

Morinda citrifolia Leaf Enhances *In Vitro* Osteogenic Differentiation and Matrix Mineralization by Human Periodontal Ligament Cells

Kanitsak Boonanantanasarn, Kajohnkiart Janebodin*, Prapan Suppakpatana, Tawepong Arayapisit, Panjit Chunhabundit, Jit-Aree Rodsutthi, Orapin Termvidchakorn and Wanida Sripairojthikoon

Department of Anatomy, Faculty of Dentistry, Mahidol University, Thailand

Abstract

Morinda citrifolia L. (noni) has been reported to have a variety of potentially therapeutic effects such as antibacterial, antiviral, antifungal, antitumor, anthelmintic, analgesic, hypotensive and anti-inflammatory, as well as immune enhancing properties. In this study, we aimed to investigate a potential effect of *Morinda citrifolia* leaf aqueous extract to induce osteogenic differentiation and matrix mineralization of human periodontal ligament (hPDL) cells *in vitro*. The optimal biocompatible dose of noni leaf aqueous extract was determined by a cell proliferation assay. The hPDL cells were cultured in growth medium alone, or medium supplemented with noni leaf aqueous extract, or β -glycerophosphate and ascorbic acid. The early osteogenic differentiation marker, alkaline phosphatase activity, was assayed using a biochemical analysis. Matrix mineralization was examined after 6 weeks *in vitro* by calcium staining and energy dispersive x-ray analysis. The noni leaf aqueous extract group significantly increased alkaline phosphatase activity, compared to either growth medium alone or β -glycerophosphate and ascorbic acid groups. Nodules containing mineralized matrices were formed only when the hPDL cells were cultured in the presence of noni leaf aqueous extract. These nodules were positively stained with Alizarin Red and were rich in calcium and phosphorus as revealed by the energy dispersive x-ray analysis. These data indicate the potential effect of noni leaf aqueous extract to promote osteogenic differentiation as well as matrix mineralization in hPDL cells and suggest that noni leaf has therapeutic benefits in bone and periodontal tissue regeneration.

Keywords: *Morinda citrifolia*; Noni; Osteoblast; Mineralization; Human periodontal ligament cells

Introduction

Periodontitis is a chronic infectious disease of the periodontium affecting children, adolescents, adults, and the elderly, and is characterized by irreversible loss of connective tissue attachment and supporting alveolar bone [1]. Restoration of damaged periodontal tissue requires a combination of cells, signaling molecules such as those found in osteoinductive materials, and osteoconductive scaffolds. Growth factors modulate the cellular activity and provide stimuli to cells to differentiate and create extracellular matrices for the developing tissue. Scaffolds guide and create a template structure to facilitate the above processes, which is critical for tissue regeneration [2].

Periodontal ligament (PDL) is a lining connective tissue located between cementum and alveolar bone and functions to sustain and constrain teeth within the jaw. This unmineralized tissue contains PDL cells which play an integral role in maintenance, repair and regeneration of periodontal tissue [3]. These PDL cells are heterogeneous, consisting of several cell types including fibroblasts and mineralized tissue forming cells such as osteoblasts and cementoblasts [4-6].

Several studies demonstrated that cells isolated from PDL have osteoblast and cementoblast-like properties. For example, PDL cells produce type I collagen and non-collagenous extracellular matrix proteins such as osteopontin and bone sialoprotein *in vitro* [7,8] as well as the expression of bone-associated markers, alkaline phosphatase (ALP) under bone inductive factors, such as parathyroid hormone, insulin-like growth factor, bone morphogenic proteins, 1,25-dihydroxyvitamin D₃ [8-12]. In addition, PDL cells are capable of forming bone-like and/or cementum-like mineralized nodules when cultured in a medium containing ascorbic acid and dexamethasone [13,14].

Various potential osteoconductive and osteoinductive materials have been suggested to promote the regeneration of alveolar bone and periodontal attachment [15,16]. However, several growth factors are

mostly capable of inducing PDL cell proliferation or migration [17,18]. Therefore, many osteoinductive materials have been extensively evaluated in order to obtain the inducing osteogenic differentiation and mineralization with low cost for periodontal tissue regeneration.

Morinda citrifolia is one of the traditional folk medicinal plants in Polynesia, and Southeast Asia, also known as noni. The major components of the whole noni plant have been found such as scopoletin, octoanoic acid, potassium, ascorbic acid (vitamin C), terpenoids, alkaloids, anthraquinones, β -sitosterol, carotene, retinoic acid (vitamin A), flavone glycosides, linoleic acid and amino acids, including calcium and phosphorus [19,20]. Noni has been reported for a broad range of usage including antibacterial, antiviral, antifungal, antitumor, anthelmintic, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects [19,21-23].

The primary usage of noni is to apply leaves as a traditional topical treatment thought to enhance wound healing. Noni leaf extract was capable of promoting wound healing in an animal model [24]. In addition, the crude extract of noni leaf has been traditionally used in patients with bone fractures or dislocation to promote tissue repair and decrease inflammation [19]. It is well established that bone and periodontal tissue repair or regeneration requires growth factors to induce progenitor/precursor cells to differentiate and produce matrix

*Corresponding author: K. Janebodin, D.D.S., 6 Yothi Street, Rajthevi, Department of Anatomy, Faculty of Dentistry, Mahidol University, Bangkok 10400, Thailand, E-mail: dtjanebk@uw.edu

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mineralization [2]. Nevertheless, there is no experimentally supporting evidence for the healing activities of noni extracts on bone or osteogenic differentiation in the literature. The present study, therefore, investigated the osteoinductive potential of the noni leaf extract on human PDL (hPDL) cells, focusing on *in vitro* alkaline phosphatase (ALP) activity and matrix mineralization as indicators of osteoblastic/cementoblastic differentiation.

Materials and Methods

Morinda citrifolia leaf extraction

Fresh noni leaves were collected from Nakhon Pathom which is a province located in the center part of Thailand, during rainy season (July to October). To obtain 1000 ml of aqueous extract, approximate 1 kg of fresh leaves were washed with sterilized de-ionized water and blended with 1500 ml of cold sterilized de-ionized water in an electrical blender at medium speed for 1 minute/round. The mixture was repeatedly blended for 10 rounds to get finely chopped contents of herb, and processed the cold infusion by incubation at 4°C. After 48 hours, the solution was collected, filtered by squeezing through fine gauze and centrifuged at 3,000 rpm for 10 minutes. The supernatant was subsequently filtered through Whatman filter paper no. 1 to remove remaining debris, freeze-dried with a lyophilizer (LYPH-LOCK6 Model 77595-01, Labconco, USA) and stored at -80°C. The lyophilized noni leaf powder was dissolved in culture medium, filtered through a 0.22 µm cellulose acetate filter (Pall Corporation, Michigan, USA), and sterilized by UV light for 30 minutes before experiments.

Cell culture

Third molar or premolar teeth were obtained from patients aged 17 to 25 years who underwent orthodontic treatments and signed a written informed consent prior to extraction. The experimental protocol was approved by the committee on human rights related to human experimentation, Mahidol University, MU 2007-087. After rinsing several times with Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, USA) supplemented with 250 IU/ml penicillin G sodium, 250 µg/ml streptomycin sulfate and 20 µg/ml antimycotic fungizone (Gibco BRL, USA), the periodontal tissues attached to the mid-third of the root were carefully removed with a surgical scalpel, washed twice with phosphate buffered saline (PBS) (Hyclone, USA), and placed in a tissue culture flask. The tissue explants were incubated in growth medium containing DMEM with 10% fetal bovine serum (Hyclone, USA) and 250 IU/ml penicillin G sodium, 250 µg/ml streptomycin sulfate under an environment of 5% CO₂ and 95% humidified air at 37°C. The culture medium was changed after 1 week and then every three days until cells grew out of the explants and reached confluence. Cells from passages 3 to 5 were used in these studies.

Mouse calvaria-derived pre-osteoblastic cell line, MC3T3-E1 (a gift from Associate Professor R. Surarit, Department of Physiology and Biochemistry, Faculty of Dentistry, Mahidol University), was cultured under conditions equivalent to those used for hPDL cells.

Cell proliferation assay

In order to assess the maximum concentration of noni leaf extract which could promote cell growth, the oxidative activity of mitochondria was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [25]. The hPDL cells were seeded at a density of 20,000 cells/well in a 96-well plate (Nunclon, Roskilde, Denmark). After incubation for 24 hours, confluent cells were treated with various concentrations; 0.025, 0.625, 1.25, 2.5, and

5% (g/ml) of noni leaf extract diluted in growth medium, at 37°C for 24 hours. Cells were washed with PBS and then incubated with 0.05% MTT (Sigma Co. St. Louis, MO, USA) at 37°C for 4 hours with light protection. At the end of the incubation, the untransformed MTT was removed by aspiration and 100 µl of DMSO (Dimethylsulfoxide, Merck, Darmstadt, Germany) was added to each well to dissolve the formazan crystals. The plate was then shaken vigorously to ensure that the blue formazan was fully solubilized. The optical density (OD) of each well was measured in wavelength mode of 540 nm using an automatic multi-well microplate spectrophotometer (Maxline Microplate Reader, Molecular Device Corp, Menlo Park, CA, USA). The higher amount of MTT converted to the formazan salt (higher OD) was relative to higher cell proliferation. The percentage of cell proliferation in the treated groups compared to control after 24-hour noni treatment was calculated by the following formula; cell proliferation (% of control) = (OD of treated cells/ OD of control cells) x 100.

Alkaline Phosphatase (ALP) Activity Assay

hPDL cells were seeded at a density of 1×10³ cells/cm² in a 24-well plate (Nunclon, Roskilde, Denmark). After 24 hours, the cells were incubated with a) growth medium alone, b) growth medium containing the 2.5% (g/ml) of noni leaf extract that was the highest non-toxic concentration to hPDL cells after 24-hour treatment, or c) growth medium supplemented with 2 mM β-glycerophosphate (β-GP) plus 50 µg/ml ascorbic acid. The hPDL cells were cultured for 6 weeks. Each week, the cells from each group were washed with PBS 3 times, collected with a rubber spatula and centrifuged at 1200 rpm for 4 minutes. The precipitate was mixed with 200 µl of a cold lysis buffer (containing NaCl, EDTA and Triton X-100 in Tris-HCl, pH 7.5) and allowed to lyse at 4°C. The ALP activity was determined by a modified Lowry method [26,27] and normalized to cellular total proteins to indicate early osteoblastic differentiation.

Total protein of lysed cells was determined with the Bradford protein assay (Sigma, USA) which is a method to measure the concentration of protein based on the formation of a complex between the dye and proteins in solution. The amount of absorption of the dye is proportional to the protein present [28]. Briefly, 5 µl of each suspension sample was added to 250 µl dye reagent in a 96-well plate, mixed on a shaker for 30 sec, and incubated at room temperature for 30 min. The optical density (OD) for total protein quantification was measured at 595 nm. Bovine serum albumin (BSA) was used as the standard. The amount of protein in the cell lysate samples was calculated by referring to the standard curve. The standard curve was drawn by plotting the concentration of the standard protein solution (0.25, 0.50, 0.75, 1, 1.25, and 1.50 mg/ml of BSA) on the x-axis and the optical density on the y-axis. The protein content in the cell lysate was calculated using the formula; protein concentration = (OD of sample/OD of standard) x concentration of standard.

For ALP activity, the reaction was started by adding 20 µl suspension of lysed cells to 100 µl of 125 mM carbonate-bicarbonate buffer (pH 10.3) at 37°C for 5 minutes, then mixed with 100 µl of substrate solution that was composed of 2 mM MgCl₂ and 2 mM *p*-nitrophenylphosphate (*p*-NPP) (Sigma, USA). After incubation at 37°C for 15 minutes, the reaction was stopped by adding 100 µl of 0.2 M NaOH. The yellow color of *p*-nitrophenol (*p*-NP) which is developed from *p*-NPP dephosphorylated by alkaline phosphatase was measured at 405 nm. The *p*-NP standard curve was used to get the amount of *p*-NP generated by ALP sample. The ALP activity was calculated from amount of *p*-nitrophenol generated from total cellular proteins per reaction time and represented as nmol/mg protein/min.

Mineralized nodule staining

Following three culture conditions (the growth medium alone, medium supplemented with noni leaf extract, or β -GP and ascorbic acid) of hPDL cells, nodule formation was observed every week. Alizarin Red staining was used to verify the calcified nodules. After 6 weeks of treatment, cells were washed 3 times with PBS, fixed with ice cold 70% ethanol for 30 minutes and rinsed with 1 ml of distilled water for 5 minutes to remove ethanol. The cells were then stained with 1% Alizarin Red solution for 30 minutes, rinsed twice with distilled water, and dehydrated by ethanol series before air dry. The calcified nodules that exhibited bright red color were identified and photographed under an inverted microscope (Nikon Eclipse TS100, Japan) with a digital camera (Nikon Coolpix 4500, Japan).

To confirm the osteogenic induction capacity of noni leaf aqueous extract in other osteogenic precursor cells, MC3T3-E1 cells were incubated with a) complete medium, b) complete medium containing the optimal concentration of noni leaf, or c) complete medium supplemented with 2 mM β -GP plus 50 μ g/ml ascorbic acid. After 6 weeks, matrix mineralization was determined as described above.

Elemental analysis

Energy dispersive x-ray mapping was done in order to detect the presence of calcium and phosphorus in mineralized nodules. After washing with PBS, the samples were fixed in 2.5% glutaraldehyde solution for 2 hours at 4°C. Subsequently, they were frozen by liquid nitrogen, and vacuum-dried immediately in a scanning electron microscope (SEM, JSM-5410LV, Tokyo, Japan). The mineralized nodules were located and subjected to elemental mapping analysis by an Oxford energy-dispersive X-ray spectroscopy (EDS) system (ISIS 300, Oxford instrument, UK) attached to SEM at an accelerating voltage of 20 kV.

Statistical analysis

Three independent experiments were performed. Experimental values in each assay were given as means \pm standard deviation (SD) from triplicate wells. The statistical significance of differences was evaluated by one-tailed Student's *t*-test for the cell proliferation assay, and one-way ANOVA for the ALP activity. *P*-values less than 0.05 were considered significant using the statistical software (SPSS version 11).

Results

Effect of noni leaf extract on hPDL cell proliferation

Cultured hPDL cells exhibited spindle and polygonal morphology regardless of noni leaf extract concentration. In order to assay the effect of noni leaf extract on hPDL cell viability, as well determine as optimal concentration of noni leaf extract for further experiments, a dose-response experiment employing the MTT assay for cell proliferation was performed. Initially, to establish the range of concentration to use in the MTT assay, the effect of noni leaf extract on hPDL cells was estimated by a 24-hour preliminary experiment of PDL cells treated with noni concentrations from 0.01% to 10% g/ml. The hPDL cells did not show any alteration when treated by noni leaf extract concentration less than 0.025%; however, cells were completely dead at 7.5% concentration. Consequently, in this experiment hPDL cells were treated with 0.025% to 5% g/ml of noni leaf extract for 24 hours. All concentrations of noni leaf extract, except the highest dose of 5% (g/ml), significantly promoted cell proliferation compared to control group levels ($* p < 0.05$) (Figure 1 and Table S1). In addition, total protein synthesis in hPDL cells, analyzed by the Bradford protein assay,

was affected by all concentrations of noni leaf extract. The 2.5% (g/ml) of noni leaf extract is the highest dose in this study that promoted hPDL cell proliferation without any toxicity, and therefore was chosen for use in subsequent experiments.

Noni leaf extract increases alkaline phosphatase activity in hPDL cells

To examine the effect of noni leaf on osteogenic induction, ALP activity was performed up to the 6th week. The results showed that the ALP activity of the untreated hPDL cells slowly increased from 5 to 8 nmol/mg protein/min need more explanation. The hPDL cells treated with 2.5% (g/ml) of noni leaf extract exhibited about 2-fold increase in the ALP activity at the end of 2nd week. This enzymatic activity reached the maximum level (4-fold) during the 3rd and 4th weeks, then gradually decreased to a similar level as that of the untreated group at the end of 6th week (Figure 2 and Table S2). In the β -GP together with ascorbic acid treated group, the ALP activity approximately increased 2 folds during the 2nd and 3rd weeks in comparison with that of cells cultured in the complete medium alone. The ALP activity of the noni treated cells was significantly higher ($* p < 0.05$) than that of the β -GP together with ascorbic acid treated and untreated groups particularly from the 3rd to the 5th week. These data indicate a potent osteogenic induction of noni leaf extract.

Noni leaf extract increases mineralized matrix formation in hPDL cells

To confirm the effect of noni leaf extract on hPDL cells, matrix mineralization that correlated with the data of ALP activity was determined for 6 weeks. After 7 days in culture, hPDL cells under all three treatment conditions were spindle-like (Figures 3A-C). Over the course of 6 weeks, hPDL cells cultured with growth medium alone did not form any nodules. Conversely, the formation of cell clusters and small nodules were observed in the noni leaf extract experimental group after 3 weeks (Figure 3D, indicated by an arrow). These nodules gradually increased in size by 4-6 weeks (Figures 3E and F). Alizarin Red staining for calcium deposition confirmed that untreated hPDL cells did not form mineralized matrices (Figure 3G). In contrast,

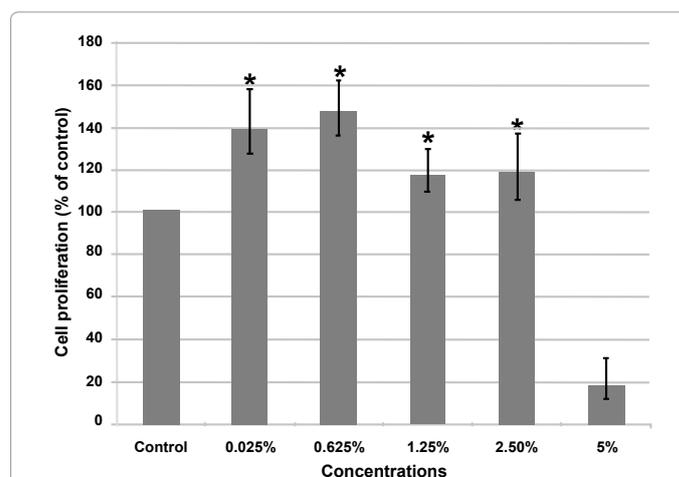


Figure 1: Dose-response effect of noni leaf extract on hPDL cell proliferation. Cell proliferation of hPDL cells treated with noni leaf extract (0.025%, 0.625%, 1.25%, 2.5%, and 5% g/ml) for 24 hours was measured by MTT assay. All concentrations of noni leaf extract except 5% (g/ml) significantly enhanced cell proliferation ($* p < 0.05$), compared to the control group. Values are displayed as percentage of control \pm SD from three independent experiments.

nodules observed in the noni treated hPDL cells were positively stained with Alizarin Red (Figures 3H and I). The group treated with β -GP and ascorbic acid formed some cell aggregations with weak Alizarin Red staining after the 6-week experiment (Figure 3J).

Noni Leaf extract enhances mineralized matrix formation in MC3T3-E1 pre-osteoblast cells

To further examine whether noni leaf extract had a similar effect on mineralized nodule formation in other osteogenic precursor cell types, the murine pre-osteoblast MC3T3-E1 cell line was employed. These cells were treated under conditions similar to hPDL cells. Nodule formation was not detected in the untreated group (Figure 4A). By the 3rd week of culture, mineralized nodules were consistently observed in the group treated by noni leaf extract, and additionally, these nodules were larger (Figure 4B) in comparison to nodules formed in the β -GP and ascorbic acid treated group (Figure 4C). After six weeks, mineralized nodules were still not found in the complete medium treated group (Figure 4D), while progressively larger, Alizarin Red stained nodules were observed in the noni leaf extract treated (Figure 4E), as well as β -GP with ascorbic acid treated groups (Figure 4F).

Elemental analysis confirms noni-treated hPDL and MC3T3-E1 derived mineralized nodules

Energy dispersive x-ray mapping under a scanning electron microscope was performed to confirm a composition of mineralized nodules deposited. Elemental analysis indicated that the nodule formation was derived from accumulation of cells and matrices (Figure 5A). Calcium (Figure 5B) and phosphorus (Figure 5C) signals were densely detected in the nodules found in the noni treated group after 6 weeks of treatment.

Discussion

Bone and periodontal tissue regeneration processes depend upon a sequence of events including cellular proliferation, osteogenic differentiation, and attachment to components of the extracellular matrices together with the organic matrix formation as well as matrix

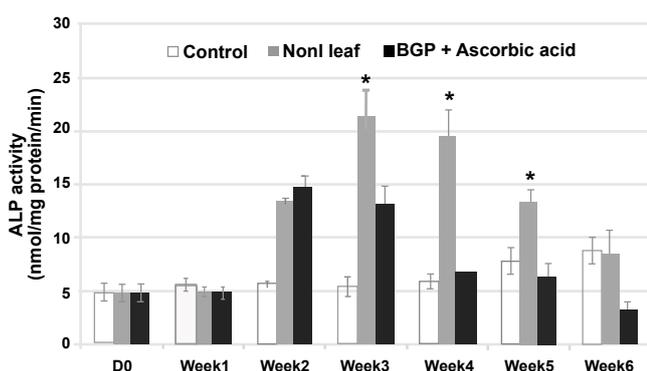


Figure 2: Noni leaf extract increases alkaline phosphatase activity in hPDL cells. ALP activity was measured in hPDL treatment groups over the course of 6 weeks in culture. ALP activity of untreated cells slowly increased over the course of 6 weeks in culture. The hPDL cells treated with 2.5% (g/ml) of noni leaf extract demonstrated increased ALP activity at the end of 2nd week, peaking during the 3rd and 4th weeks, before gradually decreasing by the end of 6th week. In the group treated by β -GP and ascorbic acid, ALP activity approximately increased during the 2nd and 3rd weeks, compared to the untreated group. The ALP activity of noni treated cells significantly increased (* $p < 0.05$), compared to both untreated and β -GP plus ascorbic acid treated cells, particularly from the 3rd to the 5th week. Values are displayed as mean \pm SD from three independent experiments.

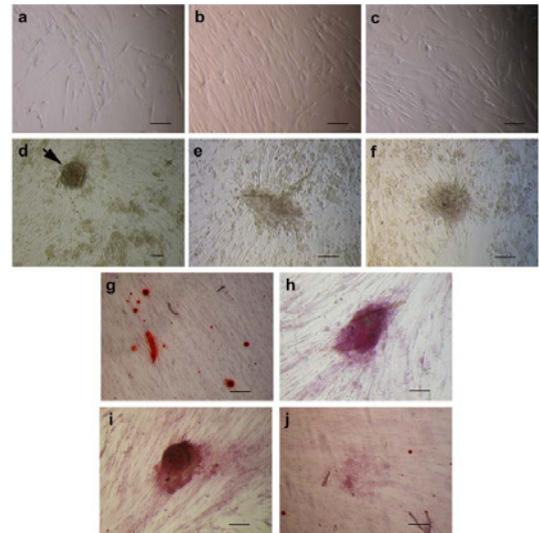


Figure 3: Noni leaf extract increases mineralized matrix formation in hPDL cells. Matrix mineralization was assayed by Alizarin Red staining after 6 weeks in culture. (A-C) Phase-contrast micrographs showed the monolayer of primary hPDL cells with spindle-like morphology after 7 days in culture with complete medium, 2.5% (g/ml) noni leaf extract, or β -GP together with ascorbic acid, respectively. (D) Formation of a cell cluster associated with a small nodule (indicated by arrow) was observed in the experimental group treated with 2.5% (g/ml) noni leaf extract after 3 weeks. (E and F) Representative nodules gradually increased in size after 4-6 weeks of the noni leaf extract treatment. (G) hPDL cells cultured in complete medium stained negative for Alizarin Red, indicating that untreated cells did not form mineralized matrix (Note that bright red spots found in the untreated group were an artifact of Alizarin Red precipitation). (H and I) Mineralized nodules formed by noni-treated hPDL cells were stained strongly positive by Alizarin Red. (J) The β -GP with ascorbic acid treated group revealed cell aggregation with only weakly positive Alizarin Red staining after 6 weeks. Scale bars indicate 300 μ m (D), and 200 μ m (A-C and E-J).

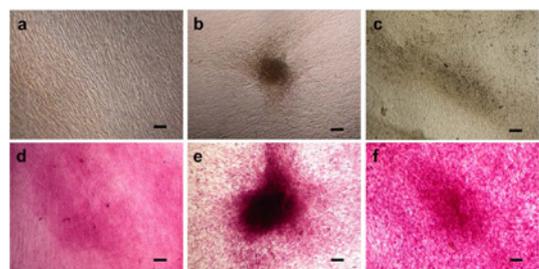
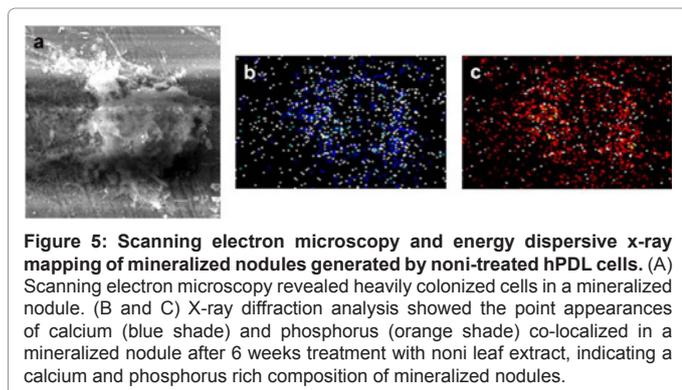


Figure 4: Noni leaf extract increases mineralized matrix formation in MC3T3-E1 pre-osteoblasts. Matrix mineralization was assayed by Alizarin Red staining after 6 weeks in culture. (A) A confluent monolayer of MC3T3-E1 cells was observed in the untreated group without any cellular nodule formation. (B and C) Formation of multilayered MC3T3-E1 cells and nodules occurred after 3 weeks with 2.5% (g/ml) noni leaf extract or β -GP with ascorbic acid, respectively. (D) Untreated cells remained unstained by Alizarin Red. (E and F) MC3T3-E1 cells treated with noni leaf extract or β -GP with ascorbic acid, respectively, produced nodules that stained positively by Alizarin Red. (E) The strongest staining was found in the noni leaf extract group at the end of the culture period. Scale bars indicate 300 μ m.

mineralization [29,30]. The ability to grow and manipulate stem cells derived from the periodontal ligament is of considerable clinical significance, especially in terms of developing novel mechanisms for achieving bone and periodontal tissue regeneration [31]. Previous findings indicated that a subpopulation of PDL cells was induced to osteoblast-like cells which were able to increase ALP activity and form calcified nodules *in vitro* when cultured in the presence of growth



factors such as transforming growth factor-beta (TGF- β) or enamel matrix derivative (EMD) [32-34]. However, results from *in vitro*, *in vivo*, and clinical studies with growth factors such as EMD have shown some variability, and also these commercially prepared and purified growth factors remain costly [34]. Therefore, we sought to determine whether a certain natural herb that has been widely and safely used in tropical folk medicines could exhibit osteogenic properties that would be potentially useful for bone and periodontal tissue regeneration.

Noni leaves have been used as an indigenous Polynesian medicine for traditional bone injury treatment [19,30], as well as a topical treatment for wound healing [24]. Some scientific studies have been performed to explore the effect of noni leaves on promoting soft tissue wound healing [24] and mitigating ultraviolet-B (UVB) light-induced injury to the skin [35]. Nevertheless, no controlled studies have been reported on the potential for noni leaves to promote bone or periodontal tissue repair and regeneration. Our study is the first investigation of noni leaf effect on tooth and bone derived cell populations, and we have demonstrated a potent ability for noni leaf extract to induce osteogenic changes in human PDL cells, *in vitro*. Noni leaf extract increased hPDL cell proliferation and protein synthesis, and additionally promoted increased ALP and mineral nodule formation. The ALP activity and mineral nodule formation is considered as the early and late markers of osteoblast cell function, respectively. In addition, noni leaf extract had a parallel effect on MC3T3-E1 pre-osteoblasts, strongly increasing mineralized matrix formation. Thus, noni leaf extract was capable of promoting differentiation and mineralization by both PDL and pre-osteoblast cell types.

Cell proliferation remains one of the cornerstone events in tissue regeneration or repair, as there must be sufficient cells to repopulate and begin to repair damaged areas. Our *in vitro* results showed that the hPDL cells treated with noni leaf aqueous extract proliferated at a rate approximately 1.2-1.5 folds higher than controls (Figure 1). This is corresponded to an *in vivo* result by Nayak et al. [24] on the wound healing effect of ethanol noni leaf extract using a rat model which showed that there were more fibroblasts in the granulation tissue when those rats treated with the noni leaf extract orally. Although our study used the different solvent to extract noni solution, we still demonstrated that the water extract of noni leaves enhanced hPDL proliferation. These results indicate that noni leaf extract has a potential effect on promoting cell proliferation not only in fibroblasts, but in this case also cells originating from PDL tissues. However, the *in vivo* effect of the water noni extract, and the comparison between the water and ethanol noni extracts will be further studied.

The present study revealed that 2.5% (g/ml) of noni leaf extract which was the highest dose in this study that enhanced hPDL cell

proliferation without any toxicity also promoted 4-fold increased ALP activity during the 3rd and the 4th weeks of the experiment (Figure 2). The result also showed that timing of peak ALP induction was consistent with mineralized nodule formation after 3 weeks of culture (Figures 2, 3D-F, 3H and I). The change of ALP activity expression of noni-treated hPDL in our study were consistent with the previous study by Aronow et al. [36] and Bellows et al. [37]. The former showed that fetal rat calvaria osteoblasts five weeks in culture expressed alkaline phosphatase was sequentially related to collagen accumulation and mineralization [36]. The latter also showed that ALP activity highly increased before mineralization, and then gradually decreased although mineralization continued in fetal rat calvaria cell cultured in osteogenic condition, indicating alkaline phosphatase plays a crucial role during early differentiation and mineralization of cells with osteoblastic phenotypes [37]. The effect of noni leaf on cell proliferation, ALP activity pattern, and mineralized nodule formation indicates their function to enhance *in vitro* osteoblast differentiation of hPDL cells.

In the β -GP together with ascorbic acid treated group, the ALP activity of hPDL cells was 2-fold greater than that of the control group during the 2nd and the 3rd weeks of experiment, and then decreased similarly to the control level (Figure 2). However, the ALP activity of this group was significantly lower than that of the noni treated group, and also no nodule formation and matrix mineralization was observed. This observation corresponded to the previous results that the nodule formation formed by PDL cells was mostly induced in certain conditions such as in the presence of dexamethasone, estradiol and EMD together with TGF- β 1 [13,34,38]. Interestingly, noni leaf treated hPDL cells were able to form mineralized nodules in culture without those inducers, suggesting an attractive property of this natural herb in mineralized tissue regeneration.

The mouse pre-osteoblast line, MC3T3-E1, was exposed to noni leaf extract in order to confirm the osteogenic differentiation and mineralized matrix induction potency of the extract on hPDL cells. Despite untreated MC3T3-E1 cells failed to produce nodules throughout the experimental period, these cells when provided with noni leaf extract or with β -GP and ascorbic acid exhibited mineralized nodules by 3 weeks (Figures 4B-C, and 4E-F). In accordance with several prior studies, β -GP together with ascorbic acid was sufficient to induce MC3T3-E1 to differentiate into osteogenic lineage and form mineralized matrices [39,40] please review ref. no. 35 & confirm did they use β -GP with ascorbic acid or not?. Consequently, our data suggest that the noni leaf extract may act as a promising osteogenic inducing material for both mouse pre-osteoblast and hPDL cells.

The noni plant consists of several phytochemical constituents that may function to enhance the osteogenic induction and matrix mineralization. Some bone stimulatory components of noni leaves have been reported, such as ascorbic acid, triterpenes, flavonoid, and others [24,30]. Ascorbic acid can promote type I collagen and α 2 β 1 integrin interaction, resulting in enhancing the expression of ALP activity [41] also, confirm did they use ascorbic acid or not?. This confirms our result that hPDL cells exhibit only a low level of ALP activity when cultured in growth medium without ascorbic acid. Therefore, ascorbic acid in noni treated group may enhance ALP activity via the pathway that induced collagen production and integrin expression in hPDL cultured cells. Subsequently, calcium and phosphorus from noni extract may accumulate in these stimulating collagen fibrils and form matrix mineralization.

The other components of noni such as triterpenes may activate cell proliferation [42], protein synthesis [43], and also ALP activity [44]

through TGF- β signaling pathway [45]. In addition, flavonoids have been shown to promote the osteogenic differentiation of human bone marrow-derived mesenchymal stem cells [46]. Consequently, analysis of some contents in the aqueous extract of *Morinda citrifolia* will be focused in our future study to confirm its active ingredients.

In conclusion, the present study provides a novel property in herbal medicine of noni leaf aqueous extract to enhance cell proliferation, ALP activity and matrix mineralization of hPDL cells *in vitro*. These data will support the further development of the noni leaf, a natural herb, as a promising osteoinductive agent for bone and periodontal tissue regeneration.

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