

Effects of *Cirsium setidens* (Dunn) Nakai on the osteogenic differentiation of stem cells

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Abstract. *Cirsium setidens* (Dunn) Nakai, commonly known as gondre, is a perennial herb that grows predominantly in South Korea. It contains several bioactive phytochemicals with antioxidant, anti-cancer, anti-tumor and anti-inflammatory properties. The present study aimed to investigate the effects of methanolic extracts of gondre on osteogenic differentiation of human periodontal ligament stem cells (hPDLSCs). As characterized by nuclear magnetic resonance spectroscopy and matrix-assisted laser deposition/ionization (time-of-flight) mass spectrometry, the methanol extract of gondre was found to be enriched with pectolinarin. After 48 h, enhanced viability of hPDLSCs was observed in the presence of gondre compared with under control conditions, suggesting the biocompatibility of gondre. Notably, biocompatibility was markedly affected by gondre concentration in cultured media. Relatively high cell viability was observed in medium containing 0.05% gondre. Furthermore, mineralization was significantly higher in hPDLSCs in the presence of gondre compared with that in control cells, indicating their mineralization potential. Increased expression of various transcription markers, such as collagen 1, runt-related transcription factor 2, bone sialoprotein and alkaline phosphatase, was also detected when hPDLSCs were stimulated with gondre compared with in the control groups, further confirming the superior osteogenic potential of gondre extract for tissue engineering applications, particularly in bone tissues.

Introduction

Medicinal plants and their derivatives have emerged as functional food materials globally because they contain diverse bioactive phytochemicals with potential immunomodulatory effects on the human body. Therefore, the demand for functional foods and beverages has rapidly increased (1,2). Active ingredients in functional foods include polyunsaturated fatty acids, peptides, flavonoids, and phenolic compounds, which help prevent obesity, diabetes, and cancer (3,4).

Cirsium setidens (Dunn) Nakai, a wild perennial herb popularly known as gondre, is mainly found in the Kangwon province of South Korea (5). Its extract is commonly used as a food product in Korea, China, Japan, Canada, USA, and Australia (6). All parts of *Cirsium spp.*, including roots, are enriched in phytochemicals, such as flavonoids, phenolics, sterols, and alkaloids, and utilized in the treatment of liver and kidney inflammation and bleeding, as well as other hepatic disorders (7-9). In addition, the aerial parts of *C. chanroenicum* are used to treat fever and to improve detoxification and blood circulation, in Chinese medicine (10). Leaves and stems are rich sources of calcium, proteins, and vitamins. In the case of gondre, pectolinarin, pectolinarigenin, apigenin, and luteolin are the essential phytochemicals that can regulate various physiological activities (11). Pectolinarin has anti-tumor, anti-cancer, anti-oxidant, anti-inflammatory, and hepatoprotective properties, whereas pectolinarigenin has anti-oxidant and anti-inflammatory effects (12-14).

Stem cells are considered a promising source for regenerative medicine and tissue engineering, because of their multi-lineage differentiation potential and self-renewal properties (15,16). For instance, human mesenchymal stem cells (hMSCs) are used in cell-based therapies to treat various diseases. Nevertheless, microenvironment conditions, such as oxidative stress, low oxygen level, inflammation, and limited nutrient supply, restrict clinical trials of stem cells (17).

Human dental mesenchymal stem cells can be obtained from different dental tissues, such as the periodontal ligament, dental pulp, periapical follicle, and apical papilla. These cells have the potential for multi-lineage differentiation, and can be exploited to regenerate a desired cell type for tissue engineering applications (18,19).

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This study aimed to investigate the differentiation potential of human periodontal ligament stem cells (hPDLSCs) in the presence of gondre powder. Thus, a methanol extraction was performed to determine the major bioactive components of the gondre powder, and the extracted material was analyzed with nuclear magnetic resonance (NMR) and matrix-assisted laser deposition/ionization (time-of-flight) MALDI-TOF mass spectrometry. The results indicated that pectolinarin is the chief component of the gondre extract. Enhanced mineralization occurred in hPDLSCs in the presence of gondre powder via the activation of osteogenesis-related genes and proteins. Therefore, gondre can be used as a functional food material for improving metabolism and cellular homeostasis.

Materials and methods

Extraction and characterization of the methanol extract of *C. setidens*. The gondre powder was obtained from the Department of Food Science and Biotechnology, Kangwon National University, Republic of Korea. The extraction of bioactive phytochemicals was performed as described by Jeong *et al* (11). Briefly, methanol was added to sufficient quantities of dried gondre powder at room temperature, with continuous mechanical stirring for 4 h. After that, the mixture was filtered to remove undesired particles, and repeated extraction was performed three times. The obtained solution was centrifuged ($4,000 \times g/10^\circ\text{C}$) for 30 min, and the supernatant was separated for further analysis. The chemical structures of the phytochemicals present in the methanol extract were elucidated with $^1\text{H-NMR}$ (JNM-ECZ400S/L1) in DMSO-d_6 solvent, and MALDI-TOF mass spectrometry (Bruker Autoflex speed TOF/TOF).

Culture of hPDLSCs. Human third molars were collected from three young males (18–22 years). This protocol was approved by the Institutional Review Board of the Dental Hospital, Seoul National University (Seoul, Republic of Korea; IRB Number 05004), and written consent was obtained from each patient. The primary cell culture was established according to the procedure described by Jin and Choung (20). In brief, hPDLSCs were gradually separated from the extracted third molar and treated with a solution of 3 mg/ml collagenase type 1 (Worthington Biochem) and 4 mg/ml dispase (Boehringer-Mannheim) at 37°C for 1 h. The cell suspension was obtained by passing the solution through a $40\text{-}\mu\text{m}$ strainer (Falcon-BD Labware). The derived cells were cultured in α -modified Eagle's medium (α -MEM, Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), $100\text{ }\mu\text{mol/l}$ ascorbic acid 2-phosphate (Sigma-Aldrich; Merck KGaA), 2 mM glutamine, 100 U/ml penicillin, and $100\text{ }\mu\text{g/ml}$ streptomycin (Biofluids), and incubated at 37°C in 5% CO_2 . The medium was changed after 24 h, followed by changes every 3–4 days. Only primary cells at passages 2 or 3 were used for the proliferation and differentiation studies.

Flow cytometry analysis. The expression of mesenchymal stem cell-associated surface markers at passage 3 was analyzed with flow cytometry, to characterize the immunophenotype. Approximately 1×10^6 cells were fixed with 3.7% paraformal-

dehyde (Sigma-Aldrich; Merck KGaA) for 10 min and then re-suspended in phosphate-buffered saline (PBS) solution (Welegene) containing 1% bovine serum albumin (BSA) (ICN Biomedicals), for 30 min to block the nonspecific antibody-binding sites. Next, the cells were incubated with specific antibodies against CD34, CD13, CD90, and CD146 at 4°C for 1 h, followed by incubation with fluorescent secondary antibodies at room temperature for 1 h. All antibodies were purchased from BD Biosciences. The percentages of CD13, CD90, and CD146-positive, as well as CD34-negative cells, were measured with a fluorescence-activated cell sorting (FACS) caliber flow cytometer (Becton Dickinson Immunocytometry Systems). The data were analyzed using the Cell Quest Pro software (BD Biosciences).

Cytotoxicity and migration assay. Cell proliferation and cytotoxicity were measured using the colorimetric 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Promega Corp.). Briefly, the cells were seeded in 96-well plates (1×10^4 cells per well), cultured for the desired periods, and then treated with various concentrations of gondre powder (0.01, 0.05, 0.1, 0.2, and 0.25%) for the required time periods. These concentrations have proliferative effects, as reported earlier (21). Medium without gondre was considered a negative control. The dye solution ($15\text{ }\mu\text{l}$) was added at the end of the treatment, followed by further incubation in 5% CO_2 at 37°C for 4 h. The solubilized formazan product was obtained by adding $100\text{ }\mu\text{l}$ of a solubilization/stop solution in the media. The formed products were quantitated using an ELISA plate reader at 595 nm (with readings at 655 nm as reference). All experiments were performed in triplicate, and values are expressed as mean \pm standard deviation (SD).

We performed migration assays to assess the defect healing potential of hPDLSCs in the presence of gondre. To this end, cells were seeded in 6-well plates and allowed to grow to 90–100% confluence. Next, the cell monolayer was wounded with a plastic tip (1 mm), and washed with PBS (twice) to remove cell debris. Scratched cells were incubated either with or without gondre. Cell migration towards the wounded area was monitored after time intervals of 0, 12, 24 and 48 h, using a light microscope (Olympus U-SPT; Olympus Corporation). Cell migration was quantified by measuring the distance traveled by migrating cells from the wound edge (starting point at $t=0$) to the furthest migration point.

Multi-lineage differentiation of hPDLSCs. The cells were cultured in osteogenic, chondrogenic, adipogenic, and neurogenic differentiation media for 21 days (Gibco; Thermo Fisher Scientific, Inc.) to examine the osteogenic, chondrogenic, adipogenic, and neurogenic differentiation potential of hPDLSCs, respectively, when stimulated with an appropriate supplement. After 21 days of treatment, cells were stained with 2% alizarin red S stain (ARS) at pH 4.2, 1% alcian blue, 0.3% oil red O dye (all three from Sigma-Aldrich; Merck KGaA), and Nissl stain. Stained cells were visualized under an inverted light microscope to detect the calcified matrix, proteoglycans, fat vacuoles, and Nissl bodies, which are indicators of osteogenic, chondrogenic, adipogenic, and neurogenic differentiation, respectively.

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	GenBank no.	Sequences
<i>Coll</i>	NM007742	F: 5'-GCTCCTCTTAGGGGCCACT-3' R: 5'-CCACGTCTCACCATTGGGG-3'
<i>Runx2</i>	NM_001146038	F: 5'-CGCACGACAACCGCACCAT-3' R: 5'-CAGCACGGAGCACAGGAAGTT-3'
<i>BSP</i>	L09555	F: 5'-AACTTTTATGTCCCCCGTTGA-3' R: 5'-TGGACTGGAAACCGTTTCAGA-3'
<i>ALP</i>	NM007431	F: 5'-CCAACCTCTTTTGTGCCAGAGA-3' R: 5'-GGCTACATTGGTGTGAGCTTTT-3'
<i>HPRT</i>	NM_000194	F: 5'-GGCTATAAGTTCTTTGCTGACCTG-3' R: 5'-CCACAGGGACTAGAACACCTGCTA-3'

Coll, collagen 1; *Runx2*, runt-related transcription factor 2; *BSP*, bone sialoprotein; *ALP*, alkaline phosphatase; *HPRT*, hypoxanthine-guanine phosphoribosyl transferase; F, forward; R, reverse.

In vitro differentiation and mineralization. Cells (4×10^4) were cultured in a 24-well plate with α -MEM containing 10% FBS until they reached 50% to 60% confluency. The cells were fixed with 10% formalin solution (Duksan Chemical Co., Gyeonggi-do), followed by an incubation with 0.1% Triton X-100 for 5 min. Then, the incubated cells were stained with a leukocyte ALP kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocols. Mineralized nodules were detected by staining with 2% ARS at pH 4.2 on treatment day 14. For mineralization, cells were cultured in an osteogenic differentiation medium with 50 μ g/ml ascorbic acid, 10 mM β -glycerophosphate, and 100 nM dexamethasone (Sigma-Aldrich; Merck KGaA) for 14 days.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. We utilized RT-qPCR to evaluate the expression of osteogenesis-related genes in hPDLSCs (22,23). Briefly, cells (1×10^6) were cultured in a 60-mm culture dish for 2 weeks under differentiation induction conditions, and RNA was isolated from the treated cells using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Next, cDNA was synthesized from 2 μ g of total RNA using reverse transcriptase (Superscript II Preamplification System; Invitrogen; Thermo Fisher Scientific, Inc.). SYBR-Green PCR Master Mix (ABI Prism 7500; Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for RT-qPCR. The experiment conditions were as follows: 40 cycles of denaturation for 15 sec at 95°C and 1 min of amplification at 60°C. All reactions were run in triplicate, and normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT). The cycle threshold values were calculated and compared to assess gene expression levels in control and gondre-treated groups. The relative mRNA expression levels in hPDLSCs and their gondre-treated counterparts were plotted in a histogram. We evaluated the expression levels of collagen 1 (*Coll*), runt-related transcription factor 2 (*Runx2*), bone sialoprotein (*BSP*), and alkaline phosphatase (*ALP*) and *HPRT*. The specific primer sets used for this analysis are listed in Table I.

Western blot analysis. Gondre-induced protein expression levels were determined with western blotting. For this, cells were lysed with RIPA buffer containing 1 mM phenyl-methylsulfonyl fluoride, centrifuged, and collected in clean Eppendorf tubes. Protein concentration was analyzed using the BSA protein assay kit (Bio-Rad Laboratories, Inc.). An equal volume of protein (25 μ g) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (GE Healthcare). Primary antibodies against *Runx2* and *osterix* (*OSX*) were purchased from Abcam. The blots were developed using horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.), and visualized using a gel-documentation imaging system (Chemi-Doc XRS+Imaging System; Bio-Rad Laboratories, Inc.). Blots were quantified using the ImageJ software (ImageJ v1.8, NIH), and normalized to α -tubulin.

Statistical analysis. Statistical analysis was performed with one-way ANOVA using the Origin Pro 9.0 software. All experiments were performed in triplicate ($n=3$), and the results are expressed as mean OD \pm SD. To compare significant differences between control and experimental groups, Tukey's post hoc analysis was performed. $P<0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

Characterization of the extract. The proton spectrum is a powerful analytical tool used to identify the different kinds of protons present in a structure. This information provides substantial support for explaining the chemical composition of a certain material. The ^1H -NMR spectrum of the methanol-extracted sample is shown in Fig. 1. The NMR spectrum resembled the previously reported pattern of pectolinarin, which suggests that the extracted material primarily consisted of pectolinarin (10,12). The proton NMR spectrum exhibits several peaks in the chemical shift (δ) region of 0.83-2.2 ppm, which correspond to the methyl

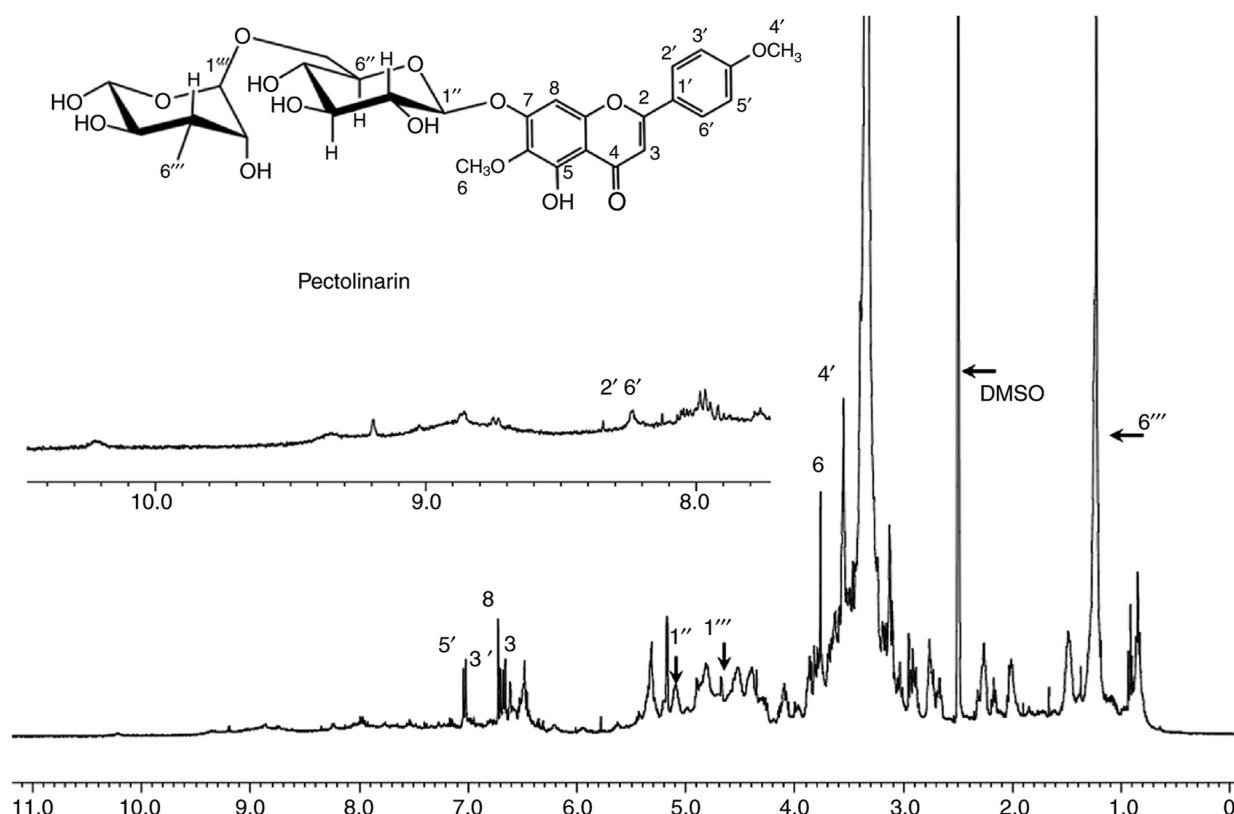


Figure 1. The ^1H -nuclear magnetic resonance spectrum of the methanol-extracted bioactive phytochemicals from *Cirsium setidens* (gondre), indicating the presence of pectolinarin chemical moiety.

and methylene hydrogen moieties (24). Chemical shifts of other protons due to different chemical environments are indicated in detail in the proton spectrum. The MALDI-TOF mass spectrum of the methanol-extracted sample with a 2,5-Dihydroxybenzoic acid (2,5-DHB)-assisted matrix is shown in Fig. 2. It is well established that the 2,5-DHB matrix enhances the intensity of the signals in the MALDI-TOF spectrometry (25). Interestingly, the gap between the two fragmented peaks is not constant. This is attributed to the presence of different chemical moieties in the extracted sample, which are associated with various linkages. The gap between the two peaks was ~ 147 and 149 Da, suggesting the presence of glucose and rhamnose units in the structure, respectively. It can also be inferred that it was linked with a glycosidic linkage, which was cleaved during the measurement. The appearance of a signal at ~ 288 shows the presence of a flavone structure in the methanol extract, which is linked to the glucose unit. It is not always necessary to consider the highest m/z peak ratio as the molecular ion species $[\text{M}+\text{H}]^+$ in the positive mode, and $[\text{M}-\text{H}]^-$ in a negative way, mainly because of the formation of molecular complexes ($[2\text{M}+\text{H}]^+$ or $[2\text{M}-\text{H}]^-$) or adducts with solvents or acid molecules (26). Based on the data obtained by ^1H -NMR and MALDI-TOF, the methanol extract of gondre predominantly consisted of the pectolinarin chemical moiety.

Characterization of the hPDLSCs. A schematic illustration of the presence of periodontal ligament cells in the human molar tooth and of their isolation for primary cell culture is presented in Fig. 3A. Cultured cell morphology after

different time intervals is shown in Fig. 3B. Stem cells are characterized by their pluripotentiality and their expression of different surface markers, and their fate is profoundly affected by growth factors, cytokines, and the surrounding microenvironments (27,28). These factors can promote self-renewal and dedifferentiation of stem cells. This ability is known as the stemness potential (29). As shown in Fig. 3C, we evaluated the stemness potential of hPDLSCs through FACS analysis. Not only can hPDLSCs be differentiated into cementoblasts, osteoblasts, adipocytes, and chondrocytes (30), but they also exhibit superior bone cell formation properties, compared with human dental pulp stem cells and human periapical follicular stem cells (31). FACS results showed enhanced expression ($\sim 90\%$) of stem cell-related surface markers, such as CD13, CD90, and CD146, indicating the stemness potential of cultured hPDLSCs. The low expression ($\sim 10\%$) of CD34 surface markers in hPDLSCs supports their stemness potential as well. Fig. 3D shows the multi-lineage differentiation potential of hPDLSCs after 21 days of culture in diverse induction media, which was determined by monitoring the expression of specific markers. The presence of mineralized matrix, proteoglycan, fat vacuoles, and Nissl bodies, as detected by staining with ARS, alcian blue, oil red O dye, and Nissl stain, indicates the osteogenic, chondrogenic, adipogenic, and neurogenic potential of hPDLSCs, respectively (10).

Cell viability and migration. Cytotoxicity is one of the important criteria that determine whether a substance can be used in the biomedical field, since materials should be nontoxic

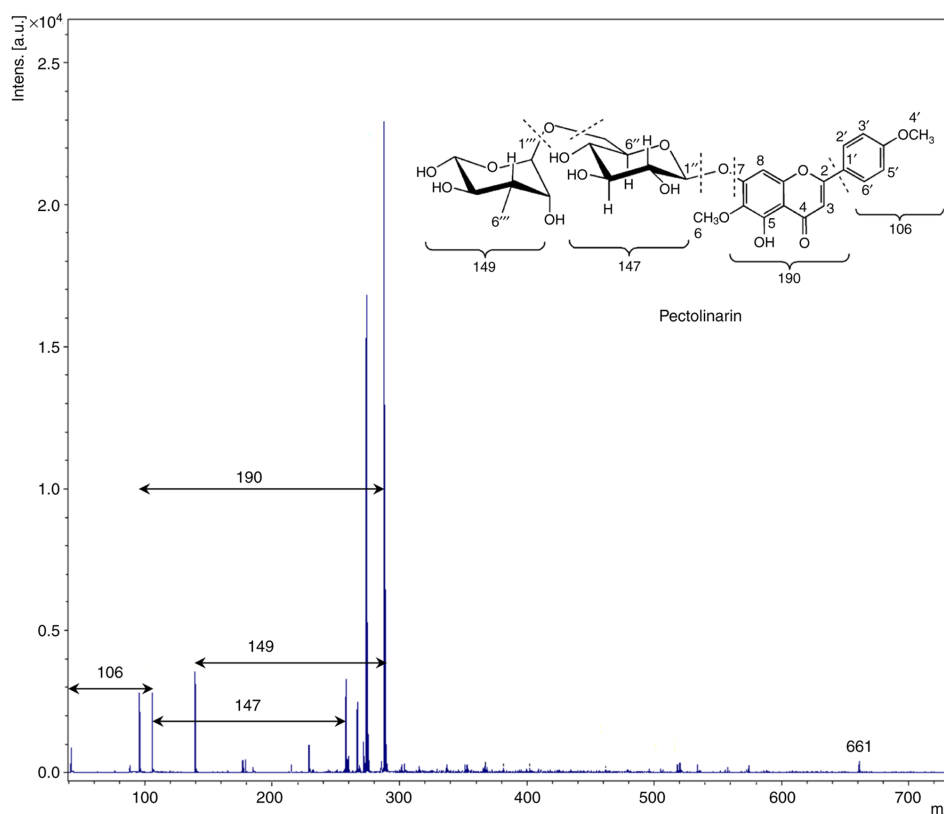


Figure 2. MALDI-TOF mass spectrum of the methanol-extracted sample of gondre, using 2,5-DHB as a matrix. The arrows indicate the gap between the two fragmented peaks in Da. The fragmented ions appeared at ~98, 106, 139, 253, and 288 Da. MALDI-TOF, Matrix-assisted laser desorption/ionization (time-of-flight).

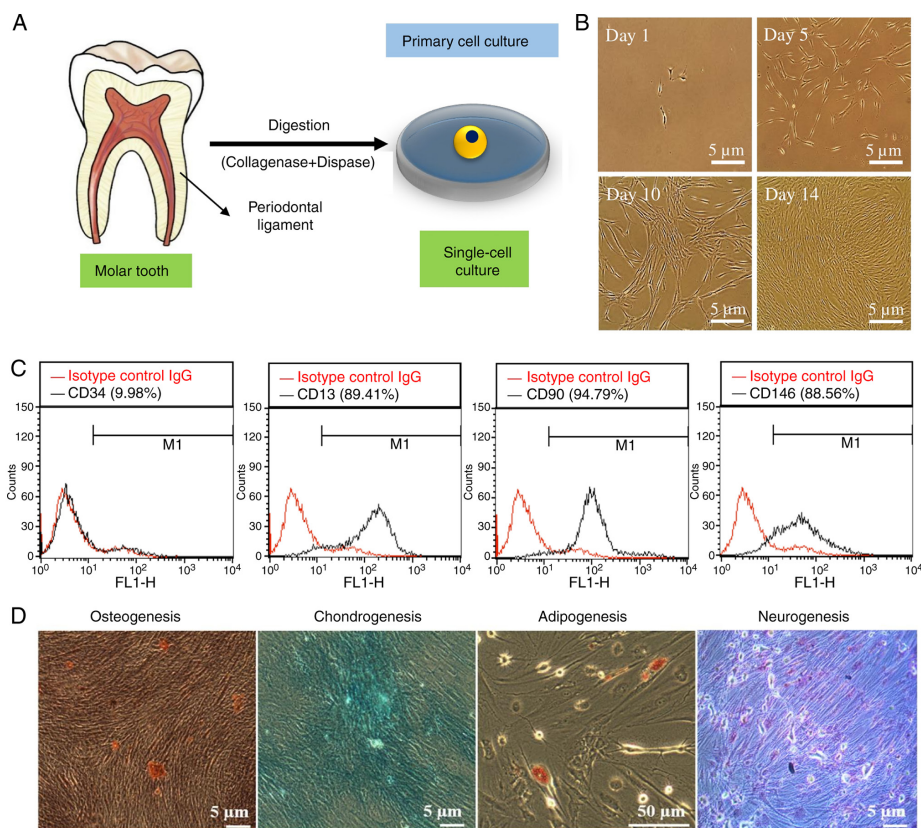


Figure 3. Isolation and characterization of hPDLSCs. (A) Schematic presentation for the presence of hPDLSCs in the human molar tooth, (B) The morphologies of the cultured stem cells after different time intervals, (C) Fluorescence-activated cell sorting analysis of hPDLSCs. (D) The multi-lineage differentiation potential of hPDLSCs after 21 days of treatment in different induction media. hPDLSCs, human periodontal ligament stem cells.

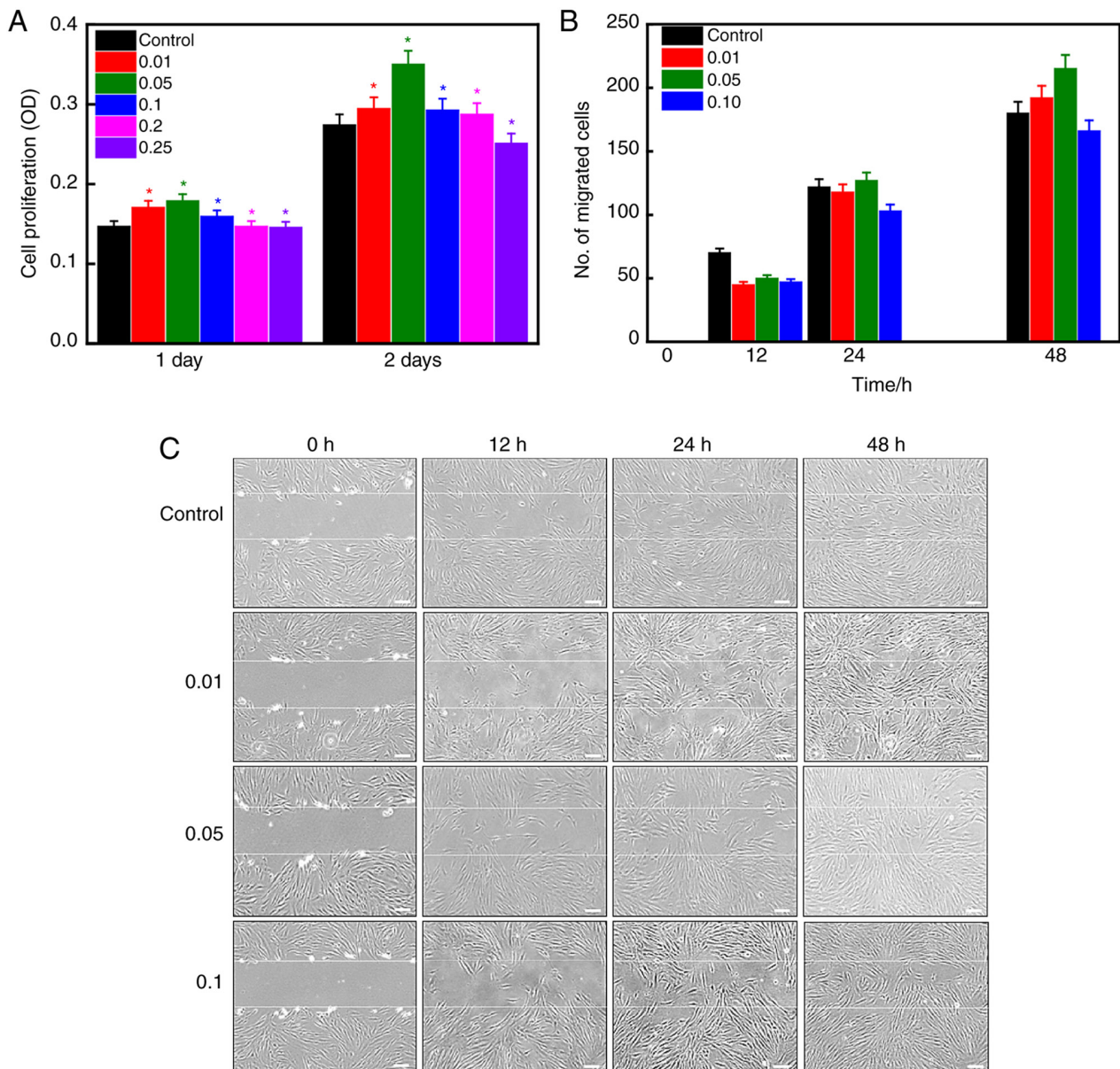


Figure 4. Evaluation of the cytotoxicity and migration potential of hPDLSCs. (A) The cell viability data of hPDLSCs in the presence of different concentrations of gondre at indicated time intervals. (B and C) The scratch healing assay in the presence of gondre at indicated time intervals (magnification, x10). The data are mean \pm SD of triplicate experiments (n=3). *P<0.05 vs Control group. hPDLSCs, human periodontal ligament stem cells.

and biodegradable (32). The cytotoxic effects of gondre powder on hPDLSCs were evaluated using the MTT assay, as presented in Fig. 4A. Cells cultured in medium without gondre were used as control. An increase in cell viability in gondre-treated hPDLSCs, compared with control ones, was evidenced after 24 h of incubation, suggesting that there is biocompatibility. Furthermore, a significant improvement in cell viability was observed after 48 h of incubation, indicating their improved biocompatibility. This shows that gondre has no adverse effects on hPDLSCs. Among 0.01, 0.05, 0.1, 0.2, and 0.25% gondre concentrations, 0.05% elicited the highest cell viability, demonstrating that it is a suitable concentration for improved biocompatibility. Lee *et al* (15) also reported increased viability of hMSCs in the presence of gondre, after inducing oxidative stress with H_2O_2 for 8 h. The authors noted that cell viability was significantly enhanced in the presence of *C. setidens*, compared to untreated conditions,

which evidenced the protective effects of *C. setidens* against H_2O_2 -induced oxidative stress (15).

Migration is essential for living cells, as it contributes to healthy development, and immune responses, as well as to disease processes, such as cancer metastasis and inflammation (33). Stem cell migration occurs not only during embryonic development but also in adult tissues to maintain homeostasis and repair damage. The migration ability of stem cells has tremendous therapeutic significance in the field of regenerative tissue applications (34). Fig. 4B and C shows the migration potential of hPDLSCs in the presence of different concentrations of gondre at indicated time intervals. Although cell migration towards the wounded area was initially slow in the presence of gondre compared with control conditions, after 24 h, this tendency increased, and a higher accumulation of cells was observed in gondre-treated conditions, compared to the control. Moreover, a 0.05% concentration of gondre

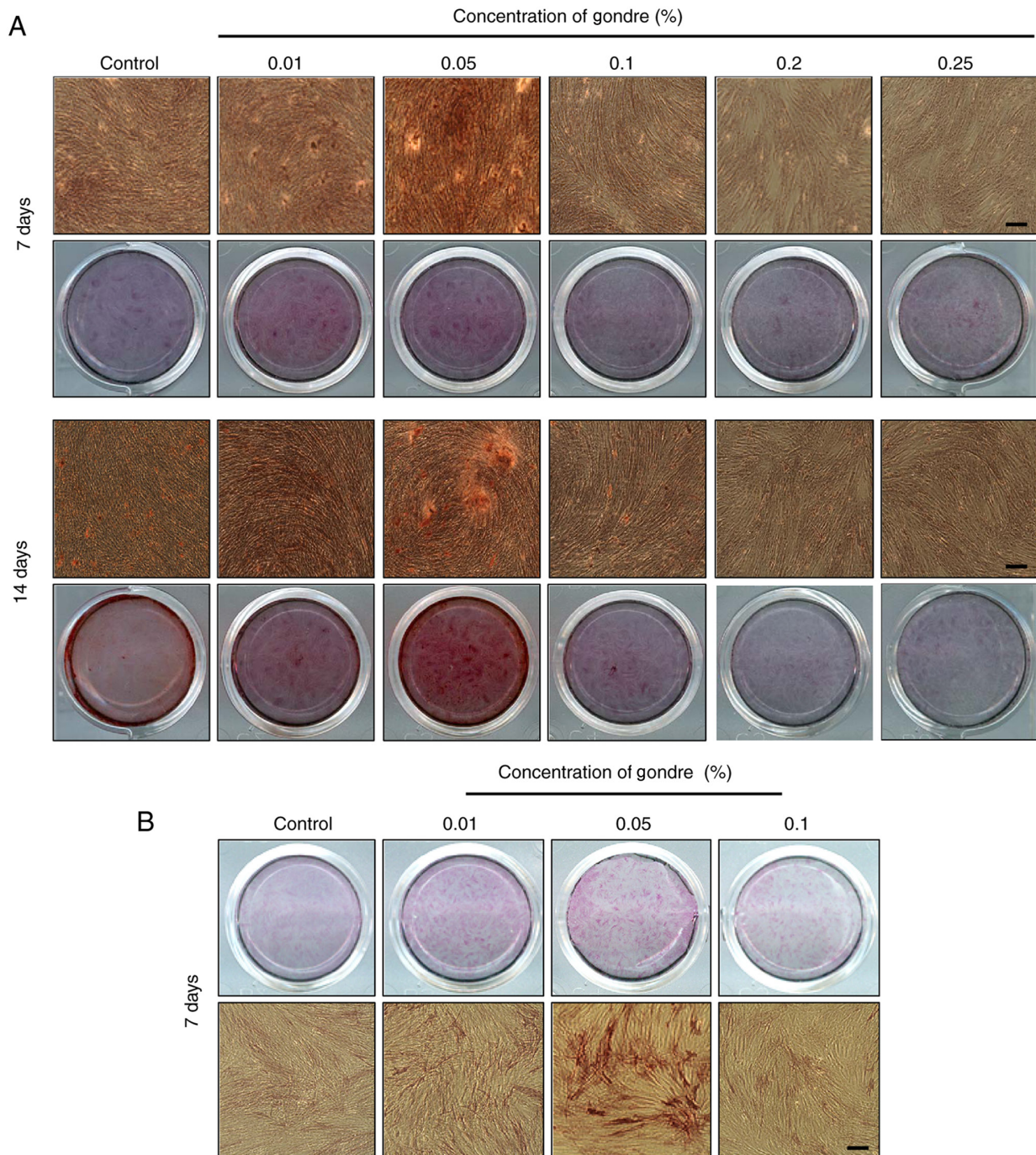


Figure 5. *In vitro* differentiation potential of hPDLSCs in the presence of gondre extract. (A) The mineralized nodule formation potential of hPDLSCs in the presence of different gondre concentrations at indicated time intervals (magnification, x10). (B) Alkaline phosphatase activity of hPDLSCs in the presence of the indicated gondre concentration after 14 days of incubation (magnification, x10). hPDLSCs, human periodontal ligament stem cells.

exhibited a high number of cells migrating towards the wound area. Cell viability and migration tendency results indicated that the 0.05% gondre concentration was the optimal concentration to improve cellular activity.

Mineralization and ALP activity. Stem cells are the most prominent cells in tissue engineering applications, owing to their differentiation potential. Even though they can be differentiated into osteoblasts, chondrocytes, adipocytes, and other cells (35,36), their differentiation ability is profoundly affected by local biological conditions (37). Gondre-induced

mineralization of hPDLSCs was examined through the ARS staining process after 7 and 14 days of incubation, and the results are shown in Fig. 5A. A more intense color was observed in gondre-treated cells compared with those in control conditions, which demonstrated better mineralization potential. This parameter is extensively affected by the biomaterial's concentration in the differentiation medium (38). Among experimental conditions (0, 0.01, 0.05, 0.1, 0.2, and 0.25%), a gondre concentration of 0.05% showed greater potential for mineralization after 7 days of treatment. A similar trend was also observed after 14 days of

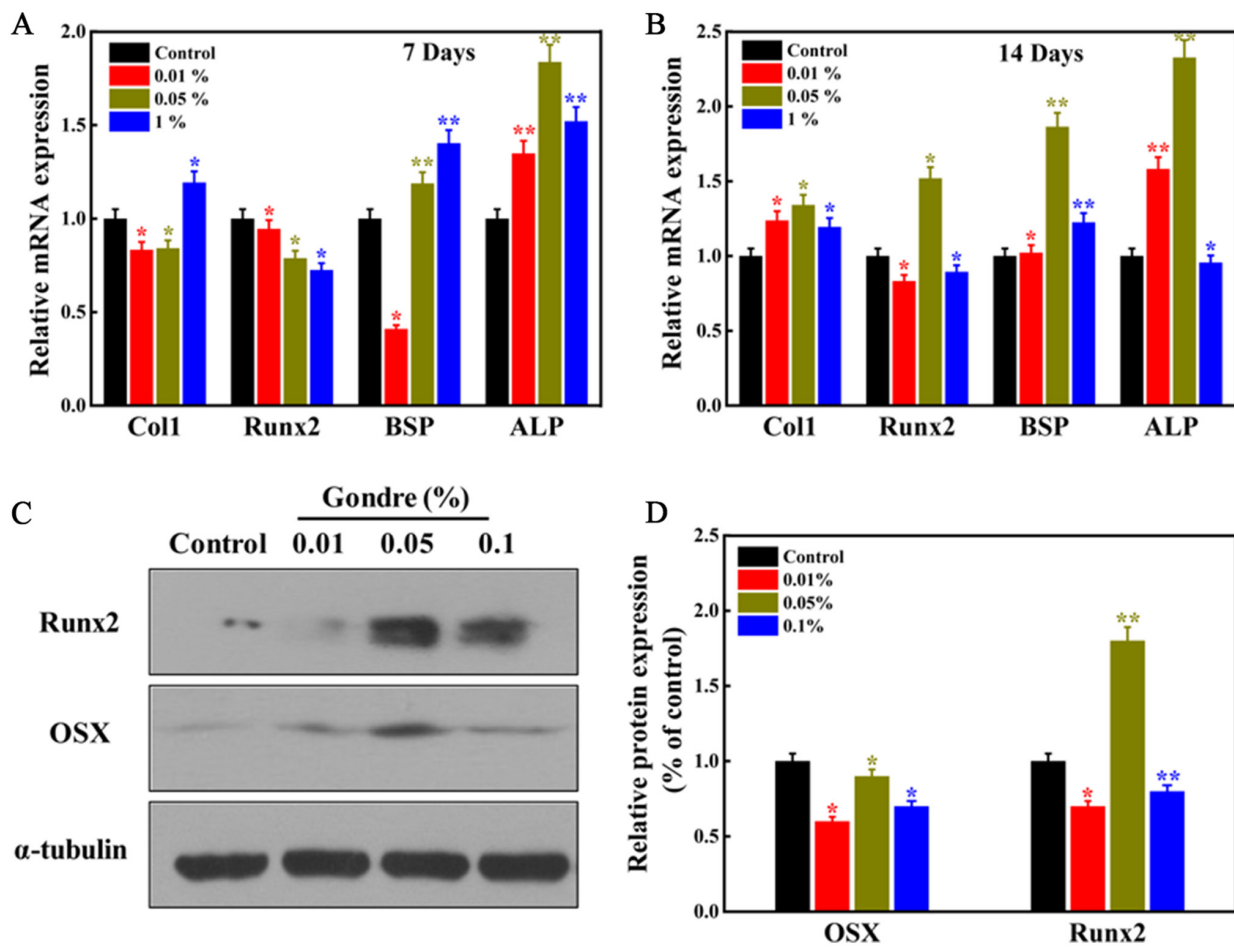


Figure 6. Evaluation of the expression of osteogenesis-specific gene markers and proteins in the presence of gondre powder. (A and B) The relative expression level of mRNA in the presence of different concentrations of gondre after 7, and after 14 days of treatment, respectively. Data were normalized to the house-keeping gene HPRT. (C) Western blot analysis of Runx2 and OSX following gondre (0, 0.01, 0.05, and 0.1%) treatment. (D) Western blotting was quantified using the ImageJ software, and data were normalized to α -tubulin. The data are mean \pm SD of triplicate experiments ($n=3$). * $P<0.05$, ** $P<0.01$ vs. Control. Col1, collagen 1; Runx2, runt-related transcription factor 2; BSP, bone sialoprotein 2; ALP, alkaline phosphatase; OSX, osterix.

treatment, indicating that 0.05% is the optimal concentration for mineralization.

Different dosages of gondre may have different effects on stem cells. For instance, it has been reported that lower concentrations of this traditional herb enhance the metabolic efficacy and alkaline phosphatase activity, whereas high concentrations remarkably decrease cell density (3). Fig. 5B shows the ALP activity of hPDLSCs in the presence of gondre after 14 days of treatment. ALP activity was higher in the gondre-treated groups than in the control group, and this difference was significant in the 0.05% gondre-treated cells. Since ALP is an important gene marker that suggests the presence of preosteoblasts and osteoblasts for bone mineral production at the time of differentiation (23,39), more intensely stained cells in the presence of gondre confirm a higher osteogenic potential of gondre-treated cells compared to control ones. It is well known that osteoclast resorption and osteoblast formation are two important processes that influence overall bone metabolic activity.

Osteogenic gene and protein expression. Bone formation is a complex biological process that involves several

osteogenesis-associated genes (22). The expression of osteogenesis-related genes (Col1, Runx2, BSP, and ALP) in the presence of gondre after 7 and 14 days of treatment is shown in Fig. 6A and B. Untreated cells (no gondre) were considered as controls. Col1, the most abundant protein found in the bone matrix, is vital during the proliferation of osteoblast cells (40). The expression of the Col1 biomarker from hPDLSCs in the presence of gondre evidences bone cell formation. Moreover, its expression was high in gondre-treated cells, compared to control ones, after 14 days of treatment, suggesting their greater osteogenic potential. Runx2 is another osteogenic marker that is expressed during osteogenesis. Since it is an early transcription marker, osteogenesis cannot occur without it. The results showed that its expression was higher in the gondre-treated cells than in the control ones. Moreover, Runx2 expression was high after 14 days of treatment with 0.05% gondre. The expression and activity of Runx2 are severely affected by other transcription factors and protein-protein or protein-DNA interactions. However, overexpression of the Runx2 factor facilitates bone resorption (35). BSP comes from a 'small integrin-binding ligand N-linked glycoprotein' (SIBLING) family, which occurs in bone and dentin. SIBLING plays a significant role in bone

development, healing, remodeling, and mineralization (41). Higher expression levels of BSP in the presence of gondre, compared to those in control conditions, indicated a higher osteogenic efficiency.

Osteogenesis is widely affected by the nature, concentration, and size of the biomaterials present in the osteogenic media (42). Bi *et al* (43) tested the osteogenic potential of surface-functionalized gold nanoparticles (Au NPs) with different surface charges in the presence of hMSCs. They observed that Au NPs with hydroxyl groups exhibited higher osteogenic properties than others due to the presence of a hydroxyl moiety, which facilitates bone formation and growth, thus inducing osteogenesis. The hydroxyl groups also promote ECM growth, which contributes to osteogenesis (43). A similar result was also reported by Chen *et al* (44), with green tea catechins. In the present work, the abundant hydroxyl groups in pectolinarin facilitate apatite deposition in the matrix, as observed in the ALP activity and mineralization test, which causes greater osteogenesis. Cellular activity was higher at the 0.05% concentration compared to the others; hence, more apatite deposition occurred at this concentration, leading to superior osteogenesis.

Understanding the interaction mechanisms between hPDLSCs and gondre at the cellular and molecular level will help utilize the herb-derived material for osteogenic differentiation of cells in tissue engineering applications. Thus, the material characteristics and mechano-transduction pathways provide the primary molecular mechanics that affect cellular activities (45). It has been reported that nanomaterial-incorporated platforms display improved cellular activities, owing to their nano-topographical surface geometry, which promote cell proliferation and differentiation (46). Therefore, the abundance of hydroxyl groups in gondre promotes the improved osteogenic differentiation of hPDLSCs *via* their surface chemistry. As presented in Fig. 6C and D, changes in protein expression of hPDLSCs in the presence of gondre were evaluated with western blotting. Results showed that protein expression of Runx2 was higher in gondre-treated cells than in control ones, further confirming their superior osteogenic potential. In particular, 0.05% gondre-treated cells presented the highest levels compared to the other groups. Runx2, also known as Cbfa1 or AML3 transcription factor, is an essential transcription factor that regulates the osteogenic differentiation of stem cells (47). Based on the improved osteogenic activity and protein expression in the presence of gondre, a hypothetical pathway is presented in Fig. 7. It has been reported that the mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinase (ERK) signaling pathway may play a crucial role in osteogenic differentiation and bone cell development by accelerating Runx2 phosphorylation and transcriptional activity (47). Previously, it was reported that gondre extract had a positive role in H₂O₂-induced oxidative damage protection in hMSCs by signaling through MAPK/ERK pathway (15). Therefore, we assumed that the MAPK/ERK signaling pathway facilitates superior osteogenic differentiation in the presence of gondre, by activating Runx2 phosphorylation and transcriptional activity. The lack of proper molecular mechanism to support

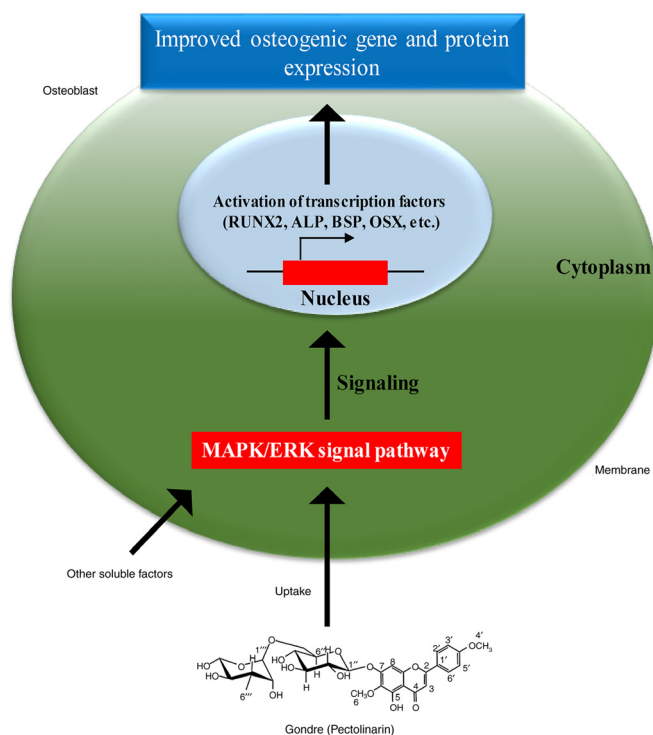


Figure 7. A hypothetical drawing for gondre-induced odontoblastic differentiation of hPDLSCs. hPDLSCs, human periodontal ligament stem cells; RUNX2, runt-related transcription factor 2; BSP, bone sialoprotein; ALP, alkaline phosphatase; OSX, osterix.

these findings will be the future direction of this work. In this study, a concentration of 0.05% gondre is optimal and can be explored as an osteogenic substrate for tissue engineering applications, especially in bone tissues.

In this study, we extracted the phytochemicals present in gondre, using methanol, and characterized them via MALDI-TOF mass spectrometry and ¹H-NMR spectroscopy. It was observed that the methanol extract of gondre primarily consisted of pectolinarin. hPDLSCs were used to evaluate gondre cytotoxicity. The stemness potential of the hPDLSCs was evaluated using the FACS technique. Interestingly, the isolated hPDLSCs maintained their stemness potential. Notably, higher cell viability was observed in the presence of gondre, compared to the control, and viability was extensively affected by gondre content in the media. Among these concentrations (0.01, 0.05, 0.1, 0.2, and 0.25%), 0.05% exhibited greater cell viability than the other concentrations. The cell migration study also supports that 0.05% is an ideal concentration for improved cellular activity. A more intense mineralized nodule formation occurred in the presence of 0.05% gondre concentration, and the control showed greater osteogenic potential. In addition, a significant enhancement in osteogenesis-related gene expression was observed in the presence of 0.05% concentration of gondre, compared to the other concentrations, after 7 and 14 days of treatment, suggesting its potential use in tissue engineering applications. Based on these findings, we concluded that gondre was effective for enhanced osteogenesis, and can be useful as a natural, edible, and osteogenic agent.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SDD and DKP conceptualized the study, designed the methodology and validated the study. SDD, BJ, SIC and OHL performed formal analysis. SDD wrote the original draft. DKP reviewed and edited the manuscript. KTL conceptualized the work, acquired funding and gave the final approval of the paper. SDD and KTL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of the Dental Hospital, Seoul National University (Seoul, Republic of Korea; IRB Number 05004), and written consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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