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In Vitro Evaluation of Alpha-asarone Induced Osteogenic Differentiation and Cytotoxicity in MC3T3-E1 Cells

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ABSTRACT

Osteoporosis affects over 200 million people worldwide, with its prevalence expected to rise due to ageing populations. Side effects associated with long-term use of synthetic treatments have driven interest in natural alternatives. Alpha-asarone, a natural metabolite found in several therapeutic plants, was identified as a major metabolite in *Piper sarmentosum* Roxb. (kaduk), a local plant known for its bone-protective properties. While Alpha-asarone is known for its neuroprotective and anti-inflammatory properties, its osteogenic role remains unexplored. This study aimed to assess the cytotoxicity of Alpha-asarone and its potential to induce osteogenic differentiation *in vitro* using MC3T3-E1 pre-osteoblast cells. Cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay across Alpha-asarone concentrations (0–800 μM). For osteogenic evaluation, cells were treated with 1–20 μM Alpha-asarone over 21 days. Osteogenic differentiation was assessed through ALP activity and von Kossa staining. Untreated cells served as the negative control, while cells treated with ascorbic acid (50 μg/mL) and β-glycerophosphate (10 mM) served as the positive control. Alpha-asarone exhibited a dose-dependent cytotoxic response in the cytotoxicity assay, with no cytotoxic effects at the concentrations used for osteogenic evaluation. After 21 days, von Kossa staining revealed a significant increase in mineralised matrix formation, indicated by intensified dark brown staining, with a 95.1% increase. ALP activity was also elevated, with the highest increase at 174%. Alpha-asarone at 1 μM produced the most significant enhancement in

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both mineralisation and ALP activity. Alpha-asarone showed its promise as a natural osteogenic agent by effectively and safely inducing MC3T3-E1 cell differentiation.

Keywords: Alpha-asarone, ALP activity, bone mineralisation, MC3T3-E1 cells, natural bone anabolic agent, osteogenic differentiation, osteoporosis prevention

INTRODUCTION

Osteoporosis is a skeletal disorder resulting from an imbalance involving osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells), leading to weakened and fragile bones, prone to fractures (Föger-Samwald et al., 2020). It is often termed a "silent disease" due to its asymptomatic progression (Wei et al., 2024). The global burden includes approximately nine million fractures annually, affecting over 200 million people (Akkawi & Zmerly, 2018; Pisani et al., 2016). In Malaysia, the number of individuals aged 60 or older is projected to grow from 1.4 million in 2000 to 3.3 million in 2050, with 24.1% prevalence in central region postmenopausal women (Lim et al., 2005; Mafauzy, 2000).

The therapeutic goal in bone-related conditions ideally involves slowing down bone loss and promoting new bone formation. However, most current treatment approaches mainly focus on targeting bone-resorption, with limited support to actively promote new bone formation. Consequently, this makes the effective therapies that stimulate new bone formation remain limited (An et al., 2016; Lippuner, 2012). The current established synthetic medicines such as bisphosphonates, estrogen, estrogen receptor antagonists, calcitonin, and teriparatide (Khosla & Hofbauer, 2017; Zhu & March, 2022), although effective in slowing bone loss, present side-effects such as myalgia, osteonecrosis, atypical femur fractures, musculoskeletal aches, adverse gastrointestinal issues, headache, nausea, leg cramps, hot flashes and increased risk of blood strokes (Skjødt et al., 2019; Zhu & March, 2022). Therefore, the therapeutic need remains for agents that actively promote bone formation (An et al., 2016; Lippuner, 2012).

One promising direction in bone-therapy is promoting osteoblast differentiation (Zeng et al., 2020). This multi-stage differentiation process involving proliferation, matrix maturation, and mineralisation is responsible for the development of stem cells into osteogenic progenitors, and, eventually, into mature osteoblasts, essential for bone formation, remodelling, and mineral homeostasis (Carluccio et al., 2020; Yazid et al., 2010). It can be evaluated using several established methods, such as alkaline phosphatase (ALP) activity; an osteoblast marker (Meesuk et al., 2022; Trivedi et al., 2020), and von Kossa staining, which assesses mineralised matrix formation (Matta et al., 2019; Shima et al., 2015).

Natural compounds are increasingly gaining interest and demand in bone-related therapies (Marcucci et al., 2023), due to their reduced incidence of adverse side effects

compared to synthetic drugs, along with their ability to enhance bone health and support long-term use (Qu et al., 2024). Among them, phenylpropanoids such as Alpha-asarone emerge as potential osteogenic agents. It has been reported to exhibit a wide range of promising therapeutic effects, including protection against neurological, liver, and kidney damage, as well as pain relief, antidepressant effects, anticancer properties, antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory activities (Das et al., 2019; Elhoby et al., 2024; Uebel et al., 2021). Alpha-asarone was reported as the major metabolite found in both aqueous and ethanolic extracts of the local plant *Piper sarmentosum* Roxb. "kaduk" (Abidin et al., 2023; Ariffin et al., 2020); traditionally used to treat joint pain, fever, and cough (Sun et al., 2020). Studies have reported its potential in enhancing bone structure and strength, as well as preventing bone loss in osteoporotic rat models (Asri et al., 2016; Asri et al., 2020; Nirwana et al., 2012). Therefore, in addition to the various pharmacological effects of Alpha-asarone, its osteogenic role remains largely unexplored and requires further investigation. This study aimed to evaluate the cytotoxicity of Alpha-asarone, as well as its osteogenic differentiation potential, using the MC3T3-E1 pre-osteoblast cell line.

MATERIALS AND METHODS

MC3T3-E1 Cell Culture

The MC3T3-E1 cell line (ATCC No: CRL-2596™) was used in this study. The cells were cultured in Alpha minimum essential medium (α-MEM; Gibco, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 2% penicillin/streptomycin (Gibco, USA), and 1 mM sodium pyruvate (Gibco, USA). The cell incubation was carried out at 37°C in a humidified environment with 5% CO_2 (Abidin et al., 2021).

Cytotoxicity of Alpha-asarone on MC3T3-E1 Cells

The cytotoxic analysis was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Using 96-well plates, 5 × 10^3 cells/well were seeded and incubated for 24 hours to allow cell attachment at 37°C in a humidified environment with 5% CO_2. The cells were then treated with Alpha-asarone at concentrations ranging from 0 to 800 µM for 24, 48, and 72 hours.

Alpha-asarone (Tokyo Chemical Industry Co., Ltd, Japan), with a supplier-certified purity of >98%, was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Japan) to prepare a stock solution, which was protected from light and stored at -20°C. Working dilutions were freshly prepared prior to each experiment, with the final DMSO concentration maintained at 0.1% (Tian et al., 2022).

On the day of measurement, the old medium was discarded and replaced with fresh medium containing 10 µL MTT (5 mg/ml; Sigma Aldrich, USA) per well, followed by incubation for 4 hours. The medium was then discarded, and 100 µL of DMSO was

added to dissolve the formed formazan salts. Absorbance was measured at 570 nm using a microplate reader (Biotek Instruments Inc., CA, USA) (Wang et al., 2015; Yazid et al., 2022). Cell viability of treated cells was normalised to that of untreated cells and reported as a percentage. The half-maximal inhibitory concentration (IC_{50}) was determined using GraphPad Prism software.

Induction of Osteogenic Differentiation using Alpha-asarone

Approximately 3 × 10^3 cells/well were cultured in 96-well plates, and various concentrations of Alpha-asarone (1–20 μM) were used to induce osteogenic differentiation. The osteogenic concentration range of Alpha-asarone (1–20 μM) was selected based on MTT-assay results confirming the absence of cytotoxicity within this range and in accordance with concentrations commonly reported for phenylpropanoid compounds in *in vitro* osteogenic studies; preliminary screening further indicated that these concentrations-maintained cell viability while eliciting measurable osteogenic response. The medium supplemented with 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate was used as a positive control. Meanwhile, cells cultured in the complete culture medium only were used as a negative control. The cell incubation was carried out in a humidified atmosphere containing 5% CO_2, at a temperature of 37°C, for a period of 21 days. The medium was changed every 3 days (Abidin et al., 2021).

Alkaline Phosphatase (ALP) Enzymatic Assay

ALP assay was conducted on days 1, 7, 14, and 21. Cells were washed with phosphate buffer saline (PBS; Gibco, USA) and lysed using 0.1% Triton X-100 (Sigma Aldrich, USA). The amount of total protein was determined by adding the cells with Bradford reagent for 5 minutes at room temperature, followed by measuring absorbance at 595 nm. ALP enzyme activity was measured by incubating the cells for 30 mins at 37°C in alkaline conditions (pH 10) with 0.1 M sodium bicarbonate–sodium carbonate buffer, 2 mM MgSO4, and 6 mM p-nitrophenyl phosphate (pNPP), i.e., the enzyme substrate. The reaction was stopped with the addition of 1.5 M NaOH, and absorbance was measured at 405nm using a microplate reader (Biotek Instruments Inc., CA, USA). ALP activity was measured as specific activity, with unit activity per total protein (mg). The activity was normalised to untreated cells and expressed as a percentage (Abidin et al., 2023; Hadzir et al., 2014; Yazid et al., 2010).

Mineralisation Analysis using Von Kossa Staining

On the day of analysis, cells were washed using PBS (Gibco, USA) and fixed using 10% (v/v) formalin (Sigma Aldrich, USA) prepared in PBS for 30 min. Following washing with

deionised water thrice and staining the cells using freshly prepared 5% (v/v) silver nitrate solution (Sigma Aldrich, USA) for 30 min under ultraviolet light, followed by washing with deionised water thrice. After which addition of fresh 5% (v/v) sodium carbonate in 25% (v/v) formalin took place for 5 min, followed by 5% (v/v) sodium thiosulfate (Sigma Aldrich, USA) for 2 min to remove any unreacted silver nitrate. The cells were washed thrice and air-dried, to be viewed under the light microscopy (Olympus Scientific Solutions, Japan), in which dark brown or black-stained cells represent mineralisation. ImageJ software was used for mineralisation quantification (Abidin et al., 2021; Abidin et al., 2023; Hadzir et al., 2014). Mineralisation quantification was performed using ImageJ software with identical threshold settings applied to all images within each experiment set. Images were acquired under consistent magnification and lighting conditions, and threshold parameters were defined based on control samples and uniformly applied across all treatment groups to minimise observer bias.

Statistical Analysis

All the experiments were conducted in triplicate (n=3) and represented as the mean ± standard deviation. Statistical analysis was conducted using GraphPad Prism software using a two-way analysis of variance (ANOVA) test followed by Dunnett's post hoc test. The result was considered statistically significant at a p-value of <0.05. No statistical outliers were excluded from the analysis. Given the exploratory nature of this *in vitro* study, formal a priori power calculations were not performed; however, reproducibility was supported through independent experimental repeats with consistent effect sizes observed across multiple time points.

RESULTS

The cytotoxicity analysis of Alpha-asarone on MC3T3-E1 cells showed a dose-dependent manner, represented by reduced cell viability percentages as the concentration increases (Figure 1). The percentage of cell viability exhibited a significant difference ($p<0.05$) compared to the negative control (0 µM), whereby Alpha-asarone showed the lowest viability percentage after 72 hours of treatment at 48.1% (600 µM) and 19.5% (800 µM). The IC_{50} values of Alpha-asarone on MC3T3-E1 cells were only determined after 48 and 72 hours of treatment with 688.7 µM (143.43 µg/mL) and 447.8 µM (93.26 µg/mL), respectively.

During differentiation, ALP enzymatic activity was measured, as depicted in Figure 2, showing the ALP activity normalised to the negative control (untreated cells), set as 100% vs the differentiation days. ALP activity exhibited a significant increment ($p<0.05$) in the treated cells compared to the control group throughout the 21 days of incubation with Alpha-asarone. The highest ALP activity was observed on day 14 at 1 µM (174%),

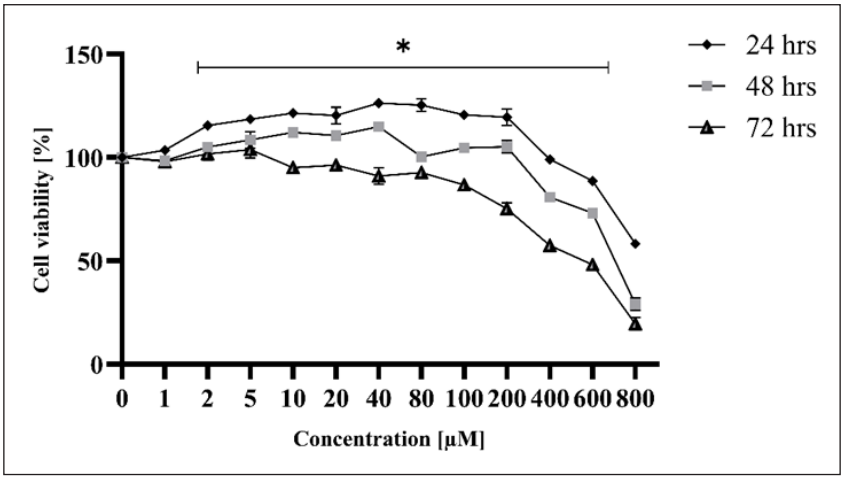


Figure 1. The MC3T3-E1 cell viability (%) during the treatment with Alpha-asarone in a range of concentrations (0–800 µM) for 24, 48 and 72 hours, normalised to untreated cells. The (*) denotes the significant difference compared to untreated cells

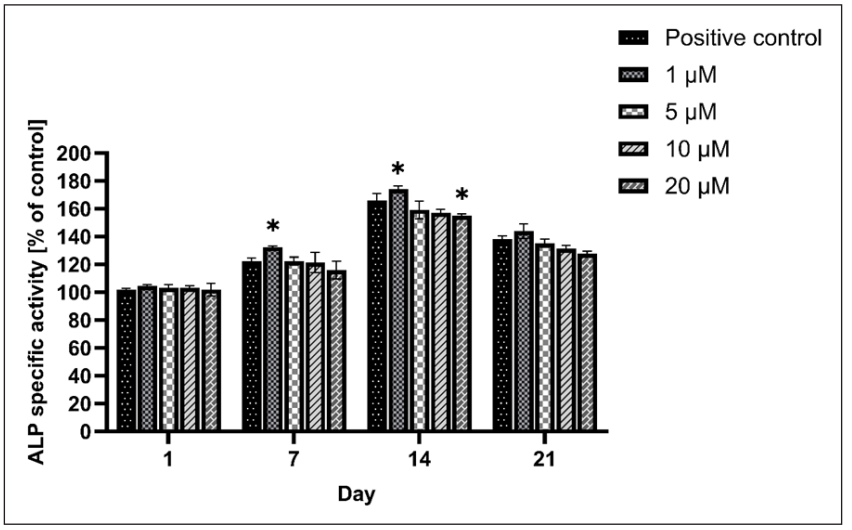
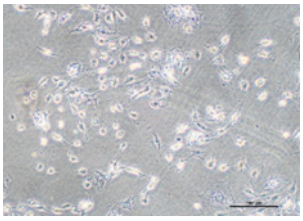
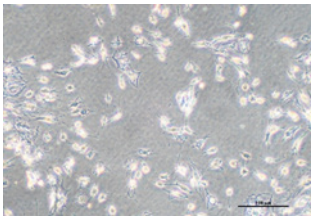
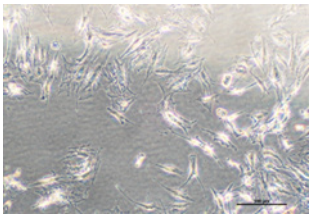
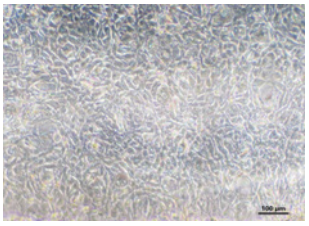
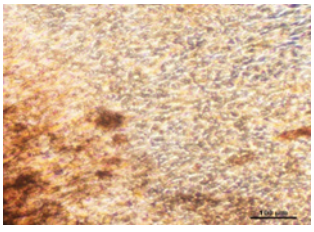
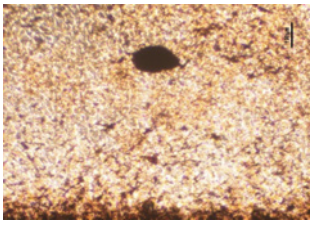


Figure 2. The ALP specific activity (%) of MC3T3-E1 cells treated with Alpha-asarone for 21 days, normalised to untreated cells. The (*) denotes the significant difference compared to the positive control

followed by 5 µM (159%), 10 µM (157%), and 20 µM (154.9%), indicating a concentration-dependent enhancement of early osteogenic differentiation, with maximal effects at lower concentrations.

Von Kossa staining was used to evaluate the effect of Alpha-asarone on the mineralised matrix formation (late stage of differentiation) during the 21-day treatment period, as depicted in Figure 3A and Figure 3B. Figure 3A shows the representative microscopic

A.

Day	Negative control	Positive control	Treated cell
1			
21			

B.

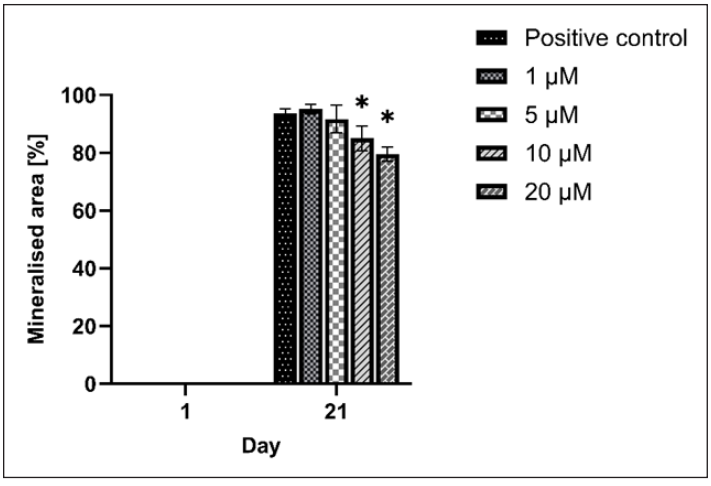


Figure 3. Mineralisation analysis of MC3T3-E1 cells treated with Alpha-asarone for 21 days. Figure (3A) shows the representative microscopic visualisation of stained MC3T3-E1 cells following von Kossa staining. Figure 3B shows the percentage of quantified mineralisation. The (*) denotes the significant difference compared to the positive control

visualisation of stained MC3T3-E1 cells, and Figure 3B shows the percentage of quantified mineralisation. The treatment with 1 µM Alpha-asarone exhibited the highest mineralisation after 21 days (95.1%), followed by 5 µM (91.7%), 10 µM (85%), and 20 µM (79.6%). Meanwhile, 5 µM showed comparable effects ($p>0.05$), 10 µM and 20 µM exhibited lower mineralisation on day 21, compared to the positive control. Meanwhile, the negative control showed no mineralised matrix formation up to day 21.

Morphological assessment further supported these findings, as Alpha-asarone -treated MC3T3-E1 cells exhibited increased cell density, enhanced extracellular matrix deposition, and the formation of mineralised nodules, particularly at lower concentrations. These morphological changes were consistent with the observed increase in ALP activity and von Kossa staining intensity.

DISCUSSION

Prior to differentiation analysis, the cytotoxicity of Alpha-asarone on MC3T3-E1 cells was assessed (Figure 1). MC3T3-E1 cells were employed due to their well-characterised and highly producible osteogenic differentiation, making them an accepted and reliable model for initial mechanistic and safety screening before any subsequent validation in human primary osteoblast and *in vivo* systems. The cytotoxicity analysis findings suggested that Alpha-asarone exhibited a weak and moderate cytotoxicity on the cells at 48 and 72 hours, respectively (Fithrotunnisa et al., 2020). The findings also showed that Alpha-asarone is deemed non-cytotoxic for the cells at the various concentrations below the reported $IC_{50.}$ The IC_{50} values obtained (447.8–688.7 µM) are substantially higher than the concentrations used for osteogenic differentiation (1–20 µM), indicating a wide therapeutic safety margin *in vitro*. While direct extrapolation to *in vivo* plasma concentrations is limited, these findings demonstrate that osteogenic effects occur well below cytotoxic levels. The study is positioned as a proof-of-concept, with pharmacokinetic and bioavailability studies required for *in vivo* relevance. Accordingly, the present work should be interpreted as an exploratory study, providing foundational evidence to guide subsequent statistically powered investigations in human-relevant and *in vivo* models. The wide concentration range (0–800 µM) was selected to comprehensively characterise the cytotoxic profile of Alpha-asarone, exhibiting low to moderate cytotoxicity across different cell types at relatively high concentrations. Therefore, extending the range ensured capture of both non-toxic and cytotoxic thresholds, allowing a clear distinction between safe osteogenic concentrations and cytotoxic levels.

Alkaline phosphatase (ALP) enzyme is one of the most well-known markers of osteoblastic differentiation. When osteoblast cells undergo the differentiation process to perform their specialised function of bone formation, ALP enzyme activity is elevated, indicating an ongoing and active bone formation taking place, given the fact that it is secreted by active osteoblast cells (Trivedi et al., 2020). In this study, Alpha-asarone at all concentrations, and the positive control induced a significant time-dependent increase in ALP activity of MC3T3-E1 cells ($p<0.05$), shown from day 7 to 14, compared to the untreated cells, which indicates osteogenic differentiation induction (Figure 2).

Alpha-asarone at 1 µM consistently resulted in the highest ALP activity throughout the differentiation period, with the highest observed after 14 days compared to the negative

control. In addition, ALP activity for 1 µM Alpha-asarone was significantly higher ($p<0.05$) than the positive control on days 7 and 14, indicating its notable potential to efficiently enhance ALP activity, crucial for bone differentiation and formation. Meanwhile, 5 µM and 10 µM showed comparable ALP activity enhancement with no significant difference ($p>0.05$), while 20 µM exhibited lower ALP activity on day 14 compared to the positive control. This signifies that at low concentrations, particularly 1 µM, Alpha-asarone was effective in regulating ALP activity, demonstrating its potency and potential to show the highest biological effect using small doses.

ALP enzyme activity of all concentrations of Alpha-asarone showed a decline after day 14, as well as the positive control. This decline is linked to the mineralisation of MC3T3-E1 cells; as besides ALP being an indicator of osteoblastic differentiation, it is also crucial for the last stage of differentiation, i.e., mineralisation, due to its function in providing inorganic phosphate (Yazid et al., 2018; Vimalraj, 2020), which together with calcium ions form hydroxyapatite crystals; the hard and strong tissue leading to bone formation (Boonrungsiman et al., 2012). This finding is consistent with other previous findings, in which MC3T3-E1 cells exhibited a decreasing trend in ALP activity, corresponding with mineralisation (Yazid et al., 2022). Another study has also confirmed these findings on differentiated MC3T3-E1 cells, whereby ALP activity decreased after 21 days (Kwon et al., 2014).

The evaluation of mineralisation is important as it represents the last stage of differentiation, when osteoblast cells lay down the calcified mineralised matrix, crucial for bone strength, structure, and function (Bourne et al., 2021). Hence, evaluating the effect of Alpha-asarone on MC3T3-E1 cell mineralisation provides valuable insights into its osteogenic potential. Upon staining with silver nitrate, silver is deposited in place of calcium ions reduced by light, resulting in the formation of metallic silver mineralised nodules, noted by osteoblasts stained in dark brown or black (Yazid et al., 2010). As observed in microscopic visualisation (Figure 3A) and quantification of mineralisation percentage (Figure 3B), no mineralisation was observed at day 1 in both the treatment and control groups. The formation of mineralised matrix in all Alpha-asarone-treated cells and the positive control was evident by day 21, signifying osteogenic differentiation induction. While von Kossa was employed as a well-established method to visualise phosphate-containing mineralised nodules and was supported by complementary ALP activity data, future studies will incorporate calcium-specific assays such as Alizarin Red S staining to further validate and extend the assessment of osteogenic mineralisation.

These findings suggest that Alpha-asarone enhances osteogenic mineralisation marked by the dark-brown stained cells, with 1 µM being the most effective, further supporting the findings of ALP enzymatic activity. Meanwhile, negative control (untreated cells) showed no staining of MC3T3-E1 cells, due to the absence of necessary differentiation factors.

Quantitative analysis of the stained areas further confirmed these findings and indicated that Alpha-asarone enhances bone matrix formation, reflecting its osteogenic potential.

The findings in this study showed the interconnected relationship between ALP activity and mineralisation in osteogenic differentiation. Both ALP activity and mineralisation analyses demonstrated that 1 µM Alpha-asarone optimally enhanced osteogenic differentiation, as evidenced by the highest ALP levels and subsequent mineralisation. As previously reported, a study on MC3T3-E1 cells treated with *Piper sarmentosum* extract demonstrated that the concentration exhibiting the highest ALP activity also resulted in the highest mineralisation (Abidin et al., 2021).

The ability of Alpha-asarone to induce the differentiation of MC3T3-E1 cells could be attributed to its pharmacological and therapeutic effects. In a previous study, Alpha-asarone exhibited potent antioxidant activity, including 2,2-Diphenyl-1-picrylhydrazyl radical scavenging, metal chelation, and lipid peroxidation inhibition, suggesting its potential as a powerful agent for combating oxidative stress. The presence of reactive oxygen species has been found to promote apoptosis of bone formation-associated cells, such as osteocytes and osteoblasts, growth and differentiation (Lu et al., 2017), favouring bone resorption and ultimately causing bone loss (Marcucci et al., 2023). Hence, the possession of strong antioxidant activity provides a favourable environment for bone formation and differentiation. Overall, the present study demonstrates that Alpha-asarone promotes osteogenic differentiation of MC3T3-E1 cells, as evidenced by increased ALP activity and mineralised matrix formation. While these findings provide robust preliminary evidence of osteogenic activity, their generalisation beyond the MC3T3-E1 murine pre-osteoblast model should be interpreted with caution, as this cell line primarily serves as a highly reproducible and well-characterised platform for early-stage screening. Consequently, future studies employing human primary osteoblast and relevant *in vivo* bone models will be essential to confirm biological relevance and translational potential.

Alpha-asarone is known to exhibit neuroactive effects at higher systemic doses, which warrants consideration when evaluating its translational potential. Importantly, the concentrations employed in this study (1–20 µM) were substantially lower than levels reported to obtain neurotoxic or behavioural effects *in vivo*. Furthermore, the present work focusses on cellular osteogenic responses under controlled *in vitro* conditions rather than systemic exposure. Nevertheless, comprehensive safety profiling, including neurotoxicity and organ-specific assessments, will be important in future *in vivo* studies prior to clinical translation.

To our knowledge, this is the first study to evaluate the effect of Alpha-asarone on ALP activity and mineralisation. Moreover, this study explored a natural compound, addressing the growing interest in alternative therapies for bone-related conditions. It also evaluated the compound's potential to promote osteogenic differentiation and bone formation,

representing an important therapeutic avenue. This finding is derived from a murine pre-osteoblast *in vitro* model and cannot be directly extrapolated to human therapy. Accordingly, the present study is a proof-of-concept investigation aimed at identifying the osteogenic activity of Alpha-asarone under controlled experimental conditions. While MC3T3-E1 cells provide a highly reproducible and well-characterised platform for early-stage screening, interspecies differences in cellular responses, metabolism, and pharmacokinetics necessitate cautious interpretation. Therefore, future studies incorporating human primary osteoblasts, pharmacokinetic and bioavailability profiling, and validation in relevant *in vivo* osteoporosis models will be essential to bridge the translational gap and establish clinical relevance.

Specific experimental refinements that would strengthen this work include validation in human primary osteoblasts, incorporation of calcium-specific mineralisation assays, molecular pathway analyses, and *in vivo* efficacy and safety studies. These refinements are planned for future investigations to build upon the current findings. The potential ethical and regulatory considerations must also be acknowledged when proposing Alpha-asarone as a therapeutic agent. These include comprehensive safety profiling, neurotoxicity evaluation, and assessment of long-term exposure risks. Despite being a naturally occurring compound, Alpha-asarone would still require full preclinical toxicology studies and adherence to regulatory compliance frameworks before any therapeutic application can be considered.

However, as preliminary research, these findings serve as a foundation for future studies. Therefore, as a future recommendation, these findings may be implied and used in other models, such as human primary cells and further *in vivo* models, for clinical validity. In addition, elucidation of the molecular mechanisms underlying its osteogenic effects is important. To minimise variability, Alpha-asarone was sourced from a single commercial supplier with certified purity, and fresh stock solutions were prepared using standardised protocols. Nevertheless, batch-to-batch variation can act as a potential limitation. Future studies should incorporate analytical verification methods such as high-performance liquid chromatography (HPLC) to confirm compound consistency and strengthen reproducibility.

FUTURE RECOMMENDATIONS

Future studies should incorporate additional assays to strengthen the claims of bone anabolic potential of Alpha-asarone. Specifically, a calcium-specific mineralisation assay such as Alizarin Red S staining would provide complementary validation to von Kossa staining by directly quantifying calcium deposition. Furthermore, osteogenic gene expression profiling of key markers, including *RUNX2*, *COL1A1*, and osteocalcin (*OCN*), would demonstrate the transcriptional regulation underlying the observed differentiation. Protein-level validation through Western blotting or immunocytochemistry, together with mechanistic pathway analyses, would further clarify the molecular basis of Alpha-asarone ’s osteogenic effects. Beyond *in vitro* assays, *in vivo* bone formation studies coupled with

micro-computed tomography (micro-CT) analyses are recommended to substantiate the anabolic potential and provide translational relevance. These approaches will collectively strengthen the evidence base and support the clinical development of Alpha-asarone as a natural bone anabolic agent.

CONCLUSION

In conclusion, Alpha-asarone demonstrated promising potential to promote osteoblastic differentiation of MC3T3-E1 cells, as evidenced by significant increases in ALP activity and mineralised matrix formation compared with the untreated and positive control groups. Alpha-asarone at a concentration of 1 μM significantly exhibited the highest osteogenic effect without inducing cytotoxicity. These findings suggest Alpha-asarone may serve as a safe and effective natural agent for enhancing osteogenic differentiation.

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