Thesis for the Degree of Doctor of Philosophy

Dynamic interplay between macrophages and gastric cancer in chemotherapy

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February 2019

Dynamic interplay between macrophages and gastric cancer cells in chemotherapy (항암화학요법에서 대식세포와 위암 세포 사이의 역동적 상호작용에 대한 연구)

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by
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A thesis submitted in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

in Department of Microbiology, The Graduate School,
Pukyong National University

February 2019

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February 22, 2019

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List of Abbreviations

5FU	Fluorouracil
AIE	*Aster incisus* extract
AIF	Apoptosis-inducing factor
CDKs	Cyclin-dependent kinases
COCL2	Cobalt (II) chloride
Cox-2	Cyclooxygenase-2
DAPI	4',6-diamidino-2-phenylindole
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H2O2	Hydrogen peroxide
HIF-1α	Hypoxia-inducible factor 1-alpha
HO-1	Heme oxygenase-1
IL	Interleukin
iNOS	Inducible nitric oxide synthetase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloprotease
NF-kB	Nuclear factor-kB
NO	Nitrc oxide
Nrf-2	Nuclear factor erythroid 2-related factor 2
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline

PI3K	Phosphatidylinositol 3-kinase
PI	Propidium iodide
TAMs	Tumor-associated macrophages
TNF-α	Tumor necrosis factor alpha
WST-1®	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium, monosodium salt

Dynamic interplay between macrophages and gastric cancer cells in chemotherapy

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Abstract

Inflammation is related to a large number of diseases like diabetes, cardiovascular disorders, Alzheimer's disease, autoimmune and pulmonary diseases, arthritis, and cancer. Immune cells, especially macrophages, play a major part by the production of inflammatory mediators such as cytokines through the activation of various pathways. Cancer is a major health burden worldwide. The incidence rate of gastric cancer is especially high in Eastern Asia. The control or regulation of cell cycle by induction of cell death through activation cell cycle arrest or induction of apoptosis are the major aim of cancer experiments. In the past decades, various bioactive compounds from plants have been investigated for the purpose of discovering a treatment for human diseases. *Aster incisus* is a common plant mostly found in Asia and has traditionally been used for medicinal purposes in South Korea. In this thesis, we evaluated the potential anti-inflammatory and anticancer effects of a methanol extract from *Aster incisus* in murine macrophages (Raw 264.7 cells), human gastric adenocarcinoma (AGS cells).

Results presented in this thesis show that *Aster incisus* successfully inhibited the production of nitric oxide (NO) and the expression of inflammatory cytokines (TNFα, IL-1β, IL-6, iNOS, Cox-2) through the regulation of NFkB, MAPK and Akt pathways in LPS-stimulated Raw 264.7 macrophages treated with *Aster incisus*. Additionally, *Aster incisus* scavenged DPPH therefore we were able to determine that *Aster incisus* has antioxidant effect. In gastric adenocarcinoma, *Aster incisus* inhibited the proliferation of AGS cells. Further analysis demonstrated that *Aster incisus* inhibited the proliferation of AGS cells by the induction of cell cycle arrest in G1 phase and by apoptosis. G1 phase cyclins (cyclin D1/3, cyclin E2) and CDK (CDK4/6, CDK2) were down-regulated while inhibitors (p16, p18, p21, p27) were over-expressed. Pro-apoptotic proteins (Bid, Bad, Bak, cytochrome c, AIF, cleaved caspase-3, -8, -9 and cleaved PARP) were also highly expressed in AGS cells treated with *Aster incisus*.

Inflammation has been proven to play a crucial role in tumor microenvironment. Macrophages (Tumor-Associated-Macrophages) are the most abundant cells in the tumor microenvironment and play a major role in the resistance of cancer cells to chemotherapy. In this thesis, we tested the effect of 5-FU treatment on AGS cells before and after coculture with tumor-associated macrophages. Tumor-associated macrophages induced the survival of AGS gastric cancer cells through PI3K/Akt pathway, inhibited H_2O_2-induced oxidation through Nrf2-HO1. Additionally, tumor-associated macrophages increased migration and invasion of 5-FU-treated-AGS cells cocultured with tumor-associated macrophages by the regulation of integrin β3, FAK, paxillin, E-cadherin proteins.

CHAPTER ONE

Introduction

Chapter 1
Introduction

Signs and consequences of inflammation were known to doctors in the ancient Sumerian and Egyptian cultures. For example, the Egyptians described abscesses and ulcers, and the Code of Hammurabi (2000 Be) contains instructions on how to treat abscesses of the eye. The first comprehensive description of inflammatory symptoms can be found in the writings of Aulus Celsus (died AD 38). In his De Medicina, he introduced four of the five cardinal symptoms of inflammation: rubor, tumor, color, and dolor (redness, swelling, heat, and pain). Galen of Pergamon (born AD 129) added a fifth sign of inflammation, functio laesa, (impaired function; Anonymous, 1978) [Ley , 2001]. He was a successful physician and surgeon to Roman Emperor Marcus Aurelius. Galen introduced the concept of the four vital humors: sanguis (blood), pituita (phlegm), chole (yellow bile), and melaine chole (black bile). Inflammation was considered a maladjustment of the ratios of these four humors. Galen's writings were influential for at least a millennium, and physicians throughout the Middle Ages were educated based on his texts [Ashley *et al*., 2012].

1.1. Inflammation

Inflammation is a biological reaction to a disrupted tissue homeostasis [Medzhitov, 2008]. At its basic level, it is a tissue-destroying process that involves the recruitment of blood-derived products, such as plasma proteins, fluid, and leukocytes, into perturbed tissue. This migration is facilitated by alterations in the local vasculature that lead to vasodilation, increased vascular permeability, and increased blood flow.

Inflammation is a pervasive form of defense that is broadly defined as a nonspecific response to tissue malfunction and is employed by both innate and adaptive immune systems to combat pathogenic intruders.

There are 2 main types of inflammation:

Acute inflammation: Immediate response. It is short-lived and of the order of a few days to a few weeks maximum. It is characterized by an important infiltrate of inflammatory cells at the site of the lesion. Its resolution is in most cases spontaneous and leaves very few tissue sequels.

Chronic inflammation: it is an inflammation that persists, evolves, and sometimes worsens, for several months or years [Medzhitov, 2008].

Inflammation is a multifactorial process in which different actors are involved (cellular, vascular, matrix, molecular).

Serum molecules and inflammatory blood cells are recruited at the site lesion because of the particular microenvironment generated by the inflammatory process.

To allow this recruitment, vasodilatation occurs at the level of the lesion, which leads to an increase in vascular permeability, thus allowing the passage of cells and the molecular exudate from the blood to the lesional site.

The stages of the inflammatory reaction will adapt according to different elements, as the nature of the pathogen, the injured organ as well as the physiological terrain of the host. These are all elements that will condition the

intensity and duration of the inflammatory reaction and the residual lesion aspect. Figure 2 below schematizes the the course of the inflammatory process as well as the possible outcomes at the onset of this process.

1.1.1. Molecular mediators of inflammation.

Cytokines are very strongly involved in the development and regulation of inflammatory processes and allow communication between cells. Cytokines are synthesized and secreted by cells that can be leukocyte or not [Figarella-Branger *et al.,* 2003; Feghali *et al.,* 1997]. These molecules correspond to soluble peptide factors. Their action is most often limited to the nearby tissue and may affect either the cells that produced them or the cells neighbors. Thanks to their attachment to their high affinity membrane receptor, cytokines are generally effective at low concentrations. There are a large number of cytokines and they are not all involved in the inflammatory reaction [Hopkins, 2003].

Different families of molecules are distinguished: amines, lipid mediators, enzymes and metabolites of polynuclear and macrophages, nitric oxide and growth factors.

There are three plasma systems known to intervene during the inflammatory process: the complement system, the kinin system and the coagulation system.

The kinin system: kinins are vasoactive polypeptides formed at from the plasma kininogen through the intervention of enzymes, kallikreins. The most well-known kinin is bradykinin. Signals that trigger production kinin are multiple. We find the factor XII of coagulation, the proteases released by polynuclear and fibrotic tissue and histamine. They will play an important role during the vasculo-exudative phase thanks to their ability to vasodilatation, thanks to the induction of capillary hyper-permeability and

increase adhesion of neutrophils to endothelial cells. The action of kinins remains limited in time [Bryant *et al.,* 2009; Moreau *et al.,* 2005].

The complement system: the complement is a set of proteins plasma levels (35 are known at present). Twelve of these molecules are directly involved in the fight against pathogens, while the others molecules in the system are used to regulate them to prevent the appearance of reaction nonspecific or autoimmune.

The coagulation system: the relationship between inflammation and the system of coagulation are complex because of the involvement of fibrin in these two process [Carroll, 2004]. Factor XII will be involved in the activation of the kinin system, complement, coagulation and fibrinolysis. The degradation products of the fibrin have vasodilating and chemotactic properties (polynuclear). Finally, thrombin may allow the activation of platelets and endothelial cells. It also has chemotactic properties for polynuclear and monocyte-macrophages.

1.1.2. Cellular mediators of inflammation.

In an inflammatory response, the immune cells are divided into 2 families that act in a complementary way:

Innate or "non-specific" immunity. This reaction is characterized by a response fast cell response to the most common microorganisms. It also includes natural barriers between the body and the external environment, such as skin and mucous membranes.

This reaction will involve the epithelial and vascular cells, in order to allow the secretion of pro-inflammatory mediators, but also the immune cells specialized in phagocytosis (neutrophils, macrophages, dendritic cells), in order to to eliminate bacteria as well as infected cells.

The speed of the innate response is based on the activation of receptors recognizing patterns Molecular: PRR (Pattern Recognition Receptor), the different types of which are the following:

 Receptors recognizing the constituents of pathogens, such as bacteria, parasites and viruses, are grouped under the term PAMP (Pathogen Associated Molecular Pattern). The most well-known PAMPs are TLRs, which recognize specifically some patterns on the surface of the various families of pathogens. TLRs play a determining role in the innate inflammatory response and are expressed by many intestinal cells among which can be mentioned epithelial cells, immune cells and resident cells endothelial.

 PRRs may also recognize danger signals released during apoptosis. These PRRs are then called DAMP (Damage Associated Molecular Protein). Proteins such as Heat Shock Proteins (HSP) or a large number of DNA-associated proteins are part of the DAMPs [Pandolfi *et al.,* 2009].

Adaptive immunity is specific to a given antigen. There are two types of Responses within adaptive immunity:

The humoral response via B lymphocytes. Their action is to block the action pathogens and facilitate their elimination through specific antibodies.

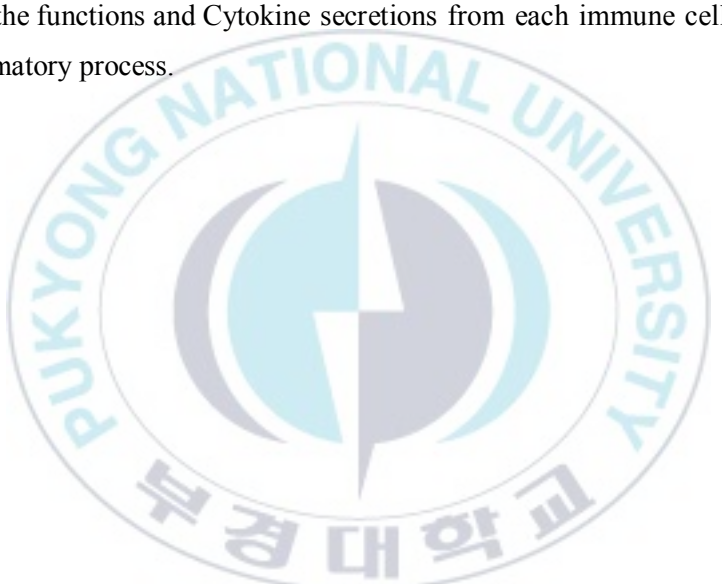
The cytotoxic response via T lymphocytes. Their action aims at eliminating the infected cells (carrying the pathogen's antigen) and to facilitate their destruction.

The adaptive response requires the presentation to B and T lymphocytes of antigens from of the pathogen by specialized cells, so-called antigen presenting cells (CPA). The main APCs of the body are dendritic cells and macrophages.

Recognition of the antigen presented on APCs by lymphocytes allows select the immunocompetent cells, which induces their clonal proliferation, and thus allows an effective fight against the target pathogen. This recognition specific comes in addition to the innate response if it is ineffective or insufficient. One of peculiarities of the adaptive response is that it allows the

generation of memory cells specific to the pathogen. These cells have a long life, which will enable the setting up a faster response during a second contact with the same pathogen [Vivier *et al.,* 2005].

All cells of the immune system may be involved in the inflammatory reaction. The cells of the innate immunity will thus constitute the first defenses against the pathogenic element. Adaptive immunity cells will intervene later, following their recruitment by the molecules secreted by the damaged tissue and by cells of innate immunity. The Table below shows the functions and Cytokine secretions from each immune cell during the inflammatory process.



1.1.3. Inflammation in diseases.

Inflammation is implicated in various organs and diseases or complications that affect them [Okin *et al.,* 2012].

Table 1. Inflammatory disorders in human

Organs	Disorders
Brain	Depression, autism, Alzheimer's disease and MS [Campbell, 2004]
Skin	Rashes, dermatitis, eczema, acne, psoriasis. [Galli *et al.,* 2008]
Cardiovascular	Strokes, anemia [Emsley *et al.,* 2002]
Bones	Osteoporosis [Armour *et al.,* 2003]
Cancer	Macrophages [Kashfi, 2009]
GI tract	Chron's disease, celiac disease [MacDonald *et al.,* 2005]
Muscle	Muscle pain, polymyalgia rheumatica. [Kreiner *et al.,* 2010]

1.2. Gastric cancer.

1.2.1. Anatomy and physiology of Normal stomach

The stomach is an organ located in the abdomen and shaped like a J [Lacy *et al.,* 2005]. It is part of the digestive system and its size varies from one individual to another. It is connected to the esophagus and the small intestine. The human stomach is divided in 5 regions: the cardia, the fundus, the body, the antrum and the pylorus [Treuting *et al.,* 2017].

Table 2. 5 regions of a stomach

Region	Location	Function
Cardia	First part right below the esophagus	Prevents stomach contents from going back up
Fundus	Lies to the left of the cardia	
Body	Largest and main part of the stomach	Where the food is mixed and starts to break down
Antrum	Lower part of the stomach	Holds the broken-down food until it is ready to be released into the small intestine
Pylorus	Part that connects to the small intestine	Acts like a valve to control the emptying of the chyme into the duodenum.

The stomach has 3 main functions: temporary storage for food, which passes from the esophagus to the stomach where it is held for 2 hours or longer, mixing and breakdown of food by contraction and relaxation of the muscle layers in the stomach and digestion of food [Matsuo *et al.,* 2008]. The stomach does not play a big role in absorption of food. It only absorbs water, alcohol and some drugs.

1.2.2. Epidemiology and Risk factors of gastric cancer.

The stomach cancer usually begins in the mucosa [Vkan Cutsem *et al.,* 2016]. As it grows, it invades deeper into the stomach wall and then through the wall. When the tumor affects only the mucosa or submucosa (the layer of the stomach wall below the mucosa), it is known as early stomach cancer. It can invade, or grow into, and destroy nearby tissue. It can also spread, or metastasize, to other parts of the body [Krejs, 2010; Alberts *et al.,* 2003; Rugge *et al.,* 2015].

The most common cancerous tumor of the stomach is adenocarcinoma. Adenocarcinoma tumors start in the gland cells that line the inner surface of some organs in the body, including the stomach. Adenocarcinoma accounts for up to 95% of all stomach cancers [Kelley *et al.,* 2003].

A risk factor is something that increases the risk of developing cancer. It could be a behavior, substance or condition. Most cancers are the result of many risk factors. *Helicobacter pylori* infection is the most important risk factor for stomach cancer [Compare *et al.,* 2010].More men than women develop stomach cancer. The risk of developing stomach cancer increases with age. It is greatest after 50 years of age. Some studies show that low socio-economic status is linked with a higher rate of stomach cancer [Torre *et al.,* 2015].

Stomach cancer is most common in Japan, China, South America and Eastern Europe. It is not as common in North America [Pourhoseingholi *et al.,* 2015; Rahman *et al.,* 2014].

Table 3. The risks factors of gastric cancer

Known risk factors	Possible risk factors
Helicobacter pylori infection	Salt and salty foods
Smoking	Alcohol
Family history of stomach cancer	Obesity
Inherited genetic conditions	Gastroesophageal reflux disease
Certain stomach conditions	Occupational exposure to lead
Previous stomach surgery	Asbestos
Epstein-Barr virus	Cystic fibrosis
Exposure to ionizing radiation	
Working in rubber industry	
Type A blood	
Smoked, cured and processed meats	

Unknown risk factors

Grilled and barbecued meats, a diet low in vegetables and fruit, lack of regular physical activity, working in the coal mining, tin mining, or iron and steel industries, bariatric surgery, which is surgery to the stomach to help you lose weight.

1.2.3. Diagnosis and Staging.

Diagnosing stomach cancer usually begins with a visit to your family doctor. The following tests are commonly used to rule out or diagnose stomach cancer. Many of the same tests used to diagnose cancer are used to find out the stage, which is how far the cancer has progressed (Pierzyk *et al.,* 2016).

Health history and physical exam.

A record of symptoms, risk factors and all the medical events or problems from the past will be required. Questions about the family history for past cases of gastric cancer or any other type of cancer in family will be asked. Additionally, a physical exam will allow your doctor to look for any signs of stomach cancer by feeling the abdomen for enlarged organs, lumps or fluid and by checking the lymph nodes in the armpits and above the collar bones.

Complete blood count (CBC)

A CBC to measure the number and quality of white blood cells, red blood cells and platelets will conducted. A CBC is done to check for anemia from long-term (chronic) bleeding into the stomach.

Blood chemistry tests

Blood urea nitrogen (BUN) and creatinine may be measured to check kidney function. Increased levels could indicate that cancer has spread to the ureters or kidneys. Lactate acid dehydrogenase, alkaline phosphatase, transaminase and bilirubin may be measured to check liver function. Increased levels could indicate that cancer has spread to the liver [Jin *et al.,* 2015].

Upper gastrointestinal (GI) endoscopy.

An upper gastrointestinal endoscopy is an examination of the upper GI tract, including the esophagus, stomach and upper part of the small intestine (duodenum). It allows to look inside these organs using a flexible tube with a light and lens on the end (an endoscope). This is the most common test used to

diagnose stomach cancer. An upper GI endoscopy is done to examine the stomach for bleeding, ulcers, polyps, tumors and inflammation (gastritis) and to take samples of tissue to be tested in the lab (biopsy). An ultrasound may be done with an endoscope (endoscopic ultrasound, or EUS) to see how far the tumor has grown into the wall of the stomach and nearby tissues. An EUS can also show if cancer has spread to nearby lymph nodes [Choi *et al.,* 2015].

Endoscopic ultrasound

Endoscopic ultrasound (EUS) uses an endoscope with an ultrasound probe at the end. Ultrasound uses high-frequency sound waves to make images of structures in the body. It can provide detailed information about the location, size and depth of the tumor (how far it has spread into the wall of the stomach) and if cancer has spread to lymph nodes or surrounding tissues. EUS is often done at the same time as an upper GI endoscopy.

The endoscopic ultrasound can also be used to guide a needle for a biopsy (EUS-guided needle biopsy) to check for cancer in the wall of the stomach, outside the stomach or in surrounding lymph nodes.

Biopsy

During a biopsy, tissues or cells are removed from the body so they can be tested in the lab. The report from the lab will confirm whether or not cancer cells are present in the sample and what type of cancer cells (such as adenocarcinoma) are present.

Tumor marker tests

Tumor markers are substances in the blood that may mean stomach cancer is present. Tumor marker tests are generally used to check your response to cancer treatment. They can also be used to diagnose stomach cancer. The following tumor markers may be measured for stomach cancer:

HER2 (human epidermal growth factor receptor 2) is a gene that can change (mutate). If it changes, it may help a tumor grow (oncogene). HER2 status testing is done to find out the amount of HER2 made by a tumor. Stomach tumors that contain HER2 are called HER2-positive [Moasser, 2007; Menard *et al.,* 2003].

Higher than normal levels of carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9) or cancer antigen 125 (CA 125) may indicate stomach cancer.

Other diagnosis methods are: CT scan, Chest x-ray, Laparoscopy, PET scan, MRI, and Ultrasound.

Stages of stomach cancer

Staging describes or classifies a cancer based on how many and where tumors or cancer cells are in the body. The results of these tests are used to find out the size of the tumor, which parts of the organ have cancer, whether the cancer has spread from where it first started. The stage can also be used to plan treatment and the estimated outcome [De Manzoni *et al.,* 2015; Washington, 2010].

The most common staging system for cancers is the TNM system. For stomach cancer there are 5 stages (from stage 0 followed by stages 1 to 4). Often the stages 1 to 4 are written as the Roman numerals I, II, III and IV. Generally, the higher the stage number, the more the cancer has spread. When describing the stage, doctors may use the words local, regional or distant. Local means that the cancer is only in the stomach and has not spread to other parts of the body. Regional means close to the stomach or around it. Distant means in a part of the body farther from the stomach.

Table 4. Stages of gastric cancer

Stage	**Characteristics (Part 1)**
Stage 0	The tumor is only within the epithelium of the mucosa
Stage 1A	The tumor has grown into the layer of connective tissue in the mucosa, the layer of muscle in the mucosa, or the layer of connective tissue that surrounds the mucosa (submucosa).
Stage 1B	The tumor has grown into the layer of connective tissue or muscle in the mucosa or into the submucosa. The cancer has also spread to 1 or 2 lymph nodes near the stomach or the tumor has grown into the thick muscle layer of the stomach (muscularis propria).
Stage 2A	The tumor has grown into the layer of connective tissue or muscle in the mucosa or into the submucosa. The cancer has also spread to 3 to 6 lymph nodes near the stomach, the tumor has grown into the muscularis propria. The cancer has also spread to 1 or 2 lymph nodes near the stomach or the tumor has grown into the area between the muscularis propria and the outer covering of the stomach (serosa).
Stage 2B	The tumor has grown into the layer of connective tissue or muscle in the mucosa or into the submucosa. The cancer has also spread to 7 to 15 lymph nodes near the stomach, the tumor has grown into the muscularis propria, the cancer has also spread to 3 to 6 lymph nodes near the stomach, the tumor has grown into the subserosa or the cancer has also spread to 1 or 2 lymph nodes near the stomach or the tumor goes through the serosa.

Stage	Characteristics (Part 2)
Stage 3A	The tumor has grown into the muscularis propria. The cancer has also spread to 7 to 15 lymph nodes near the stomach, the tumor has grown into the subserosa. The cancer has also spread to 3 to 6 lymph nodes near the stomach, the tumor goes through the serosa. The cancer has also spread to 1 to 6 lymph nodes near the stomach or the tumor has grown into nearby organs or areas, such as the spleen, colon, small intestine, liver, diaphragm or abdominal wall.
Stage 3B	The tumor has grown into the layer of connective tissue or muscle in the mucosa, the submucosa or the muscularis propria. The cancer has also spread to 16 or more lymph nodes near the stomach, the tumor has grown into the subserosa or it goes through the serosa. The cancer has also spread to 7 to 15 lymph nodes near the stomach or the tumor has grown into nearby organs or areas. The cancer has also spread to 1 to 6 lymph nodes near the stomach.
Stage 3C	The tumor has grown into the subserosa or it goes through the serosa. The cancer has also spread to 16 or more lymph nodes near the stomach or the tumor has grown into nearby organs or areas. The cancer has also spread to 7 or more lymph nodes near the stomach
Stage 4	The cancer has spread to other parts of the body (called distant metastasis), such as to the lungs, bone, peritoneum or omentum. This is also called metastatic stomach cancer.

If stomach cancer spreads, it can spread to the following organs:

Pancreas, spleen, colon, small intestine, liver, mesentery (folds of tissue that hold the abdominal organs in place), omentum, diaphragm, abdominal wall, esophagus, adrenal glands, lymph nodes in and around the abdomen or above the left collarbone (Virchow node), liver, lung, bone, skin, ovaries (Krukenberg tumour), area around the belly button (Sister Mary Joseph node), uterus, pelvic area around the rectum (Blumer shelf), brain

Prognosis and survival for stomach cancer

A prognosis is the doctor's best estimate of how cancer will affect someone and how it will respond to treatment. Prognosis and survival depend on various factors. Only a doctor familiar with your medical history, the type, stage and characteristics of your cancer, the treatments chosen and the response to treatment can put all of this information together with survival statistics to arrive at a prognosis.

Table 5. Survival by stages

Stomach cancer survival	
Stage	5-year observed survival
1A	71%
1B	57%
2A	46%
2B	33%
3A	20%
3B	14%
3C	9%
4	4%

1.2.4. Treatment of gastric cancer.

Treatment for gastric cancer

The treatment of gastric cancer is often decided according to the following criteria:

Stage of the cancer, location of the tumor in the stomach, HER2 (ERBB2) status and your overall health

You may be offered the following treatments for stomach cancer.

Surgery

Surgery is often used to treat stomach cancer. The type of surgery you have depends mainly on the size and location of the tumor. Gastrectomy is the most common surgery to treat stomach cancer. A gastrectomy is the surgical removal of all or part of the stomach through an incision in the abdomen. Lymph node dissection is often done along with a gastrectomy to remove lymph nodes around the stomach. Palliative surgery may be done to relieve the symptoms of advanced stomach cancer such as bleeding, pain or not being able to eat [Marano *et al.,* 2016; Brenkman *et al.,* 2016].

Chemotherapy

Chemotherapy may be used to treat stomach cancer at any stage. Chemotherapy uses anticancer, or cytotoxic, drugs to destroy cancer cells, to shrink a tumor before other treatments such as surgery or radiation therapy, destroy cancer cells left behind after surgery and reduce the risk of the cancer recurring (called adjuvant chemotherapy) - radiation therapy may be given at the same time (called adjuvant chemoradiation) or to relieve pain or control the symptoms of advanced stomach cancer (called palliative chemotherapy) [Orditura *et al.,* 2014; Digklia *et al.,* 2016].

Chemotherapy for stomach cancer may be given as a single drug or as a combination of 2 or more drugs. The most common chemotherapy drugs used to treat stomach cancer are:

- ✓ 5-fluorouracil (Adrucil, 5-FU) – leucovorin (folinic acid) is often given along with 5-fluorouracil to make 5-fluorouracil work better
- ✓ capecitabine (Xeloda)
- ✓ cisplatin (Platinol AQ)
- ✓ carboplatin (Paraplatin)
- ✓ epirubicin (Pharmorubicin)
- ✓ docetaxel (Taxotere)
- ✓ irinotecan (Camptosar)
- ✓ oxaliplatin (Eloxatin)
- ✓ paclitaxel (Taxol)
- ✓ doxorubicin (Adriamycin)
- ✓ mitomycin (Mutamycin)
- ✓ methotrexate
- ✓ etoposide (Vepesid)

Some examples of chemotherapy regimens (a combination of 2 or more drugs) used for stomach cancer are:

- ✓ ECX – epirubicin, cisplatin and capecitabine
- ✓ ECF – epirubicin, cisplatin and 5-fluorouracil
- ✓ DCF (TCF) – docetaxel, cisplatin and 5-fluorouracil

Radiation therapy

Radiation therapy may be used to treat stomach cancer at any stage. The type of radiation therapy used most often is external beam radiation therapy.

Chemotherapy may be given at the same time as radiation therapy (chemoradiation).

Targeted therapy

Trastuzumab (Herceptin) and ramucirumab (Cyramza) are targeted therapy drugs used to treat advanced, metastatic or recurrent stomach cancer [Xu *et al.,* 2016].

The research in stomach cancer focuses on: Diagnosis and prognosis, Targeted therapy and immunotherapy, Immunotherapy, Chemotherapy and Surgery

1.3. Macrophages in tumor microenvironment.

1.3.1. Tumor microenvironment.

Cancer stroma is a highly complex environment containing malignant and host cells as well as non-cellular elements, including metabolites, ECM, fibres, ions, secreted proteins and free acids.

Inflammatory cells are key components of the TME and are recognized as integral factors in contributing to carcinogenesis. Colotta *et al.* were the first to describe the link between inflammation and malignancy [Whiteside, 2008], and it is now an established hallmark of cancer [Mbeunkui *et al.,* 2009]. Epidemiological and experimental data indicate that a highly inflammatory TME within gastric cancer contributes to the development and progression of tumorigenesis, for example chronic inflammation is known to increase the risk of developing gastric cancer [Korniluk *et al.,* 2017].

The relationship between immune cells and cancer within the TME is complex. Inflammatory cells and mediators, including cytokines, chemokines and prostaglandins, co-ordinate a milieu of pro-inflammatory responses which act in both an autocrine and paracrine manner on malignant and non-malignant cells [Lu *et al.,* 2006]. A more immunosuppressive infiltrate, including immature myeloid cells (also known as myeloid-derived suppressor cells), tumour-associated macrophages (TAMs) and Treg cells then dominated the initial response and remained in invasive lesions. Thus, immune cell infiltrate may dictate the ability of gastric cancer cells to disseminate from the primary tumour site and consequently targeting inflammation in gastric cancer may lead to less metastasis. *In vivo*, this was demonstrated by Rhim *et al.* using the immunosuppressive agent dexamethasone, which abolished circulating gastric cancer cells [Gajewski *et al.,* 2006]. In conclusion, inflammation plays a role in driving gastric cancer tumorigenesis and pre-clinical data supports the concept

of targeting this hallmark of cancer to inhibit tumour progression and dissemination.

1.3.2. Tumor-Associated Macrophages.

Macrophages are one of the most abundant immune cells within the TME and are known to drive inflammation [Hao *et al.,* 2012]. Therefore understanding the role of these immune cells in driving cancer may aid in developing a viable anti-inflammatory treatment strategy.

Macrophages are dominant immune cells that modulate tissue homeostasis and play a vital role in host inflammation and infection in response to pathogens and disease [Sica *et al.,* 2012; Martinez *et al.,* 2008]. Their functions in normal states are to engulf invading bacteria and cell debris at inflamed and injured sites, secrete immunomodulatory cytokines, present antigen to T cells and act as accessory cells in lymphocytes activation. It is thought that two distinct populations of macrophages exist in homeostatic states; 'elicited' macrophages, recruited mainly from the bone marrow in response to inflammatory stimuli, and 'resident' macrophages, derived from embryonic (yolk sac) progenitors. Elicited macrophages are derived from blood monocytes. Monocytes themselves originate in the bone marrow from hematopoietic stem cells (HSCs) and arise from a series of sequential differentiation stages [Gordon *et al.,* 2010].

Monocytes are released into the circulation and within a few days they seed tissues by the process of extravastion through the endothelium. They eventually differentiate into either dendritic cells (DCs) or macrophages. Dependent on the tissue location and the inflammatory insult, there is evidence that both recruitment of monocytes / macrophages and local proliferation takes place to replenish and maintain the tissue-specific population of macrophages under normal pathological conditions.

'Resident' embryonic macrophages on the other hand take residence in tissues prior to birth and maintain themselves within local tissue throughout adulthood independently of bone marrow derived precursors. Recently developed fate mapping techniques have enabled the identification and tracking of different embryonic macrophage populations into adulthood, and have revealed this population of macrophages to complex and heterogeneous. This appreciation of macrophage origin and heterogeneity is vital when exploring the effects of targeting the macrophage population within gastric cancer, for example the loss on the monocyte-derived macrophages had limited effects on tumour progression compared to loss of the tissue-resident population, which significantly reduced tumour progression [Ostuni *et al.,* 2015].

1.3.2.1. Macrophage Phenotype and Activation

Macrophages are one of the most plastic cells of the hematopoietic system, showing great phenotypic and functional diversity. One way to classify tissue macrophages is according to their anatomical location, which then dictates functional phenotype.Diversity of macrophage function is greatly influenced by the surrounding microenvironment. They respond not only to inflammatory stimuli but also to signals from antigen-specific immune cells and even macrophage-derived factors. Previously, the most commonly used classification of their activation state was the 'classically activated' M1 phenotype, and 'alternatively activated' M2 phenotype [Chanmee *et al.,* 2014].

This classification arose in the 1990s, when differential effects on macrophage gene expression were noted in response to external stimuli akin to the Th1/Th2 paradigm; M1 macrophages polarized in response to bacterial moieties such as lipolysaccharide (LPS) and the Th1 cytokine interferon (IFN)-γ, whilst M2 macrophages polarized in response to the Th2 cytokine interleukin (IL)-4. These findings have been since validated both *in vitro* with peripheral

blood monocyte derived macrophages, and *in vivo*. Based on these two activation states, consistent differences in function, metabolism and subsequent cytokine production have been observed. M1 macrophages produce large quantities of pro-inflammatory cytokines (e.g. IL1β, IL12, and tumour necrosis factor (TNF)-α) that promote cell-mediated Th1 responses, have increased major histocompatibility complex class (MHC) class II expression, and are implicated in killing of pathogens and tumour cells. In contrast, M2 macrophages secrete IL10 and other cytokines that mediate Th2 responses, moderate inflammatory responses and promote tissue re-modelling and repair [Sunderkotter *et al.,* 1994].

The concept of M1/M2 however has now been updated, with the old binary definition viewed as outdated and oversimplified. Although this nomenclature is useful to describe the two extremes of population, expert consensus recognizes the complexities in macrophage activation, phenotype and plasticity. The current view is that macrophages are most likely to exist in a spectrum of activation states depending on the exact composition of the activating signals present in the microenvironment rather than one or the other. Therefore instead of fitting within the constant and dualistic definition of M1/M2 as described in it is likely that the plasticity of macrophages within the tumour microenvironment leads to constant and complex changes in phenotype driven by gene and surface marker expression leading to a heterogeneous population at any given time.

1.3.2.2. Tumour Associated Macrophages

Macrophages are a major component in leucocyte infiltration of the TME, and their role in tumorigenesis is complex. Early studies into the role of TAMs initially reported that activation by bacterial moieties and cytokines enable tumour cell kill. However, it soon became apparent that they could also promote

tumour growth and metastasis. Thus, early on in the investigation of TAMs, a dual function was demonstrated [Sounni *et al.,* 2013].

TAMs in tumour tissue are derived from tissue-resident macrophages, as demonstrated with microglial cells in glioma, and cytokine recruited blood monocytes and monocyte-related myeloid-derived suppressor cells (M-MDSCs). The dual supportive and inhibitory role of macrophages within tumours is largely driven by the TME. Granulocyte macrophage colony stimulating factor (GMCSF) driven macrophages mediate an antitumor / cytotoxic effect, whilst IL4, IL13 and macrophage colony stimulating factor (MCSF) driven macrophages are typically pro-tumorigenic, supporting several hallmarks of cancer, including angiogenesis, cell invasion and migration, and suppression of anti-tumour immune responses. The phenotype of TAMs is skewed by several different signals originating from tumour cells (e.g. chemokine secretion), B cells (immune complexes) and stromal cells (e.g. IL1), leading to a diverse and heterogeneous population of TAMs.

Despite the opposing functional populations of TAMs that exist within a primary tumour (pro- vs. anti- tumour), clinical data largely support a high density of macrophages in tumours with poor prognosis as 80% of studies indicate a higher macrophage density is associated with inferior patient prognosis [Kenny *et al.,* 2007].

In gastric cancer, TAMs are thought to be typically protumourigenic (as defined by CD163) and relate to progression and treatment resistance. This observation supports the widely accepted view that TAMs mainly promote several different aspects of tumour progression.

1.3.2.3. Macrophages and Chemoresistance

Macrophages are important determinants of the efficacy of chemotherapeutic agents, but whether they enhance or counteract the anticancer effect is highly dependent on the drug used and tumour type [Mantovani *et al.,* 2008].

Interestingly, recent clinical data in gastric cancer suggest a dual prognostic significance of TAMs in the adjuvant setting; in patients not treated with chemotherapy, density of TAMs at the tumour-stroma interface (as defined by CD206, CD163 expression and IL10 expression) was associated with worse prognosis and distant metastasis [Mantonavi *et al.,* 2017]. However, a high density was also associated with better prognosis for patients who received postsurgical adjuvant chemotherapy. *In vitro* data supported this finding, with gemcitabine treated macrophages becoming tumouricidal following treatment, implying chemotherapy're-educated' macrophages by inhibiting protumour functions and driving cytotoxic activity. This study implies therapeutic targeting of macrophages in the TME, especially in the adjuvant setting, should not only focus on decreasing TAMs density, but suggest developing agents that polarize macrophage function to improve outcomes by promoting their antitumourigenic functions.

1.3.3. Targeting macrophages in cancer therapy.

Due to the large infiltration of macrophages in cancers, their ability to influence adaptive immune cells, their association with poor prognosis and involvement in tumour progression, TAMs are emerging as targets of immunotherapy in cancer.

Existing chemotherapeutic agents may already have effects on TAMs, for example the chemotherapeutic drug trabectadin has been reported to have an additional action of selective cytotoxicity to human monocytes as well as its direct anti-cancer cell properties. In soft tissue sarcoma patients treated with

trabectadin, decreased circulating monocytes and a reduction in TAMs were seen on biopsy specimens. This drug is now approved for treatment of soft tissue sarcoma and is being tested in several other tumour types and thus its anti-macrophage properties may be further explored [De Palma *et al.,* 2013].

Currently there are no approved therapeutic agents in clinical use that target TAMs specifically, and therefore further development is needed into novel ways to target the pro-tumourigenic functions of TAMs which may then translate to better clinically efficacy. Advancing our understanding of the relationship between TAMs and cancer cells could help identify better therapeutic approaches.

Due to the pleiotropic effects of inflammatory cytokines in tumour progression, selective inhibition of specific cytokines have been trialled, with the aim of disrupting the already dysregulated tumour cytokine network in cancer to achieve both systemic as well as tumour-specific therapeutic effects [Mitchem *et al.,* 2013].

Several approved and novel agents targeting cytokines have been or are being tested in early phase cancer trials. Some of these act on TAM function also, for example CCL2 inhibitors would limit macrophage recruitment. Cytokine targeting agents have been tested in gastric cancer, but little benefit has been seen, with anti-TNFα and anti-IL6 agents. One of the reasons for lack of efficacy could be because these cytokines are not key players in driving gastric tumour progression, and thus inhibiting their activity has little clinical efficacy [Ruffel *et al.,* 2015]. It may also be due to the stage of treatment, as giving a cytokine agent in the setting of metastatic disease could be ineffective due in an already established complex network of signaling pathways compared to giving it early.

1.4. Aims of this thesis.

In this thesis, we investigated:

- The potential anti-oxidant and anti-inflammatory effects of Aster incisus extract (AIE) in Raw 264.7 macrophages stimulated with LPS and the pathways involved.

- The anticancer effect of AIE in AGS gastric adenocarcinoma and the pathways related to the induction of cell death of AGS treated with AIE.

- The pathways involved in the survival and chemoresistance of AGS cells to fluorouracil (5FU) when coculture with M2 polarized macrophages.

- The potential involvement of integrin β3/FAK and associated proteins (vinculin and paxillin) in the resistance of AGS cells to 5FU when cocultured with M2 macrophages.

1.5. References

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CHAPTER TWO

Antioxidant and anti-inflammatory effects of *Aster incisus* in RAW 264.7 cells.

CHAPTER 2

Antioxidant and anti-inflammatory effects of *Aster incisus* in RAW 264.7 cells through NFkB, MAPK and Akt pathways

2.1. Abstract

Aster incisus is a common flower found in almost all regions of South Korea. In the current study, we investigated the potential antioxidant and anti-inflammatory properties of the Aster incisus methanol extract in LPS-stimulated RAW 264.7 cells. We analyzed the phytochemicals contained in the extract by GC-MS. GC-MS results showed that the *Aster incisus* extract contains 9 known compounds. Later on, DPPH assay, WST-1 assay, nitric oxide (NO) assay, Western blot, and RT-PCR were conducted to investigate the anti-inflammatory effects of the extract. Our WST-1 assay results revealed that *Aster incisus* did not affect the viability of all tested cell lines up to a concentration of 200 μg/ml; therefore, lower concentrations (50 μg/ml and 150 μg/ml) were used for further assays. *Aster incisus* scavenged DPPH and inhibited the production of NO. *Aster incisus* also reduced significantly the production of inflammation-related enzymes (iNOS, Cox-2) and cytokines (TNFα, IL-1β, and IL-6) and the gene expression of the proinflammatory cytokines. Additionally, further Western blot results indicated that *Aster incisus* inhibited the expression of p-PI3K, p-IκBα, p-p65 NF-κB, p-ERK1/2, p-SAPK/JNK, and p-Akt. Our results demonstrated that Aster incisus suppressed the expression of the inflammation mediators through the regulation of NF-κB, MAPK, and Akt pathways.

2.2. Introduction

The inflammation is usually described as a defense mechanism used by animals to fight intruders. Inflammation can be divided into acute inflammation, which is a quick and short immune response; and chronic inflammation, which takes time to set up and may result in failure to remove the initial cause [Neuman, 2007]. It can be related to a large number of serious diseases, like diabetes, cardiovascular disorders, Alzheimer's disease, autoimmune and pulmonary diseases, arthritis, and cancer [Kundu *et al.,* 2008].

Immune cells, especially macrophages, play a major part in the non-specific immunity against foreign intruders, specifically infectious microorganisms (bacteria, viruses, mycoplasma etc.) [Beer *et al.,* 2005; Funk, 2001; Hsieh *et al.,* 2011]. Their activation during chronic inflammation is very crucial and sometimes associated with complications of chronic inflammation-related diseases by excessive production of nitric oxide (NO) and prostaglandin E2 (PGE2), or other inflammation-related cytokines [Qi *et al.,* 2012; Kolb *et al.,* 1992; Zhong *et al.,* 2015; Cho *et al.,* 2014; Jiang *et al.,* 2006].

Previously published papers have described the importance of NFkB, MAPK and Akt pathways in the expression of mediators specific to inflammation [Posads *et al.,* 2003; D'Acquisto *et al.,* 1997; An *et al.,* 2011; Taniguchi *et al.,* 2014].

Aster incisus is a species of the *Asteraceae* family and belongs to the *Aster* genus which contains approximately 248 to 706 species and belongs to the family of the (Aster Family). *Aster incisus* is native to China, Japan, and Korea [Hassler, 2017].

Recently, we reported the anticancer effects of *Aster incisus* in gastric adenocarcinoma. [Ngabire *et al.,* 2018]. Other plants belonging to the Asteraceae family have been used for traditional medicinal purposes and most recently, plants from the Aster genus were studied and reported for their anti-inflammatory effects

[Choi *et al.,* 2014] and other biological activities [Hu *et al.,* 2017; Yao *et al.,* 2017; Zhang *et al.,* 2017; Wang *et al.,* 2017; Han *et al.,* 2017].

In the current study, the potential antioxidant and anti-inflammatory effects of *Aster incisus* extract were investigated for the first time ever in RAW 264.7 macrophages stimulated with LPS. Our results demonstrated that *Aster incisus* can inhibit LPS-induced NO and inflammation-related cytokines like TNFα, IL-6, and IL-1β. We were able to demonstrate that NFkB, MAPK, and Akt pathways were significantly inhibited by treatment with *Aster incisus*.

2.3. Materials and Methods

2.3.1. Cell lines and reagents

Human kidney cells (HEK293 cells), human keratinocytes (HaCaT cells) and murine macrophages (Raw 264.7 cells) were from the ATCC (Manassas, VA, USA). DMEM media was obtained from Hyclone Laboratories (USA), Fetal bovine serum (FBS) and penicillin-streptomycin from Cellgro (Manassas, VA, USA). *Aster incisus* methanol extract (voucher no. 016-001) was purchased from Korean Plant Extract Bank (KPEB, Cheongju, Korea). EZCytox (WST-1; Daeil Lab service, Seoul, Korea), dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA), LPS (Sigma, MO), Griess reagent (Sigma, USA) and cell lysis buffer was purchased from Intron Biotechnology Inc., Gyeonggi, Korea. Antibodies: iNOS, Cox-2, TNFα, pIkBα, IkBα, p-p65 NFkB, NFkB, p-Akt, p-PI3K, p-mTOR, p-SAPK/JNK, p-ERK1/2, p-p38 and the second antibody linked to a peroxidase (Cell Signaling Technology) and ECL detection solution (Pierce, Rockford, IL, USA). DAPI, 4% formaldehyde, rabbit normal serum, anti-rabbit IgG.

2.3.2. Gas Chromatography-Mass Spectroscopy (GS-MS)

Aster incisus methanol extract (AIE) sample obtained from Korean Plant Extract Bank (voucher no. 016-001) was analyzed using a GC-MS equipment. The experimental settings were: the standard non-polar column dimensions were 30 x 0.25 µm ID x 0.25 µm df and Helium was used as a gas carrier at a flow rate of 1.0 ml/min. The oven temperature was set at 50°C and later raised to 320°C at a speed of 7°C/min. The temperature for the injector was fixed at 280°C with the volume of the injection equal to 0.1 μl. The interpretation was conducted using the database of NIST library.

2.3.3. DPPH assay

The antioxidant scavenging activity was investigated by using the scavenger and free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH). This experiment was conducted as detailed by Mahesh et al. [Patil *et al.,* 2016] with minor variations. In brief, 1 ml from various concentrations of AIE diluted in methanol (20, 50, 100 and 150 µg/ml) were mixed with 4 ml of methanol each containing 0.07 mM of DPPH. Afterwards, the mixtures were vigorously vortexed followed by an incubation in the dark at room temperature for about 30 min. The DPPH scavenging results data were collected by reading the absorbance at 517 nm. For standard, we used ascorbic acid and methanol as blank. The inhibition percentage was determined using the formula;

Percentage of Inhibition = [(Abs of control – abs of samples) / Control abs] x 100

All scavenging analyses were conducted in triplicate and the values are mentioned as the result mean values ± standard deviation (S.D.).

2.3.4. Cell culture

HaCaT cells, HEK 293 cells and Raw 264.7 cells were obtained from ATCC. They were cultured in DMEM medium containing 10% of FBS and 1% of antibiotics (penicillin-streptomycin) and subcultured every time they reached 80-90% of confluency for HaCaT cells and HEK 293 cells, 70% of confluency for Raw 264.7 cells. All cells were incubated at 37°C and 5% of CO_2.

2.3.5. Cell viability assay

The toxicity of AIE was analyzed using WST-1 reagent after treatment of HaCaT cells, HEK 293 cells and RAW264.7 cells with various concentrations of AIE. All cell lines were plated in 96 well plates separately at a concentration of 1 X 10^4 cells by well in 100 µl of DMEM for overnight. After 24h of incubation, cells were treated with different concentrations of AIE (100, 150, 200 µg/ml) for 24 h. After treatment, the supernatant in each well was removed and replaced by fresh prewarmed media. Additionally, 10 µl of WST-1 reagent was added in each well and the plates were incubated in dark at 37°C for further 3h. After incubation, viable cells were quantified using ELISA microplate reader by measuring the absorbance at 460 nm.

2.3.6. NO assay

For the NO production, RAW 264.7 macrophages were cultured in a 24 wells plate for 24h at a final concentration of 5 X 10^4 cells by well overnight. After 24h of incubation, RAW 264.7 cells were later challenged with 50 µg/ml and 150 µg/ml of AIE for 4h then stimulated with 1µg/ml LPS for further 24h. The produced NO was established by quantifying the nitrate accumulated in the collected supernatant in which we added 1:1 volume of Griess reagent. Elisa microplate reader was used for the quantification of NO at 540nm.

2.3.7. Western Blot analysis

Initially, RAW 264.7 cells were plated in 100 mm dishes and incubated for 24h or 36h at 37°C. The next day, they were treated with AIE (50 and 150 μg/ml) for 4 h previous to a 30 min or 18 h stimulation period with LPS (1 μg/mL). For the protein extraction, cells were collected in PBS and lysed in an ice-cold lysis buffer. The concentration of proteins in each sample was measured using the Bradford protein assay. During separation in gel electrophoresis, 30 μg of protein mixture from each sample were loaded in the wells of 12% polyacrylamide gels. Once the electrophoresis was finished, the proteins in the gels were transferred onto western blot nitrocellulose membranes for 2h at 50Volts. After the transfer, both sides of the membranes were blocked in 5% of skim milk diluted in PBS-Tween 20 (PBST) for 1 h. The membranes were then washed with PBST three times and incubated with diluted antibodies: p38 MAPK, GADPH, p-SAPK/JNK, Cox-2, TNFα, p-Akt, p-PI3K p85, PI3K p85, p-p38 MAPK, p-ERK1/2, SAPK/JNK, ERK1/2, p-IκBα, Akt, IkBα iNOS, NFkB and p-p65 NFkB (Ser536) overnight. All the used antibodies were obtained from CST (Cell Signaling Technology). After three consecutive washings, the membranes were incubated with second antibodies conjugated with peroxidase for 1h and half, the protein bands were revealed in a dark room.

2.3.8. RT PCR

Raw 264.7 cells were cultured in DMEM and incubated at 37°C for 24h. The following day, cells were treated with 50 and 150 μg/ml of AIE for 4h then stimulated with LPS for 6h. Total mRNA from Raw 264.7 cells was extracted using a Quiagen RNeasy plus kit as described by the manufacturer. Total mRNA (2μg) was converted to cDNA in an equal series of standard 10μl reverse transcription reactions. Obtained DNA was amplified by PCR reactions. Primer sequences that were used to amplify the targeted cDNA fragment are presented in

Table 1. For all amplifications steps, we run 30 cycles with each made by the following steps: DNA denaturing at 94°C for 30 s, primers pairing at 57°C for 30 s, and primers were allowed to elongate at 72°C for 30 s. The obtained cDNA fragments were then separated in a 1.2% agarose gel for 15 min by electrophoresis at 100 V and further revealed under UV light right after the gels were stained with ethidium bromide. GAPDH bands were used as a reference for specific genes targets.

2.3.9. Immunofluorescence

Raw 264.7 cells were seeded at 2 x 10^5 cells/well on cover glasses bottom dishes for 24h. After 24h incubation, cells were treated or not with AIE for 4h and then stimulated or not with LPS for 30 min. Following stimulation and treatment, the cover glass dishes were washed each twice with phosphate-buffered saline (PBS), stained with 1μM 4, 6-diamidino-2-phenylindole (DAPI; Thermo Scientific, Rockford, IL) diluted in methanol for 20 min at 37°C and washed twice with PBS after staining. After blocking for 2 hours with 5% dry skim milk, cells were incubated with anti-p65 first antibody at 4°C for overnight. The following day, dishes were washed twice with PBS, Alexa Fluor 555 conjugated second antibody was added for 2 hours. After incubation with the second antibody, the cover glass bottom dishes were washed with PBS and coverslips were mounted on the slides. Cells were visualized under LSM 510 laser confocal microscope from Zeiss (Jena, Germany).

2.3.10. Statistical analysis

All the experiments in this study were repeated three times before the analysis. The statistical analysis of the obtained data was conducted using GraphPad Prism7 (GraphPad Software, San Diego, CA). A probability value of $p<0.05$ was

considered significant. Our analyzed data are presented as mean value ± standard deviation (SD).

2.4. Results

2.4.1. Gas Chromatography-Mass Spectrum analysis

GC-MS analysis of the methanol extract of AIE showed 9 peaks (Fig.1 A) which indicated the presence of 9 phytochemical constituents (Fig. 1 B). After comparison with the NIST library, the 9 compounds were identified and characterized. 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol (3.95%), liliolide (2.58%), neophytadiene (5.13%), Triterpene lupeol (25.05%), trans-phytol (13.10%), palmitic acid beta-monoglyceride (6.33%), chondrillasterol (9.83%), olean-12-en-3-one (3.64%) and palmitic acid (13.31%) were present in the extract.

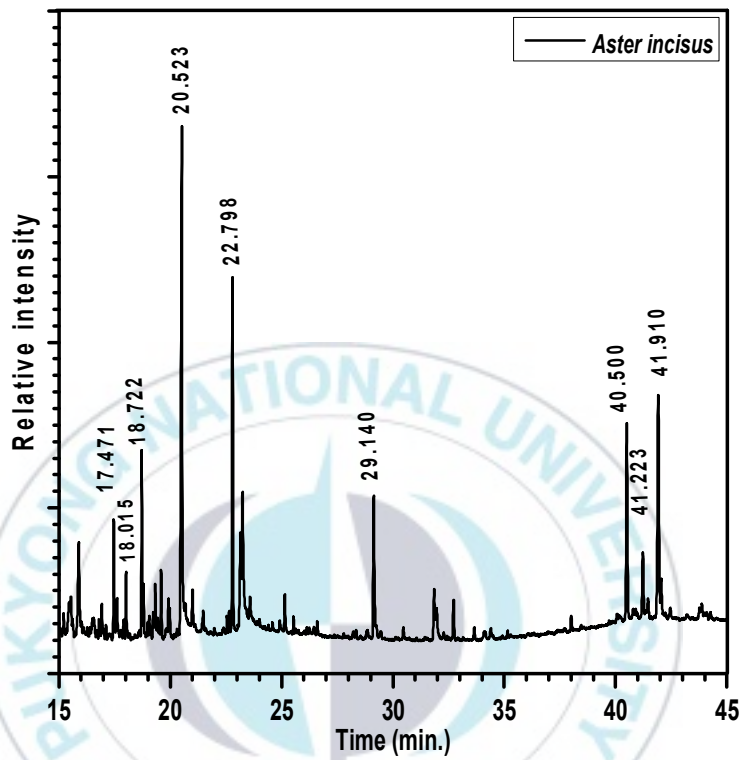

Figure 2.1. Gas Chromatography-mass spectrometry (GC-MS) of *Aster incisus* methanol extract.

RT (min)	Compound name [a]	Molecular formula	Molecular weight	Peak area (%)
17.471	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	3.95
18.015	Liliolide	$C_{11}H_{16}O_3$	196	2.58
18.722	Neophytadiene	$C_{20}H_{38}$	278	5.13
20.523	Triterpene Lupeol	$C_{16}H_{32}O_2$	256	25.05
22.798	Trans-phytol	$C_{20}H_{40}O$	296	13.10
29.140	Palmitic acid beta-monoglyceride	$C_{19}H_{38}O_4$	330	6.33
40.500	Chondrillasterol	$C_{29}H_{48}O$	412	9.83
41.223	Olean-12-en-3-one	$C_{30}H_{48}O$	424	3.64
41.910	Palmitic acid	$C_{30}H_{50}O$	426	13.31

Figure 2.2. Chemical compounds identified by GC-MS in the methanol extract of *Aster incisus*.

2.4.2. Anti-oxidant capacities of AIE

The scavenging capacity of AIE was assimilated with ascorbic acid for comparison as the standard antioxidant. The results in Fig. 2 are a representation of the radical scavenging abilities of AIE extract compared to standard. In our results, the values of radical scavenging activity for DPPH were found to be 18.28%, 61.60%, 78.17%, and 80.07% for 20, 50, 100 and 150 μg/ml of AIE respectively (Fig. 2).

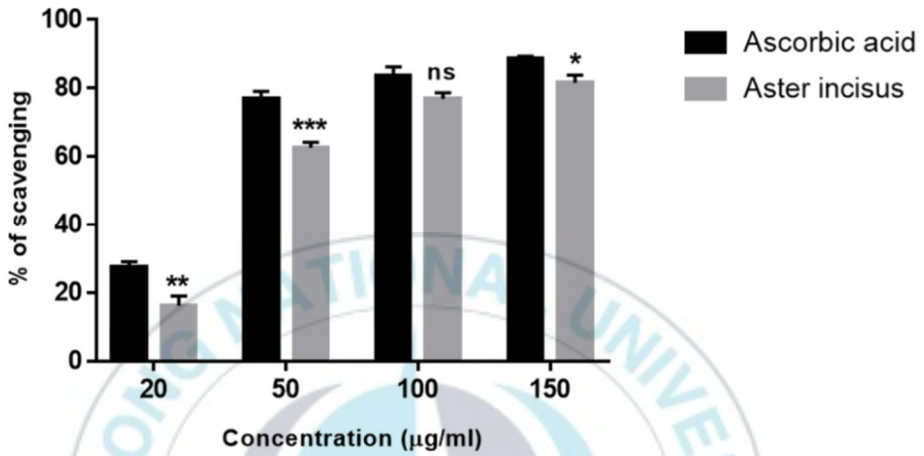

Figure 2.3. DPPH scavenging activity of AIE. DPPH scavenging graph of *Aster incisus* and of the standard, ascorbic acid. The image displays the scavenging percentage of DPPH radical by *Aster incisus*. Statistical differences between Ascorbic acid and Aster incisus were significant at the values of **P* <0.05, ***P*<0.01 or ****P*<0.001.

2.4.3. AIE effects on cells viability

The cytotoxicity effect of AIE was measured using WST-1 assay in Raw 264.7 cells, HaCaT cells, and HEK 293 cells. As shown Fig.3.A, AIE did not affect the cell viability after 24h treatment with AIE. However, AIE was cytotoxic at an increased concentration of 200 μg/ml. These results were used to determine the safe concentrations for further experiments therefore in the subsequent experiments, AIE was used at concentrations up to 150 μg/ml.

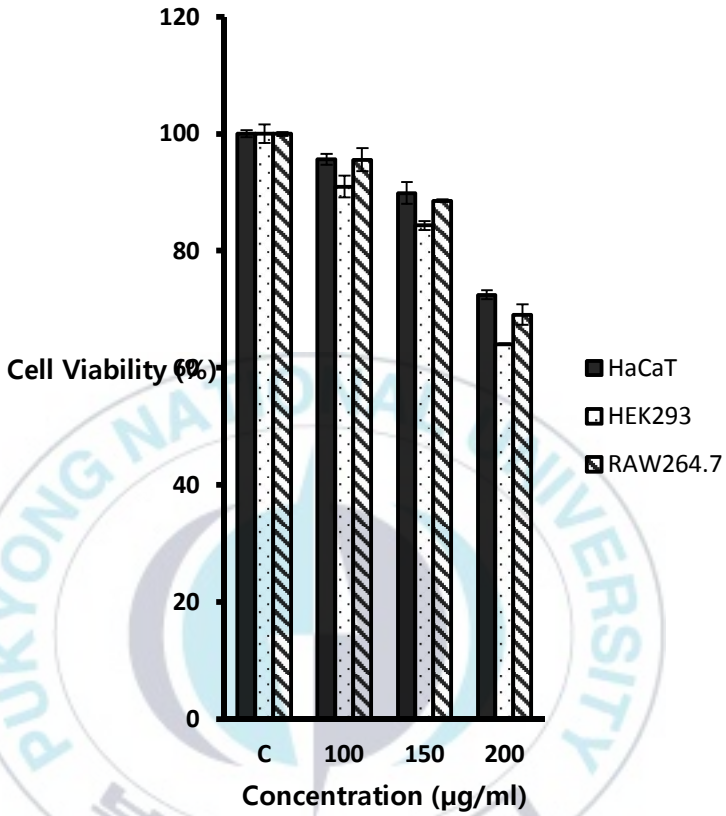

Figure 2.4. AIE cytotoxicity effects. HaCaT, HEK293, and Raw 264.7 cells were cultured for 24h, treated with *Aster incisus* as shown above and cell viability was obtained by WST-1 assay.

2.4.4. Inhibition of nitric oxide production by AIE

The effects of AIE on the production of nitric oxide in the supernatant media of Raw 264.7 cells were investigated and determined 24 hours after cells were treated with 1μg/ml LPS and different concentrations of AIE (50,150 μg/ml). As shown in Fig.3.B, AIE significantly decreased the production of nitric oxide. We can conclude from these results that AIE can inhibit in RAW 264.7 macrophages the production of nitric oxide when stimulated by LPS.

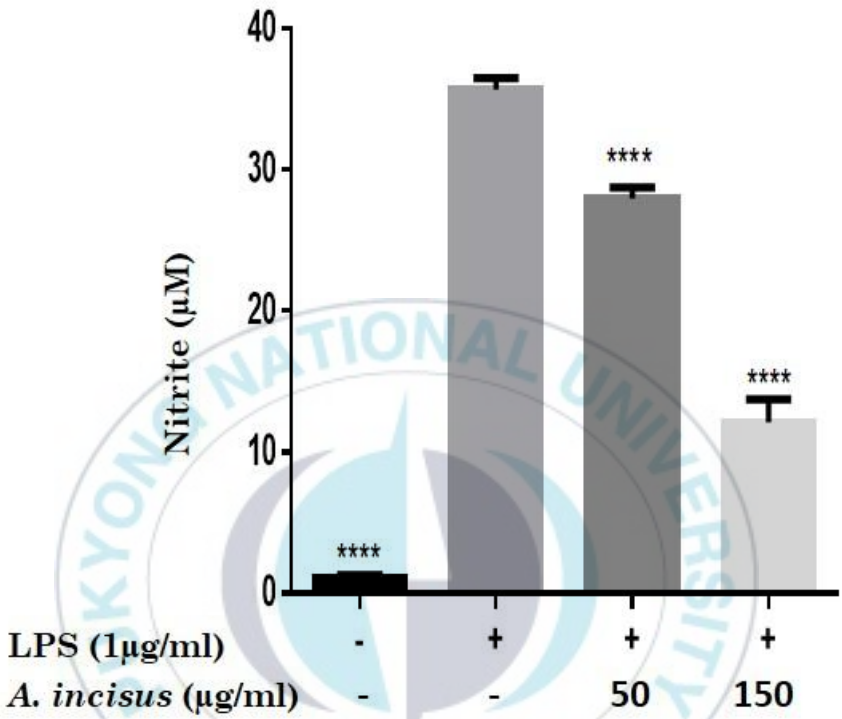

Figure 2.5. Effects of AIE on Nitric Oxide (NO) production. After 24h incubation of Raw 264.7 cells, the macrophages were treated with *Aster incisus* for 4 h followed by action with 1 μg/ml LPS for further 24h. NO concentrations were determined using Griess reagent. Statistical differences between the treatment groups and the control group compared to the LPS-stimulated non-treated group were significant at the values of **** $P<0.0001$.

2.4.5. Inhibition of inflammatory mediators by AIE.

In addition to NO production investigation, we further analyzed the action of AIE in inflammation-related enzymes iNOS, Cox-2, and the expression of cytokines. The analysis by western blot of iNOS, Cox-2 and TNF-α (Fig.4.A) showed a significant reduction in their expression. After the investigation on the expression of these inflammatory mediators we continued by conducting RT-PCR on TNFα, IL-6, IL-1β and GAPDH mRNAs (Fig.4.B) showed that AIE did also significantly inhibit the expression of cytokines at mRNA level. These results suggest that AIE can regulate the gene expression of proinflammatory cytokines.

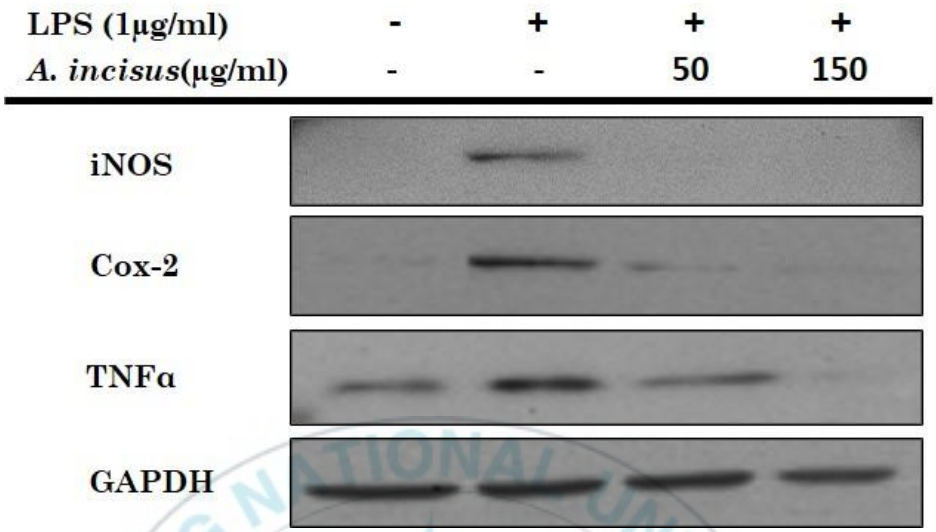

Figure 2.6. AIE effects on inflammatory enzymes. Levels of iNOS and Cox-2 enzymes and of cytokine TNFα were determined using western blot analysis after cell treatment by *Aster incisus* and activation by LPS.

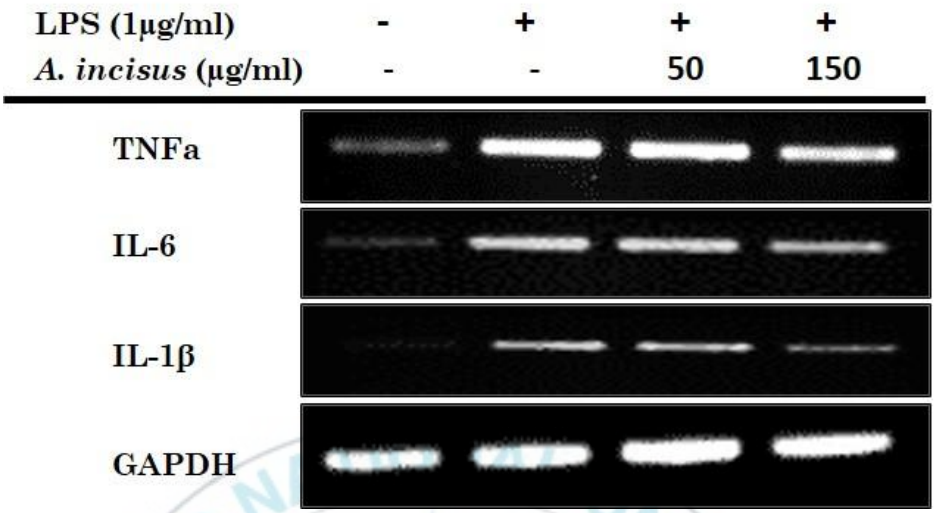

Figure 2.7. AIE effects on inflammatory cytokines. Raw 264.7 macrophage cells were treated by *Aster incisus* for 4h and activated with LPS for 6h. Messenger RNAs (mRNAs) expression of cytokines like TNFα, IL-6, and IL-1β were analyzed by RT-PCR.

2.4.6. Inhibition of NF-kB phosphorylation and translocation in LPS-stimulated Raw 264.7 cells by AIE.

NFkB proteins are heterodimers represented by two monomers p50 and p65. Its activation is prevented by IkBa. The activation of IkBα by phosphorylation induces the translocation of phosphorylated NF-kB from the cytoplasm into the nucleus for regulation of specific transcription factors. To investigate the effect of AIE on the regulation of the NFkB pathway, the p-NFkB protein and pIkBα were examined using Western blotting analysis. As shown in **Fig. 5. A,** p-NFkB and p-IkBα were negatively regulated in a dose-dependent manner then further p-NF-kB was visualized using immunofluorescence to evaluate the effect of AIE on its translocation. The results in **Fig.5. B** showed that AIE inhibited p-NFkB translocation from the cytoplasm to the nucleus. These results reveal that AIE regulates the inflammatory response of Raw 264.7 cells by inhibiting the phosphorylation of p-NFkB and p-IkBa.

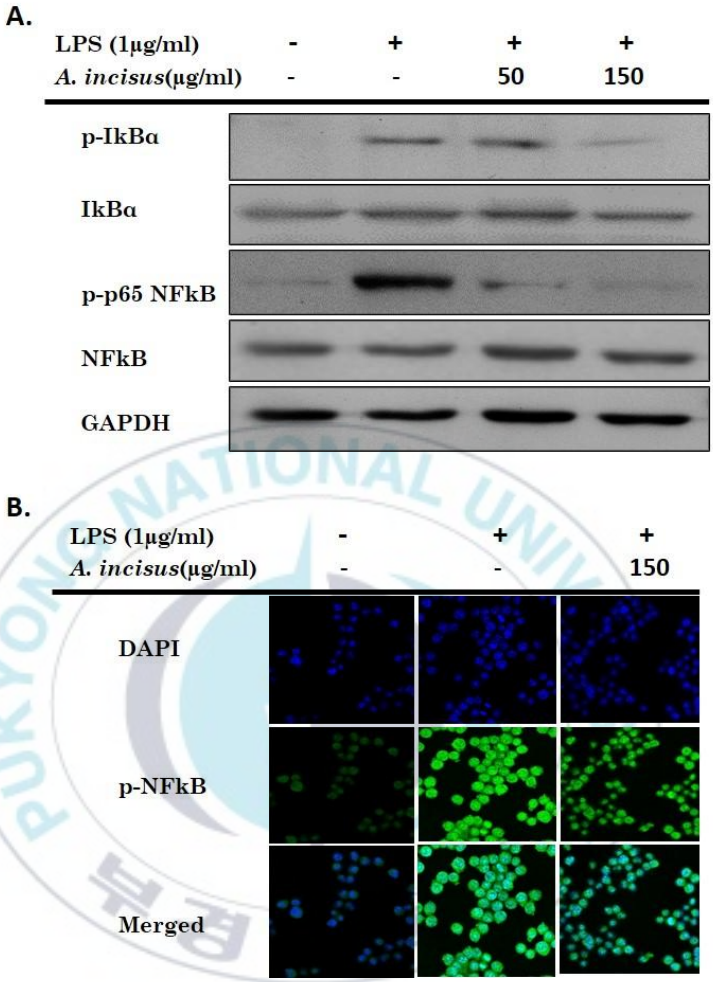

Figure 2.8. Effects of AIE on NF-kB pathway. A. For NF-kB proteins expression, Raw 264.7 macrophages cells were cultured for 36h, treated with *Aster incisus* for 4h, activated with LPS for 30 min and whole cell lysates separated by western blot. **B.** p-p65 translocation was analyzed by Immunofluorescence, cultured Raw 264.7 cells macrophages were treated with *Aster incisus* for 4h, activated with LPS for 30 min. DAPI nuclear staining and anti p-p65 NF-kB were used for p-p65 localization.

2.4.7. AIE inhibited the activation of MAPK pathway.

As MAPK proteins are also crucial in NO and proinflammatory cytokines production and are therefore potential efficient targets of AIE, we later examined the function of MAPKs pathway proteins in the inhibition of NO and cytokines by AIE. RAW264.7 macrophages cells were initially treated with AIE for 4h and later on stimulated with LPS for 30 min. ERK1/2, p38, and SAPK/JNK phosphorylated form were strongly increased by LPS treatment but when cells were treated with AIE after LPS activation, ERK1/2 phosphorylation and SAPK/JNK phosphorylation were decreased in a dose-dependent manner Fig.6.A. Western blot of p-p38 MAPK did not show any significant inhibition of the protein expression in treated LPS-activated Raw 264.7 macrophages.

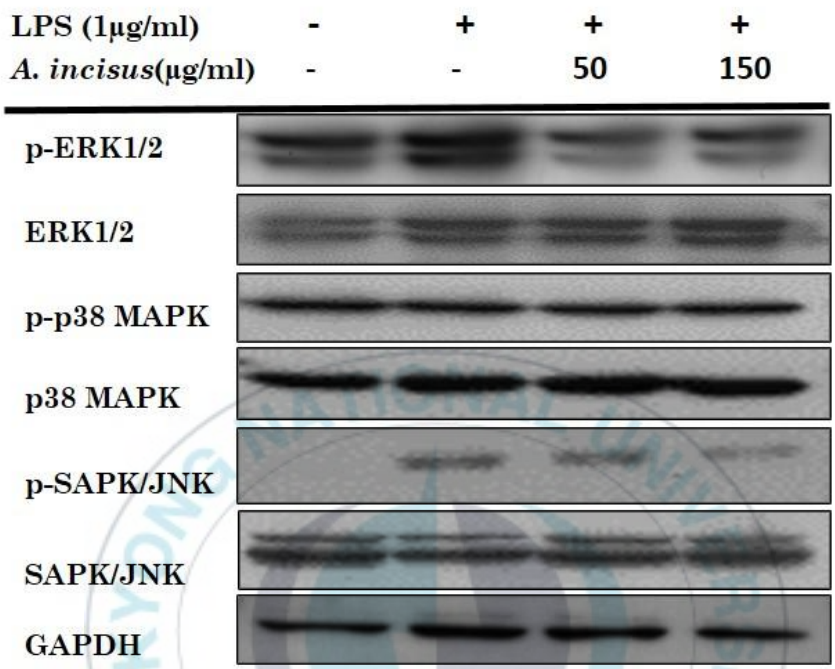

Figure 2.9. Effects of AIE on MAPK pathway. AIE effects on MAPK pathway. Raw 264.7 macrophages cells were cultured in DMEM, treated with *Aster incisus*, activated with LPS and whole cell lysates were obtained and proteins were quantified before being separated by western blot. Phosphorylated and non-phosphorylated proteins of the MAPK kinases pathway were analyzed as shown above.

2.4.8. AIE inhibited PI3K/Akt pathway activation

Activation of the PI3K/Akt pathway results in the regulation of numerous important cell processes including production of main inflammation-related mediators. Our study investigated the effects of AIE treatment on the expression of PI3K and Akt proteins in Raw 264.7 macrophages activated with LPS using western blot analysis. Our results revealed that AIE suppressed the LPS-induced phosphorylation of PI3K and Akt Fig.6.B.

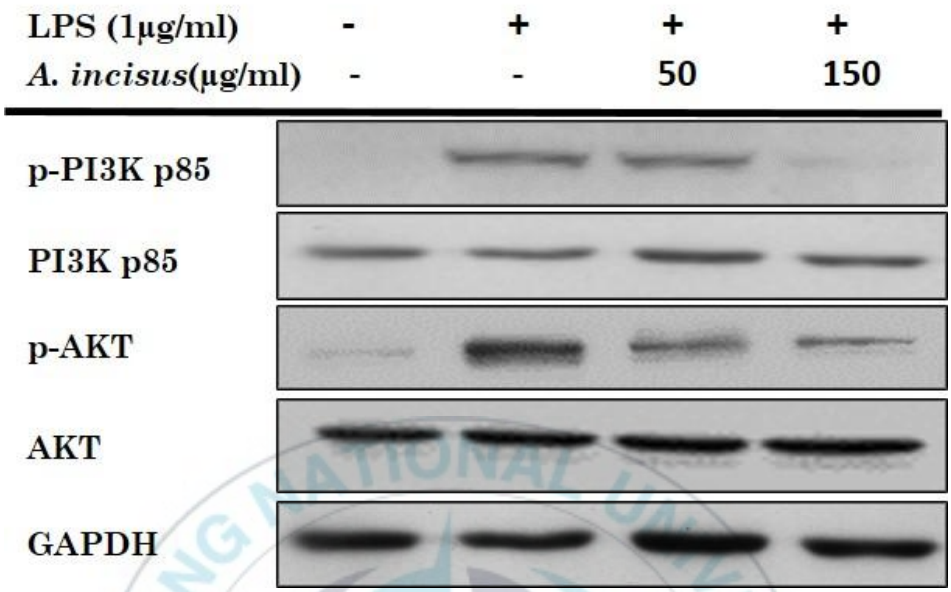

Figure 2.10. Effects of AIE on Akt pathway. *Aster incisus* effects on the PI3K-Akt pathway. Raw 264.7 macrophage cells were treated with *Aster incisus* for 24h, activated by LPS for 30 min followed by protein electrophoresis and viewed by western blot. Phosphorylated and non-phosphorylated proteins of Akt pathway were analyzed using specific antibodies and GAPDH was used as standard protein.

2.5. Discussions

Herbal medicines have been used for decades as remedies for a wide range of diseases. In recent years, published studies showed that plants have various biological activities including anti-inflammatory effects through regulation or inhibition of inflammatory mediators such as cytokines and pathways involved in their production [Cragg *et al.,* 2005; Mann *et al.,* 2001].

Inflammation is defined as an immune defensive response to infection or injury and has been shown plays a crucial and pivotal role in a variety of diseases. When the body fails to resolve the inflammation, tissue injury and loss of function can occur. Inflammation plays a major rule in the complications of many diseases like inflammatory bowel diseases (IBD), atherosclerosis and cancer. It is mostly associated with a bad prognosis and when not well treated it can precipitate the patient death. Inflammation is amplified by the production of inflammatory cytokines that mostly recruit and attract other cells to the site of inflammation [Grivennikov *et al.,* 2011]. Additionally free radicals like NO and H_2O_2 are being produced during inflammation and can lead to vasodilatation and cell damage that in the end will also amplify inflammation, therefore, the inhibition or downregulation of the inflammatory mediators is a major focus in the search for new anti-inflammatory molecules. In this study, we analyzed AIE extract with GC-MS. The results indicate that AIE contained 9 known phytochemicals. We evaluated the antioxidant and anti-inflammatory activities of AIE in LPS-activated RAW 264.7 murine macrophages. Results from our study demonstrated that AIE significantly scavenged DPPH and inhibited the production of NO together with the downregulation of iNOS enzyme expression.

Inflammatory molecules like prostaglandins and cytokines have all been shown to be supporting the malignant phenotype in cancer development. Highly expressed

IL-1β have been presented in many human cancers such as lung, breast, melanoma, colon, neck and head cancers. TNFα is destructive to tumor blood vessels and induces cell death by necrosis at high concentrations while elevated levels of IL-6 are also present in numerous tumors such as colorectal cancer, gastric carcinoma, and Hodgkin lymphoma. Considering all the data accumulated about inflammatory cytokines and their function in many pathologies specifically in cancer it is therefore very important to control inflammation by finding more efficient compounds that can inhibit triggered inflammation [Yamamoto *et al.,* 2001; Siveen *et al.,* 2009; Rossol *et al.,* 2011; Candan *et al.,* 2003]. In the current study, we investigated the effects of AIE in Raw 264.7 cells stimulated by LPS and AIE markedly suppressed the release and expression of IL-6, IL-1β and TNFα.

To further investigate the anti-inflammatory function of plants we need to understand pathways related to the production of inflammatory mediators. Numerous studies demonstrated that specific transcription factors are responsible for the regulation of a large number of molecules and proteins from activated macrophages. NFkB transcription factor family is a crucial pathway in inflammatory response processes, therefore, inhibition of activated NFkB units in the immune defense system is nowadays considered to be a major therapeutic target for the decrease of intense inflammatory responses. Beside NF-kB pathway activation, MAPK and Akt pathways have also been confirmed to be major players in the expression of numerous proinflammatory genes [Shao *et al.,* 2013; Barnes *et al.,* 1997]. After stimulation with LPS through TLR4, proteins from these pathways are activated by phosphorylation and their activated forms can further regulate their specific transcription factors targets. Therefore, both pathways signaling cascades are therapeutic targets for the development and production of efficient anti-inflammatory substances. Our results revealed that AIE partially inhibited phosphorylation of MAPK proteins: ERK and SAPK/JNK, but not p38 MAPK. We

also were able to find that AIE attenuated expression of phosphorylated p65 protein and degradation of p-IkBα. Immunofluorescence images showed that AIE is able to regulate the NFkB pathway in another way by blocking the nuclear translocation phosphorylated p65 [Caivano, 1999; Chen *et al.,* 1999; Kim *et al.,* 2013a; Kim *et al.,* 2013b; Lee *et al.,* 2012].

In conclusion, as actual scientific studies have shown the importance of controlling inflammation in various disease, we investigated the potential antioxidant and anti-inflammatory effects of AIE in Raw 264.7 cells stimulated with LPS. As shown in Figure 7, AIE successfully inhibited proinflammatory cytokines via 3 different pathways. Our results demonstrated that AIE is a promising source of anti-inflammatory compounds.

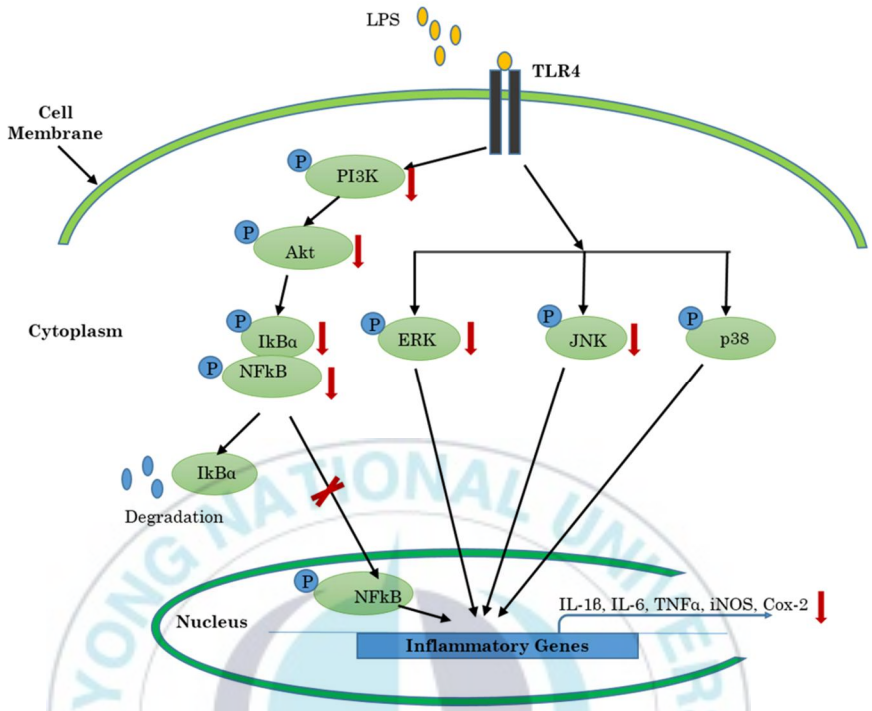

Figure 2.11. Schematic representation of the inhibition mechanisms by AIE in LPS-induced inflammation.

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CHAPTER THREE

Anticancer effects of *Aster incisus* in AGS gastric adenocarcinoma cells.

CHAPTER 3

Induction of apoptosis and G1 phase cell cycle arrest by *Aster incisus* in AGS gastric adenocarcinoma cells

3.1. Abstract

Aster incisus has traditionally been used for medicinal purposes in South Korea. In this study, we evaluated the potential anticancer effects of a methanolic extract of *Aster incisus* (AIE) in a normal human cell line (HaCaT keratinocytes) and in 4 different types of human cancer cell lines (A549, lung cancer; Hep3B, liver cancer; MDA-MB-231, breast cancer; and AGS, gastric cancer). The HaCaT, A549, Hep3B, MDA-MB-231 and AGS cells were treated with various concentrations of AIE and following treatment, cell survival was evaluated. Additional analyses, such as WST-1 assay, western blot analysis, DAPI staining, flow cytometry, immunofluorescence staining and wound healing assay were performed to elucidate the mechanisms and pathways involved in the cell death induced by AIE. Treatment with AIE induced morphological changes and considerably reduced the viability of the both normal and cancer cell lines. Further analysis of the AGS gastric cancer cells revealed that AIE led to the induction of apoptosis and a high accumulation of cells in the G1 cell phase following treatment with AIE in a dose-dependent manner. The results also revealed that AIE successfully suppressed the migration of the AIE-treated AGS cells, increased the expression of pro-apoptotic proteins, and decreased the expression of the anti-apoptotic proteins, Bcl-2 and Bcl-xL.

3.2. Introduction

In South Korea, cancer has been reported to be one of the leading health issues since the 1980s. Currently, lung cancer is the most diagnosed in Korean men followed by gastric cancer, colorectal, liver, and prostate respectively. These previously cited cancers were expected to lead as the major cause of death in men in 2016 together with pancreatic cancer [Oh *et al.,* 2016]. The situation is different in women where the five most diagnosed cancers are breast, thyroid, colorectal, gastric and lung cancers. It has been projected that in 2016 lung, colorectal, pancreatic, and stomach cancers will be the most killing cancers among women in South Korea [Kyu-Won *et al.,* 2017].

Citing the World Health Organization (WHO) reports, it is believed that 80% of the population from developing countries like Asia and Africa are still using traditional herbs and plants as medicine for their health-related trauma [WHO, 2013; Ma *et al.,* 2005]. Pharmaceutical companies rely more now on experiments conducted on various traditionally known medicinal plants for finding new molecules and bioactive substances more efficient against diseases [Padmaje *et al.,* 2002; Bais, 2013] There has been an increase of concerns associated with fatal side effects of some of the medicine available on the market and this led researchers to focus more on studying traditional medicinal plants. Considering the fact that only 1% of more than 500,000 known plant species have been investigated, the need for novel medicinal bioactive compounds is substantial [Cragg *et al.,* 2005; Mann, 2001].

Gastric cancer has been reported as the fourth most diagnosed cancer all over the world and the second most lethal in cancer patients [Parkin *et al.,* 2005; Jemal *et al.,* 2011; Siegel *et al.,* 2014]. The incidence rate of gastric cancer is remarkably elevated in Eastern Asia, Eastern Europe, and South America [Fock, 2014; Satolli *et al.,* 2015]. At present, the treatment for gastric cancer includes surgery,

chemotherapy, radiotherapy, chemoradiation and targeted therapy, but the prognosis is still poor [Yoong *et al.,* 2011; Cravo *et al.,* 2015; Davidson *et al.,* 2015; Lee *et al.,* 2012]. In addition, the effects of current chemotherapeutic drugs are not particularly good and they have various side effects. Therefore, it is important to search for new agents from natural sources, which have the ability to treat gastric cancer [Cragg *et al.,* 2009 Yin *et al.,* 2013; Bishayee, 2012; Amin *et al.,* 2009; Cragg *et al.,* 2005].

Cancer is characterized by loss of regulation in cell cycle followed by uncontrolled cell growth [Nakanishi *et al.,* 2006; Jin *et al.,* 2005]. Cancer cells will grow and constitute a tumor in some types of cancer. After a while, cancer cells can leave the primary site of establishment and invade other organs of the body. This processes is called metastasis and is one of the reason cancer is hard to cure. The control of cell cycle by the induction of cell death through activation cell cycle arrest or the activation of apoptosis is the major aim of cancer experiments. Various proteins regulate the cell cycle. The interaction between cyclins and specific cyclin-dependent kinase (CDK) allow the cell to progress from one phase to another (Morgan, 1995). The complexes which play a significant role in G1/S cell phase transition are cyclin D1,3/CDK4,6 and cyclin E/CDK2. These cyclin/CDK complexes are activated and regulated via phosphorylation.

Apoptosis is an extraordinary controlled cell death mechanism by which cells are eliminated without inducing inflammation around the dying cell [Hassan *et al.,* 2014; Cory *et al.,* 2002]. Apoptotic cell death is triggered by the interaction between ligands and membrane extrinsic death receptors or by intrinsic mitochondrial-mediated pathway [Langerak *et al.,* 2011; Al-Ejeh *et al.,* 2010]. While the extrinsic pathway is triggered by ligand-receptor interaction, the intrinsic pathway is initiated by intracellular processes like cell stress and lead to the release

of cytochrome C from mitochondria which will lead to the activation of apoptosis through a number of cascade reactions in the cell. The downstream caspases activation results in the formation of apoptotic bodies, DNA fragmentation, and chromatin condensation. Dying cells ultimately shrink and then they are removed by phagocytic cells [Branzei *et al.,* 2008; Jackson *et al.,* 2009].

Aster incisus, also known as *Kalimeris incisa*, is a flower of the Asteraceae family. It is an aromatic constant plant that mostly grows in many regions of the Northern Hemisphere. Plants belonging to Asteraceae family have been used for traditional medicinal purposes however, there is no report on the use of *Aster incisus* for the treatment of cancer or the effect of *Aster incisus* in gastric cancer.

In the current study, we evaluated the anti-cancer effect as well as the possible signaling pathways involved in the cell death of human gastric adenocarcinoma AGS cells treated with *Aster incisus* methanol extract (AIE).

3.3. Materials and Methods

3.3.1. Cell culture and cell viability assay

HaCaT, A549, Hep3B, MDA-MB231 and AGS cells, all purchased from ATCC (Manassas, VA, USA), were maintained in Dulbecco's Modified Eagle's Medium (DMEM, HyClone) and Roswell Park Memorial Institute 1640 Medium ((RPMI, HyClone) and both media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin (100 U/ml penicillin and 10 μg/ml streptomycin) (PAA Laboratories GmbH, Pasching, Austria) and incubated at 37 ℃ in a 5% CO2 in a humidified air atmosphere. For, the cell viability assay, both cells separately were plated into 96-well plates at a final concentration of 10^4 cells per well and were incubated for 24 h. Both cells were later treated with different concentrations (50, 100 or 150 μg/ml) of AIE for further 24 h. AIE (voucher no. 016-001) was purchased from Korean Plant Extract

Bank (KPEB, Cheongju, Korea) with 99.9%HPLC purity. After 24h treatment, media was removed, replaced with fresh media and after 10 μl of WST-1® solution was added to each well followed by 3h of additional incubation. Cell viability was determined by reading the absorbance using an ELISA microplate reader (Molecular Devices, Silicon Valley, CA, USA) at 460 nm and the percentages of inhibition were calculated.

3.3.2. DAPI staining

HaCaT, A549, Hep3B, MDA-MB231 and AGS cells were plated on cover-glass bottom dishes and incubated for 24h. The following day, cells were challenged with 150 μg/ml of AIE and were incubated again for additional 24 h. After the additional 24h of incubation, the cells were washed up one time with phosphate-buffered saline (PBS) buffer (135 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate, 1.4 mM potassium dihydrogen phosphate) and then stained with DAPI (4',6-diamidino-2-phenylindole) solution (1μg/ml) diluted in methanol. After incubation in the dark at 37 ℃ for 20 min, the dishes were rinsed with PBS and fixed with 4% formaldehyde for 15 min at room temperature. Fixed cells were mounted on the slide with Prolong Gold Antifade Reagent and observed under a ZEISS LSM 710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

3.3.3. Western blot analysis

AGS cells were seeded in 100mm dishes, treated with the various concentrations (80, 100 or 140 μg/ml), were harvested and later lysed with ice-cold lysis buffer. Proteins were next quantified using CBB solution and were separated in a 12% electrophoresis gel (SDS-PAGE). After electrophoresis, proteins were transferred onto nitrocellulose membranes then the membranes were blocked in PBST buffer (135 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM

sodium phosphate, 1.4 mM potassium dihydrogen phosphate, and 0.5% Tween-20) containing 5% skim milk powder. The blots were probed with the primary antibodies overnight at 4 ℃. The blots were then washed three times in PBST, followed by incubation at room temperature for 1 h with HRP conjugated anti-rabbit or anti-mouse IgG secondary antibody. The blots were then washed in PBST and visualized by ECL detection solutions.

3.3.4. Immunofluorescence staining

AGS cells were plated on cover-glass bottom dishes, incubated at 37 ℃ and later treated with 140 μg/ml of AIE for 24 h. Cells were first stained with 1 μg/ml of a solution of DAPI for 20 min at room temperature. After staining with DAPI, cells were then fixed with 4% formaldehyde for 15 min at room temperature and blocked for 1 h in a blocking solution, including 5% rabbit and mouse normal serums with 0.3% Triton X-100. Fixed and blocked cells were incubated with the primary antibodies (β-actin, cleaved caspase-3) for 3 h and washed three times with PBS buffer. After washing, the cells were treated with 0.1 μg/ml of anti-mouse IgG (H+L), F(ab')2 fragment (Alexa Fluor® 555 Conjugate) and anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor® 488 Conjugate) for 1 h. The stained cells were mounted on the slide with Prolong Gold Antifade Reagent and measured under a ZEISS LSM 710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

3.3.5. Flow cytometry analysis

After treatment with 80, 100 or 140 μg/ml of AIE for 24 h, cells were harvested by trypsinization and the cells were fixed with 70% ethanol at 4 ℃ overnight. After overnight, the cells were resuspended in PBS buffer containing 0.2 mg/ml RNase A and incubated for 1 h at 37 ℃. The cells were then stained with 40 μg/ml propidium iodide for 30 min at room temperature in the dark. The distribution of sub-G1 DNA was analyzed using a BD FACS Verse TM (Becton-Dickinson, Mountain View, CA, USA).

3.3.6. Wound healing assay

For the wound healing assay, AGS cells were seeded in culture-Insert (ibidi, Planegg/Matrinsied, Germany) and cultured until the cells reached confluence. Culture-Insert were removed and the cells were washed with PBS to remove non-adherent cells. We then provided fresh media containing 140 μg/ml of AIE and photographed the plates at 0, 12 and 24h to capture two different fields at each time point on each plate. The average wound width was measured between the two lines representative of cell migration determined by the mean of the furthest and the nearest cells at the leading edge.

3.3.7. Cell invasion assay

Cell invasion of AGS cells was investigated using 24 well plate transwell inserts (8μm pore size, Corning). The inserts were first coated with 100μl of matrigel (BD Biosciences). RPMI media containing FBS in the lower compartment was used as a chemoattractant. During and after treatment by AIE, media in the inserts were replaced with FBS free media and the plate was incubated at 37°C in 5% CO_2 for 24h. After incubation, noninvading cells were removed inside the inserts using a cotton swab and the invading cells in the lower surface were stained with crystal violet. Images were taken using an inverted microscope.

3.3.8. Statistical analysis

Data are presented as the mean ± standard deviation (SD) for the indicated number of separated experiments. The mean of the control was compared with the mean of each individual treatment group by two-way ANOVA using the statistical software GraphPad Prism 7 (Graphpad Software Inc., La Jolla, CA, USA), and a statistically significant difference was set at *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and # $P < 0.0001$.

3.4. Results

3.4.1. AIE inhibited the proliferation of human cancer cell lines.

We examined cell viability of HaCaT, A549, Hep3B, MDA-MB231 and AGS cells treated with various concentrations of AIE and found that AIE had effectively inhibited the growth of both treated cancer cell lines but did not affect the proliferation of keratinocytes normal cells HaCaT cells up to a concentration of 150 μg/ml. The results showed that AIE gradually inhibited the cell viability of cancer cells in a dose-dependent manner, whereas AIE did not significantly affect cell viability in HaCaT cells (Fig. 1A). Furthermore, the morphological changes of all treated cancer cells were visualized under the inverted microscope and the obtained image showed that AIE significantly reduced the AGS cells growth but also affected their morphology (× 100) (Fig. 1D).

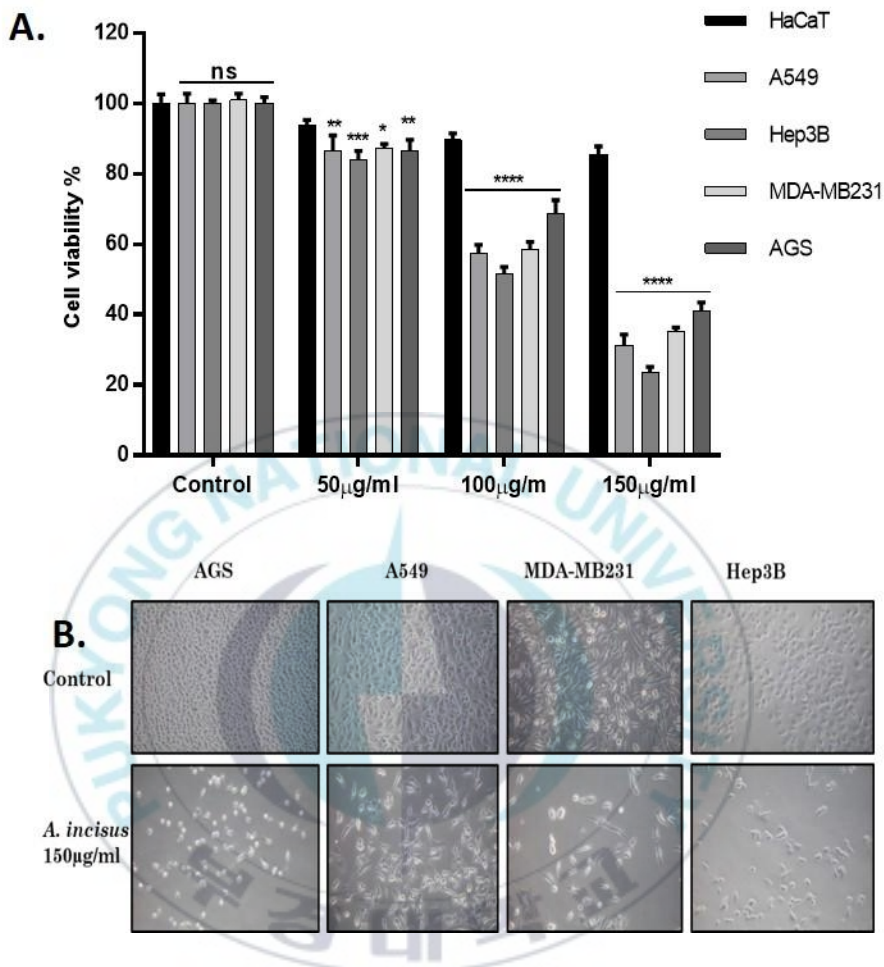

Figure 3.1. Cell viability of cancer cells treated with AIE. A. Cell viability of cancer cells treated with *Aster incisus*. **B.** Cell morphology of cancer cells treated with AIE. Statistically significant difference was set at ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and # $P < 0.0001$.

3.4.2. AIE induced apoptotic changes in treated cancer cell lines

A549, Hep3B, MDA-MB231 and AGS cells were incubated with caspase-inhibitor followed by a treatment with 150 μg/ml of AIE. The WST1 results showed that caspase-inhibitor improved significantly the proliferation of both treated cells (Fig. 1B) demonstrating that caspase-related death pathway might play a major part in the antiproliferation effects of AIE. Additionally, changes in nuclear morphology indicating apoptosis were observed after the cancer cells were treated with 150 μg/ml of AIE and stained with DAPI solution. The images were obtained and analyzed under a ZEISS LSM 710 confocal laser scanning microscope (× 1000). Our results showed that AIE-treated cells were characterized by apoptotic morphological changes such as the nuclear condensation and formation of apoptotic bodies, whereas untreated cells exhibited normal round-shaped nuclei (Fig. 1C).

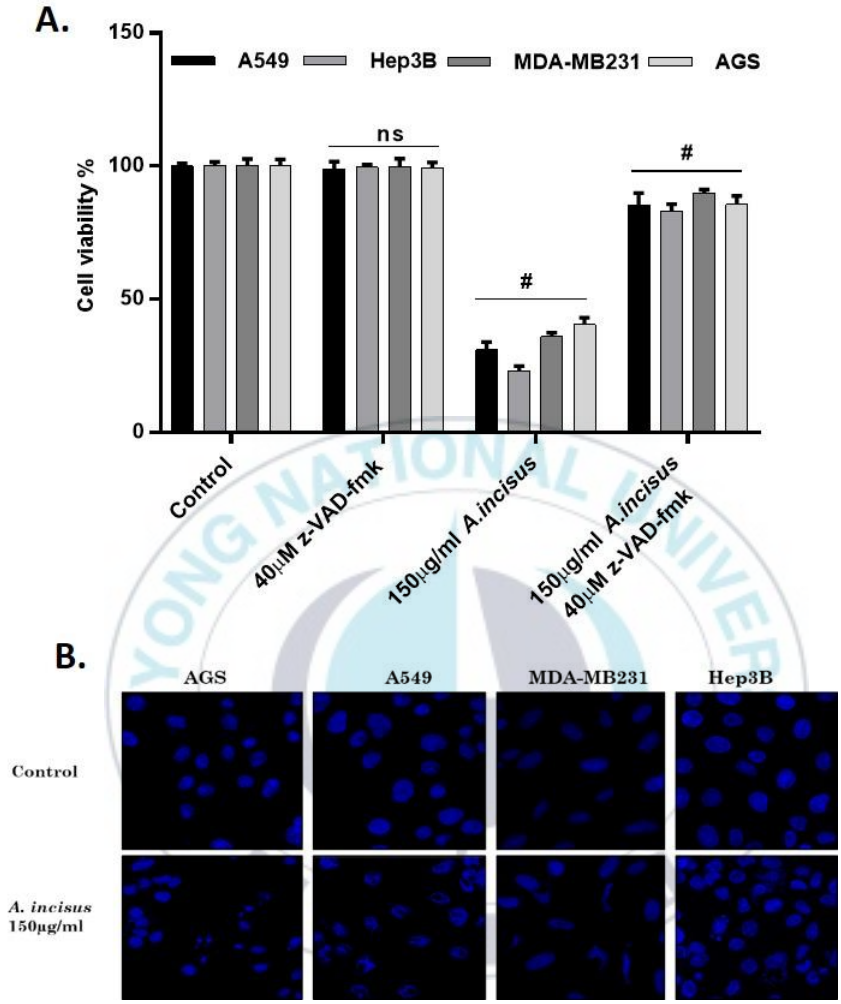

Figure 3.2. Cell viability and DAPI staining of cancer cells treated with AIE. A. Cell viability of cancer cells challenged with caspase-inhibitor and treated with AIE. **B.** DAPI staining of cancer cells treated with AIE. Statistically significant difference was set at *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and # $P < 0.0001$.

3.4.3. AIE induced apoptosis in AGS gastric cell lines.

After the initial investigation into the four different cancer cell lines, we then focused on AGS cell lines specifically.

Following the results above, we analyzed the expression of apoptosis-related proteins by western blot. Our results showed that proteins from both intrinsic and extrinsic pathways varied in their expressions in a dose-dependent manner. Proapoptotic proteins expression like Bax, Bak, tBid, Bad, AIF, EndoG, Cytochrome C, cleaved Caspase-8,-9,-3 and cleaved PARP were significantly downregulated meanwhile antiapoptotic proteins like FLIP, Bcl2 and BclxL were highly expressed in treated AGS cells compared to non-treated cells. These results confirmed that AIE induced apoptosis in AGS treated cells (Fig. 2A). To support even more these results, we conducted an immunofluorescence staining assay of AGS cells with the caspase-3 antibody. Our results revealed that caspase-3 protein was highly expressed in AGS cells treated with AIE compared to control (Fig. 2B). With all these results, we can definitely conclude that AIE inhibited the proliferation of AGS gastric cell line by the induction of apoptosis.

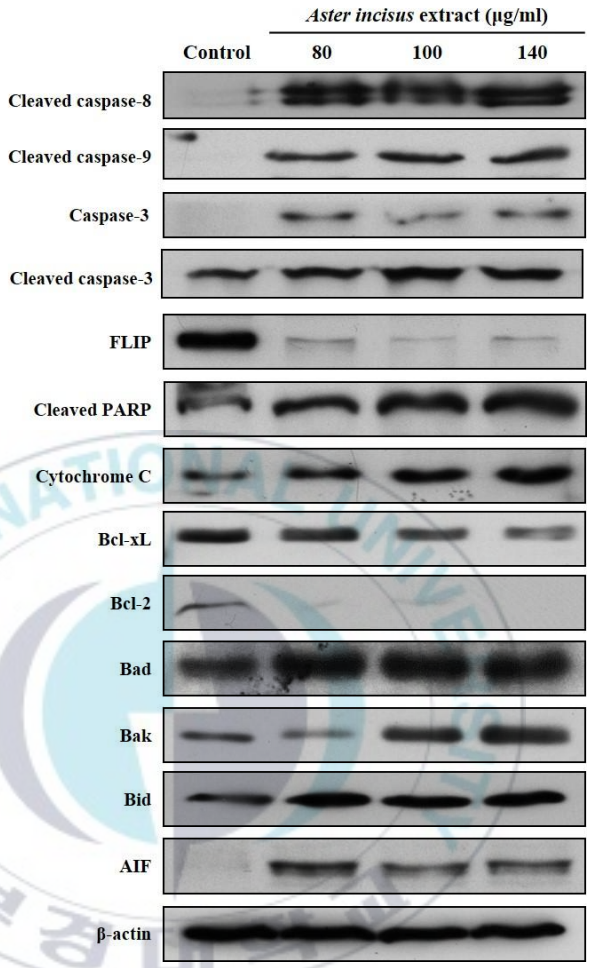

Figure 3.3. Expression of apoptotic proteins in AGS cells treated with AIE. Apoptotic proteins analyzed by western blot in AGS treated cells. AGS cells were treated with 80, 100, and 140μg/ml of AIE for 24h followed by the extraction of proteins. After quantification, proteins were separated in gel electrophoresis and obtained results revealed the level of expression of different analyzed apoptotic proteins **B.** Immunofluorescent staining of AGS treated cells with caspase-3. AGS cells were treated with 140μg/ml of AIE and stained with DAPI, caspase-3 antibody, and β-actin. Images were obtained using a confocal microscope.

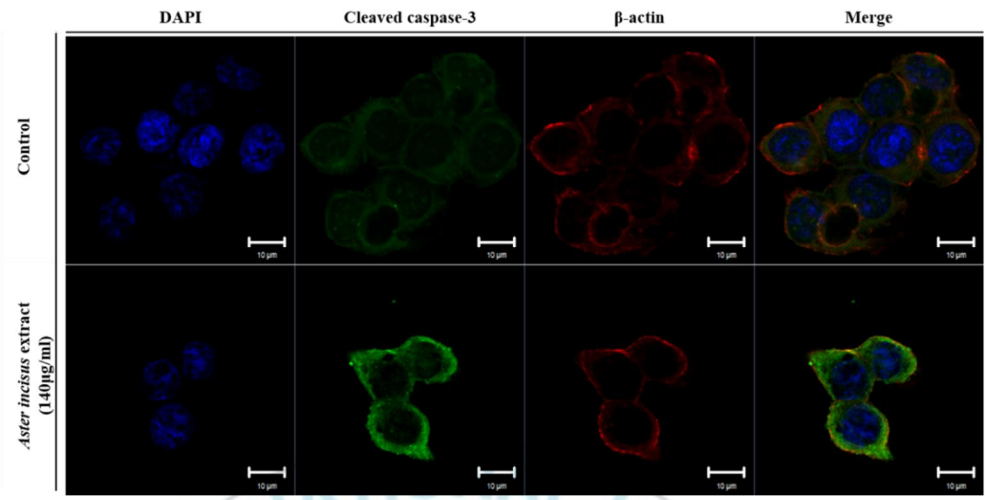

Figure 3.4. Immunofluorescence expression of apoptotic cleaved-caspase 3 in AGS cells treated with AIE. Immunofluorescent staining of AGS treated cells with caspase-3. AGS cells were treated with 140μg/ml of AIE and stained with DAPI, caspase-3 antibody, and β-actin. Images were obtained using a confocal microscope.

3.4.4. AIE induced G1/S phase cell cycle arrest in AGS gastric cancer cells.

We later investigated the effects of AIE on cell cycle phases by using PI staining in FACS analysis. This assay allowed us to quantify the percentage of the different phases of cell cycle. For this analysis, AGS cells were treated with 80, 100, 140 µg/ml of AIE for 24 h and later stained with PI. In our flow cytometry analysis, the results showed that cells were accumulating in G1/S phase in a dose-dependent manner with 21.3 % for non-treated cells and for treated AGS cells 29.6 %, 47.2 %, 73.6 % for 80µg/ml, 100 µg/ml and 140 µg/ml respectively (Fig. 3A). To support the obtained results, we conducted western blot analysis on proteins involved in G1/S phase especially cyclins, cdks, and CDK-inhibitors. Our results showed that AIE downregulated the expression of proteins involved in G1/S phase progression such as Cyclin D1, Cyclin D3, Cyclin E, CDK2, CDK4, CDK6 while increasing the expression of CDKs related inhibitors like p16, p18, p21, and p27 (Fig. 3B). Considering these results, we can conclude that AIE successfully induced cell cycle arrest in AGS treated cell causing the accumulation of the cells in G1/S phase (Fig. 5).

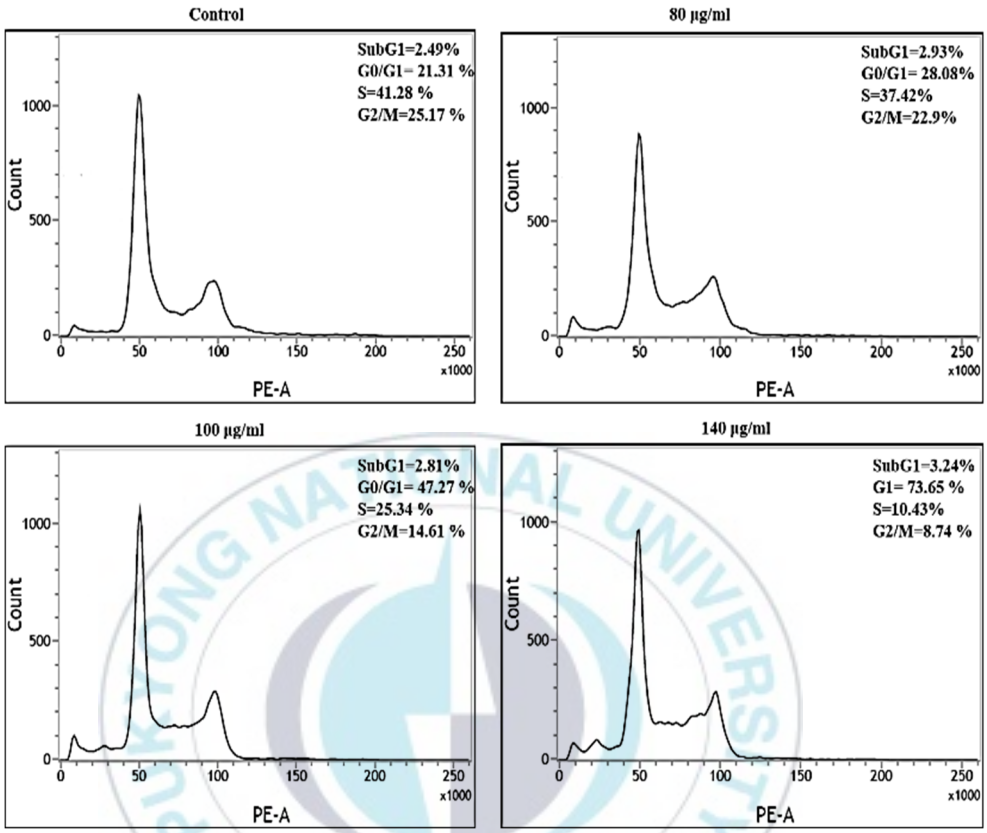

Figure 3.5. Cell cycle analysis in AGS cells treated with AIE. Flow cytometric of AGS cells treated with various concentrations of AIE. AGS cells were treated with 80, 100, and 140µg/ml of AIE and later stained with propidium iodide. Percentages of each cell cycle phase were obtained by flow cytometric analysis.

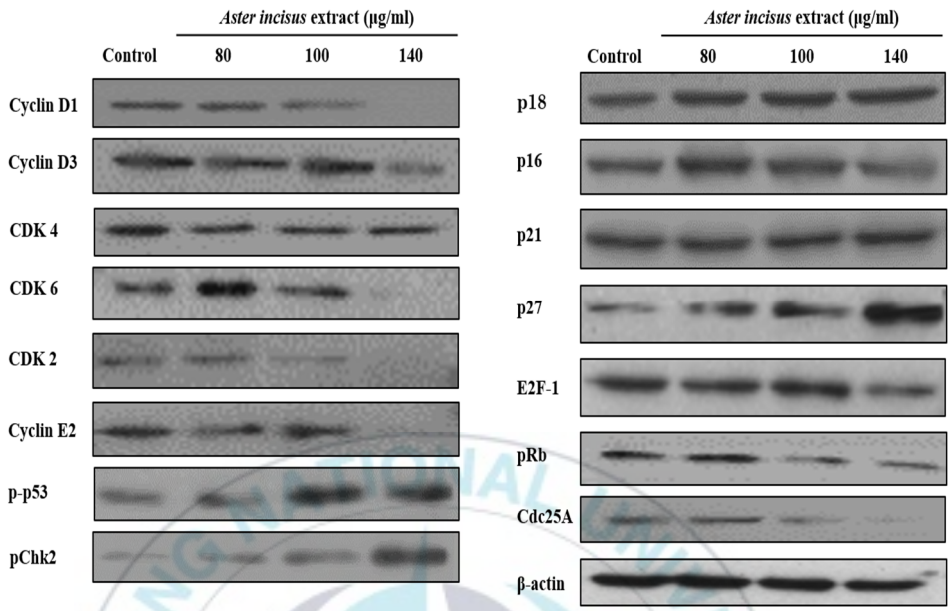

Figure 3.6. Western blot analysis in AGS cells treated with AIE. Expression of G1 phase-related proteins in western blot analysis.

3.4.5. AIE inhibited cell migration and invasion in AGS cells

To explore the effect of AIE on cell migration wound healing assay was conducted. For wound healing assay, after 24h of treatment images were taken using an inverse microscope to record the progression of cells from the moment of treatment to 24h. The results in Fig. 4A shows that AIE successfully inhibited the migration of treated AGS cells. Treated AGS cells were not able to cover the gap between cells while non treated cells filled the surface within 24h. These results attest that AIE is able to inhibit the migration of AGS cancer cells.

For invasion assay, AIE successfully inhibited the invasion of AGS cells through matrigel layer. These results suggest that AIE has the ability to inhibit the degradation of extracellular matrigel by proteases from cancer cells, therefore, preventing the cancer cells to spread in other organs (Fig. 4B).

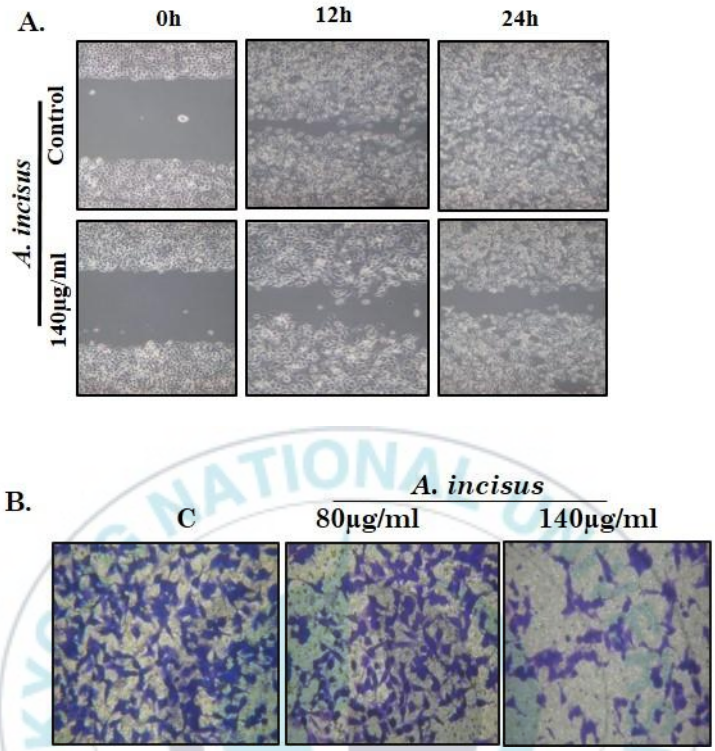

Figure 3.7. Cell migration and cell invasion assays in AGS cells treated with AIE. A. Wound Healing assay of AGS cells treated with *A. incisus*. AGS cells were treated with 140μg/ml of AIE and regularly monitored to capture the migration of cells. Pictures were taken using inverse microscope right after treatment then at 12h and finally after 24h. **B.** Cell invasion assay of AGS cells with *A. incisus*. AGS cells were plated in inserts coated with Matrigel, treated with AIE, incubated for 24h. Noninvading cells were removed and invading cells stained and observed under an inverted microscope.

3.5. Discussion

AIE belongs to Asteraceae family and has been used for medicinal purposes in South Korea. However, there is no report on the use of AIE for the treatment of cancer cells. We investigated the potential anticancer effects of AIE in four different cancer cell lines, A549, Hep3B, MDA-MB231 and AGS cells. Our results suggest that AIE reduces cell viability in a dose-dependent manner (Fig. 1A).

Apoptosis exhibits well-defined morphological changes that result from following the activation of specific signaling pathways. The apoptotic cells show DNA fragmentation, nuclear condensation and blebbing of the plasma membrane that results in the release of small membrane-enclosed particles known as apoptotic bodies [Kantari *et al.,* 2011; Brunelle *et al.,* 2009]. We found that AIE initiated nuclear condensation in both cancer cell lines treated with 150 µg/ml (Fig. 1D). MTT assay results of the cells challenged with caspase-inhibitor before treatment cleared demonstrated that the inhibitor significantly increased the survival of AGS gastric cancer cells treated with AIE (Fig. 1B)

The two major most common pathways involved in apoptosis are the extrinsic and the intrinsic pathway. The first pathway (extrinsic pathway) is initiated through the interaction between cellular death receptors and their respective ligands. Following the interaction, intracellular caspase 8 is recruited by the intracellular part of the death receptor and is activated. Activated caspase 8 can now initiate apoptosis through cleavage of effector caspases or cleavage of Bid into tBid [Kim *et al.,* 2015]. Our results revealed that AIE increases the expression of cleaved caspase-8 and Bid in AGS cells (Fig. 2A).

The second pathway (intrinsic pathway) is started by different stimuli that do not require transmembrane receptor such as cellular stresses and in which Bcl-2

family proteins play a crucial role. Bcl-2 family proteins are a large group of proteins that include anti-apoptotic proteins (Bcl-2, Bcl-XL, Mcl-1, and Bcl-w), pro-apoptotic proteins (Bax and Bak), and finally the BH3-only proteins (Bad, Bid). BH3-only proteins monitor cellular processes and transduce both extrinsic and intrinsic death signals to the Bcl-2 family proteins at the outer mitochondrial membrane (OMM) to modulate mitochondrial outer membrane permeability (MOMP). The increase of MOMP results in the release of cytochrome c, AIF, and EndoG into the cytosol. Cytochrome c and Apaf-1 complex cleave procaspase 9 and the cleaved Caspase9 cleaves Procaspase3 and activates Caspase3 [Dela Cruz *et al.,* 2014]. AIF and EndoG translocate in the nucleus where they are involved in DNA fragmentation. Many in vitro studies have confirmed that the induction of apoptosis leads to the death of cancer cells [Cazal *et al.,* 2010; Kastan *et al.,* 2004 Malumbres *et al.,* 2009].

In the present study, we observed morphological changes and nuclear condensation. Our results showed that AIE induced the mitochondrial dysfunction via increased expression of Bak, pro-apoptotic proteins and decreased expression of Bcl-2 and Bcl-XL, anti-apoptotic proteins in treated AGS cells and in a dose-dependent manner (Fig. 2A). These western blot results and immunofluorescence results of caspase 3 as shown in figure clearly indicated a significant increase of proapoptotic proteins and inhibition in antiapoptotic proteins in treated cells compared to non-treated cells and these results were confirmed with immunofluorescence results of caspase 3 expression in treated AGS cells (Fig. 2B).

The cell cycle is the most biological conserved process through the years. The cell cycle is regulated by numerous proteins and variation in the cell regulation usually results in cancer. Cancer can be caused by different reason such as dysregulation in cellular functions. The human cell cycle is divided into 4 phases:

G1 phase, S phase, G2 phase and M phase. Cyclins and cyclin-dependent kinases (CDKs) are very important in the cell division, therefore, the expression and the regulation of these proteins play a crucial role in the ignition of cancer or in cancer treatment. Each cell cycle phase is regulated by specific cyclins and CDKs. The interaction between cyclins and CDKscdks allow the cells to progress in their division. Genetic changes in cells such as mutation can evolve in loss of cell regulation. Components of the cell cycle machinery are frequently altered in human cancer. A group of inhibitory proteins, called cyclin-dependent kinases inhibitors (CKIs) or CDK inhibitors, control cyclin-CDK activity thereby restraining cell cycle progression in response to extracellular and intracellular signals. The crucial role of the CDKs has prompted great interest in the development of specific kinase inhibitors that would be expected to block cell cycle progression and induce growth arrest. CDK inhibitors are divided into two major groups: the CDK4 inhibitors named because of the capability to inhibit CDK4 and CDK6 by competing with Cyclin D and includes p16 and p18. The second family is Cip/Kip family (for CDK interacting protein/ Kinase inhibitory protein) which include p21 and p27 [Hoshino *et al.,* 2001]. As different other studies have reported before, the activation of CDKIs is a very effective pathway to inhibit the proliferation of cancer cells [Chen *et al.,* 2001; Harbour *et al.,* 2000]. G1 phase is very important in cell cycle as it gives the signal to the cell to allow it to enter in cell division.

Cyclin D-CDK4/6 complex initiates the phosphorylation of Rb during G1 to S phase transition which leads to the release of E2F-1 by Rb allowing the initiation of transcription and translation. Later in G1 phase, the complex cyclin A-CDK2 and the cyclin E-CDK2 complex in early S phase regulate the transition from G1 phase to S phase. CDKIs, by interfering in the interaction of cyclins with CDKs, trigger the arrest of cell cycle in G1 phase [Kim *et al.,* 2015; Dela Cruz *et al.,* 2014; Cazal *et al.,* 2010]. Many plants used as traditional medicine has been reported to arrest

the cell cycle of various cancer cells lines during the G1/S phase transition [Agami *et al.,* 2000; Petronelli *et al.,* 2009; Wang *et al.,* 2012; Wu *et al.,* 2012].

One of the other most important tumor suppressors is tumor protein p53 which plays a key role in the cellular response to DNA damage. Like Cyclin-CDK complexes, p53 is very important in the transition of cells during division from G1 to S phase. P53 stops the cells in G1 phase after DNA damage by activating CDKIs [Jung *et al.,* 2014, Banin *et al.,* 1998; Reinhardt *et al.,* 2012].
Chk2 (checkpoint kinase 2) protein is also phosphorylated following DNA damage and inactivates in turn cell division cycle 25A phosphatase (Cdc25A). Deactivation of Cdc25A results in the elevation of the phosphorylated (inactive) form of Cdk2, therefore, cells cannot progress into S phase and replicate their DNA [Qui *et al.,* 2011; Barket *et al.,* 2003].

Our results in the current investigation indicated the accumulation of treated AGS cells in G1 phase after flow cytometric analysis. Further results from western blot showed that AIE decreased the expression cyclins and CDKs involved in G1 phase more specifically cyclin D1, cyclin D3, cyclin E, CDK2 CDK 4, CDK 6 but increased the expression of CDKIs p16, p18, p21 and p27 (Fig. 3B).

For deepening our investigation, we analyzed the effect of AIE on cell migration and cell invasion. Migration and invasion are very important steps of metastasis. In a cancer patient, some cancer cells can spread from the initial site to secondary sites. This process called metastasis is made possible by the possibility of to migrate and to degrade the extracellular matrix and spread to other parts of the body through the bloodstream. Once cancer cells start to diffuse, the prognosis for the patient becomes worst and it reduces considerably the chances of survival. Results of our experiments showed that AIE successfully inhibited the migration of treated AGS gastric cancer cell lines in a wound healing assay. AGS cells plated in

inserts coated with matrigel then treated with AIE were not able to migrate. These results revealed that AIE reduced considerably the ability of AGS gastric cancer cells to invade tissues by degradation of extracellular matrix components. We can conclude that AIE has the ability to inhibit the migration of AGS cells and their invasion (Fig. 4).

In conclusion, our results demonstrated that AIE induced apoptosis via both intrinsic mitochondrial-mediated cell death pathway and the caspase-dependent pathway by the activation of caspases in AGS cells. AIE also significantly induced cell cycle arrest in G1/S phase and furthermore, AIE inhibited AGS cells migration and invasion. We present a schematic representation of the pathways by which AIE induces apoptosis and cell cycle arrest in AGS cells (Fig. 5). These findings suggest that AIE would be considered as a potential therapeutic agent for the treatment of gastric cancer.

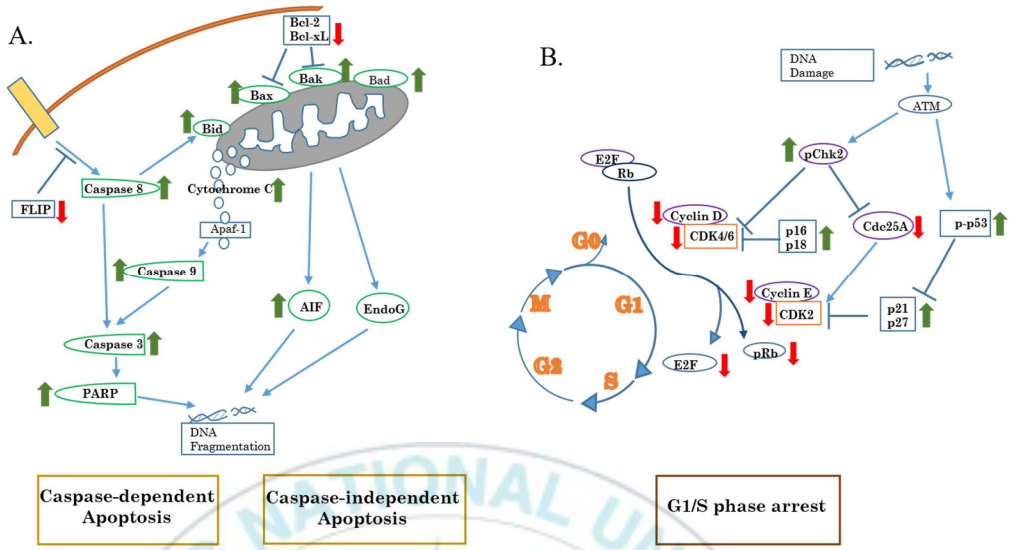

Figure 3.8. Schematic representation of anticancer effects of AIE in AGS cells. **A.** Schematic representation of pathway involved in activation of apoptosis by AIE in AGS cells. **B.** Graphic representation of the pathway involved the cell cycle arrest by AIE in treated AGS cells. AIE induced apoptosis by increasing the levels of pro-apoptotic proteins such as caspases and mitochondrial pro-apoptotic proteins. Anti-apoptotic proteins Bcl2, Bcl-xL, and FLIP were downregulated after AIE treatment. AIE treatment additionally induced cell cycle arrest in treated AGS cells. Cyclins and cyclin-dependent kinases were significantly downregulated while inhibitors proteins were increased.

3.6. References

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CHAPTER FOUR

Macrophages mediate the resistance to fluorouracil in AGS gastric adenocarcinoma

CHAPTER 4

Macrophages mediate 5-FU resistance in AGS gastric adenocarcinoma cells through upregulation of integrin β3, FAK and p-cofilin

4.1. Abstract

Tumor microenvironment (TME) defines development and progression of many cancers. Macrophages are the most predominant cells in TME and play a major role and cancer invasiveness. Gastric cancer is one of the most common cancer in Asia and recently, various cases of resistance to fluorouracil treatment has been reported. In this sudy, we investigated the role of alternatively activated macrophages in the resistance of AGS gastric cancer cells to fluorouracil. Raw 264.7 cells were polarized using IL-4 then were cocultured with AGS cells treated with fluorouracil. Cell viability, Western blot, immunofluorescence and cell invasion were performed for the analysis. Our results demonstrated that polarized macrophages initiated the survival of treated AGS cells and induced the resistantance in AGS by upregulating the expression of integrin β3, FAK and p-cofilin. These results suggested that the inhibition of integrin β3 might improve the survival of gastric cancer patients treated with fluouracil.

4.2. Introduction

The tumor microenvironment is composed by tumor cells and stromal cells like fibroblasts, endothelial cells as well as immune cells, among which macrophages are the most prominent innate immune cells [Grivennikov *et al.,* 2010; Gajewski *et al.,* 2013]. As an essential component of innate immunity, macrophages have multiple functions in both inhibiting or promoting cell proliferation and tissue repair [De Visser *et al.,* 2006]. Two phenotypes of macrophages are now known in tumor microenvironment: Classically activated macrophages or M1-type macrophages release cytokines that inhibit the proliferation of surrounding cells and damage contiguous tissue, and alternatively activated macrophages or M2-type macrophages release cytokines that promote the proliferation of contiguous cells and tissue repair [Wang *et al.,* 2014; Italiani *et al.,* 2014].

M1 and M2 macrophages produce different types of cytokines. M1 macrophages express inflammatory cytokines TNF□α, IL□1β, IL□6 and promote cytotoxic adaptive immunity by up□regulating MHC class II molecules in conjunction with co□stimulatory molecules CD40, CD80, CD86. In contrast M2 macrophages support resolution of inflammation by switching gene expression toward anti□inflammatory molecules such as IL□10, TGF□β, IL□1R type II, IL□1Ra [Burkholder *et al.,* 2014; Solinas *et al.,* 2009].

The M2 macrophages promote tumor development by enhancing proliferation [Vasievich *et al.,* 2011], metastasis [Massague *et al.,* 2016], angiogenesis [Liotta *et al.,* 2001], lymphangiogenesis [Sanchez-Tillo *et al.,* 2012] and immunosuppression [Hao *et al.,* 2012].

Therefore, targeting TAMs represents an efficient strategy for the management of multiple tumors [Hanahan *et al.,* 2011]. However, the relationship between TAMs and chemotherapy efficacy in gastric adenocarcinoma is still obscure.

5FU is an anticancer agent used in the treatment of various cancers including gastric cancer. The major mechanism of the cytotoxicity of 5-FU is the inhibition of nucleotide synthesis. This drug rapidly enters tumor cells, and one of the principal intracellular derivatives of 5-FU, fluorodeoxyuridine monophosphate (FdUMP), forms a covalent complex with thymidylate synthetase (TS), thereby inhibiting the catalytic activity of TS, leading to depletion of the intracellular pools of deoxythymidine mono- and tri-phosphate (dTMP and dTTP) and an increase in the relative levels of the normal precursor dUMP and its anabolic derivative dUTP [Longley *et al.,* 2003; Liu *et al.,* 2001].

In recent years, various studies have reported the increase of resistance of gastric cancer tumor against 5FU. Different cell processes can mediates the chemoresistance such the inhibition of apoptosis or migratory and invasiveness proprieties. EMT increases cell migration, invasion and survival in anchorage-independent conditions [Talbot *et al.,* 2012; Gomes *et al.,* 2011; Voulgari *et al.,* 2009], which are the primary properties of cancer cells that should succeed in metastasis. The critical hallmarks of EMT are the E-cadherin to N-cadherin switch, and the up-regulation of related proteins [Conacci-Sorrell *et al.,* 2002; Schmalhofer *et al.,* 2009]. Likewise, current research has pointed out that the function and cellular levels of certain integrins, including αv and β3, can facilitate metastasis in many cancers, including gastric cancer [Avraamides *et al.,* 2008; Rathinam *et al.,* 2010]. The up-regulation of αv and β3 integrins has been shown to be predominantly found in metastatic cancers [Desgrosellier *et al.,* 2010]. FAK regulates cell adhesion and migration signals in various cell lines, is involved in the engagement of integrin and assembly of focal adhesions (FA) through catalyzing several downstream signals, and mediates cell behavior. Cofilin 1 is an important regulatory factor of EMT in tumor cells, demonstrating an association with the occurrence and development of tumors [Gabarra-Niecko *et al.,* 2003].

In this study, we investigated the M2 macrophages in the resistance of AGS cells to treatment with 5FU.

4.3. Materials and methods

4.3.1. Cell culture

The gastric cancer cell line AGS and macrophage cell line RAW 264.7 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were maintained in continuous exponential growth by twice weekly passage in Roswell Park Memorial Institute medium (RPMI, Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and cultured at 37 °C with 5% CO_2. Each cell line was split regularly before attaining 70–80% confluence.

4.3.2. Cell viability assay

AGS cells and Raw 264.7 cells were plated in 96-well plates at a final concentration of 1×10^4 cells for 24h before the experiments. Cells were then treated with various concentrations of 5-Fu for 24h with (or without) conditioned media (CM) from M1 and M2 macrophages for 24 h. After treatment, the media in the wells was removed and replaced by fresh prewarmed media. 10% MTT was added to each well and the plates were incubated at 37 °C for an additional period of 4 h. The cell viability was also performed on AGS cells treated with 150 μM of H_2O_2 and 100 μM of $COCl_2$ separately. The absorbance was measured using a multiplate reader at the wavelength of 490 nm.

4.3.3. Macrophage polarization

Macrophage generation and differentiation for the obtention of M1 and M2 phenotypes from Raw 264.7 cells were performed as follow. Raw 264.7 cells were plated into 6 well plates or 6 well plate inserts and incubated for 24h at 37ºC. To

generate M1 phenotypes Raw 264.7 macrophages were activated with LPS (1 µg/ml) for 24h while for M2 macrophages, Raw 264.7 cells were activated IL-4 (40 ng/ml), conditioned media (CM) or the inserts containing previously plated Raw 264.7 cells were placed in wells containing AGS cells (Coculture). Conditioned media was collected from AGS supernatant. Briefly, AGS cells were cultured in T75 flasks till they reach 70 % of confluency. The supernatant medium was centrifuged at 1000 rpm for 5 min and filtrated through 0.2 µM pore size filter. The obtained conditioned media as stored at -20^{0}C. Like for M1 macrophages, the cells were incubated for additional 24 h at 37^{o}C. After 24h of activation and coculture, the polarization was complete and cells were used for further analysis.

4.3.4. Coculture experiments

Raw 264.7 cells were plated on the upper compartment of 0.4 µm pore size transwell of 6 well plates in 1 ml of RPMI complete medium, while AGS cells (3×10^6) were plated in the lower chamber. Initially AGS cells and Raw 264.7 cells were cultured on separate plate. Raw 264.7 cells were polarized into M1 and M2 macrophages using LPS and IL-4 like described above and after polarization the inserts were transferred on the plate containing AGS cells. Before the transfer of inserts in the wells containing AGS cells, AGS cells were treated with 10 µg/ml of 5FU. The plates were then incubated for 18h and after treatment further analysis were conducted.

4.3.5. NO assay

Raw 264.7 cells were plated in 24 well plates and incubated at 37^{0}C for 24h. After 24h of incubation, Raw 264.7 cells were activated with LPS (1 µg/ml), IL-4, conditioned media (40 % of CM and 60 % of fresh media) and coculture (0.4 µm pore size inserts of 24 well plate) for an additional 24 h. The production of NO was determined using the Griess reagent. Briefly, after 24 h of treatment with LPS (1

μg/ml), IL-4, CM and coculture, 100 μl of culture media supernatant from each sample were mixed with an equal volume of Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% phosphoric acid]. Following incubation for 15 min, the absorbance values were read at 540 nm using an ELISA microplate reader. The nitrite content was calculated compared with that of standard concentrations of sodium nitrite dissolved in RPMI.

4.3.6. Western Blot

For whole cell lysates, Raw 264.7 cells and AGS cells were cultured as described above. After activation or treatment, the cells were collected by trypsinization and protein were extracted using a cell lysis buffer. Cytoplasmic and nuclear proteins were extracted using NE-PER nuclear and cytoplasmic extraction.

Samples (20 μl) were subjected to SDS-PAGE (12% acrylamide gel) and proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Blocking was performed for 1 h at room temperature with 5% BSA in TBS 0.1% Tween-20 (TBS-T). Membranes were incubated overnight at 4°C with IL-1β, IL-6, iNOS, IL-10, Arg-1, MARCO, pPI3K, pAkt, pNrf2, HIF-1α, MMP-2, MMP-9, integrin β3, pFAK, Vinculin, Paxillin, E-cadherin, pCofilin and GAPDH first antibody diluted 1:1,000 in PBST. The secondary antibody (goat anti-rabbit IgG-HRP, Sc-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was diluted 1:10,000 in PBST and was applied for 1 h at room temperature.

4.3.7. Cell invasion assay.

In vitro cell migration assay was performed using Transwell (Costar, Lowel, MA) with 8-μm polycarbonate filter-membranes in 24-well plates following manufacturer's instructions. The experiments were conducted like previously described [Ngabire *et al.,* 2018] with minor modifications. Briefly, the lower side of the filter-membrane was coated with 100 μl of matrigel (Corning, Bedford, MA).

AGS cells were seeded in the inserts coated with matrigel and incubated for 24h at 37^0C. After 24h incubation, AGS cell were treated (or not) with 10 µg/ml of 5FU diluted in media without FBS then the inserts were placed into wells containing (or not) polarized M2 macrophages. The cells were incubated for further 24h at 37^0C and after 24 h, non-migrating cells on upper side of the membrane were removed and migrated cells on lower side of the membrane were fixed with ice-cold methanol. Fixed membranes were stained with 0.5% crystal violet (Sigma Chemicals, St. Louis, MO) for 20 min, washed twice with distilled water, and allowed to air dry. The stained cells were visualized under a microscope (magnification, 100×).

4.3.8. Immunofluorescence

Immunohistochemistry analysis was performed as described previously [Ngabire *et al.,* 2018] with minor modifications. Briefly, Raw 264.7 cells and AGS cells were plated on cover-glass bottom dishes, incubated at 37 ℃. Raw 264.7 cells, after polarization with LPS and IL-4 for 24h, were first stained with 1 µg/ml of DAPI for 20 min at room temperature, fixed with 4% formaldehyde for 15 min at room temperature, washed with PBS and then stained with F-actin for 20 min at room temperature. For AGS cells, cells were stained with DAPI then fixed with 4% formaldehyde for 15 min at room temperature and blocked for 1 h in a blocking solution, including 5% rabbit and mouse normal serums with 0.3% Triton X-100. Fixed and blocked cells were incubated with the primary antibodies (pCofilin, E-cadherin, β-actin) for 3 h and washed three times with PBS buffer. After washing, the cells were treated with 0.1 µg/ml of anti-mouse IgG (H+L), F(ab')2 fragment (Alexa Fluor® 555 Conjugate) and anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor® 488 Conjugate) for 1 h. The stained cells were mounted on the slide with Prolong Gold Antifade Reagent and observed under a ZEISS LSM 710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

4.3.9. Statistical analysis

Data are presented as the mean ± standard deviation (SD) for the indicated number of separated experiments. The mean of the control was compared with the mean of each individual treatment group by two-way ANOVA using the statistical software GraphPad Prism 7 (Graphpad Software Inc., La Jolla, CA, USA), and a statistically significant difference was set at *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and # $P < 0.0001$.

4.4. Results

4.4.1. LPS and IL-4 successfully polarized Raw 264.7 cells into M1 and M2 macrophages respectively.

We investigated in vitro polarization of Raw 264.7 cells. Macrophages can be classically (M1) or alternatively (M2) activated. M1 macrophages are characterized by the production of inflammatory cytokines (IL-1β, IL-6, iNOS) while M2 macrophages produce commonly IL-10, Arginasa-1 and TGF- β. To obtain M1 and M2 macrophages Raw 264.7 cells were activated with LPS and IL-4 respectively for 18-24h. After activation, we observed a significant difference in shape between M1 and M2 macrophages. M1 macrophages presented an irregular shape with multiple pseudopods while M2 macrophages had an elongated shape without pseudopods. The assay revealed that M1 macrophages produced high levels of nitric oxide (NO) while M2 macrophages had low levels of NO. Additionally, western blot results revealed that M1 macrophages produced higher levels of IL-1β, IL-6 and iNOS while M2 macrophages produced higher levels of Arg-1 and expressed the MARCO receptor. These results confirmed that we were able to polarize Raw 264.7 cells into M1 and M2 macrophages.

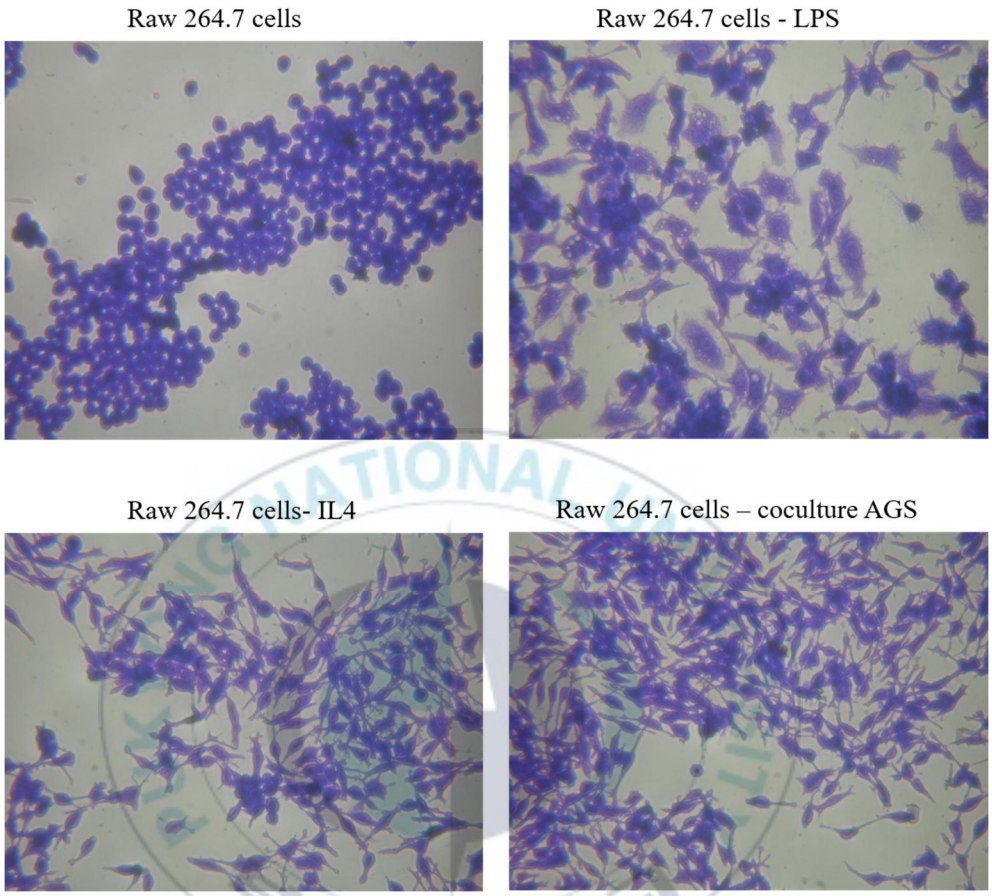

Figure 4.1. Polarization of Raw 264.7 cells into M1 and M2 macrophages with LPS, IL-4, conditioned media and coculture. Crystal violet staining of polarized Raw 264.7 cells.

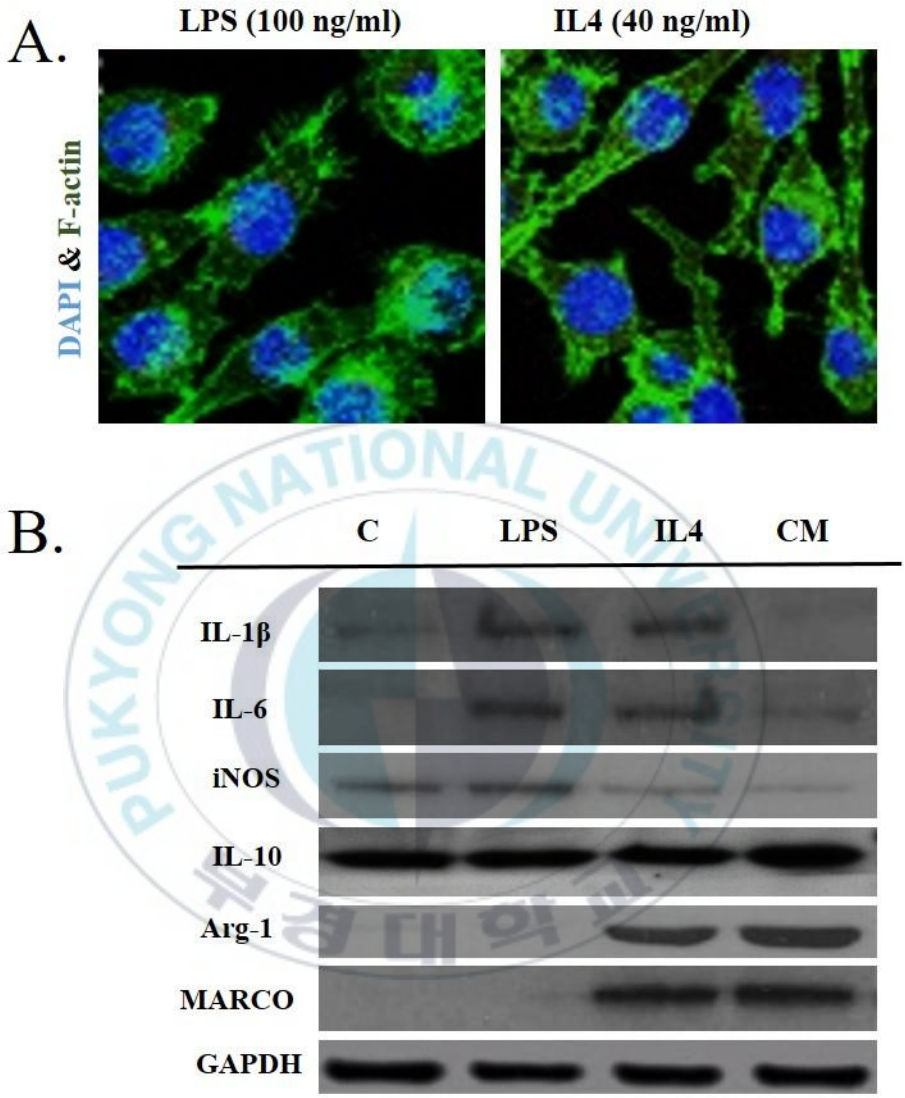

Figure 4.2. Immunofluorescence and western blot analysis of Raw 264.7 cells polarized into M1 and M2 macrophages with LPS, IL-4, conditioned media. A. DAPI and F-actin staining of polarized Raw 264.7 cells. **B.** Western blot analysis of polarized Raw 264.7 cells.

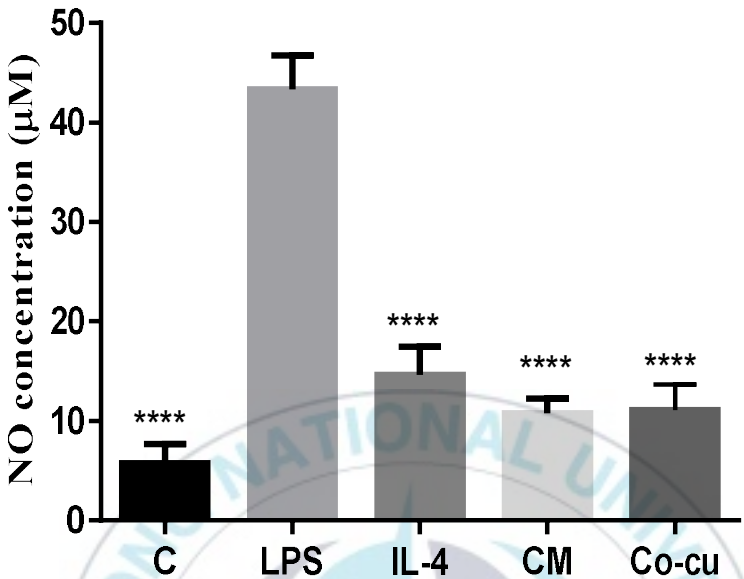

Figure 4.3. Nitrite oxide (NO) in polarized Raw 264.7 cells with LPS, IL-4, conditioned media and coculture. NO assay of polarized Raw 264.7 cells. Statistically significant difference was set at *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

4.4.2. M2 macrophages induced the inhibition of oxidative stress through Nrf-2/HO-1 and HIF1α.

Oxidative stress play a major role in cancer cell death. Macrophages produces toxic chemicals like H_2O_2 to induce cancer cells death in tumor microenvironment. In response to oxidative stress, cells respond by producing HO-1 through the activation of Nrf-2 and HIF-1α. Activated Nrf-2 and HIF-1α translocate to the nucleus were they interact with transcription factors. Previous studies were able to induce oxidative stress in vitro using H_2O_2. We induced oxidative stress in AGS cells by exposing the cells at 150 μM of H_2O_2 for 5h then cells were cocultured with alternatively activated macrophages (M2 macrophages) for 18h. Western analysis of cytoplasmic and nuclear proteins showed that pNrf2 and HIF-1α were highly expressed in both the cytoplasm and nucleus in AGS cells treated with 5FU and cocultured with M2 macrophages. Similar results were obtained for HO-1. We were able to conclude that M2 macrophages increases anti-oxidant proprieties of AGS cells treated with 5FU.

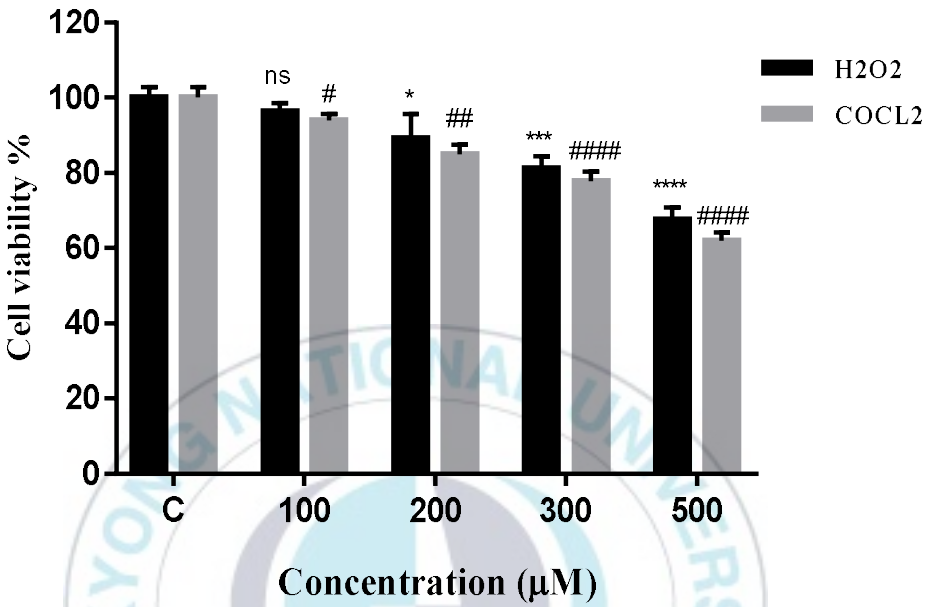

Figure 4.4. Cell viability of AGS cells treated with various concentrations of H_2O_2 and $COCL_2$. Statistically significant difference was set at *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and # $P < 0.0001$.

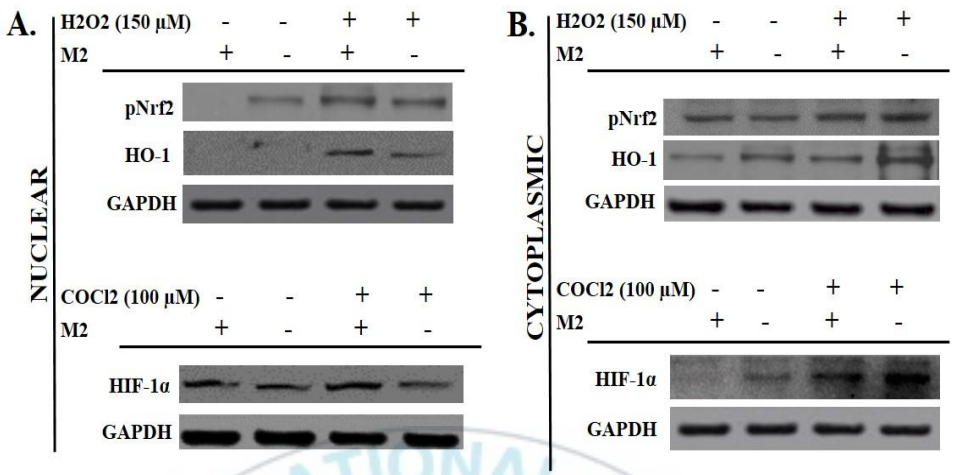

Figure 4.5. Hypoxia and oxidative stress in AGS cells coculture with Raw 264.7-M2 macrophages. A. Nuclear expression of HIF-1α, pNrf2 and HO-1 proteins in AGS cells cocultured with M2 macrophages. **B.** Cytoplasmic expression of HIF-1α, pNrf2 and HO-1 proteins in AGS cells cocultured with M2 macrophages. The western plot experiments, AGS cells were plated in 6 well plates and incubated at 37^0C for 24h. After 24h AGS cells were treated with 150 µM of H_2O_2 or 100 µM of $COCL_2$ then 0.4 µm pore size 6 well plate inserts containing polarized M2 macrophages were placed in the wells containing AGS cells. After 24h of coculture, nuclear and cytoplasmic proteins were extracted and analyzed by western blot.

4.4.3. M2 macrophages induced cell survival in AGS cells through PI3K/Akt pathway.

M2 macrophages reportedly induces proliferation of cancer cells in tumor microenvironment. We investigated if M2 macrophages played a role in the resistance of AGS cells to 5FU treated. AGS cells plated 96 well then challenged with 5-FU (or without) and conditioned media (CM) from MO (non-activated macrophages), M1 macrophages (LPS-activated macrophages) and M2 macrophages (IL-4 activated macrophages). The results showed that CM from M2 macrophages increased the proliferation of AGS cells in both non-treated and treated scenario with 5-FU. Additionally, we investigated if PI3K/Akt pathway played a role in the survival of AGS cells and uncovered that 5FU-treated AGS cells cocultured with M2 macrophages expressed higher levels of pPI3K and pAkt proteins. These results allowed us to conclude that M2 macrophages mediated the survival of 5FU-treated AGS cells through the activation of PI3K/Akt macrophages.

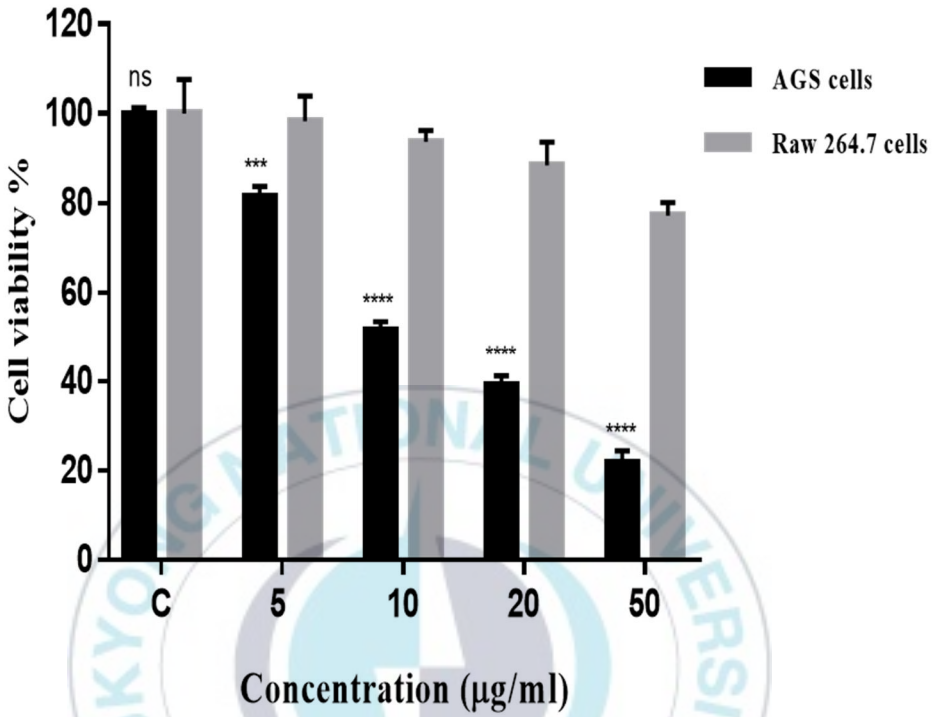

Figure 4.6. Cell viability of AGS cells and Raw 264.7 cells treated with 5FU. AGS cells and Raw 264.7 cells were cultured in RPMI media and incubated for 24h at 37^0C. After 24h of incubation, cells were treated with various concentrations of 5FU and incubated for further 24h. After treatment, the media in the plate was remove and replaced with fresh media then 10 µl of WST-1 solution was added in each well and the plate were incubated for further 4h at 37^0C. Absorbance was obtained using Elisa microplate reader at 490 nm wavelength. Statistically significant difference was set at *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and # $P < 0.0001$.

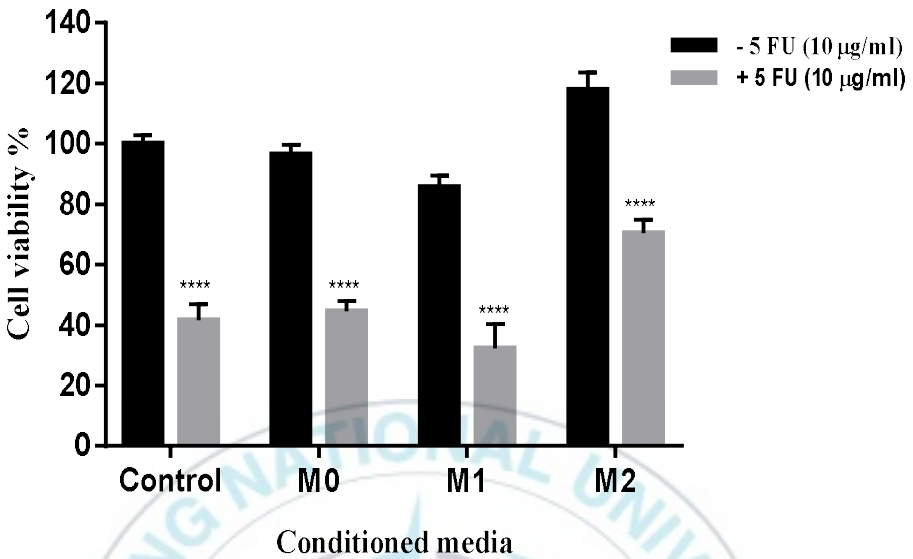

Figure 4.7. Cell viability and proliferation of AGS cells treated with 5FU and cocultured with conditioned media from M2 macrophages. AGS cells were plated in 96 well plate and incubated for 24h at 37^0C. After 24h of incubation, the media was replaced with a mixture of fresh media and conditioned media from macrophages to a final ratio of 60:40 respectively. 5FU was simultaneously added to the cell to final concentration of 10 µg/ml and the plate were incubated for 24h. After treatment, the media in the plate was remove and replaced with fresh media then 10 µl of WST-1 solution was added in each well and the plate were incubated for further 4h at 37^0C. Absorbance was obtained using Elisa microplate reader at 490 nm wavelength. Statistically significant difference was set at *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and # $P < 0.0001$.

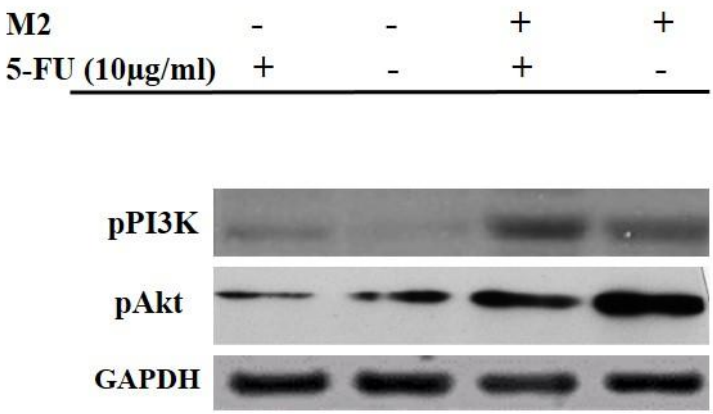

Figure 4.8. Western blot analysis of Akt pathway. AGS cells were plated in 6 well plate and incubated for 24h at 37^0C. After 24h of incubation, AGS cells were treated with 10 μg/ml of 5FU then polarized Raw 264.7-M2 macrophages cultured in 6 well plate inserts of 0.4 μm pore size were added to the AGS wells and incubated for additional 24h. After treatment, proteins were extracted from AGS cells and separated by western blot.

4.4.4. M2 macrophages induced cell migration and invasion in AGS cells.

Metastasis is major issue in cancer. This process allows cancer cells to migration and invade distant or nearby organs from the initial site of the tumor. EMT is a transition of cells from epithelial phenotype to mesenchymal is one of the initial step after the ECM degradation that allow cancer cells to reach blood circulation. MMPs proteins play a crucial function in tissue remodeling by degradation of the ECM. MMP-2 and MMP-9 break down the ECM and the resulted components interact with integrins to activate further cell processes. Integrin pathway play a major role in the EMT. EMT is characterized by low levels of E-cadherin and high levels of N-cadherin. Vinculin, Paxillin and Cofilin are related to FAK pathway and play crucial role in cancer cell migration and invasiveness. We investigated the expression of EMT proteins in 5FU-treated AGS cells cocultured with M2 macrophages. Our results also showed that the loss of E-cadherin expression and integrin β3, pFAK, pCofilin were highly expressed. However, there was no significant difference in the expression of Vinculin, Paxillin. These results confirmed that M2 macrophages stimulated invasiveness behavior in 5FU-treated AGS cells and for the first time we were able to show the role of Integrin β3/FAK pathway.

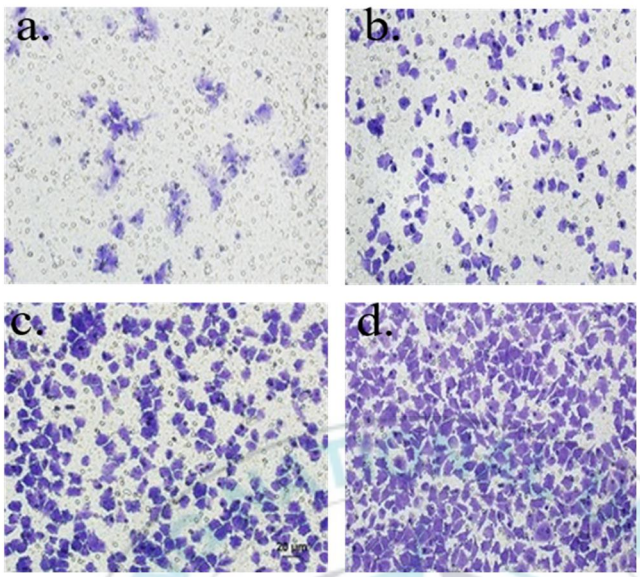

Figure 4.9. Invasion of 5FU-treated AGS cells cocultured with Raw 264.7-M2 macrophages. AGS cells were plated in 0.8 µm pore size transwell and incubated for 24h. After 24h incubation, AGS cells were treated with 10 µg/ml of 5FU in FBS free media and the inserts were added in wells containing polarized Raw 264.7-M2 macrophages or fresh media and incubated for further 24h. After 24h, non-migrating cells on upper side of the membrane were removed and migrated cells on lower side of the membrane were fixed with ice-cold methanol. Fixed membranes were stained with 0.5% crystal violet.

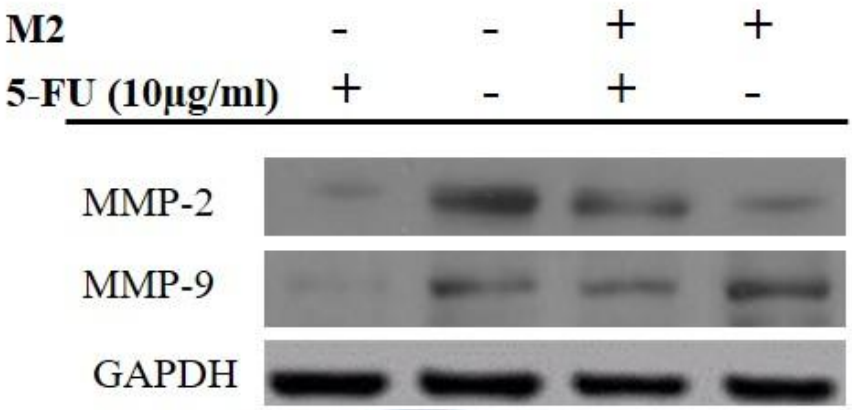

Figure 4.10. Expression of matrix metalloproteases (MMPs). Western blot analysis of MMP-2 and MMP-9 proteins.

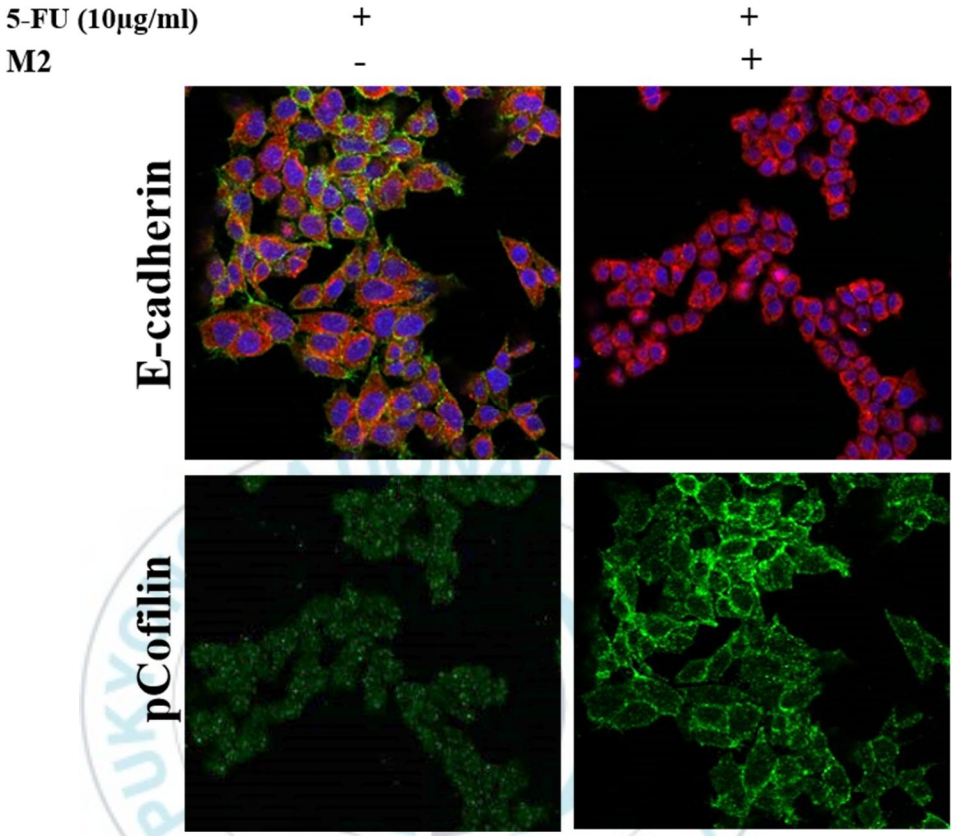

Figure 4.11. E-cadherin and pCofilin expression in 5FU-treated AGS cells cocultured with Raw 264.7-M2 macrophages. AGS cells were plated in 6 well plate containing cover glass for 24h. After 24h of incubation, AGS cells were treated with 10 μg/ml of 5FU and the inserts containing polarized Raw 264.7-M2 macrophages were added to the AGS wells. AGS cells were then stained with DAPI and with pCofilin, E-cadherin, β-actin antibodies. The stained cells were observed under confocal microscope.

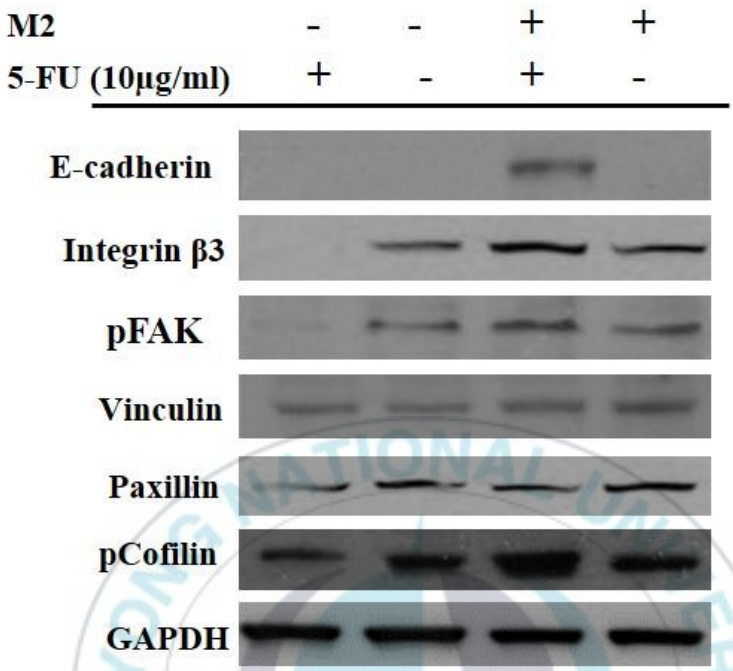

Figure 4.12. Western Blot analysis of integrin β3, pFAK, Vinculin, Paxillin, E-cadherin and pCofilin expression.

4.5. Discussion

Tumor progression is controlled by crosstalk between cancer cells and other cell types within the tumor microenvironment. The tumor microenvironment includes proliferating tumor cells, blood vessels, the tumor stroma, inflammatory cells, and a variety of associated tissue-type cells. Macrophages are known to be polarized into M1 and M2 in the tumor microenvironment. We were successfully able to polarized Raw 264.7 cells into M1 and M2 macrophages using LPS and IL-4 respectively as stimulants or activators. Significant morphological differences were observed between obtained M1 and M2 macrophages. Like already reported by previous studies, M1 macrophages produced high levels of nitric oxide compared to M2 macrophages and western blot results confirmed that M1 macrophages produced high levels of inflammatory cytokines (IL-1β, IL-6, iNOS) while M2 macrophages produced high levels of IL-10 and Arg-1 and expressed the mannose receptor MARCO. Additionally, Raw 264.7-M2 macrophages increased the proliferation of AGS cells in both treated and not treated cases with 5FU. Cell viability results showed the percentage of AGS cells cultured with conditioned media from M2 macrophages was higher compared to control AGS cells cultured just with fresh media. The treatment of AGS cells with 5FU and the addition of M2 media also increased AGS cells proliferation compared to 5FU- treated AGS cells without conditioned media. From these results we were able to establish that M2 macrophages induced the resistance of AGS against 5FU and promoted cell survival. PI3K/Akt pathway has been reported in various studies to play a crucial role in cell proliferation and cell survival. Western blot results of phosphorylated forms of PI3K and Akt showed a significant increase in the expression of PI3K and Akt proteins therefore we were able to determine that the survival of 5FU-treated AGS cultured with conditioned media from Raw 264.7-M2 macrophages was at least mediated partially by PI3K/Akt pathway.

The hallmark of hypoxia and oxidative stress is the formation of reactive oxygen species (ROS) [Gorrini *et al.,* 2013; Dennery, 2010]. The accumulation of these chemicals in cells can lead to their death. The activation of Nrf2 and HIF-1α promotes the transcription of antioxidants genes such as heme oxygenase 1 (HO-1) [Zhang *et al.,* 2015; Bryan *et al.,* 2013]. Nrf2 and HIF-1α also contributes to several steps required for tumor growth and metastasis. Owing to the fact that both HIF-1 and Nrf2 are well established as mechanisms of resistance to anticancer therapies, simultaneously targeting these pathways represents an attractive approach for therapeutic development [Loboda *et al.,* 2016; Nguyen *et al.,* 2009]. In this study, we investigated the effects of coculture of 5FU-treated AGS cells with Raw 264.7-M2 macrophages in hypoxia and oxidative stress conditions. Our results showed an increase in the expression of pNrf2, HIF-1α and HO-1. These results confirm that hypoxia, through the activation of HIF-1 α, contributes in the chemoresistance of AGS cells to 5FU when cocultured with M2 macrophages and that M2 macrophages increase the survival of 5FU-treated AGS cells to oxidative stress by the activation of Nrf2.

Metastasis is the main cause of cancer-related mortality secondary to malignancy [Alizadeh *et al.,* 2014]. It has been clearly demonstrated that inflammatory cells in the tumor microenvironment play an important role in tumor growth, progression, and metastasis. Within the tumor stroma, tumor-associated macrophages (TAMs) constitute a pivotal class of inflammatory cells, and compelling evidence has emerged to suggest that TAMs play a promoting role in processes such as carcinogenesis, tumor growth, angiogenesis, and lymphangiogenesis and are the key regulators of the metastatic phenotype of cancer cells [Vasievich *et al.,* 2011; Solinas *et al.,* 2009]. Moreover, in gastric adenocarcinoma, this pro-tumor role of TAMs is further supported by clinical studies demonstrating a correlation between high macrophages numbers in tumor tissue and poor patient prognosis. However, the activated phenotype and

polarization role of TAMs in chemotherapy and chemoresistance are not well-understood. Cell migration is an essential process for the development and maintenance of multicellular organisms and is defined as the orchestrated movement of cells in a particular direction to a specific location. To migrate, a cell must modify its shape to be able to interact with the surrounding tissue structures. Therefore, the extracellular matrix (ECM) serves as a substrate as well as a barrier for an advancing cell body [Larsen *et al.,* 2006].

Matrix metalloproteinases (MMPs) are a family of zinc-containing proteases that function to degrade extracellular matrix proteins, such as collagen, laminin and proteoglycan [Egeblad *et al.,* 2002; Kessenbrock *et al.,* 2010; Deryugina *et al.,* 2006]. We conducted a cell invasion assay in AGS cells treated or not with 5FU using M2 macrophages conditioned media as chemoattractant. The results showed that 5-FU treated AGS cells migrated more through the membrane coated with the matrigel compared to other groups in the experiment. To further understand, we analyzed the metalloproteases expression and western blot results revealed that MMP-2 and MMP-9 proteases were in 5FU-treated AGS cells cocultured with M2 macrophages therefore we can conclude that M2 macrophages are able to increase the invasion of AGS cells despite the presence of 5FU. Cofilin is an actin-binding protein that has an important role in the regulation of actin dynamics [McGough *et al.,* 1997; Bravo-Cordero *et al.,* 2013]. Cofilin-mediated severing of actin filaments at the leading edge of motile cells controls the formation of lamellipodia, which is essential cancer cell metastasis [Huang *et al.,* 2006; Mizuno, 2013]. We analyzed the expression of pCofilin in 5FU-treated AGS cells and non-treated AGS cells both cocultured with M2 macrophages and revealed that pCofilin was highly expressed in 5FU-treated AGS cells cocultured with M2 macrophages. These results were confirmed by western blot analysis therefore we can establish that the activation of Cofilin is part of the

mechanisms involved in cell migration of 5FU-treated AGS induced by coculture with M2 macrophages.

Cancer metastasis is the multistep process of cancer cells spreading from their original site to other distant sites of the body [Coghlin *et al.,* 2010]. Among several potentiating factors of metastasis, the transition of epithelial to mesenchymal phenotype, known as EMT [Voulgari *et al.,* 2009; Guarino, 2007; Yilmaz *et al.,* 2009], has garnered most attention. EMT has been shown to facilitate cancer progression and aggressive behaviors of cancer cells, such as increased cell migration and invasion. Previous studies indicated that EMT is a highly complex process requiring extensive changes in adhesion, cell morphology and protein expression [Bonnomet *et al.,* 2010; De Craene *et al.,* 2013; Yilmaz *et al.,* 2009]. Down regulation of E-cadherin and up regulation of N-cadherin are the major molecular events regarding the reduction in cell-cell adhesion and facilitation of cell movement. Our immunofluorescence results showed that E-cadherin expression was lost in 5FU-treated AGS cells cultured with M2 macrophages conditioned media and these results were confirmed by western blot analysis of E-cadherin protein expression.

Integrins are a family of transmembrane adhesion receptors comprised of 19α and 8β subunits that interact non-covalently to form up to 24 different heterodimeric receptors. Integrin binds to ECM proteins or integrin cross-linking increases the tyrosine phosphorylation of FAK [Scneider *et al.,* 2011; Zhao *et al.,* 2009]. FAK is a non-receptor tyrosine kinase primarily localized to cell-matrix adhesions which acts as a central regulator of focal adhesion influencing cell survival, differentiation, proliferation, migration and tissue remodeling. Once localized to sites of transmembrane integrin receptor clustering, tyrosine-phosphorylated FAK plays an important role in signal transduction triggered by diverse extracellular signals [Liu *et al.,* 2000] and represents a convergent point for synergistic interaction between signal pathways activated by growth factors and

integrins. Activation of FAK initiates a number of biological processes, including cell attachment, migration, invasion, proliferation, and survival. The cytoplasmic tail of β-integrin (β1, β2, and β3) facilitates FAK activation and accessory proteins like paxillin and vinculin are required for the initiation of signal transduction. Also integrins are connected to actin stress fibers through vinculin which binds to actin cytoskeleton [Van der Flier *et al.,* 2001]. In the present study, we investigated the hypothesis of a potential role of integrin β3 and its associated complex in the resistance of AGS cells to 5FU when cocultured with M2 macrophages. Our results determined that 5FU-treated AGS cocultured with RA 264.7-M2 macrophages expressed increases levels of integrin β3, and pFAK. There was no significant difference in the expression of Vinculin and Paxillin between 5FU-treated AGS cells and not treated AGS cells both cocultured with Raw 264.7-M2 macrophages. We can conclude that Integrin β3 and FAK mediate the resistance of AGS cells to 5FU when cocultured with M2 macrophages but that it doesn't require the intervention on Vinculin and Paxillin.

In conclusion, our study is the first report to establish the role of integrin β3, FAK and pCofilin in the resistance of AGS gastric adenocarcinoma cells against 5FU in presence of M2 macrophages. These results provide an additional insight in understanding mechanisms acquired by cancer cells in chemoresistance and open new strategic research targets in tumor microenvironment research.

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국문요약

염증은 당뇨병, 심혈관계 질환, 알츠하이머, 자가 면역 질환, 폐 질환, 관절염 및 암과 같은 많은 질병에 관련되어 있다. 면역 세포, 특히 대식세포는 다양한 경로를 통하여 활성화되고, cytokine 과 같은 염증매개체의 생성에 중요한 역할을 한다. 암은 전세계적으로 큰 건강 문제로 여겨지고 있으며, 특히 위암의 경우 동아시아에서 높은 발병률을 보인다. 세포 주기를 정지시키거나, 세포자살을 유도하여 세포 주기를 조절하는 것을 이용하여 세포의 사멸을 유도하는 것은 암과 관련된 실험에서의 주요 목표이다. 지난 수십 년 동안 인간의 질병에 대한 치료법을 발견하기 위해 식물이 가지고 있는 다양한 생리활성 물질들이 사용되었다. *Aster incisus* 는 아시아에서 흔히 볼 수 있는 식물로서 한국에서는 전통적으로 약초로 사용되어왔다. 본 논문에서는 쥐의 대식세포(Raw 264.7 세포주)와 인간 위암(AGS 세포주)에서 *Aster incisus* 의 메탄올 추출물의 항염증 효과와 항암 효과를 확인하였다.

이 논문의 결과에서는 *Aster incisus* 를 처리하였을 때, NFkB, MAPK 및 Akt 신호전달 경로의 조절을 통하여 LPS 에 의해 자극된 Raw 264.7 대식세포에서 산화 질소 (NO)와 염증 관련 cytokine 들 (TNFα, IL-1β, IL-6, iNOS, Cox-2) 의 생성이 성공적으로 억제되는 것을 확인하였다. 또한, *Aster incisus* 는 DPPH 의

radical 을 제거하므로 항산화 효과가 있는 것 역시 확인하였다. 위선암에 대한 효과로는 *Aster incisus* 를 처리하였을 때, AGS 세포의 증식이 억제되었다. 추가적인 분석을 진행하였을 때, *Aster incisus* 가 AGS 세포의 성장을 억제하는 것은 G1 기에서 세포 주기를 정지시키는 것과 세포자살을 유도하는 것에 의해 일어나는 것을 확인하였다. G1 기에서 발현되는 cyclin 들(cyclin D1 / 3, cyclin E2)과 CDK (CDK4 / 6, CDK2)의 발현은 억제되었고, 이들에 대한 억제 단백질들 (p16, p18, p21, p27)은 발현이 증가하였다. 세포자살 유도 단백질들(Bid, Bad, Bak, cytochrome c, AIF, cleaved caspase-3, -8, -9 및 cleaved PARP) 역시 *Aster incisus* 를 처리한 AGS 세포에서 높게 발현되었다.

염증은 종양 미세 환경에서 중요한 역할을 하는 것으로 알려져 있다. 대식세포 (종양 관련 대식세포)는 종양 미세 환경에서 가장 많이 존재하며, 화학 요법에 대한 암세포의 저항성에 깊게 관련되어있다. 이 논문에서 우리는 종양 관련 대식세포와 공동 배양하기 전후에서의 AGS 세포에 대한 5-FU 의 효과를 확인하였다. 종양 관련 대식세포는 PI3K / Akt 경로를 통해 AGS 위암 세포의 생존을 유도하고, Nrf2-HO1 을 통해 H2O2 에 의해 유도되는 산화반응을 억제하였다. 또한 종양 관련 대식세포는 integrin β3, FAK, paxillin, E-cadherin 단백질의 조절을 통하여 종양 관련 대식세포와 공동 배양되고 있는 5-FU 가 처리된 AGS 세포의 전이와 침습을 증가시켰다.

Acknowledgements

I would like to thank God for protecting me to this day, blessing me with amazing opportunities and providing me excellent health.

I would like to express my sincere gratitude to my supervisor and advisor Professor Gun-Do Kim for giving me the opportunity to join his research lab and for his guidance. My gratitude goes to members of my thesis committee: Professor Young Tae Kim, Professor Nam Gyu Park, Professor Myeong-Sook Lee and Professor Soo-Wan Nam, for their insightful comments and their encouragement.

I would particularly like to thank Dr Yeong Ae Seong for her academic advice and her incredible support during my stay in Korea. I couldn't have completed this work without my colleagues in the Cell Signaling Lab: Maheshkumar Prakash Patil, Min-Jae Kang, my sister Niyonizigiye Irvine, Nan-Hee Kim, Chang-Won Kang, Dukhyun Hwang, Min-Seok Park, Ji-Hye Kim, Ji-Huyn Lee, Hyung-Wook Choi, Boram Kim, Pham Thi Hai Ha, and the rest of the members of the Department of Microbiology. Thank you for your friendship and for creating such a joyful working environment.

I would like to thank my family. My mother Ruragahiye Thecla-Paula, who has always been there in my happiest and darkest moments, always put me in her prayers and I am extremely grateful her unconditionnal love. My cousin Dr Nkenyereye Lewis, who made it possible to me to come to Korea and accomplish my dream of completing my PhD. To my brother-in-law Dr Niyongabo Edouard, I always valued your advices and counted on you in any diffucult moment. My amazing wife Courtney Green; she always has been by my side during the completion of this work. Thank you for being a truthful and reliable partner. I will never take for granted your caring, affectionate and everlasting love. Last but not least, I dedicate this thesis to my late father Nibitanga Nestor. May your soul live in eternal peace.