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Effects of Flaxseed (*Linum usitatissimum*) Extract on the Osteoblast Differentiation Potential of Stem Cells Derived from Human Exfoliated Deciduous Teeth

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Abstract

Background: Flaxseed promotes bone health and possibly induces bone regeneration. However, the capacity of flaxseed to induce the differentiation of stem cells into osteoblasts remains unreported. Accordingly, this study aimed to determine the effects of flaxseed extract on the osteoblast differentiation potential of stem cells derived from human exfoliated deciduous teeth (SHED).

Methods: SHED cultured in osteoblast induction media (OIM) were treated with 4 mg/mL flaxseed extract. RNA was collected and extracted with Total RNA Mini Kit (Geneaid) from cells cultured at days 1, 3, 7, 14, and 21 and subjected to reverse-transcriptase PCR for osteoblast markers (*OSX*, *OCN*, and *DMP1*). Alkaline phosphatase (ALP) activity was determined by ALP assay, and Alizarin Red-S staining was performed to evaluate calcium deposition in SHED.

Results: All osteoblast markers were expressed in all samples analyzed. *OSX* expression was reduced in the SHED treated with flaxseed extract. In addition, the SHED treated with flaxseed extract had lower ALP activity than the control ($p < 0.05$). Calcium deposition was positive in the SHED cultured in OIM only.

Conclusions: Flaxseed can reduce the expression of osteoblast markers, ALP activity, and calcium deposition in SHED. Thus, flaxseed potentially inhibits the osteoblast differentiation of SHED.

Keywords: flaxseed extract, human exfoliated deciduous teeth, osteoblast differentiation, stem cells

INTRODUCTION

Flaxseed (*Linum usitatissimum*) contains beneficial compounds recognized for its health benefits. Flaxseed has attracted considerable interest because of its potential benefits associated with its biologically active components, such as approximately 59% α -linoleic acid and lignan secoisolariciresinol diglycoside (SDG).^{1–4} These compounds may exert protective effects on bone formation and bone metabolism. Consuming food rich in omega-3, such as flaxseed, improves bone health.⁵ The consumption of flaxseed also alleviates menopausal effects and osteoporosis.⁶ Flaxseed extract promotes bone health, especially in estrogen-deficient individuals, and possibly induces bone regeneration.^{6–8}

Dental pulp tissue is an attractive source of mesenchymal stem cells (MSCs) because of its readily accessible and high yield source.⁹ In addition, stem cells from dental pulp possess similar gene expression and comparable regenerative potential to bone marrow MSCs.¹⁰ Previously, we have shown that 4 mg/mL flaxseed extract influences the bioavailability and growth of stem cells from human deciduous teeth (SHED).¹¹ SHED is a type of MSCs that can differentiate into multiple cell lineages, including osteoblasts.¹² The osteogenic potential of SHED has been demonstrated previously.^{13,14} In specific, SHED can differentiate into osteoblast cells when cultured in osteoblast induction media containing β -glycerophosphate, ascorbic acid, and dexamethasone.^{15,16} Moreover, the expression levels of osteoblast-related genes increase in SHED cultured in the presence of growth factors, indicating the application of SHED in tissue engineering because of its osteogenic differentiation potential.¹⁴

Bone regeneration involves the physiological process of bone formation during normal fracture healing and is involved in continuous remodeling throughout adult

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life.¹⁷ Given their minimal side effects, natural compounds obtained from natural resources give added value in regenerative medicine. Although flaxseed extract affects bone formation, its effect in inducing stem cells to differentiate into osteoblasts has not been reported. Hence, this study aimed to determine the effects of flaxseed extract on the osteoblast differentiation potential of SHED.

METHODS

Culture of SHED

SHED (ALLCells, USA) were cultured in complete growth media (alpha-MEM; 10% FBS; 0.5% Pen-Strep) until confluence. SHED at 80%–90% confluence were subjected to osteoblast differentiation. The complete growth medium was replaced with osteoblast induction media (OIM) to initiate differentiation.^{14,18} The cells were incubated in 5% CO₂ incubator at 37°C for 21 days.

SHED cultured in complete growth media and OIM were treated with or without 4 mg/mL flaxseed extract. The flaxseed extract was prepared, and the optimum concentration of extract was determined as previously described.¹¹ Four groups were set up for the analysis: SHED cultured in growth media alone (A), SHED cultured in growth media treated with 4 mg/mL flaxseed extract (B), SHED cultured in OIM alone (C), and SHED cultured in OIM treated with 4 mg/mL flaxseed extract (D).

Alkaline phosphatase activity of SHED

SHED were seeded in a 12-well plate (1 mL of 5×10^4 cell suspension/well) and placed in 5% CO₂ incubator until confluence. For background control, 1 mL of media (without cells) was aliquoted into the same 12 well plate. The media were discarded and replaced with media consisting of growth media as negative control, OIM as positive control, growth media with 4 mg/mL flaxseed extract, and OIM with 4 mg/mL extract. Alkaline phosphatase (ALP) activity of SHED was evaluated at days 1, 3, 7, 14, and 21 by ALP assay.

Alizarin red-s staining for the calcium deposition of SHED

SHED (1×10^5 cells) were seeded in a 6-well plate in OIM and cultured in 5% CO₂ incubator until confluence. The media was discarded and replaced with media containing 4 mg/mL flaxseed extract. The cells were maintained in 5% CO₂ incubator. The media was replaced every 3 days. Calcium deposition of SHED was analyzed at days 14 and 21 by using Alizarin Red-S staining. The stained mineral deposits were viewed under an inverted phase contrast microscope (Leica, Germany). SHED

cultured in OIM without flaxseed extract served as control.

Treatment of SHED for gene expression analysis

SHED (1×10^6 cells) were cultured in OIM and treated with 4 mg/mL flaxseed extract. Non-treated SHED cultured in OIM acted as control. The cells were collected at days 1, 3, 7, 14, and 21. RNA was extracted using Total RNA Mini Kit (Geneaid, Korea). Approximately 20 ng of total RNA was used for cDNA synthesis (ReverTra Ace qPCR Master Mix, Toyobo, Japan) and subjected to reverse-transcriptase PCR analysis for osteoblast markers *OSX*, *DMP1*, and *OCN* by using primer sequences as described previously.¹⁹ Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal positive control.

Statistical analysis

All data were expressed as mean (\pm SD) from at least three independent experiments and were subjected ANOVA and t-test by using SPSS software (SPSS version 17). Statistical significance was considered at $p < 0.05$.

RESULTS

ALP activity

The ALP activity of all groups was detectable after 3 days of incubation. The ALP activity of groups A, B, and D gradually increased until day 21. The ALP activity of group C gradually increased from day 3 until day 14 and then significantly decreased at day 21 ($p < 0.05$) (Figure 1). A significant difference in ALP activity was found between groups A and B at day 14 ($p < 0.05$, t-test) but not at day 21 ($p > 0.05$). Similarly, the ALP activity of the SHED cultured in OIM (C) was significantly higher than that of the SHED treated with flaxseed (D) at day 14 ($p < 0.01$, t-test) but not at day 21 ($p > 0.05$).

Calcium deposition

The calcium deposition of SHED was assessed using Alizarin Red-S staining at days 14 and 21. The SHED cultured in OIM were positively stained at day 14, and the nodules of the mineralized matrix were strongly stained at day 21. A weak staining of Alizarin Red-S was observed in the SHED cultured in OIM treated with 4 mg/mL flaxseed extract at day 14. In addition, the formation of mineralized nodules at day 21 was lesser than that at day 14 (indicated by a weak staining of Alizarin Red-S). By contrast, Alizarin Red-S staining was negative in the other groups, whereas no visible staining of Alizarin Red-S was observed on the SHED cultured in growth media with or without flaxseed extract at days 14 and 21 (Figure 2).

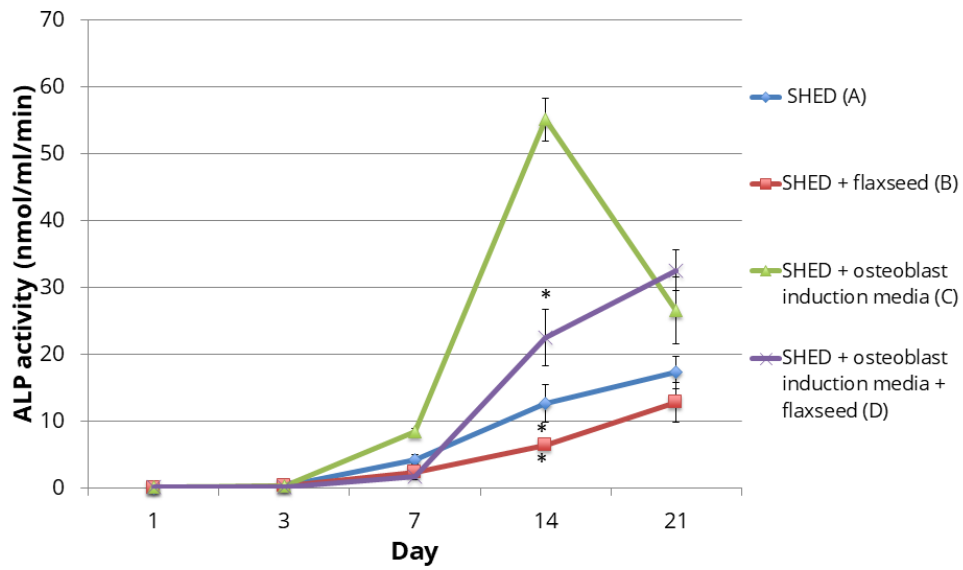


FIGURE 1. Intracellular ALP activity of SHED treated with and without flaxseed extract cultured in growth media and osteoblast induction media. Values are expressed as means \pm SD (n = 3)

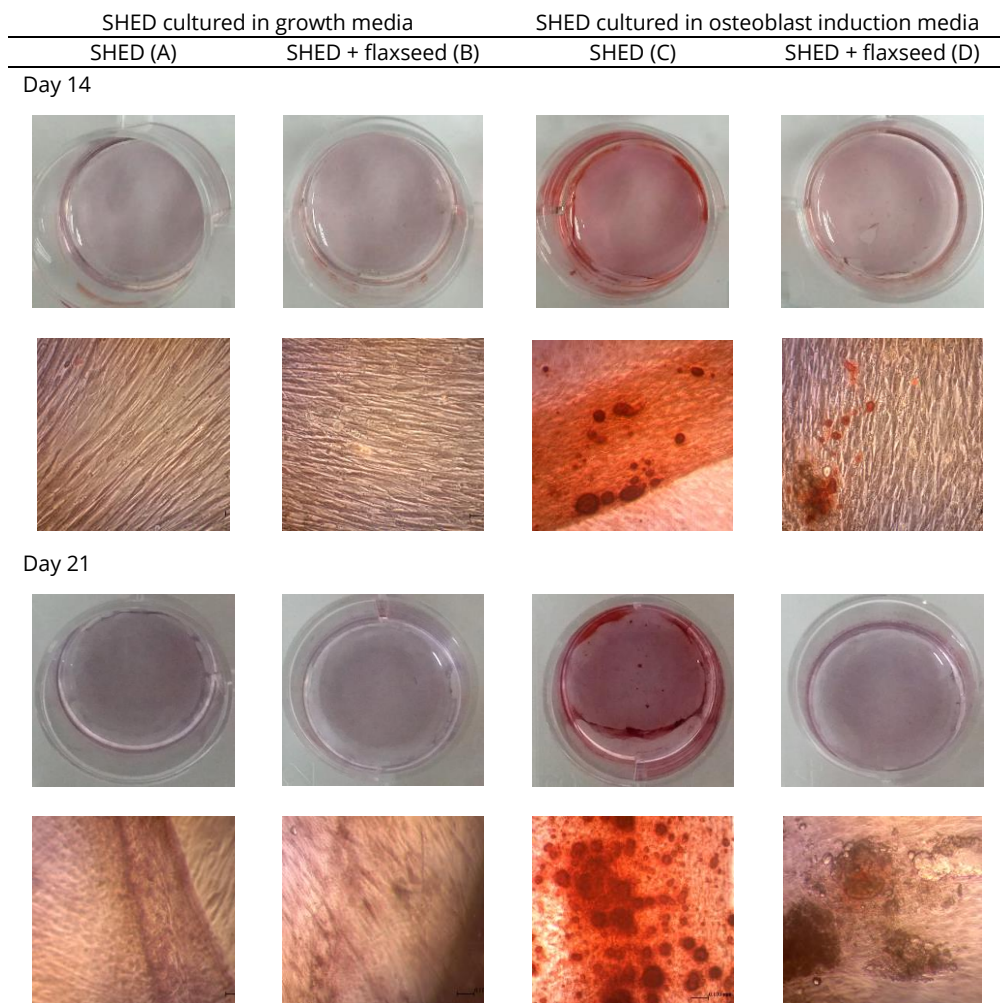


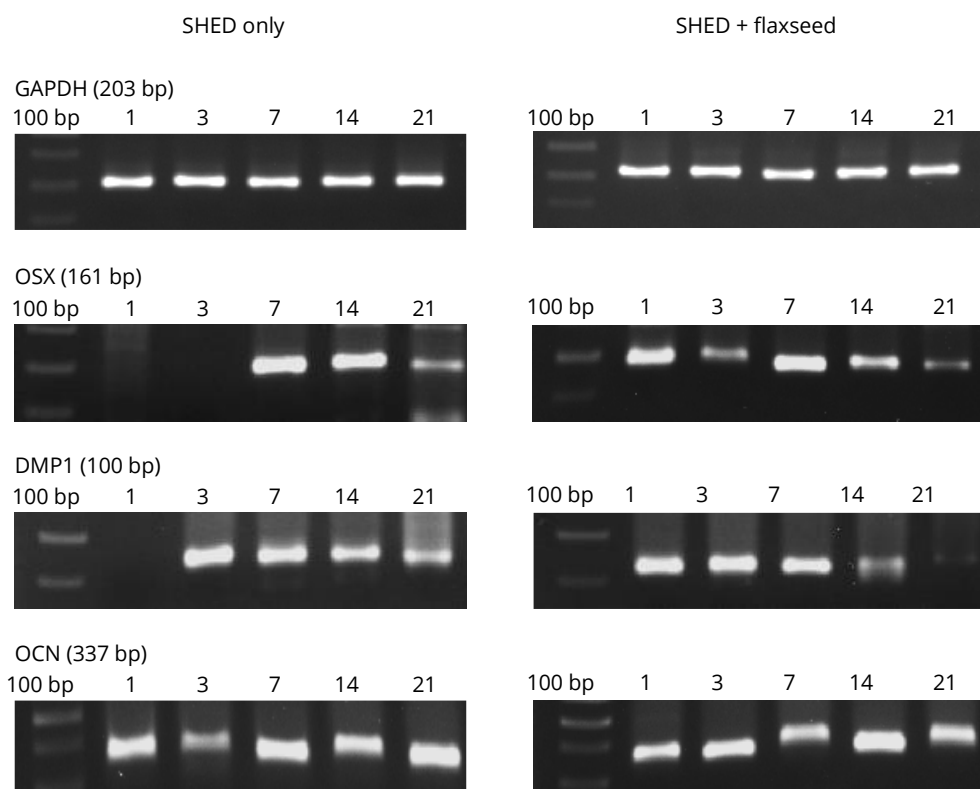
FIGURE 2. Plate view of SHED stained with Alizarin Red Solution (upper row) and microscopic view at 10 \times magnification (lower row) observed at days 14 and 21

TABLE 1. Expression of osteoblast markers on SHED cultured in osteoblast induction media (OIM) (treated with and without flaxseed)

Day	OSX		DMP1		OCN	
	SHED only (control)	SHED + flaxseed	SHED only (control)	SHED + flaxseed	SHED only (control)	SHED + flaxseed
1	N.D.	0.8141 ± 0.0320	N.D.	1.1459 ± 0.0266	1.0692 ± 0.0809	0.9365 ± 0.0305
3	N.D.	0.7931 ± 0.0389	1.1403 ± 0.0378	1.0036 ± 0.0548*	0.5201 ± 0.0193	1.2222 ± 0.0607*
7	1.3569 ± 0.0707	0.3756 ± 0.0077*	1.1113 ± 0.0397	1.2675 ± 0.0021*	1.4015 ± 0.0875	1.0532 ± 0.0328*
14	1.1759 ± 0.0548	1.1677 ± 0.0588	0.8281 ± 0.0319	0.5102 ± 0.0090*	0.9171 ± 0.1005	2.0487 ± 0.0661*
21	0.3586 ± 0.0170	1.2683 ± 0.0739*	0.9022 ± 0.0525	0.1097 ± 0.0396*	1.5837 ± 0.0841	1.0349 ± 0.0563*

Expression level of each gene was measured and normalized to GAPDH (housekeeping gene) and expressed as average intensity value (AIV) (means ± SD, n = 3). *Significant difference when compared with the control of the same day within the same group ($p < 0.05$).

N.D. = Not detected.

**FIGURE 3.** Agarose gel view of *GAPDH* and osteoblast marker (*OSX*, *DMP1*, *OCN*) on SHED cultured in osteoblast induction media treated with and without flaxseed extract (as control).

Expression of osteoblast markers

Analysis of gene expression on osteoblast markers was only performed on samples collected from the SHED cultured in OIM treated with or without 4 mg/mL flaxseed extract. The band intensities were measured using Chemidoc (BioRad, USA) and expressed as average intensity value (Table 1). Figure 3 illustrates the gene expression patterns observed in the analysis.

OSX expression was detectable at days 7 to 21 in the control, whereas day 7 recorded the highest and day 21 recorded the lowest expression. By contrast, *OSX* expression was detectable at days 1 to 21; it significantly reduced at day 7 in the treatment group but significantly increased at day 21 compared with the control ($p < 0.05$).

DMP1 was highly expressed at days 3 to 21, except for day 1, where the expression was not detected in the control. In the SHED treated with flaxseed, the expression of *DMP1* was observed from day 1 to day 21. The *DMP1* expression in the treatment group significantly reduced at days 14 and 21 compared with that in the control ($p < 0.05$).

Overall, *OCN* was strongly expressed in all five days (1, 3, 7, 14, and 21) in the control and treatment groups. Although the expression of *OCN* fluctuated in both groups, it significantly increased at day 14 and reduced at day 21 in the treatment group compared with the control ($p < 0.05$).

DISCUSSION

Osteogenesis involves the differentiation of mesenchymal cells into pre-osteoblasts and osteoblasts, leading to the synthesis and deposition of bone matrix proteins.²⁰ Osteoblast differentiation undergoes proliferation, matrix maturation, and mineralization, which are regulated by different transcription factors and signaling proteins.²¹ The current study confirmed the potential of SHED to differentiate into osteoblast-like lineage when cultured under specific induction media.^{13,14} We demonstrated the differentiation of SHED into osteoblast lineage by ALP and calcium deposition analysis and positive expression of osteoblast-related markers as presented previously.¹⁴ Nonetheless, the addition of flaxseed extract affected the ability of SHED to undergo osteoblast differentiation. ALP is a well-known marker of early osteoblast differentiation stage. ALP is a cell surface protein ubiquitously expressed by several cell types and is used for screening pre-osteoblasts.²² ALP activity peaked at the second week of culture at about day 14 and then decreased with the onset of mineralization, which is a typical positive indication.²³ In the present study, flaxseed extract reduced the osteoblast differentiation potential of SHED. The ALP activity of the SHED cultured in OIM alone resembled the normal phases of osteoblast differentiation process. In specific, ALP activity started to increase from day 7, peaked at day 14, and then significantly decreased at day 21. By contrast, the ALP activity of the SHED cultured in OIM treated with flaxseed significantly reduced at day 14 compared with that of the control. In addition, the ALP activity of the SHED treated with flaxseed cultured in normal growth media also reduced. This result might be additional evidence showing that flaxseed reduces the osteoblast differentiation potential of SHED.

Osteoblast differentiation was also analyzed by calcium deposition through Alizarin Red-S staining. Intense Alizarin Red-S staining and mineralized nodules were observed in the SHED cultured in OIM alone at day 21. Conversely, a weaker staining was observed and only few mineralized nodules were detected in the SHED cultured in OIM treated with flaxseed at day 21. This finding further supports that flaxseed extract reduces the osteoblast differentiation of SHED and thus reduces calcium deposition. Mineralization is the final phase of osteoblast differentiation, where the mineral matrix, which predominately contains calcium phosphate in the form of hydroxyapatite, is secreted and deposited by mature osteoblasts.²⁴ In the present study, SHED cultured in OIM exhibited normal osteoblast differentiation, but the addition of flaxseed extract reduced the differentiation potential of SHED into osteoblast-like lineage.

Gene expression levels of osteoblast markers *OSX*, *DMP1*, and *OCN* were analyzed to evaluate the osteoblast

differentiation potential of SHED in the presence of flaxseed extract. Osterix (*OSX*) is an osteoblast-specific transcription factor important for osteoblast differentiation and bone formation and is also an early osteoblast marker.²⁵ Although the expression of *OSX* was only detectable from day 7 onward in control, it was consistent with the normal phases of osteoblast differentiation as demonstrated in the ALP analysis results that ALP activity increased from day 7, peaked at day 14, and reduced at day 21. In the treatment group, *OSX* was expressed at all days analyzed with highest expression at day 21 compared with the control. This result might be due to the continuous osteoblast differentiation at this stage. ALP results revealed that ALP activity slightly increased at day 21 in the treatment group. In comparison to control, ALP activity reduced at day 21, which could be related to reduction of *OSX* expression.

Dentin matrix protein 1 (*DMP1*) expression is localized in the mineralized matrix of bone; it is constitutively expressed during osteoblast differentiation.^{26,27} In the present analysis, *DMP1* expression reduced from day 14 to day 21 in the flaxseed-treated group compared with the control. Osteocalcin (*OCN*) is a late-stage marker of osteogenic differentiation.²⁸ In the present study, *OCN* was expressed in all days and peaked at day 21 in the control, which can be related to the final phase of mineralization. Intense Alizarin Red-S staining was observed at day 21 in the control, confirming that mineralization is related to the high *DMP1* and *OCN* expression. By contrast, the reduction in *DMP1* and *OCN* expression in the flaxseed-treatment group at day 21 could be related to the reduced ALP activity and weak Alizarin Red-S staining, especially at days 14 and 21.

Overall, our result demonstrated that flaxseed crude extract affected the osteoblast differentiation potential of SHED by reducing the ALP level, calcium deposition, and gene expression of osteoblast-related differentiation markers. Flaxseed extract possibly modulated the activity of ALP, the deposition of calcium, and the gene expression of osteoblast-related markers. Previously, we have shown that flaxseed extract contains high fatty acid contents.¹¹ Evidence presented over recent years has shown that n-3 polyunsaturated fatty acids (PUFAs) are beneficial for bone health.^{29,30} However, PUFAs result in apoptosis at high concentrations and necrosis at even higher concentrations.³¹ The inhibitory effects of fatty acids depend on carbon chain length and double bond number.³² The high contents of fatty acids in flaxseed may inhibit SHED growth and other biological activities, such as ALP activity and osteoblast differentiation. However, further investigation is warranted in the future to clarify this issue.

Most previous studies utilized pure flaxseed oil to study the effects of flaxseed oil on bone health.⁸ The present

study focused on flaxseed crude extract; the content and ratio of the compound might vary with pure flaxseed oil and thus affect the overall process of osteoblast differentiation. The present study utilized stem cells from dental pulp for *in vitro* analysis, whereas others used osteoblast primary cell line as a starting material, which could also affect the overall outcome. Nonetheless, the current study provides additional information regarding the utilization of 4 mg/mL flaxseed extract, which can inhibit the osteoblast differentiation potential of SHED.

CONCLUSIONS

Flaxseed crude extract reduces ALP activity and calcium deposition in SHED and affects the expression of osteoblast markers. Thus, flaxseed can potentially inhibit the osteoblast differentiation of SHED, suggesting its possible application in the synthesis of natural-based drugs that can control bone development. Nonetheless, the actual mechanism must be elucidated in the future.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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REFERENCES

- Amin T, Thakur M. A comparative study on proximate composition, phytochemical screening, antioxidant and antimicrobial activities of *Linum usitatissimum* L. (flaxseeds). *Int J Curr Microbiol App Sci*. 2014;3:465–81.
- Draganescu D, Ibanescu C, Tamba B, Andritoiu C, Dodi G, Popa M. Flaxseed lignan wound healing formulation: Characterization and *in vivo* therapeutic evaluation. *Int J Biol Macromol*. 2015;72:614–23.
- Jhala AJ, Hall LM. Flax (*Linum usitatissimum* L.): Current uses and future applications. *Aust J Appl Sci*. 2010;4:4304–12.
- Imran M, Ahmad N, Anjum FM, Khan MK, Mushtaq Z, Nadeem M, et al. Potential protective properties of flax lignan secoisolariciresinol diglucoside. *Nutr J*. 2015;14:71.
- Griel AE, Kris-Etherton PM, Hilpert KF, Zhao G, West SG, Corwin RL. An increase in dietary n-3 fatty acids decreases a marker of bone resorption in humans. *Nutr J*. 2007;6:2.
- Goyal A, Sharma V, Upadhyay N, Gill S, Sihag M. Flax and flaxseed oil: An ancient medicine & modern functional food. *J Food Sci Technol*. 2014;51:1633–53.
- Nahla EA, Thabet HA, Ahmed HF. The effect of supplementation with flaxseed and its extract on bone health. *Nat Sci*. 2013;11:71–7.
- El-Saeed GSM, Elghoroury EA, Morsy S, Aly HM, Wafaey H. Phenotype of vitamin D receptor gene polymorphisms, impact of feeding flaxseed oil, and osteoporosis in ovariectomised diabetic rats. *Bull Natl Res Cent*. 2018;42:1–7.
- Kanafi MM, Pal R, Gupta PK. Phenotypic and functional comparison of optimum culture conditions for upscaling of dental pulp stem cells. *Cell Biol Int*. 2013;37:126–36.
- Aljohani H, Senbanjo, LT, Chellaiah, MA. Methylsulfonylmethane increases osteogenesis and regulates the mineralization of the matrix by transglutaminase 2 in SHED cells. *PLoS One*. 2019;14:1–19.
- Nordin NS, Mokhtar KI, Md Isa ML, Idid SZ, Lestari W, Mustafa BE, et al. Flaxseed ethanolic crude extract influences growth of stem cells from human exfoliated deciduous teeth (SHED). *J Int Dent Med Res*. 2018;11:643–9.
- Sharma R, Bhargava D, Yadav M, Rastogi P, Chandavarkar V. Review research paper dental stem cells: Harnessing newer possibilities. *J Indian Acad Forensic Med*. 2013;35:378–82.
- Chadipiralla K, Yochim JM, Bahuleyan B, Huang CY, Garcia-Godoy F, Murray PE, et al. Osteogenic differentiation of stem cells derived from human periodontal ligaments and pulp of human exfoliated deciduous teeth. *Cell Tissue Res*. 2010;340:323–33.
- Farea M, Husein A, Halim AS, Abdullah NA, Mokhtar KI, Lim CK, et al. Synergistic effects of chitosan scaffold and TGFβ1 on the proliferation and osteogenic differentiation of dental pulp stem cells derived from human exfoliated deciduous teeth. *Arch Oral Biol*. 2014;59:1400–11.
- Dahake PT, Panpaliya NP, Kale YJ, Dadpe MV, Kendre SB, Bogar C. Response of stem cells from human exfoliated deciduous teeth (SHED) to three bioinductive materials – An *in vitro* experimental study. *Saudi Dent J*. 2020;32:43–51.
- Mussano F, Genova T, Petrillo S, Roato I, Ferracini R, Munaron, L. Osteogenic differentiation modulates the cytokine, chemokine, and growth factor profile of ASCs and SHED. *Int J Mol Sci*. 2018;19:1454.
- Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: Current concepts and future directions. *BMC Med*. 2011;9:66.
- Sila-Asna M, Bunyaratvej A, Maeda S, Kitaguchi H, Bunyaratavej N. Osteoblast differentiation and bone formation gene expression in strontium-inducing bone marrow mesenchymal stem cell. *Kobe J Med Sci*. 2007;53:25–35.
- Fitri AR, Pavasant P, Chamni S, Sumrejkanchanakij P. Asiaticoside induces osteogenic differentiation of human periodontal ligament cells through the Wnt pathway. *J Periodontol*. 2018;89:596–605.
- Aubin JE, Triffitt JT. Mesenchymal stem cell and osteoblast differentiation. In Bilezikian J, Raisz LG,

- Rodan GA. Eds. *Principles of Bone Biology*. 2nd ed. San Diego: Academic Press, 2002, p. 59–82.
21. Jo S, Yoon S, Lee SY, Kim SY, Park H, Han J, *et al.* DKK1 induced by 1,25D3 is required for the mineralization of osteoblasts. *Cell*. 2020;9:236.
 22. Kannan S, Ghosh J, Dhara KS. Osteogenic differentiation potential and marker gene expression of different porcine bone marrow mesenchymal stem cell subpopulations selected in different basal media. *Biol Open*. 2020;9:bio05328.
 23. Costa-Pinto AR, Correlo VM, Sol PC, Bhattacharya M, Charbord P, Delorme B, *et al.* Osteogenic differentiation of human bone marrow mesenchymal stem cells seeded on melt-based chitosan scaffolds for bone tissue engineering applications. *Biomacromolecules*. 2009;10:2067–73.
 24. Clarke B. Normal bone anatomy and physiology. *Clin J Am Soc Nephrol*. 2008;3:131–9.
 25. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, *et al.* The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell*. 2002;108:17–29.
 26. Narayanan K, Srinivas R, Ramachandran A, Hao J, Quinn B, George A. Differentiation of embryonic mesenchymal cells to odontoblast-like cells by overexpression of dentin matrix protein 1. *Proc Natl Acad Sci U S A*. 2001;98:4516–21.
 27. Narayanan K, Srinivas R, Peterson MC, Ramachandran A, Hao J, Thimmapaya B, *et al.* Transcriptional regulation of dentin matrix protein 1 by JunB and p300 during osteoblast differentiation. *J Biol Chem*. 2004;279:44294–302.
 28. Aubin JE. Regulation of osteoblast formation and function. *Rev Endocr Metab Dis*. 2001;2:81–94.
 29. Watkins BA, Li Y, Lippman HE, Seifert MF. Omega-3 polyunsaturated fatty acids and skeletal health. *Exp Biol Med*. 2001;226:485–97.
 30. Rozner R, Vernikov J, Griess-Fishheimer S, Travinsky T, Penn S, Schwartz B, *et al.* The role of omega-3 polyunsaturated fatty acids from different sources in bone development. *Nutrient*. 2020;12:3494.
 31. Mainou-Fowler T, Proctor SJ, Dickinson AM. Gamma-linolenic acid induces apoptosis in B-chronic lymphocytic leukaemia cells in vitro. *Leuk Lymphoma*. 2001;40:393–403.
 32. Lima TM, Kanunfre CC, Pompéia C, Verlengia R, Curi R. Ranking the toxicity of fatty acids on Jurkat and Raji cells by flow cytometric analysis. *Toxicol In Vitro*. 2002;16:741–7.