สารเทอร์พีนอยด์จากหัวว่านพระฉิม (*Dioscorea bulbifera*) และสารแอนาลอกดัดแปรที่มีฤทธิ์ยับยั้งการเปลี่ยนสภาพเซลล์สลายกระดูก

นางสาวปรียาพรรณ ศรีพลอย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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TERPENOID FROM TUBER OF *Dioscorea bulbifera* AND MODIFIED ANALOGUES WITH
SUPPRESSING ACTIVITY AGAINST OSTEOCLAST DIFFERENTIATION

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ปรียาพรรณ ศรีพลอย : สารเทอร์พีนอยด์จากหัวว่านพระฉิม (*Dioscorea bulbifera*) และสารแอนา ลอกดัดแปรที่มีฤทธิ์ยับยั้งการเปลี่ยนสภาพเซลล์สลายกระดูก (TERPENOID FROM TUBER OF *Dioscorea bulbifera* AND MODIFIED ANALOGUES WITH SUPPRESSING ACTIVITY AGAINST OSTEOCLAST DIFFERENTIATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ธนาภัทร ปาลกะ , หน้า.

โรคกระดูกพรุนพบมากในคนวัยชรา สาเหตุการเกิดโรคนั้นมีหลายปัจจัย มักเกิดในเพศหญิงมากกว่า เพศชาย เนื่องจากสภาวะการลดระดับลงของฮอร์โมนเอสโตรเจนหลังหมดประจำเดือน โรคกระดูกพรุนมีลักษณะ ของโรคคือ มีการลดลงของมวลกระดูกและเนื้อกระดูก ซึ่งตามปกติกระดูกจะมีภาวะที่สมดุลกันระหว่างกระบวนการ สร้างและสลายกระดูก ดังนั้นสาเหตุหลักของการเกิดโรคกระดูกพรุน คือ ความไม่สมดุลกันระหว่างการทำงานของ เซลล์สร้างกระดูกและเซลล์สลายกระดูก ส่งผลทำให้มวลกระดูกลดน้อยลง เปราะบางและเสี่ยงต่อการแตกหักได้ ง่าย ดังนั้นการรักษาโรคกระดูกพรุนมักมุ่งเป้าในการลดการทำงานของเซลล์สลายกระดูก การรักษาในปัจจุบันมี หลายทางแต่มักมีผลข้างเคียง ผู้วิจัยจึงนำสารจากสมุนไพรมาศึกษาเพื่อโอกาสในการนำไปพัฒนาเป็นยา ในงานวิจัย นี้นำสารจากส่วนหัวของว่านพระฉิม (*Dioscorea bulbifera*) มาคัดกรองและทดสอบฤทธิ์ในการยับยั้งการเปลี่ยน สภาพเซลล์สลายกระดูกในเซลล์จากไขกระดูกจากหนูเมาส์ สารที่ได้จากส่วนหัวของว่านพระฉิมที่ใช้ในงานวิจัยนี้มี ทั้งหมด 12 ชนิด เป็นสารที่มีโครงสร้างตามธรรมชาติ 1 ชนิด และสารแอนาลอกปรับเปลี่ยนโครงสร้างอีก 11 ชนิด ซึ่งสารทั้งหมดไม่พบความเป็นพิษต่อเซลล์ RAW 264.7 และ ไม่มีผลต่อการยับยั้งการเกิดการอักเสบ จากการ ทดสอบฤทธิ์ในการยับยั้งการเปลี่ยนสภาพของเซลล์สลายกระดูก พบว่า ASTP069 ซึ่งเป็นสารแอนาลอกปรับเปลี่ยน โครงสร้างมีประสิทธิภาพในการยับยั้งการเปลี่ยนสภาพของเซลล์สลายกระดูกที่เหนี่ยวนำโดย Receptor activator of nuclear factor-kB ligand (RANKL) โดยมีค่า IC_{50} เท่ากับ 10.07±0.05 ไมโครโมลาร์ เมื่อทดสอบความสามารถ ในการยับยั้งการเปลี่ยนสภาพของเซลล์สลายกระดูกในระดับการแสดงออกของยีนส์ที่เกี่ยวข้องกับการเปลี่ยนสภาพ ของเซลล์สลายกระดูก พบว่า ASTP069 ลดการแสดงออกของ mRNA ของ *nfatc1, ctsk* และ *irf8* ซึ่งเป็นยีนส์ที่มี ผลต่อการเปลี่ยนสภาพและการทำงานของเซลล์สลายกระดูก และจากผลการทดสอบของ ASTP069 ต่อวิถีสัญญาณ ของ RANKL พบว่า ASTP069 กดการทำงานของวิถีสัญญาณ NF-kB แต่ไม่มีมีผลต่อการทำงานของวิถีสัญญาณ MAPK ที่ถูกเหนี่ยวนำโดย RANKL โดยยับยั้งการเคลื่อนที่เข้าสู่นิวเคลียสของ NF-kB p65จากผลการทดลองทั้งหมด แสดงให้เห็นว่า ASTP069 มีความสามารถในการยับยั้งการเปลี่ยนสภาพของเซลล์สลายกระดูกโดยลดการแสดงออก ของ *nfatc1* และยีนส์ที่เกี่ยวข้องผ่านทางวิถีสัญญาณ NF-kB ดังนั้นสารเทอร์พีนอยด์ที่ปรับเปลี่ยนโครงสร้างจาก สารบริสุทธิ์จากว่านพระฉิมที่มีศักยภาพนำไปพัฒนาเป็นยารักษาโรคกระดูกพรุนต่อไป

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PREEYAPAN SRIPLOY: TERPENOID FROM TUBER OF *Dioscorea bulbifera* AND MODIFIED ANALOGUES WITH   SUPPRESSING ACTIVITY AGAINST OSTEOCLAST DIFFERENTIATION. ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., pp.

Osteoporosis is often found in elderly. There are many causes of osteoporosis such as life style, aging, gender. Osteoporosis is found in females rather than males, as a result of decreasing the level of estrogen in post-menopausal female. Osteoporosis is characterized by decreasing bone mass and bone tissue. Normally, bone remodeling is maintained by balance between bone formation and bone resorption. Therefore, the main cause of osteoporosis is an imbalance between osteoblasts and osteoclasts. As a result, the condition makes bone fragile and increased risk of fracture. Therefore, osteoclasts are the target of osteoporosis treatment. Nowadays, treatments of osteoporosis have several side effects. In this study, the chemical constituents of plants were investigated to potential develop for osteoporosis drug. One terpenoid isolated from tuber of *Dioscorea bulbifera* and 11 modified analogues of natural terpenoid were used in this study. All compounds were screened for the anti-osteoclastogenic activity murine bone-marrow-derived macrophage precursors. All tester compounds did not cytoxicity and anti-inflammatory activity in RAW 264.7. From anti-osteoclastogenic activity screening, we found that ASTP069, a modified analogue, significantly suppressed the differentiation of the tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells induced by receptor activator of nuclear factor-kB ligand (RANKL) with an IC_{50} of ASTP069 10.07±0.05 µM. At the transcription level, we found that ASTP069 clearly decreased the expression of *nfatc1*, *ctsk* and *irf8* leading to inhibit osteoclastogenesis and osteoclast function. The effect of ASTP069 on signaling pathway was investigated. The results showed that ASTP069 decreased activation of NF-kB pathway but did not affect the activation of MAPK pathway by suppressing NF-kB p65 nuclear translocation. The results indicated that ASTP069 inhibited osteoclastogenesis by downregulation of nuclear factor of activated T cells (NFATc1) and the other osteoclast-related genes via NF-kB pathway. Therefore, the modified terpenoid from *D. bulbifera* has therapeutic potential for development as anti-osteoporosis drug.

Department:	Microbiology	Student's Signature
Field of Study:	Microbiology and Microbial Technology	Advisor's Signature
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LIST OF ABBREVIATIONS

%	Percentage
° C	Degree Celsius
mg	Microgram
mM	Micromolar
/	Per
:	Ratio
Ab	Antibody
AcO	Acetyl group
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CO2	Carbon dioxide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	dATP, dCTP, dGTP and dTTP
dTTP	Deoxythymidine triphosphate
ER	Estrogen receptor
FBS	Fetal bovine serum
g	Gravity (Centrifuge speed)
hr	Hour
IC50	50 % of inhibitory concentration
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
kDa	Kilo Dalton
LPS	Lipopolysaccharide
m	Murine
mA	Milliampere

MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium brominde
N	Normality (Concentration)
NED	N-(1-naphtyl)- ethylenediamide
NF-kB	Nuclear factor - kB
nM	Nanomolar
nm	Nanometer
ng	Nanogram
NO	Nitric oxide
OPG	Osteopotigerin
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline - Tween 20
PCR	Polymerase chain reaction
PVDF	Polyvinylidine fluoride
qPCR	Quantitative polymerase chain reaction
RANKL	Receptor activator of NF-kB ligand
RNA	Ribonucleic acid
rpm	Round per minute
rRANKL	Recombinant RANKL
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
sec	Second
TNF	Tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
TRAP	Tartrate-resistant acid phosphatase
U	Unit

v	Volume
w	Weight
α	Alpha
β	Beta
δ	Delta
γ	Gamma
κ	Kappa

CHAPTER I

BACKGROUND

Osteoporosis is a major health problem for both in elderly men and women. Osteoporosis is a bone disorder of lacking bone matrix and tissues. As a result, there are changes in skeletal structure, bone fragile and high risk of bone fracture (1). In addition to lifestyle and aging process, sex is a major factor influencing osteoporosis. This disease occurs more in women than men, as a result of estrogen deficiency during menopause (2). Bone remodeling has two continuous processes, bone formation mediated by osteoblast and bone resorption mediated by osteoclast. Imbalance between the two processes is the main cause of osteoporosis (3).

Osteoclast or commonly known as bone-degrading cells, is originated from hematopoietic stem cells. Osteoclast is a large cell and has specialized character as multinucleated cells. The osteoclast precursor becomes mature osteoclast with the process called osteoclastogenesis and this process requires the presence of growth factor and necessary cytokines such as macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-kB ligand (RANKL) (4).

The binding between MCSF and colony-stimulating factor-1 receptor (CSF-1R) on osteoclast precursors stimulate their proliferation, survival and differentiation of early precursors. The interaction between RANKL and RANK receptor on the cell surface of precursors stimulates the activation of RANK with the main signal transduction molecules (TNF receptor associated factor) TRAF6 in osteoclastogenesis signaling pathway. TRAF6 activates nuclear factor-κB NF-κB) pathway that include p50 and p65 and MAPK pathway, including ERK, JNK and p38. Activation of the two pathways activated nuclear factor of activated T cells (NFATc1) (4).

The main transcriptional factor, NFATc1 play a crucial role in osteoclastogenesis becauseNFATc1 regulate expression of osteoclast-related genes such as *ctsk* and DC-STAMP that control function and differentiation of osteoclasts. The activation of several signaling pathway induces bone marrow derived macrophage precursors (BMs) to differentiate into tartrate-resistant acid phosphatase (TRAP)-positive cells or activated osteoclast by nuclear fusion. The activated osteoclasts degraded bone tissue by acid and proteolytic enzymes secretion (5).

Osteoporosis treatment nowadays, uses several pharmacological agents against osteoporosis. Bisphosphonates are the main drugs for treatment of decreasing bone loss by binding to hydroxyapatite and inhibiting the activation of osteoclast (6). Denosumab and hormone replacement therapy (HRT) are other treatments for osteoporosis. On the other hand,

these drugs have severe side effect such as low plasma levels, decreasing intestinal absorption, breast cancer development and risks of heart disease (7)

This study aims to find novel therapeutic drug for osteoporosis. In previous study, compounds from plants inhibit bone resorption have been reported such as the action of NFATc1 was suppressed by arctigenin from seeds of *Arctium lappa* (8), osteoclast differentiation was inhibited via reduction of NFATc1 and NF-κB pathway by Bajijiasu from *Morinda officinalis* (9) and osteoclast formation was also suppressed via the inhibition of NFATc1 by Gymnasterkoreayne F from the leaves of *Gymnaster koraienesis* (10).

Dioscorea bulbifera has been reported to have wide medical properties such as anti-inflammatory activity (11), antimicrobial activity (12), anti-tumor activity (13) and anti-diabetic activity (14). The anti-osteoclastogenic activity of *Dioscorea alata* and *Dioscorea spongiosa* (member of *Dioscorea* species) were found in previous studies (15) (16). Therefore, the diterpenoid from tuber of *D. bulbifera* and modified analogues were investigated for the anti-osteoclastogenic activity and their inhibitory mechanism. In this research, one diterpenoid from *D. bulbifera* and eleven modified analogues were screened and tested in transcriptional and translational levels.

OBJECTIVES

This research aimed to...

1. To screen terpenoid compounds from tuber of *Dioscorea bulbifera* and its modified analogues for anti-osteoclastogenic activity.

2. To investigate its mode of action on RANK/RANKL signaling pathway.

CHAPTER II

LITERATURE REVIEWS

2.1 Osteoporosis

Osteoporosis is a common disorder condition in the bone structure by decreasing bone component such as bone matrix, bone mass, bone tissue and enhancing susceptibility to fracture (17). In Thailand, the ratio of osteoporosis population was reported that 181 per 100,000 populations (18) caused by aging and gender factors. In addition, smoking, chronic heavy alcohol consumption, lack of vitamin C and D are indirect factors for osteoporosis (19). Osteoporosis commonly occurs in both men and women during the age range 50-80 years old (20) (21) and osteoporosis affects more women than men possibly by the deficiency of estrogen in post-menopausal women (22). A balance between bone formation-osteoblasts and bone resorption-osteoclasts in bone remodeling is important. Therefore, osteoporosis is caused by an imbalance between and bone formation and bone resorption in bone remodeling cycle (23).

2.2 Bone remodeling

Bone is a strong and hard organ of body and it is regulated by bone remodeling. Bone has multiple functions such as supports skeleton of body, body motion and protects internal organs. Bone remodeling cycle is a necessary process to maintain bone mineral homeostasis and consist of bone formation and bone resorption (24). Bone formation is controlled by osteoblast (bone-forming cells) to form new bone tissue for bone replacement on degrading site. Bone resorption is controlled by osteoclast (bone-resorping cells) to transfer calcium to the blood by break down bone tissue (25). Bone remodeling is carry out in 4 phases as cycle; (A) mature osteoclasts break down bone tissue to degrade bone matrix, bone tissue and mineral, the bone on resorption site was replaced later. (B) Bone resorption site surface was prepared for bone formation by mononuclear cells. (C) Mature osteoblasts binding to bone surface and form collagen and mineral to fill degrading site, mature osteoblasts transform to osteocyte. (D) The resting phase until resorption phase begins again (26) (Figure 2.1).
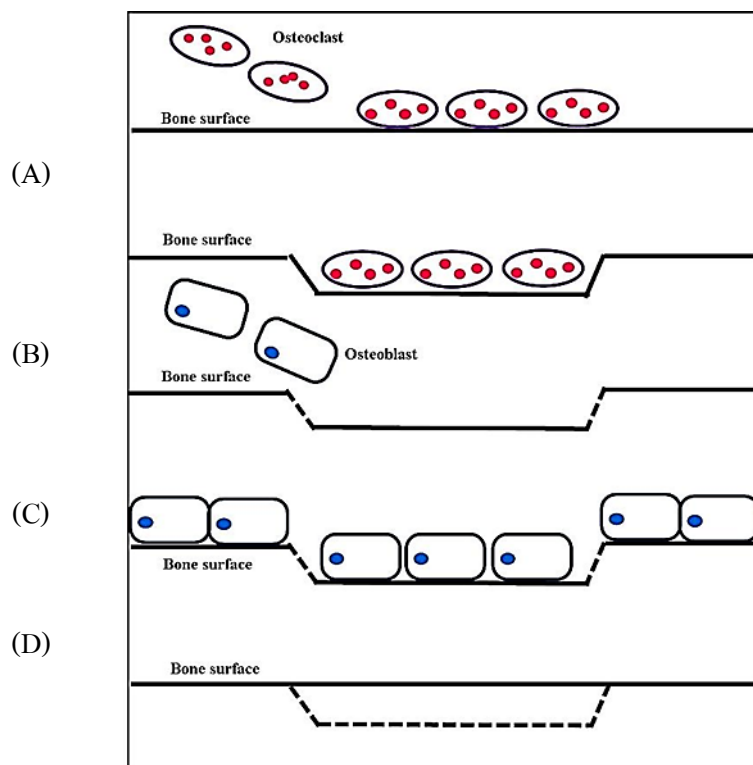
Figure 2. 1 Bone remodeling cycle. The bone mineral degradation and new bone replacement carry out in 4 phases (A) bone resorption phase by mature osteoclasts (B) reversal phase (C) bone formation phase by mature osteoblasts (D) resting phase.

2.3 Osteoclastogenesis

Bone resorption is controlled by osteoclast. Osteoclast is a giant multinucleated cell derived from hematopoietic stem cell (27) and differentiate to the tartrate-resistant acid phosphatase (TRAP)-positive cells by growth factors and necessary cytokines such as macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-kB ligand (RANKL) (28). The osteoclast differentiation or osteoclastogenesis is beginning when interaction between M-CSF and colony-stimulating factor-1 receptor (CSF-1R) and the binding of RANKL to RANK receptor on osteoclast precursors promotes their proliferation, survival and differentiation of early precursors (29).
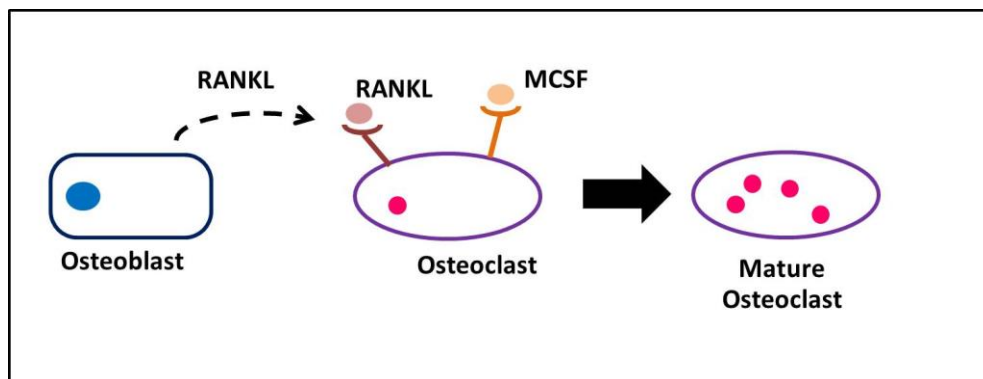
Figure 2. 2 Osteoclastogenesis. Osteoclast precursors are induced to become mature osteoclast by the binding of MCSF and RANKL to its receptor. MCSF and RANKL are released by osteoblast.

2.4 Current treatment of osteoporosis

Nowadays, osteoporosis has variety of available anti-osteoporotic drug. There are different therapies and side effects are described below.

Bisphosphonates

Bisphosphonates are used as a medicine for bone disorder conditions such as Paget's disease and osteoporosis. Its help osteoblasts to more effectively form new bone by decreasing osteoclast function. It worked by inducing osteoclast apoptosis. As a result, bone loss was decreased. But bisphosphonates are some side effect such as difficult swallow, chest pain and heartburn. Alendronate, ibandronic acid, etidronate and risedronate are drug in the bisphosphonates groups and have various brand names (30).

Denosumab

Denosumab is RANKL inhibitor, produced form human monoclonal antibody and approved in June 2010 by the Food and Drug Administration (FDA) in USA. Denosumab is used to treat bone loss and bone metastases and works by specific binding to the RANKL with high affinity. It causes decreases in bone resorption. Denosumab has several side effects such as joint and muscle pain in the arms or legs, increase risk of infection and hypersensitivity allergy reactions (31).

Calcitonin

Calcitonin or Thyrocalcitonin is polypeptide hormone that is released from parafollicular in the thyroid gland. Calcitonin treated osteoporosis by decreasing calcium in the blood (it reverses with the action of parathyroid hormone). Loss of food requirements, nausea, vomits and stomach pain are side effect of calcitonin (32).

Estrogen replacement therapy

This therapy is osteoporosis treatment in women because lack of estrogen is factor of bone loss in post-menopausal women. Estrogen replacement therapy is also has side effect such as increases risks of breast cancer and venous thromboembolic (33).

2.5 Signaling pathway of osteoclastogenesis

For specific osteoporosis treatment, understanding of the signaling pathway that regulated osteoclastogenesis is needed for appropriate treatment. The factors and signaling pathway in osteoclastogenesis are described below (Figure 2.3).

TNF receptor associated factor 6 (TRAF 6)

TRAF 6 is the main adaptor molecule associated with the osteoclasts differentiation. TRAF 6 mediates the signaling of TNF receptor superfamily and Toll/IL-1 family. Also in osteoclastogenesis signaling pathway, TRAF 6 activated the MAPK/ NF-κB early signaling pathway via RANK/RANKL activation (34).

Nuclear factor kappa B (NF-κB) signaling pathway

NF-κB is a protein complex consist of p65/RelA, p50/RelB, IκBα, IκBβ, IκBε, p105/ NF-κB 1 and p100/ NF-κB 2. The NF-κB activation depends on two pathways. The first is the canonical pathway involving activation of the IκB kinase (IKK) complex and the other is the non-canonical NF-κB signaling pathway involving RelB/p52 NF-κB complex13. NF-κB signaling pathway is involved in osteoclast formation and function (35)

Mitogen-activated protein kinases (MAPKs)

MAPKs are one of protein kinase and involved in response in cells such as inflammation and osteoclast differentiation. MAPKs pathway consists of Extracellular Signal-Regulated Kinases (ERKs), c-Jun amino-terminal kinases (JNKs) and p38 (36). MAPKs are involved in the activation of AP-1 component and therefore may have a role in osteoclastogenesis by modulating AP-1 activity. Activation of ERK and JNK can directly phosphorylate c-Fos and c-Jun, respectively. The activation of p38 by RANKL stimulation is involved the osteoclast function by the induction of *ctsk* expression (37). In addition, ERK is involved in osteoclast survival. All of the above indicated that MAPKs signaling pathway necessary for osteoclastogenesis (38).

cFos and activator protein-1 (AP-1)

AP-1 is a dimeric complex that composed of c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD. AP-1 regulates gene expression by functioning as transcription factor in response to growth factors and cytokines for survival by the activation of MAPKs signaling pathway, proliferation and apoptosis of the cells (Matsuo et al., 2004). cFos plays a crucial role in osteoclastogenesis because it activates the main transcription factor of osteoclastogenesis signaling pathway. Previously study, mice lacking cFos also lack osteoclastogenesis and leading to osteopetrosis (39).

Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1)

NFAT family is consisting of NFATc1, NFATc2, NFATc3, NFATc4 and NFATc5. NFATc1 is human protein that encoded by *NFATc1* gene. NFATc1, 2, 3 and 4 are involved in differentiation and function of many cells. NFATc1 work as a master transcription factor by RANKL stimulation in osteoclastogenesis signaling pathway (39). NFATc1 activation is regulated by cFos and NF-κB signaling pathways and function to activate osteoclast-related genes such as *ctsk* and *DC-STAMP* for osteoclast differentiation and function (40).

Interferon regulatory factor 8 (IRF8)

IRF8 is encoded by *irf8* gene and a member of IRF. IRF8 is a transcription repressor in osteoclastogenesis signaling pathway. The stimulation of RANKL promotes the activation of several signaling pathway for osteoclastogenesis and down regulation of IRF8. As a result, the level of IRF8 was decreased in the initial step of osteoclastogenesis and NFATc1 autoamplification (41). In addition to activator protein of NFATc1, interferon regulatory factor 8 (IRF8) is suppressor protein of NFATc1. IRF8 is highly expressed in osteoclast precursors before RANKL stimulation and IRF8 expression was decreased when RANKL binding to RANKL receptor on osteoclast precursors. Previous study reported that mice lack of NFATc1 expression lead to osteopetrosis condition (41).

Cathepsin K (CTSK)

Cathepsin K is a lysosomal cysteine protease enzyme encoded by *ctsk* gene and a member of the peptidase C1 protein family involved the osteoclast function to resorp bone tissue and bone matrix. Cathepsin K has potential to degrade bone and cartilage to release collagen type1 and elastin. Cathepsin K is a target treatment to reduce bone loss in osteoporosis (42).
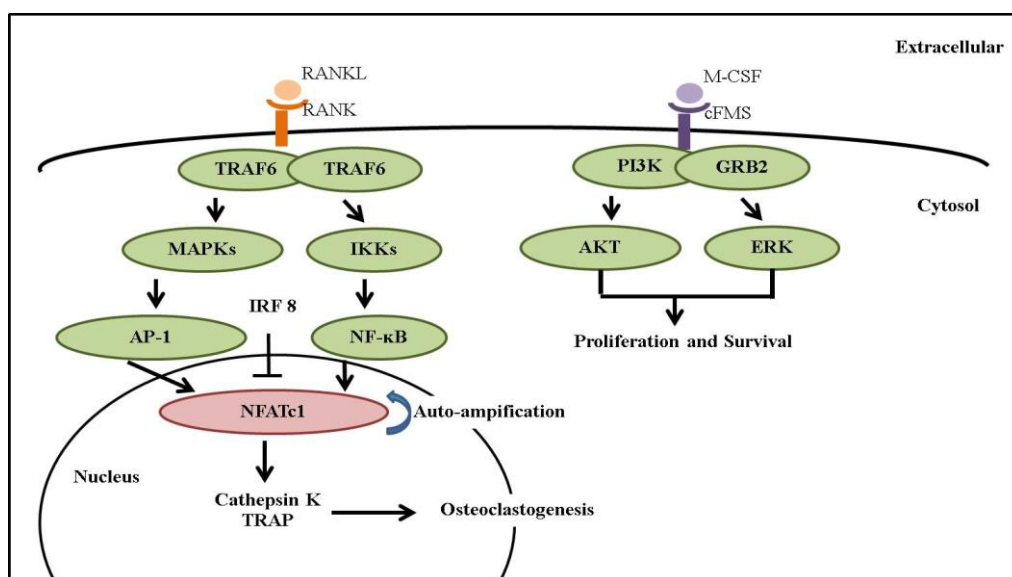

Figure 2. 3 Signaling pathways of osteoclastogenesis. The stimulation of RANKL is activated MAPK/NF-κB signaling pathway via TRAF 6 results in the activation of NFATc1 and reduced the activation of IRF8 to induce osteoclastogenesis. The stimulation of M-CSF activates the signaling of AKT and ERK pathway via PI3K and GRB2, respectively, for the proliferation and survival of osteoclasts.

2.6 Anti-osteoclastogenic activity of bioactive compounds from plants

Table 2. 1 Previous study of anti-osteoclastogenic activity of bioactive compounds from plants.

Scientific name of plants	Bioactive compounds	Anti-osteoclastogenic activity	Reference
Panax ginseng	Ginsenoside	Indirectly decreased bone loss by reducing the level of hydrogen peroxide in MC3T3-E1 cells	(43)
Cibotium barometz	The extract from *C. barometz*	Inhibited bone loss in rat	(44)
Lepidium meyenii	N-benzyl-palmitamide	Decreased bone loss by promoting osteoblast formation	(45)
Camellia sinensis	The aqueous extract	Inhibited osteoclastogenesis by increasing the level of estrogen hormones	(46)
Curcuma comosa	Diarylheptanoid	Inhibited osteoclastogenesis by decreasing the level of cFos and NFATc1 expression via MAPK signaling pathway	(47)
Xylocarpus moluccensis	7-oxo-deacetoxygedunin	Suppressed bone loss by inhibiting the activation of NF-κB and MAPK signaling pathway	(48)

2.7 *Dioscorea bulbifera*

Dioscorea bulbifera is known as air potato or wan-pra-chim in Thai and a member of *Dioscorea* species. *D. bulbifera* is a true yam native in Africa, India and Asia. *D. bulbifera* is diversely used such as food and drug (49). The compounds extracted from *D. bulbifera* have many application in medicine such as anti-fungal by dihydrodioscorine extracted from tuber of *D.*

bulbifera (50), anti-cancer by the extract from tuber of *D. bulbifera* and antibiotic resistant bacteria of 8-epidiosbulbin E acetate from *D. bulbifera* (51).

2.8 The terpenoid from *D. bulbifera* and modified analogues

Terpenoids are the large group of natural products isolated from plants and modified from plants (50, 52). Terpenoid are similar to terpenes, derived from 5-carbon isoprene units and modified structure by chemical method. The major product that isolated from tuber of *D. bulbifera* is diosbulbin B (53). In this study, diosbulbin B was modified to produce eleven modified analogues. Diosbulbin B was reported to exhibit antioxidant activity (54) and cytotoxic activity on SGC-7901 cells (55).
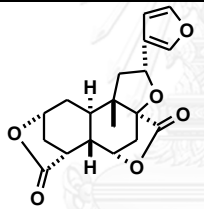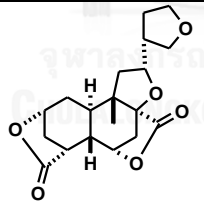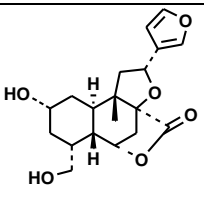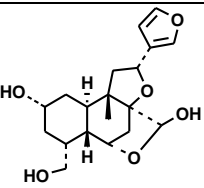
CHAPTER III
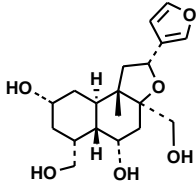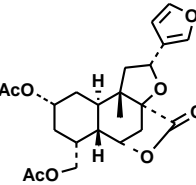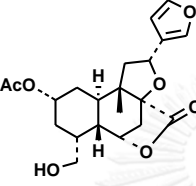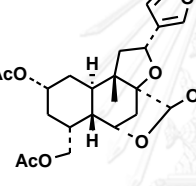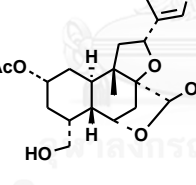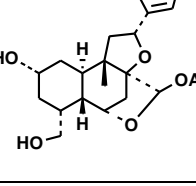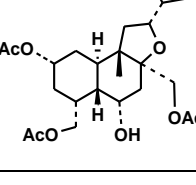
MATERIALS AND METHODS
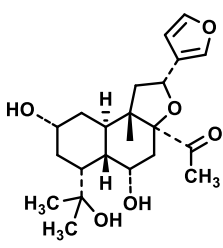
3.1 Compounds

The terpenoid isolated from tuber of *Dioscorea bulbifera* and the modified analogues were obtained from Prof. Dr. Apichart Suksamrarn (Department of Chemistry, Faculty of Science, Ramkhamhaeng University). The compounds were dissolved in DMSO and stored at -20 °C. The chemical structures of the terpenoid from tuber of *D. bulbifera* and the modified analogues

Table 3. 1 The chemical structures of the terpenoid (ASTP043) from tuber of *D. bulbifera* and the modified analogues (ASTP044, ASTP061-ASTP070).

No.	Structure	Formula	Code ASTP
1		$C_{19}H_{20}O_6$	043
2		$C_{19}H_{24}O_6$	044
3		$C_{19}H_{24}O_6$	061
4		$C_{19}H_{26}O_6$	062

5		C$_{19}$H$_{28}$O$_6$	063
6		C$_{23}$H$_{28}$O$_8$	064
7		C$_{21}$H$_{26}$O$_7$	065
8		C$_{23}$H$_{30}$O$_8$	066
9		C$_{21}$H$_{28}$O$_7$	067
10		C$_{21}$H$_{28}$O$_7$	068
11		C$_{25}$H$_{34}$O$_9$	069

12		$C_{22}H_{32}O_6$	070

3.2 RAW 264.7 cell line

A macrophage like cell line, RAW 264.7 (ATTC TIB-71) was cultured in DMEM media (Hyclone, UK) containing 10% fetal bovine serum (Gibco-Invitrogen, USA), 1% HEPES free acid, 1% sodium pyruvate and 1% penicillin-streptomycin (Hyclone, UK) at 37°C in a humidified atmosphere of 5% in CO_2 incubator (Thermo Electron Corporation, USA).

3.2.1 Cell preservations

RAW 264.7 were resuspended in 1 ml of freezing media (10% (v/v) DMSO (Sigma-Aldrich, USA) in DMEM complete media) and transferred to cryovial (SPL life sciences, Korea). Cells were stored at -80 °C.

3.2.2 Cell preparation

RAW 264.7 were collected from sterile petri dishes (Biomed, Thailand) by using cold 1x PBS and centrifuged at 1000 rpm for 5 minutes. Cell pellets were resuspended in DMEM complete media and stained by 0.4% solution of trypan blue stain (Gibco, USA). Cells were counted by hemacytometer. The number of viable cell was calculated by using the following formula:

$$\text{The number of cells} = \frac{(\text{number of counted cells in 16 large square} \times \text{dilution factor}) \times 10^4}{4}$$

3.3 Cell viability assay by MTT

RAW 264.7 were seeded at 1×10^4 cells/well in 96 well plates (Thermo Fisher Scientific, UK) overnight and treated with bioactive compounds dissolved in DMSO at various concentrations (0.316, 1, 3.162, 10, 31.62 and 100 μm). DMSO was used as vehicle control. After 24 hours, ten microliter of MTT solution (5 mg/ml) was added to cells for 4 hours at 37°C with 5% CO_2. After the treatment, 0.04N HCl in isopropanol was added to dissolve the insoluble formazan. The

absorbance was measured at 540 nm by microplate reader (Biochrom Anthos, UK) and percent of cell viability was calculated by using the following formula:

$$\text{Percent of Cell viability} = \frac{(\text{Abs of the treatment} - \text{Abs of blank}) \times 100}{\text{Abs of vehicle control} - \text{Abs of blank}}$$

3.4 Anti-inflammatory activity assay

RAW 264.7 were seeded at 2×10^5 cells/well in 96 well plates (Thermo Fisher Scientific, UK) overnight. RAW 264.7 were pre-treated with bioactive compounds at various concentrations or vehicle control (DMSO) for 1 hour and stimulated with or without lipopolysaccharide (LPS) (100 ng/ml) (Sigma–Aldrich, USA) and recombinant interferon-gamma (IFN-γ) (10 ng/ml) (Biolegend, USA) for 24 hours. The culture supernatant was collected to determine the amount of nitric oxide production, compared with nitrite standards by griess reaction. Sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride (50 μl/ well) was added to culture supernatant and incubated for 10 min in the dark at room temperature. The absorbance was measured at 540 nm by microplate reader (Biochom Anthos, UK). The relative nitric oxide productivity (%) was expressed as a percentage relative to the vehicle control and compared with nitrite standard.

3.5 Osteoclast differentiation

Bone marrow cells (BMs) were isolated from femur and tibia of 7-8 week-old BALB/c female mice (National Laboratory Animal Center, Mahidol University). BMs were cultured with of recombinant macrophage colony-stimulating factor (rMCSF, 25 ng/ml) (ImmunoTools, Germany) for 48 hours in DMEM medium containing 10% fetal bovine serum (FBS), 1% HEPES free acid, 1% sodium pyruvate and 1% penicillin-streptomycin at 37℃ in 5% CO_2. Furthermore, BMs were cultured with M-CSF (25 ng/ml) and receptor activator of nuclear factor-κB ligand (rRANKL, 100 ng/ml) (Biolegend, USA) for another 6 days to induce BMs differentiation to osteoclasts. All procedures involved laboratory animals were approved by Chulalongkorn University IACUC (Protocol No.1623003).

3.6 Anti-osteoclastogenic activity assay

BMs from mice were seeded 7.6 x 10^5 cell/well in 24 well plates (Thermo Fisher Scientific, UK) and cultured with 25 ng/ml of M-CSF (ImmunoTools, Germany) for 48 hours to generate the osteoclast precursors. After 48 hours, BMs were pre-treated with bioactive compounds or vehicle control (DMSO) for 30 minutes at indicated concentrations. After that cell

were stimulated with of recombinant RANKL (100 ng/ml) for 6 days. After 6 days, cells were washed with 1xPBS, fixed with 10% formaldehyde for 10 minutes, permeated with 95% ethanol for 2 minutes and washed with PBS again. Finally, cells were stained with a TRAP-staining solution (50 mM acetate buffer (pH 5.0), 50 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml fast red violet LB salt (Sigma Aldrich, USA)) for 30 minutes and washed with 1xPBS. Tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells with three or more nuclei were counted as osteoclast under a light microscope (Olympus, Tokyo, Japan).

3.7 RNA extraction

After BMs were cultured with M-CSF (25 ng/ml), Cells were treated with presence of ASTP069 (50 µM) or DMSO and stimulated cells to become mature osteoclast with recombinant RANKL (100 ng/ml) for o, 24, 48 hours. Total RNA were extracted by using of Trizol reagent (500 µl/well) (Thermo Fisher Scientific, UK) and collected to microcentrifuge tubes. The total RNA samples were extracted by Chloroform (100 µl) (Lab-Scan, Ireland) and vigorously shaked for 15 seconds. The samples were incubated for 2-3 minutes and centrifuged at 4 °C for 15 minutes at 12000 rpm by using eppendorf 5424R refrigerated microcentrifuge (Eppendorf, USA). The aqueous phase of sample was transferred to new microcentrifuge tubes, added isopropanol (250 µl) (Merck, Germany) and mixed 10 times. After incubation for 10 minutes at room temperature, the samples were centrifuged at 4 °C for 20 minutes at 12000 rpm and supernatant was removed. The RNA pellet was washed with cold 75 % ethanol (500 µl), gentle mixed and centrifuged at 4 °C for 5 minutes at 7500 rpm. The RNA pellets were dried for 30 minutes and resuspended in diethylpyrocarbonate (DEPC) water. The samples were incubated at 60 °C for 10 minutes and the RNA concentration was measured 260/280 nm by NanoDrop[TM] 2000/2000c Spectrophotometers (Thermo Fisher Scientific, UK).

3.8 cDNA synthesis by reverse transcription

Total RNA were mixed with random hexamers (0.5 µl) (Qiagen, Hilden, Germany), adjusted volume to 12.5 µl by diethyl pyrocarbonate-treated water and heated at 65°C for 5 minutes. The reverse transcription mixture contains reverse transcriptase, 1 mM dNTP mix and RNase inhibitor (Fermentas, Canada). The reaction was performed at 25°C for 10 minutes, 42°C for 60 minutes and 70°C for 10 minutes. The cDNA was stored at -20 °C.

3.9 Real-time reverse transcription polymerase chain reaction (qPCR)

Two µl of cDNA were prepared with 0.5 µl of the specific forward primer, reverse primer and nuclease-free water. qPCR was performed using iQ™ SYBR® Green supermix (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The reaction was performed at 95 °C for 10 minutes, 95 °C for 30 second, annealing temperature depends on each genes for 30 seconds, 72 °C for 1 minutes and 72 °C for 10 minutes in CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The relative expression of osteoclast related gene was determined and normalized, using the expression levels of β-actin and calculate by $2^{-\Delta\Delta_{Ct}}$ method (56). Primer sequences and annealing temperature of each gene are shown in Table 3.2

Table 3. 2 Primer sequences and conditions for RT-PCR.

Gene	Primer sequence		Anneal ing Temp. (°C)	Product Size. (bp.)
	Forward 5'- 3'	Reverse 5'-3'		
nfatc1	GGTAACTCTGTCTTTCTA ACCTTAAGCTC	GTGATGACCCCAGCA TGCACCAGTCACAG	62	240
irf8	GGAAAGCCTT ACCTGCTGAC	AAGGTCACC GTGGTCCTT	55	112
ctsk	GGCCAACTC AAGAAGAAA	GTACCCTCT GCATTTAGC	58	225
β-actin	ACCAACTGGGAC GACATGGAGAA	GTGGTGGTGA AGCTGTAGCC	55	380

3.10 Western blot

3.10.1 Protein extraction and preparation

Bicinchoninic acid assay (BCA protein assay) was used to measure protein concentration. One mg/ml of bovine serum albumin (BSA) (Thermo Fisher Scientific, UK) was used to be protein standard at concentration 0, 31.25, 62.5, 125, 250, 500, 1000 µg/ml in 96 well plate. The working reagent was prepared at 50:1 ratio of Reagent A and B (Thermo Fisher Scientific, UK). The protein samples were diluted at 1:10 ratio by sterile deionized water and added 200 µl of working reagent. After incubation for 30 minutes, the protein standard and sample were measured at 540

nm by using microplate reader and calculated to prepare the samples with equal amount of protein.

3.10.2 SDS-polyacrylamine gel electrophoresis (SDS-PAGE) and signal detection

After calculated protein concentration, 10-20 µg of proteins were mixed with 6x dye and heated with 99 °C for 5 minutes by using Thermomixer Compact (Eppendorf, Germany). The Color Prestained Protein Standard (New england biolabs, USA) were used to be molecular weight marker. The samples and marker were loaded into separating gel and electrophoresis was run at 80 volts for 120 minutes condition. After electrophoresis, the cellular proteins were transfer to polyvinylidene fluoride (PVDF) membrane (GE Healthcare, USA) by using Trans-Blot® SD Semi-Dry Transfer Cell (Biorad, USA) at 80 mA for 90 minutes condition. The membrane was blocked twice with blocking solution composed of 3% skim milk (BD, USA) in PBS and 0.05% Tween-20 for 5 minutes. The primary antibody was prepared in blocking solution using the working dilution in table 3.3 and the membrane was probed with the primary antibody overnight at 4 °C. After that, the primary antibody was removed and the membrane was washed with 1xPBS for 5 minutes 6 times. The membrane was probed with the secondary antibody (Sheep anti-mouse IgG or Goat anti-rabbit IgG (Cell Signaling Technology, USA)) with dilutions 1:4000 for 1 hour. After one hour, the membrane was wash with 1xPBS and incubated with chemiluminescent substrates for 1 minute. The membrane was wrapped with plastic before placed on hypercassette (Amersham Bioscience, UK) and exposed to high performance chemiluminescence X-ray film (Amersham Bioscience, UK) in the dark.

Table 3. 3 The working dilutions of primary antibodies for Western blot

Target protein	Working dilution	Exposure time
NFATc1	1:1000	5 minutes
cFos	1:2000	5 minutes
Phospho-SAPK/JNK	1:2000	5 minutes
SAPK/JNK	1:2000	3 minutes
Phospho-p38	1:2000	5 minutes
p38	1:4000	3 minutes
Phospho-p44/42	1:2000	3 minutes
P44/42	1:4000	1 minute
Phospho-p65	1:2000	5 minutes
p65	1:4000	3 minutes
IKBα	1:2000	5 minutes
β-actin	1:10000	3 second

3.11 Immunofluorescent staining

BMs were cultured with MCSF (25 ng/ml) for 2 days. Cells were treated with presence or absence of ASTP069 (50 µM) and stimulated cells with recombinant RANKL (100 ng/ml) for 0, 30 and 60 minutes. Cells were fixed with 4% paraformaldehyde for 10 minutes after washing with 1xPBS and permeabilization with 0.2% Triton-X 100 in PBS for 2 minutes. Ten percent of 2.4G2 in

10% FBS in PBS were used to blocked FC receptor for 20 minutes and washed with 1xPBS. After washing, cells were incubated with the primary antibody (Anti-p65, 1:100) in 1.5% of FBS in PBS for 1 hour and washed. The secondary antibody conjugated with fluorochrome (Goat anti-rabbit IgG Alexa fluor® 555) was added to samples and incubated for 45 minutes in the dark. Nucleus staining dye DAPI was added and incubated for 3 minutes in the dark before mounted with nail reagent. The sample was visualized under the FV10i confocal laser scanning microscope (Olympus scientific solutions America corporation, US).

3.12 Statistical analysis

All experiments were done in triplicate with two independent experiments unless otherwise indicated. The data were analyzed by using GraphPad Prism 5.03 and. The comparisons were made as specified, and P value <0.05 was considered statistical significance.

CHAPTER IV

RESULTS

4.1. Toxicity of terpenoid derived from *Dioscorea bulbifera* and the modified analogues

This experiment screened for toxicity of the tested compounds. One terpenoid constituent (ASTP043) from *D. bulbifera* and eleven modified analogues (ASTP044, ASTP61-ASTP070) were screened by using MTT assay in RAW264.7 cell line. The 50 % of inhibitory concentration (IC_{50}) of each terpenoid was calculated. The result showed that all tested compounds had no cytotoxicity in RAW264.7 cell line as shown in Table 4.1. Therefore, the concentration of each compound used in the further experiments is 100 µM.

4.2. Anti-inflammatory activity of terpenoid derived from *D. bulbifera* and the modified analogues

The production of nitric oxide (NO) is an indicator for inflammatory activity. The stimulation of NO production via the activation of MAPK and NF-kB signaling pathways is stimulated by lipopolysaccharide (LPS) and IFNγ. MAPK and NF-κB signaling pathways are related to the early signaling pathway of osteoclastogenesis. Therefore, the terpenoid that had inhibitory effect on inflammation might also inhibit osteoclastogenesis. Twelve compounds were tested for the effect on anti-inflammatory activity in RAW264.7 cell line at indicated concentrations by the griess assay. The results showed that all terpenoids did not inhibit NO production by LPS and IFNγ stimulation at the tested concentrations as summarized in Table 4.1.

Table 4. 1 The IC_{50} for cell viability and anti-inflammatory activity of the terpenoid from *D.bulbifera* and modified analogues

Bioactive compounds	IC_{50} (µM) for viability	Remark	Anti-inflammatory activity
AS-TP 043	>100	No toxicity	No activity
AS-TP 044	>100	No toxicity	No activity
AS-TP 061	>100	No toxicity	No activity
AS-TP 062	>100	No toxicity	No activity
AS-TP 063	>100	No toxicity	No activity
AS-TP 064	>100	No toxicity	No activity
AS-TP 065	>100	No toxicity	No activity
AS-TP 066	>100	No toxicity	No activity
AS-TP 067	>100	No toxicity	No activity
AS-TP 068	>100	No toxicity	No activity
AS-TP 069	>100	No toxicity	No activity
AS-TP 070	>100	No toxicity	No activity

4.3 Effects of terpenoid from *D. bulbifera* and modified analogues on osteoclast differentiation

4.3.1 Anti-osteoclastogenic activity

Twelve terpenoids described above were subjected to screening for anti-osteoclastogenic activity. BMs were cultured with M-CSF in the presence of terpenoids or DMSO (vehicle control) and stimulated by rRANKL to induce mature osteoclast. After 6 days, treated cells were assayed by TRAP staining and the TRAP positive multinucleated cells as mature osteoclast were counted (Figure 4.1A-C). The results showed that two terpenoids significantly decreased the numbers of TRAP positive multinucleated cells to less than 50% of vehicle control. ASTP064 treatment results in 21.89% of osteoclastogenesis while the treatment of ASTP069 results in 1.02% of osteoclastogenesis (Figure 4.1D). The strongest anti-osteoclastogenic activity was found in ASTP069 and it was chosen to further investigate the mode of action.
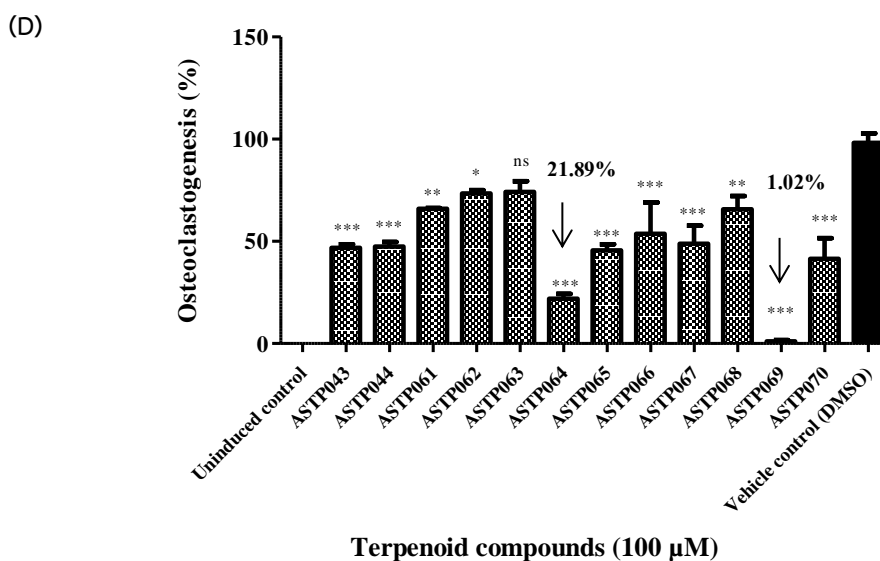
(A) Uninduced

(B) RANKL + Vehicle control

(C) RANKL + ASTP069 (100µM)

(D)
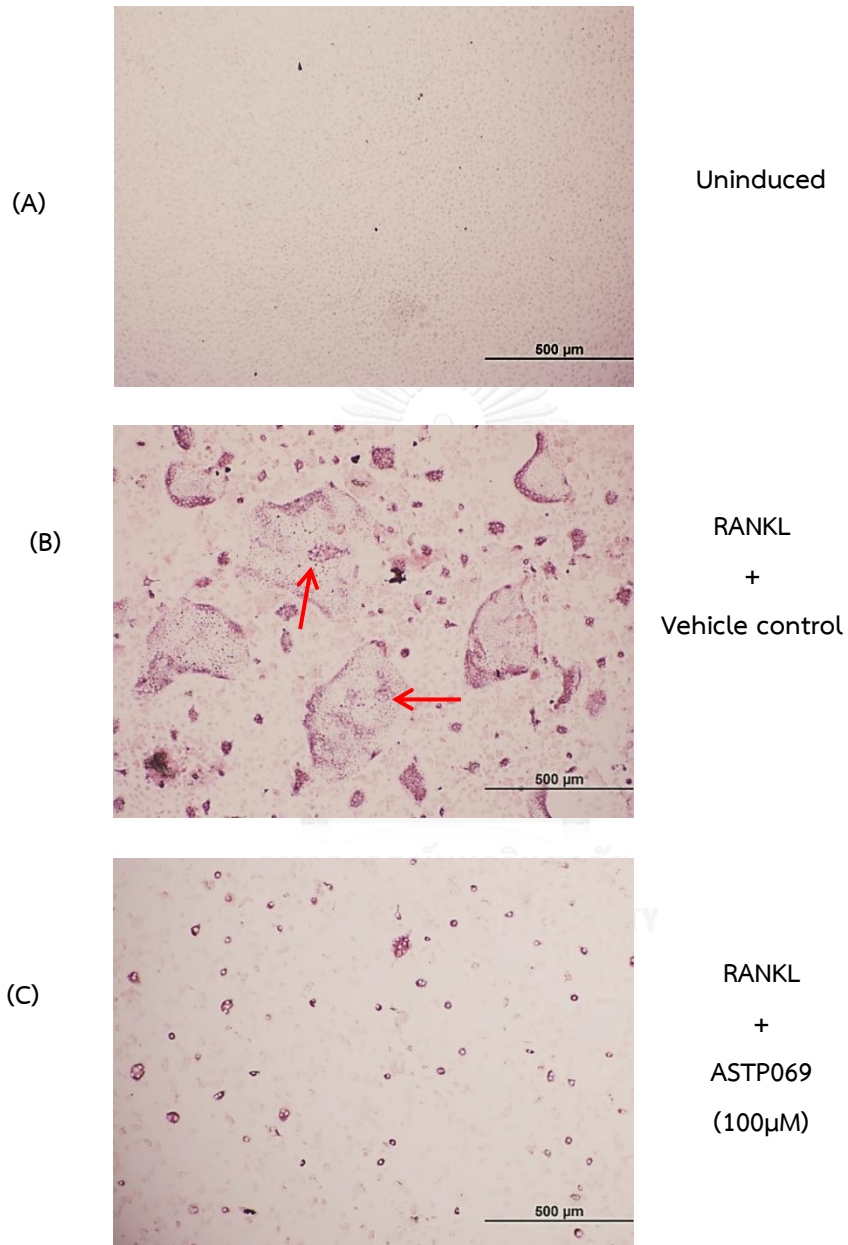
Figure 4. 1 Anti-osteoclastogenic activities of terpenoid from *D. bulbifera* and its modified analogues. (A-C) BMs cells were stimulated with rRANKL in the presence of compounds or vehicle control DMSO as described in materials and methods. Cells were stained by TRAP staining solution. (B) TRAP staining positive multinucleated cells (more than 3 nuclei) was counted under the light microscope (as indicated by the arrow heads) and (D) osteoclastogenesis (%) was calculated using vehicle control-treated cell as 100% (*$p<0.05$)(**$p<0.01$) (***$p<0.001$). ns: not significance.

4.3.2 The IC_{50} of ASTP069 for osteoclastogenesis

ASTP069 is a modified of Diosbulbin B (ASTP043) (Figure 4.2A). To evaluate the IC_{50} for osteoclastogenesis of ASTP069, BMs cells were cultured in the presence of ASTP069 at various concentrations (0, 3.33, 10, 33.3 and 100 µM) and the TRAP staining assay was performed. The result showed that the percentages of TRAP+ multinucleated cells are 100±5.05, 70.48±6.30, 53.05±4.52, 24.68±4.03 and 11.20±5.12%, respectively for each concentration. The results indicated that ASTP069 at 33.3 – 100 µM significantly decreased the numbers of TRAP positive multinucleated cells to less than 50 % in a dose-dependent manner. The IC_{50} of ASTP069 is 10.07±0.05 µM and the IC_{20} is approximately 50 µM (Figure 4.2B). Therefore, in further study, the concentration of 50 µM is used. To confirm the effect, at this concentration BMs cells were treated in the presence of ASTP069 at 50 µM to confirm the efficiency anti-osteoclastogenic activity. Consistant with the previous result, ASTP069 at 50 µM significantly decreased osteoclastogenesis to 23.26% of vehicle control (Figure 4.2C).

(A)

(B)

(C)
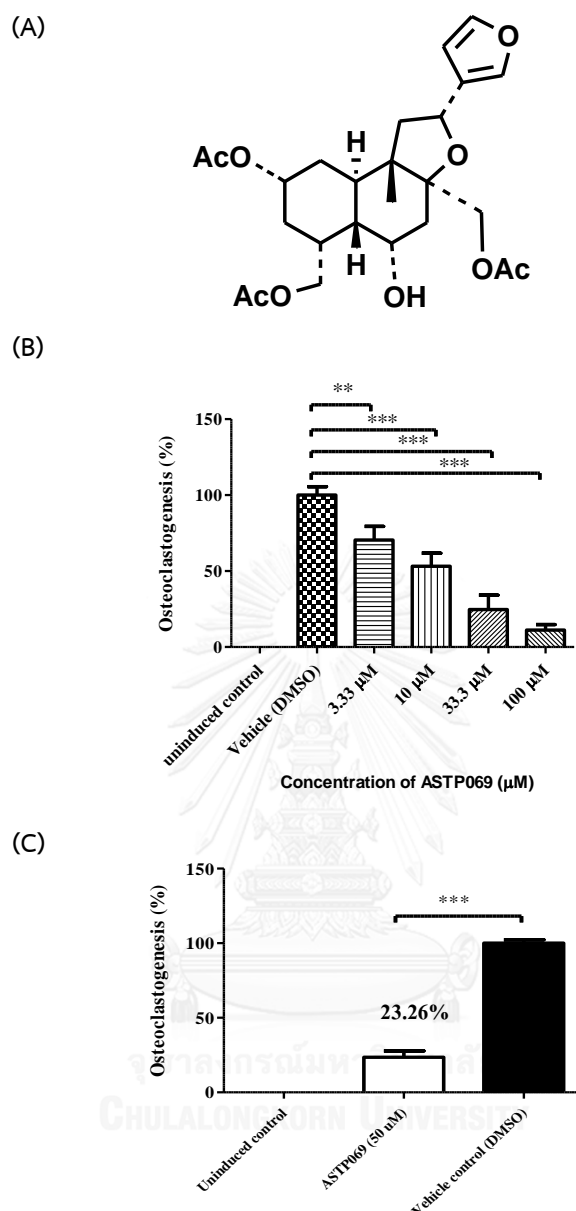
Figure 4. 2 The IC_{50} of ASTP069 for osteoclastogenesis. (A) The chemical structure of ASTP069 (B) BMs cells were stimulated with rRANKL in the presence of ASTP069 at various concentration as described above. The TRAP positive multinucleated cells (more than 3 nuclei) was counted under the light microscope and percent of osteoclastogenesis was calculated using vehicle control-treated cell as 100% (**p<0.05) (***p<0.01). (C) BMs cell were cultured in presence of ASTP069 at 50 µM to confirm the IC_{50} of ASTP069 result and the TRAP multinucleated cell were calculated and compared with vehicle control as (B).

4.4 Effect of ASTP069 on osteoclast- related mRNA expression by RT-qPCR

ASTP069 was further studied for its effect on mRNA expression in osteoclasts. The effect of ASTP069 was examined on *nfatc1, ctsk* and *irf8* mRNA expression which are involved in osteoclastogenesis by RT-qPCR. Expression of *nfatc1* mRNA was induced at 24 hours and increased at 48 hours by RANKL treatment in the vehicle control. In ASTP069 (50 µM) treatment, the level of *nfatc1* expression was compared to vehicle control at 24 hours. The level of *nfatc1* mRNA was dramatically decreased at 48 hours (Figure 4.3A). After RANKL stimulation for 24 hours, the level of *irf8* mRNA decreased and gradually increased at 48 hours in control. In contrast, by ASTP069 treatment, the expression of *irf8* was reduced at 48 hours. Therefore, ASTP069 at 50 µM concentration also interfered with the expression of *irf8* by ASTP069 treatment (Figure 4.3B). *ctsk* was normally induced at 24 and 48 hours at high level after RANKL stimulation. After ASTP069 treatment, the expression of *ctsk* was similarly expressed as the vehicle control at 24 hours but the level was strongly decreased at 48 hours (Figure 4.3C). From these results, osteoclastogenesis was inhibited at the transcriptional level for *nfatc1, irf8* and *ctsk* by ASTP069 treatment.

(A) *nfatc1*

(B)

(C)
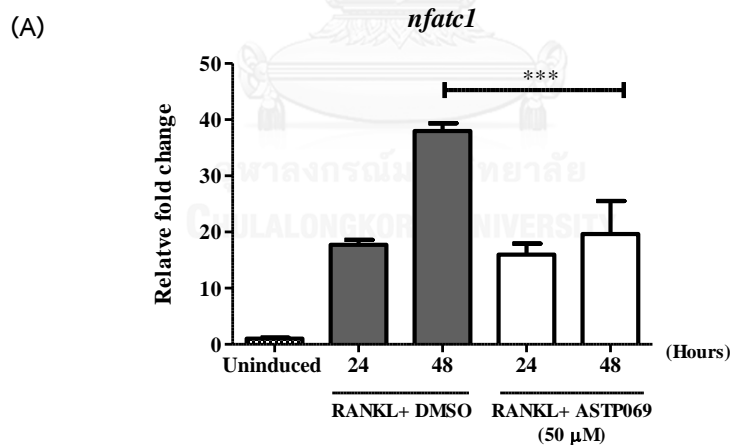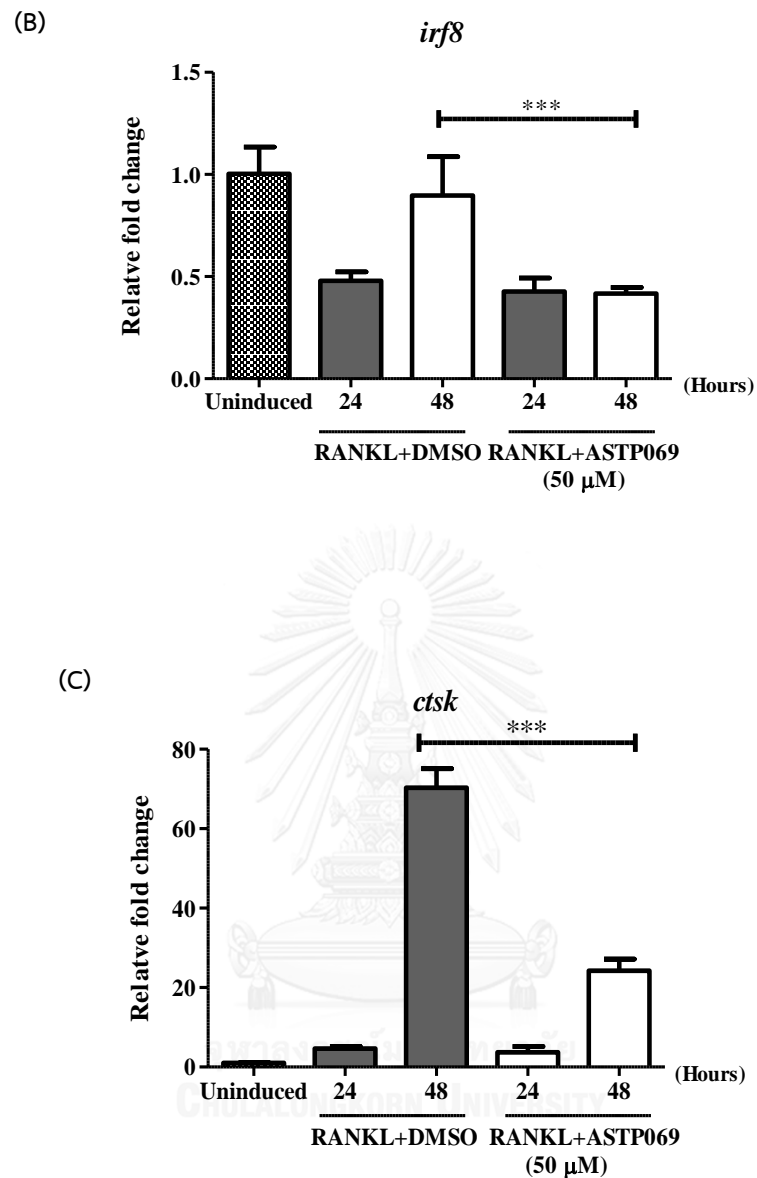
Figure 4. 3 Effect of ASTP069 on mRNA osteoclast-related genes (*nfatc1*, *irf8* and *ctsk*). BMs cells were pre-treated with ASTP069 (50 μM) and cultured with M-CSF (25 ng/ml) for 30 minutes. BMs cells were stimulated with RANKL (100 ng/ml). Total RNA were examined for the expression of *nfatc1* (A) *irf8* (B) *ctsk* (C) by qPCR. The results are representative of three independent experiments. The data are mean ± SD of triplicate (***$p<0.05$).

4.5. Effect of ASTP069 on NFATc1/cFos expression

ASTP069 was investigated for its effect on NFATc1, cFos and early signaling pathway of osteoclastogenesis at the protein level. The expression of NFATc1 was investigated at different time points. NFATc1 was induced at 24, 48, 72 and 96 hours after RANKL stimulation in the

control group. After ASTP069 treatment, the level of NFATc1 was reduced at 96 hours (Figure 4.4A, C). This result led to test the effect of cFos expression because NFATc1 was regulated by cFos. In vehicle treated control cells, the expression of cFos was increased at 12, 24 and 48 hours at the RANKL stimulation. After treatment of ASTP069, cFos was reduced at 48 hours (Figure 4.4B, D). Therefore, ASTP069 treatment decreased cFos expression.
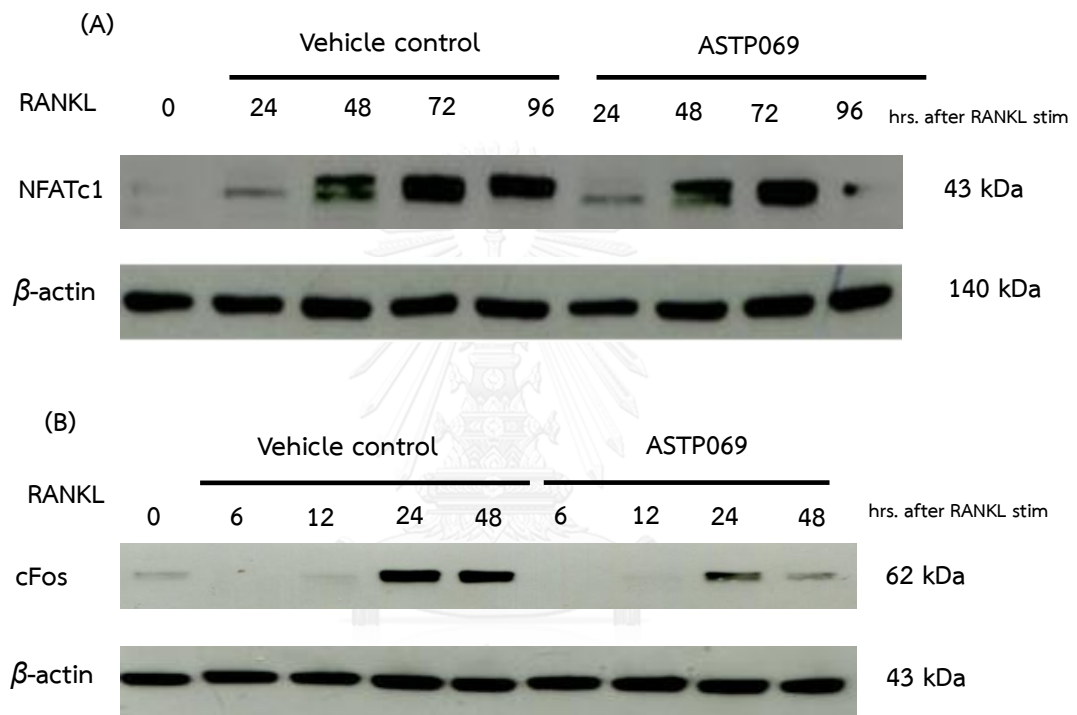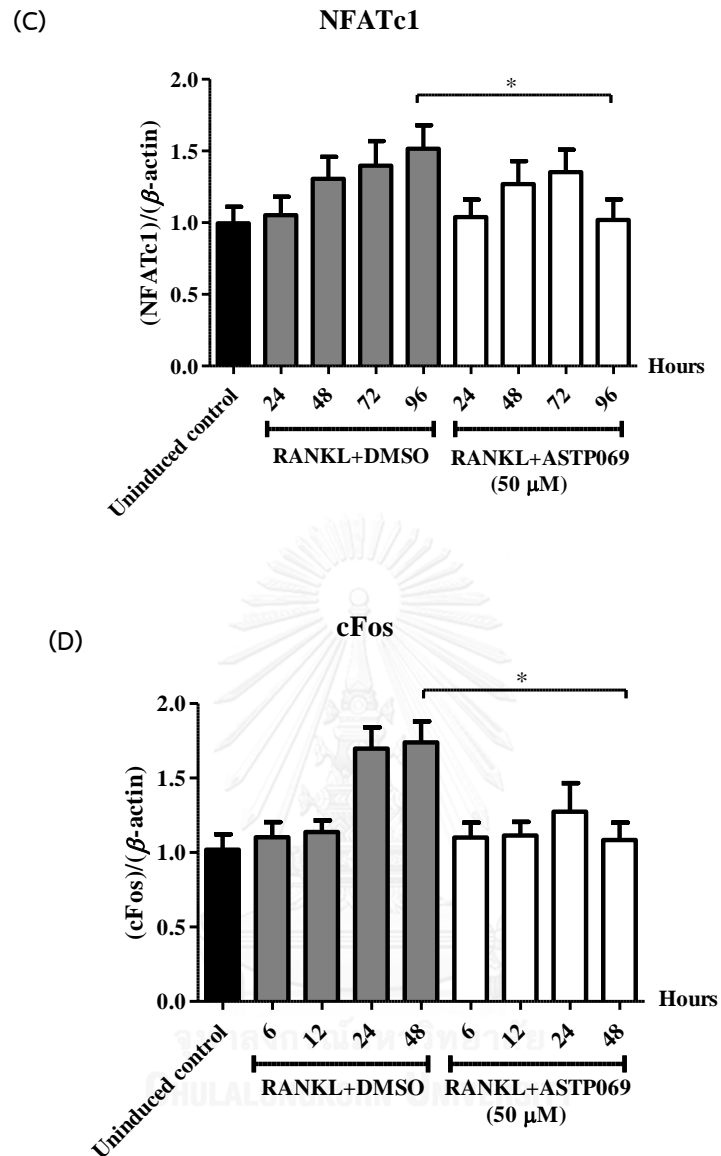
Figure 4. 4 Effect of ASTP069 on the expression of NFATc1 and cFos. BMs cells were treated with ASTP069 (50 μM) or DMSO as vehicle control and stimulated with RANKL for indicated time points. Cell lysates were examined for the expression of NFATc1 (A) and cFos (B). β-actin was used as loading control and the samples were assayed by Western blot. The normalized band density of NFATc1 and β-actin was shown in (C). The band density of cFos was normalized by β-actin and showed in (D). ns: not statistical significance (*$p<0.05$).

4.6. Effect of ASTP069 on early signaling pathways downstream of RANK/RANKL

Western blot was used to investigate the activation of MAPK/NF-κB. MAPK (phosphor-pSAPK/JNK, phospho-p38 and phospho-p44/42) and NF-κB pathway (IκBα and phospho-p65) are early signaling pathways in osteoclastogenesis and NFATc1 was controlled by the activation of MAPK and NF-κB. The results showed that the level of phospho-pSAPK/JNK, phospho-p38 and phospho-p44/42 were not significantly decreased by ASTP069 treatment when compared with the vehicle control at all tested time points (Figure 4.5A). For NF-κB pathway, the level of IκBα was not different but the level of phospho-p65 was decreased at 60 minutes when compared to the vehicle control (Figure 4.5B, C). From this result, it is indicated that ASTP069 suppress RANKL activation by interfering with NF-κB p65 activation.
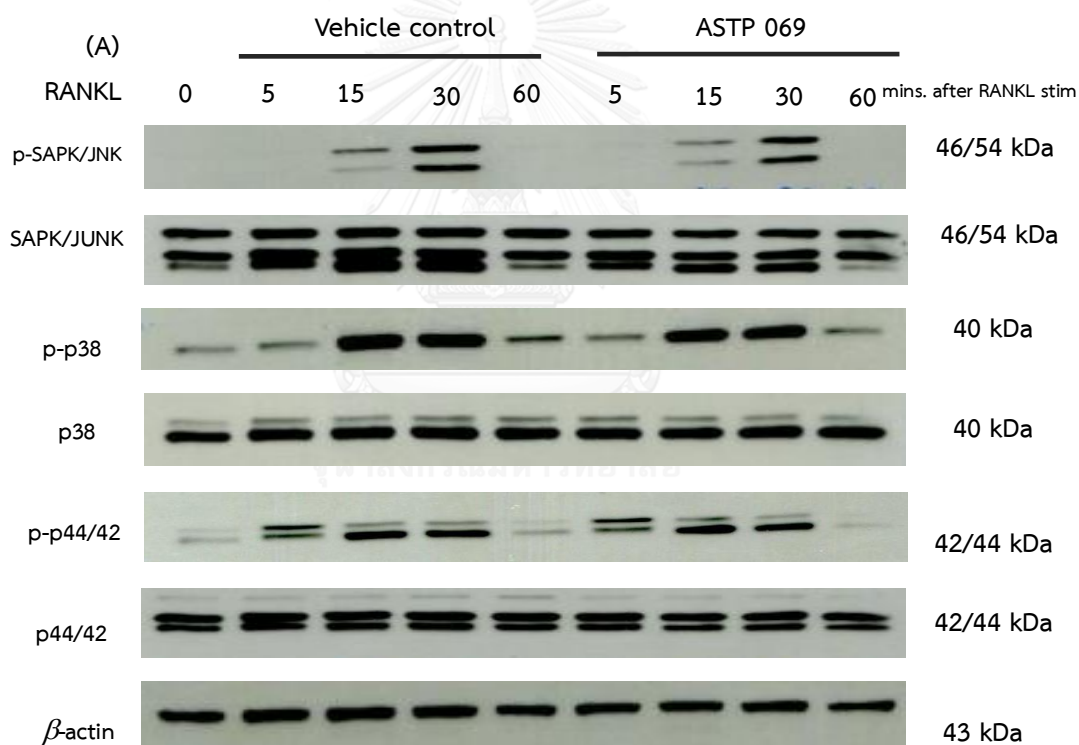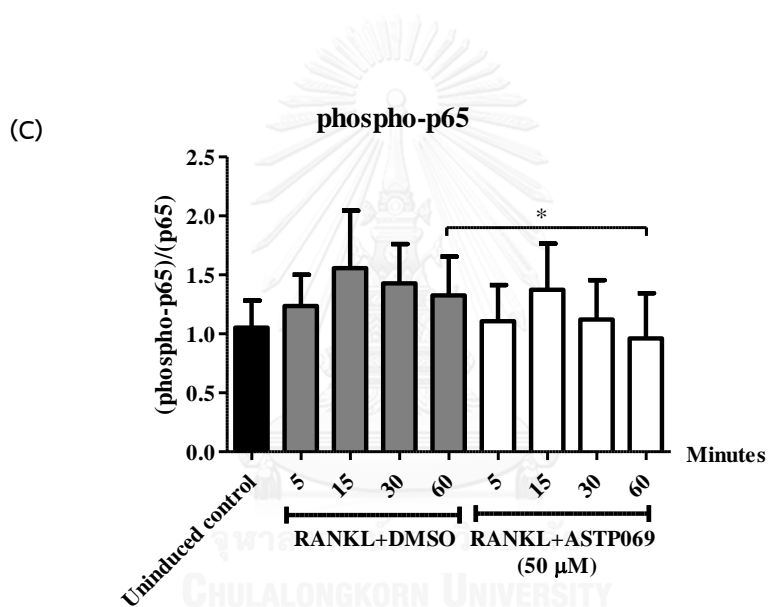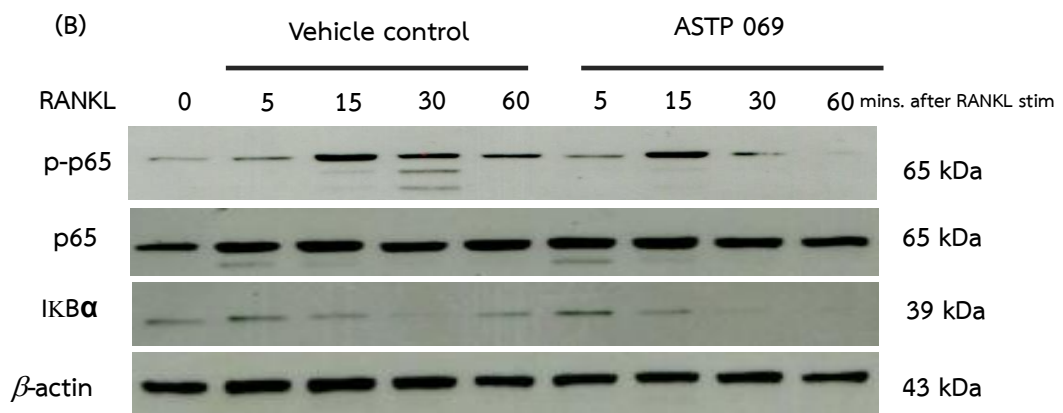
Figure 4. 5 Effect of ASTP069 on early signaling pathways (MAPK and NF-κB signaling pathway). BMs cells were treated with ASTP069 (50 μM) and stimulated with RANKL for indicated time points. Cell lysates were determined the activation of MAPK (A) and NF-κB (B). β-actin was used as loading control. The samples were assayed by Western blot. The band density of phosphor-p65 was normalized by p65 was shown in (C). ns: not statistical significance (*p<0.05).

4.7 Effect of ASTP069 on NF-κB p65 nuclear translocation

From the results in 4.6, ASTP069 treatment decreased the level of phospho-p65 upon RANKL stimulation. Therefore, ASTP069 was investigated for the effect on NF-κB p65 nuclear translocation by immunofluorescent. After RANKL stimulation, NF-κB p65 was translocated into nucleus at 30 and 60 minutes in the vehicle control. In treated cells with ASTP069, most NF-κB p65 did not translocate into nucleus at 30 minutes and 60 minutes. From these results, ASTP069 suppressed RANKL activation via suppressing nuclear translocation of the NF-κB p65. (Figure 4.6)

	DAPI	NF-κB p65	Merged

DMSO — A, B, C

ASTP 069 — D, E, F

Un induced

RANKL + DMSO — G, H, I

RANKL + ASTP 069 — J, K, L

30 minutes

RANKL + DMSO — M, N, O

RANKL + ASTP069 — P, Q, R

60 minutes

Figure 4. 6 Effect of ASTP069 on NF-κB p65 nuclear translocation. BMs cells were treated with 50 μM of ASTP069 and stimulated with RANKL for 0, 30 and 60 minutes. NF-κB p65 nuclear translocation were assayed by immunofluorescent and observed under laser scanning confocal microscope. DMSO was used as vehicle control.

CHAPTER V

DISCUSSION

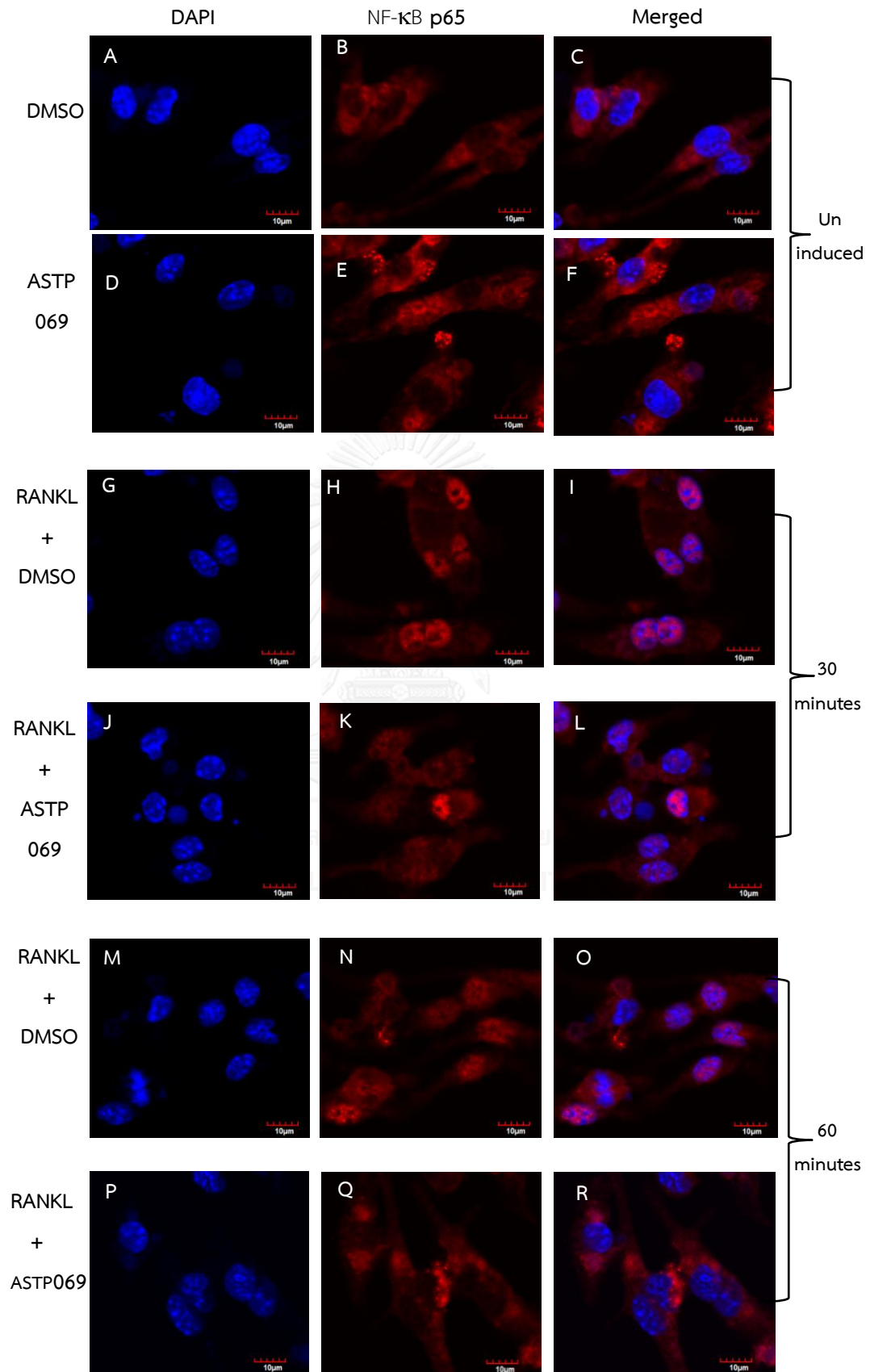*Dioscorea bulbifera* or air potato (in Thai "Wan-pra-chim" or "Wan-sam-pan-teung") is widely used as food and medicine in Thailand. *D. bulbifera* has therapeutic potential for several diseases and diterpenoids were reported as the constituents of the tuber of this plant species (57). In the present study, diosbulbin B (ASTP043) was modified by chemical method to obtain new analogues (11 compounds).

In the first experiment, all compounds were not cytotoxicity at any concentrations in RAW264.7 cell line and also no anti-inflammatory activity because they did not decrease the amount of nitric oxide production that induced by the combination of LPS and IFN𝛾 in RAW264.7 at all concentrations. In contrast with previous study in 2011, inflammation was inhibited by the aqueous and methanol extracted from *D. bulbifera* with the doses of 300 and 600 mg/kg by oral administration in mouse model (11). The different condition such as the characteristics of compounds (crude or pure compound) and dose of compounds might be the reason for the contradictory results.

The extracts from *Dioscorea* species with anti-osteoclastogenesis were reported in previous study. For example, the water extracts from *Dioscorea spongiosa* have strong inhibitory effect on anti-osteoclastogenesis via stimulated osteoblast formation (15) and the ethanol extracts from *Dioscorea alata* inhibited osteoporosis by induced osteoblast formation through increased alkaline phosphatase activity (16).However until now, there were no reported on the anti-osteoclastogenic activity on *D. bulbifera*. In the anti-osteoclastogenic acivity screening experiment, we found that ASTP064 and ASTP069 from *D. bulbifera* have the ability to suppress osteoclastogenesis at 100 µM with 21.89% and 1.02% of osteoclastogenesis, respectively (Figure 4.2D). ASTP069 has dramatically suppressed osteoclast differentiation. The structure of ASTP069 different from Diosbulbin B (ASTP043, Natural structure) by acetyl group (-AcO) and hydroxyl group (-OH) addition, this two position of ASTP069 might be effect on anti-osteolastogenesis activity.

ASTP069 was used to investigate in their molecular mechanism experiment. We investigated at the transcriptional level of the transcription factor, transcription repressor and osteoclast-related function (*nfatc1, irf8* and *ctsk*, respectively) (25). NFATc1 is the major transcriptional factor for osteoclastogenesis and its expression, it was negatively regulated by *IRF8* (58). *irf8* was decreased in initial step of osteoclastogenesis within 24 hrs and high at 48 hrs (47). ASTP069 has inhibitory effect on *irf8* and *nfatc1.ctsk* is encodes protein with the function of

mature osteoclast to resorb bone tissue and it was regulated by *nfatc1* (59). From the result, the expression of *nfatc1* was decreased and *ctsk* was also decreased by treatment of ASTP069. Therefore, expression of key gene induced in osteoclastogenesis was inhibited by in ASTP069 treatment.

The inhibitory effect of ASTP069 on signaling pathway at the translational level was investigated to confirm the result of transcriptional experiment. NF-κB signaling pathway are early signaling pathway involved in osteoclastogenesis (60) and we found that ASTP069 inhibited NF-κB pathway by reducing the level of phosphorylated p65 (Figure 4.5B). Previously, acetyl-11-keto-β-boswellic acid (a pentacyclic terpenoid) from *Boswellia serrate* had inhibitory effect on oteoclastogenesis by suppression of NF-κB signaling pathway and its regulated genes (61). Next, we focused on cFos that is the main regulator of NFATc1 (62). The activation of cFos was decreased by the treatment of ASTP069 (Figure 4.5B) and NFATc1 was reduced at both transcriptional and translational levels (Figure 4.5A). Therefore, ASTP069 inhibited osteoclast differentiation by downregulation of NFATc1 and cFos via NF-κB signaling pathway.

A stated above, ASTP069 inhibited osteoclastogenesis by inhibition of NF-κB signaling pathway. Therefore, ASTP069 may be decrease the amount of NO production which is under regulated by NF-κB signaling pathway (63) but this compound did not exhibit anti-inflammatory activity. The stimulation of LPS to promote cascade signaling pathway, the activation of MAPK and NF-κB were cascade induced by Toll-like receptor 4 which lead to pro-inflammatory mediators and inflammatory cytokines secretion to response inflammation in RAW 264.7 (64). ASTP069 may effect on upstream signaling pathway unique to RANK/RANKL and is not involved LPS/TLR4 pathway.

This study discovered the new activity of diterpenoid from *D. bulbifera*. ASTP069, the modified analogue from tuber of *D. bulbifera* has potential for therapeutic application for osteoporosis and may be developed to be a new anti-osteoporosis drug in the future.
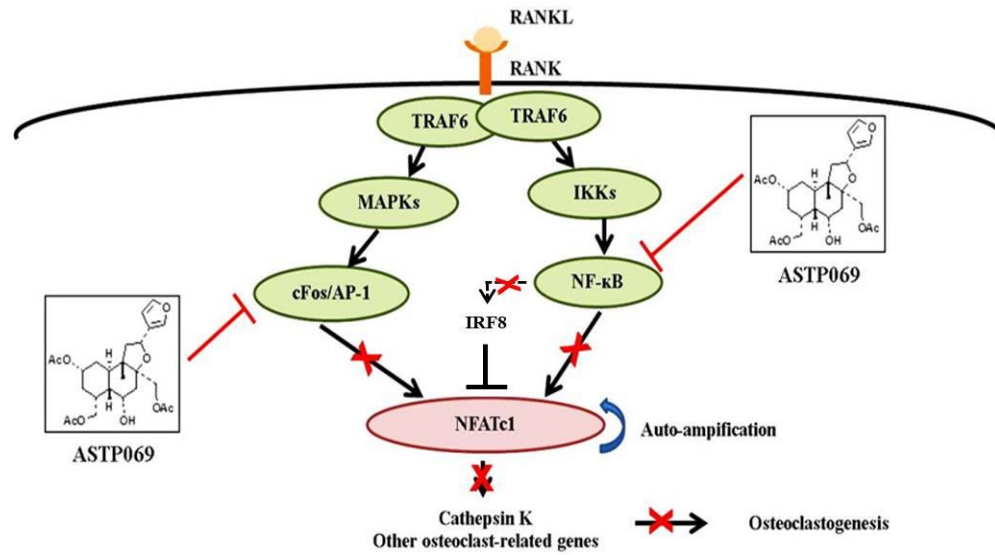
Figure 5 1 The proposed inhibition mechanism of modified analogue (ASTP069) from *D. bulbifera* in RANK/RANKL- induced osteoclast differentiation.

CHAPTER VI
CONCLUSION

(1.) ASTP069 had no toxicity and anti-inflammatory activity in RAW264.7at 100 μM.

(2.) ASTP069, a structurally modified analogue of diosbulbin B, the natural terpenoid isolated from tuber of *D. bulbifera*, has dramatically anti-osteoclastogenic activity by inhibiting the tartrate resistant acid phosphatase (TRAP) positive multinucleated cells induced by RANKL.

(3.) ASTP069 affects the osteoclastogenesis in transcription level by downregulating the expression of *nfatc1, ctsk* and *irf8*.

(4.) ASTP069 inhibited the osteoclastogenesis in translation level by suppressing NFATc1 via p65 subunit of NF-κB pathway and cFos downregulation.
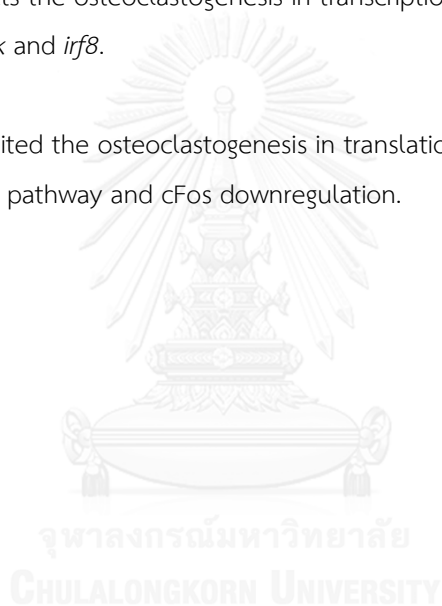
Suggestion for the future work

Osteoblast formation is involved in osteoclastogenesis. The effect of ASTP069 on their molecular mechanism in osteoblasts and the effect of ASTP069 on anti-osteoclastogenic activity in animal model should be further investigated to develop as osteoporosis drug in the future.

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