# THE EFFECT OF *SENNA ALATA* LEAF EXTRACT ON MIGRATION AND INVASION OF CHONDROSARCOMA CANCER CELL LINE

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# THE EFFECT OF *SENNA ALATA* LEAF EXTRACT ON MIGRATION AND INVASION OF CHONDROSARCOMA CANCER CELL LINE

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BY

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| Title | THE EFFECT OF *SENNA ALATA* LEAF EXTRACT ON MIGRATION AND INVASION OF CHONDROSARCOMA CANCER CELL LINE |
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Chondrosarcoma, the second most common malignant bone tumor, originates from chondrocytes and is caused by abnormal proliferation. The major cause of death among cancer patients is due to the process of metastasis. *Senna alata* (SA) or candle bush is a traditional medicinal plant with a wide range of biological activities. In this study, cell viability was determined by MTT assay. The inhibitory effects of the SA extract on cancer metastasis were determined by cell migration, cell invasion, and cell adhesion using wound healing assay, Transwell assay, and cell adhesion assay, respectively. The SA extract inhibited cell migration, cell invasion, and cell adhesion of SW1353 cells in a dose-dependent manner. Cell signaling protein expression was investigated by Western blot analysis indicated that MMP-2 and MMP-9 expression were downregulated, while TIMP-1 and TIMP-2 expression were upregulated in SW1353 treated cells. Moreover, SA extract also decreased the expression of NF-KB transcription factor that involved cancer metastasis leading to the reduction of cellular migration and invasion by decreasing the expression of p-ERK1/2, p-p38, and PI3k/Akt signaling proteins. These results revealed that inhibition of NF-KB, MAPK, and PI3K/Akt signaling pathway were associated with MMP-2 and MMP-9 downregulation leading to a reduction in cancer cell metastasis. Therefore, these results clearly suggest that SA may be a promising agent against human chondrosarcoma metastasis.

Keyword : Senna alata, cell invasion, cell migration, chondrosarcoma, MMPs, TIMPs

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# CHAPTER 1
# INTRODUCTION

## 1.1 Background

Cancer remains a major public health problem worldwide. Cancer is caused by accumulating cancer-causing substances that lead to gene damage. The substances that cause cancer are called carcinogens. The most common human carcinogen including ultraviolet ( UV) light, radiation exposure, tobacco, chemical substances. Almost 20% of cancers are related to chronic infections of virus or bacteria. The most significant ones being human papillomaviruses (HPV), or *Helicobacter pylori* (*H. pylori*) (1). The other causes of cancer including physical activity, poor diet, alcohol consumption, aging, and family history (genetic) (2). These factors may promote carcinogenesis, a normal cell changing to a tumor cell. From reported, there were 19.3 million new cancer cases in 2020 worldwide, 10 million people were estimated for total cancer deaths (3). The common types of cancer treatment are chemotherapy, radiotherapy, surgery, and alternative treatment.

Chondrosarcoma is the second most common malignant tumor of bone, representing 25% of all malignant bone (4) but this tumor is found the most in adult accounts for more than 40%. This type of cancer originates from cartilage cells (chondrocytes) that abnormal proliferation and normally grows within a bone. This type of cancer has a potent capacity to invade locally and causes metastatic to distance organs, especially to the lungs and the leading cause of high mortality of cancer. This process is called “metastasis”.

Metastasis is the cause of 90% of human cancer deaths (5). Metastasis is an extremely multistep processes including the primary tumor loss of cell-cell adhesion, migration, and then penetration to the extracellular matrix (ECM) or basement membrane (BM) into the blood vessels or lymph vessels. Then cancer cells adhere to blood or lymph vessels wall around secondary sites, exit from the circulatory system, and invade into the surrounding tissue at the distal sites. After that cancer cells proliferate and grow to secondary organs or tissues. Degradation of the ECM by

proteolytic enzymes surrounding the primary tumor is a major step of tumor cells to invade into circulatory systems. The main group of proteolytic enzymes that helping cancer invasion is the matrix metalloproteinases (MMPs) especially MMP-2 and MMP-9 which play a key roles in digesting various types of ECM molecules in the BM (6). These enzymes are a group of gelatinase enzyme, zinc ( $Zn^{2+}$) -dependent endopeptidase which degrading ECM proteins including type IV collagen of basement membrane and leading to cancer invasion. The other approach to target MMPs is through regulated by endogenous inhibitors of MMPs, tissue inhibitors of metalloproteinases (TIMPs) such as TIMP-1/-2. Thus, inhibition of MMPs activity is essential for prevent the effect of cancer metastasis.

In cancer, the essential process for metastasis is regulated by intracellular signaling pathway. It is also reported that the family members of mitogen-activated protein kinases (MAPKs) along with extracellular signal-regulated kinase (ERK), p38 MAP kinase, and stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) are well known to be activated in human chondrocytes (7). PI3K/AKT is also involved in apoptosis, angiogenesis, cell proliferation, and metastasis in many type of cancer cells (8). Furthermore, nuclear factor-**κ**B (NF-**κ**B) is also regulated the expression of MMPs (9). Inhibition of these signaling molecules may lead to the prevention of cancer metastasis as a plausible target for cancer therapy. At present, metastasis is the largest challenge in cancer treatment. Most cancer chemotherapy usually uses highly cytotoxic drugs. These agents can cause severe side effects in normal tissues or cells. Thus, natural bioactive compounds with less side effects in the regular cells are one of the compounds of choice.

*Senna alata* (SA) is a medicinal plant, belongs to the Fabaceae family which has been known in Thai language common name as Chumhetthet. The common names are candle bush and Ringworm bush (10). This plant can be found widely in Thailand, Indonesia, Philippines, India, Bangladesh, Africa and tropical America (11, 12). In many countries, fresh leaves of *Senna alata* are used in the treatment of skin diseases for example itching, ringworm, and other related skin diseases (13). Traditionally, the crude

extract of leaves possess a broad range of medicinal properties such as antiparasitic, antifungal, antibacterial, antinflammatory, and anticancer (14). However, the effect of *Senna alata* leaf extract on chondrosarcoma metastasis has not yet been reported.

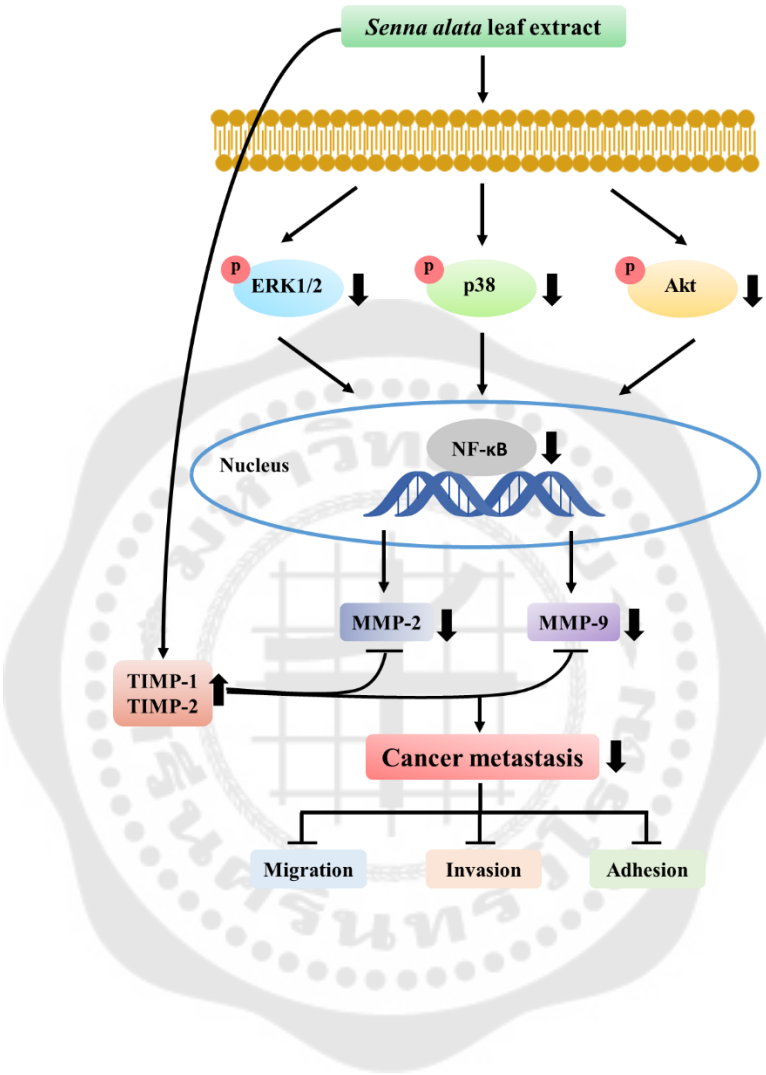
### 1.2 Scope of the study

To investigate the effect of SA leaf extract on cell proliferation, cell migration, cell invasion, cell adhesion, and the potential mechanism of action of chondrosarcoma cell line, SW1353.

### 1.3 Hypothesis

*Senna alata* leaf extract may act as the anti-metastatic activities of SW1353 cells by suppressing cell migration, invasion, and adhesion and the mechanism involved.

1.4 Conceptual framework



# CHAPTER 2
# LITERATURE REVIEW

## 2.1 Bone and cartilage

Bone and cartilage are specialized connective tissues. They are components of a skeletal system that have an extremely complex structure. The main constituents consist of natural organic and inorganic phases. There are calcium phosphate (approximately 70%), collagen (approximately 20%), water and other organic substances such as proteins, polysaccharide, and lipids approximately 10% by weight. In particular, collagen fiber represents about 90% of the total protein matrix and it forms fibrils in diameter of 100-2000 nm. The inorganic part of the bone, calcium phosphate is in the crystal form, hydroxyapatite is the main component that support rigidity of bone. Hydroxyapatite crystals are needle-shaped with a width of 20 nm, the length of 40-60 nm, and a thickness of 1.5-5 nm that parallel embedded with collagen fiber (15).

The main organ of the body framework is the bone. It provides protection to internal organs, acts as a calcium, cytokines, and growth factors reservoir and produces erythrocytes and leukocytes (16). Mainly, cartilage is a softer form of bone-like tissue which remains at the end of bone, as a part of joint. It is composed of a matrix and fiber. Cartilage is found in many areas of the body such as ankles, knees, elbows, ears, nose, ribs, spine, and airways.

Cartilage consists of specialized cells called chondrocytes. The normal functions of cartilage including produce and secrete the major components of extracellular matrix (ECM) such as proteoglycan to provide structural support (17).

It is classified into three major types include fibrous cartilage, hyaline cartilage, and elastic cartilage. Three types of cartilage are different in distribution, relative amounts of collagen and proteoglycan (18). In the joints of the body, cartilage maintains the shape and flexibility of fleshy appendages. Cartilage also acts as a shock absorber by covering the ends of the bones to prevent them from rubbing against each other (Figure 1) (19).

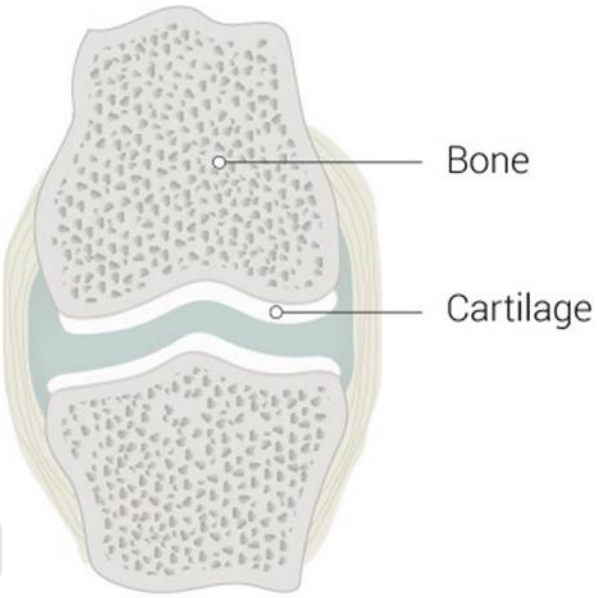



Figure 1 Bone and cartilage

Source: Palmer B. (2021). Anatomy Cartilage: StatPearls Publishing

**2.2 Cancer**

Cancer is defined as a group of diseases in which abnormal cell grow rapidly and continuously and out of control, forming a malignant tumor. However, not all tumors are cancerous. Tumors are classified into 2 types such as benign and malignant (Figure 2) (20). A benign tumor is unable to spread to other areas of the body. Thus, this type is not cancer and not dangerous. In contrast, a malignant tumor is normally known as cancer, are formed from abnormal cell proliferation that is highly able to invade to distant parts of the body via the bloodstream or lymph vessels.

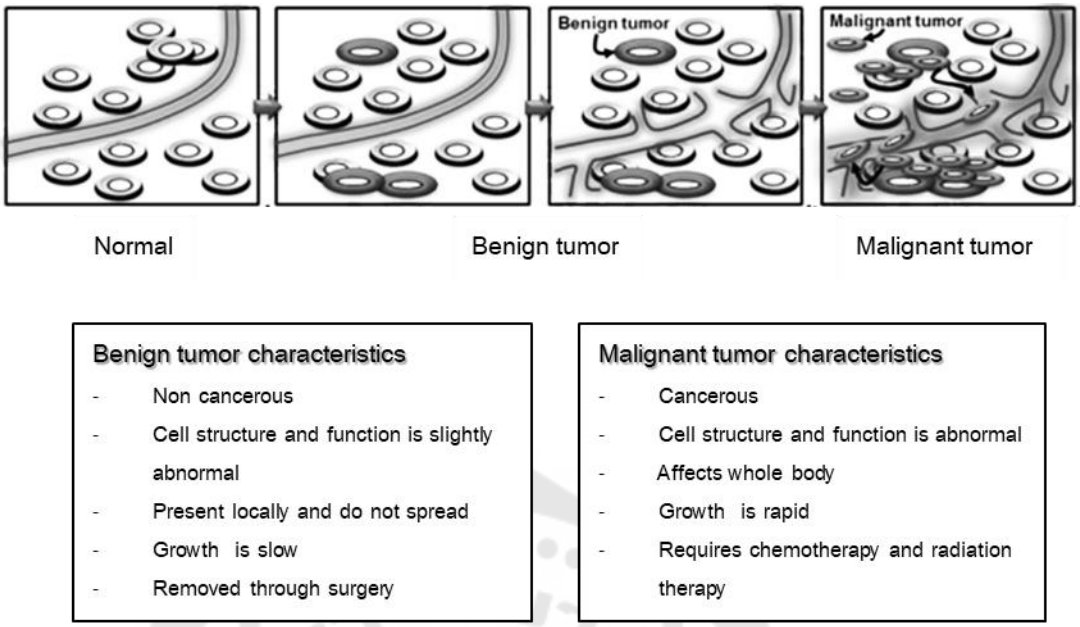



Figure 2 Characteristics of benign and malignant tumor.

Source: Kumar D, Sharma S, and et al. (2015). Molecular signalling saga in tumour biology: Journal of Tumor. p.310

Name of most cancers depend on the tissue of origins, such as brain, breast, lung, colon, or prostate cancer. Some cancers are named as a type, melanoma is cancer of the skin cells, leukemia is a type of blood cancer, and lymphoma is cancer of the lymphatic system. Carcinomas are cancers that develop in the epithelial tissue such as the lung, liver, skin, or breast. Another group is sarcomas, which develop in the supporting tissues of the body such as bone. Cartilage tissue can be developed in benign tumors called chondroma and the malignant tumors called chondrosarcoma (21).

### 2.3 Chondrosarcoma

In 2020 worldwide, the International Agency for Research on Cancer (IARC) has reported the incidence data of cancer, estimated nineteen million new cancer cases and about ten million cancer deaths. In the United States (USA), the American Cancer

Society's (ACS) estimated 3,610 people of all ages will be diagnosed with primary bone sarcoma in a year 2021 (about 0.2% of all cancer deaths). This cancer is also estimated that 2,060 deaths will occur in 2021 (about 0.3% of all new cancer cases). In adults, more than 40% of primary bone sarcomas is chondrosarcoma. Followed by 28% of osteosarcoma, 10% of chordoma, 8% of Ewing sarcoma, 4% of fibrosarcoma and others (Figure 3) (22).

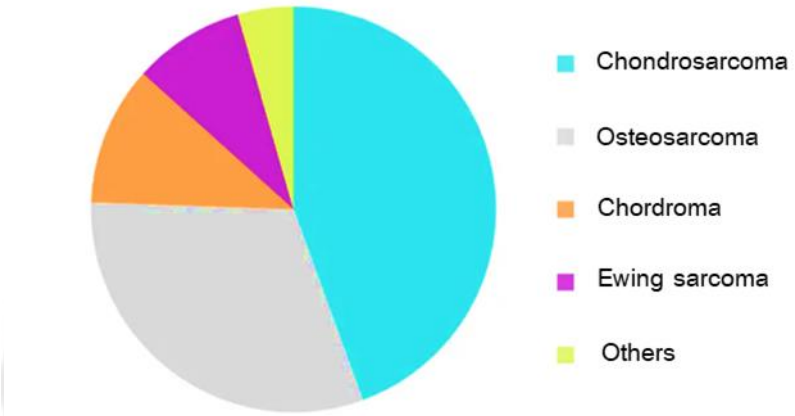



Figure 3 Estimated percentage of bone cancer types in adults, global 2020

Source: Bone Cancer treatment market-growth, trends, and forecasts (2021): Mordor Intelligence.

Chondrosarcoma, a type of bone sarcoma, is a malignant cancer of cartilage-producing cells (chondrocytes) also calls malignant cartilaginous tumors. This type of cancer occurs from abnormal proliferation of cartilage cells. A tumor usually grows on its surface and within a bone (23). The cells can develop the ability to spread to another part in the body and mainly affects the legs, upper arms, scapula, ribs, and pelvic bones. Sometimes, chondrosarcoma develops in the trachea, larynx, and chest wall.

According to the American Cancer Society 2016, chondrosarcoma has become the most common primary malignant tumor of bone, approximately 20 - 25% of bone

sarcomas (24). This type of cancer can occur in any region of bones of the body which has cartilage and can occur at any age. It is typically affected in adults, accounting for more than 40% of the adult cases and less than 5% of cases occur in patients < 20 years (25). This cancer has the potential for invasion and spreading to distal organs, especially lungs and liver (26) and resulting in mortality from cancer metastasis.

Chondrosarcoma can be divided in several ways. First, primary chondrosarcoma (conventional chondrosarcoma) usually develops locally and exit through basement membrane to invade to the other tissues. Secondary chondrosarcoma which arising from a benign for example osteochondromas, enchondromas, or divided by histologic grade, by location within the bone (central or peripheral), or syndrome (Figure 4) (27).

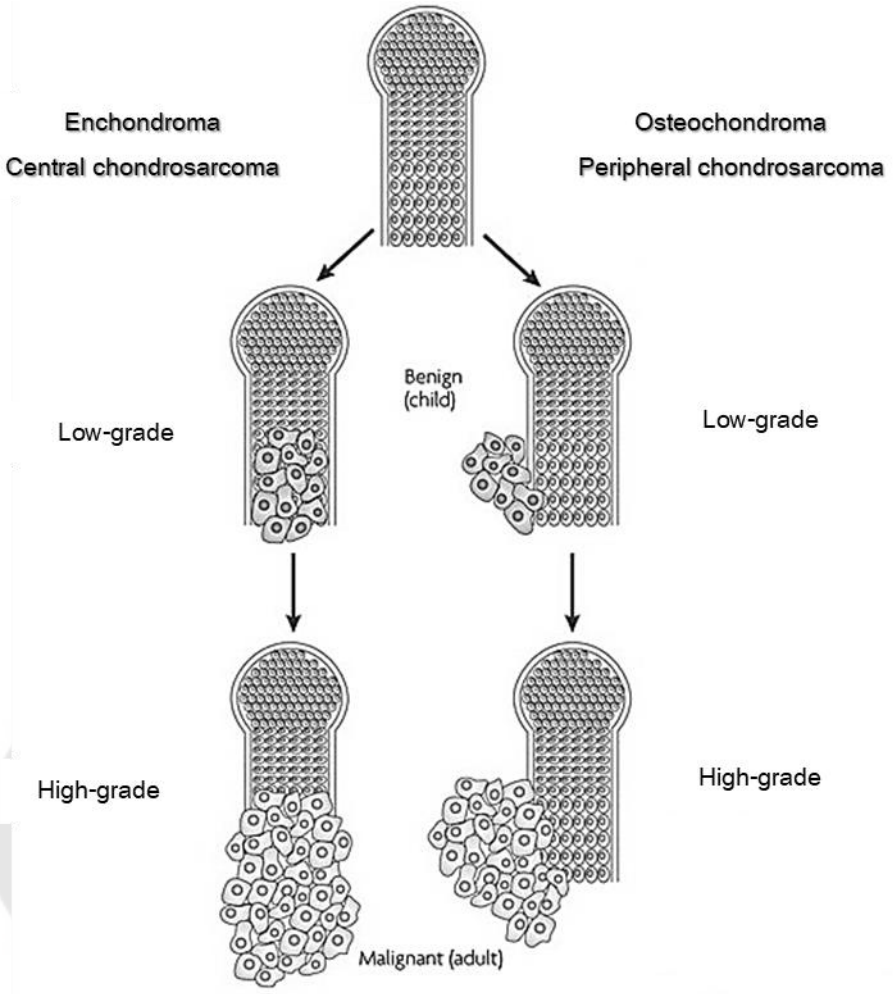



Figure 4 Development of benign cartilaginous tumors and their progression to chondrosarcoma

Source: Judith VM, Bovée G, Pancras CW, Jay S, Benjamin A. (2010). Cartilage tumours and bone development: molecular pathology and possible therapeutic targets: Nature Review Cancer. p. 484.

### 2.3.1 Grading of chondrosarcoma

Chondrosarcoma can be classified by grading under a microscope, which measures how fast cancer grows and divided by the pathologist. The low grade is grow slowly of cancer cells like a regular cells. When cancer is growing-slowly, the prospect that it will have less ability to spread. In high-grade chondrosarcomas, the cells can

grow rapidly and has ability to spread. The grades are divided from 1 to 3, grade 1 is low grade and grade 3 is high grade. Higher grade chondrosarcomas are the most likely to cause recurrent and may spread to other parts of the body (28). The grading is based on the nuclear size of tumor cells, nuclear staining, and cellularity (29).

**2.3.1.1 Grade I chondrosarcoma**

This is low-grade cancer, which has an amount of cells moderately. It is most similar to normal cartilage. In some cases, binucleated cells form (cells with two nuclei instead of one) can be present.

**2.3.1.2 Grade II chondrosarcoma**

This is intermediate grade cancer, which consists of more cells that have a greater extent of hyperchromasia, nuclear atypia, and nuclear size (30)

**2.3.1.3 Grade III chondrosarcoma**

This is high-grade cancer, which has pleomorphism areas. There are large cells with more hyperchromasia than grade 2 with abundant necrosis and occasional giant cells. (31).

**2.3.2 Type of chondrosarcoma**

There are several histologic subtypes of chondrosarcoma. The name type depended on the characteristic of cells under a microscope including conventional, dedifferentiated, clear cell, and mesenchymal chondrosarcoma (32).

**2.3.2.1 Conventional chondrosarcoma**

They are also known as central chondrosarcoma. This type is the most common subtype of chondrosarcoma and may be low, intermediate, or high grade. This type usually occurs in the central area of bone. The most common site is long bone (33).

**2.3.2.2 Dedifferentiated chondrosarcoma**

This type begin as normal chondrosarcoma and then some areas of the tumor change into cells like high-grade for example fibrosarcoma and osteosarcoma (34).

**2.3.2.3 Clear cell chondrosarcoma**

This type is very rare and low grade cartilage malignant cancer. The characteristic of this type is slowly growth (35). The lesions arise primarily in skeletally mature adults. The most common sites are humerus, tibia, and femur.

**2.3.2.4 Mesenchymal chondrosarcoma**

This type is highly aggressive tumors and rapidly growth. They arise both in bone and in the soft tissues. The most common sites are the jaw, pelvis, spine, and scapula.

**2.3.3 Causes of chondrosarcoma**

Although the causes of chondrosarcoma are unknown. Some factors let patients at a higher risk and increase a person's risk of developing to chondrosarcomas are as follows.

**2.3.3.1 Age**

The risk of bone sarcoma overall increases with age. However, some bone sarcoma subtypes occur more frequently in children and younger adults.

**2.3.3.2 Radiation**

Exposure to radiation increases bone sarcoma risk and causes of cancer are associated with receiving radiotherapy to previous cancer treatment (36).

**2.3.3.3 Previous cancer**

Chondrosarcoma can occur as a second primary tumor from metastasis of the primary site (spreading to the bones from elsewhere) (37).

**2.3.3.4 Genetics**

Genetic syndromes are caused by mutations that are considered to be risk factors for this cancer. Multiple exostoses syndrome is a rare condition associated with the formation of cartilage bump on the bones. In this condition, benign bone lesions (osteochondromas) transform to chondrosarcomas. (38).

**2.3.3.5 Other medical condition**

Several non-heritable conditions characterized by benign bone lesions can in a minority of cases transform into malignant bone tumors.

2.2.3.5.1 Ollier's disease is a disease of multiple benign bone tumors within the bones. Chondrosarcoma occurs in around 40% of this cases (39).

2.2.3.5.2 Maffucci's syndrome is a very rare condition and slightly more likely to develop a chondrosarcoma (40).

**2.3.4 The symptoms of chondrosarcoma**

The symptoms are vary depending on the size and location of cancer in the body. Symptoms include a large tumor on the bone, pain, and local swelling. The other symptoms are fatigue, fever, and weight loss. Chondrosarcoma can also spread to somewhere else in the body. Thus, the signs and symptoms of secondary bone cancer can be different (41).

**2.3.5 Treatment for chondrosarcoma**

Treatment depends on several factors including the type, size, location, age and overall health, the grade of cancer and whether it has spread. The treatments that might be used are surgery, chemotherapy, and radiotherapy (42).

**2.3.5.1 Surgery**

Surgery is the standard treatment for chondrosarcoma. The tumor is removed along with some surrounding normal tissue. For chondrosarcoma, surgical options including amputation (43), limb-sparing surgery, and biopsy (44).

**2.3.5.2 Chemotherapy**

Despite surgery remains a universal treatment option for chondrosarcoma. Chemotherapy is an important treatment to limit the proliferated number of tumor cells. High-grade chondrosarcoma may require this therapy but it can occur side effects.

**2.3.5.3 Radiotherapy**

Radiation therapy treatment by using x-ray beams to destroy tumor cells. This way of treatment affects less harm as possible to regular cells. Sometimes radiotherapy can cause side effects such as skin redness (erythema) and fatigue.

## 2.4 Metastasis

Cancer metastasis is the major cause of patient death number one of the world's population. Although the number of patients with cancer is less than patients with cardiovascular disease but the mortality rate from cancer is higher than in some high

and middle income countries. Metastasis is the primary reason for most cancer morbidity and mortality by more than 90% of patients who died from the spread of cancer to distal organs for example lungs, liver, bones, and brain. Cancer is pathological of cells that are caused by the accumulation of abnormal genes and leading to abnormal development and uncontrolled cell growth. Cancer cells will damage the surrounding cells and the function of normal cells is not working properly. During the differentiation of a normal tissue cell into invasive cells, phenotypic and biochemical are changed in cancer cells. These alterations relate to various factors include cell morphology, cell-cell adhesion molecules, growth factor, or gene expression. Cancer cells can develop metastases to move out from the organ of origin and to grow in the other distal organs through the bloodstream or lymphatic system (Figure 5)(45) . The cancer cells spread through the blood vessels or lymph vessels depending on the anatomical location of the organ of origin and direction of blood flow. However, many types of cancer often spread to the lungs. This is because when cancer cells move into the bloodstream and then through the right atrium and ventricle and to the lungs before entering to the left and right atrium to go to other organs (Figure 6). Thus, most cancer cells are restricted to the lung capillaries. Lungs are the organ with oxygen and nutrient-rich so whether cancer cells originated from any organ, they are usually grown and spread to the lungs than other organs (46).

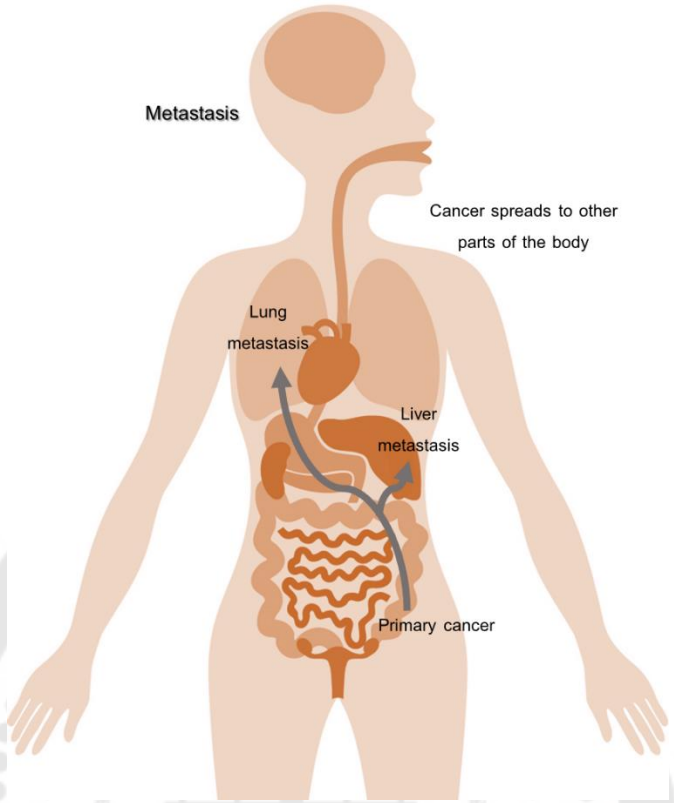



Figure 5 Overview of cancer metastasis

Source: National Cancer Institute (2020). Metastatic cancer: When cancer spreads: The National Institutes of Health.

Most cancers can spread to the lymph nodes via the lymphatic vessels and often found in the early stages of metastasis condition due to the structure of the lymph vessels contributing to the spread of cancer cells. Because of the lymph vessels have a larger size than the blood vessels and lymphatic epithelial cells are very thin single layer. Moreover, the basement membrane is a discontinuous layer and link with the connective tissue. As a result, the spread of cancer through the lymph vessels occur more easily than blood vessel.

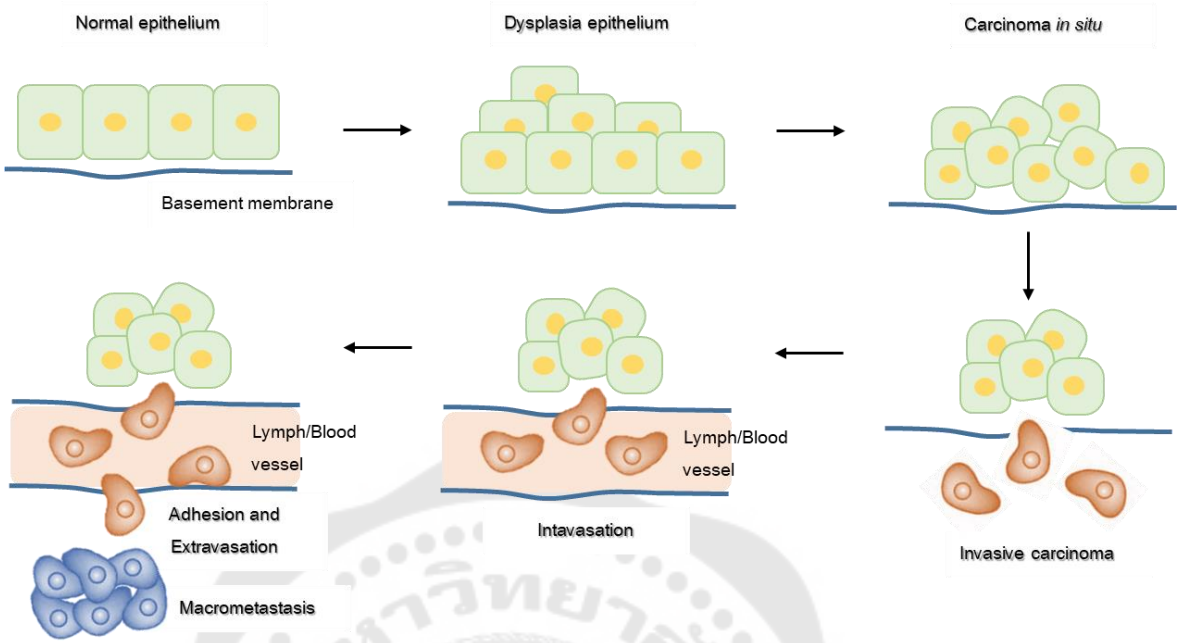



Figure 6 Cancer metastasis

Source: Seyfried TN, Huysentruyt LC. (2013). On the origin of cancer metastasis: Critical reviews in oncogenesis p. 33.

### 2.4.1 Historical development of the seed and soil theory

Metastasis has been studying for more than one hundred years by many researchers. In the middle of the nineteenth century, Stephen Paget, the English surgeon published in the title of “The distribution of secondary growths in cancer of the breast”. He studied from different primary tumors more than nine hundred autopsy records of patients and hypothesized that metastasis (the spread of cancer) did not occur in randomly pattern. Then, he proposed this theory to explain the non-random pattern of cancer metastasis. The seed refer to metastatic cancer cells. The soil refers to the organ or tissue for growing properly (47).

#### 2.4.1.1 The seed

The seed is cancer cells that eventually expand into a successful metastasis. Cancer cells can survive result of mutations oncogenic. The genome

changes include chromosomal abnormalities, DNA mutations, epigenetic alterations, which result in tumorigenesis (48). The surface molecule of a primary tumor is cadherin. The previous study reported that the melanoma cells had the ability of invasive by the loss of E-cadherin and then cancer cells can be migrated and invaded to other sites. E-cadherin is a major adhesion molecule that expressed both melanocytes and keratinocytes (49). For the cancer cells to migrate to distal site, cell migration is requires the presence of proper surface molecules that allow for adhesion to stromal proteins (50). Therefore, these are the capability of cancer cells to survive in the body.

**2.4.1.2 The soil**

The soil is organs or tissues for the seed to grow properly and orderly for tissue specificity of metastasis formation. To determine the role of soil that known as the host microenvironment, Isaiah Fidler and his postdoctoral fellow at the Barts Cancer Institute, University of London, set out to perform the experiment. They investigated the seed and soil hypothesis by implanted lung and kidney tissue into the muscle of a mice and injected melanoma cells into intravenous of mice. The results of them showed metastatic cells grew only in the lung and implanted lung tissue (51).

These studies reported how cancer cells react with the environmental. Thus, the interaction between cancer cells and environment leads to help cancer cells to spread to other sites.

**2.4.2 Metastasis process**

Metastasis is an extremely multistep process complex that is one of the hallmarks of cancer (52). Cancer cells can invade to tissue by entering the circulatory system or lymph vessel. These cells must develop the ability involving in the spread of cancer and the lack of any stage of metastasis process will make unsuccessful metastasis (Figure 6). Therefore, the metastasis process represents multi-steps including tumor cell invasion, entry into the blood system then migrate, exit from the circulation, and growth at the distal sites. The sequential steps of metastasis are shown below (51).

**2.4.2.1 Primary tumor growth**

Cancer cells at the primary site have developed the essential that cell requires spreading including secretion of proteolytic enzyme, matrix metalloproteinase enzyme to degrade components of the ECM (ECM remodeling) and evasion of the immune system.

**2.4.2.2 Vascularization or angiogenesis**

This process is about the growth of new blood vessels to obtain a blood supply which occurs when cancer cells are developing and growing. Typically, oxygen can diffuse through the blood vessels at a distance of 150-200 micrometers. The tumor is larger than 1-2 millimeters in a diameter must create new blood vessels for supplying food and oxygen to cancerous cells. Pro-angiogenic factors are produced and secreted from tumor, then bind to the specific receptor, and activate endothelial cells. The blood vessel is formed abnormally characteristics such as size, shape, cell growth, and changing normal function of blood vessels.

**2.4.2.3 Detachment from primary tumor and invasion**

Metastatic cancer cells can invade the connective tissue. Following activation of, the endothelial cells secrete enzymes to break down the BM of the blood vessel. Then endothelial cell proliferate and migrate through small pores normally. The cells of connective tissue have specific molecules on the surface and hold cell to cell adhesion. Cancer cells can detach from tumors originated by changing specific cell surface molecules. The loss of cell-cell adhesion ability allows tumor cells to secrete metalloproteinase enzymes and degrade the ECM. Then, cancer cells have the ability to invade and migrate through the blood system.

**2.4.2.4 Intravasation**

This step is the ability of cells to spread through bloodstream. If cancer cells can escape through the capillaries into the blood system to the heart and lungs, it can spread to various organs throughout the body. Therefore, passing cells must adapt to move in the blood vessels, which are much smaller than the cancer cells (2-7 micrometers size in vessels and tumor cells have an average size of 20 micrometers).

**2.4.2.5 Adhesion**

Cancer cells escape into the blood stream, migration with the pressure of bloodstream, and adhere to blood vessel walls in the secondary site. In this stage, cancer cells have an adhesion molecule such as integrin and cadherin on the cell surface that can bind to specific receptor molecules of the vessel wall to form stronger bonds. Then, the cells can migrate into distant organs.

#### 2.4.2.6 Extravasation

This step is the migration of cancer cell into the surrounding tissues at the distant site or secondary site. Cancer cells can move out of the blood vessel wall by various ways. The cancer cell may use the same mechanism to spread into the bloodstream by an enzyme that digesting endothelial tissue thus cancer cell can penetrate the endothelium and the basement membrane. In some cases, cancer cells may proliferate and penetrate the vessel wall cells to insert into the secondary site.

#### 2.4.2.7 Growth of secondary tumor

This step is threatened by cancer cells. These cells can be invaded and migrated to grow into a new tumor through lymphatic or blood circulatory systems. For example, colon cancer cells often, metastasize to the lymph nodes and liver. Patients with metastatic cancer have a group of small cancer that embedded in many organs in the body but cannot detect, this cancer cell micro metastases. For some types of cancer, blood tests can detect exact proteins releasing by cancer cells.

### 2.4.3 Routes of metastasis

In the body, cancer cells can spread in several way including lymphatic spread, hematogenous spread, and transcoelomic spread. Some types of cancer cells can circulate by multiple routes. All of these routes are categorized depending on where a cancer cell invades and moves to the other secondary tissue in the body.

#### 2.4.3.1 Lymphatic spread

In human cancer, lymphatic metastases is a key mechanism for spreading of cancer cells. The cancer cells can spread through the lymph nodes and finally spread to other areas. Due to not all cancer cells are efficient to insert into blood vessels and lymphatic system may be the possible pathway for the dissemination of cancer cells. This route is the most common of carcinomas metastasis. On the other

hand, this route is uncommon for metastasizing of sarcoma (cancers from the mesoderm origin). Breast cancer, prostate, and colon cancer are some examples using this route (53, 54).

**2.4.3.2 Hematogenous spread**

In this route, cancer cells spread through the blood circulation system. This is the typical route of metastasis for carcinomas and sarcomas. The lymphatic metastasis may finally found in later stages of cancer lead to the hematogenous spread in systemic because the duct connects the lymph to the blood vessel. For example, breast cancer can spread through the lymphatic system but is also found to commonly spread to the bone marrow through blood circulation.

**2.4.3.3 Transcoelomic spread**

In transcoelomic route, cancer cells will spread into the surface of body cavities. This is a common route of cancer metastasis that originates in the organ surrounded by body cavities. For example, ovarian and lung cancer can penetrate through the surface of the peritoneal cavity that surrounds the ovary and the pleural cavity that surrounds the lung respectively (55).

**2.4.4 Mediator of metastasis**

**2.4.4.1 Matrix metalloproteinase (MMPs)**

The interaction between cells and ECM including collagens, fibronectin, laminin, and proteoglycans are very important in physiological processes. The ECM holds cells together and plays a roles in various biological processes such as apoptosis, morphogenesis, tissue repair, cell differentiation, cell migration, and cell growth. Thus, the activities of ECM-degrading proteinases must be regulated. The system of proteolysis is generated by the cell, which is the role for molecules degradation of ECM components. Control the balance of the ECM structure will affect cell division, cell changes, and cell death. Therefore, the loss of balance of this enzyme causes abnormal development of the embryo and leads to many diseases such as arthritis, fibrosis, and cancer due to excessive degradation of ECM (56).

Matrix metalloproteinases (MMPs) are key enzymes of the proteolytic enzyme system that controlled the function between cells and ECM. MMPs protein can

degrade the ECM protein components. MMPs are a group of proteins that have many characteristics that are similar to the structure of the protein which transcribed from different genes. However, the different structure of MMPs is depended on the type of MMPs (56). At present, MMPs family have been found total 23 enzymes in human (57). MMPs are classified into 2 categories such as soluble MMPs (secreted from cells) and membrane-type MMPs (bound to the cell surface). The MMPs have been divided into five major groups such as collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-1, -8, -13), and membrane-type MMPs (MT-MMPs: MMP-14 to -17, -24, -25) according to their substrate specificity, primary structure, and cellular location (58).

Generally, MMPs are synthesized as inactive proenzymes (pro-MMPs), MMPs have a sequence of amino acids that determine the functional activity of a protein called domain. All MMPs have 3 major domains include; 1) pro-peptide domain that controls the function of MMPs, 2) haemopexin-like-domain, plays an major role in protein–protein interactions and determining enzyme specificities, and 3) catalytic domain contains a $Zn^{2+}$ binding site. In domain 3, there are zinc and calcium components that help to maintain the three-dimensional structure, which is essential to the stability and activity of some MMPs enzymes such as MMP-2 and MMP-9 have haemopexin-like-domain between active site and zinc-binding sites. In addition, MMP-9 has a type 4 collagen-like domain between the $Zn^{2+}$ binding domain and haemopexin C-terminal domain. These enzymes are specific to different substrates (59, 60). MMPs enzyme has a bonding link with cysteine in the pro-domain site (Cys-$Zn^{2+}$), whereas Cys-$Zn^{2+}$ is the part that allows the enzyme to be an inactive form. It was found that the pro-domain part was the position of the autoinhibitor. When there is a protease enzyme comes to the stimulus by cutting off the prodomain section leads to Cys-$Zn^{2+}$ bonds are cleaved separately. The active site or $Zn^{2+}$ position is stimulated lead to the active enzyme. The bonding of cysteine and $Zn^{2+}$ positions are the cysteine switch that acts to close and open an enzyme activity by keeping the condition of an inactive enzyme.

When the enzyme stimulates to bond dissociation then the enzyme can be active (Figure 7) (61).

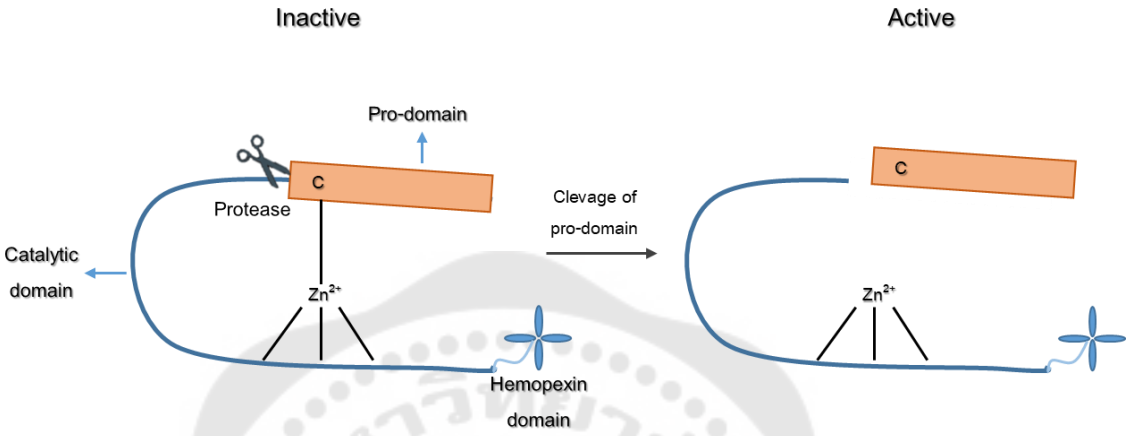



Figure 7 Activation of MMPs by pro-domain breaking down at the bonding site between Cysteine and $Zn^{2+}$

Source: Page MA, Ewald AJ, Werb Z. (2007). Matrix metalloproteinases and the regulation of tissue remodelling: Nature reviews Molecular cell biology p. 223.

Typically, all MMPs have a similar structure including amino ($NH_2$) terminal followed by pro-peptide, a zinc catalytic, and haemopexin C-terminal domain at the carboxyl end. The haemopexin-like domain is connected to the catalytic domain by the hinge region (Figure 8) (62).

Pro-peptide: at first, MMPs are synthesized as an inactive form containing the propeptide domain. Removal of this pro-peptide domain part of the MMPs will result in active MMPs. This domain has a cysteine switch, it will be conserved to handle the zinc binding site in the active site via cysteine residue and then leads to an inactive enzyme. The conserved amino acid sequence for cysteine residue is PRCGxPD and some MMPs may have a part of furin recognition site in the pro-peptide section.

Catalytic domain: In the catalytic domain, there is a 2 nm active site and in the active site at the catalytic domain, zinc ion is important for stimulation to become active enzyme. The zinc is bound to the histidine residues. The conserved amino acid sequence of this section is HExxHxxGxxH also called zinc binding motif.

Hinge region: It is the connection between the zinc catalytic and C-terminal domain. The amino acid length is about 75 and the structure is uncertain.

Haemopexin-like C-terminal domain: C-terminal domain has structural similar to haemopexin serum protein. The structure is consist of a 4-bladed β-propeller, which relate to the interaction between protein and protein. This structure determines the specificity of the substrate and TIMPs that will bind to the MMPs. In addition, MT-MMPs are attached to the BM via the transmembrane domain amino acid or GPI-anchoring domain.

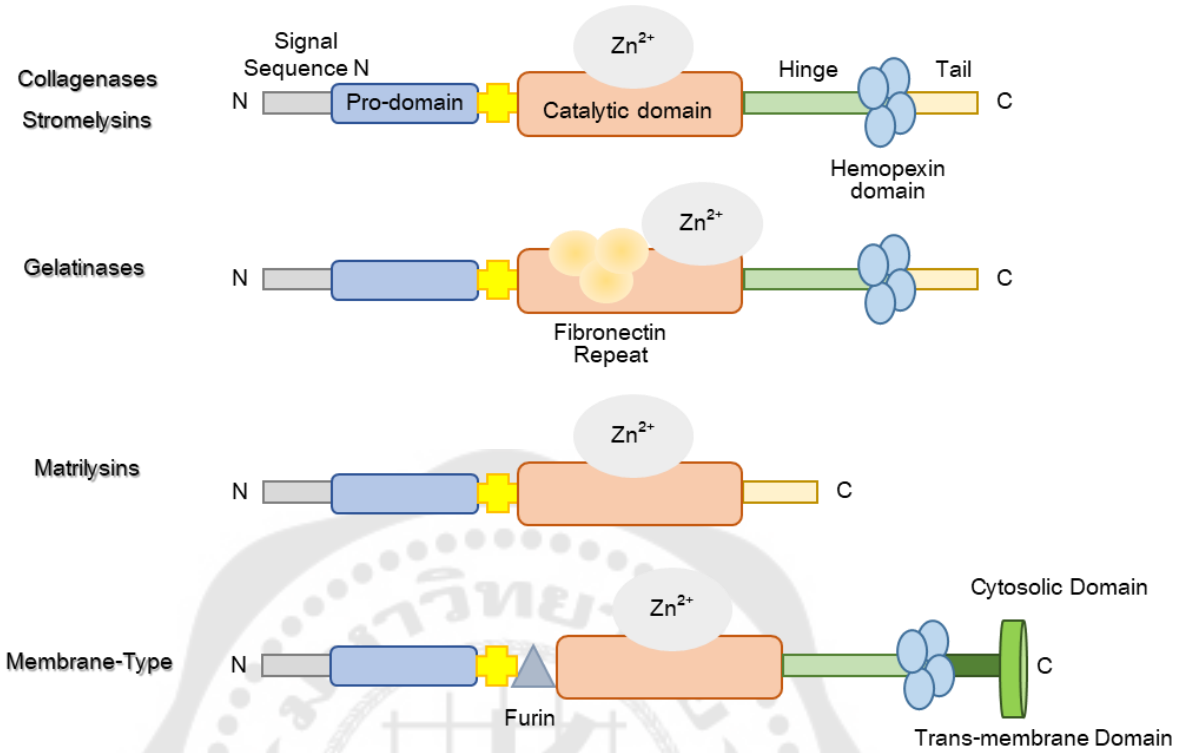



Figure 8 Schematic of the domain structures of MMPs

Source: Benjamin MM, Khalil RA. (2012). Matrix Metalloproteinase Inhibitors as Investigative Tools in the Pathogenesis and Management of Vascular Disease: Experientia Supplementum p. 57.

#### 2.4.4.2 MMPs and cancer cell invasion

MMPs are responsible for the physiological processes and causing tumor metastasis. The involvement of MMPs in metastasis are the migration of cancer cells through ECM components. There are a theory to explain how cancer can intercalate to this ECM barrier three steps. First, the cancer cells adhere to the specific cell-surface receptors of matrix macromolecules on the basement membrane or stromal and then the tumor cell secretes MMPs to degradation of ECM. Finally, the cancer cell migrates into the ECM degradation. These three steps occur in cyclic repetition for the continued invasion of tumor cells (Figure 9) (63). Currently, MMPs play the major role in

ECM breaking down and generating a pathway for cancer cells metastasis. Many studies have been reported the correlation between tumor progression and expression of a variety of MMPs in surrounding tissues. These MMPs such as MMP-1, -2, -3, -7, -9, -10, -11, -13, and -14 (61).

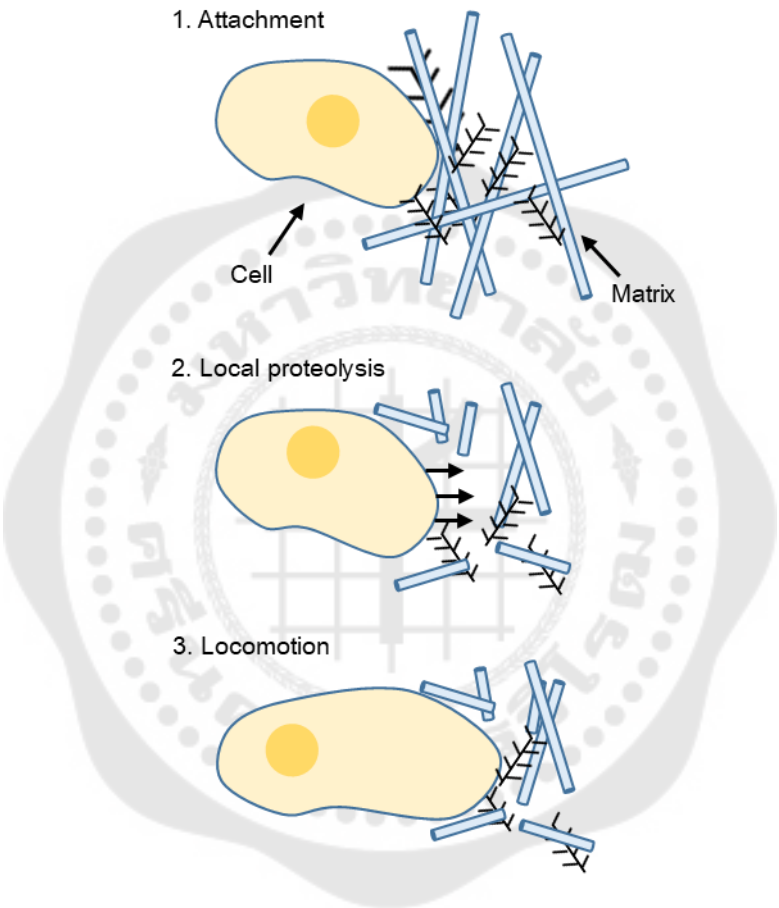



Figure 9 Three-step theory of cancer cell invasion

Source: Liotta LA, Thorgeirsson UP, Garbisa S. (1982). Role of collagenases in tumor cell invasion: Cancer Metastasis reviews p. 280.

#### 2.4.4.3 MMP-2 and MMP-9 in cancer metastasis

Many experimental studies have been demonstrated that MMPs are associated with cancer progression and metastasis. MMP-2 and MMP-9 play an

essential roles in cancer cell invasion and metastasis by degradation of a major component of the ECM such as type IV collagen (63).

MMP-2: MMP-2 (gelatinase A, 72 kDa) can be found in humans encoded by the *MMP2* gene. The activation of MMP-2 requires a complex with MT1-MMP and tissue inhibitor of metalloproteinase 2 (TIMP-2). An initiation step is the binding of TIMP-2 to active MT1-MMP on the cell membrane. Then the complex acts as a receptor to recruit pro-MMP2 produced by surrounding stromal cells (64, 65). Therefore, MMP-2 can activate by the assembly of proMMP-2, MT1-MMP, and TIMP-2 subsequently promoting cellular invasion (Figure 10) (66).

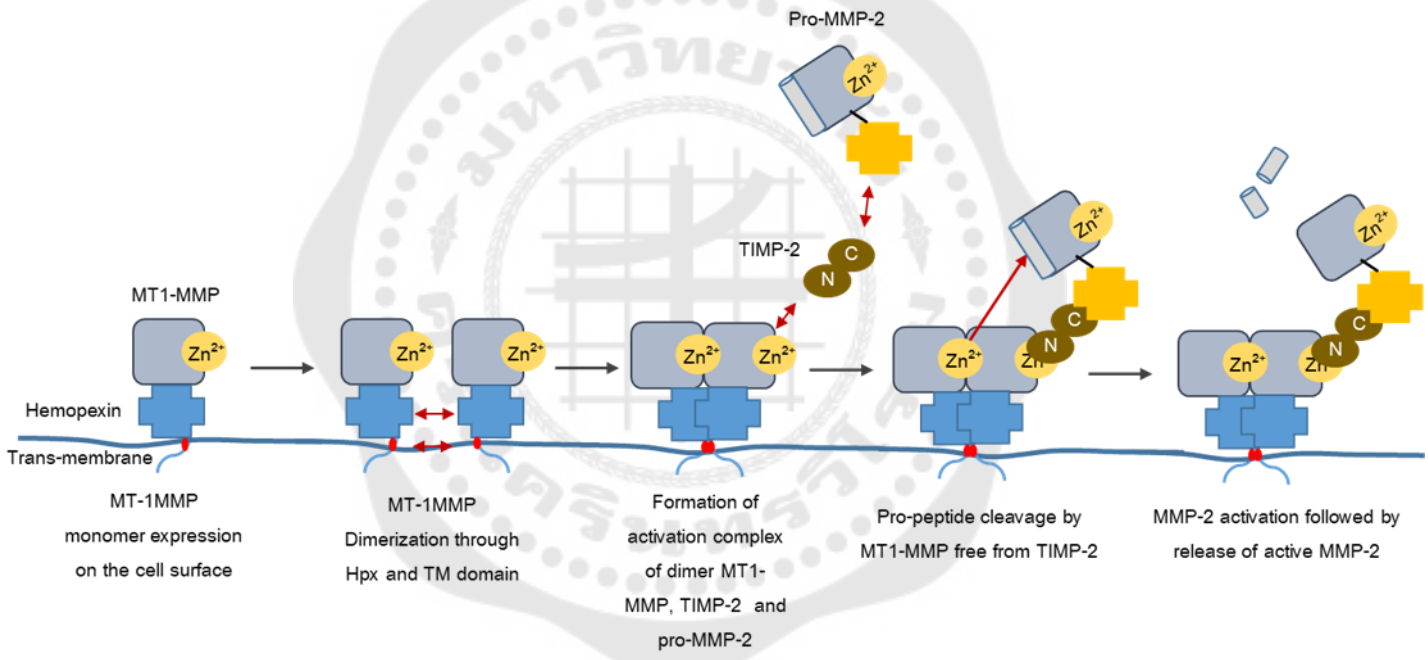



Figure 10 MMP-2 activation pathway

Source: Itoh Y. (2015). Membrane-type matrix metalloproteinases: Their functions and regulations: Society for Matrix Biology p. 210.

MMP-9: MMP-9 (gelatinase B, 92 kDa) is synthesized as inactive proenzyme and encoded by *MMP9* gene. The regulation of MMP-9 activity is also

controlled by TIMP-1. When peptide bonds between amino acids glutamine - methionine (Glu-Met) of pro-MMP-9 are broken down, MMP-9 will stimulate to an active form. Stimulation of pro-MMP-9 to inactive condition is a cascade mechanism. First, MMP-2 and MMP-13 are stimulated by MT1-MMP. After that, the MMP-2 or MMP-13 stimulated will coordinate with MMP-3 to break down pro-domain of MMP-9. When the MMP-9 enzyme is activated, TIMP-1 is released and allows the MMP-9 enzyme to work (Figure 11) (67).

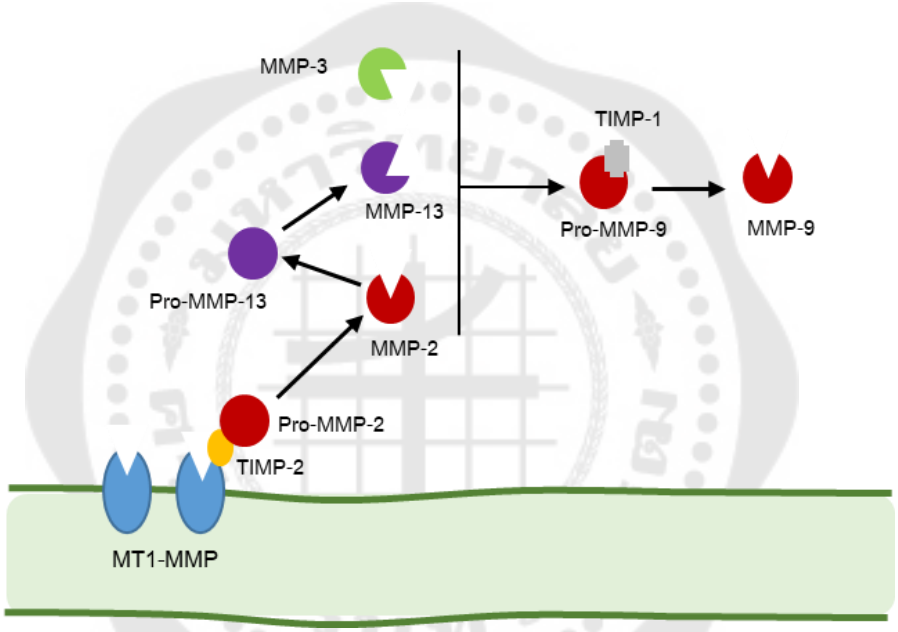



Figure 11 MMP-9 activation pathway

Source: Fridman R, Toth M, Chvyrkova I, Meroueh SO, Mobashery S (2003). Cell surface association of matrix metalloproteinase-9 (gelatinase B): Cancer metastasis reviews p. 157.

In normal tissue, MMP-9 combines with other MMPs and involve in embryonic development and wound healing whereas, overexpression of MMP-9 involves in metastasis such as invasion and migration in malignant cells. MMP-9 also promotes

angiogenesis by degrading the vascular BM and by releasing VEGF, which is a well-known angiogenic molecule.

**2.4.4.4. Tissue inhibitor of metalloproteinase (TIMPs)**

TIMPs is the major endogenous inhibitors of MMPs consist of 184-194 amino acids. TIMPs control MMPs activity by interaction with the MMPs. TIMP family are known to have numerous roles such as growth factor, cell migration, and apoptosis stimulation or inhibition (68). TIMPs have the same structure as common proteins. It consists of N-terminal and C-terminal terminals with 6 cysteine and 3 disulfide bonds. TIMPs are categorized into 4 types which are TIMP-1, TIMP-2, TIMP-3, and TIMP-4. These enzymes have special properties for binding to the MMPs such as TIMP-1 and MMP-9, TIMP-2 and MMP-2.

TIMP-1: TIMP-1 enzyme is glycoproteins and 26 kDa of molecular weight. TIMP-1 acts as MMPs inhibition such as MMP-9. TIMP-1 is expressed in fibroblasts, epithelial cells, chondrocytes, smooth muscle cells, osteoblasts and tumor cells. In addition, TIMP-1 has been shown to exert an antiangiogenic activity in tumor both *in vitro* and *in vivo* (69).

TIMP-2: TIMP-2 (22 kDa) is encoded by *TIMP-2* gene. TIMP-2 is an important role in regulating MMP-2 activity. TIMP2 may act as metastasis suppressor gene and has been reported to inhibit the invasion of tumor cell both *in vitro* and *in vivo* and. TIMP-2 and MMP-2 are formed between the C-terminal domain of the inhibitor and the C-terminal hemopexin of MMP-2 and maintain their proteolytic and inhibitory properties, respectively (70).

## 2.5 Regulation of MMPs production

In cancer, the production of MMPs can be controlled by several levels including signal transduction pathway. Gene expression can increase to response to multiple extracellular stimuli such as growth factor and cytokines. These signal stimulation from extracellular are transmitted to the nucleus to activate the transcription factor via the signal transduction pathway such as mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal

kinase (JNK), and p38 MAPK, play an important role in cell growth and metastasis. In addition, MMPs gene expression is also regulated by transcription factor such as nuclear-factor-**K**B (NF-**K**B) and the activator protein 1 (AP-1) that binding in promoter region on MMP gene (71).

### 2.5.1 MAPK signaling pathway

MAPKs family is a type of serine-threonine protein kinase. The MAPK pathway is involved in many cellular processes such as gene expression, cell proliferation, apoptosis, and cell motility (72). Moreover, MAPK plays a critical role in cell survival, and metastasis of cancer. The MAPK signaling cascade consists of a kinase that activating kinase proteins by phosphorylation sequentially including MAP3K, MAPKK and MAPK. There are three major subfamilies of MAPK include ERK1/2, JNK, and p38 MAPK. These signaling cascades lead to modulated gene expression involve in metastasis of cancer. The ERK pathway is one of the most important for cancer migration and invasion. Final step of this signaling pathway leading to the activation of transcription factors and regulating the expression of MMPs (Figure 12) (73).

After binding of extracellular stimuli such as cytokines with receptor resulting in small guanosine triphosphate (GTP)-binding proteins such as Ras which stimulates the action of MAPKKK. MAPKKK will phosphorylate to MAPKK. Then MAPKs are activated by MAPKK phosphorylation. When MAPK are activated, they translocate from the cytoplasm into the nucleus and then transcription factors are activated by phosphorylation of MAPK. The transcription factors will bind to the specific binding site in the region of gene promoter. For example, the AP-1 site is a binding position of Fos and Jun transcription factor proteins. The binding of transcription factors to the specific binding site on gene will be will be the beginning of the process will be the initiation of transcription of several genes (72).

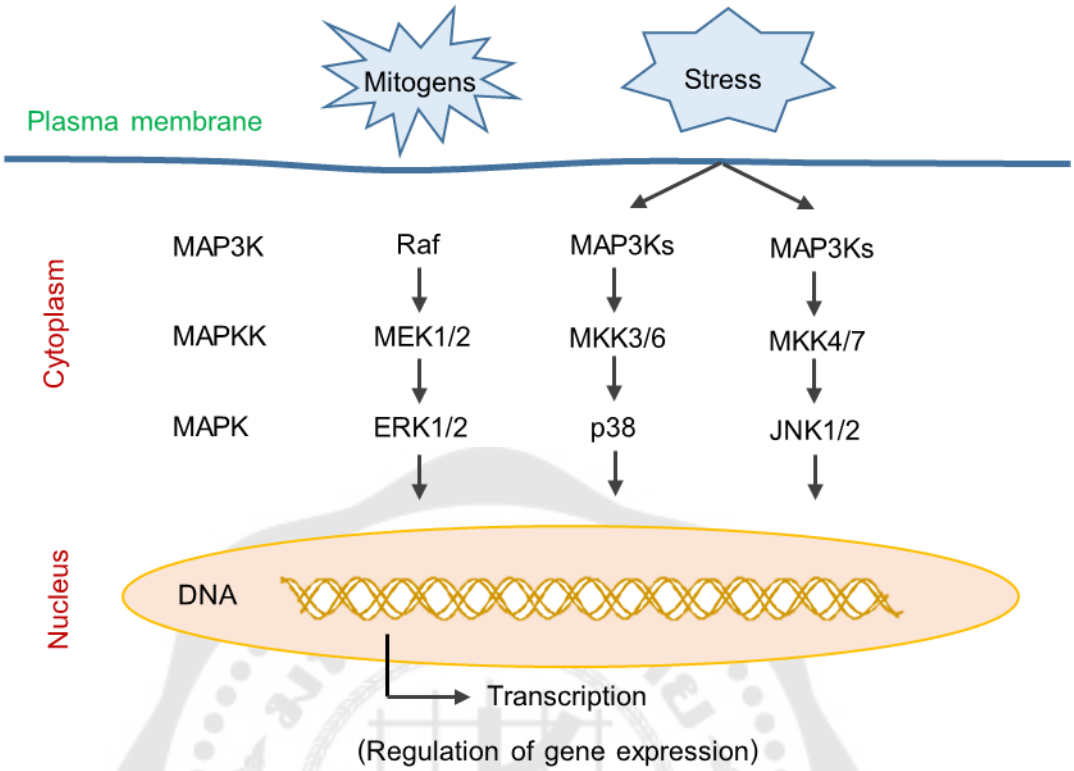



Figure 12 MAPK signal transduction pathway

Source: Plotnikov A, Zehorai E, Procaccia S, Seger R. (2011). The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation: Biochimica et Biophysica Acta p. 1621.

### 2.5.2 PI3K/Akt signaling pathways

The phosphatidyl-inositol-3-kinase (PI3K)/Akt signaling pathways is important pathways for cancer such as cell adhesion, migration, invasion, proliferation, and survival. The activation of this pathway results in disruption of normal regulation of cell growth and cell survival which lead to cancer metastatic. PI3Ks are a lipid kinase family which divided into three different classes. The mechanism of the PI3K/Akt pathway activation is triggered by the receptor protein tyrosine kinases (RPTK) on the cell surface such as fibroblast growth factor receptor (FGFR) family and epidermal growth factor receptor (EGFR) family, resulting in PI3K activation by autophosphorylation

of phosphatidylinositol-4, 5-biphosphate (PIP2) to phosphatidylinositol-3, 4, 5-tris phosphate (PIP3). PIP3 is the second messenger that play a role in several signal transduction pathway. PIP3 will recruit Akt and translocates to inner membrane. Akt is then stimulated by phosphorylation by phosphoinositide-dependent kinase-1 (PDK-1) (74, 75).

Akt or protein kinase B (PKB) is serine-threonine kinase, a downstream effector of PI3K. It is a key mediator of PI3K signaling. Akt is stimulated by PDK1 phosphorylation at threonine-308 and serine-473. Akt regulates several biological processes involved in cell growth, cell proliferation, cell survival, and angiogenesis. Abnormal regulation of PI3K/Akt signaling pathway resulting in cellular disturbance and leading to progression of cancer (Figure 16) (76).

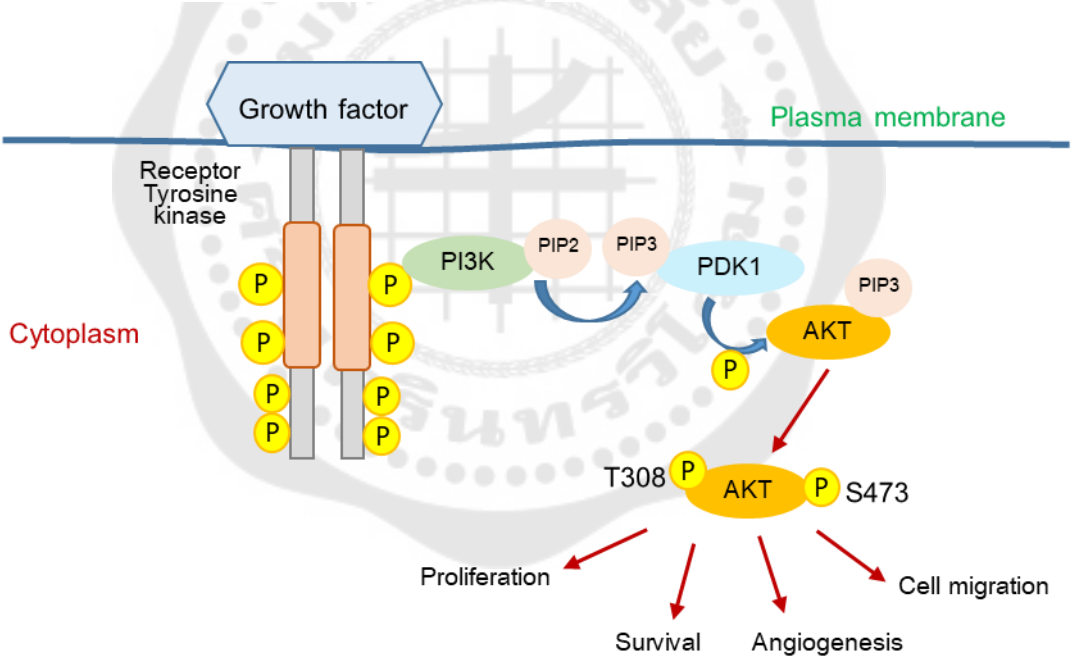



Figure 13 Schematic of the PI3K/Akt signaling pathway

Source: Echeverria CG, Sellers WG. (2008). Drug discovery approaches targeting the PI3K/Akt pathway in cancer: Oncogene p. 5512

### 2.5.3 NF-**K**B signaling pathway

NF-**K**B is a transcription factor that has an essential role in many steps of cancer progression by controlling metastasis. NF-**K**B is associated with an MMPs upregulation. Degradation of the ECM is a major process for cancer evasion. The NF-**K**B/Rel family has been identified five members include NF-**K**B1 (p50/p150), NF-**K**B2 (p52/p100), p65 (RelA), RelB, and c-Rel (77). The active forms of this transcription factor are present in the heterodimer of p65 combined with p50 or p52. When interleukin-1 (IL-1) or tumor necrosis factor alpha (TNF-$\alpha$) bind to the receptor resulting in the activation of intracellular protein via the cytoplasmic domain of the receptor. Then this protein complex activates NF-**K**B inducing kinase (NIK) and the MAPK kinase kinase (MAPKKK) protein stimulates the inhibitor of **K**B kinase (IKK-$\alpha$, IKK-$\beta$, and IKK-$\gamma$). IKKs act to phosphorylation to an inhibitor of **K**B (I**K**B), which are inhibition of NF-**K**B to enter to the nucleus (78). After I**K**B phosphorylation results in proteosome-mediated proteolytic degradation of I**K**B. Then the p50/p65 translocate to the nucleus and bind to the NF-**K**B binding site in promoter regions of MMPs (Figure 14) (79).

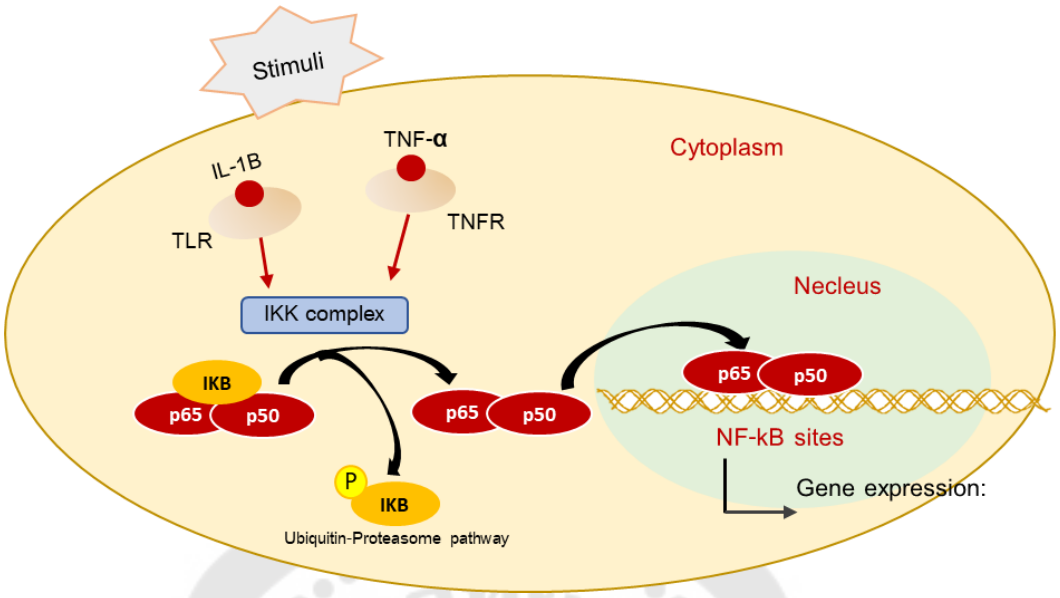



Figure 14 NF-ΚB Signal transduction pathway

Source: Soleimani A, Rahmani F, Ferns GA, Ryzhikov M, Avan A, Hassanian SM (2020). Role of the NF-ΚB signaling pathway in the pathogenesis of colorectal cancer: Gene p. 6.

## 2.6 *Senna alata*

### 2.6.1 Background

*Senna alata* (Syn. *Cassia alata*) is the medical herbal plant belongs to family Fabaceae. It has various common names known as ringworm bush, candle bush, and acapulco (80), which has been known in Thai language common name as Chumhetthet. The fresh leaf and leaf extract of the plant have been reported to possess medicinal properties against skin diseases (81).The plant is now widely distributed throughout the world and can be found in Thailand, Indonesia, Philippines, India, Bangladesh, Africa and tropical America. It is large shrub with thick downy branches and erects. Flowers are yellow. The pod is rather straight, dark brown or nearly black and contains triangular seed (Figure 15) (82).

Figure 15 *S. alata*, leaves, fruit, and flower,

Source: Fatmawati S, Yuliana, Purnomo AS, Abu Bakar MF. (2020). Chemical constituents, usage and pharmacological activity of *Cassia alata*: Heliyon p. 2.

All parts of the species are used in traditional medicine especially antimicrobial activity (83). The leaf are used for skin infections treatment and root is used against womb disorder (82). The leaf of SA has been reported a wide range of biological activity such as anti-inflammatory, antifungal, antiparasitic, antimicrobial, and anti-cancer. This study focuses on *Senna alata* ethanolic leaf extract. The activity of the plants is associated with the presence of a various chemical components. Many studies have been reported that quercetin, flavonoids (kaempferol-3-O-β-D-glucopyranoside), flavone (3, 5, 7, 40-tetrahydroxy flavone and 2,5,7,40-tetrahydroxy isoflavones, chrysoeriol, and luteolin), stearic acid, palmitic acid, hexadecanoic acid, and octadecanoic acid methyl ester), steroid, tannin, and anthraquinone (aloe-emodin, emodin, rhein, chrysophanic acid, and chrysophanol) was found in SA ethanolic extract (82). In the leaf, SA is one of the most important species of the genus *Senna* which contains a maximum content of anthraquinones glycosides. Anthraquinones are a

functional group of aromatic. It has a yellow crystalline or green-gray solid powder. The amount of anthraquinone is depended on the period of harvesting, especially in the summer and winter seasons have been found the highest content of anthraquinones glycosides (84).

**2.6.3 Medicinal research**

Many parts of SA have been widely studied. Previous studies reported the applications for medicinal use of SA extract include antibacterial, antifungal, antioxidant activities, antimicrobial, and analgesic, and anticancer.

**2.6.2.1 Antibacterial activity**

The crude water extract of SA leaves showed higher antimicrobial activity on *Stapylococcus aureus in vitro* (85). The methanolic extracts of SA root, stem, flowers, and leaves showed a broad spectrum of antibacterial activity (86). In addition, the ethanolic leaf extract of SA was also investigated for its antibactreial activities (87).

**2.6.2.2 Antifungal activity**

Many studies have been carried out on the potency of antifungal activity of the plant. The ethanolic leaf extract of SA was reported for its activity against dermatophytic fungi and nondermatophytic fungi by inhibiting *Trichophyton mentagrophytes*, *Microsporium canis, Penicillium notatum, Aspergillus niger, and Candida albicans* (88). The methanol extract of leaf showed the activities of SA against *Aspergillus niger,* Rhizopus, and Mucor are higher than petroleum ether and ethanol extracts (89).

**2.6.2.3 Antioxidant activity**

The ethyl acetate extract of SA showed a strong DPPH radical scavenging activity (90). The presence of phytochemical component of vitamin A, vitamin-C, phenol, anthraquinone, and flavonoid in the methanol extract of SA leaf showed strong DPPH radical scavenging activity (91). In addition, SA ethanolic extract showed the antioxidant activity against superoxide anion and hydrogen peroxide (92).

**2.6.2.4 Anti-skin Infections activity**

The leaf of SA was well known for the treatment skin infections diseases in humans. The ethanol leaf extracts were reported an effective in curing

bovine dermatophilosis (93). It has been reported the bioactive compounds of SA on skin infection inhibition are linked to tannins, flavonoids, phenols, anthrones, anthranols, and anthracene derivatives. (80).

2.6.2.5 Anti-inflammatory activity

The leaf of SA showed anti-inflammatory activity in TNF-α production (92). Rhein is one components of SA extract showed the anti-inflammatory activity by inhibiting *tert*-butyl hydroperoxide induced inflammatory in HaCaT skin cell line (94).

2.6.2.6 Anti-cancer activity

Various studies have been reported the cytotoxicity activity of SA leaf extract on cancer such as the methanol extract of SA showed cytotoxicity effect in lung cancer cells, A549 and this effect may be from kaempferol, natural flavonoid was identified (95). The hexane extract also showed the cytotoxic effect on MCF-7, Col2, and T-24 cancer cells (96). Furthermore, it has been reported that anthraquinone derivative as aloe-emodin has the effect on oral cancer and emodin inhibited cell invasion by inhibition of MMP-9 expression via suppressing AP-1 and NF-KB signaling pathways in human cancer cells (97).

However, the effects of *Senna alata* ethanolic leaf extracts on metastasis in human chondrosarcoma have not yet been reported.

# CHAPTER 3
# MATERIALS AND METHODS

## 3.1 Chemicals and Instruments

| | |
|---|---|
| Acrylamide | HiMedia |
| Ammonium persulfate | HiMedia |
| Bovine serum albumin | HiMedia |
| Bradford reagent | BioRad |
| Bromophenol blue | Sigma |
| Calcium chloride ($CaCl_2$) | Sigma |
| Coomassie Brilliant Blue R-250 | Bio-Rad |
| Dimethylsulfoxide (DMSO) | Sigma |
| Dulbecco's modified Eagle's medium (DMEM) | HiMedia |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma |
| Fetal bovine serum (FBS) | HiMedia |
| Glycerol | Sigma |
| Glycine | Sigma |
| Immobilon Western Chemiluminescent HRP substrate | Merck |
| 2-Mercaptoethanol | Sigma |
| Matrigel | BD Biosciences |
| Methanol | Merck |
| Thiazolyl blue tetrazolium bromide (MTT) | Sigma |
| Penicillin/streptomycin | HiMedia |
| Phenylmethylsulfonyl fluoride (PMSF) | Sigma |
| Sodium chloride (NaCl) | Sigma |
| Sodium dodecyl sulfate (SDS) | Vivantis |
| TEMED (N,N,N',N'-Tetramethylethylene) | Merck |
| Tris (Tris Hydroxymethyl aminomethane) | Vivantis |
| Triton-X 100 | Phamacia |
| Trypan blue | Sigma |

| | |
|---|---|
| Trypsin | HiMedia |
| Tween-*20* | Phamacia |
| Centrifuge-HSC 10K | Vant |
| Centrifuge-MIKRO 20 | Hettich |
| Centrifuge ICE centra-4R | Hettich |
| $CO_2$ incubator (water-jacketed) | NuAire |
| Inverted microscope | Olympus |
| Lamina flow-Nu-440 | NuAire |
| Western blot apparatus | BioRad |
| Shaking-Mini Rocker MR-I | Biosan |
| Microplate reader-Multiskan EX | Thermo electron |

### 3.2 Preparation of *Senna alata* leaf extract

The leaf powder of SA was purchased from herbal phamacy, Bangkok, Thailand. The extraction was performed by using maceration method. Briefly, leaves powder (100 g) were homogenized in 500 mL of ethanol. After 24 hours incubation, the crude extract was swirled and filtered using Whatman No.1 filter paper. Then, the SA extract was dried in vacuum at 40°C using a rotary evaporator. The extracted-leave was dried and stored at 4°C until used in the experiment. For isolation part, the crude extract was dissolved in methylene chloride or dichloromethane (DCM) and further separated by column chromatography over silica gel. The column was eluted with DCM: methanol using the gradient technique (100:0, 100:1, 100:2, 100:5, and 100:10, volume/volume). Then, the fractions which contained the similar components were combined using thin-layer chromatography (TLC) technique. All fractions were dried using a rotary evaporation. The anti-migration activity of all fractions were tested by *in vitro* wound-healing assay. Then, $^{1}$H NMR spectra of active part was investigated by Nuclear magnetic resonance (NMR) spectrometer.

### 3.3 Cell culture

SW1353, the human chondrosarcoma cell line (ATCC, USA) was maintained in DMEM contained 10% of FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% $CO_2$. The medium of cells were changed every 2-3 days after confluence about 90% by 0.25% trypsin-EDTA.

### 3.4 Cell viability assay

The cytotoxicity of SA extract was determined using MTT assay. Briefly, MTT is a standard calorimetric used for measurement of proliferation and cytotoxicity of cells by the color changing. The yellow MTT was reduced by metabolically living cells, mitochondrial reductase enzyme that conversion into insoluble purple formazan crystals.

SW1353 cells were seeded into a 96-well microtiter plate at $5x10^3$ cells/well and incubated for 24 hours. Then the cells was treated with SA at serial concentrations ranging from 7.8 to 500 µg/mL (7.81, 15.62, 31.25, 62.50, 125, 250, and 500 µg/mL) and incubated for 24 hours at 37°C with 5% $CO_2$. After incubation, 100 µL of 0.5 mg/ml MTT solution was added to each well of the plate and incubated further 2 hours at 37°C incubator. Then MTT was removed and DMSO was added to each well with shaking for 10 minutes to dissolve formazan crystals. The absorbance was quantified using microplate spectrophotometer at 570 nm and cell viability percentage (%) was analyzed using Gen5™ software compared with the control group.

### 3.5 *In vitro* wound-healing assay

SW1353 cells were seeded into 12-well plates at $9 \times 10^4$ cells/well. After incubation overnight, cells was reached to 80% confluence as a monolayer, the cells were starved with serum free medium (SFM) overnight. Then, the cells were linearly scratched using a sterile 200 µL micropipette tip. Cells were washed with PBS to remove cell debris. SA at 50, 100 and 150 µg/mL were treated for 24 hours. After treatment, the cells was monitored and photographed using the microscope. ImageJ software was used for image analysis.

### 3.6 Transwell migration and invasion assay

Transwell Boyden chamber was used for cell migration and invasion assay. For invasion assay part, transwell chamber membrane was coated with 0.3 mg of Matrigel overnight at 4°C. Cells were seeded in insert chamber at $1.5 \times 10^4$ cells/well containing complete medium for the treated conditions and SFM as the positive control. After 2 hours incubation, lower compartment was added with 10% FBS medium as chemoattractant. Then, cells were treated with a presence of SA at 50, 100, and 150 µg/mL for 24 hours. After incubation, cells were fixed with 100% methanol and stained with 0.1% crystal violet. Then, the cells were washed with PBS until the color were cleared. In upper compartment, the cells were gently scraped off with a cotton swab. The migratory and invasive cells were observed under a microscope. The percentage (%) of migratory and invasive cells were determine by ImageJ.

### 3.7 Cell adhesion assay

96-well plate was pre-coated with 0.3 mg of Matrigel at 4°C overnight before testing. Then, plate was incubated at 37°C for 1 hour and then Matrigel was removed and then washed with PBS for cell adhesion assay. In 6-well plates, SW1353 cells were seeded at $2 \times 10^5$ cells/well and then cells were pretreated at 50, 100, and 150 µg/mL of SA. After 24 hours incubation, the treated-cells were collected and seeded in a 96-well pre-coated plate and incubated further 1 and 4 hours. The non-adherent cells were removed by aspiration and washed with PBS. Then MTT assay was used to determine the adherent cells. The cell adhesion percentage (%) was calculated compared to the control group.

### 3.8 Western blot analysis

#### 3.8.1 Sample preparation

SW1353 cells were seeded in 35-mm dish at $3 \times 10^5$ cells/well. The cells were treated with SA at various concentrations for 24 hours. After incubation, cells were extracted with RIPA buffer supplement with protease inhibitor cocktail. Cell lysate was

centrifuged at 13,000 rpm at 4°C for 30 minutes. The supernatants were collected and transferred to a new tube. Then, protein concentration was determined before next step.

**3.8.2 Protein determination**

The protein concentration was determined using Bradford's assay. In addition, BSA was used as a standard protein. The supernatants were diluted to 1:10 and BSA were diluted with distilled water to 0, 0.2, 0.4, 0.6, 0.8, 1 mg/mL. Then, 10 µL of sample and each standard protein concentrations were mixed with 200 µL of Bradford's dye. The concentration of protein was determined at 595 nm using a spectrophotometer.

**3.8.3 Preparation of SDS-PAGE**

The separating gel and stacking gel were prepared for SDS-PAGE. The sample were loaded onto upper part of stacking gel. Proteins were concentrated in to a narrow band. Then, the band was entered to the lower part (separating gel) and proteins were separated by molecular size.

**3.8.4 Running condition of protein**

Sample was mixed with 5X sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mM2-mercaptoethanol, 0.1% bromophenol blue). Then, the protein sample was heated at 100°C for 5 minutes. Total proteins were separated by 10% gel and transferred to PVDF membranes. Membranes were then blocked with 5% non-fat dry milk in TBST (10 mM Tris, pH 7.5, 150 mM NaCl and 0.1% Tween-20) for 1 hour.

**3.8.5 Protein detection**

After Blocking, membranes were probed with the primary antibody (MMP-2, MMP-9, TIMP-1, TIMP-2, ERK1/2, p-ERK1/2, p38, p-p38, AKT, p-Akt (Ser473), NF-κB, GAPDH, and β-actin) overnight at 4°C. Membranes were then washed and probed with secondary antibodies conjugated with HRP for 1 hour. Protein bands were detected by chemiluminescence using ECL. The quantitative proteins were analyzed as relative to GAPDH or β-actin housekeeping genes.

### 3.9 Statistical analysis

The data were expressed as the means ± standard deviation (SD) using one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered as statistically significant.

# CHAPTER 4
# RESULTS

## 4.1 Identification of the phytochemical active fraction in SA by $^{1}$H NMR spectroscopy

The phytochemical constituents of SA were identified by NMR spectroscopy. The results investigated that the active fraction of SA mostly included unsaturated fatty acid and some of flavonoids and chlorophyll (Figure 16A and 16B). The results of $^{1}$H-NMR showed methylene and methyl protons at δ 0.80-1.40, allylic protons at δ 1.97, methine proton at δ 5.10, and olefinic protons in the region of δ 5.31, corresponding to unsaturated fatty acid proton signals.

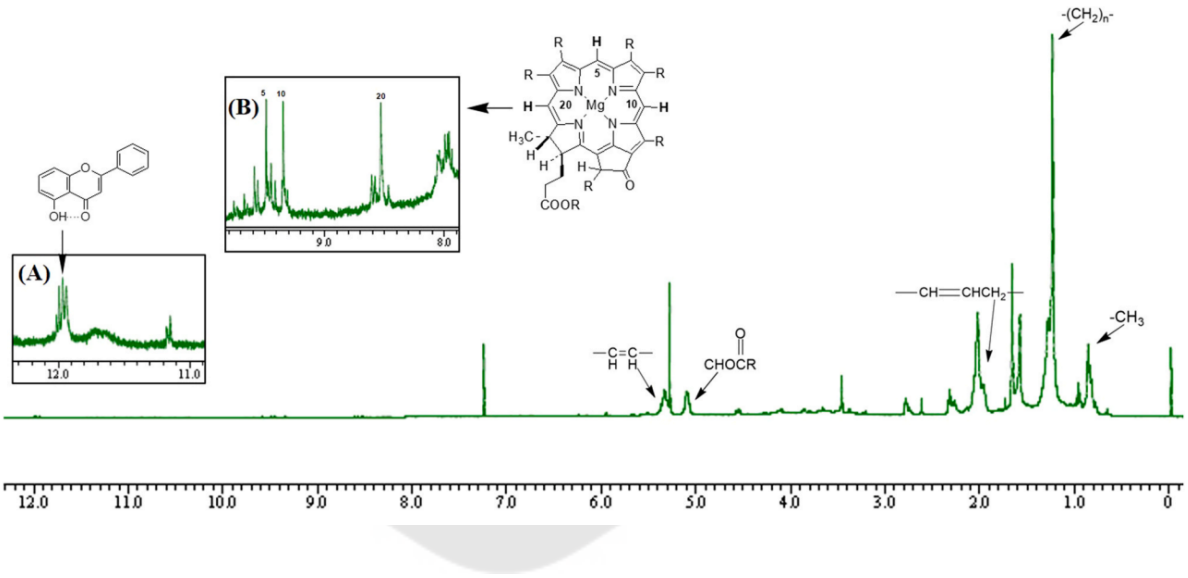



Figure 16 $^{1}$H NMR spectrum of SA

(A) Some chlorophylls (B) Flavonoids.

## 4.2 Effect of SA on chondrosarcoma cell viability

MTT assay was used to determine the cytotoxicity effect of SA on human chondrosarcoma cells. The SW1353 cell viability and cytotoxicity were investigated at 24 hours as shown in Figure 17. The results showed that treatment with the leaf extract of SA at serial dilution concentrations showed a potent inhibit the proliferation of SW1353 cells in concentration-dependent manner. The SW1353 cell viability was

reduced to 99.42 ± 0.15%, 98.70 ± 0.76%, 100.02 ± 2.22%, 92.44 ± 2.95%, 88.06 ± 4.90%, 67.09 ± 2.59%, and 28.56 ± 1.08%, respectively, compared to 100% viability in non-treated control group ($p < 0.05$). (n= 3) The $IC_{20}$ value of subtoxic concentration (> 80% cell viability) was 166.684 ± 3.18 µg/mL and the $IC_{50}$ at 346.24.28 ± 2.89 µg/mL. Thus, SA at nontoxic concentrations) at 10, 50 and 100 µg/mL were chosen for further experiments in this project.

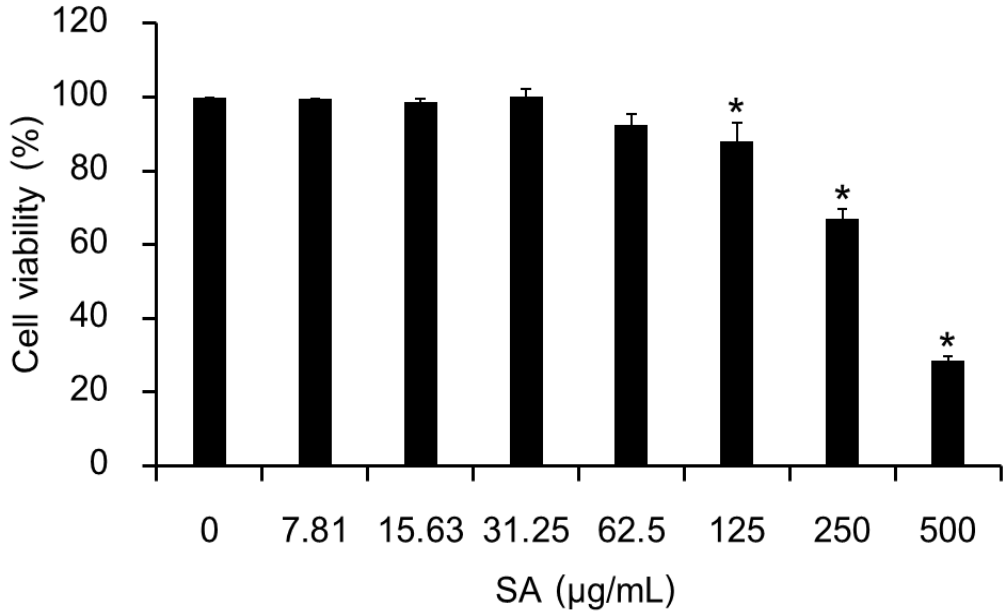


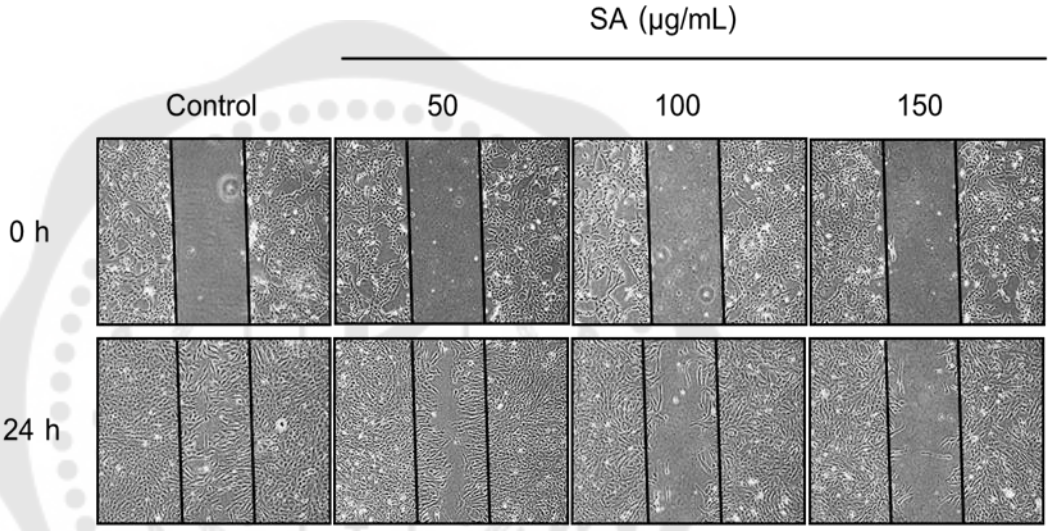

Figure 17 Relative cell viability of SW1353 cells after treatment with SA.
The cytotoxicity effect of SA at 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 µg/mL on SW1353 cell viability. Data represented the mean values ± SD. *$p < 0.05$ compared to the control group.

**4.3 Effect of SA on chondrosarcoma cell migration by In vitro wound-healing assay**

Chondrosarcoma SW1353 cells also exhibit migratory properties that essential for cancer metastasis. Wound healing or scratch assay was used to investigate the inhibitory effect of SA on the migratory activity of SW1353 cells. After 24 hours SA treated-cells at 50, 100, and 150 µg/mL, the results showed that the migratory cells were

slightly inhibited in presence of SA at 50 µg/mL while 100 and 150 µg/mL of SA highly reduced wound closure. (Figure 18A). As shown in Figure 18B, treatment with 50, 100, and 150 µg/mL SA showed percentage of cell migration at 81.39 ± 1.29%, 45.93 ± 1.68% and 37.56 ± 1.20%, respectively, compared to the non-treated control group ($p < 0.05$) (n= 3).

(A)




(B)

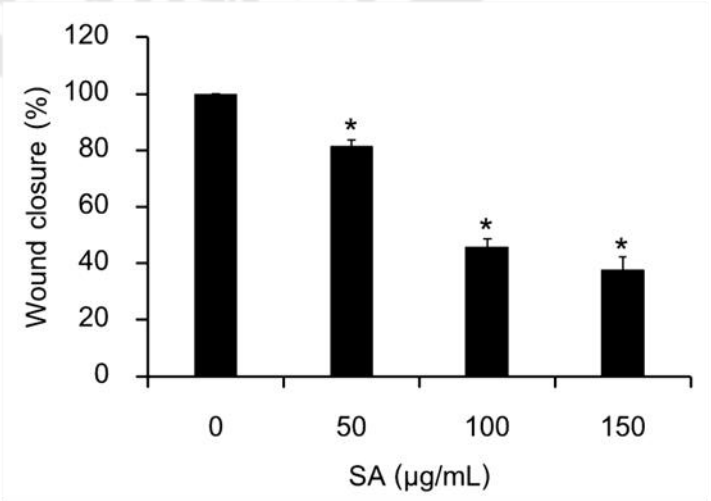


Figure 18 The effect of SA on chondrosarcoma cell migration was determined by wound healing assay.

(A) Cells were treated with SA at 50, 100, and 150 μg/mL at 24 hours. (B) The percentage of wound closure was measured by ImageJ analysis. Data represented the mean values ± SD (n = 3). *$p < 0.05$ compared to the control group.

### 4.4 Effect of SA on chondrosarcoma cell migration by *In vitro* Transwell assay

Moreover, *In vitro* transwell migration assay also showed the migratory inhibition of SA in a dose-dependent manner (Figure 19A). The results in Figure 19B showed that cell migration of SW1353 cell were significantly decreased when treated with SA at same concentrations. The percentage of cell migration inhibition was 39.69±1.99, 57.79±3.97 and 68.26±2.67%, respectively, compared to the control group. Thus, our results demonstrated that SA showed a highly repressive effect on the migration of SW1353 cells ($p < 0.05$) (n= 3).

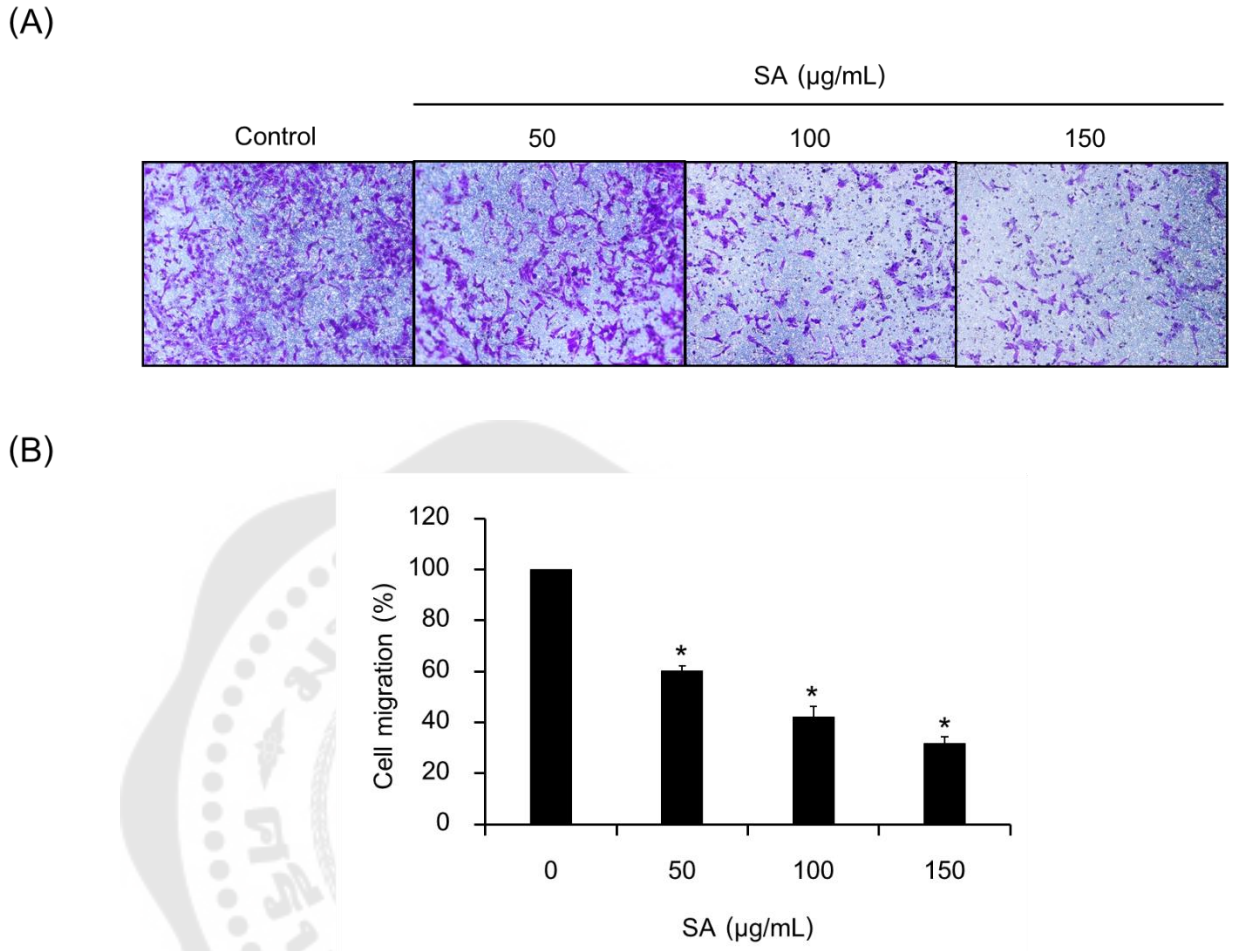



Figure 19 The effect of SA on cell migration was investigated using *In vitro* Transwell assay.

(A) Cells were exposed with SA at 50, 100, and 150 μg/mL at 24 hours. (B)The percentage of cell migration was measured by ImageJ analysis. Data represented the mean values ± SD (n = 3). *$p < 0.05$ compared to the control group.

**4.5 Effect of SA on chondrosarcoma cell invasion by *In vitro* Transwell assay**

The effect of SA on cell invasion of human chondrosarcoma cells was studied using transwell invasion assay. Treated cells with or without SA were added to Matrigel coated-chamber. In Figure 20A, the results showed that cell invasion were decreased by 50, 100, and 150 μg/mL of SA in a dose-dependent manner ($p < 0.05$). The percentage

of cell invasion inhibition was 17.34 ± 3.26%, 22.30 ± 1.18%, and 35.57 ± 0.62%, respectively, compared to the control group (Figure 20B). Thus, the cells treated with SA showed significantly change in the invasive activity in chondrosarcoma SW1353 cells ($p < 0.05$) (n= 3).

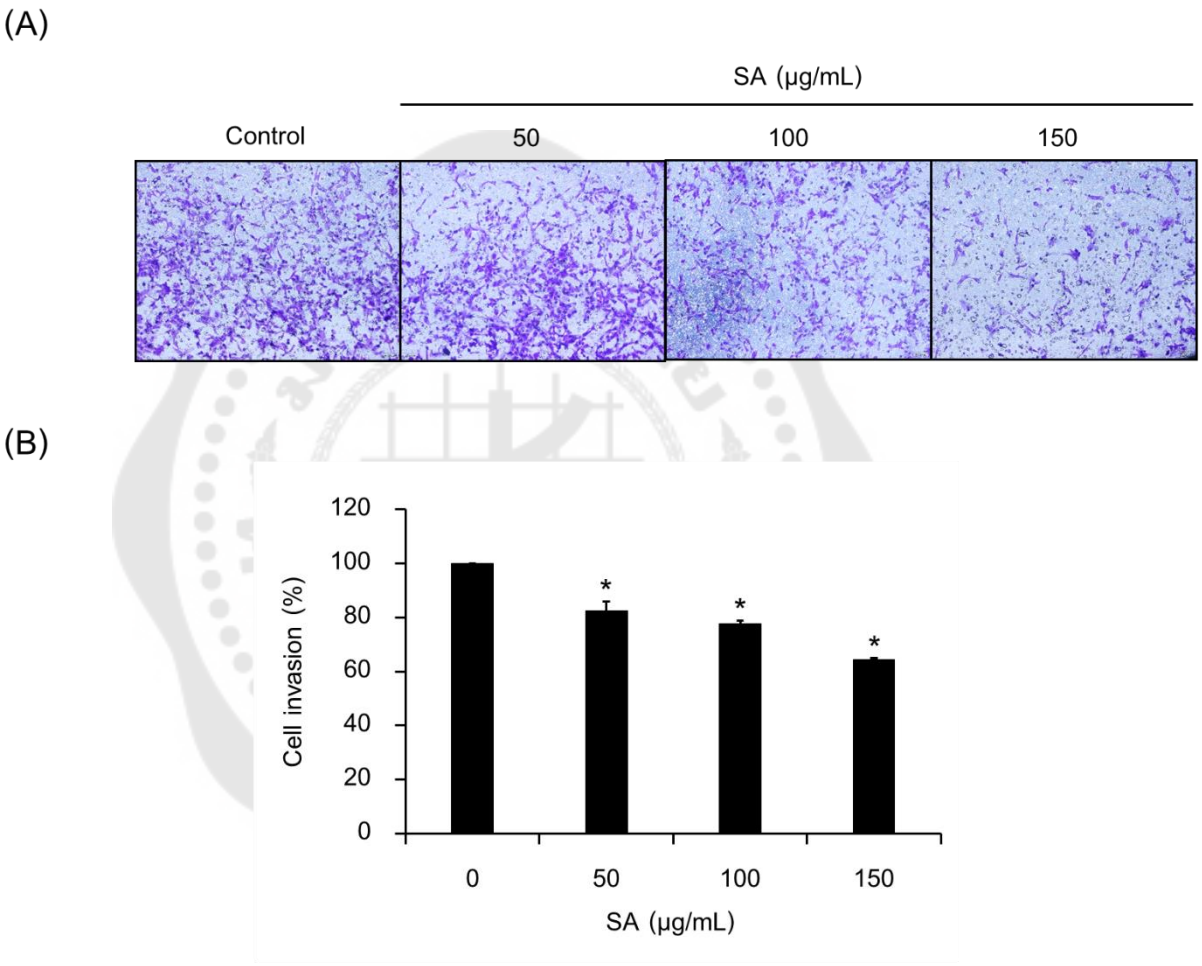
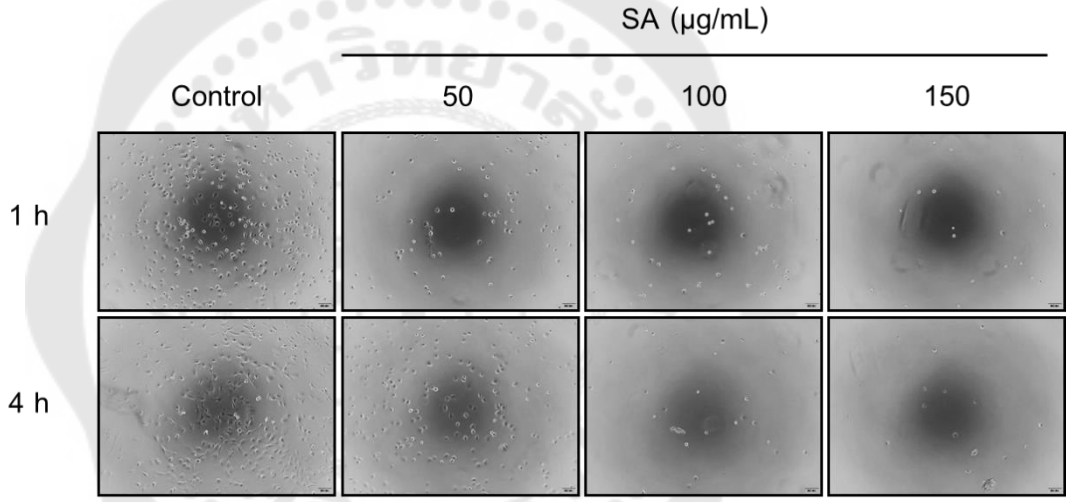



Figure 20 The effect of SA on cell invasion was determined by *In vitro* Transwell assay. (A) Cells were treated with SA at 50, 100, and 150 μg/mL at 24 hours. (B)The percentage of cell invasion was measured by ImageJ analysis. Data represented the mean values ± SD. *$p < 0.05$ compared to the control group.

**4.6 Effect of SA on chondrosarcoma cell adhesion**

The effect of SA on the adhesion of human chondrosarcoma cells was determined by Matrigel-coated plate. The results showed that when increasing the concentration of SA, unadhered cells were significantly increased both 1 and 4 hours compared with the non-treated group (Figure 21A). In Figure 21B, the percentage of cell adhesion inhibition at 1 hour was 17.34 ± 3.26%, 22.30 ± 1.18%, and 35.57 ± 0.62%, as well as, 46.13 ± 3.08%, 69.86 ± 1.60% and 76.04 ± 3.33% for 4 h, respectively ($p < 0.05$). (n= 3) Thus, these results suggested that SA could reduce adhesive ability.

(A)




(B)

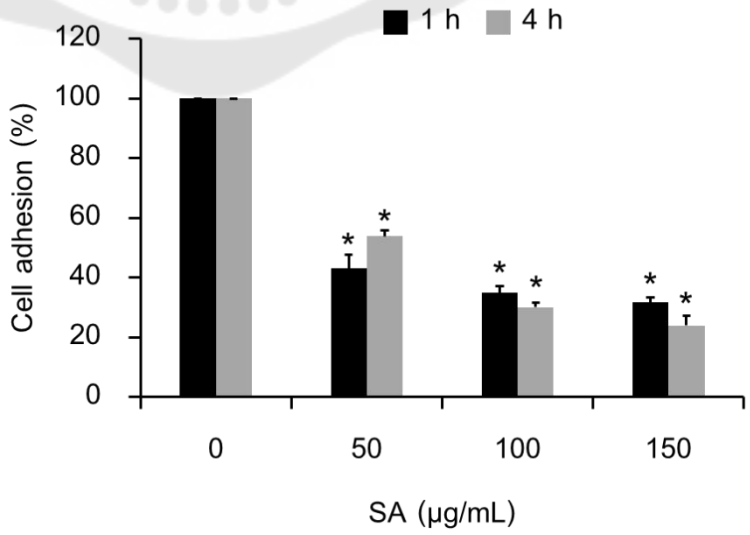


Figure 21 The effect of SA on cell adhesion was determined by cell adhesion assay. (A) Cells were exposed with SA at 50, 100, and 150 μg/mL at 1 hour and 4 hours. (B) The percentage of cell adhesion was measured by ImageJ analysis. Data represented the mean values ± SD. *$p < 0.05$ compared to the non-treated group.

**4.7 Effects of SA on MMP-9, MMP-2 and TIMP-1, TIMP-2 protein expression in chondrosarcoma cells**

MMPs such as MMP-2 and MMP-9 are well known that involved in cancer invasion and metastasis by degradation of BM. Western blotting analysis was used to determine the expression of proteins. As presented in Figure 22, SA significantly down-regulated the expression of MMP-9 and MMP-2 in a concentration-dependent manner compared with non-treated control cells. Moreover, MMP-9 and MMP-2 proteins expression levels are strongly regulated by TIMP-1 and TIMP-2 endogenous inhibitor, respectively. The results in Figure 23 showed that SA significantly upregulated the expression of TIMP-1 and TIMP-2 compared to the control cells ($p < 0.05$). According to our results, these studies suggest that down-regulation of MMP-9 and MMP-2 and up-regulation of TIMP-1 and TIMP-2 may inhibit the progression of the metastasis of SW1353 cells.

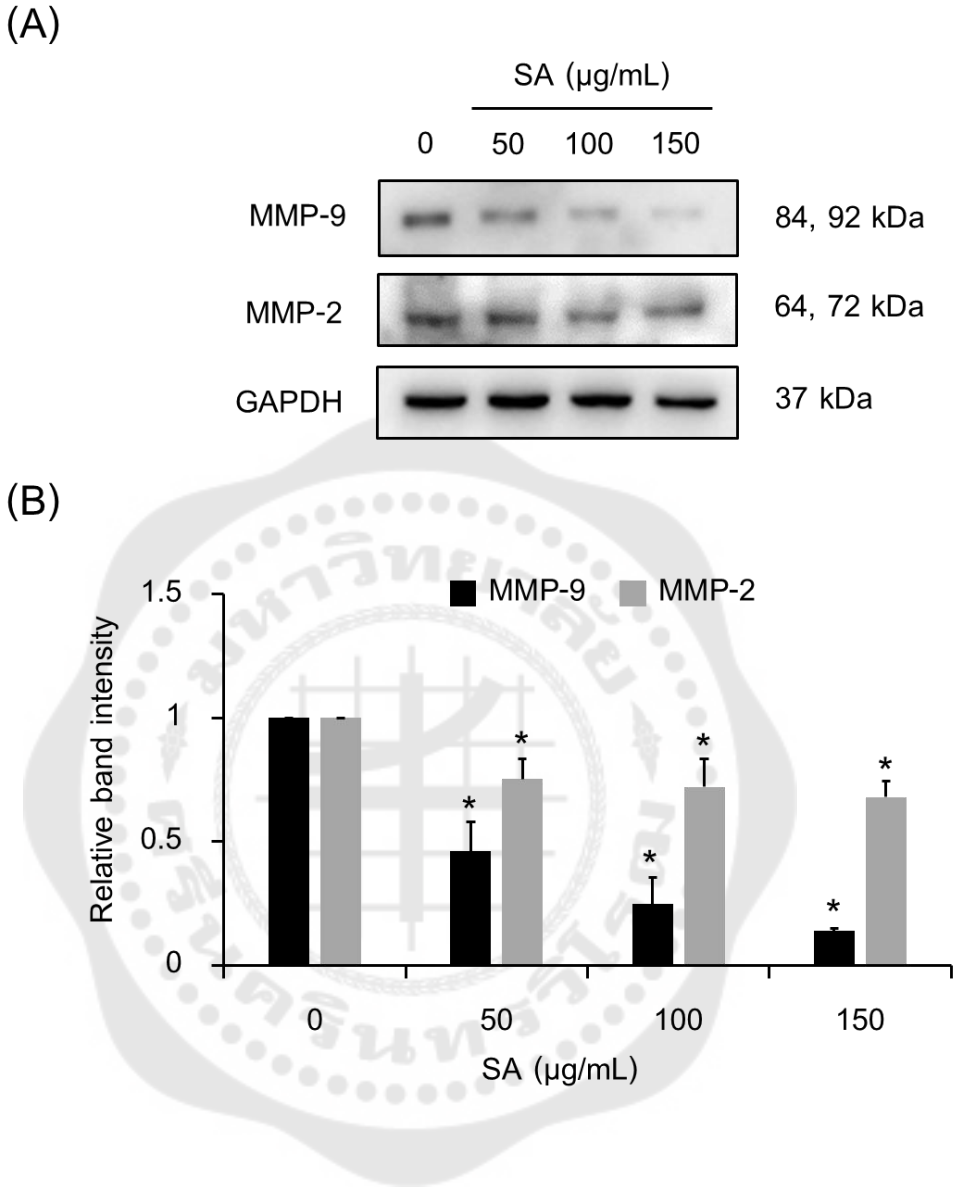



Figure 22 The effect of SA on MMP-9 and MMP-2 protein expression in SW1353 cells. (A) Cells were exposed with SA for 24 hours and proteins were dertermined by Western blotting. (B) The relative protein density was analysed by ImageJ. Data expressed as mean values ± SD. *$p < 0.05$ compared to the non-treated group.

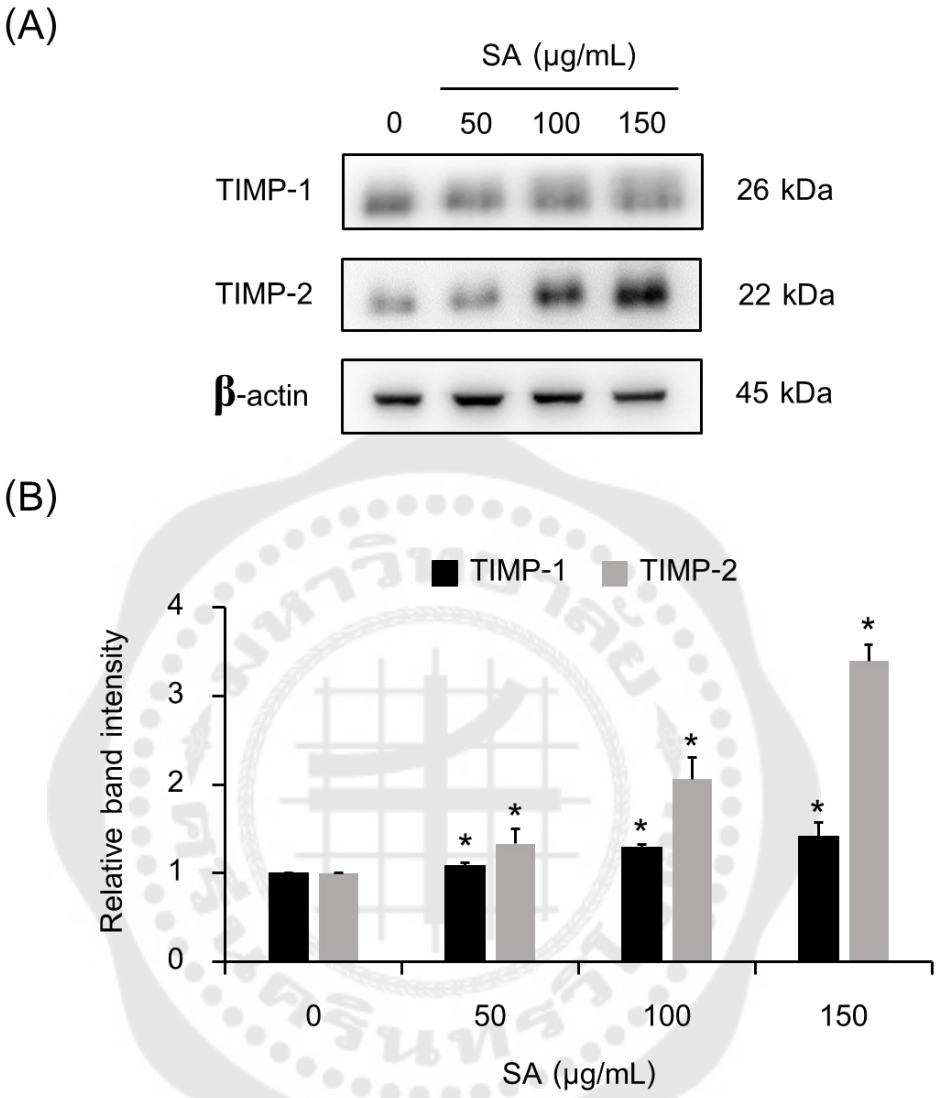



Figure 23 The effect of SA on TIMP-1 and TIMP-2 protein expression in SW1353 cells. (A) Cells were exposed with SA for 24 hours. (B) The relative protein density was analysed by ImageJ. Data expressed as mean values ± SD. *$p < 0.05$ compared to the non-treated group.

**4.8 Effects of SA on MAPK, PI3K/Akt signaling pathway and NF-κB transcription factor in chondrosarcoma cells**

MAPK and PI3K/Akt signaling has been reported to be involved in cancer migration, invasion, and metastasis. In various cancer cells, MAPK and PI3K/Akt

proteins can induce metastasis of cancer by regulation of MMPs protein via transcription factor such as NF-κB. After treatment with SA for 24 hours. In Figure 24, SA suppressed the protein phosphorylation of ERK1/2 and p38 protein expression. Accordingly, our results suggested that inhibition of MAPK members involved in the down-regulation of MMP-2 and MMP-9 proteins level.

To investigate the effects of SA on metastatic upstream PI3K/Akt signaling levels. Western blotting results showed that incubation with SA significantly reduced the protein levels of PI3K, Akt, and p-Akt (Ser47) (Figure 25). In addition, NF-κB involve in the expression of MMPs. The results in Figure 26 demonstrated that SA also inhibited NF-κB expression in a dose-dependent manner. Therefore, these studies revealed that SA could inhibit cell migration and cell invasion in SW1353 cells by suppressing PI3K/AKT as well as reducing the NF-κB protein expression.

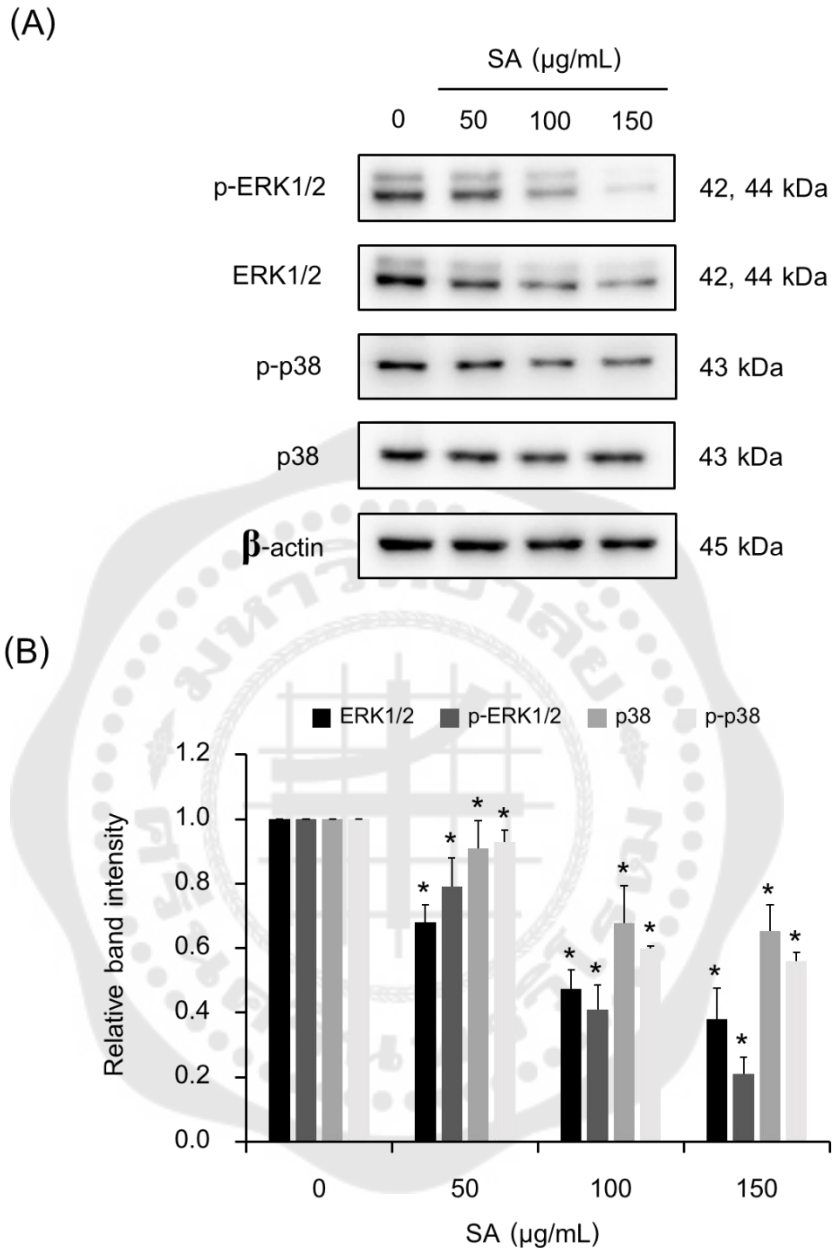



Figure 24 Effects of SA on MAPK signaling pathway in chondrosarcoma cells. (A) Cells were exposed with SA at various concentrations for 24 hours. (B) The relative protein density was analysed by ImageJ. Data expressed as mean values ± SD. *$p <$ 0.05 compared to the non-treated group.

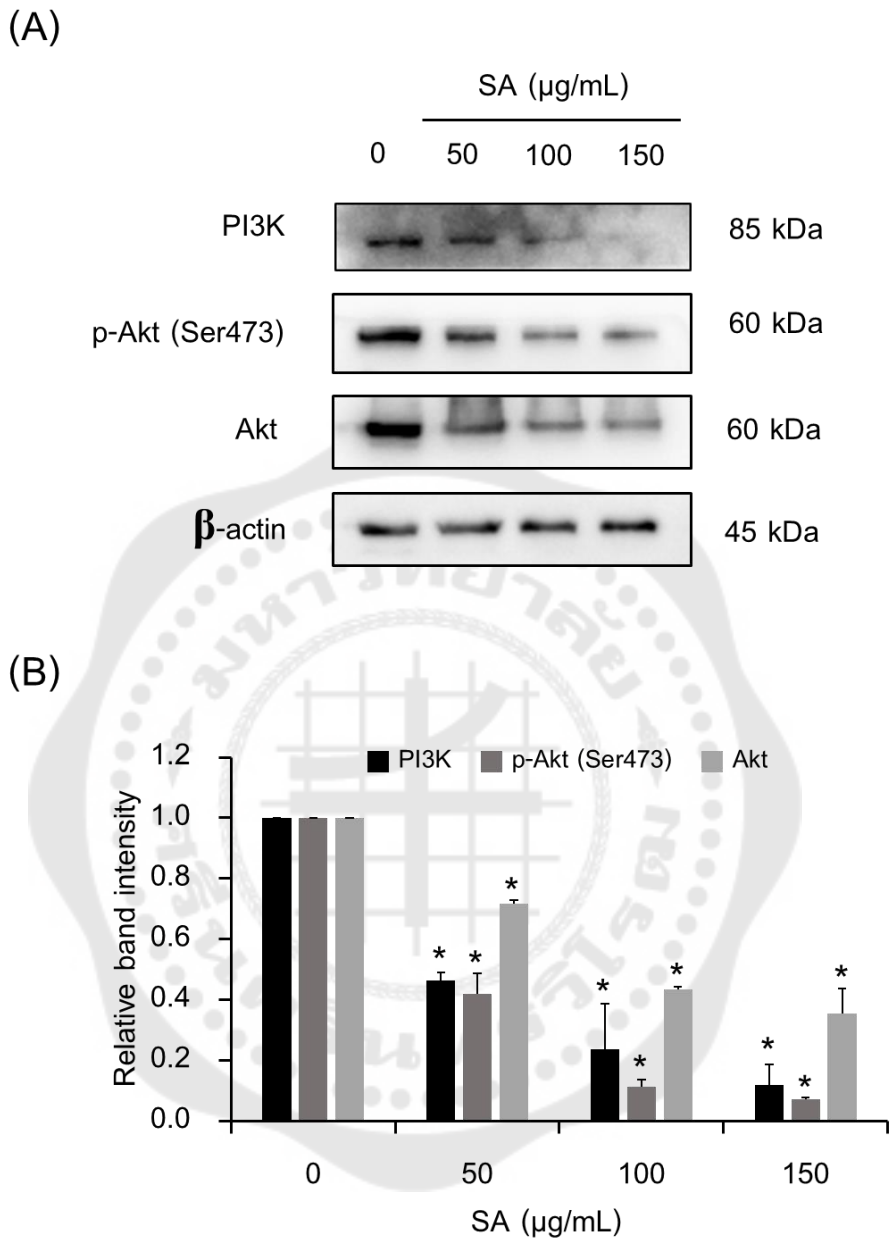



Figure 25 Effects of SA on PI3K/Akt signaling pathway in chondrosarcoma cells. (A) Cells were exposed with SA at various concentrations for 24 hours. (B) The relative protein density was analysed by ImageJ. Data expressed as mean values ± SD. *$p <$ 0.05 compared to the non-treated group.

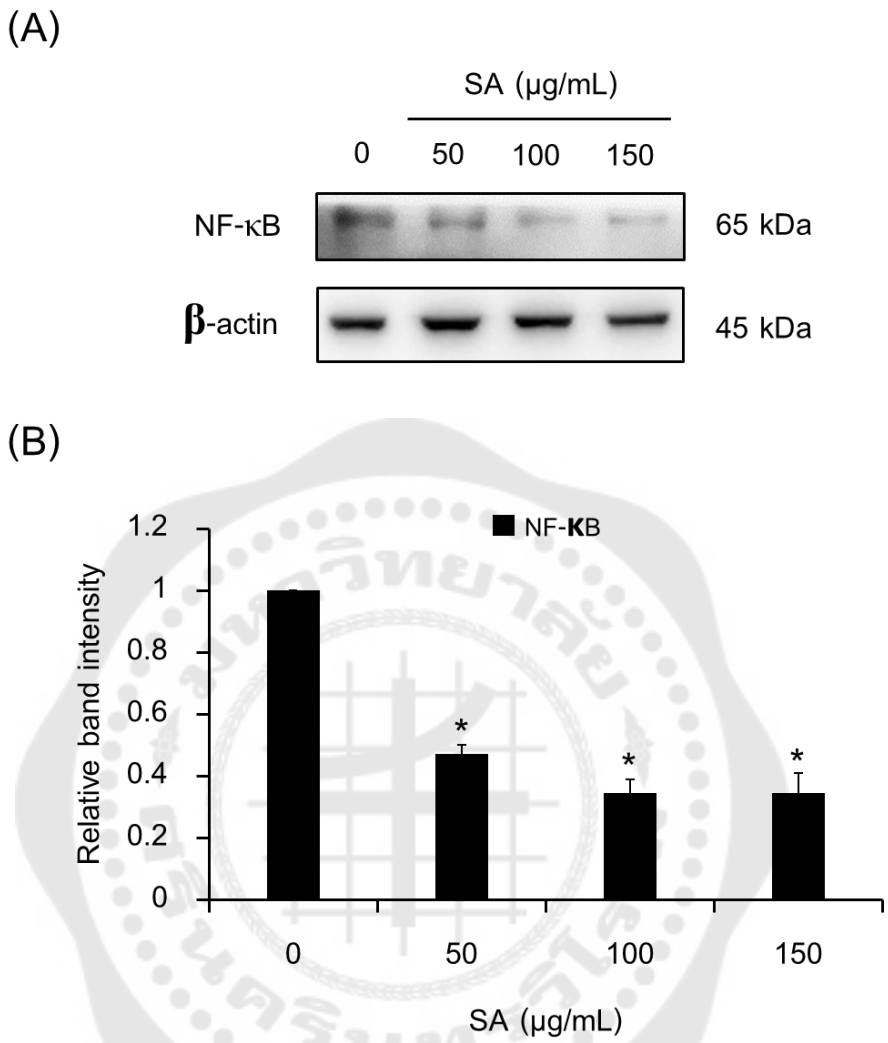



Figure 26 Effects of SA on NF-κB transcription factor in chondrosarcoma cells. (A) Cells were exposed with SA at various concentrations for 24 hours. (B) The relative protein density was analysed by ImageJ. Data expressed as mean values ± SD. *$p <$ 0.05 compared to the non-treated group.

# CHAPTER 5

# SUMMARY DISCUSSION AND SUGGESTION

Tumor metastasis is the main cause of death in patients with chondrosarcoma cancer (98). In clinical, the most common treatment for chondrosarcoma is surgical excision because chemotherapy and radiotherapy are typically unavailing remedies for this cancer. Accordingly, a novel treatment with high efficiency and safe from adverse effects are necessary to study to suppress metastasis of chondrosarcoma. Numerous studies have been reported the medicinal properties of SA extract. SA has been used as an alternative medicinal plant for a long time by various active chemical components such as fatty acids, flavonoids, anthraquinones, fatty acids, steroids, terpenes, and tannins. The ethanolic fraction of SA extract was found to possess antimicrobial, antifungal, antioxidant, anti-inflammatory, anti-skin infection. Moreover, the SA leaf extract also has anticancer activity. However, anti-metastasis activity of SA leaf extract has not been reported the data. In this study, the leaf of SA was extracted and investigated the potential anti-metastatic effects in human chondrosarcoma cancer. Additionally, the bioactive component of SA on metastatic activity was determined by NMR. We found that the ethanolic leaf extract of SA and its active fraction were unsaturated fatty acid and a few compounds of flavonoids and chlorophyll. The cytotoxicity MTT results revealed that SA induced cancer cell death in a dose-dependent manner. Similarly of recent study, Chahardehi et al. reported that SA ethanolic extract exhibited cytotoxic effect of human breast cancer cells (99). The leaf extract also inhibited human urinary bladder carcinoma (T24) and colon carcinoma (Col-2) cell lines (97). In porcine articular chondrocytes, it has been reported that SA did not show cytotoxic effects on regular cells (100). *In vivo*, Roy et al. reported that SA ethanol extract at 1, 2, and 3 g/kg showed no toxicity in mice. These results revealed the oral administration of SA leaf extract was safe (101). The non-toxic concentrations of SA were used in the anti-metastatic experiments. It was investigated that migration capacity in SW1353 cells decreased after treatment with SA leaf extract both *In vitro* scratch

assay and transwell migration assay. Levy et al. reported that kaempferol of SA leaf extract may show cytotoxic effect in A549 cancer cells (96). Moreover, rhein is also major components of SA and has many pharmacological effects such as cell migration and invasion. The results showed that SA was effective in suppressing the proliferation and migration of SW1353 cells. Furthermore, our results also found that SA significantly inhibited invasion and adhesion of SW1353 cells. Therefore, our results showed that SA leaf extract had anticancer activity by inhibiting cell proliferation, migration, invasion, and adhesion in SW1353 human chondrosarcoma cells.

The metastatic potentials of chondrosarcoma are associated with MMPs protein expression (102). MMPs are endopeptidase enzymes that enable degradation of cartilage by breaking down ECM proteins. Several studies have reported that MMP-2 and MMP-9 played an essential role in the invasion and metastasis of tumor cells. Sakamoto et al, Ueda et al. reported that the higher expression level of MMP-2 and MMP-9 in chondrosarcoma associated with the tumor cell progression (103, 104). Therefore, a regulation of MMP-2 and MMP-9 expression is important in the suppression of tumor metastasis. Our results showed that SA leaf extract decreased the expression of MMP-2 and MMP-9. On the other hand, TIMP-2 and TIMP-1 are endogenous inhibitor of MMP-2 and MMP-9, respectively. TIMP-2 and TIMP-1 also significantly upregulated in a dose-dependent manner. Previous studies have demonstrated that resveratrol inhibits MMP-2 and MMP-9 via the JNK and p38 pathways in human chondrosarcoma HTB94 cells (105). Thus, our finding revealed that the downregulation of MMP-proteolytic enzymes associated with the suppression of migration and invasion of SW1353 cells.

In order to clarify the mechanisms underlying the anti-migration and anti-invasion effect of SA on human chondrosarcoma cells. MAPK, PI3K/AKT signaling, and NF-**K**B expressions were investigated. Cancer metastasis progressions are involved in the activation of the MAPK and PI3K/Akt pathway. These signaling molecules are important in the regulation of MMPs expression. Our results show that SA leaf extract inhibited the p-ERK1/2 and p-p38 in SW1353 cells. The expression of PI3K, Akt, p-Akt (Ser473), and NF- **K**B transcription factor were also significantly reduced in a concentation-dependent

manner. Recent study reported that MMP-2 and MMP-9 expression were inhibited via suppressing the phosphorylated of MAPK members, ERK1/2 and JNK1/2, and reduced SW1353 chondrosarcoma cells invasion (106). Moreover, MAPK and PI3K/AKT signaling has been reported to regulate NF-**K**B activation and tumor metastasis (107, 108). In this study, we investigated that SA leaf extract inhibited PI3K, Akt, and NF-**K**B expression. Pan et al. and Chen et al. found that suppression of ERK/NF-**K**B and PI3K/Akt/NF-**K**B activation reduced cell migration and invasion in malignant bone sarcoma cells, respectively (109, 110).

In summary, these studies suggested that the leaf extract of SA has an anti-metastatic effect on chondrosarcoma cells by inhibiting cell migration, cell invasion, and cell adhesion. Especially, SA also reduced the expression of MMP-2 and MMP-9 via suppressing PI3K/Akt, MAPK, and NF-**K**B protein signaling pathway. Thus, this herbal plant may be necessary for further development as an alternative anticancer agent in chondrosarcoma patients.

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