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Toxicological and bioactivity evaluation of blackcurrant press cake, sea buckthorn leaves and bark from Scots pine and Norway spruce extracts under a green integrated approach

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ABSTRACT

Aqueous extracts from blackcurrant press cake (BC), Norway spruce bark (NS), Scots pine bark (SP), and sea buckthorn leaves (SB) were obtained using maceration and pressurized hot water and tested for their bioactivities. Maceration provided the extraction of higher dry matter contents, including total phenolics (TPC), anthocyanins, and condensed tannins, which also impacted higher antioxidant activity. NS and SB extracts presented the highest mean values of TPC and antioxidant activity. Individually, NS extract presented high contents of proanthocyanidins, resveratrol, and some phenolic acids. In contrast, SB contained a high concentration of ellagitannins, ellagic acid, and quercetin, explaining the antioxidant activity and antibacterial effects. SP and BC extracts had the lowest TPC and antioxidant activity. However, BC had strong antiviral efficacy, whereas SP can be considered a potential ingredient to inhibit α-amylase. Except for BC, the other extracts decreased reactive oxygen species (ROS) generation in HCT8 and A549 cells. Extracts did not inhibit the production of TNF-alpha in lipopolysaccharide-stimulated THP-1 macrophages but inhibited the ROS generation during the THP-1 cell respiratory burst. The recovery of antioxidant compounds from these by-products is incentivized for high value-added applications.

1. Introduction

Side streams of berries and woods offer diverse sources of various bioactive compounds. Especially berries are excellent and variable sources of phenolic compounds, such asphenolic acids, flavonoids, and proanthocyanidins (Ovaskainen et al., 2008). Industrial side streams from berry and wood sectors have a huge potential as they may contribute to the improvement of the circular economy, especially if cascade use models are adopted, that is, reusing and recycling products as many times as possible before their final disposal via energy generation or landfilling. In this panorama, in many EU countries, the production of sea buckthorn (SB), Norway spruce (NS), Scots pine (SP), and blackcurrant (BC) is of economic significance as a wide array of products are manufactured – from food ingredients to furniture. The recovery of

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bioactive compounds from industrial by-products using green approaches would be an alternative for the energetic use of these valuable materials.

Leaves of sea buckthorn (*Hippophaë rhamnoides* L.) are by-products of berry plant cultivation. They have been reported to contain high levels of phenolic compounds, proteins, amino acids, minerals, vitamins, fatty acids, carotenoids, and tocopherols. Further, sea buckthorn leaf extracts are reported to have antibacterial, anti-viral, antitumoral, anti-inflammatory, and antioxidative activities (Ciesarová et al., 2020; Tian et al., 2017). According to Tian et al. (2017), the sea buckthorn leaves contained more polyphenols and showed different phenolic profiles (ellagitannins above 90%) from the corresponding berries.

Blackcurrant (*Ribes nigrum*) pomace is the material that remains after juice pressing containing mainly stems, peels and seeds. According to the recent review by Cortez and de Mejia (2019), BC pomace is still not sufficiently exploited even though it is rich in highly antioxidative polyphenols and other bioactivities that may improve the consumer's overall health. In cell-based studies, BC press cake, which is a rich source of cyanidin and delphinidin rutinoside) enhanced the proliferation of the *Lactobacillus rhamnosus* and showed antimicrobial effects in relation to *Salmonella enterica* and *Streptococcus pneumonia* (Parkar et al., 2014; Ikuta et al., 2012). The antiviral activity of blackcurrant extracts against respiratory syncytial virus (RSV), influenza virus A and B (IFV-A and IFV-B), adenovirus (AdV), and herpes simplex virus type 1 has already been reported (Ikuta et al., 2012).

Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) are the most common trees in Northern Europe. Bark and knot wood are the most economically available wood residues. The phenolic compounds found in these trees include stilbenes, flavonoids, proanthocyanidins, phenolic acids, and lignans which have demonstrated antioxidant and antibacterial properties (Metsämuuronen and Sirén, 2019; Mäkinen et al., 2020).

The use of polyphenols as bioactive compounds at various business levels has urged scientists to investigate green extraction approaches to achieve eco-friendlier/sustainable, efficient and cost-effective techniques. Because the industrial side streams are still underutilized, there is a clear need to develop sustainable, green, and safe extraction methods to recover value-added compounds from these natural resources. More information about the extract's potential regarding its toxicological safety and *in vitro* bioactivities is required to understand more deeply the health benefits of industrial side-streams of BC, SB, NS, and SP. Thus, the objectives of this study are to characterize the chemical composition and antioxidant, antimicrobial and anti-inflammatory activities of BC, SB, NS, and SP aqueous extracts and their relative toxicological profile (i.e., toxicity) in different human cell lines.

2. Materials and methods

2.1. Chemical reagents

Cyanidin 3-*O*-rutinoside (≥95%), cyanidin 3-*O*-glucoside (≥95%), delphinidin 3-*O*-rutinoside (≥95%), delphinidin 3-*O*-glucoside (≥95%), petunidin 3-*O*-glucoside (≥95%), peonidin 3-*O*-glucoside (≥95%), and procyanidin B2 (>90%) were purchased from Extrasynthese (Lyon, France). Glacial acetic acid and phosphoric acid (85%) were from J. T. Baker (Mallinckrodt Baker Inc., Utrecht, The Netherlands). Folin-Ciocalteu reagent, gallic acid, ferrozine (3- (2-pyridyl) −5,6-di (2-furyl) −1,2,4-triazine-5′,5″ disodium disulfonic acid), ascorbic acid, 2-thiobarbituric acid (TBA), bacterial lipopolysaccharides (LPS), neocuproin (2,9-dimethyl-1,10-fenantroline), sodium carbonate, and 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), catechin (99%), epicatechin (98%), gallocatechin (98%), and epigallocatechin (95%), gallic acid (98%), ellagic acid (95%), cysteamine (98%), formic acid (98%), dichlorodihydro-fluorescein diacetate (DCFH-DA), 3–4,5 dimethylthiazol-2, 5 diphenyl tetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM), heneicosanoic acid, betulinol were acquired from Sigma Aldrich (St. Louis, USA). HPLC grade methanol (≥99.8%), acetonitrile (≥99.8%), ferric chloride hexahydrate, anhydrous sodium acetate, copper sulfate pentahydrate, 1,10 phenanthroline monohydrate, and copper chloride dihydrate were from VWR Chemicals BDH® (Germany). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Alfa Aesar (Ward Hill, Massachusetts, USA), while phosphate buffer pH 7.5 and potassium hexacyanoferrate (III) were obtained from Merck (Darmstadt, Germany). The other reagents were of analytical grade. Aqueous solutions were prepared using ultrapure water (Millipore, São Paulo, Brazil). Sodium pyruvate, RPMI 1640-GlutaMAX™ medium, streptomycin, 2-mercaptoethanol, L-glutamine, [+]-D-glucose were obtained from Gibco® Invitrogen™, USA. HEPES was obtained from Fisher Scientific, Göteborg, Sweden. Phorbol-12-myristate-13-acetate, PMA was obtained from Santa Cruz Biotechnology, Santa Cruz, USA. The other reagents were of analytical grade.

2.2. Plant materials

Sea buckthorn leaves var. Raisa (moisture content of 61.1%) were collected in Nurmela, Finland, in September 2016. Freeze-dried Norway spruce bark (moisture content of 3.8%) and fresh Scots pine barks (moisture content of 59.4%) were collected in a wood industry Pietarsaari in March 2020. Industrial blackcurrant press cake (moisture content of 49.6%) was obtained in 2019 from a berry processing company located in East-Finland (Suomussalmi). All samples were standardized concerning the particle size (2 mm) using an industrial mill (Fritsch GmbH, model 15903, Idar-Oberstein, Germany) and extracted within 24 h. It is important to note that the study was conducted without further drying of the raw materials to avoid degradation of bioactive compounds and to eliminate one more operation unit in the process, thus mimicking how the industrial process.

2.3. Extraction technologies

Raw materials were extracted using a classic method (e.g., maceration) compared to pressurized hot water extraction (PHWE). For maceration, 100 g of raw material was extracted with 1000 mL of distilled water, under constant magnetic agitation and temperature monitoring, for 60 min at either 40 °C (blackcurrant press cake) or 50 °C (sea buckthorn leaves, Norway spruce bark, and Scots pine bark). For PHWE, the same temperature and extraction time parameters were used in accelerated solvent extractor (ASE-350, Dionex, Sunnyvale, USA), employing a pressure of 100 bar (98.7 atm) in 2 extraction cycles. Then, extracts were filtered using qualitative paper (Whatman n.1) and directly analyzed. The total solids content, expressed as a percentage of the raw material used in the extraction, was analyzed by weighing 3 mL of the extract and let it dry until constant weight at 105 °C. Part of the liquid extracts was freeze-dried under vacuum at 1200 μL of Hg for 120 h, and the extraction yield, expressed as a percentage, was calculated concerning the raw material used in the procedure. Extractions were performed in two independent replicates.

2.4. Phenolic composition

The total phenolic content (TPC) of extracts was analyzed using the spectrophotometric method based on the Prussian Blue assay, and results were expressed as mg of gallic acid equivalent per 100 g of material (wet basis), mg GAE/100 g or per gram (freeze-dried extracts) (Margraf et al., 2015). Total condensed tannins (CT) were estimated using the spectrophotometric method that employs vanillin in acidic medium (H_2SO_4), and results were expressed as mg of (+)-catechin equivalent per 100 g of material (wet basis), mg CE/100 g or per gram (freeze-dried extracts) (Horszwald and Andlauer, 2011). Total monomeric anthocyanins (TA) of blackcurrant press cake extracts were quantified using the

pH differential method described by Lee et al. (2005), and results were expressed as mg of malvidin-3-glucoside equivalent per 100 g of material (wet basis), mg/100 g. Total flavonoid content was assayed using the aluminum chloride hexahydrate protocol described by Zhishen et al. (1999) and results were expressed as mg CE/g. Analyses were performed in triplicate.

HPLC determination of anthocyanins from BC extract was performed according to the method described by Hellström et al. (2013). Agilent 1100 (Agilent Technologies Inc., Espoo, Finland) HPLC device equipped with diode array detection (DAD) and a Gemini C_{18} column (150 × 4.6 mm, 5 μm) was used and the separation was accomplished with a gradient elution of acetonitrile into 5% formic acid (aq). Peonidin and petunidin rutinoside and coumaroyl derivatives of glucosides of cyanidin and petunidin were quantified as corresponding glucosides. Ellagitannin content was determined after acid hydrolysis, according to Mattila and Kumpulainen (2002). Ellagic and gallic acids were determined using an Inertsil ODS-3 column (150 × 4.0 mm, 3 μm) with a gradient elution of acetonitrile into 50 mmol/L H_3PO_4 (pH 2.5).

Proanthocyanidins in freeze-dried extracts was determined by HPLC after thiolytic degradation, according to Mattila et al. (2018). A Zorbax Eclipse Plus C_{18} column (50 × 2.1 mm, 1.8 μm) was used with a gradient elution of acetonitrile into 0.5% formic acid (v/v) provided by UHPLC device (Agilent 1290 Infinity, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with diode array (DAD) and fluorescence detection (FLD).

2.5. Monomeric sugar composition

The monomeric sugar composition was determined by gas chromatography-flame ionization (GC-FID) detection via acid methanolysis and derivatization as described by Raitanen et al. (2020). Analyses were conducted in triplicate and results were expressed as mg/g od freeze dried extract.

2.6. Gas chromatography-mass spectra of extracts

The GC-mass spectrometry (MS) screening and quantification of some phenolic compounds (except for anthocyanins, ellagitannins, and proanthocyanidins) was performed on a GC-MS (HP6890-5973 GC-MSD instrument, Hewlett Packard, Palo Alto, CA, USA) using a HP-5 column (Agilent Technologies, Inc., Santa Clara, CA, USA; 30 m × 0.25 mm i.d., film thickness 0.25 μm) according to the methodology and conditions described by Raitanen et al. (2020). Helium was used as carrier gas and the injection was made in splitless mode. Mass spectra were obtained in EI mode (70 eV) and the fragmentation pattern was compared to standards in commercial (NIST14/Wiley11) libraries, as well as the standards in our own MS libraries available at our laboratory. Analyses were conducted in triplicate and results were expressed as mg/g od freeze-dried extract.

2.7. Antioxidant activity (AA)

The ferric reducing antioxidant power (FRAP), the scavenging activity of DPPH radical and the cupric ion reducing antioxidant capacity (CUPRAC) were assayed according to Xu et al. (2021) and results were expressed as mg of ascorbic acid equivalent per 100 g of material (wet basis), mg AAE/100 g. Folin-Ciocalteu reducing capacity was assessed using the methodology described by Margraf et al. (2015), and results expressed as mg GAE/100 g. The Fe^{2+} chelating ability (6–75 mg/L, R^2 = 0.986) was evaluated using ferrozine as the chromogen agent, and results were expressed as mg of disodium ethylenediaminetetraacetic acid (EDTA) per 100 g of material (wet basis), mg EDTAE/100 g) (Santos et al., 2017). Oxygen radical absorbance capacity (ORAC) of the freeze-dried extracts was determined in triplicate according to the methods described by Prior et al. (2003) and results were expressed as Trolox equivalent per gram of freeze-dried extract (μmol TE/g).

The inhibition of lipid oxidation (ILP) of Wistar rat's brain homogenate was assessed using the protocol described by Fidelis et al. (2018), in which 7-month old male wild-type Wistar rats (Envigo Venray, the Netherlands) were used in the experiment. Different concentrations of freeze-dried extracts obtained via maceration were tested (500, 1000, 2000, 3000, and 4000 mg/L) and thiobarbituric acid reactive species (TBARS) were measured at 532 nm using a spectrophotometer. Water was used as the negative control (maximum oxidation), and results were expressed as mg quercetin equivalent per gram of freeze-dried extract (mg QE/g). Approval of the Ethics Committee was obtained at the University of Helsinki.

The hydroxyl radical scavenging capacity of freeze-dried extract was assessed using a modified method initially described by Mukhopadhyay et al. (2016). For 750 μL of diluted sample, 150 μL of the $FeSO_4.7H_2O$ solution at 1 mmol/L and 100 μL of a H_2O_2 solution at 15 mmol/L. After 5 min, 750 μL of 1,10-phenanthroline at 1 mmol/L were added to the test tube and the content was vortexed for 10 s. After 20 min, the absorbance was read at 536 nm and water was used as the negative control and for the blank, H_2O_2 was replaced by water. The hydroxyl radical scavenging capacity was calculated according to Equation 1 and results were compared to a standard curve using gallic acid as the standard (0.75–50 mg/L, R^2 = 0.995) and expressed as mg GAE/g:

Hydroxyl radical scavenging capacity (%inhibition) = (Abs_{sample}/Abs_{blank}) x 100 (Eq. 1).

2.8. Antimicrobial effects

2.8.1. Microorganisms and cell suspensions

The microorganisms used were *Pseudomonas aeruginosa* (IAL, 1853), *Staphylococcus aureus* (ATCC 13565), *Staphylococcus epidermidis* (ATCC 12228), grown in Nutrient agar (Kasvi, São Paulo, Brazil) during 24 h at 37 °C for *P. aeruginosa* and 30 °C for *Staphylococcus. Candida albicans* (INCQS 40071) and *Propionibacterium acnes* (INCQS 229) were cultivated in Potato Dextrose Agar (Kasvi, São Paulo, Brazil) (30 °C/48 h) and Blood Agar (Laborclin, Paraná, Brazil) (37 °C/48 h, under anaerobiosis). The concentration of cells in the suspensions were standardized at 0.5 McFarland scale using turbidimeter (MS Tecnopon, Piracicaba, Brazil).

2.8.2. Antimicrobial tests

The antimicrobial activity tests carried out in this study were done as previously proposed (Cleeland and Squires, 1991) using the plate-cavity agar diffusion method (Escher et al., 2018) using 10^6 CFU per mL of each microorganism. Dimethyl sulfoxide (DMSO) was used as a negative control while imipenem (10 μg) was used as a positive control, excluding for *P. acnes* for which Penicillin G (10 IU) was used. All tests were performed twice in triplicate and the results expressed as halo of inhibition (in cm).

The minimum inhibitory concentration was performed according to Siddiqi et al. (2011). The extracts were diluted in DMSO so that the concentration of the stock solution was equal to 1000 μg/mL. Serial dilutions were prepared to obtain 250, 125, 62.5, 31.25, 15.625 μg/mL, respectively. The lowest concentration that did not show microbial growth was considered to be the minimum inhibitory concentration (MIC).

2.9. Inhibition of α-amylase and α-glucosidase

The inhibition of α-amylase and α-glucosidase activities were determined using the protocol outlined by Johnson et al. (2011). Freeze-dried extracts were resuspended in ultrapure water and tested at different concentrations (α-amylase: 1, 5, 10, 15, and 20 mg/mL; α-glucosidase: 10, 25, 50, 100, 200 and 500 μg/mL). For the α-amylase assay, acarbose (1 mmol/L) was used as a positive control. The inhibition of the enzyme activity was expressed in percentage and, when applicable, IC_{50} values (the concentration required to inhibit enzyme

Table 1
Comparison between maceration and pressurized hot water extraction (PHWE) on the extraction yield, dry matter content, bioactive compounds, and antioxidant activity of sea buckthorn leaves, blackcurrant press cake, Scots pine bark, and Norway Spruce bark extracts.

Extraction techniques	Raw materials	Extraction yield (% w/w)	Dry matter (% w/w)	TPC (mg GAE/100 g)	Condensed tannins (mg CE/100 g)	Total anthocyanins (mg/100 g)	CUPRAC (mg AAE/100 g)	Fe^{2+} chelating ability (mg EDTAE/100 g)	DPPH (mg AAE/100 g)	FCRC (mg GAE/100 g)
Maceration	Norway spruce bark	11.59 ± 0.20^{b}	16.09 ± 0.13^{a}	2199±6^{a}	932 ± 61^{a}	ND	34414 ± 353^{a}	ND	2383±5^{b}	5388 ± 73^{a}
	Scots pine bark	2.20 ± 0.16^{e}	2.16 ± 0.04^{f}	488 ± 15^{f}	238 ± 19^{d}	ND	3051 ± 63^{d}	ND	296±3^{f}	509 ± 14^{e}
	Sea buckthorn leaves	12.74 ± 0.35^{a}	11.76 ± 0.10^{b}	2119 ± 31^{b}	218±6^{d}	ND	22992 ± 104^{b}	155 ± 25^{a}	3318 ± 30^{a}	3280 ± 90^{b}
	Blackcurrant press cake	4.90 ± 0.52^{c}	5.37 ± 0.03^{d}	944 ± 10^{e}	632 ± 48^{b}	225.80 ± 12.36^{a}	2409 ± 283^{e}	ND	543±6^{e}	656 ± 21^{d}
PHWE	Norway spruce bark	3.73 ± 0.33^{d}	5.33 ± 0.06^{d}	1173±5^{d}	551 ± 24^{c}	ND	9431 ± 492^{c}	ND	1279 ± 34^{d}	2148 ± 112^{c}
	Scots pine bark	2.18 ± 0.26^{e}	1.72 ± 0.15^{g}	194±6^{h}	51±2^{e}	ND	785 ± 18^{g}	ND	162±3^{g}	325±5^{f}
	Sea buckthorn leaves	10.90 ± 0.23^{b}	10.25 ± 0.10^{c}	1402±8^{c}	86±5^{e}	ND	9829 ± 136^{c}	61 ± 12^{b}	1663 ± 16^{c}	2087 ± 47^{c}
	Blackcurrant press cake	5.37 ± 0.26^{c}	4.65 ± 0.04^{e}	333 ± 13^{g}	510 ± 10^{c}	92.28 ± 0.36^{b}	1821 ± 84^{f}	ND	305±4^{f}	288±3^{f}

Note: Values are expressed as means followed by the standard deviation (n = 3) and are related to the raw materials (wet basis). Different letters in the same column represent statistically different results ($p < 0.05$). TPC = total phenolic content, FCRC = Folin-Ciocalteu reducing capacity, ND = not detected, GAE = gallic acid equivalent, CE = catechin equivalent, AAE = ascorbic acid equivalent, EDTAE = EDTA equivalent.

activity by 50%) were obtained in triplicate.

2.10. Anti-inflammatory activity in THP-1 cells

Kinetic measurements of THP-1 cell respiratory burst (RB) activity were conducted with a microplate reader (Hidex Sense, Turku, Finland) using white 96-well flat-bottom non-treated microtiter plates following the method fully described by Tompa et al. (2011) and LPS at 10 μg/mL was used to trigger ROS generation in the system. The freeze-dried extracts were dissolved in HBSS-buffer (30 mg/mL) and the pH was adjusted to 7.4, whereby different freeze-dried extract concentrations were tested (7–1667 μg/mL). The chemiluminescence (CL) signal was counted and integrated at 0.5 s at 2.33 min intervals for 90 min to obtain kinetic curves. The CL peak (relative light units, rlu) was regarded as the sample's CL value. The efficacies of extracts were expressed as IC_{50} values, i.e., concentrates, which inhibit 50% of the CL emission obtained from cells without extracts in the same plate.

2.10.1. THP-1 cell differentiation and cytokine production

THP-1 cells (2×10^5/mL) were differentiated for 48 h using a 50 ng/mL solution of PMA as described in Alqarni et al. (2019). After the 24 h incubation in PMA free medium, the extracts at different concentrations (7–1667 μg/mL) were added to cells and thereafter LPS (0.5 μg/mL) was added. After additional 24 h incubation, the TNF-α content was measured by ELISA kits (BioLegend® ELISA MAX™ Deluxe Set Human TNF-α, Cat No. 430204, San Diego, USA) according to the manufacturer's instructions. The plates were read at 450 nm and 570 nm and the absorbance readings were corrected by subtracting readings taken at 570 nm.

2.11. Anti-viral activity

Adenocarcinomic human alveolar basal epithelial (A549) cells and Coxsackievirus A9 strain (CVA9; Griggs strain, ATCC) were used in the assays. CVA9 was produced and purified as described before (Ruokolainen et al., 2019). The anti-viral activity was assessed using a cytopathic effect (CPE) Inhibition assay, as described before (Martikainen et al., 2015), and modified from Schmidtke et al. (2001). Cytotoxicity was also evaluated using the CPE inhibition assay, where the compounds (without any virus) were tested for their toxicity on A549 cells. Mock infection was used as a control for the assay.

2.12. Cell viability and proliferation: preliminary toxicological safety assessment

Effects of extracts on THP-1 promonocyte viability was measured as described in Alqarni et al. (2019). Briefly, the THP-1 cells were added into 96-well plates at a density of 1×10^5 cells per well and incubated for 24 h in an incubator (at 37 °C, in 5% CO_2 and 100% RH). After 24 h the buffered (pH 7.4) SP, NS, BC and SB extract solutions were added in final concentrations ranging from 7 to 1667 μg/mL (same as in the TNF-α production test), and the cells were incubated further for 24 h. Only the medium was added in negative control cells, and 2.5% and 5% DMSO was used as a positive control. Resazurin salt solution (0.1 mg/mL) was added at final concentration of 10% (v/v) and cells were incubated further. Fluorescence (544 nm excitation, 595 nm emission) was measured with Hidex Sense fluorometer (Hidex, Turku, Finland) at 6, 8 and 24 h time points. Cell viability was assessed as relative to the mean values of the negative controls.

The cytotoxicity of the extracts (SB, NS, SP, and BC) was evaluated in relation to lung adenocarcinoma epithelial cells (A549), human ileocecal adenocarcinoma cells (HCT8), and normal human lung fibroblast (IMR90). Briefly, cells were seeded into 96-well plates at a density of 6×10^3 cells/well (IMR90) and 1×10^4 cells/well (HCT8, A549). Following the treatment (100, 500, 1000, and 2000 μg/mL) for 48 h, MTT (0.5 mg/mL) was added to each well and incubated for 4 h at 37 °C. The metabolically active cells reduced the MTT to blue formazan crystals, which were dissolved in DMSO and absorbance was measured at 570 nm. The IC_{50} (50% cell viability inhibition), GI_{50} (50% growth inhibition), and LC_{50} (50% cell death) parameters were performed. Moreover, the selectivity index (SI) was calculated by the ratio IC_{50} (IMR90)/IC_{50} (cancer cell lines). Any sample with an SI value higher than 3 will be considered to have high selectivity (Boechat et al., 2014). Intracellular ROS generation was assessed using the DCFH-DA assay. Malignant (A549, HCT8) cells (6×10^4 per well) were treated for 1 h with different concentrations of anthocyanins extracts (100–1000 μg/mL) or 22.5 μmol/L H_2O_2 (positive control) or culture medium (negative control). Following the treatment, H_2O_2 was added at 22.5 μmol/L in the wells, and the fluorescence intensity (λemission = 538 nm and λexcitation = 485 nm) was measured (Escher et al., 2018).

Table 2
Chemical composition of freeze-dried extracts obtained with maceration from sea buckthorn leaves, Norway spruce bark, Scots pine bark, and blackcurrant press cake.

Chemical compounds	Norway spruce bark	Scots pine bark	Sea buckthorn leaves	Blackcurrant press cake
Arabinose (mg/g)	0.43 ± 0.09^{c}	2.03 ± 0.03^{a}	0.44 ± 0.09^{c}	1.58 ± 0.01^{b}
Fructose (mg/g)	75.26 ± 15.29^{d}	111.75 ± 15.36^{c}	138.94 ± 21.53^{b}	221.99 ± 14.61^{a}
Galactose (mg/g)	7.94 ± 1.62^{c}	24.54 ± 0.21^{b}	121.42 ± 8.26^{a}	3.16 ± 0.21^{d}
Galacturonic acid (mg/g)	1.39 ± 0.34^{c}	7.09 ± 0.61^{b}	0.54 ± 0.04^{d}	46.27 ± 2.42^{a}
Glucose (mg/g)	81.58 ± 17.31^{d}	95.13 ± 14.98^{c}	153.43 ± 1.18^{a}	137.93 ± 6.34^{b}
Glucuronic acid (mg/g)	0.52 ± 0.19^{c}	4.63 ± 0.72^{b}	6.41 ± 0.37^{a}	5.36 ± 11.11^{ab}
Mannose (mg/g)	8.34 ± 2.05^{c}	14.61 ± 2.44^{b}	11.03 ± 0.10^{b}	37.82 ± 7.17^{a}
Rhamnose (mg/g)	0.26 ± 0.05^{b}	0.86 ± 0.01^{a}	0.32 ± 0.01^{b}	0.07 ± 0.05^{c}
Xylose (mg/g)	0.30 ± 0.10^{c}	0.87 ± 0.01^{b}	1.70 ± 0.01^{a}	0.61 ± 0.04^{b}
Sucrose (mg/g)	89.58 ± 12.78^{a}	13.25 ± 1.29^{c}	24.04 ± 0.55^{b}	27.47 ± 0.75^{b}
Malic acid (mg/g)	2.70 ± 0.30^{c}	3.70 ± 0.50^{c}	31.70 ± 0.10^{a}	22.00 ± 0.10^{b}
Quercetin (mg/g)	26.20 ± 1.40^{b}	28.40 ± 5.80^{a}	21.10 ± 0.50^{c}	21.10 ± 0.10^{c}
Quinic acid (mg/g)	25.70 ± 2.20^{b}	51.10 ± 2.30^{a}	15.20 ± 0.70^{c}	ND
3,4,5-Trihydroxybenzoic acid (mg/g)	ND	ND	13.90 ± 0.30	ND
Ellagic acid (mg/g)	ND	ND	1.50 ± 0.60	ND
Pinitol (mg/g)	24.80 ± 0.60^{c}	70.60 ± 3.70^{b}	302.70 ± 11.70^{a}	ND
(+)-Catechin (mg/g)	ND	3.00 ± 0.20	ND	ND
Taxifolin (mg/g)	ND	2.60 ± 1.10	ND	ND
4-Hydroxycinnamic acid (mg/g)	2.60 ± 0.10^{a}	ND	ND	0.30 ± 0.10^{b}
3,5,4′-Trihydroxystilbene (mg/g)	0.30 ± 0.10	ND	ND	ND
Neoabietic acid (mg/g)	0.40 ± 0.10	ND	ND	ND
Isorhapontin (mg/g)	2.90 ± 0.30	ND	ND	ND
Astringin (mg/g)	3.00 ± 0.20	ND	ND	ND
Proanthocyanidins (mg/100 g)	4113 ± 39^{c}	8172 ± 210^{b}	9287 ± 711^{a}	1579 ± 42^{d}
Degree of polymerization	7.29 ± 0.61^{b}	4.92 ± 0.06^{c}	2.80 ± 0.07^{d}	25.6 ± 1.3^{a}
Procyanidins (%)	95.9	100	58.7	43.3
Prodelphinidins (%)	4.1	NA	41.3	56.7
Ellagitannins (mg/100 g)	ND	ND	3148 ± 35	ND
Ellagic acid (%)	NA	NA	78	NA
Gallic acid (%)	NA	NA	22	NA
Delphinidin 3-*O*-glucoside (mg/100 g)	ND	ND	ND	736 ± 21
Delphinidin 3-*O*-rutinoside (mg/100 g)	ND	ND	ND	1524 ± 26
Cyanidin 3-*O*-glucoside (mg/100 g)	ND	ND	ND	200 ± 6
Cyanidin 3-*O*-rutinoside (mg/100 g)	ND	ND	ND	1159 ± 15
Petunidin 3-*O*-rutinoside (mg/100 g)	ND	ND	ND	42.4 ± 1.9
Peonidin 3-*O*-rutinoside (mg/100 g)	ND	ND	ND	24.4 ± 1.0
Cyanidin-coumaroyl-glucoside (mg/100 g)	ND	ND	ND	17.3 ± 0.5
Petunidin-coumaroyl-glucoside (mg/100 g)	ND	ND	ND	6.30 ± 0.46

Note: ND = not detected, NA = Not applicable. Different letters in the same line represent different results ($p < 0.05$) between extracts.

2.13. Statistical analysis

A completely randomized experimental design was used in the experiments, in which two extractions were performed and samples were analyzed in triplicate (except for monosaccharides), totaling six results per sample. Experimental data are expressed as means followed by the standard deviation. Comparison between extraction technologies and extracts from different sources was based on one-way analysis of variances (ANOVA) and, when appropriate, the Fisher's test was used to compare the mean values. When two samples are compared, an unpaired Student-*t* test was used. Correlation coefficients between responses were calculated. Probability values below 0.05 were considered to be significant (Granato et al., 2014). The software TIBCO Statistica v. 13.3 (TIBCO Software Ltd, Palo Alto, USA) was used in the analyses.

3. Results and discussion

3.1. Physicochemical properties, phenolic composition (TPC, TA, CT), and antioxidant activity (AA): comparison between maceration and PHWE

In this study, as observed in Table 1, the maceration provided greater extraction of dry matter contents, including total phenolics (TPC), anthocyanins (TA), and condensed tannins (CT), compared to PHWE. The conventional method also provided a higher antioxidant activity for all protocols tested (CUPRAC, Fe^{2+} chelating ability, DPPH, and FCRC). Similarly, a study concerning the effects of different pre-treatment maceration techniques on the content of phenolic compounds, antioxidant capacity, and other chemical properties reported that this procedure improved the extraction of compounds from red grape skin (Wojdyło et al., 2021).

Comparing the raw materials, The NS and SB extracts presented the highest extraction yield, followed by dry matter, mean values of TPC, CUPRAC, DPPH, and FCRC. On the other hand, SP and BC extracts had the lowest mean values of antioxidant activity and TPC among the plant materials studied (Table 1). Michalska et al. (2017) reported similar TPC values for BC (701 mg/100 g). A fractionation study of SB pomace into valuable components using high pressure and enzyme-assisted extraction methods was conducted (Kitrytė et al., 2017). The authors revealed that under pressurized ethanol extraction (10.3 MPa/70 °C/15 min) recovered 158 g/kg (15.8% of extraction yield) of polar constituents from the pomace, indicating similar results to our findings. The results also agree with those reported by Kim et al. (2011) found that the aqueous extract from SB leaves could decrease oxidation products' formation (i.e., peroxides) in a TBA-based test. The extract also had a similar antioxidant activity with vitamin E and BHT. Ma et al. (2019) found that ellagitannins (peduncalagin, stacchyurin, hippophaenin B and C, casuarin, and casuarictin) were the main responsible for the antioxidant activity of SB leaves aqueous extract measured by the 2-deoxyribose assay and metal chelating ability.

3.2. Characterization of the extracts obtained by the most promising extraction process

3.2.1. Carbohydrates, phenolic composition, and antioxidant activity

The maceration process was selected to continue the study, as it was

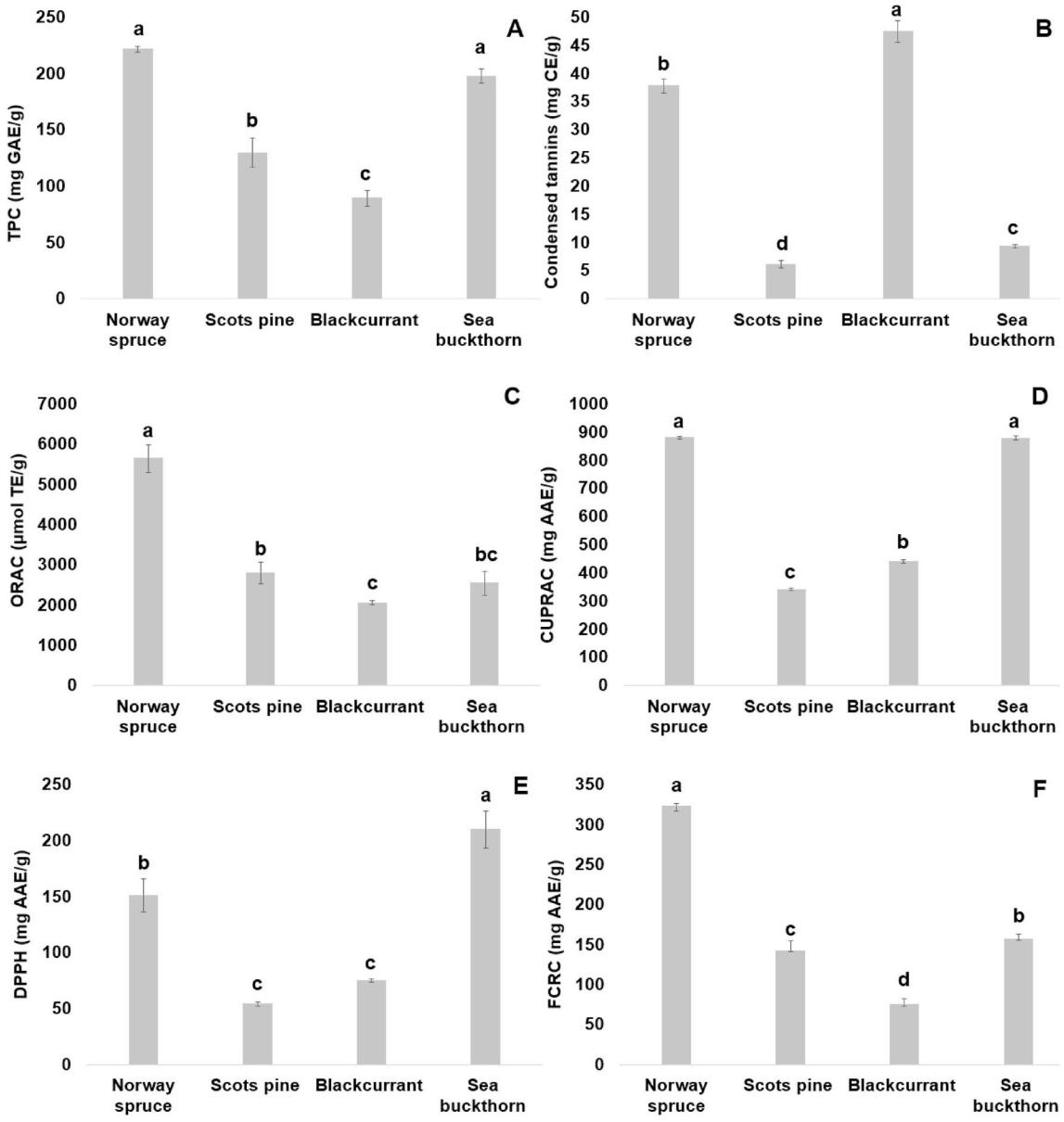

Fig. 1. Total phenolic content (TPC), condensed tannins (B), oxygen radical absorbance capacity (C), cupric ion reducing antioxidant capacity (D), free radical scavenging activity towards DPPH (E), Folin-Ciocalteu reducing capacity, FCRC (F), inhibition of lipid oxidation (ILP) using Wistar rat's brain as substrate (G), inhibition of α-amylase (H) and α-glucosidase (I), ferric reducing antioxidant power, FRAP (J), total flavonoids (K), and scavenging of hydroxyl radical (L) of freeze-dried extracts obtained with maceration from sea buckthorn leaves, Norway spruce bark, Scots pine bark, and blackcurrant press cake. Different letters represent statistically different results at $p < 0.05$. Note: AAE = ascorbic acid equivalent, CE = catechin equivalent, GAE = gallic acid equivalent, QE = quercetin equivalent, TE = Trolox equivalent.

considered the most promising approach. Regarding the carbohydrate composition shown in Table 2, 10 different compounds were identified in the extracts, in which fructose, glucose, sucrose were the most abundant ones. Fig. 1 contains the chemical composition and antioxidant activity of the freeze-dried extracts obtained by maceration. NS and SB freeze-dried extracts presented the highest mean values of TPC (Fig. 1A), CUPRAC (Fig. 1D), DPPH (Fig. 1E), FCRC (Fig. 1F), ILP (Fig. 1G), and scavenging of hydroxyl radical (Fig. 1L). As observed in Table 2, NS freeze-dried extract presented high content of proanthocyanidins, resveratrol, and some phenolic acids while SB freeze-dried extracts contained a high concentration of ellagitannins, proanthocyanidins, ellagic acid, and quercetin, explaining the high mean values for the antioxidant activity. On the contrary, SP and BC freeze-dried extracts had the lowest TPC (Fig. 1A), ORAC (Fig. 1C), DPPH (Fig. 1E), FCRC (Fig. 1F), ILP (Fig. 1G), and scavenging of hydroxyl radical (Fig. 1L). However, BC showed a comparable ($p > 0.05$) antioxidant activity measured by FRAP (Fig. 1J) with NS, while SB showed an intermediate FRAP value. The main chemical compounds found in the BC extract were anthocyanins and proanthocyanidins. Four major anthocyanins (3-rutinosides and 3-glucosides of delphinidin and cyanidin) comprised over 97% of total anthocyanins in BC agreeing with previous studies (Mattila et al., 2016; Wu et al., 2004).

Proanthocyanidins in the SP were essentially procyanidins while in other extracts CTs were mixtures of procyanidins and prodelphinidins in accordance to earlier studies on the same plant species (Gu et al., 2003; Hellström et al., 2009; Matthews et al., 1997). The average degree of

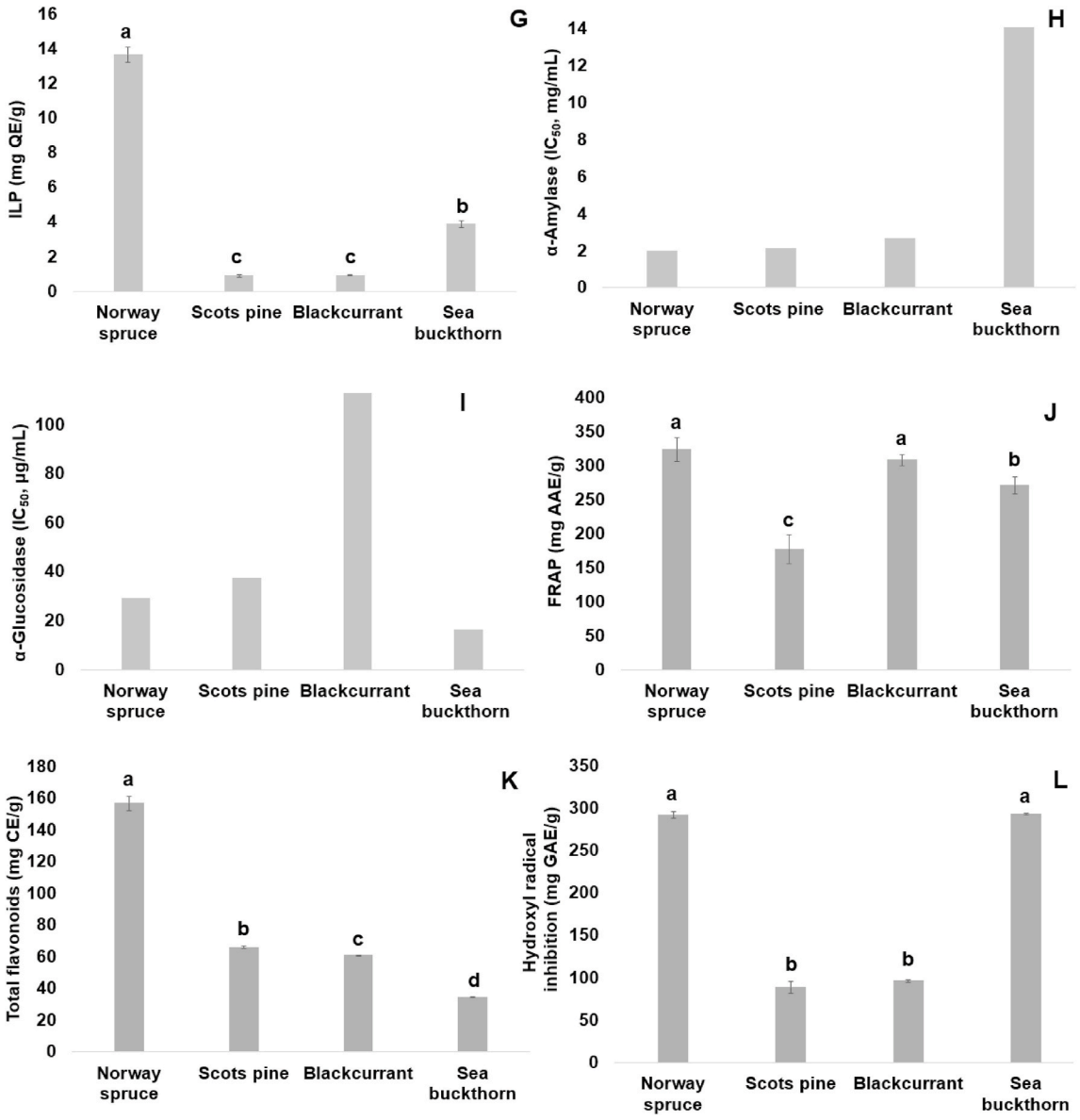

Fig. 1. (*continued*).

polymerization (DP) varied from 4.9 (SB) to 25.6 (BC). Monomeric forms were not analyzed separately, i.e. they were considered as 'terminal units' in thiolysis. Consequently, high content of free monomers, i.e. (epi)catechin and (epi)gallocatechin, would have some decreasing effect on DP. High DP of BC tannins have been reported already before (Gu et al., 2003; Hellström et al., 2009).

Correlation analysis showed an obvious result: the antioxidant capacity measured by different assays was significantly ($p < 0.05$) correlated to TOC, flavonoids, and TCT: DPPH ($r_{TPC} = 1.00$, $r_{flavonoids} = 0.935$), FCRC ($r_{TPC} = 0.847$, $r_{flavonoids} = 0.909$), and FRAP ($r_{TCT} = 0.586$). This result corroborates the findings of previous research on the bioactivity of phenolic-rich extracts (Santos et al., 2017; Margraf et al., 2015).

3.2.2. Antimicrobial activity

The results obtained for the antimicrobial activity test are shown in Table 1 – supplementary material. Among the analyzed extracts, SB showed an antimicrobial potential for some of the tested microorganisms, with the greatest activity obtained for *P. aeruginosa* IAL 1853. BC press cake, NS and SP freeze-dried extracts did not present any antimicrobial activity. These findings indicate SB freeze dried extract may be useful in inhibiting both Gram-negative and Gram-positive microorganisms. Despite this, the MIC assessment indicated that none of the studied extracts were able to inhibit the microorganisms in any of the concentrations evaluated (data not shown).

3.2.3. Inhibition of digestive enzymes

As shown in Fig. 1, all four extracts showed potent inhibition effects on two digestive enzymes at low concentrations. For the α-glucosidase inhibition, the BC freeze-dried extract had the lowest inhibition activity with IC_{50} value ($IC_{50} = 113$ μg/mL), while SB freeze-dried extract had the opposite behavior ($IC_{50} = 16.19$ μg/mL). This result corroborates the findings of Sharma et al. (2011) who observed that streptozotocin-nicotinamide induced type-2 diabetic rats had a significant decrease in blood sugar levels (up to 50%) and an increase of antioxidant activity in blood (up to 200%) after the administration of SB juice. On the contrary, our results do not agree with those obtained by Iizuka et al. (2018), who found that blackcurrant extract (obtained from the pulp) significantly reduced blood glucose concentration and improved glucose tolerance in type 2 diabetic mice, and this result is

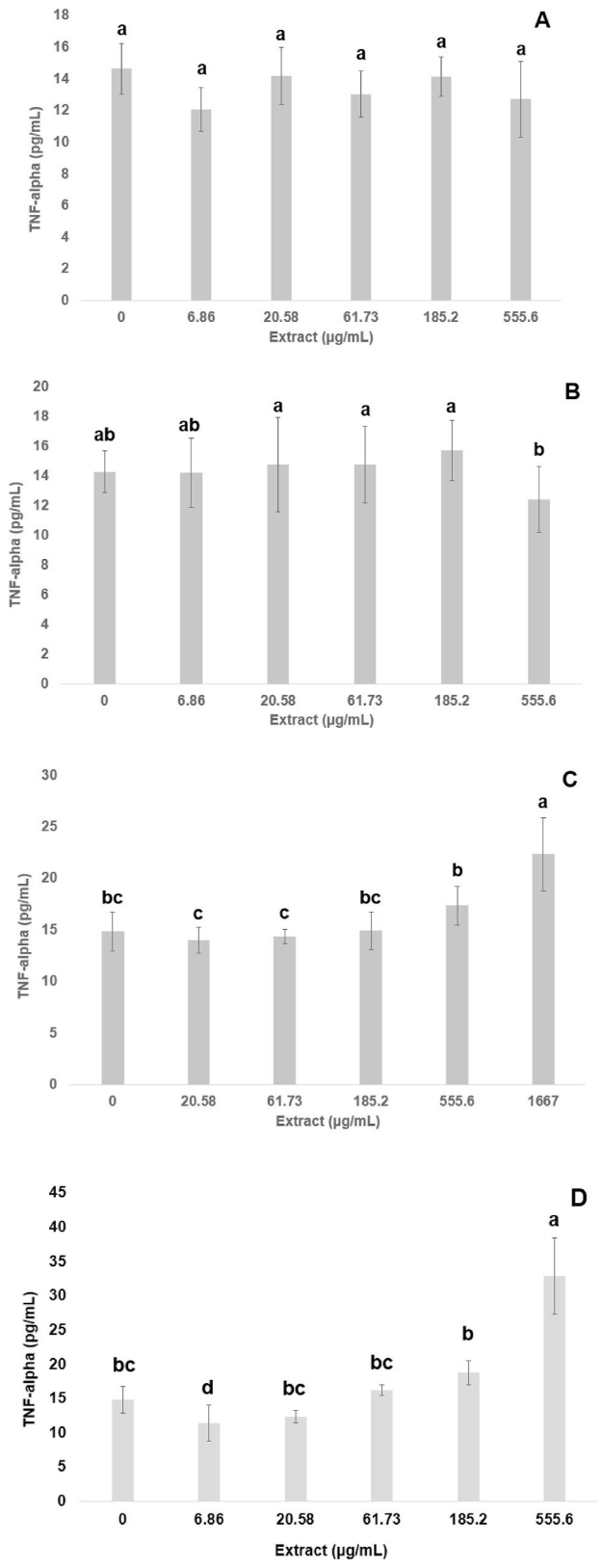

Fig. 2. The effects of freeze-dried extracts from Scots pine bark (A), Norway spruce bark (B), blackcurrant press cake (C), and sea buckthorn leaves (D) on *Escherichia coli* LPS induced TNF-alpha secretion in THP-1 macrophages. Different letters comparing the extracts represent statistically different results ($p < 0.05$).

associated with the levels of anthocyanins, especially delphinidin 3-*O*-rutinoside.

Table 1 indicated that the TPC in SB was much higher than the other extracts, which may explain its inhibition effects on α-glucosidase. On the contrary, for the α-amylase inhibition, SB presented the lowest value (IC_{50} = 14.06 mg/mL) while NS and SP extracts presented the highest and comparable inhibition (IC_{50} = 1.96 and IC_{50} = 2.10 mg/mL, respectively). Herein, the inhibition of α-amylase was correlated ($p < 0.05$) with total condensed tannins ($r = -0.487$) but not with TPC ($r = 0.375$), while the inhibition of α-glucosidase was correlated ($p < 0.05$) with TPC ($r = -0.830$). These results are in line with those obtained by Fidelis et al. (2018), who analyzed an industrial by-product from the juice industry (camu-camu seed coat), rich in ellagitannins. Kim et al. (2011) studied different aqueous and organic extracts of SB and found that quercetin, kaempferol, and isorhamnetin glucosides are responsible for the antioxidant activity and inhibition of α-glucosidase. Therefore, the results suggest that the freeze-dried extracts produced by maceration can be further tested *in vivo* for their antidiabetic potential.

3.2.4. Anti-inflammatory activity

Although many published studies have been reporting that phenolic extracts from plants can inhibit pro-inflammatory cytokines, including TNF-alpha from THP-1 macrophages, our extracts did not inhibit LPS stimulated TNF-alpha production. SP had no significant effect on the TNF-alpha activation (Fig. 2A), whereas NS had a slight inhibition effect on the TNF-alpha activation (Fig. 2B). Correlation analysis showed that higher TPC ($r = -0.670$, $p = 0.017$) and flavonoids ($r = -0.662$, $p = 0.019$) were associated with lower TNF-alpha release and higher DPPH and ORAC values ($r = -0.670$, $p = 0.017$ and $r = -0.553$, $p = 0.064$). On the other hand, BC (556 and 1667 μg/mL, Fig. 2C) and SB (62–556 μg/mL, Fig. 2D) extracts increased TNF-alpha activation, thus indicating a pro-inflammatory effect. When administered alone, chlorogenic acid increased TNF-alpha production in THP-1 cells stimulated with LPS, thus supporting the observation that depending on the matrix (i.e., crude extract or isolated compound), phenolic compounds may show ambiguous behavior toward the activation of pro-inflammatory cytokines (Bisht et al., 2020). Regarding the release of TNF-alpha in THP-1 cells challenged with LPS, although, the antioxidant effects observed using multiple chemical assays were not enough to counteract the inflammation response in LPS-challenged THP-1 cells.

Extracts showed a dose-dependent inhibition of reactive oxygen species (ROS) generation during the THP-1 cell respiratory burst (Fig. 3). The mean concentrations that inhibited 50% of ROS production (ID_{50}) were in SP, NS, BC, and SB as follows: 68.7, 57.1, 157.3, and 41.6 μg/mL, respectively. Comparatively, BC extract showed lower efficacy ($p < 0.05$) compared to the other extracts, which corroborated with the AA data. Extracts inhibited the ROS generation almost totally in the highest concentrations, 476 and 1429 μg/mL (Fig. 3). This is of importance since ROS are signaling molecules in the immune system. Interestingly, neither TPC nor flavonoids were significantly correlated ($r<0.30$, $p>0.60$, data not shown) with the decrease of ROS generation in THP-1 cell model. However, the decrease of ROS generation in THP-1 cells was strongly correlated ($p < 0.05$) with CUPRAC ($r = -0.601$) and scavenging of hydroxyl radical ($r = -0.689$), implying that multiple *in vitro* chemical antioxidant assays still can be important tools to explain cell-based anti-inflammatory/antioxidant data (Granato et al., 2018).

For the BC extract, results are in agreement with previous reports: mimicking obesity-associated chronic inflammation using RAW264.7 macrophages stimulated with LPS, Lee and Lee (2019) found that BC powder (0–100 μg/mL) was able decrease IL-1β and IL-6 mRNA, but not TNF-alpha mRNA levels. In a mouse model of obesity, authors verified that when BC powder was administered daily for 22–23 weeks, no changes were observed on the expression of pro-inflammatory genes (IL-1β, IL-6, and TNF-alpha). On the contrary, THP-1 cells treated with LPS and anthocyanin-rich BC extract (5–50 ng/mL) showed a lower activation of TNF-alpha and IL-6, showing that, hypothetically, the

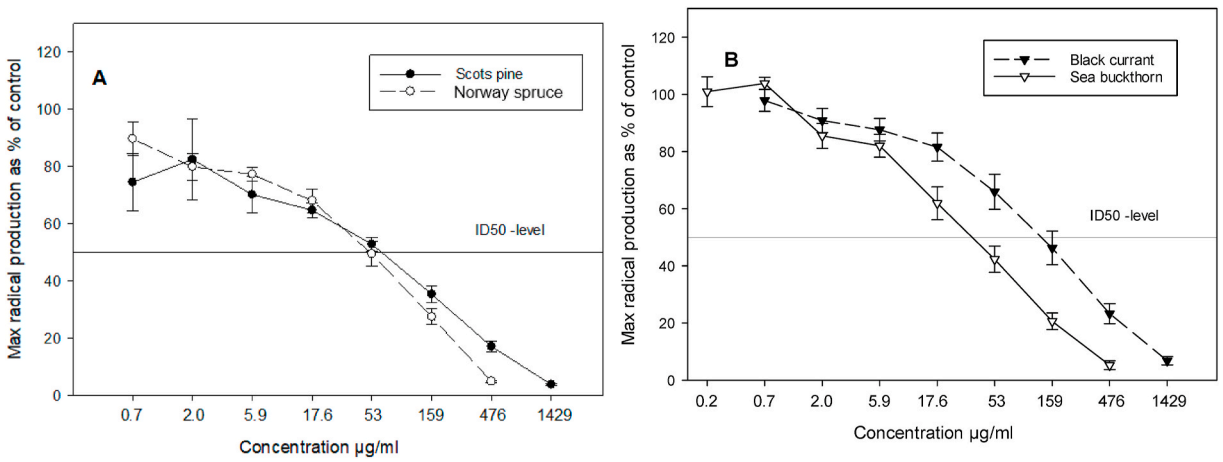

Fig. 3. The effects of extracts on the maximum radical production rate of THP-1 cells during respiratory burst. The cells were primed to an inflammatory state with *Escherichia coli* LPS, and 35 min later, the respiratory burst reaction was induced by human serum-treated yeast cell wall particles. The values are the peroxidase dependent luminol enhanced chemiluminescence reaction maxima presented as percent of that of the controls with no extract. The error bars represent the standard error of the mean (S.E., n = 9). The ID_{50} means the level of 50% inhibition.

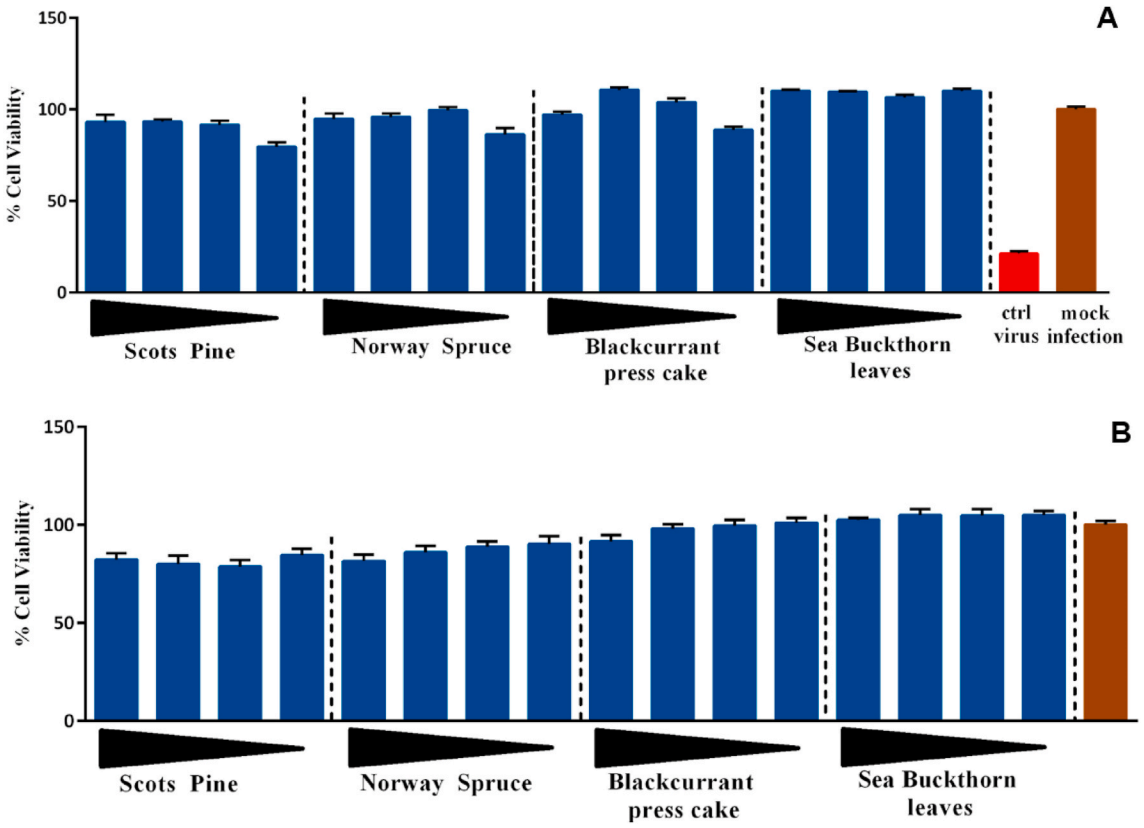

Fig. 4. Antiviral efficacy (A) and cytotoxicity (B) of the different samples at different concentrations (1, 50, 100, and 200 µg/mL) tested on human A549 cells. For the antiviral assessment, CoxsackievirusA9 (2 × 10E7 pfu) was treated with increasing concentrations from the left to the right-hand side (1, 50, 100, and 200 µg/mL).

modulation of pro-inflammatory response in cell models is mostly attributed to the anthocyanins and not by the pool of chemical compounds in crude BC extract (Lyall et al., 2009). For SB, it seems that the anti-inflammatory activity is related to the flavonoids: Jian et al. (2017) treated LPS-stimulated RAW264.7 macrophages with SB extract (12.5–100 µg/mL) and flavonoids downregulated the production and mRNA expression of pro-inflammatory cytokines (TNF-alpha, IL-6, and IL-1β) in a dose-dependent manner. A knot extract of SP rich in lignans (e.g. nortrachelogenin) was tested for its anti-inflammatory effects in murine J774 macrophages. Authors observed that SP extract downregulated the production of nitric oxide, prostaglandin E2, IL-6, and monocyte chemotactic protein-1 in J774 macrophages (Laavola et al., 2017). Similarly, Laavola et al. (2015) compared the anti-inflammatory effects of a knot extract of SP and two stilbenes found in the extract (pinosylvin and monomethylpinosylvin) in a cell-based model (J774 macrophages challenged with LPS). Results showed that the SB extract (up to 100 µg/mL) and the phenolic compounds (up to 100 µM) decreased the release of nitric oxide and IL-6. Similarly, Spilioti et al. (2014) observed that lignans (up to 100 µM) from NS knot were able to downregulate the expression of pro-inflammatory markers

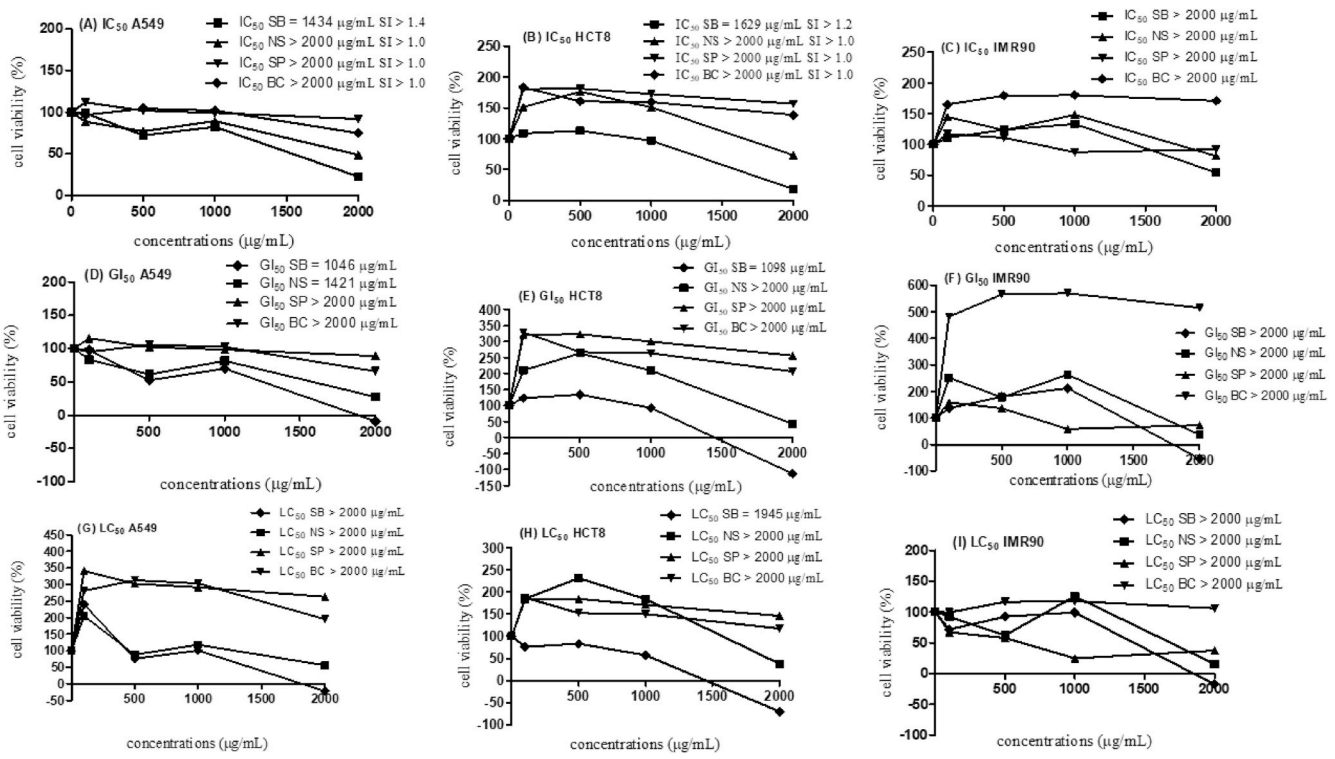

Fig. 5. Cell viability and representative evaluation of the concentration-dependent impact after 48 h exposure to the anthocyanins extracts in A549, HCT8, and IMR90 line cells. (A, B, C): IC_{50}, (D, E, F): GI_{50}, (G, H, I): LC_{50} in A549, HCT8, and IMR90 line cells. Note: SI = selectivity index based on the IC_{50} of the normal cell line (IMR90).

(TNF-alpha, JNK, VCAM-1, ICAM-1, and NF-κβ) in human aortic endothelial cells activated with TNF-alpha.

3.2.5. *Antiviral activity*

Various concentrations of the extracts from NS, SP, SB, and BC were tested for their ability to reduce infectivity in A549 cells (Fig. 4). NS and SP extracts were able to completely rescue the cells against infection at around 10 μg/mL. Those concentrations were also non-cytotoxic, which was evaluated by the CPE assay without virus in the solution. BC showed strong anti-viral efficacy with even lower concentrations and without cytotoxicity in A549 cells in a similar assay. The extracts from SB also showed antiviral potential. In the correlation analyses, both TPC ($r = -0.607$, $p = 0.036$) and flavonoids ($r = -0.538$, $p = 0.071$) seemed to be associated with the antiviral activity. Our results are in-line with those reported by Ikuta et al. (2012) who concluded that BC extract was able to decrease the adsoption and replication of respiratory syncytial virus, influenza virus A and B, adenovirus (AdV), herpes simplex virus type 1, and Haemophilus influenzae type B. Similarly, Torelli et al. (2015) found that an extract from sea buckthorn bud had anti-influenza (H1N1) virus potential *in vitro*.

Antiviral activity of the extracts was tested against the non-enveloped enterovirus coxsackievirus A9. Enteroviruses, and non-enveloped viruses in general, are known to be very stable and to resist alcohol-based solutions much better than viruses with a lipid envelope (Linnakoski et al., 2018). Enteroviruses are very prevalent viruses and cause vast amounts of acute infections around the world. They also contribute to chronic diseases such as atherosclerosis, and asthma, thus causing a substantial economic burden (Hyöty, 2016). Presently, there is a demand for anti-viral molecules that would be non-toxic, certainly less harmful than synthetic and irritable chemicals, which would efficiently and irreversibly compromise viral infectivity. For any viruses, the anti-viral action may be based on either directly acting on the virion, or attacking the cellular proteins needed for virus infection, or on the newly synthesized viral proteins in the cells (Laajala et al., 2020).

3.2.6. *Cell viability and proliferation: preliminary toxicological safety assessment*

All freeze-dried extracts did not show any apparent cytotoxicity in THP-1 cells as the cell viability was higher than 93% at concentrations ranging from 7 to 1667 μg/mL (data not shown). As freeze-dried extracts contain a high content of different chemical compounds, it is important to assess whether their interaction with human cell lines is deleterious. Our results clearly show that freeze-dried aqueous extracts from the four side-streams are not cytotoxic in this cell-based model.

Fig. 5 shows the cytotoxicity evaluation of freeze-dried extracts obtained via maceration. Results show that SB showed a slight decrease in cell viability in cancer cell lines (A549, $IC_{50} = 1434$ μg/mL; HCT8, $IC_{50} = 1629$ μg/mL), which suggests that this extract presented better anti-proliferative activity than the other extracts. Moreover, SB was the only extract that presented lethal concentration ($LC_{50} = 1945$ μg/mL) towards HCT8 cell line. NS showed a decrease in cell growth of A549 cell line, which suggests that this extract demonstrate antiproliferative activity, although it has not reduced cell viability and has not been lethal. On the other hand, the SP and BC extracts exhibited high IC_{50} and IG_{50} values for all the cell lines (>2000 μg/mL), indicating relative safety. Among the cell lines, IMR90 cells have been shown to be more resistant to all extracts, which exhibited high IC_{50} and GI_{50} values (>2000 μg/mL), meaning no cytotoxicity and no cell inhibition. Herein, the malignant cells seemed to be more susceptible to the extracts than the normal cell line indicating a possible therapeutic window as an anti-cancer agent. The SI values (IC_{50} cancer cell/IC_{50} normal cell) ranged from 1.2 to 1.4, meaning that there was no selectivity of the extracts in relation cell lines, once high selectivity is for values higher than 3.

The effects of freeze-dried extracts obtained via maceration were analyzed in relation to the ROS generation in cancer cell lines (A549, HCT8) and results are presented in Fig. 6. The levels of ROS induced by H_2O_2 were higher than the control and similar to the treatment group. in A549 cells, all the extracts exhibited a significant decrease in ROS generation to the same levels of spontaneous generation (negative

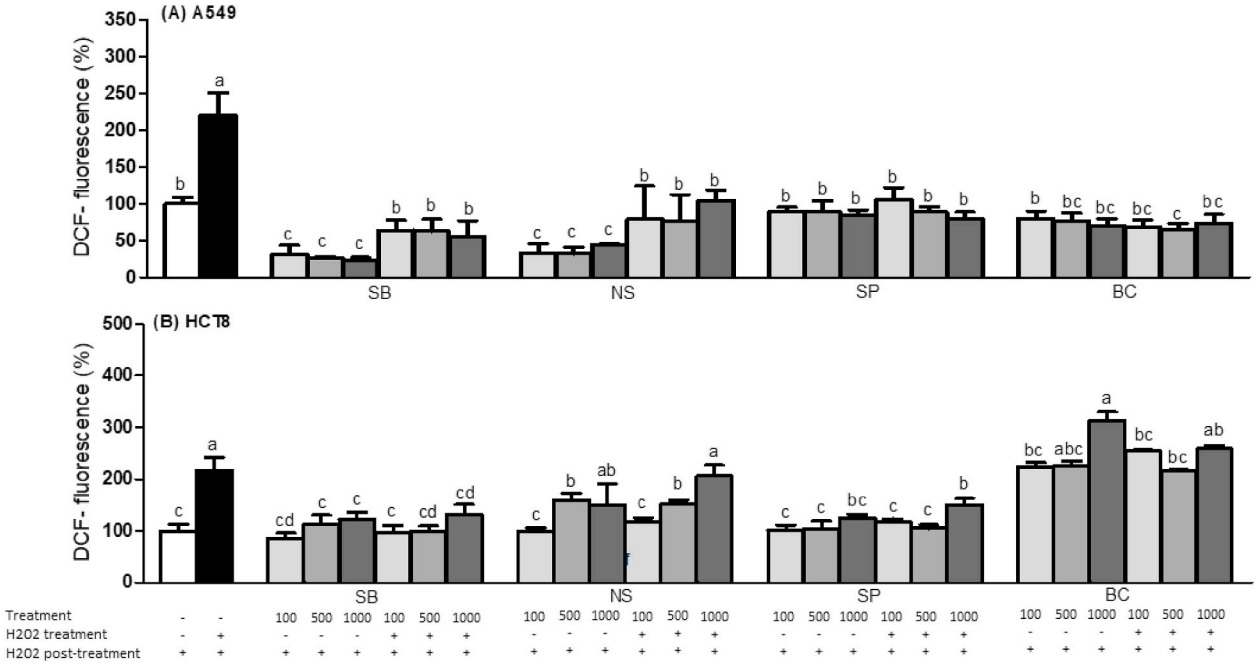

Fig. 6. Results of intracellular ROS measurement in A549, HCT8 cells by spectrofluorimetry. Treatment anthocyanins extracts at 100–1000 μg/mL. Quantitative data are the mean ± standard deviation (n = 4). Different letters represent statistically significant differences ($p < 0.05$).

control), suggesting a protective effect against H_2O_2. Similarly, in HCT8 cells, SB and SP were able to significantly reduce the ROS generation, implying higher cellular antioxidant capacity towards H_2O_2-induced oxidation. According to Kim et al. (2011) and Upadhyay et al. (2010), this antioxidant activity may be associated to isorhamnetin glucosides present in SB aqueous extract. On the contrary, higher BC and NS extract concentrations triggered intracellular ROS generation, which is somehow in-line with the data obtained using THP-1 macrophages. Similar results were found by Olejnik et al. (2018), who showed that the gastrointestinal digested BC extract at 100 μg/mL induced a substantial increase in ROS generation in colon cancer cells.

4. Conclusions

Four different side streams from the food and forestry industries were studied for their cytotoxicity in different human cell lines and their bioactivities. The recovery of bioactive compounds using maceration proved to be the most promising technology to produce extracts with biological activities. Freeze-dried extracts represent a rich source of carbohydrates and polyphenols, expressed toxicity to a cancer cell (HCT8), inhibition effects on digestive enzymes, and reactive oxygen species (ROS) generation during the THP-1 cell respiratory burst. Our results clearly show the aqueous extracts from BC press cake, SB leaves, and bark from NS and SP are suitable candidates for further *in vivo* tests to assess their toxicological profile and bioactivities.

CRediT authorship contribution statement

Nora Pap: Conceptualization, Investigation, Writing – original draft, Funding acquisition, Formal analysis. **Dhanik Reshamwala:** Formal analysis, Writing – original draft. **Risto Korpinen:** Formal analysis, Writing – original draft. **Petri Kilpeläinen:** Formal analysis, Writing – original draft. **Marina Fidelis:** Formal analysis. **Marianna M. Furtado:** Formal analysis, Writing – original draft. **Anderson S. Sant'Ana:** Formal analysis, Writing – original draft. **Mingchun Wen:** Formal analysis, Writing – original draft. **Liang Zhang:** Formal analysis, Writing – original draft. **Jarkko Hellström:** Formal analysis, Writing – original draft. **Pertti Marnilla:** Formal analysis, Writing – original draft. **Pirjo Mattila:** Formal analysis, Writing – original draft. **Tytti Sarjala:** Formal analysis, Writing – original draft. **Baoru Yang:** Writing – review & editing. **Luciana Azevedo:** Formal analysis, Writing – original draft. **Varpu Marjomäki:** Formal analysis, Writing – original draft. **Daniel Granato:** Conceptualization, Methodology, Software, Validation, Investigation, Formal analysis, Resources, Data curation, Writing – original draft, Supervision, Project administration, Funding acquisition.

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.fct.2021.112284.

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