Journal of Ayurveda and Integrative Medicine 10 (2019) 102–110

Contents lists available at ScienceDirect

Journal of Ayurveda and Integrative Medicine

journal homepage: http://elsevier.com/locate/jaim

Original Research Article (Experimental)

Eurycoma longifolia, a promising suppressor of RANKL-induced differentiation and activation of osteoclasts: An *in vitro* mechanistic evaluation

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ARTICLE INFO

Article history:
Received 30 May 2017
Received in revised form
26 June 2017
Accepted 9 July 2017
Available online 16 August 2018

Keywords:
Osteoporosis
Eurycoma longifolia
Osteoclasts
Osteoclastogenesis
Bone resorption
Molecular mechanism

ABSTRACT

Background: *Eurycoma longifolia* (*E. longifolia*) has gained remarkable recognition due to its promising efficacy of stimulating bone formation in androgen-deficient osteoporosis. Numerous *in vivo* studies have explored the effects of *E. longifolia* on osteoporosis; however, the *in vitro* cellular mechanism was not discovered yet.
Objectives: The present study was aimed to investigate the effect of *E. longifolia* on the proliferation, differentiation and maturation of osteoclasts and the translational mechanism of inhibition of osteoclastogenesis using RAW 264.7 cells as an *in vitro* osteoclastic model.
Materials and methods: Having assessed cytotoxicity, the cell viability, cell proliferation rate and osteoclastic differentiation capacity of *E. longifolia* was investigated by evaluating the tartrate-resistant acid phosphatase (TRAP) activity in receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL)-induced osteoclasts. Taken together, the time-mannered expression of osteoclast-related protein biomarkers such as matrix metallopeptidase-9 (MMP-9), cathepsin-K, TRAP, nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), superoxide (free radicals) generation and superoxide dismutase activity were also measured to comprehend the mechanism of osteoclastogenesis.
Results: *E. longifolia* did not show significant effects on cytotoxicity and cell proliferation of RAW 264.7 cells; however, a significant inhibition of cells differentiation and maturation of osteoclasts was observed. Moreover, a significant down-regulation of RANKL-induced TRAP activity and expression of MMP-9, cathepsin-K, TRAP, NFATc1 and generation of superoxide and enhanced superoxide dismutase activity was observed in *E. longifolia* treated cell cultures.
Conclusion: We anticipated that *E. longifolia* that enhances bone regeneration on the one hand and suppresses osteoclast's maturation on the other hand may have great therapeutic value in treating osteoporosis and other bone-erosive diseases such as rheumatoid arthritis and metastasis associated with bone loss.

1. Introduction

Osteoporosis is a serious health problem that is related to aging; it is characterized by decreased bone density and increased risk of fracture [1]. Bone remodeling is a continuous process between bone resorption (activity of osteoclasts) and formation (activity of osteoblasts). Deregulation of these oppositely acting processes may cause bone diseases [2–4]. Osteoclasts are activated for many reasons, one of which is the imbalance of hormones caused by the menopause [5]. The absence of estrogen, induced by the menopause, increases the formation and the activity of osteoclasts, which play key roles in bone loss, and osteoclasts ultimately increase the risk of menopausal osteoporosis [6]. Therefore, inhibiting osteoclast formation and function is an important therapeutic strategy.

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Peer review under responsibility of Transdisciplinary University, Bangalore.

https://doi.org/10.1016/j.jaim.2017.07.014

Osteoclasts are multinucleated cells generated from monocyte/macrophage precursor cells, and osteoclast formation requires RANKL. The receptor activator of NF-κB (RANK) is expressed on RAW 264.7 cell surfaces and conjugates with RANKL, which is essential for osteoclastogenesis [7]. RAW 264.7 cells have been well-recognized as osteoclast precursors. RAW 264.7 cells respond to RANKL stimulation *in vitro* to exhibit all characteristic features of fully-differentiated matured osteoclast [8–10]. Upon activation, RAW 264.7 cells stimulate activation of an important transcription factor, NFATc1, for osteoclastogenesis [11]. As a master transcription factor of osteoclastogenesis, NFATc1 regulates diverse osteoclastogenesis-related proteins such as TRAP, cathepsin-K and MMP-9 [12–14].

Various therapies are available for post-menopausal osteoporosis, such as estrogen replacement therapy (ERT), bisphosphonate and calcitonin, and it has previously been noted that ERT is most commonly employed for the management of post-menopausal osteoporosis [15]. However, long-term use of ERT has been associated with several side effects such as breast cancer and endometrial cancer [16]. An alternative therapy which is thus worthy of consideration is natural herbs, which exert positive effects on osteoporosis and have fewer harmful side effects.

Eurycoma longifolia (*E. longifolia*) has gained widespread recognition due to its pharmacological activities for the treatment of cancers [17,18], aphrodisiac properties [19], anti-malarial, anti-bacterial, anti-inflammatory, anti-anxiolytic, anti-diabetic, anti-ulcer, anti-rheumatism. In our previous studies, our research group revealed that *E. longifolia* exhibits strong bone regenerating ability [20,21]. We have also proposed the molecular and translation mechanism of *E. longifolia* that was possibly responsible for the regulation of bone formation. We evidenced that the promising bone forming capacity of *E. longifolia* is mediated by regulating the expression of various osteoblast-specific protein biomarkers including 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) [22]. In this study, we are first time evaluating the inhibitory activity of *E. longifolia* on osteoclastogenesis.

Thus, the present study was aimed to investigate and establish the effect of *E. longifolia* on the proliferation, differentiation and maturation of osteoclasts. Taken together, we aimed to explore the mechanistic pathway for the inhibitory activity of *E. longifolia* on osteoclastogenesis using RAW 264.7 cells. Having assessed cytotoxicity and proliferative activity of *E. longifolia* on RAW 264.7 cells, their cell differentiation ability was evaluated in terms of TRAP activity and the expression of specific osteoclast-related proteins including MMP-9, cathepsin-K, TRAP and NFATc1 *in vitro*. Taken together, we have also evaluated the effect of *E. longifolia* on the RANKL-induced generation of superoxide (free radicals) and superoxide dismutase (SOD) activity in osteoclasts.

2. Materials and methods

2.1. Material and reagents

RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC) Cell Bank (Manassas, VA, USA) and were used as *in vitro* model. Cell culture reagents Dulbecco modified Eagles medium (DMEM), penicillin, streptomycin and foetal bovine serum (FBS) were sourced from Gibco Laboratories (Grand Island, NY, USA). RANKL was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) assay kit was purchased from Sigma–Aldrich, USA. Lactate dehydrogenase (LDH) cytotoxicity detection kit was purchased from Roche Diagnostics, Switzerland. *E. longifolia* was gifted by Faculty of Pharmacy (University Sains Malaysia, Malaysia). The composition of the extract was same as that used in health supplements. 5α-dihydrotestosterone (5α-DHT) was purchased from Abcam, USA. ELISA kits for the expression of MMP-9, TRAP, cathepsin-K and NFATc1 were purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). All other chemicals and reagents were sourced from pharmacology and cell culture laboratories of Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Malaysia.

2.2. Standardized extract of E. longifolia

A dried root extract of *E. longifolia*, a light brown fine powder with 4–6% moisture contents was used in the present study. The aqueous extract was prepared from the standardized dried root powder (Sample: TAF 273) (batch No: 20130627TAF273) of *E. longifolia* using a patented high pressure water extraction process (US 7,132,117 B2) under the extraction condition of water: root powder ratio of 15:1 at 3 reflux cycles, each for 4 h. Briefly, the dried root powder of *E. longifolia* was pulverized and boiled in water followed by the removal of debris by the process of centrifugation. The refine extract was subjected to reverse-phase high performance liquid chromatography (RP-HPLC) and size-exclusion chromatography. The 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 the extract, can be isolated with the retention time of 17.13 min. Finally, the refined aqueous extract of *EL* was filtered through 1–4 μm and freeze dried as a light brown powder.

2.3. Drug treatment

Prior to drug treatment, a stock solution (100 μg/mL) of *E. longifolia* was prepared using either DMEM or differentiation media. Different concentrations (1, 5, 25, 50 and 100 μg/mL) of *E. longifolia* [22] were then prepared from the stock solution and were sterilized using 0.2 μm syringe filter (Sartorius, Germany). RAW 264.7 cells were then treated with different concentrations of *E. longifolia* and culture media was replaced every other day throughout the experimental period.

2.4. Cell culturing

The transformed murine monocyte/macrophage cell line (RAW 264.7) was used as the osteoclast precursor cells. Cell culturing and sub-culturing were performed by growing active RAW 264.7 cells in a growth media consisting of DMEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (Antibiotic/Antimycotic). 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 cell scrapper and the removed cells were counted using a hemocytometer. For analyzing osteoclastic differentiation, cells were sparsely seeded at a density of 1×10^4 cells/well in 24-well plate and were cultured under the same incubation circumstances. Cells were cultured for 24 h to obtain monolayers containing DMEM with 10% FCS to promote cell survival, division and metabolism. The cells were subsequently treated with or without *E. longifolia* at different concentrations (1, 5, 25, 50 and 100 μg/mL). These cultures were fed every other day by replacing the differentiation media with an identical volume of fresh media.

2.5. Cytotoxicity assay

RAW 264.7 cells were seeded at a density of 1×10^4 cells/well in 24-well plate and cultured for 24 h. After the cell adherence, culture media was changed and different doses of *E. longifolia* (1, 5, 25 and 50, 100 μg/mL) were added. Cells were cultured for additional 24 h and culture media was collected to test cytotoxicity.

Lactate dehydrogenase (LDH) cytotoxicity assay was performed according to the manufacturer's protocol. This colorimetric assay quantifies activity of LDH released from the cytosol of damaged cells into the supernatant and thus serves as an index of cell death. Results were presented relative to the LDH activity in the media of control cells (100% of cell viability) and of cells treated with 1% Triton X-100 (0% cell viability) using the following equation.

$$\textit{Cell viability}(\%) = \frac{\textit{E. longifolia treated cells} - \textit{Control cells}}{\textit{Triton treated cells} - \textit{Control cells}} \times 100$$

2.6. Cell proliferation assay

Cell proliferation ability of *E. longifolia* was performed by using colorimetric [3-(4,5-dimethylthiazol-2-yl)-5-(3-caroxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay. This colorimetric assay is based on the metabolic reduction of the MTS tetrazolium by the living cells to a formazan product. The absorbance of the formazan product was quantified at the wavelength of 490 nm. Briefly, RAW 264.7 cells were seeded at a density of 1×10^4 cells/well in 24-well culture plates with six duplicate wells per treatment for cell proliferation assay and maintained in growth media for 24 h at 5% CO_2 at 37 °C. After 24 h incubation, media was removed and the cells were treated with fresh growth media containing different concentrations (1, 5, 25, 50, 100 μg/mL) of *E. longifolia* aqueous extract and were cultured for additional 24 h. Normal growth medium (without *E. longifolia*) was used as untreated (Normal) while 5α-DHT at concentration of (100 pg/mL) was used as positive control. After 24 h of incubation, 20 μL of diluted MTS solution was added to each well and cells were further incubated at 37 °C in the dark after being covered with aluminum foil for additional 2 h. The absorbance of each well was recorded using microplate reader.

2.7. Cell differentiation

RAW 264.7 cells differentiate into mature osteoclasts after stimulating with RANKL. For that, RAW 264.7 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin and seeded in 24 wells culture plates at a density of 1×10^4 cells/well and placed in the CO_2 incubator overnight to allow the cells to adhere to the well surfaces. After 24 h, the culture media was replaced with osteoclastic differentiation media and eight groups were made: (1) first group was untreated containing RAW 264.7 cells cultured with normal growth media, (2) Second group was RANKL-treated cells (RTC) in which RAW 264.7 cells were incubated with differentiation media containing 50 ng/mL RANKL, (3) third group was named positive control in which RANKL-induced RAW 264.7 cells were treated with 100 pg/mL 5α-DHT, (4) fourth group was RANKL-induced RAW 264.7 cells that were treated with 1 μg/mL *E. longifolia*, (5) fifth group was RANKL-induced RAW 264.7 cells that were treated with 5 μg/mL *E. longifolia*, (6) sixth group was RANKL-induced RAW 264.7 cells that were treated with 25 μg/mL *E. longifolia*, (7) seventh group was RANKL-induced RAW 264.7 cells that were treated with 50 μg/mL *E. longifolia* and (8) eighth group was RANKL-induced RAW 264.7 cells that were treated with 100 μg/mL *E. longifolia*.

2.8. TRAP activity

To analyze the extent of osteoclastic differentiation, RAW 264.7 cells were cultured in 24-well cell culture plates at a density of 1×10^4 cells/well and induced with differentiation medium after being treated with *E. longifolia* at different concentrations (1, 5, 25, 50 and 100 μg/mL) or with 5α-DHT (100 pg/mL) or left untreated (Normal) for 5 days. The medium was changed every other day. Afterward, the medium was removed and the cell monolayer was gently washed twice using ice-cold PBS. The cells were fixed in 3.5% formaldehyde for 10 min and ethanol/acetone (1:1) for 1 min. Subsequently, the dried cells were incubated in a 50-mM citrate buffer (pH 4.5) containing 10 mM sodium tartrate and 6 mM PNPP. After 1 h of incubation, the reaction mixtures were transferred to new well plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at 405 nm using an enzyme-linked immunoassay reader and TRAP activity was expressed as optical density compared to the controls.

2.9. Expression of osteoclastogenesis-related protein biomarkers

In this experiment, the potential biological influence of *E. longifolia* on the expression and regulation of specific RANKL-induced osteoclast-functional protein biomarkers such as MMP-9, TRAP, cathepsin-K and NFATc1 was investigated. These proteins are major phenotypic markers for osteoclast differentiation during bone resorption.

The expression of the above bone-resorption protein markers were measured using sandwich ELISA method. 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 optical density (OD) values were recorded at 450 nm and the intensity of the color is directly proportional to expression concentration of each protein marker in the sample.

2.10. Superoxide production

Superoxide concentration was measured using a Green Chemiluminescence CD kit according to the manufacturer's instructions. Green Chemiluminescent CD is a highly sensitive chemiluminescence probe that reacts with the superoxide anion and a luminescence dye specific for the detection of superoxide. Briefly, RAW 264.7 cells were cultured in 24-well cell culture plates at a density of 1×10^4 cells/well and were left untreated, treated with 5α-DHT or different concentrations of *E. longifolia* (1, 5, 25, 50 and 100 μg/mL) as described in section 2.8. The contents of the kit were dissolved in hot methanol: water (1:1 v/v) containing 0.1% (w/v) trifluoroacetic acid. To measure superoxide generated into the media, 200 μL of the media was mixed with 150 μL of the reagent. The luminescence intensity of each sample was measured using a luminescence plate reader. The value for each treated group was converted to a percentage of the control luminescence.

2.11. SOD activity

SOD activity expressed in each cell culture media was measured using an SOD Assay kit-WST according to the manufacturer's instructions (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, RAW cells were seeded in a 24-well plate at a density of 1×10^4 cells/well and were left untreated, treated with 5α-DHT or different concentrations of *E. longifolia* (1, 5, 25, 50 and 100 μg/mL) as described in section 2.8. The SOD activity was measured by

mixing the reagents from 220 μL of the WST kit with 20 μL of the culture medium. After 20 min incubation at 37 °C, absorbance was measured at 450 nm using a microplate reader (Bio-Rad Model 680; Bio-Rad, Hercules, CA, USA). The value for each treated group was converted to a percentage of the value obtained for the control group.

2.12. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by student's t-test using SPSS version 21.0. All the experiments were performed independently three times with quadruplicate sampling (n = 12). Data were presented as mean ± standard deviation (S.D.). A significant difference was recognized by $^{\#\#}p < 0.01$ represent significant difference between normal and RTC groups and $^{**}p < 0.01$ and $^{*}p < 0.05$ represents significant differences between treated (*E. longifolia* or 5α-DHT) and RTC groups.

3. Results

In this study, a standardized aqueous root extract of *E. longifolia* has been evaluated for anti-osteoclastogenesis and bone resorption capacity of RAW 264.7 cells. The effect of *E. longifolia* on the bone resorption ability of RAW 264.7 cells was assessed in terms of cell proliferation, phase contrast microscopy, cell differentiation, TRAP activity, expression of various bone resorption biomarkers (MMP-9, Cathepsin-K, TRAP and NFATc1), and production of superoxide and SOD activity.

3.1. Effect of E. longifolia on cell viability/cytotoxicity of RAW 264.7 cells

To examine the cytotoxicity of *E. longifolia* on RAW 264.7 cells, cell viability was measured using LDH cytotoxicity assay. Raw 264.7 cells were incubated with different concentrations of *E. longifolia* and their cell viability was measured at days 1 and 5 compared to the control groups (untreated and RTC) (Fig. 1). Results demonstrated no cytotoxicity of *E. longifolia* at different concentrations (1, 5, 25, 50 and 100 μg/mL) on day-1 (Fig. 1A); however, a decrease in cell viability was observed on day-5 at 50 and 100 μg/mL concentrations in undifferentiated (Fig. 1B) and RANKL-induced (Fig. 1C) RAW 264.7 cells. The viability of cells at all the tested concentrations was >95% except at 50 and 100 μg/mL on day-5 which indicated that long-term incubation of RAW 264.7 cells with *E. longifolia* might cause considerable cytotoxicity at higher concentrations (≥50 μg/mL). These results were in line with our previous findings where we observed that higher concentrations (≥50 μg/mL) of *E. longifolia* showed cytotoxicity against bone-forming precursor cells (MC3T3-E1 cells) [20,21].

3.2. Effect of E. longifolia on proliferation of RAW 264.7 cells

Cell proliferation assay was performed to evaluate the effect of different doses of *E. longifolia* on the growth rate of RAW 264.7 cells. In this study, RAW 264.7 cells were subjected to five different concentrations of *E. longifolia* (1, 5, 25, 50 and 100 μg/mL) and their cell growth rate was measured using MTS assay and results were compared to the control groups (untreated and RTC) (Fig. 2). There was no significant ($^{*}p < 0.05$, one way ANOVA) increase in the cell proliferation was observed in RAW 264.7 cells at different time points in *E. longifolia* treated cells cultures compared to the untreated (normal) group (Fig. 2B). At higher doses (50 and 100 μg/mL), cell proliferations were weaker which was expectedly due to cytotoxicity of *E. longifolia*. Fig. 2 also depicted that cell proliferation was comparatively lower in positive control group (RAW 264.7 cells

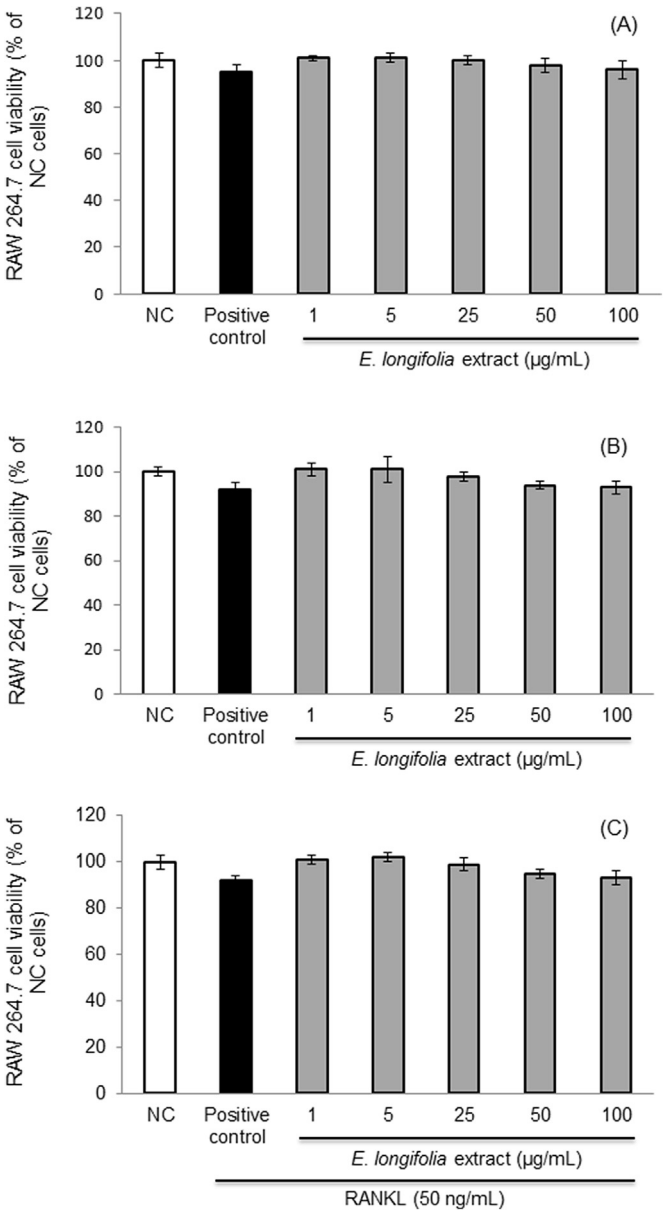

Fig. 1. Effect of *E. longifolia* on RAW 264.7 cells viability: (A) RAW cells were treated with different concentrations of *E. longifolia* for 24 h. (B) RAW cells were treated with different concentrations of *E. longifolia* for 5 days. (C) RAW cells were treated with RANKL and *E. longifolia* for 5 days. Data are representative of results from three independent experiments in quadruplicate sampling (n = 12). Data are expressed as percentages of the value of the control cells (mean ± S.D, n = 12).

treated with 5α-DHT) compared to the *E. longifolia* treated groups at different time points. These results indicated that *E. longifolia* is safe and significantly ($^{*}p < 0.05$, one way ANOVA) decrease cells growth at concentrations higher than 50 μg/mL (Fig. 2).

3.3. Effect of E. longifolia on RANKL-induced differentiation of RAW 264.7 cells

To examine the effect of *E. longifolia* on osteoclastogenesis, RAW 264.7 cells were exposed to RANKL (50 ng/mL) to fully differentiate them into mature multinucleated TRAP-positive cells. RANKL-induced differentiation potential of RAW 264.7 cells was evaluated by measuring TRAP activity of different cell cultures treated with different concentrations of *E. longifolia* compared to the

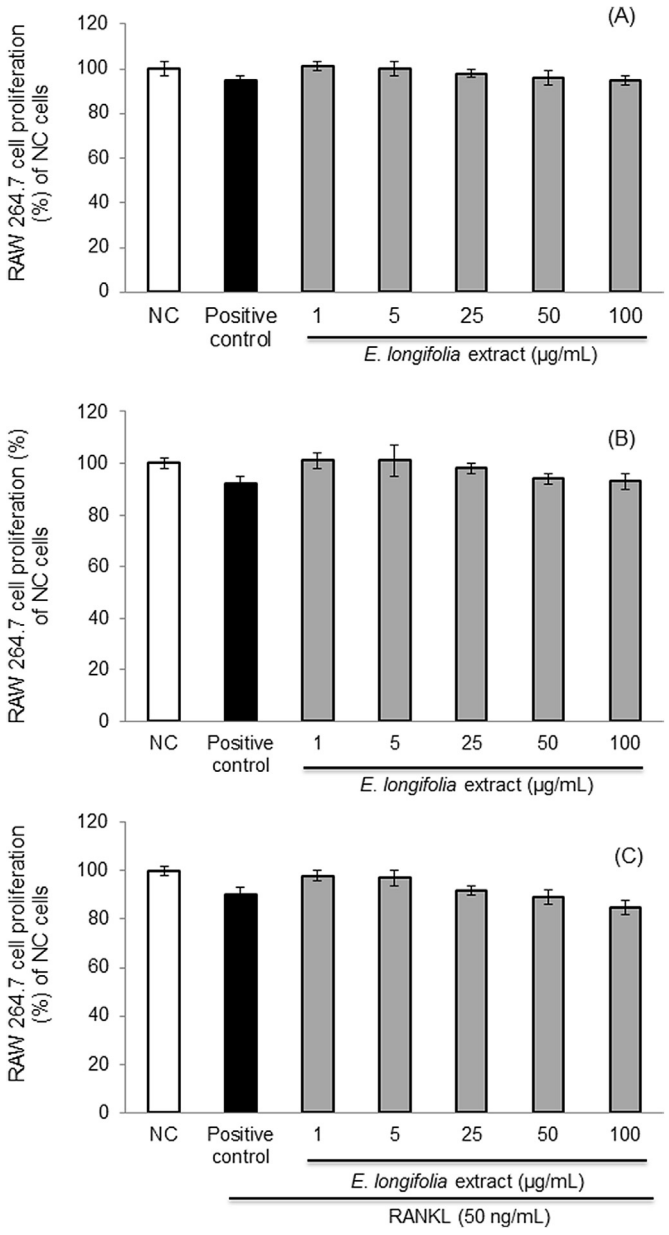

Fig. 2. Effect of *E. longifolia* on RAW 264.7 cells proliferation: (A) RAW cells were treated with different concentrations of *E. longifolia* for 24 h. (B) RAW cells were treated with different concentrations of *E. longifolia* for 5 days. (C) RAW cells were treated with RANKL and *E. longifolia* for 5 days. Data are representative of results from three independent experiments in quadruplicate sampling (n = 12). Data are expressed as percentages of the value of the control cells (mean ± S.D, n = 12).

control groups (untreated and RTC). Results are presented in Fig. 3. Analysis of tested cell cultures revealed that TRAP activity was negligibly low at day-1; however, it was enormously high at day-5 compared to the untreated cells (Normal). Resulting data demonstrated that on day-5 a significant ($^{\#\#}p < 0.05$, one way ANOVA) increase in TRAP activity was observed in RANKL-treated cells (RTC) compared to the untreated cells (Normal) (Fig. 3). Upon treatment with different concentrations of *E. longifolia*, a dose-dependent significant ($^{**}p < 0.05$, one way ANOVA) reduction in TRAP activity was observed in RAW 264.7 cell cultures compared to RTC group. The down-regulating potential of *E. longifolia* was considerably lower than cells treated with 5α-DHT; however, the decreasing potential became comparable with positive control group at higher concentrations (≥25 µg/mL) of *E. longifolia* (Fig. 3).

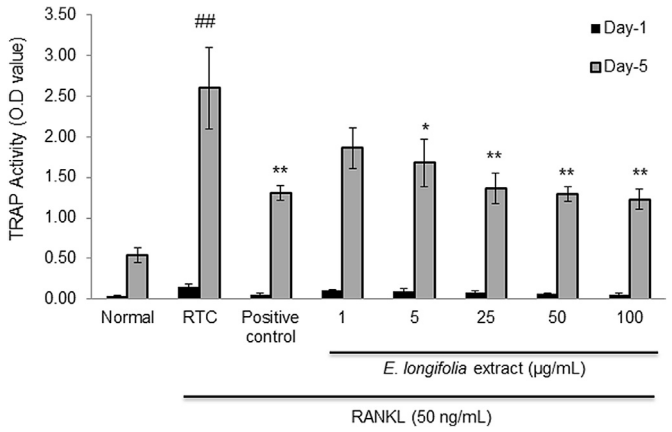

Fig. 3. Effect of *E. longifolia* on receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL)-induced osteoclastogenesis in RAW 264.7 cells. TRAP activity was measured using microplate reader (optical density, 405 nm). Data are represented as the means ± S.D of three independent experiments in quadruplicate sampling (n = 12). $^{\#\#}p < 0.01$ compared with normal; $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with RTC.

3.4. Effect of *E. longifolia* on expression of protein biomarkers

To understand the effect *E. longifolia* on the bone resorption and skeletal remodeling, we measured the expression of various phenotypic bone resorption-related proteins including MMP-9, cathepsin K, TRAP and NFATc1 in RAW 264.7 cell cultures.

3.4.1. Expression of MMP-9 in RANKL-induced RAW 264.7 cells

MMP-9, one of the bone resorption-related enzymes, is highly expressed in osteoclastic cells and plays an important role in bone remodeling [22,23]. Therefore, we investigated the effect of different concentrations of *E. longifolia* on the expression of MMP-9. Resulting data demonstrated that the expression of MMP-9 was negligibly low at day-1; however, an enormous increase in its expression was observed on day-5 in all the treated and untreated RAW 264.7 cell cultures (Fig. 4A). Comparative analysis revealed that the expression of MMP-9 was significantly ($^{\#\#}p < 0.01$, one way ANOVA) high in RTC group (421 ± 63 pg/mL) compared to the untreated group (96 ± 21 pg/mL). A significant dose-dependent decrease in the expression of MMP-9 was observed in *E. longifolia* treated RAW 264.7 cells at different concentrations compared to RTC group ($^{**}p < 0.01$, one way ANOVA). Further analysis revealed that highest reduction in MMP-9 expression was observed in positive control group; however, the expression intensity was comparable at higher doses of *E. longifolia* (≥25 µg/mL) (Fig. 4A).

3.4.2. Expression of cathepsin-K in RANKL-induced RAW 264.7 cells

Cathepsin-K is a protease, which is defined by its high specificity for kinins that is involved in bone resorption. The bone and cartilage resoprtion ability of cathespsin-K is mediated by catabolising elastin, collagen and gelatin. Therefore, this experiment was executed to evaluate the effect of *E. longifolia* on the time-mannered expression patterns of cathepsin-K in RANKL-induced RAW 264.7 cells using sandwich ELISA technique and the results were compared with RTC and normal control groups (Fig. 4B). The resulting data illuminated that the expression of cathepsin-K was enormously high at day-5 compared to day-1 in all the tested groups. The highest expression of cathepsin-K was observed in RTC group (987 ± 67 pg/mL) on day-5 compared to the normal group (132 ± 21 pg/mL) ($^{\#\#}p < 0.01$, one way ANOVA). A dose-dependent decrease in the levels of cathepsin-K was observed in *E. longifolia* treated groups; however, the decreasing trend was more obvious at

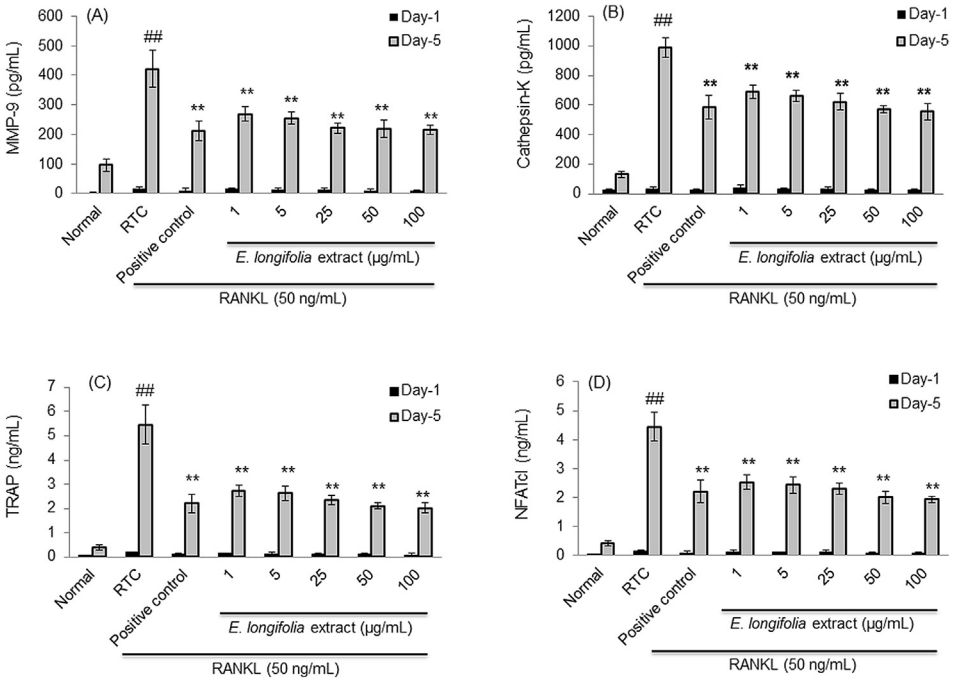

Fig. 4. Effect of *E. longifolia* on the expression of osteoclastogenesis-related proteins in RANKL-stimulated RAW 264.7 cells. (A) expression of MMP-9 using sandwich ELISA, (B) expression of Cathepsin-K using sandwich ELISA, (C) expression of TRAP using sandwich ELISA, and (D) expression of NFATc1 using sandwich ELISA. Data are represented as the means ± S.D of three independent experiments in quadruplicate sampling (n = 12). $^{\#\#}p < 0.01$ represent significant difference compared with normal; $^{**}p < 0.01$ represent significant difference compared with RTC.

50 µg/mL (571 ± 26 pg/mL) and 100 µg/mL (555 ± 36 pg/mL) concentrations ($^{**}p < 0.01$, one way ANOVA) (Fig. 4B).

3.4.3. Expression of TRAP in RANKL-induced RAW 264.7 cells

TRAP is another bone-remodeling enzyme that regulates osteoclastogenesis. In this experiment, we examined the effect of *E. longifolia* on the sequential expression of TRAP in all tested RAW 264.7 cell cultures and the results were compared with control groups (Fig. 4C). Results demonstrated that the expression of TRAP was lowest on day-1 in all the experimental groups; however, an enormous increase in the expression of TRAP was observed on day-5. Results showed that the expression intensity of TRAP in RTC group (5.45 ± 0.80 ng/mL) was significantly high ($^{\#\#}p < 0.01$, one way ANOVA) compared to the untreated group (0.40 ± 0.08 ng/mL) at day-5. The resulting pattern demonstrated a systematic physiological correlation between the expression of TRAP and osteoclastogenesis. A significant dose-dependent decrease in the expressional intensity of TRAP was observed in *E. longifolia* treated RAW 264.7 cells compared to the RTC group ($^{**}p < 0.01$, one way ANOVA). Though, highest decrease in TRAP expression was observed at 100 µg/mL but it was not significantly different from the expression intensity observed at 25 µg/mL (Fig. 4C).

3.4.4. Expression of NFATc1 in RANKL-induced RAW 264.7 cells

NFATc1, a member of the NFAT family of transcription factor, has been shown to be up-regulated after RANKL stimulation and is important for osteoclast differentiation [23,24]. Therefore, in order to confirm the inhibitory effect of *E. longifolia* on osteoclastogenesis and bone resorption, we measured the expression of one of the important osteoclast differentiation indicators, NFATc1. NFATc1 is known to be a master transcription factor in osteoclastogenesis [4]. The effect of different concentrations of *E. longifolia* on the expression of NFATc1 was evaluated using sandwich ELISA and the results were reported in Fig. 4D. Results demonstrated that RANKL induced significantly ($^{\#\#}p < 0.01$, one way ANOVA) higher expression of NFATc1 in RTC group (4.45 ± 0.53 ng/mL) compared to the untreated group (0.40 ± 0.09 ng/mL) at day-5. At day-1, there was observed negligibly low expression of NFATc1 in all the experimental groups. A significant dose-dependent decrease in the expression intensity of NFATc1 was observed in *E. longifolia* treated RAW 264.7 cell cultures compared to the RTC group ($^{**}p < 0.01$, one way ANOVA). The highest decrease in the expression of NFATc1 was observed at higher concentrations (≥25 µg/mL) of *E. longifolia* (Fig. 4D).

3.5. Effect of E. longifolia on superoxide production in RANKL-induced RAW 264.7 cells

Reactive oxygen species (ROS) have been recognized as secondary messengers and play pivotal roles in regulating the differentiation of osteoclasts. Excessive ROS production results in an abnormal osteoclastogenesis and results in overproduction of osteoclasts [25]. Therefore, we have also examined the effect of *E. longifolia* on the production of ROS and superoxide in RANKL-induced RAW 264.7 cells and the results were compared with untreated and control cells (Fig. 5A). The resulting data depicted that the production of superoxide was significantly high in RTC group (taken as 100%) on day-5. However, a dose-dependent decreasing trend was observed in superoxide production in various *E. longifolia* treated RAW 264.7 cell cultures and was varied from 78 ± 6% to 65 ± 7% compared to the RTC group. Comparative analysis revealed that the inhibition of superoxide production was more obvious at higher concentrations (≥25 µg/mL) of *E. longifolia*.

3.6. Effect of E. longifolia on SOD levels in RANKL-induced RAW 264.7 cells

SOD is an important antioxidant defense in nearly all living cells including osteoclasts cells. This an enzyme alternately catalyzes the

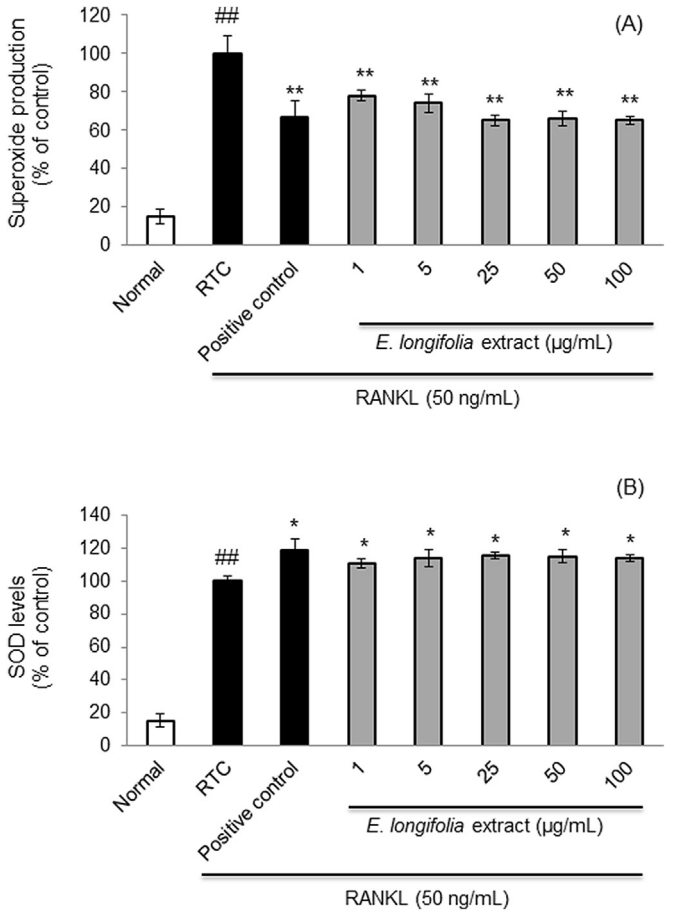

Fig. 5. Effect of *E. longifolia* on (A) superoxide production and (B) superoxide dismutase (SOD) activity. RAW 264.7 cells were cultured with RANKL and the indicated concentration of *E. longifolia* for 5 days. Superoxide production was measured by a luminescence intensity assay using a luminescence plate reader. SOD activity was measured at 450 nm using a microplate reader. Data are representative of results from three independent experiments in quadruplicate sampling (n = 12). Data are expressed as percentages of the value of the control cells (mean ± S.D, n = 9). $^{*}p < 0.05$ and $^{**}p < 0.01$ represent significant difference between tested and RTC group and $^{\#\#}p < 0.01$ represent significant difference between RTC and untreated (normal) group.

dismutation of the superoxide (O_2^-) radicals into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) and thus play an important role in maintaining and regulating homeostasis in bodily tissues including bone matrices. Hence, the activity of this enzyme was also evaluated in RANKL-induced RAW 264.7 cells with or without *E. longifolia* treatment and the results were compared with control group (Fig. 5B). Resulting data showed that the highest SOD level was observed in control positive control (RAW 264.7 cells treated with 5α-DHT) ($^{**}p < 0.01$, one way ANOVA) compared to the RTC group. On the other hand, RAW 264.7 cells treated with different concentration of *E. longifolia* showed a significant increase in SOD levels at various concentrations (1, 5, 25, 50 and 100 µg/mL) compared to RTC group ($^{*}p < 0.05$, one way ANOVA). Comparative analysis of SOD levels suggested that the effect of *E. longifolia* on SOD levels is independent of treatment dose (Fig. 5B).

4. Discussion

The major modalities currently used in osteoporosis treatment include primarily estrogen replacement therapy along with bisphosphonates, selective estrogen receptor modulators, and calcitonin. However, such therapies are associated with adverse effects, including breast cancer, hypercalcemia, and hypertension. Among various natural herbal complementry medicines, *E. longifolia* has long been well-recognised in stimulating production of androgen hormones particularly, testosterone [26] and thus predisposed to be used as a potential therapeutic alternative of testosterone replacement therapy (TRT) for the treatment of androgen deficient male osteoporosis [27].

Recently, we revealed that standardized aqueous root extract of *E. longifolia* significantly enhances bone formation by up-regulating 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 [20,21]. Taken together, we have also executed various experiments to comprehend the molecular and translational mechanism of *E. longifolia* in enhanicng bone formation. For that, we have evaluated the effect of *E. longifolia* on the time-mannered expression of bone-related mediators including BMP-2, ALP, Runx-2, OCN, type I collagen, OPN, TGF-β1 and AR [22].

Bone remodeling is tightly regulated by two processes: bone formation and bone resorption. The balance between both of these processes is the main key for maintaining bone density and health. In this study, we have first time reported that, in addition to its role in bone formation, *E. longifolia* exhibit significant potential to decrease osteoclastic activity by attenuating the differentiation of RAW 264.7 cells *in vitro* after stimulating them with RANKL.

RAW 264.7 cells were used to evaluate the effects of *E. longifolia* on osteoclastogenesis and activity of mature osteoclast. RAW 264.7 cells respond to RANKL stimulation *in vitro* to exhibit all characteristic features of fully-differentiated matured osteoclast [8–10]. Though no significant effects were observed at proliferation and viability of osteoclasts; however, our results clearly demonstrated that *E. longifolia* significantly down-regulated differentiation and maturation of RAW 264.7 cells *in vitro*.

TRAPs are expressed particularly in osteoclasts and are commonly used as phenotype markers of osteoclasts; treatment of RAW 264.7 cells with RANKL has been shown to easily induce cell differentiation into TRAP positive osteoclasts [28]. The study of TRAP-positive cell formation and activity is a well-known method of determining osteoclast formation and function [29,30]. In the present study, we evidenced that *E. longifolia* significantly inhibited TRAP activity which indicated that *E. longifolia* exhibits promising inhibitory potential on osteoclastogenesis.

Taken together, in order to analyze molecular and translation mechanism, we evaluated various key osteoclastogenesis-related protein biomarkers such as MMP-9, cathepsin-K, TRAP and NFATc1 in RANKL-induced RAW 264.7 cells. The resulting data revealed that *E. longifolia* enormously reduced up-regulation of all protein biomarkers responsible for osteoclastic activity and maturation.

MMP-9 plays critical role in initiation of the osteoclast-mediated bone resorption process by removing the collagenous layer from the bone surface prior to demineralization [31]. Furthermore, MMP-9 is one of the most important phenotypic biomarkers to anticipate the bone resorption mechanism [32,33]. In this study, the resulting data revealed that the treatment of RAW 264.7 cells with *E. longifolia* results in dose-dependent decrease in the expression of MMP-9 which indicates that *E. longifolia* exhibit promising potential to downregulate bone resorption. For digestion and solubilization of the bone matrix, the ruffled border secretes enzymes and protons after attachment to the bone. Cathepsin-K activity is required to start actin ring formation and, thus, activation and functioning of osteoclasts [34]. Our results clearly demonstrated that the treatment of RAW 264.7 cells with *E. longifolia* resulted in dose-dependent decrease in the expression of cathepsin-K *in vitro*.

Several studies have demonstrated that NFATc1 is an important transcription factor for RANKL-mediated osteoclast differentiation, fusion and activation [11,12]. It has also been noted that over-expression of NFATc1 induces differentiation into osteoclasts even in cases of RANKL deficiency [35]. Moreover, in NFATc1 knock-out mice, defective osteoclast differentiation and osteopetrosis have been noted [36]. NFATc1 plays an important role in osteoclast activation through the release of osteoclastogenesis-related genes such as TRAP and MMP-9 and the expression of these mediators is mainly responsible for the degradation of bone mineral and collagen matrices [12–14]. In the present study, we noticed that *E. longifolia* exerted significant inhibitory effects on the expression of NFATc1 *in vitro*.

ROS was recognized as a secondary messenger in the differentiation of osteoclasts and plays an important role in differentiation [37]. By contrast, excessive ROS production results in an over-activation of osteoclast functions due to an increase in the number of osteoclasts [25]. Additionally, ROS production by osteoporotic bone tissue is significantly higher compared to normal bone tissue [38]. Several studies have reported that the ROS level increases during RANKL-induced osteoclast differentiation. Thus, to investigate whether *E. longifolia* can inhibit superoxide generation during osteoclast differentiation, the production of superoxide was analyzed. Our resulting data evidenced that the treatment of RANKL-induced osteoclast with *E. longifolia* inhibited the production of ROS in a dose-dependent manner. Despite of its significant down-regulation of ROS production, *E. longifolia* showed significant upregulation in SOD activity, suggesting that *E. longifolia* has a positive effect on increasing antioxidant defense in RANKL-induced RAW 264.7 cells.

5. Conclusion

Conclusively, our results suggested that *E. longifolia* exhibit remarkable inhibitory effects on the differentiation, maturation and functioning of osteoclast. The inhibitory effects *E. longifolia* on the differentiation and functioning of osteoclasts are regulated through mechanisms involving down-regulation of RANKL-induced TRAP activity and expression of various bone-resorption related protein biomarkers including MMP-9, cathepsin-K, TRAP and NFATc1. Taken together, suppression of superoxide generation and enhanced SOD activity also mediates pivotal role in alleviating the over-activation and functioning of osteoclasts. Notably, *E. longifolia* that enhances bone regeneration on the one hand and suppress osteoclast differentiation on the other may have great therapeutic value in treating osteoporosis and other bone-erosive diseases such as rheumatoid arthritis and metastasis associated with bone loss. Our findings suggest that *E. longifolia* may potentially be useful for the treatment of bone diseases associated with excessive bone resorption.

Sources of funding

Authors would like to acknowledge Universiti Teknologi MARA for providing LESTARI grant (600-IRMI/DANA 5/3/LESTARI (0007/2016)) and support to compete the present research project. Authors would also like to acknowledge Ministry of Agriculture for providing New Application for Herbal Research Grant Scheme (NRGS) (NH1014D040).

Conflict of interest

The authors declare that they have no competing interests.

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