A Comparison of the Antiosteoporotic Effects of Cornelian Cherry (Cornus mas L.) Extracts from Red and Yellow Fruits Containing Different Constituents of Polyphenols and Iridoids in Osteoblasts and Osteoclasts

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Background and aims. Bone remodeling in which old or damaged bone cells are removed by osteoclasts, and new bone cells are developed by osteoblasts is a key target for antiosteoporotic agents. These processes can also be modulated by nutrients. In this study, we have compared the antiosteoporotic effects of three extracts from cornelian cherry (Cornus mas L.) fruits: RED EXT1 extracts from red fruits, and YL EXT2 and YL EXT3 extract from yellow fruits.

Methods. Polyphenolic and iridoid constituents of extracts were analyzed qualitatively and quantitatively using the ultraperformance liquid chromatography system coupled with a quadrupole-time of flight mass spectrometry. Primary cultured osteoblasts isolated from mouse calvarias and osteoclast-lineage primary cultured monocytes isolated from mouse bone marrow were used for the assessment of osteoblast and osteoclast differentiation. In the osteoblast culture, cellular viability, alkaline phosphatase (ALP) activity, ALP staining, and mRNA expression of Alpl and Runx2 were examined. In the osteoclast culture, the examined parameters were cellular viability, tartrate-resistant acid phosphatase (TRAP) activity and staining, and mRNA expression of Nfatc1, Ctsk, and Acp.

Results. A total of 41 main compounds of iridoids, anthocyanins, hydrolysable tannins, phenolic acids, and flavonols were identified in the three extracts. RED EXT1 contained the most of the tested polyphenols and iridoids and was the only extract containing anthocyanins. YL EXT2 contained only one iridoid, loganic acid and gallic acid. YL EXT3 comprised a mixture of iridoids and polyphenols. RED EXT1, YL EXT 2, and to a lesser extent YL EXT3 promoted osteoblast differentiation increasing significantly ALP activity and the amount of ALP-positive stained cells. All extracts upregulated mRNA expression of Alpl and Runx2. RED EXT1 caused the most significant decrease in TRAP activity and the numbers of TRAP-positive multinucleated cells. RED EXT1 caused also the most significant downregulation of mRNA expression of osteoclast related genes Nfatc1, Ctsk, and Acp. Extracts from yellow fruits, mostly YL EXT2 caused lower, but still significant inhibitory effect on TRAP and osteoclast related genes.

Conclusions. The main conclusion of our study is that all three extracts, especially RED EXT1 from red cornelian cherry fruits, possess the antiosteoporotic potential and may be a promising phytomedicine candidate for the prevention and treatment of osteoporosis.
1. Introduction

Osteoporosis is a common systemic bone disease characterized by the loss of bone mass and deterioration of the bone microstructure, leading to fractures and subsequent complications. An increased life expectancy in developed countries combined with comorbidities and drugs causing bone loss has resulted in osteoporosis, becoming more prominent in the last few decades. Moreover, increased levels of physical activity in elderly populations, offering several benefits but leading to a higher risk of pathological fractures, makes osteoporosis an increasingly greater medical, social, and economic challenge.

Bone remodeling is regulated by homeostasis between osteoclasts resorbing old or damaged bone cells and osteoblasts developing new bone structures [1]. The differentiation and function of osteoclasts and osteoblasts are key target areas for antiosteoporotic agents. It has been proven that bone metabolism can be modulated by nutrients [2, 3]. Recently published studies have reported that polyphenols, especially anthocyanins [4, 5], phenolic acids [6], and flavonoids [7], as well as iridoids [8] and hydrolysable tannins [9] can prevent bone loss through different mechanisms. All these substances are present in cornelian cherries, in varying amounts, making their extracts promising candidates for antiosteoporotic agents.

A previous in vitro study demonstrated that Cornus officinalis Sieb. et Zucc. shared a few similar iridoid and polyphenol constituents with the cornelian cherry (Cornus mas L.), and it inhibits the receptor activator of nuclear factor-kappaB ligand (RANKL)-induced osteoclast differentiation [10]. Our previous studies showed that red cornelian cherries, rich in polyphenols and iridoids, prevented both cholesterol-induced dyslipidemia and atherosclerosis via the upregulation of peroxisome proliferator-activated receptors (PPARs) expression [11–14] and prevented bone loss in osteoporotic animals by inhibiting bone resorption and increasing bone formation [15]. Another study demonstrated that the pulp of cornelian cherries partially reversed impaired microarchitecture bone quality in Zucker diabetic fatty rats [16].

In the present study, we have compared the antiosteoporotic effects of three different extracts from cornelian cherry fruits: RED EXT1 from red fruits containing exceptionally anthocyanins and substantial amounts of various other polyphenols and iridoids, YL EXT2 from yellow fruits containing a single iridoid, loganic acid and gallic acid, and YL EXT3 from yellow fruits comprising substantial amounts of various iridoids and polyphenols. To investigate the effects of extracts on bone remodeling, we used an in vitro model of mouse primary cultured osteoblasts isolated from mouse calvarias and osteoclasts isolated from mouse bone marrow monocytes. In the osteoblast culture, the analyzed parameters were cellular viability, alkaline phosphatase (ALP) activity and amount of ALP-positive stained cells, and mRNA expression of a gene (Alpl) and transcription factor (Runx2) involved in osteoblastic bone remodeling. In osteoclast culture, the analysis pertained to cellular viability, tartrate-resistant acid phosphatase (TRAP) activity, the number of TRAP-positive multinucleated cells, and mRNA expression of transcriptional factor (Cisk), as well as genes (Nfatc1, Acp5) involved in osteoclast differentiation and activity.

2. Materials and Methods

2.1. Reagents and Standards. All reagents and organic solvents were of analytical grade. Authentic standards of loganic acid, loganin, sweroside, cyanidin 3-O-glucoside, p-coumaric acid, ellagic acid, quercetin 3-O-glucoside, and kaempferol 3-O-glucoside were purchased from Extrasynthese (Genay, France). Trans-caftaric acid was purchased from the Cayman Chemical Company (Michigan, EUA, Ann Arbor, MI, USA). Trans-coutaric acid was purchased from Merck (Darmstadt, Germany). Methanol, acetonitrile, and formic acid were obtained from POC (Gliwice, Poland).

2.2. Extraction, Purification, and Fractionation of Extracts. Red (‘Podolski’) and yellow (‘Yantarnyi’ and ‘Flava’) cornelian cherries were harvested in the Arboretum in Bolestraszyce, near Przemyśl, Poland. The plant materials were authenticated by Elżbieta Zygala, M.Sc. (Arboriculture and Institute of Physiography in Bolestraszyce, Przemyśl, Poland), and adequate voucher specimens (‘Yantarnyi’ – BDPA 14131; ‘Flava’ – BDPA 8795; ‘Podolski’ – BDPA 10462) were deposited at the Herbariums of Arboretum in Bolestraszyce, Poland. After harvesting, the fruits were immediately frozen at −20°C.

Extracts were prepared as previously described by Lewandowski et al. [17] with some modifications. Frozen ripe cornelian cherries were shredded and heated for 5 min at 95°C using a Thermomix (Vorwerk, Wuppertal, Germany). The pulp was subsequently cooled to 50°C and depectinized at this temperature for 2 h by adding 0.5 mL/kg of Pectinex BE XXL (Novozymes A/S, Denmark). After depectinization, the pulp was pressed in a laboratory hydraulic press (SRSE, Warsaw, Poland). The pressed juice was filtered and run through an Amberlite XAD-16 resin column (Rohm and Haas, Chauny Cedex, France). Impurities were washed off with distilled water. RED EXT1 (red fruits) and YL EXT3 (yellow fruits) were eluted with 80% ethanol while YL EXT2 (yellow fruits) was eluted with up to 50% ethanol (v/v in water). The eluents were concentrated at 40°C under vacuum. The solvent was evaporated using a Rotavapor (Unipan, Warsaw, Poland) and freeze-dried (Alpha 1–4 LSC, Christ, Osterode am Harz, Germany). As a result, we have obtained three extracts from cornelian cherry fruits: RED EXT1 from red fruits containing substantial amounts of various anthocyanins, iridoids, and hydrolysable tannins with moderate or small amounts of phenolic acids and flavonols, YL EXT2 from yellow fruits containing a single iridoid, loganic acid and small amounts of phenolic acids, and YL EXT3 from yellow fruits comprising substantial amounts of various iridoids and hydrolysable tannins, and moderate or small amounts of phenolic acids and flavonols.
2.3. Qualitative Identification of Compounds by Liquid Chromatography-Mass Spectrometry (LC-MS). This method was previously described by Przybylska et al. [18]. Compounds were identified using the Acquity ultra-performance liquid chromatography (UPLC) system, coupled with a quadrupole-time of flight (Q-TOF) MS instrument (UPLC/Synapt Q-TOF MS, Waters Corp., Milford, MA, USA), with an electrospray ionization source. Separation was achieved using an Acquity UPLC BEH C18 column (100 x 2.1 mm i.d., 1.7 μm; Waters Corp., Milford, MA, USA). The mobile phase was a mixture of 2.0% aq. formic acid v/v (a) and acetonitrile (b). The gradient program is as follows: initial conditions: 1% B in A, 12 min; 25% B in A, 12.5 min; 100% B, 13.5 min; 1% B in A. The flow rate was 0.45 mL/min, and the injection volume was 5 μL. The column was operated at 30°C. The UV-Vis absorption spectra were recorded online during the UPLC analysis, and the spectra were measured in the wavelength range of 200–600 nm, in steps of 2 nm. The major operating parameters of the Q-TOF MS were set as follows: capillary voltage of 2.0 kV, cone voltage of 40 V, cone gas flow of 11 L/h, collision energy of 28–30 eV, source temperature of 100°C, desolvation temperature of 250°C, argon as the collision gas, desolvation gas (nitrogen) flow rate of 600 L/h, and data acquisition range (m/z) of 100–2500 Da. The compounds were monitored at 245, 280, 320, 360, and 520 nm and explored in the negative and positive (only anthocyanins) modes before and after fragmentation. The data was collected with the MassLynx V 4.1 software (Waters Corp., Milford, MA, USA).

2.4. Quantitative Determination of Anthocyanins, Flavonols, Phenolic Acids, and Iridoids Using HPLC-PDA. The HPLC analysis was performed as described by Spychaj et al. [19] using the Dionex (Germering, Germany) system equipped with an Ultimate 3000 diode array detector, LPG-3400A quaternary pump, EWPS-3000SI autosampler, TCC-3000SD thermostated column compartment, and controlled with the Chromeleon v.7.2 software. Separation was achieved using a Cadenza Imitakt column CD-C18 (75 x 4.6 mm, 5 μm). The mobile phase was composed of solvent A (4.5% aq. formic acid, v/v) and solvent B (100% acetonitrile). The gradient profile is as follows: 5% B in A, 0–1 min; 25% B in A, 1–20 min; 100% B, 20–26 min; 5% B in A, 26–30 min. The flow rate of the mobile phase was 1 mL/min, and the injection volume was 20 μL. The column was operated at 30°C. Anthocyanins were detected at 520 nm, flavonols at 360 nm, phenolic acids at 320 nm, gallic acid at 280 nm, ellagic acid at 254 nm, and iridoids at 245 nm. Calibration curves at concentrations ranging from 0.02 to 0.3 mg/mL (R² ≥ 0.9998) were determined experimentally for cyanidin 3-O-glucoside, quercetin 3-O-glucoside, kaempferol 3-glucoside, caffeic acid, trans-cafeic acid, trans-coumaric acid, p-coumaric acid, ellagic acid, and gallic acid. The results were expressed as mg/100 g of the dried extract. Results were provided as the mean ± standard deviation of three replications and expressed as mg/100 g dw (dry weight) of the extract.

2.5. Quantitative Determination of Hydrolysable Tannins by HPLC-PDA. The HPLC analysis was performed as described by Przybylska et al. [18] using the Dionex (Germering, Germany) system equipped with an Ultimate 3000 diode array detector, LPG-3400A quaternary pump, EWPS-3000SI autosampler, TCC-3000SD thermostated column compartment, and controlled with the Chromeleon v.7.2 software. Separation was achieved using a Hypersil GOLD C18-column (250 x 4.6 mm, 5 μm; Thermo Fisher Scientific Inc., UK). The following mixtures were used as eluents: (a), water-FA (98.5:1.5, v/v) and (b), acetonitrile-FA (98.5:1.5, v/v). The gradient profile is as follows: initial conditions: 100% A, 30 min; 30% B, 33 min; 70% B, 45 min; 70% B in A, 48 min; 100% B, 55–60 min; 100% A. The flow rate of the mobile phase was 1.2 mL/min, and the injection volume was 20 μL. The column was operated at 22°C. Hydrolysable tannins were detected at 280 nm. A calibration curve at concentrations ranging from 0.02 to 0.3 mg/mL was determined experimentally for gallic acid. Results are provided as the total of individual isomers of three replications and expressed as mg/100 g of the extract.

2.6. Primary Osteoblast Culture and Induction of Osteoblast Differentiation. Primary cell cultures of the mice were conducted using a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Ajou University School of Medicine (2016-0062). Primary osteoblasts of the mice were isolated from neonatal C57BL/6 mice (4–5 pups), as previously described [20]. The calvarias of the mice were dissected and digested with collagenase II (Sigma, St. Louis, MO, USA) at 37°C for 2 h. The digestive solution was filtered through a 40 μm cell strainer, and the cells were incubated with α-modified minimal essential medium (α-MEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco) for 2–3 days. After 70–80% of confluency was reached, the cells were incubated with 10 mM β-glycerophosphate (Sigma) and 50 μg/mL ascorbic acid (Sigma) for 3 days.

2.7. Isolation of Primary Monocytes and Induction of Osteoclast Differentiation. To isolate osteoclast-lineage primary-cultured monocytes, bone marrow (BM) cells from nine-week-old C57BL/6 mice were isolated as previously described [21]. The femoral bones of the mice were flushed using warm phosphate-buffered saline (Gibco), and the cellular suspension was subsequently filtered through a 40 μm cell strainer to remove the debris. BM cells were then incubated with α-MEM (Gibco) containing 10% FBS (Gibco) and 50 ng/mL of macrophage colony-stimulating factor (M-CSF) (Peprotech, Cranbury, NJ, USA) without penicillin/streptomycin (Gibco) for 3 days. M-CSF was added daily. After osteoclast differentiation, the cells were seeded in a 96-well plate and incubated with a growth medium supplemented with M-CSF (50 ng/mL; Peprotech) and RANKL (50 ng/mL; Peprotech) for 5 days.

2.8. Cell Viability Assay. The cells were incubated in 96-well plates and treated with different concentrations of Cornus mas L. extracts (2, 5, and 10 μg/mL) during differentiation. The cells were then incubated with D-Plus™ cell counting kit (CCK) cell viability assay reagent (Dongin Biotech, Seoul, Korea) at 37°C, and the cell viability was measured using a
microplate reader (Bio-Rad, Hercules, CA, USA) at an absorbance of 450 nm.

2.9. ALP/TRAP Activity Assay and Staining. The cells were collected using a lysis buffer (0.5 M Tris-hydrogen chloride (HCl), pH 8.8, containing 0.9% sodium chloride, 1% Triton X-100, and 200 mM ethylenediaminetetraacetic acid (EDTA)), and ALP activity was measured using 1-Step® p-nitrophenyl phosphate (Sigma) according to the manufacturer’s recommendations. ALP-positive cells were stained with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma) at room temperature. TRAP activity and staining were processed using an Acid Phosphatase Kit (Sigma) in accordance with the manufacturer’s instructions.

2.10. Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR). The total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction, and the complementary DNA (cDNA) was synthesized using a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, NH, USA). qRT-PCR was processed using a SYBR Green I qPCR Kit (TaKaRa, Shiga, Japan), via the CFX Connect™ Real-Time System (Bio-Rad). The gene-specific primers used in this study were as follows: forward 5′-TCC CAC GTT TTC ACA TTC GG-3′ and reverse 5′-CCC GTT ACC ATA TAG GAT AGC C-3′ for mouse Alpl, forward 5′-TAA AGT GAC AGT GGA CCG TTC C-3′ and reverse 5′-AAT GCG CCC TAA ATC ACT GAG G-3′ for mouse Runx2, forward 5′-AAT ACC ACC TTC TCT TCG GTA CTA CA-3′ and reverse 5′-TGG TTC TGTT ACT GGA GTA ACG TA-3′ for mouse Ctsk, forward 5′-TGG TAG GTG CTC GCT GGA AAC-3′ and reverse 5′-AGT TGC CAC ACA GCA TCA CTG-3′ for mouse Acp5, forward 5′-AGG TCG GTG TGA ACG GAT TTG-3′ and reverse 5′-TGT AGA AGA AGA CTA TGG-3′ for mouse Gapdh, forward 5′-GAG GAG TCC TGG TGA TGT GGC C-3′ and reverse 5′-GCG TGG CCT ATG GGA TCA TAG TGG-3′ for mouse Hprt. All gene expression levels were normalized using mouse Gapdh (osteoblast) and mouse Hprt (osteoclast), and relative expression levels were calculated using the 2−ΔΔCt method (ΔΔCt = ΔCtTreatment − ΔCtInduction).

2.11. Statistical Analysis. The data in the bar graphs is presented as the mean ± standard error of the mean (SEM) using the GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed in multiple groups using a one-way analysis of variance with Tukey’s honest significant difference post hoc test. A probability value below 0.05 (p < 0.05) was considered statistically significant.

3. Results and Discussion

3.1. A Comparison of the Chemical Composition of Cornelian Cherry (Cornus mas L.) Extracts from Red and Yellow Fruits. The polyphenols and iridoids were analyzed via LC-MS and HPLC in the three extracts. The results of qualitative and quantitative identification of the compounds of cornelian cherry extracts are presented in Table 1.

Phenolic and iridoid compounds, as well as hydrolysable tannins, were identified by their elution order, retention times, spectra of individual peaks (MS, MS/MS), and by comparison with data in the literature [22–24]. A total of 41 main compounds were identified: 4 iridoids (loganic acid and cornuisin with pseudomolecular ions [M − H]− at m/z 375 and 541, respectively, and sweroside and loganin with [M − H + 46]− at m/z 403 and 435, respectively), 7 anthocyanins (5 glycosides: delphinidin 3-O-galactoside, cyanidin 3-O-galactoside, pelargonidin 3-O-galactoside, and pelargonidin 3-O-robinobioside with [M + H]− at m/z 463, 449, 595, 433, and 579, respectively, and 2 aglycons: cyanidin and pelargonidin with [M + H]− at m/z 287 and 271, respectively), 6 phenolic acids (gallic acid, 2 isomers of caftaric acid, coumaric acid, p-coumaric acid, and ellagic acid with [M − H]− at m/z 169, 311, 295, 163, and 301, respectively), 4 flavonoids (quercetin 3-O-glucuronide, quercetin 3-O-glucoside, kaempferol 3-O-galactoside, and kaempferol 3-O-gluconuride with [M − H]− at m/z 477, 463, 447, and 461, respectively), and 20 hydrolysable tannins, including their spatial isomers. Among the hydrolysable tannins, the main compounds were one trimeric ellagittannin (cornusin C, which gave two ions, [M − 2H]2− at m/z 1176 and [M − H]− at m/z 2353), two dimeric ellagittannins (camptothin A, which displayed two ions [M − 2H]2− at m/z 708 and [M − H]− at m/z 1417 and cornusin A with two ions, [M − 2H]2− at m/z 784 and [M − H]− at m/z 1569), as well as gemin D − the simplest among the ellagitannin molecules with ion [M − H]− at m/z = 633 and its two derivatives (tellimagrandin I with [M − H]− at m/z 785 and tellimagrandin II with [M − H]− at m/z 937).

RED EXT1 contained most of the identified compounds (40 compounds), namely 16.9% iridoids, 3.5% anthocyanins, 1.2% phenolic acids, 0.6% flavonoids, and 16.8% hydrolysable tannins. The quantitative and qualitative composition of iridoids and phenolic compounds in RED EXT1 is comparable to the composition of the red fruit extract described by Dzydzan et al. [23]. Extracts from yellow fruits (YL EXT2 and YL EXT3) did not contain anthocyanins but were richer in iridoids. YL EXT2 contained only one iridoid, 22.1% of loganic acid, whereas YL EXT3 contained four iridoids, which made up a quantity of 27.7% in total and included 17% of loganic acid. YL EXT2 contained four phenolic acids which made up a quantity of only 0.9% in total. As compared to the other extracts, YL EXT3 was the richest in phenolic acids, making up a quantity of 3.5% in total. The flavonoids found in YL EXT3 were comparable to those present in RED EXT1, whereas YL EXT2 did not contain any of these compounds. A comparison between the two yellow fruit extracts showed that YL EXT2 mainly contained loganic acid and small amounts of phenolic acids, but no flavonoids or tannins, whereas YL EXT3 was more abundant in various iridoids, phenolic acids, and hydrolysable tannins. Collectively, the three extracts contained different constituents of iridoids, anthocyanins, hydrolysable tannins, phenolic acids, and flavonoids.
Table 1: Identification and content (mg/100 g dw) of main compounds of extracts from red (RED EXT1) and yellow (YL EXT2 and YL EXT3) cornelian cherry fruits by using LC-MS and HPLC.

| Compound                      | UV λ<sub>max</sub> (nm) | MS<sup>1</sup> [M−H]<sup>−</sup> / [M−H]<sup>+</sup> (m/z) | MS<sup>2</sup> Other ions (m/z) | RED EXT1 Mg/100 g DW | YL EXT2 Mg/100 g DW | YL EXT3 Mg/100 g DW |
|-------------------------------|--------------------------|--------------------------------------------------------|-------------------------------|----------------------|---------------------|---------------------|
| **IRIDOIDS**                  |                          |                                                        |                               |                      |                     |                     |
| Loganic acid                  | 245                      | 375                                                    | 213                           | 12913.51 ± 159.09     | 22052.09 ± 206.67   | 17986.41 ± 190.37   |
| Sweroside                     | 245                      | 403 [M−H + 46]<sup>−</sup>                             | 195                           | 533.64 ± 14.71        | 0.00                | 1135.86 ± 15.95     |
| Loganin                       | 245                      | 435 [M−H + 46]<sup>−</sup>                             | 227                           | 1285.98 ± 35.44       | 0.00                | 2205.69 ± 30.97     |
| Cornuside                     | 245/273                  | 541                                                    | 169                           | 2140.49 ± 17.96       | 0.00                | 6364.23 ± 50.54     |
| **ANTHOCYANINS**              |                          |                                                        |                               |                      |                     |                     |
| Delphinidin 3-O-galactoside   | 524                      | 463+                                                   | 303+                          | 663.84 ± 7.42         | 0.00                | 0.00                |
| Cyanidin 3-O-galactoside      | 515                      | 449+                                                   | 287+                          | 203.43 ± 8.49         | 0.00                | 0.00                |
| Cyanidin 3-O-robinobioside    | 516                      | 595+                                                   | 287+                          | 301.25 ± 3.26         | 0.00                | 0.00                |
| Pelargonidin 3-O-galactoside  | 501                      | 433+                                                   | 271+                          | 1509.59 ± 23.75       | 0.00                | 0.00                |
| Pelargonidin 3-O-robinobioside| 501                      | 579+                                                   | 271+                          | 268.22 ± 7.44         | 0.00                | 0.00                |
| Cyanidin 4-O-robinobioside    | 523                      | 287+                                                   |                               | 231.34 ± 6.01         | 0.00                | 0.00                |
| Pelargonidin                  | 509                      | 271+                                                   |                               | 300.75 ± 5.47         | 0.00                | 0.00                |
| **PHENOLIC ACIDS**            |                          |                                                        |                               |                      |                     |                     |
| Gallic acid                   | 272                      | 169                                                    |                               | 0.00                 | 263.59 ± 9.94       | 0.00                |
| Trans-catechin acid           | 326                      | 311                                                    | 179/149                       | 101.03 ± 5.76         | 252.58 ± 4.47       | 43.63 ± 3.42        |
| Caffeic acid isomer           | 326                      | 311                                                    | 179/149                       | 562.26 ± 9.56         | 360.82 ± 13.68      | 1102.77 ± 27.14     |
| Cauftaric acid isomer         | 312                      | 295                                                    | 163/149                       | 333.07 ± 8.82         | 36.08 ± 2.19        | 1475.38 ± 36.47     |
| p-Coumaric acid              | 310                      | 163                                                    |                               | 33.58 ± 1.70          | 0.00                | 565.88 ± 12.78      |
| Ellagic acid                  | 254                      | 301                                                    |                               | 146.24 ± 1.06         | 0.00                | 338.53 ± 7.92       |
| **FLAVONOLS**                 |                          |                                                        |                               |                      |                     |                     |
| Quercetin 3-O-glucuronide     | 354                      | 477                                                    | 301                           | 306.24 ± 10.42        | 0.00                | 816.52 ± 13.52      |
| Quercetin 3-O-glucoside       | 353                      | 463                                                    | 301                           | 23.69 ± 2.07          | 0.00                | 0.00                |
| Kaempferol 3-O-galactoside    | 348                      | 447                                                    | 285                           | 235.32 ± 9.97         | 0.00                | 0.00                |
| Kaempferol 3-O-glucuronide    | 351                      | 461                                                    | 285                           | 27.93 ± 2.76          | 0.00                | 0.00                |
| **Total flavonols**           |                          |                                                        |                               | 593.18               | 0.00                | 816.52              |
| Compound                        | UV λ<sub>max</sub> (nm) | MS<sup>1</sup> [M – H]<sup>+</sup> / [M – H]<sup>+</sup> | MS<sup>2</sup> Other ions (m/z) | RED EXT1 | YL EXT2 | YL EXT3 |
|--------------------------------|-------------------------|------------------------------------------------------|--------------------------------|----------|---------|---------|
| Total gemin D isomers          | 265                     | 633                                                  | 301/275/249/169                | 903.33   | 0.00    | 1024.41 |
| Tellimagrandin I               | 267                     | 785                                                  | 633/301/275/249/169            | 276.11   | 0.00    | 437.58  |
| Tellimagrandin II              | 271                     | 937                                                  | 785/633/301/275/249/169        | 595.47   | 0.00    | 0.00    |
| Total camptothin A isomers     | 264                     | 708<sup>–</sup>, 1417                                 | 1247/783/633/301               | 2294.22  | 0.00    | 2699.29 |
| Total cornusiin A isomers      | 273                     | 784<sup>–</sup>, 1569                                 | 935/633/313/301                | 8503.24  | 0.00    | 3385.07 |
| Total cornusiin C isomers      | 268                     | 1176<sup>–</sup>, 2353                                | 786/633/451/301                | 4249.05  | 0.00    | 1551.42 |
| Total hydrolysable tannins     |                         |                                                      | 16821.42                       | 0.00     | 9097.77 |

**HYDROLYSABLE TANNINS**

Abbreviations: (dw): dry weight; (HPLC): high-performance liquid chromatography; (LC-MS): liquid chromatography-mass spectrometry; (m/z): mass-to-charge ratio.
3.2. The Effects of Extracts from Cornelian Cherry Fruits on Osteoblast Differentiation. Further, we investigated the effects of the three cornelian cherries (Cornus mas L.) extracts (RED EXT1, YL EXT2, and YL EXT3) on osteoblast differentiation. Primary osteoblasts isolated from neonatal mouse calvarias were cultured in an osteoblastic induction media containing β-glycerophosphate and ascorbic acid and cotreated with different concentrations of Cornus mas L. extracts (2, 5, and 10 μg/mL) for three days. Treatment with the extracts did not influence the cell viability of primary-cultured osteoblasts, proving that there is no risk of cytotoxicity from these three extracts for primary osteoblasts (Figure 1).

Osteoblast differentiation was evaluated using an ALP activity assay and ALP staining. ALP plays a major role in the regulation of bone formation and mineralization during osteoblast differentiation [25]. Treatment with two of the three extracts, RED EXT1 and YL EXT2, significantly increased ALP activity at a concentration of 5 and 10 μg/mL. EXT3 increased ALP activity only at a concentration of 10 μg/mL (Figure 2). In addition, ALP staining results also showed an increase in ALP staining-positive cells in all three extracts (Figure 3). Although all three cornelian cherry extracts augmented osteoblast differentiation by increasing ALP activity, RED EXT1 and YL EXT2, compared to the YL EXT3, exhibited better efficacy on both ALP activity and staining, probably due to differences in the contents of the bioactive compounds. RED EXT1 consisted of abundant amounts of anthocyanins, a mixture of iridoids and hydrolyzable tannins, moderate amounts of phenolic acids, and small amounts of flavonols. Notably, despite YL EXT2 containing only a single iridoid, loganic acid, and a few phenolic acids, it showed better efficacy than YL EXT3 containing various iridoids and polyphenols. Among the three extracts, only YL EXT2 consisted of gallic acid and the highest amount of loganic acid, suggesting that these two compounds may be responsible for the better effect of YL EXT2 on the augmentation of osteoblast differentiation.

Next, we further investigated the effect of the three extracts on the mRNA expression changes of osteoblastogenesis biomarker genes: Alpl and runt-related transcription factor 2 (Runx2). Alpl is a bone-specific isoform localized on the surface of osteoblasts and is a sensitive indicator of bone metabolism [26]. Runx2 is an essential transcription factor for osteoblast differentiation during the early stages of bone formation [27]. These factors play an important role in the transcriptional regulation of bone formation and mineralization [28]. Primary cultured preosteoblasts were treated with the three extracts (RED EXT1, YL EXT2, and YL EXT3) at 10 μg/mL for 3 days, and thereafter the mRNA expression levels were analyzed using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). All three extracts increased the mRNA expression levels of Alpl and Runx2 compared to that in the osteoblast-induced controls (Figure 4). These results suggest that Cornus mas L. extracts may enhance osteoblast differentiation by upregulating the expression of the Alpl and Runx2 genes at the transcription level. There was no difference in the augmentation efficacy of the three extracts for the expression of Alpl and Runx2. Taken together, these results showed that the three extracts from the Cornus mas L. fruits exhibited bone formation effects.

3.3. The Effects of Cornelian Cherry Fruits Extracts on Osteoclast Differentiation. Imbalanced homeostasis between bone resorption and bone formation results in weak and fragile bones associated with the risk of developing bone diseases [1, 29]. Dysregulation of osteoclast differentiation is a key factor causing abnormal bone metabolism and leading to osteoporosis [30–33]. Previous studies have suggested that a decrease in osteoclast differentiation is one of the main strategies for the prevention and treatment of osteoporosis [34–37]. Therefore, we investigated the effects of the three Cornus mas L. extracts (RED EXT1, YL EXT2, and YL EXT3) on osteoclast differentiation. Mouse bone marrow-derived osteoclast-lineage primary-cultured monocytes were isolated from the femoral bone. To induce osteoclast
differentiation, the monocytes were treated with M-CSF and RANKL [38]. Treatment with the three extracts at three different concentrations (2, 5, and 10 μg/mL) for 5 days did not affect cell viability, indicating that there is no risk of cytotoxicity for primary monocytes from these three extracts (Figure 5).

During osteoclast differentiation, TRAP stimulates the migration of osteoclasts into the bone resorption region [39]. TRAP is considered to be an important osteoclast histochemical indicator during skeletal development [40]. To assess osteoclast differentiation, TRAP activity assays and TRAP staining were used in this study. All three *Cornus mas* L. extracts showed a significant decrease in TRAP activity in the primary monocytes of mice at 10 μg/mL, and RED EXT1 and YL EXT2 also showed a significant decrease at 2 and 5 μg/mL (Figure 6). Among the three extracts, RED EXT1 exhibited the best efficacy, higher as compared to extracts from yellow fruits (Figure 6(a)). The results of the TRAP staining further revealed that the number of TRAP-positive multinuclear cells was significantly decreased after treatment with the three extracts (Figure 7). These results indicate that the *Cornus mas* L. extracts prevented osteoclast differentiation by decreasing TRAP activity, resulting in the attenuation of osteoclastic bone resorption. The best inhibitions of TRAP activity and TRAP staining were observed...
from the RED EXT1. Anthocyanins were only detected in RED EXT1, suggesting that the pronounced inhibitory effect of RED EXT1 on TRAP activity and staining could be attributed to the anthocyanins.

Bone resorption resulting from osteoclast differentiation is regulated by osteoclast-related enzymes, such as tartrate-resistant acid phosphatase 5 (Acp5) and cathepsin K (Ctsk) [41]. The transcription factor, nuclear factor of activated T cells 1 (Nfatc1), is known to promote osteoclast differentiation [42]. To confirm the inhibitory effects of Cornus mas L. extracts on osteoclast differentiation, the mRNA expression changes of osteoclastogenesis-related genes (Nfatc1, Alpl, and Runx2) were measured by qRT-PCR using gene-specific primers. CM, Cornus mas L. extract treatment. *p < 0.05 vs. induction (Student’s t-test).

Figure 4: The effects of the Cornus mas L. extracts on the expression changes of osteoblast-specific genes in the primary osteoblasts of mice. The primary osteoblasts were incubated with ascorbic acid (50 μg/mL) and β-glycerophosphate (10 mM) with or without 10 μg/mL of Cornus mas L. extracts for 3 days. Osteoblast-specific genes including Alpl and Runx2 were measured by qRT-PCR using gene-specific primers. CM, Cornus mas L. extract treatment. *p < 0.05 vs. induction (Student’s t-test).
Ctsk, and Acp5) were investigated. The results showed that treatment with all three extracts significantly decreased the mRNA expression levels of Nfatc1, Ctsk, and Acp5 at a concentration of 10 μg/mL (Figure 8). This finding indicates that Cornus mas L. extracts inhibit osteoclast differentiation through the decreased expression of these osteoclastogenic genes. Being consistent with the results from TRAP activity assays and staining, RED EXT1 was more effective than YL EXT2 and YL EXT3 in this experiment. These results suggest that the anthocyanins contained in RED EXT1 play a crucial role in the osteoclastogenesis reduction effects of Cornus mas L. extracts.

We analyzed and compared the constituents in a total of 41 compounds of iridoids, phenolic acids, flavonols, anthocyanins, and tannins in the three extracts obtained from red (RED EXT1) and yellow (YL EXT2 and YL EXT3) Cornus mas L. fruits using LC-MS and HPLC (Table 1). Although we did not examine the identified compounds directly, comparing the antiosteoporotic efficacies of the three extracts and reviewing the literature, it is possible to interpret which compounds are responsible for the antosteoporotic effects of the extracts.

Our results are consistent with our recently published studies showing the antiosteoporotic effects of the cornelian cherry extract containing loganic acid. Park et al. [8] found that loganic acid isolated from Gentiana lutea L. roots extract significantly stimulated osteoblast differentiation by increasing ALP activity and upregulating the mRNA expression of Alpl, Bglap, and Sp7. In animal experiments, extracts from the cornelian cherry ameliorated the harmful changes in the bone turnover markers and bone mineral density (BMD) of hypercholesterolemic-diet rabbits [15] and...
counteracted the decrease in BMD and bone flexural strength of ovariectomized mice [8, 43]. In addition, phenolic acids, such as gallic acid and ellagic acid, were also revealed to have antosteoporotic effects through the RANKL-related pathway [6, 44]. Unexpectedly, the antosteoporotic effects of the YL EXT2 extract, containing only a single iridoid, loganic acid, were better than that of the YL EXT3 extract containing various iridoids including loganic acid, loganin, cornuside, and sweroside. Loganin and sweroside are known to have beneficial bone remodeling roles from previous studies [45, 46], and cornuside also reportedly has an antosteoporotic effect [47]. We may try to explain the better antosteoporotic effect of YL EXT2 because it contains uniquely gallic acid and a higher amount of loganic acid than YL EXT3. The exact molecular mechanism of the antosteoporotic effect proposed for loganic acid requires further investigation, although anti-inflammatory activity and impact on described transcription factors and genes seem to be involved. Regarding anthocyanins, although previous studies proved their possible inhibitory effect on osteoclast differentiation through the RANKL-mediated pathway [48, 49], only a few recently published studies suggest their possible enhanced effect on osteoclast differentiation and osteoblast-related mineralization through the ERK1/2 signaling pathway [50] or the inhibition of GSK-3β and subsequent activation of β-catenin [4]. Other proposed antosteoporotic mechanisms of action of anthocyanins include their anti-inflammatory and antioxidant properties [50]. Inflammatory factors, glutathione depletion, and redox stress can inhibit the synthesis and differentiation of osteoblasts and bone mineralization. Polyphenols, especially anthocyanins can reduce these processes by the activation of sirtuin type 1 deacetylase expression with subsequent upregulation of ALP and Runx2 [51].

Phenolic acids, such as gallic acid or ellagic acid showed in other studies an antosteoporotic effect through the RANKL-related pathway [6, 44]. Nevertheless, in our study, gallic acid was found only in small amounts in YL EXT2 and ellagic acid was present in small amounts in RED EXT1, and moderate amounts of it were found in YL EXT3. Cornelian cherry extracts, especially YL EXT3, contained more caftaric acid and coumaric acid but we did not find information about their potential impact on bone metabolism in the available data. Moreover, YL EXT3 proved the weakest antosteoporotic activity in tests conducted as part of this study.

In previous studies, flavonoids such as kaempferol and quercetin proved to have an antosteoporotic effect. They decreased osteoclastogenesis, mainly through RANKL-related pathways, and promoted osteoblast differentiation [7, 52]. Proposed mechanisms responsible for the benefits of kaempferol in senile osteoporosis include also anti-inflammatory and antioxidative properties and regulation of osteoblasts’ and osteoclasts’ apoptosis [53]. Kaempferol and quercetin in the forms of 3-O-glucuronide, 3-O-galactoside, or O-glucoside were found in small amounts in RED EXT1, but their potential contribution to the antosteoporotic effect is difficult to elucidate. YL EXT3 contained moderate amounts of quercetin 3-O-glucuronide but had the lowest antosteoporotic potency. YL EXT2 did not contain flavonoids. An accurate assessment of possible synergism between anthocyanin or iridoids and flavonoids would require additional studies using different compositions of extracts and higher doses of flavonols.

Hydrolysable tannins were most abundant in RED EXT1, but substantial amounts were also found in YL EXT3; they were not present at all in YL EXT2. Previous studies have shown the antosteoporotic potential of hydrolysable tannins. Corilagin inhibited osteoclastogenesis via downregulation of the NF-κB and PI3K/AKT signaling
pathways in murine bone marrow macrophage cells (BMMs) [54]. Other studies have proven that geraniin enhanced proliferation and osteoblastic differentiation of bone marrow-derived stem cells (BMSCs) in both normal and osteoporotic rats through the activation of Wnt/β-catenin signaling [55]; it also reduced bone turnover marker levels, increased osteoprotegerin (OPG), decreased RANKL, and increased the OPG/RANKL ratio in rats with ovariectomy-induced osteoporosis [9]. Hydrolysable tannins can also hydrolyze to phenolic acids, such as gallic acid and ellagic acid, and potentially exert an effect through these substances. Both RED EXT1 and YL EXT3 extracts were found to contain gemicin, tellimagrandin, camptothin, and cornusiin isomers. It is worth emphasizing that cornusiin isomers were about 3 times more abundant in RED EXT1 than in YL EXT3. Although RED EXT1 proved to have strong antiosteoporotic effects, it is difficult to understand why YL EXT3 showed a lower efficacy than even YL EXT2, which did not contain any hydrolysable tannins at all. It is difficult to explain this finding, especially that we investigated mixtures of substances; contribution of hydrolysable tannins to antiosteoporotic effects may depend on its different structures and doses, and interactions with other extracts’ constituents. It seems that among hydrolysable tannins detected in our study, the most promising substance is cornusiin, but further investigations with isolated compounds are needed.

Collectively, this study demonstrated that Cornus mas L. extracts enhanced osteoblastogenesis and reduced osteoclastogenesis. Comparison of the antiosteoporotic efficacy of the three extracts and analysis of the constituents in the polyphenols and iridoids, may help elucidate the molecular mechanisms responsible for the antiosteoporotic effects. Among the three extracts, the RED EXT1 extract uniquely

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**Figure 8**: The effects of *Cornus mas* L. extracts on the expression changes of osteoclast-specific genes in the primary osteoclasts of mice. The primary monocytes were incubated with M-CSF (50 ng/mL) and RANKL (50 ng/mL) with or without 10 μg/mL of *Cornus mas* L. extracts for 5 days. Osteoclast-specific genes including Nfatc1, Ctsk, and Acp5 were measured with qRT-PCR using gene-specific primers. CM; *Cornus mas* L. extract. *p < 0.05 vs. induction (Student’s t-test).
comprised several anthocyanins and the highest variety of polyphenols and iridoids; the YL EXT2 extract uniquely contained gallic acid and the highest amount of an irodoid, loganic acid (Table 1). RED EXT1, and YL EXT2 showed similar efficacy to each other, and better efficacy than YL EXT3, in the augmentation of osteoblast differentiation (Figures 2–4). These results strongly suggest that the iridoids, such as loganic acid, present in the extracts, may be responsible for the increase in osteoblast differentiation via the upregulation of Alpl and Runx2 expressions at the transcription level. Meanwhile, RED EXT1 showed the best efficacy in inhibiting osteoclast differentiation (Figures 6–8), strongly suggesting that the anthocyanins present in the extracts may be responsible for the decrease in osteoclast differentiation via the downregulation of Nfatc1, CtsK, and Acp5 expressions at the transcription level. A suggested molecular mechanism for Cornus mas L. extracts in the regulation of osteoblast and osteoclast differentiation is described in Figure 9.

4. Conclusions

The main conclusions of our study were that three extracts from red and yellow Cornus mas L. fruits containing different constituents of iridoids and polyphenols such as anthocyanins, hydrolysable tannins, phenolic acids, and flavonoids had antiosteoporotic effects. All these extracts significantly enhanced osteoblast differentiation and significantly reduced osteoclast differentiation. The antiosteoporotic effects of the RED EXT1 extract, containing several anthocyanins and iridoids, exerted via reduction of osteoclast differentiation, were better than that of YL EXT2 and YL EXT3. These results suggest that the red Cornus mas L. extract could be a promising phytomedicine candidate for the prevention and treatment of osteoporosis.
Data Availability

All the data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest

We confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Authors’ Contributions

Eunkuk Park contributed in drafting of the article, literature search and project administration, and critical revision of manuscript and its approval to be submitted. Alicja Z. Kucharska worked on the plant materials and samples preparation of cornelian cherry extracts, acquisition of laboratory data – identification and determination of compounds by LC-MS and HPLC-PDA, drafting in manuscript part of the methods section concerning plant materials, samples preparation and LC-MS and HPLC-PDA, and critical revision of manuscript and its approval to be submitted. Dominika Przybylska worked on the plant materials and samples preparation of cornelian cherry extracts, acquisition of laboratory data – identification and determination of compounds by LC-MS and HPLC-PDA, and critical revision of manuscript and its approval to be submitted. Narcyz Piórecki contributed in providing cornelian cherry materials and samples preparation of cornelian cherry extracts, acquisition of laboratory data – identification and determination of compounds by LC-MS and HPLC-PDA, and critical revision of manuscript and its approval to be submitted. Tomasz Sozański was assigned in the main conception of the study, participation in acquisition of laboratory data, literature search, analysis and interpretation of data collected, major contribution in drafting of the article, and critical revision of manuscript and its approval to be submitted. Seon-Yong Jeong worked on the conceptualization and design of the work, writing and editing of manuscript, and critical revision of manuscript and its approval to be submitted. Eunkuk Park, Tomasz Sozański, and Chang-Gun Lee contributed equally to this work.

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References

[1] J. M. Kim, C. Lin, Z. Stavre, M. B. Greenblatt, and J. H. Shim, “Osteoblast-osteoclast communication and bone homeostasis,” Cell, vol. 9, no. 9, p. 2073, 2020.
[2] M. Rondonelli, M. A. Faliva, G. C. Barrile et al., “Nutrition, physical activity, and dietary supplementation to prevent bone mineral density loss: a food pyramid,” Nutrients, vol. 14, no. 1, p. 74, 2022.
[3] B. Xia, R. Zhu, H. Zhang et al., “Lycopene improves bone quality and regulates AGE/RAGE/NF-κB signaling pathway in high-fat diet-induced obese mice,” Oxidative Medicine and Cellular Longevity, vol. 2022, Article ID 3697067, 14 pages, 2022.
[4] W. A. H. M. Karunarathne, I. M. N. Molagoda, K. T. Lee, Y. H. Choi, C. Y. Jin, and G. Y. Kim, “Anthocyanin-enriched polyphenols from Hibiscus syriacus L. (Malvaceae) exert anti-osteoporosis effects by inhibiting GSK-3 β and subsequently activating β-catenin,” Phytomedicine, vol. 91, article 153721, 14 pages, 2021.
[5] Z. Ren, N. A. Raut, T. O. Lawal, S. R. Patel, S. M. Lee, and G. B. Mahady, “Peonidin-3-O-glucoside and cyanidin increase osteoblast differentiation and reduce RANKL-induced bone resorption in transgenic medaka,” Phytotherapy Research, vol. 35, no. 11, pp. 6255–6269, 2021.
[6] X. Lin, G. Yuan, Z. Li et al., “Ellagic acid protects ovariectomy-induced bone loss in mice by inhibiting osteoclast differentiation and bone resorption,” Journal of Cellular Physiology, vol. 235, no. 9, pp. 5951–5961, 2020.
[7] M. Martiniakova, M. Babikova, J. Blahova, V. Kovacova, and R. Omelka, “The role of macronutrients, micronutrients and flavonoid polyphenols in the prevention and treatment of osteoporosis,” Nutrients, vol. 14, no. 3, p. 523, 2022.
[8] E. Park, C. G. Lee, E. Lim et al., “Osteoprotective effects of loganic acid on osteoblastic and osteoclastic cells and osteoporosis-induced mice,” International Journal of Molecular Sciences, vol. 22, no. 1, p. 233, 2020.
[9] X. Wang, M. Wang, X. Cui et al., “Antiosteoporosis effect of geranin on ovariectomy-induced osteoporosis in experimental rats,” Journal of Biochemical and Molecular Toxicology, vol. 35, no. 6, pp. 1–8, 2021.
[10] J. Y. Kim, Y.-K. Kim, M. K. Choi, J. Oh, H. B. Kwak, and J.-J. Kim, “Effect of Cornus officinalis on receptor activator of nuclear factor-kappaB ligand (RANKL)-induced osteoclast differentiation,” Journal of Bone Metabolism, vol. 19, no. 2, pp. 121–127, 2012.
[11] T. Sozański, A. Z. Kucharska, A. Szumnny et al., “The protective effect of the Cornus mas fruits (cornelian cherry) on hypertriglyceridemia and atherosclerosis through PPAR α activation in hypercholesterolemic rabbits,” Phytomedicine: International Journal of Phytotherapy and Phytopharmacology, vol. 21, no. 13, pp. 1774–1784, 2014.
[12] T. Sozański, A. Z. Kucharska, A. Rapak et al., “Iridoid-loganic acid versus anthocyanins from the Cornus mas fruits (cornelian cherry): common and different effects on diet-induced atherosclerosis, PPARs expression and inflammation,” Atherosclerosis, vol. 254, pp. 151–160, 2016.
Oxidative Medicine and Cellular Longevity

[13] T. Sozański, A. Z. Kucharska, J. Wiśniewski et al., “The iridoid loganic acid and anthocyanins from the cornelian cherry (Cornus mas L.) fruit increase the plasma L-arginine/ADMA ratio and decrease levels of ADMA in rabbits fed a high-cholesterol diet,” *Phytotherapy Research and Phytopharmacology*, vol. 52, pp. 1–11, 2019.

[14] T. Sozański, A. Z. Kucharska, S. Dzimira et al., “Loganic acid and anthocyanins from cornelian cherry (Cornus mas L.) fruits modulate diet-induced atherosclerosis and redox status in rabbits,” *Advances in Clinical Experimental Medicine*, vol. 27, no. 11, pp. 1505–1513, 2018.

[15] B. Nowak, A. Matuszewska, M. Tomanik et al., “Cornelian cherry extract ameliorates osteoporosis associated with hypercholesterolemia in New Zealand rabbits,” *Advances in Clinical Experimental Medicine*, vol. 29, no. 12, pp. 1389–1397, 2020.

[16] R. Omelka, J. Blahova, V. Kovacova et al., “Cornelian cherry pulp has beneficial impact on dyslipidemia and reduced bone quality in Zucker diabetic fatty rats,” *Animals*, vol. 10, no. 12, p. 2435, 2020.

[17] L. Lewandowski, I. Bednarz-Misa, A. Z. Kucharska et al., “Cornelian cherry (Cornus mas L.) extracts exert cytotoxicity in two selected melanoma cell lines-a factorial analysis of time-dependent alterations in values obtained with SRB and MTT assays,” *Molecules*, vol. 27, no. 13, p. 4193, 2022.

[18] D. Przybylska, A. Z. Kucharska, I. Cybulska, T. Sozański, N. Piórecki, and I. Fecka, “Cornus mas L. stones: a valuable by-product as an ellagitannin source with high antioxidant potential,” *Molecules*, vol. 25, no. 20, p. 4646, 2020.

[19] R. Spychaj, A. Z. Kucharska, A. Szumnny, D. Przybylska, E. Pejcz, and N. Piórecki, “Potential valorization of Cornelian cherry (Cornus mas L.) stones: Roasting and extraction of bioactive and volatile compounds,” *Food Chemistry*, vol. 358, article 129802, 12 pages, 2021.

[20] M. L. Doolittle, C. L. Ackert-Bicknell, and J. H. Jonason, “Isolation and culture of neonatal mouse calvarial osteoblasts,” *Methods in Molecular Biology*, vol. 2230, pp. 425–436, 2021.

[21] M. J. Oliva-Martin, L. I. Sánchez-Abarca, A. Carrillo-Jiménez, J. A. Pérez-Simón, and J. L. Venero, “Evaluation of a method for murine monocyte isolation by bone marrow depletion,” *Analytical Biochemistry*, vol. 480, pp. 42–48, 2015.

[22] A. Z. Kucharska, A. Szumnny, A. Sokół-Lętowska, N. Piórecki, and S. V. Klymenko, “Iridoids and anthocyanins in cornelian cherry (Cornus mas L.) cultivars,” *Journal of Food Composition and Analysis*, vol. 40, pp. 95–102, 2015.

[23] O. Drydzan, I. Bila, A. Z. Kucharska, I. Brodyak, and N. Sybirna, “Antidiabetic effects of extracts of red and yellow fruits of cornelian cherries (Cornus mas L.) on rats with streptozotocin-induced diabetes mellitus,” *Food & Function*, vol. 10, no. 10, pp. 6459–6472, 2019.

[24] M. Szandra-Bender, M. Rutkowska, A. Merwid-Ląd et al., “Cornelian cherry iridoid-polyphenolic extract improves mucosal epithelial barrier integrity in rat experimental colitis and exerts antimicrobial and antiadhesive activities *In Vitro*,” *Oxidative Medicine and Cellular Longevity*, vol. 2020, Article ID 7697851, 19 pages, 2020.

[25] Y. Sugawara, K. Suzuki, M. Koshikawa, M. Ando, and J. Iida, “Necessity of enzymatic activity of alkaline phosphatase for mineralization of osteoblastic cells,” *The Japanese Journal of Pharmacology*, vol. 88, no. 3, pp. 262–269, 2002.

[26] J. Masrour Roudsari and S. Mahjoub, “Quantification and comparison of bone-specific alkaline phosphatase with two methods in normal and Pagel’s specimens,” *Caspian Journal of Internal Medicine*, vol. 3, no. 3, pp. 478–483, 2012.

[27] T. Komori, “Regulation of proliferation, differentiation and functions of osteoblasts by RUNx2,” *International Journal of Molecular Sciences*, vol. 20, no. 7, p. 1694, 2019.

[28] W. Huang, S. Yang, J. Shao, and Y.-P. Li, “Signaling and transcriptional regulation in osteoblast commitment and differentiation,” *Frontiers in Bioscience-Landmark*, vol. 12, no. 8-12, pp. 3068–3092, 2007.

[29] K. A. Abu-Bakar, R. R. A. Atia, H. O. Mohammed, R. S. Ramanadan, and N. A. Mohammed, “The potential anti-osteoporotic effect of exercise—induced increased preptin level in ovariectomized rats,” *Anatomical Science International*, pp. 1–14, 2022.

[30] J. F. Charles and A. O. Aliprantis, “Osteoclasts: more than ‘bone eaters’,” *Trends in Molecular Medicine*, vol. 20, no. 8, pp. 449–459, 2014.

[31] H. Kitaura, A. Marahleh, F. Ohori et al., “Osteocyte-related cytokines regulate osteoclast formation and bone resorption,” *International Journal of Molecular Sciences*, vol. 21, no. 14, p. 5169, 2020.

[32] Y. Kohara, R. Haraguchi, R. Kitazawa, and S. Kitazawa, “Knockdown of Lrp1 in RAW264 cells inhibits osteoclast differentiation and osteoclast-osteoblast interactions *in vitro*,” *Biochemical and Biophysical Research Communications*, vol. 523, no. 4, pp. 961–965, 2020.

[33] Y.-H. Hwang, S.-A. Jang, A. Lee et al., “Polysaccharides isolated from lotus leaves (LLEP) exert anti-osteoporotic effects by inhibiting osteoclastogenesis,” *International Journal of Biological Macromolecules*, vol. 161, pp. 449–456, 2020.

[34] Y. Yang and B. Yang, “Anti-osteoporosis effect of Ganoderma (lingzhi) by inhibition of osteoclastogenesis,” *Advances in Experimental Medicine and Biology*, vol. 1182, pp. 263–269, 2019.

[35] Y. Nara, H. Kitaura, S. Ogawa et al., “Anti-c-fms antibody prevents osteoclast formation and bone resorption in co-culture of osteoblasts and osteoclast precursors *in vitro* and in ovariectomized mice,” *International Journal of Molecular Sciences*, vol. 21, no. 17, p. 6120, 2020.

[36] Y. Zhuang, X. Sun, B. Liu, H. Hou, and Y. Sun, “Effects of rambutan peel (Nephelium lappaceum) phenolic extract on RANKL-induced differentiation of RAW264.7 cells into osteoclasts and retinoic acid-induced osteoporosis in rats,” *Nutrients*, vol. 12, no. 4, p. 883, 2020.

[37] C. G. Lee, J. Kim, S. H. Yun et al., “Anti-osteoporotic effect of morroniside on osteoblast and osteoclast differentiation in vitro and ovariectomized mice in vivo,” *International Journal of Molecular Sciences*, vol. 22, no. 19, article 101642, 2021.

[38] D. S. Györi and A. Mócsai, “Osteoclast signal transduction during bone metastasis formation,” *Frontiers in Cell and Developmental Biology*, vol. 8, pp. 507–515, 2020.

[39] H. Drissi and A. Sanjay, “The multifaceted osteoclast: far and beyond bone resorption,” *Journal of Cellular Biochemistry*, vol. 117, no. 8, pp. 1753–1756, 2016.

[40] A. R. Hayman, “Tartrate-resistant acid phosphatase (TRAP) and the osteoclast/immune cell dichotomy,” *Autoimmunity*, vol. 41, no. 3, pp. 218–223, 2008.
L. Zhu, Y. Tang, X.-Y. Li et al., "Osteoclast-mediated bone resorption is controlled by a compensatory network of secreted and membrane-tethered metalloproteinases," Science Translational Medicine, vol. 12, no. 529, article eaaw6143, p. 16, 2020.

Q. Zhao, X. Wang, Y. Liu, A. He, and R. Jia, "NFATc1: functions in osteoclasts," International Journal of Biochemistry & Cell Biology, vol. 42, no. 5, pp. 576–579, 2010.

B. Nowak, A. Matuszewksa, A. Szeląg et al., "Cornelian cherry (Cornus mas L.) extract reduces cardiovascular risk and prevents bone loss in ovariectomized Wistar rats," Journal of Functional Foods, vol. 90, article 104974, p. 9, 2022.

S. Chauhan, A. Sharma, N. K. Upadhyay, G. Singh, U. R. Lal, and R. Goyal, "In-vitro osteoblast proliferation and in-vivo anti-osteoporotic activity of Bombax ceiba with quantification of lupeol, gallic acid and β-sitosterol by HPTLC and HPLC," BMC Complementary and Alternative Medicine, vol. 18, no. 1, p. 233, 2018.

L. Y. Choi, M. H. Kim, and W. M. Yang, "Promotion of osteogenesis by sweroside via BMP2-involved signaling in postmenopausal osteoporosis," Phytotherapy Research, vol. 35, no. 12, pp. 7050–7063, 2021.

[52] M. Li, W. Wang, P. Wang, K. Yang, H. Sun, and X. Wang, "The pharmacological effects of morroniside and loganin isolated from Liuweidihuang Wan, on MC3T3-E1 cells," Molecules, vol. 15, no. 10, pp. 7403–7414, 2010.

F. Gao, S.-L. Xia, X.-H. Wang, X. X. Zhou, and J. Wang, "Cornuside I promoted osteogenic differentiation of bone mesenchymal stem cells through PI3K/Akt signaling pathway," Journal of Orthopaedic Surgery and Research, vol. 16, no. 1, p. 397, 2021.

J. Cheng, L. Zhou, Q. Liu et al., "Cyanidin chloride inhibits ovariectomy-induced osteoporosis by suppressing RANKL-mediated osteoclastogenesis and associated signaling pathways," Journal of Cellular Physiology, vol. 233, no. 3, pp. 2502–2512, 2018.

N. Imangali, Q. T. Phan, G. Mahady, and C. Winkler, "The dietary anthocyanin delphinidin prevents bone resorption by inhibiting RANKL-induced differentiation of osteoclasts in a medaka (Oryzias latipes) model of osteoporosis," Journal of Fish Biology, vol. 98, no. 4, pp. 1018–1030, 2021.

B. Hu, L. Chen, Y. Chen, Z. Zhang, X. Wang, and B. Zhou, "Cyanidin-3-glucoside regulates osteoblast differentiation via the ERK1/2 signaling pathway," ACS Omega, vol. 6, no. 7, pp. 4759–4766, 2021.

V. Domazetovic, G. Marcucci, I. Falsetti et al., "Blueberry juice antioxidants protect osteogenic activity against oxidative stress and improve long-term activation of the mineralization process in human osteoblast-like SaOS-2 cells: involvement of SIRT1," Antioxidants, vol. 9, no. 2, p. 125, 2020.

B. Nowak, A. Matuszewksa, A. Nikodem et al., "Oral administration of kaempferol inhibits bone loss in rat model of ovariectomy-induced osteopenia," Pharmacological Reports, vol. 69, no. 5, pp. 1113–1119, 2017.

F. Tang, P. Zhang, W. Zhao et al., "Research on the mechanism of kaempferol for treating senile osteoporosis by network pharmacology and molecular docking," Evidence-Based Complementary and Alternative Medicine, vol. 2022, Article ID 6741995, 12 pages, 2022.