EFFECT OF GIGANTOL ON EPITHELIAL TO MESENCHYMAL TRANSITION IN LUNG CANCER CELLS

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ผลของไจแกนทอลต่อการเปลี่ยนแปลงลักษณะของเซลล์เยื่อบุผิวไปเป็นเซลล์มีเซนไคม์ในเซลล์มะเร็งปอด

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โรคมะเร็งปอดเป็นโรคที่มีอัตราการแพร่กระจายไปยังส่วนต่างๆ ของร่างกายสูง ซึ่งเป็นสาเหตุหลักที่ทำให้ผู้ป่วยโรคมะเร็งปอดมีอัตราการรอดชีวิตต่ำ การที่เซลล์มะเร็งปอดจะแพร่กระจายได้นั้น เซลล์มะเร็งปอดจะต้องมีการเปลี่ยนแปลงลักษณะจากเซลล์เยื่อบุผิวไปเป็นเซลล์มีเซนไคม์ (Epithelial to mesenchymal transition, EMT) ซึ่งมีคุณสมบัติทำให้เซลล์สามารถอยู่รอดในสภาวะหลุดออกจากการยึดเกาะและสามารถเคลื่อนที่ออกจากก้อนมะเร็งได้ สารไจแกนทอลเป็นสารกลุ่ม bibenzyl ซึ่งสกัดจากดอกกล้วยไม้พันธุ์เอื้องเงิน ซึ่งจากงานวิจัยที่ผ่านมาพบว่ามีความเป็นไปได้ในการพัฒนาเพื่อใช้ในการรักษาโรคมะเร็ง งานวิจัยนี้เป็นงานวิจัยแรกที่ศึกษาฤทธิ์ของสารไจแกนทอลในการยับยั้งกระบวนการ EMT และกลไกการออกฤทธิ์จากผลต่อการเปลี่ยนแปลงของระดับโปรตีนภายในเซลล์ ผลการศึกษาพบว่าสารไจแกนทอลมีผลยับยั้งกระบวนการ EMT ได้โดยลดการมีชีวิตรอดของเซลล์ในสภาวะไร้การยึดเกาะและลดความสามารถในการเคลื่อนที่ นอกจากนี้สารไจแกนทอลยังมีผลเปลี่ยนแปลงการแสดงออกของโปรตีนที่บ่งบอกถึงกระบวนการ EMT โดยเพิ่มการแสดงออกของ E-cadherin และลดการแสดงออกของ N-cadherin และ Vimentin ซึ่งการเปลี่ยนแปลงการแสดงออกของโปรตีนดังกล่าวเป็นผลมาจากการลดลงของ Transcription factor คือโปรตีน Slug โดยสารไจแกนทอลสามารถยับยั้งการทำงานของ protein kinase B (AKT) ซึ่งมีผลลดกระบวนการผลิตและเพิ่มกระบวนการทำลาย Slug ด้วยเหตุผลดังกล่าวข้างต้นสารไจแกนทอลจึงเป็นสารที่มีศักยภาพในการพัฒนาเพื่อเป็นยารักษาโรคมะเร็งได้ในอนาคต

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Lung cancer has been a disease with high fatality rate due to the high metastatic rate. One of the most essential processes of metastasis is the ability of cancer cells to undergo the epithelial to mesenchymal transition (EMT) which allows cancer cells to resist the programmed cell death in a detached condition called anoikis and to migrate into the surrounding tissue. Gigantol, a bibenzyl compound extracted from *Dendrobium draconis*, has been a promising naturally derived compound for cancer therapy due to several cytotoxic effects in cancer cells. This study has demonstrated for the first time that gigantol significantly attenuated EMT process in lung cancer cells. The results have shown that gigantol decreased lung cancer cells viability in a detached condition as well as decreased the migration and invasion. Western blotting analysis revealed that gigantol caused significant changes in EMT markers including the increase in E-cadherin expression, the decrease in N-cadherin and Vimentin expressions. These changes in EMT markers were induced by the decrease in expression of transcirption factor, Slug. It was found that gigantol was able to suppress the activity of protein kinase B (AKT). Therefore, gigantol could be a potential cancer therapeutic compound suggesting for further developed for cancer therapy.

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LIST OF ABBREVIATIONS

%	=	percentage
ºC	=	degree Celsius
µM	=	micromolar
AKT	=	protein kinase B
ANOVA	=	analysis of variance
Cis	=	cisplatin
CMA	=	concanamycin
CO_2	=	carbon dioxide
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	dimethyl sulfoxide
ECM	=	extracellular matrix
EMT	=	epithelial-mesenchymal transition
h	=	hour, hours
Lac	=	lactocystin
min	=	minute (S)
ml	=	milliliter
mM	=	millimolar
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSCLC	=	non-small cell lung cancer
PBS	=	phosphate buffer saline
PI	=	propidium iodide
RPMI	=	Roswell Park Memorial Institute's medium
S.D.	=	standard deviation
U	=	unit

CHAPTER I
INTRODUCTION

Lung cancer has been responsible for a high mortality rate in patients due to metastasis. Metastasis is a series of steps where cancer cells spread from the primary site to form the secondary tumor which contributes to cancer aggressiveness. Therefore, the discoveries of new natural compound with the ability to decrease the rate of metastasis in cancer cells have garnered most interest in cancer pharmaceutical science.

In cancer biology, the epithelial to mesenchymal transition (EMT) process has been recognized as a hallmark of cancer aggressiveness and a fundamental step driving cancer metastasis. EMT is a biological process when fully differentiated epithelial cells undergo transdifferentiation to gain mesenchymal phenotype (Voulgari & Pintzas 2009). These alterations in cell behavior potentiate cell migration and encourage a resistance in apoptosis (Chiarugi & Giannoni 2008; Kalluri & Weinberg 2009). EMT enables cancer cells to disintegrate with the basement membrane and disseminate from the neighboring cells but still sustain the viability of the cell together with gain the ability to invade the surrounding tissues (Yilmaz & Christofori 2009). This transition therefore supports a successful metastasis of the cancer cells. It is widely accepted that EMT is an initial step mediating cancer cells towards high migrative ability and anoikis resistance (Frisch et al. 2013). Anoikis is a programmed cell death induced by cellular detachment from extracellular matrix (ECM) protecting the body from an abnormal cell growth. It has been shown that EMT behavior provides the survival mechanism to suppress metastatic cell anoikis (Chiarugi & Giannoni 2008).

EMT has been observed to be correlated with poor prognosis in colon, bladder and lung cancers (Pasquier et al. 2015; Loboda et al. 2011; McConkey et al. 2009; Voulgari & Pintzas 2009). The process of EMT involves a disassembly of cell-cell

junction, loosing cell polarization, and gaining the fibroblastic phenotype (Kalluri & Weinberg 2009). In order to achieve these morphological changes, cancer cells must undergo some molecular signaling pathways. There are multiple signaling pathways that were recognized as the molecular mechanism underlying this cellular reversed transition. One of the hallmarks of EMT process is the regulation of transcription factor in Snail family and thereby an attenuation of E-cadherin expression which is a major suppressor of invasion and mesenchymal phenotype (Kalluri & Weinberg 2009; Yilmaz & Christofori 2009; Kalluri & Weinberg 2009). E-cadherin expression is mainly regulated by a group of transcription factors in Snail family including Snail and Slug (Sánchez-Tilló et al. 2012; Frisch et al. 2013; Shih & P. C. Yang 2011). These transcription factors act as molecular switch suppressing the expression of E-cadherin by repressing a set of genes that encodes E-cadherin (Lamouille et al. 2014). The Snail family transcription factors can be regulated at both transcriptional and post-translational level (Franco et al. 2010). At the transcriptional level, the translocation of β-catenin into the nucleus where it functions as a transcriptional activator together with T cell factor/Lymphoid Enhancer Factor (TCF/LEF) complex, activates the transcription of Snail and Slug genes resulting in increases in the expression of the transcription factors (Lee & Nelson 2012). Also Snail and Slug transcription factors can be post-translational regulated by degradation pathway. The Snail family transcription factors contain a serine-rich domain (SRD) which can be post-translational modified by phosphorylation. The phosphorylated transcription factors are recognized and interacted by ubiquitin ligase which is responsible for promoting Snail and Slug ubiquitination and proteasome degradation (Lamouille et al. 2014). Therefore, the discoveries of a compound that can interfere with such regulatory cascades of these transcription factors including Snail and Slug could have an impact on the process of EMT.

Gigantol is an herbal bibenzyl compound extracted from *Dendrobium draconis*. It has been reported as a promising naturally derived compound for cancer therapy

due to several cytotoxic effects in cancer cells including breast and lung cancers (Klongkumnuankarn et al. 2015). However, the potential effect of gigantol on the EMT process of non-small cell lung cancer cells has not yet been investigated. Therefore the aim of this research is to examine the mechanistic machinery of gigantol in inhibiting EMT process together with the underlying molecular pathway.

CHAPTER II

LITERATURE REVIEW

1. Lung cancer

Currently, lung cancer remains as the fundamental cause of a high mortality rate of cancer-related death. According to the American cancer society annual report (Figure 1), lung cancer was ranked as the second most diagnosed cancer following prostate cancer in men and breast cancer in women with an estimation of 224,390 new lung cancer were assumed in 2016 which account for 14 % of all cancer incidence. In addition, for the mortality rate, lung cancer was ranked as the first cause of death among all cancer in both male and female with an anticipation of approximately 158,080 more death or about 1 in 4 cancer deaths during 2016 in the United States (Siegel et al. 2014). Therefore any therapeutic discoveries that can improve the well being of lung cancer patients are in high demand.

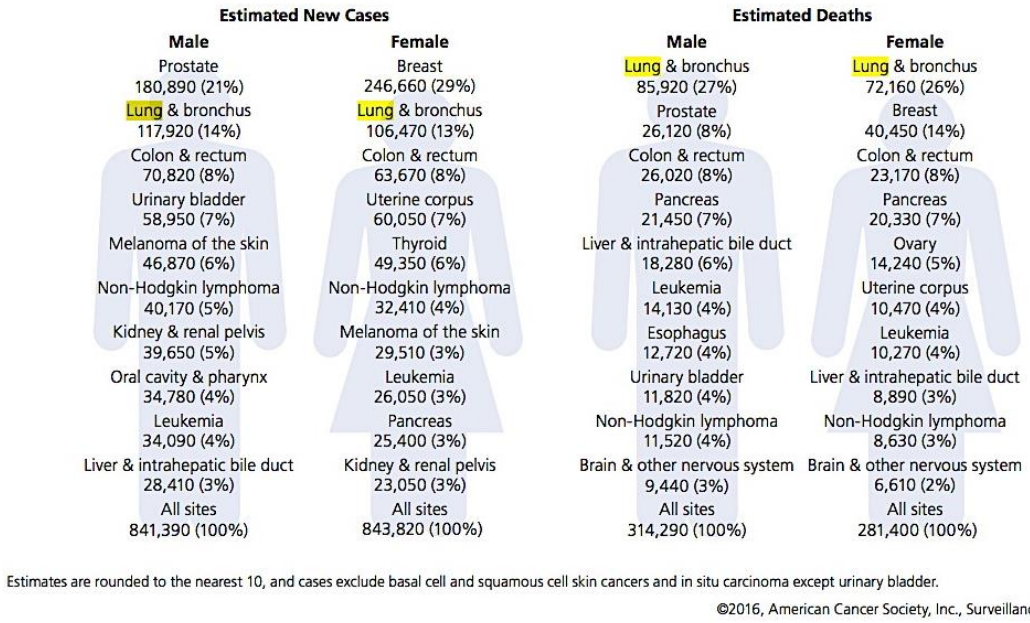


Figure 1 Cancer statistics (American cancer society, 2016)

In order to gain a better understanding of lung cancer, American Cancer Society has classified lung cancer cell type into 2 major groups, the small cell lung cancer

(SCLC) and non-small cell lung cancer (NSCLC). Among the 2 types of cancer cells, non-small cell lung cancer (NSCLC) was accused for more than 85 % of lung cancer patients (Shih & P. C. Yang 2011). Moreover, it was reported that NSCLC is highly associated cigarette smoking, often results in a negative response to chemotherapy and usually shows no symptoms until the disease is well advance. Therefore it is reasonable to examine the cellular behavior of the NSCLC cell lines to predict the clinical outcome in lung cancer patients.

Regarding to the studies in cancer disease, even though the treatment of lung cancer has been highly developed in the past recent years, the survival rate of the patients was not as well improved. Only 17 % of lung cancer patients survived a 5-year period after the cancer diagnosis (American cancer society, 2016). The cancer annual report has found that from 2008 to 2012, the mortality rate only decreased by 2.9 % in men and 1.9 % in women per year. This is due to the poor prognosis and the relapse of the disease resulting from metastasis.

2. Metastasis

Metastasis is the ability of cancer cell to spread from one organ to another organ composing of multiple steps. Although metastasis is a common complication in cancer disease, it was known that once cancer cells have metastasized to form the secondary tumor, the cancer will be a lot more aggressive and almost impossible to cure (Chambers et al. 2002). Tumor progression is resulted from an accumulation of genetic alterations from the cancer microenvironment inducing tumor expansion at the primary site forming into malignant lesions (Voulgari & Pintzas 2009). A further buildup of genetic alterations causes tumor expansion at the primary site and finally drives cancer dissemination into metastasis in order to invade other organs for the formation of secondary tumor.

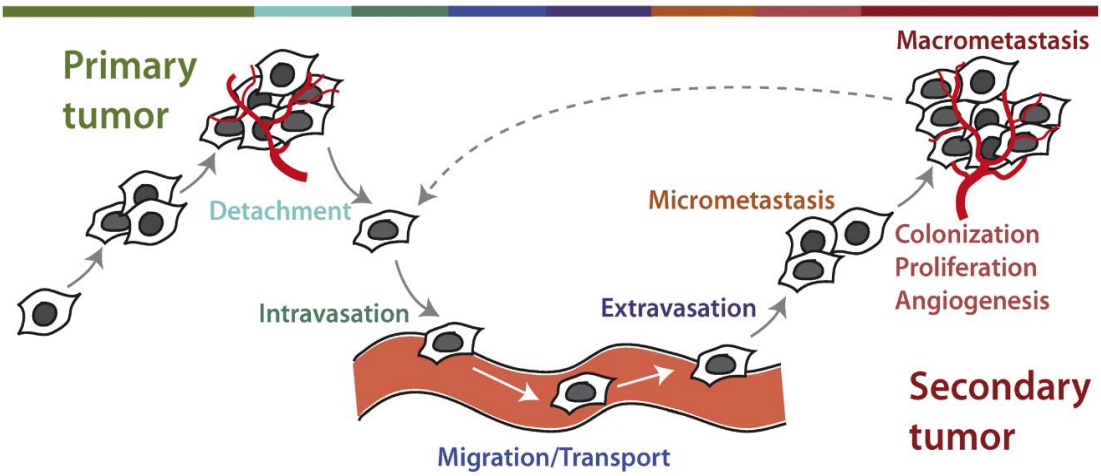


Figure 2 Cancer metastasis (Divoli et al. 2011)

Metastasis involves many cellular steps (Figure 2) starting at the primary site where cancer cells proliferate uncontrollably until a certain point is reached. Then cancer cell is disseminated from the primary site to invade through extracellular matrix to reach the blood or lymph vessel before intravasate into the vessel. At this stage, cancer cells will travel along with the blood or lymph to the distanced tissues. When the cancer cells reach a new suitable location, they will escape from the vessel and invade into the new organ or tissue to produce the secondary tumor (Divoli et al. 2011). It was claimed that secondary tumor is a lot more aggressive than the primary tumor and is the main reason behind the constant death rate in lung cancer. (Eccles & Welch 2007; Geiger & Peeper 2009).

Lung cancer cell lines are commonly used to study the behavior of cancer cell *in vitro*. NCI-H460 (ATCC HTB-177) is a non-small cell lung cancer cell line derived from the pleural effusion of a lung cancer patient which is used to represent a subpopulation of cancer cells that have the ability to metastasize. On the other hand, A549 (ATCC CCL-185) is another type of non-small cell lung cancer cell line. A549 was collected from the lung tissue of a lung cancer patient representing the population of cancer cell that has not yet metastasized. In order to establish an *in vitro* experiment

on metastasis behavior of cancer cells, it is reasonable to use these 2 non-small cell lung cancer cell lines in the study.

3. Epithelial to mesenchymal transition (EMT)

Epithelial-to-mesenchymal transition or EMT is a multistep process of cellular biochemical changes from squamous epithelial cell morphology into spindle-like mesenchymal phenotype. The process of EMT naturally occurs during embryonic development where the epithelial precursors transdifferentiate to complete gastrulation or to form neural crest (Baum et al. 2008). These morphogenesis processes requires the differentiated epithelial cells to regain their migrative fibroblastic phenotypes in order to travel a long distance to build other tissues. Recently increasing evidence has been reported that EMT also occurs in metastatic cancer cells as one of the most essential mechanisms to escape anoikis and facilitate cell movement (Floor et al. 2012; Chanvorachote 2013). In contrast, it was reported that an inhibition of EMT process leads to a reduction on cell viability due to the down-regulation of survival pathways and the up-regulation of apoptotic pathways (Geiger & Peeper 2009; Floor et al. 2012).

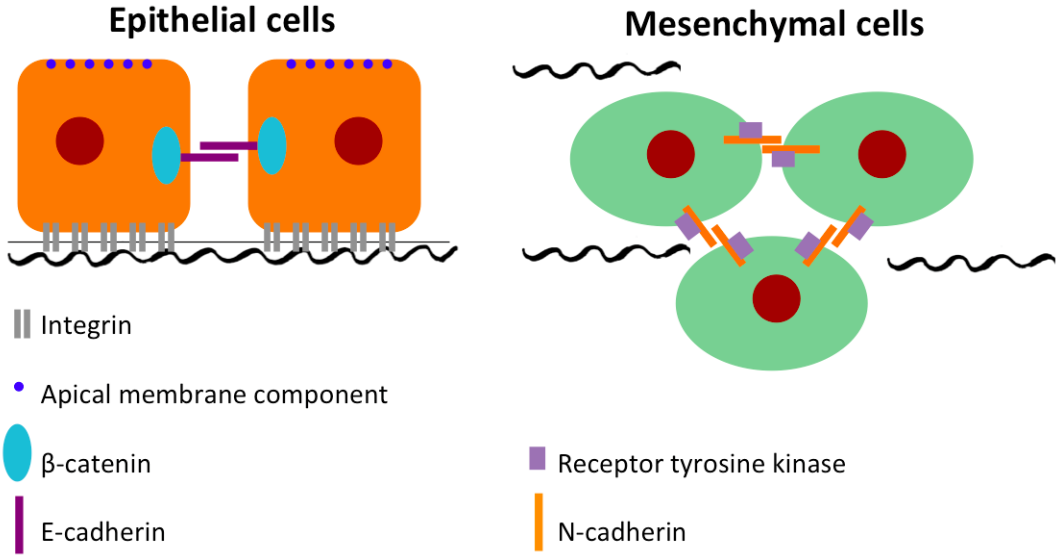


Figure 3 Epithelial and mesenchymal morphologies

There are some modifications in biological molecules that have been claimed as the hallmark of EMT process. A down regulation of E-cadherin together with and up regulation of N-cadherin expression indicates the classical conversion of epithelium into mesenchyme (Kalluri & Weinberg 2009). E-cadherin is the major molecular component in establishing stable epithelial cell-cell adhesions including desmosome, adheren junction, and tight junction (Miyoshi & Takai 2008). These intercellular junctions allow communication between cells, restrict mobility of the epithelial tissue, and preserve the apico-basal polarization (Figure 3). The down regulation of E-cadherin results in the dissemination of epithelial cell architecture by disrupting the apico-basal polarization and promoting the front-rear polarization supporting the migratory phenotype (Moreno-Bueno et al. 2008). In addition, the loss of E-cadherin also contributes to liberating of protein complex attaching to the cytoplasmic domain of E-cadherin including p120 catenin. Free p120 catenin accumulation increases cell survival and migrative behaviors (Yilmaz & Christofori 2009). On the other hand, N-cadherin is another adhesive cadherins found in mesenchymal cells permitting a binding affinity of other types of mesenchymal cells such as fibroblasts, vascular endothelial cells, and nervous tissue (Figure 3). N-cadherin also forms a complex at the intracellular domain called neural cell adhesion molecule (NCAM). This complex permits N-cadherin to interact with modulate the activities of receptor tyrosine kinases (RTKs) such as platelet-derived growth factor receptor (PDGF) and fibroblast growth factor receptor (FGF) resulting in a sustained in survival mitogen-activated protein kinases (MAPK) pathways activation and increased cell motility (Williams et al. 2001). An up regulation of N-cadherin expression has been observed to correlate with an aggressiveness in different types of cancer (Peinado et al. 2004). This cadherin switching occurrence during EMT allows cancer cells to gain higher chance to survive in an attached-free condition together with the ability to migrate and metastasize away from the primary tumor site (Geiger & Peeper 2009; Voulgari & Pintzas 2009; Kalluri &

Weinberg 2009). Moreover, vimentin is an intermediate filament found in most mesenchymal cells and is required for migration (Sabbah et al. 2008; Shi et al. 2013). Vimentin can tolerate high stress of the traction force during cellular movements. The expression of Vimentin has been found to correlate with EMT incidence and cancer progression (Heatley et al. 1993). Thus, vimentin was also claimed as a cytoskeleton maker for EMT process.

4. EMT transcription factors

Even though EMT is a highly complex process involving many cellular changes, the process is regulated by transcription factors including Snail family, Homodimeric and heterodimeric basic helix-loop-helix (bHLH), and zinc-finger E-box-binding (ZEB). These transcription factors function together to suppress the epithelial genes and induce the mesenchymal phenotypes (Lamouille et al. 2014).

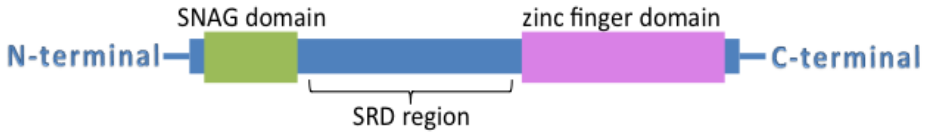


Figure 4 Structure of Snail family transcription factor

The Snail family proteins found in mammal include Snail (Snail1, SNAI1) and Slug (Snail2, SNAI2). These transcription factors share similar structure with highly conserved C-terminal domain containing C_2H_2-type zinc finger that bind to the E-box motif of the DNA in the target gene promoter (Figure 4). The N-terminal domain of Snail family transcription factors contains a SNAG domain which is important for recruiting other co-repressor enzyme which can lead to transcriptional repression of the target gene (Wang et al. 2013). The central domain of Snail and Slug transcription factors contains a serine-rich domain (SRD) which is responsible of protein stability. The expression of Snail and Slug transcription factors are mainly regulated through both the transcriptional and degradation pathways. The *in vivo* has confirmed that the up regulation of these transcription factors results in invasive and mesenchymal-like

phenotype in cell line and poor clinical prognosis (Shih & P. C. Yang 2011). Moreover, lung cancer metastasis has been most reported with the requirement of Snail family protein up regulation (Peinado et al. 2007). These Snail family members function as transcriptional repressor of EMT-related protein including E-cadherin and also have been reported to be able to regulate the apoptosis related protein at transcriptional level leading to a less susceptibility to apoptosis (Franco et al. 2010).

In addition to transcriptional repressor of E-cadherin, Snail family transcription factors also function as transcriptional activator of the bHLH and ZEB family transcription factors to further regulate the EMT process. bHLH transcription factor including E12, E47, and Twist can be activated through dimerization to regulate the E-box DNA binding and was known to be the major inducer of N-cadherin (Lamouille et al. 2014). ZEB activation normally depends on activation of Snail and Slug. ZEB in turn acts as a second repressor complex that further inhibits E-cadherin expression (Yilmaz & Christofori 2009). Nevertheless, the changes in the expression of Snail family transcription factors occur earlier in the EMT process and the Snail family transcription factors leads to activation of other transcription factors associated with EMT process. Therefore, it can be concluded that Snail family transcription factors are the key molecular target for EMT process.

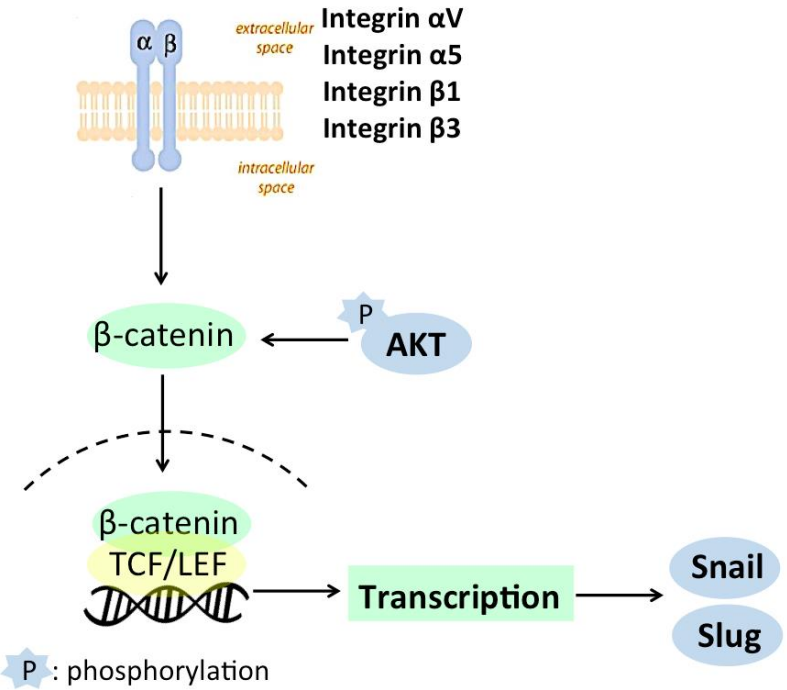


Figure 5 The Snail family transcription factors transcriptional regulation

An increase in the Snail family transcription factor expression can be induced by integrin dependent pathway (Figure 5). Based on previous knowledge that changes in cancer microenvironment can be a major factor for cancer aggressiveness and metastasis. The most well studied cell surface receptor connecting the cell to the extracellular environment is the integrins family protein. Integrins are the transmembrane glycoprotein binding to the extracellular matrix component and have the intracellular domain serving as signaling dock regulating cellular activities such as cell proliferation, cell migration, and cell survival-apoptosis (Chiarugi & Giannoni 2008). The functional integrins are heterodimers of non-covalently binding of α and β subunits. There are 18 α and 8 β subunits found in vertebrates making 24 different receptors with various binding properties leading to diverse cellular pathways activation (Campbell & Humphries 2011). The activation of integrin leads to an increase in the stability of β -catenin. In non-transformed epithelial cells, β -catenin is interacted with the cytoplasmic domain of E-cadherin forming the intracellular complex and subjected to degradation. However, during EMT process, β-catenin is accumulated in

the cytoplasm and translocated into the nucleus where it interacts with the T cell factor (TCE/LEF) complex and functions as a transcriptional activator to elevate the expression of the Snail and Slug resulting in EMT induction. An up regulation of integrin αV β3 was reported to promote the stress fiber formation and associate with an up regulation of EMT-transcription factor which further support cancer metastasis (Knowles et al. 2013). Moreover, an increase in α5 β1 is associated with cell migration and cellular plasticity (Huttenlocher & Horwitz 2011; Collo & Pepper 1999). These changes in integrins expression could be the key modulator in EMT initiation process. In addition, the cytoplasm accumulation of β-catenin was also driven by the activated ATP-dependent tyrosine kinase (AKT) (Fang et al. 2007). The activated AKT causes a dissociation of β-catenin from the cadherin complex before translocating into the nucleus to bind with the target gene. Therefore, the activity of β-catenin can be modified by both integrin dependent pathway and AKT dependent pathway, and consequently govern the transcription factor Snail and Slug

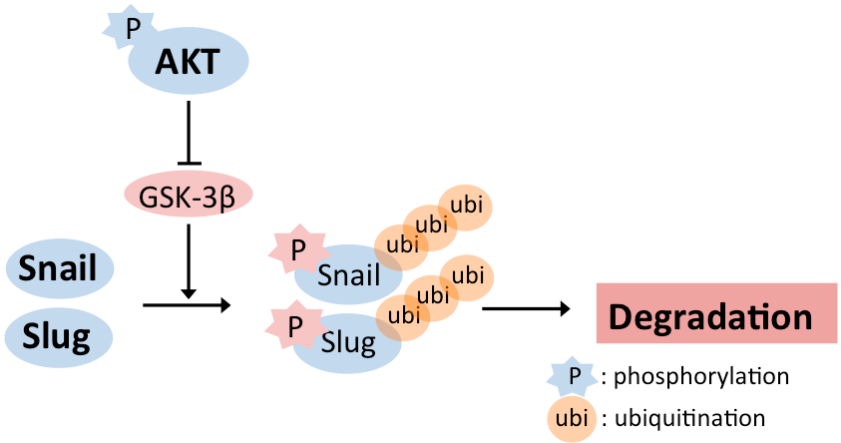


Figure 6 The Snail family transcription factor degradation pathway

On the other hand, the expression of Snail and Slug transcription factors can also be regulated through the degradation pathway (Figure 6). The SRD region in the central domain of these transcription factors can be phosphorylated by Glycogen synthase kinase-3β (GSK-3β) (Wang et al. 2013). The phosphorylated Snail and Slug are interacted with ubiquitin ligase which are then subjected to proteasome degradation

(Wu et al. 2008). An increase in the rate of the degradation, resulting in an enhancement of E-cadherin expression could inhibit the EMT process in cancer cells and further suppress cancer metastasis. However, the stability of GSK-3β can be influenced by the activity of AKT. It was reported that AKT is able to reduce the active form of GSK-3β resulting in a decrease in Snail and Slug degradation (Lamouille et al. 2014).

5. Gigantol

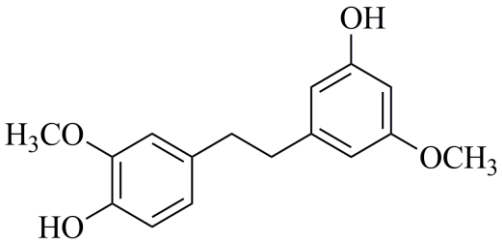


Figure 7 Chemical structure of gigantol

Gigantol (Figure 7), a 1,2-diphenylethane or bibenzyl compound, is a compound of interest in cancer research due to promising pharmaceutical activities such as anti-proliferation and anti-migration (Klongkumnuankarn et al. 2015; Charoenrungruang, Chanvorachote & Sritularak 2014a; Charoenrungruang, Chanvorachote, Sritularak & Pongrakhananon 2014b). Natural compounds with bibenzyl structure have been extensively studied including moscatilin and resveratrol. Moscatilin was reported with an anti-motility activity in lung cancer cell through a suppression of endogenous reactive oxygen species (Kowitdamrong et al. 2013). Also the *in vivo* study has proven that resveratrol could reduce tumor size and inhibit metastasis in lung cancer (Kimura & Sumiyoshi 2016). Therefore, it is highly possible that the bibenzyl structure of gigantol may be the key structure for the biological activities. Gigantol is commonly found in many therapeutic orchids including *Dendrobium draconis* or 'Ueang ngoen' which was found in the northern region of Thailand. The effect of Gigantol on anoikis and EMT processes are still not yet clarified. With the anti-migration property in lung cancer cell line of gigantol, it is possible that gigantol may have the ability to attenuate the

upstream process of EMT. The result of this study would provide information regarding EMT suppression effect of gigantol as well as the mechanism of action supporting the development of the compound as an anticancer agent.

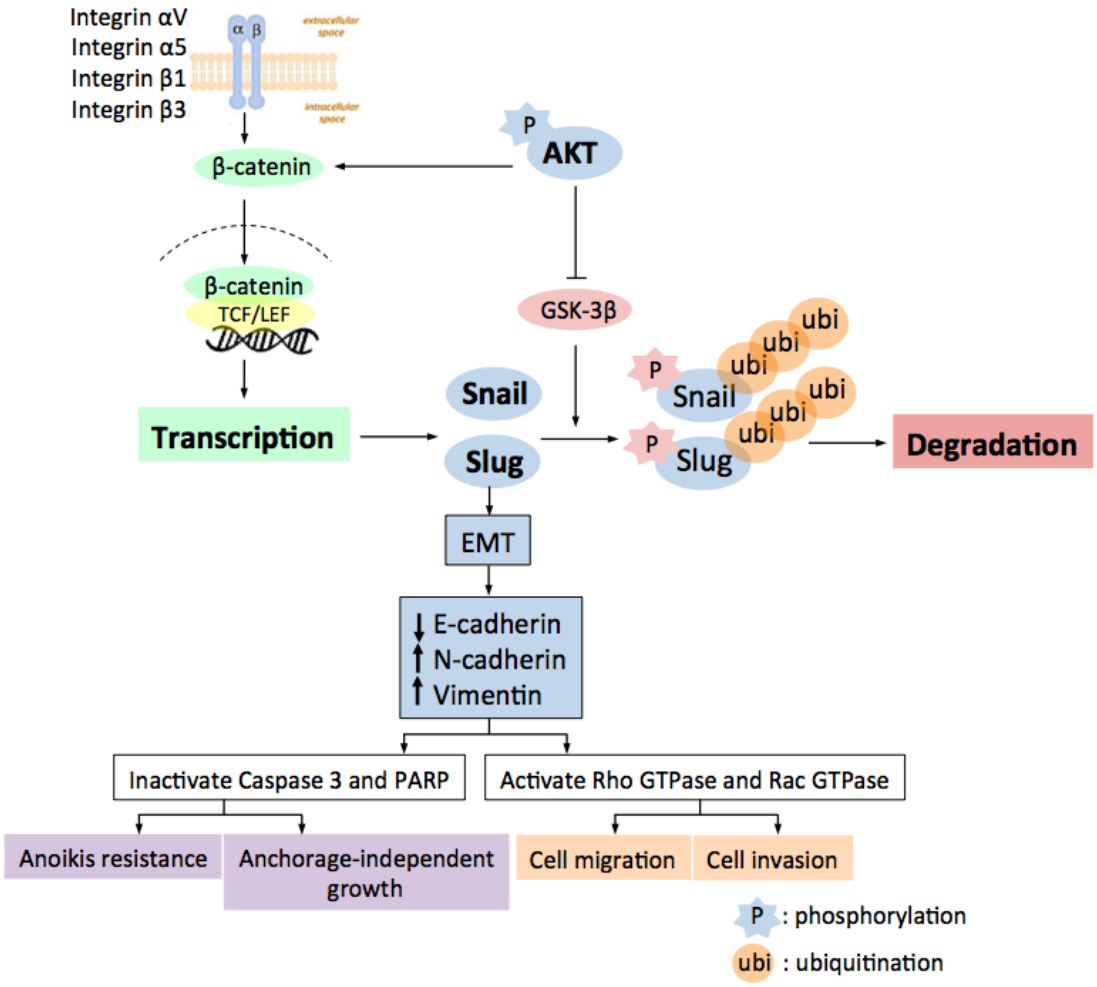


Figure 8 Pathway summary

CHAPTER III
MATERIALS AND METHODS

Materials

Human non-small cell lung cancer cell lines including H460, A549 and human dermal fibroblast cell (PCS-201-010) were obtained from the American Type Culture Collection ATCC (Manassas, VA). Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's Modified Eagle Medium (DMEM) FBS, L-glutamine, penicillin/streptomycin, phosphate-buffered saline (PBS), trypsin, and EDTA were purchased from GIBCO (Grand Island, NY). DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, Propidium iodide (PI), bovine serum albumin (BSA), and antibody for ubiquitin were purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibodies for Rho GTP and Rac GTP were purchased from NewEast Bioscience (King of Prussia, PA, USA). Antibodies for N-cadherin, E-cadherin, Vimentin, Slug, β-catenin, phosphorylated AKT (Ser473), AKT, phosphorylated GSK-3β (Ser9), GSK-3β, GAPDH, and peroxidase-conjugated secondary antibodies were purchased from Cell Signaling (Denvers, MA, USA).

Methods

Cells culture

H460 cells were cultured in RPMI medium supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin. A549 and fibroblasts were cultivated in DMEM supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin. All cells were cultured at 37°C in a humidified incubator with 5 % CO_2 and passaged at near confluence with Trypsin-EDTA.

Treatment preparation

Gigantol was isolated from *Dendrobium draconis* as previously described (Sritularak et al. 2011). Gigantol used in this study has more than 95 % purity determined by HPLC and NMR spectroscopy. Gigantol was prepared in dimethyl sulfoxide (DMSO) for stock solution and PBS was used to dilute into working concentrations. The final concentration of DMSO used in all of the experiments was 0.1 %. The results from the treatment groups were compared with the untreated control exposed to the 0.1 % final concentration of DMSO.

Cytotoxicity and Cell Proliferation assays

Cell viability and cell proliferation were examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric assay that tests the metabolic activity of mitochondria. Cells were seeded in 96-well plates at 10,000 and 2,000 cells/well for cytotoxicity and cell proliferation, respectively. After exposure to the gigantol treatments at doses 0-50 μM for indicated times, the serum was removed and 100 μl of MTT solution was added to each well. Then the plates were incubated for an additional 4 h at 37°C. After removing the supernatant, 100 μl of DMSO was added to dissolve the formazan crystals. The absorbance at 570 nm was measured using a microplate reader. The data were presented in relative cell viability as absorbance of the treatment group divided by absorbance of the control group.

Apoptosis Assay

To examine nuclear condensation during cellular apoptosis, Hoechst 33342/PI nuclear staining assay was carried out. After the similar treatments, cells were washed by PBS and incubated with 2 μg/ml Hoechst 33342 and 1 μg/ml PI for 30 min. Hoechst 33341 staining showed nuclear condensation and fragmentation in cell undergoing apoptosis and necrosis cell were stained by PI dye. Cells were then photographed and analyzed using a fluorescence microscopy (Olympus IX51). The data were presented

in % apoptosis as number of Hoechst-positive cells divided by number of total cells. The non-apoptosis doses were used in further experiments.

Anoikis Assay

To investigate the cell mortality in a detached condition, the anoikis assay was performed. After 24 h treatment of gigantol at non-apoptotic doses, lung cancer cells in the culture plate were detached and made into a single-cell suspension in serum free medium. Then the suspended cells were seeded into a poly-HEMA coated plate at a density of 1.5 X 10^5 cells/ml. The suspended cells were then be harvested at 0, 3, 6, 9, 12, and 24 h. Cell viability of each sample was analyzed using XTT assay according to the manufacturer's protocol. The cytotoxicity index was calculated by dividing the absorbance of the treated cells by that of the control cells.

Anchorage-Independent Growth Assay

For anchorage-independent growth, the cell growth was determined by soft agar colony-formation assay. After 24 h treatment of gigantol at non-toxic concentrations, the bottom layer of soft agar was prepared by using a 1:1 mixture of serum free medium and 1 % of agarose gel making the final concentration of agarose as 0.5 %. The mixture was allowed to solidify in a 24-well plate to form a bottom layer. After that an upper cellular layer containing suspended cells at concentration of 3 x 10^3 cells/ml in the agarose gel mixed with 10 % FBS and 0.3 % agarose was added on top of the bottom layer. After the upper layer was solidified, medium containing 10 % FBS and gigantol at various concentrations (0-20 μM) were added to the system and maintained at 37°C. Colony formation was determined after apporoximately 2 weeks using a phase-contrast microscope (Olympus 1X51 with DP70). Relative colony number and diameter were determined by dividing the values of the treated cells by those of the control cells.

Cell Scratch assay

To evaluate the effect of the treatment on cell migration, wound healing assay was performed. Lung cancer cells were seeded in the 24-well plate at a density of 2.5 x 10^5 cells/well, then the cells were allowed to form a monolayer at the bottom of the plate for an overnight. After that a wound space was created by a micropipette tip, then the excess cells were washed twice using PBS and replaced with serum free medium containing non-toxic concentrations of gigantol. The migrated cells closing up the wound space were photographed using an inverted microscope at 0, 24, 48, and 72 h. Then the wound size was analyzed by dividing the difference of the wound space of the sample by the wound size of the control group in each experiment.

Migration and Invasion assay

To detect the cell migration in three dimensional scale together with cell invasion, the transwell chamber was used. The lung cancer cells were seeded on the upper chamber of a 24-transwell plate with 8 μM pore at a density of 5 x 10^4 cells/well in the medium containing 0.1 % FBS. In the lower chamber medium with 10 % FBS was added. After incubating for 24, 48, 72 h, the left over cells on the upper chamber were removed and the migrated cell in the lower chamber were stained with 2 μg/ml Hoechst 33342 for 30 min. The Hoechst staining cells were photographed and analyzed using fluorescence microscopy. For the three dimensional scale migration, the filter between upper and lower chamber was not coated with Matrigel while Matrigel-coated filter was used to evaluate cell invasion.

Western blot Analysis

To detect differences in protein expressions in response to gigantol treatment, the gigantol treated cells were washed twice by PBS buffer before exposed to lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 % Triton X-100, 150 mM sodium chloride, 10 % glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail for 1 h at 4°C. The cell

lysate was separated by centrifuging at 12,000 rpm for 3 min at 4°C and the supernatant was collected as protein sample. Protein concentration was measured by the Bradford assay kit (Biorad Laboratories, Hercules, CA). Protein from each sample was denatured by heating at 95°C for 5 min prior to the gel electrophoresis. Then protein samples were separated by molecular weight using precast 5-10 % gradient SDS-PAGE gel and transferred on to nitrocellulose membranes. After blocking with 5 % skim milk for 1 h, the membranes were incubated with the indicated primary antibodies at 4°C overnight. After that the membranes were washed thoroughly with TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05 % Tween 20), then they were incubated with horseradish peroxide-conjugated secondary antibodies for an additional an hour at room temperature. Subsequently, the bands were then visualized using a film exposure with a chemiluminescence detection system and analysed with analyst/PC densitometry software by Image J.

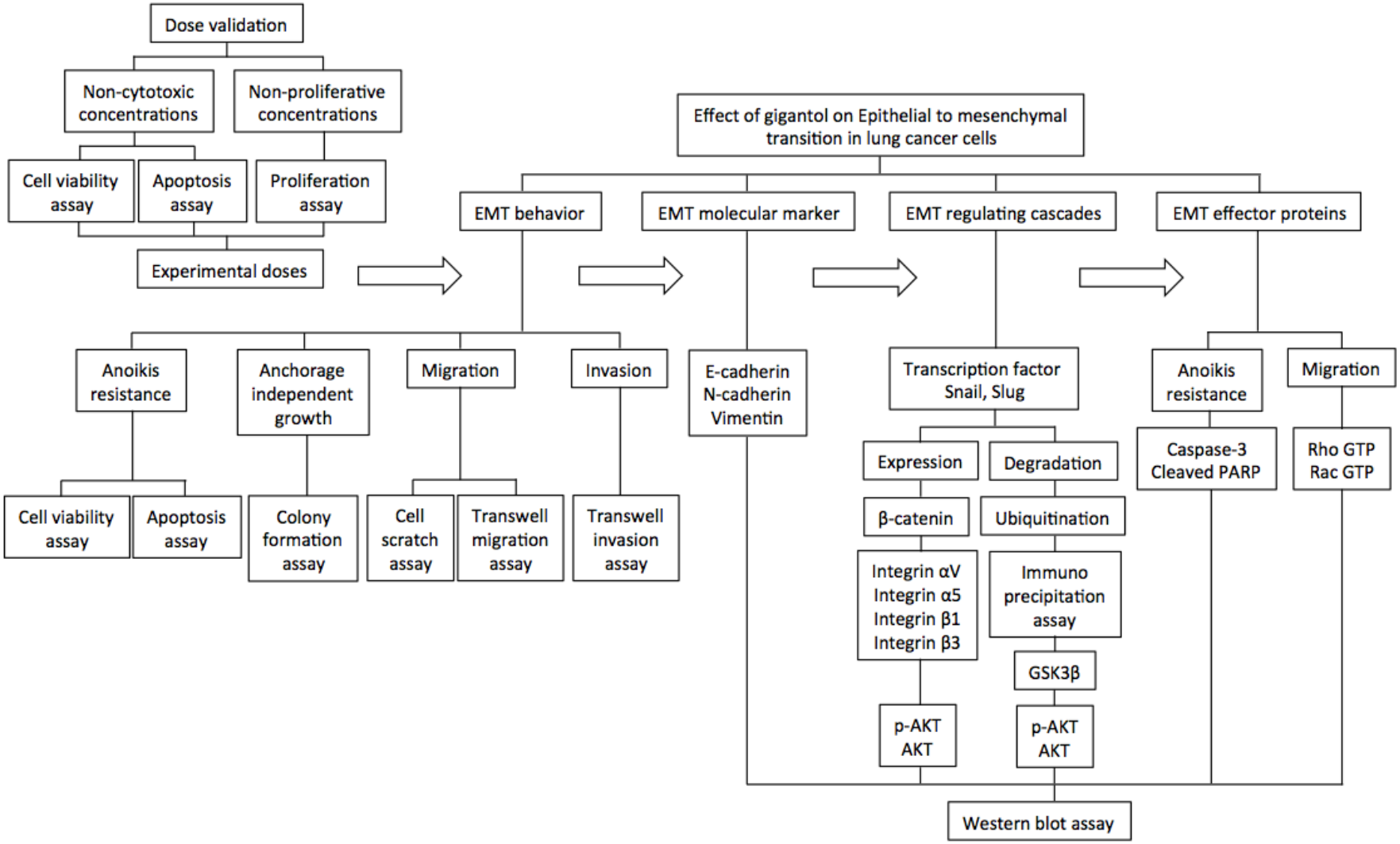
Immunoprecipitation Assay

To detect the ubiquitin protein complex, the immunoprecipitation assay was performed. Lactacystin was pretreated an hour prior to the gigantol treatment. After the treatment of gigantol, cells were lysed in a lysis buffer for 45 min at 4°C. The cell lysate was separated by centrifuging at 12,000 rpm for 3 min at 4°C and the supernatant was then collected. After that the cell lysate was pre-clear with agarose bead to prevent an unspecific binding. Then the remaining cell lysate was subjected to protein measurement by the Bradford assay kit (Biorad Laboratories, Hercules, CA). Next the specific antibody was added to bind with the specific protein for overnight at 4°C. Then the protein-antibody complex was incubated with agarose beads (Santa cruz) for 3 h at 4°C. The protein-antibody-bead complex was washed with lysis buffer and resuspended in 2x Laemmli sample buffer, then heated at 95°C for 5 min. After that protein samples were separated using precast 5-10 % gradient SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membranes. Then the Western

blot analysis was done using an anti-ubiquitin antibody. The result from this immunoprecipitation assay showed the amount of specific protein that is binding with ubiquitin as a complex for degradation.

Statistical Analysis

Data were expressed in mean ± standard deviation (S.D.) form at least four independently performed experiments. Differences between treatments were examined using the one-way analysis of variance (ANOVA) followed by post hoc test. *p*-values less than 0.05 was considered statistically significant.


Dose validation
Non-cytotoxic concentrations
Non-proliferative concentrations
Cell viability assay
Apoptosis assay
Proliferation assay
Experimental doses
Effect of gigantol on Epithelial to mesenchymal transition in lung cancer cells
EMT behavior
EMT molecular marker
EMT regulating cascades
EMT effector proteins
Anoikis resistance
Anchorage independent growth
Migration
Invasion
E-cadherin
N-cadherin
Vimentin
Transcription factor
Snail, Slug
Anoikis resistance
Migration
Cell viability assay
Apoptosis assay
Colony formation assay
Cell scratch assay
Transwell migration assay
Transwell invasion assay
Expression
Degradation
Caspase-3
Cleaved PARP
Rho GTP
Rac GTP
β-catenin
Ubiquitination
Integrin αV
Integrin α5
Integrin β1
Integrin β3
Immuno precipitation assay
GSK3β
p-AKT
AKT
p-AKT
AKT
Western blot assay

CHAPTER IV
RESULTS

Dose validation of gigantol compound

The effect of gigantol on H460 and A549 cells cell viability

To investigate cytotoxic effect of gigantol in lung cancer cells, cell viability was determined after the treatment. The non-small cell lung cancer cell lines used in this study include H460 and A549. Regarding to the American Type Culture Collection (ATCC), H460 is metastasized adenocarcinoma cell line derived from pleural fluid whereas A549 cell line represents cancer cell from the primary site. Cells were incubated with various concentrations of gigantol (0-50 μM) for 24 h, then the cell viability was measured by MTT assay. Figure 9a and 10a indicated that the cytotoxicity in H460 and A549 cells were detected with 50 μM of gigantol, whereas no detectable change was observed in both lung cancer cells treated with lower doses of gigantol (0-20 μM). The apoptosis assay also confirmed that gigantol at 0, 1, 5, 10 and 20 μM could not induce cells to undergo apoptosis (Figure 9c and 10c). Gigantol at 50 μM caused sign of nuclear condensation of apoptosis process at approximately 15 % and 20 % of H460 and A549 cells, respectively (Figure 9b and 10b). The PI-positive necrotic cells were not detectable in any treatments of gigantol. Together, these data suggested that gigantol at concentrations of 0-20 μM caused no cytotoxic effect in both cell types.

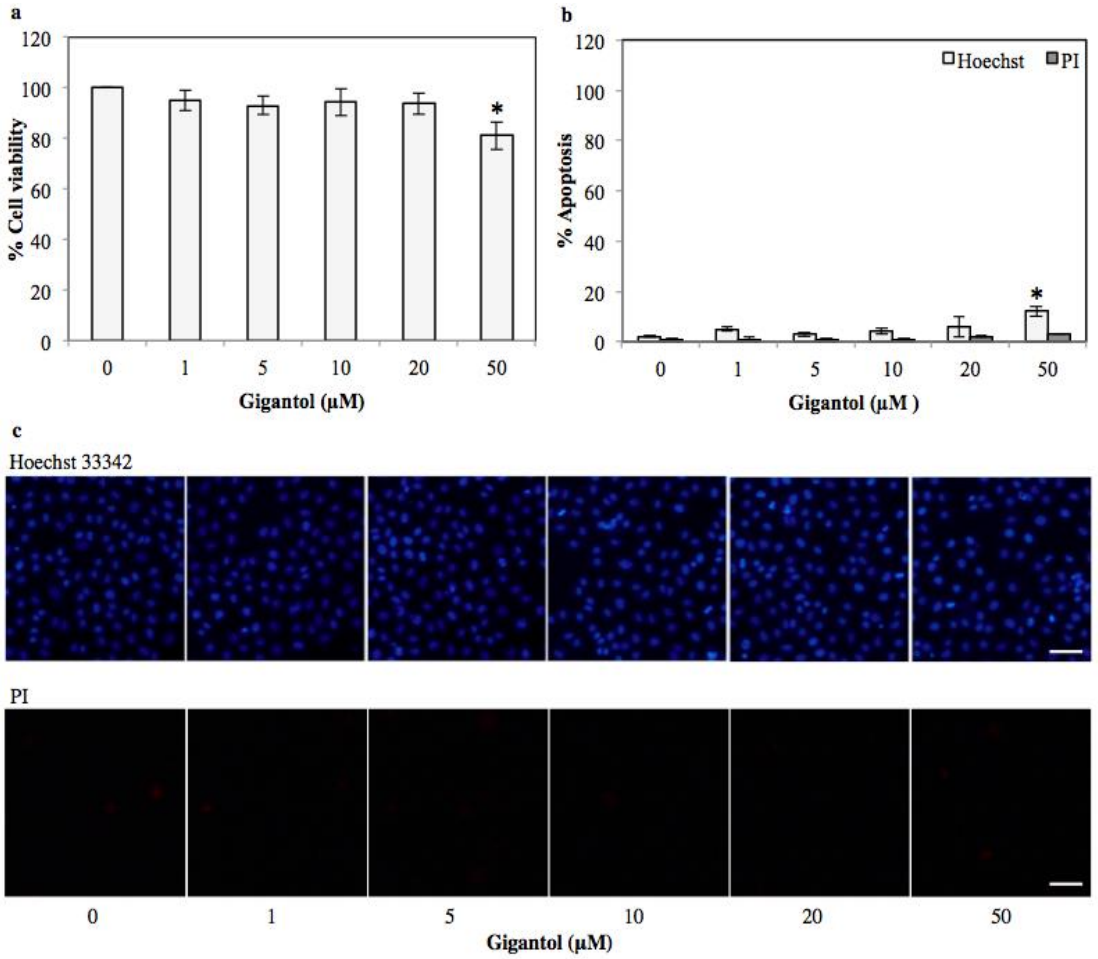


Figure 9 The effect of gigantol on H460 cell cytotoxicity. **a** H460 cells were treated with various concentrations (0-50 μM) of gigantol for 24 h, and cell viability was measured by the MTT assay. The viability of untreated control cells was represented as 100%. **b** H460 cells were treated with various concentrations (0-50 μM) of gigantol for 24 h, and apoptotic and necrotic cell death was evaluated using Hoechst 33342 nuclear and propidium iodide (PI) staining dye. The percentages of cells undergoing apoptosis were calculated comparing to the untreated control cells. **c** The fluorescence images were captured after nuclear staining assay (*scale bar* is 50 μm). The data represent mean ± SD (n=4). *$p < 0.05$ versus untreated control cells.

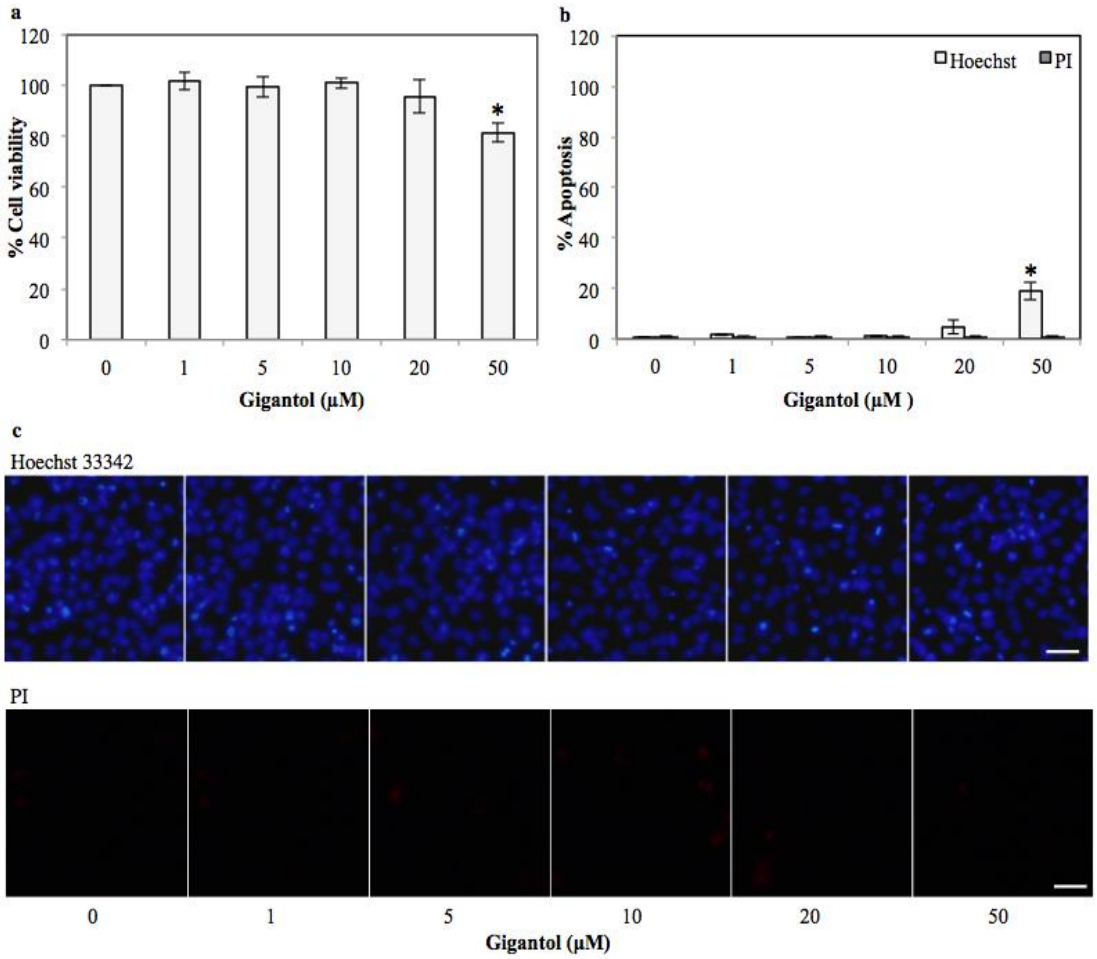


Figure 10 The effect of gigantol on A549 cell cytotoxicity. **a** A549 cells were treated with various concentrations (0-50 µM) of gigantol for 24 h, and cell viability was measured by the MTT assay. The viability of untreated control cells was represented as 100%. **b** H460 cells were treated with various concentrations (0-50 µM) of gigantol for 24 h, and apoptotic and necrotic cell death was evaluated using Hoechst 33342 nuclear and propidium iodide (PI) staining dye. The percentages of cells undergoing apoptosis were calculated comparing to the untreated control cells. **c** The fluorescence images were captured after nuclear staining assay (*scale bar* is 50 µm). The data represent mean ± SD (n=4). *$p < 0.05$ versus untreated control cells.

The effect of gigantol on fibroblasts cell viability

In order to verify the possible cytotoxic effect on normal cells with mesenchymal cell behavior and high migrating activity, fibroblasts were also exposed

to the similar treatment. According to Figure 11, it was found that there is no significant difference in the cell viability of fibroblast in response to gigantol. The non-cytotoxic concentrations of gigantol at 0-20 μM were used in further experiments.

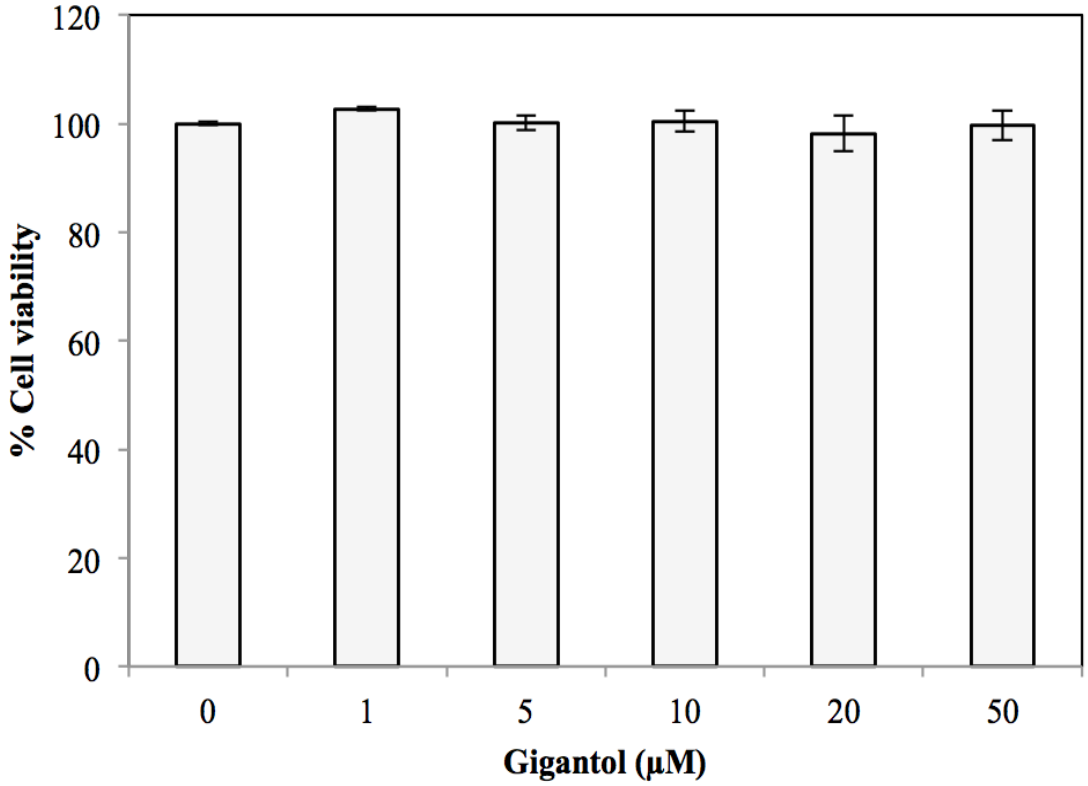


Figure 11 The effect of gigantol on fibroblast cytotoxicity. Fibroblasts were treated with various concentrations (0-50 μM) of gigantol for 24 h, and cell viability was measured by the MTT assay. The viability of untreated control cells was represented as 100%. The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

The effect of gigantol on H460 and A549 cells proliferation

To ensure that the concentrations of gigantol used in this study did not show the proliferative effect in lung cancer cells. The proliferation assay was done using MTT detecting changes in cell viability. Lung cancer cells were seeded at a low density, then they were allowed to proliferate for 24, 48, and 72 h. Figure 12 and 13 demonstrate that gigantol treatment did not significantly decrease cell proliferation in both H460 and A549 cells under attached condition. These results indicated that the

concentrations of gigantol used in this study did not cause any anti-proliferation effect that might affect the results from the further experiments.

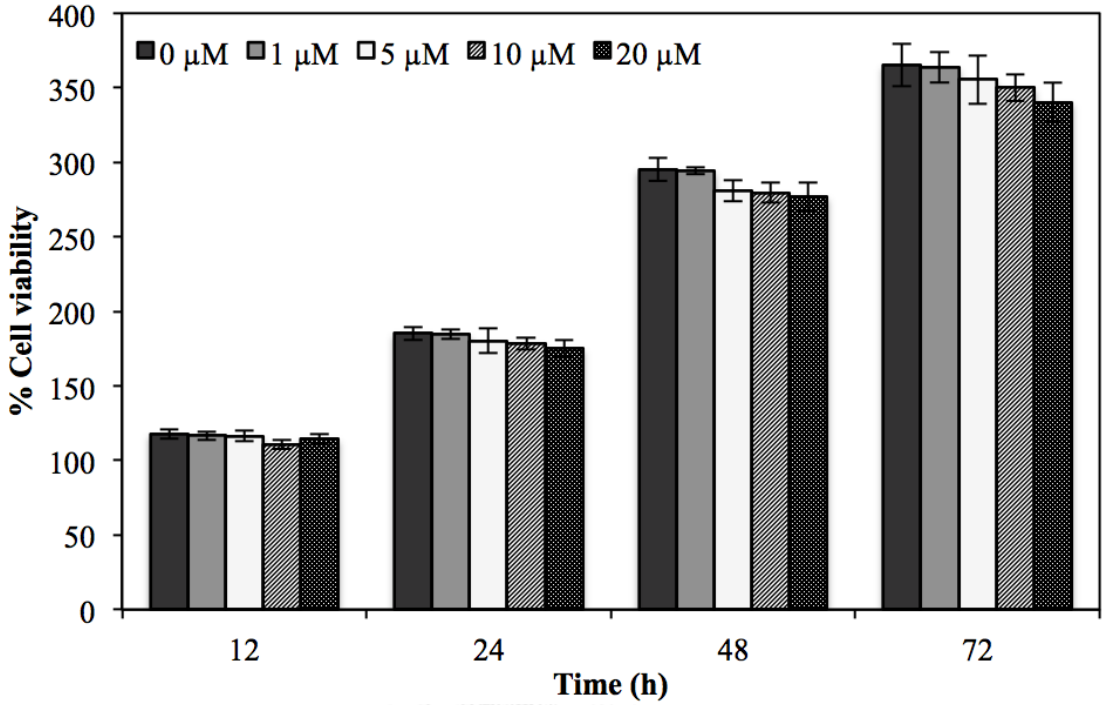


Figure 12 The effect of gigantol on H460 cell proliferation. Attached H460 cells were treated with gigantol (0-20 µM) for 12, 24, 48, and 72 h. Cell proliferation was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The viability of untreated cells at 0 h was represented as 100%. The data was presented as mean ± standard deviation (S.D.).

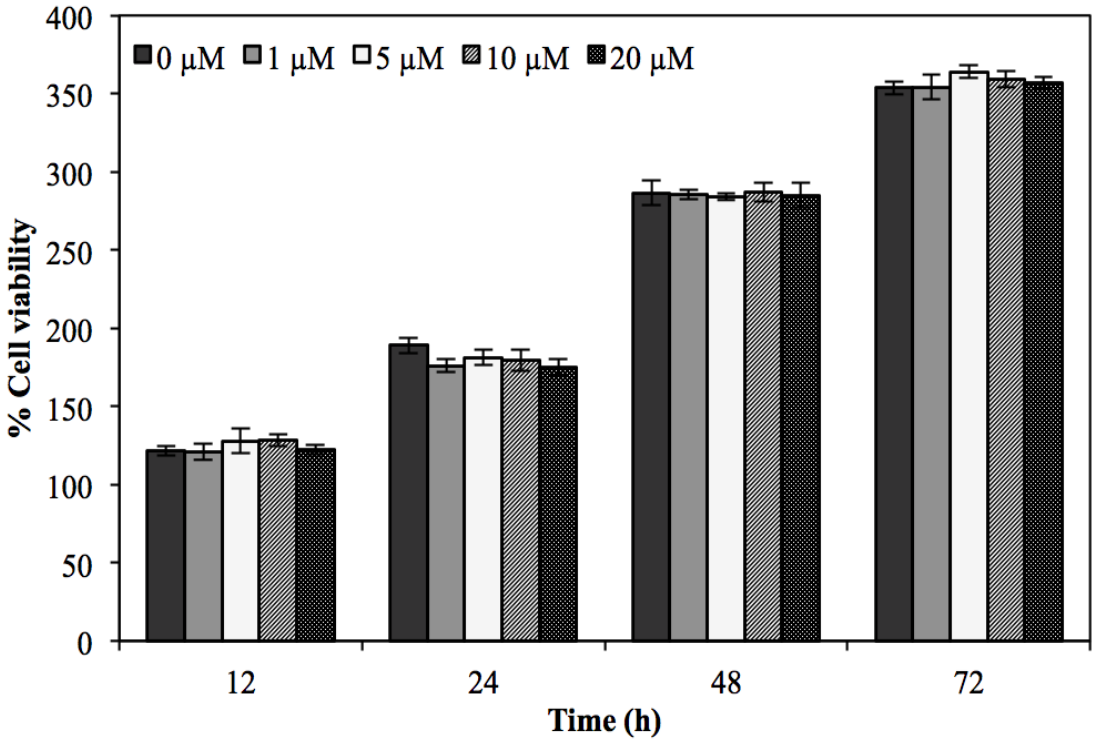


Figure 13 The effect of gigantol on A549 cell proliferation. Attached H460 cells were treated with gigantol (0-20 µM) for 12, 24, 48, and 72 h. Cell proliferation was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The viability of untreated cells at 0 h was represented as 100%. The data was presented as mean ± standard deviation (S.D.).

The effect of gigantol on EMT behavior

The effect of gigantol on H460 and A549 cells anoikis resistance

In order to examine the anoikis inhibitory effect of gigantol, lung cancer cells were subjected to non-cytotoxic treatment of gigantol for 24 h. After that lung cancer cells were detached from the cultured dish and maintained in suspended condition in non-adhesive poly-HEMA-coated plates. Then the suspended lung cancer cells were harvested at 0, 3, 6, 9, 12, and 24 h to evaluate the cell viability using MTT assay. Figure 14a showed the gradually decrease in viable of H460 cells in non-treated groups and at 24 h after detachment, only about 65 % of the cells remained viable. As early as 6 h after the detachment, treatment of gigantol at concentration of 20 µM

significantly reduced cell viability approximately to 60 % comparing to those of non-treated group with 80 % of viable cells, and the most potent effect was found in cells treated with gigantol for 24 h. This suggested that gigantol sensitized anoikis in H460 cells. The nuclear co-staining by Hoechst 33342/PI assay was used to ensure the cell viability evaluation. Consistently, Figure 14b and 14c revealed that an approximately 68 % of the detached cells treated with 20 μM of gigantol were Hoechst-positive anoikis nuclei. However no PI-positive cells were detected in all conditions. On the other hand, Figure 15a indicated that A549 cells were not resistant to anoikis type of cell death. It was shown that the cell viability of A549 cells in all treatment groups was significantly reduced as soon as 6 h after the detachment. More than 50 % of A549 cells showed sign of apoptotic cells at 9 h in the suspending condition (Figure 15b and 15c). There was no significant difference in the viability of A549 cells among the treatment groups. Also PI-positive cells were not observed in any treatment conditions.

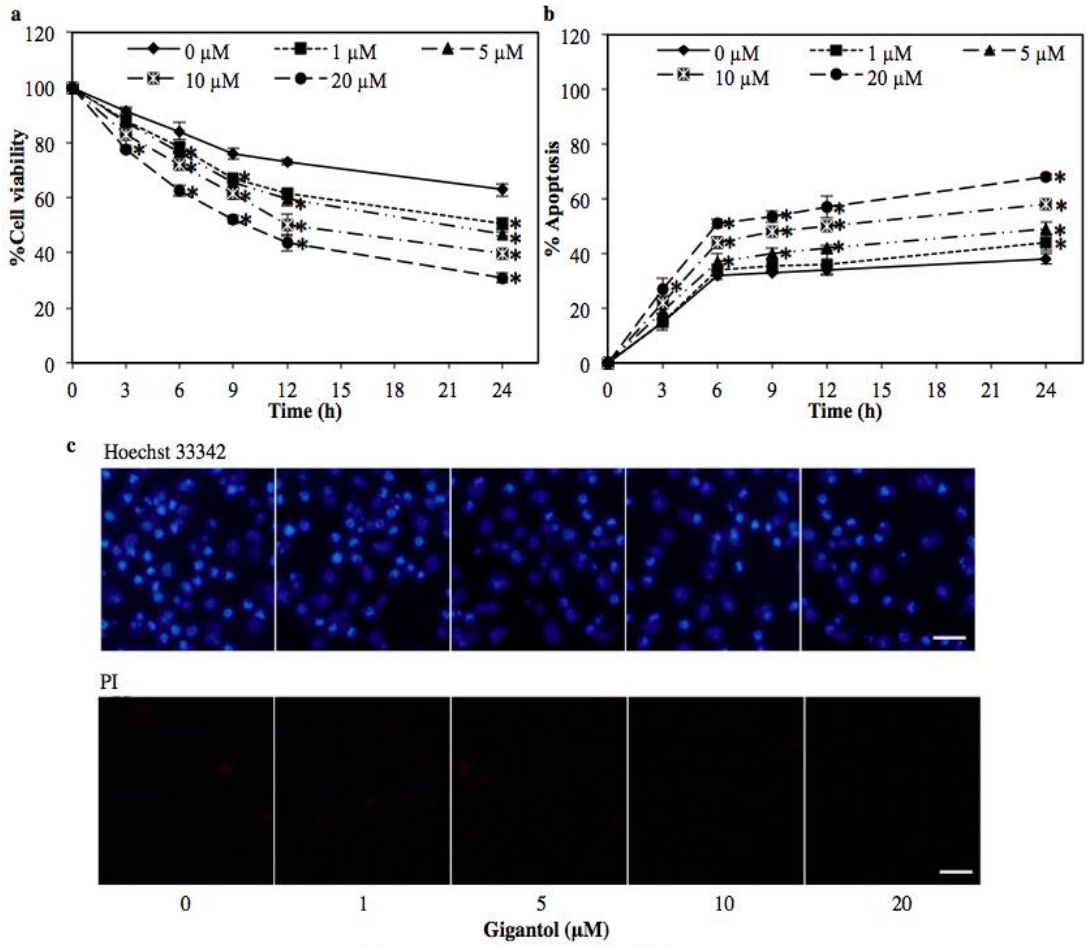


Figure 14 The effect of gigantol on H460 cell anoikis resistance. **a** H460 cells were treated with non-cytotoxic concentrations of gigantol (0-20 µM) for 24 h before suspended in the detached condition, and cell survival was determined by XTT assay. The viability of untreated cells was represented as 100%. **b** and **c** H460 cells were treated with gigantol (0-20 µM) for 24 h. Percentage of apoptotic and necrotic cell death was evaluated using Hoechst 33342/PI staining compared with non-treated control cells (*Scale bar* is 50 µM). The data presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

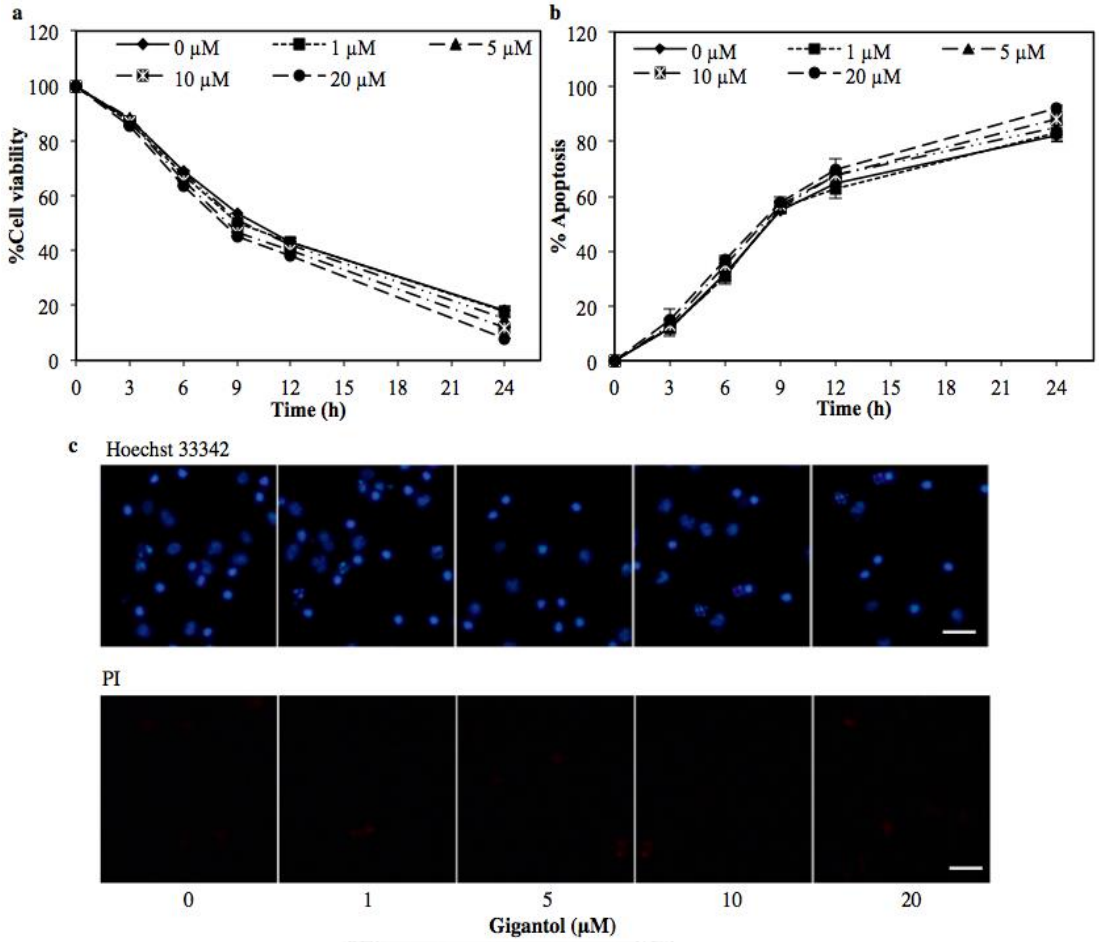


Figure 15 The effect of gigantol on A549 cell anoikis resistance. **a** A549 cells were treated with non-cytotoxic concentrations of gigantol (0-20 µM) for 24 h before suspended in the detached condition, and cell survival was determined by XTT assay. The viability of untreated cells was represented as 100%. **b** and **c** H460 cells were treated with gigantol (0-20 µM) for 24 h. Percentage of apoptotic and necrotic cell death was evaluated using Hoechst 33342/PI staining compared with non-treated control cells (*Scale bar* is 50 µM). The data presented as mean ± standard deviation (S.D.).

The effect of gigantol on H460 and A549 cells colony formation

To further confirm the anoikis sensitizing property of gigantol, another cellular behavior were be evaluated. Cells that are able to resist anoikis should have the ability to form colonies in an anchorage-independent condition. The anchorage-independent

condition can be mimicked in the *in vitro* study by using agarose gel to prevent cell attachment and aggregation. After 24 h treatment of gigantol at 0-20 μM, lung cancer cells were detached and suspended in culture medium mixed with agarose as descript previously in the method section for 14 days. The culture medium was added on top of the agarose every 3 days to prevent surface dryness. Figure 16 indicated that in H460 cells both colony number and size were substantially decreased upon gigantol treatment. Since the number of colony represents the cell survival during detach condition, whereas the size of colony indicates cell proliferation originated from single cell under detachment condition. The treatment of gigantol at 20 μM was able to reduce the colony number and colony size by 60 % and 40 % respectively (Figure 16b and 16c). These results indicated that gigantol was able to inhibit cancer cell growth in an anchorage-independent condition and further supported gigantol as a potential anti-metastasis compound. Nevertheless, according to Figure 17, it was clearly showed that A549 could not form any colony in an anchorage-independent condition. No viable colony was observed in any of the treatment groups.

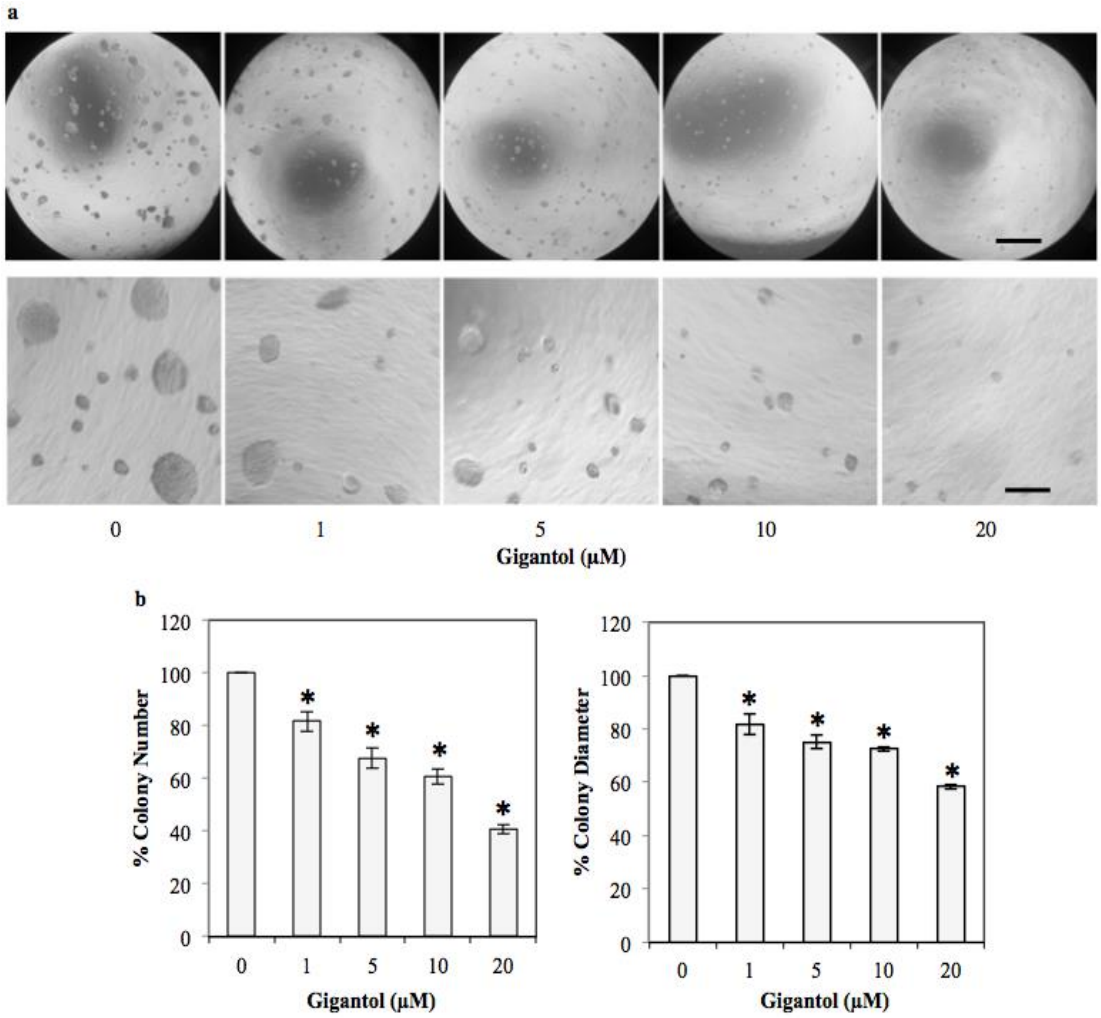


Figure 16 The effect of gigantol on H460 cell colony formation. **a** After treatment with gigantol (0-20 μM) for 24 h, H460 cells were suspended and subjected to colony-formation assay. Colony images were captured at day 14. *Scale bars* are 1000 μM (above) and 400 μM (below). **b** Colony number and **c** colony diameter were analyzed and calculated as relative to the non-treated control cells. The relative number and relative diameter of untreated cells was represented as 100%. The viability of untreated cells at 0 h was represented as 100%. The data presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

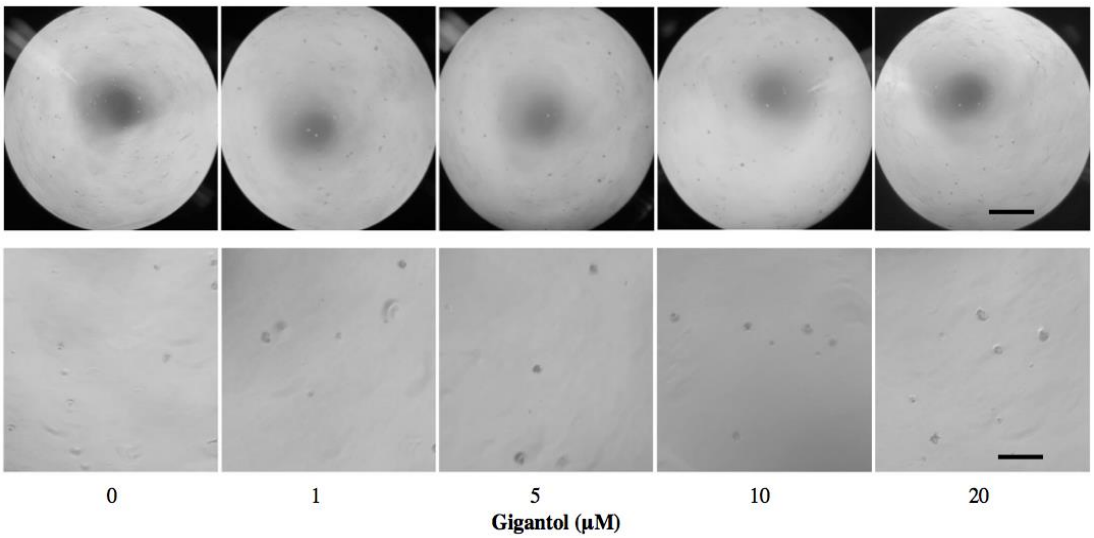


Figure 17 The effect of gigantol on A549 cell colony formation. After treatment with gigantol (0-20 μM) for 24 h, A549 cells were suspended and subjected to colony-formation assay. Colony images were captured at day 14. *Scale bars* are 1000 μM (above) and 400 μM (below).

The effect of gigantol on H460 and A549 cells migration

Cellular migration is known as another hallmark for EMT process since cellular movement represents the migrative behavior of mesenchymal cells. Cell scratch assay was used to observe the two dimensional movement of lung cancer cells. After 24 h of gigantol treatment at 0-20 μM, lung cancer cells were seeded into monolayer before wound space was created. The assay allowed cells to undergo migration across the wound space for 24, 48, and 72 h in order to see the effect of the treatment group compared with those of the control group at each day. The concentrations of the gigantol treatment that were used in this assay did not cause cell proliferation, so that the migration effect were not interfered by cell proliferation. Figure 18 showed that gigantol at concentration of 20 μM was able to suppress cell migration across the wound space about 70 % compared to the control as early as 24 h. At 72 h, gigantol at concentrations of 1, 5, 10, and 20 μM were able to significantly attenuate H460 cell motility. Migration assay was used to confirm the migrative effect observed by cell

scratch assay. This assay allowed cancer cell to migrate through the pores of the filter in a three dimensional manner. After 24 h of gigantol treatment at non-toxic concentrations, lung cancer cells were seeded into the upper compartment of the transwell plate. The cancer cells migrated from the upper chamber to the lower compartment of the well via the concentration gradient of FBS within the cultural medium. After incubated for the indicated amount of time, the cells that did not migrate on the upper chamber were removed. Then the migrated cells in the bottom chamber were stained and visualized. Consistently, the result from transwell migration assay also illustrated that gigantol was able to decrease the number of cells moving across the transwell filter within 24 h (Figure 19). For A549, the significant difference in cell migration was observed within 24 h in cell scratch assay at 10 μM and 20 μM of gigantol (Figure 20). From Figure 21, the levels of transwell migration of A549 cells were slightly dropped according to gigantol treatment. Nevertheless, there was no significant different in the migration level within 24 h after the treatment.

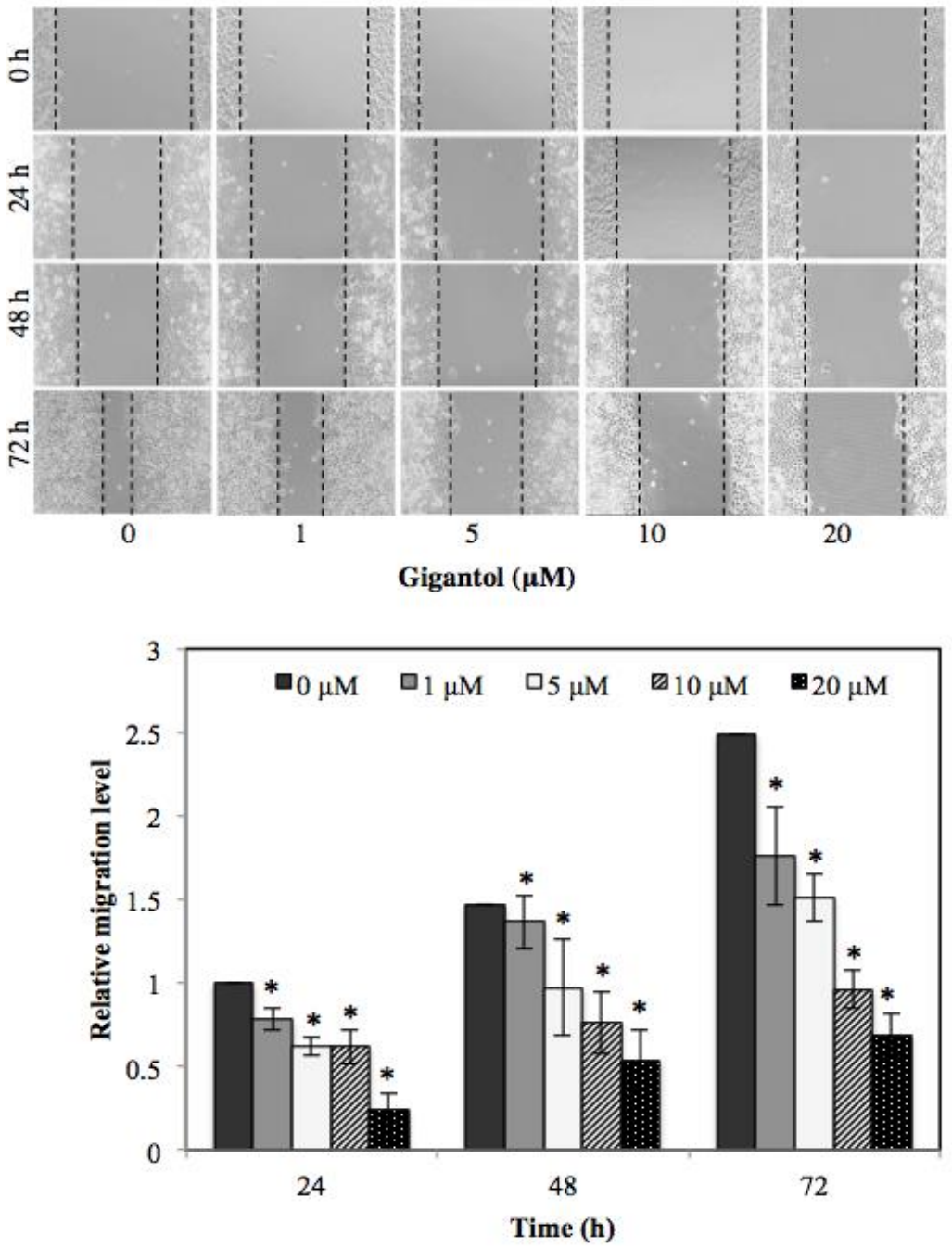


Figure 18 The effect of gigantol on H460 cell scratch assay. H460 cells were treated with non-cytotoxic doses of gigantol (0-20 μM) for 24 h. Wound space was photographed and analyzed at 0, 24, 48, and 72 h. The relative migration level was calculated as the changes of wound space of the treatment groups compared to that of the untreated control group at the indicated time. The data presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

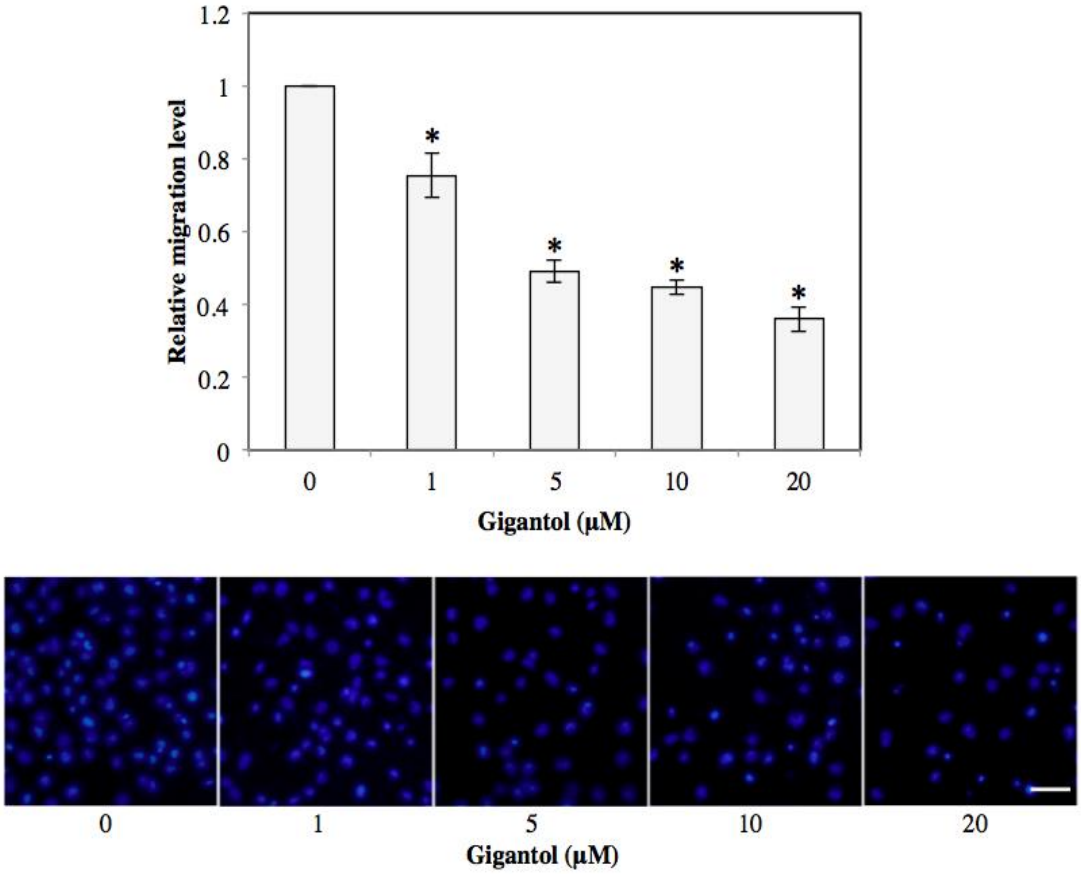


Figure 19 The effect of gigantol on H460 cell transwell migration. H460 cells migration was examined using Transwell migration assay. After 24 h the migrated cells were stained with Hoechst 33342 and visualized by fluorescence microscopy (*Scale bar* is 50 µm). The relative migration level was calculated as the number of migrated cells of the treatment groups divided by that of the untreated control group. The data presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

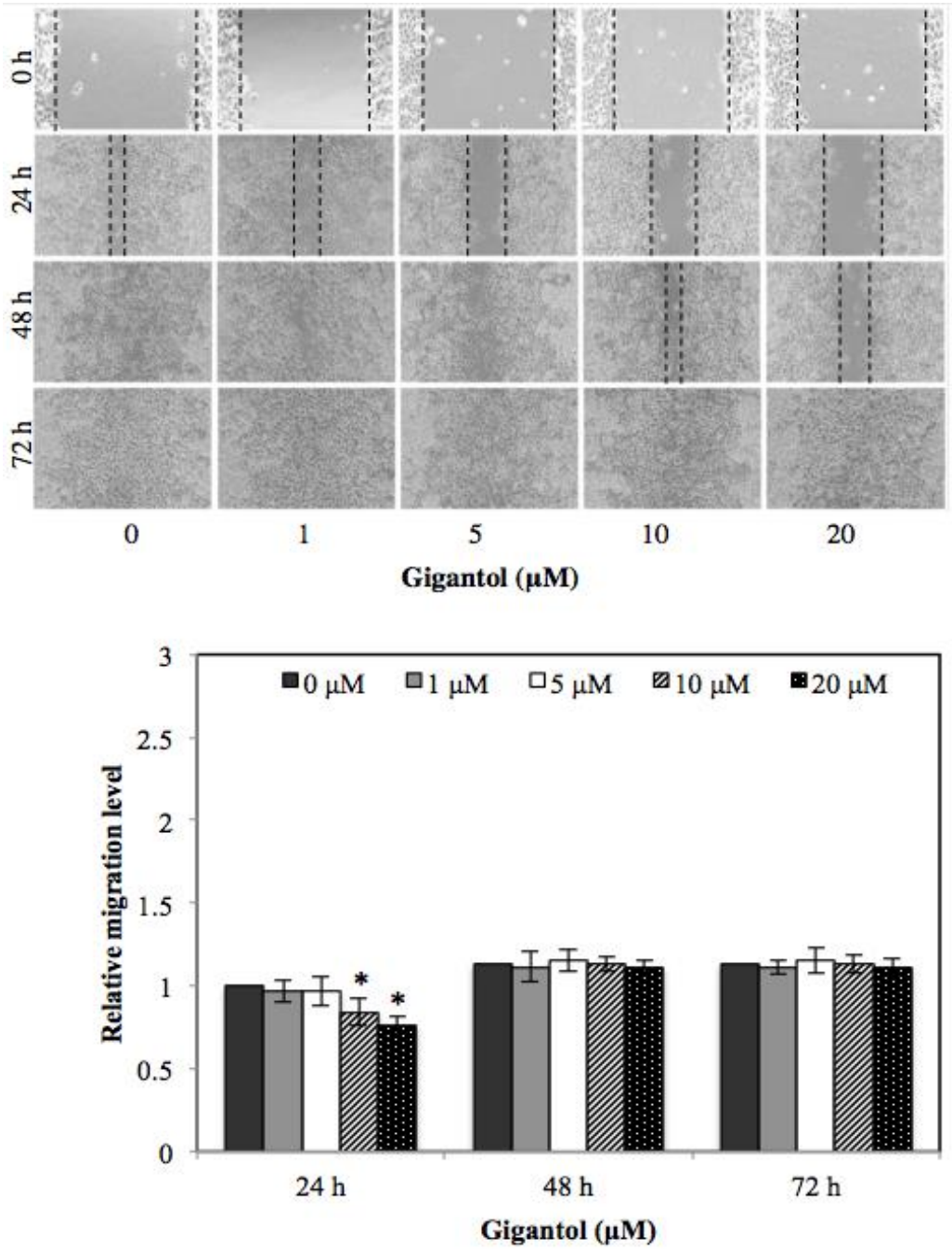


Figure 20 The effect of gigantol on A549 cell scratch assay. A549 cells were treated with non-cytotoxic doses of gigantol (0-20 μM) for 24 h. Wound space was photographed and analyzed at 0, 24, 48, and 72 h. The relative migration level was calculated as the changes of wound space of the treatment groups compared to that of the untreated control group at the indicated time. The data presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

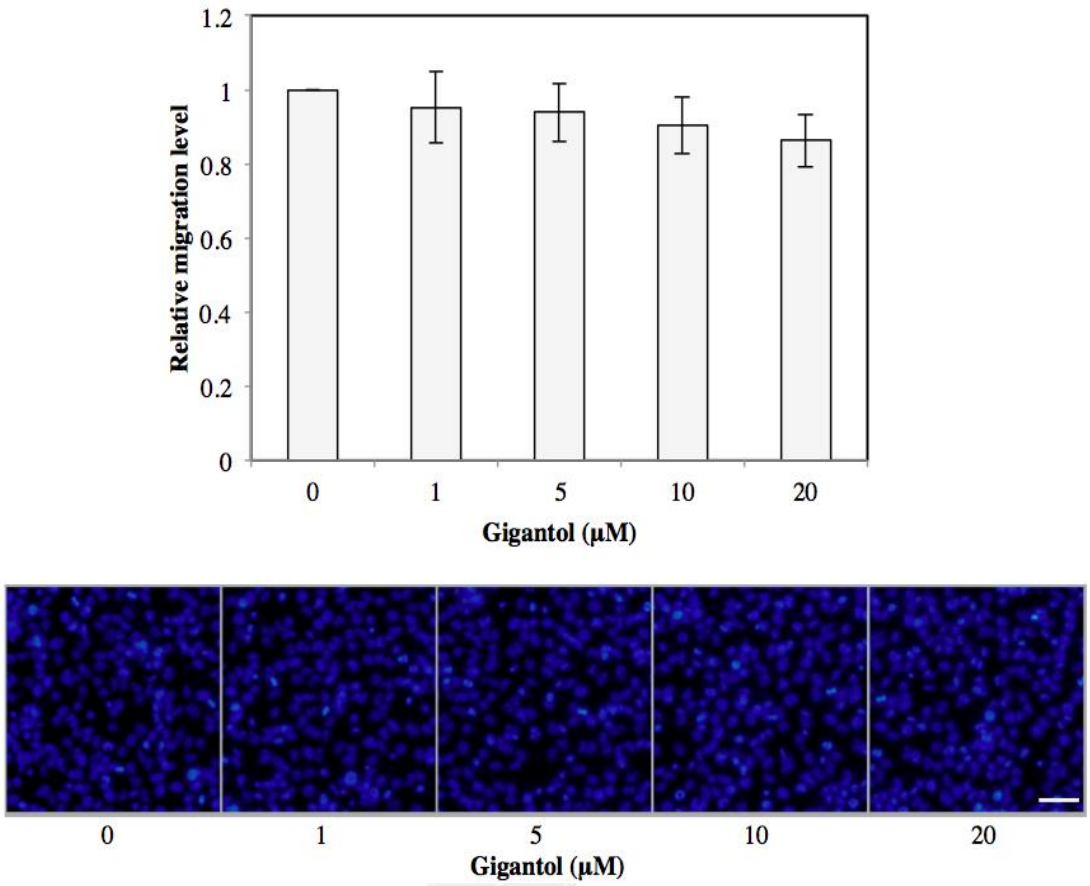


Figure 21 The effect of gigantol on A549 cell transwell migration. H460 cells migration was examined using Transwell migration assay. After 24 h the migrated cells were stained with Hoechst 33342 and visualized by fluorescence microscopy (*Scale bar* is 50 µm). The relative migration level was calculated as the number of migrated cells of the treatment groups divided by that of the untreated control group. The data presented as mean ± standard deviation (S.D.). **p*-values < 0.05 vs non-treated cells.

The effect of gigantol on H460 and A549 cells invasion

To confirm the effect of cell motility, invasion assay was done. The transwell chamber was used in this study similar to the migration assessment. However, the ability of cells to invade includes the cell movement through the extracellular matrix, therefore the filter between the top and the bottom chamber was coated with Matrigel representing the extracellular material in the basal tissue. To move across the Matrigel, cancer cells are required to digest the material along the path mimicking the *in vivo*

condition. After lung cancer cells were exposed to gigantol treatment, cells were seeded on to the upper compartment of the transwell plate. The amount of cells moved through the coated filter was then evaluated. Approximately 30 %, 35 %, 45 %, and 70 % reductions in invasion ability were recorded in H460 cells treated with concentrations 1, 5, 10, and 20 μM of gigantol for 24 h, respectively (Figure 22). On the other hand, no significant difference was observed with the invasion level of A549 cells between the treatment groups (Figure 23). It can be seen that H460 cells responded according to the treatment, therefore, only H460 cells were used in further study.

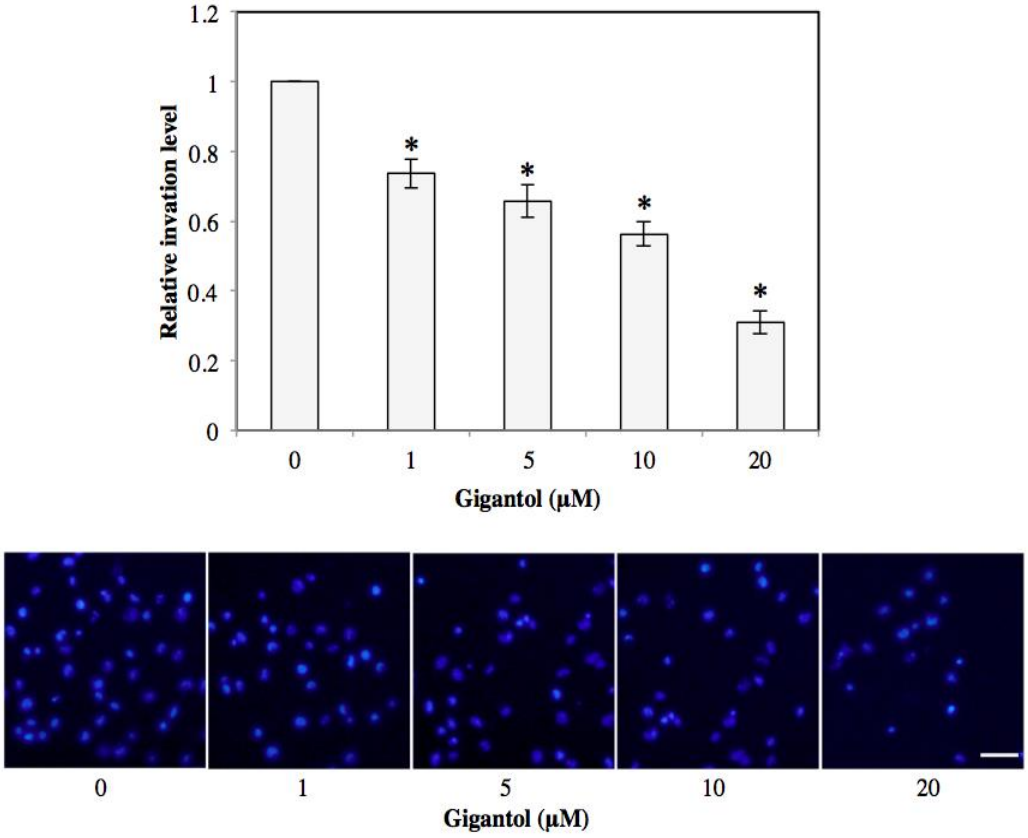


Figure 22 The effect of gigantol on H460 cell transwell invasion. H460 cells invasion was examined using Transwell invasion assay. After 24 h the invaded cells were stained with Hoechst 33342 and visualized by fluorescence microscopy (*Scale bar* is 50 μm). The relative invasion level was calculated as the number of migrated cells of the treatment groups divided by that of the untreated control group. The data presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

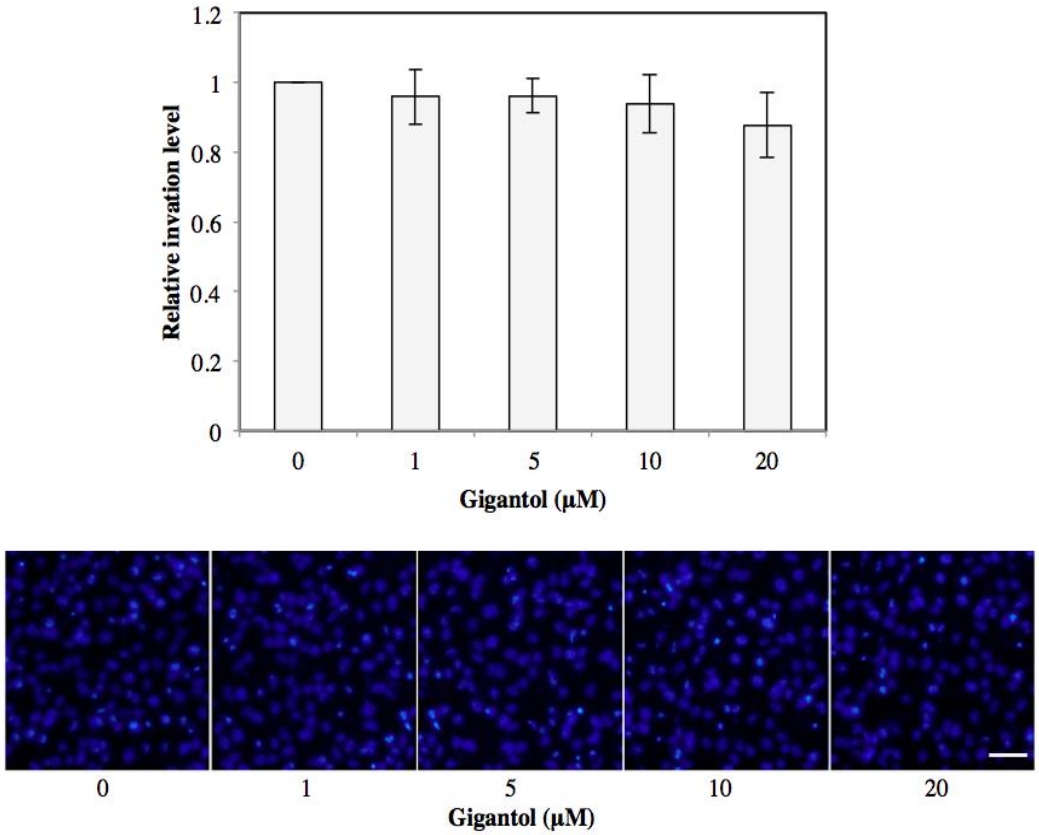


Figure 23 The effect of gigantol on A549cell transwell invasion. A549 cells invasion was examined using Transwell invasion assay. After 24 h the invaded cells were stained with Hoechst 33342 and visualized by fluorescence microscopy (*Scale bar* is 50 µm). The relative invasion level was calculated as the number of migrated cells of the treatment groups divided by that of the untreated control group. The data presented as mean ± standard deviation (S.D.).

The effect of gigantol on EMT molecular markers

EMT markers are proteins that have been proven to be up-regulated or down-regulated in response to the changes in the particular cellular phenotype. According to several reports, E-cadherin, N-cadherin, and Vimentin, are EMT marker proteins. H460 lung cancer cells were subjected to gigantol treatment for 24 h, then the treated cells were harvested for Western blot assay. Figure 24 indicated that the treatment of gigantol significantly suppressed the expression of N-cadherin and Vimentin together

with up-regulated E-cadherin. These results suggested that gigantol has an ability to suppress EMT in lung cancer cells.

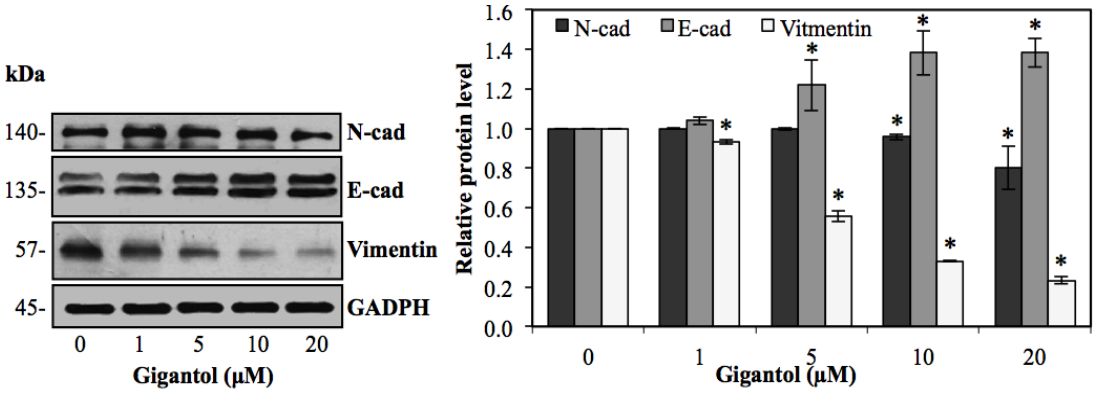


Figure 24 The effect of gigantol on EMT molecular marker in H460. Gigantol modulates EMT markers. After treatment with gigantol (0-20 μM) for 24 h, level of N-cadherin, E-cadherin, and Vimentin were determined by Western blotting assay. The immunoblotting signals were quantified by densitometry and presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

The effect of gigantol on EMT regulating cascades

The effect of gigantol on snail and slug transcription factor

As the Snail family transcription factors including Snail and Slug are claimed to be able to directly initiate EMT process, the expression of Snail and Slug proteins were investigated. The amount of protein expression was determined using Western blotting analysis. The protein sample were harvested from H460 cells 3 h and 24 h after gigantol treatment. Figure 25 and 26 illustrated that 20 μM of gigantol was able to reduce the expression level of Slug to about 80 % and 50 % compared to the control at 3 h and 24 h, respectively. However, there was no difference in the expression of Snail at either 3 h or 24 h after the treatment (Figure 25 and 26). This suggested Slug might be the main transcription factor responsible for gigantol treatment.

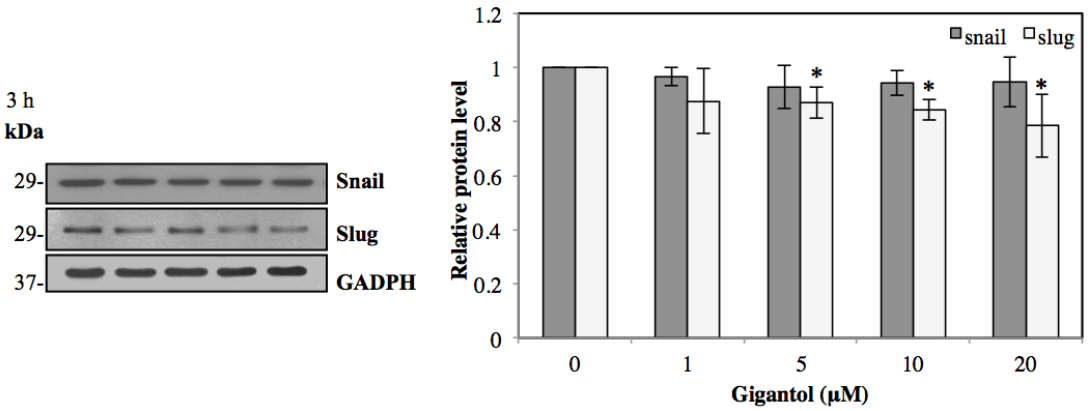


Figure 25 The effect of gigantol on snail and slug transcription factors in H460 at 3 h. Gigantol modulates EMT markers. After treatment with gigantol (0-20 µM) for 3 h, level of Snail and Slug were determined by Western blotting assay. The immunoblotting signals were quantified by densitometry and presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

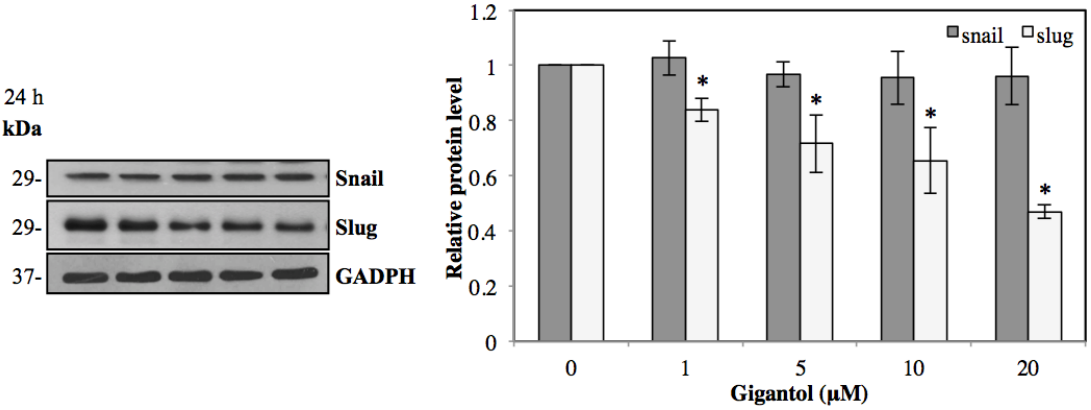


Figure 26 The effect of gigantol on snail and slug transcription factors in H460 at 24 h. Gigantol modulates EMT markers. After treatment with gigantol (0-20 µM) for 24 h, level of Snail and Slug were determined by Western blotting assay. The immunoblotting signals were quantified by densitometry and presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

The effect of gigantol on slug production pathway

The expressions of Slug transcription factors can be regulated at transcriptional level. β-catenin is responsible for the Snail family transcription by interacting with Snail and Slug encoded genes. β-catenin can be regulated through integrins and AKT

dependent pathways. It was reported that an increase in the expression of integrin αVβ3 and α5β1 correlated with EMT incidence (Knowles et al. 2013). These switches in integrin expression resulted in activation and accumulation of β-catenin. In addition, β-catenin accumulation also associated with the activity of AKT. Western blotting analysis was used to measure the expression level of integrin family proteins including αV, α5, β1, and β3, activated AKT (p-AKT), total AKT, and β-catenin. It can be seen from Figure 27 that the expressions of integrin αV, β1, β3 were increased with gigantol treatment at 20 μM whereas there was a decrease in integrin α5 expression due to the treatment at 3 h. Nevertheless, there was no significant difference in the any type of integrins expression at 24 h after the treatment (Figure 28). The expression of β-catenin was reduced at 3 h but such difference was not observed at 24 h (Figure 27 and 28). Interestingly, it clearly showed in Figure 27 and 28 that treatment of gigantol could decrease expression ratio of activated AKT at both 3 h and 24 h, respectively.

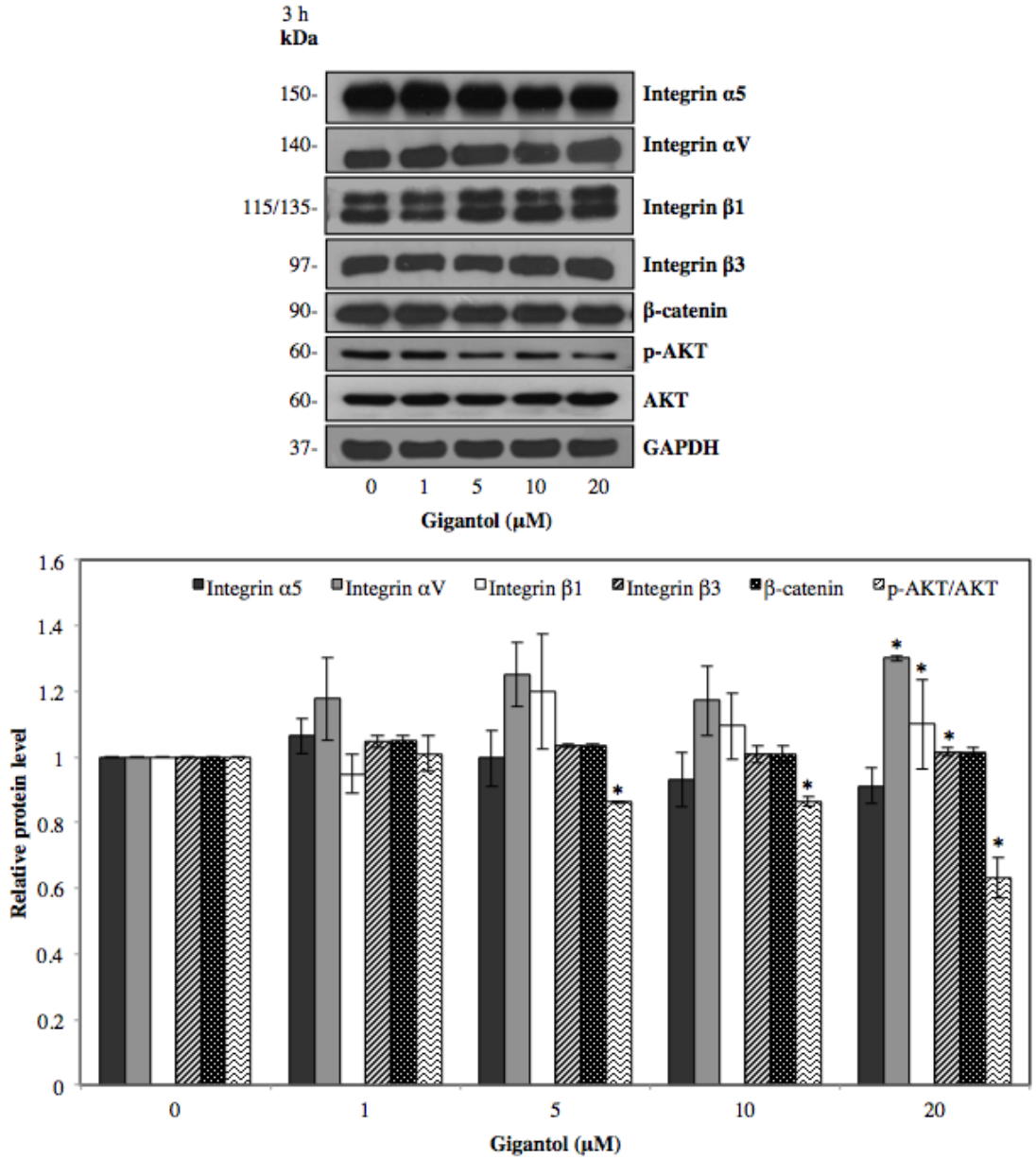


Figure 27 The effect of gigantol on slug production pathway in H460 at 3 h. After treatment with gigantol (0-20 μM), level of integrin α5, αV, β1, β3, β-catenin, p-AKT, and AKT were determined by Western blotting assay. The immunoblotting signals were quantified by densitometry and presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

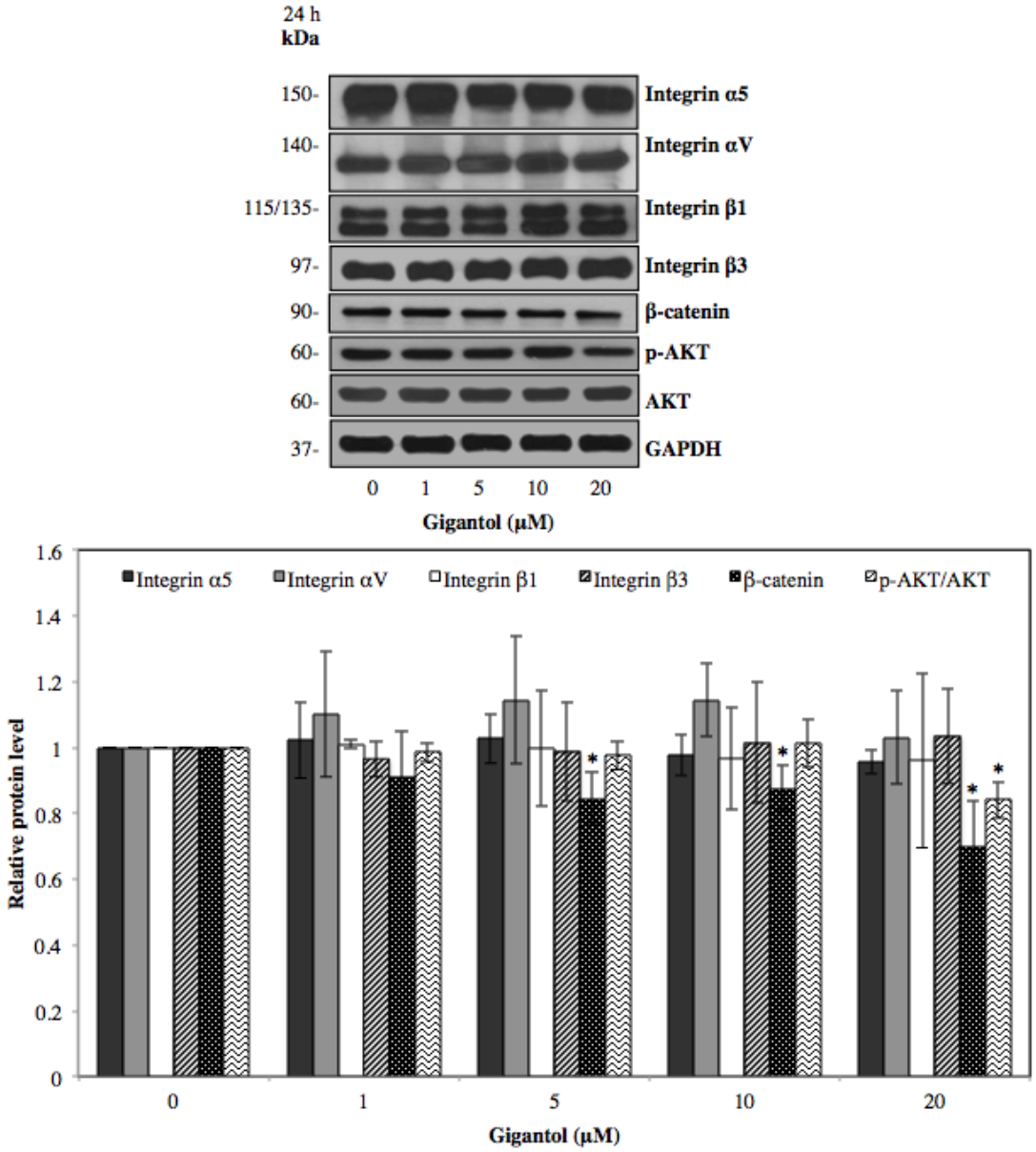


Figure 28 The effect of gigantol on slug production pathway in H460 at 24 h. After treatment with gigantol (0-20 μM), level of integrin α5, αV, β1, β3, β-catenin, p-AKT, and AKT were determined by Western blotting assay. The immunoblotting signals were quantified by densitometry and presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

The effect of gigantol on slug degradation pathway

As we have illustrated that gigantol was able to down regulate the expression of Slug, this experiment was objected to further investigate the mechanism by which

gigantol treatment caused a down regulation in Slug expression. It was reported that the stability of Slug was controlled by proteasomal degradation (Lee & Nelson 2012; Wu et al. 2008). Since protein degradation was through either proteasomal or lysosomal pathways, to verify whether the down regulation of Slug was consistent with the statement, H460 cells were treated with a specific proteasomal inhibitor, lactacystin (Lac) or a lysosomal inhibitor, concanamycin A (CMA). Figure 29a showed that Lac was able to rescue the reduction of Slug level in response to gigantol. This indicated that the degradation of Slug was prohibited in the proteasomal-suppressed cells making the accumulated Slug level in the cells higher than that of the control. In contrast, it can be seen that the CMA treatment had no effect to the level of Slug expression. This finding revealed that the proteasomal degradation was involved with the stability of Slug expression. As it was clearly known that ubiquitination is a critical prerequisite and a rate-limiting step prior to proteasomal cleavage, Slug-ubiquitin complexes in response to gigantol treatment were investigated by immunoprecipitation assay. Figure 29b indicated that the H460 cells treated with gigantol exhibited a significant increase in the level of Slug-ubiquitin complexes, revealing that gigantol was able to enhance the degradation rate of Slug through proteasomal pathway.

GSK-3β is responsible for the Snail family post-translational modification by phosphorylation leading to Slug degradation through ubiquitin proteasome system. Figure 30 presented that the accumulated level of inactivated GSK-3β (p-GSK-3β) was down regulated as soon as 3 h due to gigantol treatment. However the expression of p-GSK-3β was unchanged at 24 h after gigantol exposure. Consistently, gigantol caused significant reduction in the expression of p-AKT at both 3 h and 24 h (Figure 30 and 31).

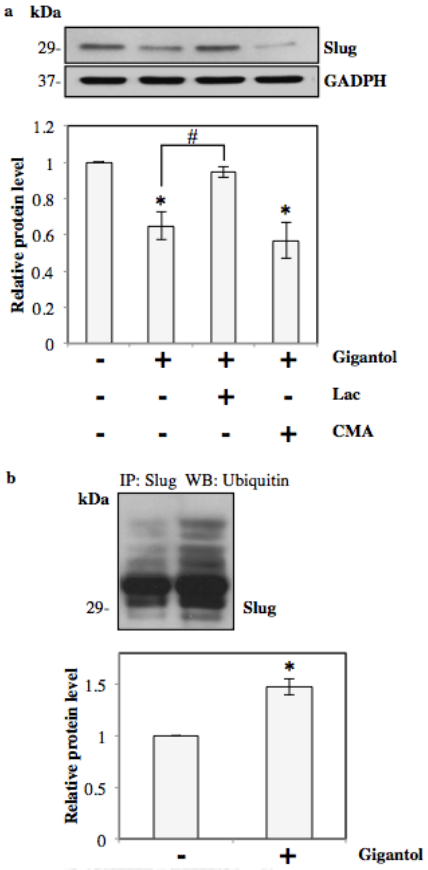


Figure 29 The effect of gigantol on slug ubiquitination **a** H460 cells were pretreated with a proteasomal inhibitor Lactacystin (Lac) 10 μM or lysosomal inhibitor Concanamycin A (CMA) 1 μM for an hour before treatment with 20 μM of gigantol for 24 h. Slug expression was analyzed using Western blotting assay. The immunoblot signals were qualified by densitometry. The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells # p < 0.05 versus gigantol treated cells. **b** H460 cells were pretreated with Lactacystin (Lac) 10 μM for an hour, then the pretreated cells were exposed to a presence of gigantol or left untreated for 3 h. The levels of immunocomplexes were analyzed for ubiquitin using anti-ubiquitin antibody. Immunoblot signals were qualified by densitometry. The data presented as mean ± SD (n=4). *p < 0.05 versus control cells.

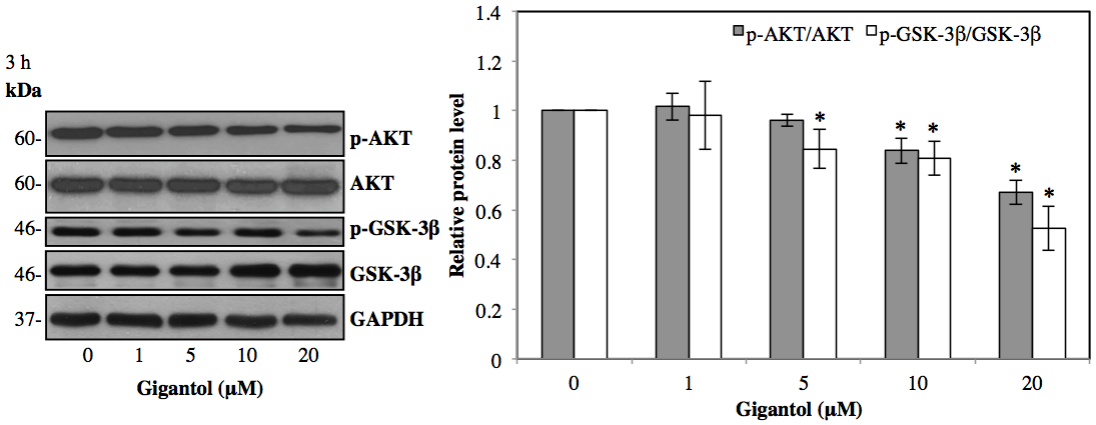


Figure 30 The effect of gigantol on slug degradation pathway in H460 at 3 h. After treatment with gigantol (0-20 μM), level of p-AKT, AKT, p-GSK-3β and GSK-3β were determined by Western blotting assay. The immunoblotting signals were quantified by densitometry and presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

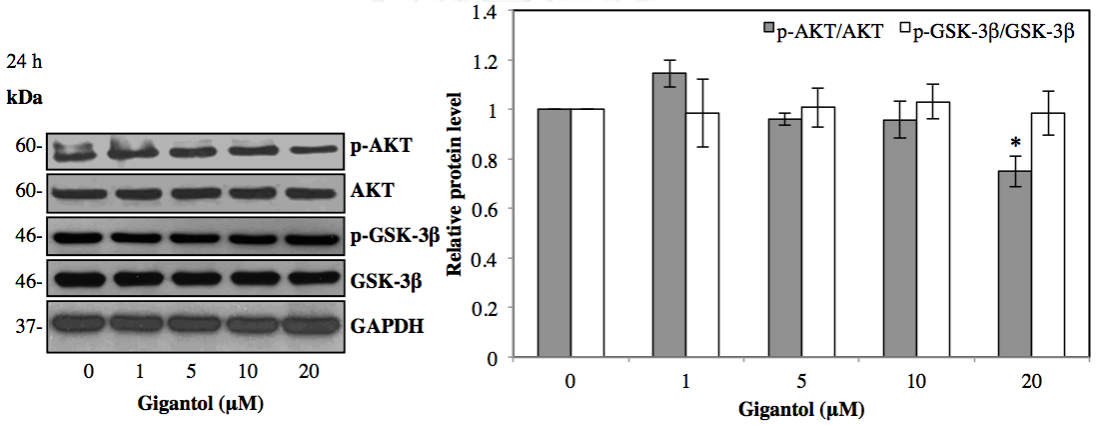


Figure 31 The effect of gigantol on slug degradation pathway in H460 at 24 h. After treatment with gigantol (0-20 μM), level of p-AKT, AKT, p-GSK-3β and GSK-3β were determined by Western blotting assay. The immunoblotting signals were quantified by densitometry and presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

The effect of gigantol on EMT effector proteins

It was well accepted that EMT process leads to a resistance to anoikis induced by cellular detachment together with an increase in cell motility. Thus, in order to

ensure that the gigantol treatment is able to attenuate EMT, some of the downstream effector proteins underlying the anoikis resistance and cell migration behavior are evaluated using Western blotting assay.

The effect of gigantol on anoikis resistance effector proteins

During the programmed cell death, Poly (ADP-ribose) polymerase (PARP) and procaspase-3 are cleaved and activated leading to an irreversible process of apoptosis mechanism. Therefore, the level activated poly (ADP-ribose) polymerase (PARP) and caspase-3 expression are able to molecularly confirm the anoikis incidence in detached cells. Western blot analysis of apoptotic markers from Figure. 32 confirmed that cleaved PARP and activated caspase-3 were gradually increased, and Procaspase-3 was in turn reduced, whereas PARP was not changed. These results illustrated that gigantol at non-cytotoxic concentration (0-20 μM) significantly sensitized anoikis in lung cancer cells.

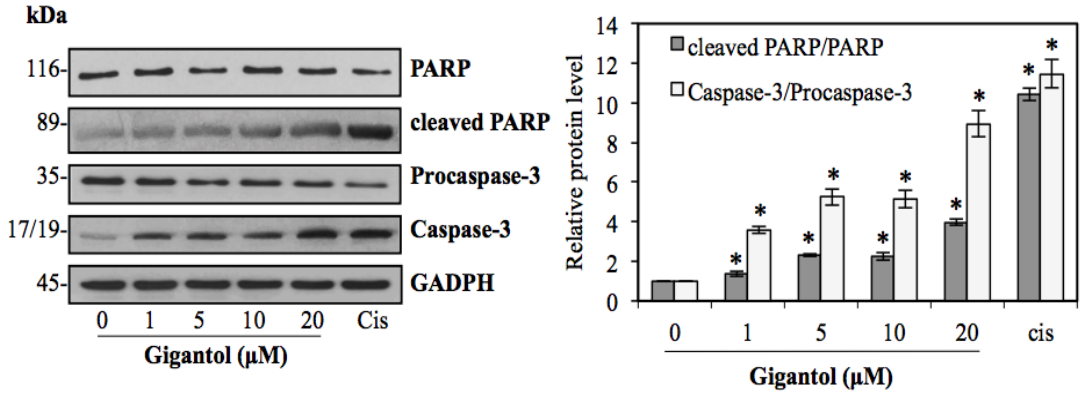


Figure 32 The effect of gigantol on anoikis resistance effector proteins in H460. After treatment with gigantol (0-20 μM) or 50 μM of cisplatin (Cis) as positive control for 24 h under detachment condition, H460 cells were collected and levels of Poly (ADP-ribose) polymerase (PARP), cleaved PARP, Procaspase-3 and activated Caspase-3 were determined by Western blotting assay. The blots were re-probed with GADPH to confirm equal loading. The immunoblotting signals were quantified by densitometry

and presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

The effect of gigantol on migration effector proteins

During cell migration, the proteins that play important roles in cellular movement are Rho family proteins including Rho GTP and Rac GTP. Therefore, the expression of Rho GTP and Rac GTP were able to molecularly indicate cell migration. The result from Western blot analysis was shown in Figure 33 that treatment of gigantol suppressed the expression of Rho GTP and Rac GTP.

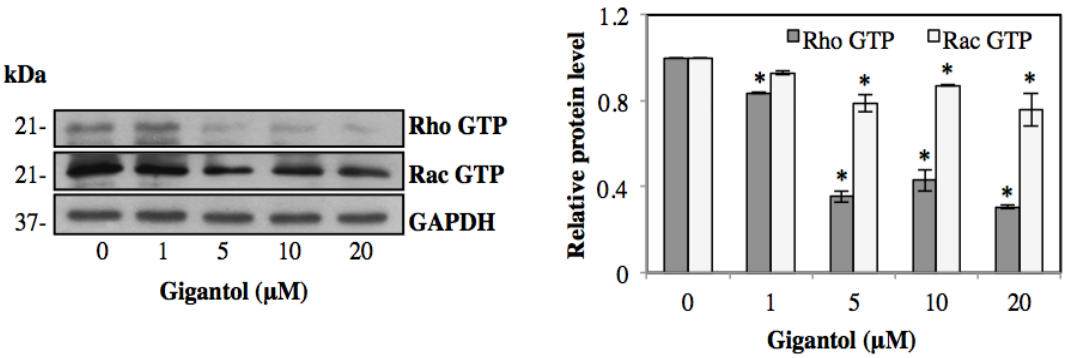


Figure 33 The effect of gigantol on migration effector proteins in H460. After H460 cells were treated with non-cytotoxic doses of gigantol (0-20 µM) for 24 h, the expression of Rho GTP and Rac GTP were evaluated using Western Blot assay. The blots were re-probed with GADPH to confirm equal loading. The immunoblotting signals were quantified by densitometry and presented as mean ± standard deviation (S.D.). *p-values < 0.05 vs non-treated cells.

CHAPTER V
DISCUSSION AND CONCLUSION

Metastasis of cancer cell from the primary site to the distant secondary tumor is accused for more than 90 % of cancer patient deaths (Mehlen & Puisieux 2006; Weigelt et al. 2005). The mechanism underlying metastasis is highly complex. However in order to pursue a successful metastasis, one of the most crucial processes that cancer cells acquired is a transdifferentiation from epithelial into mesenchymal morphologies or EMT. This transition in cellular phenotypes facilitates the aggressiveness of cancer disease by enhancing cellular migrative level and anoikis resistance. Several researches have been done to explore the natural compounds with the ability to attenuate cancer metastasis. Gigantol, a natural stilbenoid derivative extracted from *Dendrobium draconis*, was reported with promising anti-cancer properties (Charoenrungruang, Chanvorachote, Sritularak & Pongrakhananon 2014a; Charoenrungruang, Chanvorachote & Sritularak 2014b; Bhummaphan & Chanvorachote 2015; Unahabhokha et al. 2016). In this study, we have provided further molecular evidence supporting the potential of gigantol as a biological agent for cancer treatment. Our results have demonstrated that the non-cytotoxic concentrations of gigantol were able to significantly reduce the EMT behavior including anoikis resistance (Figure 14 and 16) and cellular migration and invasion (Figure 18, 19, and 21) as well as decrease the level of EMT marker proteins (Figure 24). Moreover, our findings also indicated that such an inhibitory effect was involved in the potential of gigantol to down regulate the expression of Slug which is the major transcription factor underlying EMT (Figure 25 and 26) (Lee & Nelson 2012; Barrallo-Gimeno 2005)15.

Cell death by anoikis is normally occurred in epithelial cells in order to prevent an inappropriate growth in detachment condition. Cancer cells with the ability to adapt to these aggressive phenotype lead to a poor prognosis and thus limit the survival rate

of cancer patients. Therefore a novel therapeutic method that can enhance anoikis cell death especially a reduction of the metastasis rate raises an essential challenge in the pharmaceutical research. Gigantol was reported with many promising anticancer activities (Bhummaphan & Chanvorachote 2015; Ho & C.-C. Chen 2003; Klongkumnuankarn et al. 2015). However, little was known about the effect of gigantol on anoikis cell death in lung cancer cells. In this study, it was well proven that gigantol can enhance the death rate of metastatic lung cancer cells (H460) by sensitizing anoikis. It was shown that the cancer cell viability in a detach condition (Figure 14) and the anchorage independent growth were significantly reduced when treated with gigantol (Figure 16). Moreover, Figure 32 has confirmed that gigantol was able to suppress the function of anoikis effector proteins including PARP and Caspase-3 which were detected after 24 h of gigantol pretreatment and another 6 h of cells suspension. In contrast, the non-metastatic lung cancer cells (A549) did not have the ability to resist anoikis cell death or the ability to form colony in an anchorage-independent condition. Therefore, from Figure 15 and Figure 17, it can be seen that gigantol had no effect to A549 cells. Interestingly, the concentration of gigantol used in this study was very low and has shown no cytotoxic effect to normal human fibroblasts which have mesenchymal-like behaviors, suggesting that gigantol only possessed anoikis sensitizing effect on a selected lung cancer cells.

Cellular migration and invasion are also crucial behaviors for a successful metastasis. Cell migration explains the movement of cancer cells by dynamic changes of cytoskeleton involving cycle of actin-myosin contraction (Fidler 2002). Cell invasion emphasizes on the proteolytic activities of the enzyme secreting from the cells to disrupt the basement membrane and the extra cellular matrix (ECM) surrounding the cells (Fidler 2002). Our results have demonstrated that the non-cytotoxic concentrations of gigantol were able to significantly inhibit both migration and invasion behaviors (Figure 18, 19 and 21) as well as decrease the level of migration regulating

proteins including Rho GTP and Rac GTP (Figure 33) in lung cancer H460 cells. This finding was consistent with the previous report (Charoenrungruang, Chanvorachote, Sritularak & Pongrakhananon 2014b). Interestingly, in the present study, gigantol was pretreated for 24 h before migration and invasion evaluation and the inhibitory effect was still persisted up to 72 h after the treatment was removed (Figure 18, 19, and 22). This finding led to a new evidence that gigantol possess the effect on the upstream mechanism of migration. In addition, the migration and invasion was also observed in A549 lung cancer cells. However, there was no difference in migration and invasion levels in the gigantol treated groups compared with the control group of A549 (Figure 20, 21, and 23). This might be because of the highly invasive behavior of A549 as reported by Chanvorachote and his colleagues so that the inhibition effect may not clearly detected (Chanvorachote 2013). Together with the inability of A549 to become resistant to anoikis and to form colony, it could be concluded that A549 was not an appropriate type of lung cancer cell for EMT inhibitory study. Consequently, only metastatic non-small cell lung cancer cell H460 was used in the further molecular studies.

The efficacy of cancer metastasis depends on the EMT phenotype of the cancer cells. The EMT allows cancer cells to become more migrative and invasive to move across the surrounding extracellular matrix and invade into the blood circulation (Chanvorachote 2013; Baum et al. 2008). In addition, EMT also helps maintaining the cell viability in the circulatory system by up regulating the survival mechanism of the cancer cells during the detached condition (Shi et al. 2013). In order to achieve EMT, cancer cells are required to adapt their epithelial proteins to become more mesenchymal-like behavior. E-cadherin is one of the most fundamental cell-cell adhesions in epithelial cells whereas the expression of N-cadherin represents the morphology changes to a fibroblastic phenotype (Shi et al. 2013; Larue & Bellacosa 2005). One of the most obvious EMT phenotypes is the switching of the cellular

adhesion proteins called cadherin from E-cadherin to N-cadherin (Baum et al. 2008; Winitthana et al. 2014). In this study, we observed that the N-cadherin to E-cadherin switching occurred with the gigantol treatment in lung cancer cells (Figure 24). Moreover, Vimentin is recognized as the mesenchymal cytoskeleton driving the EMT mechanism (Winitthana et al. 2014; Thiery & Sleeman 2006; Nurwidya et al. 2012). In order to emphasize on the role of gigantol suppressing EMT process, the expressions of Vimentin was further evaluated. Interestingly it was found that there was a decrease in Vimentin expression according to the gigantol treatment. Therefore, it is rational to claim that gigantol was able to reduce EMT in lung cancer cells and might lead to the weakening of anoikis resistant and migrative behaviors of such cancer.

It is widely accepted that the Snail family transcription factors including Snail and Slug are the main regulator of EMT acting as a molecular switch suppressing the expression of E-cadherin by repressing a set of genes that encodes E-cadherin (Lee & Nelson 2012). Form our results, it was shown that the expression of Slug transcription factor was significantly suppressed due to gigantol treatment at both 3 h and 24 h (Figure 25 and 26). However, the expression of Snail was unchanged. This might be due to the instability of the Snail transcription factor as it was previously reported that the half-life of Snail transcription factor was only within 30 min. Nevertheless, one of the proteins that can be encoded by Snail transcription factor includes Slug. Therefore the expression of Slug might already include the influence from Snail transcription factor. Our results have shown that the expression of Slug transcription factor was significantly suppressed due to gigantol treatment at both 3 h and 24 h (Figure 25 and 26). This finding suggested that gigantol was able to attenuate EMT process at the transcriptional level.

The expression of Slug transcription factor can be regulated through the production and degradation pathways by β-catenin and GSK-3β proteins, respectively (Lee & Nelson 2012). In epithelial cells, β-catenin is interacted to the cytoplasmic

domain of E-cadherin whereas during EMT process β-catenin is released from the complex and translocated into the nucleus to increase the expression of Slug. It was reported that an increase in the expression of integrin αVβ3 and α5β1 correlated with EMT incidence (Knowles et al. 2013). These switches in an integrin expression results in an activation and an accumulation of β-catenin, so it is possible that gigantol may able to interfere with the integrin expressions. However, from the Western blot results 3 h, the expression of integrin α5, β1, and β3 were increased while integrin αV was unchanged with 20 μM gigantol treatment. This result indeed contradicted to our expected outcome. It was possible that the expression of integrins might not be involved with the EMT inhibitory in lung cancer cells induced by gigantol. It was reported that the increase in an expression of Integrins involved with several survival pathways. Therefore, the increase in the integrin expressions might be due to the initial response of lung cancer cells to gigantol treatment. However the increase in all integrin expression seems to be subsided at 24 h indicating that the changes in integrin expression might not associate to gigantol treatment. In case of β-catenin, it was significantly decreased with gigantol treatment at 3 h. It is possible that gigantol decreased the expression of Slug via reducing the β-catenin activity to transcribe Slug. On the other hand, activated GSK-3β causes phosphorylation of Slug in the central domain leading to Slug ubiquitination and consequently proteasome degradation (Lee & Nelson 2012). GSK-3β can be inactivated through phosphorylation. Our results indicated that gigantol treatment did not only decrease the production of Slug by promoting degradation pathway, but also suppressing the expression of transcriptional activator of such protein.

Accumulative studies have demonstrated that the activated AKT plays an important role in the EMT process (Lamouille et al. 2014; Fenouille et al. 2012). The previous report also evidenced that gigantol inhibited migration through decreasing the function of AKT (Charoenrungruang, Chanvorachote, Sritularak & Pongrakhananon

2014b). However, in the present study, it was shown that the activation of AKT was significantly suppressed within the first 3 h of gigantol treatment (Figure. 27 and 30). Therefore, it was possible that gigantol may possess the effect associated with the up stream signaling pathway to attenuate migration behavior. This data is strongly consistent with previous evidence that the attenuation of AKT activity was able to inhibit mesenchymal transition through β-catenin and GSK-3β pathway (Kiratipaiboon et al. 2016; Zhao et al. 2013; Fang et al. 2007). Since the activation of AKT positively regulates Slug transcription via β-catenin *in vitro* (Huang et al. 2015), and suppresses Slug degradation (Kao et al. 2013), the inhibition of AKT would be promising therapeutic approach to attenuate EMT process.

Previous studies have been reported on compounds with bibenzyl structure having AKT inhibition activity. Resveratol (trans-3-4', 5-trihydroxystilbene), a naturally derived polyphenolic compound, has been reported with a cancer therapeutic agent due to the inhibition of Phosphoinositide kinase-3/Protein kinase B/Mammalian target of rapamycin (PI3K/AKT/mTOR) pathway leading to an increase in apoptosis in glioma (Jiang et al. 2009). Moreover, another natural compound derived from medicinal orchid, moscatilin (4,4'-dihydroxy-3,3',5'-trimethoxybibenzyl), also reported with AKT inhibitory effect. Moscatilin was reported to decrease the endogenous reactive oxygen species (ROS) and decrease AKT activity through focal adhesion kinase (FAK) pathway (Kowitdamrong et al. 2013). Together, it is highly possible that natural compounds containing bibenzyl structure are likely to have the anti-cancer property though the AKT dependent pathway.

Accumulated amount of evidence have been reported that cancer stem cells (CSCs) phenotypes are related to EMT (Scheel & Weinberg 2012; Mani et al. 2008). Cancer stem cells are a group of specialized cells within tumor that possess self-renewal ability together with tumor differentiation capability. These characteristics are accounted for tumor initiating, metastasis, and cancer recurrence (Vinogradov & Wei

2012; Lobo et al. 2007; Yongsanguanchai et al. 2015). Since the existance of CSCs provides a resource of new cancer cells which are responsible for tumor relapse after the therapy, many studies have been focusing on targeting CSCs for cancer treatment (K. Chen et al. 2013; Han et al. 2013). As EMT is a conversion of the terminal differentiated epithelial cell back to mesenchymal state, these processes can be seen as an initiation step of the 'stemness' phenotype of CSCs. However, in contrast to the previous reports, recent studies have demonstrated that there are some CSCs characteristics had been observed regardless of EMT (Jung & J. Yang 2015; Schmidt et al. 2015; Rajendran et al. 2013). Whether EMT is a required step driving cancer cell towards CSC or whether EMT is one of the CSC behavior is still debatable. According to Bhummaphan and Chanvorachote 2015, it was indicated that gigantol could reduce the expression of CSC markers after 48 h of treatment. These results are indeed correlated with our findings. It is therefore possible that gigantol was able to suppress stem cell like behavior through attenuating the EMT process.

In conclusion, this study has demonstrated that gigantol was able to attenuate EMT process in lung cancer cells through AKT activity. The reduction of AKT activity decreases the transcription as well as the stability of Slug. Gigantol was shown to reduce β-catenin activity and Slug transcription while enhance the activity of GSK-3β to ubiquitinate Slug resulting in a decrease in Slug level and, thereby, suppressing EMT process (Figure 34). This novel discovery of gigantol activity could support the future development of the compound as an anti-metastasis treatment for cancer patients.

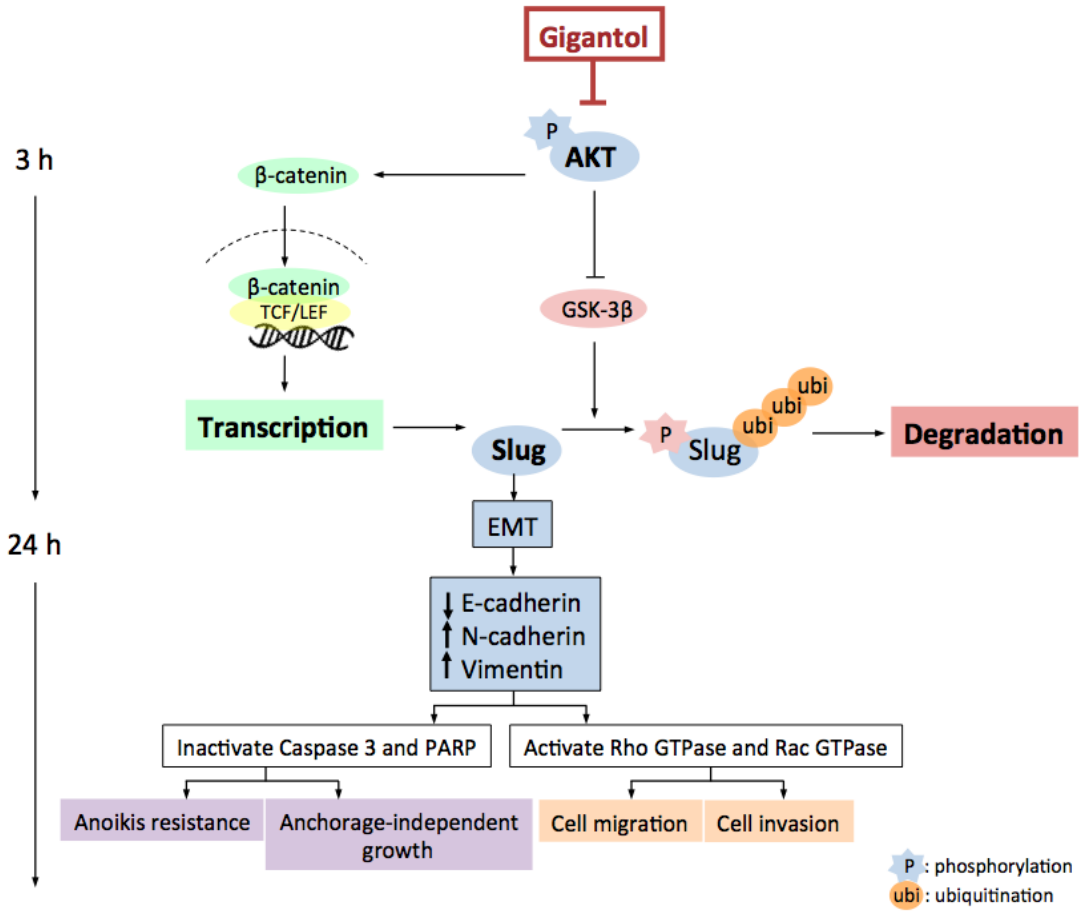


Figure 34 A schematic diagram summarizes the EMT inhibitory mechanism of gigantol on lung cancer cells. Gigantol suppresses the activation of AKT resulting in a decrease in Slug by both decreasing the production and increasing the degradation processes.

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APPENDIX

TABLES OF EXPERIMENTAL RESULTS

Table 1 The effect of gigantol on H460 cell viability

Gigantol (μM)	%Cell viability
0	100.00±0.00
1	94.91±3.99
5	93.04±3.55
10	94.40±5.35
20	93.87±4.29
50	81.18±5.52*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 2 The effect of gigantol on H460 cell apoptosis

Gigantol (μM)	%Cell viability
0	100.00±0.00
1	94.91±3.99
5	93.04±3.55
10	94.40±5.35
20	93.87±4.29
50	81.18±5.52*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 3 The effect of gigantol on A549 cell viability

Gigantol (μM)	%Cell viability
0	100.00±0.00
1	101.90±3.43
5	99.49±4.02
10	101.10±2.12
20	95.78±6.53
50	81.34±3.67*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 4 The effect of gigantol on A549 cell apoptosis

Gigantol (μM)	%Cell apoptosis
0	1.06±0.13
1	1.85±0.34
5	0.56±0.45
10	1.11±0.21
20	4.80±2.65
50	19.43±3.36*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 5 The effect of gigantol on fibroblast cell viability

Gigantol (μM)	%Cell viability
0	100.00±0.00
1	102.72±0.28
5	100.15±1.34
10	100.45±2.01
20	98.19±3.28
50	99.70±2.77

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 6 The effect of gigantol on H460 cell proliferation

Gigantol (μM)	%Cell proliferation at 12 h	%Cell proliferation at 24 h	%Cell proliferation at 48 h	%Cell proliferation at 72 h
0	117.89±3.23	185.34±4.49	295.44±5.86	365.26±14.24
1	116.53±2.73	184.57±3.10	294.24±2.15	363.74±10.21
5	116.18±3.53	180.27±8.49	280.86±4.95	360.48±16.34
10	110.90±3.12	178.31±4.17	279.51±5.88	358.11±8.93
20	114.50±3.53	175.43±5.64	277.19±8.39	355.31±13.09

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 7 The effect of gigantol on A549 cell proliferation

Gigantol (μM)	%Cell proliferation at 12 h	%Cell proliferation at 24 h	%Cell proliferation at 48 h	%Cell proliferation at 72 h
0	121.56±3.21	188.96±3.58	286.63±2.29	353.58±3.36
1	120.84±5.43	175.95±4.71	285.28±3.51	354.12±8.13
5	127.95±8.83	181.18±5.39	283.97±2.09	364.08±4.45
10	128.48±4.61	179.46±7.18	286.83±6.30	359.11±5.05
20	122.26±3.14	175.00±5.66	285.06±8.73	356.90±4.94

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 8 The effect of gigantol on H460 cell anoikis resistance

Gigantol (μM)	%Cell viability at 0 h	%Cell viability at 3 h	%Cell viability at 6 h	%Cell viability at 9 h	%Cell viability at 12 h	%Cell viability at 24 h
0	100.00±0.00	91.58±1.19	84.14±3.05	75.98±1.98	72.90±0.91	62.83±2.41
1	100.00±0.00	88.01±1.75	78.23±1.43	66.88±0.12	61.29±1.78	50.54±2.11*
5	100.00±0.00	87.53±4.79	76.25±0.34*	65.55±0.65*	59.31±2.36*	46.79±0.80*
10	100.00±0.00	82.67±3.91	71.97±1.48*	61.56±2.02*	50.09±3.93*	39.78±1.18*
20	100.00±0.00	77.52±0.57*	62.45±1.83*	52.18±1.48*	43.68±3.06*	30.87±1.71*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 9 The effect of gigantol on A549 cell anoikis resistance

Gigantol (μM)	%Cell viability at 0 h	%Cell viability at 3 h	%Cell viability at 6 h	%Cell viability at 9 h	%Cell viability at 12 h	%Cell viability at 24 h
0	100.00±0.00	87.79±0.02	68.96±0.01	53.54±0.01	43.31±0.01	18.07±0.02
1	100.00±0.00	86.98±0.01	67.46±0.05	50.53±0.00	42.91±0.07	17.85±0.01
5	100.00±0.00	88.34±0.02	68.54±0.01	51.97±0.04	42.62±0.02	15.76±0.02
10	100.00±0.00	87.10±0.01	65.34±0.01	46.81±0.01	40.33±0.02	12.75±0.01
20	100.00±0.00	85.46±0.01	63.53±0.01	45.79±0.01	38.41±0.02	8.52±0.01

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 10 The effect of gigantol on H460 cell colony formation

Gigantol (μM)	%Colony number	%Colony diameter
0	100.00±0.00	100.00±0.00
1	81.65±3.86*	81.89±3.98*
5	67.72±3.85*	75.13±2.67*
10	60.76±2.94*	72.58±0.85*
20	40.51±1.71*	58.30±0.77*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 11 The effect of gigantol on H460 cell scratch assay

Gigantol (μM)	Relative migration at 24 h	Relative migration at 48 h	Relative migration at 72 h
0	1.00±0.00	1.47±0.00	2.49±0.00
1	0.78±0.07*	1.27±0.16*	1.76±0.29*
5	0.62±0.06*	0.97±0.29*	1.51±0.14*
10	0.62±0.10*	0.76±0.19*	0.96±0.11*
20	0.24±0.10*	0.54±0.18*	0.69±0.13*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 12 The effect of gigantol on H460 transwell migration

Gigantol (μM)	Relative migration level
0	1.00±0.00
1	0.75±0.06*
5	0.49±0.03*
10	0.45±0.02*
20	0.36±0.03*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 13 The effect of gigantol on A549 cell scratch assay

Gigantol (μM)	Relative migration at 24 h	Relative migration at 48 h	Relative migration at 72 h
0	1.00±0.00	1.13±0.00	1.13±0.00
1	0.97±0.07	1.11±0.09	1.11±0.04
5	0.97±0.09	1.15±0.07	1.15±0.08
10	0.84±0.09*	1.13±0.05	1.13±0.05
20	0.76±0.05*	1.11±0.03	1.11±0.05

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 14 The effect of gigantol on A549 transwell migration

Gigantol (μM)	Relative migration level
0	1.00±0.00
1	0.95±0.1
5	0.94±0.08
10	0.90±0.08
20	0.86±0.07

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 15 The effect of gigantol on H460 transwell invasion

Gigantol (μM)	Relative invasion level
0	1.00±0.00
1	0.74±0.04*
5	0.66±0.05*
10	0.56±0.03*
20	0.31±0.03*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 16 The effect of gigantol on A549 transwell invasion

Gigantol (μM)	Relative invasion level
0	1.00±0.00
1	0.96±0.08
5	0.96±0.05
10	0.94±0.08
20	0.88±0.09

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 17 The effect of gigantol on EMT molecular marker

Gigantol (μM)	E-cadherin	N-cadherin	Vimentin
0	1.00±0.00	1.00±0.00	1.00±0.00
1	1.17±0.05	0.95±0.02	0.79±0.02*
5	1.27±0.16*	0.72±0.01	0.56±0.04*
10	1.44±0.14*	0.51±0.01*	0.60±0.04*
20	1.46±0.12*	0.50±0.03*	0.50±0.01*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 18 The effect of gigantol on snail and slug transcription factors at 3 h

Gigantol (μM)	Snail	Slug
0	1.00±0.00	1.00±0.00
1	0.97±0.03	0.88±0.12
5	0.93±0.08	0.87±0.06*
10	0.94±0.05	0.84±0.04*
20	0.95±0.09	0.78±0.12*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 19 The effect of gigantol on snail and slug transcription factors at 24 h

Gigantol (μM)	Snail	Slug
0	1.00±0.00	1.00±0.00
1	1.03±0.06	0.84±0.04*
5	0.97±0.04	0.72±0.10*
10	0.95±0.10	0.66±0.12*
20	0.96±0.10	0.47±0.03*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 20 The effect of gigantol on slug production pathway at 3 h

Gigantol (μM)	Integrin α5	Integrin αV	Integrin β1	Integrin β3	β-catenin	p-AKT/AKT
0	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
1	1.06±0.05	1.18±0.13	0.95±0.06	0.98±0.03	0.91±0.14	1.01±0.05
5	1.00±0.09	1.25±0.10	1.20±0.17	0.98±0.14	0.84±0.08*	0.86±0.00*
10	0.93±0.08	1.17±0.11	1.09±0.10	1.01±0.12	0.88±0.07*	0.86±0.02*
20	0.91±0.05*	1.30±0.01*	1.10±0.14*	1.09±0.03*	0.70±0.14*	0.63±0.06*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 21 The effect of gigantol on slug production pathway at 24 h

Gigantol (μM)	Integrin α5	Integrin αV	Integrin β1	Integrin β3	β-catenin	p-AKT/AKT
0	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
1	1.02±0.11	1.10±0.19	1.01±0.01	0.97±0.05	1.05±0.02	0.98±0.03
5	1.03±0.07	1.14±0.19	1.00±0.18	0.99±0.15	1.03±0.01	0.97±0.04
10	0.98±0.06	1.14±0.11	0.97±0.16	1.01±0.18	1.01±0.03	1.01±0.07
20	0.96±0.04	1.03±0.14	0.96±0.26	1.04±0.15	1.01±0.01	0.84±0.06*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 22 The effect of gigantol on slug degradation

Treatment	Slug
Control	1.00±0.00
Gigantol 20 μM	0.65±0.07*
Lac 10 μM	0.94±0.03#
CMA 1 μM	0.57±0.10*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells. #p < 0.05 versus gigantol treated cells.

Table 23 The effect of gigantol on slug ubiquitination

Treatment	Slug
Control	1.00±0.00
Gigantol 20 μM	1.48±0.08*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 24 The effect of gigantol on slug degradation pathway at 3 h

Gigantol (μM)	p-AKT/AKT	p-GSK-3β/GSK-3β
0	1.00±0.00	1.00±0.00
1	1.02±0.05	0.98±0.14
5	0.96±0.02	0.85±0.08*
10	0.84±0.05*	0.81±0.07*
20	0.67±0.05*	0.53±0.09*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 25 The effect of gigantol on slug degradation pathway at 24 h

Gigantol (μM)	p-AKT/AKT	p-GSK-3β/GSK-3β
0	1.00±0.00	1.00±0.00
1	1.14±0.05	0.99±0.06
5	0.96±0.02	1.01±0.00
10	0.96±0.07	1.03±0.06
20	0.75±0.06*	0.99±0.06

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 26 The effect of gigantol on anoikis resistance effector proteins

Treatment	cleaved PARP/PARP	Caspase-3/ Procaspase-3
Gigantol 0 μM	1.00±0.00	1.00±0.00
Gigantol 1 μM	1.36±0.14*	3.59±0.18*
Gigantol 5 μM	2.32±0.07*	5.26±0.42*
Gigantol 10 μM	2.24±0.19*	5.14±0.45*
Gigantol 20 μM	3.97±0.17*	8.94±0.66*
Cisplatin 50 μM	10.44±0.32*	11.47±0.70*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

Table 27 The effect of gigantol on migration effector proteins

Gigantol (μM)	Rho GTP	Rac GTP
0	1.00±0.00	1.00±0.00
1	0.84±0.00*	0.93±0.01
5	0.35±0.03*	0.79±0.04*
10	0.43±0.05*	0.87±0.00*
20	0.31±0.01*	0.76±0.08*

The data represent mean ± SD (n=4). *p < 0.05 versus untreated control cells.

VITA

Thitita Unahabhokha was born on the 4th of May 1990 in Bangkok, Thailand. She finished her elementary and secondary programs in Satit Prasarnmit School (PSM). In 2008, she started her undergraduate degree in biomedicine majoring in human structure and function at the University of Melbourne, Australia. After 3 years, she moved back to Thailand and entered the Faculty of Cosmetic science, Mae fah laung University, Chiangrai, for her Master degree together with enrolling in the International Graduate Program in Pharmaceutical Technology, Faculty of Pharmaceutical Science, Chulalongkorn University for her Doctorial study. In August 2014, she received her Master of Cosmetic Science degree with outstanding score. She has always been an enthusiastic learner and loves to gain new knowledge. She likes to teach and is interested in running her own business.