EFFECT OF *AQUILARIA CRASSNA* CRUDE EXTRACT ON OSTEOGENIC ACTIVITY OF MC3T3-E1 CELLS

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ABSTRACT

This study aimed to investigate the effect of the *Aquilaria crassna* extract (AE) on osteogenic activity including cell viability, cell proliferation, cell attachment and osteogenic differentiation of osteoblast like cells (MC3T3-E1) and to further evaluate the effect of AE on the cell proliferation and cell attachment when applied on modified titanium (Ti) surface. These were evaluated the cell viability, cell proliferation and cell attachment by MTT assays. While the methods of ALP staining and activity kits, quantitative real-time PCR of osteogenic gene expression, ELISA kit for osteocalcin product and Alizarin Red-S staining were performed to evaluate the effect of the AE on osteogenic differentiation. AE were applied on modified Ti surface by dipping method. Then, these were evaluated the surface properties (surface roughness, surface morphology and contact angle) and the AE release characteristics. After that, these were evaluated the cell proliferation and cell attachment by MTT assays. The results showed that the concentration of AE at 10, 25 and 50 µg/ml had no cytotoxicity. The AE (50 µg/ml) effectively enhanced cell proliferation at 24 h, increased cell attachment and promoted osteogenic differentiation by increasing an ALP activity, an expression of osteogenic gene markers (Col 1, ALP, BSP and OCN), a protein product of osteocalcin and a mineral deposition. There were no significant differences on surface roughness and contact angle values among acid etched Ti and acid etched Ti with applied AE by dipping method. The AE release characteristics were consistently highest concentration within the first 24 h. Dipped AE on Ti surfaces significantly enhanced cell proliferation and increased cell attachment. In conclusion, the data presented in this study showed a potential of AE to improve initial cell attachment and proliferation, and to stimulate osteogenic differentiation in MC3T3-E1 cells. Furthermore, dipped AE on Ti surfaces is the simple and effective method to enhance initial cell proliferation and cell attachment on Ti surfaces. Therefore, AE are a promising anabolic agent for bone regeneration and osteointegration.

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ABBREVIATION

AFM	=	Atomic force microscopy
ALP	=	Alkaline phosphatase
ASTM	=	American Society for Testing and Materials
BCA	=	Bicinchoninic acid
BMP	=	Bone morphogenetic protein
BSP	=	Bone sialoprotein
cDNA	=	Complementary deoxyribonucleic acid
CaP	=	Calcium phosphate
Col1	=	Collagen type I
CpTi	=	Commercially pure titanium
CO_2	=	Carbon dioxide
dNTP	=	Deoxyribonucleotide triphosphate
DTT	=	Di-thio-threitol
DMEM	=	Dulbecco's modified Eagle's medium
DMP1	=	Dentine matrix protein 1
DMSO	=	Dimethyl sulfoxide
ECM	=	Extracellular matrix
EDTA	=	Ethylene diamine tetraacetic acid
FBS	=	Fetal bovine serum
HA	=	Hydroxyapatite
HCl	=	Hydrochloric acid
HF	=	Hydrofluoric acid
H_2SO_4	=	Sulfuric acid
HNO_3	=	Nitric acid
ELISA	=	Enzyme linked immunosorbent assay
IGF	=	Insulin-like growth factor
M-CSF	=	Macrophage colony-stimulating factor
MSC	=	Mesenchymal stem cell
MTT	=	Methyl thiazolyl tetrazolium

ABBREVIATION (CONT.)

OCN	=	Osteocalcin
OD	=	Optical density!
OPG	=	Osteoprotegerin
OP-1	=	Osteogenic protein-1
Osx	=	Osterix
PBS	=	Phosphate buffer saline
PCR	=	Polymerease chain reaction
PDGF	=	Platelet-derived growth factor
pNPP	=	p- Nitrophenol phosphate
Ra	=	Roughness average
RNA	=	Ribonucleic acid
RT	=	Reverse transcriptases
Runx2	=	Runt-related transcription factors 2
SEM	=	Scanning electron microscopy
TGF-β	=	Transforming growth factor-β
Ti	=	Titanium
Ti-6Al-4V	=	Titanium alloy
Wnt	=	Wingless
α-MEM	=	Alpha-minimal essential medium

CHAPTER I

INTRODUCTION

Rationale for the study

The two major causes of oral bone loss are periodontitis and residual ridge resorption. Progressive periodontitis results in continued alveolar bone loss and residual ridge resorption occurs after tooth extraction. In severe alveolar bone loss cases could result in tooth mobility and ultimately tooth loss. Furthermore, teeth replacement in these cases may be difficult to treat because of instability prosthesis or limited bone support for dental implant placement (1).

Currently, several regenerative procedures had been introduced to reconstitute alveolar bone loss such as guided tissue regeneration, bone grafts, growth factors and tissue engineering technologies. However, there is still no ideal regenerative procedures approach to achieve predictable and optimal bone regeneration (2).

For bone renewal, osteoclast and osteoblast two major responsible cell types of a process of bone remodeling. The two principle strategies are inhibition of osteoclast activity and stimulation of osteoblast function (3). The one current method, using anti-resorptive agents inhibit osteoclast activity such as bisphosphonates (4). However, they still have some the adverse effects for example osteonecrosis of the jaw (5). Anabolic agents are considered as beneficial agents, which stimulate osteoblast activity and enhance bone formation. The current wildly anabolic agents, bone morphogenic proteins (BMPs) have been used in alveolar bone reconstruction or improving osseointegration of dental implant (6, 7, 8). Several studies reported that BMPs have some complications including severe gingival swelling and may associated with high cancer risk (9, 10). Moreover, the recombinant human BMPs for clinical using are still quite complex, costly and time consuming to produce (11). Therefore, it is a great need to discover novel anabolic agents for bone regeneration.

Recently, natural plants used in traditional medicine have been accepted as one of the main sources of drug discovery and development due to fewer side effects compared with those of synthetic compounds (12). For traditional medicines, some

natural plants have been used as an alternative drugs for bone diseases such as arthritis, gout and bone fracture. *Eurycoma longifolia* and *Labisia pumila* have been used as traditional medicines in Southeast Asian for bone fracture and osteoporosis treatment (13). Some natural plant extracts have been confirmed to have effect on osteogenic activity including *Rhizoma drynariae* and *Euodia sutchuenensis Dode* extract that enhanced the proliferation and osteoblast differentiation in vitro studies (14, 15). Thus, natural plant extract may be the good alternative choices of anabolic agents due to low adverse effects, obtainable, low cost and contain effective compounds.

Aquilaria crassna Pierre ex Lecomte or agarwood, the heartwood of tropical tree, belongs to the family Thymelaeaceae and class Magnoliosida. It can be found in many countries including Thailand, Indonesia and Malaysia. It has been used as traditional medicines for bone diseases including arthritis and gout (16). Moreover, *Aquilaria crassna* extract was also reported other effects including anticancer, antioxidative, antibacterial and analgesic activities (17-20). However, there is still no published report describing the effect of the *Aquilaria crassna* extract on osteogenic activity until now.

In missing teeth patients, dental implant treatment becomes the one treatment of choices for replacing or restoring function in teeth. Normally, success rates of dental implant treatment have quite high rate (more than 97%) in patients with good alveolar bone condition. However, the success rate was decreased when placed dental implant in patients with severe alveolar bone loss (21, 22). Because of the important factors for the initial implant stabilization and healing capacities for osseointegration (21). The bone quantity and quality of implantation sites have been affected to success rates of dental implant treatment. According to Friberg et al. (23) implant placement in cases of poor bone quality, the healing time periods were extended more than 50% (8.5 months in the maxilla and 4.5 months in the mandible).

The current strategies for dental implants treatment in patients with compromised bone sites are improving osseointegration by increasing osteoconductive and osteoinductive properties of the dental implants (24). To improve the osteoconductivity, surface modifications have been introduced. The modifying surface of the dental implant aims to change surface topography or surface chemical, which is proper for bone cell living, and promote osseointegration. Previous studies showed that

many methods for surface modifications can improve osseointegration of dental implants such as sand blasting, acid etching, anodizing, plasma spraying and biochemical coating (25).

For more effective implant surface, adding of osteoinductive molecules to the implant surfaces after surface modification will be enhance osteoinductive properties of the implants (26). Osteoinductive molecule can promote the osteoblast differentiation and promote bone formation lead to increase osseointegration of the implants. There are widely used of osteoinductive molecule for improving osseointegration such as peptide sequences (RGD), growth factors (TGF-β, IGF) and osteoinductive proteins (BMPs) (27). Moreover, incase of severe bone loss implant placement necessary combine with bone grafting, adding osteoinductive molecule can improve osseointegration and success rate of the bone grafting treatment (28).

Several studies reported that adding osteoinductive molecule promoted bone healing around the dental implant, significant improved bone apposition and increased osseointegration especially BMPs (6-8). While, some natural plants extract which have potential osteoinductive ability have been applied for dental implant. Previous study reports on the osteogenic effects of *Puerarin* that have potently induced osteogenic differentiation and mineralization in SaOS-2 cells (29). After that, Yang et al. (2012) demonstrated that *Puerarin* loaded titanium surfaces induce osteoblastic differentiation *in vitro* study, which have the potential to enhance the osseointegration (30). However, currently there are not approved *in vivo* study and clinical applications.

Therefore, this study investigated the effect of the *Aquilaria crassna* extract (AE) in various concentrations on cell viability, proliferation, morphology and attachment including osteogenic differentiation of osteoblast like cells (MC3T3-E1). And further, we determined the effect of the *Aquilaria crassna* extract when apply on titanium surface. That *Aquilaria crassna* extract may be a new alternative choice of anabolic agents. Furthermore, when applied *Aquilaria crassna* extract on the implant surface may be improve osseointegration and bone formation around implant sites.

Purpose of the study

1. To evaluate effect of *Aquilaria crassna* crude extract on the cell proliferation, cell attachment and osteogenic differentiation of MC3T3-E1 cells.

2. To evaluate effect of *Aquilaria crassna* crude extract on the cell proliferation and cell attachment of MC3T3-E1 cells on modified titanium surface.

Significant of the study

1. Knowing the effect of the *Aquilaria crassna* extract (AE) in various concentrations on cell viability, proliferation, morphology and attachment including osteogenic differentiation of MC3T3-E1 cells.

2. Knowing the effect of the *Aquilaria crassna* extract on the cell proliferation and cell attachment of MC3T3-E1 cells when applied on modified titanium surface.

3. The result of this study will be evidence base of anabolic agents that *Aquilaria crassna* extract may be a new alternative choice for bone loss treatment.

4. The result of this study will be evidence base of anabolic agents that *Aquilaria crassna* extract may be applied on the implant surface for improve osseointegration and bone formation around implant sites.

Scope of the study

This study was in *vitro* study that evaluated the effect of *Aquilaria crassna* crude extract on the cell proliferation, cell attachment and osteogenic differentiation of MC3T3-E1 cells. The second part, of this study evaluated the effect of *Aquilaria crassna* crude extract on the cell proliferation and cell attachment of MC3T3-E1 cells when applied on modified titanium surface.

Hypothesis

1. The cell proliferation, cell attachment and osteogenic differentiation of MC3T3-E1 cells treated with *Aquilaria crassna* crude extract is not different from the cells treated without *Aquilaria crassna* crude extract.

2. The cell proliferation and cell attachment of MC3T3-E1 cells treated with *Aquilaria crassna* crude extract is not different from the cells treated without *Aquilaria crassna* crude extract when applied on modified titanium surface.

CHAPTER II

REVIEW LITERATURE

Bone biology

Bone is a mineralized connective tissue. The primary function of bone is load bearing and distribution, which enables the body for locomotion, support and protection of soft tissue organs. Moreover, bone plays an important role for calcium and phosphate metabolism and storage (31, 32).

Definitions of bone biological terms

Anabolic agent: a compound which to promote bone formation (33)

Osteogenesis: the formation and development of bone (33)

Osteogenic activity: functioning in osteogenesis, producing bone (33)

Osteoinduction: the process by which osteogenesis is induced (34)

Osteoconduction: bone grows on a surface (34)

Osseointegration: direct contact between living bone and implant (35)

Bone matrix and bone cells

Bone consists mainly of matrix and cells. Bone matrix can be described as a composite biomaterial of inorganic matrix (hydroxyapatite and tricalciumphosphate 50-70%) and organic fiber material (collagen, 20-40%), water (10%) and lipids (5%). The basic bone qualities are the compact or cortical bone and the cancellous bone. Cortical bone is a compact mass of bone matrix which only porosity is a network of narrow nutritive canals. Cancellous bone is very porous. The trabecular spaces are filled with bone marrow. The variability of the bone architecture exists depending on the age, individual and location. Bone exhibits 4 types of cells including osteoblasts, bone lining cells, osteocytes, and osteoclasts. These all cells play a crucial role in bone formation and bone resorption (31, 32).

Osteoblasts

Osteoblasts, which cuboidal shape cells are comprise 4–6% of the total bone. It was located along the bone surface. The cell characteristics are as polarized cells with various secretory vesicles that secrete the osteoid toward the bone matrix. Osteoblasts also have a crucial role in the bone formation process, which the main function is mineralization of the matrix. After mineralization, some of the osteoblasts are inactive form and retained in the bone surface, which called as the bone-lining cells (36-39).

Bone Lining Cells

Bone lining cells are flat shaped cells on the bone surfaces. They have extended processes between adjacent bone lining cells and osteocytes. The function of bone lining cells depends on the bone status such as these cells can be active secretory cell by enlarge size and cuboidal shape. Bone lining cells functions are not clear understood. (40).

Osteocytes

Osteocytes are the most long-lived cells (up to 25 years), which comprise 90–95% of the total bone cells (41, 42). Osteocytes are differentiated from osteoblast. At the end of a bone formation cycle, some of osteoblasts become osteocytes embed into the bone matrix. The morphology of cell will be changed, including the smaller round osteoblast size (43). The cells entrapped within mineralized bone matrix (called lacuna), its cytoplasmic processes cross tiny tunnels. These cytoplasmic processes are connected to other surrounding osteocytes processes by gap junctions for connected to the vascular system for oxygen and nutrients supply (44, 45).

Osteoclasts

Osteoclasts are multinucleated cells formed by the fusion of the monocyte/macrophage family, which originate from mononuclear cells of the hematopoietic stem cell lineage. Osteoclasts are responsible for bone resorption, which attach on the bone surface, secreting acids and lysosomal enzymes for resorpting bone surface to control bone formation and bone mass (46-48).

Extracellular Bone Matrix

The main compositions of bone matrix are inorganic and organic matrix. The inorganic matrix of bone consists mainly of phosphate and calcium however there is also present some others such as fluorite, potassium and zinc. Hydroxyapatite crystals

are main form of calcium and phosphate, that are represented by the chemical formula $Ca_{10}(PO_4)_6(OH)_2$ (31, 49).

The organic matrix compose by collagenous proteins (90%), mainly type I collagen, and noncollagenous proteins. The noncollagenous proteins include proteoglycans, cytokines and growth factors. Major of noncollagenous proteins include osteonectin, osteocalcin, bone sialoprotein and osteopontin. Collagen and noncollagenous matrix proteins become a scaffold for hydroxyapatite before the mineralization process (31).

1. Type I collagen (Col 1)

Type I collagen is a principle extracellular matrix protein in bone. It is a right-handed helical molecule that consists of 3 polypeptide chains. Collagen is also characterized by high content of proline and hydroxyproline (20-21%). A major part of the collagen type I (300 kDa) is synthesized by fibroblasts and osteoblasts. Col 1 is considered that active collagen form play a significant role in the mineralization that are initial sites for mineral compound deposition. Thus, collagen type I synthesis and degradation can be the marker for diagnosis or assessment of osteoblastic differentiation and bone formation (31).

2. Osteocalcin (OCN)

Osteocalcin (bone gamma carboxyglutamic acid containing protein: BGLAP) located in bone and dentin. It is the most abundant noncollagenous protein in bone comprising about 20% of the noncollagenous matrix proteins. Osteocalcin produced principally by odontoblasts and osteoblasts. It is a member of a family of extracellular mineral binding proteins present in the bone. It is a low molecular weight protein of 6 kD, which contains three γ-carboxylglutamic acid residues that bind calcium, and it is vitamin K-dependent. It has been demonstrated that osteocalcin facilitated calcification. However, its physiological role in mineralization is still unclear. Osteocalcin is often used as a marker for the late stage of bone formation (50).

3. Osteopontin

Osteopontin produced by osteoblasts that belongs to the SIBLING protein family. It is a key factor in bone mineralization and resorption. The fuctions was binded with hydroxyapatite in bone. It has calcium binding sites that has a role in attachment of osteoclast and bone resorption (51). Osteopontin expression is regulated by vitamin

D, which increases its secretion. It binds to integrin receptors on the osteoclast by its RGD sequence, activating the phospholipase C pathway in the osteoclast and enhancing intracellular calcium (50).

4. Osteonectin

Osteonectin is a glycoprotein (40 kD), which 4 domains: an calcium binding domains at the amino terminus (domain I), a cysteine-rich (domain II), a hydrophilic region (domain III) and an E-F hand structure at the carboxy terminus region (domain IV). The domains at the amino and carboxy terminus are calcium-binding regions. It is expressed by osteoprogenitor cells, osteoblasts, and newly formed osteocytes. Osteonectin associated in cell attachment and supported bone remodelling and maintenance of bone mass (52). It has been reported that osteonectin promote crystal growth and also enhance the activity of matrix metalloproteinases (50).

5. Bone sialoprotein (BSP)

Bone sialoprotein is the main non-collagenous proteins in bone. BSP has been found about 8% of all non-collagenous proteins in bone (50). The functions of BSP are regulating bone formation, remodelling and repair. Bone sialoprotein bridge to calcium and hydroxyapatite, and acts as a nucleator of the induce hydroxyapatite crystals and promotes osteoblast and osteoclast differentiation. In addition, BSP has been shown to stimulate angiogenesis by mediating endothelial cell attachment and migration (53).

6. Fibronectin

Fibronectin, a unique dimeric glycoprotein, is one of the major ECM components. It is composed of two similar subunits with molecular weights of 250,000. Fibronectin is the earliest bone matrix protein locally synthesized by osteoblast but also synthesized elsewhere of many tissues and brought in by the vascularization. It has been demonstrated that fibronectin is formed in the early phase of osteogenesis and is maintained within mineralized matrix. It is closely related to the mineralization of bone matrix, induction of bone cell migration, differentiation, and the survival of bone cells, although the precise function is not definitive (54, 55).

7. Alkaline phosphatase (ALP)

Alkaline phosphatase is an enzyme produced by osteoblasts. Robison (1952) reported ALP is important role in the mineralization process (56). ALP may be involved in the degradation phosphate esters to provide a local concentration of phosphate or it may remove pyrophosphate to enable mineralization to proceed. Its distribution is before the calcification that may be act as preparative function. ALP indicated that act as an early indicator of cellular activity and differentiation (50).

Osteogenic differentiation

Osteoblasts are derived from mesenchymal stem cells. The MSCs have potential differentiation into other cell types such as myoblasts, haematocytes and possibly even neural cell (57). The commitment of MSC towards the osteoprogenitor lineage requires though the mechanism of bone morphogenetic proteins (BMPs) and members of the Wingless (Wnt) pathways (58, 59).

The osteoblast differentiation process can be divided in to three phases: proliferation, extracellular matrix synthesis and maturation and mineralization. Osteoprogenitor cells from MSCs were differentiated to preosteoblasts, osteoblasts and osteocytes. Bone morphogenetic proteins (BMPs) play crucial roles in directing fate decisions for MCSs. That strongly promotes osteoblast differentiation via the canonical Wnt pathway (59-62).

The expressions of Runt-related transcription factors 2 (Runx2 or Cbfa1) and osterix (Osx) are crucial for osteoblast differentiation (36, 63). Runx2 and Osx, are expressed during process of osteoblast differentiation. Previous reported that Runx2-null mice are devoid of osteoblasts (Figure 1) (59, 64, 65).

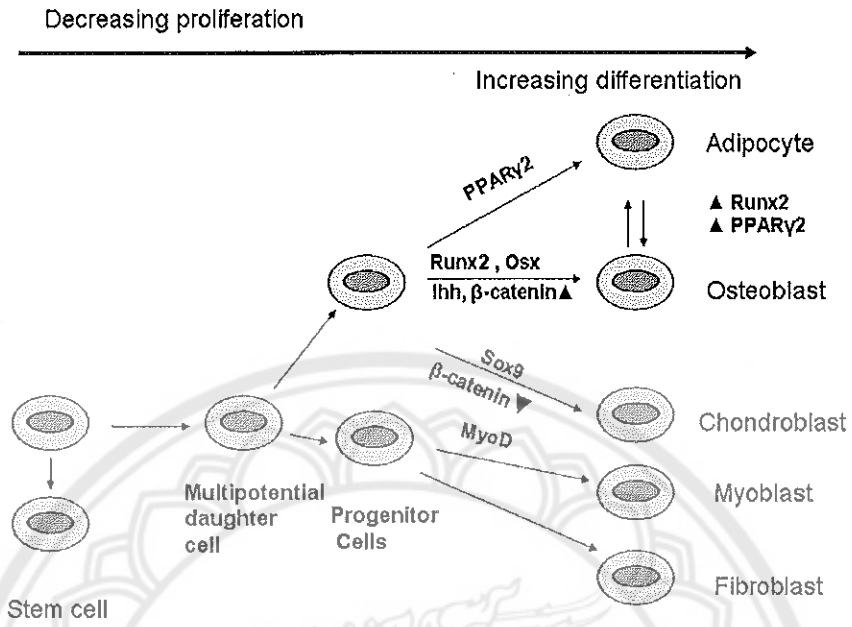

Figure 1 Commitment of mesenchymal stem cells to tissue-specific cell types (65)

After the preosteoblasts differentiated to mature osteoblasts, the osteoblasts synthesized bone matrix by secreting collagen proteins, mainly type I collagen, noncollagen proteins (OCN, BSP, osteonectin and osteopontin), and proteoglycan (decorin and biglycan). Also, the most often osteoblast differentiation key markers are Runx2, Osx, Col 1, osteopontin, BSP and OCN. In proliferation phase, osteoblast progenitors express Runx2 and Col1. Early phase of differentiation, there are expression of ALP, BSP and Col 1, while OCN appears late phase of differentiation, parallel with mineralization (Figure 2 and 3) (59, 61, 65). There are many hormones, growth factors and cytokines regulate the growth and differentiation of osteoblast including PTH, Insulin-like growth factor 1 (IGF-I) and Fibroblast growth factor 8 (FGF-8) (66, 67).

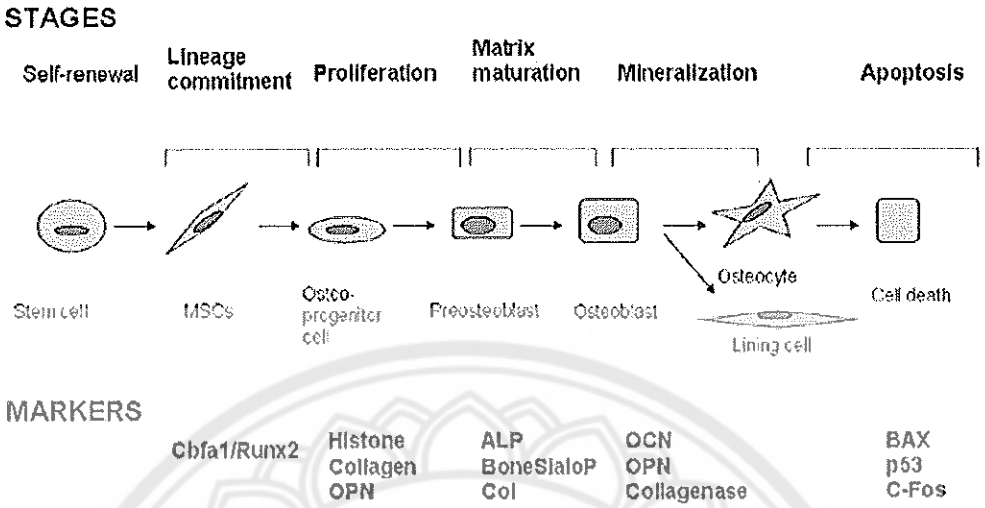

Figure 2 Stages and the key factors of osteogenesis (65)

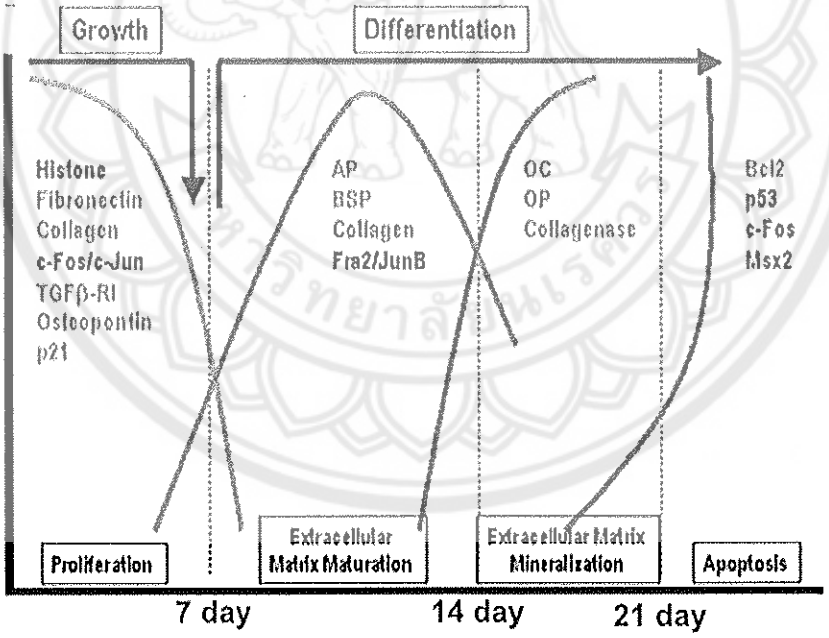

Figure 3 Level of key factors in each stage of osteogenesis (65)

Natural plant extraction

Natural plants extract have rich source of bioactive compounds for example quinine, alkaloids, cocaine, nicotine, digitalis and muscarine. Bioactive molecules contain in plant extraction have many effect activities such as antitumor, antiviral, antibacterial and antifungal activity (68).

Some natural plants extract from *Drynariae Rhizoma* (14), *Fructus psoraleae* (69), *Actaea racemosa* (70) and *Ulmus davidiana planch* (71) exhibited osteogenic activities by promoting osteoblast differentiation and mineralization. Jeong, et al. (14) reported that *Drynariae Rhizoma* extract has osteogenic effects through the promotion of differentiation in MC3T3-E1 cells. The study showed that *Drynariae Rhizoma* extract enhanced ALP activity and mineralization. Moreover, the result showed that the *Drynariae Rhizoma* extract increased mRNA expression of type I collagen, ALP and BMP-2 (181). After that, the studies founded Naringin, main effective component of *Drhizoma drynariae* enhanced the osteoblastic differentiation on MC3T3-E1 cells and human bone mesenchymal stem cells (BMSCs) (143, 182). Other study, Huh, et al. (29) founded the osteogenic effects of Puerarin that have stimulate differentiation gene markers such as ALP, OCN, osteopontin (OPN), Col 1, and mineralization in SaOS-2 cells (35). While as, Muthusami, et al. (138) reported *Cissus quadrangularis* stimulate the proliferation, differentiation, and mineralized depositon of SaOS-2 cells. The result showed that after *Cissus quadrangularis* treatment were increased ALP activities, gene expression of ALP and Col 1. A significant increases in osteocalcin protein and mineralized bone nodule formation after *Cissus quadrangularis* treatment was observed on day 21 (142). Recently, Hwang, et al. (15) reported that *Euodia sutchuenensis Dode* (ESD) extract enhanced osteogenic differentiation by activated the Wnt/β-catenin pathway. ESD extract enhanced β-catenin levels and also enhanced gene expression of RUNX2, BMP2 and Col 1, and increased ALP activity and staining with Alizarin Red S in mouse osteoblasts (15).

Some natural plants extracted which have osteoinductive ability have been applied for dental implant. Previous study reports on the osteogenic effects of *Puerarin* that have potently induced osteogenic differentiation gene markers such as ALP, OCN, OPN, Col I, and mineralization in SaOS-2 cells (29). After that, Yang, et al. (30)

demonstrated that *Puerarin* loaded titanium surfaces promote osteogenic osteoblast differentiation which have the potential to improve osseointegration (30).

Aquilaria crassna Pierre ex Lecomte

Aquilaria crassna Pierre ex Lecomte or agarwood, the heartwood of tropical tree, belongs to the family Thymelaeaceae and class Magnoliosida. It can be found in many countries in Southeast Asia including Thailand, Indonesia and Malaysia. It has been used as traditional medical treatment for bone diseases including arthritis and gout. There are more than 15 species of genus *Aquilaria.* At least 4 species are found in tropical rainforest areas of Thailand, namely *Aquilaria crassna Pierre ex Lecomte, A. subintegra, A. malaccensis, and A. rugosa* (16).

Figure 4 *Aquilaria crassna* tree, fruit and seed (72)

Studies on the chemical constituents of the genus *Aquilaria* started the past few decades. There are more than 133 the compounds that has been isolated and reported in recent years (73). Previous studies reported the main compositions of the crude extract of *Aquilaria crassna* are phenolic compounds (40.8%) followed by flavonoids (15.9%),

triterpenoids (10.5%), alkaloids (9.8%), saponins (4.1%) and tannins (3.1%) (74). Dahham et al. (2014) reported the major phenolic compounds in *Aquilaria crassna* extract are glycosides of flavonoids, benzophenones and xanthones (19, 75). *Aquilaria spp.* extract have been reports the effect on many biological activities including central nervous system (CNS) activity, antimicrobial activity, antitumor activity and antioxidative activity (73, 75, 76).

Biological activities of *Aquilaria crassna* extract

Aquilaria crassna extract was also reported many effect of biological activity including antimicrobial, antitumor, antioxidant, anti-inflammatory, anti-ischemic, antipyretic and analgesic activities.

Antimicrobial activity

Aquilaria crassna heartwood extract have been reported the antibacterial activity that investigated by zone of inhibition against the bacteria test. The results showed higher antibacterial activity against gram-positive bacteria. It was demonstrated against *S. aureus* which the minimum inhibitory concentration (MIC) at 8 μg/ml. While, the result of antifungal activity of *Aquilaria crassna* heartwood extract indicated moderate activity (75).

Wetwitayaklung, et al. (77) reported that *Aquilaria crassna* extracts by water distillation had antimicrobial activities against S. aurues with MIC at 0.5 mg/ml and C. albicans with MIC at 0.5 mg/ml, but were not sensitive to E. coli. Kamonwannasit, et al. (18) also reported the aqueous extract of *Aquilaria crassna* leaves exhibited antibacterial activity and inhibitory effect on biofilm formation of *Staphylococcus epidermidis*.

In addition, Novriyanti, et al. (78) demonstrated the antifungal activity of *Aquilaria crassna* extract by antifungal bioassay against *Fusarium solani* fungi. The result showed that ethanol-soluble extract of *Aquilaria crassna* wood exhibited low class of antifungal activity with 15.2% anti fungal activity (AFA) against *F. solani in vitro*. While, ethyl acetate-soluble extract showed the highest antifungal activity that is categorized as strong class with AFA at 52.5%.

Antitumor activity

The ethanol extract of *Aquilaria* crassna demonstrated potent anti-tumor activity which against cancer cell including pancreatic (PANC-1), prostrate (PC3) and breast (MCF-7) cancer cells with the 50 percent inhibition concentration (IC50) of 30, 72, 119 and 140 μg/ml respectively (74). Other study reported *Aquilaria crassna* extract by hydrodistillation have the effect on anti-colon cancer cells. The anticancer effects of the extract may be from the active components such as β -Caryophyllene (19).

Antioxidative activity

The antioxidant activity *Aquilaria crassna* heartwood was evaluated by the DPPH free radical scavenging assay. The results exhibited significant DPPH free radical scavenging effects which the IC50 value of the extract was 4.25 μg /ml (75).

Sattayasai, et al. (17) was also reported that an anti-oxidative activity of *Aquilaria crassna* leaf extracts was observed with an IC50 value of 47.18 μg /ml by using the DPPH anti-oxidant assay. The results are consistent with Ray, et al. (79) that *Aquilaria crassna* leaf extracts have antioxidative activity by DPPH scavenging assay which IC50 value of the extract was 32.25 μg /ml. That the main antioxidative compounds are mangiferin and genkwanin. Moreover, Tay (2004) reported antioxidant active molecules from *Aquilaria crassna* extract by ethanol are Epigallocatechin Gallate, Epicatechin Gallate and Iriflophenone 3-C-β-Glucoside. (80).

Anti-inflammatory activity

Kumphune, et al. (81) reported that the anti-inflammatory effect AE on lipopolysaccharide (LPS) induced tumour necrosis factor-alpha secretion from isolated human peripheral blood mononuclear cells. The results showed that 1.5 mg/ml ethyl acetate extract of *Aquilaria crassna* was significantly inhibited LPS-induced tumour necrosis factor factor-alpha secretion. Moreover, the mechanisms of anti-inflammation apparently resulted from selectively attenuating the p38 MAPK activation without affecting on the ERK1/2 MAPK activation.

Anti-ischemic activity

Jermsri, et al. (82) reported anti-ischemic activity of AE that 5 mg/ml of AE could reduced simulated ischemia induced cell death in cardiac myoblast cell line (H9c2), as well as isolated adult rat ventricular myocytes (ARVMs) (83).

Suwannasing, et al. (84) also reported that AE has effect on in isolated mouse heart with ischemia/reperfusion, ex vivo study, subjected to ischemia/ reperfusion. The results showed that pre-treatment with 5-mg/ml AE for 30 min prior to global ischemia significantly decreasing infarct volume. In addition, the AE (5-mg/ml) inhibited ischemia by the mechanism of induced p38 MAPK phosphorylation.

Antipyretic and analgesic activity

Sattayasai, et al. (17) reported antipyretic and analgesic of AE leaves extract in rodents. They were treated orally with an aqueous extract of AE leaves and were tested for antipyretic (Baker's yeast-induced fever in rats) and analgesic (hot plate test in mice). The results reported that, after 5 hours of injection (400 and 800 mg/kg AE extract) reduced the rectal temperature of rats.

However, until now, there are no reports about the effect of the *Aquilaria crassna* extract on osteogenic activity.

Dental implant

Currently, dental implant treatment becomes the one treatment of choices for replacing or restoring function in missing teeth patients. Since the success rates of dental implant treatment are quite high rate (more than 97%). A many variety of materials have been used to produce dental implants. An ideal implant material should be biocompatible, with adequate toughness, corrosion, strength and wear resistance. Materials used for dental implants fabrication can be categorized by the chemical composition that can be categorized into 3 groups: metals, ceramics and polymers (Table 1) (85, 86).

Titanium and its alloys are the most commonly used dental implant materials due to the good required properties. The biocompatility of titanium and its surface, are form by a native oxide layer (87, 88). The relationship of the implant with the surrounding tissue is a direct affected on the interaction between the passive titanium oxide (TiO_2) and biological elements such as collagen, osteoblasts, fibroblasts and blood constituents. Since, TiO_2 layer is very stable and corrosion-resistant which influence to good biocompatibility of titanium implant (89).

According to the American Society for Testing and Materials (ASTM), there are categorized 6 types of titanium implant. There are 4 grades of commercially pure titanium (CpTi) and two titanium (Ti) alloys. The mechanical and physical properties are showed in Table 2 (90).

Osseointegration of dental implant

Dental implant was developed and improved in recent years dealing with the replacement of the missing of the natural teeth for restored masticatory function and aesthetic appearance. Due to the effectiveness of the dental implant, biomaterials for implant necessary obtained the formation of a direct bone connection to the surface of the implants without interposition of non-bone tissue. This phenomenon, described as "osseointegration" (91). This concept has been described by Branemark, as "a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant" (92).

Table 1 Materials used for the fabrication of dental implants (85, 86)

Material types	Implant Materials
I. Metals	Titanium (CpTi)
	Titanium Alloys (Ti-6A1-4V)
	Stainless Steel
	Cobalt Chromium Alloy
	Gold Alloys
	Tantalum
II. Ceramics	Alumina
	Hydroxyapatite
	Beta-Tricalcium phosphate
	Carbon-Silicon
	Bioglass
	Zirconia
III. Polymers	Polymethylmethacrylate
	Polytetrafluoroethylene
	Polyethylene
	Polyurethane
	Polyether ether ketone

Influence of surface morphology of titanium implant on osseointegration

The long-term success of dental implants also depends on the osseointegration of the implant materials, which is determined by the responses of bone healing around dental implants. In order for dental implant osseointegration, there must be an adherence of the cells to the surface of the dental implants. The implant surface characteristic is the important factor of dental implant osseointegration. That appearance can stimulate the adsorption of proteins, lipids, sugar, and ions present in the tissue fluids. Then, the cell attached to the surface of dental implants (93, 94). Many studies analyzing the factor influence for success of implant osseointegration, surface morphology is the one of important factor. This factor influences the primary stability, the distribution of forces and mechanical properties of the implant.

Several researchers (95, 96) reported the effect of surface properties of titanium implants on bone apposition into surface. The biological response depends on the surface properties of implants including morphology, roughness, thickness of the oxide layer, impurity level and types of oxides. Previous studies reported that the implant after surface modification affect the interfacial forces, wettability, roughness, energy and adsorption capacity of the molecules those factors are involving implant and osteoblast responses (97, 98). The surface roughness and wettability are the main properties that affect on the protein adsorption and enhance osteoblasts attached on the implant surface (99).

Surface modifications of Ti implants to improve osseointegration

The rationale for the surface modification of implants is in order to achieve the desired biological responses by modifying surface layer to influence the bio-interaction and osseointegration processes which can be controlled at molecular and cellular levels of the implant surface. There are various surface modification methods which can be subdivided into physicochemical and biochemical methods (100).

Physicochemical methods

These methods alter the energy, charge and composition of the existing implant surface resulting in the implant surfaces with modified in surface morphology (especially surface roughness), surface energy surface charge and surface chemical.

Many studies reported that there are many factors of surface implant characteristics which influent to implant osseointegration. Previous studies reported roughness, surface energy, surface charge and inorganic composition of the implant surface have affect cell attachment and spreading of bone cell (101).

Surface treatment with acid

Implant surface treatment with acid is one of the most widely used methods. In general, acid treatment has performed by immersing the implants into acid solutions such as HCl, H_2SO_4, HF and HNO_3. Acid etching produces micro pits on titanium surfaces with sizes ranging from 0.5 to 2 μm in diameter (102). Acid etching has been shown to greatly enhance osseointegration (103). Previous studies found that acid etched surfaces increase the attachment of osteogenic cells, resulting in bone formation directly on the surface of the implant. It has been indicated that implants treated by acid etching have a optimal topography able to promote the cell adhesion, and thus to promote bone formation (104). Several studies have reported higher BIC value of acid etched surfaces compared to machined surfaces (104, 105). Acid etched surface provide homogeneous roughness, increased active surface area and increase wettability of the surface that hydrophilic surfaces greatly promote osseointegration and increase the torque (106). The acid etched surface morphology are varies with the treatment conditions depend on many factors including acid types, acid concentration, etching time and temperature treatment (107).

Previous study reported that etching with H_2SO_4 produced a rougher titanium surface than in HCl, H_3PO_4, HF, or HNO_3. It was also demonstrated that the increasing surface roughness of titanium surface by increasing acid temperature and etching time. Moreover, etching with H_2SO_4 was found to be a simple and effective surface modification method (108). Iwaya, et al. (109) evaluated surface roughness and the biological responses of osteoblast-like cells (MC3T3-E1) of the different treatment surface including polishing, sandblasting, etching in 48% H_2SO_4 and etching in 48% H_2SO_4 with vacuum firing. The result demonstrated that the surface roughness of titanium after etching in 48% H_2SO_4 higher roughness values than polishing and sandblasting treatment. Osteoblast-like cells attached, spread, and proliferated were no significant difference with 4 type different surface treatments. This study suggests that

etching with 48% H_2SO_4 was an effective way to roughen the surface of titanium with good biocompatibility.

Biochemical methods

The goal of biochemical methods is to stabilized peptides, proteins and enzymes on the surface of implant to induce bone cells (adhesion, signaling and stimulation) and to improve osteointegration. Several growth and differentiation factors have been used coating on the surface implants to stimulate and enhance the bone ingrowth. Some of bone morphogenetic proteins (BMP-2, BMP-7 and OP-1), growth factors such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and transforming growth factor-beta 1 (TGF-β 1) have been used coated implants (26).

The most promising anabolic agents are the members of the transforming growth factor-β (TGF-β) superfamily, such as bone morphogenic proteins (BMPs). The previous reported that the applications of BMPs have been used to improve the implant osseointegration (110, 111). Moreover, BMPs could be used for alveolar ridge augmentation before implant placement. BMP-2 is a member of the TGF-β superfamily of multifunctional cytokines. It is a homodimer of two subunits, each consisting of 114 peptides (110, 112). BMP-2 exhibits high osteoinductive properties that stimulate differentiation into osteoblasts. Previous studies reported that coating BMP-2 on implants surface promote cell proliferation and increasing the osseointegration. The main effect of BMPs is the stimulation of bone growth through an enhancing in cell differentiation (113). However, several studies reported that BMPs have some complications including severe gingival swelling and may associated with high cancer risk (9, 10). Moreover, the recombinant human BMPs for clinical using are still quite complex, costly and time consuming to produce (11).

Therefore, using of natural plants extracted for dental implant application need to discover and approve the anabolic efficiency. That may be the novel alternative choice of anabolic agents. However, currently using of natural plants extract to improve the osseointegration still has been limited evidence base and not approved *in vivo* study and clinical applications.

CHAPTER III

RESEARCH METHODOLOGY

Samples

1. Osteoblast cell line (MC3T3-E1)
2. Fibroblast cell line (L929)
3. Titanium disks (Cp titanium grade 2: Tdental Lab, Thailand)

Research instrument

1. Microplate reader (XMARK®, USA)
2. Nanodrop spectrophotometer (Nanodrop®, USA)
3. Roche Light cycler 480 real time PCR system machine (Roche®, USA)
4. Atomic Force Microscope (AFM) (NanoSurf®, USA)
5. Scanning electron microscopy (SEM) (Leo1455VP®, USA)
6. Optical contact angle measuring device (20LHT®, Germany)
7. Bright field optical microscope (Olympus®, Japan)
8. Centrifuge (Hettich®, USA)
9. Laminar airflow cabinet (ESCO®, USA)
10. CO_2 Incubator (Forma®, USA)
11. Micropipette (Gilson®, USA)
12. Eppendorf tube (Eppendorf®, USA)
13. Cuture plate (Nunc®, USA)
14. Pipette tip
15. Beaker

Research materials and chemical agents

1. *Aquilaria crassna* extraction
2. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco®, USA)
3. Alpha-minimal essential medium (α-MEM) (Gibco®, USA)
4. Fetal bovine serum (FBS) (Gibco®, USA)
5. Penicillin and streptomycin solution (Gibco®, USA)
6. Trypsin/EDTA solution (Gibco®, USA)
7. Methyl thiazolyl tetrazolium (MTT) (USB®, USA)

8. Dimethyl sulfoxide (DMSO) (Sigma®, USA)
9. L-glutamine (Gibco®, USA)
10. Ascorbic acid (Sigma®, USA)
11. Dexamethasone (Sigma®, USA)
12. ALP activity colorimetric assay kit (K412-500, Biovision®, USA)
13. Bicinchoninic Acid (BCA) protein assay kit (Pierce®, USA)
14. RNA extraction kit (NucleoSpin®, Germany)
15. Reverse transcriptase enzyme kit (iScript®, USA)
16. LightCycler 480 SYBR Green I Master mix (Roche Diagnostics®, USA)
17. Power SYBR green Master mix (ABI systems®, USA)
18. Protease inhibitors (Sigma®, USA)
19. Sodium dodecyl sulfate (SDS, Sigma®, USA)
20. Nitrocellulose membranes (BioTrace®, USA)
21. Non-fat milk (LabScientific®, USA)
22. Chemiluminescence kit (Pierce®, USA)
23. Alizarin Red-S solution (Sigma®, USA)
24. Cetylpyridinium chloride monohydrate (Sigma®, USA)
25. Folin and Ciocalteu's phenol reagent (Sigma®, USA)
26. Phosphate buffered saline (PBS) (Sigma®, USA)
27. Absolute alcohol
28. Deionized water
29. Normal saline solution

Research Methods

***Aquilaria crassna* extraction**

Aquilaria crassna Pierre ex Lecomte used in this study was obtained from Mr. Choosak Rerngrattanabhume. The plant was originally cultivated at the area in Pong Nam Ron district, Chantaburi province, Thailand. Subsequently identified by Dr. Pranee Nangngam, Department of Biology, Faculty of science, Naresuan University. The specimen voucher number 002540 was kept at Department of Biology herbarium, Faculty of Science, Naresuan University.

Briefly *Aquilaria crassna* extracted process, the heartwood was sliced into small pieces. After that, the dried plant (1kg) was extracted with ethyl acetate (800 ml reflux) for 2 days. The resulting ethyl acetate solution was concentrated under reduced pressure to yield Ethyl acetate extract (950mg). The ethyl acetate extract of *Aquilaria crassna* was dissolve in DMSO for stock solution at 1g/ml and stored at 4 °C. The ethyl acetate extraction of *Aquilaria crassna* was dissolved with serum free media for various concentrations before using in experiments (81).

Part 1 To evaluate effect of *Aquilaria crassna* crude extract on cell proliferation, cell attachment and osteogenic differentiation of MC3T3-E1 cells

1. Cell culture

L929 cells, a mouse fibroblast-like cell line, and MC3T3-E1 cells, a mouse osteoblast-like cell line were used in this study. L929 cells were maintained in DMEM (157). While, MC3T3-E1 cells were maintained in alpha-MEM (158). The medium were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml amphotericin B. The cells were maintained in humidified atmosphere and 5% CO2, at 37 °C. The medium was changed every 2 days.

2. Evaluation of cell viability and proliferation

Cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (followed ISO 10993-5 In vitro cytotoxicity test protocol). L292 cells (50,000 cells) were seeded on culture plates (n=3 for each sample) in serum free medium with added AE for different concentration including 10, 25, 50, 100, 500 and 1,000 µg/ml and without AE as control group. The cells were cultured for 24 h. After that, the cells were incubated with 0.5 mg/ml MTT at 37 °C for 30 min. Then the MTT solution was removed and dissolved the formazan crystals by DMSO. After 10 min, each sample was determined the optical density by a microplate reader at 570 nm (114).

For the cell proliferation evaluation, MC3T3-E1cells (50,000 cells) were seeded on culture plate (n=3 for each sample). The cells were culture medium, which treated with AE at 10, 25, 50 µg/ml concentration and without AE were used as control. The cells were cultured for 24, 48 and 72 h. At the specified time-points, the cells were determined the proliferation by MTT assay based on the above instructions.

3. Evaluation of cell attachment

Cell attachment was measured using a standard MTT assay (n=3 for each sample). MC3T3-E1cells (50,000 cells) were cultured in a culture plate in standard culture medium for 18 h. After that, the cells were change to culture in serum free medium for 6 h. Then, *Aquilaria crassna* crude extract was added in culture medium for 3 different concentration groups (10 µg/ml, 25 µg/ml, 50 µg/ml) and control group (with out AE). The cells were cultured for 4 and 24 h (115). At the specified time-points, the cells were incubated with 0.5 mg/ml MTT at 37 °C for 30 min. Then the MTT solution was removed and dissolved the formazan crystals by DMSO. After 10 min, the optical density was determined by a microplate reader at 570 nm (114).

The morphology of attached cells was evaluated by SEM. At 4 and 24 h time-points (115), the samples were washed with PBS (pH 7.4) to remove non-adherent cells. Then, the samples were fixed in 4% paraformaldehyde for 1 h. After that, the sample was sequential dehydration in an ethanol series (30%, 50%, 70%, 90%, 95% and 100%) for 5 minutes in each concentration. Then, the sample was coated with gold and the morphology of the attached cells was evaluated using SEM (115).

4. Evaluation of osteogenic differentiation

MC3T3-E1 cells were cultured in culture medium with AE (10, 25 and 50 µg/ml) and without of AE for 3 days. After that, the culture medium was changed to osteogenic medium (α-MEM medium supplemented 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/mL amphotericin B, ascorbic acid (50 µg/ml), dexamethasone (100 nM) and sodium phosphate (2 mM). AE were added in osteogenic medium as the same concentration of each group, which added in culture medium. The cells were cultured in osteogenic medium for 7, 14 and 21 days. The medium were changed every 48 h. At the specified time-points, ALP activity, osteogenic genes expression and mineral deposition were evaluated using methods described below.

4.1 Alkaline phosphatase activity

MC3T3-E1 cells (50,000 cells) were seeded on a culture plate (n=3 for each sample). The cells were cultured in culture media with AE (10, 25 and 50 µg/ml) and without of AE for 3 days. Then, the culture medium was changed to osteogenic medium for 7, 14 and 21 days. At the specified time-points, the ALP activity was

determined by colorimetric assay kit (K412–500, Biovision®). In brief, the cells were lysed in ALP assay buffer. Next, the samples were incubated with p-nitrophenol phosphate (pNPP) at 25°C for 60 min. Then the stop solution was added. The absorbance was determined at 405 nm by using a microplate reader. The ALP activity was calculated using standard curve and further normalized with total cellular protein concentration, which was measured by BCA protein assay kit (Pierce®). For the ALP staining assay, the cells were stained using the TRACP and ALP Double-Stain kit (Takara®) according to the manufacturer's instructions. Images were visualized with a bright field optical microscope (114).

4.2 Quantitative real-time polymerase chain reaction analysis (qRT–PCR)

MC3T3-E1 cells were seeded at density 150,000 cells/well on 6-well-plate (n=3 for each sample). The cells were cultured in culture media with AE (10, 25 and 50 µg/ml) and without of AE for 3 days. Then, the culture medium was changed to osteogenic medium for 7, 14 and 21 days. At the specified time-points, the osteogenic gene markers including Col 1, ALP, BSP and OCN were evaluated by qRT–PCR analysis. Briefly, total RNA from the cells of each group was extracted using NucleoSpin® RNA kit according to the manufacturer's instructions. The extracted RNA quantity and quality were assessed using Nanodrop® spectrophotometer. One microgram of each RNA sample was converted to cDNA by iScript® Reaction kit following the manufacturer's instructions.

The qRT–PCR reactions was performed. A 20 µl reaction mixture, each consisting of samples of cDNA, specific primer mix and LightCycler 480 SYBR Green I Master mix® were setup in each well of a reaction well plate. The plate was sealed using optical adhesive cover and was placed in Roche Light cycler 480 real time PCR system machine. The cycle conditions were set up as detailed: 50 °C for 2 min initial heating, 95 °C for 1 min, 40 cycles of 95 °C for 30 s followed by 60 °C for 30 s with 72 °C elongation for 30 s each. The reactions were run in triplicate and the results were averaged. Forward and reverse primers specific for genes are showed in table 3. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogeneous control for calculating fold differences in RNA levels of cells by the $2^{-\Delta\Delta CT}$ method (116).

4.3 Osteocalcin product evaluation by ELISA assay

MC3T3-E1 cells were seeded at density 150,000 cells/well on 6-well-plate (n=3 for each sample). The cells were cultured in culture media with AE (10, 25 and 50 µg/ml) and without of AE for 3 days. Then, the culture medium was changed to osteogenic medium for 21 days. At the specified time-points, the OCN protein was evaluated by ELISA analysis. Briefly, the cellular protein was extracted using RIPA buffer (Sigma, USA) (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris; pH8.0). Total protein concentration was measured by BCA protein assay kit. For the ELISA assay, the extracted protein was determined OCN protein using the Mouse Osteocalcin ELISA kit (Abbexa®) according to the manufacturer's instructions. The OCN protein was calculated using standard curve and normalized with total cellular protein concentration.

Table 2 Primer sequences used for quantitative real-time PCR (116)

Genes	Forward primer 5'-3'	Reverse primer 5'-3'	Product length (bp)
Col 1	CTCCTGACGCATGGCCAAGAA	TCAAGCATACCTCGGGTTTCCA	100
ALP	ACCCGGCTGGAGATGGACAAAT	TTCACGCCACACAAGTAGGCA	113
OCN	AGCAGGAGGGCAATAAGGTAGT	TCGTCACAAGCAGGGTTAAGC	118
BSP	ACCGGCCACGCTACTTTCTTTA	GGAACTATCGCCGTCTCCATTT	113
GAPDH (control)	AGCGAGACCCCACTAACATCA	CTTTTGGCTCCACCCTTCAAGT	118

4.4 Mineral deposition by alizarin red-s staining

MC3T3-E1 cells (50,000 cells) were seeded on culture plate (n=3 for each sample). The cells were cultured in culture media with AE (10, 25 and 50 µg/ml) and without of AE for 3 days. Then, the culture medium was changed to osteogenic medium for 7, 14 and 21 days. At the specified time-points, the calcium deposition was determined by Alizarin Red-S staining. Briefly, the cells were fixed with cold methanol for 10 min. Then the cells were washed with deionized water and immersed in 1% Alizarin Red-S solution in a mixture of 0.4 mL ammonium hydroxide/40 mL water

(pH = 4.2), for 3 min. Then, the cells were destained by 10% cetylpyridinium chloride monohydrate in 10 mM sodium phosphate at room temperature for 15 min. The optical density was measured at 570 nm by a microplate reader (114).

Part 2 To evaluate effect of *Aquilaria crassna* crude extract on cell proliferation and cell attachment of MC3T3-E1 cell on modified titanium surface

1. Titanium disc preparation and surface treatment

The titanium disks (10 mm in diameter) were cut from a commercial pure titanium rod (grade 2) with 1 mm thickness. Ti disks were polished with silicon carbide sandpaper No.280, 360, 400, 600, 800, and 1000 grits in series and then washed with acetone, absolute alcohol and deionized water in an ultrasonic cleaner, respectively, for 15 min each. Next, the specimens were dried at room temperature for 1 h (117). After that, the titanium disks were treated with acid etched surface modification following the previously reported procedures (109). In brief, the titanium disks were etched with 48% H_2SO_4 at 60°C for 60 min and then cleaned in deionized water for 15 min by an ultrasonic cleaner. All the specimens were dried in the air at room temperature for 24 h. Finally the specimens were sterilized by UV exposure for 30 min in a chamber.

2. Preparation of loading AE on titanium surfaces by dipping technique

For loading of AE onto the titanium surfaces, the samples were prepared by dipping technique (118). The acid etched Ti specimens were immersed into AE solutions with 50 µg/ml concentration for 24 h that the concentration had highest potential for osteoblast differentiation from the results of part 1. After that, the dipped AE Ti specimens also were investigated the surface properties including surface roughness, surface morphology and contact angle (115) by compared with acid etched Ti specimens (without AE) and polished Ti specimens (without AE) as control group (n=3 for each sample).

3. Surface analysis

3.1 Atomic force microscopy (AFM)

The titanium specimens of all groups were evaluated surface roughness by the atomic force microscope with 50 x 50 μm^2 scanning size.

3.2 Scanning electron microscopy (SEM)

The titanium specimens of all groups were sputtered with a thin layer of gold and observed by a scanning electron microscopy. The morphology of specimens was imaged at magnifications of 2500x and 10,000x.

3.3 Contact angle measurement

The titanium specimens of all groups were examined contact angle by an optical contact angle measuring device using 1 µl deionized water at 25°C and 45% humidity. Contact angle was measured with the profiles of droplets deposited on the Ti surfaces and calculated by software.

4. Release characteristic evaluation of *Aquilaria crassna crude* extract from modified titanium surface

For release characteristic evaluation, the acid etched Ti specimens were immersed into 50 µg/ml AE solutions for 24 h (118). After that, these specimens were immersed in 1 ml of PBS (pH 7.4) for 30 min, 1 h, 4 h, 6 h, 12 h, 1 day, 3 days and 7 days (n=3 for each timepoint). At the specified time points, AE concentration that release from Ti specimens were determined by detecting the present of total phenolic content (the major composition) (74) using colorimetric reactions of Folin-Ciocalteau assay (119). In brief, 100 µl of PBS were collected and mixed with 400 µl of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 400 µl of sodium carbonate solution (7.5%, w/v). Then, the specimens were incubated for 30 min at room temperature. The absorbance of specimens (blue color) was measured at 765 nm using a microplate reader. The release ratio was calculated by using the linear equation of a standard curve of *Aquilaria crassna crude extract* concentration, which prepared by Folin-Ciocalteu reagent (concentration range 1– 100 µg/ml) (120).

5. Cell proliferation evaluation on titanium

Cell proliferation was measured using a standard MTT assay. MC3T3-E1 cells (50,000 cells) were seeding on Ti samples in 24-well plates with 5 different groups (n=3 for each sample) including,

Dipped AE acid etched Ti group

Acid etched Ti treated AE (50 µg/ml) in culture medium group

Acid etched Ti (without AE) group

Polished Ti (without AE) group

Glass surface (without AE) group (as control).

Before added culture medium into each well, the cells were allowed to initially attach for 45 min. The cells were cultured for 24, 48 and 72 h (115). At the specified time-points, the cells were measured cell proliferation by MTT assay following the protocol describe in part 1.

6. Cell attachment and morphology evaluation on titanium

Cell attachment was measured using a MTT assay at 4 and 24 h (115). MC3T3-E1 cells (50,000 cells) were seeding on Ti samples in 24-well plates with 5 different groups (n=3 for each sample) in the same groups of evaluated cell proliferation as describe above. After, the cells were allowed to initially attach for 45 min and added culture medium into each well. The cells were cultured for 4 and 24 h (115). At the specified time points, the samples were rinsed with PBS (pH 7.4) to remove non-adherent cells. Then, the cells were measured cell attachment by MTT assay and evaluated cell morphology by SEM following the protocol describe in part 1.

Analysis of Data

Mean with standard deviation (SD) calculated and analyzed with SPSS software program. The normality and homogeneity of variance of the data were checked by using Kolmogorov–Smirnov and Levene's test. The differences between experimental groups were analyzed using ANOVA and followed by multiple comparison tests. The differences are assumed to be significant when $p<0.05$.

Experimental work flow part 1

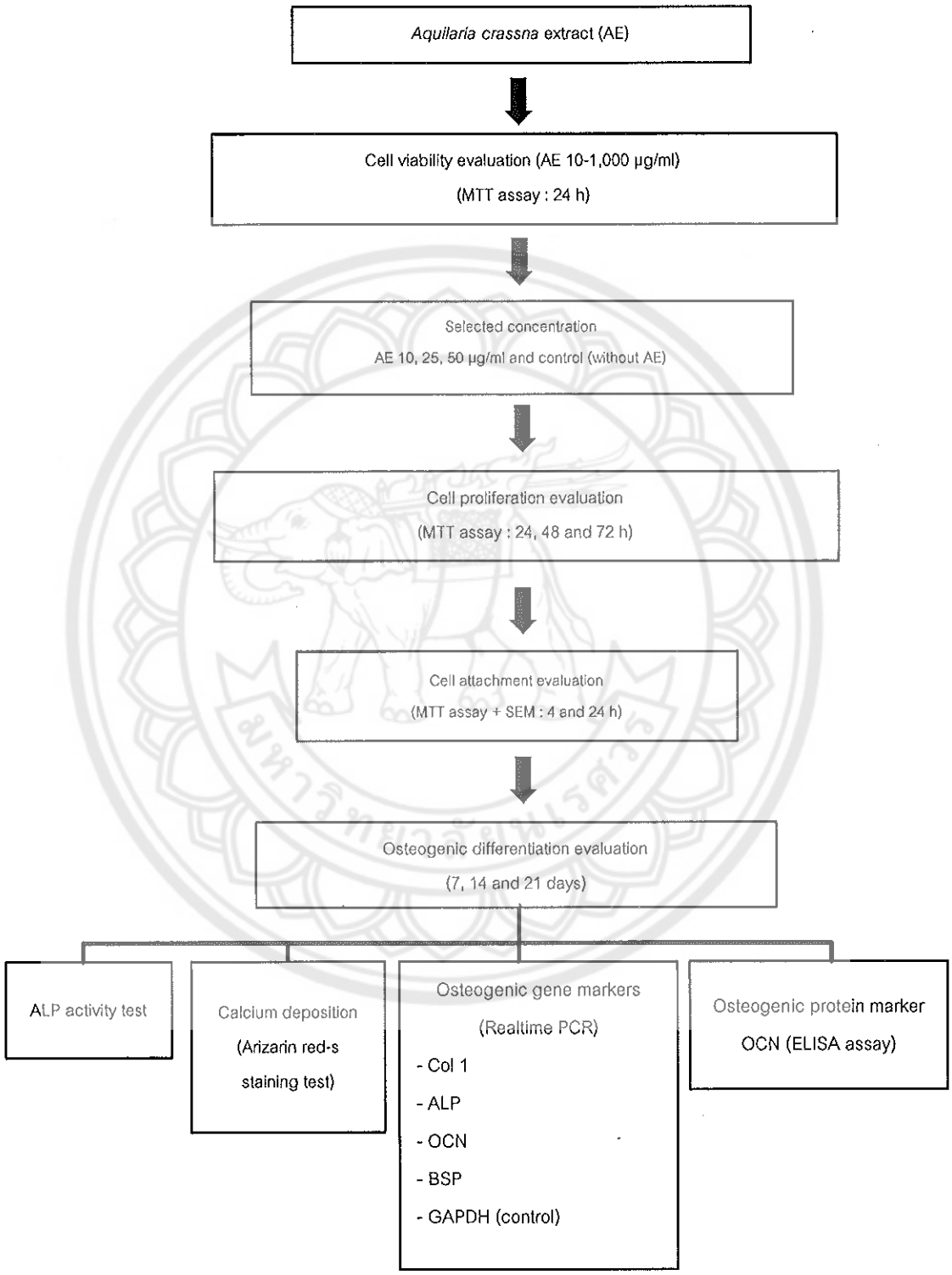

Figure 5 Experimental work flow part 1

Experimental work flow part 2

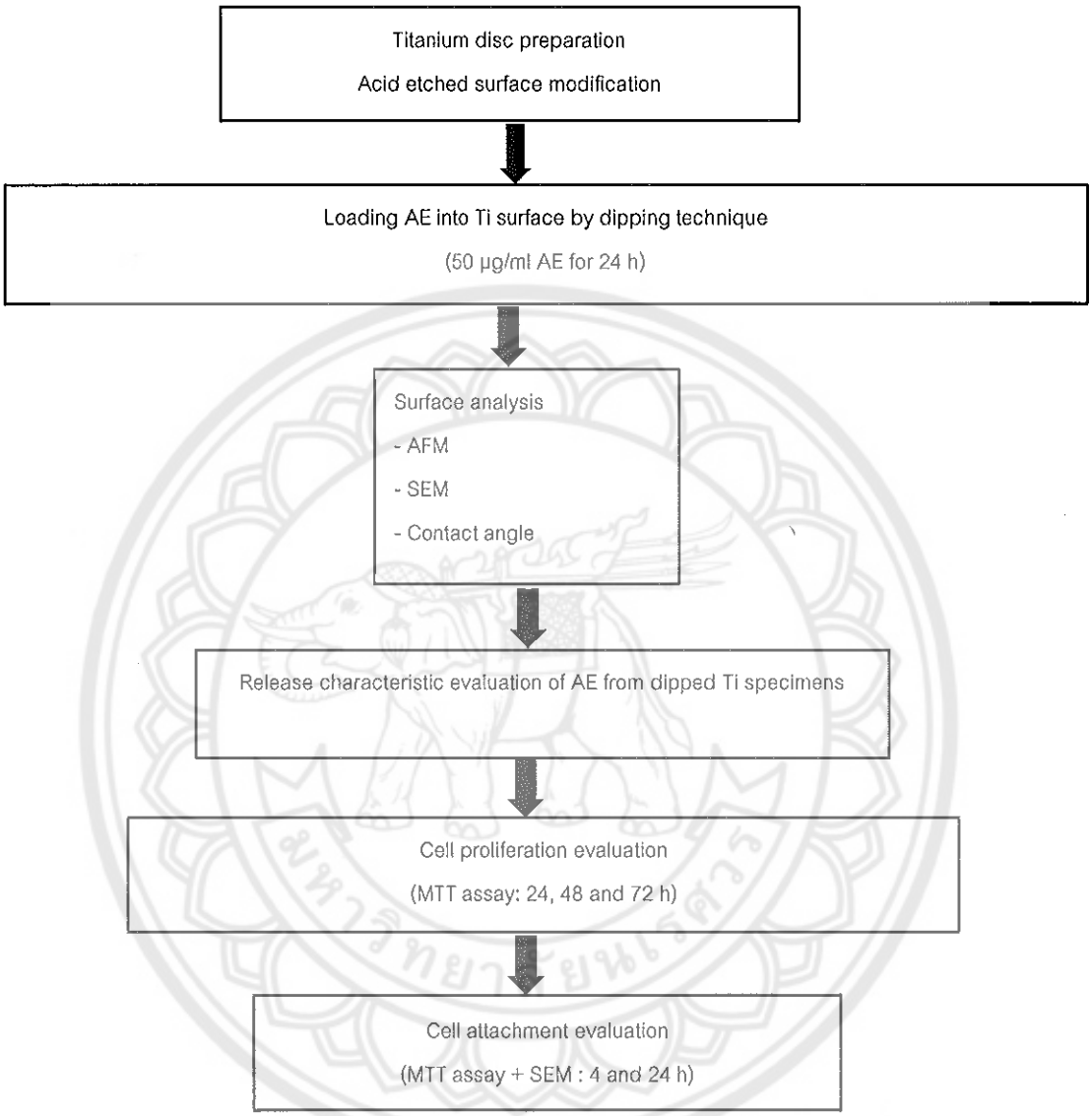

Figure 6 Experimental work flow part 2

CHAPTER IV

RESULTS

Part 1 To evaluate effect of *Aquilaria crassna* crude extract on cell proliferation, cell attachment and osteogenic differentiation of MC3T3-E1 cells

1. Cell viability and proliferation

To determine cell viability and the optimal concentration of AE, a dose-response experiment on L929 cells was performed by using MTT assay followed ISO 10993-5 In vitro cytotoxicity test protocol. The range of AE concentrations for investigation in this study were conducted using 10-1,000 µg/ml. After L929 cells were treated with varied concentrations of AE for 24 h, the cell viability results showed that there was no toxic effect on cells when treated with AE concentrations less than 50 µg/ml. On the other hand, treated with AE concentrations above 100 µg/ml, the cell viability was decrease less than 50 % when compared to control (Figure 7). It was apparent that 50 µg/ml of AE concentration was the highest concentration which had no toxicity. Therefore, the selected AE concentrations were 10, 25 and 50 µg/ml for subsequent experiments.

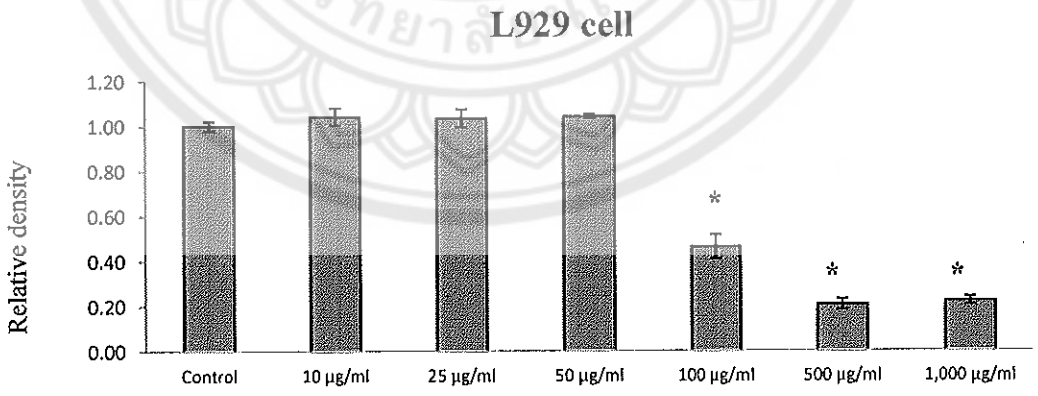

Figure 7 Dose-response effect of AE (10-1,000 µg/ml) on L929 cell viability, measured for 24 h by MTT assay. The AE over than 50 µg/ml were significantly decrease cell viability (*: $p<0.05$)

To investigate cell proliferation, MC3T3-E1cells was performed by using MTT assay. The cells were treated with AE at 10, 25 and 50 µg/ml concentrations for 24, 48 and 72 h. The results showed that the relative density of cells treated with 50 µg/ml AE concentration was statistically significant higher than those of other AE concentrations at 24 h. However, the proliferation rate was no statistically significant difference comparing with different concentrations of AE after treated for 48 and 72 h. (Figure 8).

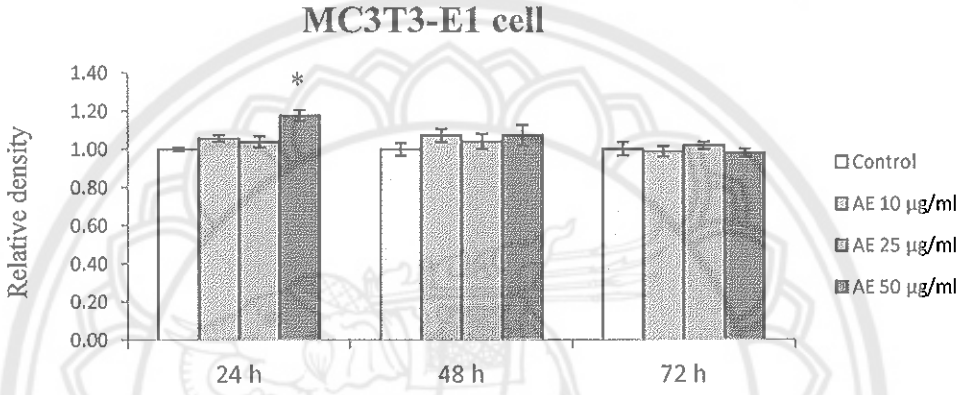

Figure 8 Effect of AE (10, 25 and 50 µg/ml) on MC3T3-E1 cell proliferation was determined by MTT assay (24, 48 and 72 h). Cell proliferation was significantly enhanced only when treated with 50 µg/ml of AE at 24 h time point (*: $p<0.05$)

2. Evaluation of cell attachment

The results showed that cells attachment was significant enhanced when treated with 50 µg/ml AE group at both 4 and 24 h time points compared to the control group (Figure 9).

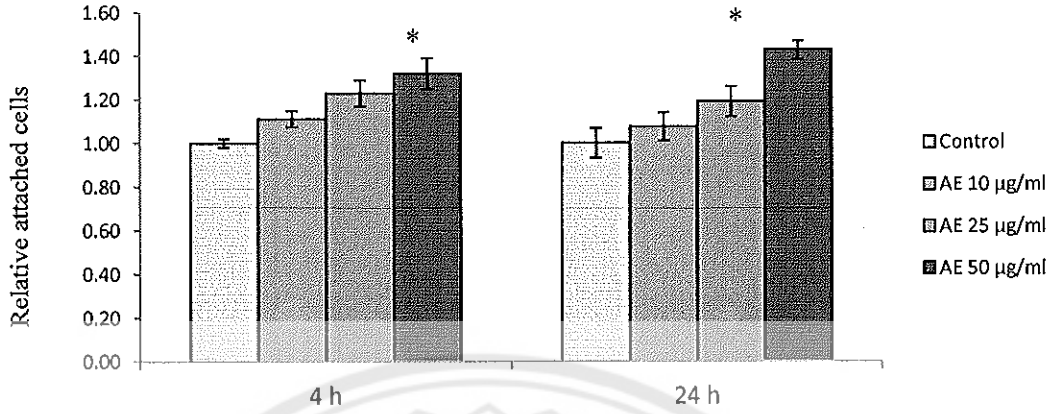

Figure 9 Effect of AE on MC3T3-E1 cell attachment was evaluated by MTT assay after treated with various concentration of AE (10, 25 and 50 µg/ml) for 4 h and 24 h. Cell attachment was significantly enhanced only when treated with 50 µg/ml of AE at both 4 and 24 h time point (*: $p<0.05$)

Morphological observation of MC3T3-E1cells attached under phase contrast microscope (Figure 10A). At 4 h, the most of cells appeared round shape in control group. In contrast, cell morphology of treated with 50 µg/ml AE group was appeared polygonal cells, which larger and flatter than those in control group however, it still have some interspersed round cell. No difference in cell morphology was obviously detectable among at 4 h and 24 h. At high magnification, the SEM examination showed that the cells attached morphology of treated with 50 µg/ml group appeared flat shape with a large and thin cytoplasmic layer and with filopodia which, was extending from the cells to the surface. While, the control group appeared round shape cell with short filopodia (Figure 10B).

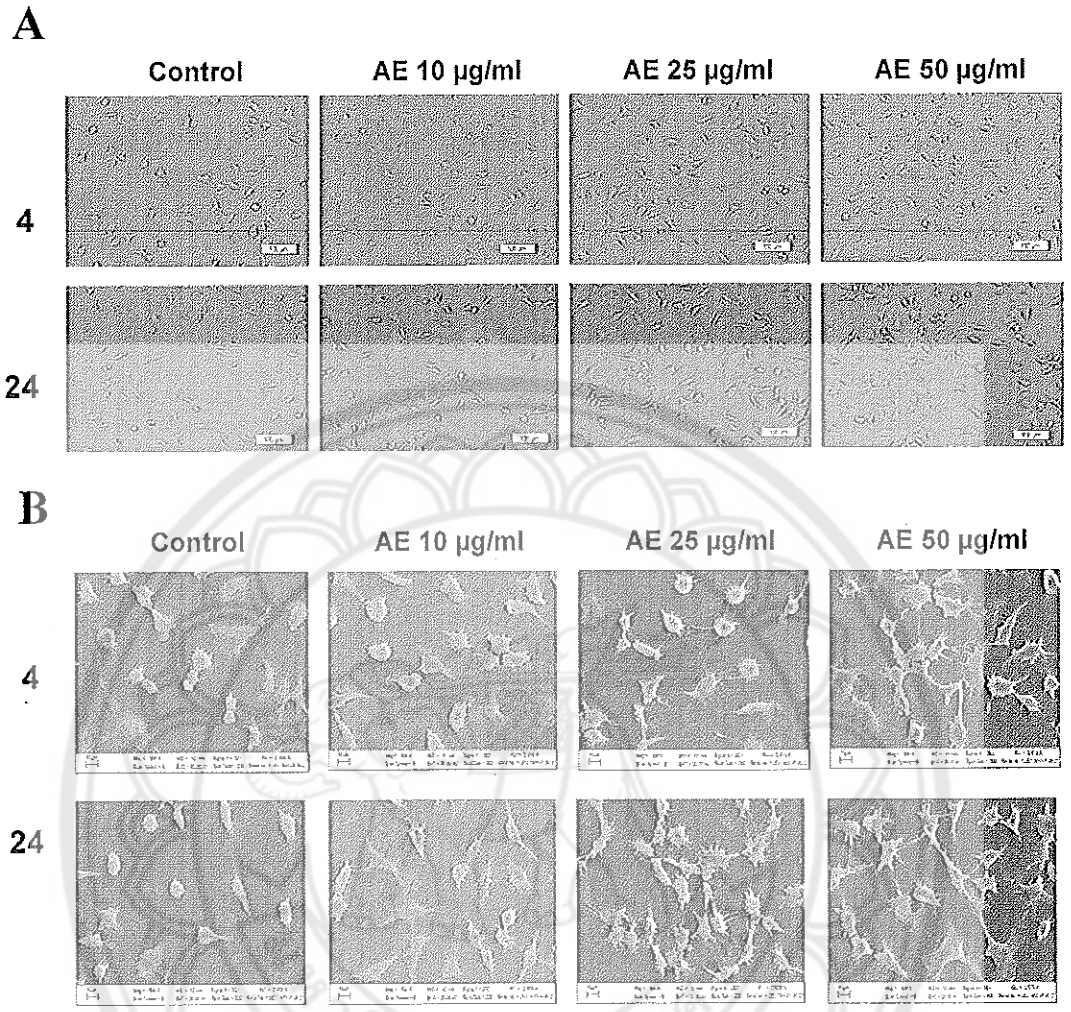

Figure 10 Morphology observation of MC3T3-E1 cell attachment after treated with AE (10, 25 and 50 µg/ml) for 4 h and 24 h using phase contrast microscopy (A) and using scanning electron micrographs for high magnification (B)

3. Evaluation of osteogenic differentiation

Alkaline phosphatase activity

The alkaline phosphatase staining at 14 days time point was shown that the active ALP stained cells of treated 50 µg/ml AE group appeared more than those of control groups (Figure 11A). The quantitative examination of ALP activity indicated

that the ALP activity of treated with 50 µg/ml AE groups was significantly highest than control groups at every time point (Figure 11B).

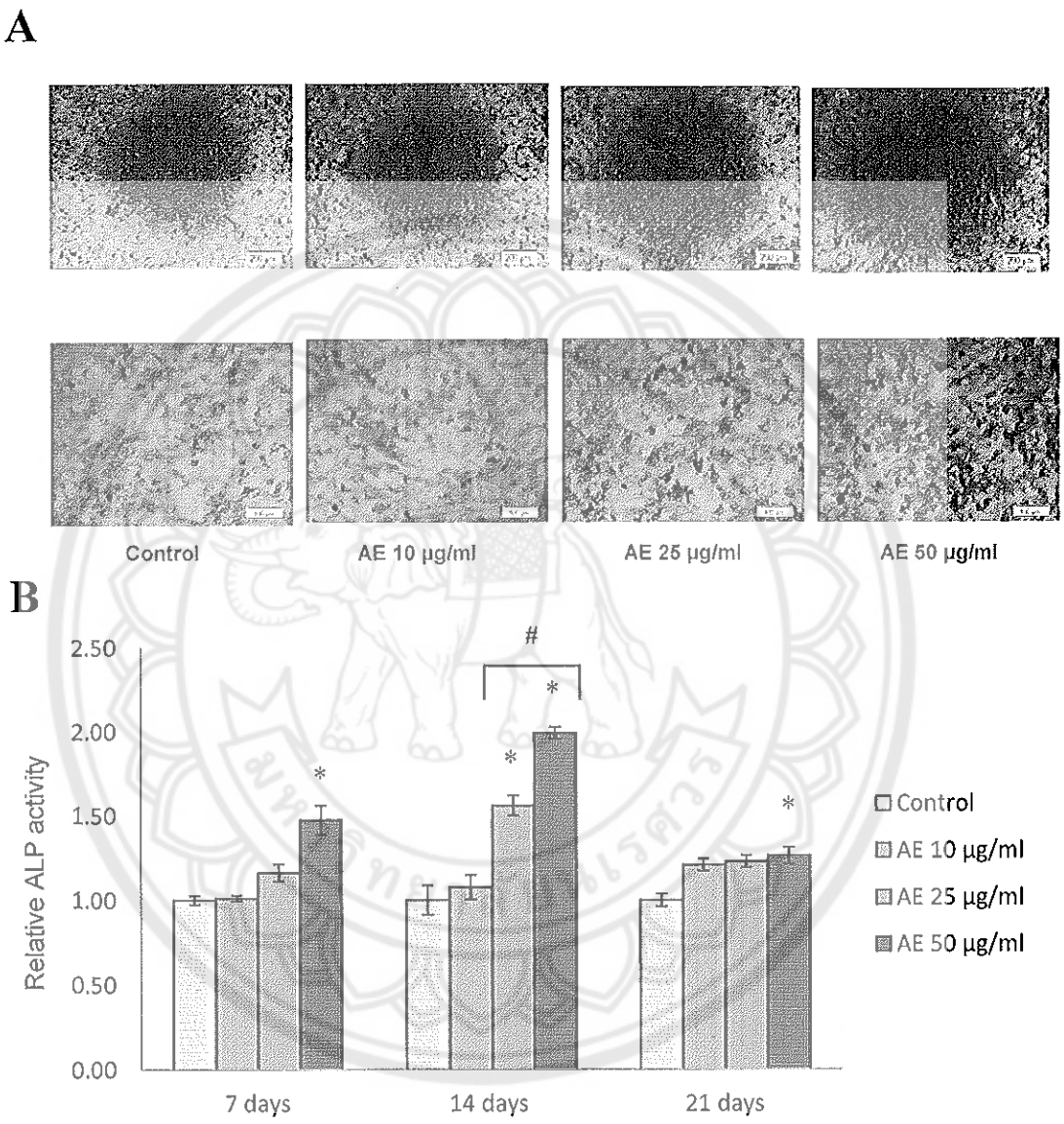

Figure 11 Effect of AE on the ALP staining and activity of MC3T3-E1 cells was evaluated after cultured in osteogenic medium. ALP staining of the cells at 14 days timepoint was shown (A). The ALP activity at 7, 14 and 21 days timepoints showed that the ALP activity of treated with 50 µg/ml AE groups was significantly highest than other groups at all time point (B). (*, # : p<0.05)

4. Osteogenic genes expression

The expressions of osteogenic genes were evaluated using qRT-PCR at 7, 14 and 21 days. The results showed that Col 1 mRNA expression was significantly higher in treated with 50 μg/ml of AE group than the control group for all time points (Figure 12). ALP mRNA expression was significantly higher in treated with 50 μg/ml of AE group than the control group for all time points. (Figure 13).

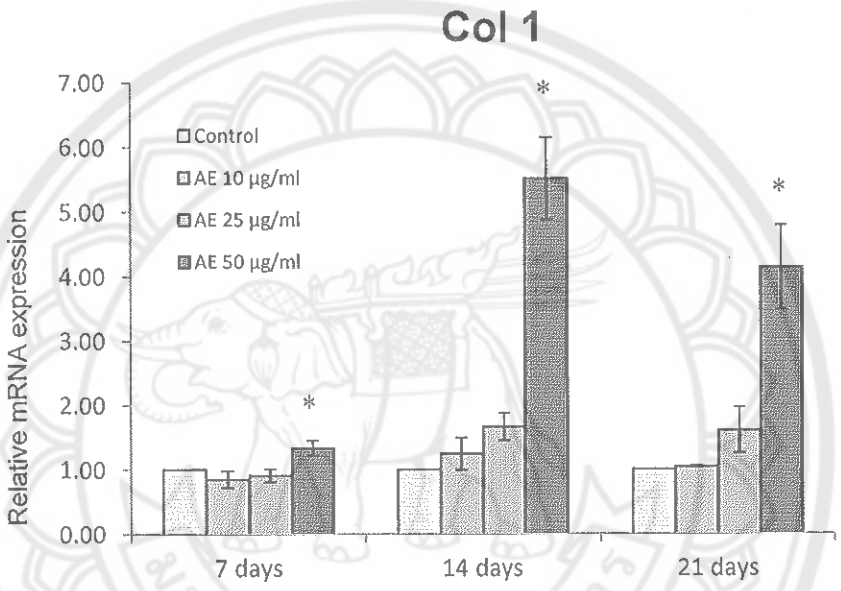

Figure 12 The expression of Col 1 gene by real-time PCR evaluation. The expression of Col 1 gene was significantly highest in treated with 50 μg/ml of AE group for all time points. (*, # : p<0.05)

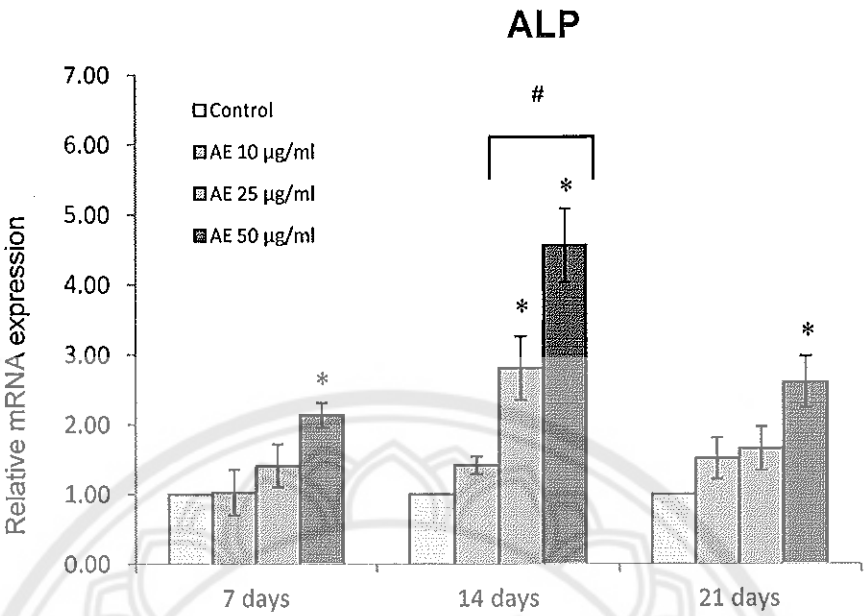

Figure 13 The expression of ALP gene by real-time PCR evaluation. The expression of ALP gene was significantly higher in treated with 50 µg/ml of AE group for all time points. (*, # : p<0.05)

In addition, BSP and OCN mRNA expression was significantly higher only in 14 and 21 days time points both of treated with 25 and 50 µg/ml of AE groups compared to the control group. However, BSP and OCN mRNA expression of treated with 50 µg/ml group was significantly higher than those of treated with 25 µg/ml group in both time points at 14 and 21 days (Figure 14 and 15).

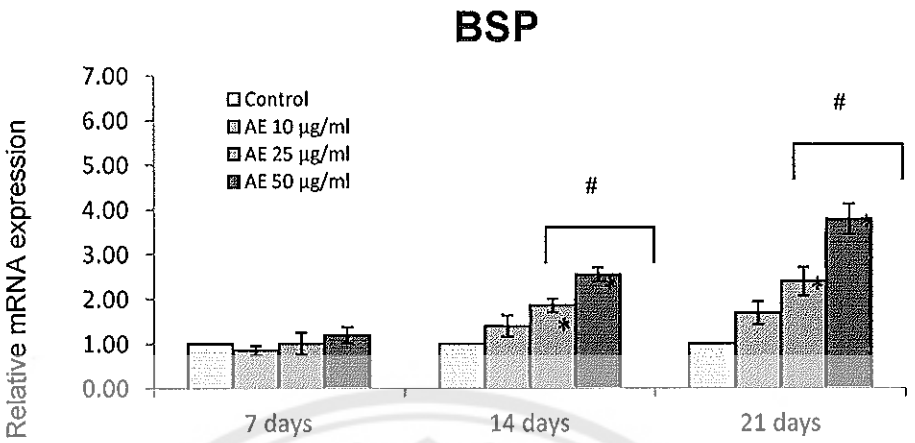

Figure 14 The expression of BSP gene by real-time PCR evaluation.

The expression of BSP gene was significantly higher in treated with 50 µg/ml of AE group at 14 and 21days time points. (*, # : $p<0.05$)

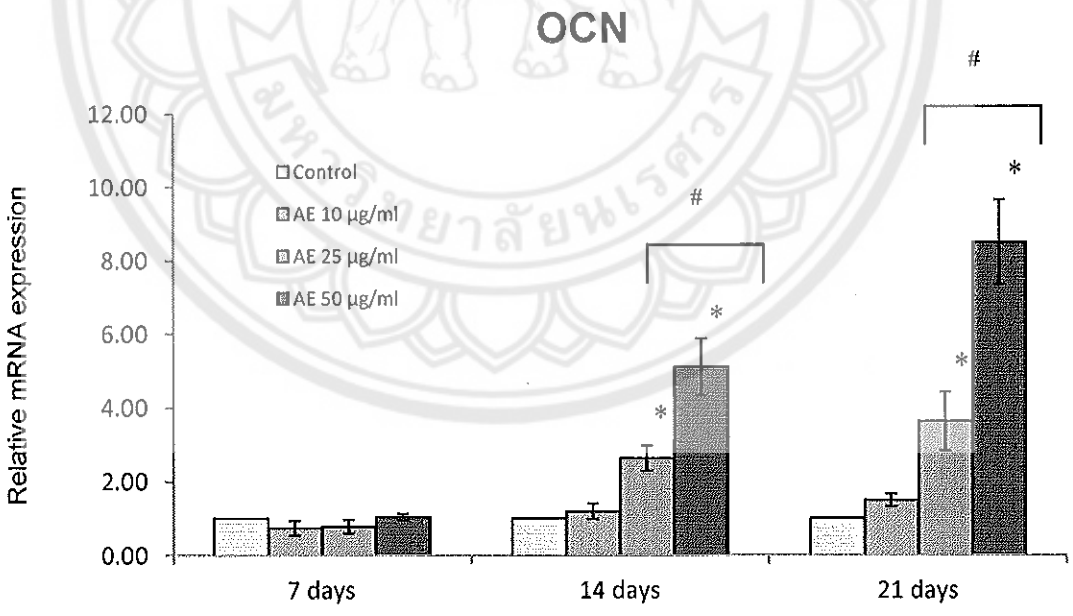

Figure 15 The expression of OCN gene by real-time PCR evaluation.

The expression of OCN gene was significantly higher in treated with 50 µg/ml of AE group at 14 and 21days time points. (*, # : $p<0.05$)

5. Osteocalcin product evaluation by ELISA assay

The osteocalcin proteins of MC3T3-E1 cells were detected by using ELISA assay at 21 days timepoint. The results showed that osteocalcin product was significantly highest in treated with 50 µg/ml of AE group compared other groups (Figure 16).

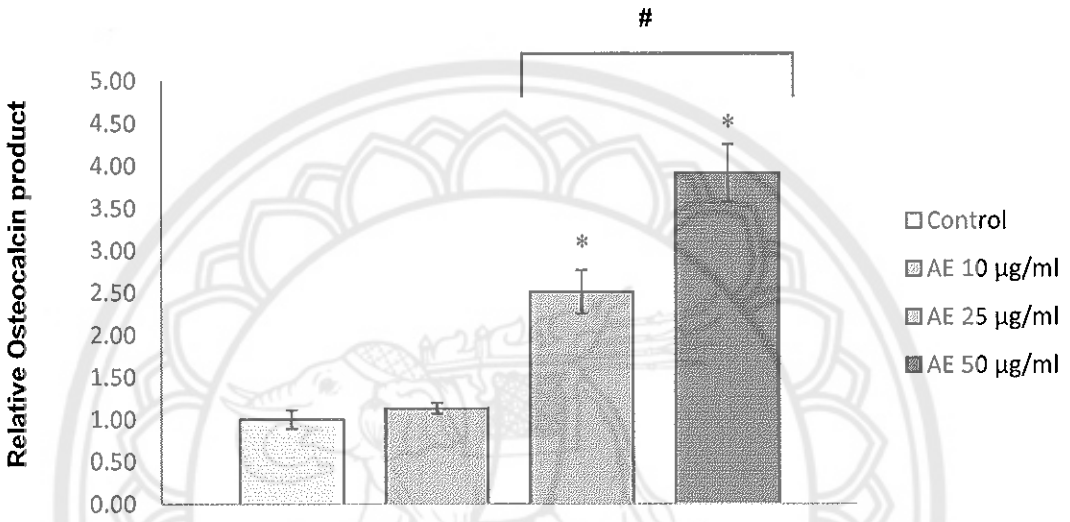

Figure 16 The osteocalcin proteins of MC3T3-E1 cells were detected by using ELISA assay at 21 days timepoint. The results showed that osteocalcin product was significantly highest in treated with 50 µg/ml of AE group compared other groups. (*, # : $p<0.05$)

6. Mineral deposition

The mineral deposition was investigated at 7, 14 and 21 days after cultured cells in osteogenic medium. The results showed that mineral deposition of 50 µg/ml AE treated group was significantly highest than other groups only at 21 days time point. The cells treated with 50 µg/ml AE exhibited faster matrix mineralization than those of other groups (Figure 17).

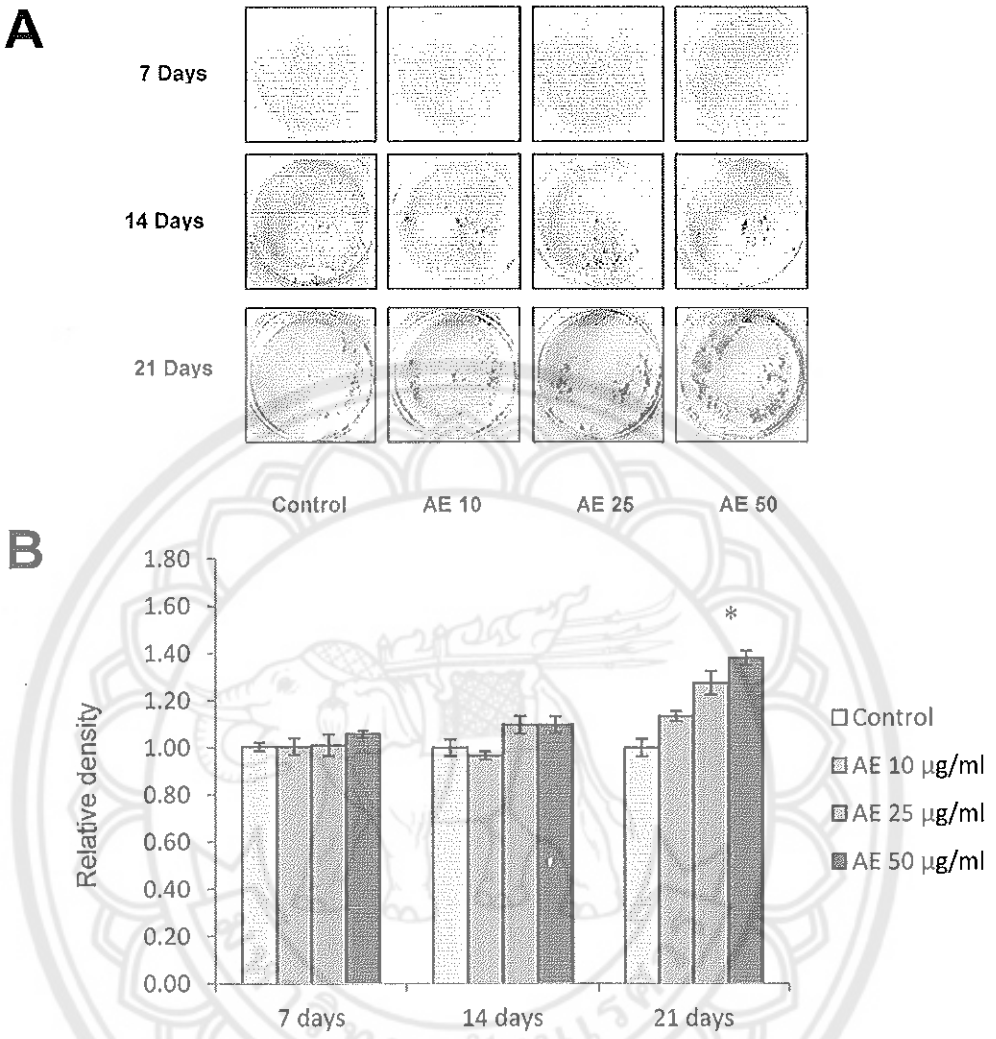

Figure 17 Effect of AE on the levels of mineral deposition of MC3T3-E1 cells. The mineral deposition was stained with alizarin red after cultured cells in osteogenic medium for 7, 14 and 21 days. The staining wells after treat with AE were shown (A). Destained quantification by cetylpyridinium chloride (B). (* : $p<0.05$)

Part 2 To evaluate effect of *Aquilaria crassna* crude extract on cell proliferation and cell attachment of MC3T3-E1 cell on modified titanium surface

1. Surface analysis

The atomic force microscopy examination (Figure 18) showed the surface roughness values (*Ra*) of acid etched Ti groups were higher than control group (polished Ti group). No significant difference in the surface roughness was observable between in dipped AE and those in none dipped AE of acid etched Ti groups. AFM topography has been shown in Figure 19.

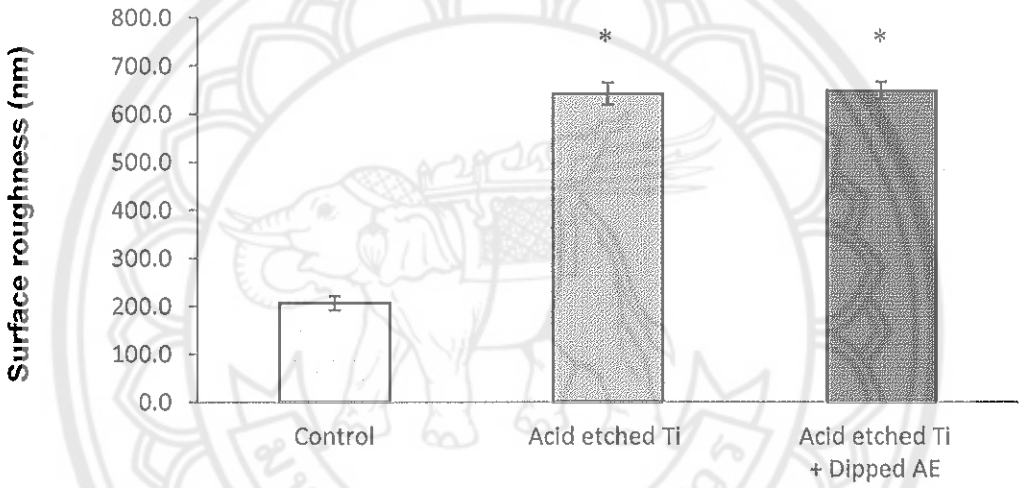

Figure 18 Surface roughness of acid etched Ti group and dipped AE acid etched Ti group was higher than polished Ti group (control) investigated with atomic force microscopy. No significant difference between those in dipped AE and those in none dipped AE of acid etched Ti groups. (* : $p<0.05$)

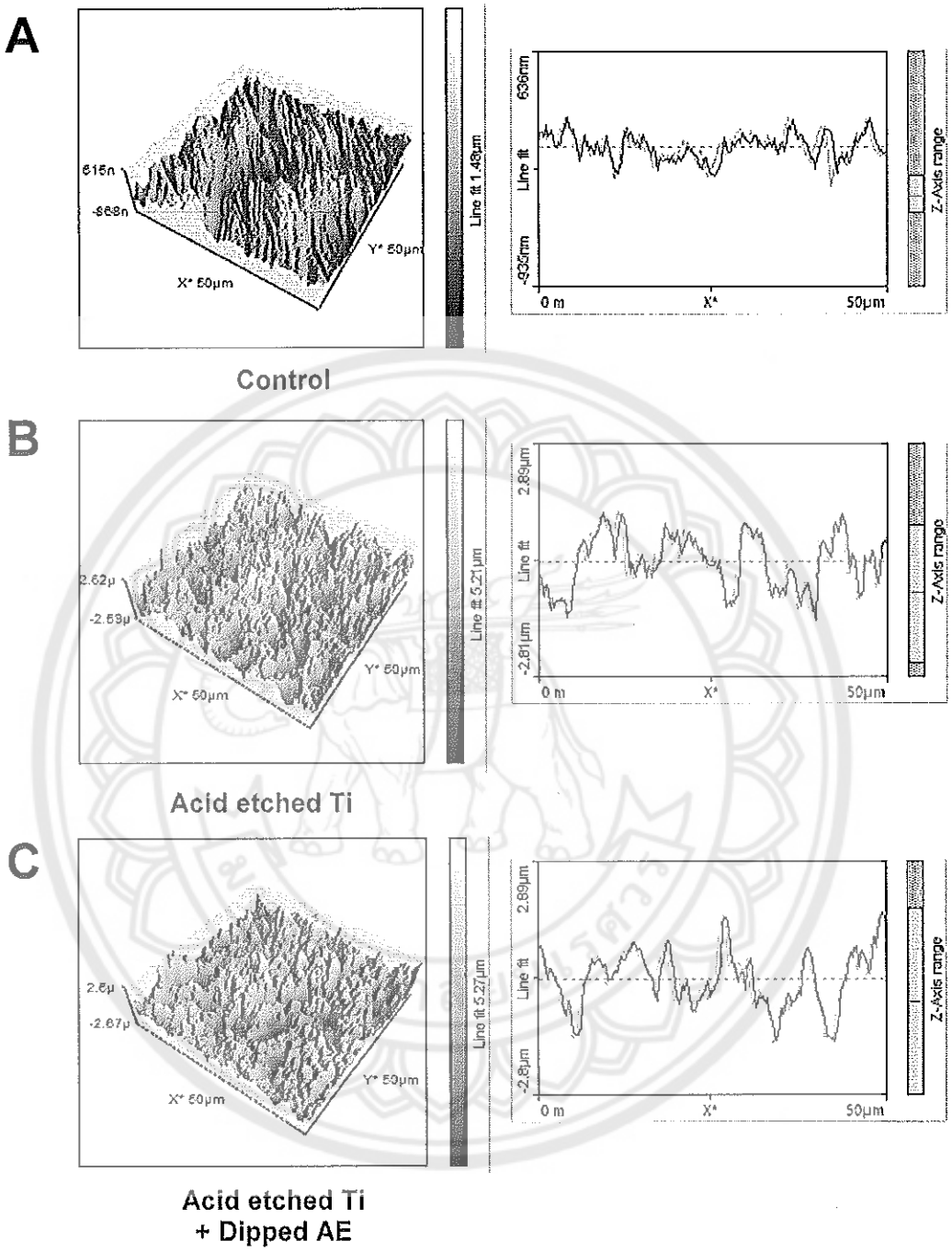

Figure 19 AFM topography (50 x 50 µm^2 scanning size) of polished Ti group (control) (A), acid etched Ti groups (B) and acid etched Ti group after dipped with AE for 24 h.

2. Scanning electron microscopy (SEM)

In an electron micrograph, the acid etched Ti groups possessed microporous structures formed by an acid etchant with some homogeneous micro-pits. Such pits seemed deeper, when compared to those in control group (polished Ti). No remarkable difference in the surface morphology between in dipped AE and those in none dipped AE of acid etched Ti groups (Figure 20).

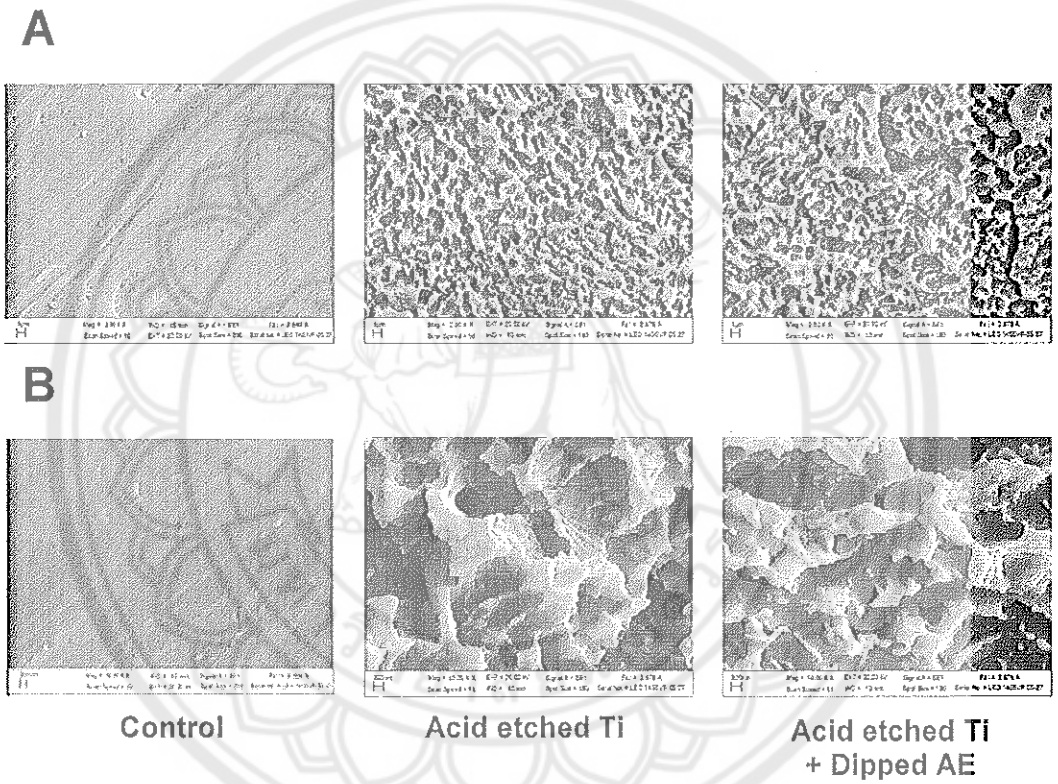

Figure 20 Morphology of polished Ti group (control), acid etched Ti groups and acid etched Ti group after dipped with AE for 24 h by using scanning electron micrographs for high magnification (A 2,500X and B 10,000X)

3. Contact angle measurement

The results showed that the contact angle values of acid etched Ti groups were higher than control group (polished Ti). No significant differences in contact angle values between dipped AE and those in none dipped AE of acid etched Ti groups (Figure 21).

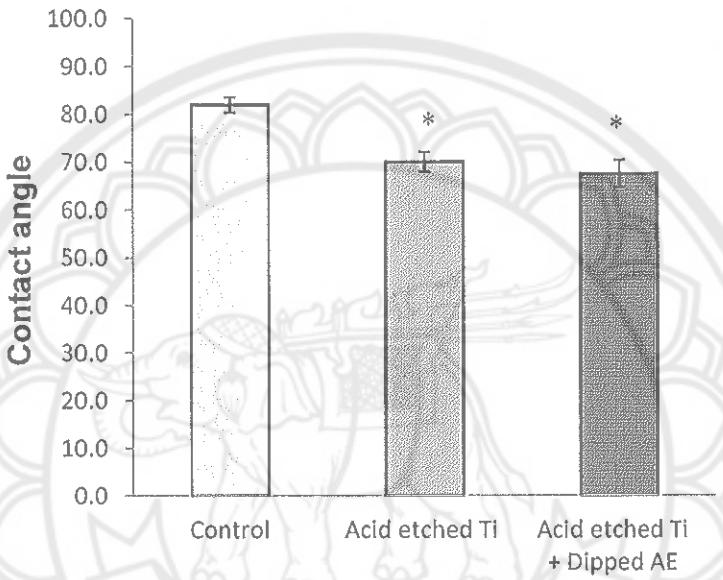

Figure 21 Contact angles of acid etched Ti group and dipped AE acid etched Ti group was lower than polished Ti group (control). No significant difference of those between dipped AE and those in none dipped AE of acid etched Ti groups. (* : $p<0.05$)

4. Release characteristics evaluation of *Aquilaria crassna crude extract* from modified titanium surface

The release characteristics of *Aquilaria crassna* crude extract from titanium surface after dipped AE for 24 h were investigated by Folin-Ciocalteu assay. The result showed that AE concentration still quite high within the first 24 h, after that it significantly reduced after 3 days. Finally, at 7 days time points it found the remained AE concentration less than 5 µg/ml (Figure 22).

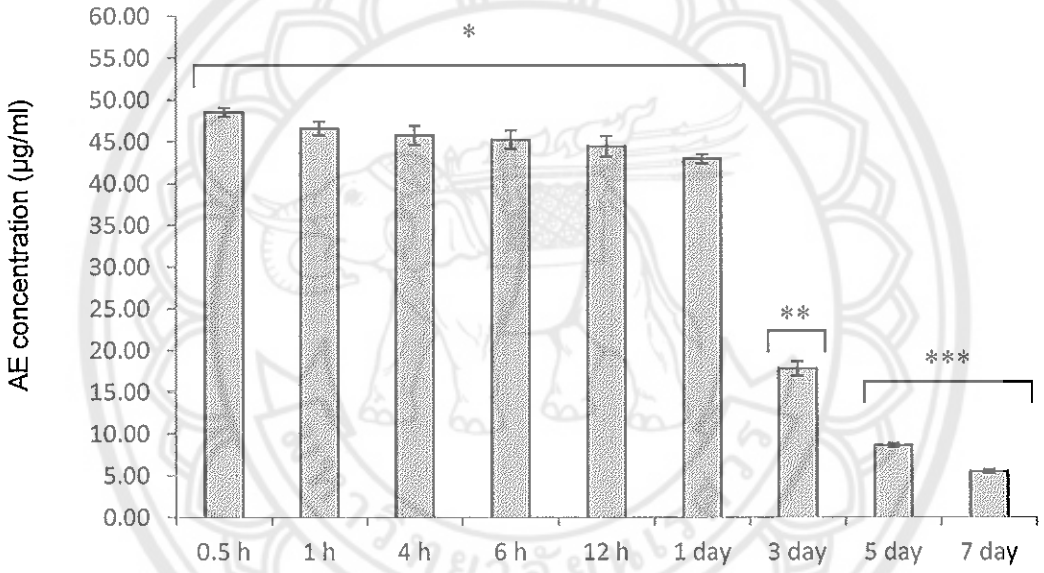

Figure 22 The release characteristics of *Aquilaria crassna* crude extract from the dipped acid etched Ti sample by Folin-Ciocalteu assay. The result showed that AE concentration still quite high at the first 24 h, after that it significantly reduced after 3 day, finally, at 7 day remained AE less than 5 µg/ml. (p<0.05 :*, **, *)**

5. Cell proliferation evaluation on titanium

Cell proliferation of MC3T3-E1cells on titanium samples was evaluated at 24, 48 and 72 h by MTT assay. The results of cell proliferation showed that the relative density of cell on acid etched Ti with treated AE in culture medium group and dipped AE acid etched Ti group were statistically significant higher than those of other groups for all timepoints (Figure 23).

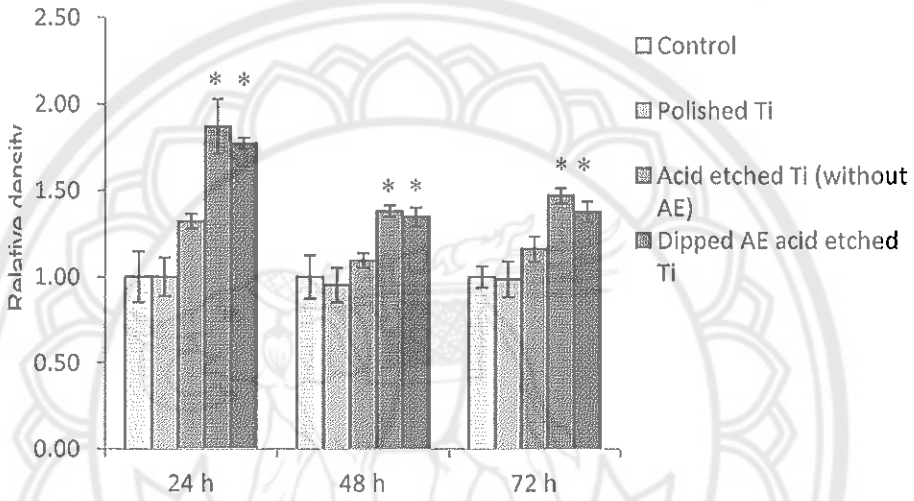

Figure 23 Cell proliferation of MC3T3-E1 cells when culture on Ti samples was evaluated by MTT assay (24 h, 48 h and 72 h). Cell proliferation on acid etched Ti with treated AE in culture medium group and dipped AE acid etched Ti group were statistically significant higher than those of other groups for all timepoints. Significant differences (p<0.05) are marked with *

6. Cell attachment evaluation on titanium

Cell attachment was evaluated after culture MC3T3-E1 cells on Ti samples for 4 and 24 h with MTT assay. The results showed that cells attachment was significant enhanced when culture on acid etched Ti with treated AE in culture medium group and dipped AE acid etched Ti group at both 4 and 24 h time points. No significant difference in cell attachment between groups (Figure 24).

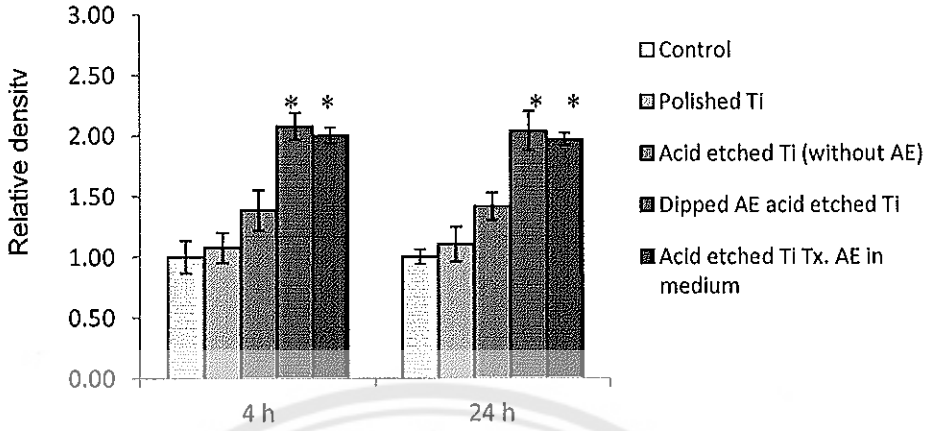

Figure 24 Cell attachment of MC3T3-E1 cells when culture on Ti samples was investigated by MTT assay. Cell attachment was significantly enhanced when culture on acid etched Ti with treated AE in culture medium group and dipped AE acid etched Ti group than those of other groups at both 4 and 24 h time points. Significant differences (p<0.05) are marked with *

7. Morphology of cell attachment on titanium evaluation by SEM

Morphological observation of MC3T3-E1cells attached under SEM examination at high magnification (350X and 10,000X) showed that the cells attached morphology of groups that culture on acid etched Ti with treated AE in culture medium group and dipped AE acid etched Ti group appeared flat shape with a large and thin cytoplasmic layer and with numerous extended filopodia from the cell body to the surface. While, the cell in control groups that cultured on acid etched Ti (without AE) group, polished Ti group and control group (glass surface) still appeared round shape-attached cell with short filopodia for both timepoints. When compare between 4 h and 24 h timepoints, the cell morphology of groups that culture on acid etched Ti with treated AE in culture medium group and dipped AE acid etched Ti group at 24 h timepoint seem appeared more flat shape and wild spreader than that at 4 h (Figure 25 and 26).

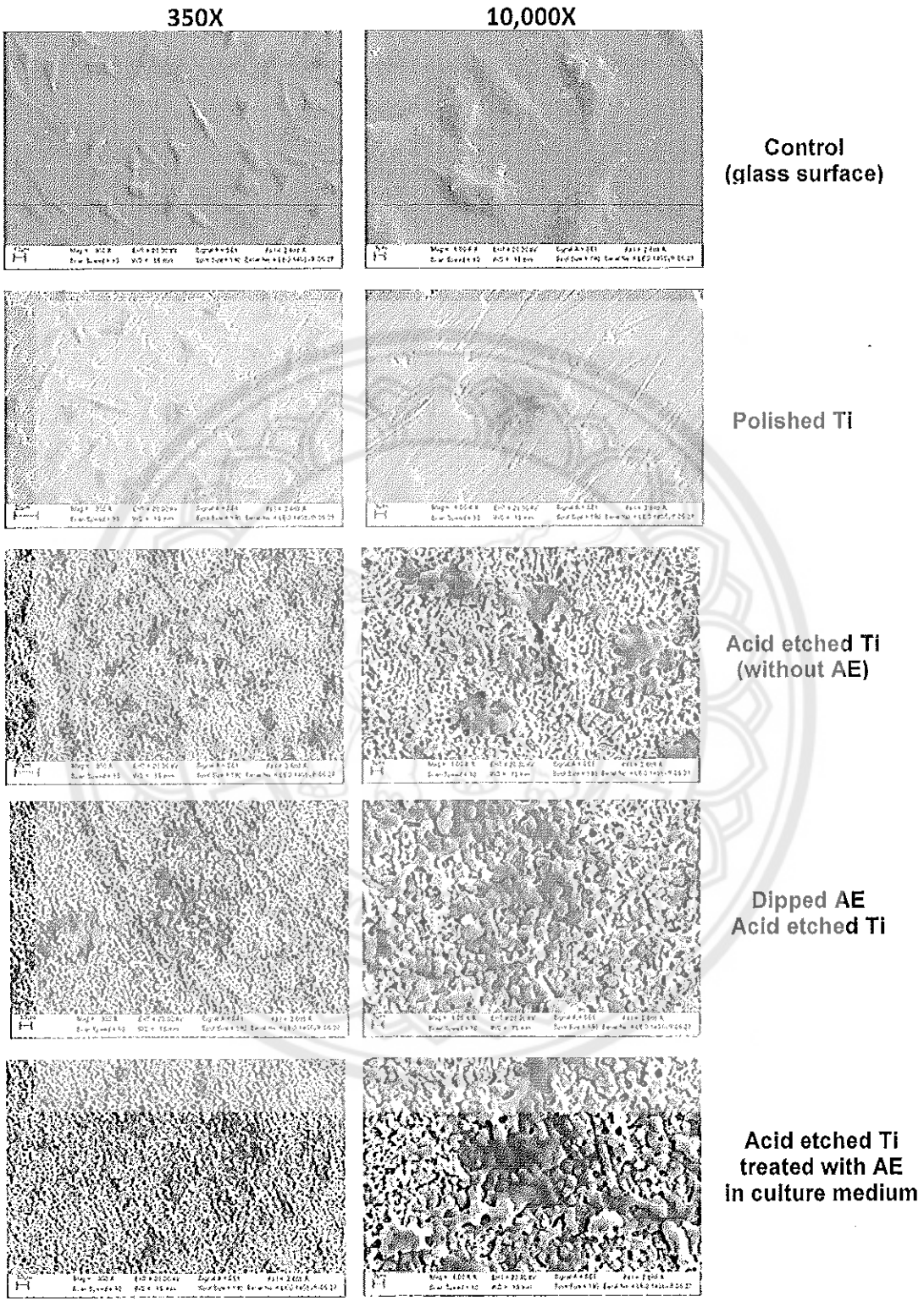

Figure 25 Morphology observation of attached cells on Ti samples at 4 h time point by SEM examination (350X and 10,000X)

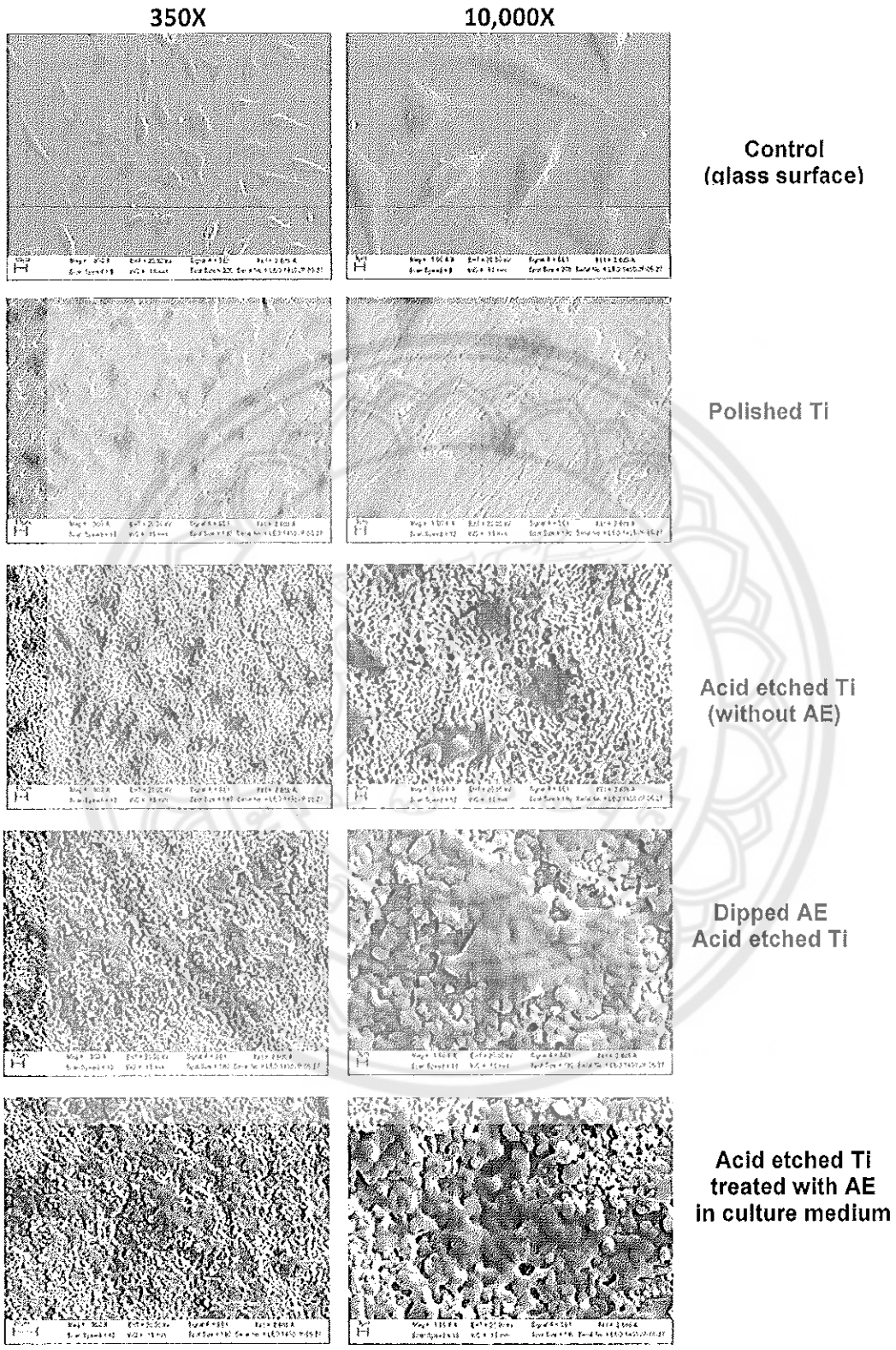

Figure 26 Morphology observation of attached cells on Ti samples at 24 h time point by SEM examination (350X and 10,000X)

CHAPTER V

CONCLUSION

Discussion

Current therapeutic approach for bone regeneration still has some limitations and adverse side effects (4). Previous studies reported the side effects of using bisphosphonates (anti-resorptive agents) such as osteonecrosis of the jaw (5). The anabolic agents are considered as beneficial agents. The recombinant human BMPs are current wildly used anabolic agents for bone regeneration in oral cavity. However, several studies reported that BMPs have some complications including severe gingival swelling and may associated with higher cancer risk (9, 10). Moreover, BMPs for clinical using are still quite complex, costly and time consuming to produce (11). Therefore, our study expected to discover new novel anabolic agents for helping bone growth and differentiation. Natural plants become the important sources of drug discovery and development. They are often fewer side effects compared with synthetic compounds (12). Therefore, in this study, we discover new anabolic agents from natural herb. *Aquilaria crassna Pierre ex Lecomte* or agarwood, a natural herb has been used for bone diseases such as arthritis and gout as folk medicine in Southeast Asian (16). There was still no of scientific publication of *Aquilaria crassna* osteogenic activity. This study is an in vitro study using MC3T3-E1 preosteoblastic cell line model. That is a well model acceptable for osteogenesis *in vitro* to test the osteoblasts differentiate capable (121, 122).

The optimal concentration of AE determined non-toxic concentration. This study was used MTT assay to determine cell viability of L929 cells followed ISO 10993-5 *In vitro* cytotoxicity test protocol. That, the L929 cells have usually used to test the cytotoxicity of natural plant extracts (123, 124). The results showed that AE was no toxic effect on L929 cells when treated with AE concentrations less than 50 µg/ml. On the other hand, treated with AE concentrations above 100 µg/ml, the cell viability was decrease less than 50 % when compared to control (Figure 5). These results indicated that the AE biologically safe concentration range between 10-50 µg/ml. Consistent with

study of Dahham, et al. (125) demonstrated the cytotoxic effect of AE on cancer cells including prostrate (PC3), colorectal (HCT 116) and breast (MCF-7) cancer cells. The cytotoxicity results demonstrated 50 % cell death or 50 % inhibition concentration (IC50) with 72, 119 and 140 μg/ml respectively. Moreover, cytotoxicity on human endothelial cells (HUVEC) demonstrated IC50 with 48 μg/ml.

Bone formation is a biological sequence of cell attachment, cell proliferation, osteogenic differentiation, organic matrix formation, and matrix mineralization (65). Cell attachment is main function of cell communication and regulation. It is a crucial consideration for biomaterial development especial in bone tissue engineering. Cell attachment involved in several signals that stimulate and regulate cell proliferation and differentiation (126). In this study, the results showed that treated with 50 μg/ml AE was significant increase cell attachment on both 4 and 24 h time points (Figure 8A). The results were confirmed cells attached morphology with SEM (Figure 8B). These results may indicate that AE stimulate cell attachment that may subsequently affect to promote cells proliferation and differentiation.

To determine cell proliferation, we used MTT assay to evaluate MC3T3-E1cells after treated with AE at 10, 25 and 50 μg/ml concentrations for 24, 48 and 72 h. These results showed that the relative density of cells treated with 50 μg/ml AE concentration was statistically significant higher than those of other AE concentrations at 24 h. Previous studies have been reported that natural plants extract stimulate cell proliferation. Suh et al. (127) reported that 20 μg/ml of *Ulmus davidiana* extract significant stimulate cell proliferation of MC3T3-E1 cells after culture for 48 h *in vitro* assay. While, Xiang et el. (2011) demonstrated that *Polygonum orientale* extract significantly stimulated the proliferation of MC3T3-E1 cells with the range of concentration at 1-10 μg/ml after culture for 24 h *in vitro* study (128). However, the results of our study show no significant difference of proliferation rate after culture for 48 and 72 h (Figure 6). Therefore, from this results may indicate that 50 μg/ml AE promoted MC3T3-E1cells proliferation in first 24 h, after that the cells may lead to stage of differentiation without any subsequent proliferation.

To evaluate osteogenic differentiation, this study measured ALP activity, expressions of osteogenic marker genes and mineral deposition. ALP activity is a key marker of early stage of osteogenic differentiation, while mineral deposition is a marker

of the late stage of osteogenic differentiation. Previous studies demonstrated that ALP played an important role in the bone formation process (129). Some studies reported natural plant extract stimulate ALP activity including *Drynariae Rhizoma* (14), *Ulmus davidiana* (127), *Polygonum orientale* (128). The results of our study showed that treated with AE at 50 μg/ml significant increased ALP activity for all time points (Figure 9B). The ALP activity pattern was increased at 1-2 weeks and decreased at 3 week. These patterns related with investigate of mineral deposition.

The gene expression patterns are key to determine the osteogenic differentiation. The common osteogenic differentiation markers are ALP, Col 1, BSP and OCN. Early phase of differentiation, there are expressions of ALP and Col 1, while BSP and OCN appears are the late phase markers of osteogenic differentiation that is represent to osteoblastic maturation. Also, the expressions of osteogenic marker genes including Col 1, ALP, BSP and OCN usually used to confirm osteogenic differentiation (130-132). This study used quantitative real-time PCR for gene expression evaluation.

Osteocalcin is a late protein marker of osteogenic differentiation that is highly related to fully osteoblastic maturation (131, 133). In this study, we evaluated osteocalcin with ELISA assay. While, the mineral deposition is a complete differentiation marker. The main composition of mineralized formation is calcium that it be the key marker involved in bone formation (134). In this study, we used Alizarin Red-S staining to detect calcium and quantify matrix mineralization.

Many previous studies repoted that natural plants extract exhibited osteogenic activities by promoting osteoblast differentiation and mineralization. Jeong et al., 2004 reported that *Drynariae Rhizoma* extract has osteogenic effects through the promotion of differentiation in MC3T3-E1 cells. The study showed that *Drynariae Rhizoma* extract enhanced ALP activity and mineralization. Moreover, the result showed that the *Drynariae Rhizoma* extract increased mRNA expression of type I collagen, ALP and BMP-2 (135). After that, the studies founded Naringin, main effective component of *Drhizoma drynariae* enhanced the osteoblastic differentiation on MC3T3-E1 cells and human bone mesenchymal stem cells (BMSCs) (136, 137). Other study, Huh, et al. (2006) reports on the osteogenic effects of Puerarin that have stimulate differentiation gene markers such as ALP, OCN, osteopontin (OPN), Col 1, and mineralization in SaOS-2 cells (29). While as, Muthusami, et al. (138) reported *Cissus quadrangularis*

stimulate the proliferation, differentiation, and mineralized depositon of SaOS-2 cells. The result showed that after *Cissus quadrangularis* treatment were increased ALP activities, gene expression of ALP and Col 1. A significant increases in osteocalcin protein and mineralized bone nodule formation after *Cissus quadrangularis* treatment was observed on day 21. Recently, Hwang, et al. (15) reported that *Euodia sutchuenensis Dode* (ESD) extract enhanced osteogenic differentiation by activated the Wnt/β-catenin pathway. ESD extract enhanced β-catenin levels and also enhanced gene expression of RUNX2, BMP2 and Col 1, and increased ALP activity and staining with Alizarin Red S in mouse osteoblasts.

In this study, our results showed that cell treated with AE at 50 μg/ml was significantly increased expression of Col 1, ALP, BSP and OCN for all time points (Figure 12-15). Consequently, it was significantly increased in the levels of osteocalcin at 21 days time point (Figure 16). While, the mineralized formation results showed that 50 μg/ml AE treated groups was significantly increased mineral deposition at 21 days time point (Figure 17). Interestingly, cell treated with 50 μg/ml AE exhibited faster matrix mineralization than those of other groups. These data also indicated that 50 μg/ml of AE is a promising anabolic agent to enhance osteogenic differentiation and matrix mineralization.

Phytochemical constituents studies reported that the natural plant extracted molecules have osteoinductive ability such as decalpenic acid, triterpenes, flavonoids, and quinones (139). Previous phytochemical analysis of the crude extract of *Aquilaria crassna* showed the presence main compositions were triterpenes and flavonoid, which may affect an enhancing bone formation (75, 125). Triterpenes reported to stimulate proliferation, protein synthesis, and ALP activity of PDL cell lineage (140). While, flavonoids reported to stimulate the bone formation of human bone mesenchymal stem cells (141). However, it has not yet analyzed the chemical compositions of AE that used in this experiments. Therefore, in future studies need more in-deep analysis the active ingredients that involve the osteogenic process.

For evaluation the effect of AE on cell proliferation and attachment when applied on modified Ti surface, in this study used dipping method for loading AE to Ti surface. The dipping method is conducted by a simple immersion of implants into some solution. Its advantage is a preservation of an implant's topography, post-introduction

of a bone-forming drug (factor) onto its surface. The AFM and SEM of this study have revealed no deterioration to the implants' roughened surfaces, after being immersed into AE solution that no significant difference in the surface roughness was observable between those in dipped AE and those in none dipped AE groups as shown in Figure 16, 17 and 18. The results in this study have coincided well with Yang et al.'s study (142), that immersed implant into simvastatin solution. Since surface roughness is a key factor that affect to osseointegration rate and biomechanical fixation of the Ti implants (95, 143). The surface roughness also affects the hydrophilicity of the surface due to biological fluids, surface and cells interaction (144, 145).

The contact angle is one of key factors that affects to the success of dental implant treatment (146). The previous studies indicated that most favorable for adhesion and growth of cells were the surfaces with water contact angles in the range of 60-80° (147). In this study, the contact angles value of acid treated surface groups were almost within that range where as, the control group were not within that range. When compared between dipped AE and none dipped AE groups, there were no significant difference in contact angle values (Figure 21). Hence, it could be suggested that the loading AE on Ti samples by dipping method in this study were simple and effective method without destroy the important surface properties including surface roughness and contact angles.

For success of osseointegration, rough surface was the principal factor through enhancement of osteoblast attachment and subsequent proliferation and differentiation, and enlargement primary stability of the implant by increasing in contacted area with the host bone (148, 149). Previous studies reported a significant enhance cell proliferation on rougher surfaces (150, 151). Consisting with this study, the cell proliferation of acid etched Ti groups was significant higher than those of polished Ti groups for all time points (Figure 23).

As demonstrated in the first part of this study, AE has affected to enhance cell attachment and proliferation and stimulate osteogenic differentiation in MC3T3-E1 cells. When applied AE to Ti surfaces, the results showed that the groups of AE treatment were statistically significant higher than those of other groups (without AE groups) for all timepoints (Figure 23). Several studies have been used natural extraction applied to implant surfaces to improve the osseointegration. Yang, et al. (142)

demonstrated that *Puerarin* applied on Ti surfaces promote accelerated osteoblastic differentiation (30). Other studies results indicate using modified pectin of *Malus domestica* coated titanium implants a better interaction, which enhanced bone cell proliferation, attachment and differentiation in *vitro* and in *vivo* (152, 153).

To compare between dipped AE group and direct treated AE in culture media, there was no significant difference of cell proliferation. It seem dipped method could be the effective method to carry the AE to Ti surfaces at first 3 days or the early stage of bone formation. Several previous studies have been using dipped method for carrying bone-forming drug to implant surfaces. Yang, et al. (142) loaded simvastatin implant surfaces by dipped method resulting in promote osteogenic differentiation of preosteoblasts. As the result of the AE release investigation, AE concentration still quite high within the first day, after that it reduced more than 50% after 3 days, finally, at 7 days only AE less than 5 μg/ml (Figure 20). The model of drug release can hardly precisely reflect *in vivo* drug release kinetics. The implant was placed in the drilling hole, which surrounded by blood or hematoma in a closed environment. The drug release kinetics was primarily dependent on the surrounding hematoma (154). Other methods have been introduced to prolong drug release from Ti surfaces, such as chitosan, gelatin or polymer loading techniques (155, 156). However, it needs more studies to improve the method to control time and drug releasing of the implant surfaces for prolong effective concentration.

It is well understood that cell attachment is essential factor for osteointegration. That involved in stimulating signals that regulate cell proliferation and cell differentiation (126). This study showed that effect of AE was significant enhances cell attachment to Ti surfaces at both 4 and 24 h time points. No significant difference between dipped AE method and direct treated AE in culture media (Figure 24). Furthermore, morphological observation using SEM showed that cell of the groups of AE treatment appeared more flat shape and wild spreader attached to the surface comparing with none AE treated groups for both 4 and 24 h time points (Figure 25 and 26). A similar cell behavior was seen by previous studies with regard to the cell attachment (116). Previous studies demonstrated the association between cell attachment and osteogenic differentiation capacity (157, 158). Therefore, dipped AE Ti

surfaces increase of cell attachment may affect to stimulate cell differentiation. However, it need more investigation in future studies.

Therefore, it could be suggest that dipped AE is the simple and effective method to enhance cell proliferation and cell attachment on Ti surface at early time point. Considering its application in dental implantology, accelerating bone formation could be the good for clinical application in patients with compromised bone healing.

Conclusion

In conclusion, the results in this study demonstrated that *Aquilaria crassna* extract was efficacious in inducing initial cell attachment and proliferation and stimulated the osteogenic differentiation and matrix mineralization *in vitro*. Furthermore, dipped AE on Ti surfaces is the simple and effective method to enhance initial cell proliferation and cell attachment on Ti surfaces. Therefore, *Aquilaria crassna* are a promising anabolic agent for bone regeneration and osseointegration.

Recommendation

For osteogenic efficiency of AE, we need to evaluate by comparing with some commercial products such as recombinant human BMP. The AE should be analyzed the chemical constituents to identify the main active compositions which stimulate osteogenic activity and in-depth analysis of mechanism pathways. For application, it will be reducing the adverse effect, which may from the other compositions of the crude extract. While, the osteogenic effect of AE on Ti surfaces still are investigated only early stage of the bone formation. The future studies need to clarify osteogenic effect in late stage. Furthermore, loading AE on Ti still have limited of the effective releasing concentration. It needs more studies to improve the method to control AE releasing from the implant surfaces for optimal concentration and time span. Consequently, further studies are *in vivo* studies.

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APPENDIX

No.	Compound class and name	Source or origin
	Agarofurans (*Sesquiterpenes*)	
1	α-Agarofuran	*A. agallocha* (India)/ *A. malaccensis* (Indonesia)
2	β-Agarofuran	*A. agallocha* (India/Vietnam)/ *A. sinensis* (China)
3	Dihydroagarofuran	*A. agallocha* (India)
4	Norketoagarofuran	*A. agallocha* (India)
5	Dihydro-4-hydroxyagarofuran	*A. agallocha* (India)
6	Dihydro-3,4-dihydroxyagarofuran	*A. agallocha* (India)
7	Baimuxinol	*A. sinensis* (China)
8	Dehydrobaimuxinol	*A. sinensis* (China)
9	Isobaimuxinol	*A. sinensis* (China)
10	Baimuxifuranic acid	*A. agallocha* (India)
11	(3*R*,5a*S*,9a*R*)-Octahydro-2,2,5a-trimethyl-2*H*-3,9a-methano-1-benzoxepine	*A. agallocha* (India)
12	(3*R*,5a*S*,9*R*,9a*R*)-Octahydro-2,2,5a-trimethyl-2*H*-3,9a-methano-1-benzoxepin-9-ol	*A. agallocha* (India)
13	Epoxy-β-agarofuran	*A. agallocha* (India)
14	(3*R*,5a*R*,9*S*,9a*S*)-Octahydro-2,2,5a-trimethyl-2*H*-3,9a-methano-1-benzoxepine-9-carbaldehyde	*A. agallocha* (India)
	Agarospiranes (*Sesquiterpenes*)	
15	Agarospirol	*A. agallocha* (India)
16	Baimuxinic acid	*A. agallocha* (India)
17	Baimuxinal	*A. agallocha* (India)
18	Oxoagarospirol	*A. malaccensis* (Cambodia)
19	Isoagarospirol	*A. malaccensis* (Cambodia)
20	Vetaspira-2(11),6-dien-14-al	*A. agallocha* (India)
21	Vetaspira-2(11),6(14)-dien-7-ol	*A. agallocha* (India)
22	2,14-Epoxyvetispir-6-ene	*A. agallocha* (India)
23	2,14-Epoxyvetispira-6(14),7-diene	*A. agallocha* (India)
24	(4*R*,5*R*,7*R*)-11-Hydroxyspirovetiv-1(10)-en-2-one	*A. agallocha* (Vietnam)
	Guaianes (*Sesquiterpenes*)	
25	Sinenofuranol	*A. sinensis* (China)
26	Sinenofuranal	*A. sinensis* (China)
27	(−)-Guaia-1(10),11-dien-14-al	*A. agallocha* (Vietnam)
28	(−)-Guaia-1(10),11-dien-14-ol	*A. agallocha* (Vietnam)
29	(−)-Guaia-1(10),11-dien-14-oic acid	*A. agallocha* (Vietnam)
30	Methyl guaia-1(10),11-dien-14-oate	*A. agallocha* (Vietnam)
31	(+)-Guaia-1(10),11-dien-9-one	*A. agallocha* (Vietnam)
32	(−)-1,10-Epoxyguai-11-ene	*A. agallocha* (Vietnam)
33	(−)-Guaia-1(10),11-dien-14,2-olide	*A. agallocha* (Vietnam)
34	(−)-Rotundone	*A. agallocha* (Vietnam)
35	(−)-2α-Hydroxyguaia-1(10),11-dien-14-oic acid	*A. agallocha* (Vietnam)
36	(+)-1,5-Epoxynorketoguaiene	*A. agallocha* (Vietnam)
37	α-Guaiene	*A. agallocha* (Vietnam)
38	α-Bulnesene	*A. agallocha* (Vietnam)
39	α-Gurjunene	Vietnam
	Eudesmanes (*Sesquiterpenes*)	
40	Jinkoheremol	*A. malaccensis* (Indonesia)
41	Kusunol	*A. malaccensis* (Indonesia)
42	(−)-10-Epi-γ-eudesmol	*A. malaccensis* (Indonesia)

Table (cont.)

No.	Compound class and name	Source or origin
43	(−)-Selina-3,11-dien-9-one	*A. agallocha* (Vietnam)
44	(+)-Selina-3,11-dien-9-ol	*A. agallocha* (Vietnam)
45	(−)-Selina-3,11-dien-14-al	*A. agallocha* (Vietnam)
46	(+)-Selina-4,11-dien-14-al	*A. agallocha* (Vietnam)
47	(−)-Selina-3,11-dien-14-oic acid	*A. agallocha* (Vietnam)
48	(+)-Selina-4,11-dien-14-oic acid	*A. agallocha* (Vietnam)
49	(+)-9-Hydroxyselina-4,11-dien-14-oic acid	*A. agallocha* (Vietnam)
50	Dehydrojinkoheremol	*A. agallocha* (Vietnam)
51	2-[(2*R*,4a*S*)-1,2,3,4,4a,5,6,7-Octahydro-4a-methylnaphthalen-2-yl]propan-2-ol	*A. agallocha* (India)
52	(8a*S*)-1,2,3,7,8,8a-Hexahydro-8a-methyl-6-(1-ethylethyl)naphthalene	*A. agallocha* (India)
53	(4a*S*)-1,2,3,4,4a,5,6,7-Octahydro-4a-methyl-2-(1-methylethylidene)naphthalene	*A. agallocha* (India)
54	(2*R*,4a*S*)-1,2,3,4,4a,5,6,7-Octahydro-4a-methyl-2-(1-methylethenyl)-naphthalene	*A. agallocha* (India)
55	Valenca-1(10),8-dien-11-ol	*A. agallocha* (India)
56	Calarene	*A. agallocha* (Vietnam)
	Eremophilanes (Sesquiterpenes)	
57	Agarol	*A. agallocha* (India)
58	Dihydrokaranone	*A. malaccensis* (Cambodia)/ *A. agallocha* (Vietnam)
59	Karanone	*A. malaccensis* (Cambodia)
60	Neopetasane	*A. agallocha* (Vietnam)
61	Eremophila-9,11(13)-dien-12-ol	*A. agallocha* (India)
62	8,12-Epoxyeremophila-9,11(13)-diene	*A. agallocha* (India)
63	Valenc- or eremophil-9-en-12-al (tentative)	*A. agallocha* (India)
	Prezizaanes (Sesquiterpenes)	
64	Jinkohol	*A. agallocha*/*A. malaccensis* (Indonesia)
65	Jinkohol-II	*A. malaccensis* (Indonesia)
	Others (Sesquiterpenes)	
66	Gmelofuran	*A. agallocha* (India)
67	8βH-Dihydrogmelofuran[a])	*A. agallocha* (India)
68	ar-Curcumene	*A. malaccensis* (Cambodia)
69	Nerolidol	*A. malaccensis* (Cambodia)
	*2-(2-Phenylethyl)-4*H*-chromen-4-one derivatives*	
70	2-(2-Phenylethyl)-4*H*-chromen-4-one	*A. agallocha* (Vietnam, Kalimantan)/*A. malaccensis* (Indonesia)/ *A. sinensis* (China)
71	6-Hydroxy-2-(2-phenylethyl)-4*H*-chromen-4-one (AH_3)	*A. agallocha* (Kalimantan)/ *A. sinensis* (China)
72	6-Methoxy-2-(2-phenylethyl)-4*H*-chromen-4-one (AH_4)	*A. agallocha* (Kalimantan)/ *A. sinensis* (China)
73	6-Methoxy-2-[2-(3-methoxyphenyl)ethyl]-4*H*-chromen-4-one (AH_5)	*A. agallocha* (Kalimantan)/ *A. sinensis* (China)
74	6,7-Dimethoxy-2-(2-phenylethyl)-4*H*-chromen-4-one (AH_6)	*A. agallocha* (Kalimantan)/ *A. sinensis* (China)
75	6-Hydroxy-2-[2-(4-methoxyphenyl)ethyl]-4*H*-chromen-4-one	*A. sinensis* (China)

Table (cont.)

No.	Compound class and name	Source or origin
96	2-(2-Phenylethyl)-6-{[(5*S*,6*R*,7*R*,8*S*)-5,6,7,8-tetrahydro-5,6,7-trihydroxy-4-oxo-2-(2-phenylethyl)-4*H*-chromen-8-yl]oxy}-4*H*-chromen-4-one (AH_{13})	*A. agallocha* (Kalimantan)
97	2-(2-Phenylethyl)-6-{[(5*S*,6*S*,7*S*,8*R*)-5,6,7,8-tetrahydro-6,7,8-trihydroxy-4-oxo-2-(2-phenylethyl)-4*H*-chromen-5-yl]oxy}-4*H*-chromen-4-one (AH_{14})	*A. agallocha* (Kalimantan)/ *A. sinensis* (China)
98	AH_{21}	*A. agallocha* (Kalimantan)
99	AH_{18}	*A. agallocha* (Kalimantan)
100	AH_{19a}	*A. agallocha* (Kalimantan)
101	AH_{19b}	*A. agallocha* (Kalimantan)
102	AH_{20}	*A. agallocha* (Kalimantan)
103	2-[2-(4-Hydroxy-3-methoxyphenyl)ethyl]-6-methoxy-4*H*-chromen-4-one	*A. malaccensis* (Indonesia)
104	6,8-Dihydroxy-2-(2-phenylethyl)-4*H*-chromen-4-one	*A. malaccensis* (Indonesia)
105	6-Hydroxy-2-[2-(4-hydroxyphenyl)ethyl]-4*H*-chromen-4-one	*A. malaccensis* (Indonesia)
106	6-Hydroxy-2-[2-(2-hydroxyphenyl)ethyl]-4*H*-chromen-4-one	*A. malaccensis* (Indonesia)
107	7-Hydroxy-2-(2-phenylethyl)-4*H*-chromen-4-one	*A. malaccensis* (Indonesia)
108	7-Hydroxy-8-methoxy-2-(2-phenylethyl)-4*H*-chromen-4-one	*A. malaccensis* (Indonesia)
109	5-Hydroxy-6-methoxy-2-(2-phenylethyl)-4*H*-chromen-4-one	*A. sinensis* (China)
110	6-Hydroxy-2-(2-hydroxy-2-phenylethyl)-4*H*-chromen-4-one	*A. sinensis* (China)
111	(5*S*,6*S*,7*S*,8*R*)-8-Chloro-5,6,7,8-tetrahydro-5,6,7-trihydroxy-2-(2-phenylethyl)-4*H*-chromen-4-one	*A. sinensis* (China)
112	(6*S*,7*R*)-5,6,7,8-tetrahydro-6,7-dihydroxy-2-(2-phenylethyl)-4*H*-chromen-4-one	*A. sinensis* (China)
113	(5*R*,6*R*,7*R*,8*R*)-5,6 : 7,8-Diepoxy-5,6,7,8-tetrahydro-2-(2-phenylethyl)-4*H*-chromen-4-one	*A. crassna* (Vienam)/ *A. sinensis* (China)
114	(5*R*,6*R*,7*R*,8*R*)-5,6 : 7,8-Diepoxy-5,6,7,8-tetrahydro-2-[2-(4-methoxyphenyl)ethyl]-4*H*-chromen-4-one	*A. crassna* (Vienam)/ *A. sinensis* (China)
115	(5*R*,6*R*,7*R*,8*R*)-5,6 : 7,8-Diepoxy-5,6,7,8-tetrahydro-2-[2-(3-hydroxy-4-methoxyphenyl)ethyl]-4*H*-chromen-4-one	*A. crassna* (Vienam)/ *A. sinensis* (China)
116	2-[2-(3-Acetoxyphenyl)ethyl]-5,8-dimethoxy-4*H*-chromen-4-one	*A. agallocha* (Cambodia)
117	6,8-Dihydroxy-2-[2-(4-hydroxy-3-methoxyphenyl)ethyl]-4*H*-chromen-4-one	*A. sinensis* (China)
118	2-[2-(4-Hydroxy-3-methoxyphenyl)ethyl]-6-methoxy-4*H*-chromen-4-one	*A. sinensis* (China)
119	6-Hydroxy-2-[2-(4-hydroxy-3-methoxyphenyl)ethyl]-4*H*-chromen-4-one	*A. sinensis* (China)
120	(5*S*,6*S*,7*S*,8*R*)-8-Chloro-5,6,7,8-tetrahydro-5,6,7-trihydroxy-2-[2-(3-hydroxy-4-methoxyphenyl)ethyl]-4*H*-chromen-4-one	*A. sinensis* (China)
121	(5*S*,6*S*,7*R*,8*S*)-5,6,7,8-Tetrahydro-5,6,7,8-tetrahydroxy-2-[2-(3-hydroxy-4-methoxyphenyl)ethyl]-4*H*-chromen-4-one	*A. sinensis* (China)

Table (cont.)

No.	Compound class and name	Source or origin
122	(5*S*,6*R*,7*S*)-5,6,7,8-Tetrahydro-5,6,7-trihydroxy-2-[2-(3-hydroxy-4-methoxyphenyl)ethyl]-4*H*-chromen-4-one	*A. sinensis* (China)
123	(5*S*,6*R*,7*R*)-5,6,7,8-Tetrahydro-5,6,7-trihydroxy-2-[2-(3-hydroxy-4-methoxyphenyl)ethyl]-4*H*-chromen-4-one	*A. sinensis* (China)
	Aromatics	
124	Benzylacetone	Review/*A. sinensis* (China)
125	(*p*-Methoxybenzyl)acetone	Review/*A. sinensis* (China)
126	Anisic acid	*A. sinensis* (China)
	Triterpenes	
127	22-Hydroxyhopan-3-one	*A. sinensis* (China)
128	Hederagenin	*A. sinensis* (China)
	Others	
129	(*E*)-Undeca-8,10-dien-2-one	*A. agallocha* (Vietnam)
130	(2*R*,3*S*)-2,3-Dimethyl-2-(3-methylbut-2-en-1-yl)-cyclohexanone	*A. agallocha* (Vietnam)
131	Methyl abieta-8(14),9(11),12-trien-19-oate	*A. agallocha* (Cambodia)
132	Aquillochin	*A. agallocha* (India)

Statistical analysis

Part 1. To evaluate effect of Aquilaria crassna crude extract on cell proliferation, cell attachment and osteogenic differentiation of MC3T3-E1 cells

Cell viability

Cell Viability MTT 24h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	3	.06533	.001528	.000882	.06154	.06913	.064	.067
10 ug/ml	3	.06767	.002517	.001453	.06142	.07392	.065	.070
25 ug/ml	3	.06733	.002887	.001667	.06016	.07450	.064	.069
50 ug/ml	3	.06767	.000577	.000333	.06623	.06910	.067	.068
100 ug/ml	3	.03000	.003464	.002000	.02139	.03861	.026	.032
500 ug/ml	3	.01333	.001528	.000882	.00954	.01713	.012	.015
1,000 ug/ml	3	.01433	.001155	.000667	.01146	.01720	.013	.015
Total	21	.04652	.024841	.005421	.03522	.05783	.012	.070

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
2.617	6	14	.065

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.012	6	.002	433.973	.000
Within Groups	.000	14	.000		
Total	.012	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE Cytotoxicity	(J) AE Cytotoxicity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	10 ug/ml	-.002333	.001773	.834	-.00839	.00372
	25 ug/ml	-.002000	.001773	.909	-.00805	.00405
	50 ug/ml	-.002333	.001773	.834	-.00839	.00372
	100 ug/ml	.035333*	.001773	.000	.02928	.04139
	500 ug/ml	.052000*	.001773	.000	.04595	.05805
	1,000 ug/ml	.051000*	.001773	.000	.04495	.05705
10 ug/ml	Control	.002333	.001773	.834	-.00372	.00839
	25 ug/ml	.000333	.001773	1.000	-.00572	.00639
	50 ug/ml	.000000	.001773	1.000	-.00605	.00605
	100 ug/ml	.037667*	.001773	.000	.03161	.04372
	500 ug/ml	.054333*	.001773	.000	.04828	.06039
	1,000 ug/ml	.053333*	.001773	.000	.04728	.05939
25 ug/ml	Control	.002000	.001773	.909	-.00405	.00805
	10 ug/ml	-.000333	.001773	1.000	-.00639	.00572
	50 ug/ml	-.000333	.001773	1.000	-.00639	.00572
	100 ug/ml	.037333*	.001773	.000	.03128	.04339
	500 ug/ml	.054000*	.001773	.000	.04795	.06005
	1,000 ug/ml	.053000*	.001773	.000	.04695	.05905
50 ug/ml	Control	.002333	.001773	.834	-.00372	.00839
	10 ug/ml	.000000	.001773	1.000	-.00605	.00605
	25 ug/ml	.000333	.001773	1.000	-.00572	.00639
	100 ug/ml	.037667*	.001773	.000	.03161	.04372
	500 ug/ml	.054333*	.001773	.000	.04828	.06039
	1,000 ug/ml	.053333*	.001773	.000	.04728	.05939
100 ug/ml	Control	-.035333*	.001773	.000	-.04139	-.02928
	10 ug/ml	-.037667*	.001773	.000	-.04372	-.03161
	25 ug/ml	-.037333*	.001773	.000	-.04339	-.03128
	50 ug/ml	-.037667*	.001773	.000	-.04372	-.03161
	500 ug/ml	.016667*	.001773	.000	.01061	.02272
	1,000 ug/ml	.015667*	.001773	.000	.00961	.02172
500 ug/ml	Control	-.052000*	.001773	.000	-.05805	-.04595
	10 ug/ml	-.054333*	.001773	.000	-.06039	-.04828
	25 ug/ml	-.054000*	.001773	.000	-.06005	-.04795
	50 ug/ml	-.054333*	.001773	.000	-.06039	-.04828
	100 ug/ml	-.016667*	.001773	.000	-.02272	-.01061
	1,000 ug/ml	-.001000	.001773	.997	-.00705	.00505
1,000 ug/ml	Control	-.051000*	.001773	.000	-.05705	-.04495
	10 ug/ml	-.053333*	.001773	.000	-.05939	-.04728
	25 ug/ml	-.053000*	.001773	.000	-.05905	-.04695
	50 ug/ml	-.053333*	.001773	.000	-.05939	-.04728

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE Cytotoxicity	(J) AE Cytotoxicity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1,000 ug/ml	100 ug/ml	-.015667	.001773	.000	-.02172	-.00961
	500 ug/ml	.001000	.001773	.997	-.00505	.00705

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

AE Cytotoxicity	N	Subset for alpha = 0.05		
		1	2	3
500 ug/ml	3	.01333		
1,000 ug/ml	3	.01433		
100 ug/ml	3		.03000	
Control	3			.06533
25 ug/ml	3			.06733
10 ug/ml	3			.06767
50 ug/ml	3			.06767
Sig.		.997	1.000	.834

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell proliferation

Cell Proliferation MTT 24h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	.05867	.000577	.000333	.05723	.06010	.058	.059
AE 10	3	.06200	.001000	.000577	.05952	.06448	.061	.063
AE 25	3	.06100	.001732	.001000	.05670	.06530	.060	.063
AE 50	3	.06900	.001732	.001000	.06470	.07330	.068	.071
Total	12	.06267	.004185	.001208	.06001	.06533	.058	.071

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
2.429	3	8	.140

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	32.364	.000
Within Groups	.000	8	.000		
Total	.000	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.003333	.001106	.065	-.00687	.00021
	AE 25	-.002333	.001106	.228	-.00587	.00121
	AE 50	-.010333*	.001106	.000	-.01387	-.00679
AE 10	control	.003333	.001106	.065	-.00021	.00687
	AE 25	.001000	.001106	.803	-.00254	.00454
	AE 50	-.007000*	.001106	.001	-.01054	-.00346
AE 25	control	.002333	.001106	.228	-.00121	.00587
	AE 10	-.001000	.001106	.803	-.00454	.00254
	AE 50	-.008000*	.001106	.000	-.01154	-.00446
AE 50	control	.010333*	.001106	.000	.00679	.01387
	AE 10	.007000*	.001106	.001	.00346	.01054
	AE 25	.008000*	.001106	.000	.00446	.01154

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05	
		1	2
control	3	.05867	
AE 25	3	.06100	
AE 10	3	.06200	
AE 50	3		.06900
Sig.		.065	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell Proliferation MTT 48h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	.16767	.005508	.003180	.15399	.18135	.164	.174
AE 10	3	.17967	.005686	.003283	.16554	.19379	.175	.186
AE 25	3	.17433	.006658	.003844	.15779	.19087	.170	.182
AE 50	3	.72900	.953532	.550522	-1.63970	3.09770	.170	1.830
Total	12	.31267	.477894	.137956	.00903	.61631	.164	1.830

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
15.790	3	8	.001

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.694	3	.231	1.017	.435
Within Groups	1.819	8	.227		
Total	2.512	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.012000	.389301	1.000	-1.25868	1.23468
	AE 25	-.006667	.389301	1.000	-1.25334	1.24001
	AE 50	-.561333	.389301	.510	-1.80801	.68534
AE 10	control	.012000	.389301	1.000	-1.23468	1.25868
	AE 25	.005333	.389301	1.000	-1.24134	1.25201
	AE 50	-.549333	.389301	.527	-1.79601	.69734
AE 25	control	.006667	.389301	1.000	-1.24001	1.25334
	AE 10	-.005333	.389301	1.000	-1.25201	1.24134
	AE 50	-.554667	.389301	.520	-1.80134	.69201
AE 50	control	.561333	.389301	.510	-.68534	1.80801
	AE 10	.549333	.389301	.527	-.69734	1.79601
	AE 25	.554667	.389301	.520	-.69201	1.80134

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05
		1
control	3	.16767
AE 25	3	.17433
AE 10	3	.17967
AE 50	3	.72900
Sig.		.510

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell Proliferation MTT 72h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	.29567	.010263	.005925	.27017	.32116	.287	.307
AE 10	3	.29167	.007767	.004485	.27237	.31096	.283	.298
AE 25	3	.30100	.005568	.003215	.28717	.31483	.296	.307
AE 50	3	.28967	.006028	.003480	.27469	.30464	.284	.296
Total	12	.29450	.007926	.002288	.28946	.29954	.283	.307

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
.699	3	8	.578

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	1.288	.343
Within Groups	.000	8	.000		
Total	.001	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	.004000	.006232	.915	-.01596	.02396
	AE 25	-.005333	.006232	.827	-.02529	.01462
	AE 50	.006000	.006232	.773	-.01396	.02596
AE 10	control	-.004000	.006232	.915	-.02396	.01596
	AE 25	-.009333	.006232	.481	-.02929	.01062
	AE 50	.002000	.006232	.988	-.01796	.02196
AE 25	control	.005333	.006232	.827	-.01462	.02529
	AE 10	.009333	.006232	.481	-.01062	.02929
	AE 50	.011333	.006232	.332	-.00862	.03129
AE 50	control	-.006000	.006232	.773	-.02596	.01396
	AE 10	-.002000	.006232	.988	-.02196	.01796
	AE 25	-.011333	.006232	.332	-.03129	.00862

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05
		1
AE 50	3	.28967
AE 10	3	.29167
control	3	.29567
AE 25	3	.30100
Sig.		.332

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell attachment

Cell Attachment 4 h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	.06267	.001155	.000667	.05980	.06554	.062	.064
AE 10	3	.06967	.002082	.001202	.06450	.07484	.068	.072
AE 25	3	.07700	.003464	.002000	.06839	.08561	.075	.081
AE 50	3	.08267	.004041	.002333	.07263	.09271	.078	.085
Total	12	.07300	.008257	.002384	.06775	.07825	.062	.085

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
3.099	3	8	.089

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	3	.000	26.745	.000
Within Groups	.000	8	.000		
Total	.001	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.007000	.002380	.072	-.01462	.00062
	AE 25	-.014333*	.002380	.001	-.02196	-.00671
	AE 50	-.020000*	.002380	.000	-.02762	-.01238
AE 10	control	.007000	.002380	.072	-.00062	.01462
	AE 25	-.007333	.002380	.059	-.01496	.00029
	AE 50	-.013000*	.002380	.003	-.02062	-.00538
AE 25	control	.014333*	.002380	.001	.00671	.02196
	AE 10	.007333	.002380	.059	-.00029	.01496
	AE 50	-.005667	.002380	.159	-.01329	.00196
AE 50	control	.020000*	.002380	.000	.01238	.02762
	AE 10	.013000*	.002380	.003	.00538	.02062
	AE 25	.005667	.002380	.159	-.00196	.01329

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05		
		1	2	3
control	3	.06267		
AE 10	3	.06967	.06967	
AE 25	3		.07700	.07700
AE 50	3			.08267
Sig.		.072	.059	.159

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell Attachment 24 h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	.06333	.004163	.002404	.05299	.07368	.060	.068
AE 10	3	.06667	.002309	.001333	.06093	.07240	.064	.068
AE 25	3	.07533	.004163	.002404	.06499	.08568	.072	.080
AE 50	3	.09033	.002517	.001453	.08408	.09658	.088	.093
Total	12	.07392	.011285	.003258	.06675	.08109	.060	.093

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
.910	3	8	.478

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	3	.000	37.647	.000
Within Groups	.000	8	.000		
Total	.001	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.003333	.002779	.644	-.01223	.00557
	AE 25	-.012000*	.002779	.011	-.02090	-.00310
	AE 50	-.027000*	.002779	.000	-.03590	-.01810
AE 10	control	.003333	.002779	.644	-.00557	.01223
	AE 25	-.008667	.002779	.056	-.01757	.00023
	AE 50	-.023667*	.002779	.000	-.03257	-.01477
AE 25	control	.012000*	.002779	.011	.00310	.02090
	AE 10	.008667	.002779	.056	-.00023	.01757
	AE 50	-.015000*	.002779	.003	-.02390	-.00610
AE 50	control	.027000*	.002779	.000	.01810	.03590
	AE 10	.023667*	.002779	.000	.01477	.03257
	AE 25	.015000*	.002779	.003	.00610	.02390

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05		
		1	2	3
control	3	.06333		
AE 10	3	.06667	.06667	
AE 25	3		.07533	
AE 50	3			.09033
Sig.		.644	.056	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Alkaline phosphatase activity

ALP 7 days

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	51.86733	2.692704	1.554633	45.17829	58.55638	49.092	54.469
AE 10	3	52.43733	1.761628	1.017077	48.06121	56.81346	50.405	53.528
AE 25	3	60.23867	5.100353	2.944690	47.56869	72.90865	54.420	63.936
AE 50	3	76.54033	8.539473	4.930267	55.32711	97.75356	69.815	86.148
Total	12	60.27092	11.316203	3.266706	53.08094	67.46089	49.092	86.148

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
3.664	3	8	.063

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1190.041	3	396.680	14.518	.001
Within Groups	218.580	8	27.323		
Total	1408.621	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.570000	4.267907	.999	-14.23734	13.09734
	AE 25	-8.371333	4.267907	.277	-22.03867	5.29601
	AE 50	-24.673000*	4.267907	.002	-38.34034	-11.00566
AE 10	control	.570000	4.267907	.999	-13.09734	14.23734
	AE 25	-7.801333	4.267907	.328	-21.46867	5.86601
	AE 50	-24.103000*	4.267907	.002	-37.77034	-10.43566
AE 25	control	8.371333	4.267907	.277	-5.29601	22.03867
	AE 10	7.801333	4.267907	.328	-5.86601	21.46867
	AE 50	-16.301667*	4.267907	.021	-29.96901	-2.63433
AE 50	control	24.673000*	4.267907	.002	11.00566	38.34034
	AE 10	24.103000*	4.267907	.002	10.43566	37.77034
	AE 25	16.301667*	4.267907	.021	2.63433	29.96901

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05	
		1	2
control	3	51.86733	
AE 10	3	52.43733	
AE 25	3	60.23867	
AE 50	3		76.54033
Sig.		.277	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ALP 14 days

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	54.30833	11.754613	6.786529	25.10826	83.50841	42.177	65.646
AE 10	3	58.48800	10.106366	5.834913	33.38240	83.59360	47.357	67.089
AE 25	3	84.76867	5.911594	3.413061	70.08345	99.45388	79.102	90.898
AE 50	3	108.21700	3.561643	2.056316	99.36939	117.06461	104.331	111.326
Total	12	76.44550	23.835747	6.880788	61.30099	91.59001	42.177	111.326

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
1.156	3	8	.385

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5673.688	3	1891.229	26.272	.000
Within Groups	575.884	8	71.985		
Total	6249.571	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-4.179667	6.927503	.928	-26.36397	18.00464
	AE 25	-30.460333*	6.927503	.010	-52.64464	-8.27603
	AE 50	-53.908667*	6.927503	.000	-76.09297	-31.72436
AE 10	control	4.179667	6.927503	.928	-18.00464	26.36397
	AE 25	-26.280667*	6.927503	.022	-48.46497	-4.09636
	AE 50	-49.729000*	6.927503	.000	-71.91330	-27.54470
AE 25	control	30.460333*	6.927503	.010	8.27603	52.64464
	AE 10	26.280667*	6.927503	.022	4.09636	48.46497
	AE 50	-23.448333*	6.927503	.039	-45.63264	-1.26403
AE 50	control	53.908667*	6.927503	.000	31.72436	76.09297
	AE 10	49.729000*	6.927503	.000	27.54470	71.91330
	AE 25	23.448333*	6.927503	.039	1.26403	45.63264

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05		
		1	2	3
control	3	54.30833		
AE 10	3	58.48800		
AE 25	3		84.76867	
AE 50	3			108.21700
Sig.		.928	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ALP 21 days

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	41.73800	3.794853	2.190959	32.31106	51.16494	37.886	45.473
AE 10	3	50.41400	3.715719	2.145272	41.18364	59.64436	46.525	53.928
AE 25	3	51.25933	3.565922	2.058786	42.40109	60.11757	47.157	53.617
AE 50	3	52.72333	4.908590	2.833976	40.52972	64.91695	47.621	57.412
Total	12	49.03367	5.649883	1.630981	45.44390	52.62343	37.886	57.412

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
.112	3	8	.951

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	221.098	3	73.699	4.534	.039
Within Groups	130.035	8	16.254		
Total	351.133	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-8.676000	3.291847	.111	-19.21765	1.86565
	AE 25	-9.521333	3.291847	.077	-20.06299	1.02032
	AE 50	-10.985333*	3.291847	.041	-21.52699	-.44368
AE 10	control	8.676000	3.291847	.111	-1.86565	19.21765
	AE 25	-.845333	3.291847	.994	-11.38699	9.69632
	AE 50	-2.309333	3.291847	.894	-12.85099	8.23232
AE 25	control	9.521333	3.291847	.077	-1.02032	20.06299
	AE 10	.845333	3.291847	.994	-9.69632	11.38699
	AE 50	-1.464000	3.291847	.969	-12.00565	9.07765
AE 50	control	10.985333*	3.291847	.041	.44368	21.52699
	AE 10	2.309333	3.291847	.894	-8.23232	12.85099
	AE 25	1.464000	3.291847	.969	-9.07765	12.00565

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05	
		1	2
control	3	41.73800	
AE 10	3	50.41400	50.41400
AE 25	3	51.25933	51.25933
AE 50	3		52.72333
Sig.		.077	.894

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Expression of Col 1 gene

Col1 gene 7 days Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	.84800	.129047	.074505	.52743	1.16857	.699	.924
AE 25	3	.90500	.102132	.058966	.65129	1.15871	.794	.995
AE 50	3	1.33300	.118655	.068505	1.03824	1.62776	1.200	1.428
Total	12	1.02150	.214441	.061904	.88525	1.15775	.699	1.428

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
3.804	3	8	.058

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.424	3	.141	13.718	.002
Within Groups	.082	8	.010		
Total	.506	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	.152000	.082828	.325	-.11324	.41724
	AE 25	.095000	.082828	.673	-.17024	.36024
	AE 50	-.333000*	.082828	.016	-.59824	-.06776
AE 10	control	-.152000	.082828	.325	-.41724	.11324
	AE 25	-.057000	.082828	.899	-.32224	.20824
	AE 50	-.485000*	.082828	.002	-.75024	-.21976
AE 25	control	-.095000	.082828	.673	-.36024	.17024
	AE 10	.057000	.082828	.899	-.20824	.32224
	AE 50	-.428000*	.082828	.004	-.69324	-.16276
AE 50	control	.333000*	.082828	.016	.06776	.59824
	AE 10	.485000*	.082828	.002	.21976	.75024
	AE 25	.428000*	.082828	.004	.16276	.69324

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05	
		1	2
AE 10	3	.84800	
AE 25	3	.90500	
control	3	1.00000	
AE 50	3		1.33300
Sig.		.325	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Col1 gene 14 days Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	1.24333	.248846	.143671	.62517	1.86150	1.019	1.511
AE 25	3	1.66200	.212671	.122786	1.13370	2.19030	1.469	1.890
AE 50	3	5.50867	.636099	.367252	3.92851	7.08882	4.842	6.109
Total	12	2.35350	1.942739	.560821	1.11914	3.58786	1.000	6.109

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
3.077	3	8	.090

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	40.493	3	13.498	105.497	.000
Within Groups	1.024	8	.128		
Total	41.517	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.243333	.292055	.838	-1.17860	.69193
	AE 25	-.662000	.292055	.185	-1.59726	.27326
	AE 50	-4.508667*	.292055	.000	-5.44393	-3.57340
AE 10	control	.243333	.292055	.838	-.69193	1.17860
	AE 25	-.418667	.292055	.515	-1.35393	.51660
	AE 50	-4.265333*	.292055	.000	-5.20060	-3.33007
AE 25	control	.662000	.292055	.185	-.27326	1.59726
	AE 10	.418667	.292055	.515	-.51660	1.35393
	AE 50	-3.846667*	.292055	.000	-4.78193	-2.91140
AE 50	control	4.508667*	.292055	.000	3.57340	5.44393
	AE 10	4.265333*	.292055	.000	3.33007	5.20060
	AE 25	3.846667*	.292055	.000	2.91140	4.78193

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05	
		1	2
control	3	1.00000	
AE 10	3	1.24333	
AE 25	3	1.66200	
AE 50	3		5.50867
Sig.		.185	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Col1 gene 21 days Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	1.19300	.275512	.159067	.50859	1.87741	1.026	1.511
AE 25	3	1.60567	.357500	.206403	.71759	2.49375	1.248	1.963
AE 50	3	4.13000	.654140	.377668	2.50503	5.75497	3.597	4.860
Total	12	1.98217	1.358152	.392065	1.11924	2.84510	1.000	4.860

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
3.853	3	8	.056

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19.027	3	6.342	40.166	.000
Within Groups	1.263	8	.158		
Total	20.290	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.193000	.324451	.931	-1.23201	.84601
	AE 25	-.605667	.324451	.313	-1.64467	.43334
	AE 50	-3.130000*	.324451	.000	-4.16901	-2.09099
AE 10	control	.193000	.324451	.931	-.84601	1.23201
	AE 25	-.412667	.324451	.603	-1.45167	.62634
	AE 50	-2.937000*	.324451	.000	-3.97601	-1.89799
AE 25	control	.605667	.324451	.313	-.43334	1.64467
	AE 10	.412667	.324451	.603	-.62634	1.45167
	AE 50	-2.524333*	.324451	.000	-3.56334	-1.48533
AE 50	control	3.130000*	.324451	.000	2.09099	4.16901
	AE 10	2.937000*	.324451	.000	1.89799	3.97601
	AE 25	2.524333*	.324451	.000	1.48533	3.56334

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05	
		1	2
control	3	1.00000	
AE 10	3	1.19300	
AE 25	3	1.60567	
AE 50	3		4.13000
Sig.		.313	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Expression of ALP gene

ALP gene 7 day Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	1.02567	.329215	.190072	.20785	1.84348	.752	1.391
AE 25	3	1.41000	.307000	.177247	.64737	2.17263	1.103	1.717
AE 50	3	2.13400	.177764	.102632	1.69241	2.57559	1.994	2.334
Total	12	1.39242	.520901	.150371	1.06145	1.72338	.752	2.334

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
2.470	3	8	.136

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.516	3	.839	14.323	.001
Within Groups	.468	8	.059		
Total	2.985	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.025667	.197582	.999	-.65839	.60706
	AE 25	-.410000	.197582	.239	-1.04273	.22273
	AE 50	-1.134000*	.197582	.002	-1.76673	-.50127
AE 10	control	.025667	.197582	.999	-.60706	.65839
	AE 25	-.384333	.197582	.283	-1.01706	.24839
	AE 50	-1.108333*	.197582	.002	-1.74106	-.47561
AE 25	control	.410000	.197582	.239	-.22273	1.04273
	AE 10	.384333	.197582	.283	-.24839	1.01706
	AE 50	-.724000*	.197582	.026	-1.35673	-.09127
AE 50	control	1.134000*	.197582	.002	.50127	1.76673
	AE 10	1.108333*	.197582	.002	.47561	1.74106
	AE 25	.724000*	.197582	.026	.09127	1.35673

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05	
		1	2
control	3	1.00000	
AE 10	3	1.02567	
AE 25	3	1.41000	
AE 50	3		2.13400
Sig.		.239	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ALP gene 14 day Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	1.41133	.127500	.073612	1.09460	1.72806	1.320	1.557
AE 25	3	2.80700	.452636	.261329	1.68259	3.93141	2.374	3.277
AE 50	3	4.56133	.525484	.303388	3.25596	5.86671	3.968	4.968
Total	12	2.44492	1.486110	.429003	1.50069	3.38915	1.000	4.968

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
4.020	3	8	.051

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23.299	3	7.766	62.472	.000
Within Groups	.995	8	.124		
Total	24.294	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.411333	.287886	.517	-1.33324	.51058
	AE 25	-1.807000*	.287886	.001	-2.72891	-.88509
	AE 50	-3.561333*	.287886	.000	-4.48324	-2.63942
AE 10	control	.411333	.287886	.517	-.51058	1.33324
	AE 25	-1.395667*	.287886	.006	-2.31758	-.47376
	AE 50	-3.150000*	.287886	.000	-4.07191	-2.22809
AE 25	control	1.807000*	.287886	.001	.88509	2.72891
	AE 10	1.395667*	.287886	.006	.47376	2.31758
	AE 50	-1.754333*	.287886	.001	-2.67624	-.83242
AE 50	control	3.561333*	.287886	.000	2.63942	4.48324
	AE 10	3.150000*	.287886	.000	2.22809	4.07191
	AE 25	1.754333*	.287886	.001	.83242	2.67624

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05		
		1	2	3
control	3	1.00000		
AE 10	3	1.41133		
AE 25	3		2.80700	
AE 50	3			4.56133
Sig.		.517	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ALP gene 21 day Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	1.51000	.294557	.170063	.77828	2.24172	1.170	1.688
AE 25	3	1.65100	.313000	.180711	.87346	2.42854	1.338	1.964
AE 50	3	2.60167	.371500	.214486	1.67881	3.52452	2.230	2.973
Total	12	1.69067	.651499	.188071	1.27672	2.10461	1.000	2.973

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
1.797	3	8	.226

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.023	3	1.341	16.622	.001
Within Groups	.645	8	.081		
Total	4.669	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.510000	.231929	.203	-1.25272	.23272
	AE 25	-.651000	.231929	.087	-1.39372	.09172
	AE 50	-1.601667*	.231929	.001	-2.34438	-.85895
AE 10	control	.510000	.231929	.203	-.23272	1.25272
	AE 25	-.141000	.231929	.927	-.88372	.60172
	AE 50	-1.091667*	.231929	.007	-1.83438	-.34895
AE 25	control	.651000	.231929	.087	-.09172	1.39372
	AE 10	.141000	.231929	.927	-.60172	.88372
	AE 50	-.950667*	.231929	.015	-1.69338	-.20795
AE 50	control	1.601667*	.231929	.001	.85895	2.34438
	AE 10	1.091667*	.231929	.007	.34895	1.83438
	AE 25	.950667*	.231929	.015	.20795	1.69338

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05	
		1	2
control	3	1.00000	
AE 10	3	1.51000	
AE 25	3	1.65100	
AE 50	3		2.60167
Sig.		.087	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Expression of BSP gene

BSP gene 7days Realtime PCR

Onewa

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	.85167	.103196	.059580	.59531	1.10802	.745	.951
AE 25	3	1.00600	.246000	.142028	.39490	1.61710	.760	1.252
AE 50	3	1.19633	.178408	.103004	.75314	1.63952	1.063	1.399
Total	12	1.01350	.187219	.054045	.89455	1.13245	.745	1.399

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
2.303	3	8	.154

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.180	3	.060	2.325	.151
Within Groups	.206	8	.026		
Total	.386	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	.148333	.131018	.682	-.27123	.56790
	AE 25	-.006000	.131018	1.000	-.42557	.41357
	AE 50	-.196333	.131018	.481	-.61590	.22323
AE 10	control	-.148333	.131018	.682	-.56790	.27123
	AE 25	-.154333	.131018	.656	-.57390	.26523
	AE 50	-.344667	.131018	.112	-.76423	.07490
AE 25	control	.006000	.131018	1.000	-.41357	.42557
	AE 10	.154333	.131018	.656	-.26523	.57390
	AE 50	-.190333	.131018	.505	-.60990	.22923
AE 50	control	.196333	.131018	.481	-.22323	.61590
	AE 10	.344667	.131018	.112	-.07490	.76423
	AE 25	.190333	.131018	.505	-.22923	.60990

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05
		1
AE 10	3	.85167
control	3	1.00000
AE 25	3	1.00600
AE 50	3	1.19633
Sig.		.112

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

BSP gene 14 days Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	1.39833	.240804	.139028	.80014	1.99652	1.229	1.674
AE 25	3	1.85633	.146118	.084361	1.49336	2.21931	1.690	1.964
AE 50	3	2.55067	.154500	.089201	2.16687	2.93447	2.396	2.705
Total	12	1.70133	.617445	.178241	1.30903	2.09364	1.000	2.705

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
3.778	3	8	.059

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.987	3	1.329	51.511	.000
Within Groups	.206	8	.026		
Total	4.194	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.398333	.131153	.063	-.81833	.02167
	AE 25	-.856333*	.131153	.001	-1.27633	-.43633
	AE 50	-1.550667*	.131153	.000	-1.97067	-1.13067
AE 10	control	.398333	.131153	.063	-.02167	.81833
	AE 25	-.458000*	.131153	.033	-.87800	-.03800
	AE 50	-1.152333*	.131153	.000	-1.57233	-.73233
AE 25	control	.856333*	.131153	.001	.43633	1.27633
	AE 10	.458000*	.131153	.033	.03800	.87800
	AE 50	-.694333*	.131153	.003	-1.11433	-.27433
AE 50	control	1.550667*	.131153	.000	1.13067	1.97067
	AE 10	1.152333*	.131153	.000	.73233	1.57233
	AE 25	.694333*	.131153	.003	.27433	1.11433

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05		
		1	2	3
control	3	1.00000		
AE 10	3	1.39833		
AE 25	3		1.85633	
AE 50	3			2.55067
Sig.		.063	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

BSP gene 21 days Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	1.68333	.254079	.146693	1.05217	2.31450	1.469	1.964
AE 25	3	2.39433	.312666	.180518	1.61763	3.17104	2.034	2.594
AE 50	3	3.78467	.338042	.195169	2.94492	4.62441	3.405	4.053
Total	12	2.21558	1.100326	.317637	1.51647	2.91470	1.000	4.053

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
3.963	3	8	.053

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.765	3	4.255	61.534	.000
Within Groups	.553	8	.069		
Total	13.318	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.683333	.214705	.051	-1.37089	.00423
	AE 25	-1.394333*	.214705	.001	-2.08189	-.70677
	AE 50	-2.784667*	.214705	.000	-3.47223	-2.09711
AE 10	control	.683333	.214705	.051	-.00423	1.37089
	AE 25	-.711000*	.214705	.043	-1.39856	-.02344
	AE 50	-2.101333*	.214705	.000	-2.78889	-1.41377
AE 25	control	1.394333*	.214705	.001	.70677	2.08189
	AE 10	.711000*	.214705	.043	.02344	1.39856
	AE 50	-1.390333*	.214705	.001	-2.07789	-.70277
AE 50	control	2.784667*	.214705	.000	2.09711	3.47223
	AE 10	2.101333*	.214705	.000	1.41377	2.78889
	AE 25	1.390333*	.214705	.001	.70277	2.07789

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05		
		1	2	3
control	3	1.00000		
AE 10	3	1.68333		
AE 25	3		2.39433	
AE 50	3			3.78467
Sig.		.051	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Expression of OCN gene

OCN gene 7 days Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	.73800	.197547	.114054	.24726	1.22874	.543	.938
AE 25	3	.78100	.181133	.104577	.33104	1.23096	.628	.981
AE 50	3	1.03900	.086000	.049652	.82536	1.25264	.953	1.125
Total	12	.88950	.182487	.052679	.77355	1.00545	.543	1.125

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
2.404	3	8	.143

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.208	3	.069	3.498	.070
Within Groups	.158	8	.020		
Total	.366	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	.262000	.114913	.182	-.10599	.62999
	AE 25	.219000	.114913	.298	-.14899	.58699
	AE 50	-.039000	.114913	.986	-.40699	.32899
AE 10	control	-.262000	.114913	.182	-.62999	.10599
	AE 25	-.043000	.114913	.981	-.41099	.32499
	AE 50	-.301000	.114913	.114	-.66899	.06699
AE 25	control	-.219000	.114913	.298	-.58699	.14899
	AE 10	.043000	.114913	.981	-.32499	.41099
	AE 50	-.258000	.114913	.191	-.62599	.10999
AE 50	control	.039000	.114913	.986	-.32899	.40699
	AE 10	.301000	.114913	.114	-.06699	.66899
	AE 25	.258000	.114913	.191	-.10999	.62599

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05
		1
AE 10	3	.73800
AE 25	3	.78100
control	3	1.00000
AE 50	3	1.03900
Sig.		.114

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

OCN gene 14 days Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	1.19100	.210000	.121244	.66933	1.71267	.981	1.401
AE 25	3	2.62533	.344500	.198897	1.76955	3.48112	2.281	2.970
AE 50	3	5.10700	.772000	.445714	3.18925	7.02475	4.335	5.879
Total	12	2.48083	1.753957	.506324	1.36642	3.59524	.981	5.879

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
2.242	3	8	.161

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32.323	3	10.774	56.799	.000
Within Groups	1.518	8	.190		
Total	33.840	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.191000	.355613	.947	-1.32980	.94780
	AE 25	-1.625333*	.355613	.008	-2.76413	-.48653
	AE 50	-4.107000*	.355613	.000	-5.24580	-2.96820
AE 10	control	.191000	.355613	.947	-.94780	1.32980
	AE 25	-1.434333*	.355613	.016	-2.57313	-.29553
	AE 50	-3.916000*	.355613	.000	-5.05480	-2.77720
AE 25	control	1.625333*	.355613	.008	.48653	2.76413
	AE 10	1.434333*	.355613	.016	.29553	2.57313
	AE 50	-2.481667*	.355613	.001	-3.62047	-1.34287
AE 50	control	4.107000*	.355613	.000	2.96820	5.24580
	AE 10	3.916000*	.355613	.000	2.77720	5.05480
	AE 25	2.481667*	.355613	.001	1.34287	3.62047

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05		
		1	2	3
control	3	1.00000		
AE 10	3	1.19100		
AE 25	3		2.62533	
AE 50	3			5.10700
Sig.		.947	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

OCN gene 21 days Realtime PCR

Oneway

Descriptives

RNA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	1.00000	.000000	.000000	1.00000	1.00000	1.000	1.000
AE 10	3	1.49033	.176684	.102009	1.05143	1.92924	1.345	1.687
AE 25	3	3.62700	.799242	.461443	1.64157	5.61243	2.705	4.123
AE 50	3	8.49400	1.156000	.667417	5.62234	11.36566	7.338	9.650
Total	12	3.65283	3.154609	.910657	1.64849	5.65718	1.000	9.650

Test of Homogeneity of Variances

RNA

Levene Statistic	df1	df2	Sig.
3.167	3	8	.085

ANOVA

RNA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	105.454	3	35.151	70.081	.000
Within Groups	4.013	8	.502		
Total	109.467	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RNA

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.490333	.578265	.831	-2.34214	1.36147
	AE 25	-2.627000*	.578265	.008	-4.47881	-.77519
	AE 50	-7.494000*	.578265	.000	-9.34581	-5.64219
AE 10	control	.490333	.578265	.831	-1.36147	2.34214
	AE 25	-2.136667*	.578265	.025	-3.98847	-.28486
	AE 50	-7.003667*	.578265	.000	-8.85547	-5.15186
AE 25	control	2.627000*	.578265	.008	.77519	4.47881
	AE 10	2.136667*	.578265	.025	.28486	3.98847
	AE 50	-4.867000*	.578265	.000	-6.71881	-3.01519
AE 50	control	7.494000*	.578265	.000	5.64219	9.34581
	AE 10	7.003667*	.578265	.000	5.15186	8.85547
	AE 25	4.867000*	.578265	.000	3.01519	6.71881

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

RNA

Tukey HSD

AE	N	Subset for alpha = 0.05		
		1	2	3
control	3	1.00000		
AE 10	3	1.49033		
AE 25	3		3.62700	
AE 50	3			8.49400
Sig.		.831	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Osteocalcin product evaluation

ELISA OCN 21 days

Oneway

Descriptives

OC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	.46333	.050501	.029157	.33788	.58878	.413	.514
AE 10	3	.52433	.030089	.017372	.44959	.59908	.493	.553
AE 25	3	1.16167	.119818	.069177	.86402	1.45931	1.029	1.262
AE 50	3	1.81267	.156513	.090363	1.42387	2.20147	1.655	1.968
Total	12	.99050	.578873	.167106	.62270	1.35830	.413	1.968

Test of Homogeneity of Variances

OC

Levene Statistic	df1	df2	Sig.
1.734	3	8	.237

ANOVA

OC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.601	3	1.200	113.497	.000
Within Groups	.085	8	.011		
Total	3.686	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OC

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.061000	.083973	.884	-.32991	.20791
	AE 25	-.698333*	.083973	.000	-.96724	-.42942
	AE 50	-1.349333*	.083973	.000	-1.61824	-1.08042
AE 10	control	.061000	.083973	.884	-.20791	.32991
	AE 25	-.637333*	.083973	.000	-.90624	-.36842
	AE 50	-1.288333*	.083973	.000	-1.55724	-1.01942
AE 25	control	.698333*	.083973	.000	.42942	.96724
	AE 10	.637333*	.083973	.000	.36842	.90624
	AE 50	-.651000*	.083973	.000	-.91991	-.38209
AE 50	control	1.349333*	.083973	.000	1.08042	1.61824
	AE 10	1.288333*	.083973	.000	1.01942	1.55724
	AE 25	.651000*	.083973	.000	.38209	.91991

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OC protein

Tukey HSD

AE	N	Subset for alpha = 0.05		
		1	2	3
control	3	.46333		
AE 10	3	.52433		
AE 25	3		1.16167	
AE 50	3			1.81267
Sig.		.884	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Mineral deposition

Alizarin Red 7 days

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	.11233	.002082	.001202	.10716	.11750	.110	.114
AE 10	3	.11233	.004041	.002333	.10229	.12237	.110	.117
AE 25	3	.11300	.005196	.003000	.10009	.12591	.107	.116
AE 50	3	.11833	.001528	.000882	.11454	.12213	.117	.120
Total	12	.11400	.004000	.001155	.11146	.11654	.107	.120

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
3.621	3	8	.065

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	2.027	.189
Within Groups	.000	8	.000		
Total	.000	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	.000000	.002887	1.000	-.00924	.00924
	AE 25	-.000667	.002887	.995	-.00991	.00858
	AE 50	-.006000	.002887	.238	-.01524	.00324
AE 10	control	.000000	.002887	1.000	-.00924	.00924
	AE 25	-.000667	.002887	.995	-.00991	.00858
	AE 50	-.006000	.002887	.238	-.01524	.00324
AE 25	control	.000667	.002887	.995	-.00858	.00991
	AE 10	.000667	.002887	.995	-.00858	.00991
	AE 50	-.005333	.002887	.320	-.01458	.00391
AE 50	control	.006000	.002887	.238	-.00324	.01524
	AE 10	.006000	.002887	.238	-.00324	.01524
	AE 25	.005333	.002887	.320	-.00391	.01458

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05
		1
control	3	.11233
AE 10	3	.11233
AE 25	3	.11300
AE 50	3	.11833
Sig.		.238

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Alizarin Red 14 days

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	.21500	.006000	.003464	.20010	.22990	.209	.221
AE 10	3	.21467	.006110	.003528	.19949	.22984	.208	.220
AE 25	3	.22567	.004726	.002728	.21393	.23741	.222	.231
AE 50	3	.22567	.004726	.002728	.21393	.23741	.222	.231
Total	12	.22025	.007313	.002111	.21560	.22490	.208	.231

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
.082	3	8	.968

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	3.980	.052
Within Groups	.000	8	.000		
Total	.001	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	.000333	.004435	1.000	-.01387	.01453
	AE 25	-.010667	.004435	.153	-.02487	.00353
	AE 50	-.010667	.004435	.153	-.02487	.00353
AE 10	control	-.000333	.004435	1.000	-.01453	.01387
	AE 25	-.011000	.004435	.138	-.02520	.00320
	AE 50	-.011000	.004435	.138	-.02520	.00320
AE 25	control	.010667	.004435	.153	-.00353	.02487
	AE 10	.011000	.004435	.138	-.00320	.02520
	AE 50	.000000	.004435	1.000	-.01420	.01420
AE 50	control	.010667	.004435	.153	-.00353	.02487
	AE 10	.011000	.004435	.138	-.00320	.02520
	AE 25	.000000	.004435	1.000	-.01420	.01420

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05
		1
AE 10	3	.21467
control	3	.21500
AE 25	3	.22567
AE 50	3	.22567
Sig.		.138

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Alizarin Red 21 days

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	3	.47767	.018009	.010398	.43293	.52240	.460	.496
AE 10	3	.54167	.010116	.005840	.51654	.56680	.530	.548
AE 25	3	.60867	.024090	.013908	.54882	.66851	.581	.625
AE 50	3	.65933	.014640	.008452	.62297	.69570	.646	.675
Total	12	.57183	.073121	.021108	.52537	.61829	.460	.675

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
1.060	3	8	.418

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.056	3	.019	61.540	.000
Within Groups	.002	8	.000		
Total	.059	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) AE	(J) AE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	AE 10	-.064000*	.014267	.009	-.10969	-.01831
	AE 25	-.131000*	.014267	.000	-.17669	-.08531
	AE 50	-.181667*	.014267	.000	-.22736	-.13598
AE 10	control	.064000*	.014267	.009	.01831	.10969
	AE 25	-.067000*	.014267	.007	-.11269	-.02131
	AE 50	-.117667*	.014267	.000	-.16336	-.07198
AE 25	control	.131000*	.014267	.000	.08531	.17669
	AE 10	.067000*	.014267	.007	.02131	.11269
	AE 50	-.050667*	.014267	.031	-.09636	-.00498
AE 50	control	.181667*	.014267	.000	.13598	.22736
	AE 10	.117667*	.014267	.000	.07198	.16336
	AE 25	.050667*	.014267	.031	.00498	.09636

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

AE	N	Subset for alpha = 0.05			
		1	2	3	4
control	3	.47767			
AE 10	3		.54167		
AE 25	3			.60867	
AE 50	3				.65933
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Part 2 To evaluate effect of Aquilaria crassna crude extract on cell proliferation and cell attachment of MC3T3-E1 cell on modified titanium surface

Surface roughness analysis

Surface roughness Ti + AE

Oneway

Descriptives

Ra

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum
					Lower Bound	Upper Bound	
control	4	205.9000	14.71947	7.35973	182.4780	229.3220	191.84
Acid etched Ti	4	641.5075	23.02489	11.51245	604.8698	678.1452	616.55
Dipped AE + Acid etched Ti	4	652.2775	14.59113	7.29556	629.0598	675.4952	640.53
Total	12	499.8950	217.78001	62.86767	361.5242	638.2658	191.84

Descriptives

Ra

	Maximum
control	223.02
Acid etched Ti	666.51
Dipped AE + Acid etched Ti	673.59
Total	673.59

Test of Homogeneity of Variances

Ra

Levene Statistic	df1	df2	Sig.
1.876	2	9	.208

ANOVA

Ra

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	518830.346	2	259415.173	810.918	.000
Within Groups	2879.128	9	319.903		
Total	521709.474	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Ra

Tukey HSD

(I) Ti	(J) Ti	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
control	Acid etched Ti	-435.60750*	12.64720	.000	-470.9185
	Dipped AE + Acid etched Ti	-446.37750*	12.64720	.000	-481.6885
Acid etched Ti	control	435.60750*	12.64720	.000	400.2965
	Dipped AE + Acid etched Ti	-10.77000	12.64720	.682	-46.0810
Dipped AE + Acid etched Ti	control	446.37750*	12.64720	.000	411.0665
	Acid etched Ti	10.77000	12.64720	.682	-24.5410

Multiple Comparisons

Dependent Variable: Ra

Tukey HSD

(I) Ti	(J) Ti	95% Confidence Interval
		Upper Bound
control	Acid etched Ti	-400.2965*
	Dipped AE + Acid etched Ti	-411.0665*
Acid etched Ti	control	470.9185*
	Dipped AE + Acid etched Ti	24.5410
Dipped AE + Acid etched Ti	control	481.6885*
	Acid etched Ti	46.0810

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Ra

Tukey HSD

Ti	N	Subset for alpha = 0.05	
		1	2
control	4	205.9000	
Acid etched Ti	4		641.5075
Dipped AE + Acid etched Ti	4		652.2775
Sig.		1.000	.682

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Contact angle measurement

Contact angle Ti + AE

Oneway

Descriptives

Contact angle

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum
					Lower Bound	Upper Bound	
control	3	81.9667	1.59478	.92075	78.0050	85.9283	80.20
Acid etched Ti	3	70.0667	2.05020	1.18369	64.9737	75.1597	68.00
Dipped AE + Acid etched Ti	3	67.5333	2.85015	1.64553	60.4532	74.6135	64.70
Total	9	73.1889	6.94702	2.31567	67.8489	78.5288	64.70

Descriptives

Contact angle

	Maximum
control	83.30
Acid etched Ti	72.10
Dipped AE + Acid etched Ti	70.40
Total	83.30

Test of Homogeneity of Variances

Contact angle

Levene Statistic	df1	df2	Sig.
.290	2	6	.758

ANOVA

Contact angle

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	356.349	2	178.174	35.946	.000
Within Groups	29.740	6	4.957		
Total	386.089	8			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Contact angle

Tukey HSD

(I) Ti	(J) Ti	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
control	Acid etched Ti	11.90000*	1.81781	.001	6.3225
	Dipped AE + Acid etched Ti	14.43333*	1.81781	.001	8.8558
Acid etched Ti	control	-11.90000*	1.81781	.001	-17.4775
	Dipped AE + Acid etched Ti	2.53333	1.81781	.401	-3.0442
Dipped AE + Acid etched Ti	control	-14.43333*	1.81781	.001	-20.0109
	Acid etched Ti	-2.53333	1.81781	.401	-8.1109

Multiple Comparisons

Dependent Variable: Contact angle

Tukey HSD

(I) Ti	(J) Ti	95% Confidence Interval
		Upper Bound
control	Acid etched Ti	17.4775*
	Dipped AE + Acid etched Ti	20.0109*
Acid etched Ti	control	-6.3225*
	Dipped AE + Acid etched Ti	8.1109
Dipped AE + Acid etched Ti	control	-8.8558*
	Acid etched Ti	3.0442

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Contact angle

Tukey HSD

Ti	N	Subset for alpha = 0.05	
		1	2
Dipped AE + Acid etched Ti	3	67.5333	
Acid etched Ti	3	70.0667	
control	3		81.9667
Sig.		.401	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Release characteristics evaluation of AE from modified titanium surface

AE Release Dipped Ti

Oneway

Descriptives

AE Release

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0.5h	3	1.78567	.030271	.017477	1.71047	1.86086	1.758	1.818
1h	3	1.77133	.026839	.015496	1.70466	1.83801	1.741	1.792
4h	3	1.77333	.043822	.025300	1.66447	1.88219	1.723	1.803
6h	3	1.75400	.042579	.024583	1.64823	1.85977	1.705	1.782
12h	3	1.75567	.010263	.005925	1.73017	1.78116	1.747	1.767
1D	3	1.74533	.022591	.013043	1.68922	1.80145	1.724	1.769
3D	3	.70400	.033956	.019604	.61965	.78835	.681	.743
5D	3	.35200	.009644	.005568	.32804	.37596	.341	.359
7D	3	.23267	.009504	.005487	.20906	.25628	.223	.242
Total	27	1.31933	.652402	.125555	1.06125	1.57741	.223	1.818

Test of Homogeneity of Variances

AE Release

Levene Statistic	df1	df2	Sig.
2.718	8	18	.037

ANOVA

AE Release

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.052	8	1.381	1698.055	.000
Within Groups	.015	18	.001		
Total	11.066	26			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: AE Release

Tukey HSD

(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0.5h	1h	.014333	.023289	.999	-.06727	.09593
	4h	.012333	.023289	1.000	-.06927	.09393
	6h	.031667	.023289	.899	-.04993	.11327
	12h	.030000	.023289	.922	-.05160	.11160
	1D	.040333	.023289	.721	-.04127	.12193
	3D	1.081667*	.023289	.000	1.00007	1.16327
	5D	1.433667*	.023289	.000	1.35207	1.51527
	7D	1.553000*	.023289	.000	1.47140	1.63460
1h	0.5h	-.014333	.023289	.999	-.09593	.06727
	4h	-.002000	.023289	1.000	-.08360	.07960
	6h	.017333	.023289	.997	-.06427	.09893
	12h	.015667	.023289	.999	-.06593	.09727
	1D	.026000	.023289	.964	-.05560	.10760
	3D	1.067333*	.023289	.000	.98573	1.14893
	5D	1.419333*	.023289	.000	1.33773	1.50093
	7D	1.538667*	.023289	.000	1.45707	1.62027
4h	0.5h	-.012333	.023289	1.000	-.09393	.06927
	1h	.002000	.023289	1.000	-.07960	.08360
	6h	.019333	.023289	.994	-.06227	.10093
	12h	.017667	.023289	.997	-.06393	.09927
	1D	.028000	.023289	.946	-.05360	.10960
	3D	1.069333*	.023289	.000	.98773	1.15093
	5D	1.421333*	.023289	.000	1.33973	1.50293
	7D	1.540667*	.023289	.000	1.45907	1.62227
6h	0.5h	-.031667	.023289	.899	-.11327	.04993
	1h	-.017333	.023289	.997	-.09893	.06427
	4h	-.019333	.023289	.994	-.10093	.06227
	12h	-.001667	.023289	1.000	-.08327	.07993
	1D	.008667	.023289	1.000	-.07293	.09027
	3D	1.050000*	.023289	.000	.96840	1.13160
	5D	1.402000*	.023289	.000	1.32040	1.48360
	7D	1.521333*	.023289	.000	1.43973	1.60293
12h	0.5h	-.030000	.023289	.922	-.11160	.05160
	1h	-.015667	.023289	.999	-.09727	.06593
	4h	-.017667	.023289	.997	-.09927	.06393
	6h	.001667	.023289	1.000	-.07993	.08327
	1D	.010333	.023289	1.000	-.07127	.09193
	3D	1.051667*	.023289	.000	.97007	1.13327
	5D	1.403667*	.023289	.000	1.32207	1.48527
	7D	1.523000*	.023289	.000	1.44140	1.60460

Multiple Comparisons

Dependent Variable: AE Release

Tukey HSD

(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1D	0.5h	-.040333	.023289	.721	-.12193	.04127
	1h	-.026000	.023289	.964	-.10760	.05560
	4h	-.028000	.023289	.946	-.10960	.05360
	6h	-.008667	.023289	1.000	-.09027	.07293
	12h	-.010333	.023289	1.000	-.09193	.07127
	3D	1.041333*	.023289	.000	.95973	1.12293
	5D	1.393333*	.023289	.000	1.31173	1.47493
	7D	1.512667*	.023289	.000	1.43107	1.59427
3D	0.5h	-1.081667	.023289	.000	-1.16327	-1.00007
	1h	-1.067333	.023289	.000	-1.14893	-.98573
	4h	-1.069333	.023289	.000	-1.15093	-.98773
	6h	-1.050000	.023289	.000	-1.13160	-.96840
	12h	-1.051667	.023289	.000	-1.13327	-.97007
	1D	-1.041333*	.023289	.000	-1.12293	-.95973
	5D	.352000*	.023289	.000	.27040	.43360
	7D	.471333*	.023289	.000	.38973	.55293
5D	0.5h	-1.433667	.023289	.000	-1.51527	-1.35207
	1h	-1.419333	.023289	.000	-1.50093	-1.33773
	4h	-1.421333	.023289	.000	-1.50293	-1.33973
	6h	-1.402000	.023289	.000	-1.48360	-1.32040
	12h	-1.403667	.023289	.000	-1.48527	-1.32207
	1D	-1.393333*	.023289	.000	-1.47493	-1.31173
	3D	-.352000*	.023289	.000	-.43360	-.27040
	7D	.119333*	.023289	.002	.03773	.20093
7D	0.5h	-1.553000	.023289	.000	-1.63460	-1.47140
	1h	-1.538667	.023289	.000	-1.62027	-1.45707
	4h	-1.540667	.023289	.000	-1.62227	-1.45907
	6h	-1.521333	.023289	.000	-1.60293	-1.43973
	12h	-1.523000	.023289	.000	-1.60460	-1.44140
	1D	-1.512667*	.023289	.000	-1.59427	-1.43107
	3D	-.471333*	.023289	.000	-.55293	-.38973
	5D	-.119333*	.023289	.002	-.20093	-.03773

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

AE Release

Tukey HSD

Time	N	Subset for alpha = 0.05			
		1	2	3	4
7D	3	.23267			
5D	3		.35200		
3D	3			.70400	
1D	3				1.74533
6h	3				1.75400
12h	3				1.75567
1h	3				1.77133
4h	3				1.77333
0.5h	3				1.78567
Sig.		1.000	1.000	1.000	.721

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell proliferation evaluation on titanium

Cell Proliferation on Ti MTT 24h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum
					Lower Bound	Upper Bound	
Control (Glass)	3	.01033	.001528	.000882	.00654	.01413	.009
Polish Ti	3	.01033	.001155	.000667	.00746	.01320	.009
Acid etched Ti	3	.01367	.000577	.000333	.01223	.01510	.013
Acid etched Ti + Tx AE	3	.01833	.000577	.000333	.01690	.01977	.018
Dipped AE + Acid etched Ti	3	.01933	.003055	.001764	.01174	.02692	.016
Total	15	.01440	.004205	.001086	.01207	.01673	.009

Descriptives

OD

	Maximum
Control (Glass)	.012
Polish Ti	.011
Acid etched Ti	.014
Acid etched Ti + Tx AE	.019
Dipped AE + Acid etched Ti	.022
Total	.022

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
3.042	4	10	.070

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	4	.000	20.146	.000
Within Groups	.000	10	.000		
Total	.000	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) Ti	(J) Ti	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
Control (Glass)	Polish Ti	.000000	.001350	1.000	-.00444
	Acid etched Ti	-.003333	.001350	.174	-.00778
	Acid etched Ti + Tx AE	-.008000*	.001350	.001	-.01244
	Dipped AE + Acid etched Ti	-.009000*	.001350	.000	-.01344
Polish Ti	Control (Glass)	.000000	.001350	1.000	-.00444
	Acid etched Ti	-.003333	.001350	.174	-.00778
	Acid etched Ti + Tx AE	-.008000*	.001350	.001	-.01244
	Dipped AE + Acid etched Ti	-.009000*	.001350	.000	-.01344
Acid etched Ti	Control (Glass)	.003333	.001350	.174	-.00111
	Polish Ti	.003333	.001350	.174	-.00111
	Acid etched Ti + Tx AE	-.004667*	.001350	.039	-.00911
	Dipped AE + Acid etched Ti	-.005667*	.001350	.012	-.01011
Acid etched Ti + Tx AE	Control (Glass)	.008000*	.001350	.001	.00356
	Polish Ti	.008000*	.001350	.001	.00356
	Acid etched Ti	.004667*	.001350	.039	.00022
	Dipped AE + Acid etched Ti	-.001000	.001350	.942	-.00544
Dipped AE + Acid etched Ti	Control (Glass)	.009000*	.001350	.000	.00456
	Polish Ti	.009000*	.001350	.000	.00456
	Acid etched Ti	.005667*	.001350	.012	.00122
	Acid etched Ti + Tx AE	.001000	.001350	.942	-.00344

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) Ti	(J) Ti	95% Confidence Interval
		Upper Bound
Control (Glass)	Polish Ti	.00444
	Acid etched Ti	.00111
	Acid etched Ti + Tx AE	-.00356*
	Dipped AE + Acid etched Ti	-.00456*
Polish Ti	Control (Glass)	.00444
	Acid etched Ti	.00111
	Acid etched Ti + Tx AE	-.00356*
	Dipped AE + Acid etched Ti	-.00456*
Acid etched Ti	Control (Glass)	.00778
	Polish Ti	.00778
	Acid etched Ti + Tx AE	-.00022*
	Dipped AE + Acid etched Ti	-.00122*
Acid etched Ti + Tx AE	Control (Glass)	.01244*
	Polish Ti	.01244*
	Acid etched Ti	.00911*
	Dipped AE + Acid etched Ti	.00344
Dipped AE + Acid etched Ti	Control (Glass)	.01344*
	Polish Ti	.01344*
	Acid etched Ti	.01011*
	Acid etched Ti + Tx AE	.00544

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

Ti	N	Subset for alpha = 0.05	
		1	2
Polish Ti	3	.01033	
Control (Glass)	3	.01033	
Acid etched Ti	3	.01367	
Acid etched Ti + Tx AE	3		.01833
Dipped AE + Acid etched Ti	3		.01933
Sig.		.174	.942

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell Proliferation on Ti MTT 48h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum
					Lower Bound	Upper Bound	
Control (Glass)	3	.02100	.002646	.001528	.01443	.02757	.018
Polish Ti	3	.02000	.002000	.001155	.01503	.02497	.018
Acid etched Ti	3	.02300	.001000	.000577	.02052	.02548	.022
Acid etched Ti + Tx AE	3	.02833	.001528	.000882	.02454	.03213	.027
Dipped AE + Acid etched Ti	3	.02900	.001000	.000577	.02652	.03148	.028
Total	15	.02427	.004131	.001067	.02198	.02655	.018

Descriptives

OD

	Maximum
Control (Glass)	.023
Polish Ti	.022
Acid etched Ti	.024
Acid etched Ti + Tx AE	.030
Dipped AE + Acid etched Ti	.030
Total	.030

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
1.319	4	10	.328

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	4	.000	16.978	.000
Within Groups	.000	10	.000		
Total	.000	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) Ti	(J) Ti	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
Control (Glass)	Polish Ti	.001000	.001430	.952	-.00371
	Acid etched Ti	-.002000	.001430	.642	-.00671
	Acid etched Ti + Tx AE	-.007333*	.001430	.003	-.01204
	Dipped AE + Acid etched Ti	-.008000*	.001430	.002	-.01271
Polish Ti	Control (Glass)	-.001000	.001430	.952	-.00571
	Acid etched Ti	-.003000	.001430	.292	-.00771
	Acid etched Ti + Tx AE	-.008333*	.001430	.001	-.01304
	Dipped AE + Acid etched Ti	-.009000*	.001430	.001	-.01371
Acid etched Ti	Control (Glass)	.002000	.001430	.642	-.00271
	Polish Ti	.003000	.001430	.292	-.00171
	Acid etched Ti + Tx AE	-.005333*	.001430	.025	-.01004
	Dipped AE + Acid etched Ti	-.006000*	.001430	.012	-.01071
Acid etched Ti + Tx AE	Control (Glass)	.007333*	.001430	.003	.00263
	Polish Ti	.008333*	.001430	.001	.00363
	Acid etched Ti	.005333*	.001430	.025	.00063
	Dipped AE + Acid etched Ti	-.000667	.001430	.989	-.00537
Dipped AE + Acid etched Ti	Control (Glass)	.008000*	.001430	.002	.00329
	Polish Ti	.009000*	.001430	.001	.00429
	Acid etched Ti	.006000*	.001430	.012	.00129
	Acid etched Ti + Tx AE	.000667	.001430	.989	-.00404

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) Ti	(J) Ti	95% Confidence Interval
		Upper Bound
Control (Glass)	Polish Ti	.00571
	Acid etched Ti	.00271
	Acid etched Ti + Tx AE	-.00263*
	Dipped AE + Acid etched Ti	-.00329*
Polish Ti	Control (Glass)	.00371
	Acid etched Ti	.00171
	Acid etched Ti + Tx AE	-.00363*
	Dipped AE + Acid etched Ti	-.00429*
Acid etched Ti	Control (Glass)	.00671
	Polish Ti	.00771
	Acid etched Ti + Tx AE	-.00063*
	Dipped AE + Acid etched Ti	-.00129*
Acid etched Ti + Tx AE	Control (Glass)	.01204*
	Polish Ti	.01304*
	Acid etched Ti	.01004*
	Dipped AE + Acid etched Ti	.00404
Dipped AE + Acid etched Ti	Control (Glass)	.01271*
	Polish Ti	.01371*
	Acid etched Ti	.01071*
	Acid etched Ti + Tx AE	.00537

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

Ti	N	Subset for alpha = 0.05	
		1	2
Polish Ti	3	.02000	
Control (Glass)	3	.02100	
Acid etched Ti	3	.02300	
Acid etched Ti + Tx AE	3		.02833
Dipped AE + Acid etched Ti	3		.02900
Sig.		.292	.989

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell Proliferation on Ti MTT 72h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum
					Lower Bound	Upper Bound	
Control (Glass)	3	.02467	.001528	.000882	.02087	.02846	.023
Polish Ti	3	.02433	.002517	.001453	.01808	.03058	.022
Acid etched Ti	3	.02867	.002082	.001202	.02350	.03384	.027
Acid etched Ti + Tx AE	3	.03400	.002000	.001155	.02903	.03897	.032
Dipped AE + Acid etched Ti	3	.03633	.001528	.000882	.03254	.04013	.035
Total	15	.02960	.005289	.001366	.02667	.03253	.022

Descriptives

OD

	Maximum
Control (Glass)	.026
Polish Ti	.027
Acid etched Ti	.031
Acid etched Ti + Tx AE	.036
Dipped AE + Acid etched Ti	.038
Total	.038

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
.274	4	10	.888

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	4	.000	22.819	.000
Within Groups	.000	10	.000		
Total	.000	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) Ti	(J) Ti	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
Control (Glass)	Polish Ti	.000333	.001606	1.000	-.00495
	Acid etched Ti	-.004000	.001606	.169	-.00928
	Acid etched Ti + Tx AE	-.009333*	.001606	.001	-.01462
	Dipped AE + Acid etched Ti	-.011667*	.001606	.000	-.01695
Polish Ti	Control (Glass)	-.000333	.001606	1.000	-.00562
	Acid etched Ti	-.004333	.001606	.124	-.00962
	Acid etched Ti + Tx AE	-.009667*	.001606	.001	-.01495
	Dipped AE + Acid etched Ti	-.012000*	.001606	.000	-.01728
Acid etched Ti	Control (Glass)	.004000	.001606	.169	-.00128
	Polish Ti	.004333	.001606	.124	-.00095
	Acid etched Ti + Tx AE	-.005333*	.001606	.048	-.01062
	Dipped AE + Acid etched Ti	-.007667*	.001606	.005	-.01295
Acid etched Ti + Tx AE	Control (Glass)	.009333*	.001606	.001	.00405
	Polish Ti	.009667*	.001606	.001	.00438
	Acid etched Ti	.005333*	.001606	.048	.00005
	Dipped AE + Acid etched Ti	-.002333	.001606	.611	-.00762
Dipped AE + Acid etched Ti	Control (Glass)	.011667*	.001606	.000	.00638
	Polish Ti	.012000*	.001606	.000	.00672
	Acid etched Ti	.007667*	.001606	.005	.00238
	Acid etched Ti + Tx AE	.002333	.001606	.611	-.00295

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) Ti	(J) Ti	95% Confidence Interval
		Upper Bound
Control (Glass)	Polish Ti	.00562
	Acid etched Ti	.00128
	Acid etched Ti + Tx AE	-.00405*
	Dipped AE + Acid etched Ti	-.00638*
Polish Ti	Control (Glass)	.00495
	Acid etched Ti	.00095
	Acid etched Ti + Tx AE	-.00438*
	Dipped AE + Acid etched Ti	-.00672*
Acid etched Ti	Control (Glass)	.00928
	Polish Ti	.00962
	Acid etched Ti + Tx AE	-.00005*
	Dipped AE + Acid etched Ti	-.00238*
Acid etched Ti + Tx AE	Control (Glass)	.01462*
	Polish Ti	.01495*
	Acid etched Ti	.01062*
	Dipped AE + Acid etched Ti	.00295
Dipped AE + Acid etched Ti	Control (Glass)	.01695*
	Polish Ti	.01728*
	Acid etched Ti	.01295*
	Acid etched Ti + Tx AE	.00762

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

Ti	N	Subset for alpha = 0.05	
		1	2
Polish Ti	3	.02433	
Control (Glass)	3	.02467	
Acid etched Ti	3	.02867	
Acid etched Ti + Tx AE	3		.03400
Dipped AE + Acid etched Ti	3		.03633
Sig.		.124	.611

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell attachment evaluation on titanium

Cell Attachment on Ti MTT 4h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum
					Lower Bound	Upper Bound	
Control (Glass)	3	.00433	.000577	.000333	.00290	.00577	.004
Polish Ti	3	.00467	.000577	.000333	.00323	.00610	.004
Acid etched Ti	3	.00600	.001000	.000577	.00352	.00848	.005
Acid etched Ti + Tx AE	3	.00867	.000577	.000333	.00723	.01010	.008
Dipped AE + Acid etched Ti	3	.00933	.000577	.000333	.00790	.01077	.009
Total	15	.00660	.002197	.000567	.00538	.00782	.004

Descriptives

OD

	Maximum
Control (Glass)	.005
Polish Ti	.005
Acid etched Ti	.007
Acid etched Ti + Tx AE	.009
Dipped AE + Acid etched Ti	.010
Total	.010

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
.308	4	10	.866

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	4	.000	33.714	.000
Within Groups	.000	10	.000		
Total	.000	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) Ti	(J) Ti	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
Control (Glass)	Polish Ti	-.000333	.000558	.972	-.00217
	Acid etched Ti	-.001667	.000558	.080	-.00350
	Acid etched Ti + Tx AE	-.004333*	.000558	.000	-.00617
	Dipped AE + Acid etched Ti	-.005000*	.000558	.000	-.00684
Polish Ti	Control (Glass)	.000333	.000558	.972	-.00150
	Acid etched Ti	-.001333	.000558	.195	-.00317
	Acid etched Ti + Tx AE	-.004000*	.000558	.000	-.00584
	Dipped AE + Acid etched Ti	-.004667*	.000558	.000	-.00650
Acid etched Ti	Control (Glass)	.001667	.000558	.080	-.00017
	Polish Ti	.001333	.000558	.195	-.00050
	Acid etched Ti + Tx AE	-.002667*	.000558	.005	-.00450
	Dipped AE + Acid etched Ti	-.003333*	.000558	.001	-.00517
Acid etched Ti + Tx AE	Control (Glass)	.004333*	.000558	.000	.00250
	Polish Ti	.004000*	.000558	.000	.00216
	Acid etched Ti	.002667*	.000558	.005	.00083
	Dipped AE + Acid etched Ti	-.000667	.000558	.754	-.00250
Dipped AE + Acid etched Ti	Control (Glass)	.005000*	.000558	.000	.00316
	Polish Ti	.004667*	.000558	.000	.00283
	Acid etched Ti	.003333*	.000558	.001	.00150
	Acid etched Ti + Tx AE	.000667	.000558	.754	-.00117

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) Ti	(J) Ti	95% Confidence Interval
		Upper Bound
Control (Glass)	Polish Ti	.00150
	Acid etched Ti	.00017
	Acid etched Ti + Tx AE	-.00250*
	Dipped AE + Acid etched Ti	-.00316*
Polish Ti	Control (Glass)	.00217
	Acid etched Ti	.00050
	Acid etched Ti + Tx AE	-.00216*
	Dipped AE + Acid etched Ti	-.00283*
Acid etched Ti	Control (Glass)	.00350
	Polish Ti	.00317
	Acid etched Ti + Tx AE	-.00083*
	Dipped AE + Acid etched Ti	-.00150*
Acid etched Ti + Tx AE	Control (Glass)	.00617*
	Polish Ti	.00584*
	Acid etched Ti	.00450*
	Dipped AE + Acid etched Ti	.00117
Dipped AE + Acid etched Ti	Control (Glass)	.00684*
	Polish Ti	.00650*
	Acid etched Ti	.00517*
	Acid etched Ti + Tx AE	.00250

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

Ti	N	Subset for alpha = 0.05	
		1	2
Control (Glass)	3	.00433	
Polish Ti	3	.00467	
Acid etched Ti	3	.00600	
Acid etched Ti + Tx AE	3		.00867
Dipped AE + Acid etched Ti	3		.00933
Sig.		.080	.754

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Cell Attachment on Ti MTT 24h

Oneway

Descriptives

OD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum
					Lower Bound	Upper Bound	
Control (Glass)	3	.00967	.000577	.000333	.00823	.01110	.009
Polish Ti	3	.01067	.001528	.000882	.00687	.01446	.009
Acid etched Ti	3	.01367	.001528	.000882	.00987	.01746	.012
Acid etched Ti + Tx AE	3	.01900	.001000	.000577	.01652	.02148	.018
Dipped AE + Acid etched Ti	3	.01967	.003215	.001856	.01168	.02765	.016
Total	15	.01453	.004549	.001175	.01201	.01705	.009

Descriptives

OD

	Maximum
Control (Glass)	.010
Polish Ti	.012
Acid etched Ti	.015
Acid etched Ti + Tx AE	.020
Dipped AE + Acid etched Ti	.022
Total	.022

Test of Homogeneity of Variances

OD

Levene Statistic	df1	df2	Sig.
3.342	4	10	.055

ANOVA

OD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	4	.000	19.673	.000
Within Groups	.000	10	.000		
Total	.000	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) Ti	(J) Ti	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
Control (Glass)	Polish Ti	-.001000	.001476	.957	-.00586
	Acid etched Ti	-.004000	.001476	.122	-.00886
	Acid etched Ti + Tx AE	-.009333*	.001476	.001	-.01419
	Dipped AE + Acid etched Ti	-.010000*	.001476	.000	-.01486
Polish Ti	Control (Glass)	.001000	.001476	.957	-.00386
	Acid etched Ti	-.003000	.001476	.318	-.00786
	Acid etched Ti + Tx AE	-.008333*	.001476	.002	-.01319
	Dipped AE + Acid etched Ti	-.009000*	.001476	.001	-.01386
Acid etched Ti	Control (Glass)	.004000	.001476	.122	-.00086
	Polish Ti	.003000	.001476	.318	-.00186
	Acid etched Ti + Tx AE	-.005333*	.001476	.030	-.01019
	Dipped AE + Acid etched Ti	-.006000*	.001476	.015	-.01086
Acid etched Ti + Tx AE	Control (Glass)	.009333*	.001476	.001	.00448
	Polish Ti	.008333*	.001476	.002	.00348
	Acid etched Ti	.005333*	.001476	.030	.00048
	Dipped AE + Acid etched Ti	-.000667	.001476	.990	-.00552
Dipped AE + Acid etched Ti	Control (Glass)	.010000*	.001476	.000	.00514
	Polish Ti	.009000*	.001476	.001	.00414
	Acid etched Ti	.006000*	.001476	.015	.00114
	Acid etched Ti + Tx AE	.000667	.001476	.990	-.00419

Multiple Comparisons

Dependent Variable: OD

Tukey HSD

(I) Ti	(J) Ti	95% Confidence Interval
		Upper Bound
Control (Glass)	Polish Ti	.00386
	Acid etched Ti	.00086
	Acid etched Ti + Tx AE	-.00448*
	Dipped AE + Acid etched Ti	-.00514*
Polish Ti	Control (Glass)	.00586
	Acid etched Ti	.00186
	Acid etched Ti + Tx AE	-.00348*
	Dipped AE + Acid etched Ti	-.00414*
Acid etched Ti	Control (Glass)	.00886
	Polish Ti	.00786
	Acid etched Ti + Tx AE	-.00048*
	Dipped AE + Acid etched Ti	-.00114*
Acid etched Ti + Tx AE	Control (Glass)	.01419*
	Polish Ti	.01319*
	Acid etched Ti	.01019*
	Dipped AE + Acid etched Ti	.00419
Dipped AE + Acid etched Ti	Control (Glass)	.01486*
	Polish Ti	.01386*
	Acid etched Ti	.01086*
	Acid etched Ti + Tx AE	.00552

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

OD

Tukey HSD

Ti	N	Subset for alpha = 0.05	
		1	2
Control (Glass)	3	.00967	
Polish Ti	3	.01067	
Acid etched Ti	3	.01367	
Acid etched Ti + Tx AE	3		.01900
Dipped AE + Acid etched Ti	3		.01967
Sig.		.122	.990

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.