ผลของเคอร์เซตินต่อการตายแบบอะโพโทซิสและออโตฟาจีผ่านทางวิถีสัญญาณ BAD และ BCL-2 ใน
เซลล์เพาะเลี้ยงมะเร็งเม็ดเลือดขาวสายมัยอีลอยด์ของมนุษย์

นางสาวอลิสา อยู่สบาย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์โลหิตวิทยาคลินิก ภาควิชาจุลทรรศน์ศาสตร์คลินิก
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

Effect of Quercetin on apoptosis and autophagy through BAD and BCL-2 signaling pathway in human acute myeloid leukemia cell lines

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Clinical Hematology Sciences
Department of Clinical Microscopy
Faculty of Allied Health Sciences
Chulalongkorn University
Academic Year 2016

Thesis Title	Effect of Quercetin on apoptosis and autophagy through BAD and BCL-2 signaling pathway in human acute myeloid leukemia cell lines
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อลิสา อยู่สบาย : ผลของเคอร์เซตินต่อการตายแบบอะโพโทซิสและออโตฟาจีผ่านทางวิถีสัญญาณ BAD และ BCL-2 ในเซลล์เพาะเลี้ยงมะเร็งเม็ดเลือดขาวสายมัยอีลอยด์ของมนุษย์ (Effect of Quercetin on apoptosis and autophagy through BAD and BCL-2 signaling pathway in human acute myeloid leukemia cell lines) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. สุพันธิตรา ชาญประเสริฐ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. นพ.พลภัทร โรจน์นครินทร์, 110 หน้า.

โรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอีลอยด์เป็นโรคมะเร็งทางระบบโลหิตวิทยาที่พบได้บ่อยในผู้สูงอายุ วิธีการรักษาโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอีลอยด์ที่ใช้ในปัจจุบันก่อให้เกิดผลข้างเคียงสูงต่อผู้ป่วย ด้วยเหตุนี้ การใช้สารสกัดจากธรรมชาติเป็นอีกการรักษาทางเลือกจึงได้รับความสนใจ ในงานวิจัยครั้งนี้ ผู้วิจัยทำการศึกษาฤทธิ์ในการยับยั้งเซลล์มะเร็งเม็ดเลือดขาวของเคอร์เซติน ซึ่งเป็นสารฟลาโวนอยด์ที่พบได้มากในพืชและผลไม้หลายชนิด โดยคณะผู้วิจัยทำการเพาะเลี้ยงเซลล์มะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอีลอยด์ชนิด U937 กับสารเคอร์เซตินเป็นเวลา 24 ชั่วโมง พบว่า สารเคอร์เซตินมีฤทธิ์ในการยับยั้งการเจริญของเซลล์มะเร็งเม็ดเลือดขาวอีกทั้งยังมีฤทธิ์ในการลดเปอร์เซ็นต์การมีชีวิตของเซลล์มะเร็งเม็ดเลือดขาวอีกด้วย การทดสอบฤทธิ์ของสารเคอร์เซตินต่อการเกิดอะโพโทซิสโดยใช้ annexin V และ PI พบว่า เคอร์เซตินมีฤทธิ์ในการชักนำให้เกิดกระบวนการอะโพโทซิสเพิ่มขึ้นอย่างมีนัยสำคัญ นอกจากนี้ คณะผู้วิจัยยังพบว่าการเพาะเลี้ยงเซลล์ชนิด U937 กับเคอร์เซตินทำให้มีการเพิ่มขึ้นของ phosphatidylethanolamine conjugated form of microtubule-associated protein light chain 3 (LC3-II) ซึ่งเป็นโปรตีนที่จำเพาะในกระบวนการออโตฟาจี อีกทั้งคณะผู้วิจัยยังพบว่าการเพาะเลี้ยงเซลล์ชนิด U937 ร่วมกับสารยับยั้งกระบวนการออโตฟาจี คือ 3-Methyladenine (3-MA) ก่อนบ่มเพาะกับสารเคอร์เซตินทำให้เกิดการเสริมฤทธิ์ของเคอร์เซตินในการชักนำให้เกิดกระบวนการอะโพโทซิสเพิ่มขึ้นซึ่งบ่งชี้ถึงบทบาทของกระบวนการออโตฟาจีในการปกป้องเซลล์จากการตายของเซลล์ชนิด U937 ที่บ่มเพาะกับเคอร์เซติน การวิเคราะห์ด้วยวิธี Western blot เพื่อศึกษาระดับการแสดงออกของโปรตีนในแฟมิลี Bcl-2 ซึ่งเป็นโปรตีนที่สำคัญในกระบวนการอะโพโทซิส ในเซลล์เพาะเลี้ยงชนิด U937 หลังบ่มเพาะกับเคอร์เซติน พบว่า เคอร์เซตินมีฤทธิ์ในการลดการแสดงออกของโปรตีนชนิด Bcl-2 และลดระดับการเติมหมู่ฟอสเฟตของโปรตีน Bad อีกทั้งยังมีฤทธิ์ในการเพิ่มการแสดงออกของโปรตีนชนิด Bad อีกด้วย จึงสรุปได้ว่างานวิจัยนี้แสดงถึงองค์ความรู้เพิ่มเติมในด้านฤทธิ์ของสารเคอร์เซตินที่เกี่ยวข้องกับกระบวนการตายของเซลล์มะเร็งเม็ดเลือดขาวและแสดงให้เห็นว่าเคอร์เซตินอาจเป็นอีกทางเลือกหนึ่งในการร่วมรักษาโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอีลอยด์ โดยเฉพาะเมื่อรักษาร่วมกับสารยับยั้งกระบวนการออโตฟาจี

ภาควิชา	จุลทรรศน์ศาสตร์คลินิก	ลายมือชื่อนิสิต .....
สาขาวิชา	วิทยาศาสตร์โลหิตวิทยาคลินิก	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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5776664337 : MAJOR CLINICAL HEMATOLOGY SCIENCES
KEYWORDS: QUERCETIN / AML / APOPTOSIS / AUTOPHAGY / BCL-2 / BAD

ALISA YOOSABAI: Effect of Quercetin on apoptosis and autophagy through BAD and BCL-2 signaling pathway in human acute myeloid leukemia cell lines. ADVISOR: SUPANTITRA CHANPRASERT, Ph.D., CO-ADVISOR: PROF. PONLAPAT ROJNUCKARIN, M.D., 110 pp.

Acute myeloid leukemia (AML) is a hematopoietic malignant disease that common in the elderly. Current therapeutic approaches for AML have many side effects. For this reason, natural compounds are considered as alternative medicine. In the present study, we focus on anti-leukemic activity of quercetin, a natural flavonoid broadly founded in many plants and fruits. Our study founded that treatment of U937 cells with quercetin resulted in growth inhibition as well as decreased in cell viability in dose-dependent manner after 24 h of incubation. Apoptosis assay using annexin V and propidium iodide (PI) showed that quercetin significantly increased in the percentage of apoptotic cells. Moreover, exposure of U937 cells to quercetin augmented the expression of phosphatidylethanolamine conjugated form of microtubule-associated protein light chain 3 (LC3-II), a hallmark of autophagy. Furthermore, pretreatment of U937 cells with autophagy inhibitor, 3-Methyladenine (3-MA), dramatically enhanced quercetin-induced apoptotic cell death, indicated the cytoprotective role of autophagy in quercetin-treated AML cells. Western blot analysis was performed to investigate the expression of Bcl-2 family proteins, well-known modulators of apoptosis, after treated cells with quercetin. Results showed that quercetin downregulated the expression of Bcl-2 and phosphorylation levels of Bad and upregulated the expression of total Bad. In conclusion, our findings provided further basis of quercetin-mediated leukemic cell death and proposed that quercetin could be considered as a potent complementary medicine for AML treatment, particularly in combination with autophagy inhibitor.

Department:	Clinical Microscopy	Student's Signature .....
Field of Study:	Clinical Hematology Sciences	Advisor's Signature .....
		Co-Advisor's Signature .....
Academic Year:	2016	

ACKNOWLEDGEMENTS

First, my gratitude is extended to my advisor, Dr.Supantitra Chanprasert for her support, beneficial advices, kindness and encouragement during my graduate study.

I would like to thank my co-advisor, Professor Ponlapat Rojnuckarin for his helpful suggestion.

I would like to thank all fellow graduated students for providing me the invaluable guidance of research laboratory techniques.

I would like to express my gratitude and appreciation to thesis committee, Dr.Sirikalaya Brimson, Assistant Professor Siriporn Chuchawankul and Dr.Moltira Promkan for their gainful suggestion for completeness of the thesis.

I would like to thank Mr. Pattarawat Thantiworasit, Faculty of Medicine, Chulalongkorn University for flow cytometry technical support.

The research was funded by the Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej and the 90th Anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund).

Finally, my sincere thanks go to my beloved family members and my friends for understanding, love, encouragement and cheerful throughout my study.

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LIST OF ABBREVIATIONS

%	Percent
/	Per
°C	Degree Celsius
3-MA	3-Methyladenine
AML	Acute myeloid leukemia
Bcl-2	B cell lymphoma 2
BSA	Bovine serum albumin
CO_2	Carbon dioxide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
FAB	French-American-British
FBS	Fetal bovine serum
FITC	Fluorescein iso-thiocyanate
h	Hour
LC3	Microtubule-associated protein light chain 3
MTT	3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium Bromide
OD	Optical density
PBS	Phosphate-buffered saline

PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween® 20

CHAPTER I
Introduction

1.1 Background and rationale

Acute myeloid leukemia (AML) is a malignant disorder of hematopoietic cells that is categorized by an accumulation of abnormal myeloid cells in the bone marrow with maturation arrest and impaired of normal blood cells production. AML is recognized as the most common type of malignant myeloid disorder in adults. The annual incidence of AML is estimated 3.6 cases per 100,000 (1, 2). Symptoms of AML may include fatigue, hemorrhage, infections, fever, paleness, and organ infiltration by leukemic cells. Currently, the major options of acute myeloid leukemia treatment are chemotherapy and bone marrow transplantation (3). However, these treatments have many side effect for patient. The adverse effects of chemotherapeutic drug are intestinal ulceration, diarrhea, oral mucositis, gram-negative septicemia, fever and elevation of hepatic enzymes (4). Due to the side effects of chemotherapeutic drug, the development of natural compounds derived from plant sources as an alternative medicine are considered.

Quercetin is the most abundant natural flavonoid isolated from many vegetables and fruits. Quercetin has been described as a flavanols with many biological properties such as antimicrobial, antioxidant, anti-inflammatory (5) and

a potential anticancer in many types of cancer including leukemia (6-13). In contrast, normal hematopoietic progenitors and peripheral blood mononuclear cells were not growth-inhibited by quercetin treatment (14, 15).

Apoptosis or type I programmed cell death, a regulated cell-suicide program, is an important process for maintenance homeostasis by the removal of damaged or unnecessary cells. Nowadays, apoptosis represents the most desirable target mechanism for leukemic cell death induction (16). However, Apoptosis is not only one type of cell death but cells may be eliminated by alternative mechanisms including autophagy.

Autophagy is an intracellular lysosomal catabolic mechanism that plays a vital role in cell homeostasis by eliminate aging or damaged proteins and organelles via their own lysosome system. Autophagy could be defined as type II programmed cell death or functions to promote cell survival depends on cellular setting (17, 18). Previous studies reported that some substances can concurrently induce apoptosis and autophagy in leukemia cell lines. In 2008, Yokoyama and et al. found that vitamin K2 can induce autophagy and apoptosis in HL-60 cell line (19). Arsenic trioxide (As_2O_3) induces autophagy and apoptosis in Molt-4 and Mutz-1 cells (20). Recently, Cao and co-workers reported that clioquinol induces apoptosis and pro-death autophagy in many types of leukemic cell lines by disrupting mTOR pathway (21). Moreover, many evidences also suggest that phytochemical can modulate programmed cell death of leukemic cells. For instance, morin, a flavonoid isolated

from plants in Moraceae family, induces apoptosis in U937 cell via caspase-dependent manner (22). Tetrahydrocurcumin, a metabolite of curcumin, induces autophagy in HL-60 cell line through regulation of MAPK and PI3K/Akt/mTOR signaling cascade (23). Interestingly, Antho 50, Anthocyanin-rich dietary bilberry extract, induces apoptosis in B-CLL cells through BAD and BCL-2 pathways (24). These finding demonstrated the anti-leukemic efficacy of natural compound by inducing cell death through apoptosis and autophagy and provided an effective application for leukemia treatment.

Bcl-2 family proteins are essential molecules of intrinsic apoptosis pathway. This proteins family consist of anti-apoptotic molecules including Bcl-2, Bcl-xL, and Mcl-1 and pro-apoptotic molecules including Bad, Noxa, and Puma (25). In addition, Bcl-2 also bind and inhibit Beclin 1, an important molecule for initiation step of autophagy. The BH3 domains of BH3-only proteins such as Bad involve in an activation autophagy process by competitively disrupt the interaction between Bcl-2 and Beclin1. Taken together, these reports reveal that Bcl-2 and Bad maybe targeted molecules for modulating programmed cell death of leukemic cells. However, mechanism of natural compound especially quercetin mediated apoptosis and autophagy of leukemic cells remain unclear. So, in this study we aim to examine the effect of quercetin on apoptosis and autophagy and its underlying mechanism. Further studies provide useful information for the application of natural compound as alternative medicine for leukemia treatment.

1.2 Research questions

1. Does quercetin has an effect on apoptosis and autophagy induction in U937 cells?

2. What are the underlying mechanism on the apoptosis and autophagy induction in U937 cells after treat with quercetin?

1.3 Research hypothesis

Quercetin has abilities to induce both apoptosis and autophagy at least through BAD and BCL-2 signaling pathway in human acute myeloid leukemia cell line.

1.4 Objectives

1.To study the effect of quercetin on cell cytotoxicity in U937 cell line.

2.To study the effect of quercetin on cell viability in U937 cell line.

3.To study the effect of quercetin on apoptotic induction in U937 cell line.

4.To study the effect of quercetin on induction of autophagy in U937 cell line.

5.To clarify the role of autophagy induction by quercetin in U937 cell line.

6.To investigate the involvement of Bad and Bcl-2 in apoptosis and autophagy signaling pathway after induction by quercetin in U937 cell line.

1.5 Expected outcomes

1.Understanding the molecular mechanism involved in apoptosis and autophagy induction by quercetin.

2.Provide scientific information of quercetin for further studies in natural compound development as an alternative medicine for treatment of acute myeloid leukemia.

1.6 Limitation

This research use U937 cell line as in vitro model of acute myeloid leukemia. So, result and conclusion from this study are limited to in vitro study. Further studies that performed ex vivo and in vivo model are required.

1.7 Scope of study

In the research, we study the effect of quercetin on cell cytotoxicity by using MTT based method. The effect of quercetin on cell viability was determined based on cell permeability to the DNA binding dyes. The effect of quercetin on apoptosis are determined by 2 methods including microscopic analysis which observe morphological feature of apoptotic cells under inverted light microscope and flow cytometry analysis by Annexin V and Propidium iodide double staining. The effect of quercetin on autophagy are determined by using Anti-LC3.

In addition, Western blot analysis was performed to investigate the involvement of Bad and Bcl-2 in apoptosis and autophagy signaling pathway after induction by quercetin in U937 cell line.

CHAPTER II

Literature review

2.1 Leukemia

Leukemia is one type of blood cancer. This disease arises from the malignant transformation of hematopoietic stem cells, resulting in uncontrolled and rapid production of abnormal blast cells, results in an accumulation of immature cells in human's body especially in the bone marrow. These leukemic cells have abnormal morphology and function.

2.1.1 Classification of leukemia

Leukemia can be simple classified into 4 group based on cell lineage and disease progression. There are

Acute lymphoblastic leukemia (ALL)

Acute myelogenous leukemia (AML)

Chronic lymphocytic leukemia (CLL)

Chronic myelogenous leukemia (CML)

In addition, acute leukemia can be divide into subtype M0-M7 in AML following French-American-British (FAB) criteria that based on blood cell morphology and cytochemistry staining (26-29).

Table 1 FAB classification of acute myeloid leukemia (30)

FAB type	Granulocytopoiesis [%]	Monocytopoiesis [%]	Erythropoiesis [%]	Immune markers
M 0	< 10 POX < 3	< 20	< 50	Lymphoid neg. Myeloid pos.
M 1	< 10			
	POX > 3	< 20	< 50	
M 2	> 10	< 20	<50	
M 3	Hypergranular, Auer rods	< 20	< 50	HLA-DR neg.
M 3v	Microgranular, monocytoid nuclei	< 20	< 50	HLA-DR neg.
M 4	> 20	> 20	< 50	
M 4Eo	> 20	> 20	< 50	
	Abnormal Eo			
M 5a	< 20	>80, immature	< 50	
M 5b	< 20	>80, mature	< 50	
M 6	>30% of NEC are blasts; variable	Variable	> 50	
M 7	> 30 megakaryoblasts	Variable	< 50	CD 41 / CD 61 pos.

M0 Undifferentiated acute myeloblastic leukemia

M1 Acute myeloblastic leukemia with minimal maturation

M2 Acute myeloblastic leukemia with maturation

M3 Acute promyelocytic leukemia

M4 Acute myelomonocytic leukemia

M5 Acute monocytic leukemia

M6 Acute erythroid leukemia

M7 Acute megakaryocytic leukemia

However, The FAB classification had a superficial correlation with clinical outcome. So, the World Health Organization (WHO) classification had combined cytogenetic abnormalities, immunophenotyped and molecular genetic data that become an important criteria of the definition of hematopoietic neoplasms (31). A main difference between WHO classification and FAB classification is the percentage of blast cutoff for AML diagnosis. The percentage of blast cutoff for AML is 20% in WHO classification and 30% in FAB classification (32).

Table 2 2016 WHO classification of myeloid neoplasm and acute leukemia (31) .

WHO myeloid neoplasm and acute leukemia classification
Myeloproliferative neoplasms (MPN)
Chronic myeloid leukemia (CML), BCR-ABL11
Chronic neutrophilic leukemia (CNL)
Polycythemia vera (PV)
Primary myelofibrosis (PMF)
PMF, prefibrotic/early stage
PMF, overt fibrotic stage
Essential thrombocythemia (ET)
Chronic eosinophilic leukemia, not otherwise specified (NOS)
MPN, unclassifiable
Mastocytosis

Myeloid/lymphoid neoplasms with eosinophilia and rearrangement of PDGFRA, PDGFRB, or FGFR1, or with PCM1-JAK2
Myeloid/lymphoid neoplasms with PDGFRA rearrangement
Myeloid/lymphoid neoplasms with PDGFRB rearrangement
Myeloid/lymphoid neoplasms with FGFR1 rearrangement
Provisional entity: Myeloid/lymphoid neoplasms with PCM1-JAK2
Myelodysplastic/myeloproliferative neoplasms (MDS/MPN)
Chronic myelomonocytic leukemia (CMML)
Atypical chronic myeloid leukemia (aCML), BCR-ABL12
Juvenile myelomonocytic leukemia (JMML)
MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T)
MDS/MPN, unclassifiable

Myelodysplastic syndromes (MDS)
MDS with single lineage dysplasia
MDS with ring sideroblasts (MDS-RS)
MDS-RS and single lineage dysplasia
MDS-RS and multilineage dysplasia
MDS with multilineage dysplasia
MDS with excess blasts

MDS with isolated del(5q)
MDS, unclassifiable
Provisional entity: Refractory cytopenia of childhood
Myeloid neoplasms with germ line predisposition
Acute myeloid leukemia (AML) and related neoplasms
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
APL with PML-RARA
AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A
AML with t(6;9)(p23;q34.1);DEK-NUP214
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
Provisional entity: AML with BCR-ABL1
AML with mutated NPM1
AML with biallelic mutations of CEBPA
Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms

AML, NOS
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (TAM)
Myeloid leukemia associated with Down syndrome

2.2 Acute myeloid leukemia (AML)

Acute myeloid leukemia (AML) is a highly proliferative disorder of hematopoietic cells. AML is categorized by an accumulation of abnormal myeloid blast cells, mainly in the bone marrow with rapid progression and impaired of normal blood cells production, resulting in symptoms of hematopoietic insufficiency.

2.2.1 Epidemiology

Acute myeloid leukemia is rare cancer, accounting for about 1.3 % of all new cancer patients in the United States. The annual incidence of AML is estimated 4.0 cases per 100,000 with 5-years survival rate of 25.9 %. The median age at diagnosis is approximately 67 years (33) . The probability of remission in AML treatment is approximately 80% in children and lower than 25% in elderly (1) .

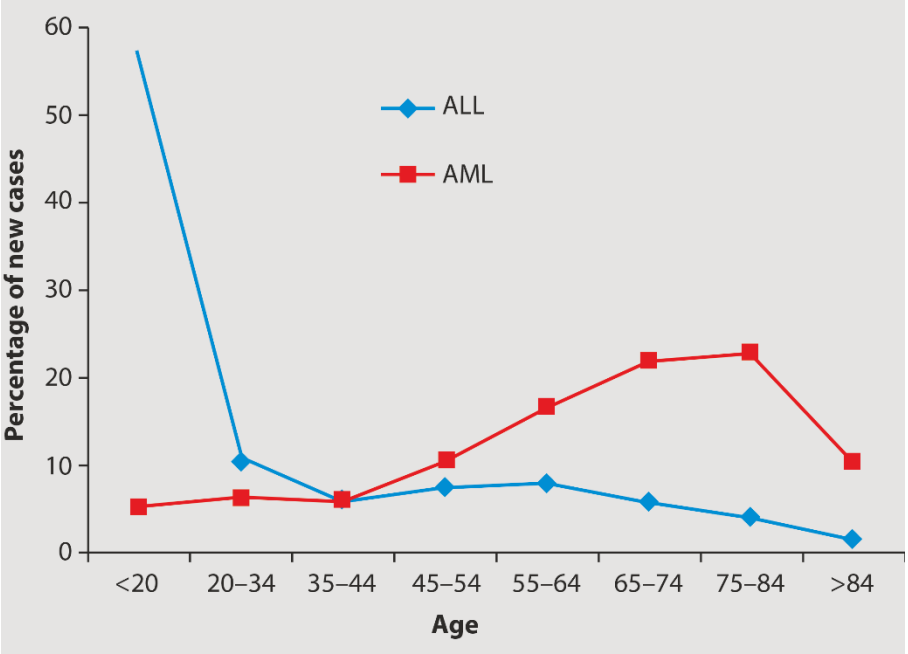


Figure 1 Age-related incidence of acute leukemia (33)

2.2.2 Etiology

The exact cause of leukemia is currently still unclear. However, many studies provide information about the possible causes of human leukemia.

2.2.2.1 Radiation

High-dose radiation exposure has been reported as a risk factor of acute myeloid leukemia. Radiation cause DNA double strand breaks in hematopoietic stem cells and lead to DNA mutations (33). An increased incidence of leukemia was observed in atomic bomb survivors in Japan (34). Radiation alone as therapeutic approach for cancer therapy could slightly increase risk for AML but radiation could highly increase risk for AML in the individuals who received alkylating agents (32).

2.2.2.2 Chemical carcinogens

Chronic exposure to many chemical substances have been reported the association with the development of acute leukemia. Previous study reported the significant relationship between high dose benzene exposure and AML incidence (35). Heavy smoking also increases the risk of AML. The risk of AML incidence are increased 2-3 times in smokers who exposed more than 20 packs per year (2, 36) . Moreover, an augmented risk of AML has been described in persons who chronic exposed to ethylene oxide, pesticide, paint and petroleum (2) .

2.2.2.3 Drugs

Certain cytotoxic drugs have been reported as an etiology of AML. Treatment with alkylating agent such as melphalan and cyclophosphamide has been reported as significant risk factors of AML. Moreover, exposure of DNA topoisomerase II inhibitors is associated with chromosome breakage that increase risk of AML (37, 38).

2.2.2.4 Genetic disorder and chromosome abnormalities

Chromosome abnormalities result in activation or inactivation of transcription factor. For example, t (8;21) (q21;q22) translocation causing AML1gene fused to a novel gene called Eight-Twenty One oncoprotein (ETO) became to AML1/ETO fusion gene and then translation to the chimeric protein called AML1/ETO fusion protein. AML1/ETO fusion protein has been reported its involvement in repress transcription and block myeloid differentiation(39, 40).

In addition, many evidences suggested that genetic abnormality diseases such as Klinefelter syndrome and Down syndrome are important risk factors associated with AML.

2.2.3 Clinical Features

The common clinical presentations in patients with acute myeloid leukemia are caused by leukemic cells infiltration of the bone marrow that results in cytopenia. The typical symptoms of AML may include fatigue, tachycardia, hemorrhage, petechia, mucosal bleeding, spontaneous bruising, menorrhagia, infections, fever, paleness.

Moreover, leukemic cells can infiltrate into other tissues and organs, including lymph nodes (lymphadenopathy), skin (leukemia cutis), liver (hepatomegaly), spleen (splenomegaly), central nervous system (normally found in cerebrospinal fluid (CSF), bone (bone pain) and gum hypertrophy (common in AML subtype M4, M5). Leukemic blast cells infiltration of extramedullary organs is found in 2.5–9% of AML patients (33, 41).

2.2.4 Diagnostic testing

2.2.4.1 Morphology

The primary step in the diagnostic testing of patients with suspected acute myeloid leukemia is cell morphological evaluation of bone marrow smear and/or peripheral blood smear under microscope. A diagnosis of AML is typically based on morphologic finding of ≥ 20% blast cells in myeloid lineage evaluated by a differential count of at least 500 hematopoietic cells in the bone marrow smear (33).

2.2.4.2 Immunophenotyping

Multiparameter flow cytometry analysis (MFC) is a recommended diagnostic testing for acute leukemia. MFC determines concurrently many surface markers on a single cell. Flow cytometry analysis with panels of antibodies against specific marker on hematopoietic cells allow for precise phenotypic characterization of cell populations and classify into leukemia subtypes with different prognosis and treatment requirements (42).

Table 3 Immunophenotypic profiles acute Leukemias (43)

	B-ALL	T-ALL	AML (M0-M2)	AML with t(8;21)	APL (AML-M3)	AML-M4		AML-M5	AML-M6	AML-M7
						Blasts	Monocytes			
CD1a	–	–/+	–	–	–	–	–	–	–	–
CD2	–	+	–	–	–	–	–	–/+	–	–
		(rarely–)	(rarely+)		(rarely+)					
cCD3	–	+	–	–	–	–	–	–	–	–
CD4	–	–/+[a]	+/–	–	–	+/–	+	+/–	–	–
CD5	–	–/+	–	–	–	–	–	–	–	–
CD7	–	+	–/+	–	–	–/+	+/–	–/+	–	–
		(rarely–)								
CD8	–	–/+[a]	–	–	–	–	–	–	–	–
CD10	+/–	–/+	–	–	–	–	–	–	–	–
								(rarely +)		
CD11b	–	–	–	–	–	–	+	+	–	–
CD11c	–	–	–/+	–	–	+/–	+	+	–	–
CD13	–	–	+	+	+	+	+	+(dim)	–	–/dim+
	(rarely+)	(rarely+)								
CD14	–	–	–	–	–	–	+	–/+	–	–
CD19	+	–	–	+	–	–	–	–	–	–
CD20	–	–	–	–	–	–	–	–	–	–
	(rarely+)									
CD22	+	–	–	–	–	–	–	–	–	–
	(rarely–)									
CD33	–	–	+	+	+	+	+	+	–	–
	(rarely+)	(rarely+)	–/+	–/+	(bright)	+/–	–	(bright)	(rarely+)	(rarely+)
CD34	+	+/–			–			–	+/–	–
	(rarely–)				(rarely+)			(rarely+)		(rarely+)
CD41	–	–	–	–	–	–	–	–	–	+
CD45	–/dim+	+	+	+	+	+	+	+	–	+
			(rarely–)					(bright)	(rarely+)	(rarely–)
CD56	–	–	–/+	+	–	–	–/+	+/–	–	–
				(rarely–)	(rarely+)				(rarely+)	(rarely+)
CD61	–	–	–	–	–	–	–	–	–	+
CD64	–	–	+/–	–	+(dim)/–	–	–	+	–	–
CD79a	+	–	–	–	–	–	–	–	–	–
CD117	–	–	+	+	+	+	–	–/+	dim+	dim+
		(rarely+)								
cCD79a	+/–	–	–	–	–	–	–	–	–	–
GPHA	–	–	–	–	–	–	–	–	+	–
									(rarely–)	
HLA–DR	+	–	+	+	–	+	+	+	+	–/dim+
	(rarely–)	(rarely+)	(rarely–)	(rarely–)				(rarely–)		
MPO	–	–	+	+	+	+	–	–	–	–
TdT	+	+/–	–/+	–/+	–	–	–	–	–	–
	(rarely–)									

[a]Usually dual CD4/CD8 positive or negative.

Abbreviations: T-ALL, T-cell acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; AML-M4, acute myelomonocytic leukemia; AML-M5, acute monoblastic leukemia; AML-M6, acute erythroid leukemia; AML-M7, acute megakaryoblastic leukemia; GPHA, glycophorin A; MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase.

2.2.4.3 Cytogenetics and molecular testing

Metaphase karyotyping is an important technique for the detection of chromosomal alterations that involved in several subtypes of AML. At least 20 metaphases should be evaluated.

Fluorescence in situ hybridization (FISH) is normally used to rapidly detect specific chromosomal alterations such as PML-RARA in patients with suspected acute promyelocytic leukemia (APL).

Table 4 Cytogenetic abnormalities observed in acute myelogenous leukemia (1)

Chromosome Abnormality	Genes Affected	Clinical Correlation
Loss or gain of chromosome		
Deletions of part or all of chromosome 5 or 7	Not defined	Frequent in patients with acute myelogenous leukemia (AML) occurring *de novo* and in patients with history of chemical, drug, or radiation exposure and/or previous hematologic disease.[282,283,286,287]
Trisomy 8	Not defined	Very common abnormality in acute myeloblastic leukemia. Poor prognosis, often a secondary change.[283,289]
Translocation		
t(8;21) (q22;q22)	*RUNX1(AML1)–RUNX1T1(ETO)*	Present in ~8% of patients <50 years old and in 3% of patients >50 years old with AML.[288] Approximately 75% of cases have additional cytogenetic abnormalities, including loss of Y in males or X in females. Secondary cooperative mutations of *KRAS, NRAS, KIT* common. Present in ~40% of myelomonocytic phenotype. Higher frequency of myeloid sarcomas.[263–266]
t(15;17) (q31; q22)	*PML-RAR-α*	Represents ~6% of cases of AML.[288] Translocation involving chromosome 17, t(15;17), t(11;17), or t(5;17) is present in most cases of promyelocytic leukemia.[290,291]
t(9;11); (p22; q23)	*MLL (especially MLLT3)*	Present in ~7% of cases of AML. Associated with monocytic leukemia.[292,293] 11q23 translocations in 60% of infants with AML and carries poor prognosis. Rearranges *MLL* gene.[292–296] Many translocation partners for 11q23 translocation.[295–298] *MLL1, MLL4, MLL10* may also result in AML phenotype.
t(9;22) (q34; q22)	*BCR-ABL1*	Present in ~2% of patients with AML.[299,300]
t(1;22)(p13;q13)	*RBMIS-MKL1*	<1% of cases of AML. Admixture of myeloblasts, megakaryoblasts, micromegakaryocytes with cytoplasmic blebbing, dysmorphic megakaryocytes. Reticulin fibrosis common.[301]
t(10;11) (p12-13;q14-21)	*PICALM-MLLT10*	Outcome similar to that of intermediate prognosis group; more extramedullary disease and CD7 expression.[302]
Inversion		
Inv(16) (p13.1;q22) or t(16;16) (p13.1;q22)	*CBF-β MYH11*	Present in ~8% of patients <50 years of age and in ~3% of patients >50 years of age with AML[288]; often acute myelomonocytic phenotype; associated with increased marrow eosinophils; predisposition to cervical lymphadenopathy,[303] better response to therapy.[304–307] Predisposed to myeloid sarcoma.
Inv(3) (q21q26.2)	*RPN1-EVI1*	~1% of cases of AML. Approximately 85% of cases with normal or increased platelet count. Marrow has increased dysmorphic, hypolobulated megakaryocytes. Hepatosplenomegaly more frequent than usual in AML.[308]

On the molecular scale, testing for NPM1 mutations, Fms-like tyrosine kinase 3 (FLT3) internal tandem duplication (ITD), and CEBPA mutations should be considered as the minimum requirement for molecular genetic testing mainly in patients with normal cytogenetics. For patients with FLT3-ITD, the mutant-to-wild type ratio should be reported. Testing for additional gene mutations should be considered optional in routine practice (33).

Table 5 common gene mutations in AML patients with normal cytogenetics (1)

Mutated Gene	Approximate Frequency in AML with Normal Karyotype (%)	Implication	Comments
NPM1	50	More-favorable outcomes	Most frequently mutated gene in AML. Allogenic transplantation not needed in first remission if this mutation occurs in absence of mutated *FLT3*-ITD
FLT3 ITD	40	Less-favorable outcomes	
DNMT3A	20	Less-favorable outcomes	Seen more often in AML patients with normal cytogenetics. Mutant *NPM1*, *FLT3-ITD*, and *IDH1* have been found more frequently in AML patients with *DNMT3A* mutations compared to those with wild-type *DNMT3A*
RUNX1	15	Less-favorable outcomes	
TET2	15	Less-favorable outcomes	Coincidence of mutated *TET2 with NPM1* mutation in the absence of *FLT3-ITD* mutation predicts a less-favorable outcome
CEBPA	15	More-favorable outcomes	Only cases with double mutations associated with favorable outcomes
NRAS	10	Little effect on prognosis	
IDH1 or IDH2	10	Little effect on outcomes	More frequent in AML patients with normal cytogenetics. Frequently associated with *NPM1*. Adverse prognostic factor if present with mutated *NPM1* without *FLT3-ITD*. Serum 2-hydroxyglutarate levels indicate high probability of *IDH* mutation
MLL-PTD	8	Less-favorable outcomes	
WT1	6	Less-favorable outcomes	More frequent in females than in males (6.6 vs. 4.7%; P = 0.014) and in patients <60 than in patients >60 years (P <0.001)
FLT3-TKD	6	Little effect on outcomes	May appear after use of FLT3-ITD inhibitor

2.2.4.4 Lumbar puncture

Lumbar puncture is commonly optional only in AML patients with cerebrospinal fluid (CSF) involvement that present neurologic symptoms such as confusion and focal neurological defects.

Table 6 Diagnostic testing for patients with acute leukemia (33)

Diagnostic test	Recommendation
Complete blood count with differential count	Mandatory
Bone marrow aspiration: • Morphological evaluation of May-Grünwald-Giemsa-, Wright-Giemsa- or Pappenheim-stained slides • Myeloperoxidase and non-specific esterase stains • Iron staining in cases with multilineage dysplasia	Mandatory
Bone marrow core biopsy: • Morphological evaluation (H&E stain) • Immunohistochemistry	Recommended/mandatory in patients with a dry tap
Flow cytometry: • Can be performed on bone marrow or blood	Mandatory
Cytogenetics: • Karyotyping of G-banded metaphase chromosomes	Mandatory
Genetics (AML):	
• Rapid testing for *PML-RARA* by FISH or PCR	Mandatory if APL suspected
• Testing for *NPM1*, *FLT3*, and *CEBPA* gene mutations	Mandatory in patients with normal cytogenetics
• Testing for KIT gene mutations	Recommended in patients with CBF leukemias
• Other molecular markers	Optional
Genetics (ALL):	
• Testing for *BCR-ABL1* rearrangement by FISH and/or PCR	Mandatory
• Testing for clonal rearrangement of immunoglobulin or TCR genes	Recommended for later MRD monitoring
• Assessment for hypo-/hyperdiploidy by flow cytometry	Optional
Lumbar puncture	ALL: Mandatory AML: Optional / mandatory in patients with clinical symptoms suspicious of CNS involvement
Biobanking of pretreatment bone marrow and / or blood	Recommended

2.2.5 Treatment of acute myeloid leukemia

2.2.5.1 Chemotherapy

The primary objective of induction therapy in AML is achievement of complete remission. The major of AML conventional induction chemotherapy regimens is the antimetabolite cytarabine (ara-C) in combination with anthracycline including daunorubicin, doxorubicin, idarubicin and epirubicin. Cytarabine is generally administered as a continuous infusion of 100 to 200 mg/m^2/day for 7 days, in combination with an anthracycline administered in an appropriate dose by intravenous for 3 days. The ancestor of this combination is the so-called '7+3'. Cytarabine is a cell cycle S-phase–specific antimetabolite that involved in interference of DNA synthesis whereas anthracyclines are DNA intercalators which their mode of action is thought to be inhibition of topoisomerase II, resulting in DNA breaks. (2, 33)

2.2.5.2 Stem cell transplantation

Allogeneic stem cell transplantation is important in the post-remission treatment of younger patients with AML. Many evidences reported that stem cell transplantation confer a survival advantage for AML patients. HLA-identical related and unrelated stem cell donors can be used but because of earlier availability and less costs, sibling donor is the main choice. Both peripheral blood and bone marrow can be used as source of stem cells with similar outcome. If HLA-identical or

compatible donor is not available, alternative stem cell sources from cord blood or haploidentical donor can be used (33).

2.2.5.3 Drug conjugated antibody

Drug conjugated antibody therapy with CD33 is the best target because CD33 is express on AML cells in 90% of cases and is expressed on normal hematopoietic cells but not on normal hematopoietic stem cells. The most noteworthy anti-CD33 antibody therapy in clinical application is gemtuzumab ozogamicin, which uses anti-CD33 monoclonal antibody conjugated with calicheamicin, toxin-mediated cytotoxicity that incorporated to the DNA minor grooves, inducing DNA double strand breaks and apoptosis (2, 33).

2.2.5.4 Molecular targeting agents

Various molecular targeting agents including FLT3 Inhibitors, Kit Tyrosine Kinase Inhibitors, Nuclear Factor-Kappa B Inhibitor, Antisense agents to anti-apoptotic proteins, Farnesyltransferase Inhibitors and Geranylgeranyltransferase-1 Inhibitors are in clinical trial for combination of these agent with standard chemotherapy for further improve the survival rate of AML patients (44, 45).

2.3 Plant-derived compounds as alternative medicine

The development of anti-cancer agent form natural source started in 1950s. The first compounds that succeed for clinical used are vinblastine (VLB) and vincristine

(VCR), the vinca alkoloids isolated from *Catharanthus roseus* G. Don. Semi-synthetic analogs of these agents are vinorelbine (VRLB) and vindesine (VDS). The compounds are used in combination with current chemotherapeutic agents for cancer treatment, including testicular cancer, breast cancer, lung cancers, and Kaposi's sarcoma, leukemia and lymphoma. Etoposide (VM 26) and teniposide (VP 16-213), semi-synthetic derivatives epipodophyllotoxin, have also been reported as active compounds for treatment of skin cancer, testicular cancer and lung cancer. Paclitaxel (Taxol®) isolated from the *Taxus* species is used in the treatment of ovarian cancer, breast cancer, Kaposi sarcoma, non-small cell lung cancer (NSCLC), rheumatoid arthritis, multiple sclerosis, and psoriasis while docetaxel (Taxotere®), an active paclitaxel analogs, is used in the treatment of breast cancer and non-small cell lung cancer (NSCLC). Homoharringtonine, isolated from the Chinese tree *Cephalotaxus harringtonia* are used for AML and CML treatment in China. Flavopiridol is a synthetic flavone, isolated from *Dysoxylum binectariferum*. Flavopiridol is currently in clinical trials against leukemia and lymphomas (46, 47).

Currently, many studies suggested that quercetin, the most common flavonols in various fruits and vegetables exhibit anticancer properties in many types of cancer including leukemia (5, 13).

2.4 Quercetin

Quercetin (3,3',4',5,7-pentahydroxyl-flavone) is the most common flavonols that present in various fruits and vegetables. Quercetin is found in plants in many different glycosidic forms as its derivatives. The chemical structure of quercetin and its derivatives are shown in Figure 3 and 4. (5, 48, 49)

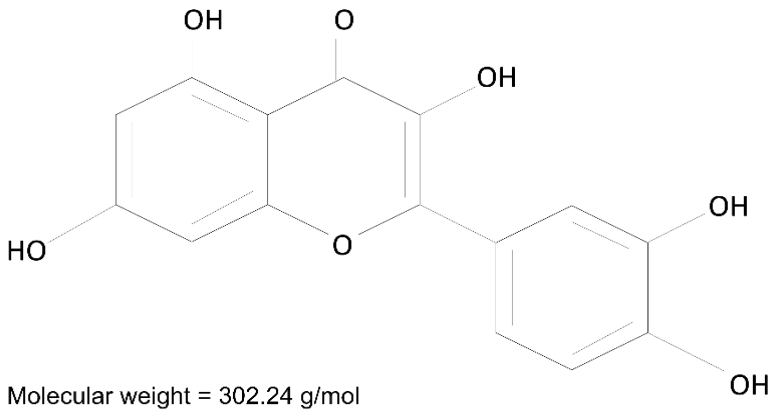


Figure 2 Chemical structure of quercetin

Many evidences reported that quercetin exhibit several beneficial biological properties such as antioxidant (50), antimicrobial (51), anti-inflammatory (52), neuroprotective effect and a potential anticancer in many types of cancer including leukemia (13, 53).

Anti-leukemic activities of quercetin have been reported in many studies. For example, Larocca et al. reported that quercetin possess growth inhibitory effect on

several ALL and AML primary cells from acute leukemia patients (14). Teofili et al. found that when used cytarabine, standard chemotherapeutic agent combination with 1 μM of quercetin produced an increase inhibitory effect than that observed at the corresponding dose of the drug alone in HL-60 cell. These result indicated that the combination of quercetin with cytarabine resulted in a synergistic inhibitory activity on leukemic cells (54). Yoshida et al. demonstrated that quercetin is an effective agent that arrests cell proliferation in the G1 phase of the cell cycle by suppress the expression of cyclin A and p34 cdc2 in CEM leukemic human T-cells (55). Xiao et al. reported that quercetin induced apoptosis in human leukemia HL-60 cell line via downregulation of bcl-2 gene expression level (56). Maso et al. found that quercetin induced apoptosis in MDS-chronic myelomonocytic leukemia (CMML) P39 cell line through downregulation of anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1 proteins), upregulation of Bax protein, activation of cytochrome-c release to cytosol and caspases activation as well as induction of autphagosome formation (57). These finding suggested that quercetin is a desirable bioactive compound that modulated programmed cell death in leukemic cells.

2.5 Programmed cell death

Programmed cell death (PCD) is a terminal process for removal of long lived, abnormal and damaged cells to maintain homeostasis. Cell death can be classified

into many types according to morphological characteristic, enzymological criteria, functional features or immunological characteristics(58).

One of the hallmarks of cancer is resisting cell death (59). So, finding new therapeutic approaches to activate programmed cell death in cancer cell are important for the combating cancers including leukemia.

There are two prominent of programmed cell death that represents the most interesting target mechanism for the induction of cell death in leukemia, apoptosis and autophagy.

2.5.1 Apoptosis

Apoptosis, also known as type I programmed cell death is an evolutionally conserved form of cell death process that characterize by cell shrinkage, chromatin condensation, nuclear collapse, plasma membrane blebing, apoptotic bodies formation and phagocytosis of the apoptotic bodies by macrophages. Cells undergo apoptosis by using at least 2 pathways. There are the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (60).

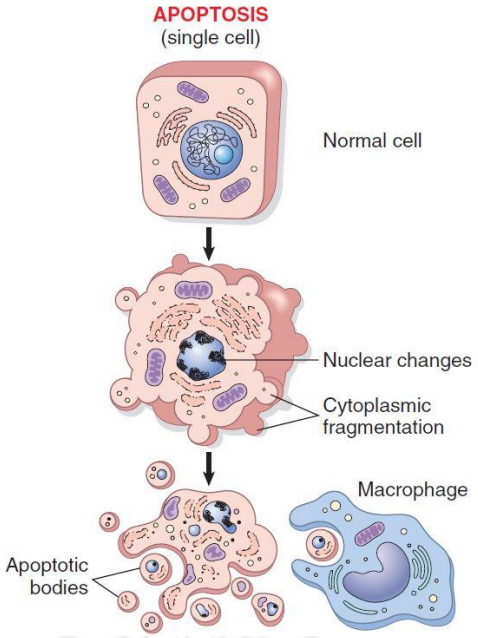


Figure 3 Characteristics of apoptosis mechanism (61).

2.5.1.1Extrinsic Pathway

The extrinsic pathway of apoptosis is activated by the interaction of extracellular protein and cell surface death receptors in tumor necrosis factor (TNF) receptor gene superfamily. These death receptors contain death domain that plays a crucial role in conducting the cell death signal from the cell surface to the intracellular signaling pathways.

A well-known example of death receptors is Fas death receptors. The interaction of Fas ligand to Fas receptor results in the recruitment of the adapter protein FADD followed by the recruitment of procaspase-8 or procaspase-10 to form death-inducing signaling complex (DISC) that lead to auto-catalytic activation of

procaspase-8 and procaspase-10. Then, activated procaspase-8 and 10 trigger downstream executioner procaspase to induce apoptotic process.

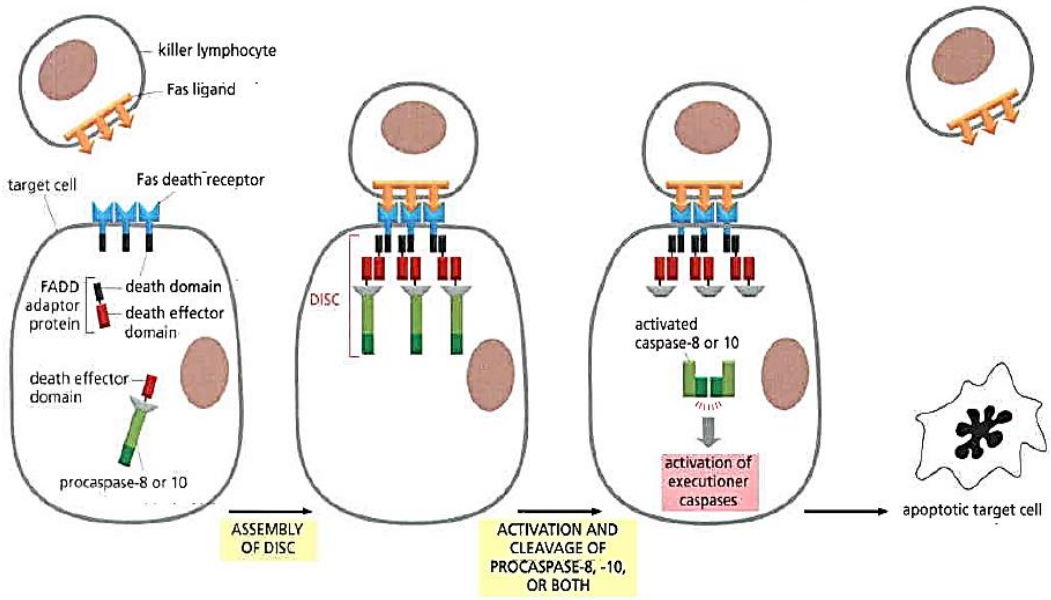


Figure 4 Extrinsic pathway of apoptosis activated through Fas death receptors (25)

2.5.1.2 Intrinsic pathway

The intrinsic pathway of apoptosis is triggered by non-receptor-mediated stimuli that produce intracellular signals that causes a decrease in mitochondrial membrane potential and release of cytochrome c proteins from the intermembrane space of mitochondrial into the cytosol. When cytochrome c are released into cytosol, cytochrome c binds and activates Apaf-1 as well as procaspase-9 to form an apoptosome that leading to an activation of procaspase-9. Then, activated procaspase-9 trigger downstream executioner procaspase to induce apoptosis.

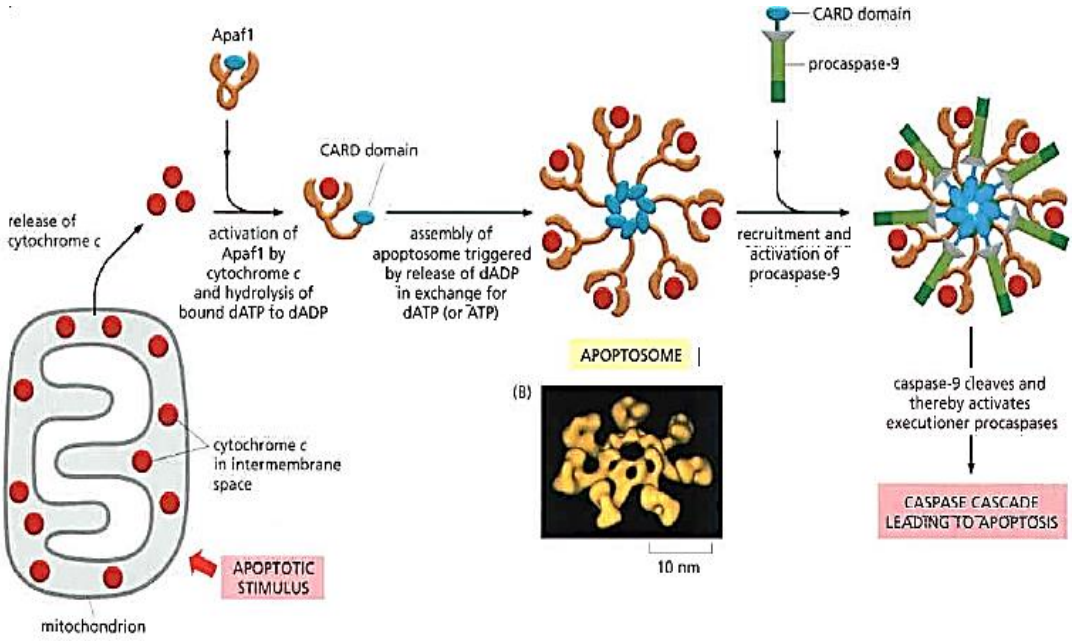


Figure 5 The intrinsic pathway of apoptosis (25)

2.5.1.3 The Bcl-2 family in apoptosis regulation

Bcl-2 family including anti-apoptotic proteins, pro-apoptotic BH123 proteins and pro-apoptotic BH3 only proteins are major class of intracellular regulators of apoptosis.The anti-apoptotic subfamily such as Bcl-2, Bcl-xL, and Mcl-1 contain four BH domains. The pro-apoptotic BH123 proteins including Bax and Bak contain three BH domains; BH1, BH2 and BH3. The pro-apoptotic BH3 only proteins including Bad, Bid, Bim, Noxa and Puma contain one BH3 domain as shown in figure 6. (62, 63)

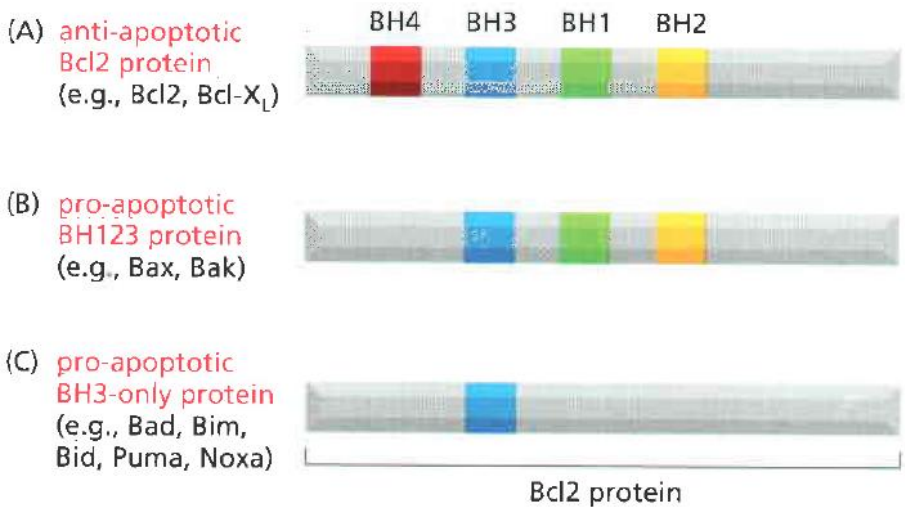


Figure 6. The three subfamilies of BCL-2 family protein (25)

Bcl-2 family proteins regulate apoptosis through their abilities to regulate cytochrome c release from the intermembrane space of mitochondrial into the cytosol. In response to apoptotic stimuli, pro-apoptotic BH123 proteins including Bax and Bak translocates to the mitochondrial membrane and aggregate on the outer mitochondrial membrane lead to facilitating the release of cytochrome c from the mitochondrial intermembrane space into the cytosol resulting in an activation of the intrinsic apoptosis pathway.

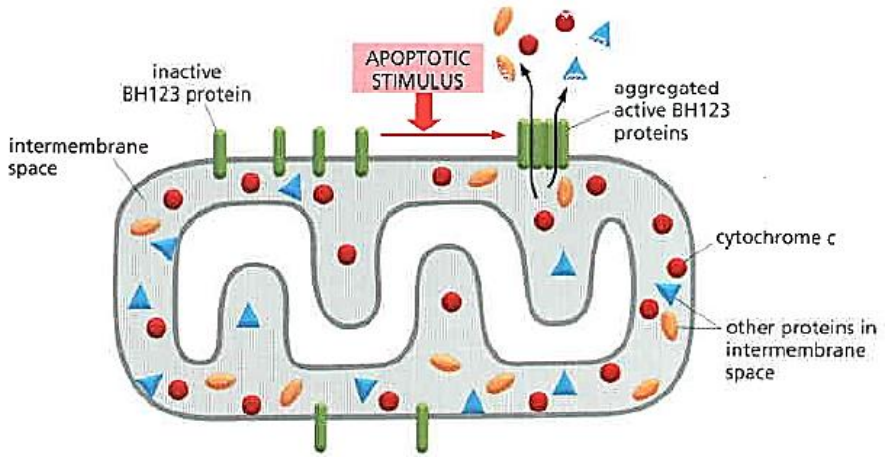


Figure 7 The role of BH123 proteins in the release of cytochrome c (25)

The anti-apoptotic proteins such as Bcl-2 and Bcl-xL can inhibit apoptosis pathway by binding to pro-apoptotic BH123 proteins and prevent pro-apoptotic BH123 from oligomerization, thereby inhibiting the release of cytochrome c from the mitochondrial intermembrane space into the cytosol.

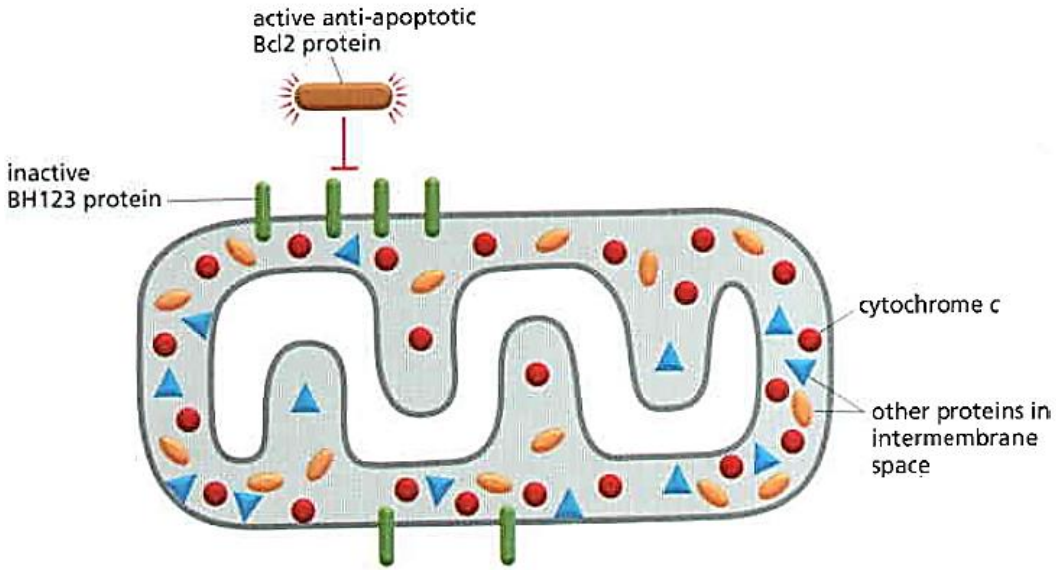


Figure 8 The role of anti-apoptotic proteins in inhibiting the release of cytochrome c (25)

The BH3-only proteins such as Bad and Noxa preferentially binding to anti-apoptotic Bcl-2 proteins result in dissociating Bcl-2 from pro-apoptotic BH123 proteins. When Bcl-2 are dissociated from pro-apoptotic BH123 proteins, they can no longer inhibit pro-apoptotic BH123 proteins which now become activated adnd aggregate on the outer mitochondrial membrane lead to facilitating the release of cytochrome c.

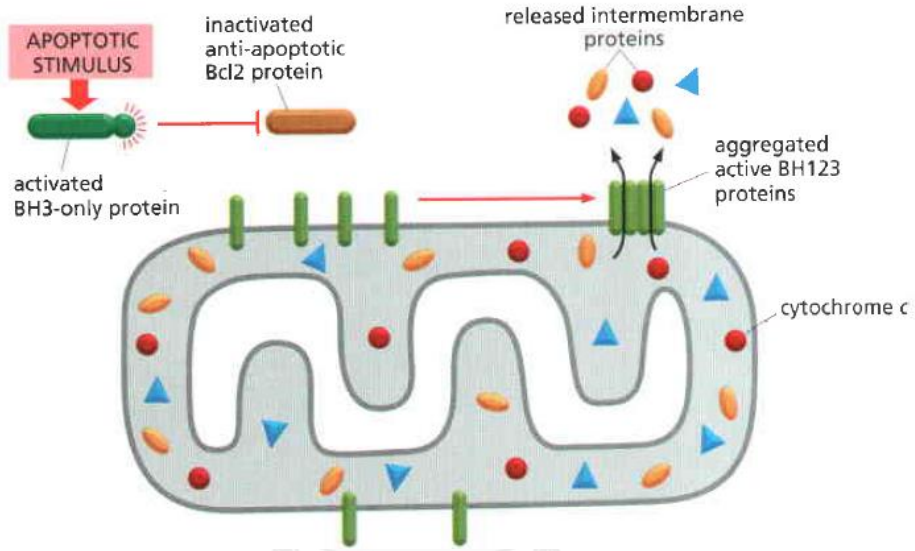


Figure 9 The role of BH3 only proteins in the release of cytochrome c (25)

2.5.2 Autophagy

Autophagy is a catabolic process that associated with lysosomal degradation of long- lived and damaged proteins and organelles. Autophagy process is defined by the sequestration of aged, damaged or unwanted proteins and organelles in double or multimembrane vesicles and delivery and fuse with their own lysosomes system for subsequent degradation.

The process of autophagy can be divided: 1) induction 2) autophagosome nucleation 3) elongation and completion 4) lysosomal fusion 5) degradation.

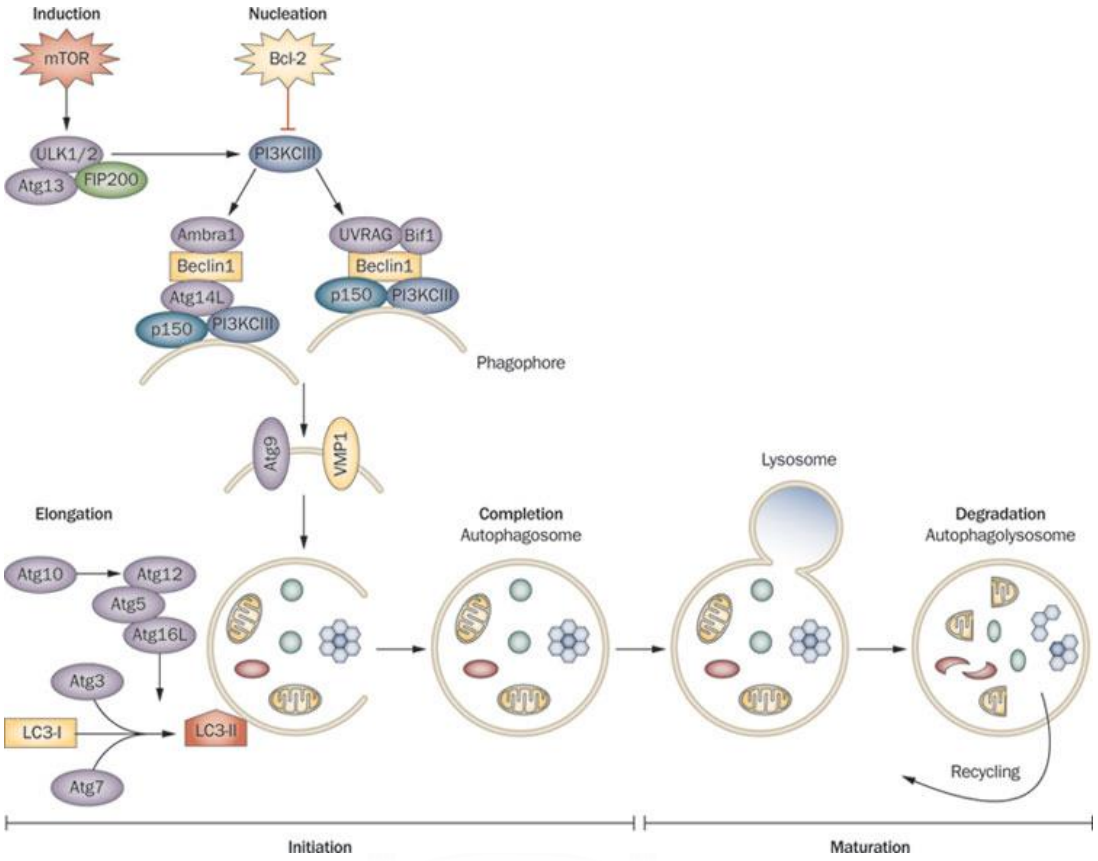


Figure 10 Various step of autophagy process (64)

In the induction step, autophagy related gene-1 (ATG1) complex, consist of Atg1(ULK1/2), Atg13(mAtg13) and Atg17(FIP200) are activated controlling by mammalian target of rapamycin complex 1(mTORC1). Atg13 is phosphorylated by mTORC1 that modulates its binding to Atg1 and Atg17 whereas inactivation of mTORC1 leads to de-phosphorylation of Atg13 lead to increasing Atg1-Atg13-Atg17 complex formation and trigger autophagy pathway (65).

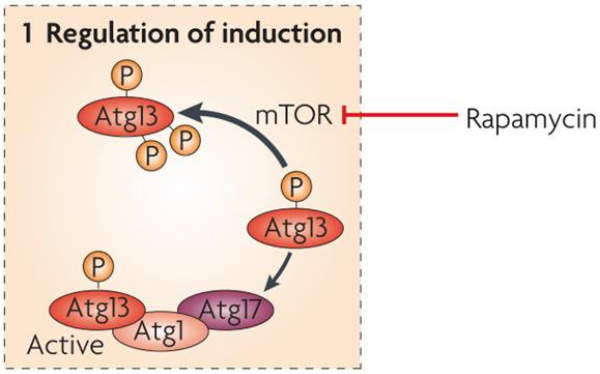


Figure 11. Induction step of autophagy process (17)

Following induction step, autophagosome nucleation requires the activation of Beclin-1 core complex, compose of Beclin-1, class III PI3K/hVps34 and p150/hVps15 to recruit lipids and proteins for autophagosome formation (18).

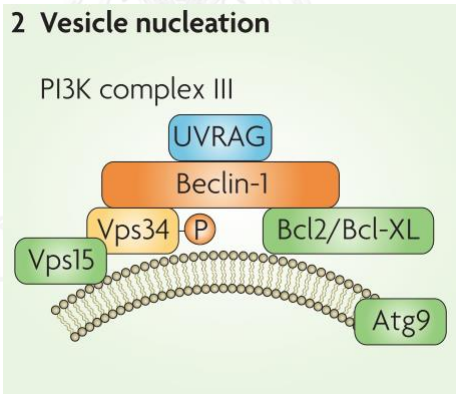


Figure 12. Nucleation step of autophagy process (17)

After that, elongation and completion are required two ubiquitin-like conjugation systems. The first system consists of ATG7 (E1-like enzymes) and ATG10 (E2-like enzymes) which required for the conjugation of ATG12 to ATG5, followed by cooperates with ATG16L to form bulky multimeric called ATG12–ATG5–ATG16L

complex. Vesicle membrane binding with the ATG12–ATG5–ATG16L complex is needed for determining the sites of Atg8/LC3 lipidation. The second system consist of ATG7 (E1-like enzymes) and ATG3 (E2-like enzymes). In this system, Atg8/LC3 is cleaved at C terminal by Atg4 to originate the cytosolic LC3-I with C-terminal glycine residue. Then, cytosolic LC3-I is conjugated to phosphatidylethanolamine (PE) by Atg7 and Atg3 activities. Lipidation with PE converts LC3-I into LC3-II which is stably binding to the autophagosome membrane. LC3-II is associated with the accumulation of organelles and proteins and into the autophagosome. So, for these reason, LC3-II is one of hallmark of autophagy process (17, 18, 65).

Next, autophagosome fuses with lysosome, result in autophagosome degradation by acid hydrolase.

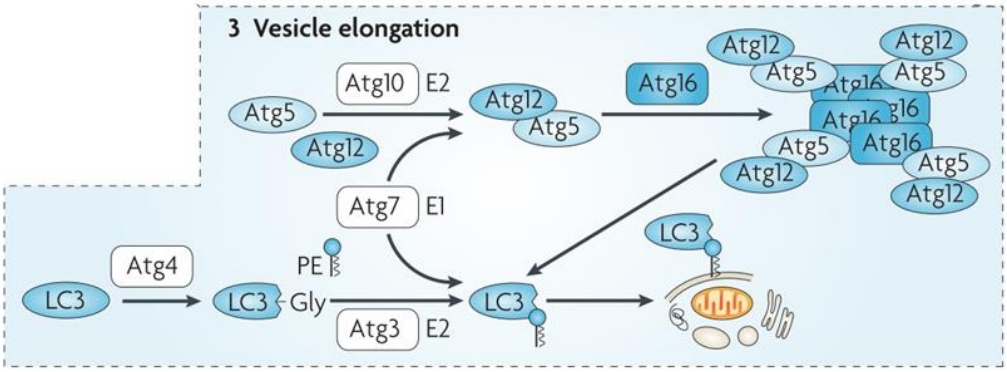


Figure 13. Elogation step of autophagy process (17)

2.5.2.1 The Bcl-2 family in autophagy regulation

As described above, Beclin 1 is a crucial molecule that participates in autophagosome formation in autophagy process. Beclin 1 can be present in two different complexes, one that stimulates autophagy by an interaction with class III PI3K/hVps34 and another that inhibits autophagy by an interaction with Bcl-2 and Bcl-xL. The BH3 domains of BH3-only proteins such as Bad competitively disrupt the interaction between Beclin 1 and Bcl-2 that lead to an activation of the kinase activity of class III PI3K/hVps34, thereby stimulates autophagy process (17, 62, 63) (66).

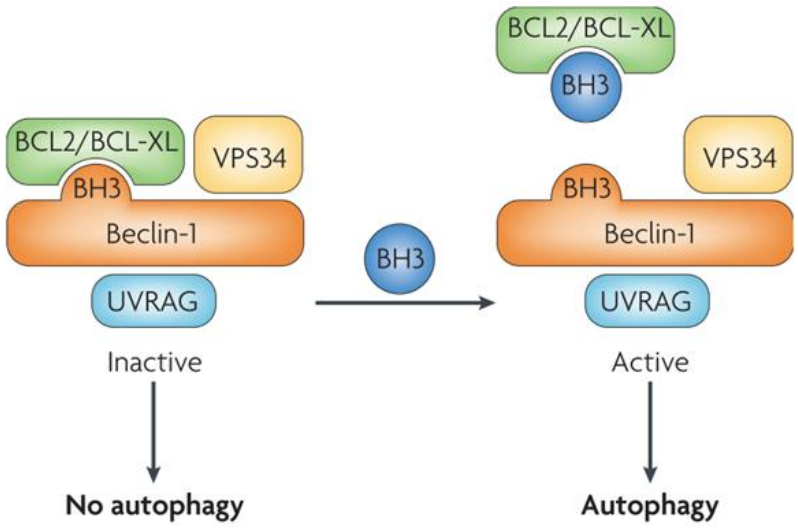


Figure 14. The Bcl-2 family in autophagy regulation (17)

CHAPTER III

Materials and methods

3.1 Materials

3.1.1 Human acute myeloid leukemia cell line

U937 (Human leukemic monocyte lymphoma cells)

(ATCC® CRL-1593.2™)

3.1.2 Reagents

N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Bio Basic Canada Inc., Canada
3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium Bromide (MTT)	AppliChem GmbH, Germany
3-Methyladenine (3-MA)	TOCRIS Bioscience Inc., USA
Acrylamide	Bio Basic Canada inc., Canada
Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit	Thermo Fisher Scientific, USA
Ammonium persulphate (APS)	Bio Basic Canada Inc., Canada
Anti-rabbit IgG, HRP-linked Antibody #7074	Cell Signaling Technology, USA
Bad (D24A9) Rabbit mAb #9239	Cell Signaling Technology, USA

Bcl-2 (D55G8) Rabbit mAb (Human Specific) #4223	Cell Signaling Technology, USA
Beta actin (D6A8) Rabbit mAb #8457	Cell Signaling Technology, USA
Bio-Rad protein assay	Bio-Rad Laboratories, Inc., USA
Bovine serum albumin (BSA)	Sigma Aldrich, USA
Coomassie blue G-250	Bio Basic Canada Inc., Canada
Cytosar®(Cytarabine)	Pfizer Inc, USA
Dimethyl sulfoxide (DMSO)	Thermo Fisher Scientific, USA
Disodium hydrogen phosphate (Na_2HPO_4)	Merck Millipore, Germany
Dithiothreitol (DTT)	Bio Basic Canada Inc., Canada
Ethylenediaminetetraacetic acid (EDTA)	May & Baker Nigeria Plc., UK
Fetal bovine serum (FBS)	Thermo Fisher Scientific, USA
FlowCellect™ Autophagy LC3 Antibody-based Assay Kit	Merck Millipore, Germany
Glycerol	Amreaco Inc., USA
Glycine	Ajax Finechem, Australia
Hydrochloric acid (HCl)	Merck Millipore, Germany
Hypersensitive ECL chemiluminiscence substrate	Boster Biological Technology, USA
Methanol	Tedia, USA

Muse® Count &Viability reagent	Merck Millipore, Germany
N, N'-methylenebisacrylamide	Bio Basic Canada Inc., Canada
Nonfat Dry Milk	Anlene, Thailand
Nonidet-P40 (NP-40)	AppliChem GmbH, Germany
Penicillin-Streptomycin	Thermo Fisher Scientific, USA
Phenylmethylsulfonyl fluoride (PMSF)	Merck Millipore, Germany
Phospho-Bad (Ser112) (40A9) Rabbit mAb #5284	Cell Signaling Technology, USA
Potassium chloride (KCl)	Merck Millipore, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck Millipore, Germany
Protein ladder	Thermo Fisher Scientific, USA
Quercetin	Sigma Aldrich, USA
RPMI 1640 medium	Thermo Fisher Scientific, USA
Sodium dodecyl sulfate (SDS)	Vivantis Technologies, Malaysia
Sodium chloride (NaCl)	Merck Millipore, Germany
Sodium fluoride (NaF)	Sigma Aldrich, USA
Tris-base	Vivantis Technologies, Malaysia
Trypan blue solution	Thermo Fisher Scientific, USA
Tween-20	Bio Basic Canada Inc., Canada

3.1.3 Instruments

-20 ๐C freezer	Sanyo, Japan
-80 ๐C freezer	Ilshin Lab, Netherlands
4 ๐C refrigerator	Mitsubishi, Japan
Analytical balance	Mettler Toledo, Switzerland
Autoclave	Tomy, Japan
Autopipette 0.5 – 10 ul	Labnet International Inc., USA
Autopipette 2.0 – 20 ul	Gilson, French
Autopipette 20 – 200 ul	Gilson, French
Autopipette 100 – 1000 ul	Gilson, French
Benchtop centrifuge	Hettich Lab Technology, Germany
Micro high speed refrigerated centrifuge	Vision scientific, Korea
CO_2 incubator	Thermo Fisher Scientific, USA
Digital differential cell counter	Modulus, USA
ELISA plate reader	BioTek, USA
Flow cytometer	BD Biosciences, USA
Hemocytometer	HBG Henneberg-Sander GmbH, Germany
Inverted microscope	Olympus, Japan
Laminar flow	Faster, Italy

Light microscope	Olympus, Japan
Liquid nitrogen tank	Air Liquide, French
Vortex mixer	Labnet, USA
Waterbath	Mgw Lauda, Germany
Vertical Electrophoresis cell	Bio-Rad Laboratories, Inc., USA
Power supply	Hoefer, USA
Tank blotting cells	Bio-Rad Laboratories, Inc., USA
Chemiluminescent imaging system	Syngene, United Kingdom
pH meter	Mettler Toledo, Switzerland
Pipette boy	Scilogex, USA

3.1.4 Glassware and plastic ware

25 cm2 culture flasks	SPL Life Sciences, Korea
75 cm2 culture flasks	SPL Life Sciences, Korea
6 wells cell culture plates	SPL Life Sciences, Korea
12 wells cell culture plates	SPL Life Sciences, Korea
96 wells cell culture plates	SPL Life Sciences, Korea
Centrifuge tube (15, 50 ml)	SPL Life Sciences, Korea
Serological pipette 10 ml	SPL Life Sciences, Korea
Beaker glass (50, 250, 500, 1000 ml)	Schott Duran, Germany

Bottle glass (100, 500, 1000 ml)	Schott Duran, Germany
Cylinder (100, 1000 ml)	Witeg Diffico, Germany
Micro centrifuge tube 1.5 ml	Eppendorf, Germany
Pipette tip 10, 200 and 1000 ul	KIRGEN Bioscience Inc., China
Cryovial tube 2.0 ml	Nunc, Denmark
BD Falcon™ Round-Bottom Tubes	BD Biosciences, USA
Polyvinylidene difluoride (PVDF)	Merck Millipore, Germany
Whatman filter paper	GE Healthcare, UK

3.2 Methods

3.2.1 Cell culture condition

U937 (Human leukemic monocyte lymphoma cells) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/mL streptomycin and 100U/mL penicillin at 37°C in and 5% CO_2. Culture medium was changed every 3 days.

3.2.2 Cell count and viability

U937 cells were counted and calculated the percentage of cell viability before started the experiments using hemocytometer and stained with 0.4 % trypan blue solution. All experiments were performed when the percentage of cell viability of U937 cells reached more than 90 %. Cell density and the percentage of cell viability were calculated using the following formula:

Cell density (cells/ml) = Average cell number per square (1 x 1 x 0.1 mm^3) x dilution factor x 10^4

$$\textbf{\% Viability (\%)} = \frac{\text{The number of viable cells}}{\text{The number of total cells}}$$

3.2.3 Quercetin preparation

Quercetin was dissolved in DMSO and diluted with RPMI-1640 medium to reach indicated concentrations that contain lower than 0.1 % DMSO. Stock solution of

quercetin was stored at -20 °C. Quercetin at final concentrations of each experiment were prepared freshly prior used.

3.2.4 Treatment cell with quercetin

U937 cells were treated with various concentrations of quercetin for 24 h in 5% CO_2 incubator. 0.1% DMSO treated cells were used as negative control. Cytarabine 200 ug/ml was used as positive control.

3.2.5 Cell cytotoxicity assay

The effect of quercetin on cell cytotoxicity in AML cell line was determined by using MTT based assay. The principle of MTT assay is based on the activity of specific mitochondrial enzymes which are inactivated after cell death. 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium Bromide (MTT) is a yellow compound that when reduced by mitochondrial reductase of viable cells, the tetrazolium ring of MTT, becomes purple formazan crystals, which are water insoluble. The formazan products were dissolved in solubilizing reagent. The amount of formazan products is directly proportional to cell number, indicating cell cytotoxic effect of tested compound. The absorbance of the solution is measured by spectrophotometer at 570 nm (67).

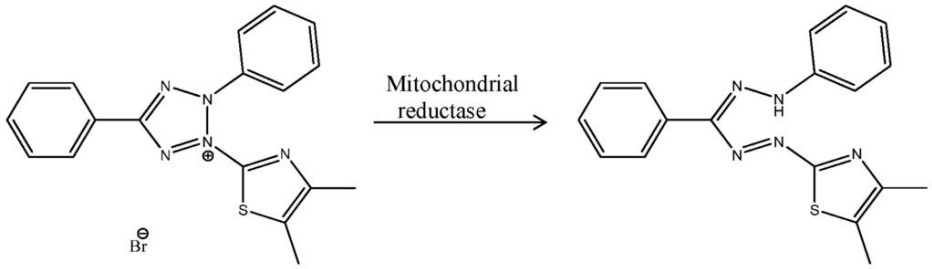


Figure 15 Formazan crystals formation by the reduction of mitochondrial reductase enzyme (68)

U937 cells were seeded in 96 well plates at a density of 5 x 10^5 cells/ml. Cells were treated with 25-200 uM (final concentrations) of quercetin for 24 h in 5% CO_2 incubator. 0.1% DMSO treated cells were used as negative control and cytarabine 200 ug/ml was used as positive control. After incubation time, MTT solution (5mg/ml in Phosphate-buffered saline (PBS) pH 7.4) was added into each well and incubated for 4 h at 37°C in 5% CO_2 incubator. Then, formazan products were dissolved in 10% SDS-HCl solution, and the optical density (OD) at 570 nm was measured by microplate reader. The percentage of cell growth inhibition was calculated with the following formula:

$$\% \text{ Growth inhibition} = \frac{(OD_{\text{negative control}} - OD_{\text{test}})}{OD_{\text{negative control}}} \times 100\ \%$$

The half maximal inhibitory concentration (IC50) is calculated from percent growth inhibition graph using linear regression analysis.

3.2.6 Cell viability assay

U937 cells (5 x 10^5 cell/ml) were incubated with different concentrations of quercetin for 24 h in 5% CO_2 incubator. Then, quercetin-treated cells were stained with two DNA binding dyes presented in Muse® Count &Viability reagent (Merck Millipore) according to the manufacturer's protocol (69). In brief, after treatment with quercetin for 24 h, diluted the cells sample 1:10 with Muse® Count &Viability reagent in microcentrifuge tubes and gently mixed. Allowed cells to stain for 5 minutes at room temperature. Finally, analyzed data and calculated the percentage of cell viability by Muse™ Cell Analyzer (Merck Millipore).

3.2.7 Microscopic analysis

Morphological changes in cell death were investigated by inverted light microscopy. U937 cells were cultured in six-well plates and treated with different concentration of quercetin (25 and 50 uM) for 24 h at 37°C in 5% CO_2 incubator, after that quercetin-treated cells were subjected to photography by CCD camera.

3.2.8 Cell apoptosis assay

The principle of apoptosis assay by Annexin V and Propidium iodide staining is based on the alteration of plasma membrane and DNA fragmentation during apoptotic process.

In living cells, phosphatidylserine (PS) is located on inner leaflet of the plasma membrane. However, in early stage of apoptosis, phosphatidylserine is translocated

from the inner to the outer leaflet of the plasma membrane. Annexin V binds specifically to phosphatidylserine. So, Fluorescent- labelled Annexin V can be used to detect apoptotic cells.

The cell membrane integrity of living cell and early apoptotic cell have ability to excludes propidium iodide(PI), whereas the membranes of late apoptotic and necrotic cells are permeable to PI. Thus, dual staining using Annexin V and propidium iodide allows for the discrimination between viable, early apoptotic and late apoptotic/necrotic cells (70, 71).

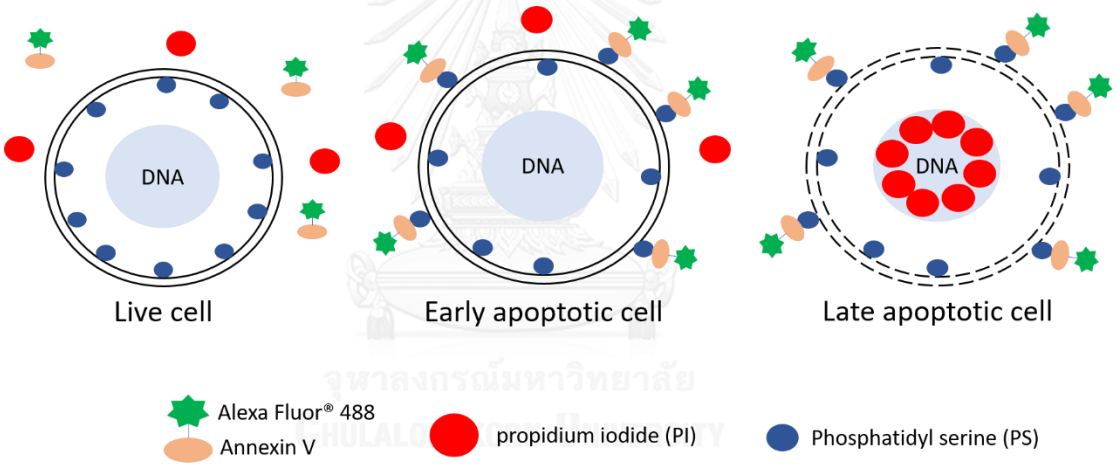


Figure 16 Principle of apoptosis assay using Annexin V and Propidium iodide

U937 cells were seeded in six-well plates (5 x 10^5 cell/ml) and cells were treated with different concentrations of quercetin (25 and 50 uM) for 24 h at 37 °C in 5% CO_2 incubator in the presence or absence of 3-MA, autophagy inhibitor. Apoptosis assay was performed using Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit following manufacture's protocol (Thermo Fisher Scientific, USA) (72).

Preparation of reagents

1.Dilute 5x to 1X annexin-binding buffer with deionized water.

2. Prepare working solution of propidium iodide (PI) by diluting 1 part of component B in 9 parts of 1X annexin-binding buffer.

Protocol

1) Transfer treated-cells to BD Falcon™ Round-Bottom Tubes.
2) Spin for 5 minutes at 1500 rpm to pellet the cells.
3) Discard media and wash cells twice with 200 µL of cold PBS, pH 7.4.
4) Discard the supernatant carefully.
5) Resuspend the cells in 100 ul of 1X annexin-binding buffer.
6) Add 5 µL Alexa Fluor® 488 annexin V and 2 µL PI working solution to each tube.
7) Incubate the cells for 15 minutes at room temperature in the dark.
8) Add 400 µL 1X annexin-binding buffer, mix the samples softly and keep on ice.
9) The stained cells were analyzed by flow cytometer (FACS Calibur, BD Biosciences, USA).

3.2.9 Autophagy detection

As described in literature review, microtubule-associated protein 1 light chain 3 (LC3) is one of hallmark of autophagy process. So, the principle of autophagy

detection is based on measuring LC3 using Anti-LC3 antibody conjugated with FITC and analyze by flow cytometry (73-76).

U937 cells were treated with different concentrations of quercetin (25 and 50 uM) for 24 h in 5% CO_2 incubator. Autophagy induction was determined using FlowCellect™ Autophagy LC3 Antibody-based Assay Kit (Merck Millipore). The assay was based on the measurement of autophagosome associated LC3 (Lipidated LC3-II), well-known autophagy marker, using flow cytometry analysis. Autophagy assay was performed according to the manufacturer's instructions (77).

Preparation of reagents

1) Reagent A: reconstituted the contents of the vial in 250 µL deionized water, stored at -20°C
2) Reagent B: diluted 10x to 1X with deionized water, Stored at 2 - 8°C.
3) Autophagy assay Buffer: diluted 5x to 1X with deionized water, Stored at 2 - 8°C.

Protocol

1) Thirty minutes before the end of incubation time, autophagy reagent A was added to each well and incubated for 30 minutes at 37 °C.
2) Transfer treated-cells to BD Falcon™ Round-Bottom Tubes.
3) Spin for 5 minutes at 1500 rpm to pellet the cells.
4) Discard the supernatant carefully.

5) Add 100 µL of 1X Reagent B to each tube and immediately spin at 1500 rpm for 5 minutes
6) Carefully discard supernatant from each tube
7) Resuspend cells in 95 µL 1X Assay Buffer + 5 µL of 20X optimized anti-LC3/FITC antibody
8) Incubate 30 minutes at room temperature in the dark
9) Wash once with 200 ul 1X Assay Buffer and spin at 1500 rpm for 5 minutes. Carefully discard supernatant from each tube
10) Resuspend cells in each tube with 200 µL 1X Assay Buffer.
11) The fluorescence intensity of anti-LC3/FITC antibody was measured and analyzed using flow cytometer (FACS Calibur, BD Biosciences, USA)

3.2.10 Western blot analysis

1) Protein extraction

U937 cells are cultured in six well plates and treated with quercetin at 25 and 50 uM for 24 h at 37°C in 5% CO_2 incubator. After incubation time, quercetin-treated cells were harvested and washed twice with cold PBS buffer, pH 7.4. Whole cell proteins were extracted in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM PMSF and 1 mM DTT (PMSF and DTT were added immediately prior use) at 4 °C for 1 h. After that, lysed cells

were centrifuged at 14000g at 4 °C for 20 minutes. Supernatants were collected and stored at -20 °C.

2) Evaluation of protein concentration

The protein content of supernatant was measured by Bio-Rad protein assay according to the manufacturer's standard procedure for microtiter plates.

1.Prepare working dye reagent by diluting 1 part of 5X dye reagent concentrate with 4 parts of distilled water and filtered through Whatman filter paper #1 to remove dye precipitate.

2.Prepare 6 dilutions of protein standard, Bovine serum albumin (BSA) 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml in distilled water.

3.Pipet 10 ul of each protein standard and protein sample into each well of 96 well plates. 10 ul of distilled water is used as a blank. Each protein solution was performed in duplicate.

4.Add 200 ul of diluted dye reagent into each well and mix thoroughly.

5.Incubate for 5-60 minutes at room temperature and measure absorbance at 595 nm by microplate reader.

6.Protein concentration of each samples are calculated by generating standard curve of BSA protein standard.

3) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A. Casting polyacrylamide gels

Resolving gel and stacking gel solutions were prepared without APS and TEMED according to following recipes.

Table 7 Recipes for preparation of resolving gel and stacking gel solution

	Resolving gel (15%)	**Stacking gel (4%)**
40% Acrylamide: bisacrylamide solution	3 ml	0.5 ml
0.5 M Tris-HCl, pH 6.8	-	1.25 ml
1.5 M Tris-HCl, pH 8.8	2 ml	-
10 % SDS	80 ul	50 ul
diH_2O	2.8 ml	3.1 ml
10% APS	80 ul	50 ul
TEMED	8 ul	5 ul
Total volume	8 ml	5 ml

APS and TEMED were immediately added to the gel solution and pour the gel solution to the glass cassette system. Overlay the resolving gel solution layer with distilled water and allow gel to polymerize for 45 minutes. Remove distilled water above polymerized resolving gel and then pour stacking gel solutions instead. Place the comb in glass cassette system and allow gel to polymerize for 30 minutes

B. Performing protein electrophoresis

a. Prepare 1X running buffer and 5X sample buffer (see appendix)

b. Dilute 1 part of 5X sample buffer to 4 parts of protein samples (30 ug) and heat samples at 95 °C for 10 minutes and shock samples on ice for 5 minutes.

c. Equal amounts of protein (30 ug) were loaded into gel wells. Protein ladder was loaded into one well as molecular weight marker. Protein electrophoresis was performed according to following conditions:

Initial condition: 80 V for 30 minutes

Final condition: 120 V for 45-60 minutes

4) Blotting

After separated proteins through SDS-PAGE, proteins were transferred to PVDF membrane using Towbin transfer buffer system following the steps below.

a. Prepare transfer buffer (see appendix).

b. Separated gels were rinsed in distilled water and equilibrate gels in transfer buffer for 15 minutes at room temperature to remove contaminated salts from electrophoresis step.

c. Soaked PVDF membrane in 100 % methanol for 5 minutes at room temperature.

d. Equilibrate PVDF membrane, filter papers and foam pad in transfer buffer for 10 minutes at room temperature.

e. Set up the gel, PVDF membrane, filter paper and foam pad sandwich in transfer cassette following the figure below.

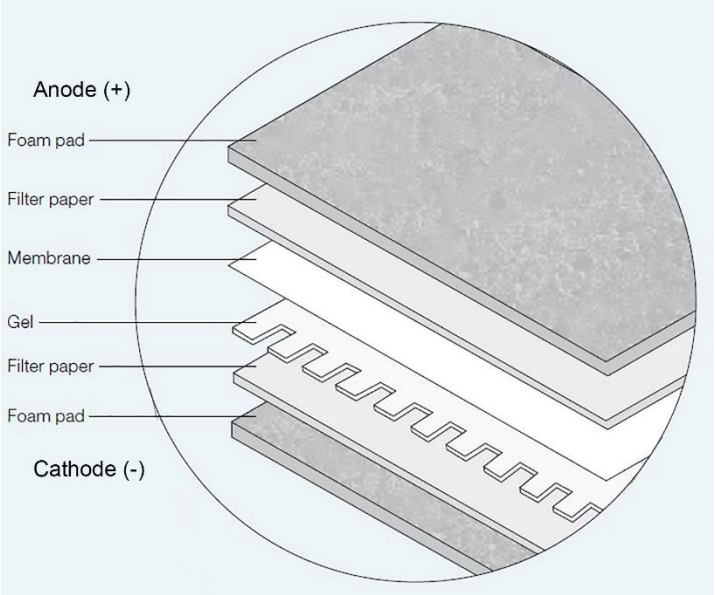


Figure 17 Gel and membrane set up for protein transfer (78).

f. Assemble protein transfer systems and perform electro-transfer using Towbin transfer buffer at 110 V for 2 hours in cooling condition.

5) Blocking

After transferred proteins to PVDF membrane, washed the membrane for 10 minutes in TBS and then the membrane was blocked in blocking buffer, 5 % skimmed milk in TBST (see appendix), for 1 h at room temperature with gentle shaking to reduce non-specific binding.

6) Primary and secondary antibodies incubation

After blocking step, the membrane was washed twice with TBST for 10 minutes per wash and then incubated membrane with primary antibodies (1:1000) overnight at 4 °C with gentle shaking.

After primary antibodies incubation, the membrane was washed 4 times with TBST for 6 minutes per wash and probed with secondary antibodies (1:2500) for 1 h at room temperature with gentle shaking.

After secondary antibodies incubation, the membrane was washed 4 times with TBST for 6 minutes per wash and washed once with TBS for 6 minutes to remove tween 20.

Table 8 List of primary and secondary antibodies used in Western blot analysis

Primary antibodies		Secondary antibody	
Anti-Bcl-2	(1:1000)	Anti-Rabbit IgG	(1:2500)
Anti-Bad	(1:1000)	Anti-Rabbit IgG	(1:2500)
Anti-P-Bad (Ser112)	(1:1000)	Anti-Rabbit IgG	(1:2500)
Anti-beta-actin	(1:1000)	Anti-Rabbit IgG	(1:2500)

7) Detection and quantification of protein band

The immunoblot was visualized using Hypersensitive ECL chemiluminiscence substrate (Boster Biological Technology, USA) and protein band quantification was measured by GeneSnap and GeneTools analysis software (Syngene, USA).

3.2.11 Statistical analysis

All experiment was performed in triplicates. Quantitative data are reported as mean ± standard deviation (SD). Data comparisons of each individual group were analyzed by the one-way analysis of variance (ANOVA) with Tukey's post hoc test using SPSS program. A *P-values* less than 0.05 was considered as statistically significance.

CHAPTER IV

Results

4.1 The effect of quercetin on cell cytotoxicity in AML cell line (U937)

To determine the cytotoxicity of quercetin in AML cell line, MTT based assay was performed. U937 cells were incubated with quercetin (25-200 uM) for 24 h.

As shown in Fig.19, treatment with quercetin for 24 h led to significantly increased in growth inhibition of U937 cells compared with negative control. The 50% concentration inhibition (IC50) value of quercetin in U937 cells was estimated by linear regression analysis. Results founded that, the IC50 values of quercetin in U937 cells for 24 h was 55.70 uM (Fig.18).

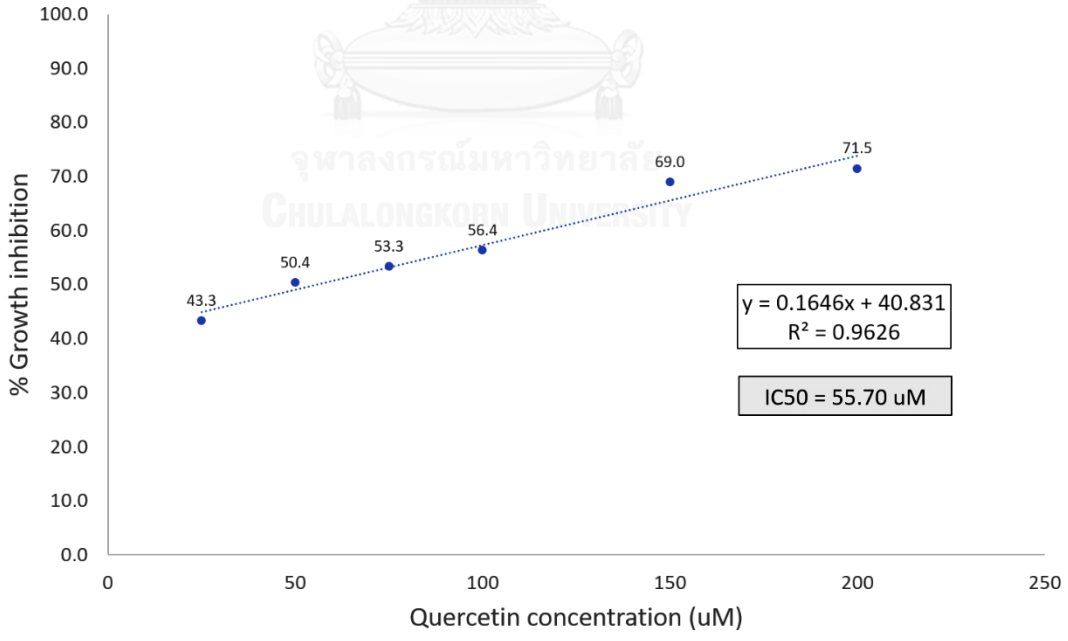


Figure 18 The IC50 value of quercetin in U937 cells was estimated by linear regression analysis

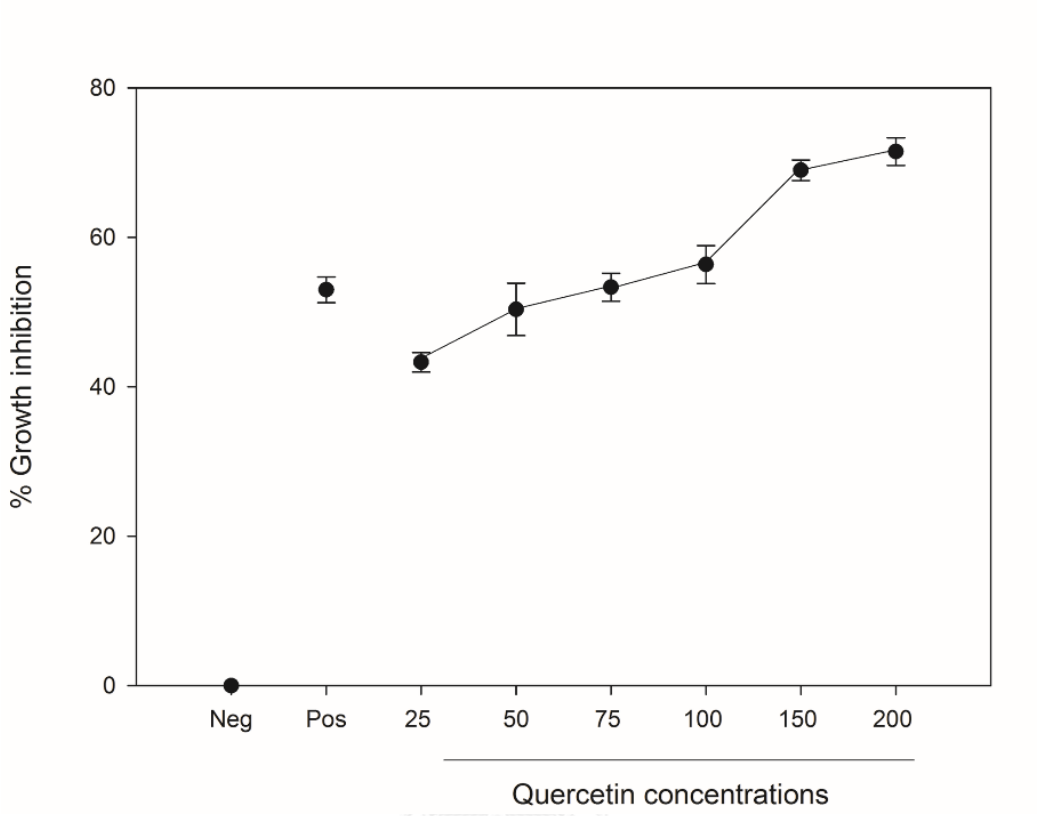


Figure 19.Cytotoxic effect of quercetin on U937 cells. U937 cells were treated with different concentrations of quercetin for 24 h and MTT assay was performed to determined growth inhibitory effect of quercetin on U937 cells. 0.1 % DMSO was used as negative control. Cytarabine 200 ug/ml, chemotherapeutic drug for AML treatment, was used as positive control.

4.2 The effect of quercetin on cell viability in AML cell line.

The effect of quercetin on cell viability in U937 cells was evaluated by Muse® Count &Viability Assay Kit (Merck Millipore). Discrimination between live and dead cells were based on cell permeability to the DNA binding dyes in the reagent kit. U937 cells were treated with quercetin (25-200 uM) for 24 h. The percentage of cell viability in quercetin-treated cells were calculated by Muse™ Cell Analyzer (Merck Millipore). As shown in Fig.20, quercetin significantly reduced the percentage of cell viability of U937 cells in a dose-dependent manner. Dot plots obtained from Muse™ Cell Analyzer were reported in supplementary data.

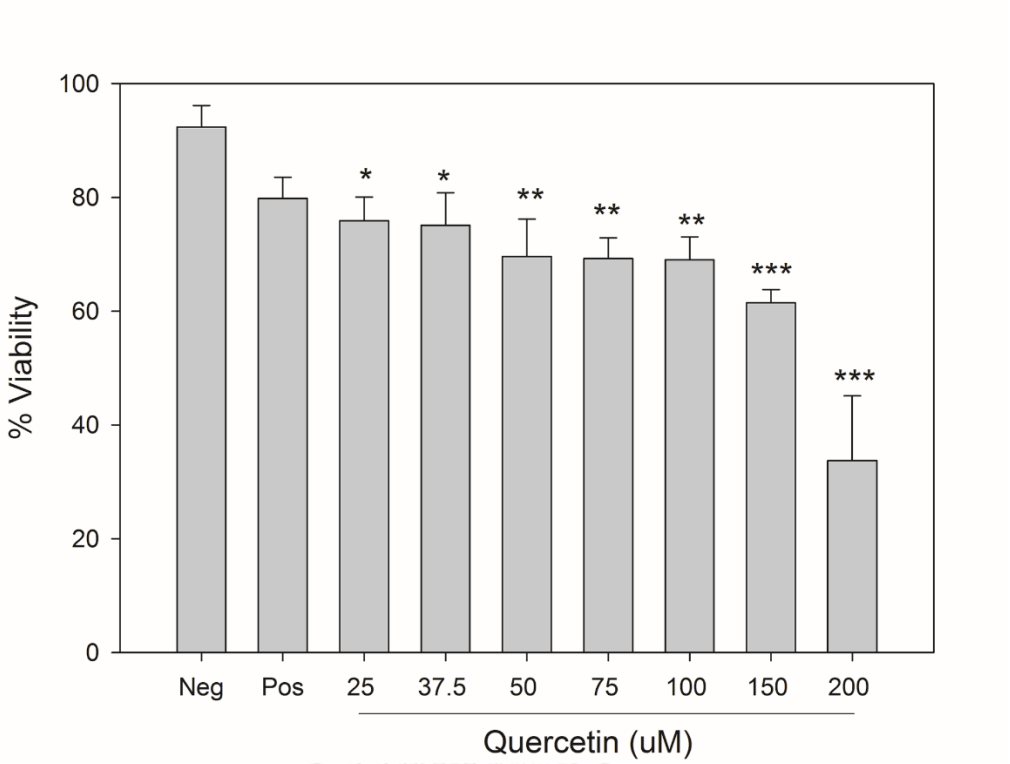
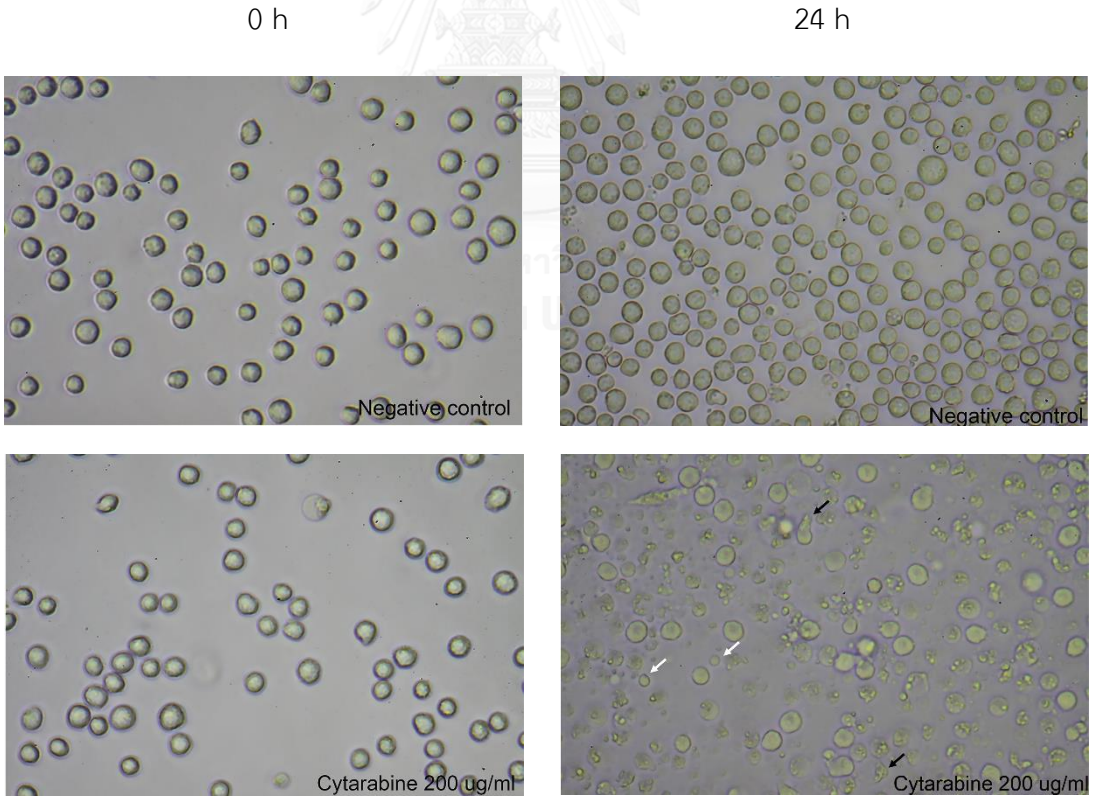


Figure 20 Cell viability of U937 cells was determined after treatment for 24 h with quercetin. U937 cells were treated with different concentrations of quercetin for 24 h and cell viability assay was performed by Muse® Count &Viability reagent to determine the reduction of cell viability in quercetin-treated U937 cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with negative control group (0.1 % DMSO). Cytarabine 200 ug/ml, chemotherapeutic drug for AML treatment, was used as positive control.

4.3 The effect of quercetin on cell morphology alterations in AML cell line.

The effect of quercetin on cellular morphology change in U937 cells was observed under inverted light microscope. After exposure for 24 h, quercetin induced apparent morphological alteration in human AML U937 cells. Noticeably, cellular morphology observation of quercetin-treated cells founded that compared with negative control group (0.1% DMSO), quercetin-treated group showed obviously morphological features of apoptotic cells including membrane blebbing and apoptotic bodies formation (Fig.21).



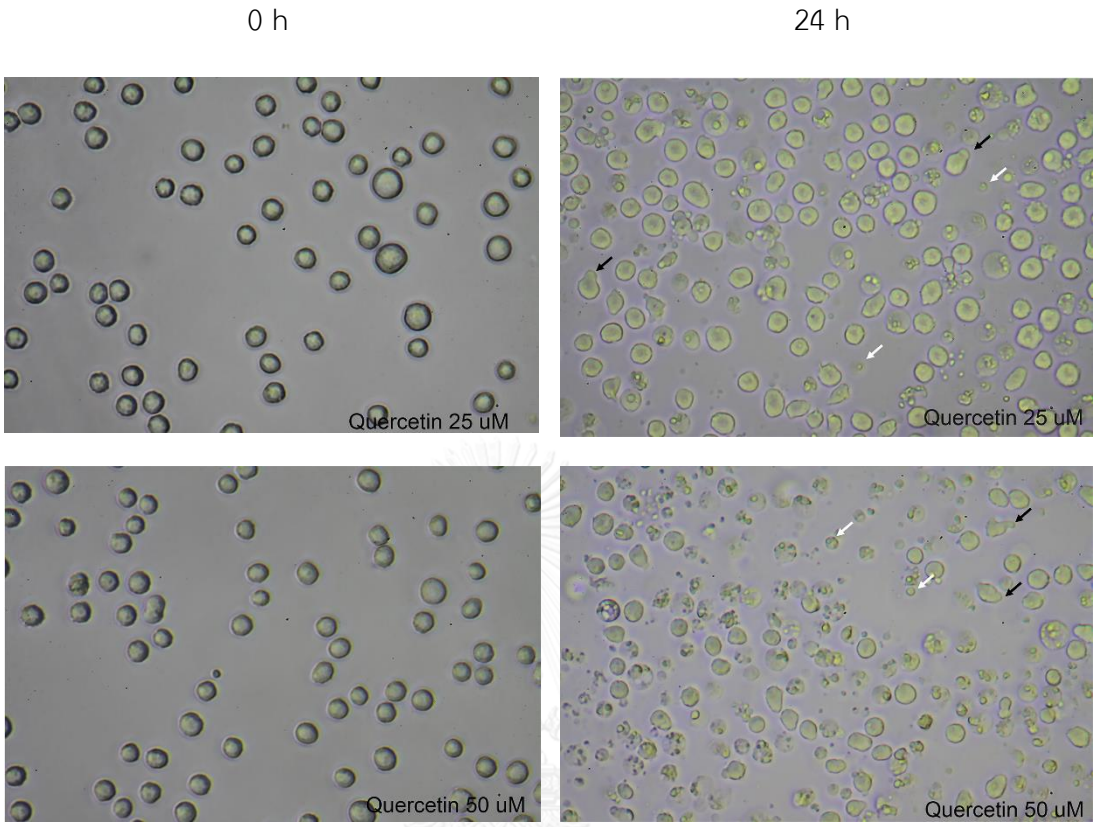


Figure 21 Effect of quercetin on cellular morphology of U937 cells by microscopic analysis. U937 cells were exposed to quercetin 25 and 50 uM for 24 h. Cell morphology of quercetin-treated cells were observed under inverted light microscope and captured by CCD camera (magnification x200). Black arrows indicated membrane blebbing and white arrows indicated apoptotic bodies formation. 0.1 % DMSO was used as negative control and 200 ug/ml cytarabine was used as positive control.

4.4 The effect of quercetin on apoptosis induction in AML cell line.

To further investigate whether the growth inhibitory effect and the reduction of cell viability after treatment with quercetin accompanied by apoptosis induction, apoptosis assay was performed by Annexin V/PI double staining. After quercetin treatment for 24 h, the percentage of apoptotic cells was significantly increased (Fig. 22,23). Data showed that the percentage of total apoptotic cells was increased from 7.63 % to 62.63% and 74.41% after treatment with quercetin 25 uM and 50 uM respectively compared with negative control (0.1 % DMSO). These data suggested the that cytotoxicity of quercetin in AML cell lines was associated with apoptosis induction.

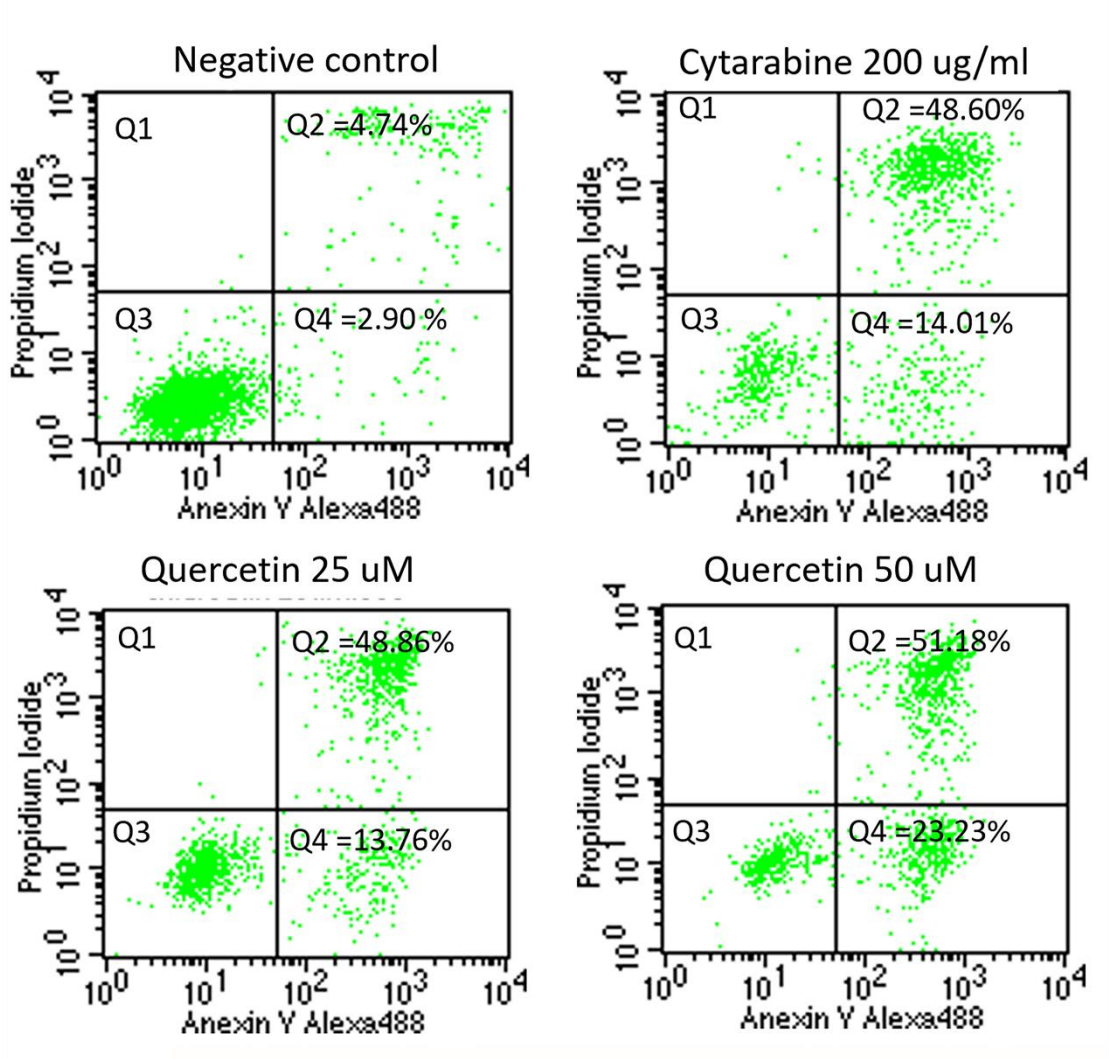


Figure 22 Representative scatter plot of apoptosis assay using Annexin V/PI. U937 cells were treated with quercetin 25 uM and 50 uM for 24 h. Cells were stained using annexin V/PI and analyzed by flow cytometry. Q3 represented viable cells (Annexin V -/PI-), Q4 represented early apoptotic cells (Annexin V +/PI-) and Q2 represented late apoptotic cells (Annexin V +/PI+). Quantitative data were expressed as mean (n = 3).

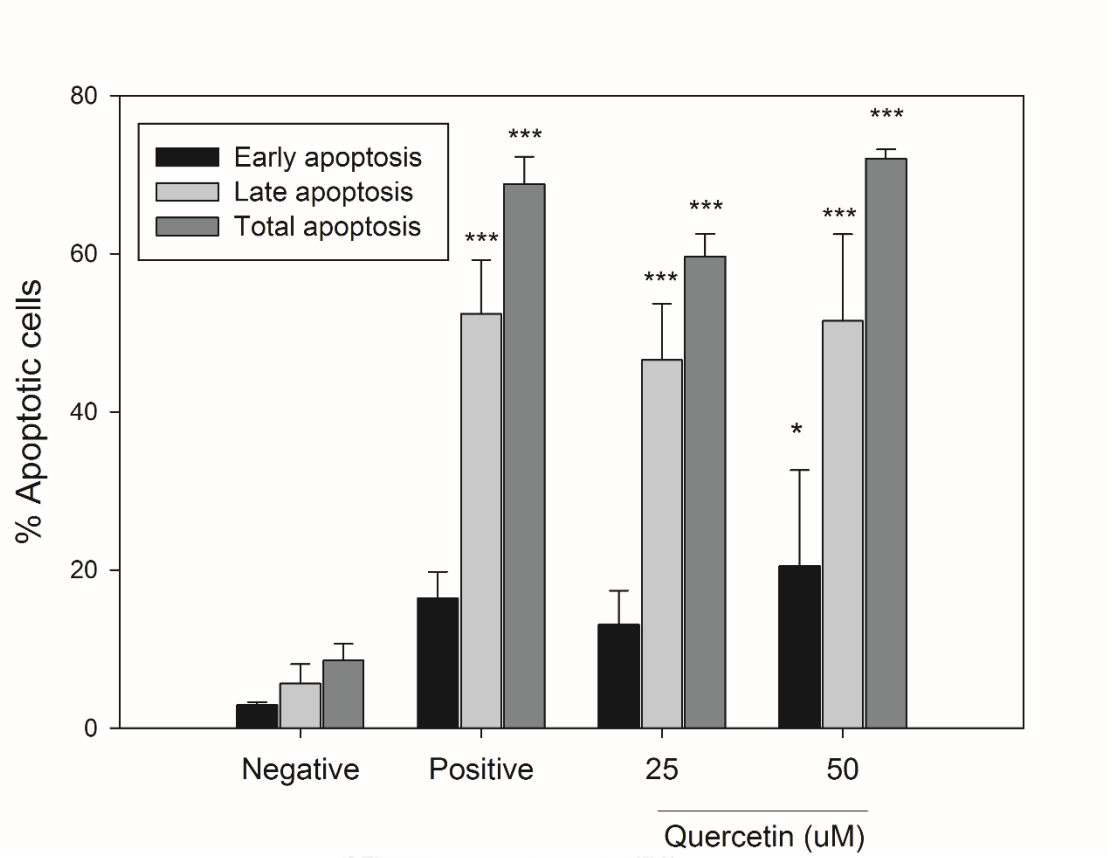


Figure 23 Effect of quercetin treatments on apoptosis in U937 cells. The percentages of apoptotic cells were presented in bar charts. * $P < 0.05$, *** $P < 0.001$ compared with negative control group (0.1 % DMSO). Cytarabine 200 ug/ml, chemotherapeutic drug for AML treatment, was used as positive control (n=3).

4.5 The effect of quercetin on autophagy induction in AML cell line.

FlowCellect™ Autophagy LC3 Antibody-based Assay Kit (Merck Millipore) were used to analyzed the effect of quercetin treatment on autophagy. As shown in Fig.24,25, treatment with quercetin alone for 24 h increased the accumulation of LC3-II as indicated by an increase of mean fluorescence intensity from 32.51 to 347.64 and 579.44 after treatment with quercetin 25 uM and 50 uM respectively compared with negative control (0.1 % DMSO). In addition, there were no difference in mean fluorescence intensity between negative control and pre-treatment with 5 mM 3-MA for 1 h in combination with quercetin. These results revealed that quercetin possessed autophagy induction effect in U937 cells and the ability of quercetin-induced autophagy was reduced by 3-MA.

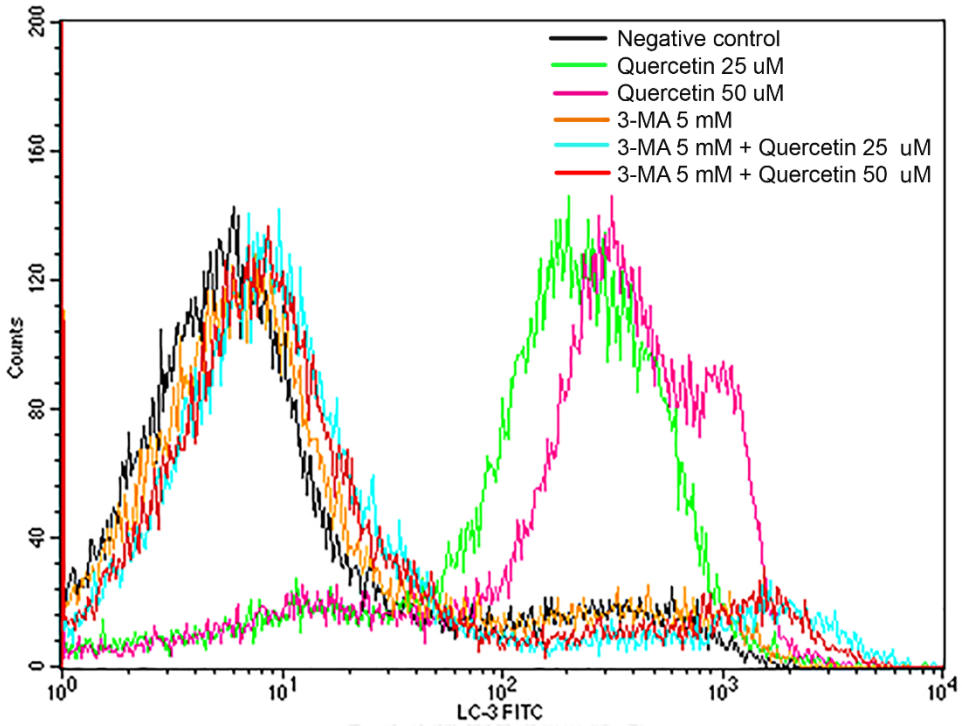


Figure 24 Representative histogram of autophagy detection. U937 cells were incubated with quercetin 25 uM and 50 uM in the presence or absence of 3-MA, autophagy inhibitor, for 24 h. FlowCellect™ Autophagy LC3 Antibody-based Assay Kit (Merck Millipore) was used to measure the amount of autophagosome associated LC3 (Lipidated LC3-II), autophagy marker, using flow cytometry.

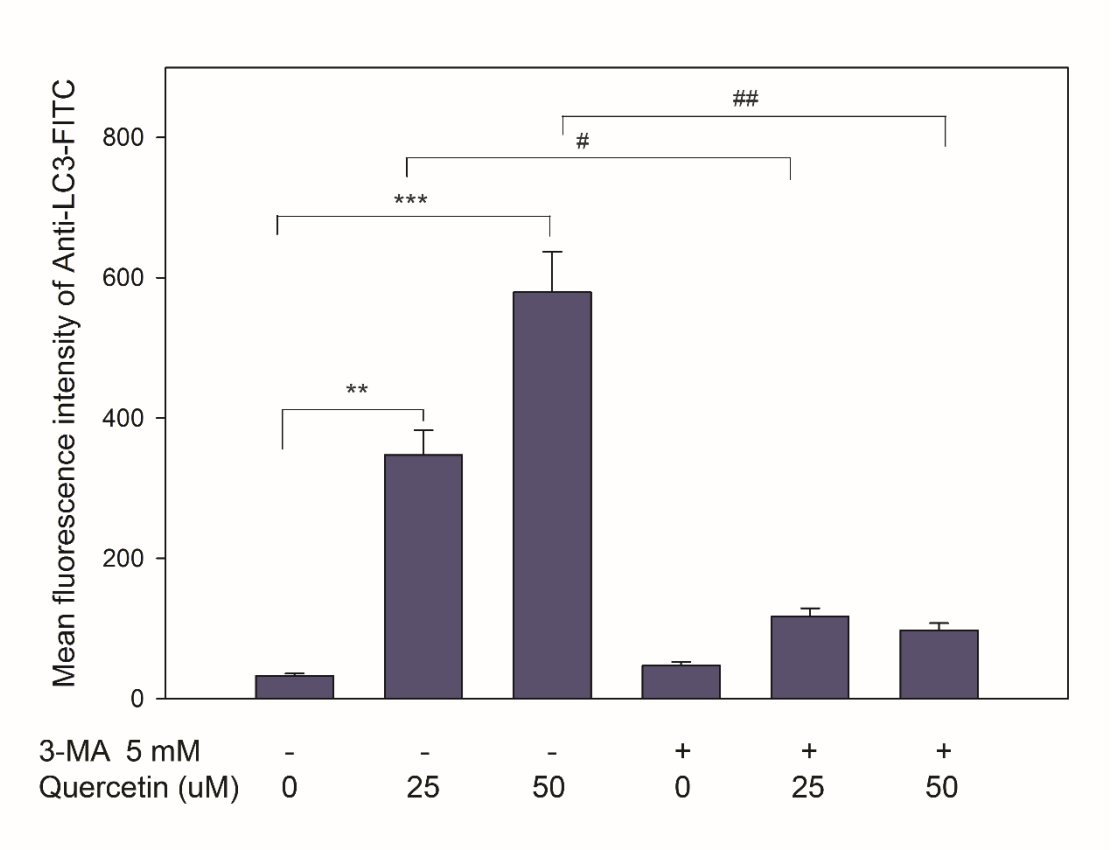


Figure 25 Quercetin induced autophagy in U937 cells. Mean fluorescence intensity of LC3-II were shown in bar charts. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with negative control group (0.1 % DMSO). # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ compared with quercetin-treated group (n=3).

4.6 The role of autophagy induction by quercetin in AML cell line.

To further clarify whether autophagy induction after quercetin treatment was a prosurvival or prodeath mechanism, we performed cytotoxic assay using MTT based method, viability assay using trypan blue exclusion method and apoptosis assay by flow cytometry analysis.

After treatment with quercetin for 24 h in combination with 3-MA, there were no significantly difference in the percentage of growth inhibition between treatment with quercetin alone and pre-treatment with 5 mM 3-MA for in combination with quercetin (Fig.26).

After treatment with quercetin for 24 h in combination with 3-MA, the percentage of cell viability was significantly decreased from 60.49 % to 24.54 % and 38.52 % to 16.79 % after pre-treatment with 5 mM 3-MA for 1 h in combination with quercetin 25 uM and 50 uM respectively compared with quercetin treatment alone (Fig. 27).

After treatment with quercetin for 24 h in combination with 3-MA, autophagy inhibitor, the percentage of apoptotic cells was dramatically increased (Fig. 28,29). Results showed that the percentage of total apoptotic cells was increased from 62.63 % to 94.64 % and 74.41 % to 97.20 % after pre-treatment with 5 mM 3-MA for 1 h in combination with quercetin 25 uM and 50 uM respectively compared with quercetin treatment alone. These data suggested that the inhibition of autophagy by

3-MA enhanced quercetin-induced apoptosis in AML cell line, indicated a prosurvival role of autophagy induction by quercetin in U937 cells.

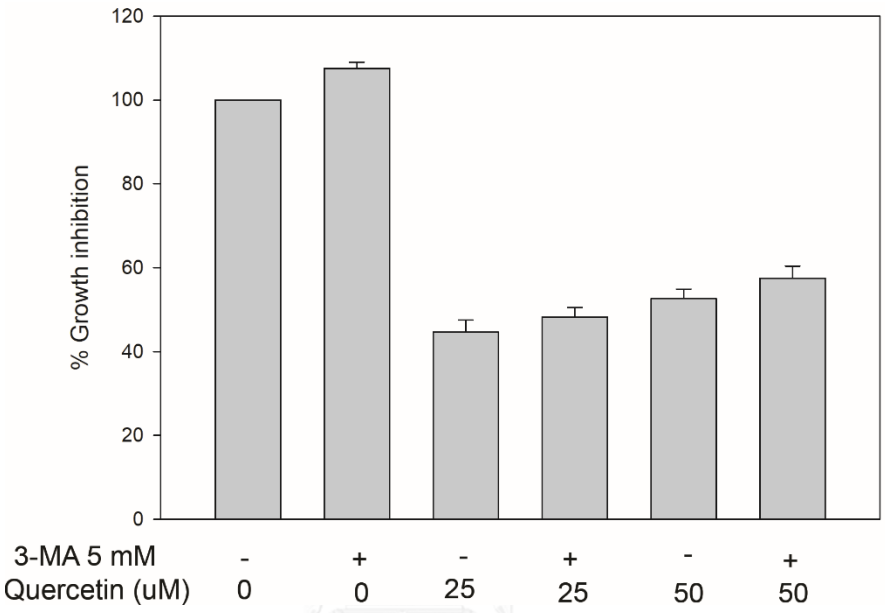


Figure 26 Cytotoxic effect of quercetin in combination with 3-MA. U937 cells were incubated with quercetin 25 uM and 50 uM in the presence or absence of 3-MA, autophagy inhibitor, for 24 h and MTT assay was performed to determined growth inhibitory effect of quercetin in combination with 3-MA on U937 cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with quercetin-treated group (n=3).

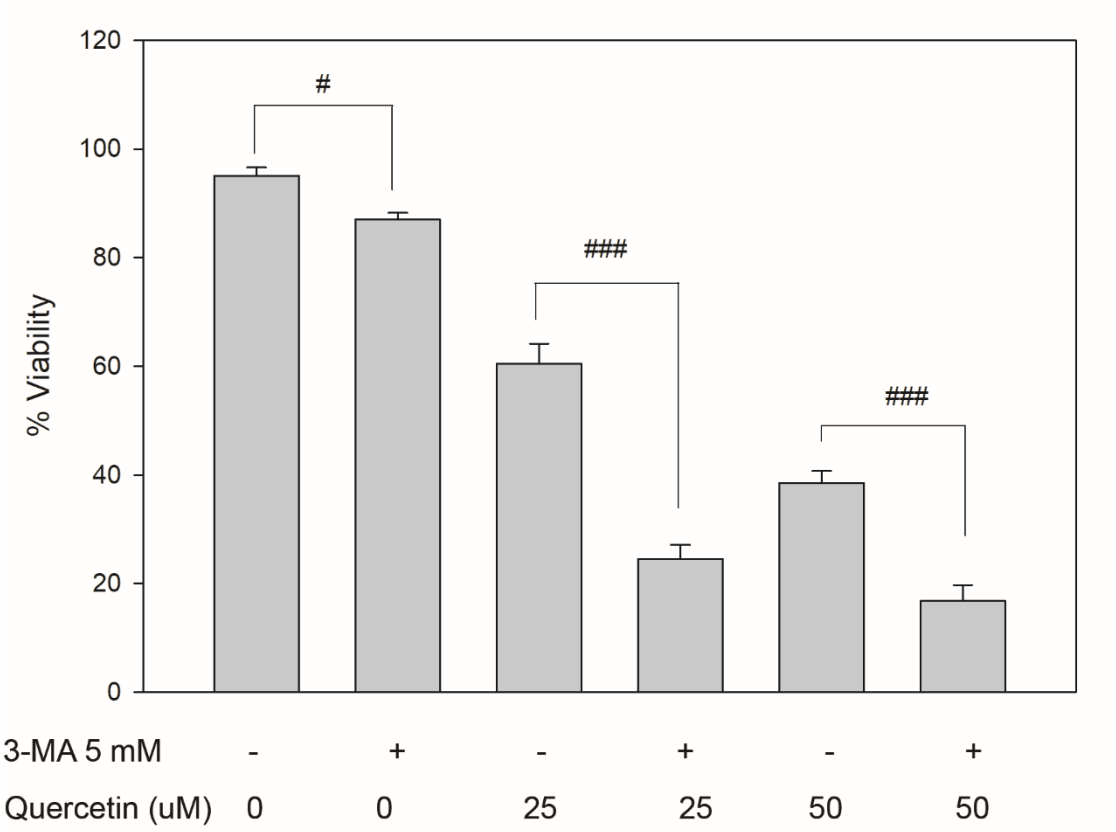


Figure 27 Cell viability effect of quercetin in combination with 3-MA. U937 cells were incubated with quercetin 25 uM and 50 uM in the presence or absence of 3-MA, autophagy inhibitor, for 24 h and trypan blue exclusion assay was performed to determined effect of quercetin in combination with 3-MA on cell viability. $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ and $^{\#\#\#}$ $P < 0.001$ compared with quercetin-treated group (n=3).

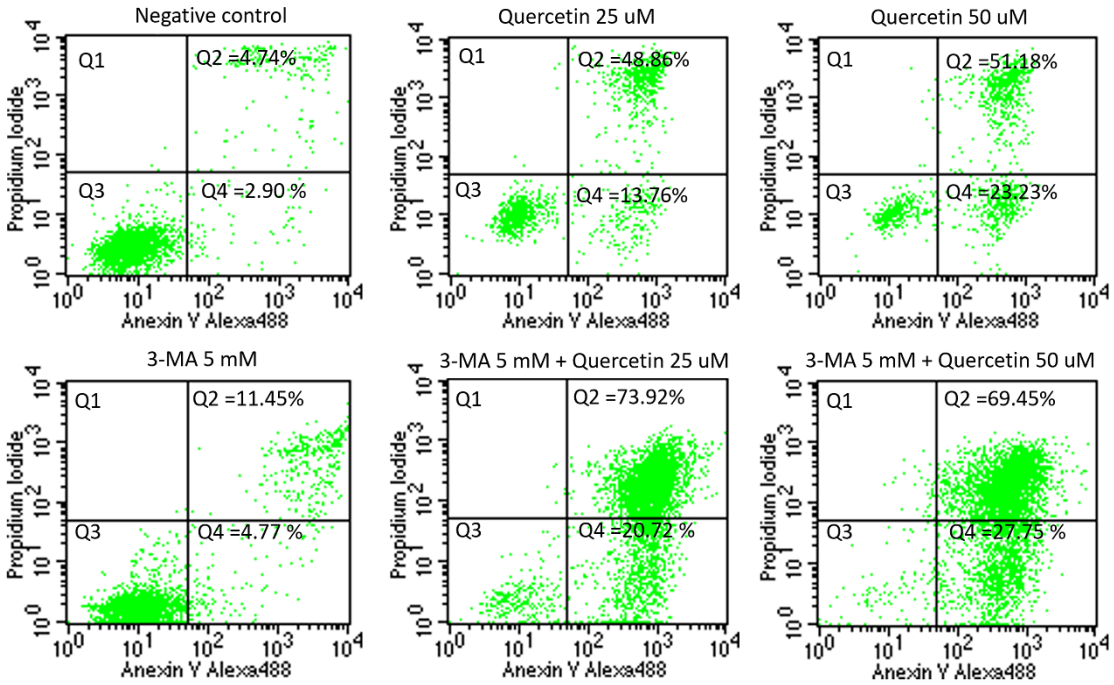


Figure 28 Representative scatter plot of apoptosis assay showed that inhibition of autophagy by 3-MA enhanced quercetin-induced apoptotic cell death. U937 cells were incubated with quercetin 25 uM and 50 uM in the presence or absence of 3-MA, autophagy inhibitor, for 24 h. Cells were stained using annexin V/PI and analyzed by flow cytometry. Q3 represented viable cells (Annexin V -/PI-), Q4 represented early apoptotic cells (Annexin V +/PI-) and Q2 represented late apoptotic cells (Annexin V +/PI+). Quantitative data were expressed as mean (n = 3).

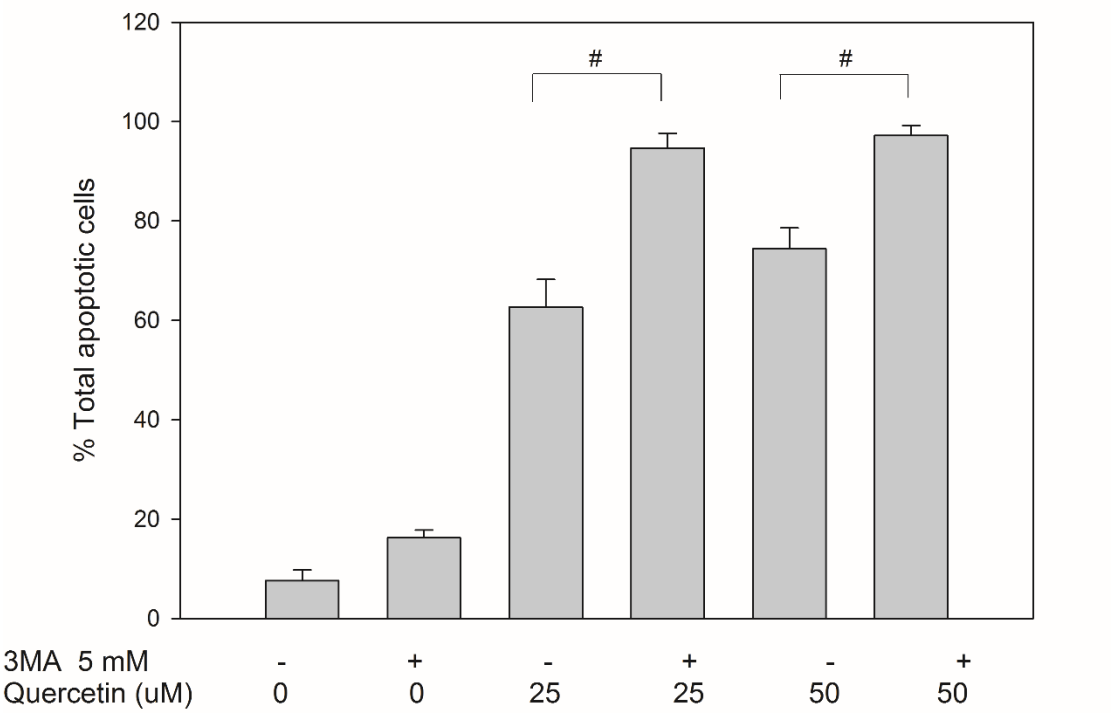


Figure 29 Inhibition of autophagy by 3-MA enhanced quercetin-induced apoptotic cell death. The percentages of total apoptotic cells were presented in bar charts. # $P < 0.05$ compared with quercetin-treated group.

4.7 Quercetin-induced leukemic cell death was accompanied by Bcl-2 and Bad signaling pathway.

To further clarify the underlying molecular mechanisms of quercetin on AML cell line, we performed Western blot analysis to measure the expression of proteins related with apoptosis and autophagy.

Results from Western blot analysis revealed that protein expression of Bcl-2 was decreased after treatment with quercetin (25 and 50 uM) for 24 h. On the other hand, Bad protein level was slightly increased in quercetin-treated cells. Furthermore, the exposure of U937 cells to quercetin down-regulated the phosphorylation of Bad protein (Fig.30,31).

These results indicated that quercetin induced apoptosis and autophagy were involved in the regulation of Bcl-2 and Bad signaling pathway in U937 cells.

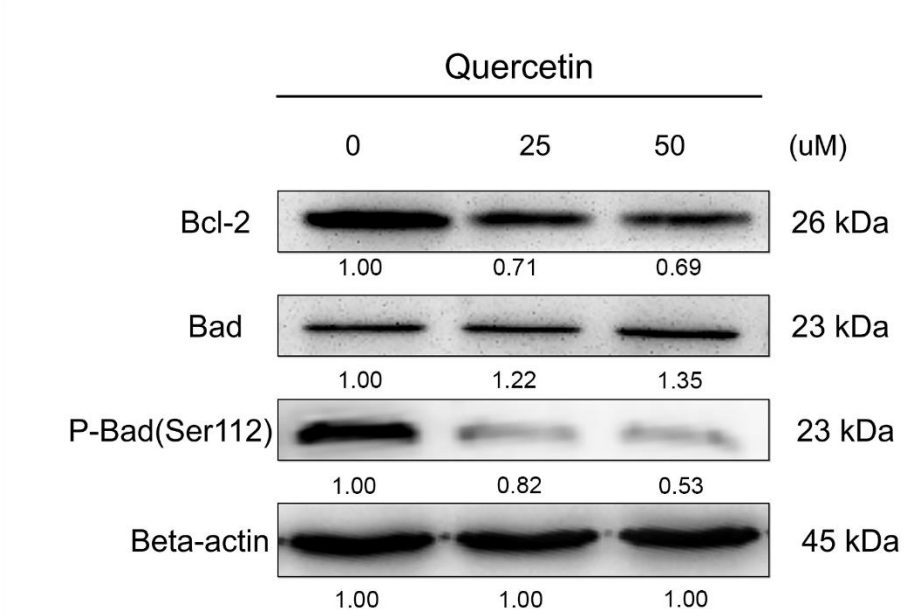


Figure 30 Effect of quercetin treatment on the expression level of apoptosis and autophagy related proteins in U937 cells. U937 cells were treated with quercetin 25 uM and 50 uM for 24 h and Western blot analysis was performed to evaluate protein expression level of Bcl-2, Bad and phosphorylated form of Bad. Beta actin served as an internal control.

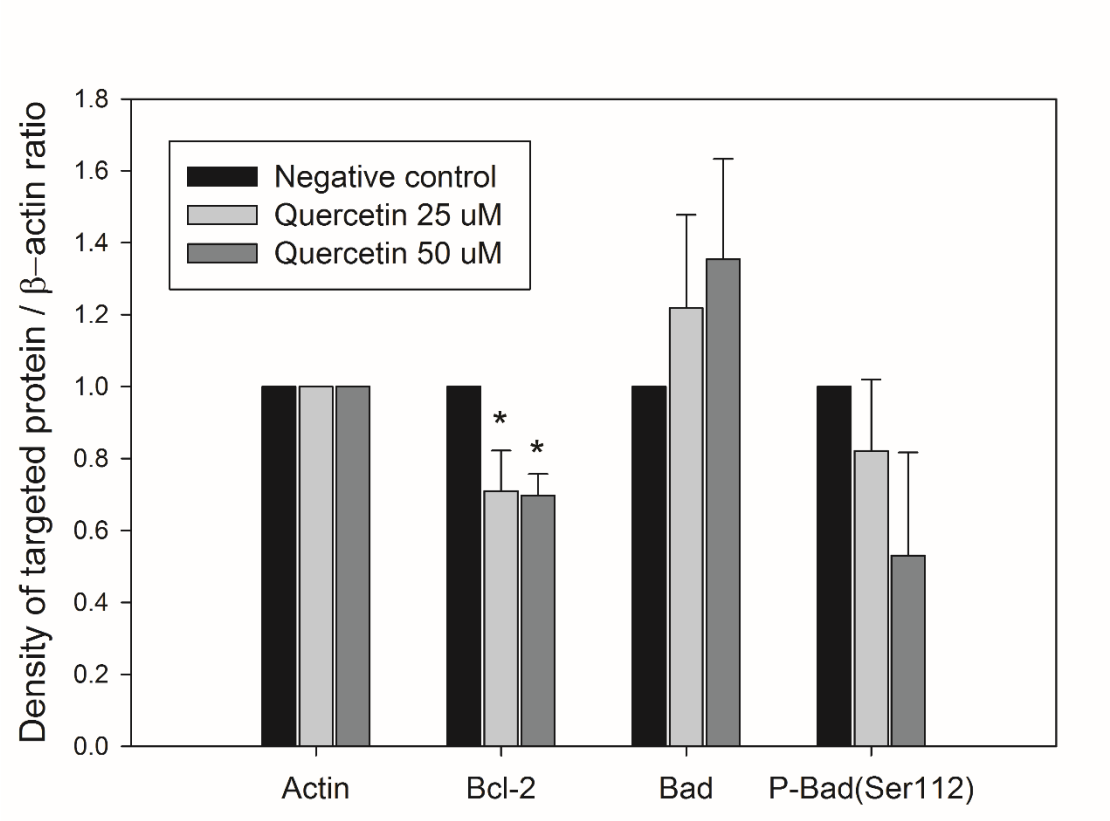


Figure 31 Relative density of apoptosis and autophagy related proteins in quercetin-treated U937 cells. U937 cells were treated with quercetin 25 uM and 50 uM for 24 h and Western blot analysis was performed. Protein band quantification was measured by GeneSnap and GeneTools analysis software (Syngene, USA). Beta actin served as an internal control. * $P < 0.05$ compared with negative control group (0.1 % DMSO) (n=3).

CHAPTER V

Discussion and conclusion

Over the past decades, phytomedicines, plant-derived compounds, are becoming popular as alternative therapeutic approaches to improve clinical outcomes of cancer therapy especially in developing countries because phytochemicals have less cytotoxicity against normal cells and fewer adverse effects. Furthermore, certain natural substances exhibit beneficial biological properties including anti-oxidant and anti-inflammatory effects (79, 80). Although the antileukemic effect of natural products have been broadly examined in recent years, their underlying mechanisms have remained to be completely explained. Quercetin is a bioactive flavonoid derived from many dietary sources. Currently, many studies have been reported that quercetin exhibits growth inhibition properties in several cancer such as gastric cancer (6) , breast cancer (7) , ovarian carcinoma cells (8) , colon cancer (9) , non-small cell lung cancer (10) esophageal squamous cell carcinoma (11) , pancreatic cancer (12) and leukemia (13, 81, 82) , but not against normal hematopoietic cells (14, 15) . In the present study, the dietary flavonoid quercetin exhibits growth inhibitory effect on U937 cells with the IC50 value of 55.70 uM. Our results are in agreement with the IC50 values reported by Lee et al. in many leukemia cells (83) . Concomitantly, the present study also demonstrates that quercetin treatments resulted in the reduction of the percentage of cell viability in U937 cells in concentration-dependence manner.

Apoptosis induction is one of the most desirable strategy for AML therapy. Previous studies have been confirmed that many conventional chemotherapeutic agents effectively induced apoptosis in AML cells. Ibrado et al. reported that cytarabine, etoposide and mitoxantrone induced apoptosis in HL-60 cells determined by DNA fragmentation assay and cleavage of poly (ADP-ribose) polymerase (84) . Daunorubicin induced apoptosis in HL-60, K562 and leukemic cells isolated from AML patients evaluated by caspase-3 activity assay, DNA fragmentation assay and PI staining (85) . Idarubicin induced apoptosis in HL-60, U937 and KG1 cells assessed by DNA fragmentation assay (86) . Apoptotic cells manifest characteristic morphological aspects including plasma membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies that contain organelles or small fragments of the nucleus (87) . In the present study, we observe the cellular morphology of U937 cell after treatment with quercetin for 24 h under inverted light microscope. Microscopic analysis showed that quercetin-treated cells obviously display characteristic morphological features of apoptotic cells including membrane blebbing and apoptotic bodies formation. The morphological features alteration of quercetin-treated U937 cells was correlated with the significantly increased in the percentage of apoptotic cells evaluated by Annexin V/PI double staining. Our results revealed that cytotoxic effects of quercetin were accompanied by the induction of apoptotic cell death in human AML cell line. Consistently, quercetin induced apoptosis was also investigated in several cancer cells. Chien et al. reported that

quercetin induced apoptosis in human breast cancer MDA-MB-231 cells via caspase-3, -8 and -9 activation (88) . Lim et al. founded that quercetin induced apoptosis in colon carcinoma HCT116 cells through NAG-1 up-regulation in EGR-1 and p53-dependent manner (89) . Kuo et al. demonstrated that quercetin induced apoptosis in human lung carcinoma cells via survivin and p53 regulations (90) .

Recently, autophagy is considered as a novel alternative therapeutic strategy for combatting cancer cells. Autophagy can promote cancer cell survival but under certain conditions autophagy plays a role as cell death mechanism called autophagic cell death or type II programmed cell death. During autophagy process, the soluble form of LC3 called LC3-I is conjugated to phosphatidylethanolamine (PE) by two ubiquitin-like conjugation systems to form autophagosome associated LC3 named LC3-II which firmly bind to the autophagosome membrane. LC3-II is involved in the recruitment of damaged or unwanted proteins and organelles into the autophagosome to sequentially eliminate by lysosome system. Accordingly, LC3-II is one of marker of autophagy process (17) . In the present study, we investigated whether quercetin can induce autophagy process in human AML cell line by the evaluation of LC3-II levels. Our data showed that treatment with quercetin for 24 h increased the accumulation of LC3-II as indicated by an increase of mean fluorescence intensity of anti-LC3-FITC. Taken together, our findings indicated that quercetin simultaneously induced apoptosis and autophagy in human leukemia U937 cells.

Previously, many compounds have been confirmed to trigger both apoptosis and autophagy process. However, the role of autophagy-mediated leukemic cell death remains extremely controversial. Certain substances such as Lapatinib (91) , Platonin (92) , Vitamin K2 (19) , Arsenic trioxide (As_2O_3) (93) and Sodium selenite (94) induced autophagic cell death in leukemic cells, whereas some agents such as Perifosine (95) , OSI-027 (mTOR kinase inhibitor) (96) , MK-2206 (Akt inhibitor) (97) and Triciribine (Akt inhibitor) (98) induced cytoprotective autophagy that protected leukemic cells from apoptotic death. For this reason, we evaluated apoptosis assay after quercetin treatment in combination with autophagy inhibitor to clarify whether autophagy induction by quercetin in human AML cell line characterized as a mechanism for autophagic cell death (type II programmed cell death) or cytoprotective manner for preventing cell death. Our findings revealed that autophagy inhibitor 3-MA, which inhibits class III phosphatidylinositol 3-kinases (Class III PI3K) lead to the inhibition of autophagosome nucleation step of autophagy process (99), dramatically enhanced the induction of apoptotic cell death in quercetin-treated U937 cells. These findings suggested that the role of autophagy in quercetin-mediated leukemic cell death is cytoprotective manner for rescue leukemic cells from apoptotic cell death. Our results are consistent with previous studies, quercetin-induced cytoprotective autophagy has been reported in gastric cancer (100) , glioblastoma cells (101) , MDS-chronic myelomonocytic leukemia (CMML) (57) , cervical cancer (102) , ovarian cancer (103) and acute myeloid leukemia (AML) (104) .

However, up to now, the underlying molecular mechanisms of quercetin-mediated leukemic cell death has not been fully explored in acute myeloid leukemia. Bcl-2 family proteins are well-known regulators in apoptotic cascade. This family of protein can be divided into 3 subfamilies including anti-apoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1), pro-apoptotic BH123 proteins (Bax and Bak) and pro-apoptotic BH3 only proteins (Bad, Bid, Bim, Noxa and Puma). Bcl-2 family proteins regulate apoptosis via their abilities to regulate cytochrome c release from the intermembrane space of mitochondrial into the cytosol that resulting in an activation of the intrinsic apoptosis pathway (25). In addition, some of Bcl-2 family proteins can be key modulators of autophagy process. Bcl-2 and Bcl-xL, members of anti-apoptotic subfamily proteins, can inhibit autophagy by an interaction with Beclin 1, a crucial molecule that contributes to autophagosome formation in autophagy process, thus preventing the interaction between Beclin 1 and class III PI3K/hVps34. The BH3 domains of BH3-only proteins such as Bad competitively disrupt the interaction between Beclin 1 and Bcl-2 that lead to an activation of the kinase activity of class III PI3K/hVps34, hence stimulates autophagy process.

In 2006, Lee et al. reported that the exposure of U937 cells for 24 h with quercetin at concentrations lower than 20 uM did not change the protein expression level of Bcl-2 (105). Interestingly, the present study founded that quercetin treatment at concentrations of 25 and 50 uM for 24 h resulted in downregulation of the expression of Bcl-2 protein in U937 cells. Concurrently, the present study also proved

that quercetin downregulated of phosphorylation levels of Bad protein and upregulated the expression of total Bad protein. Our results from Western blot analysis are consistent with previous studies. Niu et al. demonstrated that after treatment with quercetin at concentration 25, 50 and 100 uM for 48 h, the expression of Bcl-2 protein levels were decreased in human leukemia cell (HL-60) (106) . Dai et al. founded that quercetin induced apoptosis in hepatocellular carcinoma cell (HepG2 and SMCC-7721) via upregulation of Bad protein and downregulation of Bcl-2 (107) . Lee et al. reported that quercetin treatment decreased the phosphorylation of Bad proteins in human prostate cell line (LNCaP) (108) . Nguyen et al. founded that quercetin downregulated Bcl-2 level as well as upregulated total Bad protein expression in lung cancer cell line (A549) (109) .

In conclusion, current study demonstrates that quercetin possesses growth inhibitory effect and reduces cell viability in U937 cells. The cytotoxicity of quercetin in U937 cells was associated with the induction of apoptotic cell death. Moreover, our data showed that quercetin induces cytoprotective autophagy in U937 cells. The underlying mechanisms by which quercetin-mediated leukemic cell death are involved in the regulation of Bcl-2 and Bad signaling pathway. Our findings provide considerable evidences indicating the potential anti-leukemic effects of quercetin. A better understanding of the underlying mechanisms that associated with quercetin-induced AML cell death will be valuable for the development of natural compounds especially

quercetin as complementary and alternative approaches for target molecular abnormalities in AML treatment.

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APPENDIX

Supplementary data

Effect of quercetin on cell viability in U937 cells.

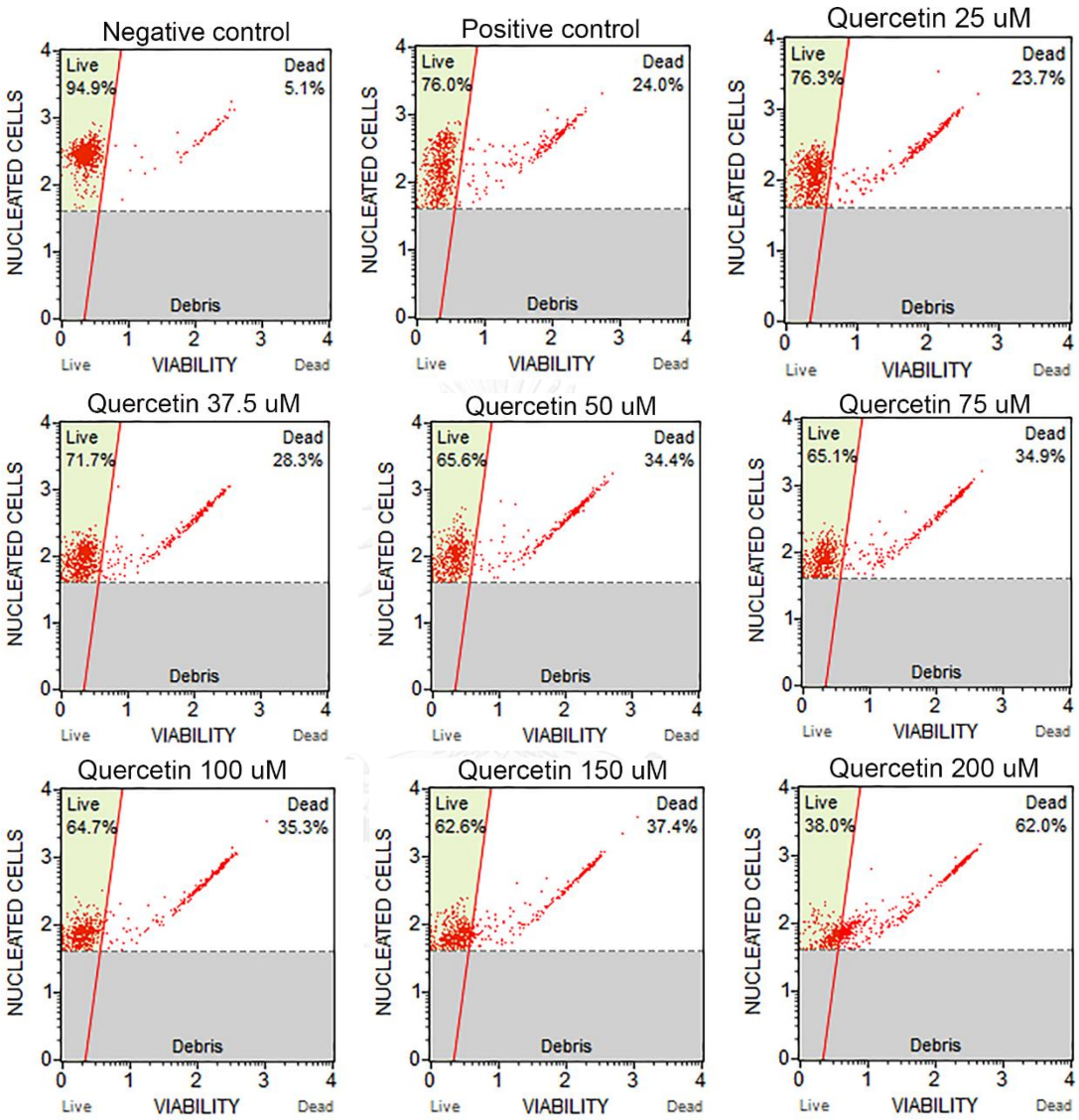

Figure 32 Cell viability dot plots obtained from Muse™ Cell Analyzer (Test No.1).

U937 cells were treated with different concentrations of quercetin for 24 h and cell viability assay was performed by Muse® Count &Viability reagent. The percentage of cell viability was calculated by Muse™ Cell Analyzer.

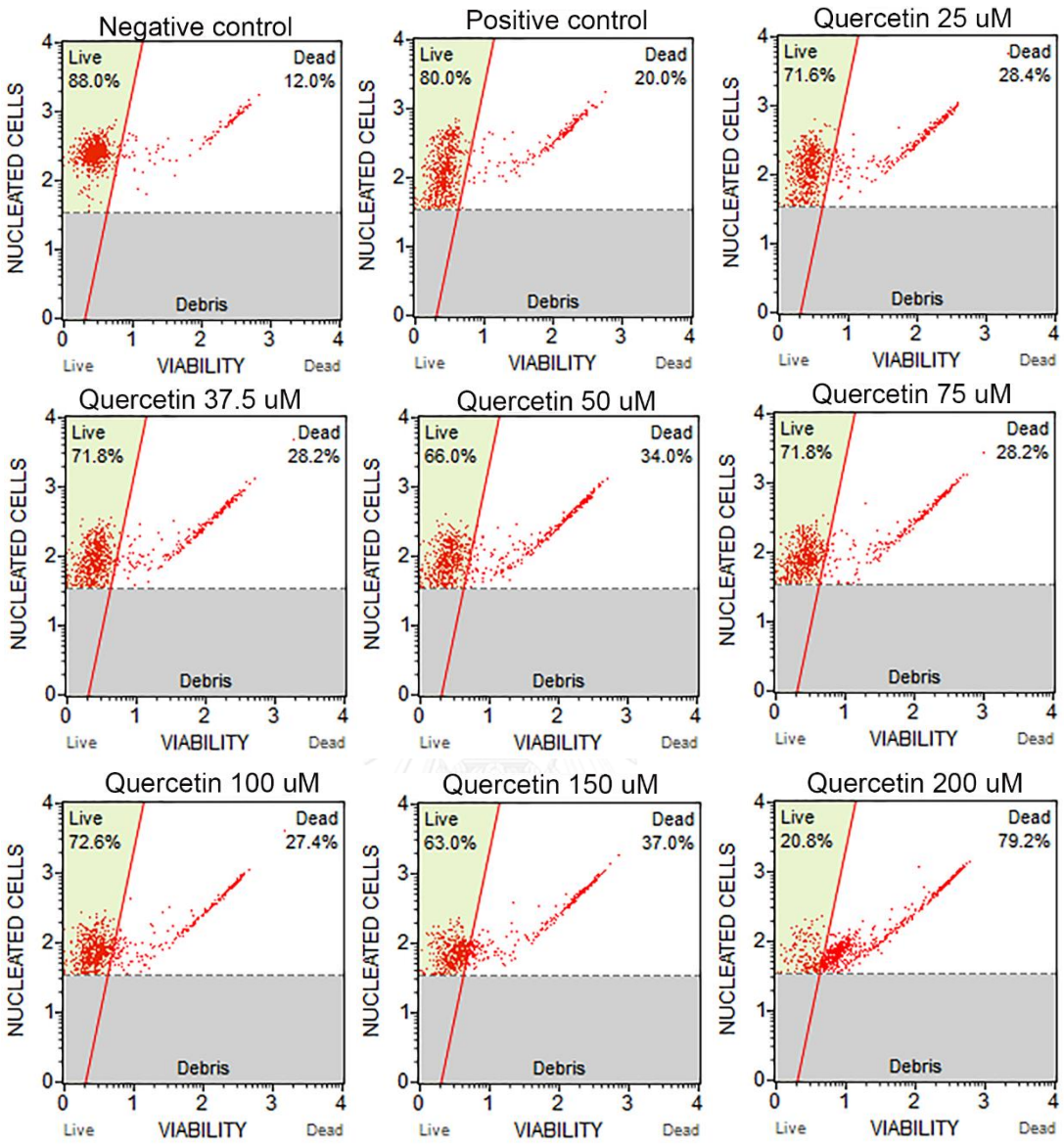

Figure 33 Cell viability dot plots obtained from Muse™ Cell Analyzer (Test No.2).

U937 cells were treated with different concentrations of quercetin for 24 h and cell viability assay was performed by Muse® Count &Viability reagent. The percentage of cell viability was calculated by Muse™ Cell Analyzer.

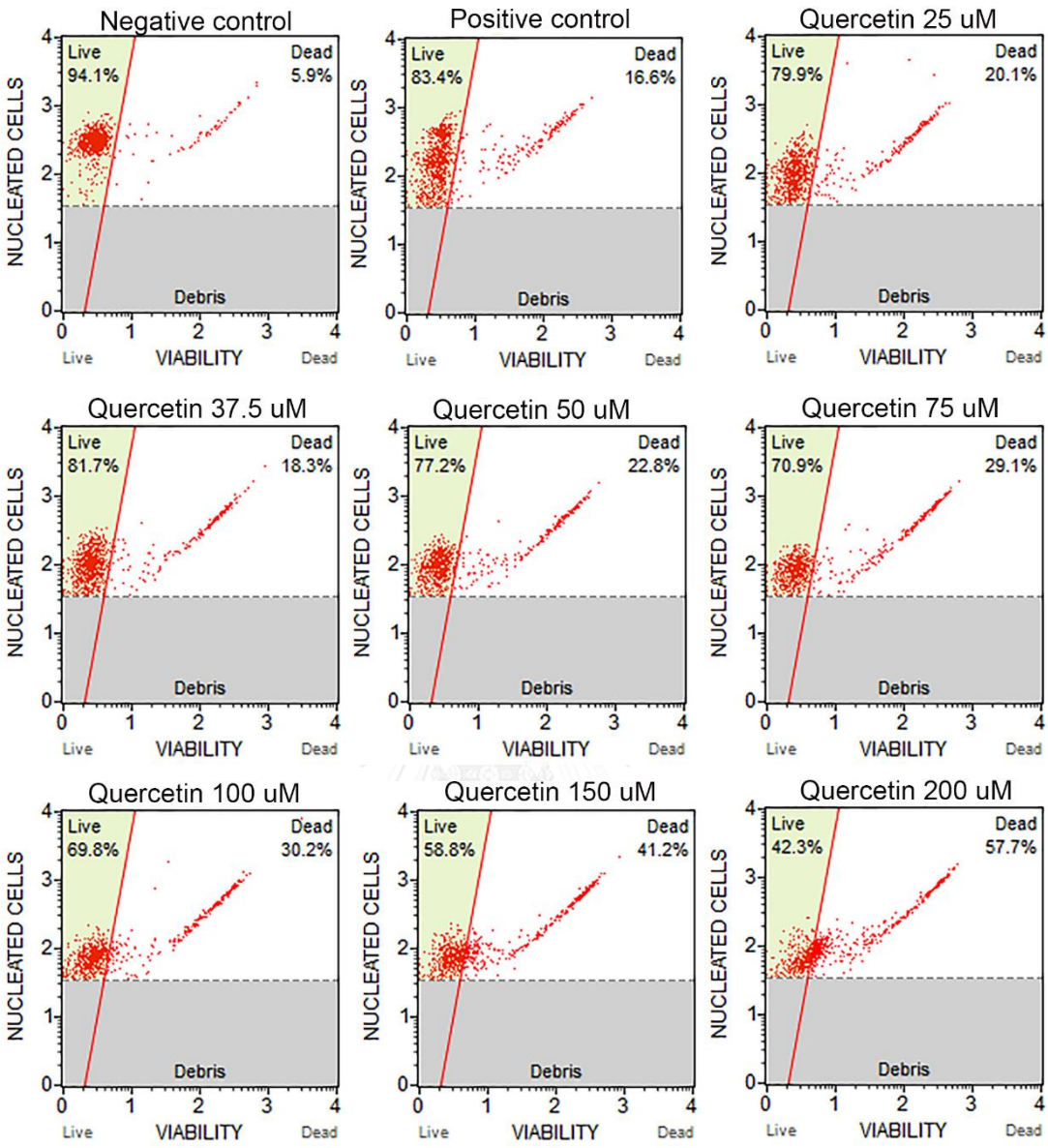

Figure 34 Cell viability dot plots obtained from Muse™ Cell Analyzer (Test No.3).

U937 cells were treated with different concentrations of quercetin for 24 h and cell viability assay was performed by Muse® Count &Viability reagent. The percentage of cell viability was calculated by Muse™ Cell Analyzer.

Preparation of buffer and reagents

Complete culture medium for U937 cells	**50**	**ml**
RPMI-1640 medium	44.5	ml
Fetal bovine serum (FBS)	5	ml
100X Penicillin-Streptomycin	0.5	ml
Stored culture medium at 4 °C		
MTT solution 5 mg/ml	**5**	**ml**
MTT	25	mg
Phosphate-buffered saline (PBS) pH7.4	5	ml
Stored solution at 4 °C and protected from light.		
1X Phosphate buffer saline (PBS), pH 7.4	**1000**	**ml**
NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.02	g
Deionized water	800	ml

Adjusted pH to 7.4 and adjusted the total volume to 1000 ml with deionized water.

Protein lysis buffer **500 ml**

50 mM Tris-HCl, pH 7.4	25 ml of 1M	
1% NP-40	5	ml
0.1% SDS	0.5	g
150 mM NaCl	15 ml of 5M	
2 mM EDTA	2 ml of 0.5M	
50 mM NaF	1.05	g
Deionized water	up to 500	ml

Stored protein lysis buffer at 4 °C and 1 mM PMSF and 1 mM DTT were added immediately prior used.

1 M Tris-HCl, pH 7.4 **100 ml**

Tris-base	12.11	g
Deionized water	80	ml

Adjusted pH to 7.4 with HCl and adjusted the total volume to 100 ml with deionized water.

40% Acrylamide:bisacrylamide solution **100 ml**

Acrylamide	39.2	g
N, N'-methylenebisacrylamide	0.8	g
Deionized water	80	ml

Dissolved the solution for 10 minutes at 65 °C and filtered through Whatman

filter paper #1 and adjusted the total volume to 100 ml with deionized water. Stored solution at 4 °C and protected from light.

10 % (w/v) Sodium dodecyl sulphate (SDS)	**10**	**ml**
Sodium dodecyl sulphate (SDS)	1	g
Deionized water	10	ml

Filtered the solution through Whatman filter paper #1 and stored solution at 4 °C.

10% (w/v) Ammonium persulphate (APS)	**1**	**ml**
Ammonium persulphate (APS)	0.1	g
Deionized water	1	ml

Stored solution at 4 °C.

1.5 M Tris-HCl, pH 8.8	**150**	**ml**
Tris-base	27.23	g
Deionized water	80	ml

Adjusted pH to 8.8 with 6N HCl and adjusted the total volume to 150 ml with deionized water. Stored solution at 4 °C.

0.5 M Tris-HCl, pH 6.8	**100**	**ml**
Tris-base	6.00	g
Deionized water	60	ml

Adjusted pH to 6.8 with 6N HCl and adjusted the total volume to 100 ml with deionized water. Stored solution at 4 °C.

5X Sample buffer		**50**	**ml**
1 M Tris-HCl, pH 6.8		3.125	ml
10% (w/v) SDS		20	ml
Glycerol		10	ml
Bromophenol blue		0.05	g
Deionized water	up to	50	ml

10 ul of 1 M DTT were added to 90 ul of 5X sample buffer immediately prior used.

1 M Tris-HCl, pH 6.8	**100**	**ml**
Tris-base	12.11	g
Deionized water	80	ml

Adjusted pH to 6.8 with HCl and adjusted the total volume to 100 ml with deionized water.

10X Protein running buffer	**1000**	**ml**
Tris-base	30.2	g
Glycine	141.41	g
Sodium dodecyl sulphate (SDS)	10	g
Deionized water	1000	ml

Filtered the solution through Whatman filter paper #1

1X Protein running buffer	**1000**	**ml**
10X Protein running buffer	100	ml

Deionized water		900	ml
Stock Coomassie blue G250 solution		**1000**	**ml**
Coomassie blue G250		1	g
Ammonium sulfate		100	g
Phosphoric acid		20	ml
Deionized water	up to	1000	ml

The solution was stirred overnight in the dark

Working Coomassie blue G250 solution		**1000**	**ml**
Stock Coomassie blue G250 solution		800	ml
100% Methanol		200	ml
1X Transfer buffer		**1000**	**ml**
Tris-base		3	g
Glycine		14	g
Methanol		200	ml
Deionized water		800	ml
10X Tris buffer saline (TBS), pH 7.6		**1000**	**ml**
Tris-base		24.2	g
NaCl		80	g
Deionized water		800	ml

Adjusted pH to 7.6 with HCl and adjusted the total volume to 1000 ml with deionized water.

1X Tris buffer saline (TBS)	**1000**	**ml**
10X Tris buffer saline (TBS), pH 7.6	100	ml
Deionized water	900	ml
1X Tris buffer saline, 0.05% Tween 20 (TBST)	**1000**	**ml**
10X Tris buffer saline (TBS), pH 7.6	100	ml
Deionized water	900	ml
Tween-20	0.5	ml
1X Blocking solution	**100**	**ml**
Nonfat dry milk	5.0	g
1X TBS	100	ml
Stirred the solution to dissolve.		
Antibody dilution buffer	**50**	**ml**
Bovine serum albumin (BSA)	2.5	g
1X TBST	50	ml
Mixed the solution to dissolve.		

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