EFFECTS OF BIOSYNTHESIS *OROXYLUM INDICUM* / SILVER NANOPARTICLES ON BIOLOGICAL BEHAVIORS OF THE ENCAPSULATED STEM CELLS

By

JARUTAI PRAPAIPITTAYAKHUN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE PROGRAM IN DENTAL IMPLANTOLOGY FACULTY OF DENTISTRY THAMMASAT UNIVERSITY ACADEMIC YEAR 2022

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THAMMASAT UNIVERSITY
FACULTY OF DENTISTRY

Thesis

BY

MS. JARUTAI PRAPAIPITTAYAKHUN

ENTITLED

EFFECTS OF BIOSYNTHESIS *OROXYLUM INDICUM* / SILVER NANOPARTICLES ON BIOLOGICAL BEHAVIORS OF THE ENCAPSULATED STEM CELLS

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Thesis Title	Effects of *Oroxylum indicum*/silver nanoparticle biosynthesis on biological behavior of the encapsulated stem cells
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ABSTRACT

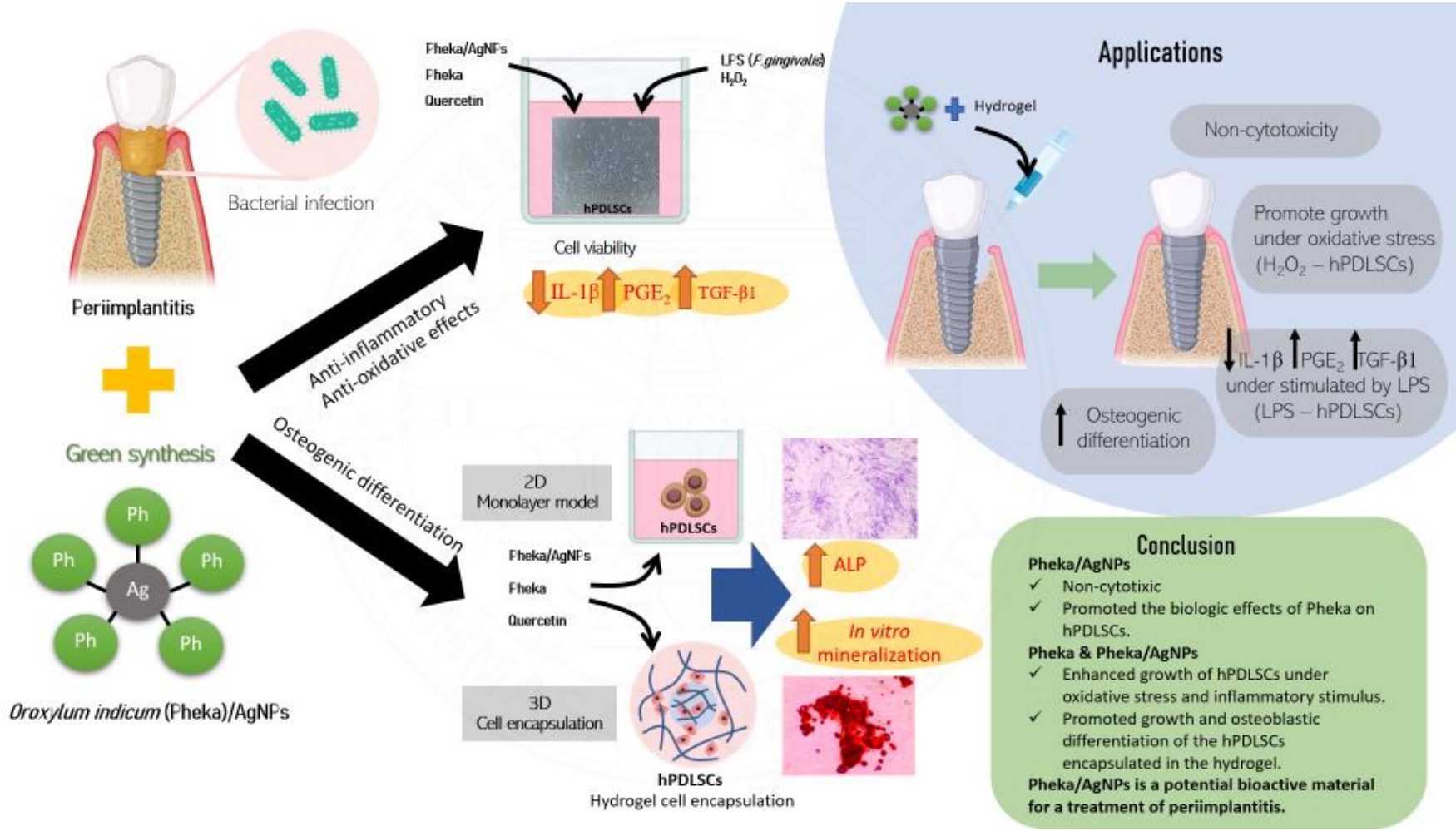
Background: Biosynthesized silver nanoparticles (AgNPs) with *Oroxylum indicum* (Pheka) as reducing agent (Pheka/AgNPs) were introduced as alternative biomaterial to combat tissue destruction caused by bacterial toxins and inflammatory cytokines and promote bone regeneration in peri-implantitis defects. The goal was to improve treatment outcomes for peri-implantitis and promote alveolar bone regeneration. **Objective:** To investigate and compare biologic effects of the extracts from the stem bark of the *Oroxylum indicum* (Pheka) and biosynthesis silver nanoparticles (Pheka/AgNPs) on human periodontal ligament stem cells (hPDLSCs) that were under oxidative stress and inflammatory stimulus, including growth and osteogenic differentiation of hPDLSCs on two-dimensional cell culture and the hydrogel encapsulated cells. **Materials and methods:** Results were compared with effects of quercetin (QT) as a reference flavonoid. Cytotoxicity, antioxidative effects, and anti-inflammatory effects and effects on growth and osteogenic differentiations of Pheka and Pheka/AgNPs on hPDLSCs were investigated in two- and three-dimensional cell cultures. The present research was divided into two parts: *I)* two-dimensional (2D) cell culture and *II)* three-dimensional (3D) cell culture or cell encapsulation. According to different cell culture medium supplements, four study groups were created: A) growth medium alone (negative control); B) growth medium with *Oroxylum indicum* crude extract (Pheka); C) biosynthesized Pheka/AgNPs; and D) Quercetin (positive

control). Approved by the Ethical Review Sub-Committee Board for Human Research Involving Sciences, Thammasat University, No.3 and patient informed consent, third molars and premolars were collected from 10 healthy patients aged 18 to 25 undergoing surgical removal or tooth extraction at the Dental Clinic, Thammasat University Hospital. In Part I, hPDLSCs were exposed to culture media supplemented with Pheka or Pheka/AgNPs according to study groups, and cell viability assay was performed to determine working concentrations for each supplement. Human PDLSCs were stimulated with oxidative stress and inflammatory stimulus caused by hydrogen peroxide (H_2O_2) and purified *Porphyromonas gingivalis* lipopolysaccharide (LPS), respectively. In addition, Pheka/AgNP endocytosis was examined by transmission electron microscopy (TEM). In Part II, hPDLSCs were encapsulated in an in-house thermosensitive 2% (w/v) nano-hydroxyapatite – 2% (w/v) calcium carbonate microcapsules - 10% beta-glycerophosphate- 4:1 (w/w) chitosan/collagen hydrogel (nHA-$CaCO_3$-Chitosan/collagen hydrogel) to recapitulate natural restricted environment of human mesenchymal stem cells (hMSCs) during stem cell transplantation. Then hydrogel-cell suspensions were seeded on a 24-well cell culture plate and developed in group A, B, C and D culture mediums for 21 days. After that, live/dead cell staining and cell viability assays, TEM and enzyme-linked immunosorbent assays (ELISA) of secreted inflammatory cytokines, interleukin-1 beta (IL-1β), prostaglandin E_2 (PGE_2) and transforming growth factor-beta 1 (TGF-β1) in culture mediums and characterizations of osteogenic differentiation of hPDLSCs were performed (p<0.05, N=3-5, Mean±SD). **Results:** 1) Working concentrations of Pheka, Pheka/AgNPs were 5 parts per million (ppm) and 20 ppm and for QT, 2.5 ppm, and 5 ppm; 2) biosynthesized Pheka/AgNPs were non-cytotoxic and internalized into cell cytoplasm and endosomes. Human PDLSCs with AgNPs endocytosis exhibited strong cell viability, intact cell and nuclear membranes, and exosomes for AgNPs exportation from cells as exhibited by TEM; 3) Pheka and Pheka/AgNPs could decrease adverse effects of oxidative stress and inflammatory stimulus on cell viability of hPDLSCs similarly to a reference flavonoid, QT; 4) Pheka and Pheka/AgNPs could promote growth and osteogenic differentiation of hPDLSCs on cell culture plates and hydrogel encapsulated cells; and 5)

Pheka/AgNPs enhanced biological functions of Pheka on hPDLSCs. **Conclusions:** Biosynthesized Pheka/AgNPs were non-cytotoxic to hPDLSCs, and biosynthesized silver nanoparticles could enhance bioactivity of the flavonoid Pheka. It was hypothesized that hydrogel impregnated with Pheka/AgNPs could decrease degrees of inflammatory response and tissue damage caused by bacterial toxins and inflammatory cytokines in periodontal defects as well as promote osteogenic differentiation of host and transplanted cells in skeletal defects. Effects on bone regeneration of biosynthesized Pheka/AgNPs in peri-implantitis in an animal model should be further investigated.

Keywords: Anti-inflammatory effects, Biosynthesized silver nanoparticles, Cell encapsulation, Flavonoids, Human periodontal ligament stem cells, *Oroxylum indicum,* Osteogenic differentiation

GRAPHIC ABSTRACT



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Ms. Jarutai Prapaipittayakhun

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List of abbreviations

Abbreviations	Description
AgNPs	Silver nanoparticles (AgNPs)
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
$CaCO_3$	Calcium carbonate
CISR	Cumulative Interceptive Supportive Therapy
CND	chronic noncommunicable diseases (CND)
CO_2	Carbon dioxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
h	Hours
hMSCs	Human mesenchymal stem cells
hPDLSCs	Human periodontal ligament stem cells
H_2O_2	Hydrogen peroxide
IL-1β	Interleukin-1 beta
LPS	Lipopolysaccharide
min	Minutes
MTT assay	Cell viability assay
nHA	nanohydroxyapatite

OD	Optical density
PDL	periodontal tissue (PDL)
PGE_2	Prostaglandin E2
Pheka	*Oroxylum indicum* (*O. indicum*)
Pheka/AgNPs	Biosynthesized silver nanoparticles using Pheka as a reducing agent
ppm	Part per million
TGF-β1	Transforming growth factor-beta 1
Ti-AgNP	AgNP-modified titanium surfaces
TiO_2/PA66	titanium nanoparticles-polyamide 66
TNF-α	Tumor necrosis factor-alpha
UV	Ultraviolet

CHAPTER 1
INTRODUCTION

1.1 Rationale of the study

Nowadays, dental implant treatment has been widely used to restore masticatory function and facial esthetic that are affected by tooth loss in all age groups from young adults to elderly. To ensure osteointegration and survival of the dental implants, a dental implant placement requires a sufficient amount of bone in good quality (1). An inevitable progressive bone resorption following tooth loss and after dental implant placement, together with a limited availability and morbidities associated with autologous bone grafting increase a need for bone augmentation and an intervention by bone tissue engineering (2). As a result, various bone substitutes have been developed and are commercially available. However, it is necessary to improve bioactive property of bone substitutes to promote faster and stronger bone regeneration that would shorten treatment time and enhance satisfactory outcomes, particularly in compromised cases.

Furthermore, another important aspect of post-treatment complication of dental implant treatment is peri-implantitis. Peri-implantitis is a critical biological complication of dental implant that could cause implant failure. Causes of peri-implantitis associate with bacterial toxins and inflammation of the supporting gingiva, periodontal ligament and bone around the implants (3). According to a Cumulative Interceptive Supportive Therapy (CIST) protocol 2002, a treatment protocol of peri-implantitis includes mechanical debridement with or without systemic or local antibiotic treatment, and a need for additional regenerative bone surgery are determined upon the extent of bone resorption (4).

Therefore, with an aim to improve treatment outcomes of peri-implantitis and promote alveolar bone regeneration, the biosynthesized silver nanoparticles (AgNPs) using *Oroxylum indicum* (Pheka) as a reducing agent (Pheka/AgNPs) was introduced as an alternative biomaterial that would help to combat bacteria, support

antibacterial effects of antibiotics, and promoted bone regeneration. Based on antibacterial property of the AgNPs (5), anti-inflammatory and antioxidant properties of the flavonoids in the Pheka's stem bark extracts and together with the enhanced biologic effects of the biosynthesized flavonoid/AgNPs (6, 7), it was postulated that the biosynthesized Pheka/AgNPs would improve treatment outcomes of peri-implantitis treatment by decreasing local inflammatory responses caused by bacterial toxin and also decreasing a need for antibiotics to control local infections in the periodontal pockets, as well as promoting alveolar bone regeneration.

Green synthesis or biosynthesis of AgNPs, using plant extracts as a reducing agent is an alternative to chemical synthesis method. While chemical synthesis has high cost and toxicity caused by chemical used during the synthesis, green synthesis of AgNPs is simple, cost-effective, environment friendly, reproducible and safe (8, 9). An additional advantage is that the flavonoids, leading chemical components in plant extracts, can be found in high concentrations in several plants such as Pheka, bamboo, citrus, cherries, berries, broccolis, apples, onions and etc. Moreover, the biosynthesized AgNPs enhance biologic effects of flavonoids by increasing interactions between flavonoids and cell or bacteria through high surface area of the nanoparticles (6). Silver nanoparticles adhere to cell membrane of bacteria and disrupt permeability and respiration functions of cell membrane and thus causing cell dead (10). The AgNPs are effective against gram negative and positive bacteria, which are a pathognomonic cause of peri-implantitis (11). Besides, AgNPs can promote mesenchymal stem cells (MSCs) differentiation and proliferation (12).

Up to date, an antibacterial effect of silver nanoparticle is well understood, but there are a few studies on how flavonoids, particularly Pheka and Pheka/AgNPs affect growth and differentiation of human mesenchymal stem cells (hMSCs). Additionally, to make the study more relevance to treatment of peri-implantitis, human periodontal ligament stem cells (hPDLSCs) were used in the current study. The investigation on the biologic effects of Pheka/AgNPs on the encapsulated hPDLSCs was an initial step to observe biologic effects of the flavonoids on cells in the

restricted environment of the hydrogel cell encapsulation during the stem cell transplantation.

Regarding sources of flavonoid used, In the current study focused on *Oroxylum indicum or* Pheka, a Thai traditional medicinal plant that exhibits antibacterial, antioxidative and anti-inflammatory effects and inhibits adipogenic different (7, 13-15). Based on a previous report, an inhibitory effect on adipogenic differentiation of mesenchymal stem cells of Pheka could promote differentiation potential of MSCs and osteoprogenitor cells toward osteogenic differentiation (16). Therefore, the current study aimed to investigate biologic effects of the Pheka, and Pheka/AgNPs on growth, and osteogenic differentiations of hPDLSCs in a mono layer cell culture and the three-dimensional cell culture of the hydrogel cell encapsulation.

1.2 Conceptual framework and hypotheses

1.2.1 Conceptual framework

A conceptual framework of the present study was to apply flavonoids (Pheka) and the biosynthesis flavonoid/silver nanoparticles (Pheka/AgNPs) for decreasing inflammation and promoting growth and osteogenic differentiation of the human periodontal ligament stem cells (hPDLSCs) in monolayer cell culture and the hydrogel encapsulation model. For further clinical application, it was expected that by delivering the Pheka/AgNPs into periodontal defects it would decrease degrees of inflammation and tissue damages caused by bacterial toxins and autogenous inflammatory reactions, and at the same time promoted osteogenic differentiation of host and the transplanted cells in skeletal defects.

The fundamental benefit was to maximize healing and bone regeneration of the periodontium in peri-implantitis cases because it was reported that natural flavonoids not only exhibited anti-inflammatory, anti-oxidative and anti-bacterial effects, but also could stimulated differentiation and bone regeneration (17). Flavonoids from *Oroxylum indicum* (L.) Kurz (Pheka) demonstrates anti-adipogenic differentiation effects (18) that might on the other hand promote osteogenic differentiation (19, 20). Additionally, the biosynthesized silver nanoparticles

(flavonoid/AgNPs) was not only exhibited antibacterial function but also enhanced antioxidative and other biological effects of the natural flavonoids (21). Additionally, hPDLSCs were selected as tested cells because of their primary roles in regenerating periodontium, tooth supporting bone and connective tissue (22).

Therefore, the current study explored a possibility to apply an extract from a Thai medicinal plant to improve bioactivity of bone substitute for treating peri-implantitis and promoting bone regeneration. Under a support from an interdisciplinary collaboration of researchers from Faculty of Science and Technology and Faculty of Dentistry, Thammasat University, effects of *Oroxylum Indicum* bark extracts (Pheka), and the biosynthesized silver nanoparticles using Pheka as a reducing agent (Pheka/AgNps) on antioxidative and anti-inflammatory reactions and growth and differentiation of hPDLSCs were investigated (Figure 1.1).

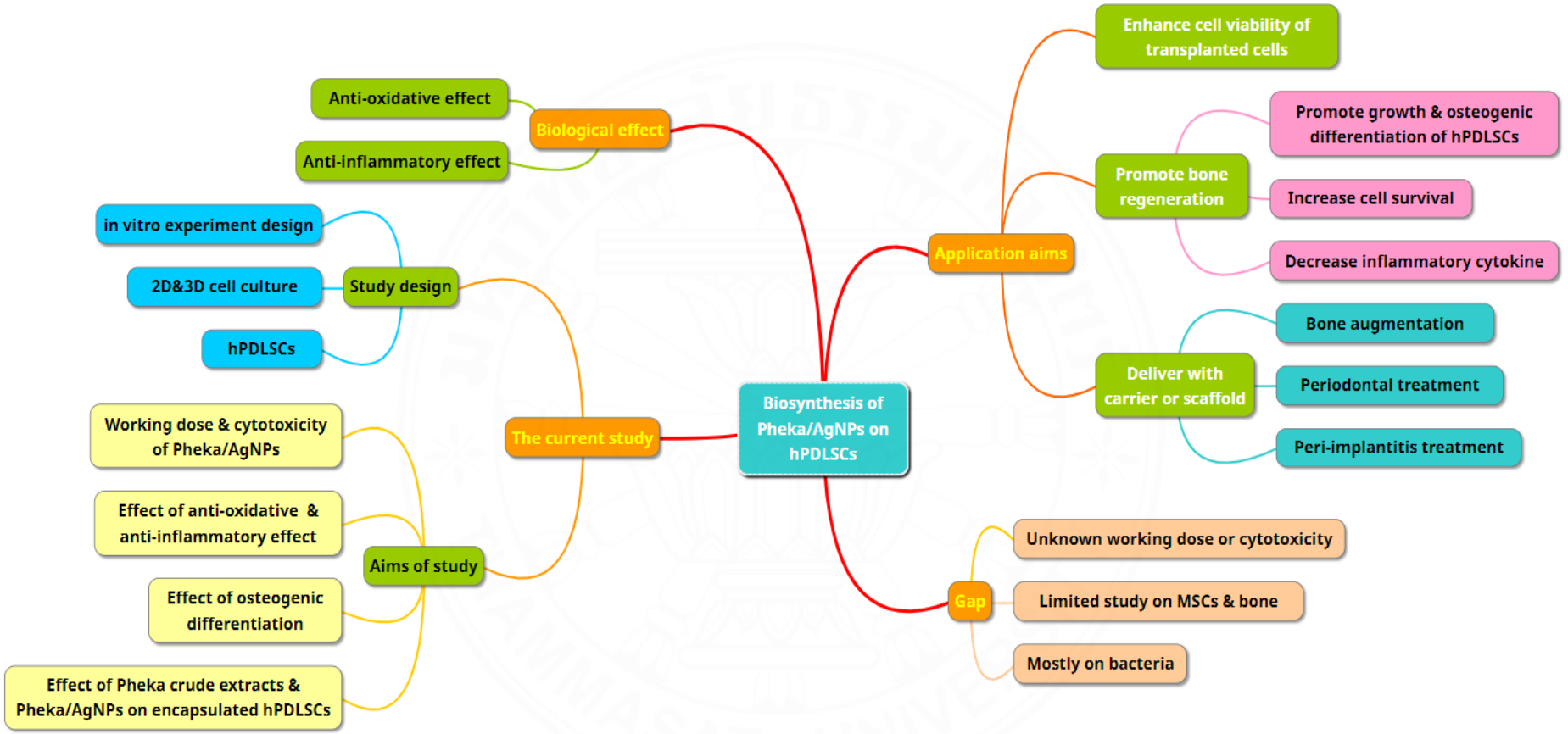

Figure 1.1 Conceptual framework

1.2.2 Hypotheses

1. The Pheka and Pheka/AgNPs was non-cytotoxic to hPDLSCs.

2. The Pheka and Pheka/AgNPs were able to decrease adverse effects of oxidative stress, inflammatory response stimulated by bacterial toxin on cell viability of the hPDLSCs.

3. The Pheka and Pheka/AgNPs were able to promote growth and osteogenic differentiation of the hPDLSCs in mono layer cell culture and the encapsulated cells culture.

4. The biosynthesized Pheka/AgNPs enhanced biologic functions of Pheka on the hPDLSCs.

1.2.3 Research questions

1. Would Pheka and Pheka/AgNPs be toxic to hPDLSCs and what was working concentrations of Pheka and Pheka/AgNPs?

2. Would Pheka and Pheka/AgNPs be able to decrease adverse effects of oxidative stress, inflammatory response stimulated by bacterial toxin on cell viability of the hPDLSCs?

3. Would Pheka and Pheka/AgNPs be able to promote growth and osteogenic differentiation of the hPDLSCs in mono layer cell culture and the encapsulated cells culture?

4. Would Pheka/AgNPs enhance biologic functions of Pheka on the hPDLSCs?

1.3 Objectives

Aims of the present study were

1. To investigate the effects of the Pheka and Pheka/AgNPs on cell viability and determine working concentrations.
2. To investigate and compare effects of the Pheka and Pheka/AgNPs on anti-oxidative and anti-inflammatory response stimulated by bacterial toxin on cell viability of the hPDLSCs.
3. To investigate effects of the Pheka and Pheka/AgNPs on growth and osteogenic differentiations of the hPDLSCs in mono layer cell culture and the encapsulated cells culture.
4. To compare biologic functions of Pheka and Pheka/AgNPs on the hPDLSCs.

1.4 Research design

In vitro experimental study

1.5 Scope of the study

The current study was an *in vitro* study that aimed to investigate and compare biologic effects of the Pheka and Pheka/AgNPs on growth and osteogenic differentiation of the hPDLSCs on cell culture plates and the encapsulated cells, and then compared with those of a reference flavonoid, quercetin (QT). Cytotoxicity, antioxidative and anti-inflammatory effects and the effects on growth and osteogenic differentiations of the Pheka and Pheka/AgNPs on hPDLSCs were investigated in two- and three-dimensional cell cultures. The current study was separated into 2 parts, (I) two-dimensional (2D) cell culture on mono layer cell culture and (II) three-dimensional (3D) cell culture of cell encapsulation. According to the different supplements in cell culture mediums, there were 4 groups of the study, Group A: Growth medium alone (negative control), Group B: Pheka, Group C: Pheka/AgNPs, and Group D: Quercetin (positive control) (Table 1.1).

Under a permission from the Ethical Review Sub-Committee Board for Human Research Involving Sciences, Thammasat University, No.3 and patient informed

consent, third molars and premolar were collected from 10 healthy patients aged 18-25 years old undergoing a surgical removal or tooth extraction at the Dental Clinic, Thammasat University Hospital. Then, periodontal ligament stem cells (hPDLSCs) were isolated and expanded. After that **Part I,** hPDLSCs were exposed to culture mediums supplemented with the Pheka or Pheka/AgNPs according to groups of the study, and then cell viability assay was performed to determine working concentrations of each supplement. Next, antioxidative and anti-inflammatory effects of Pheka and Pheka/AgNPs on cell viability and effects of Pheka and Pheka/AgNPs on secretion levels of inflammatory cytokines, interleukin-1 beta (IL-1β) and prostaglandin E_2 (PGE_2) and an anti-inflammatory cytokine, transforming growth factor-beta 1 (TGF-β1) in culture mediums, and growth and osteogenic differentiations of hPDLSCs were investigated. A purified *Porphyromonas gingivalis* LPS was used as a representative bacterial toxin to stimulate inflammatory reaction of the hPDLSCs.

Part II of the study, the hPDLSCs were encapsulated in an in-house thermosensitive nano-hydroxyapatite-calcium cabornate-chitosan/collagen hydrogel to recapitulate natural restricted environment of the hMSCs during stem cell transplantation and the hydrogel delivery. Then the hydrogel-cell suspensions were seeded on 24 well cell culture plate and cultured in culture mediums of Groups A, B, C and D for 21 days. After that live/dead cell staining and cell viability assays and the characterizations of osteogenic differentiation of the hPDLSCs were performed. Descriptive data were described, and quantitative data were compared among groups. Significant different results were set at $p<0.05$ (N=3-5, Mean±SD) (Figure 1.2 and 1.3) (Tables 1.2 and 1.3).

Table 1.1 Groups of the study

Sets of study	Groups	Descriptions
Negative control group	A	Cell culture medium alone
Experimental groups	B	Cell culture medium supplemented with Pheka
	C	Cell culture medium supplemented with Pheka/AgNPs
Positive control group	D	Cell culture medium supplemented with Quercetin

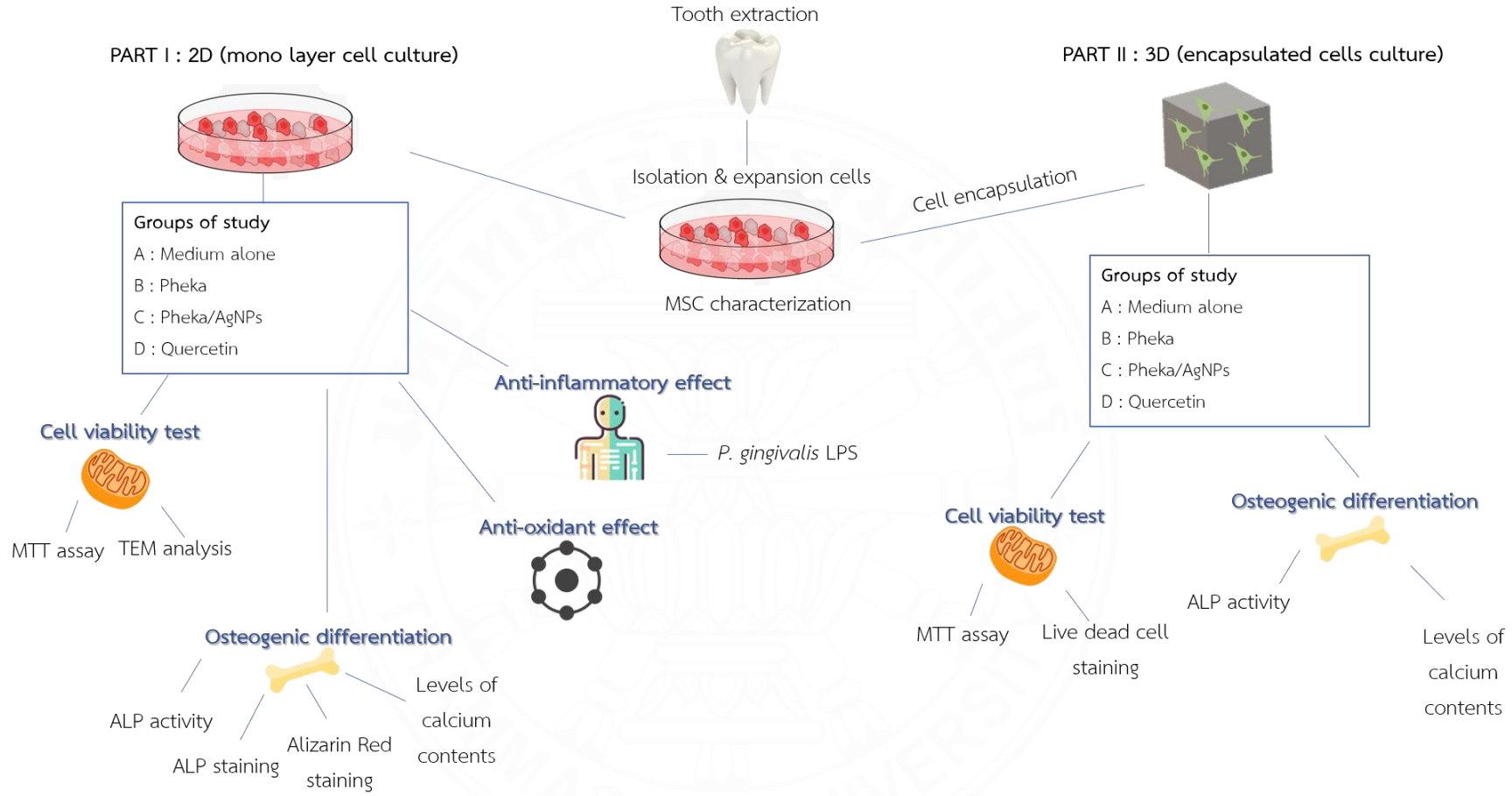

Figure 1.2 Scope of study

Figure 1.3 Diagram of scope of the study

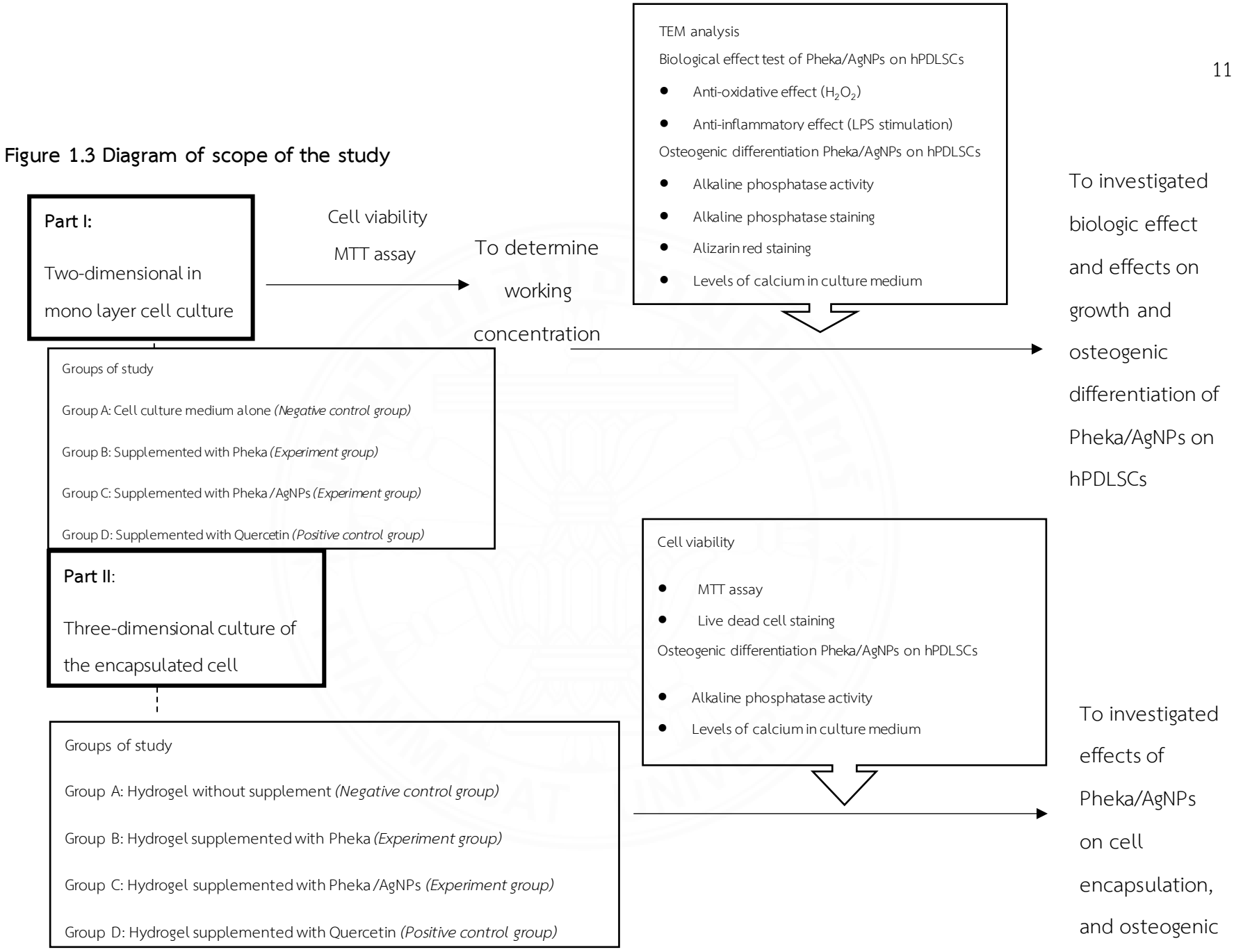

Table 1.2 Dependent variables

Groups of Dependent Variables	Details of Dependent Variables
Type of cells	Human periodontal ligament stem cells
Cell culture models	**Part I:** Cells in mono layer cell culture **Part II:** Hydrogel encapsulated cells
Groups of the study according to supplements in the culture mediums:	
Negative control groups	**Part I:** Cell culture medium alone **Part II:** Hydrogel in cell culture medium alone
Experimental groups	**Part I:** Cell culture medium with Pheka Cell culture medium with Pheka/AgNPs **Part II:** Hydrogel cell encapsulation in culture medium with Pheka Hydrogel cell encapsulation in culture medium with Pheka/AgNPs
Positive control groups	**Part I:** Cell culture medium with Quercetin **Part II:** Hydrogel in cell culture medium with quercetin
Oxidative stimulators	Hydrogen peroxide (H_2O_2)
Inflammatory stimulator	Lipopolysaccharide of *Porphyromonas gingivalis* (LPS)

Table 1.3 Independent variables

Groups of Independent Variables	Details of Independent Variables
Cell cytotoxicity test	
Cell viability assay (MTT assay)	Optical density
Live dead cell staining	Vital cells in Green & Dead cells in Red
Particle endocytosis	TEM images of intracellular AgNPs
Biologic effects	
Anti-oxidative effect	Cell viability, optical density
Anti-inflammatory effect	Levels of inflammatory cytokines, IL-1β PGE_2, and TGF-β1
Osteogenic differentiation	
Early stage	- Levels of ALP enzyme ○ ALP staining ○ ALP activity
Late stage	- Levels of *In vitro* mineralization ○ Alizarin red staining on mono layer cell culture ○ Levels of calcium in culture medium in cell encapsulation model

CHAPTER 2
REVIEW OF LITERATURE

2.1 Literature reviews

2.1.1 Principles of tissue engineering

Tissue engineering is firstly defined by Langer, R. and Vacanti, J. P. (1993) as quoted "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ", which could be conceptualized by a triad of cells, matrix (scaffolds, bone substitute) and bioactive proteins or signaling molecules (i.e., Growth factor) (Figure 2.1).

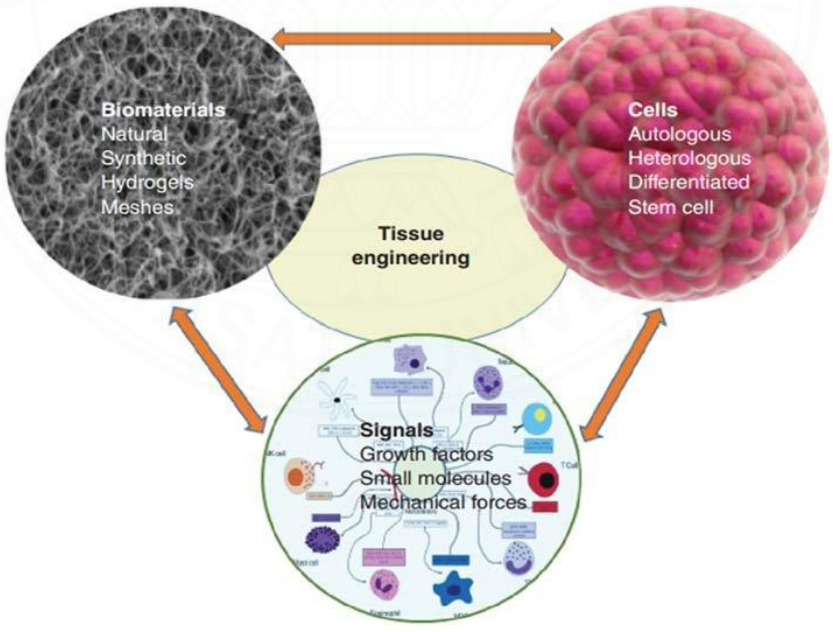

Figure 2.1 Tissue engineering triad (reproduced from Almouemen et al.) (23).

2.1.2 Peri-implantitis

Peri-implantitis is a critical biological complication of dental implant that could cause implant failure. Causes of peri-implantitis associate with inflammation of the supporting gingiva, periodontal ligament and bone around the implants (3). Adhesion of pathogenic biofilms on dental implant surface and peri-implant tissues results in bone loss and destruction of periodontium (24). Pathogenesis of peri-implantitis is similar to periodontitis, in which bacteria in the biofilm are predominantly gram-negative anaerobes such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Aggregatibacter actinomycetemcomitans* (25). As a result, inflammation and endotoxin activated by bacteria promote progressive alveolar bone loss leading to mobility and finally the loss of dental implant (24).

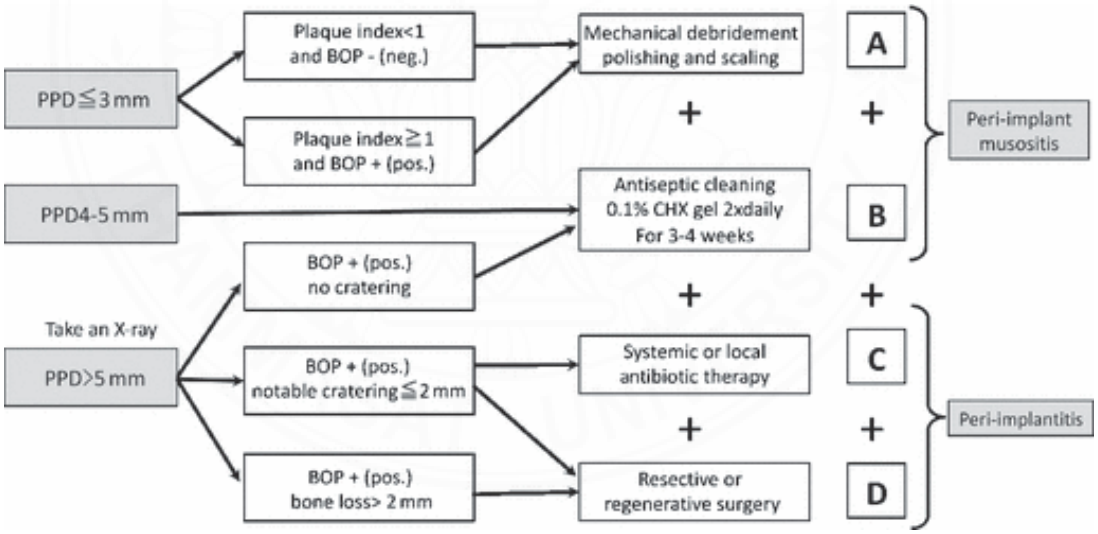

Figure 2.2 Cumulative Interceptive Supportive Therapy (CIST) protocol (reproduced from Panche et al.) (26).

Empirical treatment of peri-implantitis is to remove the pathogenic biofilms from dental implant surface combined with application of the tissue regeneration approach. According to a Cumulative Interceptive Supportive Therapy (CIST) protocol, treatment protocol of peri-implantitis includes mechanical debridement with or without systemic or local antibiotic treatment, and a need for additional regenerative bone surgery depending on the extent of bone resorption (Figure 2.2) (4). The CIST protocol classifies peri-implantitis based on severity of the disease into four levels from mild to severe or A to D, respectively. At level D, the defects are positive with bleeding on probing, having peri-implant pockets deeper than 5 mm. and exhibiting greater than 2 mm bone resorption shown on the x-ray. Thus, according to CIST protocol, treatment of Group D would include mechanical debridement, systemic or local antibiotic treatment and regenerative bone surgery. A common regenerative surgery is guided bone regeneration using barrier membrane and bone substitute materials (27).

2.1.3 Flavonoids

2.1.3.1 Definition

Flavonoids are polyphenolic compounds containing aromatic ring with hydroxyl group. Flavonoids are natural compounds found in plant, fruit and vegetable such as bamboo, citrus , cherries , berries , broccolis , apples , and onions (28). Beneficial actions of flavonoids include antioxidant, anti-inflammation and anti-allergenic effects.

2.1.3.2 Principle chemical structure and functions

A primary structure of flavonoids consists of 15-carbon atoms. The carbon atoms comprise two phenyl rings (A and B) and a heterocyclic ring (C) and arrange in a C6–C3–C6 configuration. A configuration of B ring on the C ring and degrees of unsaturation and oxidation of C ring significantly influences property of flavonoids and thus flavonoids could be categorized into 5 subgroups, flavones, flavanols, chalcones, anthocyanins and isoflavones (Figure 2.3) (29).

2.1.3.3 Functions of flavonoid in plant

Flavonoid is essential molecules maintaining important functions in plants. Pigmentations of flavonoids in yellow, red or blue pigmentation in different parts of plants such as leaves and flowers help to stimulate germination of spores and seeds, decrease levels of UV light and attract pollinator (30).

2.1.3.4 Special characteristics of flavonoids

Flavonoids is the largest class of polyphenolic compounds. Components of flavonoid could be identified using spectroscopy analysis based on different UV absorbance wavelengths of different functional groups (Figure 2.4) (28).

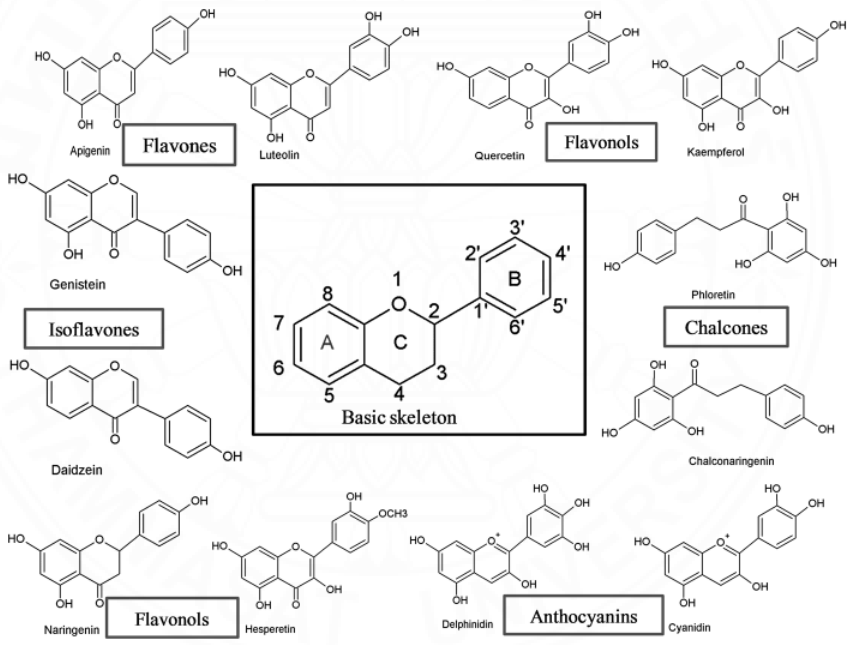

Figure 2.3 Demonstrating basic chemical structures of flavonoids and their classes (reproduced from Sato et al.) (29).

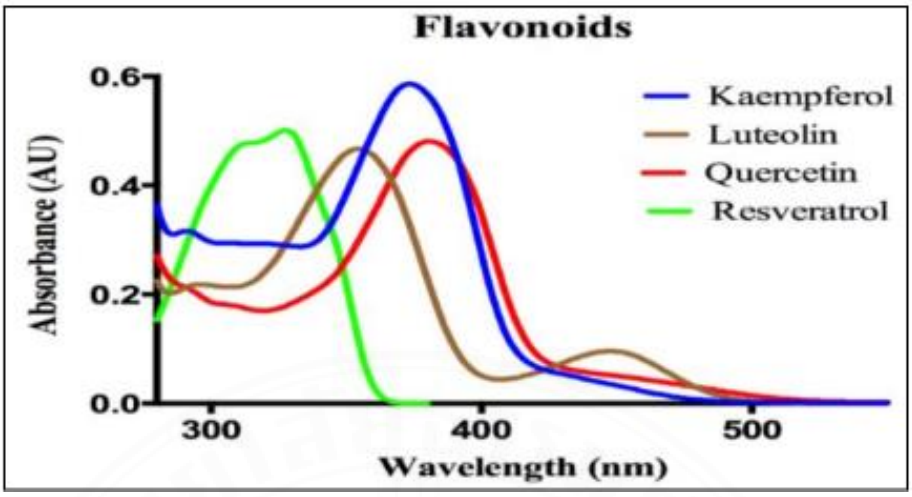

Figure 2.4 Demonstrating spectrophotometric analysis of different absorbance wave lengths of the different flavonoid contents (reproduced from Karak P et al.) (28).

2.1.4 Biological activity of flavonoids

2.1.4.1 Antioxidative activity

Antioxidants are substances that can prevent oxidative reaction by being able to reduce free radical oxygen. Oxygen free radical is a by products from various biochemical process in living tissue that could damage normal tissue. There are several natural antioxidants in the tissue such as coenzyme Q10 and alpha-lipoic acid. However, under stressful condition, additional antioxidants are needed to remove excess free radical oxygen to prevent cellular damage. Damages caused by excess oxidative effects could weaken immune system leading to chronic noncommunicable diseases (CND) such as diabetes, cancer, CVD, neurodegenerative disorders, and aging. Therefore, antioxidative property of flavonoids is useful for improving human health (31).

Flavonoids are natural antioxidants presenting in most parts of plants, such as roots, leaves, flowers, and fruits. Antioxidative function of flavonoids could interact with free radical in different forms and directly scavenge free radical to prevent cellular damage. Oxidative molecules will interact with hydroxyl group of

flavonoids to stabilize the reactive oxygen species (ROS), as explained in the following equation:

Flavonoid (OH) + R → Flavonoid (O) + RH

R represents free radical and O, oxygen free radical (32)

An antioxidative property of flavonoids has been clearly reported. Vison and Hontz in 1995 show that antioxidant and free radical scavenging function of plant flavonoids are stronger than vitamin C and vitamin E (33). Later, Kerry and Abbey in 1997 reported that flavonoids can inhibit low-density lipoprotein (LDL) oxidation in an *in vitro* study. Lately, Dinda and co-workers in 2015 reported that *Oroxylum indicum* (Pheka) could scavenge a stable free radical molecule, DPPH (2,2-diphenyl-1-picrylhydrazyl), super oxide anion, hydroxyl, nitric oxide and Fe^{3+}radicals. Flavonoids from different parts of plant exhibit antioxidation property differently, such as Pheka from leaves and stem bark extracts exhibit greater reductive ability and higher free radical scavenging activity than the extracts from bark, stem, and fruit extracts. It is reported that Pheka (*Oroxylum indicum*) in ethyl acetate and methanol extracted shows the highest inhibitory effects on lipid-peroxidation (14).

2.1.4.2 Anti-inflammatory activity

Inflammation is a responding process of the body to noxious stimuli such as infection, trauma, or cellular damage. Tissue damage is caused by over expression and functions of TNF-α and IL-1β and toll-like receptors (TLRs, microbial pattern recognition). Inflammatory cytokines cause cellular apoptosis, tissue fibrosis and chronic degenerative change. Therefore, by down-regulating inflammatory cytokines would help to decrease levels of inflammatory response and severity of tissue damage (34, 35).

It has been reported that flavonoids could decrease the production of inflammatory cytokines, including isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipoxygenase, that lead to a reduction of inflammatory

mediators such as nitric oxide, prostaglandins, leukotrienes and other mediators and thus a decreasing of tissue damage. For an example, ethanol extracted chrysin, a flavonoid component of *Oroxylum indicum*, has been reported to suppress activation of inflammatory cytokines and decrease function of inducible cyclooxygenase II (Cox-II) (36).

2.1.5 *Oroxylum indicum*

2.1.5.1 Description

Oroxylum indicum (*Pheka*) is a tree commonly found in tropical countries such as India, Japan, China, Sri Lanka, Malaysia, and Thailand. It is called in different names in Thailand including Pheka. Leaves of Pheka are large up to 2 meters long. It could be pinnate, bipinnate or tripinnate (37).

The pinkish flowers of the plant are long arrangement and night blooming and that are pollinated by bats (Figure 2.5A). The fruits are long and sword-like up to 90 cm x 9 cm and covered with kidney-shaped capsules. Inside the capsule contains, yellowish-green seeds that are surrounded by brown wing. On the other hand, fresh root bark is soft and juicy and has creamish yellow to greyish in colors. Pheka is also called "Broken bones tree" because the accumulating of dry long leaf and flower bearing stalks under the tree is resemble to a pile of broken bones (Figure 2.5B).

2.1.5.2 Phytochemistry

Pheka is a rich source of flavonoids. Various parts of Pheka, such as leaves, root bark and stem bark contain flavonoids namely chrysin, oroxylin-A, scutellarin, baicalein, quercetin, apigenin, ellagic acid and ursolic acid (37).

Figure 2.5 Demonstrating *Oroxylum indicum* (A) flowers and (B) tree (reproduced from Ahad et al.) (37).

2.1.5.3 Traditional use

Pheka is an important herbal medicine in many Asian countries. The plant is edible and various parts of Pheka has been used as traditional medicine to treat multiple diseases as followed. Root bark is for treating fever, diarrhea, bronchitis asthma, and inflammation. Seed extracts exhibit antimicrobial, analgesic and anti-inflammatory properties. Mature fruits of Pheka are useful in treating cough, bronchitis, jaundice, and dyspepsia. Stem bark possesses anti-inflammation, anti-arthritis, antifungal and antibacterial activities. To prepare stem bark extract for traditional medicine, it will be boiled in water and concentrated till color changed and then, a glass of cooled extract will be taken with 2 tablespoons sugar for many times a day. In India stem bark, fruits, and leaves are used as medicines to cure jaundice (37).

2.1.6 Quercetin (QT)

Quercetin (3,3',4',5,7-pentahydroxyflavone) (Figure 2.6), comes from the Latin word "Quercetum" which means Oak Forest. Quercetin is the one of the most widely used bioflavonoids for the treatment of metabolic and inflammatory disorders. Quercetin is one of the most abundant dietary flavonoids found in fruits and

vegetables. The highest concentrations of flavonols were found in vegetables such as onions and broccoli, fruits such as apples, cherries, and berries, and drinks such as tea and red wine (38).

2.1.7 Pharmacological activity of Quercetin

2.1.7.1 Antioxidant properties of Quercetin

Quercetin, a plant-derived aglycone form of flavonoid glycosides, has been used as a nutritional supplement and may be beneficial against a variety of diseases (38) and can also protect against environmental causes of free radicals such as smoking. It was also found that quercetin could protect erythrocytes from the membranous damage that is caused by cigarette tar (39).

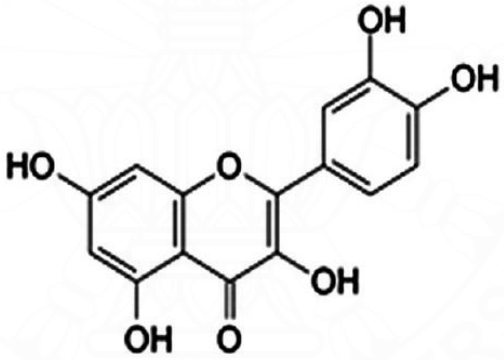

Figure 2.6 Chemical structure of Quercetin (reproduced from Anan David et al.) (38).

2.1.7.2 Anti-inflammatory properties of Quercetin

In vitro studies, quercetin showed a significant reduction in the levels of inflammatory mediators such as NO synthase, COX-2, and down-regulation of the nuclear factor kappa B pathway in liver cells (40). In vivo studies, in rats quercetin 80 mg/kg inhibited both acute and chronic inflammation and also showed significant antiarthritic activity against adjuvant-induced arthritis (41).

2.1.7.3 Osteogenic differentiation properties of Quercetin

Osteogenic differentiation properties in in vitro studies, quercetin promoted osteogenic differentiation and inhibited adipogenic differentiation of mouse bone mesenchymal stem cells by enhanced the phosphorylation of AMPK protein and upregulated the expression of SIRT1, thus activating the AMPK/SIRT1 signaling pathway (42). In vivo, in rat models of osteoporosis, quercetin enhance osteogenic differentiation of bone marrow mesenchymal stem cells to increase the bone mineral density (43).

2.1.8 Silver nanoparticles (AgNPs)

Bactericidal of silver-based compound has been well known for over 2,000 years and used to decrease infection in burn and wound therapies and as disinfectant in other medical purposes (44). On the other hand, nanoparticle is a microscopic particle with particle size smaller than 100 nanometers. Silver nanoparticles has gain wide interest because of their superior electrical and thermal conductivity, surface-enhanced Raman scattering, chemical stability, catalytic activity and non-linear optical behavior. It is used in the productions of inks, microelectronics and medical imaging devices (45). For biomedical applications, silver nanoparticles are known to exhibit a broad spectrum of anti-bacterial, antifungal and antiviral activities (46). Silver nanoparticles are incorporated in various products including cosmetic, health care products, antimicrobial textiles and wound dressings (47).

2.1.8.1 Antibacterial effect of silver nanoparticles (AgNPs)

Silver nanoparticles are broad spectrum bactericidal to both gram-negative and gram-positive bacteria. The mechanisms of action are a penetration of AgNPs inside the bacteria. Silver nanoparticles could sustain release silver, free radicals and oxidative stress. Silver ion (Ag^{+}) interacts with sulfur-containing proteins and phosphorus containing compounds of cell membrane and DNA, respectively (48, 49) and thus interfere with respiratory chain in bacterial mitochondria and cause cell dead

(50). In addition, nano particles promote bacterial cell dead by increasing particle/bacteria surface contact and thus promoting penetration of nanoparticles and disruption of integrity and permeability of the cell membrane (48).

2.1.8.2 Toxicity of nanoparticles (NPs)

Among various advantages of NPs in various applications, toxicity of the particles to mammalian and bacterial cells have been reported (51, 52). Factors contributing to cytotoxicity of NPs are size, surface area and solubility of the particles. Because of an extremely small particle size of less than 100 nm, NPs can penetrate cell membrane and accumulate inside the cells and interfere with normal functions of organs (53). Larger surface area of NPs may enhance toxicity of the particles by increasing surface contact or particle/cell interactions that enhance chemical reactivity and intracellular or organ accumulation (54). In addition, low solubility of the particles heightens the accumulation of NPs inside the cells and thus increases cytotoxicity of NPs (55).

Castiglioni and co-workers in 2017 studied the cytotoxicity of AgNPs size 50 nm at the antibacterial concentrations on osteoblasts and osteoclasts. Authors found dose dependent cytotoxic effects of AgNPs on growth and differentiation of osteoblasts and osteoclasts. It is reported that antibacterial doses of AgNPs are 2-4 times higher than that requires to inhibit growth and differentiation of osteoblasts and osteoclasts. Therefore, at the antibacterial levels, AgNPs have a potential to adversely effects osteogenesis and bone remodeling and this could raise concerns on biological safety of the silver-releasing implantable materials (56). Pauksch and co-workers in 2014 reported that AgNPs at a concentration of 10 µg/mL adversely affect cell viability of human mesenchymal stem cells and osteoblasts after 21 days cell culture. However, authors suggest that although NPs could increase stress on cells, and cytotoxicity of AgNPs is dose and time dependent, there is a therapeutic window for the application of AgNPs in clinical applications (57).

2.1.8.3 Cellular autophagy of silver nanoparticles

Nanomaterials, including AgNPs, are able to induce cellular autophagy. The cellular autophagy is able to promote osteogenesis differentiation of

stem cells. He and co-worker in 2020 studied the cellular autophagy is triggered by the AgNPs in hMSCs and AgNPs could promote osteogenesis of hMSCs through activation of autophagy with 26.8 nm AgNPs sized at 2.5 and 5 μg/ml were found to rise level of Alkaline phosphatase, Type I collagen, Osteopontin and Osteocalcin and enhanced ECM mineralization of hMSCs (Figure 2.7) (58).

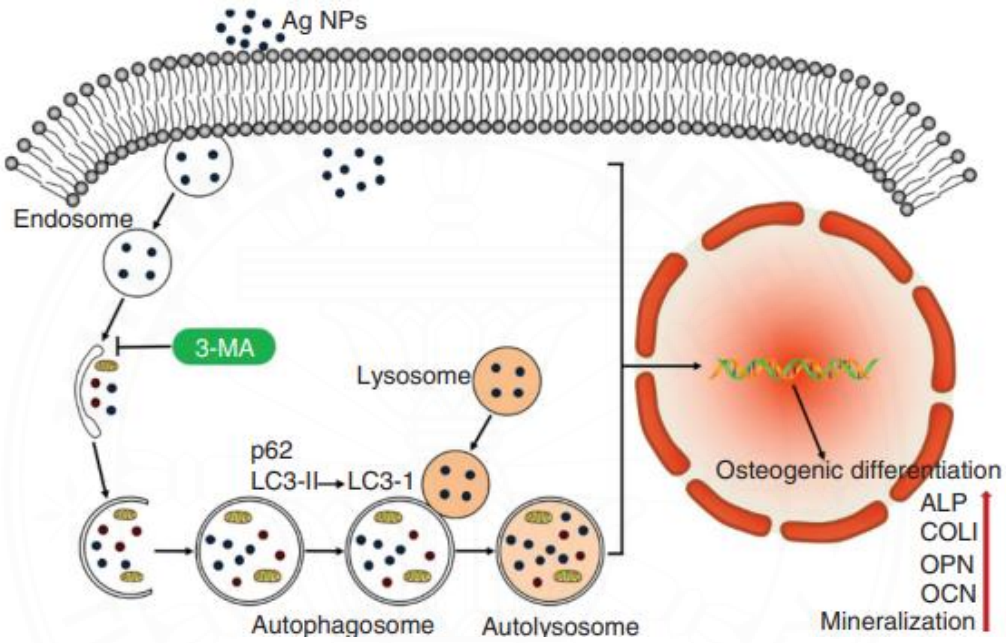

Figure 2.7 Silver nanoparticles stimulate osteogenesis of stem cells through activation of autophagy (reproduced from He et al.) (58).

2.1.8.4 Clinical use of silver nanoparticles (AgNPs)

Antibacterial property promotes applications of silver nanoparticles for various aspects in dentistry, particularly dental periodontology, implantology, and alveolar bone regeneration such as in guided bone regeneration (GBR), scaffolds for bone regeneration, and dental implant coatings (59).

Ye and co-workers in 2011 study effects of silver ion-substituted nanohydroxyapatite (Ag-nHA) expanded polytetrafluroethylene (e-PTFE) membranes (Ag-nHA-e-PTFE) in comparison to titanium nanoparticles-polyamide 66 (AgnHA-TiO_2/PA66) on osteoblastic differentiation of osteoblast cell line MG63. Authors found

that Ag-nHA-nTiO2/PA66 membrane exhibited excellence biocompatibility. The particles had no negative effects on growth of osteoblast like cells and promoted cell adhesion. While *in vivo* study, osteogenic activity of Ag-nHA-nTiO_2/PA66 membranes was comparable to that of conventional e-PTFE membranes in a rat model, while Ag-nHA-nTiO2/PA66 membranes could decrease inflammatory response (60).

Furthermore, AgNPs could be applied to create antibacterial activity on the titanium surface (47). Juan and co-workers in 2010 investigated AgNP-modified titanium surfaces (Ti-AgNP) using silanization method. Authors reported that the titanium (Ti) plates with Ag-NPs exhibit antibacterial activity, as it is found that the particles exhibit higher than 90% bactericidal effects on *Staphylococcus aureus* and *Escherichia coli* adhering on titanium surface. Additionally, scanning electron microscope (SEM) analysis shows antiadhesive properties of the Ti-AgNP surface as fewer bacteria attached on the Ti-AgNP surface and the numbers were markedly higher on an untreated Ti control surface (61).

2.1.9 Synthesis of silver nanoparticles (AgNps)

AgNPs can be prepared by physical, chemical, and biological methods.

2.1.9.1 Physical method

The physical method is the most effective method yielding a high-purity AgNPs. The most popular method is a laser irradiation that generate AgNPs by blasting laser beam on the silver targets placed in the solution. Nanoparticle size of colloids depends on the laser wavelength, ablation time, and duration of laser pulses but the limitation of this process is the high cost of laser facility (51).

2.1.9.2 Chemical method

The chemical route is the most used and economic method to generate AgNPs because the process is simple and cost-effective. The sequential procedures require 3 essential components, silver salt and reducing and stabilizing agents. Silver nitrate is the most widely used salt precursor, followed by, the use of

sodium borohydride ($NaBH_4$), ascorbic acid, glucose, hydrazine, sodium citrate, or ethylene glycol (EG) as reductants to reduce silver ion (Ag^+) to silver (Ag^0). Among several agents, sodium borohydride is a strong reducing agent that yields fined and monodispersed AgNPs. After that to stabilize the reduction reaction, polymer-based such as poly (vinyl alcohol), polyvinylpyrrolidone, poly (ethylene glycol), poly (methacrylic acid) and poly (methyl methacrylate) have been used as a stabilizing agent. Stabilizing agents function as the charge repulsion to counteract the van der Waals attraction between colloidal nanoparticles. Chemical method requires several chemical agents that could be harmful to living tissues and environment and increase production cost. Therefore, alternative synthesis methods are necessary to increase biomaterial safety in medical applications (51) and that lead to biological method.

2.1.9.3 Biological method

To increase biocompatibility of silver ion, biosynthesis method has been applied to reduce the applications of chemical in AgNP production process. Natural products are used to replace chemical reducing and stabilizing agents (52). Biosynthesis is environmentally safe, low cost and able to produce large numbers of nanoparticles. Various natural products such as bacteria, plant extracts and fungi have been used as the reducing and stabilizing agents. However, the use of bacterial is limited in medical application because of health safety concerns.

Regarding safety issues, utilizing plant derived agents has gained high interests in the synthesis process. Various parts of plants could be a source of natural reducing and stabilizing agents to form green AgNPs, in which the common parts of plants are leaves, stems, fruits and seeds (52). This is because natural plants generally contain carbohydrates, fats, proteins, nucleic acids and pigments that can act as effective reducing agents and stabilizers for silver ions (62). Various functional groups of polysaccharides such as a hydroxyl group play roles in both the reduction and the stabilization of the metallic nanoparticles. Examples of plant polysaccharides and their derivatives are chitosan, cellulose, starch, hyaluronic acid and heparin. Besides, leaf extracts are not only rich in polyphenols such as flavonoids, but also are effective reductant (63).

2.1.10 Biosynthesis of nanoparticles using Flavonoids from plant extracts

Plant phytochemicals exhibit high reduction and stabilization capacities that could control size and prevent agglomeration of metallic nanoparticles. Flavonoids from plants have been used as reducing agents in green synthesis of nanoparticles. Hydroxyl groups of flavonoids play roles in the reduction and the stabilization of metallic nanoparticles as demonstrated in Figure 2.8 (21).

Kumar and co-workers in 2009 used Neem (*Azadirachta indica*) leaf broth as reducing agents in silver and gold nanoparticle syntheses and found that the synthesizing process was rapid and yielded high concentrations of stable silver and gold nanoparticles (64).

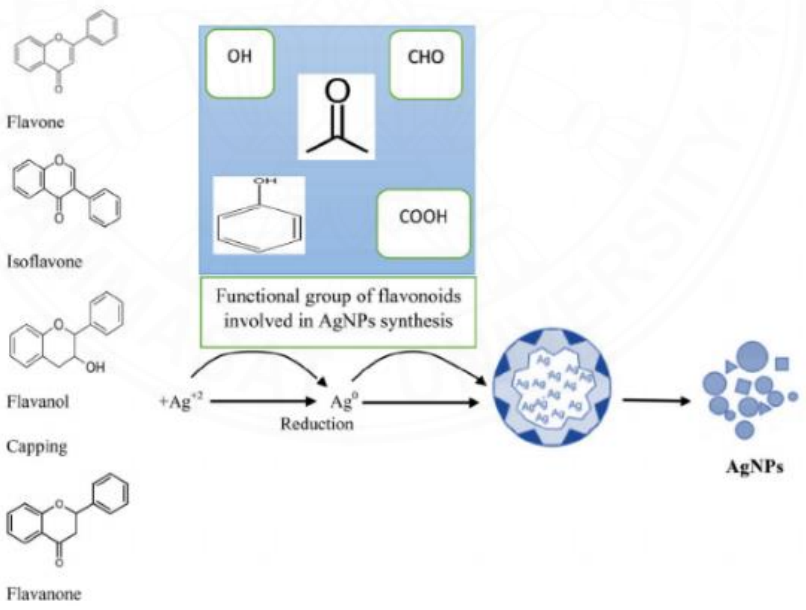

Figure 2.8 Demonstrating functions of flavonoids as reducing and capping agents in the biosynthesis of silver nanoparticles (AgNPs) (reproduced from Hussain et al.) (21).

Hu and co-workers in 2019 reported that biosynthesis *Bauhinia acuminate*/AgNPs was non-cytotoxic and able to promote osteogenic differentiation of mouse mesenchymal stem cells. It is postulated that plant extract polyphenols apart from functioning as reducing agents, also act as supplementary nutrients for cell growth and differentiation. Taken together, biosynthesis AgNPs using plant extracts could provide a possibility to avoid the use of harmful chemicals and at the same time promote biologic functions of the plant extracts, flavonoids (65).

2.1.11 Human periodontal ligament stem cells (hPDLSCs)

Human mesenchymal stem cells (hMSCs) are multipotent adult progenitor cells that can differentiate into different cells types in mesenchymal lineage, particularly osteoblasts, chondrocytes, and adipocytes (66). Advantages of applying MSCs in tissue engineering and regenerative medicine are accessibility, uncomplicated isolation methods and low immunogenicity of hMSCs. It has been reported that a periodontal space may contain MSCs that are able to differentiate into cementoblasts and osteoblasts that could form periodontal tissues (67). Periodontal ligament (PDL) is a fibrous connective tissue that surrounds and supports the tooth. It is secured by connective tissue fibers (Sharpey's Fibers) embedded between the thin mineralized outer layer of cementum and the inner wall of the alveolar bone (67). Human PDL contains PDL cells which possess characteristic properties of stem cells, such as self-renewal and multipotency, and express MSC markers, CD105, CD166, and STRO-1, on their cell surface (68). These cells are generally termed periodontal ligament stem cells (PDLSCs). Seo and co-worker in 2004 demonstrate capacity of hPDLSCs to generate a thin layer of cementum-like tissue along with condensed collagen fibers on surface of hydroxyapatite/tricalcium phosphate ceramic particles in immunocompromised rat (69). Kim and co-worker in 2012 reported that after culturing PDL cells and gingival fibroblasts in osteogenesis-inducing media for 21 days, *in vitro* mineralization was observed in both cell types, but levels of mineralization of hPDLSCs was higher than the gingival fibroblasts (70). In a rodent fenestration model, PDLSCs

tend to exhibit greater bone formation capacity than bone marrow mesenchymal stem cells, as indicated by a higher bone volume ratio and trabecular thickness, number, and separation in subcutaneous and calvaria transplants (71). Hence, hPDLSCs exhibit high osteogenic potential and thus are a potential source of progenitor cells for regenerative periodontal therapy (72). Together with their greater accessibility PDLSCs may be an alternate cell source to bone marrow mesenchymal stem cells (BMMSCs) for tissue regeneration and inflammatory-mediated diseases (73).

2.1.12 Hydrogels-based scaffolds for alveolar bone regeneration

Tissue engineering in alveolar bone regeneration has developed new alternatives for the treatment of bone defects. The alveolar bone is essential to guarantee the success of dental organ preservation, and also dental implants. It is necessary to develop hydrogel in combination with biomolecules (74). Alveolar scaffolds must be osteoinductive or osteoconductive, have mechanical resistance, adequate degradation, avoid the collapse of the area to be regenerated, good flexibility, adapt to different morphologies, and be easily managed (75).

Arpornmaeklong and co-workers in 2019 reported that the effects of quercetin-bGP-2:1 (wt/wt) chitosan/collagen hydrogel was non-cytotoxic of hPDLSCs supported by live/dead cell staining assay on hPDLSCs seeded on and encapsulated in the hydrogels. Thermosensitive bGP-2:1 (wt/wt) chitosan/collagen hydrogel was porous, degradable with high swelling capacity and noncytotoxic scaffold that would promote bone regeneration. The thermosensitive 2:1 chitosan-collagen hydrogel was a potential injectable hydrogel for natural flavonoid delivery and stem cell transplant for bone regeneration (76).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Investigations

List of investigations, methods and investigation time were summarized in Table 3.1

3.1.1 Biosynthesis of the flavonoid/silver nanoparticles

The initially refined *Oroxylum indicum* stem bark extracts (Pheka) and biosynthesized silver nanoparticles (Pheka/AgNPs) were prepared by Assoc. Prof. Dr. Supakorn Boonyeun, the Department of Chemistry, Faculty of Science and Technology, Thammasat University. In brief, barks of *Oroxylum indicum* from Buriram province were peeled, minced and air dried. Then they were washed several times with deionized (DI) water, air-dried and crushed into powder. Then, about, 10 g of the cleaned powder was dissolved in 100 mL of DI water and boiled for about 20 mins. Then the initially refined Pheka were diluted in the DMSO to the final concentrations of 12,000 part per million (ppm) and stored at 4°C for further use or kept at -80°C for a long-term storage.

After that, the crude extract was purified by column chromatiography (silica gel : hexane : ethanol), resulting in 4 fractions (F1-F4). After that the initially refined Pheka F2 (2400 ppm) was collected and used as reducing agent for synthesis of the Pheka/AgNPs. To prepare the biosynthesized silver nanoparticles, 10 mL of the Pheka's extract was mixed with 100 ml of 0.01M $AgNO_3$ and incubated in room temperature (RT). After 30 min incubation, color of the solution was changed from clear to light yellow and dark brown colors indicating a complete reaction of the Pheka/AgNPs synthesis. Under a transmission electron microscopy (TEM), an average size of the Pheka/AgNPs was of 21.49±0.32 nm.

3.1.2 Human periodontal ligament stem cells isolation and culture

Following an approval from the Ethical Review Sub-Committee Board for Human Research Involving Sciences, Thammasat University, No.3 and under a patient written informed consent, human periodontal ligament stem cells (hPDLSCs)

were collected from periodontal ligament tissue (PDL) of the third molar and premolar teeth obtained from simple extraction or surgical removal of the whole teeth from 10 healthy patients, age between 18-25 years old from the Dental clinic, Thammasat University Hospital were collected for the study.

3.1.3 Human periodontal ligament stem cell isolation and expansion

Teeth were washed in phosphate buffer saline (PBS) containing 2% penicillin/streptomycin (10,000 U/mL) and then periodontal tissue (PDL) were gently scraped from the middle third of roots using surgical blade No. 15 and then minced. (Figure 3.1). After that the minced PDL were digested with a digesting enzyme solution, 3 mg/ml collagenase type I (Worthington Biochemical, NJ, USA) and 4 mg/ml dispase (Sigma-Aldrich, St Louis, MO) solution for 1 hour at 37° C. Subsequently, single cells and the digested PDL pieces were seeded on T25 cell culture flasks (Corning, Tewksbury, MA) two tooth for one flask, and cultured in 4 ml growth media till reach 80% confluence or around 12 – 14 days. Then, the expanded cells were passaged and seeded at $3x10^3$ cells/cm^2 and passaged at 80% confluence. The hPDLSCs at passages 3-5 were used for the investigations. Growth media comprised of phenol red DMEM-F12, 10% (Corning, Tewksbury, MA), fetal bovine serum (FBS), 1% penicillin/streptomycin, 0.5% fungizone and (FBS) (All from Gibco, Thermo Fisher Scientific, Waltham, MA). Osteogenic medium is growth medium supplemented with 10 mM β-glycerophosphate, 50 mM ascorbic acid and 100 μM Dexamethasone (All from Sigma-Aldrich) (77).

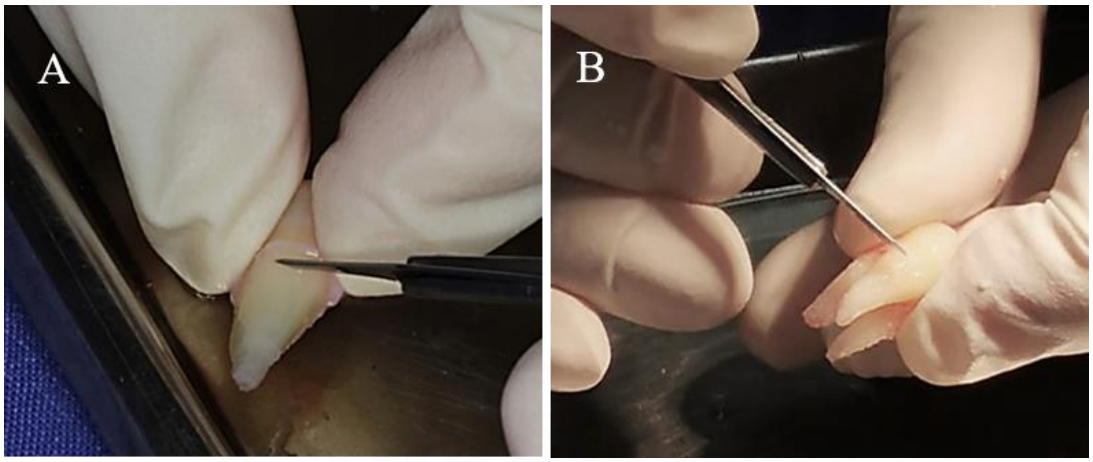

Figure 3.1 Demonstrating periodontal tissue harvesting by gently scraping the tissue from the middle third of roots of a (A) premolar and (B) third molar using a surgical blade No.15.

3.1.4 Flow cytometry analysis of characteristic cell surface antigens of mesenchymal stem cell

Human PDLSCs were cultured in a growth medium. At 80% confluence, the hPDLSCs were detached with 0.05% (w/v) trypsin in 0.05 mM (w/v) in ethylenediaminetetraacetic acid (EDTA) (Gibco). Then, expression of the characteristic cell surface antigens of the mesenchymal stem cells (MSCs) CD73, CD90 and CD105, and hematopoietic stem cells, CD34, CD11b, CD45 and HLR-DR were examined using the Human MSC Analysis Kit (BD Stemflow, BD Biosciences, San Diego, CA), following manufacturer's instruction using fluorescence activated cell sorter (FACS) (FACSAria™ III, BD Life Sciences, San Jose, CA) at the Center of Scientific Equipment for Advanced Research, Thammasat University (TUCSEAR). Numbers of cells for each antigen test were $3x10^5$ cells/sample and the flowcytometry analysis was performed on 20,000 events. The Positivity expression of the markers was set at the levels higher than 90% of a total cell population (78).

3.1.5 Human periodontal ligament stem cells (hPDLSCs) culture

A mono layer cell culture

Human PDLSCs were seeded at $3x10^3$ cells/cm^2 for investigations on flowcytometry, cell cytotoxicity, antioxidative stress and anti-inflammation properties and $5x10^3$ cells/cm^2 for an osteogenic differentiation study in a monolayer cell culture (79). For experiments on the anti-oxidative stress and antiinflammation properties, cells were grown in a growth medium with 10% FBS overnight and then the medium was changed to a growth medium with 1% FBS with the supplements according to groups of the study. For osteogenic differentiation and cell encapsulation studies, the hPDLSCs were continuously cultured in culture mediums with 10% FBS.

Hydrogel cell encapsulation

For cell encapsulation, the hPDLSCs were encapsulated in the in-house thermosensitive 2% (w/v) nanohydroxyapatite - 2% (w/v) calcium carbonate - 10% (w/v) β-glycerophosphate - 4:1 (w/v) chitosan/collagen hydrogel (all from Sigma-Aldrich, excepting for the collagen from Koken, Tokyo, Japan) at a concentration of $1x10^6$ cells/ml (80). Subsequently, the hydrogel cell suspension, 300 µl/well ($3x10^5$ cells/well) was added in 24 well cell culture plate. Then, the hydrogels were incubated in an incubator at 37°C in 5% CO_2, 95% humidity for 15 min to stimulate sol-gel transition, and then an osteogenic culture medium 500 µl/well was added in to each well. Then 24 h later, the culture medium was changed to osteogenic medium supplemented with the Pheka, Pheka/AgNPs and QT and osteogenic medium alone according to groups of the study and cultured for 21 days. Culture medium was changed every 2-3 days. After that, live/dead cell staining and cell viability assays, and measuring of levels of alkaline phosphatase (ALP) activity and calcium contents were performed (Figure 3.2).

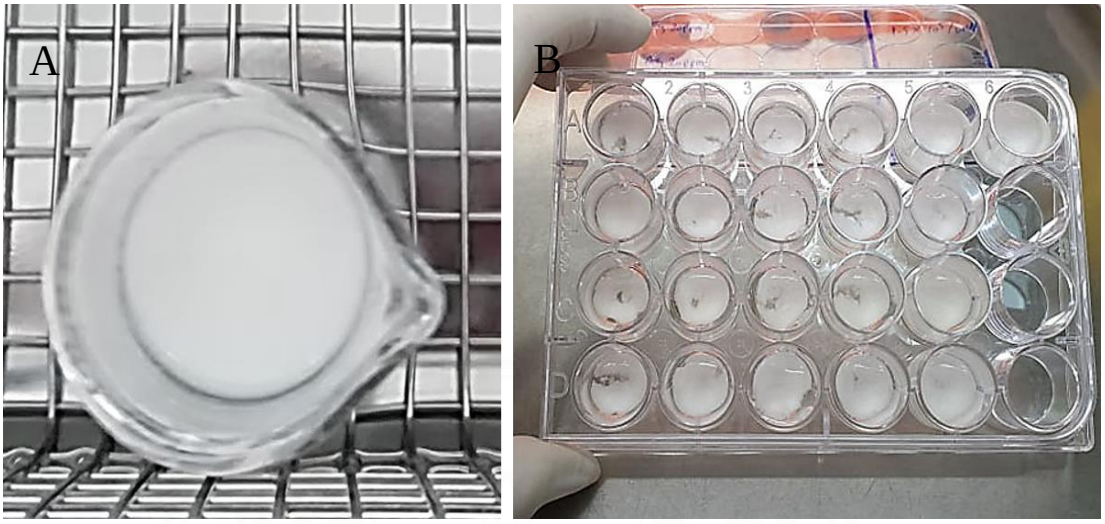

Figure 3.2 Demonstrating three-dimensional cell culture of the hydrogel cell encapsulation, (A) A hydrogel cell suspension of the in-house thermosensitive 2% (w/v) nanohydroxyapatite - 2% (w/v) calcium carbornate - 10% (w/v) betaglycerophosphate- 2:1 (w/v) chitosan/collagen hydrogel with hPDLSCs on ice before plating, (B) The hydrogel cell suspension in a gel stage after 15 min incubation at 37°C in an incubator, 300 µl/well.

3.1.6 Osteogenic differentiation

To determine osteogenic differentiation potential, the hPDLSCs were seeded at $5x10^3$ cells/cm^2 on a cell culture plate or encapsulated in the hydrogel at $1x10^6$ cells/ml and cultured in growth medium with 10% FBS for 24 h. Then, the growth medium was changed to osteogenic medium supplemented with Pheka, Pheka/AgNPs and QT according to groups of the study for up to 21 days. After that, ALP and alizarin red staining and measuring levels of ALP activity in protein lysates and osteocalcin in culture medium were performed to determine early and late stages of osteoblastic differentiation of the hPDLSCs on cell culture plates and the encapsulated cells (16, 81) .

3.1.7 Characterizing property of the extracts from the stem barks of the *Oroxylum indicum*

3.1.7.1 Total Flavonoid Content (TFC) Assay

A total flavonoid content (TFC) assay uses an aluminium chloride colorimetric method to estimate levels of flavonoids in the extracts. The analysis was performed on the 1200 ppm samples, the Pheka, crude Pheka and Pheka/AgNPs. In brief, the samples, initially refined extracts (Pheka) and crude extract (Crude) in DI water were mixed with the 5% (w/v) sodium nitrite, followed by the 10% (w/v) aluminium chloride and 4% (w/v) sodium hydroxide, respectively (All chemicals from Sigma-Aldrich). Then, the solutions were changed to brown and dark brown colors and the absorbance was read at 510 nm using a microplate reader (Varioskan Flash, Thermo Fisher scientific, Vantaa, Finland). After that the total flavonoid content of samples were extrapolated from a standard curve of QT and expressed as equivalent to quercetin concentration in ppm (ppm QT) (82, 83).

3.1.7.2 DPPH Radical Scavenging Assay

The free radical activity was determined by measuring the ability of the extracts to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The principle is based on the reduction of the DPPH in the presence of a hydrogen donating antioxidant that the reduction reactions decrease color of the DPPH in relation to the proton donating ability or antioxidant capacity of the samples (84, 85).

In brief, the samples were mixed with the 120 μM DPPH solution (Sigma-Aldrich) and allowed to stand for 30 min in the dark at RT. Then the absorbance was measured at 515 nm wavelength using a microplate reader (Varioskan Flash), and methanol was use as a blank solution (84). The radical scavenging activity was expressed as the inhibition percentage using the equation, where A_0 and A_1 were absorbances of the control and sample solutions, respectively as follows.

$$\text{DPPH scavenging activity (\%)} = [(A_0 - A_1) / A_0] \times 100.$$

3.1.8 Investigations on the biologic effects of the extracts and biosynthesized silver nanoparticles

Human PDLSCs were seeded on 24 well cell culture plate at $5x10^3$ cells/cm^2 ($1.25x10^4$ cells/well) in a growth medium with 10% FBS overnight and then the medium was changed to mediums with 1% FBS for 24 h washed out period. Then, the culture medium was changed to control and experimental mediums, according to groups of the study (Group A-D) for 72 h.

3.1.9 The effect on cell viability under an oxidative stress

The hPDLSCs were seeded on 96 well cell culture plate, $3x10^3$ cells/well and cultured in a control and culture mediums supplemented with Pheka, Pheka/AgNPs, and QT according to groups of the study for 72 h. After that the pre-treated cells were incubated with the 12.5 μM hydrogen peroxide (H_2O_2) in growth medium at 37°C in 5% CO_2, 95% humidity for 45 min. Then, the H_2O_2 medium was removed, and cell viability assay was performed to determine levels of cell viability using CellTiter 96® AQueous One Solution (Promega, Madison, WI), following the manufacturing's protocol. In brief, cells were incubated with the 20% AQeous One Solution in a phenol red free medium at 37°C in 5% CO_2, and 95% humidity for 2 h. Then, the absorbance was measured at 490 nm using a microplate reader (Varioskan Flash) (86).

3.1.10 An anti-inflammatory effect

3.1.10.1 Exposing to *Porphyromonas gingivalis* lipopolysaccharide (LPS)

To activate inflammatory response, hPDLSCs were cultured with 1 μg/ml purified lipopolysaccharide (LPS) of *Porphyromonas gingivalis* (InvivoGen, California, USA).(87) Human PDLSCs were seeded on 24 well cell culture plate at $5x10^3$ cells/cm^2 ($1.25x10^4$ cells/well) in growth medium for 24 h. Then the culture media was changed to the control and experimental mediums, according to groups of the

study with and without 1 μg/mL LPS (InvivoGen) for 72 h. Therefore, two sets of parallel cell cultures were set, first groups *with* and second *without* LPS (87). After that, levels of cell viability and secretion levels of IL-1β in culture medium were measured.

3.1.10.2 Measuring levels of cell viability and inflammatory cytokines

After exposing to the bacterial LPS for 24 h, 48 h, 120 h the supernatants were collected for measuring levels of IL-1β and then cell viability assay was performed. The collected culture medium was centrifuged at 2,000 rpm for 5 min at 4°C, and then the supernatants were collected and kept at -80°C for further used. Subsequently, the cell viability assay was performed as described in an oxidative stress section. The collected culture mediums were subjected to an ELISA assay to measure levels of IL-1β, an inflammatory cytokine using a Quantikine ELISA kit (R&D systems, Minneapolis, MN), PGE_2 ELISA (Enzo, Farmingdale, NY) and Quantikine ELISA human TGF-β1 kits (R&D systems, Minneapolis, MN) following the manufacturers' protocols. Absorbances were read at450 nm, 405 nm and 450 nm for IL-1β, PGE_2, and TGF-β1, respectively using a microplate reader (Varioskan Flash), and then the concentrations of the cytokines were extrapolated from the standard curves and reported as pg/ml (87).

3.2 Methodology

3.2.1 Determining cell Viability

3.2.1.1 Live/dead cell staining assay

The cell viability of the hPDLSCs in mono layer cell culture and hydrogel cell encapsulation was examined using the LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Thermo Fisher scientific) following the manufacturer's protocol. In brief, on culture day 14 in mono layer cell culture and day 21 in hydrogel cell encapsulation, the incubating medium was prepared by adding 4 µl of the 4 mM calcein and 20 µl of 2 mM the ethidium homodimer-1 (EthD1) in dimethylsulfoxide (DMSO) in 10 ml phenol red free culture medium. Then, the hydrogels were incubated with the incubating medium, 0.5 ml per well of 24 well cell culture plate, for 30 min. After that, replaced the incubating medium with a phenol red free culture medium and examined the fluorescence staining of the encapsulated cells under the confocal laser scanning microscope (CLSM) (Nikon ECLIPSE, Nikon, Melville, NY). The cytoplasmic green staining of calcein represented vital cells and red staining of the EthD1, non-vital cells (76, 81, 88).

3.2.1.2 Cell viability (MTT) assay

Part I: In mono layer cell culture, the hPDLSCs were seeded at $3x10^3$ cells/well on 96-well cell culture plate in growth medium for overnight. After that the culture medium was changed to growth mediums supplemented with 1.25 – 20 ppm Pheka, Pheka/AgNPs and QT according to groups of the study and cultured for 72 h and then the cell viability assay was perform, as described earlier in section 3.1.9 The effect on cell viability under an oxidative stress (86). The percentages cell viability was calculated relatively to the optical density of a control in growth medium without supplements, as follows.

Percentages of cell viability = (OD of sample/OD of control) * 100

Part II: Hydrogel cell encapsulation, the hydrogel/cell suspensions were seeded in 24 well cell culture plate and cell viability assay was performed on a culture-day 21 by incubating the hydrogels with 300 µl of the 20% CellTiter 96® AQueous One Solution (CellTiter 96® AQueous One Solution Cell Proliferation Assay) in a phenol red free culture medium for 2 h, as described earlier in section 3.1.9 The effect on cell viability under an oxidative stress (86). After that the incubated culture mediums were transferred to the 96 well plate, 200 µl/well in duplicate and then the absorbance was read at 490 nm (Varioskan Flash). Then the percentages of cell viability were calculated relatively to the optical density of a control in growth medium without supplements, as described in the earlier section. A control group was a group of growth medium without supplementation.

3.2.1.3 Transmission electron microscopy

The hPDLSCs 1 × 10^6 cells that were cultured in growth medium and growth medium with the 20 ppm Pheka/AgNPs for 14 days, and then the internalization of the AgNPs were examined under transmission electron microscope (TEM) (JEM-1400, JEOL, Peabody, MA) at the Research Service Section, Research Office, Faculty of Dentistry, Mahidol University. In brief, the particle exposed cells were trypsinization and centrifuged at 1500 rpm for 5 min at 4°C. The pellets were washed once with PBS, then fixed with 4% phosphate buffer glutaraldehyde (Sigma-Aldrich), rinsed 3 times with distilled water and pre-stained with 2% (w/v) osmium tetroxide (Electron microcopy sciences, Hatfield, PA) in PBS for 45 min. After that, the pellets were rinsed with the 1% (w/v) uranyl acetate (Electron microcopy sciences) in PBS for 30 min, rinsed twice with DI water, then dehydrated with ethanol dilutions from 70% to 100%. After the dehydration, the samples were infiltrated with the 50% (w/v) propylene oxide in resin for 30 min, followed by the 100% (w/v) resin for 2 h, and then the resin was polymerized in an oven at 70°C for 12 h. Then, the resin blocks were sectioned using a Boeckeler instrument Power Tome XL (RMC Products, USA) at 80 nm per section and the imaging was conducted using a JEOL JEM-1400 TEM (JEM-1400) operating at 100 kV (Figure 3.3) (89, 90).

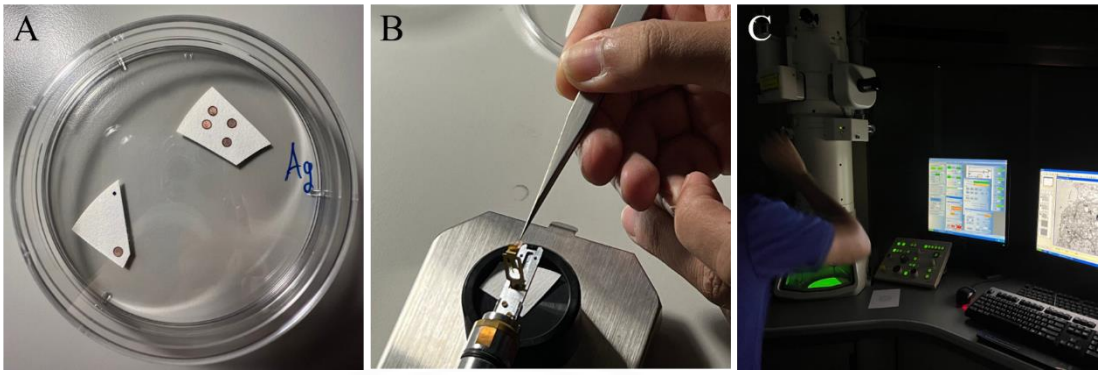

Figure 3.3 Demonstrating the preparation samples for transmission electron microscopy (TEM) imaging, (A) resin-embedded specimens sectioned with an ultramicrotome at 80 nm per section, (B) Mounting the resin embedded specimens on the TEM slot grid for imaging and (C) the TEM (JEM-1400, JEOL) operating at 100 kV.

3.2.2 Determining osteogenic differentiation potential

To determine early and late stages of osteogenic differentiation the alkaline phosphatase (ALP) and in vitro mineralization, respectively were examined (16).

3.2.2.1 Alkaline Phosphatase staining

The hPDLSCs in monolayer cell culture were washed by PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 5 min, then washed 2 times with DI water. After that, 1 ml of the BCIP/NBT liquid substrate system (Sigma-Aldrich) was added into each well and incubated in the dark at RT for 10 min and then washed 2 times in DI water. Later, the staining was observed under a light microscope (Evos XL core, Invitrogen, Thermo Fisher Scientific) (81).

3.2.2.2 Alizarin red S staining

The alizarin red S staining was performed to detect the in vitro mineralization of the extracellular matrix (ECM). The hPDLSCs on cell culture plate on culture-day 21 were washed in PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich)

for 5 min, washed again 2 times with DI water, incubated with 40 mM alizarin red S solution (pH 4.2) for 20 min, then rinsed 2 times in DI water and observed under light microscope (Evos XL core) (91).

3.2.2.3 Measuring levels of alkaline phosphatase (ALP) activity

3.2.2.3(1) Cell lysis and total protein content measurement

On culture day 21, cells were washed twice with PBS, then incubated in ice cold 1% Triton X-100, kept at -20°C for 24 h, then frozen and thawed 2 times, on ice for 1 h and at -20°C for 1 h, then the cells were scraped and transferred with the incubated Triton X-100 to 1.5 ml tubes, vortexed vigorously for 30 seconds and centrifuged at 10,000 rpm for 10 min at 4°C (5424 R, Eppendorf AG, Hamburg, Germany) at 4°C for 10 min. Then the supernatants were collected as cell lysate solutions and cell pellets were collected for measuring levels of calcium contents. After that the concentrations of the total protein contents in the cell lysate solutions were measured using a DC protein assay kit (Bio-rad, Hercules, CA) following the manufacturer's protocols. An optical density of the assay was read at 680 nm in duplicate using a microplate reader (Varioskan Flash). Then, the amount of protein contents was extrapolated from a standard curve and reported as mg proteins/ml (92).

3.2.2.3(2) Measuring levels of alkaline phosphatase activity

At each investigation time, levels of ALP activity in cell lysate solutions were measured using the ALP yellow liquid substrate system for ELISA (Sigma-Aldrich) according to manufacturer's protocols. In brief, 50 µl of cell lysate solutions were added in duplicate in a 96 well plate followed by 200 µl of the ALP yellow liquid substrate, mixed and incubated in the dark at 37°C for 1 h. Subsequently, the optical density was determined at 405 nm using a microplate reader (Varioskan Flash). After that concentrations of the ALP activity were extrapolated from a standard curve of p-nitrophenol (Sigma-Aldrich) and reported as nanomolar p nitrophenol/mg total protein contents (81).

3.2.2.4 Determining levels of calcium contents in the extracellular matrix

Cell pellets from the protein lysis samples were washed two times in PBS and incubated in 0.5 M hydrochloric acid (Sigma-Aldrich) in PBS at 37°C for 12 h. After that, the mixtures were centrifuged (Eppendorf 5424R) at 10,000 rpm for 10 min at 4°C, and then the supernatants were collected for the analysis. The amount of the calcium contents in the dissolved solutions were measured using a calcium colorimetric assay kit (Sigma-Aldrich) following the manufacturer's instructions. The optical density was measured at 575 nm in duplicate using a microplate reader (Varioskan Flash). The amount of calcium contents was extrapolated from a standard curve and reported as nanogram calcium per milligram protein (ng/mg protein) (Table 3.1) (77, 81).

Statistical analysis

Qualitative data such as staining results were described. Quantitative results were quantified and compared. Numbers of samples at each investigation time were 3-5 samples and reported as an average $\pm$ standard deviation (N=5, Mean$\pm$SD). Data were analyzed using the SPSS Statistics for Windows, Version 26.0 (IBM Corp, Armonk, NY). The Kolmogorov-Smirnov test was used to examine the data distribution. One-way ANOVA and Scheffe's test (Tukey's HSD) were used for the normal distribution data. For the data with non-normality distribution, Kruskal–Wallis and Dunn-Bonferroni post hoc tests were used for multiple comparisons. Significant difference was set at p-value < 0.05.

Table 3.1 List of investigations, methods, and investigation time

Investigations	Methods/assays	Parameters	Timing
Characterization of the initially refined extracts	Total flavonoid contents	quercetin equivalent in part per million (ppm)	On day1
	An antioxidant activity	2,2-diphenyl-1-picrylhydrazyl (% DPPH)	On day1
Anti-oxidative effect	MTT assay	NAD-dependent dehydrogenase activity (Optical density, OD)	On day1
Anti-inflammatory effect, LPS stimulations	MTT assay	NAD-dependent dehydrogenase activity (Optical density, OD)	On day 1
	ELISA	Inflammatory cytokine levels, IL-1β, PGE_2 and TGF-β1 (extrapolating OD values with standard curve of cytokine concentrations, pg/ml)	
Cell viability	Live dead cell staining	Qualitative evaluation: Vital & dead cells stained green & red, respectively	On day 14 on mono layer cell culture and day 21 on hydrogel cell ncapsulation
	MTT assay Microplate reader	Optical density (OD)	On day 3
	TEM images	Particle endocytosis of AgNPs	On day 14

Table 3.1 (Cont.) List of investigations, methods, and investigation time

Investigations	Methods/assays	Parameters	Timing
Osteogenic differentiation (a) Early stage	Alkaline phosphatase (ALP) staining	Semi-qualitative analysis: observing staining intensity	On day 21
	Cell lysate solution	Quantitative: Amount of total protein contents (mg proteins/ml)	On day 21
	ALP activity assay	Quantitative: ALP level analysis μM of p nitrophenol / mg of total protein contents (μM/mg proteins)	On day 21
(b) Late stage	In vitro mineralization (Alizarin Red staining)	Qualitative analysis: observing staining intensity of ECM	On day 21
	Levels of calcium contents in extracellular matrix	Quantitative: nanogram of calcium / ml of total protein contents (ng/mg protein)	On day 21

CHAPTER 4

RESULTS

4.1 Characterization of the initially refined extracts

4.1.1 Total flavonoid contents

The total flavonoid contents (TFC) of the extracts and crude from the stem barks of Pheka was shown as quercetin equivalent in ppm. The TFC levels of the extract fraction 3 (F3) at 3.84±0.16 ppm (12.7 μM of QT) were significantly higher than the fraction 4 (F4) and crude (Crude) ($p<0.05$), but it was not significantly higher than the fraction 2 (F2) ($p>0.05$) (Figure 4.1).

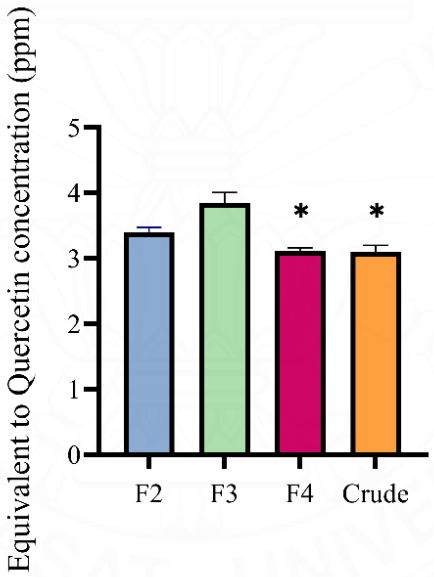

Figure 4.1. Exhibiting total flavonoid contents of the initially refined and crude extracts from stem barks of the *Oroxylum indicum* (Pheka). Abbreviations F2 denote the extract from the chromatography fraction 2, F3, fraction 3, and F4, fraction 4 (F4) and Crude, the crude extract. The total flavonoid contents were expressed as the quercetin equivalent in part per million (ppm). A symbol * represents significantly lower than the F3 group $p<0.05$ (N=5, Mean ± SD).

4.1.2 An antioxidant activity

The initially refined extracts, fractions 2, 3 and 4 (F2, F3 and F4) and crude from the stem barks of the Pheka exhibited high levels of the antioxidant activity. The average antioxidant activity of the extracts, F2, 3 and 4 was 78.46±0.59 %DPPH, which was significantly higher than the activity of crude at 77.18 ± 2.15 %DPPH ($p<0.05$) (Figure 4.2).

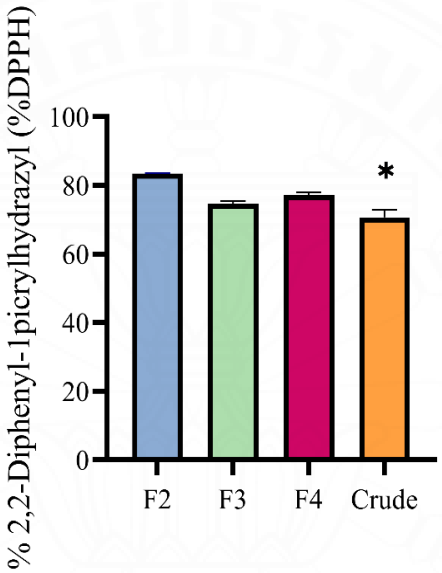

Figure 4.2 The antioxidant activity of the extracts from stem barks of the *Oroxylum indicum* (Pheka). Abbreviations F2 denote the extract from the chromatography fraction 2, F3, fraction 3, and F4, fraction 4 and Crude, the crude extract. The antioxidant activity was reported as percentages of the antioxidant activity of the 2,2-diphenyl-1-picrylhydrazyl (%DPPH). A symbol * represents significantly lower than the other groups (F2, F3 and F4) (N=5, Mean ± SD).

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4.2 Human periodontal ligament stem cells isolation and expansion

On days 5 – 7 after cell seeding at the primary passage, the hPDLSCs proliferated from the individual seeded cells and propagated from the dissociated periodontal tissues (Figure 4.3A). At the primary passage, proliferated cells took 10-14 days to grow and cover 80% of the surface area of the 25 cm^2 cell culture flasks (Figure 4.3B). The expanded hPDLSCs exhibited homogenous fibroblast-like cell morphology (Figure4.3B and C). At first and later passages, the hPDLSCs extended their cell bodies anteriorly and posterior created homogenous elongated spindle shaped cells (Figure 4.3C).

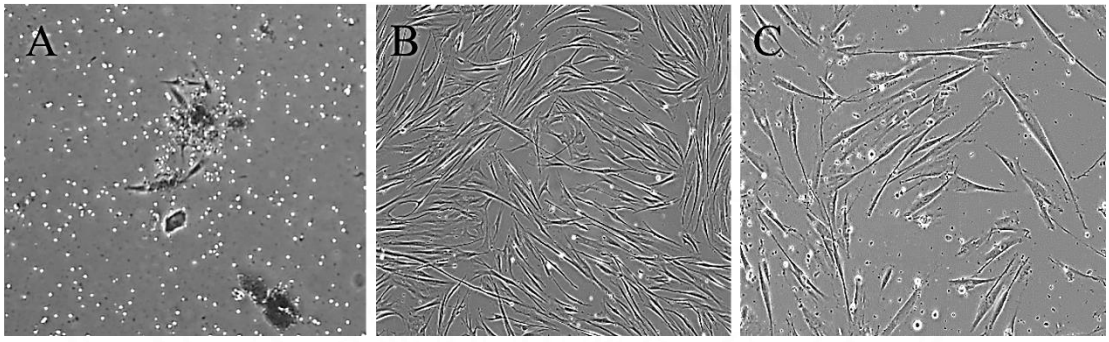


Figure 4.3 Demonstrating the expansion and morphology of the human periodontal ligament stem cells at primary and first passage and the spindle shaped cells on (A) at primary passage on day 5 and (B) on day 10, and (C) at the first passage on day 3 in growth medium (magnification x10).

4.3 Expressions of the characteristic cell surface antigens of mesenchymal stem cells

Flow cytometry analysis exhibited high expression levels of the characteristic MSC cell surface antigens, CD73, CD90, and CD105. The expression levels of the CD73, CD90, and CD105 were higher than 90% of a total cell population, 99.9%, 99.5% and 99.3%, respectively (Figure 4.4C, D, E). The expression levels of the negative control cocktail of the hematopoietic stem cell surface antigens CD34, CD45, HLA-DR, CD11b and CD19 were 0.4 % of the total cell population (Figure 4.4B).

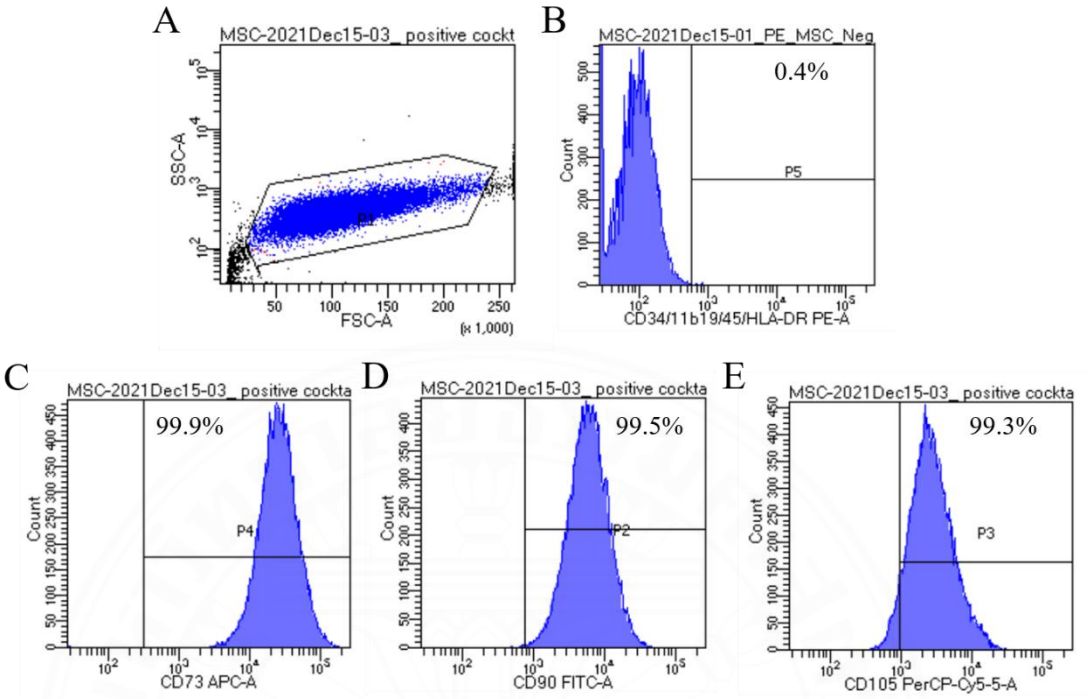


Figure 4.4 Flow cytometry analysis of the characteristic cell surface antigens of mesenchymal stem cells (MSCs) of the human periodontal ligament stem cell (hPDLSCs), (A) Flow cytometry analyses of a total cell populations, (B) negative expression of the hematopoietic stem cell markers (CD34, CD45, HLA-DR, CD11b, CD19) that were lower than 2%, (C, D, E) Positive expressions of the MSC surface markers, CD73, CD90, and CD105, respectively. The Y-axis represents intensity of the staining (Count) and X-axis, the numbers of positive cells in each designated gate. A negative control cocktail was conjugated with phycoerythrin (PE) (B). A positive cocktail, CD73 was conjugated with allophycocyanine (APC), CD90, fluorescein isothiocyanate (FITC) and CD105, PerCP-Cyanine 5.5. Abbreviations FSC-A denote Forward versus side scatter analysis and P, flowcytometry analysis gate designated for the analysis of samples.

4.4 Cell viability

4.4.1 Cell cytotoxicity and working concentrations of the extracts and biosynthesized silver nanoparticles

Based on the maximum concentration at 20 ppm, the toxicity of the supplements was not found, the average cell viability levels of all groups were higher than 90% of the control in growth medium alone. The averages of the percentages of cell viability of the Pheka and Pheka/AgNPs and QT were 107.36±4.08, 100.51±4.41 and 90.37± 5.96respectively. The levels of cell viability of the 5, 10 and 20 ppm Pheka and 5 ppm Pheka/AgNPs were significantly higher than a control in growth medium alone ($p<0.05$), but not from the other groups. Levels of cell viability of the 1.25 – 20 ppm Quercetin groups were not significantly different within groups and with a control ($p>0.05$) (Figure 4.5).

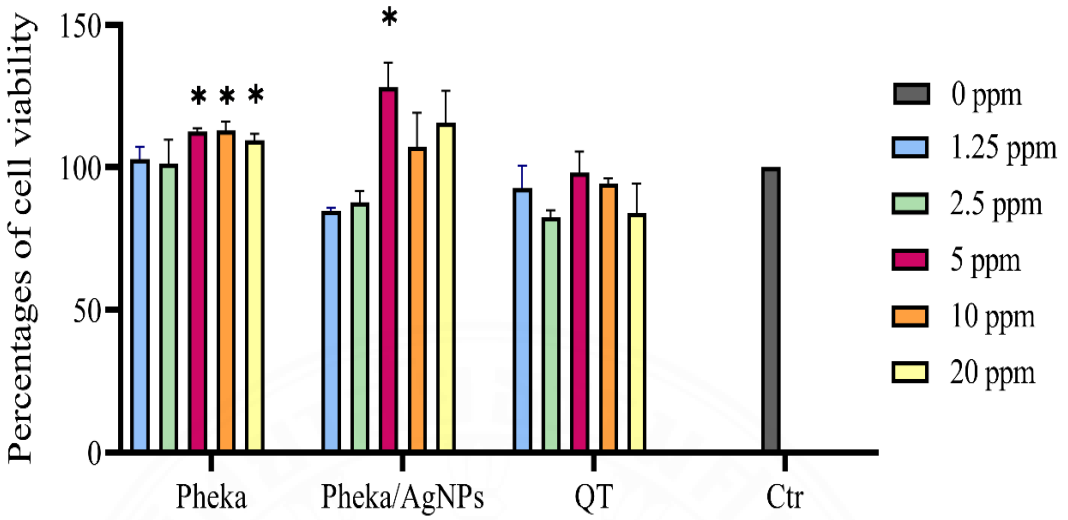


Figure 4.5 Demonstrating cell cytotoxicity and working concentrations of the initially refined extract (Pheka), the biosynthesis Pheka/silver nanoparticles (Pheka/AgNPs) and quercetin (QT) on human periodontal ligament stem cells (hPDLSCs). The hPDLSCs were incubated with the supplements in growth medium for 72 h. The percentages of cell viability were calculated relatively to the distinct optical density (OD) of a control group in growth medium supplemented with the 1:1000 dimethylsulfoxide (DMSO) (Ctr). A symbol * represents significantly higher than a control group (Ctr) (N=5, Mean ± SD).

4.4.2 Live/Dead cell staining assay

The hPDLSCs cultured on cell culture plate in the growth mediums supplemented with 1:1000 DMSO and 20 ppm Pheka and Pheka/AgNPs for 14 days exhibited fluorescence bright green calcein staining of the vital cells and growth of the spindle shaped cells in confluence (Figure 4.6A-C).

4.4.3 Transmission electron microscopic images of the silver nanoparticle endocytosis.

The hPDLSCs cultured in the growth mediums supplemented with 1:1000 DMSO and 20 ppm Pheka and Pheka/AgNPs on cell culture plate for 14 days. The intracellular accumulation of the silver nanoparticles (AgNPs) in those cells were examined under the TEM. The TEM demonstrated agglomeration of the AgNPs in cell cytoplasm, endosomes and exosomes. The particles were not found in nucleus and intracellular organelles and cell and nuclear membranes were intact (Figure 4.6D-I).

4.4.4 Levels of cell viability under oxidative stress

Oxidative stress could impair cell viability and functions. Pheka and Pheka/AgNPs protected hPDLSCs from injury induced by oxidative stress from the hydrogen peroxide (H_2O_2). The hPDLSCs were in growth medium alone (Ctr-FBS) and pre-treated with the Pheka, Pheka/AgNPs and QT for 48 h before being stimulated with 12.5 μM H_2O_2 for 45 min. It was found that the percentage of cell viability of the hPDLSCs in a control group with H_2O_2 alone (Ctr- H_2O_2) group was decreased to 80% of the non-treated group (Ctr-FBS) and it was significantly lower than those were pre-incubated with the 20 ppm Pheka, 5 and 20 ppm Pheka/AgNPs and 2.5 and 5 ppm QT ($p<0.05$). The percentages of cell viability among the pre-incubated groups, Pheka, Pheka/AgNPs and QT and with the Ctr-FBS were not significantly different ($p>0.05$). A strength of 20 ppm Pheka and 5 and 20 ppm Pheka/AgNPs were comparable to the 2.5 and 5 ppm QT groups. The findings suggested that the protective effect on cell viability of the Pheka/AgNPs tended to be higher than the Pheka (Figure 4.7).

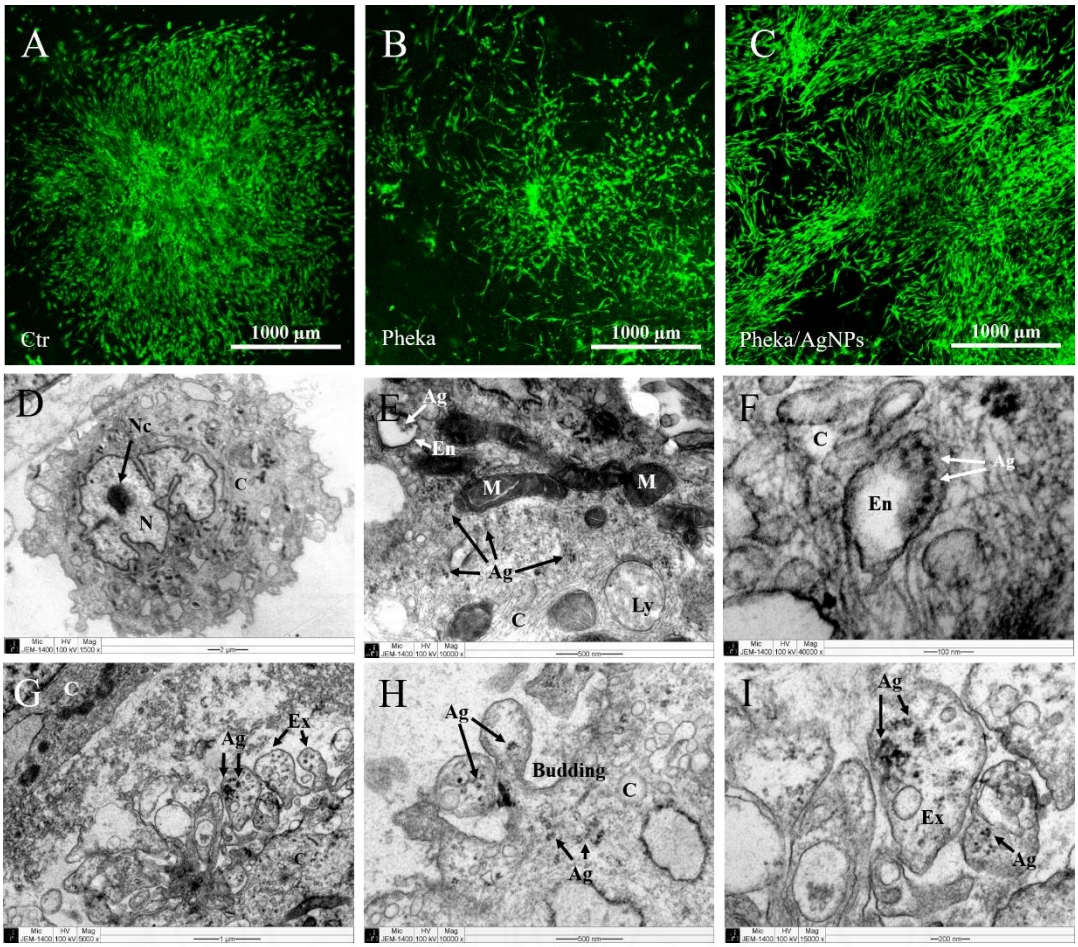

A
Ctr
1000 µm
B
Pheka
1000 µm
C
Pheka/AgNPs
1000 µm
D
Nc
C
N
E
Ag
En
M
M
Ag
C
Ly
F
C
Ag
En
G
C
Ag
Ex
C
H
Ag
Budding
C
Ag
I
Ag
Ex
Ag

Figure 4.6 Demonstrating the live/dead cell staining and transmission electron microscopy analyses of the human periodontal ligament stem cells (hPDLSCs) cultured on cell culture plate in growth mediums supplemented with the 1:1000 DMSO and 20 ppm Pheka and Pheka/AgNPs for 14 days, (A-C) Confocal laser scanning microscopy (CLSM) images of the live/dead cell staining assay (magnification x4), (A) in growth medium with the 1:1000 DMSO alone (growth medium), and (B) with 20 ppm Pheka and (C) 20 ppm Pheka/AgNPs, in which a bright green staining exhibits live cells and red staining of non-vital cells are not seen. (D-I) the TEM images of the hPDLSCs incubated (D) in growth medium, and (E) with 20 ppm Pheka and (E-I) 20 ppm Pheka/AgNPs. The TEM images (E) exhibits accumulation of the Pheka/AgNPs in cell cytoplasm (black arrows), (F) endosomes (white arrows) and (G-I) in exosomes and the budding exosomes on the cell membrane and out of cells, respectively. Abbreviations Ag signify silver nanoparticles, N, nucleus, Nc, nucleolus, C, cytoplasm, M, mitochondria, En, endosomes, Ly, lysosomes, and Ex, exosomes.

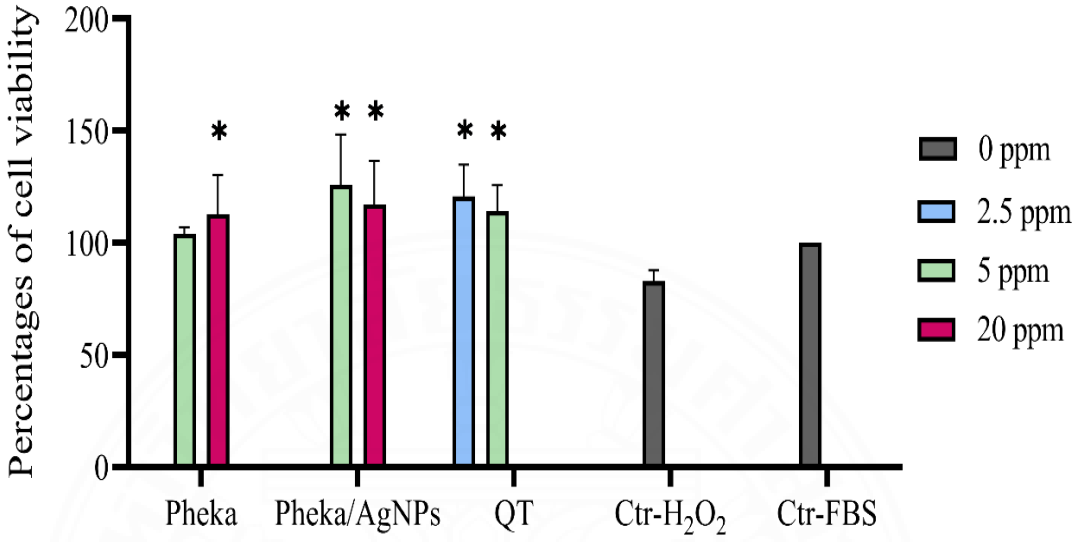


Figure 4.7 Demonstrating protective effects of the Pheka extract and biosynthesized silver nanoparticles (Pheka/AgNPs) on cell viability of the hydrogen peroxide treated human periodontal ligament stem cells (hPDLSCs). The hPDLSCs were pre-treated with Pheka, Pheka/AgNPs and QT for 48h and then followed by the stimulation with the 12.5 μM hydrogen peroxide (H_2O_2). Control groups were hPDLSCs in growth medium alone (Ctr-FBS) and growth medium with 12.5 μM H_2O_2 (Ctr-H_2O_2). A symbol * represents significantly higher than the Ctr-H_2O_2 group (N=5, Mean ± SD).

4.5 Anti-inflammatory effects

4.5.1 Effects of the Pheka extract and biosynthesized silver nanoparticles on cell viability under an inflammatory stimulus caused by a bacterial lipopolysaccharide

When the hPDLSCs were treated with the 1 — 8 µg/ml Lipopolysaccharide of *Porphyromonas gingivalis* (LPS) for 5 days in a growth medium. It was found that on each investigation day, day 1, 2 and 5, the percentages of cell viability of the hPDLSCs with 0, 1, 2, 4 and 8 ug/ml LPS were not significantly different and higher than 95% of a control in growth medium alone (0µg/ml LPS), with an average of the percentages of cell viability on days 1 and 2 at 101.34±3.76%. A significant increase of the levels of cell viability was found on day 5, when the average cell growth of all groups at 142.76±4.86% were significantly higher than the levels on days 1 and 2 ($p<0.05$). Therefore, the findings demonstrated that the 1 – 8 µg/ml LPS was non-cytotoxic to the hPDLSCs and the lowest working concentration was 1 µg/ml (Figure 4.8).

Furthermore, it was found that the 5 and 20 ppm Pheka and Pheka/AgNPs could promote cell growth and the 1 µg/ml LPS did not adversely affect the stimulating effects of those supplements. As it could be seen that the levels of cell viability of those groups were significantly higher than a control in growth medium alone (Ctr) ($p<0.05$). At the same time, the stimulating effects of the 2.5 and 5 ppm QT and adverse effects of the LPS on cell growth were not see. The percentages of cell viability of the QT groups were not significantly different from the controls without and with LPS (hPDLSCs and LPS-hPDLSCs). The differences between the groups with and without LPS of all supplement groups were not significantly different ($p>0.05$) (Figure4.9).

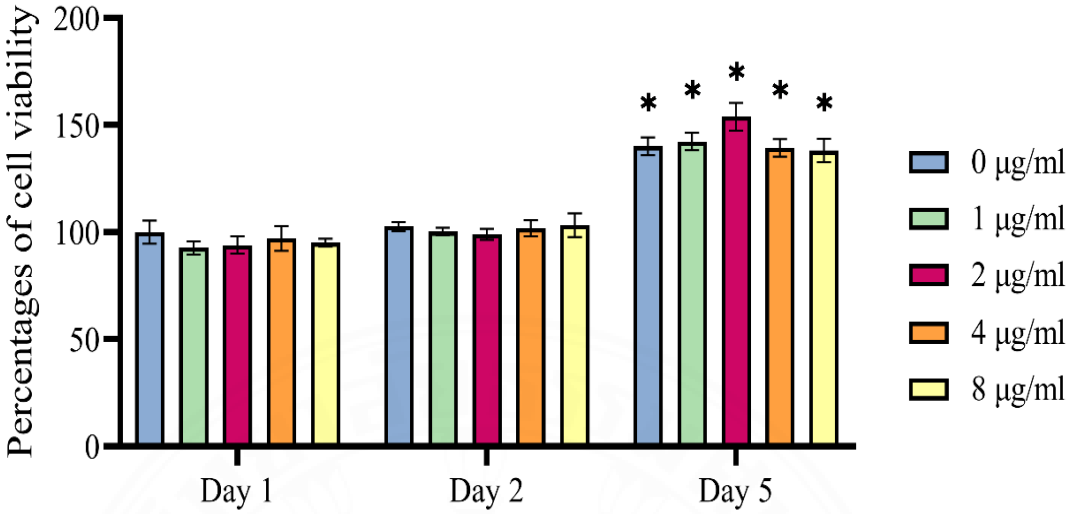


Figure 4.8 Demonstrating cell cytotoxicity of the *Porphyromonas gingivalis* (LPS) on the human periodontal ligament stem cells (hPDLSCs). Cell viability assay demonstrated non cytotoxicity of the 0-8 µg/ml LPS on hPDLSCs. The percentages of cell viability were calculated relatively to the cell viability of a control in 0 µg/ml LPS. A symbol * represents significantly higher than the groups on days 1 and 2 ($p<0.05$) (N=5, Mean ± SD)

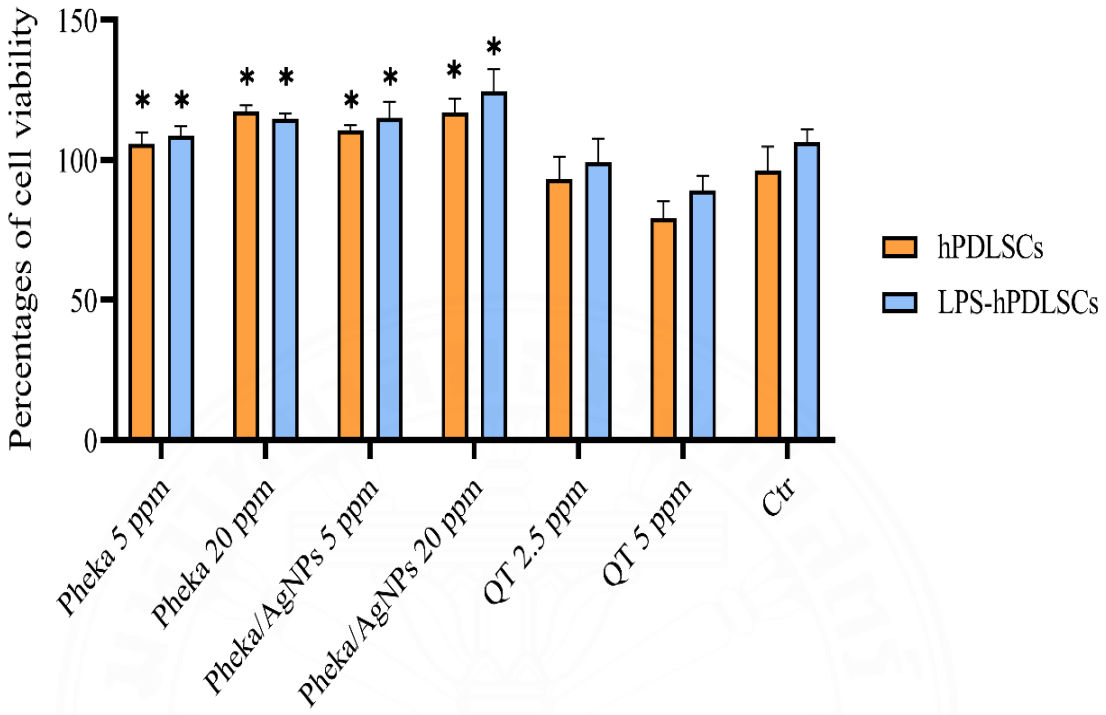


Figure 4.9 Demonstrating effects of the lipopolysaccharide of *Porphyromonas gingivalis* (LPS) and the initially refined extracts (Pheka), biosynthesized silver nanoparticles (Pheka/AgNPs) and quercetin (QT) on cell viability of the hPDLSCs. The control groups were the hPDLSCs in growth medium alone (hPDLSCs) and with 1 µg /ml lipopolysaccharide (LPS) (LPS-hPDLSCs). A symbol * represents significantly different from the control with LPS, LPS-hPDLSCs (N=5, Mean ± SD).

4.5.2 Effects of the Pheka extract and biosynthesized silver nanoparticles on a secretion of an interleukin-1 beta, prostaglandin E2 and Transforming growth factor-beta 1

4.5.2.1 Secretion levels of interleukin-1 beta

The bacterial LPS stimulated a secretion of an inflammatory cytokine, Interleukin-1 beta (IL-1β) from the hPDLSCs. Levels of the IL-1β of the hPDLSCs in growth medium with 1 μg/ml LPS (LPS-hPDLSCs) were significantly higher than a control group in growth medium alone (hPDLSCs) ($p<0.05$). When the hPDLSCs were pre-treated with the Pheka, Pheka/AgNPs and QT, the LPS did not increased the secretion levels of IL-1β in those groups ($p>0.05$). However, it could be noticed that the levels IL-1β of 5 ppm Pheka and 2.5 ppm QT without and with LPS were significantly lower than the controls (Ctr), hPDLSCs and LPS-hPDLSCs ($p<0.05$) (Figure 4.10).

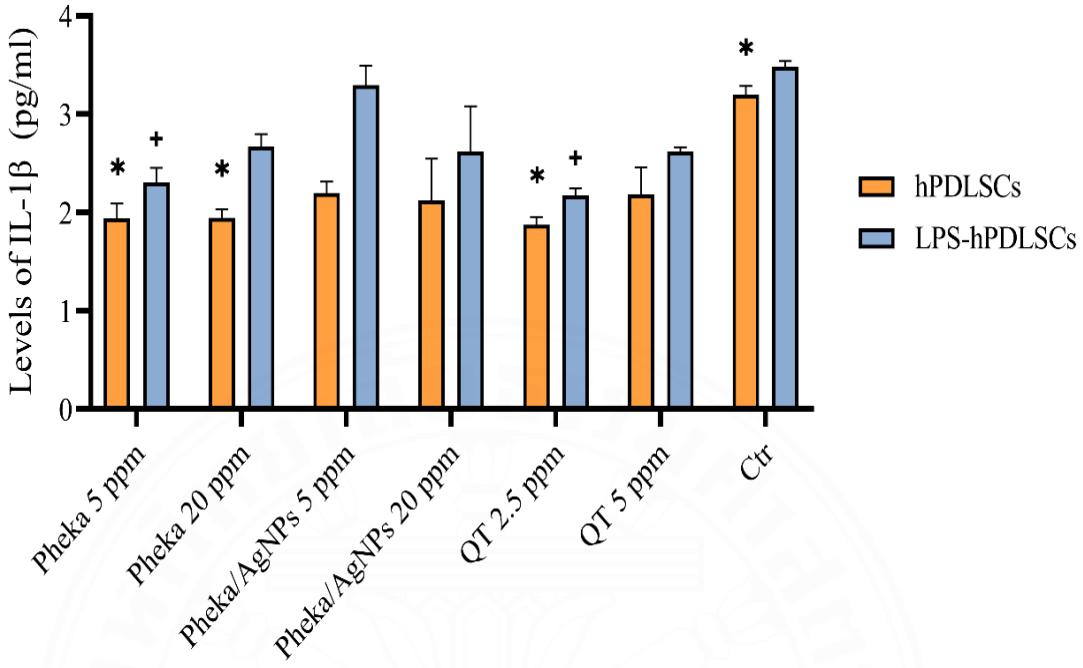


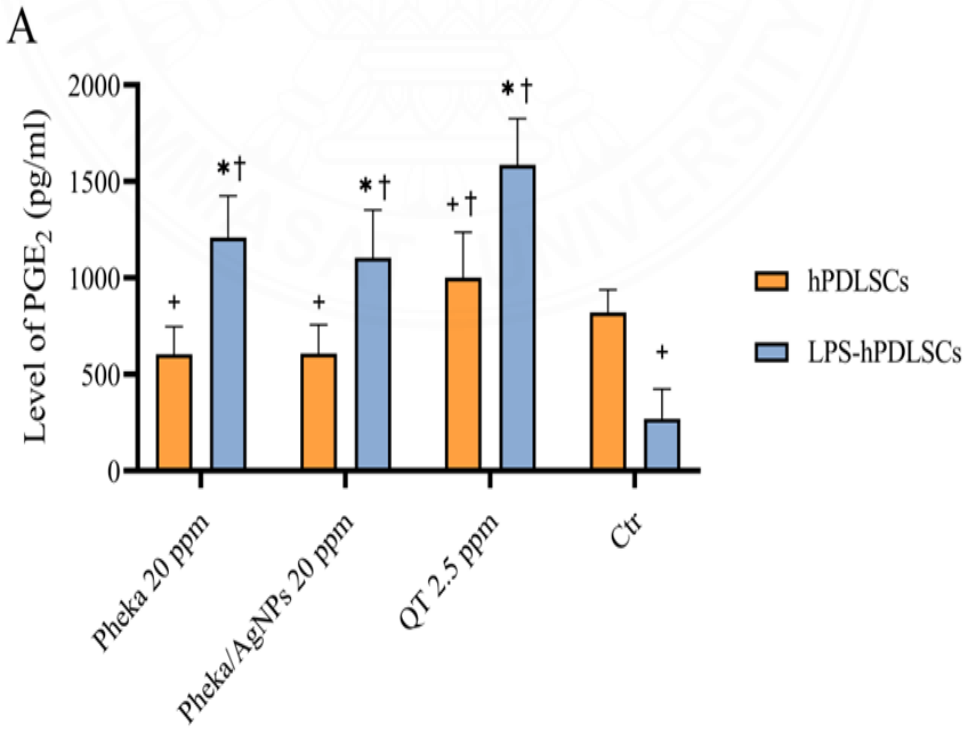
Figure 4.10 Demonstrating effects of Pheka extract, biosynthesized silver nanoparticles and quercetin on secretion levels of interleukin-1 beta (IL-1β) of the human periodontal ligament stem cells (hPDLSCs) under the bacterial lipopolysaccharide (LPS) stimulation. Human PDLSCs were cultured with Pheka extract (Pheka), biosynthesized silver nanoparticles (Pheka/AgNPs) and quercetin (QT), with and without 1 μg/ml lipopolysaccharides (LPS) (LPS-hPDLSCs and hPDLSCs) for 48 h. Symbols * represents significantly lower than the control with LPS (LPS-hPDLSCs) and +, significantly lower than the control without LPS (hPDLSCs) ($p<0.05$) (N=5, Mean ± SD).

4.5.2.2 Secretion levels of prostaglandin E2

In the current study, the hPDLSCs were cultured with 1 μg/ml LPS and Pheka, Pheka/AgNPs and QT in a growth medium for 48 h, and groups in growth medium without (hPDLSCs) and with LPS (LPS-hPDLSCs) were control groups (Ctr). It was found that the levels of PGE_2 of the LPS-hPDLSCs was significantly lower than the hPDLSCs group ($p<0.05$). In contrast, the levels of PGE_2 of the LPS-hPDLSCs with 20 ppm Pheka and Pheka/AgNPs and 2.5 ppm QT were significantly higher than the other groups with supplements and the control in growth medium alone (hPDLSCs) ($p<0.05$) (Figure 4.11A).

4.5.2.3 Secretion levels of transforming growth factor-beta 1

On the other hand, it was found that the 1 μg/ml LPS stimulated the secretion of the transforming growth factor-beta 1 (TGF-β1) from the control with LPS, LPS-hPDLSCs and the experiment groups, LPS-hPDLSCs with Pheka, Pheka/AgNPs and QT ($p<0.05$) (Figure 4.11B).



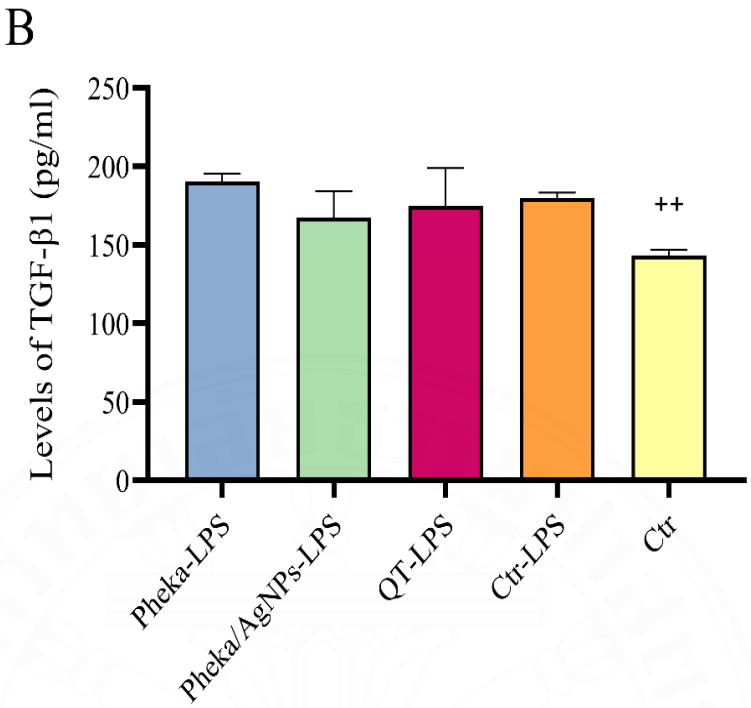


Figure 4.11 Demonstrating effects of the Pheka extract, biosynthesized silver nanoparticles and quercetin on the secretion levels of prostaglandin E2 and transforming growth factor-beta1 from the human periodontal ligament stem cells (hPDLSCs) under the stimulation from the bacterial lipopolysaccharide (LPS), (A) Secretion levels of prostaglandin E2 (PGE_2) and (B) transforming growth factor-beta1 (TGF-β1). Human PDLSCs were cultured with the Pheka extract (Pheka), biosynthesized silver nanoparticles (Pheka/AgNPs) and quercetin (QT) Pheka, with and without 1 μg/ml lipopolysaccharides (LPS) (hPDLSCs and LPS-hPDLSCs) for 48 h. Symbols * represent significantly higher than a control, LPS-hPDLSCs, †, higher than a control without any supplements (hPDLSCs), $^{+}$, lower than the other group with the same concentration and $^{++}$, lower than other groups ($p<0.05$) (N=5, Mean ± SD).

4.6 Osteogenic differentiation of hPDLSCs on mono layer cell culture

4.6.1 Osteogenic differentiation

4.6.1.1 Alkaline phosphatase activity

It was found that the ALP levels of the 5 and 20 ppm Pheka/AgNPs were significantly higher than the control without supplements (Ctr) ($p<0.05$), while the ALP activity of 5 ppm Pheka tended to be higher than the control ($p>0.05$) (Figure4.12). The promoting effects of the 5 and 20 ppm Pheka/AgNPs and 5 ppm Pheka were supported by the ALP staining, which was demonstrated by the cytoplasmic blue staining of the hPDLSCs in osteogenic mediums supplemented with the Pheka and Pheka/AgNPs for 21 days (Figure 4.13).

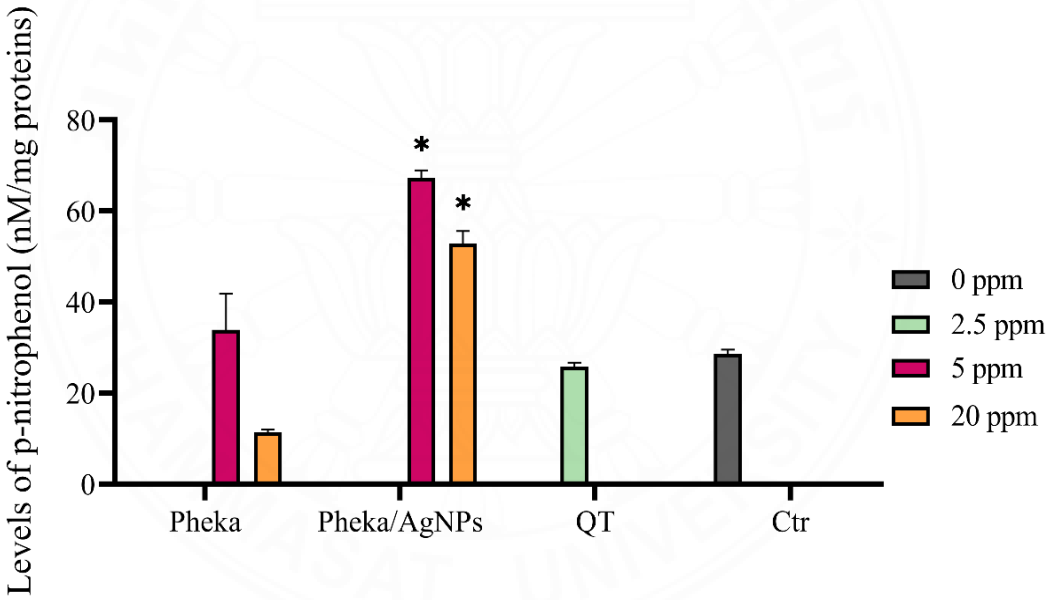


Figure 4.12 Demonstrating alkaline phosphatase activity (ALP) of the human periodontal ligament stem cells in an osteogenic medium supplemented with Pheka extract (Pheka), biosynthesized silver nanoparticles (Pheka/AgNPs) and quercetin (QT) for 21 days. A symbol * represents significantly higher than other groups (Pheka, QT and Ctr) ($p<0.05$) (N=5, Mean ± SD).

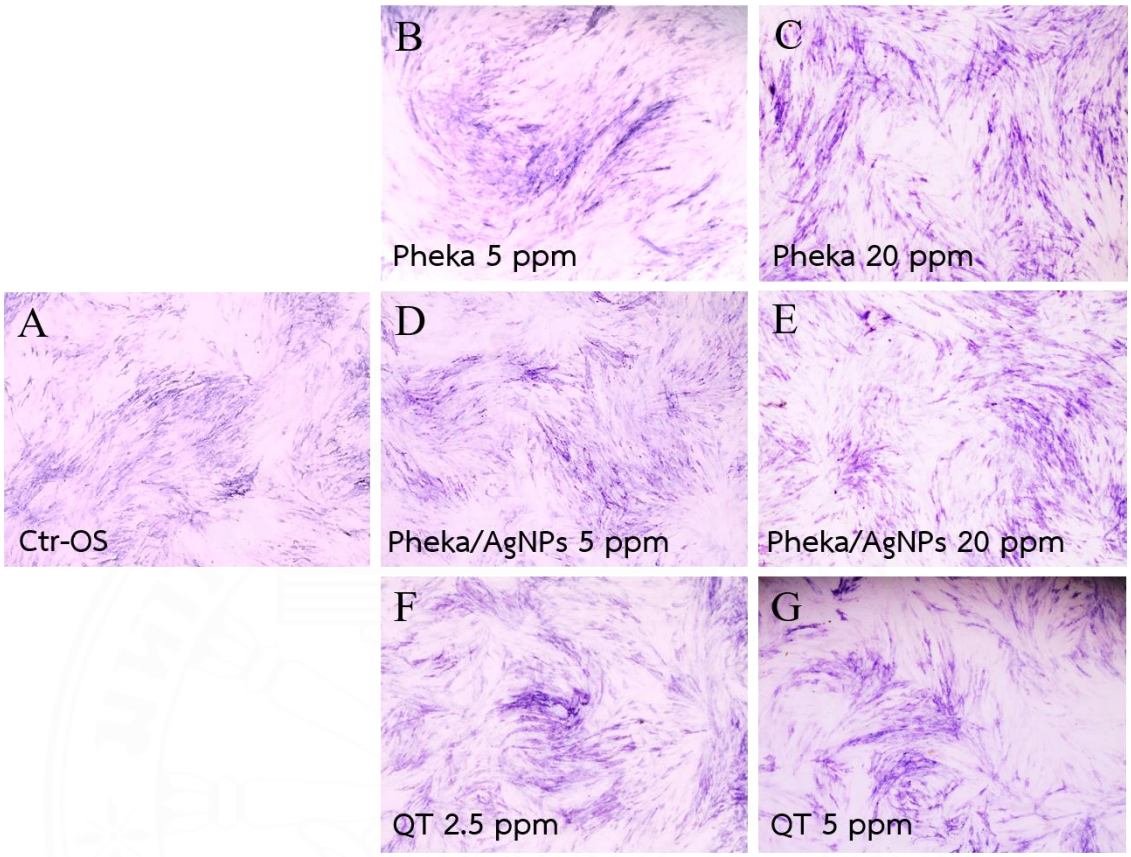


Figure 4.13. Demonstrating alkaline phosphatase staining of the human periodontal ligament stem cells (hPDLSCs) in an osteogenic medium supplemented with Pheka extract (Pheka), biosynthesized silver nanoparticles (Pheka/AgNPs) and quercetin (QT) for 21 days. A control group is an osteogenic medium alone (Ctr-OS). Blue staining exhibits alkaline phosphatase staining of the hPDLSCs (magnification x4).

4.6.2 *In vitro* mineralization

4.6.2.1 Levels of calcium contents in extracellular matrix

Levels of *in vitro* mineralization were quantified by measuring levels of calcium contents in extra cellular matrix of the hPDLSCs cultured in osteogenic medium with and without Pheka extract (Pheka), biosynthesized silver nanoparticles (Pheka/AgNPs) and quercetin (QT) for 21 days. It was found that the levels of calcium contents of 5 ppm Pheka, 5 ppm Pheka/AgNPs and 2.5 ppm QT were significantly higher than control in osteogenic medium only (Ctr) (0 ppm), and they were also higher than the other groups in the 20 ppm and 5 ppm groups ($p<0.05$) (Figure 4.14).

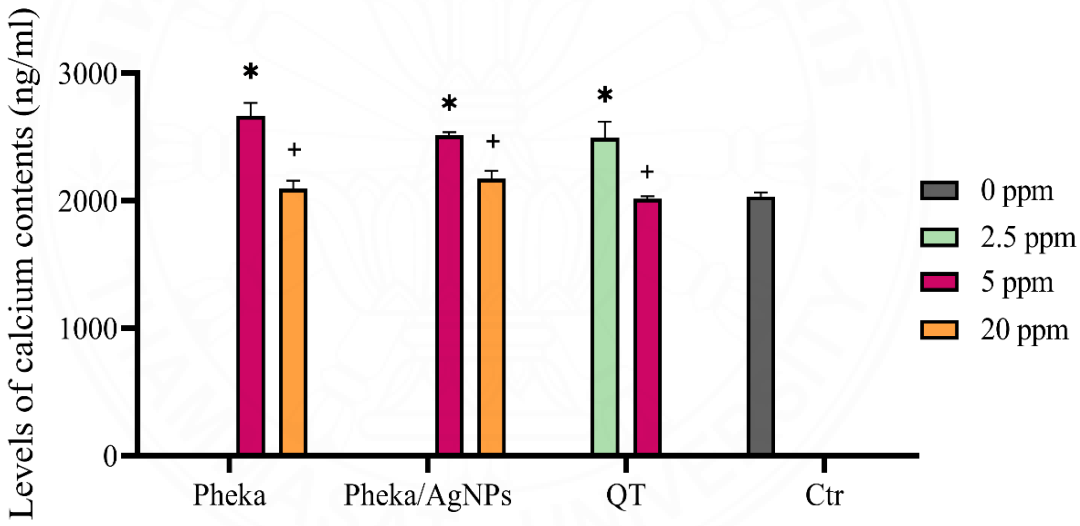


Figure 4.14 Demonstrating levels of calcium contents in extracellular matrix of the human periodontal ligament stem cells (hPDLSCs) in an osteogenic medium supplemented with the Pheka extract (Pheka), biosynthesized silver nanoparticles (Pheka/AgNPs) and quercetin (QT) for 21 days. A control group is an osteogenic medium alone (Ctr). Symbols * represent significantly higher than a Ctr group and $^{+}$, significantly lower than another concentration in the same groups ($p<0.05$) (N=5, Mean ± SD).

4.6.2.2 Alizarin red staining

Alizarin red staining exhibited an in vitro mineralization of the extracellular matrix in red. The intensity and area of red staining represent the degrees of mineralization. A strong staining was found in 5 ppm Pheka, Pheka/AgNPs and QT and control groups (Figure 4.15).

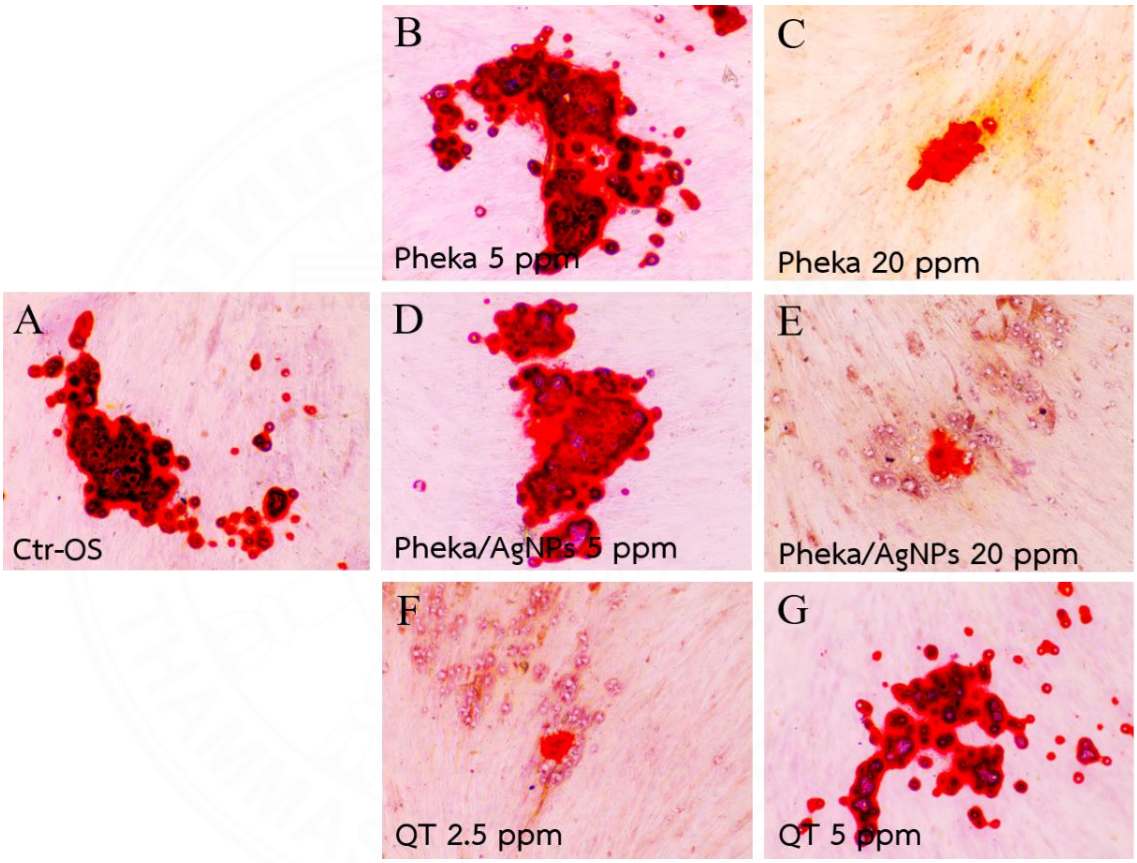


Figure 4.15 Demonstrating alizarin red staining of the human periodontal ligament stem cells (hPDLSCs) in osteogenic medium supplemented with the Pheka extract (Pheka), biosynthesized silver nanoparticles (Pheka/AgNPs) and quercetin (QT) for 21 days. Red staining of cell nodules and extracellular matrix indicates calcium depositing in the extra cellular matrix (magnification x20).

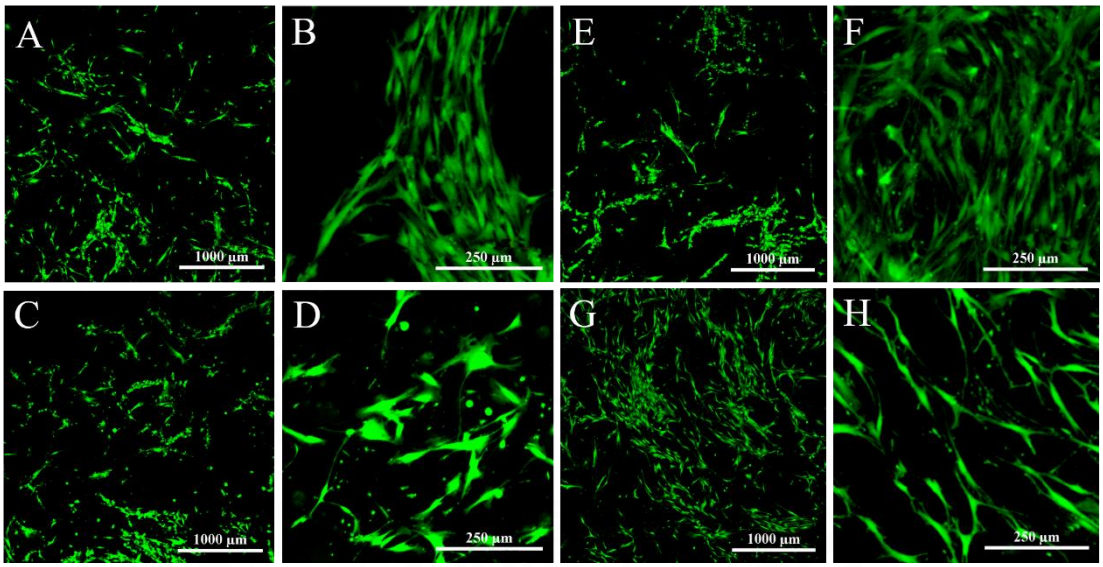
4.7 Effect of Pheka on hPDLSCs of the encapsulated cells

Human periodontal ligament stem cells (hPDLSCs) were encapsulated in a thermosensitive 2% nHA-2%$CaCO_3$-10%bGP-4:1 (w/w) chitosan/collagen hydrogel and cultured in osteogenic medium for 21 days. Levels of cell viability, alkaline phosphatase activity and calcium contents were measured to determine effects of the supplements on cell growth and osteogenic differentiation. It was found that Pheka and Pheka/AgNPs promoted cell growth and osteogenic differentiation of the encapsulated hPDLSCs.

4.7.1 Live dead cell staining assay of the encapsulated cells

Live dead cell staining was performed to examine effects of Pheka extract (Pheka), biosynthesized silver nanoparticles (Pheka/AgNPs) and quercetin (QT) on cell morphology and cell growth of the encapsulated hPDLSCs cultured in an osteogenic medium with and without the supplements. The fluorescence green staining of the vital cells demonstrated that the encapsulated hPDLSCs were able to survive, proliferate, and extend their cytoplasmic process forming intercellular contact on the three-dimensional structure of the hydrogel. A red staining of non-vital cells was not found (Figure 4.16).

Pheka 20 ppm QT 2.5 ppm



Pheka/AgNPs 20 ppm Ctr-OS

Figure 4.16 Confocal laser scanning microscopic images of a live/dead cell staining assay of the encapsulated human periodontal ligament stem cells (hPDLSCs). The hPDLSCs were encapsulated in the thermosensitive 2% (w/v) nano hydroxyapatite-2% (w/v) calcium carbonate-chitosan/collagen hydrogel and cultured for 21 days in the osteogenic mediums supplemented with 20 ppm Pheka extract (Pheka), 20 ppm biosynthesized silver nanoparticles (Pheka/AgNPs), and 2.5 ppm quercetin (QT), and osteogenic medium alone as a control group (Ctr). The staining demonstrates effects of the supplements on cell viability, growth and morphology of the encapsulated hPDLSCs, (A & B) 20 ppm Pheka, (C & D) 20 ppm Pheka/AgNPs, (E & F) 2.5 ppm QT and (G & H) osteogenic medium alone. (A, C, E, G) x4 magnification and (B, D, F, H) x 20 magnification. Green staining exhibits live cells ad a red staining of non-vital cells were not seen.

4.7.2 Cell viability assay of the encapsulated cells

It was found that the percentages of cell viability of the encapsulated hPDLSCs cultured in an osteogenic medium supplemented with the 20 ppm Pheka extract (Pheka) and 20 ppm biosynthesized silver nanoparticles (Pheka/AgNPs) for 21 days were significantly higher than a control group in osteogenic medium alone and a group with 2.5 ppm quercetin (QT) ($p<0.05$) (Figure 4.17).

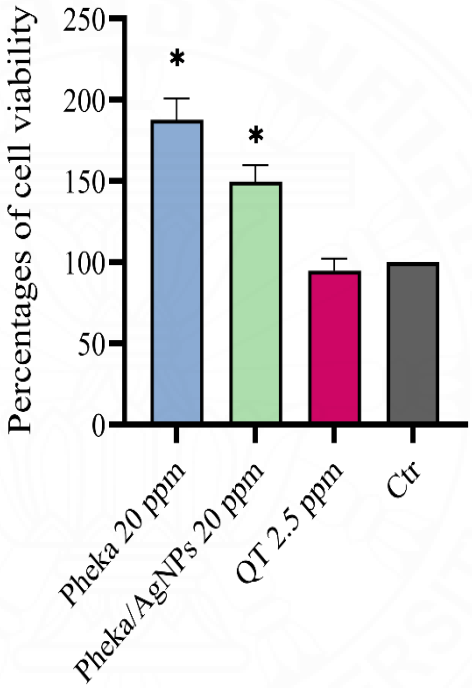


Figure 4.17 Demonstrating effects Pheka extract (Pheka), biosynthesized silver nanoparticles (Pheka/AgNPs) and quercetin (QT) on cell viability of the encapsulated human periodontal ligament stem cells (hPDLSCs). The encapsulated hPDLSCs were cultured in the osteogenic mediums with and without supplements for 21 days. A symbol * represents significantly higher than the control group (Ctr) and QT 2.5 ppm ($p<0.05$) (N=5, Mean ± SD).

4.7.3 Alkaline phosphatase activity of the encapsulated cells

Promoting effects of the Pheka, Pheka/AgNPs and QT on osteogenic differentiation of the encapsulated hPDLSCs were clearly demonstrated. The levels of ALP activity of those groups at 5 ppm Pheka, 5 ppm Pheka/AgNPs and 2.5 and 5 ppm QT were significantly higher than a control in osteogenic medium alone (Ctr-FBS) ($p<0.05$). The levels of 5 ppm Pheka and 2.5 ppm QT groups were significantly higher than the other groups ($p<0.05$). A promoting effect of QT 2.5 and 5 ppm tended to be stronger than Pheka and Pheka/AgNPs but they were not significantly different ($p>0.05$) (Figure 4.18).

4.7.4 *In vitro* mineralization of the encapsulated cells

An *in vitro* mineralization of the encapsulated cells was quantified by measuring levels of calcium contents in extracellular matrix. In case of the encapsulated cells, levels of calcium contents of the encapsulated hPDLSCs were also compared with the level of gel only. Calcium levels in extracellular matrix were quantified and compared with a control without supplements and hydrogel alone groups.

It was found that levels of calcium contents of the encapsulated cells cultured for 21 days in osteogenic mediums supplemented with the 5 and 20 ppm Pheka extract (Pheka), 5 and 20 ppm biosynthesized silver nanoparticles (Pheka/AgNPs), and 2.5 and 5 ppm quercetin (QT) and osteogenic medium alone as a control group (Ctr). It was found that the levels of calcium contents of the 5 ppm Pheka, 5 and 20 ppm Pheka/AgNPs and 2.5 and 5 ppm Quercetin were significantly higher than group of a hydrogel alone without cells and without supplements (Ctr-Gel) ($p<0.05$). They tended to be higher than the control of the encapsulated cells in an osteogenic medium alone (Ctr) but they were not significantly different ($p>0.05$) (Figure 4.19).

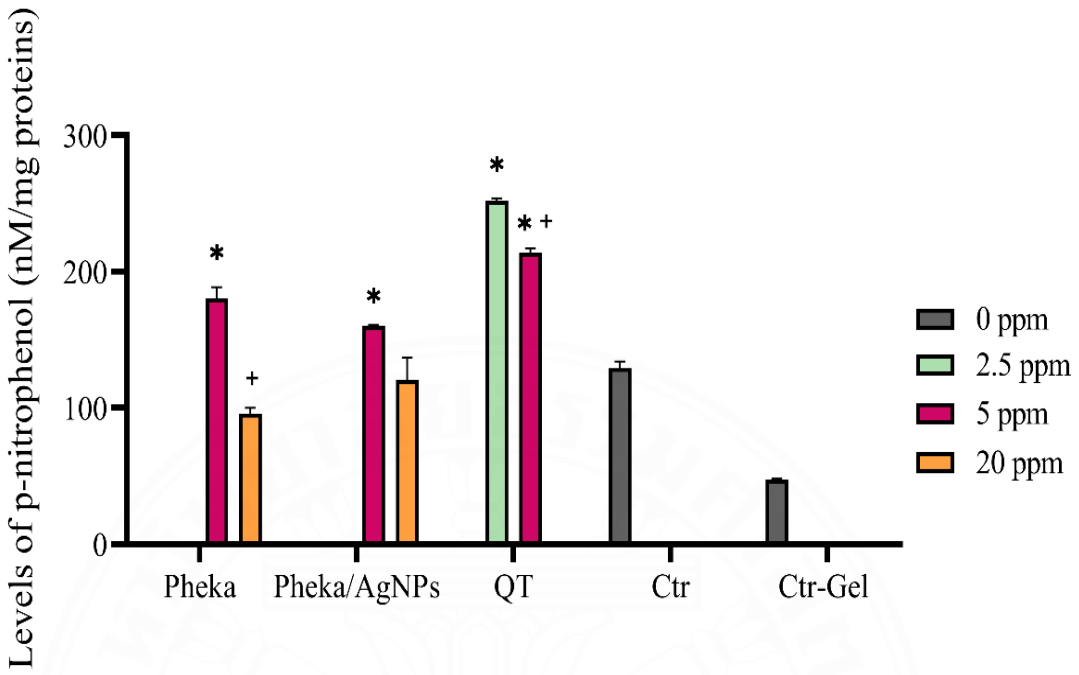


Figure 4.18 Demonstrating levels of alkaline phosphatase activity of the human periodontal ligament stem cells (hPDLSCs) encapsulated in the thermosensitive 2% (w/v) nano hydroxyapatite-2% (w/v) calcium carbonate-chitosan/collagen hydrogel and cultured for 21 days in osteogenic mediums supplemented with 20 and 5 ppm Pheka extract (Pheka) and biosynthesized silver nanoparticles (Pheka/AgNPs), and 2.5 and 5 ppm quercetin (QT) and osteogenic medium alone as a control group (Ctr). Symbol * represents significantly higher than Ctr and $^{+}$, significantly lower than the other concentration in the same groups ($p<0.05$) (N=5, Mean ± SD).

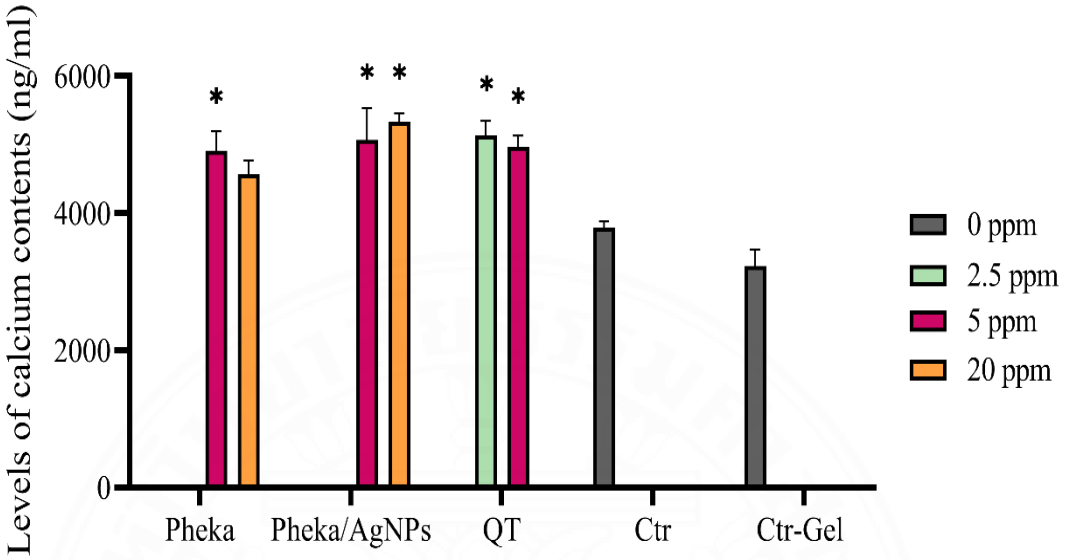


Figure 4.19 Demonstrating levels of calcium contents of the human periodontal ligament stem cells (hPDLSCs) encapsulated in the thermosensitive 2% (w/v) nano hydroxyapatite-2% (w/v) calcium carbonate-chitosan/collagen hydrogel and cultured for 21 days in osteogenic medium supplemented with 20 and 5 ppm Pheka extract (Pheka) and biosynthesized silver nanoparticles (Pheka/AgNPs), and 2.5 and 5 ppm quercetin (QT). The encapsulated cells in an osteogenic medium alone (Ctr) and the hydrogel alone without cells and without supplement in osteogenic medium (Ctr-Gel) were set as control groups. A symbol * represents significantly higher than the Ctr-Gel ($p<0.05$) (N=5, Mean ± SD).

CHAPTER 5
DISCUSSION

Anti-oxidative stress and anti-inflammatory properties of Pheka has been reported (19), but those effects on hPDLSCs including the effects on growth and osteogenic differentiation of the hPDLSCs have not been investigated. Human PDLSCs exhibit key characteristics of MSCs, such as self-renewal, multipotency, and immunomodulation (93) and are progenitor cells for the periodontal tissue regeneration including alveolar bone, cementum, and periodontal ligament (69, 94). Therefore, this study investigated effects of Pheka and Pheka/AgNPs on the hPDLSCs. New findings from the current study were that flavonoids extracted from the stem bark of *Oroxylum indicum* (Pheka) and biosynthesized Pheka/AgNPs exhibited a protective effect on the hPDLSCs under an oxidative stress, inhibited secretion of an inflammatory cytokine, IL-1β from the hPDLSCs, and promoted growth and osteogenic differentiation of the hPDLSCs on cell culture plates and the hydrogel encapsulated cells. The hPDLSCs cultured with the 20 ppm Pheka/AgNPs in osteogenic medium for 14 days were able to form cell sheet at confluence and the encapsulated cells extended cell cytoplasm and filopodia to exhibit a spindle shaped cell morphology and created three-dimensional intercellular contact on the three-dimensional structure of the hydrogel, while the internalization of Pheka/AgNPs was found. The biologic effects of the Pheka/AgNPs tended to be stronger than those of Pheka on the hPDLSCs.

Silver nanoparticle biosynthesis is environmentally safe and low cost and produces large numbers of nanoparticles (8). The capping of flavonoid molecules, Pheka on the AgNPs of the Pheka/AgNPs helped to protect the neutral silver molecules from the oxidation to silver ions (Ag^{2+}). Thus, the reduction decreased the production of free metal ions and radical oxygen species (ROS), main causes of AgNP toxicity (95). A traditional Indian medicine has used a similar principle to remove toxicity of the metal ions for centuries (96). Additionally, a high surface area of the silver nano particles could increase concentrations of the flavonoids on the particles and promote

intracellular internalization of the Pheka/AgNPs (97). As a result, the biologic effects of Pheka would be enhanced.

Furthermore, the current findings support a previous study that the biosynthesized silver nanoparticles Pheka/AgNPs enhanced biologic effects of Pheka. The findings demonstrated that biologic effects of Pheka/AgNPs tended to be higher than those of Pheka, as it was found that the protective effects of Pheka/AgNPs on cell viability under an oxidative stress induced by the H_2O_2 and osteogenic differentiation in an early stage of hPDLSCs on a monolayer cell culture and late osteogenic differentiation stage of the encapsulated hPDLSCs tended to be higher than those of Pheka. Hu and co-workers in 2019 report that biosynthesis *Bauhinia acuminate*/AgNPs is non-cytotoxic and able to promote osteogenic differentiation of mouse mesenchymal stem cells (98). In corresponding to a previous study, the Pheka/AgNPs were non-cytotoxicity and average cell viability levels of all groups were higher than 90% of the control in growth medium alone. Additionally, the results demonstrated that the Pheka/AgNPs did not interfere with cell growth and osteogenic differentiation of mesenchymal stem cells (MSCs). The accumulation of the AgNPs in cytoplasm and endosomes, and an intact nuclear and cell membranes and growth and osteogenic differentiation of the hPDLSCs suggested non-cytotoxicity of the Pheka/AgNPs. The findings support previous reports that the AgNPs agglomerates were typically located in the perinuclear region, found mainly in the endo-lysosomal structures (57, 99).

A non-cytotoxicity of the biosynthesize AgNPs might be because of a non-cytotoxic concentration and antioxidative effects of the Pheka/AgNPs. A reductive interaction between Pheka and AgNPs or capping of Pheka on the AgNPs helped stabilize the Pheka/AgNPs (8). The hypothesis was supported by the accumulation of the particles in the exosomes at the cell membrane and the budding exosomes. The exosomes suggested autophagy reaction of the hPDLSCs that expelled the Pheka/AgNPs from the cells, and thus decrease adverse effects of the nanoparticles to cell viability and functions (100). Together with the promoting effects of the 5 and 20 ppm Pheka/AgNPs on an osteogenic differentiation of the hPDLSCS, the findings

supports a previous report that the autophagy help promote osteogenic differentiation of the hMSCs (58). Moreover, He and co-workers in 2020 report that the cellular autophagy of human mesenchymal stem cells is triggered by the AgNPs and consequently an autophagy promotes osteogenic differentiation of hMSCs. Therefore, the findings exhibited that the biosynthesized silver nanoparticles (Pheka/AgNPs) do not only exhibit antibacterial function, but also enhance biological effects of the natural flavonoids.

Additionally, an ability to export the nanoparticle could be contributed by the spherical shape and small particle size at 21 nm of the Pheka/AgNPs that assisted a well-off of the particle in endosome and transportation of the particles out of the cells in exosome (101). However, a high surface area of the nanoparticles could enhance toxicity of the particles by increasing particle/cell surface contact and enhancing chemical reactivity and intracellular or organ accumulations (102). The findings agree with a previous report that the biosynthesized AgNPs size 30 nm in diameter as well as the current Pheka/AgNPs size 21 nm in diameter are non-cytotoxic and do not interfere with growth and differentiation of hMSCs (103). As it could be seen that despite cell endocytosis of AgNPs following a 14-days incubation with the 20 ppm Pheka/AgNPs in osteogenic medium, the biosynthesized Pheka/AgNPs could promote cell growth and osteogenic differentiation of the hPDLSCs on cell culture plates and the encapsulated hPDLSCs. Cell cytotoxicity and promoting effects on osteogenic differentiation of the Pheka/AgNPs support the applicability of the particles for clinical use for promoting bone tissue engineering. Growth and cell morphology of the encapsulated cells also indicated that a thermosensitive nanohydroxyapatite-calcium carbonate-chitosan/collagen hydrogel provided supporting environment for cell growth and differentiation (80, 104, 105).

Free radical oxygens caused by oxidative stress and inflammatory cytokine secretion stimulated by bacterial toxin such as LPS could adversely affect cell viability and functions (106). The biologic effects of the Pheka and Pheka/AgNPS corresponded to the effects of quercetin, a commonly found flavonoids in fruits and vegetables (107), in which the antioxidative, anti-inflammatory and promoting effects

on osteogenic differentiation of MSCs of quercetin have been well reported (108). In the current study, the protective effects of the Pheka/AgNPs on the hPDLSCs that were under an oxidative stress and an inhibitory effect on secretion of an inflammatory cytokine were clearly shown.

Bacterial endotoxin LPS has been recognized as a trigger factor of inflammatory response in gingivitis and periodontitis (109). Prostaglandin E2 (PGE_2) is an important inflammatory cytokine secreted following cell membrane injuries that stimulates inflammatory cascades and promotes vasodilation and osteoblastic differentiation by activating cAMP signaling through G protein coupled EP4 receptor (110). The increasing of levels of PGE_2 in the LPS-hPDLSCs with and without Pheka, Pheka/AgNPs and QT, while it was significantly decreased in the LPS-hPDLSCs without supplements suggested that the Pheka and Pheka/AgNPs could promote PGE_2 secretion and osteogenic differentiation in normal and inflammatory environments. This is because PGE_2 could promote osteoblastic differentiation of hMSCs and enhanced vasodilation (110). Moreover, transforming growth factor beta 1 (TGF-β1) which is an important anti-inflammatory growth factors with various stimulating effects on regeneration, including bone regeneration and osteoblastic proliferation and maturation (111). Sun and co-workers in 2018 demonstrate that the bacterial LPS modulates secretion of the TGF-β1 and the TGF-β1 significantly increases synthesis of type I collagen (112). Therefore, the findings that the LPS stimulated secretion of the TGF-β1 from hPDLSCs regardless of flavonoid supplements suggested that the supplements, Pheka, Pheka/AgNPs and QT did not adversely affect regenerative capacity of the hPDLSCs under an inflammatory stimulus (113, 114).

The current study clearly demonstrated a protective effect on oxidative stress and anti-inflammatory effects of the Pheka and Pheka/AgNPs, including theirs promoting effects on growth and osteogenic differentiation of hPDLSCs. Antioxidative and anti-inflammatory effects might enable promoting effects of the Pheka and Pheka/AgNPs on cell growth and osteogenic differentiation of the hydrogel encapsulated hPDLSCs. This was because a low oxygen level and limited nutrient diffusion and effusion of cellular waste in the restricted environment of the

encapsulated cells could increase cellular stress on the encapsulated cells (115). Thus, the hydrogels impregnated the Pheka and Pheka/AgNPs would enhance cell viability and promote osteogenic differentiation of the transplanted cells and local host cells.

As periodontitis and peri-implantitis are inflammatory diseases that an excessive inflammatory cytokines in the periodontal pocket cause damages to the periodontium leading to attachment loss and bone resorption (116), the applications of the Pheka/AgNPs in the periodontal pockets would decrease cellular damage and severity of bone loss, and promote bone regeneration. The use of hPDLSCs for testing the biologic effects of the Pheka/AgNPs in the current study supported future application of Pheka and Pheka/AgNPs for the treatment of peri-implantitis. This is because hPDLSCs could promote periodontal tissues formation, cementum-like and new bone formation on titanium surface of dental implants *in vivo* model (117) while osteogenic differentiation potential of the hPDLSCs is comparable to bone marrow stem cells (BMSCs) (118). Additionally, the periodontal tissue is accessible in routine clinical practices, isolation and expansion methods of hPDLSCs are uncomplicated and hPDLSCs exhibit osteogenic differentiation potential for clinical periodontal regenerative therapy (119).

Furthermore, a thermosensitive hydrogel would facilitate the delivery of Pheka and Pheka/AgNPs into a small and narrow space such as periodontal pockets and provide supporting environment for cell growth and differentiation (76). Furthermore, the synergistic effects of an antibacterial property of Pheka (37) and silver nanoparticles (48, 120) would further enhance antibacterial property of the Pheka/AgNPs for the treatment of periodontitis. The findings suggested that by delivering Pheka/AgNPs in the periodontal pockets of natural teeth or dental implants, the biosynthesized Pheka/AgNPs would decrease bacterial accumulation and minimize cellular damage caused by bacterial toxin and inflammatory cytokines as well as promote bone regeneration. At the same time, it would promote bone regeneration. The promoting effects of the Pheka and Pheka/AgNPs on growth and osteogenic differentiation of the encapsulated hPDLSCs would facilitate stem cell transplantation

and promote cell survival and functions of the transplanted cells in the defect sites. Additionally, the anti-inflammatory effects of Pheka and Pheka/AgNPs could be applied for decreasing patient discomfort and promote tissue healing of oral mucositis patients in forms of mouth rinse and oral paste for topical applications.

CHAPTER 6
CONCLUSION

The current study focused on finding a new biomaterial for treatment of peri-implantitis using traditional medicinal plants. Peri-implantitis is a critical biological complication leading to dental implant failures and causes of peri-implantitis associate with bacterial toxins and inflammation of the supporting gingiva and bone surrounding the implants. The utilization of local materials and plants to produce medical materials help decrease cost of medical care and thus increase the accessibility to high quality medical care for Thai patients, particularly the elderly and disadvantage groups. Furthermore, the using of local products could promote Thai economy in micro-and macro scales, starting from farmers to the manufacturers of the medical products and the employment of educated skilled personnel for the advanced material productions.

The current study achieved its aims on investigating biological activities of the Thai traditional medicinal plant, *Oroxylum indicum* L. Kruz (Local name, Pheka) as a bioactive material for improving treatment outcomes of peri-implantitis and promoting alveolar bone regeneration. The flavonoid contents from the stem barks of Pheka were extracted and partially refined. Then the silver nanoparticles (Pheka/AgNPs) were biosynthesized using the flavonoid contents in the Pheka extracts as a reducing agent. The green synthesis of the Pheka/AgNPs is an innovation that employs local plants and traditional wisdom to synthesize new bioactive materials for medical applications. The investigations demonstrated that the Pheka extracts and Pheka/AgNPs were not only exhibited anti-inflammatory and antioxidant effects but also promoted osteogenic differentiation, in which those advantages were applicable for the treatment of peri-implantitis and regenerative medicine.

The unique advantage properties of the Pheka/AgNPS for bone regeneration are simple, cost-effective, environment friendly, reproducible, and safe productions. The findings demonstrated that the initially refined Oroxylum indicum crude extracts (Pheka) and biosynthesized Pheka/AgNPs exhibited protective effects on

cell viability under oxidative stress, decreased secretion of an inflammatory cytokine and promoted growth and osteogenic differentiations of the hPDLSCs on a monolayer cell culture and the encapsulated cells. The biosynthesized Pheka/AgNPs tended to enhance biological effects of the Pheka and were non cytotoxic to the hPDLSCs. They were potential bioactive molecules for the prevention and treatment of periodontitis and peri-implantitis. Effects of the hydrogel impregnated with the biosynthesized Pheka/AgNPs in the periodontal pockets in an animal model should be further investigated.

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APPENDIX

An acceptance letter of the 19th International Scientific Conference of Dental Faculty Consortium of Thailand (DFCT 2022) "Novel Challenges in Dental Practice and Research", proceedings.

ACCEPTANCE OF YOUR FULL MANUSCRIPT FOR ORAL PRESENTATION

Dear Jarutai Prapaipittayakhun

Congratulations! It is our pleasure to accept your manuscript/proceedings, ID number **OM-00016**, for an **oral** presentation at the 19th International Scientific Conference of Dental Faculty Consortium of Thailand (DFCT2022). The details of your preparation for the **oral** presentation are available on our website.

***Important: For only ONLINE oral presenter or POSTER presenter, please send your file by emailing to support@micerent.com and CC Anupong.mak@mfu.ac.th before October 17th, 2022 according to the dead line from the presentation guideline.

Best Regards,
Clinical Professor Emeritus Dr. Varunee Kerdvongbundit
Editor-in-chief
DFCT2022
P.S.: This is an automatic electronic mail in response to your submission; there is no need to reply to this electronic mail.

BIOGRAPHY

Name	Ms. Jarutai Prapaipittayakhun
Date of Birth	July 30, 1991
Educational Attainment	Academic Year 2015: Doctor of Dental Surgery, Faculty of Dentistry, Naresuan University, Thailand 2019 - present: A Master's student in a Master of Science Program in Dental Implantology, Faculty of Dentistry, Thammasat University, Thailand
Work Position	General dental practitioner
Scholarship (If any)	-
Publications	Proceeding in the 19th International Scientific Conference of Dental Faculty Consortium of Thailand (DFCT 2022) "Novel Challenges in Dental Practice and Research" proceedings, Jarutai PRAPAIPITTAYAKHUN, Supakorn BOONYUEN, Alvin Lim Teik ZHENG, Komsan APINYAUPPATHAM and Premjit ARPORNMAEKLONG. An *Oroxylum indicum* Extract, a Potential Biomaterial for the Treatment of Peri-implantitis. at the 19th International Scientific Conference of Dental Faculty Consortium of Thailand (DFCT 2022), School of Dentistry, Mae Fah Luang University, Thailand, November 2 - 4, 2022 (Appendix).

Work Experiences	2019 - present: General dental practitioner, Private dental clinic, Bangkok, Thailand 2017 - 2019: General dental practitioner, Private dental clinic, Chiang Rai, Thailand 2015 - 2017: General dental practitioner, Doi Luang hospital, Chiang Rai, Thailand