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Sustainable Bioactive Composite of *Glehnia littoralis* Extracts for Osteoblast Differentiation and Bone Formation

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Abstract: Different bone-related diseases are mostly caused by the disruption of bone formation and bone resorption, including osteoporosis. Traditional medicinal literature has reported the possible anti-osteoporotic properties of *Glehnia littoralis*. However, the chemical compounds in extracts that are responsible for bone metabolism are poorly understood. The present study aimed to explore and compare the coumarin-based compounds present in *G. littoralis* extracts, the antioxidant activities, and the anti-osteoporotic properties of different extracts of *G. littoralis* (leaf and stem, fruit, whole plant, and root extracts) on bone metabolism. This study analyzed *G. littoralis* extract effects on the proliferation and osteoblastic differentiation of MC3T3-E1 osteoblasts. Among the different tested samples, stem extracts had the highest scopoletin (53.0 mg/g), and umbelliferone (1.60 mg/g). The significantly ($p < 0.05$) highest amounts of imperatorin (31.9 mg/g) and phellopterin (2.3 mg/g), were observed in fruit and whole plant extracts, respectively. Furthermore, the results confirmed alkaline phosphatase activity, collagen synthesis, mineralization, osteocalcin content, and osterix and RUNX2 expression. *G. littoralis* extracts at concentrations greater than 20 µg/mL had particularly adverse effects on MC3T3-E1 cell viability and proliferation. Notably, cell proliferation was significantly elevated at lower *G. littoralis* concentrations. Comparatively, 0.5 µg/mL stem had a higher osteocalcin content. Of the four extract types, stem showed a higher collagen synthesis effect at concentrations of 0.5–5 µg/mL. Except for fruit extracts, *G. littoralis* extract treatment significantly elevated osterix gene expression. All *G. littoralis* extracts increased RUNX2 gene expression. The results described here indicate that *G. littoralis* ethanolic extracts can effectively prevent osteoporosis.

Keywords: *Glehnia littoralis*; osteoblast differentiation; collagen synthesis; alkaline phosphatase activity; osteoblast; osterix expression; RUNX2 expression

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1. Introduction

Osteoporosis is an osteometabolic disorder caused by an imbalance in bone formation by osteoblasts and bone resorption by osteoclasts that make up bones [1,2], which leads to fragility, increased risk of fractures, and threatens mobility in the elderly [3]. According to the International Osteoporosis Foundation (IOF), approximately 200 million people worldwide suffer from osteoporosis [4,5]. Recent studies suggest that increases in inflammation-related cytokine secretion, the number of macrophages, and leukotriene B4, an inflammation-inducing factor [6–9], cause this disease. In women, osteoporosis is associated with a sharp decline in estrogen secretion in the postmenopausal stage, known as postmenopausal osteoporosis [10]. Synthetic therapeutic agents, such as parathyroid hormones, bisphosphonates, selective estrogen receptor modulators (SERMs), and hormone replacement therapy (HRT), are used to treat osteoporosis [11]. However, these drugs can have side effects, including hypercalcemia and osteosarcoma in postmenopausal

women [12], esophageal gastric irritation, and cancer [13], and they can increase the chance of strokes, breast cancer, and coronary heart diseases [14]. Thus, it would be beneficial to discover natural anti-osteoporotic agents that minimize bone loss in postmenopausal women.

Osteoblast cells are critical in bone metabolism and are responsible for bone matrix synthesis and mineralization [15]. Cell culture is widely used to assess the activity of a substance in vitro, such as its osteoinductive potential. In particular, MC3T3 pre-osteoblasts have methodological advantages and facilities [16,17], meaning the differentiation of these cells into mature osteoblasts can be easily recognized by markers of osteoblastic metabolism, such as alkaline phosphatase (ALP), and by the degree of extracellular matrix (ECM) mineralization [18]. ALP is the most widely used biochemical marker for estimating osteoblastic activity [19]. This enzyme is associated with the ECM mineralization process and osteoblastic differentiation, as it is responsible for the maturation of the matrix, which will later be mineralized [18,20].

Current research focuses on natural materials and phytoestrogens for bone formation and resorption pathways related to bone metabolism. There is a constant search for alternatives that can help bone healing, in cases of injury, or favor rehabilitating the quality of the bone tissue. Several recent reports have indicated that phytoestrogen compounds in food and plants can effectively suppress the secretion of inflammation-inducing factors and inflammation-related cytokines associated with osteoporosis [21,22], and enhance or stimulate osteoblast activity [23]. Phytoestrogens act like estrogen and exist in the form of flavonoids, lignins, isoflavones, and coumestans, which share structural and functional similarities with synthetic estrogens [24,25]. The present study aimed to research therapeutic alternatives to treat osteoporosis based on drugs obtained from natural sources, mainly by observing their lower cost and incidence of adverse effects when compared to synthetic drugs.

Glehnia littoralis Fr. Schmidt et Miquel, a perennial marine herbaceous plant belonging to the Apiaceae family, is native to the sandy coastal area of eastern Asia, mainly Japan, Korea, China, Manchuria, Sakhalin, Okhotsk, the Kuril Islands, and North America from California to Alaska [26,27]. The leaves and flower buds are edible, their rhizomes and roots are used traditionally to treat lung diseases, tuberculosis, coughs, hemoptysis, and dyspnea [28,29], and they have diaphoretic, antipyretic, and analgesic effects [30,31]. Previous studies have reported the presence of phytochemicals such as phenolic acids, flavonoids, pyranocoumarins, and polysaccharides in *G. littoralis* extracts, which have various biological activities, including antitumor, antimicrobial, antioxidant, blood circulation-promoting and immunomodulatory properties [29]. Moreover, several previous studies have shown that these phytochemicals are effective in bone formation [32]. To our knowledge, no studies have reported the use of *G. littoralis* extracts as potential natural therapeutic agents to prevent osteoporosis.

Therefore, the main objectives of the present study were to identify and select the toxicity of different *G. littoralis* plant parts for use in the differentiation analysis of an MC3T3-E1 cell culture through a cell viability assay. In addition, this study investigated the proliferative potential of MC3T3-E1 cells treated with *G. littoralis* using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This study also compared the degree of osteoblastic differentiation between control cells and those treated with different *G. littoralis* concentrations by quantifying ALP. Finally, we evaluated the degree of matrix mineralization formed by MC3T3-E1 cells treated with different *G. littoralis* plant parts and concentrations to confirm its applicability as a natural material for osteoblast differentiation and bone formation.

2. Materials and Methods

2.1. Chemicals

All the chemicals and solvents used in the experiments were of analytical grade. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant Material and Extract Preparation

The *G. littoralis* used in the experiment was grown and harvested from a field in Gangneung-si, Gangwon-do (Slonaemall, Gangneung, Republic of Korea) at 37°45′06″ N latitude and 128°52′38″ E longitude, in October 2020. The leaves, roots, fruits, and stems of the *G. littoralis* were washed with purified water and separated. The collected samples were dried at room temperature for 72 h. Approximately 2 g of the finely ground samples and placed in a conical flask containing 40 mL of 80% (*v/v*) ethanol. The mixture was filtered through filter paper to remove debris, and the solvents were evaporated at 41 °C in a rotary vacuum evaporator (Eyela, SB-1300, Shanghai Eyela Co., Ltd., Shanghai, China) and then lyophilized using a freeze dryer (PVTFD 300R) (IlShinBioBase, Yangju, Republic of Korea). The collected extracts were mixed with 80% methanol and stored in a refrigerator at 4 °C until further analysis.

2.3. Determination of Total Phenolic Content (TPC)

The total phenolic content of different samples was measured by following the Folin-Ciocalteu procedure, the method Singleton and Rossi [33] described previously. An aliquot of 100 µL of the plant extract (at a concentration of 1 mg mL^{-1}) and 500 µL of the Folin-Ciocalteu reagent (1:3 *v/v*) were mixed with 500 µL distilled water in a test tube and shaken for 5 min at room temperature. Then, 500 µL sodium carbonate (10%) was added to the solution, and the mixture was left to rest for 1 h. Deionized distilled water served as a blank. Then, the absorbance of the obtained mixture was taken using a ultraviolet-visible (UV-Vis) spectrophotometer (Jasco V530 UV-VIS spectrophotometer, Tokyo, Japan) at 725 nm against the blank. The results are expressed as mg of the gallic acid equivalent (GAE) per g of the dry weight (DW). A calibration curve was prepared using 20–500 mg/L gallic acid (R^2 = 0.9980).

2.4. Determination of Total Flavonoid Content (TFC)

Total flavonoid content was measured following the method described by Moreno et al. [34]. An aliquot of 500 µL of plant extract (at a concentration of 1 mg mL^{-1}) was mixed with 100 µL KCH_3COO (1 M) and 100 µL of 10% $Al(NO_3)_3$ in a 10 mL test tube and homogenized manually, and incubated for 50 min at room temperature. Then, the absorbance was measured using a spectrophotometer (Jasco V530 UV-VIS spectrophotometer, Tokyo, Japan) at 415 nm against the blank. The experiment was carried out in triplicate, and the results are expressed as the mean ± standard deviation in mg of the quercetin equivalent (QE) per g of the dry sample. A calibration curve was prepared using 20–500 mg/L quercetin (R^2 = 0.9970).

2.5. LC/UVD Quantitative Analysis of Coumarin-Based Compounds

Quantitative analysis of coumarin-based compounds was performed by using LC/UVD with various solvents. An UltiMate 3000 HPLC system (Thermo Fisher Scientific Lin., San Jose, CA, USA) coupled with a UV detector (Thermo Fisher Scientific, San Jose, CA, USA) was applied for quantitative analysis of coumarin-based compounds. The separation of each compound was achieved by a column (5 µm, 250 mm × 4.6 mm, Bischoff Analysentechnik und-geräte GmbH., Leonberg, Garmany). The mobile phase comprised water (A) and acetonitrile (B). The gradient elution conditions were: 0–5 min, 80% A; 5–60 min, 80–0% A; 60–70 min, 0% A; 70–71 min, 0–80% A; 71–80 min, 80% A. The temperature of the column oven was maintained at 30 °C. A flow rate of 1.0 mL/min was used and the injection volume was 10 µL. The chromatograms of the compounds were acquired at 203 nm. Standard chemical compounds such as scopoletin, umbelliferone, phellopterin, and imperatorin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock standard chemicals (1000 ng/mL) were prepared in 100% methanol. The stock solutions were maintained at 4 °C and used to construct calibration curves after appropriate dilution. Amounts of different quantified compounds were calculated as mean values from HPLC analyses based on the calibration

curves of the corresponding standard compounds. The regression equation of the standard compounds were as follows.

$$\text{Scopoletin, } y = 0.482x + 0.077, R^2 = 0.999$$

$$\text{Umbelliferone, } y = 1.005x + 0.027, R^2 = 0.999$$

$$\text{Imperatorin, } y = 0.996x + 0.017, R^2 = 0.999$$

$$\text{Phellopterin, } y = 0.993x + 0.010, R^2 = 0.999$$

2.6. Antioxidant Activity

2.6.1. Evaluating the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The free radical scavenging capacity of *G. littoralis* samples was measured using a DPPH radical scavenging assay, following the method Chung et al. [35] described previously. In triplicate, an aliquot of 200 µL of different sample extracts was mixed with 4.5 mL DPPH (0.004% in methanol). The reaction mixture was homogenized manually and incubated at room temperature (25 °C) for 40 min. The mixture was shaken and kept in dark conditions for 45 min. Then, the absorbance value was taken using a spectrophotometer (Jasco V530 UV-VIS spectrophotometer, Tokyo, Japan) at 517 nm. A blank was prepared by replacing DPPH with 80% methanol in the reaction medium. BHT was used as the positive control.

The free radical scavenging ability of the sample was measured from the following equation:

$$\text{DPPH scavenging activity (\%)} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}} \times 100\%$$

where $\text{Abs}_{\text{control}}$ represent the absorbance of the mixture + methanol, and $\text{Abs}_{\text{sample}}$ represent the absorbance of the mixture + plant extract. The antioxidant activity was expressed as the capacity to scavenge or reduce the DPPH radical by 50%; that is, the amount of antioxidant compounds required to scavenge or reduce the initial concentration of DPPH by 50%.

2.6.2. Evaluation of the 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) Assay

The antioxidant activity was determined using the ABTS radical method described elsewhere [35]. Briefly, the ABTS solution used in the experiments was formed by mixing 7.4 mM ABTS and 2.6 mM potassium persulphate (1:1, *v/v*). The reaction solution was incubated for 12 h at room temperature. The solution was then diluted with 80% methanol until a solution with an absorbance of 0.70 ± 0.01 was achieved. Then, 1 mL diluted ABTS was mixed with 100 mL of the sample. Then, the absorbance was taken using a spectrophotometer (Jasco V530 UV-VIS spectrophotometer, Tokyo, Japan) at 734 nm. Trolox was used as a positive control. The standard curve was prepared from various concentration of trolox (500 µM, 600 µM, 700 µM, 800 µM, 900 µM, and 1000 µM).

The ABTS radical scavenging ability of the samples was measured from the following equation:

$$\text{ABTS scavenging activity} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}} \times 100$$

where $\text{Abs}_{\text{control}}$ represent the absorbance of the ABTS solution + methanol, and $\text{Abs}_{\text{sample}}$ represent the absorbance of the ABTS solution + test sample.

2.7. Cell Culture

Osteoblast MC3T3-E1 cells derived from mouse bones were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Osteoblast MC3T3-E1 cells were prepared in α-minimum essential medium (α-MEM, Gibco, Grand Island, NE, USA) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NE, USA), 100 U/mL penicillin (CO_2 incubator (MCO-230AIC-PK, Panasonic, Kadoma, Japan) using a culture medium supplemented with Gibco (Grand Island, NE, USA)), and 100 U/mL streptomycin (Gibco, Grand Island, NE, USA) at 37 °C, 5% CO_2, and 95% air. The cells were washed with phosphate-buffered saline (PBS; pH 7.4; Gibco, Grand Island, NE, USA) and 0.25% trypsin-2.65 mM EDTA (Gibco, Grand Island, NE, USA), and sub-cultured by changing the culture medium every two days.

2.8. Cell Viability

Cell viability was analyzed according to the protocol described by Denizot and Land [36] by dispensing osteoblast MC3T3-E1 cells in a 96-well plate at 2×10^3 cells/well and subsequently pre-incubating them in a CO_2 incubator for 24 h. After incubation, the MC3T3-E1 cells were treated with different concentrations of plant sample for 72 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT solution) at 5 mg·mL^{-1} was added to each well and maintained at 37 °C for 4 h. Then, the resultant formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance of the mixture was taken using a microplate reader (Thermo Fisher Scientific Instrument Co., Ltd., Shanghai, China) at 570 nm.

2.9. Cytotoxicity

The cytotoxicity of different plant parts was evaluated according to a previously described protocol [37,38]. Osteoblast MC3T3-E1 cells were dispensed in a 96-well plate at a density of 2×10^3 cells/mL, then pre-incubated in a CO_2 incubator for 24 h. After incubation, the cells were treated with various concentrations of samples (ranging from 0 to200 μg/mL) and incubated for 48 h. The cell culture solution (10 μL) was added to a 96-well plate containing 40 μL PBS. After placing the LDH reagent in each well, the mixture was placed in a dark place for 45 min at 25 °C. After the reaction was terminated by mixing 50 μL of stop solution, the cytotoxicity was evaluated by quantifying the plasma membrane damage. LDH is a stable enzyme present in all cell types and is rapidly released into the culture medium when the plasma membrane is damaged. Cell membrane integrity was evaluated by measuring the LDH leakage levels from the cells using the LDH Cytotoxicity Assay Kit (BioVision, Inc., Milpitas, CA, USA). The absorbance value of the samples was measured using a microplate reader (Thermo Fisher Scientific Instrument Co., Ltd., Shanghai, China) at 492 nm.

2.10. ALP Activity

MC3T3-E1 cells were seeded as described above, and the ALP activity of the media was measured by following the method described by Liu et al. [39]. Initially, MC3T3-E1 cells were dispensed in a 48-well plate at 5×10^4 cells/well and pre-incubated in a CO_2 incubator for 24 h. Then, the MC3T3-E1 cells were treated with different sample concentrations (0–20 μg/mL). Subsequently, the culture medium was replaced and supplemented with 100 μL chemiluminescent substrate for alkaline phosphatase (CSPD; Roche, Basel, Switzerland), added to 20 μL total cell lysate, and reacted for 30 min. The amount of protein in the total cell lysate was measured using CSPD (Life Technologies, Carlsbad, CA, USA) according to the Protein Assay Kit (Bio-Rad, Hercules, CA, USA), and the ALP activity value was expressed as the fold change per μg of total protein.

2.11. Collagen Synthesis Rate

Collagen content was quantified by a Sirius Red-based colorimetric assay as described by Park et al. [40]. The MC3T3-E1 cells were cultured in an osteogenic medium containing

10 mM β-glycerophosphate, 5 nM dexamethasone, 50 g/mL ascorbic acid, and *G. littoralis* (0 µg/mL and 20 µg/mL). After seven days, the cells were cultured in a medium containing bovine serum albumin (BSA) (3%) for two days. The cultured cells were gently washed twice with PBS, followed by fixation with Bouin's fluid (8.3% formaldehyde and 4.8% acetic acid in saturated aqueous picric acid, $(O_2N)_3C_6H_2OH$) for 1 h. After fixation, the fixative fluids were removed, and the cultured cells were washed with tap water for 10 min. The cultured cells were air-dried and stained with Sirius red dye (0.1% saturated picric acid) for 1 h on a shaker. The stained cells were washed with PBS and observed under a microscope. For quantitative analysis, the stained cell layer was washed extensively with 0.01 N HCl to remove the non-bound dye. The stained cells were dissolved in 0.2 mL of 0.1 N and shaken for 30 min. Then, the absorbance was measured at 540 nm.

2.12. Mineralization Content

Calcium content was measured in osteoblasts using the method described by Zakłos-Szyda et al. [41]. Osteoblast MC3T3-E1 cells were aliquoted in a 24-well plate at 2×10^4 cells/well in a CO_2 incubator at 37 °C. The cells were treated with plant samples at a concentration of 0–20 µg/mL and placed in an incubator for seven and 14 days to induce mineral deposition. The culture medium was changed at three-day intervals. For harvesting, the MC3T3-E1 cells were washed with PBS twice and fixed with 4% paraformaldehyde for 2 h. The cells were then stained with 40 mM Alizarin Red S (pH 4.5). The stained cells were then rinsed four times with distilled water and observed under an inverted microscope. Calcified nodules appearing bright red were confirmed, and 100 mM cetylpyridinium chloride (Sigma) solution was added to each well to elute the stain. The eluted stain (100 µL) was added to a 96-well microplate. The absorbance of solubilized calcium-bound Alizarin Red S was taken at 570 nm using a spectrophotometer (Eppendorf AG 22331; Hamburg, Germany). Calcium deposition was expressed as the molar equivalent of calcium.

2.13. Osteocalcin Content

Osteocalcin concentration was determined by the test method of the OCN ELISA kit (Takara Bio, Kusatsu, Japan) by following the process described by Bukhari et al. [42]. Initially, 100 µL of the cell culture solution was added to a 96-well plate coated with antibodies and incubated for 1 h at 37 °C. After removing unbound material with washing buffer (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20), horseradish peroxidase (HRP)-conjugated streptavidin was added to each well and incubated at 37 °C for 1 h to bind to the antibodies. After washing the wells five times, tetramethylbenzidine (TMB) solution was added to each well and incubated at room temperature for 20 min in the dark. HRP catalyzed the conversion of a chromogenic substrate (TMB) to a colored solution, with a color intensity proportional to the amount of protein present in the sample. After adding the stop solution, the absorbance of the sample in each well was measured at 450 nm. Results are presented as the percentage change in activity compared to the untreated control. The concentration of osteocalcin in the serum was measured using the same method as that used for cultured cell samples.

2.14. mRNA Expression Rate

Total RNA was isolated from cells at specific times using TRIzol reagent (Invitrogen, Waltham, MA, USA) with DNase treated with RNase-free DNase (35 U/mL) for 50 min at 37 °C according to the method described by Liu et al. [43] and Matsubara et al. [44]. The total RNA present in each sample was quantified by measuring the absorbance at 260 nm using a spectrophotometer (Eppendorf AG 22331; Hamburg, Germany). Gene expression was measured by adding cDNA to a PCR mixture containing EXPRESS SYBR Green qPCR Supermix (Bio Prince, Seongsu, Seoul, Republic of Korea). Real-time PCR was performed using the Rotor-Gene Q (Qiagen, Düsseldorf, Germany). The reaction was carried out at 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 25 s for 40 cycles of amplification. Relative

mRNA expression of specific genes was standardized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and sequencing was performed using PCR primer sequences (Table 1).

Table 1. Forward and reverse primers sequences used in this study.

Primer Name		Sequence
Osterix	Forward	5′-AGCGACCACTTGAGCAAACAT-3′
	Reverse	5′-GCGGCTGATTGGCTTCTTCT-3′
RUNX2	Forward	5′-CGGCCCTCCCTGAACTCT-3′
	Reverse	5′-TGCCTGCCTRGGGATCTGTA-3′

2.15. Statistical Processing

The results were presented as the mean ± standard deviations of three trials independent (n=3). The data was compared by one-way ANOVA using the SPSS program (Statistical Package for Social Science, Version 24), followed by Duncan's multiple range test, considering significant differences at P values < 0.05.

3. Results

3.1. The Total Phenolic Contents (TPCs) and Total Flavonoid Contents (TFCs)

The total phenolic contents (TPCs) and total flavonoid contents (TFCs) of stems, leaves, roots, and whole plant extracts of *G. littoralis* extracts were calculated from the linear regression equation of the gallic acid standard calibration curve. The TPC of different plant parts ranged from 4.27 ± 0.03 to 23.29 ± 0.43 mgGAE/g dry sample. As Figure 1 shows, the leaf and stem (GLSE) and root (GLRE) extracts had the highest and lowest TPCs, respectively. The total flavonoid contents (TFCs) of stems, leaves, roots, and whole plant extracts of *G. littoralis* extracts were determined using colorimetric analysis. The flavonoid content of different *G. littoralis* extract parts ranged from 0.28 ± 0.04 to 4.93 ± 0.30 mgQE/g dry sample. The results showed that fruit (GLFE) extracts contained a higher TFC, followed by GLFE, whole plant (GLAE), and GLRE extracts.

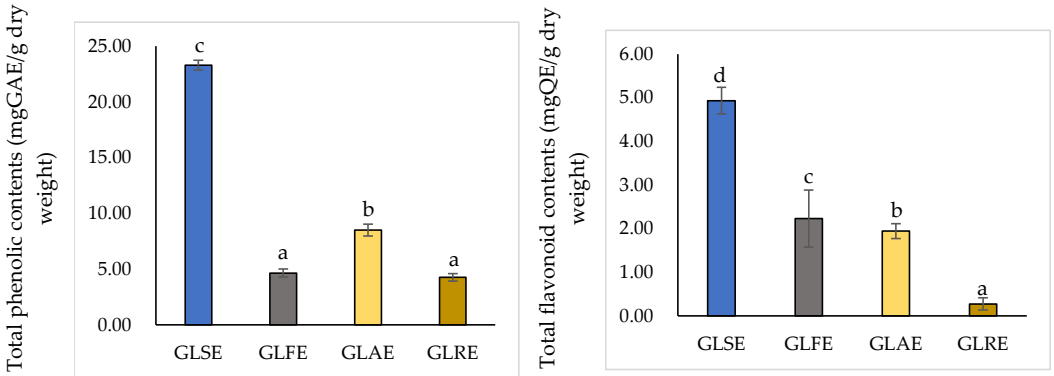

Figure 1. (**a**) Total phenolic contents (TPC) and (**b**) total flavonoid contents (TFC) in *G. littoralis* extracts. The mean values followed by the same letter are not significantly different based on the DMRT ($p < 0.05$). GLSE: *G. littoralis* leaf, stem extracts, GLFE: *G. littoralis* fruit extracts, GLAE: *G. littoralis* all extracts, GLRE: *G. littoralis* root extracts.

3.2. LC/UVD Quantitative Analysis of Coumarin-Based Compounds

LC/UVD quantitative analysis of four coumarin components varied widely with the leaf, stem, fruit, whole plant, and root extracts of *G. littoralis* (Table 2). Among the different tested samples, GLSE extracts had the highest scopoletin (53.0 mg/g), and umbelliferone (1.6 mg/g). The lowest amount of scopoletin was observed in GLRE (8.5 mg/g), and

of umbelliferone, in GLFE (0.8 mg/g). The significantly ($p < 0.05$) highest amount of imperatorin (31.9 mg/g) and phellopterin (2.3 mg/g) were observed in GLFE and GLAE extracts, respectively (Table 2). It was confirmed that the total content of four types of coumarin was higher in the order of GLSE, GLRE, GLAE, and GLFE (Figure 2).

Table 2. LC/UVD quantitative analysis compounds of extracts from *G. littoralis* (Unit: mg/g, dry weight).

Sample [1]	Scopoletin	Umbelliferone	Imperatorin	Phellopterin	Total
GLSE	53.0 ± 0.2 [d]	1.6 ± 0.0 [d]	2.0 ± 0.0 [a]	N.D. [2]	56.6 ± 0.2 [d]
GLFE	17.7 ± 0.0 [b]	0.8 ± 0.0 [a]	8.9 ± 0.1 [b]	1.1 ± 0.4 [b]	28.5 ± 0.5 [a]
GLAE	24.5 ± 1.1 [c]	1.0 ± 0.0 [b]	15.1 ± 0.6 [c]	0.6 ± 0.1 [a]	41.1 ± 1.8 [b]
GLRE	8.5 ± 0.0 [a]	1.4 ± 0.1 [c]	31.9 ± 0.1 [d]	2.3 ± 0.0 [c]	44.2 ± 0.1 [c]

[1] GLSE: *G. littoralis* leaf, stem extracts, GLFE: *G. littoralis* fruit extracts, GLAE: *G. littoralis* plant extracts, GLRE: *G. littoralis* root extracts. [2] N.D.: not detected. Each value is means ± SD of three replicate tests. The mean values followed by the same letter are not significantly different based on the DMRT ($p < 0.05$).

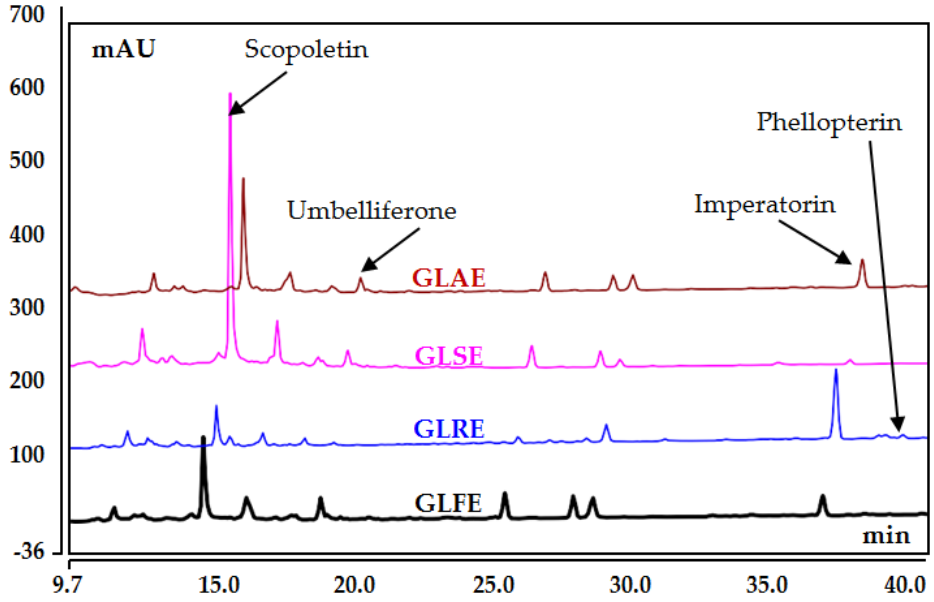

Figure 2. Representative chromatograms of compounds of extracts from *G. littoralis*.

3.3. Antioxidant Activity

Figure 3 presents the radical scavenging potential of the GLSE, GLFE, GLAE, and GLRE extracts. The results showed that the antioxidant activity of different plant parts varied significantly and appeared in a concentration-dependent manner. Likewise, GLAE extracts had higher antioxidant properties, as represented by the lower half-maximal inhibitory concentration (IC_{50}) (718.40 ± 14.025 μg mL^{-1}). Furthermore, all other plant part extracts, except GLRE extracts, possessed a better radical scavenging potential, indicating the presence of higher amounts of antioxidant compounds. The results showed that the $ABTS^{+}$ radical scavenging potential of different *G. littoralis* extract parts varied significantly ($p < 0.05$), with GLAE extracts exhibiting higher ABTS radical scavenging activity (16,348.41 ± 1315.26 μg mL^{-1}). Comparatively, the GLRE extract showed the lowest antioxidant activity (Figure 3). Two different antioxidant assays were carried out, and these antioxidant assays were positively and significantly correlated with TPC and TFC of *G. littoralis* (Table S1).

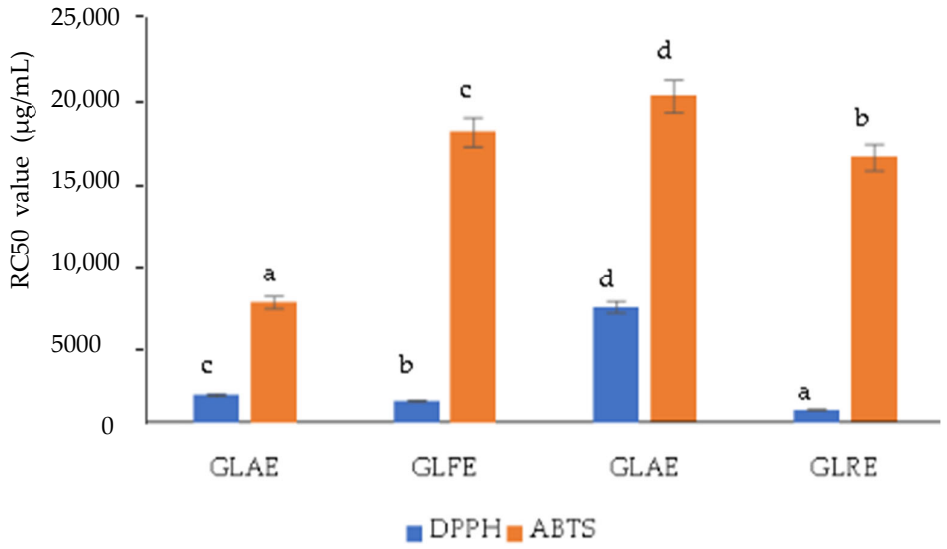

Figure 3. Antioxidant activity (DPPH and ABTS radical assay) in *G. littoralis* extracts. Mean values followed by the same letter are not significantly different based on the DMRT ($p < 0.05$). GLSE: *G. littoralis* leaf, stem extracts, GLFE: *G. littoralis* fruit extracts, GLAE: *G. littoralis* all extracts, GLRE: *G. littoralis* root extracts.

3.4. Cell Viability Test

We used osteoblastic precursor cell lines (MC3T3-E1 cells) derived from *Mus musculus* to induce the expression of osteoblast markers and investigate the effect of *G. littoralis* on bone metabolism. In this study, we attempted to determine the effect of *G. littoralis* extract on in vitro osteogenic induction using MC3T3-E1 cells. We performed a pilot study in which *G. littoralis* extract concentrations varied from 0.5 to 200 µg/mL to determine the optimal concentration. Cell viability studies were performed for different *G. littoralis* parts, including GLSE, GLFE, GLAE, and GLRE extracts. Initially, the purified *G. littoralis* ethanol extracts were re-suspended in 70% ethanol, and various plant extract concentrations were added to the MC3T3-E1 cell culture. The viability of cells varied considerably among the different plant parts used. The viable cell number increased in up to 5 µg/mL of *G. littoralis* treatment. Then, the higher concentrations showed a significant decrease in the cell population in a concentration-dependent manner (Figure 4), indicating *G. littoralis*'s cellular toxicity properties. Lower concentrations of the plant extracts (between 0 and 2 µg/mL) did not significantly affect the cell viability. Among the different extraction samples, GLSE at a concentration of 0.5–10 µg/mL showed 88.5 ± 1.0% to 96.6 ± 3.9% cellular viability, while these values varied in GLFE (90.7 ± 1.2% to 94.5 ± 0.9%), GLAE (85.8 ± 1.6% to 95.4 ± 1.2%), and GLRE (80.0 ± 0.7% to 97.7 ± 0.4%) at the same concentrations (Figure 4). In addition, the lactate dehydrogenase (LDH) cytotoxicity assay proved that plant extracts beyond 5 µg/mL were toxic to the cell lines (Figure 5). We used 5 µg/mL of *G. littoralis* extract in the subsequent experiments to avoid cytotoxicity and promote MC3T3-E1 cell growth.

3.5. ALP Activity

We evaluated ALP activity to assess how *G. littoralis* extracts affect osteogenic induction (Figure 6). All the sample extracts (GLSE, GLAE, GLRE, and GLFE) significantly increased ALP activity in MC3T3-E1 cells. Comparatively, higher ALP activities were observed at sample concentrations of 0.5 µg/mL. Increasing the treated sample concentration resulted in a decrease in ALP activity. Among the treated samples, GLSE at a concentration of 0.5–20 µg/mL showed higher ALP activity ranging from 158.4 ± 7.9% to 125.9 ± 11.5%, respectively, while the lowest ALP activity was observed in GLFE at a concentration of 0.5–20 µg/mL ranging from 127.4 ± 2.9% to 84.1 ± 3.7%, respectively. The results indicate

that *G. littoralis* extract enhanced the ALP activity required for osteoblast formation and ECM mineralization in MC3T3-E1 cells.

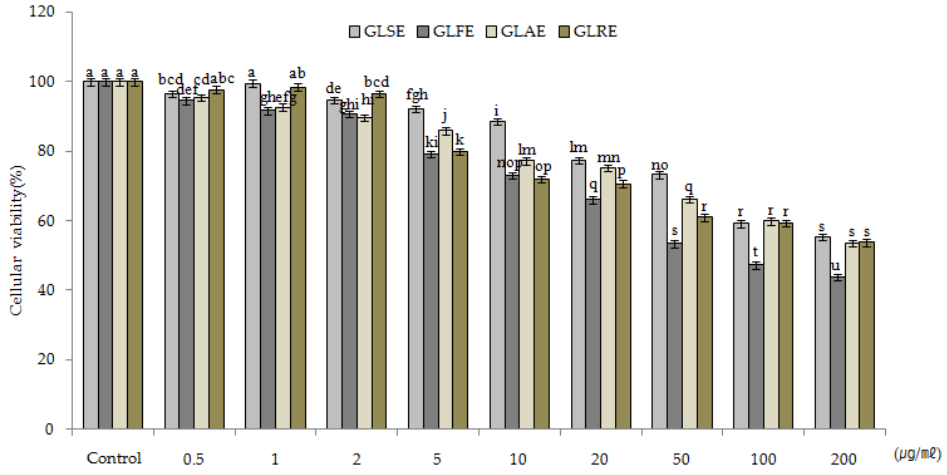

Figure 4. Cell viability of extracts from each part of *G. littoralis* in osteoblastic MC3T3-E1 cell line. Each value is the mean ± standard deviation of nine replicate tests. The mean values followed by the same letter are not significantly different based on the DMRT ($p < 0.05$). GLSE: *G. littoralis* leaf, stem extracts, GLFE: *G. littoralis* fruit extracts, GLAE: *G. littoralis* all extracts, GLRE: *G. littoralis* root extracts.

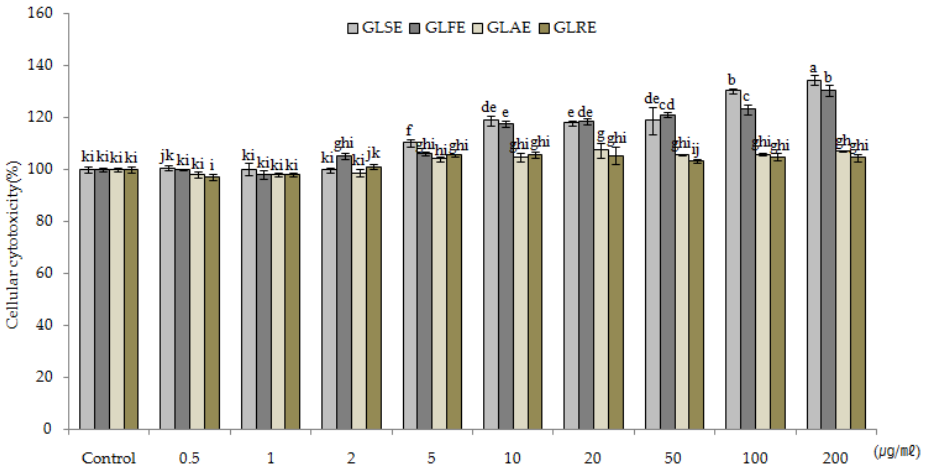

Figure 5. Cell cytotoxicity of extracts from each part of *G. littoralis* in osteoblastic MC3T3-E1 cell line. Each value is the mean ± standard deviation of nine replicate tests. Mean values followed by the same letter are not significantly different based on the DMRT ($p < 0.05$). GLSE: *G. littoralis* leaf, stem extracts, GLFE: *G. littoralis* fruit extracts, GLAE: *G. littoralis* all extracts, GLRE: *G. littoralis* root extracts.

3.6. Collagen Synthesis Rate

GLSE, at a concentration of 0.5–5 μg/mL, showed a higher collagen synthesis effect than the other extract types (Figure 7). The results indicate that the GLFE methanolic extract had phytochemicals that inhibited the collagen synthesis effect. As Figure 1 shows, GLFE extracts showed lower collagen synthesis effects at concentrations ranging from 1 to 20 μg/mL, which decreased in a concentration-dependent manner. GLFE extract concentrations higher than 2 μg/mL did not affect collagen synthesis. However, no significant difference was observed between GLAE and GLRE extracts in collagen synthesis at any of the concentrations used in the experiment.

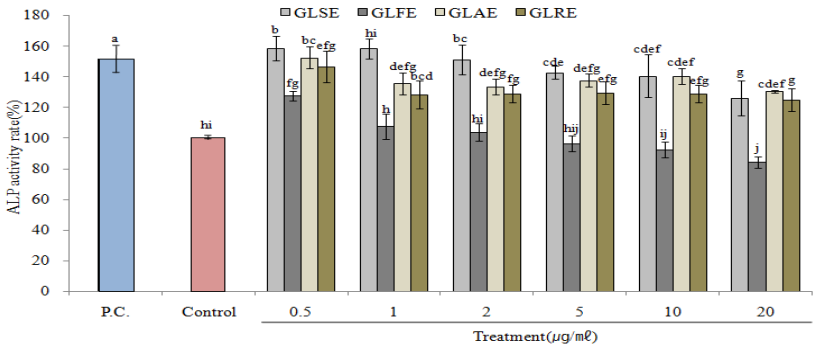

Figure 6. ALP activity of extracts from each part of *G. littoralis* in osteoblastic MC3T3-E1 cell line. Each value is the mean ± standard deviation of nine replicate tests. Mean values followed by the same letter are not significantly different based on the DMRT ($p < 0.05$). P.C.: Positive control (ascorbic acid (50 μg/mL), β-glycerophosphate (100 mM). GLSE: *G. littoralis* leaf, stem extracts, GLFE: *G. littoralis* fruit extracts, GLAE: *G. littoralis* all extracts, GLRE: *G. littoralis* root extracts.

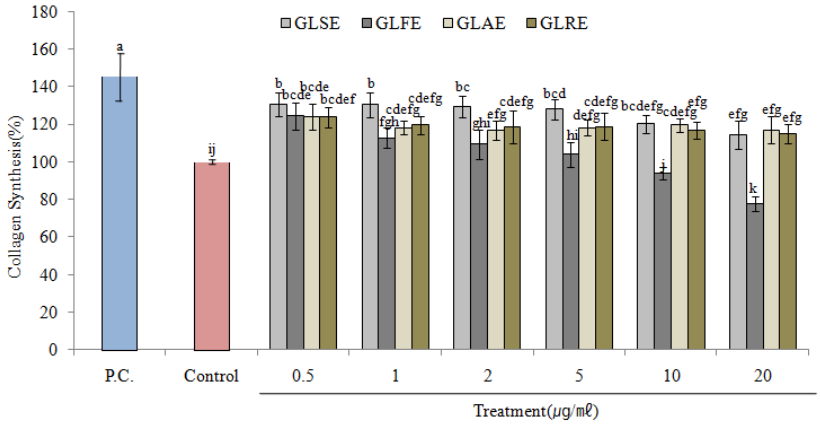

Figure 7. Collagen synthesis effects of extracts from each part of *G. littoralis* in osteoblastic MC3T3-E1 cell line. Each value is mean ± standard deviation of nine replicate tests. Mean values followed by the same letter are not significantly different based on the DMRT ($p < 0.05$). P.C.: Positive control (ascorbic acid (50 μg/mL), β-glycerophosphate (100 mM). GLSE: *G. littoralis* leaf, stem extracts, GLFE: *G. littoralis* fruit extracts, GLAE: *G. littoralis* all extracts, GLRE: *G. littoralis* root extracts.

3.7. GL Extracts Enhanced Osteoblast Mineralization

After seven days of treatment with *G. littoralis* extracts, we measured the mineralization content in MC3T3-E1 cells. Among the different samples, GLAE showed a higher mineral content (131.9 ± 4.7%), followed by GLSE (130.8 ± 2.9%), GLRE (127.8 ± 3.4%), and GLFE (119.9 ± 5.6%), at a concentration of 0.5 μg/mL. *G. littoralis* extracts at a concentration of 0.5 μg/mL induced higher mineralization levels in all the groups compared to 20 μg/mL treatments (Figure 8). GLFE showed lower mineralization and decreased in a concentration-dependent manner. GLAE showed higher mineralization at all concentrations used (0.5–20 μg/mL), indicating that the whole plant extracts contain more bioactive compounds responsible for osteoblast formation.

3.8. Osteocalcin Content

We investigated the effects of *G. littoralis* extracts on the degree of osteocalcin production during the late stage of osteoblast differentiation. As Figure 9 shows, *G. littoralis* extract significantly increased osteocalcin production in MC3T3-E1 cells. This is the first report describing the inhibitory osteoporotic properties of *G. littoralis* using the MC3T3-E1 in vitro system. Osteocalcin content levels varied with different concentrations of the tested plant parts. Except in the case of GLAE, an increase in the sample concentration

resulted in reduced osteocalcin content. Comparatively, 0.5 µg/mL GLSE resulted in a higher osteocalcin content, indicating that the phytochemical responsible for producing the protein in osteoblast cells is more present in this extract. However, increasing the GLFE concentration resulted in a decrease in osteocalcin content in a concentration-dependent manner. Comparatively, 20 µg/mL GLFE displayed lower osteocalcin production than the negative control, indicating that a higher GLFE concentration is cytotoxic and inhibits osteoblast formation.

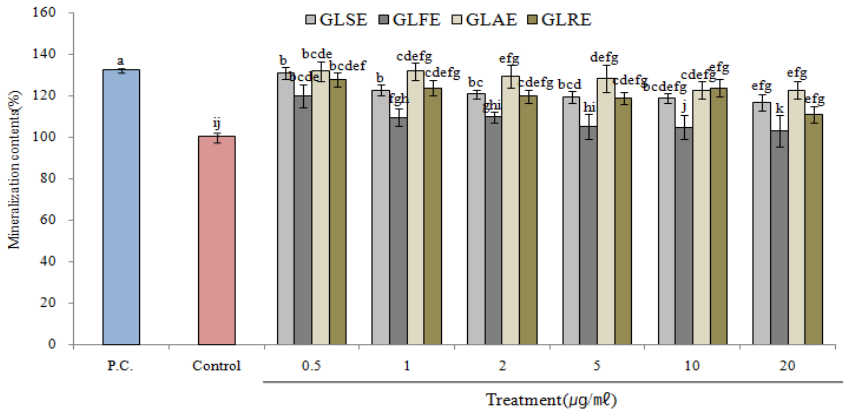

Figure 8. Mineralization contents of extracts from each part of *G. littoralis* in osteoblastic MC3T3-E1 cell line. Each value is the mean ± standard deviation of nine replicate tests. Mean values followed by the same letter are not significantly different based on the DMRT ($p < 0.05$). P.C.: Positive control (ascorbic acid (50 µg/mL), β-glycerophosphate (100 mM). GLSE: *G. littoralis* leaf, stem extracts, GLFE: *G. littoralis* fruit extracts, GLAE: *G. littoralis* all extracts, GLRE: *G. littoralis* root extracts.

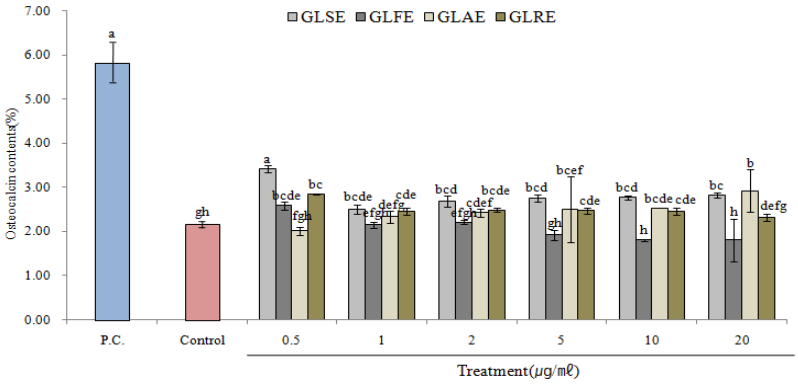

Figure 9. Osteocalcin contents of extracts from each part of *G. littoralis* in osteoblastic MC3T3-E1 cell line. Each value is mean ± standard deviation of nine replicate tests. The mean values followed by the same letter are not significantly different based on the DMRT ($p < 0.05$). P.C.: Positive control (ascorbic acid (50 µg/mL), β-glycerophosphate (100 mM). GLSE: *G. littoralis* leaf, stem extracts, GLFE: *G. littoralis* fruit extracts, GLAE: *G. littoralis* all extracts, GLRE: *G. littoralis* root extracts.

3.9. mRNA Expression Rate

We confirmed how *G. littoralis* extracts affect osteoblast differentiation by analyzing the expression patterns of prominent osteoblast marker genes Table 3. After seven days of treatment with different *G. littoralis* extracts, a real-time polymerase chain reaction (PCR) was performed to investigate the effect on osterix and RUNX2 mRNA expression. As the results show, the expression patterns of both osteoblastic genes changed when treated with the *G. littoralis* extracts. The findings showed that, except in the case of GLFE extracts, *G. littoralis* extract treatment significantly elevated osterix gene expression, and RUNX2 gene expression was increased by all *G. littoralis* extracts. Comparatively, RUNX2 expression significantly increased after treatment with lower *G. littoralis* extract concentrations (ranging from 0.5 to 2 µg/mL).

Table 3. Effect of *G. littoralis* extracts on Osterix and RUNX2 mRNA expression in osteoblastic MC3T3-E1 cells.

Sample	Concentration (μg/mL)	mRNA Expression Rate (Fold) Osterix [1]	mRNA Expression Rate (Fold) RUNX2
P.C.	AA (50 μg/mL) + BGP (100 mM)	1.820 ± 0.072 ^{c}	2.531 ± 0.050 ^{a}
Control	-	1.000 ± 0.000 ^{ki}	1.000 ± 0.000 ^{k}
GLSE	0.5	1.510 ± 0.046 ^{ef}	2.327 ± 0.023 ^{ab}
	1	1.530 ± 0.041 ^{de}	2.220 ± 0.090 ^{b}
	2	1.620 ± 0.033 ^{d}	2.190 ± 0.250 ^{b}
	5	1.820 ± 0.053 ^{c}	1.940 ± 0.150 ^{c}
	10	1.520 ± 0.021 ^{ef}	1.533 ± 0.029 ^{fg}
	20	1.430 ± 0.003 ^{f}	1.410 ± 0.017 ^{gh}
GLFE	0.5	0.990 ± 0.020 ^{ki}	1.920 ± 0.080 ^{cd}
	1	0.970 ± 0.070 ^{lm}	1.830 ± 0.070 ^{cde}
	2	0.960 ± 0.010 ^{lm}	1.800 ± 0.090 ^{cde}
	5	0.937 ± 0.055 ^{lm}	1.700 ± 0.120 ^{def}
	10	0.900 ± 0.070 ^{lm}	1.310 ± 0.040 ^{ghij}
	20	0.880 ± 0.050 ^{m}	1.110 ± 0.050 ^{jk}
GLAE	0.5	1.280 ± 0.020 ^{gh}	1.280 ± 1.113 ^{jk}
	1	1.250 ± 0.070 ^{gh}	1.250 ± 1.517 ^{fg}
	2	1.240 ± 0.080 ^{gh}	1.240 ± 1.660 ^{ef}
	5	1.200 ± 0.080 ^{hi}	1.200 ± 1.353 ^{ghi}
	10	1.130 ± 0.060 ^{ij}	1.130 ± 1.190 ^{hijk}
	20	1.080 ± 0.020 ^{jk}	1.080 ± 1.073 ^{jk}
GLRE	0.5	1.080 ± 0.050 ^{jk}	1.150 ± 0.030 ^{jk}
	1	1.320 ± 0.030 ^{g}	1.060 ± 0.040 ^{k}
	2	1.430 ± 0.080 ^{f}	1.230 ± 0.120 ^{hijk}
	5	1.850 ± 0.020 ^{c}	1.190 ± 0.150 ^{hijk}
	10	2.020 ± 0.120 ^{b}	1.080 ± 0.070 ^{jk}
	20	2.220 ± 0.050 ^{a}	1.010 ± 0.030 ^{k}

[1] Each value is mean ± standard deviation of nine replicate tests. Mean values within a column followed by the same letter are not significantly different based on the DMRT ($p < 0.05$). P.C.: Positive control (ascorbic acid (50 μg/mL), β-glycerophosphate (100 mM), AA: Ascorbic acid, BGP: β-glycerophosphate. GLSE: *G. littoralis* leaf, stem extracts, GLFE: *G. littoralis* fruit extracts, GLAE: *G. littoralis* all extracts, GLRE: *G. littoralis* root extracts.

4. Discussion

Plants contain various antioxidants; these antioxidants play an important role in protecting plants from oxidative stress and signals, when ingested, and they can act as natural antioxidants to help prevent disease [45]. Among these compounds, the phenolic compounds present in the plants are mostly responsible for the antioxidant properties [46]. In the present study, the DPPH and ABTS radical scavenging assays presented wide variations in antioxidant activity values for different plant parts. We studied the relationship between the different parameters by obtaining Pearson's correlation coefficients. The antioxidant potentials estimated by both assays were somewhat different and showed a high correlation between them ($p < 0.05$, r = 0.894). The variation in antioxidant potential could be due to the various antioxidant compound responses to the different radicals present in each assay. Excessive reactive oxygen species (ROS) production can cause lipid and protein oxidation, damage DNA integrity, and simultaneously cause tissue damage [47]. Several previous studies have shown the involvement of ROS in bone remodeling by enhancing osteoclastic bone resorption and decreasing osteoblast cell formation [48,49]. In the present study, all *G. littoralis* extracts effectively scavenged the DPPH and ABTS radicals, indicating that the *G. littoralis* extracts had potential antioxidant activity and protected the MC3T3-E1 cells from degeneration and death. Moreover, Pearson's correlation analysis revealed a significant correlation between DPPH and TPC and TFC in *G. littoralis* extracts. A simi-

lar correlation was also observed between ABTS and TFC. Phenolic compounds, such as coumarins and their derivatives, have been reported as dominant *G. littoralis* phytochemical components [48,49]. In *G. littoralis* extracts, phenolic compounds, including caffeic acid, vanillic acid, ferulic acid, chlorogenic acid, rutin, quercetin, kaempferol, and coumarins and their derivatives, have been identified [50–52]. In the present study, coumarin-based flavonoids such as scopoletin, umbelliferone, imperatorin, phellopterin have been detected in *G. littoralis* extracts. These compounds are mostly responsible for the antioxidant properties. For instance, scopoletin has been involved in considerable antioxidant activities by scavenging ROS, especially hydrogen peroxide (H_2O_2) scavenging activity, ferrous ion ($Fe2^+$) chelating activity, and activity against superoxide anion radicals ($O_2^{\bullet -}$), and OH-radicals [53]. Moreover, Um et al. [53] isolated scopoletin and umbelliferone from *Glehnia littoralis* and demonstrated a reactive oxygen species (ROS) scavenging ability of about 60% or more compared to a control. Imperatorin (IMP) has been reported in several plants with antioxidant properties [54–57]. Methanolic extracts containing umbelliferone have been shown to exhibit an efficient pro-oxidant activity [58] and inhibit lipid peroxidation [59]. Others observed that the treatment of umbelliferone has been shown to inhibit the intracellular ROS production in irradiated lymphocytes and effectively restore the mitochondrial membrane and inhibited gamma radiation-induced DNA damage [60].

Numerous studies observed that oxidative stress enhances the differentiation and function of osteoclasts [61]. ROS-induced oxidative stress has been shown to involve the suppression of bone formation and the stimulation of osteoclast resorption [62]. Present results indicate that phenolic compounds such as scopoletin, umbelliferone, imperatorin, and phellopterin present in the *G. littoralis* extracts inhibited ROS formation to suppress the excessive bone breakdown by osteoclasts. Moreover, several studies have reported the anti-osteoporotic properties of coumarins by suppressing the interaction of advanced glycation end-products (AGE) and their receptors [63]. Treatment using scopoletin prevented bone loss in diabetic mice by increasing bone turnover of bone-degrading osteoclasts and bone-forming osteoblasts. It has been shown that treatment with imperatorin in rats promoted osteogenesis and suppressed the osteoclast differentiation [64]. The authors found that the imperatorin activates AKT that leads to the inactivation of GSK3β that causes the activation of β-catenin and accumulation of β-catenin in the nucleus [65–68]. It was believed that the activation of β-catenin plays an important role in the suppression of osteoblast differentiation [69]. Thus, it can be inferred that imperatorin could induce osteogenesis via the AKT/GSK3β/β-catenin pathway [64], indicating that imperatorin present in the GL extracts could be responsible for bone growth and inhibition of resorption. Furthermore, umbelliferone prevented trabecular bone matrix degradation and osteoclast formation in bone tissue [70]. The authors reported that umbelliferone is closely associated with the dysfunction of osteoclasts attributed to defects in osteoclast survival and differentiation [65]. In addition, Li et al. [71] reported that phellopterin inhibits Ca^{2+} influx induced by the stimulation of voltage-gated and receptor-dependent calcium channels [72,73]. Therefore, in the present study, these compounds, together with the other polyphenols present in the *G. littoralis* extracts, strongly favored MC3T3-E1 cell differentiation. Furthermore, it can be suggested that *G. littoralis* effectively contributes to the prevention of oxidative damage to bone tissues via antioxidant action and its phytochemicals.

ALP, a typical protein product, is associated with osteoblast growth and differentiation and is expressed and increased during the active matrix maturation phase immediately after the cell proliferation period [18,20,74]. Although the exact ALP mechanism of action is poorly understood, it is believed that these enzymes are responsible for bone mineralization [75]. Therefore, it is important to examine the effect of *G. littoralis* extracts on MS3T3-E1 cell ALP activity. In the present study, all *G. littoralis* extracts effectively accelerated ALP activity in a dose-dependent manner. Moreover, some *G. littoralis* extracts showed higher ALP activity than the positive control, indicating that the different phytochemicals present in the extracts may be necessary for osteoblast differentiation. We hypothesize that

G. littoralis extracts are associated with osteoblast proliferation and differentiation from a newly synthesized protein component.

In this study, maximum ALP activity was observed at the lowest *G. littoralis* extract concentration (0.5 µg/mL), which was confirmed in GLSE and GLAE extracts. Similar to our findings, the aqueous extracts of rooibos promoted ALP activity and mineralization [76]. Moreover, there is abundant evidence that dietary phytochemicals have osteoprotective effects. Caffeic acid regulates bone remodeling by inhibiting osteoclastogenesis, bone resorption, and osteoblast apoptosis [77]. Chlorogenic acid extracted from *Cortex Eucommiae* inhibited a decrease in bone mineral density [78]. Quercetin inhibits osteoblast apoptosis, osteoclastogenesis, and oxidative stress [79]. Jang et al. [80] reported similar results in *A. rugosa*, stating that some of the phenolic compounds present in *A. rugosa* effectively suppressed osteoclasts [81,82]. Flavonoids, such as orientin, quercetin, and luteolin, have shown blastogenic effects by increasing ALP activity and mineralization in rooibos [83,84]. Treating osteoblast cells with various phenolic compounds increases ALP synthesis and decreases the expression of antigens involved in osteoblast immune functions, which may improve bone mineral density [85]. Flavonoids, such as icariin and naringin, were found to regenerate bone tissues by increasing ALP activity and osteopontin content [86–89]. Another study observed increases in osteoblast proliferation, and several other reports have provided convincing data about phytochemicals and their association with osteoclast formation in vitro [90]. Although this study did not determine the exact composition of phenolic compounds in *G. littoralis*, the phenolic compounds from *G. littoralis* could be crucial in modulating the bone formation process through the osteoblast formation process and ALP production.

It has been reported that osteoblasts produce biochemical markers, such as type I collagen, ALP, and osteopontin, which are important components for matrix maturation and mineralization [74]. In the present study, phenotypic markers, such as collagen and osteocalcin, mainly associated with the later stage of osteoblast differentiation and were elevated in the MS3T3-E1 cells treated with *G. littoralis* extract. This indicated that *G. littoralis* extracts were vital in osteoblast differentiation. GLSE showed greater collagen synthesis and upregulated osteoblastic MC3T3-E1 cell proliferation and differentiation by enhancing ALP activity and mineralization compared to the other extract types. There is increasing interest in both in vitro and in vivo research that phenolic compounds may favorably improve osteoporosis. Sparse experimental data show that phenolic acids may have in vitro estrogenic activity. Bioactive compounds, such as β-estradiol, reportedly significantly increase osteoblastic cell proliferation, DNA and protein content, and ALP activity [91]. Phenolic acids may act on osteoblasts by binding to their estrogen receptors, found in osteoblastic cells [92].

Bone mineralization refers to the deposition of calcium and minerals in cells. It acts as a reservoir for calcium and phosphorus in the bone, maintains bone elasticity and flexibility, and provides compactness and mechanical resistance to the bone [93]. During the postmenopausal period, estrogen deficiency causes a decrease in the absorption of micronutrients in the body [94]. In the present study, different GL extracts showed various degrees of elevation in calcium levels, possibly due to affecting calcium absorption and contributing to matrix deposition during osteogenesis [95]. In this test, the maximum mineralization content was observed at the lowest concentration (0.5 µg/mL) of GLSE, GLFE, and GLRE extracts. Our results are consistent with those reported by Yun et al. [96], who observed an increase in calcium deposition in osteoblast MC3T3-E1 cells treated with lower concentrations of chrysanthemum extract. Prak et al. [97] reported similar results in 10 µg/mL of seaweed extracts. Osteocalcin is a non-collagenous protein in the bone secreted into osteoblasts and used as a biochemical marker for bone formation [98]. Osteocalcin is associated with changes in bone turnover rate in bone metabolism and is reflected in the rate of bone formation. The osteocalcin carboxyl group is removed and released into the circulation due to pH acidification of the bone when osteoclasts resorbed it [99].

Several transcription factors are involved in osteoblast differentiation. Most importantly, RUNX2 and osterix are genes that differentiate mesenchymal stem cells into immature osteoblasts and are osteoblast-specific transcription factors required for osteoblast differentiation and bone formation [100]. RUNX2, the earliest identifiable marker, is known as "a master gene" for osteoblast differentiation and is associated with ALP and osterix upregulation [101,102]. It has been argued that RUNX2 triggers osteocalcin expression by binding to the cis-acting elements of the osteocalcin promoter region of osteogenic genes to initiate the expression of ALP, osteopontin, bone sialoprotein (BSP), and osteocalcin [103,104]. Osterix is an osteoblast-specific transcription factor containing a zinc finger. It maintains strong expression in mesenchymal cells and the periosteum and is expressed in cells, such as chondrocytes and the bone matrix [105].

In the present study, we determined the gene expression patterns of osteoblast differentiation markers to understand how *G. littoralis* extracts induce mineralization. The results showed that *G. littoralis* extract treatments significantly elevated osterix and RUNX2 gene expression and enhanced the production of proteins involved in osteoblast production, such as type 1 collagen and osteocalcin. RUNX2 gene upregulation by the cells and their ALP activity and mineralization have also been reported in *Davallia formosana* extracts [106]. Previous studies have shown that phenolic compounds of different plant species extracts can induce the proliferative capacity and maturation of osteoblastic cells by improving ALP activity and increasing calcium ion deposition in the ECM [107,108]. It has been reported that changes in osteoblastic cell activity by phenolic compounds occur through the modulation of different transcription factors, such as osterix, osteocalcin, and bone morphogenic proteins (BMPs), by activating the genes involved [108]. The phenolic compounds of various plant species induce osteoblast cell differentiation through the expression of osterix and RUNX2 markers, which are associated with bone maturation [109–113]. In another study, daidzein, present in soy, acted as a phytoestrogen via osteoblast proliferation and differentiation by activating the BMP/Smad signaling pathway [114]. In the present study, all the GL extracts showed higher expression levels of mRNA expression rate of RUNX2 than control. Furthermore, it has been shown that imperatorin promotes the maturation and differentiation of osteoblast by increasing the expression of RUNX2; thus, it is closely associated with early stage osteogenic differentiation [115–117]. In the present study, all *G. littoralis* extracts increased RUNX2 gene expression. The results described here indicate that *G. littoralis* ethanolic extracts can effectively prevent osteoporosis. These results indicate that the phenolic compounds in GL extracts may synergistically induce osteoblastic cell proliferation to a greater extent than a single compound. Moreover, the results further suggest that phytochemicals other than phenolic compounds may be present in the *G. littoralis* extracts, causing the osteoblastic proliferation of MC3T3-E1 cells. Because the extracts of GLAE with stronger antioxidant activity show stronger anticancer activities, it is implied that the contents of flavonoids in GL are responsible not only for its antioxidant activities but also effectively prevent osteoporosis.

5. Conclusions

This study is the first to demonstrate that *G. littoralis* extracts can enhance osteoblast cell formation. The data produced at the molecular level suggest that *G. littoralis* extracts effectively induced osteoblast cell ALP production and mineralization. However, further research is required to establish the detailed mechanism involved in *G. littoralis*'s anti-osteoporotic potential by identifying the active components present in *G. littoralis* extracts. This study suggests that *G. littoralis* extracts have phytoestrogenic properties that may enable the development of therapeutic agents to prevent osteoporosis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11051491/s1. Table S1. Pearson's correlation coefficient among antioxidant activities, total phenolic contents, and total flavonoid contents.

Author Contributions: C.J.K. and B.K.G. contributed by doing experiments and writing the manuscript. C.Y.Y. supervised the experiments. S.K.C. and J.G.L. contributed by analyzing phenolic compounds and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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