting 2 to 6 fold greater values than PPEs from non-pigmented varieties. The PPE from the purple variety (CCS1385) presented the highest antioxidant activity: 1765.73 ± 207.17 µg trolox equiv. / gfw. All PPEs from white or yellow fleshed varieties (52.1-10, CCS1283 and CCS1307) presented lower and similar values, showing no significant differences between them (Figure 3).

Correlation between different groups of polyphenols and antioxidant activity

Correlation between total phenolic compounds, flavan-3-ols, CGA, CA, FA, CoA and the antioxidant activity (DPPH) of the five PPEs was analyzed. Table 3 shows the R^2 and p values for the correlation analyses. A positive and significant relationship was found between total phenolic compounds and antioxidant activity. Also, the analysis of the main phenolic acids presented in all varieties demonstrated that CGA and CA exhibited a significant correlation with the antioxidant activity (DPPH).

Table 1. Characterization and quantification of PPEs phenolic acids and flavan-3-ols content.

Phenolic acid	52.1-10	CCS 1283	CCS 1307	CC 1418	CCS 1385
CGA	21.55 ± 2.86 [a] (71.29)	17.44 ± 5.62 [a] (70.32)	26.89 ± 8.52 [a] (77.54)	143.27 ± 13.79[b] (96.01)	390.44 ± 65.66 [c] (94.64)
CA	3.44 ± 0.75 [a] (11.38)	2.09 ± 0.59 [a] (8.43)	N/D	3.52 ± 1.24 [a]	13.61 ± 1.33 [b] (3.3)
CoA	3.8 ± 0.38 [a] (12.57)	4.33 ± 1.45 [a] (17.46)	4.37 ± 0.48 [a] (12.6)	N/D	6.69 ± 2.34 [a] (1.61)
FA	1.44 ± 0.16 [a] (4.63)	0.94 ± 0.29 [a] (3.79)	1.42 ± 0.56 [a] (4.09)	2.44 ± 1.09 [a] (1.64)	1.8 ± 0.58 [a](0.44)
CT	N/D	N/D	N/D	N/D	N/D
ECT	N/D	N/D	N/D	N/D	N/D
TOTAL	30.23 ± 4.15	24.8 ± 7.95	34.68 ± 9.56	149.23 ± 16.12	412.54 ± 69.97

Metabolite levels were determined by HPLC-DAD and expressed as µg/gfw ± SD from at least three independent extractions. Numbers in parentheses indicate the % of each compound(s) with respect to the corresponding total. CGA: chlorogenic acid; CA: caffeic acid; CoA: coumaric acid; FA: ferulic acid; CT: catechin; ECT: epicatechin. N/D: not detected. Different letters indicate significant differences by ANOVA, followed by Bonferroni's multiple comparison test ($p< 0.05$).

Table 2. Characterization and quantification of anthocyanidin content from pigmented varieties.

	Anthocyanidins concentration (µg/gfw)	
Anthocyanin	**CC 1418**	**CCS 1385**
Delphinidin	N/D	1.44 ± 0.29(0.65)
Cianidin	N/D	0.79 ± 0.67(0.36)
Pelargonidin	14.83 ± 4.21 [a](77.93)	1.36 ± 0.13 [b](0.61)
Peonidin	4.2 ± 0.22 [a](22.07)	21.04 ± 8.29 [b](9.48)
Malvidin	N/D	197.39 ± 8.72(88.91)
Petunidin	N/D	N/D
TOTAL	19.03 ± 4.43	222.02 ± 18.1

Metabolite levels were determined by HPLC-DAD and expressed as µg/gfw ± SD from at least three independent extractions. Numbers in parentheses indicate the % of each compound(s) with respect to the corresponding total. N/D: not determined. a, b: Different letters indicate significant differences by Student's t-test ($p < 0.05$).

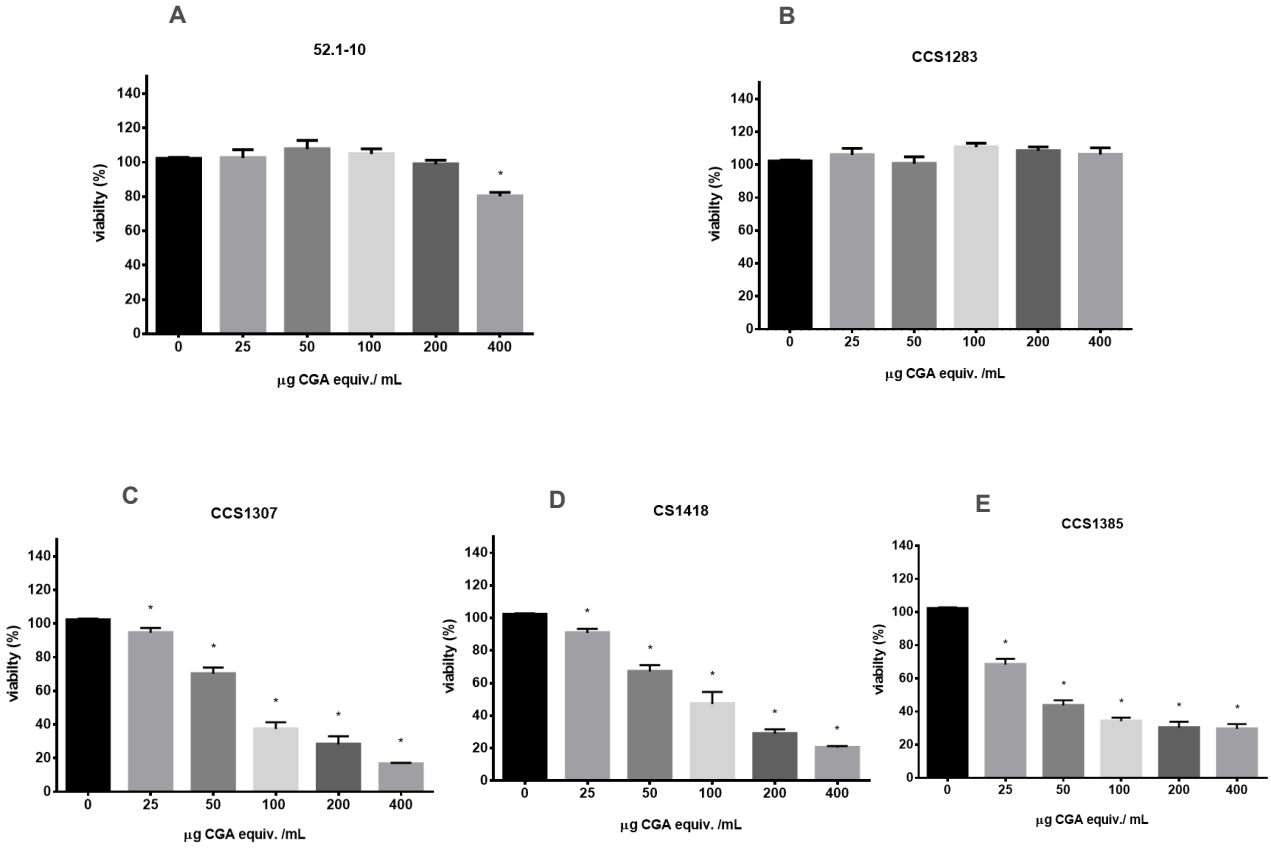

Figure 4. Cytotoxic effect of PPEs of Andean and industrial varieties against Hep3B cell line. Cells were treated with different concentrations (25, 50, 100, 200, and 400 µg CGA equiv/mL) of five potato varieties PPEs **(A)** 52.1-10, **(B)** CCS1283, **(C)** CCS1307, **(D)** CS1418, **(E)** CCS1385; for 24 h. Viability was measured using the MTT assay and expressed relative to the viability of untreated control cells. Viability was determinated by MTT assay and calculated as: (Abs treated cells/Abs control cells) x 100. Results are expressed as the media ± SEM of four independent experiments. Significant differences ($p < 0.05$) with respect to the control are indicated with (*).

Cytotoxic activity of PPEs on human hepatocarci-noma cells

The cytotoxic activity of the five PPEs was evaluated on a human hepatocellular carcinoma cell line, Hep3B. Cells were treated with different concentrations of PPEs (0, 25, 50, 100, 200, and 400 µg CGA equiv./mL) for 24 h, and cytotoxicity was measured by the MTT assay. Figure 4 illustrates the viability rate of Hep3B cells treated with the PPEs. From the five studied PPEs, three of them, (CCS1307, CS1418, and CCS1385) resulted in a significant reduction of Hep3B cell viability in a concentration dependent manner after 24 h of treatment (Figure 4C, D and E). Cells treated with the maximum concentration of the 52.1-10 variety PPE showed low but significant cytotoxicity after 24 h treatment (Figure 4A). CCS1283 showed no differences in viability, compared to the control at any of the concentrations tested (Figure 4B). All the experiments were validated by the absence of cytotoxic effects in cells incubated with 30% methanol (extraction solvent), compared to non-treated cells. Similar results were observed on another human hepatocarcinoma cell line Huh-7 (data not shown). The cytotoxic concentration 50 (CC_{50}) was calculated for the three cytotoxic PPEs; representing a low CC_{50} value a higher cytotoxic activity. CCS1385 presented the lowest CC_{50} = 37.28 µg CGA equiv. / mL, followed by CS1418 = 54.55 µg CGA equiv. / mL and CCS1307 = 66.71 µg CGA equiv. / mL. According to these results, the purple variety CCS1385, exhibited the highest cytotoxic activity by reducing cell viability in human malignant hepatocytes (Hep3B cell line), followed by the white fleshed variety CS1418 and the yellow fleshed variety CCS1307.

Table 4. Correlation between total phenolic compounds and the antioxidant activity.

.	Antioxidant activity	
	R^2	**P**
Total phenolic	0.97	0.0003
Flavan-3-oles	0.24	0.9732
CGA	0.86	0.0078
CA	0.78	0.0198
FA	0.16	0.4358
CoA	0.18	0.4083

CGA: chlorogenic acid; CA: caffeic acid; FA: ferulic acid; CoA: coumaric acid.

DISCUSSION

Polyphenols are a broad group of plant molecules, which main function is to protection from different types of stresses. Beside their relevance in plants, these compounds have been extensively studied for their beneficial effects on human health. Polyphenols can be incorporated to the diet by ingesting different fruits and vegetables, with potato being an important source of these compounds due to its vast consumption.

The results of this work showed that the content of total phenolics, total anthocyanins, and flavan-3-ols varied among the five studied *S. tuberosum* varieties (4 *andigena* and 1 *tuberosum*). Furthermore, the obtained data is in agreement with results of previous studies from this laboratory and other research groups [23, 25-27], finding that pigmented variety contained the highest levels of the different groups of polyphenolic compounds. A direct association between total phenolic content and the pigmentation of the tuber has been established in several reports; being the quantity of polyphenols higher in purple or red varieties than in yellow or white ones [28, 45, 46]. Similarly, flavan-3-ols and anthocyanin contents have been previously reported to be higher in purple tubers [19, 22, 23, 27].

Different factors can affect the polyphenolic content in the potato tuber, including genotype and type of tissue (peel or flesh), different growing location, method of extraction or sample preparation [47, 48]. Related to the mentioned factors, the non-pigmented varieties 52.1-10 (*S. tuberosum ssp. tuberosum*- industrial) and CCS1283 and CCS1307 (*S. tuberosum ssp. andigena*- Andean), presented similar quantities of compounds, suggesting that neither the genotype nor the growing location had a great effect on the polyphenol levels of these varieties. Peels and flesh of potato tubers might differ not only in quantity but also in the diversity of polyphenolic compounds present in them [22, 23, 45, 49]. Also, phenolic content in purple-fleshed varieties could be between 6 to 8 fold greater than in white or yellow fleshed ones; and this could be explained by the presence of both anthocyanins and phenolic acids [27]. This might explain the results observed for the purple variety CCS1385, which showed the highest levels of total phenolic, total anthocyanin, and flavan-3-ols content, probably due to the combination of peel and flesh used in these PPEs.

In accordance with previous studies from this and other groups [22, 23, 40, 45, 50]; HPLC analysis showed that CGA is the main phenolic acid present in the five studied PPEs, representing between 70-90 % of the total phenolic acids in EPPs. Also, pigmented varieties presented higher quantities of CGA, with CCS1385 containing the highest levels. The relationship between CGA content and tuber pigmentation has been well documented [28, 51]; with 8 to 22 fold high-

er CGA content observed in pigmented varieties. Moreover, the HPLC results showed that CA, CoA, and FA appear in a minimum proportion in relation to CGA in all the PPEs. In agreement with our results, CA was described as the second most abundant phenolic acid present in potato cultivars, showing higher levels in the pigmented clones than in non-pigmented clones [27, 49]. In contrast, previous studies in this lab showed that neither CT nor ECT could be detected in the PPEs [23]. This difference between the spectrophotometric quantification and the HPLC analysis might be due to the fact that only two compounds of this group were analyzed by HPLC. Finally, in agreement with previous studies [45, 52] the HPLC analysis performed for anthocyanins showed pelargonidin as the main anthocyanidin in red varieties and petunidin and malvidin as the main anthocyanin in the purple ones.

Antioxidant activity was evaluated by the DPPH method for all the PPEs. The results showed that pigmented varieties have higher antioxidant capacity, exhibiting 2 to 4 fold higher levels than those observed in non-pigmented ones. These results suggest that not only the phenolic content but also the presence of anthocyanin contribute to the antioxidant activity of PPEs. Also, the results showed a positive significant correlation between total phenolic, CGA and CA content and the antioxidant activity. This correlation between phenolic composition and the antioxidant activity was previously reported [21]. Furthermore, it has been reported that the antioxidant potential of pigmented cultivars can be 2 to 8 fold higher than that of non-pigmented ones, which can be explained by the presence of anthocyanins along with phenolic acids [21, 28]. The present study only included two pigmented varieties, making it impossible to calculate the statistical correlation between anthocyanins and antioxidant activity.

Plant polyphenol activity has been largely studied against several types of human cancers such as colon, prostate, and breast [53-55]. Particularly, numerous publications have reported the effects of PE from different fruits, vegetables and beverages against HCC [21, 56-58] but also, for many indigenous herbs used in traditional medicine [59-62]. Furthermore, the obtained results demonstrated that PPEs significantly reduced cell viability in a human HCC cell line, although the mechanisms by which PPEs exert their effect need to be determined. Cytotoxic and pro-apoptotic effects against Hep3B of different polyphenolic extracts from different sources like *Iponema batatas* Lam., green tea polyphenols and *Vitex negundo,* have been previously reported [63-65]. Furthermore, not only total phenolic extracts but also anthocyanidins and anthocyanin fractions from different plants have been studied for their anti-HCC activity [66, 67]. It is important to mention that the Hep3B cell line (analyzed in this work) is p53 defective, suggesting that PPEs may induce cell death by a p53 independent pathway.

The results of this work are the first that demonstrate the cytotoxic effect of PPEs in Hep3B cells. From the five studied PPEs, three of them reduced cell viability, with the purple variety CCS1385 exhibiting the strongest anti-HCC activity, followed by the white with red spots variety CS1418, and the non-pigmented variety CCS1307. In accordance with previous studies, the purple potatoes presented higher levels of phenolic compounds and antioxidant activity that correlated with higher anticancer effect [27]. The relevant cytotoxic activity of the potato varieties detected in Hep3B cells, justifies further analysis in other human malignant hepatocyte cell lines. Also, the mechanisms underlying PPE-induced hepatocyte cell death should be characterized in a future study.

There are numerous reports about effects of the PPE or their anthocyanin fraction from pigmented varieties against different types of cancer *in vitro* [38, 40, 68]. For example, the anthocyanin fraction from *S. tuberosum* L. var. Vitelotte is cytotoxic in cervical, breast, and prostate cancer cell lines [69]. Recently, it was described that anthocyanin from purple-fleshed potatoes significantly suppressed the proliferation of colon cancer stem cells with or without p53 expression [36]. However, it is noteworthy that treatment with CCS1307, a non-pigmented cultivar, resulted in cytotoxicity against the Hep3B cell line in a similar extent as pigmented varieties (CS1418 and CCS1385). Although cytotoxic activity has been mainly described for PPEs from pigmented varieties, there are some previous reports that showed anti-cancer effects of PPEs from white or yellow tubers [21, 48]. Furthermore, the cytotoxic effect of CCS1307 could not be explained based on spectrophotometric or HPLC characterization, as it presented similar quantities and phenolic acid profiles as the other two non-pigmented varieties. Taken together, these results indicate that other factors besides the presence of anthocyanins might be involved in the cytotoxic activity against Hep3B cells. These characteristics make CCS1307 an interesting variety to continue analyzing in order to find potential novel compounds with anti-HCC activity.

In conclusion, the phenolic acids were the main group of phenolic compounds in all the studied varieties, with CGA being the main compound in all the PPEs. Generally, pigmented potatoes presented higher levels of the different compounds than non-pigmented ones. A similar trend was observed for the antioxidant activity. Finally, three of the studied PPEs presented relevant anti-HCC activity, one of them being from a non-pigmented variety, suggesting that this activity might be driven by other compounds besides anthocyanins. Further studies are needed to determine which are the active compounds present in PPEs and to characterize the molecular pathways involved in the

cytotoxic activity of PPEs on human malignant hepatocytes. Finally, these results demonstrate the biological activity of PPEs, and suggest their potential use as a source for new anti-HCC molecules.

AUTHOR CONTRIBUTIONS

L.B. and A.B.A designed and instructed the research work. M.J.M. performed the experiments. M.J.M. and A.B.A. wrote the manuscript.

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