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PRODUCTION AND CYTOTOXICITY AGAINST CANCER CELLS OF RECOMBINANT CECROPIN B-POLYPROLINE

Miss Sirin Sarunyutanon

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	PRODUCTION AND CYTOTOXICITY AGAINST CANCER CELLS OF RECOMBINANT CECROPIN B-POLYPROLINE
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Field of Study	Biotechnology
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ศิรินทร์ ศรัณยุตานนท์ : การผลิตและความเป็นพิษต่อเซลล์มะเร็งของรีคอมบิแนนต์ซีโครพินบี-พอลิโพรลีน (PRODUCTION AND CYTOTOXICITY AGAINST CANCER CELLS OF RECOMBINANT CECROPIN B-POLYPROLINE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.ศรินทิพ สุกใส, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.กิตตินันท์ โกมลภิส, 93 หน้า.

ซีโครพินบี (CB) เป็นเพพไทด์ต้านจุลชีพและมะเร็งจากธรรมชาติที่มีฤทธิ์ในการฆ่าแบคทีเรียที่ดีที่สุดในกลุ่มของซีโครพินด้วยกัน ในงานวิจัยนี้ ซีโครพินบีพอลิโพรลีน (CB-PP) ได้ถูกสร้างด้วยการเติมพอลิโพรลีน (PP) ที่เป็นส่วนสำคัญของตัวรับโปรเจสเตอโรน (PR) ต่อกับปลาย C ของลำดับของ CB ชิ้นยีน CB และซีโครพินบีพอลิโพรลีน (CB-PP) ถูกนำเข้าสู่เวกเตอร์พาหะ pPICZαA จากนั้นรีคอมบิแนนต์พลาสมิด CB และรีคอมบิแนนต์พลาสมิด CB-PP ถูกส่งถ่ายยีนเข้าสู่เซลล์ยีสต์ *Pichia pastoris* สายพันธุ์ KM71H ด้วยวิธีการชักนำด้วยกระแสไฟฟ้า การผลิตรีคอมบิแนนต์โปรตีน CB (rCB) และรีคอมบิแนนต์โปรตีน CB-PP (rCB-PP) ประสบความสำเร็จโดยการเหนี่ยวนำการผลิตด้วยเมธานอลที่ความเข้มข้น 0.5% ทุกๆ 24 ชั่วโมงเป็นเวลา 48 ชั่วโมง และทำบริสุทธิ์โดยใช้นิกเกิลคอลัมน์โครมาโทกราฟี ขนาดของ rCB และ rCB-PP ที่ได้ประมาณ 4.9 และ 6.6 กิโลดาลตัน ตามลำดับ เซลล์ 6 ชนิดได้แก่เซลล์มะเร็งเต้านม (BT-474), เซลล์มะเร็งปอด (ChaGo-K1), เซลล์มะเร็งตับ (Hep-G2), เซลล์มะเร็งกระเพาะอาหาร (Kato-III), เซลล์มะเร็งลำไส้ใหญ่ (SW-620) และเซลล์ไฟโบรบลาสต์ของปอด (WI-38) ถูกนำมาทดสอบความเป็นพิษต่อเซลล์ เซลล์ทั้งหมดถูกบ่มด้วย rCB และ rCB-PP ที่ความเข้มข้น 0.078–20 ไมโครโมลาร์เป็นเวลา 72 ชั่วโมง ผลการทดลองแสดงให้เห็นว่าหลังจากทดสอบความเป็นพิษต่อเซลล์ระหว่างเซลล์ทั้ง 6 ชนิดกับ rCB และ rCB-PP เซลล์มะเร็งมีการตายมากกว่า 50% ที่ความเข้มข้นของโปรตีนในช่วง 7 - 20 ไมโครโมลาร์ ในขณะที่เซลล์ปกติยังมีชีวิตรอดมากกว่า 80% ที่ความเข้มข้นของโปรตีนเดียวกัน จากผลการทดลองทั้งหมดชี้ให้เห็นว่า rCB และ rCB-PP สามารถถูกสร้างได้จาก *Pichia pastoris* ที่ถูกส่งถ่ายยีนโดยรีคอมบิแนนต์โปรตีนที่ได้แสดงความสามารถในการต้านมะเร็ง

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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SIRIN SARUNYUTANON: PRODUCTION AND CYTOTOXICITY AGAINST CANCER CELLS OF RECOMBINANT CECROPIN B-POLYPROLINE. ADVISOR: SARINTIP SOOKSAI, Ph.D., CO-ADVISOR: ASST. PROF. KITTINAN KOMOLPIS, Ph.D., 93 pp.

Cecropin B (CB) is a natural anticancer antimicrobial peptide which has the highest antibacterial activity among the Cecropin family. In this work, Cecropin B - polyproline (CB-PP) was constructed by adding polyproline (PP) which is a part of progesterone receptor (PR) to the C-terminus of CB sequence. The CB and CB-PP gene fragments were cloned into the pPICZ**α**A expression vector. Then, the recombinant plasmid CB and CB-PP were transformed into *Pichia pastoris* strain KM71H by electroporation. The recombinant protein CB (rCB) and CB-PP (rCB-PP) were successfully produced by inducing with 0.5% methanol for 24 and 48 hours and purified using nickel column chromatography. The size of rCB and rCB-PP were found to be approximately 4.9 and 6.6 kDa, respectively. The cytotoxicity against six cell lines including human breast carcinoma (BT-474), human lung bronchus carcinoma (ChaGo-K1), human liver hepatocellular carcinoma (Hep-G2), human gastric carcinoma (Kato-III), human colon carcinoma (SW-620) and human lung fibroblast (WI-38) were investigated. All cell lines were treated with recombinant protein CB and CB-PP with the protein concentration between 0.078 μM – 20 μM for 72 hours. The results revealed that more than 50% of those five cancer cells were dead at the rage of 7 μM - 20 μM protein concentrations while more than 80% of normal cells was alive even treated with the same protein concentration. Those resultsmindicated that rCB และ rCB-PP could be produced by the transformed *Pichia pastoris* and the obtained recombinant proteins exhibited anticancer activity.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST of ABBRIVATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram(s)
µL	Microliter(s)
µM	Micro molar
$\mu g.mL^{-1}$	Microgram per milliliter
×g	Multiply by gravitational force (×9.80665 $m.s^2$)
AOX1	Alcohol oxidase 1 gene
$_P$ *AOX1*	Alcohol oxidase 1 promoter
AOX2	Alcohol oxidase 2 gene
$_P$ *AOX2*	Alcohol oxidase 2 promoter
g	Gram(s), (Unit of mass)
h	Hour(s)
kb	Kilobase pair(s)
M	Molar
mg	Milligram(s)
mL	Milliliter(s)
ng	Nanogram(s)
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RNase	Ribonuclease A
rpm	Revolution per minute
TAE	Tris-acetate-EDTA
Tm	Melting temperature (°C

CHAPTER I

INTRODUCTION

In many countries, a trend of mortality rate due to cancers has significantly increased (1). While, the most commonly diagnosed cancers worldwide were breast, lung and colorectal cancers, breast cancer was one of the most diagnosed cancer in Thai woman (2). This incident remains the major leading causes of the loss of life influence millions of people and a lot of suffering. Currently, there are several approaches to treat cancers including surgery, chemotherapy, radiation therapy, hormone therapy and transplantation. However, each method inherits its own unavoidable flaws. These treatments can destroy or get rid of cancer cells but usually affect normal cells to a certain degree. In some cases, they also pose unwanted side effects such as blood disorder, painful and diarrhea to the patients (3). Alternative approaches based on DNA-alkylating agents, antimetabolites and natural products were studied. However, they were still insufficient in selectivity. These agents damage not only cancer cells but also normal cells (4, 5). Therefore, a new approaches or agent that can destroy only the cancer cells still definitely in need. In this research, a new type of agents was proposed and investigated. This new agent, called as CB-PP, consisted of two parts including Cecropin B (CB) and Polyproline (PP).

CB is an antimicrobial peptides which have been reported to have anticancer activity against various types of cancer cells such as colon carcinoma, leaukemia. hepatocellular carcinoma, bladder cancer, breast cancer and gastric carcinoma (6-10). While PP is a group of proline, located as a part of progesterone receptor (PR), which has been reported to activate breast cancer cell proliferation by interacting with cell sarcoma (c-Src) through SH3 (11, 12). Therefore, it was expected that the PP of the CB-PP will interfere the binding between PP of the PR and the SH3 domains, thus inhibiting the proliferation of the cancer cells while the CB of the CB-PP will directly damage the cancer cells. As a result, this new conjugate can be used at low

concentration to avoid the undesired normal cell damage but it still effective against the cancer cells. Therefore, in this research, the recombinant protein CB-PP were constructed and produced in *Pichia pastoris* KM71H. Subsequently, the obtained recombinant protein were purified and tested for their toxocoties to human breast carcinoma cell (BT-474), human lung bronchus carcinoma cell line (ChaGo-K1), human liver hepatocellular carcinoma cell line (Hep-G2), human gastric carcinoma cell line (Kato-III), human colon carcinoma cell line (SW-620) and human lung fibroblast cell line (WI-38). The information obtained from this work could be used in order to develop a potent anticancer peptide or drug in the future.

Objective

1. To produce recombinant protein CB and CB-PP in *Pichia pastoris* KM71H
2. To study cytotoxicity activity of recombinant protein against some human cancer cells

Outcome

1. To obtain the recombinant protein CB and CB-pp
2. To gain information on the cytotoxicity of the obtained recombinant protein to some human cancer cells

CHAPTER II

LITERATURE REVIEW

2.1 Cancer statistics

Cancer is the major public health and is one of the most leading causes of global disease. Each year, tens of millions of people around the world are diagnosed with cancer, and more than half of the patients ultimately die from it (13). In 2012, there were an estimated 14.1 million new cases of cancer in the world: 7.4 million (53%) in males and 6.7 million (47%) in females, giving a male: female ratio of 10:9. The most common causes of cancer death are cancers of lung (1.59 million deaths), liver (745,000 deaths), stomach (723,000 deaths), colorectal (694,000 deaths), breast (521,000 deaths) and oesophageal cancer (400,000 deaths) (14).

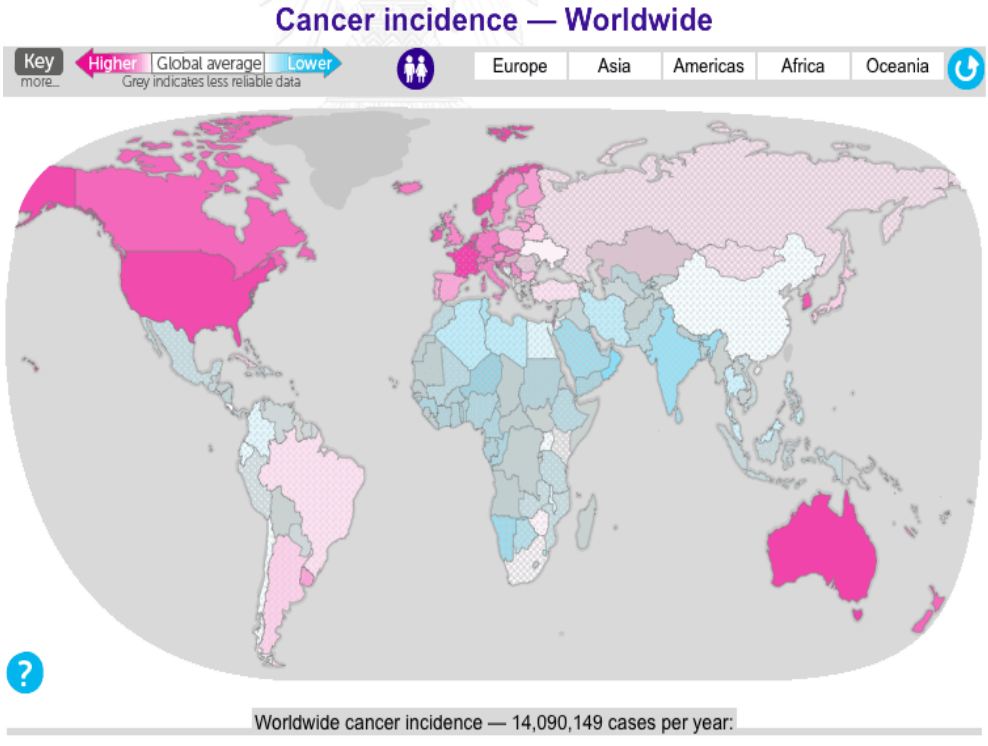


Figure 2. 1 Interactive Map of Cancer Incidence, World, 2012 Estimates

(Source: www.cancerresearchuk.org)

2.2 Cancer

According to National Cancer Institute, cancer is defined as “Cancer is the name given to a collection of related diseases. In all types of cancer, some of the body’s cells begin to divide without stopping and spread into surrounding tissues.” Cancer can start almost anywhere in the human body, which is made up of trillions of cells. Normally, human cells grow and divide to form new cells as the body needs them. When cells grow old or become damaged, they die, and new cells take their place. Cancer can be defined with uncontrolled cellular proliferation and dedifferentiation (15). It can occur from abnormal proliferation of the different kinds of cells in the body, thus there are more than a hundred distinctive types of cancer, which can vary extensively in their behaviour and response to treatment. Not all tumors are cancerous (16), the most important cancer issue is the distinction between benign and malignant tumors. A tumor is any abnormal proliferation of cells, which may be either benign or malignant. A benign tumor, such as a common skin wart, remains confined to its original location, invading neither surrounding normal tissue nor spreading to distant body sites. A malignant tumor, on the other hand, is competent of both invading surrounding normal tissue and spreading throughout the body via the circulatory or lymphatic systems. As a consequence, only malignant tumors are appropriately referred to as cancers, and it is their ability to invade and metastasize that makes cancer so perilous. Whereas benign tumors can usually be removed surgically, the development of malignant tumors to distant body sites generally makes them resistant to such localized treatment. Both benign and malignant tumors are categorized according to the type of cell from which they originate. Most cancers fall into one of three main groups: carcinomas, sarcomas, and leukemias or lymphomas. Carcinomas, include approximately 90% of human cancers, are malignancies of epithelial cells. Sarcomas, which are rare in humans, are solid tumors of connective tissues, such as muscle, bone, cartilage, and fibrous tissue. Leukemias and lymphomas, which account for approximately 8% of human malignancies, arise from the blood-forming cells and from cells of the immune system, respectively. Tumors are further classified according to tissue of origin

(e.g., lung or breast carcinomas) and the type of cell involved. For example, fibrosarcomas arise from fibroblasts, and erythroid leukaemia from precursors of red blood cells (17).

2.2.1 Risk factors for Cancer

Development of cancer is attributed to various factors including exposure to environmental carcinogens, unhealthy lifestyle and genetic predisposition. These factors contribute to tumorigenesis by damaging genetic material, which is normally repaired or triggers senescence or elimination of the cell depending on extent damage (18).

2.2.2Cancer treatment and side effects

Current possible therapeutic approaches are determined by an interacting set of factors including the status of the patient, the behavior of the disorder and the cancer burden of the body. The most well-established treatment modalities for cancer elimination are surgery, radiation therapy, hormone therapy and chemotherapy. While the usage of surgery and radiation therapy is dependent on the progression of the disorder, presence of metastases and additional crucial factors chemotherapy is utilized in a substantially wider clinical spectrum. Traditional chemotherapeutic agents exhibit proven efficacy with inefficient cancer selectivity which explains their well characterized side effects on relatively fast growing normal cells (vomitus, fatigue, loss of hair). Based on the recent advent of more selective anticancer agents it can be 7 speculated that all tumor type possesses different suitable points for intervention and therefore a unique agent could be developed for the treatment of each cancer types.

2.3 Breast cancer

As reported by National Cancer Institute at the National Institute of Health, breast cancer is defined as, “Cancer that forms in tissues of the breast, usually the ducts (tubes that carry milk to the nipple) and lobules (glands that make milk). It occurs in both men and women, although male breast cancer is rare.” Breast cancer is the leading cause of cancer related mortality in women, it is the most common malignancy among females affecting approximately one out of ten women (19). The vital signals of breast cancer may involve in a change in breast shape, a lump in the breast, dimpling of the skin, fluid coming from the nipple, or a red scaly patch of skin (20). In those with distant dissemination of the disease, there may be bone pain, swollen lymph nodes, shortness of breath, or yellow skin (21).

2.3.1 Risk factors for breast cancer

Breast cancer developing resulting from several risk factors comprise of female gender, age, race, obesity, lack of physical exercise, night-shift work, drinking alcohol, using hormone replacement therapy during menopause for long periods, treatment of radiation therapy with breast or chest, early age at first menstruation, do not have children or having lately, and older age (20). The breast cancer cases are owing to genetic inheritance from parents or relatives about 5–10%, containing BRCA1 and BRCA2 among others. The development of breast cancer most ordinary occurs in cells from the lining of milk ducts and the lobules that supply the ducts with milk. Cancers progressing from the ducts are noticed as ductal carcinomas, whereas those developing from lobules are recognized as lobular carcinomas (20).

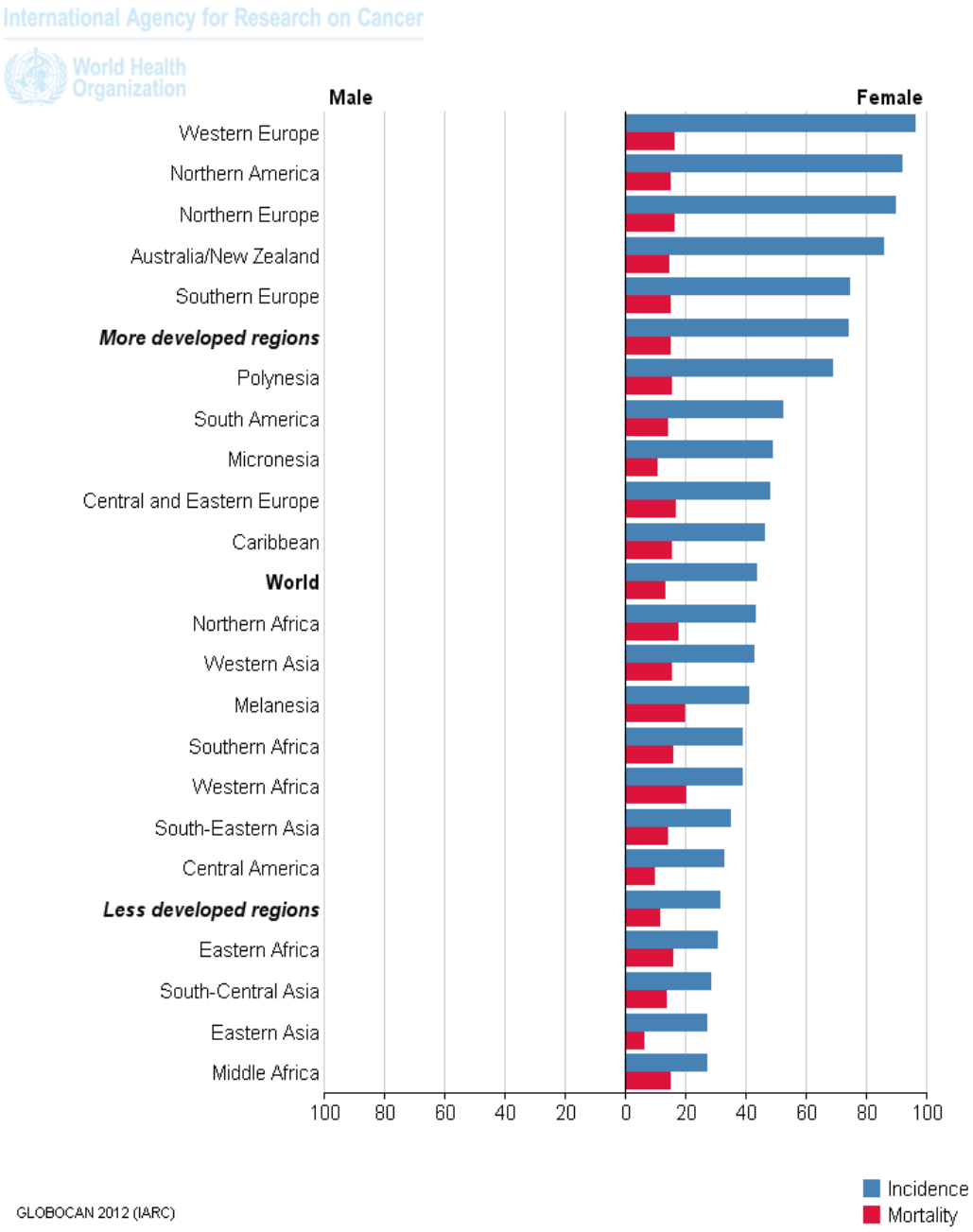


Figure 2. 2 Incidence and Mortality Rate of Breast Cancer (GLOBOCAN 2012)

(Source: wwwglobocan.iarc.fr)

2.3.2 Breast cancer classification

To classify breast cancer, it can be commonly categorized into three distinct subtypes found on the presence or absence of three receptors based on cancer cells (22). First is hormone receptor (HR) positive breast cancer cell, where cancer cells express estrogen and/or progesterone receptors (ER/PR). Second, the oncogene human epidermal growth factor receptor 2 (HER-2/neu) is over-expressed. While in the third type of breast cancer, cancer cells are negative for the expression of ER, PR and HER-2/neu; this is also known as triple negative breast cancer (23).

2.3.3 Symptoms of breast cancer

Having a new lump or mass is the most common symptom of breast cancer. However, according to the Mayo Clinic, a lump in the breast is generally associated with breast cancer, nevertheless for the most part; a lump in the breast is not cancer. From hormonal changes in teenagers to damaged fat tissue, all breast lumps in women in their early age of 20 to early 50 are noncancerous (benign) approximately more than 90 percent. A painless, hard mass that has irregular edges is more likely to be cancerous, but breast cancers can be tender, soft, or rounded. They can even be painful. In addition, other feasible symptoms of breast cancer consist of swelling of all or part of a breast, breast or nipple pain, redness, or thickening of the nipple or breast skin, skin irritation or dimpling, nipple retraction and discharge (24, 25).

2.3.4 Breast cancer treatment and side effects

Treatment therapy for acute breast cancer includes excision, hormone therapy, chemotherapy and radiation therapy. For ongoing breast cancer therapy and if it is an estrogen and/or progesterone receptor-positive breast cancer; chemotherapy is included with other treatment modalities such as mastectomy, reconstruction, re-excision and radiation therapy (26). All currently available treatments have undesirable limitations. Chemotherapy is one of the advance tool used in cancer treatment, but uncompromising side effects resulting from chemotherapeutics agents are always toxic on normal cells remains an important

hindrance in clinical application (27, 28). Surgery removal is limited to patients with small tumors before metastasis. Even though tumors are removed, the inherent problem is not necessarily eliminated and deteriorate is highly possible (29). All above mentioned therapies are assorted with stern side effects such as severe nausea, bone marrow suppression, vomiting, and hair loss. Further medication usually needed for patients in an endeavor to ameliorate the side effects of their treatment, compounding patient strain in terms of treatment complexity and cost (30).

2.4 Progesterone receptor

The breast cancer will be progesterone-receptor-positive (PR+) if it has progesterone receptors; this means that the cancer cells may receive signals from progesterone that could promote their growth. Progesterone receptor (PR) is member of the nuclear receptor superfamily, act as ligand-activated transcription factors and initiators of c-Src kinase and mitogenic-activated protein kinase signaling (MAPK). This PR functions as direct activators of cytoplasmic signal transduction molecules (12) (Figure 1.3). In the amino-terminal domain of conventional PR has a specific polyproline motif (Figure 1.4) that directly interacts progestin-dependent interaction of PR with the SRC Homology 3 (SH3) domains of various cytoplasmic signaling molecules consist of c-Src tyrosine kinases. PR-SH3 domains interaction shows potential effect on rapid progestin-induced activation of Src and downstream MAPK in mammalian cells, this ability is not occur on PR transcriptional activity (11).

Moreover, PR is an effective activator of Src kinase working by SH3 domain, this former study also revealed that PR signaling pathway via regulatory SH3 domains was influenced on progestin-induced growth arrest of breast cancer epithelial cells. The previous reported that SH3 domains recognize short proline-rich motifs in PR which is the important sequences for PR and SH3 domains interaction and this mechanism inducing signaling pathway for cell proliferation.

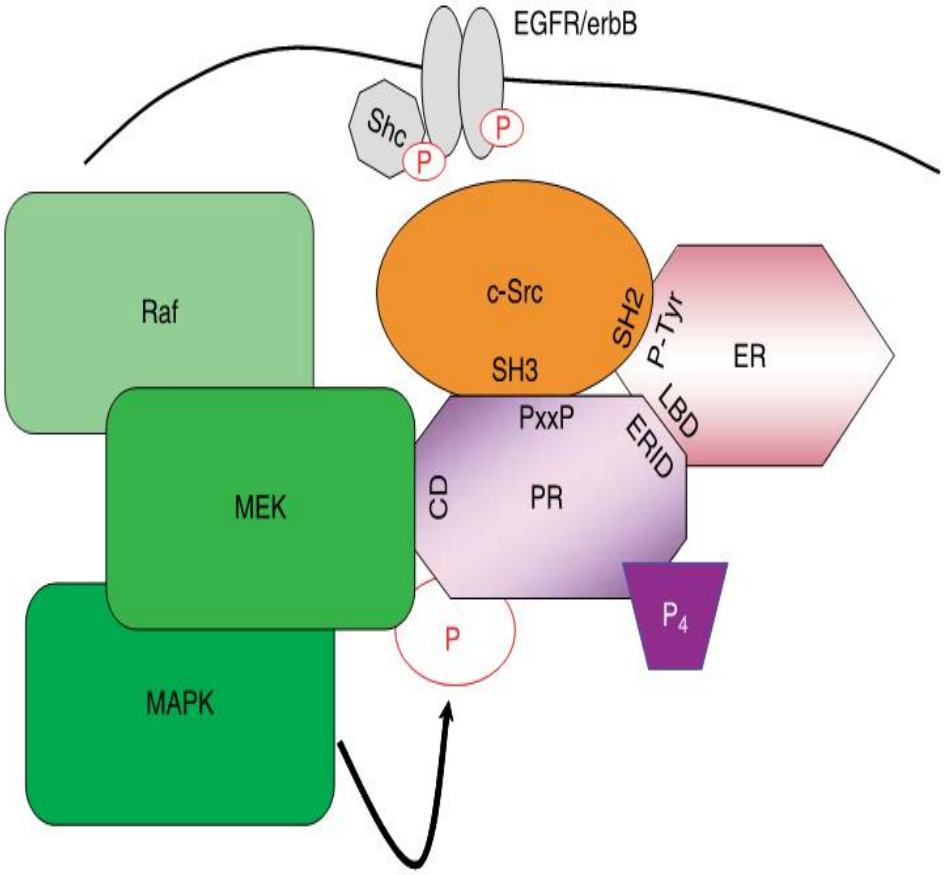


Figure 2. 3 PR – scaffolding interaction, the interaction between PR and c-Src has been shown to be essential for progesterone-induced c-Src/MAPK activation (Arnold *et al.* 1998, Boonyaratanakornkij *et al.* 2001).

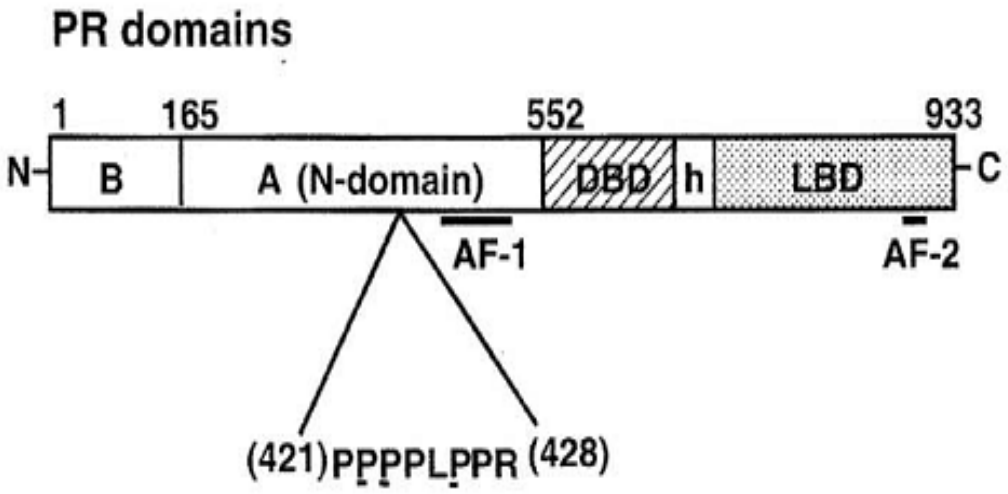


Figure 2. 4 A schematic of human PR domains including ligand binding domain (LBD), hinge (h), DNA binding domain (DBD), transcriptional activation domain 1 and 2 (AF-1, AF-2) and polyproline motis (421-428) (Boonyaratanakornkij *et al.* 2001)

2.5 Antimicrobial peptide

Antimicrobial peptides (AMPs) act as an initial role of defensing against broad spectrum of microorganisms such as bacteria, virus, yeast, parasite, and fungi (31-33). They were firstly found in invertebrates with typically small size, generally less than 150-200 amino acid residues (34), They are widely disseminated in nature, synthesized and later also found in vertebrates, including humans (35). Many previous studies reveal that AMPs directly act on the membrane of the target cell (36). The AMPs with net positive charge causes their special binding to more than one negatively charged target on bacteria, which may account for the selectivity of antimicrobial peptides (37).

Each of AMPs has its own character and unique. They can be divided into four sub-groups rely on their amino acid composition and structure. The first one is anionic antimicrobial peptides with small size (~ 721-823 kDa) (38); they can act against Gram-positive and Gram-negative bacteria. This group of AMPs rich in glutamic and aspartic acid that are required zinc as a cofactor for antimicrobial activity such as

Maximin-H5 from amphibians (39), Dermcidin from humans (40). The second sub-group is linear cationic α-helical peptides which lack in cysteine and containing approximately < 40 amino acid residues. They sometimes have a hinge in the middle (41) such as cecropin, magainin, CAP18 and LL37. The third sub-group is cationic peptides which are rich in proline and arginine and lack in cysteine (42) such as hymenoptaecin, bactenecins, coleoptercin and indolicidin. The fourth sub-group is anionic and cationic peptides that contain cysteine residues. They form disulphide bonds and stable β-sheet such as a diverse family of defensins, protegrin and brevinin (43).

2.5.1 Mechanisms of antimicrobial peptide activity

The mechanism can be divided into two mechanisms; First is intracellular antimicrobial peptide activity and another is transmembrane pore-forming, shown as table 1.1 (44, 45).

The AMPs killing mechanism in mode of intracellular antimicrobial peptide activity reveal that AMPs penetrate into the cell and bind the intracellular molecules which critical to cell living. The peptides can inhibit cell wall synthesis, nucleic acid synthesis, protein synthesis, enzymatic activity or alter cytoplasmic membrane septum formation (Figure 1.5).

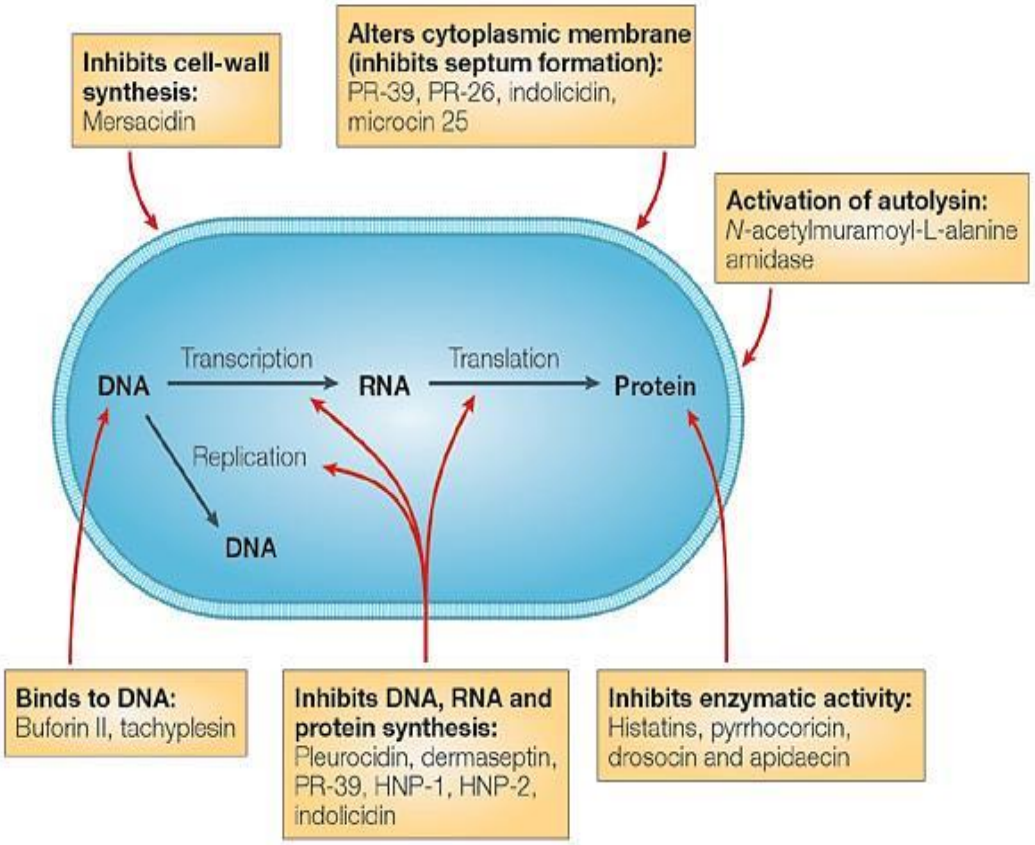


Figure 2. 5 Mode of action for intracellular antimicrobial peptide activity (46)

Several AMPs play a role on bacteria using a transmembrane pore-forming mechanism via interaction with the cell membrane components leading to pore-formation and bacterial cytoplasmic contents leakage. The following models are barrel-stave, carpet and toroidal-pore.

First, the barrel-stave model which is the action of peptides aggregating in the membrane by aligning parallel to the phospholipids eventually forming an ion-channel pore (47). The secondary structure conformation and amphipathic property of the peptides play a crucial role in pore formation. Peptides using this action are able to perform interacting strongly with both zwitterionic and negatively-charged membranes and are consequently non-selective (48).

Second, the carpet model involves peptides accumulate and bind parallel on the surface of the phospholipid membrane to form clusters that cover the surface with a ‘carpet’ of peptide and then integrate into the membrane when reaching

threshold level. The type of peptide is influence on permeation diversity, with mechanisms such as detergent-like disintegration and channel aggregate formation. The interaction between the membrane and peptides is destined by hydrophobic charges (49). The membrane integrity is disturbed and making cytoplasmic contents leak. The disturbance of the membrane potential and membrane disintegration, leads to rapid death (50).

Third, toroidal model which peptides insert perpendicularly in the bilayer, but instead of packing parallel to the phospholipid chains, peptides induce a local membrane curvature in such a way that the pore lumen is lined partly by peptides and partly by phospholipid head groups. Continuity between inner and outer leaflets is established.

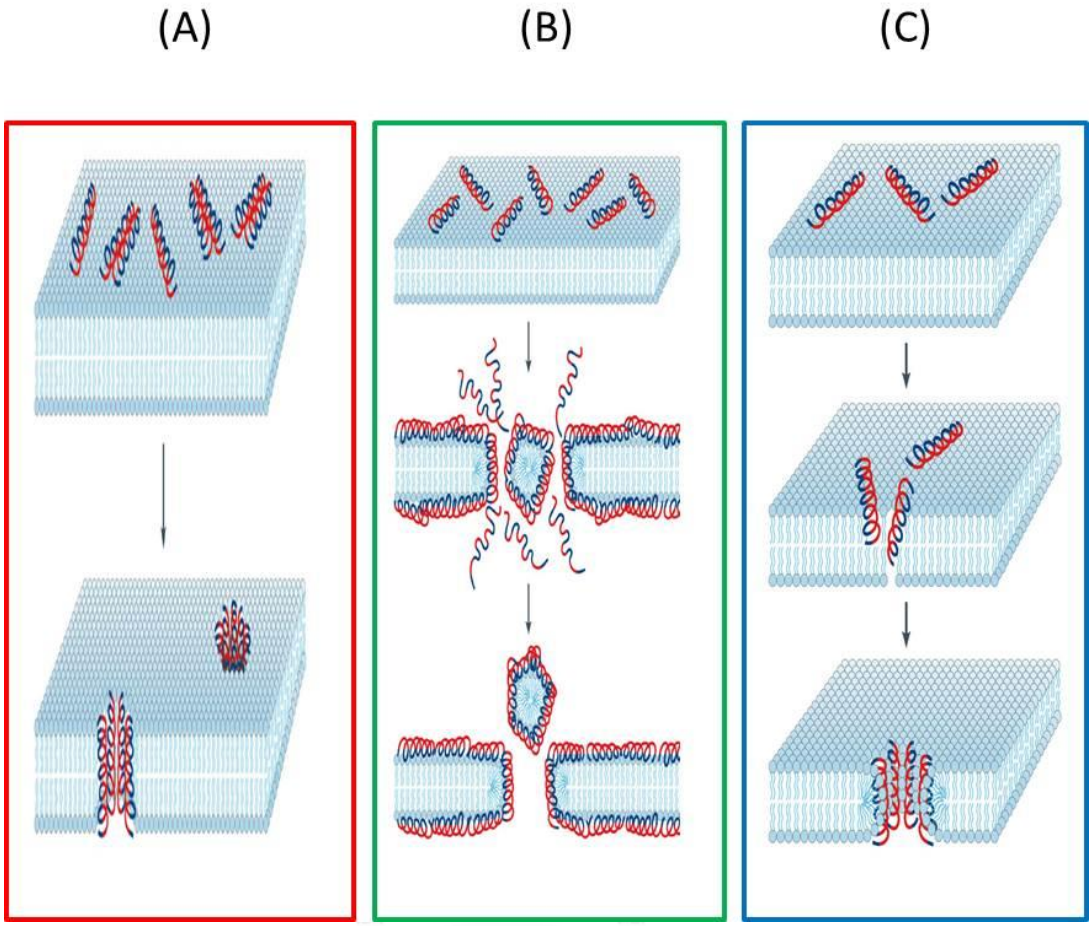


Figure 2. 6 Pore-forming mechanisms of antimicrobial peptide; (A) The barrel-stave model, (B) The carpet model, and (C) The toroidal model (51, 52)

Table 2. 1 Membrane and intracellular models of antimicrobial peptide killing and lysis (52)

Model of antimicrobial activity	Synonym	Examples of peptides
Transmembrane pore-forming mechanisms		
Toroidal pore	Wormhole, disc	Maginin, Protegrin, Mellitin, LL-37, MSI-78
Carpet		Dermaseptin, Mellitin, Caerin 1.1, Ovispirin and Cecropin
Barrel-steve	Helical-bundle model	Alamethicin
Modes of intracellular killing		
Flocculation of intracellular contents		Anionic peptides
Alters cytoplasmic membrane septum formation		Indolicidin, PR-39, PR-26 and Microcin
Inhibits cell wall synthesis		Mersacidin
Inhibits protein synthesis		Pleurocidin, Dermaseptin, PR-39, HNP-1 and Indolicidin
Binds nucleic acids		Buforin II and Tachyplesin
Inhibits enzymatic activity		Pleurocidin, Dermaseptin, PR-39, HNP-1 and Indolicidin

2.5.2 Cationic antimicrobial peptides

Cationic antimicrobial peptides (CAPs) are found in many diverse species playing a part in the innate immune system (31). CAPs are able to kill a broad spectrum of bacteria as well as fungi, enveloped viruses and protozoa (53). CAPs are generally short with around < 40 amino acids, positively charged and able to form amphipathic structures in nonpolar solvents. Little sequence conservation between these peptides are found and they are consequently categorized depend on their secondary structure (54). CAPs are offered to perform by interrupting negatively charged microbial membrane because of electrostatic interaction, leading to membrane permabilization and disruption (46). Some CAPs such as bovine lactoferricin, defensins, magainin and cecropins also display cytotoxic activity against many different types of human cancer cells directly (55, 56). Those CAPs can be divided into two categories based on their anticancer activity. The first group are CAPs that potent to microorganisms, cancer cells and normal mammalian cells, for example bee venom, melittin (57, 58), tachyplesin isolated from horseshoe crab (59, 60), human neutrophil defensins (61) and human LL-37 (62). Another group are CAPs highly cytotoxic to microorganisms and cancer cells, but not harmful to non-cancer cells such as magainins isolated from the frogs skin (55, 63), insect cecropins (9, 64), BMAP-28 (65), bovine lactoferricin (56) and others. Nonetheless, because of their fundamental amphipathic design, most CAPs at higher concentration become cytotoxic against normal cells (66, 67).

2.5.3 Interaction of CAPs with cancer cells

Related to bacteria, many cancer cells carry an increased net negative charge due to an elevated expression of anionic molecules, such as phosphatidylserine in the outer membrane leaflet (68-70), compared to non-malignant cells. CAPs with highly positive charge will interact with and interrupt the highly negative charged cancer cells by electrostatic interaction. Other different points between the cellular membranes of the target cells that may have a potential on the interaction of CAPs with cancer cells is membrane fluidity which is typically increased in cancer cells

relative to their healthy analogue (71, 72). The cancer cell membrane will destabilize by membrane-bound CAPs with this property. Another feasible clarification for the selective effect of the peptides is that on cancer cells surface tend to have more ample microvilli in comparison to normal cells (73). This increased the surface area of the tumorigenic cell membranes and may facilitate CAP-mediated cytotoxicity by allowing a greater number of CAP molecules to interact with the surface of the cancer cell (74). Thus, for many reasons, the cancer cells are more susceptible to CAPs compared to normal cells.

2.5.4 Mechanisms of cell killing by CAPs

The presence of killing mechanism was first proved in magainin and its synthetic analogues, in which irreversibly lysis of hematopoietic and solid tumor cells at concentrations having little effect on normal cells was shown (32). Several models including the carpet model, the barrel-stave model, and the toroidal model (Figure 1.6) have been suggested to describe the cytotoxic peptide-membrane interactions.

2.6 Cecropins

Cecropins are family of AMPs, firstly found in the immune hemolymph of silkworm pupae *Hyalophora cecropia* (75), insects, and mammals (76) and contribute to the innate immunity in animal. Cecropins family consist of A, B, C, D, E and F composed of 35 to 39 amino acids in length which is highly homologous sequences (7). The sequences of natural Cecropins are constructed with α-helical structure which has amphipathic in N-terminal segments and hydrophobic property in C-terminal segments. These peptides have a broad spectrum with anti-bacterial, anti-viral and anti-fungal activity (77-79). Because Cecropins are able to form specific amphipathic α-helixes, they directly attach on the non-polar lipid cell membranes and form transmembrane channels. These channels permit free circulation of electrolytes, metabolites and water through the phospholipid bilayers, therefore leading to irreversible cytolysis and finally to cell death (9, 80, 81). Among the

Cecropins group, Cecropin A and B possess strong activity to against several Gram-positive, Gram-negative bacteria in micromolar concentrations (1, 6). In addition, several previous studies have reported anticancer activity of both Cecropin A and B against various cancer cell lines (9, 10, 82).

2.6.1 Cecropin B

Natural Cecropin B (CB) is compose of 35 amino acids and possess the highest anti-microbial activity among the Cecropins family (83). The structure of cecropin B predicted by sequence analysis, Nuclear Magnetic Resonance (NMR) and circular dichroism spectroscopy is superlative NH2-terminal amphipathic α-helix, and a COOH-terminal hydrophobic α-helix joined by a flexible hinge at positions 23 and 24 (Figure. 1.5, Gly and Pro) (9, 84, 85). Both a hydrophilic and a hydrophobic group are contained in amphipathic α-helix (86). Cecropin B influences rapid lysis of bacterial cell membranes (87). Previous report has shown that the rate of lysis is dependent on the concentration of Cecropin B (88).

Lys (K) – Trp (W) – Lys (K) – Val (V) – Phe (F) – Lys (K) – Lys (K) – Ile (I) – Glu (E) – Lys (K) – Met (M) – Gly (G) – Arg (R) – Asn (N) – Ile (I) - Arg (R) – Asn (N) - Gly (G) – Ile (I) – Val (V) – Lys (K) – Ala (A) – Gly (G) – Pro (P) - Ala (A) – Ile (I) – Ala (A) – Val (V) – Leu (L) – Gly (G) – Glu (E) - Ala (A) - Lys (K) – Ala (A) – Leu (L) - NH2

Figure 2. 7 Cecropin B sequence consisting of 35 amino acids

2.6.2 Cytotoxicity of Cecropin B on cancer cell lines

Cecropin B are able to lyse different types of human cancer cells at peptide concentrations that are not harmful to normal eukaryotic cells (10). Since the COOH-terminal α-helix is hydrophobic while the NH2-terminal α-helix of cecropin B is highly amphipathic, Cecropin B also has anticancer activity. The effect of Cecropin B on the AGS human stomach carcinoma cell line analysed by patch-clamp analysis shown that peptide treatment caused short outward currents that were consistent with the formation of transient channel-like pores (89). However, the Cecropin B3 analogue

consisting of two hydrophobic α-helixes, declined to induce pore-formation. Surprisingly, the Cecropin B1 analogue with two amphipathic α helixes shows potent cytotoxic activity against several human leukemia cell lines at peptide concentrations that do not lyse normal fibroblasts or erythrocytes (90). What's more, Cecropin B1 is a more efficient toxic than Cecropin B against HL-60 human promyelocytic leukemia cells (91). From several researches point out that the amphipathic NH2-terminal α-helix of Cecropin B considered to interact with anionic membrane through its basic amino acid residues and enable cytotoxic activity against cancer cells. On the other hand, the hydrophobic COOH-terminal α-helix ought to help peptide inserting into the membrane resulting in positive curvature strain on the membrane that creates an toroidal pore (92). Cecropin B may be a promising drug for using in the human cancers treatment as this anticancer peptide reveals in vivo antitumor activity in mice bearing ascetic colon adenocarcinoma cells, as well as in vitro cytotoxic activity against human breast cancer cell lines (82), ovarian cancer cell lines (10), leukemia cancer cell lines (9), bladder cancer cell lines (7), hepato carcinoma cell lines (8), stomach carcinoma AGS, lung cancer cell lines (64).

2.6.3 Production of Cecropin B

High potential of Cecropin B in killing microorganisms and anticancer activity induce researchers explore more activity of this peptide. Several company have synthesized ready-to-use Cecropin B powder, however the cost of synthesis peptide are still expensive. Many experiments utilize prokaryote as hosts for production (93, 94). Nevertheless, recombinant protein from prokaryote also has limited condition with post-translational modification which peptide may become malfunction because of unstable protein folding (95). According to eukaryotic organisms that have such function of post-translational modification, correct glycosylation and protein folding, peptides production in eukaryote can be the alternative way to solve this problem. From previous studies, Cecropin B was successfully produced in *Pichia pastoris* strain SMD 1168 and studied its bioactivity (96). Although recombinant protein Cecropin B was expressed and determined some bioactivity, however study

of anticancer activity using recombinant Cecropin B produced in eukaryotic cells should be improved for further application.

To produce the recombinant protein CB and CB-PP, the developed eukaryotic expression system in methylotrophic yeast was used to increase the production. The methylotrophic yeast is a group of yeast that has ability to use methanol as carbon source such as *Pichai pastoris, Hansenula polymorphas, Cadida boidinii* (97, 98) *Pichia pastoris* is a single-celled microorganism that is easy to manipulate and culture. However, it is also a eukaryote and capable of many of the post-translational modifications performed by higher eukaryotic cells such as proteolytic processing, folding, disulfide bond formation and glycosylation. *Pichai pastoris* contains two genes which is potential inducible promoters which are $_{p}$*AOX1* and $_{p}$*AOX2* promoters, these promoters will introduce yeast produce more recombinant protein. *Pichai pastoris* has been defined into three groups; methanol utilization plus (Mut plus), methanol utilization slow (Mut slow) and methanol utilization minus (Mut minus). Mut plus are X-33 wild type strain and GS115 which needs a histidine supplement to grow, both strain have two promoters. Mut slow is KM71H, inserting with argininosucinate lyase (*ARG4*), *AOX1* was disable resulting in slow utilize methanol. Mut minus, cannot grow in media containing methanol resulting from the two promoters were disrupted.

As a result, many recombinant proteins that come to close as inactive inclusion bodies in bacterial systems such as *E. coli* are produced as biologically active molecules in *Pichia pastoris* instead. The *Pichia pastoris* system is also generally considered because it is faster, easier, and less expensive to take advantage of than other higher eukaryotic expression systems such as insect and mammalian tissue culture cell systems and usually gives higher expression levels (99)

CHAPTER III

MATERIALS AND METHODS

3.1 Equipment and chemicals

3.1.1 Equipment

4°C Freezer	(Panasonic, Thailand)
Autoclave HV-50	(Amerex Instrument, Inc., U.S.A.)
ÄKTA start	(GE healthcare, U.K.)
Automatic micropipette P2, P20, P200 and P1000 HTL LABMATEPRO	(HTL LAB SOLUTIONS, Poland)
Automatic micropipette 1-5 ml	(Thermo Scientific, U.S.A.)
Balance Adventure™, ARC 120	(Ohaus., U.S.A)
Balance Adventure™, ARC 2140	(Ohaus., U.S.A)
Biological safety cabinet, Heal force®, Hfsafe-1200	(Shanghai Lishen, China)
Gene Pulser® Cuvette 0.2 cm	(Bio-Rad Laboratories, China)
High speed micro refrigerated centrifuge MTX-150	(Tony Seiko Co., Ltd., Japan)
High speed micro refrigerated centrifuge 6500	(Kubota, Japan)
Hot plate PC-101	(Corning, U.S.A.)
Incubator MIR 152	(Sanyo Electric, Japan)
Microplate reader Multiskan Fc Type 357	(Thermo Fisher scientific, China)
Micropulser™	(Bio-Rad, U.S.A.)
Microwave oven National®	(Matsushita Electric, Japan)
NanoDrop™ 2000C UV-Vis Spectrophotometer	(Thermo scientific, U.S.A)
PCR	(Bio-Rad, U.S.A.)
pH meter Accumet® AB15	(Fisher scientific, Singapore)
Power supply, Power PAC3000	(Bio-RAD Laboratories, U.S.A.)
UV-Visible recording spectrophotometer UV-160	(Shimadzu, Japan)
Vortex mixer KMC-1300V	(Vision scientific, Korea)
Water proplus	(Labconco, U.S.A.)

3.1.2 Chemicals and reagents

3-(4, 5-dimethylthiazol-2-yl)-2, 5 Bio Basic INC diphenyltetrasolium bromide (MTT) (Bio-Basic, Canada)

Absolute ethanol (Merk, U.S.A.)

Absolute methanol (Merk, U.S.A.)

Ammonium persulfate (Sigma Aldrich, U.S.A.)

Glacial acid 100% anhydrous (Merk, Germany)

Agar Microbiology grade (Merk, Germany)

Agarose Molecular biology grade (Research organics, U.S.A.)

Bacto™ Peptone powder (Becton, Dickinson and company, France)

Biotin (Fluka, Germany)

Calcium chloride anhydrous (Merk, Germany)

di-Potassium hydrogen phosphate (Carlo Erba Reagenti, Italy)

di-Sodium hydrogen phosphate (Carlo Erba Reagenti, Italy)

Dimethyl sulfoxide, DMSO (Fluka, Switzerland)

Dithiothreitol, DTT (Bio-Basic, Canada)

DNA ladder 1 kb, M11 (SibEnzyme, Russia)

D-sorbitol (Fluka, Germany)

Ethidium bromide (Bio-Basic, Canada)

Ethylenediaminetetraacetic acid, EDTA (Carlo Erba Reagenti, Italy)

Fetal calf serum, FCS (Biochrom, U.K.)

Glycerol (Ajex Chemicals, Australia)

HEPES, free acid (Bio-Basic, Canada)

Hydrochloric acid 37% (Merk, Germany)

Roswell Park Memorial Institute medium, RPMI 1640 (Biochrom, U.K.)

Sodium chloride (Ajex Chemicals, Australia)

Sodium dodecyl sulfate, SDS (Bio-Basic, Canada)

Sodium hydroxide (Ajex Finechem, Australia)

TAE buffer free mix powder (Bio-Basic, Canada)

Tetramethylethylenediamine, TEMED (Bio-Rad, U.S.A.)

Tris, molecular biology grade (Research organics, U.S.A.)

Tryptone powder (Bio-Basic, Canada)

Yeast nitrogen base powder with ammonium sulfate, YNB (Bio-Basic, Canada)

Yeast extract powder (Bio springer, France)

Zeocin™ (Invitrogen, U.S.A.)

3.1.3 Kits

E.Z.N.A. gel extraction kit (OMEGA Biotek, Georgia)

3.1.4 Enzymes

EcoR I (Roche, Germany)

i-*Taq* DNA polymerase (iNtRON Biotechnology, Korea)

Kpn I (Roche, Germany)

Pst I (Vivantis, Malasia)

RNase A (New England Biolab, U.K.)

T4 DNA Ligase (Promega, U.S.A.)

3.1.5 Microorganisms

Escherichia coli Top10F' used as a host for recombinant plasmid construction and *Pichia pastoris* strains; KM71H, X-33 and GS115 purchased from Invitrogen, U.S.A.

3.1.6 Cell lines

Cells line were obtained from the American Type Culture Collection (Rockville, MD)

Human breast carcinoma cell (BT-474), ATCC® HTB-20™

Human lung bronchus carcinoma cell line (ChaGo-K1), ATCC® HTB-168™

Human liver hepatocellular carcinoma cell line (Hep-G2), ATCC® HTB-8065™

Human gastric carcinoma cell line (Kato-III), ATCC® HTB-103™

Human colon carcinoma cell line (SW-620), ATCC® HTB-227™

Human lung fibroblast cell line (WI-38), ATCC® CCL-75™

3.2 General protocol

3.2.1 Agarose gel electrophoresis

The agarose gel was prepared by dissolving the agarose gel powder in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Then, melted the agarose gel and cooled down solution. The solution was poured into a tray equipped with comb for a well-forming. After the gel was polymerized, it was loaded into the chamber with 1× TAE running buffer. The DNA samples mixed with 1X loading dye (final concentration) (50 mM Tris-HCl, 2.5 mg/ml bromophenol blue, 2.5 mg/ml xylene cyanol, 60% glycerol at pH 7.6) were loaded into the wells. Next, the standard DNA markers (1kb DNA ladders) were loaded into the well. The DNA was separated by electrophoresis at 100 volts for 25 min in 1× TAE buffer. The gel was stained with ethidium bromide solution for 10 minute and de-stained in the water for 2 min. The DNA bands were visualized under the UV transilluminator.

3.2.2 Protein analysis

3.2.2.1 Analysis of recombinant protein by SDS-PAGE

The gel solutions for 18% separating gel (2.50 ml of 30% acrylamide, 1.27 ml of 1.5 M Tris-HCl pH 8.8, 50 µl of 10% SDS, 50 µl of 10% APS, 2 µl of TEMED and 1.13 ml of distilled water) and 5% stacking gel (500 µl of 30% acrylamide, 380 µl of 1 M Tris-HCl pH 6.8, 30 µl of 10% SDS, 30 µl of 10% APS, 3 µl of TEMED and 2.06 ml of distilled water) were prepared. The separation gel solution was poured into the glass plates with 0.75 mm spacer and covered on top with distilled water. After 30 min, the gel was completely polymerized. Then, the stacking gel solution was poured on top of the separating gel and comb was immediately put between the glass plates. After the stacking gel was polymerized, the comb was removed and the wells were rinsed with distilled water to remove excess un-polymerized acrylamide. The protein samples were prepared by resuspending the proteins in 1× SDS loading buffer (12 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 0.02% bromophenol blue, and 2.88 mM 2-mercaptoethanol). The samples were then boiled for 10 min and spun down. The samples and protein standard marker were loaded into gel. Electrophoresis was run

in 1× SDS running buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 0.1% (w/v) SDS) with a constant current of 25 mA per gel until the dye font search the end of the gel. After electrophoresis, the gel was stained in the Coomassie brilliant blue R250 staining solution (0.1% (w/v) Coomassie brilliant blue R250, 10% (v/v) acetic acid, 45% (v/v) methanol) at room temperature with gentle shaking for 1 hour. Next, the gel was destained using the destaining solution (10% (v/v) acetic acid, 10% (v/v) methanol) and shaken at room temperature until the gel background was clear.

3.3 Methods

3.3.1 Construction of Cecropin B (CB) and Cecropin B-Polyproline (CB-PP) into pGEM®-T Easy Vector

3.3.1.1 Amplification of CB and CB-PP genes

The double stand DNA of CB-PP gene was synthesize and cloned into pUC57 vector by BIO BASIC Int. (Canada) to become pUC57::CB-PP plasmid. The CB and CB-PP gene was amplified by PCR using pUC57::CB-PP as template. For CB gene, CB-Fp (5′-GAA TTC AAG TGG AAG GTC TTC AAG-3′) which contained *Eco*R I restriction site (underlined) as forward primer, CB-Rp (5′- ATG CTG CAG TGC CTT CGC TTC GCC CAG -3′) which contained *Pst* I restriction site (underlined) as reverse primer. For CB-PP gene, CB-Fp (5′-GAA TTC AAG TGG AAG GTC TTC AAG-3′) which contained *Eco*R I restriction site (underlined) as forward primer, CB-PP-Rp which contained *Pst* I restriction site (5′-G CTG CAG GCG GGA CGG GGT AGC GCG-3′) as reverse primer. PCR reactions were performed according to the manufacturer's instruction of the iTaq™ DNA polymerase master mix (Intron Biotechnology), which the amplification procedure consisted of an initial incubation for 2 min at 94°C followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 30 s at 72°C. The reaction was then maintained at 72°C for 7 min. The amplified PCR products were analysed using 1% agarose gel electrophoresis preparing with 1× TAE buffer.

3.3.1.2 Ligation of CB and CB-PP into pGEM®- T Easy Vector

PCR products of CB and CB-PP were ligated into a pGEM®-T Easy Vector (Promega), with overhang T-A cloning technique. The total 20 µl of ligation mixture contained 2 µl of 10× T4 ligation buffer, 100 *ng* of pGEM®-*T Easy Vector*, 50 ng of PCR products of CB and CB-PP fragments, 1 µl of T4 DNA ligase and adjusted to expected total volume with sterilized water. The reaction was incubated at 16°C for 3-5 h. Afterwards, 10 µl of the ligation mixture was transformed into freshly prepared *E. coli* Top10F' competent cells as described in the section below.

3.3.1.3Transformation of pGEM::CB and pGEM::CB-PP into competent E. coli

3.3.1.3.1Preparation of fresh competent *E. coli*

Pick up a fresh single colony of ***E. coli*** (Top10F'), inoculated in 5 ml of LB broth in 250 ml Erlenmeyer flask with shaking at 200, grown for 16-20 hours at 37 $^{\circ}$C. Transferred the culture about 0.5 ml to 100 ml LB broth, incubated the culture about 3 hours at 37 $^{\circ}$C, 200 rpm. The cell culture was transferred aseptically to a sterile ice-cold 50 ml tubes, the culture was cooled to 0 $^{\circ}$C for 10 minutes. The cells was recovered by centrifugation at 4000 rpm for 10 minutes at 4 $^{\circ}$C. The media was decanted from cell pellets and resuspended each pellet in 10 ml ice-cold 0.1 M $CaCl_2$ and store on ice. The cells was recovered again by centrifugation at 4000 rpm for 10 minutes at 4 $^{\circ}$C, decanted the supernatant and resuspended each pellet in 2 ml ice-cold 0.1 M $CaCl_2$ and store on ice. A chilled, sterile pipette tip was then used to transfer 200 µl of competent cells to a sterile microfuge tube, kept the competent cells until use.

3.3.1.3.2Transformation of *E. coli* by heat shock

The ligation reaction 10 µl was added to each tube, mix the tubes gently by swirling then stored on ice for 30 minutes. The tubes were transferred to a rack place in a circulating water bath which had been preheated to 42°C, leaved the tubes in the rack for exactly 90 seconds without shaking. After that, rapidly

transferred the tubes on ice, allow the cells to chill for 3 minutes. Adding each tube with 800 µl of LB broth incubated the cultures for 45 minutes at 37 °C. Transfer appropriate volume of transformed competent cells onto LB agar containing 100 µg/ml ampicillin, gently spread plate on agar plate, incubated plate at 37 °C, colonies will appear in 12-16 hours. The selected clones were selected using blue-white screening method and were sequenced using T7 promoter primer with an automated sequencer by a commercial service (Macrogen Inc., Korea), The DNA sequences were analyzed, and a BLAST search was performed using NCBI portal. The pGEM®-T Easy Vector fused with CB and CB-PP was named as pGEM::CB and pGEM::CB-PP.

3.3.2 Construction of CB and CB-PP into pPICZαA expression vector

3.3.2.1 Extraction and purification of plasmid DNA

CB and CB-PP in pGEM®-T Easy Vector was extracted using the protocol from molecular cloning, second edition (E.F. Fritsch, J. Sambrook and T. Maniatis, 1989)

3.3.2.1.1 DNA extraction

A single bacterial colony was transferred into 5 ml of LB medium containing 100µg/ml ampicillin. Incubate the culture overnight at 37°C, 200 rpm. The culture was poured into a microfuge tube, centrifuged at 12,000g for 30 seconds at 4°C in a microfuge. Store the remainder of the culture at 4°C. The medium was then removed, leaving the bacterial pellet as dry as possible. The bacterial pellet was resuspended in 100 µl of ice-cold solution I by vigorous vortex. The freshly prepared solution II was added of 200 µl, mixed by inverting the tube rapidly 5 times. The 150 µl of ice-cold solution III was added, vortexed gently or inverted for 10 seconds, stored on ice for 3-5 minutes. Centrifuge at 12,000g for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh tube. Precipitate the double-stranded DNA with 2 volumes of ethanol at room temperature. Mix by vortexing Allow the

mixture to stand for 2 minutes at room temperature. Centrifuge at 12,000g for 5 minutes at 4°C in a microfuge. Remove the supernatant, rinse the pellet with 1 ml of 70**%** ethanol at 4 °C. Remove the supernatant and allow the pellet to dry in the air for 10 minutes. Redissolve the nucleic acids in 30-50 µl of TE pH 8.0 or sterile water containing DNAase free pancreatic RNAase 20 µg/ml, vortex briefly and store the DNA at -20 °C.

3.3.2.1.2 DNA purification

The plasmids or PCR products were purified from agarose gel by E.Z.N.A. gel extraction kit, OMEGA Biotek. The agarose gel band was excised from agarose gel with a clean sharp scalpel. Extra agarose was removed to minimize the size of the gel slice. The gel slice was weighted and transferred to a clean microcentrifuge tube. Assuming a density of 1 g/mL of the volume of gel is derived by adding 1 volume Binding Buffer (XP2) to the tube, incubated at 60°C for 7 minutes or until the gel has completely melted. The tube was shaking every 2-3 minutes, inserted a HiBind® DNA Mini Column in 2 ml Collection Tube then added no more than 700 µl DNA/agarose binding buffer to the HiBind® DNA Mini Column. The tube was centrifuged at 10,000 x g for 1 minute at room temperature, discarded the filtrate and reuse collection tube, repeated again and the sample has been transferred to the column. Binding Buffer (XP2) was added to the tube 300 µl, centrifuged at maximum speed (≥13,000 x g) for 1 minute at room temperature. The filtrate was discarded, added 700 µL SPW Wash Buffer and centrifuged at maximum speed for 1 minute at room

temperature, discarded the filtrate. The empty HiBind® DNA Mini Column was centrifuged for 2 minutes at maximum speed to dry the column matrix. Then, transferred the HiBind® DNA Mini Column to a clean 1.5 mL microcentrifuge tube, added 30-50 µL Elution Buffer or deionized water directly to the center of the column membrane and centrifuged at maximum speed for 1 minute. DNA was kept at -20°C until used.

3.3.2.1.3 Ligation and transformation of CB and CB-PP into pPICZαA expression vector

The pPICZαA::CA-PP constructed by inserting *Pst* I site and 6-histidine after Cecropin A (CA) nucleotides sequence was used to be a host vector (constructed by Dr.Sarintip Sooksai). The pGEM::CB and pGEM:CB-PP were extracted and digested with *Eco*R I and *Pst* I to get CB and CB-PP fragments. After that, the CB and CB-PP fragments with the size of 114 and 159 bp respectively were purified, and ligated with the modified host vector, pPICZαA::CA-PP which digested with *Eco*R I and *Pst* I to generate the pPICZαA::CB and pPICZαA::CB-PP plasmid. The total 20 µl of ligation mixture contained 2 µl of 10× T4 ligation buffer, 100 *ng* of *Eco*RI/*Pst*I digested pPICZαA*, 200 ng of Eco*RI/*Pst*I digested CB and CB-PP*,* 1 µl of T4 DNA ligase and adjusted to expected total volume with sterilized water. The reaction was incubated at 16°C for 3-5 hours. After that*,* 10 µl of the ligation mixture was transformed into freshly prepared *E. coli* Top10F' competent cells as described in the section 3.3.1.3.1. The reaction will be transformed into *E. coli* Top10F' competent cells by heat shock method (described in section 3.3.1.3.2) and plate on low salt LB solid medium containing 25 µg.ml^{-1} Zeocin. Pick up a transformant clones, growth and extract plasmids (described below) for checking the expected size by 1% agarose gel electrophoresis, then sequenced by Macrogen (Korea). The

DNA sequences were analyzed, and a BLAST search was performed using NCBI portal. The pPICZαA vector fused with CB and CB-PP was named as pPICZαA::CB and pPICZαA::CB-PP.

3.3.3 Transformation of recombinant plasmid pPICZαA::CB and pPICZαA::CB-PP into *Pichia pastoris* strain KM71H

3.3.3.1 Preparation of competent yeast

Grew *Pichia pastoris* strain KM71H in 5ml YPD at 30 °C, 250 rpm overnight. Inoculate 1 ml of culture in 50 ml YPD broth (see Appendix A), shaking with 250 rpm, 30 °C about 3 hours. Centrifuge at 5000 rpm, 4 °C, 5 minutes. Resuspend the pellet in 10 ml YPD and 2 ml 1 M HEPES pH8.0 Add 250 µl 1M DTT, gently mix. Incubate at 30 °C, 5 minutes. Chill on ice 5 minutes. Centrifuge at 5000 rpm, 4 °C, 5 minutes. Wash with 25 ml cold sterile water. Centrifuge at 5000 rpm, 4 °C, 5 minutes. Wash with 2 ml cold 1M sorbital. Resuspend in 100 µl cold 1M sorbital.

3.3.3.2 Transformation of fresh competent yeast

The CB and CB-PP were linearized using Kpn I restriction enzymes then, mix 10 µl DNA (approximately 5 -10 ng DNA) with 80 µl competent cell and transfer to 2-mm gap cuvette, held on ice 5 minutes. Pulse the cells with 2.5 kV. Add 500 µl of 1M cold sorbital to cuvette and transfer to 1.5 ml microtube. Incubate the cells at 30 °C for 1 hour without shaking. Add 500 µl YPD broth and incubate at 30 °C for 1 hour, 200 rpm. Gently spread plate on YPD plate containing 100 µg/ml zeocin antibiotic. Incubate at 30 °C for 3 days.

3.3.4 Expression of recombinant protein CB and CB-PP

Fresh single colony of each *Pichia pastoris* KM71H recombinant clones was inoculated in 10 ml of BMGY medium (see Appendix A) for starter culture and growth overnight at 30°C with shaking at 280 rpm to an OD_{600} of 2-6. Inoculated 10% of

starter culture in 100 ml of BMGY medium in 250 ml baffle flask and cultivate at 30°C for 24 h with shaking at 280 rpm. After 24 hours, Cells were collected by centrifugation at 5,000×g for 5 minutes at room temperature, decant the media and pellets resuspend in 20 ml of BMMY expression medium (see Appendix A) for induction and 100% methanol was added to a final concentration of 0.5% methanol every 24 hours to maintain an induction. The cell cultures were took at 24, and 48 hours and analysed by SDS-PAGE analysis.

3.3.5 Purification of recombinant protein CB-PP

The His_6-tag rCB-PP protein was purified by nickel affinity chromatography (Histrap Fast Flow column) using Akta start. First, filtrated the supernatant with 0.45 micron filter, then equilibrated protein by mixing with binding buffer (20 mM Sodium Phosphate buffer pH7.4 containing 0.5 M NaCl, and 5 mM imidazole) in the ratio 1 : 2 (supernatant : binding buffer). After that, the Histrap Fast Flow bead (GE Healthcare) was equilibrated with the same binding buffer for 10 column volumes and 100 ml supernatant was loaded into the column. Then, the Ni-bead was washed by the binding buffer for 10 column volumes to remove unbound proteins. Next, the recombinant protein was eluted by the elution buffer (20 mM Tris-HCl supplement with, 0.5 M NaCl, pH 7.4) containing 500 mM imidazole and collected the fractions. The purified fractions were analyzed by 18% SDS-PAGE. The fractions containing the expected recombinant protein were pooled and the imidazole was removed by dialysis one hundred thousand fold at 4 ℃ against phosphate-buffered saline (PBS), pH 7.4. The protein quantified using NanoDrop 2000C UV-Vis spectrophotometry.

3.3.6 Cytotoxicity assay using MTT

3.3.6.1 Cell lines culture and treatment condition

The cell lines including human breast carcinoma cell (BT-474), human lung bronchus carcinoma cell line (ChaGo-K1), human liver hepatocellular carcinoma cell line (Hep-G2), human gastric carcinoma cell line (Kato-III) and human colon carcinoma cell line (SW-620) were cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplement with 5-10 % fetal calf serum (FCS). While human

lung fibroblast cell line (WI-38) were cultured in *Dulbecco's Modified Eagle's Medium (DMEM)* supplement with 5-10 % fetal calf serum (FCS). Cells cultured were incubating at 37°C in a 5 % CO_2 atmosphere. After 24-48 hours incubation period, the attached cell was trypsinized by trypsin for 3-5 minutes and centrifuges a 380 x g for 5 minutes. The cell were counted and distributed in 96-well plates at $5x10^3$ cell per well for further treatment.

All cell lines including both normal cell line and cancer cell lines were treated with several concentration of peptide. For the first experiment, the synthetic CB and recombinant protein CB were used in the concentration of 0.019μM - 10μM. The second experiment, the recombinant CB and CB-PP were used in the concentration of 0.039μM - 20μM. All samples were diluted by serial dilution of culture media. Cell lines were counted and distributed in 96-well plates at 5x103 cell/well and incubate for 24 hours, then culture medium were remove from 96-well plates and added 200 μl, serial dilution of peptide for each well. The plate was incubating 72 h at 37° C in a fully humidified atmosphere of 5% CO_2 atmosphere.

3.3.6.2 Cytotoxicity test by MTT assay

Cytotoxicity assay was performed using tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a mitochondrial-based cell viability assay. This assay is a colorimetric assay for assessing the activity of mitochondrial dehydrogenase enzyme that can reduce MTT to produce insoluble formazan in living cells which has a purple colour (Figure 3.1). This reaction allows determining the viability of cells. The procedure is starting with 5×10^3 cells per well was plate in 96-well plate and, after 24 hours the cell lines were treated with different concentration of peptide. The plate was incubated 72 hours at 37°C in 5% CO_2 atmosphere. After 72 hours, cells were incubated with 10 μl of 5 mg/ml MTT solution for 4 hours at 37°C in 5% CO_2 atmosphere. The MTT solution was then discarded and 150 μl of DMSO was added to each well. The absorbance of this solution was quantified by measuring with microplate reader at the wavelength of

540 nm. The percentage of cell viability was calculated according to the following formula.

$$\text{Percentage of cell viability} = \frac{\text{OD of treated cell} \times 100}{\text{OD of control cells}}$$

The IC_{50} values were obtained by plotting the percentage of cell viability versus the peptide concentration.

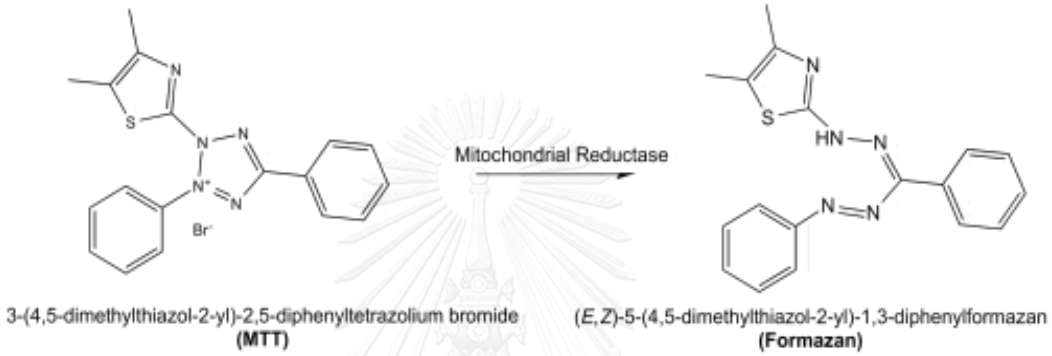


Figure 3. 1 The scheme of reduction of the MTT to formazan by mitochondrial reductase enzymes.
(Source: www.interchopen.com)

1

CHAPTER IV
RESULTS

4.1 Construction and transformation of pGEM::CB and pGEM::CB-PP

Previously, the pUC57::CB-PP plasmid synthesized by BIO BASIC Int. (Canada) was used as a template for amplifying CB and CB-PP genes which both will have 6 histidine tag (6x His - tag) at C-terminus. The CB gene was amplified by primer CB-Fp which introduced an *Eco*R I site at N-terminus, and CB-Fp which introduced a *Pst* I site at C-terminus. The CB-PP gene was amplified by primer CB-Fp which introduced an *Eco*R I site at N-terminus, and CB-PP-Rp which introduced a *Pst* I site at C-terminus. Size of the purified PCR products was analysed by 1% agarose gel electrophoresis (figure 4.1). It was found that the sizes of CB and CB-PP genes were approximately 114 and 159 bp respectively.

After receiving the CB and CB-PP genes, pGEM::CB and pGEM::CB-PP were generated by cloning these genes into pGEM® T-easy vector. The PCR products of CB and CB-PP fragments and were directly ligated with the pGEM® T-easy vector, then transformed into *E. coli* Top10F'. Size of the recombinant plasmid was analysed by 1% agarose gel electrophoresis to ensure that the correct fragment was inserted into pGEM® T-easy vector as shown in figure 4.4 and 4.5. The sizes of pGEM::CB was approximately 3129 bp and pGEM::CB-PP was approximately 3174 bp and the purified recombinant plasmid was confirmed by sequencing analysis (figure 4.2 and 4.3).

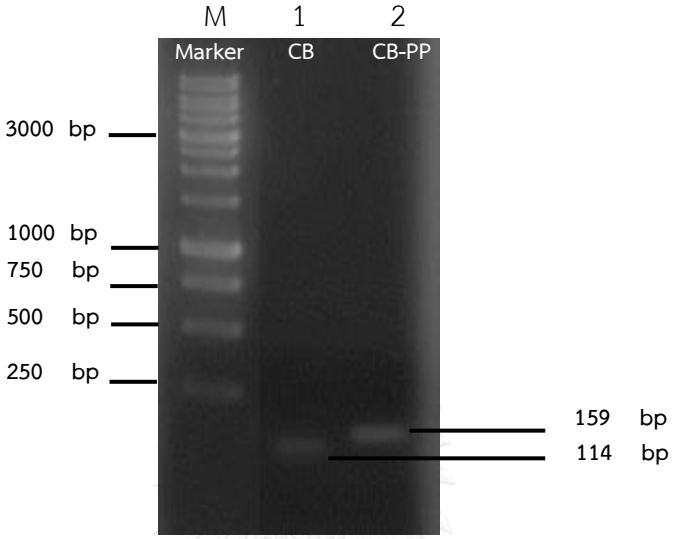


Figure 4. 1 1% agarose gel electrophoresis of CB and CB-PP genes. The CB-Fp, CB-Rp and CB-PP-Rp were used to amplify CB and CB-PP genes. Lane M is a 1 kb DNA ladder marker. Lane 1 is the CB gene with the size of 114 bp. Lane 2 is the CB-PP gene with the size of 159 bp.

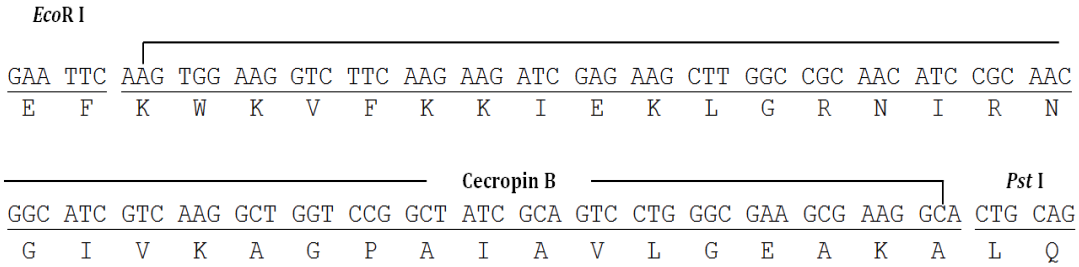


Figure 4. 2 The sequence of CB gene digested with *Eco*R I and *Pst* I enzymes consist of 114 nucleotides, 38 amino acids.

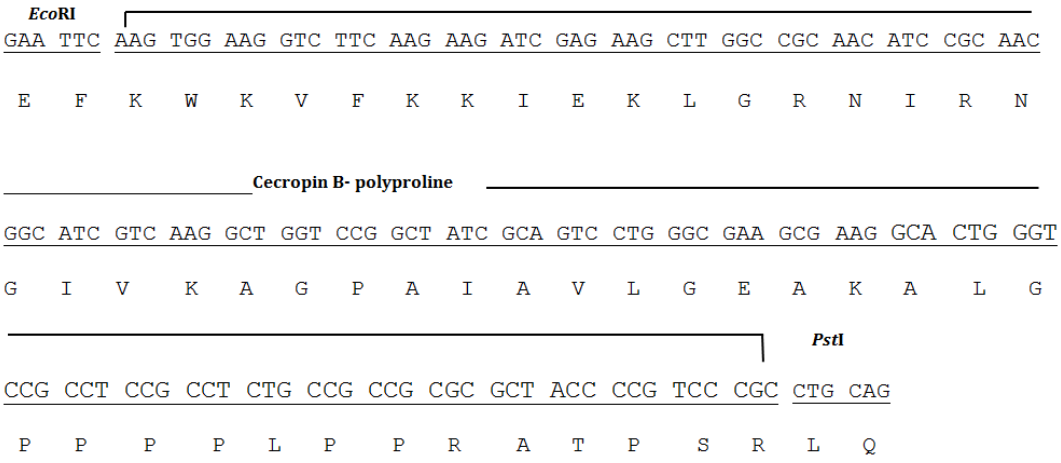


Figure 4. 3 The sequence of CB gene digested with *Eco*R I and *Pst* I enzymes consist of 159 nucleotides, 53 amino acids.

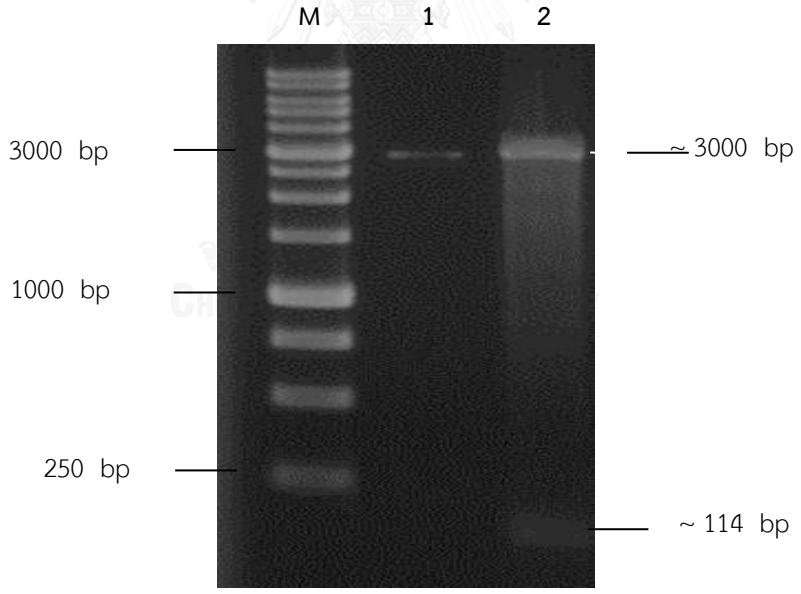


Figure 4. 4 1% agarose gel electrophoresis of pGEM::CB. The CB genes were ligated into pGEM® T-easy vector to generate the pGEM::CB and 1% agarose gel electrophoresis was used to analysed. Lane M is a 1 kb DNA ladder marker. Lane 1 is pGEM® T-easy vector with the size of 3015 bp. Lane 2 is pGEM::CB digested with *Pst* I and *Eco*R I restriction enzymes, the upper band was pGEM with the size approximately 3000 bp and the lower band was CB with the size approximately 114 bp.

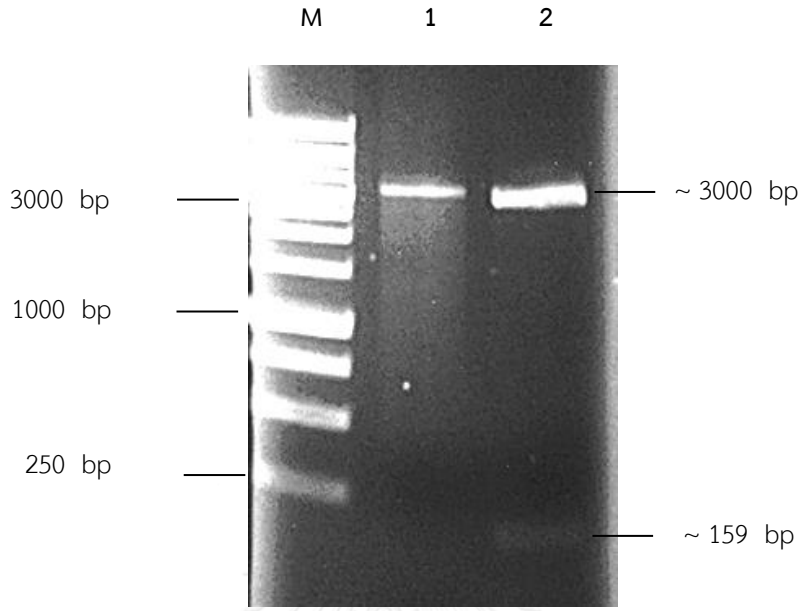


Figure 4. 5 1% agarose gel electrophoresis of pGEM::CB-PP. The CB-PP genes were ligated into pGEM® T-easy vector to generate the pGEM::CB-PP and 1% agarose gel electrophoresis was used to analysed. Lane M is a 1 kb DNA ladder marker. Lane 1 is pGEM® T-easy vector with the size of 3015 bp. Lane 2 is pGEM::CB-PP digested with *Pst* I and *Eco*R I restriction enzymes, the upper band was pGEM with the size approximately 3000 bp and the lower band was CB with the size approximately 159 bp.

4.2 Construction and transformation of pPICZαA::CB and pPICZαA::CB-PP

The pGEM::CB and pGEM::CB-PP were digested with restriction enzymes *Eco*R I and *Pst* I to obtain CB and CB-PP fragments. These CB and CB-PP DNA fragments were ligated into the modified pPICZαA expression vector which has been modified by inserting *Pst*I recognition site, 6-histidine and stop codon in front of the *Kpn*I of the recognition site in origin pPICZαA expression vector. The recombinant plasmids pPICZαA::CB and pPICZαA::CB-PP were transformed into *E. coli* Top10F' competent cells by chemical method. The transformants were selected on low salt LB containing 25 μg.ml^{-1} of Zeocin agar plate. Transformant clones were grown and the

recombinant plasmids, pPICZ**α**A::CB and pPICZ**α**A::CB-PP, were extracted. The size of the recombinant plasmid was analysed by 1% agarose gel electrophoresis to ensure that the correct fragment was inserted into the pPICZ**α**A expression vector as shown in figure 4.6 and 4.7. The sizes of pPICZ**α**A::CB and pPICZ**α**A::CB-PP was found to be 3697 bp and 3742 bp respectively. Then, the purified recombinant plasmid was confirmed by sequencing analysis (Figure 4.8 and 4.9) to assure that the fragments were inserted into the expression correctly as shown in the genetic map figure 4.10 and 4.11.

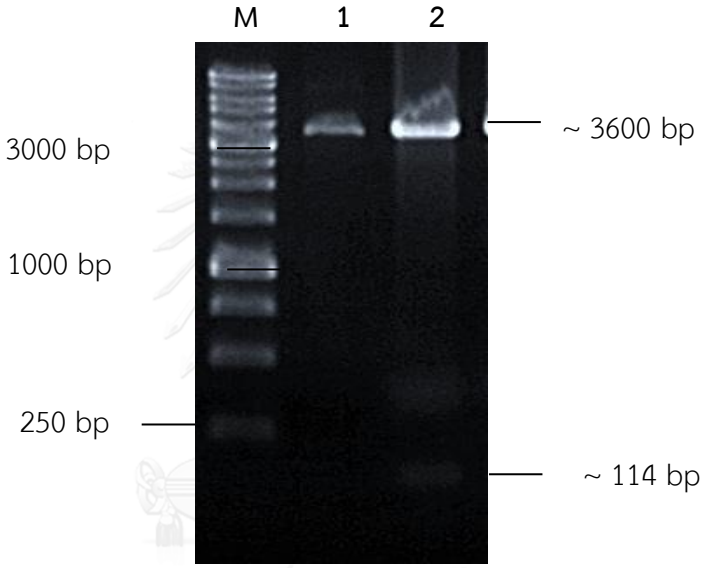


Figure 4. 6 1% agarose gel electrophoresis of pPICZ**α**A::CB. The CB genes digested with EcoR I and *Pst* I from pGEM® T-easy vector was ligated into pPICZ**α**A expression vector which was digested with same enzymes to generate the pPICZ**α**A::CB and 1% agarose gel electrophoresis was used to analysed. Lane M is a 1 kb DNA ladder marker. Lane 1 is pPICZ**α**A with the approximate size of 3600 bp. Lane 2 is pPICZ**α**A::CB digested with *Pst* I and *Eco*R I restriction enzymes, the upper band was pPICZ**α**A with the size approximately 3600 bp and the lower band was CB with the size approximately 114 bp.

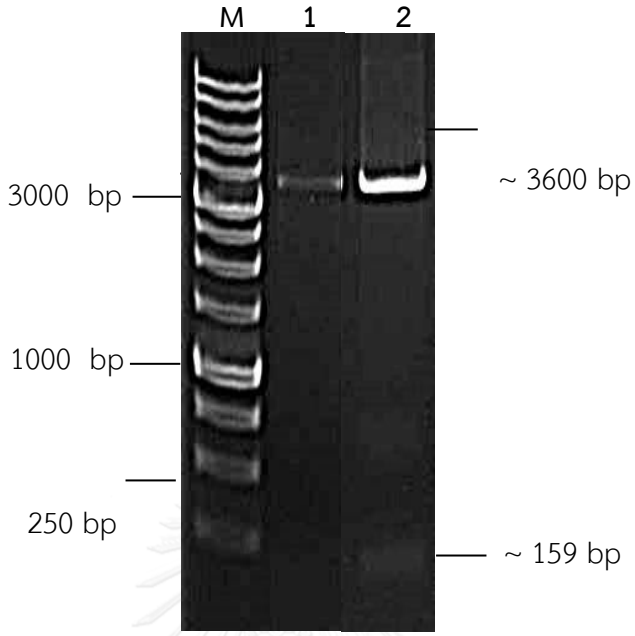


Figure 4. 7 1% agarose gel electrophoresis of pPICZαA::CB-PP. The CB-PP genes digested with EcoR I and *Pst* I from pGEM® T-easy vector was ligated into pPICZαA expression vector which was digested with same enzymes to generate the pPICZαA::CB-PP and 1% agarose gel electrophoresis was used to analysed. Lane M is a 1 kb DNA ladder marker. Lane 1 is pPICZαA with the approximate size of 3600 bp. Lane 2 is pPICZαA::CB-PP digested with *Pst* I and *Eco*R I restriction enzymes, the upper band was pPICZαA with the size approximately 3600 bp and the lower band was CB-PP with the size approximately 159 bp.

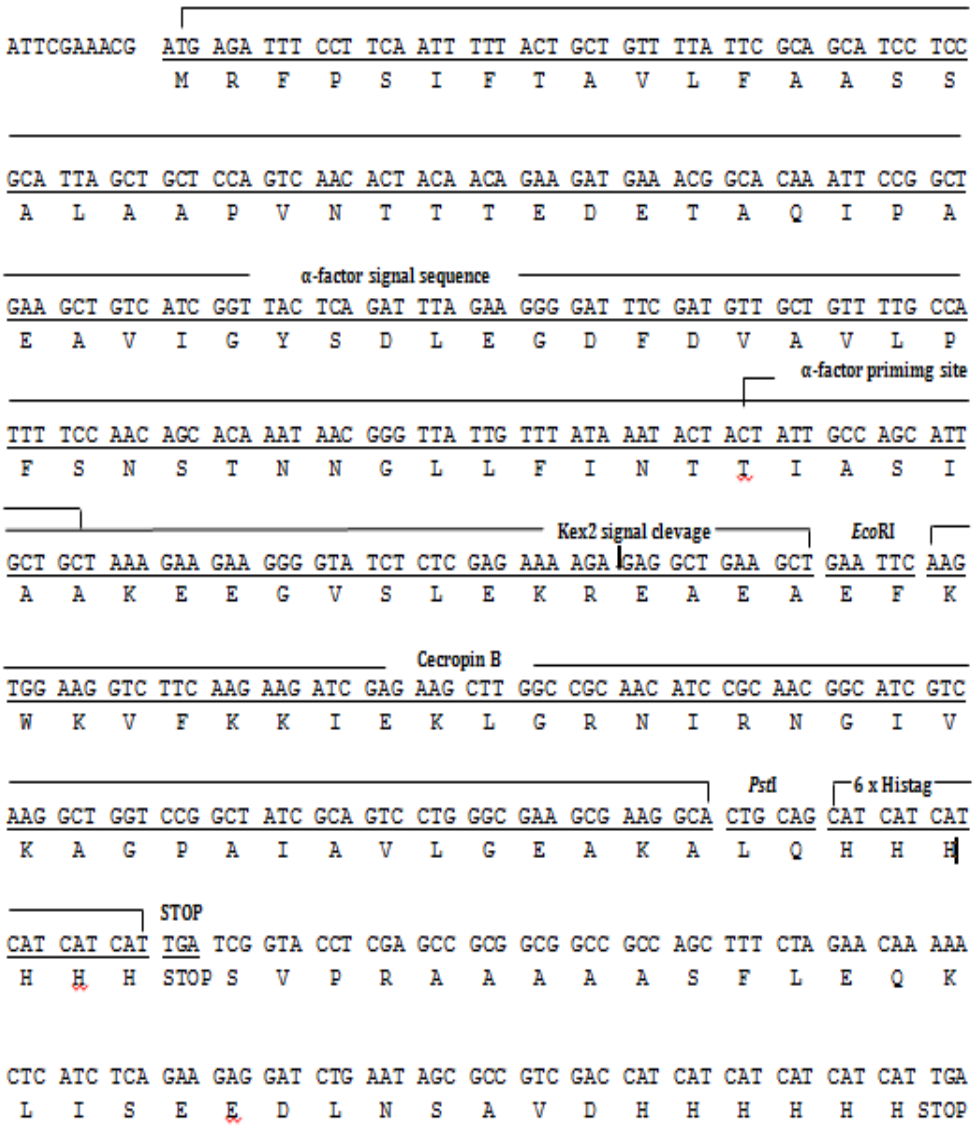


Figure 4. 8 The sequence of CB gene in pPICZαA expression vector with α-factor signal sequence and 6-histidine tag after *Pst* I restriction site.

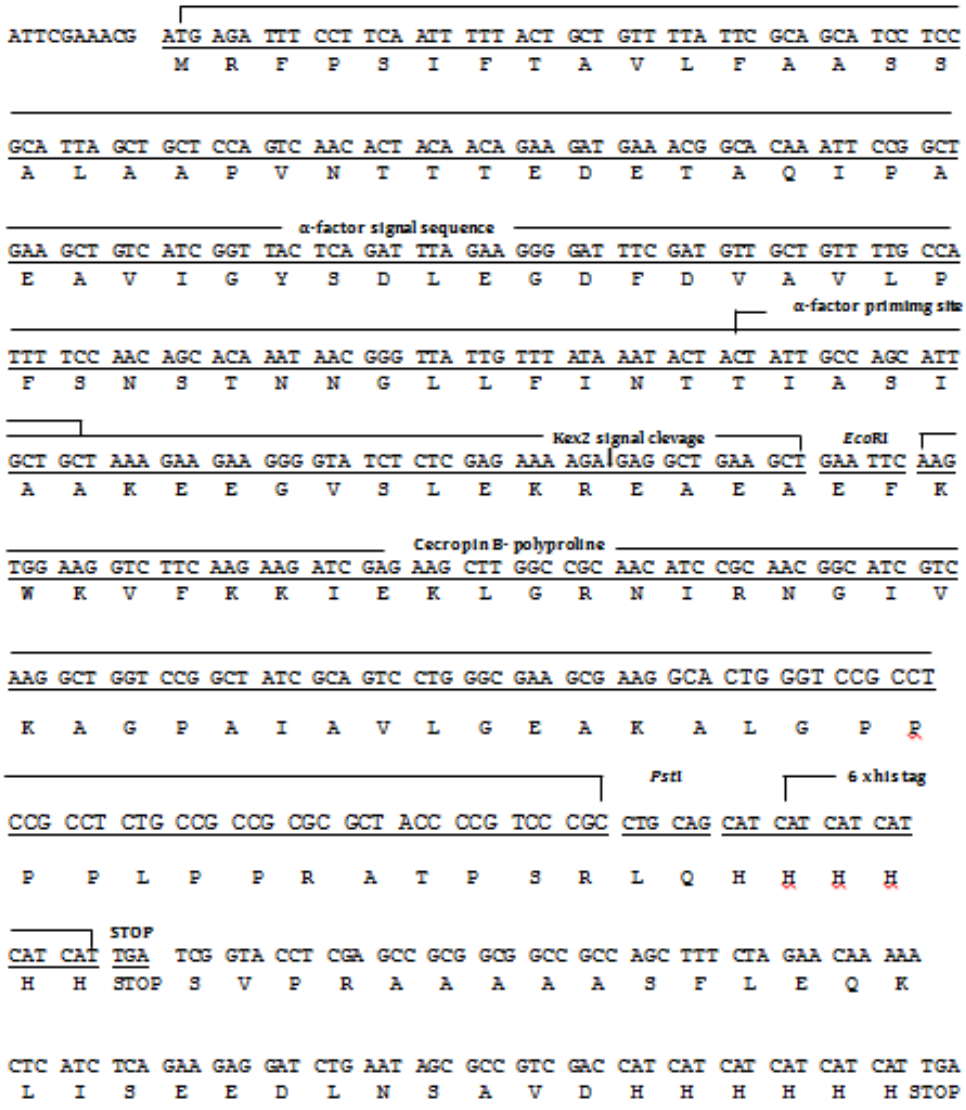


Figure 4. 9 The sequence of CB-PP gene in pPICZαA expression vector with α-factor signal sequence and 6-histidine tag after *Pst* I restriction site.

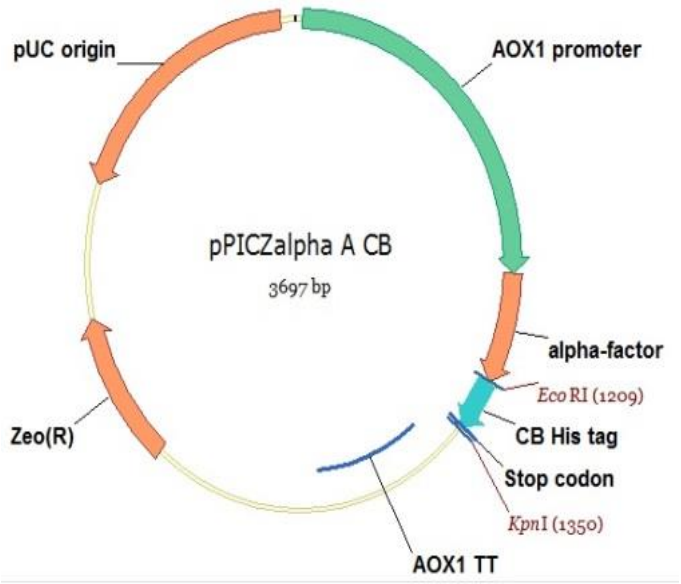


Figure 4. 10 Genetic map of recombinant plasmid of pPICZαA::CB. The size of pPICZαA::CB is approximately 3697 bp.

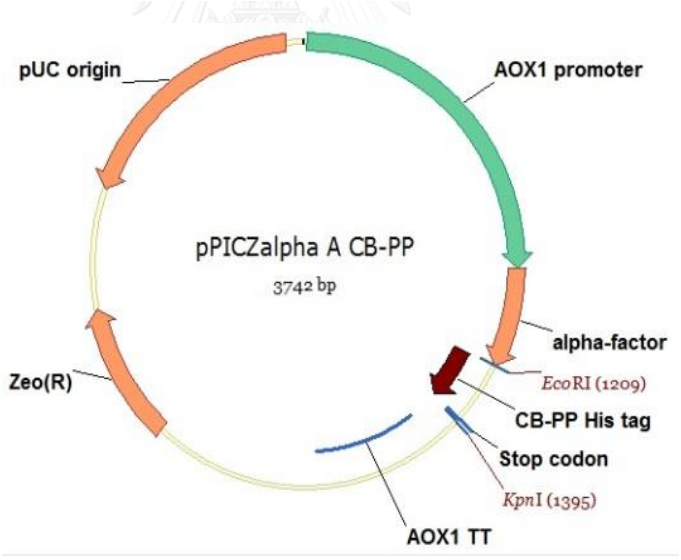


Figure 4. 11 Genetic map of recombinant plasmid of pPICZαA::CB-PP. The size of pPICZαA::CB-PP is approximately 3742 bp.

4.3 Expression and purification of recombinant protein CB and CB-PP

Transformants of *P. pastoris* KM71H which harbour pPICZ**α**A::CB or pPICZ**α**A::CB-PP were cultured in 100 ml BMGY medium in 500 ml baffled flasks for increase the cell population. After that cell pellet was collected and cultured in 20 ml BMMY for inducing protein expression. At every 24 h point during the expression process, 0.5% methanol was added to induce the strong expression of AOX1 promoter for 48 hours. The supernatant was collected every 0, 24 and 48 hours, stored under the temperature of -70^{o}C and analysed by 18% SDS-PAGE. The expected size of rCB and rCB-PP were found to be 4.9 and 6.5 kDa, respectively (Figure 4.12). Subsequently, the SDS-PAGE bands of recombinant protein CB-PP were sent to identify by mass spectrometry (MS/MS) (Figure. 4.13)**.** The supernatant of the recombinant protein was purified by nickel fast flow column (GE Healthcare) using Akta start (GE Healthcare). The recombinant proteins were eluted by 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4 and 500 mM imidazole. The typical chromatogram was shown in Figure 4.14, 4.15. After that the purified recombinant protein was analyzed by 18% SDS-PAGE, the expected size of rCB and rCB-PP were approximately 4.9 and 6.5 kDa respectively.

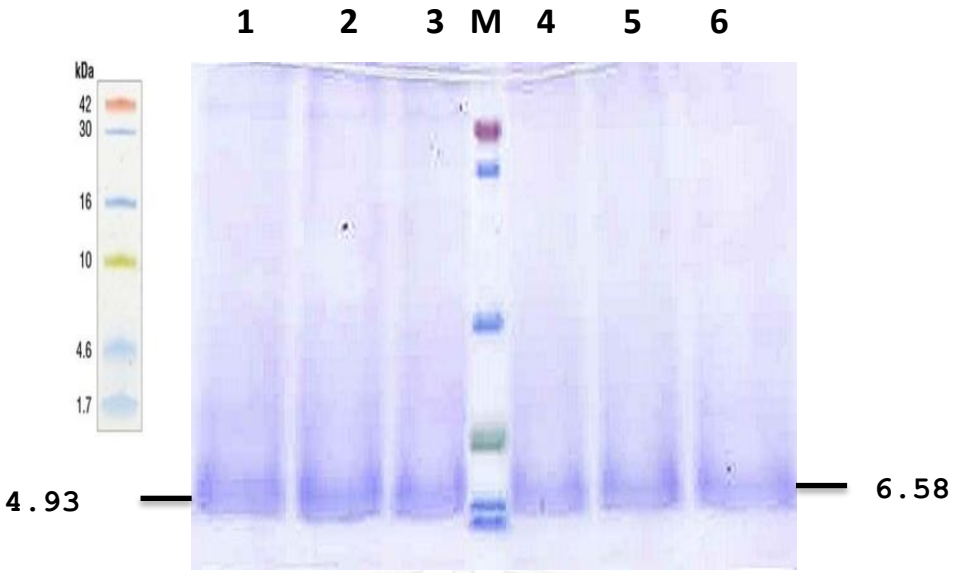
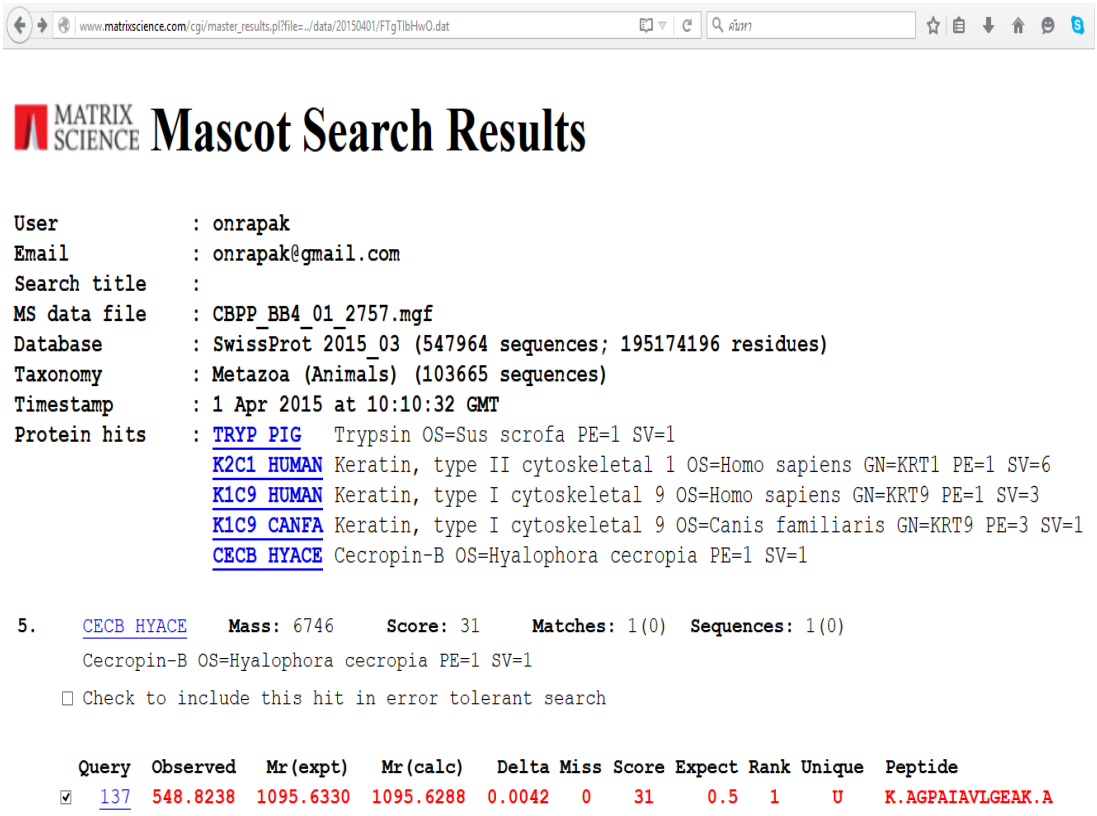


Figure 4. 12 18% SDS-PAGE analysis of recombinant protein CB and CB-PP from *P. pastoris* KM71H staining with Coomassie blue. Lane M, protein marker; Lane 1, 2 and 3, supernatant of recombinant protein CB collected in 0, 24, 48 hours respectively with the expected size approximately 4.9 kDa; Lane 4, 5 and 6, supernatant of recombinant protein CB-PP collected in 0, 24, 48 hours respectively with the expected size of approximately 6.6 kDa.



www.matrixscience.com/cgi/master_results.pl?file=../data/20150401/FTgTIbHwO.dat

MATRIX SCIENCE **Mascot Search Results**

User : onrapak
Email : onrapak@gmail.com
Search title :
MS data file : CBPP_BB4_01_2757.mgf
Database : SwissProt 2015_03 (547964 sequences; 195174196 residues)
Taxonomy : Metazoa (Animals) (103665 sequences)
Timestamp : 1 Apr 2015 at 10:10:32 GMT
Protein hits : TRYP_PIG Trypsin OS=Sus scrofa PE=1 SV=1
K2C1_HUMAN Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
K1C9_HUMAN Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3
K1C9_CANFA Keratin, type I cytoskeletal 9 OS=Canis familiaris GN=KRT9 PE=3 SV=1
CECB_HYACE Cecropin-B OS=Hyalophora cecropia PE=1 SV=1

5. CECB_HYACE **Mass:** 6746 **Score:** 31 **Matches:** 1(0) **Sequences:** 1(0)
Cecropin-B OS=Hyalophora cecropia PE=1 SV=1
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑ 137	548.8238	1095.6330	1095.6288	0.0042	0	31	0.5	1	U	K.AGPAIAVLGEAK.A

Figure 4. 13 Maspectromtry result of recombinant CB-PP. The recombinant CB-PP was analysed by mass spectrometry (MS/MS), then the database was searched by SwissProt program.

(http://www.matrixscience.com/cgi/master_results.pl?file=../data/20150401/FTgTIbHwO.dat.)

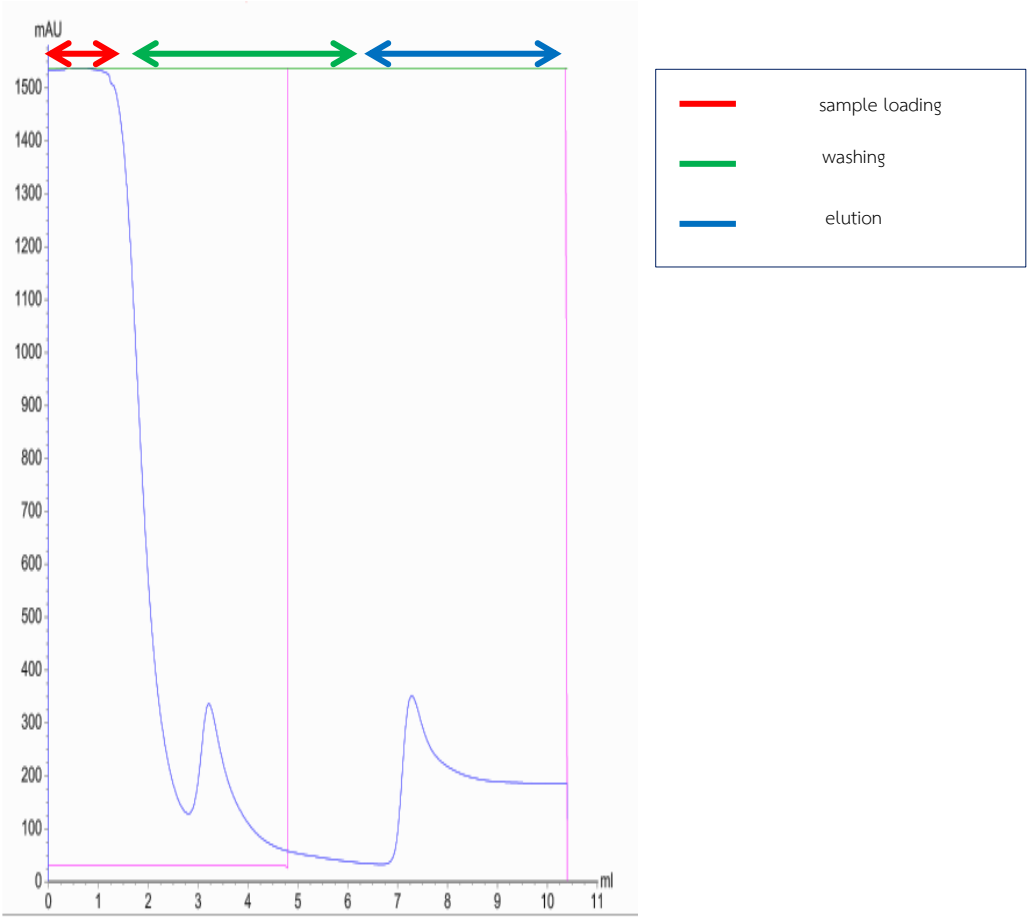


Figure 4. 14 The chromatogram of purified rCB. 100 ml of supernatant was equilibrated with binding buffer in the ratio 1:2 then, applied to the Histrap column. Subsequently, 3 ml (twice elution) of purified recombinant protein was eluted with elution buffer.

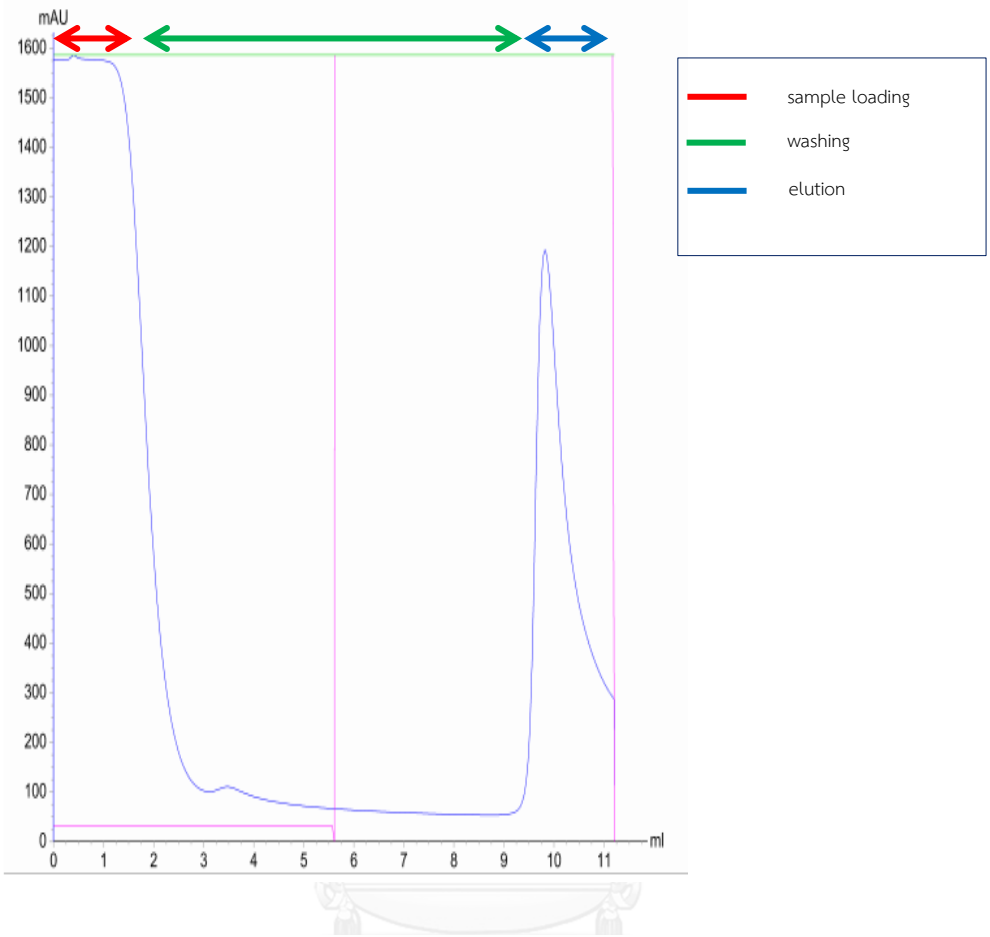


Figure 4. 15 The chromatogram of rCB-PP. 100 ml of supernatant equilibrated with binding buffer in the ratio 1:2 then, applied to the Histrap column. Then, 3 ml of purified recombinant protein was eluted with elution buffer.

Total concentration of recombinant protein CB and CB-PP was determined using NanoDrop2000C UV-Vis spectrophotometer (Thermo scientific, U.S.A.). The concentration of recombinant protein CB and CB-PP were approximately 0.4 mg/ml and 0.5 mg/ml respectively.

4.4 Cytotoxicity test by MTT assay

Cytotoxicity effect of the recombinant protein CB and CB-PP on the cell viability of human breast carcinoma cell line (BT-474), human lung bronchus carcinoma cell line (ChaGo-K1), human liver hepatocellular carcinoma cell line (Hep-G2), human gastric carcinoma cell line (Kato-III), human colon carcinoma cell line (SW-620) and human lung fibroblast cell line (WI-38) was studied. All cell lines were treated separately with each peptide in different concentration for 72 hours. Then, MTT assay was performed to determine the percentage of cell viability. The graphs of cell survival (%) versus the protein concentration were shown in Figure 4.16 and 4.17.

In the first experiment, , six cell lines were treated with synthetic CB and rCB-PP at the concentration between 0.019 μM – 10 μM. The results illustrated that the cell viability decreased as the concentration of protein increased. In addition, it was found that the viability of human breast carcinoma cell lines (BT-474), human lung bronchus carcinoma cell line (ChaGo-K1) and human colon carcinoma cell line (SW-620), treated with 10 μM of synthetic CB was lower than treated with rCB-PP at the same protein concentration. The IC_{50} values of cell lines could not be calculated because the protein concentration used in the experiment was not high enough. Interestingly, cell viability of human lung fibroblast cell line (WI-38) did not decreased when treated with both proteins. On the contrary, the cell numbers increased with respect to the protein concentrations as compared with untreated cells.

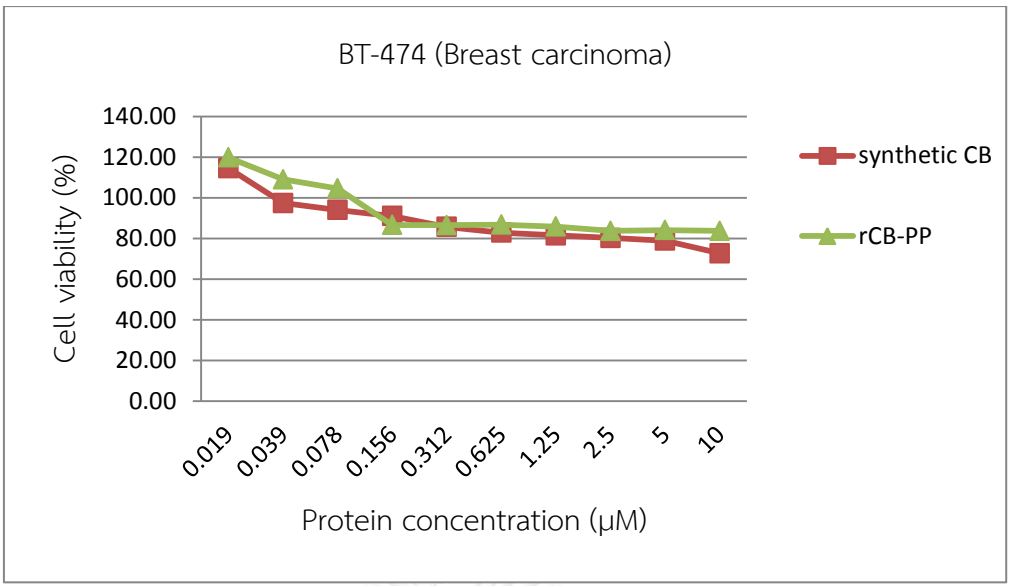


Figure 4. 16 Cell viability of human breast carcinoma cell (BT-474) after 72 hours treated with synthetic CB and recombinant CB-PP.

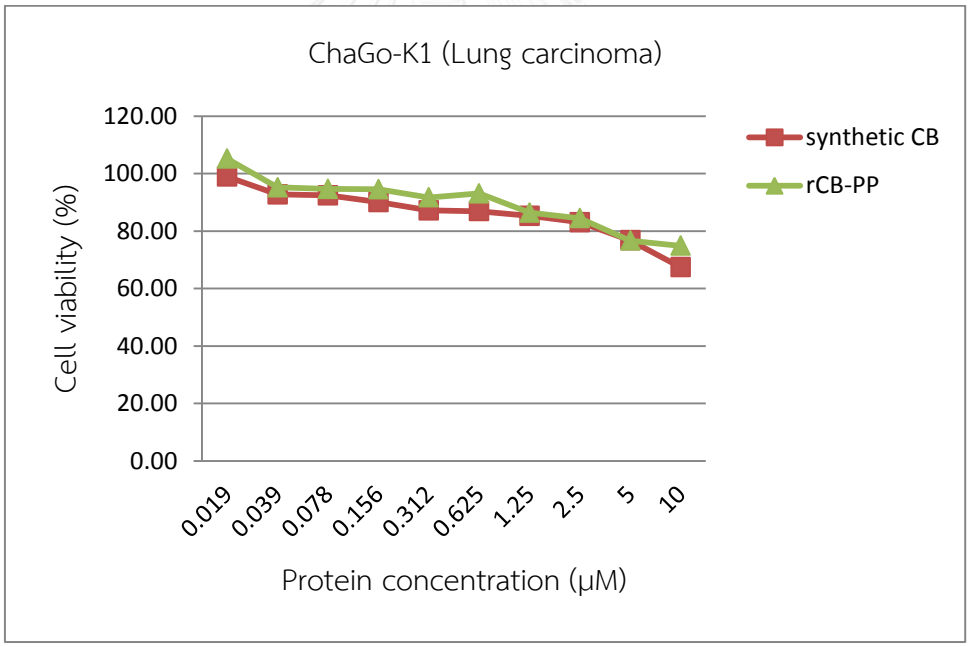


Figure 4. 17 Cell viability of human lung bronchus carcinoma cell line (ChaGo-K1) after 72 hours treated with synthetic CB and recombinant CB-PP.

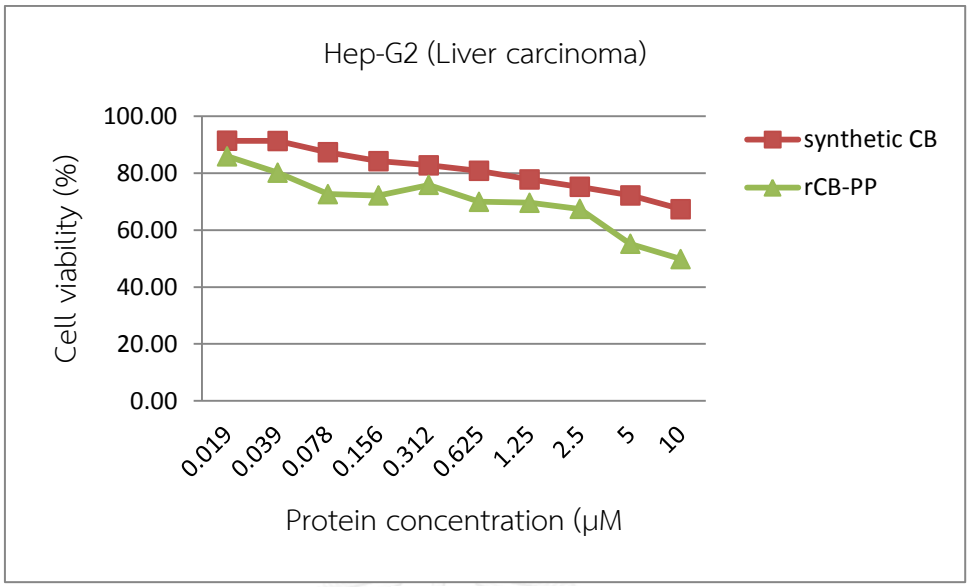


Figure 4. 18 Cell viability of human liver hepatocellular carcinoma cell line (Hep-G2) after 72 hours treated with synthetic CB and recombinant CB-PP.

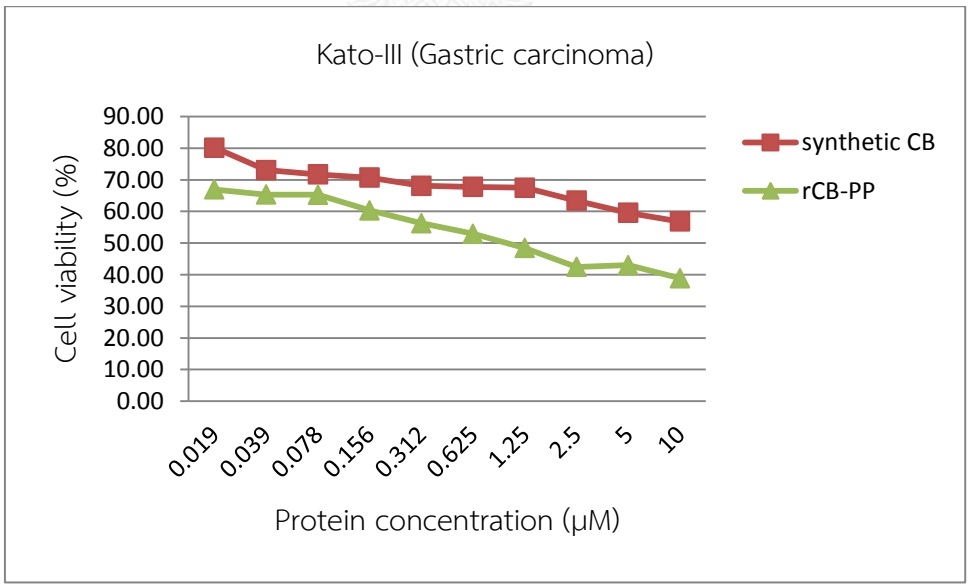


Figure 4. 19 Cell viability of human gastric carcinoma cell line (Kato-III after 72 hours treated with synthetic CB and recombinant CB-PP.

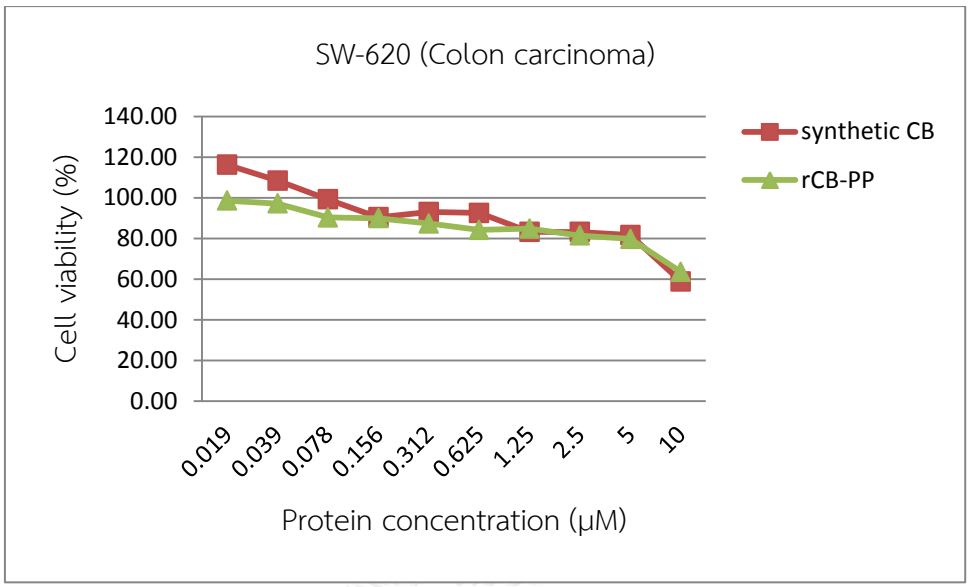


Figure 4. 20 Cell viability of human colon carcinoma cell line (SW-620) after 72 hours treated with synthetic CB and recombinant CB-PP.

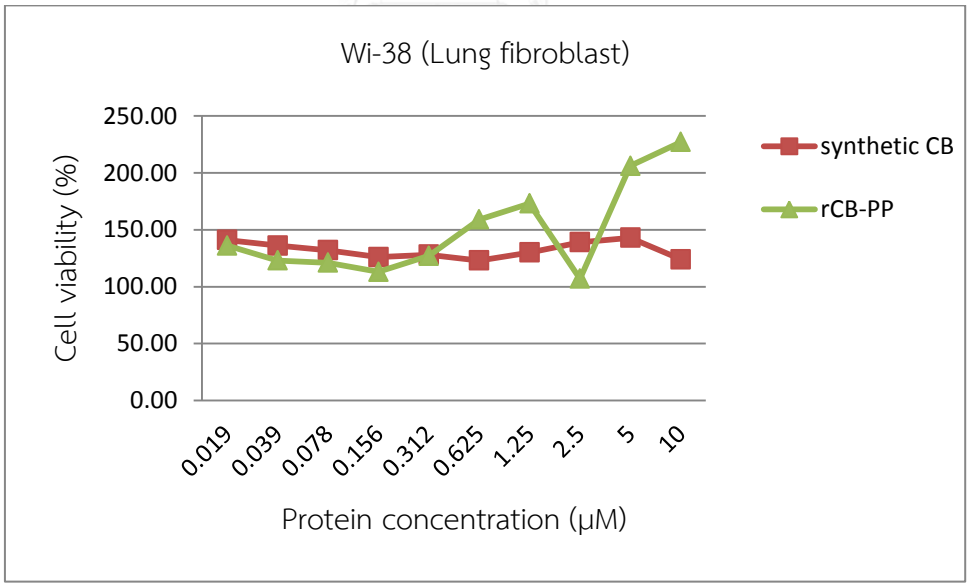


Figure 4. 21 Cell viability of human lung fibroblast cell line (WI-38) after 72 hours treated with synthetic CB and recombinant CB-PP.

The second experiments, cell lines were treated with recombinant protein CB and CB-PP with the protein concentration between 0.078 μM – 20 μM for 72 hours. The results showed that the cell viability of cancer cells decreased when the protein concentrations increased. Five cancer cell lines treated with recombinant protein CB and CB-PP showed IC_{50} values at various protein concentrations. The 50% inhibitory concentration (IC_{50}) was calculated as shown in table 4.1. While the cell number of human lung fibroblast cell line (WI-38) did not decrease even though it was treated with the same protein concentration. The pictures of cells after 72 hours treated with recombinant protein CB and CB-PP compared with cells did not treated with protein were shown in the figure 4.22-4.27. The results of the morphological change of six cell lines treated with rCB and rCB-PP under the microscope were observed with respect to control. According to the picture, it was found that the morphology of treated cancer cells was different from untreated cells. The treated cancer cells became apoptotic. The picture showed that the amount of five cancer cell lines after treated with 20 μM of rCB and rCB-PP were decreased compared with control, however the amount of Wi-38 cells before and after treated with rCB and rCB-PP were not change because the growing of Wi-38 was slower than other cell lines.

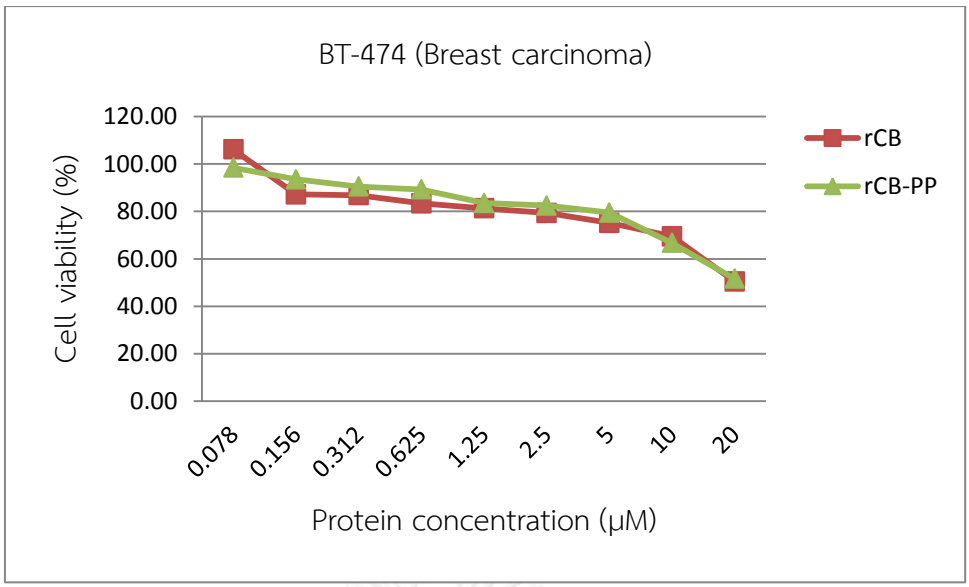


Figure 4. 22 Cell viability of human breast carcinoma cell line (BT-474) after 72 hours treated with recombinant CB and CB-PP.

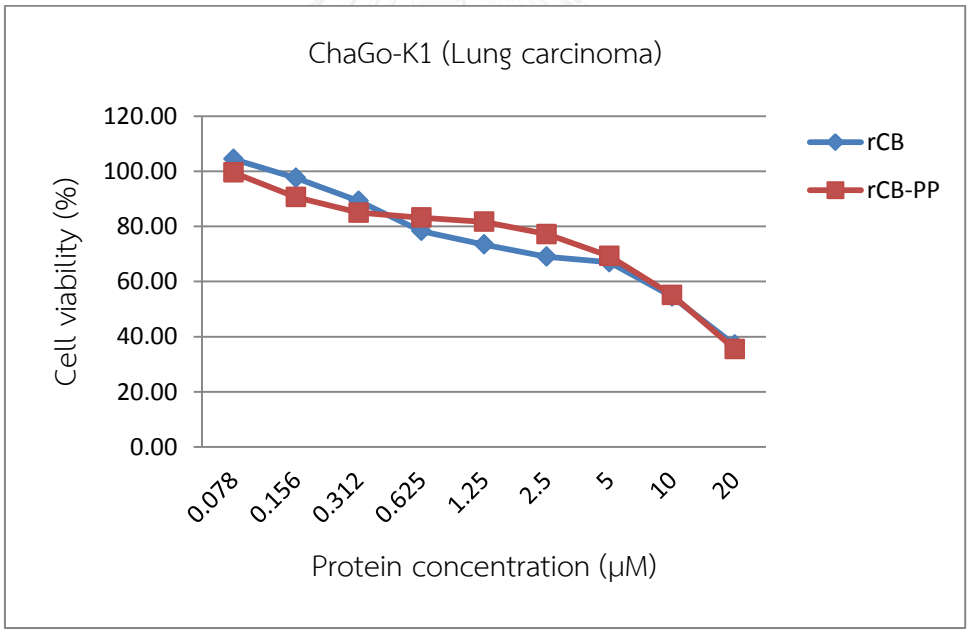


Figure 4. 23 Cell viability of human lung carcinoma cell line (Chago-K1) after 72 hours treated with recombinant CB and CB-PP.

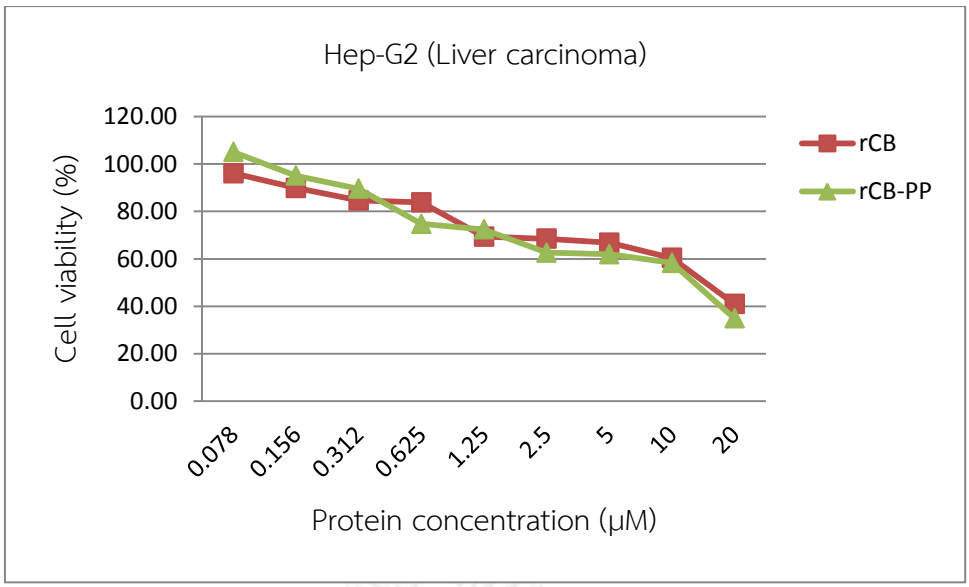


Figure 4. 24 Cell viability of human liver carcinoma cell line (Hep-G2) after 72 hours treated with recombinant CB and CB-PP.

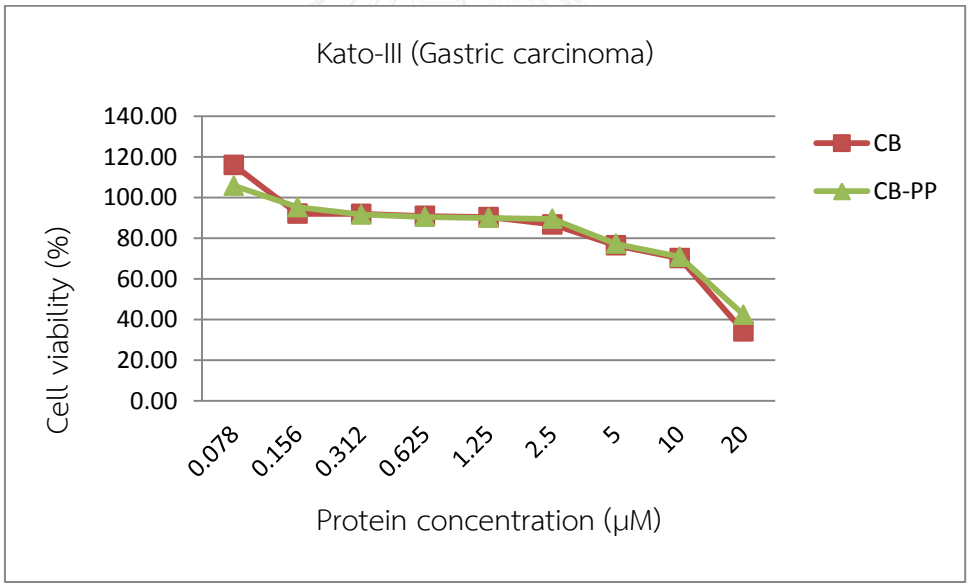


Figure 4. 25 Cell viability of human gastric carcinoma cell line (Kato-III) after 72 hours treated with recombinant CB and CB-PP

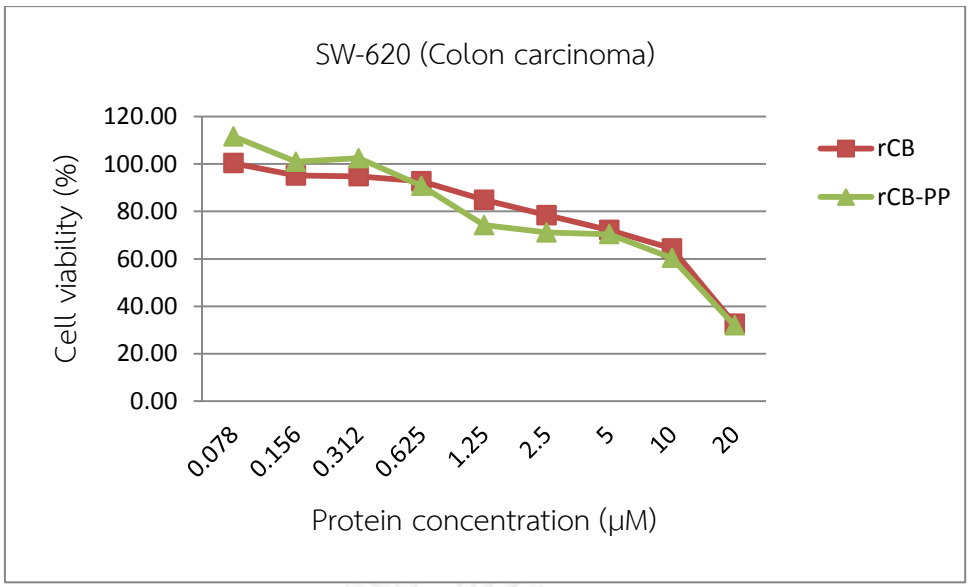


Figure 4. 26 Cell viability of human colon carcinoma cell line (SW-620) after 72 hours treated with recombinant CB and CB-PP.

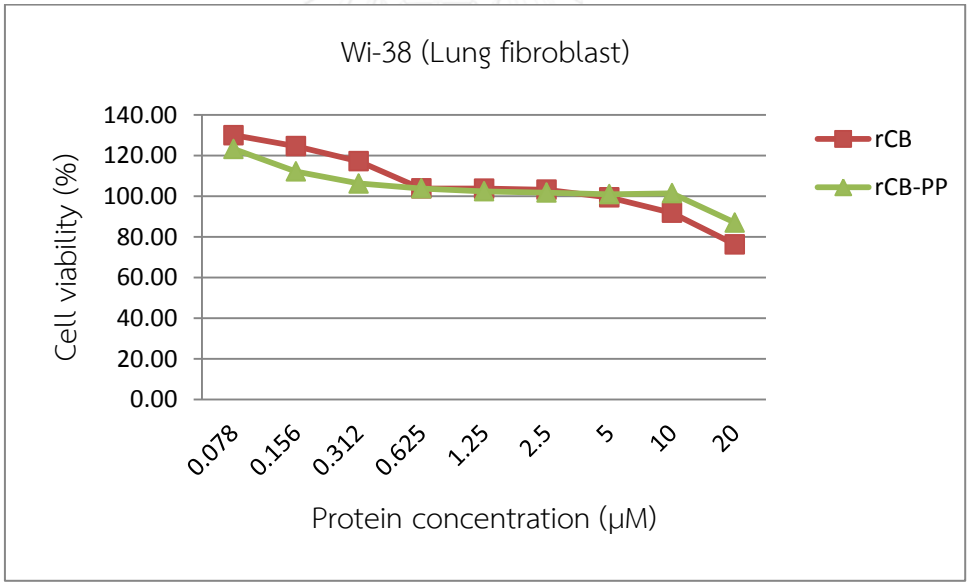


Figure 4. 27 Cell viability of human lung fibroblast cell line (Wi-38) after 72 hours treated with recombinant CB and CB-PP.

Table 4. 1 Cytotoxicity of rCB and rCB-PP against various cell lines

Type of cell lines	50 % inhibition concentration (IC_{50})*	
	rCB (μM)	rCB-PP (μM)
BT-474	20	20
ChaGo-K1	14.80	16.20
Hep-G2	17.15	14.45
Kato-III	16.59	18.97
SW-620	16.20	14.01
Wi-38	>20	>20

*The 50% inhibition concentration (IC_{50}) of the recombinant protein; rCB and rCB-PP against cell lines were determined using the MTT assay.

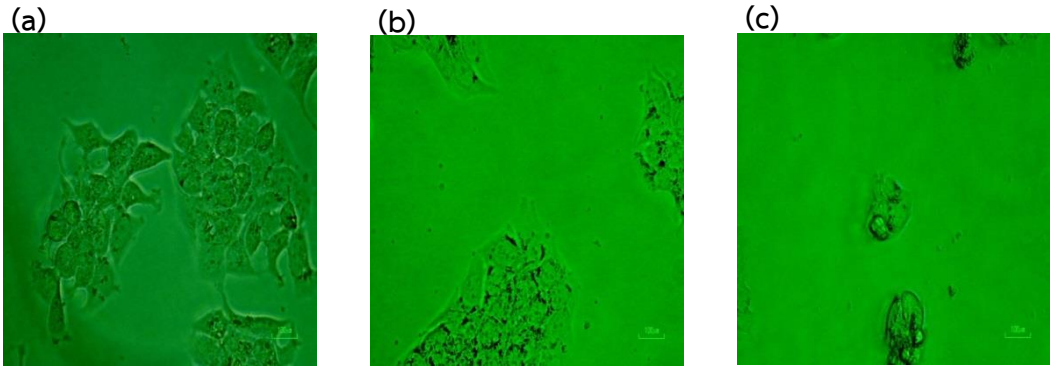


Figure 4. 28 Human breast carcinoma cell (BT-474) after 72 hours; (a) cells without treating with protein, (b) cells treated with 20 μM rCB and (c) cells treated with 20 μM rCB-PP.

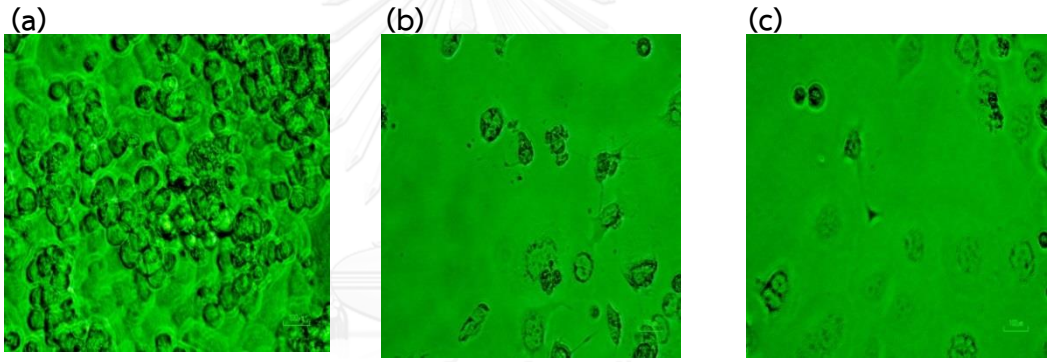


Figure 4. 29 Human lung bronchus carcinoma cell line (ChaGo-K1) after 72 hours; (a) cells without treating with protein, (b) cells treated with 20 μM rCB and (c) cells treated with 20 μM rCB-PP.

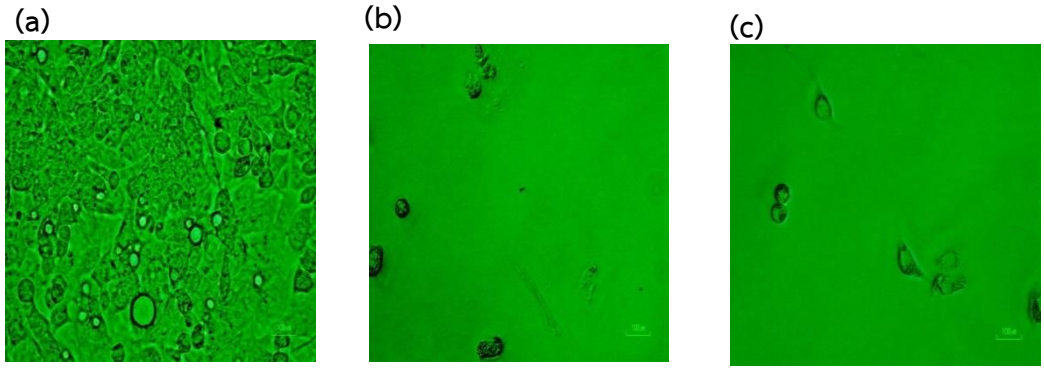


Figure 4. 30 Human liver hepatocellular carcinoma cell line (Hep-G2) after 72 hours; (a) cells without treating with protein, (b) cells treated with 20 μM rCB and (c) cells treated with 20 μM rCB-PP.

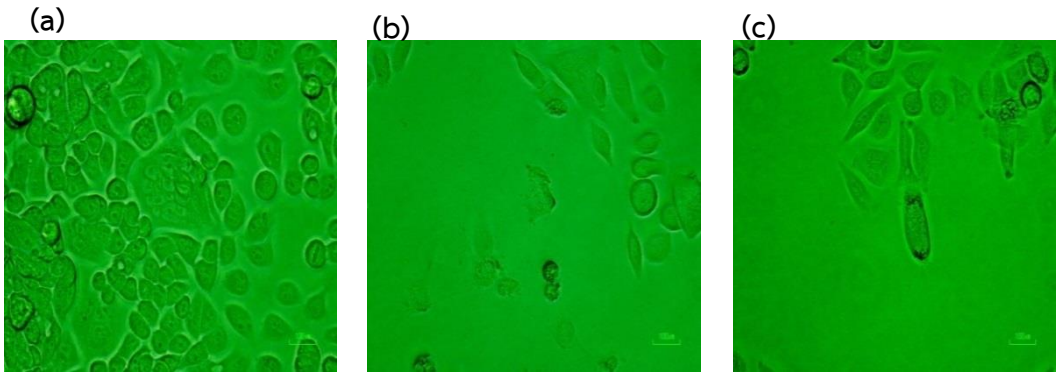


Figure 4. 31 Human gastric carcinoma cell line (Kato-III), after 72 hours; (a) cells without treating with protein, (b) cells treated with 20 μM rCB and (c) cells treated with 20 μM rCB-PP

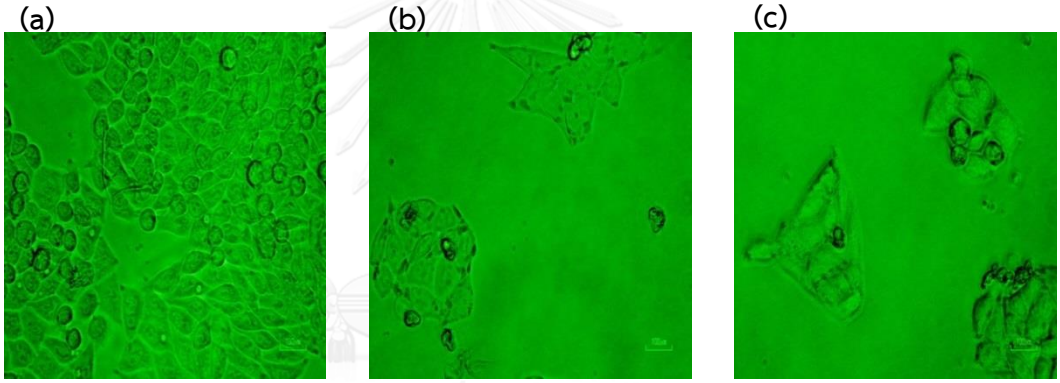


Figure 4. 32 Human colon carcinoma cell line (SW-620), after 72 hours; (a) cells without treating with protein, (b) cells treated with 20 μM rCB and (c) cells treated with 20 μM rCB-PP.

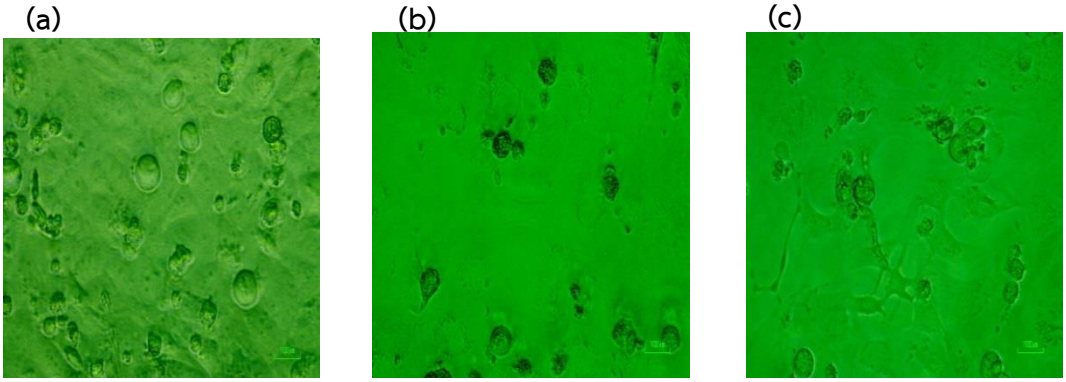


Figure 4. 33 Human lung fibroblast cell line (WI-38), after 72 hours; (a) cells without treating with protein, (b) cells treated with 20 μM rCB and (c) cells treated with 20 μM rCB-PP.

CHAPTER V
DISCUSSION AND CONCLUSION

Cancers have had massive impact on many people worldwide because they cause million deaths, burden of money and many severe incidents. Although current treatments are able to treat various types of cancer, however these solutions also have many harmful side effects which remain the major factors of patient distress and most of them passed away. Although, the chemical therapeutic drugs are designed to specifically target to cancer cells, they can act on both normal cells and other rapidly dividing cells. In contrast, many investigations reported that most anticancer peptides specifically acted on cancer cells rather than noncancerous cells. Therefore, anticancer peptides are one of the promising alternative drugs for cancer treatment. Among the anticancer peptides reported, Cecropin B is the choice of interest since it has been reported to effect only on several cancer cells. Therefore, to obtain a large amount of CB, an effective production method must be developed. One approach is to produce CB as a recombinant protein by yeast.

In this study, we produced recombinant protein Cecropin B (rCB) and Cecropin B-Polyproline (rCB-PP) *in vitro*. We also exhibited the cytotoxic effect of on six cell lines including human breast carcinoma cell lines (BT-474), human lung bronchus carcinoma cell line (ChaGo-K1), human liver hepatocellular carcinoma cell line (Hep-G2), human gastric carcinoma cell line (Kato-III), human colon carcinoma cell line (SW-620) and human lung fibroblast cell line (WI-38). From all previous reports, the cytotoxicity activity against many cancer cell lines were assayed with synthetic purified CB. Therefore, to obtain the large amount of CB, recombinant protein expression is one of the best ways to study. Thus, *Pichia pastoris* strain KM71H was used as the host for recombinant protein production.

The rCB and rCB-PP were successfully produced in *Pichia pastoris,* 0.4 and 0.5 mg/ml of purified rCB and rCB-PP were obtained. After that, the cytotoxicity was tested using MTT assay. In the preliminary study, the comparative cytotoxicity assay of synthetic CB and rCB-PP at different concentrations on six cell lines was

performed to investigate whether the rCB-PP was more effective than synthetic CB. The growth ability of cancer cell lines including BT-474, ChaGo-K1 and SW-620 started to decrease significantly at 10 μM of synthetic CB and rCB with the percentage of cell viability between 60% - 80%. Interestingly, Hep-G2 and Kato-III were killed more than 50% at 1.25 μM concentration of rCB-PP. The normal cells treated with synthetic CB and rCB-PP at the concentration between 0.019 μM –10 μM still had growing ability more than 100%. These results revealed that the cytotoxicity of both peptides was not significantly different because the length of PP in CB might be short.

Later, we also used the protein from the same production technique, which were rCB and rCB-PP obtained from *Pichia pastoris* strain KM71H to treat the same series of cell lines at 72 hours as well as the first experiment, protein concentration between 0.078 μM – 20 μM were used. The result of cell viabilities show that five cancer cells treated with the range of above concentration of rCB and rCB-PP showed IC_{50} values at various protein concentrations on each cells. However, Wi-38 showed more than 80% viability even it was treated with rCB and rCB-PP at 20 μM. The IC_{50} values of tested cells were not significantly different since the amount of PP in CB was not high enough to play the outstanding role.

In case of BT-474, the IC_{50} of rCB and rCB-PP on this cell lines were 20 μM, compared with human breast cancer cell lines (MDA-MB-231) treating with synthetic CB. The anticancer efficiency of synthetic CB was significant at least 60 μM (82). The cytotoxicity effect of ChaGo-K1 treated with rCB and rCB-PP showed the IC_{50} at 14.80 μM and 16.20 μM respectively. The rCB and rCB-PP also killed human liver hepatocellular carcinoma cell lines (Hep-G2) at 20 μM and exhibited outstanding IC_{50} values at 17.15 μM and 14.45 μM, while cytotoxicity of synthetic CB on human hepatocellular carcinoma BEL-7402 cell lines revealed IC_{50} values at 25 μM (8). The cytotoxicity of rCB and rCB-PP against Kato-III showed IC_{50} values at 16.59 μM and 18.97 μM respectively while Chan SC, Hui L and Chen HM studied antitumor activity of synthetic CB against gastric carcinoma cells, they showed IC_{50} values at 13 μM (74). The IC_{50} values for human colon carcinoma cell lines (SW-620) performed with rCB and rCB-PP were 16.20 μM and 14.01 μM respectively, however; in 1994, Moore and

his colleagues studied anticancer activity of synthetic CB against murine ascetic colon adenocarcinoma and exhibited IC_{50} values at 3.2 μM (10). While human lung fibroblast cell lines (WI-38) still alive more than 80% at 20 μM protein concentration. Many previous studies also showed cytotoxicity against leukemia cell lines by including in 1997, Hueih Min Chen and his colleagues found that synthetic CB, CB-1 (possess two N-terminals) and CB-2 (identical to CB-1 except insertion of some amino acids in the sequences), had the potential to harm leukemia cancer cells (HL-60, K-562, Jurkat E6-1 and CCRF-CEM) and showed IC_{50} of CB at 8 μM - 17 μM, CB-1 at 2 μM – 10 μM and CB-2 at 3 μM – 11 μM, respectively. Nonetheless the cytotoxicity needed more than 50 μM concentration of these peptide to toxic to normal fibroblast cells (9). The cytotoxicity activity o CB-1 was also studied on leukemia cell lines and fibroblast cells by S. Srisailam and team in 2000. This experiment introduced erythrocytes to treat with CB-1 and the result showed that CB-1 must be used more than 100 μM to gain the IC_{50} value (101). The antitumor activity against bladder cancer cells was studied by Ulrike Suttmann and colleagues in 2008. They found that at least 50 μM of lyophilized synthetic CB had antitumor activity against bladder cancer cells while high concentration of CB was needed to treat mouse and human fibroblast cells to against proliferative of these normal cells (7).

The percentages of cell cytostasis of ChaGo-K1, Hep-G2, Kato-III and SW-620 treated with rCB and rCB-PP were seem to be satisfied since they show low percentage of alive cells. These outcomes have clearly proved that rCB and rCB-PP had anticancer activity against cancer cells and did not harmful to the tested mammalian normal cell lines. Currently, the mechanism by which CB kills cells are not clearly understood, however the one feasible reported mechanism is that CB demonstrated membrane disruption and attacked cell membranes with electrostatic action using its positive net charge (7, 102). According to cholesterol which is the major components of eukaryotic cell membranes, it may protect membranes from peptide intervention (54) but having a high population of the irregular microvilli on the cell surface could help increasing peptide attraction before lysis in membrane bilayers can be efficiently initiated (74). Furthermore, the mechanism by which CB is

operational is also relying on amphipathicity, hydrophobicity as well as its concentration.

To consider the difference between anticancer efficiency of rCB and rCB-PP. The study did not reveal the significantly difference of these peptides. It could be explained by the reason that polyproline may be too short. Therefore, more copies of polyproline should be inserted to show effectively activity against cancer cells. However, the results revealed that cancer cell lines are much more sensitive to rCB and rCB-PP than the normal cell lines.

The cytotoxicity activity of rCB and rCB-PP compared with other anticancer peptide such as maginin, melittin or LL-37 were also different. The previous studies found that the those anticancer peptides showed cytotoxicity activity against not only cancer cells but also damage normal cells at the same protein concentrations (64). While Cecropin B needed more concentration of protein to toxic to normal cells.

To summarize, the recombinant protein CB and CB-PP were successfully constructed, cloned and expressed in *Pichia pastoris* expression system in soluble form. They showed toxicity activity against BT-474, ChaGo-K1, Hep-G2, Kato-III and SW-620 but not harmful to normal human lung fibroblast cell line (WI-38). The anticancer activity results indicated that to obtain wide range of cytotoxicity activity, so more concentrations of protein were needed. Therefore, to gain high protein concentration, optimization of recombinant protein production and purification will be needed for further experiment. Moreover, more copies of polyproline should be introduced into the recombinant protein for increasing a chance of peptide to inhibit engagement between progesterone receptor and c-Src in breast cancer proliferation pathway.

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APPENDIX A

Culture Media

1. Low salt LB medium

- 1% Tryptone
- 0.5% Yeast extract
- 0.5% Sodium chloride (NaCl)
- 2% Agar (Solid medium)

Adjust the pH value to 7.5 with 1 N NaOH and sterilized by autoclave at 121°C, 15 lb.in^{-2} for 20 minutes. Low salt LB with Zeocin™, cool the solution to ~ 50°C and add Zeocin™ to a final concentration of 25 µg.mL^{-1} and store at +4°C in the dark.

2. Luria-Bertani (LB) medium

- 1% Tryptone
- 0.5% Yeast extract
- 1% Sodium chloride (NaCl)
- 2% Agar (Solid medium)

Adjust the pH value to 7.5 with 1 M NaOH and sterilized by autoclave at 121°C, 15 lb.in^{-2} for 20 minutes. Store at +4°C.

3. Buffered complex Glycerol Medium (BMGY)

(1 liter)

- 1% yeast extract
- 2% peptone
- 100 mM potassium phosphate, pH 6.0
- 1.34% YNB 0.00004% biotin
- 1% glycerol or 0.5% methanol

Dissolve 10 g of yeast extract, 20 g peptone in 700 ml water. Autoclave 20 minutes on liquid cycle, then cool to room temperature, add the following and

mix well: 100 ml 1 M potassium phosphate buffer, pH 6.0 100 ml 10X YNB 2 ml 500X B 100 ml 10X GY For BMMY, add 100 ml 10X M instead of glycerol and store media at +4

4. Buffered complex Methanol Medium (BMMY)

(1 liter)

- 1% yeast extract
- 2% peptone
- 100 mM potassium phosphate, pH 6.0
- 1.34% YNB 0.00004% biotin
- 0.5% methanol

Dissolve 10 g of yeast extract, 20 g peptone in 700 ml water. Autoclave 20 minutes on liquid cycle, then cool to room temperature, add the following and mix well: 100 ml 1 M potassium phosphate buffer, pH 6.0 100 ml 10X YNB 2 ml 500X B 100 ml, 100 ml 10X M instead of glycerol and store media at +4

Stock solution preparation

- 10×YNB; 13.4% YNB w/o amino acid w/ ammonium sulfate, 100 mL
  - YNB without amino acid W ammonium sulfate powder 13.4g

  Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by filtration (membrane, pore size 0.22 μm) and stored at +4°C.
- 500×Biotin; 0.02% Biotin, 50 mL
  - Biotin powder 10 mg

  Dissolved with sterilized double distilled water and adjust the volume to 50 mL using volumetric flask. Sterilized by filtration (membrane, pore size 0.22 μm) and stored at +4°C.
- 100×Histidine; 0.4% Histidine, 100 mL
  - Histidine powder 0.4 g

Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by filtration (membrane, pore size 0.22 μm) and stored at +4°C.

- 1 M potassium phosphate buffer, pH 6.0

Combine 132 mL of 1 M K2HPO4, 868 mL of 1 M KH2PO4 and confirm that the pH = 6.0 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH). 2. Sterilize by autoclaving and store at room temperature.

5. Yeast Peptone Dextrose (YPD) medium

- 1% Yeast extract
- 2% Peptone
- 2% Dextrose (Glucose)
- 2% Agar (Solid medium)

Sterilized by autoclave at 121°C, 15 $lb.in^{-2}$ for 20 minutes. In case of YPD with Zeocin™, add Zeocin™ to a final concentration of 100 $\mu g.mL^{-1}$ and store at +4°C in the dark.

APPENDIX B

Solutions Preparation

1. 1% Agarose for agarose gel electrophoresis

- Agarose (molecular grade) 1 g
- 10×TAE buffer solution 10 mL
- Double distilled water 90 mL

Melt the agarose solution by microwave oven until the gel completely melted. Cool the solution to +50°C and pour the gel to the gel boxes, inserts the combs and wait for the gel has been set.

2. 0.1 M $CaCl_2$ for fresh competent *E. coli* preparation

- $CaCl_2.H_2O$ powder (MW = 147.02 g.mole^{-1}) 14.702 g

  Dissolved in double distilled water and adjust the volume to 100 mL using volumetric flask. The solution was sterilized by autoclave and stored at +4°C.

3. 1 M DTT, Yeast competent cells preparation

- DTT powder (MW = 154.25 g.mole^{-1}) 0.2314 g

  Dissolved in sterilized double distilled water and adjust the volume to 1.5 mL and sterilize by filtration (pore size 0.22 µm). Store at 0°C.

4. 1 M HEPES buffer (pH 8.0), Yeast competent cells preparation

- HEPES free acid powder (MW = 238.30 g.mole^{-1}) 2.383 g
- Dissolved in sterilized double distilled water and adjust the pH value to 8.0 with 5 M NaOH. Adjust the volume to 10 mL and sterilize by filtration (pore size 0.22 µm). Store at +4°C.

5. Lysis by alkali for plasmid extraction

Solution I

- 50 mM glucose (Glucose monohydrate; MW = 198.17 g.mole^{-1})
- 25 mM Tris·Cl (pH 8.0), (Tris base; MW = 121.1 g.mole^{-1})

- 10 mM EDTA (pH 8.0), (EDTA; MW = 292.25 g.mole^{-1})

autoclaved for 15 minutes and stored at +4°C.

Stock solution preparation

- 1 M Glucose, 100 mL
  - Glucose monohydrate powder 19.817 g

Adjust the volume to 100 mL by sterilized double distilled water using volumetric flask. Stored at +4°C.

- 0.5 M Tris·Cl (pH 8.0), 100 mL
  - Tris·base powder 6.055 g

Dissolved with sterilized double distilled water and adjust the pH to 8.0 with 1 M HCl. Adjust the volume to 100 mL by double distilled water using volumetric flask. Stored at +4°C.

- 0.5 EDTA (pH 8.0), 100 mL
  - EDTA powder 14.6125 g

Dissolved with sterilized double distilled water and adjust the pH to 8.0 with 5 M NaOH. Adjust the volume to 100 mL by double distilled water using volumetric flask. Stored at +4°C.

Compositions of Solution I from stock solution as the following

- 1 M Glucose 5 mL
- 0.5 M Tris•Cl (pH 8.0) 5 mL
- 0.5 EDTA (pH 8.0) 2 mL
- Adjust the volume with double distilled water to 100 mL

Solution II (freshly prepared before use)

- 0.2 N NaOH (NaOH; MW = 40 g.mole^{-1})
- 1% SDS (SDS; MW = 288.38 g.mole^{-1})

Dilute with sterilized double distilled water.

Stock solution preparation

- 2 N NaOH, 100 mL
  - NaOH pellet 4 g

Dissolved with sterilized double distilled water and adjust the volume to 100 mL using volumetric flask and stored at +4°C.

- 10% SDS, 100 mL
  - SDS powder 28.84 g

Dissolved with sterilized warm double distilled water and adjust the volume to 100 mL and stored at +4°C.

Compositions of Solution II from stock solution

- 2 N NaOH 1 mL
- 10% SDS 1 mL
- Sterilized double distilled water 8 mL

Solution III

- 5 M Potassium acetate (MW = 98.14 g.mole^{-1}) 60 mL
- Glacial acetic acid 11.5 mL
- Double distilled water 28.5 mL

Stock solution preparation

- 5 M Potassium acetate, 100 mL
  - Potassium acetate powder 49.07 g

Dissolved with double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilized by autoclave and stored at +4°C.

Compositions of Solution III from stock solutions above

- 5 M Potassium acetate (Sterilized) 60 mL
- Glacial acetic acid 11.5 mL
- Sterilized double distilled water 28.5 mL

6. 0.01 M Phosphate Buffer Saline (PBS), pH 7.4

- 200 mM Phosphate buffer, pH 7.4 1 L
- Sodium chloride (NaCl) 175.2 g
- Double distilled water 18 L

Stock solution preparation

- Stock solutions, 200 mM each for 1 L
  - Stock A: $NaH_2PO_4.H_2O$ (MW = 137.99 g.mole^{-1}) 27.6 g
  - Stock B: $Na_2HPO_4.12H_2O$ (MW = 358.135 g.mole^{-1}) 71.63 g
  - Each $NaH_2PO_4.H_2O$ and $Na_2HPO_4.12H_2O$ powder was separately dissolved in double distilled water and adjusts the volume to 1 L using volumetric flask. Stored at +4°C.
- 200 mM Sodium phosphate buffer (pH 7.4), 1 L
  - Stock A (774 mL) and B (226 mL) were mixed together and adjust the pH value to 7.4 by titration with 5 M HCl. Store at room temperature.

7. Tris-EDTA (TE) buffer pH 8.0

- 10 mM Tris·Cl (pH 8.0); Stock solution 0.5 M Tris·Cl (pH 8.0) from Solution I
- 1 mM EDTA (pH 8.0); Stock solution 0.5 M EDTA (pH 8.0) from Solution I

Compositions of TE buffer (pH 8.0) from stock solutions above

- 0.5 M Tris•Cl (pH 8.0) 20 mL
- 0.5 EDTA (pH 8.0) 2 mL

Adjust the volume with double distilled water to 1000 mL

Sterilize by autoclave and store at +4°C.

8. 1 M D-Sorbitol

- D-Sorbital (D-Sorbital; MW = 182.18 g.mole^{-1}) 18.2 g

Dissolved in sterilized double distilled water and adjust the volume to 100 mL using volumetric flask. Sterilize by filtration (0.22 µm, membrane) and store at +4°C in the dark.

9. Binding and elution buffer for fast flow nickel column

Stock solution

- 5 M Imidazole 1.702 g imidazole add to5 ml water

Sterilize by filtration (0.22 µm, membrane) and store at +4°C.

- 5 M NaCl 146.1 g NaCl

  Mix 146.1 g of NaCl with 450 ml of H_2O by stirring, add H_2O until final volume is 500 ml. Sterilize by autoclave, store at room temperature.

- 1 M Sodium phosphate buffer pH 7.4

  Mixing 1 M NaH_2PO_4 (monobasic) and 1 M Na_2HPO_4 (dibasic) stock solutions in the volumes of 22.6 and 77.4 ml respectively. To prepare for stock solution; dissolve 138 g of $NaH_2PO_4 \cdot H_2O$ (mw = 138) in sufficient H_2O to make a final volume of 1 L and dissolve 142 g of Na_2HPO_4 (mw = 142) in sufficient H_2O to make a final volume of 1 L.

APPENDIX C

Cytotoxicity test using MTT assay

The cell lines treated with synthetic CB and recombinant CB-PP

rCB4.9 kDa	20μM=98 μg/ml	10μM=49 μg/ml	5μM=24.5 μg/ml	2.5μM=12.25 μg/ml	1.25μM=6.125 μg/ml	0.625μM=3.063 μg/ml	0.312μM=1.53 μg/ml	0.156μM=0.76 μg/ml	0.078μM=0.38 μg/ml	0.039μM=0.19 μg/ml
rCB-PP 6.5 kDa	20μM=65 μg/ml	10μM=65 μg/ml	5μM=32.5 μg/ml	2.5μM=16.25 μg/ml	1.25μM=8.13 μg/ml	0.625μM=4.06 μg/ml	0.312μM=2.03 μg/ml	0.156μM=1.02 μg/ml	0.078μM=0.5μ g/ml	0.039μM=0.25 μg/ml

BT474

rCB	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	Control	
1	0.6851	1.0211	0.9347	0.9029	0.9967	1.1204	1.0327	0.8529	0.8979	1.3751	1.4953
2	0.7562	0.8873	0.8451	0.8455	1.0684	0.8818	0.9913	1.3544	1.5601	1.3903	1.9471
3	0.5969	0.8842	1.2425	1.4418	1.2044	1.3501	1.4675	1.2981	1.8167	1.0234	1.7951
Average	0.6794	0.9309	1.0074	1.0634	1.0898	1.1174	1.1638	1.1685	1.4249	1.1303	1.3230
SD	0.0798	0.0782	0.2084	0.3290	0.1055	0.2342	0.2638	0.2747	0.4741	0.9721	1.1885
% Viability	50.47	69.47	75.18	79.35	81.33	83.39	86.85	87.20	106.16	1.1854	1.1588
% Cytostasis	49.53	30.53	24.82	20.65	18.67	16.61	13.15	12.80	0.00	Average= 1.34	
										SD= 0.30	

rCB-PP	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM
4	0.7563	0.8141	1.1040	0.8558	1.0128	1.1515	1.4280	1.3788	1.1982
5	0.6718	0.9757	1.0452	1.3274	1.1873	1.0588	1.1598	1.2327	1.6002
6	0.6465	0.8918	1.0461	1.1316	1.1579	1.3775	1.0507	1.1472	1.1585
Average	0.6915	0.8939	1.0651	1.1049	1.1193	1.1959	1.2128	1.2529	1.3190
SD	0.0575	0.0808	0.0337	0.2369	0.0934	0.1639	0.1942	0.1171	0.2444
% Viability	51.6	66.7	79.49	82.45	83.53	89.25	90.5	93.5	98.45
% Cytostasis	48.4	33.3	20.51	17.55	16.47	10.75	9.5	6.5	1.55

Chago-K1

rCB	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	Control	
1	0.4823	0.8112	0.7874	0.7227	0.9715	0.9381	1.3875	1.4283	1.7068	1.2395	1.7455
2	0.5455	0.7429	0.7957	1.1545	1.1046	1.2476	1.2789	1.5429	1.4075	1.6018	1.3012
3	0.4843	0.6714	1.1502	0.9392	0.9211	1.0108	0.9754	1.0114	1.1484	1.5494	1.0740
Average	0.5040	0.7418	0.9111	0.9388	0.9991	1.0655	1.2139	1.3275	1.4209	1.3191	1.2011
SD	0.0359	0.0699	0.2071	0.2159	0.0948	0.1618	0.2136	0.2797	0.2794	1.2302	1.4204
% Viability	37.06	54.54	66.99	69.03	73.46	78.35	89.26	97.61	104.48	1.2851	1.3518
% Cytostasis	62.94	45.46	33.01	30.97	26.54	21.65	10.74	2.39	0	Average= 1.3599	
										SD= 0.1897	

rCB-PP	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM
4	0.5230	0.7015	0.8322	1.0843	0.8788	1.2923	1.0881	1.0076	1.1695
5	0.4610	0.7494	1.1807	0.9828	1.1421	0.9415	1.4247	1.1357	1.1995
6	0.4651	0.7993	0.8134	1.0822	1.3117	1.1600	0.9564	1.5557	1.6940
Average	0.4830	0.7501	0.9421	1.0498	1.1109	1.1313	1.1564	1.2330	1.3543
SD	0.0347	0.0489	0.2068	0.0580	0.2181	0.1772	0.2415	0.2867	0.2945
% Viability	35.51	55.15	69.27	77.19	81.68	83.18	85.03	90.66	99.58
% Cytostasis	64.49	44.85	30.73	22.81	18.32	16.82	14.97	9.34	0.42

Hep-G2

rCB	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	Control	
1	0.6810	0.9021	0.8520	1.1743	1.1247	1.0539	1.1740	1.0132	1.5108	1.4322	1.8105
2	0.5547	0.9093	1.0445	1.1583	1.2550	1.0631	1.4630	1.7125	1.4556	1.3773	1.6255
3	0.6786	1.0094	1.0634	1.1903	0.8101	1.7985	1.3150	1.3612	1.5235	1.3372	1.2155
Average	0.6381	0.9403	0.9866	0.9866	1.1743	1.3052	1.3173	1.0633	1.4966	1.5757	1.6872
SD	0.0722	0.0600	0.1170	0.0160	0.2287	0.4273	0.1445	0.3497	0.0361	1.9195	1.3861
% Viability	40.97	60.38	66.80	68.50	69.33	83.81	84.58	89.86	96.10	1.6204	1.6994
% Cytostasis	59.03	39.62	33.20	31.50	30.67	16.19	15.42	10.14	3.90	Average= 1.5573	
										SD= 0.2098	

rCB-PP	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM
4	0.5111	0.8615	0.8546	1.0576	0.9135	1.5114	1.4801	1.4373	1.6837
5	0.5132	0.8971	0.9627	1.0279	1.0182	0.8262	1.0649	1.3154	1.5416
6	0.6040	0.9634	1.0776	0.8406	1.4497	1.1563	1.6386	1.6885	1.6840
Average	0.5428	0.9073	0.9650	0.9754	1.1271	1.1646	1.3945	1.4804	1.6364
SD	0.0530	0.0517	0.1115	0.1177	0.2842	0.3427	0.2963	0.1902	0.0821
% Viability	34.85	58.27	61.96	62.63	72.37	74.78	89.54	95.06	105.07
% Cytostasis	65.15	41.73	38.04	37.37	27.63	25.22	10.46	4.94	0

KATO-III

rCB	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	Control	
1	0.6369	1.1535	0.9118	1.1323	1.1510	1.5599	1.6458	1.4057	2.1709	1.0875	2.1363
2	0.5321	0.9499	1.3594	1.9360	1.6115	1.5054	1.5762	1.7779	1.6360	1.3838	2.0315
3	0.4566	1.5306	1.0645	1.0577	1.5345	1.2595	1.1501	1.1914	1.7979	1.3854	1.5971
Average	0.5419	1.2113	1.1119	1.3753	1.4323	1.4416	1.4574	1.4583	1.8683	1.3854	1.5971
SD	0.0905	0.2946	0.2275	0.4870	0.2467	0.1600	0.2684	0.2968	0.2743	1.3668	1.8025
% Viability	34.19	70.15	76.42	86.77	90.37	90.95	91.95	92.01	115.98	1.5062	1.7930
% Cytostasis	65.81	23.58	29.85	13.23	9.63	9.05	8.05	7.99	0	Average= 1.5849	
										SD= 0.3038	

rCB-PP	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM
4	0.4566	1.5306	1.5345	1.0645	1.1914	1.1501	1.0577	1.2595	1.7979
5	0.6752	1.0664	1.2955	1.3588	1.5301	1.2929	1.4865	1.7203	1.6792
6	0.6060	1.1635	1.5308	1.1692	1.4079	1.5937	1.3868	1.4726	1.6780
Average	0.6406	1.1150	1.4132	1.2640	1.4690	1.4433	1.4367	1.5965	1.6786
SD	0.1117	0.2448	0.1369	0.1492	0.1715	0.2264	0.2244	0.2306	0.0689
% Viability	42.33	70.82	77.24	89.51	89.99	90.47	91.63	95.26	105.83
% Cytostasis	57.67	29.18	22.76	10.49	10.01	9.53	8.37	4.74	0

SW620

rCB	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	Control	
1	0.4500	1.0689	1.1049	0.9719	1.5660	1.2252	1.4159	0.9176	1.6264	1.3643	1.2123
2	0.4382	0.8662	1.1376	1.1331	1.1601	1.3468	1.3598	1.1536	1.3341	1.2922	1.7463
3	0.5437	0.8935	0.9274	1.3427	1.0063	1.5045	1.3920	2.1100	1.4507	1.2139	1.5792
Average	0.4773	0.9429	1.0566	1.1492	1.2441	1.3588	1.3892	1.3937	1.4704	1.6734	1.8723
SD	0.0578	0.1100	0.1131	0.1859	0.2892	0.1400	0.0282	0.6314	0.1471	1.4459	1.0135
% Viability	32.57	64.34	72.10	78.42	84.90	92.73	94.80	95.11	100.34	1.6700	1.4999
% Cytostasis	67.43	35.66	27.90	21.58	15.10	7.27	5.20	4.89	0	Average= 1.4653	
										SD= 0.2551	

rCB-PP	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM
4	0.4674	0.7472	1.4312	1.3504	1.4087	1.4884	1.7632	1.6721	1.4801
5	0.4772	0.9695	0.7338	0.9993	0.9852	1.1957	1.3873	1.5788	1.2838
6	0.4569	0.9369	0.9281	0.7765	0.8687	1.3093	1.3510	1.1851	2.1420
Average	0.4672	0.8845	1.0310	1.0421	1.0875	1.3311	1.5005	1.4787	1.6353
SD	0.0102	0.1200	0.3599	0.2893	0.2842	0.1476	0.2282	0.2585	0.4497
% Viability	31.88	60.36	70.36	71.11	74.21	90.84	102.4	100.91	111.6
% Cytostasis	68.12	39.64	29.64	28.89	25.79	9.16	0	0	0

Wi-38

rCB	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	Control	
1	0.6922	0.7972	1.0087	0.7813	1.0581	0.6943	0.9750	0.8610	0.6995	0.9310	1.1107
2	0.6586	0.6626	0.6819	0.8130	0.6480	0.7013	1.0055	1.0273	1.3151	0.6475	0.8847
3	0.6770	0.7764	0.7294	0.9177	0.8192	1.1317	0.8715	1.1449	1.1505	0.7849	0.6846
Average	0.6759	0.7454	0.8067	0.8373	0.8418	0.8424	0.9507	1.0111	1.0550	0.7261	0.9191
SD	0.0168	0.0725	0.1766	0.0714	0.2060	0.2505	0.0702	0.1426	0.3187	0.6983	0.7340
% Viability	76.36	91.88	99.44	103.21	103.77	103.84	117.19	124.64	130.05	0.7534	0.8616
% Cytostasis	23.64	8.12	0.56	0	0	0	0	0	0	Average= 0.8112	
										SD= 0.1336	

rCB-PP	20μM	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM
4	0.6860	0.8212	0.7768	0.8739	0.8293	0.8227	0.7806	0.8141	0.9720
5	0.7332	0.8244	0.9048	0.9115	0.8444	0.7693	0.9696	1.1724	0.9899
6	0.6989	0.8234	0.7756	0.6920	0.8187	0.9356	0.8360	0.7429	1.0384
Average	0.7060	0.8230	0.8191	0.8258	0.8308	0.8425	0.8621	0.9098	1.0001
SD	0.0244	0.0016	0.0742	0.1174	0.0129	0.0849	0.0972	0.2302	0.0344
% Viability	87.03	101.45	100.97	101.79	102.41	103.85	106.27	112.15	123.28
% Cytostasis	12.97	0	0	0	0	0	0	0	0

The cell lines treated with recombinant CB and CB-PP

synthetic CB 3.8 kDa	10μM=38 μg/ml	5μM=19 μg/ml	2.5μM=9.5 μg/ml	1.25μM=4.75 μg/ml	0.625μM=2.38 μg/ml	0.312μM=1.2 μg/ml	0.156μM=0.6 μg/ml	0.078μM=0.3 μg/ml	0.039μM=0.1 5μg/ml	0.019μM=0.07 μg/ml
CB-PP(His) 6.5 kDa	10μM=65 μg/ml	5μM=32. 5μg/ml	2.5μM=16.2 5μg/ml	1.25μM=8.13 μg/ml	0.625μM=4.06 μg/ml	0.312μM=2.0 3μg/ml	0.156μM=1.02 μg/ml	0.078μM=0.5 μg/ml	0.039μM=0.2 5μg/ml	0.019μM=0.13 μg/ml

BT474
(Breast)

ynthesized CI	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM	Control	
A	0.7111	0.8549	0.9310	0.9471	0.8936	0.7910	1.0630	1.0620	1.0915	1.1933	1.0548	1.3493
B	0.8100	0.8691	0.7161	0.8641	0.9357	0.8588	0.8781	0.9730	0.9275	0.9761	0.8944	0.9162
C	0.5985	0.6085	0.6579	0.7078	0.5124	0.7824	0.7563	0.7259	0.7686	1.1551	0.9549	0.7150
D	0.5848	0.6047	0.6805	0.5128	0.7395	0.7604	0.6902	0.7361	0.8364	0.9370	0.7388	0.9444
Average	0.6761	0.7343	0.7464	0.7580	0.7703	0.9118	0.8469	0.8743	0.9060	1.0654	0.8779	1.0255
SD	0.1057	0.1476	0.1254	0.1912	0.1915	0.0424	0.1637	0.1694	0.1398	0.1276	0.9539	0.9057
% Viability	72.72	78.98	80.28	81.53	82.85	85.73	91.09	94.04	97.45	114.60	0.8938	1.1377
% Cytostasis	27.28	21.02	19.72	18.47	17.15	14.27	8.91	5.96	2.55		1.2527	0.2609
											Average=0.92974375	
											SD=0.2429	

rCB-PP	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM
E	0.7764	0.9404	0.8229	0.8665	0.6385	0.7802	0.7686	1.0563	0.8312	1.0698
F	0.7400	0.5922	0.7807	0.8077	1.0192	1.3134	0.8240	1.1253	1.5531	1.3203
G	0.8043	0.6482	0.8434	0.6169	0.7463	0.4555	0.8867	0.8412	1.0792	0.8628
H	0.7936	0.9476	0.6676	0.9018	0.8245	0.6702	0.7421	0.8686	0.9938	0.8066
Average	0.7786	0.7821	0.7787	0.7982	0.8071	0.8048	0.8054	0.9729	1.1143	1.0149
SD	0.0282	0.1884	0.0785	0.1270	0.1606	0.3649	0.0641	0.1395	0.3101	0.2330
% Viability	83.74	84.12	83.75	85.85	86.81	86.56	86.63	104.64	119.85	109.16
% Cytostasis	16.26	15.88	16.25	14.15	13.19	13.44	13.37	0.00	0.00	0.00

Chago-K1

(Lung)

synthetic CB	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM	Control	
1	0.7892	1.0199	1.0487	1.1859	1.1616	1.2366	0.7769	1.0208	0.6548	1.1134	0.9152	1.0289
2	0.9033	0.8400	0.6194	0.8505	0.7339	0.8031	1.1026	1.3217	1.0296	1.5887	0.9675	0.9897
3	0.6910	0.8193	0.9312	0.9652	1.3109	1.0086	1.1719	0.9806	1.1701	0.9335	1.2095	0.8888
4	0.7088	0.8440	1.2112	0.9123	0.7806	0.9503	1.0827	0.9175	1.4020	0.9034	1.2387	1.0455
Average	0.7731	0.8808	0.9526	0.9785	0.9968	0.9997	1.0335	1.0602	1.0641	1.1348	1.2568	0.8576
SD	0.0968	0.0934	0.2501	0.1460	0.2838	0.1801	0.1753	0.1795	0.3131	0.3165	1.3878	0.9742
% Viability	67.41	76.81	83.07	85.33	86.92	87.18	90.12	92.45	92.79	98.96	1.2053	1.6605
% Cytostasis	32.59	23.19	16.93	14.67	13.08	12.82	9.88	7.55	7.21	1.04	1.1614	1.5605
											Average=1.1467	
											SD=0.2360	

rCB-PP	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM
5	0.6789	0.9214	0.9271	1.0530	0.8723	1.0167	1.1020	0.9629	1.2446	1.0755
6	0.9056	0.8137	0.9964	0.8327	1.1825	1.0876	1.3452	1.0096	1.0664	1.0323
7	0.9497	0.8853	0.7709	0.9481	1.2111	0.9277	0.5442	1.4211	0.9407	1.4783
8	0.9002	0.8978	1.1811	1.1306	1.0049	1.0316	1.3453	0.9487	1.1159	1.2393
SD	0.1218	0.0464	0.1700	0.1294	0.1590	0.0663	0.3778	0.2252	0.1257	0.2020
Average	0.8586	0.8795	0.9688	0.9911	1.0677	1.0519	1.0842	1.0855	1.0919	1.2063
% Viability	74.87	76.69	84.48	86.43	93.11	91.73	94.54	94.66	95.22	105.19
% Cytostasis	25.13	23.31	15.52	13.57	6.89	8.27	5.46	5.34	4.78	0

Hep-G2 (Liver)

synthetic CB	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM	Control	
1	1.0256	1.3486	1.2885	1.3319	1.2016	0.8351	1.4430	1.1406	1.5720	1.3460	1.6453	1.2595
2	0.8602	1.2807	1.3306	1.1981	1.7146	1.5565	1.4036	1.1435	1.4901	1.4003	1.4379	1.6861
3	1.0347	1.0858	1.0860	1.3091	1.1105	1.4515	1.4705	1.5600	1.2642	1.4503	1.4182	2.2226
4	1.4645	1.1991	1.1943	1.2290	1.1232	1.5440	1.1659	1.8428	1.5472	1.7527	1.6053	1.6969
Average	1.0962	1.1751	1.2235	1.2670	1.3149	1.3467	1.3707	1.4217	1.4863	1.4873	1.8257	1.6576
SD	0.2583	0.1131	0.1087	0.0637	0.2876	0.3443	0.1393	0.3430	0.1404	0.1820	2.4503	1.1583
% Viability	67.35	72.19	75.17	77.84	80.78	82.74	84.21	87.34	91.31	91.37	1.3701	1.3972
% Cytostasis	32.65	27.81	24.83	22.16	19.22	17.26	15.79	12.66	8.69	8.63	1.6342	1.5767
											Average=1.6276	
											SD=0.3304	

rCB-PP	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM
5	0.9892	1.0631	1.1239	0.7608	0.9632	1.1601	1.0332	1.3218	1.4729	1.5040
6	0.6664	1.0345	1.1227	1.3576	0.9568	1.0523	1.0950	0.8749	1.1416	1.0217
7	0.7634	0.7405	0.9855	1.1202	1.3785	1.5345	0.7145	1.4807	1.4372	1.4680
8	0.8236	0.7512	1.1567	1.2945	1.2570	1.1934	1.8855	1.0539	1.1676	1.5997
Average	0.8106	0.8973	1.0972	1.1332	1.1388	1.2350	1.1744	1.1828	1.3048	1.3983
SD	0.1355	0.1754	0.0761	0.2678	0.2124	0.2085	0.4977	0.2705	0.1744	0.2572
% Viability	49.8	55.13	67.41	69.62	69.96	75.87	72.15	72.67	80.16	85.91
% Cytostasis	50.13	44.87	32.59	30.38	30.04	24.13	27.85	27.33	19.84	14.09

KATO-III

(Stamach)

synthetic CB	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM	Control	
1	1.4234	1.5477	1.8036	1.6795	1.5874	1.7974	1.7859	1.8020	1.3040	1.7483	2.2319	2.6915
2	1.3742	1.2000	1.4119	1.6288	1.4495	1.7114	1.1967	1.4831	1.5475	1.9778	2.5035	2.9371
3	1.1330	1.0968	1.1004	1.3946	1.3818	1.2580	1.9714	1.4481	1.7462	1.5740	1.1459	2.8878
4	1.1567	1.4861	1.3582	1.3366	1.6448	1.3269	1.3713	1.6831	1.9407	1.8718	2.7292	2.2595
Average	1.2718	1.3327	1.4185	1.5099	1.5158	1.5234	1.5813	1.6041	1.6346	1.7930	2.6765	2.8719
SD	0.1483	0.2183	0.2905	0.1695	0.1213	0.2705	0.3587	0.1677	0.2727	0.1735	2.6330	1.4737
% Viability	56.85	59.57	63.41	67.49	67.76	68.10	70.68	71.70	73.07	80.15	2.2712	1.8017
% Cytostasis	43.15	40.43	36.59	32.51	32.24	31.90	29.32	28.22	26.93	19.85	1.0894	1.5896
											Average=2.237	
											SD=0.6264	

rCB-PP	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM
5	0.6805	1.1162	1.1324	1.0807	1.2299	1.4670	1.1944	1.3431	1.8086	2.5472
6	0.9634	0.8645	0.8749	1.1288	1.2052	0.9979	1.1278	1.4776	1.6335	1.0364
7	0.8443	0.8020	0.7439	1.1310	1.2853	0.7912	1.4972	1.7750	1.3119	1.2773
8	0.9910	1.0641	1.0483	0.9916	1.0146	1.7789	1.5778	1.2465	1.0906	1.1294
Average	0.8698	0.9617	0.9499	1.0830	1.1838	1.2588	1.3493	1.4606	1.4612	1.4976
SD	0.1413	0.1520	0.1742	0.0652	0.1176	0.4474	0.2215	0.2301	0.3215	0.7067
% Viability	38.88	42.99	42.46	48.41	52.91	56.27	60.31	65.29	65.31	66.94
% Cytostasis	61.12	57.01	57.54	51.59	47.09	43.73	39.69	34.71	34.69	33.06

SW620

(Colon)

synthetic CB	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM	Control	
1	0.8495	1.5108	1.3298	1.1672	1.1715	0.9374	1.2049	1.2079	1.1277	1.2972	1.3824	1.7592
2	0.9456	0.9544	1.4200	1.3726	1.4543	1.8455	1.1746	1.5392	2.1706	1.8302	1.2897	1.4329
3	0.9724	0.9270	1.2427	1.2800	1.3274	1.6872	1.8768	1.7466	1.8943	2.5134	1.4734	1.4413
4	1.0701	1.9313	1.4296	1.6035	2.0755	1.5928	1.6357	1.9740	1.8748	1.9337	2.4775	1.3141
Average	0.9594	1.3309	1.3555	1.3558	1.5072	1.5157	1.4730	1.6169	1.7669	1.8936	2.5456	1.4913
SD	0.0907	0.4823	0.0876	0.1853	0.3961	0.3994	0.3418	0.3254	0.4470	0.4985	1.8318	1.0086
% Viability	58.90	81.72	83.23	83.24	92.54	93.06	90.44	99.28	108.49	116.27	1.6962	1.4459
% Cytostasis	41.06	18.28	16.77	16.76	7.46	6.94	9.56	0.72	0.00	0.00	1.7340	1.7343
											Average=1.6286	
											SD=0.4045	

rCB-PP	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM
5	1.1415	1.4471	1.2595	1.2111	1.0872	1.7355	1.2538	1.3369	1.6521	1.4491
6	0.8454	1.8272	1.1959	1.2591	1.3681	1.9609	1.6548	1.2257	1.6186	1.3991
7	0.9414	1.1413	1.1446	1.3007	1.8782	1.0421	1.4898	1.7248	1.9684	1.7055
8	1.2153	0.7827	1.7078	1.7574	1.1544	0.9560	1.4630	1.6014	1.0939	1.8702
Average	1.0359	1.2996	1.3270	1.3821	1.3720	1.4236	1.4654	1.4722	1.5833	1.6060
SD	0.1718	0.4444	0.2582	0.2529	0.3581	0.5001	0.1646	0.2306	0.3623	0.2215
% Viability	63.6	79.79	81.48	84.86	84.24	87.41	89.97	90.39	97.21	98.61
% Cytostasis	36.32	20.21	18.52	15.14	15.76	12.59	10.03	9.09	7.29	1.39

Wi-38 (Fibroblast
from lung tissue)

synthetic CB	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM	Control	
1	0.2390	0.3320	0.3241	0.3220	0.2975	0.3101	0.3085	0.3188	0.3334	0.3253	0.2111	0.1914
2	0.2893	0.3299	0.3220	0.2815	0.3026	0.2426	0.3049	0.2937	0.3033	0.3152	0.2135	0.2113
3	0.3076	0.2959	0.3032	0.2670	0.2568	0.2985	0.2793	0.2826	0.2932	0.3000	0.2560	0.2106
4	0.2523	0.3014	0.2723	0.2698	0.2200	0.2760	0.2130	0.2621	0.2619	0.3008	0.2272	0.2061
Average	0.2721	0.3148	0.3054	0.2851	0.2692	0.2818	0.2764	0.2893	0.2980	0.3103	0.3050	0.1991
SD	0.0319	0.0188	0.0240	0.0254	0.0387	0.0297	0.0442	0.0236	0.0295	0.0122	0.3106	0.2240
% Viability	124.00	143.00	139.00	130.00	123.00	128.00	126.00	132.00	136.00	141.00	0.2414	0.2088
% Cytostasis	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0997	0.1864
											Average=0.2188	
											SD=0.4809	

rCB-PP	10μM	5μM	2.5μM	1.25μM	0.625μM	0.312μM	0.156μM	0.078μM	0.039μM	0.019μM
5	0.6338	0.4094	0.3512	0.3634	0.3812	0.2927	0.2532	0.2844	0.2744	0.3096
6	0.4088	0.4462	0.4143	0.4967	0.4018	0.2349	0.2672	0.2576	0.2633	0.2994
7	0.4348	0.4464	0.3745	0.3285	0.2832	0.2988	0.2679	0.2705	0.2707	0.2810
8	0.4968	0.4513	0.3737	0.3798	0.3483	0.2791	0.2489	0.2650	0.2694	0.2981
SD	0.1009	0.0386	0.0289	0.0796	0.0537	0.0297	0.0286	0.0160	0.0046	0.0122
% Viability	227.0	206.0	107.0	173.0	159.0	127.0	113.0	121.0	123.0	136.0
% Cytostasis	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

VITA

Miss Sirin Saranyutanon was born on November 29th, 1988 in Phatthalung. She graduated bachelor degree of Science from the department of Biochemistry, Faculty of Science, Chulalongkorn University since 2011. In 2015, she presented a poster entitle “Cloning and Expression of Recombinant Cecropin B – Polyproline in Pichia pastoris” at the National Genetic Conference 2015 (NGC 2015) at Centara Grand Convention Center, Khonkaen, Thailand in July 14-16, 2015.