# THE EFFECTS OF ARTONIN E ON CELL GROWTH INHIBITION AND APOPTOSIS INDUCTION IN COLON CANCER LoVo CELLS

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# ผลของ Artonin E ต่อการยับยั้งการเจริญและเหนี่ยวนำกระบวนการอะพอพโทซิส ในเซลล์มะเร็งลำไส้ใหญ่ชนิด LoVo

กัญญาลักษณ์ ยางนอก

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# THE EFFECTS OF ARTONIN E ON CELL GROWTH INHIBITION AND APOPTOSIS INDUCTION IN COLON CANCER LoVo CELLS

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THE EFFECTS OF ARTONIN E ON CELL GROWTH INHIBITION
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BY

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Nowadays, colon cancer is the leading cause of cancer deaths among men and women. In Thailand, colon cancer is in the third rank of cancer among men and the second rank of cancer among women. Currently, the treatments of colon cancer include surgery, chemotherapy, radiation therapy, immunotherapy, hormone therapy, targeted drug therapy and stem cell therapy. However, some treatments had side-effects for cancer patients with unwanted symptoms. In addition, targeted therapy comes with a high cost for patients. Therefore, bioactive compounds might be a good choice for colon cancer treatment. In this study, the effects of artonin E on cytotoxic induction in colon cancer LoVo cells by MTT assay were investigated. In addition, nuclear morphological changes and the loss of mitochondrial membrane potential ($\Delta\Psi$m) were determined by Hoechst 33342 staining and JC-1 staining (fluorescence dye) respectively. The cell cycle distribution was determined by flow cytometry. In order to examine the expression of mediator proteins, Western blot analysis was carried out. The results showed that artonin E decreased cell viability in a dose-dependent manner and induced apoptotic bodies. In addition, artonin E stimulated mitochondrial membrane potential changes until damage, as well as increased sub-G1 population in the cell cycle. The Western blot test showed that artonin E induced apoptosis via p-ERK1/2/ERK1/2, p-p38/p38, and p-c-Jun/c-Jun ratio expression in LoVo cells. The results suggested that artonin E induced apoptosis through MAPKs signaling pathway. Therefore, artonin E might be used as a potential anticancer drug for colon cancer in the future.

Keyword : colon cancer, artonin E, apoptosis, MAPK

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# CHAPTER I
# INTRODUCTION

## Background

Cancer is a group of complicated genetic diseases in which cells in slightly part of the body start to grow out of control signal [1]. These cancer cells can distribute into other parts of the body over the lymphatic and bloodstream system [2]. The cancer starts in the first place of the body is called the primary site, and then it breaks apart and leads to a spread on a secondary site within the host's body. These cells begin to grow up and form or become other types of new tumor at the part of normal tissue by themselves [3]. These mechanisms are called metastasis, which is the major considered property of cancers [4]. The mechanism of cancer suggests that all cancers are originated from both environmental and genetics associated with multiple external carcinogenic agent factors combined with internal genetics changes, carcinogenic agents are including ultraviolet, X-rays, viruses, products of tobacco, pollution, chemicals reagents, and other sources from outside the body [5]. Moreover, the mutation of genetic is a cause of cancer which relates with prolonged exposure to the carcinogenic agents and, finally, lead to the occurrence of the gene mutant [6]. The mutations of genes are correlated with inherited and considered to be internal factors of the disease mutation. In fact, most cancers grow unhurriedly and use multiple steps and take many years for development. It was found that cancer was the third rank causes of cancer death in men and women is second rank in Thailand [7].

Colon cancer is caused by the abnormal growth of epithelial cells which form the lining of the colon or rectum. The treatments of this cancer are including surgery, chemotherapy, radiation therapy, targeted drug therapy, immunotherapy and stem cell therapy. The cost of this cancer treatment is more expensive and getting more, and these things are trending to increase every year [8].

Anticancer drugs are used to destroy, kill, shrink or control the growth of cancerous cells. And have cytotoxic to normal or healthy cells by killing cells that divide quickly such as hair, skin, and the lining of the intestinal tract [9]. As describe above, anticancer drugs show some side effects to cancer patients that include hair loss, dry skin, weight loss and diarrhea [10]. Presently, many studies show that the bioactive compounds from plant extract have anticancer properties in many types of cancer which inhibit cell growth, cell proliferation as well as induce cell death. Therefore, the bioactive compounds are a well choice to use as an anticancer agent.

Artonin E is one of effective bioactive compounds that has been used in many topics in cancer research. Artonin E is a prenylated flavonoid that was isolated the stem bark of the genus *Artocarpus* [11]. In this study, we used artonin E extracted from *Artocarpus elasticus* Reinw. ex Blume that the most found in the Southern part of Thailand and is known by the local name, 'Ka Ok', 'Ka O' or 'Tue Ka' [12]. Previous studies, artonin E has been shown to inhibition of cell proliferation, migration, invasion and has the ability to cause cytotoxicity. Finally, it leads to induces apoptosis in a variety of cancer such as lung cancer [13], breast cancer [14] ovarian cancer [12] and skin cancer [15]. However, the effects of artonin E on human colon cancer LoVo cells through the MAPKs signaling pathways have not yet been reported. Therefore, in this study, we looked into the effect of artonin E on MAPK pathway.

Mitogen-activated protein kinase (MAPK) consists of proteins that are a serine/threonine kinase group. That binds to receptors on the cell membrane. It is a receptor for extracellular change. MAPK is involved in a wide range of signaling pathways, in and kinase cascades control different intracellular processes depending on the cell type and stimulus. The major of MAPK proteins are composed of three separate regulatory major groups: extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun, NH2-terminal kinase1/2/3 (JNK1/2/3), and p38 $\alpha$, $\beta$, $\lambda$ and $\delta$. The functions of each group were as follows: ERK1/2 were involved with pathogenesis and response to growth and survival factors and induced cell growth, cell differentiation and cell death inhibition.

At the same time, JNK and p38 play roles in cell proliferation, cell survival, cell cycle regulation and apoptosis [16].

## Objectives

1. To investigate the effects of artonin E on on cell growth inhibition and apoptosis induction in colon cancer LoVo cells.
2. To investigate the mechanism involved with apoptosis induction.

## Conceptual framework

We hypothesized that artonin E affected cell viability, nuclear morphological changes, mitochondrial membrane potential ($\Delta\Psi m$), cell cycle as well as signaling proteins e.g., MAPK. The conceptual framework is showed in Figure 1.

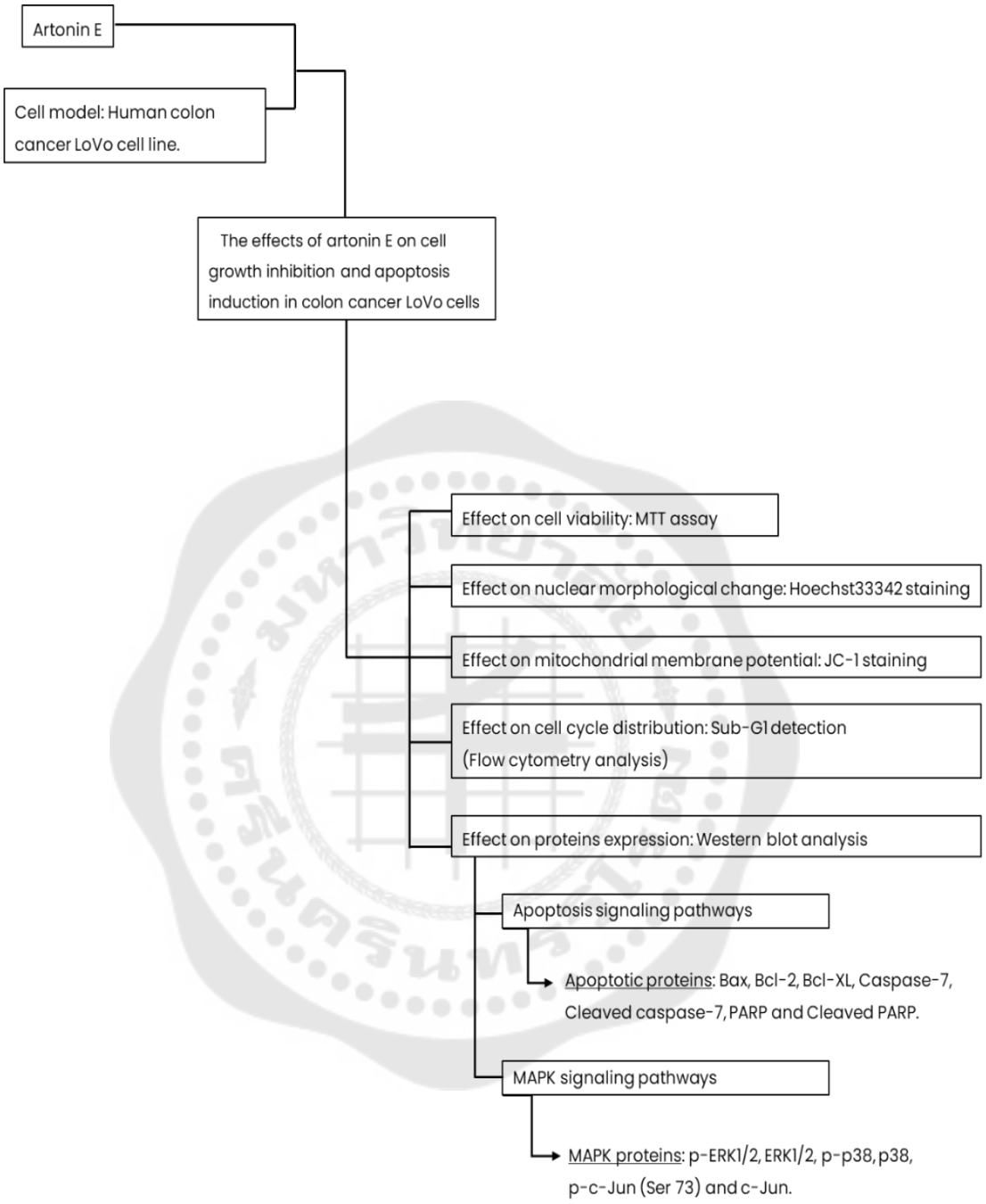



Figure 1 Conceptual framework

# CHAPTER II
# LITERATURE REVIEW

## Colon cancer

Colon cancer is a cancer originated from colon or rectum which can be called colon or rectum cancer. They are often grouped together as they have many features in common [17]. This type of cancer is classified as a high-risk type. And can be found most frequently around the world. Approximately 1 million new cases occur annually, with the average risk group aged 50 years and over.

In 2020, the American Cancer Society reported that colon cancer is the third most common cancer diagnosed in both males and females and ranking second for cancer-related deaths [18]. The United States reports cancer cases in 2021, with new cases and deaths expected to account for 45,230 cases of rectal cancer and 104,270 cases of colon cancer. And finally, it is estimated that a total of 52,980 cases will die from these cancers [19].

Colon cancer in Thailand is the third most common cancer, accounting for 11% of cancer burden in Thailand [20]. And the statistics of colon cancer in 2020 found a total of 21,103 new cases of colon cancer occurring in both sexes (11.1%), which was found the second in women with 10,443 cases (10.7%) and as third in males with 10,660 cases (11.4%) respectively.

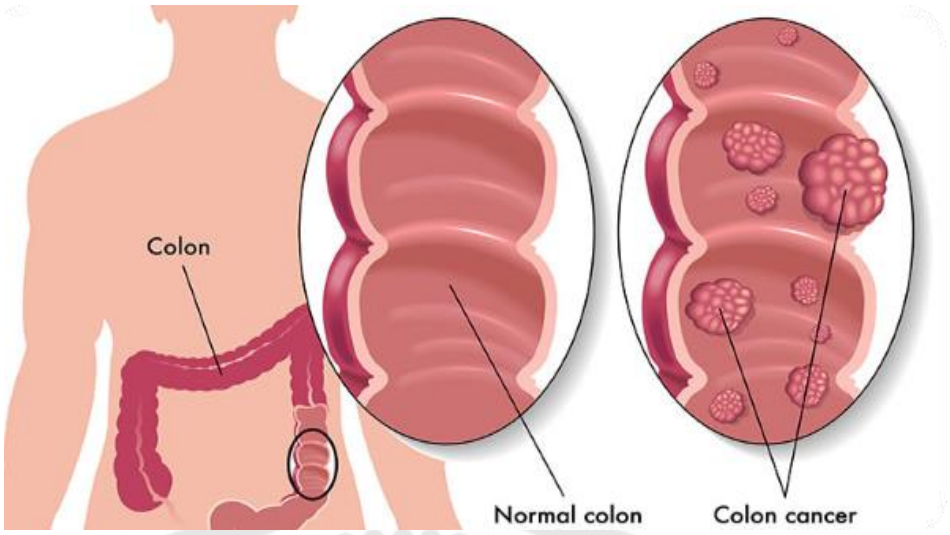



Figure 2 Colon cancer.

Source: Labianca R, Beretta GD, Kildani B, Milesi L, Merlin F, Mosconi S, et al. Colon cancer. Crit Rev Oncol Hematol. 2010;74(2):106-33.

**Symptoms of colon cancer**

There are no specific symptoms of colon cancer. But it will be the same symptoms as symptoms of the general intestinal disease such as the behavior of defecation changes from normal may be defecation frequency or more constipation, fresh bloody stools or black like charcoal, severe stomach pain, nausea, colic, abdominal discomfort, fatigue, unexplained weight loss and so tired that they can't work normally.

**Risk factors of colon cancer**

Colon cancer in the United States is attributable to potentially modifiable risk behaviors, with more than half (55%) of these factors including: eating a high-fat diet, low fiber food, overcooked grilled foods, pickled food, heavy alcohol use, smoking, lack of exercise, having digestive system problems such as inflammatory bowel disease, chronic constipation, irritable bowel syndrome and have a family history of colon cancer or different types of cancer.

### Treatments

The treatment depends on a diversity of factors [21]. Surgery, the main treatment, is more likely to be useful for earlier stage of colon cancer. Moreover, colon cancer can also be treated by using drug to kill cancers via injection into bloodstream or oral intake known as chemotherapy. These are classified as a systemic treatment because they can reach almost all cancer cells in the body [22]. For colon cancer patient, the chemotherapy generally takes place before and/or after surgery, alone or in combination with radiation therapy for it is used to kill any remaining cancerous cells as well as control the growth of tumors. In addition, there are some treatments including, immunotherapy, targeted drug and stem cell therapy are newer options for some advanced cancers [23].

### Anti-cancer drugs

Nowadays, there are many types of anti-cancer, for example, cisplatin. DDP can making DNA damage and apoptosis in different types of cancer cells. And had inhibitory effect on cancer cell proliferation and induction of G0/G1 cell cycle arrest and enhanced the expression and activation of caspase-3, caspase-8 [24]. Recently, many bioactive compounds can be effective as anti-cancer, such as cinnamon which shows pharmacological functions that contain activities of anti-microbial, anti-oxidant and anti-cancer. Furthermore, cinnamon has effects of anti-cancer through apoptosis-related pathways in cancer cells [25].

Apoptosis is the programmed cell death in the process of growth and development of an organism, which is linked to the development of the shape and organs of the embryo, such as the growth of fingers and toes. The growth of an organ or tissue usually begins with extreme cell division or cell deformation. This is followed by apoptosis to reshape the tissues to standard size and shape. This cell death process is characterized by cell shrinkage and fragmentation, which are not caused following inflammation [26].

**Apoptosis signaling pathway**

Apoptosis is a programmed cell death (PCD) according to the normal functioning state of the body in which it occurs. When the embryo begins to grow in the womb until adult. The process of cell death is regulated by specific genes for the elimination of the following: unwanted cells, abnormal cells or cells that can cause harm to the body. This is to maintain the balance between the number of new born cells and the number of cells death in the body.

Apoptosis plays an essential role in the development and cellular homeostasis in complex organisms. Therefore, irregularities in the regulation of apoptosis can cause many types of diseases or pathologies, including defects in the induction of cellular apoptosis that can lead to cancer, autoimmune diseases. Excessive apoptosis induction may lead to neurodegenerative diseases and ischemic diseases. Apoptosis can be divided into two main pathways, which are extrinsic and intrinsic pathways [(27)].

**The extrinsic pathway**

The extrinsic pathway, begins when the binding of extracellular death ligands such as tumor necrosis factor (TNF)-α, Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL) with death receptors on the cell membrane, such as tumor necrosis factor receptor (TNFR)-.1, Fas, DR5 induces the binding of death domains of a receptor with adapter molecules such as FADD, TRADD[(28)].

Therefore, the combination of death ligands and death receptors results in a death inducing signaling complex (DISC), in which DISC activates the procaspase-8 to caspase-8. Then activated caspase-8 activates procaspase-7 to transform into caspase-7. This caspase digests its substrate such as PARP (DNA repairing) and results in cell apoptosis [(29)].

**The intrinsic pathway.**

This pathway has processes including an intracellular signal that acts on target within the cell and events initial from mitochondria. This pathway is initial from abnormal cells, such as DNA damage, and oncoprotein expression, then the cells signal the protein p53 activation molecule to activate BH3 protein to inhibit the activity of Bcl-2 protein and activate Bax and Bak proteins to tear the membranes of mitochondria and releasing cytochrome c via permeability transition pore (PT pore). Then cytochrome c binds to Apaf-1 and then Apaf-1 binds to procaspase-9, causing procaspase-9 to cleave off the prodomain and convert it to caspase-9. After that caspase-9 activates procaspase-7 to translate into caspase-7. That caspase-7 is the effector caspase to be activated in order to cells signal apoptosis [(30)].

The regulation of this mitochondrial pathway is mainly through the activity of proteins in the Bcl-2 family, which control the release of cytochrome-c from mitochondria. Overexpression of proapoptotic proteins such as Bax protein modifies the structure of Bax proteins and transmigration to the mitochondrial membrane. At the same time, cytochrome-c release occurs, attended by reduced membrane potential of the mitochondria. In addition, activation through the death receptor pathway can be connected to the mitochondrial pathway. It can induce cytochrome-c release as well, starting from caspase-8 and also stimulating the BH3-interacting domain death agonist (Bid) cleaved part of prodomain to convert tBid. The tBid activates the Bak protein, releasing cytochrome-c from the mitochondria and eventually allowing the cell to enter apoptosis through the mitochondrial pathway [(31)].

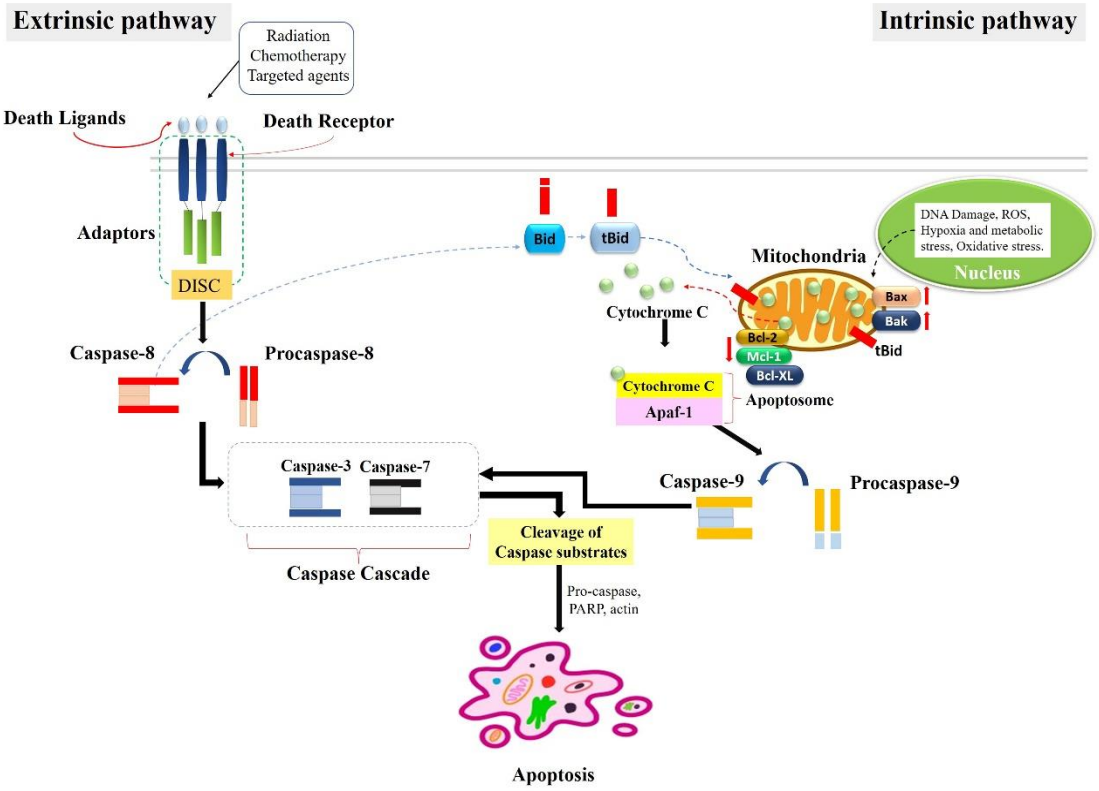



Figure 3 The extrinsic and intrinsic apoptotic signaling pathways.

Source: Bjelakovic G, Nagorni A, BjelakoviĆ M, StamenkoviĆ I, ArsiĆ R, Vuka K. Apoptosis: Programmed cell death and its clinical implications. Med and Bio. 2005;12.

## Signal transduction

Signal transduction is the responding process of cells to external stimuli. These molecular signals are produced from signaling cells and detected by target cells. All cells contain proteins that can recognize the molecules on the cell membrane, like receptor proteins, in which these proteins act on incoming signals (extracellular signal) into signals that can be transmitted into cells (intracellular signal). These molecular signals regulate the behavior of the cell's response to external stimuli [32].

There are many different types of external molecular signals which can bind to specific receptor protein. As cells have a limited number of receptor proteins allowing them regulate and stimulate molecular signals in a complicate way and could be divided as follows.

Firstly, one molecular signal binds to one receptor, causing several stimuli on target cells such as movement, shape, metabolism and regulating gene expression. The signals that are transmitted through the receptor protein are sent into the cell. The proteins inside the cells will generate a series of signal transduction to stimulate the cells. This causes many forms of intracellular signaling depending on the intracellular composition.

Secondly, stimulation of cells occurs when the cells produce a large amount of receptor proteins for different molecular signals, which stimulates the cells to respond better, when any one of signals. The interaction of each type of signal is very important for cell survival and cell mutations. If no signal stimulates the cell, the cell will undergo programmed cell death or apoptosis [33].

Currently, there are many pathways that cause cancer progression or apoptosis but the main known the pathways is MAPK signaling pathway.

**Mitogen-activated protein kinase (MAPK) signaling pathway**

MAPK cascades containing three sequentially activated protein kinases. Its function is to control a biological process over mechanisms in cellular including cell proliferation, cell differentiation, cell migration, stress responses and cell death. In mammalian cells, MAPK can be grouped into three main families. Including ERKs, JNKs and p38 [34].

The activation of a MAPK cascade occurs in a module of consecutive phosphorylation, once activated, each MAPK is phosphorylated by an upstream MAPK. A MAPK module comprises a MAPKKK that receives an external stimulation signal, then MAPKKK activates a MAPKK by phosphorylation to serine and threonine region, which then MAPKK, in turn activates a MAPK, respectively [35].

The signaling mechanism of this pathway is cascade form, starting when a receptor protein receives a signal from outside the cell, Ras protein is activated. Then activated Ras protein activates Raf protein by phosphorylation, resulting in activated Raf protein. After that, Raf protein activates the MEK1/2 protein by phosphorylation. The activated MEK1/2 protein activates the ERK1/2 protein by phosphorylation. Finally, ERK1/2 protein is responsible for inducing targeted molecular responses such as activation of the transcription factor for the expression of genes involved in cell division, cell differentiation and inhibition of cell death. The classic ERK1 and ERK2 were found to be the main compounds in the regulation of underlying mechanisms and cancer cell division and control the stimulation of the phosphorylation within the cell. Both proteins have different signaling, ERK2 plays a role in fundamental regulation and signaling to ras-dependent proteins in cell division, whereas ERK1 protein affects all the signals that are from the cell and resist the stimulation of ERK2. Moreover, ERK is found to activate cell from G1 to S phase and work with PI3K enzyme to activates cells from G2 to M phase [(36)].

JNK pathway is activated by environmental conditions such as osmotic stress, oxidative stress, UV light and cytokines such as tumor necrosis factor-α and interleukin-1ß. In stimulating this pathway, the proteins MEKK1/2/3/4, MKK4/7 and JNK1/2/3 were sequenced by phosphorylation. After that, activated JNK1/2/3 activates the transcription factors including c-Jun, ATF-2, and Elk-1 by phosphorylation. Then JNK activates a c-Jun by phosphorylation to c-Jun. C-Jun is the transcription factor of JNK downstream. When the c-Jun was activated will have an effect on controlling the expression of genes connected to cell growth, cell division, apoptosis and cell survival in conditions where cells have to respond to stressful conditions [(37)].

p38 family members include p38α, p38β, p38γ and p38δ, respectively. That modules are 38 kDa of molecular weight. The p38 activation is activated in sequence. That starts with external stimuli signal of the cell such as stress, UV light, DNA damage, inflammation and ischemia. The MEKK1/4, ASK1/2 were activated at first by phosphorylation. And then activates MKK3/6 and MKK4 by phosphorylation as well.

The activated MKK3/6 and MKK4 activate at all isoforms of p38 by phosphorylation and activated p38. Then activates transcription factors such as ATF-2, CREB, CHOP and MEF2C to transcribe genes linked to cell growth and cell division including apoptosis [38].

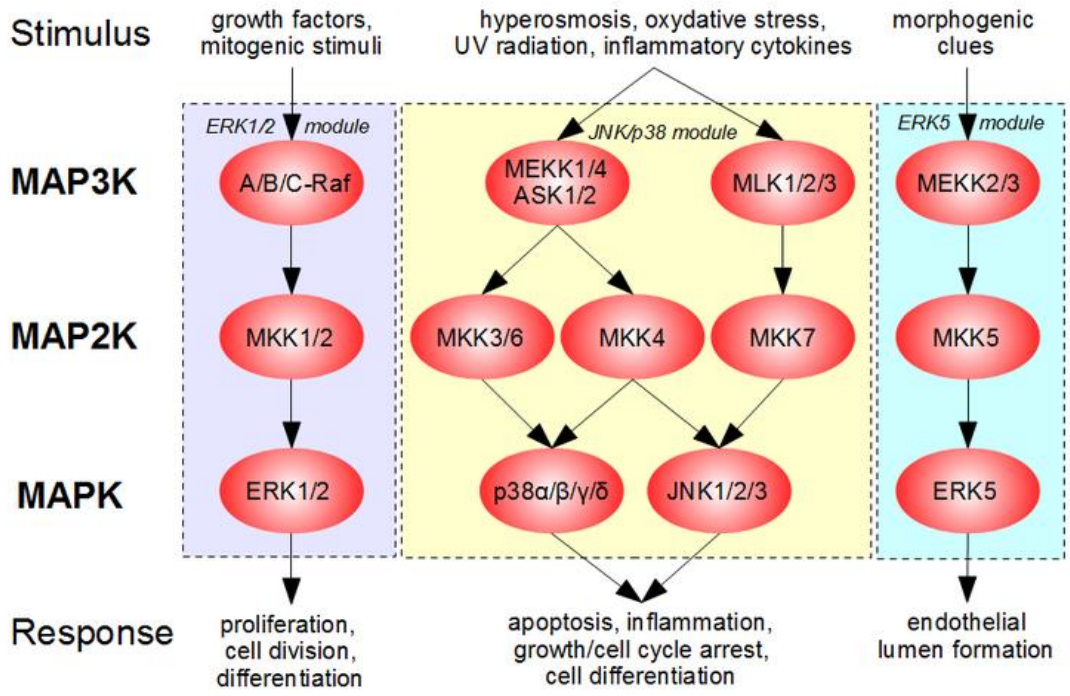



Figure 4 Mitogen-activated protein kinase (MAPK) pathways.

Source: Morrison DK. MAP kinase pathways. Cold Spring Harb Perspect Biol. 2012;4(11).

## Artonin E

Artonin E is a prenylated flavonoid isolated from *A. elasticus* Reinw. (Moraceae) [6]. The extreme diversity of which is in Southeast Asia. Many species of these plants have been used in many countries as folk medicine [11]. In the southern part of Thailand that the local names are “Ka Ok”, “Ka O” and “Tue Ka” [7].

In previous study it is reported that artonin E enhances anoikis of human lung cancer H460 cells in a dose-dependent manner. And also alerted the cells by downregulating of MCL-1 protein [39].

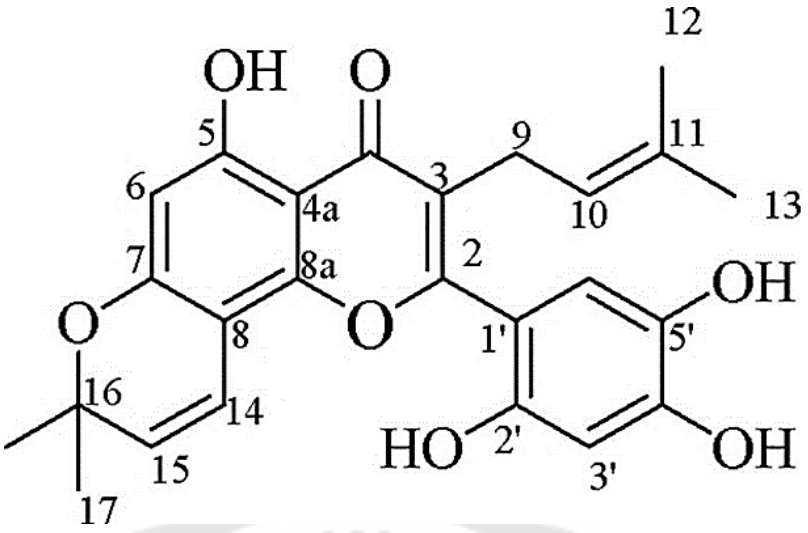



Figure 5 The chemical structure of artonin E.

Source: Suhartati T, Hernawan H, Suwandi J, Yandri Y, Hadi S. Isolation of artonin E from genus Artocarpus, synthesis of Artonin E acetate and evaluation of anticancer activity. Macedonian Journal of Chemistry and Chemical Engineering. 2018; 37:35.

In lung cancer, Plaibua et al (2013) reported that artonin E exhibited anti activities about migration and invasion in lung cancer H460 cells. Morphology of cells exposed that artonin E at nontoxic concentrations led to FAK and AKT activation were decreased and inhibition of migration and invasion of other lung cancer H292, H23 and A549 cells, respectively [(40)].

In ovarian cancer, Rahman et al (2016) demonstrated that presented that inhibition of artonin E on SKOV-3 cells growth and showed a reduced amount of toxicity toward an average human ovarian T1074 cell line. All results exhibited that artonin E induced cell cycle arrest at the S phase and induced apoptosis via the regulation of the intrinsic pathway [(7)].

In breast cancer, Etti et al (2017) revealed that artonin E induced apoptosis by caspase activity from extrinsic and intrinsic pathways. It also boosted the total of ROS that was released which polarized the mitochondrial membrane, compounding the cytochrome C release. All of these, artonin E exposed the potential as a promising candidate to fight the aggressive TNBC or triple negative breast cancer [(9)].

In colon cancer, Sophonnithiprasert et al (2019) demonstrated that artonin E in combination with TRAIL boosted cytotoxicity and led to chromatin condensation and apoptotic bodies in LoVo cells. In contrast, artonin E and TRAIL treatment alone was not. In the last one, they exhibited that artonin E enhanced TRAIL-induced apoptosis in LoVo cells via downregulation of cFLIP and DR5 upregulation [(41)].

In skin cancer, Innajak et al (2017) demonstrated that artonin E presented anti-proliferation and induction of apoptosis in skin cancer A431 cells. Furthermore, the activation of cleaved caspase-7 and cleaved-PARP proteins were induced by artonin E treatment in A431 cells, allowing apoptosis cell death [(15)].

# CHAPTER III
# MATERIALS AND METHODS

## Materials

### Chemicals

All of the chemicals that used in this study and listed alphabetically were including

| Name of chemicals | Company |
|---|---|
| Acrylamide | Bio-Rad |
| Ammonium persulfate (APS) | HiMedia |
| Bovine serum albumin (BSA) | VWR |
| Dimethyl sulfoxide (DMSO) | RCI Labscan |
| Disodium hydrogen phosphate ($Na_2HPO_4$) | Panreac |
| Ethylenediaminetetraacetic acid (EDTA) | HiMedia |
| Fetal bovine serum (FBS) | HiMedia |
| Glycine | Vivantis |
| Guava® Cell Cycle Reagent | Merck |
| HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) | USB |
| Hoechst 33342 | Thermo Fisher |
| JC-1 | Wako |
| 2-Mercaptoethanol | ACROS |
| Methanol | VWR |
| MTT | USB |
| Penicillin/Streptomycin | HiMedia |
| PMSF (Phenylmethylsulphonyl fluoride) | Sigma-Aldrich |
| Potassium chloride (KCl) | BDH |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | Ajax |
| RPMI-1640 medium | HiMedia |

| | |
|---|---|
| Sodium chloride (NaCl) | HiMedia |
| Sodium dodecyl sulfate (SDS) | Vivantis |
| TEMED | HiMedia |
| Tris | HiMedia |
| Tris-HCl | HiMedia |
| Triton X-100 | Panreac |
| Trypsin | CAPRICORN |
| Tween 20 (Polysorbate 20) | Vivantis |

**Plant extraction**

In this study artonin E was obtained from Associate Professor Wilawan Mahabusarakum, Faculty of Science, Prince of Songkla University, Thailand in purified powder form with a molecular weight was 436.5. And IUPAC name was 5-hydroxy-8,8-dimethyl-3-(3-methylbut-2-enyl)-2-(2,4,5-trihydroxyphenyl) pyrano (2,3-h) chromen-4-one] [42].

**Cell culture**

The LoVo cell was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The first step cells were maintained in medium was RPMI formula in culture flasks. The supplemented was added in medium consist of 10% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin after that cell were incubated in 5% CO2 at 37°C of humidified atmosphere. Then every 2-3 days, the medium was renewed. Finally, cells were subcultured with 0.25% trypsin-EDTA, when amount of cells were 90% confluence.

**Detection of cell viability and proliferation**

Cell growth and proliferation were determined by MTT assay. This assay is a detection about quantitative and sensitive of cellular metabolic activity in proliferative cells by resulting in a relationship of linear between activity of cell and absorbance. The reductase enzyme inside mitochondrial in living cells can lessen yellow MTT to purple formazan [43].

Effect of artonin E on a viability of cell was studied by MTT assay. LoVo cells were seeded at a density of 7x103 cells/well and allowed to grow for 24 hours. And then cells were treated by artonin E at 1.5625, 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/ml. And control group was treated with DMSO. After 24 hours, MTT solution at 0.5 mg/ml was added to each well and incubated for 2 hours at 37°C. MTT solution was removed and added DMSO to each well to solubilize water insoluble formazan crystals (purple colour). Then absorbance was measured at 570 nm by a microplate reader (Multiskan Sky Microplate Spectrophotometer, Waltham, Massachusett, USA). Finally, IC50 value was calculated by using GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA, USA).

**Detection of nuclear morphological changes**

The changes of nuclear morphological in the cells with apoptotic character were detected by Hoechst33342 staining. The Hoechst33342 dye that used for labeling of DNA [44]. It specially binds to A-T regions at minor groove on DNA double-stranded and excited by UV light and emits blue fluorescence at 460 to 490 nm [45].

LoVo cells were seeded at 2.2x104 cells/well and allowed to grow 24 hours. Cells were treated with artonin E at 3, 5, 10 and 30 µg/ml for 24 hours, we chose the concentration between the IC50 values or to cover the IC50 values and take this concentration for testing in the next experiment. And cells in control group were treated with DMSO. The incubated cells, after these cells were stained with Hoechst33342 at 5 µg/ml and incubated at 37°C for 30 minutes. Finally, cells were detected under the fluorescence microscope (DP73+IX71 Olympus, Japan).

**Detection of mitochondrial membrane potential ($\Delta\Psi m$)**

The apoptosis of unhealthy cells is a cellular process which involves genetical events causing cell death. Several major events occur in mitochondrial health associated with low mitochondrial membrane potential ($\Delta\Psi m$) [46]. The mitochondrial disruption including the membrane is changes and assumed to be connected with the

mitochondrial permeability transition pore is opening and led to changes in electron transport and cytochrome C release.

JC-1 is a lipophilic cationic dye. That selectively enter into mitochondria and reversibly change color from green to red as the membrane potential increase. The healthy cells with high level of $\Delta\Psi m$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence (absorption/emission 585/590 nm). In contrast, in unhealthy cells with low $\Delta\Psi m$, JC-1 remains in the monomeric form, that show the green fluorescence (absorption/emission 514/529 mm) [47].

LoVo cells were seeded at $3.2x10^4$ cells/well and allowed to grow for 24 hours. Then cells were treated with 3, 5, 10 and 30 µg/ml of artonin E for 6 hours. The control cells were treated with DMSO at 0.3%. After incubation, cells were stained with JC-1 dye and incubated at 37°C for 10 minutes. To finish, cells were detected by fluorescence microscope (DP73+IX71 Olympus, Japan).

**Flow cytometry**

The induction of apoptosis through accumulation of DNA content in sub-G1 phase was detected by flow cytometry. That used to cell counting that have undertaken apoptosis. Then cells were detected by PI (propidium Iodide) staining and allowed by flow cytometry analysis [48].

The Guava® Cell Cycle Reagent can be used for measuring cell cycle distributions. Based on stained PI on the of whole-cell [49].

LoVo cells were seeded at 5 × 104 cells/well and grow for 24 hours. Then, LoVo cells were treated with 3, 5, 10, and 30 µg/ml of artonin E, while cells in control group were treated with DMSO. During treatment, the cells were harvested, PBS washed, then fixed cells with 70% ethanol (cold), and kept cell at 4 °C. The fixated cells were stained with reagent of cell cycle (Guava Cell Cycle® reagent from Merck KGaA, St. Louis, MO, USA). Lastly, the Guava easyCyte™ flow cytometer and GuavaSoft™ software version 3.3 (Merck KGaA, St. Louis, MO, USA) was using observed the content of DNA.

**Western blot analysis**

This method analysis is a well-accepted and widely used technique for the detection and analysis of proteins. The SDS-PAGE or sodium dodecyl sulfate-polyacrylamide gel electrophoresis is used isolated proteins conferring to their MW of proteins. SDS used as a buffer in order to make all proteins in a uniform negatively charge. The separated proteins are then transferred onto a PVDF membrane, where they are probed by using primary antibody of the target proteins. To detect the complex of antibody-antigen, the biochemically detectable marker was used to tagged with secondary antibody. In this study, the ECL or enhanced chemiluminescence was selected of which secondary antibody is conjugated with HRP or horseradish-peroxidase. The band on the membrane was imagined by loaded with ECL reagent results in signal of chemiluminescence that can be captured with CCD camera (AllianceQ9 advanced chemiluminescence imaging system from Uvitec, UK) [50].

**Sample preparation**

LoVo cells were seeded in 6-wells plate at 20x104 cells/well and grow for 24 hours at 37°C. Then cells were treated with artonin E at 3, 5, 10 and 30 µg/ml and 0.3% DMSO was added to the control group for 24 hours. After that the incubated cells were harvested and allowed cool PBS washed. After that, cells were lysate in lysis buffer (RIPA), supplement with 100 mM PMSF and complete mini protease inhibitor cocktail. Then the lysate cell was centrifuged at 13,000g in 4°C. The supernatants were transferred to a new eppendorf and the protein content was defined before loading to the gel each well.

**Protein determination**

The Bio-Rad protein assay (Bio-Rad Laboratories, USA) was used for determined the concentration of total protein. Which originated on the Bradford protein assay. Bovine serum albumin (BSA) was used as protein standard. To determine the protein concentration, the BSA was diluted with distilled water to 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml. The measurement protein concentration was determined at 595 nm by a spectrophotometer (Multiskan Sky Microplate Spectrophotometer, USA).

**Preparation of SDS-PAGE**

The SDS-PAGE consists of two layers of gel including stacking and separating gels. The stacking gel (upper layer) has large of pores size that let the proteins to migrate freely and get stacked at the interface among stacking and separating gels. So that they were started migrating in the separating gel all together. The gel at lower layer was separating gel, used to separate the proteins in the sample affording to molecular weight.

**Separating gel**

The separating gel is a lower layer gel, that the protein was separated according to the molecular weight depends on this layer gel. Smaller proteins were resolved at higher acrylamide concentrations. The preparation of separating gel was according to the table below.

Table 1 Recipes for preparation of separating gel

| Stock solution | 10% | 12% |
|---|---|---|
| $dH_2O$ | 2.42 ml | 2.17 ml |
| 1.5 M Tris-HCl, pH 8.8 | 1.25 ml | 1.25 ml |
| 10% SDS | 50 µl | 50 µl |
| 40% acrylamide | 1.25 ml | 1.5 ml |
| 10% ammonium persulfate (APS) | 25 µl | 25 µl |
| TEMED | 2.5 µl | 2.5 µl |
| **Total volume** | **5 ml** | **5 ml** |

**Stacking gel**

The preparation of stacking gel was according to the table as below, after the separating gel was polymerized. After pouring the gel, then inserted for comb to the gel and let it polymerized

Table 2 Recipes for preparation of stacking gel

| Stock solution | Volume (per 1 gel) |
|---|---|
| $dH_2O$ | 1.52 ml |
| 1.5 M Tris-HCl, pH 6.8 | 0.25 ml |
| 10% SDS | 20 µl |
| 40% acrylamide | 0.196 µl |
| 10% ammonium persulfate (APS) | 10 µl |
| TEMED | 2 µl |
| **Total volume** | **2 ml** |

**Running condition**

The 5X sample was (60 mM Tris-HCl; pH6.8, glycerol at 25%, SDS at 2%, 2-mercaptoethanol at 14.4 mM and bromophenol blue at 0.1%) was mixed with total proteins and then boiled for 5 minutes. Protein samples were loaded into the gel. The gel was run at 100 V for 1:30 hours in running buffer (Tris-HCl at 0.025 M, glycine at 0.192 M, and SDS at 0.1%). And then, the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, USA) used for transferred gel onto PVDF membranes at 25 V for 30 minutes in transfer buffer.

**Protein detection**

After protein transferring, the membrane was blocked with 5% BSA in 1X PBS for 1 hour and incubated with specific primary antibodies for overnight at 4°C. The primary antibody was a rabbit immunoglobulin G that specific to the mediator proteins were including Bax (dilution 1:1,000), Bcl-2 (dilution 1:1,000), Bcl-xL (dilution 1:1,000), Cleaved caspase-7 (dilution 1:1,000), Caspase-7 (dilution 1:1,000), Cleaved PARP (dilution 1:1,000), PARP (dilution 1:1,000), phosphorylated-ERK1/2 (dilution 1:1,000), ERK1/2 (dilution 1:1,000), phosphorylated-p38 (dilution 1:1,000), p38 (dilution 1:1,000), phosphorylated-c-Jun (Ser 73) (dilution 1:1,000) and c-Jun (dilution 1:1,000) thirteen proteins were expressed in pathways. Then the primary antibody excess was TBST washed for 10 minutes for 3 times, then incubated with the anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies (dilution 1:10,000) for 1 hour at room temperature. The secondary antibody excess was removed out of membranes by washed with TBST 10 minutes for 3 times. And next, the ECL reagent was used for detected immunoreactive protein bands by chemiluminescence principle. Once ECL reagent is a substrate was added, it reacts with HRP and emits light. After that the emitted of light was detected by using chemiluminescence imaging system. (chemiluminescence using ImmobilonTM Western chemiluminescent HRP substrate (Merck Millipore Corporation, Merck KGaA, Darmstadt, DE)).

**Statistical analysis.**

The One-way analysis of variance or ANOVA was evaluated the statistical significance. The analysis of statistical was operated by using SPSS statistical software package (version 15; SPSS, Inc., Chicago, IL, USA) also carried out using the GraphPad Prism 3.03 (GraphPad Software, Inc., La Jolla, CA, USA). The band intensity of protein was quantified by Image J densitometer. The differences were speculated statistically significant at $P<0.05$.

# CHAPTER IV
# RESULTS

**Artonin E inhibited cell proliferation of LoVo cells.**

MTT assay used to determine the activity about anti-proliferation of artonin E in LoVo cells. The artonin E inhibited cell proliferation in a dose-dependent manner in LoVo treated cells that presented in results. The IC50 was 11.73±1.99 µg/ml (Fig. 5).

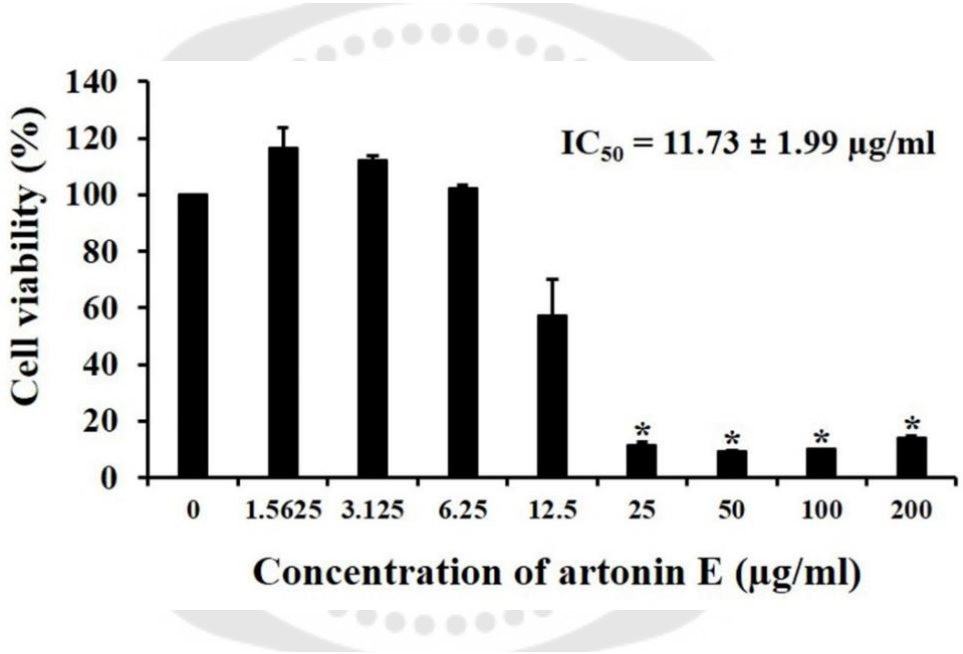



Figure 6 Effect of artonin E on the percentage of cell viability in LoVo cells.

Effect of artonin E on cell viability after treatment with artonin E in colon cancer LoVo cells for 24 hours. The data are presented as mean ± SD (n = 3), * $p < 0.05$ compared with control group.

**Effect of artonin E on cell apoptosis induction in LoVo cells.**

The morphological changes of nuclear in apoptotic cells were detected by staining of Hoechst33342 dye. The Hoechst33342 is a fluorescence dye was used for labeling DNA [12]. This study, the results verified that the induction of chromatin condensation and apoptotic bodies in a dose-dependent manner of artonin E in LoVo treated cells (Fig. 6A-B).

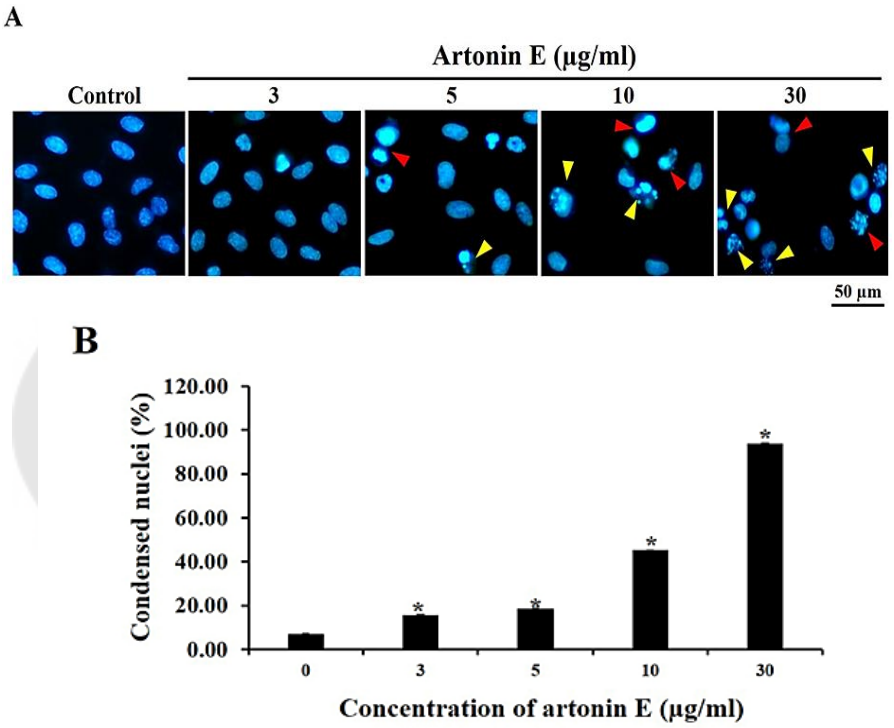



Figure 7 Chromatin condensation and apoptotic bodies in LoVo cells treated with artonin E.

The observation of chromatin condensation and apoptotic bodies in LoVo cells. (A) The LoVo cells were treated with artonin E for 24 hours, after that the condensation of chromatin and apoptotic bodies were observed by Hoechst33342 staining. The red and yellow arrows indicated chromatin condensation and apoptotic bodies respectively, compared with the control group. (B) Quantification of percentage of condensed nuclei was determined by Hoechst 33342 staining. The data are presented as mean ± SD (n = 3). * $p < 0.05$ compared with control group.

**Mitochondrial membrane potential ($\Delta\Psi$m).**

The mitochondria were disrupted by activation of pro-apoptotic proteins in all through apoptosis and leading to loss of $\Delta\Psi$m. Untreated cells with high $\Delta\Psi$m, JC-1 formed J-aggregates complexes as shown that brighter red fluorescence intensity while apoptotic cells with low $\Delta\Psi$m, JC-1 remained in form of monomeric as shown green fluorescence. This results, artonin E induced the loss of $\Delta\Psi$m in a dose-dependent manner in LoVo cells. Therefore, artonin E induced apoptosis through injury of mitochondria in LoVo cells (Fig. 7A-B).

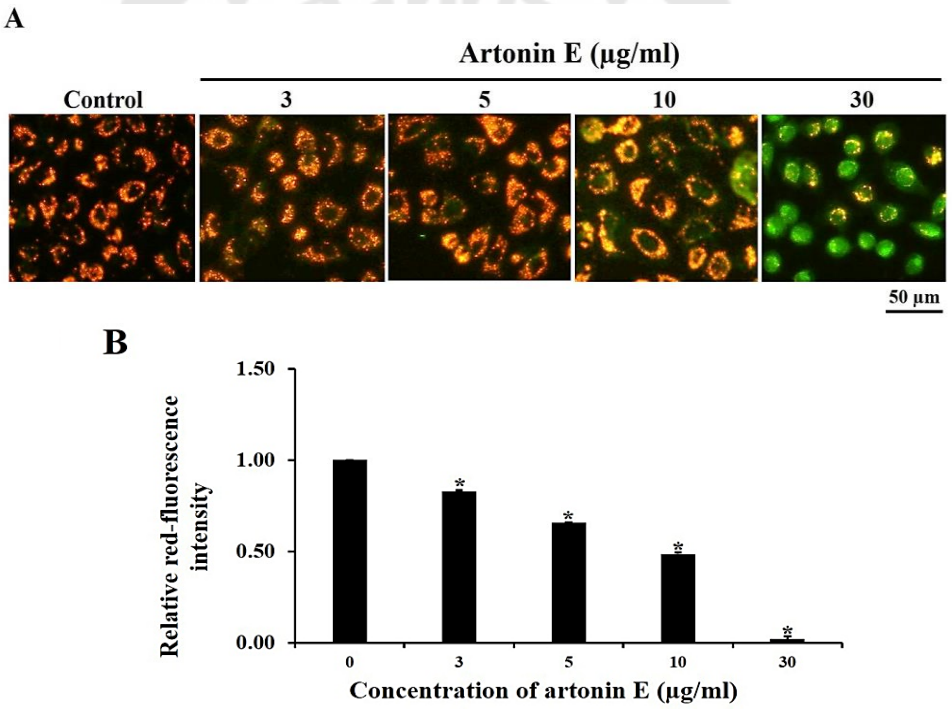



Figure 8 Effect of artonin E on $\Delta\Psi$m in LoVo cells.

The effect of artonin E on $\Delta\Psi$m in LoVo cells. (A) LoVo cells were treated with artonin E for 6 hours. Artonin E showed decreased red fluorescence while increased green fluorescence as compared with control cells. (B) Quantification of relative red-fluorescence intensity was determined by JC-1 staining. The presented of data as mean ± SD (n = 3). * $p < 0.05$ compared with control group.

**Cell cycle distribution.**

To confirm the induction of apoptosis by the effect of artonin E, LoVo cells were stained with cell cycle reagent and histogram analysis-related the contents of DNA were measured by flow cytometry. LoVo cells treated with artonin E at 30 µg/ml exhibited maximum of population in sub-G1 phase at 12.61%. While control cells showed 0.35% (Fig. 8A-B). These results exposed that the artonin E induced apoptosis induction in LoVo treated cells through accumulate of sub-G1 population.

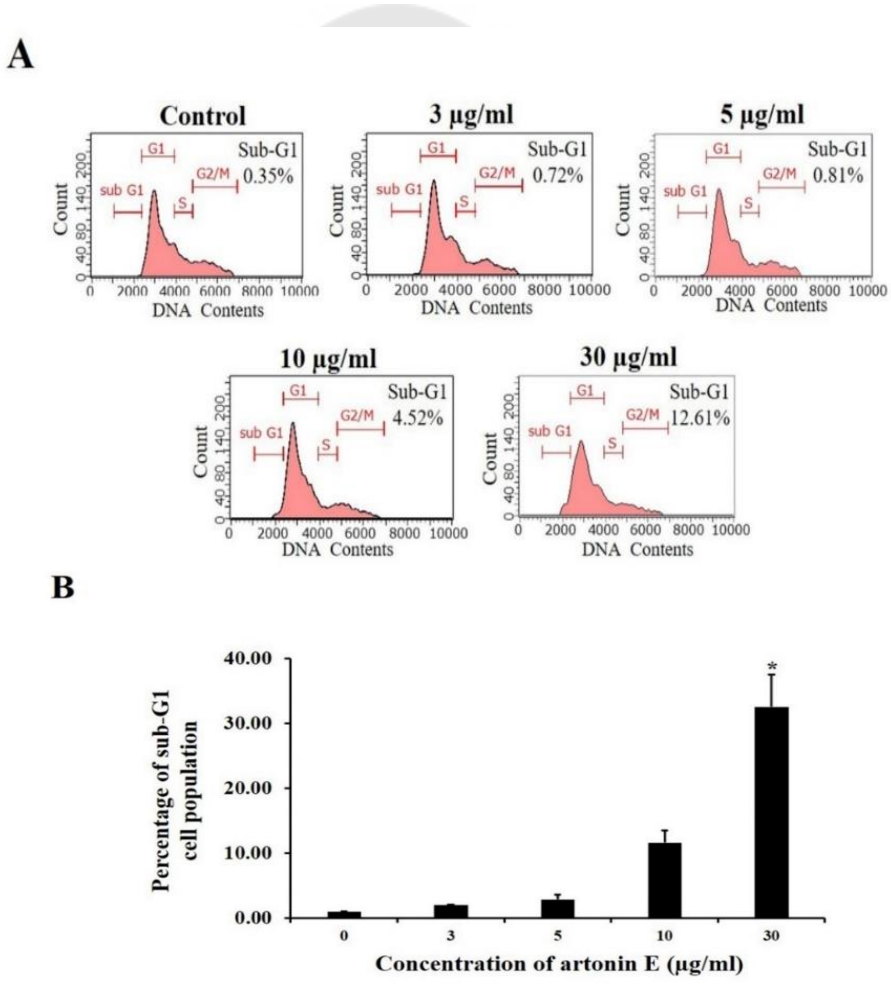


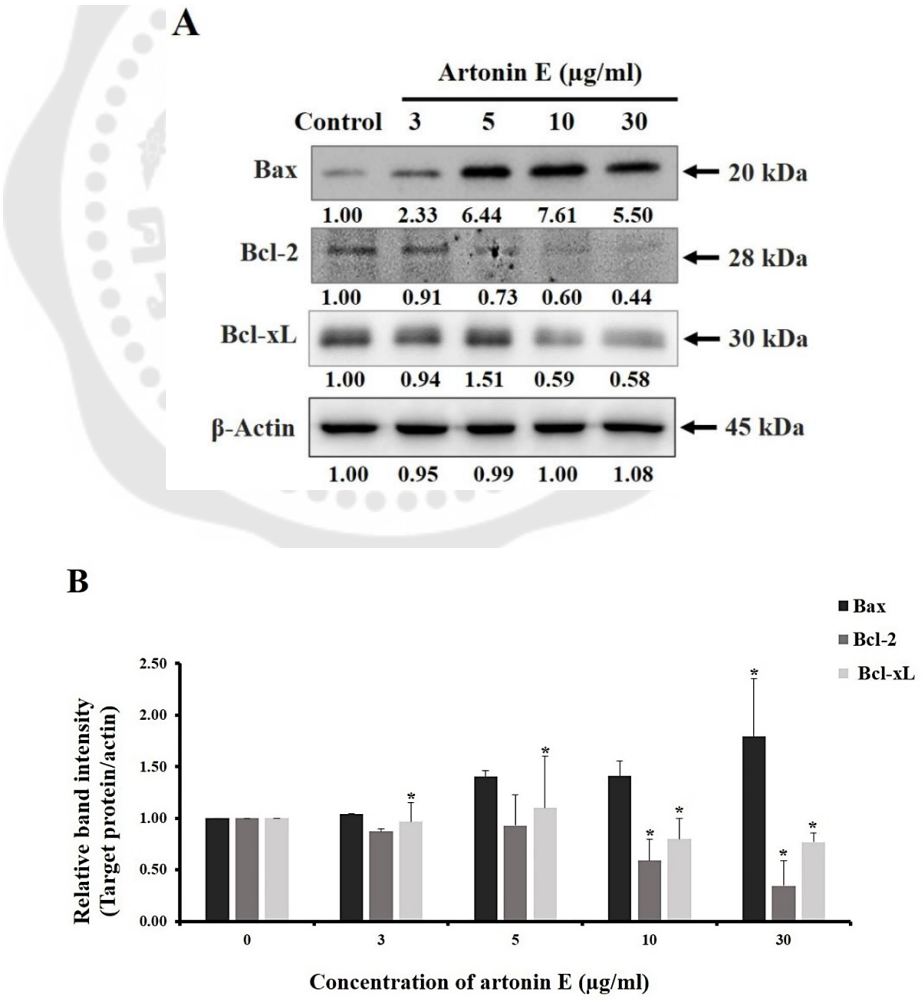

Figure 9 Effect of artonin E on cell cycle distribution in LoVo cells.

Artonin E on cell cycle distribution. The sub-G1 population peak was the reduction of DNA content formed that represented the apoptotic cells were presence. The mean apoptotic population of LoVo cells were 0.35% under control condition and

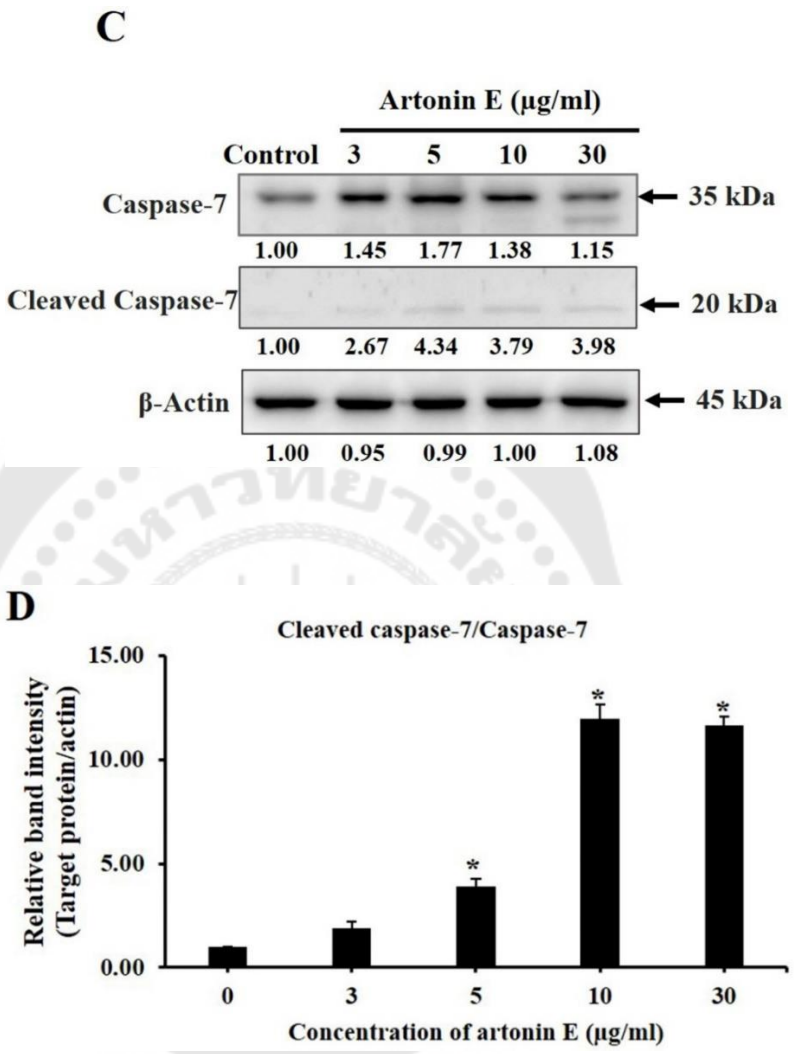
these cells were increased 0.72% upon treatment with 3 μg/ml of artonin E for 24 hours. (B) Quantification of percentage of cells under sub-G1 peak in LoVo treated cells. The data are presented as mean ± SD (n = 3). * $p < 0.05$ compared with the control group.

**The expression of Bcl-2 Family, Caspase-7, and Cleaved-PARP proteins in LoVo Cells.**

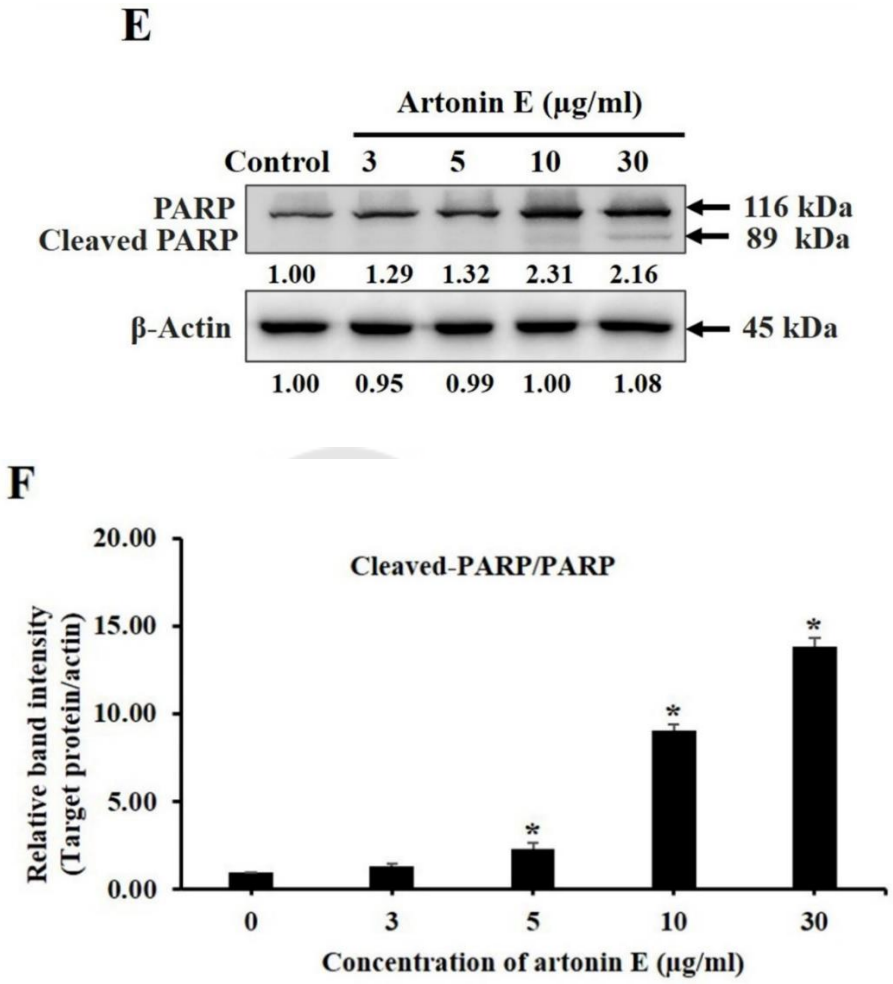
The activation of apoptosis mediator protein was detected by Western blotting. The results established that artonin E increased the level of Bax (proapoptotic protein) expression and declined the level of Bcl-2 and Bcl-xL (anti-apoptotic proteins) in LoVo cells. Furthermore, artonin E induced activation of caspase-7 and inactivated PARP (DNA repair protein) also (Figure 9A-F). Therefore, these results indicated that artonin E induced apoptosis connected with the activation of caspase-7 in LoVo cells.




Effects of artonin E on level of Bcl-2 family proteins and relative band intensity.




Effects of artonin E on level of caspase-7, cleaved caspase-7 proteins and relative band intensity of cleaved caspase-7/caspase-7 ratio.



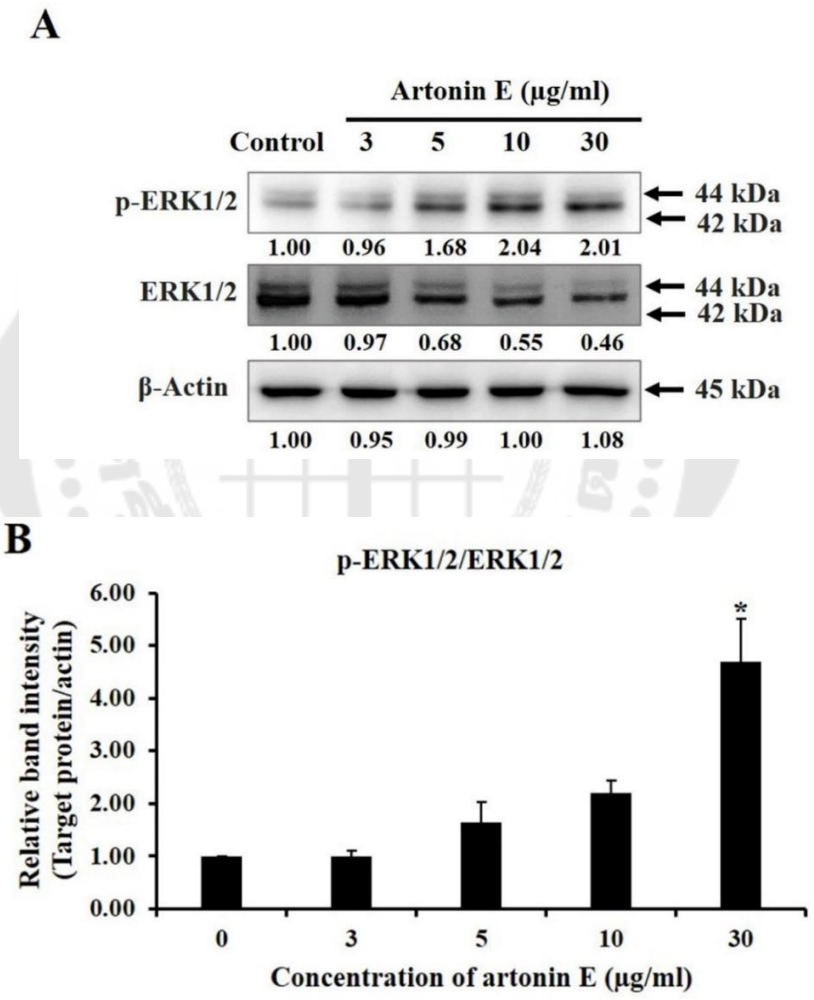

Effects of artonin E on level of PARP, cleaved PARP proteins and relative band intensity of cleaved PARP/PARP ratio.

Figure 10 Effect of artonin E on the expression of apoptosis protein.

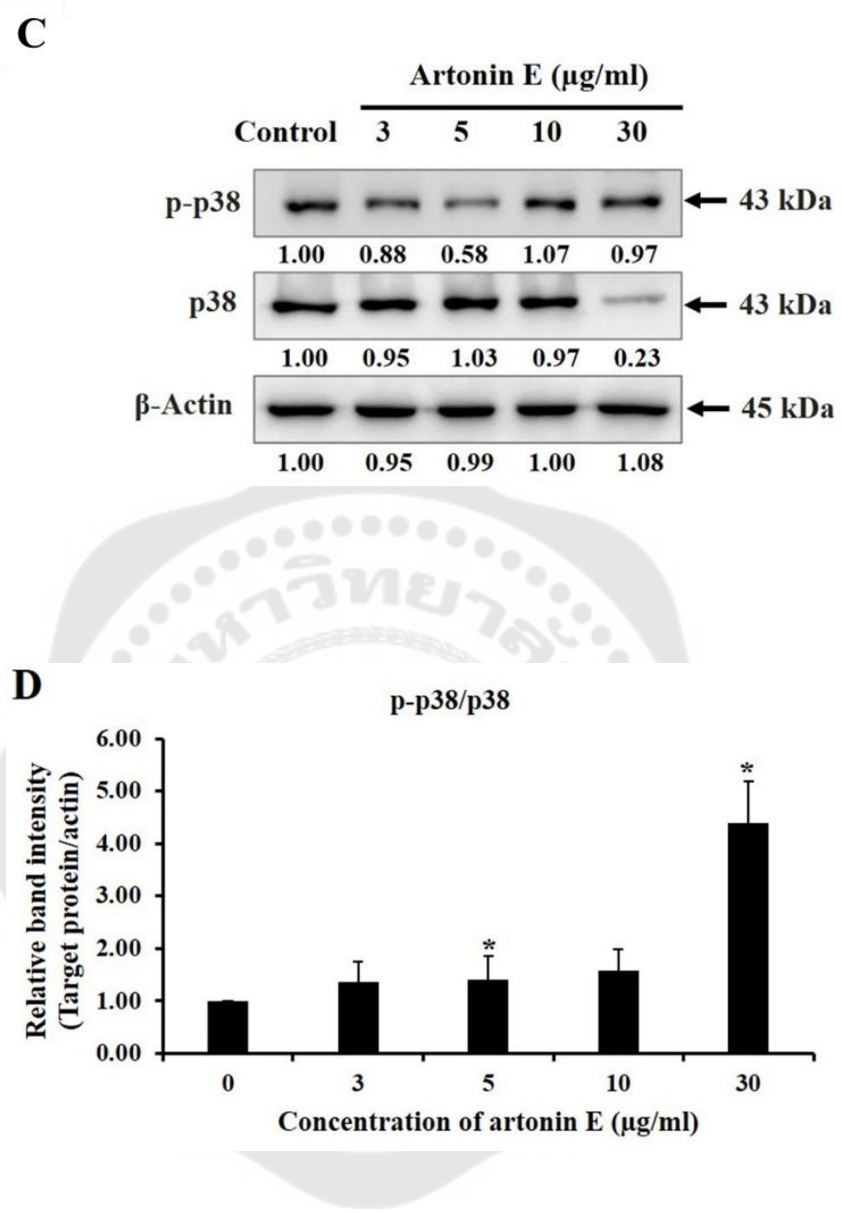
The cells were treated with artonin E for 24 hours. A-B) Expression of the Bcl-2 family proteins and relative band intensity. C-D) The level of caspase-7, cleaved caspase-7 proteins and relative band intensity of cleaved caspase-7/caspase-7 ratio and E-F) The level of PARP, cleaved PARP proteins and relative band intensity of cleaved PARP/PARP ratio. Examined by western blot analysis (* $p < 0.05$).

**The effect of artonin E on MAPK signaling pathway.**

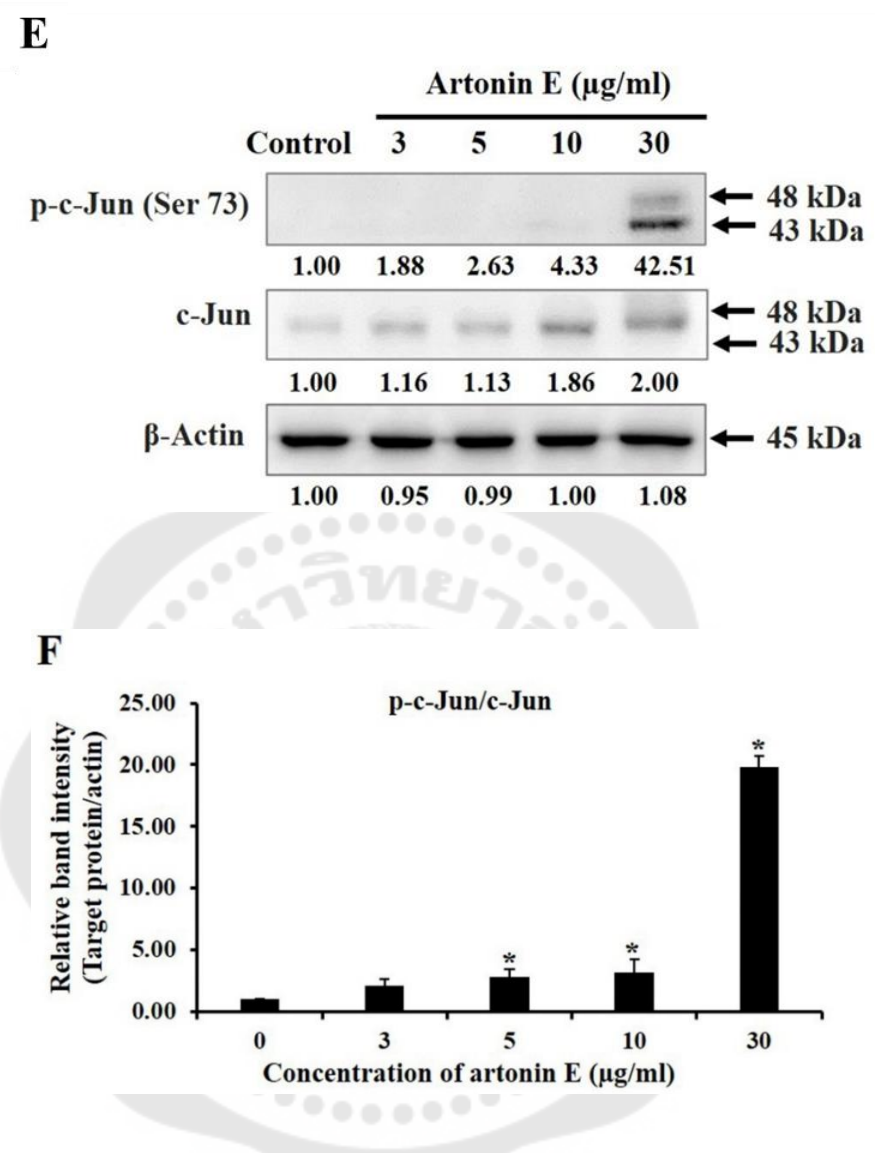
This pathway that involved cell survival and apoptosis. The important role of MAPK was transduction extracellular signals to responses of cellular. When treatment with artonin E. The level of phosphorylated-ERK1/2/ERK1/2, phosphorylated-p38/p38 and phosphorylated-c-Jun/c-Jun ratios were increased. These results presented that artonin E induced apoptosis through MAPK signaling pathway (Fig. 10A-F).




Effects of artonin E on level of p-ERK1/2, ERK1/2 proteins and relative band intensity of p-ERK1/2/ERK1/2 ratio.




Effects of artonin E on level of p-p38, p38 proteins and relative band intensity of p-p38/p38 ratio.




Effects of artonin E on level of p-c-Jun, c-Jun proteins and relative band intensity of p-c-Jun/c-Jun ratio.

Figure 11 Effects of artonin E on phosphorylation of MAPK signaling in LoVo cells.

Effects of artonin E on phosphorylation of MAPK proteins in LoVo cells. A) The level of p-ERK1/2, ERK1/2 proteins and relative band intensity of p-ERK1/2/ERK1/2 ratio. B) The level of p-p38, p38 proteins and relative band intensity of p-p38/p38 ratio and C) The level of p-c-Jun, c-Jun proteins and relative band intensity of p-c-Jun/c-Jun ratio, respectively, determined by Western blot analysis (* $p < 0.05$).

# CHAPTER V
# DISCUSSION

Colon cancer is one of the most widespread and terrible types of cancer in both of men and women worldwide. The patients about 90% with metastatic cancer had treatment disappointment is believed to be learned resistance from chemotherapy, including fight to epidermal growth factor receptor (EGFR) antagonists [51]. In contrast, only 10-20% of patients exhibit a primary response to EGFR-targeted therapies, cetuximab or panitumumab [52],[53]. Therefore, effective treatments require more discovery and inferior side effects for this type of cancer treatment. In recent years, the discovery of new cancer medication in preclinical experiment has turned to products of natural with little drug resistance and low toxicity. Previous studies have exposed that the artonin E inhibited the growth of SKOV-3 cells and showed not as much of toxicity toward a normal human ovarian cell line T1074 [6]. Additionally, artonin E inhibited breast cancer growth and proliferation over beginning of the intrinsic apoptotic pathway while suppressing the mechanism of antiapoptotic, accordingly circumventing the relative immortality of cancer cells [54]. Here study, effect of artonin E, extracted from *A. elasticus* Reinw. (Moraceae). was experiential in LoVo cells. The results exposed that artonin E could inhibit cell growth with $IC_{50}$ of 11.73±1.99 μg/ml [55]. That correlated with previous research by Sophonnithiprasert et al, revealed that treatment at 10, 20, 30, 40 and 50 μM of artonin E in LoVo cells exhibited more than 80% of cell viability indicating that the LoVo cells insensitive to artonin E activated cell death [41].

Hoechst33342 staining was used to confirm on apoptosis and check nuclear morphological changes of cell over induction of apoptosis. These results indicated that artonin E induced chromatin condensation and led to apoptotic bodies in LoVo cells [41]. Furthermore, the JC-1 staining assay showed that the treated cells led to unhealthy mitochondria that decreased brighter red fluorescence intensity while increased green fluorescence intensity suggesting the loss of $\Delta\Psi$m. Cells released some

pro-apoptotic proteins from mitochondria pores to cytosol and induced apoptosome formation and followed by caspase-9 activation [56]. Then active caspase-9 can activate the effector caspase-7, that cleave vital substrates including PARP, leading to apoptosis induction [57]. These results linked with previous study by Innajak et al, that upon treatment with artonin E, epidermoid carcinoma cell line, A431 showed chromatin condensation and apoptotic bodies. In addition, effect of artonin E on the $\Delta\Psi$m in A431 cells was detected by increased green fluorescence [15].

The cell cycle is a major mechanism that involved with cell growth. For the time of apoptosis, DNA was released by endonuclease in cells resulting in fewer DNA population compared with healthy cells, representing the peak of sub-G1 phase in flow cytometry analysis [55],[58]. Previous report presented that natural compounds could inhibit LoVo cells by induced population in sub-G1 phase [59]. Our results discovered that LoVo cells treated with artonin E had an increased sub-G1 population compared with control cells.

To confirm the apoptosis signaling pathway and MAPK signaling pathway were studied by Western blotting. The apoptosis signaling pathways have two main are including, extrinsic and intrinsic pathways. For LoVo cells treated with artonin E, that results presented increased of Bax protein expression. In contrast, Bcl-2 and Bcl-xL proteins were decreased. Moreover, cleaved caspase-7 and cleaved-PARP proteins expression were increased by induced from artonin E. These results correlated with the previous research that artonin E showed the activation about anti-proliferation and induction of apoptosis by induced cleaved of caspase-7 and PARP activation in skin cancer A431 cells, resulting in apoptosis cell [56].

In normal, the caspases consist of two types, initiator and effector caspase; the caspase cascade, initiator caspase such as caspase-9 can activate effector caspase such as caspase-7, and led to cleaves essential substrates, for example PARP that plays an important role in repairing of DNA [60]. These present results linked to previous study representing that artonin E induced apoptosis in more than a few cancer cell types including MDA-MB231, MCF-7, SKOV-3, H460, and A431 [12], [14], [54], [15], [61].

The proteins in MAPK signaling pathway play important roles both in cell survival and cell death, [62] and also in transduction extracellular signals to cellular responses. Conventional MAPKs in mammalian include the ERK1/2, JNK1/2 and p38. The ERK1/2 activates Bax and caspase proteins and leads to apoptosis. In part of JNK1/2, JNK1/2 can activate the transcriptional factors including c-Jun. p38 is a tumor suppressor, which induces apoptosis and inflammation. Our results presented that artonin E induced p-ERK1/2/ERK1/2, p-p38/p38 and p-c-Jun/c-Jun ratio were upregulation in LoVo treated cells leading to apoptosis. Moreover, ERK1/2 is vital in cell proliferation, cell growth, cell differentiation and cell survival, correlated with previous report about ERK1/2 induced apoptosis by Tangchirakhaphan et al, that ERK1/2 could activate caspase and pro-apoptotic protein in Bcl-2 family proteins, leading to eventual apoptosis induction [63]. Therefore, our results showed that artonin E induced apoptosis in LoVo cell.

In conclusion, we showed that artonin E induced apoptosis connected with MAPK signaling pathway by promoting cell death through the expression of p-ERK1/2, p-p38 and p-c-Jun proteins and decreased cells survival instantaneously in LoVo cells. Therefore, the effect of artonin E should be further studies in an animal model.

APPENDIX

molecules

MDPI

Article

# Effects of Artonin E on Cell Growth Inhibition and Apoptosis Induction in Colon Cancer LoVo and HCT116 Cells

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**Abstract:** Today, colon cancer is the leading cause of cancer death. In Thailand, colon cancer is the third most common cancer in men and the second in women. Currently, the treatments for colon cancer include surgery, chemotherapy, radiation therapy, immunotherapy, hormone therapy, targeted drug therapy, and stem cell therapy. However, some treatments have side effects for cancer patients, causing unwanted symptoms. In addition, targeted therapy comes with a high cost for patients. Therefore, bioactive compounds might be a good choice for colon cancer treatment. In this study, we investigated the effect of artonin E on apoptosis induction in colon cancer LoVo and HCT116 cells. The concentration ranges of artonin E at 3, 5, 10, and 30 µg/mL in LoVo cells and 1, 1.5, 2, and 3 µg/mL in HCT116 cells were examined. The results implied that artonin E decreased cell viability and increased apoptotic cells in a dose-dependent manner. In addition, artonin E stimulated mitochondrial membrane potential ($\Delta\Psi m$) changes associated with apoptosis by increasing the sub-G1 population analyzed by flow cytometry. Western blotting showed that artonin E increased the proapoptotic protein, Bax, and decreased anti-apoptotic proteins' (Bcl-2 and Bcl-x) expression. Moreover, artonin E also increased cleaved caspase-7 and cleaved-PARP expression in both LoVo and HCT116 cells. Interestingly, artonin E induced apoptosis through p-ERK1/2, p-p38/p38, and p-c-Jun expression in both cells. Our results suggested that artonin E induced apoptosis via caspase activation associated with the MAPKs signaling pathway. Therefore, artonin E might be used as a potential anticancer drug for colon cancer in the future.

**Keywords:** colon cancer; artonin E; apoptosis; MAPK

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Citation: Yangnok, K.; Innajak, S.; Sawasjirakij, R.; Mahabusarakam, W.; Watanapokasin, R. Effects of Artonin E on Cell Growth Inhibition and Apoptosis Induction in Colon Cancer LoVo and HCT116 Cells. *Molecules* 2022, 27, 2095. https://doi.org/10.3390/molecules27072095

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## 1. Introduction

Cancer is a group of complex genetic diseases in which cells in any part of the body start to grow out of control [1]. These cancer cells can spread into other parts of the body through the bloodstream and lymphatic system [2]. The first place that cancer starts in the body is called the primary site; then, it breaks apart and spreads to a secondary site. These cells begin to grow and form tumors as part of normal tissue [3]. This mechanism is called metastasis, which is considered the main characteristic of cancers [4]. Most cancers are caused by multiple external carcinogenic factors, called environmental factors, combined with internal genetic changes, called genetic factors. All cancers originate from both environment and genetics, due to a combination of multiple external carcinogenic factors combined with internal genetic changes. The carcinogenic agents include UV light, X-rays, viruses, tobacco products, pollutants, chemical reagents, and any sources from outside the body [5]. Furthermore, genetic mutation is a cause of cancer that correlates with long time exposure to the carcinogenic agents, which finally leads to the occurrence of the mutant

genes [6]. In fact, most cancers grow slowly, following multiple steps, and take many years to develop. In Thailand, for men, cancer is the third leading cause of death, and it is the second leading cause of death for women [7]. In 2020, the USA reported that the most common type of cancer was breast cancer, while the next most common cancers were lung cancer, prostate cancer, and colon cancer. Colon cancer is caused by an abnormal growth of epithelial cells which form in the lining of the colon or rectum. Treatments for colon cancer include surgery, chemotherapy, radiation therapy, targeted drug therapy, and immunotherapy. The cost of colon cancer treatment is expensive, and it is expected to increase further [8].

Apoptosis, programmed cell death, is a genetically determined process to destroy cells to maintain cellular homeostasis in the tissue. It is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, and embryonic development. In the case of defective apoptotic signals, it may eventually promote carcinogenesis [9].

Artonin E is a prenylated flavonoid that was isolated from the stem bark of the genus Artocarpus. It has been examined in much cancer research [10]. In this study, artonin E was extracted from *Artocarpus elasticus* Reinw. ex Blume found in the Southern part of Thailand, known by the local name, 'Ka Ok', 'Ka O', or 'Tue Ka' [11]. The artonin E was purified by Associate Professor Wilawan Mahabusarakum, Faculty of Science, Prince of Songkla University, Thailand in a previous study. Briefly, *A. elasticus* was immersed in hexane and $CH_2Cl_2$ before being chromatographed on a quick column chromatography and eluted with a solvent of increasing polarity from hexane through acetone, respectively. After that, the extract was purified by Sephadex™ LH-20 column [12]. The artonin E compound is a yellow powder, with a molecular weight of 436.5. Previous studies reported that artonin E has shown efficiency in inhibition of cell growth, proliferation, migration, and invasion and presented a cytotoxic activity leading to apoptosis induction in many cancer cells, such as lung cancer [13], breast cancer [14], ovarian cancer [15], and skin cancer [16]. Our previous study showed that LoVo cells sensitized TRAIL-induced apoptosis in combined treatment with artonin E and TRAIL [17]. However, the effect of artonin E on human colon cancer LoVo and HCT116 cells through the MAPKs signaling pathways has not been reported.

## 2. Results

### *2.1. Artonin E Inhibited Cell Proliferation in LoVo and HCT116 Cells*

An MTT assay was used to determine the antiproliferation activity of artonin E in LoVo and HCT116 cells. The results showed that artonin E inhibited cell proliferation in a dose-dependent manner in both LoVo and HCT116 cells with an $IC_{50}$ value of $11.73 \pm 1.99$ μg/mL and $3.25 \pm 0.24$ μg/mL, respectively (Figure 1). Based on the $IC_{50}$ value on each cell line, we chose the concentration of artonin E at 3, 5, 10, and 30 μg/mL for LoVo cells and 1, 1.5, 2, 2.5, and 3 μg/mL for HCT116 cells for the following experiments.

### *2.2. Effect of Artonin E on Apoptosis Induction in LoVo and HCT116 Cells*

Nuclear morphological changes of apoptotic cells were determined by Hoechst 33342 staining, which is a fluorescent dye used for DNA labeling [19]. It preferentially binds to the adenine–thymine (A–T) regions of double-stranded DNA excited by ultraviolet light and emits blue fluorescence at 460 to 490 nm [20]. In this study, the results demonstrated that artonin E induced chromatin condensation and apoptotic bodies in a dose-dependent manner in both LoVo and HCT116 cells (Figure 2).

### 2.3. Mitochondrial Membrane Potential (ΔΨm)

During apoptosis, mitochondria were disrupted by proapoptotic protein activation leading to loss of ΔΨm. For the untreated cells with high ΔΨm, JC-1 formed J-aggregated complexes as red fluorescence, while apoptotic cells with low ΔΨm showed green fluorescence. In this study, artonin E induced loss of ΔΨm in a dose-dependent manner in LoVo and HCT116 cells with decreased red fluorescence and increased green fluorescence compared with the control group (Figure 3). This result implied that artonin E induced apoptosis through mitochondrial damage in both LoVo and HCT116 cells.

### 2.4. Cell Cycle Distribution

To confirm the effect of artonin E on apoptosis induction, LoVo and HCT116 cells were stained with PI, and the DNA contents were measured by flow cytometry. LoVo and HCT116 cells treated with 30 μg/mL and 2.5 μg/mL artonin E, respectively, showed a maximum sub-G1 population of 12.61% and 6.80% (Figure 4). In this study, the results revealed that artonin E induced apoptosis in LoVo and HCT116 cells through the accumulation of the sub-G1 population.

### 2.5. Expression of Bcl-2 Family, Caspase-7, and Cleaved-PARP in LoVo and HCT116 Cells

Western blotting was performed to detect the activation of apoptosis mediator proteins. The results demonstrated that artonin E increased the level of proapoptotic protein Bax expression and decreased the level of anti-apoptotic proteins Bcl-2 and Bcl-xL in LoVo and HCT116 cells. Moreover, artonin E induced caspase-7 activation and inactivated a DNA repair protein PARP, in both cells (Figure 5). These results indicated that artonin E induced apoptosis associated with caspase-7 activation in both LoVo and HCT116 cells.

### 2.6. Effect of Artonin E on MAPK Protein Expression

MAPK signaling is involved in both apoptosis and cell survival processes. It plays an important role in the transduction of extracellular signals to cellular responses. In this study, we found that artonin E induced increased levels of p-c-Jun, p-ERK1/2, and p-p38/p38 ratios indicating that artonin E induced apoptosis through the MAPK signaling pathway (Figure 6).

## 3. Discussion

Colon cancer is one of the most prevalent and deadly types of cancer in both men and women worldwide. About 90% of patients with metastatic cancer had treatment failure due to acquired resistance to chemotherapy, including resistance to epidermal growth factor receptor (EGFR) antagonists [21]. In contrast, only 10–20% of patients exhibited a primary response to EGFR-targeted therapy, cetuximab, or panitumumab [22,23]. Therefore, more effective treatments with lower side effects for colon cancer need to be discovered. In recent

years, the search for novel cancer therapeutics in preclinical trials has turned to natural products with low toxicity and low drug resistance. Previous studies have revealed that artonin E inhibited the growth of SKOV-3 cells and showed less toxicity toward a normal human ovarian cell line T1074 [6]. In addition, artonin E inhibited breast cancer growth and proliferation through the initiation of an intrinsic apoptotic pathway while suppressing an antiapoptotic mechanism, thus circumventing the relative immortality of cancer cells [24]. In this study, the anticancer effect of artonin E was observed in LoVo and HCT116 cells. The results revealed that artonin E could inhibit cell growth in a dose-dependent manner, with an $IC_{50}$ of 11.73 ± 1.99 μg/mL and 3.25 ± 0.24 μg/mL, respectively.

Hoechst 33342 staining was used to confirm apoptosis and nuclear morphological changes of cells through apoptosis induction. These results revealed chromatin condensation and apoptotic bodies after treatment with artonin E at 3, 5, 10, and 30 μg/mL and 1, 1.5, 2, 2.5 m and 3 μg/mL in LoVo and HCT116 cells, respectively. Furthermore, JC-1 staining showed that artonin E stimulated the loss of ΔΨm, released some proapoptotic proteins from mitochondria to cytosol and induced apoptosome formation, followed by activation of caspase-9 [25]. The active caspase-9 then activated the effector caspase [3,6,7] that cleaves crucial substrates including PARP, resulting in apoptosis induction [26]. These results correlated with a previous study showing condensed chromatin and apoptotic bodies in the epidermoid carcinoma cell line A431 after treatment with artonin E. In addition, the effect of artonin E on the ΔΨm in A431 cells was observed by increased green fluorescence [27].

To verify the apoptosis signaling pathway, Bcl-2 family proteins, caspase proteins, and the MAPK signaling pathway were analyzed by Western blotting. There are two main apoptosis signaling pathways, the extrinsic and intrinsic pathways. For artonin E-treated LoVo and HCT116 cells, the results showed increased proapoptotic proteins Bax expression. In contrast, anti-apoptotic proteins Bcl-2 and Bcl-xL were decreased. Moreover, artonin E-increased cleaved caspase-7 and cleaved-PARP expression. These results correlated with the previous study that artonin E showed anti-proliferation and apoptosis induction by induced cleaved caspase-7 and cleaved-PARP activation in A431 skin cancer cells, resulting in apoptosis cell death [26]. In general, there are two types of caspases, initiator and effector caspase; the caspase cascade, caspase-9 (initiator caspase) can activate caspase-7 (effector caspase), which then cleaves vital substrates, such as PARP that plays an important role in DNA repairing [30]. These present results related to previous research demonstrating that artonin E induced apoptosis in several cancer cell types including MDA-MB231, MCF-7, SKOV-3, H460, and A431 [11,15,24,27,31].

Proteins in the MAPK signaling pathway play an important role both in cell survival and cell death [32]. Conventional MAPKs in mammalian cells include ERK1/2, JNK1/2, and p38. ERK1/2 activates Bax and caspase leading to apoptosis. JNK1/2 can activate the transcriptional factors, such as p-c-Jun. p38 is a tumor suppressor, which induces apoptosis and inflammation and inhibits cell proliferation. This result indicated that artonin E induced p-ERK1/2, p-p38/p38 ratio, and p-c-Jun upregulation in LoVo and HCT116 cells leading to apoptosis. In addition, ERK1/2 is important in cell proliferation, cell differentiation, cell growth, and cell survival; however, we found that artonin E upregulated p-ERK1/2 in LoVo and HCT116 cells correlated with the previous report that ERK1/2 could activate caspase and proapoptotic proteins in the Bcl-2 family leading to apoptosis induction [33]. Therefore,

## 4. Materials and Methods

### 4.1. Materials

Artonin E [IUPAC name: 5-hydroxy-8,8-dimethyl-3-(3-methylbut-2-enyl)-2-(2,4,5-trihydroxyphenyl) pyrano (2,3-h) chromen-4-one] was obtained from Associate Professor Wilawan Mahabusarakum, Faculty of Science, Prince of Songkla University, Thailand in purified powder form; the extraction and purification were reported in a previous study [12]. Chemicals for cell viability assay including MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and Dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorescent dye Hoechst 33342 was purchased from Thermo Fisher Scientific, Inc. (InvitrogenTM, Waltham, MA, USA) and the fluorescent dye JC-1 was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Guava Cell Cycle® reagent and antibodies for Western blotting analysis including β-Actin were purchased from Merck Millipore (Merck KGaA, Darmstadt, Germany). Rabbit monoclonal primary antibodies and anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

### 4.2. Cell Culture

The human colon cancer cell lines, LoVo and HCT116, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI1640 medium in sterile culture flasks. The medium was supplemented by 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin; after that, the cells were incubated in a humidified atmosphere of 5% $CO_2$ at 37 °C. The medium was renewed every 2–3 days.

### 4.3. Cell Proliferation and Cell Viability Assays

The MTT assay was used to determine the cytotoxicity of artonin E. Firstly, LoVo and HCT116 cells were seeded at a density of $7 \times 10^3$ and $6 \times 10^3$ cells/well and treated with artonin E at 3, 5, 10, and 30 μg/mL and 1, 1.5, 2, 2.5, and 3 μg/mL, respectively, while the control group was treated with DMSO. After 24 h, 0.5 mg/mL MTT solution was added to each well and incubated for 2 h at 37 °C. MTT solution was added to each well and incubated at 37 °C for 2 h, then removed; next, 100 uL of DMSO was added to each well to solubilize water insoluble purple formazan crystals. The absorbance was measured at 570 nm by using a microplate reader (Multiskan Sky Microplate Spectrophotometer, Waltham, MA, USA), and the $IC_{50}$ value was calculated by GraphPad Prism 3.03 (GraphPad Software, Inc., San Diego, CA, USA).

*4.4. Detection of Nuclear Morphological Changes*

Nuclear morphological changes of apoptotic cells were stained by Hoechst 33342, which is a fluorescence dye used for DNA labeling [19]. Firstly, LoVo and HCT116 cells were seeded at a density of $2.2 \times 10^4$ cells/well and $5 \times 10^4$ cells/well and treated with artonin E at 3, 5, 10 and 30 µg/mL and 1, 1.5, 2, 2.5 and 3 µg/mL, respectively, while the control cells were treated with DMSO for 24 h. After incubation, both cells were stained with 5 µg/mL Hoechst 33342 and incubated at 37 °C. The fluorescence signal was measured at 460 to 490 nm [20]. Cells were investigated under a fluorescence microscope (DP73 Olympus, Tokyo, Japan).

*4.5. Detection of Mitochondrial Membrane Potential (ΔΨm)*

Mitochondrial membrane potential (ΔΨm) was stained by JC-1, which is a lipophilic cationic dye that can selectively enter into mitochondria and reversibly change the color from green to red as the membrane potential increases. Untreated cells with healthy mitochondria showed brighter red fluorescence intensity, while apoptotic cells showed green fluorescence intensity with low ΔΨm. LoVo and HCT116 cells were seeded at a

*4.6. Cell Cycle Analysis*

To study apoptosis induction via accumulation of the sub-G1 population, flow cytometry was carried out. LoVo and HCT116 cells were seeded at $5 \times 10^4$ cells/well and allowed to grow for 24 h. Then, LoVo and HCT116 were treated with artonin E at 3, 5, 10, and 30 µg/mL and 1, 1.5, 2, 2.5, and 3 µg/mL, respectively, while the control cells were treated with DMSO. Upon treatment, both cells were harvested, washed with PBS, fixed with ice cold 70% ethanol, and then kept at 4 °C. After fixation, cells were stained according to the manufacturer's instructions (Guava Cell Cycle® reagent from Merck KGaA, St. Louis, MO, USA). The DNA content was observed using a Guava easyCyte™ flow cytometer and GuavaSoft™ software version 3.3 (Merck KGaA, St. Louis, MO, USA).

*4.7. Western Blot Analysis*

LoVo and HCT116 cells were seeded at a density of $20 \times 10^4$ cells/well and $35 \times 10^4$ cells/well, respectively, and treated with artonin E at 3, 5, 10, and 30 µg/mL and 1, 1.5, 2, 2.5, and 3 µg/mL, respectively, while the control cells were treated with DMSO. After incubation, cells were harvested, washed with cold PBS, and lysed in RIPA lysis buffer (50 mM Tris-HCL, pH 7.5, 5 mM EDTA, 250 mM NaCI, 0.5% Triton X-100) supplemented with 100 mM PMSF, and a complete mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The cell lysate was centrifuged at 13,000× *g* for 30 min. The supernatant was transferred to a new microcentrifuge tube, and the protein content was determined by Bradford's method before loading to the gel. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes by using the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, Hercules, CA, USA); then, they were blocked with 5% BSA in 1X PBS at room temperature. The primary antibody was a rabbit immunoglobulin G, specific to the mediator proteins including Bax, Bcl-2, Bcl-xL, caspase-7, cleaved caspase-7, ADP-ribose polymerase (PARP), p-ERK1/2, ERK1/2, p-p38, p38, p-c-Jun (Ser73), and β-actin. Then, the anti-rabbit lgG horseradish peroxidase-conjugated secondary antibodies were added for 1 h at room temperature and detected by chemiluminescence using an enhanced chemiluminescence (ECL) reagent. The emitted light was detected by using a chemiluminescence imaging system (Chemiluminescence using Immobilon™ Western chemiluminescent HRP substrate (Merck KGaA, Darmstadt, Germany)).

*4.8. Statistical Analysis*

Statistical significance was assessed by one-way analysis of variance (ANOVA). Statistical analysis was performed using SPSS statistical software package (version 15; SPSS, Inc., Chicago, IL, USA) and using the software GraphPad Prism 3.03 (GraphPad Software, Inc., La Jolla, CA, USA). The Western blotting band intensity was quantified by an Image J densitometer. The differences were considered statistically significant at $p < 0.05$.

**5. Conclusions**

In conclusion, artonin E induced apoptosis associated with the MAPK signaling pathway by promoting apoptosis cell death through p-ERK1/2, p-p38/p38, and p-c-Jun, and it decreased cell survival simultaneously in both types of human colon cancer LoVo and HCT116 cells. Therefore, in the future, artonin E may be further studied in animal models.

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