ผลของอาร์โทนินอีต่อการเคลื่อนที่ของเซลล์มะเร็งปอดชนิด เอช460

นายกนก พลายบัว

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EFFECTS OF ARTONIN E ON MIGRATION OF H460 LUNG CANCER CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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กนก พลายบัว: ผลของอาร์โทนินอีต่อการเคลื่อนที่ของเซลล์มะเร็งปอดชนิด เอช460 (EFFECTS OF ARTONIN E ON MIGRATION OF H460 LUNG CANCER CELL) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ภก.ดร.ปิติ จันทร์วรโชติ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ภก.ดร.บุญชู ศรีตุลารักษ์, 70 หน้า.

การศึกษาเกี่ยวกับสารที่สามารถลดการเคลื่อนที่ของเซลล์มะเร็งได้รับความสนใจเพิ่มมากขึ้นอย่างมีนัยสำคัญ เนื่องมาจากสารเหล่านี้จะเป็นประโยชน์ต่อการพัฒนาวิธีการในการรักษาโรคมะเร็ง การแพร่กระจายเป็นสาเหตุสำคัญของการตายในผู้ป่วยมะเร็งปอด การแพร่กระจายของเซลล์มะเร็งประกอบด้วยหลายขั้นตอนโดยที่การเคลื่อนที่ของเซลล์มะเร็งถือเป็นปัจจัยสำคัญปัจจัยหนึ่งที่ทำให้การแพร่กระจายของเซลล์มะเร็งเกิดขึ้นได้สำเร็จ การวิจัยนี้มีเป้าหมายเพื่อศึกษาผลของอาร์โทนินอีในการยับยั้งการเคลื่อนที่ของเซลล์มะเร็งปอดชนิด เอช 460 โดยสารอาร์โทนินอี เป็นสารในกลุ่มฟลาโวนอยด์ที่สกัดได้จากเปลือกต้นของ *Artocarpus gomezianus* และการวิจัยนี้เป็นงานวิจัยชิ้นแรกที่แสดงให้เห็นว่า สารอาร์โทนินอีสามารถยับยั้งการเคลื่อนที่ของเซลล์เอช460 ซึ่งเป็นมะเร็งปอดประเภทที่ไม่ใช่เซลล์เล็ก จากการศึกษาโครงสร้างของเซลล์หลังจากที่เซลล์ได้รับอาร์โทนินอีในระดับความเข้มข้นที่ต่ำกว่าระดับความเป็นพิษพบว่า อาร์โทนินอีลดการสร้างฟิโลโพเดีย ลดระดับโปรตีน focal adhesion kinase (FAK)ที่อยู่ในสภาวะถูกกระตุ้น, ลดระดับโปรตีน protein kinase B (Akt) ที่อยู่ในสภาวะถูกกระตุ้นและลดระดับโปรตีน cell division cycle 42 (Cdc42) ซึ่งโปรตีนที่กล่าวนี้มีความสำคัญต่อการยับยั้งการเคลื่อนที่ของเซลล์มะเร็งที่เป็นผลจากการได้รับอาร์โทนินอี นอกจากนี้อาร์โทนินอียังสามารถยับยั้งการเคลื่อนที่ของเซลล์มะเร็งปอดชนิดอื่นๆ ได้แก่ เซลล์มะเร็งปอดชนิด H292, H23 และ A549 จากผลการศึกษาทั้งหมดนี้แสดงให้เห็นว่า อาร์โทนินอีเป็นสารที่มีความเป็นไปได้ในการนำไปพัฒนาใช้ในแง่การยับยั้งการแพร่กระจายและต้านเซลล์มะเร็งเพื่อเพิ่มอัตราการรอดชีวิตของผู้ป่วยโรคมะเร็งปอด

ภาควิชา เภสัชวิทยาและสรีรวิทยา	ลายมือชื่อนิสิต .....
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KANOK PLAIBUA: EFFECTS OF ARTONIN E ON MIGRATION OF H460 LUNG CANCER CELL.

ADVISOR: ASSIST PROF. PITHI CHANVORACHOTE, Ph.D., THESIS

CO-ADVISOR: ASSOC. PROF. Boonchoo Sritularak, Ph.D., 70 pp.

Knowledge regarding substances that attenuate motility of cancer cells has garnered significant attention as they may benefit a development of novel anti-cancer strategies. In lung cancer, the metastasis has become an important cause of cancer-related death. Metastasis of cancer cells composes of several steps and the migratory activity of cancer cells is the prominent factor for successful metastasis. The present study aimed to investigate the anti-migration effects of artonin E in H460 lung cancer cells. Artonin E, an active flavonoid from bark of *Artocarpus gomezianus*, was reported herein for the first time to exhibit anti-migration activity of H460 human non-small cell lung cancer cells. Cell morphology observation revealed that treatment of the cells with non-toxic concentrations of artonin E resulted in a decrease of filopodia formation. Artonin E was shown to inhibit activated focal adhesion kinase (FAK), suppress downstream protein kinase B (Akt) activation, and decrease cell division cycle 42 (Cdc42) levels, which were responsible for anti-motility effect of such a compound. Also, artonin E inhibited migration in other lung cancer cells, namely H292, H23 and A549 cells. These results reveal that artonin E may be a promising candidate for anti-metastasis and anti-cancer approaches to improve survival of lung cancer patients.

Department : Pharmacology and Physiology	Student's Signature……
Field of Study : Pharmacology	Advisor's Signature ……..
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%	=	percentage
°C	=	degree Celsius (centigrade)
µg	=	microgram
µl	=	microliter
µM	=	micromolar
ABP	=	actin-binding proteins
Akt	=	protein kinase B
ARP2/3	=	actin relating protein 2/3
BSA	=	bovine serum albumin
Cdc42	=	cell cycle division 42
DMEM	=	Dulbecco's Minimal Essential Medium
DMSO	=	dimethylsulfoxide
ECM	=	extracellular matrix
EGFR	=	Epidermal Growth Factor Receptor
et al.	=	et alii, and other people
Fak	=	focal adhesion kinase
FAT	=	focal-adhesion targeting
FBS	=	fetal bovine serum
FRNK	=	FAK-related non kinase
GDP	=	guanosine diphosphate
GEFs	=	guanine-nucleotide exchange factors
GTP	=	guanine triphosphate
GTPase	=	guanine triphosphatase
h	=	hour
IC_{50}	=	Median Inhibition Concentration
min	=	minute

ml	=	milliliter
MLC	=	myosin light-chain
MLCK	=	myosin light-chain kinase
MLCPtase	=	myosin light-chain phosphatase
mM	=	mill molar
MMPs	=	matrix metalloproteinase proteins
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSCLC	=	non-small cell lung cancer
Nm	=	nanometer
OD	=	optical density
pAkt	=	phosphorylated Akt
PBS	=	phosphate buffer saline
pFAK	=	phosphorylated FAK
pH	=	poteintial of hydrogen
PI	=	propidium iodide
PI3K	=	phosphatidylinositol 3-kinase
PI45K	=	phosphatidylinositol 4, 5-kinase
PIPs	=	phosphoinosites
PTK	=	protein tyrosine kinase
Rac	=	Rho-related C3 botulinum toxin substrate
Rho	=	Ras homologous proteins
RNA	=	ribonucleic acid
ROCK	=	Rho-associated coiled coil- containing protein kinase
RPMI	=	Roswell Park Memorial Institute
SCLC	=	small cell lung cancer
SD	=	standard deviation
SDS	=	sodium dodecyl sulfate
SiRNAs	=	small interfering RNAs

TBST	=	tris-buffered saline, tween 20
Tyr	=	tyrosine
WASP	=	Wiscott-Aldrich syndrome protein

CHAPTER I

INTRODUCTION

Metastatic lung cancer is causative for more than 90% of lung carcinoma related deaths worldwide (Ray and Jablons, 2009). Although the earliest stage of disease presenting as only pulmonary nodule without involved lymph nodes at resection, some of these patients will finally die from undetectable micrometastases (Maslyar *et al*., 2004). Cancer metastasis is a complex process of cell spreading which can be divided into several steps including migration, invasion, intravasation, survival in the circulation, extravasation, and metastatic colonization (Hanahan and Weinberg, 2000; Mina and Sledge, 2011). Although a growing body of study suggests that migration is a crucial step for successful metastasis (Harlozinska, 2005), at present, there are no approved drugs that inhibit such behaviors of cancer cells.

Even though the molecular mechanisms by which cancer cells use for migration are not fully understood, based on previous researches, they involve abilities of cancer cells to change their affinity and avidness for the extracellular matrix (ECM) and such alterations are due to modifications of various cellular signaling pathways including focal adhesion kinase (FAK; Bolos *et al*., 2010). Indeed, the activation of FAK through phosphorylation rendering its kinase activity is important for FAK-induced focal adhesion turnover and cell movement (Vicente-Manzanares *et al*., 2009). Activated FAK can then transduce the signal through the phosphorylation of protein kinase B (Akt) resulting in cellular responses such as cell invasion and migration (Bolos *et al*., 2010). Recently, the Rho families of small guanosinetriphosphatases (GTPases), especially cell division cycle 42 (Cdc42), were

shown to play an essential role in modulating actin reorganization associating with cell motility and filopodia formation (Raftopoulou and Hall, 2004). The expression level of Cdc42 was found to be up-regulated in many cancers (Jiang, Zhang and Qu, 2011; Kamai *et al*., 2004) and its overexpression was shown to be associated with an enhanced migration and cancer aggressiveness (Kamai *et al*., 2004; Yoshioka, Nakamori and Itoh, 1999). In lung cancer, Cdc42 was shown to be highly overexpressed in primary lung cancer cells (Chen *et al*., 2012). Also, the study indicated that either curcumin-mediated Cdc42 down-regulation or Cdc42 knockdown could attenuate cancer cell motility (Chen *et al*., 2012).

Artonin E, an active flavonoid, obtained from a stem bark of *Artocarpus gomezianus* Wall. exTréc. (Moraceae), known as "Hat-Nun" in Thailand (Sritularak *et al*., 2010). Artonin E was shown to exhibit promising growth inhibition action against breast cancer cells (Shajarahtunnur, 2006). Artonin E also exhibited anoiskis sensitization property on lung cancer cells (Wongpankam, 2012). Moreover, artonin E was shown to possess many pharmacological activities such as antiplatelet aggregation (Shajarahtunnur, 2006), antioxidant effect (Sritularak *et al*., 2010). However, effect of artonin E on cancer cell migration is unknown. In our view, the knowledge regarding such activities of the compound would benefit the development of novel anti-metastasis drug as well as strategy to overcome cancer.

Research Questions

1. Does artonin E affect migration of lung cancer cells?
2. What is the underlying intracellular mechanism that artonin E inhibit migration of lung cancer cells?

Objectives

1. To investigate the effect of artonin E on migration of lung cancer cells.
2. To study mechanism that artonin E inhibit migration of lung cancer cells.

Hypothesis

Artonin E can inhibit migration of lung cancer cells by inducing alteration of the protein regulating cell migration.

CHAPTER II

LITERATURE REVIEW

Lung cancer

Pulmonary carcinoma is the main cause of cancer death in patients from the many countries around the world (Jemal *et al*., 2011). Lung cancer can be divided into two groups including non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). About 85% of all lung cancer patients are NSCLC. Non-small-cell lung cancer also can be sub-divided into 3 main subtypes namely squamous-cell carcinoma, adenocarcinoma, and large-cell lung cancer (Panov, 2005).

Lung cancer can be caused by many factors but the common cause is smoking. The biology of lung cancer among smokers and non-smokers is different. For example, non-smoker patients, common type of lung cancer is adenocarcinomas, have improved survival compare to smokers (Ray and Jablons, 2009). Current treatments of pulmonary carcinoma are surgery, radiotherapy and chemotherapeutic treatment. Choosing treatment depends on histology and stage of cancer.

In USA, about 75% of patients with lung cancer already have locally advanced or metastatic disease after initial diagnosis (Ray and Jablons, 2009). Global cancer statistic also show that more than 90% of patients with lung carcinoma died from metastatic lung cancer (Ray and Jablons, 2009). NSCLC and SCLC own the highly invasive and metastatic properties. The most common sites of lung cancer metastasis are extrathoracic lymph nodes, brain, bone and liver. Furthermore, most patients with advance lung cancer were found to have brain metastasis (Hirsch *et al.*, 1982; Kelly

and Bunn, 1998). In patient with NSCLC, 40% of them already have hematogenous metastases after initial diagnosis (Ray and Jablons, 2009). Consequently, almost of them will finally die from such disease.

SCLC causes the worst clinical course in any type of lung cancer if it has not been treated. Survival time of SCLC patients is 2-4 months after diagnosis. Likewise, SCLC tends to increase at a greater rate of metastasis compare to the other types of lung tumour. However, SCLC has more responsive to cytotoxic chemotherapy and radiation therapy compare to NSCLC (Timbrell, 2008).

The use of chemotherapeutic agents in patients with NSCLC remains arguable due to impact on overall survival of patient still unclear. Cisplatin, carboplatin, paclitaxel gemcitabine and the vinca alkaloids (vinblastine and vindesine) were classified as chemotherapeutic drugs of choice for lung cancer (Komaki, Tsao and Mehran, 2013). Nevertheless, only 20% of response rates using single agent were observed (Ihde, 1992). Chemotherapy cocktails were used and have improved the response rates (Ihde, 1992). From this review, the way to treat metastatic NSCLC is controversial and very hard to combine chemotherapeutic agents with the other treatments (Ihde, 1992). Despite of development of many cytotoxic drugs for advance NSCLC, overall 5 years survival of patients after diagnosis is 14% (Haura, 2001). New therapies, specific for lung cancer and safe for patients, are needed.

Metastasis

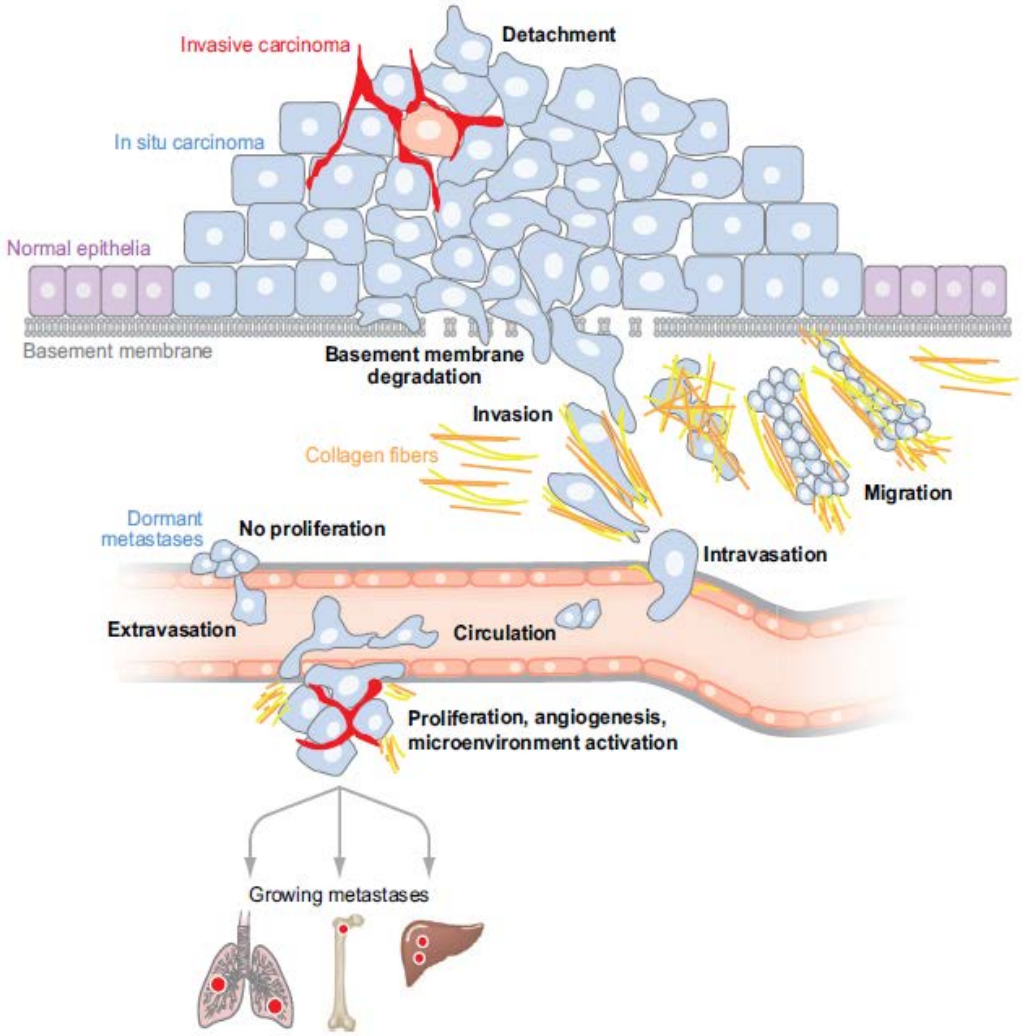


Figure 1. Metastasis of cancer cells (Onkal and Djamgoz, 2009)

Metastasis is the major cause of death in patients with cancer. Moreover, 5-year survival of patients with early-stage cancers is 49%. However, 5-year survival patient with end-stage cancer is 3% (Jemal *et al*., 2011). The metastatic process begins when cancer cells separate from primary tumor site, migrate and invade to surrounding area *via* local lymphatic and blood vessels, some of them can survive in

circulation then attach to capillaries or venules of remote organs and they finally adapt to new microenvironment and establish a new tumor (Bacac and Stamenkovic, 2008).

There are some essential functions of cancer cell to achieve the events as mentioned previously including tumors-microenvironment interactions, migration, invasion, anoikis resistance and angiogenesis capability (Bacac and Stamenkovic, 2008). These functions are modulated by adhesion and proteolysis which are necessary for metastatic tumor cell (Bacac and Stamenkovic, 2008).

Migration is the pivotal parameters in the metastatic cascade. The lymph nodes are the one of major routes which cancer cell uses them to migrate through the body *via* the lymphatic system. Therefore, lymph node metastases in cancer are a good indicator for patient's survival and prognostic indicator of whether distal metastases will develop. A previous study indicated that NSCLC show characteristic of stem cells (Krystal *et al*., 1996) which could migrate and lead to metastasis then successful colonization at target organ. For beginning of migration, cancer cell need changes in cell motility and cytoskeletal reorganization to separate from primary tumor. The most common form of morphogenesis is epithelial-mesenchymal transition (EMT) which is the process that leads to cell elongation, secretion of extracellular enzymes to degrade ECM and migrate out (Ray and Jablons, 2009).

Molecular mechanism of migration

Mechanism of cancer migration can be divided into 5 stages including

First: Cell membrane was forced in a specific direction by connecting between adaptor proteins and stretching actin filaments. The actin connects to the

actin relating protein 2/3 (ARP2/3) complex that links to an adaptor protein called Wiscott-Aldrich syndrome protein or WASP to form actin polymerization (Rohatgi *et al*., 1999). Complex of ARP2/3/WASP can attach to clustered phosphoinosites (PIPs) to stay inside plasma membrane (Rohatgi *et al*., 1999). Moreover, there is the interaction between ARP2/3 and pre-existing actin filament to form branching of the actin filament network organization (Blanchoin *et al*., 2000). The ability of PIPs also activates guanine-nucleotide exchange factors (GEFs) which regulate Rac, CDC42 and RHO which are the family of small GTPases protein (Kaibuchi, Kuroda, and Amano, 1999). Furthermore, CDC42 can link both of PIPs and WASP that lead to stimulate formation of the filopodia or pseudopod extensions which are essential structure for cancer motility (Guillou *et al*., 2008; Nobes and Hall, 1995). PIPS can be produced by two enzymes, phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol 4, 5-kinase (PI45K; Ren *et al*., 1996; Tsutsumi, Gupta, Hogan *et al*., 2002).

Second: ECM ligands link to integrins result in creating of clustered integrin in inner of plasma membrane (Zamir and Geiger, 2001). After integrin clustering, the adaptor and signaling protein are recruited and phosphorylated for signal generation into the cell (Hynes, 2002). Integrin in the inner part of plasma membrane can interact with FAK or several important proteins (Miyamoto *et al*., 1995). These essential proteins can connect to the adaptor protein to induce the actin-binding proteins or ABP with target protein including PI3K enzyme or RHO GTPases (Cdc42) to focal contact (Zamir and Geiger, 2001). Therefore, signaling pathway from PI3K and RHO GTPases impact the alteration of the assembly of focal contact (Hynes, 2002; Degani *et al*., 2002).

Third: Focal contacts recruit the surface proteases to cleave ECM composition including collagen, fibronectin and pro-matrix metalloproteinase proteins (MMPs) which produce active MMPs namely MMP-2 (Ohuchi *et al*., 1997).

Fourth: Active myosin (Actomyosin) links to actin filaments for contraction of cell (Cramer, 1999). Myosin light chain or MLC can be activated by myosin light-chain kinase (MLCK) lead to stimulation of myosin II (Kamm and Stull, 2001). MLC phosphatase (MLCPtase) also regulates function of MLC by dephosphorylation result in deactivation of MLC (Kamm and Stull, 2001). Actomyosin contraction is modulated by ROCK, an effector of the small GTPase Rho, for phosphorylation and inhibition of MLCPtase (Katoh *et al*., 2001).

Fifth: Various mechanisms cause focal adhesion disassembly at trailing edge such as phosphorylation of FAK at tyrosine 397 can induce focal adhesion turnover (Hamadi *et al*., 2005). As cell moves forward, there are proteolytic cleavage of adhesion receptor to weaken focal contact and the gathering of collagen fragments (Carragher, Levkau, Ross *et al*., 1999). Finally integrins can internalize by endocytosis for detachment from substrate and recycling to the leading edge or accumulate onto the substrate after focal adhesion disassembly (Bretscher, 1996).

As the stage that mention above, we have focused on three proteins that play an important role for migration including FAK, Akt and Cdc42.

Focal adhesion kinase (FAK)

Focal adhesion kinase, which is a member of non-receptor protein tyrosine kinase (PTK), is found in many cell types or tissues and also expressed in the mammal and the lower eukaryotic organisms. Structure of FAK consists of 3 domains including a central catalytic domain, non-catalytic domain including N- terminal

domains and C-terminal domains (Parsons, 2003). There is a section of N-terminal domain shows the same sequence of a family of protein called FERM domain and its role is unclear in FAK (Parsons, 2003). Generally, members of this protein connect transmembrane glycoproteins to the actin cytoskeleton. The N-terminal domain is linked to the tail of ß-integrin subunits in the cytoplasmic domain (in vitro model; Parsons, 2003). Moreover, previous evidence shows that the adhesion protein called talin uses its FERM domain connects to the cytoplasmic part of ß3 integrin tails then modulate the integrin stimulation (Hynes, 2002).

The C-terminal domain of FAK is essential for FAK signaling and has many protein-protein interaction sites (Sieg *et al*., 1999). There is a ~100 residue sequence called FAT (focal-adhesion targeting) which designates FAK to form adhesion complexes (Bertolucci, Guibao and Zheng, 2005). So this sequence is important and necessary for targeting FAK to adhesion complexes (Bertolucci, Guibao and Zheng, 2005).

Phosphorylation of FAK at Tyr397 or various areas within kinase and the C-terminal domains can cause integrin clustering (Calalb *et al*., 1995). Furthermore, phosphorylation of Tyr397 results from temporary dimerization of FAK proteins as well as associates with increased catalytic activity of FAK (Bertolucci, Guibao and Zheng, 2005). Interestingly, phosphorylation at Tyr576 and Tyr577, which are two conserved residues sited in central kinase domain, are found to be important as well as phosphorylation at Tyr397 (Calalb *et al*., 1995). Phosphorylation of these tyrosine positions is essential for FAK signaling and activation to the following effect proteins (Calalb *et al*., 1995).

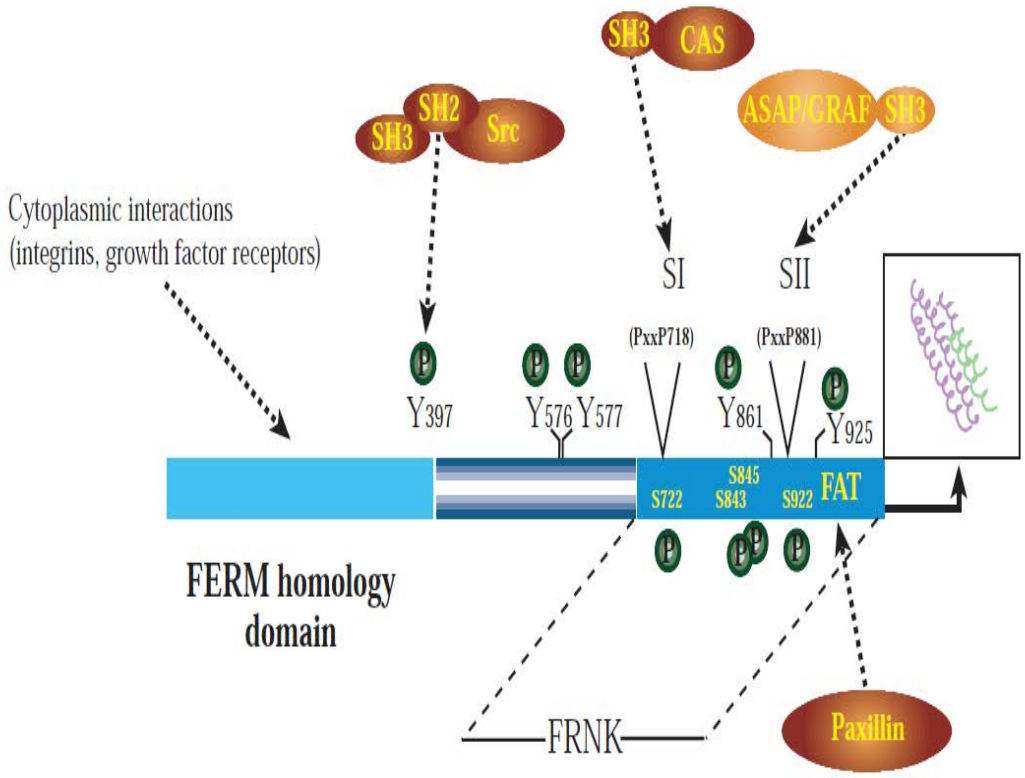


Figure 2. Molecular structure of FAK (Parsons, 2003)

Phosphorylation of Tyr397 is also found to be a critical step to recruit many SH2-containing proteins such as the subunit of phosphoinositide 3- kinase (PI 3-kinase; Chen *et al*., 1996) which can activate its downstream effector, protein kinase B or Akt.

As mentioned above, the C-terminal domain has many protein-protein interactions sites. There are two important sites that bind to protein paxillin in the FAT domain and consist of the sites for SH3 containing proteins (SI and SII in Figure 2; Parsons, 2003). The small GTPase proteins including CDC42 connect to FAK at the site II motif and play an essential role in cytoskeleton organization (Liu *et al*., 2002).

FAK in cell migration

Over expressions of pFAK were found in many type of cancer such as lung, and ovarian cancers (Imaizumi et al., 1997; Grisaru-Granovsky *et al.*, 2005). Many reasonable studies indicate the essential role of FAK in the control of cell motility. Interestingly, Knockdown of FAK exhibits slow spread of cell on the extracellular matrix protein, an accumulated number of obvious focal contacts as well as a slow of migration process in the response to haptotactics and chemotactic attractants (Sieg *et al*., 1999; Sieg *et al*., 2000). The FRNK (FAK-related non kinase) section is found in non-catalytic C-terminal domain of FAK. An increased expression of FRNK can slow down the rate of cell spreading, haptotaxis and chemotaxis of cell migration (Sieg *et al*., 1999). Therefore, effective outcomes of FAK signaling require essential key regulatory proteins. Moreover, the study of FAK in Chinese hamster ovary (CHO) cells show that overexpression of FAK in this cell can increase cell motility (Cary *et al*., 1996). In FAK siRNA transfected cells, wildtype FAK can restore cell motility of these cells by reconstitution technique, however, using of FAK that lack kinase activity or capability to link Src family kinase or Site I mutation cannot improve motility (Sieg *et al*., 1999). Previous study also indicates that there is relation between FAK-deficient cells and the regulation of Rho-regulated contractility (Chen *et al*., 2002).

Protein kinase B (Akt)

Protein kinase B or known as Akt family members are downstream of the PI3K signaling. Akt can be activated by several agonists such as growth factors or many cytokines (Stambolic and Woodgett, 2006). After Akt is stimulated, it can

control many essential functions of the cell namely proliferation (increased number of cell), growth (increased size of cell), survivability of cells or many aspects of intermediary metabolism (Stambolic and Woodgett, 2006).

Protein kinase B in cell migration

The role of protein kinase B/Akt in regulation of cell migration has garnered increased researcher attention in many years. For example, the study of the role of Akt in squamous cell carcinoma reveal that Akt can promote the epithelial to mesenchymal transition (EMT) and also increase the motility and invasion of this lung cancer cell (Grille *et al*., 2003). Akt knockdown in murine thyroid cancer model exhibits slow tumor progression (Saji *et al.* 2012). Down-regulation of pAkt also decreases cancer migration (Lee *et al.* 2010). Moreover, Akt regulate stabilization of microtubule, which has an essential role for mobile cell (Onishi *et al*., 2007).

Cell cycle division 42 (Cdc42)

Cdc42, a member of the Rho family small GTPase, has regulatory functions for cytoskeleton organization, intercellular communication for many physiological procedures including the proliferation of cell, migration, invasion, control the direction of cell and the growth of cell (Sinha and Yang, 2008). Interestingly, there are many disorder conditions that have malfunction of Cdc42 namely degenerative disorder of neuron, cardiac disorder and carcinoma (Schmidt and Hall, 2002). Cdc42 is activated by guanine nucleotide exchange factors or GEFs which exchange GDP (guanosine diphosphate)-Cdc42 form to GTP (guanosine triphosphate)-Cdc42 form for Cdc42 stimulation (Schmidt & Hall, 2002; Sinha and Yang, 2008). Following

activated Cdc42, it can send signal that affect many downstream effectors lead to many physiological processes as mentioned above.

Cell cycle division 42 in cell migration

Cdc42 affects the motility of cell by various processes such as modulation of cytoskeleton structure namely actin and tubulin, and membrane dynamics by stimulation many effector proteins like many protein kinases (Sinha and Yang, 2008).

Cdc42 also implicates in filopodia formation by actin polymerization. Filopodia locate at the front of mobile cell and have a critical function for direction of cell motility (Raftopoulou and Hall, 2004).

Overexpression of Cdc42 is found to increase motility of many cancers from many studies (Jiang *et al*., 2011; Kamai *et al*., 2004).

Cdc42-knock down cell has generated less metastasis compared to the control cells (Reymond *et al.*, 2012).

Filopodia

Filopodia are the protrusion structures of actin containing cell membrane and found at the outer edge of a mobile cell (Mattila and Lappalainen, 2008). Characteristics of filopodia are thin filament about 0.1-0.3 μm long, finger-like arrangements which are consist of polymerization of actin (Mattila and Lappalainen, 2008). Filopodia play an essential role in many basic physiological functions of cells including cell movement or cell migration. Filopodia also use many receptor for direct the signal transduction of several molecules and extracellular matrix molecules

(Mattila and Lappalainen, 2008). Recent study of the relationship between filopodia and Cdc42 exhibit that Cdc42 has been implicated in the formation of filopodia (Guillou *et al*., 2008).

Artonin E

Artonin E is a prenylflavone as shown in figure 3A. It was isolated as a yellow powder from stem bark of Hat-nun (*Artocarpus gomezianus*) in Moraceae family as shown in figure 3B. This plant is a tree widely distributed throughout in Thailand (Sritularak *et al*., 2010) and its stem bark has been used as traditional medicine in many indications such as relieving fever, deworming, and antiflatulent (Seepin, 2009).

Chemical formula of artonin E is $C_{25}H_{24}O_7$ and its molecular weight is 436. Artonin E can be dissolved in ethanol. Artonin E's melting point is 244-248 °C at 760 mmHg respectively (Hano *et al.*, 1990).

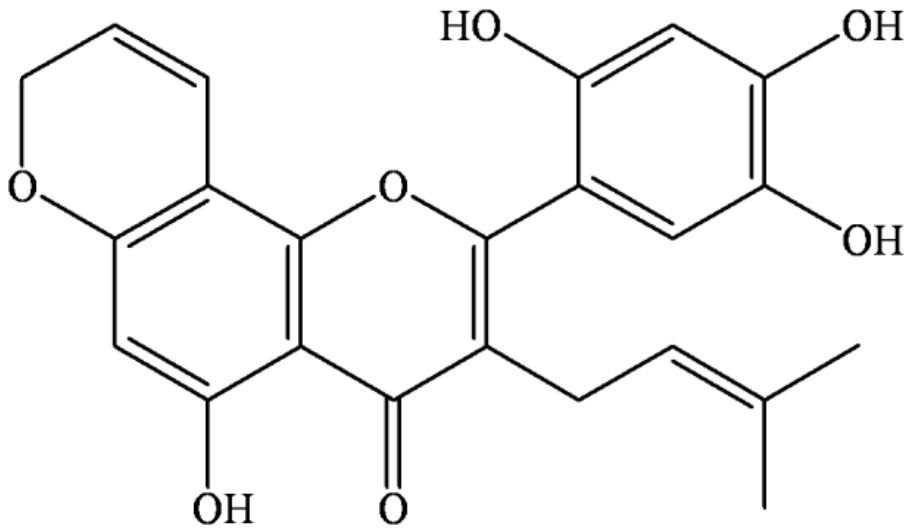


Figure 3. A. Basic Structure of Artonin E

B. Artocarpus gomezianus (Seepin, 2009)

Research of Artonin E

Antioxidant activity of artonin E was investigated in vitro assay using DPPH assay and nitric oxide inhibitory assay (Sritularak *et al*., 2010).

Artonin E also has been found in *Artocarpus lowii*, *A. scortechinii*, *A. teysmanii* (Shajarahtunnur, 2006), *A. altilis* (Boonphong *et al*., 2007), *A. rigida* (Suhartati, Yandri and Hadi, 2008). Artonin E was shown to inhibit the platelet

aggregation by block adenosine diphosphate activity compared to aspirin (Shajarahtunnur, 2006).

Moreover, antitubercular and antimalarial ability of artonin E have been demonstrated (Boonphong *et al*., 2007). Artonin E also exhibits promising property for antiasthma activity by inhibition of arachidonate 5-lipoxygenase (Reddy *et al*., 1991). In antibacterial activity, artonin E can against some bacteria namely *Escherichia coli* and *Bacillus subtilis* (Suhartati, Yandri and Hadi, 2008).

Interestingly, recent studies have demonstrated anticancer activity of artonin E. Artonin E can against the growth of breast carcinoma cell line compared to tamoxifen (Shajarahtunnur, 2006), inhibit the proliferation of leukemia P-388 cell (Suhartati, Yandri and Hadi, 2008), also show toxic effect against oral squamous carcinoma (KB cell) and breast carcinoma (Boonphong *et al*., 2007).

Finally, Artonin E exhibits anti-metastasis activity of cancer cell by anoikis sensitization (Wongpankam, 2012).

However, the ability of artonin E on cancer cell migration which is the early step of metastasis is waiting for investigation.

CHAPTER III

MATERIALS AND METHODS

1. Chemicals and reagents

Artonin E, a yellow powder, was obtained from Associate Professor Boonchoo Sritulalak. Hoechst 33342, propidium iodide (PI), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical, Inc. (St. Louis, MO). Primary and secondary Antibodies for western blot including Cdc42, pFAK, FAK, pAkt, Akt and β-actin rabbit antibody were purchased from Cell Signaling Technology, Inc. (Danver, USA).

2. **Instruments**

- Analyst/PC densitometry software (Bio-Rad, USA)
- Automated cell counter (Bio-Rad, USA)
- Autopipette: 0.2-2 µl, 2-20 µl, 20-200 µl and 100-1,000 µl
- Cell culture plate: 6-well and 96-well (Costar)
- Centrifuge
- Chemiluminescence substrate (Supersignal West Pico; Pierce)
- Fluorescence microplate reader (SpectraMax® M5, Molecular Devices)
- Fluorescence microscope (Olympus IX51 with DP70, NY)
- Laminar flow cabinet, humidified incubator
- Mini Trans-Blot cell and PowerPac Basic Power Supply (Bio-Rad, USA)
- Nitrocellulose membranes (Bio-Rad, USA)

- pH meter
- Vertex mixer

3. Test compound preparation

Artonin E was dissolved in ethanol and Roswell Park Memorial Institute medium (RPMI) 1640 to achieve designed concentrations containing less than 0.5% ethanol.

4. Cells culture

Human lung cancer cells namely Human lung cancer H460 (Large cell lung cancer), H23 (adenocarcinoma), H292 (mucoepidermoid pulmonary carcinoma) and A549 (adenocarcinoma) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). H460, H292, H23 cells were cultured in RPMI 1640 and A549 cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units/mL penicillin/streptomycin in under certain circumstance (5% carbon dioxide at 37 °C).

5. Experimental design

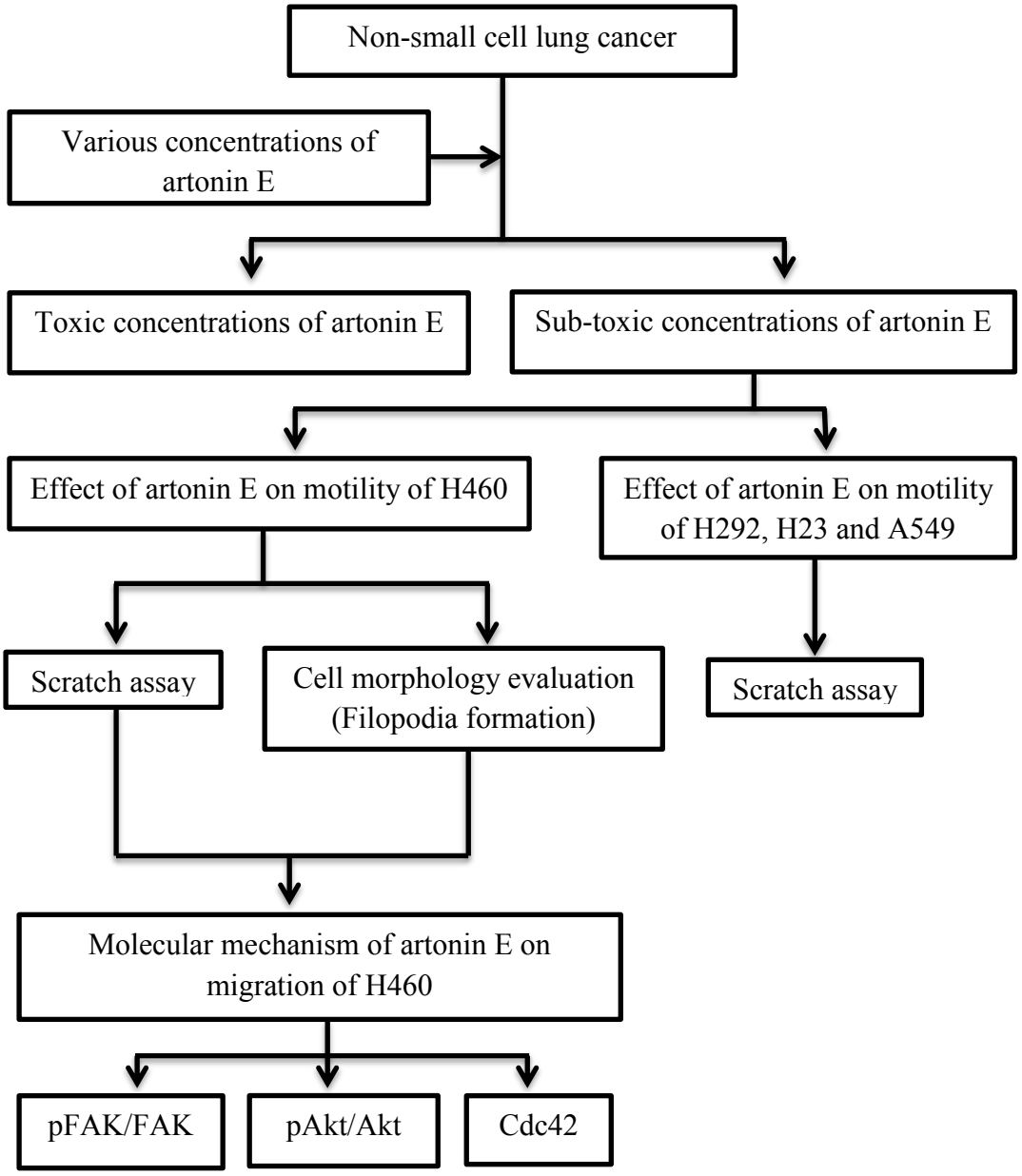


Figure 4. Experimental design of this study

5.1. Investigation on cellular toxicity induced by artonin E in human lung cancer H460 cells

To determine sub-toxic concentrations of artonin E that didn't significantly affect H460 cell viability compare to untreated group, cell survival was examined by MTT assay which measures activity of mitochondria dehydrogenase enzyme. Cells were seeded at 1 x 10^4 cells per well in a 96-well plate 12 h. Cells were treated with various concentrations of artonin E (0-50 μg/ml) and incubated for 24 h. Cells were incubated with 100 μl of 500 μg/ml MTT solution for 4 h at 37 °C. Then, MTT solution was replaced with 100 μl of DMSO which dissolved MTT formazan crystal. Finally, fluorescence microplate reader was performed to measure fluorescence intensity of formazan product at optical density (OD) 570 nm. Rate of cell viability (%) was calculated as this formula

$$\text{Cell viability (\%)} = \frac{OD_{570} \text{ of treatment} \times 100}{OD_{570} \text{ of control}}$$

5.2. Investigation on nuclear morphology induced by artonin E in human lung cancer H460 cells

To determine whether artonin E caused apoptosis or necrosis in H460 cell, Hoechst 33342 and PI co-staining assay were used to detect mode of cell death. Cells were seeded at 1 x 10^4 cells per well in a 96-well plate 12 h. Cells were treated with sub-toxic concentrations of artonin E (0-0.5 μg/ml) for 24 h cells were then incubated with 10 μM of the Hoechst 33342 and 5 μg/mL PI dye for 30 min at room temperature. The characteristics of the apoptotic cells were chromatin condensation or

fragmentation of nuclei. For analyzing apoptotic or necrotic cells, a fluorescence microscope was used to distinguish these cells.

5.3. Investigation effect of artonin E on proliferation of human lung cancer H460 cells

To study effect of artonin E on cell proliferation, MTT assay was used as describe previously. Cells were seeded at 5 x 10^3 cells per well in a 96-well plate 12 h. Cells were treated with sub-toxic concentrations (0-0.5 µg/ml) of artonin E and incubated for 12, 24, 48 and 72 h. Then, cell viability was examined by MTT assay.

5.4. Investigation effect of artonin E on migration of human lung cancer H460 cells

To study effect of sub-toxic concentrations of artonin E on cell migration, the ability of H460 cells on cell migration was evaluated using scratch assay at various times. A space between cells was created by using 1-mm width tip on a monolayer of cells in 96-well plate. After washing with phosphate buffer saline (PBS), cells were incubated with sub-toxic concentrations of artonin E (0-0.5 µg/ml) and allowed to migrate for indicated time (24, 48 and 72 h). Pictures were taken under a phase contrast microscope and the distance between cells at each time was measured by using Olympus DP controller software. The analysis of cell migration was done by using an average space from those random fields of view, and % change at various times was calculated using the following formula:

% change at time 24, 48 and 72 h

$$= \frac{\text{(Average space at time 0 h) - (Average space at time 24, 48 and 72 h)} \times 100}{\text{Average space at time 0 h}}$$

Relative cell migration was calculated by dividing the percentage change in the wound space of treated cells by that of the control cells in each experiment

5.5. Investigation of cell morphology induced by artonin E in human lung cancer H460 cells

To study the effect of artonin E on cell morphology, H460 cells were stained with phallodin. Cells were seeded at 5 x 10^3 cells per well in a 96-well plate 12 h. Cells were treated with sub-toxic concentrations of artonin E (0-0.5 μg/ml) for 24 h. After rinsing with PBS, cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were then permeabilized by 0.1% Triton-X100 in PBS for 4 min, washed with PBS 3 times and blocked with 0.2% BSA for 30 min. After washing cells with PBS 3 times, cells were labeled with rhodamine-phalloidin diluted 1:100 in PBS for 15 min and washed with PBS 3 times 5 min each. Picture of stained-cell were taken under fluorescence microscope.

5.6. Investigation on molecular mechanisms that induced by artonin E in human lung cancer H460 cells

To identify the underlying mechanism of artonin E-inhibited migration in H460 cells, western blot analysis was performed to evaluate p-FAK, FAK, p-Akt, Akt and Cdc42 expression. Cells were seeded at the number of 5 × 10^5 cells per well onto 6-well plate 12 h and treated with sub-toxic concentrations of artonin E for 24 h and

72 h. Cells were lysed with lysis buffer (20 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and cocktail protease inhibitor) for 1 h on ice. After collecting cell lysate, protein content was quantified using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Proteins (60-80 μg) were resolved on 10% SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes using wet transfer method. The membranes were blocked for 1 h in 5% skim milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20). After washing twice with TBST for 7 min each, the transferred membranes were incubated with the primary antibodies at 4°C for 10 h and washed 3 times with TBST for 7 min each then incubated with horseradish peroxidase–coupled isotype-specific secondary antibodies for 2 h at room temperature. The immune complexes were detected by enhanced with chemiluminescence substrate and quantified using analyst/PC densitometry software normalized to the level of β-actin protein.

5.7. Investigation effect of artonin E on migration of other type of human lung cancer cells

To study effect of sub-toxic concentrations of artonin E on cell migration in the other types of human lung cancer cells including H292, H23 and A549, the ability of these cells on cell migration was evaluated using scratch assay as describe previously at 24, 48 and 72 h.

6. Statistical Analysis

The data were presented as the mean ± SD from at least three independent experiments and were normalized to result in the untreated control cells. The

differences between group was performed using one way ANOVA (analysis of variance) followed by Duncan's test at a significance level of p-values less than 0.05.

7. Conceptual framework

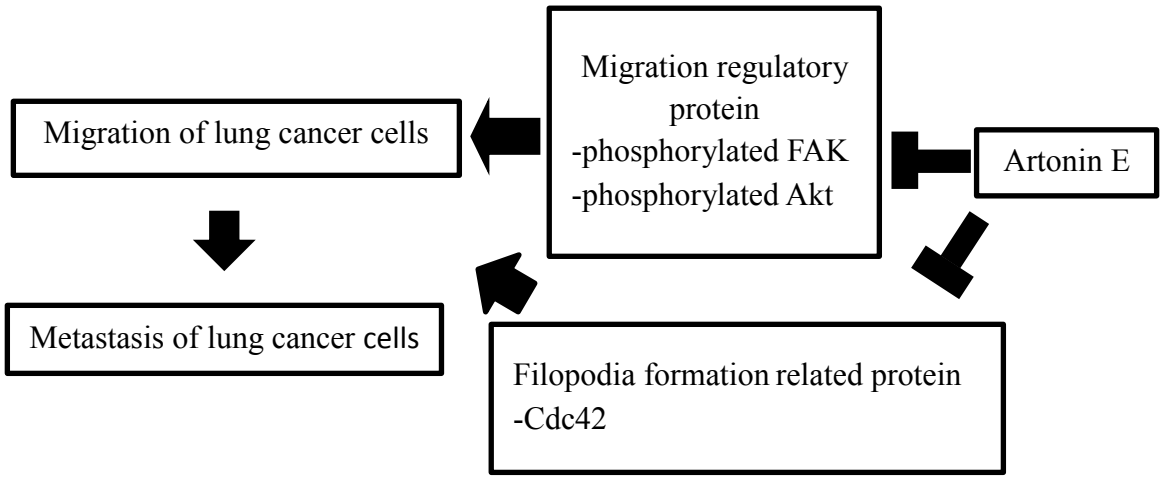


Figure 5. Conceptual framework of this study

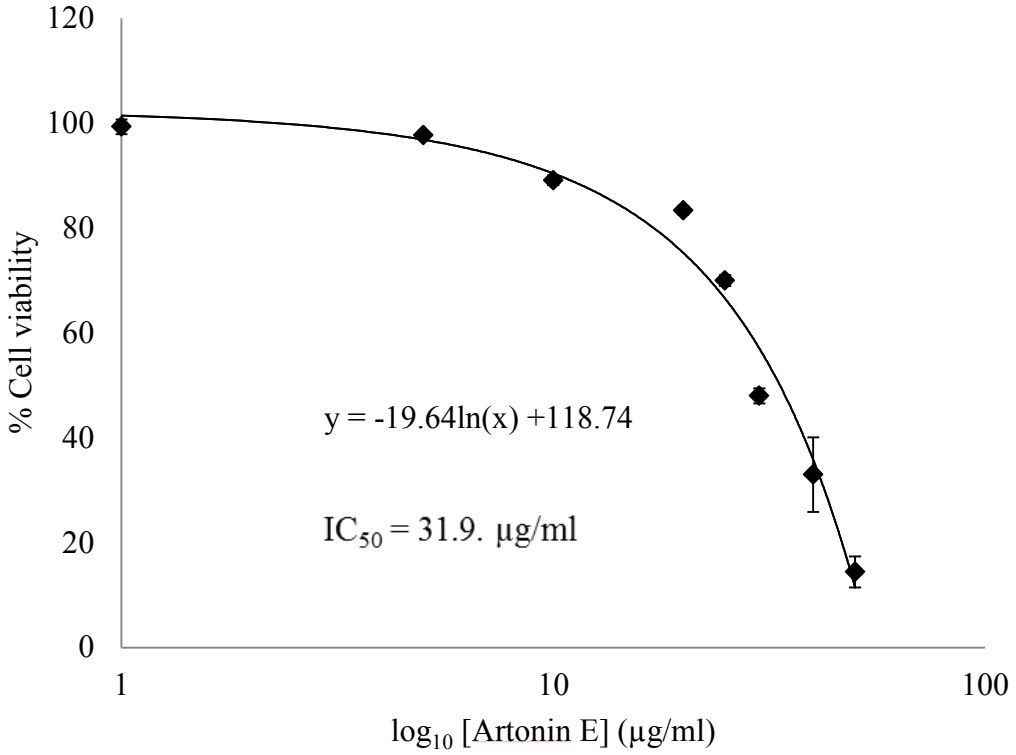
CHAPTER IV

RESULTS

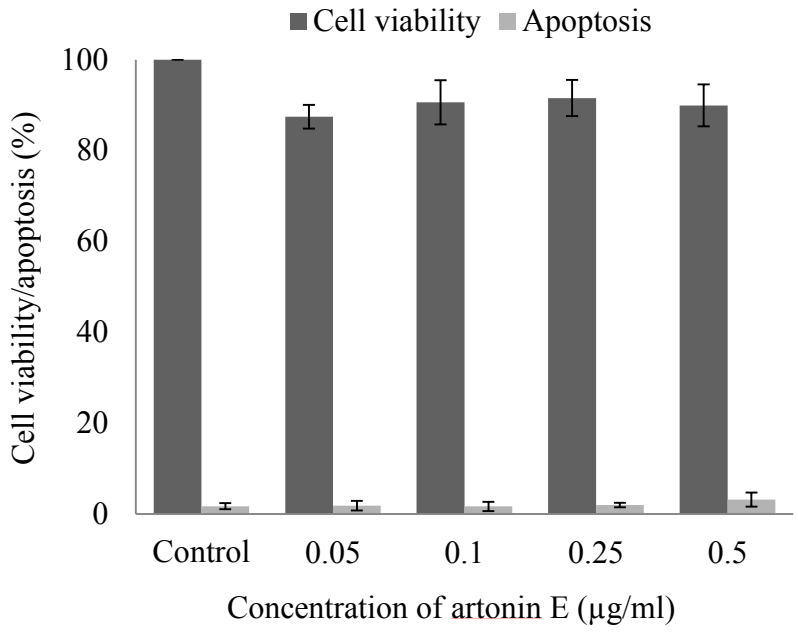
1. Artonin E induces apoptosis and does not affect proliferation on human non-small cell lung cancer H460 cells.

The present study investigated anti-cancer activity of artonin E using H460 human lung cancer cells by incubating the cells in the presence or absence of artonin E (0-50 μg/ml) for 24 h, and cell viability was analyzed. Figure 6A shows that treatment with artonin E caused a concentration-dependent decrease in cell survival and 50 % inhibition (IC_{50}) was observed in response to artonin E at the concentration of 31.93 μg/ml. Because the study aimed to investigate anti-migration activity of the compound, concentrations of artonin E which cause neither toxic nor proliferative effects were clarified. Results indicated that treatment of artonin E at the concentrations 0.05–0.5 μg/ml for 24 h caused no significant effect on H460 cell viability (figure 6B). The nuclear morphology study shown in figure 6C supported the above finding that no apoptotic and necrotic cell death was detected in response to artonin E at indicated concentrations. In addition, artonin E at the concentrations of 0.05-0.5 μg/ml, did not significantly alter proliferation of the cells up to 72 h (figure 6D).

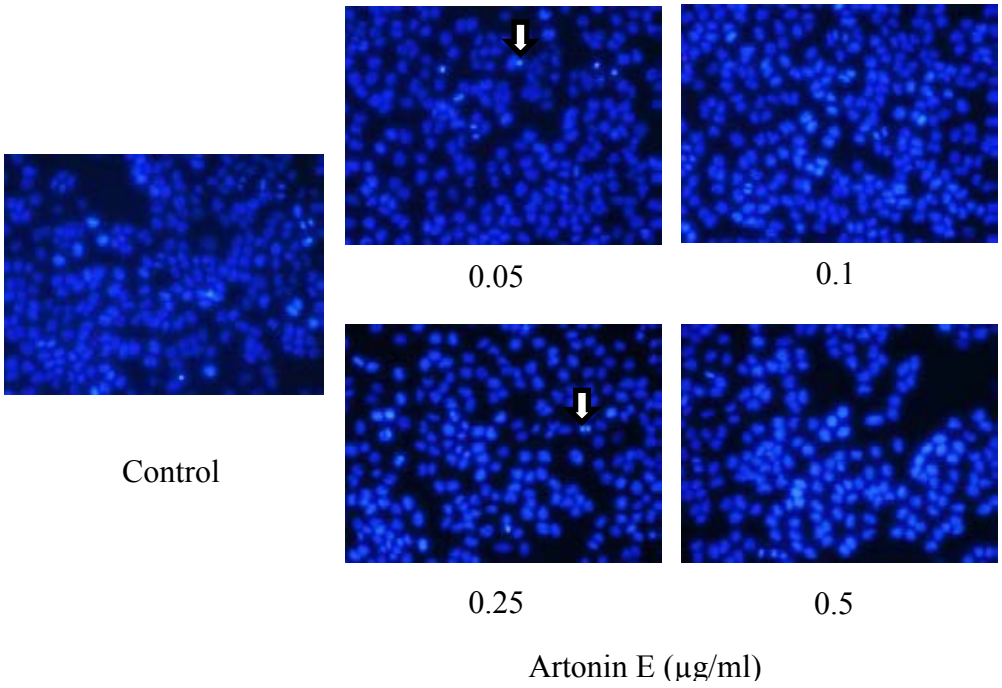
A


120
100
80
60
40
20
0
% Cell viability
y = -19.64ln(x) +118.74
IC50 = 31.9. µg/ml
1
10
100
log10 [Artonin E] (µg/ml)

B


Cell viability
Apoptosis
100
80
60
40
20
0
Cell viability/apoptosis (%)
Control
0.05
0.1
0.25
0.5
Concentration of artonin E (µg/ml)

C


0.05
0.1
Control
0.25
0.5
Artonin E (μg/ml)

D

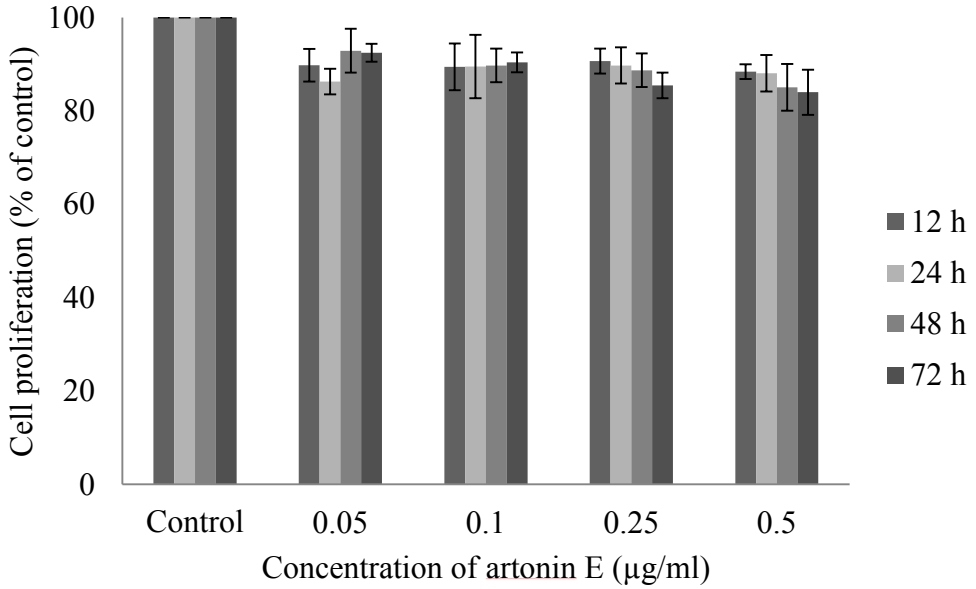
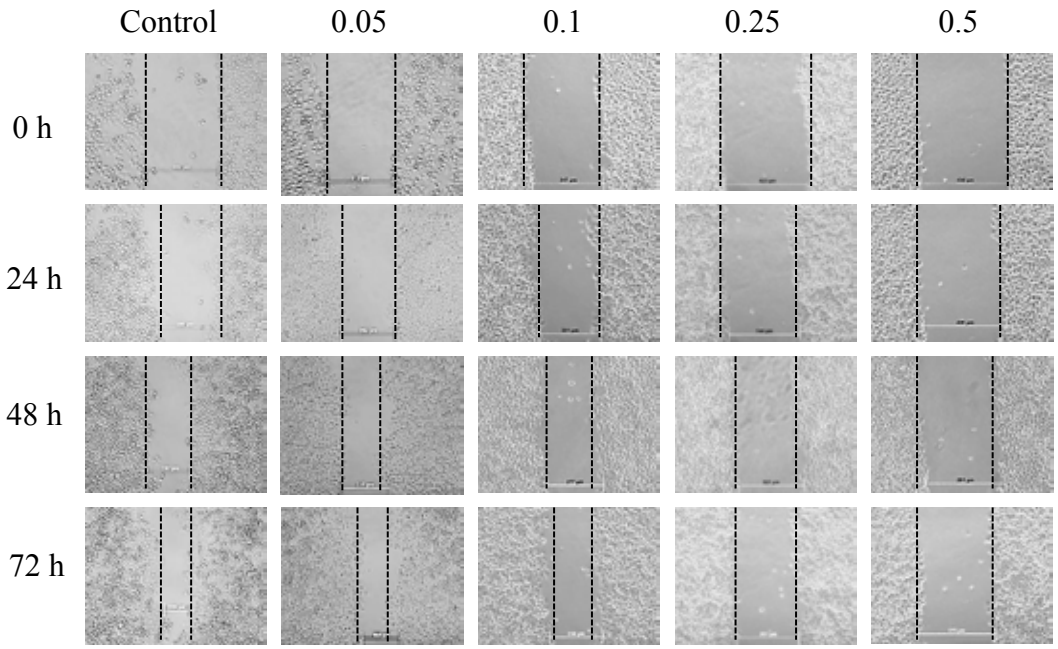

100
80
60
40
20
0
Cell proliferation (% of control)
12 h
24 h
48 h
72 h
Control
0.05
0.1
0.25
0.5
Concentration of artonin E (μg/ml)

Figure 6. Effect of artonin E on cell viability of human lung cancer H460 cells. Cells were treated with various concentrations of artonin E (0-50 μg/ml) for 24 h. A: Cytotoxicity was determined by MTT assay and concentration for 50% cell survival (IC_{50}) was determined. B: Percentage of cell viability and cell apoptosis were analyzed by MTT assay and Hoechst 33342 staining assays, respectively. C: Morphology of apoptotic nuclei stained with Hoechst 33342 and PI (Apoptotic cells are indicated by arrow). D: Proliferation of H460 cells in response to artonin E 0.05-0.5 μg/ml for 12, 24, 48, and 72 h was investigated by MTT assay. Values are means of triplicate samples ± SD.

2. Artonin E inhibits migration of H460 cells.

To examine the effect of artonin E on migration of the cells, scratch assay was performed. Briefly, the confluent monolayer of H460 cells was scratched and cultured with or without sub-toxic concentrations of artonin E (0.05-0.5 μg/ml) for 24, 48, and 72 h. Figure 7A and 7B show that the incubation of artonin E at the concentration of 0.5 μg/ml significantly decreased the spreading of H460 cells to the wound area as early as 24 h whereas artonin E at 0.05 μg/ml had no significant effect on cell migration in comparison to that of non-treated control. In addition, artonin E at the concentrations of 0.1 and 0.25 μg/ml significantly inhibited migration of H460 cells at 72 h. These results indicated that artonin E possess ability to inhibit cancer cell migration.

A


Control
0.05
0.1
0.25
0.5
0 h
24 h
48 h
72 h

B

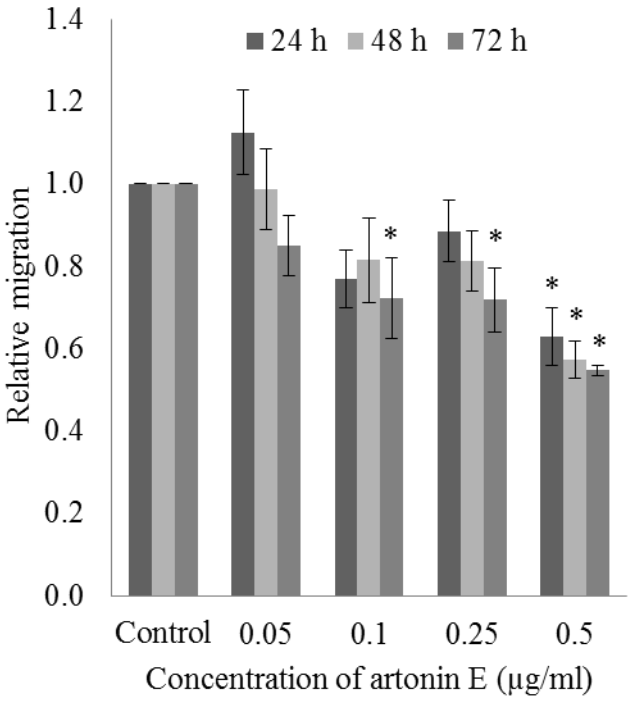

24 h
48 h
72 h
Relative migration
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
0.05
0.1
0.25
0.5
Concentration of artonin E (μg/ml)

Figure 7. Effects of artonin E on H460 cell migration. For migration assay, scratch assay was performed. Wound space was made and the cells were treated with sub-toxic concentrations of artonin E for various times. A: Wound space was visualized under a phase contrast microscope at indicated times. B: The relative cell migration was analyzed by comparison of the relative change in wound space of the treated groups over untreated control.

3. Artonin E inhibits filopodia formation.

Having shown that artonin E at the sub-toxic concentration significant inhibited lung cancer cell migration, we further tested whether the compound could have an effect on filopodia formation in these cells. Cells were treated with 0-0.5 μg/ml of artonin E and the phalloidin-labeled filopodia were detected under fluorescence microscope. Figure 8 shows that in non-treated control, cells exhibited several membrane protrusions of filopodia. Interestingly, treatment with artonin E dramatically decreased directional stress fiber and filopodia in H460 cells in comparison to that of un-treated cells. These data and above findings suggested that artonin E have a negative effect on cell migration.

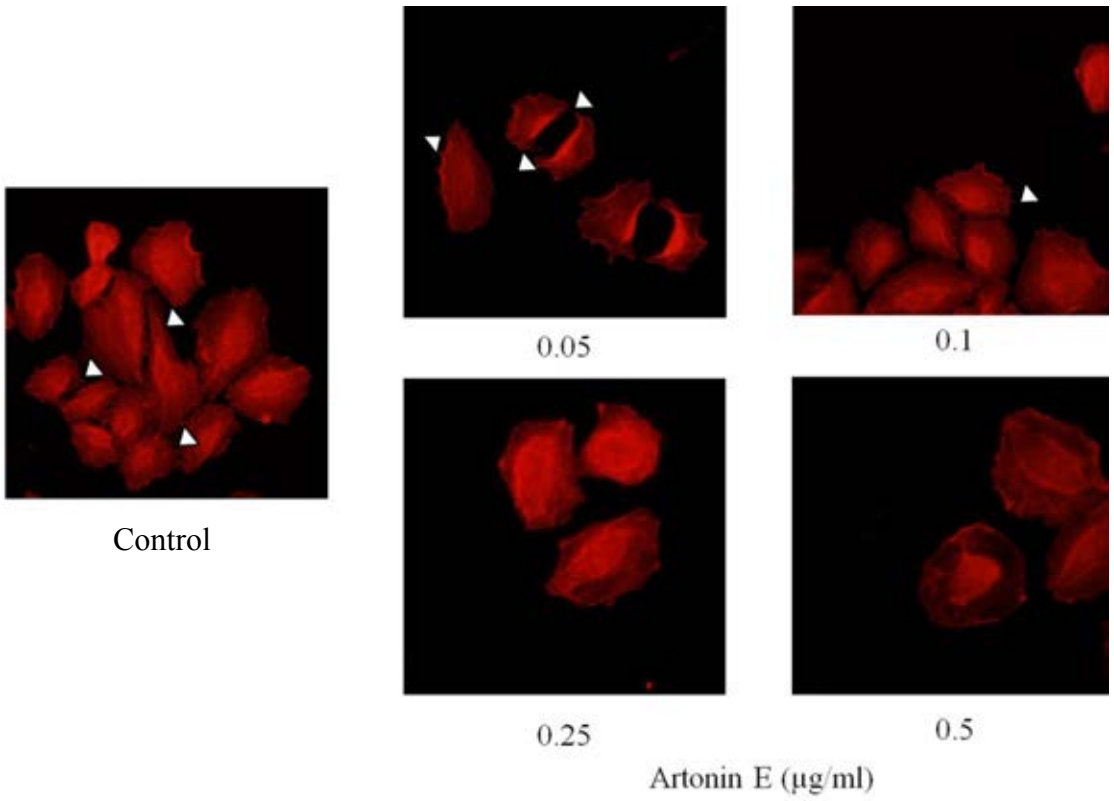


Figure 8. Effect of artonin E on filopodia alteration. After treating with sub-toxic concentrations of artonin E for 24 h, cells were stained with phalloidin and filopodia was examined under fluorescent microscope (n=3). Filopodia are indicated by arrow.

4. Artonin E inhibits FAK signaling and suppresses Cdc42 expression in H460 cells.

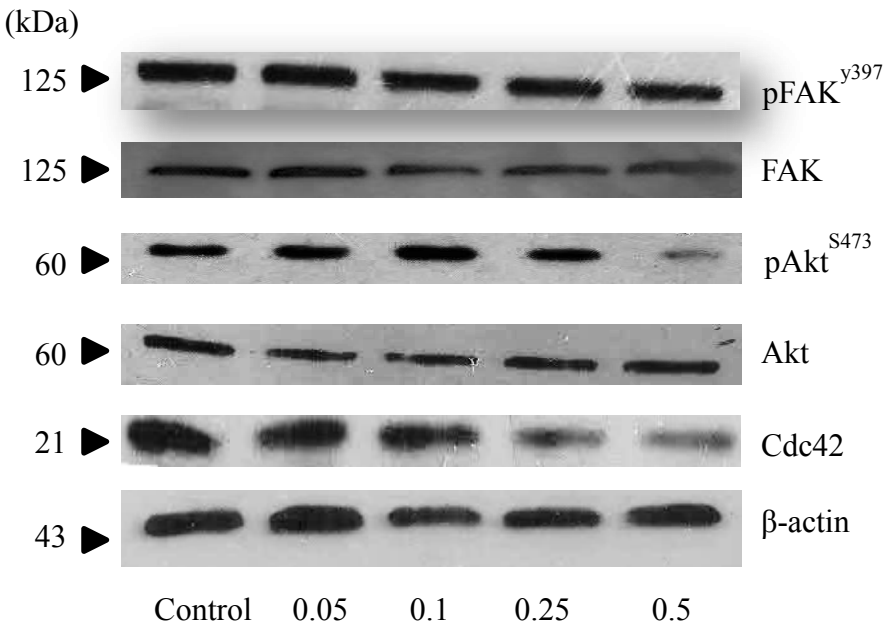
In order to clarify the mechanism of artonin E in suppression of cancer cell motility, the expression level and activation status of protein regulators of cell motility including FAK and Akt were investigated. Cells were seeded and incubated in the presence or absence of artonin E (0.05-0.5 μg/ml) and the expressions of proteins were determined by western blotting, as mentioned in Materials and Methods. After 24 h treatment with artonin E (0.5 μg/ml), it significantly decreased the level of phosphorylated FAK at Tyr 397 (Activated FAK) and did not effect on total FAK as shown in figure 9. We investigated the effect of artonin E on Akt activation. Figure 9 demonstrates that artonin E at the concentrations of 0.5 μg/ml also significantly decreased phosphorylation of Akt (Ser 473) while had no significant effect on total Akt expression.

We assessed the effect of artonin E on Cdc42 level by western blot analysis. Figure 9 shows that the level of Cdc42 protein was found to be down-regulated in response to artonin E treatment at the concentrations of 0.25 and 0.5 μg/ml in comparison to that of untreated control.

Furthermore, we investigated effect of artonin E on the expressions of related proteins at 72 h as shown in figure 10. While artonin E had only minimal effect on the level of total FAK and Akt, treatment of artonin E at the concentrations of 0.1-0.5 μg/ml significantly decreased the levels of phosphorylated FAK at Tyr 397 (Activated FAK) and phosphorylated Akt at Ser 473. These findings indicated that artonin E inhibits cell migration via FAK-Akt-dependent mechanism.

Together, these results suggest the role of artonin E in attenuation of lung cancer cell migration through FAK-Akt-dependent mechanism and *via* reduction of Cdc42.

A


(kDa)
125
pFAK y397
125
FAK
60
pAkt S473
60
Akt
21
Cdc42
43
β-actin
Control
0.05
0.1
0.25
0.5

B

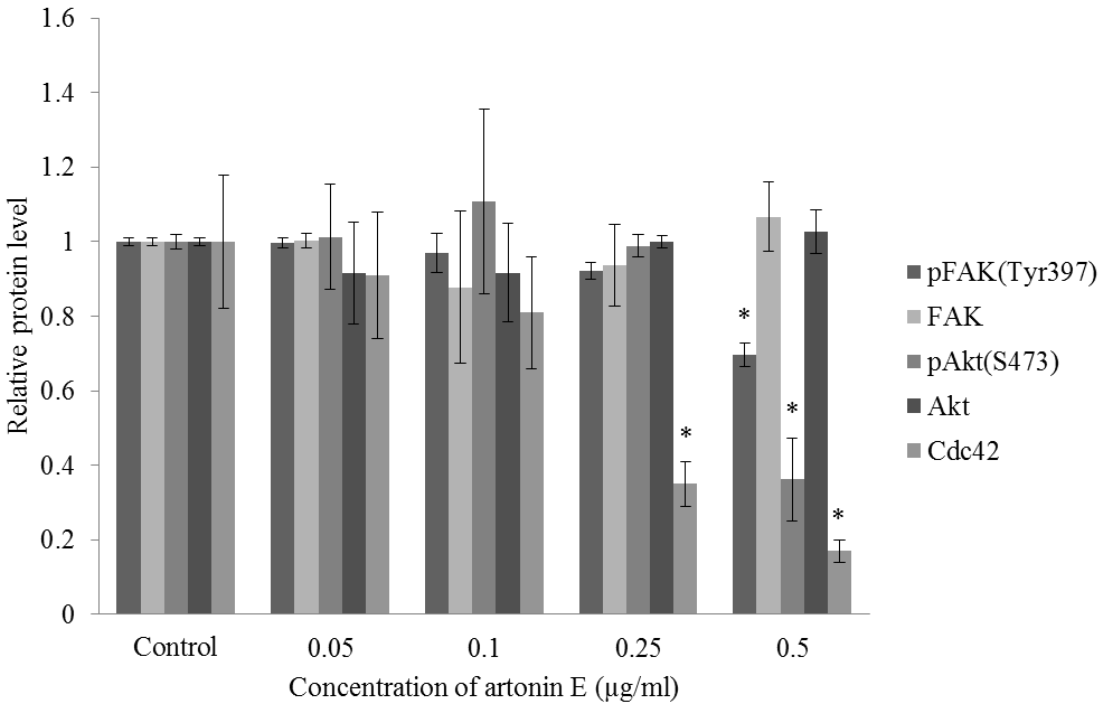
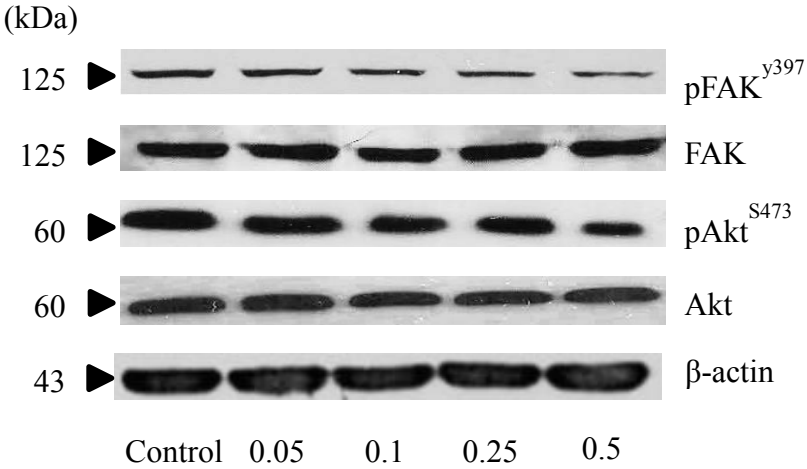

Relative protein level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
0.05
0.1
0.25
0.5
Concentration of artonin E (μg/ml)
pFAK(Tyr397)
FAK
pAkt(S473)
Akt
Cdc42
*
*
*
*

Figure 9. Effect of artonin E on FAK, Akt and Cdc42 proteins for 24 h. A: Cells were seeded and treated with various concentrations of artonin E (0–0.5 μg/ml) for 24 h and the expressions of pFAK (Tyr 397), FAK, pAkt (Ser 473), Akt and Cdc42 proteins were determined by Western blotting. To confirm equal loading of samples, blots were re-probed with β-actin antibody. B: The immunoblot signals were quantified by densitometry and mean data from four independent experiments were presented. Values are means of samples ± SD. *$p < 0.05$ versus untreated group.

A


(kDa)
125
pFAK y397
125
FAK
60
pAkt S473
60
Akt
43
β-actin
Control
0.05
0.1
0.25
0.5

B

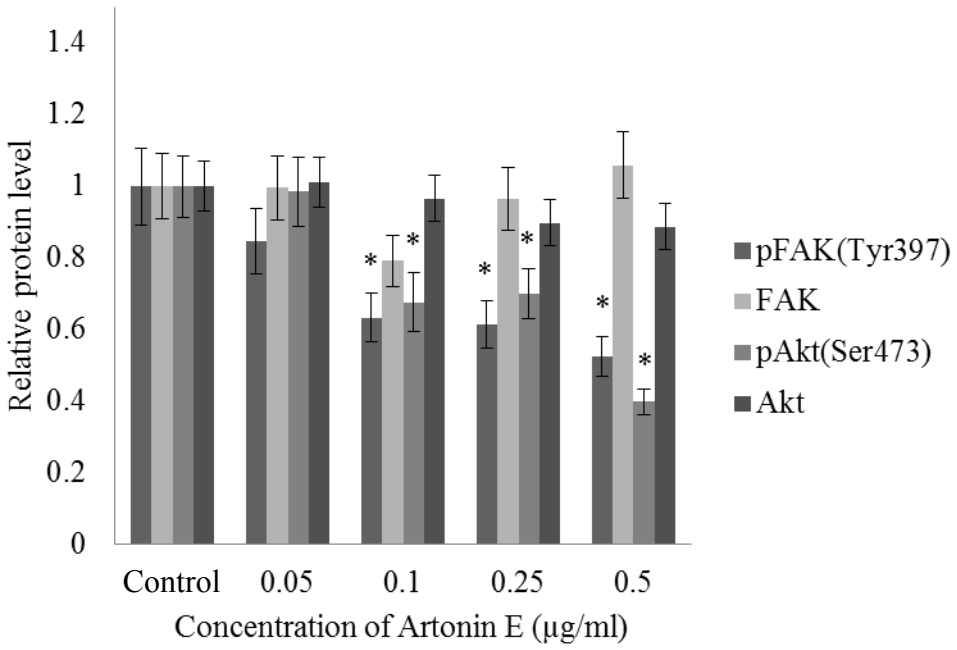

Relative protein level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
0.05
0.1
0.25
0.5
Concentration of Artonin E (μg/ml)
pFAK(Tyr397)
FAK
pAkt(Ser473)
Akt

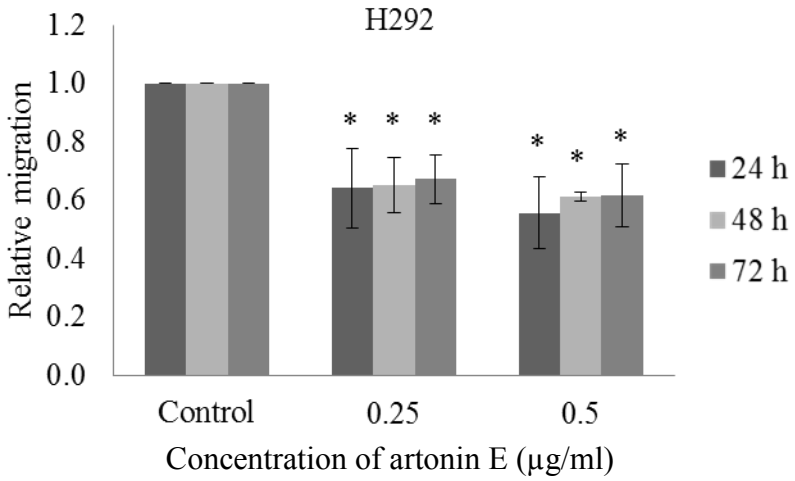
Figure 10. Effect of artonin E on FAK, Akt proteins for 72 h. A: Cells were seeded and treated with various concentrations of artonin E (0–0.5 μg/ml) for 72 h and the expressions of pFAK (Tyr 397), FAK, pAkt (Ser 473), Akt proteins were determined by Western blotting. To confirm equal loading of samples, blots were re-probed with β-actin antibody. B: The immunoblot signals were quantified by densitometry and mean data from four independent experiments were presented. Values are means of samples ± SD. *$p < 0.05$ versus untreated group.

5. Artonin E inhibits migration of other lung cancer cells.

In order to support the negative regulatory activity of artonin E on lung cancer cell migration and invasion, human lung cancer cells, namely H292, H23 and A549 cells.

H292 cells

Human lung cancer H292 cells were treated with the non-toxic concentrations of artonin E (0-0.5 μg/ml) and subjected to migration assays. Our results shown in the figure 10A showed that artonin E at the concentrations of 0.25 and 0.5 μg/ml significantly inhibited migratory behavior of H292 cells in comparison to that of non-treated control of each cells.



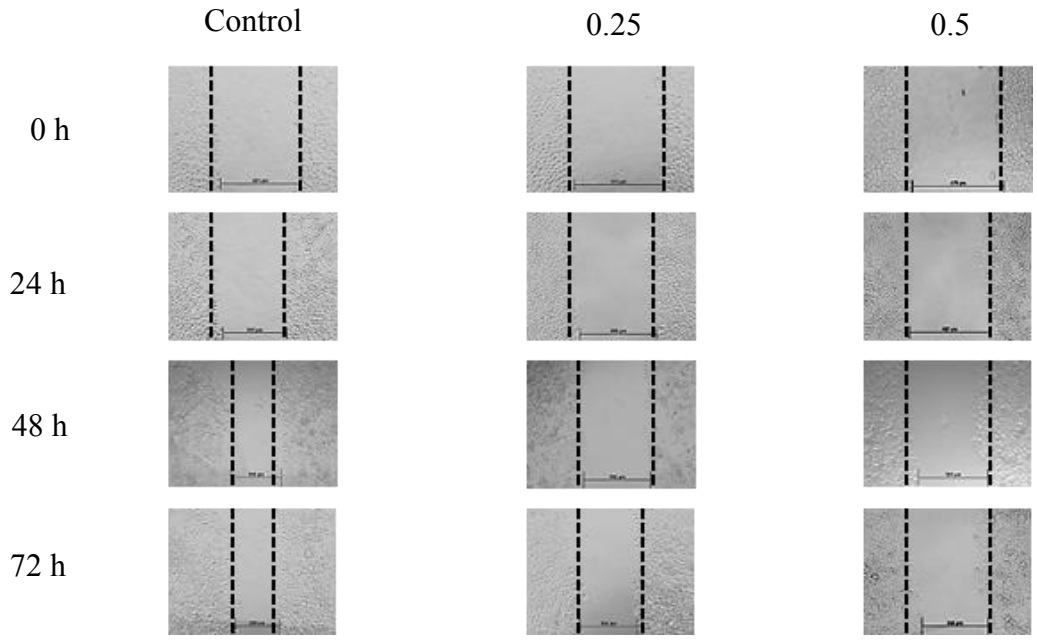
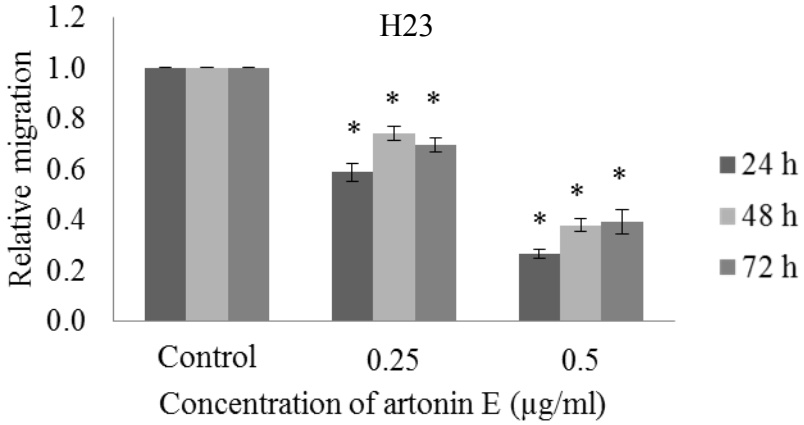


Figure 11. Effect of artonin E on migration of H292 cells. H292 cells were subjected to migration assay and visualized under a phase contrast microscope. The relative cell migration of H292 cells was shown. Values are means of triplicate samples ± SD. **p*<0.05 versus untreated control.

H23 cells

Human lung cancer H23 cells were treated with the non-toxic concentrations of artonin E (0-0.5 µg/ml) and subjected to migration assays. Our results shown in the figure 11 showed that artonin E at the concentrations of 0.25 and 0.5 µg/ml significantly inhibited migratory behavior of H23 cells in comparison to that of non-treated control of each cells.



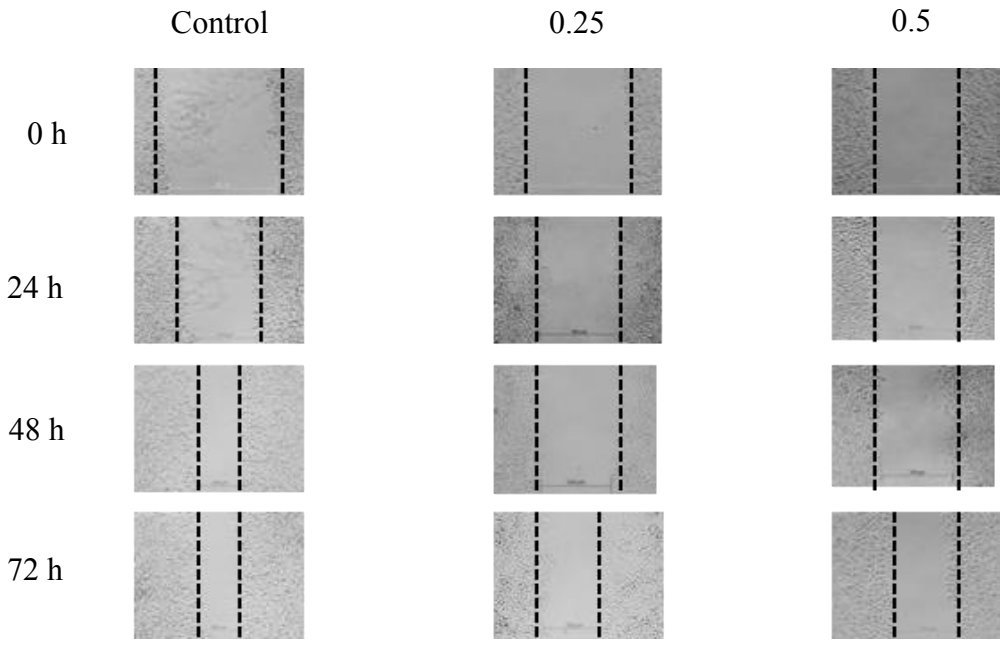


Figure 12. Effect of artonin E on migration of H23 cells. H23 cells were subjected to migration assay and visualized under a phase contrast microscope. The relative cell migration of H23 cells was shown. Values are means of triplicate samples ± SD. *p<0.05 versus untreated control.

A549 cells

Human lung cancer A549 cells were treated with the non-toxic concentrations of artonin E (0-0.5 µg/ml) and subjected to migration assays. Our results shown in the figure 12 showed that artonin E at the concentrations of 0.25 and 0.5 µg/ml significantly inhibited migratory behavior of A549 cells in comparison to that of non-treated control of each cells.

These data have strengthen the observations of the present study that artonin E possess ability to inhibit migration of lung cancer cells.

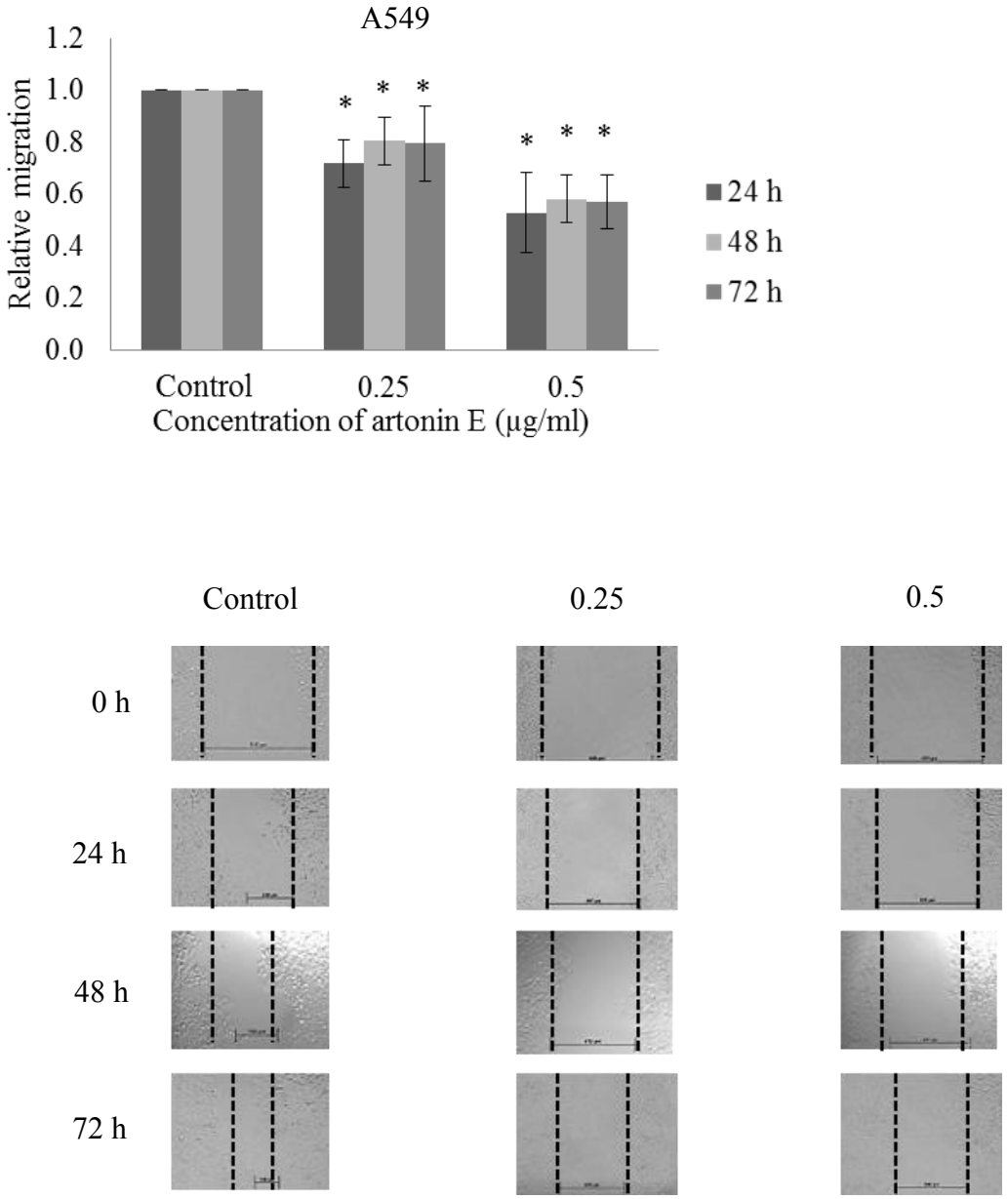


Figure 13. Effect of artonin E on migration of A549 cells. A549 cells were subjected to migration assay and visualized under a phase contrast microscope. The relative cell migration of A549 cells was shown. Values are means of triplicate samples ± SD. *p<0.05 versus untreated control.

CHAPTER V

DISCUSSION AND CONCLUSION

Advance and novel strategies for cancer therapies involving those inhibit metastasis of cancer have garnered increasing attention these days. So far, limited efficacy has been obtained from the available therapy resulted in only 68 % 5-year survival of cancer patients in the United States (Merrill and Hunter, 2010), and the major cause of death found in such patients involves metastasis. In patient with NSCLC, 40% of them already have hematogenous metastases after initial diagnosis (Ray and Jablons, 2009). Consequently, almost of them will finally die from such disease. As a hallmark of cancer metastasis, ability of cancer cells to migrate away from original tumor and invade to the blood or lymphatic circulations is considerably important (Hanahan and Weinberg, 2000). Moreover, migration is the critical parameters in the metastatic cascade. The lymph nodes are one of the major routes which cancer cell uses them to migrate through the body *via* the lymphatic system. Therefore, lymph node metastases in cancer are a good indicator for patient's survival and prognostic indicator of whether distal metastases will develop.

Although anti-cancer activity of artonin E was previously demonstrated in breast cancer cell model (Shajarahtunnur, 2006), it is not clear whether such a compound cause cytotoxic to the lung cancer cells. Furthermore, artonin E was reported to have many pharmacological properties such as antiasthma activity by inhibition of arachidonate 5-lipoxygenase (Reddy *et al*., 1991), antioxidant activity by inhibition of DPPH radical activity (Sritularak *et al*., 2010). Herein we demonstrated

for the first time that artonin E a plant-originated pure compound has a promising ability to inhibit lung cancer cell migration.

PTK families, including FAK, own the potential role in metastasis of cancer cells such as angiogenesis, tumor cell distribution, and cell motility (Germanov, Berman, and Guernsey, 2006). Overexpression or deregulations of PTK mostly occur in several cancer cells (Eccles, 2005; Onn, Tsuboi, and Thatcher, 2004).

From western blot analysis, we found that artonin E had different effect on the expressions of pFAK and pAkt proteins at 24 and 72 h. These results were consistent with effect of artonin E on scratch assay at 24 and 72 h. Artonin E (0.5 μg/ml) could inhibit migration of H460 cells and suppress the expressions of pFAK and pAkt at 24 h. Moreover, artonin E (0.1-0.5 μg/ml) could decrease migration of H460 cells and the expressions of pFAK and pAkt at 72h.

Activation of cancer cell movements involves several mechanistic pathway including FAK (Bolos *et al*., 2010), Akt (Kim *et al*., 2001) and Cdc42 (Sinha and Yang, 2008). Tumor progression and metastasis can be stimulated by FAK signaling pathways through the regulation of cell migration. It has been well established that the phosphorylation of FAK at Try 397 activated its kinase activity (Bolos et al., 2010). Mutagenesis of FAK gene by insertion technique at 397 site eliminated ability of FAK to activate motility (Cary *et al*., 1996). Likewise, many studies demonstrate that phosphorylation of FAK at Tyr 397 is necessary for capability of FAK to promote cell migration (Niwa *et al*., 2005; Sieg *et al*., 2000; Zhao and Guan, 2009). Moreover, FAK can transduce the signal through the activation of PI3K-Akt pathway. Akt activation was shown to be associated with cell migration by several means (Grille *et*

al., 2003; Kim *et al*., 2001). Akt can regulate the stabilization of microtubules, which has an essential role for mobile cell (Onishi *et al*., 2007) and also increase invasiveness of cell by promoting matrix metalloproteinase production (Kim *et al*., 2001).

During cell movement, the membrane protrusions called filopodia were shown to be increased and the formation of filopodia was shown to tightly involve with cancer cell migration and invasion (Arjonen et al., 2011). Cdc42 protein, a member of Rho GTPase families, has been shown to regulate many cellular processes including actin reorganization, cell polarity (Schmitz *et al*., 2000). Cdc42 is also implicated in filopodia formation which leads to migration and invasion of cancer cell (Machesky, 2008). Some evidences exhibit that cdc42 has been overexpressed in many types of human carcinoma resulting in aggressiveness of cancer (Jiang *et al*., 2011; Kamai *et al*., 2004). Evidence indicated that knockdown of Cdc42 resulted in inhibition of cancer cell migration and invasion suggesting significance impact of this protein on cell motility (Raymond *et al*., 2012). Previously, curcumin was shown to down-regulated Cdc42 expression and inhibited migration and invasion of lung cancer cell lines (Chen *et al*., 2012). Since the Cdc42 was shown to be up-regulated in primary lung cancer cells and such an increase of the protein was shown to associate with high TNM stage and lymph node metastasis, it is interesting to investigate whether artonin E treatment could affect cellular Cdc42 level. In the present study, we found that treatment of the lung cancer H460 cells with artonin E resulted in the reduction of cellular Cdc42 level (Figure 5A and B), along with the finding indicated that filopodia protrusions were decrease in the cells treated with artonin E (Figure 8), this substance may, at least in part, inhibit migration through Cdc42 suppression.

In addition, we have provided evidence indicating that the inhibitory effect of artonin E on cancer cell motility can also be observed in other lung cancer cell models. Three human lung carcinoma cell lines, namely, H292, H23 and A549, were tested with sub-toxic concentrations of artonin E and the results indicated that artonin E had similar activities in these cells as those found in H460 cells. Nevertheless, the treatment of artonin E (0.25 μg/ml) for 24 h did not affect migration of H460 cells, this effect was due to characteristic and aggressiveness of H460 (Large cell lung cancer; Lynne, 2012).

Migration inhibitory of artonin E by down regulation of pFAK, pAkt and Cdc42 proteins might be due to anti-EGFR properties (Kandaswami *et al*., 2005) or anti-oxidant effect like many flavonoids (Adhikary *et al*., 2010; Lai *et al*., 2013). Previous study shows that the inhibition of hydroxyl radical can inhibit migration of lung cancer cells (Kowitdamrong *et al*., 2013).

This investigation reveals that artonin E can inhibit migration of lung cancer cells *via* FAK signaling pathways and suppression of Cdc42 therefore artonin E may be a promising agent for anti-metastasis therapy or used as adjuvant with standard therapies to improve survival of cancer patients.

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APPENDIX

APPENDIX

TABLE OF EXPERIMENTAL RESULTS

Table 1. Percentage of H460 cell viability after treatment with various concentrations of artonin E at 24 hours was determined by MTT assay**.**

Artonin E (μg/ml)	Cell viability (%)
control	100±1.41
1	99.32±0.12
5	97.66±0.90
10	89.11±0.80
20	83.37±1.04
25	70.04±1.41
30	48.01±7.11
40	33.05±2.94
50	14.42±0.16

Each value represents the mean ± S.D. of three independent experiments.

Table 2. Percentage of H460 cell viability after treatment with sub-toxic concentrations of artonin E at 24 hours was determined by MTT assay.

Artonin E(μg/ml)	Cell viability (%)
control	100±0.04
0.05	87.43±2.59
0.1	90.58±4.87
0.25	91.51±3.98
0.5	89.92±4.62

Each value represents the mean ± S.D. of three independent experiments.

Table 3. Percentage of H460 cell apoptosis after treatment with sub-toxic concentrations of artonin E at 24 hours was determined by Hoechst assay.

Artonin E(μg/ml)	Cell apoptosis (%)
control	1.72±0.69
0.05	1.83±1.04
0.1	1.67±1.04
0.25	2.00±0.50
0.5	3.17±1.53

Each value represents the mean ± S.D. of three independent experiments.

Table 4. Relative cell proliferation of H460 cell after treatment with sub-toxic concentrations of artonin E at various time points was determined by MTT assay.

Artonin E(μg/ml) / Time (h)	Relative cell growth (% of control)			
	0.05	0.1	0.25	0.5
12	89.74±3.50	89.40±2.73	90.67±4.71	88.37±1.93
24	86.27±5.01	89.48±6.79	89.70±3.61	88.04±2.14
48	92.85±2.68	89.72±3.88	88.69±3.60	85.05±2.73
72	92.43±1.59	90.36±3.88	85.45±5.01	83.98±4.84

Each value represents the mean ± S.D. of three independent experiments.

Table 5. Relative cell migration of H460 after treatment with sub-toxic concentrations of artonin E at various time points was determined by scratch assay.

Artonin E(μg/ml) / Time (h)	Relative cell migration (of control)			
	0.05	0.1	0.25	0.5
24	1.13±0.10	0.77±0.07	0.86±0.07	0.63±0.07
48	0.97±0.1	0.81±0.1	0.81±0.07	0.57±0.05*
72	0.85±0.07	0.72±0.10*	0.72±0.08*	0.54±0.01*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from control: *, $p < 0.05$ determined by One-way ANOVA followed by Duncan's test.

Table 6. Relative of various proteins were determined by western blot analysis after treatment with sub-toxic concentrations of artonin E at 24 hours for pFAk, FAK, pAkt Akt and Cdc42 proteins.

Artonin E(μg/ml) / Proteins	Relative protein level				
	control	0.05	0.1	0.25	0.5
pFAK	1.00±0.01	0.99±0.01	0.97±0.05	0.92±0.02	0.69±0.03*
FAK	1.00±0.01	1.00±0.01	0.87±0.20	0.93±0.11	1.07±0.09
pAkt	1.00±0.02	1.01±0.14	1.11±0.25	0.99±0.03	0.36±0.11*
Akt	1.00±0.01	0.91±0.14	0.92±0.13	0.99±0.02	1.03±0.06
Cdc42	1.00±0.18	0.91±0.17	0.81±0.15	0.35±0.06*	0.17±0.03*

Each value represents the mean ± S.D. three independent experiments. Asterisks refer significant difference from control: *, $p < 0.05$ determined by One-way ANOVA followed by Duncan's test.

Table 7. Relative of various proteins were determined by western blot analysis after treatment with sub-toxic concentrations of artonin E at 72 hours for pFAk, FAK, pAkt and Akt proteins.

Artonin E(μg/ml) / Proteins	Relative protein level				
	control	0.05	0.1	0.25	0.5
pFAK	1.00±0.11	0.85±0.09	0.63±0.07*	0.62±0.07*	0.53±0.06*
FAK	1.00±0.09	1.00±0.09	0.79±0.07	0.96±0.09	1.06±0.09
pAkt	1.00±0.09	0.99±0.10	0.68±0.08*	0.71±0.07*	0.40±0.03*
Akt	1.00±0.07	1.01±0.07	0.97±0.06	0.90±0.06	0.89±0.06

Each value represents the mean ± S.D. three independent experiments. Asterisks refer significant difference from control: *, $p < 0.05$ determined by One-way ANOVA followed by Duncan's test.

Table 8. Relative cell migration of H292 after treatment with sub-toxic concentrations of artonin E at various time points was determined by scratch assay.

Artonin E(μg/ml) / Time (h)	Relative cell migration (of control)	
	0.25	0.5
24	0.64±0.14*	0.56±0.12*
48	0.65±0.09*	0.61±0.02*
72	0.67±0.08*	0.61±0.11*

Each value represents the mean ± S.D. three independent experiments. Asterisks refer significant difference from control: *, $p < 0.05$ determined by One-way ANOVA followed by Duncan's test.

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Table 9. Relative cell migration of H23 after treatment with sub-toxic concentrations of artonin E at various time points was determined by scratch assay.

Artonin E(μg/ml) / Time (h)	Relative cell migration (of control)	
	0.25	0.5
24	0.59±0.05*	0.26±0.02*
48	0.74±0.04*	0.37±0.04*
72	0.70±0.04*	0.39±0.06*

Each value represents the mean ± S.D. three independent experiments. Asterisks refer significant difference from control: *, $p < 0.05$ determined by One-way ANOVA followed by Duncan's test.

Table 10. Relative cell migration of A549 after treatment with sub-toxic concentrations of artonin E at various time points was determined by scratch assay.

Artonin E(μg/ml) / Time (h)	Relative cell migration (of control)	
	0.25	0.5
24	0.72±0.09*	0.53±0.15*
48	0.80±0.09*	0.58±0.09*
72	0.79±0.14*	0.57±0.10*

Each value represents the mean ± S.D. of three independent experiments. Asterisks refer significant difference from control: *, $p < 0.05$ determined by One-way ANOVA followed by Duncan's test.

VITA

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