PRODUCTION AND CHARACTERIZATION OF ANTI-RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA-B LIGAND MONOCLONAL ANTIBODY FROM *NICOTIANA BENTHAMIANA*

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Pharmaceutical Sciences and Technology
Common Course
FACULTY OF PHARMACEUTICAL SCIENCES
Chulalongkorn University
Academic Year 2020

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การผลิตและลักษณะสมบัติของโมโนโคลนอลแอนติบอดีต่อรีเซบเตอร์แอกทิเวเตอร์นิวเคลียร์แคปปาบีลิแกนด์จากต้น *Nicotiana benthamiana*

น.ส.วนัชฐา บุณยโยธิน

จุฬาลงกรณ์มหาวิทยาลัย
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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชศาสตร์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2563
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	PRODUCTION AND CHARACTERIZATION OF ANTI-RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA-B LIGAND MONOCLONAL ANTIBODY FROM *NICOTIANA BENTHAMIANA*
By	Miss Wanuttha Boonyayothin
Field of Study	Pharmaceutical Sciences and Technology
Thesis Advisor	Associate Professor WARANYOO PHOOLCHAROEN, Ph.D.

Accepted by the FACULTY OF PHARMACEUTICAL SCIENCES, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

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วนัชฐา บุณยโยธิน : การผลิตและลักษณะสมบัติของโมโนโคลนอลแอนติบอดีต่อรีเซบเตอร์แอกทิเวเตอร์นิวเคลียร์แคปปาบีลิแกนด์จากต้น *Nicotiana benthamiana*. (PRODUCTION AND CHARACTERIZATION OF ANTI-RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA-B LIGAND MONOCLONAL ANTIBODY FROM *NICOTIANA BENTHAMIANA*) อ.ที่ปรึกษาหลัก : รศ. ดร.วรัญญู พูลเจริญ

แอนติบอดี Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) เป็นโมโนโคลนอลแอนติบอดีที่มีความสามารถในการยับยั้งการเจริญของเซลล์สลายกระดูก (Osteoclast) ในการศึกษาครั้งนี้จึงมีวัตถุประสงค์ที่จะพัฒนาการผลิตแอนติบอดีต่อ RANKL จากต้นยาสูบ *Nicotiana benthamiana* โดยวิธีการแสดงออกแบบชั่วคราวด้วยเจมมิไนไวรัลเวคเตอร์ (Geminiviral vector) จากนั้นตรวจสอบโครงสร้างของแอนติบอดีต่อ RANKL ที่ผลิตจากต้นยาสูบและทดสอบประสิทธิภาพในการยับยั้งการเจริญของเซลล์สลายกระดูกอีกด้วย จากผลการทดลองพบว่า ต้นยาสูบสามารถผลิตแอนติบอดีต่อ RANKL ได้ผลผลิตที่สูงสุด ถึง 0.5 มิลลิกรัมต่อกรัมน้ำหนักใบพืช ในระยะเวลา 8 วัน จากนั้นทำบริสุทธิ์แอนติบอดีที่ผลิตจากพืชด้วยเทคนิคโครมาโทรกราฟฟีแบบจำเพาะกับโปรตีน A และตรวจสอบความสามารถในการจับจำเพาะกับโปรตีน RANKL ด้วยวิธี ELISA จากผลการทดลอง พบว่า แอนติบอดีที่ผลิตจากพืชนั้นสามารถจับกับโปรตีน RANKL ได้อย่างจำเพาะ และในการศึกษาประสิทธิภาพการยับยั้งการเจริญเติบโตเซลล์สลายกระดูก โดยกระตุ้นเซลล์เม็ดเลือดขาวชนิดโมโนไซท์ (monocytes) ด้วยโปรตีนสำคัญที่ทำให้เกิดการเจริญของเซลล์สลายจากกระดูก ได้แก่ โปรตีน RANKL และ macrophage colony-stimulating factor (M-CSF) จากนั้นทดสอบการยับยั้งการเจริญเติบโตของเซลล์สลายกระดูก จากผลการทดลองพบว่า แอนติบอดีที่ผลิตจากพืชสามารถยับยั้งการเจริญเติบโตของเซลล์สลายกระดูกได้ในระดับ *in vitro* เมื่อเปรียบเทียบกับยา Denosumab ที่ใช้ในการปรับสมดุลสภาวะกระดูกพรุน ดังนั้นในการศึกษานี้จึงแสดงให้เห็นว่าแอนติบอดีต่อ RANKL ที่ผลิตจากต้นยาสูบนั้นมีประสิทธิภาพในยับยั้งการเจริญเติบโตของเซลล์สลายกระดูกได้ และอาจนำไปพัฒนาเพื่อวัตถุประสงค์ในการรักษาโรคกระดูกพรุนได้ในอนาคต

สาขาวิชา เภสัชศาสตร์และเทคโนโลยี ลายมือชื่อนิสิต .....

ปีการศึกษา 2563 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6176461033 : MAJOR PHARMACEUTICAL SCIENCES AND TECHNOLOGY

KEYWORD: Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL), plant-produced mAb, Nicotiana benthamiana, osteoporosis, osteoclastogenesis

Wanuttha Boonyayothin : PRODUCTION AND CHARACTERIZATION OF ANTI-RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA-B LIGAND MONOCLONAL ANTIBODY FROM *NICOTIANA BENTHAMIANA*. Advisor: Assoc. Prof. WARANYOO PHOOLCHAROEN, Ph.D.

Anti-RANKL monoclonal antibody is a fully human monoclonal antibody (mAb) available for treatment of osteoporosis. In this study, anti-RANKL mAb was transiently expressed using the Geminiviral expression system in *Nicotiana benthamiana*. Moreover, the structure of plant-produced mAb was characterized and functional activity was also determined. The result indicated that anti-RANKL mAb was produced with the maximum expression level on 8 days post infiltration and estimated to be 0.5 mg/g leaf fresh weight. The recombinant mAb from crude extracts was purified by using protein A affinity column chromatography. The plant-produced mAb provided *in vitro* affinity binding with human RANKL, determined by RANKL-ELISA binding. The function of plant-produced mAb was evaluated *in vitro*. CD14 positive cells isolated from human peripheral blood mononuclear cells (PBMCs) were cultured in vitro in the presence of human RANKL and macrophage colony-stimulating factor (M-CSF) in order for stimulating osteoclastogenesis. The results demonstrated that plant-produced mAb could significantly decrease the number of osteoclasts compared to commercially available denosumab. These results demonstrated that the plant-produced mAb has the potential to inhibit the osteoclast differentiation and can be applied for the osteoporosis treatment.

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Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my special thank of gratitude to my research advisor, Associate Professor Dr. Waranyoo Phoolcharoen, Ph.D. from the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for her patience, motivation, valuable suggestion and encouragement and she also provided the opportunities to study in Ph.D. program and perform this interesting research. Besides my advisor, I would like to thank to the members of the Research Unit for Plant-Produced Pharmaceuticals (RU-PPP), for supporting me and always help each other.

Furthermore, I am thankful to a Pharmaceutical Sciences and Technology (PST) program, Faculty of Pharmaceutical Sciences, Chulalongkorn University for the attractive courses of my Ph.D. study. I would also like to thank the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphisek Somphot Endowment Fund) for a financial support in this research.

Last but not the least, I would like to express my extreme sincere gratitude and appreciation towards my parents, family and friends who always cheer me up and support me in all my pursuits.

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CHAPTER 1

INTRODUCTION

1.1 Rationale and significant

Osteoporosis is known as the most common skeletal disease due to an imbalance of bone remodeling process (or bone metabolism) (E. Michael Lewiecki, 2010b) which is a normal dynamic process that is necessary to balances between bone formation via osteoblasts and bone resorption via osteoclasts (Hadjidakis & Androulakis, 2006b). According to the bone disorder, the risk of fracture significantly increased because of the high rate of bone resorption, low bone mineral density (BMD). The disease is an important health tissue that impact on several group of people such as all sexes, all races and especially older people. In Unites States, the study of osteoporosis show that the rate of patient dramatically raised from approximately 10 million people to more than 14 million people in 2020 (Burge et al., 2007). Bone remodeling occurred continuously including replaced the new bone by osteoblast while resorbed by osteoclast. In term of imbalance activity, the process of resorption exceeds the formation that regulated by the important factors including receptor activator of nuclear factor kappa B ligand (RANKL) presented by osteoblast. These factors can be activated the osteoclast precursor become an activated osteoclast by the interaction between RANKL and RANK.

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Nowadays, a new drug that developed for the treatment of osteoporosis is anti-RANKL monoclonal antibody or Denosumab. The antibody is a complete human monoclonal antibody of the IgG2 immunoglobulin isotype with a high affinity and specificity for RANKL and can prevent the binding between RANKL and RANK, similar to OPG, to suppress osteoporosis. Denosumab has recently been approved in Japan,

Europe and the US for the treatment of postmenopausal osteoporosis as well as bone metastasis. In the past, recombinant therapeutic proteins have been produced using various transgenic systems such as mammalian cells, yeast, and bacteria (Giddings et al., 2000). Nevertheless, there are many drawbacks which showed in these platforms. To overcome the limitations, plant-based production system was an alternative platform for recombinant therapeutic protein production.

This research aims to produce recombinant ant-RANKL mAb with transient expression using *N. benthamiana* as an alternative platform. This is because plant expression platform can provide several beneficial sides over traditional expression platforms. For instances, plant presents the lower production cost, plant-produced protein provide only 0.1–1% of the cost of recombinant protein production from mammalian cell culture (Chen et al., 2005), and also have the pathway of protein synthesis which is closed to mammalian cell culture pathway. Furthermore, plant-produced protein production platform is easy to scale up and plants have ability to perform the post-translational modifications, such as glycosylation (Xu et al., 2011). This system is able to decrease the chance of contaminated a human or animal pathogen in plant-based products (Pogue et al., 2010; Xu et al., 2011). In addition to many beneficial sides greater than other expression system, plant expression system is an alternative platform which suitable to produce recombinant protein for the future therapeutic purpose.

1.2 Research hypothesis

- *N. benthamiana* can be used as the alternative production platform for producing anti-RANKL mAb.
- The plant-produced anti-RANKL mAb can inhibit the interaction of RANK and RANKL to prevent osteoclast differentiation.

1.3 LITERATURE REVIEW

1.3.1 Osteoporosis

Osteoporosis is the common skeletal disease due to an imbalance of bone remodeling process (E. Michael Lewiecki, 2010b). This disease indicated by bone mass reduction and alteration of bone architecture. This disease is a major public health problem, and it also leads to the bone fragility and increases risk of fracture (Akkawi & Zmerly, 2018; Ensrud & Crandall, 2017). This disease mainly occurs in many groups of people such as Caucasian, postmenopausal women, and elderly people and the rate of osteoporosis will rise according to the age of populations. It is known as asymptomatic disease without any evidence until fractures happen (Cosman, de Beur, LeBoff, Lewiecki, Tanner, Randall, Lindsay, et al., 2014). The common positions of osteoporosis-related fracture represent at the spine, hip, and distal forearm. The osteoporotic fracture causes important public health concerns because it may lead to acute pain, physical function loss, hospitalization, and even mortality. Cooper et al. (1992) reported that the amount of patient worldwide that associated with osteoporotic hip fractures was approximately more than 200 million. According to Europe and the United States, the amount of about 30% of woman are the osteoporosis patient while 30% of men have osteoporosis-related fractures and the 40% remaining is menopause (Cooper et al., 1992; Reginster & Burlet, 2006). In woman, osteoporotic fractures illustrated the larger number of cases than stroke, heart attack and breast cancer (Figure 1).

In the United States, National Health and Nutrition Examination Survey (NHANES) data shows the estimated 10.2 million of osteoporosis patients who as after the age of 50 years from 2005-2010. Moreover, the number of elderly people with osteoporosis will rise by more than 30% from 2010 to 2030 (Williams et al., 2020).

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In the European Union, the osteoporosis patients were approximately 22 million woman and 5.5 million in men in 2010. Moreover, the number of osteoporotic fractures reported around 3.5 million cases, including 17% of hip fracture 15% of vertebral fracture 16% of forearm fracture and 52% of others. Two-third of all cases observed from women. According to the population changes, the percentages of fracture are projected to increase by 28% from 3.5 million between 2010 and 2025 (Kanis et al., 2019).

Moreover, the number of elder in Turkey is increasing, osteoporosis also become an important health issue. The rate of hip fracture is a significant increase at the age around 50 years old, the study shows that 3.5% of men and 14.6% of women probably get a hip fracture in their remaining lifetime (Tuzun et al., 2012).

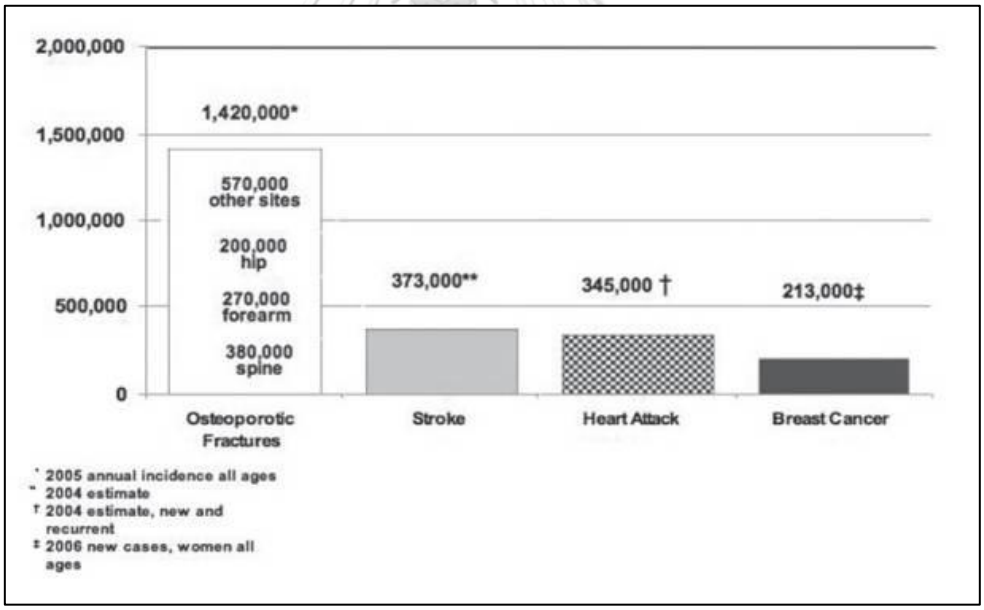

Figure 1 The comparison of the number of osteoporotic fractures, stroke, heart attack and breast cancer of women in the United States, relied on statistics data from 2004 to 2006.

Source: Tümay Sözen et al. (2017)

Classification

There are several factors that caused osteoporosis (Table 1) such as aging, female sex, postmenopausal status, hypogonadism, prolonged use of glucocorticoids, smoking and alcohol (Riggs et al., 1982). There are to 2 groups of osteoporosis that categorized by the factors affecting bone remodeling process (T. Sözen et al., 2017; Tu et al., 2018).

1. Primary osteoporosis

The primary osteoporosis is the common form of the disease and it can be divided into 2 groups.

- Involutional Osteoporosis Type I known as postmenopausal osteoporosis.

  This type is occurred by the reduction of estrogen in postmenopausal woman leading to increase the bone resorption. Therefore, women have more possibility to face the osteoporosis than men. According to the (Cosman, de Beur, LeBoff, Lewiecki, Tanner, Randall, & Lindsay, 2014), the ratio of osteoporosis in men/woman is 4/5.7.

- Involutional Osteoporosis Type II known as senile osteoporosis.

  Age-related osteoporosis presents the loss of cortical and trabecular bones mass by the reduction of stem-cell precursors.

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2. Secondary osteoporosis

The osteoporosis is induced by diseases, medication, and lifestyle. For instances, the excess of glucocorticoid production in Cushing syndrome affects the bone turnover with the higher bone resorption than formation. Moreover, long-term glucocorticoid therapy in many diseases also induces the bone resorption rate. In addition, the excessive alcohol use related to the osteoporosis type 2.

Table 1 Risk factors of osteoporosis

Age-related osteoporosis	50 years or over
	Low calcium absorption rate
	High level of parathyroid hormone (PTH)
	Calcitonin reduction
Genetic	More possibility in female
	Familial prevalence
	Races
Lifestyle	Insufficient calcium intake
	Excessive alcohol intake
	High caffeine intake
	High salt intake
	High animal protein
	Cigarette use
	Deficient physical activity
Endocrine disorder	Postmenopausal status
	Obesity
	Hypogonadism

Modified from Dobbs et al. (1999)

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1.3.2 Bone remodeling process

Bone remodeling is a continuous mechanism to replace the old and damaged bone with the new bone tissue to preserve bone integrity and maintain mineral homeostasis (Hadjidakis & Androulakis, 2006a; Kenkre & Bassett, 2018). According to the remodeling process, osteoclasts continuously resorb the old and damage bone while osteoblasts reconstruct the new bone (Hadjidakis & Androulakis, 2006b). The rate of bone formation should be the same proportion as the bone resorption. This process

give the effect on the mechanical strength and repair to make the stable bone mass (Tu et al., 2018). In term of remodeling imbalance, the amount of resorption rate is greater than formation and then osteoporosis will occur. If this imbalance activity prolongs, bone mass will loss and the risk of fracture will increase.

According to Frost (1990), the bone remodeling process occurred related with a Basic Multicellular Unit (BMU). The specific bone cells that related to BMU are bone-forming osteoblasts and bone-resorbing osteoclasts.

Osteoblast: Bone Formation

Osteoblasts differentiated from mesenchymal stem cells. The active osteoblasts produced a type I collagen-rich matrix known as osteoid matrix and then it plays an important role in osteoid mineralization in the bone formation process. After completing mineralization, the osteoblasts attached into the bone matrix and differentiated to osteocytes. In addition, the osteoblast can provide the main factors which are a critical for osteoclast differentiation.

Osteoclast: Bone Resorption

Osteoclast is a giant multinucleated cell that differentiated from hematopoietic cells. The active osteoclast can resorb the bone matrix by attached with the bone surface and secreted hydrochloric acid and proteolytic enzymes including tartrate-resistant acid phosphatase (TRAP) and cathepsin K. (Hadjidakis & Androulakis, 2006a; Kenkre & Bassett, 2018)

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Bone resorption

The action of osteoclasts can be activated by the main regulators including receptor activator of nuclear factor kappa B ligand (RANKL) and macrophage-colony stimulating factor (M-CSF). The osteoclast can be generated form hematopoietic precursor known as the granulocyte-macrophage colony forming cell (GM-CFU). M-CSF is expressed by osteoblasts and stromal cells. This factor can stimulate the GM-CFU

proliferation by attached with the colony-stimulating factor-1 receptor (c-fms) (Hodge et al., 2007; Ross, 2006). RANKL is highly presented by osteoblasts, T cells, and endothelial cells. The interaction between RANKL and its receptor on the cell surface of osteoclast precursor, receptor activator of nuclear factor kappa-B (RANK), can generated the activated osteoclast, increasing the bone resorption (Collin-Osdoby, 2004; Hsu et al., 1999) (Figure 2). To reduce the bone resorption, osteoprotegerin (OPG) expressed by osteoblasts which can prevent the interaction of RANKL/RANK, suppress the osteoclast formation and activity (Burgess et al., 1999; Lacey et al., 1998; Simonet et al., 1997). OPG also represents that the bone remodeling has the process to control the balance of bone formation and resorption. Therefore, the ratio of RANKL and OPG can be used to indicate pathogenesis the of resorption (Mizuno et al., 1998) and another bone disease (Hofbauer & Schoppet, 2004; Nagy & Penninger, 2015) .

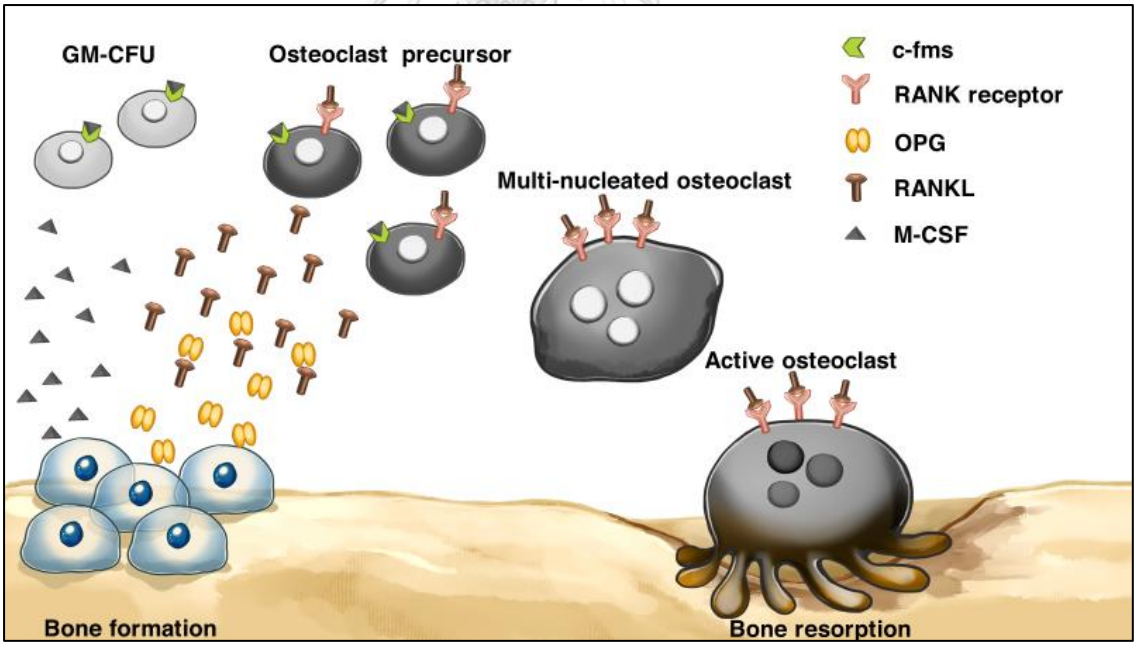

Figure 2 Bone remodeling mechanism: The binding of RANKL/RANK and M-CSF direct/GM-CFU sequentially activated the osteoclast precursor.

1.3.3 Treatment of osteoporosis

The goal of osteoporosis therapy is to prevent the risk of fractures. There are two type of treatments including (i) anti-resorptive agent to decrease the bone resorption and (ii) anabolic agent to improve the bone formation (Akkawi & Zmerly, 2018).

Nowadays, the common treatment of osteoporosis focused on the reduction of bone resorption to avoid the osteoporosis, the osteoporotic fracture risk and increase the bone mass. There are many antiresorptive agents. For instance, selective oestrogen receptor modulators (SERMS) or Raloxifene is used for post-menopausal osteoporosis (Philipp et al., 2013). Strontium ranelate (SR) also reported as the drugs that can reduce the risk of fracture (Seeman, 2006). The most widely used drugs in the osteoporosis treatment are bisphosphonates that stimulated the apoptosis of osteoclast cell and impair osteoclast function to suppress the resorption (Harvey et al., 2017; E. Michael Lewiecki, 2010a). Nonetheless, the drug should be used with caution such as after taking the drugs, patients must remain upright for 30–60 min because it can damage the upper gastrointestinal tract. This drug presented the important side effect on nephrotoxicity which are not recommended for patients with a creatinine clearance <30-35 mL/min. Moreover, the drug is reported the rare case of the osteonecrosis of the jaw (ONJ) and atypical femoral fractures (AFF) in patients who consumed bisphosphonate for long term (Gedmintas et al., 2013; Khosla et al., 2007; E. Michael Lewiecki, 2010a; Miller et al., 2013; Vives et al., 2015). According to these adverse events, a drug holiday is recommended by the American Society for Bone and Mineral Research, USPSTF, and the American College of Physicians (ACP). Denosumab or anti-human RANKL monoclonal antibody is the biologic drug. This mAb used as first-line therapy for the patient who are unable to get the therapy by oral and postmenopausal osteoporosis patients with the high fracture risk (Tu et al., 2018). It can be reduced the

risk of the spine, hip, and non-vertebral fractures (Bone et al., 2013) and improve the bone mineral density (BMD) at all skeletal sites (Anastasilakis et al., 2018).

1.3.4 Denosumab or anti-human RANKL monoclonal antibody

Denosumab is a fully human monoclonal IgG2 antibody which produced form Chinese Hamster Ovary (CHO) cells with approximately size 147 kDa. It has a specificity binding to RANKL. RANKL provided the ability to promote the osteoclasts differentiation function and survival by binding to its receptor on the osteoclast precursor (Faienza et al., 2018; Makras et al., 2015) (Figure 3). The antibody indicated for osteoporosis treatment in postmenopausal women with high risk of fracture to reduce the risk of fracture at vertebral, non-vertebral and hip. Furthermore, this antibody provided the ability to reduce the vertebral fracture risk in men with prostate cancer and in patients who long-term systemic glucocorticoid therapy. The recommended dose is 60 mg via a single subcutaneous injection in the upper arm, the upper thigh, or the abdomen for every 6 months. According to Warnings and Precautions, calcium 1000 mg and at least 400 IU vitamin D should be received in daily.

The function of Denosumab mimics the role of native OPG. OPG, a soluble member of the tumor necrosis factor (TNF) receptor superfamily, is secreted by osteoblast cells, marrow stromal cells and other cells (Simonet et al., 1997). It is known as the natural antagonist of RANKL that can prevent the interaction between RANK and RANKL. The imbalance of RANK/OPG ratio resulted in osteoporosis and other skeletal diseases (Kong et al., 1999; Lacey et al., 1998; Yasuda et al., 1998). According to Bekker et al. (2001), the effect of Fc-OPG was investigated in postmenopausal women with a single dose injection. The results demonstrates that administration of Fc-OPG rapidly decreases the biochemical marker of bone resorption. Based on this study, the single injection of Fc-OPG indicated the rapid reduction of bone turnover and the protein may be the alternative treatment to decrease the bone resorption in osteoporosis

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therapy. Moreover, Bekker et al. (2004) study indicated that the effect of a single dose of AMG 162 (anti-RANKL mAb) are similar to Fc-OPG. However, AMG162 represented the longer duration time of antiresorptive effect in the same doses. Furthermore, the mAb provided the greater reduce in bone resorption markers at lower doses when compared to Fc-OPG. The major concern of Fc-OPG administration is anti-OPG antibodies induction. This may interfere with the endogenous OPG and inhibit their activity (Hamdy, 2006). In phase 1 study, the expression of anti-OPG antibodies was observed in one subject (P Bekker, unpublished observation, 2001).

Therefore, the use of anti-RANKL mAb can avoid the cross-react with endogenous OPG. Another potential risk is the binding potential between OPG and TNF-related apoptosis-inducing ligand (TRAIL) which may interfere the natural defense mechanism against tumorigenesis. Based on the Emery et al. (1998) study, the result suggested that OPG provided a low affinity to TRAIL although this can be a safety issues when used OPG at high doses. On the other hand, AMG162 presented the affinity binding with RANKL and it did not represent the binding signal with TRAIL, TNF-α, TNF-β, and CD40L (Kostenuik et al., 2009). These results suggested that the AMG162 or anti-RANKL mAb provided the ability to use for osteoporosis therapy without TRAIL binding concern.

The efficacy of denosumab represents the ability to rapidly decrease the bone turnover marker (BTM) in healthy postmenopausal women within 12 h after injection and profound (up to 84%) and remain suppressed for 6 months and reversible effect was found after discontinuation in phase 1 study (Bekker et al., 2004).

In Phase 2 study, the efficacy and safety of denosumab was evaluated in postmenopausal women with low bone mineral density (BMD). After 12 months administration, denosumab indicated the efficiency to increase BMD for 3.0-6.7% and continue increasing until 24 months. The 24-month study was extended for more 24 months. The result indicated that BMD was increased for 9.4-11.8% when compared with baseline and the 4-year study was continued an additional 4 years for 8-year study

to evaluate the efficacy and safety. The preliminary result at 6 years indicated 13% increasing of BMD comparing to baseline and the BTM was sustained reductions c(Brown et al., 2013; Miller et al., 2008).

FREEDOM (Fracture REduction Evaluation of Denosumab in Osteoporosis every 6 Months) was conducted for phase 3 study. 7868 postmenopausal women with osteoporosis were injected with 60 mg of denosumab (Prolia®) every 6 months. The results demonstrated that denosumab can improve the BMD up to 9.2% compared to placebo. Moreover, the efficacy of anti-fracture at all skeletal sites was also proved. The risk of fracture was reduced up to 68% at 36 months of treatment. The FREEDOM study was continued for 10 years. 2343 women from the study increased BMD around 9.2% and the fracture rate remained low throughout the study duration. According to 10-year clinical trials and more than 7 years of post-marketing surveillance, denosumab provided an acceptable safety profile for osteoporosis treatment (Anastasilakis et al., 2018; Brown et al., 2009; Kendler et al., 2010; E. M. Lewiecki, 2010; Lorentzon, 2019; Roux et al., 2014).

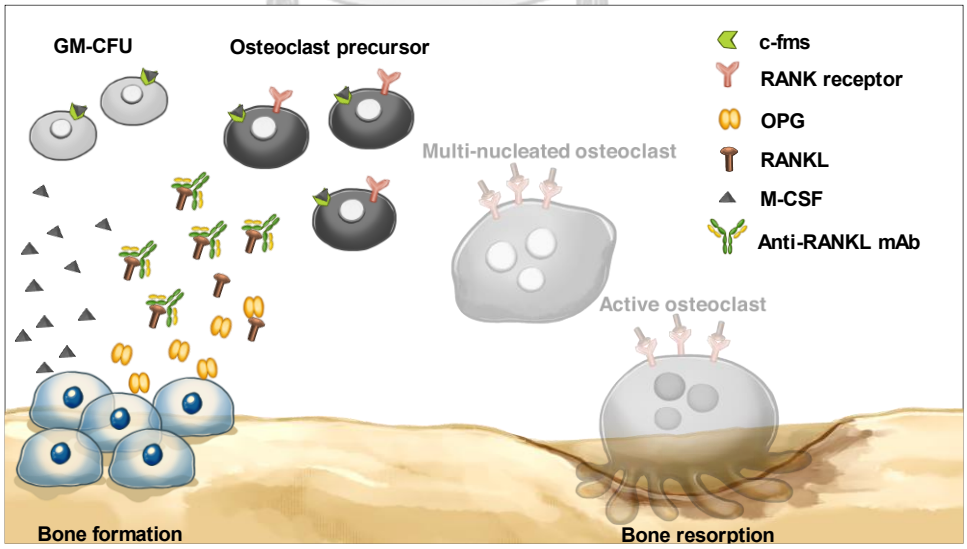

Figure 3 The activity of Denosumab blocks the interaction of RANK/RANKL on the bone remodeling mechanism.

1.3.5 Recombinant therapeutic protein expression systems

Recombinant therapeutic proteins have been produced using prokaryotic and eukaryotic expression system such as bacteria, mammalian cells, yeast, insect cells and plant (Giddings et al., 2000; Hejnaes & Ransohoff, 2018; Owczarek et al., 2019; Tripathi & Shrivastava, 2019).

Bacteria expression system, generally *E. coli*, are widely used as a host for producing recombinant therapeutic protein. Although bacterial culture is required the simple nutrient supplements, easy and inexpensive to perform in large scale production, well-known genetics, rapid growth rate, and good productivity, there are some drawbacks presented in product such as the prokaryote cell are not capable to modify the protein structure after translation or post-translational modifications (PTMs). This may affect to the improper folding of protein structure and their function (Balamurugan et al., 2006; Gupta & Shukla, 2017; Mohammed et al., 2015).

Mammalian cells (such as Chinese hamster ovary (CHO) cells) become an attractive platform over the past years. The mammalian cells are predominant in the production of various approved recombinant protein-based biopharmaceuticals among the other expression systems (Dumont et al., 2016; Khan, 2014). This system represents many beneficial sides in recombinant therapeutic protein production such as the capacity to express large and complex recombinant proteins, providing the desired protein folding and PTMs (Khan, 2013). Nevertheless, this process has a high production cost such as media supplements, complex nutrient requirements, limited of scalability and the possibility contaminated the product by human and animal pathogen (Houdebine, 2009; Leuzinger et al., 2013; Zhang, 2010).

Yeast system is the one optional platform for recombinant protein expression. It provides many advantages such as rapid growth rate, required the simple growth medium, well-known genetics, non-endotoxin releasing and be able to perform post-translational modifications (Ahmad et al., 2014; Fletcher et al., 2016; Huertas & Michán,

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2019). However, this system has some limitation such as complicated downstream process (Gomes et al., 2016), hyper-glycosylation that interfere protein activity. Moreover, the hypermannosylation pattern presents faster blood clearance when used as therapeutics and might induce allergic reactions in humans (Ahmad et al., 2014).

Insect cells represent the steadily increased over several decades. Baculovirus expression vector system (BEVS) was used for the production of recombinant proteins in insect cells by infected with a recombinant baculovirus containing the gene of interest. One of the beneficial sides is the quick turnaround time for the expression of recombinant proteins which provided the bioactivity similar to native proteins. Moreover, it showed possibility to contaminate with mammalian virus infection. Protease was observed in the cell which can degrade the protein of interest. For the glycosylation, the pattern is different from the mammalian cell but the introduction or co-expression of mammalian glycosyltransferases together with gene of interest into insect can overcome this problem (Contreras-Gómez et al., 2014; Gecchele et al., 2015; Le et al., 2018).

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Table 2 Comparison of recombinant protein expression systems

Expression platform	Overall cost	Production period	Scale-up ability	Propagation	Product yield	Product quality	Contamination issues	Cost of Downstream process
Bacteria	Low	Low	High	Easy	Medium	Low	Medium	High
Mammalian cells	High	High	Very low	Hard	Medium-High	Very high	Very high	High
Yeast	Medium	Medium	High	Easy	High	Medium	Low	Medium
Insect cell culture	Medium	Medium	High	Feasible	High	Medium	Very low	Medium
Whole plants	Very low	Medium	Very high	Easy	High	High	Low	High

Modified from Gharelo et al. (2016); Owczarek et al. (2019).

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1.3.6 Plant Molecular Pharming (PMF)

Among the various recombinant expression platforms, plant-based expression system was an alternative platform for recombinant therapeutic protein production (Table 2). Plant systems do not show only the lower production cost (Chen et al., 2005), but also have the pathway of protein synthesis which is closed to mammalian cell culture pathway. Furthermore, plant expression platform is easy to scale up by growing the high number of plants liked an agricultural scale. In addition, Plants are a eukaryote cell which have ability to perform the post-translational modifications, such as glycosylation, for producing many complex proteins (Xu et al., 2011). The one advantage of plant-based production system is this system be able to decrease the chance of contaminated a human or animal pathogen in plant-based products (Pogue et al., 2010; Xu et al., 2011). In June 2009, there is the report of the contamination issue in Cerezyme®, the commercial biologic drug for Gaucher disease, which produced from Chinese hamster ovary (CHO) cell. The cell line which used for producing the product are contaminated with a calicivirus (Rybicki, 2010). In addition to the greater beneficial sides, the development of plant-produced protein production is required to response the increasing demand in many applications (Table 3) and to improve the efficiency and quality of therapeutic proteins in the future.

Nowadays, there are many plant-derived pharmaceuticals which are at the different stage of clinical trial and some are approved by FDA for therapeutic purpose (Table4).

Table 3 Recombinant proteins production in plant

Product	Host
Recombinant protein	
Human serum albumin	*Nicotiana tabacum*
Human erythropoietin	*N. tabacum* cv. BY-2
Bryodin 1	*N. tabacum* cv. NT-1
Human growth hormone	*Oryza sativa* cv. Bengal
Cytotoxic T cells surface antigen	*O. sativa*
Recombinant antibody	
Human anti-rabies virus mAb	Tobacco
Anti-phytochrome single-chain Fv (scFv)	Tobacco
LO/BM2, IgG	Tobacco
MAK33, IgG	Potato
H10, IgG1λ	Tobacco
Recombinant vaccine	
Hepatitis B surface antigen	Potato
VP1 epitope of hand foot and mouth disease virus	Potato
B subunit of the heat liable toxin of *E. coli*	Lettuce
Rheumatoid arthritis type II collagen	Rice
Capsid precursor polypeptide of HFMDV	Rice

Modified from Gharelo et al. (2016)

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Table 4 Plant-derived pharmaceuticals and their status

Product	Application	Status
Recombinant human intrinsic factor	Vitamin B12 deficiency	Phase II completed 2006
Recombinant human lactoferrin	Anti-inflammatory conditions in HIV patients	Phase I and II, completed 2006, under Phase III
Locteron™	Hepatitis B and Hepatitis C	Phase I and II, completed 2009, under Phase III
P2G12 antibody	HIV	Phase I completed 2011
HAI-05	H5N1 vaccine	Phase I, 2011
Recombinant interferon	Antiviral treatment	Phase II
Human α-galactosidase	Fabry disease	Phase I
PRX-102	Fabry disease	Phases I and II, 2014
ZMApp	Ebola virus	Phases I and II, 2015
Newcastle disease virus protein	Poultry vaccine	USDA approved
Human Epidermal growth factor	Burns treatments	Commercialization
Human growth hormone	Deficiency treatments	Commercialization
Glucocerebrosidase enzyme (ELELYSO)	Gaucher's disease	Commercialization

Modified from Owczarek et al. (2019)

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1.3.7 Recombinant therapeutic protein production in plant

There are currently two approaches for plant-produced recombinant therapeutic protein (Chen et al., 2013). For Stable expression, the gene of interest (GOI) was inserted into an expression vector and transformed into plant cell. The transgenic plants were generated by stably integrated of GOI into plant nuclear or chloroplast genome, which is able to transfer a new trait to the next plant generations. This method represents some beneficial sides such as high scale-up ability and convenience for large scale manufacturing (Commandeur et al., 2003). However, this method consumed long time to develop and generate the transgenic plant or genetically modified (GM) plants. The major concern is the ability to cross with food crops, only limiting field that accept for transgenic plants (Chen, 2011; Davies, 2010).

Transient expression provided the alternative approach to recombinant protein production during the short period with the high yield. To generate transient expression, an expression vector harboring the GOI is delivered into plant cell excepted the stably integrating into plant genomes (Sainsbury & Lomonossoff, 2014). The transformed GOI begins transiently expressing the desired protein which can produce the recombinant protein in a short production period. This method offers many beneficial sides such as high amount of protein accumulation in a short production time, transformed plant can be collected in 2-7 days after infiltration (dpi), and none generate transgenic plants cell line (Komarova et al., 2010).

In this study, *N. benthamiana* or tobacco was used as a plant-based bioproduction platform. This plant is widely used for many recombinant protein expressions because considerable biomass, non-food crops, rapid growth well-known genetic rate and scalability.

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Agrobacterium- mediated gene transformation in tobacco

Gene transformation in plants is the techniques which can manipulate the genetic of plant cell and stimulate the plant for producing desired recombinant protein. The gene of interest (GOI) can be obtained from the same plant species, bacterial, virus, animal and human. *Agrobacterium* mediated gene transformation is the method for transferring the expression system with GOI into plant cell to produce the recombinant therapeutic protein.

Agrobacterium tumefaciens is a gram-negative bacterium which are known as a causal agent of crown-gall disease in plants. *A. tumefaciens* presents the ability to naturally transfer the transferred DNA (T-DNA) form their tumor-inducing (Ti) plasmid into plant cells. According to *Agrobacterium*-mediated gene transformation, the bacterial virulence (*vir*) genes are induced by the plant phenolic compound which release from plant wound and then, *vir* gene delivered T-DNA into the host-cell. T-DNA harboring the oncogenes and opine-catabolism genes to generate the neoplastic growth and produce the of amino acid derivatives, opines, as a nitrogen source of *Agrobacterium* (Figure 4) (Tzfira & Citovsky, 2006). For recombinant protein production, the native T-DNA was replaced with GOI and *Agrobacterium* was used as a mediator for the introduction of foreign genes into plants.

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To generate the gene transformation in plant, agroinfiltration was most common methods to indirect delivery GOI into plant cell by using *A. tumefaciens* (Rivera et al., 2012). To perform a small-scale production, the common method of agroinfiltration is syringe infiltration that use of a syringe without needle to deliver *Agrobacterium* suspension into plant leaves. Vacuum infiltration was developed for large-scale production and for some plant species that cannot be successfully agroinfiltrated by syringe infiltration. The intact plants were submerged into a vacuum chamber containing *Agrobacterium* suspension under a negative atmospheric pressure. The air was draws out form the interstitial spaces of the submerged plant leaf. When the

vacuum is released, *Agrobacterium* suspension is filled and replace in the space (Chen et al., 2013; Rivera et al., 2012; Vaghchhipawala et al., 2011).

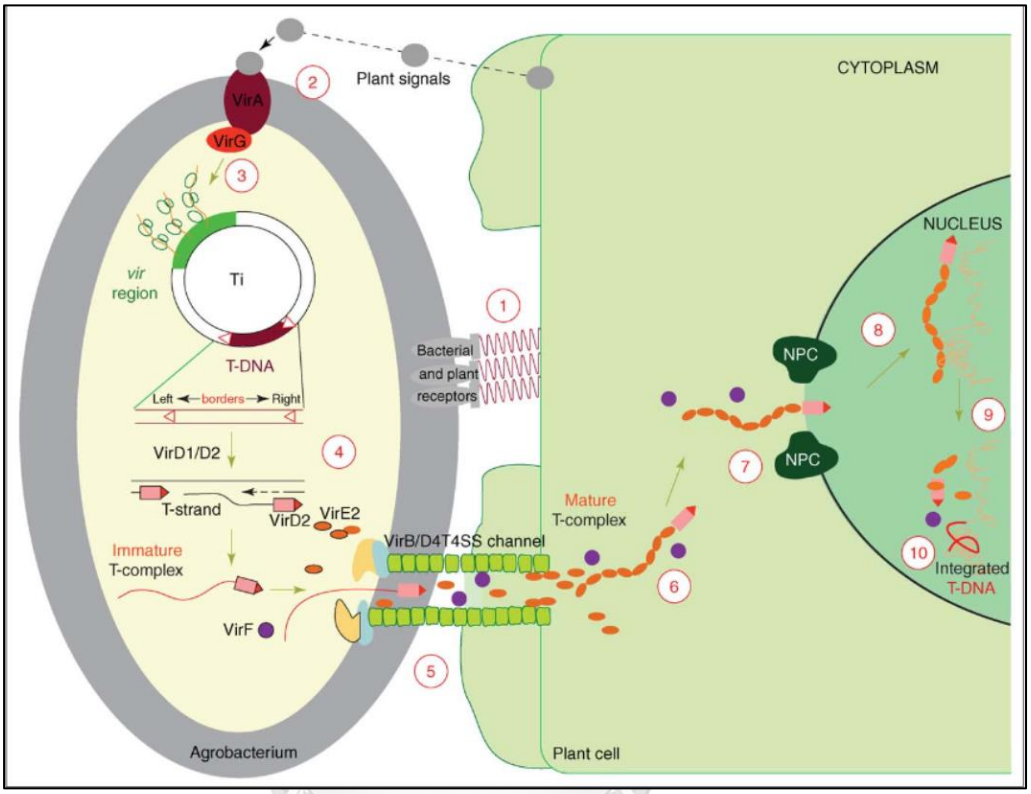

Figure 4 A mechanism for the *Agrobacterium*-mediated genetic transformation.

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Source: Tzfira and Citovsky (2006)

1.3.8 Geminiviral expression system

Nowadays, plant viral expression systems are widely used for gene transformation into plant. This system provided advantages such as the high rapid change of viral system improving the transient expression level (Kusnadi et al., 1997; Lico et al., 2008). There are many viral systems that develop for expression vector such as Cucumber mosaic virus (CMV) (Hwang et al., 2012), Tobacco mosaic virus (TMV) (Liu & Nelson, 2013), Potato virus X (PVX) (Lico et al., 2015), Cowpea mosaic virus (CPMV) (Sainsbury et al., 2010). On of the effective system is magnI-CON system which can produce very high levels of target gene mRNA (Gleba et al., 2005).

Geminiviruses are a family of plant virus in the *Mastrevirus* genus that contained the circular single-stranded DNA genome with the approximately size 2.5 kb to 3.0 kb (Francki, 1991). The genomic DNA can be replicated in the nucleus of infected cell through a rolling circle replication mechanism. *Mastreviruses* are all monopartite genome, and it can infect both monocot plants (such as wheat dwarf virus, maize streak virus) and dicot plants (such as tobacco yellow dwarf virus, bean yellow dwarf virus (BeYDV)).

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The BeyDV genome consists of two part of Intergenic region (IR) including smaller intergenic region (SIR) and larger intergenic region (LIR). For the viral-sense (V) strand, MP encoded the movement protein, CP encoded the capsid protein. Rep encoding the Replication initiator protein and RepA protein genes are occur on the complementary-sense (C) strand (C1/C2 genes) (Figure 5).

Intergenic region (IR) is only the cis-element which used for genome replication. The LIR harboring a bi-directional promoter and a stem-loop structure that initiated rolling-circle replication. SIR is presented the origin of C-strand synthesis and contains transcription termination and polyadenylation signals.

For Geminiviral DNA genome initial replication, the viral single-stranded DNA are converted into double-stranded DNA in the nucleus of infected cell. This DNA are provided for viral open reading frame (ORF) transcription and the further rolling circle replication mechanism. The Rep presents the function of nicking and ligating the DNA. In addition, the 3′-OH terminus is served as the primer for the synthesis of nascent DNA. DNA synthesis is processed by host replication proteins, including DNA polymerase. Rep plays an important role on rolling circle replication by nicking the stem-loop structure to release nascent DNA and then ligating to form circular molecules (Chen et al., 2011; Gutierrez et al., 2004; Mor et al., 2003).

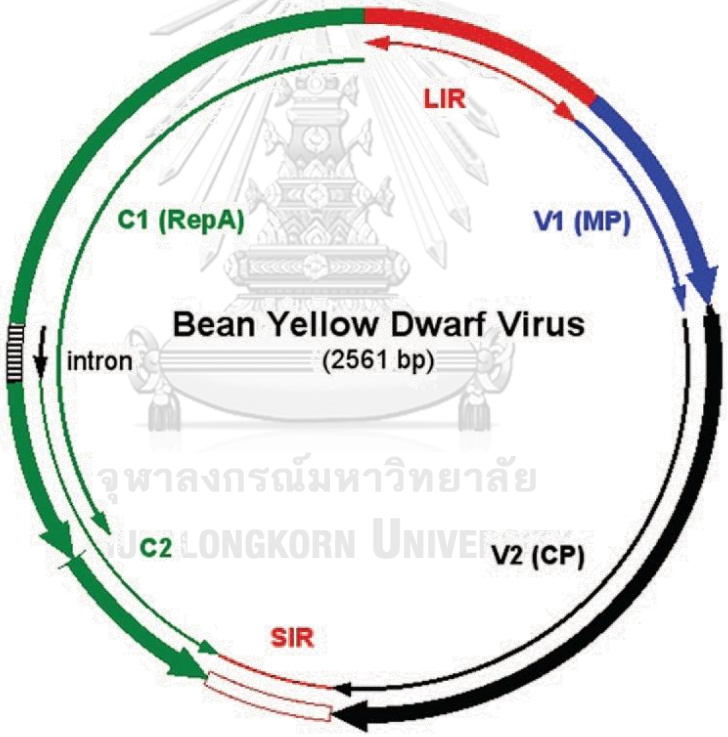

Figure 5 Genome structures of Bean yellow dwarf virus

Source: Chen et al. (2011)

The Geminiviral expression system was develop from the genome structure of the Gemini virus to improve the protein expression level and the production period. Previous study shows that there are many recombinant proteins producing form Geminiviral replicon systems (Table 5).

Table 5 Recombinant proteins expression by using Geminiviral expression system.

Proteins	Replicons	Expression level
Hepatitis B viral (HBcAg)	BeYDV	0.8 mg/g FW
Norwalk virus capsid (NVCP)	BeYDV	0.34 mg/g FW
mAb 6D8	BeYDV	0.5 mg/g FW
HPV L1 capsid	BeYDV	0.55 mg/g FW
HIV p24	BeYDV	0.03 mg/g FW

Modified from Chen et al. (2011)

According to Mor et al. (2003), the Gemini viral expression system was used for producing beta-glucuronidase (GUS) in plant. In addition, Kim et al. (2007) demonstrated that the Gemini viral expression system could improve the expression level of the green fluorescence protein when compared with the traditional plant expression system. Furthermore, the Gemini viral expression system also produce Enterotoxin B in plant cell (Hefferon & Fan, 2004). Moreover, Phoolcharoen et al. (2011) developed Gemini viral expression system or pBYR vector for producing recombinant Ebola immune complex (EIC) in *N. benthamiana*. The result suggested that EIC could be produced in plant by using Gemini viral expression system. According to Rattanapisit et al. (2017) study, the result has demonstrated that plant-derived human OPN transiently expressing by geminiviral expression system could induce

osteogenic differentiation of stem cells from periodontal ligament in vitro and could potentially be used for bone tissue engineering applications.

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CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and Equipment

2.1.1 Genetic materials

1. The synthesize gene of heavy-chain (HC) and light-chain (LC) of anti-RANKL mAb (Bioneer company, South Korea)
2. pBYR2eK2Md; pBYK-2e vector (Geminiviral expression system) (Chen et al., 2011)

2.1.2 Plant materials

1. Tobacco plant (*Nicotiana benthamiana*)

2.1.3 Microorganisms

1. *Escherichia coli* strain DH10B
2. *Agrobacterium tumefaciens* strain GV3101

2.1.4 Equipment

1. Autoclave (TOMY by Amuza Inc., Japan)
2. Blender (OTTO®)
3. Gel Electrophoresis (Mupid Co., Ltd, Japan)
4. Gene Pulser Electroporator (Bio-Rad®, USA)
5. Hettich® Universal 320/320R centrifuge (Andreas Hettich GmbH & Co. KG, Germany)
6. ImageQuant™ *LAS 4000* (GE Healthcare, USA)
7. Micropipette 2-1000 *μL* (Pipetman®, USA)
8. Microplate incubator (Hercuvan Lab systems, UK)
9. MicroPulser Electroporator™ (Bio-Rad®, USA)
10. Mini Centrifuge (Bio-Rad®, USA)

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11. Mini Trans-Blot® cell (Bio-Rad®, USA
12. Mini-PROTEAN® Tetra handcast systems (Bio-Rad®, USA)
13. Multichannel pipette (Cleaver scientific, UK)
14. PCR Machine (Bio-Rad®, USA)
15. SpectraMax® M5 Microplate Reader (Molecular Devices LLC, USA)
16. Spectrophotometer (Thermo Fisher Scientific, USA)
17. WIS-20 Precise Shaking Incubator (WiseCube®)

2.1.5 Materials

1. 96-well microplate (Greiner bio-one, Austria)
2. Amintra® Protein A Resin (Expedeon Ltd., UK)
3. Centrifuge tube 0.2-50 mL (Axygen®, USA)
4. Cuvette
5. DNA-spin™ Plasmid DNA Purification Kit (iNtRON Biotechnology, Inc., Korea)
6. Ficoll-Paque PLUS density gradient media (GE Healthcare, USA)
7. MEGA quick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, Inc., Korea)
8. MACS CD14+ microbeads (Miltenyi Biotec, Germany)
9. Membrane filter 0.45 µm (MilliporeSigma, USA)
10. Nitrocellulose Membrane, 0.45 µm (Bio-Rad®, USA)
11. PCR tubes/strips (Axygen ®, USA)
12. Petri dishes
13. Pipet Tip sizes: 10, 200, 1000 µL and 5 mL (Axygen®, USA)
14. Purification column
15. TRAP Staining Kit (Kamiya Biomedical Company, USA)

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2.1.6 Chemical reagents

1. 2-(N-morpholino) ethanesulfonic acid monohydrate (MES) (Bio Basic Inc., Canada)
2. 50X Tris-Acetate-EDTA (TAE) Buffer, pH8.0, Ultra-Pure Grade (Vivantis, Malaysia)
3. Acrylamide/Bisacrylamide 40% (HiGenoMB®, India)
4. Agar powder (Titan Biotech Ltd., India)
5. Agarose gel (Vivantis, Malaysia)
6. Amersham ECL prime western blotting detection reagent (GE Healthcare, UK)
7. Ammonium Persulfate (APS) (HIMEDIA®, India)
8. Antibiotics
   - Ampicilin (Panreac AppliChem®, USA)
   - Gentamycin (Panreac AppliChem®, USA)
   - Kanamycin sulfate (Panreac AppliChem®, USA)
   - Rifampicin (Bio Basic Inc., Canada)
9. Bromophenol blue (Honeywell FlukaTM, Finland)
10. Coomassie Blue blue R-250 (AppliChem®, USA)
11. Denosumab (AMGEN, United States)
12. Deoxynucleoside triphosphate (dNTP): dATP, dCTP, dGTP, dTTP
13. Di -Sodium hydrogen phosphate (Na_2HPO_4) (EMSURE®, Germany)
14. Ethanol (EMSURE®, Germany)
15. Ethylenediaminetetraacetic acid (EDTA) (HIMEDIA®India)
16. Fetal bovine serum (FBS) (Thermo Fisher Scientific, United States)
17. Glycerol (HIMEDIA®, India)
18. Glycine (HIMEDIA®, India)
19. HRP-conjugated anti-human gamma antibody (The Binding Site, UK)
20. HRP-conjugated anti-human kappa antibody (Southern Biotech, United States)
21. Human IgG1 kappa isotype antibody (Abcam, UK)

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22. Human RANKL protein (ProSpec-Tany TechnoGene Ltd.,Israel)
23. Magnesium sulfate ($MgSO_4$) (KEMAUS®, Australia)
24. Methanol (Merck, Germany)
25. Peptone (Himedia Laboratories Pvt. Ltd., India)
26. Potassium chloride (KCL) (Carloerbareagets, Italy)
27. Potassium dihydrogen phosphate (KH_2PO_4) (Carloerbareagets, Italy)
28. Protein ladder (Bio-red®, USA)
29. Restriction enzyme (New England Biolabs, UK): *Xba*I, *Sac*I
30. Safe green Loading Dye (New England Biolabs, USA)
31. Skim milk (Difco™)
32. Sodium chloride (NaCl) (Ajax Finechem Pty., Ltd, New Zealand)
33. Sodium dodecyl sulfate (SDS) (Carloerbareagets, Italy)
34. β-merceptoethanol (Applichem, Germany)
35. SureBlue™ TMB 1-Component Microwell Peroxidase Substrate (Promega, United States).
36. T4 DNA ligase (New England BioLabs, UK)
37. Taq DNA polymerase (Vivantis, Malaysia)
38. Tetramethylethylenediamine (TEMED) (Affymetri®, USA)
39. Tris -base (Vivantis®, Malaysia)
40. Tween -20 (Vivantis®, Malaysia)
41. VC 1 kb DNA Ladder (Vivantis®, Malaysia)
42. ViSafe Red Gel Stain (Vivantis, Malaysia)
43. Yeast Extract (Himedia Laboratories Pvt. Ltd., India)
44. α-minimal essential medium (Thermo Fisher Scientific, United States)

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2.1.7 Software and database

1. Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)
2. ExPAsy Bioinformatics Resource Portal (https://web.expasy.org/translate/)
3. GenBank NCBI (https://www.ncbi.nlm.nih.gov/genbank/)
4. GeneArt Gene Synthesis Portal
5. (https://www.thermofisher.com/order/geneartgenes/projectmgmt)
6. NCBI (https://www.ncbi.nlm.nih.gov/)
7. NEBcutter V2.0 (http://nc2.neb.com/NEBcutter2/)

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2.2 Experimental Procedures

2.2.1 Plasmid construction

Gene optimization for N. benthamiana

Amino acid sequences of heavy-chain (HC) and light-chain (LC) of anti-RANKL mAb were derived from drugbank database (DrugBank accession number: DB06643). The amino acid sequences were changed into nucleotide sequences and optimized to the codon-usage of *N. benthamiana.* The optimized nucleotide sequences were added with signal peptide (MGWSCIILFLVATATGVHS) in the amino-terminal (N-terminal) and SEKDEL were added in the carboxyl-terminal (C-terminal) of both HC and LC. The restriction enzyme sites including *Xba*I and *Sac*I were also added into the optimized sequences for the process of plasmid construction. The sequences were synthesized into cloning vector (pBHA).

Construction of plant expression vectors for producing anti-RANKL mAb

The cloning vector containing genes of HC and LC of anti-RANKL mAb were digested with restriction enzyme including *Xba*I and *Sac*I as shown in Table 6.

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Table 6 Component for enzyme digestion

COMPONENT	20 µl REACTION
Restriction Enzyme	10 units is sufficient, generally 1 µl is used
DNA	1 µg
10X NEB buffer	2 µl
Total Reaction Volume	20 µl

*incubate at 37°C 1 hours or overnight.

The digested products were separated on 1% Agarose gel electrophoresis. After that, the gel was staining with Safegreen dye to visualize the DNA band

comparing the size with 1 kb DNA ladder (VC 1kb DNA Ladder, Vivantis). The gel with the expected DNA fragments were extracted and purified using MEGA quick-spin™ Plus Total Fragment DNA Purification Kit followed the instruction of suppliers. To produced assembly antibody including 2HC and 2LC, the gene of interest (GOI) was ligated into geminiviral expression vector (pBYR2eK2Md; pBYK-2e) (Table 7) (Chen et al., 2011) which was kindly provided by Hugh S. Mason, Arizona State University, United States (Figure 5). After that, the ligated product was transformed into *E. coli* strain DH10B competent cells via Heat shock transformation.

Table 7 Component for enzyme ligation

COMPONENT	**10 µl REACTION**
10X T4 DNA Ligase Buffer (10X)	1 µl
Vector DNA	1 µl
Insert DNA	7 µl
T4 DNA ligase	0.5 µl
Nuclease-free water	to 20 µl

*incubate at 16°C overnight or room temperature for 10 min for cohesive ends.

**incubate at 16°C overnight or room temperature for 2 hours for blunt ends or single base overhangs, (alternatively, high concentration T4 DNA Ligase can be used in a 10-min ligation).

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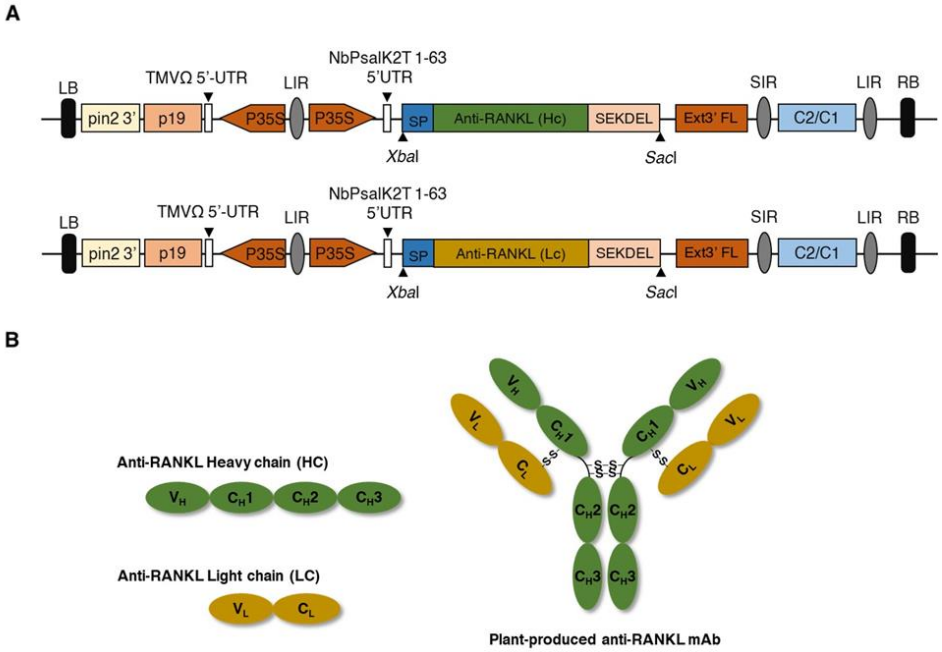

Figure 6 (A) Schematic representation of Geminiviral expression vector and (B) Schematic and structural elements of heavy chain (HC), light chain (LC) and assembled plant-produced anti-RANKL mAb.

E. coli strain DH10B competent cell preparation

E. coli Strain DH10B competent cells were prepared by inoculated a single colony into 3 mL LB media and then incubated at 200 rpm 37°C for overnight. After that, 0.5 mL bacterial suspension was transferred into 100 mL LB medium and incubated at 200 rpm 37°C until the optical density at 600 nm (OD600) reached 0.3-0.4. The bacterial suspension was chilled on ice for 10 min and centrifuged at 6,000 rpm for 10 min at 4°C for collecting bacterial cell. Then, the cell pellet will be resuspended with 0.1M $MgCl_2$ and centrifuged at 6,000 rpm for 10 min at 4°C (Repeating this step). The cell pellet was resuspended with 0.1 M $CaCl_2$ containing 10% Glycerol. After that, the bacterial suspension was aliquoted into each microcentrifuge tubes and stored at -80°C.

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Heat shock transformation

E. coli Strain DH10B competent cells competent cell was thaw on ice. Then, 1 - 5 µl of ligated product or DNA was added into 50-100 µl of competent cells and then incubated on ice for 20 min. After that, the competent cell was incubated at 42°C for 90 seconds and then put back on ice for 2 min. 500 µl of LB media (without antibiotic) will be added to the bacteria and incubated at 200 rpm 37°C for 30 min. After incubation, the transformed competent cell was grown on LB agar plate containing the 50 µg/ml of Kanamycin sulfate at 37°C for overnight. The bacterial colonies on the LB agar plate were selected and confirmed by polymerase chain reaction (PCR) with primer that specific to the gene. The PCR reaction was prepared following table 8 and 9. The PCR products were analyzed using 1% agarose gel electrophoresis to compare the DNA band with the positive control. The positive colonies containing the recombinant vectors were grown in LB broth with 50 µg/ml of Kanamycin sulfate at 200 rpm 37°C for overnight. After that, the recombinant *E. coli* cell was collected and extracted using DNA-spin™ Plasmid DNA Purification Kit, according to the instruction of suppliers to obtain the recombinant expression vectors. Then, the expression vector was transformed into *A. tumefaciens* strain GV3101 competent cell via electroporation.

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Table 8 Component for Polymerase Chain Reaction

COMPONENT	50 µl REACTION
Reaction Buffer	1X
dNTPs Mix	100 µM
$MgCl_2$	1 µl
Forward Primer Reverse Primer	0.2-1 µM
Template DNA	0.02-0.2 ng
DNA Polymerase	2 µl
Nuclease-Free Water	to 50 µl

Table 9 Condition for Polymerase Chain Reaction

STEP	CONDITION
Initial Denaturation	94°C for 2 min
Denaturation	94°C for 30 sec
Annealing	50-68 °C for 30 sec
Extension/1 kb	72°C for 30 sec
Final Extension	72°C for 7 min

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A. tumefaciens strain GV3101 competent cell preparation

A. tumefaciens strain GV3001 was grown on LB agar plate containing the 50 µg/ml Rifampicin and 50 µg/ml Gentamicin incubating at 28°C for 48 hours. A single colony was inoculated into 3 mL of LB media containing antibiotics and then incubated at 200 rpm 28°C for overnight. After that, 0.5 mL bacterial suspension was transferred into 100 mL LB medium and incubated at 200 rpm 28°C until reached 0.3-0.4 (OD_{600}). The bacterial suspension was chilled on ice for 20 min and centrifuged at 6,000 rpm for 10 min at 4°C for collecting bacterial cell. Then, the

cell pellet will be resuspended with cold sterile water and centrifuged at 6,000 rpm for 10 min at 4°C for collecting bacterial cell (Repeating this step). The cell pellet was resuspended with cold sterile water containing 10% glycerol. After that, the bacterial suspension was aliquoted into each microcentrifuge tubes and stored at -80°C.

Gene transformation by Electroporation

After plasmid extraction form recombinant *E. coli*, the recombinant vector was added into *A. tumefaciens* strain GV3101 competent cell and then transferred into electroporation cuvettes incubating at 4°C. After that, the cuvettes were electroporated with appropriated condition by using Gene Pulser Electroporator. Then, the transformed bacteria were grown in 0.5 mL of LB media (without antibiotic) at 200 rpm 28°C 2 hours. After incubation, the transformed competent cell was grown on LB agar plate containing 50 µg/ml each of Kanamycin sulfate, Rifampicin and Gentamicin incubating at 28°C for 48 hours. The bacterial colonies on LB agar plate containing antibiotic were selected and confirmed by PCR with primer that specific to the gene. The PCR products were analyzed using 1% agarose gel electrophoresis to compare the DNA band with the positive control.

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2.2.2 Agrobacterium-mediated transient transformation

Plant materials preparation

Nicotiana benthamiana wild-type seeds will be germinated in soil for two weeks and then the seedlings will be transferred into 10 cm pots. The tobacco plants will be grown at 28°C under a cycle of sixteen hours light and eight hours dark for further experiments.

Agroinfiltration

A single colony of *A. tumefaciens* strain GV3101 harboring pBYK-2e- anti-RANKL HC and LC was inoculated into LB media containing 50 µg/ml each of kanamycin sulfate, rifampicin and gentamicin incubated at 200 rpm 28°C for overnight. The bacterial suspension was centrifuged at 6,000 rpm for 10 min at 4°C for collecting bacterial cell. Then, the cell pellet will be resuspended with infiltration buffer (10 mM MES pH 5.5 and 10 mM $MgSO_4$) and delivered into *N. benthamiana* wild-type leaves using 1 mL syringe without needle to optimize the appropriated condition. The infiltrated plants were incubated under controlled environment at 28°C under a cycle of sixteen hours light and eight hours dark. After optimization, the large-scale production was performed by using vacuum infiltration. Intact plants were submerged into the chamber that contain the *A. tumefaciens* suspension. A vacuum pump drawn air out of the plant leaves via the stomata. After releasing the vacuum, *A. tumefaciens* suspension was replaced into the leaves due to the difference pressure. The infiltrated plants were also incubated at 28°C under a cycle of sixteen hours light and eight hours dark.

Verification of anti-RANKL heavy-chain (HC) and light-chain (LC) expression in N. benthamiana

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A. tumefaciens strain GV3101 harboring pBYK-2e- anti-RANKL HC and LC were cultured and resuspended with infiltration buffer (OD_{600} at 0.4). Then, the suspension was infiltrated into tobacco leaves. To verify the expression, the infiltrated leaves were harvested and extracted with 1x PBS (137 mM NaCl, 2.68 mM KCl, 10.1 mM Na2HPO4 and 1.76 mM KH2PO4, pH 7.4). Crude extract was centrifuged at 13,000 rpm for 45 min to remove cell debris. The supernatant was measured total soluble protein (TSP) by Bradford assay and adjusted the equal amount of protein in supernatant. The supernatant was determined by SDS-PAGE and western blot analysis to investigate the expression of HC and LC of anti-RANKL mAb.

Co-expression of anti-RANKL heavy-chain (HC) and light-chain (LC) in N. benthamiana

To produce anti-RANKL mAb, *A. tumefaciens* strain GV3101 harboring the expression construct for encoding HC and LC were co-infiltrated into tobacco leaves. *A. tumefaciens* harboring expression construct were cultured and resuspended with infiltration buffer (OD_{600} at 0.4). The ratio of *A. tumefaciens* suspension between HC and LC were compared before infiltrating into leaves. The ratio of HC:LC was 1:1, 1:2, 1:3, 1,4, 2:1, 3:1 and 4:1. The co-infiltrated leaves were harvested and analyzed by western blot analysis to investigate the optimal ratio of *A. tumefaciens* suspension.

Determination of days post infiltration of anti-RANKL mAb expression in N. benthamiana

To investigate the production period, *A. tumefaciens* suspension (OD_{600} at 0.4) harboring the expression construct with the optimal ratio was co-infiltrated into tobacco leaves. Days post infiltration (dpi) at 2, 4, 8, 10 was optimized to determine the highest expression level. The co-infiltrated leaves were harvested on several days and then the extracts were examined using ELISA to investigate the optimal production period that can express anti-RANKL mAb with the highest level.

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2.2.3 Purification of plant-produced anti-RANKL mAb

Large-scale production of anti-RANKL mAb in N. benthamiana

After determining the optimal condition, the large-scale production of antibody was performed. *A. tumefaciens* strain GV3101 harboring pBYK-2e- anti-RANKL HC and LC were cultured and resuspended with infiltration buffer (OD_{600} at 0.4). Intact plants were submerged into vacuum chamber that contain the *A.*

tumefaciens suspension. After vacuum co-infiltration, the infiltrated plants were incubated 28°C under a cycle of sixteen hours light and eight hours dark. The plants were harvested at the optimal dpi and extracted with 1x PBS. Then, crude extract was centrifuged to remove the cell debris at 13,000 rpm for 45 min and clarified by a sterile 0.45 -micron membrane filter.

Protein A affinity column chromatography for antibody purification

Protein A presents the high affinity binding to FC region of IgG type antibodies. Due to its capability, protein A affinity chromatography purification is commonly used in antibody downstream process. To conduct the protein A purification, Amintra® protein A resin was packed into a purification column. Firstly, the packed column was flowed with deionized (DI) water to remove 20% ethanol from the beads. After that, 1x PBS was flowed through the column for 5 column volumes (CV) to equilibrate the beads. The clarified extracts form large-scale production was applied into the column to allow the interaction between plant-produced antibody and protein A beads. Then. 1x PBS was flowed through the column to remove unbound material. In elution step, elution buffer (0.1 M glycine, pH 2.7) was added into the column to elute the bound plant-produced antibody and neutralized with 1.5 M Tris-HCl (pH 8.8). The purified samples were verified by SDS -PAGE and western blot analysis. The concentration of plant-produced antibody was quantified by sandwich enzyme -linked immunosorbent (ELISA) assay. For further study, the purified plant-produced antibody was concentrated with AMICON® ULTRA-15 15ML - 30 KDa cutoff columns and sterile filtered through a 0.2-micron filter membrane.

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2.2.4 Determination and quantification of plant-produced anti-RANKL mAb

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

To determine plant-produced mAb, samples were analyzed under reducing and non-reducing condition. For non-reducing condition, the loading buffer (125 mM Tris-HCl pH 6.8, 12% (w/v) SDS, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue) was added into samples and then denatured at 95°C for 5 min. The denature samples were loaded and separated on 6%-10% of gradient polyacrylamide gels. For reducing condition, the loading buffer containing 22% ß-mercaptoethanol was added into samples. Then, the samples were denatured at 95°C for 5 min and separated on 15% polyacrylamide gels. The protein bands were visualized by stained with Coomassie brilliant blue.

For western blot analysis, the polyacrylamide gels containing the separated protein were electro-transferred into a nitrocellulose membrane at 100 V for 90 min or 30 V for overnight. After that, the membranes were blocked with 5% non-fat skim milk in 1x PBS (pH 7.4) for at least 30 min at room temperature or overnight at 4 °C to block non-specific binding sites on membranes. Then, the membranes were probed with HRP-conjugated anti-human gamma antibody or HRP-conjugated anti-human kappa antibody at 1:5,000 dilution in 3% non-fat skim milk prepared in 1x PBS (pH 7.4) for at least 2 hr at room temperature or overnight at 4°C. After incubation, the membranes were washed three times with 1x PBS (pH 7.4) containing 0.05% tween -20. and then the membranes were developed with Amersham ECL prime western blotting detection reagent to determine the protein bands.

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Antibody quantification by sandwich enzyme-linked immunoassay (ELISA)

To quantify the amount of plant-produced mAb, 96-well ELISA plate was coated with 50 µl anti-human IgG-Fc fragment specific prepared at 1:1,000 dilution in 1x PBS (pH 7.4) and incubated for 4 hr at 37°C or overnight at 4 °C. After that, the coated 96-well ELISA plate was washed three times with 200 µl 1x PBS (pH 7.4) containing 0.05% tween -20 and blocked with 200 µl 5% non-fat skim milk in 1x PBS (pH 7.4) for 2 hr min at 37°C or overnight at 4 °C. after washing three times, the plate was coated with the commercially available denosumab and plant-produced mAb with varying dilutions in 1x PBS (pH 7.4) and incubated at 37 °C for 2 hr. Then, the plate was washed three times and coated with HRP-conjugated anti-human kappa antibody at 1:1,000 dilution in 1x PBS (pH 7.4) and incubated for 2 hr at 37°C. Lastly, the plate was washed three times and developed using SureBlue™ TMB 1-Component Microwell Peroxidase Substrate before stopping by acidification and then the absorbance was measured using microplate reader at the optical density of 450 nm.

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2.2.1 N-Glycan analysis of plant-produced mAb

Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)

To investigate the N-Glycan analysis, the heavy chain of purified plant-produced anti-RANKL mAb and commercially available Denosumab was separated on 15% of polyacrylamide gels (reducing condition) and stained with Coomassie brilliant blue to visualize the protein band. The heavy chain band was excised from the polyacrylamide gel *S*-alkylated and digested with trypsin. Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) was used for analyzing tryptic glycopeptides as described previously (Strasser et al., 2008).

2.2.2 Antibody structure characterizations

Circular dichroism (CD) spectroscopy

To investigate the secondary structure of plant-produced antibody, purified plant-produced anti-RANKL mAb (10 µM) was be dissolved in 1x PBS buffer (pH 7.4). The CD spectra of samples were recorded at room temperature using a quartz cell of 1 mm optical path length on a J-720W CD spectropolarimeter. Triplicate measuring in each sample.

Nuclear Magnetic Resonance (NMR) spectroscopy

To study tertiary structures of plant-produced antibody, NMR spectra of purified plant-produced anti-RANKL mAb (100 µM) was dissolved in 1x PBS buffer (pH 7.4) containing 10% v/v D_2O. NMR spectra were recorded on a Varian Unity INOVA 600 spectrometers. Triplicate measuring in each sample.

2.2.3 Plant-produced mAb to human RANKL protein binding assay

To investigate the binding activity, 96-well ELISA plate will be coated with 3 µg/mL human RANKL protein prepared at 1:1,000 dilution in 1x PBS (pH 7.4) and incubated for 4 hr at 37°C or overnight at 4 °C. After that, the coated 96-well ELISA plate was washed three times with 200 µl 1x PBS (pH 7.4) containing 0.05% tween -20 and blocked with 200 µl 5% non-fat skim milk in 1x PBS (pH 7.4) for 2 hr min at 37°C or overnight at 4 °C. After washing three times, the plate was coated with the serial dilutions of plant-produced antibody and commercially available human IgG1 kappa isotype antibody (negative control) in 1x PBS (pH 7.4) and incubated at 37 °C for 2 hr. Then, the plate was washed three times and coated with HRP-conjugated anti-human gamma antibody at 1:1,000 dilution in 1x PBS (pH 7.4) and incubated for 2 hr at 37°C. Lastly, the plate was washed three times and developed using SureBlue™ TMB 1-Component Microwell Peroxidase Substrate before

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stopping by acidification and then the absorbance was measured using microplate reader at the optical density of 450 nm.

2.2.4 Functional evaluation of plant-produced anti-RANKL mAb

Isolation of CD14+ monocytes

To isolate the human peripheral blood mononuclear cells (PBMCs), buffy coats obtained from The Thai Red Cross Institute were diluted 1:1 of Dulbecco's phosphate-buffered saline (DPBS) containing 2% fetal bovine serum (FBS). The diluted buffy coats were gently overlay onto the Ficoll-Paque Plus and centrifuged at 400 g for 30 min without brake off. After centrifugation, PBMCs were collected from the interface between the plasma and the Ficoll-Paque Plus and then the cells were washed with ice-cold DPBS containing 2%FBS. To isolate the CD14+ monocytes, PBMCs were incubated with MACS CD14+ microbeads for 15 min at 4°C and washed with buffer (DPBS containing 0.5% BSA and 2 mM EDTA) and passed through a MACS cell separator. CD14+ monocytes were collected for further experiments.

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Induction of osteoclast and inhibition

For osteoclast differentiation, CD14+ monocytes were seeded at density1x10^6 cells/well in 24-well plates and cultured in 1 ml of **α**-minimal essential medium (**α**-MEM) containing 50 ng/ml M-CSF and 100 ng/ml human RANKL protein. To investigation the inhibitory efficiency, the plant-produced mAb and commercially available denosumab were added into the culture obtain the final concentration of 500 ng/ml. The anti-SARS-CoV mAb CR3022 (500 ng/ml) (Rattanapisit et al., 2020b) was also used as a negative control. The 50% of the medium containing 50 ng/ml M-CSF and 100 ng/ml human RANKL protein and 500 ng/mL each of plant-produced mAb, denosumab and mAb CR3022 was changed

every 3 days and the cultures were then maintained for 15 days to investigate the inhibitory efficiency.

Tartrate-resistant acid phosphatase (TRAP) staining

To determine the active osteoclast, TRAP staining was performed following followed the instruction of suppliers. Osteoclast cells were identified from TRAP-positive multinucleated cells containing more than 3 nuclei. The cells were counted under a microscope. Four fields were randomly selected, and images were taken for each well.

Statistical analysis.

All data were presented as the mean ± standard error of the mean (SEM). To assess statistical significance of differences, the unpaired two sample t-test was performed with a P value less than 0.0001 (p-values ≤ 0.0001) considered as statistically significant.

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CHAPTER 3

RESULTS AND DISCUSSION

3.1 Construction plant expression vector

To produce anti-RANKL mAb, amino acid sequences of HC and LC of anti-RANKL mAb were derived from drugbank database (DrugBank accession number: DB06643) and reversed into nucleotide sequences. The nucleotide sequences were optimized to the codon-usage of *N. benthamiana* to enhance the recombinant protein expression and fused with 5' signal peptide sequences at N-terminal. The nucleotide sequences of SEKDEL were added in C-terminal of both HC and LC to retain the plant-produced mAb in endoplasmic reticulum (ER). The ER retention has been presented many advantages such as the proper folding and assembly antibody structure witth correct disulfide bond formation and glycosylation (Aebi, 2013; Braakman & Bulleid, 2011; Kleizen & Braakman, 2004). Furthermore, it could improve the antibody accumulation (Petruccelli et al., 2006) because the ER compartment was lack of proteases which can degrade the recombinant protein. The restriction sites of *Xba*I and *Sac*I were also added in the sequences for the plasmid construction. The HC and LC gene were synthesized in to pBHA vector. For plasmid construction, the HC and LC gene and plant expression vector were digested with the restriction enzymes including *Xba*I and *Sac*I. The digested products including HC, LC and plant expression vector were run on 1% agarose gel electrophoresis to visualize the DNA band with approximately size 1500, 750 and 12,400 bp, respectively (Figure 7). The gel with the expected DNA fragments were extracted and purified. The purified products of HC and LC were ligated into plant expression vector. In this study, geminiviral expression vector (pBYR2eK2Md; pBYK-2e) (Chen et al., 2011) was used as an expression vector which was kindly provided by Hugh S. Mason, Arizona State University, United States. Geminiviral expression system was developed based on bean yellow dwarf virus (BeYDV).

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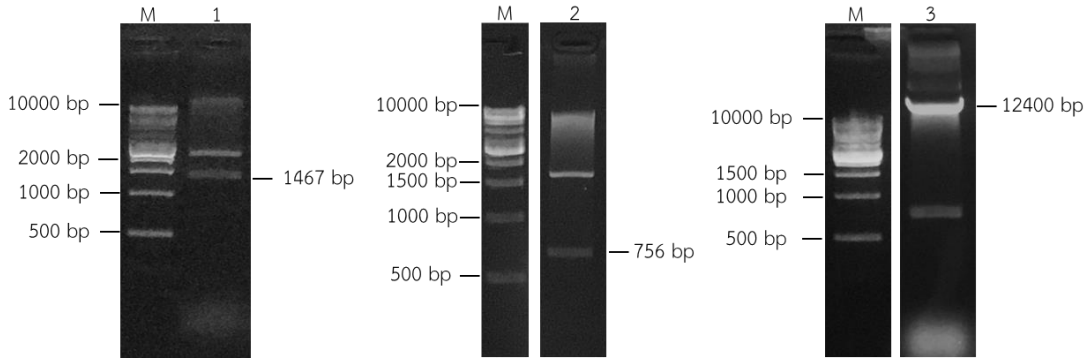

Figure 7 1% Agarose gel electrophoresis of *Xba*I and *Sac*I restriction enzyme digestion

Lane M: VC 1kb DNA Ladder

Lanes 1: pBHA-anti-RANKL-HC vector

Lanes 2: pBHA-Anti-RANKL-LC vector

Lanes 3: pBYK-2e vector

This system represented that there are many recombinant proteins rapidly transient expressed with very high production level (Ahmad et al., 2019; Hefferon & Fan, 2004; Peyret & Lomonossoff, 2013; Phoolcharoen et al., 2011; Rattanapisit et al., 2017; Rattanapisit, Phakham, et al., 2019; Rattanapisit et al., 2020b; Shanmugaraj et al., 2020). Then, the ligated product was transformed into *E. coli* strain DH10B competent cells via Heat shock transformation. The transformed competent cell was grown on selective medium. The bacterial colonies on the LB agar plate were selected and confirmed by PCR. The results showed that the selected bacterial colonies contained the gene of interest when compared with the control as shown in the figure 8. After that, the construct was extracted and confirmed by digested with the restriction enzymes (*XbaI*/*Sac*I). The results showed that the size of HC and LC gene was at approximately size 1500 and 750 bp, respectively (Figure 9). Lastly, the plant expression vector was electro-transformed into *A. tumefaciens* and then the transformed bacterial was confirmed by PCR and compared with the control to obtain the positive clone for anti-RANKL mAb expression (Figure 10).

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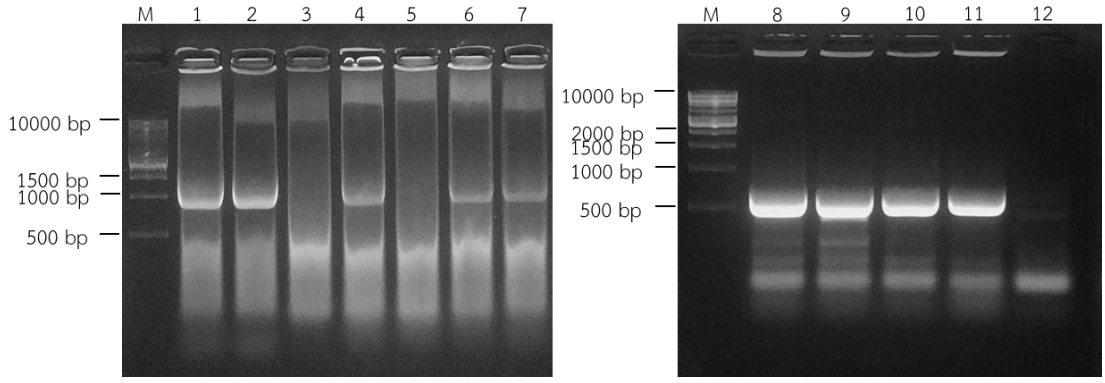

Figure 8 1% Agarose gel electrophoresis of *E. coli* colony PCR

Lane M: VC 1kb DNA Ladder

Lanes 1-2: pBHA-anti-RANKL-HC vector (Positive control)

Lane 3, 12: pBYK-2e vector (Negative control)

Lane 4-7: Transformed Colony (HC)

Lanes 8-10: Transformed Colony (LC)

Lanes 11: pBHA-Anti-RANKL-LC vector (Positive control)

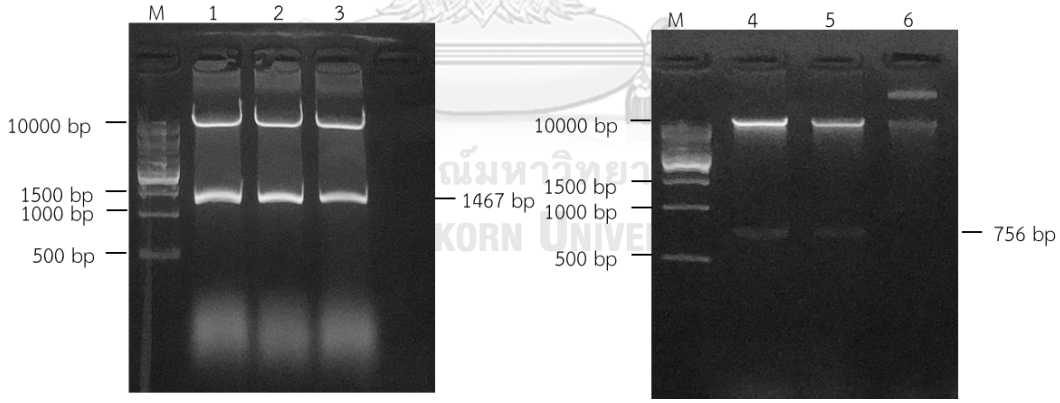

Figure 9 1% Agarose gel electrophoresis of *Xba*I and *Sac*I restriction enzyme digestion

Lane M: VC 1kb DNA Ladder

Lanes 1-3: pBYK-2e -anti-RANKL-HC vector

Lanes 4-6: pBYK-2e -anti-RANKL-LC vector

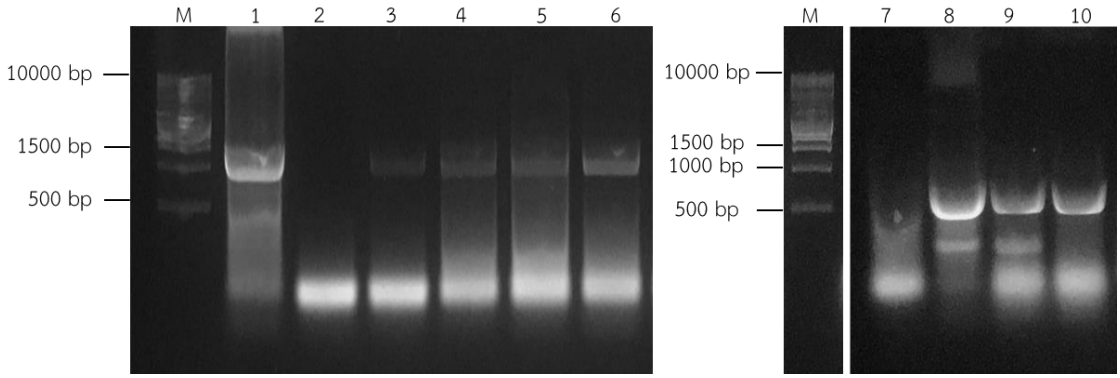

Figure 10 1% Agarose gel electrophoresis of *A. tumefaciens* colony PCR

Lane M: VC 1kb DNA Ladder

Lane 1: pBHA-anti-RANKL-HC vector (Positive control)

Lane 2,7: pBYK-2e vector (Negative control)

Lanes 3-6: Transformed Colony (HC)

Lane 8: pBHA-anti-RANKL-LC vector (Positive control)

Lanes 9-10: Transformed Colony (LC)

3.2 Verification of anti-RANKL heavy-chain (HC) and light-chain (LC) expression in *N. benthamiana*

Agrobacterium-mediated gene transformation of delivery agroinfiltration is the one of widely used methods for transient expression in plant. For small scale production, agrobacterium harboring expression vector was infiltrated into plant leaf to deliver the agrobacterial suspension through plant's stomata by gently pressed a syringe without needle on the leaf (Chen et al., 2013; Leuzinger et al., 2013; Natorajan et al., 2010; Vaghchhipawala et al., 2011).

To express the plant-produced mAb, the *Agrobacterium* harboring the construct of HC and LC were evaluated. The tobacco leaves were agroinfiltrated with each construct, harvested and extracted to determine the expression. The supernatant of crude extracts was measured the amount of TSP and analyzed by SDS -PAGE and western blot analysis. According to the results, the supernatant was separated on 8%

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and 15% polyacrylamide gels under reducing condition. The total protein band was visualized by stained with Coomassie brilliant blue. To verify the HC expression, the separated protein was transferred to nitro cellulose membrane and detected by HRP-conjugated anti-human gamma antibody. The results showed that the protein band of HC was observed at approximately 50 kDa (Figure 11). Moreover, the expression of LC was verified by detected with HRP-conjugated anti-human kappa antibody. The protein band of LC was observed at approximately 25 kDa as shown in the Figure 12.

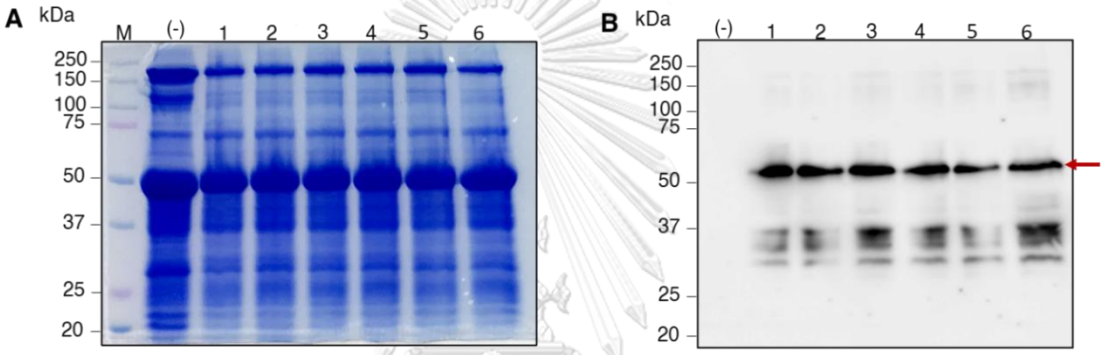

Figure 11 (A) 8% polyacrylamide gels and (B) western blotting of HC expression in *N. benthamianna.* Equal amounts of total soluble protein were loaded in each lane.

Lane M: Precision Plus Protein™ Standards

Lanes (-): Wild type leaves (Negative control)

Lanes 1-2: agroinfiltrated leaves with pBYK-2e -anti-RANKL-HC vector (1)

Lanes 3-4: agroinfiltrated leaves with pBYK-2e -anti-RANKL-HC vector (2)

Lanes 5-6: agroinfiltrated leaves with pBYK-2e -anti-RANKL-HC vector (3)

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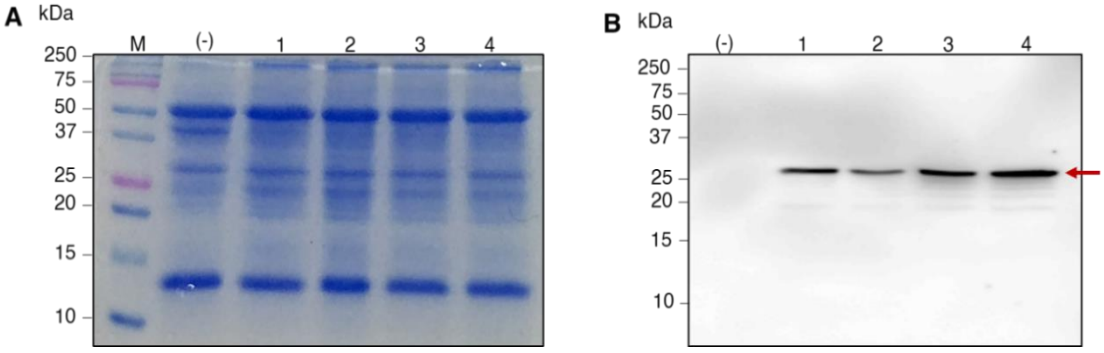

Figure 12 15% polyacrylamide gels and western blotting of LC expression in *N. benthamianna.* Equal amounts of total soluble protein were loaded in each lane.

Lane M: Precision Plus Protein™ Standards

Lanes (-): Wild type leaves (Negative control)

Lanes 1-2: agroinfiltrated leaves with pBYK-2e -anti-RANKL-LC vector (1)

Lanes 3-4: agroinfiltrated leaves with pBYK-2e -anti-RANKL-LC vector (2)

3.3 Co-expression of anti-RANKL heavy-chain (HC) and light-chain (LC) in *N. benthamiana*

A. tumefaciens strain GV3101 harboring the expression construct for encoding HC and LC were co-infiltrated into tobacco leaves to observe the expression of anti-RANKL mAb. The assembly mAb are tetrameric form with the molecular size of approximately 150 kDa. There are four polypeptides including 2 HC and 2 LC (Figure 6B) (Vidarsson et al., 2014). Commonly, the expression of mAb in plant was infiltrated with the equivalent ratio of *Agrobacterium* concentration (OD_{600}). Therefore, the optimal ratio of *Agrobacterium* suspension was determined in this study. The cell suspension harboring the expression construct containing either HC or LC (OD_{600} at 0.4) was mixed in 1:1, 1:2, 1:3, 1,4, 2:1, 3:1 and 4:1 ratio and co-infiltrated into the leaves. The crude from co-infiltrated leaves was harvested and processed for by western blot analysis. According to figure 13, western blotting represented the strongest protein band form co-infiltrated leaves with 1:1 ratio of the cell suspension. This result suggested that a 1:1 ratio could express the higher expression of mAb than others at the same TSP

amount. To our knowledge, this is the first report demonstrated the optimal ratio of *Agrobacterium* suspension to produce anti-RANKL mAb in *N. benthamiana.*

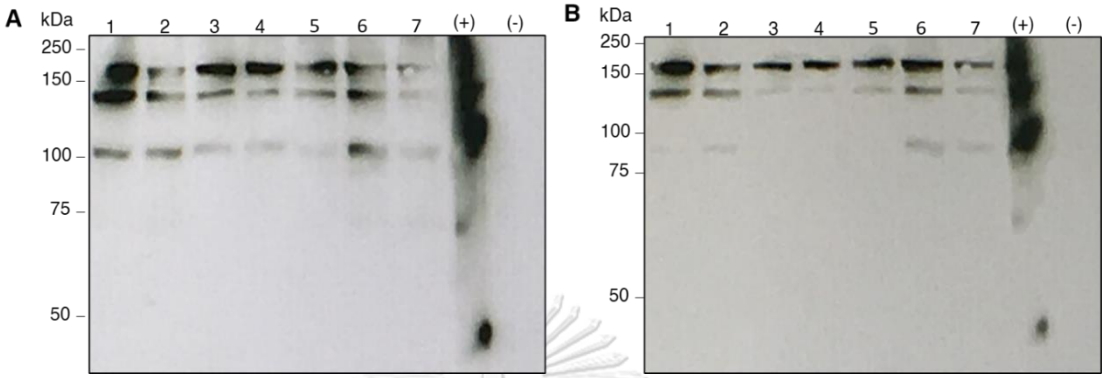

Figure 13 Western blotting of co-expression of anti-RANKL in *N. benthamianna* with various ratio of *Agrobacterium* suspension harboring the expression construct containing either HC or LC (OD_{600} at 0.4) was mixed in 1:1 (1), 1:2 (2), 1:3 (3), 1,4 (4), 2:1 (5), 3:1 (5) and 4:1 (7) ratio. (A) Detected with HRP-conjugated anti-human gamma antibody and (B) HRP-conjugated anti-human kappa antibody. Equal amounts of total soluble protein were loaded in each lane. Lanes (-): Wild type leaves (Negative control); Lanes (+): Commercially available denosumab (Positive control)

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3.4 Optimal days post infiltration (dpi) of anti-RANKL mAb expression in *N. benthamiana*

A time-course experiment was performed in order to determine the optimal expression condition. The tobacco leaves were co-infiltrated with *Agrobacterium* harboring harboring pBYK-2e- anti-RANKL HC + LC (a 1:1 ratio, OD_{600} at 0.4). The infiltrated leaves were harvested on 2, 4, 6,8 and 10 dpi. After agroinfiltration, strong leaf necrosis was observed as shown in the figure 14A. According to (Diamos & Mason, 2019), they generated a geminiviral expression system from BeYDV which could produce the very high-level of recombinant proteins. This system might be activated the hypersensitive response in plant and defense the pathogen infection resulting in cell death. The results showed that the leaf necrosis rapidly increased related to the days post infiltration (dpi). In this study, the optimal dpi was analyzed by sandwich ELISA. Based on the result, the plant-produced mAb was expressed with the optimal production time on 8 dpi with the maximum expression level up to 0.5 mg/g fresh weight (Figure 14B). This result demonstrated that anti-RANKL mAb was rapidly transient expressed in N. *benthamiana* with very high-level production within 8 day after agroinfiltration.

However, many studies have demonstrated that production time of plant-produced recombinant proteins depend on many factors including protein expression system and protein molecules. For instances, Sheludko et al. (2007) indicated that the maximum rGFP expression level in plants obtained from the CaMV 35S promoter system with co-expression of TBSV P19 at 3 dpi and remained constant until 8 dpi. Furthermore, transient expression of recombinant Ebola immune complex in *N. benthamiana* using geminiviral vector was found to attain maximum level at 4 dpi (Phoolcharoen et al., 2011). Rattanapisit, Phakham, et al. (2019) represented that the plant-produced anti-PD1 mAb was transiently expressed with the highest levels on 6 dpi. In addition, SARS-CoV-2 receptor binding domain (RBD) and spike specific mAb

CR3022 were transiently produced with the highest expression level at 3 dpi (Rattanapisit et al., 2020a) and Shanmugaraj et al. (2020) created the assembled plant-produced mAbs B38 and H4 that could neutralized SARS-CoV-2 *in vitro* within 4 dpi.

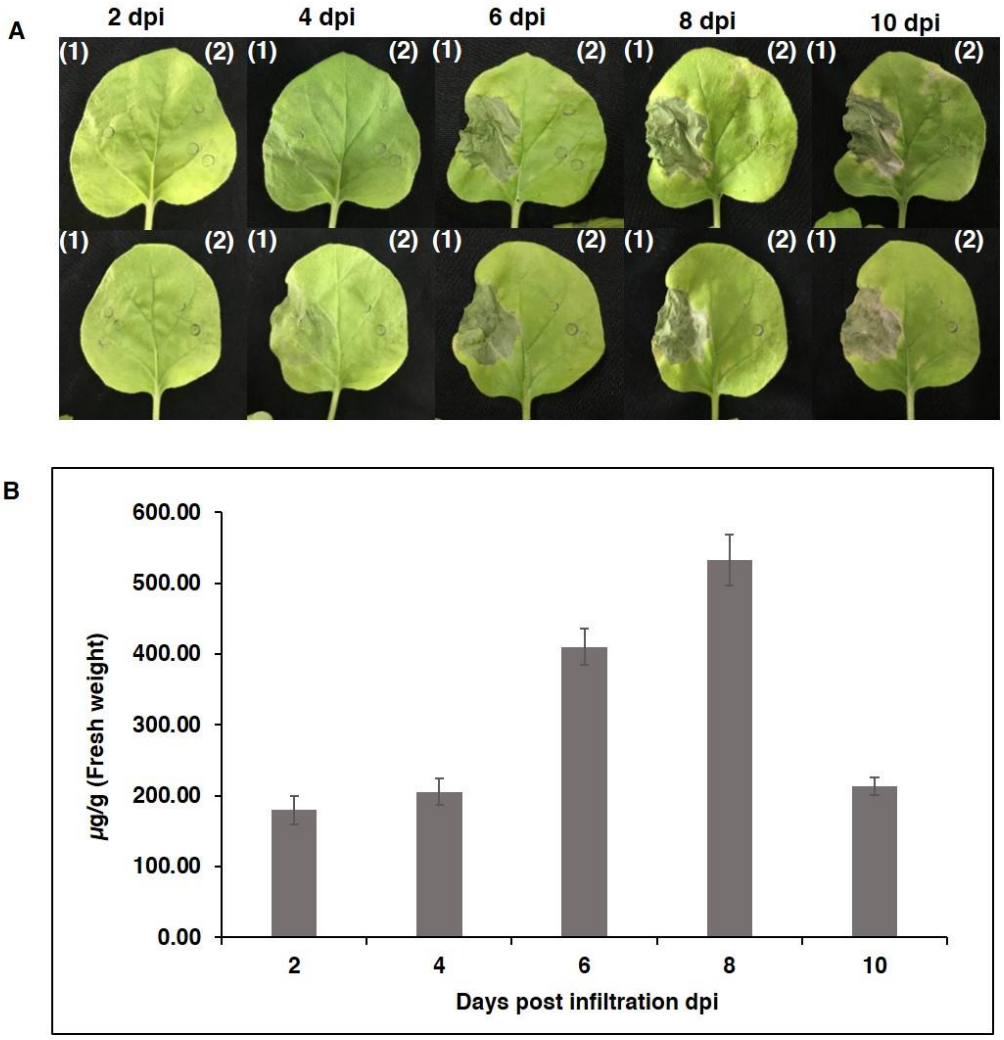

Figure 14 Transient expression of anti-RANKL mAb in N. benthamiana leaves. (A) Phenotype of leaves on days 2, 4, 6, 8 and 10 after agroinfiltration. (1) *N. benthamiana* leaves were co-infiltrated with A. tumefaciens harboring pBYK-2e- anti-RANKL HC + LC and (2) *A. tumefaciens* without expression vector. (B) Infiltrated leaves were harvested on days 2, 4, 6, 8 and 10 and quantified by sandwich ELISA resulting in micrograms of anti-RANKL mAb per gram fresh weight of leaves.

3.5 Purification of plant-produced anti-RANKL mAb

Based on our results, plant-produced mAb represented the maximum expression level at approximately 0.5 mg/g fresh weight on 8 dpi when infiltrated with *Agrobacterium* concentration of 0.4 (OD_{600}) in a 1:1 ratio of HC and LC. In this study, the vacuum infiltration was conducted with the previous optimal condition for plant-produced mAb large-scale production. The whole plants were immersed into *Agrobacterium* suspension and then the vacuum pump was generated a negative atmospheric pressure to draw the air out from the plant leaves. The *Agrobacterium* suspension could replace into the plant tissue when released vacuum condition (Chen et al., 2013; Diego-Martin et al., 2020; Loh & Wayah, 2014). This high throughput method represents the rapidly scalable protein production platform, economical and safe industrial manufacturing. Previous study showed that there are many recombinant protein producing in plants via vacuum agroinfiltration method (Ahmad et al., 2019; Laughlin et al., 2019; Phoolcharoen et al., 2011; Ramirez-Alanis et al., 2018; Rattanapisit, Phakham, et al., 2019; Shanmugaraj et al., 2020; Tottey et al., 2018; Tuse et al., 2015; Vanmarsenille et al., 2018; Zhumabek et al., 2018)

The vacuum agroinfiltrated plants were harvested at 8 dpi and then processed for the purification step. The infiltrated leaves were extracted with 1x PBS (pH 7.4). Then, the crude extracts were purified through single-step protein A affinity column chromatography. For the downstream processing of mAb, protein A was widely applied for the affinity purification system. The protein can provided the high affinity interaction with Fc-part of IgG (Hober et al., 2007). Previous studies indicated that plant-produced mAb successfully purified using single-step protein A purification. For instances, plant-produced anti-SARS-CoV-2 mAbs were purified by protein A (Rattanapisit et al., 2020b; Shanmugaraj et al., 2020). This method also used for anti-EV71 mAb and anti-PD1 mAb purification (Rattanapisit, Chao, et al., 2019; Rattanapisit, Phakham, et al., 2019). Moreover, recombinant protein fusing with Fc region of human immunoglobulin IgG1

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were purified using protein A (Rattanapisit, Srifa, et al., 2019; Siriwattananon et al., 2020). After purification, plant-produced mAb was confirmed by SDS-PAGE and western blotting. The SDS-PAGE gel was stained with Coomassie brilliant blue stain to visualize the separated protein band (Figure 15-16 A and D). For non-reducing condition, Coomassie Blue-stained gels showed that the purified plant-produced mAb represented tetrameric form of the assembled antibody with the molecular size of approximately 150 kDa. For reducing condition, the protein bands were observed at molecular size of approximately 50 and 25 kDa which corresponds to HC and LC of antibody. Western Blot was performed by detected with anti-human gamma-HRP and anti-human kappa-HRP. For non-reducing condition, the assembled antibody was confirmed in tetrameric form with the molecular size of approximately 150 kDa when compared to the commercially available denosumab (Figure 15, Lanes 5-6 and (+)) and no protein band was observed in the wildtype control leaf extract (Figure 15, Lane (-)). For reducing condition, the protein bands were detected at molecular size of approximately 50 and 25 kDa which corresponds to HC and LC of antibody compared to commercially available denosumab (Figure 16 E-F, Lanes 5-6 and (+)) and no protein band was observed in the wildtype control leaf extract (Figure 16, Lane (-)). According to the blotting results, crude extracts showed unwanted native protein impurities that could be eliminated after purification.

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These results indicated that the co-infiltration of HC and LC produced the assembled anti-RANKL mAb in *N. benthamiana* leaves and the single-step protein A purification could purify the plant-produced mAb for or further analysis.

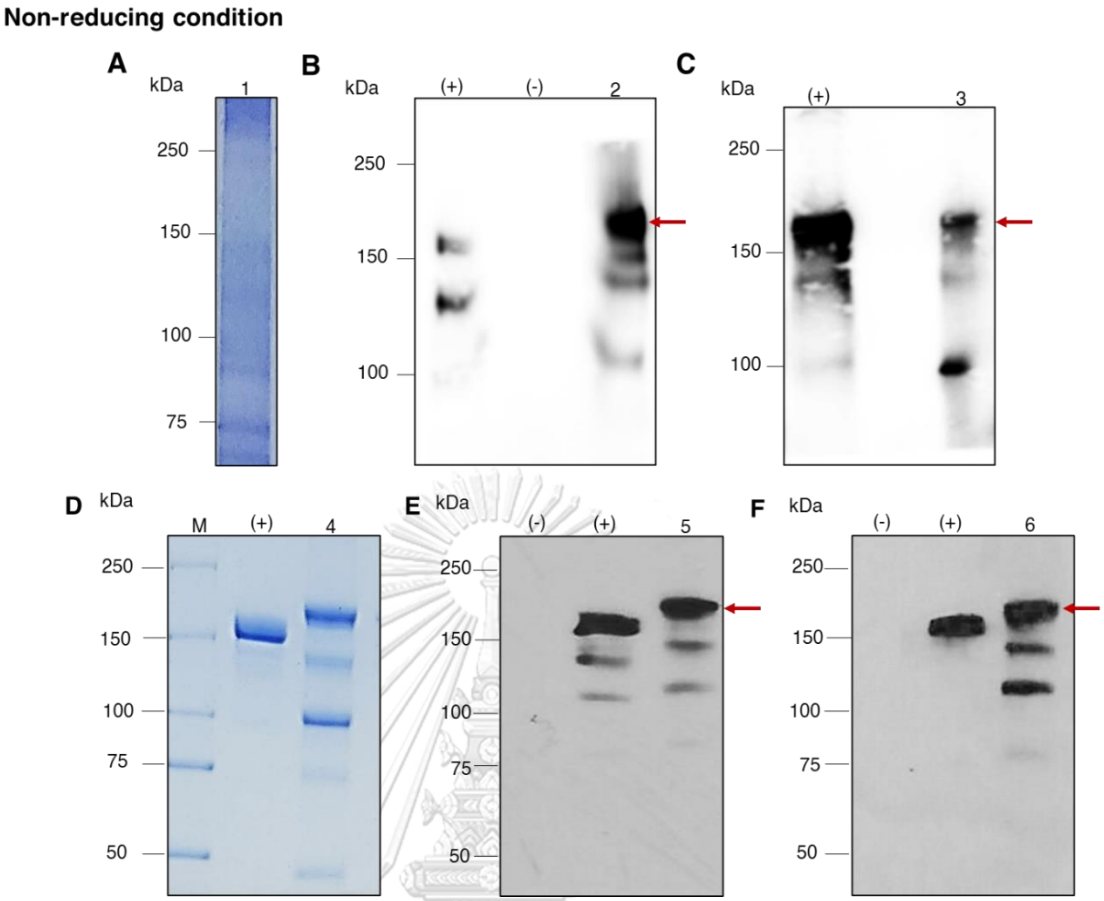

Figure 15 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and of purified plant-produced anti-RANKL mAb under non-reducing condition. The crude extracts and purified product were analyzed by SDS-PAGE staining with Coomassie blue (A, D). For western blot analysis, the membrane was detected with either HRP-conjugated anti-human gamma chain antibody (B, E) or anti-human kappa chain antibody (C, F). Lane M: Protein ladder; Lane (-): Wildtype crude extracts; Lane (+): Commercially available denosumab; Lane 1-3: Crude extracts from infiltrated leaves; Lane 4-6: Purified plant-produced anti-RANKL mAb. Equal amounts of total soluble protein were loaded in each lane.

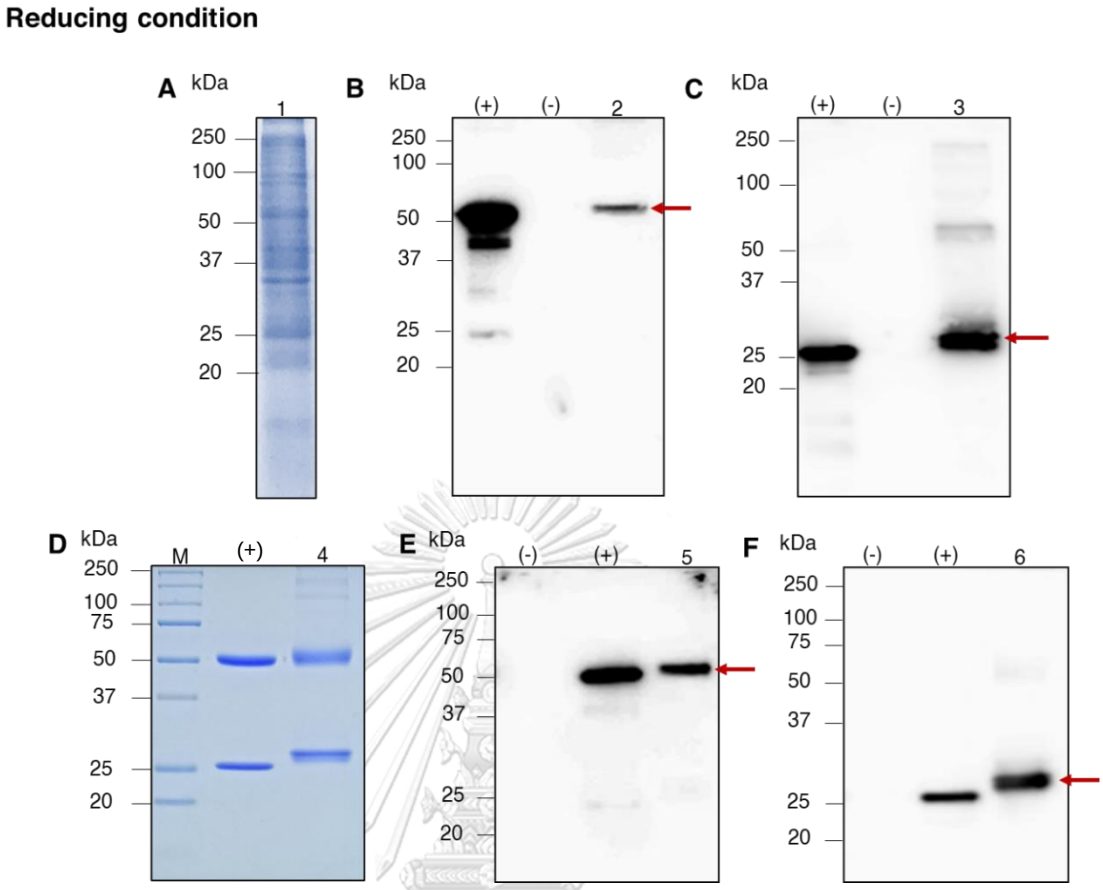

Figure 16 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and of purified plant-produced anti-RANKL mAb under reducing condition. The crude extracts and purified product were analyzed by SDS-PAGE staining with Coomassie blue (A, D). For western blot analysis, the membrane was detected with either HRP-conjugated anti-human gamma chain antibody (B, E) or anti-human kappa chain antibody (C, F). Lane M: Protein ladder; Lane (-): Wildtype crude extracts; Lane (+): Commercially available denosumab; Lane 1-3: Crude extracts from infiltrated leaves; Lane 4-6: Purified plant-produced anti-RANKL mAb. Equal amounts of total soluble protein were loaded in each lane.

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3.6 N-Glycan analysis of plant-produced mAb

Liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) was performed for the analysis of glycopeptides from plant-produced mAb. The result indicated that the mAb displays mainly oligomannosidic N-glycans (Man5-Man9) and small amounts of complex-type and truncated N-glycans (e.g., GnGnXF) (Figure 17). Based on the result, plant-produced mAb provided the different glycosylation patterns from commercially available denosumab that produced from CHO cell. Although plant and mammalian glycoproteins presented the same pattern of a core complex *N* -glycan structure, the terminal residues of complex-type N -glycans in plant and mammals are difference. Based on plant- N -glycans, β 1,2-xylose substituted the β -mannose that is not presented in mammalian N -glycans. In addition, plant N- -glycans also attached plant-specific α 1,3-fucose residues, instead of an a1,6 fucose in mammals (Gomord et al., 2004; Montero-Morales & Steinkellner, 2018; Saint-Jore-Dupas et al., 2007; Schähs et al., 2007; Schoberer & Strasser, 2018; Strasser, 2016). According to Stelter et al. (2020), plant glycosylation presented the limitation of binding capacity to FcγR interactions and it may effect on biological function of plant-produced mAbs (Gomord et al., 2010; Ko et al., 2003). According to IgG rapid clearance from the circulation, the interaction with neonatal Fc receptor (FcRn) which prevent the antibody form endosomal degradation are influenced (Junghans & Anderson, 1996). However, Stelter et al. (2020) reported that the binding capacity to the neonatal FC receptor (FcRn) between plant-made glycovariants and HEK cell-derived antibody have a similar affinity. These results demonstrated that the plant-produced mAb might have the low binding capacity to Fc receptors but the half-life of the plant-produced mAb is comparable to mammalian cell-derived antibody.

However, the bioactivity of both the plant-produced anti-RANKL mAb and commercially available denosumab should be investigated in the further study.

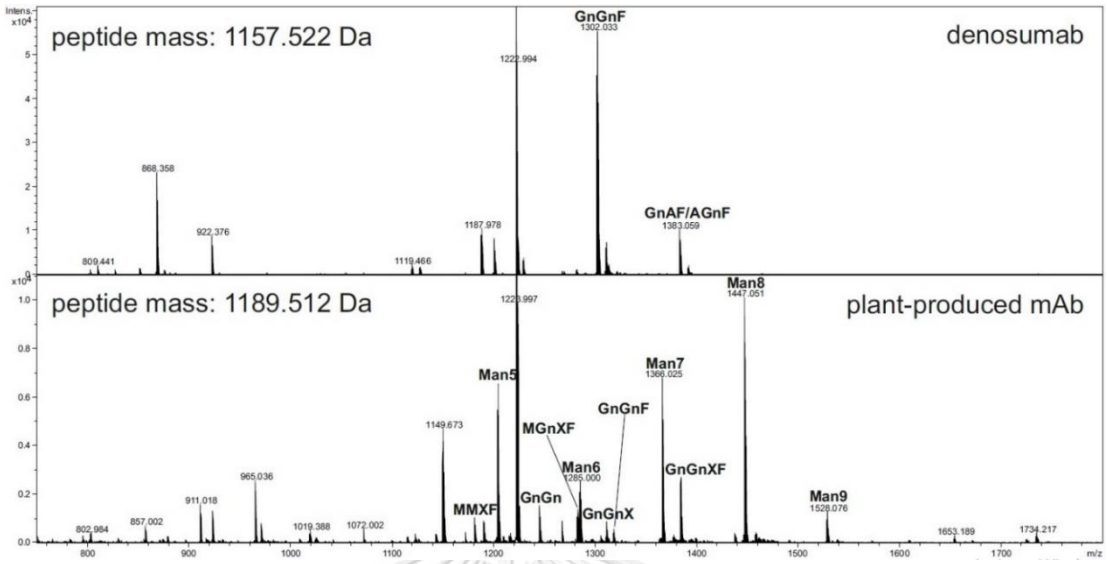

Figure 17 Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) of tryptic glycopeptides from anti-RANKL mAb produced in wild-type *N. benthamiana* plants. (See www.proglycan.com for a detailed explanation of glycan structure abbreviations.)

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3.7 Antibody higher-order structure characterizations

Circular dichroism (CD) is a common technique to evaluate the secondary structure of antibody. These methods can be used to determine the difference between left and right circularly polarized light. In this study, the far-UV CD spectra was used to estimate the plant-produced mAb secondary structure. The result indicated that CD spectrum analysis represented a negative absorbance at 218 nm (Figure 18A) which correlated with the previous study of plant-produced mAb. According to Rattanapisit, Phakham, et al. (2019), the secondary structure of plant-produced anti-PD1 and commercial mammalian cell-produced antibodies was analysed by CD technique. Both spectra presented a negative absorbance at 218 nm (Figure 19A). Moreover, the secondary structure of rituximab (Rituxan®) or infliximab (Remicade®) demonstrated the strong negative absorbance around 217 nm which corresponded with the dominant β -sheet structure in both mAbs (Figure 20A) (Chen et al., 2016). These results demonstrated that the plant-produced anti-RANKL mAb has mainly β -sheet structure that is typically found in IgG antibodies structure (Bakshi et al., 2014; Chelius et al., 2010; Greenfield, 2006; Joshi et al., 2014; Li et al., 2011).

For the tertiary structure of plant-produced mAb, nuclear magnetic resonance (NMR) spectroscopy was used to examine the tertiary structures. The result indicated the signals of up-field methyl and dispersed aromatic protons in ^{1}H-NMR spectroscopy (Figure 18B). Moreover, the tertiary structure of plant-produced anti-PD1 and commercial nivolumab was also analysed by NMR spectroscopy. The results showed the dispersed aromatic protons (Figure. 19B) and up-fielded methyl protons (Figure 19C) which indicated the retention of the tertiary structures. Both NMR spectra were confirmed the similar profiles of plant-produced anti-PD1 and commercial nivolumab. Therefore, the NMR spectra of plant-produced anti-RNKL mAb showed the correlation with the plant-produced anti-PD1 and commercial nivolumab in presiovus study and

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it can be concluded that the tertiary structure of plant-produced anti-RANKL mAb was retained.

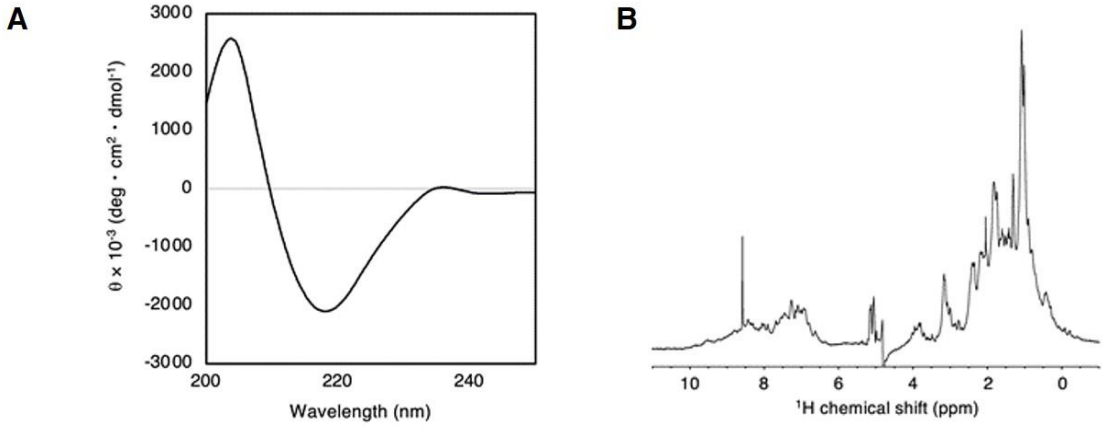

Figure 18 The characterization of plant-produced mAb structure (A) Far-UV CD spectrum of plant-produced anti-RANKL mAb was recorded on a J-720W CD spectropolarimeter using a quartz cell of 1 mm optical path length at 25°C. (B) ^{1}H NMR spectrum of plant-produced anti-RANKL mAb at pH 7.5 and 25°C.

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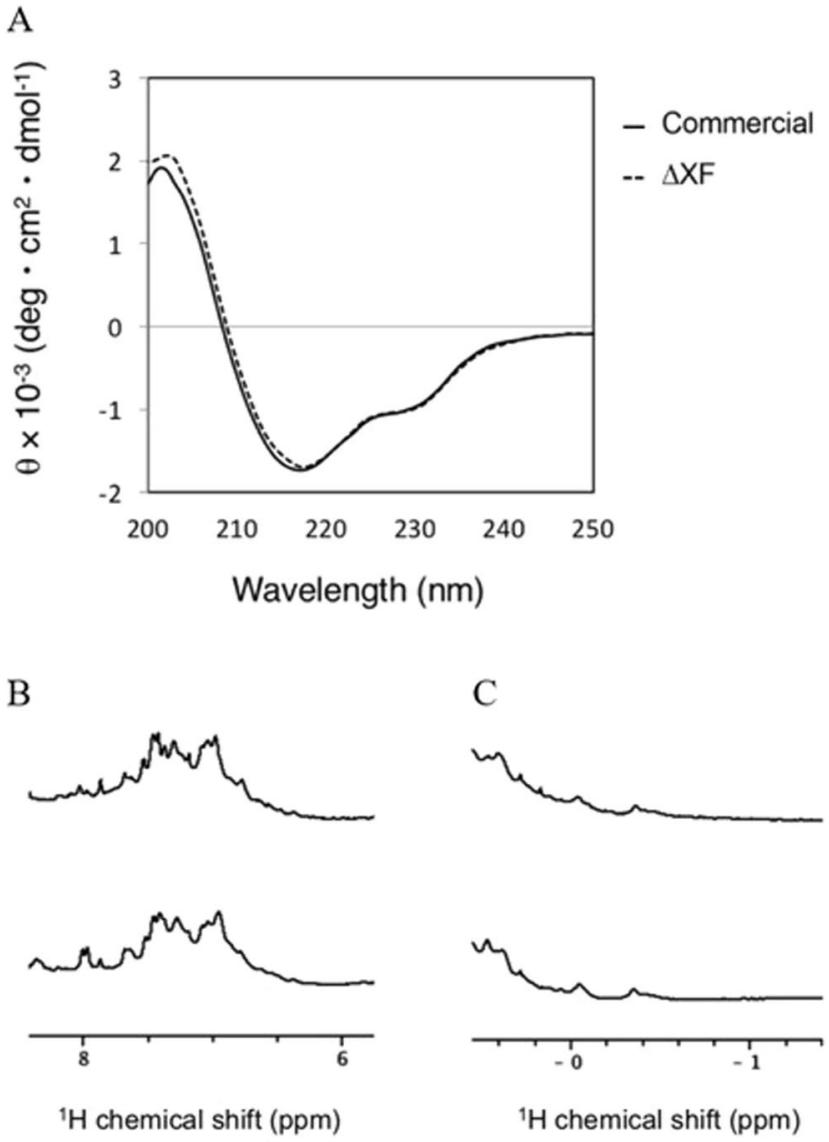

Figure 19 Structural analysis of plant-produced anti-PD1 and commercial mammalian cell-produced antibodies. (A) Far-UV CD spectrum was recorded on a J-720W CD spectropolarimeter using a quartz cell of 1 mm optical path length at 25°C. (B,C) NMR spectra in (B) aromatic region and (C) methyl region of commercial mammalian cell produced antibody (upper) and plant-produced anti-PD1 (lower).

Source: Rattanapisit, Phakham, et al. (2019)

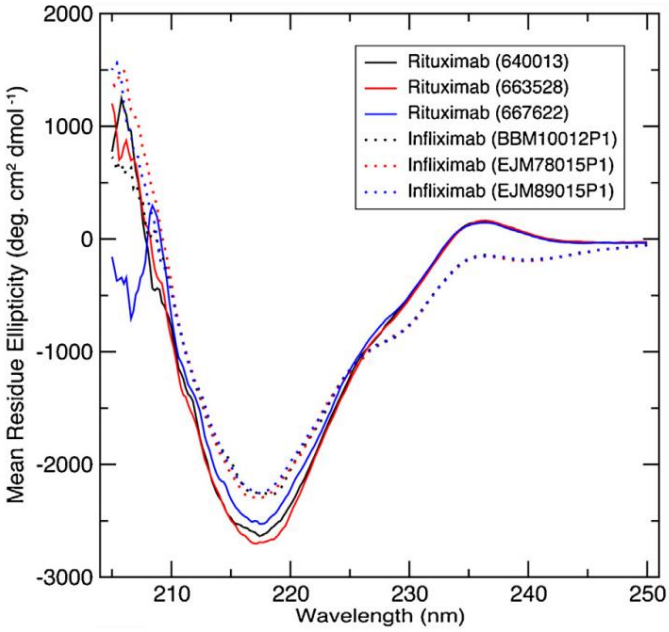

Figure 20 Far-UVCD spectral region from 205 to 250 nm of 3 lots each of rituximab (Rituxan®) or infliximab (Remicade®).

Source: Chen et al. (2016)

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3.8 Plant-produced mAb to human RANKL protein binding assay

To investigate the ability of plant- produced mAb, RANKL-ELISA binding analysis was performed to examine the specific binding between the plant-produced mAb and human RANKL. The serial dilutions of purified plant- produced mAb, standard human IgG1 (as a negative control) and commercially available denosumab (as a positive control) were incubated on the captured human RANKL on 96- well plate. The commercially available denosumab and plant- produced mAb presented signal when detected with anti-human gamma- HRP. Our results showed that plant-produced anti-RANKL mAb provided the binding potential to human RANKL while the commercial antibody presented the significant higher binding signal. However, the standard human IgG1 (as s negative control) did not show the binding signal (Figure 20). The statistical significance of difference between an experimental group and the control were evaluated with Two-way ANOVA (GraphPad Prism 9.1.2) (APPENDIX D). As shown in the figure 19, the anti-RANKL mAb amino acid sequences that obtained from DrugBank (accession number: DB06643) were different form the commercialized antibody in patent (Robblee et al., 2017). Therefore, the difference of amino acid sequences might have an impact on binding capacity. However, the bioactivity on osteoclast inhibition of plant-produced mAb compared commercially available denosumab should be performed to investigate the potential of plant-produced mAb in further study.

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HC:

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Drugbank-HC     -------------------------EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMS
Patent-HC       -------------------------EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMS

Drugbank-HC     WVRQAPGKGLEWVSGITGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYY
Patent-HC       WVRQAPGKGLEWVSGITGSGGSTYYADSVKGRFIISRDNSKNTLYLQMNSLRAEDTAVYY

Drugbank-HC     CAKDPGTTVIMSWFDPWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
Patent-HC       CAKDPSATVLMSWFDPWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYF

Drugbank-HC     PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK
Patent-HC       PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTK

Drugbank-HC     VD-KKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
Patent-HC       DVDKTVERKCC---VECPPCPAPPV-AGPSVFLFPPKPKDTLMISRTPEVICVVVDVSHE

Drugbank-HC     DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
Patent-HC       DPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLP

Drugbank-HC     APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN
Patent-HC       APIEKTISKTKGQPREPQV-TLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN

Drugbank-HC     NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK--
Patent-HC       NYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK--
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LC:

```
Drugbank-LC     -------------------------EIVLTQSPGTLSLSPGERATLSCRASQSVRGRYLA
Patent-LC       -------------------------EIVITQSPGTLSLSPGERAILSCRASQSVRGRYLA

Drugbank-LC     WYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVFYCQQYGSS
Patent-LC       WYQQKPGQAPRILLYGASSRATGLPDRFSGSGSGTDFTLTISRNKPEDFAVFYCQQYGSS

Drugbank-LC     PRTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNAL
Patent-LC       PRTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNAL

Drugbank-LC     QSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
Patent-LC       QSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
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Figure 21 The comparison of amino acid sequences between anti-RANKL mAb from DrugBank (accession number: DB06643) and the commercially available denosumab (Robblee et al., 2017)

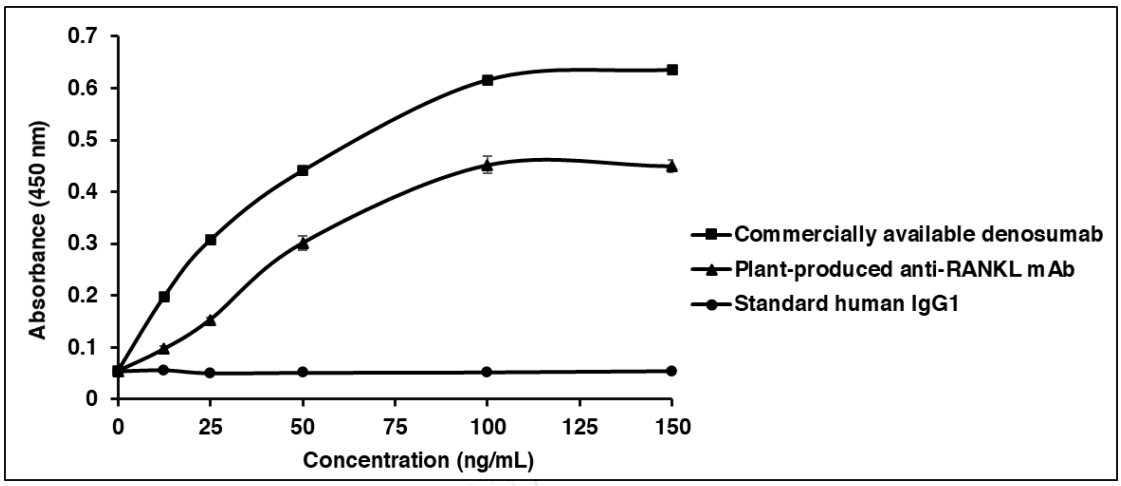

Figure 22 Binding properties of both plant-produced anti-RANKL mAb and commercially available denosumab (AMGEN, United States) to human RANKL. Indirect ELISA was used for investigated the specific binding. The purified plant-produced mAb, standard human immunoglobulin G (IgG)1 (as negative control) and commercially available denosumab (as positive control) were added into the plates coated with commercial human RANKL. The bound antibody was detected with HRP-conjugated anti-human gamma chain antibody. The data were showed as mean ± standard deviation (SD) of triplicates.

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3.9 Functional of plant-produced anti-RANKL mAb on osteoclast inhibition

According to the purpose of this study, the osteoclast inhibition was performed to determine the functional activity of plant-produced mAb. Human peripheral blood mononuclear cells (PBMCs) were isolated and induced the differentiation of osteoclast. To investigate the effect of plant-produced mAb on osteoclastogenesis, CD14 + monocytes derived from PBMCs were cultured with the essential factor including M-CSF and human RANKL protein. As shown in figure 21A, CD14 + cells were differentiated into osteoclast as judged by the multinucleated appearance and the positive staining of tartrated resistant acid phosphatase (TRAP). Our preliminary study confirmed that at the various concentration of the antibodies, the plants-produced mAb are able to reduce the osteoclast differentiation at the same dose with the commercial antibody. The result was provided in APPENDIX E. These results demonstrated that addition of both plant-produced mAb and commercially available denosumab (AMGEN, United States) could significantly reduce the osteoclast differentiation with dose 500 ng/ml ($p \leq 0.0001$), when compared with the control condition of adding M-CSF and RANKL. Comparable effect of both antibodies was found. On the contrary, addition of plant-produced mAb CR3022 had no effect on osteoclast formation (Figure 21B). This result was correlated with the earlier study which demonstrated that the commercially available denosumab can inhibit the osteoclast differentiation *in vitro*, with the concentration up to 3 nM (approximately 500 ng/mL) (Kostenuik et al. (2009).

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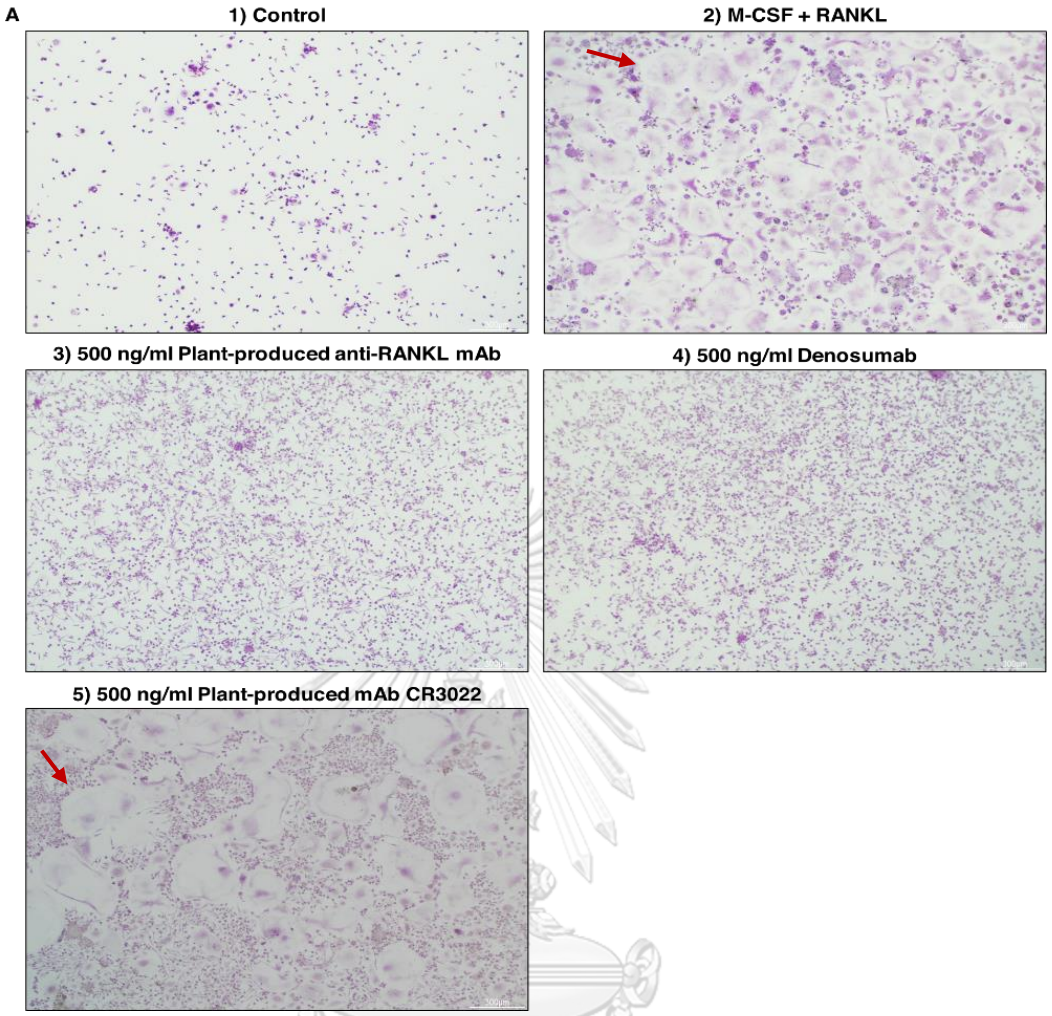

Figure 23 (A) Plant-produced anti-RANKL mAb suppresses osteoclast differentiation. $CD14^{+}$ monocytes were cultured in different condition for 15 days following 1) control, 2) M-CSF and RANKL, 3) 500 ng/ml plant-produced anti-RANKL mAb, 4) 500 ng/ml commercially available denosumab, 5) 500 ng/ml Plant-produced mAb CR3022. Representative images of TRAP staining. The red arrow indicates the multinucleated cells. The multinucleated cells were counted under a microscope. Four fields were randomly selected, and pictures were taken by using Axio Observer Z1 and ZEN pro (ZEISS International, Oberkochen, Germany).

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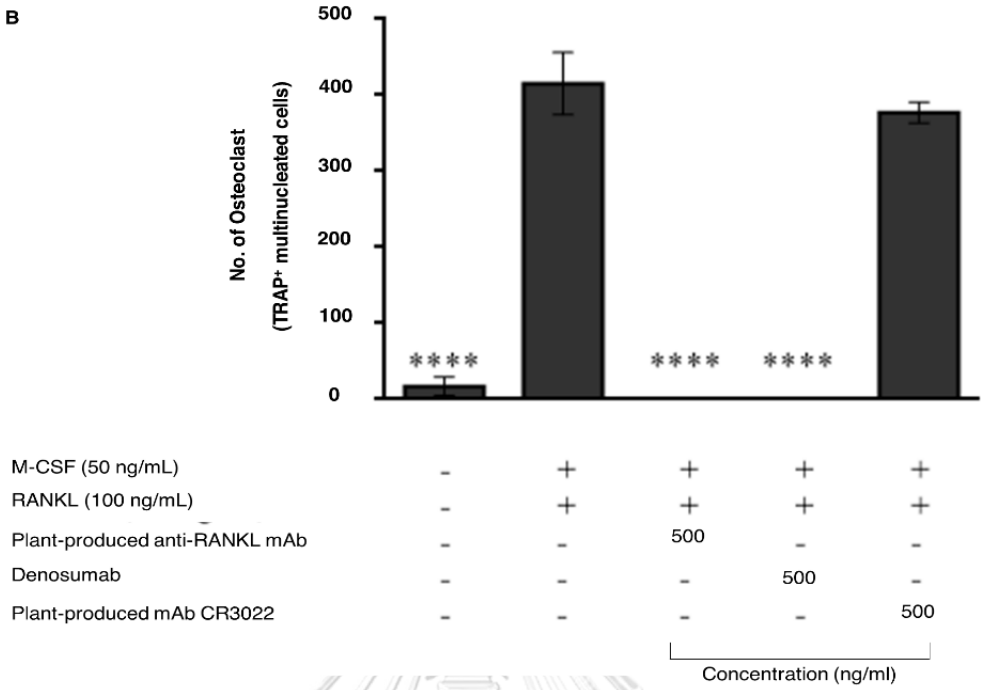

Figure 24 (B) Quantification of TRAP$^+$ osteoclasts were showed as mean and SEM of triplicates. ****$p \leq 0.0001$

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CHAPTER 4

CONCLUSION

Currently, majority of therapeutic protein including vaccine, antibody and biologics that used for the therapeutic purposes are commonly produced from mammalian and microbial platforms. However, there are some drawbacks presented in the production platforms such as lack of post-translational modifications (PTMs), high production cost and scalability limitation. Recently, plants are considered as a bioreactors which can overcome the limitation because it can produce large volumes of recombinant proteins efficiently, be able to perform post-translational modifications and production cost effectiveness (Chen, 2018; Chen & Davis, 2016; Shanmugaraj & Phoolcharoen, 2021; Tusé et al., 2014; Uversky, 2013). There are many plant-produced-proteins which are at the different stages of clinical trial and few are approved by FDA. For instances, alpha-galactosidase-A (moss-aGal) for therapy of Fabry disease (phase 1) (Veen et al., 2020), HIV-neutralizing human mAb 2G12 (phase 1) (Tremouillaux-Guiller et al., 2020), HA VLP influenza vaccine (phase 2) (Pillet et al., 2019), anti-Ebola IgG cocktail (ZMApp) for treatment of Ebola infection (phase 2/3) (Davey et al., 2016) and glucocerebrosidase enzyme (ELELYSO) for therapy of Gaucher's disease (Fox, 2012). *N. benthamiana* is the preferable system for recombinant protein expression. This plant is genetically well known and easily manipulated, non-food/non-feed crop and also produces high biomass (Xu et al., 2012). In this study, anti-RANKL mAb was produced in *N. benthamiana* and its structure biological activity was investigated.

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Based on the results, anti-RANKL mAb was successfully produced in *N. benthamiana* using agroinfiltration method delivered the geminiviral expression

system. The optimal production condition represented the maximum yield of the plant-produced mAb at approximately 0.5 mg/ g fresh weight on 8 dpi. Moreover, the vacuum infiltration was preformed to scale up the production of plant-produced mAb. This method represents the rapidly scalable protein production platform, economical and safe industrial manufacturing. The plant-produced mAb could be successfully purified by single-step protein A affinity chromatography. According to many studies, this method was widely used to purify many plant-produced mAbs (Phoolcharoen et al., 2011; Rattanapisit, Chao, et al., 2019; Rattanapisit et al., 2020b; Shanmugaraj et al., 2020) and also recombinant protein fusing with Fc region of human immunoglobulin IgG1 (Rattanapisit, Srifa, et al., 2019; Siriwattananon et al., 2020). However, the product from single-step protein A purification showed some protein impurities. So, purification optimization needs to be evaluated to eliminate the impurities prior to for industrial scale manufacturing. Subsequently, the purified plant-produced mAb was investigated the bioactivity. Our study demonstrated that the plant-produced mAb presented the ability in affinity binding and the efficacy of both plant-produced mAb and the commercial antibody on the osteoclastogenesis inhibition is comparable. Our proof-of-concept study demonstrated that *N. benthamiana* could be the cost-effective and rapidly production platform for anti-RANKL mAb and other biopharmaceutical products. However, *in vivo* study should be performed further to confirm the efficacy and safety of the plant-produced mAb.

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APPENDICES

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APPENDIX A

The nucleotide sequences of anti-RANKL mAb

The amino acid sequences were changed into nucleotide sequences and optimized to the codon-usage of *N. benthamiana.* The optimized nucleotide sequences were added with signal peptide (MGWSCIILFLVATATGVHS) in the amino-terminal (N-terminal) and SEKDEL were added in the carboxyl-terminal (C-terminal) of both HC and LC. The restriction enzyme sites including *Xba*I and *Sac*I were also added into the optimized sequences.

Anti-RANKL mAb heavy chain nucleotide sequences

TCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACTCTGAT
GTTCAACTTCTCGAGGAAGTGCAGCTGCTTGAATCTGGTGGTGGTCTTGTTCAACCTGGTGGTTCTCTT
AGGCTTTCTTGTGCTGCGTCTGGCTTCACCTTCAGCTCTTATGCTATGAGCTGGGTGAGACAGGCTCCT
GGTAAGGGTCTTGAATGGGTGTCAGGTATTACCGGTTCTGGTGGCTCTACCTACTACGCTGATTCTGTG
AAGGGCCGTTTCACCATCAGCAGGGACAATTCTAAGAACACCCTGTACCTGCAGATGAACAGCCTGAGA
GCTGAGGATACTGCTGTGTACTACTGCGCTAAGGATCCTGGAACCACCGTGATCATGTCATGGTTTGAT
CCTTGGGGTCAGGGAACCCTTGTGACCGTTTCTTCTGCTAGCACTAAGGGCCCATCTGTGTTTCCACTT
GCTCCGTCATCTAAGAGCACCTCTGGTGGTACTGCTGCTCTTGGTTGCCTTGTGAAGGATTACTTCCCT
GAGCCTGTGACCGTGTCATGGAATTCTGGTGCTTTGACTAGCGGCGTGCACACTTTTCCAGCTGTGCTT
CAATCTAGCGGCCTGTACTCTCTTTCTTCTGTGGTGACTGTGCCCTCCTCTTCACTTGGTACTCAGACC
TACATCTGCAACGTGAACCACAAGCCGAGCAATACCAAGGTGGACAAGAAGGTCGAGCCTAAGTCTTGC
GATAAGACCCATACTTGTCCTCCGTGTCCTGCTCCTGAACTTCTTGGTGGTCCTAGCGTTTTCTTGTTC
CCTCCGAAGCCTAAGGATACCCTGATGATTTCTAGGACCCCTGAGGTTACCTGCGTTGTGGTTGATGTG
TCTCATGAGGATCCCGAGGTGAAGTTCAATTGGTACGTTGACGGTGTCGAGGTGCACAACGCTAAGACT
AAGCCTAGAGAGGAACAGTACAACAGCACCTACAGGGTTGTGTCTGTGCTTACCGTGCTTCACCAGGAT
TGGCTGAACGGCAAAGAGTACAAGTGCAAGGTGTCCAACAAGGCTCTGCCTGCTCCTATCGAAAAGACC
ATCTCTAAGGCTAAGGGTCAGCCTAGGGAACCGCAAGTTTACACTCTTCCACCTTCTAGGGATGAGCTG
ACCAAGAACCAGGTGTCACTTACCTGCTTGGTGAAGGGCTTCTACCCTTCTGATATCGCTGTTGAGTGG
GAGTCTAATGGCCAGCCAGAGAACAACTACAAGACTACCCCTCCTGTGCTGGATTCCGATGGTTCATTC
TTCCTGTACAGCAAGCTGACCGTGGACAAGTCTAGATGGCAGCAGGGTAATGTGTTCAGCTGCTCTGTT
ATGCATGAGGCCTTGCACAATCACTACACCCAGAAGTCCCTTTCTCTGTCCCCTGGTAAGTCTGAAAAG
GATGAGCTTTGAGAGCTC

Anti-RANKL mAb light chain nucleotide sequences

TCTAGAACA**ATG**GGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCA
CTCTGATGTTCAACTT**CTCGAG**GAAATCGTGCTGACCCAGTCTCCTGGTACTCTTTCTTTGT
CACCTGGTGAGAGGGCTACCTTGTCTTGTAGAGCTTCTCAGTCTGTGAGGGGCAGATACCTT
GCTTGGTATCAACAAAAGCCTGGTCAGGCTCCTAGGCTGCTTATCTATGGTGCTTCTTCTCG
TGCTACTGGCATCCCTGATAGGTTCTCTGGTTCTGGCTCTGGAACCGATTTCACCCTTACCA
TCTCTAGGCTTGAGCCTGAGGATTTCGCTGTGTTTTACTGCCAGCAGTACGGCTCATCTCCT
AGGACTTTTGGTCAGGGCACCAAGGTTGAGATTAAG**CGTACG**GTTGCTGCTCCGTCCGTGTT
CATTTTTCCACCATCTGATGAGCAGCTGAAGTCCGGTACTGCTTCTGTTGTGTGCCTGCTGA
ACAACTTCTACCCTAGGGAAGCTAAGGTGCAGTGGAAGGTTGACAATGCTCTGCAGTCCGGT
AACAGCCAAGAGTCTGTTACTGAGCAGGACAGCAAGGACTCCACCTACTCACTTTCTTCTAC
TCTGACCCTGAGCAAGGCCGATTACGAGAAGCACAAGGTTTACGCTTGCGAGGTTACCCACC
AGGGACTTTCTTCACCTGTGACCAAGTCTTTCAACAGGGGAGAGTGTTCTGAAAAGGATGAG
CTT**TGA**GAGCTC

XbaI
T/ CTAGA
AGATC /T

XhoI
C/ TCGAG
GAGCT /C

NheI
G/ CTAGC
CGATC /G

BsiWI
C/ GTACG
GCATG /C

SacI
GAGCT /C
C/ TCGAG

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APPENDIX B

Preparation of the LAB reagents

1. LB (Luria-Bertani) medium (1 Liter)

— Yeast Extract	5	g
— Peptone	10	g
— Sodium chloride	10	g
*For LB agar plate adding Agar powder	1.5%	

2. 10x Infiltration Buffer (pH 5.5, 1 Liter)

— 100 mM MES

*Dissolved in DI water 400 mL and then adjusted the pH to 5.5.

**Adjusted the volume to 500 ml.

— 100mM MgSO4

*Dissolved in DI water 500 ml.

— Mixed the solution together.

2.1 1x Infiltration Buffer (pH 5.5, 1 Liter)

— 10 x Infiltration Buffer	100 ml
— DI water	900 ml

3. 10x Phosphate buffer saline, PBS (pH 7.4, 1 Liter)

— 137 mM Sodium chloride

— 2.68 mM Potassium chloride

— 10.1 mM Di -Sodium hydrogen phosphate

— 1.76 mM Potassium dihydrogen phosphate

3.1 1x PBS (pH 7.4, 1 Liter)

— 10 x PBS	100 ml
— DI water	900 ml

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3.2 1x PBST (pH 7.4, 1 Liter)

— 1 x PBS	100 ml
— Tween 20	0.05%
— Adjusted the volume to	1 l with DI water.

4. Preparation of polyacrylamide gel electrophoresis reagent

4.1 1.5 M Tris-HCl pH8.8

— 1.5M Tris-base

— Adjusted pH to 8.8 with 1 M HCl.

— Adjusted volume to 100 mL with distilled water.

4.2 10% w/v SDS solution

— SDS 10 g

— Adjusted volume to 100 mL with distilled water.

4.3 10% w/v Ammonium persulfate (APS)

— Ammonium persulfate 10 g

— Adjusted volume to 100 mL with distilled water.

4.4 10x Non-reducing loading buffer

— 0.125 M Tris-HCl, pH7.4

— 12% w/v SDS

— 10% v/v Glycerol

— 0.001% w/v bromophenol blue

*For reducing loading buffer, adding 22% v/v beta-mercaptoethnol.

4.5 10x running buffer (1L)

— Tris-base (MW 121.14)	30 g
— Glycine (MW 75.07)	144 g
— SDS (MW 288.08)	10 g

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*Adjusted volume to 1000 mL with distilled water.

1x running buffer (1L)

— 10 x running buffer	100 ml.
— DI water	900 ml

4.6 Coomassie Blue staining (4 l)

— Coomassie Blue blue R-250	1 g
— DI water	1600 ml
— Methanol	2000 ml
— Glacial acetic acid	400 mL

4.7 Distaining solution (4L)

— DI water	2800 ml
— Methanol	800 ml
— Glacial acetic acid	400 mL

5. Preparation of 6-10% gradient polyacrylamide gels

Ingredients	Total volume (5 ml)		
	6%	8%	10%
H_2O	2.850	2.600	2.100
Acrylamide/Bisacrylamide 40%	0.750	0.980	1.280
1.5 M Tris (pH 8.8)	1.300	1.300	1.300
10% SDS	0.050	0.050	0.050
10% APS	0.050	0.050	0.050
TEMED	0.004	0.003	0.002

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6. Preparation of 15% polyacrylamide gels

Ingredients	Stacking gel 6% (2.5 ml)	Resolving gel 15% (10ml)
H_2O	1.425	3.440
Acrylamide/Bisacrylamide 40%	0.375	1.960
1.5 M Tris (pH 8.8)	0.650	2.600
10% SDS	0.025	0.100
10% APS	0.025	0.100
TEMED	0.002	0.006

7. Preparation of western blot analysis reagents

7.1 20x transfer buffer

— Tris-base (MW 121.14) 30 g

— Glycine (MW 75.07) 144 g

*Adjust volume with distilled water to final volume of 1000 ml.

7.2 1xtransfer buffer

— 20x transfer buffer 50 ml.

— Methanol 150 ml.

*Adjust volume with distilled water to final volume of 1000 ml.

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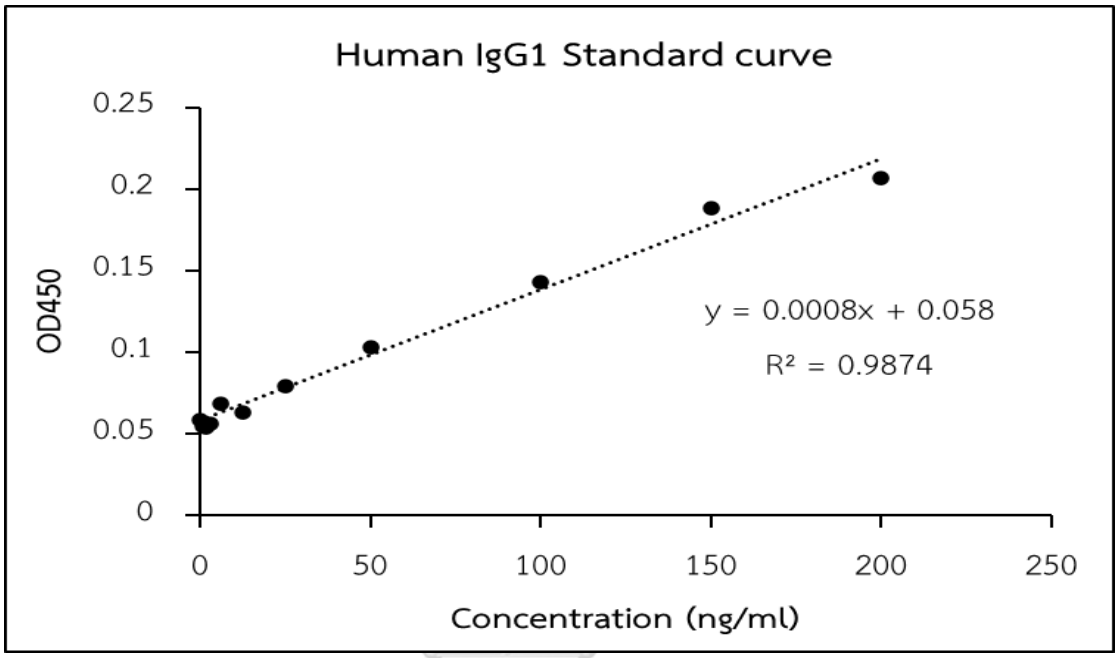
APPENDIX C

Time-course experiment of plant-produced anti-RANKL mAb quantified by sandwich ELISA.



Standard human IgG1 concentration (ng/ml)	OD_{450}		
	Rep 1	Rep 2	Average
0	0.062	0.056	0.059
0.78	0.055	0.055	0.055
1.56	0.051	0.057	0.054
3.13	0.053	0.06	0.0565
6.25	0.064	0.074	0.069
12.50	0.063	0.064	0.0635
25.00	0.079	0.08	0.0795
50.00	0.105	0.101	0.103
100	0.149	0.137	0.143
150	0.186	0.191	0.1885

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200	0.211	0.203		0.207	
Days post infiltration	Plant-produced anti-RANKL mAb concentration in mg/g (Fresh weight)				
	Rep 1	Rep 2	Rep 3	Average	STDEV
2	0.166	0.194	0.084	0.148	0.057
4	0.174	0.219	0.192	0.195	0.023
6	0.392	0.338	0.429	0.386	0.046
8	0.507	0.559	0.554	0.540	0.028
10	0.205	0.222	0.188	0.205	0.205

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APPENDIX D

Two-way ANOVA table

Concentration (ng/mL)	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjusted P Value
150	Denosumab vs. Plant-produced anti-RANKL mAb	0.1639	0.1497 to 0.1781	****	<0.0001
	Denosumab vs. Standard human IgG1	0.559	0.5448 to 0.5733	****	<0.0001
	Plant-produced anti-RANKL mAb vs. Standard human IgG1	0.3951	0.3809 to 0.4094	****	<0.0001
100	Denosumab vs. Plant-produced anti-RANKL mAb	0.1638	0.1496 to 0.1780	****	<0.0001
	Denosumab vs. Standard human IgG1	0.5635	0.5493 to 0.5777	****	<0.0001
	Plant-produced anti-RANKL mAb vs. Standard human IgG1	0.3997	0.3855 to 0.4139	****	<0.0001
50	Denosumab vs. Plant-produced anti-RANKL mAb	0.139	0.1248 to 0.1533	****	<0.0001
	Denosumab vs. Standard human IgG1	0.3893	0.3751 to 0.4036	****	<0.0001
	Plant-produced anti-RANKL mAb vs. Standard human IgG1	0.2503	0.2361 to 0.2645	****	<0.0001

Two-way ANOVA table (continued)

Concentration (ng/mL)	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjusted P Value
25	Denosumab vs. Plant-produced anti-RANKL mAb	0.1532	0.1390 to 0.1674	****	<0.0001
	Denosumab vs. Standard human IgG1	0.256	0.2418 to 0.2703	****	<0.0001
	Plant-produced anti-RANKL mAb vs. Standard human IgG1	0.1028	0.08860 to 0.1171	****	<0.0001
12.5	Denosumab vs. Plant-produced anti-RANKL mAb	0.1006	0.08640 to 0.1149	****	<0.0001
	Denosumab vs. Standard human IgG1	0.1414	0.1272 to 0.1556	****	<0.0001
	Plant-produced anti-RANKL mAb vs. Standard human IgG1	0.04077	0.02653 to 0.05500	****	<0.0001
0	Denosumab vs. Plant-produced anti-RANKL mAb	0	-0.01423 to 0.01423	ns	>0.9999
	Denosumab vs. Standard human IgG1	0	-0.01423 to 0.01423	ns	>0.9999
	Plant-produced anti-RANKL mAb vs. Standard human IgG1	0	-0.01423 to 0.01423	ns	>0.9999

APPENDIX E

Determination of binding capacity of plant-produced anti-RANKL mAb quantified by indirect ELISA.

Concentration ng/mL	Commercially available denosumab				
	Rep 1	Rep 2	Rep 3	Average	STDEV
150	0.608	0.622	0.611	0.636	0.008
100	0.617	0.616	0.615	0.616	0.001
50	0.437	0.447	0.439	0.441	0.006
25	0.311	0.295	0.315	0.307	0.011
12.5	0.201	0.191	0.201	0.198	0.005
0	0.054	0.055	0.054	0.054	0.001
Concentration ng/mL	Plant-produced anti-RANKL mAb				
	Rep 1	Rep 2	Rep 3	Avr	STDEV
150	0.436	0.454	0.459	0.450	0.012
100	0.437	0.469	0.449	0.452	0.016
50	0.318	0.291	0.296	0.302	0.014
25	0.153	0.157	0.151	0.154	0.003
12.5	0.093	0.103	0.095	0.097	0.005
0	0.054	0.055	0.054	0.054	0.001
Concentration ng/mL	Standard human IgG1				
	Rep 1	Rep 2	Rep 3	Avr	STDEV
150	0.051	0.053	0.060	0.055	0.005
100	0.050	0.053	0.055	0.052	0.002
50	0.050	0.052	0.052	0.052	0.001
25	0.050	0.050	0.052	0.051	0.001
12.5	0.057	0.058	0.054	0.056	0.002
0	0.054	0.055	0.054	0.054	0.001

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APPENDIX F

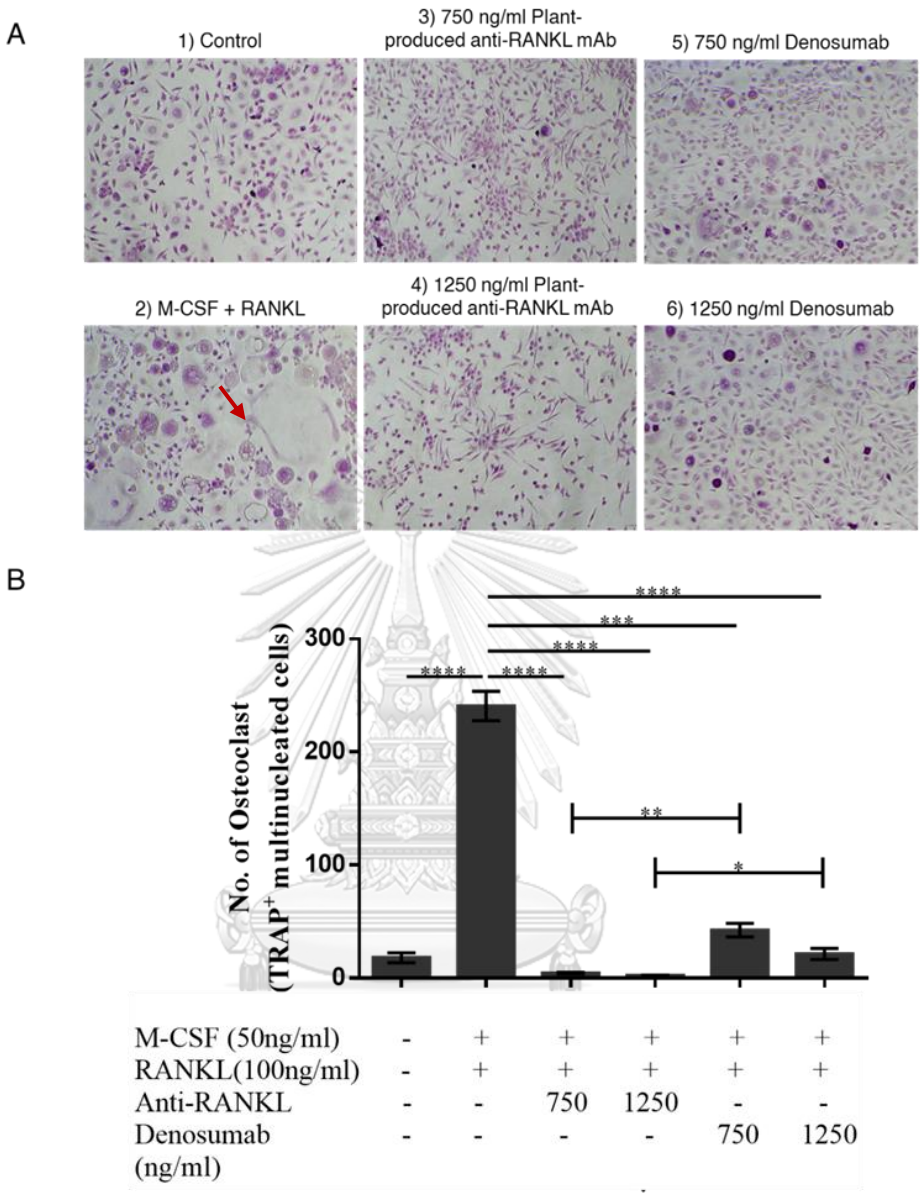

Figure 25. Plant-produced anti-RANKL mAb suppresses osteoclast differentiation. CD14+ monocytes were cultured in different condition for 15 days following 1) control, 2) M-CSF and RANKL, 3) 750 ng/ml plant-produced anti-RANKL mAb, 4) 1250 ng/ml plant-produced anti-RANKL mAb, 5) 750 ng/ml commercially available denosumab and 6) 1250 ng/ml commercially available denosumab. (A) Representative images of TRAP staining. (B) Quantification of TRAP+ osteoclasts were showed as mean and SEM of triplicates. ****p ≤ 0.0001

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