THE EFFECTIVENESS OF ASIATICOSIDE ON OSTEOGENIC DIFFERENTIATION ABILITY IN AGING HUMAN PERIODONTAL LIGAMENT CELLS *IN VITRO*

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Thesis Title	THE EFFECTIVENESS OF ASIATICOSIDE ON OSTEOGENIC DIFFERENTIATION ABILITY IN AGING HUMAN PERIODONTAL LIGAMENT CELLS *IN VITRO*
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อดิศา สุธิราธิกุล : ประสิทธิภาพของสารสกัดจากใบบัวบกต่อความสามารถในการแปรสภาพไปเป็นเซลล์สร้างกระดูกของเซลล์เอ็นยึดปริทันต์ชรา. (THE EFFECTIVENESS OF ASIATICOSIDE ON OSTEOGENIC DIFFERENTIATION ABILITY IN AGING HUMAN PERIODONTAL LIGAMENT CELLS *IN VITRO*) อ.ที่ปรึกษาหลัก : รศ. ทพญ. ดร.ปิยมาศ สำเร็จกาญจนกิจ

การมีฟันคงเหลือในช่องปากส่งผลต่อคุณภาพชีวิตของประชากรสูงอายุ อย่างไรก็ตามความชราภาพส่งผลต่อการแบ่งตัวเพิ่มจำนวนและการฟื้นฟูสภาพของเอ็นยึดปริทันต์ เอเชียติโคไซด์ (Asiaticoside) ซึ่งเป็นสารสกัดจากใบบัวบก ถูกพบว่ามีความสามารถในการกระตุ้นเซลล์เอ็นยึดปริทันต์ให้แปรสภาพไปเป็นเซลล์สร้างกระดูกและเกิดการสร้างแร่ธาตุซึ่งเป็นองค์ประกอบของกระดูกขากรรไกร การวิจัยนี้จึงมีจุดประสงค์เพื่อศึกษาผลของเอเชียติโคไซด์ต่อการแปรสภาพไปเป็นเซลล์สร้างกระดูกและเกิดการสร้างแร่ธาตุของเซลล์เอ็นยึดปริทันต์ชรา โดยเซลล์ชราจะถูกสกัดจากฟันที่ถอนจากคนไข้อายุตั้งแต่ 60 ปีขึ้นไป และยืนยันสถานะชราด้วย Senescence-Associated β -Galactosidase assay เซลล์เอ็นยึดปริทันต์ชราจะถูกเลี้ยงในเอเชียติโคไซด์ความเข้มข้นต่างๆ เป็นเวลา 72 ชั่วโมงเพื่อทดสอบความเป็นพิษต่อเซลล์ด้วย MTT assay หลังจากนั้นเซลล์จะถูกเลี้ยงในเอเชียติโคไซด์เป็นเวลา 1 และ 7 วัน เพื่อดูระดับการแสดงออกของยีนที่เกี่ยวข้องกับการแปรสภาพไปเป็นเซลล์สร้างกระดูก โดยใช้เทคนิกปฏิกิริยาลูกโซ่โพลิเมอเรสชนิด Real time การเกิดแร่ธาตุจะถูกยืนยันด้วยการย้อมสี alizarin red จากผลการวิจัยพบว่า เอเชียติโคไซด์ความเข้มข้น 12.5 และ 25 μM ไม่ส่งผลต่อการมีชีวิตและรูปร่างของเซลล์ พบการเพิ่มขึ้นอย่างมีนัยสำคัญของยีน *BMP9, RUNX2* และ *OSX* ในเอเชียติโคไซด์ความเข้มข้น 2.5 μM เมื่อเซลล์ถูกเลี้ยงไปได้ 1 วัน ($P<0.05$) ในวันที่ 7 พบการเพิ่มขึ้นอย่างมีนัยสำคัญของยีน *DMP1* และ *BMP2* อย่างมีนัยสำคัญ ($P<0.05$) ที่เอเชียติโคไซด์ความเข้มข้น 25 μM ในทางกลับกัน ระดับของยีน *COL1* มีระดับการแสดงออกลดลงอย่างมีนัยสำคัญ เมื่อเซลล์เอ็นยึดปริทันต์ชราถูกเลี้ยงไปจน 14 วันพบการเพิ่มขึ้นของแร่ธาตุอย่างมีนัยสำคัญ จากผลการวิจัย แสดงให้เห็นว่าเอเชียติโคไซด์สามารถกระตุ้นการแปรสภาพไปเป็นเซลล์สร้างกระดูกของเซลล์เอ็นยึดปริทันต์ชรา

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KEYWORD: Aging, Asiaticoside, Osteogenic differentiation, Periodontal ligament

Adisa Suthirathikul : THE EFFECTIVENESS OF ASIATICOSIDE ON OSTEOGENIC DIFFERENTIATION ABILITY IN AGING HUMAN PERIODONTAL LIGAMENT CELLS *IN VITRO*. Advisor: Assoc. Prof. PIYAMAS SUMREJKANCHANAKIJ, D.D.S., Ph.D.

Retaining teeth is essential for the quality of life of the aging population. However, aging has biological effects in periodontal ligament cells with significant changes in their proliferative rate and regenerative function. Asiaticoside, extracted from *Centella asiatica*, was found to have potential in osteogenic differentiation and mineralization in human periodontal ligament cells (HPDLCs). The aim of this study is to investigate the effect of asiaticoside on the aging HPDLCs osteogenic differentiation and mineralization. Aging HPDLCs, retrieved from patients age 60 and above, were confirmed aging status using Senescence-Associated β -Galactosidase assay. The cells were incubated with various concentrations of asiaticoside to test cell viability by MTT assay for 72 hours. After treating cells with asiaticoside for 1 and 7 days, the mRNA expression of osteogenic genes was analyzed by real-time polymerase chain reaction (PCR). Alizarin red was performed to evaluate the osteogenic differentiation and matrix mineralization. The results showed asiaticoside at concentrations 12.5 and 25 μM has no effect on cell viability and morphology. On day 1, the expression of *BMP9, RUNX2* and *OSX* in 2.5 μM asiaticoside was significantly upregulated ($P<0.05$). Interestingly, *DMP1* and *BMP2* mRNA expressions in 25 μM asiaticoside were significantly enhanced at day 7 ($P<0.05$), while *COL1* expression was decreased markedly. On day 14, Matrix mineralization was also significantly promoted ($P<0.05$). The results suggest asiaticoside can promote osteogenic differentiation in aging HPDLCs.

Field of Study:	Geriatric Dentistry and Special Patients Care	Student's Signature .....
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My last salutations are to my family who knew me imperfect and loved me.

Adisa Suthirathikul

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CHAPTER I
INTRODUCTION

Background and Rationale

Elderly individuals tend to retain more teeth due to improvements in oral health care in developed societies such as Thai society. Retaining teeth is essential for proper quality of life among elderly individuals (1). Noncommunicable chronic diseases, such as diabetes mellitus, cardiovascular disease, metabolic disease and neurodegenerative disease, alongside medications affect oral health status (2). There are also strong evidences showing a relationship between these chronic diseases and periodontal disease (2, 3). The periodontal ligament (PDL) is one of the periodontal structures that plays an important role in supporting teeth and transmitting and absorbing mechanical stress (4). The PDL also regulates nutritive function, homeostasis function and reparative function (5-7). Human Periodontal ligament cells (HPDLCs) are mesenchymal origin, can differentiate into osteoblasts, express bone–associated proteins and synthesize mineralization nodules (8). However, aging has biological effects in HPDLCs with significant changes in their proliferative rate and cellular function (9). From several studies of aging HPDLCs *in vitro*, the expression rate of alkaline phosphatase and the genes involved in osteogenic differentiation are decreased when comparing to younger HPDLCs (10-13). The lower rate of osteogenic differentiation of aging HPDLCs affects the efficiency of periodontium repair. Hence, elderly individuals with periodontitis have a high tendency toward tooth loss, accomplished by the destruction of periodontal attachment and alveolar bone support. These incidents combined with poor oral hygiene care due to insufficient oral health literacy or impaired motor function can result in tooth loss. Tooth loss can lead to low chewing efficiency, poor nutritional status, frailty and mortality.

To protect PDLC secretory function and associated bone remodeling upon pathogenic stimuli during aging, there is a critical need to find natural products or bioproducts that have the potential to rescue the expression of essential proteins involved in osteogenesis. Asiaticoside, extracted from *C. asiatica.* is a natural bioproduct that can improve the maturation of matrix status in *in vitro* studies. Asiaticoside possess good wound healing activity, remodels scar tissue and improves cognitive deficit (14-17). Asiaticoside can also protect neurons and has antidepressant activity (18, 19). Asiaticoside can inhibit proinflammatory mediators and promote antitumor activity (20). Additionally, asiaticoside can increase the level of type I collagen synthesis in skin fibroblasts (16). In terms of the periodontal ligament, asiaticoside in osteogenic medium can increase alkaline phosphatase (ALP) activity,

which is a marker of bone calcification, *in vitro* (21). There is marked evidence showing that the level of the genes essential for osteogenic differentiation are increased when HPDLCs are treated with asiaticoside (22). However, there is no evidence to support the effect of asiaticoside on aging HPDLCs. Therefore, this study aims to investigate the potential of asiaticoside on osteogenic differentiation and mineralization of aging HPDLCs.

Research questions

Question 1: Can asiaticoside promote osteogenesis- related gene expression in aging periodontal ligament cells *in vitro?*

Question 2: Will the mineralization nodule capability increase after treating aging periodontal ligament cells with asiaticoside *in vitro*?

Research objectives

1. To determine the expression of osteogenesis-related genes upon asiaticoside *in vitro* treatment in aging HPDLCs.
2. To determine the capability of mineral deposition upon asiaticoside *in vitro* treatment in aging HPDLCs.

Research hypotheses

1. Asiaticoside induces osteogenesis-related gene expression in aging HPDLCs.
2. Asiaticoside induces mineralized nodule formation in aging HPDLCs.

Scope of research

This research aims to investigate the osteogenic differentiation of aging human periodontal ligament cells *in vitro*. Aging HPDLCs were retrieved from patients over 60 years old. The aging status was confirmed by senescence-associated β-galactosidase assay (SA-β-gal) using a β-galactosidase staining kit (Cell Signaling Technology, Inc., Danvers, MA). Then, the cells were treated with the asiaticoside. The viability of cells in asiaticoside at various concentrations was determined using the MTT assay at 72 hours. Osteogenic gene biomarkers that play important roles in osteoblastic differentiation and bone formation, including runt-related transcription factor 2 (*RUNX2*), osterix (*OSX*), dental matrix protein 1 (*DMP1*), alkaline phosphatase (*ALP*), bone morphogenic protein 2 (*BMP2*), bone morphogenic protein 9 (*BMP9*) and osteocalcin (*OCN*) mRNA expression, were determined using real-time polymerase

chain reaction (PCR) at day 1 and 7. Mineralized nodule formation was determined using Alizarin Red staining at day 14. All outcomes were compared with a control group without asiaticoside media supplementation.

Limitation

Although the primary cell culture model can give results relevant to human physiology, it is still a preliminary study. The results of the experiments need to be confirmed in *in vivo* experiments.

Expected outcome

As per previous studies (26,35), it has been demonstrated that asiaticoside can be an effective treatment for inducing osteogenesis differentiation of normal HPDLCs (at early passage) *in vitro*. Thus, I can expect that asiaticoside can also enhance osteogenesis differentiation in aging PDL cell *in vitro*. All *in vitro* the data generated from this project will lead to translational research to test the asiaticoside in *in vivo* periodontal disease models in small animals.

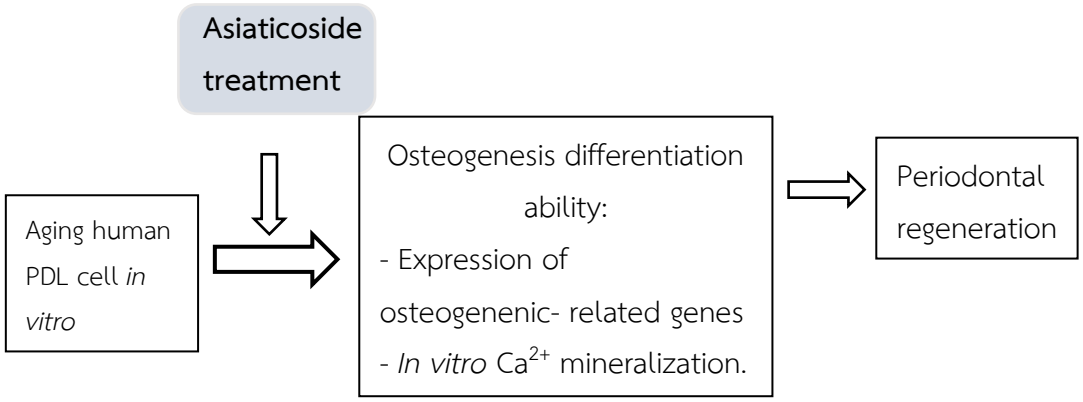
Keywords

aging, asiaticoside, osteogenesis differentiation, periodontal ligament

Research design

Laboratory research

Conceptual framework



CHAPTER II
LITERATURE REVIEW

Aging population and dentition

The aging population has become a challenge in oral health promotion due to gradual changes in population demographics. The World Health Organization (WHO) reported that there are 600 million people aged 65 and above. This number is expected to double by 2025 and will reach 2 billion people by 2050 (23). In contrast with the increasing elderly population, with effective oral health promotion and prevention tactics, edentulism status is dramatically decreased (24). Noncommunicable chronic diseases such as diabetes mellitus, cardiovascular disease, metabolic disease and neurodegenerative disease, have become trends in the aging population. These diseases along with medications affect oral health status. Diabetes mellitus and cardiovascular disease are recognized to be associated with periodontal disease, which is a common oral problem in the aging population (3). There is also strong evidence showing a relationship between respiratory disease, Alzheimer's disease, and periodontal disease (2). Drugs used in hypertension and antidepressants can cause xerostomia, leading to caries (25). These incidents combine with poor oral hygiene care due to insufficient oral health literacy or impaired motor function, resulting in tooth loss. Oral health problems can affect quality of life. Nutritional status will be compromised as an effect of chewing efficiency decline. Poor masticatory function, as a result of dental pain or reduction in masticatory ability, is also associated with frailty progression.(1) Poor esthetic and speech function problems will also be a consequence (3, 26).

The Periodontal Ligament (PDL)

The periodontal ligament (PDL) is a dense fibrous connective tissue located between the cementum and the alveolar bone (7). Before dental eruption, the PDL is produced from fibroblasts in dental follicles. It starts to develop into collagenous fibers during tooth development. The fibers insert themselves in the cementum matrix, apically to the cementoenamel junction and align horizontally within the coronal- third of the root surface. In the middle third of the root, the fibers change to run obliquely to the alveolar bone. At the apical-third, the fibers run apically to the alveolar bone (6). The width of the periodontal ligament, crossing the ligament space is ~0.15-0.38 mm. depending on the tooth type.(27) The PDL gains blood

supply from the superior and inferior alveolar arteries, which pass through the alveolar bone. It also has venous drainage and lymphatic vessels. PDL vascularization is important in wound healing and homeostasis. The nerve fibers, superior and inferior branch alveolar nerves, are generally found in the outer part of the ligament space. The branch of single nerve fibers will develop from the main nerve bundles and run toward the cementum to supply mechanoreceptors (6).

The PDL has an important role in supporting teeth and transmitting and absorbing mechanical stress. The viscoelastic behavior of the ligament modifies the fibers to withstand the transmitted occlusal load (4). The PDL also regulates nutritive function, homeostasis function and reparative function (5-7). The PDL provides nutrition to the cells in the periodontium through blood vessels. The absorption of occlusal force protects the nerves and blood vessels from injury and tooth movement. Homeostasis between the osteogenic fibroblasts and the root surface is one way to maintain the width of the PDL. If this hemostasis is interfered with, ankylosis may result. Shimono et al. (1998) stated that the PDL has intensive regenerative capability. PDL cells can proliferate, migrate and differentiate into osteoblasts, cementoblasts and fibroblasts, producing alveolar bone, cementum and periodontal ligament, respectively (5, 8).

Periodontal ligament cells are heterogeneous. They possess many diverse cell-type characteristics. Those typical for fibroblasts will produce collagen and extracellular matrix components. Fibroblasts are the main cell type in the PDL (~50-60% of the cell popularity) (6). Fibroblasts responsible for building up the ligament and the fibers, allow the regeneration of the PDL to occur upon injury. PDL cells also showed an osteoblastic phenotype. The cells can differentiate into osteoblasts, express bone- associated proteins and synthesize mineralization nodules.(28) The subpopulation of the cells also contains stem cell-like traits that can differentiate into cementoclasts and cementoblasts (6). This property is responsible for resorbing and repairing the cementum and root dentine (9).

In addition to their fibroblastic and osteogenic properties, PDL cells also function similarly to leukocytes and leukocyte-like cells which are involved in innate immunity such as macrophages. The cells produce inflammatory cytokines and chemokines in response to inflammatory stimulation (29, 30). Jonsson, D., et al. (2010) reviewed cytokine and chemokine expression in proinflammatory stimulated human periodontal ligament cells. After treatment with low, intermediate and high levels of bacterial lipopolysaccharide (LPS), the periodontal ligament produces IL-1β, IL-8, IL-6 and TNFα at the RNA and protein levels. (28)

Aside from PDL cells, the PDL also consists of osteoclasts, which originate from monocytes within the blood vessel and are found in areas where alveolar bone and cementum are reabsorbed, and epithelial cells rest of Malassez, which occur near the cementum and are responsible for maintaining the PDL width (6).

The potential of PDL to differentiate osteoblasts is essential in bone formation. Osteoblasts are bone specific mesenchymal cells. They also expresses genes and proteins that are necessary to induce mineralization (31). Osteoblastic differentiation is undergoing three phases and various genes compromising one development are expressed as follows:

(a) Cell proliferation - During this stage, there is an increase in the expression of growth-regulating genes (*histone, c-fos, c-myc*)(32) and extracellular matrix genes (procollagen-1, fibronectin, transforming growth factor (*TGF- β1*). Runt-related transcription factor 2 (*Runx2/Cbfa1*), an essential gene in osteoblastic differentiation also plays a potential role in this stage. *Runx2* promotes the expression of major bone genes such as alkaline phosphatase (*ALP*), osteocalcin (*OCN*), osteopontin (*OPN*) and bone sialoprotein (*BSP*) (33).

(b) Extracellular matrix maturation and collagen synthesis – In this stage, DNA synthesis and the proliferation rate decline. The extracellular matrix modified its composition, making it competent for mineralization. ALP activity is maximum in this stage (34). ALP is a metalloenzyme, that is believed to increase inorganic phosphate. It is one of the earliest markers of osteoblastic differentiation (35).

(c) Matrix mineralization – There is an increase in the expression of bone cell-specific proteins, such as *OCN*, *BSP* and *OPN*, in concert with the accumulation of minerals (31).

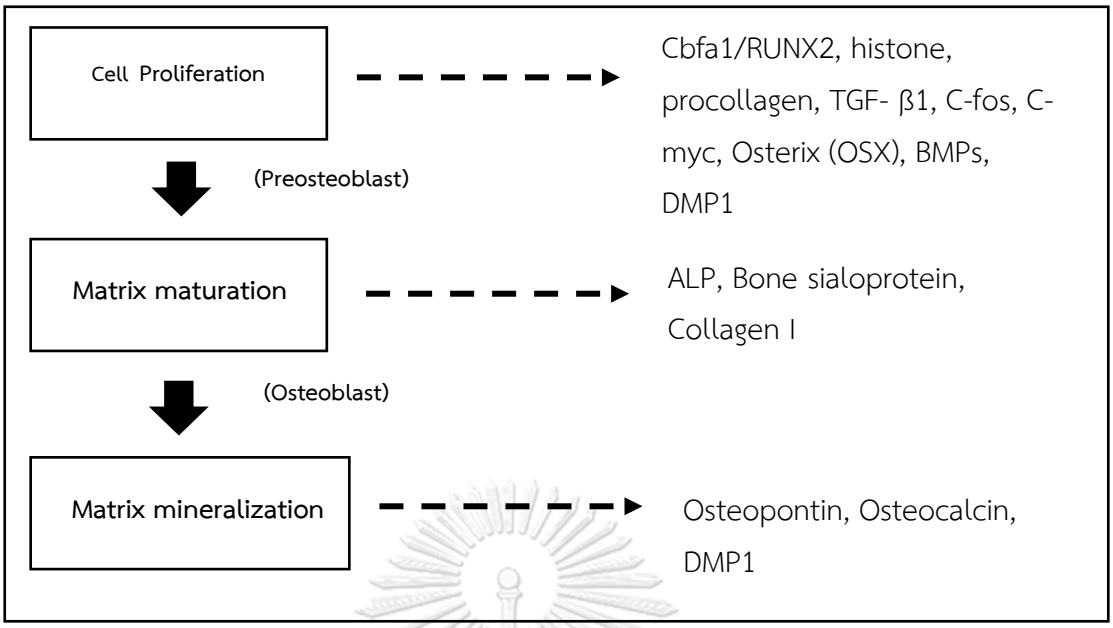


Figure 1 *Gene expression during the osteogenic differentiation process*

Several signaling pathways play roles in the complete process of osteoblast development. Wnt/β-catenin is considered to be an important signaling pathway for bone formation via stimulation of osteoblast development. Higher levels of β-catenin increase the expression of osteoblast-specific genes. It is essential for the preosteoblast stage and for mature osteoblast differentiation (31). Bone morphogenic proteins (BMPs) as well as TGF- β, transmit signals through the Smad pathway to regulate the transcription of osteoblastic genes (Figure 2). Smad 1, Smad 5 and Smad 8 are activated and phosphorylated after binding with BMP type I receptor and then form heterodimers to enter the nucleus and upregulate osteoblast transcription factors such as RUNX2 or OSX(36). BMPs are regulating factors that induce mesenchymal stem cells to differentiate into osteoblasts and increase the number of mature osteoblasts through membrane receptors and the BMP signaling pathway. BMP2 plays a significant role in regulating the transcription of osteogenic genes such as *RUNX2, ALP, Collagen I (COL1), OCN* and *OSX* (37). The *BMP2* gene was found to have the highest level expression on day 7 (34). BMP9, known as growth different differentiation factor 2 (GDF2), has been demonstrated to be one of the most osteogenic BMPs. Peptides derived from BMP9 increase ALP activity in a dose-dependent manner and effectively activate other osteogenic genes (38). The aging population was found to have high expression levels of Pleckstrin homology domain-containing family O member 1 (PLEKHO1), a ubiquitination-related molecule that negatively regulates the levels of SMAD 1/5 and the BMP signaling pathway (39).

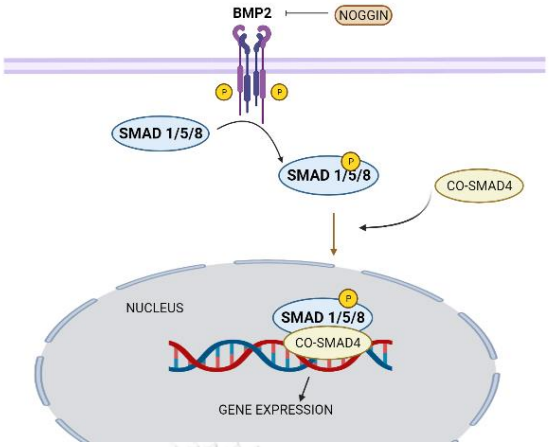


Figure 2 *BMP2/SMAD pathway*

Mineralization process

The mineralization process starts when calcium phosphate hydroxyapatite is formed in matrix vesicles. The calcium transporter, annexin V and sodium-dependent phosphate cotransporter, Pit-1, play the main role in transporting extracellular inorganic phosphate (Pi) and calcium to the vesicle (40). Ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1), ankylosis progressive protein in human (ANKH), and isoenzyme of ALP, known as tissue nonspecific ALP (TNAP) are crucial proteins in generating Pi. ENPP1 is a membrane-bound enzyme that hydrolyzes ATP to produce inorganic pyrophosphate (PPi) (41, 42). ANKH is a nonenzymatic transmembrane protein that transports intracellular PPi to the extracellular matrix (43). Inorganic pyrophosphate is hydrolyzed and degraded to Pi by TNAP and transported into vesicles, initiating mineralization (41, 44). The accumulation of Pi and PPi ratio regulate calcification. PPi inhibits hydroxyapatite formation by downregulating ALP activity and the expression of osteogenic genes such as *RUNX2, OSX* and dental matrix protein 1 (*DMP1)* (45). Excessive levels of PPi can lead to pathologic calcification of nonbone tissue (42). Aside from the TNAP hydrolyzing mechanism, extracellular Pi can be taken up by systemic regulation of diet, parathyroid and renal function. Pi promotes the formation of hydroxyapatite in matrix vesicles and upregulates collagen content (40). Excessive levels of Pi can lead to calcification overgrowth. However, a high level of Pi can present negative feedback on TNAP by downregulating ALP activity to balance the accumulation of the Pi/PPi ratio (40, 41).

Since ENPP1 and ANKH generate PPi, the level of these proteins affects the differentiation and mineralization. Loss of ENPP1 function enhances collagen I

synthesis.(45) Loss of ANKH function suppresses osteoblastic differentiation by reducing osteogenic genes and extracellular PPi reduction (43, 46). Genetically engineered *ENPP1*-deleted mice that consume high phosphate diet can suppress the anti-aging protein, Klotho, and exhibit premature aging phenotypes such as calcification in the kidney, arteriosclerosis and osteoporosis, reducing bone mass (47). For periodontal tissue, the lack of PPi due to ENPP1 and ANKH deficiency led to drastic changes in the periodontium. There is significant growth of the cementum and enhanced periodontal ligament volume and thickness (48-51). In contrast, the PDL width is maintained due to an insignificant reduction in alveolar bone volume with no alteration of bone density (49, 50). The change in cementum level, alveolar bone and PDL space as mentioned can imply that loss of ANKH and ENPP1 does not result in dental ankylosis. Nevertheless, few reports show that the PDL space has a tendency to be reduced (48). The alveolar bone density is significantly reduced but the osteoid volume is increased significantly (51).

Aging and periodontal ligament cells

Oral aging is characterized by diminished keratinization and a thinner oral epithelium, resorption at the cementum surface and a reduced number of cells in the osteogenic layer of the alveolar bone (52). Moreover, aging further decreases the number of cycling cells (cells entering the DNA synthesis phase), particularly those derived from periodontal tissues. The low number of cycling cells, results in a significant reduction in the proliferative periodontal ligament cell population (9), which will impact collagen matrix production and limit periodontium regeneration.

Evidence has shown that aged HPDLCs have a decreased rate of cell proliferation. Aging HPDLCs have lower level of the c-fos protooncogene which is essential for DNA synthesis and regulates cell proliferation and apoptosis, than younger HPDLCs (53). This results in a lower growth rate of aging HPDLCs (54).

There are alterations in inflammatory mediators at different levels of PDL protein contents by aging. The levels of *IL-1β, IL-4* and *IL-6* in HPDLCs from aging donors are significantly higher than those from young donors (54). When compressive force is applied, aging HPDLCs show greater levels of prostaglandin E_2 (*PGE_2*) and cyclooxygenase-2 (*COX-2*) (55). PGE_2 has a recognized role as a proinflammatory agent and has an indirect effect on alveolar bone resorption. It is produced by arachidonic acid through COX (9).

A consistent number of studies have shown that aged HPDLCs lose their wound healing and osteogenic potential properties. The chemotactic response which

is an essential mechanism in the wound healing process against basic fibroblast growth factor (b-FGF) in aging HPDLCs is significantly decreased (10). The expression rate of b-FGF in aging HPDLCs is also decreased, resulting in declining healing ability and promoting regeneration and vascularization of HPDLCs (56). Studies have reported a decrease in the expression rate of alkaline phosphatase (12, 13). Osteopontin, osteocalcin and collagen type I also decrease in aging HPDLCs (13, 52). However, bone resorption inhibitor (Osteoprotegerin- OPG) is significantly increased and the level of RANKL (osteoclast activated protein) is not affected by aging (54). This may indicate that the aged PDL still has potential against bone resorption.

As a result of the lower proliferative rate of cells, the higher level of cytokines responsive to inflammation and the declining rate in collagen and osteogenic production, along with the long-term accumulation of dental plaque and calculus, lead to accelerated periodontium destruction in aging. Periodontitis is a disease manifesting in the irreversible destructive periodontium and alveolar bone caused by bacteria that colonize the subgingival area. This noncommunicable chronic disease ranks in the top ten most prevalent chronic conditions in the world. The prevalence of the disease is related to aging, lower income and education, as well as several systemic diseases (57, 58). Poor oral hygiene or lower capability in oral hygiene care with age is leads to the deposition of bacteria on teeth. The metabolic products of bacteria trigger the adaptive and innate immune system. Inflammatory cytokines and inflammatory-related cells such as neutrophils, macrophages and plasma cells stimulate inflammation of the gingiva and periodontium and enhance the irregularity of blood flow. Some of the inflammatory cytokines, IL-1ß and TNF-α promote osteoclastogenesis (59). The ultimate result of the whole lesion process is tooth loss, which is the initiation of poor masticatory function, malnutrition and low quality of life.

To promote oral hygiene care, in addition to mechanical cleansing, we used chemical agents. Mouth rinses contain fluorides, antimicrobial substances and alcohol. We also used antimicrobial agents such as antibiotics (e.g., erythromycin, amoxycilin, metronidazole) and toothpaste which contains fluoride, calcium carbonate and sodium lauryl sulfate. However, some of these chemical agents cause side effects, such as mucosal burning and tooth staining or develop resistance to antibiotics (60). The rise of herbal medicine or natural products has attracted interest. Many natural substances improve periodontal health status. Honey and Raisins have activity against *Porphyromonas gingivalis*, the main bacteria in periodontitis (61). Ginko and Propolis can promote fibroblast synthesis and reduce the inflammatory

cytokines involved in osteoclastogenesis (62). Pomegranate and chamomile extract mouthwash can reduce gingival inflammation (63).

Centella Asiatica and Asiaticoside

Centella Asiatica (C. asiatica), commonly known as Indian Pennyworth, has been used in traditional medicine for hundreds of years in China, India, Malaysia, Madagascar, Nepal and Sri Lanka. *C. asiatica,* belonging to the family Apiaceae or Umbelliferae, is 15-20 cm. long perennial herb, with a glabrous, striated stem (Figure 3). The leaves are green, round or fan-shape with 15.5-5 cm. long and 2-6 cm. wide. This plant is commonly seen in damp, moist, shady and wet places such as riverbanks. It can be found in Africa, Australia, Cambodia, Central America, Madagascar, Thailand, Vietnam, South America and the Pacific Islands. The flowers are white, purple or pink and occur in July- September. The fruits are approximately 2 inches long, globular shape with a thickened pericarp. The most commonly used parts for medical purposes are leaves, stems and dried whole plants (18, 64, 65). In addition to being used as an herbal medicine, *C. asiatica* is also used as a vegetable and drinks, such as tea and juice, for cooling inner heating and thirsty quenching (66). *C. asiatica* contains triterpenoids (saponins) composed of Asiaticoside, Asiatic acid, Madecassoside and Madasiatic acid. Other biological compounds are volatile compounds, fatty oils, alkaloids, and flavanoids and present some amino acids such as glycine, glutamic acid and aspartic acid. *C. asiatica* also consists of vitamins B and C, vallerine (a bitter component), phytosterols and abundant tannins (65, 67, 68). These extracts from *C. asiatica* have been found to be useful in wound healing by enhancing collagen type I synthesis. They also have potential in cytotoxic, antitumor, antioxidant, anti-allergic, anti-pruritic, anti-inflammatory, anti-convulsant, anti-depressant and neuroprotective activities (14, 18, 69).

Asiaticoside is a triterpenoid derivative, that is a natural compound extracted from *C. asiatica*. It has pentacyclic configuration formed by a 30-carbon skeleton and a glycosylated side chain (Figure 4). Asiaticoside possess good wound healing activity. Asiaticoside promotes the migration rates of dermal cells and increases the number of normal human dermal fibroblasts, contributing to remodeling of scar tissues (15). Asiaticoside also induces the Smad signaling cascade, leading to an increase in type I collagen synthesis in fibroblasts (16). Asiaticoside has been shown to improve cognitive deficits by reducing acetylcholinesterase activity in aging mice and decreasing the free radicals such as nitric oxide which can cause neurodegenerative process (17). Asiaticoside can also protect neurons and has antidepressant activity

(18, 19). For anti-inflammatory purposes, asiaticoside can inhibit serum TNF-α and IL-6 which are proinflammatory cytokines and can also upregulate anti-inflammatory molecule (IL-10) and heme oxygenase-1 (HO-1) (70). Moreover, asiaticoside can promote anti-tumor activity by decreasing the expression of tumor necrosis factor-alpha (TNF-α) and IL-1β which are cytokines involved in regulating tumor-associated inflammation and tumor cell proliferation, differentiation and apoptosis (20).

In terms of osteogenic purposes, HPDLCs treated with asiaticoside have increased ALP activity. Other osteogenic genes such as *BSP, COL1, OSX* and *DMP1*, were also upregulated in HPDLCs (21, 22). Asiaticoside can also inhibit RANKL-induced osteoclast formation by suppressing RANKL- induced osteoclastogenesis via Ca^{2+} signaling, NFAT and the nuclear factor- kappa b pathway (71). Furthermore, the numbers of cells treated with asiaticoside at various concentrations were not altered compared with the control. The results suggested that asiaticoside is not cytotoxicity or cytoproliferative (21, 22, 71). Thus, it may be used as a potent osteoinduction agent. However, there have been no studies on the effect of asiaticoside in aging HPDLCs. To elucidate its capability, this research is performed.

Figure 3 *Centella Asiatica (72)*

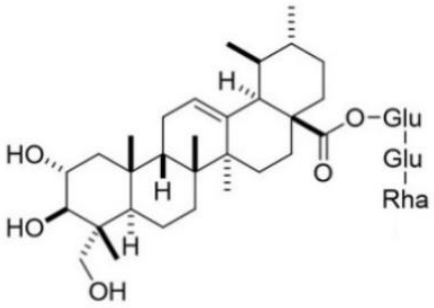


Figure 4 *Structure of asiaticoside*

CHAPTER III
RESEARCH METHODOLOGY

Asiaticoside preparation

Asiaticoside powder (Sigma-Aldrich, St Louis, MO) was dissolved in Dimethyl sulfoxide (DMSO) for cell culture (Sigma-Aldrich, St Louis, MO) to prepare the stock solutions. When used, the stock solutions was diluted in culture medium to final concentrations of 12.5, 25 and 50 μM(22). Cells incubated with the highest concentration of DMSO were used as control in the experiments.

Aging HPDLCs isolation and culture

Human periodontal ligament cells (HPDLCs) were isolated from the teeth of patients aged 60 and above who had their teeth extracted according to the dental treatment plan from April 2020 – December 2020. Based on clinical and radiographic examination, the teeth with healthy periodontium without the presence of tooth mobility, periodontal infection, alveolar bone loss were included (Figure 5).

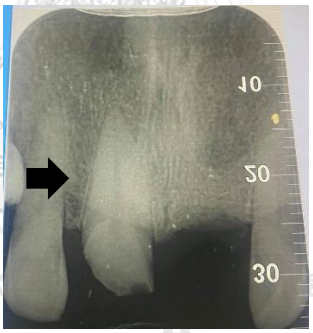


Figure 5 *Example of selected tooth*

The upper right incisor showed tooth fracture with clinically pulp exposure without any symptoms. Patient preferred extraction to root canal treatment. Radiographic showed normal periodontium.

All experimental procedures and biosafety were approved from the Human Research Ethics Committee (HREC-DCU 2020-033) and the Institute biosafety committee, Faculty of Dentistry, Chulalongkorn University (DENT CU-IBC 009/2020). Briefly, the extracted tooth was rinsed with phosphate buffered saline (PBS). Periodontal ligament tissue was scraped from the middle third of root surface and transferred to 35 mm. culture dish. The tissue will be covered with Dulbecco's modified Eagle's medium (DMEM) supplemented containing with 10% fetal bovine

serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and 5 mg/ml amphotericin B (Gibco, Carlsbad, CA) and incubated in an 85% humidified incubator at 37°C and 5% CO_2 to permit attachment of explants. The medium was replaced until sufficient HPDLCs proliferation was evident. Then, the cells were seeded in 60 mm. culture dish and cultured in an 85% humidified incubator at 5% CO_2, and 37°C. The medium will be changed every 2 days. Each of the experiments was performed in triplicate using cells from three individual teeth (Passage 4-7).

Identify senescence status

The senescence status was confirmed by Senescence-Associated β - galactosidase assay (SA- β - gal) using the β -galactosidase staining kit (Cell Signaling Technology, Inc., Danvers, MA). According to the manufacturer's protocol, HPDLCs were plated in 24 well plates. Cells were washed with PBS and fixed in 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 10-15 minutes. Then HPDLCs were washed twice with PBS. The staining solution was added to a culture dish (1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactosidase, 40 mM citric acid [pH 6.0], 40 mM sodium phosphate [pH 6.0], 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, and 2 mM magnesium chloride). The stained HPDLCs were incubated at 37°C without CO_2 overnight. The blue color will be detected under a light microscope (ImageJ software, version, NIH, USA)(73).

Viability test

Cell viability was determined using the MTT assay. First, HPDLCs were trypsinized using 2.5% trypsin-EDTA. After stopping trypsin activity with 10% DMEM, the number of viable cultured HPDLCs was counted with a hemocytometer chamber (Thermo Fisher Scientific, Waltham, MA). HPDLCs were seeded in 24 well plates and incubated for 1 day at 10^5 cells per well. HPDLCs were treated with asiaticoside at concentrations of 12.5, 25 and 50 μM in 10% DMEM for 3 days. The control was cells incubated with DMSO. Then, the medium was removed and the cells were washed with phosphate buffered saline (PBS). The PBS was substituted with 300 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (USB Corp., Cleveland, OH, USA) solution and incubated for 30 minutes to 1 hour at 37°C. After that, the formazan crystals were eluted by 1 ml. of 1:9 DMSO/glycine buffer, including the control. The absorbance will be measured at 570 nm using a microplate reader

(Elx800; Biotek, Winooski, VT). The percentage of cell viability compared with the control was calculated.

Osteogenic gene expression

HPDLCs were seeded in 12 well plates and treated with 2.5 and 25 μM asiaticoside. The cells were cultured in osteogenic medium containing 50 μg/mL ascorbic acid (Sigma-Aldrich, St Louis, MO) and 5 mM β -glycerophosphate (Sigma-Aldrich, St Louis, MO) for 1 and 7 days. The medium was changed every 2 days. The optimized concentration of asiaticoside was added from the cell viability experiments. Total cellular RNA was extracted using TRIZOL® reagent (Thermo Fisher Scientific, Waltham, MA). RNA samples 1 μg was converted to cDNA using a reverse transcriptase kit (ImProm-II Reverse Transcription System, Promega, USA). Oligonucleotide primers of the specific genes, as in Table 1, were designed and synthesized by Thermo Fisher Scientific. Real-time PCR was performed to detect these target genes using a SYBR green detection system (Fast Start Essential DNA Green Master; Roche Diagnostic, Indianapolis, IN). *18S* ribosomal RNA primer was used as housekeeping gene. The PCR protocol is as follows: denaturation at 94°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s for 45 cycles. The relative gene expression was analyzed using software (CFX Manager software, Bio-Rad)

Table 1 *Oligonucleotide primers*

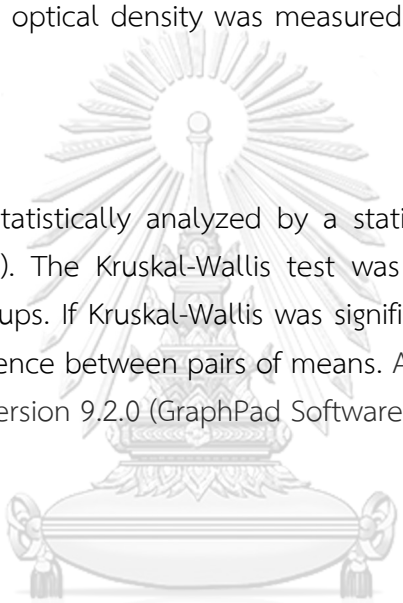
Gene	Primer sequence 5′⟶ 3′	Accession No.
ALP	Forward: CGA GAT ACA AGC ACT CCC ACT TC Reverse: CTG TTC AGC TCG TAC TGC ATG TC	NM_000478.3
BMP2	Forward: GCG TGA AAA GAG AGA CTG Reverse: CCA TTG AAA GAG CGT CCA C	NM_001200.4
BMP9	Forward: CCT GGG CAC AAC AAG GAC Reverse: CCT TCC CTG GCA GTT GAG	NM_016204.4
OCN	Forward: TGA CGA GTT GGC TGA CCA Reverse: GCC GTA GAA GCG CCG ATA GGC	NM_199173.4
OSX	Forward: GCC AGA AGC TGT GAA ACC TC Reverse: GCT GCA AGC TCT GCA TAA CC	NM_001300837.1
RUNX2	Forward: CAG ACC AGC AGC ACT CCA TA Reverse: CAG CGT CAA CAC CAT CAT TC	NM_001278478.1
DMP1	Forward: ATG CCT ATC ACA ACA AAC C Reverse: CTC CTT TAT GTG ACA ACT GC	NM_004407.3
18S	Forward: GGC GTC CCC CAA CTT CTT A Reverse: GGG CAT CAC AGA CCT GTT ATT	NR_003286.2

Analysis of mineralized nodule formation

Alizarin Red S staining was conducted to evaluate mineralized nodule formation. Briefly, the cells maintained in the asiaticoside and osteogenic induction medium for 14 days were fixed in 4% formalin for 10 minutes. Then, the cells were washed with deionized water and stained with 1% Alizarin Red S solution (Sigma-Aldrich, St Louis, MO) at room temperature. After 5 minutes, the solution was discarded and the cells were rinsed with deionized water. The plate was left until dry. Then, 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich, St Louis, MO) in 10 mM sodium phosphate was added to quantify the amount of red-stained deposited calcium. The optical density was measured at 570 nm using a microplate reader.

Statistical analysis

The data was statistically analyzed by a statistical software program (SPSS Version 22, Chicago, IL). The Kruskal-Wallis test was performed to determine the difference between groups. If Kruskal-Wallis was significant ($P<0.05$), Dunn's test was used to analyzed difference between pairs of means. All illustrations were performed using GraphPad Prism version 9.2.0 (GraphPad Software, CA, USA)



CHAPTER IV
RESULT

SA- β – gal confirmed the aging status of the HPDLCs

The aging status was confirmed using SA- β - gal. The aging HPDLCs expressed a dark blue color precipitate in the cytoplasm under a microscope (Fig. 5A-B). Contrast with the young HPDLCs that explant from 18-year-old patient's tooth, express the faint blue color in lessen number of cells (Fig. 3C-D). The magnification is labeled in the picture.

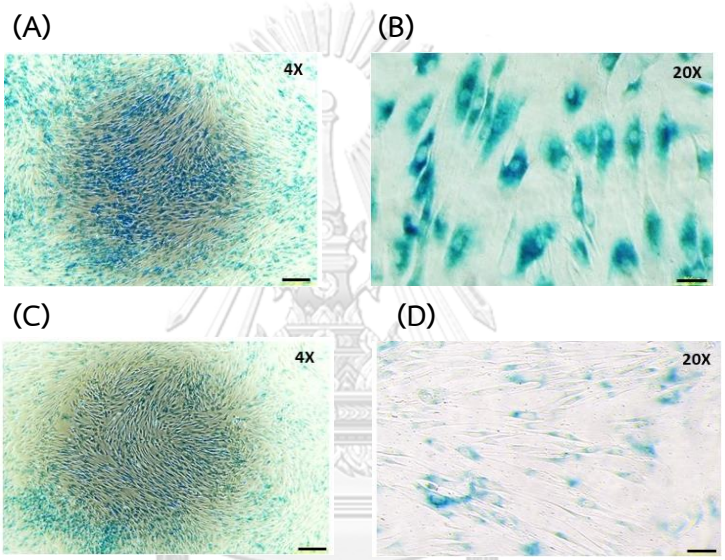


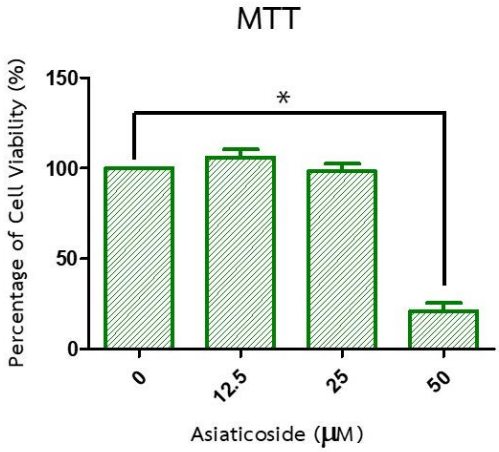
Figure 6 *SA - β - galactosidase staining of aging HPDLCs compared with young HPDLCs.*

Aging HPDLCs (donor's age = 60 y.o.) and young HPDLCs (donor's age = 18 y.o.) were cultured in cultured medium and test the aging status with SA - β – galactosidase assay. (A, B) Aging HPDLCs present dark blue color in cytoplasm. (C, D) Young HPDLCs present with none or having faint blue color in cytoplasm. The difference of cells morphology can be seen under micrography (Scale bar at 4X = 300 μm. Scale bar at 20X = 70 μm.)

Effect of asiaticoside on the viability of aging HPDLCs

After the cells were incubated with asiaticoside for 3 days, the MTT assay showed that asiaticoside did not affect aging HPDLCs when they were treated at concentrations of 12.5 and 25 μM (Fig. 6A). The cell morphology remained unchanged. However, at a concentration of 50 μM, the cell viability decreased significantly ($P<0.05$). The cells were crooked and unattached in the plates (Fig. 6B).

(A)



(B)

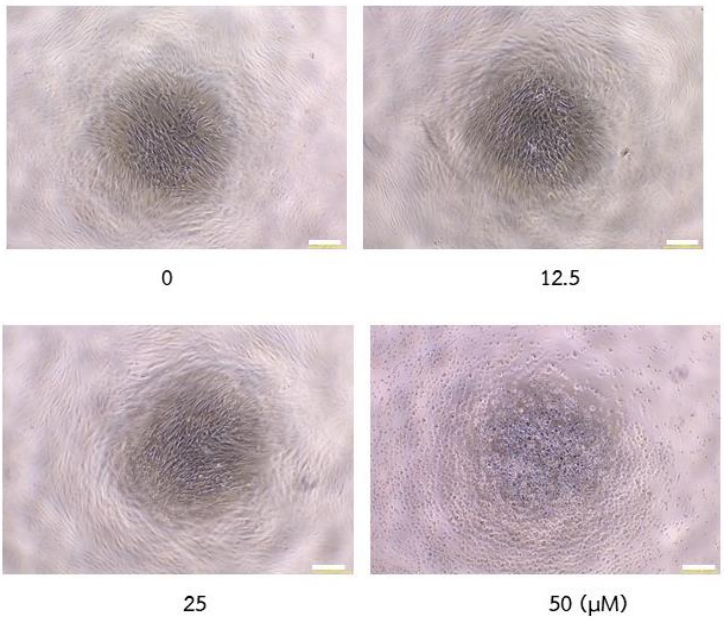


Figure 7 *Low concentrations of Asiaticoside did not affect the viability of aging HPDLCs*

(A) Cell viability was measured after incubating cells with asiaticoside for 3 days using MTT.

(B) Micrograph of aging HPDLCs after incubation with asiaticoside for 3 days. At 50 μM asiaticoside, the cells were crooked and scattered.

Asterisk (*) indicate the statistical significance compared with the control ($P < 0.05$). Scale bar = 300 μm.

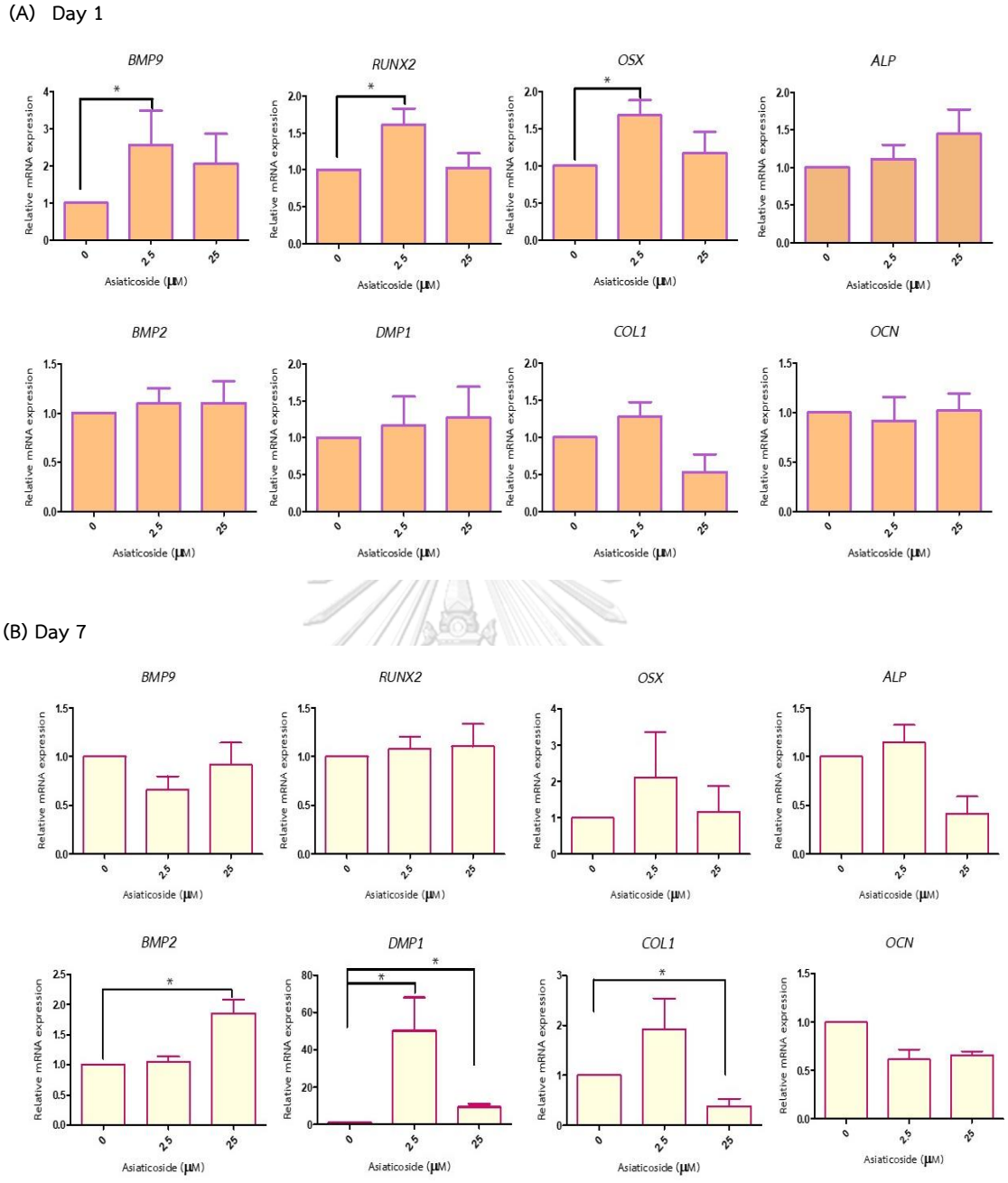


Figure 8 *Asiaticoside enhanced osteogenic differentiation in aging HPDLCs*

Aging HPDLCs were treated with 2.5 and 25 μM asiaticoside for 1 and 7 days. The level of *BMP2, BMP9, RUNX2, OSX, DMP1, COL1, ALP* and *OCN* expression was analyzed using real-time PCR. Asterisks (*) indicate the statistical significance compared with the control.

Effect of asiaticoside on regulatory osteogenic gene expression

To elucidate the effect of asiaticoside on osteogenic gene expression, aging HPDLCs were treated with asiaticoside at non-toxic concentrations (2.5 μM and 25 μM) in osteogenic medium for 1 and 7 days. The gene expression level was evaluated using real-time PCR. After exposure to asiaticoside for 1 day, the expression levels of *BMP9*, *RUNX2* and *OSX* in 2.5 μM asiaticoside were significantly enhanced (Fig. 7A). On day 7, the expression of BMP2 and *DMP1* was significantly upregulated ($P<0.05$). In contrast, the expression of *COL1* was significantly downregulated by asiaticoside at a concentration of 25 μM ($P<0.05$) (Fig. 7B). The other osteogenic genes expression, *ALP and OCN*, were not influenced by asiaticoside at either day 1 or day 7.

Effect of asiaticoside on matrix mineralization

Aging HPDLCs were incubated in osteogenic medium with asiaticoside for 14 days. The result showed that asiaticoside significantly promoted mineralization when aging HPDLCs were treated at a concentration of 25 μM ($P<0.05$). The micrograph presents the red staining of the mineralized nodule (Fig. 8).

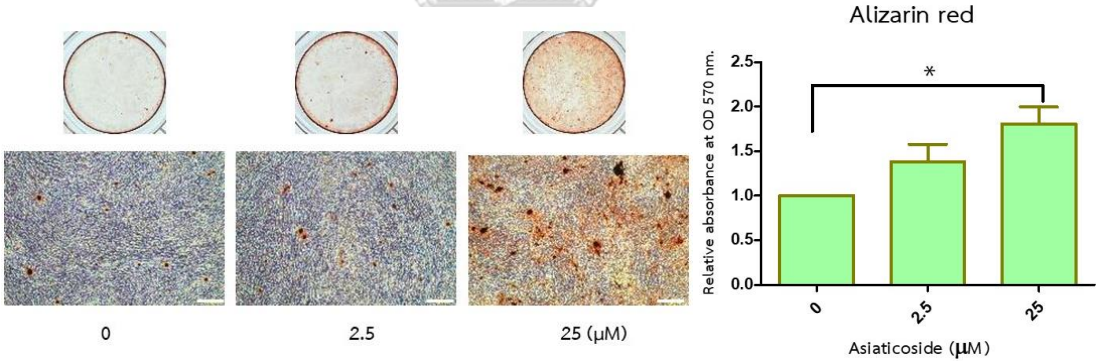


Figure 9 *Asiaticoside promoted mineralization of aging HPDLCs*

Alizarin red staining was used to determine the mineralization in aging HPLDCs after treating cells in asiaticoside for 14 days. (Right) The red color of calcified nodules accumulation was detected under a microscope (magnification 10X, scale bar = 100 μm). (Left) The quantity of mineralization nodule was measured. Asterisks (*) indicate statistical significance compared with the control.

CHAPTER V
DISCUSSION

Tooth loss not only affects the masticatory function but also leads to poor physical performance, loss of muscle mass and increase risk of frailty (1, 74). Regeneration of the periodontal structure in aging individuals in which the function of the cells is decreased due to physiological aging is quite challenging. With the rise of plant-based materials, several natural products have been investigated for their effects on periodontium regeneration and bone mineralization (75). However, the number of experiments on aging HPDLCs is limited. The current study is the first to investigate the effect of asiaticoside on osteogenic differentiation and mineralization in aging HPDLCs.

There are two experimental models in the investigation of aging HPDLCs. The first is to derive the cells directly from aged donors (52, 53, 76). The others subcultivate the cells from juvenile donors until reaching the maximum population doubling level or senescent status (11, 77, 78). In this experiment, we decided to follow along the first model to explore the actual aging cells. We found that it takes time to obtain enough amounts of aging HPDLCs for the experiment due to its low proliferative rate. In the early passage, the young HPDLCs took 3 days to reach confluence in the plate when a 1:4 split ratio was used. However, the aging HPDLCs took at least 7 days to reach confluence when they were subcultivated in the same split ratio. We also used the SA- β – gal staining assay to confirm that the cells from aged donors were truly in aged condition. The enzyme β – galactosidase is expressed distinguishably in the cytoplasm of senescent cells at pH 6.0. The expression of this enzyme was proven to have the strong correlation with the donor's age of cells.(13, 79) As a result, our experiment exhibited the distinct differences in the intensity of blue color from positive SA- β – gal cells under microscope between young and aging cells. From the low proliferative rate of the cells and the result of SA- β – gal staining assay, the aging status of our cells was confirmed.

To confirm mesenchymal characteristics of aging HPDLCs, immunophenotype analysis and cell differentiation assay need to be performed including the expression of mesenchymal associated markers (CD29, CD44, CD90 and CD166) and hematopoietic markers (CD31, CD45) determined by flow cytometry (80, 81) and differentiation capacity by osteogenic, adipogenic, and chondrogenics differentiation assays (82). Although our study did not identify the mesenchymal cell markers, the cells morphology was spindle, suggesting mesenchymal cell characteristic. The osteogenic differentiation capacity was observed in the aging HPDLCs with and

without asiaticoside treatment. Thus, we may imply that the aging cells we retrieved from PDL have mesenchymal cell characteristics.

Cell viability refers to the number of living cells. When the environment of cells is changed, the viability of the cells must be tested to explore the compatible habitat. Asiaticoside was proven to have no cytotoxic effect on HPDLCs when treated with concentrations lower than 100 μM (22). In our study, we found that at 50 μM, aging HPDLCs were shrunken and unattached with the plate. This may be the result of aging cell vulnerability. Not only the concentration of asiaticoside that affects the cells but also the medium condition. In previous reports, both used serum-free medium when testing the viability of the cells. However, our aging cells did not survive in that environment. This finding indicated that aging HPDLCs are more vulnerable than young HPDLCs. Interestingly, we used 10% DMEM with FBS for MTT assay instead of serum free medium, inconsistent with previous studies (21, 22). The reason we need to change the medium was that the aging HPDLCs could not survive in serum free medium with asiaticoside treatment. This incidence supports the vulnerable state of aging HPDLCs.

According to the MTT assay result, we selected 25 μM asiaticoside - the highest concentration without toxicity to aging HPDLCs, for subsequent experiments. We decided to use 2.5 μM asiaticoside, as a comparative concentration to determine the effectiveness of asiaticoside in high and low concentration. The reason we selected the highest effective dose, 25 μM of asiaticoside not 12.5 μM used in the viability test, because the 25 μM was expected to produce the most desirable effect. In addition, with the reduced active state of aging cells, the low concentration of asiaticoside and the narrow gap between different concentrations might not show any significant differences.

Compared with young HPDLCs, aging HPDLCs exhibit significantly less mineralization nodule area, less ALP activity and less expression of osteogenic gene markers such as *RUNX2, COLI,* and *OCN* (12, 13, 52, 80, 83, 84). DMP1, BMP2 and BMP7 proteins were found to gradually diminish with increasing age (85). These discoveries indicated that the genes involved in all 3 stages of osteogenic differentiation, proliferation (preosteoblastic), matrix maturation and matrix mineralization, were altered by the influence of age. In this regard, we hypothesized that asiaticoside may improve the level of osteogenic gene expression. The gene expression analyzed by PCR at day 1 showed the upregulation of *RUNX2*, *OSX* and *BMP9* genes after treatment with 2.5 μM asiaticoside. *RUNX2* (*Cbfa1*), a preosteoblastic gene marker, is regulated through the Wnt pathway and BMP/Smad

signaling pathway. RUNX2 acts as a transcription factor targeting the expression of *OSX, ALP, OCN, COL1* and *BSP* (86, 87). *RUNX2* knockedout mice showed an absence of calcification in the skull and mandible (88). *RUNX2* working with Smad5 can induce the expression of *ALP* (89). *OSX*, following the expression of *RUNX2*, also upregulated the osteogenic genes that were expressed during the matrix maturation and mineralization stages (90). The upregulation of these 2 genes corresponds with the results of a previous study (22), indicating that asiaticoside promotes osteogenic differentiation from the early stage.

Bone morphogenic proteins (BMPs) are regulating factors that bind with type I and II receptors and phosphorylate pathway-specific R-SMADs (SMAD1/5/8). The phosphorylated SMADs translocate into the nucleus and target osteogenic genes (36). In our study, *BMP9* was found to be significantly upregulated at day 1. BMP9 is one of the most potent BMPs member in osteoinduction (91). BMP9 regulates various osteogenic genes although it is believed that the BMP9 osteoinduction pathway is different from the others BMPs members (92, 93). BMP3 and noggin, BMPs antagonists, do not inhibit BMP9 (38, 93). The enhancement of BMP9 expression may also support the asiaticoside ability in promoting early osteogenic gene markers.

In contrast with BMP9, which was upregulated at day 1, *BMP2* was found to be enhanced by asiaticoside at day 7. This may be associated with the pattern of gene expression. Choi *et al*. (2011) studied the pattern of gene expression in HPDLCs during osteogenic differentiation at days 1, 7 and 14 and found that *BMP2* showed maximum active expression at day 7 (34). *BMP2* can stimulate the expression of *COL1, OCN, ALP, OSX* and *OPN* in HPDLCs (34, 94). However, at a concentration of 25 μM asiaticoside, although the expression of *BMP2* was significantly upregulated, the *COL1* expression level was downregulated remarkably. This may be the consequence of the anti-inflammatory effect of asiaticoside. Asiaticoside suppressed collagen by promoting BMP7/Smad1/5 pathway in pulmonary fibrosis and in keloid fibroblasts, which were both in inflammatory conditions (95, 96). Hence, we hypothesized that asiaticoside suppressed *COL1* in aging HPDLCs through BMP7/Smad1/5 pathway, that need to be further investigated.

DMP1 is the transcription factor that regulates osteoblastic genes during the early and late stages of osteoblastic differentiation and induces hydroxyapatite formation (97, 98). In the preosteoblastic stage, DMP1 is localized in the nucleus, binding with Ca^{2+}. DMP1-Ca^{2+} undergoes phosphorylation by casein kinase II and is exported to the extracellular matrix during matrix mineralization stage to regulate calcium phosphate formation and morphology (99, 100). *DMP1* knockout mice

(*DMP1*$^{-/-}$) exhibited low serum phosphate (101) and low hydroxyapatite concentrations (102). Interestingly, as in previous studies, asiaticoside induced the expression of *DMP1* at day 1 and day 14 (21, 22), while in our study, asiaticoside upregulated *DMP1* at day 7. This may be suggested that asiaticoside affects the expression of *DMP1* in every stage of the osteogenic differentiation process: however, the duration when asiaticoside will take action may vary.

ALP and *OCN* were unaltered when cells were treated with asiaticoside. Both are gene markers expressed during the matrix maturation and mineralization stage (34). OCN is a noncollagenous hormone in bone, produced by osteoblasts (103). Carboxylated osteocalcin has high affinity for Ca^{2+} in extracellular matrix and can bind with hydroxyapatite (103, 104). *OCN* does not regulated bone formation since *OCN*-deficient mice (*OCN*$^{-/-}$) have presented normal bone formation and normal bone volume (104, 105). However, *OCN*$^{-/-}$ mice were found to have disrupted hydroxyapatite alignment, leading to low bone strength (105). The unchanged expression level of OCN in our study is in consistency with the results of Fitri *et al*. (2018) (22).

The mineralization process starts when ALP hydrolyzes PPi, which is hydrolyzed from ATP by ENPP1 and transport extracellularly by ANKH, to Pi (35, 42-44). Pi combines with Ca^{2+} and forms calcium phosphate hydroxyapatite in matrix vesicles and accumulate in collagen fibrils to form mineralized nodules (40). Loss of ALP activity decreases the Pi/PPi ratio, leading to a reduction in mineralization (106). In our study, at the same concentration level of asiaticoside, although *ALP* expression was not altered, the formation of mineralization nodules was induced remarkably. We hypothesized that 1) asiaticoside may decrease the expression of *ENPP1* and *ANKH* genes, which will affect the level of PPi, since Pi was proven to still be maintained when *ENPP1* was suppressed (107), and *DMP1* was also found to be upregulated when *ENPP1* and *ANKH* were attenuated (51). 2) asiaticoside possibly induces the level of Pi since the increasing level of Pi will send negative feedback to suppress ALP (40), which needs to be further investigated.

The induction of osteogenic gene markers in the preosteobalstic stage (*BMP9, RUNX2* and *OSX*) matrix maturation and mineralization stage (*BMP2* and *DMP1*) in our study suggested that asiaticoside can stimulate osteogenic differentiation throughout the process. However, the exact mechanism of action needs to be further investigated. Asiaticoside was once proven to promote osteogenic differentiation and mineralization through the Wnt signaling pathway (22). Asiaticoside was also reported to act through the TGF- ß/Smad independent signaling pathway (16) and

BMP7/Smad1/5 signaling pathway (95). The increase of *BMP2* level and the upregulation of mineralized nodules at the same concentration in our study suggested that asiaticoside may also induce osteogenic differentiation and mineralization of aging HPDLCs through BMP/Smad pathway. Additionally, the promotion of mineralized deposits may be the consequence of the upregulation of *DMP1* level.

The number of teeth remaining in aging individuals not only impacted the rate of frailty but also impacted the quality of life. Several reports have presented the relationship between the number of missing teeth and the impairment of oral health-related quality of life (OHRQoL) (108). Surgical or aggressive periodontal treatment to regenerate hard and soft periodontal tissue to preserve the teeth may not be suitable for elderly individuals. Thus, an alternative remedy is important for this group of patients. The ability of asiaticoside to promote osteogenic differentiation and mineralization in aging HPDLCs may lead to appropriate regenerative treatment for the aging population.

CHAPTER VI
CONCLUSION

The current study is the first to investigate the effect of asiaticoside in aging HPDLCs. Asiaticoside can enhance gene expression during osteogenic differentiation and induce mineralization. Further studies are required to precisely identify its potency *in vivo* treatment to elucidate the potential of asiaticoside in periodontal regeneration of the aging population.

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