ผลเหนี่ยวนำอะพ็อพโทซิสของสารสกัดเปปไทด์/โปรตีนจากเห็ดขอนขาวใน
เซลล์มะเร็งปอดชนิดเอช460 ของมนุษย์

นางสาวอริสรา ประทีป

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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APOPTOSIS INDUCING EFFECT OF PEPTIDE/PROTEIN EXTRACTS FROM
LENTINUS SQUARROSULUS MONT. IN HUMAN H460 LUNG CANCER CELLS

Miss Arisara Prateep

A Thesis Submitted in Partial Fulfillment of the Requirements

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Department of Biochemistry and Microbiology

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Thesis Title	APOPTOSIS INDUCING EFFECT OF PEPTIDE/PROTEIN EXTRACTS FROM *LENTINUS SQUARROSULUS* MONT. IN HUMAN H460 LUNG CANCER CELLS
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อริสรา ประทีป : ผลเหนี่ยวนำอะพ็อพโทซิสของสารสกัดเปปไทด์/โปรตีนจากเห็ดขอนขาวใน
เซลล์มะเร็งปอดชนิดเอช460 ของมนุษย์ (APOPTOSIS INDUCING EFFECT OF
PEPTIDE/PROTEIN EXTRACTS FROM   LENTINUS SQUARROSULUS MONT. IN HUMAN
H460 LUNG CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ภก. ดร. ฉัตรชัย เชาว์ธรรม, อ.
ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภญ. ดร. มณีวรรณ สุขสมทิพย์, 87 หน้า.

เพื่อค้นหาสารใหม่ที่มีประสิทธิภาพและความปลอดภัยสำหรับรักษาโรคมะเร็งปอด การศึกษานี้ได้
แสดงให้เห็นถึงฤทธิ์ต้านมะเร็งและกลไกการทำงานของโปรตีนที่แยกได้จากเห็ดขอนขาว (*Lentinus
squarrosulus* Mont.) ซึ่งใช้บริโภคในประเทศไทย โดยการตกตะกอนโปรตีนจากส่วนของดอกเห็ดขอนขาว
โดยใช้แอมโมเนียมซัลเฟตความเข้มข้นสุดท้าย 40-80% (น้ำหนัก/ปริมาตร) แล้วนำไปทำให้บริสุทธิ์โดย
ไอออนเอ็กซ์เชนจ์โครมาโทกราฟีบนตัวกลางไดเมทิลอะมิโนเอทิล-เซลลูโลส, และเจลฟิลเตชันโครมาโทกราฟี
บนตัวกลางเซ็ฟาเด็กจี-50 หรือการกรองผ่านเมมเบรน ตามลำดับ โปรตีนที่ไม่จับกับไอออนบนตัวกลาง
(แฟรกชัน 1) และโปรตีนที่จับกับไอออนบนตัวกลางซึ่งถูกชะด้วยเกลือโซเดียมคลอไรด์ความเข้มข้น
0.3   โมลาร์ (แฟรกชัน 4) จากการใช้ไอออนเอ็กซ์เชนจ์โครมาโทกราฟีมีความเป็นพิษสูงสุดต่อเซลล์มะเร็ง
ปอดของมนุษย์โดยมีค่าความเข้มข้นในการยับยั้งการอยู่รอดของเซลล์ร้อยละ 50 (IC_{50}) ประมาณ 22.68 ±
1.85 ไมโครกรัมต่อมิลลิลิตร และ 21.15 ± 6.14 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ความพยายามที่จะทำให้
ได้โปรตีนเพียงชนิดเดียวไม่ประสบความสำเร็จ ดังนั้นโปรตีนจากแฟรกชัน F1 และ F4 ถูกนำมาใช้ใน
การศึกษาต่อไป โปรตีนจากแฟรกชัน 4 เหนี่ยวนำให้เกิดการตายแบบอะพ็อพโทซิสของเซลล์โดยทำให้เกิด
การลดลงของโปรตีนต่อต้านการตายแบบอะพ็อพโทซิสชนิด Bcl-2 และ Mcl-1 ซึ่งเป็นโปรตีนที่ควบคุมเยื่อ
หุ้มไมโทคอนเดรียรวมถึงการลดลงของโปรตีน c-FLIP ที่เป็นตัวยับยั้งวิถีของตัวรับสัญญาณการตาย ในขณะ
ที่การเหนี่ยวนำให้เกิดการตายแบบอะพ็อพโทซิสและการเปลี่ยนแปลงของโปรตีนที่ควบคุมการตาย
แบบ   อะพอพโทซิสไม่ปรากฏชัดเจนในเซลล์มะเร็งปอดที่ถูกเพาะเลี้ยงร่วมกับโปรตีนแฟรกชัน 1 แม้ว่า
โปรตีนจาก แฟรกชัน 1 และ 4 ความเข้มข้น 25 ไมโครกรัมต่อมิลลิลิตร จะก่อให้เกิดภาวะเครียดที่เกิดจาก
ออกซิเดชันในเซลล์มะเร็งปอดของมนุษย์ แต่ฤทธิ์ต้านมะเร็งไม่ขึ้นกับอนุพันธ์ออกซิเจนที่ว่องไว โดยสรุป
การศึกษานี้นำเสนอข้อมูลใหม่เกี่ยวกับฤทธิ์ต้านมะเร็งและกลไกของโปรตีนที่แยกได้จากส่วนของดอกเห็ด
ขอนขาวในเซลล์มะเร็งปอด ซึ่งอาจเป็นประโยชน์ต่อการพัฒนาเป็นสารต้านมะเร็งต่อไป

ภาควิชา ชีวเคมีและจุลชีววิทยา	ลายมือชื่อนิสิต	....
สาขาวิชา ชีวเวชเคมี	ลายมือชื่อ อ.ที่ปรึกษาหลัก	....
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ARISARA PRATEEP: APOPTOSIS INDUCING EFFECT OF PEPTIDE/PROTEIN EXTRACTS FROM *LENTINUS SQUARROSULUS* MONT. IN HUMAN H460 LUNG CANCER CELLS. ADVISOR: CHATCHAI CHAOTHAM, Ph.D., CO-ADVISOR: ASSOC. PROF. MANEEWAN SUKSOMTIP, Ph.D., 87 pp.

In searching for the novel compounds with improving efficacy and safety profile for the treatment of lung cancer, anti-cancer activity and underlying mechanism of proteins extracted from Thai edible mushroom, *Lentinus squarrosulus* Mont. were firstly presented in this study. Proteins from fruiting body of *L. squarrosulus* Mont. were isolated by precipitation with solid ammonium sulfate at 40-80% (W/V) final concentration and further purified by ion-exchange chromatography on Diethylaminoethyl (DEAE)-cellulose, and gel filtration chromatography on Sephadex G-50 or membrane filtration, respectively. The unbound protein (F1 fraction) and the bound protein eluted with 0.3 M NaCl (F4 fraction) from ion-exchange chromatography showed the highest toxicity to human lung cancer cells with 50% inhibitory concentration (IC_{50}) approximately 22.53 ± 0.22 µg/ml and 21.15 ± 6.14 µg/ml, respectively. The attempt to further purify to homogeneity did not succeed. So proteins from F1 and F4 fractions were used for further studies. The proteins from F4 fraction induced apoptosis cell death through the reduction of anti-apoptosis proteins including Bcl-2 and Mcl-1 which regulated mitochondrial membrane as well as the reduction of c-FLIP, an inhibitor of death receptor pathway. Meanwhile, the induction of apoptosis and the alteration of apoptosis-regulating proteins were not obviously shown in lung cancer cells incubated with proteins from F1 fraction. Although 25 µg/ml of protein from F1 and F4 fraction generated oxidative stress in human lung cancer cells, their anti-cancer activity was reactive oxygen species (ROS) independent. In summary, this study provided the novel information regarding anti-cancer activity and mechanism of proteins from fruiting body of *L. squarrosulus* Mont. in lung cancer cells which will be beneficial for further development as anti-cancer agent.

Department:	Biochemistry and Microbiology	Student's Signature	....
		Advisor's Signature	....
Field of Study:	Biomedicinal Chemistry	Co-Advisor's Signature	....
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จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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LIST OF ABBREVIATIONS

%	=	percentage
$^{\circ}C$	=	degree Celsius
µM	=	micromolar
ANOVA	=	analysis of variance
Apaf-1	=	apoptosis protease activating factor-1
Bad	=	BCL2-associated death promoter
Bak	=	BCL2 homologous antagonist/killer
Bax	=	BCL2-associated X
Bcl-2	=	B-cell lymphoma 2
Bcl-XL	=	B-cell lymphoma-extra large
Bid	=	BH3 interacting domain death agonist
Bim	=	BCL2-like protein 11
c-FLIP	=	Cellular-FLICE inhibitory protein
CO_2	=	carbon dioxide
$DCFH_2$-DA	=	2, 7-dichlorofluorescein diacetate
DISC	=	Death Inducing Signaling Complex
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	dimethyl sulfoxide
FADD	=	Fas-associated protein with death domain
FBS	=	fetal bovine serum
GPXs	=	glutathione peroxidase
GR	=	glutathione reductase
GSH	=	glutathione
h	=	hour, hours
H_2O_2	=	hydrogen peroxide

JNK	=	c-Jun N-terminal kinases
KCl	=	Potassium Chloride
Mcl-1	=	myeloid cell leukemia sequence 1
min	=	minute (S)
ml	=	milliliter
mM	=	millimolar
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NAC	=	N-acetylcysteine
NaCl	=	Sodium Chloride
NO$^\bullet$	=	Nitric oxide
Noxa	=	PMA-induced protein
NOS	=	nitric oxide synthase
NSCLC	=	non-small cell lung cancer
$O_2{}^{\bullet-}$	=	superoxide anion radical
$^\bullet$OH	=	hydroxyl radical
ONOO$^-$	=	peroxynitrite
PBS	=	phosphate buffer saline
PI	=	propidium iodide
PUMA	=	p53-upregulated modulator of apoptosis
ROS	=	reactive oxygen species
RPMI	=	Roswell Park Memorial Institute's medium
S.D.	=	standard deviation
SOD	=	superoxide dismutases
TNF	=	tumor necrosis factor
TRAIL	=	TNF-Related Apoptosis Inducing Ligand

CHAPTER I

INTRODUCTION

Lung cancer is a leading cause of mortality in cancer patients, worldwide [1]. The endeavor of searching for the novel active compounds with improved efficacy and safety profile has been continued for several decades [2]. Although many chemotherapeutic drugs are currently prescribed for the treatment of lung cancer, their usages are frequently limited by severe side effects as well as drug resistance [3]. Recently, many promising anti-cancer compounds have been identified from natural resource [4-6].

Apoptosis induction is the most focused activity of anti-cancer drugs nowadays [7-10]. Apoptosis is a programmed cell death that maintains the homeostasis of cellular function and cell population through eliminating of unwanted, harmful, and damaged cells [11]. The disruption of apoptosis cascade leads to aberrant of cell population, tumor initiation, and eventually cancer pathology [12]. Therefore, apoptosis is considered as a key mechanism for inhibition as well as elimination of cancer [13]. Apoptosis is mechanistically approached through two major pathways, intrinsic and extrinsic machinery. The intrinsic or mitochondrial pathway causes the alteration in balance of Bcl-2 family proteins such as Bcl-2 (B-cell lymphoma 2), Mcl-1 (Myeloid Cell Leukemia 1), Bcl-xL (B-cell lymphoma-extra large) and Bax (BCL2 Associated X Protein) [14-16]. The shift in cellular Bcl-2 family proteins toward an increase of pro-apoptotic proteins and/or a decrease of anti-apoptotic resulted in the instability of mitochondrial membrane causing the release of cytochrome c to cytoplasm [17, 18]. The cytosol cytochrome c then stimulates caspase activation cascade [19, 20]. The interaction between ligand and death receptor on cell membrane stimulates the extrinsic apoptosis pathway through caspase-8 activation [21]. Failure to execute apoptosis frequently involves with the high cellular level of c-FLIP (FLICE-like inhibitory protein),

a potent inhibitor for caspases-8 [22, 23]. Modulation on apoptosis-regulated proteins in both intrinsic and extrinsic machinery have been reported to exert anti-cancer activity of several natural product-derived compounds [24, 25]. Recent study has shown an important role of reactive oxygen species (ROS) in cancer development [26]. The evidences indicate that ROS regulate the expression of Bcl-2 family proteins. The accumulation of ROS causes down-regulation of Mcl-1 and Bcl-2 in lung cancer cells [27, 28]. Moreover, ROS mediates extrinsic pathway of apoptosis through the suppression of c-FLIP level following with increase of apoptotic cell death [29-31].

As a unique natural source of diverse bioactive compounds, mushrooms are potential for novel anti-cancer drug discovery. Apoptosis induction has been demonstrated in cancer cells cultured with peptides isolated from various edible mushrooms [32, 33]. Interestingly, these anti-cancer peptides also possess anti-microbial activity [34, 35]. The biological activity of peptide extracted from *Lentinus squarrosulus* Mont., Thai edible mushroom has recently gained an attention. Peptides purified by ion-exchange and size-exclusion chromatography from *L. squarrosulus* demonstrates anti-fungal activity [36]. However, there is no study about anti-cancer activity of peptides from *L. squarrosulus*. This study aims to evaluate anti-cancer activity and underlying mechanism of peptide extracts from Thai edible mushroom, *L. squarrosulus* in human lung cancer cells.

Research Questions

1. Does peptide/protein extracts from *Lentinus squarrosulus* Mont. induce apoptosis in human lung cancer cells?

2. What are the possible mechanisms of peptide/protein extracts from *Lentinus squarrosulus* Mont. in apoptosis induction in human lung cancer cells?

3. Does apoptosis inducing effect of peptide/protein extracts from *Lentinus squarrosulus* Mont. depend on the alteration of intracellular ROS level?

Objectives

1. To evaluate the effect of peptide/protein extracts from *Lentinus squarrosulus* Mont. in apoptosis induction in human non-small cell lung cancer cells.

2. To investigate the underlying mechanisms of peptide/protein extracts from *Lentinus squarrosulus* Mont. in induction of apoptosis in human non-small cell lung cancer cells.

3. To evaluate the effect of peptide/protein extracts from *Lentinus squarrosulus* Mont. on the level of intracellular ROS.

Hypothesis

Peptide/protein extracts from *Lentinus squarrosulus* Mont. can induce apoptosis in human non-small cell lung cancer cells through alteration of cellular ROS associating with down-regulation of anti-apoptotic proteins.

CHAPTER II

LITERATURE REVIEWS

Lung cancer

Lung cancer is a leading cause of mortality in cancer patients worldwide [1]. Although, the intensive research and development of effective treatment has been preceded for several decades, cancer statistic still demonstrates the highest death rate in lung cancer patients (Fig. 2.1) [37]. Tumor dissection is recommended for non-spreading lung cancer at the beginning stage of the disease. On the other hand, radiotherapy and chemotherapy have been administrated in the patients with advance stage presenting metastasis of cancer pathology into other vital organs [1]. Lung cancer is categorized into two major types including small and non-small cell. Small cell lung cancer possesses metastatic phenotype. Most of patients with small cell lung cancer usually are diagnosed at late stage of the disease. Despite the good response to chemotherapeutic drugs, the high rate of recurrence is still presented in small cell lung cancer [38]. According to different histopathology, non-small cell lung cancer can be categorized as adenocarcinoma, large cell carcinoma, squamous cell carcinoma and others. Approximately, 85-90% of lung cancer patients are non-small cell lung cancer in which adenocarcinoma is the mostly prevalence [39]. Diagnosis at metastasis stage and chemotherapeutic resistance are major cause of low 5-years survival rate in patients with non-small cell lung cancer [40]. Various studies report about the failure of chemotherapy treatment in advance stage of non-small cell lung cancer [41, 42].

Estimated Deaths

			Males	Females			
Lung & bronchus	85,920	27%		Lung & bronchus	72,160	26%
Prostate	26,120	8%		Breast	40,450	14%
Colon & rectum	26,020	8%		Colon & rectum	23,170	8%
Pancreas	21,450	7%		Pancreas	20,330	7%
Liver & intrahepatic bile duct	18,280	6%		Ovary	14,240	5%
Leukemia	14,130	4%		Uterine corpus	10,470	4%
Esophagus	12,720	4%		Leukemia	10,270	4%
Urinary bladder	11,820	4%		Liver & intrahepatic bile duct	8,890	3%
Non-Hodgkin lymphoma	11,520	4%		Non-Hodgkin lymphoma	8,630	3%
Brain & other nervous system	9,440	3%		Brain & other nervous system	6,610	2%
All Sites	**314,290**	**100%**		**All Sites**	**281,400**	**100%**

Figure 2.1 Estimated death rate from distinct cancer types in 2016 [37]

Low achievement of lung cancer treatment is indicated with 30-70% of patients with recurrence stage of the disease [43]. Several studies revealed the less susceptibility to current chemotherapeutic drugs in lung cancer cells isolated from patients [44-46]. The up-regulation of anti-apoptotic proteins such as Bcl-2, Mcl-1 and Bcl-XL plays a critical role in manipulating drug resistance in lung cancer [47]. The study reveals the correlation between c-FLIP, an inhibitor of apoptosis cell death and progression of cancer pathology in patient with non-small cell lung cancer [48]. Recently, c-FLIP become one of potential targeted molecules for apoptosis induction in non-small cell lung cancer [49, 50]. Not only low susceptibility rate but also serious side-effects such as nephrotoxicity and cardiotoxicity that limit the administration of the current chemotherapeutic drugs [51, 52].

<u>Apoptosis</u>

Mode of cell death mainly comprises apoptosis, necrosis and autophagy. These three types of cell death are differentiated via specific alteration of morphology and molecular biology as well as the effect to neighboring cells [53]. Induction of inflammation is presented in the area of necrotic cell death. The inflammatory

cytokine and immune cells that are provoke to get rid of debris from necrosis cell can cause the damage to surrounding cells [54]. On the other hand, apoptosis and autophagy are programed cell death which is less damage to other cells. Autophagy comprises formation of autophagosome, a double membrane vesicle enclosing injured cellular organelles or foreign substance. Starvation and exposure to toxic environment can initiate autophagosome that may resulted in recovery of cell survival or irreversible cell death [55]. Due to non-toxic to surrounding cell and extensive investigation of molecular mechanisms, apoptosis become highlighted in anti-cancer research [7-10].

Apoptosis is a programed cell death that maintains the homeostasis of cell population. It is a normal process during differentiation and immune defensive mechanism [13]. Dysregulation of apoptosis leads to many disorders such as autoimmune disease, immune deficiency syndrome, viral infections, neurodegenerative disorders and cancer [56]. Distinct morphological change including cell shrinkage, membrane blebbing and DNA fragmentation are presented in apoptotic cells [13]. Intrinsic mitochondria-mediated pathway and extrinsic death receptor–induced pathway are two major apoptotic pathways [15].

Extrinsic pathway

The extrinsic pathway of apoptosis is usually initiated at the surface of cell membrane. After specific ligand binding to death receptors including death receptor and tumour necrosis factor-related apoptosis inducing ligand receptor (TRAIL receptor) locating on cell membrane, there is the activation Fas-associated protein with death domain (FADD) and caspase 8. Then, activated caspase 8 triggers caspase cascade which degrades target proteins resulting in apoptotic cell death [8, 57]. Death receptor stimulating apoptosis is successfully suppressed by c-FLIP (Cellular-FLICE inhibitory protein). c-FLIP is an anti-apoptotic regulator that inhibits FADD-mediated caspase 8 activation (Fig. 2.2) [58]. Evidence indicates the resistance to death receptor ligands

and chemotherapeutic agents by c-FLIP in various cancer cells [59]. Moreover, overexpression of c-FLIP involves with failure to induce apoptosis by cisplatin, a recommended chemotherapy for melanoma, hepatocellular carcinoma, non-small cell lung carcinoma, endometrial, colon, and prostate cancer [60].

Not only interaction between ligand and death recepter but cellular proteins also trigger the extrinsic apoptosis mechanism. Cytoplasmic domain of intergin, an adhesion molecule activates the cleavage of caspase 8 associating with apoptotic cell death [61, 62].
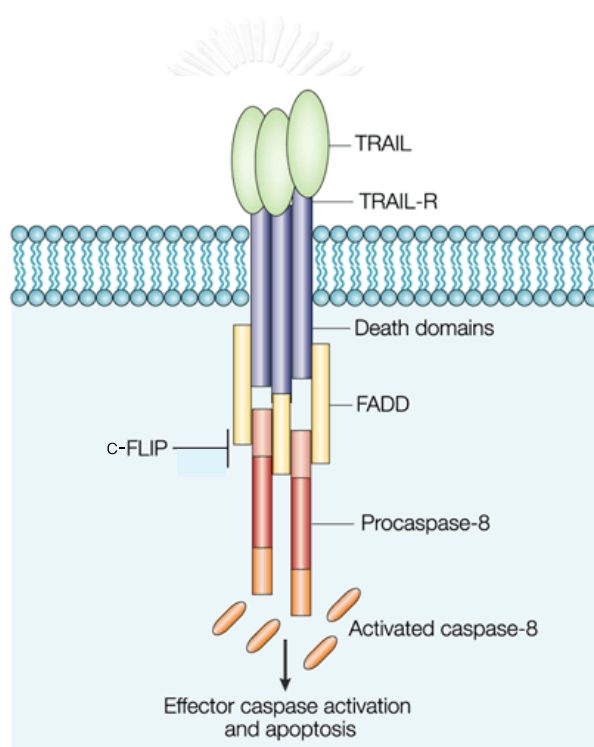
Figure 2.2 Machinery of death receptor-induced apoptosis [58]

Intrinsic pathway

The mitochondria-mediated pathway is one of the most important pathway for apoptosis induction. This intrinsic pathway is regulated by proteins of Bcl-2 family [8, 57]. Pro-apoptotic protein, Bax (BCL2 Associated X Protein) and Bak (Bcl-2

antagonist/killer) translocate to mitochondrial membrane for initiation of apoptosis. Bax and Bak can oligomerize to make pores on mitochondrial membrane leading to release of cytochrome c into cytoplasm. The interaction between cytochrome c and Apaf-1 (Apoptotic protease activating factor 1) causes a formation of apoptosome which activate caspase-9 following with stimulation of executing caspases-3, -6, and -7. These processes are inhibited by anti- apoptotic Blc-2 family proteins including Bcl-2 (B-cell lymphoma 2), Bcl-XL (B-cell lymphoma-extra large) and Mcl-1 (Myeloid Cell Leukemia 1) (Fig.2.3). These anti-apoptosis proteins inactivate Bax and Bak and prevent pore formation on mitochondrial membrane through binding with BH3-only proteins including Bid, Bad, Bim, Puma and Noxa. Chemotherapeutic drugs can trigger intrinsic apoptosis pathway through different mechanisms. Cisplatin and doxorubicin also cause DNA damage and generation of reactive oxygen species (ROS) associating with apoptosis cell in both cancer and normal cells [63-65]. Damaged and un-repaired DNA strain activates p53 which mediates transcription of pro-apoptosis proteins and oligomerization of Bax on mithochondrial membrane [66]. Meanwhile, oxidative stress increase the degradation of anti-apoptosis protein consequence with induction of caspase cascade [67]. The evidence shows that overexpression of anti-apoptotic Bcl-2 or Bcl-xL probably occurs in more than 50% of all cancers [68]. Moreover, Bcl-2 sufficiently ceases apoptosis generated by current chemotherapy such as paclitaxel [69].
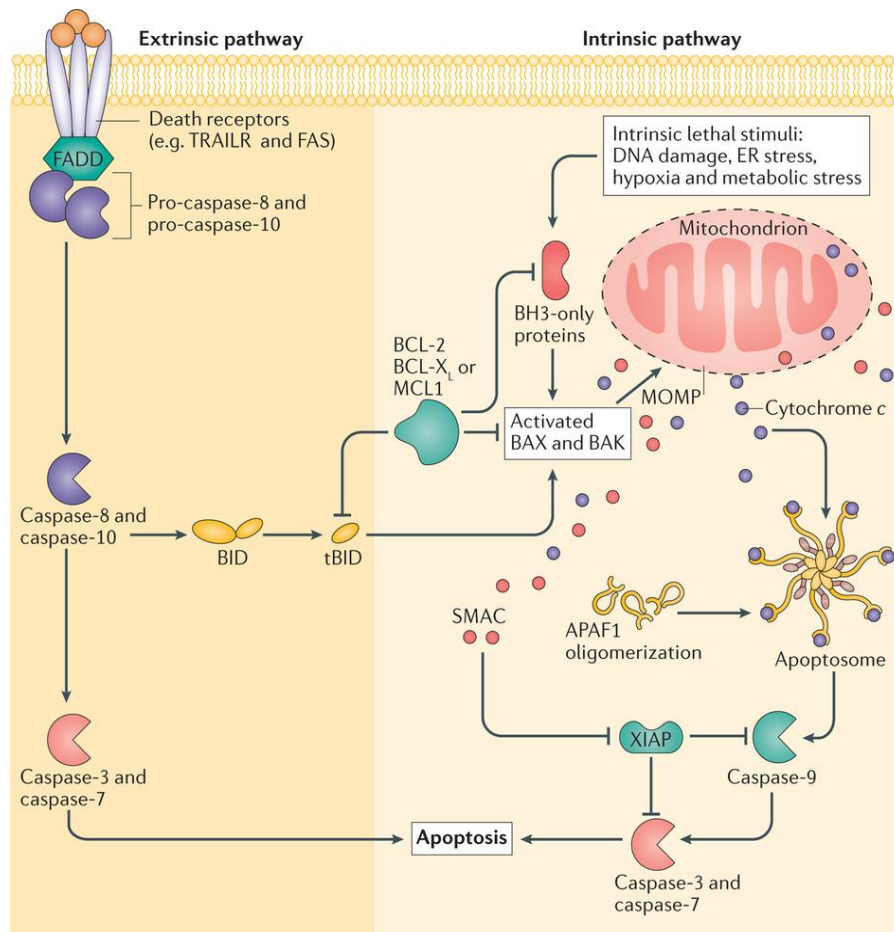
Figure 2.3 The relation between intrinsic and extrinsic signaling of apoptotic pathway [70]

Reactive oxygen species (ROS)

ROS are active chemical substance or free radical derived from oxygen molecule such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$). These oxygen molecules normally contain one or two unpaired electrons that are ready for activation of chemical reaction [71]. Cellular ROS are endogenously generated in mitochondrial membrane during oxidative phosphorylation of aerobic respiration [72]. Not only mitochondria, but also endoplasmic reticulum and NADPH oxidase complex in cytoplasm involve in cellular ROS production [73]. The initial ROS generated by both mitochondrial and cytoplasmic system is $O_2^{\bullet-}$ which is converted to

H_2O_2 by superoxide dismutases (SOD) enzyme. Then, H_2O_2 rapidly change to $^\bullet OH$, the most dangerous ROS [74]. Nitric oxide ($NO^\bullet$) is a reactive radical generated from arginine amino acid by nitric oxide synthase (NOS). Nitric oxide has a short half-life and can react with superoxide to produce peroxynitrite ($ONOO^-$). ROS play important role in regulating of various transcription factors, mediating cellular signaling and activating/deactivating protein function [75]. Cellular homeostasis is normally maintained by a balance between generation and elimination of intracellular ROS. This balance is managed by ROS-scavenging enzymes such as SOD, catalase, glutathione peroxidase (GPXs), glutathione reductase (GR) and ROS scavenging molecules including glutathione (GSH) [76].

Role of ROS in apoptosis

The biological function of ROS in cancer development and progression has been well established. ROS have both beneficial and harmful effects to cancer cells. High level of ROS triggers DNA damage, protein degradation and eventually cell death. Many chemotherapeutic agents potentiate apoptosis via the generation of ROS [77, 78]. Increase of $O_2^{\bullet-}$ and H_2O_2 down-regulate anti-apoptotic proteins in cancer cells via stimulating of degradation and inhibition of protein synthesis [79-81]. Moreover, ROS also play as a crucial signaling molecule in JNK and death receptor-mediated apoptosis [82]. The regulation of mitochondrial membrane potential corresponding with releasing of cytochrome c are involved with the increase of cellular oxidative stress [83]. However, dramatical increase of ROS, especially H_2O_2 can initiate the rupture of cell membrane resulting in necrosis cell death [84, 85]. Meanwhile, low and moderate level of ROS stimulates proliferation, survival and development of cancer cells [86]. ROS-induced mutation is one of critical machineries of various carcinogenic agents [87, 88]. It is worth mentioning that various antioxidant substances potentially prevent cancer initiation but their underlying mechanisms are still unclear [89].
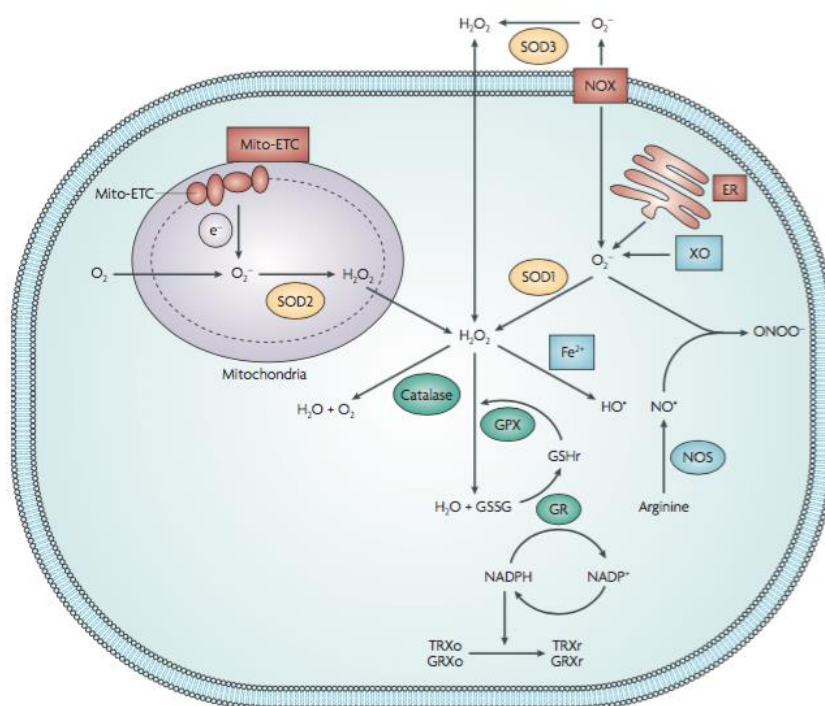
Figure 2.4 Homeostasis of cellular oxidative stress [76]

Anti-cancer peptide

Peptide is small molecule (less than 50 amino acids) associated with decreasing of high molecular weight protein to polypeptide formation. Polypeptide is normally degraded by peptidyl peptidases and aminopeptidases to produce small peptides [90]. Recently, the biological activity of small peptide isolated from natural resources has been highlighted [91]. One of many advantages of these biological peptides is potentially safe for human usage. Thus, there is an attempt to isolate and develop anti-cancer peptide with low toxicity to normal cells from natural substance including animals, plants and mushrooms [92, 93]. Interestingly, most of antimicrobial peptides possess anti-cancer activity in various tumor cells [34, 35].

The selective toxic effect of peptide on cancer cells may be due to different in composition of cell membrane [94]. Net negative charge on cell membrane of

cancer cells results from O-glycosylated mucins, sialylated gangliosides, heparin sulfate and phosphatidylserine (PS). Normally, phosphatidylserine is phospholipid locating at inner cell membrane but there is a lot of phophatidylserine presented on outer cell membrane of cancer cells [95]. Moreover, the structure of cancer cell membrane is less stable than normal cells. These can be targeted by peptide with anti-cancer activity to bind with receptor or charge on cancer cell membrane. Pore formation is one possible mechanism of anti-cancer peptide [96]. The small peptide (less than 1 kDa) has cytotoxicity against cancer cells by an efficient penetration and uptake into cancer cells [97]. Arginine containing in cationic peptide are necessary for cellular uptake [98]. Some studies report that amino acids of arginine, glycine, aspartic acid and asparagine can bind to integrins or cell surface molecules that express on cell membrane of cancer cells [99]. The hydrophobic property of peptide can also influence are anti-cancer activity [100]. Furthermore, peptide also induces apoptosis through ROS production [101, 102] and down-regulate anti-apoptotic proteins such as Bcl-2 and Mcl-1 [103, 104].

Antimicrobial peptides (AMPs) from Lentinus squarrosulus Mont.

As a unique natural source of diverse bioactive compounds, mushrooms are potential resource for novel anti-cancer drug discovery. Apoptosis induction has been demonstrated in cancer cells cultured with peptides isolated from various edible mushrooms [32, 33]. *Lentinus squarrosulus* Mont. (Fig. 2.5) is an edible mushroom from northern region of Thailand. *Lentinus* species are found on fallen tree trunks and decaying timber. They are cultivated and consumed in all region of Thailand [105]. Beside carbohydrate, protein, vitamin and mineral, *L. squarrosulus* also contain various bioactive compounds including phenolic compound, immunostimulatory glucans and lectins [106-108]. Peptide in the family of AMPs was isolated from *L. squarrosulus* and the activity against various pathogenic fungal was demonstrated [36]. However, the

anti-cancer activity of peptide extracted from *L. squarrosulus* has not been investigated.

Figure 2.5 *Lentinus squarrosulus* Mont.
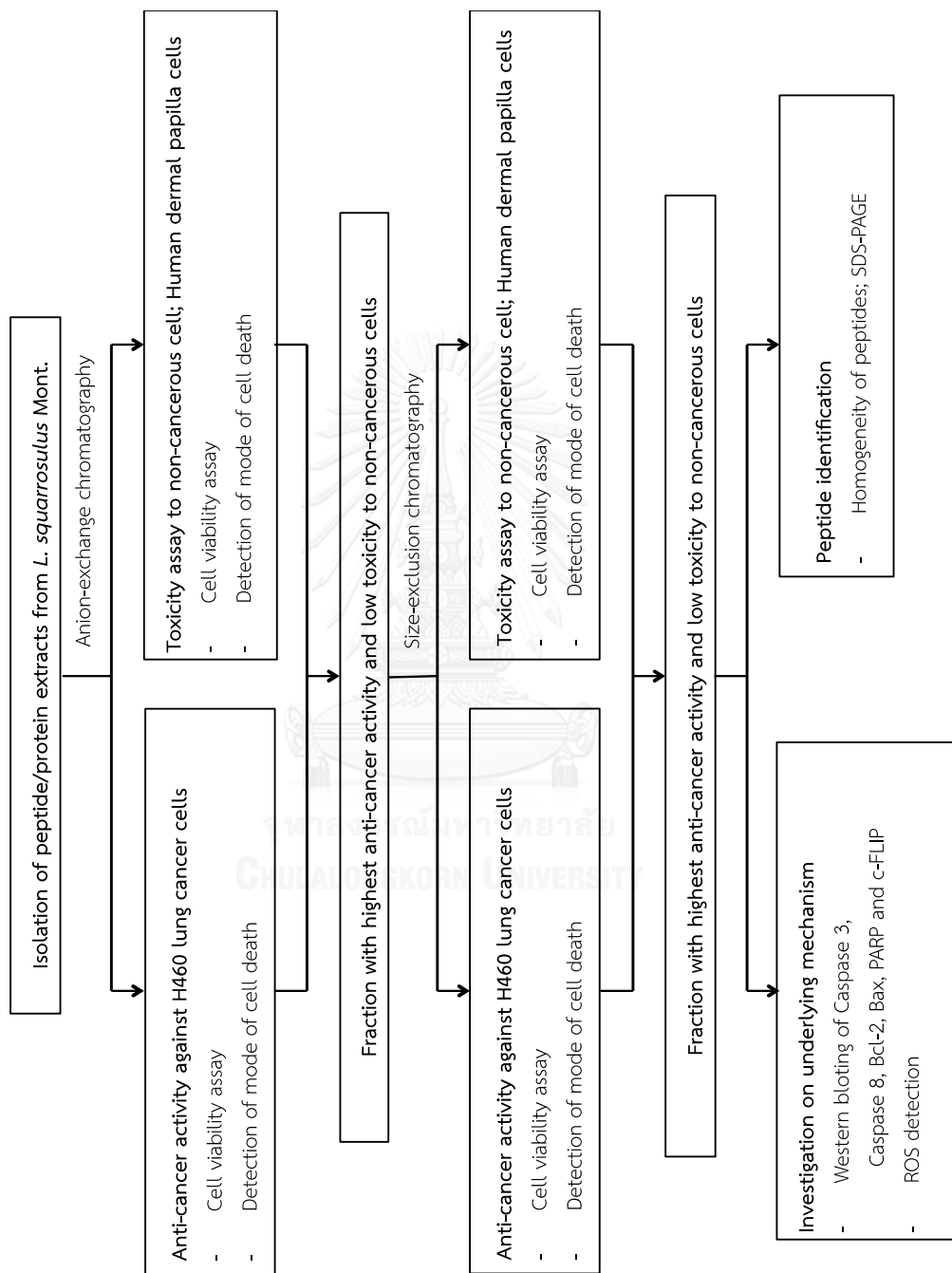
Figure 2.6 Experimental design

The flowchart shows the following sequence:

Isolation of peptide/protein extracts from *L. squarrosulus* Mont.
- Anion-exchange chromatography

Anti-cancer activity against H460 lung cancer cells
- Cell viability assay
- Detection of mode of cell death

Toxicity assay to non-cancerous cell; Human dermal papilla cells
- Cell viability assay
- Detection of mode of cell death

Fraction with highest anti-cancer activity and low toxicity to non-cancerous cells
- Size-exclusion chromatography

Anti-cancer activity against H460 lung cancer cells
- Cell viability assay
- Detection of mode of cell death

Toxicity assay to non-cancerous cell; Human dermal papilla cells
- Cell viability assay
- Detection of mode of cell death

Fraction with highest anti-cancer activity and low toxicity to non-cancerous cells

Investigation on underlying mechanism
- Western bloting of Caspase 3, Caspase 8, Bcl-2, Bax, PARP and c-FLIP
- ROS detection

Peptide identification
- Homogeneity of peptides; SDS-PAGE

Figure 2.7 Conceptual framework

CHAPTER III
METERIALS AND METHODS

Materials

1. Cell culture

All cell lines were cultured in the optimum incubator supplied with 5% CO_2 at 37°C. Human lung cancer H460 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in RPMI (Roswell Park Memorial Institute's) 1640 medium. Meanwhile, human dermal papilla cells (Applied Biological Materials Inc., Richmond, BC, Canada) were maintained in DMEM (Dulbecco's Modified Eagle Medium) medium. The mediums were supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 100 units/ml of penicillin/streptomycin (Gibco, Gaithersburg, MA, USA).

2. Chemicals and Reagents

Ammonium sulfate $(NH_4)_2SO_4$ and potassium chloride (KCl) were obtained from Merck (Darmstadt, Germany). Sodium chloride (NaCl) derived from Univar (Ajax Finechem, Australia). Hoechst33342, propidium iodide (PI), trypsin, dimethysulfoxide (DMSO), ethyl alcohol, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetylcysteine (NAC), glutathione (GSH) and 2,7-dichlorouorescein diacetate (DCFH2-DA) were purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibodies for Bcl-2, Mcl-1, Bax, caspase-3, caspase-8, PARP, FLIP, β-actin and peroxidase-labeled secondary antibodies were obtained from Cell Signaling Technology, Inc. (Denver, MA, USA). Immobilon Western chemiluminescent HRP substrate were purchased from Millipore, Corp (Billerica, MA, USA)

Methods

1. Preparation of protein extracts from _Lentinus squarrosulus_ Mont.

1.1) Isolation of crude proteins

Lentinus squarrosulus Mont. mushrooms were bought from Klong Toey market. Mushroom identification was performed by National Center for Genetic Engineering and Biotechnology (BIOTEC). The nucleotide sequence analyses of mushroom sample (Figure 3.1) were comparable 99% with nucleotide sequences of _Lentinus squarrosulus_ Mont. in databases on Genbank, CBS.

ลำดับนิวคลีโอไทด์			
Nucleotide sequence(s)			
ลำดับที่	รหัสตัวอย่าง	บริเวณของลำดับนิวคลีโอไทด์	ลำดับนิวคลีโอไทด์ (5' -> 3')
No.	Sample No.	Nucleotide region of	Nucleotide sequence (5' -> 3')
1	PT1	ITS rDNA	TAGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGTTTCAGGAGCTT CGAAAGCGAGAAAGGGGCCTTCCCGGGCTTTTTTCTTGCCTAGTTGTTACTGGGCCTACGTTTCACTACAAACACTTA TAAAGTATCAGAATGTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCG ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTG CGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTAACGAGTTCTTAACGGGACTTG CTTTAGGCTTGGACTTGGAGGTTCTTGTCGGCTTGCTTCAATGTCAAGTCGGCTCCTCTTAAATGCATTAGCTTGGTT CCTGTGCGGATCGGCTCACGGTGTGATAATTGTCTACGCCGCGACCGTTGAAGCGTTTTTATAGGCCAGCTTCTAGTC GTCTCTTTACGAGACAATAATCATCGAACTCTGACC

Figure 3.1 Nucleotide sequence of mushroom sample

Crude peptide extracts from _L. squarrosulus_ were prepared and kept at -80°C as previously described [36]. Briefly, fresh fruiting bodies of edible mushroom, _L. squarrosulus_ were homogenized in deionized sterile water (3 ml/g). The clear supernatant was collected after centrifugation at 12,000×g at 4°C for 30 min. Solid ammonium sulfate was slowly added to reach 40-80% saturation. After 1 h at 4°C, the protein pellet was collected by centrifugation at 12,000×g, 4°C for 30 min. The pellets were re-solubilized in 50 ml of phosphate buffer (pH 7.4) containing 8.06 mM KH_2PO_4 and $Na_2HPO_4 \cdot 2H_2O$ then dialyzed overnight with phosphate buffer (pH 7.4) at 4°C to eliminate ammonium sulfate. Freeze-drying method was used to obtain the concentrated crude protein.

1.2) Protein purification

1.2.1) Ion exchange chromatography

For further purification, ion exchange chromatography on diethylaminoethyl (DEAE)-cellulose (Sigma Chemical, St. Louis, MO, USA) column (5 × 30 cm) pre-equilibrated with phosphate buffer (pH 7.4) was used. The bound proteins were eluted with step-wise salt concentration gradient (0, 0.1, 0.2, 0.3, 0.4, 0.5 M NaCl) in phosphate buffer (pH 7.4) at a flow rate of 0.2 ml/min. The eluted fractions with the UV absorbance at 280 nm were pooled and concentrated using freeze-drying method. The concentrated proteins were further determined for homogeneity, cytotoxicity and mode of cell death.

1.2.2) Size-exclusion chromatography

Further purification of these pooled fractions were carried out through size-exclusion chromatography on Sephadex G-50 (Amersham Bioscience, Piscataway, NJ, USA) column (5 × 30 cm) pre-equilibrated with PBS (pH 7.4). The proteins were eluted with PBS (pH 7.4) at flow rate of 0.2 ml/min. All steps of purification was performed at 4°C. Fractions with the UV absorbance at 280 nm was pooled and concentrated using freeze-drying method. The concentrated proteins were further determined for homogeneity, cytotoxicity and mode of cell death.

1.2.3) Membrane filtration

Membrane filtration was optionally used for further purification of pooled fraction from ion-exchange chromatography. The peptide extracts were loaded into minicolumn with filter membrane of molecular weight cut off at 30 kDa (Merck Millipore, Cork, Ireland) and spin by using fixed-angle rotor at 5,000xg at 4°C for 30 min. The filtrate was further purified via minicolumn with filter membrane of molecular weight cut off at 10 kDa. The different fractions containing proteins of larger than 30 kDa, between 30-10 kDa and lower than 10 kDa were concentrated for further determination of homogeneity, cytotoxicity and mode of cell death.

1.3) Determination of protein content

The concentrated purified fractions were further determined for total protein content by bicinchoninic acid (BCA) protein assay kit (Thermo scientific, Waltham, MA USA). The concentrated purified fractions were freshly dissolved in deionized sterile water, then incubated with the mixture of BCA Reagent A and B at the ratio 50:1 in dark place at 37°C for 30 min. The optical density of purple color product was evaluated via microplate reader (Anthros, Durham, NC, USA) at 562 nm. The protein concentration was calculated from the calibration curve of bovine serum albumin (BSA) at 0-12 µg/µl.

1.4) Evaluation on homogeneity of purified fractions

The homogeneity of purified fractions was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The method was carried out as described by Laemmli and Favre, (1973), using a 15% (w/v) gel [109]. The gels were stained with 0.1% Coomassie brilliant blue R-250 solution and destained with methanol: acetic acid: water (30: 10: 60 %v/v).

2. Cell viability assay

After treatment with various concentrations (0-100 µg/ml) of protein extract for indicated time point, cell viability was determined by MTT assay. The cells were incubated with 0.4 mg/ml of MTT in dark place at 37°C for 4 h. Then, the supernatant was replaced with dimethyl sulfoxide (DMSO) to dissolve the formazan product. The intensity of formazan color was examined by microplate reader (Anthros, Durham, NC, USA) at 570 nm. The optical density ratio of the treated to the non-treated control cells were calculated and presented in terms of relative cell viability.

3. Detection of mode of cell death

Apoptotic and necrotic cells death was evaluated by co-staining of Hoechst33342 and propidium iodide (PI). After incubation with protein extracts (0-100

µg/ml) for indicated time, the cells were stained with 10 µM of Hoechst33342 and 5 µg/ml PI dyes for 30 min at 37 °C. The apoptotic and necrotic cells were visualized under a fluorescence microscope (Olympus IX51 with DP70) as condensed chromatin and/or fragmented nuclei and red fluorescence-positive cells, respectively.

4. Western blot analysis

After treatment with protein extracts for indicated time, the cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 45 minutes at 4°C. Then, the supernatant was collected, and the protein content was determined using the BCA protein assay kit. Equal amounts of protein from each sample were denatured by heating at 95°C for 5 min with Laemmli loading buffer and subsequently loaded onto a 12% SDS-PAGE. After separation, proteins were transferred onto 0.45 µM nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl pH 7.5, 125 mM NaCl, and 0.05% Tween 20) and incubated with appropriate primary antibodies at 4°C overnight. Then, the membranes were washed three times with TBST for 5 min and incubated with horseradish peroxidase-labeled isotype-specific secondary antibodies for 2 h at room temperature. The immune complexes were detected by enhancement with chemiluminescence substrate and quantified by analyst/PC densitometric software (Bio-Rad Laboratory, Hercules, CA, USA).

5. Cellular ROS detection

Reactive oxygen species (ROS) were determined by 2,7-dichlorouorescein diacetate (DCFH$_2$-DA) fluorescent probe (Sigma, St. Louis, MO, USA). Cells were pre-

incubated with 10 µM DCFH$_2$-DA for 15 min at 37℃ prior treatment with protein extracts for 1 h. The alteration on cellular ROS level was evaluated through the measurement of DCFH$_2$-DA fluorescence intensity using a microplate reader at the excitation and emission wavelengths of 488 and 538 nm, respectively.

6. The effect of ROS on anti-cancer activity

To determine ROS-mediated cytotoxicity, H460 cells were pretreated with ROS scavenger including N-acetyl cysteine (NAC) and glutathione (GSH) for 30 min. Then, the cells were cultured with culture media containing protein extract for 24 h. MTT assay was used to determine cell viability.

7. Statistical analysis

Mean data were averaged from at three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. A *p-value* < 0.05 will be considered as statistical significance.

CHAPTER IV

RESULTS

1. Partially-purified protein extracts isolated from *Lentinus squarrosulus* Mont.

The crude proteins of *L. squarrosulus* were obtained from crude extracts of fruiting bodies utilizing ammonium sulfate precipitated with 40-80% (w/v) saturation. The crude proteins were further purified by anion-exchange chromatography on a DEAE-cellulose column. After eluting with phosphate buffer (pH 7.4), the unbound proteins have passed through the column as fraction 1. Then, bound crude proteins were eluted with a step-wise salt concentration gradient of NaCl (0.1, 0.2, 0.3, 0.4 and 0.5 M) phosphate buffer (pH 7.4) as fraction F2 to F6, respectively at a flow rate of 0.2 ml/min. The amount of protein in each fraction was estimated by UV absorbance at 280 nm. Six different elution peaks corresponding to fraction 1 to 6 were obtained in the chromatogram (Fig. 4.1A). Only five fractions, F1 to F5 contained high protein content. The collected volume in each fraction was pooled and concentrated before further analysis. The SDS-PAGE analysis of concentrated protein extracts showed various bands of proteins at molecular mass range between 10 to 100 kDa (Fig. 4.1B). The %yield of fraction F1 to F5 were 0.36 ± 0.05, 0.33 ± 0.04, 0.12 ± 0.02, 0.19 ± 0.06, and 0.16 ± 0.05, respectively.
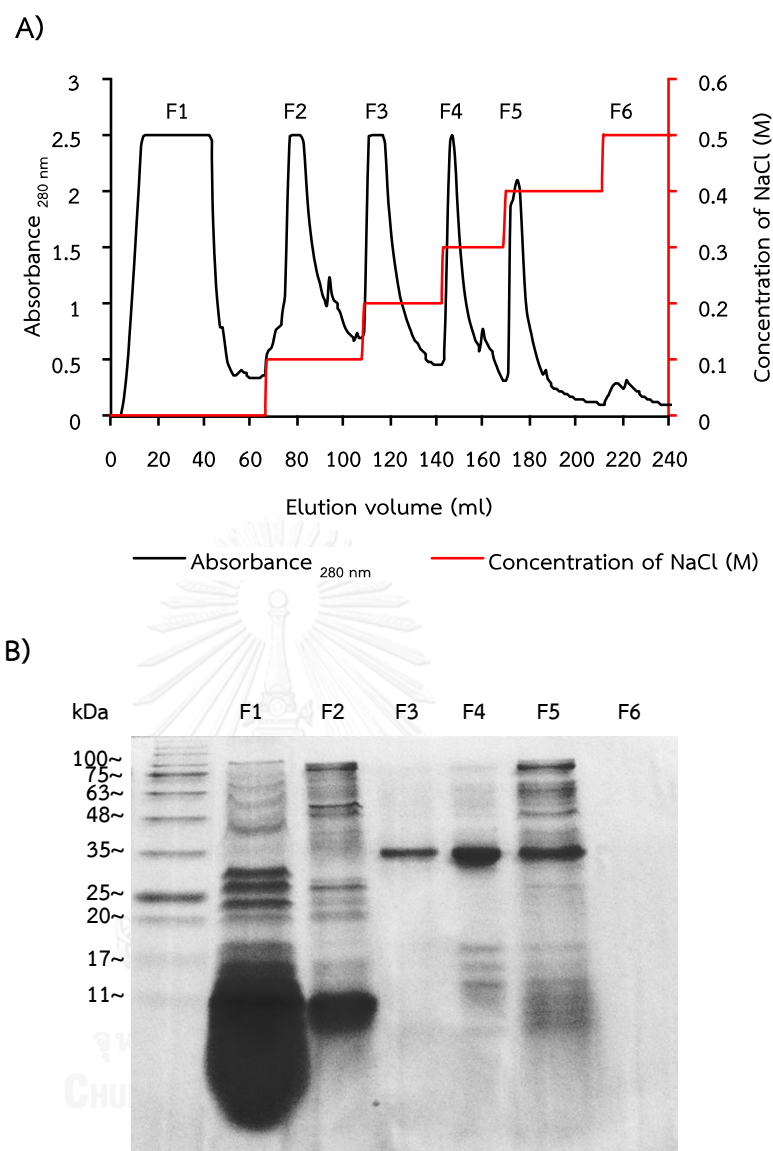
Figure 4.1 Partially-purified proteins isolated from fruiting bodies of *L. squarrosulus* Mont., **A)** Chromatogram from ion-exchange chromatography of crude proteins on a DEAE-cellulose column (5x30 cm) equilibrated with phosphate buffer (pH 7.4) at a flow rate of 0.2 ml/min. The unadsorbed protein have pass through the column and appeared as F1. The adsorbed proteins were eluted sequentially with 0.1-0.5 M NaCl in the same buffer to yield fraction F2, F3, F4, F5 and F6. **B)** Homogeneity of proteins in different fraction from ion-exchange column chromatographic purification as determined by SDS-PAGE.

2. Effect of partially-purified proteins on cell viability in human lung cancer and human dermal papilla cells

In order to select the fraction that had the highest toxicity to human lung cancer cells and low toxicity to non-cancer cells, human lung cancer H460 cells and human dermal papilla cells were cultured in suitable medium containing 25 μg/ml of protein from F1, F2, F3, F4 or F5 fraction. After 24 h of incubation, cell viability was determined by MTT assay.

The results showed the reduction of cell viability after treatment of human lung cancer H460 cells with 25 μg/ml proteins from F1 to F5 for 24 h. Nevertheless, the statistical significant difference was not observed with protein from F3 fraction. The highest toxicity to H460 cell was observed for treatment with F1 and F4 (Fig. 4.2A). The 50% reduction in viability was found in the cell treated with 25 μg/ml protein extracts of F1 and F4. Interestingly, the non-toxicity to normal human cell, human dermal papilla cells was also observed after treatment with F1 and F4 fraction (Fig. 4.2B). Thus, the fraction F1 and F4 were selected for next experiments.

Figure 4.3A and 4.4A present dose-dependent cytotoxicity (0-100 μg/ml) of the protein extracts from F1 and F4 fraction in human lung cancer H460 cells, respectively. Mode of cell death was detected by co-staining of Hoechst33342/PI. The process of apoptosis involves with chromatin condensation and DNA fragmentation. The results presented the bright blue fluorescence of condensed chromatin and fragmented DNA stained by hoechst33342 after incubation of H460 cells with F4 protein extracts at 0-50 μg/ml for 24 h. Meanwhile, there was no notification of necrosis (Fig. 4.4B). Although, F1 protein fraction significantly reduced cell viability in dose-dependent manner (Fig 4.3A), co-staining with Hoechst33342/PI did not obviously show apoptosis induction in H460 cells (Fig. 4.3B).

Dose response effect of proteins from fraction F1 and F4 in non-cancer cells was also investigated. Figure 4.5A and 4.6A indicate small reduction of cell viability

only when incubation of human dermal papilla cells with 50-100 μg/ml of F1 and F4 fraction. There were no significant increase of apoptosis or necrosis cell death in human dermal papilla cells treated with 0-100 μg/ml of proteins from fraction F1 (Fig. 4.5b) and F4 (Fig. 4.6B). Dose-dependent effect of other fraction on human lung cancer H460 cells and human dermal papilla cells was shown in appendix (Fig. 6.3 and 6.4, respectively).

A)

B)
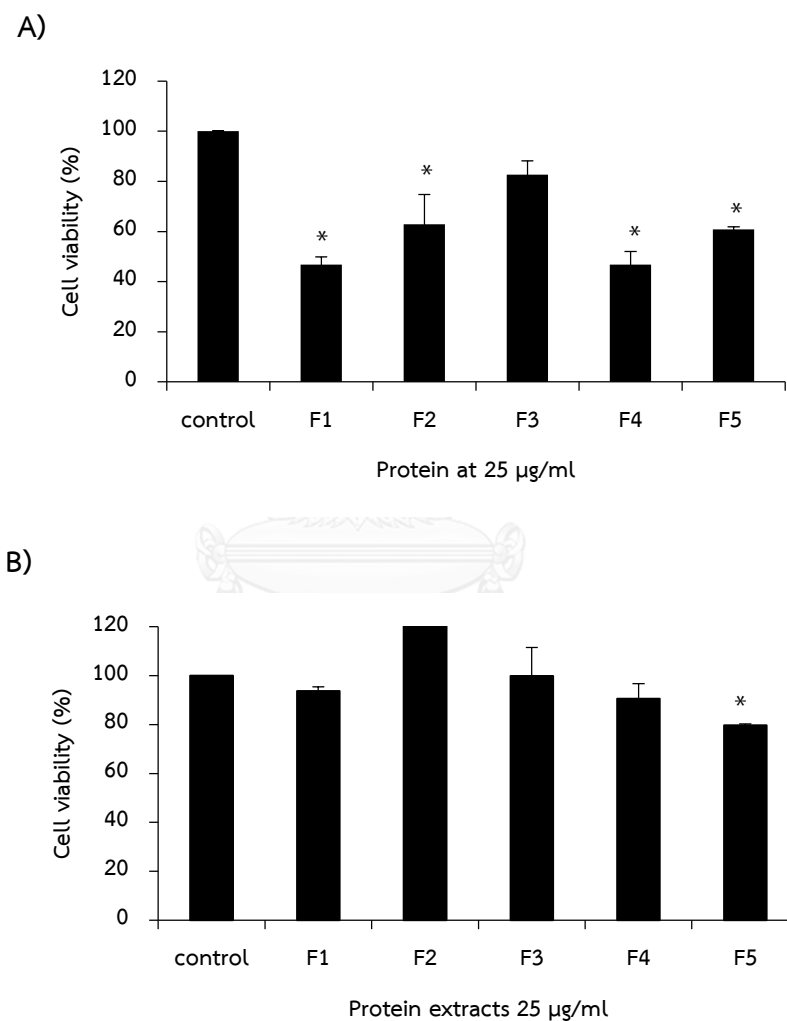
Figure 4.2 Effect of partially-purified protein extracts from *L. squarrosulus* Mont. fruiting bodies on cell viability. **A)** human lung cancer H460 cells and **B)** human dermal papilla cells after incubation with 25 μg/ml of protein extracts from F1 to F5 for 24 h. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

A)

B)
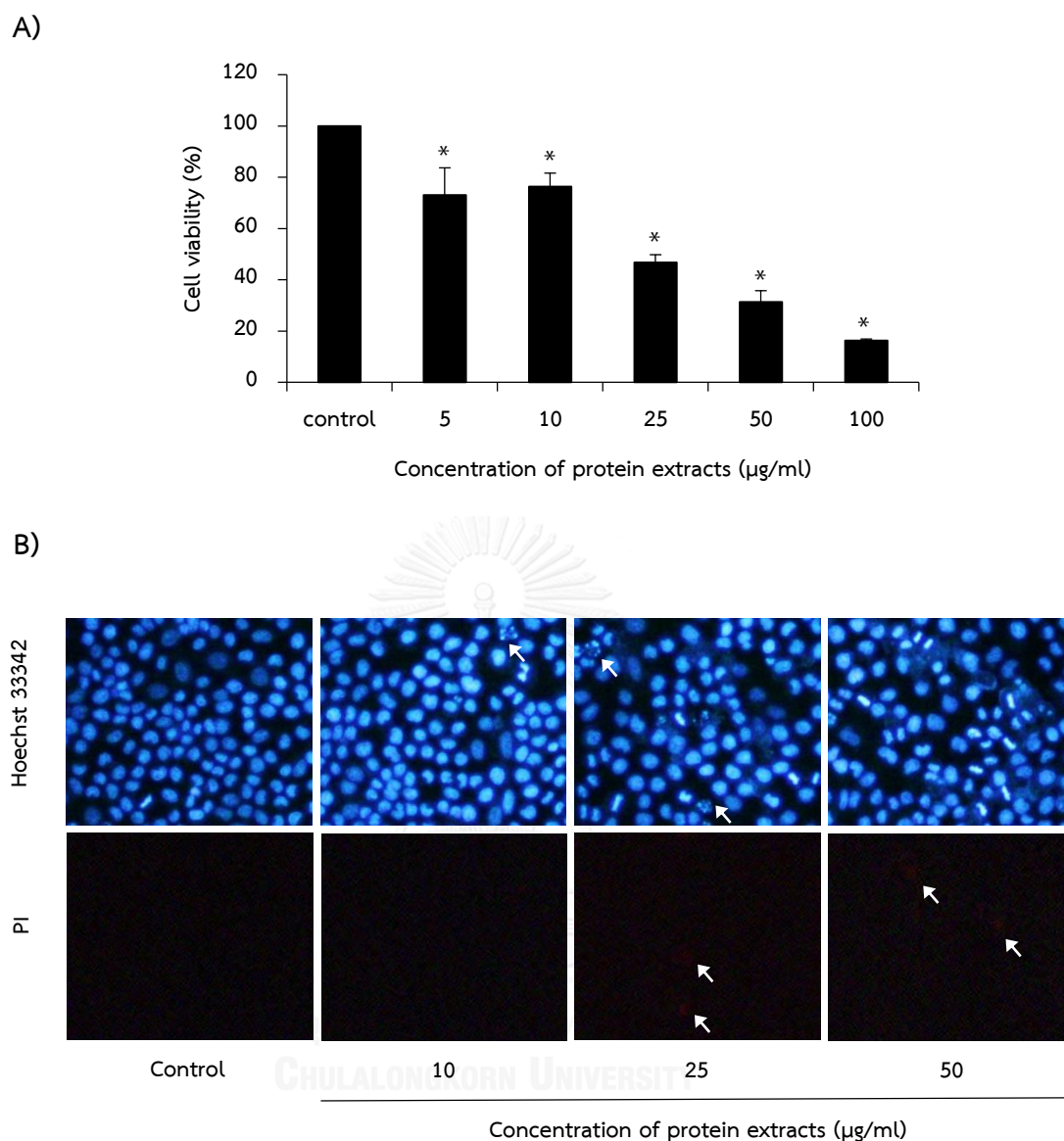
Figure 4.3 Dose dependent effect of partially-purified protein from fraction F1 in human lung cancer cells. **A)** Cell viability of H460 lung cancer cells was evaluated after treatment with 0-100 µg/ml of protein from fraction F1 for 24 h. **B)** There was no obvious apoptotic morphology in H460 cells treated with 10-50 µg/ml of protein from fraction F1 for 24 h. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

A)
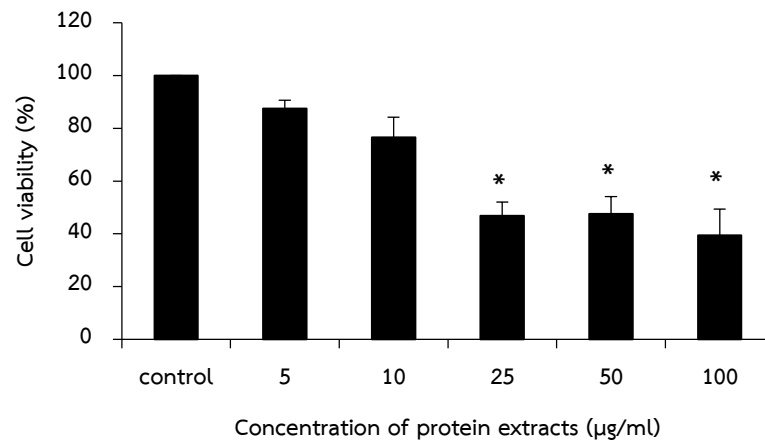
B)
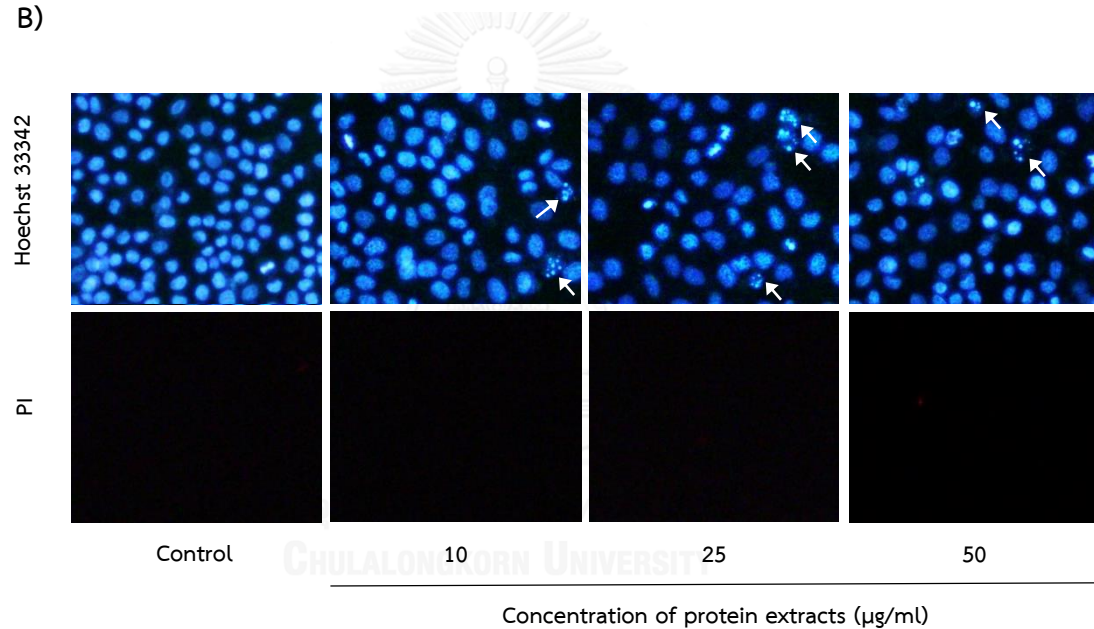
Figure 4.4 Apoptosis induction of partially-purified protein from fraction F4 (eluted with 0.3 M NaCl) of ion-exchange chromatographic purification of crude protein from *L. squarrosulus*. **A)** The reduction of 50% cell viability was notified in H460 cells incubated with 25-50 µg/ml of proteins from fraction F4. **B)** Additionally, there was a significant increase of apoptotic cell death in the incubation of protein extracts at 10-50 µg/ml for 24 h. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

A)
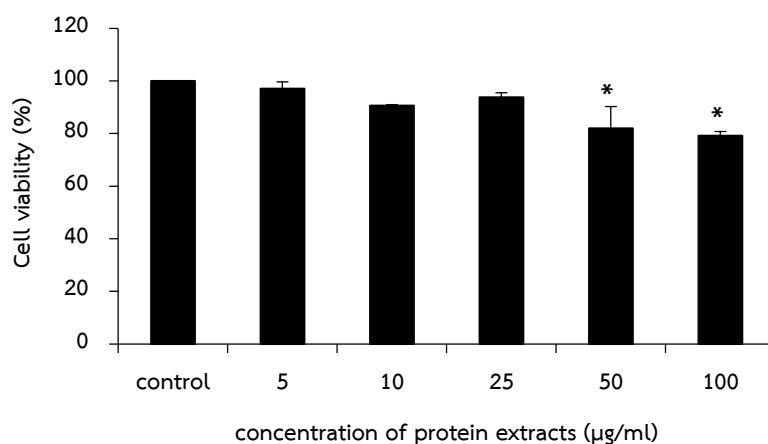
B)
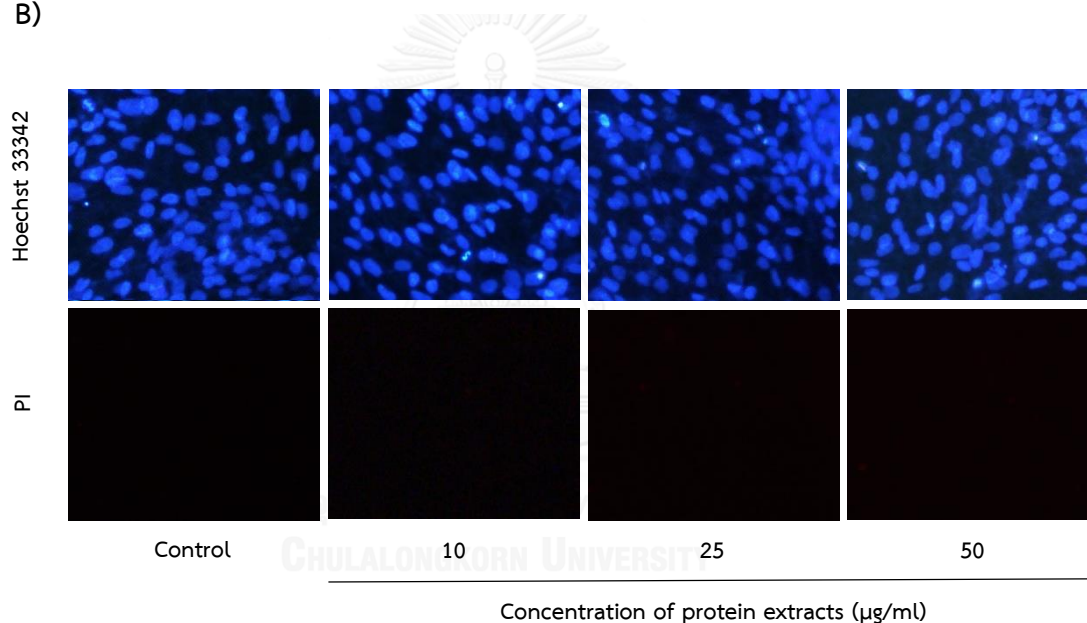
Figure 4.5 Effect of protein from fraction F1 on cell viability of human dermal papilla cells. **A)** The reduction of 20% cell viability was only found in treatment of human dermal papilla cells with high concentrations (50-100 µg/ml) of F1 for 24 h. **B)** There was no apoptosis and necrosis detected by co-staining of Hoechst3342/PI in human dermal papilla incubated with 10-50 µg/ml of protein from fraction F1 for 24 h. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

A)
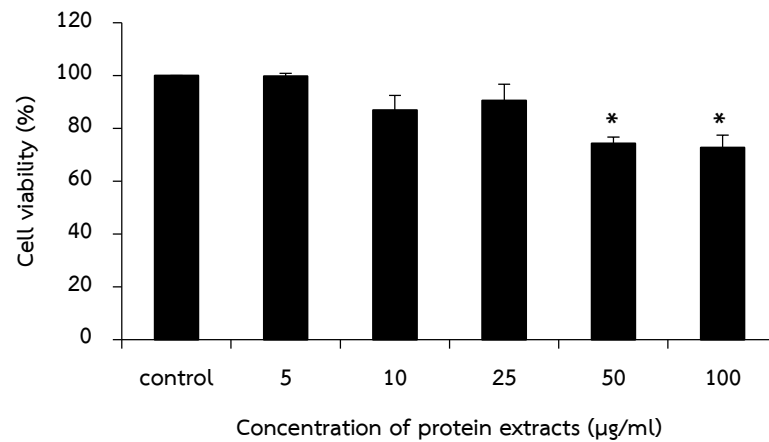
B)
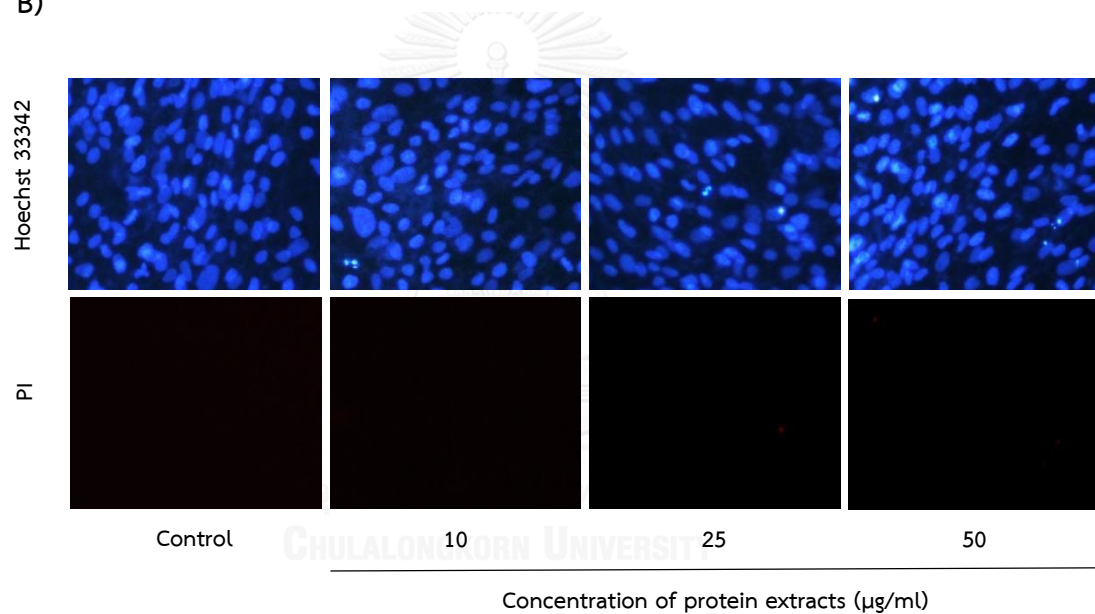
Figure 4.6 Low toxicity of protein from fraction F4 in human dermal papilla cells. **A)** MTT assay presented small reduction of %cell viability in the cell incubated with 50-100 µg/ml of F4 proteins for 24 h. **B)** After 24 h of treatment with protein from fraction F4 (10-50 µg/ml), apoptosis and necrosis cell death were not detected in human dermal papilla cells. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

3. Partially-purified protein extracts obtained from gel filtration and centrifugal filtration

Partially-purified protein fractions obtained from ion-exchange chromatography were further purified by gel filtration on a Sephadex G-50 column. Figure 4.7A shows chromatogram of size-exclusion column chromatographic purification of F1 on Sephadex G-50 column. The elution of 3 ml was collected in each separated tube. There are high concentration of protein in collection tube no. 13-21 as estimated by UV absorption at 280 nm. SDS-PAGE analysis showed various protein bands between 10 to 100 kDa in the colleting tube no. 13 to 16 (Fig. 4.7B). Meanwhile, high amount of small proteins (approximately 10 kDa) and small amount of moderate (20-48 kDa) and large (>48 kDa) proteins was observed in collecting tube no. 17 to 21. In order to identify the most effective anti-cancer protein, cytotoxicity of proteins in each collecting tube was investigated in human lung cancer H460 cells. Figure 4.7C demonstrates the significant reduction of cell viability in H460 cells after treatment with 25 µg/ml of proteins from collecting tube no. 13 to 21. It was worth to note that 50% reduction of cell viability was found in treatment of proteins from collecting tube no. 15 to 17 which contained higher amount of proteins of moderate size (20-48 kDa) more than other collecting tubes.

Because single band of protein was not obtained by gel filtration chromatography, membrane filtration was used as an alternative method for further purification of F1 and F4 protein fraction. According to the result of cytotoxicity effect of proteins at moderate size (20-48 kDa), the membrane with molecular weight cut off at 30 and 10 kDa were respectively used for protein purification. The result of protein homogeneity after membrane filtration was shown in figure 4.8A and 4.9A. Fraction (a) was protein extracts that did not pass through filter membrane of 30 kDa cut off while fraction (b) was retentate from membrane filtration of molecular weight cut off 10 kDa. The results showed that after purification by membrane filtration, proteins with

molecular weight >30 kDa and 10-30 kDa were obtained in fraction (a) and (b) and protein with molecular weight <10 kDa are in the filtrate after pass through membrane filtration with molecular weight cut off 10 kDa were obtained in fraction (c). Various sizes of proteins were gained from both fraction (a) and (b) of F1 fraction (Fig. 4.8A). Although, better separation of protein compositions was presented in fraction (b) of F4 protein fraction, similar pattern of protein compositions as F4 were collected in fraction (a) (Fig. 4.9A).

To identify the most potent anti-cancer proteins in F1 and F4 fraction, cell viability of human lung cancer H460 cells was determined after culture with medium containing with 25 µg/ml of protein fraction. The comparable reduction of cell viability was demonstrated after treatment with 25 µg/ml of F1, F1(a) and F1(b) proteins (Fig. 4.8B). These results corresponded with similar pattern of protein compositions in F1, F1(a) and F1(b) fraction (Fig. 4.8A). Meanwhile, the different pattern of protein composition was observed between F4, F4(a) and F4(b) (Fig. 4.9A). Comparable with highest toxicity to H460 lung cancer cells as determined by MTT assay was observed in F4 and F4(a) fraction (Fig. 4.9B) with protein of larger size more than 30 kDa was observed. This result suggested that proteins with size larger than 30 kDa may responsible for this cytotoxicity.

Taken together, potent anti-cancer proteins were not completely purified from both gel filtration chromatography and membrane filter. Moreover, the highest toxicity profiles were observed in protein extracts obtained from ion-exchange chromatography. Thus, F1 and F4 protein fraction were selected for further experiments.

A)

B)

C)
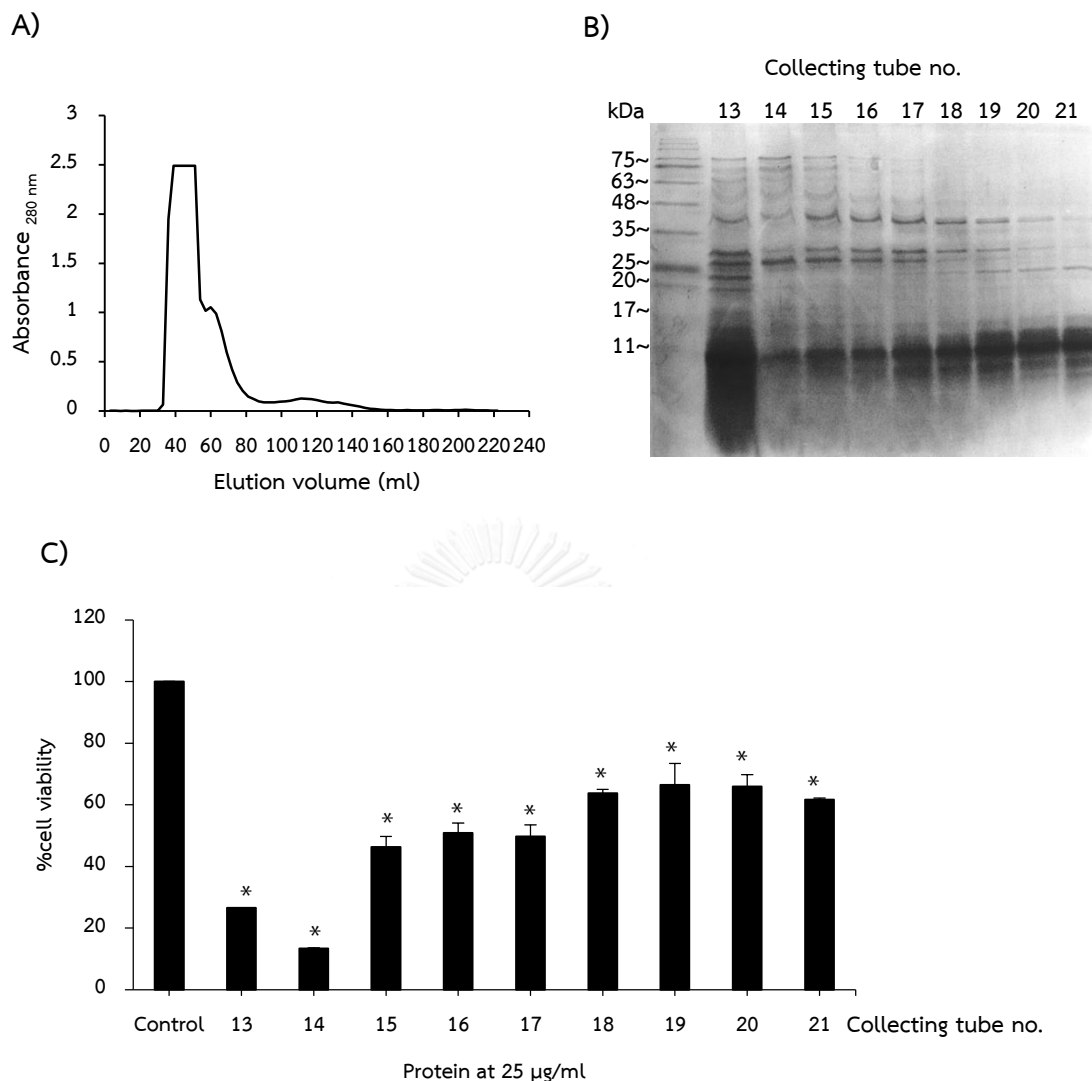
Figure 4.7 Gel filtration of fraction F1 (from DEAE-cellulose column) on a Sephadex G-50 column equilibrated with PBS (pH 7.4) at a flow rate of 0.2 ml/min. **A)** Protein elution profile detected by UV absorption at 280 nm. **B)** SDS-PAGE analysis result for heterogeneity of proteins eluted in different collecting tube. **C)** Cytotoxic effect of purified protein eluted in different collecting tubes as determined by MTT assay. The result showed that proteins from all collecting tubes have anti-cancer activity against human lung cancer cells. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.
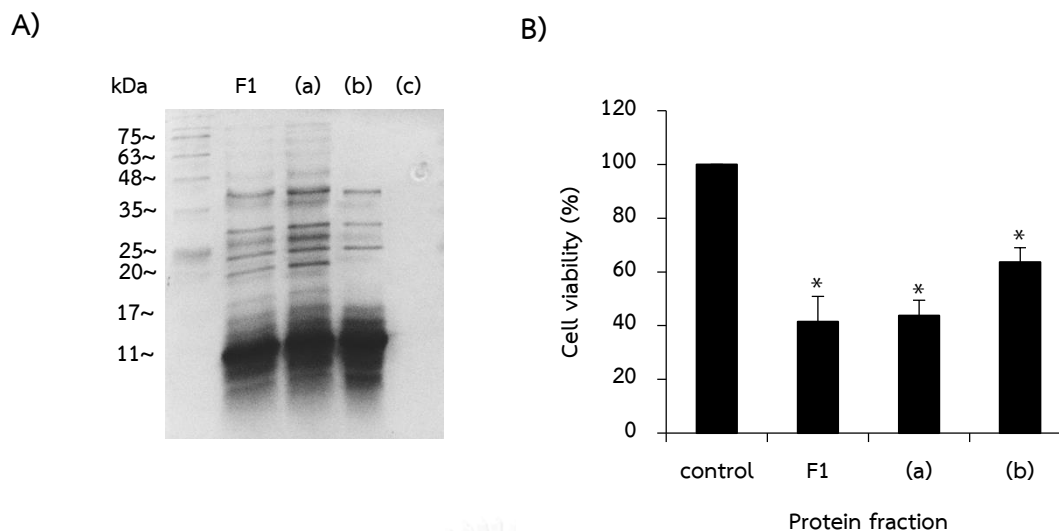
Figure 4.8 Protein homogeneity and anti-cancer activity of purified proteins from membrane filtration. **A)** Partially-purified protein extracts with similar composition of proteins with F1 fraction were evaluated by SDS-PAGE in (a) and (b) fraction from membrane filtration. (a) protein composition from retentate of membrane filtration with molecular weight cut off 30 kDa. (b) protein composition from retentate of membrane filtration with molecular weight cut off 10 kDa. (c) protein composition from filtrate of membrane filtration with molecular weight cut off 10 kDa **B)** Anticancer activity of F1, (a) and (b) fraction. Comparable anti-cancer activity was indicated in lung cancer cells treated with 25 µg/ml of either F1, (a) or (b) fraction for 24 h. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.
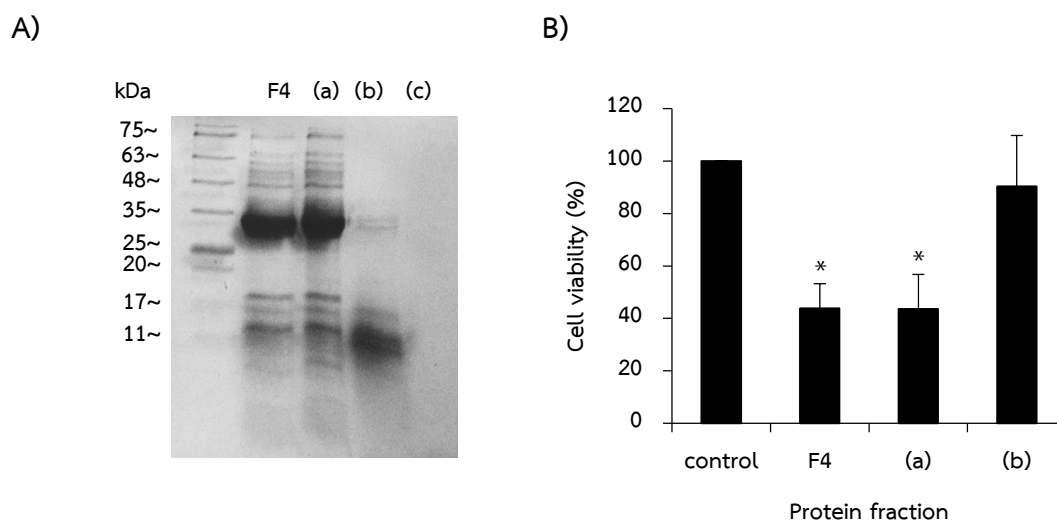
Figure 4.9 Potent anti-cancer protein in from fraction F4 **A)** Different profile of protein composition was demonstrated in fraction (b) obtained from retentate of membrane filtration with molecular weight cut off 10 kDa. **B)** Low cytotoxic effect of (b) fraction was detected in H460 cells. Cell viability determined by MTT assay after incubation with protein extracts at concentration of 25 µg/ml for 24 h. Values are means of the independent triplicate experiments ± SD. * p < 0.05 versus non-treated control.

4. Apoptosis induction in lung cancer cells treated with protein extracts

To determine whether the partial-purified protein can induced apoptosis, the evaluation on apoptosis marker proteins was performed via western blot analysis. The increase of cleaved-caspase3 was observed after treatment of H460 cells with partially-purified protein extracts (25 µg/ml) of F1 and F4 fraction for 24 h (Fig. 4.10A) suggested for the activation of caspase 3. The increase of cleavage PARP (as substrate of activated caspase 3) [110] in H460 cells treated with 25 µg/ml of partially-purified proteins from fraction F4 was also observed (Fig. 4.10B). Although the activation of caspase 3 in the cells treated with 25 µg/ml of protein from fraction F1 was observed, there was no elevated level of cleaved-PARP. This might be due to lower level of cleaved-caspase3 in lung cancer cell incubated with protein from fraction F1 compared with fraction F4 (Fig. 4.10).

A)

B)
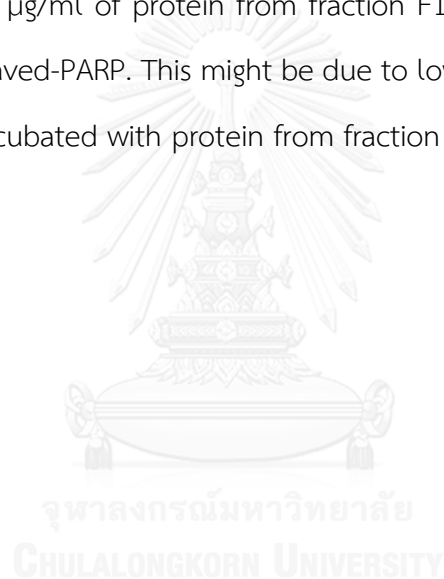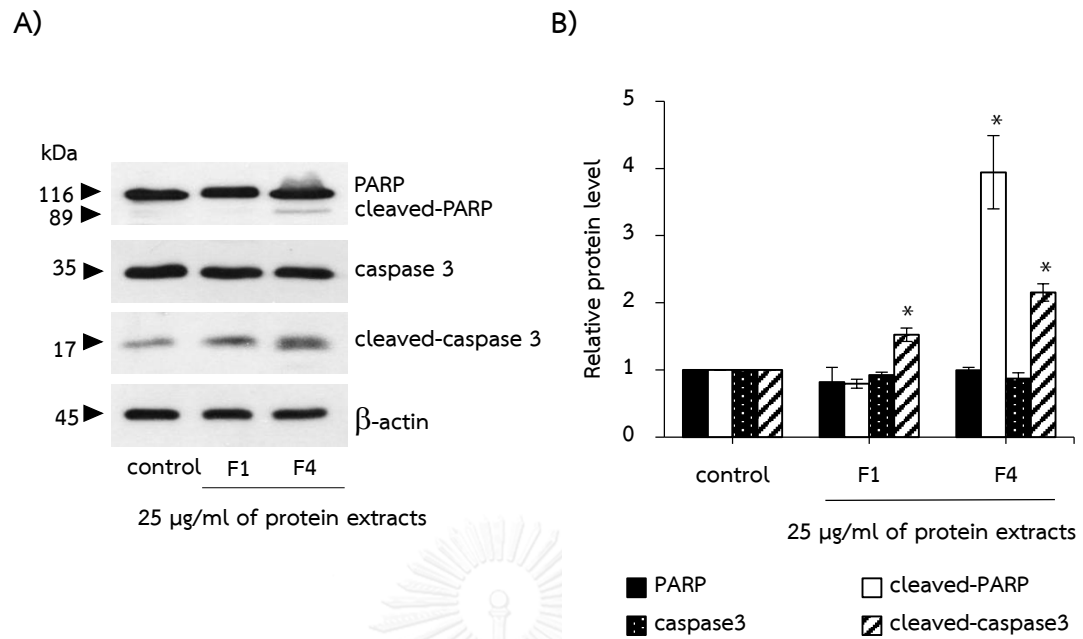
Figure 4.10 The apoptosis cells death was confirmed by the alteration of apoptosis marker proteins. **A)** Western blot analysis indicated the increasing of active caspase3 (cleaved-caspase3) in F1 and F4-treated H460 cells. **B)** As substrate of activated casapase3, the significant increase of cleaved-PARP was associated with the level of cleaved-caspase3 in treatment of H460 cells with protein extracts F4. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

**5. Proteins extracted form *L. squarrosulus* stimulate both intrinsic and extrinsic apoptosis pathway**

There are two major apoptotic pathways, intrinsic and extrinsic [14]. The alteration of Bcl-2 family proteins involving in intrinsic or mitochondrial pathway was significantly observed in H460 cells treated with the partially-purified protein from F1 and F4 fraction. Figure 4.11 shows that there was a reduction of Bcl-2, an anti-apoptotic protein after incubation of lung cancer cells with 25 μg/ml of protein fraction from F1 and F4 for 12 h. Meanwhile, a pro-apoptotic protein, Bax was not different compared with non-treated control. Moreover, protein from fraction F4 also decreased level of Mcl-1, an anti-apoptosis protein in H460 cells. On the contrary, there was significant augmentation of Mcl-1 in the cells treated with 25 μg/ml proteins from fraction F1 (Fig. 4.11b).

The induction of extrinsic or death receptor signaling in protein extracts-treated H460 cells was presented by the reduction of c-FLIP, an inhibitor of death receptor-activated caspase associating with the augmentation of cleaved-caspase8 (Fig. 4.12A). Interestingly, the stimulation of extrinsic apoptosis pathway was observed only in culture of human lung cancer cells with proteins from fraction F4 at 25 μg/ml for 12 h (Fig. 4.12 B).
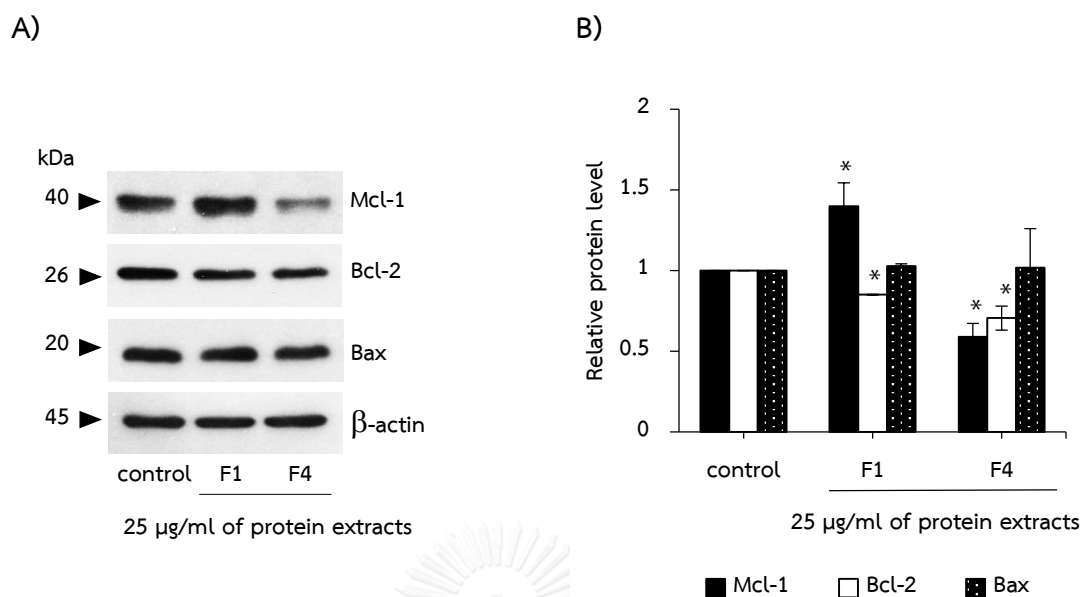
Figure 4.11 Partially-purified protein extracts from *L. squarrosulus* Mont. stimulated mitochondrial apoptotic pathway. **A)** Apoptosis-mediating proteins were analyzed in H460 cells incubated with 25 μg/ml of either F1 or F4 fraction for 12 h. There were significant reduction of Bcl-2 and Mcl-1 after treatment of lung cancer cells with 25 μg/ml of F4 proteins. **B)** Proteins from F1 fraction significantly up-regulated Mcl-1 level in lung cancer cells. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.
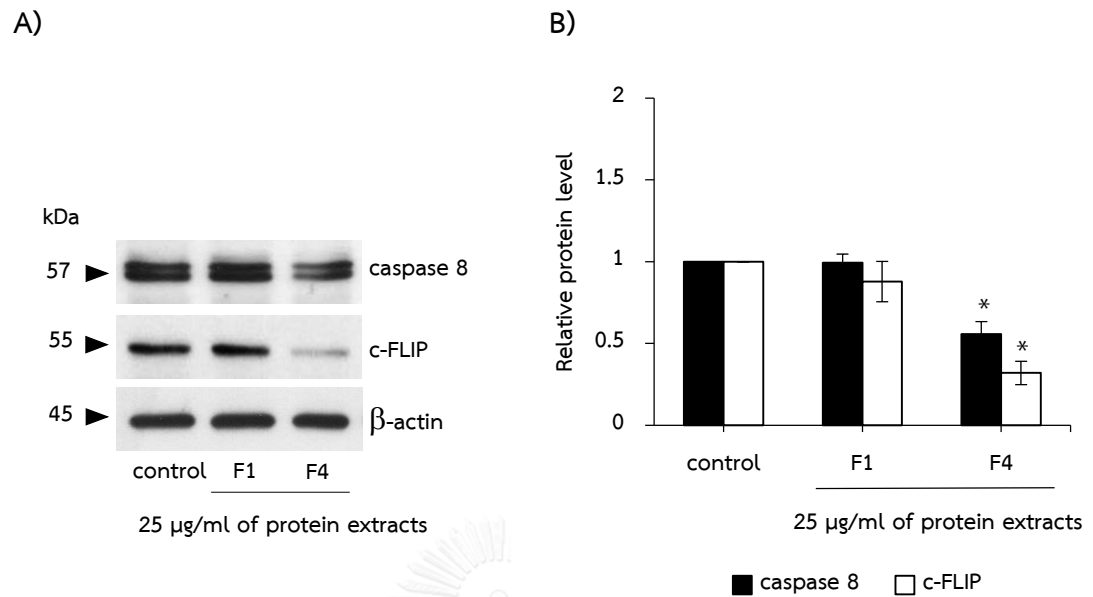
Figure 4.12 Partially-purified protein extracts from *L. squarrosulus* Mont. stimulated protein-relating death-receptor pathway. **A)** c-FLIP and caspase8 were obviously decreased in F4 proteins-treated H460 cells. **B)** There was no significant alteration of proteins involving with extrinsic apoptosis pathway in H460 cultured with medium containing protein F1 fraction at 25 µg/ml for 12 h. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

6. Protein extracts from *L. squarrosulus* generates cellular ROS in human lung cancer H460 cells

To investigate the possible anti-cancer mechanisms of protein extracts from *L. squarrosulus*, cellular ROS was evaluated in human lung cancer H460 cells. Lung cancer H460 cells at density of 10,000 cells/well in 96 well-plate were pre-incubated with 10 µM DCF, a ROS fluorescent probe at 37ºC for 15 min. Then, the cells were further incubated with 25 µg/ml of either F1 or F4 protein fraction for 1 h. There was the obvious increment of cellular ROS level in H460 cells-treated with protein from fraction F1 and F4 (Fig. 4.13A and B). Well known antioxidant, NAC and GSH could restrain F4 protein-induced ROS in human lung cancer cells (Fig. 4.13B). However, pre-treatment with 5 mM of NAC or GSH for 30 min prior exposed to F1 proteins did not repress ROS level in H460 cells. It was noted that F1 proteins dramatically induced intracellular ROS level approximately 20 times higher than non-treated control cells (Fig. 4.13A).

A)

B)
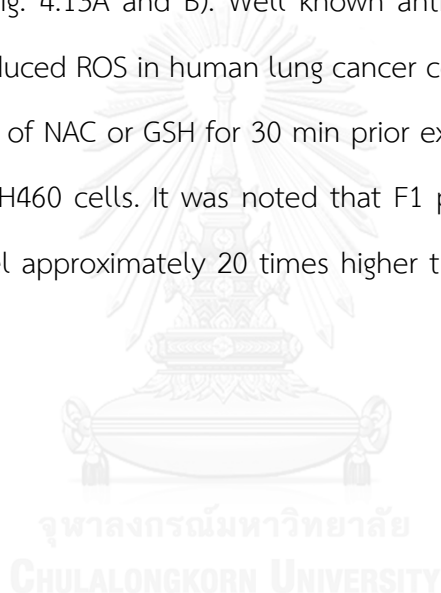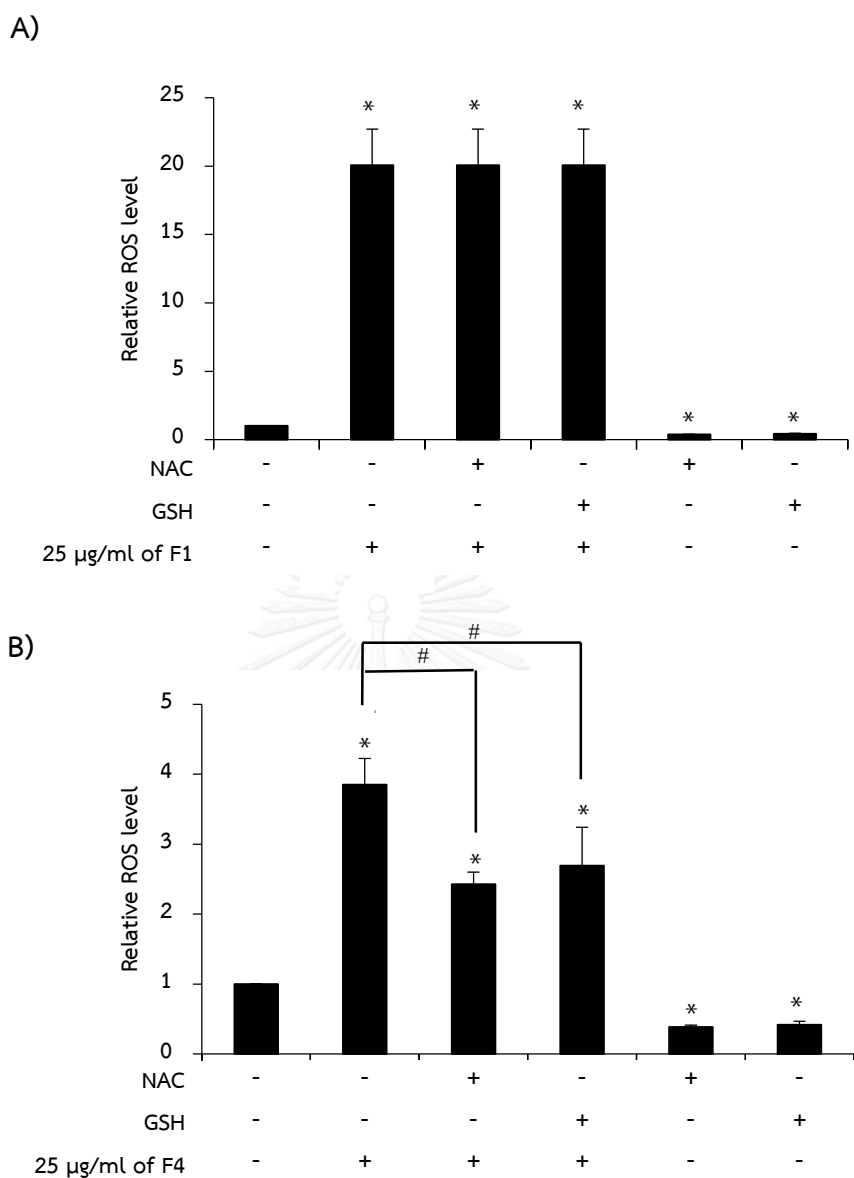
Figure 4.13 Relative cellular ROS level in human lung cancer H460 cells treated with proteins extract from *L. squarrosulus* Mont. **A)** Ultimate increasing of cellular ROS level in human lung cancer cells was detected in treatment with F1 proteins (25 μg/ml) for 1 h with or without antioxidant agents **B)** F4 protein significantly induced ROS in lung cancer H460 cells. Pre-treatment with ROS scavenger, NAC (5 mM) or GSH (5 mM) successfully restrained F4 proteins-induced oxidative stress in human lung cancer cells. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control and # $p < 0.05$ versus peptide extracts-treated cells.

7. Effect of proteins generated-ROS on anti-cancer activity in human lung cancer cells

Because protein extracts from *L. squarrosulus* induced oxidative stress, the relation between ROS and cell viability in human lung cancer H460 cells was investigated. Pre-incubation H460 cells with ROS scavenger, NAC (5 mM) or GSH (5 mM) for 30 min prior treatment of the protein extracts did not preserve cell viability in human lung cancer cells (Fig. 4.14 A and B). These results corresponded with sustained cellular ROS level in the cell incubated with F1 protein fraction with or without antioxidant (Fig. 4.13). Interestingly, significant decrease of %cell viability was observed in lung cancer cells pre-treated with NAC or GSH prior treatment with F4 proteins (Fig. 4.14B) although the lower ROS level was indicated in these cells (Fig. 4.13B). The above results imply that anti-cancer activity of F1 and F4 protein extracts did not depend on the generation of ROS.

A)

B)
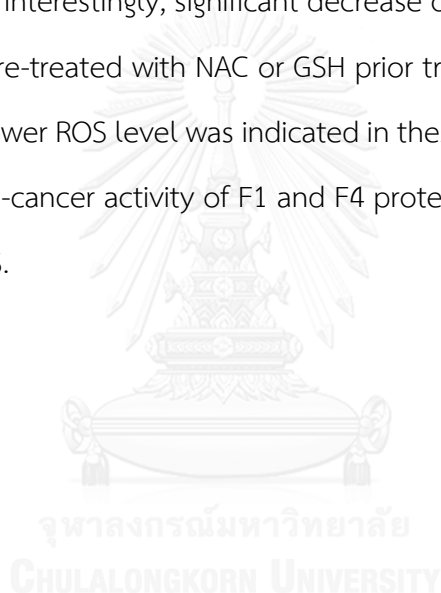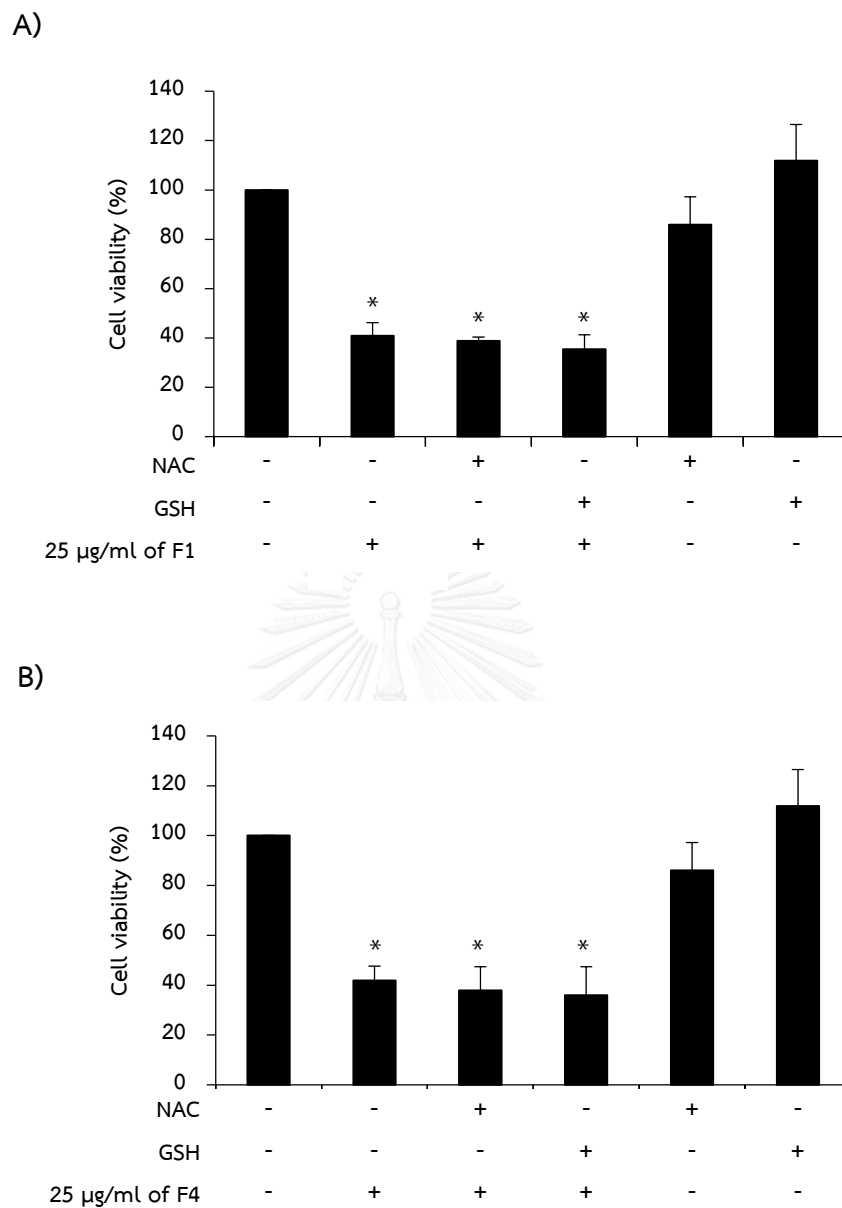
Figure 4.14 Effect of ROS on protein extracts-induced toxicity in human lung cancer cells. After treatment with 5 mM of NAC or 5 mM GSH for 30 min, the cells were further cultured in medium with or without protein extracts for 24 h. MTT assay revealed that anti-cancer activity of protein from both **A)** Fraction F1 and **B)** Fraction F4 was not related with the generation of ROS. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

CHAPTER V

DISCUSSION AND CONCLUSION

Among leading cancers, lung cancer is recognized as an important cause of cancer-related deaths, with significant part of the death due to failure in drug treatment [44]. Cancer research has focused on the searching for the novel active compounds with high efficacy and low toxicity. Herein, the promising anti-cancer effect of proteins extracted from *L. squarrosulus* Mont. mushroom against human lung cancer has been demonstrated. Although the drug resistance in lung cancer is complex and frequently caused by several factors, the increase of anti-apoptotic proteins such as Bcl-2, Mcl-1 and c-FLIP has been shown to be dominant [14, 111]. The overexpression of Bcl-2 and accumulation of cellular Mcl-1 proteins have been shown to mediate lung cancer cell resistant to several chemotherapies as well as death stimuli [112-114]. Likewise, the increase in the level of c-FLIP was shown to inhibit the death of cancer cells in response to immune cell-mediated apoptosis [23, 115, 116].

In the present study, the proteins have been isolated from fruiting bodies of the edible mushroom *L. squarrosulus* Mont. and purified by ion-exchange chromatography on DEAE-cellulose column. Purified proteins from fraction F4 (eluted with 0.3 M NaCl) at concentrations of 25-100 µg/ml significantly decreased viability and induced apoptosis of human lung cancer H460 cells. The significant increase of cleaved-caspase3 and cleaved-PARP confirmed apoptosis inducing activity of protein from fraction F4. The mechanism of apoptosis was demonstrated in H460 lung cancer cells. The down-regulation of anti-apoptosis protein in both intrinsic, Bcl-2 and Mcl-1 as well as extrinsic pathway c-FLIP was mediated by protein from fraction F4. These results are consistent with many studies showing anti-cancer effect of natural-derived compounds by suppressing Bcl-2 and Mcl-1 protein [76-78]. Although c-FLIP has been perceived the principle role as a death receptor-mediating apoptosis, studies also suggested that this protein increase cell survival and proliferation [23, 79]. For examples, the increase of cellular c-FLIP has been shown to activate NF-ĸB [80], and inhibition of such a pathway by dominant expression of its inhibitory subunit IĸB decreased cell survival [81]. Western blotting has shown that treatment of the cells

with protein from fraction F4 extracted from *L. squarrosulus* resulted in a significant depletion of c-FLIP. Together with above context, the protein extract may not only induce apoptosis, but also suppress cancer cell survival and proliferation.

The disruption on the interaction between integrins, cell adhesion molecules and extra-cellular matrix (ECM) can induces apoptosis via mitochondrial and death-receptor machinery. The interaction between integrins on cell membrane and component of ECM initiates various cellular signals including cell survivals via up-regulation of anti-apoptosis protein, Bcl-2 and c-FLIP. Unligated integrins or free integrins activate caspase8 following with extrinsic pathway-mediated apoptosis [117]. Evidence indicated that some amino acid composition of peptide molecule can bind to integrins or cell surface molecules on cancer cells [101]. According to the obvious reduction of Bcl-2, c-FLIP and caspase8, protein from fraction F4 might disturb the ligation between integrins and their ligand resulting in activation of apoptosis cell death in both intrinsic and extrinsic pathway.

Because F1 fraction can pass through DEAE-cellulose column without eluting with NaCl, the unbound protein in fraction F1 should be a neutral or positive net charge protein. Many anti-cancer peptides possess the net positive charge which selectively targets to the negative charge molecules on cell membrane of cancer cells [95]. Cationic peptides cause selective toxicity to cancer cells via damage cell membrane integrity resulting in cell death [96]. Although, there was no obvious apoptotic morphology, the leakage of cell membrane was demonstrated in lung cancer cells treated with protein from fraction F1 at 25 and 50 µg/ml. This indicated that anti-cancer activity of protein from fraction F1 might come from the disruption of cell membrane integrity. There is a report about positively charged protein cause apoptotic and necrotic cell death in cancer cells [118]. Moreover, protein can induce cell death via activate caspase 3 as an apoptotic marker and increase Mcl-1 as an anti-apoptotic protein [4] but the mechanism of protein still not clear. It was worth to note that the increase of Mcl-1 protein and decrease of Bcl-2 protein in lung cancer cells incubated with protein from fraction F1 (25 µg/ml) for 12 h might be a consequent compensation in damaged cells. Cell growth and survival are mediated by cellular Mcl-1 level which can be up-regulated in stress condition [119, 120].

Cellular oxidative stress is caused by the unbalance between cellular ROS and antioxidant mechanisms. The accumulation of cellular ROS leads to different types of cell death. High level of ROS cause damage on cell membrane following with necrosis cell death while low and moderate level of cellular ROS augment apoptosis via diminution of anti-apoptosis proteins [27, 30]. Many studies have shown that mushrooms are rich in antioxidant compounds. Peptides from *Ganoderma lucidum* and *Pleurotus abalonus* have been used to reduce oxidative stress [121, 122]. However, there are the studies reporting that anti-tumor mushroom extracts induce apoptosis cell death via ROS-mediated mitochondrial apoptotic pathway [123-125]. These particular findings are consistent with the results of this study whereby the protein extract caused the dramatic increase of cellular ROS in cancer cells. The induction of oxidative stress by proteins from F1 and F4 fraction in human lung cancer cells was indicated with significant increase of cellular ROS level. However, effective ROS scavengers could not preserve cell viability in lung cancer cells treated with the protein extracts. The unsuccessful preservation of cell viability by well-known antioxidant, NAC and GSH might arise from the extreme production of ROS in F1 treated-lung cancer cells. Moreover, the failure of antioxidant on inhibition of F4 protein induced-toxicity was also observed even there was lower cellular ROS level in pretreatment with NAC or GSH before expose to F4 proteins. The present study suggests that proteins extracted from *L. squarrosulus* induced toxicity in human lung cancer cells independently on the generation of oxidative stress.

In this experiment the crude protein was obtained from crude water extract of fruiting bodies of *L. squarrosulus* by ammonium sulfate precipitation. The crude protein was further purify by ion-exchange chromatography on DEAE-cellulose column. After evaluating for purify of protein from the first step of purification the result showed heterogeneity of the purified protein. To increase the purity of the protein, gel filtration chromatography on Sephadex G-50 or membrane filtration with MW cut off at 30 and 10 kDa were utilized. After the second step of purification, the heterogeneity property of protein still exist. The attempt to purify protein to unique molecular mass was not succeed. The molecular mass of the proteins composition in the purified fraction F4 and F1 are not big different. So it will be more difficult to optimize only the condition

of the gel-filtration. Some other factors such as changing to high resolution column resin or technique utilizing other property of protein such as hydrophobicity should be taking into consideration. Nevertheless, the cytotoxicity of different fraction obtained from both gel filtration chromatography and membrane filtration indicated that the potent anti-cancer protein of F1 and F4 fraction was the proteins with molecular weight approximately 25 kDa and >30 kDa, respectively.

Bioactive peptide isolated from edible mushrooms was reported in previous studies. Among these includes 40kDa purified lectin peptide from *Lentinus cladopus* Lév [126], 55 kDa lectin from *Lentinus squarrosolus* [127], laccase from *Tricholoma mongolicum* [128] and 66 kDa peptide from *L. squarrosolus* [129]. Anti-cancer activity is reported in the study of lectin and laccase peptide [130, 131]. Upon the purification process of the *L. squarrosolus* Mont. proteins in this study, it was found that the effective anti-cancer protein for lung cancer cells was the protein with molecular mass of about 25-48 kDa. Because the molecular weight of this protein is different from lectins previously extracted from *L. squarrosulus*, the anti-cancer protein extracted from this study might be a novel protein.

The modification of extraction and purification process to obtain purified anti-cancer protein should be further studied. The purified peptide has been successfully isolated from many edible mushrooms via different methods. Rully H. et al. extracted a lectin (40 kDa) peptide using protein precipitation, Q-Sepharose chromatography, DEAE-cellulose chromatography, continuous gradient Q-Sepharose chromatography and identification by mass spectrometry [126]. Ngai, P.H.K., et al. extracted a 9 kDA Agrocybin, an anti-fungal peptide from *Agrocybe cylindracea* by ion-exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, ion-exchange chromatography by fast protein liquid chromatography (FPLC) and FPLC-gel filtration on a Superdex 75 column [132]. These studies imply that many purification techniques should be adapted to isolate a single band of purified protein. Additionally, mass spectrometry technique could be used to identify the active anti-cancer protein in this study. An option of purification by using fast protein liquid chromatography (FPLC) and FPLC-gel filtration on a Superdex 75 column are also proposed.

In conclusion, our findings highlighted the potent effects of proteins extracted from fruiting bodies of *L. squarrosulus* Mont. mushroom, in mediating apoptosis in lung cancer cells through the decrease of anti-apoptotic Bcl-2, Mcl-1 and c-FLIP proteins (Fig. 5.1).
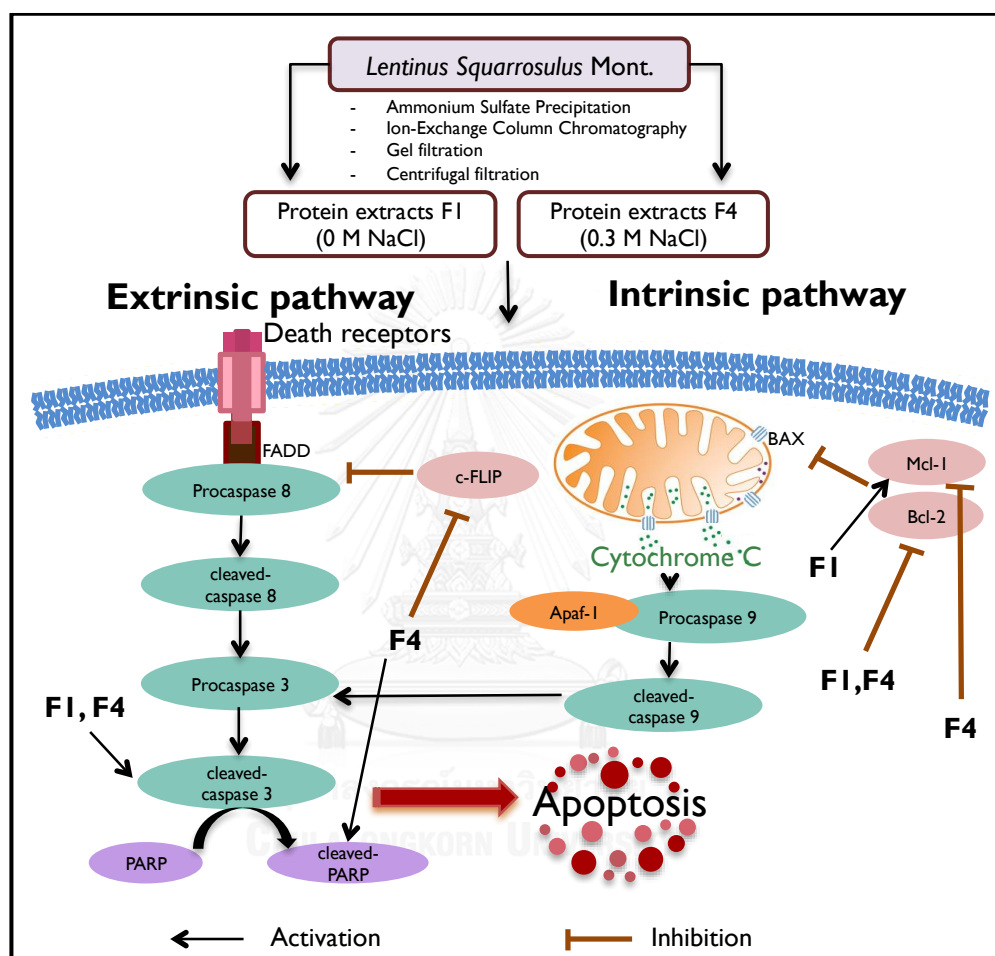
Figure 5.1 Proposed mechanistic scheme of anticancer activity of protein extracted from *Lentinus squarrosulus* Mont. in human lung cancer cells. The extracts mediated mitochondrial or extrinsic apoptotic pathway through the reduction of Bcl-2, Mcl-1 and activated caspase 3. Meanwhile, the extracts also decrease c-FLIP, an inhibitor for death-receptor regulating apoptosis.

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APPENDIX

APPENDIX

TABLES AND FIGURES OF EXPERIMENTAL RESULTS

**Figure 6.1** Comparison of nucleotide sequences with reference strains

	ข้อมูลการเปรียบเทียบความเหมือนของลำดับนิวคลีโอไทด์ Comparison of nucleotide sequences with reference strain(s)					

Strain: PT1

	Description	Max score	Total score	Query cover	Ident	Accession
1	*Lentinus* sp. 5-D-3-A(br)-42 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1064	1064	100%	99%	KJ654561.1
2	*Lentinus squarrosulus* clone 66 18S ribosomal RNA gene, partial sequence	1059	1059	100%	99%	KT120054.1
3	*Lentinus squarrosulus* clone 63 18S ribosomal RNA gene, partial sequence	1059	1059	100%	99%	KT120051.1
4	*Lentinus squarrosulus* clone 1 18S ribosomal RNA gene, partial sequence	1059	1059	100%	99%	KT120037.1
5	*Lentinus squarrosulus* voucher WARRIPt 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1059	1059	100%	99%	KT273380.1
6	*Lentinus squarrosulus* voucher WARRI34 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1059	1059	100%	99%	KT273379.1
7	*Lentinus squarrosulus* voucher UNIP13 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1059	1059	100%	99%	KT273373.1
8	*Lentinus squarrosulus* voucher Odi26 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1059	1059	100%	99%	KT273370.1
9	*Lentinus squarrosulus* voucher IBD43 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1059	1059	100%	99%	KT273364.1
10	*Lentinus squarrosulus* strain WCR1201 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1059	1059	100%	99%	KT956127.1
11	*Lentinus squarrosulus* isolate SIMuthu2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1059	1059	100%	99%	KR150734.1
12	*Lentinus squarrosulus* strain AO-DEBCR-3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1059	1059	100%	99%	KT207470.1

Table 1 Percent yield of protein extracts of fraction F1 to F5

Fraction of protein extracts	Yield (%)
F1 (0 M NaCl)	0.36 ± 0.05
F2 (0.1 M NaCl)	0.33 ± 0.04
F3 (0.2 M NaCl)	0.12 ± 0.02
F4 (0.3 M NaCl)	0.19 ± 0.06
F5 (0.4 M NaCl)	0.16 ± 0.05

Figure 6.2 Partially-purified protein extracts of *L. squarrosulus* Mont. were separated by SDS-PAGE
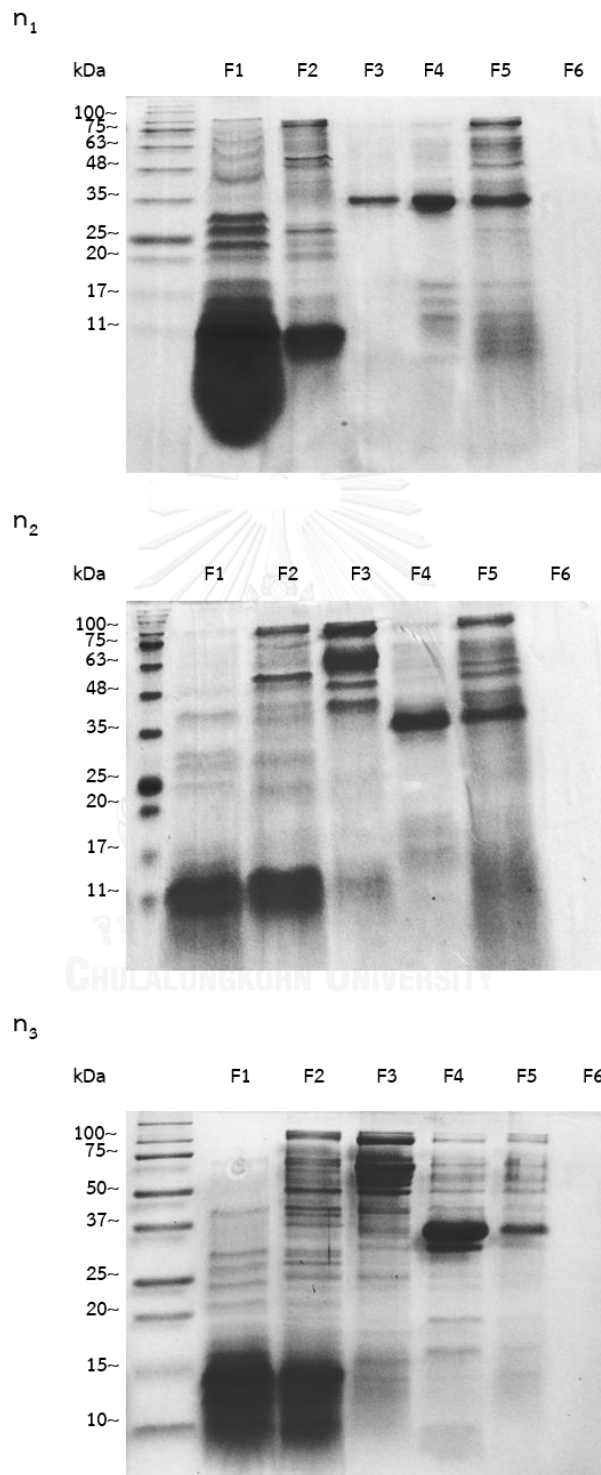
n₁, n₂ and n₃ are representative of the independent triplicate experiments.

Table 2 The percentage cell viability of human lung cancer H460 cells after treatment with 25 µg/ml of protein extracts F1 to F5

Fraction of protein extracts 25 µg/ml	Cell viability (%)		
	n_1	n_2	n_3
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
F1	46.81 ± 3.05*	40.94 ± 2.23*	36.37 ± 2.73*
F2	62.89 ± 11.81*	61.63 ± 4.86*	70.52 ± 7.92*
F3	82.67 ± 5.50	62.22 ±1.54*	66.60 ± 9.07*
F4	46.78 ± 5.22*	45.35 ± 1.00*	45.12 ± 3.40*
F5	60.89 ± 0.96*	68.26 ± 0.21*	59.93 ± 10.94*

Values are means of the independent triplicate experiments ± SD.

* $p < 0.05$ versus non-treated control.

Table 3 The percentage cell viability of human dermal papilla cells after treatment with 25 µg/ml of protein extracts F1 to F5

Fraction of protein extracts 25 µg/ml	Cell viability (%)		
	n_1	n_2	n_3
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
F1	93.75 ± 1.70	75.76 ± 4.15*	85.31 ± 3.52
F2	123.44 ± 2.58	85.88 ± 6.14	85.30 ± 1.34
F3	99.85 ± 11.72	76.71 ± 5.60*	111.51 ± 15.64
F4	90.61 ± 6.14	75.22 ± 4.90*	92.98 ± 5.72
F5	79.73 ± 0.54*	72.36 ± 4.03*	77.53 ± 6.94*

Values are means of the independent triplicate experiments ± SD.
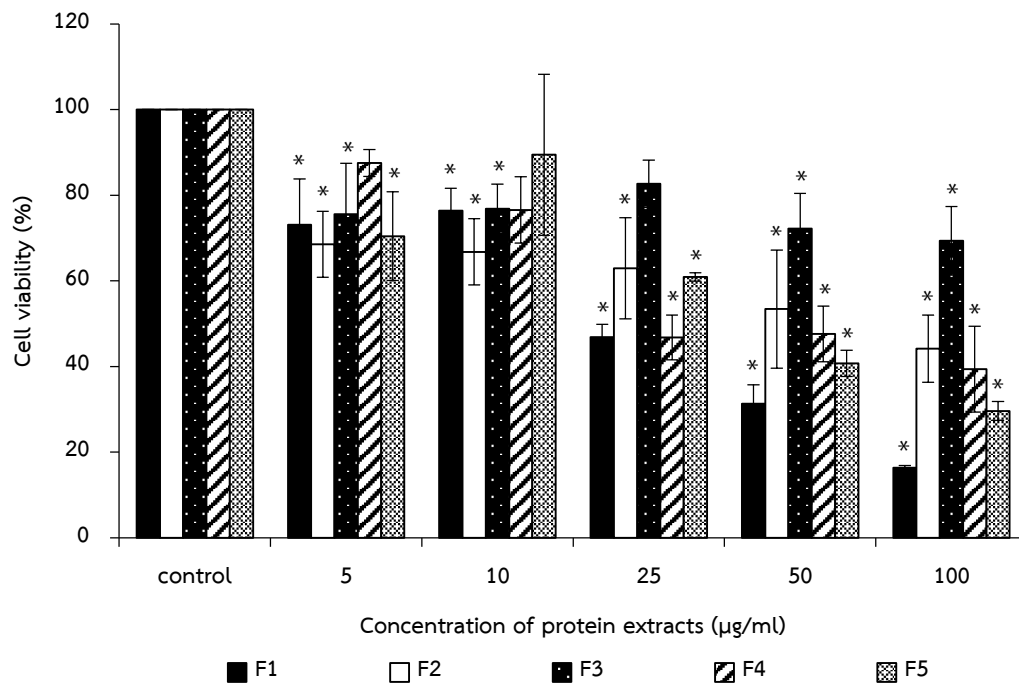
* $p < 0.05$ versus non-treated control.

Figure 6.3 Dose dependent effect of partially-purified protein from fraction F1 to F5 in human lung cancer cells.

* *p* < 0.05 versus non-treated control.

Table 4 The IC_{50} of human lung cancer cells after treatment with various concentration of partially-purified protein from fraction F1 to F5

Fraction of protein extracts	Half maximal inhibitory concentration; IC_{50} (µg/ml)
F1	22.68 ± 1.85
F2	69.26 ± 14.64
F3	>100.00
F4	21.15 ± 6.14
F5	36.06 ± 1.75

Values are means of the independent triplicate experiments ± SD.

Figure 6.4 Dose dependent effect of partially-purified protein from fraction F1 to F5 in human dermal papilla cells.
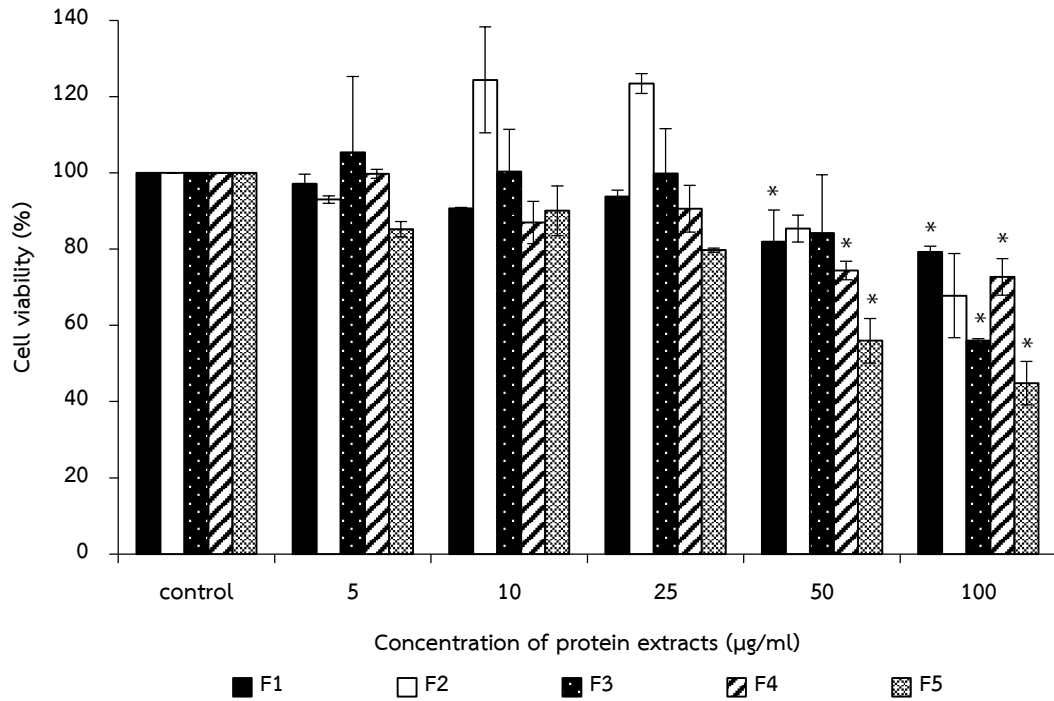
* $p < 0.05$ versus non-treated control.

Table 5 The IC_{50} of human dermal papilla cells after treatment with various concentration of partially-purified protein from fraction F1 to F5

Fraction of protein extracts	Half maximal inhibitory concentration; IC_{50} (µg/ml)
F1	>100.00
F2	>100.00
F3	>100.00
F4	>100.00
F5	75.47 ± 6.73

Values are means of the independent triplicate experiments ± SD.

Table 6 The percentage cell viability of human lung cancer H460 cells after treatment with various concentration of protein extracts F1

Protein extracts F1 (μg/ml)	Cell viability (%)
Control	100.00 ± 0.00
5	73.12 ± 10.65*
10	76.39 ± 5.22*
25	46.81 ± 3.05*
50	31.32 ± 4.43*
100	16.36 ± 0.51*

Values are means of the independent triplicate experiments ± SD.

* $p < 0.05$ versus non-treated control.

Table 7 The percentage cell viability of human lung cancer H460 cells after treatment with various concentration of protein extracts F4

Protein extracts F4 (µg/ml)	Cell viability (%)
Control	100.00 ± 0.00
5	87.51 ± 3.16
10	76.55 ± 7.72
25	46.78 ± 5.22*
50	47.60 ± 6.50*
100	39.40 ± 10.01*

Values are means of the independent triplicate experiments ± SD.

* $p < 0.05$ versus non-treated control.

Table 8 The percentage cell viability of human dermal papilla cells after treatment with various concentration of protein extracts F1

Protein extracts F1 (µg/ml)	Cell viability (%)
Control	100.00 ± 0.00
5	97.10 ± 2.57
10	90.69 ± 0.28
25	93.75 ± 1.70
50	81.94 ± 8.29*
100	79.23 ± 1.55*

Values are means of the independent triplicate experiments ± SD.

* *p* < 0.05 versus non-treated control.

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Table 9 The percentage cell viability of human dermal papilla cells after treatment with various concentration of protein extracts F4

Protein extracts F4 (μg/ml)	Cell viability (%)
Control	100.00 ± 0.00
5	99.75 ± 1.17
10	86.99 ± 5.52
25	90.61 ± 6.14
50	74.35 ± 2.44*
100	72.71 ± 4.77*

Values are means of the independent triplicate experiments ± SD.

* $p < 0.05$ versus non-treated control.

Table 10 The percentage cell viability of human lung cancer H460 cells after treatment with protein extracts F1 of gel filtration chromatography at concentration of 25 µg/ml for 24 h

Number of elution protein extracts	Cell viability (%)
control	100.00 ± 0.00
13	26.62 ± 0.01*
14	13.36 ± 0.26*
15	46.32 ± 3.44*
16	50.91 ± 3.16*
17	49.75 ± 3.72*
18	63.81±1.25*
19	66.49 ± 6.98*
20	65.94 ± 3.89*
21	61.74± 0.44*

Values are means of the independent triplicate experiments ± SD.

* $p < 0.05$ versus non-treated control.

Table 11 The percentage cell viability of human lung cancer H460 cells after treatment with 25 µg/ml of protein extracts F1 from centrifuge filtration

Protein extracts F1 (µg/ml)	Cell viability (%)
Control	100.00 ± 0.00
F1	41.51± 9.34*
>30 kDa	43.76 ± 5.69*
10-30 kDa	63.67 ± 5.32*

Values are means of the independent triplicate experiments ± SD.

* p < 0.05 versus non-treated control.

Table 12 The percentage cell viability of human lung cancer H460 cells after treatment with 25 µg/ml of protein extracts F4 from centrifuge filtration

Protein extracts F4 (µg/ml)	Cell viability (%)
Control	100.00 ± 0.00
F4	43.86 ± 9.35*
>30 kDa	43.62 ± 13.15*
10-30 kDa	90.38 ± 19.43

Values are means of the independent triplicate experiments ± SD.

* $p < 0.05$ versus non-treated control.

Table 13 The relative protein level of apoptotic protein after treatment with 25 μg/ml of protein extracts for 24 h

25 μg/ml of protein extracts	Relative protein level			
	PARP	cleaved-PARP	caspase 3	cleaved-caspase 3
control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
F1	0.82 ± 0.22	0.58 ± 0.14	0.92 ± 0.04	1.52 ± 0.10*
F4	0.99 ± 0.04	3.94 ± 0.55*	0.87 ± 0.09	2.15 ± 0.13*

Values are means of the independent triplicate experiments ± SD.

* *p* < 0.05 versus non-treated control.

Table 14 The relative protein level of mitochondrial apoptotic pathway after treatment with 25 µg/ml of protein extracts for 12 h

25 µg/ml of protein extracts	Relative protein level		
	Mcl-1	Bcl-2	BAX
control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
F1	1.40 ± 0.14*	0.85 ± 0.00*	1.03 ± 0.01
F4	0.59 ± 0.08*	0.71 ± 0.08*	1.02 ± 0.24

Values are means of the independent triplicate experiments ± SD.

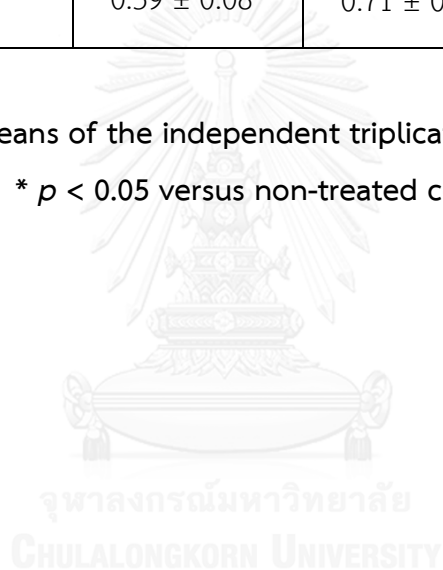* $p < 0.05$ versus non-treated control.

Table 15 The relative protein level of death-receptor pathway after treatment with 25 µg/ml of protein extracts for 12 h

25 µg/ml of protein extracts	Relative protein level	
	caspase 8	c-FLIP
control	1.00 ± 0.00	1.00 ± 0.00
F1	0.99 ± 0.05	0.88 ± 0.12
F4	0.80 ± 0.07	0.32 ± 0.07*

Values are means of the independent triplicate experiments ± SD.

* *p* < 0.05 versus non-treated control.

Table 16 The relative cellular ROS level of human lung cancer H460 cells after treatment with 25 µg/ml of protein extracts, 5 mM NAC and 5 mM GSH for 1 h

Treatment groups	Relative cellular ROS level	
	Protein extracts F1	Protein extracts F4
control	1.00 ± 0.00	1.00 ± 0.00
25 µg/ml of protein	20.07 ± 2.64*	3.85 ± 0.38*
NAC + 25 µg/ml of protein	20.07 ± 2.64*	2.43 ± 0.17*,#
GSH + 25 µg/ml of protein	20.07 ± 2.64*	2.69 ± 0.55*,#
NAC	0.38 ± 0.03*	0.38 ± 0.03*
GSH	0.42 ± 0.05*	0.42 ± 0.05*

Values are means of the independent triplicate experiments ± SD.

* $p < 0.05$ versus non-treated control and
# $p < 0.05$ versus peptide-treated cells.

Table 17 The percentage cell viability of human lung cancer H460 cells after treatment with 25 μg/ml of protein extracts, 5 mM NAC and 5 mM GSH for 1 h

Treatment groups	Cell viability (%)	
	Protein extracts F1	Protein extracts F4
control	100.00 ± 0.00	1.00 ± 0.00
25 μg/ml of protein	40.86 ± 5.37*	41.81 ± 5.89*
NAC + 25 μg/ml of protein	38.87 ± 1.52*	37.90 ± 9.46*
GSH + 25 μg/ml of protein	35.44 ± 5.91*	36.03 ± 11.38*
NAC	85.99 ± 11.24	85.99 ± 11.24
GSH	111.85 ± 14.67	111.85 ± 14.67

Values are means of the independent triplicate experiments ± SD.

* *p* < 0.05 versus non-treated control

VITA

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