EFFECTS OF INTEGRIN SUBTYPES ON MIGRATION AND INVASION IN PRIMARY LUNG CANCER CELLS IN ADVANCED STAGE AND INHIBITORY EFFECT OF MILLETTOCALYXIN B

Miss Pennapa Lafauy

A Thesis Submitted in Partial Fulfillment of the Requirements
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

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By	Miss Pennapa Lafauy
Field of Study	Physiology
Thesis Advisor	Professor PITHI CHANVORACHOTE, Ph.D.

Accepted by the GRADUATE SCHOOL, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

.... Dean of the GRADUATE SCHOOL
(Associate Professor YOOTTHANA CHUPPUNNARAT, Ph.D.)

THESIS COMMITTEE

.... Chairman
(Associate Professor THONGCHAI SOOKSAWATE, Ph.D.)

.... Thesis Advisor
(Professor PITHI CHANVORACHOTE, Ph.D.)

.... Examiner
(Associate Professor VARISA PONGRAKHANANON, Ph.D.)

.... External Examiner
(Associate Professor Uraiwan Panich, Ph.D.)

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สารออกฤทธิ์มุ่งเป้าต่ออินทีกรินมีประโยชน์ในทางคลินิกในผู้ป่วยหลายราย ในการศึกษานี้ได้ทำการศึกษาฤทธิ์ของสารมิลเล็ทโทคาลีซิน บี ที่สกัดจากเปลือกลำต้นของ *Millettia erythrocalyx* ต่อเซลล์มะเร็งปอด ความอยู่รอดของเซลล์มะเร็งปอดถูกทดสอบด้วยวิธี 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazoliumbromide (MTT assay) สำหรับความสามารถในการเคลื่อนที่ (migration) และการรุกรานของเซลล์มะเร็งปอด (invasion) ทดสอบโดยการย้อมสี Phalloidin-rhodamine เพื่อดูการเกิด filopodia อีกทั้งวิเคราะห์โปรตีนและสัญญาณภายในเซลล์ที่เกี่ยวข้องกับการควบคุมการเคลื่อนที่ ผลการศึกษาพบว่า ความเข้มข้นที่ไม่เป็นพิษ ของ millettocalyxin B (0-25 ไมโครโมลาร์) สามารถลดการเคลื่อนที่และรุกรานของเซลล์มะเร็งปอดชนิด A549 ได้ และสารยังสามารถลด จำนวน Filopodiaต่อเซลล์ได้อย่างมีนัยสำคัญ นอกจากนี้ระดับของโปรตีนที่ควบคุมการเคลื่อนที่คือโปรตีน integrin α5, active FAK, active Akt และ Cdc42 ลดลงอย่างมีนัยสำคัญในเซลล์ที่ได้รับสารมิลเล็ทโทคาลีซิน บี ผลการวิจัยในครั้งนี้แสดงให้เห็นฤทธิ์ในการยับยั้งการเคลื่อนที่และการรุกรานของเซลล์มะเร็งปอดและกลไกในการออกฤทธิ์ของสารมิลเล็ทโทคาลีซิน บี ซึ่งอาจนำไปใช้ในการรักษามะเร็งปอดได้ในอนาคต

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สาขาวิชา	สรีรวิทยา (สหสาขาวิชา)	ลายมือชื่อนิสิต ....
ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

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Integrin-targeting compounds have shown clinically significant benefits in many patients. Here, we examined the activity of millettocalyxin B, extracted from the stem bark of *Millettia erythrocalyx*, in lung cancer cells. The viability of human lung cancer cell was investigated by the 3-(4,5- dimethylthiazol-2-yl)-2,5diphenyl tetrazoliumbromide (MTT) assay. Migration and invasion assays were performed. Phalloidin-rhodamine staining was used to determine the formation of filopodia. Western blot analysis was used to identify the signaling proteins involved in migration regulation. Non-toxic concentrations (0-25 μM) of millettocalyxin B reduced migration and invasion of lung cancer A549 cells. Filopodia were significantly reduced in millettocalyxin B treated cells. The migration regulatory proteins including integrin α5, active FAK, active Akt, and Cdc42 were significantly decreased in Millettocalyxin B-treated cells. Our findings revealed a novel anti-migration and anti-invasion effects and the underlying mechanism of millettocalyxin B, which may be exploited for cancer treatment.

Field of Study:	Physiology	Student's Signature ....
Academic Year:	2021	Advisor's Signature ....

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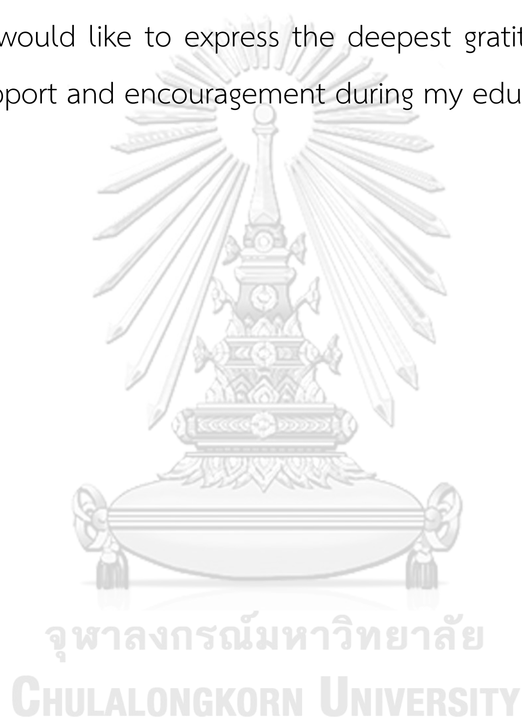

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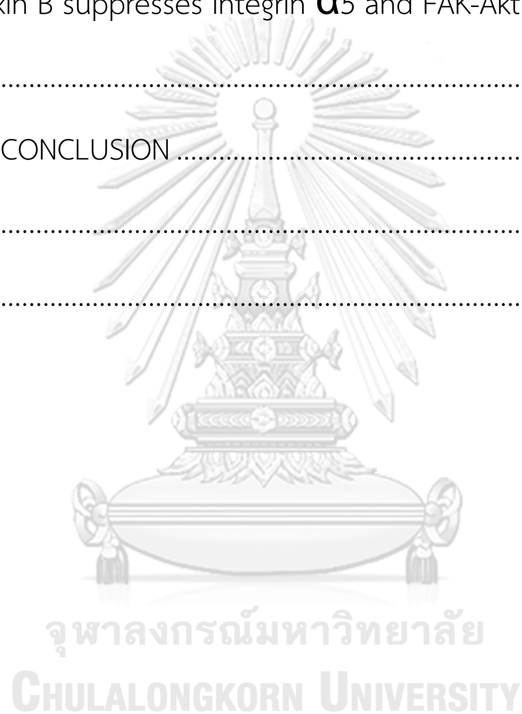

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CHAPTER I
INTRODUCTION

1.1 Background and rational

Lung cancer has become a major public health issue worldwide, with a high incidence and mortality rate (1). Cancer metastasis is responsible for the great majority of cancer-related deaths (2), (3). Cell migration is a multi-step process controlled by signaling molecules such as integrin, focal adhesion kinase (FAK), Akt, and RhoGTPases (4), (5), (6), (7). Focal adhesion kinase (FAK) can regulates cell proliferation, survival, and cell migration (8). FAK expression links to the ability of cancer cells to migrate and invade (9). A previous study found that FAK can be activated via an Integrin-dependent mechanism and plays a key role in cell migration (10). Integrins are also known to regulate cancer cell survival and motility and have been recognized as important anti-metastatic drug targets (11). Integrins α5, and integrin β1 have been shown to augment the survival and metastatic potential of lung cancer cells (12). In lung cancer, integrins α5 and β1 have been demonstrated to induce FAK activation, leading to cancer cell movement (13). In addition, integrin α5 and β1 promote cell contraction and transmission are resulted from integrin α5 and β1 activation(14). These integrin types are considered as highly motile cell markers(11). The activation of protein kinase B (Akt) is triggered from integrin-dependent activation of FAK. It promotesthe phosphorylation at serine 473 on Akt protein which is the functional form(15), (16), (17). Rho-GTPase and cell division cycle 42 (Cdc42) are down-stream targets of FAK, regulating cell migration and filopodia (18). In lung cancer, Akt (protein kinase B) is phosphorylated and the active Akt is correlated with cancer metastasis (15). Several reports have shown that Akt is a downstream

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target of FAK (19). The characteristics of the integrin pattern in advanced stage lung cancer cells are interesting as they may represent the regulatory signal of migration and can be novel molecular targets for drug discovery. Unfortunately, the specific targets in anti-metastasis are under-extrapolated and present drugs are not sufficient to prevent metastasis activities of cancers .

Consequently, these types of target alterations can ultimately lead to drug and can prevent cancer metastasis. Therefore, the new compounds from several types of medicinal plants with anti-cancer activities as well as antimetastatic effects would be beneficial for the disease managements. Natural compounds obtained from plants have recently received increased interest as possible medications or lead compounds in drug development (20). Several studies have been revealed that flavonoids such as cycloartobiloxanthone, Artonin E and Phoyunnanin E, have shown the potential inhibits cell migration and invasion in lung cancer (21), (22), (23). This study focuses on the activity of millettocalyxin B as a flavonoid. Millettocalyxin B is a phenolic compound, group flavonoid from the stem bark of *Millettia erythrocalyx* Gagnep (24). This compound has a variety of pharmacological effects, including antioxidant, anti-herpes simplex virus, anti-allergy, and anti-inflammatory properties (25), (26), (27). However, the effect of millettocalyxin B on migration and its underlying mechanism have not been investigated. To reveal the potential use of millettocalyxin B for anti-metastasis approaches, this study aimed at investigating its inhibitory effect on integrin mediated lung cancer cell migration and invasion.

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1.2 Objective

1.2.1 To investigate expression of integrin subtypes that relate to migration and invasion of primary lung cancer cells.

1.2.2 To investigate the effect of millettocalyxin B on the integrin subtypes in lung cancer cells.

1.3 Research question

1.3.1 Which subtypes of integrin that promote migration and invasion in primary lung cancer cells in advanced stage?

1.3.2 How does millettocalyxin B affect expression of integrin subtypes in lung cancer cells?

1.4 Hypothesis

1.4.1 Primary lung cancer cells in advance stage could overexpress integrin $\alpha5$ which lead to aggressive migration and invasion.

1.4.2 Millettocalyxin B could inhibit the integrin $\alpha5$ level and suppress migration and invasion.

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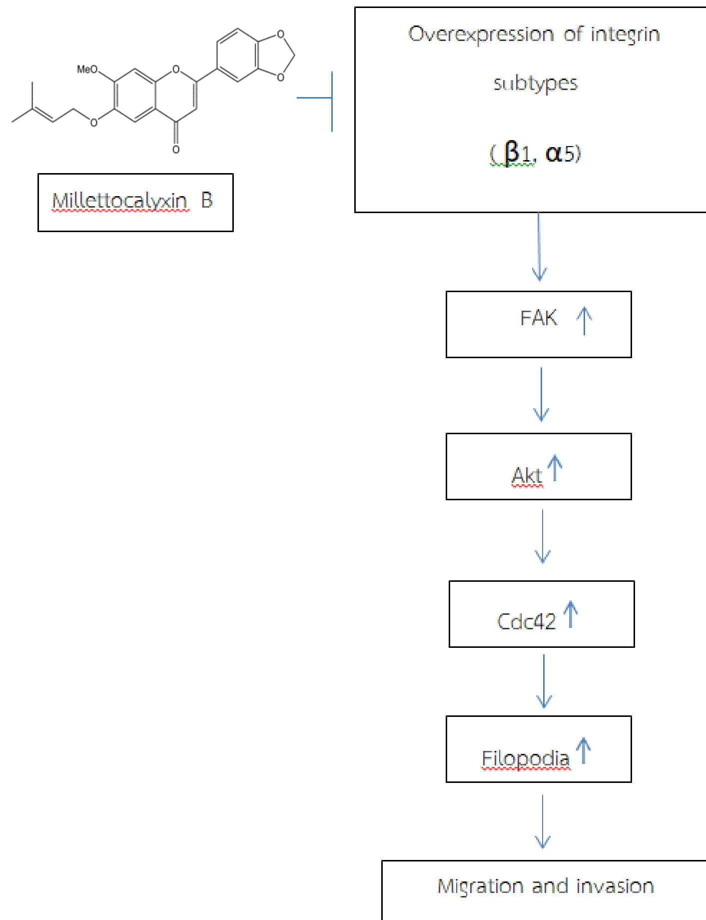
1.5 Conceptual framework



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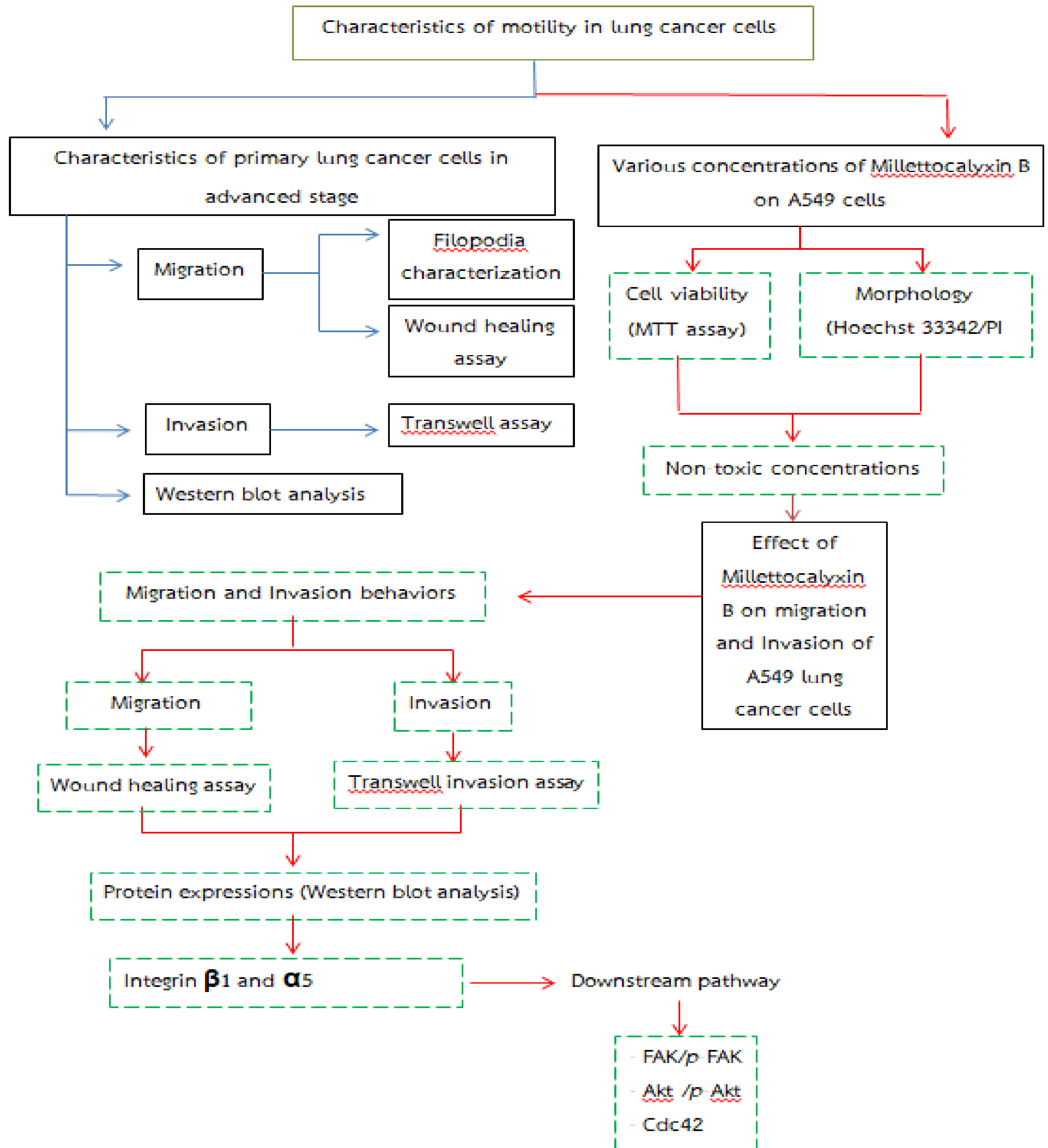
1.6 Research design



CHAPTER II

LITERATURE REVIEWS

2.1 Lung cancer

Lung cancer is the leading cause of cancer mortality in people from all over the world (28). There were 17 million new cases and 9.6 million cancer deaths from cancer worldwide. Lung cancer is the most common diagnosed cancer accounted for approximately 11.6% of all cancer cases (29). Importantly, cancer cells can invade into nearby tissue, and spread through the blood-stream to other organs in the body, also known as metastatic cancers. Cancer metastasis has long been proposed as one important factor for high mortality. Therefore, the novel anticancer strategy focusing on inhibition of cancer cell metastasis is in need and is believed to improve the clinical outcome for cancer treatment.

2.2 Type of lung cancer

Histologically, the World Health Organization (WHO) classified lung cancer into two broad categories based on its biology: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which develops from bronchial epithelial progenitor cells (30). NSCLC can be further divided into 3 major histological subtypes: squamous-cell carcinoma (which accounts for around 25%–30% of all cancer cases), adenocarcinoma (which accounts for approximately 40% of all cancer cases), and large-cell carcinoma (which accounts for approximately 5–10% of lung cancer cases) (31).

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2.3 Cell migration

Cancer cell migration is the integral components of metastatic disease, which is the main cause of death in cancer patients (32). Cell migration can be divided into two main types, single-cell and collective cell migration(33). Dynamically, new interactions between cells and basement matrix are made in front of protrusion areas, and the old adhesions are disassembled in the rear bodies of cells that drive the cell to move forward in response to a gradient of stimulating factors or ECM proteins.

2.4 Molecular mechanism of migration

Mechanism of cancer migration can be divided into 4 stages including

First: The engagement of adaptor proteins and the actin filament cause the arrangement of cell membrane in a specific direction. The protein actin interacts to the protein complex named ARP2/3 (actin relating protein 2/3) and link the actin-complex to WASP (Wiscott-Aldrich syndrome protein) that activates the polymerization of actin (34). ARP2/3/WASP complex then recruits PIPs (phosphoinosites) at the cell membrane (34). ARP2/3 and actin filament can also form the actin network branches (35). For mechanism, PIPs was shown to activate GEFs (guanine-nucleotide exchange factors) that intern regulates Cdc42, a protein belong to the family of small GTPases protein (36). Cdc42 can function with the PIPs and WASP and initiate filopodia formation as well as pseudopod extensions which are important of the cell movement (37). PIPS production is a result of PI3K (phosphatidylinositol 3-kinase) and phosphatidylinositol 4, 5-kinase (38).

Second: the interaction and link of ECM components to protein integrins cause the clustered integrin at the cell membrane (39). Integrin cluster induces the

formation of complex of adaptor and signaling proteins. Activity of kinases in the complex generate the cell signal via phosphorylation (40). These kinases include FAK and proteins that connect to adaptor and form actin-binding proteins and recruit PI3K enzyme or Rho GTPases (Cdc42) to focal contact(39), (41), (42).

Third: Active myosin binds to actin filaments and activates the cell contraction. MLCK (myosin light- chain kinase) activates Myosin light chain and stimulates myosin II. The contraction of active myosin is regulated by ROCK (43).

Fourth: the dissociation of focal adhesion at trailing edge. This process can induce by several mechanisms including phosphorylation of FAK at tyrosine 397 (44). When cell moves forward, the old cell adhesion complex will be proteolytic cleavage to destroy the focal contact. The integrins at old focal contact will be internalized via endocytosis and recycling to the leading site and form the new focal contact(45).

2.5 Integrins

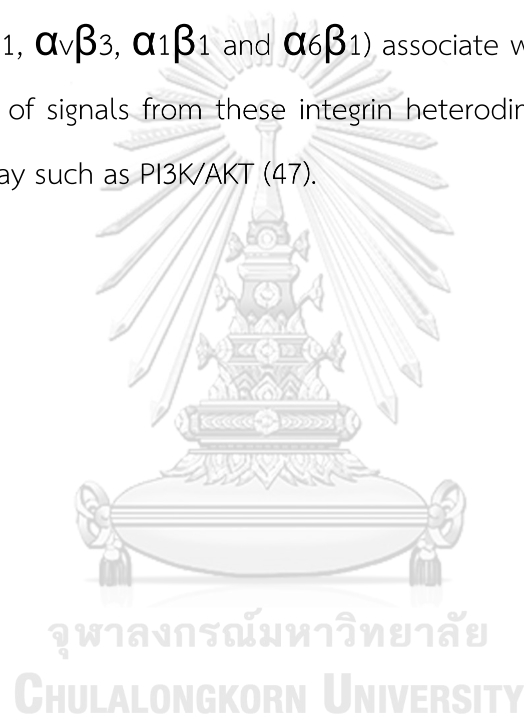
2.5.1 Integrin structure and family

Integrins are protein family of transmembrane glycoprotein receptors. Integirns are known for the control and activation of cell interaction and adhesion to ECM and activate the arrangement of actin cytoskeleton during cellular movement. Integrins can form 24 different heterodimers from 18 α-subunits and 8 β-subunits (40). The binding of heterodimer of integrins is mediated via non-covalent bond link between subunit (40).

2.5.2 Function of integrins

The main functions of integrins are binding the actin cytoskeleton to the extracellular matrix (ECM) and transduction of signal to the cell. These signals are important for cell migration, cell invasion, and survival.

In terms of cell migration, integrin binds to ECM protein and provide the traction force for the movement. In the first steps, a ligand of integrin activates focal adhesion kinase (FAK), It can stimulate the Rho GTPase family such as Rho, Rac, and Cdc42 (46). Furthermore, previous reports showed that heterodimers of integrins ($\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha 1\beta 1$ and $\alpha 6\beta 1$) associate with increased cell survival. The activation of signals from these integrin heterodimers are able to stimulate survival pathway such as PI3K/AKT (47).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2.6 Phytochemistry

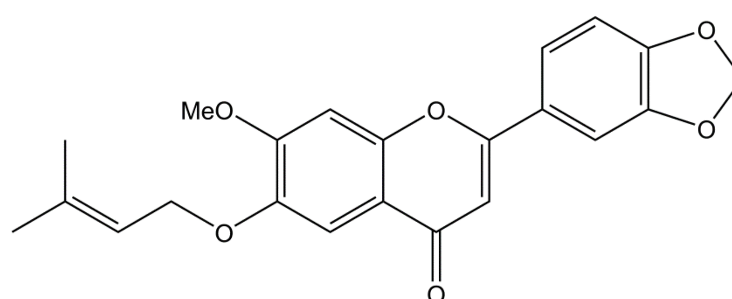

Figure 1. structure of millettocalyxin B

Millettocalyxin B is a phenolic compound, extracted from the stem bark of *Millettia erythrocalyx* Gagnep, with molecular weight of 380.4 g/mol (48). The *Millettia* species have been used for antibacterial, anti-tumor, insecticidal, pesticide (49) anti-spasmodial, chemopreventive (50) joint pain, rheumatoid arthritis, amenorrhea, tuberculosis, etc. *Millettia erythrocalyx Gagnep* has a local name as Jun. It is a medium-sized tree that grows to a height of 7-8 meters. Bark: grayish. Rusty strigose branchlets that grows glabrescent with conspicuous spotted lenticels. 7–11 leaflets, ovate-or elliptic-lanceolate, papery, 3-6 x 1.5-2 cm, broadly cordate base, caudate apex, glabrous and shiny adaxially, crimson strigose on midrib and edges abaxially, glabrous and shiny adaxially, crimson strigose on midrib and edges abaxially. Pseudoracemes: axillary, 6-7 cm, thickly dark brown strigose; flower 8-9 mm, calyx 3 mm, deep red, sparsely hairy, teeth truncate, ciliated; corolla lilac, vexillum glabrous, round, tapering at base, with 2 minute callus; ovary villose, ovules 4-5. Pod: linear-oblong, 9-10 x 2 cm, flat, slightly curved, tapering to the base, brown tomentose when young, glabrescent when mature, woody, spirally twisted valves.

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Seed: 2-3, chestnut brown in color, 13 x 10 mm, lens-shaped, and smooth. Thailand, Laos, and Cambodia have all discovered it (51).

Traditional Uses and Biological Activities of *Millettia* Constituents.

Evidences show that several parts of the *Millettia* plants have been widely applied for intestinal parasite treatment and colic treatment in children (52). The gargle made from the bark pulp of M. zechiana, Guinea grains, and sea water has been used to relieve rhinopharyngal and bronchial troubles (53). Moreover, the seed and the other parts of *Millettia* plants are reported to have insecticidal, piscidal and molluscicidal activities. According to activities, Ethiopian used the seeds of M. ferruginea as fish poisoning (54). Extracts and aqueous suspension of M. pachycarpa seeds possess an insecticidal activity against several insects, e.g. bean aphids, houseflies, pentatomids, bean aphids, cabbage worms, and leaf beetles. In addition, they also have stomach toxicity, contact poisons and ovicidal activity (55).

Bioactive flavonoids found in *Millettia* plants as shown in Table 1 have been studied in breast cancer cells and found that they can inhibit cancer cell migration and invasion significantly. Herein, we have demonstrated that millettocalyxin B extracted form stem bark of *Millettia erythrocalyx* Gagnep, also exhibited the inhibitory effect on cell migration and invasion in lung cancer cells (A549).

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Table 1. Biological compounds that have an anti-cancer ability.

Compound	Anti- cancer properties	Reference
Cycloartobiloxanthone	Inhibit migration and invasion of lung cancer cells	(21)
Artonin E	Inhibit migration and invasion of lung cancer cells	(22)
Phoyunnanin E	Inhibit migration of lung cancer cells	(23)

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CHAPTER III

MATERIALS AND METHODS

3.1 Study population

Thirty-four patients with histologically or cytological confirmed non-small cell carcinoma of the lung with 75 malignant pleural effusion receiving treatment at the King Chulalongkorn Memorial Hospital were prospectively 76 enrolled during February to June 2019. Pleural effusion was collected as part of standard practice for diagnosis or 77 supportive treatments to relieve symptoms. This study was approved by the 79 Ethical Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB 365/62). All 80 patients agreed to participate with signed inform consent.

3.1.2 Protocol for cancer cell preparation from malignant pleural effusion.

Primary cancer cells were obtained from pleural effusion (500–1000 ml) by thoracentesis and collected aseptically in heparinized. Samples were centrifuged at 300 g for 10 min, at 4 °C, and cell pellets were resuspended in 10 % FCS-RPMI (Invitrogen). Viability was determined by Trypan Blue exclusion dye. After a harvesting and 2 rounds of washing step, the cells were subjected to 10-15 passages of cell cultivation

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3.2 Cell cultures

Primary lung cancer cells in advanced stage (ELC08, ELC16, ELC17) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), and streptomycin and penicillin (Gibco, MD, USA) 100 unit/ml at temperature 37 °C with 5% CO_2 in incubator. The

cells were washed with phosphate buffer saline (PBS) 1 ml and trypsinized by trypsin-EDTA. Cells will be changed medium every 2-3 days.

Table 2. Primary lung cancer cells and lung cancer cell line description

Cells	Tissue	Pathology	Molecular alteration	Patient character
Primary lung cancer cells				
ELC08	Pleural effusion	Lung adenocarcinoma	N/A	82-year-old male,post Rx:erlotinib
ELC16	Pleural effusion	Lung adenocarcinoma	EGFR exon 19 deletion	33-year-old female ,post Rx:CK101
ELC17	Pleural effusion	Lung adenocarcinoma	N/A	73-year-old female ,post Rx:Carboplatin
lung cancer cell line				
A549	lung	Lung carcinoma	Kras mutation	51-year-old male

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Primary lung cancer cells have been used to test the concept of tumor biology, and identified potential new treatments. Malignant pleural effusion (MPE) was found in one-sixth clinical presentation at the initial diagnosis of advanced stage NSCLC. Primary lung cancer cells from malignant pleural effusion has been showed to have high efficiency in in-vitro growth and propagation (56).

The lung cancer cell, A549 cell was used as a cell culture models in the present study. A549 cells contains Kras mutation. Kras mutation in lung cancer was show to associate with increased metastatic potentials (57). Ras mutations are found in around 30% of lung cancer and Kras being the most common

member of the mutated family (58). Kras actions in the transducing signal of receptor epidermal growth factor receptor (EGFR), AKT and ERK pathways that in turn affect cell growth, differentiation and survival (59). As we focus on the migration process, Kras is known to activate migration, resulting in tumor progression and metastasis. In terms of cell migration, several studies have utilized A549 cell lines for investigating migratory activity. For instance, Xia Rongmu et al. showed the anti-migration and anti-invasion of hesperidin compound in A549 cells (60). Also, Ophiopogonin B was demonstrated to inhibit metastasis-related activities of A549 cells (61).

3.3 Isolation of millettocalyxin B

Millettocalyxin B was isolated from the stem bark of *M. erythrocalyx*. Millettocalyxin B will be dissolved in DMSO and media to achieve the desired concentrations, the stock solution will be diluted with DMSO concentrations less than 0.1% at final concentrations.

Dried and powdered stem bark of *Millettia erythrocalyx* (2 kg) was macerated with ethyl acetate at room temperature, giving an ethyl acetate extract (37 g). The ethyl acetate extract was fractionated by vacuum liquid chromatography (silica gel, ethyl acetate-hexane, gradient) to give 9 fractions (A-I). Fraction I (8.8 g) was separated by a polyamide column eluted with the mixture of ethanol and water (1:4) and further isolated on Sephadex LH-20 (methanol) to give 13 fractions (IA-IM). Fraction IL (1.8 g) was purified by medium-pressure liquid chromatography (silica gel, ethyl acetate-petroleum ether, 1:4) to give 22 fractions (IL1-IL22). Fraction IL11 (235 mg) was separated by column chromatography (silica gel, ethyl acetate- petroleum ether, 3:7) and further repeated column chromatography (silica gel, ethyl acetate-dichloromethane, 1:9) to afford millettocalyxin B (19 mg).

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Plant Material.

The stem bark of *M. erythrocalyx* Gagnep. was collected from Tayang district, Petchaburi Province, Thailand, in April 1999. Authentication was performed at the Royal Forest Department, Ministry of Agriculture and Cooperatives, and a voucher specimen (KL-032542) is on deposit at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

3.4 Reagents

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
- Propidium iodide (PI)
- Hoechst 33342
- Triton X-100
- Phalloidin-rhodamine fluorescent
- Bovine serum albumin (BSA)
- glycerol
- Fetal bovine serum (FBS)
- antibodies for integrin α5, β1, p-Akt (Tyr308), Akt, p-FAK (Tyr397), FAK, β-actin and secondary antibodies
- Chemiluminescence substrates

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3.5 Instruments

- Auto-pipette 0.2-2 µl, 2-20 µl, 20-200 µl and 100-1,000 µl (Corning®, USA)
- Automated cell counter (TC20™ Bio-Rad, Singapore)

- Boyden chamber / transwell plate (BD Bioscience, USA)
- Cell culture plate: 6-well and 96-well (Corning®, USA)
- Centrifuge (CF-10 Wise spin®, Korea)
- Conical tube 15 ml and 50 ml (Corning®, USA)
- Carbon dioxide humidified incubator (Thermo scientific, USA)
- Duran bottle 100 ml, 250 ml, 500 ml and 1000 ml (Mainz Germany)
- Fluorescence microscope (Olympus IX51 with DP70, Japan)
- Fluorescence microplate reader (Anthros, Durham, USA)
- Laminar air flow cabinet (Boss tech, Thailand)
- Vortex mixer (Bohemia, USA)

3.6 Method

Cell viability assay

Cells viability was detected by MTT assay. A549 cells (1×10^4 cells/well) were seeded onto 96-well plates and incubated overnight at 37 °C. The cells were then administered with various concentrations of Millettocalyxin B (0-100 µM) for 24 h, followed by treated with 100 µl of MTT solution for 4 h. MTT solution were removed and added 100% DMSO to dissolve the formazan product. The optical density was measured by a microplate reader (Anthros, Durham, NC, USA) at wavelength 570 nm. The percentage of cell viability was calculated form absorbance of Millettocalyxin B treated cells comparative to untreated cells. The next experiment used a non-toxic concentration because the dose needs to be carefully optimized to avoid toxic effects that may affect cell migration.

Nuclear staining assay

A549 cells (1 × 10^4 cells/well) were seeded onto 96 well plates and incubated overnight for cell attachment. After cell attachment, cells were indicated with various concentrations of Millettocalyxin B (0-100 μM) for 24 h. Then, cells were stained with 10 μM of Hoechst 33342 and propidium iodide (PI) (Sigma Chemical, St. Louis, MO, USA) for 15 min at 37 °C. Cell morphology was imaged using a fluorescence microscopy (Nikon ECLIPSE Ts2, Tokyo, Japan) and four random fields were taken a picture at 20× magnification. The percentages of apoptotic and necrotic cells were comparative to non-treatment cells.

Migration assay

Migration was evaluated by wound healing assay. Primary lung cancer cells in advanced stage were cultured as monolayer in 96-well plates at a density 2.5 × 10^5 cells/well. After that, cells were created space of wound using sterile 1-mm-wide pipette tip at the bottom of plate. Then, removed old medium, washed with 1× PBS (Gibco, Grand Island, NY, USA) and then added a fresh medium. Cells were incubated at 37 °C for 24, 48 and 72 h. The migration areas were captured using a phase contrast of microscope (Nikon ECLIPSE Ts2, Tokyo, Japan).

Additionally, A549 cells were cultured as monolayer in 96-well plates. After that, using sterile 1-mm-wide pipette tip (P200 micropipette tip) scratched the bottom of each well. Then, removed medium and washed with 1× PBS (Gibco, Grand Island, NY, USA). Cells were then administering with non-toxic concentrations of Millettocalyxin B (0-25 μM) for 24, 48 and 72 h, respectively. The wound areas were photographed using a phase contrast of microscope (Nikon ECLIPSE Ts2, Tokyo, Japan). The relative cell migration was photographed and analyzed as follows:

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$$\text{Wound closure (\%)} = [(A_0 - A_{\Delta h}) / A_0] \times 100$$

A_0 is the area of the wound measured immediately after scratching (0 h).

$A_{\Delta h}$ is the area of the wound measured at 24, 48 or 72 h after treatment.

Cell invasion assay

Invasion was performed by Trans-well Boyden chamber. Prior to seeding the primary lung cancer cells in advanced stage was applied with 0.5% Matrigel for overnight at 37 ˚C. Thereafter, ELC08, ELC16, and ELC17 cells were seeded at a density 2.5×10^5 cells/well containing with serum free medium in the upper chamber, followed by 10% serum medium were added into the lower chamber and incubated at temperature 37 °C for 24 h. Then, cells were fixed with methanol (Gibco, Grand Island, NY, USA) for 10 min and stained with 10 μM of Hoechst 33342 (Sigma Chemical, St. Louis, MO, USA) for 10 min. Invading cells were captured and visualized by a fluorescence microscopy (Nikon ECLIPSE Ts2, Tokyo, Japan).

Furthermore, A549 cells were pre-treated with Millettocalyxin B at non-toxic concentrations (0-25 µM) for 24 h. The surface upper chamber was coated with 0.5% Matrigel dilution in serum free medium and incubated overnight. After treatments, cells were seeded at a density 2.5×10^5 cells/well containing with serum free medium in the upper chamber and 10% serum media were added into the lower chamber. Then, incubated at 37 °C for 24 h, fixed with methanol (Gibco, Grand Island, NY, USA) for 10 min and stained with 10 μM of Hoechst 33342 (Sigma Chemical, St. Louis, MO, USA) for 10 min. Invading cells were imaged and visualized by a fluorescence microscopy (Nikon ECLIPSE Ts2, Tokyo, Japan). The relative invade cells was calculated as the number of invaded cells of treated cells / the number of invaded cells of non-treated cells.

Filopodia evaluation assay

The morphology and filopodia characterization were examined by a phalloidin-rhodamine staining assay. Primary lung cancer cells in advanced stage were seeded at a density 3×10^4 cells/well in 96-well plates and incubated overnight. After that, washed with 1× PBS (Gibco, Grand Island, NY, USA) and fixed with 4% paraformaldehyde (Sigma Chemical, St. Louis, MO, USA) dilution in 1× PBS (Gibco, Grand Island, NY, USA) for 10 min at room temperature. Then, cells were permeabilized with 0.1% Triton X-100 (Sigma Chemical, St. Louis, MO, USA) for 4 min and blocked non-specified with 0.2% bovine serum albumin (BSA) (Merck, DA, Germany) for 30 min. Finally, stanning with a phalloidin-rhodamine (Sigma Chemical, St. Louis, MO, USA) for 15 min and mounted with 50% glycerol. Cell morphology was observed under fluorescence microscope (Nikon ECLIPSE Ts2, Tokyo, Japan).

In addition, A549 cells were indicated with Millettocalyxin B at non-toxic concentrations (0-25 μM) for 24 h. After treatments, wash with 1× PBS (Gibco, Grand Island, NY, USA) and fixed with 4% paraformaldehyde (Sigma Chemical, St. Louis, MO, USA) dilution in 1× PBS (Gibco, Grand Island, NY, USA) for 10 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 (Sigma Chemical, St. Louis, MO, USA) for 4 min and blocked non-specified with 0.2% bovine serum albumin (BSA) (Merck, DA, Germany) for 30 min. Finally, stanning with a phalloidin-rhodamine (Sigma Chemical, St. Louis, MO, USA) for 15 min and mounted with 50% glycerol. Cell morphologic alterations were visualized under fluorescence microscope (Nikon ECLIPSE Ts2, Tokyo, Japan). Relative spike of filopodia were reported as the number of filopodia divided by number of cells.

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Filopodia are small cellular projections that help cells to move through and sense their environment. Filopodia play crucial roles in processes such as development and wound-healing. Also, increases in filopodia number or size are

characteristic of many invasive cancers and are correlated with increased rates of metastasis. Filopodia can be detected by eye using confocal fluorescence microscopy, and they can be manually annotated in images to quantify filopodia parameters. Manual detection is a significant barrier to the discovery and quantification of new factors that influence the filopodia system (62).

Western blot analysis

Primary lung cancer cells in advanced stage were seeded at density 5×10^5 cells/well and incubated overnight. Thereafter, cells were washed twice with 1× PBS (Gibco, Grand Island, NY, USA) and incubated with lysis buffer on ice for 1 h. Then, all the pellets were placed in the Eppendorf, and centrifuged 5000 rpm for 10 min. The protein concentrations were measured by BCA protein assay kit.

For A549 cells, after treatment with Millettocalyxin B (0-25 μM) for 24 h. Lysis buffer was applied for 1 h on ice. The cellular lysates were collected and the protein concentrations were measured by BCA protein assay kit.

Each protein samples were heated at 95 °C for 5 min. After that, the amounts of proteins approximately 80 μg were loaded into 7.5-10% SDS-polyacrylamide gels. After separation, proteins were transferred into nitrocellulose membranes and blocked with 5% skim milk dilution (Gibco, Grand Island, NY, USA) in TBS-T for 1 h. Then, washed membrane three times with TBS-T for 5 min and probe with primary antibodies against , Integrin α5, Integrin β1, phosphorylated Akt (p-Akt; Ser 473), Akt, p-FAK (p-FAK; Tyr 397), FAK, Cdc42 and β-actin (Cell Signaling, Danvers, MA, USA) for overnight at 4 °C, as well as incubated specific secondary antibodies (Cell Signaling, Danvers, MA, USA) at room temperature for 2 h. Finally, the blots were detected by chemiluminescence (Pierce, Rockford, IL, USA) and quantified using the analyst/PC densitometry software.

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3.7 Statistical analysis

All data are presented as the mean ± SD, derived from at least three independent experiments. Statistical differences between multiple groups were analyzed by analysis of variance (ANOVA) with a post-hoc test for comparing multiple groups at *p*-value significance levels of ≤ 0.05 were considered.

3.8 Expected benefit and application

The results of this study will demonstrate that millettocalyxin B could inhibits migration and invasion in lung cancer cells. This report is beneficial for finding new drug targets, improving and developing in lung cancer therapy.

CHAPTER IV

RESULTS

Part I investigation of migration and invasion behaviors of primary lung cancer cells in advanced stage

4.1 Cell behavior analysis: Migration assay

Migration assay was used to determine the ability of cell to migrate. The primary lung cancer cells from advanced stage lung cancer were culture and evaluated for migration. The cells from different patients were named ELC08, ELC16, ELC17.

Figure 2 Shows that ELC08 and ELC16 were significantly more migratory than A549 cells.

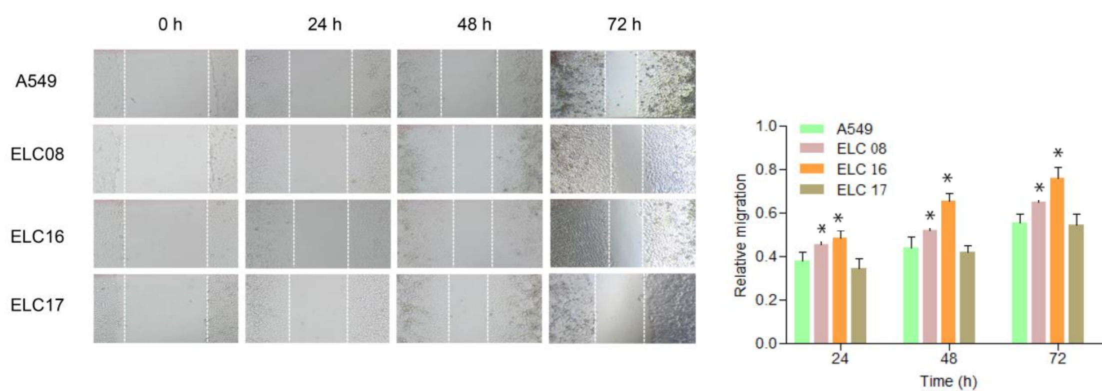

Figure 2. Migratory behavior of A549 and primary lung cancer cells.

The wound healing assay was performed to evaluate cell migration at 24, 48, and 72 h. The values are means of triplicate measurements ±SD (n=3).

4.2 Analysis of filopodia formation

During cell movement, the formation of filopodia relates to increased migration of cancer cells (63). This study aim to investigate filopodia formation of primary lung cancer cells compared to lung cancer cell line. The figure shows that ELC16 and had more filopodia than A549 cells, while ELC17 had the least number of filopodia formation when compared to A549 cells.

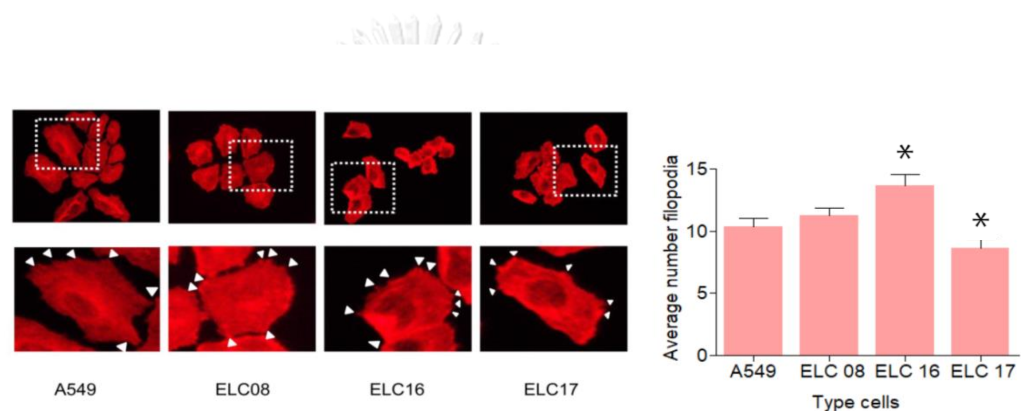

Figure 3. Filopodia formation of A549 and primary lung cancer cells.

The number of filopodia was evaluated. The values are means of triplicate measurements ±SD (n=3).

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4.3 Analysis for invasion activity

Cell invasion was determined using the transwell Boyden chamber assay. Primary lung cancer cells and A549 were added to a 24-well transwell pre-coated with Matrigel. As shown in Figure 4, ELC16 had the ability to invade more than A549, while ELC17 exhibited the lowest ability to invade compared to A549 cells.

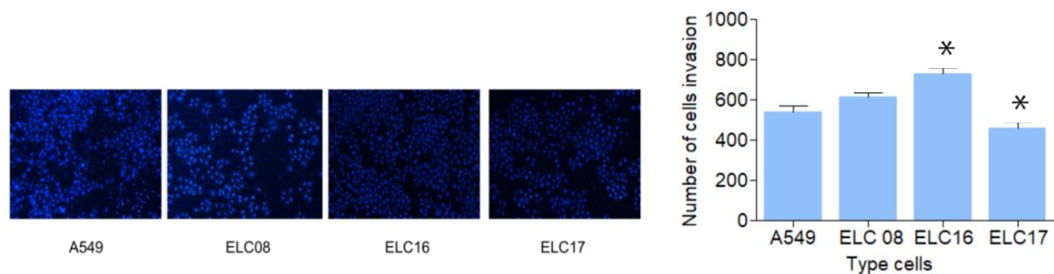

Figure 4. Invasion analysis of A549 and primary lung cancer cells.
The cells were seeded onto Matrigel coated membrane of tranwell for 24 h. The invaded cells were stained with Hoechst 33342 and captured under a fluorescence microscopy. The number of invaded cells was determined. The values are means of triplicate samples±SD (n=3).

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4.4 Evaluation of integrin expressing pattern.

The expression of integrin α5 and β1 in lung cancer cell line and primary lung cancer cells were determined by western blot analysis. As integrin α5 and β1 were linked with high metastasis potential in cancers.

The results showed that ELC16 had the highest level of integrin α5, while ELC17 had the lowest integrin α5 level when compared to A549 cells. However, the levels of integrin β1 were found to be comparable in all cells.

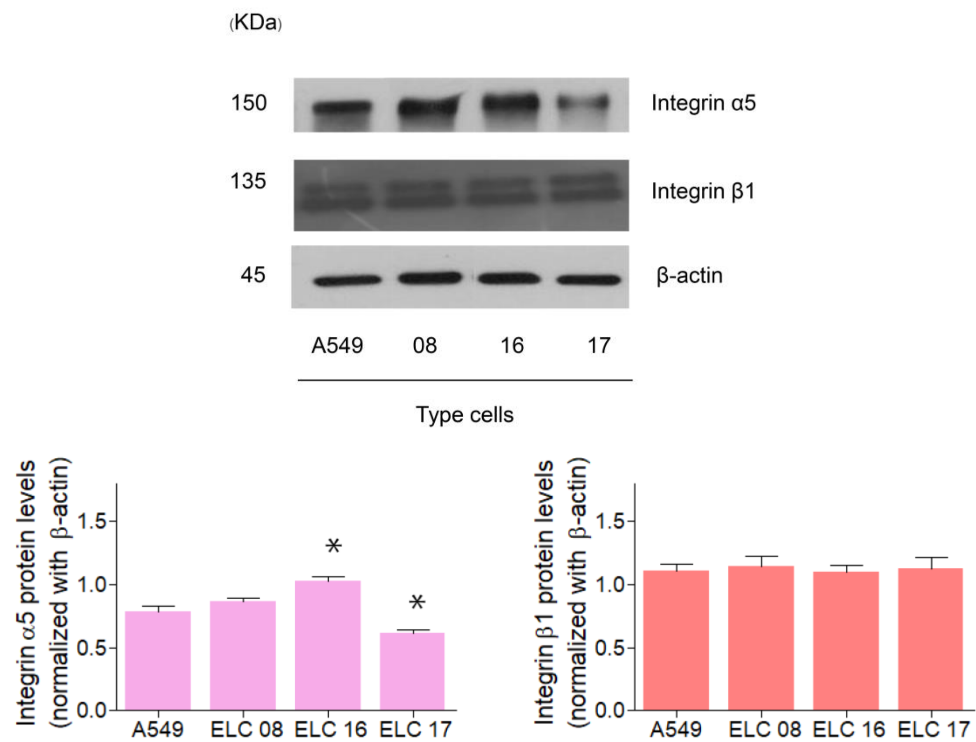

Figure 5. The expression of integrin α5 and β1 were determined in A549 and primary lung cancer cells.

The protein levels were evaluated by western blot analysis followed by densitometry. Values are means are means of triplicate samples±SD (n=3).

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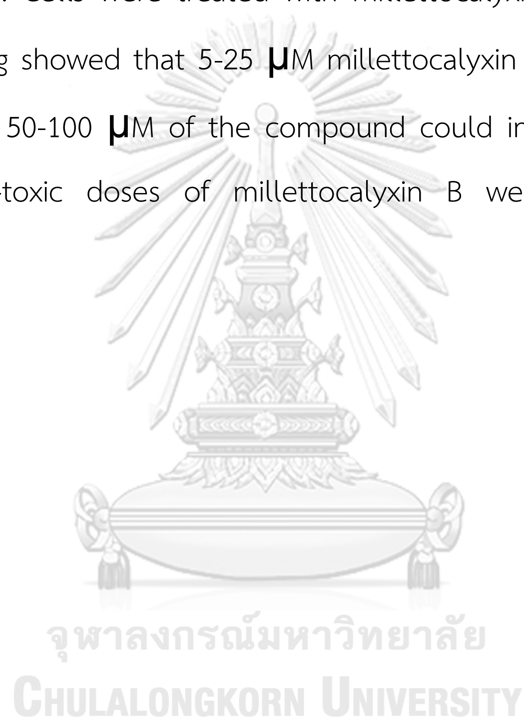
Part II Cytotoxic effect of millettocalyxin B on A549 cells

4.1 The cytotoxicity of millettocalyxin B on A549 cells

We first determined the cytotoxicity of millettocalyxin B on A549 cells by the MTT assay. Cells were treated with millettocalyxin B (0-100 μM) for 24 h. Cell viability assay revealed that millettocalyxin B was not cytotoxic at concentrations less than 25 μM (Figure 6B). A nuclear co-staining assay was used to detect apoptotic and necrotic cells. Cells were treated with millettocalyxin B (0-100 μM) for 24 h. Hoechst/PI staining showed that 5-25 μM millettocalyxin B caused no apoptosis or necrosis, whereas 50-100 μM of the compound could induce significant apoptosis (Figure 6C). Non-toxic doses of millettocalyxin B were used for the further experiments.



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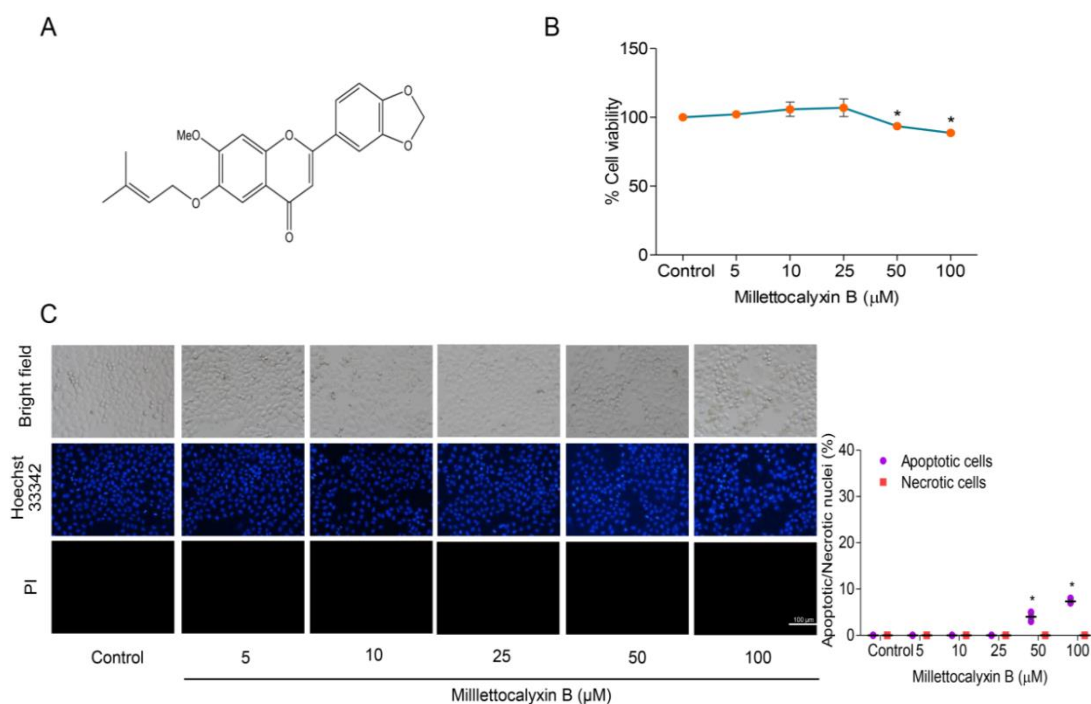

Figure 6. Effect of millettocalyxin B on cell viability A549 cells

(A) Chemical structure of millettocalyxin B. (B) Effect of millettocalyxin B on the viability of A549 cells. The cells were treated with 0- 100 μM of millettocalyxin B for 24 h and cell viability was analyzed by the MTT assay. (C) Apoptosis and necrosis was determined with the Hoechst 33342 and propidium iodide staining assay. The values are means of triplicate measurements ± SD.; *p< 0.05 versus control.

4.2 Millettocalyxin B suppresses migration and invasion of the A549 cells.

Cell migration activity was determined by wound-healing assay in response to the treatment with 0-25 μM millettocalyxin B at 24, 48 and 72 h. The results showed that 5-25 μM millettocalyxin B significantly inhibited A549 cell migration at all time points, compared with the non-treated control (Figure 7A). Additionally, cell invasion was determined using the transwell Boyden chamber assay. A549 cells were treated with 0-25 μM millettocalyxin B for 24 h and then added to the upper chamber. Millettocalyxin B significantly inhibited cell invasion at 24 h (Figure 7B). These results indicate that millettocalyxin B possesses the ability to inhibit human lung cancer cell migration and invasion

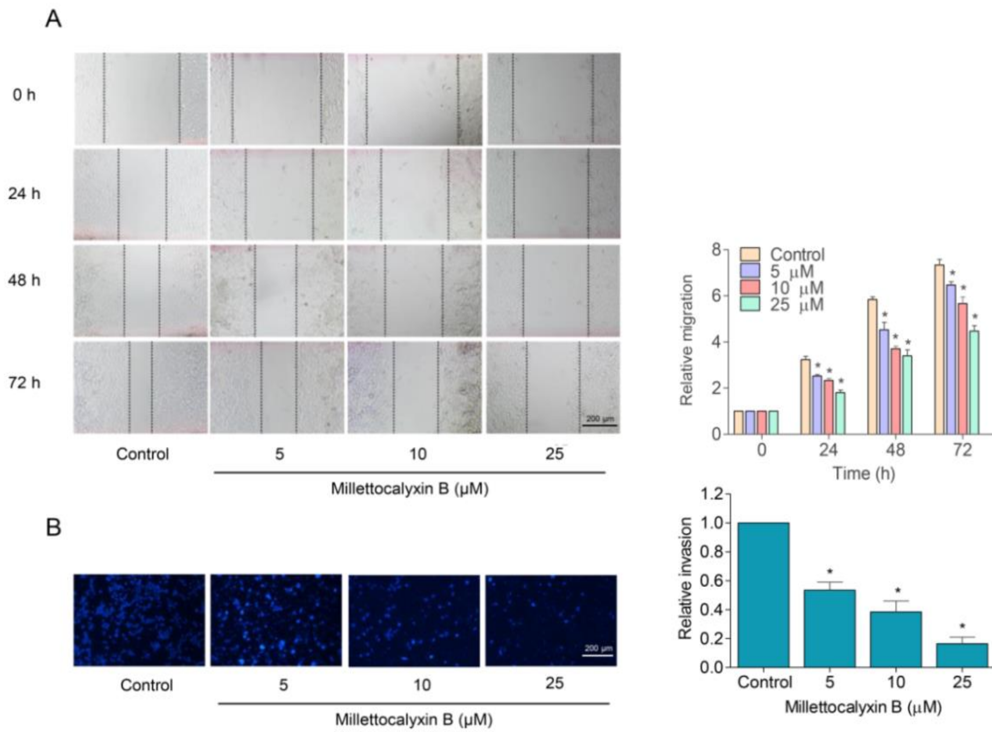

Figure 7. The effect of millettocalyxin B on the migratory behavior of A549 cells

(A) The wound healing assay was performed to evaluate cell migration after treatment with millettocalyxin B for 24, 48, and 72 h. The relative cell migration was calculated by comparing with the control. (B) For the invasion assay, the cells were seeded onto Matrigel and treated with millettocalyxin B (0-25 μM) for 24 h. The values are means of triplicate measurements ±SD.; *p< 0.05 versus control.

4.3 Millettocalyxin B suppresses filopodia formation and downregulates Cdc42.

The formation plasma membrane protrusion termed filopodia associates with increased cell movement (64). We next analyzed the effect of millettocalyxin B on filopodia formation. A549 cells were treated with 0-25 μM millettocalyxin B and phalloidin-labeled filopodia were determined. Figure 8A shows that the untreated control cells exhibited several membrane protrusions of filopodia which was significantly diminished following treatment with 5-25 μM millettocalyxin B. As Cdc42 is a known regulator of filopodia formation (64), we further investigated the expression levels of Cdc42 in millettocalyxin B-treated cells. Compared to untreated cells, Cdc42 was strongly down-regulated in cells treated with 5-25 μM millettocalyxin B (Figure 8B).

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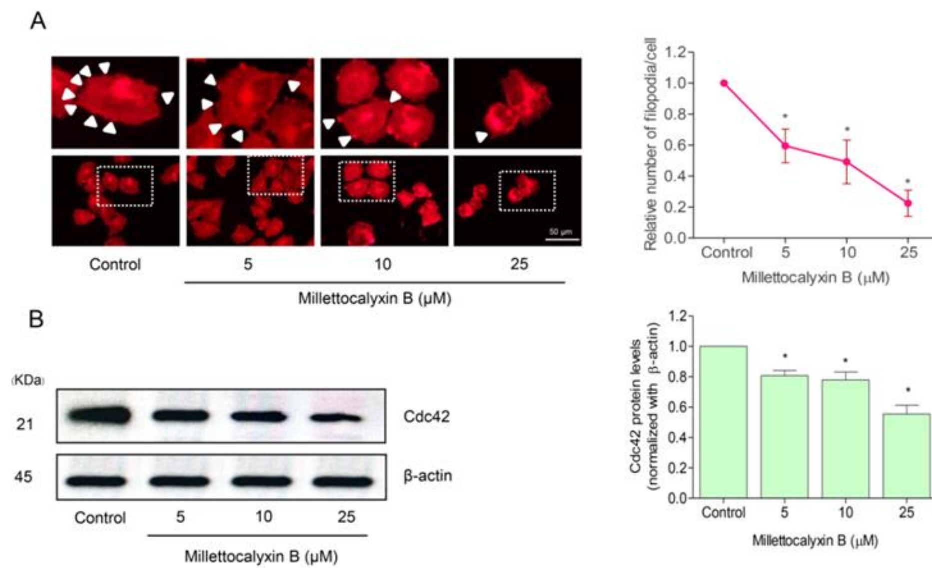

Figure 8. Millettocalyxin B reduces filopodia formation and Cdc42 levels.

(A) After treatment with millettocalyxin B for 24 h, the relative number of filopodia was evaluated. The values are means of triplicate measurements ±SD.; *p<0.05 versus control. (B) The effect of millettocalyxin B on Cdc42 protein levels. The levels of Cdc42 were evaluated by western blot analysis. The relative protein levels were quantified by densitometry. The data was presented in mean±SD. (n=3); *p**<0.05.**

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4.4 Millettocalyxin B suppresses integrin α5 and FAK-Akt signaling.

Integrins control cancer cell invasion and migration via activating several pathways including FAK/Akt signaling. Cells were incubated with the compound (0- 25 μM) for 24 h and migration-related proteins were examined by western blot analysis. Figure 9 shows that millettocalyxin B minimally affected integrin β1, while it significantly reduced the levels of integrin α5. The activation status of proteins regulating cell motility down-stream of integrins such as FAK and Akt was further elucidated by western blotting. In parallel with suppressed integrin α5, millettocalyxin B significantly reduced the cellular levels of active FAK indicated by the reduction of p-FAK/FAK ratio at the concentrations of 5-25 μM. Figure 5 further reveals that millettocalyxin B could decrease the levels of active Akt (phosphorylation at Ser 473). These results suggested that millettocalyxin B exhibited anti-migratory effect through the suppression of integrin α5 and its down-stream FAK-Akt signaling pathway.

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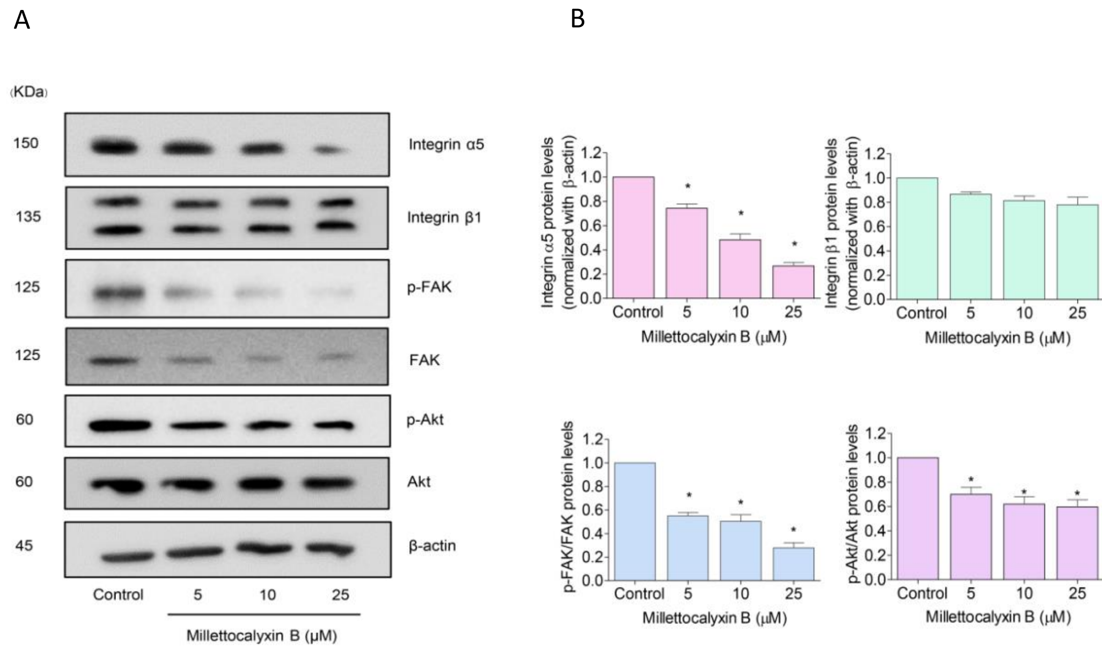

Figure 9. The effect of millettocalyxin B on integrin α5, integrin β1 and migratory proteins.

The cells were treated with 0-25 μM millettocalyxin B for 24 h. A: The protein levels were evaluated by western blot analysis followed by densitometry. B: The relative proteins were quantified by densitometry and mean data from three independent experiments were presented. Values are means of measurements ±SD. (n=3); *p<0.05 versus control.

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CHEAPTER IV

DISSCUSSION AND CONCLUSION

Cancer metastasis is known as important process resulting in the death of lung cancer patients (65). Previous studies have reported the anti-metastasis potential of several compounds via integrin targeting (66), (21), (23). In line with such studies, we showed the integrin α5 targeting activity of millettocalyxin B in lung cancer cells. In lung cancer, it has been demonstrated that the expression levels of integrin α5 are important for tumor growth and cancer progression (67). It is interesting that integrin α5 could be promising target as it is not generally expressed in normal lung cells (68). Furthermore, integrin α5 overexpression is associated with cancer aggressiveness and poor prognosis (69). Cancer cell migration and invasion involve multiple pathways that are associated with integrins, such as FAK, Akt, and Cdc42 (70). It is known that integrins are important to elevate cell motility (71), (72).

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Recently, natural compounds from plants have been receiving increasing attention either as the potential drugs or lead compounds in drug discovery (73). *Millettia erythrocalyx*, which is widely distributed in the tropical and subtropical regions of the world, such as China and Thailand (74), is one natural species that has attracted interest and is a source of millettocalyxin B. millettocalyxin B was isolated from *M. erythrocalyx* has been used in traditional medicines, such as for treating stomach pain (24). In this study, millettocalyxin B treatment suppressed integrin α5 (Figure 5) and reduced the expression of p-FAK, p-Akt and Cdc42 (Figure 9 and 8B). Cdc42 belongs to the Rho GTPase family of proteins and is involved in the formation of filopodia, which facilitate migration and invasion (75), (76). In addition, decreased Cdc42 expression has been demonstrated to inhibit

cell migration (77), (78), (79). Cycloartobiloxanthone has been reported to have an inhibitory effect on cancer migration and invasion by suppressing integrin α5, integrin αV, FAK and Cdc 42 (21) . Also, phoyunnanin E and a bibenzyl have been reported to have an inhibitory effect by inhibiting integrin α5, integrin αV, α4, β3, FAK and Cdc 42 (66), (23).

The non-small cell lung cancers A549,were mostly applied as a model in anti-metastasis research (60) (61). This cell type represents some mutation proteins on Kras protein that associates to metastatic potentials of cancer cells (57). Approximately 32% of metastasis cancer cells have been found Kras mutation (58). Kras is a small guanine triphosphatase (GTPase) which plays a role in signal transduction between EGFR and tyrosine kinases. Overexpression of Kras can promote the Akt and ERK signaling pathway resulting in cell growth, proliferation and differentiation. Moreover, the activation of Kras can trigger cell migration and motility (59).

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In conclusion, the migration activity of the cells was inhibited by targeting integrin α5 leading to the downregulation of active FAK, active Akt and Cdc42. We provided information regarding anti-migratory activity of millettocalyxin B that may benefit the future development of this compound for anti-metastasis therapy or its use as an adjuvant with standard therapies to improve clinical outcome.

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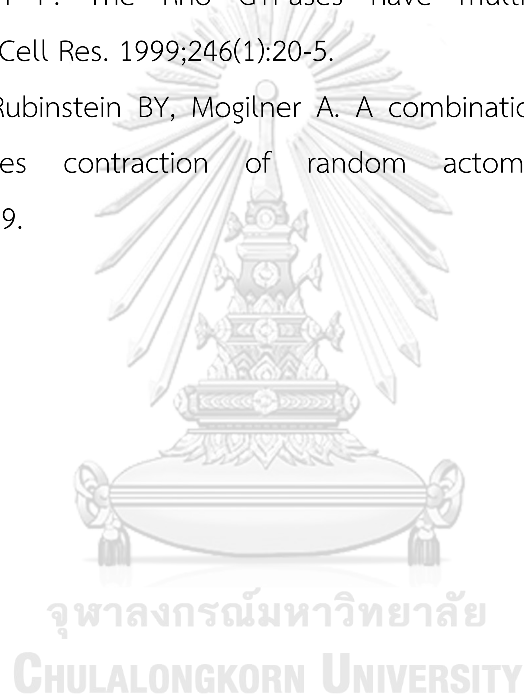
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Chulalongkorn University

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

VITA

NAME	นางสาวเพ็ญนภา ล่าเฟือย
DATE OF BIRTH	20 พฤศจิกายน 2537
PLACE OF BIRTH	กรุงเทพมหานคร
INSTITUTIONS ATTENDED	ปริญญาตรี
HOME ADDRESS	165/107 หมู่ 11 ซอยสุขาภิบาล 6/1 ต.บางพลีใหญ่ อ. บางพลี จ. สมุทรปราการ 10540
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Chulalongkorn University