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Advanced Extraction of Blackcurrant Pomace Polyphenols: Chemical Characterization and Biological Insights

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Abstract

This study evaluated the efficiency of advanced extraction techniques for recovering polyphenols from blackcurrant pomace (BP), with particular emphasis on anthocyanins. Pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and the use of cryogrinding (CG) as a pretreatment were investigated under selected experimental conditions and compared with the conventional extraction technique (CT). Among the investigated extraction techniques, UAE provided relatively high anthocyanin recovery, while CG significantly enhanced extraction efficiency. Under the investigated conditions, CT yielded the highest total phenolic content (TPC) among the extraction techniques compared, but not the highest total monomeric anthocyanin content (TMAC), whereas CG combined with UAE (CG + UAE; 2 min CG, 75% amplitude, 5 min, 1:60 g/mL) provided a favorable balance between TPC and TMAC. UPLC–ESI–MS^2 profiling confirmed high recovery of delphinidin- and cyanidin-based glycosides, flavonols, phenolic acids, and flavan-3-ols. The optimized extract showed strong antioxidant capacity in FRAP, DPPH, ABTS, and ORAC assays. Zebrafish embryotoxicity and cell-based assays showed concentration-dependent effects, indicating that further assessment is needed before conclusions regarding extract safety can be drawn. Overall, CG + UAE showed potential for improving the recovery of bioactive compounds from BP under the investigated conditions.

Keywords: *Ribes nigrum*; by-product valorization; antioxidants; PLE; MAE; UAE; cryogrinding

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1. Introduction

The valorization of fruit and vegetable by-products has gained increasing attention due to their richness in bioactive compounds with health-promoting properties, while simultaneously addressing the environmental challenges posed by the large volumes of residues generated by the food industry [1]. The efficient utilization of by-products, which are rich in dietary fibers, pigments, essential oils, vitamins, enzymes, and fatty acids, is an

important approach to achieving sustainable production and generating value-added products [2–4]. Among fruit residues, blackcurrant (*Ribes nigrum*) pomace (BP), the main by-product of blackcurrant juice production, represents a substantial fraction of the processed fruit, accounting for approximately 20–30% of the original berry mass. It consists of skins, seeds, and pulp, and retains a large proportion of the fruit's bioactive compounds such as polyphenols, including anthocyanins, flavonols, phenolic acids, and proanthocyanidins [4]. However, a large proportion of these bioactives remain trapped in cell wall matrices or seeds after juice pressing, which limits their bioavailability and requires efficient extraction strategies [5,6].

Conventional extraction techniques such as maceration and Soxhlet extraction have frequently been employed for bioactive molecule isolation. However, these approaches are associated with limitations, including prolonged extraction times, excessive solvent use, and degradation of thermolabile compounds (e.g., anthocyanins and vitamin C), which ultimately lowers the biological activity of produced extracts [3,7]. To overcome these drawbacks, advanced green extraction techniques have been introduced, providing greater efficiency, selectivity, and sustainability, while at the same time reducing the possibility of compound degradation [8]. Among them, pressurized liquid extraction (PLE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) stand out as efficient techniques due to their shorter extraction times, reduced solvent consumption, and higher extraction yields compared to conventional techniques. PLE enhances solvent penetration into dense skin matrices due to the application of elevated pressure and temperature, MAE accelerates mass transfer through rapid cell wall disruption, while UAE employs acoustic cavitation to disrupt skin and seed structures, ultimately leading to the more efficient release and solubilization of target compounds [9,10]. However, extraction parameters such as temperature, extraction time, and solid-to-solvent ratio (SSR) strongly affect extraction yield, selectivity, and compound stability. Therefore, their proper optimization is necessary to achieve high efficiency while preventing thermal and oxidative degradation. In addition, extraction pretreatment is a critical but often neglected step that can significantly affect extraction efficiency by altering the physical properties of the plant matrix. Among pretreatment methods, cryogenic grinding (CG) reduces particle size and increases surface area while preserving thermolabile compounds by processing the material at very low temperatures [7]. This limits enzymatic activity and degradation, leading to higher extraction yields of sensitive compounds. Although many studies have examined green extraction techniques such as MAE, PLE, and UAE, as well as mechanical pretreatments, these techniques are usually studied separately and under different conditions. Consequently, the results are difficult to compare and provide limited information on the interaction between pretreatment and extraction parameters. In addition, the combined effects of optimized extraction conditions and CG on the recovery of BP polyphenols and anthocyanins have rarely been evaluated, indicating the need for more integrated approaches.

This study examined the effects of key extraction parameters of PLE, MAE and UAE on the recovery of BP polyphenols and anthocyanins, including the potential of CG pretreatment to improve extraction performance. Extracts were analyzed for their phenolic profile and antioxidant capacity, while zebrafish embryo and cell-based assays were used as complementary assessments of concentration-dependent biological effects, including embryo development, cellular responses, and DNA damage. By relating chemical composition to functional properties, this research provides further information on the recovery and characterization of bioactive compounds from BP and their potential for further valorization within a circular bioeconomy framework.

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2. Materials and Methods

2.1. Reagents and Chemicals

The chemicals used for the extractions and analyses were the HPLC-grade solvents ethanol (96%) and methanol, obtained from Gram-Mol Ltd. (Zagreb, Croatia); acetonitrile was purchased from J.T. Baker Chemicals (Deventer, Netherlands); formic acid (98–100%) was obtained from T.T.T. Ltd. (Sveta Nedelja, Croatia); glacial acetic acid (99–100%) and hydrochloric acid (37%) were obtained from Carlo Erba (Val-de Reuil, France). Used liquid nitrogen and nitrogen (99.999%) were purchased from Messer (Messer Croatia Plin d.o.o., Zaprešić, Croatia). Other chemicals and reagents used for the reactions were 2,4,6-tripyridyl-S-triazine (TPTZ, ≥98%), 2,2-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 97%), diammonium salt of 2,2′-azino-bis(3-ethylbenzthiazolin-6-yl)-sulfonic acid (ABTS), gallic acid (>97.5%), and 6-hydro-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox solution, 97%). DPPH (2,2-diphenyl-1-picrylhydrazyl) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium persulfate (>98%), sodium carbonate (99.5%) and sodium acetate trihydrate were purchased from Kemika (Zagreb, Croatia). Folin–Ciocalteu reagent and sodium chloride were obtained from Merck KgaA (Darmstadt, Germany), iron (III) chloride hexahydrate was obtained from Gram-mol (Zagreb, Croatia), while fluorescein sodium salt was purchased from Honeywell Riedel-de-Haën (Bucharest, Romania). The commercial standards used for UPLC analysis—gallic acid, chlorogenic acid, caffeic acid, protocatechuic acid, ellagic acid, kaempferol-3-rutinoside, quercetin-3-glucoside, cyanidin-3-glucoside chloride, delphinidin 3-glucoside chloride and myricetin—were purchased from Sigma-Aldrich (St. Louis, MO, USA), and catechin was purchased from Extrasynthese (Genay, France). Distilled water used in experiments was Milli-Q grade (Millipore Corp., Bedford, NY, USA). Ham's F-12 medium supplemented with L-glutamine and 10% fetal bovine serum (FBS) used for cell cultivation (Capricorn, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, Taufkirchen, Germany), sodium dodecyl sulfate (SDS) (PlusOne, VWR International, West Chester, PA, USA), glacial acetic acid (Kemika, Zagreb, Croatia), crystal violet (Merck, Germany), 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) (Sigma-Aldrich, Germany), low-melting-point agarose (LMP) (Sigma Aldrich, USA), normal-melting-point agarose (NMP) (Sigma Aldrich, USA), Na_2EDTA (Sigma Aldrich, USA), Tris (Sigma Aldrich, USA), Triton X-100 (Sigma Aldrich, USA), GelRed® Nucleic Acid Stain (10,000× Water) (Merck KGaA, Darmstadt, Germany), trichloracetic acid (TCA), ≥99.5% analytical reagent grade (Thermo Fisher Scientific, USA), thiobarbituric acid (TBA; Merck, Germany), and 1,1,3,3-tetramethoxypropane (TMP; Sigma-Aldrich, Germany) were used in toxicological assays.

2.2. Plant Material

The blackcurrant (*R. nigrum*) berries were collected in June and July 2024 in the orchard of Donja Zelina at the Center for Fruit and Vegetable Cultivation of the Croatian Agency for Agriculture and Food (Croatia). Immediately after harvesting, the berries were processed into the juice using a juice extractor (VerVita HU-100, Dong Ah Ind. Co., Ltd., Busan, South Korea). The remained BP was freeze-dried at −55 °C/48 h (CHRIST Alpha 1-4 LSCplus, Osterode am Harz, Germany), hermetically sealed and stored at −18 °C until further analysis. Prior to extraction, one part of the freeze-dried BP was crushed using an electric grinder (Waring WSG30, Sprzęt Laboratoryjny i Medyczny Labpartner KBS, Warszawa, Poland) and the second part was cryogenically ground (SPEX 6875D SamplePrep, Metuchen, NJ, USA). Dry matter content was determined using AOAC method [11] (AOAC, 2000) and was 98.93% in freeze-dried BP.

2.3. Cryogenic Grinding

For each cryogenic treatment, 15 g of freeze-dried BP was placed into a 50 mL grinding cell. The samples were then individually inserted into a cryogenic grinder (Spex 6875D Freezer/Mill, Metuchen, NJ, USA) where they were pre-cooled in a stream of liquid nitrogen for 2 min. CG was performed at a rate of 14 cycles/s for the designated durations (1, 2, 3, 4, and 5 min), while the sample labeled as "0" represented the conventionally ground control. After grinding, the resulting powders were carefully collected and stored at −18 °C until further analysis.

Particle Size Determination

The particle size distribution of BP powders obtained by conventional and cryogenic grinding was analyzed using laser diffraction with a Malvern Panalytical Mastersizer 2000 equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK). The measurement, based on the laser scattering method, covers a particle size range from 20 nm to 2 mm. We introduced approximately 5 g of each powder sample into the dry dispersion system, and measurements were conducted at an air pressure of 1.5 bar with a feed rate set to 100%. Collected data were processed using the Mastersizer 2000 software (versions 5.60 and 5.61) with the following settings: refractive index of 1.5, absorption of 0.1, and obscuration level of 1.4%. Particle size results were expressed as d(0.5), representing the particle diameters at 50th percentile (μm).

2.4. Extractions

2.4.1. Pressurized Liquid Extraction (PLE)

PLE of BP phenolic compounds was performed using the ThermoScientific™ Dionex™ ASE™ 350 extractor (Thermo Fisher Scientific, Sunnyvale, CA, USA). A pressure of 10.34 MPa, 30 s nitrogen purge, 30% purge volume and 3 extraction cycles were constant extraction parameters, and 1% formic acid in 50% ethanol (*v*/*v*) was an extraction solvent. Varied extraction parameters were: temperature (75, 100 and 125 °C), static extraction time (5 and 10 min) and (SSR) (1:40, 1:60 and 1:80 g/mL). The following were placed in the stainless-steel extraction cell: two cellulose filters (Dionex™ 350/150 Extraction Cell Filters, Thermo Fisher Scientific Inc.) at the bottom, the proper amount of sample mixed with 2 g of diatomaceous earth, and a cellulose filter on the top. The extraction procedure was performed according to the experimental design (Table 1) and the extracts were collected, filtered into 50 mL volumetric flasks, made up to the mark and stored at −18 °C until analysis. The investigated parameters and their ranges were selected based on previous studies [3,8,12,13], preliminary experiments, and the technical capabilities of the respective extraction systems for all three extraction techniques.

Table 1. TPC and TMAC in BP extracts obtained under different PLE, MAE and UAE conditions.

Extraction Technique	Extraction Parameters			TPC (mg GAE/g dw)	TMAC (mg C3GE/g dw)
	Temperature (°C) for PLE, MAE Amplitude (%) for UAE	Time (min)	SSR (g/mL)		
PLE	75	5	1:40	22.99 ± 1.83	12.86 ± 0.71
			1:60	21.07 ± 0.46	13.01 ± 0.39
			1:80	22.20 ± 0.76	13.19 ± 0.54
		10	1:40	19.04 ± 0.43	10.95 ± 0.34
			1:60	21.63 ± 1.87	12.78 ± 0.43
			1:80	23.58 ± 1.90	13.83 ± 0.38
	100	5	1:40	37.17 ± 0.65	15.21 ± 0.04
			1:60	32.44 ± 0.61	12.86 ± 0.02

			1:80	40.30 ± 0.97	14.69 ± 0.07
		10	1:40	37.01 ± 0.08	9.90 ± 0.00
			1:60	40.91 ± 0.61	10.94 ± 0.43
			1:80	36.86 ± 2.09	11.52 ± 0.35
		5	1:40	40.68 ± 0.41	8.87 ± 0.14
	125		1:60	44.88 ± 3.32	11.10 ± 0.09
			1:80	42.61 ± 1.13	10.48 ± 0.19
		10	1:40	52.57 ± 0.08	10.18 ± 0.11
			1:60	47.13 ± 1.97	7.53 ± 0.20
			1:80	45.18 ± 0.16	7.22 ± 0.33
		5	1:40	33.02 ± 0.32	14.18 ± 0.15
			1:60	35.49 ± 0.48	12.98 ± 0.05
	40		1:80	34.37 ± 0.16	13.64 ± 0.38
		10	1:40	35.37 ± 1.37	15.95 ± 0.13
			1:60	36.76 ± 0.49	15.34 ± 0.21
			1:80	34.71 ± 0.82	13.59 ± 0.02
		5	1:40	37.27 ± 1.21	15.66 ± 0.50
			1:60	36.76 ± 2.06	15.79 ± 0.12
MAE	60		1:80	51.51 ± 0.01	17.77 ± 0.36
		10	1:40	37.02 ± 0.49	16.60 ± 0.11
			1:60	41.93 ± 0.72	15.23 ± 0.19
			1:80	44.52 ± 1.31	16.77 ± 0.43
		5	1:40	45.71 ± 0.16	16.43 ± 0.62
			1:60	50.29 ± 1.46	17.82 ± 0.09
	80		1:80	48.57 ± 2.13	15.06 ± 0.26
		10	1:40	41.01 ± 0.97	15.26 ± 0.36
			1:60	45.13 ± 0.97	14.70 ± 0.34
			1:80	53.21 ± 0.49	15.06 ± 0.02
		5	1:40	35.37 ± 0.65	22.89 ± 0.38
			1:60	47.90 ± 0.67	27.20 ± 0.07
	50		1:80	51.18 ± 3.25	29.96 ± 0.17
		10	1:40	41.95 ± 0.69	25.40 ± 0.06
			1:60	42.23 ± 0.18	24.39 ± 0.37
			1:80	46.72 ± 4.01	24.97 ± 0.05
		5	1:40	39.91 ± 0.97	27.22 ± 0.44
			1:60	45.88 ± 1.94	29.90 ± 0.99
UAE	75		1:80	46.14 ± 0.33	27.86 ± 0.65
		10	1:40	44.68 ± 2.38	29.07 ± 0.66
			1:60	45.49 ± 2.54	29.54 ± 0.85
			1:80	44.83 ± 2.06	26.88 ± 0.72
		5	1:40	36.51 ± 0.32	21.15 ± 0.05
			1:60	39.09 ± 3.51	23.77 ± 0.25
	100		1:80	45.91 ± 0.66	25.24 ± 0.24
		10	1:40	34.37 ± 1.17	19.95 ± 0.04
			1:60	42.93 ± 0.30	25.15 ± 0.12
			1:80	43.87 ± 1.39	24.27 ± 0.07

TPC—total phenolic content; TMAC—total monomeric anthocyanin content; PLE—pressurized liquid extraction; MAE—microwave-assisted extraction; UAE—ultrasound-assisted extraction; SSR—sample to solvent ratio. Results are expressed as mean ± SD.

2.4.2. Microwave-Assisted Extraction (MAE)

MAE was carried out in an Ethos Easy microwave reactor (Milestone, Sorisole, Italy) according to the experimental design (Table 1). The extraction was performed using an acidified aqueous ethanol solution (1% formic acid in 50% ethanol, *v*/*v*) as an extraction solvent. The variable extraction parameters were temperature (40, 60 and 80 °C), irradiation time (5 and 10 min) and SSR (1:40, 1:60, 1:80 g/mL), while the constant extraction parameters were preheating time (2 min for 40 °C, 4 min for 60 °C, and 6 min for 80 °C min), ventilation and cooling after the extraction (2 min), stirring level (50%) and microwave power (400 W). Obtained extracts were filtered into a volumetric flask (25 mL), filled with the solvent, and stored at −18 °C until analysis.

2.4.3. Ultrasound-Assisted Extraction (UAE)

The UAE was performed using the ultrasonic processor UP200Ht (Dr. Hielscher GmbH, Teltow, Germany) with a frequency of 26 kHz and a maximum output power of 200 W equipped with a titanium ultrasound probe (154 mm^2). Extractions were performed according to the experimental design (Table 1), varying the sonication amplitude (50, 75 and 100%), time (5 and 10 min) and SSR (1:40, 1:60, 1:80 g/mL). Depending on the experimental conditions, 0.63, 0.42, or 0.31 g of sample was extracted with 20 mL of extraction solvent consisting of 1% formic acid in 50% ethanol (*v*/*v*). An ice-cooling bath was used to keep the temperature below 30 °C during extraction. The prepared extracts were filtered, quantitatively transferred to 25 mL volumetric flasks and stored at −18 °C until analysis.

2.4.4. Conventional Extraction Technique

Thermal reflux was used as the conventional extraction technique (CT) [7]. The freeze-dried sample (0.5 g) was mixed with the extraction solvent (40 mL; 1% formic acid in 50% ethanol, *v*/*v*) in a round bottom flask and heated for 30 min on a reflux condenser. The resulting extract was filtered, made up to 50 mL and stored at −18 °C until analysis.

2.5. Determination of the Total Phenolic Content

The total polyphenol content (TPC) was measured using the method described by Repajić et al. [13]. The procedure was performed as follows: 100 µL of the extract (or solvent as blank) was mixed with 2 mL of distilled water and 200 µL of Folin–Ciocalteu reagent. After a 3 min incubation, 1 mL of a 20% (*w*/*v*) sodium carbonate solution was added. The mixture was then heated to 50 °C/25 min. The absorbance was measured at 765 nm using a VWR UV-1600PC spectrophotometer (VWR, Wayne, PA, USA) with a 1 cm path-length cuvette. Gallic acid was used as the calibration standard, with a concentration range of 50–500 mg/L ($y = 0.0035x$, $R^2 = 0.9997$). TPC was expressed as mg of gallic acid equivalents (GAE)/g dry weight (dw) of the initial BP used for extraction.

2.6. Determination of the Total Monomeric Anthocyanin Content

The total monomeric anthocyanin content (TMAC) was determined using a method adapted from Lee et al. [14]. The procedure was carried out as follows: the first test tube contained 1 mL of the extract and 4 mL of buffer at pH of 1.0, the second test tube contained 1 mL of the extract and 4 mL of buffer at pH 4.5. After incubating the samples at room temperature for 20 min, the absorbance was measured at 520 and 700 nm. The corresponding buffers were used as blanks for each sample. The anthocyanin content was then expressed in mg of cyanidin-3-glucoside equivalents (C3GE)/g dw.

2.7. UPLC–ESI–MS² Analysis

The profile of individual polyphenols in the BP extracts was characterized using UPLC–MS². Measurements were conducted on an Agilent 1290 RRLC system coupled with a 6430 triple quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) equipped with an ESI source, following the procedure described by Elez Garofulić et al. [8,15]. Electrospray ionization was performed in both positive and negative ion modes (*m*/*z* 100–1000), with high-purity nitrogen as the nebulizing and collision gas. The operating parameters included capillary voltages of 4000 V (positive) and 3500 V (negative), a drying gas temperature of 300 °C, a gas flow rate of 11 L/h, and a nebulizer pressure of 40 psi. Separation of compounds was achieved using a Zorbax Eclipse Plus C18 column (Agilent, 100 × 2.1 mm; 1.8 µm particle size) maintained at 35 °C, with an injection volume of 2.5 µL, a flow rate of 0.3 mL/min, and a binary mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Data acquisition and processing were performed with Agilent MassHunter software (version B.04.01), while additional validation and quality parameters are provided in the referenced studies [8,15].

Identification and quantification of polyphenols were carried out using available authentic standards or, where not commercially accessible, by evaluating precursor/product ion transitions and comparing mass fragmentation data with literature reports. Among phenolic acids, gallic, caffeic, protocatechuic, and ellagic acids were quantified using its authentic standards, whereas *p*-hydroxybenzoic acid was determined using gallic acid calibration. For anthocyanins, cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-rutinoside, and cyanidin-3-*O*-glucoside were quantified against cyanidin-3-glucoside chloride calibration, while delphinidin-3-*O*-glucoside and delphinidin-3-*O*-rutinoside were quantified against delphinidin-3-glucoside chloride calibration. Flavonols including myricetin-3-*O*-arabinoside, myricetin hexoside, and myricetin were quantified using myricetin; quercetin, rutin, and isorhamnetin rutinoside were quantified using quercetin-3-β-D-glucopiranoside calibration, while kaempferol was quantified using kaempferol-3-rutinoside. Finally, catechin was quantified using its authentic standard, and epicatechin was determined against catechin calibration. The results were expressed as mg/g dw.

2.8. Antioxidant Capacity Determination

The antioxidant capacity of the obtained extract was evaluated using FRAP, DPPH, ABTS, and ORAC methods. For the FRAP, DPPH, and ABTS methods, specific reagents were applied, and the resulting colorimetric changes were measured against blank controls. The FRAP assay was conducted following the protocol by Benzie and Strain [16]. A mixture was prepared containing 240 µL of distilled water, 80 µL of the extract, and 2080 µL of FRAP reagent (comprising 20 mM $FeCl_3 \times 6H_2O$, 0.01 M TPTZ in 0.04 M HCl, and 0.3 M sodium acetate buffer (pH 3.6) in a 1:1:10 volume ratio). After 5 min incubation, absorbance was measured (593 nm). For the DPPH assay, as described by Braca et al. [17], 0.75 mL of the extract was combined with 1.5 mL of 0.2 mM DPPH solution. The mixture was kept in the dark for 20 min before the absorbance was recorded (517 nm). In the ABTS assay [18], 40 µL of the sample was added to 4 mL of a 1% $ABTS^{+\bullet}$ solution. After 1 min, absorbance was determined (734 nm). The ORAC assay was performed using a 96-well microplate format on an automated plate reader (BMG LABTECH, Offenburg, Germany), with data processed using MARS 2.0 software. Diluted samples were added to wells containing a fluorescein solution (70.3 nM) and incubated at 37 °C/30 min. Following three baseline readings, 240 mM AAPH was injected to generate peroxyl radicals. A range of Trolox concentrations (3.12–103.99 µM) was used as a standard on each plate. Fluorescence was measured every 90 s for a total 120 min duration (excitation at 485 nm, emission at 528 nm). All the results for all used methods were expressed as µmol of Trolox equivalents (TE)/g dw.

2.9. Zebrafish Embryotoxicity Test (ZET)

Adult *Danio rerio* (wild-type WIK strain) were obtained from the European Zebrafish Resource Center, Karlsruhe Institute of Technology, Karlsruhe, Germany and maintained under standard laboratory conditions [19]. Animal housing and spawning were carried out in aquaria units approved by the Croatian Ministry of Agriculture (HR-POK-023). All experiments in this study were performed on non-protected embryonic stages (up to 120 hpf), which do not require approval from animal welfare committees [20]. Embryotoxicity was assessed using the zebrafish embryotoxicity test [21]. In dose range-finding experiments, embryos (N = 10) were exposed to a wide range of sample concentrations in serial dilutions (4.68–0.02 mg/mL). Once a relevant range of concentration was identified, a refined concentration range (0.1–0.7 mg/mL) was tested using 30 embryos per concentration. The E3 medium was used as a negative control. Embryos were incubated under controlled conditions (27 ± 1 °C, 12/12 h light/dark photoperiod) using an Innova 42 incubator shaker (New Brunswick, Canada). At 120 h post-fertilization, survival and developmental abnormalities were recorded using an Olympus CKX41 inverted microscope equipped with LAS EZ 3.2.0 software and a Leica EC3 digital camera, and results were expressed as the percentage of mortality and incidence of developmental defects relative to the control group.

2.10. Cellular Response to BP Extract Treatment

In vitro biological activity was investigated using tongue epithelial carcinoma cells (CAL 27; ATCC CRL-2095) and human hepatocellular carcinoma cells (HepG2; ATCC HB-8065). The BP extract was evaporated under vacuum (Vacuum concentrator SpeedVac Concentrator SPD2010-230, Thermo Fisher Scientific, USA; 50 °C) until dry, followed by freezing at −80 °C/24 h. The concentrated extract was then freeze-dried at −55 °C/48 h. The final working concentrations used were 0.1, 0.2, 0.3, 0.38, 0.48, 0.5 and 0.7 mg/mL, with 0.3 mg/mL corresponding to the recommended daily polyphenol intake for an average adult (70 kg). Both cell lines were treated for 24 h. Absorbance and fluorescence readings were taken using a FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA).

2.10.1. Cell Lines

Both human cell lines were maintained in Ham's F-12 medium supplemented with L-glutamine and 10% FBS at 37 °C in 5% CO_2. The CAL 27 and HepG2 cell lines were sourced from the Ruđer Bošković Institute (Zagreb, Croatia) and the Institute for Medical Research and Occupational Health (Zagreb, Croatia), respectively.

2.10.2. Metabolic Activity and Cell Survival

MTT Assay

The viability of the cells was determined using the MTT assay, which assesses metabolic activity via the oxidoreductase enzyme pathway [22]. After treatment, 100 µL of 0.5 mg/mL MTT reagent was added to each well and incubated (4 h). The generated formazan was solubilized by adding 100 µL of 10% SDS and incubating overnight in the dark. Absorbance was measured (595 nm), and cell survival was calculated as a percentage of the control.

Clonogenic Assay

Cell survival was evaluated using the clonogenic assay, a technique based on the ability of individual cells to proliferate and form macroscopic colonies [23]. Briefly, 50 CAL 27 and HepG2 cells were seeded per well in a 24-well plate and cultured in standard growth medium. Following 24 h incubation, cells were treated with the specified BP extract concentrations. After a 7-day incubation period in a fresh medium, the cells were fixed using a methanol:glacial acetic acid mixture (3:1) and stained with 0.1% crystal violet

solution prepared in 25% methanol. Plating Efficiency (PE) and Survival Fraction (SF) were calculated by counting the colonies in the control and treated samples.

DCFH-DA Assay

The generation of reactive oxygen species (ROS) in the cell lines was quantified using the 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) fluorometric assay [24]. After treatment, cells were washed with phosphate-buffered saline (PBS) and incubated with 100 µL of DCFH-DA solution for 30 min. Fluorescence intensity was measured at excitation 485 nm and emission 520 nm. As a positive control, cell treatment was performed with 10 mM H_2O_2 for 15 min [25]. The results are presented as the relative change in fluorescence intensity compared to the control.

Thiobarbituric Acid-Reactive Substances (TBARS) Assay

Lipid peroxidation was assessed using the thiobarbituric acid-reactive substances (TBARS) assay, following Koç and Karayiğit [26], with minor modifications. After treatment, cells were washed twice with ice-cold PBS, resuspended in 1.15% KCl, and lysed at −80 °C/30 min. TBARS formation was induced by adding 20% TCA and heating at 85 °C/20 min. After cooling at −20 °C/20 min, 0.67% TBA was added, and samples were incubated again at 85 °C/20 min. Absorbance was measured at 532 and 600 nm. A calibration curve was prepared with TMP (precursor of malondialdehyde, MDA) standards (0–4 µM). Results were expressed as µM MDA corrected for cell viability, and presented as a percentage relative to the negative control. A 200 µM H_2O_2 treatment for 2 h served as a positive control.

2.10.3. DNA Damage

Comet Assay

The Comet assay was used to detect oxidative DNA damage and corresponding repair mechanisms [27]. Following treatment, cells were prepared as a single-cell suspension (10^5 cells/mL), centrifuged (1000× *g*, at 4 °C/5 min), and resuspended in 100 µL of 0.5% LMP. This suspension was layered onto pre-coated microscope slides (Surgipath, Richmond, IL, USA; 1.5% NMP), solidified on ice, and covered with a final layer of 0.5% agarose gel. The slides were submerged at 4 °C/1 h in a lysis solution (100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Tris, enhanced with 1% Triton X-100). Following lysis, slides were washed with distilled water and subjected to electrophoresis (25 V for 20 min) in a cold denaturation and electrophoresis buffer (10 mM NaOH, 200 mM Na_2EDTA, pH 13). After neutralization with Tris/HCl (pH 7.5), the DNA was stained for 10 min with a 3× concentration of GelRed® nucleic acid stain. For each experimental condition, two parallel slides were analyzed per independent experiment, with 50 comets scored per slide (100 comets per experiment). Comets were scored using a fluorescence microscope (Olympus BX51, Tokyo, Japan; 200× magnification) and an image analysis system (Comet Assay IV, Perceptive Instruments Ltd., Instem, UK). The median of each comet parameter was calculated for each slide, and the two slide-level values were combined to obtain one representative value per independent experiment. Three independent experiments were performed and considered biological replicates, with the independent experiment serving as the statistical unit. A 50 µM H_2O_2 treatment for 5 min served as a positive control, whereas untreated cells served as negative control. DNA damage was quantified using three key markers of genotoxicity [28]: tail length (µm), tail intensity (% DNA in tail), and tail moment (tail length × % DNA in tail). Data normalization was achieved by applying a natural logarithm (ln) transformation prior to statistical analysis [29].

2.11. Statistical Analysis

Statistical analysis was performed using Statistica software ver. 10.0 (Statsoft Inc., Tulsa, OK, USA). In the first experimental phase, a mixed-level full factorial design (including 2- and 3-level factors) was employed to determine optimal parameters of PLE, MAE, and UAE. The independent variables were extraction temperature or amplitude, duration, and the SSR, while the dependent variables included TPC and TMAC. For each experimental condition, a single extraction was performed using the respective extraction technique, and the resulting extracts were analyzed in triplicate for TPC and TMAC. Accordingly, the results are expressed as mean values ± standard error (SE), reflecting the variability among the analytical measurements. Normality and homogeneity of variance for the data and residuals were verified by Shapiro–Wilk and Levene's tests, respectively. Statistical significance was further assessed either via analysis of variance (ANOVA) followed by Tukey's HSD post hoc test, or, where applicable, by the non-parametric Kruskal–Wallis test, with subsequent multiple comparisons of mean ranks. Differences in individual polyphenolic profiles and antioxidant activities between optimized PLE, MAE and UAE extracts were analyzed using one-way ANOVA with Tukey's HSD test. In the second phase of the study, the efficiency of PLE, MAE, UAE, and reflux extraction, as well as the effect of CG duration, were evaluated using a 4- (extraction techniques) and 5-level (CG time) single-factor design with the same dependent variables as in the first phase. The effects on polyphenol and anthocyanin yields and antioxidant capacity (FRAP, DPPH, ABTS, ORAC) were analyzed following the statistical procedures described previously and results are expressed as mean ± standard deviation (SD). UPLC analyses and antioxidant capacity assays were performed in triplicate using the respective extracts. Statistical results for these measurements are reported as mean values ± SD. The relationships between the determined polyphenols and the antioxidant capacity were tested by calculating the Pearson's correlation coefficients. All tests were considered significant at $p \leq 0.05$. In the third phase, the normality of the data distribution for cellular responses to BP extract treatment was assessed by Kolmogorov–Smirnov test. Since most data were normally distributed, one-way ANOVA followed by Tukey's HSD post hoc test was applied to evaluate statistical differences. For the Comet assay results, which did not meet the assumptions for parametric testing, the non-parametric Kruskal–Wallis test was used, followed by Dunn's post hoc test for pairwise comparisons between treatment groups. All treatments in this phase were performed in at least four replicates, and the entire experiment was repeated three times to ensure reproducibility. Concentration–response curves for zebrafish mortality and developmental abnormalities were fitted using nonlinear regression with a variable-slope four-parameter logistic model in GraphPad Prism 6.01 (GraphPad Software, San Diego, CA, USA). The median lethal concentration (LC_{50}) and median effective concentration (EC_{50}), together with their corresponding 95% confidence intervals (CI), were estimated from the fitted curves. Results were expressed as mean ± SD. The significance in all statistical tests was set at $p \leq 0.05$.

3. Results and Discussion

3.1. TPC and TMAC in BP Extracts Obtained by PLE, MAE and UAE

The TPC ranged from 19.04 to 52.57 mg GAE/g dw for PLE, from 33.02 to 53.21 mg GAE/g dw for MAE, and from 34.37 to 51.18 mg GAE/g dw for UAE, while TMAC values ranged from 7.22 to 15.21 mg C3GE/g dw in PLE extracts, from 12.98 to 17.82 mg C3GE/g dw in MAE extracts, and from 19.95 to 29.96 mg C3GE/g dw in UAE extracts (Table 1).

Although the phenolic content may be influenced by several factors such as the applied extraction technique and sample characteristics, the findings in this study are within the range of previously reported values. Blejan et al. [1] reported a TPC of 26.2 mg GAE/g

dw in BP extracted using methanol in an ultrasonic bath, along with a TMAC of 3.68 mg/g dw, which was lower than the values obtained in this study; however, the comparison should be interpreted with caution because different extraction solvents and procedures were employed. Sójka and Król [30] observed slightly lower TPC (18.55–22.41 mg GAE/g dw) in seedless BP extracted by UAE, as well as TMAC ranging from 3.44 to 10.46 mg/g dw depending on the harvest year. These values were lower than those reported in this study. In contrast, Gagneten et al. [31] reported higher TPC (37.5 mg GAE/g dw) in BP using conventional solvent extraction with acidified ethanol, highlighting the strong influence of extraction and processing conditions. Overall, these variations are consistent with previous studies [1,32], indicating that anthocyanin and other secondary metabolite contents in BP may vary depending on cultivar, environmental conditions, agronomic practices, juice extraction procedures, and drying methods. As shown in Table 2, each extraction technique demonstrated distinct effects on TPC and TMAC depending on applied extraction parameters.

PLE implementation revealed temperature as statistically significant factor affecting both TPC and TMAC. While an increase in temperature positively influenced TPC, lower TMAC values were observed at the highest temperature. Furthermore, extraction time significantly affected TMAC, while the SSR did not significantly affect either TPC or TMAC. PLE was performed at relatively high temperatures (up to 125 °C), which may have enhanced the extraction of non-anthocyanin phenolics through improved solvent diffusivity and matrix disruption. However, these elevated temperatures may also have contributed to the lower TMAC observed at higher temperatures, considering the thermal sensitivity of anthocyanins. Similar effects have been reported for chokeberry pomace under elevated-temperature extraction conditions [7]. Considering the recovery of both response variables, the optimal PLE process parameters were selected as 100 °C, 5 min, and SSR 1:40 g/mL. Under these conditions, the extract exhibited a TPC of 37.17 mg GAE/g dw and a TMAC of 15.21 mg C3GE/g dw, providing a balance between the recovery of phenolic compounds and anthocyanins. In the case of MAE, temperature was the only statistically significant parameter affecting both TPC and TMAC, while irradiation time and SSR did not significantly influence either response. Previous studies have demonstrated that increasing extraction temperature can improve extraction efficiency by enhancing solubility and mass transfer. However, the effect of temperature may also depend on extraction duration [8]. It is well established that in MAE, prolonged extraction times, especially those exceeding 10 min, can cause significant degradation of both TPC and TMAC. Supporting this, Elez Garofulić et al. [8] reported that the highest extraction yield from black chokeberry pomace was obtained with a short irradiation time of only 4 min, which supports the use of a short extraction time in the present study. Therefore, 60 °C, 5 min irradiation time, and SSR 1:40 g/mL were selected as the optimal MAE conditions, under which the extract exhibited a TPC of 37.27 mg GAE/g dw and a TMAC of 15.66 mg C3GE/g dw, providing a high recovery of both response variables under the investigated conditions. Regarding UAE, increasing amplitude from 50 to 75% significantly enhanced TMAC, whereas its effect on TPC was not statistically significant ($p = 0.054$). The SSR significantly affected TPC, while extraction time did not significantly affect either response. However, increasing amplitude to 100% resulted in numerically lower TPC and TMAC values than those observed at 75% amplitude; however, the effect of amplitude on TPC was not statistically significant. The lower values observed at the highest amplitude may indicate that excessive ultrasonic intensity does not necessarily improve compound recovery. This may be related to the greater cavitation intensity generated at higher amplitudes, which can enhance cell disruption but may also promote degradation of sensitive compounds under certain conditions. The UAE conditions (75% amplitude, 5 min, and a SSR of 1:60 g/mL) were selected based on a combined evaluation of TPC and TMAC. An

amplitude of 75% was selected because it provided significantly higher TMAC than the 50 and 100% amplitude levels, whereas the effect of amplitude on TPC was not statistically significant ($p = 0.054$). Although the 1:80 g/mL ratio yielded a higher mean TPC, its effect on TMAC was not significantly different from that of the 1:60 g/mL ratio ($p = 0.070$). Therefore, the 1:60 g/mL ratio was selected to reduce solvent consumption while maintaining a high TMAC yield, and the shorter extraction time (5 min) was preferred because extraction time had no significant effect on either response. Thus, the selected conditions represent a practical compromise between TPC and TMAC recovery and solvent consumption, rather than the conditions yielding the highest numerical values for both responses. This observation aligns with findings by Mane et al. [33], who reported that higher amplitudes combined with prolonged extraction times can lead to lower recovery of polyphenols and anthocyanins. UAE conditions of 75% amplitude for 5 min with SSR 1:60 g/mL were selected as optimal, under which the extract exhibited TPC of 45.88 mg GAE/g dw and TMAC of 29.90 mg C3GE/g dw. Under the investigated conditions, these parameters provided a relatively high recovery of both response variables.

Table 2. Effects of extraction parameters on total phenolic content (TPC) and total monomeric anthocyanin content (TMAC) of blackcurrant pomace extracts obtained by PLE, MAE, and UAE.

PLE			MAE			UAE		
Source of Variation	**TPC (mg GAE/g dw)**	**TMAC (mg C3GE/g dw)**	**Source of Variation**	**TPC (mg GAE/g dw)**	**TMAC (mg C3GE/g dw)**	**Source of Variation**	**TPC (mg GAE/g dw)**	**TMAC (mg C3GE/g dw)**
Temperature (°C)	$p < 0.001$ *	$p < 0.001$ *	**Temperature (°C)**	$p < 0.001$ *	$p < 0.001$ *	**Amplitude (%)**	$p = 0.054$	$p < 0.001$ *
75	21.75 ± 0.53 [a]	12.77 ± 0.29 [b]	40	34.95 ± 0.38 [a]	14.28 ± 0.32 [a]	50	44.23 ± 1.60 [a]	25.80 ± 0.68 [a]
100	37.46 ± 0.86 [b]	12.52 ± 0.58 [b]	60	41.50 ± 1.62 [b]	16.30 ± 0.26 [b]	75	44.49 ± 0.75 [a]	28.41 ± 0.38 [b]
125	45.51 ± 1.19 [c]	9.23 ± 0.45 [a]	80	47.32 ± 1.21 [b]	15.72 ± 0.33 [b]	100	40.45 ± 1.29 [a]	23.25 ± 0.61 [a]
Time (min)	$p = 0.531$	$p < 0.001$ *	**Time (min)**	$p = 0.901$	$p = 0.842$	**Time (min)**	$p = 0.950$	$p = 0.525$
5	33.82 ± 2.18 [a]	12.47 ± 0.46 [b]	5	41.44 ± 1.72 [a]	15.48 ± 0.39 [a]	5	43.10 ± 1.30 [a]	26.13 ± 0.71 [a]
10	36.00 ± 2.76 [a]	10.54 ± 0.50 [a]	10	41.07 ± 1.37 [a]	15.39 ± 0.23 [a]	10	43.01 ± 0.89 [a]	25.52 ± 0.66 [a]
SSR (g/mL)	$p = 0.723$	$p = 0.591$	**SSR (g/mL)**	$p = 0.181$	$p = 0.752$	**SSR (g/mL)**	$p < 0.001$ *	$p = 0.070$
1:40	34.93 ± 3.37 [a]	11.33 ± 0.64 [a]	1:40	38.23 ± 1.25 [a]	15.68 ± 0.26 [a]	1:40	38.80 ± 1.15 [a]	24.28 ± 0.92 [a]
1:60	34.68 ± 3.18 [a]	11.37 ± 0.58 [a]	1:60	41.06 ± 1.63 [a]	15.31 ± 0.43 [a]	1:60	43.92 ± 0.96 [b]	26.66 ± 0.74 [a]
1:80	35.12 ± 2.73 [a]	11.82 ± 0.75 [a]	1:80	44.48 ± 2.28 [a]	15.31 ± 0.47 [a]	1:80	46.44 ± 0.86 [b]	26.53 ± 0.60 [a]

TPC—total phenolic content; TMAC—total monomeric anthocyanin content; PLE—pressurized liquid extraction; MAE—microwave-assisted extraction; UAE—ultrasound-assisted extraction; SSR—sample to solvent ratio. Results are expressed as mean ± SE. * $p \leq 0.05$. Means with the same letter within the column are not significantly different at $p \leq 0.05$.

3.2. Comparison of Extraction Effectiveness

After establishing the optimal extraction conditions for PLE, MAE, and UAE, a comparative evaluation of their extraction efficiency was conducted, including a comparison with conventional reflux technique. The results obtained (Table 3) revealed significant differences in the recovery of TPC and TMAC among the applied extraction techniques.

Table 3. Effectiveness of conventional and advanced extraction techniques (PLE, MAE, and UAE) in recovering TPC and TMAC under the selected extraction conditions.

Extraction Technique and Conditions	TPC (mg GAE/g dw)	TMAC (mg C3GE/g dw)
	$p = 0.002$ *	$p < 0.001$ *
PLE (100 °C/5 min/1:40 g/mL)	37.17 ± 0.65 [a]	15.21 ± 0.04 [a]
MAE (60 °C/5 min /1:40 g/mL)	37.27 ± 1.21 [a]	15.66 ± 0.50 [a]
UAE (75% amplitude/5 min/1:60 g/mL)	45.88 ± 1.37 [b]	29.90 ± 0.70 [c]
CT (30 min/1:100 g/mL)	53.01 ± 1.80 [c]	24.17 ± 0.50 [b]

TPC—total phenolic content; TMAC—total monomeric anthocyanin content; PLE—pressurized liquid extraction; MAE—microwave-assisted extraction; UAE—ultrasound-assisted extraction; CT—conventional technique. Results are expressed as mean ± SD. * $p \leq 0.05$. Means with the same letter within the column are not significantly different at $p \leq 0.05$.

The highest TPC was obtained by the CT (53.01 mg GAE/g dw), followed by UAE (45.88 mg GAE/g dw), MAE (37.27 mg GAE/g dw), and PLE (37.17 mg GAE/g dw). These results suggest that prolonged extraction time, as applied during CT (30 min), may enhance the solubilization of phenolic compounds through improved solvent penetration and diffusion. Despite the intensified thermal and pressure conditions applied in PLE, this technique resulted in the lowest TPC. This outcome may be attributable to thermal degradation of heat-sensitive phenolic compounds or limited matrix disruption within the applied extraction time. In contrast, UAE demonstrated the highest TMAC (29.90 mg C3GE/g dw), significantly outperforming CT (24.17 mg C3GE/g dw), MAE (15.66 mg C3GE/g dw), and PLE (15.21 mg C3GE/g dw). It should be noted that the TMAC/TPC ratio should not be interpreted as the actual proportion of anthocyanins in the total phenolic content, since TPC and TMAC are expressed using different reference standards (GAE and C3GE, respectively). The higher numerical ratio observed for UAE therefore reflects relatively greater recovery of anthocyanins in relation to total phenolic compounds under these extraction conditions. The superior performance of UAE in anthocyanin recovery is attributed to the cavitation effect of ultrasonic waves, which enhances cell wall disruption and facilitates the release of intracellular compounds. Moreover, the moderate operating temperature, which was maintained constant throughout UAE despite inherent ultrasonic induced heating, helps preserve thermolabile compounds such as anthocyanins, which are susceptible to degradation under prolonged heating. While CT yielded the highest TPC, it was less effective in retaining anthocyanins compared to UAE. This difference may reflect the combined effects of extraction efficiency and the thermal sensitivity of anthocyanins under the applied conditions [34]. Similar observations have been reported for the blueberry, raspberry, blackcurrant, and chokeberry pomace, where UAE and MAE showed favorable performance for phenolic compound recovery compared with conventional techniques [35]. MAE demonstrated high TPC recovery comparable to CT but was less effective in preserving TMAC. This may be related to the thermal conditions generated during microwave treatment, although the contribution of thermal degradation was not directly evaluated in the present study. Microwave energy can enhance solvent penetration and cell disruption; however, the resulting thermal effects may influence the recovery of heat-sensitive compounds such as anthocyanins.

Overall, these findings highlight the different performance characteristics of both conventional and advanced extraction approaches. CT resulted in the highest TPC under the investigated conditions, whereas UAE provided the highest TMAC and a relatively high TPC. These results indicate that UAE may offer a suitable balance between the recovery of phenolic compounds and anthocyanins under the investigated conditions. Under optimized conditions, UAE was therefore selected for further investigation, including its combination with CG pretreatment.

Effects of Cryogrinding

Further analyses included evaluation of CG as a pretreatment to enhance the extraction of BP bioactive compounds. To our best knowledge, this is the first study investigating the effect of CG combined with UAE on the recovery of polyphenols and anthocyanins from BP. The cryoground BP was subjected to UAE under previously optimized conditions (75%/5 min/1:60 g/mL) and the resulting TPC and TMAC values were then compared to those obtained from conventionally ground control sample (0 min). Prior to UAE, the particle size of freeze-dried BP samples was evaluated to assess the effect of CG. The median particle diameter d(0.5) ranged from 50.22 to 275.00 μm, with the highest value observed in the conventionally ground sample and progressively lower values obtained with increasing CG duration (Table 4).

Table 4. Effect of cryogrinding (CG) pretreatment on median particle diameter, total phenolic content (TPC), and total monomeric anthocyanin content (TMAC) of blackcurrant pomace extracts obtained by UAE.

Cryogrinding Time (min)	d(0.5) (μm)	TPC (mg GAE/g dw)	TMAC (mg C3GE/g dw)
	$p < 0.001$ *	$p < 0.001$ *	$p < 0.001$ *
0	275.00 ± 2.56 ^{e}	45.88 ± 1.37 ^{c,d,e}	29.90 ± 0.70 ^{c}
1	247.45 ± 2.53 ^{d}	42.47 ± 1.49 ^{a,b,c,d}	17.91 ± 0.51 ^{a}
2	129.28 ± 0.74 ^{c}	50.16 ± 0.84 ^{e}	33.15 ± 0.26 ^{d}
3	78.32 ± 0.42 ^{b}	43.64 ± 1.78 ^{b,c,d,e}	22.07 ± 0.62 ^{b}
4	74.61 ± 0.48 ^{b}	35.99 ± 0.13 ^{a}	18.71 ± 0.43 ^{a}
5	50.22 ± 1.90 ^{a}	38.15 ± 1.91 ^{a,b}	19.38 ± 0.46 ^{a,b}

TPC—total phenolic content; TMAC—total monomeric anthocyanin content; d(0.5)—median particle diameter. Results are expressed as mean ± SD. * $p \leq 0.05$. Means with the same letter within the column are not significantly different at $p \leq 0.05$.

All CG-treated samples showed significantly reduced d(0.5) values compared to conventional grinding. The most pronounced reduction occurred within the first minutes of CG, while further decreases at longer grinding times were less pronounced, indicating diminishing efficiency of prolonged cryogenic treatment. Marčac et al. [36] reported that the d(0.5) of conventionally ground fennel seeds was 658 μm, while 7 min CG reduced it by more than half (301 μm). With each additional 2 min CG, particle size further decreased by approximately 15–30%. The application of CG prior UAE under optimal conditions resulted in TPC ranging from 35.99 to 50.16 mg GAE/g dw and TMAC values ranging from 17.91 to 33.15 mg C3GE/g dw (Table 3). The highest TPC (50.16 mg GAE/g dw) was obtained after 2 min CG, whereas the lowest value (35.99 mg GAE/g dw) was recorded following 4 min of treatment. A similar trend was observed for TMAC, with the maximum content (33.15 mg C3GE/g dw) also detected at 2 min CG, while the lowest value (17.91 mg C3GE/g dw) was observed after 1 min. Although the overall effect of CG duration on TPC was statistically significant ($p < 0.001$), the TPC values obtained after 0, 1, 2, and 3 min CG were not significantly different from each other. In contrast, TMAC showed a clearer

improvement following CG, with the highest value observed after 2 min CG. Although particle size decreased progressively with increasing CG duration, this reduction did not translate into a consistent increase in TPC and TMAC. Instead, both responses reached their highest measured values after 2 min of CG, indicating that the most favorable extraction performance was achieved at an intermediate grinding duration rather than at the smallest particle size. Thus, 2 min CG was selected as the pretreatment duration for subsequent experiments, based on the recovery of both TPC and TMAC. Extending the grinding time beyond 2 min resulted in lower TPC and TMAC values, although the underlying mechanisms were not directly investigated in the present study. These findings are in line with previous reports; for instance, Repajić et al. [7] observed a maximum TPC of 115.1 mg GAE/g dw after 1 min CG, corresponding to a 17.6% increase over conventional grinding (94.8 mg GAE/g dw). In the study by E.J et al. [37], the CG application (20 min) led to an increase in both TPC and TMAC in mango peel powder, compared to conventional grinding. TPC increased from 180 to 204.71 mg GAE/g dw, while TMAC rose from 81.00 to 90.21 mg C3GE/g dw. The differences among studies may be related to differences in matrix characteristics, grinding conditions, and extraction procedures. In the study by Cvitković et al. [38], the TPC in myrtle leaves steadily increased with longer CG times, showing a 22% rise after 9 min compared to the conventionally ground sample. These findings suggest that the relationship between grinding duration and extraction yield may depend on the characteristics of the plant matrix and the extraction conditions. In the present study, the continued reduction in particle size beyond 2 min of CG was not accompanied by a further increase in TPC or TMAC. Grinding time had a statistically significant impact on all compounds, confirming that reducing particle size, and thereby increasing surface area for solvent contact, enhances bioactive molecule extraction [38]. In contrast, the lack of consistent improvement in this study beyond 2 min CG may be attributed to differences in the physicochemical properties of BP and the applied extraction protocol. Its softer and more delicate matrix may not benefit from prolonged cryogenic treatment to the same extent as more rigid plant materials. Although the improvement obtained with CG may not fully justify the additional processing cost, the observed increase in TMAC and the highest measured values of both TPC and TMAC at 2 min CG indicate that CG may improve extraction performance under the investigated conditions.

3.3. Comparative Profiling of BP Extracts Obtained by Optimized PLE, MAE, UAE, CT, and CG + UAE

Table 5 presents a detailed overview of the individual polyphenolic compounds identified in BP extracts obtained by PLE, MAE UAE, CT, and CG + UAE, analyzed using UPLC–ESI–MS^2. The findings show that the extraction technique influenced both the yield and the composition of the recovered polyphenols. In total, 19 compounds were detected, comprising 5 phenolic acids, 7 flavonols, 5 anthocyanins and 2 flavan-3-ols. Their fragmentation patterns have been described previously by [12,13,15]. Among the tested techniques, CT provided the highest overall content of identified polyphenols (20.98 mg/g dw), followed closely by CG + UAE (20.04 mg/g dw), and UAE alone (17.90 mg/g dw). In contrast, MAE and PLE yielded markedly lower total polyphenol contents (12.42 and 7.35 mg/g dw), under the applied conditions. Anthocyanins represented the most abundant class of polyphenolic compounds in all examined extracts. Among individual compounds, delphinidin-3-*O*-rutinoside and delphinidin-3-*O*-glucoside were predominant, with peak values found in CT and CG + UAE. Brennan et al. [39] reported delphinidin-3-rutinoside (25.59 mg/g) as the dominant anthocyanin in freeze-dried blackcurrant extracts, alongside delphinidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3-glucoside. In

CG + UAE extract examined in this study, the same anthocyanin compounds were identified, consistent with previous reports that delphinidin- and cyanidin-based glycosides are major anthocyanins in blackcurrant.

Differences in absolute concentrations are difficult to compare directly due to variations in sample preparation, extraction parameters, cultivar, and growing conditions, although both studies consistently highlight delphinidin- and cyanidin-based glycosides as the predominant anthocyanins. Although CT exhibited slightly superior results, CG + UAE showed statistically comparable yields for many anthocyanins, indicating comparable recovery of these compounds under the investigated conditions. The improved performance of CG + UAE may be related to the matrix disruption induced by CG, which increased the accessibility of intracellular compounds during subsequent UAE. Among flavonols, rutin was the most abundant compound, with maximum concentrations found in CT (5.69 mg/g dw), followed closely by CG + UAE. Rutin was detected at 5.34 mg/g dw in CG + UAE extracts, indicating its substantial presence in BP, while differences in extraction and sample preparation can influence levels reported in the literature [40]. Among phenolic acids, ellagic acid was dominant across all extraction techniques, with the highest concentration observed in CT (0.296 mg/g dw). Notably, *p*-hydroxybenzoic acid was detected exclusively in the CG + UAE extract (0.117 mg/g dw), although the reason for its exclusive detection under this condition cannot be established from the present data. CT yielded the highest total phenolic acid content (0.613 mg/g dw), but CG + UAE also demonstrated relatively high recovery (0.509 mg/g dw) under the investigated conditions. Similar to the results in this study, Untea et al. [40] reported the presence of gallic acid (0.113 mg/g) and ellagic acid (0.041 mg/g) among individual phenolic acids in BP. They also reported presence of hydroxycinnamic acids (0.123 mg/g), with ferulic acid (0.050 mg/g) and caffeic acid (0.023 mg/g) as the main constituents. Furthermore, the highest total flavonol concentration was obtained by CT (8.36 mg/g dw), followed by CG + UAE (7.91 mg/g dw), and UAE alone (7.10 mg/g dw). Total flavan-3-ol content followed a similar trend: CT>CG + UAE>UAE. The relatively high recovery obtained with CG + UAE indicates that this approach may facilitate the recovery of several polyphenol classes under the investigated conditions. Although CT resulted in higher yields for several polyphenol classes, differences among extraction techniques should be interpreted in the context of their distinct extraction conditions. These results are in line with previous studies suggesting that CG can enhance phenolic recovery by improving the effectiveness of subsequent extraction processes [7], particularly UAE. The main difference in the identified compounds between this study and that of Blejan et al. [1] may be related, at least in part, to differences in extraction conditions. While this study highlighted rutin, delphinidin-3-*O*-rutinoside, and delphinidin-3-*O*-glucoside as dominant, Blejan et al. [1], using ultrasonic bath extraction for 1 h with methanol, found catechin (12.53 mg/g dw), quercetin-3-β-D-glucoside (9.25 mg/g dw), and chlorogenic acid (4.29 mg/g dw) to be the main constituents. The differences in anthocyanin abundance may reflect the different extraction conditions [34]; however, anthocyanin degradation was not directly evaluated in either study.

Table 5. Individual polyphenols identified in blackcurrant pomace extracts obtained under the optimized conditions of PLE, MAE, UAE, CT, and CG + UAE.

Individual Polyphenols	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	PLE (mg/g dw)	MAE (mg/g dw)	UAE (mg/g dw)	CT (mg/g dw)	CG + UAE (mg/g dw)
Anthocyanins							
Cyanidin-3-*O*-arabinoside	419	287	0.010 ± 0.000 ^{a}	0.014 ± 0.001 ^{b}	0.023 ± 0.001 ^{c}	0.026 ± 0.001 ^{d}	0.026 ± 0.001 ^{d}
Cyanidin-3-*O*-rutinoside	595	287	0.772 ± 0.002 ^{a}	1.03 ± 0.03 ^{b}	1.46 ± 0.03 ^{c}	1.81 ± 0.06 ^{e}	1.64 ± 0.02 ^{d}
Cyanidin-3-*O*-glucoside *	449	287	0.757 ± 0.001 ^{a}	1.17 ± 0.02 ^{b}	1.55 ± 0.04 ^{c}	1.85 ± 0.02 ^{e}	1.80 ± 0.020 ^{d}
Delphinidin-3-*O*-glucoside *	465	303.1	1.08 ± 0.06 ^{a}	2.21 ± 0.03 ^{b}	3.45 ± 0.07 ^{c}	3.95 ± 0.05 ^{d}	3.93 ± 0.18 ^{d}
Delphinidin-3-*O*-rutinoside	611	303.1	1.01 ± 0.05 ^{a}	2.46 ± 0.05 ^{b}	3.71 ± 0.14 ^{c}	4.08 ± 0.14 ^{e}	3.95 ± 0.07 ^{d}
Total anthocyanins			3.62 ^{a}	6.88 ^{b}	10.19 ^{c}	11.71 ^{d}	11.35 ^{d}
Flavonols							
Myricetin-3-*O*-arabinoside	451	319	0.023 ± 0.001 ^{a}	0.026 ± 0.000 ^{b}	0.039 ± 0.001 ^{d}	0.040 ± 0.001 ^{d}	0.030 ± 0.001 ^{c}
Myricetin hexoside	481	319	0.808 ± 0.009 ^{b}	0.965 ± 0.010 ^{a}	1.37 ± 0.06 ^{d}	1.36 ± 0.03 ^{c}	1.36 ± 0.04 ^{c}
Myricetin *	319	152.9/273.1	0.124 ± 0.004 ^{a}	0.152 ± 0.006 ^{b}	0.202 ± 0.010 ^{d}	0.226 ± 0.005 ^{e}	0.182 ± 0.002 ^{c}
Quercetin	303	303	0.081 ± 0.002 ^{a}	0.156 ± 0.006 ^{b}	0.223 ± 0.007 ^{c}	0.257 ± 0.004 ^{e}	0.243 ± 0.004 ^{d}
Isorhamnetin rutinoside	625	317	0.157 ± 0.008 ^{a}	0.216 ± 0.008 ^{b}	0.413 ± 0.006 ^{c}	0.470 ± 0.028 ^{d}	0.476 ± 0.014 ^{e}
Kaempferol	287	287	0.145 ± 0.002 ^{a}	0.215 ± 0.005 ^{b}	0.250 ± 0.012 ^{c}	0.314 ± 0.008 ^{e}	0.282 ± 0.013 ^{d}
Rutin	611	465/303	1.80 ± 0.03 ^{a}	3.33 ± 0.10 ^{b}	4.60 ± 0.08 ^{c}	5.69 ± 0.19 ^{e}	5.34 ± 0.193 ^{d}
Total flavonols			3.13 ^{a}	5.06 ^{b}	7.10 ^{c}	8.36 ^{e}	7.91 ^{d}
Phenolic acids							
Protocatehuic acid *	152.9	109	0.098 ± 0.003	-	-	-	-
Gallic acid *	169	124.9	0.150 ± 0.006 ^{e}	0.030 ± 0.001 ^{b}	0.034 ± 0.001 ^{c}	0.115 ± 0.007 ^{d}	0.027 ± 0.001 ^{a}
Caffeic acid *	179	135	0.053 ± 0.002 ^{a}	0.053 ± 0.002 ^{a}	0.091 ± 0.005 ^{b}	0.202 ± 0.006 ^{d}	0.097 ± 0.002 ^{c}
Ellagic acid *	302.8	274.9	0.120 ± 0.005 ^{a}	0.181 ± 0.010 ^{b}	0.270 ± 0.011 ^{c}	0.296 ± 0.012 ^{d}	0.269 ± 0.013 ^{c}
p-Hydroxybenzoic acid	137	93	-	-	-	-	0.117 ± 0.002 ^{b}
Total phenolic acids			0.421 ^{c}	0.263 ^{a}	0.395 ^{b}	0.613 ^{e}	0.509 ^{d}
Flavan-3-ols							
Epicatechin	291	139/123	0.096 ± 0.003 ^{b}	0.109 ± 0.005 ^{c}	0.094 ± 0.003 ^{a}	0.152 ± 0.007 ^{e}	0.113 ± 0.004 ^{d}
Catechin *	291	123/139	0.081 ± 0.001 ^{a}	0.114 ± 0.004 ^{b}	0.125 ± 0.004 ^{c}	0.141 ± 0.007 ^{d}	0.148 ± 0.002 ^{d}

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Total flavan-3-ols	0.177 [a]	0.223 [c]	0.219 [b]	0.293 [e]	0.261 [d]
Total UPLC ESI MS^2-identified polyphenols	7.35 [a]	12.42 [b]	17.90 [c]	20.98 [e]	20.04 [d]

PLE—pressurized liquid extraction (100 °C, 5 min, 1:40 g/mL); MAE—microwave-assisted extraction (60 °C, 5 min, 1:40 g/mL); UAE—ultrasound-assisted extraction (75% amplitude, 5 min, 1:60 g/mL); CT—conventional technique (30 min, 1:100 g/mL), CG—cryogrinding (2 min cryogrinding followed by UAE under the conditions specified above). * Identified using authentic standards; compounds without authentic standards were tentatively identified based on MS/MS fragmentation patterns and comparison with literature data. Values are expressed as mean ± SD. Values with the same letter with in a row are not significantly different at $p \leq 0.05$.

Although MAE and PLE are widely recognized for their speed and efficiency in other matrices, they did not demonstrate superior performance in this study. The lower recovery of thermolabile anthocyanins and flavonols with MAE may be related to the thermal conditions during microwave treatment, while the relatively modest performance of PLE could be influenced by the complex nature of the sample matrix, which might limit the release of target compounds. In this study, the results obtained on the same extracts by UPLC–ESI–MS^2 and spectrophotometric methods (TPC and TMAC) showed similar trends, although absolute values differed. However, these results should not be considered as independent confirmation or validation of one analytical approach by the other. The differences in absolute TPC and TMAC values between UPLC–ESI–MS^2 and spectrophotometric methods can be attributed to the nature of the analyses. While UPLC quantifies individual compounds identified under the applied analytical conditions [15], spectrophotometric assays provide an overall response to the compounds present in the extracts and may also be influenced by other matrix constituents. In particular, the Folin–Ciocalteu assay is based on the reducing capacity of the sample and is not entirely specific to phenolic compounds, which may contribute to higher TPC values than the sum of individually quantified polyphenols [41]. These differences may also reflect the distinct analytical principles and quantification approaches of the two methods, as UPLC–ESI–MS^2 quantifies individual compounds identified under the applied analytical conditions, whereas spectrophotometric assays provide an overall response. Similarly, TMAC and UPLC–ESI–MS^2 provide complementary estimates of anthocyanin content, as the results are expressed using different quantification approaches and reference standards [42]. Therefore, differences in the reported values should be interpreted in the context of the methods used rather than as direct quantitative discrepancies. For instance, the CG + UAE extract exhibited a total UPLC–ESI–MS^2-identified polyphenol content of 20.04 mg/g dw, while spectrophotometrically, TPC was 50.16 mg GAE/g dw. The total anthocyanins quantified by UPLC for CG + UAE were 11.35 mg/g dw, while TMAC obtained spectrophotometrically was 33.15 mg C3GE/g dw. These differences reflect the distinct quantification principles and reporting bases of the two methods and should not be interpreted as direct discrepancies in the concentration of the same compounds. The UPLC–ESI–MS^2 profile further showed that anthocyanins were the predominant polyphenolic group, with delphinidin-3-*O*-rutinoside and delphinidin-3-*O*-glucoside among the most abundant individual compounds, reaching 3.95 and 3.93 mg/g dw, respectively. Together, the five identified anthocyanins accounted for approximately 57% of the total UPLC–ESI–MS^2-identified polyphenols in this extract. The relatively high TPC and TMAC values are consistent with the polyphenol-rich composition revealed by UPLC–ESI–MS^2, although the different analytical principles prevent direct quantitative comparison. Overall, the combined analysis provided complementary information on the composition and extractability of polyphenols in BP and indicated the potential of CG + UAE for improving bioactive compound recovery under the investigated conditions.

3.4. Antioxidant Capacity Determination

The results indicate that the BP extracts exhibited considerable antioxidant capacity across the applied assays (Table 6).

Table 6. Antioxidant capacity of blackcurrant pomace extracts obtained using different extraction methods.

Sample	Antioxidant Capacity (μmol TE/g dw)			
	FRAP	**DPPH**	**ABTS**	**ORAC**
	$p < 0.001$ *	$p < 0.001$ *	$p < 0.001$ *	$p < 0.001$ *
PLE	315.05 ± 11.83 [a]	117.96 ± 0.14 [a]	227.90 ± 2.56 [a]	258.49 ± 16.69 [a]
MAE	278.40 ± 2.18 [a]	114.64 ± 2.48 [a]	211.98 ± 1.42 [a]	304.28 ± 6.63 [a]
UAE	538.94 ± 3.27 [b]	574.78 ± 6.38 [c]	214.70 ± 4.25 [a]	431.85 ± 9.62 [b]
CT	627.43 ± 21.91 [c]	865.51 ± 24.92 [d]	299.08 ± 14.24 [b]	489.58 ± 34.12 [b]
CG + UAE	562.34 ± 3.27 [b]	450.20 ± 8.51 [b]	460.53 ± 8.72 [c]	482.51 ± 6.36 [b]

PLE—pressurized liquid extraction (100 °C, 5 min, 1:40 g/mL); MAE—microwave-assisted extraction (60 °C, 5 min, 1:40 g/mL); UAE—ultrasound-assisted extraction (75% amplitude, 5 min, 1:60 g/mL); CT—conventional technique (30 min, 1:100 g/mL); CG—cryogrinding (2 min cryogrinding followed by UAE under the conditions specified above). Values are expressed as mean ± SD. Values with the same letter within a column are not significantly different at $p \leq 0.05$.

Among the assays, CT showed the highest FRAP (627.43 μmol TE/g dw) and DPPH (865.51 μmol TE/g dw) values, whereas CG + UAE showed the highest ABTS value (460.53 μmol TE/g dw). For ORAC, CT showed the numerically highest value (489.58 μmol TE/g dw), although it did not differ significantly from CG + UAE (482.51 μmol TE/g dw). These results indicate that the relative performance of the extraction methods varied depending on the antioxidant assay applied. FRAP measures the capacity to reduce Fe^{3+} through electron transfer, whereas DPPH evaluates radical-scavenging activity. ABTS also measures radical-scavenging activity and can respond to both hydrophilic and lipophilic antioxidants, complementing the results obtained by DPPH [43]. ORAC provides complementary information on the capacity to neutralize peroxyl radicals and inhibit oxidation through a chain-breaking mechanism. The different responses observed across the assays may reflect their distinct chemical principles and the complex composition of the extracts; however, these results alone do not identify the compounds responsible for the measured activity. Compared with the results reported by Blejan et al. [1], the antioxidant capacity observed in the present study was higher for the selected CG + UAE extract; however, this comparison should be interpreted with caution because the studies employed different extraction conditions. The BP extract obtained by CG + UAE in the present study exhibited DPPH and ABTS values of 450.20 and 460.53 μmol TE/g dw, respectively, which are within the range of previously reported antioxidant activities for BP extracts [40]. Petrov Ivanković et al. [44] reported FRAP, DPPH, and ABTS activities of 53.56, 46.31, and 72.81 μmol TE/g dw, respectively, for BP extracts. The values obtained in the present study were higher; however, differences in extraction technique, solvent system, and experimental conditions may contribute to the observed variation. The antioxidant capacity observed in the present study was accompanied by the presence of polyphenolic compounds, particularly anthocyanins and flavonols, identified by UPLC–ESI–MS^2. The TPC and TMAC values, together with the UPLC–ESI–MS^2 profile, provide complementary information on the chemical characteristics of the extracts but do not, on their own, establish the contribution of individual compounds or phenolic groups to the measured antioxidant capacity. To further examine the associations between the phenolic composition and antioxidant capacity of the extracts, Pearson's correlation analysis was performed using total UPLC-identified polyphenols, individual phenolic groups (anthocyanins, flavonols, phenolic acids, and flavan-3-ols), spectrophotometrically determined TPC and TMAC, and the results of the antioxidant assays (FRAP, DPPH, ABTS, and ORAC) (Table 7).

Table 7. Pearson's correlation coefficients for blackcurrant pomace polyphenols (UPLC–ESI–MS²) and antioxidant capacity in optimized PLE, MAE, UAE, CT and CG + UAE extracts.

Polyphenols	FRAP	DPPH	ABTS	ORAC
TPC	0.9018 *	0.9014 *	0.3975	0.9759 *
TMAC	0.7310	0.5673	0.4263	0.8675
Total UPLC–ESI–MS²-identified polyphenols	0.7361	0.8131	0.2705	0.9859 *
Anthocyanins	0.7174	0.7957	0.2576	0.9814 *
Flavonols	0.7370	0.8193	0.2665	0.9852 *
Phenolic acids (mg/g dw)	0.9094 *	0.8203	0.4954	0.7115
Flavan-3-ols (mg/g dw)	0.6122	0.7703	0.3344	0.8649

TPC—total phenolic content; TMAC—total monomeric anthocyanin content. * Statistically significant at $p \leq 0.05$.

The correlation analysis revealed varying associations between the phenolic parameters and antioxidant capacity across the applied assays. TPC was strongly positively associated with FRAP and DPPH, with statistically significant correlation coefficients of r = 0.9018 and r = 0.9014, respectively. TPC also strongly and significantly correlated with ORAC (r = 0.9759), indicating a close association between TPC and peroxyl radical-scavenging capacity. A similar relationship between TPC and antioxidant activity was reported by Azman et al. [45] for dried BP extracts, in which TPC strongly correlated with both DPPH activity (r = 0.996) and FRAP (r = 0.919). The authors also highlighted the role of phenolic compounds, particularly anthocyanins, in radical scavenging through hydrogen donation, which may partly explain the associations observed between phenolic content and antioxidant capacity in the present study. Among the UPLC-identified phenolic groups, phenolic acids showed a strong positive association with FRAP. The strong association between phenolic acids and FRAP may be related to the electron-donating properties of phenolic hydroxyl groups, which are relevant to the electron-transfer mechanism assessed by this assay [46]. In contrast, UPLC-identified polyphenols strongly and significantly correlated with ORAC (r = 0.9859). Anthocyanins and flavonols also showed high positive correlation coefficients with ORAC (r = 0.9814 and r = 0.9852, respectively). Szydłowska et al. [47] reported associations between individual phenolic groups and antioxidant activity in smoothies containing black- or redcurrant pomace. Anthocyanins and flavonols showed moderate correlations with ABTS and ORAC, whereas flavan-3-ols and procyanidin polymers moderately correlated with FRAP. These findings provide a useful comparison, although the sample matrix differed from that of the extracts examined in the present study. The strong correlations with ORAC observed in our study indicate that higher levels of these phenolic parameters were closely associated with greater peroxyl-radical-scavenging capacity in the extracts. This pattern may reflect the combined contribution of multiple phenolic compounds and their structural characteristics to antioxidant activity, rather than the effect of a single compound class [3]. Anthocyanins' structure is based on the flavylium ring, and their antioxidant properties depend on the number and position of hydroxyl groups, as well as other structural characteristics. Two main anthocyanidins in blackcurrant are cyanidin and delphinidin, characterized by catechol and pyrogallol structures, respectively. These groups can facilitate hydrogen-atom donation and stabilization of the resulting radical through resonance, which complements with ORAC hydrogen-atom transfer mechanism [48]. Furthermore, strong correlation of flavonols with ORAC can also be explained through structural characteristics of the dominant compounds identified in BP. The structure of main flavonols is characterized by presence of hydroxyl groups, particularly the 3′,4′-catechol moiety in the B-ring, together with conjugated C2=C3 double bond and 4-keto group, all of which facilitate radical stabilization and contribute to peroxyl-radical scavenging [49].

Overall, the observed patterns varied across the assays, which may reflect differences in their underlying chemical principles and the complex composition of the extracts. It is important to acknowledge that chemical antioxidant assays provide complementary information on antioxidant capacity but may not fully reflect biological effects. Therefore, the zebrafish embryo and cell-based assays were included to provide additional insight into the biological effects of the extract under the tested conditions.

3.5. Embryotoxic Effects Evaluated Using the Zebrafish Model

To the best of our knowledge, this study is the first to investigate the effects of BP extract on zebrafish embryos. Exposure of embryos to CG + UAE extract resulted in concentration-dependent effects on embryo mortality and developmental abnormalities, with an LC_{50} of 0.409 mg/mL (95% CI: 0.398–0.420 mg/mL) and an EC_{50} of 0.349 mg/mL (95% CI: 0.339–0.360 mg/mL), respectively (Figure 1).

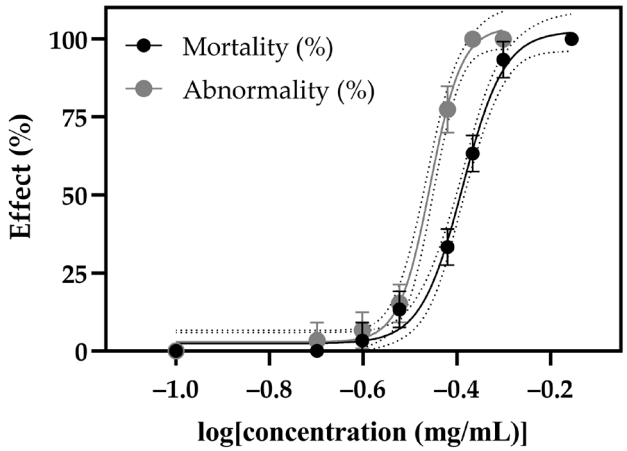


Figure 1. Dose–response curves showing cumulative mortality and developmental abnormality of zebrafish (*D. rerio*) embryos at 120 h of exposure to BP extract obtained by CG + UAE. The dotted lines represent 95% confidence intervals. LC_{50}—half-maximal lethal concentration; EC_{50}—half-maximal effective concentration. Error bars indicate SD.

The most frequently observed developmental abnormalities were pericardial and yolk sac edema, while the control group showed minimal mortality and developmental defects (<10%). Chemical characterization of the CG + UAE extract by UPLC–ESI–MS^2 revealed a complex phenolic profile, including flavan-3-ols, flavonols, and phenolic acids (Table 5). The observed embryotoxic effects may be related to the combined effects of multiple compounds present in the extract; however, the contribution of individual constituents or specific mechanisms of toxicity was not investigated in the present study. Comparable dose-dependent embryotoxic responses have been reported for grape pomace extracts in zebrafish, with LC_{50} and EC_{50} values of 0.46 and 0.49 mg/mL, respectively [50]. The LC_{50} and EC_{50} values observed for the BP extract were numerically lower than those reported for the grape pomace extract; however, direct comparison should be interpreted with caution because differences in extract composition, exposure conditions, and experimental protocols may influence embryotoxicity. Overall, the results indicate concentration-dependent effects of the BP extract on zebrafish embryo survival and development and highlight the need for further assessment of the biological effects and safety of the extract.

3.6. Cellular Effects of BP Extract Treatment

3.6.1. Cell Viability and Metabolic Activity

In the concentration range of 0.1–0.7 mg/mL, the BP extract had no significant impact on cell viability of both tested cell lines measured by the MTT assay (Figure 2a). In contrast, the clonogenic assay (Figure 2(b1)) demonstrated that the two highest concentrations tested (0.5 and 0.7 mg/mL) significantly inhibited proliferation of CAL 27 cells, while no effect was observed in HepG2 cells. This reduction is also clearly visible in the crystal violet stained wells (Figure 2(b2)). The difference between MTT and clonogenic assay results reflects the distinction between acute and long-term effects. Although the extract did not significantly affect short-term metabolic activity in either cell line, the clonogenic assay revealed a concentration-dependent reduction in the colony-forming ability of CAL 27 cells at 0.5 and 0.7 mg/mL, while HepG2 cells showed no significant changes. These findings indicate that the absence of an acute effect on metabolic activity does not exclude longer-term effects on cellular survival and proliferation. According to Pap et al. [51], BP extracts (48 h treatment) showed no apparent cytotoxicity in THP-1 cells (>93% viability at 0.007–1.667 mg/mL), and the IC_{50} values for A549, HCT8 and IMR90 cells were >2 mg/mL, indicating very low acute toxicity. These results support our finding that short-term cell viability was not affected at any of the tested concentrations.

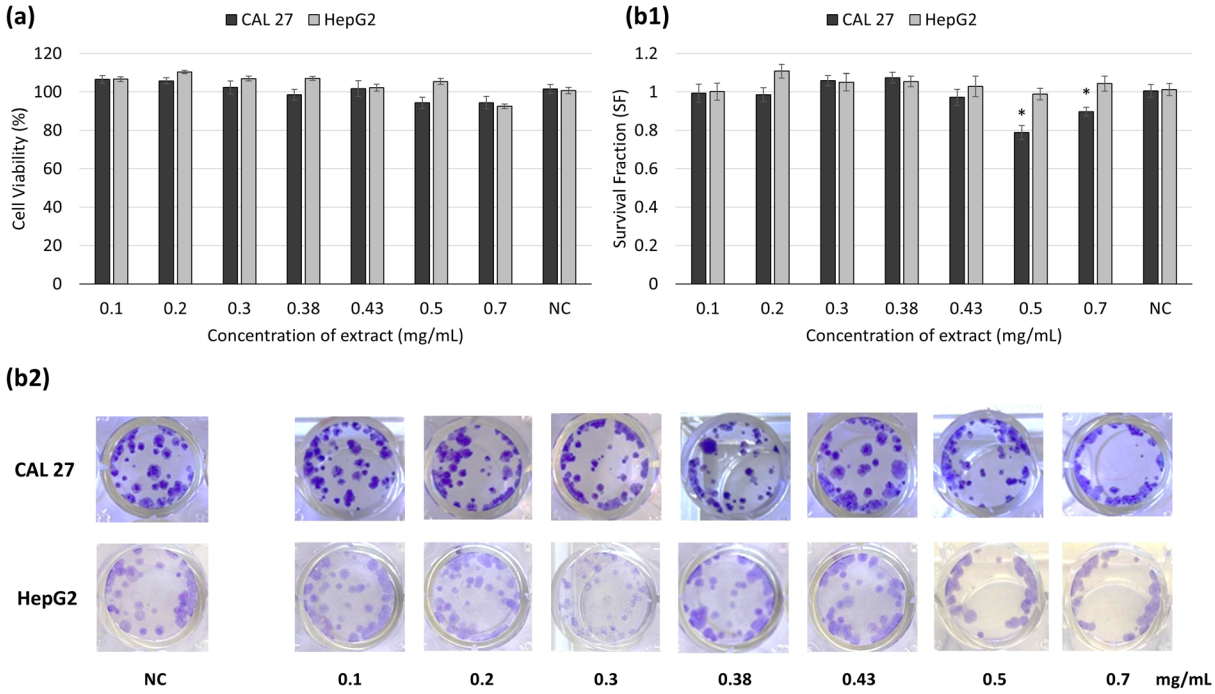


Figure 2. Effect of BP extract on CAL 27 (30 min) and HepG2 cells (24 h): (**a**) cell viability assessed by MTT assay; (**b1**) colony-forming ability assessed by clonogenic assay, with (**b2**) representative images of colonies after 7 days of growth (crystal violet staining). Data are presented as mean ± SD from three independent experiments. * Statistically significant difference compared to the untreated control ($p \leq 0.05$).

The results of the DCFH-DA assay and lipid peroxidation (LPO) measurements showed that BP extract (0.1–0.7 mg/mL) exhibited antioxidant activity in HepG2 cells. In contrast, for CAL 27 cells, only the lowest concentrations tested (0.1 and 0.2 mg/mL) showed a statistically significant reduction in ROS (Figure 3a) and LPO levels (Figure 3b). Pap et al. [51] showed strong antioxidative activity of BP extract (0.1–1 mg/mL) in lung adenocarcinoma epithelial cells (A549) by reducing ROS levels to those of the negative

control, demonstrating antioxidant activity similar to the antioxidant effect we observed in HepG2 cells across the full concentration range. Interestingly, the same study reported that BP extract (0.1–1 mg/mL) induced ROS production in human ileocecal adenocarcinoma cells (HCT8) and the monocyte cell line (THP-1), which parallels our finding that CAL 27 cells exhibited antioxidant effects only at the lowest concentrations (0.1–0.2 mg/mL). These results show that differences in natural antioxidant levels and how cells absorb and process polyphenols can make polyphenol-rich extracts act either as antioxidants or as prooxidants, depending on the type of cell [52].

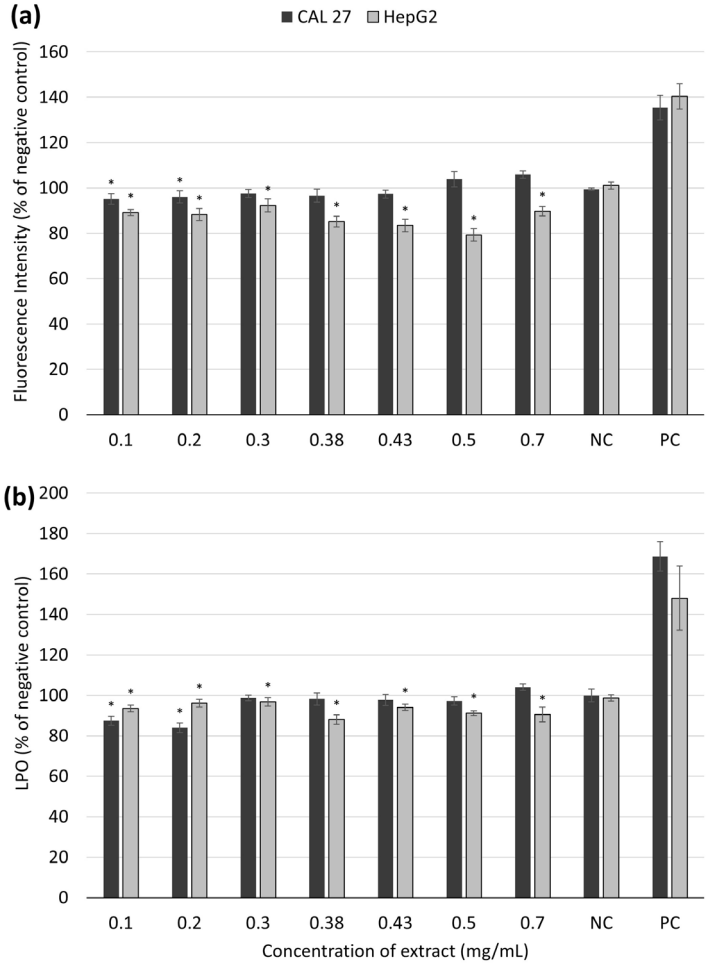


Figure 3. Effects of BP extract on CAL 27 (30 min) and HepG2 cells (24 h): (**a**) intracellular ROS levels (DCFH-DA assay) and (**b**) lipid peroxidation levels. Results are expressed as mean value ± SD (3 independent experiments). The positive control (PC) consisted of cells treated with 10 mM H_2O_2, 15 min (DCFH – DA assay) and 200 µM, 2h (LPO assay). * Statistically significant difference compared with untreated cells (negative control, NC; $p \leq 0.05$).

3.6.2. DNA Damage Assessment

In CAL 27 cells, tail length was significantly reduced for all treatments (0.1–0.5 mg/mL) compared to the negative control, except for the highest concentration (Figure 4a). No statistically significant differences were observed in HepG2 cells. Also, in CAL 27 cells, the highest concentration (0.7 mg/mL) significantly increased tail intensity and tail moment compared to the negative control, but values were still lower than the positive control (Figure 4b,c). Brown et al. [53] showed that after simulated gastrointestinal digestion and colonic fermentation, the blackcurrant extracts did not induce DNA damage in HT29 cells but exhibited an antigenotoxic effect by reducing H_2O_2-induced DNA strand

breaks. Similarly, *Castanea sativa* leaf extracts were shown to decrease DNA damage caused by aflatoxin B1 and ochratoxin A, highlighting the protective effects of polyphenol-rich plant extracts against genotoxic stress [54]. These findings support our results, where BP extract induced only moderate DNA damage at the highest concentration (0.7 mg/mL) in CAL 27 cells, with no genotoxic effects observed in HepG2 cells. These findings further highlight the importance of considering both cell type and extract concentration when interpreting the biological effects of BP extract.

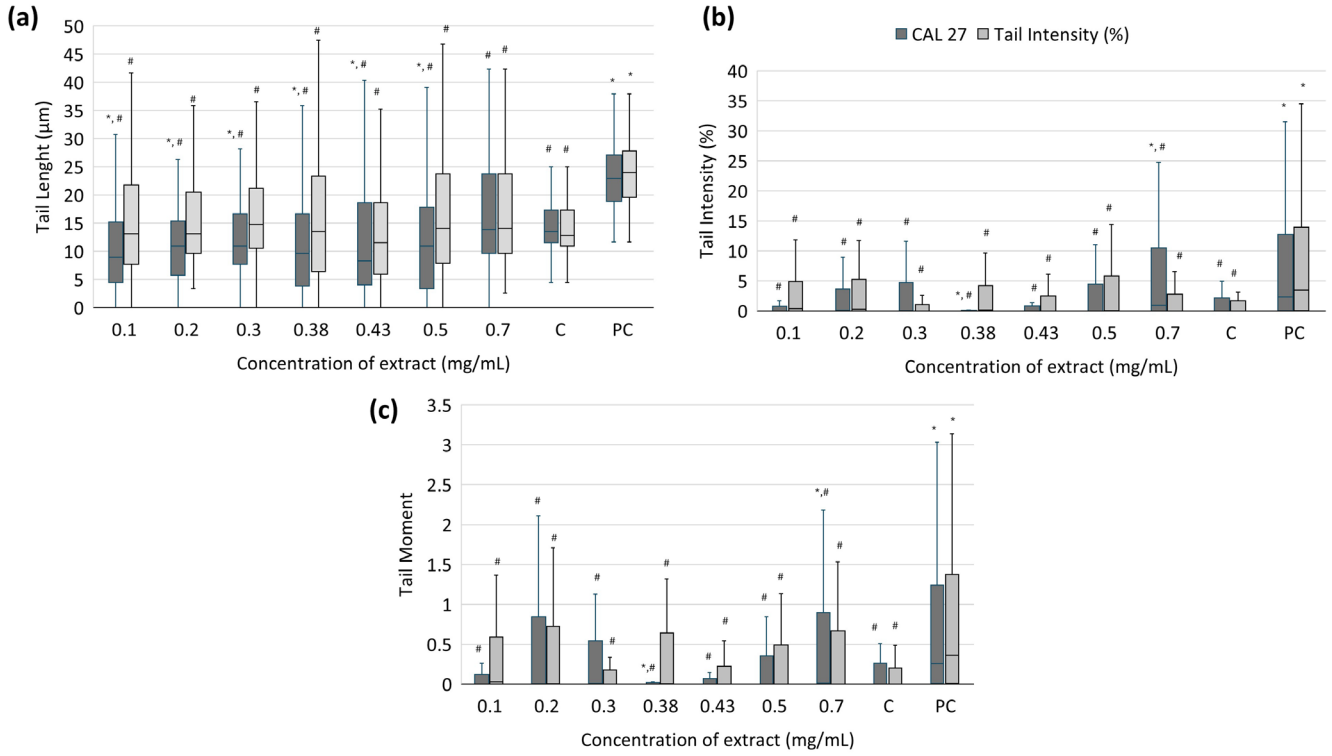


Figure 4. Genotoxic effects of BP extract on CAL 27 tongue carcinoma cells and HepG2 hepatocellular carcinoma cells using the comet assay. DNA integrity was quantified through: (**a**) tail length (µm), (**b**) percentage of DNA in the tail, and (**c**) tail moment (product of tail length and tail DNA%). Data are shown as median values with interquartile ranges (25th–75th percentile) and whiskers representing the full distribution across 100 scored nucleoids. Statistical comparisons were performed using the independent experiment as the experimental unit. Significant differences relative to the negative control (C, untreated cells *) and positive control (PC, 50 µM H_2O_2, 5 min #).

In summary, BP extract exhibited antioxidant activity, while its biological effects varied depending on the experimental model, concentration, and cell type. Zebrafish embryo assays revealed concentration-dependent effects on survival and development. In cellular assays, no significant changes in short-term metabolic activity were observed in either cell line, whereas clonogenic survival was reduced in CAL 27 cells at higher concentrations (0.5 and 0.7 mg/mL). Antioxidant effects were observed across the tested concentration range in HepG2 cells, while significant reductions in reactive oxygen species (ROS) and lipid peroxidation (LPO) levels in CAL 27 cells were limited to the lowest concentrations (0.1 and 0.2 mg/mL). Furthermore, moderate DNA damage was observed in CAL 27 cells at the highest concentration (0.7 mg/mL), whereas no significant changes in DNA damage parameters were detected in HepG2 cells. Given the complex phenolic composition of the extract, the present findings do not establish direct causal relationships between individual compounds and the observed biological responses or elucidate the underlying mech-

anisms. Further research is therefore needed to clarify the contributions of individual constituents to these effects. It should also be noted that chemical antioxidant assays may not fully reflect antioxidant effects in biological systems, and the models used do not allow direct prediction of effects in humans. Overall, these results provide preliminary insights into the biological activity of BP extract under the tested conditions but do not support definitive conclusions regarding its safety or potential health benefits.

4. Conclusions

This study demonstrated that combining UAE with CG is a promising approach for recovering bioactive compounds from BP. Optimal conditions (2 min CG + UAE at 75% amplitude for 5 min, 1:60 g/mL) resulted in the highest TPC and TMAC values among the investigated conditions. UPLC–ESI–MS^2 profiling showed the presence of key anthocyanins (delphinidin- and cyanidin- based glycosides), flavonols (rutin, quercetin derivatives), phenolic acids (ellagic, caffeic, gallic acid), and flavan-3-ols (catechin, epicatechin), providing complementary information on the phenolic composition of the extracts. In vitro antioxidant assays indicated high antioxidant capacity of the extracts, which is consistent with the high polyphenol content. Complementary in vivo testing in zebrafish embryos showed concentration-dependent effects on embryo survival and development, with higher concentrations producing effects such as pericardial and yolk sac edema. In vitro assays with CAL 27 and HepG2 cells revealed concentration- and cell-dependent responses, including reduced CAL 27 clonogenic survival at higher concentrations and moderate DNA damage at the highest concentration (0.7 mg/mL). Overall, CG + UAE showed potential for improving the recovery of bioactive compounds from BP and obtaining extracts with antioxidant capacity under the investigated conditions. These findings support the potential of CG-assisted UAE for the valorization of berry processing by-products. The biological assessments provided complementary information on the effects of the BP extract under the tested conditions; however, further studies are needed to clarify the contributions of individual constituents to the observed biological responses and to assess the extract's stability, safety, and practical applicability.

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Abbreviations

The following abbreviations are used in this manuscript:

BP	Blackcurrant pomace
PLE	Pressurized liquid extraction
MAE	Microwave-assisted extraction
UAE	Ultra-sound-assisted extraction
CG	Cryogrinding
CT	Conventional extraction technique
TPC	Total polyphenol content
TMAC	Total monomeric anthocyanin content
GAE	Gallic acid equivalents
C3GE	Cyanidin-3-glucoside equivalents
ZET	Zebrafish Embryotoxicity Test

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