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Plant cell cultures of Nordic berry species: Phenolic and carotenoid profiling and biological assessments

Heiko Rischer [a,*], Liisa Nohynek [a], Riitta Puupponen-Pimiä [a], Joselin Aguiar [b], Gabriele Rocchetti [c], Luigi Lucini [c], José S. Câmara [b,d], Thiago Mendanha Cruz [e], Mariza Boscacci Marques [e], Daniel Granato [f,g,*]

[a] VTT Technical Research Centre of Finland Ltd., Tietotie 2, P.O. Box 1000, 02044 VTT, Finland
[b] CQM – Centro de Química da Madeira, Universidade da Madeira, Campus Universitário da Penteada, 9020-105 Funchal, Portugal
[c] Department for Sustainable Food Process, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy
[d] Departamento de Química, Faculdade de Ciências Exatas e da Engenharia, Universidade da Madeira. Campus da Penteada, 9020-105 Funchal, Portugal
[e] Department of Chemistry, State University of Ponta Grossa, Av. Carlos Cavalcanti, 4748, 84030-900 Ponta Grossa, Brazil
[f] Food Processing and Quality, Natural Resources Institute Finland (Luke) – Latokartanonkaari 9, FI- 00790 Helsinki, Finland
[g] Department of Biological Sciences, Faculty of Science and Engineering, University of Limerick, V94 T9PX Limerick, Ireland

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ABSTRACT

Plant cell cultures from cloudberry (CL), lingonberry (LI), stone berry (ST), arctic bramble (AB), and strawberry (SB) were studied in terms of their polyphenol and carotenoid composition, antioxidant activity, antihemolytic activity and cytotoxicity effects on cancerous cells. High-resolution mass spectrometry data showed that LI, presented the highest antioxidant activity, contained the highest contents of flavones, phenolic acids, lignans, and total carotenoids, while CL, ST and SB presented the opposite behavior. AB and SB presented the lowest FRAP and CUPRAC values, while AB and CL presented the lowest reducing power. SB presented the lowest antioxidant activity measured by single electron transfer assays and the lowest content of lignans, phenolic acids, and flavones. CL and LI decreased the viability of *in vitro* mammary gland adenocarcinoma while only LI decreased the viability of *in vitro* lung carcinoma and showed protective effects of human erythrocytes against mechanical hemolysis.

1. Introduction

Higher plants synthesize a diverse range of bioactive, low-molecular-weight secondary metabolites described as small molecules, natural products or specialized metabolites, offering enormous commercial potential as nutraceuticals, therapeutics, flavors and fragrances with potential applications on pharmaceutical, cosmetic and food industries, among others (Câmara et al., 2021). Among the major groups of metabolites, special attention has been devoted to phenolics which exhibit remarkable bioactivities, including their antimicrobial, anti-inflammatory and antioxidant activity by scavenging free radicals, chelating trace metals and by binding proteins with suppression of their enzymatic activity associated with beneficial effects on human health protection (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005; Granato, Mocan, & Câmara, 2020). Cultivated and wild Nordic berries are highly recommended for human consumption due to their health-promoting potential, as they accumulate phenolics such as flavonoids, anthocyanins, flavonols, and flavan-3-ols, including proanthocyanidins, phenolic acids, and ellagitannins (Hannum, 2004; Puupponen-Pimiä et al., 2005).

The cosmetic industry is likewise building on the bioactivities of these compounds for cosmeceutical products (Papaioanou et al., 2018) and tailor-made skin care delivery systems (Oksman-Caldentey et al., 2017). However, the contents of bioactive compounds in fruits vary considerably from one harvesting season to another (Hykkerud, Uleberg, Hansen, Vervoort, Mølmann, & Martinussen 2018) and the pressure on limited wild resources is mounting due to climate change and habitat destruction. Recently, both the cosmetic and the food industry are increasingly turning their attention to plant cell culture technology (Eibl, Meier, Stutz, Schildberger, Huehn, & Eibl, 2018) as a more

* Corresponding authors at: Food Processing and Quality, Natural Resources Institute Finland (Luke) – Latokartanonkaari 9, FI- 00790 Helsinki, Finland (D. Granato); VTT Technical Research Centre of Finland Ltd., Tietotie 2, P.O. Box 1000, 02044 VTT, Finland (H. Rischer).
E-mail addresses: Heiko.Rischer@vtt.fi (H. Rischer), daniel.granato@ul.ie (D. Granato).

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sustainable and consistent raw material. The concept is rooted in the Cellular Agriculture approach i.e. the biotechnological production of traditional agricultural commodities with the help of cell cultures rather than farmed animals or crops (Rischer, Szilvay, & Oksman-Caldentey2020). Plant cell culture technology on the commercial level is enjoying a revival since 'plant stem cells' were widely adopted in cosmetics some years ago (Barbulova, Apone, & Colucci, 2014; Nohynek et al., 2014). Nonetheless, industrial applications in the food sector are lacking because of legislation issues. Products made of cultured plant cells are considered 'novel foods' in Europe. To base any decisions of food authorities about food applications of plant cell cultures, research on the chemical composition, bioactivity, and possible interactions with human cells are necessary. Considering the need of novel foods for feeding the world and the expectations of more natural ingredients for the industry, the main objective of the present research is to characterize the polyphenol composition, antioxidant activity, antihemolytic activity and antiproliferative activity of plant cell cultures from cloudberry, lingonberry, stone berry, arctic bramble, and strawberry.

2. Materials and methods

2.1. Chemicals, reagents, and cell lines

Methanol and dimethyl sulfoxide 99.9% (DMSO) were obtained from Fischer Scientific (Loughborough, UK). 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) salt diammonium (ABTS), aluminum chloride, dibasic sodium phosphate dodecahydrate, potassium ferricyanide and iron (III) chloride were purchased from Riedel-de-Haën (Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tri-(2-pyridyl)-s-triazine (TPTZ), 2-thiobarbituric acid, ascorbic acid, trichloroacetic acid, ferric chloride ($FeCl_3$), sodium chloride, neocuproin, trisodium citrate dihydrate, sodium pyruvate, human insulin, phosphate buffer saline (PBS), kinetin, α-naphthaleneacetic acid (NAA), sodium pyruvate and trichloroacetic acid were obtained from Sigma-Aldrich (Germany). Anhydrous sodium carbonate, sodium acetate trihydrate, sodium hydroxide and dihydrogen potassium phosphate were obtained from Panreac Quimica S.A (Spain). Folin-Ciocalteu reagent was purchased from Fluka (Switzerland), and glacial acetic acid (was obtained from Merck (Germany). Thiazolyl Blue Tetrazolium Bromide (MTT) 98% was obtained from PanReac AppliChem (Spain). MS medium, thidiazuron (TDZ) and Woody Plant Medium were obtained at Duchefa Biochemie (The Netherlands). The other reagents were of analytical grade. The human breast adenocarcinoma cell line MCF-7 and non-small cell lung carcinoma HCC-44 were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

2.2. Plant cell cultures and extraction step for spectrophotometric assays

Five lines of fully established cell suspension cultures (http://culturecollection.vtt.fi/) were used. Cell suspensions were grown in 250 mL Erlenmeyer flasks containing 70 mL of culture on an orbital shaker at 110 rpm, 24 ± 1 °C and a day-night illumination regime (photoperiod 16:8h, irradiation 40 $\mu molm^{-2}\ s^{-1}$). Regularly, after ten days, 20 mL of cell suspension were transferred to a flask with 50 mL fresh medium. Cloudberry (*Rubus chamaemorus*, VTT P-120083, yellow), stone berry (*Rubus saxatilis*, VTT P-120091, green), lingonberry (*Vaccinium vitis-idaea*, KAS 377/i-9r, red), arctic bramble (*Rubus arcticus*, VTT P-120088, yellow) and strawberry (*Fragaria* × *ananassa* 'Senga Sengana', VTT P-120010, green) cultures were maintained on media described earlier (Nohynek et al., 2014; Suvanto, Nohynek, Seppänen-Laakso, Rischer, Salminen, & Puupponen-Pimiä, 2017; Nordlund et al., 2018; Häkkinen et al., 2020). Initially, all germplasm had been sourced from Finland according to national and international legislation. Shortly, cloudberry, strawberry, arctic bramble and strawberry cells were cultivated in MS medium (Murashige & Skoog, 1962) containing 3% (w/v) sucrose, 0.1 mg/L kinetin and 1 mg/L NAA. In the case of arctic bramble and stone berry, the pH was adjusted to 4.0 before autoclaving, for strawberry to pH 4.0 and for cloudberry to pH 5.8. Lingonberry cells were grown in Woody Plant Medium (Lloyd & McCown, 1981) containing 3% (w/v) sucrose, 2.2 mg/L TDZ and 1.95 mg/L NAA adjusted to pH 4.8 before autoclaving. Plant cells were harvested by vacuum filtration using a Büchner funnel and Miracloth (Calbiochem, San Diego, USA) filtration tissue. Cells were washed twice with sterile MilliQ water and lyophilized. The powder was stored frozen (-20 °C) until extraction.

Maceration under magnetic stirring was used as the extraction procedure, and a citric acid solution (0.10 mol/L, pH 2.10) was used as the solvent. A material-to-solvent ratio of 1:30 (w/v) and a temperature of 45 °C were used in the extraction procedure. Extracts were filtered using a qualitative paper (Whatman #1) and stored in plastic tubes and microcentrifuge tubes for up to 48 h before analysis. The experiment was repeated twice, and all analyses were performed in triplicate.

2.3. Spectrophotometric determination of bioactive compounds

The total phenolic content (TPC) of the extracts was assayed using the Folin-Ciocalteu reducing capacity (Margraf et al., 2015) and results were expressed as mg gallic acid equivalent per 100 g of sample (mg GAE/100 g). The total flavonoid content (TFC) of samples was determined using the aluminum chloride colorimetric assay described by Aguiar et al. (2020) and results were expressed as mg catechin equivalent per 100 g (mg CE/100 g) of sample. The total monomeric anthocyanin content (TAC) of cell extracts was measured using the pH differential method proposed by Giusti and Wrolstad (2001) and results were expressed as mg cyanidin-3-glucoside equivalent per 100 g (mg C3GE/100 g). Total *ortho*-diphenolic content (TOC) was determined using the sodium molybdate assay (Maestro Durán et al., 1991), and results were expressed as mg chlorogenic acid equivalent per 100 g (mg CAE/100 g). Total condensed tannin content (TCT) was determined using the vanillin-H_2SO_4 assay and results were expressed as mg CE/100 g. All analyses were performed in triplicate.

2.4. Extraction procedure for high-resolution-mass spectrometry (HRMS) analyses

Dried samples were extracted (using a material to solvent ratio of 1:20 w/v) with a modified version of the method previously described by Matyash et al. (2008). Briefly, samples were dissolved in a mixture of 80% aqueous methanol and methyl-*tert*-butyl ether (MTBE) (1:1, v/v), mixed by vortexing for 2 min and then ultrasonic processed for 5 min. Thereafter, upon 10 min of incubation at room temperature, samples were centrifuged at 900 *g* for 10 min. The upper (organic) phase (500 μL) was filtered using 0.22 μm cellulose-syringe filters and 300 μL were collected in a 1.5 mL microcentrifuge tube and then dried. The extracted lipids were finally dissolved in 300 μL of isopropanol/methanol/water (60:35:5, v/v/v) and collected in amber vials for untargeted lipidomic analysis. Regarding the hydrophilic fraction, 500 μL of the lower phase (i.e., the aqueous methanol fraction) following the centrifugation step were filtered using 0.22 μm cellulose-syringe filters in amber vials until further untargeted analysis.

2.5. Untargeted profiling of bioactive compounds

2.5.1. UHPLC-QTOF analysis of polyphenols

A metabolomics-based approach was used to comprehensively annotate phenolic compounds in the studied samples, as previously reported by Rocchetti et al. (2020). Briefly, the separation was achieved using an Agilent Zorbax Eclipse Plus C18 column (50 × 2.1 mm, 1.8 μm) with water-acetonitrile linear gradient elution from 6 to 94% of acetonitrile in 35 min. Samples were acquired in positive polarity (ESI^+), using a full scan mode with an *m/z* range of 100–1200, with a scan rate of 0.8 spectra/s, and a nominal mass resolution of 30,000 FWHM. The

injection volume was 6 μL and the sequence was randomized. Pooled Quality Control samples (QCs) were injected throughout the sequence and analyzed in data-dependent MS/MS mode using 10 precursors per cycle (1 Hz, 50–1200 *m/z,* positive polarity, active exclusion after 2 spectra), with collision energies of 10, 20, and 40 eV.

The raw MS data were processed using the Agilent Profinder B.07 (Agilent Technologies) software, according to the "find-by-formula" algorithm, namely combining monoisotopic mass information and isotope profile to achieve the highest confidence in the annotation, using a 5-ppm tolerance for mass accuracy. Mass and retention time alignment were carried out and then annotation of mass features was based on the comprehensive database Phenol-Explorer 3.6 (www.phenol-explorer.eu). Therefore, in our experimental conditions, a Level 2 of annotation was achieved (i.e., putatively annotated compounds), as reported by COSMOS Metabolomics Standards Initiative (Salek, Steinbeck, Viant, Goodacre, & Dunn, 2013).

Post-acquisition data filtering, baselining and normalization of phenolic compounds was done using the software Mass Profiler Professional (version: B.12.06, from Agilent Technologies), as previously reported (Rocchetti et al., 2020). In this regard, compounds were filtered by abundance (peak area > 5000 counts) and by frequency (compounds needed to be present in 100% of replications within at least one condition), thereafter, they were Log2 transformed, normalized at 75th percentile, and baselined for the median.

Additionally, the cumulative intensity values of the different phenolic classes were converted into semi-quantitative data, using methanolic solutions of pure standard compounds (Extrasynthese, Lyon, France) analyzed under the same conditions. In particular, ferulic acid (phenolic acids), quercetin (other flavonoids), sesamin (lignans), cyanidin (anthocyanins), luteolin (flavones), resveratrol (stilbenes), and tyrosol (tyrosols, alkylphenols and low molecular weight phenolics) were used as representatives of their respective classes. A linear fitting ($R^2 > 0.98$) was built and used for quantification, and results were expressed as mg equivalents (Eq.)/g dry matter (DM).

2.5.2. Untargeted UHPLC-Orbitrap analysis of carotenoids

The identification of carotenoids in the samples was carried out using a UHPLC-MS lipidomics-based workflow, based on a Q Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) coupled to a Vanquish ultra-high-pressure liquid chromatography (UHPLC) pump and equipped with a HESI-II probe (Thermo Scientific, USA). The chromatographic separation was achieved by using a BEH C18 (2.1x100 mm, 1.7 μm) analytical column maintained at 40 °C. The mobile phases consisted of (A) 5 mM ammonium formate and 0.1% formic acid in water/methanol (95/5, v/v), and (B) 5 mM ammonium formate and 0.1% formic acid in 2-propanol/methanol/water (65/30/5, v/v/v). The linear gradient and flow rate increased linearly as follows, considering time (min), %B, flow rate (μL/min): (0,10,200), (5, 50, 200), (15, 80, 250), (28, 100, 250), (30, 100, 250), (30.9, 10, 250), and (35,10, 250). For the full scan MS analysis, acquisition was performed using both positive and negative ionization with a mass resolution of 70,000 at *m/z* 200. Automatic gain control target (AGC target) and maximum injection time (IT) were 1e6 and 100 ms, respectively. Additionally, separate and randomized injections of pooled quality control (QC) samples were performed in a data-dependent (Top N = 3) MS/MS mode with full scan mass resolution reduced to 17,500 at *m/z* 200, with an AGC target value of 1e5, maximum IT of 100 ms, and isolation window of 1.0 *m/z*, respectively. For the stage of data-dependent MS/MS, the Top N ions were selected for further fragmentation under stepped normalized collision energy (i.e., 10, 20, 40 eV). The injection volume was 6 μL and the *m/z* range for the full scan analyses was 150–1500 *m/z*. Heated electrospray ionization (HESI) parameters were as follows: sheath gas flow 30 arb (arbitrary units) auxiliary gas flow 10 arb, spray voltage 3.5 kV for ESI^+ and 2.8 kV for ESI^-, capillary temperature 320 °C. Prior to data collection, the mass spectrometer was calibrated using Pierce™ positive and negative ion calibration solutions (Thermo Fisher Scientific, San Jose CA, USA). To avoid possible bias, the sequence of injections was randomized. The collected UHPLC-HRMS data (.RAW file) were converted into .abf file using the Reifycs Abf Converter and then further processed using the software MS-DIAL (version 4.38) and MS-Finder (Tsugawa et al., 2016). The annotation via spectral matching (against the database LipidBlast) was performed in MS-Dial, and retention time information was excluded from the calculation of the total identification score. The identification step was based on mass accuracy, isotopic pattern, and spectral matching. The software MS-Finder was used to provide *in silico* fragmentation of the not fully annotated MS/MS features, considering both Lipid Maps and FoodDB libraries available in the same software.

Finally, to provide semi-quantification of the major carotenoids annotated, the cumulative intensity values were converted into semi-quantitative data using MTBE-standard solutions of pure beta-carotene (Sigma-Aldrich, CAS number: 7235–40-7) analyzed under the same conditions. A linear fitting ($R^2 > 0.98$) was built and used for quantification and results were expressed as mg equivalents (Eq.)/g dry matter (DM).

2.6. Biological activities

2.6.1. In vitro antioxidant activity

The ferric reducing antioxidant power (FRAP), cupric-ion antioxidant capacity (CUPRAC), scavenging of DPPH and ABTS radicals were assayed using the conditions and protocols described elsewhere (Santos et al., 2018; Schechtel et al., 2019; Alcántara et al., 2020) and results were expressed as mg ascorbic acid equivalent per 100 g, mg AAE/100 g, for FRAP, DPPH and CUPRAC, respectively, while ABTS was expressed as mg Trolox equivalent per 100 g (mg TE/100 g). The reducing power was assayed using the Prussian Blue assay (Margraf et al., 2015) and results were expressed as mg GAE/100 g.

2.6.2. Antihemolytic activity

The antihemolytic activity, under hypotonic conditions, was evaluated with erythrocytes isolated from O^+ blood samples obtained from Regional University Hospital Wallace Thadeu de Mello e Silva. The assays were performed according to Migliorini et al. (2019), with hematocrit 0.8%, [NaCl] = 0.1%, 0.4% and 0.8% (w/v) and the extract concentrated to 5.0, 7.5 and 10.0 μg GAE/mL, and the hemolysis rate was measured by the absorbance at 540 nm. The hemoglobin oxidation was carried out by the absorbance measure at 630 nm (Morabito et al., 2017). The entire experimental procedure was previously approved by the State University of Ponta Grossa Ethics Committee (CAAE 94830318.1.0000.0105).

2.6.3. Cell viability and antiproliferative assessments

The MCF-7 were cultured in 90% RPMI (Roswell Park Memorial Institute) 1640 medium containing 10% (v/v) fetal bovine serum (FBS, Gibco, UK) and 1% (v/v) of an antibiotic–antimycotic solution (AA, Gibco,UK). The medium was supplemented with 1% (v/v) of MEM Non-Essential Amino Acids 100x solution (Gibco, UK), 1 mM sodium pyruvate and 10 μg/mL human insulin. The HCC-44 were grown in 90% RPMI 1640 medium supplemented with 10% of FBS, 1% Antibiotic-Antimycotic. Cells were maintained in 25 cm petri dish monolayer incubated in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Afterward, the cells were harvested at 70–80% confluence, using 0.25% trypsin-EDTA solution, for the enzymatic detachment of the cells from the plastic substrate.

The extraction of bioactive compounds present in each plant matrix for the study of cytotoxicity was performed according to the procedure described by Annegowda, Bhat, Min-Tze, Karim, and Mansor (2012) with some modifications. Briefly, samples were placed in an amber glass vial and methanol was added at a ratio of 1:10 (m/v). After homogenization, the mixture was placed in an ultrasonic bath (42 kHz) at 30 °C for 30 min. This method is often used to facilitate the extraction of

metabolites from plant material, since the application of high intensity ultrasound generates mechanical stresses in the cell suspension, sufficient to cause the rupture of cells, thus increasing the solubilization of metabolites in the solvent and consequently the extraction efficiency (Demirci, 2007). The extract obtained was filtered and evaporated under a stream of nitrogen to dryness and resuspended in DMSO.

For cytotoxicity, the experiments were conducted by MTT assay. The cells were seeded in a 96 well microplate and cultured in the presence of 200 at 600 μg/mL of the dried methanolic extracts and then were incubated for 48 h. The cells treated with 100 μL MTT reagent (5 mg/mL) and incubated for 3 h at 37 °C to obtain purple-coloured formazan. The colour was dissolved in 100 μL of DMSO and estimated by measuring the absorbance at 570 nm in an ELISA microplate reader. All values were calculated as a percent of the unviable cell number compared to the control from three independent experiments performed in triplicate. Half maximal effective concentration (EC_{50}) values, which refer to the concentration of the plant cell extract which induces the inhibition of 50% of cells after a specified exposure time was calculated non-linear regression analysis.

2.7. Statistical analysis

Data were expressed as means followed by the standard deviation (n = 3). Comparison between plant cells was performed using analysis of variances (ANOVA) followed by the Duncan's multiple range test, considering $p < 0.05$ as significant. Pearson's correlation coefficients were calculated to measure the degree of association between bioactive compounds and bioactivity, considering $p < 0.05$ as significant correlations (Granato, Calado, & Jarvis, 2014). The software TIBCO Statistica v.13.3 (TIBCO Statistica Ltd, USA) was used.

Metabolomics and lipidomics-based data on phenolic and carotenoid profiling were elaborated in Agilent Mass Profiler Professional B.12.06 (Rocchetti et al., 2020). In particular, a combined dataset was used for multivariate statistical processing based on unsupervised hierarchical cluster analysis (HCA) and principal component analysis (PCA). In addition, the combined dataset was exported into SIMCA 13 (Umetrics, Malmo, Sweden), Pareto scaled, and elaborated for orthogonal partial least squares discriminant analysis (OPLS-DA) supervised modeling, as previously described (Rocchetti et al., 2020). In this regard, the variables importance in projection (VIP) approach was used to evaluate the discriminant compounds between the different plant extracts, selecting those having the highest discrimination potential (VIP score > 1). Finally, Pearson's correlations coefficients between the metabolomic/lipidomic profiling and the different *in vitro* assays were calculated using the software IBM PASW Statistics 26.0 (SPSS Inc.).

3. Results and discussion

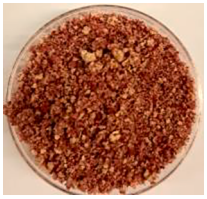
3.1. Content of bioactive compounds

Table 1 contains the results of phenolic (sub)classes assayed by UV–Vis spectrophotometry of the selected plant cell cultures. Lingonberry presented the highest ($p < 0.05$) TPC, TFC, TCT and TOC, and was the only sample with detectable TAC. Stone berry and strawberry presented the lowest TPC and TFC, whereas arctic bramble presented the lowest mean values for TOC and TCT. On the other hand, cloudberry presented intermediate phenolic contents.

The HRMS analysis based on both polyphenols and terpenoids (i.e. carotenoids) allowed the detection of 385 compounds. In this regard, several phenolic classes were detected with a great abundance of flavonoids (193 compounds), followed by phenolic acids (77 compounds), lower-molecular-weight phenolics (61 compounds), lignans (25 compounds), terpenoids (21 compounds), and stilbenes (8 compounds). Regarding the phenolic composition of the different plant-cell cultures, great differences were observed (Data set 1 – supplementary material). Overall, lingonberry showed a more complex compounds profile when considering each phenolic class annotated and when compared to the other plant-cell cultures. This was particularly evident when considering the semi-quantitative results reported in Table 2. In fact, HRMS semi-quantitative data showed that lingonberry contained the highest contents of flavones (29.85 mg/g DM), phenolic acids (25.32 mg/g DM), lignans (27.53 mg/g DM), and total carotenoids (0.64 mg/g DM), while cloudberry, strawberry and stone berry contained the lowest mean values for flavones, phenolic acids, and total carotenoids. Anthocyanins were detected in very low levels in AB, CL, and SB (on average: 0.14 mg/g DM). On the other hand, arctic bramble was highlighted as the most suitable source of stilbenes (1.8 mg/g DM), followed by lingonberry (0.95 mg/g DM) and strawberry (0.98 mg/g DM). Additionally, arctic bramble showed the highest ($p < 0.05$) content of lower-molecular-weight phenolics (33.39 mg/g DM).

In fruit pulp, phenolic compounds of berries are well described and known (Häkkinen et al., 1999). Phenolic acids and flavonoids, such as caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic and gallic acids, kaempferol, quercetin, myricetin and their glycosylated forms are very

Table 1
Total phenolic content and chemical antioxidant activity of freeze-dried arctic bramble, lingonberry, cloudberry, stoneberry, and strawberry cell cultures.

Responses	*Arctic bramble*	*Lingonberry*	*Cloudberry*	*Stone berry*	*Strawberry*
					
Total phenolic content (mg GAE/100 g)	1099 ± 12^{b}	8711 ± 50^{a}	686 ± 15^{c}	517 ± 16^{d}	525 ± 5^{d}
Total flavonoids (mg CE/100 g)	25 ± 1^{b}	5143 ± 56^{a}	22 ± 1^{b}	23 ± 1^{b}	13 ± 1^{b}
Condensed tannins (mg CE/100 g)	13 ± 1^{c}	4786 ± 27^{a}	34 ± 4^{c}	18 ± 1^{c}	78 ± 2^{b}
ortho-Diphenols (mg CAE/100 g)	44 ± 0^{d}	1460 ± 9^{a}	53 ± 1^{c}	50 ± 0 ^{cd}	74 ± 2^{b}
Total anthocyanins (mg/100 g)	ND	264 ± 16	ND	ND	ND
FRAP (mg AAE/100 g)	139 ± 8^{b}	9003 ± 52^{a}	159 ± 5^{b}	176 ± 10^{b}	140 ± 18^{b}
DPPH (mg AAE/100 g)	54 ± 1^{b}	643 ± 2^{a}	52 ± 1^{b}	81 ± 1^{b}	19 ± 1^{c}
Reducing power (mg GAE/100 g)	29 ± 1^{b}	765 ± 1^{a}	17 ± 0^{d}	28 ± 1^{c}	14 ± 1^{e}
CUPRAC (mg AAE/100 g)	138 ± 4^{c}	30515 ± 224^{a}	310 ± 4^{c}	611 ± 4^{b}	133 ± 6^{c}
ABTS (mg TE/100 g)	697 ± 18^{c}	1317 ± 2^{a}	1138 ± 13^{b}	685 ± 7^{c}	1238 ± 9^{a}

Note: Values are expressed as means ± standard deviation (n = 3). Different letters in the same line represent statistically different results according to the Duncan's multiple range test ($p < 0.05$). CAE = chlorogenic acid equivalent, AAE = ascorbic acid equivalent, CE = (+)-catechin equivalent, GAE = gallic acid equivalent, TE = Trolox equivalent, ND = not detected (<5 mg/g).

Table 2
Semi-quantitative determination of phenolic compounds and total carotenoids in plant cell cultures using high-resolution-mass spectrometry (HRMS) analyses.

Class(mg Eq./g DM)	Arctic bramble	Lingonberry	Cloudberry	Stone berry	Strawberry
Anthocyanins	0.12 ± 0.01^{c}	7.07 ± 0.11^{a}	0.16 ± 0.02^{c}	0.70 ± 0.03^{b}	0.15 ± 0.03^{c}
Flavones	2.87 ± 0.68^{b}	29.85 ± 1.92^{a}	1.78 ± 0.15^{b}	2.42 ± 0.06^{b}	1.79 ± 0.25^{b}
Other flavonoids	0.82 ± 0.10^{c}	21.54 ± 0.37^{a}	0.64 ± 0.07^{c}	1.97 ± 0.14^{b}	0.69 ± 0.05^{c}
Phenolic acids	21.98 ± 3.31^{b}	25.32 ± 0.55^{a}	5.44 ± 0.34^{c}	6.11 ± 1.66^{c}	5.24 ± 0.35^{c}
Lignans	7.89 ± 1.54^{c}	27.53 ± 1.09^{a}	10.72 ± 1.26^{b}	2.32 ± 0.08^{d}	10.12 ± 1.08^{b}
Stilbenes	1.84 ± 0.19^{a}	0.95 ± 0.02^{b}	0.26 ± 0.02^{c}	0.54 ± 0.01^{c}	0.98 ± 0.08^{b}
Other phenolics	33.39 ± 3.46^{a}	27.90 ± 1.03^{ab}	25.96 ± 5.76^{ab}	22.72 ± 6.76^{b}	22.07 ± 1.55^{b}
Total carotenoids	0.55 ± 0.00^{b}	0.64 ± 0.01^{a}	0.35 ± 0.00^{e}	0.46 ± 0.02^{c}	0.41 ± 0.01^{d}

Note: Different superscript letters in the same row represent statistically different results ($p < 0.05$).

common in fruit pulps. For instance, Häkkinen et al. (1999) found that arctic bramble and cloudberry fruits are rich sources of ellagic acid and contain a small fraction of flavonoids and phenolic acids, whereas lingonberry fruit contains flavonoids as major compounds followed by hydroxycinnamic acids. Strawberry fruit contains a balanced proportion between ellagic acid derivatives and hydroxycinnamic acids, and a small proportion of flavonols. These results are in-line with the data obtained herein (Table 2). Suvanto et al. (2017) studied the phenolic composition of strawberry, arctic bramble, cloudberry and lingonberry cell cultures and found that gallic acid derivatives (cloudberry), procyanidins (cloudberry, lingonberry and strawberry) were the main phenolic classes. lingonberry also presented cyanidin, delphinidin, petunidin, and peonidin glucosides, galactosides, and/or arabinosides.

Accordingly, from the phenolic composition of plant cell cultures reported in Data set 1 – supplementary material, lingonberry was highlighted to be a source of isomeric glycosylated forms of cyanidin, peonidin, and delphinidin followed by different glycosylated isomers of luteolin and quercetin. Interestingly, arctic bramble was found to be abundant in exclusive phenolic compounds, such as piceatannol (stilbenes), 5-nonadecylresorcinol (alkylphenols), isorhamnetin 3-*O*-glucuronide and methylgalangin (flavonols), 6-prenylnaringenin (flavanones), and petunidin 3-*O*-rhamnoside (anthocyanins). Analogously, acetyl-glycosidic forms of malvidin (anthocyanins) and 1-sinapoyl-2,2′-diferuloylgentiobiose (hydroxycinnamic acid) exclusively characterized cloudberry plant cell cultures (Data set 1 – supplementary material). Finally, resveratrol (stilbenes) was detected only in stone berry, whilst pterostilbene (stilbenes), epicatechin-(2a-7)(4a-8)-epicatechin 3-*O*-galactoside (flavan-3-ols), and some hydroxyphenylpropenes (such as anethole and estragole) exclusively characterized in strawberry. Finally, regarding terpenoids annotated with a lipidomic-based approach, we found a great abundance of some compounds, namely violaxanthin (detected in each sample under investigation with high abundance values), 10′-Apo-beta-caroten-10′-al, 2′-Apo-beta-carotenal (showing the greatest abundance in AB), 3′,4′-dihydrorhodovibrin, apo-10′-violaxanthal, and (3R,3′R,6′R,9-cis)-beta, epsilon-carotene-3,3′-diol (showing the greatest abundance in LI).

3.2. Multivariate statistical discrimination of the different samples

In this work, both unsupervised and supervised multivariate statistical approaches were used to visualize the differences and analogies in the phytochemical profiles of the different plant-cell cultures. The not-averaged unsupervised hierarchical cluster analysis (HCA) allowed to produce a heat-map based on the fold-change variations of the different bioactive compounds across each sample (i.e., strawberry, lingonberry, stone berry, arctic bramble, and cloudberry). The heat-map is provided as Fig. 1A. As can be observed, 2 main clusters and 4 sub-clusters could be discriminated; strawberry was characterized by the most characteristic phytochemical profile, whilst arctic bramble and cloudberry presented similar fold-change variations for specific clusters of metabolites (Fig. 1A). In addition, stone berry and lingonberry (although belonging to the same main cluster of arctic bramble and cloudberry) showed the most exclusive phytochemical fingerprint. In addition, the same output was obtained when considering the PCA score plot (supplementary material), with two principal components able to explain>70% of the variability. Therefore, both the HCA and PCA were particularly able to differentiate plant cells according to the different genus; in fact, it was evident that those plant-cell cultures belonging to the *Rubus* genus clustered together, whilst lingonberry (belonging to the *Vaccinium* genus) and strawberry (*Fragraria* genus) possessed more different phenolic profiles. Therefore, the unsupervised statistical approach allowed us to confirm the ability of phytochemical fingerprinting for discrimination purposes.

Thereafter, a supervised multivariate statistical approach, namely orthogonal projection to latent structures discriminant analysis (OPLS-DA) was carried out in order to extrapolate those compounds mostly explaining the discrimination power. The OPLS-DA score plot is provided as Fig. 1B. As can be observed, the introduction of an orthogonal signal allowed to separate samples in 3 main groups; the first one (on the left of the score plot) consisted in lingonberry, whilst the second one (upper-right zone of the score plot) was composed of arctic bramble, cloudberry, and strawberry. Finally, stone berry were found to possess a more specific phytochemical profile, although being included on the right panel by the orthogonal vector (Fig. 1B). The OPLS-DA model built was characterized by more than acceptable validation parameter, being the goodness-of-fit (R^2Y) = 0.99 and the goodness-of-prediction (Q^2) = 0.98. Additionally, the model was cross-validated by using a cross-validation ANOVA (p-value: 1.22×10^{-17}), whilst a permutation test (number of random permutations: 100) allowed to exclude model overfitting phenomena (supplementary material). Finally, a list of discriminant marker compounds of each plant-cell culture was obtained when considering the VIP method following the OPLS-DA modelling. The list of discriminant compounds can be found in Data set 1 – supplementary material. Overall, 165 compounds showed a VIP score (degree of discrimination) higher than 1; in this regard, the most represented classes were: flavonoids (69 marker compounds), followed by phenolic acids (30 compounds), and other compounds (including xanthophylls and tocotrienols). The highest VIP score was highlighted for the flavan-3-ol epicatechin-(2a-7)(4a-8)-epicatechin-3-*O*-galactoside, being 1.389) and exclusively characterizing strawberry extracts.

3.3. Antioxidant activity

Different antioxidant assays were used to assess the bioactivity potential of berry cell cultures. Table 1 shows that lingonberry presented the highest antioxidant capacity using FRAP, DPPH, CUPRAC, reducing power, and ABTS assays. FRAP results show that the other plant cell extracts display similar values, while for the free-radical scavenging effects (DPPH), strawberry presented the lowest mean value.

This is the first report on the antioxidant activity measurement of cell cultures of the berry species studied herein, except the cloudberry cell culture (VTT P-120083), for which the antioxidant activity reported earlier (Nohynek et al., 2014). However, for other *in vitro* cultivated species, the antioxidant activity measured by different assays is well known. For instance, Makowczyńska et al. (2015) studied the chemical composition and antioxidant potential of black horehound (*Ballota*

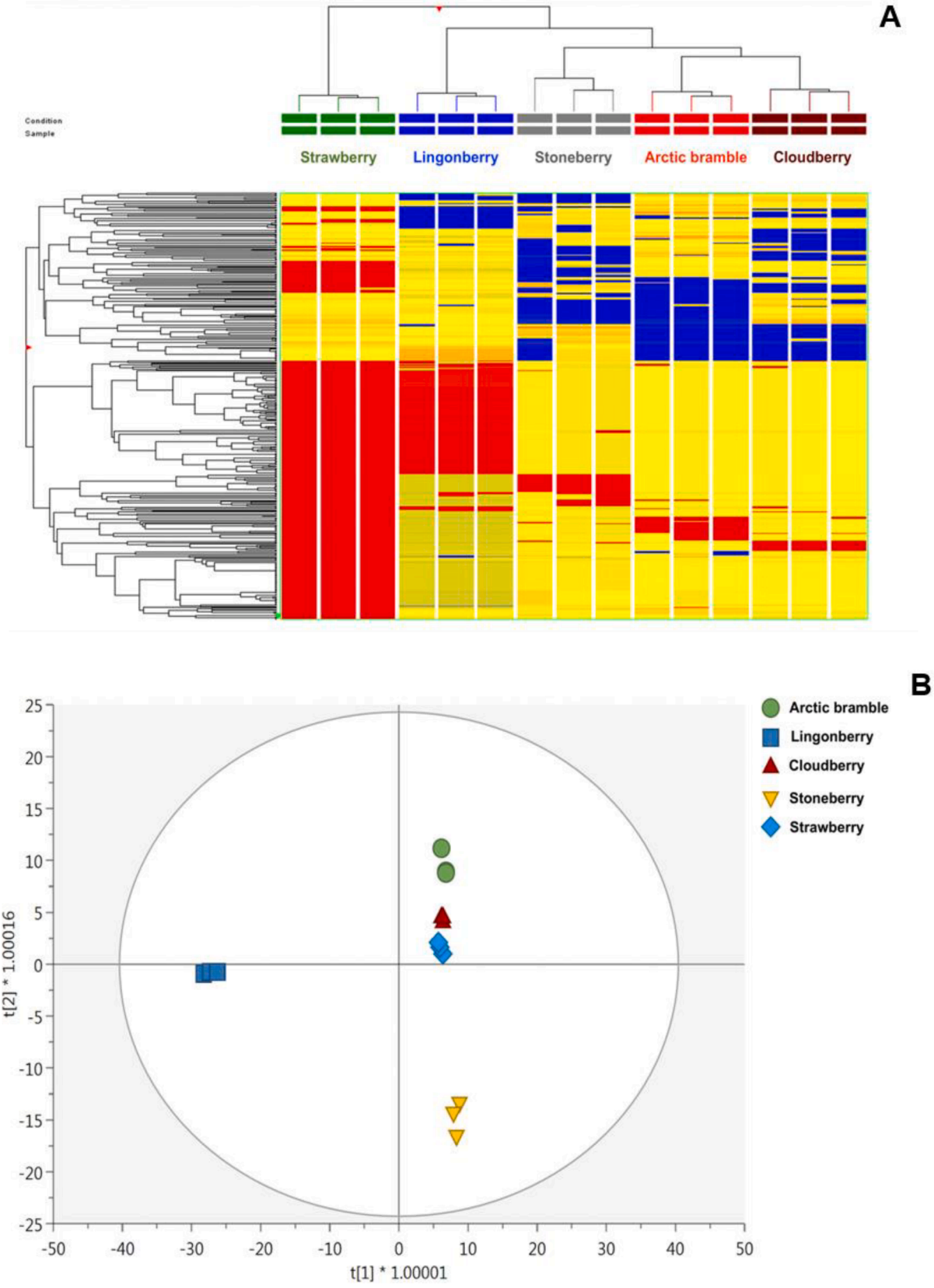


Fig. 1. Unsupervised non-averaged hierarchical cluster analysis (A) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) score plot (B).

nigra) and found 62 mg GAE/g and 17 mg CE/g of TPC and TFC. Shoots of *in vitro* propagated plant presented antioxidant activity using the DPPH (IC_{50} = 56 μg/mL), FRAP (642 μmol Fe(II)/g), and inhibition of linoleic acid oxidation (30%). Overall, the extract was less effective than butylated hydroxytoluene (BHT) and tocopherol. Similarly, Kumar et al. (2020) cultivated Indian spinach (*Basella rubra*) cell cultures using different lightening conditions and obtained a TPC of 74 mg GAE/100 g, TFC of 25 mg rutin equivalent/100 g, and FRAP of 336 mg AAE/100 g. The main phenolic compounds identified in the species were gallic, *trans*-cinnamic, and protocatechuic acids, catechin, quercetin, and rutin. The production of betaxanthins and betacyanins from red-purple pitaya (*Hylocereus costaricensis*) cell culture was conducted by Winson et al. (2020). A total betalanin content of 16.9 mg/g was achieved (betaxanthins = 9.30 mg/g, and betacyanins = 7.6 mg/g), TPC of 33 mg GAE/g, and TFC of 4.7 mg quercetin equivalent/g. The antioxidant activity measured by the DPPH assay showed an inhibition of 71%, which was far below the activity shown by ascorbic acid (93% inhibition).

3.4. Antihemolytic activity

The toxicity of the plant cell extracts in relation to O^+-type erythrocytes was evaluated in isotonic condition ([NaCl] = 0.8% w/v) (Fig. 2A). LI was the only sample that did not present hemolytic activity at the tested conditions. Cloudberry presented toxicity only at the lowest concentration tested (5.0 μg GAE/mL), while stone berry, arctic bramble and strawberry significantly increased ($p \leq 0.05$) the hemolysis rate in a dose-dependent manner. This toxicity could be justified by the pro-oxidant activity, in the biological medium and at the tested concentrations, of cloudberry, stone berry, arctic bramble and strawberry, highlighted by the oxyhemoglobin (Fe^{2+}) oxidation into methemoglobin (Fe^{3+}), which was verified by the absorbance increase at 630 nm (Fig. 2D). This is because compounds considered to be antioxidants, i.e. carotenoids and polyphenolic compounds, when in a biological environment, may present pro-oxidant behavior, depending on their structure (position of hydroxyls in the structure, presence of alkyl groups linked to oxygen, among other factors) or the condition of the medium (pH, oxygen concentration, etc.) (Kessler et al., 2003; Ribeiro et al., 2018).

In hypotonic condition (NaCl] = 0.4% w/v) (Fig. 2B), lingonberry reduced significantly ($p \leq 0.05$) the hemolysis rate, showing similar efficiency to quercetin at 5.0 μg/mL. This effect probably derives from its phenolic compounds (Table 1), mainly the anthocyanins, since anthocyanins-rich extracts usually present antihemolytic activity most likely by creating a physicochemical protection for erythrocytes (Migliorini et al., 2019). When compared to the negative control (0.0 μg/mL), the other samples did not change ($p > 0.05$) the hemolysis rate,

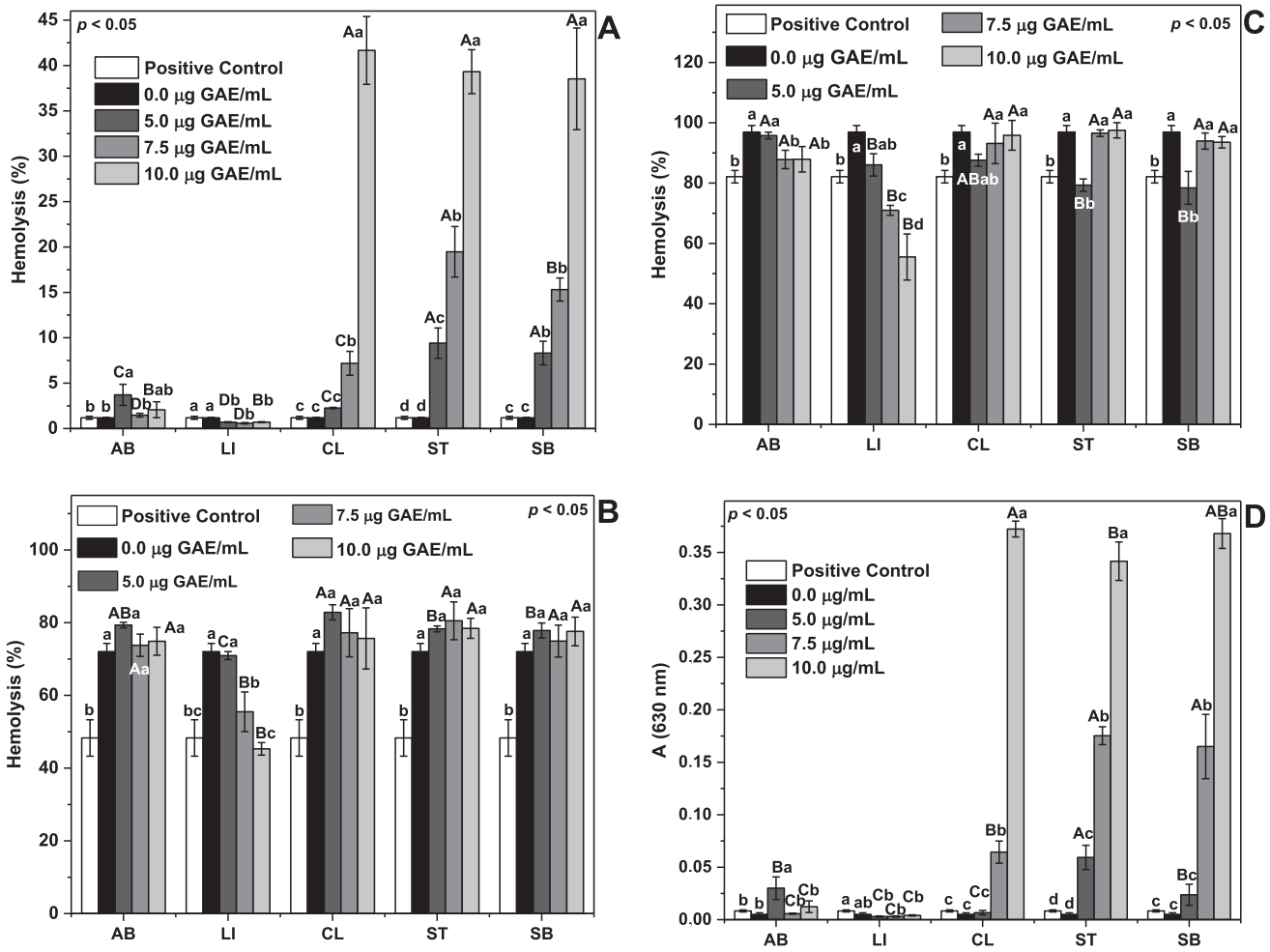


Fig. 2. Hemolysis rates verified at the presence of 5.0, 7.5 e 10.0 μg GAE/mL, in comparison to quercetin 5 μg/mL (positive control) and absence of extracts, at (A) isotonic ([NaCl] = 0.8% w/v), (B) hypotonic ([NaCl] = 0.4% w/v) and (C) harsh hypotonic ([NaCl] = 0.1% w/v) conditions. Absorbance measured at 630 nm, pointing the hemoglobin oxidation in the presence of the samples and quercetin 5 μg/mL in (D) isotonic ([NaCl] = 0.8% w/v), (E) hypotonic ([NaCl] = 0.4% w/v) and (F) harsh hypotonic ([NaCl] = 0.1% w/v) conditions. Different capital letters represent statistically different mean values ($p \leq 0.05$) in the presence of different samples at the same concentration. Different lower-case letters are responses statistically different ($p \leq 0.05$) of each sample at different concentrations. Note: AB = arctic bramble, SB = strawberry, ST = stoneberry, LI = lingonberry, CL = cloudberry.

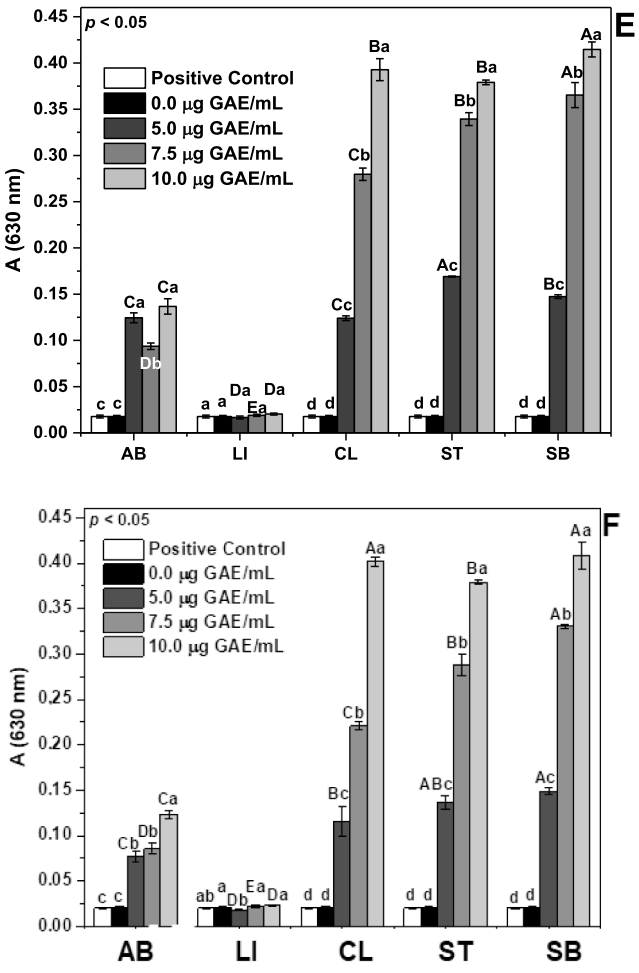


Fig. 2. (*continued*).

despite promoting the hemoglobin oxidation (Fig. 2E), which, once again, demonstrates the pro-oxidant activity of samples arctic bramble, cloudberry, stone berry and strawberry.

The LI's antihemolytic activity *in vitro* was confirmed by the results obtained in harsh hypotonic condition ([NaCl] = 0.1% w/v) (Fig. 2C). This protection is dose-dependent and, at the 7.5 e 10.0 µg GAE/mL concentrations, is even greater than the antihemolytic effect of quercetin at 5.0 µg/mL. In this osmotic condition, stone berry and strawberry, at the 5 µg GAE/mL, and arctic bramble, at the 7.5 e 10.0 µg GAE/mL, reduced significantly ($p \leq 0.05$) the hemolysis rate, despite causing the hemoglobin oxidation (Fig. 2F). This is because, in this osmotic condition, erythrocytes are swollen and cell membranes become more permeable, which facilitates the interaction between the pro-oxidant compounds and the hemoglobin.

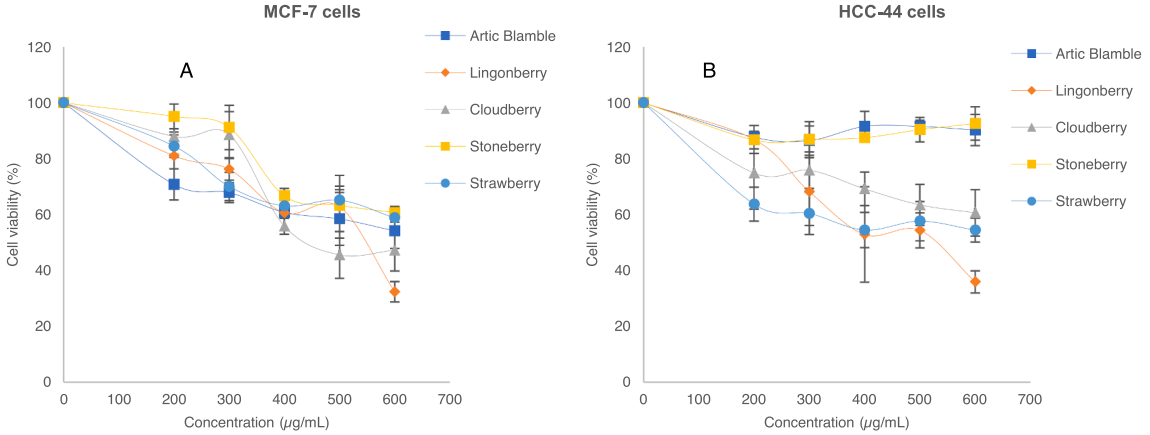


Fig. 3. Effects of different plant cell extract concentrations on the cell viability of MCF-7 (mammary gland adenocarcinoma) (A) and HCC-44 (non-small cell lung carcinoma) cell lines (B).

3.5. Cell viability and antiproliferative assessments

The results obtained showed that the extracts decreased moderately the proliferation of both breast cancer cells MCF-7 and lung cancer cells HCC-44 (Fig. 3). The inhibition of the proliferation is concentration dependent, and the degree of inhibition was found different between extracts. For MCF-7 cells (Fig. 3A), the cloudberry extract had the highest cytotoxicity, with an EC_{50} value of 513.0 ± 66.1 μg/mL, followed by lingonberry with an EC_{50} value of 526.2 ± 53.6 μg/mL. Extracts of arctic bramble, stone berry, and strawberry showed very little effect on the proliferation of MCF-7 cells. The viability of HCC-44 lung cancer cells was also inhibited in a dose-dependent manner by these berry extracts (Fig. 3B). Lingonberry extract had the highest inhibition effect for the proliferation of HCC-44 cells with an EC_{50} value of 462.0 ± 66.7 μg/mL, while arctic bramble and stone berry showed very little effect. For the highest tested concentration (600 μg/mL), the cell viability for these last two extracts decreased from 100% (control) to 90.3% and 92.6%, respectively.

Based on the literature, the antiproliferative activities (EC_{50}) of the extracts are generally categorized into four groups: ≤20 μg/mL, active; >20–100 μg/mL, moderately active; >100–1000 μg/mL, weakly active; and > 1000 μg/mL, inactive (Baharum et al., 2014; Nordin et al., 2018). Most plant cell cultures have been grown because of the interest in phenolic compounds that display antioxidant activity (Piątczak et al., 2014). However, Skorić et al. (2012) cultivated plant cells (roots and shoots) from *Cistus creticus* and showed that the ethanolic extract, rich in phenolic compounds (TPC = 1.4–1.7 mM GAE/g, TFC = 0.2–0.4 mM rutin equivalent/g), was effective against the proliferation of HeLa (cervix), MDA-MB-453 (breast) and FemX (melanoma) cancer cells, with IC_{50} values below 100 μg/mL.

Many previous studies have demonstrated the antiproliferative effect of fruits and berries in cancer cell lines. The study developed by Olloson et al. (2004), showed the effects of 10 different extracts of fruits and berries on cell proliferation of colon cancer cells HT29 and breast cancer cells MCF-7. The extracts of rosehips, blueberries, sea buckthorn, apple peel, plum, blackcurrant, and lingonberry were effective inhibitors of HT29 and MCF-7 cells whereas raspberry, black chokeberry, and cherry extracts were considerably less effective. The inhibition effect for the highest concentration of the extracts varied 2–3-fold among the species, and it was in the ranges of 24–68% for the MCF-7 cells and 46–74% for the HT29 cells.

Seeram et al. (2006) also evaluated the antiproliferative effect of six popularly consumed berries, namely blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry, against human oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT116), and prostate (LNCaP) tumor cell lines. With increasing concentration of berry extract, increasing inhibition of cell proliferation in all cell lines were observed, with different degrees of potency between extracts. In a study conducted by McDougall et al. (2008), polyphenol-rich berry extracts, particularly those from the *Rubus* genus (cloudberry, arctic bramble, and raspberry) but also lingonberry and strawberry showed to be effective in preventing the proliferation of human cervical (HeLa) and colon (CaCo-2) cancer cells. The most effective extracts (strawberry, arctic bramble, cloudberry and lingonberry) gave EC_{50} values in the range of 25–40 μg GAE/mL. In this study, all the extracts were enriched in polyphenols and depleted of organic acids, sugars, carotenoids, and vitamin C, and therefore the relative contribution of different polyphenol components could be observed.

3.6. Correlation analyses

Correlation coefficients were calculated to assess the degree of association between the chemical composition and the bioactivity of the plant cell culture extracts (Table 1 – supplementary material). Regarding the antioxidant potential, the reducing power, ABTS, FRAP, CUPRAC and the free-radical scavenging activity in relation to DPPH was positively and significantly ($p < 0.05$) correlated to TPC, TFC, TOC, TCT, total carotenoids, TAC, in which lignans, phenolic acids, and flavones were the main responsible agents. Major phenolic classes and subclasses (i.e., flavonoids) are well known to contribute to the *in vitro* antioxidant activity using chemical-based protocols, thus our results are in-line with the reported literature (Santos et al., 2018; Schechtel et al., 2019). Considering the viability of cancerous cells when extract concentration was 600 μg/mL, flavones (r = −0.829, r = −0–673), lignans (r = −0.878 and r = 0.852), and anthocyanins (r = −0.811, r = −0–662) seemed to be the main contributors to the cytotoxicity. Phenolic acids (r = −0.615) and total carotenoids (r = −0.526) also correlated significantly to the viability in MCF-7 cell. Stilbenes did not show any obvious correlation neither with the viability of both cell lines nor with the antioxidant activity. For the hemolysis of human erythrocytes, an inverse and significant correlation was found between phenolic classes/subclasses and the hemolysis rate in isotonic condition, indicating that the phenolic compounds are correlated to the reduction of the hemolysis rate. More specifically, flavones (r = -0.653), phenolic acids (r = -0.994), lignans (r = -0.589), anthocyanins (r = -0.604), and stilbenes (r = -0.742), and carotenoids (r = -0.909) were the observed classes of bioactive compounds correlated to the hemolysis rate. More interestingly, the protection of human red blood cells was correlated to the antioxidant activity measured by different methodologies – CUPRAC (r = -0.624), DPPH (r = -0–630), FRAP (r = -0.628), and reducing power (r = -0.639). These results corroborate data obtained with different plant-based extracts containing phenolic acids, flavonoids with or without anthocyanins (Santos et al., 2018; Migliorini et al., 2019).

4. Conclusions

Results obtained herein suggest that berry *in vitro* cultures provide an attractive route to produce high-value plant-derived secondary metabolites, i.e. phenolics, and therefore can be an alternative source of bioactive ingredients for food/beverage models with antioxidant potential. From the plant cell cultures studies, lingonberry showed more interesting results as the content of bioactive substances, especially anthocyanins, and bioactivities were higher compared to the other plant cells. On the other hand, arctic bramble and strawberry cell cultures presented the lowest contents of bioactive compounds and antioxidant activity.

Further studies should focus on the use of different normal human cell models (i.e., white blood, intestinal and liver cells) and, possibly, animal models to assess the toxicological profile of food grade extracts from the plant cells selected herein. The concept of cultivating plant cells to recover bioactive compounds may be an alternative to using synthetic compounds in different food applications. Due to the very beneficial nutritional composition in addition to the bioactive compounds plant cell cultures constitute also attractive “integrated” food ingredients.

Author contributions

T.M.C., M.B.M., L.N., R.P-P. and H.R.: Conceptualization, Funding acquisition, Formal analysis, Writing - original draft, Writing - review & editing. G.R., L.L., J.A and J.S.C.: Resources, Formal analysis, Writing - original draft, Writing - review & editing. D. G: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Software, Supervision, Writing - original draft, Writing - review & editing. All authors helped in the writing of the original draft and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130571.

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