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Original Research Article (Experimental)

Exploring molecular mechanism of bone-forming capacity of *Eurycoma longifolia*: Evidence of enhanced expression of bone-related biomarkers

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ABSTRACT

Background: Among the numerous well-documented medicinal herbs, *Eurycoma longifolia* (*EL*) has gained remarkable recognition due to its promising efficacy of stimulating bone formation in androgen-deficient osteoporosis. Though numerous animal studies have explored the bone-forming capacity of *EL*, the exact mechanism was yet to be explored.
Objective(s): The present study was aimed to investigate the mechanism of bone-forming capacity of *EL* using MC3T3-E1 as an *in vitro* osteoblastic model.
Materials and methods: The cell differentiation capacity of *EL* was investigated by evaluating cell growth, alkaline phosphatase (ALP) activity, collagen deposition and mineralization. Taken together, time-mannered expression of bone-related mediators which include bone morphogenic protein-2 (BMP-2), ALP, runt-related transcription factor-2 (Runx-2), osteocalcin (OCN), type I collagen, osteopontin (OPN), transforming growth factor-β1 (TGF-β1) and androgen receptor (AR) were measured to comprehend bone-forming mechanism of *EL*.
Results: Results demonstrated a superior cell differentiation efficacy of *EL* (particularly at a dose of 25 μg/mL) that was evidenced by dramatically increased cell growth, higher ALP activity, collagen deposition and mineralization compared to the testosterone. Results analysis of the bone-related protein biomarkers indicated that the expression of these mediators was well-regulated in *EL*-treated cell cultures compared to the control groups. These findings revealed potential molecular mechanism of *EL* for the prevention and treatment of male osteoporosis.
Conclusion: The resulting data suggested that *EL* exhibited superior efficacy in stimulating bone formation *via* up-regulating the expression of various mitogenic proteins and thus can be considered as a potential natural alternative therapy for the treatment of osteoporosis.

1. Introduction

Osteoporosis, one of the prevalent metabolic bone disorders in aging populations, is mainly associated with abnormal decrease in bone mass, malfunctioning bone tissues and greater risk of fracture [1,2]. Osteoporosis occurs in both sexes, but women are more prone to serious complications of osteoporosis (approximately 80%) [3]. There are several critical genetic and environmental factors involved in osteoporosis. Aging, menopause (in women) and low testosterone levels (in men) are primarily associated with the development of osteoporosis. The consequences of this disease are always associated with loss of independence, high morbidity and financial cost, and even excess mortality, which can occur at any age and in any racial or ethnic group [4]. Although osteoporosis can be partially prevented and treated today, its pathophysiology is not completely understood, yet. The key to this most common metabolic bone disease is to restore and maintain balance between bone formation and resorption. Hormone replacement therapy (HRT), selective androgen receptor modulators (SARMs), bisphosphonates

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(BPH) and calcitonin are the most commonly employed anti-osteoporotic therapies. However, due to multiple side effects associated with the use of these agents, their therapeutic feasibility and clinical applicability are limited.

For centuries, plant-derived materials and various compounds isolated from natural sources have gained outstanding recognition in treating bone-related disorders across the globe. Their therapeutic feasibility, efficacy and low side effects are well documented, making them suitable for long-term use compared to chemically synthesized medications [5,6]. Among the wide range of naturally-sourced herbs, *Eurycoma longifolia* (*EL*), a medicinal herb from the family of Simaroubaceae, has been reported to demonstrate potent androgen hormone stimulating properties [6]. Therefore, *EL* can be a potential therapeutic alternative to testosterone replacement therapy (TRT) for the treatment of androgen deficient osteoporosis in male [7]. *EL* has also been investigated for their bone mass enhancing and bone resorption diminishing abilities [8]. Though, the bone forming capacity of *EL* is well-studied in orchidectomised rats the exact molecular and translational mechanism has yet to be explored.

The present study was thus aimed to investigate and establish the mechanistic pathway for the proliferative and osteogenic effects of *EL* using MC3T3-E1 cells as *in vitro* osteoblastic model. Having assessed the proliferative activity of *EL* in MC3T3-E1 cells, their cell differentiation ability was evaluated in terms of cell growth, alkaline phosphatase (ALP) activity and collagen synthesis. To gain further insight into the molecular mechanism of the anti-osteoporotic effects of *EL*, a variety of bone-related protein markers which include bone morphogenic protein-2 (BMP-2), ALP activity, runt-related transcription factor-2 (Runx-2), osteocalcin (OCN), type I collagen, osteopontin (OPN), transforming growth factor-β1 (TGF-β1) and androgen receptor (AR) were evaluated in *EL*-treated MC3T3-E1 cells. It is anticipated that *EL* could regulate bone-related protein markers that are critically imperative for cell proliferation and regeneration and thus can be used as an alternative anti-osteoporotic therapy.

2. Materials and methods

2.1. Materials

Mouse calvariae origin osteoblastic cell line subclone 4 (CRL-2594) was purchased from American Type Culture Collection (ATCC) Cell Bank (Manassas, VA, USA). Cell culture reagents- Alpha modified minimal essential medium (α-MEM)), penicillin, streptomycin and fetal bovine serum were sourced from Gibco Laboratories (Grand Island, New York, USA). Ascorbic acid, β-glycerophosphate and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) dyes were purchased from Sigma—Aldrich, USA. *Eurycoma longifolia* Jack (*EL*) was sourced from Faculty of Pharmacy, University Sains Malaysia (Malaysia). Enzyme-linked immunosorbent assay (ELISA) kits for the expression of ALP, Runx-2, AR, OPN, OCN, BMP-2, collagen type 1, and TGF-β were purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). All other chemicals used were of analytical grade and were sourced from pharmacology and cell culture laboratories of Universiti Kebangsaan Malaysia (Malaysia).

2.2. Standardized extract of EL

In this study, aqueous extract of *EL* was prepared from standardized dried root powder of *EL* (Sample: TAF 273) (batch No: 20130627TAF273) using a patented high pressure extraction process (US 7,132,117 B2). Briefly, the dried root powder of *EL* was pulverized and boiled in water, followed by removal of debris by centrifugation process. For further refining, the resulting extract was then subjected to reverse-phase high performance liquid chromatography (RP-HPLC) and size-exclusion chromatography. RP-HPLC and size-exclusion analyses revealed several bioactive components which include proteins (30.75%), eurypeptide (21%), glycosaponins (40.3%), and eurycomanone (7.46%). Further analysis of the extract showed that eurycomanone, which is the most active component of *EL* extract, could be isolated with the retention time of 17.133 min. Finally, the refined aqueous extract of *EL* was filtered through 1–4 μm and was freeze dried as a light brown powder.

2.3. Cell culturing and sub-culturing

In the current study, highly differentiating MC3T3-E1 subclone 4 (MC-4) cells were used. These cells exhibit optimum levels of expression of all the characteristic molecular markers and form extensively mineralized ECM when grown in ascorbic acid containing medium for several days. The cell culturing and sub-culturing were performed by growing active MC3T3-E1 cells in a growth medium consisting of α-MEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (antibiotic/antimycotic). The cells were then incubated in a humidified chamber (95% air and 5% CO_2) at 37 °C until they reached 80% confluence. The adhered cells were then released from the flask using an aqueous solution of 0.2% trypsin and 0.02% EDTA (ethylene-diamine tetra acetic acid). The cells were counted using a hemocytometer and were seeded at a density of 1×10^3 cells/cm^2 in 96-well plate and were cultured under the same incubation circumstances. For experiments, cells were cultured for 24 h to obtain monolayers containing α-MEM with 10% FCS to promote cell survival, division and metabolism. Prior to initiating osteogenic differentiation, cells were sparsely seeded into 96-well plate and were cultured in an incubator overnight. For cell differentiation experiments, MC3T3-E1 cells were cultured in osteogenic differentiation medium which contains additional 50 μg/L ascorbate analog which can resist hydrolysis (ascorbate-2-phosphate) to permit collagen type I fibril assembly and 10 mM β-glycerophosphate to promote mineralization of collagen fibrils. During the pre-determined experimental period, cultured cells were typically fed twice weekly for 2–3 weeks with an osteogenic differentiation medium. Cells were not used beyond passage 15. Treatments commenced 24 h later, at which time the cultures had become confluent.

2.4. Drug treatment

Prior to the drug treatment, a stock solution (100 μg/mL) of *EL* was prepared using either α-MEM or differentiation media. Later, three pre-selected [11] concentrations (5, 25, and 50 μg/mL) of *EL* were prepared from the stock solution and were sterilized using 0.2 μm syringe filter (Sartorius, Germany). MC3T3 cells were then treated with these different concentrations of *EL* and the culture media was replaced every three days throughout the experimental period.

2.5. Cell viability

Prior to the evaluation of pharmacological effects of *EL*, cell viability was assessed. For that, MC3T3-E1 cells were treated with different concentrations (5, 25, and 50 μg/mL) of *EL* for six days and assessed using MTS assay. Briefly, MC3T3-E1 cells were seeded at a density of 1×10^3 cells/well in 96-well plate and maintained in growth media for 24 h at 5% CO_2 at 37 °C. After 24 h incubation, the media was replaced with fresh medium until day 6. The cells were segregated into three groups: 1) cells treated with the fresh growth media (without adding *EL*) were termed as negative control group, 2) cells treated with 5α-dihydrotestosterone (5α-DHT)

(100 pg/mL) were used as positive control, 3) cells treated with different concentrations (5, 25, and 50 μg/mL) of *EL* were termed as treatment groups. At the end of exposure period, 20 μL of diluted MTS solution was introduced to each well and the cells were incubated at 37 °C in the dark while being covered with aluminum foil for 2 h. The absorbance was recorded at 490 nm using microplate reader.

2.6. Cell differentiation

At the end of culture period (day 21), cell growth was determined by using crystal violet staining as described previously. Briefly, the matured MC3T3-E1 cells were divided into five groups and cultured in 96-well culture plate. All the groups were induced with differentiation media throughout the experimental period. The first group was left untreated (negative control), the second group was treated with 5α-DHT (100 pg/mL) (positive control), and the third, fourth and fifth groups were treated with 5, 25 and 50 μg/mL of *EL* concentrations, respectively. Afterwards, the treated and untreated cells were stained with 0.5% crystal violet solution for 10 min. The excess stain was removed by washing with PBS thrice and the cell growth of MC3T3-E1 cells was examined, quantitatively. The stained cells were dissolved into 200 μl of 10% acetic acid and the cell growth was then measured spectrophotometrically at 590 nm.

In this study, the levels of ALP (an early differentiation marker) in all the tested groups were also estimated at the end of the differentiation period (at day 21) using ALP colorimetric assay. The assay was performed according to the manufacturer's instructions. Briefly, 1×10^3 cells from each tested group were homogenized using assay buffer. The homogenized cells were then centrifuged at 13,000 *g* for 3 min to remove insoluble cellular debris. Afterward, 80 μL of each test sample was introduced into 96-well plate and mixed with 50 μL of 5 mM *p*NPP solution. The 96-wells plates were then incubated at 25 °C in dark for 60 min followed by the addition of 20 μL of stop solution into each well, barring the background control wells. The optical density (OD) was measured using microplate reader at 405 nm.

The differentiation propensity of *EL* was also evaluated at the end of the differentiation period (at day 21) by evaluating the magnitude of ECM collagen synthesis compared to the control groups. For that, cells from different treatment groups were stained with Sirius red/fast green staining kit. Briefly, cells from each tested group were washed with PBS thrice and fixed with Kahle fixative solution for 10 min at room temperature. The fixed cells were then incubated with Sirius red/fast green stain for 30 min at room temperature and rinsed with deionized water repeatedly until the fluid became colorless. A dye extraction buffer was then added to each well and gently mixed by pipette until color was eluted from the cells. Finally, the eluted dye solution was collected and OD was recorded at 540 and 605 nm using a spectrophotometer.

2.7. Cell morphology

2.7.1. Phase contrast microscopy

The morphology of MC3T3-E1 after being treated with different concentrations (5, 25 and 50 μg/mL) of *EL* was examined using trinocular inverted phase contrast microscopy (Leica, Tokyo). The morphology of *EL*-treated cells was also compared with those treated with 100 pg/mL of 5α-DHT (positive control) and untreated (negative control) cells.

2.7.2. Transmission electron microscopy (TEM)

The morphology of treated and untreated MC3T3-E1 cells was also examined using TEM analysis. For that, cells from each sample were washed with PBS, fixed with 0.1 M sodium cacodylate buffer (pH, 7.2) containing 2% glutaraldehyde, and stored at 4 °C for one week. After that, the cells were fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at room temperature. Subsequently, the specimen were dehydrated in an ethanol series, embedded in polybed 812 resins (Polyscience, Warrington, PA) and polymerized for 72 h at 60 °C. Samples were sectioned into thin slices using Sorvall MT 5000 ultramicrotome (Sorwall, Norwalk, CT). The thin sections were then post-stained with saturated uranyl acetate and Reynold's lead citrate, each for 8 min before viewing with a JEOL 1200 EX TEM microscope (JEOL, Peabody, MA).

2.8. Bone specific protein markers

In this study, the potential biological influence of *EL* on the sequential expression and regulation of specific bone-forming biomarkers such as ALP, Runx-2, BMP-2, AR, collagen type I, OCN, OPN, and TGF-β were also investigated. These proteins are major phenotypic markers for pre-osteoblast differentiation during bone formation. During early stages of osteoblast differentiation, osteoblasts synthesize Col1α1 and other matrix proteins, followed by production of ALP and other osteoblastic biomarkers that ultimately lead to induction of extracellular matrix (ECM) calcification.

The expression of each bone-formation specific protein markers were estimated using sandwich ELISA. In this technique, the culture plates were pre-coated with antibodies specific to the protein marker. These pre-coated antibodies specifically bind to their protein markers in the sample. The biotinylated detection antibody specific for each protein markers was added to sandwich the bound protein markers and were detected as changes in color. The OD was recorded at 450 nm and the intensity of the color being directly proportional to expression concentration of each protein marker in the sample.

2.9. Data analysis

In this study, data analysis was performed using one-way analysis of variance (ANOVA) followed by student's *t*-test and Duncan's new multiple range test using SPSS version 21.0. All the experiments were performed independently thrice with quadruplicate sampling ($n = 4$). Data were presented as mean and standard deviation (mean ± S.D.). A significant difference was recognized by $^*p < 0.05$.

3. Results

3.1. Effect of EL on MC3T3-E1 cells viability

MC3T3-E1 cells treated with different concentrations of *EL* showed remarkable increase ($p < 0.05$, ANOVA) in the cell growth compared to the untreated (negative control) group (Fig. 1). The increased cell viability was more obvious in cells treated with 25 μg/mL of *EL* when compared to other *EL*-treated and control groups. It was also observed that cell viability was comparatively higher in positive control group (MC3T3-E1 cells treated with 5α-DHT) compared to the *EL*-treated and negative control groups.

3.2. Effect of EL on MC3T3-E1 cells differentiation

In the present study, cell growth, ALP activity and collagen deposition were also measured (Table 1). MC3T3-E1 cells treated with different concentrations of *EL* showed significantly higher cell growth, particularly at 25 μg/mL concentration compared to the negative control group; however, their cell growth was moderately less as compared to 5α-DHT-treated cells (Table 1). The results also

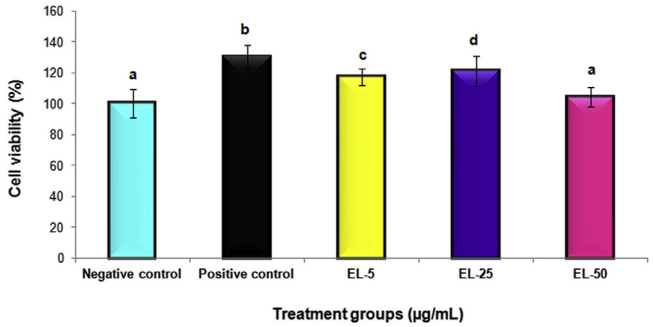

Fig. 1. Cell viability of MC3T3-E1 cells treated with different concentrations (5, 25, and 50 µg/mL) of *EL* compared to the untreated (negative control) and 5α-DHT-treated cells. Results were obtained from three independent experiments in quadruplicate ($n = 4$) and were expressed as mean ± S.D. Different letters indicate significant difference ($p < 0.05$, ANOVA).

identified a significantly ($p < 0.05$, ANOVA) higher potential of *EL* in up-regulating ALP expression compared to the control groups. The expression of ALP was particularly greater at 5 µg/mL (129.8 ± 2.47%) and 25 µg/mL (142.5 ± 3.56%) concentrations compared to the negative control (100 ± 1.64%) and positive control (123.4 ± 1.11%) groups at day 21.

Taken together, the resulting data revealed that the extent of collagen synthesis was significantly higher in *EL*-treated groups when compared to the negative and positive control groups at day 21 (Table 1). Further analysis revealed that the increase in collagen concentration was more obvious at 5 µg/mL (109.5 ± 2.52 µg/mL) and 25 µg/mL (112.2 ± 1.97 µg/mL) concentrations of *EL* when compared to the negative control (100 ± 2.02 µg/mL) and positive control (104.2 ± 1.21 µg/mL) groups (Table 1).

3.3. Morphological examination

The effects of different concentrations of *EL* on morphology and other characteristic features of bone forming cells were evaluated using phase contrast microscopy and TEM.

3.3.1. Phase contrast microscopy

The morphological assessment and relative mineral deposition in *EL*-treated MC3T3-E1 cells in contrast to the control groups were assessed on day 21 using phase contrast microscopy (Fig. 2). Prior to performing a phase contrast microscopic analysis, MC3T3-E1 cells were serially cultured and differentiated until day 21. After 21 days of culture, MC3T3-E1 cells were widely spread and formed extensive cell sheets with typical flattened morphology for both *EL*-treated and control groups. The untreated and treated cells exhibited star-like (numerous cytoplasmic extensions) morphology which indicated that MC3T3-E1 cells have shown substantial growth after 21 days. Further elucidation of the resulting micrographs revealed numerous globular masses of mineral deposits (identified as white color crystals) in the ECM of all cultured cells (Fig. 2). Moreover, in many other areas, the small globular foci appeared as coalesced masses, representing large deposits of minerals.

3.3.2. TEM analysis

In this experiment, the sub-cellular structures of the differentiated MC3T3-E1 cells were studied. The resulting monographs mainly focused on the ultra-structures of the cultured bone cells after the mineralization occurred in *EL*-treated (EL-25) cells (Fig. 3). Numerous indented nuclei with well-defined membranes were observed in these MC3T3-E1 cells at day 21. Other characteristic ultra-structures observed include Golgi apparatus (GA), vacuoles (VC), mitochondria (MC) endoplasmic reticulum (ER), collagen fibrils (CF) and mineral crystals (MC). TEM analysis of the mineralizing cultures showed abundant minerals in the MC3T3-E1 cells culture which were clearly associated with collagen fibrils (Fig. 3F). The minerals in the cell cultures produced a lattice-like fractal appearance and were clearly localized in the extracellular matrix (ECM) (Fig. 3E).

3.4. Effect of EL on bone-related protein biomarkers

3.4.1. BMP-2

Results showed a consistent increase in the levels of BMP-2 in all the tested groups from day 3 to 15; however, the increasing trend was more obvious ($p < 0.05$, ANOVA) on days 9 and 15 (Fig. 4A). Comparatively, the expression levels of BMP-2 were significantly ($^*p < 0.05$, ANOVA) higher in MC3T3-E1 cells treated with *EL*, particularly with 5 pg/mL (128 ± 8 pg/mL) and 25 pg/mL (131 ± 7 pg/mL) concentrations compared to the negative control (69 ± 4 pg/mL) and positive control (106 ± 12 pg/mL) groups throughout the experimental period. Data also showed that the levels of BMP-2 were reduced in all the tested groups at day 21; however, the concentration of this bone-related specific protein marker was comparatively higher in *EL*-treated cells (Fig. 4A).

3.4.2. ALP activity

Fig. 4B depicted that ALP activity was lowest at day 3 in all the experimental groups; however, a consistent increase in ALP activity was observed from days 3 to 15. Results demonstrated that the increase in ALP activity was significantly ($p < 0.05$, one way ANOVA) high at days 9 and 15; however, it subsequently declined on day 21 in all the experimental groups (Fig. 4B). A ccomparative analysis revealed that amongst all the tested groups, ALP activity was significantly ($p < 0.05$, ANOVA) higher in MC3T3-E1 cells treated with different *EL* concentrations, particularly at the dose of 5 µg/mL (495 ± 17 pg/mL) and 25 µg/mL (515 ± 21 pg/mL) compared to the negative control (311 ± 11 pg/mL) and positive control (434 ± 32 pg/mL) groups (Fig. 4B).

Table 1
Effects of *EL* on cell growth, ALP activity, and collagen deposition of MC3T3-E1 cells compared to the control groups at day 21.

Cell parameters	Data set	Tested groups				
		Negative control	Positive control	EL-5	EL-25	EL-50
Cell growth	Optical density	0.20 ± 0.010	0.33 ± 0.011	0.27 ± 0.009	0.29 ± 0.008	0.22 ± 0.016
	% Control	100.0 ± 2.66	164.2 ± 3.24	134.8 ± 2.11	144.6 ± 1.32	106.3 ± 5.66
ALP activity	Optical density	0.06 ± 0.001	0.07 ± 0.003	0.08 ± 0.002	0.09 ± 0.002	0.06 ± 0.003
	% Control	100.0 ± 1.64	123.4 ± 1.11	129.8 ± 2.47	142.5 ± 3.56	111.6 ± 8.69
Collagen deposition	Amount (µg/mL)	9.10 ± 0.29	9.37 ± 0.11	9.85 ± 0.18	10.10 ± 0.10	9.50 ± 0.18
	% Control	100.0 ± 2.02	104.2 ± 1.21	109.5 ± 2.52	112.2 ± 1.97	104.9 ± 1.99

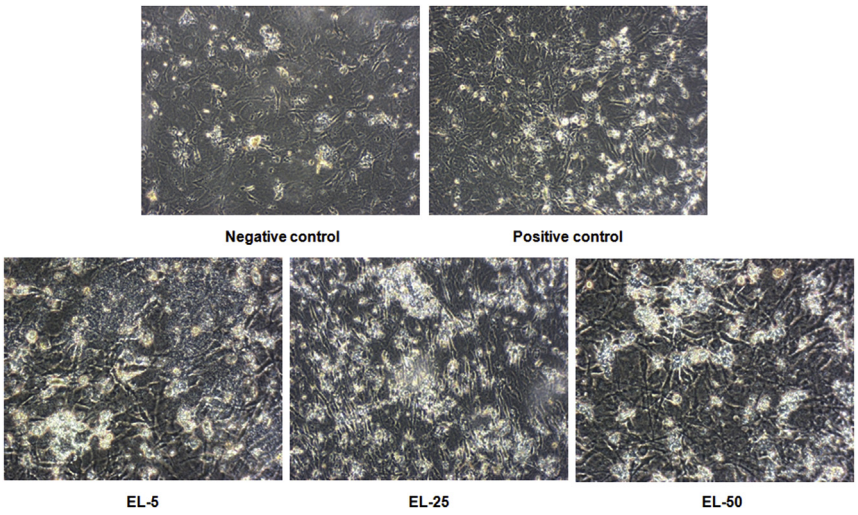

Fig. 2. Microscopic analysis of *EL*-treated MC3T3-E1 cells at day 21 compared to the negative control (untreated cells) and positive control (5α-DHT-treated) groups. Resulting monographs evidently promising differentiation and mineralization potential of *EL* (particularly in EL-5 and EL-25 groups). These micrographs were imaged using phase contrast microscopy (original magnification, ×10).

3.4.3. Expression of Runx-2

It was observed that Runx-2 gradually increased from days 3 to 15 and subsequently declined on day 21 in all the experimental groups; however, the increased expression was more obvious on days 9 and 15 (Fig. 5A). Runx-2 expressions of EL-5 (944 ± 72 pg/mL) and EL-25 (998 ± 62 pg/mL) groups were significantly ($^*p < 0.05$, ANOVA) high compared to the negative control (467 ± 32 pg/mL) and positive control (832 ± 71 pg/mL) groups at day 15 (Fig. 5A). The expression intensity of Runx-2 declined in all the experimental groups on day 21; however, it was relatively high in *EL*-treated groups compared to the control groups.

3.4.4. Expression of OCN

Results showed that the levels of OCN were not detectable in the negative control and EL-50 groups until day 9; however, low expressions of OCN were detected in EL-5, EL-25 and positive control groups during this period (Fig. 5B). The OCN levels progressively increased from days 9 to 21 in all the tested groups. The comparative analysis indicated that although the levels of OCN progressively increased in all the tested groups; however, they were significantly ($^*p < 0.05$, ANOVA) higher in EL-5 (2778 ± 71 pg/mL) and EL-25 (2896 ± 82 pg/mL) groups compared to EL-50 (1566 ± 53 pg/mL), negative control (1255 ± 41 pg/mL) and positive control (2265 ± 65 pg/mL) groups on day 21 (Fig. 5B).

3.4.5. Expression of Type I collagen

Results showed that synthesis of collagen was relatively lower on days 3 to 6 in all the tested groups; however, a progressive increase in the collagen deposition was observed in a time-dependent manner from day 9 to 21 (Fig. 6A). The resulting data further revealed that *EL*-treated groups, predominantly EL-5 (301 ± 25 pg/mL) and EL-25 (318 ± 21 pg/mL), showed significantly ($^*p < 0.05$, ANOVA) higher magnitude of collagen synthesis compared to the negative control

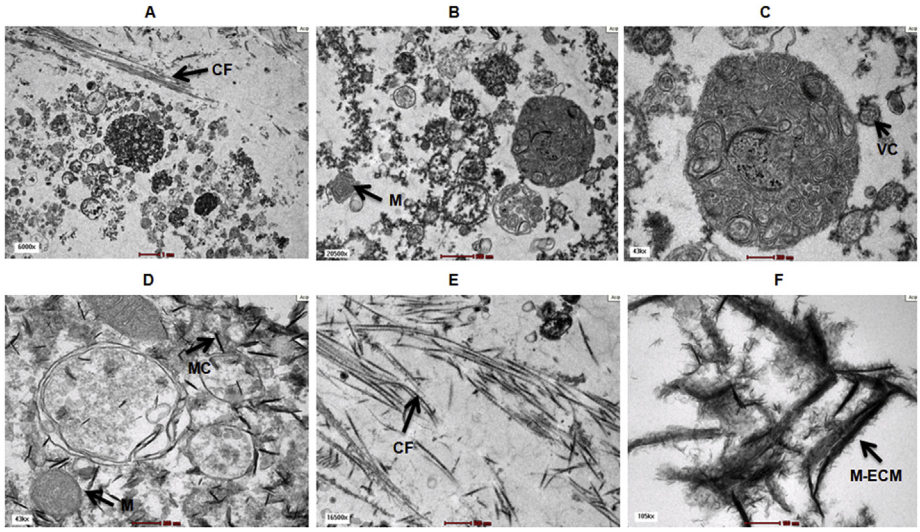

Fig. 3. TEM analysis of MC3T3-E1 cells treated with 25 μg/mL concentration of *EL* at day 21. Resulting micrographs clearly showed various characteristic sub-cellular structures which include collagen fibrils (CF) (A), mitochondria (M) (B), vacuoles (VC) (C), mineral crystals (MC) (D), and mineralized-extracellular matrix (M-ECM) (F). These micrographs were imaged under high magnification (40,000×), scale bar, 0.1–1 μm.

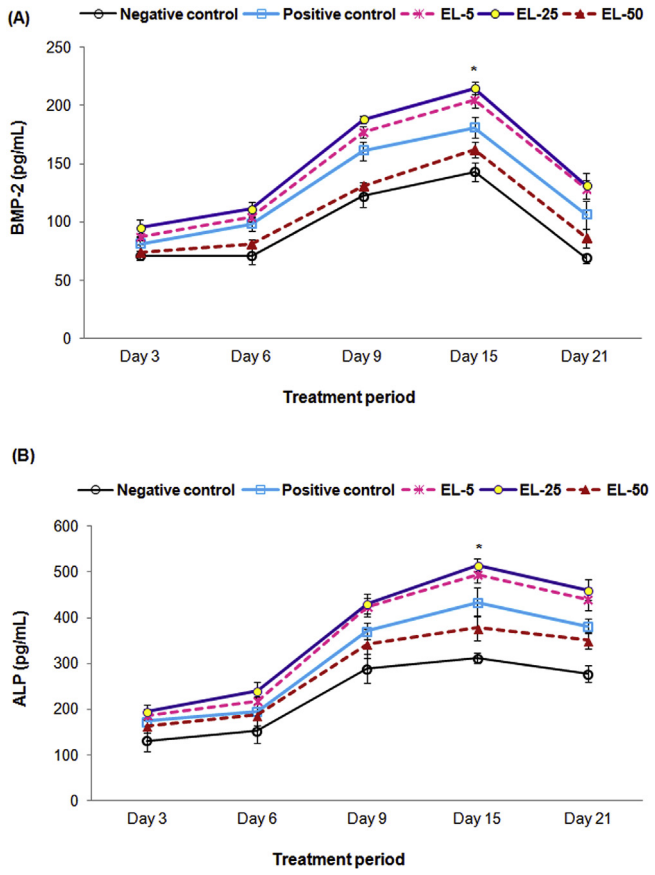

Fig. 4. Effects of different concentrations of *EL* (5, 25, and 50 μg/mL) on the expressions of BMP-2 (A) and ALP activity (B) compared to the untreated (negative control) and 5α-DHT-treated (positive control) groups. Results were obtained from three independent experiments in quadruplicate ($n = 4$) and were expressed as mean ± S.D. The significance between the tested groups was represented as $^{*}p < 0.05$, one way ANOVA and student's *t*-test.

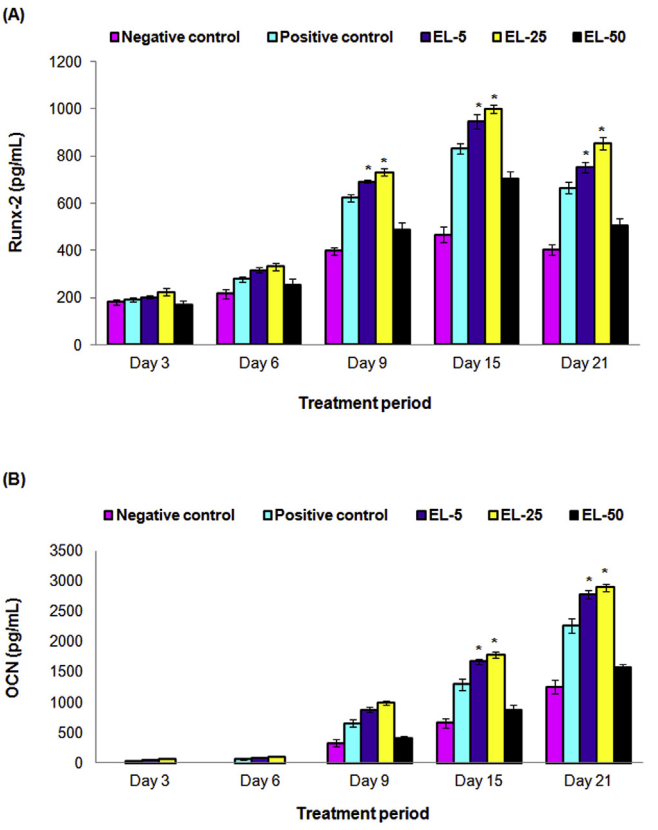

Fig. 5. Effects of different concentrations (5, 25, and 50 μg/mL) of *EL* on the expressions of Runx-2 (A) and osteocalcin (B) compared to the untreated (negative control) and 5α-DHT-treated (positive control) groups. Results were obtained from three independent experiments in quadruplicate ($n = 4$) and were expressed as mean ± S.D. The significance between the tested groups was represented as $^{*}p < 0.05$, one way ANOVA and student's *t*-test.

(211 ± 18 pg/mL) and positive control (276 ± 16 pg/mL) groups on day 21.

3.4.6. Expression of OPN

Results demonstrated that OPN expression started to appear at day 3 in all the tested groups and increased gradually until day 15 (Fig. 6B). The expression of OPN was highest in the negative control group on day 15 with approximately 7-fold increase in its level from days 3 to 15. A considerable drop in OPN expression was noticed on day 21 in all the experimental groups. The amount of OPN detected in EL-5 (69 ± 8 pg/mL) and EL-25 (73 ± 7 pg/mL) were significantly ($^{*}p < 0.05$, ANOVA) attenuated compared to the negative control (151 ± 4 pg/mL) and positive control (98 ± 8 pg/mL) groups at day 21 (Fig. 6B).

3.4.7. Expression of TGF-β1

Results showed that the expression of TGF-β1 consistently increased from day 3 to 15 in all the tested groups; however, the increasing potential of TGF-β1 was more obvious in *EL*-treated MC3T3-E1 cells compared to all the experimental groups (Fig. 7A). The amounts of TGF-β1 detected in EL-5 (231 ± 18 pg/mL) and EL-25 (242 ± 25 pg/mL) groups were significantly ($^{*}p < 0.05$, ANOVA) higher compared to the negative control (165 ± 12 pg/mL) and positive control (182 ± 19 pg/mL) groups at day 15 (Fig. 7A). The expression intensities of TGF-β1 abruptly declined in all the experimental groups at day 21. However, the levels were relatively higher in *EL*-treated groups compared to the control groups.

3.4.8. Expression of AR

Results showed that the expression levels of AR were robustly up-regulated approximately ten-fold during the differentiation period from days 3 to 21 in all the experimental groups (Fig. 7B). The amounts of AR expressed in EL-5 (509 ± 31 pg/mL) and EL-25 (543 ± 29 pg/mL) groups were significantly ($^{*}p < 0.05$, ANOVA) higher compared to the negative control (312 ± 17 pg/mL) and positive control (411 ± 28 pg/mL) groups at day 21 (Fig. 7B).

4. Discussion

Bone remodeling (or bone metabolism) is a lifelong process where mature bone tissues are removed from the skeleton (a process called bone resorption) and new bone tissues are formed (a process called ossification or new bone formation). The balance between both of these processes is the key for maintaining bone health. Osteoporosis is a seriously prevalent bone disorder characterized by fragile bones with an increased susceptibility of fractured bones [1,2]. Among various management modalities, an enhanced bone formation activity of osteoblasts and down-regulated activation, maturation, and functioning of osteoclasts is anticipated to be crucial to improve bone density and maintain bone health.

A wide range of pharmacological modalities including estrogen replacement therapy (ERT), testosterone replacement therapy

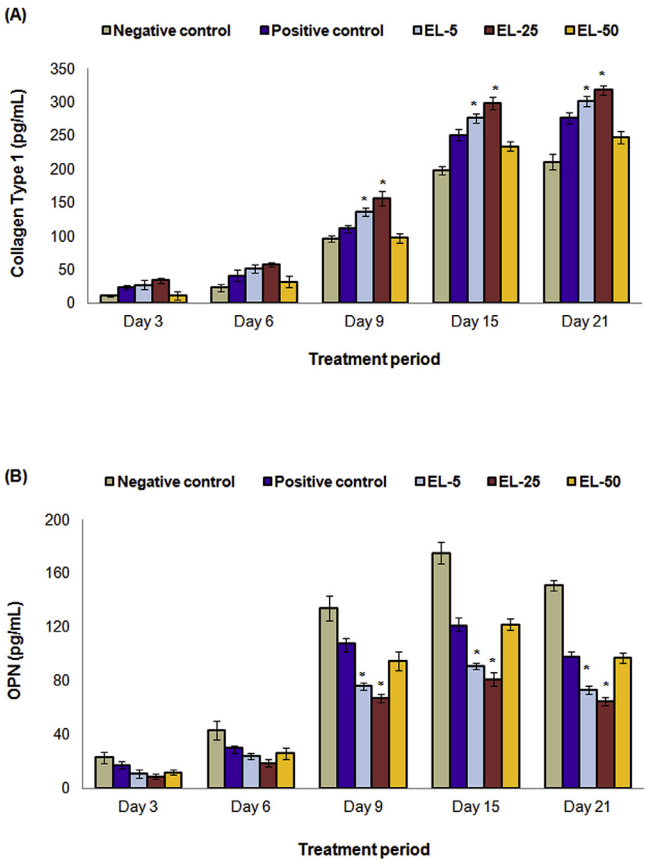

Fig. 6. Effects of different concentrations (5, 25, and 50 μg/mL) of *EL* on the expressions of collagen type I (A) and osteopontin (B) compared to the untreated (negative control) and 5α-DHT-treated (positive control) groups. Results were obtained from three independent experiments in quadruplicate ($n = 4$) and were expressed as mean ± S.D. The significance between the tested groups was represented as $^{*}p < 0.05$, one way ANOVA and student's *t*-test.

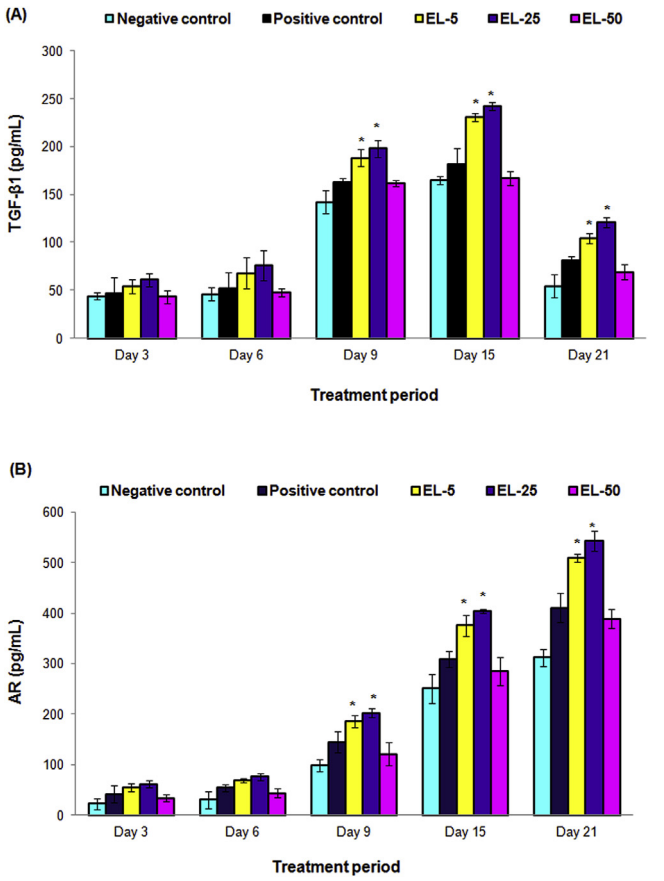

Fig. 7. Effects of different concentrations (5, 25, and 50 μg/mL) of *EL* on the expressions of TGF-β1 (A) and androgen receptors (B) compared to the untreated (negative control) and 5α-DHT-treated (positive control) groups. Results were obtained from three independent experiments in quadruplicate ($n = 4$) and were expressed as mean ± S.D. The significance between the tested groups was represented as $^{*}p < 0.05$, one way ANOVA and student's *t*-test.

(TRT), bisphosphonates, selective estrogen receptor modulators (SERM), and calcitonin are currently being employed for the management of osteoporosis. However, several adverse effects such as breast cancer, hypercalcemia, and hypertension are associated with these intensive therapies. While searching for natural herbal alternative treatment with minimal side effects and improved patient compliance, researchers have identified a promising natural plant, *Eurycoma longifolia (EL). EL* has long been recognised in stimulating production of androgen hormones particularly, testosterone and thus predisposed to be used as a potential therapeutic alternative of TRT for the treatment of androgen deficient male osteoporosis [6–8].

Recently, we revealed that standardized aqueous root extract of *EL* significantly enhances bone formation by up-regulating the osteoblastic activity. We have provided numerous evidences of up-regulation of osteoblast proliferation, differentiation, extracellular matrix (ECM) formation, minerals (calcium and phosphate) deposition, alkaline phosphatase (ALP) activity and collagen deposition [11,14]. Hence, to comprehend the molecular and translational mechanism of *EL* in enhanicng bone formation, we executed a series of *in vitro* experiments to evaluate the effect of *EL* on time-mannered expression of bone-related protein biomarkers such as BMP-2, ALP, Runx-2, OCN, type I collagen, OPN, TGF-β1 and AR.

Prior to evaluation of the efficacy of *EL* in regulating various protein biomarkers, cell viability analysis was conducted to evaluate the safe dose of *EL* that could produce desirable effects on the functioning of pre-osteoblasts (MC3T3-E1). Our results indicated that *EL* is safe and could significantly enhance cells growth at 5 and 25 μg/mL; however, their cell growth tendency was comparatively lower than that of 5α-DHT-treated cells. Besides establishing safety of *EL* on cell growth, cell differentiation is another imperative activity of bone forming cells to maintain bone strength. The appropriate deposition of extracellular matrix (ECM) and collagen, ALP activity and mineralization are mandatory for bone formation. ALP, an early marker of bone formation and osteogenic differentiation, is secreted by osteoblasts into the extracellular matrices (ECM) together with calcium salt to promote mineralization of ECM. Interestingly, the higher intensity of ALP expression and collagen deposition observed in *EL*-treated cells clearly indicated the promising potential of *EL* in stimulating osteoblast's maturation and differentiation (bone matrix formation). The collagen matrix-induced osteoblastic differentiation was expected to be due to the interaction between collagen matrix and integrin receptors [9]. Results indicated that bone cell specific proteins such as ALP and collagen appeared to have an integral role in inducing osteoblastic differentiation and formation of mineralized bone matrix. Enhanced bone mineralization in *EL*-treated cell cultures was also evident by phase contrast microscopic analysis. The intensity of mineral deposition was abundantly high in *EL*-treated (particularly in EL-5 and EL-25) groups compared to the control groups (Fig. 2). Based on the results, we anticipated that *EL* exhibits promising

potential to enhance cell differentiation and mineralization in MC3T3-E1 cells compared to the control groups.

To gain further insight into the molecular mechanism of *EL* in promoting osteoblastic differentiation and mineralization, the sequential expression of various osteoblasts-related protein biomarkers were quantitatively monitored. The major phenotypic bone-related protein markers responsible for regulation of osteoblastic differentiation including BMP-2, ALP, type I collagen, Runx-2, AR, OCN, OPN and TGF-β [10] were evaluated in the present study. Among various protein biomarkers, BMP-2 is one of the most potent inducer of osteoblast's differentiation, especially during the early stage of osteogenesis [12,13]. Our results demonstrated that marked increase in BMP-2 levels in *EL*-treated cells could be the reason for the higher osteoblastic differentiation which was observed in our previous study [11,14]. These findings suggested that *EL* may enhance osteoblastic proliferation and differentiation *via* the up-regulation of BMP-2 expression (a potent inducer of osteoblast differentiation) [15–19].

Upon reaching confluence, MC3T3-E1 cells initiate programmed multi-layering and expression of BMP-2 followed by the induction of ALP activity [10]. ALP is among the key players that exhibits primary role in osteogenesis [20] and thus, adequate levels of ALP are vital to induce and promote mineralization, differentiation and protein expression in MC3T3-E1 cells [11,21–24]. Our results clearly evidenced a remarkable potential of *EL* in up-regulating the levels of ALP in MC3T3-E1 cells which demonstrates a systematic physiological correlation between the expression pattern of ALP activity and osteoblastic differentiation. Our results were also in agreement with previous studies [25,26].

The expression level of Runx-2, a master regulatory protein, which regulates osteoblastic differentiation and maturation during early stages, was also assessed in the present study. It was anticipated that the higher expression of Runx-2 observed in *EL*-treated MC3T3-E1 cells could be a molecular basis for the higher differentiation, maturation and mineralization of MC3T3-E1 cells [11,14]. Komori [27] demonstrated that Runx-2 is involved in the regulation of type I collagen and OCN expressions, which ultimately resulted in higher differentiation and mineralization of bone forming cells. OCN, a non-collagenous and most abundant bone matrix protein synthesized by the bone cells, is among the most vital bone biomarkers responsible for the maintenance of bone mineralization [10,28]. A remarkably higher expression of OCN observed in MC3T3-E1 cells treated with different concentrations of *EL* (particularly EL-5 and EL-25) revealed that *EL* has strong potential to promote the formation of mineralized nodules in MC3T3-E1 cells [29,30]. Together, collagen (predominantly type I) synthesis is another imperative cellular differentiation biomarker [31]. Several studies have reported the biological significance of type-I collagen in regulating the bone matrix formation, osteoblastic differentiation and mineral deposition [10,27,32]. The resulting higher intensity of collagen synthesis observed in *EL*-treated MC3T3-E1 cells clearly indicated the potential of *EL* in provoking osteoblastic maturation and differentiation as signs of cellular differentiation and bone matrix formation [9].

Another well-studied protein biomarker to regulate the homeostasis in bone remodelling is OPN [33–35]. OPN is a negative regulator of cell proliferation, differentiation and bone mineralization, most likely through inhibition of mineral crystal growth, at the later stages of osteogenic differentiation [36,37]. Therefore, the effects of *EL* on OPN expression were examined during the different stages of differentiation (Fig. 6B). Our results demonstrated a significant down-regulation in the expression of OPN in *EL*-treated cell cultures which is anticipated to be one of the reasons to up-regulate the differentiation and mineralization of osteoblasts [35–38].

Among other bone regulatory proteins, TGF-β displays imperative mitogenic functions in regulating the proliferation, early differentiation and mineralization of bone cells [39–41]. Thus, we have also examined the effects of different concentrations of *EL* on the expression of TGF-β1, an important member of TGF-β superfamily and it was anticipated that higher expression of TGF-β1 observed in *EL*-treated MC3T3-E1 cells could be the molecular basis for the remarkably high proliferation, differentiation, and mineralization of MC3T3-E1 cells [41,42]. AR is a single receptor protein that plays pivotal roles in regulating proliferation, differentiation and mineralization in bone-forming cells (MC3T3-E1 cells). The expression of AR in active MC3T3-E1 cells has been well-documented [43,44]. The comparative analysis of all the tested groups revealed that MC3T3-E1 cells treated with different concentrations of *EL* (particularly EL-5 and EL-25) showed higher expression of AR compared to the control groups at all the predetermined time points. A rare physiologically expressed under the influence of steroid hormone and thus promotes osteoblastic proliferation, differentiation and mineralization in bone forming cells [45–47]. In this study, higher expression of AR in *EL*-treated cell cultures and consequently higher proliferation, differentiation and mineralization suggested that *EL* might bind to AR and consequently stimulate their cell proliferation and differentiation.

5. Conclusion

Conclusively, our results indicated that *EL* (at a dose of 25 μg/mL) significantly up-regulated the proliferation of MC3T3-E1 cells. However, their proliferation potential was slightly inferior to 5α-DHT. Resulting data also revealed that *EL* showed a superior cell differentiation potential compared to the testosterone in terms of cell growth, ALP activity, collagen deposition and mineralization. Analyses of the various bone-related biomarkers which include BMP-2, ALP, Runx-2, OCN, type I collagen, OPN, TGF-β1 and AR revealed that cells treated with different concentrations of *EL* (particularly EL-5 and EL-25) produced greater expressions of these bone-forming proteins compared to the control groups. These results provide the molecular basis for the osteogenic potential of *EL* in prevention and treatment of osteoporosis. It was also noted that the expression patterns of these bone-related proteins were regulated in temporal manner during the successive developmental stages of proliferation, bone matrix formation/maturation, and mineralization. The present study strengthened the concept of *EL* in promoting bone mass. In conclusion, *EL* promoted bone formation *via* up-regulation of various mitogenic proteins expressions and thus may provide alternative treatments of osteoporosis.

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Conflict of interest

None.

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