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Enzyme-assisted extraction of anthocyanins and other phenolic compounds from blackcurrant (*Ribes nigrum* L.) press cake: From processing to bioactivities

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ABSTRACT

The effects of commercial enzymes (pectinases, cellulases, beta-1-3-glucanases, and pectin lyases) on the recovery of anthocyanins and polyphenols from blackcurrant press cake were studied considering two solid:solvent ratios (1:10 and 1:4 w/v). β-glucanase enabled the recovery of the highest total phenolic content – 1142 mg/100 g, and the extraction of anthocyanins was similar using all enzymes (~400 mg/100 g). The use of cellulases and pectinases enhanced the extraction of antioxidants (DPPH – 1080 mg/100 g; CUPRAC – 3697 mg/100 g). The freeze-dried extracts presented antioxidant potential (CUPRAC, DPPH), which was associated with their biological effects in different systems: antiviral activity against both non-enveloped viruses (enterovirus coxsackievirus A-9) and enveloped coronaviruses (HCoV-OC43), and cytotoxicity towards cancer cells (A549 and HCT8). No cytotoxic effects on normal human lung fibroblast (IMR90) were observed, and no anti-inflammatory activity was detected in lipopolysaccharides-treated murine immortalised microglial cells.

1. Introduction

Blackcurrants are famous worldwide with a production of 153 028 metric tons (t), with Poland (105 000 t), the United Kingdom (12 600 t) and Germany (4520 t) the most prominent European producers (Cortez & Gonzalez de Mejia, 2019; International Blackcurrant Association, 2021). The berries are generally rich in different phenolics, and secondary plant metabolites. They can be divided into various groups, such as the anthocyanins, which give a purple-blue colour and are associated with beneficial health effects. The processing of berries should be carried out using mild processing concepts in which the utilisation of different enzymes first intensifies the liberation of these valuable compounds. Second, the level of these anthocyanin compounds is maintained exceptionally during each process stage to ensure the most benefit for consumers.

Blackcurrant juice is usually produced using cold pressing, preceded by enzymatic treatment with pectinases to break down the cell wall composition, release valuable compounds, and increase juice yield. Other valuable fractions also can be obtained: the remaining press cake can be valorised in a biorefinery concept. In the biorefinery, the press cake undergoes novel extraction, separation, concentration, and drying techniques to recover phenolic or volatile organic compounds and use them as value-added ingredients for functional food production. The amount of press cake in the juice production process is significant:

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Sandell et al. (2009) reported 24 ± 4% of berry mass with 38% dry matter content, and it is still rich in anthocyanins, flavonols, and hydroxycinnamic acids. The distribution of the anthocyanin and other phenolics was determined in the dried skin (Azman, House, Charalampopoulos, & Chatzifragkou, 2021), dried press cake (Grimm, Nyström, Mossing, Östman, & Geladi, 2020), from the clarified and concentrated juice (Pap et al., 2010, 2012), and the extracts of blackcurrant fruits and powders (Hui et al., 2020; Zhao et al., 2021). Cortez and Gonsales de Mejia (2019) reviewed the literature data on the phenolic compounds of blackcurrants and derivatives, finding that the rutinosides and glucosides of cyanidin and delphinidin, and glucosides of myricetin, quercetin, and kaempferol, were the most common compounds.

Barreca et al. (2021) recently published a review article on flavonoids occurring in various foods, including their bioavailability and health effects. There is evidence from pre-clinical and *in vitro* studies that flavonoids display antioxidant, cardioprotective, antiviral, antibacterial, and anticancer properties. Therefore, they should be included as part of a healthy diet, and they make excellent components for various functional food products. Furthermore, Yousefi, Shadnoush, Khorshidian, and Mortazavian (2021) described the potential of flavonoids for mitigating hypertension. Ohguro, Ohguro, and Yagi (2013) used blackcurrant anthocyanins in a double-blind placebo-controlled trial, stating that consuming 50 mg of blackcurrant anthocyanin for four weeks reduced the intraocular pressure. Still, it did not reduce the systolic blood pressure.

The value of blackcurrant flavonoids has been appreciated mainly by the food and pharmaceutical industries and even by the packaging industry due to their antimicrobial capacity (de Araújo, de Paulo Farias, Neri-Numa, Pastore, & G.m., 2021; Yousefi et al., 2021). The volume of the press cake is relevant because even with the best available technique, the amount that remains accounts for about 20–25%, and it contains valuable potential ingredients such as phenolics and dietary fibre (Alba, Campbell, & Kontogiorgos, 2019), proteins and fats (Reißner et al., 2019).

Enzymatic treatments under a green technology approach are favourable since the processing temperature is relatively mild, usually around 40–50 °C, while the inactivation is 65–80 °C depending on the nature of the enzyme, but the keeping time is short. Opposite to this, in extraction techniques like microwave-assisted extraction, the temperature rises quickly up to around 60–70 °C. It remains during the whole processing time, which may induce the degradation of the valuable compounds that are heat sensitive. The enzyme-assisted extraction of blackcurrant is not a novel approach: Landbo and Meyer (2001) tested different enzymes (Grindamyl pectinase, Macer8 FJ, Macer8 R, and Pectinex BE, and Novozym 89 protease) to extract bioactive compounds from the blackcurrant press cake devoid of seeds. The authors obtained a recovery of 1.6–5-fold more antioxidants than the untreated extraction, and the polyphenol-rich extracts inhibited the oxidation of human low-density lipoproteins (LDL) *in vitro*. The use of new extraction technologies, such as ultrasound (UAE), to recover bioactive compounds from blackcurrant is not a novel procedure either: González, Carrera, Barbero, and Palma (2022) used UAE combined with either ethanol or methanol to recover the bioactive compounds. The authors obtained 22 mg of anthocyanins per gram of freeze-dried blackcurrant fruit pulp. Although these contributions show that enzymes and extraction techniques are feasible to obtain phenolic-rich extracts from blackcurrant press cake or pulp, no scale-up study has been conducted to demonstrate innovative extraction methods to recover phenolic compounds with bioactivity in different biological media.

Most of the results in the literature use isolated compounds for measuring bioactivities and potential health effects. Separation and purification of valuable compounds like phenolics may not be feasible in industrial food applications due to the high costs of production. This research's aim was, therefore, to carry out the processing of the blackcurrant press cake into blackcurrant extract and dried extract using enzymatic hydrolyses, and i) to study the effect of different enzyme preparations on the content of different phenolics in the extract, and ii) to measure the antioxidant, antiviral, and antioxidative activities, as well as study the extracts' antiproliferative and anti-inflammatory potential.

2. Material and methods

2.1. Blackcurrant press cake

The blackcurrants were harvested in 2019 in various Finnish regions. The press cake was received as a mixture of varieties from Kiantama Ltd in Suomussalmi, Finland. A 150-kg batch of the raw material was transported in frozen form and was stored at −16 °C until further use.

2.2. Chemical reagents and enzymes

Commercial reference standards were applied for the determination of phenolic compounds. Cyanidin 3-*O*-rutinoside (≥95%), cyanidin 3-*O*-glucoside chloride (≥95%), delphinidin 3-*O*-rutinoside (≥95%), delphinidin 3-*O*-glucoside (≥95%), petunidin 3-*O*-glucoside (≥95%), and peonidin 3-*O*-rutinoside (≥95%) were purchased from Extrasynthese (Lyon, France) for anthocyanin analyses. HPLC grade methanol (≥99.8%) and acetonitrile (≥99.8%) were supplied by VWR Chemicals BDH® (Bruchsal, Germany). Glacial acetic acid was provided by J. T. Baker (Mallinckrodt Baker Inc., Utrecht, the Netherlands). Hydrochloric acid, ferric chloride hexahydrate, potassium ferricyanide, gallic acid, neocuproin, methanol, DPPH● (2,2-diphenyl-1-picrylhydrazyl), ammonium acetate, ascorbic acid, and copper (II) chloride dihydrate were purchased from VWR Chemicals BDH. Dichloro-dihydro-fluorescein diacetate (DCFH-DA), 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), lipopolysaccharides (LPS), adenosine triphosphate (ATP), and Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM) were purchased from Sigma-Aldrich (São Paulo, Brazil). Aqueous solutions were prepared using ultrapure water. Enzymes were kindly provided by AB Enzymes (Darmstadt, Germany), and fulfil the purity specifications of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and Food Chemicals Codex (FCC) for food-grade enzymes.

2.3. Enzyme-assisted extraction of blackcurrant press cake

Five different commercial enzyme preparations were used in the study to enhance the liberation of anthocyanins and other phenolics from the blackcurrant press cake into the extracts. These enzymes differed in their primary and side activities. They were either hydrolysing non-starch polysaccharides or the residual pectin in the press cake with a different mechanism of action. The detailed description of the enzymes and the treatments are illustrated in **Table 1 – Supplementary material and Supplementary material 2.**

The experimental runs were coded as A, B, C, D, E, F, G, and H in the figures, and in several runs, the different enzymes were combined (A + D, C + D, A + E, C + E, and G + F). The detailed description is shown in Table 1 – Supplementary material. The screening of the enzymes' efficiency in liberating anthocyanins and phenolic compounds was undertaken on a smaller scale, while the optimisation of the enzymatic hydrolyses was excluded from this study. The trials showing the highest anthocyanin content were used to produce extracts on a larger scale (2 L/batch) to validate the scalability and ensure a sufficient quantity of extracts for all bioactivity and functionality measurements.

Two different setups were used, in which the solid to liquid (S:L) ratio was changed from 1:10 to 1:4 w/v. Previous studies by Pap et al. (2012) showed that microwave-assisted extraction could be successfully carried out using the 1:20 ratio compared to the 1:40 required in the Armfield extractor setup. The present S:L ratio was selected further to decrease the amount of solvent over the solid material but to be able to

stir with 140 r/min throughout the hydrolyses processes. Four general setups of experiments were carried out after the frozen press cake was thawed overnight on a small scale. Firstly, a suspension with water was prepared using initially 50 g of press cake in a ratio of 1:10 w/v. After that, the suspension was heated, and when the suspension reached the temperature determined according to the enzyme type, the enzyme was added to a concentration of 200 mg/L in the first set of the test.

In the second set of experiments, the quantity of the press cake was increased to 100 g; the S:L ratio was decreased to 1:4. The enzymes were added in 100 mg/L, whose concentrations were selected according to the manufacturer's instructions. Additionally, in the third setup, the effect of pH adjustment to a pH of 5.5 was studied compared to the second setup, because enzyme activity was expected to be higher in these circumstances (personal discussion).

The temperature in all setups was chosen according to the enzyme type, the cellulases were processed at 50 °C, and the pectinases were processed at 40 °C. The samples were continuously agitated during the processing using a blade stirrer at a 140 r/min speed. The enzymes were inactivated after the length of the hydrolyses was reached – 1 h for the cellulases and 4 h of pectinases at 80 and 60 °C, respectively. The extracts and their control pairs showing the highest total anthocyanin content were freeze-dried and used to test the bioactivities (samples I, J, K, and L in the list).

2.4. Chemical composition and antioxidant activity

2.4.1. Anthocyanins composition and recovery yield

Total anthocyanins (TA) and individual anthocyanin composition were determined from the blackcurrant press cake, all hydrolysates of the small-scale trials, and the freeze-dried samples of the scale-up experiments. To this end, solid samples were extracted three times with extraction solvent (4% acetic acid in 65% aqueous methanol), while liquid samples required only filtration. Press cake (0.5–1 g) was homogenised with 10 mL of extraction solvent and the mixture was sonicated for 20 min and centrifuged, and the supernatant was poured into a 50 mL volume flask. Extraction was repeated twice, and the final volume of the extract was adjusted to 50 mL with the extraction solvent. The extracts were filtered before HPLC analyses.

An 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 applied. The separation was accomplished with a gradient elution of acetonitrile into 5% formic acid (aq) according to the method described by Hellström, Mattila, and Karjalainen (2013). Authentic reference standards were used for the quantification. The rutinosides of peonidin and petunidin and coumaroyl derivatives of the cyanidin and petunidin glucosides were quantified as corresponding glucosides.

The anthocyanin composition (mg/100 g fw) in the final liquid and freeze-dried samples was determined in triplicate using the UHPLC-DAD-MS/MS Shimadzu Nexera X2 system (Shimadzu, Japan). Individual anthocyanins were identified by comparing retention times, UV spectra, and parent and daughter ion masses with those of standard compounds and quantitated at 520 nm, based on the respective calibration curves of the standard compounds. Each sample (0.2 g) was dissolved in 10 mL 1% HCl 50% ethanol, followed by 15 min sonication in an ultrasonic bath at room temperature and shaken for 30 min. The ethanol extracts were filtered by hydrophilic Sartorius Minisart® Syringe Filters (0.2 μm) before injection to the HPLC system. The chromatographic separation of anthocyanins was implemented on an ACE Excel 3 C_{18}-PFP (100 mm × 2.1 mm × 3 μm) column maintained at 40 °C. The total flow of the mobile phase was 0.25 mL/min, and the injection sample size was 0.5 μL. The mobile phase gradient was a mixture of two solvents: 1% formic acid in Milli-Q water (A) and 1% formic acid in methanol (B). The following multistep gradient programme was used to separate the compounds: linear 15%–80% of mobile phase B from 0.01 to 27 min, linear 80%–95% B from 27 to 29 min, isocratic 95% from 29 to 33 min. The column was returned to its initial conditions with linear 95%–15% B from 38 to 44 min. The anthocyanin yield was calculated using Eq. (1):

$$Anthocyanin\ yield(\%) = 100\% \times \left(C_{total\ anthocyanin\ in\ the\ extract} \times m_{extract}/m_{sample}\right)/C_{total\ anthocyanin\ in\ the\ press-cake} \quad (1)$$

2.4.2. Total phenolic content (TPC) and antioxidant activity

TPC was assessed using the Prussian Blue assay (Margraf, Karnopp, Rosso, & Granato, 2015), and the results were expressed as mg of gallic acid equivalent 100 g of blackcurrant press cake (mg GAE/100 g on a dry weight basis - dw). The DPPH free-radical scavenging activity was measured following the method described by Brand-Williams, Cuvelier, and Berset (1995), and the results were expressed as mg of ascorbic acid equivalent per 100 g (mg AAE/100 g, dw). Cupric ion reducing antioxidant capacity (CUPRAC) was estimated utilising the copper (II)-neocuproine (Cu(II)–Nc) reagent as the chromogenic oxidant (Apak, Güclü, Özyürek, & Celik, 2008). The results were expressed as mg mg AAE/100 g, dw.

2.5. Biological assessments

2.5.1. In vitro cell-based viability evaluation and reactive oxygen species (ROS) generation

The lung adenocarcinoma epithelial cells (A549), human ileocecal adenocarcinoma cells (HCT8), and normal human lung fibroblast (IMR90) were obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil). The cells were cultured in DMEM supplemented with heat-inactivated fetal bovine serum (Gibco, EUA) to final concentrations of 10% (HCT8, A549) and 20% (IMR90). These cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Cell viability and metabolic activity were assessed using the MTT colourimetric assay. The cytotoxicity of the blackcurrant press cake extracts, the same as were used in the antiviral tests, was evaluated for A549, HCT8 (cancer cells), and IMR90 (normal cells). 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 treatment (100–2,000 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, dissolved in DMSO (Ionta, 2015). 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 calculated. Moreover, the selectivity index (SI) was estimated using the IC_{50} ratio (normal cell)/IC_{50} (cancer cell lines) (Boechat, 2014).

Intracellular ROS generation was assessed using the DCFH-DA assay. Malignant (A549) cells (6×10^4 per well) were treated for 1 h with different concentrations of blackcurrant press cake extracts (100–1,000 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 all wells, and the fluorescence intensity ($\lambda_{ex/em}$ = 485/538 nm) was measured (Escher, 2018).

2.5.2. Antiviral activity

To complete the antiviral tests, the A549 cells and MRC-5 cells were purchased from ATCC. DMEM was used for the A549 cells seeding, while Minimum Essential Medium (MEM) supplemented with 10% FBS (Gibco), 1% L-GlutaMAX (Gibco) and 1% penicillin/streptomycin (Gibco) was used in the case of the MRC-5 cells. A549 cells (1.2×10^4 cells/well) and MRC-5 cells (1.5×10^4 cells/well) were seeded on the previous day into a 96-well plate. The freeze-dried blackcurrant extracts were diluted in ddH_2O. The following day, Coxsackievirus A9 (CVA-9; 2×10^6 pfu/mL, MOI = 1) and human coronavirus HCoV-OC43 (OC43; 1.8×10^4 pfu/mL, MOI = 0.1) purchased from ATCC were treated with blackcurrant samples having concentrations of 0.1 and 1 μg/mL (dry extract). CVA-9 was purified as described before (Myllynen, Kazmertsuk, & Marjomäki, 2016; Ruokolainen et al., 2019) with the only

exception of adding 0.1% (v/v) TWEEN® 80 (Sigma-Aldrich) during the freeze–thaw cycle. Virus-sample mix was prepared in 10% DMEM with CVA-9 and in 2% MEM with OC-43. CVA-9-sample mix was incubated for 1 h or 5 min at room temperature or 37 °C, while the OC-43-sample mix was incubated for 5 min at room temperature or 34 °C. After pre-incubation, the CVA-9-sample mix was added to A549 cells and incubated for 48 h at 37 °C until the cytopathic effect was observed. Pre-incubated OC-43-sample mix was added onto MRC-5 cells and incubated for 2 h at 34 °C. The mixture was then removed and replaced with 2% MEM, and the cells were incubated for 5 d at 34 °C until the cytopathic effect was observed using a light microscope. The cells were then washed twice with PBS and stained with CPE stain (0.03% crystal violet, 2% ethanol and 36.5% formaldehyde) for 10 min at room temperature. The excess stain was washed away with water. The stained cells were finally treated with lysis buffer (0.8979 g of sodium citrate and 1 N HCl in 47.5% ethanol) to homogenise the sample. Finally, the absorbance of the stain was measured at 570 nm to determine the presence of cells still alive in the well, which indirectly shows the level of infection (Victor X4 2030 multilabel reader, PerkinElmer).

2.5.3. Anti-inflammatory effect in IMG cells

IMG cells (mouse microglial cell line) were purchased from Kerafast Inc. (Boston, USA) and cultivated in high glucose DMEM (Sigma-Aldrich), supplemented with 10% FBS (Sigma-Aldrich, #F0804), 1x Penicillin-Streptomycin, and 2 mM GlutaMax (both from Gibco, Thermo Fisher Scientific).

The effect of blackcurrant extracts on the cellular viability of the IMG cells was studied using the MTT assay. For this purpose, the IMG cells were seeded on a 96-well plate at $3x10^4$/well in triplicates in DMEM supplemented with 10% FBS, 1x Penicillin/Streptomycin and 2 mM GlutaMax. On the next day, the media was replaced with fresh FBS-free media. The freeze-dried blackcurrant press-cake extracts (J, L) and the control sample after 1 h processing (I) were dissolved in ultrapure water (stock solution 5 mg/mL) and added to the cell culture medium in a final concentration of 100 μg/mL and incubated for 5.5 h. Control cells were kept in FBS-free media. After incubation, the culture medium was replaced with fresh 100 μL of FBS-free medium. An aliquot (10 μL) of MTT working solution (5 mg/mL) was added to each well and the plate was incubated for 1 h at 37 °C in a CO_2 incubator. The medium was aspirated and formazan crystals were solubilized by adding 200 μL of DMSO to each well. OD was measured at 540 nm.

To study the possible anti-inflammatory effects of blackcurrant extracts, IMG cells were seeded on 12-well plates at $3.2x10^5$/well in triplicates in DMEM supplemented with 10% FBS, 1x Penicillin/Streptomycin and 2 mM GlutaMax. On the next day, the media was replaced with a fresh FBS-free medium and supplemented with I, J, and L extracts (100 μg/mL). The cells were pre-incubated with test substances for 1 h prior to the addition of LPS. To prime the NLRP3 inflammasome, LPS was added at a concentration of 1 μg/mL for 4 h. To activate the NLRP3 inflammasome ATP was added to cell culture at a concentration of 3 mM for 30 min. After ATP incubation, the cells were either lysed in 50 μL of lysis buffer (50mM Tris-HCL ph7.5, 150mM NaCl, 1% Triton X-100, 1x protease inhibitor (Roche)) for SDS-PAGE or lysed in 350 μL of RLT buffer for RNA extraction with RNeasy Mini Kit. For Western blot analyses, 25 μg of protein was loaded on 13% sodium dodecyl sulfate-polyacrylamide gel, separated by electrophoresis and then transferred to polyvinylidene difluoride membrane (Thermo Scientific, Rockford, IL, USA).

Membranes were blocked in Intercept Blocking buffer (LI-COR) and then incubated with primary antibodies (**Table 2 – Supplementary material**) overnight at 4 °C and with secondary antibodies for 1 h at room temperature. Membranes were scanned with the LI-COR Odyssey CLx Infrared Imaging System (LI-COR), and the protein bands were analysed with ImageJ. For the quantitative real-time PCR, cDNA was prepared from 1 μg of RNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), and qPCR was performed using the Maxima Sybr Green/Rox qPCR Master Mix (Thermo Fisher Scientific) and the QuantStudio 12 K Flex™ Real-Time PCR System (Thermo Fisher Scientific). The primers are listed in **Table 3 – Supplementary material**.

2.6. Data analysis

Analyses were performed in triplicate, and data were expressed as means followed by the standard deviation. Data were tested for homoscedasticity (Brown-Forsythe test), and the differences ($p < 0.05$) among treatments were performed using a one-way analysis of variances (ANOVA), followed by Tukey's test. Where applicable, a paired Student-*t* test was used to compare the initial and final hydrolysis processing time. The correlation analysis was calculated based on Pearson's correlation coefficient to assess the association between responses (Granato, Calado, & Jarvis, 2014). The statistical analyses were performed using the TIBCO Statistica v.13.3 software (TIBCO Software Ltd., Palo Alto, USA).

A statistical analysis of the antiviral experiments was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). The result data are presented as mean ± standard error of the mean (SEM). The statistical significance was calculated by performing one-way/two-way ANOVA, followed by the Bonferroni test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

3. Results and discussion

3.1. Effect of enzymatic treatment on total anthocyanins, total phenolic content, anthocyanin yield, and antioxidant activities

As the main objective of the study was to recover the anthocyanins from blackcurrant press cake, we have initially quantified the total anthocyanin content in the raw material, which was found to be 450 ± 18 mg/100 g of fresh weight (fw), equating to 744 ± 29 mg/100 g dw, indicating that a considerable amount of anthocyanins remains in solids during juice pressing. Tables 1A and 1B contain the results of the TA, TPC, DPPH, and CUPRAC, assessed after 1 and 4 h of enzymatic processing time. As expected, the enzymatic treatment improved the extraction of phenolic compounds and, consequently the antioxidant activity of blackcurrant press cake extracts. Moreover, the treatment time also contributed to the extraction of bioactive compounds and antioxidant activity showing a significant difference between one vs 4 h of hydrolysis. The differences in the efficiency of the selected enzymes can be attributed to their nature; A and C (G) belong to cellulosic enzymes, while B (F), D, E to the pectic enzymes. Even within the groups, there is a difference in the mechanism of action. Enzyme A breaks cellulose, pectin, glucans, and gums, while C (G) degrades cellulose, xylans, and β-glucans. The selected pectic enzymes either degrade pectin without going through the de-esterification phase, while the other enzymes act as pectinesterases and polygalacturonases to catalyse the hydrolysis of methylated carboxylic ester in pectin to form pectic acid and methanol and hydrolyse the 1,4-α-D-galactosyluronic linkages between the galacturonic residues (Wong, 1995).

The mean TA content in the extracts ranged from 304 to 430 mg/100 g dw in the press cake at 1 h, and from 346 to 424 mg/100 g dw in the press cake after 4 h, of enzymatic treatment. The prolonged enzymatic treatment in an S:L ratio of 1:10 increased the anthocyanins' recovery towards the end of the hydrolyses. The increase was statistically significant in some cases ($p < 0.05$). Considering that both enzymes increased in TA and TPC, pectin lyase and cellulase were selected for the second set of experiments. The results diverged in this case when the ratio was reduced to S:L of 1:4 w/v. Cellulase enzyme treatment seems to peak in anthocyanin concentration after 1 h of hydrolysis, whereas the total phenolic content increased until the end of the hydrolysis. This indicated that other phenolics than anthocyanins were also extracted during the treatment efficiently over time. On the other hand, the pectin

Table 1A
Comparison between the enzymatic treatments: total anthocyanins and total phenolic content at the initial (1 h) and final time (4 h) of hydrolyses (per juice press cake, dry matter).

Treatment code	Total anthocyanins (mg cya-3-glu Eq./100 g) Hydrolysis time		TPC (mg GAE/ 100 g)	
	Initial	Final	Initial	Final
A	319 ± 24^{deB}	396 ± 10^{abA}	848 ± 97^{abcB}	1142 ± 51^{bA}
B	345 ± 15^{bcdeB}	424 ± 8^{aA}	913 ± 38^{aB}	1050 ± 18^{cA}
C	319 ± 7^{deB}	405 ± 9^{aA}	791 ± 22^{cdeB}	938 ± 14^{efA}
D	324 ± 23^{deB}	386 ± 22^{abA}	836 ± 2^{abcB}	913 ± 11^{fgA}
E	336 ± 8^{cdeB}	401 ± 19^{aA}	787 ± 11^{cdeB}	898 ± 2^{fgA}
A + D	338 ± 28^{cdeB}	401 ± 20^{aA}	696 ± 21^{fghB}	840 ± 4^{hiA}
C + D	326 ± 21^{deB}	401 ± 27^{aA}	726 ± 4^{efghB}	879 ± 5^{ghA}
A + E	334 ± 23^{cdeB}	407 ± 15^{aA}	817 ± 11^{bcdB}	998 ± 4^{cdA}
C + E	312 ± 11^{eB}	383 ± 11^{abA}	891 ± 5^{abB}	911 ± 51^{fgA}
F	391 ± 19^{abc}	394 ± 16^{ab}	739 ± 16^{defg}	736 ± 3^{k}
G	430 ± 21^{a}	382 ± 23^{ab}	665 ± 3^{ghiB}	816 ± 13^{ijA}
G + F	376 ± 9^{abcd}	392 ± 21^{ab}	376 ± 2^{jB}	495 ± 2^{mA}
Control 1	320 ± 14^{deB}	410 ± 10^{aA}	776 ± 26^{cdeB}	1349 ± 8^{aA}
Control 2	304 ± 15^{eB}	391 ± 12^{abA}	640 ± 18^{hiB}	979 ± 4^{deA}
Control 3	388 ± 23^{abcB}	346 ± 8^{bA}	766 ± 3^{cdeB}	802 ± 4^{gA}
Control 4	401 ± 15^{ab}	396 ± 16^{ab}	731 ± 4^{defgB}	780 ± 4^{gA}
Control 5	362 ± 39^{bcde}	408 ± 19^{a}	586 ± 3^{iB}	643 ± 4^{hA}
p-Value	<0.001	0.003	<0.001	<0.001

Note: Values are expressed as means followed by the standard deviation (n = 3) and are related to the raw materials dry matter basis). Different lowercase letters in the same column for the TPC represent statistically different results ($p < 0.05$) among enzymatic treatments. Different uppercase letters in different columns for the same response represent statistically different ($p < 0.05$) results comparing initial (1 h) and final (4 h) hydrolysis time. TPC = total phenolic content, GAE = gallic acid equivalent.

Table 1B
Comparison among treatments on the chemical antioxidant activity of blackcurrant press-cake extracts at the initial and final time of hydrolyses (per juice press cake, dry matter).

Treatment code	DPPH (mg AAE/100 g) Hydrolysis time		CUPRAC (mg AAE/ 100 g)	
	Initial	Final	Initial	Final
A	494 ± 10^{efB}	527 ± 2^{fghiA}	1835 ± 158^{de}	1640 ± 60^{j}
B	484 ± 6^{efB}	554 ± 2^{defgA}	1580 ± 47^{efgB}	2723 ± 98^{efgA}
C	470 ± 2^{fB}	552 ± 2^{defghA}	2200 ± 69^{abcB}	3531 ± 284^{abA}
D	495 ± 3^{efB}	557 ± 4^{defA}	2411 ± 26^{aB}	3697 ± 205^{aA}
E	808 ± 83^{bB}	1080 ± 33^{aA}	1971 ± 34^{cdB}	3155 ± 150^{bcdA}
A + D	430 ± 9^{fB}	496 ± 2^{ijA}	1794 ± 47^{defB}	2892 ± 222^{defA}
C + D	437 ± 3^{fB}	520 ± 3^{hijA}	2020 ± 85^{cdB}	2990 ± 73^{defA}
A + E	475 ± 3^{efB}	537 ± 3^{efghA}	2418 ± 158^{aB}	3148 ± 91^{bcdA}
C + E	457 ± 3^{fB}	524 ± 5^{ghijkA}	2065 ± 69^{bcdB}	3103 ± 107^{cdeA}
F	545 ± 11^{e}	536 ± 3^{efgh}	1459 ± 138^{fgB}	1715 ± 69^{ijA}
G	459 ± 8^{fB}	576 ± 25^{dA}	1384 ± 90^{gB}	2648 ± 119^{fgA}
G + F	117 ± 3^{gB}	149 ± 3^{kA}	323 ± 45^{iB}	970 ± 69^{iA}
Control 1	722 ± 12^{cB}	905 ± 3^{bA}	1948 ± 130^{cdB}	3001 ± 111^{defA}
Control 2	443 ± 8^{fB}	514 ± 1^{ijA}	2219 ± 102^{abcB}	3430 ± 47^{abcA}
Control 3	625 ± 12^{d}	646 ± 7^{d}	2407 ± 227^{ab}	2437 ± 69^{gh}
Control 4	1139 ± 9^{aB}	663 ± 12^{cA}	1504 ± 145^{efgB}	2121 ± 204^{hiA}
Control 5	541 ± 7^{eB}	566 ± 3^{deA}	1813 ± 116^{de}	1807 ± 24^{ij}
p-Value	<0.001	<0.001	<0.001	<0.001

Note: Values are expressed as means followed by the standard deviation (n = 3) and refer to the raw materials dry matter basis). Different lowercase letters in the same column represent statistically different results among enzymatic treatments ($p < 0.05$). Different uppercase letters in different columns, for the same response, represent statistically different ($p < 0.05$) results comparing initial (1 h) and final time (4 h). AAE = ascorbic acid equivalent.

lyase worked inefficiently either due to the unfavourable pH adjustment or small temperature change compared to the initial results. The TPC ranged from 376 ± 2 to 913 ± 38 mg/100 g (1 h), and from 495 ± 2 to 1349 ± 8 mg GAE/100 g (4 h), revealing that a longer enzymatic processing time increased the extraction of polyphenols. During the 4 h treatments, Control 1 and β-glucanase (A) enhanced the recovery of polyphenols.

In various industrial processes, cell-wall-digesting enzymes, such as pectinases, cellulases, amylases, and proteases, are commonly applied before extraction to improve the extract yield and polyphenol content (Hong et al., 2013). Regarding the antioxidant activity (Table 1B), DPPH values varied from 117 ± 3 to 1139 ± 9 mg AAE/100 g (1 h) and from 149 ± 3 to 1,080 ± 33 mg AAE/100 g (4 h) among the samples tested. In contrast, the CUPRAC values ranged from 323 ± 45 to 2418 ± 158 mg AAE/100 g (1 h), and 970 ± 69 to 3697 ± 205 mg AAE/100 g (4 h). Overall, the antioxidant activity (DPPH and CUPRAC) values increased towards the end of the processing time (4 h), and C (cellulase), D (pectinase), and E (pectinase, *A. oryzae*) treatments exhibited the highest mean values. However, the same enzymes showed a slight decrease in the TA and TPC content during the processing, revealing that phenolics might demonstrate competitive interaction with other components or even greater sensitivity under the conditions chosen for those treatments.

Therefore, once different factors and treatment conditions can affect the polyphenol composition, optimising the extraction temperature to increase solubility and mass transfer is crucial to minimise compound degradation (Rätsep, Bleive, Kaldmäe, Kahu, & Kikas, 2020; Sandri, Lorenzoni, Fontana, & da Silveira, 2013). Comparatively, Rätsep et al. (2020) analysed the impact of enzymatic treatment on the blackcurrant polyphenols in juice and press-residue. Results reported that polyphenols in enzymatic press residue increased up to 29%. The TA of the enzyme-treated samples was significantly higher than that of non-treated samples, meaning that press cake is a valuable source of polyphenols, especially in insufficient enzyme treatment blackcurrant mash.

Basegmez et al. (2017) optimised the extraction processes of blackcurrant pomace to reach high-value functional ingredients. The authors used three extraction techniques to achieve different purposes. Firstly, supercritical carbon dioxide extraction (SFE-CO_2) was optimised using response surface methodology, based on the central composite design, to isolate the lipophilic fraction effectively. Next, pressurised liquid (PLE) and enzyme assisted (EAE) extractions were employed to isolate higher polarity substances. Results showed that the products obtained using different extractions generate valuable lipophilic and hydrophilic polyphenols for formulating food supplements and functional foods. Furthermore, in a study conducted by Kumar, Tomar, Punia, Amarowicz, and Kaur (2020), different extraction techniques were compared, including enzyme-assisted microwave extraction (EMAE), enzyme-assisted extraction (EAE) and microwave-assisted extraction (MAE), to obtain the maximum phenolic content extraction with antioxidant activity of the pomegranate (*Punica granatum*) peel (PP). The obtained data confirmed the auxiliary role of cellulolytic enzyme preparation (Viscozyme) and microwave treatment in achieving high phenolic content and AOA from the extract.

3.2. Scale-up of the enzymatic treatment

The scale-up aimed to validate the processing efficiency in

recovering anthocyanins and other phenolic compounds. As cellulase breaks down the non-starch polysaccharides in the blackcurrant press cake, it was selected to test against the mixture of β-glucanase, and pectin lyase enzymes acting on the residual starch content of the side-streams, in general, were chosen for the second setup.

The first experiment was carried out with cellulase, and the reaction was terminated after a hydrolysis time of 1 h (J). In comparison, the second experiment was run until 4 h using the β-glucanase in the first 2 h of hydrolysis and then pectin lyase (L), as shown in Fig. 1. Additionally, control samples at 1 h (I) and control at 4 h (K) were prepared in the same conditions for comparison purposes. The length of the hydrolyses was chosen based on the findings in a screening phase and based on the recommendation of the enzyme manufacturer, while the enzyme concentration was the same as in the preliminary trials to be able to compare the efficiency. A slight increase in the hydrolysis temperature was implemented compared to pre-trials, rising from 40 to 50 °C, while the inactivation temperature raised from 65 to 75 °C due to non-pectinase enzymes. These parameters followed the range recommended by the enzyme's manufacturer. Herein, TPC, anthocyanins, and antioxidant activity for J and L enzyme-treated samples were compared to I and K control samples and illustrated in Fig. 1.

The concentration of anthocyanins in the larger scale experiments showed different results than the experimental findings in the screening phase. Nor the enzymatic treatment with cellulase nor the mixture with β-glucanase and pectin lyase show higher TA than their control pairs. However, the cellulase treatment showed higher TA content than the combined β-glucanase and pectin lyase treatment, 1484.3 mg/100 g dw and 1224.5 mg/100 g dw, respectively. The anthocyanin composition was not altered in any of the treatments, showing the similar distribution of the significant anthocyanin compounds of delphinidin-, cyanidin- and peonidin rutinosides, and delphinidin and cyanidin glucosides, as shown in Fig. 2**.** These findings are different from those reported by Dinkova et al. (2014), who described the enzymatic treatment of the bilberry skin to recover anthocyanins and phenolic compounds in an extract. In their study, the content of delphinidin glucoside increased more intensively in the extracts. The content of cyanidin-3-*O*-glucoside decreased with the enzyme treatment, which the authors attributed to the differences in side-enzyme activity.

However, the results for TA (Fig. 1B) contrast with the TPC (Fig. 1A) results, as treatment J with cellulase (1559 mg GAE/100 g) and

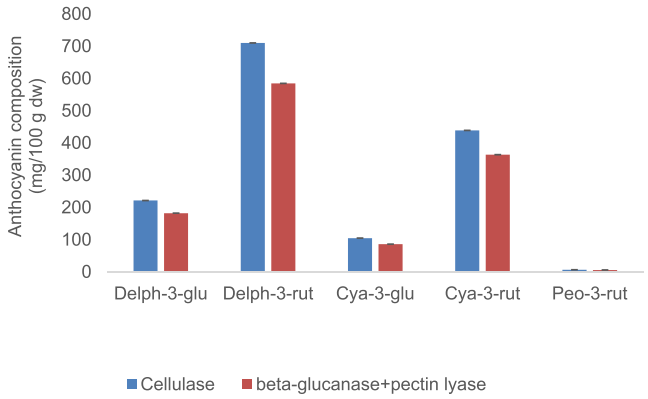

Fig. 2. Composition of anthocyanins in blackcurrant press cake extracts obtained with different enzymes. Note: Delph-Delphinidin, Cya-Cyanidin, Peo-Peonidin, glu-glucoside, rut-rutinoside.

β-glucanase + pectin lyase, L (1526 mg GAE/100 g) showed similar ($p > 0.05$) contents, and both presented higher TPC than their pair of control samples. This phenomenon may be explained by the temperature of the hydrolyses reaching a value at which the anthocyanins start degrading more intensely than the other phenolics. Patras, Brunton, O'Donnell, and Tiwari (2010). described the partial or complete degradation of anthocyanins at a temperature of 50 °C, and additional stress on the heat-sensitive anthocyanins usually arises at the inactivation phase, when the temperature is between 65 and 80 °C, depending on the nature of the enzyme. Additional adverse effects on the anthocyanins may be caused by adjusting the pH or combining the processing temperature and pH. Sui, Dong, and Zhou (2014) investigated the combined effect on the individual anthocyanin compounds. Although the temperature was much higher in their work than in this study, they determined that cyanidin-3-*O*-glucoside and cyanidin-*O*-rutinoside were more prone to degradation in the pH 5.0–6.0 range, which is in the range these enzymes are the most effective.

Data from DPPH (Fig. 1C) and CUPRAC (Fig. 1D) assays indicated that the β-glucanase + pectin lyase (L) treatment had the highest value (1086 mg AAE/100 g for DPPH; 3024 mg AAE/100 g for CUPRAC), followed by cellulase (J, 1019 mg AAE/100 g for DPPH; 1068 mg AAE/

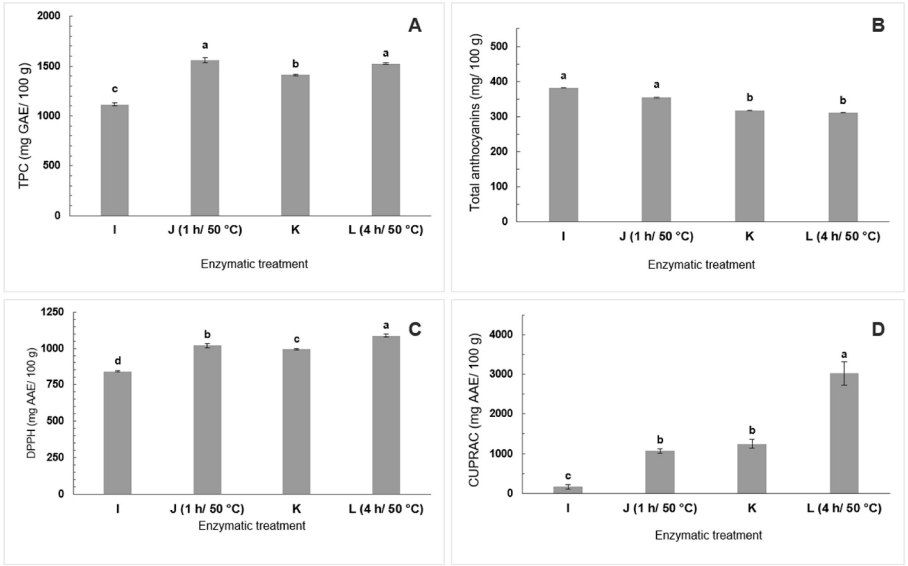

Fig. 1. Effect of enzymatic treatment (J and L) compared to control samples I (1 h) and K (2 h) on the total phenolic content (1A), total anthocyanins (1B), and antioxidant activity (1C and 1D) in a second-phase experiment (up-scaling). Different letters represent statistically significant differences ($p < 0.05$). See Table 1 – Supplementary material for sample codes.

100 g for CUPRAC), control 4 h (993 mg AAE/100 g for DPPH; 1249 mg AAE/100 g for CUPRAC), and control 1 h (841 mg AAE/100 g for DPPH; 165 mg AAE/100 g for CUPRAC). Therefore, these results indicate that using enzymatic treatment in a prolonged processing time increased the TPC, associated with higher antioxidant activity.

Kumar, Dahuja, Tiwari, Punia, Tak, Amarowicz, Bhoite, and Singh (2021) reviewed novel extraction techniques for bioactive plant compoundsmainlymentioned. Those mostly mentioned green extraction techniques such as microwave-assisted extraction, ultrasound-assisted extraction, accelerated solvent extraction, etc., but not enzyme technology as a single technology for the extraction of these compounds. In this instance, enzymatic hydrolyses of the planned biomass might be a

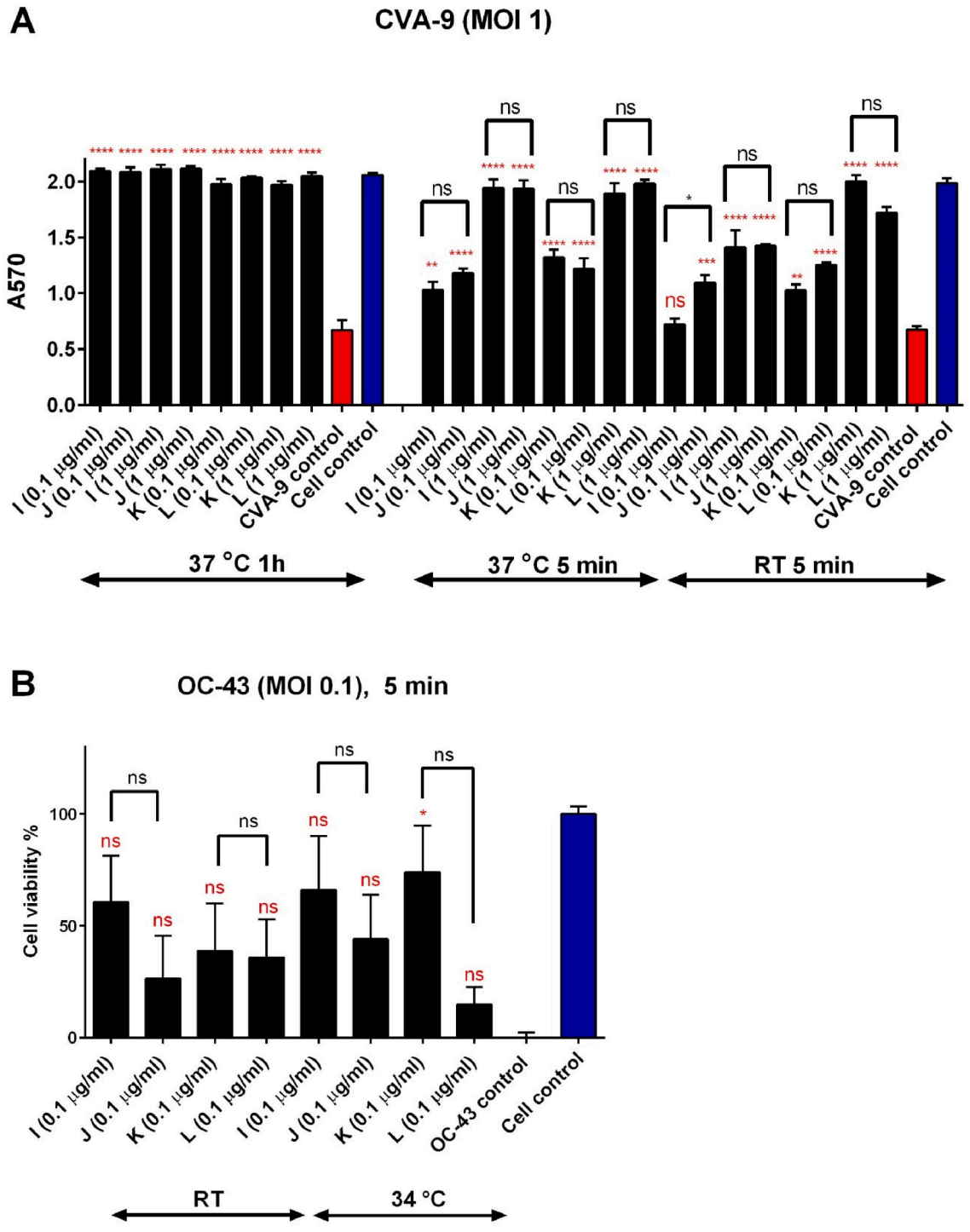

Fig. 3. Effect of enzymatic treatments (J and L) and control treatments (I and K) of the blackcurrant press-cake on the antiviral activities towards Coxsackievirus A9 and human coronavirus HCoV-OC-43. (A) Antiviral activity of blackcurrant freeze-dried extracts against CVA-9 after incubating extracts in 0.1 and 1 µg/mL with the virus for 1 h at 37 °C and for 5 min at 37 °C and room temperature. (B) Antiviral activity of blackcurrant extracts against HCoV-OC-43 after incubating extracts in 0.1 µg/mL concentration with the virus for 5 min at 34 °C and room temperature. Results are expressed as the means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ versus the virus control. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ with enzyme treatments in comparison to process control samples. See Table 1 – Supplementary material for sample codes.

part of hybrid novel technology, when enzymatic treatment could be the first step combined with some of the novel extraction to increase the overall efficiency of the valuable compound liberation into the extract. Kumar et al. (2020) described a similar set-up using cellulolytic enzyme-assisted extraction coupled with microwave treatment to extract phenolics from pomegranate peel and overcome the disadvantage the conventional extraction, such as the long processing time. These authors reported that cellulase-assisted extraction coupled with microwave-assisted extraction, followed by microwave-assisted single extraction, was the most efficient strategy to recover phenolic compounds. Conversely, conventional solvent extraction was the least efficient in recovering polyphenols. Similar findings were also described in the case of the black soybean seed coat extraction conditions (Kumar, Dahuja, Sachdev, Kaur, Varghese, Saha, & Sairam, 2019), enzyme-assisted extraction being the second-best after microwave-assisted extraction, while conventional extraction is the least efficient anthocyanins and total phenolics under optimised process conditions. Overall, these findings underline the importance of enzymatic treatment in recovering polyphenols from the plant matrices.

Although anthocyanins can be used as food colourings, and the extracts obtained from the blackcurrant press cake showed antioxidant activity, the question remains concerning the applicability of these biologically active extracts in foods. Gagneten et al. (2021) studied and showed that the blackcurrant press cake was suitable for manufacturing biscuits: the addition of 3.5% of blackcurrant press cake increased the antioxidant activity of the biscuit by 70%, while the TPC increased it by 67%. Additionally, in a static digestion simulation model, a substantial amount of the bioactive compounds reached the large intestine and displayed antioxidant activity, revealing the compounds' bioaccessibility. Freeze-dried extracts from blackcurrant press cake are water-soluble and can be applied in different technological applications in the food industry.

3.3. Antiviral activities

The results of the antiviral activity of blackcurrant extracts against Coxsackievirus A9 and human coronavirus HCoV-OC-43 are shown in Fig. 3. All tested blackcurrant extracts showed antiviral activity against the virus (Fig. 3**A**) without any cytotoxicity in A549 cells with the tested low amounts of press cake samples (data not shown). All tested extracts inhibited the infectivity of CVA-9 at the concentration of 1 μg/mL (dry extract) already after a 5-minute incubation at 37 °C. The infectivity of CVA-9 was not completely inhibited when the concentration of extracts was reduced to 0.1 μg/mL during 5-min incubation, but the antiviral effect was still considerable (Fig. 3**A**). However, there was no difference ($p > 0.05$) in the efficacy between the extracts. Only a slight difference was observed when the effectiveness was measured for 5 min at room temperature, the treatment F being better than the control extract (Fig. 3**A**). Furthermore, blackcurrant extracts showed antiviral activity against the seasonal coronavirus, HCoV-OC43. Cell viability was over 50% after treating HCoV-OC43 with control extracts at 0.1 μg/mL (Fig. 3**B**). Most extracts showed more antiviral activity at 34 °C than at room temperature. The results thus altogether show that blackcurrant extracts in this study are antiviral, but, remarkably, have very high efficacy against the very stable non-enveloped enteroviruses, which are generally challenging to decontaminate.

The antiviral effect of blackcurrant extracts against viruses, including HSV-1 and Influenza A and B, has been stated in earlier studies (Haasbach et al., 2014; Ikuta et al., 2012). In addition, the relationship between antiviral and antioxidant activities of polyphenols has been discussed (Montenegro-Landívar et al., 2021). It is likely that components in the blackcurrant extract bind directly to enteroviruses and may prevent both cell binding and RNA release from the viruses, as we have found for other polyphenols recently (Pap, 2021; Reshamwala et al., 2021). Furthermore, the extract from blackcurrant leaves was shown to act very early in influenza infection, most probably directly on the virions (Haasbach et al., 2014). However, more studies with blackcurrant press cake are needed to resolve the exact mechanism of enteroviruses and coronaviruses.

Notably, the antiviral activity of blackcurrant extracts was not significantly enhanced after enzymatic treatments. Likely, the number of bioactive compounds to induce a potent antiviral effect was already enough as the result was already quite strong at 0.1 μg/mL. Furthermore, the total anthocyanins content was not increased due to enzymatic treatments, thus suggesting that the anthocyanin level in all the fractions was high enough to induce antiviral activity. We have previously shown that willow bark extracts contain excellent antiviral activities against enteroviruses (Tienaho et al., 2021). However, this activity correlated only with an antioxidative activity that fluctuated between different willow preparations, which all contained excellent antiviral activities. It is likely that tannins in high amounts in willow bark contribute to antiviral action in many other extracts, such as those from blackcurrant press cake. It has also been proposed that certain tannins inhibit SARS-CoV-2 infection by interacting with the $3CL^{pro}$ receptor binding site (Khalifa, Zhu, Mohammed, Dutta, & Li, 2020).

3.4. In vitro cell-based viability evaluation and ROS generation.

Regarding the cytotoxic assay (Fig. 4), the control sample (I) of blackcurrant extract, treated for 1 h without an enzyme, seemed to be more sensitive to A549 cells than other cells (HCT8 and IMR90), showing a 50% reduction in cell viability at 1544 μg/mL and growth at 381.5 μg/mL. The other samples showed no reduction in the viability of the cells tested. Moreover, the lethal concentration (LC_{50}) was not determined, suggesting relative safety. Considering that the SI value higher than 3 indicates selectivity of the extract to the cell lines tested (Boechat, 2014), the sample I did not have selectivity over cancer cells once the SI values were 1.3 for A549 and 1.0 for HCT8. Jia (2014) tested blackcurrant extract with a cyanidin concentration of 74.5 ± 26.5 mg/100 g, which decreased the cell viability by 30% as the tested concentration increased. Sample I presents a cyanidin content of 604.6 ± 22 mg/100 g, justifying the similar data observed in the cytotoxicity assay. The intracellular ROS generation in the cell line was exposed to various concentrations of blackcurrant press cake extract (Fig. 1 – **Supplementary material**). H_2O_2 is generated from nearly all sources of oxidative stress, causing oxidative injury in cell membranes. Exogenous H_2O_2 can also induce cytotoxicity because of its high membrane permeability (Jia, 2014). Here, H_2O_2 was used to cause oxidative stress in the model with A549 cells after this cell line was the only one that reduced the proliferation in the cell viability assay. The results provided direct evidence of the protective effect of blackcurrant press cake extracts against H_2O_2-induced injury. In agreement with the cell viability data, the control sample showed a more significant reduction in ROS levels and achieved the basal cell level (without the stress caused by H_2O_2). These findings are consistent with the results of our previous study, in which blackcurrant extract obtained by maceration exhibited a decrease in ROS levels when tested on the same cell line (Pap, 2021). Overall, these results indicate that the control sample of the blackcurrant press cake processed without cellulase for 1 h acted as an antioxidant agent, which may correlate with its total concentration of anthocyanins and TPC. In the trials, these were comparable with the results from the enzymatic treatment with cellulase or cellulase + pectin lyase enzymes.

3.5. Anti-inflammatory properties of the anthocyanin extract

First, it was tested whether enzyme-treated samples with cellulase (J) and β-glucanase+pectin lyase (L), and the control sample at 1 h (I) processing time had any effect on the viability of the murine microglial cell line. The results are shown in Fig. 2 – **Supplementary material** confirmed that the extracts had no protective effect on the cell viability in the IMG microglial cells at 100 μg/mL. Thereafter, the test substrates

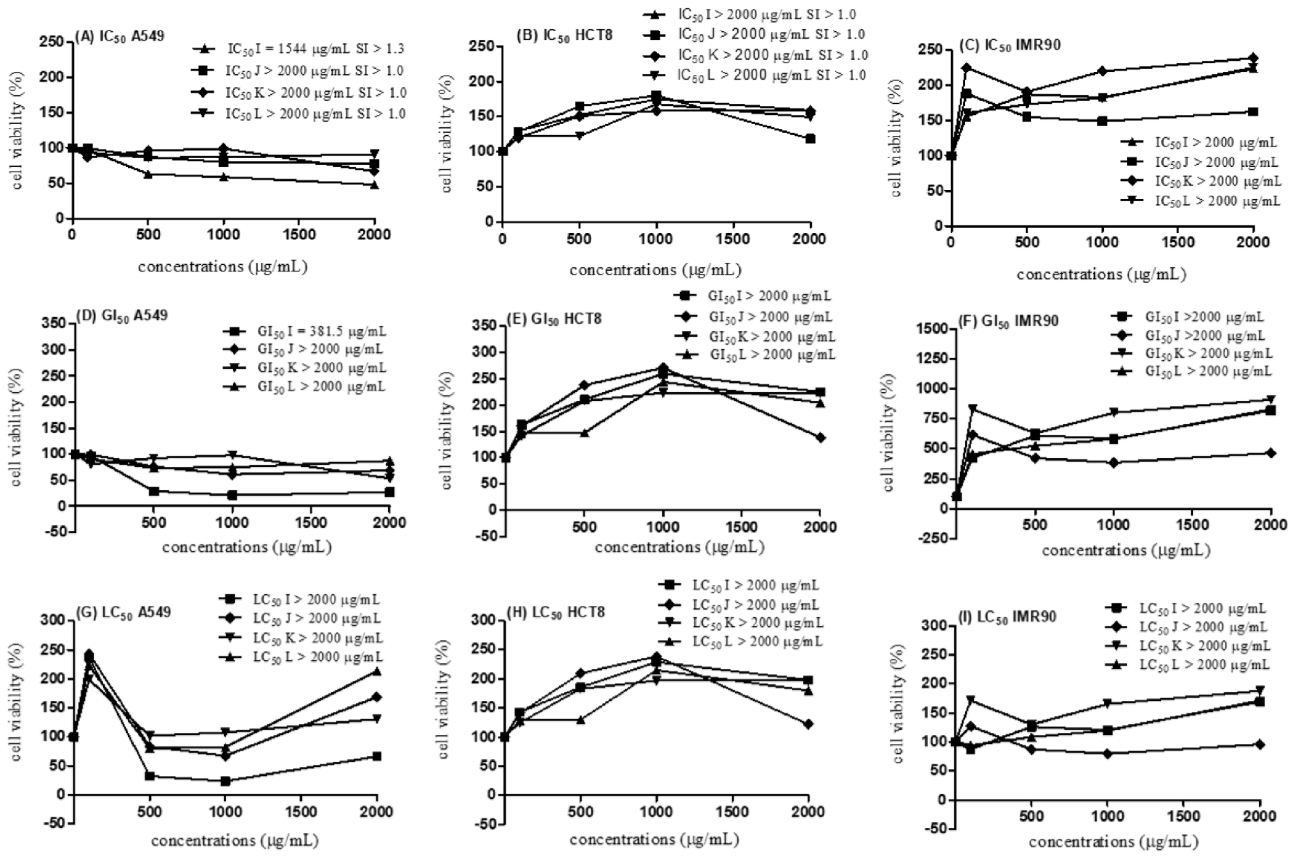

Fig. 4. Cell viability and representative evaluation of the concentration-dependent impact after 48 h exposure to the blackcurrant press-cake freeze-dried extracts after enzymatic treatment and control in A549, HCT8 and IMR90 line cells, where: I, Control 1 h; J, Cellulase 1 h; K, Control 4 h; L, β-glucanase + Pectin lyase 4 h. (A, B, C): IC_{50}; (D, E, F): GI_{50}; (G, H, I): LC_{50} in A549, HCT8 and IMR90 cell lines. SI = selectivity index based on the IC_{50} of the normal cell line, IMR90. See Table 1 – Supplementary material for sample codes.

were studied for their ability to inhibit inflammasome formation and activation in IMG cells. In summary, the inflammasome was induced by the exposure of IMG cells to LPS (1 μg/mL) for 4 h, followed by the activation of the inflammasome by ATP (3 mM) for 30 min. The addition of LPS + ATP induced inflammasome formation and activation, evidenced by a significant ($p < 0.01$) increase in the mRNA levels of NLRP3, pro-interleukin-1β, and TNF-α. On the other hand, blackcurrant extracts at 100 μg/mL did not significantly affect the mRNA levels of proinflammatory cytokines, caspase-1, and NLRP3, which further shows no anti-inflammatory effects. Additionally, Western blot analyses were performed to assess the possible impact of the blackcurrant extracts on the protein levels of proinflammatory cytokines and NLRP3 inflammasome components. Extracts from the cellulase + pectin lyase, control sample 1 h, and cellulase treatments at 100 μg/mL had no efficacy in expressing NLRP3 inflammasome-related proteins (pro-IL-1ß, procaspase-1, and cleaved caspase-1), as is aptly demonstrated in Fig. 2 – **Supplementary material**. Although the anti-inflammatory properties of blackcurrant extracts and their anthocyanins in IMG cells were not observed, there are previous reports of blackcurrant extracts having such anti-inflammatory effects (Desjardins, Tanabe, Bergeron, Gafner, & Grenier, 2012; Lyall et al., 2009). More specifically, it has been shown that the secretion of IL-6 and TNFα decreased in LPS-induced THP-1 cells when pre-treated with anthocyanin-rich blackcurrant extract even though the concentrations that were used (5 ng/mL and 50 ng/mL) were considerably lower than the concentration we used (100 μg/mL) in our study (Lyall et al., 2009). Interestingly, a significant inhibitory effect on the levels of TNF-α and IL-6 was first detected after 6 and 12 h of LPS incubation, but not after 1 or 3 h incubation, respectively. It is possible that the 4 h of LPS incubation used in the current study was not long enough to detect the anti-inflammatory effects of blackcurrant extracts. In a study by Desjardins et al. (2012), the pre-treatment with blackcurrant extract (25 μg/mL) and cyanidin-3-*O*-glucoside (5 and 25 μg/mL) decreased the secretion of IL-6 in human macrophages after incubating cells with LPS for 24 h (Desjardins et al., 2012). At the same time, the secretion levels of other pro-inflammatory proteins (e.g., IL-1ß, IL-8, and TNF-α) remained unaffected. In the current study, only the effects of blackcurrant extracts on the changes of intracellular inflammation-related proteins and mRNA were analysed. Future studies should explore the levels of secreted pro-inflammatory proteins and apply more prolonged LPS incubation times.

4. Conclusions

The enzyme-assisted extraction of blackcurrant press cake showed positive results in the recovery of anthocyanins and their phenolic compounds compared with untreated samples. The study's screening phase gave preliminary information on the most suitable enzymes or enzyme combinations, processing times, and conditions to best enhance the liberation of anthocyanins and other phenolic compounds into a liquid extract. In the scale-up process, cellulase was the most efficient enzyme in recovering anthocyanins and other phenolics and increased the antioxidant activity. Blackcurrant press cake treated with cellulase showed excellent antiviral activity against the CVA-9 enterovirus and most effectively reduced ROS in A549 cells. This study's findings open new possibilities for blackcurrant press cake extracts treated with different enzymes as value-added ingredients for the food industry.

CRediT authorship contribution statement

Daniel Granato: Conceptualization, Methodology, Project administration, Funding acquisition, Methodology, Data curation, Writing – review & editing. **Marina Fidelis:** Investigation, Formal analysis, Writing – review & editing, Data curation. **Marjo Haapakoski:** Methodology, Conceptualization, Data curation. **Amanda dos Santos Lima:** . **Janeli Viil:** Conceptualization, Methodology, Formal analysis, Data curation. **Jarkko Hellström:** Formal analysis, Data curation. **Reelika Rätsep:** Conceptualization, Formal analysis, Data curation. **Hedi Kaldmäe:** Conceptualization, Formal analysis, Data curation. **Uko Bleive:** Conceptualization. **Luciana Azevedo:** . **Varpu Marjomäki:** Methodology, Data curation, Writing – review & editing. **Alexander Zharkovsky:** Conceptualization, Methodology, Data curation, Writing – review & editing. **Nora Pap:** Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Writing – original draft.

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.2022.133240.

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