กลไกที่เกี่ยวข้องในการกระตุ้นการแปรสภาพเป็นเซลล์สร้างกระดูกจากสารสกัดเอเชียติโคไซด์ใน
เซลล์เอ็นยึดปริทันต์ของมนุษย์

นางสาวอะติกา เรสติ ฟิตริ

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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MECHANISMS INVOLVED IN ASIATICOSIDE-INDUCED OSTEOGENIC DIFFERENTIATION IN HUMAN PERIODONTAL LIGAMENT CELLS

Miss Atika Resti Fitri

A Thesis Submitted in Partial Fulfillment of the Requirements
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อะติกา เรสติ ฟิตริ : กลไกที่เกี่ยวข้องในการกระตุ้นการแปรสภาพเป็นเซลล์สร้างกระดูกจากสารสกัดเอเชียติโคไซด์ในเซลล์เอ็นยึดปริทันต์ของมนุษย์ (MECHANISMS INVOLVED IN ASIATICOSIDE-INDUCED OSTEOGENIC DIFFERENTIATION IN HUMAN PERIODONTAL LIGAMENT CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทญ. ดร. ปิยมาศ สำเร็จกาญจนกิจ, 64 หน้า.

เอเชียติโคไซด์เป็นสารสกัดจากสมุนไพรบัวบก ที่มีชื่อวิทยาศาสตร์ว่า *Centella asiatica* (L.) Urban ซึ่งพบว่ามีผลในการกระตุ้นการแปรสภาพของเซลล์เอ็นยึดปริทันต์มนุษย์ไปเป็นเซลล์สร้างกระดูก อย่างไรก็ตาม กลไกการทำงานของเอเชียติโคไซด์ยังไม่ทราบแน่ชัด การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษากลไกการทำงานของเอเชียติโคไซด์ในการกระตุ้นการแปรสภาพของเซลล์ โดยเซลล์เนื้อเยื่อเอ็นยึดปริทันต์มนุษย์จะถูกกระตุ้นด้วยเอเชียติโคไซด์ที่ความเข้มข้น 10, 25, 50, 100 ไมโครโมลาร์ ความมีชีวิตของเซลล์จะถูกทดสอบด้วยเทคนิกเอ็มทีที การแสดงออกของอาร์เอ็นเอนำรหัสจะถูกตรวจสอบด้วยเทคนิคปฏิกิริยาลูกโซ่โพลิเมอเรสชนิดรีลไทม์ และการสะสมแร่ธาตุในจานเลี้ยงเซลล์จะถูกวิเคราะห์ด้วยการวัดการทำงานของเอ็นไซม์อัลคาไลน์ฟอสฟาเทสและการย้อมสีอะลิซาลินเรด การย้อมสีฟลูออเรสเซนซ์และเทคนิกเวสเทิร์นบลอท จะใช้เพื่อบอกตำแหน่งของโปรตีนเบต้า-แคทีนินภายในเซลล์ ข้อมูลจะถูกทดสอบด้วยสถิติอะโนวา ที่ระดับความเชื่อมั่น 0.05 จากผลการศึกษาพบว่า เอเชียติโคไซด์ไม่มีผลต่อการมีชีวิตของเซลล์ เซลล์ที่ถูกกระตุ้นด้วยเอเชียติโคไซด์จะมีการแสดงออกของอาร์เอ็นเอนำรหัสของออสเทอริก ดีเอ็มพี1 และดีเอสพีพี เพิ่มขึ้นอย่างมีนัยสำคัญ การทำงานของเอ็นไซม์อัลคาไลน์ฟอสฟาเทสและการสะสมแร่ธาตุในจานเลี้ยงเซลล์ก็เพิ่มขึ้นด้วยเช่นกัน เป็นที่น่าสนใจว่าเอเชียติโคไซด์สามารถกระตุ้นการแสดงออกของอาร์เอ็นเอนำรหัสของ วินท์3เอ ตามความเข้มข้นที่เพิ่มขึ้น ในขณะที่ไม่มีผลต่อ วินท์5เอ และวินท์-10บี การกระตุ้นการส่งสัญญาณผ่านวินท์ถูกยืนยันด้วยการเพิ่มปริมาณของโปรตีนเบต้า-แคทีนินในนิวเคลียส การใส่รีคอมบิแนนท์ดีเคเค1 สามารถยับยั้งการส่งสัญญาณผ่านวินท์ได้ โดยการยับยั้งการเคลื่อนย้ายตำแหน่งของเบต้า-แคทีนินไปยังนิวเคลียส นอกจากนี้ผลของเอเชียติโคไซด์ในการกระตุ้นการแสดงออกของยีนบ่งชี้การแปรสภาพไปเป็นเซลล์สร้างกระดูกจะลดลงในภาวะที่มีดีเคเค1 จากผลการศึกษาดังกล่าวนี้แสดงให้เห็นว่าเอเชียติโคไซด์มีบทบาทในกลไกการกระตุ้นการแปรสภาพไปเป็นเซลล์สร้างกระดูกผ่านการกระตุ้นการส่งสัญญาณผ่านวินท์/เบต้า-แคทีนินในเซลล์เอ็นยึดปริทันต์ของมนุษย์ ซึ่งองค์ความรู้ที่ได้จะมีประโยชน์ในการพัฒนาสารสกัดที่ใช้ส่งเสริมการเจริญทดแทนในทางคลินิกต่อไป

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ATIKA RESTI FITRI: MECHANISMS INVOLVED IN ASIATICOSIDE-INDUCED OSTEOGENIC DIFFERENTIATION IN HUMAN PERIODONTAL LIGAMENT CELLS. ADVISOR: ASSOC. PROF. DR. PIYAMAS SUMREJKANCHANAKIJ, Ph.D., 64 pp.

Asiaticoside is an active compound isolated from Herb *Centella asiatica* (L.) Urban, which has been shown to induce osteogenic differentiation in human periodontal ligament (hPDL) cells. However, the mechanism of action of asiatioside remains poorly understood. The aim of this study was to investigate the mechanism involved in asiaticoside-induced osteogenic differentiation in hPDL cells. hPDL cells were treated with 10, 25, 50, 100 μM of asiaticoside and cell viability was tested by MTT assay. The mRNA expression levels were analyzed by using quantitative real-time PCR. Osteogenic differentiation was determined by alkaline phosphatase activity assay and alizarin red staining. The subcellular localization of β-catenin was demonstrated by immunofluorescence and western blot analysis. Data were analyzed using one-way analysis of variance (ANOVA) at $p < 0.05$. Asiaticoside did not influence the cell viability. Following asiaticoside stimulation, *OSX*, *DSPP* and *DMP1* mRNA were significantly enhanced. ALP activity and mineralized nodule formation were also markedly induced. Interestingly, asiaticoside dose-dependently increased *WNT3A*, but not *WNT5A* and *WNT10B*. The activation of Wnt signaling was confirmed by the increase of nuclear β-catenin localization. Recombinant DKK1 could inhibit the Wnt signaling by blocking the translocation of β-catenin into the nucleus. Moreover, asiaticoside-induced osteoblastic gene expression was significantly diminished by DKK1. These results demonstrate that asiaticoside is likely involved in osteogenic differentiation by activating Wnt/β-catenin signaling pathway and as a potential agent to promote tissue regeneration in clinical application.

Field of Study: Oral Biology — Student's Signature ....

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CHAPTER I

INTRODUCTION

1.1 Background and rationale

Nowadays, many herbal plants containing active compounds are widely used to treat various diseases in humans. *Centella asiatica* (L.) Urban is a well-known herb that belongs to the family *Apiaceae,* commonly found in some tropical areas (James and Dubery 2009; Bylka et al. 2014). Numerous studies showed that this tropical plant has diverse medical functions including wound healing acceleration, antidepressant, antibacterial, antifungal and treatments such as leprocy, lupus, and asthma (Hashim et al. 2011). The majority of *Centella asiatica* in skin treatment has been investigated in dermatoses patients such as hypertrophic scars, burns, and excoriations (Bylka et al. 2014).

Centella asiatica yields secondary metabolite product known as triterpenoid saponin that determine the biological activities of this herb (James and Dubery 2009). Asiaticoside belongs to triterpenoid saponin which acts as one of the bioactive components. It has shown to have roles in substantial wound healing (Lee et al. 2012), anti-inflammatory, anti-oxidative, and anti-ulcerative activity (Tang et al. 2011). Lee et al. found that asiaticoside promoted wound healing through increased cell migration and proliferation in human dermal fibroblasts (Lee et al. 2012). Asiaticoside has shown to stimulate wound healing in fibroblasts by increasing synthesis of collagen type I. The induction of collagen type I synthesis was confirmed via Smad activation in the transforming growth factor-β (TGF-β) pathway (Lee et al.

2006). Moreover, it has been shown that asiaticoside stimulated collagen type I synthesis and induced osteogenic differentiation in human periodontal ligament (hPDL) cells (Nowwarote et al. 2013). Recently, in particular conditions such as progressive periodontal defect or extensive bone loss, in which tissue regeneration is required to restore tissue function, a tissue engineering approach can be an alternative to reconstruct the damaged tissues. Progenitor cells with the capacity to differentiate into specific tissue are important to achieve this purpose (Han J. et al. 2014). It has been demonstrated that PDL tissue contains heterogenous cell populations with potential stem cell properties. PDL cells were able to form colonies from single-cell and proliferate rapidly, indicating its high rate replicative potential. Mineralized nodule formations were also observed in these cells when cultured in osteogenic differentiation medium (Seo et al. 2004; Nagatomo et al. 2006; Ohta et al. 2008; Alvarez et al. 2015).

Many signaling pathways have been investigated for their roles in stem cell differentiation. Growth factors act as ligands binding to the receptors to initiate cell signaling activation. Bone morphogenetic protein (BMP)/TGF-β pathways are the mainly well-known signaling pathways involved in osteogenesis of mesenchymal cells. BMP 2, 4, 6, 7, and 9 were shown to induce osteogenic activity in C2C12 cell line (Luu et al. 2007). Wnt signaling pathway was involved in bone formation and regulate the expression of osteoblast gene markers (Glass et al. 2005; Takada et al. 2007; Felber et al. 2015). Loss function of Wnt developed cementum impairment and root resorption in the molars of genetically mutated mice (Lim et al. 2014).

Transcription factors such as Osterix (Osx) and Runt-related transcription factor 2 (Runx2) which are known as master transcription factors in osteogenesis have

critical functions in differentiation of mesenchymal cells (Kozhevnikova et al. 2008). The mutated genes resulted in severe bone malformation in mice (Otto et al. 1997; Nakashima et al. 2002). During tooth development, both of Runx2 and Osx are indispensable in odontogenesis at different stages of development. Runx2 regulates other related genes required in osteoblast and odontoblast differentiation. Similar to Runx2, Osx is strongly expressed during bone and teeth formation in the mesenchyme (Chen et al. 2009).

Non collagenous proteins including dentin sialophosphoprotein (DSPP) and dentin matrix protein1 (DMP1) are known to have essential roles in bone and dentin mineralization. DSPP is constitutively expressed in odontoblasts thus indispensable to dentin mineralization. DSPP is also expressed in bone to regulate bone remodeling, but its expression is much lower than in dentin (Verdelis et al. 2008). DMP1 is not only highly expressed in mineralized dentin, but also by osteoblasts and osteocytes. Knockout of *DMP1* gene resulted in the reduction of mineralized bone in mice with low mineral content and the increase of crystal size (Ling et al. 2005). Deletion of *Dspp* gene in mice caused severe alveolar bone loss both in the furcation and interdental region as well as periodontal ligament destruction. Cementum devastation was observed in the periapical area of the mandibular first molar compared with wild type (WT) mice and mineralization around the osteocyte lacunae was also affected (Gibson et al. 2013).

Asiaticoside has been known to induce osteogenic differentiation in hPDL cells. However, the molecular mechanism of asiaticoside to promote osteogenic differentiation in hPDL cells remains unclear. The aim of this study was to investigate which signaling pathway involved in asiaticoside action in hPDL cells. Therefore it

may allow the use of asiaticoside as a potentially biocompatible material to stimulate tissue regeneration in periodontal therapy.

1.2 Research Question

Does asiaticoside induce osteogenic differentiation in human periodontal ligament cells via Wnt/β-catenin signaling pathway?

1.3 Research Objectives

Objective 1: To demonstrate the effect of asiaticoside on hPDL cell viability

Experimental design: Cells were seeded at a density 6 x 10^4 cells/well in a 24-well plate. After reaching confluent, medium was changed into serum free medium. Cells underwent serum starvation for 6 hour before treated with asiaticoside at concentration of 10, 25, 50, and 100 µM for 1, 2 and 3 day. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed to show cell viability and the absorbance was measured at 570 nm.

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Objective 2: To investigate the effects of asiaticoside in osteogenic differentiation of hPDL cells

Experimental design: Cells were harvested in a 12-well plate after treatment with asiaticoside (10 and 100 µM) for 1 and 3 days in growth medium (10% DMEM). Total cellular RNA was extracted with Trizol reagent and converted into cDNA by using reverse transcriptase system. Quantitative real time PCR was performed to show the mRNA expression level of osteogenic gene markers in asiaticoside-treated hPDL cells. To show the effects of asiaticoside in the late stage of osteogenic differentiation,

cells were seeded at a density 6 x 10^4 cells/well in a 24-well plate. The cells were treated with osteogenic induction medium containing asiaticoside. The medium was changed every 2 day. After 10 and 14 day, alkaline phosphatase enzymatic activity and alizarin red staining were performed respectively.

Objective 3: To show the involvement of Wnt associated genes and the activation of Wnt/β-catenin signaling pathway in asiaticoside stimulated cells

Experiment design: Cells were harvested in a 12-well plate after treatment with asiaticoside (10 and 100 μM) for 1 and 3 days in growth medium (10% DMEM). Total cellular RNA was extracted with Trizol reagent and converted into cDNA by using reverse transcriptase system. Quantitative real time PCR was performed to show the mRNA expression level of *WNT3A*, *WNT5A*, *WNT10B*, *DKK1*, and *AXIN2*. The activation of Wnt/β-catenin signaling was confirmed by β-catenin nuclear translocation to the nucleus. Nuclear and cytoplasmic proteins were extracted by cell lysis buffer, whereas the whole cell lysate was extracted by RIPA buffer. Translocated β-catenin proteins in the nucleus were determined by western blot and immunocytochemistry.

Objective 4: To show the inhibition of Wnt/β-catenin signaling pathway by DKK1 in asiaticoside-induced expression of osteoblastic gene markers

Experiment design: To confirm whether cell differentiation induced by asiaticoside is mediated via Wnt/β-catenin signaling cascade, recombinant human DKK1 (rh-DKK1) as an inhibitor of Wnt was added to block β-catenin translocation leading to the reduced expression of target genes. Cells were treated with rh-DKK1 100 ng/ml

60 minutes prior to asiaticoside. The inhibition of β-catenin localization in the nucleus was confirmed by western blot and immunocytochemistry. The mRNA expression of downstream targets was analyzed by real time PCR, whereas the protein expression was detected by immunocytochemistry.

1.4 Research Hypothesis

Asiaticoside induces osteogenic differentiation in human periodontal ligament cells by stimulating Wnt/β-catenin signaling pathway

1.5 Expected outcomes

This study will improve our understanding of the molecular mechanism in asiaticoside-induced osteogenic differentiation, thus allowing asiaticoside as a potential agent to clinically use in tissue regeneration.

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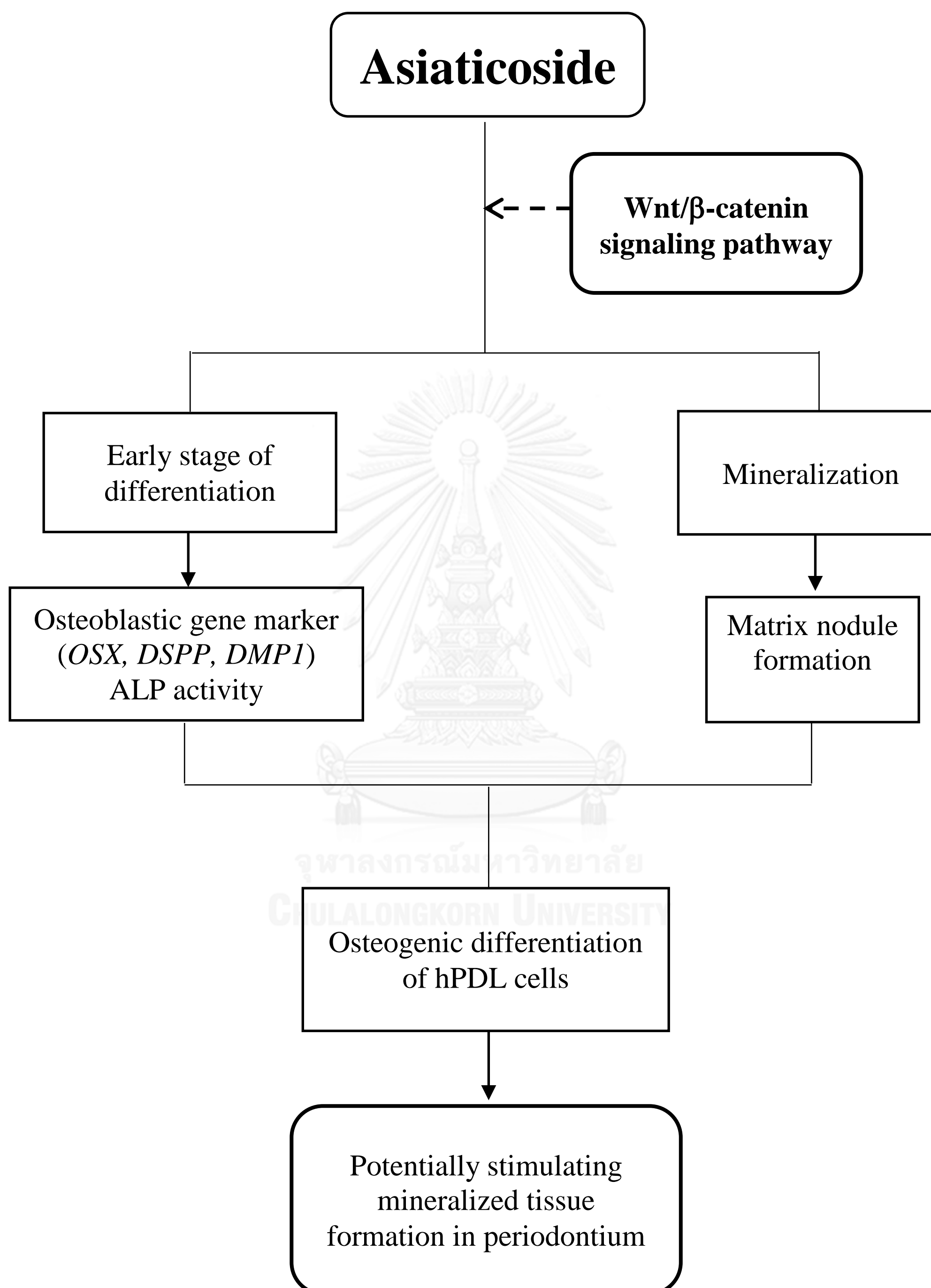
1.6 Keywords

- Asiaticoside
- Human periodontal ligament cells
- Osteogenic differentiation
- Wnt signaling
- β-catenin

1.7 Research Design

- Laboratory experimental study

1.8 Conceptual framework



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CHAPTER II

LITERATURE REVIEW

2.1 Biological properties of *Centella asiatica* and its compounds

Centella asiatica has been well known as a medicinal plant in most of Asian countries for a hundred years (Hashim et al. 2011). Several names of this herb plant are known such as *Gotu kola*, Indian pennywort, pegaga, or *mandukparni* (Gohil et al. 2010; Hashim et al. 2011; Bylka et al. 2014). *Centella asiatica* has approximately 50 species, which can grow either in tropical or subtropical areas (James and Dubery 2009; Alfarra and Omar 2013). The herbaceous *Centella asiatica* sprout abundantly in humid and swampy areas, which include India, Malaysia, Indonesia, Thailand, Madagascar, Sri Lanka, Australia and some areas in Southern part of Africa (James and Dubery 2009; Mato et al. 2011).

The major compound of *Centella asiatica* is pentacyclic triterpenoid consisting of four main chemical constituents, namely asiaticoside, madecasosside, asiatic acid and madecassic acid (James and Dubery 2009; Bylka et al. 2014). The pentacyclic triterpenoid of *Centella asiatica* is arranged in a steroid-like structure composed of a triterpene aglycone skeleton consisting of two subtypes, *oleanane* and *ursane* (James and Dubery 2009). Two bioactive components of *Centella asiatica* are triterpenoid saponins consisting of asiaticoside and madecassoside, and triterpenoid sapogenins comprising madecassic and asiatic acid (Mangas et al. 2008; James and Dubery 2009). Triterpenoid saponin as secondary metabolites synthesized in *Centella asiatica* is chemically structured as a 30-carbon skeleton that forms a pentacyclic

configuration with a glycosylated side chain, but sapogenin (aglycone) does not have a glycoside chain on its structure (Figure 2.1) (James and Dubery 2009; Dubery 2011).

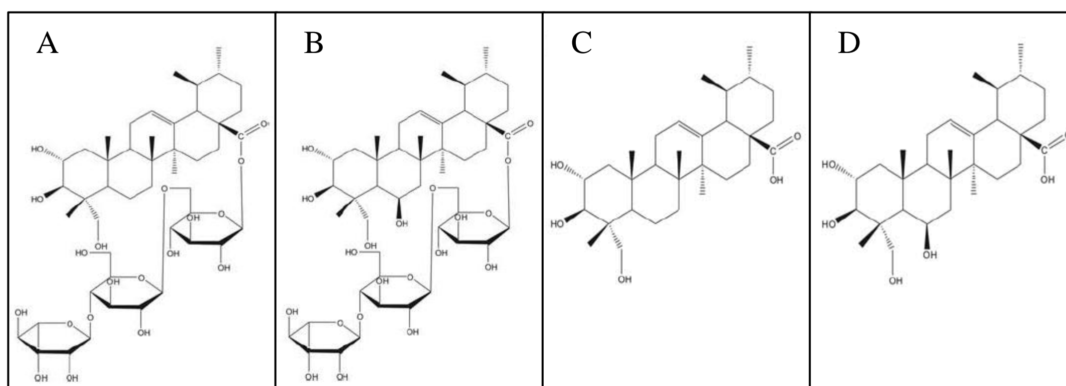

Figure 2.1. Chemical structures of asiaticoside (A), madecassoside (B), asiatic acid (C), and madecassic acid (D) (Modified from James J and Dubery I, 2011)

However, these triterpenoids may have distinct compound content levels in *Centella asiatica* plants in different regions. An investigation conducted in Madagascar exhibited an asiaticoside content of this herb of approximately 2.6-6.42% dry weight. Each part of the plant similarly contains diverse levels of triterpenoids, with the highest quantity (asiaticoside and madecassoside) found in the leaves and the lowest concentration of asiaticoside in the roots (James and Dubery 2009).

Several studies showed the beneficial effects of *Centella asiatica* extract. It might induce the synthesis of collagen on fibroblast cells (Hashim et al. 2011). Bonte et al. discovered that a mixture of asiaticoside, madecassic and asiatic acid compounds stimulated the synthesis of collagen type I secretion on human dermal fibroblasts (Bonte et al. 1994). Similarly, Maquart et al. found that titrated extract from *Centella asiatica* enhanced the synthesis of collagen in a dose-dependent manner, but that asiatic acid was the solely active constituent participating in the

stimulation of collagen synthesis (Maquart et al. 1990). A clinical trial was performed randomly in 20 female volunteers of approximately 45-60 years old in order to investigate the effect of 0.1% madecassoside application combined with 5% vitamin C on skin treatment. After a six-month therapy, the skin restored its elasticity, firmness, gentleness and hydration, which showed an appealing interaction of a madecassoside/vitamin C combination on skin remodeling (Haftek et al. 2008). Bian et al. found that the protective effect of madecassoside in human umbilical vein endothelial cells against lipid peroxidation and H_2O_2-induced apoptosis was achieved via the raised mitochondrial membrane potential, the suppression of caspase-3 activation and p38 MAPK phosphorylation (Bian et al. 2012). Because of the valuable role of *Centella asiatica* in collagen synthesis stimulation, this herb plant has been mostly applied in human skin treatment.

Asiaticoside has been considerably studied for its various biological activities and demonstrated to have anti-oxidative, anti-inflammatory as well as anti-ulcerative effects (Tang et al. 2011). A number of investigations related to the efficacy of *Centella asiatica* Urban on wound healing acceleration has been accomplished by many researchers (Rosen et al. 1967; Wu et al. 2012; Shukla et al. 1999). Lu et al. demonstrated that either mRNA or protein expression levels of particular genes (e.g. collagen type I and III, biglycan, etc.) involved in the synthesis of extracellular matrix was enhanced in asiaticoside-induced fibroblasts (Lu et al. 2004). Asiaticoside exhibited wound healing properties in human skin cells through migration, as well as cell proliferation ability. Either fibroblasts or keratinocytes showed enhanced migration and adhesion after asiaticoside treatment (Lee et al. 2012). Various concentrations of asiaticoside (10 pg, 1 ng, or 100 ng/wound area) topically applied in

burn wound-treated mice demonstrated an increased production of interleukin (IL)-1β, monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor (VEGF) in exudates of burn wounds. Similarly, burn wound healing was found in asiaticoside-treated cells through the initiation of angiogenesis by induction of MCP-1 expression in keratinocytes and IL-1β expression in macrophages that drive to the increase of VEGF production (Kimura et al. 2008).

Repression of fibroblast proliferation and collagen production in keloid were demonstrated after asiaticoside treatment through suppressing TGF-βRI and TGF-βRII expression and increasing Smad7 expression (Tang et al. 2011). In addition, asiaticoside stimulated the synthesis of type I collagen and promoted osteogenic differentiation in hPDL cells after treatment with asiaticoside for 14 day (Nowwarote et al. 2013). Another investigation in asiaticoside-injected rats showed that asiaticoside alleviated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease through maintenance of dopamine turnover, as well as its antioxidant property (Xu et al. 2012). Asiaticoside showed potential antipyretic and anti-inflammatory activities of LPS-induced inflammation in rats by blocking inflammatory mediators, such as COX-2, PGE2, TNF-α and IL-6 (Wan et al. 2013). Likewise, asiaticoside might protect against LPS/D-GalN-induced liver injury via reduction of TNFα expression levels as well as blockage of the MAPK signaling pathway (Zhang et al. 2010).

2.2 Regenerative capacity of periodontal ligament tissue

PDL serves as a connective tissue embedding the tooth in the alveolar bone. PDL tissue has a pivotal role to preserve teeth from any likely destructive effect, such

as excessive mechanical loading or periodontal disease that may occur during its presence in jaws (Choi et al. 2011; Bueno et al. 2013). It also has a function to maintain cellular homeostasis required in periodontal healing. The abilities of the PDL include proliferative property, collagen synthesis, cementum or root resorption and repair, and even alveolar bone remodeling (Shimono et al. 2003). PDL contains a large amount of blood vessels required for vascularization of the tooth. It also contains many types of cells, such as fibroblasts as the main population, as well as osteoblasts and osteoclasts, macrophages, mesenchymal cells, cementoblasts and endothelial cells (Lekic and McCulloch 1996; Choi et al. 2013).

In dentistry, some particular conditions require new tissue generation, such as periodontal tissue loss due to periodontal disease, or augmented bone requirement for dental implant treatment (Kao et al. 2009). This is known as tissue engineering, which utilizes the regenerative ability of human living tissue to restore the tissue loss or defect (Vinaya Bhat 2011). There are three components that directly take part in tissue engineering: cells, signaling molecules, and supporting matrix. These three factors are manipulated in a proper condition to establish tissue regeneration (Kao et al. 2009).

It has been shown that PDL cells can have characteristics of stem cells including self-renewal capacity, differentiation ability into other cell types (e.g. cementoblast-like cells, adipocytes, and collagen-forming cells), as well as expression of stem cell markers (Seo et al. 2004; Nagatomo et al. 2006). PDL cells were positively expressed CD105, CD166, and STRO-1, which assert the mesenchymal stem cell markers (Nagatomo et al. 2006). Furthermore, PDL cells might also have potential to differentiate into cementoblastic/osteoblastic cells, adipocytes, and collagen-forming cells under particular culture condition (Seo et al. 2004; Nagatomo

et al. 2006). PDL cells cultured in osteogenic medium for 4 weeks produced positively mineralized nodules formation on alizarin red staining. Oil red O-positive cells were observed in PDL cells within 3 weeks induction of adipogenic medium. An in-vivo experiment was conducted by transplanting PDL cells in immunocompromised mice to demonstrate the cells ability to transform into functionally cementoblast-like cells (Seo et al. 2004). Other several studies showed that mineralized matrix formation in hPDL cells was observed in osteogenic medium-cultured cells (Seo et al. 2004; Hakki et al. 2014; Ye et al. 2014). Nagatomo et al. found that mineralized nodules were formed in PDL cells after culturing in growth medium for 14 days and changing into osteogenic medium until day 21 (Nagatomo et al. 2006).

2.3 Osteogenic differentiation of mesenchymal cells

Mesenchymal cells can undergo osteogenic differentiation through three different stages, which consist of proliferation, early differentiation, and terminal differentiation followed by matrix maturation. In the first stage, the cells start proliferating until day 4, at which the cell count is highest. The number of cells eventually reaches a plateau phase lasting up to day 14, after which it reduces due to cell apoptosis. Secondly, the early differentiation stage may also involve some growth factors as initiator proteins in cellular signaling to promote osteogenic differentiation in human mesenchymal cells. In this step, the cells release a vast amount of extracellular matrix protein, collagen type I, that subsequently go through matrix mineralization (Huang et al. 2007).

Transcription factors regulate and play critical roles in this differentiation stage. Runx2, Osx and Dlx5 have been recognized as master of transcription factors in osteogenesis. Runx2, which is also known as Cbfa1/Pebp2aA/AML3, is the main transcription factor in osteogenic differentiation. It is implicated either in endochondral or intramembranous bone formation. Runx2 regulates other proteins in the extracellular matrix, which include type I collagen, osteopontin (OPN), bone sialoprotein (BSP), fibronectin, and ALP (Kozhevnikova et al. 2008). A mutated *RUNX2* in mice was established to define the function of this gene. The ossification of skeletal system was absent in homozygous mutated mice. Alkaline phosphatase was poorly expressed but OPN and osteocalcin (OCN) genes were not detected in immature osteoblasts. No mesenchymal cells and vascular were discovered in the cartilage, but a few immature osteoclast emerged at the pericondrium (Komori et al. 1997). *RUNX2*-knocked out mice showed severe skeleton malformations in the bone development stage. The osteoblasts and bone were not present in homozygous mutant skeletons, whereas heterozygous mice had specific abnormalities in skeleton known as cleidocranial dysplasia (CCD) (Otto et al. 1997). Osx, a zinc finger-containing transcription factor, has been investigated regarding its role in differentiation of preosteoblastic cells to become mature osteoblasts (Lee et al. 2003; Park and Kim 2013). The *OSX* gene is found downstream of the *RUNX2* gene and is therefore not expressed in *RUNX2* null mice. However, *RUNX2* expression is observed with no strikingly changed phenotype in *OSX*-knockout mice. When *OSX* was inactivated in E17.5 mice embryos, the newborn homozygous mutants were found died immediately caused by inability of breathing resulting from severe inward bending of limbs. The cartilage skeleton was completely formed but had a delayed mineralization in skull

bones. Osteoblast differentiation markers including BSP, osteonectin and OPN were not observed in the condensed mesenchyme (Nakashima et al. 2002).

During tooth development, both of Runx2 and Osx are important in odontogenesis at different stages of development. Runx2 plays an important role to regulate other related genes required in osteoblast and odontoblast differentiation. Runx2 was early detected in dental mesenchyme, which its expression increased during matrix formation in dental papilla, dental follicle, and alveolar bone at the cap stage. The Runx2 expression was decreased at the later stage in the differentiated odontoblast, ameloblasts and dental pulp cells (Chen et al. 2009). The abnormal phenotypes of *RUNX2*$^{-/-}$ mice tooth were prominently observed. The smaller molars with poorly differentiated enamel organ and the hypoplastic incisors were confirmed at E16.5 mice (D'Souza et al. 1999). Similar to Runx2, Osx was strongly expressed during bone and teeth formation in the mesenchymal, yet its expression was comparatively lower than those of Runx2 at E12 mice. Until the bell stage (E16), *RUNX2* and *OSX* mRNA expressions were observed in osteogenic mesenchyme, ameloblasts, odontoblasts, and dental pulp cells. Nevertheless, the expression of *OSX* gene was intensely detectable in odontoblasts and dental pulp cells compared to *RUNX2* at the advanced stage, from E18 to PN14 (Chen et al. 2009).

The last stage of osteogenic differentiation is matrix maturation, which occurs approximately after 15 days, lasting around 14 days. The markers for this late stage are OCN and OPN, followed by mineral deposition that represent various processes of bone formation in vivo (Huang et al. 2007). Calcium and phosphate formations can be detected in this stage (Hoemann et al. 2009).

2.4 Wnt signaling in osteogenesis

The Wnt signaling pathway has been known to regulate many biological developmental processes, not only during embryonic development but also in adult tissue homeostasis. The role of Wnt signaling in bone formation has become great concern in the last few decades (Kim et al. 2013). A report showed that β-catenin, the key regulator protein of the canonical Wnt pathway, is indispensable to control cell differentiation in 3T3-L1 mouse fibroblast cell line. The suppressed adipogenesis by WNT10B, WNT10A, WNT6 and WNT3A was thoroughly inhibited when β-catenin was eliminated. Moreover, exogenous Wnt-induced osteoblastogenesis was repressed in the lack of β-catenin indicating the vital function of β-catenin to regulate mesenchymal stem cell fate (Cawthorn et al. 2012).

To initiate signaling activation, Wnt molecules that serve as a ligand has to bind to a complex receptor on cell surface to transmit signal transduction. The Frizzled family receptors and co-receptors lipoprotein receptor–related protein (LRP)-5/6 are the main receptors in Wnt signaling. Binding of Wnt ligands to its receptors subsequently triggers two major distinct intracellular cascades; the canonical Wnt/β-catenin pathway and the noncanonical Wnt pathway consists of the Wnt/Ca^{2+} (calcium-dependent) and the Wnt/PCP (planar cell polarity) pathways (Liu et al. 2009; Rao and Kuhl 2010; Kim et al. 2013). These intracellular responses are stimulated by different Wnt proteins, e.g. WNT1, WNT3A, and WNT8 through the canonical pathway while WNT5A and WNT11 for the noncanonical pathway (Kim et al. 2013).

In the canonical pathway, β-catenin is the key protein take part in intracellular signal activation. In the presence of Wnt ligands, the Dishevelled (Dvl) protein is

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recruited and phosphorylated to the receptor complex that can inhibit glycogen synthase kinase 3β (GSK3β) protein to phosphorylate cytoplasmic β-catenin. The accumulation of stabilized β-catenin in the cytoplasm subsequently translocates into the nucleus, establishing an assembly of protein complex with T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) to activate the transcription of target genes e.g *CYCLIN D1*, *AXIN2*, *C-MYC* and peroxisome proliferator-activated receptor (*PPARδ*) (Valenta et al. 2012; Kim et al. 2013). Wnt signaling can be blocked by extracellular antagonists of Wnt such as secreted frizzled-related proteins (sFRPs), Dickkopf1 (DKK1) or WIF1. sFRPs and WIF1 block the Wnt signaling by binding to the Wnt ligands, whereas DKK1 interferes the interaction of the ligands by binding to the LRP-5/6 receptors (Issack et al. 2008). When the Wnt ligand is absent, Dvl protein is dissociated from the receptor so that β-catenin becomes phosphorylated mediated by a protein destruction complex comprised of GSK3β, AXIN and (Adenomatous polyposis coli) APC. This action leads to marking β-catenin for ubiquitination and protein degradation (Figure 2.2) (Kim et al. 2013).

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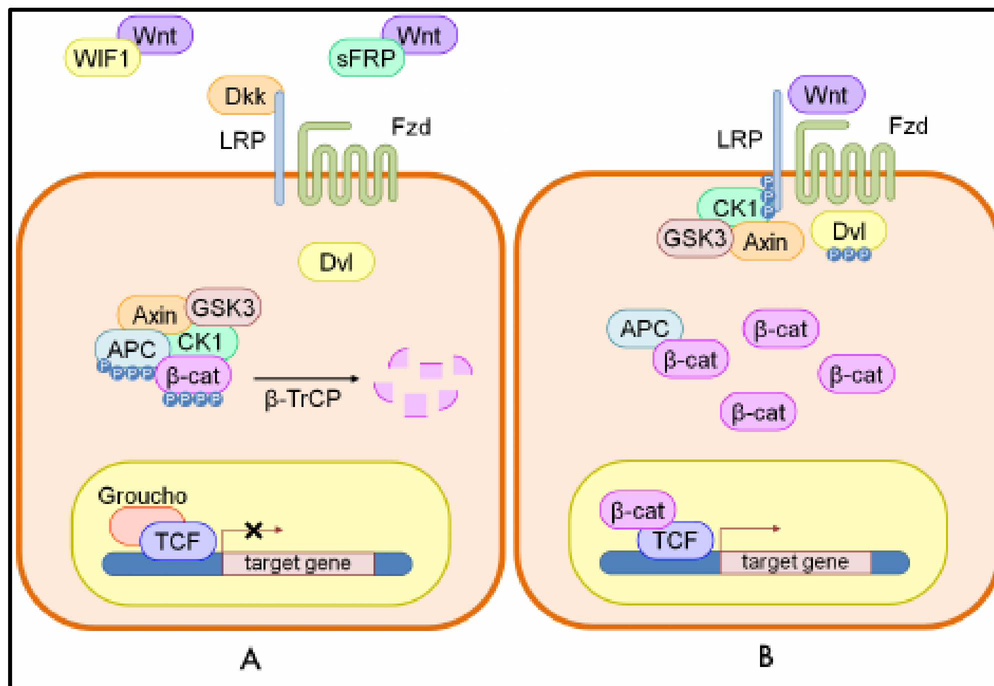

Figure 2.2 The Wnt/ β-catenin signaling pathway action. The binding of Wnt ligand to the receptors on cell surface leads to the recruitment of Dvl to the receptor complex that prevents GSK3β along with AXIN and APC protein to phosphorylate β-catenin. This action results in the accumulation of cytosolic β-catenin thus subsequently translocate into the nucleus. When exogenous inhibitors of Wnt such as DKK1, WIF1, or sFRP exist, Wnt signal is blocked resulting in the dissociation of Dvl protein from the receptors. The destructive protein complex phosphorylates β-catenin to later undergoing degradation. (Adapted and modified from Sinha S, 2016)

In the Wnt/Ca^{2+} pathway, the signal starts with the binding of Wnt ligands to the Frizzled receptors thus switching on the intracellular cascade. Induction of this pathway brings the calcium release from the endoplasmic reticulum which activates downstream mediators such as protein kinase C, calcineurin and calcium calmodulin-

dependent protein kinase II leading to the activation of transcription factors NF-κB, NFATc and cAMP response element binding protein. In contrast, the Wnt/PCP pathway involves GTPases and Frizzled receptors interaction to activate c-jun NH2 kinase (Kim et al. 2013).

Disrupting of Wnt components affected bone formation. Mutation of *Lrp-5* diminished bone thickness in mouse calvarial explant cultures (Gong et al. 2001). Similarly, overexpressed *DKK1* resulted in reduction of mineralized matrix in osteoblasts (Li et al. 2006). Heterozygous *Dkk1*-deficient mice showed an enhanced osteoblast differentiation thus increased bone formation but had no effect on bone resorption (Morvan et al. 2006). In contrast, $Dkk1^{-/-}$ mice displayed an anterior head defect and forelimb malformation (Mukhopadhyay et al. 2001). Bone mineral density and trabecular bone were attenuated in $Wnt3a^{-/-}$ mice (Takada et al. 2007). Correspondingly, Qiu et al. reported that WNT3A could promote osteoblast differentiation via Wnt/JNK pathway in human bone marrow mesenchymal stem cells (Qiu et al. 2011).

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Several studies have been demonstrated the role of Wnt/β-catenin signaling in tooth development. The diminished Wnt signaling has a significant impact in cementum formation. Irregular root surface and a large crater-like structure particularly at the cementoenamel junction were noticeable by 3-dimensional reconstruction of tissue sections. The eroded cementum was also present with penetrated dentin on the root surfaces (Lim et al. 2014). Investigation by Lu et al. showed that WNT3A, WNT10B, and β-catenin had higher expression on the tension side than on the compression side during orthodontic tooth movement in rat model, whereas DKK1 showed reversely. In addition, the increased expression of WNT3A,

WNT10B, β-catenin and DKK1 was observed on the loaded teeth compared with the unloaded teeth. These evidences suggest that the Wnt/β-catenin signaling may be related to tissue remodeling in tooth movement stimulated by mechanical force from orthodontic appliances (Lu et al. 2016).

2.5 The role of DSPP and DMP1 in bone and tooth

DSPP and DMP1 belong to the small integrin-biding ligand N-linked glycoproteins (SIBLINGs) family with the other three members are BSP, OPN and matrix extracellular glycophosphoprotein (MEPE). Among the five family members, DSPP and DMP1 have a number of similarities in genomic organization or protein structure. Both DSPP and DMP1 have critical functions during hard tissue development such as dentin and bone (Suzuki et al. 2012). Initially known as a dentin-specific gene, DSPP has been recently found to express in bone, cementum and other non-mineralized tissues. Deletion of *DSPP* gene resulted in defect of the dentin that similar to dentinogenesis imperfect-III phenotype, characterized by large pulp chambers, wide predentin zone and hypomineralization that may cause the exposed pulp (Verdelis et al. 2008). DSPP is also important to maintain periodontal tissue homeostasis. The massive destructions of cementum and alveolar bone were observed in the furcation and interproximal area of the molars in *Dspp* null mice (Gibson et al. 2013). DSPP was detected in osteoblasts of rat long bone (Qin et al. 2003)

Firstly identified in odontoblast, DMP1 has been found to have high expression in other cells such as preosteoblasts, osteocytes, and chondrocytes. DMP1 particularly takes part in the early and late stages of odontogenesis. Tooth phenotype from $Dmp1^{-/-}$ mice resembles to the one from $Dspp^{-/-}$ mice. *Dmp1* knockout mice

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showed low mineral-matrix ratio of the bone indicating the role of DMP1 to promote bone mineralization. In addition, the reduced calcium phosphate concentration was spotted in the lack of DMP1 suggesting the involvement of DMP1 in metabolism of calcium and phosphate (Ling et al. 2005). Severe defects in cartilage formation developed in *Dmp1* null mice during chondrogenesis (Ye et al. 2005). Interestingly, overexpressed DMP1 in transgenic mice could rescue the bone phenotypes of $Dmp1^{-/-}$ mice (Lu et al. 2009).

CHAPTER III

RESEARCH METHODOLOGY

3.1 Ethical considerations

Healthy third molars extracted from patients undergoing dental surgery were selected in this study. All procedural protocols were notified to the patients in advance, after which informed consent was acquired. The procedures were approved by the Human Research Ethics Committee (HREC-DCU 2016-011) from Faculty of Dentistry, Chulalongkorn University.

3.2 Samples

Periodontal ligament tissue from the middle third of the root of third molars was scraped and maintained in Dulbecco's modified Eagle's medium [(DMEM), Gibco, Carlsbad CA, USA] containing 10% fetal bovine serum (Gibco, USA), 2 mM L-glutamine (Sigma-Aldrich, Darmstadt, Germany), 100 units/ml penicillin (Sigma-Aldrich, Germany), 100 mg/ml streptomycin (Thermofisher, Carlsbad, CA, USA) and 5 mg/ml amphotericin B to allow cells to be released from the tissue. Thereafter, cells were cultured in the same medium, which were changed every 48 h. Cells were subcultured at a 1:3 ratio after cell confluent around 80%. Cell passages 3 to 6 were selected in this experiment.

3.3 Asiaticoside preparation

Asiaticoside was purchased from Sigma (#43191 Sigma-Aldrich). The purified compound was in powder form and dissolved in dimethylsulfoxide [(DMSO); Sigma-Aldrich, Germany].

3.4 Cell viability assay

To demonstrate cell viability, MTT (USB Corporation, Cleveland, OH, USA) assay was performed. hPDL cells were seeded with a density of 6 x 10^4 cells/well in a 24-well plate and incubated at 37° C overnight. The medium was changed into serum free medium containing asiaticoside at concentration 10, 25, 50, and 100 µM. Cells treated with vehicle (DMSO) only was considered as control. MTT powder was dissolved in DMEM without phenol red in a concentration 0.5 mg/ml until become yellowish. After 1, 2 and 3 day treatment, the medium was removed and substituted with MTT solution to incubate for about 30 min at 37° C. As the formazan crystal was formed, the MTT solution was eluted by a solution composing of DMSO and glycine buffer to dissolve the crystal so the color changed into purple. The optical density was then measured at absorbance 570 nm in a microplate reader (Elx800; Biotech, Winooski, VT, USA). The viable cell numbers were calculated as relative to control group.

3.5 RNA extraction

Cells at a density of 2 x 10^5 cells/well were seeded in a 12-well plate and treated with asiaticoside (10 and 100 µM) in growth medium for 1 and 3 days. To extract the total cellular RNA, Trizol reagent (Roche Diagnostics, Indianapolis, IN,

USA) was used to lyse the cells. After adding 1 ml of Trizol, the cells were scraped and mixed by using the pipette tip. The lysed cells were then incubated at 25° C for 5 min and mixed with 200 μl chloroform. The mixture was shaken vigorously for 15 s and incubated for 2-3 min at room temperature. The samples were centrifuged at 14,000 rpm for 15 min at 4° C. Three layers in solution were formed and the aqueous phase was transferred into a new 1.5 ml tube. Isopropyl alcohol (500 μl) was added, then centrifuged at 14,000 rpm for 15 min during which a pellet was formed at the bottom of the tube. The supernatant was removed gently from the tubes to avoid pellet disruption, and replaced by 1 ml of 75% ethanol. The samples were centrifuged at 8,000 rpm for 5 min at 4° C. The RNA pellet formed was dissolved in RNAse-free water and the concentration of RNA was quantified by Spectrophotometer [(NanoDrop2000), Thermo Scientific, Rockford, IL, USA].

3.6 Reverse transcription-polymerase chain reaction (RT-PCR)

A total of 1 μg of RNA samples was converted to cDNA by Improm II reverse transcriptase system (Promega, Madison, WI, USA) for 1 h 30 min at 42° C followed by incubating at 99° C for 2 min. To perform real-time quantitative PCR, SYBR green detection system (Fast Start Essential DNA Green Master; Roche Diagnostic, USA) on Mini Opticon real time PCR system (Bio-Rad, Hercules, CA, USA) was used to detect the target genes (primer sequences shown in Table 1) from the amplified cDNA. The expression of each gene was normalized to GAPDH expression.

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Table 1. PCR primer sequences of quantitative real-time PCR

Primers	Sequence 5’→ 3’	Accession No.
AXIN2	Forward – GAG TGG ACT TGT GCC ACT TCA Reverse - GTT GGC TGG TGC AAA GAC ATA G	NM_001289746.1
DSPP	Forward - ATA TTG AGG GCT GGA ATG GGG A Reverse - TTT GTG GCT CCA GCA TTG TCA	NM_004655.3
DKK1	Forward - GCC TCA GGA TTG TGT TGT GC Reverse - ATC CGG CAA GAC AGA CCT TC	NM_012242.2
DMP1	Forward - ATG CCT ATC ACA ACA AAC C Reverse - CTC CTT TAT GTG ACA ACT GC	NM_004407.3
GAPDH	Forward - CAC TGC CAA CGT GTC AGT GGT G Reverse - GTA GCC CAG GAT GCC CTT GAG	NM_014208.3
OSX	Forward – GCC AGA AGC TGT GAA ACC TC Reverse – GCT GCA AGC TCT CCA TAA CC	NM_001278478.1
RUNX2	Forward – CAG ACC AGC AGC ACT CCA TA Reverse – CAG CGT CAA CAC CAT CAT TC	NM001300837.1
WNT3A	Forward - CTG TTG GGC CAC AGT ATT CC Reverse - GGG CAT GAT CTC CAC GTA GT	NM_033131.3
WNT5A	Forward - TCA GGC ACC ATT AAA CCA CA Reverse - AAT TCA CAG AGG TGT TGC AGC	NM_003392.4
WNT10B	Forward – TTG TGC AGT CGG GCT CTA AG Reverse – GAT GTG CAG ACC CTG AAG CG	NM_003394.3

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3.7 Alkaline Phosphatase Assay

hPDL cells were cultured in 24-well plates at a density of 6 x 10^4 cells/well. After reaching confluence, the cells were cultured in osteogenic medium (OM) containing 50 µg/ml ascorbic acid (Sigma-Aldrich, USA) and 5 mM β-glycerophosphate (Sigma-Aldrich, USA) in the presence of asiaticoside (10 and 100 µM). The medium was changed every 2 days. The ALP enzymatic activity was measures at 10 day. The cells were lysed in alkaline lysis buffer and then incubated in the substrate mixture of 2 mg/ml of p-nitrophenol phosphate [(PNPP), Invitrogen, USA], 0.1 M of 2-amino-2-methyl-1-propanol (Sigma-Aldrich, USA), and 2 mM of $MgCl_2$ at 37° C for 30 min. To stop the reaction, 50 mM of NaOH was added and the optical density was measured using a microplate reader at an absorbance of 410 nm. BCA assay (Thermo Scientific) was utilized to define total cellular protein and used to normalize the enzymatic activity.

3.8 Matrix mineralization assay

Mineralized nodules were detected by using Alizarin red staining. Cells were seeded at a density 6 x 10^4 cell/well in 24-well plate. The medium was changed into OM containing asiaticoside (10 and 100 µM). After 14 day, cells were washed with PBS and fixed in cold methanol for 10 min. The cells then were washed with deionized water twice, and stained with 1% Alizarin Res S solution (Sigma Aldrich, USA) at room temperature for 5 min. The dye was completely removed, washed with deionized water and dried by air.

3.9 Immunofluorescence

hPDL cells grown on coverslips (5 x 10^4 cell/well) in 24-well plate were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton x100 for 10 min each. The cells were blocked with 3% BSA (Sigma-Aldrich, USA) in PBS for 1 hour at room temperature. Primary antibody of rabbit anti β-catenin 1:250 (#9582 Cell signaling, Danvers, MA, USA), mouse anti DSPP 1:100 (sc-73632 Santa Cruz Biotechnology, Dallas, Texas, USA) and goat anti DMP1 1:100 (ab81985 Abcam, Cambridge, UK) was diluted in 1% BSA in PBS and incubated overnight at 4° C. The cells were subsequently incubated with biotinylated secondary antibody of goat anti-rabbit (AP132B Millipore, Temecula, CA, USA), goat anti-mouse (B2763 Life technologies, Carlsbad, CA, USA) and rabbit anti-goat (B7014 Sigma-Aldrich, USA) for 1 hour at room temperature. Streptavidin-FITC (**S3762** Sigma-Aldrich, USA) was used to detect secondary antibody. The cells were washed with PBS 3 times for 5 minutes each after every step of incubation. Mounting medium fortified with DAPI (Vectashield, Burlingame, CA, USA) were applied to preserve fluorescence of cells. Images were analyzed by a fluorescent microscope (Axiovert 40CFL, Carl Zeiss, Gottingen, Germany).

3.10 Protein isolation and Western blot

Cells seeded in 100 mm culture dish were extracted to isolate cytoplasmic and nuclear proteins. Nuclear extraction was prepared as previously described (Sumrejkanchanakij et al. 2003) using extraction buffer A (10mM HEPES, 2.5mM $MgCl_2$, 10mM KCL, 0.5 mM DTT) and buffer C (20mM HEPES, 25% (v/v) glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA). Briefly, the cells were detached from

the culture dish, transferred to 1.5ml tube, and centrifuged at 14,000rpm. Pellet was formed and buffer A was added to dissolve the pellet. After centrifugating, the first supernatant was collected (cytoplasmic protein), thus pellet formed was dissolved in buffer C and incubated for 2 hour followed by centrifugation. The second supernatant was subsequently collected (nuclear protein).

The whole cell lysate were extracted by using radioimmunoprecipitation assay (RIPA) buffer (150mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50mM Tris) containing a cocktail of protease inhibitors (Sigma-Aldrich, USA). Protein concentration was determined by BCA protein assay (Pierce, Rockford, IL, USA) at 595 nm absorbance. A total of 30 μg protein extracts in each sample was separated by 7.5% SDS–polyacrylamide gel electrophoresis and blotted on PVDF membranes (Immobilon-P, Millipore, USA). The membranes were blocked with 5% skim milk in 0.1% PBS/Tween20 for 1 hour at room temperature. The blots were incubated with primary antibodies, mouse anti β-catenin 1:1000 (#05-613 Cell signaling, USA), mouse anti DSPP 1:500, goat anti DMP1 1:500 and mouse anti Actin 1:2000 (MAB1501 Millipore, USA) overnight at 4° C and then with biotinylated-conjugated secondary antibody of goat anti-mouse (B2763 Life technologies, USA) and rabbit anti-goat (B7014 Sigma-Aldrich, USA) for 1 hour at room temperature. The membrane was then incubated with horseradish Streptavidin-HRP (#3999 Cell signaling, USA). The signal was detected by an enhanced chemiluminescence kit (Pierce, Thermofisher, USA).

3.11 Statistical analysis

The data were reported as mean ± SD relative to the control from three independent experiments. Data were statistically analyzed using one-way ANOVA followed by Tukey posthoc test to compare the difference between groups. A significant difference was considered as $p<0.05$.

CHAPTER IV

RESULT

4.1 Objective 1: To demonstrate the effect of asiaticoside on hPDL cell viability

4.1.1 Effect of asiaticoside on viability of hPDL cells

hPDL cells treated with asiaticoside for 1, 2 and 3 day were tested by MTT assay to show the effect of asiaticoside on cell viability. Asiaticoside did not cause cell death or cell proliferation at any tested concentration. In addition, there was no difference in cell number at all time points (Figure 4.1). Next, asiaticoside 10 and 100 μM were selected for further experiments.

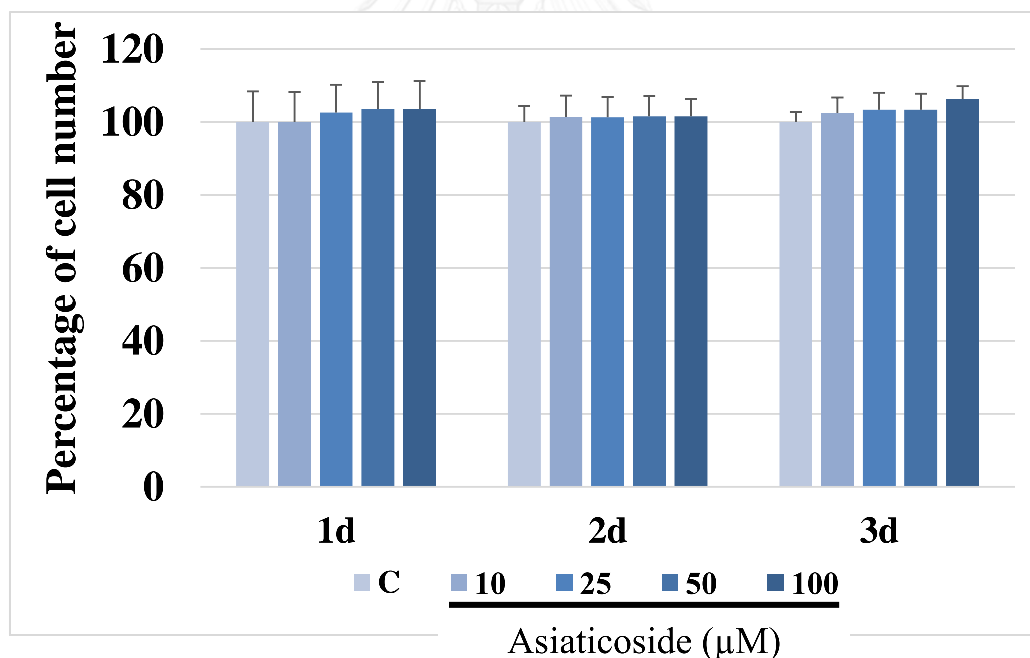


Figure 4.1 Effect of asiaticoside on hPDL cell viability. hPDL cells were starved for 6 hour and then treated with asiaticoside (10, 25, 50, 100 μM) in serum free medium for 1, 2 and 3 day. The cell viability was analyzed by MTT assay. Cell treated with

DMSO was considered as control. The data were presented as percentage of cell number and relative to control. Mean values ± SD are presented.

4.2 Objective 2: To investigate the effects of asiaticoside in osteogenic differentiation by hPDL cells

4.2.1 Asiaticoside induces osteogenic differentiation in hPDL cells

To investigate the effect of asiaticoside in osteogenic differentiation, hPDL cells were cultured in osteogenic medium in the presence or absence of asiaticoside. Asiaticoside at concentration 100µM but not 10 µM, significantly upregulated *OSX* mRNA expression at day 1 and 3, althought the expression level at day 3 was lower than at day 1. Surprisingly, the mRNA expression of *RUNX2* was unchanged (Figure 4.2A).

ALP enzymatic activity was also significantly induced by asiaticoside 100µM after 10 day cultured in OM, but remained unchanged at asiaticoside 10µM (Figure 4.2B). Correspondingly, mineralized nodules formation was more highly detected in asiaticoside-stimulated cells at concentration 100µM rather than 10µM at 14 day (Figure 4.2C). These evidences indicate that asiaticoside can promote osteogenic differentiation.

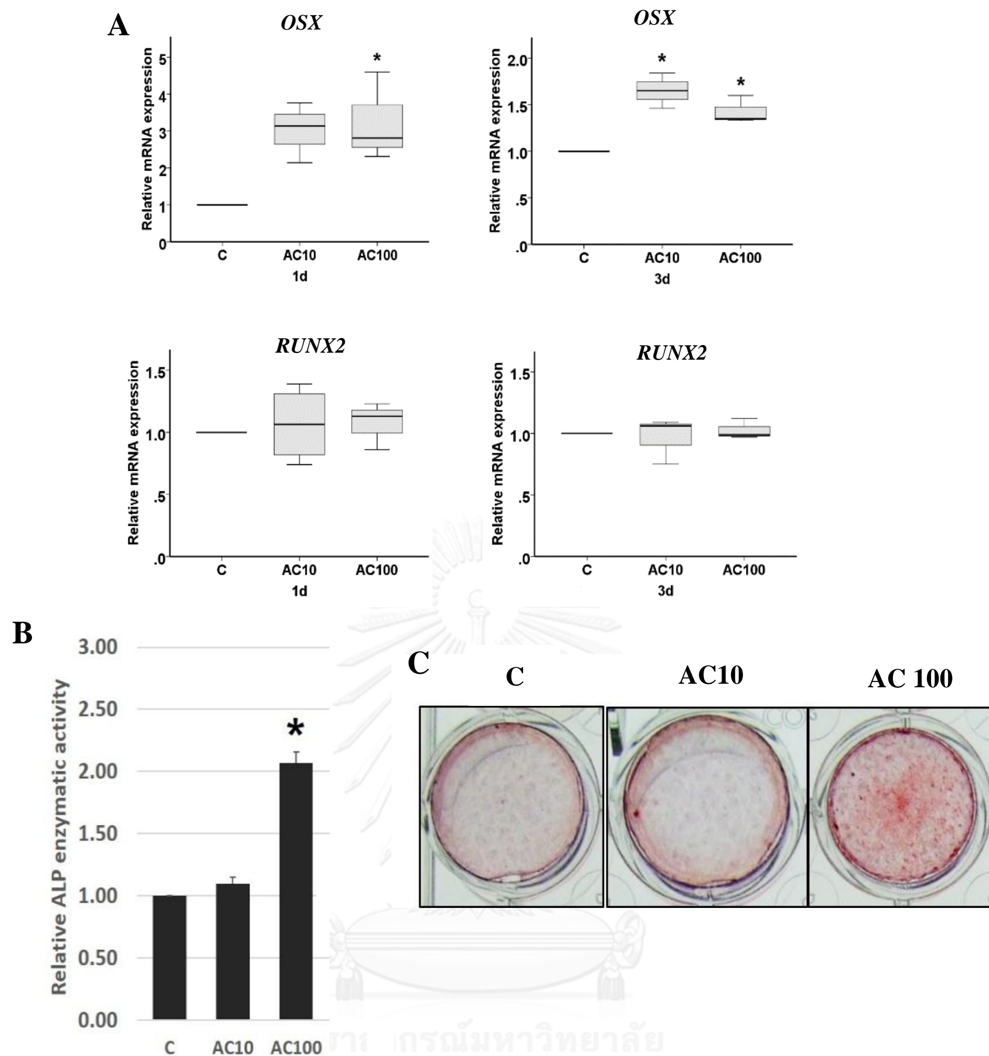
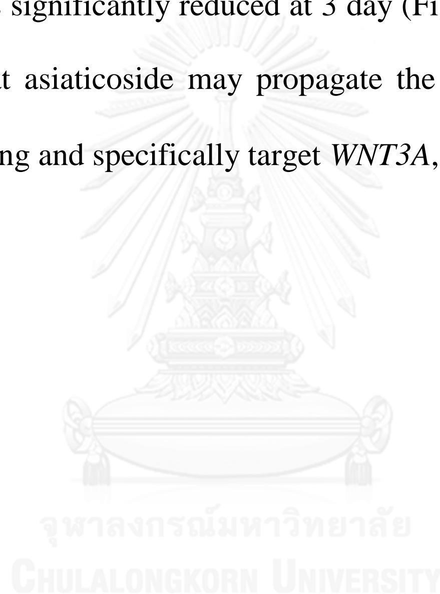


Figure 4.2 Asiaticoside (AC) stimulates osteogenic differentiation on hPDL cells. Cells were treated with asiaticoside (10 and 100 μM) in growth medium for 1 and 3 day. The expression of osteoblastic gene markers was performed by real time PCR. The gene expression was normalized to *GAPDH* (A). To investigate in vitro mineralization, cells were cultured in OM in the presence of asiaticoside (10 and 100 μM). ALP enzymatic activity was detected by ALP assay at 10 day (B). Mineralized nodules formation on hPDL cells was detected at 14 day by alizarin red staining (C). Mean values ± SD are presented with *$*p<0.05$* indicating a significant difference compared with control.

4.3 Objective 3: To show the involvement of Wnt associated genes and the activation of Wnt/β-catenin signaling pathway in asiaticoside stimulated cells

4.3.1 The effect of asiaticoside on mRNA expression of Wnt-associated genes

To elucidate which molecular mechanism might be involved asiaticoside induced cell differentiation. Asiaticoside significantly upregulated *WNT3A* gene expression dose dependently at indicated time points, but not *WNT5A* and *WNT10B*. The Wnt inhibitor, *DKK1* was not affected by asiaticoside stimulation, but *AXIN2*, a negative regulator of Wnt, was significantly reduced at 3 day (Figure 4.3). The upregulation of *WNT3A* indicates that asiaticoside may propagate the signal through the canonical Wnt/β-catenin signaling and specifically target *WNT3A*, not *WNT10B*.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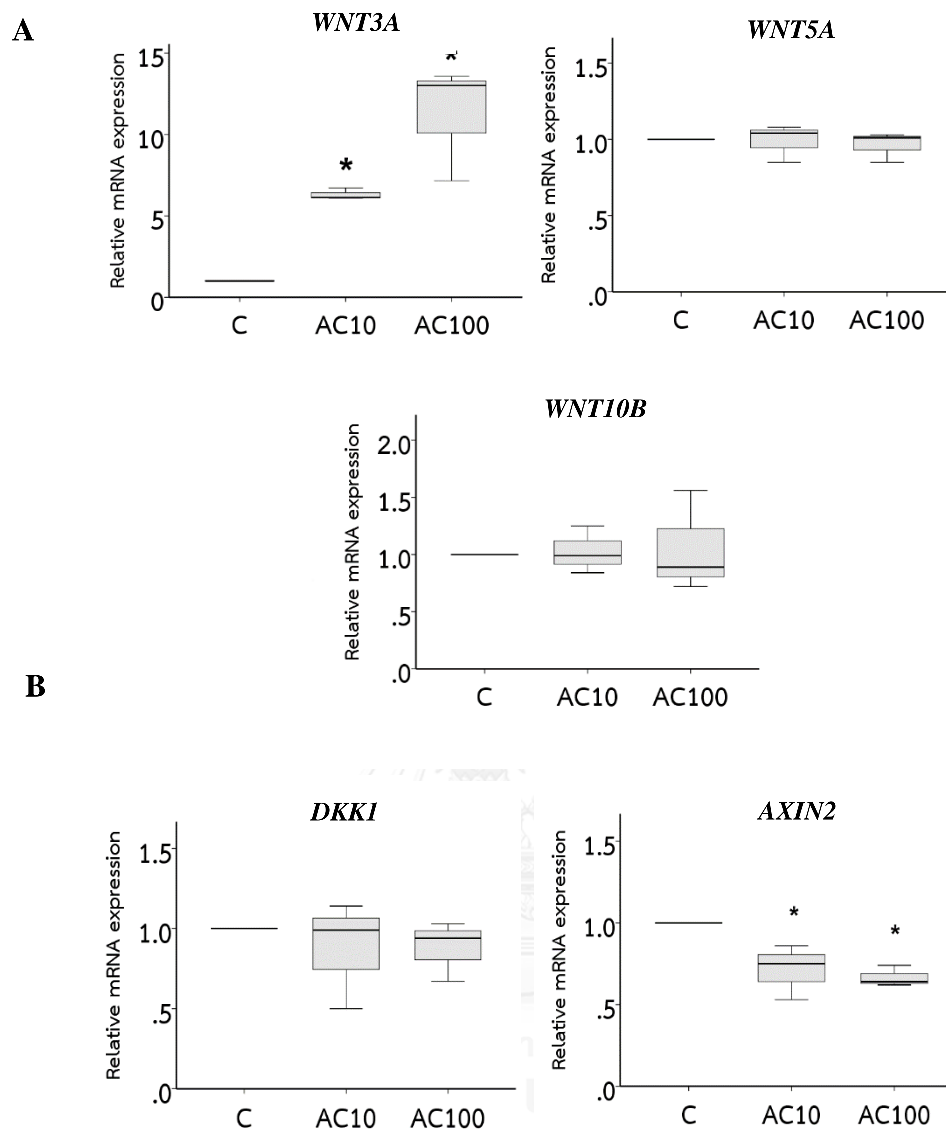

Figure 4.3 Effect of asiaticoside (AC) on the expression of Wnt-related genes. mRNA expression *WNT3A*, *WNT5A*, and *WNT10B* at 1 day (A), *DKK1* and *AXIN2* at 3 day (B) were measured by real time PCR in the presence or absence of asiaticoside (10 and 100 μM). *GAPDH* was used as housekeeping gene. Mean values ± SD are presented with *$p<0.05$ indicating a significant difference compared with control.

4.3.2 Asiaticoside-stimulated hPDL cells leads to the Wnt signaling activation

β-catenin is a direct target of the canonical Wnt pathway (Kim et al. 2013), therefore the activation of Wnt signaling was assessed by the translocation of β-catenin into the nucleus induced by asiaticoside. Fluorescent immunocytochemistry showed that β-catenin homogenously stained in the cell of control, while it predominantly localized to the nucleus in asiaticoside-treated cells (Figure 3B). The lithium chloride (LiCl), an activator of canonical Wnt signaling, was included as the positive control of β-catenin nuclear accumulation (Figure 4.4A). Correspondingly, western blotting analysis of nuclear and cytoplasmic extracts revealed the increase of β-catenin in the nuclear fraction in asiaticoside-treated cells compared to the control. The significant increase in nuclear-to-cytoplasmic (N/C) ratio of β-catenin was also shown (Figure 4.4B). To assess whether the gene expression of *β-CATENIN* was affected by asiaticoside, we performed a qualitative PCR analysis and found the expression was not difference with control (Figure 4.4C). Consistent with mRNA expression, the total protein of β-catenin in a whole cell lysate was not changed (Figure 4.4D). These results indicate that asiaticoside activates Wnt/β-catenin signaling through the translocation of cytoplasmic β-catenins into the nucleus.

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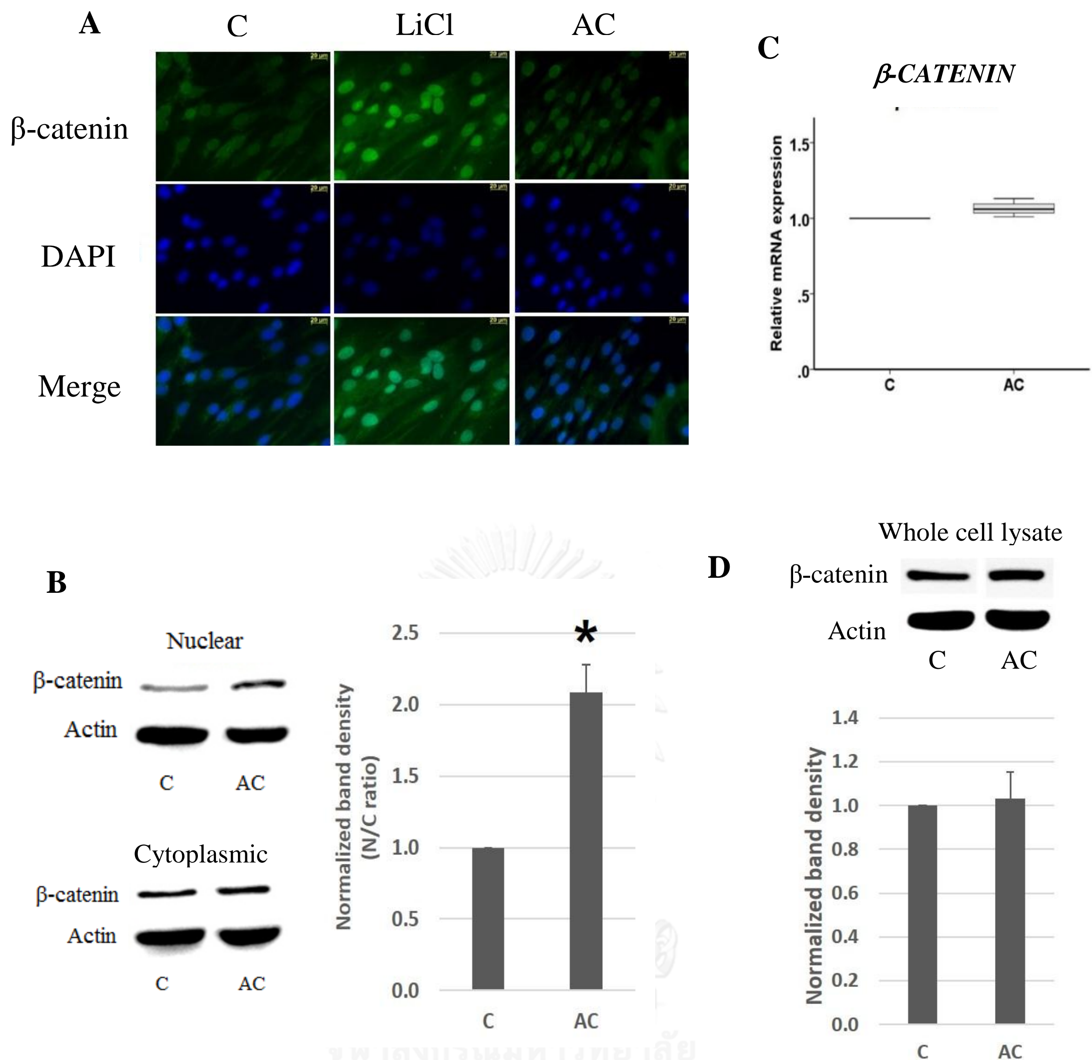

Figure 4.4 Asiaticoside-stimulated hPDL cells leads to the Wnt signaling activation. Nuclear β-catenins were detected by immunofluorescence staining after 1 day asiaticoside (100 µM) stimulation. DAPI was used to stain the nucleus. LiCl as positive control. Scale bar in micrographs represents 20 µm (A). Level of β-catenin from cytoplasmic and nuclear fraction were also confirmed by western blot to identify β-catenin localization in the nucleus after cells treated with asiaticoside 100 µM for 2 day. Actin was used as internal control. β-catenin nuclear/cytoplasmic (N/C) ratio was quantitatively assessed by measuring band density and the expression was normalized to actin (B). mRNA expression of *β-CATENIN* was shown by real time PCR (C).

Cells treated with asiaticoside for 2 day were subsequently extracted to examine the total protein of β-catenin by western blot. The quantitative data of protein expression is presented (D). Mean values ± SD are presented $*p<0.05$ indicating a significant difference compared with control.

4.3.3 Asiaticoside upregulates downstream targets of Wnt/β-catenin signaling

As previously shown (Han N. et al. 2014), *DSPP* and *DMP1* are the target genes of Wnt/β-catenin signaling in odontoblastic differentiation. To examine whether *DSPP* and *DMP1* are targeted by asiaticoside, the expression of these genes were measured by real time PCR and the protein level was detected by western blot analysis. Asiaticoside significantly increased both *DSPP* and *DMP1* mRNA expression dose dependently at 1 day. At 3 day, the highest expression was significantly seen at asiaticoside 10 µM, whereas the expression level started to decrease at concentration 100 µM (Figure 4.5A). The level of DSPP and DMP1 protein was correspondingly raised at any tested concentration of asiaticoside at 2 day (Figure 4.5B).

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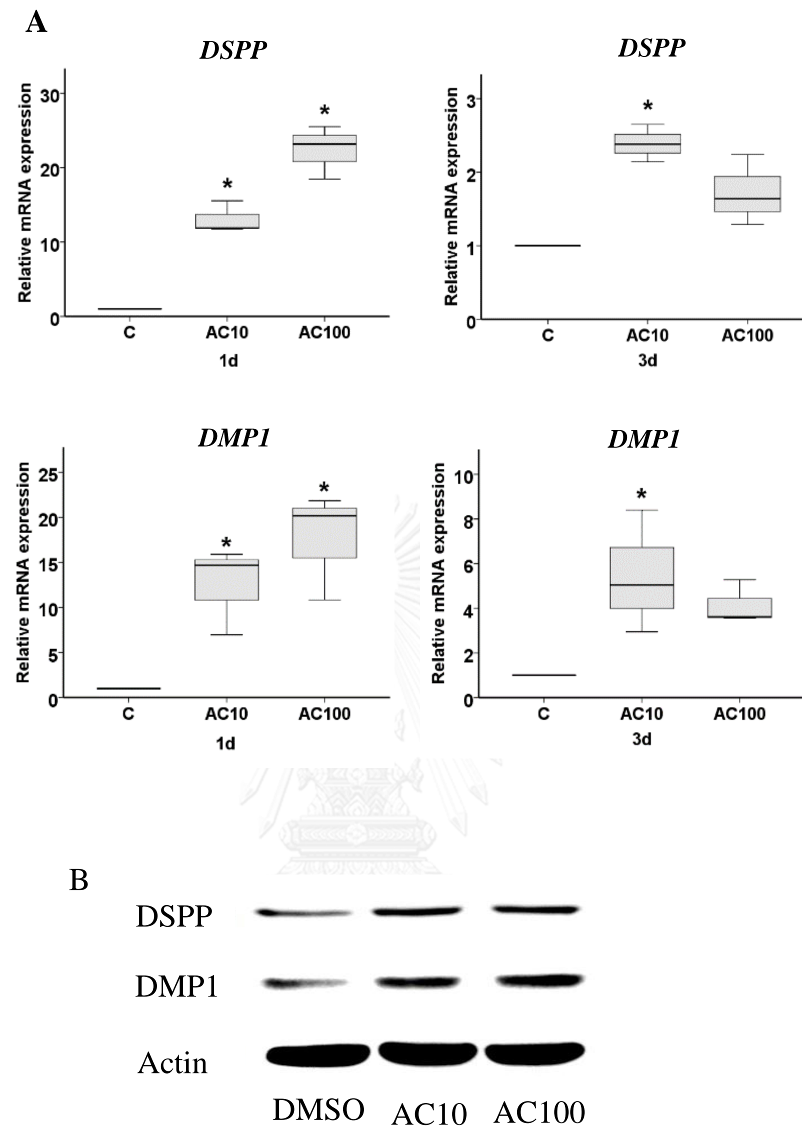

Figure 4.5 The expression of *DSPP* and *DMP1* gene by asiaticoside. Cells treated with asiaticoside (AC) in growth medium for 1 and 3 day were subsequently analyzed the level of mRNA expression by real time PCR. Relative mRNA expression of *DSPP* and *DMP1* was normalized to *GAPDH* (A) and the protein expression by western blot at 2 day. Actin was used as internal control (B). Mean values ± SD are presented $*p<0.05$ indicating a significant difference compared with control.

4.4 Objective 4: To show the inhibiton of the targets of Wnt/β-catenin signaling pathway by DKK1 in asiaticoside-treated hPDL cells

4.4.1 rh-DKK1 inhibited asiaticoside-stimulated β-catenin nuclear translocation

The Wnt signal transduction was blocked by adding recombinant human DKK1 1 hour before treating the cells with asiaticoside. Localized β-catenin in the nucleus was observed in asiaticoside group. Otherwise, DKK1 efficiently repressed asiaticoside-induced β-catenin translocation to nucleus (Figure 4.6).

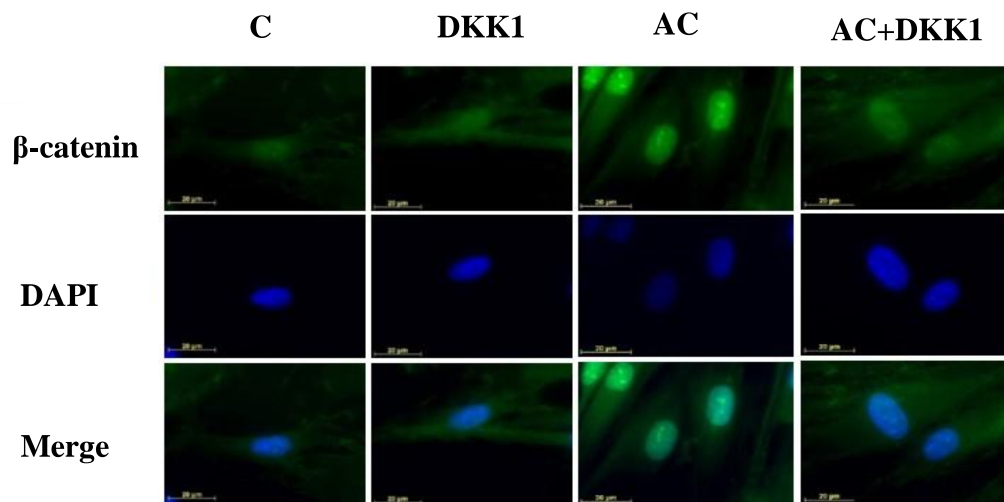

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Figure 4.6 DKK1 attenuated nuclear β-catenin in hPDL cells. Immunofluorescence staining showed β-catenin expression after 1 day asiaticoside (AC) 100µM with or without rh-DKK1 100ng/ml. DAPI was used to stain the nucleus. LiCl as positive control. Scale bar in micrographs represents 20 µm.

4.4.2 rh-DKK1 attenuated asiaticoside-induced expression of osteoblastic gene markers

The results showed that *OSX*, *DSPP* and *DMP1* mRNA expression were significantly increased following asiaticoside treatment. DKK1 significantly diminished the expression of *OSX*, *DSPP* and *DMP1* compared with asiaticoside group (Figure 4.7A). Moreover, the increased protein level of DSPP and DMP1 after asiaticoside stimulation was decreased by DKK1 (Figure 4.7B). These data indicated that the Wnt/β-catenin pathway is involved in differentiation of hPDL cells stimulated by asiaticoside.

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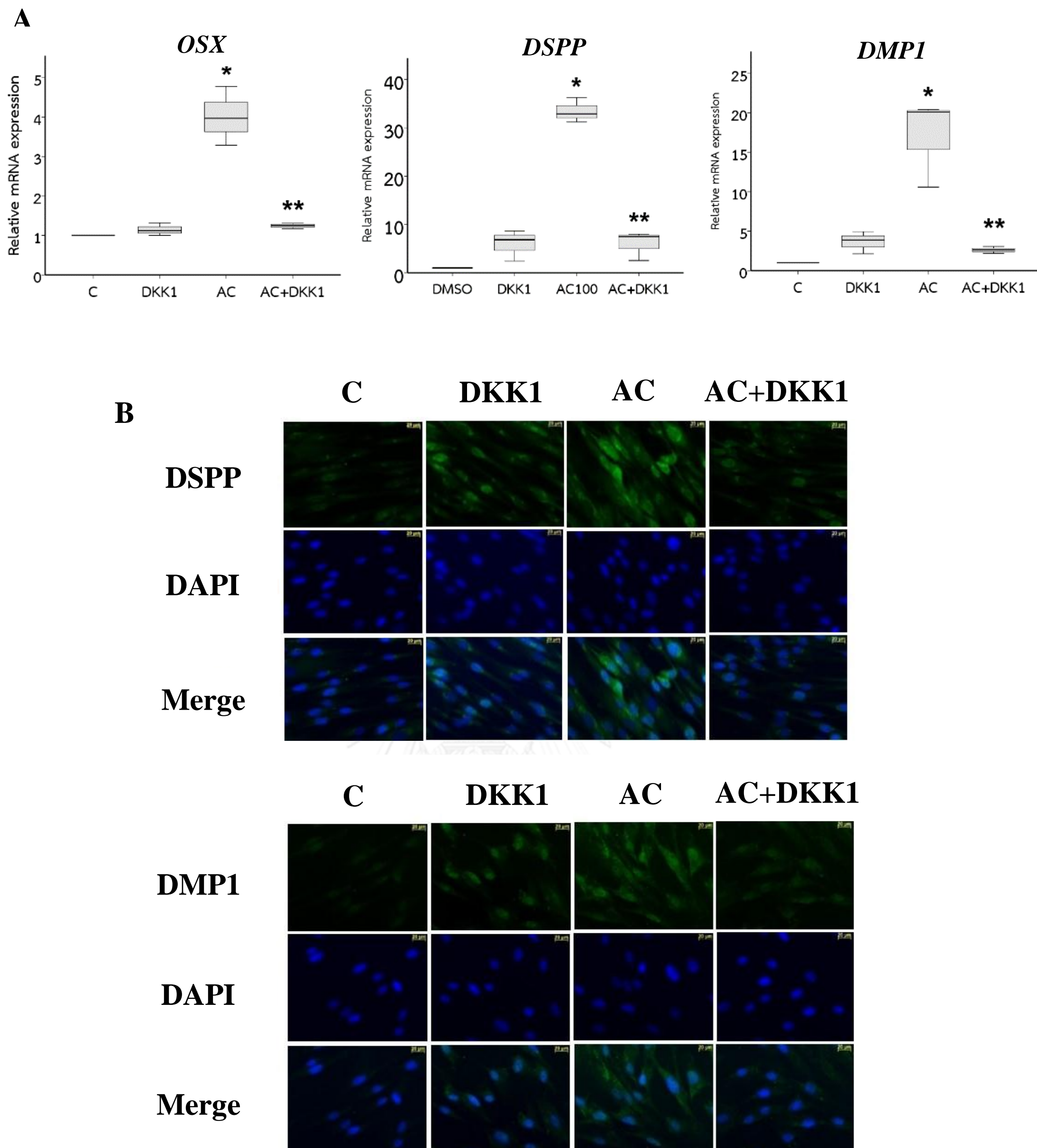

Figure 4.7 DKK1 attenuated the expression of osteogenic gene marker in asiaticoside-treated cells. After 1 day cultured in the presence or absence of asiaticoside (AC) 100µM or rh-DKK1 100ng/ml, the mRNA expression of *OSX*, *DMP1* and *DSPP* were performed by real time PCR (B). DSPP and DMP1 protein expression were detected by using immunofluorescence. Nucleus was stained with DAPI and merge image was also shown. Scale bar in micrographs represent 20 µm

(C). Mean values ± SD are presented *$p<0.05$ indicating a significant difference compared with control; **$p<0.05$ compared with asiaticoside group

CHAPTER V

DISCUSSION AND CONCLUSION

Our results are consistent with the previous study that demonstrated the effect of asiaticoside to promote osteogenic differentiation in hPDL cells (Nowwarote et al. 2013). Asiaticoside belongs to the triterpenoid saponin with pentacyclic terpene skeleton (aglycone) and glycoside side chain (James and Dubery 2009). Triterpenoid saponin is known as an amphiphilic agent with the hydrophilic sugar chains and the hydrophobic triterpene backbone. Due to these properties, saponin can interact with cholesterol in the cell membrane that forms insoluble complexes (Moses et al. 2014) creating aqueous pores thus increasing cell permeability. The increase of membrane permeability enables ions and other molecules such as proteins entering the cell (Augustin et al. 2011). Permeabilization may possibly allow Ca^{2+} influx into the cells or Ca^{2+} release from endoplasmic reticulum (Lukyanenko and Gyorke 1999) to activate the Ca^{2+} signaling through protein kinase C, calcineurin and calcium calmodulin-dependent protein kinase II that subsequently activate transcription of NF-κB, NFATc and cAMP response element binding protein (Kim et al. 2013). The similarity of saponin aglycones and steroids structure also suggests another fascinating mode of saponin action by potentially binding to receptors of glucocorticoids (Augustin et al. 2011). Hence, it is hypothesized that asiaticoside exerts its activity by penetrating cell membrane and is possibly recognized by intracellular glucocorticoid receptors. However, it remains unclear of how asiaticoside

directly acts and influences the cell. Therefore, further study is required to prove this mechanism.

Asiaticoside has been extensively known as a wound healing agent. It was found to inhibit the hypertrophic scars and keloid formation via TGF-β/Smad pathway (Tang et al. 2011). The protective effect of asiaticoside against cognitive dysfunctions associated with diabetes has been revealed through PI3K/Akt/NF-κB pathway (Yin et al. 2015). However, the molecular mechanism involved in asiaticoside-induced osteogenesis remains unclear. It is indisputable that Wnt/β-catenin signaling pathway regulates bone remodeling and the activation of Wnt leads to osteoblast differentiation (Kramer et al. 2010; Cawthorn et al. 2012). Wnt signaling particularly has a pivotal role to maintain periodontium homeostasis and regeneration. Periodontal ligament is a Wnt-responsive tissue and PDL derived cells are responsive to endogenous Wnt. When Wnt signaling was blocked, the thickness of alveolar bone was decreased and periodontal ligament space became wider (Lim et al. 2014). Downregulation of Wnt signaling leads to cementum devastation and root resorption (Lim et al. 2014). Ectopic Wnt enhanced ossification and osteogenic markers including Osx and osteoclacin, but not Runx2 (Day et al. 2005). Interestingly, stimulating PDL tissue with the Wnt signaling inducers caused new cementum and PDL fibers formation in rat periodontal defect model (Han et al. 2015).

The present study showed asiaticoside typically induced *WNT3A* gene expression, not the other Wnt ligands. Juan Lu et al. also demonstrated that WNT3A was more highly expressed on the tension side in the PDL tissue of tooth receiving orthodontic force (Lu et al. 2016). It was also shown that WNT3A could partially rescue *Porphyromonas gingivalis* LPS-reduced osteogenic activity of rat bone

marrow-derived mesenchymal cells (Tang et al. 2014). Investigation by Nemoto et al. demonstrated that WNT3A induced ALP expression which was mediated by *OSX* gene in dental follicle cells and the inhibition by DKK1 diminished its expression (Nemoto et al. 2016). In addition, WNT3A was recently reported to be involved in the promotion of mineralization by dental follicle cells when co-culture with Hertwig's epithelial root sheath cells in the presence of decalcified dentin (Yang et al. 2014). These evidences indicate the importance of WNT3A in osteoblast differentiation. Although, some reports stated the involvement of WNT5A (Wang et al. 2014; Baschant et al. 2016) and WNT10B (Lu et al. 2016) in osteogenesis or tooth development, but WNT3A may be specifically targeted by asiaticoside action.

Other studies have been reported that inhibition of Wnt signaling could promote osteoblast differentiation in PDL cells from periodontitis patients (Liu et al. 2011; Liu et al. 2015), which is contrary to this study. The possible reason to elucidate this circumstances is the distinct condition of cells used in the study. The isolated PDL cells in this study obtained from healthy patient, whereas their studies collected the samples from inflamed periodontal tissue. The microenvironment alteration definitely leads to the change of cell response and behavior.

DSSP expression was increased by WNT3A-activated canonical Wnt signaling in dental pulp cells (DPCs) (Rahman et al. 2012). In the absence of β-catenin, *DSPP* and *DMP1* expression were diminished in DPCs (Han N. et al. 2014) indicating these two genes are the target of β-catenin signaling. This indicates that the involvement of Wnt signaling is inevitable to promote *DSPP* and *DMP1* expression. In the present study, asiaticoside-induced Wnt signaling activation confirmed by β-catenin translocation to the nucleus was suppressed by DKK1. The activated Wnt pathway

resulted in the increased *DSPP* and *DMP1* expression. Similar to β-catenin, both the expression of DSPP and DMP1 were attenuated in the presence of DKK1. These results demonstrated that DSPP and DMP1 are the downstream targets of asiaticoside-activated the Wnt/β-catenin pathway.

DSPP and DMP1 are the extracellular matrix proteins that act as positive regulators in hard tissue mineralization. Mutated *DSPP* and *DMP1* gene give the impacts on dentin or bone phenotype (Suzuki et al. 2012). *DMP1* knockout mice caused the reduction of mineralized bone in mice with low mineral content and the increase of crystal size (Ling et al. 2005). The defect of dentin phenotype in *DMP1*$^{-}$/$^{-}$ and *DSPP*$^{-}$/$^{-}$ mice was observed with widened predentin and hypomineralized dentin (Sreenath et al. 2003; Ye et al. 2004). Those data proved that both DSPP and DMP1 are required for hard tissue development and maturation. Our study also showed the induction of *DSPP* and *DMP1* gene at 1 day, which was decreased at 3 day. Similarly, *OSX* was shown the higher mRNA expression at 1 day than at 3 day. It seems these osteogenic markers have stronger expression at the early time point to run their function. However, the increased expression of those genes suggests that asiaticoside has the ability to potentially promote mineralized tissue formation in hPDL cells.

We propose a schematic model of the activation of Wnt signaling by asiaticoside is possibly mediated by WNT3A through the canonical β-catenin pathway. Activation of the canonical Wnt results in the accumulation of cytoplasmic β-catenin and the subsequent translocation to the nucleus to activate the transcription of *OSX*, *DSPP* and *DMP1* gene. Induction of these genes promotes hPDL cells to differentiate into osteoblast lineage cells (Figure 5.1).

In conclusion, we demonstrated that asiaticoside could induce osteogenic differentiation in hPDL cells by activating Wnt/β-catenin signaling. By understanding the molecular mechanism of asiaticoside induced osteogenic differentiation in hPDL cells through the Wnt/β-catenin signaling, asiaticoside can be a novel therapeutic agent to induce tissue or bone regeneration by mesenchymal cells in inflammatory condition. However, further study is required to precisely identify how asiaticoside directly interacts with PDL cells and exerts its biological function. Furthermore, it is necessary to possibly modify the structure of asiaticoside to improve the outcome for clinical treatment by increasing its efficacy and efficiency.

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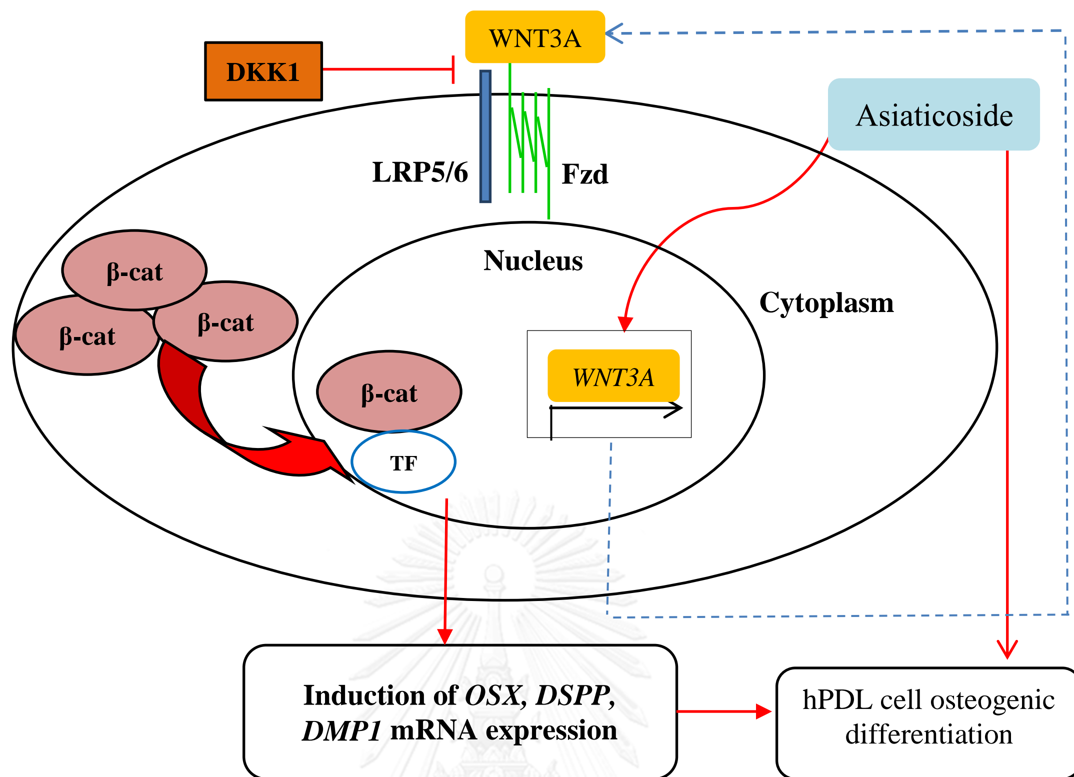

Figure 5.1 Schematic model of the proposed mechanism of asiaticoside inducing osteogenic differentiation by hPDL cells.

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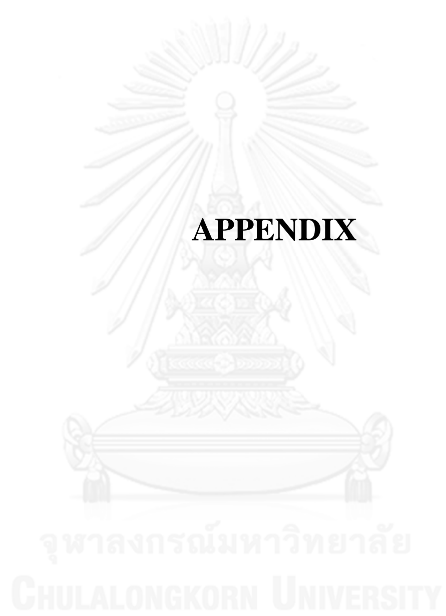

APPENDIX

VITA

Miss Atika Resti Fitri was born on April 20, 1991 in Medan, Indonesia. She graduated the degree of Doctor Dental Surgery (D.D.S) in 2014 from Faculty of Dentistry, University of Sumatera Utara. She started to study in Oral Biology program for master degree in 2014 at Faculty of Dentistry, Chulalongkorn University. The research was performed at the Research Unit of Mineral Tissue, Faculty of Dentistry, Chulalongkorn University.

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