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COMPARATIVE ANALYSIS OF OIL PALM LEAF EXTRACTS AND VITAMIN C ON OSTEOBLASTIC ACTIVITY AND MINERALISATION IN MC3T3-E1 CELLS AS POTENTIAL ANTI-OSTEOPOROTIC AGENTS

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ABSTRACT

Osteoporosis is a widespread bone disorder characterised by impaired bone remodelling and increased oxidative stress. Oil palm leaf extracts (OPLEs), a by-product of the palm oil industry, are rich in bioactive flavonoids and phenolic compounds with antioxidant and phytoestrogenic properties, yet their effects on bone health remain underexplored. This study investigated the osteogenic potential of methanolic (MEOPL) and ethanolic (EEOPL) oil palm leaf extracts in murine pre-osteoblast MC3T3-E1 cells, with vitamin C as a comparator. Cell viability, osteoblastic differentiation and mineralisation were assessed using MTT, Alizarin red S, alkaline phosphatase (ALP) activity, Sirius red, crystal violet, von Kossa staining and RT-qPCR for RUNX2 gene expression. MEOPL significantly enhanced cell viability, osteoblastic differentiation, mineralisation and RUNX2 expression compared with vitamin C, which showed dose-dependent cytotoxicity at higher concentrations. These findings highlight MEOPL as a promising plant-based therapeutic candidate for the management of osteoporosis. By demonstrating superior osteogenic effects to vitamin C in vitro, *MEOPL provides a rationale for further preclinical and clinical investigations into its potential as a safe, natural alternative to conventional osteoporosis therapies.*

Keywords: methanolic extract of oil palm leaves (MEOPL), oil palm leaves, osteoblastogenesis, osteoporosis, vitamin C.

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INTRODUCTION

Osteoporosis is a prevalent skeletal disorder characterised by an imbalance between bone formation and resorption, resulting in decreased bone mass, compromised bone structure and an increased risk of fractures, particularly in postmenopausal women due to oestrogen deficiency (Kim et al., 2023). Globally, it affects an estimated 200 million people and is further exacerbated by elevated pro-inflammatory cytokines and leukotriene B4 (Sözen et al., 2017). The condition is further aggravated by elevated pro-inflammatory cytokines and mediators such as leukotriene B4, which accelerate bone resorption.

Current pharmacological treatments, including bisphosphonates, selective oestrogen receptor modulators, parathyroid hormones and hormone replacement therapy, primarily target bone resorption but have limited ability to restore bone density. In addition, these drugs are associated with adverse effects such as hyperkalaemia, cardiovascular complications, osteosarcoma and increased risks of stroke and cancer (Abrahamsen,

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2010; Hamadeh et al., 2015; Oršolić et al., 2018). These limitations highlight the urgent need for safer, more sustainable alternatives that not only protect against bone loss but also stimulate new bone formation.

Natural products and phytoestrogens, such as flavonoids, lignans and isoflavones, are gaining increasing attention for their potential as bone-protective agents. These compounds mimic oestrogen and modulate bone metabolic pathways, offering anti-inflammatory and osteogenic effects (Kim et al., 2023). Flavonoid- and phenolic-rich extracts have been shown to enhance osteoblast function and bone matrix mineralisation. For example, rutin promotes osteogenic differentiation in MC3T3-E1 pre-osteoblasts by increasing alkaline phosphatase (ALP) activity and RUNX2 expression (Liu et al., 2021), while astragalin stimulates osteogenic markers both *in vitro* and *in vivo* (Liu et al., 2019). Similarly, icariin enhances osteoblast proliferation and mineralisation through the miR-153/RUNX2 pathway (Zhang et al., 2020), and anthocyanin-rich blackcurrant extracts have demonstrated effects on collagen synthesis and mineralisation in both cell and animal models (Yamaguchi et al., 2024). These findings collectively suggest that flavonoids and related natural compounds hold promise as safer alternatives or adjuncts to conventional osteoporosis therapies.

Furthermore, phytoestrogens such as genistein have demonstrated significant osteogenic effects by upregulating osteoblast-specific genes and increasing ALP activity and mineral deposition (Wang et al., 2023). Other natural compounds like baicalein and pinoresinol have been demonstrated to stimulate osteoblast differentiation and bone matrix mineralisation, offering potential natural alternatives for osteoporosis treatment (Chen et al., 2021; Kim et al., 2019). These recent findings collectively emphasise the promise of flavonoid- and phenolic-rich extracts in bone regeneration strategies.

Oil palm (*Elaeis guineensis*) leaves, an abundant by-product of the palm oil industry, are rich in bioactive constituents such as flavonoid C-glycosides and O-glycosides, ferulic acid and chlorogenic acid (Sasidharan et al., 2012). These compounds exhibit antioxidant, phytoestrogenic and anti-inflammatory properties and have shown wound healing (Jaffri et al., 2011) and cardiovascular benefits (Fatima et al., 2022). Despite these pharmacological attributes, the osteogenic potential of oil palm leaf extracts (OPLEs) remains poorly explored. In particular, no prior study has systematically evaluated the comparative effects of methanolic and ethanolic OPLEs against vitamin C, a recognised osteogenic enhancer, using a comprehensive panel of *in vitro* osteogenesis assays.

This study addresses that gap by investigating the osteoinductive effects of methanolic and ethanolic OPLEs on murine MC3T3-E1 pre-osteoblast cells, with vitamin C as a reference control. The study evaluates multiple parameters of osteoblast function, including ALP activity, collagen synthesis, calcium and phosphate deposition and *RUNX2* gene expression, to provide a holistic assessment of osteogenic potential.

Osteoblasts are central to bone metabolism, synthesising extracellular matrix and regulating mineralisation, thereby driving bone regeneration and remodelling. The murine MC3T3-E1 pre-osteoblast cell line, derived from calvaria, is a well-established *in vitro* model for studying osteogenesis (Kodama et al., 1981). This system enables detailed evaluation of proliferation, differentiation and matrix mineralisation, with ALP activity serving as a key marker of early osteogenic maturation (Jeong et al., 2005). Its relevance to bone biology provides a robust platform for assessing the therapeutic potential of OPLEs in comparison with vitamin C.

MATERIALS AND METHODS

Materials

Methanol, ethanol, hexane, ethyl acetate and other analytical grade solvents were purchased from Merck, Germany. α-Minimum essential medium (α-MEM; Nacalai Tesque, Japan), foetal bovine serum (FBS; Biowest, France) and antibiotics (penicillin G, Nacalai Tesque, Japan and streptomycin, Sigma Aldrich, USA). Alizarin red S, Sirius red, crystal violet, von Kossa staining kits and ALP assay kits were obtained from Abcam, UK. All reagents were of analytical grade and used without further purification. Unless otherwise stated, all other chemicals were obtained from Sigma-Aldrich, USA.

Extraction of OPLEs

Leaves of *E. guineensis* were harvested from six-year-old palm trees at the Universiti Putra Malaysia research farm (Serdang, Selangor, Malaysia). The leaves were washed, air-dried at 40°C for 48 hr and ground into a fine powder. Approximately 100 g of powdered leaves were soaked in methanol or ethanol (1:10, w/v) for 48 hr at room temperature with continuous agitation using a Gyro-Rocker Shaker (IKA, Germany). The mixtures were filtered through Whatman No. 1 filter paper (Whatman, UK) and concentrated under reduced pressure using a rotary evaporator (Rotavapor R-210; Büchi Labortechnik, Switzerland) at 40°C until

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solvent evaporation was complete (Zain et al., 2021). The resulting crude extracts (methanolic and ethanolic OPLEs) were stored at -20°C until further use.

Cell Culture

Murine calvaria pre-osteoblast cells (MC3T3-E1, subclone 4; ATCC, USA) were used as the *in vitro* model. Cells were cultured in α-MEM, supplemented with 10% FBS and antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin). They were then incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells between passages 5–15 were used for experiments. Media were replaced every three days, with prior washing using phosphate-buffered saline (PBS, pH 7.4).

Cell Viability

The cytotoxic effects of OPLEs and vitamin C on MC3T3-E1 cells were assessed following the Liu et al. (2019) method. Cells were detached with a 0.25% trypsin-EDTA solution containing 2.65 mM EDTA and seeded in 96-well plates at a density of 1×10^4 cells per well and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hr. After incubation, cells were treated with varying concentrations of methanolic and ethanolic OPLEs (0.05–0.25 mg/mL) and vitamin C (0.05–0.25 mg/mL) for 24, 48 and 72 hr.

Following treatment, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 hr. The resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and absorbance was measured at 595 nm using an Infinite M200 microplate reader (TECAN, USA). Cell viability was expressed as a percentage relative to the untreated control.

Pre-Osteoblastic Cell Culture and Mineralisation Assays

MC3T3-E1 cells were cultured in α-MEM supplemented with 10% FBS and antibiotics. Cells were maintained at 37°C in a humidified incubator supplemented with 5% CO_2. After two days, the culture medium was replaced with differentiation medium (DM) consisting of α-MEM supplemented with 10 mM β-glycerophosphate 50 μg/mL ascorbate-2-phosphate and 10 nM dexamethasone. Cells were cultured for up to 21 days, with the DM changed every three days (Coelho et al., 2023; Thu et al., 2017).

Osteogenic differentiation was evaluated using multiple assays, including crystal violet staining, ALP activity, collagen staining (Sirius red), Alizarin red S staining for calcium deposition and von Kossa staining for phosphate mineralisation.

ALP Activity

ALP activity in MC3T3-E1 cells was assessed using both staining and colorimetric assays. Cells were seeded in 24-well plates and cultured until reaching confluence, then treated with DM containing serial dilutions of methanolic extract of oil palm leaves (MEOPL) (0.05, 0.1, 0.15 and 0.2 mg/mL) and vitamin C (0.05 mg/mL). Cultures were maintained for 14 and 21 days, while the medium was replaced every three days.

On days 14 and 21, cells were rinsed with PBS, fixed for 1 min with 100% ethanol and stained with a BCIP/NBT solution (Sigma-Aldrich, USA) for 5–10 min. After staining, cells were washed, air-dried and visualised with a phase-contrast microscope (CKX41; Olympus, Japan).

For quantitative analysis, ALP activity was measured using a colorimetric ALP Assay Kit, based on the enzymatic conversion of p-nitrophenyl phosphate (pNPP) into p-nitrophenol. Absorbance was recorded at 405 nm using a microplate reader (Infinite M200).

Collagen Synthesis

Collagen quantification was conducted using the Sirius red staining method, as described in several studies (Kim et al., 2023; Park et al., 2014). MC3T3-E1 cells were cultured in osteogenic medium supplemented with 10 mM β-glycerophosphate, 5 nM dexamethasone and 50 μg/mL ascorbic acid. Cells were treated with varying concentrations of MEOPL (0.05–0.2 mg/mL) or vitamin C (0.05 mg/mL) and maintained for 14 and 21 days.

At each time point, cells were rinsed with phosphate-buffered saline (PBS) and fixed in ethanol. Samples were then stained with 0.1% Sirius red solution in picric acid for 18 hr at room temperature with agitation. After staining, cells were washed, air-dried and observed microscopically. The bound dye was subsequently eluted with 0.1 N NaOH, and absorbance was measured at 550 nm using an Infinite M200 microplate reader (Hwang et al., 2023).

Mineralisation Content

Calcium deposition in MC3T3-E1 osteoblast cells was assessed following the method demonstrated by Kim et al. (2021). Cells were seeded at a density of 1×10^4 cells per well in 96-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were treated with varying concentrations of MEOPL

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(0.05–0.2 mg/mL) and vitamin C (0.05 mg/mL) and cultured for 14 or 21 days with medium changed every three days to facilitate mineral deposition.

At each time point, cells were rinsed twice with PBS, fixed with ethanol, and stained with Alizarin red S stain. Calcified nodules were visualised using a phase-contrast light microscope (CKX41). The bound dye was then extracted with cetylpyridinium chloride, and absorbance was measured at 570 nm using an Infinite M200 microplate reader (Wang et al., 2023).

Inorganic Phosphate Deposition

MC3T3-E1 cells underwent treatment for 14 and 21 days, followed by Alizarin red S staining. After treatment, cells were rinsed with PBS, fixed in 70% ethanol for 15 min and rinsed three times with deionised water. The cells were subsequently incubated in 5% silver nitrate solution under ultraviolet (UV) light for 1 hr until a brownish precipitate appeared, indicating mineralised nodules. Following staining, cells were rinsed and neutralised with a 5% sodium thiosulfate solution for 10 min, then air-dried. The stained cells were imaged using a phase-contrast microscope (CKX41) at room temperature (Sim et al., 2023).

Quantitative RT-PCR

Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Germany) to evaluate osteoblastogenesis. The purity and concentration of RNA were measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) at a 260/280 nm ratio. Complementary DNA (cDNA) was synthesised from 1 µg of total RNA using a Quant Inova Internal Control RNA Assay kit (Qiagen, Germany). Quantitative real-time PCR (qRT-PCR) was performed by a 2 × SYBR Green qPCR master mix (Qiagen, Germany) on a Mastercycler® ep realplex 4 (Eppendorf, Germany). Primers specific to RUNX2 were used to assess osteoblast differentiation, with gene expression levels normalised to mouse GAPDH. Fold changes were evaluated using the ΔΔ-Ct method.

Statistical Analysis

The data was evaluated by one-way ANOVA followed by Tukey's multiple comparisons test to compare with the negative group, and two-way ANOVA followed by Šidák test for comparing between days within the same group, using GraphPad Prism version 9. A *p*-value of <0.05 was considered statistically significant. All experiments were performed in triplicate and repeated at least three times to ensure reproducibility of results.

RESULTS AND DISCUSSION

Effects of OPLEs and Vitamin C on Osteoblastic Cell Viability

The cytotoxic activities of OPLEs and vitamin C on MC3T3-E1 cells were assessed at various dosages (0.05, 0.10, 0.15, 0.20 and 0.25 mg/mL) over 24, 48 and 72 hr. MTT assay results indicated that both methanolic and ethanolic extracts of oil palm leaves, maintained cell viability at above 100% for concentrations up to 0.2 mg/mL at 24 hr. Notably, MEOPL (0.05–0.20 mg/mL) and ethanolic extract of oil palm leaves (EEOPL) (0.05–0.10 mg/mL) significantly enhanced cell viability compared to the control at lower doses. In contrast, higher doses of vitamin C reduced cell viability relative to the control. *Figure 1* illustrates these temporal effects, summarising the percentage of cell viability for each concentration and time interval. Based on these findings, MEOPL (0.05, 0.10, 0.15, 0.20 mg/mL) and vitamin C (0.05 mg/mL) were selected for further studies due to their non-toxic, proliferative properties.

MEOPL emerges as a promising natural alternative due to its rich bioactive compounds, including flavonoids and tannins, which offer a combined mode of action that both suppresses bone resorption and facilitates bone formation (Tow et al., 2021). This study demonstrated that MEOPL maintains and enhances cell viability in MC3T3-E1 cells across a range of concentrations, confirming its biocompatibility and potential as a safer alternative to existing osteoporosis treatments. This is consistent with previous findings supporting the non-cytotoxic nature of oil palm leaf extracts (Syahmi et al., 2010; Yusof et al., 2018; Zain et al., 2021).

Although vitamin C is generally recognised as an antioxidant, at high concentrations it can paradoxically exert pro-oxidant effects *in vitro* by generating hydrogen peroxide and reactive oxygen species. This shift contributes to oxidative stress and reduced cell viability, as observed in MC3T3-E1 cells at elevated doses (Kim et al., 2018; Li et al., 2021). Li et al. (2021) further demonstrated that vitamin C can be more toxic than MEOPL even at lower concentrations, highlighting vitamin C's narrow therapeutic window.

Importantly, although MEOPL maintained high cell viability at concentrations up to 0.20 mg/mL, exposure to ≥0.25 mg/mL resulted in reduced viability (data not shown), suggesting potential cytotoxic effects at elevated doses. This observation underscores the importance of careful dose optimisation in future applications of MEOPL. The combination of high phenolic content and strong antioxidant activity likely contributes to the

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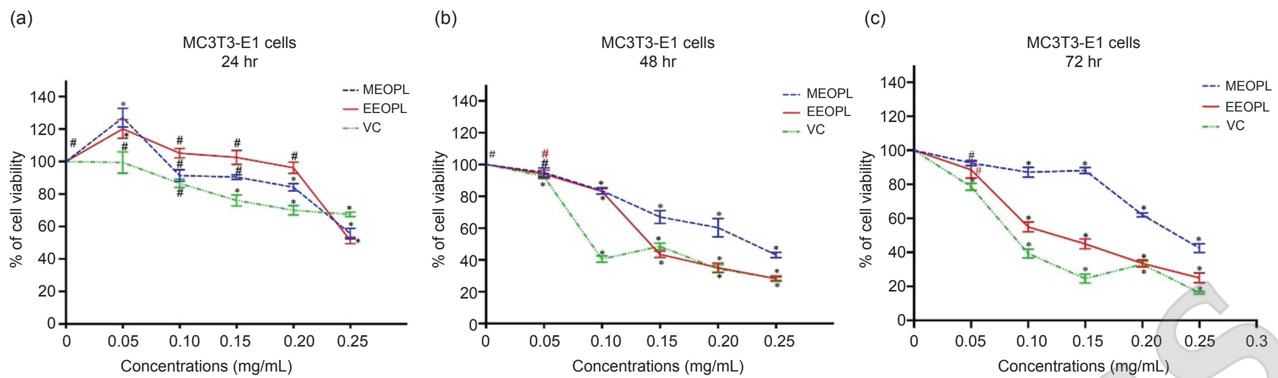

Figure 1. Cell viability of MC3T3-E1 cells following treatment with various concentrations of methanolic (MEOPL) and ethanolic (EEOPL) oil palm leaf extracts and vitamin C (VC) for (a) 24, (b) 48 and (c) 72 hr. Viability was assessed by the MTT assay and is indicated as the mean percentage of control ± standard deviation (SD). (*) indicate statistical significance ($p<0.05$) *compared to the untreated control, as obtained by one-way ANOVA and Tukey's test.*

improved viability at lower doses, reinforcing its potential as a natural agent to promote osteoblast proliferation and support bone tissue engineering.

ALP Activity

ALP activity serves as an essential biomarker for early osteogenesis. The study investigated the effect of the MEOPL and vitamin C on osteoblastic differentiation by measuring ALP activity after day 14 and 21. The findings revealed a significant increase in ALP activity in both MEOPL- and vitamin C-treated cells compared to the control. On day 14, vitamin C-treated cells had increased ALP activity, but no significant differences were observed across the MEOPL concentrations. By day 21, MEOPL at 0.05 and 0.15 mg/mL showed a marked increase in ALP activity compared to vitamin C, indicating a concentration-dependent response. All MEOPL concentrations showed enhanced ALP activity on day 21 relative to the control, highlighting its osteoinductive potential (*Figure* 2).

ALP is a crucial enzyme involved in osteoblast differentiation and bone matrix maturation. The significant increase in ALP activity observed with MEOPL and vitamin C treatments indicates their roles in promoting osteogenic differentiation. While vitamin C showed an initial increase in ALP activity at day 14, MEOPL's effect became more pronounced by day 21, particularly at doses of 0.05 and 0.15 mg/mL. This concentration-dependent response suggests that MEOPL may be more effective in sustaining and enhancing osteoblast activity over time compared to vitamin C.

These findings are consistent with other studies demonstrating the potential of natural extracts, such as those from *Drynariae rhizoma* and *Ulmus davidina* (Sirimethawong et al., 2018), *Blumea acmella* (Widyowati et al., 2020) and *Eucommia* leaves (Guan et al., 2021), in increasing ALP activity and promoting osteoblastic differentiation. Similarly, *Petasites japonicus* extracts have been reported to boost ALP in MC3T3-E1 cells (Kim et al., 2021). These results support MEOPL's comparability to vitamin C and other natural antioxidants in enhancing osteoblastic activity, which is essential for bone engineering.

Effect of MEOPL and Vitamin C on Collagen Synthesis Activity

Collagen synthesis, a key marker of osteoblastic differentiation, was assessed using Sirius Red staining, which binds to type I and III collagen fibres. Both MEOPL and vitamin C treatments showed increased collagen synthesis compared to the negative control, with the effect being more pronounced by day 21, indicating collagen maturation (*Figure 3*). A dose-response was observed, with the highest MEOPL concentration (0.2 mg/mL) resulting in the greatest collagen deposition, outperforming vitamin C. *Figure 3* illustrates these findings, showing collagen intensity and quantifying content across different MEOPL and vitamin C concentrations.

Collagen is essential for bone mineralisation, providing the structural scaffold necessary for bone matrix development (Thu et al., 2017). The dose-dependent increase in collagen synthesis observed with MEOPL treatment, particularly at 0.2 mg/mL, indicates its strong potential in promoting osteoblast maturation and bone matrix formation. This effect was more pronounced than that of vitamin C, suggesting that MEOPL may be more effective in enhancing the structural integrity and strength of bone tissue.

These findings align with existing literature, which highlights the role of collagen in bone cell development and structural bone robustness (Fujisawa et al., 2018; Thu et al., 2017). Additionally,

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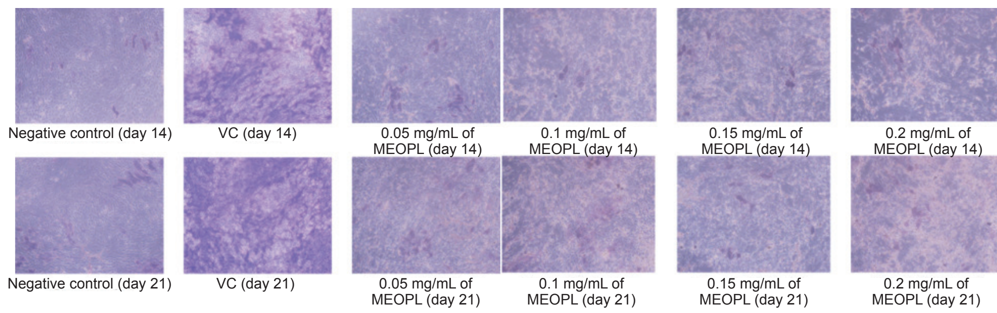
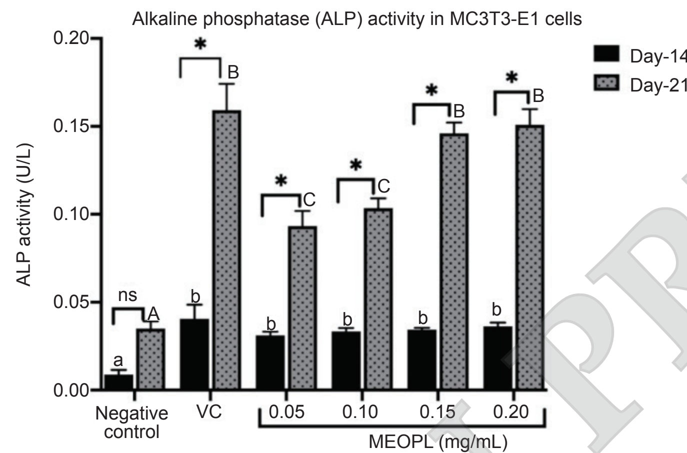
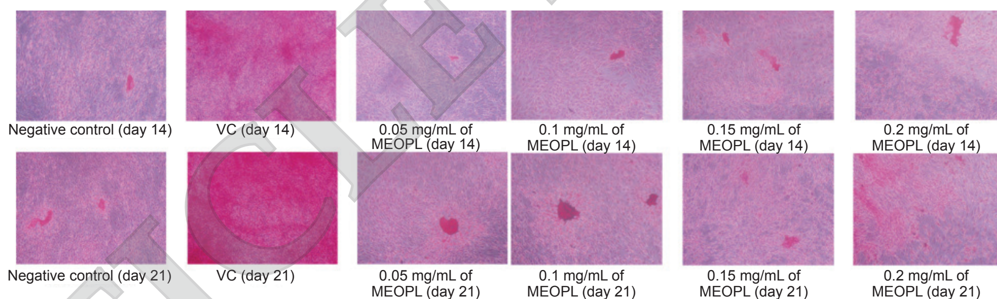
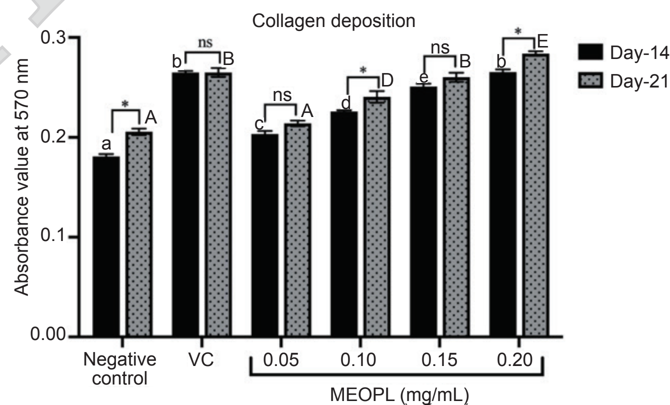




Note: Representative ALP staining images (100×); Quantified ALP activity (U/L); Data are presented as mean ± SD (n = 3); p<0.05 versus control by one-way ANOVA (Tukey's test); day 14 vs. day 21 comparisons were analysed by two-way ANOVA (Šidák test).

Figure 2. ALP staining and activity in MC3T3-E1 cells treated with MEOPL (0.05–0.20 mg/mL) and vitamin C (VC) (0.05 mg/mL) for 14 and 21 days.





Note: Representative Sirius red-stained images showing collagen fibres (100×); Collagen content quantified by absorbance at 570 nm; Bars represent mean ± SD (n = 3). Different letters indicate significant differences among concentrations (one-way ANOVA, Tukey's test). (*) denote significant time differences between 14 and 21 days (two-way ANOVA, Šidák test; * p< 0.05).

Figure 3. Collagen deposition and quantification following treatment with MEOPL (0.05–0.20 mg/mL) and vitamin C (VC) (0.05 mg/mL) for day 14 and 21.

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the phenolic compounds in MEOPL likely contribute to its ability to enhance collagen production, supporting the bone matrix and promoting overall bone health. The enhanced collagen synthesis reinforces the potential of MEOPL as a natural strategy for osteoporosis prevention and bone tissue engineering, similar to the effects observed with *P. japonicus* extracts, which are also rich in phenolic content and linked to increased osteoblast activity and collagen production (Kim et al., 2021).

Effect of MEOPL and Vitamin C on Nodule Formation

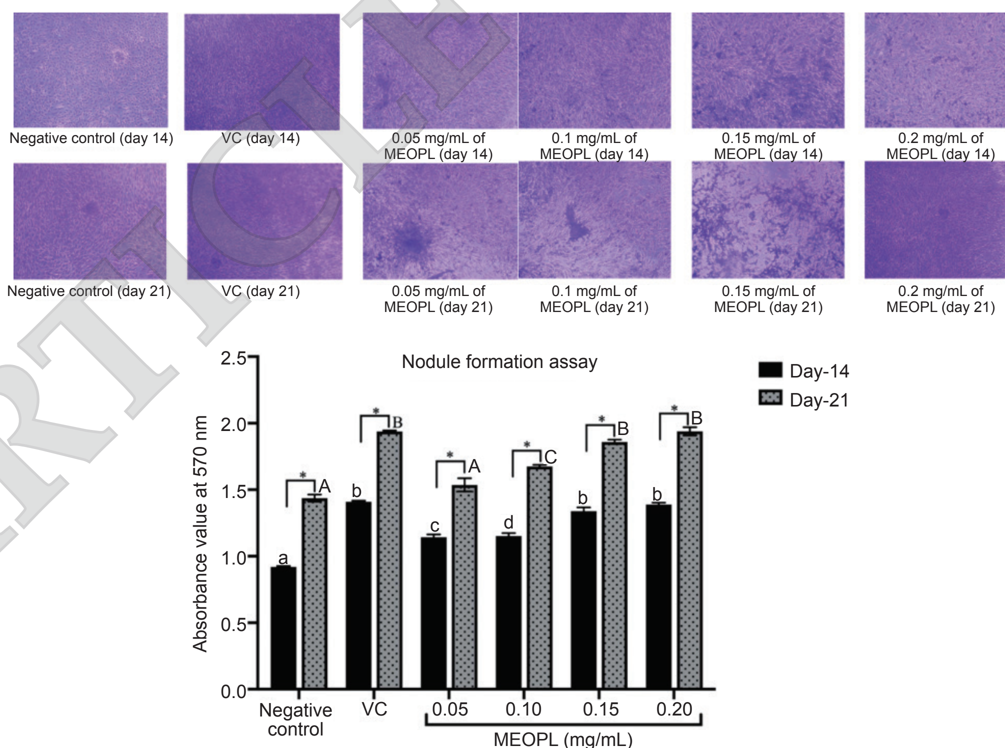
Nodule formation, a marker of early osteoblastic differentiation, was assessed in MC3T3-E1 cells treated with MEOPL and vitamin C over 14 and 21 days. Crystal violet staining showed significant nodule enhancement, especially on day 21. While vitamin C had the highest nodule development on day 14, MEOPL at 0.2 mg/mL surpassed vitamin C's effects by day 21, suggesting a dose-dependent osteogenic response. These results, illustrated in *Figure 4*, highlight MEOPL's potential to promote osteoblastic differentiation.

The significant increase in nodule formation observed with MEOPL treatment, particularly at 0.2 mg/mL, reflects enhanced matrix mineralisation and demonstrates its potent osteoinductive capability. The initial increase in nodule development with vitamin C at day 14 suggests that vitamin C can stimulate early stages of osteoblast differentiation. However, MEOPL's superior performance by day 21 indicates that it not only promotes early differentiation but also sustains and enhances osteoblast activity over time.

This dose-dependent response aligns with previous studies, such as those by Thu et al. (2017), who reported enhanced cell proliferation and differentiation with testosterone and 5α-DHT treatments, and Liu et al. (2019), who found that the flavonoid astragalin promotes dose-dependent nodule formation in osteoblastic cells. The ability of MEOPL to outperform vitamin C in nodule formation underscores its potential as a more effective natural agent for promoting bone regeneration and osteoporosis management.

Effect of MEOPL and Vitamin C on Calcium Deposition in Osteogenesis

Calcium deposition, essential for osteogenesis and bone strength, was evaluated using Alizarin red S staining to measure calcium nodule formation in MC3T3-E1 cells treated with MEOPL and vitamin C. Both treatments significantly enhanced calcium nodule formation at day 14 and 21 compared with the control ($p<0.05$).



Note: Representative crystal-violet-stained images of nodules (100×); Quantitative analysis of nodule formation by absorbance at 570 nm; Results are mean ± SD (n = 3). Different letters denote significant differences among concentrations (one-way ANOVA, Tukey's test). (*) indicate significant differences between days 14 and 21 (two-way ANOVA, Šidák test; * $p< 0.05$).

Figure 4. Nodule formation following treatment with MEOPL (0.05–0.20) mg/mL and vitamin C (VC) (0.05 mg/mL) for day 14 and 21.

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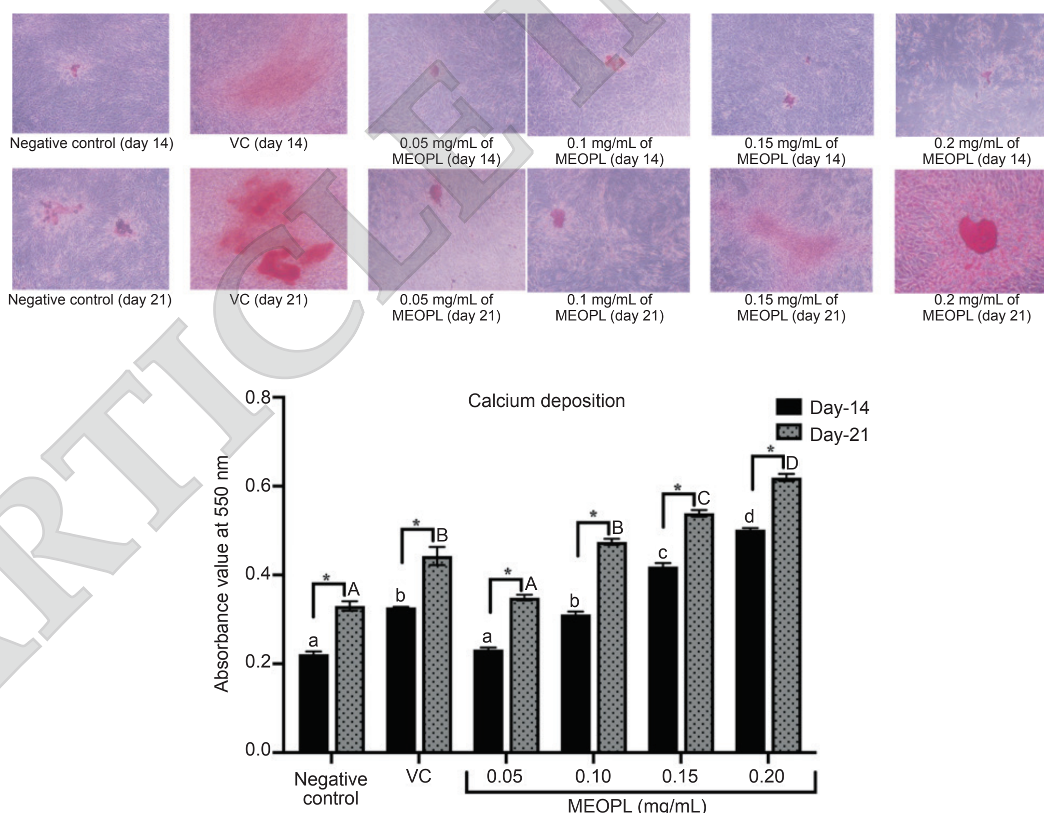
Although vitamin C demonstrated greater mineralisation on day 14, MEOPL at a concentration of 0.2 mg/mL showed the highest calcium deposition by day 21, surpassing the effect of vitamin C (p<0.05). Additional MEOPL concentrations (0.10, 0.15 mg/mL) also resulted in substantial improvements. These findings underscore the potential of MEOPL to support osteogenic differentiation, particularly at elevated concentrations (*Figure 5*).

Calcium deposition is a hallmark of osteogenic differentiation, signifying the formation of a bone matrix and the integrity of bone tissue (Owen & Reilly, 2018). The enhanced calcium deposition observed with MEOPL treatment, particularly at higher concentrations, indicates its strong osteogenic potential. Although vitamin C showed greater mineralisation at the earlier time point of day 14, MEOPL's superior performance by day 21 suggests a more sustained and effective promotion of mineralisation over time. This effect is reminiscent of established osteogenic agents like alendronate and curcumin, which are known to enhance proliferation and differentiation of bone cells (Dong et al., 2018).

The dose-dependent increase in calcium deposition with MEOPL further supports its role as a potent natural therapeutic agent for osteoporosis, aligning with studies that demonstrate the mineralisation-promoting effects of various phytochemicals and natural extracts (Park et al., 2019; Sirimethawong et al., 2018; Tasadduq et al., 2017). Hence, MEOPL's ability to promote calcium deposition underscores its potential in osteoporosis management.

Impact of MEOPL and Vitamin C on Phosphate Mineralisation

Phosphate mineralisation, a key step in osteoblast maturation, was assessed using von Kossa staining to visualise mineralised nodules. The method, applied on days 14 and 21, showed clear differences in mineral deposition between the control and treated groups (*Figure 6*). MEOPL-treated cells, especially at 0.2 mg/mL, exhibited the most intense staining and highest mineral deposits by day 21, indicating that MEOPL may enhance phosphate deposition and support osteoblast maturation.



Note: Representative stained images of calcium nodules (100×); Quantitative calcium deposition measured at 570 nm; Data are expressed as mean ± SD (n = 3). Letters denote significant differences among treatment concentrations (one-way ANOVA, Tukey's test). (*) represent significant time effects (two-way ANOVA, Šidák test; * p< 0.05).

Figure 5. Calcium mineralisation assessed by Alizarin Red S staining after treatment with MEOPL (0.05–0.20 mg/mL) and vitamin C (VC) (0.05 mg/mL) for day 14 and 21.

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Phosphate mineralisation is crucial for osteoblast maturation and the formation of a robust bone matrix. The enhanced phosphate deposition observed with MEOPL treatment, particularly at the highest dose of 0.2 mg/mL, indicates its significant role in supporting osteoblast maturation and bone tissue formation. The von Kossa staining results corroborate the Alizarin Red S findings, showing that MEOPL not only promotes calcium deposition but also enhances phosphate deposition, which is crucial for the structural integrity and functionality of bone tissue (Tasadduq et al., 2017).

This dual enhancement of calcium and phosphate mineralisation by MEOPL aligns with the mineralisation effects of established osteogenic agents like alendronate and curcumin (Dong et al., 2018), reinforcing MEOPL's potential as a comprehensive natural agent for osteoporosis treatment. The ability of MEOPL to sustain and amplify phosphate deposition over time highlights its efficacy in promoting complete osteogenic differentiation and bone health.

Modulation of Osteoblast-differentiation Gene Expression by MEOPL and Vitamin C

The genetic mechanisms by which MEOPL and vitamin C enhance osteoblast differentiation were examined using quantitative real-time PCR, focusing on RUNX2, a key transcription factor in bone development. MC3T3-E1 cells were treated with varying MEOPL concentrations (0.050–0.20 mg/mL) and a constant VC dose (0.05 mg/mL) for 14 and 21 days. MEOPL at 0.20 and 0.15 mg/mL significantly elevated RUNX2 expression at both time points ($p<0.01$), surpassing other concentrations, vitamin C and the control. This supports the hypothesis that MEOPL promotes early osteoblast differentiation, which is consistent with previous findings on ALP

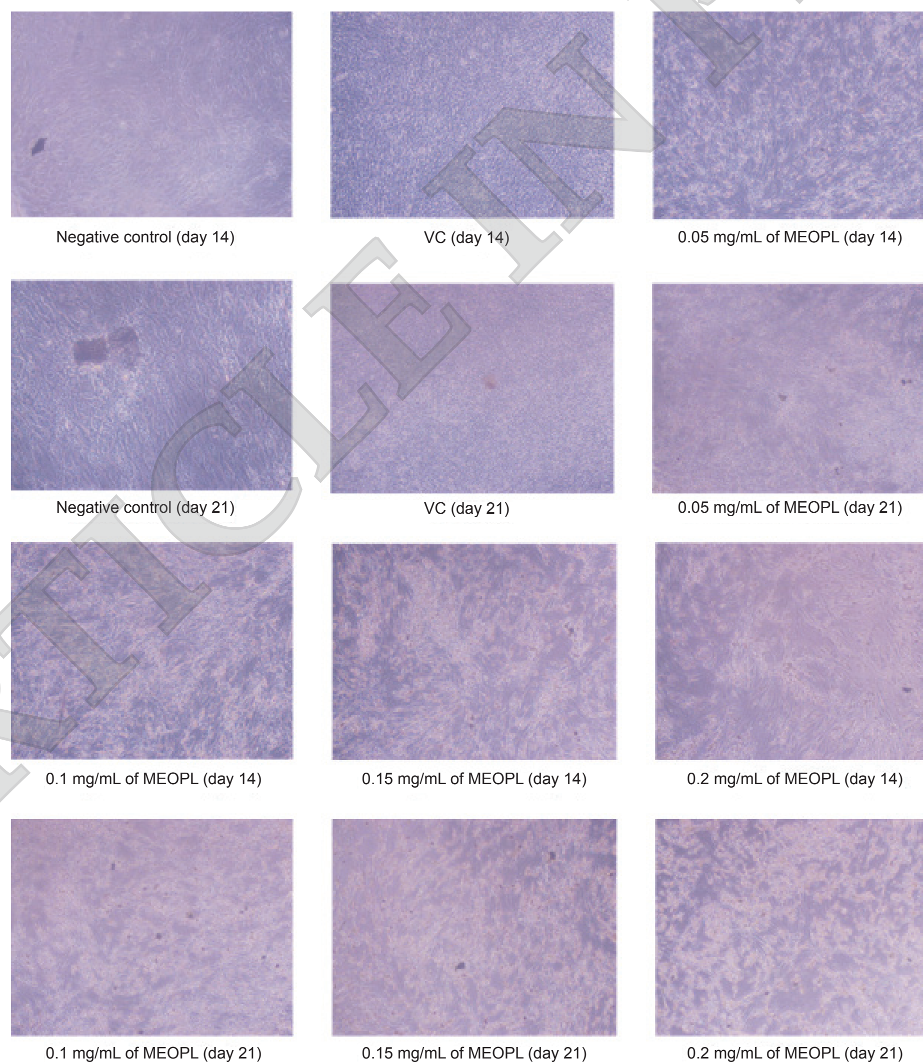

Figure 6. Phosphate deposition visualised by Von Kossa staining in MC3T3-E1 cells treated with MEOPL (0.05-0.20 mg/mL) and vitamin C (VC) (0.05 mg/mL) for day 14 and 21. Representative images show progressive mineralised nodule formation and phosphate accumulation, with more intense staining at higher MEOPL concentrations and at day 21.

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activity, collagen synthesis, calcium and phosphate deposition and nodule formation (*Figure 7*).

RUNX2 is a vital transcription factor that differentiates bone marrow stromal cells into osteoblasts and regulates their maturation, serving as a key marker for osteogenic differentiation (Sun et al., 2018; Yao et al., 2020). The marked upregulation of RUNX2 expression with MEOPL treatment, especially at higher concentrations, suggests that MEOPL effectively activates the genetic pathways critical for osteoblast differentiation and bone formation. This increase in RUNX2 expression aligns with enhanced ALP activity, collagen production, calcium and phosphate deposition, and nodule formation, collectively highlighting MEOPL's strong osteogenic potential (*Figure 7*).

The robust expression of RUNX2 with MEOPL treatment, compared to vitamin C, likely stems from MEOPL's rich flavonoid and phenolic content, which contributes to its antioxidant capacity and further supports osteoblast function (Jeong et al., 2021; Liu et al., 2021; Yao et al., 2020). Thus, MEOPL modulates osteoblast differentiation and mineralisation by upregulating RUNX2, reinforcing its potential as a natural therapeutic agent for osteoporosis and bone tissue engineering.

Comparative Analysis

The comparative analysis of the osteogenic potential of MEOPL and vitamin C on MC3T3-E1 osteoblast cells highlighted MEOPL's superior efficacy across key parameters. MEOPL not only maintained and enhanced cell viability, particularly at lower concentrations, but also demonstrated a stronger osteoinductive effect than vitamin C. This was reflected in its higher alkaline phosphatase activity, increased collagen synthesis, more significant nodule formation and greater calcium and phosphate mineralisation. Additionally, MEOPL showed a greater upregulation of the *RUNX2* gene, crucial for bone development. These findings, summarised in *Table 1*, confirm MEOPL's potent osteogenic properties, making it a promising candidate for osteoporosis management and bone tissue engineering. The table provides a concise comparison of MEOPL and vitamin C across various osteogenic parameters, highlighting their impacts on osteoblast differentiation and activity.

When integrated across all assays, MEOPL demonstrated a broader and more sustained osteogenic response compared to vitamin C and showed efficacy that is comparable to, and in some cases exceeds, other phytochemicals reported in the literature. For example, flavonoid-rich compounds such as astragalin, rutin and icariin have been shown to enhance ALP activity, collagen production and mineralisation in osteoblast models (Jeong et al., 2021; Liu et al., 2021; Yao et al., 2020), yet MEOPL achieved similar or stronger outcomes across multiple markers within a single extract. Furthermore, the performance of MEOPL aligns

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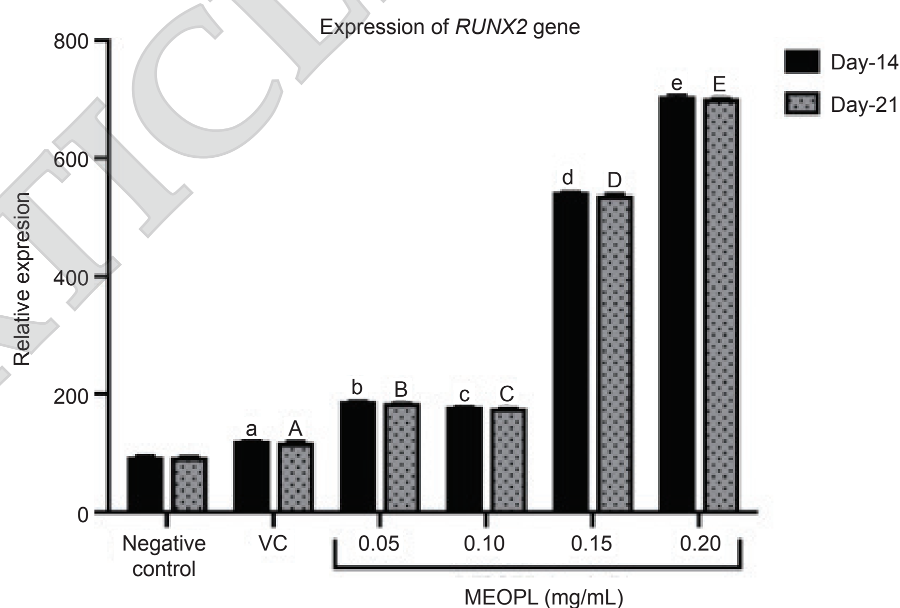

Note: mRNA levels were measured by quantitative PCR and normalised to GAPDH. Data are shown as mean ± SEM (n=5). Groups with different letters are statistically significant (ANOVA, Tukey's, $p < 0.05$). Comparisons between days 14 and 21 within the same group used two-way ANOVA, Šidák (*p<0.05).

Figure 7. Effects of MEOPL and vitamin C (VC) on RUNX2 mRNA expression in MC3T3-E1 cells after day 14 and 21.

TABLE 1. COMPARATIVE EFFICACY OF MEOPL AND VITAMIN C ON OSTEOBLAST DIFFERENTIATION AND ACTIVITY IN MC3T3-E1 CELLS

Parameter	MEOPL	VC
Cell viability	Enhanced across all concentrations	Reduced at higher doses
ALP activity	Higher, especially at higher concentrations and later stages	Less pronounced
Collagen synthesis	Stronger, particularly at the highest concentration	Less effective
Nodule formation	More pronounced, especially at higher concentrations and later stages	Initially higher, but later surpassed by MEOPL
Calcium deposition	Significantly higher, especially at higher concentrations	Initially higher, but later surpassed by MEOPL
Phosphate mineralisation	More substantial, especially at the highest concentration	Less substantial

Note: ALP - alkaline phosphatase; MEOPL - methanolic extract of oil palm leaves; VC - vitamin C.

with the effects of conventional osteogenic agents such as alendronate and curcumin, both of which are widely recognised for their bone-protective roles in osteoporosis treatment (Dong et al., 2018). This integrative comparison highlights MEOPL as not only an effective natural alternative but also a promising candidate with translational potential comparable to established pharmacological interventions.

It is important to note, however, that the present study is limited by its use of the murine MC3T3-E1 pre-osteoblast model. While this model is well established and widely applied in osteogenesis research, it does not fully replicate the complex physiological environment of human bone, where systemic hormones, osteoclast-osteoblast interactions and biomechanical loading play crucial roles (Sun et al., 2018; Yao et al., 2020). As such, although MEOPL exhibited strong osteogenic effects *in vitro*, further validation through *in vivo* studies in animal models, followed by clinical investigations, will be necessary to confirm its therapeutic potential and translational relevance in osteoporosis management.

CONCLUSION

The study highlights the osteogenic potential of the MEOPL. The results demonstrate that MEOPL enhances cell survival in murine MC3T3-E1 pre-osteoblast cells in a dose-dependent manner, outperforming both vitamin C and its ethanolic counterpart. MEOPL also significantly promotes the differentiation and mineralisation of MC3T3-E1 cells, with a notable upregulation of the osteoblast differentiation marker RUNX2 compared to vitamin C. Collectively, these findings indicate that MEOPL holds considerable promise as a natural osteogenic agent, with potential applications in promoting osteoblast activity and regeneration, offering a novel, plant-based therapeutic approach for osteoporosis management. Future studies should extend these findings to *in vivo* animal models to evaluate systemic safety, bioavailability, and long-term efficacy, followed by well-designed clinical trials to establish its therapeutic relevance in human osteoporosis.

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