INVESTIGATION OF CYTOTOXIC ACTIVITY OF FULL-LENGTH AND TRUNCATED COLICIN N ON HUMAN CANCER CELLS

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การสืบค้นฤทธิ์ความเป็นพิษต่อเซลล์ของโคลิซินเอ็นทั้งสายและปลายตัดต่อเซลล์มะเร็งมนุษย์

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Field of Study	Pharmaceutical Sciences and Technology
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แบคทีเรียที่เกิดความเครียดจากสภาพแวดล้อมหรือการแข่งขันกันเพื่อปล่อยสารพิษออกมานั้นถูกเรียกว่า แบคเทอริโอซิน โดยแบคเทอริโอซินถูกจำแนกประเภทตามความเป็นพิษต่อเซลล์ เช่น สารพิษที่ก่อให้เกิดรูพรุนหรือเอนโดนิวคลีเอส เป็นต้น โคลิซินเป็นแบคเทอริโอซินที่ผลิตจาก *Escherichia coli (E.coli)* และโคลิซินหลายประเภทมีคุณสมบัติเป็นสารพิษที่ก่อให้เกิดรูพรุน ซึ่งมีรายงานมากมายกล่าวถึงความเป็นพิษทั้งต่อแบคทีเรียและเซลล์มะเร็ง โคลิซินเอ็นก็เป็นโคลิซินที่มีคุณสมบัติเป็นสารพิษที่ก่อให้เกิดรูพรุนเช่นกัน และโคลิซินเอ็นยังไม่มีการรายงานเกี่ยวกับฤทธิ์การต้านการเจริญเติบโตต่อเซลล์มะเร็งอย่างแพร่หลายในการศึกษานี้พวกเราทำการทดสอบฤทธิ์ความเป็นพิษของโคลิซินเอ็นและโดเมนของโคลิซินเอ็นที่มีผลต่อเซลล์มะเร็ง โดยเริ่มจากการแสดงออกและการทำบริสุทธิ์ของโคลิซินเอ็นทั้งสายและปลายตัดผ่านการผลิตจาก *E. coli* และโครมาโตกราฟีแบบแอฟฟินิตี จากนั้นจึงทดสอบคุณสมบัติทางเคมีกายภาพของโปรตีนที่ผ่านการทำบริสุทธิ์ด้วยเทคนิค SDS-PAGE, Western blot, Circular Dichroism และ Mass spectrometry จากนั้นทำการทดสอบความเป็นพิษของโคลิซินทั้งสายต่อเซลล์มะเร็งด้วยเทคนิค MTT โดยใช้เซลล์มะเร็ง ได้แก่ HCT-116, HT-29, MCF-7, MDA-MB-231 และ A549 นอกจากนี้ยังมีการทดสอบความเป็นพิษของโคลิซินเอ็นแบบปลายตัดที่ประกอบไปด้วยหนึ่งหรือสองโดเมนกับเซลล์มะเร็ง HCT-116 ด้วย จากผลการทดลองโคลิซินเอ็นทั้งสายและปลายตัดสามารถผลิตได้สำเร็จและแสดงคุณสมบัติทางกายภาพตามที่คาดไว้ได้ เช่น น้ำหนักโมเลกุลและโครงสร้างแบบทุติยภูมิ สำหรับความเป็นพิษต่อเซลล์มะเร็ง พบว่า เซลล์ HCT-116 (เซลล์มะเร็งลำไส้ใหญ่) มีความไวต่อโคลิซินเอ็นทั้งสายมากที่สุด ในทำนองเดียวกันเซลล์ HT-29, MDA-MB-231 และ A549 ก็มีความไวต่อโคลิซินเอ็นทั้งสายเช่นกัน แต่ในทางตรงกันข้ามกับเซลล์มะเร็งเหล่านี้พบว่าเซลล์ MCF-7 ได้รับการส่งเสริมการเจริญเติบโตเมื่อทดสอบด้วยโคลิซินเอ็นทั้งสาย สำหรับความเป็นพิษของโคลิซินเอ็นทั้งสายน่าจะขึ้นอยู่กับชนิดของเซลล์มะเร็ง และสำหรับโคลิซินเอ็นปลายตัดนั้นไม่ก่อให้เกิดความเป็นพิษต่อเซลล์มะเร็ง ดังนั้นความเป็นพิษของแต่ละโดเมนในโคลิซินเอ็นจึงไม่เพียงพอสำหรับนำมาใช้ในฤทธิ์การต้านมะเร็ง นอกจากนี้ยังมีการทดลองสำหรับการกลายพันธุ์โคลิซินเอ็นด้วยการเพิ่มจำนวนประจุบวกบนพื้นผิวของโคลิซินเอ็น และผลการศึกษาเบื้องต้นแสดงให้เห็นการกลายพันธุ์นี้มีฤทธิ์ต้านการเจริญเติบโตของเซลล์มะเร็งมากกว่าโคลิซินเอ็นทั้งสาย ซึ่งอาจเกิดจากประจุที่แตกต่างกันหรือการเปลี่ยนแปลงตามรูปแบบของการกลายพันธุ์ จากผลการศึกษาทั้งหมดนี้สามารถนำไปใช้เพื่อเป็นข้อมูลพื้นฐานเพิ่มเติมเกี่ยวกับโคลิซินเอ็นสำหรับฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งและเพื่ออาจพิจารณาเป็นทางเลือกที่ดีในการนำมาใช้เป็นยาต้านมะเร็งในอนาคต

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Bacteria, exposed to stress from the environment or competition, release toxins called bacteriocins. Bacteriocins are classified by their mode of cytotoxicity such as pore former, endonuclease, etc. Colicins are the member of bacteriocins produced by *Escherichia coli (E.coli*). Among several types of colicins cytotoxicity, Pore-forming colicins has been reported in both bacteria and various type of cancer cells. Colicin N is also a pore-forming colicin and the anticancer activity of colicin N has not been extensively reported. Here we examined the cytotoxic effects of colicin N and its domain on cancer cells. The expression and purification of recombinant full-length and truncated colicin N were performed in *E. coli* and an affinity chromatography. The physicochemical properties of purified proteins were then assessed by SDS-PAGE, western blot, Circular Dichroism and mass spectrometry. Then, HCT-116, HT-29, MCF-7, MDA-MB-231 and A549 cells were treated with full-length Colicin N for anticancer test by MTT assay. Furthermore, a series of truncated colicin N with a deletion of one or two domains were tested on HCT-116 cells. We show that, full-length and truncated colicin N were produced successfully and showed the expected physical properties such as molecular weight and secondary structures. Regarding the cytotoxicity against cancer cells, we found that HCT-116 cells (colon cancer cells) was the most sensitive to full length colicin N. Likewise, HT-29, MDA-MB-231 and A549 cells were also sensitive to full-length colicin N. In contrast to these cancer cells, the cell viability of MCF-7 was promoted in the presence of full-length colicin N. The effect of full-length colicin N on cancer cells seemed to be dependent on types of cancer cells. The truncated colicin N did not cause cytotoxicity to cancer cells hence the toxic domain of colicin N is not sufficient for anticancer activity. Furthermore, colicin N mutant with increased number of positive charges on its surface was constructed. The preliminary results demonstrated that this mutant was more cytotoxic than wild type colicin N. This could be due to the difference in charges or conformational changes of colicin N mutant. The results from this study can improve the basic knowledge about colicin N related cytotoxic activity on cancer cells and suggestions that colicin N may be considered for its promising application of therapeutic and natural antitumor drugs.

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LIST OF ABBREVIATIONS

α	=	Alpha
β	=	Beta
°C	=	Degree Celsius
%	=	Percentage
µM	=	Micromolar
µl	=	Microliter
ANOVA	=	Analysis of variance
BCA	=	Bicinchoninic Acid
ColN	=	Colicin N
ColN-WT	=	Colicin N wild type
ColN-T	=	Colicin N T domain
ColN-R	=	Colicin N R domain
ColN-P	=	Colicin N P domain
ColN-TR	=	Colicin N TR domain
ColN-RP	=	Colicin N RP domain
$deg{\cdot}cm^2{\cdot}dmol^{-1}$	=	Unit of molar ellipticity [θ]
DMEM	=	Dulbecco's Modified Eagle Medium
DMEM/Ham's F-12	=	Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture.

DMSO	=	Dimethyl sulfoxide
FBS	=	Fetal bovine serum
h	=	Hour
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	=	The half maximal inhibitory concentration
LSD	=	Least Significant Difference
Min	=	Minute
mg/ml	=	Milligram per milliliter
ml	=	Milliliter
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
nm	=	Nanometer

CHAPTER I
INTRODUCTION

Nowadays, cancer is among main causes of death worldwide. International Agency for Research on Cancers has reported in 2018 that the number of new cancer cases has reached 18.1 million people and the number of deaths from cancer has increased to 9.6 million people (1). In the next 20 years, Ferly *et al.*, 2015 estimated that the cancer patients will increase more than 70% from 2018. Common types of cancers diagnosed in patients are lung, liver, colorectal, gastric and breast cancers (2). Cancers were caused by the abnormal cells in parts of the body and diffused to nearby areas or different parts of the body via blood or lymphatic systems. Among the available cancer treatments such as radiation, surgery, and chemotherapy (3), the most often used therapeutic approach is chemotherapy. Although chemotherapy is widely used for cancer treatment, most chemotherapeutic agents are not specific to cancer cells. The unspecific treatment leads to harmful side effects for patients (4). These side effects originated from the chemotherapeutic agents attacking normal cells. As a result, chemotherapy may have an effect on other organ functions such as nausea, vomiting, stomatitis, anorexia, low immunity, diarrhea and hair loss leading to decrease in the quality of life of patients. Therefore, the development of new anticancer drugs that are non-toxic to normal cells is interesting (5). The severity of those side effects will be dependent on the types of medication and the psychological readiness of patients. Novel cancer therapies have been developed to overcome the limitations of chemotherapies.

At present, the development of better cancer therapy by improving specificity to cancer cells and reducing side effects is needed. It will be more potential than other chemotherapeutic treatments in the market. Although, there are many approaches developing for cancer therapy (6). In recent year, bacteriocins are increasingly becoming one of alternative therapeutic agents for malignant cells.

Bacteriocins can be exploited as anticancer due to not attack the normal cells (5) and reducing serious disadvantage and side effects hence proteins in this family could be a good candidate for cancer therapeutic agent (7). Most of bacteriocins are cationic antimicrobial peptides (AMPs). Most AMPs show the positive charge and can interact with lipid membranes and stop the cell growth (5). Bacteriocins are classified by the cytotoxic mode of action such as pore forming toxins. Pore-forming toxins were used in various anticancer activities such as Microcins M-E492 by *Escherichia coli (E. coli)*, had a toxic effect on colorectal carcinoma cells (8). Nisin A and Nisin Z from *Lactococcus lactis* subspecies *lactis* were efficient for treating tumors (9). Some Nisin showed the cytotoxic effect on SW48, HT29, CaCo-2 cells (colon cancer cells) (10). Colicins, pore-forming toxins from *E. coli*, also showed the similar effect on cancer cells. There are several types of pore-forming colicins and some types have shown to inhibit cancer cells such as colicin A affects tumor cells such as HS913T, MRC5 (lung cancer cells and normal cells), SKUT-1 (uterus cancer cells), BT474, ZR75 and SKBR3 (breast cancer cells) (11) and colicin E1 affects cancerous cells such as MCF7 (breast cancer cells) and HS913T cells (lung cancer cells) (7). Recently, colicins were reported to be associated with colorectal neoplasia (12). A previous study, were reported about colicin N effectively with lung cancer cells (13).

Colicin N is a pore-forming colicins produced by *E.coli* in order to kill susceptible gram-negative bacteria (9). Colicin N has three domains, the C-terminal domain (P domain), the central domain (R domain), the N-terminal domain (T domain), and is efficient to kill bacteria when interacting on the bacterial cell surface(14). The R domain binds to the outer membrane protein and T domain translocate across the membrane and helps P-domain generating pores to killing the cells. Colicin N is a good model for understanding the pore-forming colicins because colicin N is the smallest pore-forming of colicins that easy fused to the cell. Currently, previous research used colicin N test with lung cancer cells (13) and have a cytotoxic effect. Moreover, colicin N are pore-forming colicins and have structure

like colicin A and E1(15) and colicin N will also have efficiency to inhibit the growth of cancer cells. However, the anticancer activity of colicin N has not been extensively reported and the colicin N domains involving in inhibiting eukaryotic cells is unknown (16). The treatment of colicin N with the other cancer cell types are also unknown.

The antibacterial activity of colicin N by using full-length colicin N is effectively cytotoxic into the cell death (14) because all constructs help to generated pores in bacteria. Therefore, the idea being that if using some truncated colicin N in anticancer activity, there will be any difference in its effectiveness. Here we are interested in testing full-length and truncated colicin N with HCT-116, HT-29, MCF-7, MDA-MB-231 and A549 cells. This study will allow us to understand the cytotoxicity of full-length colicin N to specific types of cancer cells. Furthermore, the study of truncated colicin N will give a better understanding of which domains are required for anticancer activity. The expression and purification of recombinant colicin N will be carried out and the purified proteins will be tested in five human cancer cell lines. This study may be an alternative strategy to inhibit the growth of cancer cells. The benefit of this study may represent a promising application of using colicin N in cancer therapeutic and biopharmaceutical drugs in the future.

Research question

What kind of cancer cells is susceptible to colicin N and which domains of colicin N are responsible for its anticancer activity?

Objectives

1. To produce and purify recombinant full-length and truncated colicin N in *Escherichia coli*.

2. To investigate the cytotoxicity of full-length and truncated colicin N against human cancer cells.

3. To improve colicin N for anticancer activities.

Hypothesis

Recombinant full-length and truncated colicin N have different cytotoxic effects on human cancer cells and colicin N can inhibit the growth of some cancer cells.

Expected benefits

This study can be the basic knowledge of full-length and truncated colicin N which possess cytotoxicity against human cancer cells.

CHAPTER II
LITERATURE REVIEWS

1. Bacterial toxin for cancer treatment

1.1 Bacteriocins

Bacteria exposed to stress from the environment or competition will release toxins called bacteriocins (17). Bacteriocins are ribosomally-synthesized cationic peptides secreted by almost all groups of bacteria. Bacteriocins can kill or inhibit the growth of other kinds of bacteria, inhibit closely species of bacteria, narrow spectrum and produced by a wide array of bacteria (9). In this study, Bacteriocins are classified by the cytotoxicity activity into three types including pore-forming toxin, nuclease types and peptidoglycan synthesis (18).

1.2 Bacteriocins and anticancer activities

The targets of cancer treatment use of antibodies specific to the outer membrane protein of cancer cells and combination with toxic compounds such as small molecule drugs or protein toxins(9). Immunotoxins using plant toxins or bacteria that attack eukaryotic cells. The idea is toxins are specific to cancer cells. The natural cell binding with toxins and prevent the normal cells. After that, the toxins were fused to target cells and causes of cell death. Immunotoxins from bacteria such as bacteriocins.

Bacteriocins have previously been reported anticancer activity. These bacteriocins are microcins, pediocin, pyocins, nisin, etc (19). This paragraph will show examples of some types of bacteriocins that have been reported on anticancer. First, Microcins are small size (<10 kDa) mostly produced by *E. coli*. Microcins are secreted by endobacteria and have potent antibacterial activity. Microcins acted by forming pore into the cell membrane (7). Microcins E492 produced by *Klebsiella pneumoniae* RYC492 and has reported the toxic effect in HeLa cells, Jurkat, RJ2.25 and colorectal

carcinoma cells. Microcins E492 was not toxic to normal bone marrow cells, splenocytes, KG-1, human tonsil cells and non-tumor macrophage derived (8). Second, Bovicins produced by *Streptococcus bovis*. They exhibited antibacterial activity against closely related *Streptococcus bovis.* It creates a pore in the cell membrane and fused to the target cell. Bovicins HC5 also has reported toxicity in cancer cells such as MCF7 and HepG2 cells (7). Third, Pediocins are small in size (>5 kDa) and produced by pediococci. They inhibited the growth of various cancer. Pediocin PA-1 produced by *P. acidilactici* PAC1.0 reported the anticancer activity against A-549 and DLD-1. Pediocin CP2 produced by *P. acidilactici* had a cytotoxic effect on HeLa, MCF7, Sp2/0-Ag14, and HepG2. Fourth, Pyocins are mostly produced by *Pseudomonas Aeruginosa* and were used as pore-forming causing the depolarization of cell membranes in the target bacteria. Pyocins have cytotoxic effects in tumor cell lines such as -HepG2, Im9 and HeLa cells. Last, Nisin produced by *Lactococcus lactis* subspecies *lactis* (9). Nisin A and Nisin Z have efficient treatment with tumors such as Nisin A with MCF-7, HepG2, HNSCC, HT29, CaCo-2 and SW480 and Nisin Z with HNSCC and HUVEC. Some Nisins were toxic to SW48, HT29, and Caco2 cells (10). All the examples of bacteriocins with anticancer activity were summarized in Table 1.

Table 1 Lists of Bacteriocins displaying the anticancer activity against various cancer cells. (7) and (19)

Bacteriocin	Producer organism	Size (kDa)	Antibacterial activity	Cancer cells
Microcin E492	*K. pneumoniae*	7.9	Gram-	Hela, Jurkat, RJ2.25
Bovicin HC5	*S. bovis* HC5	2.4	Gram+	MCF7, HepG2
Pediocin PA-1	*P. acidilactici* PAC1.0	4.6	Gram+	A-549, DLD-1
Pediocin CP2	*P. acidilactici*	4.1	Gram+	HeLa, MCF7, Sp2/0-Ag14, HepG2
Pyocin S2	*P. aeruginosa* 42A	74	Gram+	HepG2, Im9HeLa, AS-II, mKS-ATU-7
Nisin	*L. lactis*	3.5	Gram+/Gram-	MCF7, HepG2
Nisin A	*L. lactis*	3.49	Gram+/Gram-	MCF-7,HepG2, HNSCC, HT29, CaCo-2, SW480.
Nisin Z	*L. lactis* SIK-83	3.47	Gram+/Gram-	HNSCC, HUVEC

1.3 Pore-forming toxins

Pore-forming toxins are a group in bacteriocin that is expressed by bacteria and release toxins out of cells (20). Pore-forming has to attach to the membrane and combined to assembly into the membrane proteins that attack the membrane (20). Toxin conformation changed into an active membrane form with a hydrophilic interior and a hydrophobic exterior. Membrane disruption can efficiently alter cellular function, leading to cell death and inhibit the growth of cells. Several of the research was performed to develop immunotoxins based on pore-forming toxins (pore-forming immunotoxins) which are specifically toxic to tumor cells. Immunotoxins are used to inhibit the growth of cancer cells that produce by chemically and genetically, combination with toxins and antibodies against cell lines (21). Consequently, pore-forming immunotoxins will use for cancer therapy because their

ability can help to be an alternative cancer cell drug (22). Pore-forming toxins groups have a previous study of the cytotoxic activity on cancer cells such as Microcins, Bovicins, Colicins (7).

2. Colicins

2.1 Colicins

The first bacteriocins obtained from *Escherichia coli (E. coli)* are colicins in 1925 by A. Gratia (23). They are toxic to nearly some strains of *E. coli* (7). The size of colicins is 40-60 kDa. They are important in the colonization of the intestines by new species. Their killing mechanisms are diverse such as pore- formation, DNase, RNase activity, inhibition of peptidoglycan biosynthesis. Colicins are divided into three domains. The C-terminal domain (T-domain) has a lethal activity. The central domain (R-domain) is required for receptor binding recognition. The N-terminal domain (C-domain) for translocation to move across the outer membrane (Figure 1). It has been assumed that the C-terminal cytotoxic domain is required for bacterial activity (16). Each domain of colicins involved in a different step of the bactericidal killing process.

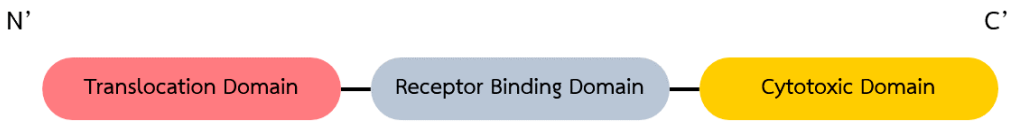


Figure 1 The three domains structure of all colicins

The arrangement of colicins, controls the synthesis and release of the mature protein. The uptake of colicin begins with the capture and attachment of the binding partners in the outer membrane. Colicins translocate through cell periplasm and pass into the inner membrane causing toxicity (24).

Colicins are classified into three groups according to the cytotoxicity activity. First, pore-forming including colicin Ia, A, E1, N, 5-10 and B. Second, nuclease types

including colicin D, Cloacin DF13 and colicin E2-9. Final, peptidoglycan synthesis such as colicin M (23). All colicins were summarized in Table 2 abovementioned group of colicins.

Table 2 Lists of the Colicins group base on the cytotoxicity

Colicin types	Cytotoxicity
Colicin Ia	Pore-forming
Colicin A	Pore-forming
Colicin E1	Pore-forming
Colicin N	Pore-forming
Colicin 5-10	Pore-forming
Colicin B	Pore-forming
Colicin D	Nuclease
Cloacin DF13	Nuclease
Colicin E2-9	Nuclease
Colicin M	Peptidoglycan synthesis

2.2 Colicin N

Colicin N is classified into a pore-forming group of bacteriocins. The size of colicin N is approximately 42 kDa. Colicin N has 387 residues. First, the T domain position is 1-90 residues. The R domain position is 91-183 residues. Last, the P domain position is 184-378 residues. Bacterial sensitivity to colicins is the result of various steps including recognition and association with a specific receptor, translocation to the membrane and the lethal action. Colicin N is a similarity to colicin A in that a wide range of C-terminal implies the ends of colicin N, identical location of the pore-forming domain (25).

2.3 Colicin N structure and activities

2.3.1 Domains and Antibacterial activity of Colicin N

Colicin N has the smallest size of the pore-forming colicins (23) and has three domains also. Colicin N has one domain that will have cytotoxicity in bacterial activity (Arunmanee et al., 2020). The research was reported that the 90-residue unstructured N-terminal domain of colicin N is cytotoxic to cells (25), which is the T-domain located at the N-terminus of colicin N. It is composed of a large unstructured region and a glycine-rich linkage with the R-domain. R-domain (red ribbon structure in Figure 2) containing a 6-stranded β-sheet structure and connect with the P-domain (blue ribbon crystal structure in Figure 2) by hydrogen bonding interactions (Figure 3). Colicin N belongs to the pore-forming toxin and they are called C-terminal is P domain that containing a 10 α-helical structure. Antibacterial activity of colicin N started at R domain interact with OmpF for recognition and transport across the outer membrane and the T domain interacts with TolA in the periplasm through to porin pores. After that, the P-domain inserts into the inner membrane and their forming voltage-gated channels which result in cell death (26) and (27) as shown in Figure 4.

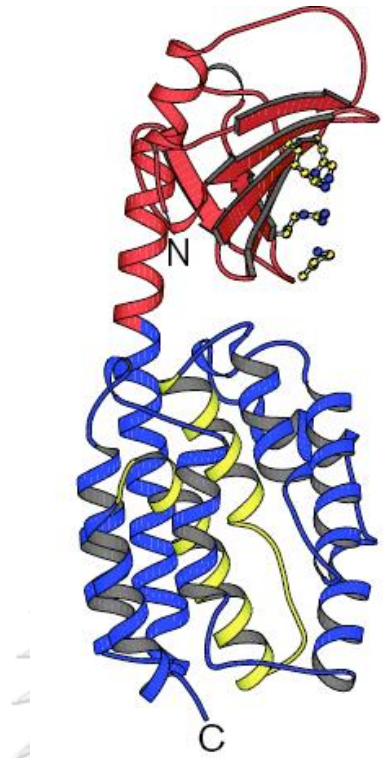


Figure 2 The crystal structure of colicin N (27)

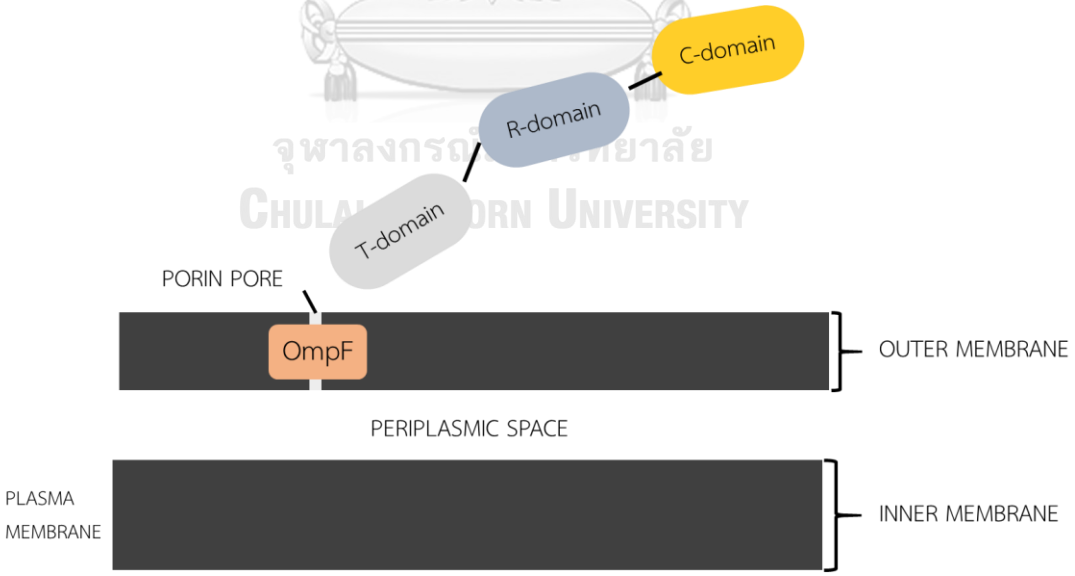


Figure 3 Domains of Colicin N are binding

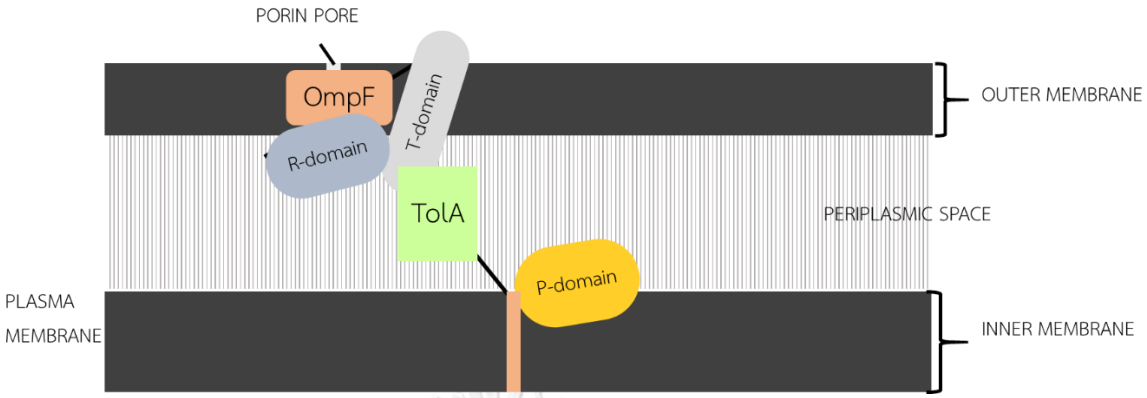


Figure 4 Colicin N mechanism was translocated into the inner membrane

1.3.2 Colicins against cancer cells

In recent years, bacteriocins are increasingly becoming one of the alternative therapeutic agents for malignant cells. Bacteriocins can be exploited as anticancer due to not attack the normal cells (5) and will have cause reducing serious disadvantages and side effects hence proteins in their family could be a good candidate for cancer therapeutics (7). Most bacteriocins are antimicrobial peptides (AMPs). Then, most AMPs show a positive charge and can interact with lipid membranes and cause their stop growth (5). The research was reported about colicins and cancer cells, have an idea about a charge on the surface. The negative charges on the surface of cancer cells originated from two factors are 1) lactate anions are secreted to remove the positive ions and leaving the negative changes on the cell surfaces. It's a glucose metabolic activity that affects the charge level of cancer cells. And 2) negatively charged phospholipids are increased on cancer cells (28). Previous research their idea is cancer cell surface has a more negative charge than the normal cell surface (28), a positive charge on protein can attack the cancer

cell surface. Protein will have the potential to affect inhibited the growth of tumor cells.

Research on colicins and tumor cell toxicity started 30 years ago. Colicin E3 has been first reported to affect cell toxicity of human HeLa cells (29). Colicins have anticancer activities against various human tumor cell lines such as breast cancer, colon cancer, bone cancer and uteri cell line HeLa (7). It has been reported that the proliferation of murine leukemia cells P388 was inhibited by colicin E3, and colicins A, E1, E3 and U ever study on 11 human tumor cell lines (9) (Table 3.). Recently, colicins were reported to be associated with colorectal neoplasia. Since colorectal neoplasia is reported in large intestinal mucosa patients can produce colicins. They were found cancer patients produce colicins and microcins in their intestinal organs (12). In this year, recombinant colicin N causes cytotoxicity and oxidative stress in human lung cancer cells (13), reported colicin N concentrations at 10-15 μM against lung cancer cells as well.

Table 3 Lists of colicins that exhibited anticancer activity against various cancer cells.

Type of colicins	Cancer cell lines	Year
Colicin E3	HeLa cell	1997 (29)
Colicin E3	P388	1979 (30)
Colicin B and M	Colorectal cancer	1991 (31)
Colicin A, E1, E3 and U	Human tumor cells (HS913T, MRC5, SKUT-1, BT474, ZR75, SKBR3)	2003 (11)
Colicin N	H460, H292 and H23	2020 (13)

CHAPTER III

MATERIALS AND METHODS

1. Chemical reagent

1.1 Protein Expression, Protein Purification, SDS-PAGE and Western blot experiments

Chemical reagent	Supplier
Luria-Bertani broth and agar	Hardy Diagnostics, Santa Maria, CA, USA
L-(+)- arabinose	TCI, Tokyo, Japan
Imidazole for buffer solutions, DNase I and Ampicillin Sodium Salt	PanReac Applichem (Darmstadt, DE, USA).
Presto™ Mini plasmid Kit	Geneaid (CA, USA)
PierceTM protease inhibitor tablets and bicinchoninic acid (BCA) protein assay kit	Thermo Scientific (Waltham, MA, USA).
Monobasic sodium phosphate from Vivantis Technologies	Selangor Darul Ehsan, MY, USA
Dialysis tubing cellulose membrane	Sigma-aldrich (St. Louis, MO, USA)
Sodium Chloride	Ajax Finechem (Seven Hills, NSW 2147, Australia).
Skim milk Powder	Criterion (Santa Maria, CA, USA)
10X Phosphate Buffered Saline (PBS)	Vivantis Technologies (Selangor Darul Ehsan, MY, USA)
Tween®-20	Lobal Chemie, Mumbai, India

1.2 Cell culture experiment

Chemical reagent	Supplier
DMEM/Ham's F-12, DMEM high glucose and 1X HEPES	HyClone (Logan, Utah)
10% FBS from Merck	Merck KGaA, Darmstadt, Germany
1%penicillin/streptomycin and Roswell Park Memorial Institute (RPMI) medium	Gibco (Gaithersburg, MA, USA)
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Abcam (Cambridge,CB2 0AX, UK)
Cisplatin and Doxorubicin	Sigma Chemical, Inc. (St. Louis, MO, USA).
Methanol and Formaldehyde	Merck (Merck KGaA, Darmstadt, Germany
Crystal violet	Himedia (Himedia, India)

1.3 Gibson cloning technique

NEBuilder® HiFi DNA Assembly Master Mix and PCR Using Q5® High-Fidelity DNA Polymerase from Biolabs (Biolabs, UK).

2. Expression of his-tagged colicin N

Colicin N constructs with a c-terminal 6xhistidine tag including ColN-WT, ColN-T, ColN-R, ColN-P, ColN-TR and ColN-RP (14) in pET3a plasmid (kindly provided by Prof. Dr.Jeremy Lakey, Newcastle University, UK). Colicin N constructs were expressed by BL21-AI™ One Shot® chemically competent *E. coli* (Invitrogen). The transformed cells from overnight cultures were grown at 37 °C in LB broth with ampicillin selection and shaking at 200 rpm. The expression of recombinant proteins were induced by 0.2% (w/v) L-(+)-Arabinose at exponential growth phase (OD_{600} = 0.6

- 0.8). Cells were continuously incubated for a further 3 h. The cell pellets were harvested by centrifugation at 8000×g for 10 min at 4°C.

3. Purification of his-tagged colicin N

The cell pellets were harvested and resuspended in 50 mM sodium phosphate buffer, pH 8.0 and 300 mM sodium chloride (NaCl) with 10 mM Imidazole that containing DNaseI and RNase. Then, the resulting mixture was lysed by sonication for 15 min and was centrifuged at 17,000xg for 20 min at 4°C. Next, the crude supernatant was purified by nickel-sepharose HisTrap™ FF affinity column (GE Healthcare Technologist, West Milwaukee, WI, USA) in Fast Protein Liquid Chromatography (FPLC) ÄTKA start (GE Healthcare Technologist, West Milwaukee, WI, USA). Then, the column was washed with a Ni column wash buffer and eluted by 50 mM Sodium Phosphate buffer, pH 8.0 and 300 mM NaCl containing 250 mM Imidazole. the proteins in elution fractions were pooled. The buffer of purified proteins was then exchanged by dialysis technique in Phosphate Buffer Saline (PBS; 50 mM Sodium Phosphate buffer, pH 7.4 and 300 mM NaCl) at 4°C overnight. The concentration of proteins were measured by BCA assay and Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Waltham, MA, USA) at 280 nm.

4. Physicochemical properties and activities of protein

4.1 SDS-PAGE

Purified proteins were mixed with sample loading buffer and heat at 100°C for 15 min. The mixture protein was loaded to SDS-PAGE gel and run at 100 V for 90 min using the Mini-PROTEAN Tetra Cell device (Biorad, Italy). The SDS-PAGE gel was stained with coomassie blue (Isopropanol, acetic acid and 0.025% Coomassie Brilliant Blue R250) overnight and followed by destaining (Isopanol and acetic acid) to remove excess the matrix gel.

4.2 Western blot

Western blot is a technique to immunologically identify a protein by antibodies. The proteins from SDS-PAGE gel were transferred onto the nitrocellulose membrane (Merck KGaA, Darmstadt, Germany) using a semi-dry transfer device Trans-Blot® SD System and PowerPac™ HC Power Supply System (Biorad, Singapore) under 100 V for 30 min. The nitrocellulose membrane was blocked by 5% (w/v) skim milk in PBS buffer for 2 h and incubated with 6x-Histag Monoclonal Antibody (HIS.H8) (Thermo Fisher Scientific, IL 61105, USA) diluted 1:4000 in 5% (w/v) skim milk in PBS buffer overnight at 4°C. The nitrocellulose membrane was washed three times for 15 min incubated with PBS with 0.1 % Tween-20 (PBST buffer) and was incubated with goat anti-Mouse IgG secondary antibody conjugated with Alkaline Phosphatase (AP) (Seracare KPL, MD, USA) diluted 1:4000 in 5% (w/v) skim milk in PBS buffer for 1 h and 30 min at room temperature. The nitrocellulose membrane was washed three times for 15 min and incubated with PBST buffer. The AP Conjugate Substrate Kit (Biorad, USA) was added to the nitrocellulose membrane for 2 min to develop a band of proteins and stopped the reaction by water.

4.3 Antibacterial activity

The antimicrobial activity of colicin N will be determined by the agar overlay technique. The culture of *E. coli* NCTC 10538 with 0.35% LB agar was added on top of solidified LB agar plate. 2 µl of purified colicin N at different concentrations were spotted on the surface of LB agar. Ampicillin 0.956 µg and PBS buffer were used as a positive and negative control, respectively. The plates were incubated for 16-18 h at 37°C. A zone of inhibition on the agar plate was measured.

4.4 Protein identification by Mass Spectrometry (MS)

The samples were sent to analyze at Dr. Sittiruk Roytrakul's Lab (National Center for Genetic Engineering and Biotechnology: BIOTEC, Thailand). Mass Spectrometry (MS) to identify proteins, the band excision of purified proteins from SDS-PAGE were cut and washed in DI water. Next, the proteins on gels were alkylated by reduction and then digested by trypsin to obtain the peptides. Then, the peptides were extracted and desalting from the supernatant of extraction. The peptides were then analyzed by Liquid Chromatography-Mass Spectrometry (LC-MS/MS) (Bruker Daltonics GmbH, Germany). The peptide mass fingerprint was compared to the results by protein sequence database searches from NCBI.

4.5 Secondary structure analysis by Circular Dichroism (CD)

The folding of protein was investigated by Circular Dichroism Spectroscopy (CD). Far-UV was carried out on a CD spectrophotometer (j-815 CD Spectrophotometer, Jasco, Tokyo, Japan). In this study, a path length circular cuvette was used a 0.10 cm and the wavelength is 190-260 nm. Proteins concentration were used of 0.4-2.0 mg/ml. Proteins were contained in Phosphate Buffer Saline (PBS buffer). Measurements were carried out at 25°C. The results were converted to the molecular ellipticity unit (deg·cm^2·dmol^{-1}).

5. Cell culture and treatment

5.1 Screening for anticancer activities of ColN-WT

The cytotoxicity activities of all the experiments in this part sent samples to an analysis by MTT assay at Excellent Center for Drug Discovery: ECDD, Mahidol University, Thailand. The cancer cells were chosen for the testing of colicin N activity against cancers including HT-116 (Colon cancer cells/ATCC® CCL-247™, USA), HT-29 (Colon cancer cells/ATCC® HTB-38™, USA), MCF-7 (Breast cancer cells/ATCC® HTB-22™, USA), MDA-MB-231 (Breast cancer cells/ATCC® HTB-26™, USA), and A549 (Lung

cancer cells/ATCC® CCL-185™, USA). The cell culture were grown in different mediums and supplements, HCT-116, MCF-7 and MDA-MB-231 were grown in high glucose DMEM with 10% FBS, 1%penicillin/streptomycin and 1X HEPES. HT-29 was grown in DMEM/Ham's F-12 with 10% FBS, 1%penicillin/streptomycin and 1X HEPES. A549 was grown in Ham's F-12K with 10% FBS, 1%penicillin/streptomycin. The cell culture was incubated at 37°C.

MTT assay was utilized to observe the cytotoxicity of colicin N towards these cell lines. Cells were seeded into a 96-well plate at a density of 10,000 cells/well and were grown in complete medium. Then, cells were incubated at 37°C for 24 h. After that, ColN-WT at various concentrations was added to the cell culture medium. Cells were incubated for 72 h at 37°C. Then, the MTT solution (0.5 mg/ml) was added to each well and incubate for 3 h at 37°C. Next, after removing the culture medium with MTT solution. The formazan was dissolved by 200 µl of DMSO. MTT reduction was measured at 570 nm by a microplate reader (Perkin Elmer, Victor3, Massachusetts, USA). The absorbance results were analyzed to estimate IC_{50} cell viability.

5.2 The cytotoxicity with full-length and truncated colicin N

The most susceptible cancer cell was chosen for anticancer activities of all colicin N constructs. The concentration of protein at 0, 10, 40 and 60 µM were used. The anticancer activity was tested by MTT assay as previously described in the method for screening for anticancer activities.

6. Gibson cloning and the cytotoxicity colicin N mutant with H460 cells.

6.1 Construction of colicin N mutant by Gibson cloning

Gibson cloning or Gibson assembly is a technique to assemble a segment of DNA with overlap sequences. The gene fragments were designed from colicin N sequence to change an amino acid. In the previous study, six positions of colicin N including E96, D105, E129, D153, D157 and D162 were recommended to change to

lysine (Table 4). Primer fragment is designed to forward and reverse primer for the Gibson cloning technique. First, Vector prepared by Polymerase Chain Reaction (PCR) using Q5® High-Fidelity DNA Polymerase (Table 5), ColN-WT(Vector) and forward-reverse vector primer (ColNmut_PF(-GTGAACTGGAAGGGACCG) and ColNmut_PR (-CCTTAGCACTCGCTCCATCACCGTTATTACCTCGATTTC)). Then, the Electrophoresis gel technique was used to check the size of DNA and was purified agarose gel by using Gel extraction to use a QIAquick kit (Qiagen).

Second, Insert fragment prepared and used the Q5® High-Fidelity DNA Polymerase also. But insert fragment changed to using forward-reverse insert primer (ColNmut_IF (-CGAGGTAATAACGGTGATGGAGCGAGTGCTAAGGTTG) and ColNmut_IR (-CAAGTTTATTGTTATATTTCGGTCCCTTCCAGTTCAC)). All fragments were used under PCR conditions by PCR Using Q5® High-Fidelity DNA Polymerase (Table 6) that mixed and using PCR Gradient Touch Thermal Cycler (Hercuvan Lab System, UK).

Table 4 The sequence of colicin N with mutations is designed for the synthesis of a gene fragment. Underlined bases are the sites of colicin N mutations.

Gene fragment	DNA sequences
ColN-WT (pET3a)	GAGTGCTAAGGTTGGAGAGATAACAATCACACCTGACAACTCGAAACCAG GTCGTTATATTTCGTCAAATCCTGAATATTCATTGTTGGCAAAATTAATTG ATGCGGAATCAATTAAAGGTACAGAGGTATATACTTTTCACACCAGAAAAG GTCAGTATGTTAAGGTTACTGTTCCAGATAGTAATATTGATAAAATGAGAG TTGATTATGTGAACTGGAAGGGACCGAA
ColN-WT (Vector) (pET3a Change amino acid six position to lysine)	GAGTGCTAAGGTTGGAAAGATAACAATCACACCTAAAAACTCGAAACCAGG TCGTTATATTTCGTCAAATCCTGAATATTCATTGTTGGCAAAATTAATTGAT GCGAAATCAATTAAAGGTACAGAGGTATATACTTTTCACACCAGAAAAGGT CAGTATGTTAAGGTTACTGTTCCAAAAAGTAATATTAAAAAAATGAGAGTT AAATATGTGAACTGGAAGGGACCGAA

Table 5 The component of PCR reaction for Q5® High-Fidelity DNA Polymerase

Component	50 µl Reaction
5X Q5 reaction buffer	10 µl
10 mM dNTPs	1 µl
10 µM Forward Primer	2.5 µl
10 µM Reverse Primer	2.5 µl
Template DNA	Calculation by NEBiocalculator program
Q5 High-Fidelity DNA Polymerase	0.5 µl
Nuclease-Free water	To 50 µl

Table 6 PCR conditions for Q5® High-Fidelity DNA Polymerase

Step	Temp	Time
Initial Denaturation	98°C	2 min
35 cycles	98°C T_a 72°C	10 sec 20 sec 20-30 sec/kb
Final extension	72°C	2 min
Hold	4-10 °C	-

Third, the ratio of vector and insert was calculated by the NEBiocalculator program version 1.12.0 at the ratio of 2:1 (insert: vector). Then, the mixture of vector and insert in a total volume of 2.5 µl were mixed with 2.5 µl of the NEBuilder® HiFi DNA Assembly Master Mix. After that, all mixture spin down and heated at 50 °C for 15 min. Next, Gibson cloning product was transformed into One Shot™ Mach1™ T1 Phage-Resistant Chemically Competent *E. coli.* 5 µl Gibson cloning product was mixed with 50 µl *E. coli* Mach1 and incubated on ice for 30 min. Next, the mixture was submerged at 42 °C for 30 seconds and incubate on the ice again for 2 min. 950 µl SOC media were added to the mixture and incubated at 37 °C, 200 rpm for 1 h. After incubation, the mixture was spread on LB agar media containing ampicillin and incubated at 37 °C overnight.

Fourth, the colony was selected to LB broth media containing ampicillin and incubated at 37 °C overnight. The plasmids were extracted from bacterial culture by the Mini plasmid kit. The mutation in colicin N gene was confirmed by DNA sequencing.

Finally, when getting the sequencing results and transferred plasmid that to *E.coli* BL21-AI for expression protein by using the transformation method as previously in *E.coli* MACH1. The cell culture from overnight culture was expressed and purified ColN mutant as previously described in the method for Expression and Purification of his-tagged colicin N. Moreover, purified Colicin N was checked physicochemical properties and activities of protein including SDS-PAGE, Western blot and antibacterial activity. Colicin N mutations was successful that we call ColNmut-K.

6.2 The cytotoxicity of colicin N mutant to H460 cells.

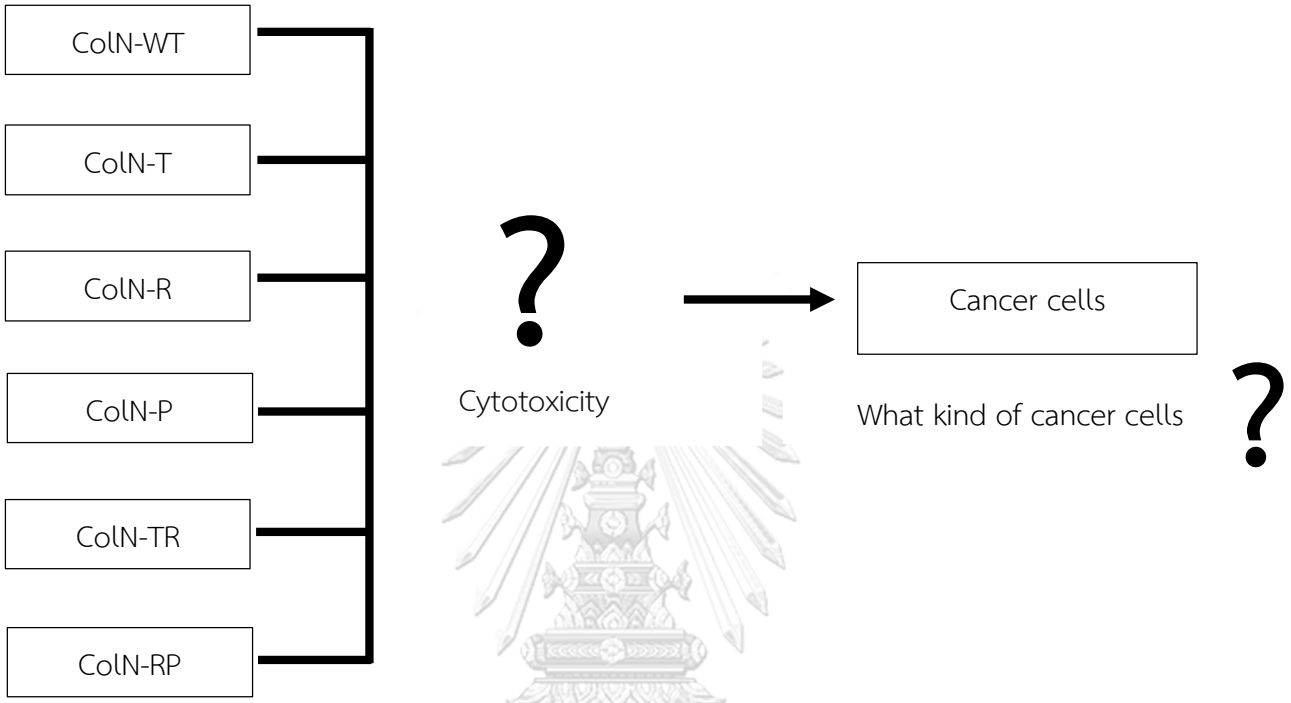
The crystal violet staining assay was a collaboration with Assistant Professor Chatchai Chaotham, Ph.D's Lab for preliminary study. The crystal violet staining assay of the lung cancer cells (H460 cells, ATCC, Manassas, VA, USA) was used to the testing of colicin N activity against cancers. In this experiment, Colicin N mutant-K

(ColNmut-K) compared with full-length colicin N (ColN-WT) concentrations at 0 - 25 µM. H460 cells (ATCC® HTB-177™, lung cancer cells) were seeded into the 6-well plate at a density of each 10,000 cells/well and were grown in complete medium. Cells were incubated at 37°C for 24 h. Whereat, ColN-WT and ColNmut-K were added to complete the cell culture medium. Cells were incubated for 24 h at 37°C. Next, 0.05% (w/v) crystal violet solution was added to each well and incubate for 30 min at room temperature. Next, after removing the culture with a crystal violet solution. The cell culture was washed by water and drying in the fume hood overnight at room temperature. The crystal was solubilized by 200 µl of Methanol and was shaken for 15 min. The solution was measured at 570 nm by a microplate reader. The absorbance results were analyzed to observe cell viability.

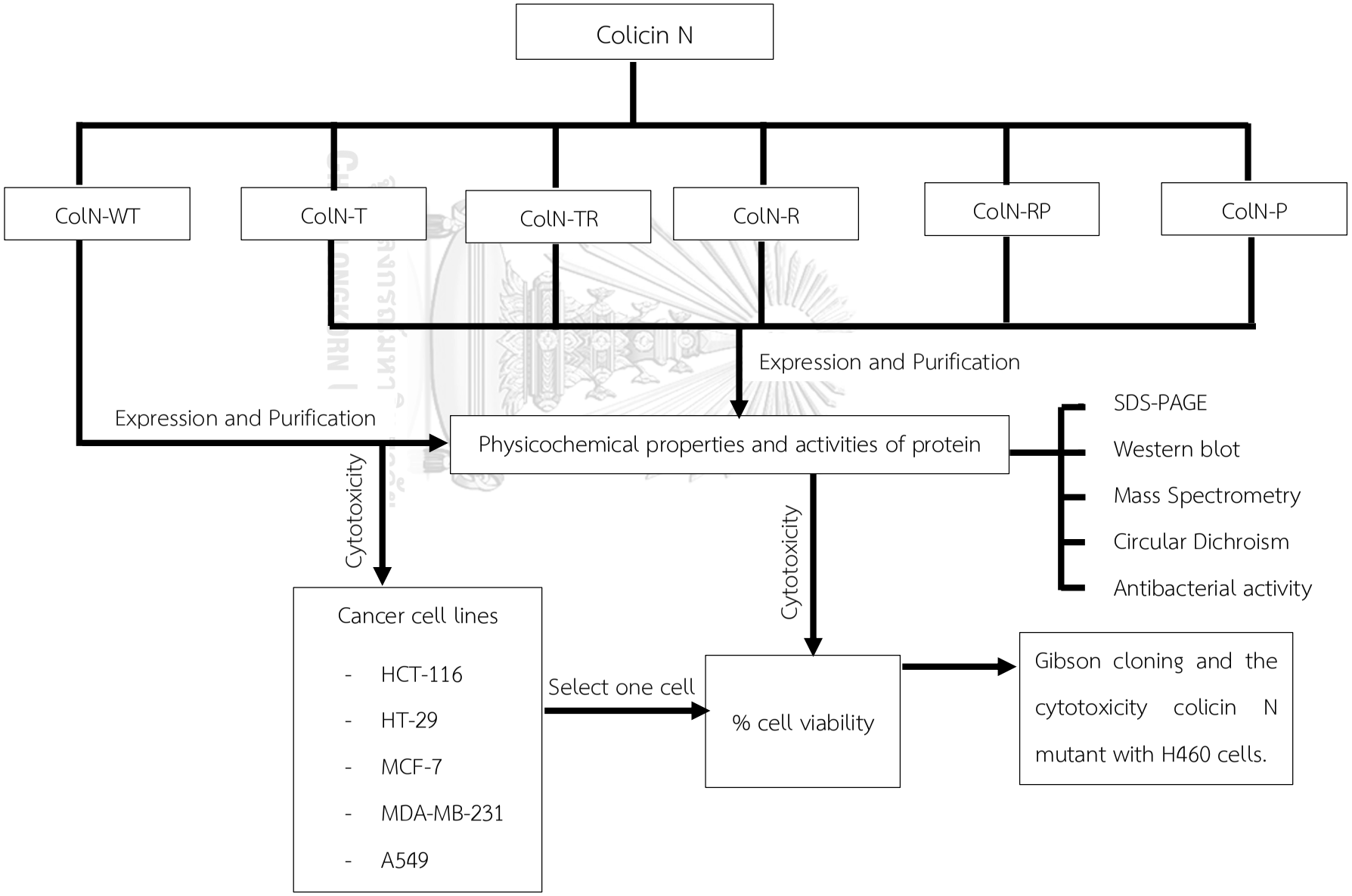
7. Statistics Analysis

The data from the MTT assay was used Least Significant Difference (LSD) Test, One-Way ANOVA in the IBM SPSS Statistics 25 program. The results were shown $*p \leq 0.05$. All experiments were reported as the mean and the standard error (±Standard Deviation, SD) from three dependent experiments and versus non-treated control.

Conceptual framework



Experimental design



CHAPTER IIII

RESULTS

1. Protein expression and purification of full-length and truncated colicin N

Colicin N can be separated into three domains. The first domain in residues 1-90 is the translocation domain (T domain, unstructured and invisible in the crystal structure). The second domain in residues 91-183 is the receptor binding domain (R domain, blue ribbon in the crystal structure in Figure 5). The last domain residues 184-387 is the pore-forming domain (P domain, red ribbon in the crystal structure in Figure5). The colicin N used in this study is composed of 6 constructs, full-length colicin N (ColN-WT) and truncated colicin N (ColN-T, ColN-R, ColN-P, ColN-TR and ColN-RP). The composition of each construct was shown in Figure 5.

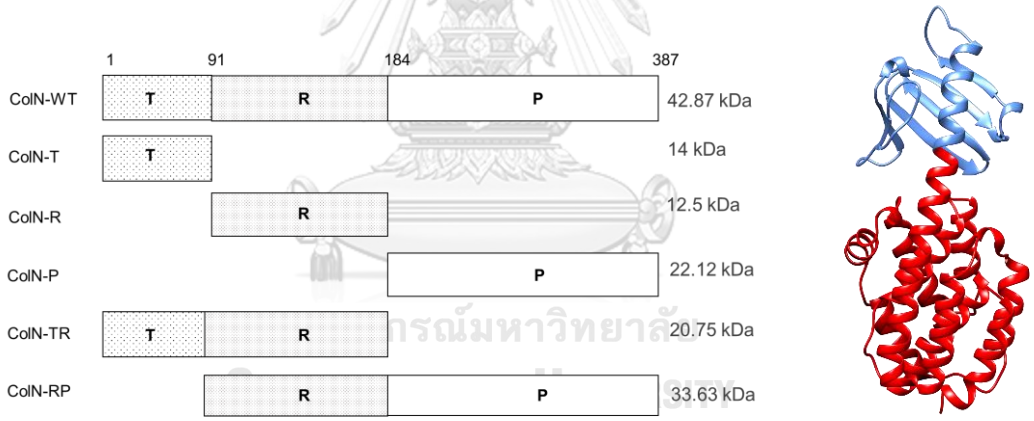
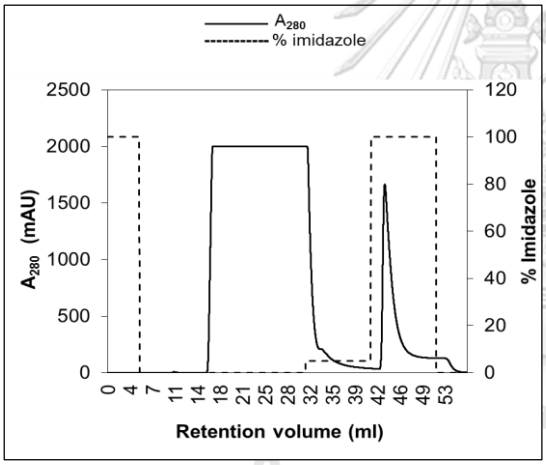


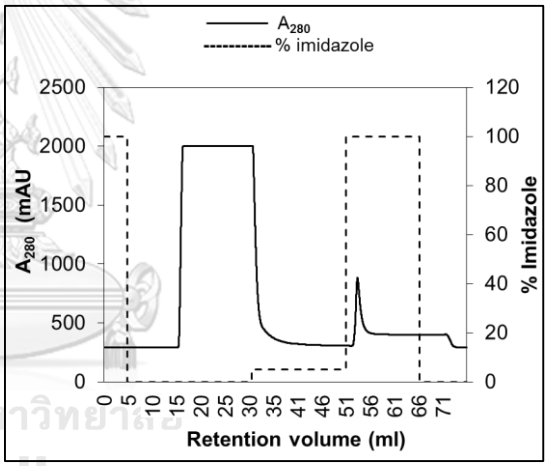
Figure 5 Domain structure of colicin N (full-length and truncated colicin N) and crystal structure of colicin N (PDB code: 1A87)

The protein colicin N were collected from *E. coli* strain BL21-AI after induced by arabinose and purified by FPLC, the chromatogram as shown in Figure 6. In the purifying process, the expressed protein, colicin N with a c-terminal 6xhistidine tag, can bind and elute with the affinity column. The early peak showed the proteins that cannot bind to the Histrap FF affinity column, called flow-through fraction (FT). The second peak showed proteins that can bind to the Histrap FF affinity column,

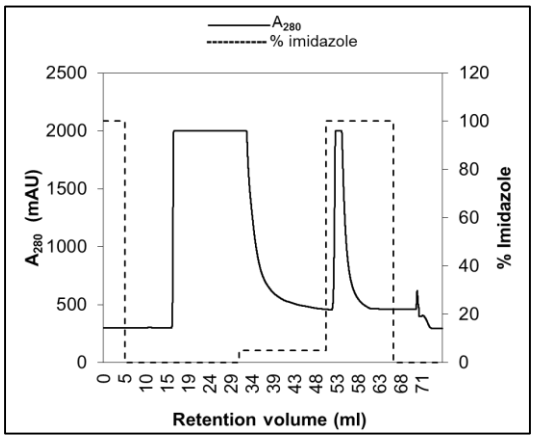
called eluted fraction (EF). The height of each EP fraction peak as shown in Figure 6 was not equal due to the low protein yield of some constructs. Then, the calculated protein yield were as follows : ColN-WT is 2.28 mg/L culture, ColN-T is 1.59 mg/L culture, ColN-R is 14.60 mg/L culture, ColN-P is 7.35 mg/L culture, ColN-TR is 8.26 mg/ L culture and ColN-RP is 7.86 mg/ L culture. The concentration of purified colicin N were measured by UV-Vis spectroscopy at 280 nm and BCA assay. ColN-R obtained the highest yield followed by ColN-TR, ColN-RP, ColN-P and ColN-WT, respectively. ColN-T had the lowest yield when produced by the same protein expression and purification process as it had the lowest peak in the second peak of the FPLC chromatogram. Due to the low yield of protein, expression and purification processes were repeated to get a sufficient amount of protein for testing with cancer cells.



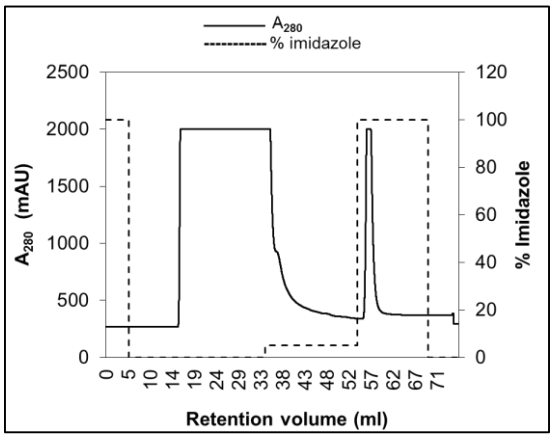
ColN-WT



ColN-T



ColN-R



ColN-P

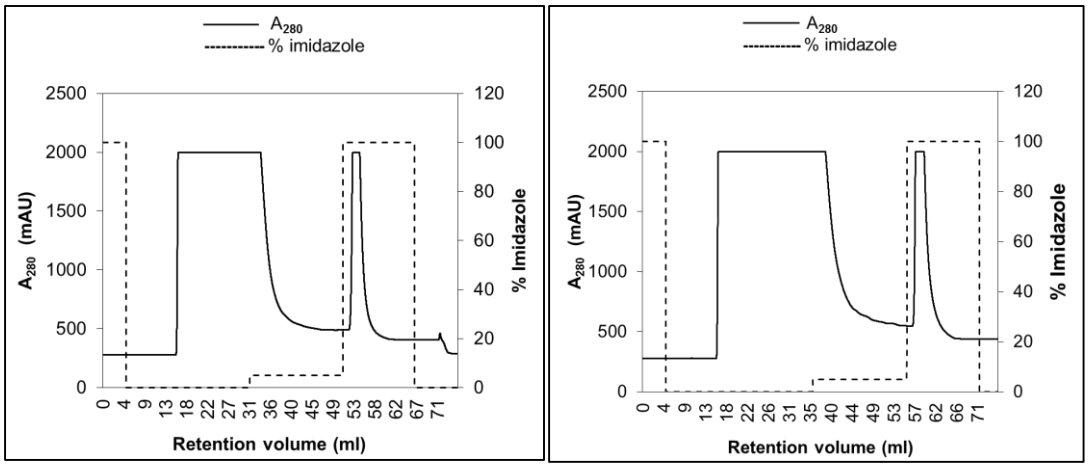


Figure 6 FPLC Chromatogram

Full-length and truncated Colicin N purified by Fast Protein Liquid Chromatography (FPLC). Purifying proteins were carried out by a Histrap FF affinity column his-tagged proteins specifically attach to a column. The first peak is proteins without his-tagged and the second peak is eluted proteins having his-tags.

After successful protein expression and purification, SDS-PAGE analysis was carried out to confirm the size and purity of proteins. The samples were collected before and after induction, crude supernatant and purification. The samples were boiled to denature proteins and loaded with the 12% SDS-PAGE gels. After coomassie blue staining, the band in SDS-PAGE gel expected to be in pre-induction lane is a protein not expression protein that have most contaminants. Post-induction lane showed a nearly correct size of protein band that means the arabinose can induce expression of a protein, in the crude supernatant lane is supernatant after break the cell and band should be same in post-induction but clearer more than post-induction, in the flow-through lane should be don't have colicin N band that expected to be correct size of colicin N because protein cannot be binding with the column and eluted fraction lane is protein can binding with the column and removed the most contaminants. Purified colicin N sizes are expected to be 42.87 kDa for ColN-WT, 12.5 kDa for ColN-R, 22.12 kDa for ColN-P, 20.75 kDa for ColN-TR and 33.63 kDa for ColN-RP (Figure 7). All eluted protein (EP) bands were at the correct size corresponding to

their theoretical molecular weight mentioned above, but the band of ColN-T did not appear in the SDS-PAGE due to very low concentration.

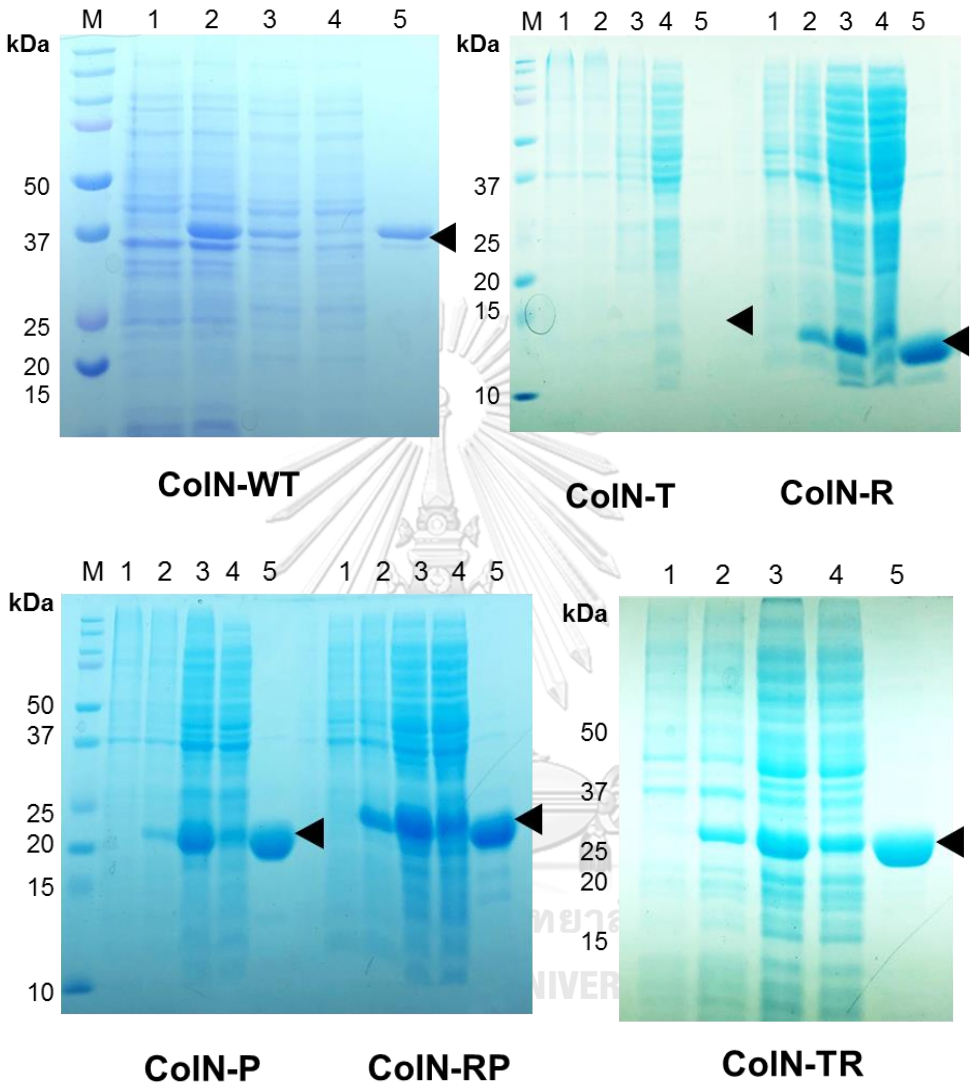


Figure 7 SDS-PAGE analysis of Full-length and truncated Colicin N expression in *E. coli.*

The samples were collected before and after induction and purification. The 12% SDS-PAGE gel was stained by Coomassie blue after electrolysis. Lane M is a protein marker, Lane 1 is pre-induction, Lane 2 is post-induction by arabinose, Lane 3 is crude supernatant, Lane 4 is flow-through from FPLC and Lane 5 is eluted protein from FPLC. ColN size is expected to be 42 kDa for ColN-WT, 14 kDa for ColN-T, 12.5

kDa for ColN-R, 22 kDa for ColN-P, 20 kDa for ColN-TR and 32 kDa for ColN-RP. All elution protein bands were at the correct size.

2. Physicochemical characterization and activities of full-length and truncated colicin N

2.1 Identification of recombinant colicin N

To identify whether purified recombinant proteins were expressed or not, immunoblotting techniques using antibodies against a c-terminal 6xhistidine tag on recombinant proteins were performed.

In this part, the purified proteins were checked by SDS-PAGE and western blot also. SDS-PAGE and Western blot showed all bands of full-length and truncated colicin N that can detect with the antibody and possessing correct the size of the proteins (Figure 8).

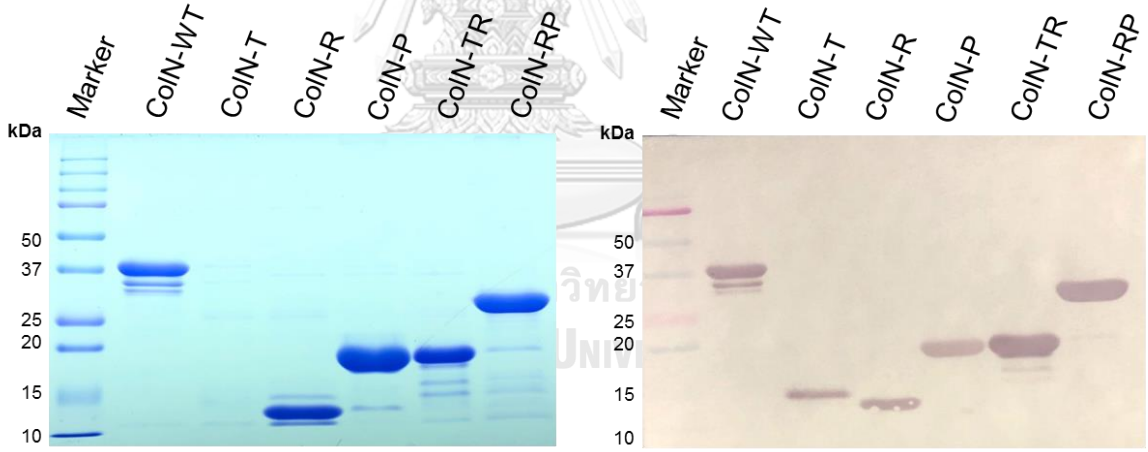


Figure 8 SDS-PAGE and western blot analysis of all recombinant proteins produced by *E. coli*.

SDS-PAGE and western blot analysis of purified full-length and truncated Colicin N. Anti-histag antibodies were used for protein detection in western blot.

To identified protein of full-length and truncated colicin N by Liquid Chromatography Mass spectrometry (LC-MS/MS) analysis. The samples were sent to analyze at BIOTEC lab, Thailand. Purified proteins were digested lysine and alanine by trypsin digestion and running in LC-MS/MS machine. After digestion and running, the results were showed the peptide mass fingerprint of protein. The peptide mass fingerprint data were checked by UniProt from NCBI, NCBIprot 20191120 (227181163 sequences; 82759882099 residues) that found ColN-WT, ColN-T, ColN-R, ColN-P, ColN-TR and ColN-RP are bacteriocin were produced by *E. coli* and showed a count of the peptide matches with the database (Table 7). Last, the results can show all purified proteins are colicin N.

Table 7 Identification of protein by Liquid Chromatography Mass spectrometry (LC-MS/MS) analysis.

Protein name	**Entry name (Uniprot)**	**Protein name**	**Peptide matches**
ColN-WT	WP_001749638.1	bacteriocin colicin N [*Escherichia coli*]	14(14)
	WP_032084302.1	helix-turn-helix transcriptional regulator [*Escherichia coli*]	1(1)
ColN-T	WP_000003373.1	protein-export chaperone SecB [*Escherichia coli*]	1(1)
	PBU81701.1	colicin-N, partial [*Escherichia coli*]	1(1)
ColN-R	PBU81701.1	colicin-N, partial [*Escherichia coli*]	11(11)
ColN-P	GDV25678.1	colicin E1 protein [*Escherichia coli*]	7(7)

	WP_001749638.1	bacteriocin colicin N [*Escherichia coli*]	7(7)
ColN-TR	PBU81701.1	colicin-N, partial [*Escherichia coli*]	9(9)
ColN-RP	WP_001749638.1	bacteriocin colicin N [*Escherichia coli*]	19(19)

2.2 Secondary structure analysis of full length and truncated colicin N by Circular Dichroism

To analyze the secondary structure of full-length and truncated colicin N, Circular Dichroism (CD) spectroscopy technique was utilized to measure the structural content of protein. The CD spectrum were converted to molecular ellipticity unit (deg·cm^2·dmol^{-1}) showed a peak as show in Figure 9. The CD curve were compared with a previous study (32). The all full-length and truncated colicin N CD curve showed nearly the reference but ColN-P not the same in the reference CD chromatogram. The results from the CD machine were compared according to the secondary structure of full-length colicin N (P08083) from the Uniprot database (https://www.uniprot.org/) that has the secondary structure contents in colicin N at Table--. The results from the CD machine were uploaded the data to BeStSel program (https://bestsel.elte.hu/index.php) that used the widest wavelength range for which the absorption within the acceptable limit and calculated the structural elements to secondary structure. The secondary structure results in Table 9 were compared with data from the database. ColN-WT, ColN-R, ColN-P and ColN-RP had a percentage of the secondary structure corresponding to the database. But ColN-T and ColN-TR are a lower signal of the secondary structure.

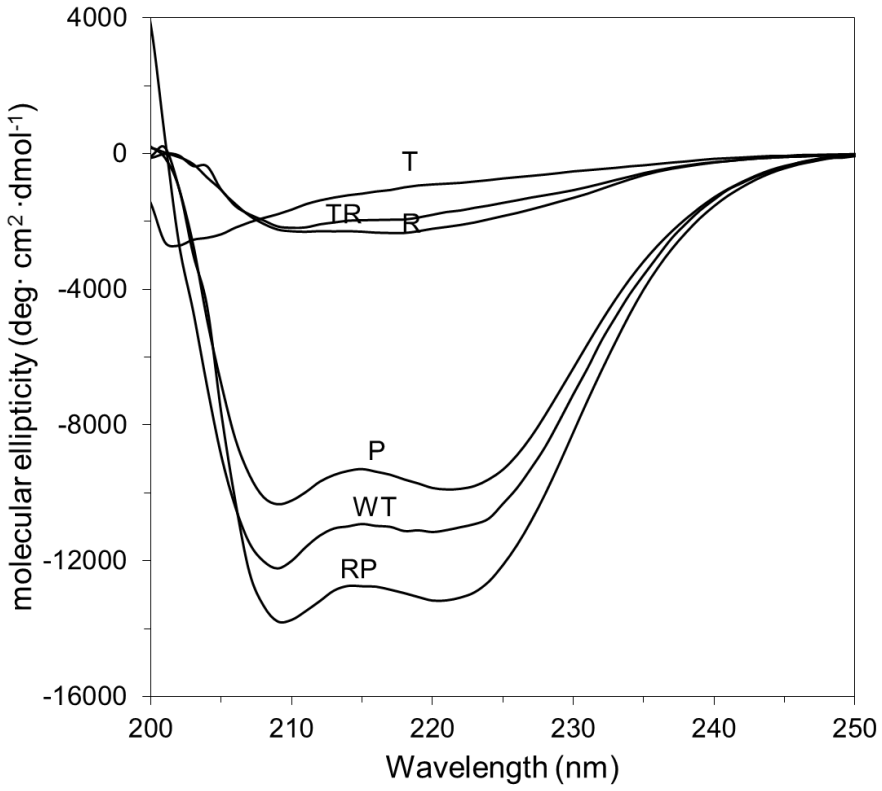


Figure 9 Secondary structure analysis of full length and truncated colicin N by Circular Dichroism (CD)

The CD spectra were measured at a protein concentration of 0.4-2.0 mg/ml at 25 °C by using a 1 mm path length cell. All samples were measured at 190-260 nm.

2.3 Antimicrobial test

The agar overlay technique for antimicrobial activity was used to test the antimicrobial activity of full-length and truncated colicin N. Colicin N has antimicrobial activity with closely strain. Then, Colicin N should be inhibit the growth of other strain of *E. coli* and observed the clear zone on an agar plate that were confirmed to colicin N activities. Full-length and truncated colicin N concentrations at 0.244, 0.492 and 0.956 µg were dropped 2 µl colicin N on LB agar plate on the lawn of *E. coli* NCTC 10538 cells and incubated overnight. ColN-WT (Figure 10A) and ColN-T (Figure 10B) concentrations at 0.244, 0.492 and 0.956 µg were observed from the clear zone on the lawn of *E. coli* NCTC 10538. ColN-WT and ColN-T inhibited the

growth of bacteria. A clear zone of other constructs did not appear. In this experiment, the positive control was ampicillin and the negative control was PBS buffer. The diameters of the clear zone were measured and shown in Table 9.

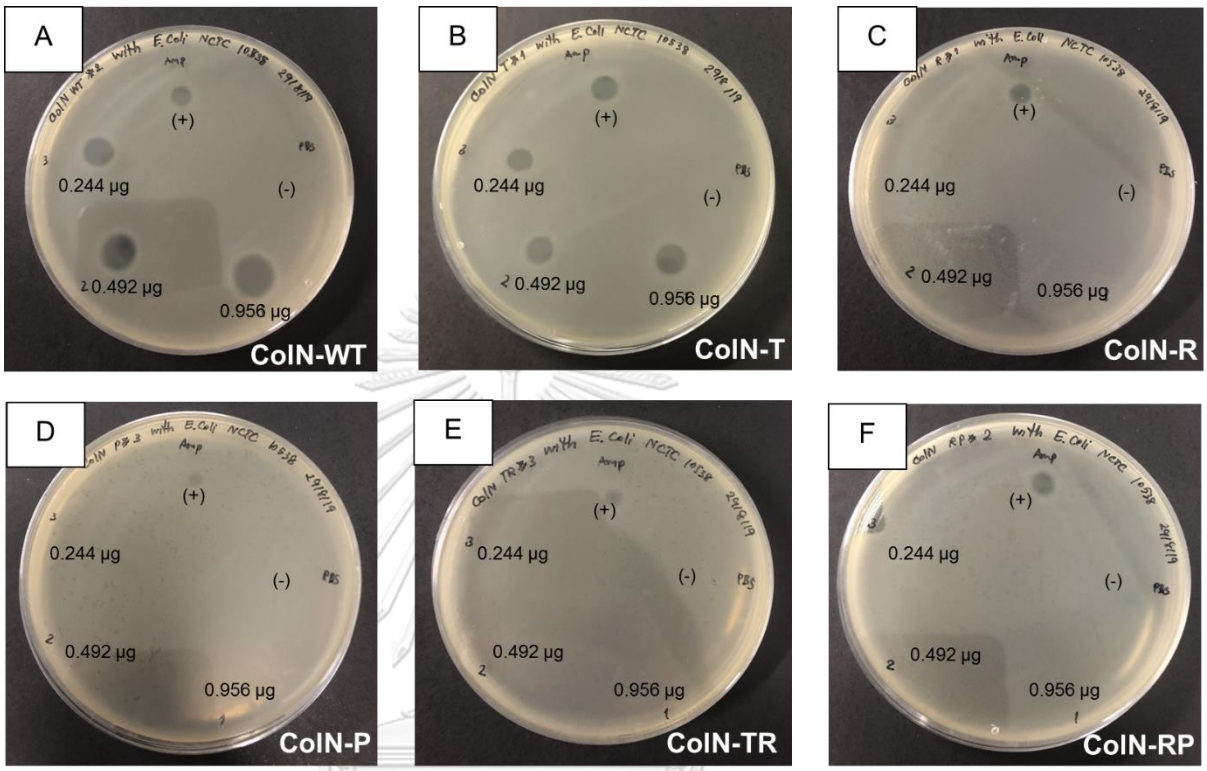


Figure 10 Antibacterial activities of colicin N.

The clear zone on the lawn of *E. coli* indicate the antibacterial function. In this experiment, positive control is ampicillin and negative control is PBS buffer.

Table 8 The antibacterial activities of colicin N

The clear zone was observed on the plate of *E. coli* NCTC 10538 after Colicin N treatment for 24 h.

Construct name	Antibacterial activity	ColN concentrations
ColN-WT	Yes	0.244, 0.492 and 0.956 μg
ColN-T	Yes	0.244, 0.492 and 0.956 μg
ColN-R	No	-
ColN-P	No	-
ColN-TR	No	-
ColN-RP	No	-

3. The cytotoxicity activity of full-length and truncated colicin N on cancer cells.

The cytotoxicity activities of all of the experiments in this part were sent to an analysis by MTT assay at Excellent Center for Drug Discovery: ECDD, Mahidol University, Thailand. HCT-116 cells, HT-29 cells, MCF-7 cells, MDA-MB-231 cells and A549 cells treated with full-length colicin N (ColN-WT) were done for screening the anticancer activities of ColN-WT. After exposure of ColN-WT concentrations at 0 - 42 μM to cancer cells, MTT assay were used to check the cell viability of cell cultures. The cytotoxicity study showed the cell viability of HCT-116 cells, HT-29 cells and MDA-MB-231 cells, was started to decrease at the concentration of ColN-WT 3.4 μM. The cell viability of A549 cells was started to decrease at a concentration of ColN-WT 1.7 μM. HCT-116, HT-29, MDA-MB-231, and A549 cells showed the reduction in cell viability more than the first ColN-WT concentration that was treated with cancer cells, respectively. Meanwhile, the cell viability of MCF-7 cells was decreased at the concentration of ColN-WT 1.7 μM and was started to grow at 8.4 μM of concentration of ColN-WT, respectively. The cell viability of MCF-7 cells has higher than control at 42.0 μM of ColN-WT. According to the graph in Figure 11, ColN-WT has a good cytotoxicity activity in HCT-116 cells. HCT-116 cells (Colon cancer cells) were shown

the lowest cell viability at 42.0 μM. When estimating IC_{50} of ColN-WT to these cancer cells, HCT-116 is the lowest among other cancers. HCT-116 cells will be decreased to 50% cell viability at around 45 μM of ColN-WT (Data show in Table 12). Therefore, HCT-116 cells were chosen for treatment with full-length and truncated colicin N that finding the constructs of colicin N are responsible for its anticancer activity.

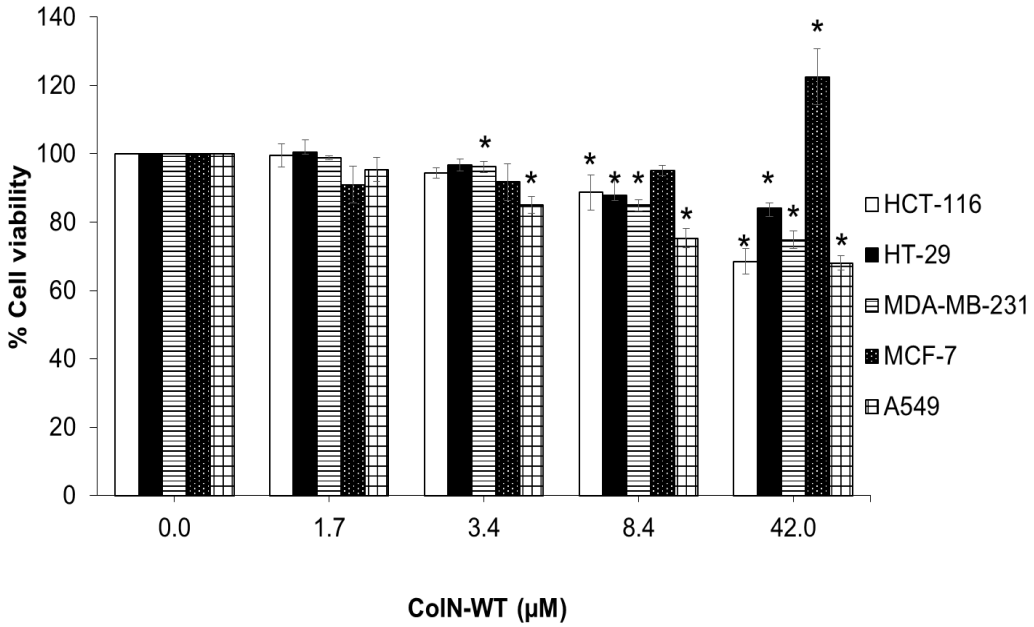


Figure 11 The cytotoxicity of wild type colicin N by MTT assay

The cytotoxicity of wild type colicin N by MTT assay against 5 cancer cells. Data are reported as the mean ± standard deviation.

The cytotoxicity activity of HCT-116 cells was treated with full-length and truncated colicin N (ColN-WT, ColN-T, ColN-R, ColN-P, ColN-TR and ColN-RP) that find the construct of colicin N are responsible for its anticancer activity. HCT-116 cells were treated with full-length and truncated colicin N at 0 - 60 μM and incubated for 72 h. In this study, MTT assay was also used for check the cell viability of cell culture. All constructs of colicin N were decreased cell viability and dependent on low to a high concentration of protein (Figure 12). But the cell viability of HCT-116 cells were stopped to decrease when treat at ColN-P concentrations 40 μM and the

cell viability was grown at ColN-P concentrations 60 μM. Moreover, when ColN-TR and ColN-RP treated HCT-116 cells, the cell viability of HCT-116 cells was stopped the growth of cell cultures at 40 – 60 μM. At last, the cell viability of HCT-116 cells that were treated with ColN-WT has the lowest among other constructs of colicin N and significant at *$p \leq 0.05$.

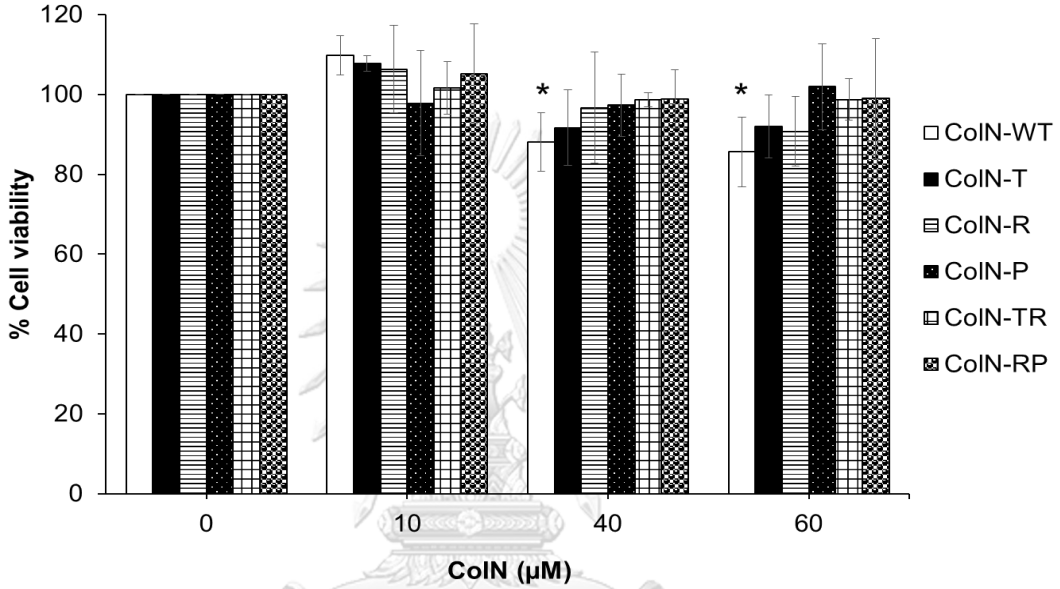


Figure 12 The cytotoxicity activity of full length and truncated colicin N with HCT-116 cells.

Data are reported as the mean ± standard deviation from three dependent experiments. Statistically significant was set at *$p \leq 0.05$.

4. Improvement of Colicin N for anticancer activities

After the cytotoxicity activity experiments, showed full-length colicin N (ColN-WT) has good toxicity in colon cancer cells. The researcher doesn't optimize of his-tagged colicin N expression because ColN-WT has a good yield of protein. Thus, the researcher used Gibson cloning to mutation of ColN-WT that improvement of colicin N for anticancer activities.

Gibson assembly is a method have efficient to use for cloning large DNA molecules and can combine many DNA fragments by fewer steps in one tube

reaction. This method has been proven to be an efficient and effective method of assembly plasmids.

Figure 13 The sequencing of amino acid results

The sequencing was read by T7promotor and T7terminator.

Figure 14 The sequencing of protein results

The sequencing was read by T7promotor and T7terminator.

In this study, Gibson cloning was successfully and showed the correct of six positions to change in the sequencing results that read by T7promotor and T7terminator (Figure 13 and 14). Colicin N these mutations were called ColNmut-K. After, the plasmid mutation was transferred to *E. coli BL21-AI* and expression protein by induced with arabinose. In the purification protein step, the FPLC chromatogram showed the first peak that protein did not bind with the Histrap FF affinity column, call flow-through fraction (FT). The second peak showed protein bound to the column called eluted fraction (EP). The FPLC chromatogram was shown the eluted fraction (Figure 16). Purified ColNmut-K were checked physicochemical properties and activities of proteins. SDS-PAGE technique was carried out to confirm the size of

protein, the results, purified ColN-WT compared with ColNmut-K in SDS-PAGE gel. The size of ColNmut-K is expected to be 42.87 kDa and the correct size of protein as shown in Figure 15.

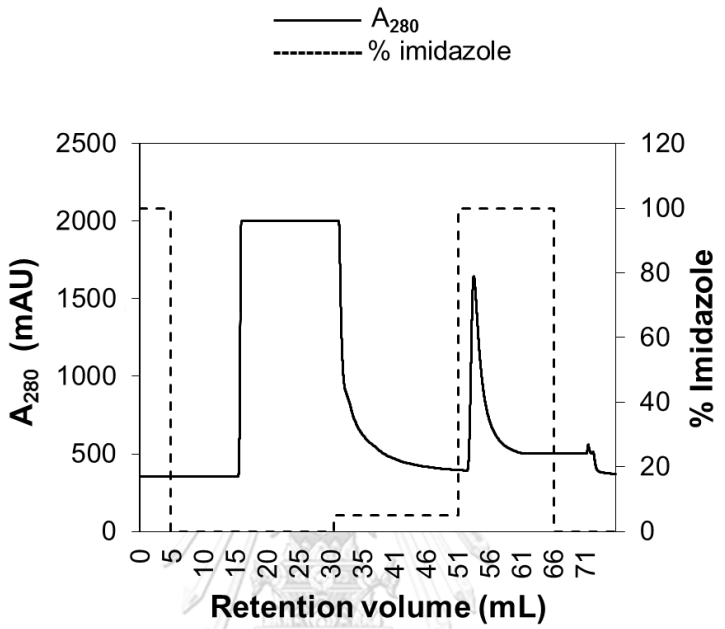


Figure 15 Purification of ColNmut-K by Fast Protein Liquid Chromatogram (FPLC). Purification protein was used Histrap FF affinity column to bind his-tagged protein**.** The first peak is proteins without his-tagged and the second peak is eluted protein that was obtained his-tagged to binding with the column.

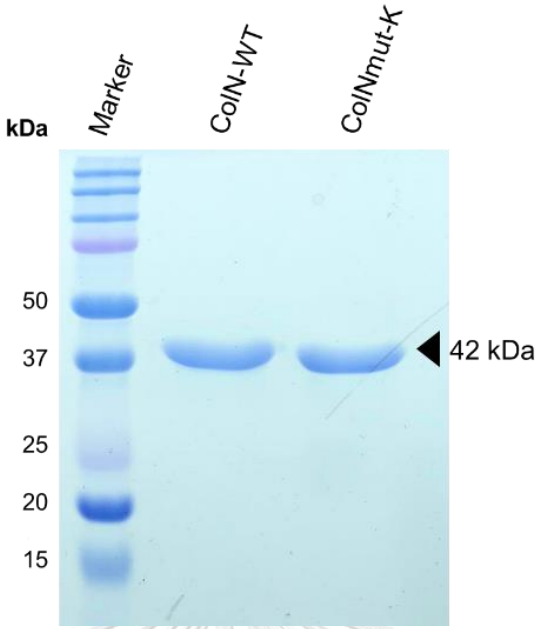


Figure 16 SDS-PAGE and western blot analysis
SDS-PAGE and western blot analysis of purified ColN-WT and ColNmut-K.

The agar overlay technique for antimicrobial activity was used to test the antimicrobial activities of ColN-WT and ColNmut-K. ColN-WT and ColNmut-K that have concentrations at 2, 0.2 and 0.02 µg on LB agar plate with *E. coli* NCTC 10538 cells and incubated overnight. ColN-WT and ColNmut-K can be inhibited bacterial and were observed from the clear zone on the lawn of *E. coli* NCTC 10538. ColN-WT and ColNmut-K were showed the clear zone on an agar plate that were confirmed to colicin N activities at concentrations 1, 0.1 and 0.01 mg/ml (Figure 17). The clear zone of ColNmut-K showed a smaller than the clear zone of ColN-WT. In this experiment, the positive control was ampicillin and the negative control was PBS buffer. The diameters of the clear zone were measured and shown in Table 14.

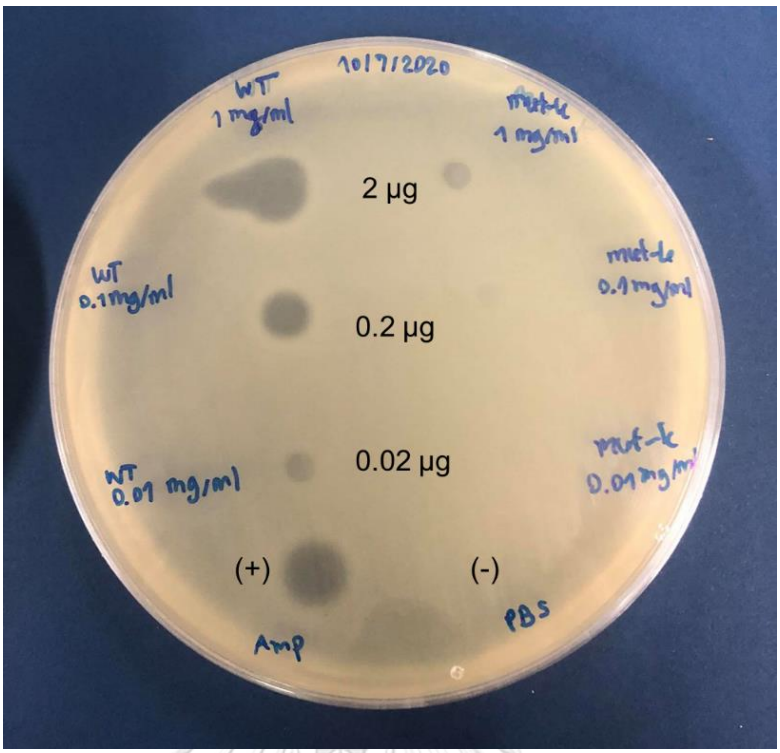


Figure 17 Antibacterial activity

Antibacterial activity of ColN-WT and ColNmut-K were observed from the clear zone on the lawn of *E. coli* NCTC 10538. ColN-WT and ColNmut-K can inhibit bacterial growth that is the biological function of ColN. In this experiment, positive control is ampicillin and negative control is PBS buffer.

The crystal violet staining assay was a collaboration with Assistant Professor Chatchai Chaotham, Ph.D's Lab for preliminary study. The crystal violet staining assay was used to counting the cell viability. After, purified ColN-WT and ColNmut-K have tested the cytotoxicity activities on H460 cells (lung cancer cells). After exposure with ColN-WT and ColNmut-K concentrations at 0 - 25 µM and incubated for 24 hours that were used crystal violet to check the cell viability of cell cultures. A graph was showed the effect of H460 cells were treated with ColN-WT and ColNmut-K. The cytotoxicity activity results showed cell viability of H460 cells with ColNmut-K is lower than ColN-WT (Figure 18). The cell viability of H460 cells with ColNmut-K decreased with treatment 5-25 µM. But the cell viability of H460 cells was grown at concentrations of ColN-WT 10-25 µM.

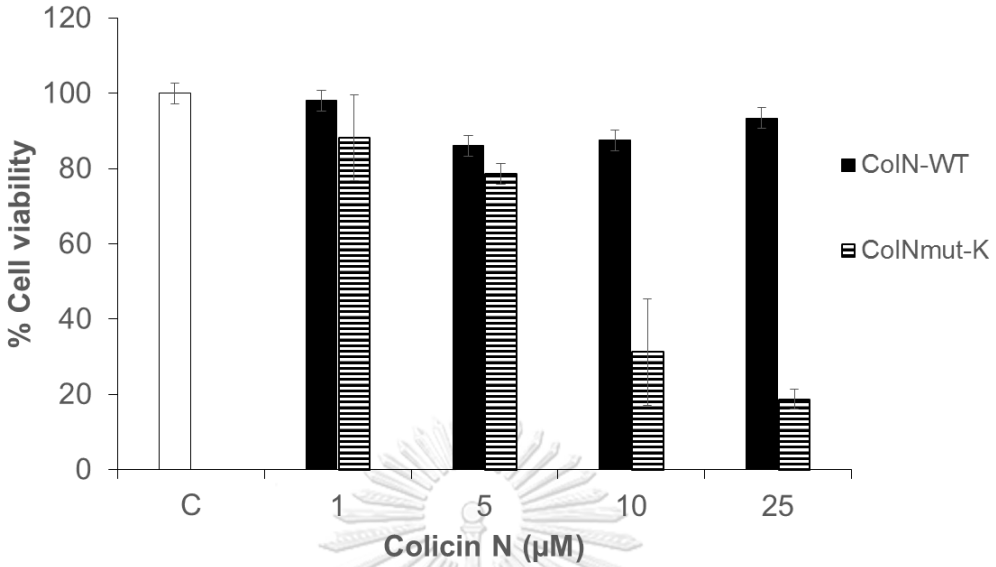


Figure 18 Graph showed the cytotoxicity activity of ColN-WT and ColNmut-K with H460 cells.

ColNmut-K has good cytotoxicity activity in H460 cells (Lung cancer cells) more than ColN-WT because ColNmut-K is lower cell viability more than ColN-WT.

CHAPTER V
CONCLUSION AND DISCUSSION

To overcome the limitation of conventional chemotherapy for cancer, protein-based therapeutic agents are the alternative that we are interested in this study. In this work, we explored a novel therapeutic protein, colicin N, which is categorized into the family of pore-forming toxins. The data presented here provide additional information about the anticancer activity of Colicin N against several cancer cells and the essential Colicin N domains. Moreover, the modification of colicin N was carried out to improve their cytotoxicity towards cancer cells.

Full-length and truncated colicin N produced by *E. coli* BL21-AI, induced by 0.2% (w/v) L-(+)-Arabinose were successfully expressed and purified by the affinity Ni-NTA column with FPLC chromatography. The identity, purity and size of purified proteins was confirmed by SDS- PAGE and Western blot. All constructs except ColN-T showed the correct size of proteins (ColN-WT 42 kDa, ColN-R 12.5 kDa, ColN-P 22 kDa, ColN-TR 20 kDa and ColN-RP 32 kDa) in SDS-PAGE gel (14). The band of ColN-T was not observed because ColN-T concentration was too low. After that, western blot was used to detect specific protein with histidine-tagged and the western blot result showed ColN-T band. Therefore, full-length and truncated colicin N were shown the correct size of proteins corresponding to its theoretical molecular weight. Full-length and truncated colicin N showed in the antibacterial activities experiment. ColN-WT and ColN-T were observed from the clear zone on the lawn of the close strain of *E. coli*. ColN-WT and ColN-T have a previous report that showed antibacterial activities also (14). This study can confirm colicin N which were active. Proteins were identified by Mass Spectrometry technique (Liquid Chromatography Mass Spectrometry, LC-MS/MS). The purified full-length and truncated colicin N after expression and purification were compared with the following peptide mass fingerprint as filed with the NCBI database. The peptide mass fingerprint of full-length and truncated colicin N match with the database. Even though ColN-P has been shown to colicin N and Colicin E1 matched, but Colicin E1 is closely related in

pore-forming bacteriocins using the TolA translocation system. TolA system forms is a multi-protein complex in the cell envelope of most gram-negative bacteria (33). The identity of the full-length and truncated colicin N is thus verified.

Moreover, circular dichroism was used to check the secondary structure of proteins. Full-length and truncated colicin N was converted to molecular ellipticity unit ($deg \cdot cm^2 \cdot dmol^{-1}$) and analyzed by using current software to calculate the percentage of the secondary structure. The percentage of all constructs after analysis, its related to the percentage of the secondary structure of colicin N references from the Uniprot database. The CD chromatograms showed some constructs including ColN-WT, ColN-R and ColN-TR relate with CD chromatograms reference in the reported by (32). ColN-T is an unstructured domain that has a weaker signal more than ColN-TR in CD chromatogram. ColN-P is a pore-forming domain has a high-resolution structure (10-helix bundle) and ColN-R has a β-sheet fold around a central helix (34) that should behave the molecular ellipticity ColN-P > ColN-WT > ColN-R (32). ColN-WT and ColN-RP are the correct positions following the above mentioned but ColN-P has a weaker signal more than ColN-WT and not correct when compared with the CD chromatograms reference that may be caused by a buffer that contains protein because salt concentration is cause to different analyses to error the CD spectrum (35).

The major important finding accomplished through this study is the cytotoxicity activity of colicin N. A previous study reported colicin A exhibited and E1 are pore-forming toxins and Colicin A and E1 showed inhibited the growth of human tumor cells and standard fibroblasts (11). Consequently, the cytotoxicity activity of full-length colicin N (ColN-WT) was tested with 5 cancer cells (HCT-116, HT-29, MCF-7, MDA-MB-231 and A549 cells) in this study and showed good toxicity against HCT-116 cells (Colon cancer cells) and have good potential in HT-29 cells (colon cancer cells). Since colorectal neoplasia is reported in large intestinal mucosa patients can produce colicins. They were found cancer patients produce colicins and microcins in their intestinal organs (12). As a result, we discuss to colicin N may have any relation with intestinal cancer cells. Thus, ColN-WT also inhibits the growth of A549 cells

(lung cancer cells) as well and previous research reported colicin N showed effective inhibition of lung cancer H460, H292 and H23 cells (13) and colicin E1 affects lung cancer HS913T cells. Other cancer cells, after treatment ColN-WT with MDA-MB-231 cells (breast cancer cells) also decrease the cell viability and have reported about colicin A that affects tumor cells including BT474, ZR75 and SKBR3 (breast cancer cells) (11). We think, the anticancer activity with colicin N like colicin A and E1 because there have the same structure. Whereas, cell viability of MCF-7 cells was increased when treating with ColN-WT. In this study, colicin N has effective against colon cancer cells that respond better than among other cancer cells and all this point can confirm ColN-WT has good sensitivity against tumor cells, seemed to be dependent on types of cancer cells. Another point to investigate in this study is the cytotoxicity activity of full-length and truncated colicin N treated with HCT-116 cells. The result showed ColN-WT is effective against colon cancer cells among other constructs. Full-length colicin N construct has effective anticancer activity on cancer cells like antimicrobial activity. Therefore, we can be confirmed that full-length colicin N is a suitable construct to inhibit the growth of cancer cells.

ColN-WT is an interesting construct of colicin N for the cytotoxicity activity hence the ability of colicin N to cross the lipid bilayer of cells and access the cells, should be developed for efficiency. At present, there are significant barriers to treating cancer is large molecules of cancer drugs. Although this problem has been modified and possible to use the larger molecule drugs into cells, but still difficult to penetrate the cell membrane. Therefore, have been developed Cell-penetrating peptides to increase the efficiency of intracellular uptake of the cell (36). Cell-penetrating peptides (CPPs), cationic peptides that have usually have arginine and lysine amino acids, can quickly change their position into almost any live cells (37), are the most popular and efficient techniques for the conventional specific tumor cell surface. CPPs intracellular uptake is not fully understood. Nevertheless, the CPPs mechanism may activate the entry of CPP cells exist and may coexist, depending on the physicochemical properties, concentration, charge, and length of CPPs (38). CPPs have been used for therapeutic, such as oligonucleotides, peptide nucleic acid, peptides and proteins. A previous study was used TP10-5 (TK) to the

designed analogs of transportan 10 (TP10). In their idea is cancer cell membranes have a more negative charge than normal cell membranes. Then, TK and TP10 can bind to the cancer cell membranes by electrostatic attraction and access the cells (39). Therefore, ColN-WT was mutant by Gibson cloning and was designed to increase the cation (positive charge) on the protein. In preliminary results, the mutation protein had a better inhibitory effect than the ColN-WT on lung cancer cells (H460 cells). This result supported the idea, the cancer cell surface is most of an anion (negative charge) (28), a positive charge on protein can attack the cancer cell surface. Protein has inhibited the growth of cancer cells. In that case, we speculate the charge on protein and tumor surfaces that affect to inhibit the growth of tumor cells.

In conclusion, colicin N was produced by *E. coli.* Colicin N showed the most effective inhibition with colon cancer cells and the optimal construct colicin N to inhibit the growth of colon cancer is ColN-WT. The results from this study can be used for the basic knowledge about colicin N for the cytotoxicity activity on cancer cells and may consider a promising application of therapeutic and natural antitumor drugs.

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APPENDIX

APPENDIX

TABLES AND FIGURE OF EXPERIMENTAL RESULTS

Table 9 The percentage of secondary structure

The percentage of secondary structure content (α-helix, β-stand, turn and others) of full-length and truncated colicin N analyzed by BeStSel (Beta Structure Selection) program.

Protein name	α- helix (%)	β-stand (%)	Turn (%)	Others (%)
ColN (reference)	42.03	9.56	0.78	47.63
ColN-WT	34.3	30.9	6.8	27.9
ColN-T	10.2	21.5	13.0	55.3
ColN-R	20.4	20.0	12.5	47.1
ColN-P	41.0	31.1	5.3	22.6
ColN-TR	6.5	11.4	34.4	47.7
ColN-RP	39.7	27.9	5.8	26.7

Table 10 The diameter of the clear zone observed on the plate of *E. coli* NCTC 10538 after all colicin N treatment for 24 h.

Protein name	[0.956 µg] (cm)	[0.492 µg] (cm)	[0.244 µg] (cm)	(+) (cm)	(-) (cm)
ColN-WT	0.70	0.58	0.45	0.57	-
ColN-T	1.03	0.93	0.83	0.57	-
ColN-R	-	-	-	0.7	-
ColN-P	-	-	-	0.4	-
ColN-TR	-	-	-	0.3	-
ColN-RP	-	-	-	0.75	-

Table 11 The cytotoxicity of wild type colicin N by MTT assay against 5 cancer cells. Data are reported as the mean ± standard deviation.

ColN conc. (µM)	Cell viability (%)				
	HCT-116 cells	HT-29 cells	MDA- MB-231 cells	MCF-7 cells	A549 cells
0.0	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
1.7	99.45 ± 1.49	100.39 ± 1.68	98.88 ± 1.67	90.97 ± 5.40	95.43 ± 2.44
3.4	94.32± 5.12	96.75 ± 4.41	96.16 ± 1.70	91.72 ± 1.63	84.97 ± 2.85
8.4	88.70 ± 3.78	87.93 ± 1.44	84.92 ± 2.61	95.10 ± 8.16	75.26 ± 2.18
42.0	68.49 ± 1.06	84.2 ± 2.65	74.86 ± 0.78	122.50 ± 8.32	68.06 ± 1.46

Table 12 The cytotoxicity of wild type colicin N by MTT assay against 5 cancer cells. Data are reported as the estimated colicin N concentrations to IC_{50}.

Cancer cells	IC_{50} (μM)
HCT-116 cells	45.07
HT-29 cells	159.53
MDA- MB-231 cells	72.93
MCF-7 cells	-
A549 cells	99.37

Table 13 The cytotoxicity activity of full length and truncated colicin N with HCT-116 cells.

Data are reported as the mean ± standard deviation from three dependent experiments. Statistically significant was set at $*p \leq 0.05$.

ColN conc. (μM)	Cell viability (%)					
	ColN-WT	ColN-T	ColN-R	ColN-P	ColN-TR	ColN-RP
0	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
10	109.79 ± 4.93	107.78 ± 1.91	106.34 ± 10.90	97.74 ± 13.18	101.65 ± 6.60	105.18 ± 12.55
40	88.08 ± 7.30	91.68 ± 9.42	96.66 ± 13.92	97.28 ± 7.80	98.63 ± 1.76	98.77 ± 7.31
60	85.61 ± 8.73	91.98 ± 7.81	90.72 ± 8.74	101.96 ± 10.75	98.73 ± 5.16	98.98 ± 15.00

Table 14 The antibacterial activities

The table showed size of the clear zone that ColN-WT and ColNmut-K can be inhibited bacterial (*E. coli* NCTC 10538)

Protein name	[2 µg] (cm)	[0.2 µg] (cm)	[0.02 µg] (cm)	(+) (cm)	(-) (cm)
ColN-WT	0.93	0.60	0.27	0.87	-
ColNmut-K	0.37	0.30	0.20	0.87	-

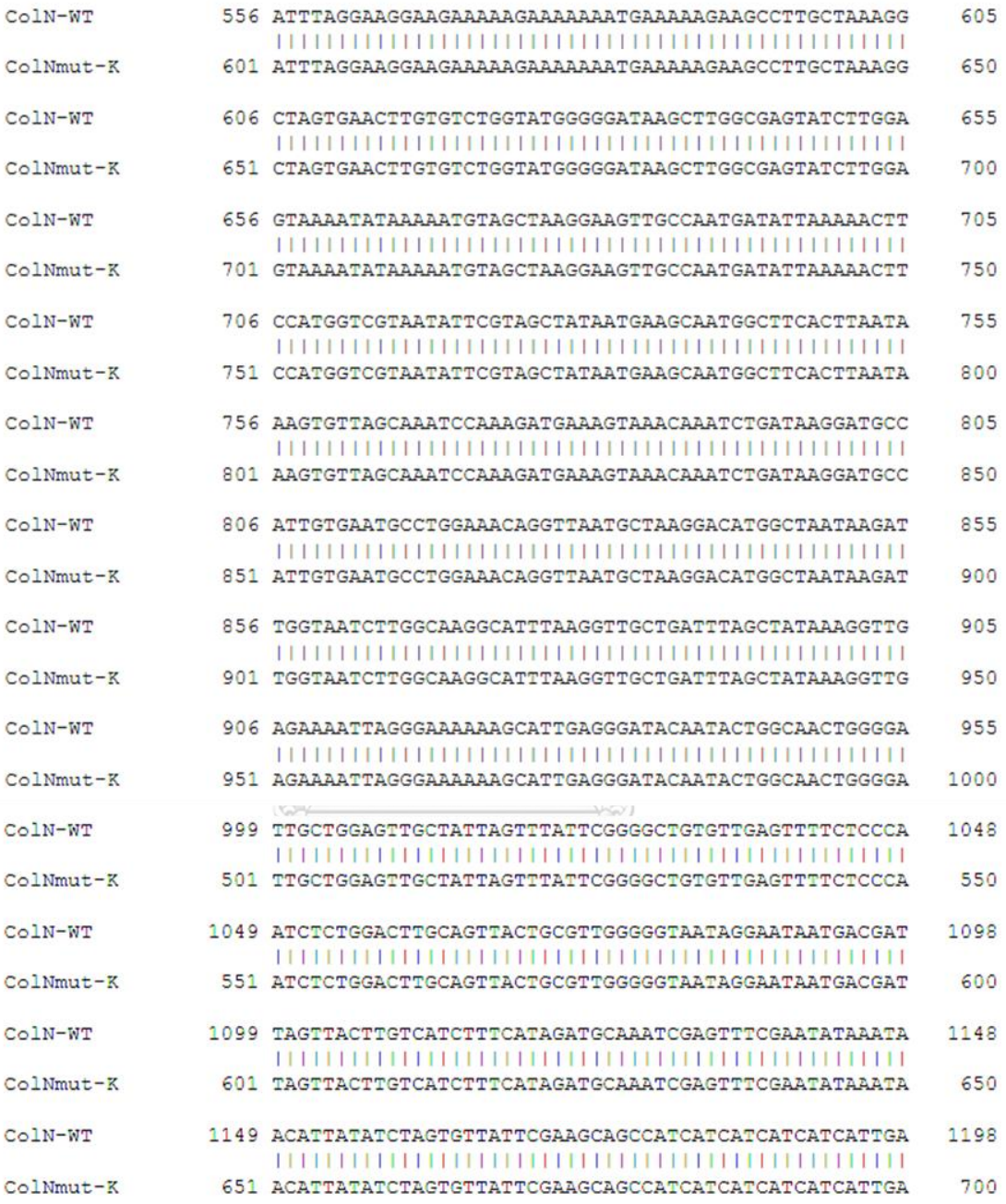
Table 15 The cytotoxicity activity of ColN-WT and ColNmut-K with H460 cells by crystal violet straining assay

Data are reported as the mean ± standard deviation.

Colicin N conc. (µM)	Cell viability (%)	
	ColN-WT	ColNmut-K
Control	100 ± 0.00	100 ± 0.00
1	98.114 ± 0.34	88.2 ± 3.51
5	86.071 ± 11.49	78.65 ± 0.77
10	87.591 ± 2.70	31.326 ± 10.71
25	93.491 ± 14.14	18.674 ± 4.15

Figure 19 The sequencing of amino acid results (full sequencing result)
The sequencing was read by T7promotor and T7terminator.

(1/3)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ColNmut-K 601 ATTTAGGAAGGAAGAAAAAGAAAAAAATGAAAAAGAAGCCTTGCTAAAGG 650
ColN-WT 606 CTAGTGAACTTGTGTCTGGTATGGGGGATAAGCTTGGCGAGTATCTTGGA 655
ColNmut-K 651 CTAGTGAACTTGTGTCTGGTATGGGGGATAAGCTTGGCGAGTATCTTGGA 700
ColN-WT 656 GTAAAATATAAAAATGTAGCTAAGGAAGTTGCCAATGATATTAAAAACTT 705
ColNmut-K 701 GTAAAATATAAAAATGTAGCTAAGGAAGTTGCCAATGATATTAAAAACTT 750
ColN-WT 706 CCATGGTCGTAATATTCGTAGCTATAATGAAGCAATGGCTTCACTTAATA 755
ColNmut-K 751 CCATGGTCGTAATATTCGTAGCTATAATGAAGCAATGGCTTCACTTAATA 800
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ColNmut-K 801 AAGTGTTAGCAAATCCAAAGATGAAAGTAAACAAATCTGATAAGGATGCC 850
ColN-WT 806 ATTGTGAATGCCTGGAAACAGGTTAATGCTAAGGACATGGCTAATAAGAT 855
ColNmut-K 851 ATTGTGAATGCCTGGAAACAGGTTAATGCTAAGGACATGGCTAATAAGAT 900
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ColNmut-K 901 TGGTAATCTTGGCAAGGCATTTAAGGTTGCTGATTTAGCTATAAAGGTTG 950
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ColN-WT 1049 ATCTCTGGACTTGCAGTTACTGCGTTGGGGGTAATAGGAATAATGACGAT 1098
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ColNmut-K 651 ACATTATATCTAGTGTTATTCGAAGCAGCCATCATCATCATCATTGA 700

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ColN-WT        1199 TATTAGTATTAGTGTCGACGGAGTATTCCTCCCCTGGTAAATAGCTTATA   1248
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ColNmut-K       701 TATTAGTATTAGTGTCGACGGAGTATTCCTCCCCTGGTAAATAGCTTATA    750

ColN-WT        1249 TGTTCCTATAAGGAACCAAAAGTACAAATATGTGAATATATATATCCCAA   1298
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ColNmut-K       751 TGTTCCTATAAGGAACCAAAAGTACAAATATGTGAATATATATATCCCAA    800

ColN-WT        1299 TATATAGTGTTATATAGAAAAAAGACAAAAACAGATCGTTTTTTGACATT   1348
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ColNmut-K       801 TATATAGTGTTATATAGAAAAAAGACAAAAACAGATCGTTTTTTGACATT    850

ColN-WT        1349 AATACAAATGTTTTTGATGACAAGGTTAGTTCTGTGTCAGTTAGCAAGAA   1398
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ColNmut-K       851 AATACAAATGTTTTTGATGACAAGGTTAGTTCTGTGTCAGTTAGCAAGAA    900

ColN-WT        1399 ACAGTATATCATGGATATATAAAGAATAGTGAAAGGAGTTAATATCTTTA   1448
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ColNmut-K       901 ACAGTATATCATGGATATATAAAGAATAGTGAAAGGAGTTAATATCTTTA    950

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ColN-WT        1749 GGATGGTTGAAAATTGGATCC-----TTAATTAA----------------   1777
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ColN-WT        1778 ------------   1777

ColNmut-K      1301 GAAGTTGTGTCC   1312
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