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ORIGINAL ARTICLE

Caffeic Acid Treatment Augments the Cell Proliferation, Differentiation, and Calcium Mineralization in the Human Osteoblast-Like MG-63 Cells

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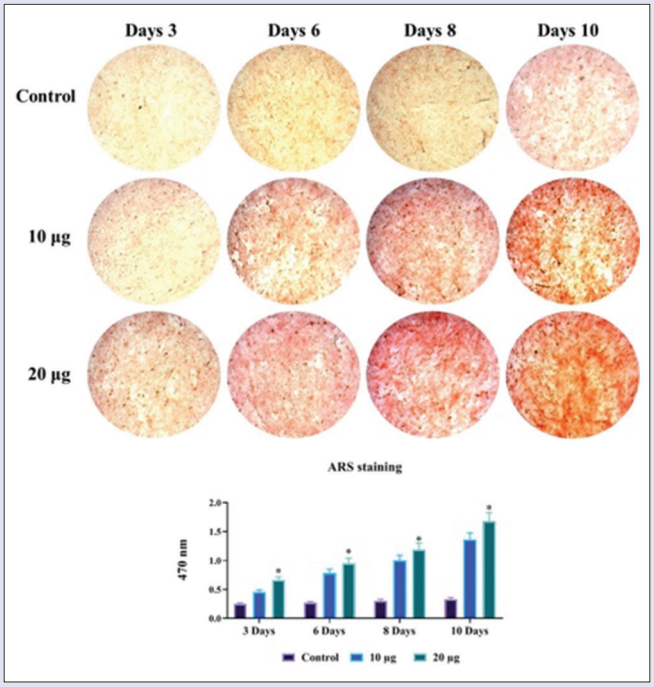
ABSTRACT

Background: Osteoporosis is an imperative health problem that extremely distresses the public that leads to a higher risk to the bones from both spontaneous and accidental bone fractures. Caffeic acid is a polyphenol compound that happens naturally in numerous vegetables such as coffee beans, potatoes, propolis, olives, and carrots with many pharmacological aids. **Objectives:** The current study was planned to examine the potential of Caffeic acid in proliferation, differentiation, and calcium mineralization of osteoblast-like MG-63 cells. **Materials and Methods:** The cell viability of Caffeic acid-supplemented MG-63 cells was examined through the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity test. The *m*RNA expression of alkaline phosphatase (ALP) and osteocalcin was reviewed through reverse transcription polymerase chain reaction study. The calcium deposition rate in the Caffeic acid-treated MG-63 cells was studied through Alizarin Red S (ARS) staining. **Results:** The result of the MTT test exposed that the 10 and 20 mg/kg of Caffeic acid supplementation did not show any cytotoxicity to the osteoblast-like MG-63 cells; instead, it helped the viability of MG-63 cells. The expression of ALP is particularly increased in the Caffeic acid-supplemented MG-63 cells, whereas the osteocalcin expression was noticeably diminished. The ARS staining was exhibited that the Caffeic acid treatment was noticeably enhanced the calcium mineralization rate in the osteoblast-like MG-63 cells. **Conclusion:** Based on the findings of investigation, it was proved that the Caffeic acid treatment was significantly enhanced the cell proliferation, differentiation, and calcium mineralization in the osteoblast-like MG-63 cells. Hence, it was clear that Caffeic acid can be engaged as the potential agent for the purpose of bone regeneration.

Key words: Caffeic acid, calcium, MG-63 cells, osteoblast, osteocalcin, osteoporosis

SUMMARY

- The bone is an active connective tissue that progresses a constructive structure with the inherent ability of regeneration
- The Caffeic acid supplementation to the MG-63 cells was clearly improved the MG-63 cell survival, as evidenced by the enhanced cell proliferation.



Abbreviations used: MAPKs: Mitogen-stimulated protein kinases; ARS: Alizarin Red S; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide.

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INTRODUCTION

The bone tissue in the body is persistently remodeled and the whole bone mass was regulated invariably via equilibrium between osteoblastic bone development and osteoclastic bone resorption. This equilibrium depends in the relations among osteoblasts that encourage new bone creation and osteoclasts, which stimulate bone resorption. Constant remodeling permits the bone to adapt to changes in heaviness and renovates the minor injuries caused by trifling accidents. The remodeling of bone in strappingly mediated via a diverse osteogenic modulator, growth factors,

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and hormones.[1] The bone is metabolically energetic, and tissues were enormously vital in the continuous creation and the resorption via the osteoblasts and osteoclast cells and function equally through paracrine signaling cascade and essential multi-cellular elements. The osteoblasts are mono-nucleate cells that distinguished from bone marrow mesenchymal stem cells and are liable for the bone matrix deposition and osteoclasts regulation. The osteoclasts are multi-nucleate cells, which distinguished from the hematopoietic stem cells and responsible for the resorption of bones.[2]

The progressions of the distinction of osteoblast are essential for bone power and remodelling. This mechanism is subdivided into three consecutive phases such as proliferation, extracellular matrix formulation, maturation, and mineralization. At the time of osteoblast differentiation, the transcription factors osterix and Runx2 activate the expression of oestrogenic indicators such as alkaline phosphatase (ALP), type-I collagen, and osteocalcin alongside with the mineralization is greatly expressed. The progression of osteoblast differentiation is modulated via numerous signaling cascades like nuclear factor kappa light chain promoter stimulated via B cells (NF-ƙB), mitogen-stimulated protein kinases , and BMP-Smads.[3,4] At the stage of extracellular matrix development, the osteoblasts produce osteocalcin, osteopontin, and alkaline that markedly expands their function in the instigation of mineralization of bone matrices. The augmented ALP function leads to the elevated deliverance of phosphate that develops a mineral segment of the bone with free calcium ions. At the beginning of osteogenic differentiation, ALP is elevated, while osteocalcin is principally expressed at an afterward the mineralization phase. The regeneration is bone tissues that modulated by a fine equilibrium between cellular and biochemical events, which finally promotes the osteoblasts to generate new tissues, especially a new extracellular matrix mainly composed of collagen. The collagen matrix is mineralized through the enzymatic actions of ALP that promote the development of calcium phosphate crystals.[5]

The recurrent deficits in bone remodeling ultimately trigger numerous inabilities and bone-related diseases like osteoporosis. Osteoporosis is a systemic skeletal disease that distinguished via lessened bone mineral concentration and the destruction of micro tissue architecture of bones and eventually resultant in elevated bone weakness and vulnerable to fracture.[6] Besides, few drugs are typically used to treat the numerous ailments have possessed poisonous effects in skeletal and also cause osteoporosis. The osteoporosis is a disease in that the resorption rate was higher than the bone development. Osteoporosis is an imperative health issue that mainly affects the public, which leads to the augmented risk to bones from both spontaneous and accidental bone fractures. This disease can be treated through agents that having strong osteoclast and osteoblast functions. By this means, exploring the novel agents that can activate and promotes bone development through preventing bone resorption was a vital task for remedial strategy.[7,8]

Caffeic acid, also termed as 3,4-dihydroxycinnamic acid, is a polyphenol compound secreted through secondary metabolism of vegetables like coffee beans, potatoes, propolis, olives and carrots and it is major cinnamic acid that occurs in the human diet.[9-11] This phenolic compound occurs in a monomer form as glycosides, amides, sugar esters, and organic acid esters. It also befalls in the form of dimmers, trimmers and other polymers in the vegetable's cell wall.[12] Preceding *in vitro* and *in vivo* investigations done in Caffeic acid showed the numerous pharmacological benefits such as antibacterial, antioxidant, antiviral, anti-inflammatory, immuno-stimulatory, anti-atherosclerotic, cardio-protective, immuno-stimulatory, hepato-protective, anti-diabetic, and anti-cancer potentials.[12-20] Equally, there are null scientific reports to declare the bone regeneration efficacy of Caffeic acid. Therefore, this scientific exploration is designed to examine the effect of Caffeic acid in proliferation, differentiation, and calcium mineralization of osteoblast-like MG-63 cells.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), antibiotics, i.e., penicillin and streptomycin, trypsin-Ethylenediaminetetraacetic acid, dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), Alizarin Red S (ARS) dye and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, USA). The extraction and test kits were acquired in Santa Cruz Biotech, CA, USA. Whole other chemicals used in this investigation were as experimental and diagnostic range and purchased from HiMedia, USA.

Culture collection and maintenance

The osteoblast-like MG-63 cell line was purchased from ATCC (USA). The cells were grown on DMEM medium supplemented with FBS (10%), Streptomycin, penicillin (1%) and sustained at the moisturized situation (37°C), along with 5% of CO_2.

Assay of cell viability

The viability of Caffeic acid supplemented MG-63 cells was examined via MTT cytotoxicity test. The MG-63 cells were loaded at the 6×10^3 cells per well in a 96-well plate for a night then shifted to a medium that containing FBS (1%) and maintained for 24 h. Afterward, the cells were added with 10 and 20 µL/mL of Caffeic acid and the cells were incubated for 3, 6, 8, and 10 days in a moistened chamber with 5% CO_2. Later than the 10 days of treatment, the 20 µL/mL of MTT solution was mixed to every well and the cells were preserved for 4 h at 37°C. After that, the cells were centrifuged, and the upper aqueous phase was removed and 100 µL/mL of DMSO was mixed, and finally, the absorbance was taken at 490 nm with the aid of micro plate reader.[21]

Mineralization assay

The mineralization status MG-63 cells were examined in a 24-well plate via ARS staining technique after the 7 days of Caffeic acid supplementation. The cells were fixed with 70% of ethanol for 1 h and then stained with 40 mM of ARS in distilled water for 15 min at 37°C. The images of ARS stained MG-63 cells were taken under the fluorescent microscope. Then the ARS suspension was eradicated, and the cells were sustained in a phosphate-buffered saline (PBS) for 15 min at 37°C in a shaker. Then the cells were washed through PBS and then de-stained for 15 min with cetylpyridinium chloride (10%) in 10 mM of sodium phosphate. The separated stain suspension was then trans-located to 96-well plate and then the absorbance was taken at 562 nm in a microplate reader.[22] The activity was determined via the following formula, in that "A" and "A_0" means the absorbance with and without sample, in that order. Mineralization level (%) = $A\text{-}A_{0/}A_0 \times 100$.

Reverse transcription polymerase chain reaction analysis

The whole RNA was extracted from the Caffeic acid (10 and 20 µg/mL) supplemented MG-63 cells with the aid of the Trizol RNA extracting kit (Santa Cruz Biotech, CA, USA) in accordance with the manufacturer protocol. 1 µg of RNA was mixed to attain the total volume of 19 µg and the cDNA was constructed by utilizing a commercial polymerase chain reaction (PCR) test kit (Santa Cruz Biotech, CA, USA).

The primers for ALP sense 5'-CCCAAAGGCTTCTTCTTG-3'; anti-sense 5'-CTGGTAGTTGTTGTGAGCAT-3', Osteocalcin sense ATGAGAGCCCTCACACTCCTC-3', anti-sense 5'-GCCGTAGAAGCG CCGATAGGC-3' and β-actin sense 5'-TGACCCAGAT CATGTTTGAGA-3', anti-sense 5'-ACTCCATGCCCAGGAAGGA-3'. The reaction was continued with initial denaturation for the 30s at 95°C; afterward 40 PCR cycles with 5s of denaturation at 95°C, annealing for 30s at 60°C and extension for 15s at 95°C. The complete examination was done in triplicate for exact values.

Statistical examination

The statistical examination was done through SPSS (SPSS Inc., Chicago, Illinois, USA) statistical tool (version-16). Data was exemplified as mean ± standard deviation. One-way ANOVA subsequently Duncan's Multiple Range Test (DMRT) quantity test was adopted to scrutinize the statistical relevance among the different groups. Data are regarded as statistically relevant if the $P < 0.05$.

RESULTS

Caffeic acid treatment enhances the MG-63 cell viability

The effect of Caffeic acid in MG-63 cell viability was examined via the MTT test and the result is shown in Figure 1a and b. The Caffeic acid supplementation showed nearly null toxicity to the MG-63 cells equally enhanced the cell viability, proliferation, and growth of MG-63 cells. The supplementation of Caffeic acid (10 and 20 µg/mL) to the MG-63 cells was illuminated the noticeable increase in the cell mass concerning the 3, 6, 8, and 10 days of treatment. The viability of cells on day 3 is almost noticeably different on day 10 of Caffeic acid supplementation that demonstrating the enhancement in the cell viability and multiplication of MG-63 cells.

Effect of caffeic acid on alkaline phosphatase and osteocalcin expressions in the MG-63 cells

The *m*RNA expression patterns of ALP and osteocalcin in the MG-63 cells were examined via reverse transcription PCR study. As portrayed in Figure 2, the *m*RNA expressions of ALP were noticeably increased; conversely, the osteocalcin expression was markedly diminished in the 10 and 20 µg/ml supplementation of Caffeic acid to 3 to 10 days. Likely, the augmented *mRNA* expression of ALP in the Caffeic acid-treated MG-63 cells was exposed to augmented phosphate mineral deliverance, which forms bone mineral portions with calcium ions. The result was exactly evidenced that the Caffeic acid (10 and 20 µg/mL) treated osteoblast-like MG-63 cells were displayed a noticeable augmentation in the ALP expression and reduced Osteocalcin expression [Figure 2] that proved the enhanced mineralization and calcium binding in the MG-63 cells.

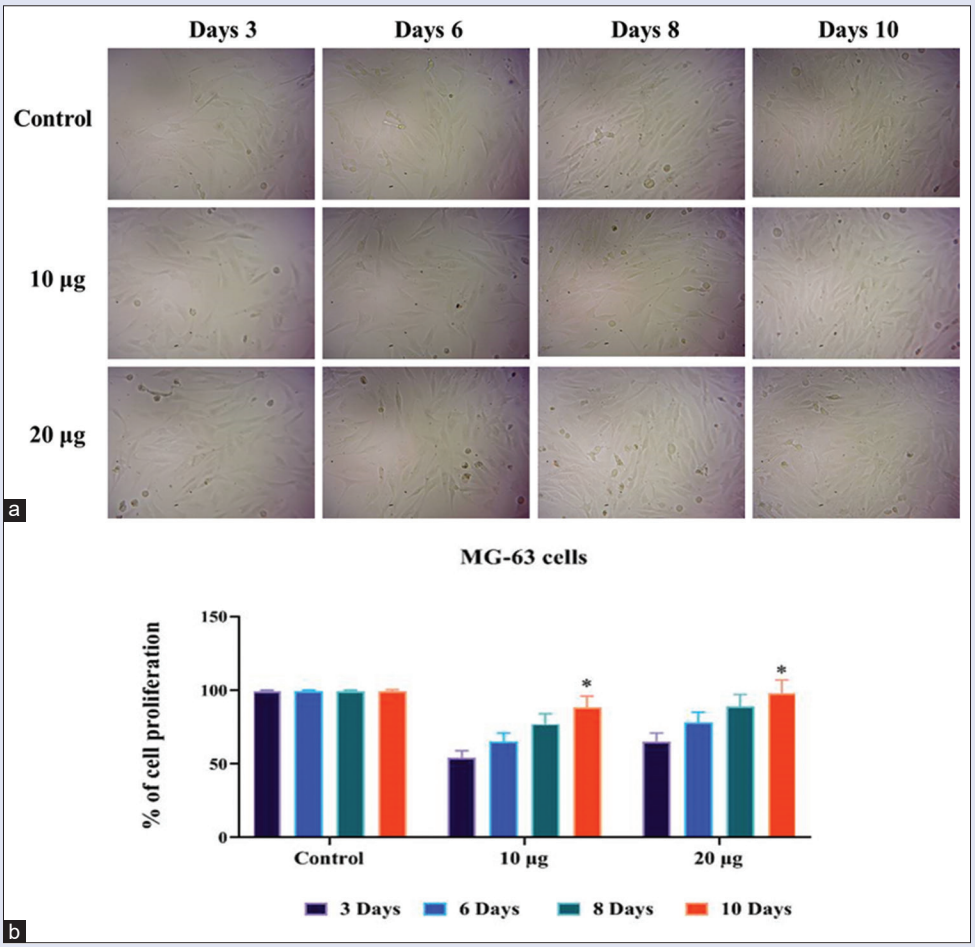

Figure 1: (a and b): Effect of caffeic acid on cell viability of osteoblast-like MG-63 cells in 3, 6, 8 and 10 days of treatment. Illustrating the result of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium cell viability assay of caffeic acid supplemented MG-63 cells. The 10 and 20 mg/kg of caffeic acid treatment enhanced the cell viability of osteoblast-like MG-63 cells in 3, 6, 8 and 10 days of treatment (a). The viability of MG-63 cells viability was displayed in a graph (b)

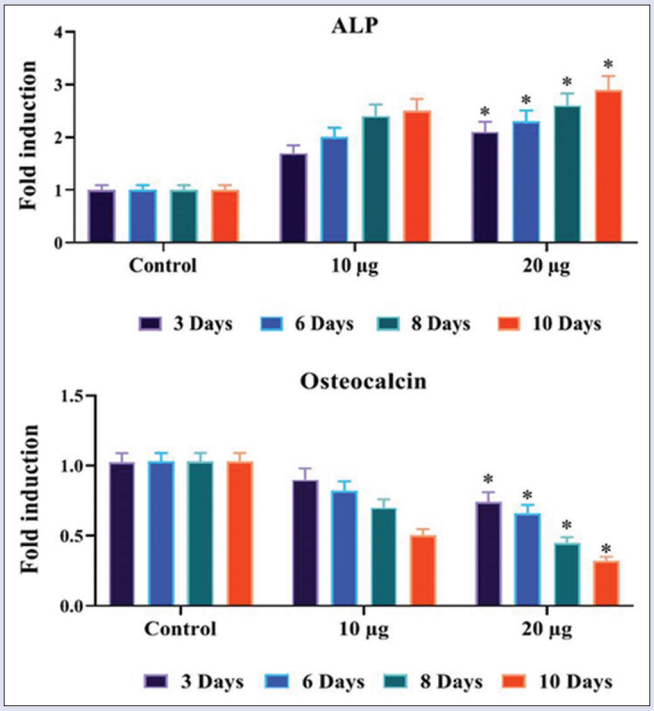

Figure 2: Effect of caffeic acid on mRNA expression of alkaline phosphatase and osteocalcin in MG-63 cells by reverse transcription polymerase chain reaction analysis. Displaying the effect of caffeic acid treatment in the expressions of alkaline phosphatase and osteocalcin in the osteoblast-like MG-63 cells. The 10 and 20 mg/kg of caffeic acid treatment improved the mRNA expression of alkaline phosphatase and osteocalcin in the osteoblast-like MG-63 cells in 3, 6, 8 and 10 days of treatment

Caffeic acid treatment enhanced the calcium deposition in the MG-63 cells

The calcium mineral deposition rate in the Caffeic acid (10 and 20 µg/mL) supplemented MG-63 cells were examined via ARS staining technique and the result is shown in Figure 3. It was showed that the calcium mineralization rate in the Caffeic acid (10 and 20 µg/mL) supplemented MG-63 cells were augmented by time-dependently. The calcium deposition rate in the MG-63 cells was markedly enhanced via the 10 and 20 µg/ml supplementation of Caffeic acid in 3, 6, 8, and 10 days. There are noticeable variations in the calcium deposition rate between day 3 and day 10 of Caffeic acid (10 and 20 µg/mL) treatment, which divulges that the Caffeic acid-treated MG-63 cells were time-dependently, enhanced the calcium mineral deposition rate [Figure 3].

DISCUSSION

The bone is an active connective tissue that grows a constructive structure with the characteristic ability of regeneration. Bone contains the mineralized inorganic constituents made up of calcium, water, magnesium, citrates, sodium, carbonates, and some other elements in trace quantity that forms the scaffold for bioorganic elements contains of collagen and non-collagen proteins such as osteopontin, osteocalcin, osteonectin, morphogenic proteins, thrombospondin, osteogenic tissues, which includes osteocytes, osteoblasts, and osteoclasts.[23] The injuries to the bone tissues may end in frequent pathological complications such as periodontitis, osteochondral degenerative diseases, osteomyelitis, and bone tumors.[24] The bone repair and regeneration mechanisms may improve via exploiting the natural and synthetic elements to speed up the restorative functions through enhanced osteoblast proliferation, differentiation, and regeneration.[25] The recreation of bone cells requires the scaffold along with bio-compatibility of imitating the natural bone extracellular matrix niches, osteogenic cells, and signaling molecules for the tissue-specific differentiation.[26,27] The regeneration of new bone tissues in the faulty portions of bone needs frequent mechanisms such as adhesion of osteogenic cells consequently their proliferation and survival.[28]

The recovery of huge bone defects due to trauma or accidents are extremely complicate and often end with failure. The application of bone tissues gathered from humans form transplantation like autografts (gathered from genetically matching individuals), allografts (gathered from genetically non-matched individuals), bone marrow cells, collagen matrices and osteocytes enhances the curative process.[29] There are huge restrictions related to these techniques, like autografts has very limited convenience, expensive, and several complications to the donor like infection and pain. The autograft transplant is normally linked to the donor site morbidity, increased risk of infections, inadequate closing of the gaps and higher cost for the two surgical procedures needed at the donor and also the host site. The allografts are also linked with frequent insufficiencies like long-term immunological rejections and elevated possibilities of transmission of diseases.[30,31] For this reason, the exploration of conventional strategies like the utilization of herbal derived active phyto-compounds is vital for healing bone-related diseases. The novel agents with the stupendous osteo-inducing and osteogenesis capacity in the site of low bone density and implant site are gradually enhanced for the bone tissue engineering benefits.[32] Caffeic acid is such an active compound with numerous pharmacological profits and in this current examination, an attempt has been made to inspect the potential of Caffeic acid in multiplication, differentiation, and calcium mineralization in osteoblast-like MG-63 cells.

The bone regeneration and remodeling underwent the manifold mechanisms, while harmonized cellular events need the connection of numerous bone cell types such as osteoblasts, osteoclasts, bone marrow mesenchymal stem cells, and osteocytes. The bone formation mechanisms were strongly linked to the viability of osteoblasts afterward augmented functions of ALP and collagen, the progression and maturation of the extracellular matrix and mineralization. The functions of ALP and type-I collagen are the advanced indicators of differentiation of the osteoblast phenotype and imperative for the modulation of cell maturation and mineralization. The outcomes of the current examination evidencing that the supplementation of 10 and 20 µg/mL of Caffeic acid was remarkably enhanced the multiplication of osteoblast-like cells as exhibited via the improved cell viability of MG-63 cells. The utmost growth stimulatory potential of Caffeic acid was noted at the 20 µg/mL dose. The ALP is expressed at the time of earlier progression in the cell surface and in the matrix vesicle. While the afterward progression phases, the other genes like osteocalcin were increased, and the *m*RNA expression of ALP was deteriorated.[33] The current results were proved that the Caffeic acid treatment was appreciably enhanced the expression pattern of *m*RNA of ALP in a time reliant mode.

The bone regeneration and repair are categorized into several phases like the inflammatory phase, hematoma development, granulation of tissue generation, callus development, and remodeling.[34] The formation of bone, regeneration, and metabolism is modulated via some genes like osteocalcin and COL-1 that has a straight association with calcium accessibility. Osteocalcin is supposed to link the hydroxyapatite and calcium that greatly expressed in escalating skeletal tissues serving in mineral depositions in the bone and bone

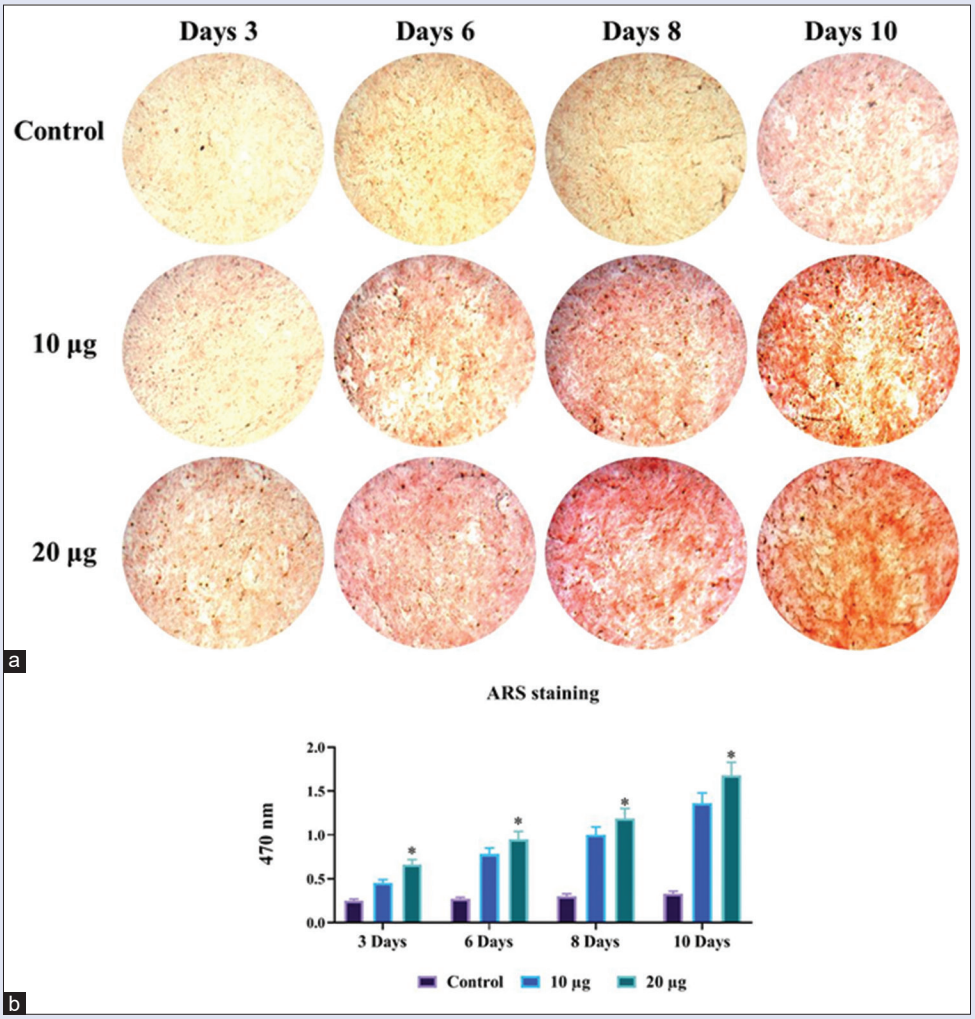

Figure 3: (a and b) Effect of caffeic acid on calcium mineralization in MG-63 cells. Showing the caffeic acid treatment induced calcium deposition rate in the osteoblast-like MG-63 cells. The supplementation of 10 and 20 mg/kg of caffeic acid appreciably improved the calcium mineralization in the osteoblast-like MG-63 cells in 3, 6, 8 and 10 days of treatment (a). The graphical view of calcium mineralization rate on the caffeic acid-treated osteoblast-like MG-63 cells was depicted in (b)

differentiation mechanisms.[35,36] The substitution of lost bone cells due to trauma or accidents shows a vital challenge in a bone tissue transplant and tissue engineering. By this means, exploring the novel bioactive compounds with massive osteogenesis capacities is highly imperious for bone tissue regeneration and tissue engineering. The bio-constituents have been used for clinical applications to aid the regeneration of bone tissues.[37]

The ALP is the vital factor to form the bone minerals, as it initiates and enhances the development of apatite in the osteoblast vesicles that functions to construct the extracellular matrix.[38] Equally, the deposition of calcium in the extracellular matrices is the main indicator of bone formation as well as osteoblast differentiation. Later than osteoblasts maturation, it produces a mineralized bone matrix, and the constituents of the matrices it might be used as indicators of the end phases of osteoblast differentiation.[39] The results of this examination are revealing that the 10 and 20 µg/mL of Caffeic acid treatment was remarkably improved the calcium mineralization in the osteoblast-like MG-63 cells. There are strong links were exist between the ALP function and the calcium mineralization, in that the ALP expression level disseminates the production of phosphates subsequently mineralization. Afterward, the mineralization mechanism, the ALP secretion is lessened constantly and it is no longer required for the mineralized matrices.[40] Many preceding reports are highlighted that the ALP is an essential enzyme that is responsible for calcium mineralization. It is also imperative to deliver the free phosphate ions via lysis of phosphates. As well, the osteoblasts are engaged in bone metabolism and contribute to the calcium and phosphorus homeostasis. It was already highlighted that the deliverance of phosphate and calcium ions always promotes the enhanced enzymatic function of ALP. This eventually exhibits that the calcium can promote the cell proliferation and phenotypic expressions of osteoblasts through the membrane regulated ion exchange.[41,42] In the current examination, it was proved that the 10 and 20 µg/mL of Caffeic acid treatment was enormously improved the multiplication, survival, and calcium deposition in the osteoblast-like MG-63 cells.

CONCLUSION

The findings of the current examination showed that the Caffeic acid was markedly improved the cell proliferation, differentiation, and calcium deposition in the osteoblast-like MG-63 cells. The Caffeic acid

supplementation to the MG-63 cells was evidently improved the MG-63 cell survival, as evidenced by the enhanced cell proliferation. Caffeic acid is also stimulated the calcium mineralization in the osteoblast-like MG-63 cells. Nevertheless, further investigation was mandatory in future to elucidate the exact bone regeneration mechanisms of Caffeic acid in MG-63 cells as well as *in vivo* models.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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