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ORIGINAL ARTICLE

Citral Promotes the Cell Proliferation, Differentiation, and Calcium Mineralization in Human Osteoblast-like MG-63 Cells

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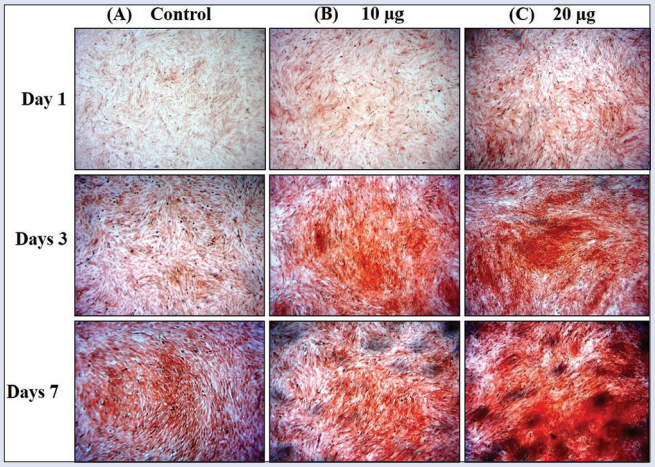
ABSTRACT

Background: Osteoporosis tends to be the major consequence of fractures in women above the age of 50 years and men are prone above the age of 65 years. The structure of bone is severely deteriorated by the reduction in bone density which eventually causes both nonfatal and fatal fractures in geriatric population. Recent researches were targeted to discover a drug which increases bone resorption and inhibits bone osteoporosis, thereby preventing fracture risk in older population. **Methods:** Citral is an aliphatic unsaturated aldehyde present in lemongrass and it renders the lemon fragrance to the lemongrass plant. Citral possesses various pharmacological properties such anti-adipogenic, anti-inflammatory, antimicrobial, and anti-carcinogenic and it also acts as a diuretic agent and stimulates the central nervous system. Therefore, we endeavor to investigate the osteogenic property of citral *in vitro* condition. Human osteoblast-like MG-63 cells were chosen for the current investigation and treated with various doses of citral for different time durations. **Results:** Citral cytotoxicity effects on MG-63 cell lines and their proliferation were assessed using MTT assay and optical microscopical analysis. Further to analyze the osteoblastic activity of citral on MG-63 osteoblast-like cells, we estimated the levels and mRNA expression of bone biomarkers alkaline phosphatase, osteocalcin, and collagen in control and citral-treated cells. To confirm the osteoblastic activity of citral, the MG-63 osteoblast-like cells were subjected to staining with Alizarin red S. The results of MTT assay and microscopic analysis of citral-treated cells proved that citral induces osteoblast-like MG-63 cell line proliferation. Osteogenic bone biomarkers such as alkaline phosphatase, osteocalcin, and collagen were significantly increased in citral-treated which corroborate the osteogenic activity of citral. **Conclusion:** Further, the Alizarin red S staining confirmed the induction of mineral deposition in MG-63 cells by citral. Overall, our results authentically proved that citral induces proliferation, maturation, and mineralization in human osteoblast-like MG-63 cells.

Key words: Bone biomarkers, citral, MG-63 cells, osteogenic drug, osteoporosis

SUMMARY

- Osteoporosis is a systemic bone disease which prominently affects millions of older population worldwide
- The osteogenic property of drug is determined by the stimulation of mineralization in bone matrix which in the later stage of osteoblast differentiation
- Citral possesses osteogenic property and it promotes MG-63 human-like osteoblast cells' differentiation, proliferation, and maturation *in vitro*.



Abbreviations used: NSAD: Nonsteroidal anti-inflammatory drugs; FBS: Fetal bovine serum; ALP: Alkaline Phosphatase

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INTRODUCTION

Osteoporosis is a systemic bone disease which prominently affects millions of older population worldwide. Osteoporosis increases hip and wrist fracture risk in old people resulting in hospitalization which eventually increases the economic burden on geriatric health care. Osteoporosis is characterized by low bone density and deteriorated skeletal tissue occurs due to the imbalance in osteoblastic and osteoclastic activities of bone tissue.[1-3] Bone tissue acts as a reservoir of calcium and continuous calcium absorption and resorption occur in the bone tissues. Excessive calcium resorption of a bone or impaired bone formation leads to osteoporosis.[4] Osteoporosis is more prevalent in older population and postmenopausal women are more prone to osteoporosis than men.[5]

Various factors play a key role in the induction of osteoporosis such as hormone like parathyroid hormone, calcitonin, estrogen, and Vitamin D, calcium balances the osteogenic and osteoclastic activities in bone tissue. The prescribed drug of choice for osteoporosis is classified as

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antiresorptive agents such as bisphosphates, estrogen, calcitonin, and anabolic agent, teriparatide.[6-8] Both antiresorptive and anabolic drugs render substantial side effects and efficacy is also not significant; hence, it is a need of today to discover a drug with high efficacy and low or nil side effects to treat osteoporosis.

Phytochemicals are promising agents which possess various pharmacological properties and are currently proven to cure various ailments such as cancer, nervous disorders, inflammatory diseases, diabetes, and pulmonary diseases. Since osteoporosis is a multifactorial disease, treating it with phytochemicals may render substantial effects.[9] Traditional medicine prescribes herbs such as *Ligustrum lucidum*, *Cissus quadrangularis*, and *Onobrychis ebenoids* to treat osteoporosis.[10] Instead of crude extract treating with bioactive compound increases the potency of the drug; therefore; we assessed the efficacy of bioactive compound citral as an osteogenic stimulator. Citral is a volatile aliphatic aldehyde responsible for the lemon fragrance of Cymbopogon citrates which belongs to Gramineae family and is commonly known as lemongrass. Various *in vivo* and *in vitro* researches confirmed that citral possesses numerous pharmacological properties such as antibacterial, anticarcinogenic, diuretic, central nervous stimulant, anti-inflammatory, and antiadipogenic[11-13] and does not cause side effects in rat and mice models.[14]

Bone cells are differentiated into osteoblasts, osteoclasts, and osteocytes which regulate the formation and remodeling of skeletal tissue. Osteoblast cells are skeletal stem cells which secrete bone osteogenic markers such as alkaline phosphatase, osteocalcin, and collagen thereby induces osteogenic activity and inhibits the osteoclast cells and prevents bone resorption. Osteoblast expresses bone nonspecific isoform of alkaline phosphatase, a glycosylphosphatidylinositol-anchored cell surface protein which induces bone mineralization and acts as a biogenic indicator of bone formation.[15,16] Osteocalcin, an osteoblast, secreted hormone which promotes the mineralization of bone matrix and had been used as a serum biomarker to assess the osteoblastic activity of bone tissue.[17,18] The other vital protein which regulates the bone formation is collagen. Collagen maintains the ratio of mineral deposition, osteopontin, and osteocalcin secretion.[19] In the current investigation, we hypothesized to analyze the osteogenic effect of phytochemical citral on human osteoblast-like MG-63 cells.

MATERIALS AND METHODS

Chemicals

Citral [Figure 1], EEM, fetal bovine serum (FBS), trypsin-EDTA, antibiotic-antimycotic solution, Alizarin red stain, cetylpyridinium chloride, and all other fine chemicals were obtained from Sigma Aldrich, USA. All other chemicals and reagents utilized for the current research were of supreme quality.

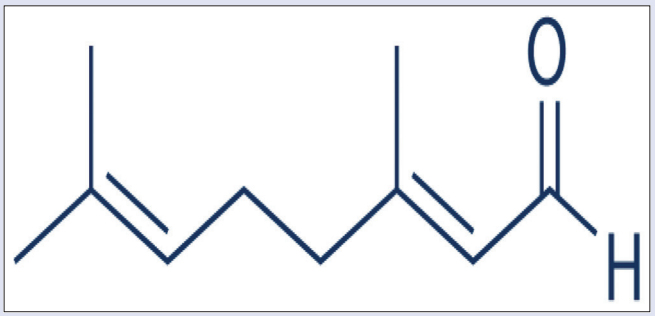

Figure 1: Chemical structure of citral

Culturing of MG-63 cell line

MG-63 cell line resembling human osteoblast cells were procured from ATCC. The cells were cultured using Eagle's Essential Medium along with 2 mM glutamine, 1% nonessential amino acids, 10% FBS, and 1% penicillin-streptomycin solution at 37°C and 5% CO_2. The culture medium was replaced for alternative days or the pH of medium changes to acidic. Upon obtaining 80% confluence, the cells were subculture using 0.25% trypsin-EDTA solution.

Cell viability assay

2×10^4 MG-63 cells/well were seeded on to the 96-well culture plates and incubated at 37°C, 5% CO_2 for 24 h. After 24 h incubation, the cells were treated with 10, 20, and 30 µg/ml of citral (dissolved in DMSO) for different durations of time such as 24, 48, and 72 h. Before subjecting to cell viability assay, the cells were viewed and inverted light microscope and the morphology of the control and treated cells were photographed. The treated MG-63 cells were then incubated with 0.5 mg/ml of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) salt solution to each well for 4 h at 37°C in dark. The reduction of tetrazolium salts into formazan crystals by the mitochondrial enzymes released by citral treated MG-63 cells were dissolved with 1 ml of DMSO. The optical density of the solution was quantified at 570 nm using Shimadzu UV spectrophotometer.[20] The experiment was repeated thrice and the viable cells percentage were determined using the below formula.

$$\text{Percentage of viable cells} = \frac{\text{Optical Density of the test sample}}{\text{Optical Density of the control sample}} \times 100$$

Evaluation of Alkaline Phosphatase activity

MG-63 cells were incubated with citral (10 and 20 µg/ml) for a period of 24, 48, and 72 h and were subjected to alkaline phosphatase assay using an Alkaline phosphatase (ALP) assay kit procured from HiMedia, India. The rate of p-nitrophenyl phosphate to p-nitrophenol hydrolysis was measured at 405 nm using a UV spectrophotometer. The control and citral-treated cells were lysed with cell lysis buffer and subjected to centrifugation at 4°C, 1500 rpm for 20 min. The cell supernatant was collected and subjected to ALP activity assay as per the instruction of the manufacturer.

Assessment of osteocalcin production

The production of late osteogenic differentiation marker osteocalcin was detected using an ELISA kit purchased from MyBioSource, USA. The supernatant of cell lysate was subjected to estimation of osteocalcin synthesis as per the instruction of the manufacturer. The optical density of the final product was quantified at 450 nm with a UV spectrophotometer. The concentration of each sample was interpolated from the standard curve plotted with subjective OD to the standard concentrations.

Estimation of collagen synthesis

The synthesis of collagen in control and citral-treated cells was detected according to the protocol of Tullberg-Reinert and Jundt.[21] MG-63 cell lines were incubated with citral (10 and 20 µg/ml) for a period 24, 48, and 72 h. The cells were fixed for 1 h with Bouin's fluid after rinsing with ice-cold PBS. After the incubation period, Bouin's fluid was drained and the cells were rinsed with distilled water, air-dried, and then stained for 1 h with 1 mg/ml of Sirius red dye dissolved in picric acid. The cells were washed with 0.01 M hydrochloric acid to remove excess dye and the cells were dissolved with 350 µl of 0.1 M sodium hydroxide. The sample was then subjected to UV spectroscopic analysis at 550 nm. The

concentration of each sample was interpolated from the standard curve plotted with subjective OD to the standard concentrations.

Quantitative polymerase chain reaction analysis

The late and early osteogenic differentiation markers such as ALP, osteocalcin, and collagen synthesis in control and citral-treated MG-63 cells were assessed using qPCR analysis. The control and citral-treated MG-63 cells were subjected to total RNA isolation using TriZol reagent and the GC content and the quantity of RNA isolated was assessed using a nanodrop spectrophotometer. The extracted RNA was reverse transcribed to cDNA as per the instruction of the manufacturer using Cells-to-cDNA™ II Kit, Thermo Fischer Scientific, USA. The synthesized cDNA was then subjected to RT PCR analysis with the respective primers ALP, osteocalcin, collagen, and internal control β-actin using a real-time PCR kit (Takara, USA). The relative gene expression was calculated using Ct analysis and normalized with internal control β-actin relative gene expression. The forward and reverse primers used in the current investigation are shown in Table 1.

Mineralization assay

The quantification of mineralization in control and citral-treated MG-63 cells was assessed using Alizarin red S staining. Alizarin red S dye binds to calcium salts in cells which is assessed under a light microscope and the mineralization of MG-63 cells was quantified using spectrophotometric analysis. MG-63 cells were incubated with 10 and 20 µg/ml of citral for different durations 1, 3, and 7 days. After the incubation period, the medium of control and citral-treated cells was removed and the cells were washed with PBS and fixed with 70% ethanol for 1 h at 4°C. After 1 h, the cells were washed with distilled water at stained with 40 mM Alizarin red solution (pH 4.2) for 10 min. The excess stain was drained by washing the cells with distilled water for five times and the cells were incubated with phosphate-buffered saline for 15 min. The cells were then incubated with 10 mM sodium phosphate (pH 7.0) containing 10% (w/v) cetylpyridinium chloride for 15 min and the absorbance of control and citral-treated cells was quantified at 562 nm with a microplate reader.[22] A graph was plotted with incubation time Vs absorbance index.

Statistical analysis

Each experiment was executed in triplicates and the data were expressed as mean ± standard deviation (SD) values. One-way ANOVA and *post hoc* Tukey's tests were used to assess the data statistically. The error bars represent SD of three independent experiments and the significance was represented as $^{*}P < 0.05$, $^{\#}P < 0.01$.

RESULTS

Citral effect on MG-63 cell viability

Effect of citral on MG-63 cells viability was assessed and the representative microscopic images of control and citral-treated MG-63 cells are depicted in Figure 2. Significant increase in the growth rate of MG-63 cells treated with citral was observed both in dose and time-dependent manners which signifies the potential of citral to induce the growth of MG-63 cells.

Cytotoxicity effect of citral

The cytotoxicity effect of citral on MG-63 cells was analyzed using an MTT assay. Figure 3 depicts the results of cytotoxic effect of citral on MG-63 cells, the viability of citral-treated MG-63 cells was increased in both dose ($P < 0.05$) and time-dependent manners than the control cells. Citral does not induce cytotoxicity in MG-63 cell line even when treated with a high dosage of 30 µg/ml ($P < 0.01$) and incubated for a duration of 72 h.

Citral stimulatory effect on Alkaline phosphatase activity

The initiation of osteoblast differentiation by citral in MG-63 cells was evaluated by estimating the levels of alkaline phosphatase activity. ALP is a membrane-bound glycoprotein which initiates the mineralization of matrix, the gene expression of ALP was significantly ($P < 0.05$) increased in both 10 and 20 µg/ml citral-treated cells on 24 h treatment, whereas the expression was decreased on 48 and 72 h treatment [Figure 5a]. The activity of ALP is vice versa to the gene expression maximum amount of ALP activity was exhibited by citral-treated cells ($P < 0.01$) on the prolonged duration of 72 h [Figure 4a].

Citral effect on osteocalcin production

Osteocalcin, osteoblast-secreted bone γ-carboxyglutamic acid protein, promotes the deposition of minerals in the bone matrix. Figures 4b and 5b depict the protein and gene expression levels of osteocalcin in control and citral-treated MG-63 cells. The osteocalcin gene expression is significantly ($P < 0.05$) decreased in citral-treated cells in a time-dependent manner [Figure 5b], whereas the protein expression osteocalcin is significantly ($P < 0.01$) increased in citral-treated MG-63 cells [Figure 4b].

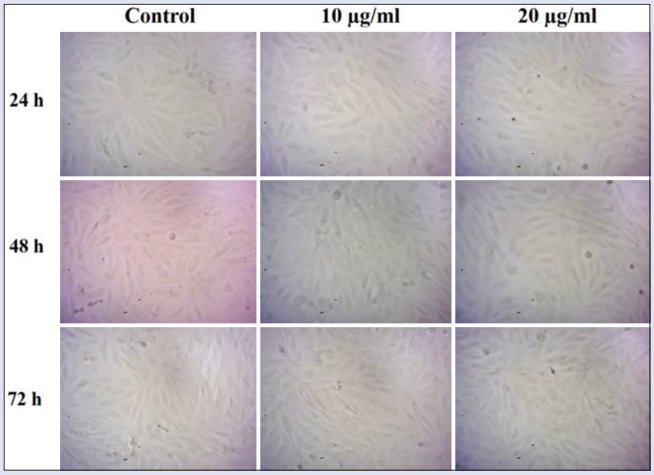

Figure 2: Citral effect on human osteoblast-like MG-63 cell viability. MG-63 cells were treated with 10 and 20 µg/ml of non-saturated aldehyde citral and incubated for 24 h, 48 h, and 72 h. The control and citral-treated cells were microscopically analyzed and the simages were depicted. The experiments were performed in triplicates

Table 1: Shows the forward and reverse primer details for target molecules.

Gene	Forward primer	Reverse primer
ALP	5'-CCCAAAGGCTTCTTCTTG-3'	5'-CTGGTAGTTGTTGTGAGCAT-3'
Osteocalcin	5'-ATGAGAGCCCTCACACTCCTC-3'	5'- GCCGTAGAAGCGCCGATAGGC-3'
Type I collagen	5'-TGACCTCAAGATGTGCCACT-3'	5'-ACCAGACATGCCTCTTGTCC-3'
β-actin	5'-TGACCCAGATCATGTTTGAGA-3'	5'-ACTCCATGCCCAGGAAGGA-3'

ALP: Alkaline phosphatase

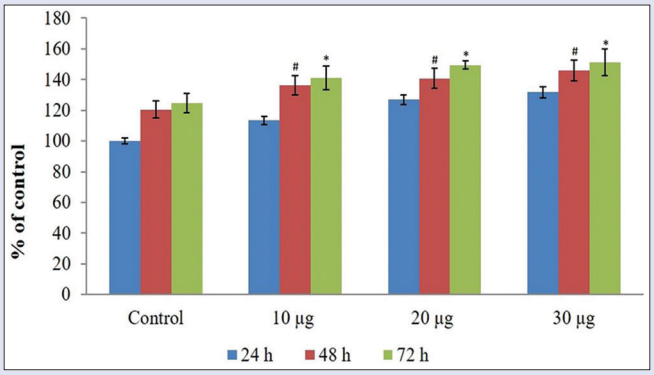

Figure 3: Cytotoxicity effect of citral on human osteoblast-like MG-63 cells. MG-63 cells were treated with 10 and 20 µg/ml of non-saturated aldehyde citral and incubated for 24 h, 48 h, and 72 h. The control and citral-treated cells were subjected to MTT assay and the results were statistically analyzed with one-way ANOVA followed by *post hoc* Tukey's test. Each bar represents mean ± standard error mean of three independent observations. $^{*}P < 0.05$ and $^{\#}P < 0.01$ were considered as statistically significant.

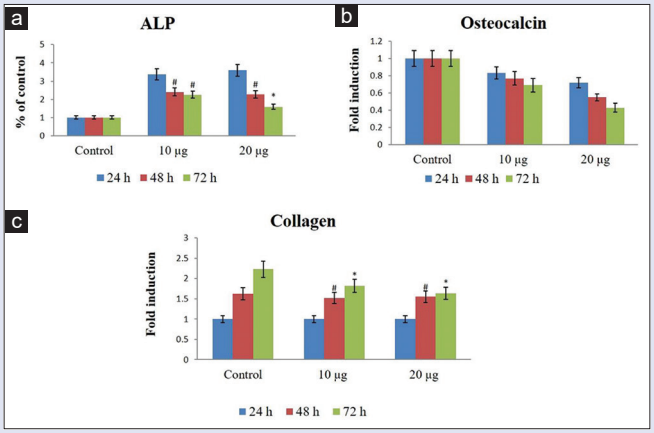

Figure 5: Citral stimulatory effect on bone biomarkers gene expression in human osteoblast-like MG-63 cells. MG-63 cells were treated with 10 and 20 µg/ml of non-saturated aldehyde citral and incubated for 24 h, 48 h, and 72 h. The gene expression of ALP (a), Osteocalcin (b), and collagen (c) was assessed using qRT-PCR analysis. The control and citral-treated cells were subjected to total RNA isolation with TriZol reagent. 2 µg of total RNA was converted to cDNA and subjected to RT-PCR analysis with primers ALP, Osteocalcin and collagen. The experiments were performed in triplicates

Effect of citral on collagen synthesis

Figures 4c and 5c signify the levels of collagen synthesized in control and citral-treated MG-63 human osteoblast-like cells. Collagen predominant substance present in bone matrix act as a marker of bone maturation was extensively ($P < 0.05$) increased in the citral-treated cells compared to control MG-63 [Figure 5c], whereas the gene expression of collagen was significantly ($P < 0.01$) decreased in time-dependent manner in citral-treated MG-63 cells [Figure 4c].

Citral osteoblastic effect on mineralization

The induction of calcium deposition in MG-63 human osteoblast-like cells by citral was assessed using Alizarin red staining and the results are depicted in Figures 6 and 7. Compared to control cells, 10 and 20 µg/ml citral-treated MG-63 cells have shown darkened red-stained spots. The

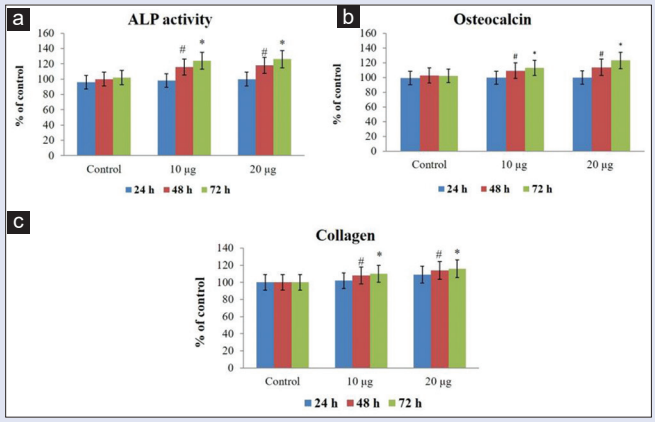

Figure 4: Citral stimulatory effect on bone biomarkers in human osteoblast-like MG-63 cells. MG-63 cells were treated with 10 and 20 µg/ml of non-saturated aldehyde citral and incubated for 24 h, 48 h, and 72 h. The control and citral-treated cells were subjected to (a) alkaline phosphatase activity, (b) osteocalcin production, and (c) collagen synthesis. The results were statistically analyzed with one-way ANOVA followed by *post hoc* Tukey's test. Each bar represents mean ± standard error mean of three independent observations. $^{*}P < 0.05$ and $^{\#}P < 0.01$ were considered as statistically significant

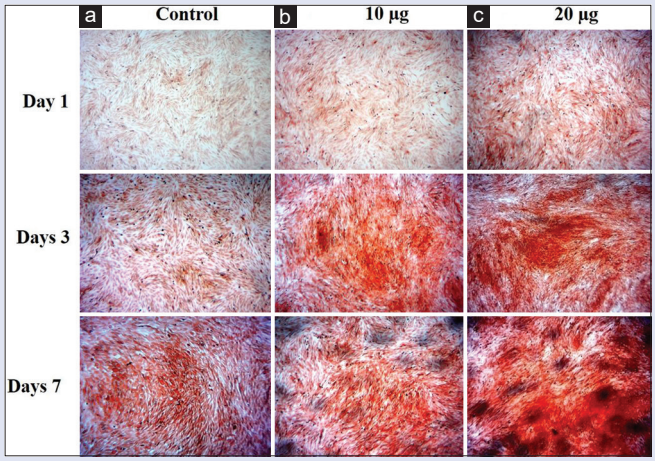

Figure 6: Citral osteoblastic effect on mineralization in human osteoblast-like MG-63 cells. MG-63 cells were treated with 10 and 20 µg/ml of non-saturated aldehyde citral and incubated for 1, 3, and 7 days. The control and citral-treated cells were stained with Alizarin red S stain and the stained cells were assessed microscopically. (a) Control MG-63 cells. (b) 10 µg/ml Citral treated cells. (c) 20 µg/ml citral-treated cells. The experiments were performed in triplicates

intensity of staining was increased ($P < 0.05$) in time-dependent manner and the high mineralization was absorbed in the 20 µg/ml citral-treated MG-63 cells incubated for 7 days [Figure 6c]. The absorbance of Alizarin-stained cells was prominent in citral-treated MG-63 cells than the control MG-63 cells and the absorbance increased ($P < 0.01$) in time-dependent manner [Figure 7].

DISCUSSION

Imbalance in osteogenesis and bone resorption activity of bone tissue leads to osteoporosis and increases the fracture risk in older population and postmenopausal women. Osteoblasts play a decisive role in maintaining the bone microarchitecture via secreting various proteins.[19]

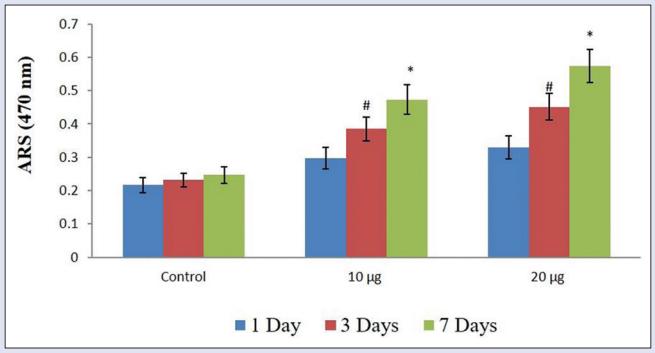

Figure 7: Citral osteoblastic effect on mineralization in human osteoblast-like MG-63 cells. MG-63 cells were treated with 10 and 20 µg/ml of non-saturated aldehyde citral and incubated for 1, 3, and 7 days. The control and citral-treated cells were stained with Alizarin red S stain and the absorbance of stained cells was assessed using a UV spectrophotometer. The results were statistically analyzed with one-way ANOVA followed by *post hoc* Tukey's test. Each bar represents mean ± standard error mean of three independent observations. $^{*}P < 0.05$ and $^{\#}P < 0.01$ were considered as statistically significant

According to 2010 data, more than 50 lakhs men and 220 lakhs European women were at risk of reduced life expectancy due to osteoporosis.[23] Stimulating the osteoblastic activity with phytochemicals may decrease the induction of osteoporosis. Hence in the current investigation, we assessed the osteoblastic stimulatory effect of aliphatic aldehyde citral in *in vitro* condition. We estimated the efficacy of citral on osteoblasts proliferation, differentiation, and maturation by assessing the osteoblasts cell viability and various bone biomarkers in human osteoblast-like MG-63 cells.

MG-63 human osteoblast-like cell line was chosen for the current study since compared to various osteosarcoma cells such as Saos-2, U-2 OS the MG-63 cell lines were characterized with both mature and immature osteoblastic morphology and the cell is one of the most heterogeneous types of osteoblastic cell line.[24] Significant reduction in MG-63 cell viability was observed when treated with 10 µM nonsteroidal anti-inflammatory drugs in both presence and absence of osteogenic stimulators,[25] whereas in the current investigation, our optical microscopic analysis and cytotoxicity analysis clearly evidence that aliphatic aldehyde citral treatment with different doses and different duration of time does not inhibit the growth of MG-63 and it does not impart any cytotoxic effect on osteoblast-like MG-63 cell.

Numerous studies have reported the critical role of alkaline phosphates in bone mineralization. ALP is the foremost initiator gene expressed in the calcification of bone, bone, bone tissues, and the cartilages.[26] Patients with hypophosphatasia who tend to have missense mutated tissue nonspecific alkaline phosphatase gene were reported to possess low or nil ALP activity confirms that ALP plays a key role in the calcification process.[27] The expression of ALP is stimulated with various drugs such as retinoic acid, parathyroid hormone, and Vitamin D which upholds the calcification of bone.[28-30] In our analysis, the phytochemical citral significantly increased the mRNA expression of ALP during 24 h treatment with both doses of 10 µg/ml and 20 µg/ml and the expression was declined with 48 h and 72 h, respectively. The reduction in ALP expression during 48 and 72 h treatment may be due to upregulation of osteocalcin since ALP is an osteogenic factor responsible for the initial phase of mineralization it decreased with time duration. The considerable amplification in the activity of ALP confirms that citral induced osteoblast differentiation via stimulating the osteogenic stimulator ALP in human osteoblast-like MG-63 cells.

Pre-osteoblasts differentiate into osteoblast were stimulated by various substances such as extracellular matrix protein, collagenous protein such as collagen, and non-collagenous proteins such as osteocalcin, osteopontin, and alkaline phosphatase.[31] During proliferative phase of osteoblasts, the expression of fibronectin, collagen, and osteopontin was increased, whereas the expression of alkaline phosphatase and collagen was prominent during the maturation phase of osteoblast.[32] The ALP activity and collagen synthesis are considered as a biomarker for osteoblast maturation.[33] Since in our study citral increased the expression of ALP protein, we further assessed the potency of citral to induce the expression of collagen protein. Collagen is the most profuse protein present in the matrix of bone which accounts for about 90% of the bone matrix.[34] Collagen along with non-collagenous proteins such as alkaline phosphatase, osteonectin, and osteocalcin regulates the osteoblast cell proliferation, differentiation, maturation, and mineralization. Our qPCR results of collagen mRNA expression confirm that citral significantly increased the expression of collagen and its activity was also increased. This clearly states that citral induced collagen genesis in human osteoblast-like MG-63 cells.

The osteogenic property of drug is determined by the stimulation of mineralization in a bone matrix which in the later stage of osteoblast differentiation.[35] Deposition of minerals on the organic scaffold is enhanced when there is increased expression of osteocalcin.[36] Osteocalcin is the second abundant protein secreted by the osteoblast and it is incorporated in the bone matrix. In our present study, osteocalcin expression was significantly enhanced at 20 µg/ml dose of citral, 72 h. The mineralization property of citral was proved with our Alizarin red S staining, 20 µg/ml dose of citral-treated MG-63 human osteoblast-like cells shows the increased density of red staining which authentically proves the induction of mineralization. This may be due to an increase in the activity of non-collagenous protein osteocalcin. Citral, aliphatic aldehyde, which consists of monoterpenoids possesses various pharmacological properties such as anti-arthritis, anti-inflammatory, antioxidant, and antimicrobial. It is reported to be antiadipogenic, carminative, sedative, and expectorant.[11,37,38] In the induction of osteogenic proteins in MG-63 human osteoblast like cells is due to the citral monoterpenoids.

CONCLUSION

In the current investigation, we assessed the osteogenic effect of citral, a predominant bioactive compound present in Cymbopogon citrates commonly known as lemongrass. Citral is responsible for the lemon aroma of Cymbopogon citrates; apart from this, it possesses various pharmacological properties. We tend to analyze its osteogenic property; therefore, we examined its osteogenic stimulating effect in human osteoblast-like MG-63 cell by estimating the levels of osteogenic biomarkers such as alkaline phosphatase, collagen, and osteocalcin. Further to confirm the induction of matrix mineralization by citral, we performed Alizarin red staining and an MTT assay was done to verify whether citral possesses cytotoxicity property. Our overall results authentically confirm that citral possesses osteogenic property and it promotes MG-63 human-like osteoblast cells' differentiation, proliferation, and maturation *in vitro*.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Canton G, Giraldi G, Dussi M, Ratti C, Murena L. Osteoporotic distal femur fractures in the elderly: Peculiarities and treatment strategies. Acta Biomed 2019;90:25-32.
2. Ducy P, Schinke T, Karsenty G. The osteoblast: A sophisticated fibroblast under central surveillance. Science 2000;289:1501-4.
3. Raisz LG, Rodan GA. Pathogenesis of osteoporosis. Endocrinol Metab Clin North Am 2003;32:15-24.
4. Legroux I, Cortet B. Factors influencing bone loss in anorexia nervosa: Assessment and therapeutic options. RMD Open 2019;5:e001009.
5. Black DM, Rosen CJ. Clinical practice. Postmenopausal osteoporosis. N Engl J Med 2016;374:254-62.
6. Camacho PM, Petak SM, Binkley N, Clarke BL, Harris ST, Hurley DL, *et al.* American Association of Clinical Endocrinologists and American College of Endocrinology Clinical Practice Guidelines for the diagnosis and treatment of postmenopausal osteoporosis – 2016. Endocr Pract 2016;22:1-42.
7. Buckley L, Guyatt G, Fink HA, Cannon M, Grossman J, Hansen KE, *et al.* 2017 American College of Rheumatology Guideline for the Prevention and Treatment of Glucocorticoid-Induced Osteoporosis. Arthritis Care Res (Hoboken) 2017;69:1095-110.
8. Brommage R. New Targets and Emergent Therapies for Osteoporosis. In: Stern P.H. (eds) Bone Regulators and Osteoporosis Therapy. Handbook of Experimental Pharmacology, vol 262. Springer, Cham 2019. https://doi.org/10.1007/164_2019_329. [Last accessed on 2020 Jul 29].
9. Jia M, Nie Y, Cao DP, Xue YY, Wang JS, Zhao L, *et al.* Potential antiosteoporotic agents from plants: A comprehensive review. Evid Based Complement Alternat Med 2012;2012:364604.
10. Singla C, Drabu S, Verma R, Dhiman A, Sharma A. Recent update on proficient Bone fracture revivifying herbs. IRJP 2011;2:3-5.
11. Modak T, Mukhopadhaya A. Effects of citral, a naturally occurring antiadipogenic molecule, on an energy-intense diet model of obesity. Indian J Pharmacol 2011;43:300-5.
12. Ekpenyong CE, Akpan E, Nyoh A. Ethnopharmacology, phytochemistry, and biological activities of *Cymbopogon citratus* (DC.) Stapf extracts. Chin J Nat Med 2015;13:321-37.
13. Martins HB, Selis ND, Souza CL, Nascimento FS, de Carvalho SP, Gusmão LD, *et al.* Anti-inflammatory activity of the essential oil citral in experimental infection with *staphylococcus aureus* in a model air pouch. Evid Based Complement Alternat Med 2017;2017:2505610.
14. Vinitketkumnuen U, Puatanachokchai R, Kongtawelert P, Lertprasertsuke N, Matsushima T. Antimutagenicity of lemon grass (*Cymbopogon citratus* Stapf) to various known mutagens in salmonella mutation assay. Mutat Res 1994;341:71-5.
15. Golub E, Battaglia K. The role of alkaline phosphatase in mineralization. Basic Sci 2007;18:444-8.
16. Gerald J, David A, Findlay, Paul, Anderson, Howard, Morris. Target Genes: Bone Proteins Vitamin D (Third Edition) Vitamin D; 2011. p. 411-24.
17. Moser SC, van der Eerden BC. Osteocalcin – A versatile bone-derived hormone. Front Endocrinol (Lausanne) 2018;9:794.
18. Delmas PD, Eastell R, Garnero P, Seibel MJ, Stepan J; Committee of Scientific Advisors of the International Osteoporosis Foundation. The use of biochemical markers of bone turnover in osteoporosis. Committee of Scientific Advisors of the International Osteoporosis Foundation. Osteoporos Int 2000;11 Suppl 6:S2-17.
19. Jafary F, Hanachi P, Gorjipour K. Osteoblast differentiation on collagen scaffold with immobilized alkaline phosphatase. Int J Organ Transplant Med 2017;8:195-202.
20. Tominaga H, Ishiyama M, Ohseto F, Sasamoto K, Hamamoto T, Suzuki K, *et al.* A water-soluble tetrazolium salt useful for colorimetric cell viability assay. Anal Commun 1999;36:47-50.
21. Tullberg-Reinert H, Jundt G. *In situ* measurement of collagen synthesis by human bone cells with a sirius red-based colorimetric microassay: Effects of transforming growth factor beta2 and ascorbic acid 2-phosphate. Histochem Cell Biol 1999;112:271-6.
22. Sims NA, Martin TJ. Coupling signals between the osteoclast and osteoblast: How are messages transmitted between these temporary visitors to the bone surface? Front Endocrinol (Lausanne) 2015;6:41.
23. Hernlund E, Svedbom A, Ivergård M, Compston J, Cooper C, Stenmark J, *et al.* Osteoporosis in the European Union: Medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). Arch Osteoporos 2013;8:136.
24. Pautke C, Schieker M, Tischer T, Kolk A, Neth P, Mutschler W, *et al.* Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. Anticancer Res 2004;24:3743-8.
25. De Luna-Bertos E, Ramos-Torrecillas J, García-Martínez O, Guildford A, Santin M, Ruiz C. Therapeutic doses of nonsteroidal anti-inflammatory drugs inhibit osteosarcoma MG-63 osteoblast-like cells maturation, viability and biomineralization potential. Sci World J 2013;2013:809891.
26. Golub EE, Harrison G, Taylor AG, Camper S, Shapiro IM. The role of alkaline phosphatase in cartilage mineralization. Bone Miner 1992;17:273-8.
27. Whyte MP. Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. Endocr Rev 1994;15:439-61.
28. Pacifici M, Golden EB, Iwamoto M, Adams SL. Retinoic acid treatment induces type X collagen gene expression in cultured chick chondrocytes. Exp Cell Res 1991;195:38-46.
29. Wang BL, Dai CL, Quan JX, Zhu ZF, Zheng F, Zhang HX, *et al.* Parathyroid hormone regulates osterix and Run×2 mRNA expression predominantly through protein kinase A signaling in osteoblast-like cells. J Endocrinol Invest 2006;29:101-8.
30. Zhou YS, Liu YS, Tan JG. Is 1, 25-dihydroxyvitamin D3 an ideal substitute for dexamethasone for inducing osteogenic differentiation of human adipose tissue-derived stromal cells *in vitro*? Chin Med J (Engl) 2006;119:1278-86.
31. Rutkovskiy A, Stensløkken KO, Vaage IJ. Osteoblast differentiation at a glance. Med Sci Monit Basic Res 2016;22:95-106.
32. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, *et al.* Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997;89:755-64.
33. Zur Nieden NI, Kempka G, Ahr HJ. *In vitro* differentiation of embryonic stem cells into mineralized osteoblasts. Differentiation 2003;71:18-27.
34. Reffitt DM, Ogston N, Jugdaohsingh R, Cheung HF, Evans BA, Thompson RP, *et al.* Orthosilicic acid stimulates collagen type 1 synthesis and osteoblastic differentiation in human osteoblast-like cells *in vitro*. Bone 2003;32:127-35.
35. Wei J, Karsenty G. An overview of the metabolic functions of osteocalcin. Rev Endocr Metab Disord 2015;16:93-8.
36. Guldberg RE, Caldwell NJ, Guo XE, Goulet RW, Hollister SJ, Goldstein SA. Mechanical stimulation of tissue repair in the hydraulic bone chamber. J Bone Miner Res 1997;12:1295-302.
37. Rabbani SI, Devi K, Khanam S, Zahra N. Citral, a component of lemongrass oil inhibits the clastogenic effect of nickel chloride in mouse micronucleus test system. Pak J Pharm Sci 2006;19:108-13.
38. Nishijima CM, Ganev EG, Mazzardo-Martins L, Martins DF, Rocha LR, Santos AR, *et al.* Citral: A monoterpene with prophylactic and therapeutic anti-nociceptive effects in experimental models of acute and chronic pain. Eur J Pharmacol 2014;736:16-25.